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Effector T cells co-expressing PD1 and CD39 are enriched in colorectal tumors: implications for cancer immunotherapy

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Acronyms and abbreviations

ACT	adoptive cell therapy
ADK	adenocarcinoma
ADK-MID	medullary adenocarcinoma
ADK-MUC	mucinous adenocarcinoma
APC	antigen presenting cell
bh-SNE	barnes-hut stochastic neighbor embedding
CRC	colorectal cancer
DC	dendritic cell
HD	healthy donor
iBc	immortalized B cells
IRs	inhibitory receptors
mCRC	metastatic colorectal cancer
MID	medullary
MP	muscolaris propria
MSI	microsatellite instability
MSS	microsatellite stability
MUC	mucinous
PBMCs	peripheral blood mononuclear cells
PCA	principal component analysis
pCRC	primary colorectal cancer
sgRNA	single guide RNA
SIER	serosa
SOM	self-organizing map
SM	submucosa
SS	subserosa
TCR	T cell receptor
TILs	tumor infiltrating lymphocytes
TME	tumor microenvironment

ABSTRACT

Effector T cells co-expressing PD1 and CD39 are enriched in colorectal tumors: implications for cancer immunotherapy

Colorectal cancer (CRC) is the 2nd cause of cancer-related death. Despite standard therapies, more than 50% of patients experience relapse, eventually with metastatic disease. The CRC microenvironment is densely infiltrated by T cells, which have a role in immune surveillance and modulation of tumor progression, and their presence correlates with improved overall survival. To counteract immune exhaustion, inhibitory receptors (IRs) blockade has been exploited, but efficacy was limited to a small group of CRC patients characterized by high mutational burden. Adoptive T cell therapy (ACT) with genetically engineered T cells could represent an innovative strategy to harness T cell function and specificity. Initially relying on isolation, *ex vivo* expansion and re-infusion of tumor-infiltrating T cells (TILs), the development of ACT cellular products has now been prompted by the newest gene transfer and genome editing techniques. However, the widening of ACT with genetically engineered T cells is still limited from by paucity of anti-tumoral T cell receptors (TCRs) and by the need to counteract the immune-suppressive tumor microenvironment (TME).

This work aims at setting the basis for the development of effective T cell products for the ACT of CRC, endowed with the

capacity to specifically recognize cancer cells and counteract the immune-suppressive CRC TME.

We employed high-dimensional flow cytometry coupled with an advanced pipeline of data handling by dimensionality reduction and clustering algorithms to describe the phenotype and the exhaustion features of TILs retrieved from the healthy, peritumoral and neoplastic tissue of treatment-naïve primary CRC patients and from the peritumoral and tumoral tissue of CRC patients undergoing surgery for liver metastasis. Unsupervised analyses highlighted the co-expression of multiple IRs and activations markers in T cells within the tumors. Populations of TILs described by a peculiar IRs signature were enriched both in primary CRC and liver metastasis. Of note, the signatures retrieved from primary and metastatic CRC overlapped for the upregulation of PD1 and CD39 thus underlining these molecules as relevant targets for T cells engineering. With the aim of exploiting this exhaustion signature to retrieve new anti-tumor specificities, we performed TCRαβ sequencing on $PD1^{+}CD39^{+}$ T cells isolated from primary CRC samples, obtaining different results from MSS and MSI tumors, where the repertoire was more oligoclonal. Of note, a small but consistent subpopulation of $PD1^{+}CD39^{+}$ T cells was also enriched in the peripheral blood of CRC patients compared to healthy donors (HDs), suggesting that exhausted tumor-specific T cells might circulate although at low frequencies. This signature could thus be used to isolate CRC-specific T cells and TCRs. As an alternative approach, we exploited a strategy to isolate CRC-

specific T cells starting from HD peripheral blood mononuclear cells (PBMCs). We repetitively stimulated HD PBMCs with autologous antigen-presenting cells loaded with a pool of peptides selected to be immunogenic and expressed by CRC. We efficiently expanded T cells specific for tumor-associated antigens and neoantigen epitopes and we sequenced their TCR. To set up a T cell genetic modification pipeline, we employed a published MUC-1 TCR. We used CRISPR/Cas9 to render T cells completely devoid of the endogenous TCR, and we redirected T cells specificity by lentiviral transduction, obtaining MUC-1 specific T cells functionally able to kill target cells. Moreover, we set up the efficient disruption of PD1 and CD39 by CRISPR/Cas9 with the aim of rendering T cells selectively resistant to CRC TME. Overall, by coupling these findings we aim at generating a library of T cell products able to specifically recognize tumor antigens and to counteract the immune-suppressive TME, to be tested in adoptive T cell therapy trials for the treatment of CRC.

CHAPTER 1

1. Colorectal cancer

1.1 Epidemiology

Accounting for 147,950 estimated new cases in 2020, colorectal cancer is the fourth most common type of malignancy and the second leading cause of cancer-related death[1]. The median age at diagnosis is 66 years[2]. Although a slight decline of CRC incidence rate per year, projections suggest a burden increase up to 2.2 million new cases worldwide by 2030, with an enhancement of the cases affecting young adults[3]. The capacity to early detect the cancerous lesions led to a decrease of mortality for CRC over the years, estimating a 5-years survival for 64% of the diagnosed patients[4]. Still, half of the cases will eventually recur, frequently with a metastatic disease, which is accompanied by a very poor prognosis.

1.2 Etiology

The vast majority of CRC diagnoses arise in developed countries[5,6]. Accordingly, risk factors for its development are represented by diet and lifestyle: vast consumption of unsaturated fats, red meat, excessive alcohol, tobacco, reduced physical activity[7,8,9]. On the other side, a diet rich in fruits and vegetables, fibers, fish, the use of vitamins, the use of non-steroidal anti-inflammatory drugs, statins, calcium and estrogen appear to be protective factors[2]. Energy balance, inflammation and gut microbiome have been also identified as relevant factors for the development of CRC[10]. All these elements are relevant for the sporadic form of cancer, accounting for 70% of the

diagnoses. Sporadic CRCs are often characterized by somatic alterations which could hamper the function of oncogenes or could dampen the expression of tumor suppressor genes. Oncogenes can be altered by point mutations or gene and chromosome rearrangements dysregulating gene expression. Tumor suppressor genes can be inactivated by gene loss or epigenetic alterations. Better defined are the factors defining CRC related to inherited predisposition[11,12]. Among familial cases, there is a small percentage of patients suffering from hereditary nonpolyposis colorectal cancer (HPCC) – also called Lynch – syndrome or familial adenomatous polyposis (FAP), accounting for 5% of all CRC cases. Nonetheless, gut pathologies as inflammatory bowel disease, ulcerative colitis and Chron's disease may increase the risk for developing CRC.

1.3 Histopathology

CRC is a very heterogeneous disease. Based on histopathologic characteristics, different CRC subtypes have been defined by the World Health Organization (WHO). More than 90% of CRCs are adenocarcinomas, which can be further discriminated as well, moderately, and poorly differentiated based on the frequency of gland formation. When more than half of the glands produce mucus, the mass is defined as mucinous carcinoma, as happens in 10% of all CRC, prevalently localized in the right colon[13,14]. When tumors present poor differentiation degree and solid nests of malignant cells, associated with major intraepithelial immune cell infiltration, they are categorized as medullary. Accounting for

the minority of CRCs, medullary tumors are more frequently described in the right colon and associated with a high mutational burden[15]. Being associated with the presence of lymphocytes within the tumor site, medullary carcinomas frequently result in a favorable prognosis[16]. Signet ring cell carcinoma is another CRC subtype, accounting for less than 1% of all CRCs and diagnosed in younger patients. Its peculiarity resides in the proliferation of signet ring cells, with the nuclei forced at the periphery by mucus production[17]. Despite the high quantity of mucus produced is related to a better overall survival[18], these tumors frequently metastasize to multiple sites[14]. Understanding the histological characteristic of CRCs is fundamental for the diagnosis since recent advances lead to think that each subtype is the result of different developmental pathways, and therefore could be targeted in a specific way.

1.4 Molecular pathways

Three major pathways have been recognized relevant for the development of CRC: chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylation (CIMP) [19,20]. Chromosomal aberrations, either numerical or structural, represent the main feature characterizing the CIN subtype. These tumors often present mutations in proto-oncogenes, such as KRAS, and tumor suppressor genes, among which TP53, the adenomatous polyposis coli (APC), and the deleted in colon cancer (DCC) genes. Accounting for around 10% of the cases, the MSI pathway is characterized by a

deficiency in the DNA mismatch repair system, thus causing microsatellite instability. Microsatellites are repetitive DNA sequences present throughout the genome, thus prone to mutations and mismatches for the ease for DNA polymerases to reach them, thus resulting in the accumulation of errors within the genome. The exonuclease activity which excises nucleotides is the result of the work of the mismatch repair (MMR) system, which is composed of six enzymes: MLH1, MLH3, MSH2, MSH3, MSH6, PMS1 and PMS2. If a mutation occurs in these enzymes, it will result in DNA replication errors, different lengths of the microsatellite repeats or production of frameshift peptides. MMR genes were demonstrated to be hypermethylated in sporadic forms of CRCs while harboring inactivating mutations in those cancers arising from familial syndromes[21,22]. The MSI phenotype is more frequently found in tumors of the right colon and is associated with a robust immune infiltrate, thus resulting less prone to metastasize to lymph nodes or distant sites[23].

CIMP phenotype defines poorly differentiated tumors presenting high levels of methylation of the CpG islands in promoter regions, thus inactivating tumor suppressor genes. This phenotype is often associated with mutations in BRAF and hypermethylation of the MLH1 gene promoter[24].

According to these pathways, four consensus molecular subtypes (CMS) have been distinguished[25].

CMS1 comprehends tumors with a good prognosis, frequently MSI and with a strong immune infiltration. These tumors frequently harbor BRAF mutations, which effect is mitigated in

MSI patients by the presence of an important immune infiltrate, while results in a worse prognosis in MSS patients[26]. CMS1 tumors harbor also frequently CpG island methylation, which is thought to be the principal mechanism by which MLH1 hypermethylation leads to sporadic MSI cancer[27].

High chromosomal instability characterizes CMS2 tumors, of epithelial origin and with an intermediate prognosis. In CRC, somatic copy number alterations affect the WNT and MAPK pathways[28,29]. WNT signaling is a pivotal pathway in the initiation of CRC, due to driver mutations in the APC gene, responsible for the development of non-invasive polyps, the initial stage of cancer. The MAPK pathway, instead, has as main actor KRAS. Among other functions, KRAS regulates the nuclear localization of beta-catenin, which upregulation is crucial for the progression of CRC and its presence in the nucleus represents a sign of worse prognosis[30,31]. Beta-catenin regulates the proliferation rate, and its upregulation is a peculiar characteristic of the CMS2 subgroup[32,33].

75% of CMS3 tumors harbor KRAS mutation, but it cannot be considered a marker of this phenotype since it is present also in tumors of other subtypes. Nonetheless, KRAS mutations have been recently discovered to be associated with metabolism in cancer cells, and metabolic dysregulation is what better characterizes CMS3 tumors. KRAS, together with other proto-oncogenes, promotes the Warburg effect which reprograms the metabolism of the cells to a glycolytic state so they can survive a hostile microenvironment[34]. Phenotypically, these tumors have a

low proliferation rate, rarely invade the stroma and are poorly infiltrated by immune cells.

Characterized by a poor prognosis, CMS4 are called mesenchymal tumors for their capacity to invade the stroma and promote angiogenesis. In these tumors CpG island methylation is frequent. CMS4 CRCs are characterized by the dysregulation of the TGF-β signaling pathway[28]. In CRC, stromal cells represent the main source of TGF- β, thus a high amount of TGF-β is found when there is a major stromal invasion and, as a consequence, increased metastasis rate[35].

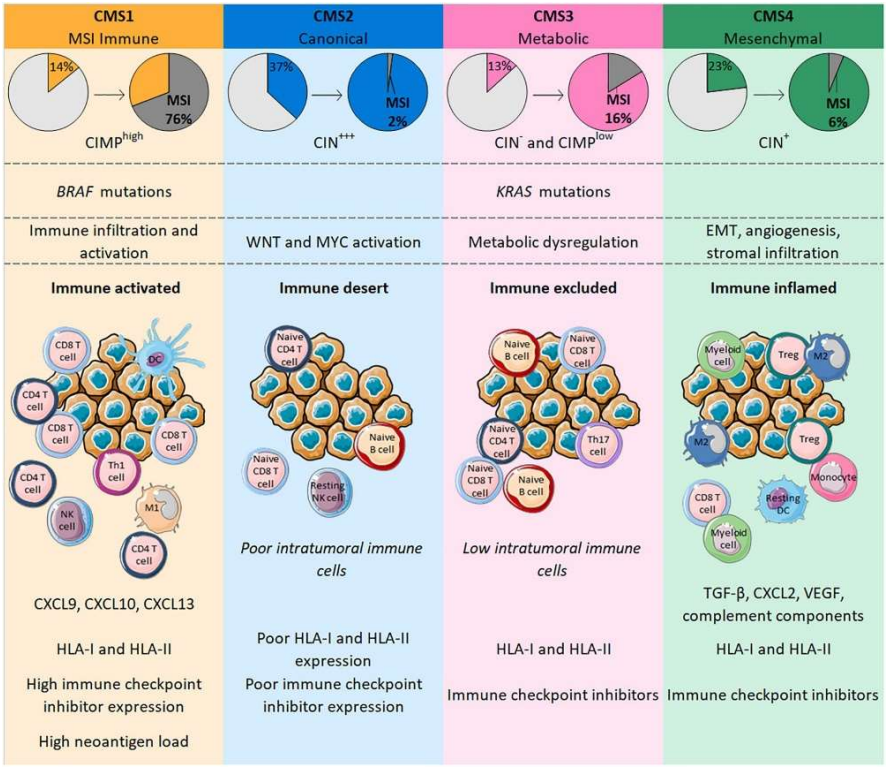

Figure 1 CMS in CRC are classified according to genetic modifications and intratumoral immune phenotype.[36]

2. CRC generation and microenvironment

2.1 Colonic epithelium and colorectal cancer stem cells

The colon is composed of four histologically different layers. The luminal surface is represented by the epithelial layer, in which the functional unit of the intestine, the crypts of Lieberkühn, reside in the spaces between the columnar epithelial cells. At the bottom of the crypt are located the multipotent stem cells, which harbor the capacity to divide asymmetrically and self-renew. The existence of intestinal stem cells with the capacity to generate all other intestinal cell subsets is still controversial. Despite well documented in mouse bowel[37], it has not been really identified in humans. While differentiating, the cells reach the top of the crypt and are extruded into the lumen[38].

The terminally differentiated epithelial cells of the colon can be distinguished in colonocytes, goblet cells and enteroendocrine cells. Not subject to a migratory pathway, the Paneth cells are the “neutrophils” of the colon and reside only in the small intestine, at the bottom of the crypt, and have a role in producing mucosal defense barriers, modulate the microflora and produce growth factor[39] for the maintenance of the stem cell niche[40]. Attached to the basal lamina surrounding the crypt reside the mesenchymal cells, which produce the Wnt signaling ligands, the main regulators of the migratory pathways of the colonic cells. Other factors contributing to the functional characteristics of colonic stem cells are the bone morphogenetic protein (BMP) antagonists gremlin1 (GREM1) and gremlin2 (GREM2), Notch

signaling pathways, ephrin-B1 (Eph-B1) and its receptors Eph-B2 and Eph-B3[41]. The non-neoplastic colonic epithelium present ordered and regular shaped crypts, which can be easily distinguished from the branched one arranged in the tumoral tissue according to a chaotic architecture. The model of colorectal tumorigenesis is still controversial. Older studies suggested a bottom-up flow, envisaging the direct mutation of stem or progenitor cells, but recent investigation demonstrated the presence of randomly distributed actively dividing cells at the periphery of colon tumors, suggesting a top-down carcinogenesis model[42]. In fact, in the initial stages of tumor development, dysplastic cells appeared localized at the top of the crypt. To sustain the previous bottom-up hypothesis, it could be suggested that normal-shaped cells already harboring tumorigenic mutations migrate to the top of the crypt before cell transformation. Nowadays, two hypotheses of colorectal carcinogenesis still stand: (i) the tumoral transformation is the consequence of stochastic events and microenvironmental factors leading to clonal selection; (ii) the tumor initiate from a small pool of cancer stem cells (CSCs) with abnormal differentiation properties. CSCs were firstly isolated in acute myeloid leukemia[43] and subsequently in other tumors, including CRC, in which CD133 was used as a phenotypic marker for CSCs. In a first study, it was demonstrated that $CD133^+$ cells were the only subset able to reproduce the original tumor in immunodeficient mice. After serial transplantation, no substantial variation was observed in the phenotype of these cells, which

continued to boost tumor growth. Moreover, $CD133^+$ cells were able to exponentially grow *in vitro* as undifferentiated tumorspheres, maintaining the ability to engraft and still reproducing the phenotypic pattern of the tumor of origin[44]. Contemporarily, another study enforced these results demonstrating that CSCs are 200 times enriched in the $CD133^+$ population[45]. Also known as prominin-1, CD133 is a five transmembrane domain molecule located in the apical plasma membrane protrusions of embryonal epithelial structures[46] and its function is largely unknown. In both studies, $CD133^+$ cells were identified at lower frequency also in non-neoplastic tissue. CD133 expression was also shown to correlate with poor prognosis for CRC[47,48,49]. CD44 and EpCAM were also used to isolate CSCs[50]. CD44 is a cell surface glycoprotein involved in cell-cell interactions, cell migration and adhesion of the cytoskeleton to the extracellular matrix[51]. Its transcription is in part activated by Wnt signaling, thus it is overexpressed in early carcinogenesis[52]. However, the expression of CD44 occurs as in the stem cell compartment as in the proliferative compartment, so its specificity to identify colonic stem cells is yet to be determined. In fact, studies on its relevance for CSCs are conflicting. On one side, it has been demonstrated that a very small amount of $CD44^+$ cells is sufficient to initiate tumor formation in mice and that the genetic KO of CD44 prevents tumorigenesis[53,54]. On the other side, it has been observed that $CD133^+$ cells usually display a low expression of CD44[55], hypothesizing that the $CD133^+$ CSCs may originate a more

aggressive subset of cancer-initiating cells which lose CD44 expression.

Lgr5 was suggested as another candidate gene to identify CSCs. Clevers et al. showed that the genetic deletion of the APC gene in $Lgr5^{+}$ cells leads to their neoplastic transformation to a macroscopic adenoma[56]. In association with Lgr5, Prom1 was indicated to mark cells located at the base of the crypts with the ability to generate the entire intestine[57]. Understanding the nature of cancer-initiating cells has a major clinical relevance. In fact, this could lead to the definition of a panel of biomarkers for both diagnosis and prognosis. Isolating cancer-initiating cells could enhance the understanding of tumor processes, both in terms of progression and metastasis and in resistance to treatments. For example, cytotoxic agents commonly used in the clinics are designed to target actively proliferating cells and this could not include CSCs, which are in fact found enriched in colon tumors following chemotherapy[58].

Further understanding of the heterogeneity of CRC CSCs could make possible novel CSC-directed therapy, potentially enhancing the efficacy of conventional treatment.

2.2 CRC immune microenvironment

In solid tumors, like CRC, the microenvironment is a complex interplay of immune cells, fibroblasts, endothelial and many other cell types[59,60,61]. Innate and adaptive immune cells infiltrating the tumor microenvironment play a dual role in tumor progression[62]. According to the cancer-immunity cycle theory[63], during the

“elimination” phase effector cells kill malignant cells apart from the rare variants which enter a dormant state, leading to the “equilibrium” phase.
When the tumor starts again to proliferate, the “escape” phase will occur; this is facilitated by an immune suppressive microenvironment[64].
Cancer progression is associated to chronic inflammation[65], which is a hallmark of the pro-tumoral environment[66]. This is particularly evident in some forms of CRC that develop in patients with pre-existing diseases, such as inflammatory bowel disease[67]. The inflammatory signals, given by apoptotic cells, free DNA molecules, damage-associated molecular pathways, cytokines or toll like receptors are fueled by the immune system or by the tumor, and can lead to non-specific activation of immune cells[68,69,70]. Some of the major cellular actors of the microenvironment in solid tumors are macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), mast cells, cancer-associated fibroblasts (CAFs), monocytes, neutrophils, CD8 and CD4 T-cells, dendritic cells (DCs), natural killer (NK) cells, endothelial cells, endothelial progenitor cells (EPCs), platelets, and mesenchymal stem cells (MSCs).

When chemokines, growth factors, vascular endothelial growth factors and other angiogenic factors are released, monocytes are recruited from the blood to the tumor microenvironment[71], where the presence of high levels of IL-10 together with low IL-12 induce their differentiation into macrophages[72]. The polarization

toward an M1 (anti-tumoral) or M2 (pro-tumoral) phenotype is driven by a complex pattern of chemokines[73]. M2 macrophages highly infiltrate the tumor microenvironment and orchestrate pro-tumoral inflammatory responses[66,74]. In colorectal cancer, tumor-associated macrophages induce angiogenesis[75] and are responsible for epithelial-to-mesenchymal transition[76], thus enhancing cell migration and invasion.

Myeloid-derived suppressor cells in colorectal cancer can be present in a consistent number, even superior to lymphocytes[77]. The presence of high numbers of myeloid cells has been often associated with a poor prognosis, possibly due to their suppressive activity on adaptive immune responses[78] and on their facilitating role on FoxP3$^+$ Tregs[79].

Already well described to be fundamental in allergic and autoimmune diseases, mast cells have been recently identified as associated with poor outcome in solid tumors. In CRC patients, the number of mast cells is much higher in tumors than in adjacent healthy tissues[80]. In fact, they have been demonstrated to contribute to tumor growth, invasion and vasculature development[81,82].

Another important player is represented by mesenchymal cells, such as fibroblasts[83]. In CRC, CAFs are crucial for CRC development[84], and are responsible for hypoxia and the migration of cancer cells[85] causing metastases[86]. High levels of

CAFs in the stroma of CRC lesions are a sign of more aggressive and potentially metastatic disease[87].

Neutrophils, known to be present in inflammatory settings, have been also found in patients with cancer, gastric and colon tumors included[88] especially in patients with pre-existing inflammatory syndromes such as inflammatory bowel disease[89]. In CRC, neutrophils have been described to promote angiogenesis[90] and epithelial to mesenchymal transition[91].

Studies investigating the presence of dendritic cells within the CRC microenvironment are controversial. Some groups demonstrated that the presence of immature DCs was related to longer disease-free survival, while mature DCs showed an opposite behavior[92,93]. On the contrary, other works suggested that the disease outcome is not affected by the presence of DCs[94]. However, it was shown that colorectal tumors contain a higher number of DCs when compared to the non-neoplastic colon tissue or with the metastasis from the same tumors[95]. A consistent reduction of circulating DCs was observed in CRC patients compared to healthy controls[96]. Nonetheless, functional impairment was also reported for DCs in the peripheral blood of patients[97]. This numerical and functional impairment was completely restored after tumor resection[98].

NK cells, despite being fundamental for gut differentiation and homeostasis, are significantly reduced in primary CRC tissues compared to normal mucosa[99].

In various solid tumors, CRC included, the tumor microenvironment accommodates T cells with various antigen specificities, not only directed to cancer cells[100,101]. TILs in CRC are mainly located in the tumor mass, in the peritumoral stroma and at the invasive margin[102]. Their presence is determinant for the course of the disease; in fact, they have been demonstrated to inhibit tumor growth[103]. The good prognosis associated with the presence of TILs has been verified in both primary tumors and metastatic ones, possibly due to their ability to inhibit tumor dissemination to lymph nodes, lymphovascular and perineural structures[104]. MSI tumors are greatly infiltrated by T cells with respect to MSS ones, and this observation can be ascribed to the increased tumor immunogenicity associated to a high mutational burden and consequent expression of frameshift peptides[105]. Galon et al. attempted to discriminate how the type and density of immune cells in a large cohort of CRC patients correlate with disease progression. They observed that a low rate of tumor recurrence occurred when a high density of cytotoxic and memory T cells was present[106]. However, the phenotype of the T cells infiltrating the tumor microenvironment has not been fully elucidated as well as their specificity for cancer antigens.

3. T cell exhaustion

3.1 T cell exhaustion in cancer

T cells play a crucial role in preventing tumor spreading and growth[107], but within the tumor microenvironment, they are exposed to a continuous antigen stimulation that could lead to functionally impaired T cells defined as “exhausted”[108,109]. T cell exhaustion is a condition due to the activation of both cell-extrinsic and intrinsic negative regulatory pathway and was first identified in chronic infections, where pathogens are not rapidly cleared but rather persist, leading to inflammation and chronic antigen stimulation[110,111]. Exhaustion represents a differentiation state in which T cells lose their effector and killing capacity in a hierarchical manner[111]. The first-occurring events are the reduction of proliferative capacity and the inability to kill the antigen-bearing targets. In the intermediate state of dysfunction, T cells cannot produce pro-inflammatory cytokines and tumor necrosis factor[110]. When T cells can no more produce Interferon-ɣ and lose the ability to degranulate, the exhaustion state is severe, in some cases leading to the physical deletion of antigen-specific T cells[112]. The co-expression of several inhibitory molecules is a hallmark of exhausted T cells. However, the latest findings support the hypothesis that the expression of these molecules, at least in healthy donors, is not synonymous of lower functionality, but rather correlates with differentiation to a T_{EM} phenotype[113]. In fact, it was demonstrated that $PD1^+$ $CD8^+$ T cell function is not always impaired[114], suggesting that the chronic expression and not the mere upregulation of PD1 might be relevant for T cell function. Moreover, a broad analysis of the

expression of IRs and cytokine production in human $CD8^+$ T cells indicates that although the upregulation of inhibitory molecules is a marker of T cell dysfunction, cytokine production capacity is variable and highly dependent on the activation and differentiation status of the cells[115].

The Chronic Lymphocytic Choriomeningitis Virus (LCMV) infections were the first context in which T cell exhaustion was observed. The induction of specific anti-LCMV $CD8^+$ T cell response was observed without antigen clearance, demonstrating impaired effector functions in this model[112]. In the same setting, the contemporary actions of different mechanisms acting to silence antiviral T cell responses were observed: $CD8^+$ T cells lose the ability to produce pro-inflammatory cytokines and to exert effector functions, and this phenotype was more evident in association with CD4 T cell deficiency[116]. Also, the deletion of LCMV-specific cytotoxic clonotypes was observed in the presence of excessive and persistent antigen stimulation[117]. T-cell exhaustion has also been reported in various human chronic viral infections such as hepatitis B (HBV)[118], and hepatitis C (HCV)[119] and human immunodeficiency virus (HIV)[120], where it was noticed that the severity of the exhaustion is directly proportional to the viral load and the duration of infection. Upon chronic infection, viral-specific $CD8^+$ T cells do not develop into memory cells with the ability to persist long term in the presence of IL-7 and IL-15: this is in line with the finding that exhausted $CD8^+$ T cells have low expression of the α-chain of the IL-7 receptor and the β-chain of the IL-2 and IL-15 receptors, and also

explains why these cells do not survive when adoptively transferred into infection-free mice[111]. In contrast, when the infection is completely cleared, highly functional memory T cells develop. Memory cells are able to rapidly acquire effector functions after antigen re-encounter, to home in secondary lymphoid tissues, and display high proliferative potential and long term persistence[121]. In 2007, Wherry et al. described the molecular signature of $CD8^+$ T cells undergoing exhaustion during chronic viral infections by comparing the gene expression profiles of dysfunctional LCMV-specific $CD8^+$ T cells from chronic or acute infection. Exhausted $CD8^+$ T cells displayed the overexpression of several inhibitory molecules, and this was accompanied by major changes in T cell receptor and cytokine signaling pathways. Exhausted T cells harbored major metabolic and bioenergetic deficiencies. Moreover, gene expression was altered in pathways involving chemotaxis, adhesion and migration[122].

The comparison between exhausted T cells in chronic viral infections and cancer showed several similarities but also some exclusive characteristics. In both settings is important to notice that exhausted T lymphocytes are not inert, since they still produce a small quantity of IFN-ɣ[123]. Regarding the transcriptional profile, it was demonstrated that Melan-A tumor-specific $CD8^+$ T cells isolated from melanoma patients and gp33 virus-specific T cells in chronic LCMV infection displayed a substantial overlapping functional phenotype[108]. As in chronic infections, tumor-specific T cells are defective in homeostatic

proliferation, as suggested by the evidence, in melanoma patients, of low levels of the IL-7 receptor in Melan-A specific $CD8^+$ TILs[109]. In cancer, the identification of patterns responsible for functional impairment of tumor-specific T cells is fundamental to unravel mechanisms underlying T cell exhaustion and thus reveal novel targets for cancer therapies. A hallmark of exhausted T cells is the contemporary expression of several inhibitory molecules, defined as immune checkpoints. In CRC, inhibitory receptors that are expressed at high frequencies in normal colon tissue may have the function to maintain immune homeostasis in the gut, while inhibitory receptors expressed on lymphocytes within the tumor tissue may prevent immune eradication of the tumor itself.

3.2 PD1

PD1, also known as CD279, is a transmembrane protein receptor belonging to the CD28 family. Human PBMCs express four different splice variants of the protein[124], all induced by stimulating T cells *in vitro* with anti-CD3 and anti-CD28. In both human and mice, the PDCD1 gene consist of five exons, encoding a signal sequence (exon 1), an IgV-like domain (exon 2), a stalk and transmembrane domain (exon 3) and a cytoplasmic domain (exons 4 and 5). The cytoplasmic tail of PD1 is composed of an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM)[125,126]. Both ITIM and ITSM domains tyrosine residues undergo phosphorylation when PD1 binds its ligands and is thus

recruited to the immunological synapse[127]. Tyrosine phosphorylation leads to the recruitment of SHP2 (Src homology 2 domain-containing tyrosine phosphatase 2), which consequently dephosphorylates several mediators of T cell activation[128]. However, if CD28/B7 are not involved in the immunological synapse, SHP-2 can target the TCR and its downstream molecule to dampen T cell activation through PD1 signaling[129].

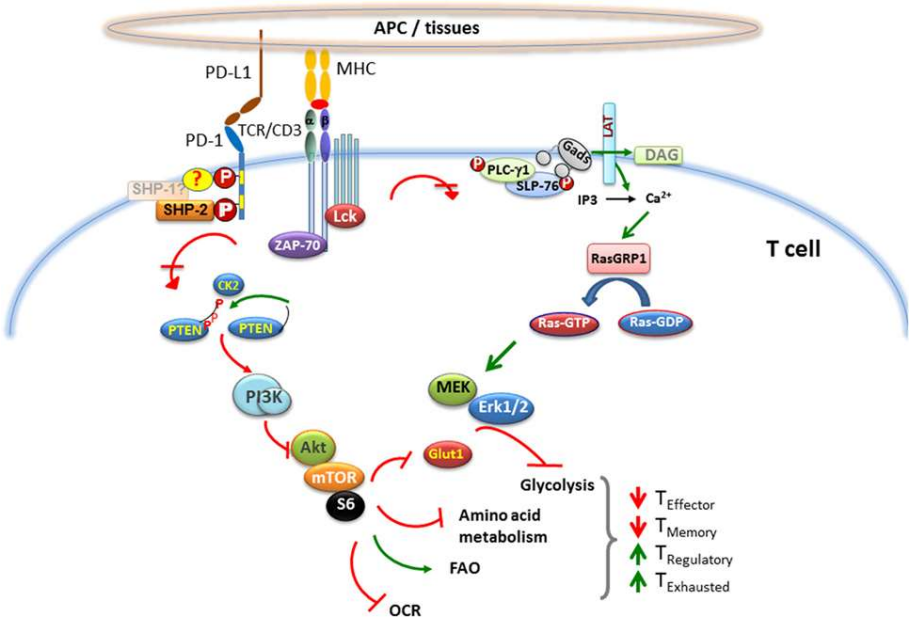

Figure 2 Effect of PD1 on major signaling pathways and subsequent metabolic reprograming in T cells.[130]

PD-L1[131] (B7-H1, CD274) and PD-L2[132] (B7-DC, CD273) are the two ligands described for PD1. They harbor similar structures and are closely linked in the genome. PD-L1 is constitutively expressed by a broad range of cell types, but its overexpression can be induced upon activation of key oncogenic pathways, such

as phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), or by T cell response upon release of IFN and other cytokines within the tumor microenvironment[133]; nonetheless, upregulation of PD-L1 upon IFN-ɣ production has been described to dampen active immune responses[134]. PD-L2 expression, on the other side, is more restricted to dendritic cells and macrophages upon IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) release[132]. PD-L2 cross-compete with PD-L1 to bind PD1, being its affinity for the receptor higher than that of PD-L1[135], but still is unfavored due to its rare expression. T cell function is affected by PD1 in many ways. The function of PD1 was firstly studied in PD1-deficient mice, which present several autoimmune conditions, such as autoantibody-induced cardiomyopathy, arthritis, lupus-like disease[136] and type 1 diabetes[137], meaning that PD1 is crucial in maintaining peripheral tolerance[138]. This activity is exploited through the binding to PD-L1[139] and has fundamental importance for tumor cells, which need to escape from host immune surveillance, by promoting T cell anergy, exhaustion[140] and apoptosis[141]. Within the tumor microenvironment, also the cells' metabolism is altered by PD1. Its signaling lead to the reduction of Akt activation, causing a switch of T cell metabolisms toward fatty acid oxidation, which promotes the generation of regulatory T cells[142] and inhibiting glycolysis, which is required for T cell effector function[143,144,145].

The upregulation of PD1 on TILs was described in several studies, correlating with a reduced frequency of IFN-γ^{+}IL-2^{+} T

cells. In CRC patients that underwent surgery, an increased population of T cells expressing PD1 and TIM-3 with an impaired capacity of producing IFN-y has been found[146]. CRC tumors harboring microsatellite instability were demonstrated to counterbalance their pro-inflammatory microenvironment with the upregulation of multiple inhibitory receptors, among which PD1[147]. $PD1^+$ T cells were also found in tumor-draining lymph nodes and tumor lesions, being impaired in functions compared to $PD1^+$ T cells found in tumor-free lymph nodes from CRC patients[148]. Hence, the expression of PD1 as well as other inhibitory receptors is non-synonymous of decreased functionality, but rather correlates with differentiation to an effector memory phenotype[113]. Accordingly, completely functional PD1 positive T cells have been described[114], suggesting that chronic exposure to antigens, more than the mere upregulation of PD1, is relevant for T cell function. On the other hands, checkpoint inhibitors interfering with the PD1 axis are successfully used to treat selected cancer types.

3.3 CD39

Recently, studies proposed that the exhaustion signature could be linked to an anti-tumoral specificity. In this context, CD39 is a molecule of interest since it appears to discriminate tumor-specific CD8 T cells from bystander lymphocytes[149]. CD39 is an endothelial ectonucleoside triphosphate diphosphohydrolase (NTPDase) which converts ATP into ADP, further hydrolyzed by CD73 into adenosine, ultimately switching the environment into an immune suppressive one. Adenosine- mediated

immunosuppression has been extensively reviewed[150,151]. Within the tumor microenvironment, cells release ATP in response to stimuli such as hypoxia or tissue damage and remodeling[152]. These phenomena, as well as chronic inflammation, lead to the upregulation of CD39[153], which high levels have been described in various solid (pancreatic, ovarian, lung, kidney, thyroid, testicular) tumors[154] and hematological (lymphoma, sarcoma, chronic lymphocytic leukemia) malignancies[155,156]. CD39 can be upregulated by tumor cells, but the main effect on the conversion of the microenvironment from immunostimulatory (with the presence of ATP) to immune suppressive is led by its upregulation on immune cells[150]. In T cells, the redirection of transcription, dependent on cyclic-AMP response element is induced by high-affinity A2A and low-affinity A2B adenosine receptors[157]. In a melanoma mouse model A2A deficiency has been associated to increased antitumor $CD8^+$ T cell responses, thus highlighting the importance of adenosine signaling in anti-tumor immunity[158]. Accordingly, immune checkpoint blockade has been observed to be enforced by blocking the formation of adenosine within the tumor microenvironment. In fact, in mouse models of colon, prostate and metastatic breast cancer, the anti-CD73 monoclonal antibody enhanced the functionality of anti-PD1 and anti-CTLA-4 mAbs demonstrating that the response was dependent on IFN-y production and CD8 T cells activity[159]. Moreover, the blockade of CD73 was found effective in ameliorating the activity of anti-HER2/ErbB2 mAb trastuzumab in a model of breast cancer as well as to treat lung metastases[160].

The pathway has shown to be relevant also for other immune cell types. For example, CD39 was demonstrated to prevent ATP-dependent differentiation of tumor-infiltrating myeloid cells into dendritic cells[161]. Of note, dendritic cells from both human and mouse liver express higher levels of CD39 compared to other dendritic cells and are more resistant to the pro-stimulatory effects of ATP[162]. In general, when ATP is present, DCs increase their antigen presentation capability and can induce T cell proliferation in vitro[163,164]. In fact, CD39 inhibition allowed recruitment of dendritic cells toward the tumor microenvironment, as well as of CD4 and CD8 T cells in response to the enhanced levels of ATP[165]. A work on KRAS mutated lung tumors further supported these results showing that using a CD39 inhibitor abolished the accumulation of Tregs to the tumor microenvironment and reduced the number of tumor foci[166]. More recently, the expression of CD39 on activated T cell has been interpreted as a sign of T cell exhaustion, since it has been linked to functional impairment and inhibitory receptor upregulation. Accordingly, subjects carrying a polymorphism causing low levels of CD39 protein have been demonstrated to be more susceptible to Chron's disease[167] and better responders when vaccinated against influenza or varicella-zoster, harboring a pool of vaccine-specific memory T cells[168]. CD39 expression was investigated in several human solid tumors. Canale et al. described the presence of $CD8^{+}CD39^{+}$ T cells in breast cancer and melanoma lesions but not in non-invaded lymphoid organs and peripheral blood. These T cells were identified as exhausted

for the observation of an impairment in the ability to produce IFN-y, TNF-α and IL-2 and for the upregulation of immune checkpoint molecules[169]. $CD39^{+}CD8^{+}$ T cells were also found in head and neck squamous cell carcinoma, melanoma, lung cancer, ovarian cancer, colorectal cancer[170], renal cell carcinoma, non-small cell lung cancer and gastric adenocarcinoma while being absent in normal mucosa[171]. Gene-set enrichment analysis showed significant enrichment of transcripts associated with T-cell exhaustion phenotype, such as PDCD1 (PD1), CTL4 (CTLA-4), HAVCR2 (TIM-3). The expression of CD39 in these cells was associated with CD103, a marker of tissue-resident memory T cells. $CD39^{+}CD103^{+}$ T cells displayed also a distinct TCR repertoire, with tumor-specific T cell clones[170]. The capacity of CD39 to discriminate among tumor-specific and bystander T cells was highlighted by the work of Simoni et al., describing a subset of CD8 TILs in lung and colorectal cancers in which tumor specificity was associated to the presence of CD39[149].

4. Therapeutic approaches for CRC

4.1 Conventional therapies

When selecting the best clinical option for colorectal cancer, the aim is to reduce the chance of disease progression and recurrence while increasing patient survival. When cancer is early diagnosed, thus being localized and non-metastatic, the ideal treatment remains the resection of the primary tumor. Surgical resection aims at eliminating the tumor while restoring bowel continuity. Surgery can be enough for patients with stage I disease. However, for patients with stage III cancer, metastatic

to the lymph node, surgical resection alone leads to a 5-year survival rate of 20-50%. When resection is not an option, the treatment decision is guided by the pathological and molecular characteristics of each patient[172]. In general, chemotherapy regimens based on the combination of oxaliplatin and fluoropyrimidine have been demonstrated to improve the outcome both in a neoadjuvant or adjuvant setting[173,174,175]. Adjuvant therapy with 5-fluorouracil has been demonstrated to be effective for patients diagnosed with stage II CRC[176], but the results coming from several randomized clinical studies are controversial. 5-fluorouracil has been proven efficacious for treating stage II MSS patients, while no benefits in overall survival has been reported for MSI patients[177]. Poorly differentiated tumor with venous invasion appear sensitive to adjuvant therapy, but the benefit of oxaliplatin in these patients remains unclear[178]. On the other side, oxaliplatin appeared beneficial in the treatment of stage III tumors[179]. In both cases, early initiation of adjuvant treatment improves success rates[180,181]. This might be also due to the possibility for surgery to activate dormant micro-metastases through angiogenesis stimulation, thus facilitating tumor growth and spreading[182,183]. Irinotecan has been also evaluated in the metastatic setting, despite failing to demonstrate advantages in improving the overall survival[184,185].

4.2 Targeted therapies

Beyond chemotherapy, monoclonal antibodies blocking the vascular endothelial growth factor (VEGF; bevacizumab) and the endothelial growth factor receptor[186] (EGFR; cetuximab or panitumumab) have been explored. As KRAS mutation represents a negative predictor for anti-EGFR therapy, cetuximab and panitumumab are utilized as a first-line treatment only for KRAS WT metastatic CRC[187]. Bevacizumab is approved as first-line therapy and is often continued in the second-line setting, but predictors for its efficacy remain elusive. Acquired resistance to these therapies is not rare. One of the main causes of this phenomenon could be tumor molecular heterogeneity of CRC. Lack of response to cetuximab and panitumumab was described in the presence of a mutation in genes involved in cell proliferation, differentiation, apoptosis (KRAS, BRAF), or when effectors of the EGFR pathway cascade (PIK3CA, PTEN, NRAS) or EGFR modifiers (HER-2, EGFR ligands) are impaired[188,189,190]. The development of resistance to VEGF blockade can be explained by the activation of other signaling pathways (PIGF, angiopoietin-2, FGF/FGFR) in a compensative manner[191,192,193,194,195]. Other pathways that have been targeted in CRC are BRAF/MEK and HER-2. BRAF is found mutated in a little percentage of CRC, but its selective blockade is thought to reactivate the EGFR cascade[196,197]. For this reason, BRAF blockade has been explored also in combination with EGFR inhibitors[198,199]. HER-2 shares many downstream pathways with EGFR, thus being causative of anti-EGFR resistance. In fact, a combined targeting of HER-2 and EGFR inhibits tumor cell

proliferation and improves outcome significantly more than single targeting[200,201]. The state-of-the-art targeted therapies for CRC have been recently fully reviewed[187].

4.3 Immunotherapies

In the last decade, immunotherapy represented a breakthrough in cancer treatment[202]. Different approaches have been proposed to treat CRC, among which monoclonal antibodies, checkpoint inhibitors, vaccines and adoptive T cell therapy.

