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

Pedro Afonso Galrão Batalha

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 anti-tumour biology of γδ T-cells along with expansion protocols might be considered in new immunotherapy approaches. One of γδ T-cell-based alternatives are 'Delta One T' (DOT) cells, an immunotherapeutic product consisting in more than 65% Vδ1+ γδ 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 favored 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.

Ageing has 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 [1]. 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 [2]. Unique contributions by prevention and surgery technical development contributed for 90% success in highly localized colon cancer cases [2]. Strikingly, 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) [3]. Thus, the development of more effective treatment options for patients diagnosed with CC is an urgent unmet need.

Long-term durable responses in previously difficult-to-treat solid tumours, such as lung cancer and melanoma [4], has kindled tremendous excitement in immunotherapy. These important contributions of new therapies have fueled scientific interest to explore immunotherapy potential for the majority of colon cancers which are non-hypermutated (more than 80% of the cases) [5]. In fact, immunotherapy has already demonstrated that is capable of generating positive responses in several hypermutated CC cases, mainly through the treatment with immune checkpoint inhibitors [3].

γδ T cells have co-evolved alongside αβ T cells and B cells for 450 million years of vertebrate evolution [6] and are increasingly recognized as having important roles in immune responses to both microbial and non-microbial stress challenges [7]. Although γδ T cell relative proportion in circulation is low, their abundance in mucosal sites is higher, which makes them an important tissue surveillance system. Secondly, by sensing endogenous stress induced-ligands, these cells can monitor other cells for abnormal changes, establishing a 'lymphoid stress-surveillance response' [8]. Their major histocompatibility complex (MHC)-unrestriction also confers a low risk for alloreactivity development. Thirdly, specific γδ T cells subsets, namely Vδ1 T cells, display a natural tropism towards mucosal tissues, thereby being better equipped to infiltrate solid tumours when compared to their αβ T cells counterparts [9]. Fourthly, γδ T cells display on their surface natural killer receptors, allowing them to respond to transformed cells [10].

The lack of suitable expansion/differentiation methods has precluded therapeutic application of Vδ1 subset of γδ T cells. However, the protocol from Almeida et al. allowed to overcome these problems and to generate a Vδ1+ T-cell-enriched adoptive cell therapy product – DOT cells – yielding high numbers of cells with increased expression of NKRs [11]. In fact, DOT cells already exhibited high cytotoxicity against *in vitro* and *in vivo* models of chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML), as well as AML primary samples [11,12].

We hypothesized that DOT cells, considering their anti-tumour functions and natural tropism to epithelial tissues, could be re-purposed to target colon cancer. We started by evaluating DOT cell cytotoxicity against colon cancer models HCT116 and SW620 and study the mechanisms of colon cancer recognition by DOT cells. Then, in order to clarify the interactions between DOT cells and colon cancer cells, we explored how the secretome of colon cancer cell lines impacted DOT cell function. Finally, being colon cancer a solid tumour, which encompasses a tumour microenvironment, we dissected the mechanisms of DOT cell regulation by factors present in the tumour microenvironment, such as the immunosuppressive molecules IL-10, TGF-β and neutrophils.

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 [19] representative of 23% of colon cancer cases [20]. 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 [21], 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 [5]. 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 NCRs 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 [21], already proved to be upregulated in expanded Vδ1 T cells by Wu et al [21]. 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 [19], 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 [19], 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 NKT cells and γδ 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 [19], 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 [19], 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 αβ T cells, thereby potentiation αβ T cell 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 αβ 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. This effect of CC 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 crosstalk 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 [17]. Our findings support a role for neutrophils in modulation of DOT cell functionality. Previous studies indicate that neutrophils inhibit *in vitro* activation of human γδ T cells, more specifically by decreasing levels of CD25 and CD69 expression, the production of IFN-γ and proliferation levels [22]. 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 in 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 [23].

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, 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 [17]. 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 γδ T cells cell inhibition by neutrophils [22, 24] 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-β, 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 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 [25]. 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.

Beyond 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 [26] was not detected with DOT cells. Moreover, our findings are also aligned with the general idea that TGF-β suppresses other cytotoxic cells like CD8 T cell effector function through inhibition of perforin, granzyme b and IFN-γ [27].

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 [28]. 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 [29]. 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 [30]. 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.

