UNIVERSITÀ DEGLI STUDI DI PAVIA

Dipartimento di Scienze Clinico-Chirurgiche, Diagnostiche e Pediatriche

Dottorato di ricerca in Medicina Sperimentale – Ciclo XXXV

Coordinatore: Ch.mo Prof. Stefano Perlini

"Isolation, expansion and characterization of tumor infiltrating lymphocytes derived from metastatic colorectal carcinoma patients for adoptive immunotherapy"

Tesi di:

Dott.ssa Agnese Rumolo

Relatore:

Prof. Lorenzo Cobianchi

Correlatori:

Prof.ssa Daniela Montagna

aa 2021/2022

"La vita è una tempesta, mio giovane amico. In un momento potreste scaldarvi al sole, in uno successivo andare a frantumarvi contro gli scogli. Che cosa vi rende un uomo, è ciò che si fa, quando arriva quella tempesta."

Alexandre Dumas, Il Conte di Montecristo

"Come mai, prima, non m'accorgevo di questo cielo così alto?
E come sono felice d'averlo riconosciuto, finalmente!
Sì, tutto è vano, tutto è inganno, fuorché questo cielo sconfinato.
Nulla, nulla esiste al di fuori di esso. Ma neanche questo esiste,
nulla esiste, al di fuori della quiete, del sentirsi placato.
E grazie a Dio!"

Lev Tolstoj, Guerra e Pace

"Come possiamo dormire di notte con tutta la sofferenza che c'è nel mondo?"
"Spegnendo la luce, cara."

I Simpson, stagione I, episodio VII

Alla mia mamma

ABSTRACT

Colorectal carcinoma (CRC) is one of the most common type of cancer and the second cause of cancer death in the world. Conventional treatments appear to slow the tumor progression, but do not bring the patients to a disease-free state. Adoptive cellular immunotherapy is emerging as a new clinical strategy after the results obtained with patients with metastatic melanoma.

In our study, we developed a new strategy for the extraction and expansion of both autologous TILs and tumor cells (TCs) from liver metastases of colorectal carcinoma. This was done by mechanical and enzymatic dissociation of tumor samples resected in the operating room. TCs were used for functional assays as well to determine TILs' potency. Three protocols for TILs expansion were developed using different cocktails of cytokines (i.e. IL-2, IL-15, IL-21 and IL-7) and all showed promising results in terms of cytotoxic activity and expansion rate. We demonstrated how it is possible to obtain and expand TILs from metastatic colorectal carcinoma (mCRC) even when TILs' recovered are low in number. TILs expanded with all three protocols were able to determine cytolysis on both an immortalized human CRC cell line (SW480) and against TCs and such activity remains sizeable even when a second round of antigen independent expansion is performed. We also observed how TILs which had prevalently expanded CD3+/CD4+ cells had a cytotoxic capacity compared to TILs which were prevalently CD3+/CD8+, showing the importance of CD3+/CD4+ in mediating CD3+/CD8+ cells anti-tumor activity and in endowing an anti-tumor response as well. Analysis of cell activation and exhaustion markers expressed by TIL in both CD3+/CD4+ and CD3+/CD8+ population documented a high heterogeneity of expression between TIL, with little difference between the different expansion protocols.

Molecular assessment were performed on both TILs and TCs to further define the role of Ca2+ signalling pathways. In particular, our findings determined how Store Operated Ca2+ Entry in TILs is up-regulated and mainly depends on diacylglycerol kinase (DGK), which prevents the protein kinase C-dependent inhibition of Ca2+ entry in normal T cells. Notably, the pharmacological blockade of SOCE with the selective inhibitor, BTP-2, during target cell killing significantly increases cytotoxic activity at low TIL density, i.e., when TILs-mediated cancer cell death is rarer. We also determined how exogenous administration of H_2S-releasing compounds has been shown to exert a strong anticancer effect by suppressing proliferation and/or inducing apoptosis in several cancer cell types,

including CRC. We provided the evidence that NaHS – a H_2S donor- induced extracellular Ca2+ entry in mCRC cells by activating the Ca2+-permeable channel Transient Receptor Potential Vanilloid 1 (TRPV1) followed by the Na+-dependent recruitment of the reverse-mode of the Na+/Ca2+ (NCX) exchanger. NaHS reduced mCRC cell proliferation, but did not promote apoptosis or aberrant mitochondrial depolarization. Our findings support the notion that exogenous administration of H_2S may prevent mCRC cell proliferation through an increase in [Ca2+]i.

Abbreviations

- TME: tumor microenvironment
- CTL: cytotoxic T lymphocyte
- APC: antigen presenting cells
- MHC: major histocompatibility complex
- PD-1: programmed cell death-1
- PD-L1: programmed cell death ligand-1
- OS: overall survival
- CRC/mCRC: colorectal carcinoma/metastatic colonrectal carcinoma
- WHO: world health organization
- TTR: time to recurrence
- CT: center of tumor
- IM: invasive margin
- DFS: disease free survival
- MSI: deficient mismatch repair
- MSS: proficient mismatch repair
- TGF-β: transforming growth factor- β
- VEGF: vascular endthotelial growth factor
- IDO: indoleamine-pyrrole 2, 3-dioxygenase
- Tregs: regulatory T cells
- CAFs: cancer associated fibroblasts
- M1/M2: type 1 macrophage/type 2 macrophage
- IFN-γ: interferon-γ
- TNF-α:tumor necrosis factor-α
- TAA: tumor associated antigens
- TSA: tumor specific antigens
- AI: active immunotherapy
- DC: dendritic cells
- GM-CSF: granulocyte macrophage-colony stimulating factor
- MoDCs: monocyte-derived dendritic cells
- mAbs: monoclonal antibodies

- HER2: human epidermal growth factor 2
- ADCC: antibody-dependent cell cytotoxicity
- EGFR: epidermal growth factor receptor
- FDA: food and drug administration
- CLL: chronic lymphocytic leukemia
- BiTEs: bispecific T-cell engagers
- ACT: adoptive cell transfer
- LAK: lymphokine activated killer cells
- CIK: cytokine induced killer cells
- TCR: T cell receptor
- CAR-Ts: chimer antigen receptor T cells
- TIL: tumor infiltrating lymphocytes
- PBMC: peripheral blood mononuclear cells
- TRUCK: T cell Redirected for Universal Cytokine Killing
- CEA: carcinoembryonic antigen
- ALL: acute lymphoblastic leukemia
- CLL: chronic lymphocytic leukemia
- $T_{EM:}$ effector memory T cells
- T_{CM}: central memory T cells
- DLI: donor lymphocytes
- GvHD: graft versus host
- AML: acute myeloid leukemia
- LBs: leukemia blasts
- MDS: myelodysplastic syndrome
- TBI: total body irradiation
- GMP: good manufacturing practice
- ICI: immunocheckpoint inhibitor therapy
- MSI-H: microsatellite instability
- SOCE: store operated calcium entry
- TRPV1: Transient Receptor Potential Vanilloid 1
- T_{EM}: Effector Memory T cells
- T_{CM}: Central Memory T cells
- T_{emRA}: Terminal Memory T cell

1 INTRODUCTION

1.1 Colorectal adenocarcinoma

Colorectal cancer (CRC) is the third most common malignant neoplasm worldwide (6.1%) and second in terms of mortality (9.2%). The overall 5-year survival rate is 65%, and deaths from colorectal adenocarcinoma represent 15% of all cancer-related deaths, second only to lung cancer. The tumor's incidence peaks around 60-70 years of age, whilst under 50 patients represents 20% of cases. The disease is slightly more diffused in male patients [2].

CRC is a multifactorial disease involving genetic, environmental and lifestyle risk factors. Although there is a strong hereditary component, most cases of CRC are sporadic and develop slowly. In fact, the time frame within which the CRC develops spans several years, for this reason it is very important to be able to diagnose it at the beginning of the development of the disease. CRC appears to be a non-homogeneous disease, endowed with multilevel molecular complexity; in addition, individual cases differ from each other in terms of location, degree of histological malignancy or type of neoplasm. Four molecular subtypes of colorectal cancer (CMS) have been found: CMS1-immune-activating MSI, CMS2-canonical, CMS3-metabolic, and CMS4-mesenchymal. In turn, these individual subtypes differ in their clinical course and in the different response to chemotherapy and biological treatments[1].

Despite advances in surgical techniques and adjuvant therapy, there has been only a modest improvement in survival for patients affected by advanced metastatic neoplasms. Hence, effective primary and secondary preventive approaches must be developed to reduce the morbidity and mortality from CRC.

1.1.1 Pathogenesis

Genetics, experimental, and epidemiologic studies suggest that CRC results from complex interactions between inherited susceptibility and environmental factors. It is possible to include these multiple factors into three large groups: i) factors related to a family and medical history, ii) factors closely related to lifestyle such as alcohol use,

cigarette smoking and obesity, and iii) other factors which include age, gender, race, socio-economic status and the state of the intestinal microbiome[3].