4.3.1 Checkpoint inhibitors

Pioneered by the 2018 Nobel prize winners James Allison and Tasuku Honjo, studies on the behavior of T lymphocytes within the tumor microenvironment led to the discovery of inhibitory mechanisms regulating T cell function[203,204]. T cells recognize peptides in an MHC-restricted manner. This recognition by the TCR is not enough for T cell activation: in fact, TCR-MHC signaling pathways are modulated by co-stimulatory or co-inhibitory signals, which are exploited by tumor cells to modulate immune response[205]. Given the promising results obtained using immune checkpoint blockers in non-small cell lung cancer[206], melanoma[207] and renal cell carcinoma[208], their use has been widened to other malignancies. The first monoclonal antibody targeting an immune checkpoint molecule approved by the FDA was ipilimumab[209] (anti-CTLA-4), in 2011. As already described, CTLA-4 is a negative regulator of T cell functions, able to disrupt essential costimulatory signals required for T cell activation. When tumor antigens are presented by APCs via the MHC and recognized by the TCR, simultaneous co-stimulation of CD28 on

T cells by CD80/CD86 on APCs occurs. However, CD80 and CD86 are ligands for CTLA-4, which binds these molecules with greater affinity thus preventing their binding with CD28. Ipilimumab acts by preventing this negative cascade and allowing CD28 to bind CD80/CD86 by blocking CTLA-4. Initially studied in the treatment of unresectable or metastatic melanoma[210,211], ipilimumab was evaluated in combination with other ICB for advanced CRC[212,213]. Another anti-CTLA-4 mAb is tremelimumab, a fully human IgG2 which did not show a significant single-drug activity in patients with metastatic CRC who failed standard treatments[214], but improves relapse-free survival rates when combined with chemotherapy[215]. The first anti-PD1 mAb, pembrolizumab, was approved in 2014. It is a humanized IgG4 with a high binding affinity for PD1, thus preventing the activation of negative regulation caused by the binding of PD1 with its ligands. PD1 blockade proved efficacy only in a small subset of CRC patients harboring microsatellite instability, while being ineffective in patients with MSS tumors[216]. In a study including 20 patients with PD-L1$^+$ CRC tumors, pembrolizumab was shown to have a good safety profile, despite being able to mediate anti-tumor activity in only one patient[217]. For this reason, nowadays pembrolizumab is approved for MSI patients whose cancer has progressed after treatment with adoptive chemotherapy. Another mAb blocking PD1 is nivolumab, approved at first as a second-line treatment for non-small cell lung cancer and then translated to other solid malignancies[218]. It proved effective in blocking the PD1/PD-L1

signaling pathway in phase I clinical trials in metastatic CRC with mismatch repair deficiency[219]. Nivolumab was also tested for safety, tolerance and efficacy in a phase I clinical trial (NCT00441337) involving 14 patients with CRC: one of them achieved prolonged survival. These trials suggested PD-L1 to be a prognostic factor for PD1 blockade therapy success in patients with advanced cancers. Nivolumab was tested also in patients with metastatic CRC, in which response lasting at least 12 weeks was achieved in 51/74 patients, while 8/71 achieved 12 months and longer responses[220]. In the attempt to block the other end of the string, anti-PD-L1-targeting IgG1 mAb atezolizumab was developed[221] and proved able to inhibit tumor growth in mouse models[222]. MDX-1105 is another fully human anti-PD-L1 mAb, which gave no clinical response when tested in CRC patients[223]. Despite being not that successful as a monotherapy to treat CRC, the combinatory blockade of multiple IRs could be beneficial. Accordingly, a 100% response rate was achieved when nivolumab plus ipilimumab were administered as neoadjuvant therapy in CRC patients[224]. Moreover, the combination of pembrolizumab, atezolizumab and subsequently ipilimumab and nivolumab have been used in a patient who relapsed 10 years after the first diagnosis with metastasis to the liver, lymph nodes and local urothelial cancer[225], providing long term control of the disease. Interestingly. the combination of the FOLFOX chemotherapeutic regimen with anti-PD-L1 and bevacizumab showed some clinical effects in patients with MSS CRC[226]. Following this observation, systematic analyses of the

mutational burden in CRC showed the existence of a subgroup of patients with a high mutational rate but not harboring microsatellite instability[227], but whether these patients could benefit from ICB remains a matter of debate.

4.3.2 Vaccines

Cancer vaccination has been explored for a long time, despite not gaining a great impact on cancer treatment compared to the one it has against infectious diseases. Being cancer a highly heterogeneous disease originated from human cells, with an antigenic repertoire to which the host is mainly tolerant, the identification of the most significant tumor antigens to target is a major hurdle. For this reason, most cancer vaccine strategies are based on primary tumor cells as antigen source. However, most tumors are poorly immunogenic, thus limiting the ability of the immune system to produce a strong response against their antigens, underlining the need for improvements. The first generation of anti-cancer vaccines was based on killed unmodified tumor cells or tumor lysates with adjuvants. More frequently used are peptide-based or DNA-based vaccines. For CRC, vaccines targeting the tumor-associated antigens CEA and beta-human chorionic gonadotropin (hCG) have been explored. Yeast-CEA is a therapeutic cancer vaccine genetically modified to express recombinant CEA protein, using heat-killed yeast (Saccharomyces cerevisiae) as a vector. A strong immune response to CEA was observed in preclinical studies. When administered to patients with CEA-expressing carcinomas,

minimal toxicity was observed but only a few patients showed an increase in antigen-specific CD8 and CD4 T cells and a decrease in Tregs, with no substantial benefit in the overall survival[228]. The glycoprotein hormone hCG is frequently expressed by colorectal tumors, being reported in 52% of CRC patients, and 41% of the patients also present elevated levels of circulating hCG[229]. Differently from CEA and other oncofetal antigens being targeted, hCG does not appear to be produced by normal colorectal cells[230] and its presence has been associated with reduced survival[231,232,229], enhanced tumor invasiveness[233] and metastatic rate[234]. A synthetic vaccine targeting hCG composed of the COOH terminal peptide conjugated to diphtheria toxoid as an adjuvant was administered to CRC patients. Immunization was safe and 56/77 patients developed anti-hCG antibodies, but no improvement in the overall survival was observed[235]. Other strategies for vaccination in CRC patients included vaccines based on dendritic cells. Tumor-specific immune responses were observed after vaccination with autologous DCs cultured with CEA HLA-A2 binding peptide or CEA electroporated mRNA in patients with CRC liver metastases. CEA-specific T cells were observed in 8/11 patients from the CEA-peptide group and in none from the CEA-mRNA one, despite the progression-free survival was higher in this last group[236]. Immunotherapy using DC vaccination was also used in combination with cytokine-induced killer cells, with the autologous tumor lysate strategy slightly improving the survival of CRC patients[237]. Further advancements were represented by the introduction of the

personalized peptide vaccination (PPV), a novel approach in which human leukocyte antigen (HLA)-matched peptides are individually selected from a panel of cytotoxic T lymphocyte (CTL) epitope peptides derived from diverse tumor-associated antigens[238,239]. A phase 2 study was conducted to examine the feasibility of PPV in CRC patients who had failed standard chemotherapies or targeted therapies. The vaccine, consisting of 2-4 HLA-matched peptides selected from a pool of peptide vaccine candidates, was administered to CRC patients. Boosted cytotoxic T cell responses to the peptides were observed in 63% of the patients and were predictive of improved overall survival[240]. Vaccination is safe, since no toxicities were observed in any trial; unfortunately, no encouraging results were achieved with such a strategy for CRC either.

4.3.3 Adjuvant immunotherapy and cytokines

In cancer therapy, adjuvants have the role to sustain and boost the immune response generated in the patient after treatment; in fact, adjuvants are mainly employed in the case of poorly immunogenic tumors. In the CRC setting, adjuvants have been employed with encouraging outcomes. Cytokines have been used to prevent lymphocyte depletion due to chemotherapy. Combination of IFN-α with gefitinib, an EGFR inhibitor, was used on a panel of colon cancer cell lines and demonstrated ability to significantly upregulate EGFR expression, suggesting IFN-α potential to enhance gefitinib efficacy[241]. When IFN-α was administered in vivo combined with 5-fluorouracyl, the treatment

was toxic and not successful. On the contrary, the addition GM-CSF and IL-2 to standard chemotherapy increased the overall survival rate in CRC patients[242,243]. IL-15 was also used as an adjuvant to immune checkpoint blockade. In a murine model of colon carcinoma, IL-15 was administered with anti-PD1 and anti-CTLA-3 mAb. The treatment resulted in higher IFN-y secretion and significantly prolonged survival of tumor-bearing mice[244].

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CHAPTER 2

REVIEW ARTICLE

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TCR Redirected T Cells for Cancer Treatment: Achievements, Hurdles, and Goals

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ABSTRACT

Adoptive T cell therapy (ACT) is a rapidly evolving therapeutic approach designed to harness T cell specificity and function to fight diseases. Based on the evidence that T lymphocytes can mediate a potent anti-tumor response, initially ACT solely relied on the isolation, in vitro expansion, and infusion of tumor-infiltrating or circulating tumor-specific T cells. Although effective in a subset of cases, in the first ACT clinical trials several patients experienced disease progression, in some cases after temporary disease control. This evidence prompted researchers to improve ACT products by taking advantage of the continuously evolving gene engineering field and by improving manufacturing protocols, to enable the generation of effective and long-term

persisting tumor-specific T cell products. Despite recent advances, several challenges, including prioritization of antigen targets, identification, and optimization of tumor-specific T cell receptors, in the development of tools enabling T cells to counteract the immunosuppressive tumor microenvironment, still need to be faced. This review aims at summarizing the major achievements, hurdles and possible solutions designed to improve the ACT efficacy and safety profile in the context of liquid and solid tumors.

INTRODUCTION

Adoptive T cell therapy for cancer (ACT) is a branch of cancer immunotherapy that relies on the ability to redirect T cell specificity to selectively target tumor antigens. ACT stemmed from two remarkable clinical observations: (i) The magnitude of T cells infiltrating tumor masses often correlates with response to treatment (1) and (ii) Allogeneic donor T cells infused in the context of hematopoietic stem cell transplantation promote clinical response in hematological malignancies (2). Initially, ACT solely relied on tumor-specific T cells isolated from the tumor masses and expanded in vitro (3). This approach was limited to resectable tumors from which enough T cells could be harvested and expanded. The development of gene engineering technologies dramatically changed the landscape of the ACT field, rapidly making this treatment accessible to an unprecedented number of patients and tumor types. By inserting an exogeneous T cell receptor (TCR) into cells, T cells specificity could be precisely redirected toward selected tumor antigens

(Figure 1). This new opportunity shifted the research focus and raised some novel questions: the main issue was no more how to harvest a sufficient number of tumor-specific T cells from each single patient, but how to isolate and harness high avidity tumor-specific TCRs, and how to proficiently generate and expand the most fit engineered T cells.

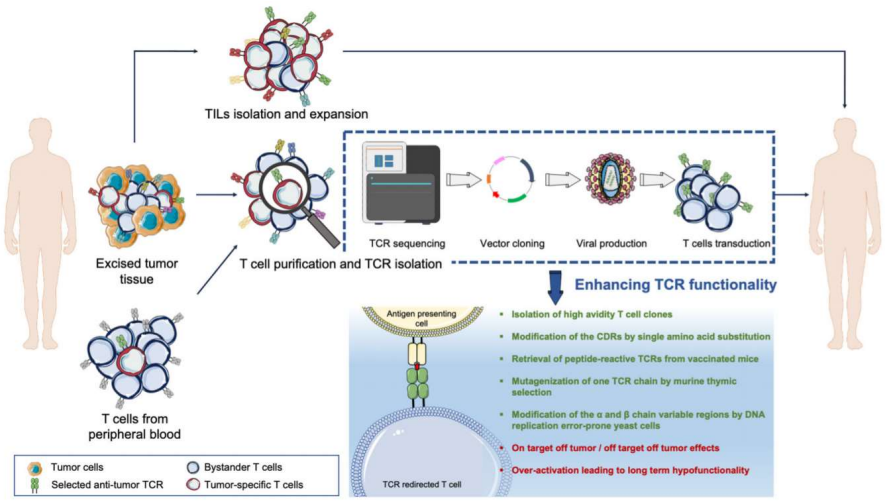

FIGURE 1 | *Overview of the TCR adoptive T cell therapy. Tumor-reactive lymphocytes can be isolated from either the tumor mass (tumor-infiltrating lymphocytes, TILs) or from the T cell pool circulating in patients' peripheral blood. T cells can be expanded in vitro and then re-infused back into the patient such as in TILs therapy. Else, the tumor-reactive T cell Receptor (TCR) genes can be isolated, sequenced, and transferred into acceptor T cells via vectors to redirect T cell specificities against tumor epitopes.*

The flexibility of the genetic modification tools offered the chance to insert and/or remove different genes in T cells and to permanently express, in the therapeutic products, entirely synthetic molecules. A striking deliverable produced by these efforts is represented by T cells expressing Chimeric Antigen

Receptors (CARs), that generated astonishing clinical results against blood malignancies (4–9). The outcomes of the first ACT clinical trials contributed to further elucidate the complex interplay between the immunosuppressive tumor microenvironment and the cellular players of immunity. Results suggested that modulation of ex vivo T cell expansion protocols and additional engineering of the T cell genome could be used to tweak T cell qualities, improving persistence and functionality of the therapeutic products. Several strategies were proposed to improve engineered T cell persistence, homing ability to the tumor site, capacity to recognize and eliminate tumor cells, and represent today's intense research lines. The possibility to modulate TCR affinity and T cell costimulatory and inhibitory signal pathways opens up novel therapeutic scenarios. The following review has the scope to summarize the cornerstones and the most relevant hurdles and efforts currently pursued to improve ACT.

FROM ALLOGENEIC STEM CELL TRANSPLANTATION TO ADOPTIVE T CELL THERAPY

Allogeneic Hematopoietic Stem cell transplantation (AlloHSCT) is a therapeutic modality relying on the infusion of hematopoietic stem and progenitor cells, harvested from a healthy donor, to a patient previously conditioned with high-doses chemo-radiotherapy. Although initially developed to regenerate the bone marrow of patients with genetic diseases or with hematological malignancies requiring strong myeloablative chemotherapy (10, 11), Allo-HSCT proved able to control malignant cells largely

through an immunological mechanism, as delineated by two major observations. Firstly, T-lymphocyte depleted grafts had a decreased efficacy in eradicating malignant diseases, suggesting that the donor to host immune response, and in particular the activity of allogeneic T cells, had per se an effect in abating the risk of relapse after transplant (2, 12, 13). Secondly, the infusion of circulating mature lymphocytes harvested from the donor (donor lymphocyte infusion, DLI) (14) correlated with the anti-leukemic effect (graft vs. leukemia, GvL) in a dose-dependent fashion (15). The efficacy of Allo-HSCT and DLI in restoring a state of disease remission represents one of the first compelling evidences of the potential of adoptive T cell therapy. Unfortunately, the benefits of allogeneic transplant and DLI against cancer are counterbalanced by toxicities, mainly due to the presence of a heterogeneous TCR repertoire with unknown specificities in the infused T cell population. Indeed, it has been calculated that ~10% of the T cell repertoire circulating in healthy donors is alloreactive (16). The most common manifestation of such toxicities is graft vs. host disease (GvHD), an immune reaction against the host's healthy tissues, occurring with varying degrees of severity but potentially fatal. The efforts to reduce toxicity while preserving the efficacy of DLI, and to export this therapeutic opportunity beyond the HSCT context, were the driving forces in promoting innovative ACT approaches. The first ACT strategies tested with autologous T lymphocytes were based on the isolation of T cells infiltrating primary lesions resected from patients with melanoma (tumor-infiltrating

lymphocytes, TILs), followed by their in vitro expansion with high-doses of interleukin-2 (IL-2) (17). The infusion of these cellular products, composed of an oligoclonal T cell repertoire incorporating CD4+ and CD8+ T cells, mediated potent antitumor responses with no toxicities in cell types other than melanocytes (3, 18, 19). The Objective Response Rate (ORR) observed was 41% across various clinical trials for patients with metastatic melanoma (20). Based on these encouraging results, the approach was widened and offered to patients affected by other solid tumors with variable outcomes, promising in some settings [e.g., sarcoma (21), cervical and ovarian cancer (22, 23)] but rather modest in others [e.g., renal (24), metastatic renal (25) and, colorectal (26, 27) cancer, Table 1]. The inconsistent efficacy of TILs may be linked to various causes: (i) the technical difficulties in isolating T cells from immune- cold tumors (44); (ii) the poor reactivity of the screened T cells, especially in tumors characterized by a low mutational burden (45, 46), and (iii) the overall low frequency of tumor-specific T cells infiltrating cancer lesions when compared to bystander T cells (47, 48). The high success rate of TILs therapy in melanoma can be in fact explained by the melanoma cells high tumor mutational burden, resulting in a heightened immunogenicity and a consequent enrichment of tumor-specific T lymphocytes (49). To expand the beneficial effect of TILs while overcoming the hurdles intrinsically associated with this therapy, the use of circulating T cells, harvested from patients and stimulated in vitro with immunogenic cancer epitopes, was proposed. This approach promotes the

selective expansion of the tumor-specific T cell fraction, in numbers sufficient to enable their re-infusion to patients, resulting in clinical benefits (50, 51).

Disease	Epitope	Antigen	Antigen type	HLA restriction	Vector (nuclease)	Number of treated patients	ORR (%)	Infusion toxicities	References
Melanoma	AAGIGILTV	MART-1	TAA (tissue restricted)	HLA-A*0201	Retrovirus	17	2 (12%)	none	(28)
Melanoma	AAGIGILTV	MART-1	TAA (tissue restricted)	HLA-A*0201	Retrovirus	20	6 (30%)	14 (skin rash), 11 (uveitis), 10 (hearing loss)	(29)
Melanoma	KTWGQYWQV	gp100	TAA (tissue restricted)	HLA-A*0201	Retrovirus	16	3 (19%)	15 (skin rash), 4 (uveitis), 5 (hearing loss)	(29)
Melanoma and synovial sarcoma	SLLMWITQC	NY-ESO-1	TAA (cancer/testis antigen)	HLA-A*0201	Retrovirus	11 and 6	5 (45%) and 4 (67%)	none	(30)
CRC metastatic and synovial sarcoma	IMIGVLVGV	CEA	TAA (tissue-restricted)	HLA-A*0201	Retrovirus	3	1 (33%)	3 (severe colitis)	(31)
Melanoma	EVDPIGHLY	MAGE-A3	TAA (Cancer/testis antigen)	HLA-A*01	Lentivirus	2	n.a.	2 (death due to cardiac toxicity)	(32)
Metastatic melanoma, sinovial sarcoma and esophageal cancer	KVAELVHF	MAGE-A3	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus	7, 1 and 1	5 (56%)	2 (death), 2 (CNS symptoms)	(33)
Metastatic melanoma	EAAGIGILTV	MART-1	TAA (tissue restricted)	HLA-A*0201	Retrovirus	13	9 (69%)	2 (skin rash), 2 (CRS)	(34)
Esophageal cancer	KVAELVHF	MAGE-A4	TAA (cancer/testis antigen)	HLA-A*2402	Retrovirus	10	0 (0%)	None	(35)
Multiple Myeloma	SLLMWITQC	NY-ESO-1	TAA (cancer/testis antigen)	HLA-A*0201	Lentivirus	20	16 (80%)	None	(36)
Sarcoma plus myeloma	SLLMWITQC	NY-ESO-1	TAA (cancer/testis antigen)	HLA-A*0201	Retrovirus	18 and 20	11 (61%) and 11 (55%)	None	(37)
Leukemia	CMTWNQMNL	WT1	TAA (Transcription Factor)	HLA-A*2402	Retrovirus	8	2 (25%)	None	(38)
Metastatic synovial sarcoma	NY-ESO-1^{c259}	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus	12	6 (50%)	11 (BM suppression)	(39)
Leukemia	RMFPNAPYL	WT1	TAA (Transcription Factor)	HLA-A*0201	Lentivirus	12	12 (100%)	9 (GvHD)	(40)
Synovial sarcoma, osteosarcoma, liposarcoma, peripheral malignant nerve sheet tumor	SLLMWITQC	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Retrovirus	10	2 (20%)	1 (CRS)	(41)
Myeloma/liposarcoma	SLLMWITQC	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus (CRISPR-Cas9)	3	0 (0%)	None	(42)
Synovial sarcoma	SLLMWITQC	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus	30	9 (30%)	n.a.	(43)

BM, Bone Marrow; CEA, Carcino Embrionic Antigen; CNS, central nervous system; CRS, Cytokine Release Syndrome; gp100, glycoprotein 100L; MAGE-A3/A4, Melanoma-Associated Antigen A3/A4; MART-1, Melanoma Antigen Recognized by T cells 1; NY-ESO-1, New York Esophageal Squamous Cell Carcinoma-1; ORR, Overall Response Rate; TAA, Tumor-Associated Antigen; GvHD, Graft vs. Host Disease; TCR, T cell Receptor; WT1, Wilms Tumor 1.

TABLE 1 | *Overview of TCR-engineered T cell-based clinical trials*

Nonetheless, the use of TILs and circulating T cells lead to the generation of a T cell population for which the affinity and functionality of the TCR could not be predicted a priori and whose ability to effectively induce clinical responses was tightly linked to the expansion potential of harvested cells. Gene editing and gene transfer technologies greatly boosted the ACT field, allowing modification of the T cell genome and redirection of T lymphocytes specificities by inserting highly functional, tumor-specific TCRs (52) into patients' T cells, that could be subsequently expanded in vitro. In the 90s the discovery that the Fab region of an antibody could be efficiently fused to the CD3

zeta chain and to other costimulatory intracellular domains to create Chimeric Antigen Receptors (CARs), further revolutionized the T cell-based immunotherapy field. CARs are able to activate T cells upon binding to a surface receptor expressed by the target tumor cell (53) and their use proved instrumental in widening the therapeutic window of blood tumor treatment in otherwise poor survivors (4–9), thus confirming TCR-engineered T cells as a new therapeutic.

MAKING TUMOR SPECIFIC T CELLS: FROM TCR GENE TRANSFER TO TCR GENE EDITING

The ability of T cells to respond to a wide spectrum of foreign antigens relies on the high variety of TCRs, heterodimeric glycoproteins composed of one α and one β chain associated to the CD3 complex (54), able to specifically interact with antigenic peptides bound to human leukocyte antigen (HLA) restriction elements (55). A series of genetic rearrangements in the α and β chain genes occur slightly differently in every single cell, thus creating a heterogeneous TCR repertoire that can recognize a vast epitope array. Hence, to fully characterize the T cell specificity it is necessary to determine the rearranged α and β chain sequences. In the 80s, the progression of genomics allowed the isolation of TCR genes (56, 57) and the study in detail of their sequences. The advent of next generation sequencing technologies rendered feasible a comprehensive identification of tumor specific TCR sequences that are today used to genetically engineer T lymphocytes in adoptive T cell therapy studies.

Gene Transfer at the Service of ACT

Most TCR-based gene therapy approaches rely on the ex-vivo transduction of T cells with viral vectors. The first vectors used in gene therapy were adenoviruses (58), vectors endowed with high cargo capacity (up to 30 kb) but unable to foster transgene integration in the host genome. This feature reduced the adenoviruses utility for ACT: since T cells robustly proliferate upon antigen encounters, integration of the transferred TCR genes in their genome is critical to the preservation of transgenic specificity in T cell progeny. Furthermore, the immunogenicity of adenoviral proteins, highlighted by the high incidence of adenovirus-specific neutralizing antibodies in humans, potentially leads to viral inactivation (59) or to life-threatening inflammatory responses (60), thus limiting their exploitation. Retroviral vectors (RV), instead, have been broadly used because of their wide cell tropism (61, 62), good integration capacity, and for the high and stable gene expression they convey. Cell division is required for RV transduction, but this limitation does not impact their use since T cells are highly proliferating in vitro. RV have been widely used to deliver a variety of molecules, including suicide genes (63–65), TCRs (28, 66), and CARs (53) in T lymphocytes. Lentiviral vectors (LV) gained interest more recently, particularly for their efficiency profile and their capacity for transducing dividing as well as non-dividing cells, a feature particularly relevant for the genetic manipulation of stem cells (67). The safety profile of RV and LV is guaranteed by a vector design ensuring replication incompetence (68, 69) and has been proven in human trials (70–

72). Adeno-Associate Viruses (AAV) (73) have been widely used in cancer gene therapy and proven to be well-tolerated and safe. Still, the need to synthesize the complementarity strand to promote transgene integration represents a limitation. To circumvent the process, both strands can be packaged as a single molecule to pair and form a dsDNA as a self-complementary AAV vector (scAAV). While this technological advancement allowed AAV to be independent from host cell complementary strand synthesis (74), it almost halved the vector packaging capacity. Nonetheless, scAAV outperformed conventional AAVs in terms of efficacy in preclinical models (75, 76). Integrating viral vectors insert the genetic cassette semi-randomly into the host genome, thus potentially leading to unwanted insertions in exons, that leads to disruption of the gene hit, or in enhancer regions, potentially altering gene regulation. Theoretically, viral integrations in oncogene regulatory elements or in tumor suppressor genes may contribute to oncogenic transformation (77), a rare event that, most importantly, has never been reported in engineered T cells. Nevertheless, several strategies have been implemented to increase the safety profile of integrating vectors. These include the elimination of viral genes responsible for virulence (78), splitting packaging genes into different plasmids (79), and the introduction of inactivation switches in the vectors' constructs (80). In addition, chromatin insulator elements can be added to the flanking regions of the insertion cassette, acting as physical barriers to hamper the interactions between viral enhancers and other regulatory

elements (81). To increase the safety profile of viral-mediated gene delivery, it is nowadays possible to instruct viral vectors to integrate into specific “safe harbors,” genomic regions distant from transcribed genes, enhancers, regulatory RNA, or microRNA regions to minimize the risk of perturbing gene expressions (82, 83). Identified safe harbors are either housekeeping genes, e.g., AAVS1 (84, 85) and ROSA26 (86), or specific loci not affecting gene expression and identified by mapping the viral vectors integration sites (87). In addition to viral vectors, a variety of non-viral gene transfer methods have been explored to transfer transgenes into T cells (Figure 2). Transposons are mobile elements composed of a transposase gene flanked by inverted terminal repeats (ITRs) (88). For the purpose of gene therapy, two plasmids are transfected together, one encoding for the transposase and the other one containing the expression cassette flanked by ITRs; upon entry, the transposase integrates the gene of interest in the genome. The so-called “Sleeping Beauty” transposon system gained the widest application, being able to transfer up to 6 kilobases into mammalian cells (89). This system may be considered as efficient as viral gene transfer, at least in vitro, if the transposon/transposase ratio is tightly controlled (90) to avoid the formation of functionally inactive transposase oligomers (91). In general, transient expression of the transposase is usually preferred, and ensured by mRNA electroporation (92).

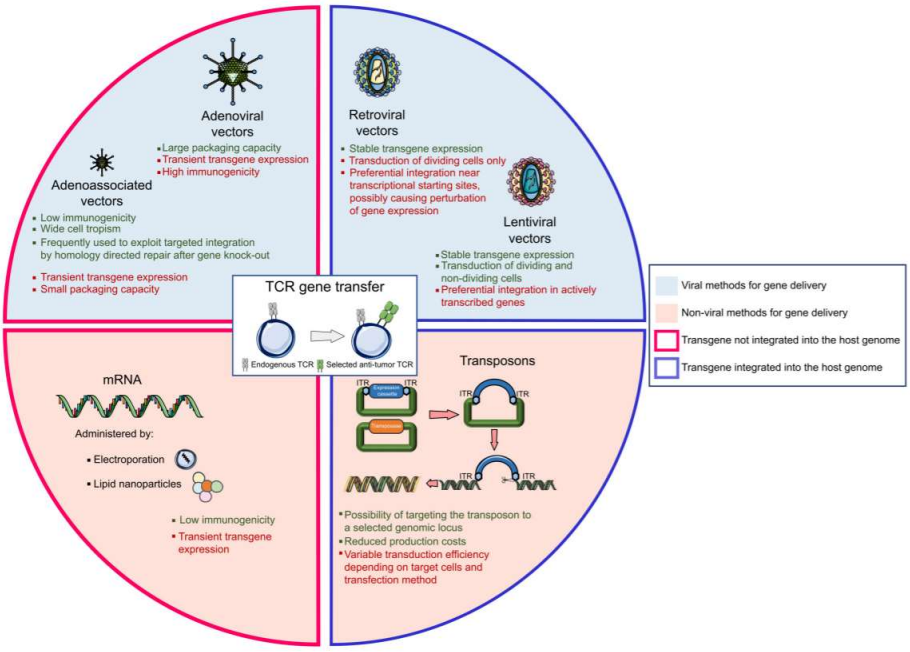

FIGURE 2 | *The landscape of gene delivery methods. The genetic transfer of an exogeneous T cell receptor (TCR) into a donor T cell can be obtained with different vectors, the most widely used being viral vectors, mRNA, and transposons systems. Strengths and weaknesses are listed for each technology.*

Transposons have an acceptable production cost and a low immunogenicity potential. Nonetheless, gene transfer efficiency varies according to the target cell and is sensitive to the size of the expression cassette. In the ACT field, transposons have been efficiently utilized to express functional CARs (9, 93–95) and TCRs (96–98) but their exploitation in clinical practice is still limited. Messenger RNA-based gene transfer, usually achieved by mRNA electroporation or by enclosing the mRNA into lipid nanoparticles (99), is devoid of insertional mutagenesis risk. However, mRNA can convey only transient transgene

expression and in vitro transcribed mRNAs could trigger cellular inflammatory reactions, whose incidence can be mitigated by introducing base modifications in the synthetic RNA (100). In ACT studies, CAR-T cells have been generated using mRNA gene transfer; still, multiple administrations of the engineered T cells were necessary to mediate tumor regression. Of interest, in a phase I clinical trial, CAR-T cells targeting mesothelin generated upon mRNA electroporation and administered to patients with advanced cancers proved safe and mediated anti-tumor activity despite transient persistence (101).

TCR Gene Transfer

In 1986, a murine exogenous α and β TCR gene pair was successfully transferred into another cytotoxic T cell, endowing the recipient cell with a new TCR specificity (52). The efficacy of the TCR gene transfer was tested in immortalized T cells, where the cDNA of a MART-1-specific TCR isolated from melanoma TILs was stably expressed (102) and, shortly after, on primary human T cells, granting recipient T cells cytolytic activities specifically toward their target epitope. The encouraging safety and efficacy pre-clinical results observed by targeting MART-1 (103, 104), the murine MDM2 oncoprotein (105) and the EBV-associated LMP protein (106), prompted the approval of TCR gene transfer in human clinical trials. In the context of metastatic melanoma, TCR-transferred T cells successfully induced tumor regression in two out of 15 patients and persisted in vivo for at least 2 months after infusion (28). The safety profile of TCR-transferred T cells specific for MART-1 or gp100 was similar to

that of TILs, with on-target off-tumor toxicities toward skin and eye melanocytes (107). Thanks to these seminal results, the TCR transfer clinical application was widened to other relevant targets, such as the New York esophageal squamous cell carcinoma (NYESO)-1, expressed in Melanoma and Synovial Sarcoma (30, 43) and the carcinoembryonic antigen (CEA), expressed in colorectal cancer (31). The broader use of TCR transfer, however, underlined some of the limitations of this new technology. Firstly, endogenous and exogenous TCRs competed for assembly with the CD3 subunits (108), thus resulting in suboptimal surface expression of the transferred receptor. Secondly, α and β chains from exogenous and endogenous TCRs could mis-pair, further diluting the expression of the correctly paired tumor-specific receptor and introducing new specificities, potentially leading to unwanted toxic reactivities (109). TCR mispairing has been described in vitro by using human cells (109) and was associated to immune-mediated toxicities in murine models (110). So far, no events potentially associated to TCR mispairings have been reported in clinical trials. Nonetheless, to address this safety issue and to increase the expression level of the exogenous TCR, several strategies have been proposed: (i) the replacement of the human TCR constant region, essential for pairing, with a murine-derived sequence (111), (ii) the introduction of cysteine residues to stabilize proper pairing of the TCR chains via disulphide bonds (112, 113), (iii) the generation of a human TCR incorporating the CD3ζ chain (114), (iv) the swapping of TCR constant domains

between the α and β chains (115), and (v) the incorporation in the vector cassette of small interfering RNA sequences able to reduce the expression of the endogenous TCR genes (116). To overcome the limitations of TCR gene transfer, nascent genome editing technology has been exploited to develop the TCR gene editing approach (117).

Genome Editing in the Service of ACT

The use of artificially modified nucleases enables the disruption of the genes encoding α and β chains of the endogenous TCR, thus completely and permanently avoiding the risk of TCR mispairing and the mutual dilution effect resulting from the expression of four TCR chains in a single cell. Artificial nucleases bind DNA in selected genomic regions, in which they mediate a DNA double-stranded break (DSB), either repaired by the high-fidelity homologous direct repair (HDR) system or by the mutagenic non-homologous end joining repair machinery (NHEJ). HDR uses a DNA template, usually the sister allele, to correct the break and restore gene function, while NHEJ introduces or erases a variable number of nucleotides upon repair, with the chance of creating premature stop codons and frameshift mutations. Both repair mechanisms can be exploited for gene therapy purposes, with different aims: HDR is suitable for gene correction when an exogenous donor DNA template is delivered with the nuclease (118), while NHEJ is preferred if a gene has to be disrupted (119). The zinc fingers nucleases (ZFNs), among the first efficient gene editing tools developed, are large multimeric molecules, each monomer targeting a 3-4

DNA base pair sequence, linked to the FokI endonuclease (120). While the multimers confer ZFNs specificity, that can be increased even by elongating the length of the multimers, the endonuclease mediates DNA cleavage. This gene editing tool supported the first genome editing clinical applications (121) and the first TCR gene editing approach. In fact, ZFN-mediated disruption of the endogenous TCR has been combined with LV gene transfer to efficiently generate WT1-specific TCR-edited T cells that outperformed TCR gene transferred T cells in safety, specificity, and efficacy in vitro and in vivo (117). Despite these encouraging results, the first protocol reported required 40 days of manufacturing to be completed and multiple manipulation steps. To improve feasibility, a single editing strategy, based on the sole disruption of the TCR α chain gene was proposed resulting in optimal expression of a NY-ESO1-specific TCR and efficient tumor rejection in animal models, in the absence of adverse events (122). An alternative to the ZFNs system is represented by transcription activator-like effector nucleases (TALENs), small (33–35 amino acids) transcription factors fused with an endonuclease domain (123). TALENs specificity is modified by mutating the two hyper-variable residues that bind the DNA helix. The nucleotide sequence recognized by TALENs is fairly short, increasing the likelihood of off-target binding sites throughout the genome and potentially leading to unwanted DNA breaks. To overcome this limitation, the DNA binding regions can be elongated by multiplying the hyper-variable residues, hence increasing TALENs specificity at the expense of a more

complicated protein design. In the ACT context, TALENs have been proficiently used to disrupt endogenous TCR genes in preclinical models and in clinical trials (124, 125). Meganucleases represent alternative genome editing tools originating from naturally occurring endonucleases that directly bind DNA (126). Meganucleases present some advantages, such as the generation of a 3′overhang at the cleavage site that favors HDR when compared with 5′overhang, and their overall small size, suitable for several delivery methods (127). Still, the difficulty in separating the endonuclease cleavage domains from the DNA binding site limits the number of DNA sequences that can be targeted. To circumvent this obstacle, chimeric proteins have been generated by fusing meganucleases with ZFNs and/or TALENs DNA binding domains, at the expense of increased manufacturing complexity (128, 129). The introduction of the CRISPR/Cas9 nucleases, bacterial proteins adapted to excise phage DNA fragments (130), completely revolutionized the genome editing field. While ZFNs and TALENs recognize the target DNA sequence via protein-DNA interaction, the CRISPR/Cas9 system relies on a short RNA sequence (single guide RNA, sgRNA). The RNA interacts with the Cas9, conferring the binding specificity and guiding the nuclease activity (131). The CRISPR/Cas9 platform is highly efficient and versatile, since the specific DNA binding is entirely mediated by the sgRNA, short enough to be easily synthetized in vitro but long enough to ensure high specificity. Compared to the previously developed nucleases, the CRISPR/Cas9 system provides three major

advantages: (i) rapid and relatively inexpensive manufacturing, (ii) the possibility of multiplex genome engineering obtained by simultaneously targeting several genes, and (iii) compliance with several delivery systems adapted to different cell types (132). Multiplex genome engineering is a remarkable feature of CRISPR/Cas9, not easily achieved with other nucleases. The possibility of disrupting genes in a single step streamlined different editing procedures (133) and had a direct impact on ACT manufacturing processes, where the synchronous disruption of the α and β TCR chains (42, 134) can sensibly decrease the in vitro manipulation time. In addition, a template strand can be delivered together with the CRISPR/Cas9 system, allowing the integration of the genetic material exactly at the cleavage site (135). Different tools can be employed to deliver CRISPR/Cas9 complexes into cells. Plasmid delivery has been used (136), but with suboptimal efficiency and with an increased risk of plasmid integration in the host genome. Furthermore, the expression of the Cas9 protein is retained for a fairly long amount of time, increasing the likelihood of adverse immune responses or off-target gene editing. An alternative approach depends on delivering the sgRNA together with the in vitro transcribed Cas9 mRNA (137), ensuring transient Cas9 expression but posing the risk of decreased cleavage efficiency. Lastly, the native Cas9 protein can be pre-assembled in vitro with sgRNA in a ribonucleoprotein complex and then electroporated into the target cells (138). This transfer method overcomes the need for transcription/translation and the risk of intracellular degradation

of the free sgRNA (139), thus improving safety and reducing off-target mutagenesis risks. As for ZFNs (140–142) and TALENs (143, 144), a side effect of the CRISPR/Cas9 system is the risk of editing off-target genes. The nuclease activity can potentially cause DNA strand breaks in other genomic regions, knocking-down unwanted genes or promoting genome translocations (145, 146). Off-target editing can also affect the RNA transcriptome, with toxic consequences for the cell (147). Three methods have been employed to minimize off-targets while increasing on-target activity when using CRISPR/Cas9: (i) the use of modified sgRNAs with higher specificity for the target site, (ii) the titration of the sgRNA and Cas9 ratio (148), and (iii) the introduction of a single point mutation in the Cas9 (149). Furthermore, additional enzymes have been incorporated in the system to mediate base editing without affecting the transcriptome (150). Several techniques have recently been optimized to map off-target cleavage sites. Mutation detection assays using T7 endonuclease followed by deep sequencing of the resulting amplicons have been initially used, but their sensitivity is limited, especially when dealing with large deletions (151). The tendency of integrase-defective lentiviral vectors to incorporate into DSBs can be exploited to barcode regions of Cas9 activity (152). BLESS [direct in situ Breaks Labeling, Enrichment on Streptavidin and next-generation Sequencing (153)] can map double-strand breaks by using biotinylated linkers that are incorporated at the DSB site; the biotinylated DNA regions are then purified and the captured DNA fragments sequenced. One

of the most sensitive off-target detection assay is GUIDEseq (genome-wide, unbiased identification of DSBs enabled by sequencing), where short phosphorylated double-stranded oligo-deoxynucleotides are incorporated into DSBs to detect Cas9 cleavage sites (154). More rarely, nucleases can cause chromosomal translocation that can be detected using high throughput, genome-wide translocation sequencing (HTGTS) methods (155), chromatin immunoprecipitation sequencing (ChIP-seq) (156), and digenome-seq or the recently proposed CIRCLE-Seq (157). All described genome editing technologies have been employed for the modification of either hematopoietic stem cells or T cells. As summarized in Figure 3, genome editing has been exploited with several purposes in the ACT field: to completely redirect T cell specificity (42, 117, 124, 158–160), to avoid the risk of GvHD or fratricide effects mediated by CAR-T cells (161–165), to make adoptively transferred T cells resistant to the immunosuppressive environment (42, 166–173) or to lymphodepleting drugs (174, 175). The high efficiency of gene editing and the overall flexibility of the CRISPR/Cas9 system makes it the most relevant tool to precisely and rapidly edit high numbers of T cells to be used in ACT. Nonetheless, issues remain to be addressed regarding the manufacturing, the delivery, and the broad accessibility of genome editing products to patients (176).

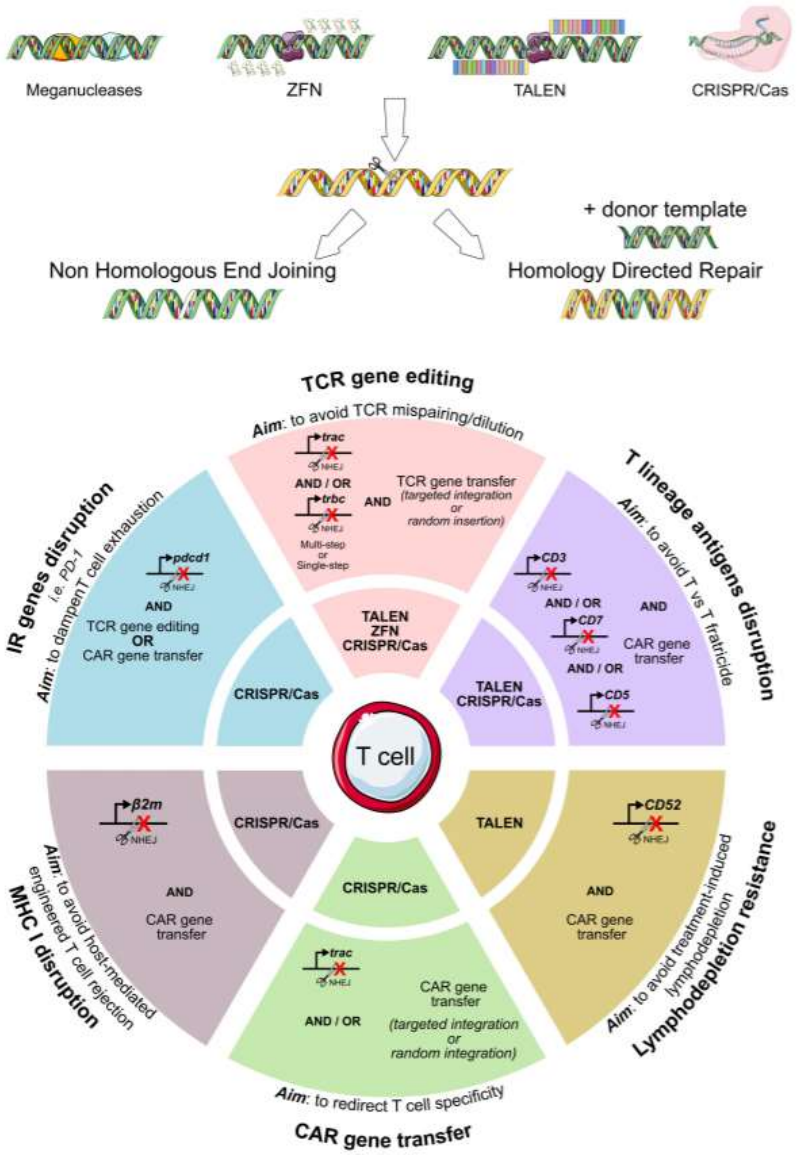

FIGURE 3 | *Genome editing exploitation for adoptive T cell therapy. To eliminate the expression of T cell genes, meganucleases, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs), the CRISPR/Cas9 system can be employed. A summary of the genes edited in the context of adoptive T cell therapy (TCR- or CAR-T cell immunotherapies) is reported, together with the specific nuclease system used. CAR, chimeric antigen receptor; TCR, T cell receptor.*

ANTIGEN IDENTIFICATION AND TCR GENE HUNTING

Different Classes of Target Antigens

One of the major questions in today's adoptive T cell gene therapy is the choice of the target antigen. Theoretically, the ideal candidate should be (i) expressed on tumor cells and not on healthy tissues (to avoid toxicities), (ii) expressed on cancer stem cells (to promote tumor eradication), (iii) associated with the oncogenic process (to reduce the risks of tumor immune evasion), (iv) able to elicit an immune response, and (v) efficiently processed and presented in the context of a common HLA allele (177). Unlike CAR-T cells, TCR-redirected T cells can target antigens independently of their intracellular localization, as soon as they are processed and presented by HLA molecules. For the majority of cancer types, the ideal antigen is yet to be identified, and the search is proving more difficult than expected, particularly for those tumors with undefined clonal evolution and not fully understood in terms of molecular pathogenesis. The two major classes of antigens in the context of ACT are tumor-associated antigens (TAAs) and neoantigens. TAAs are epitopes originated from endogenous wild-type proteins whose expression is increased in tumors and limited in magnitude or in spatial expression in healthy tissues; neoantigens are instead epitopes derived from somatic DNA alterations. Different TAAs have been investigated for their potential therapeutic relevance (178): cancer/testis antigens such as melanoma-associated antigen (MAGE)-A3 (179, 180), MAGEA4 (35, 181), and New York esophageal squamous cell carcinoma (NY-ESO)-1 (182);

oncogenes/ onco-suppressors such as WT1 (50) and p53 (183); tissue-restricted/differentiation antigens such as MART-1 (28, 34), gp100 (29), or CEA (31). The toxicity and safety profile of T cell therapies targeting TAAs seem to be heterogeneous, depending on the chosen epitope. Several TAA-specific TCRs showed important side effects: cardiovascular and neurological toxicities [with MAGE-3 specific T cells (32, 33)], undesired recognition of melanocytes (with MART-1 specific T cells) (28), and severe transient colitis (with CEA specific T cells) (31). TCR targeting NY-ESO-1, instead, conveyed no toxicities but limited clinical response in a small cohort of patients affected by melanoma and synovial sarcoma (30) and of sarcomas/nerve sheet tumors (41). Its application appeared promising in various pre-clinical tumor models, both in terms of efficacy and safety [bladder (184), ovarian (185), esophageal (186) and prostate cancers (187), multiple myeloma (188), medulloblastoma (189), non-small cell lung carcinoma (190) mesenchymal tumors (191), and breast cancer (192)] but clinical studies are needed to validate these results. The expected good toxicity profile may reside in the limited NY-ESO-1 expression in healthy tissues, essentially restricted to the gonads, an immune-privileged site. Focusing on neoantigens appears to be another efficient choice for cancer immunotherapy (193). Neoantigens represent the main target of autologous T cell responses in patients treated with TILs (194). Since neoantigens peptides are not presented to thymocytes, T cells specific for those epitopes are not deleted by central tolerance mechanisms, thus the chance of retrieving high

affinity TCRs is enhanced. However, the clinical exploitation of neoantigens in TCR-mediated ACT is hindered by their intrinsic qualities: (i) neoantigen-forming mutations tend to differ among patients, making difficult the development of a widely applicable immunotherapeutic product, and (ii) neoantigen expression might be heterogeneous across the tumor tissue. This is particularly true for passenger mutations, random alterations caused by genome instability and not homogeneously spread in the tumor mass (45). In addition, the mutational rate is highly variable in different tumors. In fact, the likelihood of identifying neoantigens from tumors with a low mutational load is poor and their relevance as therapeutic targets is limited (195). Despite these limitations, initial reports highlighting the occurrence of immunogenic neoantigens widely shared in tumor cells and among patients are emerging (196). Additionally, thanks to their broad expression in cancer cells and to their involvement in oncogenesis, founder mutations may be considered promising ACT candidate targets (197). However, epitopes arising from founder mutations may be poorly immunogenic or differ among patients, thus reducing their appeal. Overall, the interest in neoantigens has increased in recent years, and more than 100 clinical trials exploiting these candidates are currently in progress. However, the ongoing trials largely rely on in vivo peptide vaccination rather than on engineered T cell infusions, further underlining the difficulties in validating a proper target for adoptive T cell therapy (198). A third possible choice of targets is represented by Minor Histocompatibility antigens (MiHA),

peptides derived by polymorphic intracellular proteins, potentially overexpressed by tumor cells. The most known examples are HA-1 and HB1 (199–201), expressed selectively by hematopoietic cells and by different liquid cancers, thus constituting highly relevant targets (202, 203). Since MiHA derive from coding regions of polymorphic genomic sites, coupling HA-1-directed T cell therapy with Allo-HSCT from a recipient not harboring the same single nucleotide polymorphism (SNP) could provide a valuable therapeutic strategy, able to spare the donor hematopoietic stem cell population while eradicating cancer. So far, HA-1-specific engineered T cells have been tested in vitro and in preclinical models, showing an optimal efficacy and safety profile (204). However, MiHA targeting requires specific combination of SNPs and HLA allele between the donor and recipients, which reduces the broad applicability of this approach. A fourth source of antigens are proteins encoded by oncogenic viruses. Being involved in tumorigenesis, viral epitopes are shared uniformly by the tumor mass and, due to their nature of foreign molecules, they’re potentially highly immunogenic. Hereby, the isolation of high-avidity viral-specific T cells is easier than with other antigen classes, making these targets particularly appealing for ACT. Unfortunately, few tumors have a clear viral pathogenesis, thus this antigen source is currently mostly limited to some HPV and EBV-associated malignancies (205, 206).