References

[1] Kennedy, Brian K., et al. "Geroscience: linking aging to chronic disease." Cell 159.4 (2014): 709-713.
[2] Miller, Kimberly D., et al. "Cancer statistics for hispanics/latinos, 2018." CA: A Cancer Journal for Clinicians 68.6 (2018): 425-445.
[3] Franke, Aaron J., et al. "Immunotherapy for colorectal cancer: a review of current and novel therapeutic approaches." JNCI: Journal of the National Cancer Institute 111.11 (2019): 1131-1141.
[4] Zugazagoitia, Jon, et al. "Current challenges in cancer treatment." Clinical therapeutics 38.7 (2016): 1551-1566.
[5] Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337 (2012).
[6] Hayday, Adrian C. "γδ cells: a right time and a right place for a conserved third way of protection." Annual review of immunology 18.1 (2000): 975-1026.
[7] Bonneville, Marc, Rebecca L. O'brien, and Willi K. Born. "γδ T cell effector functions: a blend of innate programming and acquired plasticity." *Nature Reviews Immunology* 10.7 (2010): 467-478.
[8] Chien, Yueh-hsiu, Christina Meyer, and Marc Bonneville. "γδ T cells: first line of defense and beyond." *Annual review of immunology* 32 (2014): 121-155.
[9] Nussbaumer, Oliver, and Michael Koslowski. "The emerging role of γδ T cells in cancer immunotherapy." *Immuno-Oncology Technology* 1 (2019): 3-10.
[10] Ribeiro, Sérgio T., Julie C. Ribot, and Bruno Silva-Santos. "Five layers of receptor signalling in γδ T cell differentiation and activation." *Frontiers in immunology* 6 (2015): 15.

[11] Almeida, Afonso R., et al. "Delta one T cells for immunotherapy of chronic lymphocytic leukemia: clinical-grade expansion/differentiation and preclinical proof of concept." *Clinical Cancer Research* 22.23 (2016): 5795-5804.
[12] Di Lorenzo, Biagio, et al. "Broad cytotoxic targeting of acute myeloid leukemia by polyclonal delta one T cells." *Cancer immunology research* 7.4 (2019): 552-558.
[13] Todaro, Matilde, et al. "Efficient killing of human colon cancer stem cells by γδ T lymphocytes." *The Journal of Immunology* 182.11 (2009): 7287-7296.
[14] Simões, André E., Biagio Di Lorenzo, and Bruno Silva-Santos. "Molecular determinants of target cell recognition by human γδ T cells." *Frontiers in Immunology* 9 (2018): 929.
[15] Gentles, Andrew J., et al. "The prognostic landscape of genes and infiltrating immune cells across human cancers." *Nature medicine* 21.8 (2015): 938-945.
[16] Pizzolato, Gabriele, et al. "Single-cell RNA sequencing unveils the shared and the distinct cytotoxic hallmarks of human TCRVδ1 and TCRVδ2 γδ T lymphocytes." *Proceedings of the National Academy of Sciences* 116.24 (2019): 11906-11915.
[17] Governa, Valeria, et al. "The interplay between neutrophils and CD8+ T cells improves survival in human colorectal cancer." *Clinical Cancer Research* 23.14 (2017): 3847-3858.
[18] Qiao, Jian, and Yang-Xin Fu. "Cytokines that target immune killer cells against tumors." *Cellular & Molecular Immunology* (2020): 1-6.
[19] Berg, Kaja CG, et al. "Multi-omics of 34 colorectal cancer cell lines-a resource for biomedical studies." *Molecular cancer* 16.1 (2017): 1-16.
[20] Guinney, Justin, et al. "The consensus molecular subtypes of colorectal cancer." *Nature medicine* 21.11 (2015): 1350-1356.
[21] Wu, Dang, et al. "Ex vivo expanded human circulating Vδ1 γδT cells exhibit favorable therapeutic potential for colon cancer." *Oncoimmunology* 4.3 (2015): e992749.
[22] Fazio, J., et al. "Inhibition of human γδ T cell proliferation and effector functions by neutrophil serine proteases." *Scandinavian journal of immunology* 80.6 (2014): 381-389.
[23] Heeb, Lukas EM, et al. "Regulation of neutrophils in type 2 immune responses." *Current Opinion in Immunology* 54 (2018): 115-122.
[24] Towstyka, Nadia Yasmín, et al. "Modulation of γδ T-cell activation by neutrophil elastase." *Immunology* 153.2 (2018): 225-237.
[25] Mocellin, S., et al. "IL-10 stimulatory effects on human NK cells explored by gene profile analysis." *Genes & Immunity* 5.8 (2004): 621-630.
[26] Regis, Stefano, et al. "NK cell function regulation by TGF-β-induced epigenetic mechanisms." *Frontiers in Immunology* 11 (2020).
[27] Dahmani, Amina, and Jean-Sébastien Delisle. "TGF-β in T cell biology: implications for cancer immunotherapy." *Cancers* 10.6 (2018): 194.
[28] Schmidt-Weber, Carsten B., et al. "TGF-β signaling of human T cells is modulated by the ancillary TGF-β receptor endoglin." *International immunology* 17.7 (2005): 921-930.
[29] Bizet, Albane A., et al. "The TGF-β co-receptor, CD109, promotes internalization and degradation of TGF-β receptors." *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1813.5 (2011): 742-753.
[30] Peters, Christian, et al. "TGF-β enhances the cytotoxic activity of Vδ2 T cells." *Oncoimmunology* 8.1 (2019): e1522471.