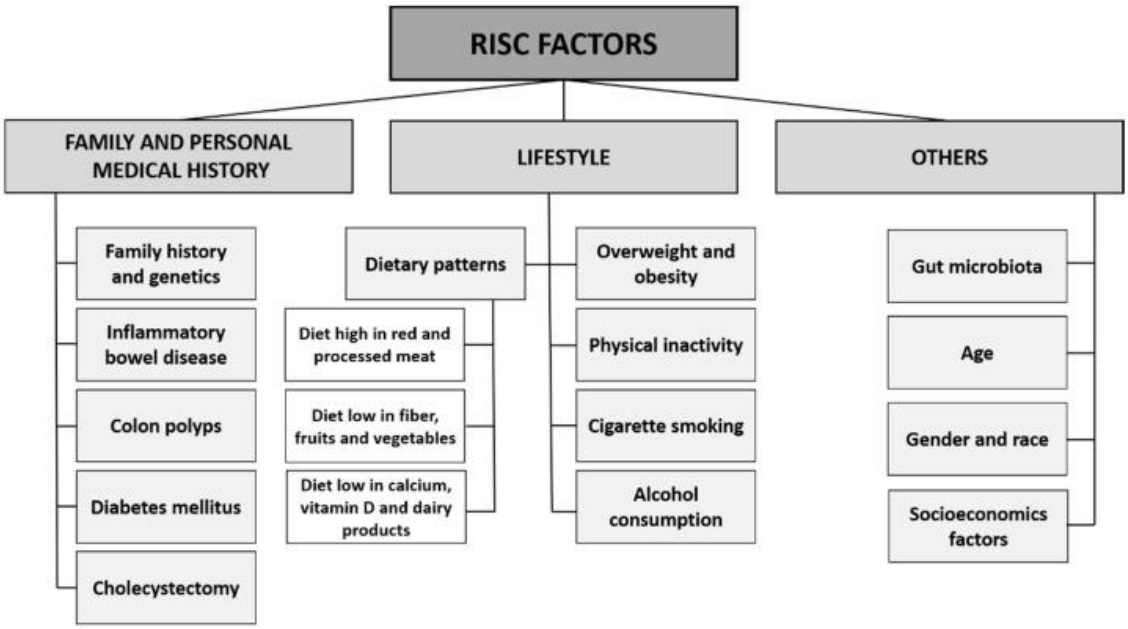

Figure 1: Risks factors associated to CRC. (Sawicki et al.,2021) [4]

In the last twenty years, researchers have found out several mechanisms related to CRC carcinogenesis. At least two distinct pathways have been studied and described: the APC/β-catenin pathway and the microsatellite instability pathway.

- **APC/β-catenin pathway**: *APC* is a tumor suppressor gene which exerts anti-proliferative effects. It encodes a cytoplasmatic protein whose main feature is the degradation of β-catenin. β-catenin, other than binding to E-Cadherin, is a key component of the WNT signaling pathway. WNTs are soluble factors that bind WNT receptors, which in turn mediate signals that prevent the APC-mediated β-catenin degradation, allowing it to traslocate to the nucleus and functioning as a transcriptional activator. With loss of APC, β-catenin degradation is prevented and the WNT signalling response is inappropriately activated even in the absence of WNT factors. In Coloninc epithelium this loss leads to increased transcription of growth promoting genes such as cyclin D1 and MYC and transcriptional regulators such as TWIST and SLUG, which repress E-cadherin expression and thus reduce contact inhibition (E-cadherin, when binding to β-catenin, causes

contact inhibition). *APC* gene dues its name to the disease it causes: adenomatous polyposis coli. The illness causes development of numerous adenomatous polyps in the colon that have a high incidence of transforming into colonic cancers.

- **The microsatellite instability pathway**: patients with DNA mismatch repair deficiency accumulate mutations on the microsatellite regions. Normally, these areas are not encoded but other microsatellite regions are in coding or promoter regions of genes involved in regulation of cell growth, such as those encoding the type II TGF-β receptor and the proapoptotic protein BAX. Normally, TGF-β inhibits colonic epithelial cells proliferation, therefore its mutant can contribute to uncontrolled cell growth and loss of BAX may ensure the survival of abnormal clones.
- The oncogene *KRAS* seems to develop mutations as well, however data showed how it appeared later in the disease. Such observation has been done after finding out that the mutations are found in 50% of CRC larger than 1 cm in diameter, 50% in invasive adenocarcinoma and fewer than 10% of CRC less that 1 cm in diameter. . Several mutations have been found of these oncogene on different codons, however the most frequent seems to be on codon 12, where a glycine substituted by aspartic acid. However, such mutation may be a target for immunotherapy strategies which shall be discussed further in this work.
- Other tumor suppressor genes whose mutation cause disease progression are *SMAD2* and SMAD4, which encode effector of the TGF-beta signaling. The signaling normally inhibits the cell cycle, therefore loss of these genes may allow unrestrained cell growth.
- Tumor suppressor gene *TP53* is mutated in 70-80% of colon cancers but is unaffected in adenomas (which are benign) suggesting that this mutation occurs in the late stage of cancer progression.
- Lastly we have CIMP, which stands for CpG islands hypermethylation phenotype. In a subset of colon cancers with microsatellite instability, there are no mutations in mismatch repair enzymes. In these tumors, the MLH1 promoting regions is often hypermethylated, reducing MLH1 expression and therefore its repair functions. Activating mutations in the BRAF oncogene are common in these kinds of cancers, whilst KRAS and TP53 are not commonly mutated [5].

1.1.2 Immune infiltrate: a prognostic predictor

Extensive data acquired over the years have documented the role of the immune infiltrate in patient outcome, suggesting a favorable prognosis in patients harboring a immune cell infiltrate *in situ* in tumors. Overall survival (OS) and time to recurrence (TTR) seem to be strongly correlated to the presence in situ of adaptive immune reaction.

Mlecnik et al. of the international Society for Immunotherapy of Cancer Immunoscore showed how the immune infiltrate, both at center of tumor (CT) and invasive margin (IM) is strongly correlated to clinical outcome. Therefore, they decided to develop an Immunoscore, to predict the clinical outcome of patients with early and advanced mCRC. 763 patients with stage III mCRC were enrolled. The primary end point was TTR – from time to surgery to occurrence -, with the second endpoints being OS, disease-free survival (DFS) and prognosis in microsatellite stable (MSS) status.CD3 and CD8 densities were calculated by using an Immunoscore software. Results demonstrates that in all categories patients with high immunoscore showed higher survival [6].

Immunoscore was also used as an element to evaluate if patients with high immunoscore might benefit from adjuvant chemotherapy. TTR was evaluated in patients with High Immunoscore which received or didn't receive adjuvant chemotherapy. It was showed how, in patients with high immunoscore, adjuvant chemotherapy showed a significantly difference with TTR, with the latter being higher. In OS, the interaction between chemotherapy and immunoscore was significant, strengthening the idea that Immunoscore is a valid instrument to predict clinical outcome. In low-immunoscore, chemotherapy was not associated with TTR both in high risk and low risk patients. Patients with High Immunoscore and low risk disease benefited the most, as well as patients with intermediate/high immunoscore and high risk disease[6].

1.2 Cancer and immune system

The immune system plays a dual role in tumor evolution: on the one hand it can recognize and control the growth of tumor cells in the process defined as " immunosurveillance" and, on the other hand, it can promote tumor progression through immunosuppression by different mechanisms [7]. In fact, data obtained in the last decade, first from mouse models and then from cancer patients, have shown that the immune system is able to recognize and eliminate transformed cells. Conversely, the immune system is also able to promote tumor progression by supporting chronic inflammation, modulating tumor immunogenicity, and suppressing antitumor immunity. These host-protective and tumor-promoting actions are integrated with tumor immunoediting which comprises three sequential phases.

- the first phase of ***elimination.*** During this phase immunogenic tumors are eradicated by immune cells that recognize and destroy most of the cells with abnormal growth initiating the process of apoptosis. In this stage, innate immunity and adaptive immunity cooperate to destroy developing tumors long before they become clinically apparent. This stage has been clearly documented in murine models. In particular, studies have shown that immunodeficient mice (deficient in effector molecules such as IFN and perforin; or cell types such as T and NK cells) exhibited increased frequencies of spontaneous tumors compared to that observed in wild type mice.
- the ***equilibrium phase***, in which tumor cells survived the previous phase coexist during which the adaptive immune system prevents the invasion and growth of the tumor and finally
- the ***escape phase***, in which the tumor cells, as a consequence of the constant immune selection to which they are subjected, adopt a strategy which leads them to acquire the ability to circumvent immune recognition thus avoiding their destruction and thus emerging as malignant cells. Different mechanisms are used including downregulation or loss of expression of MHC I molecules, essential for the recognition of CD8+ cytotoxic T lymphocytes, and increased expression of cytotoxic T lymphocyte inhibitory ligands, such as programmed death ligand 1 (PD-L1) which suppresses the attack of cytotoxic T lymphocytes. [8].

A broader understanding of cancer immunosurveillance and immunoediting is therefore essential to make the action of the immune system more efficient. Recent studies have demonstrated that the success of immunotherapy depends on several factors, including the type of therapy, the level of expression of targeted agents, the strategies adopted, the stage of the tumor and the duration of therapy [9].