The Challenges in Selecting Tumor Antigens and Tumor-Specific TCRs

Apart from choosing the antigen class of interest, tumor screening for immunologically relevant epitopes is a particularly laborious process. The most used and standardized strategy relies on paired whole-exome sequencing or, alternatively, the comparative RNA sequencing analysis of tumor and of healthy tissues to determine differently expressed genes. Once the most promising hits are identified, in silico assessment of HLA presentation and binding (207) is required. In this regard, prediction algorithms are still suboptimal (208), not always accurate and they very often lead to false positive results (209). Thus, extensive validation of the results is always required. The analysis of the tumor ligandome can now be considered a good alternative approach. This technology is based on mass spectrometry typing of all the peptides eluted from the HLAs of a specific tumor type or tumor cell line (210). Here too, the amino-acidic sequences retrieved need to be validated for their relevance in the tumor setting. Despite the great potential of this methodology, some limitations still remain, namely the high number of tumor cells needed, an endpoint is not always attainable when using primary tumor samples, and the low number of epitopes retrieved upon in silico and in vitro screening (211, 212). Finding the perfect immunogenic epitope is only half of the issue, since the typing of epitope reactive TCRs may prove challenging as well. To isolate a tumor-reactive T cell, a proper source must be selected, the clone of interest must ideally harbor a high avidity TCR and reach a significant level of frequency and

purity. These prerequisites are fundamental for the successful retrieval of functional TCR αβ nucleotide sequences. T cell isolation is particularly challenging especially for poorly immunogenic tumors and for those in which the T cell infiltrate is scant. Recent works showed that, in different cancers, tumor-reactive TILs represent only a minimal fraction of the total T cell subpopulation infiltrating the tumor (47) and that reinvigoration of tumor immunity is associated with recruitment of new T cell clones (213). These observation lead to the hypothesis that selecting T cell clones on the basis of their abundance in the tumor may be misleading. In addition, the poor efficiency of the T cell ex vivo expansion procedures may be taken in consideration as a limiting factor in the retrieval of tumor-specific T cells, especially when studying lymphocytes originated from tumors characterized by a microenvironment known to blunt T cell proliferation (214). When tumor specific T cells are retrieved, the greatest challenge of the TCR sequencing step is the correct pairing of the cancer-specific α and β TCR chains for each T cell clone. The initial approach for the identification of the TCR repertoire was based on single cell cloning coupled with Sanger sequencing (215, 216), which makes it difficult to estimate the overall repertoire diversity. The field greatly benefited from the introduction of high-throughput sequencing technologies which enabled researchers to profile the diversity of millions of TCR molecules in the analyzed samples. With this approach a complete overview of the TCR sequences constituting the repertoire of the sample is obtained (217–219). However, to

successfully characterize and select the TCR αβ pair of interest among the numerous sequences retrieved, the specimen needs to be enriched in anti-tumor specificities in order to obtain an oligoclonal population. To this aim sequencing can be preceded by enriching steps, such as co-culturing TILs with tumor cells harvested ex vivo or performing serial stimulations with professional antigen-presenting cells pulsed with the peptide (or peptide library) of interest. In the latter case, an entire protein sequence can be screened by epitope scanning (220). An interesting approach that avoids the ex vivo enrichment step is PAIRseq: the sample is split into parallel PCR runs, each one tagged with a specific barcode, and then the results are deconvoluted to identify proper TCR pairs (221). An evolution of this laborious setting is perhaps single cell RNA sequencing, where the use of a cell-specific oligo-DNA barcode allows researchers to retrieve TCRs at single cell resolution (222). The typical output of these systems is generally hundreds or thousands of TCR pairs, questioning whether or not faster sequencing translates to a more laborious validation phase. Alternatively, recent advances in the proteogenomic field may speed up the selection of tumor-specific TCRs. In fact, it is now possible to combine single-cell resolution TCR sequencing with barcoded multimers loaded with a specific HLA molecule and with a selected tumor epitope (222). This approach is extremely helpful in characterizing, in a single step, both the tumor specific TCR sequence and its epitope specificity. This new technology may greatly speed up the isolation of TCRs directly from human

samples, avoiding any enriching steps and jumping directly to the functional validation of newly retrieved TCR sequences in vitro. Another point of discussion is the choice of the ideal specimen to be used for the isolation of tumor-reactive T cell clones. Hunting for tumor-specific T cell receptors directly from the tumor site in patients has historically been the most straightforward choice (17) since anti-tumor reactivities can be intuitively more abundant in the tumor mass. However, the development of a tumor implies an escape from immune surveillance, suggesting either that the infiltrating T cells present at the tumor site were not efficient enough to eradicate the disease, thus questioning their use, or that these highly specific cells were blunted in their activity by the tumor microenvironment (223). In the latter case, the anti-tumor efficacy of the tumor-specific T lymphocytes assessed by in vitro functional assays upon isolation may not be informative, but the TCR is worth isolating and employing in TCR transfer approaches. Recent reports (224, 225) also demonstrated that the exhaustion signature could be exploited, defining a T cell subset enriched with neoantigen-specific T cells. Since tumor-reactive T cells also circulate throughout the body, patients' peripheral blood and lymph nodes from tumor patients are suitable sites to harvest these cells, and often the only available sites for tumors that cannot be excised. Tumor-specific T cells have been found in tumor-draining lymph nodes, and efficiently used for ACT (226). Melanoma-specific T cells have been enriched and expanded ex vivo starting from peripheral blood (227). However, at least in some tumor types, the low frequency

of circulating tumor-reactive T cell clones (228) might impair their retrieval.

Trading Toxicity With Efficacy

In the process of hunting for new TCR specificities, the aim is to define the TCR sequences most efficiently mediating tumor lytic functions. These highly promising TCRs can be collected and used for off-the-shelf immunotherapeutic approaches readily accessible for each candidate patient. The leading TCRs are the ones with the highest binding affinity and avidity (229) toward the HLA-peptide complex, the fastest association rate and the slowest dissociation speed (230, 231). According to this concept, isolated TCRs were screened for strong and fast killing efficacy and the most suitable ones were further developed. In addition, TCRs were modified in their complementary determining regions (CDRs) in vitro to artificially increase their affinity for the target, thus overcoming the barrier of thymic selection, that deletes thymocytes harboring autoreactive high avidity TCRs. Several approaches have been recently exploited with the ultimate aim of generating high affinity TCRs: (i) mutations in complementarity determining regions by sequential single amino acid substitutions (232–234), (ii) vaccination of mice and consequent retrieval of peptide-reactive TCRs (235), (iii) murine thymic selection to mutagenize one of the TCR chains (236), (iv) transfer of the entire human TCR αβ gene loci into mice to educate T cells against human self-antigens (237, 238), and (v) DNA replication error-prone yeast cells to modify the α and β chain variable regions (239). These techniques have been particularly useful

when dealing with TAAs, since highly avid T cell clones recognizing these antigens in a specific and efficient manner have been difficult to isolate. The advantages of TCR affinity enhancement have been demonstrated in vitro (240–242) and in clinical trials (30, 36, 37, 39). The opposite side of the coin, though, is the risk of enabling engineered T cells to respond to tissues displaying low antigen expression, fostering on- or off-target off-tumor toxicities (243). A clinical trial with an artificially enhanced TCR directed against MAGE-A3 proved highly efficient in eradicating tumor cells but was also endowed with a remarkable off-target off-tumor cardiac toxicity (32, 244), leading to the suspension of the trial. On the same line, an artificially-enhanced TCR directed against CEA was associated with the occurrence of on-target off-tumor reactions and strong systemic inflammation, underlining the limitation of procedures aimed at enhancing TCR avidity when targeting TAAs (31). As a matter of fact, it proved very challenging to predict any possible cross-reactivity of engineered T cells against human tissues. The most commonly used techniques, in vitro testing and epitope alanine scanning, have been further refined in recent years (241, 244) and extended to scan all the possible amino acid substitutions in the target epitope (245). Nevertheless, concerns about affinity enhancing techniques still persist. In this context it might prove safe to introduce a kill switch in engineered T cells, enabling their ablation if necessary (63, 246). Otherwise, the conditioning regimen prior to T cell infusion can be modulated, reducing therapy-induced tissue damage and antigen spreading, two

phenomena potentially fostering off-target reactions. In addition to the adverse events observed in clinical trials, the modification of TCR affinity may also convey excessive activation signals to T cells, that could lead to hypo-functionality and/or premature T cell death. An extensive and continuous activation is indeed detrimental for T cell function (247–249). Reports in the context of TCR engineering are still scant (40) but new insights from the field of CAR-T cell therapy have highlighted this issue (250). Since TCRs is even more sensitive to antigen density variations than CARs (251), it's reasonable to suppose that this mechanism could be relevant in the context of TCR engineering. The picture might even be more complex with TCRs, since costimulatory signals are more tightly involved in the immunological synapse (252) than in CAR-T cells, a feature that provides more flexibility but that requires greater attention to signal tuning.

PERSISTENCE OF ADOPTIVELY TRANSFERRED T CELLS AND CLINICAL RESPONSES

Nowadays, it's still unclear which variables impact long-term persistence in adoptively transferred T cells the most. This scientific question is particularly relevant because T cell persistence is a fundamental requisite for durable immunosurveillance. Whether immunosurveillance is required for the maintenance of clinical remission is still a matter of debate. However, reports indicate that the sustained and prolonged in vivo expansion of engineered T cells correlates with relapse-free survival and tumor control (39, 40). Several measures have been implemented to foster ACT persistence, including the use of

preconditioning regimens and the choice of manufacturing protocols able to enrich memory cells. The use of a lymphodepleting conditioning regimen prior to ACT inhibits host immune cells, including regulatory T cells (Tregs), and favors the accumulation of homeostatic cytokines (253), critical in sustaining engineered T cell engraftment and expansion (254, 255). The administration of low-doses IL-2 can also sustain adoptively transferred T cell proliferation in vivo (17, 19) and has been included in several ACT protocols. The use of IL-2, however, conveys the risk of toxic reactions related to the activation of bystander host cells. Furthermore, prolonged administration of this cytokine was shown to preferentially expand Tregs (256). The cellular composition, in terms of subsets and differentiation of the therapeutic product, has a direct impact on efficacy. Long telomeres (257), CD27, and CD28 co-expression (258) on TILs were associated to clinical responses in initial ACT trials. With engineered T cell products, the co-infusion of CD4 and CD8 cells fosters T cell persistence (8). In some ACT applications, manufacturing includes a selection step to enrich the product in CD8 central memory (TCM) lymphocytes (259). The polyfunctionality of adoptively transferred engineered T cells correlated with clinical responses in several clinical trials, targeting NY-ESO-1 (36), MART-1 (29), and WT1 (38). The relevance of the intrinsic qualities of the infused T cells was further highlighted by the observation that even low numbers (105) of highly fit engineered T cells were sufficient to mediate anti-tumor responses (260, 261). Based on

these observations and with the final aim of improving the fitness of the infused T cell products, different T cell expansion protocols have been developed and compared. T cell activation with phytohaemagglutinin (PHA) was shown to promote T cell expansion, but also T cell terminal differentiation (262), whereas stimulation with an anti-CD3 monoclonal antibody coupled with high doses of IL-2 reduced the TCR repertoire diversity and enhanced apoptosis (263). The combination of TCR triggering with co-stimulation, obtained thanks to the use of anti-CD3 and anti-CD28 antibodies, followed by the culture of the T cells in the presence of high-doses of IL-2, improved the fitness of the cellular products (264). Despite showing potent tumor killing abilities in vitro, effector T cells were paradoxically less effective than early differentiated T cells when transferred in tumor-bearing mice (265). These results can be explained by the progressive model of mature T lymphocyte differentiation (266). Upon antigen encounter naïve T cells differentiate into stem cells memory T cells (TSCM), TCM and subsequently in effector memory and terminally differentiated cells, progressively losing proliferating and persistence ability. Recent studies demonstrated that TSCM, originating directly from naïve T cells (267, 268), are endowed with stem cell-like properties and with the ability to persist for decades in vivo (269, 270). The persistence capacity of TSCM was confirmed in patients treated with genetically engineered T cells, in the context of both malignant (271) and non-malignant (272) diseases. In the attempt to preserve this early-differentiated T cell subset, the in

vitro protocol used for TILs expansion was shortened (273) and the anti-CD3/anti-CD28 antibodies were conjugated to cell-size beads (274) or nano-matrixes (275). The cytokine cocktail used to sustain T cell expansion in vitro also plays a major role in determining the fitness of cellular products. The introduction of Interleukin-21 in the culture medium promotes a TCM phenotype (276) while Interleukin7 and Interleukin-15 supplementation, in the absence of IL-2, expands the TSCM pool (277, 278). As already mentioned, the intrinsic T cell fitness has an impact on the persistence and thus on the efficacy of the T cells used in ACT. In CAR-T cell therapy trials, CAR-T cells isolated from poor responders expressed genes associated with effector memory differentiation and apoptosis, a glycolytic metabolism, and hypo-functionality. Conversely, efficient anti-tumor activity was associated with an early-memory differentiation signature, expression of CD27, and absence of the exhaustion marker PD-1 (259, 279, 280). Similar observations were reported in TCR-based studies. The transfer of a WT1-specific TCR into Epstein-Barr virus-specific donor CD8 T cells has been exploited to generate functional, memory-like cellular products (281). Using this manufacturing procedure, high levels of engraftment and long-term persistence were observed in humans (40). Furthermore, in an ACT trial with TCR engineered T cells, the extent of cytokine release was associated with anti-tumor activity (107). Once adoptively transferred, T cells interact with the host immune system. Competition of infused and unmodified T cells for proliferative signal accessibility may decrease cell survival.

Furthermore, engineered T cells may be recognized and rejected by the host immune system, thus abrogating ACT efficacy. In the autologous setting, rejection could be due to an immune response against the transgene products, as observed in preclinical models (282). An immune response against the murine-derived CD19 CAR was described before and after cell therapy. In the JULIET study (283), the majority of treated patients showed detectable levels of pre-existing anti-murine CD19-specific antibodies, that further increased upon CAR-T cells infusion. Nonetheless, the kinetics of engraftment was unmodified, and rejection barely detected, questioning the relevance of these markers in predicting CAR-T cells persistence. T cell mediated immune responses against Herpes Simplex Virus-derived Thymidine Kinase (TK) epitopes were described in patients treated with TK-DLI, often leading to the elimination of genetically engineered T cells (284). The immunogenicity of TK could be overcome in the HSCT context by infusing transduced T cells during the immunosuppressive phase that follows transplantation (285). For ACT applications that do not involve HSCT, the minimization of transgene immunogenicity remains a desirable and relevant goal.

OVERCOMING BARRIERS TO T CELL HOMING AT TUMOR SITE

The efficacy of ACT is strictly dependent on the ability of the infused product to infiltrate neoplastic lesions. This is particularly difficult in solid tumors, often characterized by a dense stromal architecture, an abnormal vessel structure, and by alteration of

chemo-attractants that impinge T cell homing (286–288). In recent years, different strategies have been developed to counteract these factors and hence, increase the ability of T cells to migrate inside neoplastic lesions, where they can properly exert their anti-tumor activity.

Interfering With Cancer Metabolism and Chemokines to Increase ACT Infiltration

The connection between metabolism and oncogenesis is welldocumented. Metabolic reprogramming, a hallmark of cancer, does not only impact on cancer cell survival and proliferation, but also on the immunological microenvironment. The presence of reactive nitrogen species (RNS), produced by several human tumors, can induce nitration of different proteins present in the tumor microenvironment (TME) with consequences on T cell functions (289). As an example, the nitration of the CCL2 chemokine decreases its binding affinity for CCR2, thus reducing T cell recruitment. In mouse models, preconditioning of the tumor microenvironment with small molecules blocking RNS production increased the CCL2-mediated recruitment of adoptively transferred tumor-specific CD8 T cells (290), making it an interesting target for further therapeutic development. Tumors can alter the fucosylation of T cell surface glycoproteins (291), again impinging T cell homing at tumor sites. The ex vivo glycoprotein fucosylation increases in vivo migration and cytotoxic abilities of tumor-specific T cells in leukemia, breast cancer, and melanoma models (292). The CXCL12/CXCR4 is an additional relevant axis activated by

neoplastic cells and cancer-associated fibroblasts in several human tumors. CXCR4 expression correlates with desmoplasia, metastases formation, and immunosuppression (293–298). In murine models of leukemia, melanoma and ovarian cancer, CXCR4 inhibition, obtained with blocking antibodies or with the CXCR4 antagonist AMD3100, increased the effector to Tregs ratio at the tumor site and reduced tumor growth (298–301). Stemming from these observations, several clinical trials are now exploring the efficacy of CXCR4 blockade in solid tumors (302).

Exploiting Cancer Vasculature to Foster ACT Infiltration

Tumor neo-angiogenesis involves the formation of a disorganized network of irregular and leaky vessels, inefficient in delivering oxygen, drugs, and immune cells to the neoplastic microenvironment. This process is largely orchestrated by the vascular-endothelial growth factor (VEGF) and results in tumor growth promotion and altered inflammation (303). VEGF inhibitors are currently used in the treatment of several cancers (304) owing, in particular, to their ability to increase T cell tumor homing (305–309). By promoting vessel maturation, VEGF inhibitors positively impact on immunotherapy and ACT (310). Vascular-targeting peptides represent additional effective tools for the precise delivery of small molecules capable of inducing tumor vessels normalization and increased T cells infiltration. In mouse models, tumor-necrosis factor-targeted (TFN) delivery to the tumor vasculature by linking the TNF protein with the CNGRCG angiogenic vessel-homing peptide (NGR-TNF fusion protein) enhanced the local production of immunomodulatory

cytokines, favoring the extravasation of immune cells and improving ACT therapeutic activity (311). The fusion of the TNF superfamily member LIGHT with a vascular targeting peptide (LIGHT-VTP) (312, 313) and the specific delivery of IFN-γ and TNF-α by the homing peptide TCP-1 (314) enhanced endothelial permeability and T cell infiltration in mouse models. The cyclic peptide iRGD (315) and the targeting of the vascular integrity regulator VE-cadherin by CD5-2, a specific inhibitor, facilitated T cell homing to the TME in tumor-bearing mice treated with ACT (316). Although only some of these therapies have been tested in association with ACT, their effect in increasing T cell migration into tumors strongly suggests that strategies aimed at normalizing the neoplastic vasculature could potentially increase ACT efficacy.

Enforcing Chemokine Receptor Expression in T Cells

The interaction of specific chemokines and cytokines with their receptors is a key determinant of immune cell migration, and a mismatch between the chemokines secreted by neoplastic or stromal cells and the receptors expressed by T lymphocytes strongly limits T cell homing in tumors (317–320). In recent years, attempts have been made to correct this mismatch by engineering T cells with receptors for chemokines or cytokines abundant in the TME, showing preliminary encouraging results. In TRAMP mice with metastatic prostate adenocarcinoma expressing high levels of CCL2, the expression of CCR2 in SV40 Tag-specific CD8 lymphocytes increased T cell homing to the tumor (321). In xenograft models, T cell transduction with a RV

encoding CX3CR1 enhanced migration toward human cell lines expressing Fractalkine, the CX3CL1 ligand, and inhibited tumor growth (322). In a lymphoma murine model, adoptively transferred CD8 T cells, overexpressing CXCR4, were preferentially recruited by CXCL12 expressing cells in the bone marrow, to promote tumor control (323). Anchoring IL-4 receptors to the membrane of adoptively transferred T cells increased in vivo tumor homing, cytokine secretion, and the killing of melanoma (324). Lentiviral T cell transduction to express CXCR2 enhanced in vivo homing toward human melanoma in xenograft models, by exploiting the high IL-8/CXCL8 secretion levels (325). Classical chemo-radiotherapy is also able to modulate T cell recruitment at the tumor site, suggesting that its use with ACT can have additive effects. In murine models, chemo-radiotherapy has been shown to increase the local release of CCL5, CXCL9, and CXCL11 in the TME, improving ACT-induced tumor growth control (326). Treatment with doxorubicin in mouse models bearing either murine or human melanoma induced CXCL9 and CXCL10 expression by neoplastic cells and increased the infiltration of adoptively transferred T lymphocytes. The effect was further enhanced if ACT followed a combined treatment with doxorubicin and IL-2 (327). Unfortunately, the involved chemokine receptor pattern was not deeply investigated, leaving undemonstrated a causal relation between the increased chemokine secretion by tumor cells and the increased T cell homing. Overall, these studies prompt further investigation and possible exploitation of

chemokine receptors in the context of ACT. Interestingly, a large analysis of 142 patients enrolled in ACT trials revealed the association of genetically determined alterations in chemokine receptors expression with response to therapy (328), underlining the knowledge gap on the impact of chemokines in ACT.

Generating Genetically Engineered T Resident-Memory Cells

Among TILs, T resident-memory (TRM) cells are permanent tissue-resident T cells able to mount an immune reaction; they've been proposed to be key determinants in the magnitude of antitumor immune responses, and their presence in different human cancers correlates with survival (329–336). It is tempting to assume that the induction and/or manipulation of this T cell subset might improve anti-tumor immunity and disease control. Indeed, in murine models, the administration of anti-cancer vaccines through routes that enhance the induction of TRM is associated to tumor growth inhibition and, importantly, provides protection also at distant sites (330, 337–339). In a melanoma mouse model, ACT with T cells lacking RUNX3, a transcription factor essential for TRM development, strongly reduced TILs accumulation and treatment efficacy (340). These results suggest that the association of ACT with strategies directed at promoting tissue residency of the infused cells might be beneficial to improve tumor control.

SURVIVING THE TUMOR MICROENVIRONMENT (TME)

As already demonstrated in several disease contexts, a longlasting protective memory is a critical requirement for a successful ACT. Despite improvement in manufacturing protocols and fitness of the final cellular products, adoptively transferred T cells have to face a harsh environment while interacting with cancer cells (Figure 4). Different immunosuppressive mechanisms act at the tumor site, with the potential to reduce or even dampen the curative action of ACT. If a T cell encounters the cognate antigen in an immune active environment with plenty of additional co-stimuli, the resultant immune reaction is most likely to be efficient in clearing antigen-bearing cells. However, at the tumor site a variety of signals make T cells chronically exposed to antigenic stimulation in the absence of appropriate co-stimuli. Different TME resident cells and tumor cells are responsible for this, either by directly interacting with T cells or by releasing soluble factors. As a consequence, T cell depletion, anergy, exhaustion, and accumulation of Tregs (341–345) promote tumor immune escape (214).

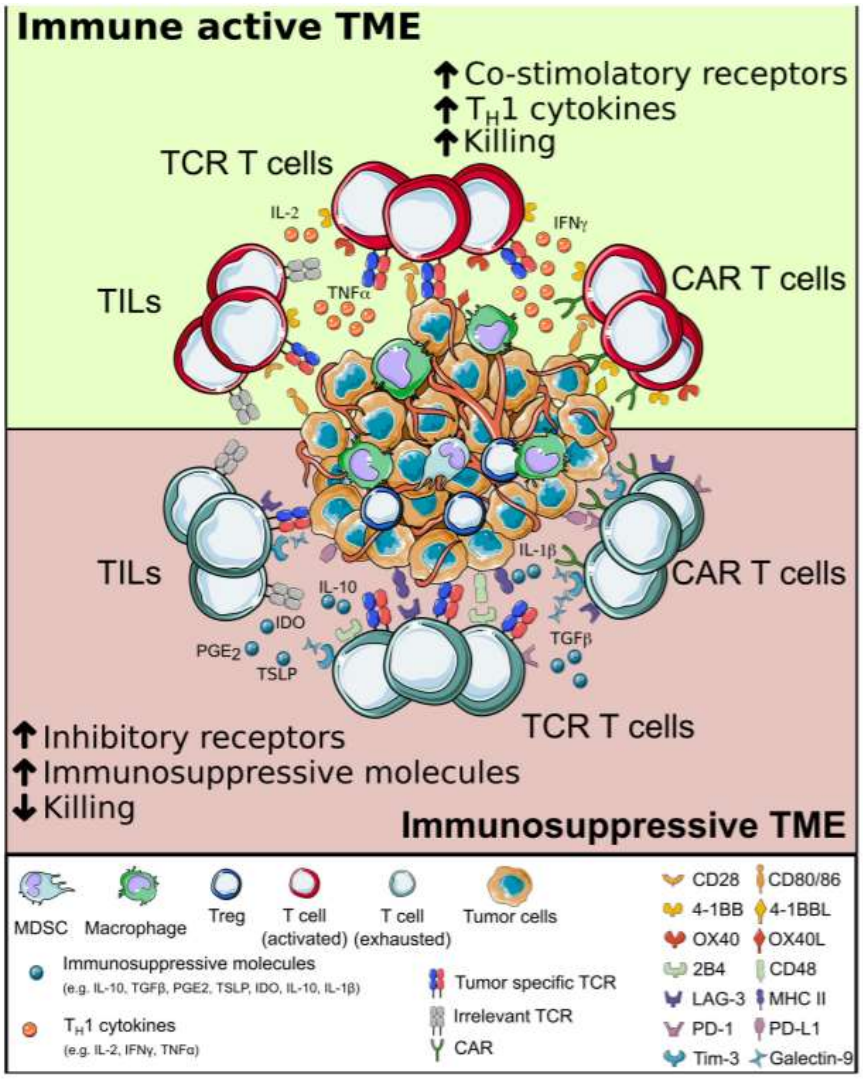

FIGURE 4 | *The interplay between T cells and tumor microenvironment. When adoptively transferred T cells (either tumor-infiltrating lymphocytes, Chimeric Antigen Receptor or T cell Receptor-redirected T cells) infiltrate the tumor, they interact with a complex environment, in which a combination of intracellular signals compete. When inflammatory signals dominate, T cells can perform effector functions and potentially eradicate cancer cells; else, they may become exhausted, have limited survival and fail in killing tumor cells. CAR, chimeric antigen receptor; TCR, T cell receptor.*

Checkpoint Blockade and ACT

Tumor-specific T cells infused to patients and chronically exposed to tumor antigens often enter a dysfunctional state defined as T cell exhaustion that hampers the anti-tumor response and fosters tumor escape (346). T cell exhaustion is a process of progressive and hierarchical loss of effector functions (e.g., chemokines production and cytolytic activities), resistance to activation through TCR engagement, metabolic deregulation, and failure to acquire an antigen-independent memory state (347). The sustained co-expression of multiple inhibitory receptors (IRs) such as PD-1, CTLA-4, LAG-3, Tim-3, 2B4, CD39, CD160, BTLA, and TIGIT was identified as a hallmark of exhausted T cells (TEX) (249) in solid and hematological tumors (e.g., melanoma, leukemia, breast, prostate, ovarian, renal, lung, and hepatocellular carcinoma (348–358). Interestingly, the pattern of inhibitory receptors expressed by TEX significantly varies among different tumor types. This indicates that exhaustion mechanisms are differentially shaped by various tumor microenvironments (248) and suggests that ACT approaches need to be tailored according to the specific features of each tumor. Blocking the interactions between the IRs expressed by tumorreactive T cells and their cognate ligands leads to the reversal of T cell exhaustion (359, 360). Monoclonal antibodies impeding the PD-1/PD-L1 binding (361) and affecting the CTLA-4 axis (362, 363) are able to restore tumor T cell recognition and tumor regression in a relevant subset of terminally ill patients. Due to their efficacy, the use of monoclonal antibodies in association with ACT could greatly benefit T cell

resistance toward the tumor microenvironment. Although still exploratory, this combinatory approach showed encouraging preclinical results in the context of CAR-T cells (364). The use of an immune checkpoint blockade is associated with significant toxicity, indicating that the indiscriminate blockage of inhibitory receptors on the entire T cell repertoire may be deleterious. Indeed, immune-related adverse events (irAEs) occurred in up to 80% of treated patients, and were life threatening in a significant fraction of cases (365). irAEs mainly occurred because potential autoreactive T cells were unleashed and Tregs functionality was dampened (366). In order to maintain the benefit of inhibitory receptor blockade while reducing toxicities, it may be beneficial to counteract inhibitory axes selectively on tumor-specific T cells. This is currently one of the focuses in the T cell-based immunotherapy field. The increased anti-tumor activity of CAR- and TCR-engineered T cells in which an inhibitory receptor gene has been disrupted was shown in different preclinical models (161, 166, 171, 367). Recently, the results of the first-in-human phase I trial with PD-1 disrupted TCR-edited autologous T cells in patients with refractory tumors have been published, highlighting the feasibility and safety of multiplex gene-editing in tumor-specific T cells (42). T cell exhaustion can also be exploited for tumor-reactive T cell isolation purposes. In fact, in the context of melanoma, it was shown that PD-1 expressing TILs are enriched in melanoma-specific T cells. These cells, despite being exhausted, could be isolated and their function restored (224). In addition, circulating PD-1 positive T cells

showed to be enriched in neoantigen-specificities (368) when compared to the PD-1 negative counterparts.

Counteracting Immune Suppressive Cells Accumulating in TME

Besides T cell exhaustion, the presence of immunosuppressive cell subpopulations or soluble cytokines may also dampen antitumor T cells responses. In tumors, monocytes have been described to preferentially polarize into M2-tumor associated macrophages (M2-TAM) (369) or Tie-2-expressing monocytes (TEM) (370). M2-TAM and TEM sustain tumor survival and blunt immune reactions. Myeloid-derived suppressor cells (371) and different components of the stroma have been implicated as well in tumor progression, through different mechanisms: (i) the expression of inhibitory receptor ligands (372), (ii) the production of metabolites or soluble factors [e.g., indoleamine 2,3-dioxygenase (IDO) (373), Interleukin-1β and thymic-stromal lymphopoietin (TSLP) (374, 375), and prostaglandin E2 (376)], or (iii) alteration of pH and oxygen levels (377–380). Among the soluble cytokines, the role of TGF-β gained particular attention. TGF-β is released by neoplastic cells of different origins (381) and its secretion is linked to common cancer genetic mutations (382). At the tumor site, TGF-β acts as a local immunosuppressor, thus reducing the effect of immunotherapy on cancer cell growth. In ACT models, the infusion of CD8 T cells genetically manipulated to resist TGF-β outperformed TGF-β sensitive cells in mediating tumor control (383–387). Interestingly, in a recent clinical study 4 out of 8 patients with

Hodgkin lymphoma treated with tumorspecific T cells engineered to express a dominant negative form of TGF-β receptor type II (388) experienced an objective clinical response (389). To help engineered T cells in counteracting the immunosuppressive microenvironment, different strategies are currently under scrutiny. CAR-T cells have been genetically modified to secrete Interleukin-12 or Interleukin-18 (TRUCK cells) upon CAR engagement (390). A deeper understanding of the expression profile associated to functional CAR-T cells has been now translated in new manipulation processes, involving the overexpression of transcription factors, such as c-Jun (391), or the deletion of inhibitory molecules such as REGNASE1 (392). These newly proposed strategies could lead to the generation of T cell products endowed with early differentiated phenotypes and enhanced anti-tumor functionality.

FINAL REMARKS

Adoptive T cell therapy represents a unique and innovative therapeutic pillar for cancer treatment. T cells couple the ability to circulate and home at different sites, to sense and respond to the surrounding environment and to persist long-term, thus providing immunosurveillance against residual malignant cells. Each of these characteristics, intrinsic to T cell biology, is however challenged by several immune escape mechanisms active in cancer patients. Table 1 summarizes the efficacy and toxicity profiles of TCRredirected T cells reported in clinical trials. Results demonstrate the feasibility of the approach, indicating its therapeutic potential, but also underline the challenges that TCR-

based ACT needs to face. Firstly, a suboptimal efficacy of TCR-based studies has currently been observed in patients and no clinical results have been published yet with engineered T cells targeting neoantigens or MiHA. Both evidences underline the difficulty in isolating high affinity tumor-specific TCRs that might be exploited for treating a large number of patients. Secondly, extensive in vitro and in vivo assays are necessary to lower the incidence of adverse events and increase the safety profile of the infused T cell products. An interesting point of discussion is whether CAR-T cell therapy should be preferred to TCR-based T cell therapy or vice versa. The answer likely lies in the middle and might envisage the alternated or even the combined use of TCR- and CAR-T cells in different clinical settings, according to the antigenic profile of each tumor type. Combinations could exploit the strength of both strategies. Despite CAR-T cells can count on the extensive level of knowledge acquired on cancer cell phenotypes, testable targets remain few. It's possible that this effect underlies the intrinsic limitation of CAR-T cells, able to target surface proteins but at present unable to recognize all the intracellular mutated or overexpressed proteins in cancer cells. An engineered TCR, instead, has the ability to virtually recognize every tumor antigen, independently of intracellular localization, including mutated molecules, intracytoplasmic proteins, and transcription factors. Hence, finding suitable targets for TCR-engineered T cells is theoretically easier. However, the HLA-restriction of TCR-based immunotherapies needs to be taken into consideration. In terms of efficacy, CAR-T cells showed

optimal results in the context of relapsed/refractory ALL. TCR therapy seems promising in liquid tumors, but both CAR and TCR-engineered T cell therapies showed less than satisfactory results in solid tumors other than melanoma when compared to TILs therapy. In terms of signaling, TCRs are sensitive to much smaller epitope densities than CARs (393) and T cell activation is finely tuned by the affinity and the avidity for the ligand itself. These differences may prove important when dealing with low antigen density and also in promoting immunological memory while avoiding T cell exhaustion. Even if the precise role of the different signals conveyed in the immunological synapse are still not completely understood, TCR signaling might be more rewarding in the ability to balance T cell activation, possibly solving the limitation in T cell persistence and functionality reported in ACT with CARs. The possibility of genetically engineering T cells by redirecting their specificity toward cancer by employing CARs or TCRs has already produced relevant clinical results in patients affected by selected tumor types. By combining T cell therapy with alternative therapeutic approaches (i.e., checkpoint blockades) and by implementing multiple genetic manipulation (i.e., by genome editing) in T cells, the efficacy of cancer immunotherapy is further increasing, and we might envisage its successful extension to a larger range of tumor types. As the field progresses, several challenges, including manufacturing complexity, regulatory issues, and sustainability will need to be faced, with the ultimate aim of offering this new therapeutic tool to all patients who could benefit.

SCOPE OF THE THESIS

This work aims at setting the basis for the development of effective T cell products for the ACT of CRC, endowed with the capacity to specifically recognize cancer cells and counteract the immune-suppressive CRC TME. Thus, we aimed at:

(i) Investigating the phenotype and the inhibitory receptors expression in T cells infiltrating primary and metastatic CRCs, thus identifying the main molecules responsible for T cell exhaustion;

(ii) Retrieving novel CRC-directed TCR specificities;

(iii) Generating a genetically modified T cell product, endowed with a CRC-specific TCR and resistant to immune exhaustion.

CHAPTER 3

High dimensional flow cytometry as a tool to dissect the exhaustion signature of T cells infiltrating colorectal tumors and colorectal liver metastasis

1. Introduction

Colorectal cancer is a highly heterogeneous malignancy, each subtype harboring a diverse immune infiltrate[1]. Within the tumor microenvironment, immune cells play a pivotal role in regulating tumor progression and metastatic spread. T cells have been described as fundamental players within the tumor mass: the presence of a robust infiltrate of CD8 T cells is a hallmark for a better prognosis. However, the infiltrate is not always sufficient to mediate an effective immune response. In fact, tumors modulate the immune system by attenuating T cell responses. In particular, upon constant and persistent antigen stimulation, T cells progressively start losing their ability to proliferate and produce pro-inflammatory cytokines and upregulate immune checkpoint molecules. These inhibitory receptors have been the target for the most recent immunotherapeutic strategies, aimed at blocking the triggering of the inhibitory signals by monoclonal antibodies binding the receptor itself or its cognate ligand. Despite immune checkpoint blockade (ICB) represented a breakthrough in cancer immunotherapy, its clinical exploitation in CRC is still limited. In fact, ICB proved effective only in a reduced fraction of tumors displaying a high mutational burden. Thus, T cell-based immunotherapy has been proposed as a strategy to treat

advanced colorectal cancer. The first attempts were based on TILs, which are a rare and heterogeneous population of cells difficult to isolate, functionally impaired and with a restricted TCR repertoire specificity, limiting their broad applicability. Adoptive T cell therapy with TCR redirected T cells could overcome the paucity of tumor-reactive T cells but would still suffers from the inhibition of the tumor microenvironment. For this reason, a comprehensive description of the exhaustion profile of T cells infiltrating CRCs is fundamental to guide treatment decision and to better design therapeutic approaches.

2. Results

2.1 High-dimensional flow cytometry analysis of primary CRC infiltrating T cells identified tumor-enriched subpopulations

We used high dimensional flow cytometry as a technique to dissect the inhibitory profile of T cells within the colorectal cancer microenvironment. Polychromatic flow cytometry allows to characterize multiple parameters simultaneously on the same cell, thus capturing the co-expression of surface receptors at the single-cell level. We collected samples from the healthy (non-neoplastic), peritumoral and tumoral colon tissue of 21 treatment-naïve patients diagnosed with primary colorectal cancer (**Table 1**).

Parameter	Cathegory	Patients (n=21)
Sex - n°(%)	Female	12 (57,1)
	Male	9 (42,9)
Age - mean (range)		69,8 (47-91)
Site of primary tumor - n°(%)	Right Colon	14 (66,7)
	Left Colon	7 (33,3)
Histology - n°(%)	ADK	11 (52,4)
	ADK-MUC	5 (23,8)
	ADK-MID	2 (9,5)
	MUC	2 (9,5)
	MID	1 (4,8)
Tissue infiltration - n°(%)	SS	16 (76,2)
	SIER	2 (9,5)
	SM	1 (4,8)
	MP	1 (4,8)
	Other	1 (4,8)
Microsatellite status - n°(%)	MSS	14 (66,7)
	MSI	7 (33,3)

Table 2. Cohort of patients affected by primary CRC. ADK=adenocarcinoma; ADK-MUC=mucinous adenocarcinoma; ADK-MID=medullary adenocarcinoma; MUC=mucinous; MID= medullary; SS=subserosa; SIER=serosa; SM=submucosa; MP=muscolaris propria; MSS=microsatellite stability; MSI=microsatellite instability.

In our cohort, 7/21 tumors were localized in the left colon and 6/7 were adenocarcinomas. 14/21 tumors, localized in the right colon, had more heterogeneous histology. 15/21 tumors infiltrated the subserosa and 7/21 were microsatellite unstable, harboring mutations in MLH1 and PMS genes.

All analyzed tumor samples displayed a T cell infiltrate. The ratio between CD4 and CD8 T cells was similar between healthy, peritumoral and tumor samples, with 50-60% of CD4 and 20-30% of CD8 T cells, slightly enriched in the tumor compared to the healthy tissue (**Fig.1a**). To discriminate the T cell memory subsets, we analyzed the expression of the surface markers CD45RA, CD62L and CD95 to distinguish naïve T cells (T_N, $CD45RA^+CD62L^+CD95^-$), memory stem T cells (T_{SCM}, $CD45RA^+CD62L^+CD95^+$), central memory T cells (T_{CM}, $CD45RA^-CD62L^+$), effector memory T cells (T_{EM}, $CD45RA^-CD62L^-$) and terminal effectors (T_{EMRA}, $CD45RA^+CD62L^-$) (**Fig.1b**). As shown by representative plots (**Fig.1c**), the majority of T cells within the tissues harbored a T_{EM} phenotype, which was further enriched in the tumor compared to the peritumoral tissue both in the CD4 (**Fig.1d**) and CD8 (**Fig.1e**) compartments. Interestingly, a small but consistent proportion of $T_{CM/SCM}$ characterized the peritumoral tissue only.

