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Re-purposing Delta One T cells for immunotherapy of solid tumours: Colon cancer as proof-of-principle

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Preface

The work presented in this thesis was performed at Bruno Silva-Santos Lab, at IMM, during the period February-October 2019, under the supervision of Dr. Sofia Mensurado. The thesis was co-supervised at Instituto Superior Tecnico by Prof. Dr. Cláudia Alexandra Martins Lobato da Silva.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Agradecimentos

A vós, pais, por todo o apoio.

Abstract

Globally, colon cancer (CC) is the third most commonly diagnosed malignancy and the second leading cause of cancer death. 50% to 75% of CC patients display only 14% of 5-year survival rate. Therefore, developing new treatment approaches for patients with this disease is an urgent unmet need.

Harnessing the unique biology of $\gamma\delta$ T-cells along with expansion protocols might be considered in new immunotherapy approaches. One of $\gamma\delta$ T-cell-based alternatives are 'Delta One T' (DOT) cells, an immunotherapeutic product consisting in more then 65% Vδ1+ $\gamma\delta$ T-cells that overexpress natural killer receptors (NKRs) and display potent anti-tumour functions.

In this work, we explored the therapeutic potential of DOT cells in CC. We found that DOT cells were cytotoxic against both CC cell lines tested, HCT116 and SW620, although SW620-targeting was 3,6 times more efficient that HCT116. Mechanistically, DOT cells recognized SW620 through the NKRs NKG2D and DNAM-1. Interestingly, the secretome of CC cells enhanced DOT cell anti-tumour and migration potential, thus suggesting that DOT cell activity *in vivo* might be favoured in CC. We also found two negative regulators of DOT cells. Peripheral blood neutrophils, when cultured with DOT cells lead to a decrease in DOT cell NKR expression and cytotoxic potential. The same pattern was observed upon DOT cell incubation with the immunosuppressive cytokine TGF-β, but not IL-10. Collectively, our work provides new insights that support further investigations for application of DOT cells in adoptive cell therapy of CC.

Keywords: Colon cancer, DOT cells, Natural cytotoxicity receptor, Neutrophils, TGF-β, IL-10

Resumo

Mundialmente o cancro do colon é o terceiro tipo de cancro mais diagnosticado e o segundo que mais mortes causa. 50% a 75% dos doentes com cancro do colon têm somente 14% de probabilidade de sobreviver 5 anos. Assim, é urgente a necessidade de desenvolver novos tratamentos para estes doentes.

Considerar a capacidade anti-tumoral de linfócitos T $\gamma\delta$ e associá-la a protocolos seguros de expansão permitiu a criação de um novo produto imunoterapêutico - as células DOT (do inglês "Delta One T"). Este produto é composto em mais de 65% por células T Vδ1+, que são expandidas e diferenciadas em potentes efectoras anti-tumorais.

Neste trabalho investigou-se o potencial terapêutico das células DOT contra o cancro do colon. As células DOT mataram eficientemente as duas linhas celulares de cancro estudadas, HCT116 e SW620 (3,6 vezes mais eficientes SW620 do que HCT116). A nível de mecanismo celular, as células DOT reconheceram as células SW620 através dos recetores NKG2D e DNAM-1. Curiosamente o secretoma das linhas celulares do colon promoveram um fenótipo anti-tumoural e a migração das células DOT, sugerindo a atividade das células DOT em estudos in vivo. Foram também aferidos dois reguladores negativos das células DOT. Neutrófilos oriundos de sangue periférico levaram a um decréscimo do potencial citotóxico das células DOT. O mesmo foi observado após incubação das células DOT com a citocina imunossupressora TGF-β, mas não com IL-10.

Coletivamente este estudo providencia interessantes desenvolvimentos sobre o potencial terapêutico das células DOT em cancro do cólon e suporta investigações futuras.

Palavras-chave: Cancro Colon, Células DOT, Recetores de células NK, Neutrófilos, TGF-β, IL-10

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Acronyms

AML acute myeloid leukemia

AICD activation-induced cell death

APC antigen-presenting cell

ARG arginase

BrHPP bromohydrin pyrophosphate

BTN butyrophilin

CAR chimeric antigen receptor

CC colon cancer

CD3 cluster determinant 3

CD8 cluster of differentiation 8

CLL chronic lymphocitic leukemia

CIMP CpG island methylator phenotype

CMS consensus molecular subtype

CTLA-4 cytotoxic T-lymphocyte antigen type 4

CXCR5 chemokine receptor type 5

CTL cytotoxic CD8 T cell

DC dendritic cell

DOT delta one T

DN double negative

DNAM-1 DNAX accessory molecule-1

EGFR epidermal growth factor receptor

ICAM-1 intercellular cell adhesion molecule type 1

IEL intra-epithelial lymphocyte

IFN interferon

IL-10 interleukin 10

IL-15R interferon 15 receptor

IPP isopentenyl pyrophosphate

MAPK Microtubule-Associated Protein Kinase

MHC major histocompatibility complex

MICA/B MHC Class I-related Chain A And B

MSI microsatellite instability

MSS microsatellite stability

NCR natural cytotoxicity receptor

NK natural killer

NKG2D receptor for natural killer cells type D

NKR natural killer receptor

PBMC peripheral blood mononuclear cell

PD-1 programmed cell death protein 1

PD-L1 programmed cell death protein 1 ligand

PHA phytohemagglutinin

PI3K phosphatidylinositol 3-kinase

ORR overall response rate

ROS reactive oxygen species

TCR T-cell receptor

TFH foliccular T helper cells

TGF-β transforming growth factor

TLR toll-like receptor

TIL tumour infiltrated lymphocyte

TNF tumor necrosis factor

TNF-α tumor necrosis factor alfa

TRAIL apoptosis-inducing ligand

TRD receptor gamma delta

TRG receptor gamma locus

ULBP UL16-binding Protein

WNT wingless/integrated

$\gamma\delta$ **T cell** gamma delta T cell

$\alpha\beta$ **T cell** alfa beta T cell

nTreg natural regulatory T cell

iTreg induced regulatory T cell

TME tumour microenvironment

TAM tumour-associated macrophages

Treg regulatory T cell

TAN tumour-infiltrating neutrophil

Chapter 1

Introduction

1.1 Ageing diseases as 21st century biggest threat

The past 200 years of the human species on earth have doubled the average human life expectancy in most developed countries [1]. Better quality of food, water, hygiene, immunization against infectious diseases, housing and lifestyle allowed physical and cognitive functioning improvements, however mean life expectancy increased the percentage of elderly people. Whereas increasing human lifespan naturally has predisposed civilization to celebrate these achievements, its lack of direct correlation with disease-free lifespan has hampered it. Ageing has also been perceived as the primary cause of many chronic diseases of later life, including chronic kidney disease, Alzheimer disease, coronary artery disease, stroke, type 2 diabetes mellitus and common cancers, such as prostate, breast and colon cancer [2]. Higher cancer incidence has been extremely associated with advancing adult age and cancer has been presented as the number one cause of death in people ranging from 60 to 79 years: it is estimated that, by 2050, more than 20% of world's population will be over the age of 60, accounting for 2 billion people [3, 4].

Despite the dramatic increase in our understanding of aetiology, epidemiology and molecular biology of cancer over the past decades, 18 million new cases were diagnosed worldwide in 2018 [5]. From those, solid tumours encompassed 90% and about 10.8% of total cancers located in the colon[5]. Approximately 900 000 deaths due to colon cancer were reported in the same year [5]. Globally, colon cancer is the third most commonly diagnosed malignancy and the second leading cause of cancer death [6].

1.2 Colon cancer and its clinical management

The majority of colon cancers arise sporadically (60 to 65% of total) through acquired somatic genomic (either genetic or epigenetic) alterations [5].

Colon cancer progression share the common steps responsible for cancer development: initiation, promotion, progression and metastasis [7]. Initiation comprehends accumulation of cells with irreversible

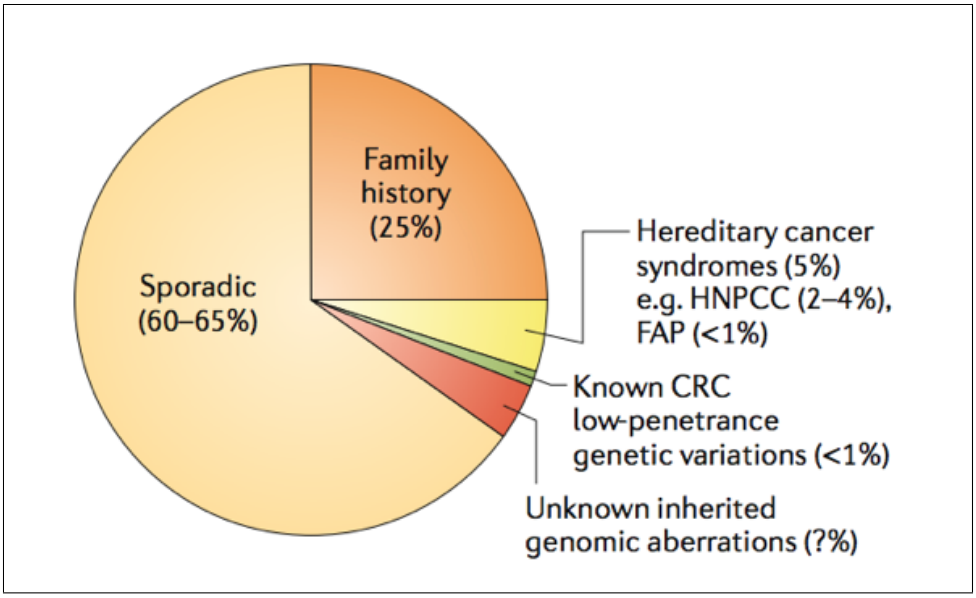

Figure 1.1: Proportion of colorectal cancer cases associated with sporadic and hereditary factors. The majority of CC arise sporadically through acquired somatic genomic alterations, whereas 35–40% of cases are associated with inherited CC susceptibility. Adapted from [5].

genetic damage, which is followed by the promotion phase - abnormal cell growth [7]. After being exposed to growth stress, cells further acquire further alterations that endow them with immune escape capabilities and metastatic potential (progression) [7]. In the metastasis phase, malignant cancer cells spread from the primary tumour to other body parts, organs or tissues through the lymphatic system and bloodstream [7].