1.2.1 Tumor escape and tumor microenvironment

The different mechanisms by which tumor cells evade the host immune response can be classified into three categories.

i) ***Loss of antigen expression*** and ***resistance to cytotoxicity*** are the two ways cancer cells overcome immunity, as they cannot be efficiently recognized by T lymphocytes.

ii) Tumor cells can also change their microenvironment into an immunosuppressive state through the ***expression of immunosuppressive cytokines*** such as transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), or indoleamine-pyrrole 2, 3-dioxygenase (IDO). They can then actively induce immunosuppression through the recruitment of regulatory T cells (Tregs; a subpopulation of CD4 T cells) and myeloid-derived suppressor cells (MDSCs).

In particular, CD8+ T lymphocytes are the key immune cells for killing cancer cells presenting MHC class I molecules. These cells, after priming, are activated in effector T lymphocytes, or CTLs, able to kill cancer cells. However, their function can be suppressed by immunosuppressive cross-talking between cancer cells with tumor stromal cells such as cancer-associated fibroblasts (CAFs), Tregs and type 2 macrophages. These cross-talking interaction allow the tumor to dampen the immune response to proliferate in an uncontrolled manner and attain invasive features.

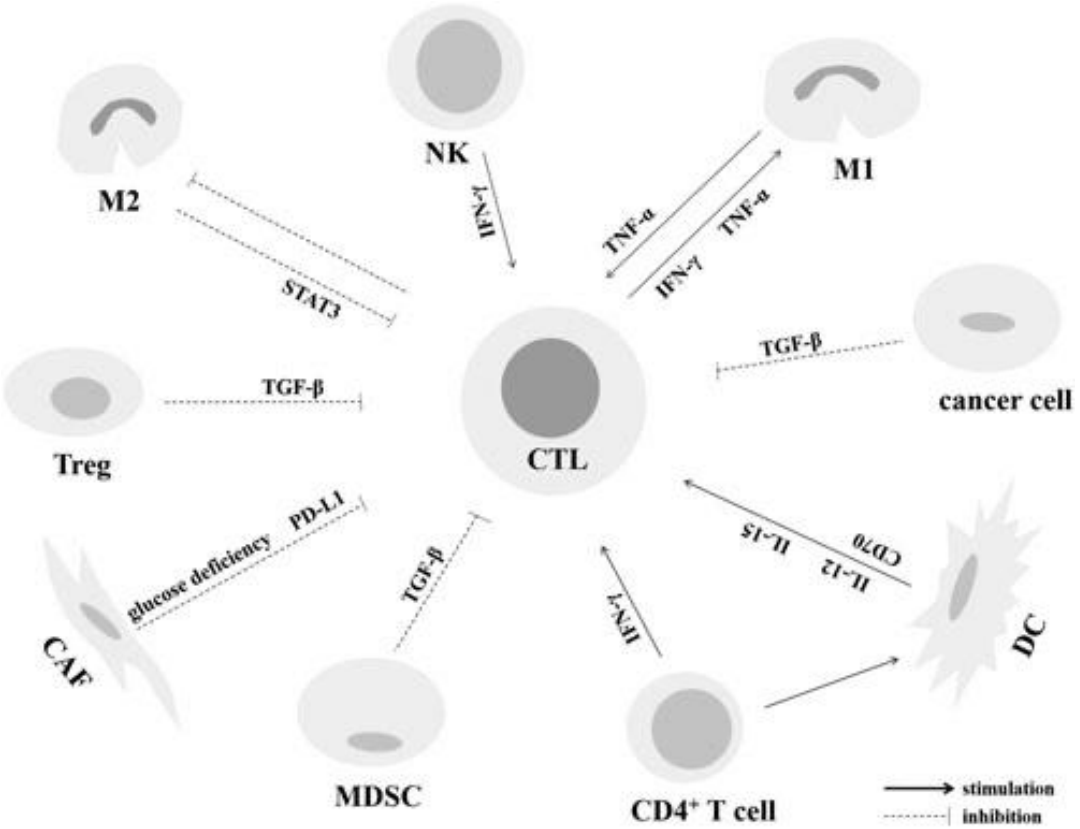

Figure 2: cross talking of CTLs with several cell types in the tumor stroma, determining activation or dampening of anti-tumor activity [10]

CAFs are the most frequent cells of tumor stroma that their activity is cardinal for mediating an immunosuppressive tumor microenviroment (TME). The cells promote all tumorigenesis stages and their number indicates poor patient survival. The position of CAFs is within the invasive border of cancers where intratumoral, inflamed CTLs are located, and they release a myriad of factors to direct tumor progression. The secretory products from CAFs are for influencing themselves and nearby cells in an autocrine and paracrine. Their position in the invasive border of the tumor is for regulation of CTLs in which CAFs are responsible for suppressing intratumoral migration of CTLs leading to an immunosuppressive state within the TME. CAFs induce an increased rate of glycolytic metabolism within the TME that further leads to glucose deficiency in this milieu. This deficiency promotes a competitive struggling between cancer cells with effector T cells. CTLs when are encountered to such conditions, tend to decrease their number, which is a mechanism for regulating the number of these cells by CAFs. CAFs can also contribute to CTL killing in an antigen-dependent mechanism via activating PD-L2 an FasL.

Immune suppressive cross talk interaction include innate immunity cells as well, such as macrophages or, in particular, type 2 macropaghes – M2.

Macropaghes are one of the leader cells of immune systems that include up to 50% of infiltrated cells to the stroma of tumor and are considered versatile cell types as they're able to tailor their responses into two diverse ways depending on the dominant signals they receive from tumor stroma. There are two types of macrophages called M1 and M2 cells. Proinflammatory M1 cells are defined as classically activated macrophages having the capacity to promote inflammatory responses and to evoke immune system. Alternatively, activated cells repress the immune system and their presence within the tumor is associated with poor prognosis. M1 cell recruitment and CTL production are the two simultaneous processes occurring in the tumor stroma under the influence of IFN release to the TME. CTLs within the tumor stroma send paracrine signals for changing macrophage polarity toward the antitumor M1 cells, and that the cells have negative cross-talking with M2 cells in which a reduction in the number of M2 cells in the TME would cause a rise in the recruitment of CTLs to this milieu to exert antitumor immunity for regression of tumor. When the number of M2 cells in the stroma is increased, the cells secrete STAT3 to the TME for impairing responses from CTLs. Both CTLs and M1 cells produce IFN-γ to induce M1 polarity. In addition, both CTLs and M1 cells release TNF-α to the TME. TNF-α is a potent anti-M2 polarizing cytokine that is tightly related to M1 phenotype, but TNF-α also stimulates the suppressor activity of CTLs.

Other immune cells present in TME which determine dampening of CTLs anti-tumor activity are Tregs. Tregs are an immunosuppressive subset of CD4+ T cell family, representing approximately 4–5% of the total CD4+ T cell population under normal situations. An increase in the number of Tregs is common in cancer patients, as they are prevailing T cells that respond to the early stages of cancers outpacing CTLs during early cancer growth. Tregs would dampen CTL translocation to the core of tumor through a corroboration work with other immune suppressor cells including M2 macrophages and CAFs . Tregs through the release of TGF-β suppresses the activity of CTLs within the TME. TGF-β would do this through inhibition of the expressions for cytolytic gene products from CTLs, including granzymes A and B, perforin, FasL, and IFN-γinvolved in determining tumor cytotoxicity and proliferation of CTLs.

Based on the data it appears evident that tumor and its microenvironment constantly interact and influence each other, either positively or negatively. TME is a complex and continuously evolving entity, and their composition varies between tumor types [11].