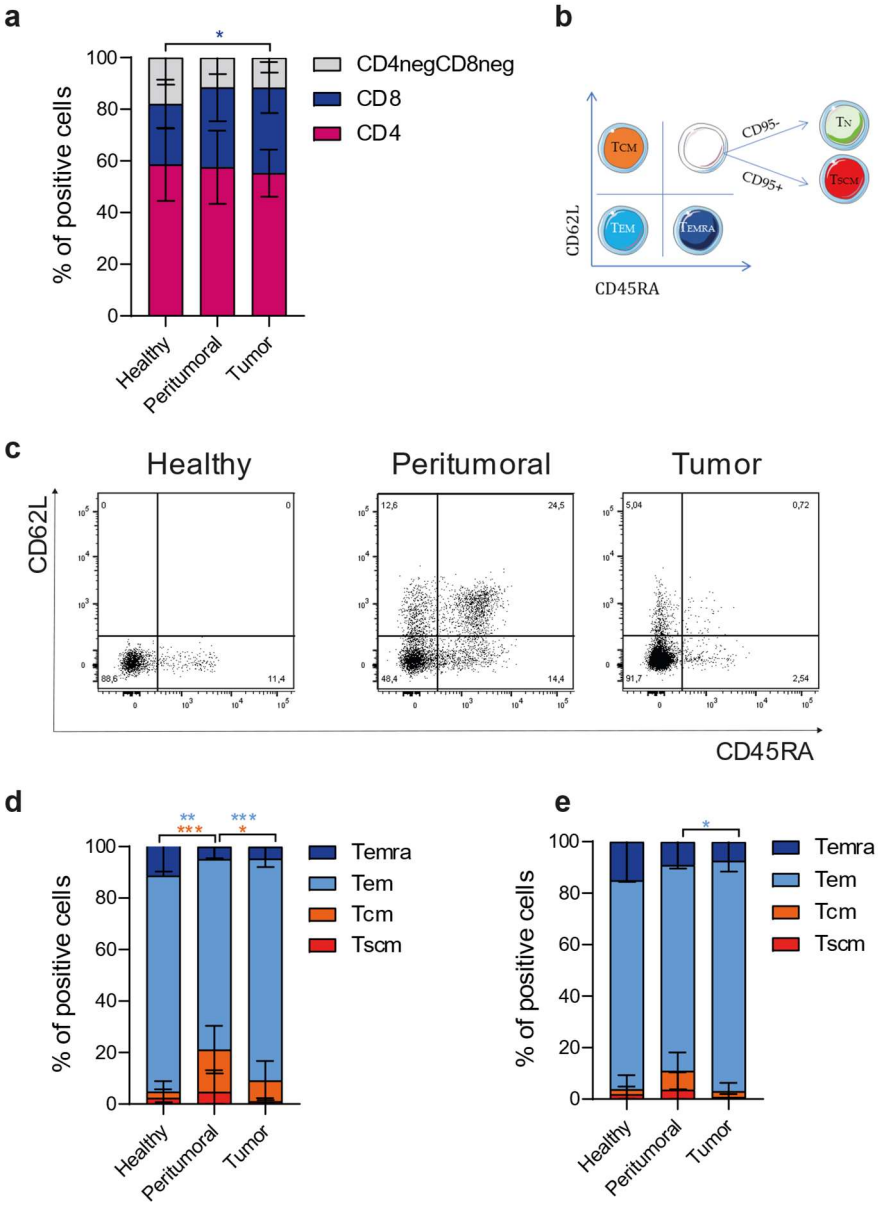

Figure 1 T cells infiltrating primary CRC display a prevalent effector memory phenotype. The ratio of CD4/CD8 T cells was evaluated in healthy, peritumoral and neoplastic colon tissue (**a**). The relative proportion of T_{CM} ($CD45RA^{-}CD62L^{+}$), T_{SCM} ($CD45RA^{+}CD62L^{+}$), T_{EMRA} ($CD45RA^{+}CD62L^{-}$), T_{EM} ($CD45RA^{-}CD62L^{-}$) (**b**) was evaluated in CD4 (**d**) and CD8 (**e**) T cells, as shown by representative plots (**c**) (n=21). Data are plotted as mean ± SEM.

When analyzing by manual gating the expression of inhibitory receptors, we noticed a higher frequency of CD4 (**Fig.2a**) and CD8 (**Fig.2b**) T cells expressing PD1 and CD39 within the tumor tissue compared to healthy and peritumoral specimens. A significant upregulation of CD4 T cells expressing GITR was also observed within the tumor tissue (**Fig.2a**), while CD8 T cells from the peritumoral tissue were characterized by a highly variable expression of 2B4, with a lower mean compared to T cells from the healthy and the tumor tissues (**Fig.2b**). However, we realized that analyses through a classical binary gating strategy were not sufficient to unravel a specific tumor signature since they permit to study the co-expression of only 2 markers at a time, failing to capture the complexity of the phenotype.

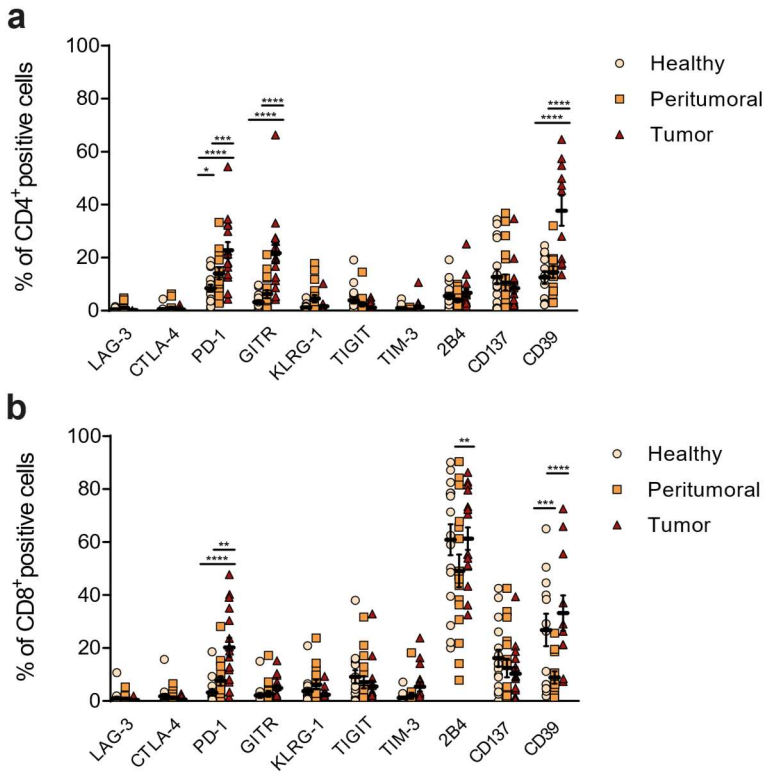

Figure 2 PD1 and CD39 are upregulated in tumor infiltrating T cells. Inhibitory receptors expression on CD4 (**a**) and CD8 (**b**) T cells harvested from healthy, peritumoral and neoplastic colon tissue (n=21). Data are plotted as mean ± SEM.

2.2 Cytochain revealed a tumor-enriched subpopulation defined by a specific exhaustion signature

Polychromatic flow cytometry analyses produce very large datasets, difficult to handle with the standard flow cytometry analysis tools; dimensionality reduction and clustering algorithms are therefore necessary to dissect the complexity of datasets and as a discovery tool for rare, specific subpopulations. This need led our group to develop a new web-application, named

Cytochain (Manfredi*, Abbati*,…, Potenza, et al, *submitted*), designed to facilitate the interpretation of complex flow cytometry data by integrating different R packages. Cytochain workflow starts with the pre-analytical check of the .fcs compensated data. The first step concerns data “cleaning”: an evaluation of the acquisition stability is performed by Flow_iQC function from the FlowAI R package to exclude data oscillations outside a determined range, considered as artifacts. A “down-sampling” step is then performed by selecting a stable acquisition timeframe. This step is necessary to avoid the introduction of biases in the analysis, since the differences in size between samples would likely reduce the variance weight of the samples with a lower number of events. To optimize the flow set, a “scaling” process is also performed. This allows the range of the measured expression variable to be standardized. After the pre-analytical data handling, dimensionality reduction and clustering analysis are performed, allowing to determine the features of the dataset qualitatively and quantitatively. As an unsupervised approach we used flowSOM, which is based on the Self Organized Map (SOM) algorithm. We performed the analysis separately on CD4 and CD8 T cell compartments.

2.2.1 PD1^{+}CD39^{+}GITR^{+}CD137^{+} T cells are enriched in tumor-specific CD4 T cells

FlowSOM resemble an artificial neural network in which nodes can be represented in a two-dimensional way. It uses hierarchical clustering to group cell clusters basing on phenotypic similarity, each visualized as a single node in a minimum spanning tree (**Fig.3a**). For each individual marker, a subplot is shown displaying its diverse expression. Both for the spanning tree (**Fig.3b**) and the bh-SNE (**Fig.3c**) visualizations, the higher is the expression level of the marker, the more saturated is the color.

FlowSOM clusterized the events into 100 clusters, automatically collapsed into 30 meta-clusters according to minimal spanning tree proximities by the ConsensusClusterPlus algorithm. A Barnes-Hut distributed Stochastic Neighbour Embedding (BH-SNE) map of the concatenated flowSet, comprehending events from the healthy, peritumoral and tumor specimens, was calculated and the 30 clusters were superimposed to the map (**Fig.4a**). Most clusters were shared between healthy, peritumoral and tumor samples, despite in a different percentage (**Fig.4b**).

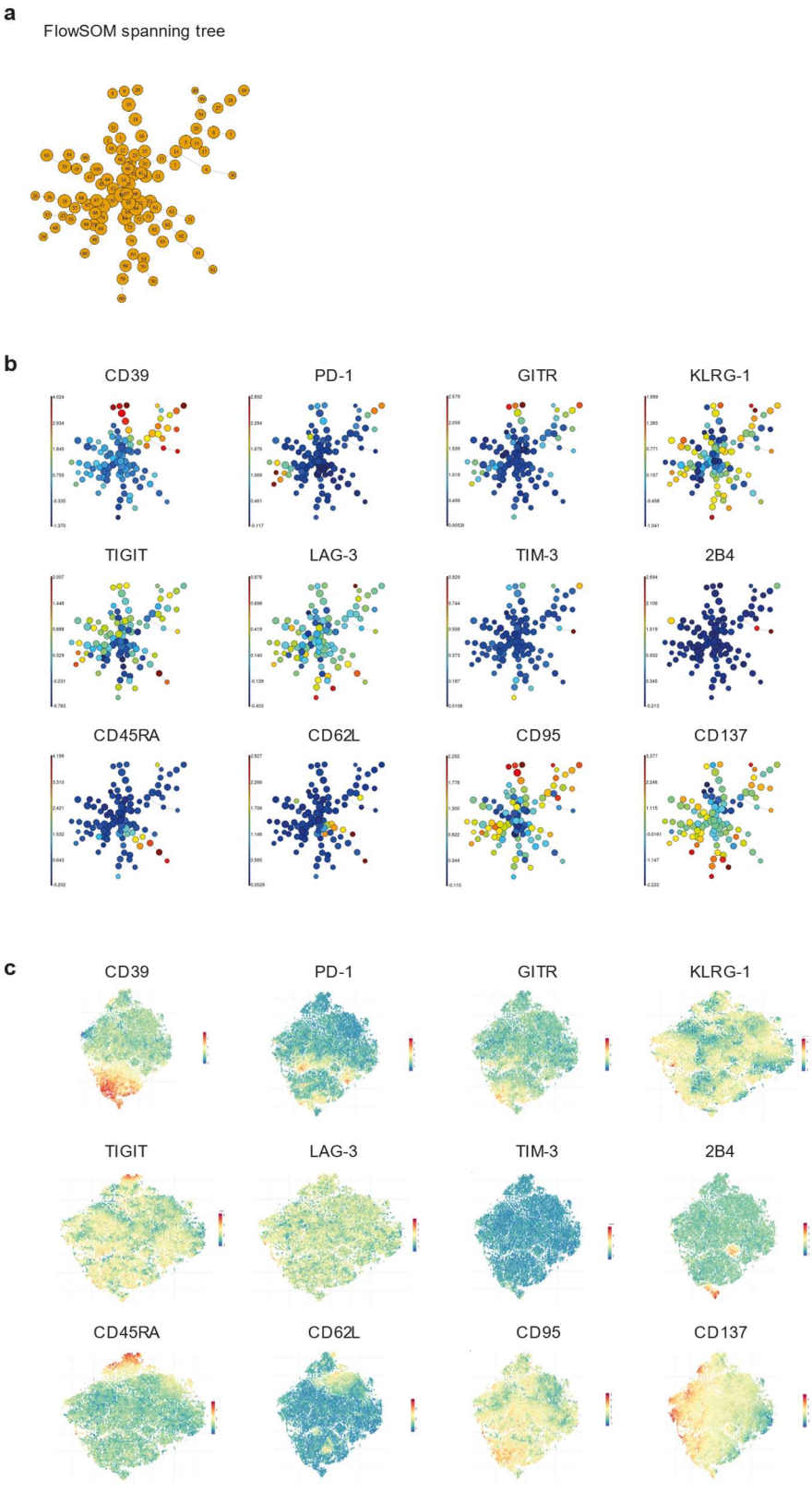
a
FlowSOM spanning tree
b
CD39
PD-1
GITR
KLRG-1
TIGIT
LAG-3
TIM-3
2B4
CD45RA
CD62L
CD95
CD137
c
CD39
PD-1
GITR
KLRG-1
TIGIT
LAG-3
TIM-3
2B4
CD45RA
CD62L
CD95
CD137

Figure 3 Markers expression on CD4 T cells visualized as minimum spanning tree and bh-SNE maps. Similar clusters are grouped and visualized in a minimum spanning tree (**a**). Markers' expression is shown for each cell cluster (**b**). The bh-SNE visualization, instead, shows markers expression by plotting each cell individually in a two-dimensional map where similar events are represented by nearby points (**c**).

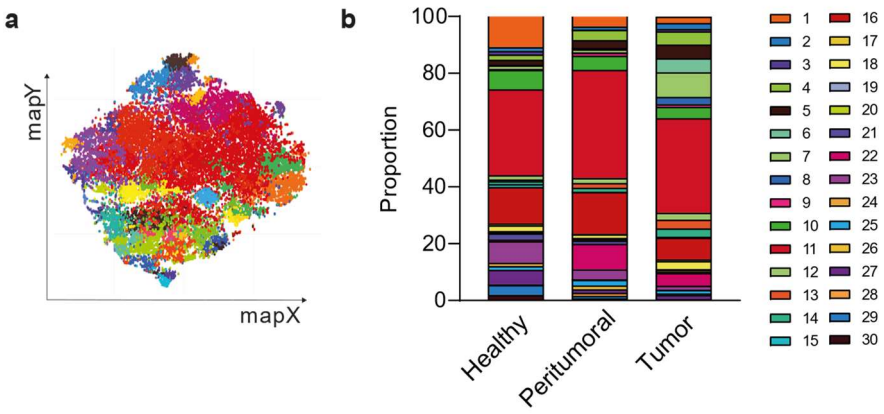

Figure 4 CD4 T cells cluster differently in healthy, peritumoral and neoplastic colon tissues. The bh-SNE map shows the distribution of the 30 meta-clusters (**a**), differently frequent in the healthy, peritumoral and neoplastic colon tissue (**b**).

For each marker analyzed, the ratio of fluorescence intensity with respect to the maximum is reported in the heatmap (**Fig.5**). For example, cluster 11 was present in >30% samples but it was not characterized by a peculiar expression phenotype. On the contrary, some clusters were significantly enriched only in one compartment, thus characterizing tissue-specific T cells.

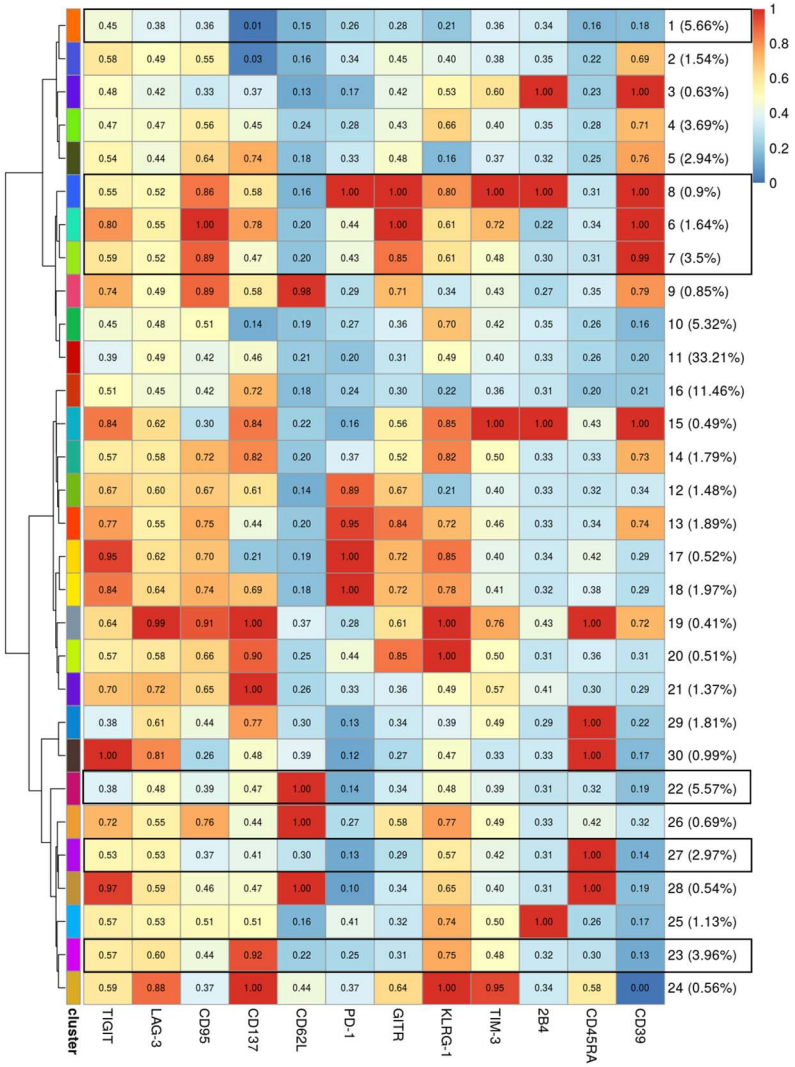

Figure 5 CD4 T cells from healthy, peritumoral and neoplastic colon tissue express different levels of IRs. Heatmap displaying the different levels of IRs expression in the 30 T cells meta-clusters. Meta-clusters 1, 6, 7, 8, 22, 23 and 27 (black rectangles) are enriched within the three specimens.

Clusters 1, 23 and 27 defined the healthy tissue. T cells belonging to these clusters do not express high levels of inhibitory molecules or activation markers (**Fig.6a**). Peritumoral-specific T cells, grouped in cluster 22, are not defined by high expression of inhibitory markers (**Fig.6b**). Clusters 6,7,8, representing 6% of tumor-specific events, are instead characterized by high expression of both inhibitory and activation molecules (**Fig.6c**) and >30% of events belonging to tumor-specific clusters co-express up to 10 markers (**Fig.6d**).

To validate the precise signature characterizing tumor-specific T cells, we evaluated by manual gating the expression of the surface markers in tumor samples compared to the whole flow set. This back-gating strategy highlighted that tumor-specific T cells were 100% positive for CD39 and PD1, 80% positive for GITR and 50% positive for the activation marker CD137 (**Fig.7a**). The bh-SNE map helped to visualize how these markers are highly co-expressed (**Fig.7b**). This $CD39^{+}PD1^{+}GITR^{+}CD137^{+}$ subpopulation represents the 6% of T cells infiltrating the tumor mass and is not present within the healthy or the peritumoral tissue (**Fig.7c**).

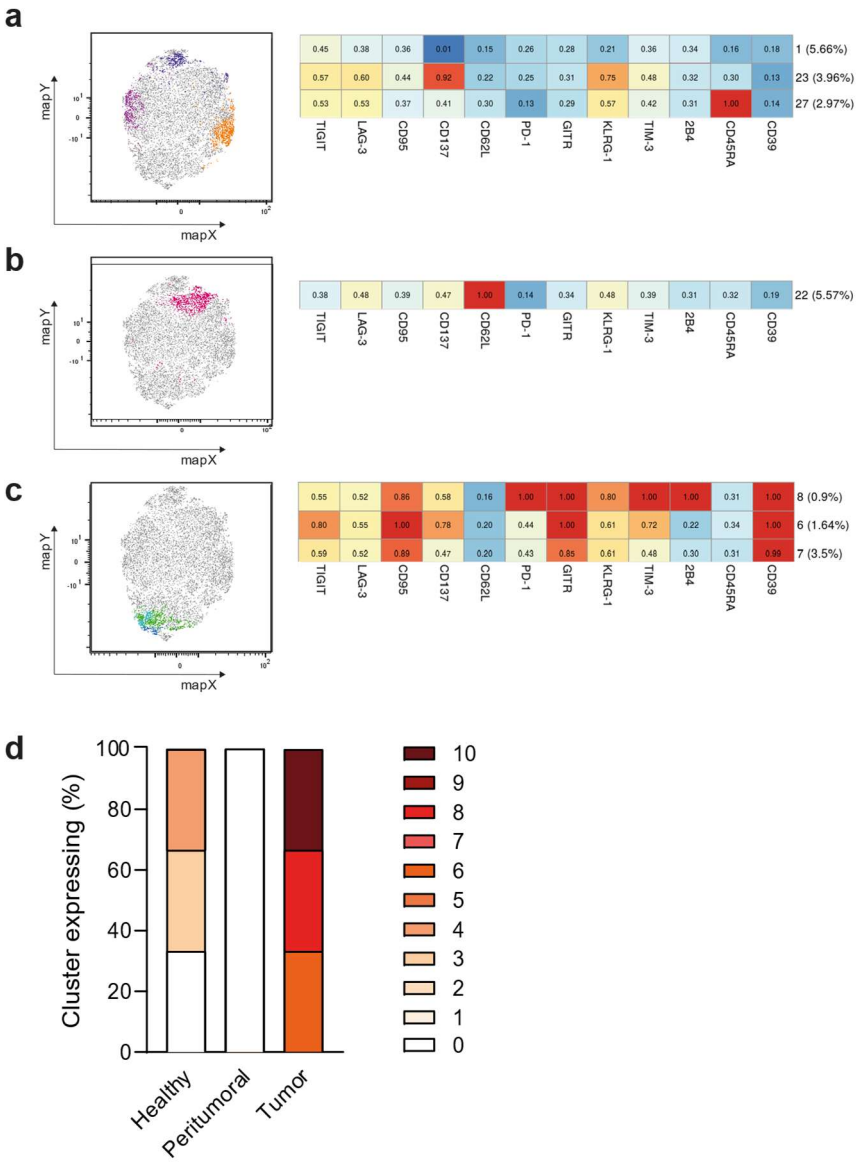

Figure 6 Tumor-specific CD4 T cell clusters are defined by the co-expression of up to 10 IRs. Selected clusters are specific for the healthy (**a**), peritumoral (**b**) and tumor (**c**) tissue and are defined by different IRs expression levels. CD4 T cells enriched within the tumor display the co-expression of up to 10 inhibitory molecules (**d**).

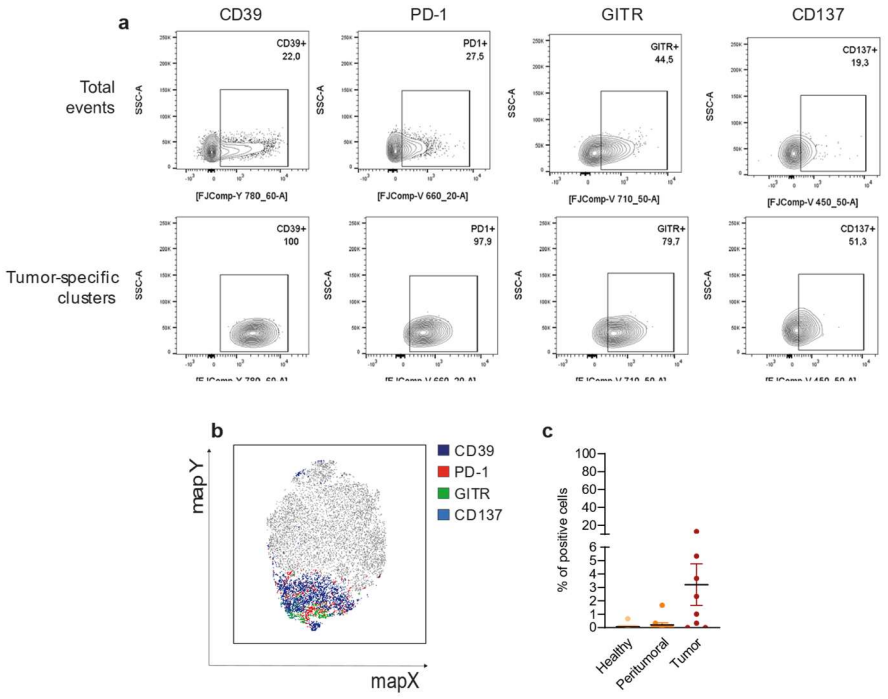

Figure 7 CD39⁺PD1⁺GITR⁺CD137⁺ CD4 T cells are enriched within the tumor tissue. The frequency of CD4 T cells positive for CD39, PD1, GITR and CD137 was manually gated and plotted among total events as well as in tumor-specific clusters (**a**). These markers are co-expressed in a specific cluster of T cells, as shown by the bh-SNE map (**b**) and define a group of tumor-enriched T cells that are absent within the healthy and peritumoral tissues (**c**).

2.2.2 PD1⁺CD39⁺GITR⁺CD137⁺ T cells are enriched in tumor-specific CD8⁺ T cells

The same analytical workflow was applied to CD8⁺ T cells. The minimum spanning tree shows the similarity between clusters (**Fig.8a**) and the expression of the markers, according to color saturation, is represented in clusters (**Fig.8b**) and in a bh-SNE map plotting single events (**Fig.8c**).

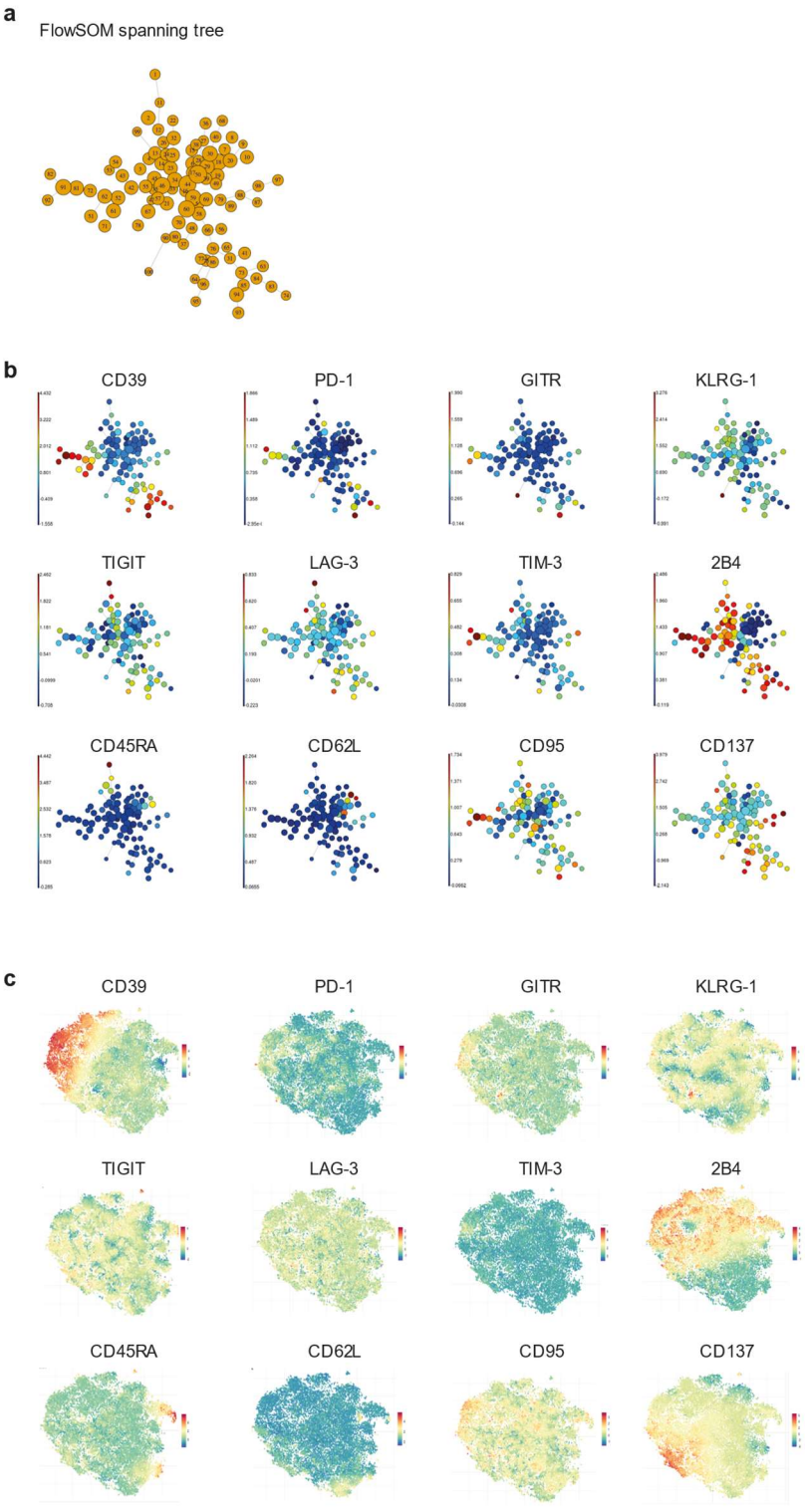
a
FlowSOM spanning tree
b
CD39
PD-1
GITR
KLRG-1
TIGIT
LAG-3
TIM-3
2B4
CD45RA
CD62L
CD95
CD137
c
CD39
PD-1
GITR
KLRG-1
TIGIT
LAG-3
TIM-3
2B4
CD45RA
CD62L
CD95
CD137

Figure 8 Markers expression on CD8 T cells visualized as minimum spanning tree and bh-SNE maps. Similar clusters are grouped and visualized in a minimum spanning tree (**a**). Markers' expression is shown for each cell cluster (**b**) or each single event in bh-SNE maps (**c**). The more saturated the color, the more expressed the marker.

The generated 30 meta-clusters as distributed on the concatenated flowSet are represented on the bh-SNE map (**Fig.9a**). Despite being more heterogeneous in terms of cluster distribution compared to CD4 T cells, some clusters were shared between healthy, peritumoral and tumor samples, such as cluster 4 (**Fig.9b**).

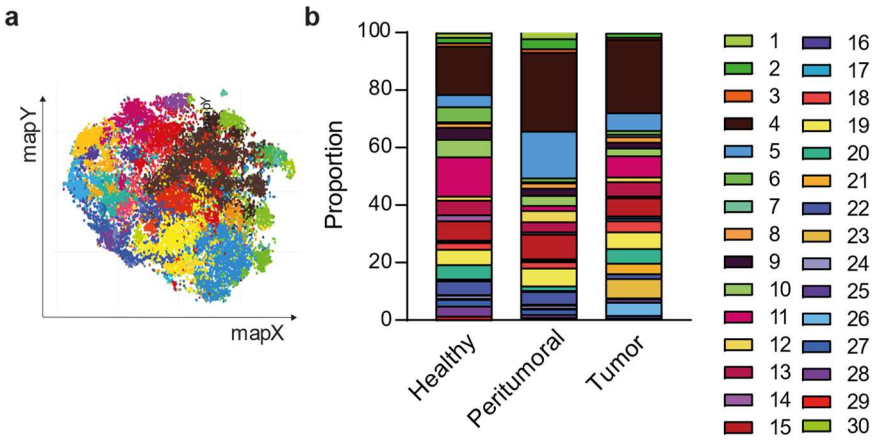

Figure 9 CD8⁺ T cells cluster differently in healthy, peritumoral and neoplastic colon tissue. The bh-SNE map shows the distribution of the 30 meta-clusters (**a**), present with different frequencies in the healthy, peritumoral and neoplastic colon tissue (**b**).

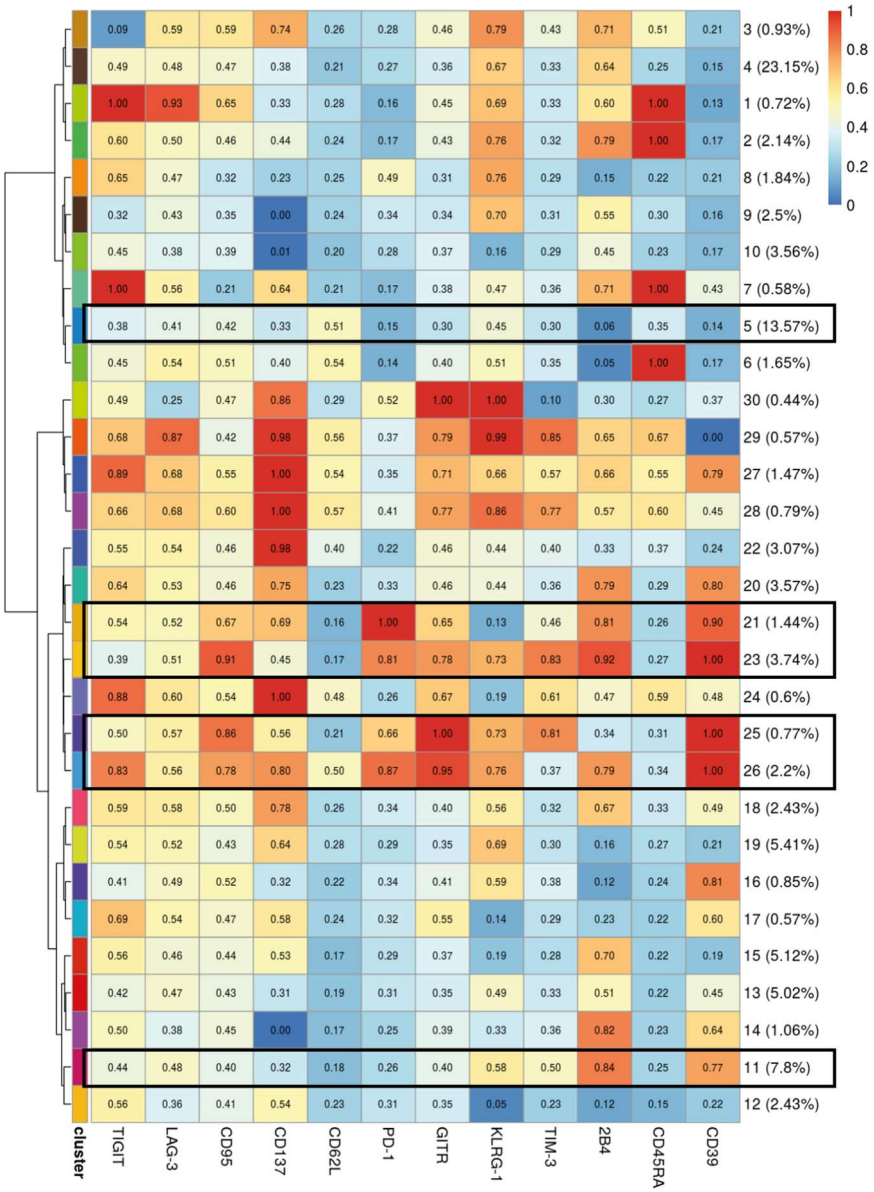

Figure 10 CD8⁺ T cells from primary CRCs express different levels of IRs. The bh-SNE map shows the distribution of the 30 meta-clusters (**a**), present with different frequencies in the healthy, peritumoral and neoplastic colon tissue (**b**). Meta-clusters 5, 11, 21, 23, 25 and 26 (black rectangles) are enriched within the three specimens.

Cluster 11 was representative of the healthy tissue (**Fig.11a**). As shown by the heatmap (**Fig.10**), it is characterized by a moderate expression of KLRG-1 and CD39 and a high expression of 2B4. Nevertheless, these receptors are not co-expressed with activation markers. Cluster 5, specific for the peritumoral tissue, is representative of T cells not expressing inhibitory receptors nor activation molecules (**Fig.11b**). Clusters 21, 23, 25 and 26 defined 8,15% of tumor-specific events (**Fig.11c**) and were characterized by the co-expression of up to 9 markers (**Fig.11d**).

Interestingly, when manually validating the exhaustion signature of tumor-specific T cells, we ended up with the same hallmarks defined for CD4 T cells. Tumor-specific CD8 T cells were indeed 100% positive for CD39 and PD1, 74% positive for GITR and 86% positive for the activation marker CD137 (**Fig.12a**), which are co-expressed as shown by the bh-SNE map (**Fig.12b**). This marker set defined 15% of T cells uniquely present within the neoplastic tissue, while absent within the healthy colon and only defining 3% of T cells from the peritumoral tissue (**Fig.12c**).

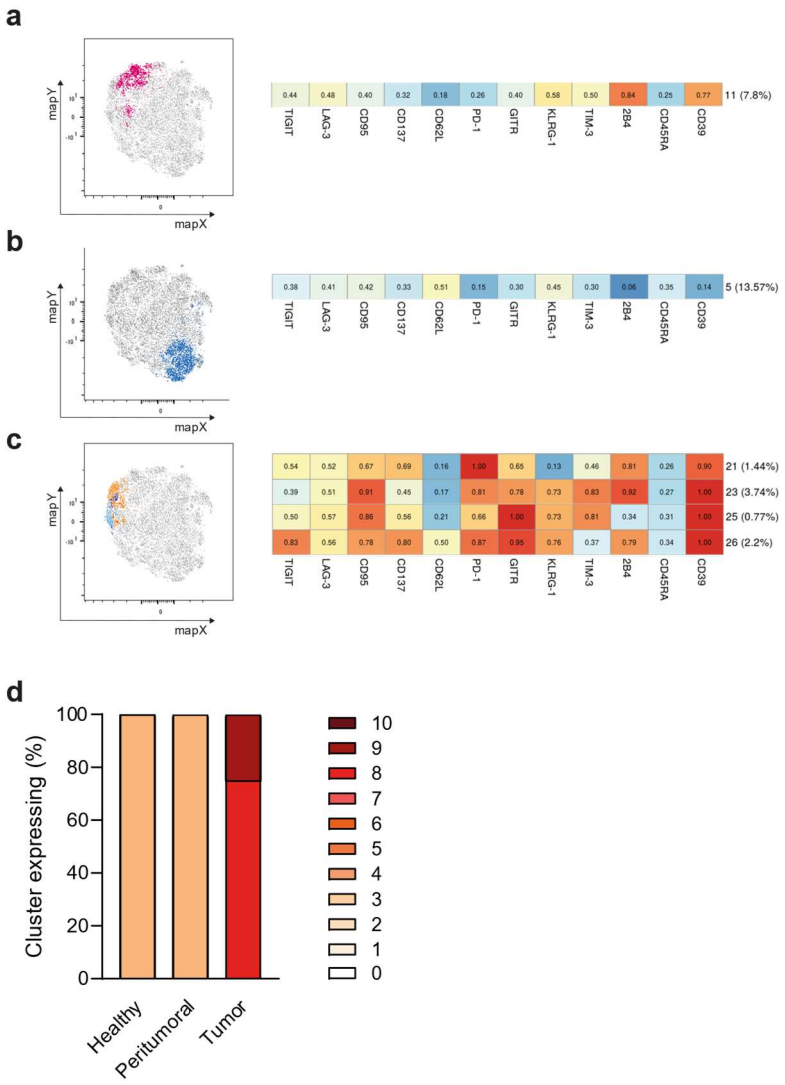

Figure 11 Tumor-specific CD8⁺ T cell clusters are defined by the co-expression of up to 9 IRs. Selected clusters are specific for the healthy (**a**), peritumoral (**b**) and tumor (**c**) tissue and are defined by different levels of IRs expression. Tumor-enriched clusters display the co-expression of up to 9 inhibitory molecules (**d**).

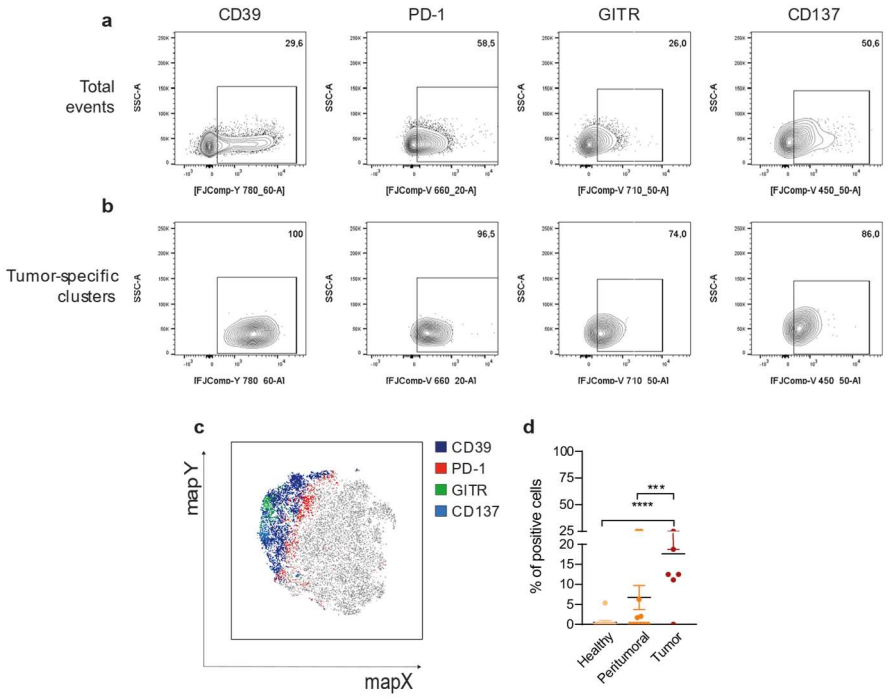

Figure 12 CD39⁺PD1⁺GITR⁺CD137⁺ CD8 T cells are enriched within the tumor tissue. The frequency of CD8 T cells positive for CD39, PD1, GITR and CD137 was manually gated and plotted among total events as well as in tumor-specific clusters (**a**). These markers are co-expressed in a specific cluster of cells, as shown by the bh-SNE map (**b**) and define a cluster of CD8 T lymphocytes significantly enriched within the tumor while being absent within the healthy and peritumoral tissues (**c**).

2.2.3 Microsatellite instability does not impact on the exhaustion phenotype

Microsatellite instability, caused by a non-proficient DNA mismatch repair system, characterizes about 15% of all colorectal cancers. MSI is associated with a robust lymphocytic infiltrate and represents the only CRC subsets benefitting from

immune checkpoint blockade. For this reason, we wondered whether the inhibitory pattern was different between MSI and MSS CRCs. By separately analyzing T cells from the tumor tissue of MSI and MSS patients, we noticed a similar expression of all the inhibitory molecules both in CD4 (**Fig.13a**) and CD8 (**Fig.13b**), with only a slight increase in the mean value of cells expressing CD39 in the MSI group. Cluster distribution was also similar between the two CRC phenotypes in both CD4 (**Fig.13c**) and CD8 (**Fig.13d**) T cells.

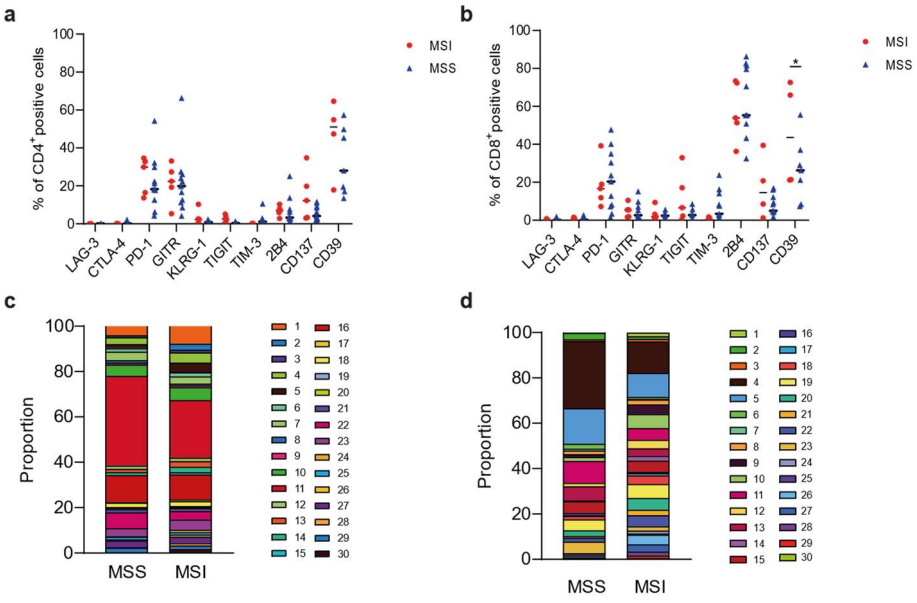

Figure 13 Microsatellite instability does not impair the exhaustion phenotype. Expression of inhibitory receptors according to the infiltration site and cluster distribution in CD4 (**a**,**c**) and CD8 (**b**,**d**) T cells. MSI=microsatellite instability; MSS=microsatellite stability.