Colon carcinogenesis is usually a well-defined process that starts with a benign precursor lesion, a polyp [8]. There are two types of polyps: adenomatous polyps, which are adenomas, a benign tumor originating in a secretory gland; and serrated polyps, which combine the saw-toothed morphological appearance of hyperplastic polyps and dysplastic features of adenomas. These two types of polyps epitomize the two major direct precursors to the majority of colon cancers [8]. From adenomas, gradual stepwise accumulation of genetic and epigenetic alterations that support uncontrolled cell growth contributes to the generation of carcinomas [9]. First, mutation in oncogenes promote adenoma growth and then inactivation of tumour suppressor genes contribute to the progression to carcinomas [9].

Colon cancer standard of care aims to remove total tumour through surgical intervention [9]. Although surgical procedures may lead to total cancer removal, the preoperative state of the patient is key [9]. Patients' diagnosis stage determines specific treatments, being surgical the only efficient one for early diagnosed cancer. 25% of cancers are diagnosed at advanced stages with metastasis, whereas 20-50% develop metastasis during preoperative instances [9]. According to Bonjer et al., 75% of the patients elected that undergo surgical procedures exhibit 3-year disease-free survival [10]. Enhancement of surgery outcome may be achieved by combination with neoadjuvant and adjuvant therapies [9].

However, patients with unresectable lesions and intolerance to surgery (accounting for the majority of the cases) need to follow alternative treatments, aiming at shrinkage of tumor and metastasis restraint [11]. The state of the art of clinical trials involving treatments for metastatic disease allowed overall

survival enlargement in about 30 months by means of chemotherapeutics and targeted therapies that exploit either tumour characteristics or tumour microenvironment properties [11].

Multiple evidences about doublet and triplet forms of chemotheraphy generated high efficacy results in addition to increased growth control of tumours over single-agents [12]. Despite particular benefits, when applying several chemotherapeutic agents, neutropenia and diarrhoea often arise as adverse effects [12]. Targeted therapies are an alternative. Various pathways mediating the initiation, progression, and migration of colon cancer, such as Wnt/β-catenin, Notch, Hedgehog, and TGF-β/SMAD, as well as those capable of activating signaling cascades, such as phosphatidylinositol 3-kinase (PI3K)/AKT or RAS, include ideal sites for targeted therapy [13, 14]. Successes with anti-EGFR agents cetuximab, panitumumab [15] and the anti-angiogenesis agent bevacizumab [16], new agents blocking different critical pathways, as well as immune checkpoints, emerged at an unprecedented rate.

Immune checkpoint inhibitors are one type of immunotherapy consisting on antibodies that aim to target co-inhibitory signals, which tumour cells exploit to escape immune-mediated destruction [17]. These inhibitors can target co-inhibitory receptors, such as CTLA-4 and PD-1 on T-cells and other immune cell subpopulations, or their ligands, such PD-L1 on tumour cells and various immune cells [17]. The fast success of CTLA-4, PD-1 and PD-L1 in some cancer types led to the active investigation of immune checkpoint inhibitors in several other cancer types. Initial studies considering unselected patients with metastatic colon cancer only showed 3% of positive responses upon anti-CTLA-4 or anti-PD-1/PD-L1 treatment [18, 19]. Interestingly, the patients that responded to this theraphy were CpG island methylator phenotype (CIMP) positive and exhibited microsatellite instability (MSI) [18, 19]. Based on the interesting results of these therapies in specific patients other studies followed a similar path. KEYNOTE-016, a proof-of-concept phase II study consisting on the administration of pembrolizumab to both CIMP positive/MSI and CIMP positive/microsatellite stability (MSS) metastatic patients, resulted in akin results. Only patients displaying CIMP positive/MSI phenotype had an overall response rate (ORR) of 40% (4 out of 10 patients) [20]. Another phase II study, CheckMate-142, evaluated the efficacy of nivolumab and ipilimumab in metastatic CIMP positive/MSI patients (previous treated or treatment-naive) [21, 22, 23]. The results of this study constitute important evidence of immune checkpoint inhibitors efficacy. 1-year overall survival rates ranged from 60 to 85% [21, 22, 23]. Nivolumab and ipilimumab also led to enhanced responses as adjuvant of surgery in a preoperative setting. Once more, improvements in metastatic CIMP negative/MSS patients were not observed [21, 22, 23]. Hence, randomized phase III trials ongoing are focused on metastatic CIMP positive/MSI patients, and are evualuating anti-PD-1, anti-PD-L1, and anti-CTLA-4 alone and in combination with chemotheraphy and targeted therapies. However, patients exhibiting a CIMP negative/MSS phenotype, which comprises the majority of colon tumours, do not respond to immune checkpoint inhibitors, requiring alternative approaches [19].

Unique contributions by prevention and surgery technical development contributed for 90% success in highly localized colon cancer cases [3]. However, there is an extremely low rate of 14% of 5-year survival rate in metastatic cases (accounting for 50 to 75% of all-around colon cases) [24]. Thus, the development of more effective treatment options for patients with this disease is an urgent unmet need. In the past decade, immunotherapy has kindled tremendous excitement owing to its success in achieving long-

term durable responses in previously difficult-to-treat solid tumours, such as lung cancer and melanoma [25]. Moreover, immunotherapeutic agents were approved for all advanced microsatellite instability-high and DNA mismatch repair–deficient solid tumors, including metastatic colon cancers [22, 20]. These important contributions of new therapies have fueled scientific interest to explore immunotherapy potential for the majority of colon cancers which are non-hypermulated (more than 80% of the cases). Importantly, the development of a mutation load-independent 'off-the-shelf' product would be the *holy grail* for colon cancer treatment.

1.3 Colon cancer models: SW620 and HCT116 cell lines

Colon cancers are molecularly heterogeneous [26] and most investigators divide them biologically according to their DNA level aberrations [27]. At DNA level, aberrations include hypermethylation at repetitive CG dinucleotides of promoter regions of tumour suppressor genes (named as CIMP), hypermethylation at repetitive CG dinucleotides of promoters of DNA mismatch-repair genes (named as microsatellite instability - MSI), and abnormalities in chromosomal copy structure and number (chromosomal instability - CIN) [28]. Hence, Guinney et al. proposed an comprehensive cross-classification of colon cancer subtypes based on their mutation levels, copy number, methylation, microRNA and pro-teomics [29]. This comprehensive analyses lead to categorize colon cancers into one of four consensus molecular subtype (CMS) based on their distinguishing features: CMS1, CMS2, CMS3 and CMS4.

The CMS1 group is characterized by MSI, hypermutation and displays immune infiltration, thereby leading to good prognosis [29]. Conversely, CMS2 group has high somatic copy number alterations but low TILs, which might explain their intermediate prognosis [29]. CMS3 group includes the less prevelant colon cancers, with significant metabolic deregulation, KRAS mutations but low TIL infiltration [29]. Importantly, CMS4 group exhibit an immunosuppressive signature, characterized by overexpression of cancer-associated fibroblasts and their coregulatory chemokines, resulting in a TME favoring tumor-associated inflammation, angiogenesis, and activation of TGF-β [29]. Besides that, CMS4 group is not generally hypermutated but has high somatic copy number alterations [29]. CMS4 group has the worse relapse-free and overall survival [29], thereby should be a point of interest to researchers, which ultimately could enhance this statistics by enhancing i) study models and ii) treatments available.

HCT116 and SW620 colon cancer cell lines, the two models used in the present work, were classified as being part of CMS4 group [26]. HCT116 cell line is MSI and CIMP-positive, whereas SW620 is MSS and CIMP-negative [26]. HCT116 cell line displays a higher level in CIN aberrations compared to SW620 [26]. According to somatic acquired aberrations (MSI/CIMP/CIN), HCT116 shows a higher level of DNA level aberrations than SW620. Direct comparisons depict HCT116 cell line with higher levels of ERK/MAPK activation, PI3K and expression of TGF-β induced genes [26]. ERK/MAPK and PI3K are important for cell proliferation, cell survival ans translation levels [27]. Moreover, HCT116 is originated from a primary tumour site whilst SW620 was originally isolated from a metastatic lymph node. In fact, SW620 mutations on TP53 and APC make them a theoretically less agressive cancer cell line in comparison to HCT116[27]. Overall, HCT116 and SW620 encompass different molecular

characteristics within the CMS group that displays the worst prognosis.