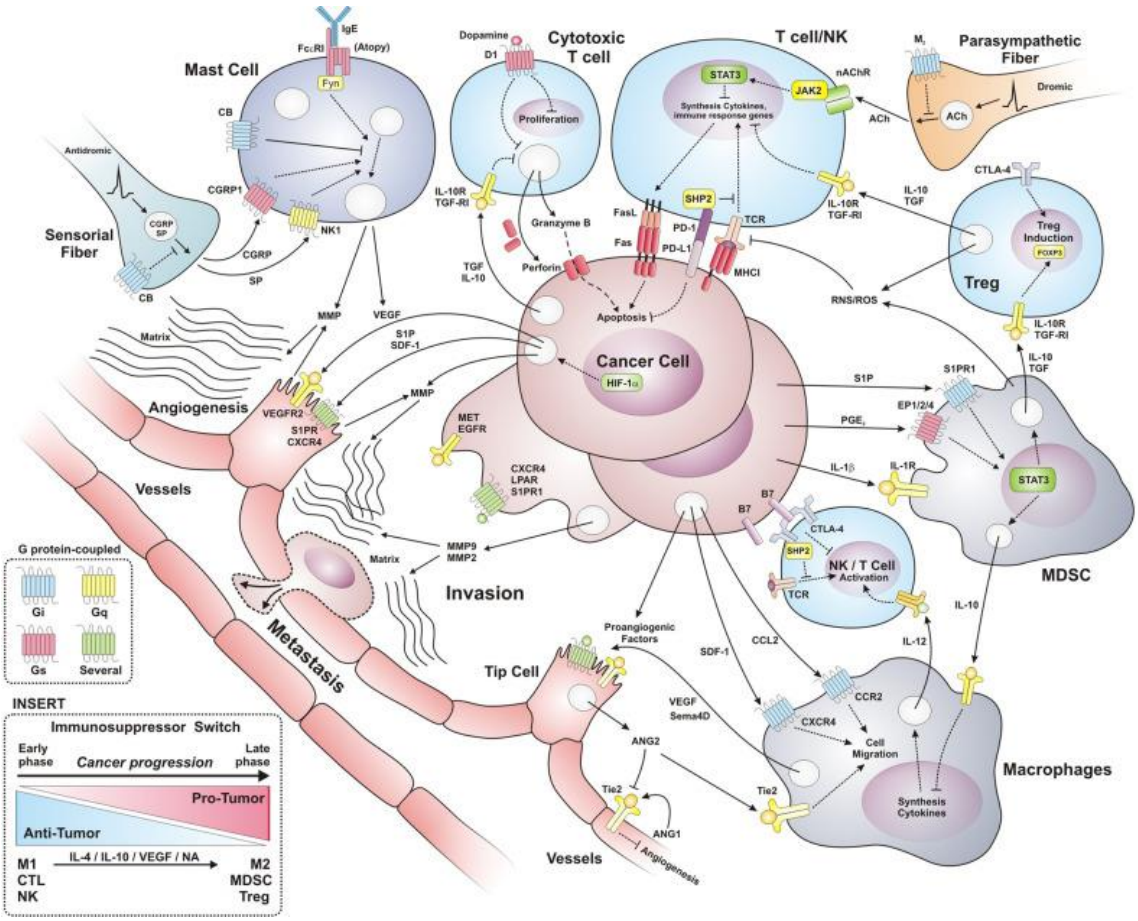

Figure 3 : Oncogenic communication between tumor cells and tumor-associated stromal cells. (Rodolfo Daniel Cervantes-Villagrana et al., 2020)[12]

TME is a highly hypoxic environment, with abnormal blood vessels – due to uncontrolled angiogenesis - rapidly growing and therefore showing structural malformations, a leaky endothelium, and an abnormal, heterogeneous blood flow, not only aggravating the supply of nutrients but also chemotherapeutic agents.

It has been described that the irregular endothelium compromises immune penetration for endogenous as well as adoptively transferred T cells, resulting in non-inflamed, cold tumors. The environment appears hostile for autologous infiltrating T cells, however another obstacle is the immune infiltrate itself: continuous TCR signaling in the TME – being the T cell continuously exposed to the TAAs - , in fact, brings the T cell to exhaustion. This is the same mechanism shared with chronic viral infections. The T-cell exhaustion status is generally defined by functional impairment associated with an upregulation of inhibitory surface receptors such as PD-1, Tim3 and Lag3 – even if it was recently demonstrated how CD8+ PD1+ T cells showed great anti-tumor activity after *ex-*

vivo expansion and *in vivo* administration [13]. [14]. Therefore, an active, non-exhausted T population must be defined and selected for expansion and adoptive immunotherapy.

1.2.2 Cancer immunotherapy

Cancer immunotherapy is probably the most promising strategy to determine tumor regression in patients refractory to conventional therapies. The goal of cancer immunotherapy is to enhance anti-tumor immunosurveillance, by providing passive or active immunity against malignant tumors whilst escaping and overcoming the tumor immune suppressions strategies.
Several immunotherapy strategies have been developed for the treatment of tumors, which can be divided in three main categories:

1) Non-specific immunomodulation
2) Active immunization approaches –(i.e. cancer vaccines)
3) Passive immunization approaches –
   - i.e Monoclonal antibodies
   - Adoptive cellular immunotherapy

1.2.2.1 Non-specific immunomodulation

This strategy consists in stimulating autologous effector T cells in vivo to promote tumor rejection with cytokines endowed of anti-tumor activity.
The first administration of high dose IL-2 was performed in 1985 in patients with metastatic melanoma [15] and renal cell cancer, determining complete regression and durable response. Responses were seen in all sites of the disease and more than 80% of complete responses appeared durable and were on-going after a median follow up of 7 years. However, IL-2 alone does not appear to be sufficient for tumor regression in other solid metastatic cancers.
In melanoma patients treated with IL-2, organ specific autoimmunity – vitiligo - was observed in 20% of patients, whilst development of autoimmune thyroiditis in 55%. Patients with renal carcinoma, instead, do not seem to develop vitiligo with this treatment

and autoimmune thyroid is rarer in this disease, meaning that probably a different set of T cells is activated by IL-2 administration.
Interferon-a2b (IFN-a2b) also has been used for the treatment of melanoma and renal cell cancer. Its role in the adjuvant therapy after surgery in patients at high risk of recurrence is controversial, since improvement in overall survival was not showed.
Long term administration of pegylated IFN-a2b, expected to maintain maximum exposure to IFN-a2b compared to injection with unpegylated formula, has given good results in a randomized clinical trial. Relapse free survival improved of 9,3 months, however it had no impact in overall survival.

1.2.2.2 Active immunization

Active immunotherapy (AI) is defined as the process of inducing an effective immune response in the host based on endogenous innate and adaptive immune effector mechanisms. [16]
AI's objective consists in stimulating the body's own immune system in order to amplify both a humoral and cytotoxic immune response to target tumor cells. It works either by boosting the immune system entirely or by training the immune system to attack the tumor based on specific antigenicity. An example of AI are cancer vaccines, and dendritic cells (DCs)- based immunotherapy.

1.2.2.2.1 Cancer vaccines

The administration of cancer vaccines consists in exposing the host's immune system to TAAs or Tumor Specific Antigens (TSAs), determining an adequate response and function of immune effectors [17].
The main goal of cancer vaccine administration is to determine a broad stimulation of CTLs and T helper cells through two mechanisms:

- Selection of appropriate antigens that induce both T cell populations.
- Rational vaccine designs that achieve concentrated delivery of tumor antigens to activated DCs, where epitopes derived from exogenous tumor antigens can be

loaded onto both MHC class I (through the cross-presentation pathway) and MHC class II molecules to stimulate CTLs and T helper cells, respectively.

A variety of therapeutic immunization strategies against putative cancer antigens has been tested. These approaches include the use of peptides, proteins, whole cells, tumor lysate, and recombinant viruses that encode the desired tumor antigens.

Peptide-based vaccines are relatively easy to manufacture, but combination with potent immune adjuvants is often needed to boost immunogenicity, and the number of people who may benefit from a given peptide vaccine is restricted by human leukocyte antigen (HLA) haplotype. Several clinical trials for peptide-based vaccines did not show any clinical benefit.[17]

Whole cell vaccines include irradiated autologous or allogeneic tumor cells sometimes engineered to secrete cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF) or to express T-cell costimulatory surface molecules, stimulated autologous antigen-presenting cells that may be loaded with peptides or tumor lysates, and autologous tumor-derived heat-shock protein gp96. The efficacy of these approaches, assessed by tumor shrinkage defined by standard response criteria, has been consistently low. [18].

Viruses or plasmids can act as vectors for DNA or RNA encoding TAAs and trigger DCs response by triggering pattern recognition receptors, however data available did not show any significant improvement in patients which received these vaccines, with or without immunocheckpoint blockade therapy. [17]

1.2.2.2.2 DC-based immunotherapy

Several forms of DC-based immunotherapy have been developed, most of which involve the isolation of patient- or donor-derived circulating monocytes and their differentiation *ex vivo*, in the presence of agents that promote DC maturation, such as granulocyte macrophage colony-stimulating factor (GM-CSF). This is fundamental, as immature DCs exert immunosuppressive, rather than immunostimulatory, functions and most often autologous DCs are re-infused into cancer patients upon exposure to a source of TAAs, including:

(1) TAA- derived peptides
(2) mRNAs coding for one or more specific TAAs
(3) expression vectors coding for one or more specific TAAs
(4) bulk cancer cell lysates (of either autologous or heterologous derivation) or bulk cancer cell-derived mRNA

As an alternative, DCs are allowed to fuse *ex vivo* with inactivated cancer cells, generating so-called dendritomes. The rationale behind all these approaches is that DCs become loaded *ex vivo* with TAAs or TAA-coding molecules, hence becoming able to prime TAA-targeting immune responses upon reinfusion. Additional DC-based anticancer immunotherapies include the targeting of specific TAAs to DCs *in vivo.* The use of DC-derived exosomes, and the (re-)administration of autologous or allogeneic DCs amplified, matured and optionally genetically modified *ex vivo*, but not loaded with TAAs.. In the former setting, TAAs are fused to mAbs, polypeptides or carbohydrates that selectively bind to DCs, encapsulated in DC-targeting immunoliposomes or encoded by DC-specific vectors. In the latter scenarios, DCs or their exosomes are administered as a relatively non-specific immunostimulatory intervention. [19]
There are two major hurdles for the success of DC-based immunotherapies: tumor-mediated immunosuppression and the functional limitation of the commonly used monocyte-derived dendritic cells (MoDCs). [20]

1.2.2.3 Passive immunization

Passive immunization is based on the administration of preformed antibodies directed towards tumor antigens, or cells of the immune system manipulated *in vitro* and directed, towards tumor antigens. Classic examples of passive immunotherapy are tumor-targeting monoclonal antibodies (mAbs) and adoptive cellular therapy approaches. The concept of passive immunotherapy consists in using molecular or cellular products which possess intrinsic anti-tumor activity and do not rely on the host's immune response. [21]

1.2.2.3.1 Monoclonal Antibodies

The purpose of this specific passive immunization approach is to administer an antibody, usually IgG, specific for one or more surface molecules expressed only on a tumor cell (TAA) and not on other cell lines and which does not allow the reaction to cross-linked with surface molecules on healthy cells. The identification and selection of such unique TAAs has proven very difficult as many of the tumor cell surface antigens are also found on normal cells. However, particularly in the case of tumors, these TAAs, although not unique, are overexpressed.
Several tumor antigens have been discovered through the years, but they may be classified in two different categories: tumor-specific antigens and tumor-associated antigens.