2.3 Characterization of the tumor microenvironment by high-dimensional flow cytometry in metastatic CRC

Despite in the last decades the availability of new targeted therapies enhanced the life expectancy of CRC patients, the presence of metastasis still represents an unmet medical need. The preferential site for metastasis arising from CRC is the liver, peculiar for its state of liver tolerance, shaped by the various cell subpopulations composing the liver anatomy and by recruited immune cells. To deeply characterize T cells infiltrating liver metastasis from CRC, we analyzed peritumoral liver and liver metastasis samples from 26 patients diagnosed with CRC metastatic to the liver (mCRC). Our cohort included patients with a primary tumor localized within the right (19,2%) or left colon (38,5%) and rectum (42,3%). 4/26 patients were chemotherapy-naive; on 8 out of 26 patients the effect of chemotherapy was considered negligible, since at least 3 months separated the surgery (and thus the sample collection) from the last chemo administration. The remaining 14 patients underwent chemotherapy in the last 3 months before liver surgery. 7/26 patients harbored KRAS mutation. Of the 18 treated patients, 13 showed a response to chemotherapy in terms of histologically measured tumor regression grade (TRG). According to the clinical risk “Fong” score, taking into account node positivity, disease-free interval, number of metastasis, pre-operative serum CEA levels and size of largest tumors, the patients were categorized into low (42,3%) and high (50%) risk (**Table 2**).

A difference in the ratio between CD4 and CD8 T cells was observed when comparing T cells retrieved from liver metastasis with T cells isolated from the peritumoral liver, displaying 50% and 20% of CD4 T cells, respectively (**Fig.14a**). As observed in primary CRC samples, T cells within the liver displayed a T_{EM} phenotype (**Fig.14b**), with a negligible fraction of T_{CM} within the CD4 (**Fig.14c**) but absent in the CD8 T cell compartment (**Fig.14d**).

Parameter	Cathegory	Patients (n=26)
Sex - n°(%)	Female	11 (42,3)
	Male	15 (57,7)
Age - mean (range)		61,6 (39-78)
Site of primary tumor - n°(%)	Right Colon	5 (19,2)
	Left Colon	10 (38,5)
	Rectum	11 (42,3)
Chemotherapy - n°(%)	Naive	4 (15,4)
	>3months	8 (30,8)
	<3 months	14 (53,8)
K-Ras status - n°(%)	Wt	16 (61,5)
	Mut	7 (26,9)
TRG - n°(%)	Low (1,2,3)	6 (23)
	High (4)	13 (50)
Fong Score - n°(%)	Low (0,1,2)	11 (42,3)
	High (3,4,5)	13 (50)

Table 3. Cohort of patients affected by metastatic CRC. TRG=tumor regression grade.

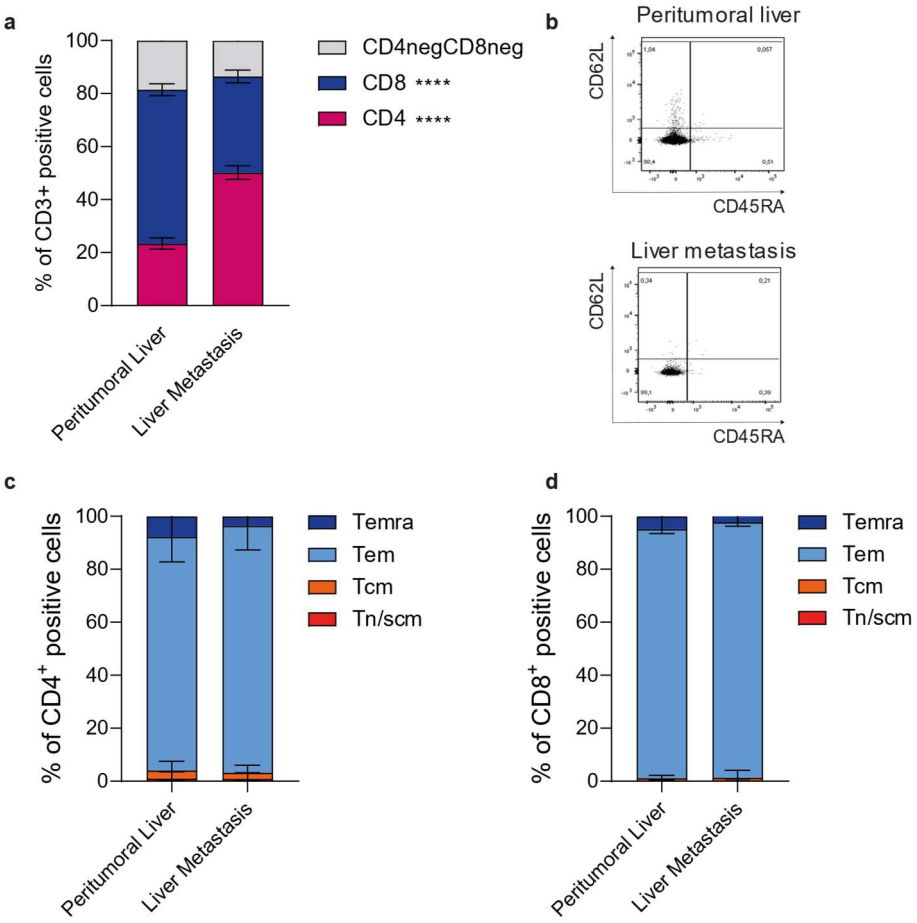

Figure 14 T cells infiltrating CRC liver metastasis display a prevalent effector memory phenotype. The ratio between CD4 and CD8 T cells was evaluated in healthy, peritumoral and neoplastic tissue in the liver (**a**). The relative proportion of T_{CM} ($CD45RA^{-}CD62L^{+}$), T_{SCM} ($CD45RA^{+}CD62L^{+}$), T_{EMRA} ($CD45RA^{+}CD62L^{-}$), T_{EM} ($CD45RA^{-}CD62L^{-}$) was evaluated in the CD4 (**c**) and CD8 (**d**) T cells, as shown by representative plots of CD8 T cells (**b**) (n=26). Data are plotted as mean ± SEM.

2.3.1 CD39^{+}TIM-3^{+}HLA-DR^{+}2B4^{-} CD4 T cells are enriched within CRC liver metastases

Unsupervised analyses of the CD4 compartment showed a heterogeneous cluster distribution (**Fig.15a**) between peritumoral and metastatic liver (**Fig.15b**), with enriched and contracted clusters in the two specimens.

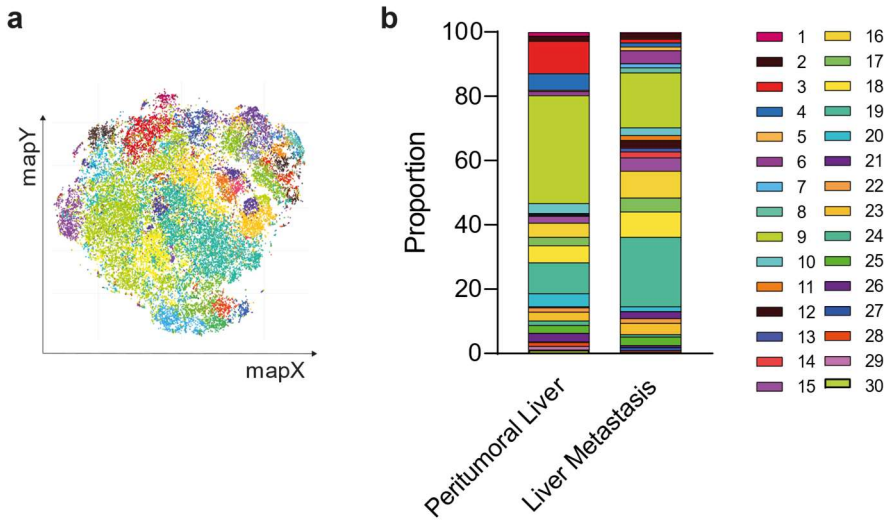

Figure 15 CD4 T cell clusters differently in peritumoral and metastatic liver. The bh-SNE map shows the distribution of the 30 meta-clusters (**a**), present with different frequencies in the peritumoral liver and liver metastasis (**b**).

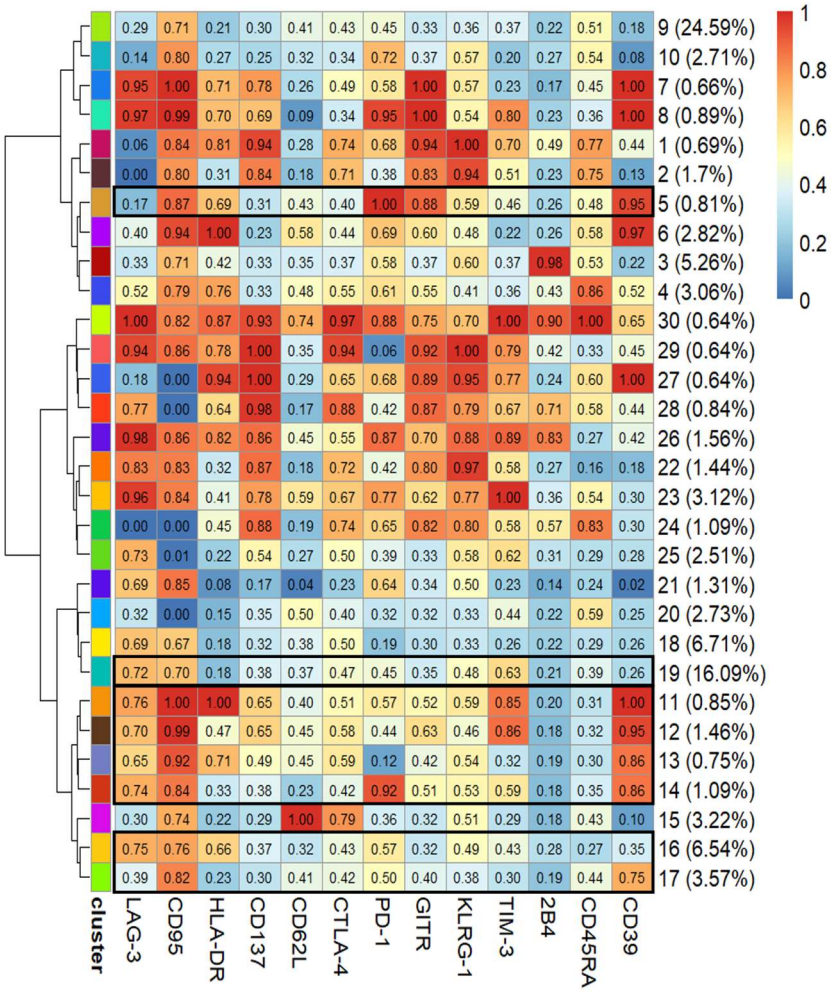

Figure 16 CD4 T cells from CRC liver metastases express different levels of IRs. Heatmap displaying the different levels of IRs expression in the 30 T cells meta-clusters. Meta-clusters 5, 11, 12, 13, 14, 16, 17 and 19 (black rectangles) are enriched within liver metastases.

We focused our analysis on clusters significantly enriched within the liver metastases, namely clusters 5, 11, 12, 13, 14, 16, 17, 19. According to the expression pattern defined by the heatmap (**Fig.16**), three different populations of T cells characterized the metastases: the majority (22,63%, clusters 16 and 19, **Fig.17a, top**) were lymphocytes expressing low levels of inhibitory receptors, except for a moderate expression of LAG-3. Clusters 5 and 17, representing 4,38% of the events, shared a high expression of CD39, while differing for the levels of PD1, GITR and HLA-DR (**Fig.17a, mid**). The most interesting population was represented by clusters 11, 12, 13 and 14 (4,08%), since it was characterized by high levels of CD39 and LAG-3 and moderate levels of PD1, HLA-DR and GITR (**Fig.17a, bottom**). When compared to the peritumoral liver, showing no IRs co-expression in the 50% of relevant clusters, >60% metastasis-specific clusters co-expressed at least 6 IRs (**Fig.17b**).

When validating the expression signature by back-gating, CD4 T cells infiltrating liver metastases were shown to be 100% positive for CD39, TIM-3 and HLA-DR as activation markers, while 100% negative for 2B4 (**Fig.18a**). 20% of $CD4^+$ $CD39^+TIM\text{-}3^+HLA\text{-}DR^+2B4^-$ T cells (**Fig.18b**) were localized within the liver metastasis, significantly enriched with respect to the peritumoral liver (**Fig.18c,d**).

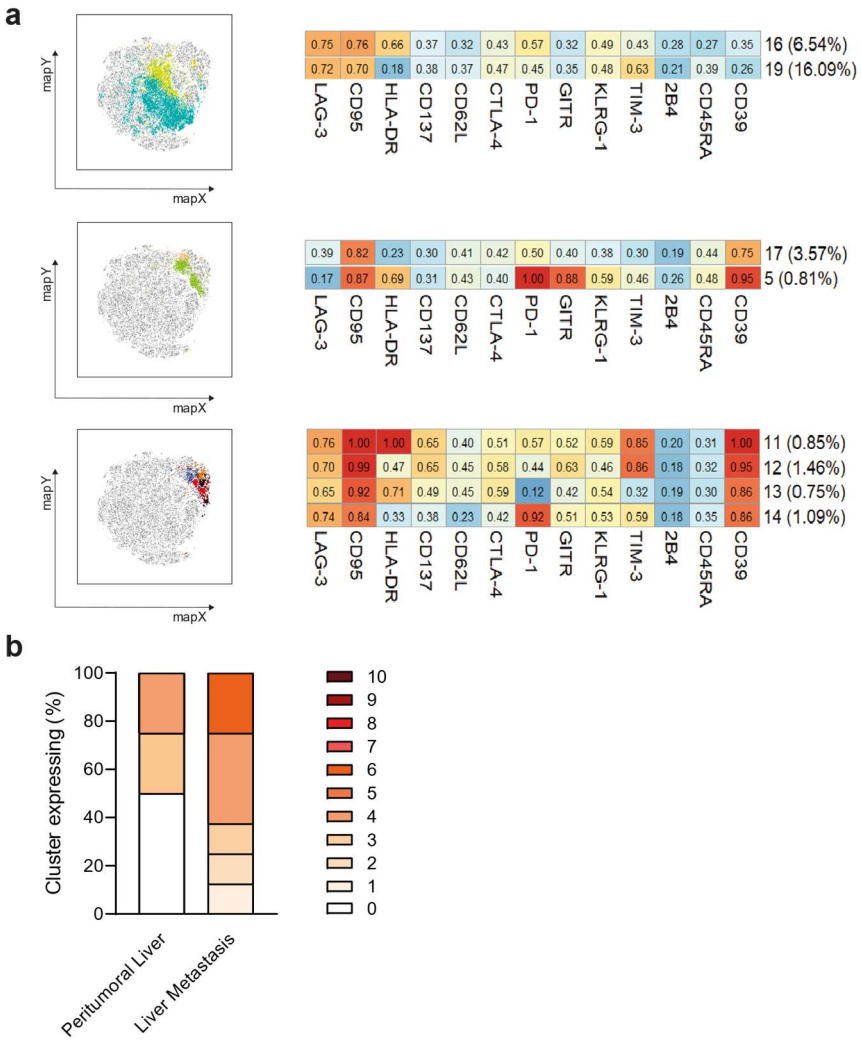

Figure 17 CRC liver metastases specific CD4 T cell clusters are defined by the co-expression of up to 6 IRs. Clusters enriched within liver metastases present three different levels of IRs co-expression (**a**). Overall, clusters enriched within the liver metastasis co-express more frequently IRs than clusters enriched within the peritumoral liver (**b**).

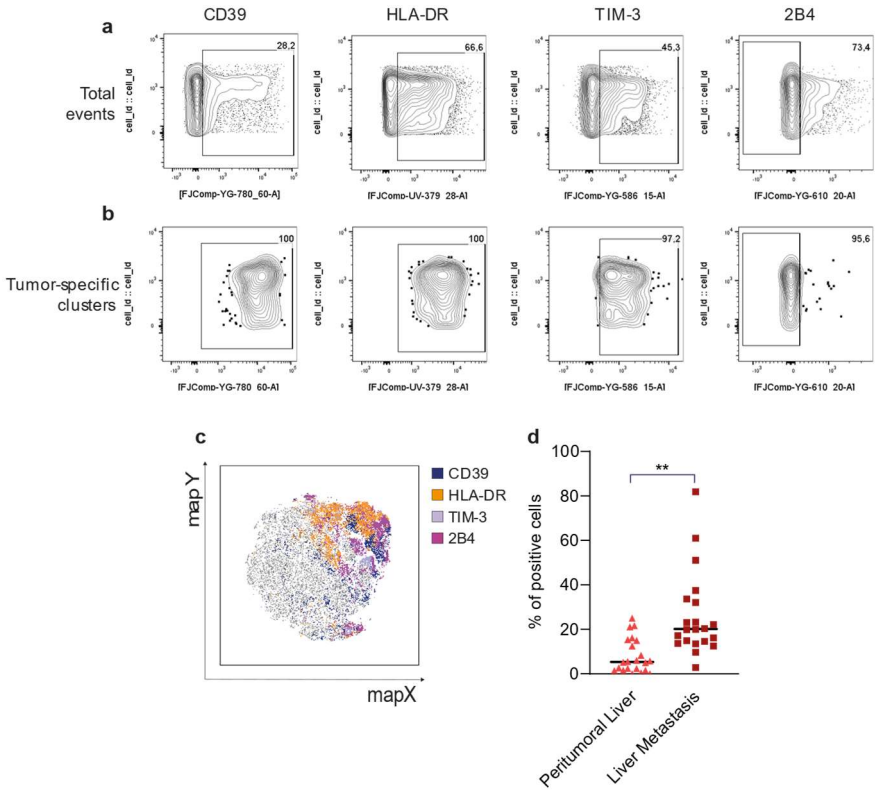

Figure 18 CD39^{+}HLA-DR^{+}TIM3^{+}2B4^{-} CD4 T cells are enriched within CRC liver metastasis. The frequency of T cells positive for CD39, PD1, GITR and CD137 was manually gated and plotted among total events as well as in tumor-specific clusters (**a**). These markers are co-expressed in a specific cluster of cells, as shown by the bh-SNE map (**b**) and define a cluster of CD4 T lymphocytes significantly enriched within the tumor while being absent within the healthy and peritumoral tissues (**c**).

2.3.2 CD39+PD1+TIM3+LAG3+2B4- CD8 T cells are enriched within CRC liver metastases

Cluster distribution (**Fig.19a**) within CD8 T cells was more heterogeneous compared to that of CD4 T cells, both in peritumoral and metastatic liver (**Fig.19b**).

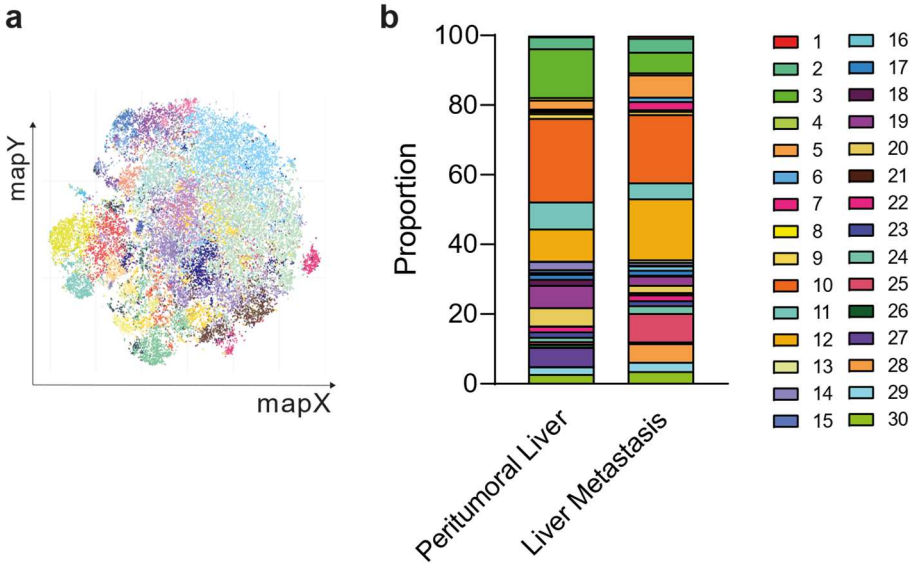

Figure 19 CD8 T cells clusters differently in peritumoral and metastatic liver. The bh-SNE map shows the distribution of the 30 meta-clusters (**a**), present with different frequencies in the peritumoral liver and liver metastasis (**b**).

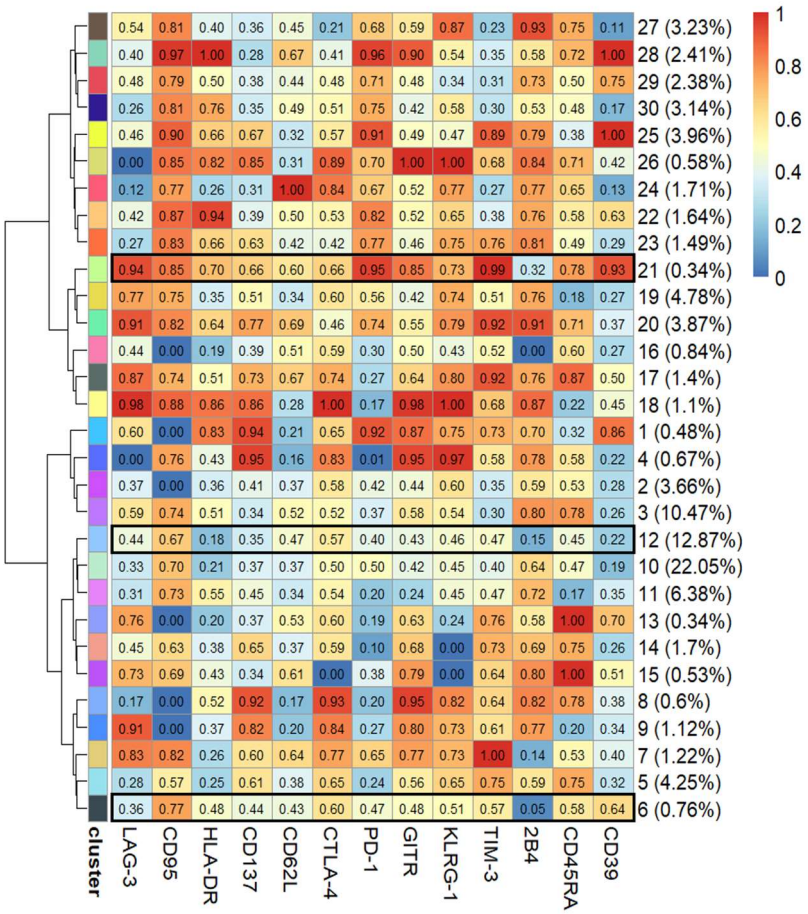

Figure 20 CD8 T cells from CRC liver metastases express different levels of IRs. Heatmap displaying the different levels of IRs expression in the 30 T cells meta-clusters. Meta-clusters 6, 12 and 21 (black rectangles) are enriched within liver metastases only.

Still, similarly to CD4 T cells, the metastases-enriched clusters represented a gradient of inhibitory receptors' expression, as shown by the heatmap (**Fig.20**). In particular, cluster 12 did not express any IR (**Fig.21a**); cluster 6 displayed an intermediate level of IRs expression (**Fig.21a**) and cluster 21 highly expressed all inhibitory and activation molecules, with particular relevance

for PD1, CD39, TIM-3 and LAG-3 (**Fig.21a**). While clusters contracted within the metastasis co-expressed at least 3 IRs, cluster 21 co-expressed 9 IRs (**Fig.21b**).

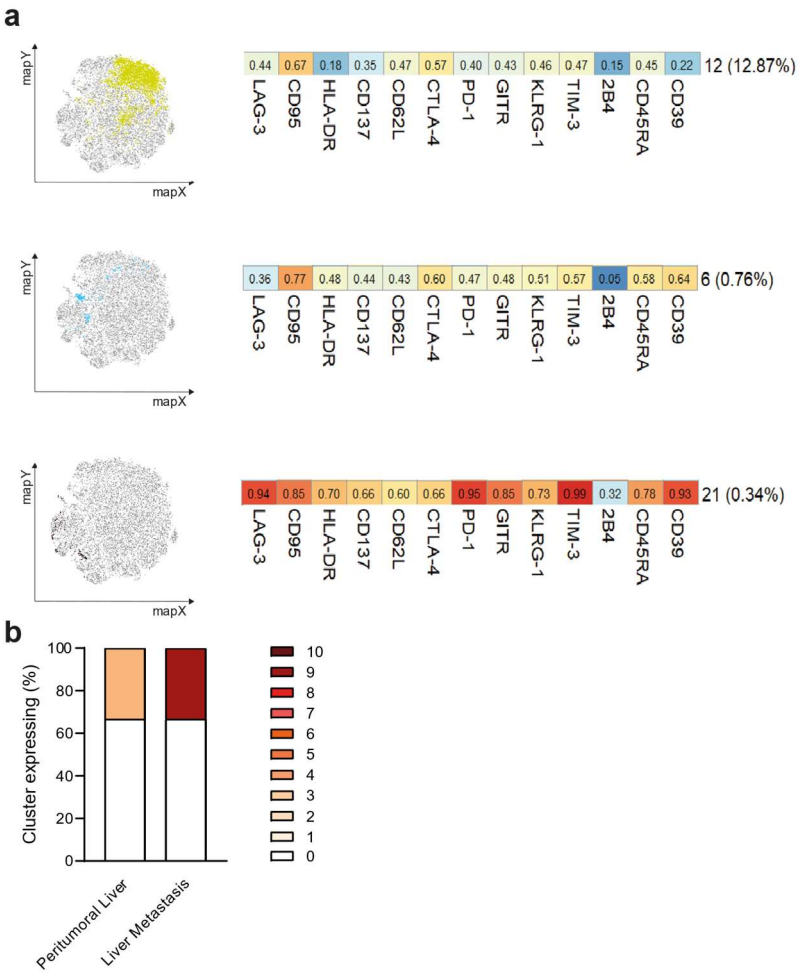

Figure 21 CRC liver metastases specific CD8 T cell clusters are defined by the co-expression of up to 9 IRs. Clusters enriched within liver metastasis present three different levels of exhaustion (**a**). Overall, clusters enriched within the liver metastasis co-express more frequently IRs than clusters enriched within the peritumoral liver (**b**).

As confirmed by manual gating (**Fig.22a**), $CD39^{+}PD1^{+}TIM3^{+}LAG3^{+}2B4^{-}$ T cells (**Fig.22b**) were significantly enriched in liver metastasis compared to peritumoral liver.

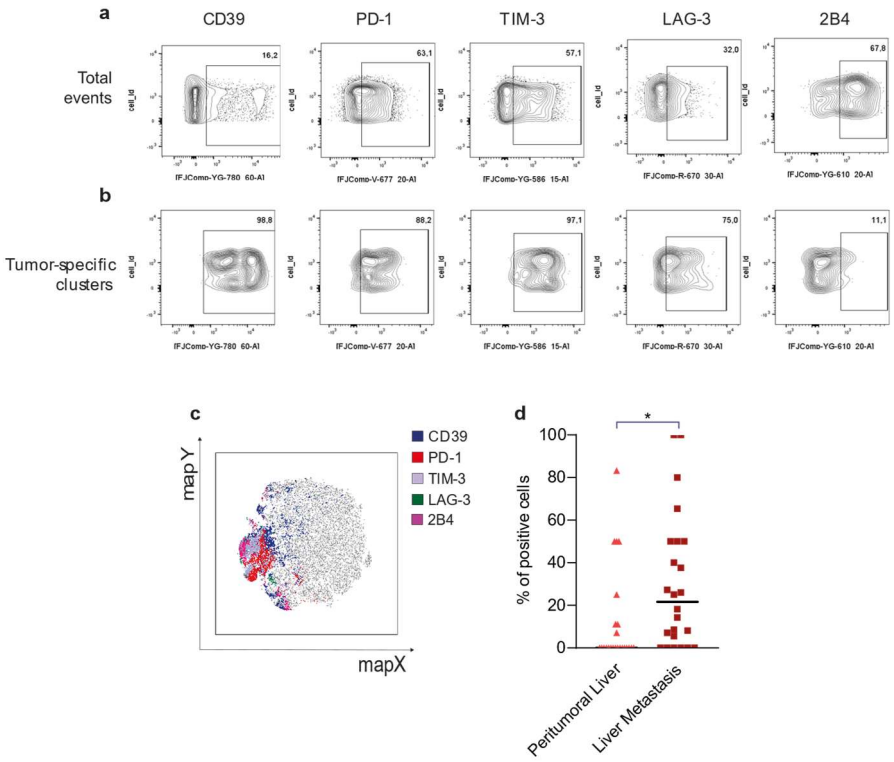

Figure 22 $CD39^{+}PD1^{+}TIM3^{+}LAG3^{+}2B4^{-}$ CD8 T cells are enriched within CRC liver metastasis. The frequency of T cells positive for CD39, PD1, GITR and CD137 was manually gated and plotted among total events as well as in tumor-specific clusters (**a**). These markers are co-expressed in a specific cluster of cells, as shown by the bh-SNE map (**b**) and define a cluster of CD8 T lymphocytes significantly enriched within the tumor while being absent within the healthy and peritumoral tissues (**c**).

2.3.3 Clinical and biological variables are not predictive of cluster distribution

To investigate a putative correlation between biological and clinical variables, we analyzed cluster distribution in the peritumoral and metastatic liver according to tumor-regression grade (**Fig.23a**), Fong score (**Fig.23b**) and KRAS status (**Fig.23c**). These parameters did not impair cluster distribution among the two compartments in our cohort. On the contrary, the effect of chemotherapy could really shape T cell phenotype and distribution. We performed a principal component analysis (PCA) on the cluster frequency in each sample: 49% of the variance is explained by PC1 and PC2 in CD4 T cells, (**Fig.24a,b**) and 39% by PC1 and PC3 in CD8 T cells (**Fig24c,d**), clustering patients in two and three groups, respectively.

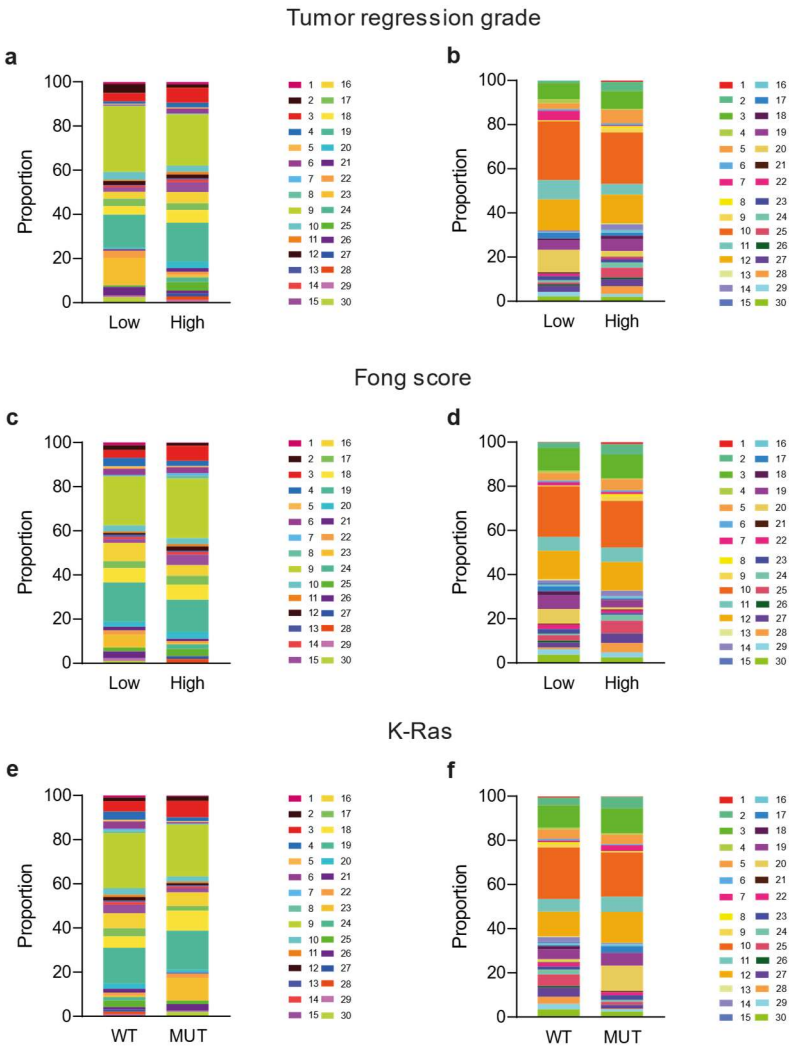

Figure 23 Cluster distribution is not driven by biological or clinical variables. Cluster distribution of both CD4 (**a**,**c**,**e**) and CD8 (**b**,**d**,**f**) does not vary depending on response to chemotherapy, measured as tumor regression grade (**a**,**b**), clinical survival probability, indicated as Fong score (**c**,**d**) and KRAS mutation (**e**,**f**).

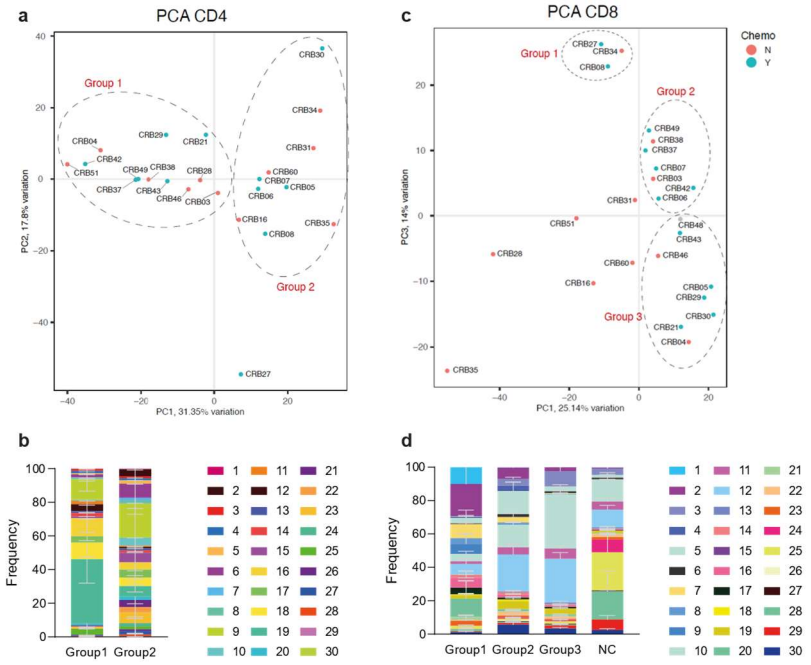

Figure 24 Neo-adjuvant chemotherapy clusters patients according to IR expression patterns. Principal component analysis discriminates two groups of patients (**a**) according to cluster distribution in CD4 T cells (**b**) and three groups of patients (**b**) according to cluster distribution in CD8 T cells (**d**). In CD4 T cells, the two groups are not different in terms of chemo-treatment. In CD8 T cells, group 2 and group 3 are characterized by a prevalence of chemo-treated patients.

2.4 PD1$^+$CD39$^+$ T cells are detectable within the peripheral blood of CRC patients

Antigen-specific T cells are usually detected and isolated from the tumor tissue. This procedure limits the broad applicability of personalized T cell therapies since it requires long and invasive procedures. The peripheral blood could represent an alternative source of tumor-specific T cells, and it has already been retrospectively demonstrated to contain tumor-reactive T cells. Since PD1 and CD39 were shared hallmarks for primary and metastatic CRC-specific T cells, we searched for PD1$^+$CD39$^+$ T cells within the peripheral blood (PB) of CRC patients compared to healthy donors (HD). Being absent in HD PB, PD1$^+$CD39$^+$ are detectable at low frequency in the PB of patients affected by primary CRC, while their proportion is significantly increased in metastatic CRC patients both in the CD4 (**Fig.25a**) and CD8 (**Fig.25b**) T cell compartments. When analyzing T cell memory subsets, a significant reduction in the memory compartments (T_{CM}/T_{SCM}) is observed in patients compared to HDs in CD4 (**Fig.25c**) as well as in CD8 (**Fig.25d**) T cells, concomitantly with an enrichment in effector T cells. Overall, these results suggest that PD1$^+$CD39$^+$ T cells circulating in patients could be enriched in tumor-specific T cells and represent an interesting source of tumor specific TCRs.

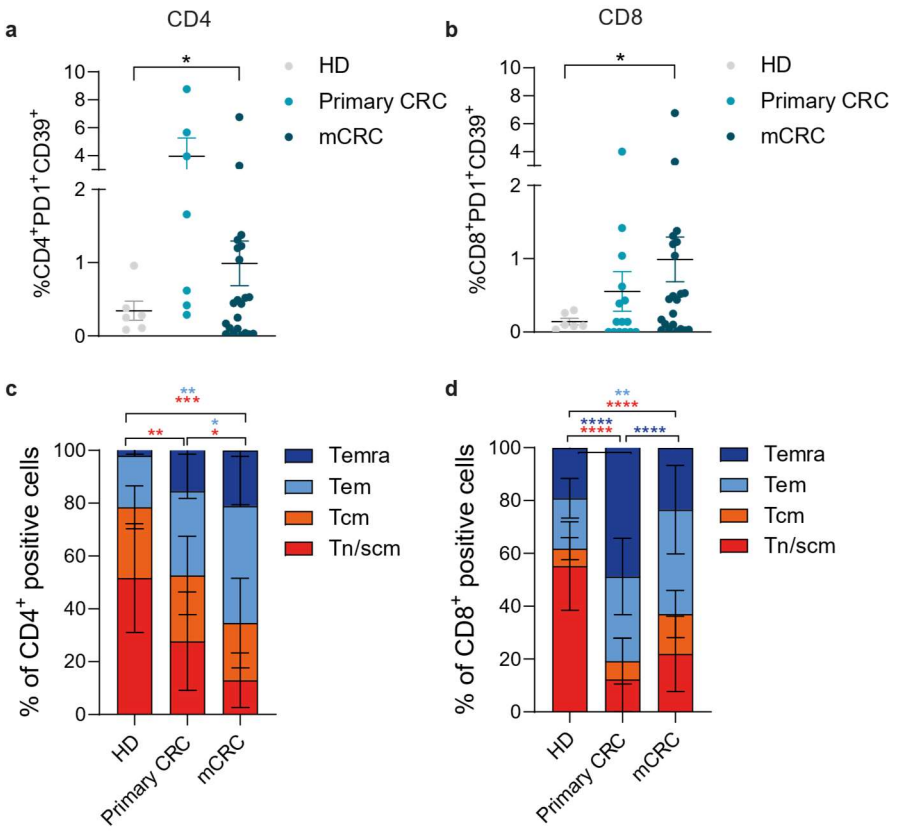

Figure 25 PD1⁺CD39⁺ T cells are enriched in CRC patients' peripheral blood. Frequency of PD1⁺CD39⁺ in CD4 (**a**) and CD8 (**b**) T cells in the peripheral blood of healthy donors (HD) and patients affected by primary or metastatic CRC. Relative proportion of circulating T_{CM} ($CD45RA^{-}CD62L^{+}$), T_N/T_{SCM} ($CD45RA^{+}CD62L^{+}$), T_{EMRA} ($CD45RA^{+}CD62L^{-}$), T_{EM} ($CD45RA^{-}CD62L^{-}$) CD4 (**c**) and CD8 (**d**) T cells from HD and CRC patients is shown. (HD n=6; pCRC n=15, mCRC n=24). Data are plotted as mean ± SEM.

3. Materials and methods

3.1 Human samples

3.1.1 Sample collection

Patients' samples were collected upon written informed consent, after Institutional Ethical Committee approval. In this study, we collected samples from 21 treatment-naïve patients affected by primary CRC undergoing hemicolectomy and 26 patients affected by CRC metastatic to the liver, undergoing liver resection. For primary CRC patients, non-neoplastic, peritumoral and tumoral colon tissues were collected from the same surgical specimens. For mCRC, peritumoral and metastatic liver were obtained. Peripheral blood was collected before surgery for each patient. Detailed patients' characteristics are described in tables 1 and 2. Healthy donors' peripheral blood was obtained at San Raffaele Hospital upon informed consent.

3.1.2 Sample processing

Tissue samples were repetitively washed with phosphate-buffered saline (PBS) and collected in Tissue Storage Solution (Miltenyi Biotec) at 4°C until processing. Tissues were manually reduced in small fragments and further processed into a single cell suspension by combining mechanical and enzymatic dissociation using gentleMACS dissociator (Miltenyi Biotec). Dissociated samples were filtered through a 70μ cell strainer (Falcon) and washed with PBS. After centrifugation, red blood cell lysis was performed when necessary by incubating cells in ACK solution for 5 minutes at room temperature.

After wash and centrifugation, cell number was determined. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood by Ficoll-Hypaque gradient separation (Lymphoprep™, Fresenius). Cells were either freshly used for flow cytometry or frozen in liquid nitrogen according to standard procedures.

3.2 Flow cytometry

3.2.1 Staining and data acquisition

Cells were washed with PBS and centrifuged (1500rpm, 5 min). Cells were stained with the Zombie Green Fixable Viability dye (Biolegend) for 15 min at room temperature. Then, cells were washed with PBS supplemented with 1% FBS and stained with a mix of antibodies listed in table 3 (primary CRC) and 4 (mCRC) and previously resuspended in BD brilliant stain buffer (BD bioscience). After a 10 min incubation at RT, samples were further washed and centrifuged. All antibodies were previously titrated to determine the optimal concentration. Data were acquired at the institutional BD LSRFortessa™ and BD Symphony™ cytometers.

Manufacturer	Clone	Specificity	Fluorophore
Biolegend	-	Zombie Green	FITC
Biolegend	C1.7	2B4	PE-Dazzle594
Biolegend	A1	CD39	PE-Cy7
Biolegend	A161A1	CD4	AlexaFluor700
Biolegend	4B4-1	CD137	BV421
Biolegend	BNI3	CTLA-4	BV605
Biolegend	EH12.2H7	PD-1	BV650
Biolegend	108-17	GITR	BV711
Biolegend	2F1	KLRG-1	BV785
Miltenyi	REA635	TIM-3	PE
Miltenyi	REA351	LAG-3	APC
Miltenyi	REA738	CD95	APC-Vio770
BD	DREG-56	CD62L	BV480
BD	SP34-2	CD3	BUV395
BD	SK1	CD8	BUV737
Fisher Scientific	MBSA43	TIGIT	PerCP-eFluor710
Thermofisher	MEM-56	CD45RA	PE-Cy5.5

Table 4 List of antibodies used to stain primary CRC samples.

Manufacturer	Clone	Specificity	Fluorophore
Biolegend	-	Zombie Green	FITC
Biolegend	C1.7	2B4	PE-Dazzle594
Biolegend	A1	CD39	PE-Cy7
Biolegend	A161A1	CD4	AlexaFluor700
Biolegend	4B4-1	CD137	BV421
Biolegend	BNI3	CTLA-4	BV605
Biolegend	EH12.2H7	PD-1	BV650
Biolegend	108-17	GITR	BV711
Biolegend	2F1	KLRG-1	BV785
Thermofisher	MEM-56	CD45RA	PE-Cy5.5
Miltenyi	REA635	TIM-3	PE
Miltenyi	REA351	LAG-3	APC
Miltenyi	REA738	CD95	APC-Vio770
BD	741182	TIGIT	BB700
BD	DREG-56	CD62L	BV480
BD	G46-6	HLA-DR	BUV395
BD	SK1	CD8	BUV737
BD	OKT3	CD3	BUV496

Table 5 List of antibodies used to stain metastatic CRC samples.