1.4 Introduction to $\gamma\delta$ T cells

$\gamma\delta$ T cells have co-evolved alongside $\alpha\beta$ T cells and B cells for 450 million years of vertebrate evolution [30] and are increasingly recognized as having important roles in immune responses to both microbial and non-microbial stress challenges [31]. $\gamma\delta$ T cells comprise only 1–10% of circulating T-cells [32], diverging from $\alpha\beta$ T cells in the thymus, during double negative 2 (DN2) and DN3 stages of thymic development [33]. Several aspects of development and anatomical pressures make $\gamma\delta$ T cells differ between each other within human body. By undergoing somatic recombination of T cell receptor gamma locus (TRG) and receptor gamma locus (TRD) gene loci, a functional $\gamma\delta$TCR surface receptor is generated. In fact, γ- and δ- chains of T cell receptor are able to ascertain $\gamma\delta$ T cells specific abilities [34]. Together with differential environmental stimulation, TCR repertoire guide $\gamma\delta$ T cells to perform established functions at certain anatomical sites [34]. TCRγ chain arises from recombination of variable, diversity and joining gene segments of TRG locus, whilst V and J TRD locus segments recombination assemble TCRδ chain. Upon TCR locus rearragments, $\gamma\delta$TCR assembles with CD3 proteins on cell surface, allowing signal transduction [35]. Mathematically, $\gamma\delta$TCR diversity is around $10^{17} : 10^{13}$ possible configurations emerged from TRD gene rearrangements multiplied by 10^4 possibilities incurred from TRG rearrangements [36].

Although $\gamma\delta$ T cell relative proportion in circulation is low, their abundance in mucosal sites is higher, which renders them an important tissue surveillance system. Moreover, $\gamma\delta$ T cells can recognize endogenous stress induced-ligands in a MHC-unrestricted manner. This type of cells are also capable of monitoring other cells for abnormal changes, establishing a 'lymphoid stress-surveillance response' [37, 38]. Furthermore, $\gamma\delta$ T cells display surface cytotoxic receptors, allowing them to respond to nfected or transformed cells [39]. All these characteristics summed to other immunological processes (such as cytokine and chemokine production, antigen-presenting and regulatory features) contribute to the strengthening of $\gamma\delta$ T cells as potential candidates to be used in colon cancer adoptive cell therapy [40, 41].

1.5 $\gamma\delta$ T cell subsets and activation

According to TCR δ chain usage, human $\gamma\delta$ T cells can be classified as V$\delta1$, V$\delta2$ or V$\delta3$ T cells [42]. Depending on δ rearrangement, certain γ chains are preferred over others - Vγ2,3,4,5,8 usually pair up with V$\delta1$ and Vγ9 with V$\delta2$ [43]. It is clear that these correspond to the majority of combinations observed by research groups on the field, albeit other TCR rearrangements should not be dismissed [44].

Vγ9Vδ2 $\gamma\delta$ T cells are generated in thymus at 8.5-15 weeks of human embryonic development [45] and usually migrate to peripheral blood, accounting for 70% of arising $\gamma\delta$ T cells [44]. Microbes and transformed cells unleash Vγ9Vδ2 activation in a TCR-dependent manner through phospoantigens [46].

In fact, the fundamental difference in TCR-mediated recognition between $\alpha\beta$ T cells and $\gamma\delta$ T cells is that $\gamma\delta$ T cells do not require antigen presentation by MHC molecules. The mevalonate pathway is altered in transformed cells leading to intracellular accumulation of isopentenyl pyrophosphate (IPP) [47, 48]. Although IPP is also produced by normal cells under normal conditions, its detection by $\gamma\delta$ T cells occurs upon accumulation of high concentrations, thereby justifying the organism well-functioning in normal conditions despite the already existing natural affinity of TCR chains for IPP [47]. Intentionally enabling IPP higher concentrations within transformed cells can be accomplished by aminobisphosphonate administration such as zoledronic acid, which inhibit farnesyl pyrophosphate synthase, an IPP-downstream enzyme of the mevalonate pathway, creating the desired increase in IPP concentrations required for $\gamma\delta$ T cells recognition [49, 50, 51]. Understanding $\gamma\delta$TCR stimulation and its inherent differences to $\alpha\beta$TCR signal behaviour could allow to identify more TCR ligands, seen as an *500-million- year-old mystery* [52]. Vγ9Vδ2 T cells activation can be exploited by *indirect TCR stimulation*, toll-like (TLRs) and natural killer receptors (NKRs) co-stimulation [53, 54]. *Indirect TCR stimulation* highlights phospoantigen fundamental behaviour of interaction with specific proteins that induce TCR reactivity. Butyrophilins (BTNs) and butyrophilin-like (BTNL) molecules have emerged as intriguing candidates to $\gamma\delta$TCR ligands, with BTN3A1 already scientifically proven to undergo conformational change and recruit molecules that directly bind to TCR upon phospoantigen binding to Vγ9Vδ2 T cells [51, 55]. Moreover, BTN3A1 is expressed by the majority of cells within the human body, being encoded by chromosome 6 and, as such, increasing $\gamma\delta$ T cells capacity of detecting abnormalities. Mitochondrial F1-ATPase expressed on the cell surface also constitutes an antigen-recognition molecule (activated upon complex formation with apoliprotein AI), as well as MutS homologue 2, a DNA repair-related protein that migrates to the cell membrane where it is detectable by the TCR [56]. Conversely, co-stimulation encompasses both activation of TLRs by pathogen associated molecular patterns, leading to cytokine/chemokine production [54], and specific NKRs - NKG2D sense MICA/B and ULBPs [57], DNAM-1 recognizes Nectin-2 and PVR [58].

In fact, NKG2D is expressed in Vδ1, Vδ2 T cells, NK cells and some $\alpha\beta$ T cells [59]. Importantly, human carcinoma samples from several organs expressing MICA and MICB were associated with the presence of Vδ1 T cells capable of killing the tumour cells [60]. NKG2D also exhibit a co-stimulatory in T cells [61]. Upon MICA engagement, activated human CD8 T cells produce more IFN, TNF, and IL-2 in response to TCR stimulation [61]. Moreover, NKG2D also mediates sensitization of other receptors. NKG2D can potentiate IL-15R signaling in memory CD8 T cell precursors [62]. In fact, T cell responsiveness to chemokines is changed upon NKG2D stimulation [62]. However, the correct mechanism associated with this modulation is not known. Suprisingly, characterization of T cells infiltrating human tumors overexpressing NKG2D ligands showed that they downregulate the NKG2D receptor, suggesting an an increased susceptibility to increased tumour growth [63].

Usually $\gamma\delta$ T cells respond to stimulation and co-stimulation signals in sequence rather than simultaneously as $\alpha\beta$ T cells [38]. Additionally, Vγ9Vδ2 $\gamma\delta$ T cells analysis reported that these cells share similar lengths and sequences of CDR3 regions (which are TCR components responsible for sensing different antigens) between clonotypes, suggesting that Vγ9Vδ2 subset display a more innate-like phe-

notype [38, 64].

Antagonistically to Vγ9Vδ2 $\gamma\delta$ T cells, Vδ1 $\gamma\delta$ T cell subset expresses a more diverse TCR repertoire, suggesting a broader range of detected antigens (diverse lengths and sequences of CDR3 regions), which may be suggestive of an adaptive phenotype of this subset [38]. As such, and with further research required to clarify TCR relation with anatomical site presence, Vδ1 T cells are mainly present in tissues: epithelia, dermis, colon, liver and spleen [31]. In fact, these cells are key in leveraging epithelial tissue integrity [31]. With TCR rearrangement completed 4 to 6 months after birth, Vδ1 T cells are entailed in responses to viral infection and cancer [45]. Nevertheless, and recalling MHC-unrestricted antigen recognition, Vδ1 T cells are able to respond to antigenic peptides, lipids and microbial metabolites presented by CD1 family molecules, thereby broadening the assortment of self and foreign antigens amenable of being encountered [65, 66]. Phospholipid antigens are presented by CD1a, CD1c or CD1d and sensed via TCR [66, 67]; this type of recognition has already been reported in myeloid leukaemic cells [68]. However, their relevance to tumour cell targeting remains unclear. MICA, a NKG2D ligand, is also acknowledged as Vδ1 TCR ligand which is overexpressed by tumour cells [69]. Albeit, its interaction is way stronger with NKG2D than with Vδ1 TCR [69]. Furthermore, it was found that not only MHC-related molecules are liable of activating Vδ1 $\gamma\delta$ T T subset, but also annexin A2, protein involved in cell motility, activate Vδ1 $\gamma\delta$ T clones [70]. Other NKG2D ligands, ULBPs are recognized by Vδ1 T cells [71]. Much more NKRs can be induced through expansion pressures, and both NKp30 and Nkp46 have already proved to recognize tumour cells, in hematological as well as solid cancers [72].