1) normal proteins which are overexpressed
2) unmutated differentiation antigens
3) cancer-testis antigens – which are unmutaded genes expressed during fetal development which are silent in adults and reactivated in cancer cells across several malignancies
4) mutated antigens unique to a single tumor or shared by a group of tumors

Monoclonal antibodies are engineered molecular structure designed to specifically target a neoplastic molecule expressed by the tumor.
Tumor targeting monoclonal antibodies may:

- specifically alter the signaling functions of receptors expressed on the surface of malignant cells.
- bind to, and hence neutralize, trophic signals produced by malignant cells or by stromal components of neoplastic lesions.
- selectively recognize cancer cells based on the expression of a TAA.

An example is trastuzumab, directed against the Human Epidermal Growth Receptor 2 (HER2), which is overexpressed in 25–30% of females with metastatic breast cancer.
Blocking this receptor by antibody retards malignant cell proliferation and as part of a multi- modality treatment approach has been shown to delay disease progression. However, it appeared clear that the action of trastuzumab was more complex than receptor blockade is from the observation that antibody dependent cell cytotoxicity -

ADCC - is more likely to occur in malignant breast cells overexpressing HER2 than in cells that do not overexpress HER2. [21]

A mAbs approved by FDA for the treatment of head and neck cancer and colorectal carcinoma is cetuximab, which is specific for the epidermal growth factor receptor (EGFR); by blocking EGFR, it inhibits a signaling pathway required for the survival or progression of neoplastic cells, but not of their non-malignant counterparts.

There are also immune conjugates, i.e., TAA-specific mAbs coupled to toxins or radionuclides, such as gemtuzumab ozogamicin; an anti-CD33 calicheamicin conjugate currently approved for use in acute myeloid leukemia patients and TAA-specific mAbs that opsonize cancer cells and hence activate antibody- dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity, such as the CD20-specific mAb rituximab, which is currently approved for the treatment of chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma. A new generation of mAbs consists in the so-called "bispecific T-cell engagers" (BiTEs), *i.e.*, chimeric proteins consisting of two single-chain variable fragments from distinct mAbs, one targeting a TAA and one specific for a T-cell surface antigen (*e.g.*, blinatumomab, a CD19- and CD3 BiTE recently approved for the therapy of Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukemia). [19]

1.2.2.3.2 Adoptive cellular immunotherapy (ACT)

Adoptive T-cell immunotherapy refers to the isolation, modification and re-infusion of autologous or allogenic T lymphocytes for therapeutic purposes. Historically, the first clinically effective adoptive transfer approach has been represented by allogenic hematopoietic stem cell transplant, whose anti-leukemic activity is mediated by a donor T cells. [22] ACT involves the *in vitro* generation of large numbers of autologous or allogenic lymphocytes with antitumor activity, which are then infused into cancer patients after appropriate immune preparation and along with growth factors to support the survival of the transferred cells.

Different types of autologous or allogeneic cells were used over time, including:

- Lymphokine activated Killer cells (LAK)
- Cytokine induced Killer Cells (CIK)

- Genetically modified T cells: TCR engineered T cells
- Chimeric Antigen Receptor T cells (CAR-Ts)
- Cytotoxic T lymphocytes (CTLs)
- Tumor infiltrating Lymphocytes (TILs)

1.2.2.3.2.1 Lymphokine activated killer cells -LAK

LAK cells are nonspecific effector cells derived from peripheral blood mononuclear cells (PBMC) and activated *ex vivo* with high-concentration interleukin 2 (IL-2).The LAK cell's cytolytic properties against numerous tumor types have been demonstrated in various models. Furthermore, it was suggested that the use of IL-2/LAK cell immunotherapy may possess preventative properties against metastasis and recurrence of disease because intraventricular administration can induce a systemic response. Yet, given the high toxicity of intravenous IL-2, local administration of this cytokine has been adopted for an increased therapeutic response and decreased morbidity. LAK cells are unable to migrate to tumor sites, necessitating local therapeutic administration at the surgical resection cavity .[23,24] However, the LAK immunotherapy was abandoned as clinical trials showed how the use of IL-2, which was necessary both for the cells' activation *in vitro* and which was also administrated for keeping LAKs *in vivo*, caused an high level of severe toxicities to the patients, without a sufficient and efficient clinical response. [25]

1.2.2.3.2.2 Cytokine induced killer cells - CIKs

Cytokine induced killer cells (CIKs) are a heterogeneous population of immune effector cells with a T/NK phenotype (CD3+/CD56+), which can be obtained from PBMC, umbilical cord blood – UCB – and bone marrow and are generated *in vitro* through the exposure of lymphocytes to an anti-CD3 Ab and cytokines such as IL-2 and IFN-γ.[26]
Various clinical trials are currently underway which combine CIKs with chemotherapy, monoclonal antibodies, DCs, other cytokines and immune checkpoint inhibitors.
In the National Health Institute databases there are several clinical trials registered using CIK cells against several kind of tumors, both solid and hematological. A retrospective study included 252 patients with metastatic colorectal cancer who received first-line

chemotherapy or first-line chemotherapy plus CIK cells. Both the median OS and PFS resulted significantly higher in patients treated with CIK immunotherapy as compared with the chemotherapy alone group. [22]

Anti-tumor strategies of CIKs include two signaling pathways: the first starts with CIKs receptors binding to its ligands and the second determines tumor cells apoptosis through the Fas signaling pathway. CIKs directly kill tumors cells then and they also release a large number of cytokines – IFN-gamma and TNF-alfa - , determining the host's immune response, both innate and adaptive. This feature is crucial, since CIKs may be used to transport cytokines directly in the TME.

1.2.2.3.2.3 Genetically modified T cells: TCR-engineered cells

TCR-engineeered cells are genetically modified T cells to express TCRs targeting with high affinity and avidity several members (i.e., NY-ESO-1, MAGE-A3, CEA, MART-1). The antigen specificity of peripheral blood T cells can be redirected to antigens expressed in tumor cells by genetic modification. The discovery of TSAs – i.e. cancer-testis antigens and tissue-differentiation antigens - prompted the engineering of T cells to express TCRs specifically targeting the chosen molecule. Early attempts with TCR-engineered T cells directed against tissue-differentiation antigens resulted in frequent "on-target, off- tumor" toxicities limiting the clinical applicability of these products, however investigations of T cells redirected against cancer testis antigens succeeded in achieving clinical efficacy in the absence of serious toxicities.[22] The first report of ACT regard the use of MART-1 TCR gene-engineered T cells for the treatment of metastatic melanoma, TCR genes were inserted into a gammaretroviral vector, and the transduced lymphocytes displayed a high level of antitumor activity in vitro[27] . Treatment with after a preconditioning immunodepleting chemotherapy along with IL-2 administration resulted in sustained tumor regression was observed 13% of patients. 3 patients affected by mCRC refractory to standard treatment were treated with T lymphocytes engineered to express a murine TCR against human CEA. One of 3 patients demonstrated an objective response. Although serum CEA levels dropped by 74% to 99% after ACT in all 3 patients, these decreases were transient. All 3 patients experienced severe colitis approximately 1 week post cell transfer that recovered by 2 to 3 weeks post cell transfer. This emphasized the need to identify and choose antigens most restricted to tumor targets, given the potency of the gene-engineered cells and the risks of autoimmune complications

when even low levels of antigens are expressed on normal tissues. The cancer-testis antigen family members appear to represent ideal tumor antigen candidates because they are expressed by a wide range of solid malignancies, found only in germ cell tissues but not in other normal tissues. NY-ESO-1, discovered in 1997, is a cancer-testis antigen known to elicit spontaneous antibody and T-cell responses in cancer patients. As recently reported, the adoptive transfer of NY-ESO-1 TCR-engineered T- cell mediated objective cancer regressions in patients with metastatic synovial sarcoma and with metastatic melanoma, without organ-specific toxicities. Other cancer-testis antigens, such as the MAGE family, presented by HLA-A2 and other class-I MHC subclasses, are also being targeted to increase the pool of eligible patients with diverse tumor types and to optimize the efficacy of this approach [28]. However, activation of TCR- engineered T cells requires the presentation of antigens through specific MHC molecules, thus substantially narrowing the current breadth of clinical utility of these products. [22].

1.2.2.3.2.4 Chimeric Antigen Receptor T cells - CAR-T

CAR-T cells are genetically engineered T cells able to recognize specific tumor antigens irrespective of their presentation by the MHC complex; they possess an extracellular antigen recognition domain, a hinge, spacer and transmembrane domain and an intracellular activating domain. The extracellular domain imparts target specificity and is conventionally constituted by the single-chain variable fragment (scFv) derived from a tumor-reactive antibody (Ab). The hinge domain affects the flexibility of the scFv thereby controlling the function of the CAR, and is commonly constituted by immunoglobulin-, CD28 or CD8-derived peptides. The transmembrane domain is constituted by a hydrophobic alpha helix that anchors the CAR in the T cell membrane and transfers the activation signals from the extracellular domain into the T cell. The intracellular domain drives the activation of the T cells upon CAR engagement.