3.2.2 Data analysis

Flow cytometry data were analyzed using Flow Jo software (Tree star Inc) and Cytochain web-app. The samples from each dataset were first separately evaluated for acquisition stability using Flow_iQC algorithm. Successively, the optimized flowSet was concatenated in a single flowFrame and data pertaining experimental variables linked to the file. Flow-SOM-based clustering followed, and the resulting 100 clusters were collapsed into 30 meta-clusters by ConsensusClusterPlus. Clusters and experimental groups were then super-imposed on the BH-SNE map and organized into a proximity tree heatmap according to fluorescence similarities. The dataset was then studied using cytoChain tools. (Manfredi*,Abbati*,…,Potenza et al., *submitted*). Statistical analyses were performed using Prism8 software (GraphPad). Student-t test and two-way ANOVA were performed throughout the study.

4. Discussion

T cells are fundamental in the CRC microenvironment to regulate tumor development and progression. However, the inhibitory signals deriving from the immune suppressive microenvironment limit their ability to harness a potent immune response. In this study, we comprehensively described the memory phenotype and the exhaustion profile of T cells infiltrating colorectal tumors and colorectal liver metastasis. To this aim, we used high dimensional flow cytometry, allowing the simultaneous visualization of multiple parameters at a single-cell resolution. By comparing T cells from healthy (non-neoplastic), peritumoral and tumoral tissue we observed that the ratio between CD4 and CD8 T cells is comparable within the three specimens. Interestingly, a small but consistent proportion of $T_{CM/SCM}$ cell is present within the peritumoral tissue only, but the largest proportion of T cells are effectors. This is in line with the known function of $T_{CM/SCM}$ cells, that preferentially home in secondary lymphoid tissues and represent long-term memory T cells[2]. When looking at the expression of inhibitory receptors by analyzing flow cytometry data by manual gating, we noticed an increased proportion of T cells expressing PD1 and CD39 within the tumors compared to the peritumoral tissue. However, it was difficult to define a precise signature. In fact, multiparametric flow cytometry produces large datasets that are difficult to handle by standard analysis. Moreover, the mere overexpression of markers is not sufficient to define T cell exhaustion, while accumulating date point to the co-expression of multiple molecules as major determinants

impaired T cell function. For this reason, we analyzed our datasets by using Cytochain, a web-app that our group developed to evaluate, optimize and investigate flow cytometry datasets by integrating different dimensionality reduction and clustering algorithms, originally developed to handle genomic and transcriptomic set of data (Manfredi*, Abbati*,…, Potenza, et al. *submitted*). Our analysis showed that inhibitory receptors co-expression is peculiar of T cells retrieved from tumor samples, independently from being primary tumors or liver metastases, while being less evident in the peritumoral or healthy tissues. Unsupervised analyses showed the enrichment of a population of $PD1^{+}CD39^{+}GITR^{+}CD137^{+}$ T cells infiltrating CRC, which is absent within the healthy and peritumoral tissues. This complex signature characterized both CD4 and CD8 T cells and was independent from the microsatellite status, thus suggesting that probably microsatellite instability is not the only determining factor for achieving response from checkpoint blockade. Of note, when compared to lymphocytes from the peritumoral liver, T cells present in the metastatic liver were also overexpressing PD1 and CD39, however this co-expression did not associate to that of GITR and CD137, but instead to the expression of TIM-3 and LAG-3, 2 additional inhibitory receptors. These results led us to hypothesize that there are tumor-specific pathways and tissue-related pathways leading to the upregulation of different molecules. It will be important to widen the study, by including in the analyses other cells present in the microenvironment, such

as myeloid, endothelial and epithelial cells, to further investigate the mechanism underlying this observation.

Clinical variables such as the tumor regression grade, Fong score or KRAS status were not impairing cluster distribution. On the contrary, chemotherapy could act as a modifying factor for the TME, since different groups of patients could be clustered basing on the time interval between the last chemotherapy and the sample collection. Longitudinal studies on samples harvested before and after chemotherapy, from the same patients, could allow to further corroborate these results and to identify potential mechanisms underlying these observations. Given the sharing of PD1 and CD39 co-expression on T cells from both colon and liver tissues, we searched for PD1 and CD39 co-expression in T cells retrieved from the peripheral blood of CRC and mCRC patients. The presence of circulating T cells harboring the same characteristics of TILs is relevant for two reasons: (i) as a prognostic factor, since the presence of $PD1^{+}CD39^{+}$ T cells could serve as a liquid biopsy to screen patients in a non-invasive way; (ii) to identify new TCR specificities starting from an easily available source. Despite at very low frequencies, we observed a slight enrichment of $PD1^{+}CD39^{+}$ T cells in patients affected by primary CRC compared to healthy donors, and a significant enrichment in mCRC patients. Nonetheless, memory T cells contract as the disease progress, favoring the expansion of effector T cells.

Overall, our data demonstrate the presence, in the CRC microenvironment, of T cells displaying exhaustion features, determined by a specific pattern of expression of inhibitory receptors. Fine mapping of the IRs pathway involving transcriptomic data from the same patients is ongoing to better define the relevance of PD1 and CD39 as targets for immunotherapeutic strategies.

CHAPTER 4

Discovering new CRC-directed TCR specificities

1. Introduction

TCR-redirected T cells harbor the potential to induce effective and durable clinical responses against tumors. However, one of the hurdles limiting the wide application of this adoptive T cell therapy strategy is the identification of novel TCRs specific for tumor epitopes. In fact, a proper target antigen needs to be expressed selectively by tumor cells, processed and presented in the context of an HLA molecule, and possibly necessary for the oncogenic process, in order to reduce the risk of tumor escape through antigen downregulation. For CRC, there is still no overexpressed tumor antigen reflecting all these characteristics, and neoantigens are hardly shared between patients, thus pointing at the generation of cost-effective personalized therapies. On the other hand, neoantigens are recognized by the immune system as foreign, thus in principle, neoantigen-specific T cells are not eliminated or dampened by tolerance mechanisms, even when they harbor high affinity TCRs[3]. The isolation of tumor-reactive T cells is a challenging process also for the choice of the proper T cell source. Starting from the consideration that tumor-reactive clones should reside inside the tumor mass, initially the preferential source for the identification of anti-tumor TCRs was thought to be TILs[4]. Unfortunately, tumor-reactive clones represent only a minimal frequency of the total tumor-infiltrating T cell population[5], thus the

ex vivo TILs culture could favor the expansion of non-specific clones. Subsequently, peripheral blood started to be considered as a source of anti-tumor TCRs, with the limitation that circulating tumor-reactive T cells are present at a very low frequency[6], hence possibly impairing their isolation. In recent years, with the upcoming concept of T cell exhaustion, studies started to explore the possibility of using the exhaustion signature to identify a subset of T cells with enriched tumor specificity[7], leading also to the enrichment of neoantigen-specific T cells from the peripheral blood. In fact, PB is a non-invasive and easy source of autologous lymphocytes of particular relevance for non-resectable tumors[8].

In this project, we decided to exploit two alternative strategies to retrieve novel anti-tumor TCR specificities: (i) selecting tumor-specific TILs by leveraging on their specific exhaustion signature and (ii) taking advantage of healthy donors' PBMCs stimulated with APCs loaded with immunogenic peptides as a source of novel TCRs specific for *a priori* selected antigens.

2. Results

2.1 Discovering new TCR specificities by exploiting TILs exhaustion signature

PD1 and CD39 positive T cells are enriched within the CRC microenvironment and are characterized by a complex exhaustion signature. IRs co-expression is a negative regulator of naturally occurring T cell responses to tumor antigens. Thus, exploiting the IRs signature could be beneficial for (i) the expansion of TIL subpopulations potentially harboring anti-tumor specificity, thus enhancing the probability of achieving tumor response after *ex vivo* expansion and re-infusion to patients, and (ii) for identifying novel anti-tumor TCR specificities to be used for adoptive T cell therapy with genetically engineered T cells. To assess whether a specific inhibitory signature could allow for the identification of a population of T cells with a tumor-driven TCR repertoire, we selected two CRC patients, respectively harboring (CRC9) or not (CRC13) microsatellite instability. We performed TCRαβ sequencing on $PD1^{+}CD39^{+}$ (IR positive), $PD1^{+}CD39^{-}$ or $PD1^{-}CD39^{+}$ (IR intermediate) and $PD1^{-}CD39^{-}$ (IR negative) subpopulations sorted from bulk TILs. TCR sequencing results were intriguing. In CRC9, we observed a strong oligoclonality in the repertoire of the IR positive population, different from the more polyclonal IR intermediate and IR negative subsets (**Fig.26a**). This is in line with the evidences indicating that inhibitory receptors are upregulated on tumor specific T cells[9,10].

However, we observed a different picture in CRC13, where the repertoire of IR positive T cells was comparable or even more polyclonal than the ones displayed by the other two sorted populations (**Fig.26b**).

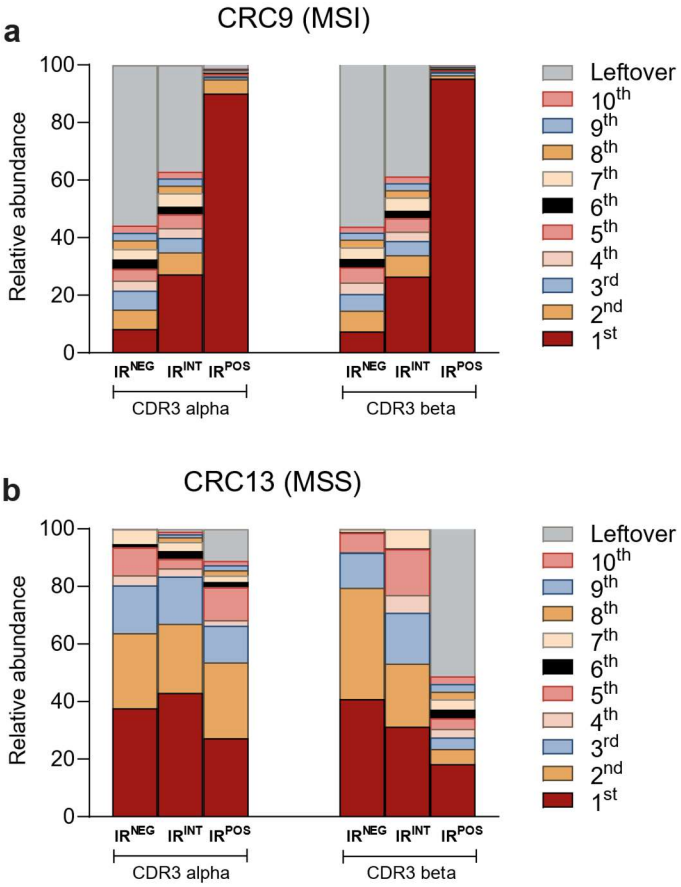

Figure 26 Assessment of TCR repertoire clonality in T cell subsets expressing different levels of IRs in CRC patients. TCR sequencing was performed on IR pos ($PD1^{+}CD39^{+}$), IR int ($PD1^{+}CD39^{-}$/$PD1^{-}CD39^{+}$) and IR neg ($PD1^{-}CD39$-) TILs from one MSI (a) and one MSS (b) CRC patient. Colors indicate the 10 most abundant TCR clones for each specimen, while the grey indicates the remaining clones of the repertoire. The highest the grey bar, the more polyclonal the repertoire. IR=inhibitory receptors; CDR= complementarity determining region.

The TCR repertoire in CRC is heterogeneous, differing between regions within the same tissue. Also, evidence showed the presence of CRC-specific T cells also within the peripheral blood[7], despite at limited frequencies. Therefore, we wanted to study the TCR patterns in T cells infiltrating healthy, peritumoral and neoplastic tissue as well as in circulating T cells to explore the possibility to use PB as a source of anti-tumor specificities.
We selected three patients, two MSS (CRC13 and CRC20) and one MSI (CRC24).

In CRC13, the repertoire of T cells infiltrating the healthy tissue was even more skewed than the one retrieved from the peritumoral or the tumor tissue (**Fig.27a,b**). As shown by the heatmaps, there was a minimal sharing of poorly represented TCR clones between the peripheral blood and any of the analyzed tissues (**Fig.27c,d**).

In CRC20, the peritumoral tissue displayed an almost monoclonal TCR repertoire both in terms of α (**Fig.28a**) and β chain (**Fig.28b**) CDR3 sequences. Few CDR3α were shared among the different specimens (**Fig.28c**). Of note, several CDR3β sequences were overlapping between the healthy and tumor tissue, and some of them were present in T cells from the peritumoral zone and in the peripheral blood (**Fig.28d**). Overall, the two MSS tumors did not lead to a particular expansion of TCR clones in TILs.

In CRC24, peritumoral TILs harbored the most various TCR repertoire. Within the healthy and the neoplastic tissue, the repertoire of TILs for both CDR3α (**Fig.29a**) and β (**Fig.29b**) was covered by the ten most abundant clones. However, the TCR repertoire of TILs in this MSI tumor was more skewed than the one observed in TILs from MSS patients. The heatmaps indicate that each specimen display its own repertoire, with nearly absence of shared clones (**Fig.29c,d**).

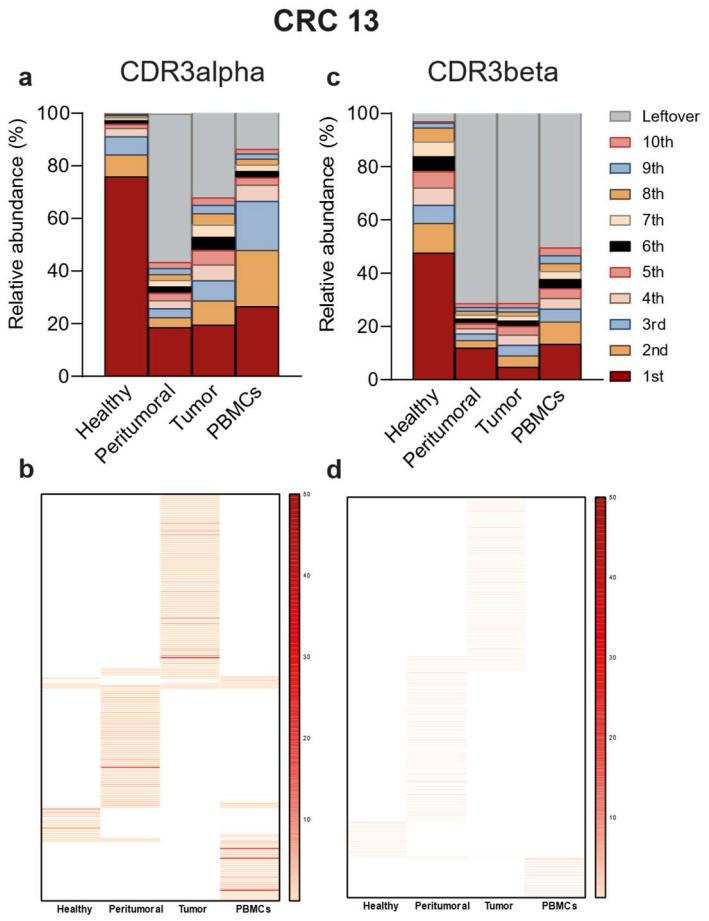

Figure 27 TCR repertoire of TILs and PB-T cells from MSS patient CRC 13. TCR sequencing was performed on T cells infiltrating the healthy, peritumoral and tumor tissue as well as from peripheral blood T cells. Colors indicate the 10 most abundant CDR3 α (a) and β (b) sequences, while the grey indicates the remaining part of the repertoire, so that the highest the grey bar, the more polyclonal the repertoire. Heatmaps highlight CDR3 α (c) and β (d) sequences sharing among the different specimens, with the color scale indicating the frequency of the CDR3 amino-acid sequences, reported in rows.

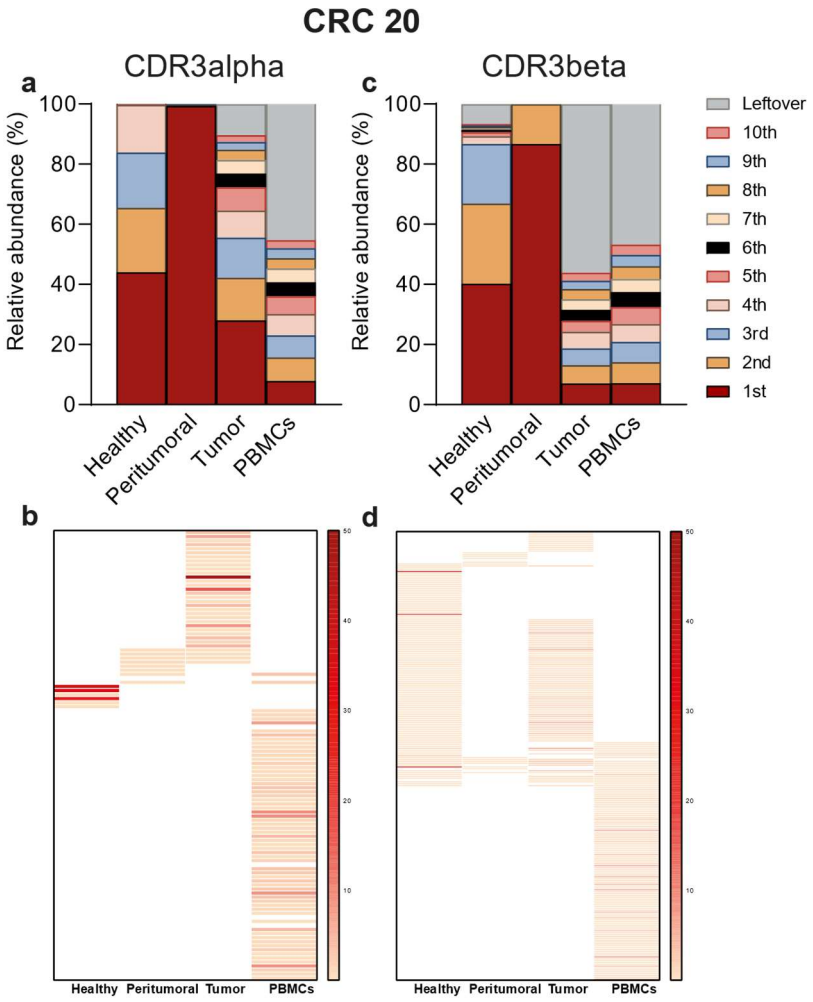

Figure 28 TCR repertoire of TILs and PB-T cells from MSS patient. CRC20. TCR sequencing was performed on TILs from healthy, peritumoral and tumor tissue as well as from peripheral blood T cells. Colors indicate the 10 most abundant CDR3 α (a) and β (b) sequences, while the grey indicates the remaining part of the repertoire, so that the highest the grey bar, the more polyclonal the repertoire. Heatmaps highlight CDR3 α (c) and β (d) sequences sharing among the different specimens, with the color scale indicating the frequency of the CDR3 amino-acid sequences, reported in rows.

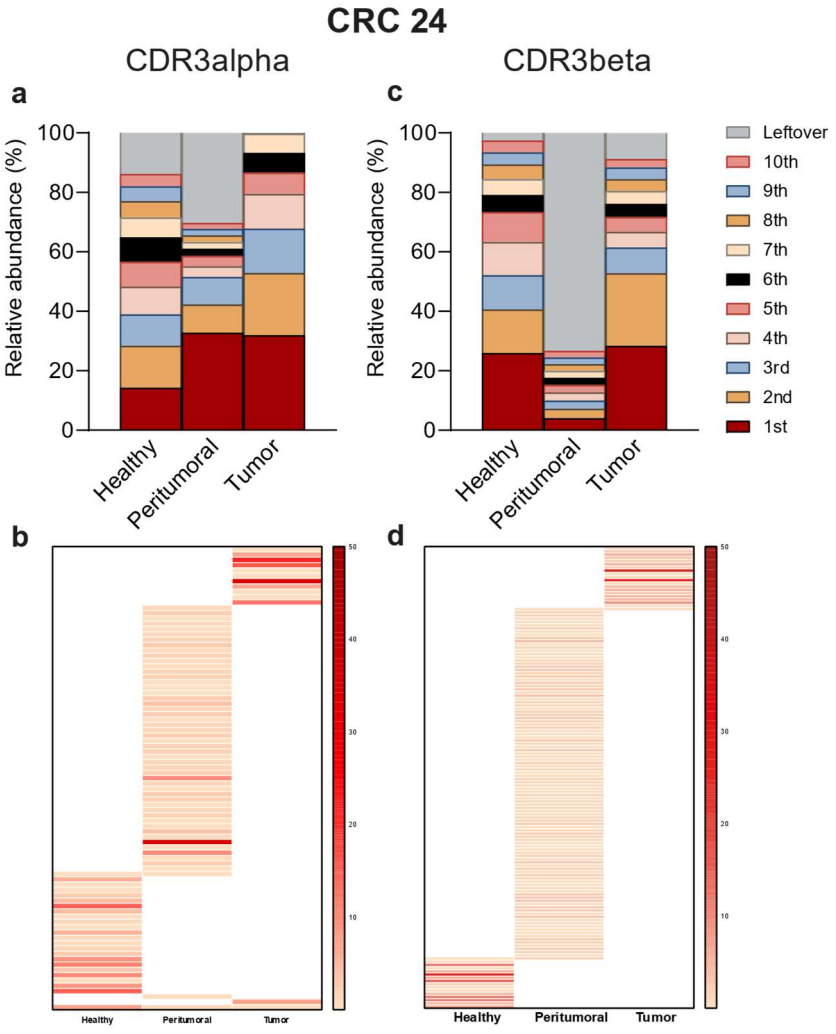

Figure 29 TCR repertoire of TILs and PB-T cells from MSI patient CRC24. TCR sequencing was performed on TILs from healthy, peritumoral and tumor tissue. Colors indicate the 10 most abundant CDR3 α (a) and β (b) sequences, while the grey indicates the remaining part of the repertoire, so that the highest the grey bar, the more polyclonal the repertoire. Heatmaps highlight CDR3 α (c) and β (d) sequences sharing among the different specimens, with the color scale indicating the frequency of the CDR3 amino-acid sequences, reported in rows.

2.2 Discovering new TCR specificities starting from healthy donors' PBMCs

A prerequisite for the development of cancer therapies is the identification of highly immunogenic tumor antigens. To screen for immunologically relevant epitopes, whole-exome and RNA sequencing represent nowadays the most standardized technologies to compare mutations and expression levels among the tumoral and the healthy tissues. Complex bioinformatic tools allow for *in silico* validation[11] of the defined epitope prior to *in vitro* screening. In a seminal work, epitope prediction algorithms followed by high throughput validation analyses led to the identification of candidate tumor antigens, revealing that a single CRC accumulates on average 7 novel and unique HLA-A*02:01 epitopes[12]. More recently, the antigenome of human CRC was characterized by examining genomic data sets from 598 tumors[13]. Results highlighted the presence of cancer-germline antigens as well as of some public (shared among 7-28% of the patients) neo-antigens[14]. Another approach to retrieve tumor epitopes relies on the study of the tumor peptidome composition[15,16]. Such a strategy was applied to investigate the

ligandome of non-malignant and neoplastic colon tissue and display the advantage to identify truly immunogenic and HLA-presented peptides[17]. Along with being naturally processed by the proteasome, peptides are presented in the context of an HLA, thus is necessary to select peptides restricted in an HLA allele common in the Caucasian population. The identification of immunogenic peptides is critical for strategies employing healthy donor peripheral blood to retrieve novel TCR specificities, allowing to overcome the limitation of the paucity of tumor-reactive repertoires in patients (Ruggiero, …, Potenza et al., *submitted*).

To identify novel TCRs starting from HD PBMCs, we selected 34 peptides described to be immunogenic for CRC, either derived from tumor-associated antigens or from neoantigens (**Table 5**).

P#	Antigen	Peptide	HLA restriction	Reference
1	EpCAM	GLKAGVIAV	A2	Int. J. Cancer: 105, 221–225 (2003)
2	HER-2	ALCRWGLLL	A*0201	Hum Immunol. 1998 Jan; 59(1):1-14.
3	HER-2	KIFGSLAFL	A*0201	Hum Immunol. 1998 Jan; 59(1):1-14.
4	HER-2	IISAVVGIL	A2	Int. J. Cancer: 105, 221–225 (2003)
5	HER-2	RWGLLLALL	A*24	British Journal of Cancer (2001) 84(1), 94–99
6	MUC-1	LLLLTVLTV	A*0201	Cancer Immunol Immunother (2007) 56:287–301
7	MUC-1	TLAPATEPA	A*0201	Int J Cancer. 2001 Feb 1;91(3):385-92.
8	MUC-1	ALGSTAPPV	A*0201	Int J Cancer. 2001 Feb 1;91(3):385-92.
9	MUC-1	FLSFHISNL	A*0201	Int J Cancer. 2001 Feb 1;91(3):385-92.
10	K-RAS	YKLVVVGAV	A2	Cell Immunol. 1998 Aug 1;187(2):103-16.
11	TGF-bRII	RLSSCVPVA	A*0201	Cancer Immunol Immunother. 2001 Nov;50(9):469-76
12	RNF43	TQLARFFPI	n.a.	Genome Biol. 2015 Mar 31;16:64.
13	RNF43	RFFPITPPV	n.a.	Genome Biol. 2015 Mar 31;16:64.
14	RNF43	MQLCTQLARFF	n.a.	Genome Biol. 2015 Mar 31;16:64.
15	RNF43	ARFFPITPPV	n.a.	Genome Biol. 2015 Mar 31;16:64.
16	RNF43	QLARFFPI	n.a.	Genome Biol. 2015 Mar 31;16:64.
17	KRAS/NRAS	GADGVGKSAL	n.a.	Genome Biol. 2015 Mar 31;16:64.
18	PIK3CA	FFDETRQL	n.a.	Genome Biol. 2015 Mar 31;16:64.
19	PIK3CA	SEITKQEKDFL	n.a.	Genome Biol. 2015 Mar 31;16:64.
20	PIK3CA	FFDETRQLCDL	n.a.	Genome Biol. 2015 Mar 31;16:64.
21	DOT1L	VTDPEKLNNY	A*0101	Cancer Res. 2018 Aug 15;78(16):4627-4641.
22	VAMP7	PSENNKLTY	A*0101	Cancer Res. 2018 Aug 15;78(16):4627-4641.
23	IFT43	LLDLDEELRY	A*0101	Cancer Res. 2018 Aug 15;78(16):4627-4641.
24	CCRL2	FLDGTFSKY	A*0101	Cancer Res. 2018 Aug 15;78(16):4627-4641.
25	CTDP1	YLNKEIEEA	A*0201	Cancer Res. 2018 Aug 15;78(16):4627-4641.
26	NDC80	ALNEQIARL	A*0201	Cancer Res. 2018 Aug 15;78(16):4627-4641.
27	AVL9	FYISPVNKL	A*2402	Cancer Res. 2018 Aug 15;78(16):4627-4641.
28	NOP16	YYQDTPKQI	A*2402	Cancer Res. 2018 Aug 15;78(16):4627-4641.
29	CTDP1	LYELHVFTF	A*2402	Cancer Res. 2018 Aug 15;78(16):4627-4641.
30	UTP20	AYIPKLLQL	A*2402	Cancer Res. 2018 Aug 15;78(16):4627-4641.
31	C2CD4A	VLRPPGTAL	B*0702	Cancer Res. 2018 Aug 15;78(16):4627-4641.
32	PTGFRN	RLASRPLLL	B*0702	Cancer Res. 2018 Aug 15;78(16):4627-4641.
33	SULF1	IRPNIILVL	C*0702	Cancer Res. 2018 Aug 15;78(16):4627-4641.
34	TACC2	YRNSYEIEY	C*0702	Cancer Res. 2018 Aug 15;78(16):4627-4641.

Table 6. List of the 34 immunogenic peptides composing the peptide pool. Peptides 1-9 belong to tumor-overexpressed antigens. Peptides 10-20 are neoantigens, and peptides 21-34 are ligandome-derived neoepitopes.

We stimulated T cells from two healthy donors, HD3 and HD7, with a pool of the 34 peptides. Since tumor-specific TCRs are very rare in the repertoire of HDs, several re-stimulations are needed to obtain an enriched antigen-specific T cell subpopulation. Thus, it's necessary to dispose of an unlimited reservoir of autologous APCs. Therefore, we generated immortalized autologous B cells (**Fig. 30a,b**) and we tested the

specificities of the T cell culture by loading APCs with the peptide pool.

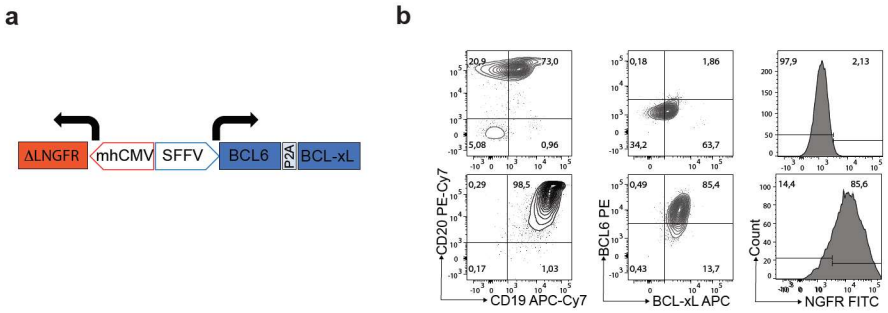

Figure 30 Schematic overview of the lentiviral vector with bidirectional promoter used to generate immortalized B cells. *BCL-6* and *BCL-XL* transgenes are oriented in sense and a reporter gene (*ΔLNGFR*) in antisense (**a**). Transduction efficiency was assessed by measuring the expression of the reporter gene on the surface of B cells and the intra-cytoplasmatic presence of Bcl-6 and Bcl-xL in immortalized B cells (lower panels) compared to non-immortalized B cells (upper panels) (**b**). *(Ruggiero, …, Potenza et al., submitted)*

For HD3, a response to the peptide pool was detected by measuring the expression of the degranulation marker CD107a (**Fig.31a**), despite low levels of IFN-y secretion (**Fig.31b**), already after two rounds of stimulation (**Fig.31a,b,c**). The immune response was augmented in terms of IFN-γ secretion after the 3rd restimulation of the T lymphocytes with the peptide pool (**Fig.31d,e,f**).

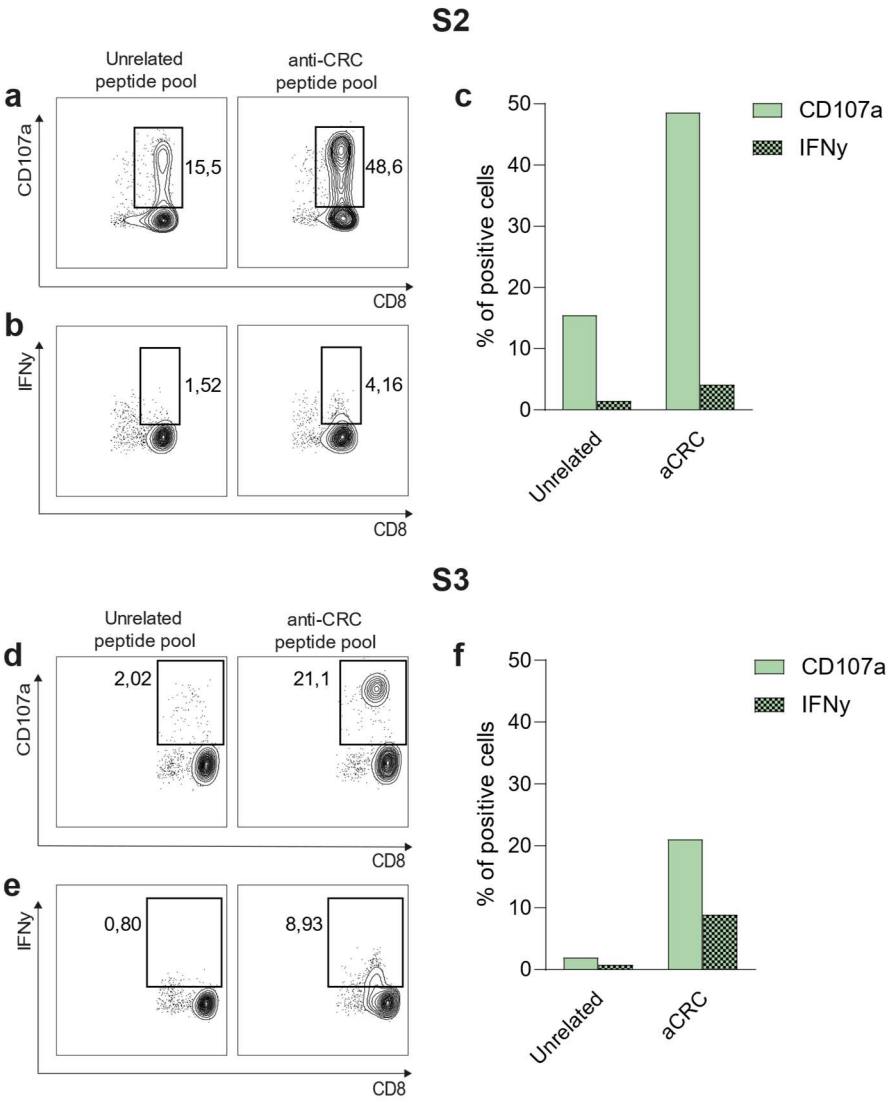

Figure 31 Peptide-specific T cell expansion from HD3 after the 2nd and 3rd round of stimulation with the peptide pool. The frequency of CD107a$^+$ and IFN-γ$^+$ cells is shown in T cells co-cultured for 6 hours with autologous APCs loaded with unrelated peptides or with anti-CRC peptide pool after two (**a,b,c**) and three (**d,e,f**) rounds of stimulation. IFN=interferon; S=stimulation.

To identify the recognized epitope, we divided peptides into sub-pools in the way that each peptide is present in only to sub-pools, as shown by the mapping grid (**Table 6**).

	SP-A	SP-B	SP-C	SP-D	SP-E	SP-F
SP-G	1	2	3	4	5	6
SP-H	7	8	9	10	11	12
SP-I	13	14	15	16	17	18
SP-J	19	20	21	22	23	24
SP-K	25	26	27	28	29	30
SP-L	31	32	33	34		

Table 7 Mapping grid. The mapping grid defines sub-pools of peptides, with each peptide being present only in two intersecting sub-pools (SP).

When stimulating T cells with APCs pulsed with the sub-pools, we observed multiple CD8-mediated T cell responses: a stronger one towards sub-pools C and G, and a milder one towards sub-pools A, B and D (**Fig.32a**), as shown by representative plots (**Fig.32b**).

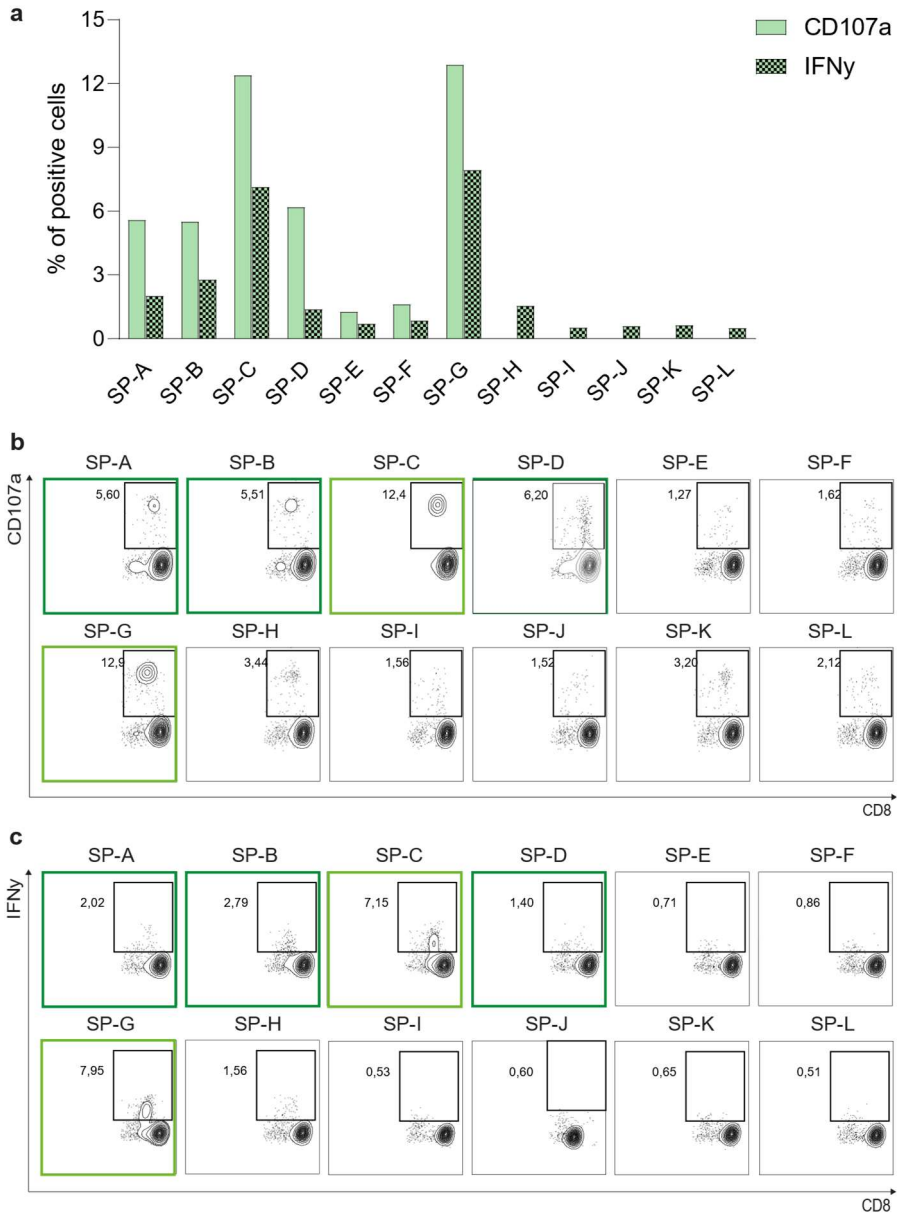

Figure 32 Sub-pool-specific T cell enrichment. Frequency of CD107a$^+$ and IFN-γ$^+$ T cells after 6 hours co-culture with autologous APCs loaded with peptide sub-pools (**a**); representative plots (**b**). SP=subpool.

Therefore, the culture was split in two: (i) a portion of cells was stimulated with APCs pulsed with P3, obtained by deconvoluting the mapping grid according to the response to sub-pools C and G; (ii) a portion was stimulated with APCs pulsed with sub-pools A, B and D. We challenged T cells from the “ABD” sub-culture with autologous APCs loaded with individual peptides belonging to sub-pools A, B and D. We observed a frequency higher than 10% of degranulating cells when stimulated with peptides 25 and 34 (**Fig.33a,b**), achieving a total of 3 peptides-specific cultures from a single HD (**Fig.33c**).

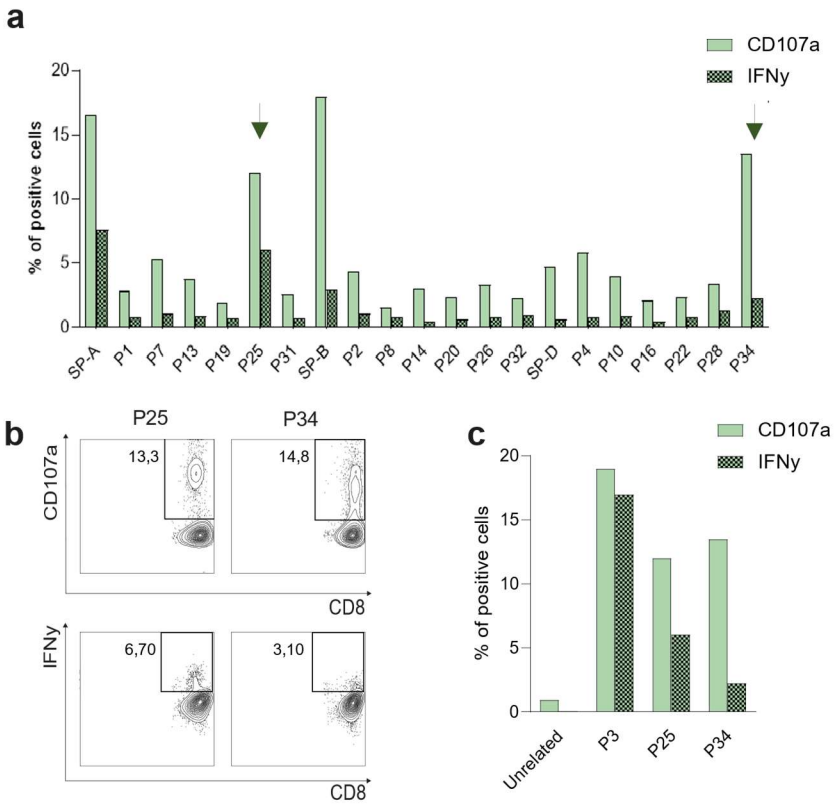

Figure 33 Expansion of T cells recognizing P3, P25 and P34 from HD3. Frequency of CD107a$^+$ and IFN-γ$^+$ T cells after 6 hours co-culture with autologous APCs loaded with peptides belonging to sub-pools A, B and D (**a**); representative plots showing degranulating (upper panel) and IFN-γ$^+$ T cells (lower panel) after co-culture with APCs loaded with peptide 25 or with peptide 34 (**b**); bar plots indicating the enrichment of T cells specific for peptides 3, 25 and 34 (**c**). Data are plotted as mean ± SEM. P=peptide; SP=subpool; IFN-γ=interferon-γ.

P25 and P34 are ligandome-derived neoepitopes of the CTDP1 and TACC2 proteins respectively, both involved in carcinogenesis. In particular, CTDP1 (RNA polymerase II subunit A C-terminal domain phosphatase) has a significant role in gastrointestinal cancer, since it is responsible for increased cell proliferation and apoptosis. Inhibition of CTPD1 expression has been demonstrated to dampen tumorigenicity in gastrointestinal cancer[18]. TACC2 (transforming acidic coiled-coil protein 2) is associated with the centrosome-spindle apparatus during cell cycle, and its gene is located in a chromosomal region associated with tumorigenesis[19]. Differently, peptide 3 is an epitope of the tumor-overexpressed antigen HER-2[20], already known to be HLA-A*02:01 restricted[21] and naturally processed by the proteasome[22]. To confirm this, we challenged the P3-stimulated subculture with peptide-pulsed autologous B cells (HLA-A*02:01,*02:17; HLA-B*15:01,*51:0; HLA-C*03:03,*16:02) (**Fig.34a,b**) as well as with peptide-pulsed T2 (HLA-A*02:01) cells (**Fig.34c,d**). Results showed the ability of P3-expanded T cells to specifically recognize HLA-A*02:01^{+} target cells only when pulsed with the cognate peptide, through a CD8-mediated T cell response.