1.6 $\gamma\delta$ T cells functions

Circulating $\gamma\delta$ T cells are highly biased towards IFNγ production [73, 74], accounting for 50% to 90% of all $\gamma\delta$ T cells [32]. Generally, IFNγ production is associated with TNF [75, 76, 77, 78], thus constituting a way of contributing to pathogen clearance. This clearence can also be induced by the release of cytotoxic perforin and granzymes [79, 80]. Priming under environmental conditions like i) IL-12 plus anti-IL-4 monoclonal antibodies with IPP [81], ii) IL-2 and IL-15 [73], iii) IL-12, IL-18 and IL-21 [82, 83], iv) IL-2 and IL-21 [83] or v) non peptide antigens and IL-21 [84] induces an IFNγ-producing profile that is also characterized by potentiation of cytotoxicity leading to slowing of tumour growth in transplantable melanoma and mammary tumour mouse models [85].

$\gamma\delta$ T cells can also exert antigen-presenting capabilities [86]. Upon microbial activation, Vγ9Vδ2 $\gamma\delta$ T cells displayed higher levels of CD69, HLA-DR, CD80, CD86 among others, thereby enabling leukocyte activation, antigen presentation and T cell co-stimulation [86]. Upregulation of CD36 by $\gamma\delta$ T cells enables the killing of liver cancer cells, followed by debris uptake, processing and presentation of antigens to CD8 T cells [87]. Moreover, TNF-α produced by $\gamma\delta$ T cells promotes CD4 T cell priming as well as DC maturation, overall describing $\gamma\delta$ T cells as key in stimulating other immune cells [88, 89].

B cell antibody secretion is perceived as a meaningful part of an effective adaptive immune response [90]. Interactions established between T and B cells impact the abundance and characteristics of antibodies that are produced [90]. $\gamma\delta$ T cells modulate antibody production of pre-immune peripheral B cells

population [91]. By being stimulated in an antigen and IL-21 dependent manner, $\gamma\delta$ T cells display a follicular Th cells (Tfh) phenotype, which is defined by high expression of the marker CXCR5 - responsible for TFH cells migration into B-cell follicles - and the signature cytokine IL-21 - that predominantly acts as a paracrine factor for germinal center B lymphocytes - [90].

In fact, a TFH phenotype associated with high levels of Bcl-6 was assessed upon antigen and IL-21 stimulation of Vγ9Vδ2 $\gamma\delta$ T cells [92, 93]. CXCR5 expression was measured and its interaction with B cells was observed, thus clarifying the helper phenotype of $\gamma\delta$ T cell population [92, 93]. Contrary to CD4 TFH, $\gamma\delta$ TFH express a Th2 phenotype, characterized by IL-2, IL-4 and IL-10 secretion [93].

$\gamma\delta$ T cells are also able to display immunosuppressive and regulatory activities during immune responses [94]. Treg-specific transcription factor Forkhead box P3 (FoxP3) is constitutively expressed by natural regulatory T cells (nTreg) and induced regulatory T cells (iTreg) [95]. Despite the fact that $\gamma\delta$ T cells freshly isolated from peripheral blood do not display a suppressive phenotype and do not express FoxP3 [96], in vitro data [97, 98] and reports from cancer cases [99, 100] suggest that Foxp3+ $\gamma\delta$ T cells can be generated and can display suppressive activity under certain conditions.

Vδ2 $\gamma\delta$ T cells exposed to strong antigens in the presence of APCs prior to IPP-stimulation, dampen the proliferation of both CD4 and CD8 $\alpha\beta$ T cells [98]. A similar result can be achieved through exposure of $\gamma\delta$ T cells to anti-CD3 mAb in combination with a co-stimulatory signal [99].

TGF-β and IL-2/IL-15 stimulation can endow $\gamma\delta$ T cells with Foxp3 expression, leading to a regulatory $\gamma\delta$ T cell phenotype. Some researchers also pinpointed anti-CD3 mAb and Staphylococcus aureus enterotoxin stimuli as key to achieve the same results, ultimately leading to $\gamma\delta$ T cell-mediated inhibition of $\alpha\beta$ T cells proliferation [99]. All in all, the presence and strength of a co-stimulatory APC-signal as well as anti-CD3 mAb stimulation seem to play an important role in the induction of $\gamma\delta$ T cell suppressive capacity.

However, it is important to note that FoxP3 and $\gamma\delta$ T cell regulatory phenotype are not fully correlated , consequently requiring further research to scrutinize the field [94]. Thus, breast tumor-infiltrating FoxP3 negative $\gamma\delta$ T cells actively suppressed T-cell responses and DC maturation [94, 101]. Similarly, CD39 positive FoxP3 negative $\gamma\delta$ T cell subset contributed to strong immunosuppression in human colorectal cancer [94, 101].

IL-17A is a pro-inflammatory cytokine which exacerbates organ-specific autoimmune inflammation, promotes mobilization of neutrophils and cytokine production by epithelial cell for protective immunity to extracellular pathogens and contributes to maintenance of tight junction integrity and angiogenesis promotion [102]. Recent studies in mice have shown that $\gamma\delta$ T cell are a considerable source of IL-17, along with $\alpha\beta$ T cells, iNKT cells and LTi-like cells [102].

However, the amount of IL-17-producing $\gamma\delta$ T cells in humans is less expressive than IFN-γ-producing $\gamma\delta$ T cells. In contrast to mice, circulating human $\gamma\delta$ T cells are highly biased towards IFN-γ production [74]. The rare situations where human IL-17-producing $\gamma\delta$ T cells have been reported include accumulation in disease settings, such as meningitis [103] and some cancers [104].

1.7 Anti-tumour functions of $\gamma\delta$ T cells

Human $\gamma\delta$ T cells recognize transformed cells through NKG2D machinery [59]. In fact, several tumour cells from either solid or hematological tumours express MICA/B or ULBPs [59]. Notably, other NKRs such as Nkp44, Nkp30 and DNAM1 also play a key role in cancer cells recognition [105]. The mechanisms on which human $\gamma\delta$ T cells rely to kill cancer cells are the same employed by conventional cytotoxic T cells [59]. Upon engagement of NKG2D the release of perforin and granzyme B induce target cell apoptosis through creation of pores in the membrane, which is followed by the entry of granzyme B responsible for inducing apoptotic cascades [59]. Other mechanisms comprise the expression of tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or FAS ligand [106]. $\gamma\delta$ T cells also express IFN-γ in B16 melanoma, leading to i) IFN-γ production by $\alpha\beta$ T cells and MHC-I expression by tumour-cells. This way, contribute to potentiate anti-tumour responses [79, 107].

1.8 Rationale for harnessing $\gamma\delta$ T cells in immunotheraphy

On the basis of their described molecular anti-tumour functions, $\gamma\delta$ T cells are a possible therapeutic strategy to treat colon cancer. This approach arise as a consequence of disappointments related with tumour immunotherapy either based on immune checkpoint inhibitors or CAR T-cell technology [108]. Overcoming the drawbacks of these two strategies - efficacy restricted to haematological neoplasms and solid tumours with high mutational burdens, limited effectiveness, frequent acquired resistance and tumour relapse, amongst others - becomes essential when launching new off-the-self immunotherapy options [109, 110]. Immune checkpoint blockers and CAR-T cell efficacy is dependent on the expression of tumour-associated antigens, MHC molecules and co-stimulatory signals [108] which are usually downregulated, strongly hampering $\alpha\beta$ T cell anti-tumour performance and even disabling it [111].

Harnessing the unique $\gamma\delta$ T cell biology to improve cell-based immunotherapy comprises several key points. As aforementioned, $\gamma\delta$ T cells do not recognize and kill tumor cells depending on the expression of a single antigen [112]. Secondly, specific $\gamma\delta$ T cells subsets, namely Vδ1 T cells, display a natural tropism towards mucosal tissues, thereby being better equiped to infiltrate solid tumours when compared to their $\alpha\beta$ T cells counterpoints [112]. Thirdly, their MHC-unrestriction confers a low risk for alloreactivity development [112]. Fourthly $\gamma\delta$ T cells interact with antigen-presenting cells and other adaptive immune components promoting secondary immune responses [112].