CAR is a fusion proteins that links variable portions of the heavy and light chains of an antibody to the intracellular signaling domains of a TCR. Introduced into a T cell, CAR enable the lymphocyte to recognize tridimensional proteins found at the surface of tumor cells without MHC restriction, rather than a short peptide nested in MHC molecules recognized by conventional TCR. By combining the antigen specificity of an antibody and the cytotoxic properties of a T cell in an HLA-unrestricted manner, CAR can be

resistant to tumor-immune evasion mechanisms, such as downregulation of MHC molecules and failure to process antigens to the cell surface. [29]

In order to obtain ever more refined CAR-T cells, researchers had to go through the evolution over time of five generations of CAR-T cells, through the exploration and continuous improvement of the effects of intracellular signaling domains.

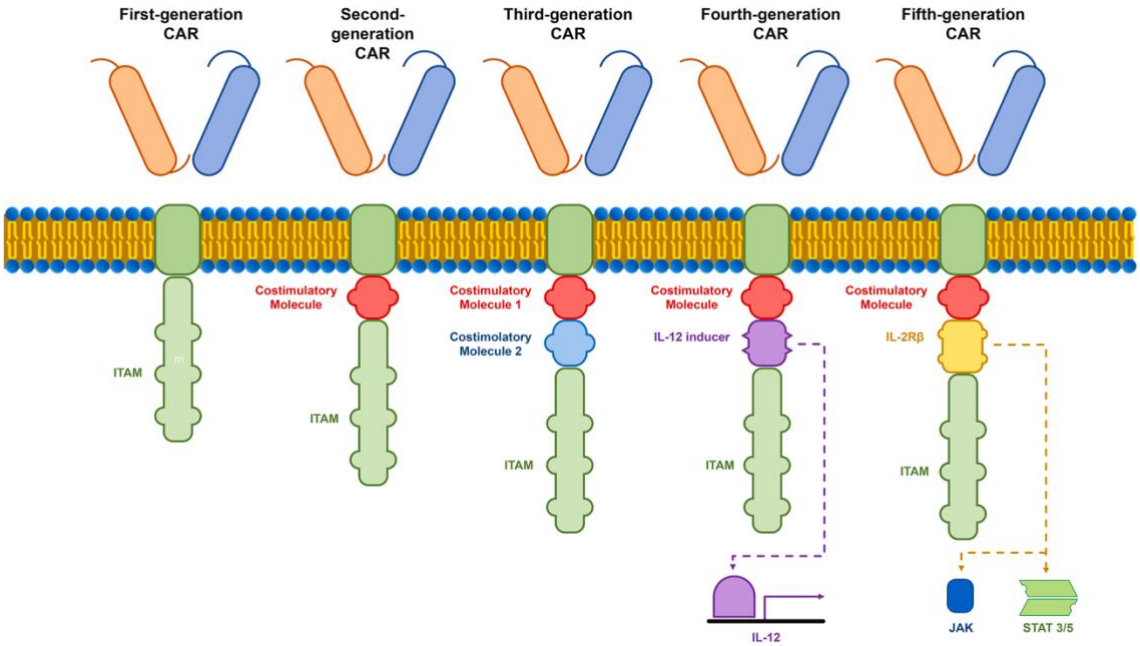

Figure 4: the five generations of CARs [22]

First-generation CARs contain the CD3ζ chain of the TCR in their intracellular portion, however they demonstrated low levels of proliferation, persistence and cytotoxicity.

Second- and third-generation CAR attempted to improve persistence and function of transduced cells by incorporating one or more costimulatory intracellular signaling molecules, such as CD28, OX40, and 4-1BB – also known as CD137. The first successful treatment of a patient with a highly refractory lymphoma targeting the CD19 antigen using a CAR containing CD28 and CD3f intracellular signaling chains was reported in 2010. [30] In the last decade, a fourth and a fifth generation of CAR-Ts have been developed, showing good results versus B-cell malignancies. Fourth-generation CAR-T cells, also named T cell Redirected for Universal Cytokine Killing (TRUCK), have been recently generated and are characterized by the ability to produce cytokines such as IL-12 or IL-15 to hack the tumor microenvironment. Fifth-generation CAR-T cells contain an intracellular domain capable of triggering signals otherwise induced by cytokines, thus potentially enhancing T cell persistence and antitumor activity .

CAR-T therapy has mostly been used in the treatment of hematologic malignancies, such as relapsed/refractory B-cell acute lymphoblastic leukemia (ALL), B-cell Hodgkin's lymphoma, and chronic lymphocytic leukemia (CLL). Currently the most studied antigen and which is leading to the greatest results of CAR-T cell therapy is the B-cell specific CD19, expressed on over 90% of B lymphocyte malignant tumors and on B lymphocytes at all stages of differentiation excluding plasma cells. The first successful clinical application of anti-CD19 CAR-T gene therapy in humans was reported in 2010, when autologous cells expressing anti-CD19 CAR were administered to a patient with refractory lymphoma resulting in regression. Later on, other groups demonstrated the efficacy of this therapy in patients with follicular lymphoma, large cell lymphomas, CLL and B-ALL. Despite the good results obtained with CAR-T infusion, this therapy is not free from the incidence of serious side effects such as the development of cytokine release syndrome (CRS) with the risk of multiple organ failure. [31] Furthermore, beyond CD19, there are few antigens present on the surface of cells and not expressed even on essential normal tissues. This severely limits the application of this therapy to a larger number of both solid and hematological malignancies.

In fact, despite extensive research, CAR-T cell therapy for solid tumors has not been as nearly as successful as in hematologic malignancies. Many factors involved in CAR-T resistance by solid tumor still need to be understood, however a possible explanation may be the low specificity of TAAs, with normal cells expressing low levels of such protein and leading to the risk of on-target off-tumor toxicities.

In addition, the expression of TAAs is heterogenous within solid tumors, therefore antigen escape mechanism and the tumor's ability of immunoediting may affect the efficacy of CAR T. While many factors are still poorly understood, several barriers appear to substantially limit the efficacy of CAR-T cells against solid tumors. First, the specificity of tumor associated antigens (i.e., CEA, HER2, EGFR among others) is generally low, with normal cells expressing low levels. While in hematological malignancies CAR-T cells encounter their targets in the bloodstream and/or lymphatic system, the homing of engineered lymphocytes towards solid tumor sites may pose some challenges, thus potentially resulting in a suboptimal infiltration of the tumor. Lastly, solid tumors are characterized by a hypoxic, acidic, low in nutrients, immunosuppressive microenvironment that can affect both survival, proliferation and differentiation of CAR-T cells. [32]

1.2.2.3.2.5 Cytotoxic T lymphocytes - CTLs

CD8+ T cells are a subset of lymphocytes developing in the thymus and are committed to detecting antigenic peptides presented by MHC class I molecules expressed by all tumor cell types. CTLs play a critical role in host defense against intracellular pathogens and in tumor surveillance, as they are capable of reacting to pathogens by extensive expansion and differentiation into cytotoxic effector cells that migrate to all corners of the body. Most studies in cancer immunotherapy have therefore focused on $CD8^+$ T lymphocytes. The major advantage of using $CD8^+$ T lymphocytes for adoptive cancer therapy is their ability to specifically target tumor cells through the recognition of differentially expressed tumor antigens on their surface. There are two mechanisms of CTL-mediated killing: calcium-dependent killing by secretion of perforin and granzymes, and calcium-independent killing mediated by Fas ligand (FasL) binding to Fas (CD95) on target cells. Both lead to the triggering of cellular apoptosis, but the former has the advantage of being a faster route. CTLs also release interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) to induce cytotoxicity in the cancer cells. IFN-γ produced by both CD8+ T cells and CD4+ T cells stimulates M1 macrophages to exert antitumoral effects.

The adoptive transfer of *ex vivo* generated antigen-specific CTLs represents a very promising approach in cell immunotherapy and has been evaluated in the treatment of some infectious diseases and cancers. The first major obstacle encountered when thinking about this type of approach is the generation of sufficient numbers of functional antigen-specific T cells.

Emerging results from both mouse models and clinical trials indicate that intrinsic properties related to the differentiation status of adoptively transferred T-cell populations are crucial for ensuring the success of ACT-based approaches. $CD8^+$ T cells have been shown to trigger tumor rejection in both mice and humans and can be distinguished into memory subpopulations classified according to their states of differentiation. $CD8^+$ T cells follow a progressive pathway of differentiation from naïve T cells to effector memory T cells (T_{EM}) to T Central Memory (T_{CM}) T cell populations. The extent of differentiation is determined by the strength of the TCR signal and also by the cytokine environment that the T cell encounters during antigen-specific activation.