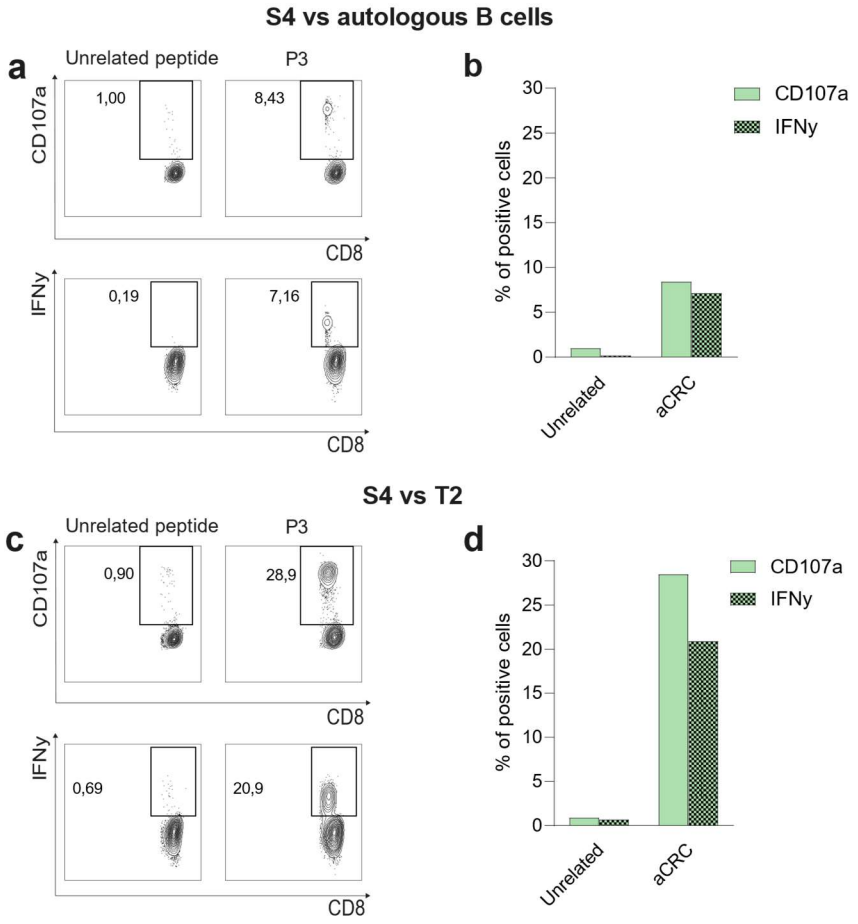

Figure 34 Validation of the HLA-A*02:01 restriction of P3-recognizing T cells. Frequency of CD107a$^+$ and IFN-γ$^+$ T cells after 6 hours co-culture with autologous APCs (**a,b**) or T2 cells (**c,d**) loaded with P3. P=peptide; IFN-γ=interferon-γ.

To verify the presence of expanded T cell clones in the different T cell subcultures, at first we evaluated overtime the Vβ repertoire by flow cytometry. TRBV6-6 was the predominant TRBV expressed by the T cells along all the stimulations, except

for the T cell subculture specific for P3 in the S5 stimulation. In this sample, we observed the enrichment of the TRBV6-5/6/9, too. (**Fig.35a,b**).

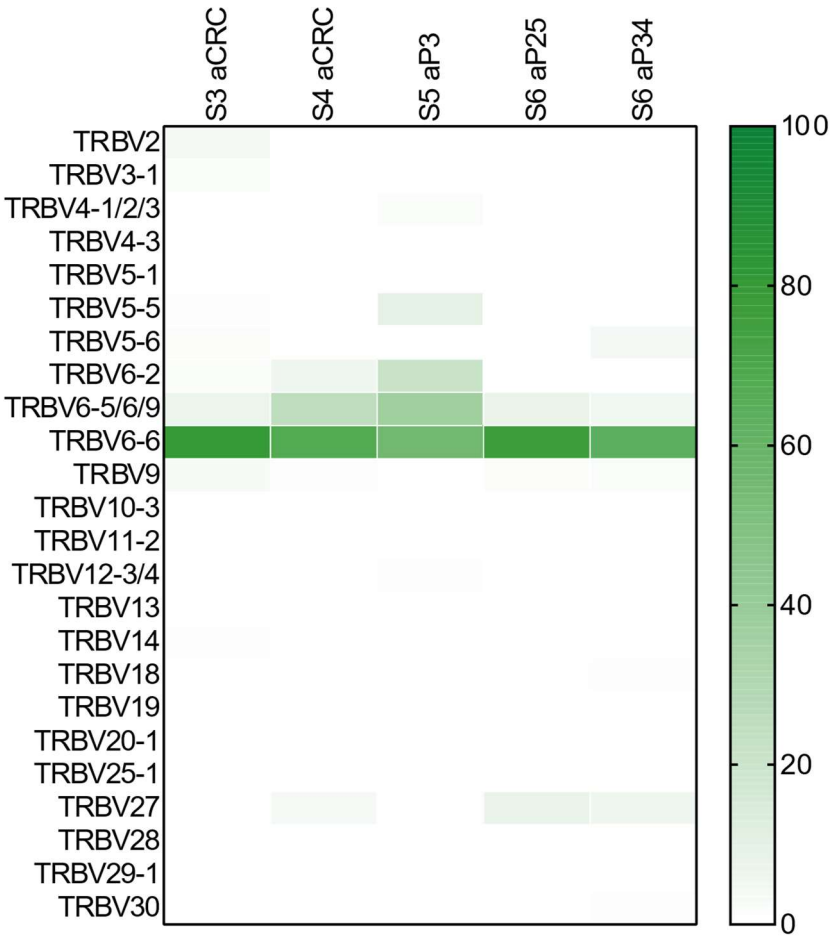

Figure 35 Cytofluorimetric assessment of the TRBV families partially allowed to evaluate the clonal T cell expansion overtime. Heatmap displaying TRBV enrichment in T cells stimulated with APCs loaded with the peptide pool after three and four re-stimulations, P3 after the 5th re-stimulation and P25 and P34 after the 6th re-stimulation. S=stimulation.

Afterwards, we performed TCRαβ sequencing, with the aim to identify and reconstruct epitope-specific TCRαβ clonotypes. To do so, we longitudinally tracked the clonality and the dynamics of the T cell clones in the different T cell subcultures. Overtime analysis revealed a progressive contraction of the TCR repertoire clonality. Specifically, for P3 we observed the predominance of two CDR3α aa sequences (frequencies: 37% and 35.5%, respectively) (**Fig.36a**), and two CDR3β aa sequences, (frequencies: 52,2% and 41,2%, respectively). The TRBV regions of the expanded TCR clones matched the ones highlighted by the flow cytometry kit (**Fig.36b**). For P25, we identified a dominant TCR pairing, with a CDR3α aa sequence present with a frequency of 73% (**Fig.36a**) and a CDR3β aa sequence present with a frequency of 85%. Of note, the TRBV region expressed by this highly dominant T cell clone could not be identified by the flow cytometry analysis, probably due to the limitations of the kit which covers only 70% of the entire Vb repertoire (**Fig.36b**). For P34 an enrichment of different αβ clonotypes was observed after the 6^{th} stimulation, with a 57% prevalent CDR3α aa sequence (**Fig.36a**) and a 47% prevalent CDR3β aa sequence. In this case, the TRBV region expressed

by the dominant T cell clone corresponded to the β sequence identified by the flow cytometry kit (**Fig.36b**).

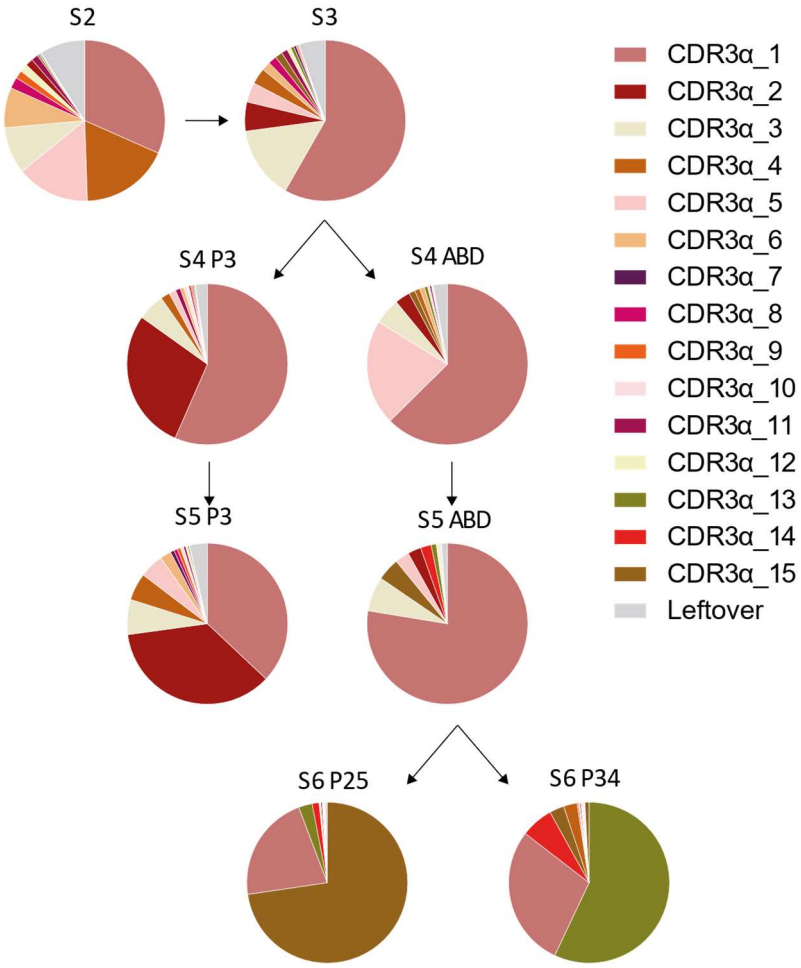

Figure 36a Clonal tracking of the TCRα chains in antigen-specific T cells from HD3. The clonality of the TRAV repertoire was longitudinally tracked by TCR sequencing. The 15 more frequent CDR3 sequences along the stimulations are plotted in the pie charts, where each color represent a unique CDR3 amino acid sequence. **S=stimulation.**

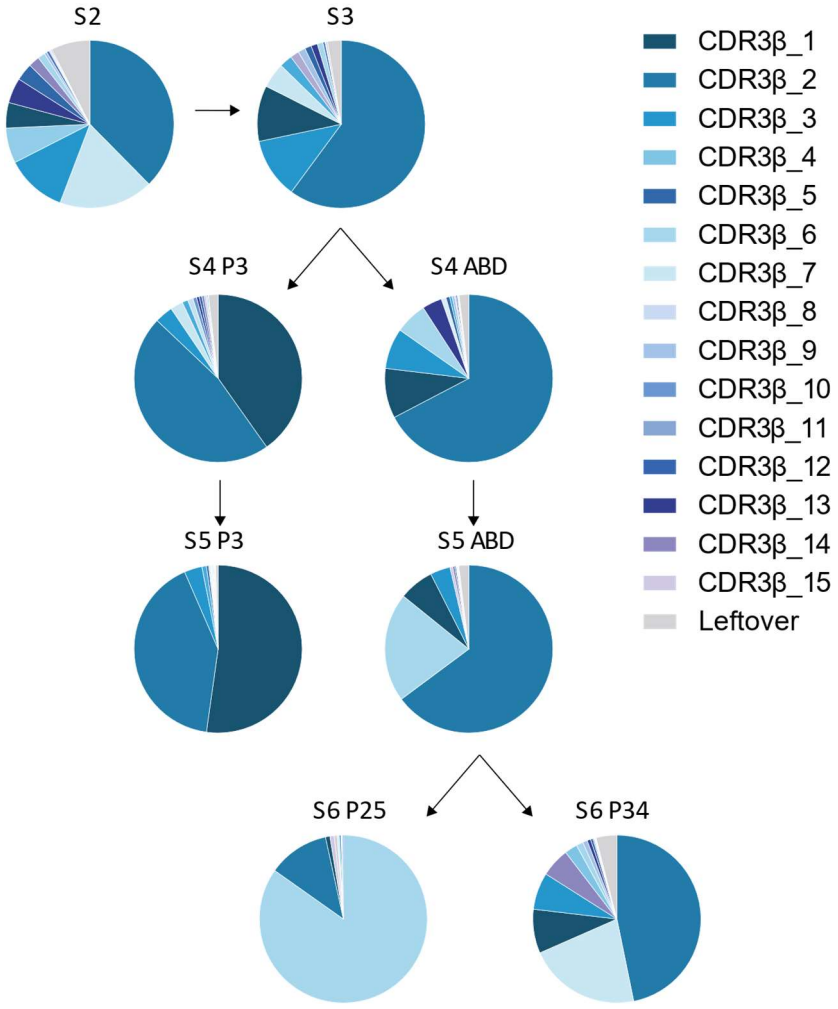

Figure 36b Clonal tracking of the TCRβ chains in antigen-specific T cells from HD3. The clonality of the TRBV repertoire was longitudinally tracked by TCR sequencing. The 15 more frequent CDR3 sequences along the stimulations are plotted in the pie charts, where each color represent a unique CDR3 sequence. S=stimulation.

We followed the same workflow for a second healthy donor (HD7). After 3 rounds of stimulation, we observed a clear T cell response in terms of CD107a when T cells were challenged with autologous APCs pulsed with the peptide pool, while little IFN-y production was detectable (**Fig.37a,b**).

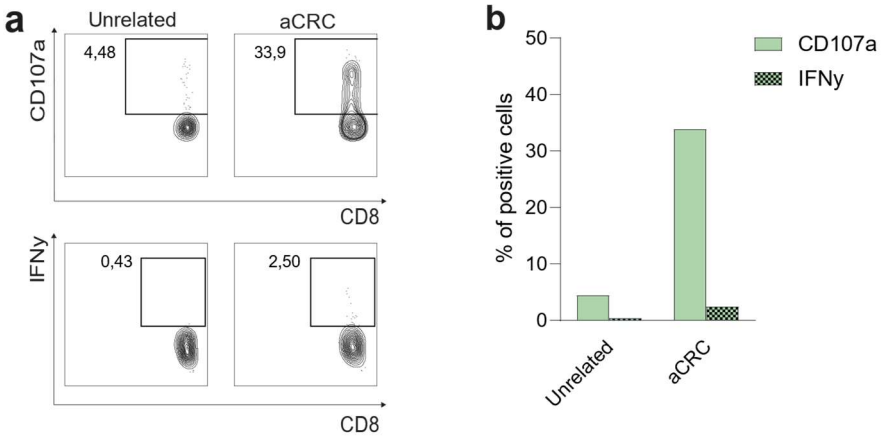

Figure 37 Tumor-specific T cells response after 3 rounds of stimulation in HD7. Representative plots (**a**) of the frequency of CD107a^{+} and IFN-γ^{+} T cells after 6 hours co-culture with autologous APCs loaded with the peptide pool (**b**).

When challenging the culture with autologous APCs pulsed with the 12 sub-pools, a 40% CD107a positive cells was observed against SP-B and SP-L (**Fig.38a,b**).

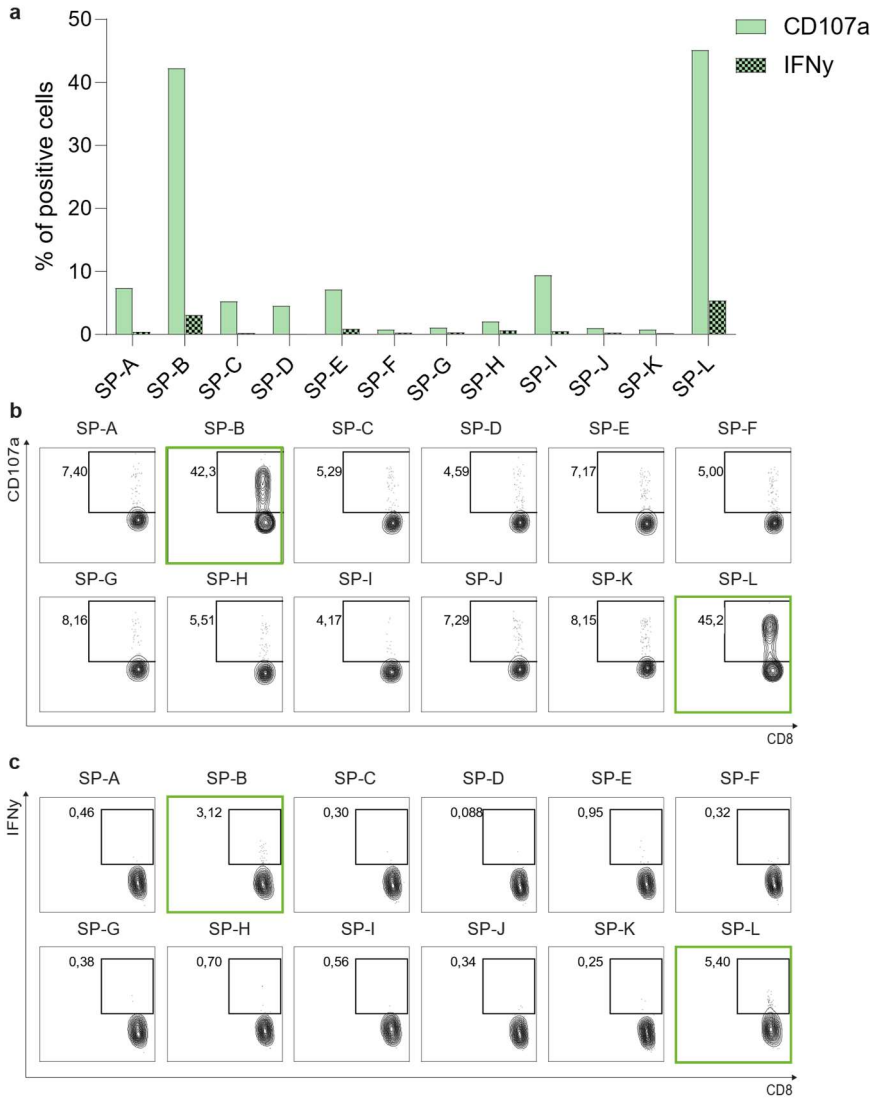

Figure 38 Enrichment of T cells responding to sub-pools B and L. Frequency of CD107a$^+$ and IFN-γ$^+$ T cells after 6 hours co-culture with autologous APCs loaded with sub-pools (**a**); representative plots (**b**). SP=subpools; IFN=interferon.

By deconvoluting the mapping grid, we identified a T cell response directed towards peptide 32 (**Fig.39**), a ligandome-derived neoepitope of the PTGFRN (Prostaglandin F2 receptor negative regulator) protein, whose overexpression is associated with a bad prognosis in various malignancies.

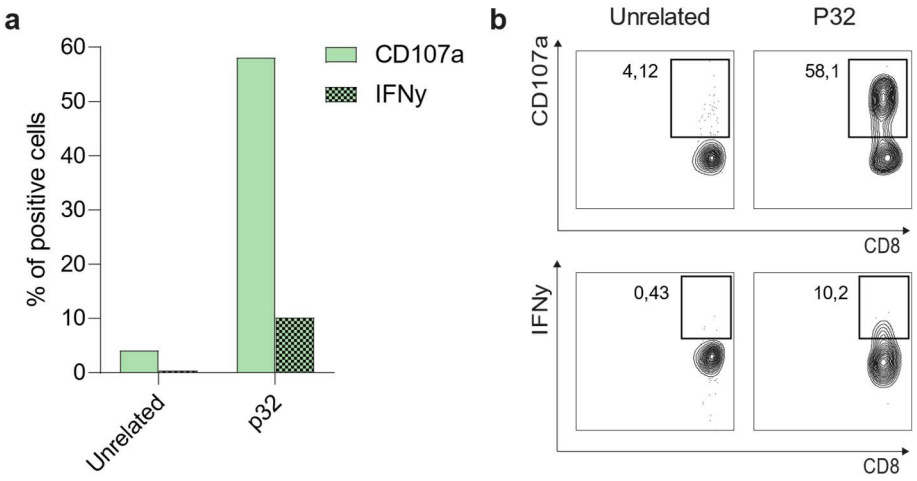

Figure 39 Specific T cells response to P32 in HD7 after 4 rounds of stimulation. Frequency of CD107a^{+} and IFN-γ^{+} T cells after 6 hours co-culture with autologous APCs loaded with an unrelated peptide or P32 (**a**); representative plots (**b**). P=peptide; IFN=interferon.

The flow cytometry assay to evaluate the Vβ repertoire did not lead to a clear indication of the T cell clonality, since multiple TRBVs were weakly present (**Fig.40**).

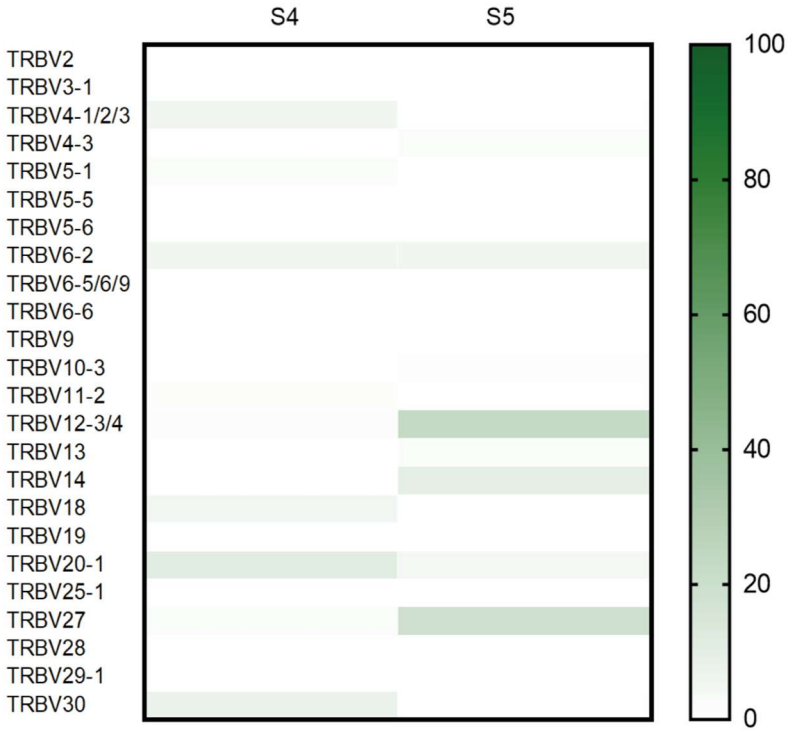

Figure 40 Cytofluorimetric assessment of the TRBV families did not allow to evaluate the clonal T cell expansion overtime. Heatmap displaying TRBV expression in T cells stimulated with APCs loaded with the peptide pool after four and five re-stimulations with P32. S=stimulation.

Differently, TCRαβ sequencing allowed to identify a strong oligoclonality, with a highly predominant CDR3α amino acid sequence present with a frequency of 87,16% (**Fig.41a**) and CDR3β aa sequence present with a frequency of 82% (**Fig.41b**),

The TRBV region expressed by this highly dominant T cell clone could not be identified by the flow cytometry kit.

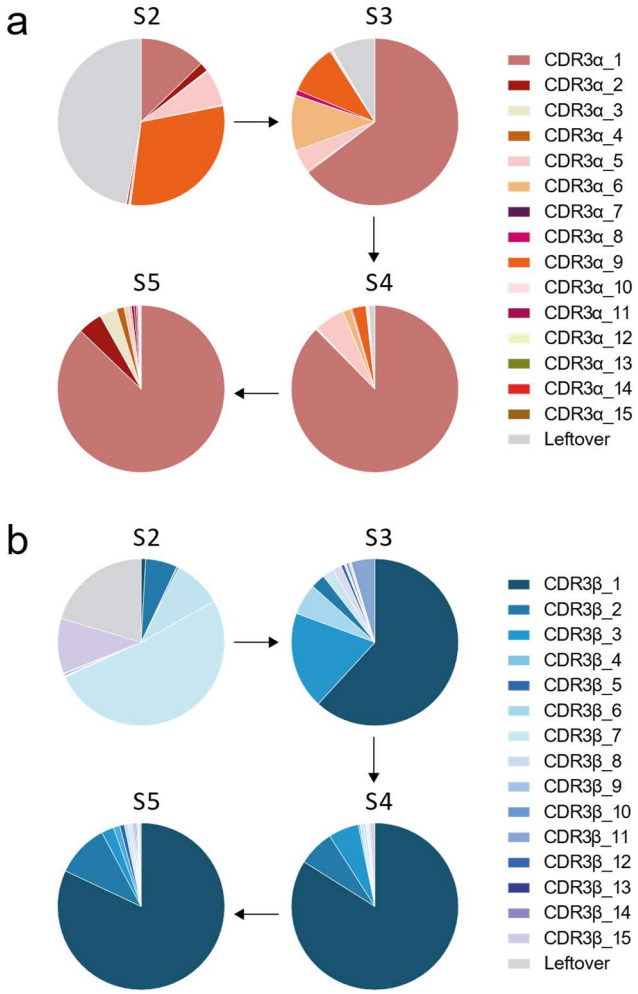

Figure 41 Clonal tracking of the P32-specific TCRαβ chains. The clonality of the TRAV (**a**) and TRBV (**b**) repertoire was longitudinally tracked by TCR sequencing. The 15 more frequent CDR3 sequences along the stimulations are plotted in the pie charts, where each color represent a unique CDR3 sequence.

Overall, this innovative pipeline allowed us to identify from two healthy donors multiple TCRs: two different alpha and beta chains specific for an epitope of the HER-2 tumor-overexpressed antigen, and 3 TCRs, each for a different ligandome-derived neoepitope, to be functionally validated for exploitation in the adoptive T cell therapy of CRC.

3. Materials and methods

3.1 Cells and media

3.1.1 Healthy donors PBMCs

Peripheral blood was obtained from healthy donors (HDs) at San Raffaele Hospital (OSR) upon informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (Fresenius) density gradient centrifugation.

3.1.1.1 Generation of immortalized B cells

Autologous B cells were isolated from PBMCs of HD3 and HD7 by using the CD19 Microbeads (Miltenyi Biotec). Subsequently, cells were transduced with the H/F LV vector encoding for the BCL-6/BCL-XL transgenes and cultured on γ-irradiated (80 Gy) mouse L-cell fibroblasts stably expressing CD40L (3T3-CD40L) at a B cell:3T3-CD40L ratio of 10:1. B cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Euroclone/Lonza) supplemented with 10% fetal bovine serum (FBS; BioWhittaker), 1% penicillin-streptomycin (Euroclone/Lonza), 2 mM glutamine and 50 ng/mL of IL21 (Miltenyi Biotec).

3.1.2 TILs from patients with primary CRC

We collected tumor samples from treatment-naïve patients affected by primary CRC undergoing hemicolectomy. Tissue samples were repetitively washed with phosphate-buffered saline (PBS) and collected in Tissue Storage Solution (Miltenyi Biotec) at 4°C until processing.

Tissues were manually reduced into small fragments and further processed into a single cell suspension by combining mechanical and enzymatic dissociation using gentleMACS dissociator (Miltenyi Biotec). Dissociated samples were filtered through a 70µ cell strainer (Falcon) and washed with PBS. After centrifugation, red blood cell lysis was performed when necessary by incubating cells in ACK solution for 5 minutes at room temperature. After wash and centrifugation, cell number was determined, and cells were stored in liquid nitrogen according to standard procedures.

3.1.3. T2 cell line

T2 cells were cultured in IMDM supplemented with 1% penicillin-streptomycin (BioWhittaker/Lonza), 2 mM glutamine (BioWhittaker/Lonza) and 10% FBS (Carlo Erba).

3.2 Stimulation and expansion of peptide-specific T cells

Freshly isolated PBMCs from HD3 and HD7 were resuspended in X-VIVO 15 medium supplemented with 5% human serum, 1% penicillin-streptomycin, 2mM glutamine and 1 ug/ml CD28 monoclonal antibody (BD Biosciences). Cells were seeded at a density of 10^7 cells/ml and stimulated with the peptide pool. After 26-30 hours, antigen-specific T cells were sorted according to CD137 expression. Briefly, cells were stained with the PE-conjugated CD137 antibody and enriched using anti-PE microbeads (Miltenyi Biotech). Subsequently, the CD137-negative fraction was depleted of the CD3 cells using CD3-Microbeads (Miltenyi Biotec), γ-irradiated at 30 Gy and used as

peptide-loaded antigen-presenting cells (APCs) in a co-culture with the CD137-positive fraction at a ratio of 100:1 and seeded at a final density of 5 x 10^6 cells/ml in X-VIVO 15 supplemented with 5% human serum, 1% penicillin-streptomycin, 2mM glutamine, 5 ng/mL IL-7 (Peprotech), 5 ng/mL IL-15 (Peprotech) and 10 ng/mL IL-21 (Miltenyi Biotec). Cells were re-stimulated every 14-20 days with y-irradiated (30 Gy), peptides-pulsed autologous APCs.

3.3 Assessment of T cell recognition of the target cells

The percentage of peptide-specific T-cells was measured by performing a co-culture of effector cells with autologous APCs pulsed with the desired peptide pool/sub-pool or unrelated peptide as control, at a 1:1 effector to target ratio. Co-cultures were seeded in X-VIVO 15 supplemented with 5% human serum, 1% penicillin-streptomycin, 2mM glutamine, CD28 monoclonal antibody (1 µg/mL), Golgi Stop Protein transport inhibitor (BD Biosciences; 1 µg/mL) and the fluorochrome-conjugated antibody CD107a antibody (BD Biosciences). In the stimulated wells only, PMA (50 ng/mL; Sigma) and Ionomycin (1 µg/mL; Sigma) were added. After 4-12 h at 37 °C, cells were stained with fluorochrome-associated monoclonal antibodies specific for surface molecules CD3, CD4, CD8. Next, cells underwent fixation and permeabilization for intracellular staining with monoclonal antibodies specific for IFN-ɣ, TNF-α and IL-2 cytokines.

3.4 Flow cytometry

3.4.1. Staining

Surface staining was performed by incubating samples with antibodies for 10 minutes at RT and washed with phosphate-buffered saline (PBS) containing 1% FBS. For the assessment of T cell clonality, the IO Test Beta Mark TCR V beta repertoire kit (Beckman Coulter) was used according to manufacturer's recommendations. Samples were acquired through a fluorescence-activated cell sorter (FACS) Canto II flow cytometer (BD Biosciences), and data were analyzed by Flow Jo software (Tree star Inc).

3.4.2 FACS sorting of TILs

For the isolation of IRpos, IRint and IRneg populations, thawed cells from primary CRC specimens were stained with fluorochrome-conjugated antibodies CD3, CD39, PD1 and a viability dye. Cells were sorted at the Institutional Facility using a FACSAria^{TM} Fusion Cell Sorter (BD). Immediately after sorting, TILs underwent a round of *in vitro* rapid expansion protocol (REP): TILs were activated with anti-CD3 OKT3 antibody (30 ng/ml, Miltenyi) in RPMI (Lonza) with FBS 10%, penicillin/streptomycin 1% (BioWhittaker/Lonza), glutamine 1% (BioWhittaker/Lonza) supplemented with recombinant human IL-2 (600 UI/ml, Novartis), and in the presence of irradiated (30 Gy) allogeneic PBMCs (1 × 10^6 cell/ml) obtained from a pool of three different healthy donors, and irradiated (100 Gy) ROSI-EBV cell line (0.2 × 10^6 cell/ml).

3.5 TCR sequencing

Cell pellet from IRpos, IRint and IRneg was collected before and after REP and stored at -80°C in Extraction Buffer (Life Technology). RNA was extracted by using the Arcturus Pico Pure RNA extraction kit (Life Technology). Complementarity determining region (CDR) 3 sequences of TILs were amplified by using a modified RACE approach[23]. Sequencing was performed using an Illumina MiSeq sequencer and CDR3 clonotypes were identified using the MiXCR software[24].

3.6 HLA typing

B cells originated from HD3 and HD7 were typed for HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ alleles at high resolution by the Institutional HLA-typing laboratory.

3.7 Statistical analysis

Statistical analyses were performed with Prism 8 software (GraphPad).

4. Discussion

TCR gene transfer and TCR gene editing techniques harbor the potential to redirect T cells specificity against virtually any tumor antigen. However, apart from picking the proper target antigen, one of the principal challenges remains the identification of anti-tumor TCRs. In fact, the TCR repertoire in patients is limited, since high-avidity cancer-specific T cells are present at low frequency. In addition, the selection of the proper source for retrieving tumor-specific T cells is still a matter of debate. In this study, we leveraged on two different approaches to retrieve novel anti-tumor TCRs: (i) by taking advantage of the exhaustion signature that we identified on TILs retrieved from primary CRCs and CRC liver metastasis to select tumor-specific T cells, and (ii) starting from HD PBMCs stimulated with immunogenic peptides. First, we sorted $PD1^{+}CD39^{+}$(IR positive), $PD1^{+}CD39^{-}$/$PD1^{-}CD39^{+}$(IR intermediate) and $PD1^{-}CD39^{-}$ (IR negative) T cells out of the TILs bulk of one MSI and one MSS primary colorectal tumors, and we sequenced their TCR. We observed a strong oligoclonality in the IR positive population in the MSI patients, suggesting that inhibitory mechanisms could act specifically on anti-tumor T cells. On the contrary, the clonality of the TCR repertoire was similar among healthy, peritumoral and neoplastic tissues in the MSS tumor. We are currently validating this finding by enlarging the cohort of TCR sequenced patients. Moreover, by sequencing the TCR of bulk T cells infiltrating healthy, peritumoral, and tumor tissue from three patients, we observed that T cell clones are hardly shared among different tissues, even

though some low frequency clones were also retrieved in the peripheral blood. This is in line with a previous report suggesting compartment-specific TCR distribution rather than alterations due to tumor-T-cell interaction in CRC[25]. We are currently addressing whether clones present at low frequencies within the bulk TILs from the tumor are enriched in IR positive sorted population, to further support the importance of exploiting inhibitory receptor expression for the selection of tumor-reactive T cells. Moreover, the IRs-sorting strategy coupled with a deeper coverage of the sequencing could allow to retrieve tumor-specific TCRs also from patients' PB. This could be of particular relevance for metastatic patients, were tumor-specific T cells are enriched in the circulation[26,27]. Accordingly, we detected a higher frequency of PD1 and CD39 positive T cells within the PB of mCRC patients compared with healthy donors or primary CRC patients, further supporting the relevance of these molecules as hallmarks for tumor-specific T cells[9,10]. Functional assays with T cells engineered to express TCRs enriched in IR^{high} cells will allow to reveal whether this approach can indeed generate relevant specificities for ACT of CRC. As an alternative approach to retrieve novel anti-tumor TCRs, we exploited a pipeline set up in our lab (Ruggiero, …, Potenza et al., *submitted*) to isolate epitope-specific T cells starting from healthy donors. HD T cells undergo negative selection in the thymus: for this reason, retrieving TCRs from HDs appears to be a potentially safer strategy[28]. We repetitively stimulated HD PBMCs with autologous antigen-presenting cells loaded with a pool of

peptides. We selected immunogenic peptides, expressed by CRC and restricted in the HLAs most common in the Caucasian population. After several re-stimulations, we tested the ability of T cells to degranulate and to secrete IFN-γ when challenged with peptides. We efficiently expanded T cells specific for the HER-2 tumor-overexpressed antigen and for three different ligandome-derived neoepitopes. Still, the natural processing and the HLA restriction of P25, P32 and P34 remain to be addressed. Moreover, we longitudinally monitored the TCR clonality of peptide-specific T cells. We were able to identify CDR3 alpha and beta sequences in all cultures, thus having the possibility to reconstruct at least 4 different tumor-specific TCRs in the absence of the time-consuming cloning procedure. Coupled with genome editing, these findings will allow to generate a redirected T cell product which will be functionally tested for the ability to eliminate CRC.

CHAPTER 5

Editing primary T cells for the adoptive T cell therapy of colorectal cancer

1. Introduction

Adoptive T cell therapy initially relied on tumor-infiltrating T cells, expanded *ex vivo* and infused back to the patient. With the recent advances in genome engineering techniques, new cellular products were envisaged for ACT, with the aim of generating T cells able to recognize the target and persist long term. The first attempts to redirect T cell specificity were based on gene transfer through viral or non-viral vectors. However, two major hurdles limited the broader use of this technology: (i) a suboptimal surface expression of the transferred receptor, caused by the competition of the endogenous and the exogenous TCRs for assembly with the CD3 subunits[29]; (ii) the potential unwanted reactivities caused by the possible mispairing between α and β chains from exogenous and endogenous TCRs, further diluting the expression of the correctly paired tumor-specific receptor[30]. The TCR gene editing approach was developed to overcome TCR gene transfer limitations by taking advantage of artificially modified nucleases. Zinc finger nucleases (ZFN) supported the development of the first TCR gene editing approaches[31,32]. CRISPR/Cas9 nucleases technology, which revolutionized the genome editing field, had a direct impact on ACT cell manufacturing. The TCR editing procedure was shortened by the possibility to synchronously disrupt the α and β TCR chains[33,34]

thus decreasing the *in vitro* manipulation time. Nonetheless, the exploitation of multiplex genome engineering, easily achieved with CRISPR/Cas9, could open the room to further modify T cells to generate a T cell product redirected against a tumor-specific antigen, persisting long-term and selectively overcoming the inhibitory signals active in the immune-suppressive tumor microenvironment.

2. Results

2.1 T cells redirected against MUC-1 tumor-associated antigen specifically eliminate target cells

With the aim of exploiting CRISPR/Cas9 technology coupled with lentiviral gene transfer to generate TCR-redirected T cells, we selected MUC-1 as a first tumor-overexpressed antigen to target. Among tumor-overexpressed antigens in CRC, MUC-1 is particularly relevant because of its peculiar distribution and glycosylation profile in cancer cells which differs from normal epithelial cells[35]. We evaluated MUC-1 expression in our cohorts of patients diagnosed with primary or metastatic CRC. Of interest, the enhanced expression of this molecule has been associated to cancer invasiveness and metastasis development[36]. Accordingly, MUC-1 expression was found to be slightly increased in primary CRCs compared to healthy or peritumoral colon tissue (**Fig.42a**), while its overexpression in our cohort of CRC liver metastasis was significantly superior to the peritumoral liver (**Fig.42b**), supporting the relevance of a MUC-1 redirected cellular product for CRC. Thus, we screened a panel of CRC cell lines to identify the proper target to be used in functional assays with MUC-1 redirected T cells. We selected SW480 to have the highest (83,8%) expression of the antigen, compared to SW620 (49,3%), HT-29 (31%), LoVo (15,6%) CRC cell lines and U87-MG glioblastoma cells used as a negative control (**Fig.42c**).

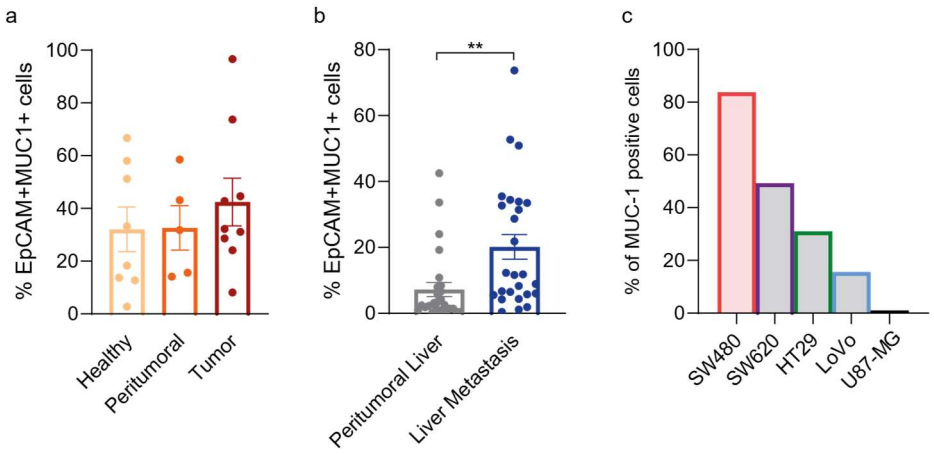

Figure 42 MUC-1 is overexpressed in primary CRCs and liver metastasis and in colorectal cancer cell lines. MUC-1 expression was evaluated by flow cytometry in healthy, peritumoral and tumor tissue from primary CRC patients (a, n=8), peritumoral and tumoral tissue from liver metastasis (b, n=25; p=0,0062) and in colorectal cancer cell lines, compared with negative control (U87-MG, glioblastoma cell line) (c). Data are reported as mean ± SEM.

We selected from the literature a non-MHC restricted, single-chain TCR recognizing the PDTRP epitope of MUC-1[37], localized at the tip of the "immunodominant knob" of the extracellular domain of the protein, composed of tandemly repeated 20 amino acid–long structures[38,39]. We reconstructed the alpha and beta chains of the MUC-1 TCR described from Alajez. et al. and we cloned it into a bidirectional lentiviral vector as a conventional double chain TCR (**Fig.43**).

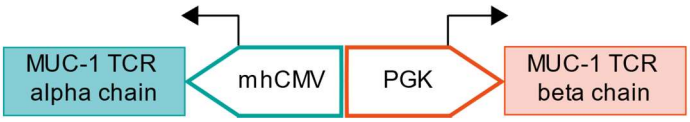

Figure 43 Schematic view of the lentiviral vector with a bidirectional promoter used to transduce T lymphocytes with MUC-1 TCR αβ chains.

We used gRNAs already available in the lab and an RNP approach to simultaneously disrupt the α and β chains of the endogenous TCR, located on chromosomes 14q11.2 and 7q34, respectively. Briefly, we stimulated cells with anti-CD3/anti-CD28-conjugated magnetic beads to enrich and activate T cells in the presence of low doses of IL-7 and IL-15. On day 2 after stimulation, we performed RNPs nucleofection for the contemporary disruption of TCR α and β genes. We achieved 99% efficiency, obtaining a T cell pool completely devoid of the endogenous TCR (**Fig.44a**). The day after, we transduced edited T cells with the lentiviral vector encoding for the MUC-1 TCR, obtaining a transduction efficiency of 52,8%, as observed by the double positivity of the cells for the CD3 and TCR αβ antibodies (**Fig.44b**). We sorted $CD3^+$ T cells to enrich for T cells expressing the MUC-1 TCR and obtained a cellular product with a purity of 98,7% (**Fig.44c**).

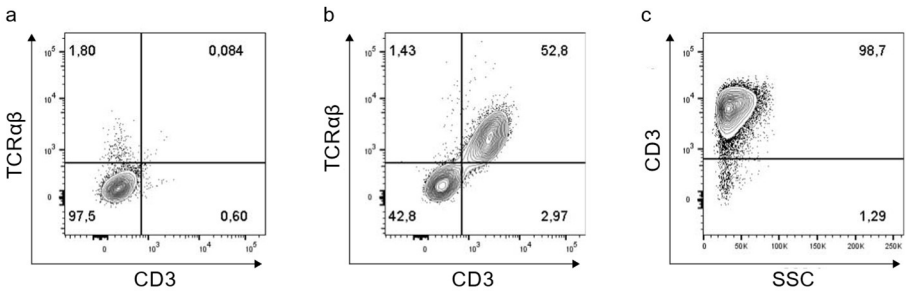

Figure 44 Efficient disruption of the endogenous TCR and MUC-1 TCR replacement in primary T cells. CRISPR/Cas9 technology was used to disrupt both α and β chains of the endogenous TCR (a). Edited T cells were transduced with a lentiviral vector encoding the MUC-1 TCR (b) and redirected T cells were further enriched by $CD3^+$ cell sorting (c).

MUC-1-engineered T cells were functional in specifically recognizing and killing MUC-1^+ target cells, as shown after 6 hours coculture in terms of Caspase 3, displaying the induction of apoptosis, (**Fig.45a**) and as elimination index upon 48 hours coculture (**Fig.45b**).

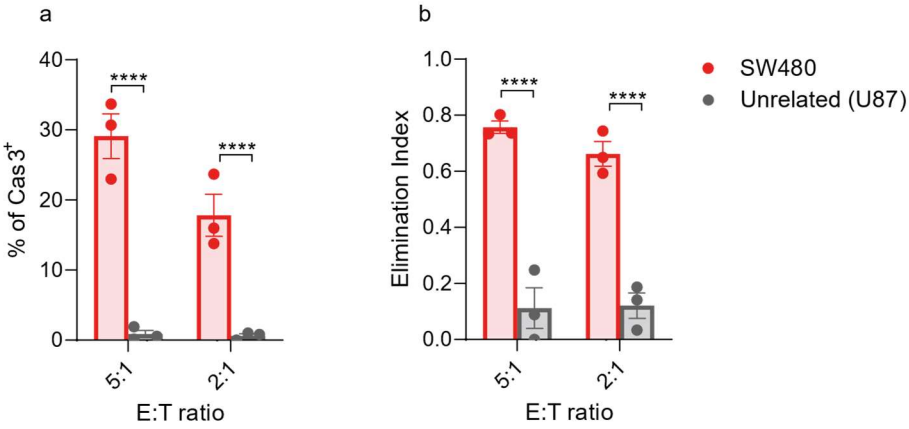

Figure 45 MUC-1-engineered T cells recognize and eliminate MUC-1^+ target cells. Cytotoxicity of MUC-1-engineered T lymphocytes against SW480 cell line was evaluated by measuring Caspase 3 expression in living

target cells by flow cytometry upon a 6 hours co-culture (a) and by evaluating killing capacity after 48 hours co-culture (b) at different effector to target ratios (E:T ratio); n=3. Data are reported as mean ± SEM. ****p<0,0001.