1.9 Cancer clinical trials results based on activated $\gamma\delta$ T cells

Boosting Vδ2 T cell anti-tumour phenotype through systemic stimulation with aminobisphosphonates comprises the first approach regarding Vδ2-based immunotherapy [112]. As such, this framework has already been used in eight phase 1 clinical trials and ascertains the upregulation of endogenous phospoantigens by tumour cells. Despite the scarce efficacy - only 7% of patients showed tumour size reduction, an objective response -, aminobisphosphonates-based expansions of Vδ2 T cells, in combina-

Year	Disease	Treatment	n	OR	CR
2003	MM NHL	Pamidronate + IL-2	19	3/ 19	0/ 19
2003	Prostate cancer Breast cancer	Zoledronate	9	0/9	0/ 9
2007	Prostate cancer	Zoledronate vs zoledronate + IL-2	18	3/ 18	0/ 18
2010	Breast cancer	Zoledronate + IL-2	10	0/ 10	0/ 10
2010	RCC Colon cancer Esophagus cancer Gastric cancer Ovarian cancer Breast cancer	BrHPP + IL-2	28	0/ 28	0/ 28
2011	RCC	Zoledronate + IL-2	12	0/ 12	0/ 12
2012	RCC MM AML	Zoledronate + IL-2	21	2/ 21	0/ 21
2016	Neuroblastoma	Zoledronate + IL-2	4	0/4	0/ 4

Figure 1.2: Pilot/Phase 1 trials evaluating safety and clinical activity of in vivo activation of Vγ9Vδ2 T cells. MM, multiple myeloma; NHL, non-Hodgkin lymphoma; RCC, renal cell cancer; AML, acute myeloid leukemia. Adapted from [112].

tion with IL-2 as stimulation factor, gathered attention owing to the all-around successful IFNγ phenotype promoted in $\gamma\delta$ T cells, in a safe way [112].

Another alternative considers adoptive transfer of autologous Vδ2 T cells upon ex vivo expansion supported by aminobisphosphonates. 9 clinical trials arose in this field, being the ex vivo expansions performed either with BrHPP or zoledronate with IL-2 [112]. As a result of the well-supported infusions on patients, the clinical trials final assessment ensued 8% of objective and 2% of complete responses.

Allogeneic Vδ2 T cells have also been used in pilot studies [113]. A patient reported tumour volume decrease and fostered CD4 and CD8 functional cells levels, while dropping exhaustion levels of the same cells [113]. New yet-to-be-developed studies in the field will provide further insights into the reliability of allogeneic Vδ2 T cells [113].

Overall, despite their promise in clinical settings, Vδ2 T cells clinical performance may be hindered by undergoing activation-induced cell death (AICD) and being strongly prone to T cell exhaustion upon continuous stimulation, therefore entailing an impaired ability to perform cytotoxicity functions and secrete cytokines.

Harnessing Vδ1 T cells has been limited by researchers' ability of isolating this cell subset [114]. Isolation of Vδ1 T cells from PBMCs or through enzymatic digestion of tissues yields low numbers of this cell type, thus emphasizing the importance of developing expansion protocols specifically to this subset [112]. Considering that Vδ1 T cells are long lived and display natural tropism to organs that often contain tumours, these cells are an attractive subset for clinical studies testing safety and efficacy of both autologous or allogeneic adoptive cell transfer [115]. In a study testing autologous transfer of TILs in

Year	Disease	Treatment	n	OR	CR
2007	RCC	Vγ9Vδ2 T cells + zoledronate + IL-2	7	3/7	0/7
2008	RCC	Vγ9Vδ2 T cells + BrHPP + IL-2	10	0/10	0/10
2009	MM	Vγ9Vδ2 T cells + zoledronate + IL-2	6	0/6	0/6
2010	NSCLC	Vγ9Vδ2 T cells + zoledronate + IL-2	10	0/10	0/10
2011	RCC	Vγ9Vδ2 T cells + zoledronate + IL-2	11	1/11	1/11
2011	Melanoma Colon cancer Breast cancer Cervical cancer Ovarian cancer Gastrointestinal cancer	Vγ9Vδ2 T cells + zoledronate	18	3/12	1/12
2011	NSCLC	Vγ9Vδ2 T cells + zoledronate + IL-2	15	0/12	0/12
2013	Colon cancer	Vγ9Vδ2 T cells	6	0/6	0/6
2014	NSCLC	Vγ9Vδ2 T cells	15	0/12	0/12
2014	Gastric cancer	Vγ9Vδ2 T cells + zoledronate	7		

Figure 1.3: Pilot/phase 1 trials evaluating safety and clinical activity of adoptively transferred autologous ex vivo expandedVγ9Vδ2 T cells. BrHPP, bromohydrin pyrophosphate; CR, complete response; IL, interleukin; MM, multiple myeloma; NSCLC, non-small cell lung cancer; OR, objective response; RCC, renal cell cancer. Adapted from [112].

metastatic melanoma, the results were impressive with objective rates of 50% and complete responses of 20% [116, 117]. Vδ1 T cells were within the population of transfered TILs, which may suggest an in vivo anti-tumour role of this subset [116, 117]. However, since the cell products also contained other subtypes, effective conclusions about Vδ1 T cells cannot be drawn, even though in vitro assays of Vδ1 T cells against melanoma cells displayed high cytotoxic responses [112]

1.10 Therapeutic opportunities for Vδ1 T cells: all-around and specific colon cancer cases

Vδ1 T cells are highly present in human tissues as the intestine, colon, dermis, amongst others [118]. In some cases, Vδ1 T cells represent the predominant tumour-infiltrating sub type [119, 120], pinpointing and empowering the conclusions of the transcriptome analysis from 39 human tissues, wherein the presence of tumour-infiltrating $\gamma\delta$ T cells on tumour sites was identified as the most favorable prognostic factor [121].

In contrast with Vδ2 T cells, Vδ1 T cells are able to persist in circulation for many years, which can

be explained by their low susceptibility to AICD and exhaustion. On the basis of these considerations, we believe that Vδ1 T cells are a promising subset to be further evaluated for ACT in colon cancer.

Besides the interest in hematological malignancies [71, 122], Vδ1 T cell-mediated recognition and cytotoxicity against of colon tumours have been recently explored. In particular, Wu et al. designed an optimized protocol to expand Vδ1 T cells in vitro from peripheral blood through PHA and IL-7 [123]. Importantly, Vδ1 T cells showed higher efficiency in in vitro killing of colon cancer cells, when compared with Vδ2 T cells [123]. In mice, tumour growth reduction was observed upon administration of Vδ1 T cells, reinforcing their potential against human solid tumors such as colon cancer [123]. Lung and liver are well-known as the most frequent metastasis locations for colon cancers, mainly due to the definite circulation patterns [124, 125]. Through bioluminescence imaging, Devaud and colleagues endorsed the Vδ1 T cells already-known ability to infiltrate colon carcinomas, their efficiency in delaying tumour development and their resourcefulness to hamper the emergence of secondary tumor foci in the lungs and liver [125].

It is known that human gut epithelium is home for a large number of $\gamma\delta$ T cells, seemingly accounting for the majority of intraepithelial lymphocytes [126]. Apart from controlling antimicrobial defense, organ homeostasis and tissue damage repair, intraepithelial $\gamma\delta$ T cells' influence in physiology of cancer should be pinpointed [126]. Indeed, several facts should be properly emphasized i) gut-resident Vδ1 T cells IELs with high expression of NKp46 were assessed as the largest subset of $\gamma\delta$ T cells amongst intestinal IELs, ii) IL-2 and IL-15 endow Vδ1 T cells with specific gut tropism and iii) NKp46+ Vδ1 T cells are associated with a better prognosis in colon cancer [127].

1.11 Delta One T cells (DOT): why and how?

Besides preclinical studies with Vδ1 T cells in colon cancer, clinical-grade scalable expansion protocols are vital to develop safety and efficacy assessments in clinical trials, which ultimately will dictate the future of Vδ1 T cell ACT therapy. However, there are several limitations to achieve such effective clinical grade expansion - i) the high level of variation in the final ACT product, ii) the use of unsafe materials and reagents in the manufacturing process and iii) the low antitumour activity of the final ACT product - [72]. For example, previous protocols relied on the use of mitogenic plant lectins, which are materials that can not be used in clinic [115].

As a result of a thorough analysis of Vδ1 T cell stimulation, Correia et al. in 2011 identified a mean to selectively stimulate natural cytotoxicity expression of Vδ1 T cells upon culturing them with TCR agonists and cytokines [128]. Later on, the host laboratory also conceived a two-step method capable of unleashing the de novo expression of specific NCRs on Vδ1 T cells as well as expanding the number of Vδ1 T cells up to 2,500-fold, envisioning a way of prompting a clinically selective increase (65% enrichment) in the number of Vδ1+ cells originated from the peripheral blood of both healthy donors and cancer patients [72]. Importantly, Vδ1 T cells become the dominant cell subset present on this product (DOT cells) and are polarized towards a type 1 cytotoxic response translated into IFNγ production [72].

Characterization of DOT cell maturation phenotype was also performed. A high potential to migrate

and circulate from tissues to blood and vice versa was suggested due to the upregulation of multiple molecules related with cell adhesion (e.g., CD56, CD96, CD172a, CD31, and ICAM-1) and chemokine receptors (CXCR3, CCR6, and CX3CR1) [72]. Besides migration features, DOT cells significantly increased NK-associated activating/cytotoxicity receptors expression - such as NKp30, NKp44, NKG2D, DNAM-1, and 2B4 -, which, in addition to the exhibited enhanced expression of IL18Ra and Notch1, foster type 1 responses [72]. On the other hand, CD161 expression levels, which are usually correlated with IL17-producing cells decreased significantly after the 21 days of the protocol [72]. Moreover, low propensity for exhaustion already exhibited by Vδ1 T cells was reinforced in DOT cells, as suggested by the low expression levels of PD-1, CTLA-4, or CD94, even after 21 days of culture [72].