In the field of hematological diseases, a number of studies have demonstrated the possibility of expanding CTLs directed against minor histocompatibility antigens. The

latter are expressed on hematopoietic cells, including leukemia cells, and represent a suitable target to enable the antitumor activity of T cells. Non-polymorphic differentiation antigens, such as proteinase 3-derived peptides, have also been investigated, which are over-expressed on myeloid cells and peptides derived from transcripts of fusion genes specific to leukemic cells (BCR/ABL or PML) as potential targets for T cell lines. [33–37]
Infusion of CTLs has been shown to be of proven benefit in the treatment of some virus-related malignancies, such as Epstein-Barr virus (EBV)-related post-transplant lymphoproliferative disease.
As far as solid tumors are concerned, it has been demonstrated that CD8+ T cells, derived from peripheral blood or from tumor tissues of patients with various tumors (melanoma and lung carcinoma), showed a specific antitumor activity directed towards known TAAs. However, the evidence that such cell therapies can achieve meaningful control of common solid tumors is more limited .
In the laboratory of Immunology of the Pediatric Clinic of the IRCCS Policlinico San Matteo/University of Pavia, researchers have developed and optimized over the years an approach to efficiently induce and expand *in vitro* autologous or allogeneic anti-tumor CTL able to *in vitro* lyse both leukemia blasts and solid tumor cells for adoptive immunotherapy approaches.[38–42]
After translation in the Cell factory of the IRCCS Policlinico San Matteo, in compliance with Good manufacturing practice guidelines (GMP), anti-leukemia CTL derived from hematopoietic stem cell transplantation donors are currently use in a Phase I/II trial for the prevention of leukemia recurrence in high-risk pediatric patients with acute leukemia.

1.2.2.3.2.6 Tumor infiltrating lymphocytes - TILs

In the 1980s, it was initially documented that tumor infiltrating lymphocytes (TILs) represent a concentrated source of cells able to *in vitro* recognize and kill tumor cells and studies on murine models showed that adoptive transfer of these tumor-infiltrating syngeneic lymphocytes IL-2-expanded TILs mediated regression of lung and liver tumors.
Rosenberg's group demonstrated that human TILs obtained from metastatic melanomas contained T cells capable of specific recognition of autologous tumors. TIL populations growing from tumors are mixtures of $CD8^+$ and $CD4^+$ with few or no major contaminating cells in mature cultures. However, responses were often short-lived and the transferred

cells did not persist long after administration. A dramatic improvement in the application of the TIL-based ACT in the treatment of metastatic melanoma occurred in the early 2000s, when Rosenberg's group demonstrated that causing immunosuppression in patients by a nonmyeloablative chemotherapy regimen - given immediately prior to TIL transfer increased overall responses, with persistent oligoclonal repopulation of the host with transferred lymphocytes.

Patients were treated with lymphodepleting chemotherapy consisting of high-dose cyclophosphamide and fludarabine, infusion of large numbers of TILs (approximately 1 x 10^{11} cells) followed by high-dose IL-2. This conditioning regimen is capable of causing brief but profound leukopenia resulting in both the creation of a physical space for the infused TILs and much less competition from the host lymphocytes for the homeostatic cytokines IL-7 and IL-15, thus giving the infused cells an advantage [43].

These results were strengthened by Rosenberg and colleagues in 2011 [44], as they demonstrated that condioning regimen based ontotal body irradiation (TBI) led to complete regression in 22% of patients enrolled. Impacts of the lymphodepletion are also likely due to transient elimination of regulatory T cells and enhancement of the activity of APC cells.

It was documented a significant association complete response and the infusion of TIL with longer telomeres, with more $CD8^+$ $CD27^+$cells. There is an inverse correlation of telomere length with time in culture [45], as though there was no maximum time in culture that precluded administration of cells. Generation of less-differentiated cells might also be accomplished by minimizing time in culture and use of alternative cytokines. The transferred cells expanded *in vivo* and persisted in the peripheral blood in many patients, sometimes achieving levels of 75% of all $CD8^+$ T cells at 6–12 months after infusion. Persistence of the transferred T-cell clonotypes correlated with cancer regression.

The use of TILs in clinical trials was complicated by the long lead times required to establish, test and expand TIL cultures (4-6 weeks). Tran K. and colleagues [45] proposed a strategy to improve the production time and obtaining a tumor-reactive TIL population: called “Young TILs”. Data demonstrated how minimally cultured TILs showed the same anti-tumor activity as standard TILs, and features associated with *in vivo* persistence, proliferation, and antitumor activity, such as a high proportion of CD4 cells, long telomere length and high expression of CD27 and CD28 – all features that standard TILs missed.[45]

Despite the success of TILs as ACT in melanoma patients, the same results were not obtained in other solid tumors, probably due to the different characteristics of immune population present solid neoplasia, or their low immunogenicity compared to melanoma. The high rate of cancer regression seen convincingly demonstrates the anti-tumor efficacy of ACT therapy and the importance of transferring cells with a high degree of antigen recognition and a high proliferative potential. Other issues of importance that might account for the lack of response in some patients relate to the traffic of TIL to draining lymph nodes or to poor expression of antigens by the tumor, making this strategy an highly personalized. [46].

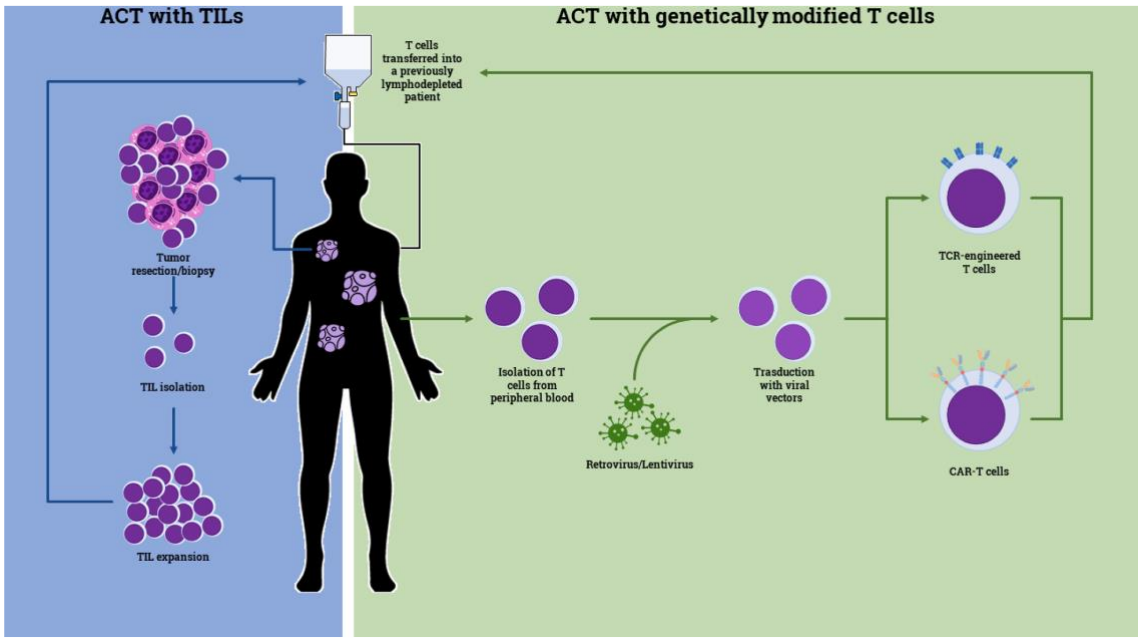

Figure 5: Different strategies of adoptive immunotherapy (Mandriani B. et all) [22]

1.3 ACT in colorectal cancer

It is known that death-related CRC represent 15% of all cancer-related deaths and OS in patients receiving chemotherapy and radiotherapy did not improve through the years. For this reason, several immunotherapies strategies have been evaluated over years in metastatic CRC patients..

CAR-Ts, despite the promising results showed in hematological malignancies, were a huge disappointment for CRC. Clinical trial with anti-HER2 CAR-T cells documented

the occurrence of respiratory distress and death as result of the low-density expression of HER2 in the lungs. [47]

A clinical trial using first generation CAR-T were tested on both systemic and regional administration, but no difference was noted: toxicity occurred and there was no disease improvement. [48] Two different second-generation CAR-T cells products have been investigated systemically or through percutaneous hepatic artery infusion. By systemic administration, escalating doses CAR-T cells induced stable disease in 70% patients with heavily-pretreated, advanced CRC, in the absence of severe toxicities. Disease stabilization persisted for more than 7 months in 2 patients. When subjected to intrahepatic delivery, CAR-T cells did not result in severe side effects.[49] At present, multiple antigen targets are being investigated in clinical trials of CAR-T cells recruiting CRC patients. [22]

Lymphocytes engineered to express a murine TCR against human CEA – carcinoembryonic antigen, a colon tumor marker - have been investigated in three patients with advanced, heavily pretreated CRC [50], however all treated patients experienced severe transient colitis occurring, with an objective response developed only by one of the three patients enrolled. many studies on TCR-based T cells in solid tumors have been launched. [51]

An ideal adoptive immunotherapy strategy would be identifying infiltrating T cells harboring the ability of targeting specific tumor marker due to the presence of a highly specific genetic mutation, saving normal tissues. This would be the beginning of a highly personalized treatment against cancer.