2.2 Counteracting the immune-suppressive CRC TME by genetically disrupting PD1 and CD39 in primary T cells

In CRC, we observed the upregulation of PD1 and CD39 on T cells retrieved from the tumor tissue as a result of the exhaustion process caused by the immune-suppressive tumor microenvironment. We hypothesized that the genetic disruption of one or both molecules on TCR-redirected T cells could be beneficial for counteracting the exhaustion signature of the tumor-specific T cell product. Therefore, we took advantage of CRISPR/Cas9 nucleases to disrupt these molecules in T cells.

2.2.1 Efficient *pdcd1* disruption in primary T cells by CRISPR/Cas9

First, we reasoned on how to determine a phenotypic assessment of *pdcd1* disruption by flow cytometry. To this aim, we needed to identify a time point in which the vast majority of T cells were positive for PD1. Accordingly, we evaluated over time the expression kinetic of PD1 in T cells stimulated *in vitro* with anti-CD3/CD28-conjugated magnetic beads in the presence of low doses of IL-7 and IL-15. This allowed us to select day 7 after stimulation as the time-point in which more than 80% of CD4 and CD8 T cells express PD1, making feasible the observation of a dampened expression of the molecule on edited cells (**Fig.46a**). As previously done with the TCR loci, we performed disruption of the *pdcd1* gene at day 2 after T cell stimulation. *pdcd1* gene is

located on chromosome 2q37.3 and is composed of 6 exons. We delivered Cas9 in a ribonucleoprotein (RNP) complex with a sgRNA by nucleofection (**Fig.46b**). By comparing the percentages of PD1^{+} cells in edited and non-edited T cells at day 7, we calculated a 50% gene disruption efficiency (**Fig.46c**) by flow cytometry, as shown in representative plots (**Fig.46d**). Of note, the genetic disruption of *pdcd1* did not impair the memory phenotype of T cells (**Fig.47a**), which were prevalently T_{SCM} in both CD4 (**Fig.47b,c**) and CD8 (**Fig.47d,e**) T cell compartments.

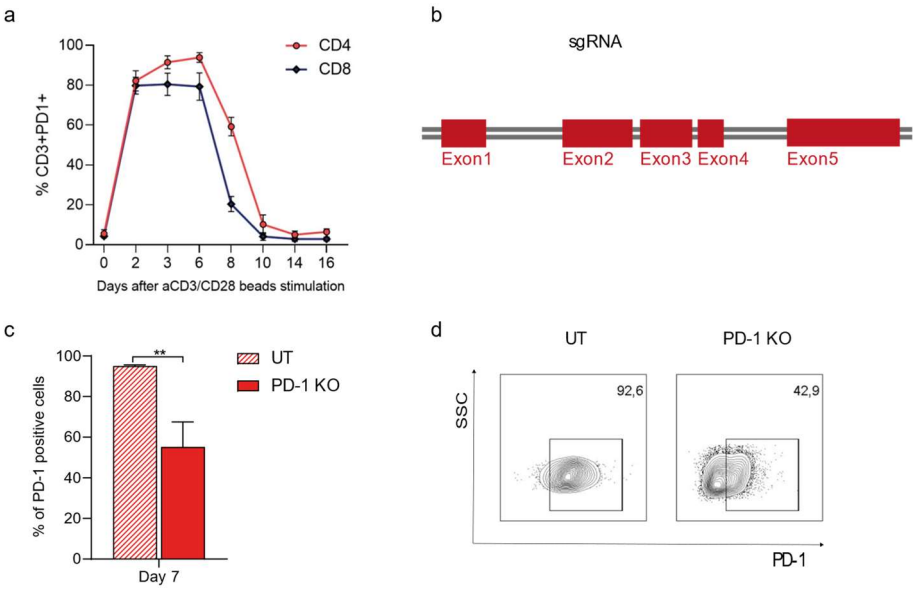

Figure 46 Efficient disruption of pdcd1 gene in primary T cells. PD1 expression kinetic in T cells after stimulation with anti-CD3/CD28-conjugated magnetic beads was measured by flow cytometry (a, n=3); schematic representation of the pdcd1 gene (b); frequency of PD1^{+} T cells after pdcd1 disruption in control and edited cells (c, n=3; p=0,0021), as shown by representative plots (d). Data are reported as mean ± SEM.

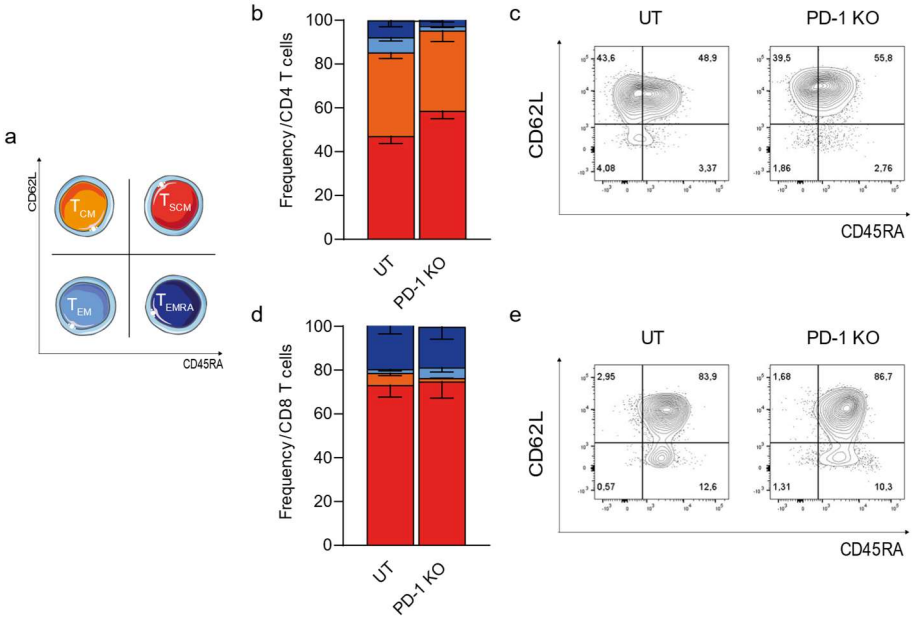

Figure 47 *pdcd1* gene disruption does not impair T cell memory phenotype. Schematic representation of T cell memory phenotypes, described according to CD45RA and CD62L expression markers as T_{CM} ($CD45RA^-$,$CD62L^+$), T_{SCM} ($CD45RA^+CD62L^+$), T_{EMRA} ($CD45RA^+CD62L^-$) and T_{EM} ($CD45RA^-CD62L^-$) (a). Distribution of $CD4^+$ (b,c) and $CD8^+$ (d,e) T cells memory subsets in untreated and PD1 KO T cells on day 15 after stimulation (n=3). Data are reported as mean ± SEM.

2.2.2 Efficient *entpd1* disruption in primary T cells by CRISPR/Cas9

As for PD1, we measured the expression kinetics of CD39 to facilitate a phenotypic assessment of gene disruption. Only 40% of CD8 T cells expressed the molecule 8 days after activation, while CD4 cells didn't express it in culture. (**Fig.48a**). This low expression is not sufficient to evaluate the frequency of CD39-

edited T cells by flow cytometry. Consequently, we forced the upregulation of CD39 by further stimulating T cells with OKT3 in the presence of IL-2 and irradiated feeder cells (rapid expansion protocol – REP[40]), a culture protocol that force T cell differentiation to an effector phenotype. With this approach, 80% of T cells expressed CD39 up to 8 days after REP. Interestingly, CD39 expression was detected also in CD4 T cells (**Fig.48b**).

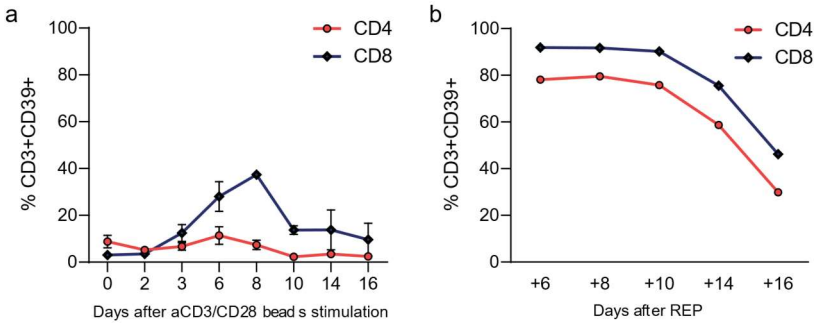

Figure 48 Expression kinetics of CD39 in different culture conditions. CD39 expression kinetic was measured by flow cytometry after anti-CD3/CD28-conjugated beads activation in the presence of IL-7 and IL-15 (a, n=3) and after OKT3 stimulation in the presence of IL-2 and irradiated feeder cells (b; n=1). Data are reported as mean ± SEM.

The *entpd1* gene, encoding for CD39, is located on chromosome 10q24.1 and is composed of 10 exons (**Fig.49**). To disrupt *entpd1*, we employed two different approaches. The first one relied on canonical sgRNA. We decided to screen three different sgRNAs, designed in exon 2 to reduce the likelihood of obtaining a truncated protein (sg4,5,6; **Table 7**). The second approach was based on the use of a multi-guide system, consisting of three pre-mixed sgRNA designed to target different sites of the same gene

region, thus producing a fragment deletion and *per se* avoiding the generation of a truncated protein (sg1,2,3; **Table 7**).

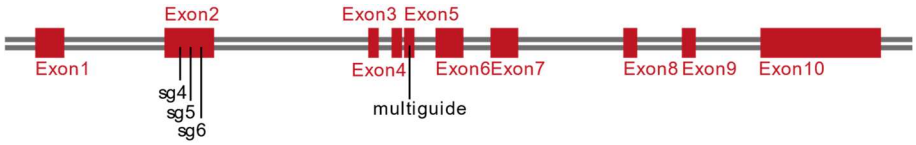

Figure 49 Schematic representation of the *entpd1* gene. The *entpd1* gene is composed of 10 exons. sgRNAs 4, 5 and 6 were designed to target exon 2, while sg1, 2 and 3, used for the multi-guide approach, target exon 5.

sg#	Sequence	PAM
sg1	AAGUGAAGAGUUGGCAGACA	GGG
sg2	AACUACCCCUUUGACUUCCA	GGG
sg3	UCUGCUGGGCAAAUUCAGUC	AGG
sg4	AAGAAUAUCCUAGCCAUCCU	TGG
sg5	AAGGAUGGCUAGGAUAUUCU	TGG
sg6	AGGAGAAGCCAAGGAUGGCU	AGG

Table 8 Sequences of sgRNAs targeting the *entpd1* gene.

When disrupting *entpd1* with sg4, sg5 and sg6, each delivered as a ribonucleoprotein complex with the Cas9 in a ratio of 5:1, we observed only a 15% efficiency (**Fig.50a**), which remained stable also when forcing CD39 upregulation with OKT3 and IL-2 (**Fig.50b**). We selected sg4 and sg5, since sg6 showed a lower KO efficiency, to optimize the experimental setting. Since enhancing the quantity of Cas9 could lead to off-target events possibly affecting the transcriptome[41], we tried to maintain stable

the amount of Cas9 while increasing the sgRNA quantity to reach a sgRNA:Cas9 ratio of 9:1. When only 30% of control cells were positive for CD39, a 25% efficiency was calculated (**Fig.50c**), but after REP we could appreciate up to 50% knock-out efficiency (**Fig.50d**).

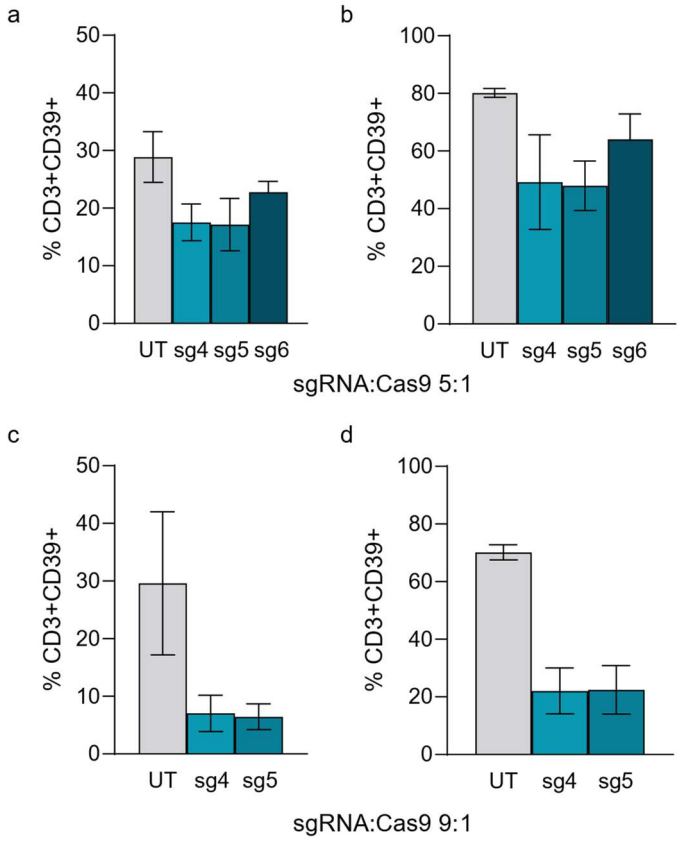

Figure 50 Efficient *entpd1* gene disruption in primary T cells by CRISPR/Cas9. Frequency of $CD39^{+}$ T cells after *entpd1* disruption with sg4, sg5 or sg6 coupled with Cas9 in a 5:1 ratio, in control and edited cells stimulated with aCD3/CD28 beads (a) or after REP (b); T cells electroporated with sg4 or sg5 as a RNP complex with Cas9 in 9:1 ratio, in control and edited cells stimulated with aCD3/CD28 beads (c) or after REP (d); n=3. Data are reported as mean ± SEM.

By analyzing CD39 expression over time in different experimental settings, we noticed that it is highly variable depending on the metabolic state of the cell rather than on activation. Consequently, to gain a more precise insight on the efficiency of *entpd1* disruption, we decided to perform the analysis at the genetic level. Therefore, we used an *in silico* tool analyzing the Inference of CRISPR Edits (ICE, Synthego) to provide a quantitative assessment of the knock-out efficiency by comparing the Sanger Sequencing traces of amplicons generated from edited and control cells. As expected, the traces of the control and edited cells (orange and green line, respectively) are superimposable in the sequence region located before the cut site, while they start to be discordant at the cut site and in its downstream genomic region (**Fig.51a**). The ICE analysis showed a knock-out efficiency up to 45% and 30% for sg4 and sg5, respectively, when used in a 9:1 ratio with Cas9, and an indel score of 50% and 60%, respectively (**Fig.51b**). It is important to underline that, while the knock-out score only includes sequences harboring a frameshift mutation or a large (+21 bp) fragment deletion, the indel score comprehends all detected sequences that are different from the wild type. This explains why sg5 displays an indel score higher than its knock-out score. On the contrary, for sg4 the knock-out and indel score are comparable, meaning that all the insertion and deletions resulted in a functional disruption.

The inferred sequences present in the edited population and their relative proportions were also analyzed for the nature of the indels, as shown for sg5 in one HD in **Fig.51c**. For sg4, the majority of indels were represented by one base deletion (20%), ten bases deletion (12%) or one base insertion (5%). For sg5, one base deletion represented 26% of indels, followed by eight (17%), twenty-five (10%) or ten (5%) bases deletions. One base insertion represented only 1% of indels (**Fig.51d**).

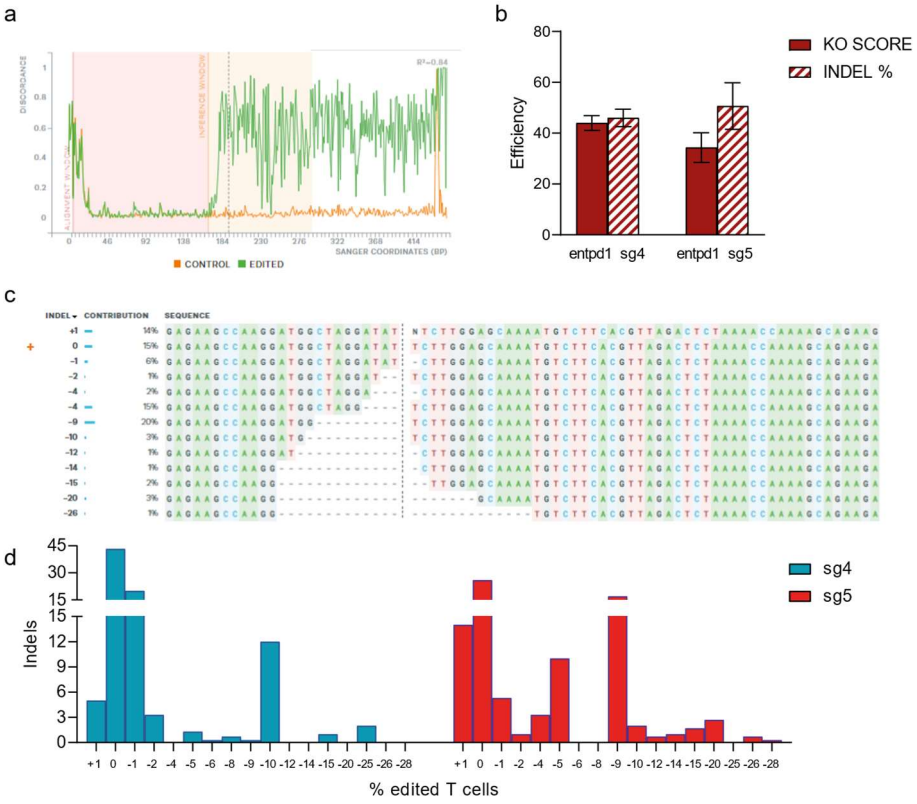

Figure 51 *entpd1* gene disruption evaluation at the genomic level confirmed flow cytometry results. Traces of control and edited cells after Sanger Sequencing (a); knock-out and indels frequencies in T cells edited

with sg4 and sg5 (b); evaluation of indels compared to control sequence (c); indels profile in T cells edited with sg4 and sg5 (d); n=3.

To further improve the knock-out efficiency, we decided to try a multi-guide strategy. By targeting the same genomic region with up to three guides, a fragment deletion is induced, ensuring a non-functional protein. The absence of CD39 was observed in edited T cells, compared to 25% (**Fig.52a,c**) and nearly 80% (**Fig.52b,d**) CD39 expression in control cells stimulated with aCD3/CD28 beads or after REP, respectively. Overall, the multi-guide system ensured a significantly higher efficiency of *entpd1* knock-out.

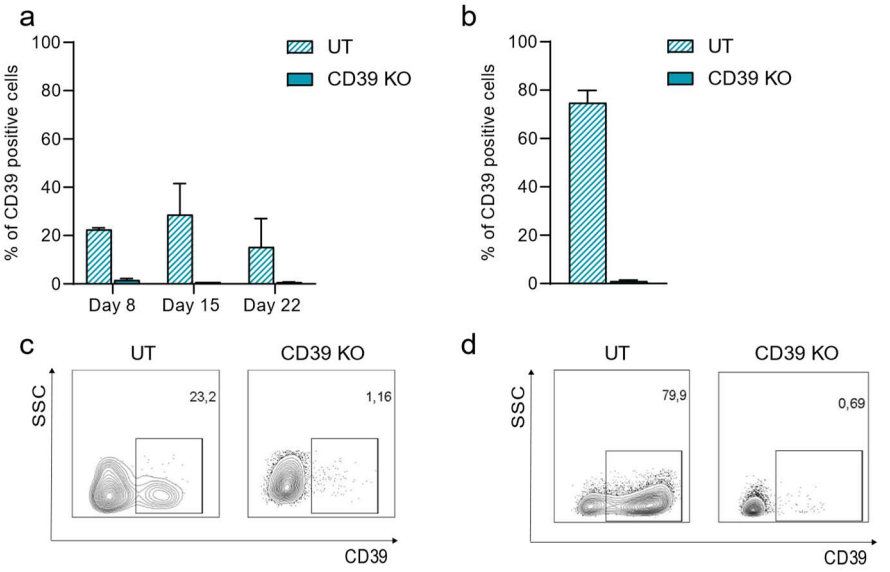

Figure 52 *entpd1* gene disruption in primary T cells reached nearly 80% efficiency with a multi-guide approach. Frequency of CD39$^+$ T cells after *entpd1* disruption with the multi-guide system including 3 sgRNAs coupled with the Cas9 protein, in control and edited cells stimulated with aCD3/CD28 beads (a,c, n=3) or after REP (b,d; n=2). Data are reported as mean ± SEM.

3. Materials and methods

3.1 Cells and media

3.1.1 Primary T cells

Peripheral blood was obtained from healthy donors (HDs) at San Raffaele Hospital (OSR) upon informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque (Fresenius) density gradient centrifugation.

3.1.2 Cell lines

SW480 (colorectal cancer cell line) and U87-MG (glioblastoma cell line) were cultured in RPMI supplemented with 1% penicillin-streptomycin (BioWhittaker/Lonza), 2 mM glutamine (BioWhittaker/Lonza) and 10% FBS (Carlo Erba).

3.2 Lentiviral vector

For the redirection of T cell specificity, we produced a lentiviral vector encoding for the MUC-1-specific TCR alpha and beta chains under a bidirectional promoter. More in detail, α chain was cloned in antisense orientation under the control of the human minimal CMV promoter (mhCMV), while β chain was cloned in sense orientation under the PGK (human phosphoglycerate kinase) promoter. LVs were packaged by an integrase-competent third-generation construct and pseudotyped by the vescicular stomatitis virus (VSV) envelope.

3.3 Evaluation of the expression kinetics of PD1 and CD39 on T cells

T lymphocytes were enriched from HD PBMCs and stimulated with anti-CD3/anti-CD28-conjugated magnetic beads (Dynabeads™) in a 3:1 beads/T cells ratio for 6 days. After 6 days of stimulation, beads were magnetically removed and cells were cultured in X-VIVO 15 (Lonza) supplemented with 5% FBS (fetal bovine serum, Carlo Erba), 1% penicillin/streptomycin (BioWhittaker/Lonza), 2mM glutamine (BioWhittaker/Lonza), hIL-7 and hIL-15 (5ng/ml, PeproTech). Cells were incubated at 37°C, 5% CO2 in a humidified cell culture incubator. Surface staining with antibodies binding CD3, CD4, CD8, PD1 and CD39 was performed before stimulation (day 0) and on days 2, 3, 6, 8, 10, 14 and 16 after stimulation.

3.4 Rapid expansion protocol

Control or edited T cells were stimulated with irradiated (40Gy) allogeneic feeder cells and irradiated ROSI-EBV cell line and cultured in RPMI-1640 medium supplemented with 10% FBS (Carlo Erba), 1% penicillin/streptomycin (BioWhittaker/Lonza), 2mM glutamine (BioWhittaker/Lonza), soluble anti-CD3 mAb (OKT3, 100 ng/ml, Biolegend) and IL-2 (600U/mL, Novartis). On day 3 after stimulation, fresh medium was added to the culture to wash out OKT3.

3.5 Genetic modification of primary T cells

PBMCs from healthy donors were activated and enriched using anti-CD3/CD28 magnetic beads (ClinExVivo CD3/CD28; Invitrogen) and seeded at a concentration of 10^6 cells/mL in X-VIVO supplemented with 1% penicillin-streptomycin, 2mM glutamine, 5% FBS, 5 ng/mL IL-7 (Peprotech), 5 ng/mL IL-15 (Peprotech). Two days after stimulation, T cells were electroporated with ribonucleoprotein complexes (consisting of Cas9 nuclease [Synthego or Intellia Therapeutics] duplexed with synthetic guide RNA, purchased commercially [Synthego] or chemically synthesized [Intellia Therapeutics]). The Lonza Nucleofector 4D Electroporation System was used for the nucleofection procedure. Upon electroporation, T cells were seeded in X-VIVO supplemented with 5% FBS in the presence of IL-7 (5ng/mL) and IL-15 (5 ng/mL). On day 3 after stimulation, TRAC+TRBC edited T cells were transduced with a lentiviral vector encoding for the MUC-1-specific TCR chains. On day 6 post-stimulation, beads were detached, and cells were seeded at a concentration of 10^6 cells/mL in X-VIVO 15 supplemented with 5% FBS and with IL-7 (5 ng/mL) and IL-15 (5 ng/mL).

3.6 Assessment of the knock-out efficiency at the genetic level

We used the Inference of CRISPR Editing (ICE) algorithm (Synthego) to assess the effect of *entpd1* KO at the genetic level. Briefly, we extracted the DNA (DNeasy blood and tissue Kit, Qiagen) from edited and control cells, and amplified by PCR the target sequence including the cut site. We run the PCR product

on a 1% agarose gel to verify the presence of a single fragment of the correct size, and we submitted the PCR-purified product (QIAquick PCR Purification Kit, Qiagen) for Sanger Sequencing.

	Primer sequence
PCR_Fw	CCTTGCCACAGCCACTAACT
PCR_Rev	TGAGGTTCTAACAGCCCAGG
SEQ_Rev	GGTTCTAACAGCCCAGGAACAAG

Table 9 Sequences of primers used for ICE analyses.

3.7 Functional assays

The cytotoxic ability of MUC-1-redirected T cells was evaluated by measuring the percentage of living target cells expressing the activated form of Caspase 3. SW480 (MUC-1^{+}) and U87MG (MUC-1^{-}) cell lines were incubated in a flat-bottom 96 wells plate at 5:1 and 2:1 E:T ratios. After 6 hours of coculture, cells were stained with a viability dye and with markers specific for effector and target cells. After fixation and permeabilization (Fix/Perm buffer set, Biolegend), cells were stained with an anti-active Caspase 3–antibody (BD Biosciences). Data were acquired at the FACS Canto II flow cytometer (BD Biosciences). The killing capacity of MUC-1-engineered T cells was evaluated in a 48 hours coculture with target cells. Cells were stained with markers specific for T cells and target cells, and cell count was performed at Canto II flow cytometer (BD Bioscience) using FlowCount

Fluorospheres (Beckman Coulter). Elimination index was calculated as follows: 1 – (number of residual target cells in presence of transduced T cells/number of residual target cells in presence of untransduced T cells).

3.8 Flow cytometry

Surface staining was performed by incubating samples with antibodies for 10 minutes at RT and washed with phosphate-buffered saline (PBS) containing 1% FBS. Samples were acquired through a fluorescence-activated cell sorter (FACS) Canto II (BD Biosciences) or Symphony (BD Bioscience) flow cytometer, and data were analyzed by using the Flow Jo software (Tree Star Inc).

3.9 Statistical analysis

Statistical analyses were performed with Prism9 software (GraphPad). Student t-test and two-way ANOVA were performed throughout the study.

4. Discussion

Adoptive T cell therapy showed promising results to treat hematological malignancies, but its wide exploitation in solid tumors is still limited by the choice of the proper target antigen, the long-term persistence of T cell products and the exhaustion induced by the tumor microenvironment. Attempts have been made by targeting tumor-overexpressed antigens (TAA) with redirected T cells for the adoptive T cell therapy of CRC. Several groups investigated CEA as a target for ACT, due to its overexpression in CRCs. In a study administering CEA-redirected T cells to three patients, despite objective response in metastasis and CEA serum level reduction, severe colitis occurred in all the patients[42]. CEA-CAR T cells were also explored. A phase I clinical trial in CRC patients displaying CEA-positive metastasis led to long-term reduction of serum CEA levels, stabilization of the disease and tumor shrinkage in two patients, in the absence of adverse effects[43]. However, CEA is expressed, despite at lower levels, also by normal cells of the gastrointestinal tract. MUC-1 is an interesting TAA since its aberrant glycosylation profile in tumors renders the protein different from its variant expressed in healthy tissue. Indeed, MUC-1 is normally expressed at low levels at the apical surface of glandular epithelial cells, but upon tumorigenesis, it loses polarity and becomes upregulated[44]. In our CRC patients' cohorts, we observed MUC-1 overexpression in primary tumors and particularly in liver metastasis. The immunogenicity of MUC-1 was first characterized by the isolation of a T cell line

established from draining lymph nodes of a patient with pancreatic cancer[45], and its specific recognition as a whole protein expressed by epithelial adenocarcinomas was MHC-unrestricted[46]. Here, we decided to focus on MUC-1 as a potential antigen for TCR-redirected T cells in the context of CRC. We reconstructed the MUC-1 specific TCR described by Alajez et al.[47]. The TCR described by O. Finn's group was generated as a single chain TCR, harboring the advantage to avoid mispairing with the endogenous receptor[48]. We decided to use a full-length TCR while taking advantage of the CRISPR/Cas9 technology to disrupt both the α and β chains of the endogenous TCR and obtained a pure (97,5%) population of T cells completely devoid of their repertoire. Afterward, we completely redirected T cell specificity towards the tumor by employing the MUC-1 TCR. When challenged with a MUC-1^{+} colorectal cancer cell line, edited T cells proved highly functional in specifically eliminating the proper target.

To address the long term-persistence of edited cells[49], we stimulated T lymphocytes with a protocol set up to instruct the memory stem T cells phenotype, ensuring self-renewal capability and high proliferative potential[50] and being able to survive long-term when adoptively transferred in patients[51,52].

Still, a major challenge remains and is represented by the immune exhaustion driven by the tumor microenvironment. To counteract this negative immune regulation, different approaches have been employed over the years. Monoclonal antibodies specifically blocking immune checkpoint molecules have been

developed and proven successful in heavily mutated tumors, such as melanoma, preventing T cell exhaustion and promoting durable response. In CRC, only a small fraction of patients harboring microsatellite instability could benefit from this therapy. Accordingly, a 40% objective response rate was observed after the administration of pembrolizumab in dMMR-MSI-H patients only[53]. Patients with the same characteristics also benefitted from nivolumab, which administration led to a 31% objective response rate and 12-months overall survival in 73%[54]. These results paved the way for the FDA approval of pembrolizumab and nivolumab to treat dMMR-MSI-H CRC patients. The use of checkpoint inhibitors is not free from toxicities; in fact, these monoclonal antibodies act in a non-selective manner of the entire T cell repertoire, resulting in immune-related adverse events. To overcome the toxicity caused by the indiscriminate blockade of IRs on the entire T cell repertoire, a strategy is represented by the use of artificial nucleases to “remove the brake” from redirected T cells to be adoptively transferred for ACT. By studying the inhibitory receptors expression profile in T cells retrieved from tumoral and peritumoral/healthy tissue of patients with primary or metastatic CRC, we observed that, besides PD1, CD39 has a pivotal role in regulating the CRC microenvironment. Therefore, we explored the possibility to use CRISPR/Cas9 nucleases to disrupt *pdcd1* and *entpd1* genes in primary T cells. We evaluated the kinetics of expression of PD1 and CD39 on the surface of healthy donors’ T lymphocytes, stimulated with aCD3/CD28 magnetic beads in the presence of IL-7 and IL-15 to

instruct the T_{SCM} phenotype, to determine a suitable time point at which their expression is at its maximum level, thus simplifying the evaluation of the knock-out efficiency by flow cytometry. When performing the genetic disruption of *pdcd1*, we observed a 50% knock-out efficiency on $CD3^+$ T lymphocytes on day 7 after activation, when up to 80% of control T cells expressed PD1. The T cell memory phenotype was not impaired after *pdcd1 disruption.* To edit *entpd1* in primary T cells, we compared the efficiency of three single guides RNA to a multi-guide system consisting of 3 mixed sgRNAs, targeting multiple sites within the same sequence, thus causing a fragment deletion. Compared to 50% disruption with sgRNAs, also confirmed at the genetic level, the multi-guide approach allowed to increase the efficiency of disruption up to 80%. Functional assays are ongoing to assess whether the absence of PD1 and/or CD39 confers an advantage to T cells in exerting their effector functions.

CHAPTER 6

Summary, Conclusions and Future Perspectives

Colorectal cancer (CRC) is a highly aggressive malignancy, affecting almost 150 000 people yearly and ranking second in the most frequent deaths for cancer[55]. When the tumor is localized and diagnosed at early stages, surgery is still the standard of care, together with chemotherapy or radiotherapy. Still, more than half of patients relapse with metastatic disease, representing the real unmet medical need behind CRC. New targeted treatments, such as monoclonal antibodies blocking EGFR (cetuximab, panitumumab) or VEGF (bevacizumab), have been introduced recently in the clinical practice to treat metastatic CRC (mCRC), but acquired resistance and severe toxicities limit their broad applicability.

The CRC microenvironment is heavily infiltrated by T cells, and their presence correlates with improved overall survival[55,57], underpinning their role in controlling tumor progression and supporting the rationale for immunotherapy strategies in CRC. However, within the tumor microenvironment (TME), T cells are exposed to chronic inflammation and continuous antigen stimulation leading to exhaustion, causing progressive loss of T cells effector functions and thus impaired capacity to control tumor growth[58].

To counteract exhaustion, immune checkpoint blockade has been proposed, but it demonstrated efficacy only in a small fraction of patients displaying microsatellite instability (MSI), related to a massive mutational burden.

Adoptive T cell therapy (ACT) could represent an innovative approach to treat CRC. Initially based on the collection of T cells infiltrating the tumor mass and their re-infusion to the patients after *ex vivo* expansion[59], ACT leverages nowadays the potential of the newest genome editing techniques[60].

A cellular product for ACT must meet some fundamental criteria: it has to survive the immune-suppressive TME, it must specifically target cancer cells and it possibly needs to be endowed with the capacity to survive long-term, to patrol for tumor recurrence. Given these premises, understanding the exhaustion features of T cells infiltrating the CRC microenvironment is pivotal. To this aim, we employed high-dimensional flow cytometry to dissect the memory phenotype and the inhibitory receptors (IRs) expression on T cells infiltrating primary CRCs compared to the healthy and the peritumoral tissue from the same patients. T cells within the tissues displayed an effector memory phenotype, with a small proportion of $T_{CM/SCM}$ cells in the peritumoral tissue only.

When looking at the expression of inhibitory receptors by analyzing flow cytometry data by manual biaxial gating, we noticed an increased proportion of T cells expressing PD1 and CD39 within the tumors compared to the peritumoral tissue.

However, to pinpoint a complex exhaustion signature for TILs, we employed a pipeline of unsupervised data handling by means of dimensionality reduction and clustering algorithms packed into Cytochain, a new web-app that our group developed to analyze large datasets (Manfredi*, Abbati*, …, Potenza et al., *submitted*).

Cytochain allowed identifying a population of exhausted $PD1^{+}CD39^{+}GITR^{+}CD137^{+}$ CD4 and CD8 T cells infiltrating exclusively primary CRCs, while being absent within the healthy and peritumoral colon tissues. The presence of an exhausted population in the TME sustains the rationale for ICB, which employment in CRC proved efficacy only in a small group of MSI patients. However, our data suggest that the inhibitory profile expression is not different between MSI and MSS patients, leading us to hypothesize that mechanisms other than the mutational burden are responsible for thwarting the ICB activity. Accordingly, ICB proved effective when used to treat tumors other than MSS-CRC but carrying a similar median number of mutations per mega-base[61]. Nonetheless, neoantigen-specific TILs have been described in MSS as well as in MSI tumors[5,62,63,64]. Since the preferential site for CRC metastasis is the liver, we wondered whether TILs infiltrating CRC liver metastasis harbored the same features of TILs infiltrating primary tumors. We observed the enrichment of $CD39^{+}HLA\text{-}DR^{+}TIM3^{+}2B4^{-}$ CD4 T cells and $CD39^{+}PD1^{+}TIM3^{+}LAG3^{+}2B4^{-}$ CD8 T cells in liver metastasis compared to the peritumoral liver. These observations raised the hypothesis that there could be tumor-specific factors as well as tissue-related factors leading to the upregulation of different IRs. Importantly, besides the co-upregulation of PD1 and CD39, TILs within primary and metastatic tumors also shared the more frequent upregulation of IRs, up to 10, compared to T cells infiltrating non-neoplastic tissues.

$PD1^+CD39^+$ T cells were also enriched, despite at very limited frequencies, in the PB of primary and metastatic CRC patients compared to healthy controls. A recent report showed how PD1 could serve as a marker to select tumor-specific T cells from PB, able to recognize the autologous tumors[7], but given the limited frequency of tumor-specific T cells within the PB, using it as a source for retrieving novel specificity requires long screening procedures[65]. Nonetheless, it was demonstrated that the repertoire of neo-antigens-recognizing T cells is discordant between TILs ad PB-derived T cells, with a lower functional avidity characterizing the latter[66].

In view of these findings, we wanted to investigate the TCR repertoire of lymphocytes infiltrating healthy, peritumoral and neoplastic tissues as well as circulating T cells. We observed that each compartment owns a different TCR repertoire, with few shared clones present at very low frequency. Our results are in line with previous reports demonstrating the diversity of T cell receptor sequences from the T cells infiltrating the tumor and the adjacent mucosal tissue[67].

Given the vast diversity of clones present in the repertoire, the missed identification of shared sequences could be due to limited coverage of the sequencing and to the little amount of starting material, limiting for example in the peripheral blood the likelihood to retrieve low-frequency T cell clones shared with the tumor site[68].

Another factor to consider when analyzing the TCR repertoire of TILs infiltrating the colon tissue is the anatomical site where the

tumor is located. Within the lumen of the gut, commensal bacteria are present and tolerated but considered as non-self from the immune system when breaching the mucosal barrier[69,70]. The presence of bacterial-reactive T cells[71] could impact the overall clonality of the repertoire, acting as a confounding factor in the analysis of the repertoire and contributing to the observed polyclonality.

To enrich for tumor-specific clones, leveraging on the exhaustion signature could represent an advantage, given that both PD1[7,9] and CD39[10], that we found upregulated on TILs, have been described to define cancer-reactive T cells.

We sorted three populations out of the TILs bulk: IR positive ($PD1^{+}CD39^{+}$), IR intermediate ($PD1^{+}CD39^{-}$/ $PD1^{-}CD39^{+}$) and IR negative ($PD1^{-}CD39^{-}$) T cells. We performed TCR sequencing on the three populations from one MSI and one MSS primary CRC. Of note, the IR positive population from the MSI tumor was sharply oligoclonal, with a 95% prevalent CDR3alpha and CDR3beta amino-acid sequence, raising the hypothesis that inhibitory mechanisms act on tumor-specific T cells. This difference was not present among the three populations from the MSS tumor, where different clones were enriched in the IR positive as well as in the IR intermediate and negative populations. Further investigation on the repertoire of more MSI and MSS patients is ongoing to address the relevance of this observation.

To test whether $PD1^{+}CD39^{+}$ T cells harbor tumor-specific TCRs, functional assays are needed to observe the recognition of the

autologous tumor. To this aim, the setting of a proper *in vitro* model is mandatory. We recently started to establish organoids from the tumoral tissue, given their potential to fully recapitulate the phenotypic and genotypic features of the tumor of origin[72].

As an additional approach to retrieve novel TCRs to be used for re-addressing T cells specificity for ACT, we used a pipeline set up in our lab starting from healthy donors' PBMCs (Ruggiero, …, Potenza et al., *submitted*). By stimulating T cells with autologous APCs loaded with a pool of peptides selected to be immunogenic for CRC, we isolated populations of T cells specific for HER-2 tumor-associated antigen and CTDP1, TACC2 and PTGFRN ligandome-derived[17] neoepitopes, related with tumorigenesis. The advantage of using HDs to retrieve novel specificities resides in the physiological negative selection T cells underwent in the thymus, rendering these TCRs potentially safer[28]. TCR αβ tracking of the peptide-specific populations' clonality allowed to observe a progressive contraction of the repertoire, with a prevalently enriched CDR3alpha and CDR3 beta sequence after several restimulations. The obtained novel TCRs will be further characterized and used to redirect T cell specificity.

To overcome the limitations of TCR gene transfer, among which the suboptimal surface expression of the transferred receptor[73] and the potential harmful reactivities caused by the possible mispairing between α and β chains from exogenous and endogenous TCRs[30], we set up TCR gene editing by leveraging the potential of CRISPR/Cas9 nucleases. We produced T cells devoid of the endogenous TCR by disrupting both α and β chains

with 98% efficiency, and we redirected their specificity against MUC1 tumor-overexpressed antigen by lentiviral transduction with a MUC-1 TCR cloned by taking advantage of the CDR3 α and β genes from a published, MHC-unrestricted single-chain TCR[47]. MUC-1 redirected T cells were able to exert killing capacity when challenged with a MUC-1$^+$ CRC cell line. For generating long-term persisting cellular products, we stimulated our edited cells with a protocol developed to instruct the memory stem T cells (T_{SCM}) phenotype[50]. T_{SCM} are endowed with self-renewal capabilities and high proliferative potential[74], and have shown the ability to survive long-term when adoptively transferred[51,52]. Still, TCR-redirected T_{SCM} have to counteract the immune-suppressive TME. Being the exhaustion signature in CRC driven by PD1 and CD39, we explored the feasibility to disrupt these molecules in T cells. In fact, the use of monoclonal antibodies or small molecules to block these factors would act on the entire T cell repertoire, rather than tumor-specific T cells only, possibly causing adverse events. By using a sgRNA nucleofected in a ribonucleoprotein complex with Cas9, we achieved up to 50% disruption efficiency in both genes. To enhance the disruption frequency, we employed a multi-guide system targeting CD39, which ensured a fragment deletion and up to 75% of knock-out. The *in silico* ICE validation of CD39 disruption on Sanger-sequenced amplicons confirmed the results obtained by flow cytometry analysis of protein expression. Several other technologies are available to validate the disruption at the genomic level: mismatch detection assays[75],

mismatch cleavage assays such as T7E1 or Surveyor[76], next-generation sequencing to detail the mutations occurred after cleavage[77,78]. One of the most sensitive methods for detecting editing efficiency is digital droplet PCR[79] (ddPCR): we compared results obtained by ddPCR to results from ICE analyses, obtaining overlapping outcomes, thus verifying the reliability of the ICE approach.

Still, the most important thing to consider when using CRISPR/Cas9 is the likelihood of off-target events occurrence, which can be evaluated by different technologies, spanning from Sanger sequencing after single clones amplifications[75] to next-generation sequencing, avoiding the time-consuming single-cell cloning[80]. Among the most used assays, the integrase-defective lentiviral vector (IDLV) allows capturing off-targets by detecting LV integration sites after the repair of the nuclease-induced double-strand break via non-homologous end joining[81]. Many more technologies have been developed in recent years[82], since off-target evaluation is fundamental when purposing a cellular product for clinical use.

To our project, CRISPR/Cas9 is the most suitable technology for the possibility to exploit multiplex genome editing, thus contemporary disrupting the endogenous TCR and inhibitory receptors. However, functional assays are needed to evaluate whether the absence of PD1 and/or CD39 confers T cells a functional advantage in eliminating target cells.

Taken together, these results provide insights into the inhibitory profile of T cells in primary colorectal tumors as well as in liver

metastasis. We planned immunohistochemistry studies to visualize the spatial location of exhausted T cells within the tumor mass and we are currently performing RNA sequencing analysis on primary tumors as well as on liver metastasis to explore immune cell heterogeneity and widely understand which are the microenvironmental factors defining the tumoral tissue.

Also, we aim at further characterizing the library of TCRs we retrieved from both patients or healthy donors by challenging redirected T cells with HLA-matched organoids or CRC cell lines expressing the antigen. Once prioritized the most promising TCRs, we will couple TCR editing with IRs disruption and test the safety and the efficacy of the obtained cellular products, to select to fittest product for a future clinical trial.

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