To assess DOT cell efficiency in tumour-targeting was assessed in vitro and in vivo assays were performed in models of chronic lymphocitic leukemia (CLL) [72]. DOT cells were highly cytotoxic against CLL cell line MEC-1 and against both allogeneic and autologous CLL primary samples [72]. Regarding molecular mechanisms of CLL cancer cell recognition by DOT cells, the biggest contribution was by NKp30 and NKp44 [72]. Using a xenograft model of human CLL the host laboratory found that: i) DOT cells inhibited tumor growth and prevented tumor dissemination, ii) 2 months after ACT, DOT TILs produced type I cytokines but not IL17, thereby keeping a functional type 1 response and iii) recovered DOT cells were 98% to 100% Vδ1 T cells, while the administrated product had 65% of Vδ1 T cells [72]. In fact, these results corroborate the theoretical propositions that DOT cells are widely resistant to exhaustion and that the administered Vδ1 T cells were able to thrive in vivo.

DOT cells were also studied against acute myeloid leukemia (AML) models [129]. Thus, DOT cell efficiently targeted AML lineages and primary samples [129]. The recognition of AML cells was performed through NKp30, suggesting once again the importance of natural cytotoxicity receptors in cancer cell recognition [129]. In vivo experiments indicated that DOT-cell treatment reduced tumour burden and increased host survival [129].

Finally, although DOT cells were firstly tested against CLL and AML, their potential to target solid tumours, specially colon cancer should be investigated, for all the reasons mentioned above.

1.12 Delta One T cells (DOT) and tumour micro environment (TME)

Despite the promising results of DOT cell ACT in models of CLL and AML, these studies were performed in immunodeficient mice, which compromises the study of cellular crosstalks between DOT cells and other immune cells present in the TME.

In fact, tumour cells orchestrate a series of interactions between different subsets of immune cells, signalling components, tissue-resident and tissue-non-resident cells, taking advantage of the inherent plasticity of several cell subsets to enhance chronic inflammatory yet immunosuppressive state [130, 131]. This state sets the stage for immune evasion and metastasis induction. However, as naturally expected from an inflammation situation, myeloid cell development and mobilization are enhanced in an attempt to hamper an inflammatory state that 'never ends' - similar to several autoimmune disease triggers [132].

Myeloid cells accumulate systemically and in tumours under context-dependent polarization conditions [133] and usually correlate with poor patient outcomes [134]. These cells are often modulated by cancer cells, which influence their polarization towards a pro-tumour subversion and T cell suppression functions.

Whilst tumour-associated macrophages (TAMs) are usually abundant within the tumour milieu - about 30% of TME cells -, their capacity to stimulate T cell function is usually hampered [134]. Antigen-presenting cells (APCs) are fundamental for cytotoxic CD8 T cells (CTLs) priming, leading the way to the generation of effective T cell responses [135]. In several studies, TAMs have been shown to induce tumour cell invasiveness and motility [135]. Classical dendritic cell (DCs) known as cDC1, on which the immune system rely on to effectively transport antigens to lymph nodes, have their IL-12 production impaired by TAM-derived IL-10 [135]. Moreover, TAM-derived CCL17 and CCL22 induce regulatory T (Treg) cell migration to tumour environment [136]. Treg cells are strong T-cell suppressors via Il-10 and TGF-β production [136]. Importantly, TAMs also upregulate inhibitory molecule expression such as PD-L1, which actively inhibit CTL action [137]. Treg cells naturally dampen antitumour immune responses. In addition to to-tumour TAM-induced Treg cells migration, TAM-independent migrations of Treg cells happen and correlate with worse outcomes, supported either by studies in preclinical mouse models and in patients [138]. Treg cell–APC interactions promote downregulation of the co-stimulatory molecules CD86 and CD80 on APCs, leading to decreased capacity of CTL stimulation [139]. Moreover, Treg cells also impact NK cell apoptosis [140] and induce CD8 T cell inhibition via IL-2 consumption [141].

Besides PD-L1, several other molecules characteristic of the tumour microenvironment are produced by a wide-variety of cells [142]. As stated, IL-10 is usually linked with a suppressive function. Thus, IL-10 contributes to inhibition of TH1 immune responses and T-cell cytotoxic activity [143]. IL-10 hampers the proliferation, cytokine production, and migratory capacities of effector T cells [143]. IL-10 also leads to downregulation of MHC-II and costimulatory molecules CD80, CD86 and IL-12 in DC [144]. Importantly, DC can produce IL-10 and create an autocrine cycle, thereby maintaining an immature state associated with pro-tumour functions [143]. Furthermore, IL-10 sustains the expression of Foxp3 and TGF-β receptor in Tregs, further contributing to tumour growth [145].

Other important molecule in TME is TGF-β. TGF-β released by cancer cells, stromal fibroblasts, among others prompts cancer progression by shaping the architecture of the tumour as well as suppressing the antitumour activities of immune cells, thereby generating an immunosuppressive environment that attenuates the efficacy of anticancer immunotherapies. Importantly, TGF-β represses NKG2D expression in NK and CD8 T cells [146], an important factor for DOT cell anti-tumour function. TGF-β enhances DC immunosuppressive activity, represses antigen presentation, hampers reactive oxygen species and nitric oxide production by macrophages and suppresses DC cell migration [147, 148]. TGF-β affects CD8 T cell proliferation as well as effector function, by impairing Perforin, Granzyme B and Fas Ligand expression [149, 150]. Effects on CD4 T cells are similarly negative [151]. Considering the immunossupressive features of TGF-β it is of outmost importance to study the effect of this molecule on DOT cell performance.

Tumour development leads to release of neutrophils from bone marrow due to the increase pro-

duction of G-CSF [133, 152, 153]. Importantly, the release pressure is so high that immature myeloid cells are also released [153]. Importantly, systemically released neutrophils are polarized towards an immunossupressive phenotype - mainly due to TGF-β exposure -, and migrate into tumours [154]. Interestingly, an enlightening study where 18000 human tumours across 39 different malignacies were analysed identified tumour-infiltrating neutrophils (TANs) as the most negative prognostic immune cell population [121].

TGF-β-dependent N2-polarized TANs [154] are characterized by production of nitric oxide (NO), reactive oxygen species (ROS) and arginase 1,2 (ARG1,2) according to transcriptomic analysis [121, 152, 154]. TAN-derived ROS have been correlated with DNA damage and genetic instability in epithelial cells [155]. Besides genetic instability, ROS favors T cell supression [133], also promoted via inducible NO synthase (iNOS) [152]. In addition, production of pro-angiogenic factors (such as MMP9, BV8, and VEGFA) by neutrophils fosters tumour angiogenesis [156]. Research studies identified MMP9 as VEGFA activator and BV8 as endothelial cells mobilizer, thereby supporting the initial angiogenic switch [156]. The expression of arginase 1 by TANs hamper T cell responses by decreasing L-arginine levels in the TME, which is essential for T cell proliferation [157]. Collectively, these studies support a role for neutrophils as immunosuppressive thus pinpointing the importance of studying possible interactions between neutrophils and DOT cells.

Various tumour types, including colon cancer , generate a suppressive environment, which constitutes a big hurdle to achieve efficient immunotherapy therapies. How these immunosuppressive factors impact DOT cell functions remains to be fully elucidated and is a subject investigated in this thesis.

1.13 Motivation and Thesis Aims

Delta One T (DOT) cells established a new cellular product with great promise for cancer immunotherapy. DOT cells are generated by an in vitro two-step protocol that expands the rare population of peripheral blood Vδ1 T cells up to 5,000-fold and differentiates them into potent cytotoxic effectors. DOT cells are characterized by the expression of natural cytotoxicity receptors, such as NKp30 and NKp40, that synergize with the T cell receptor to mediate tumour-specific cell-targeting, while sparing healthy human cell types [129, 72]. DOT cell treatment in vivo led to reduced tumour burden and dissemination in two xenograft models of hematological cancers, chronic lymphocytic leukaemia [72] and acute myeloid leukaemia [129]. However, to date, the therapeutic potential of this cellular product has not been tested in solid tumours. In contrast to, Vδ1 T cells, the major component of DOT cells, account for around 20% of peripheral blood $\gamma\delta$ T cells, but representing the largest fraction (around 50%) of $\gamma\delta$ T cells in intestinal intraepithelial lymphocytes of human healthy colon [127]. Moreover, Vδ1 IELs expressing specific NCRs exhibit an increased anti-tumour potential upon contact with tumour cells [127]. Furthermore, Vδ1 T cells expressing anti-tumour molecules were associated with increased disease-free survival in colon cancer patients [120]. Thus, we question whether DOT cell adoptive cell transfer would be a promising treatment for colon cancer.

The three main objectives of this project are to:

1. Test the efficacy and study the mechanisms of colon cancer recognition by DOT cells, *in vitro*.
2. Understand how the secretome of colon cancer cell lines impact DOT cell function.
3. Dissect mechanisms of DOT cell regulation by factors of the tumour microenvironment.

Chapter 2

Discussion

The work developed in this thesis supports the idea that DOT cells can potentially be re-purposed to target solid tumours, specifically colon cancer. Here we used two colon cancer cell lines, HCT116 and SW620, whose molecular features are similar to those of consensus molecular subtype 4 (CMS4), a subgroup of colon cancers [26] representative of 23% of colon cancer cases [29]. This subgroup of CRC is characterized by displaying the worse relapse-free and overall survival and low hypermutation rate. Our findings showed that DOT cells readily killed HCT116 and SW620 colon cancer cell lines. These results are consistent with the ones from Wu et al. reporting HCT116-targeting by Vδ1 T cells [123], despite the different protocol used to expand the cells. Although HCT116 and SW620 belong to the same CMS, these two cell lines display some differences. Whereas SW620 has a metastatic origin, HCT116 is originated from a primary site [27]. Besides, HCT116 is microsatellite instable (MSI) and CpG island methylator phenotype (CIMP) positive while SW620 is microsatellite stable (MSS) and CIMP negative. Importantly, by killing both HCT116 and SW620 cell lines, DOT cells show increased clinical relevance as it might suggest that DOT cells could target metastatic or primary tumours, with or without aberrations at the DNA level.

Although DOT cells targeted both cell lines, SW620 cells were more efficiently killed than HCT116 cells. This could be due to differential i) levels of tumour cell recognition by DOT cells, ii) susceptibility to killing mechanisms or iii) colon cancer cell line intrinsic features that may increase their survival. Regarding i) recognition of cancer cells, it is possible that DOT cells recognize HCT116 less efficiently than SW620 cells, therefore leading to a less efficient killing. DOT cells recognize SW620 cells through NKG2D and DNAM-1, but in this study we were unable to identify any contribution of NK-receptors (NKR) in HCT116 recognition. One possibility is that DOT cells recognize HCT116 via the T cell receptor (TCR), which would mean that tumour cell recognition through the TCR is less efficient than NKG2D and DNAM-1. Conversely, assuming an equal level of recognition of colon cancer cells by DOT cells, the difference might occur in ii) tumour susceptibility to killing mechanisms. It is possible to assume that DOT cells employ killing mechanisms to which SW620 cells are more sensitive than HCT116 - for example TRAIL or Fas ligand [123], already proved to be upregulated in expanded V1 T cells by Wu et al [123]. Thus, it would be interesting in the future to address the level of colon cancer cells susceptibility to different

killing mechanisms.

Moreover, iii) intrinsic cell line features may impact their ability to survive. HCT116 cells display increased PI3K and RAS signaling pathway compared to SW620 [26], ultimately inhibiting apoptosis at a higher extent. Therefore, this may turn HCT116 more resistant to DOT cell-induced apoptosis. Moreover, TGF-β activation is significantly increased in HCT116 in comparison with SW620 [26], which, adding to our findings showing that DOT cell is negatively modulated by TGF-β, could also be contributing to targeting efficiency differences. However, since we also observed that HCT116 supernatant induces DOT cell proliferation and cytotoxic potential it is unlikely that a soluble factor produced by these tumour cells, such as TGF-β, is responsible for the differences in HCT116- and SW620-targeting by DOT cells. Considering other cell lines from all consensus molecular subtypes (CMS) of colon cancer groups is also important to empower future conclusions about DOT cell targeting of colon cancer.

Recently, several problems were uncovered in ACT of CAR T cells, mainly due to off-target events. Hence, knowing DOT cell recognition mechanisms of cancer cells is important to increase the knowledge about DOT cell regulation, therefore engineering control mechanisms and to select patients for ACT therapy whose tumours could be recognized by DOT cells. As aforementioned, we identified an important role for NKG2D in SW620 cell recognition by DOT cells. NKG2D provides co-stimulatory signals in activated T cells and is expressed by most CD8 and a small subset of CD4 cytotoxic T cells, as well as in iNKT cells and $\gamma\delta$ T cells. Moreover, we found that even with NKG2D and DNAM-1 blockade, the apoptotic amount of SW620 cells did not drop dramatically, suggesting that other mechanisms should be also involved in tumour cell recognition. Thus, DOT cells are empowered since they do not show a high dependency on one specific mechanism. Regarding HCT116 cell line, the host laboratory has shown that this tumour line displays higher mRNA and protein levels of some NKR ligands (data not shown), which is somehow paradoxical with the lack of role for NKR in DOT cell recognition. It is also known that HCT116 cell line displays higher levels of acquired somatic mutations than SW620 [26], which would be in line with an increased potential relevance of TCR-mediated recognition of HCT116 by DOT cells. However, HCT116 killing is less efficient than SW620 killing which may suggest that even though HCT116 demonstrates a higher level of somatic acquired changes compared SW620 [26], this level may not be sufficient to achieve the threshold required for very efficient TCR-mediated DOT cell recognition and thus, killing.

In this study, we also demonstrated that HCT116 secretome promotes DOT cell proliferation and enhances their cytotoxic potential, which may also seem paradoxical given the lower ability of DOT cells to kill this cell line. However, it is possible that although DOT cells become more activated in the presence of HCT116 secretome, their ability to recognize HCT116 as target cells and form immunological synapse to deliver cytotoxic molecules might be hampered. Moreover, as discussed above, it may also be that the tumour cells themselves are more resistant to DOT cell killing mechanisms. HCT116 secretome led to increased levels of the activation marker CD69 and NKG2D in DOT cells, however, since co-incubation of DOT cells with this cell line did not translate into a higher killing (compared to SW620 and HEL cell lines), it reinforces the idea that NKG2D is not a relevant molecule in HCT116 recognition by DOT cells. Importantly, the increased levels of IFN-γ, TNF-α and proliferation of DOT cells upon incubation with

HCT116 secretome might be event more relevant in an in vivo setting, as it may unleash an indirect anti-tumour response. This indirect effect may be trough influence in other cells such as: induction of IFN-γ production of $\alpha\beta$ T cells, thereby potentiation $\alpha\beta$ T cells cytotoxicity or also induction of MHC-I expression on tumour cells, which could enhance their potential to be recognized by other cytotoxic T cells.

One limitation of the simplistic in vitro assay we have performed is precisely the inability to assess the indirect influence in other immune cells, and also the limiting time of the assay may not be sufficient to allow MHC-I modulation and therefore observe the possible impact in ab T cell-mediated tumour targeting. Interestingly, the fact that HCT116 and SW620 belong to CMS4 group, characterized by some immune infiltration is consistent with the fact that we observe increased migration of DOT cells towards the secretomes of these two cell lines [29]. This effect of CRC secretomes on DOT cell migration is an exciting observation as it may suggest that DOT cell effectiveness in ACT may be promoted, as ACT requires migration of transferred cells to the tumour microenvironment upon intravenous delivery.

Another important aspect to consider for DOT cell ACT is the crosstalks that may occur between these cells and other immune subsets . Human neutrophils interact with innate immune cells, such as NK cells, as well as with adaptive immune cells, such as T cells [158]. Our findings support a role for neutrophils in modulation of DOT cell functionality. Previous studies indicate that neutrophils inhibit in vitro activation of human T cells, more specifically by decreasing levels of CD25 and CD69 expression, the production of IFN-γ and proliferation levels [159]. In fact, we also observe a decrease in the levels of CD69, IFN-γ and proliferation of DOT cells, but also demonstrated the same pattern the levels of recognition molecules (NKG2D and DNAM-1) and cytotoxic molecules (granzyme B and perforin). However, the use of neutrophils isolated from the peripheral blood can be a limitation of this assay. Ultimately, to really dissect mechanisms of DOT cell regulation by factors, such as neutrophils, of the tumour microenvironment it would be more relevant to use neutrophils isolated from colon cancer biopsies or blood of colon cancer patients, or even neutrophils differentiated into a N2 phenotype, as this is the most prevalent in the tumour microenvironment [160]. Since neutrophils clearly lead to the downregulation of NKG2D expression by DOT cells it is possible that neutrophils negatively impact DOT cell ability to recognize SW620. Interestingly, neutrophils lead to an increase in CD107a expression, suggesting that DOT cell degranulation may be positively impacted by neutrophils. However, the levels of granzyme B (not statistically) and perforin decreased in the presence of neutrophils, thus it is unclear if DOT cell cytotoxic potential would be affected. To test this, it would be necessary to perform a killing assay of tumour cell lines with DOT cells that had been pre-incubated with neutrophils.

Notably, it would be interesting to ascertain the influence of neutrophils in the killing capacity of non-NCR recognized HCT116, to reinforce this point (or dismiss it).

Although it important to study the impact of neutrophils on DOT cells comparisons of the level of neutrophil inhibition with other immune subsets is more informative. Therefore, we have compared the effect of neutrophils on CD8 T cells, NK cells and DOT cells, as these are all cytotoxic cells. We have observed that whereas CD8 T cells increased the expression of CD107a, IFN-γ and TNF-α, upon contact with neutrophils, both NK cells and DOT cells exhibited lower levels of these molecules, with NK

cells exhibiting a more marked inhibition than DOT cells. It was previously reported that neutrophils enhance CD8 T cell activation, proliferation, and cytokine release [161]. Notably, neutrophils in this report encompass neutrophils from healthy donor and colon cancer patient peripheral blood (PB) as well as tumour-associated neutrophils (TANs), all of them promoting an upregulation in CD8 T cells effector function. The fact that the same pattern of interaction between CD8 T cells and neutrophils is observed either from experiments performed with PB or TANs can indirectly also allow us to postulate some similarity between DOT cell modulation by neutrophils from peripheral blood or from TANs. Although this point might slightly overcome the limitation of our essay, it does not constitute a clear evidence of the relationship between DOT cells and neutrophils from different origins and further experiments should be performed. Regarding NK cells several studies highlight the suppression of their survival, cytotoxicity and proliferation by neutrophils-derived molecules or mechanisms, thus corroborating our findings. The mechanisms of NK cell inhibition by neutrophils have been shown to involve neutrophil-derived microparticles, which modulate IFN-γ and TNF-α expression on NK cells. It is possible that neutrophils microparticles are also negatively modulating DOT cells, however other well-known mechanisms involved in gd T cell inhibition by neutrophils [162, 163], such as production of reactive oxygen species should be investigated. Of note, the duration of our co-incubation of lymphocyte subsets and neutrophils was 3 days, while the expected life time of neutrophils is 8 hours, which may suggest that the effect observed might be mediated by neutrophil death and consequent release of neutrophil extracellular traps (NETs). If that proves to be true it may suggest that DOT cells and NK cells are more susceptible than CD8 T cells to neutrophil-released molecules upon death, such as NETs.

Besides studying the impact of neutrophils on DOT cells we also investigated the role of two immunosuppressive cytokines, IL-10 and TGF-b, on DOT cell phenotype and killing ability. We observed that, in general, DOT cell functionality was not impaired by IL-10, as the only significant effect observed was a reduction of IFN-γ and TNF-α upon culture with high levels of this cytokine. Conversely, although preliminary and lacking statistical significance, we observed that this same incubation with high levels of IL-10 led to a tendency of increased SW620-killing. Interestingly, previous studies investigating the effect of IL-10 on NK cells showed a similar pattern., Incubation with IL-10 did not affect proliferation levels or gene expression of IFN-γ, TNF-α and Granzyme B in NK cells, however, these cells displayed higher cytotoxicity levels in a cytotoxicity assay upon incubation with IL-10 [164]. This ultimately suggests that IL-10 may promote other mechanisms of tumour cell-killing by both NK and DOT cell, thus prompting us to broaden the study of mechanisms of tumour cell-killing by DOT cells. It is particularly important to mention that although before DOT cell expansion, Vδ1 T cells exhibit low percentages of IL-10 receptor positivity, upon expansion this percentage increased, thus suggesting that DOT cells are equipped with the receptor to directly respond to this cytokine and strengthening the idea that the lack of inhibitory effects of this cytokine on DOT cells is not due to the absence of machinery to respond to this molecule.

Besides IL-10 we also studied the impact of TGF-β. Our findings suggest an impact of TGF-β on DOT cell proliferation, cytokine and cytotoxic molecule production, however there was no impact observed in the expression of activation markers. Importantly, it seems (although preliminary and lacking statistical significance) that this culminates with a decreased ability of DOT cells to kill SW620 tumour cells. These

findings agree with the previously reported decrease in IFN-γ production by NK cells, but it contrasts with the fact that the observed decrease in NKG2D levels in NK cells upon culture with TGF-β [165] was not detected with DOT cells. Moreover, our findings are also aligned with the general idea that TGF-β suppresses CD8 T cell effector function through inhibition of perforin, granzyme b and IFN-γ [166]. We do not have data on expression of TGF-β receptors on DOT cells, but since we observe an effect on their phenotype it is very likely that DOT cells express these receptors. However, two other cell-surface proteins can interact with TGF-β: CD105 and CD109. Previous studies demonstrated that CD105 is expressed in the cell surface upon T cell activation and antagonizes the suppressive capacity of TGF-β [167]. CD109, a component of TGF-β ligand co-receptor complex, is also responsible for the attenuation of TGF-β signaling, through promotion of internalization of TGF-β receptor II [168]. Although these molecules are expressed, to some extent, by DOT cells, TGF-β still impacts DOT cell function. This may be explained by i) insufficient expression levels of these molecules, to antagonize TGF-β, ii) high susceptibility of DOT cells to TGF-β, independently of CD105 and CD109 expression or iii) these levels of CD105 and CD109 are sufficient to antagonize TGF-β action on the expression of activation markers, but not to a level that impedes the impact on proliferation and expression of cytotoxic markers. This third hypothesis would mean that what we observe upon culture of DOT cells with TGF-β is an intermediate level of inhibition, due to the incomplete protection provided by the expression of CD105 and CD109. Previous studies have reported TGF-β-mediated increase of CD103 levels, an integrin important for direct contact with epithelia; and decrease of KLF2 levels, a transcription factor that favors the egress from secondary lymphoid organ. Thus, TGF-β may potentially play opposing roles in the migration of immune cells to tumour sites. Interestingly, culture of Vδ2 T cells and CD8 T cells with TGF-β led to increased expression of CD103 and interaction of this molecule with E-cadherin on tumour cells, which ultimately contributed to higher levels of tumour cell-killing [169]. Thus, it is also important to understand the effects of TGF-β on the migration of DOT cells. Taken together, these findings suggest that TGF-β acts as a regulator of DOT cell activity, which is important not only because it may consist a “safety switch” for DOT cell therapy but also because it provides the rationale to combine DOT cell ACT with TGF-β blockade, thus providing important insights for future combination strategies.

Chapter 3

Conclusions and Future Work

In this thesis we provided new insights on the potential application of DOT cells in adoptive cell therapy of colon cancer. We evaluated the efficacy of DOT cells to kill two colon cancer cell lines, in vitro. However, the limitations of this assay encompass i) incubation time, which was limited to 3 hours and ii) proportion of effector:target cells used, which was 5:1. Other incubation times and effector:target ration should be tested to better understand if the killing capacity observed is close to the maximum, or is sub optimal. Moreover, in order to broaden the knowledge of colon cancer-targeting by DOT cells, it would be useful to consider colon cancer cell lines from others CMS groups: CMS1 (e.g. Co115, HCC2998), CMS2 (e.g. EB, LS1034), CMS3 (e.g. CL-34, CL-40). Importantly, even within each CMS group, studying different cell lines based on their CIMP/MSI status is key to empower future conclusions. Ultimately, studies with organoids, primary samples and in vivo models are essential to proceed in the preclinical evaluation of DOT cell efficacy.

Here we identified two NKRs important for SW620 cell recognition by DOT cells. Since antibody-based assays display some caveats, such as uncertainty of the extent of blockade/activation, the contribution of these NKRS should also be confirmed with CRISPR-Cas9-mediated gene-editing tools. Moreover, it is crucial to understand if the pattern of primary sample recognition by DOT cells is similar or not. Identification of a general pattern of colon cancer recognition by DOT cells may help to select patients for DOT cell ACT.

We also found that HCT116 secretome promotes DOT cell proliferation and enhances their cytotoxic potential. Pinpointing the molecule(s) responsible for the increase in DOT cell functionality is central, as it may lead to improvements to the current expansion protocol or provide candidates for future adjuvant therapy to achieve increased effectiveness of DOT cell ACT.

This study also uncovered potential negative regulators of DOT cells that may be present in the tumour microenvironment. We found that neutrophils inhibit DOT cell function, which has important implications, not only for the success of the expansion protocol – since it highlights that the starting PBMC population should be devoid of neutrophils - but also for the future efficacy of DOT cell ACT. Dissecting the mechanisms employed by neutrophils to hamper DOT cell activity might provide useful information for combination strategies aiming at increasing DOT cell efficiency in neutrophil-rich tumours. More-

over, testing this inhibition with tumour-associated neutrophils, isolated from tumour biopsies, although technically challenging, would empower our conclusions.

Our results suggest that DOT cells are suppressed by TGF-β but not IL-10, which for one hand identifies TGF-β as a potential "safety-switch", but on the other hand highlights that DOT cells are not easily suppressed, which may contribute to an increased efficacy in vivo. In the future it would be relevant to test DOT cell function in the presence of a broad panel of potential immunossupressive molecules, present in the tumour microenvironment.

Although immunotherapy has dramatically changed the landscape of treatment for many advanced cancers, the benefit in colon cancer has thus far been limited to patients with MSI and CIMP positive tumors [170]. SW620 is a MSS and CIMP negative cell line that was very efficiently targeted by DOT. If such efficiency is confirmed with other similar cell lines and tumour samples, this may pave the way for the use of DOT cells for the treatment of MSS and CIMP negative colon cancers, which affect the vast majority of patients.

Taken together, our work provides interesting evidence that DOT cells might be a therapeutic option for colon cancer, and lays the foundation for further preclinical studies of DOT cells for the treatment of this type of cancer. Studying DOT cell functionality and targeting of other solid tumours (e.g. pancreatic, prostate, liver) in several in vitro and in vivo models, will be the next big challenge in the field.

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