TILs expanded *ex vivo* determined complete regression in melanoma and some clinical response in other solid tumors – pancreatic, renal, lung, ovarian – because these cells target somatic mutations expressed by the patients' tumor. Using next-generation sequencing and immunologic screening, Tran et al. [52] identified mutation specific CD4+ cells with and observed regression of lung and liver metastases after infusion of a highly enriched population of these cells. The patient' tumor contained only 26 mutations, therefore indicating how the immune system can mount a clinically relevant T cells response. However, failure to do so shows how mutation-reactive T cells may be rare or absent in tumors of the gastrointestinal tract. Therefore, an identification of the specific mutation harbored by the tumor and the selection of the correct T cell population able to specifically identify it may bring a solution. Mutation of the KRAS oncogene are frequent in most human cancers and the vast majority are frequent and repetitive on codon 12, 13

or 61, the first being the most frequent site of mutation. In particular, on codon 12, a glycine is substituted by aspartic acid. This mutation is showing in 13% of CRC. Tran et al. [53] reported in 2016 the complete regression of lung metastases in a woman having CRC after administration of a TIL population specifically targeting KRAS G12D. TILs were obtained after tumor resection using and the population which showed the highest frequency of $CD8^+$ T cells reactive to KRAS G12D was selected, expanded and adoptively transferred. The patient showed regression of lung metastases 40 days after TIL infusion and she had a partial response for 9 months until only one lesion showed progression. The population selected for adaptive response showed mainly a Central Memory Phenotype ($CD45RO^+CD162L^+$) and expressed CD28 and CD57 as well.
This provides evidence of the presence of T cells in the tumor microenvironment and how they are de-activated or in low number to perform an anti-tumor immune response. Zhao *et al* [54], demonstrated the presence of naturally occurring $CD8^+$ TIL able to specifically recognize autologous metastatic gastric, bile duct, and colon adenocarcinomas by generating new cancer cell lines and TIL cultures. Three main observations can be made from their findings:

1. Tumor-reactive $CD8^+$ TIL were found at low frequency.
2. Metastatic GI cancer recognition by $CD8^+$ TIL was seen only in the autologous setting, in absence of shared recognition across allogeneic cancer cell lines.
3. Cancer cell lines generated from given patients were not equally recognized by T cells, and in other patients were deficient in MHC-I expression.

This study defines at the clonal and molecular level the existence of naturally occurring cytotoxic $CD8^+$ TIL specifically reactive to autologous metastatic GI cancers highlighted an additional difference with melanoma, which is the absence of shared recognition across allogeneic tumors. Further studies should also aim at clarifying whether tumor reactive $CD8^+$ TIL represents dominant clonotypes in freshly resected tumors, and if current in vitro TIL expansion protocols lead to overgrowth of non-tumor reactive bystander rather than tumor-reactive T cells.

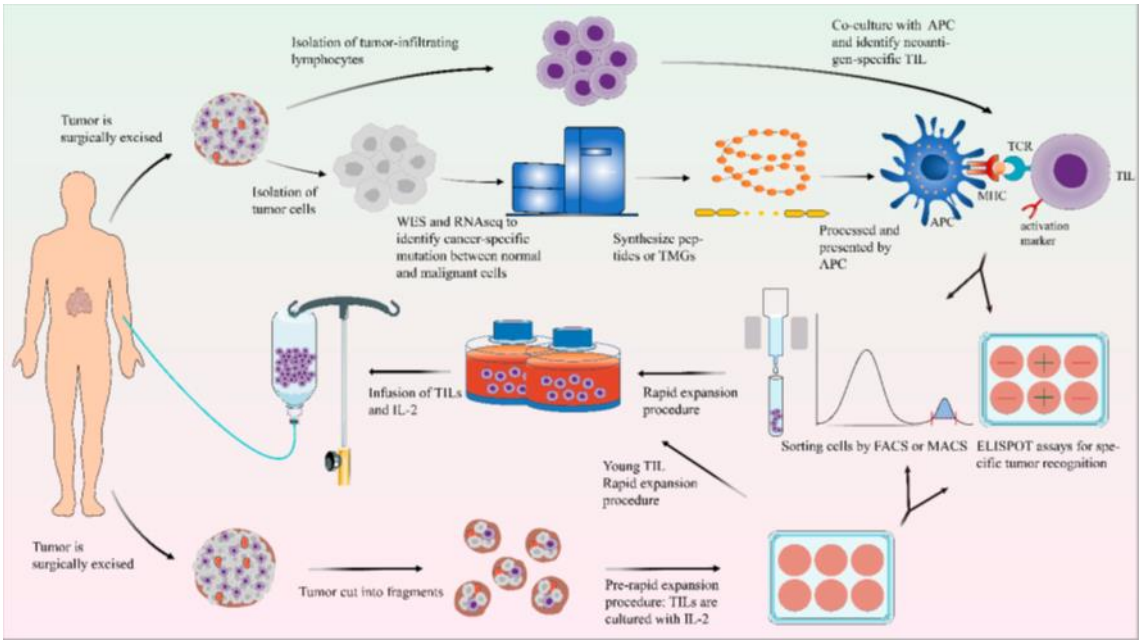

Figure 5: a summary of TIL preparation (Zhao et al.)[55]

1.4 Background

CRC the second cause of death in developed countries, after lung cancer, is potentially curable with surgical resection of the primary tumor. The clinical problem of CRC, however, is the spread and outgrowth of metastases. Over the last decade, the development of new combinations of chemotherapeutic agents along with the introduction of targeted therapies improved survival of a cohort of mCRC patients. Moreover, treatment of advanced disease is still associated with a poor prognosis and significant morbidity. Further intensification of chemotherapy would increase the incidence of treatment-related toxicity and non-relapse-related mortality. The failure of conventional treatment to significantly improved outcomes in mCRC has prompted the search for alternative molecular targets with the goal of improve the prognosis of these patients. There are, currently, multiple systemic treatments available for advanced/metastatic CRC cancers but none of them is curative. In recent years, immune checkpoint inhibitor (ICI) therapy has been shown to be active in several gastrointestinal malignancies, including gastroesophageal adenocarcinoma and hepatocellular carcinoma. However most of GI cancers have been shown to be resistant to checkpoint inhibitors, especially CRC, where ICI treatment is approved only in the case of mCRC harboring microsatellite instability (MSI-H). The efficacy of anticancer therapy could be increased by exploiting the ability of the immune system to selectively recognize and kill neoplastic

cells, thus limiting treatment-related toxicities. Cancer immunotherapy allows the selective elimination of tumor cells without increasing the toxicity linked to the therapies. Optimal T-cell based immunotherapy requires targeting an antigen that is both highly expressed and restricted to the tumor, but most tumor antigens do not meet these criteria; TILs are the simplest starting point of ACT as a personalized approach, as they're isolated from tumor specimens and harnessed to be used as a direct treatment. TILs expanded *in vivo* from a surgically resected tumor, and re-infused back into the patient have given excellent results in the treatment of metastatic melanoma. For this reason, TILs' development has been tested for other solid tumors, but an established expansion protocol has not been found yet. To obtain clinical responses by TILs transfer, it is necessary to optimize TILs expansion strategies and better analyze the molecular and functional characteristics of both TILs and TC. Several obstacles need to be overcome, however, such as the low immune infiltrate present in some solid tumors, the presence of an exhausted TIL population or a low immunogenic neoplasia, determining few TAA that can be recognized by T cells. Therefore, a protocol that allows to select and expand TILs, while preserving their cytotoxic and homing capacity is needed. In CRC patients it was observed that the ability of $CD8^+$ TILs to recognize tumors cells can be variable and limited to 10% of TILs, therefore it is necessary all the possible information about these cells and their interaction with tumor cells. Recently, new reagents and mediums are available for isolation and expansion of TIL, making this approach feasible even in solid tumors other than melanoma.

It is known that intracellular $Ca2^+$ signaling may control the susceptibility of tumor cells to lysis and their ability to grow and disseminate. Then, addressing this issue in CRC cells is of compelling urgency to validate SOCE as potential target for anticancer therapies in mCRC patients. SOCE appears to be a Ca2+ entry pathway sustaining cytotoxic lymphocyte function. SOCE is activated following depletion of the endoplasmic reticulum (ER) $Ca2^+$ reservoir and is mediated by the interaction between stromal interaction molecules (STIM1 and STIM2), which sense ER $Ca2^+$ levels, and Orai1, the $Ca2^+$ entry channel on the plasma membrane. While the role played by SOCE in the immune system under healthy conditions is clear, its contribution to antitumor immunity is unknown. Studies revealed that STIM1 and STIM2 control the degranulation of CD8+ cells, their expression of Fas ligand and production of TNF-α and IFN-γ in a murine model of melanoma[56]. Surprisingly, the cytotoxic efficiency of $CD8^+$ cells is increased by a reduction in Orai1 levels and SOCE.[57] It has, therefore, been suggested that partial

SOCE inhibition could represent a suitable strategy to increase antitumor activity and reduce cancer growth and metastasis. Unfortunately, these studies were not carried out in patients-derived $CD8^+$ cells, which might present a rather different behavior as compared to healthy or mice-derived cells. Investigating the signal transduction pathways, e.g. SOCE, that control TIL-mediated cytotoxic activity in human patients, could unveil additional strategies to improve TIL-therapy

1.5 Scope of the thesis

Cell therapy with adoptive transfer of T lymphocytes derived from the tumor site and capable of killing neoplastic cells after expansion in vitro, represents one of the most promising approaches to overcome tumor escape mechanisms and to exploit the immune system by limiting toxicities related to treatment. To date, little data is available on the feasibility of this therapy in cancers other than melanoma. In particular, in the case of CRC these studies are limited by the difficulty of expanding *in vitro* large quantities of TILs capable of recognizing and lysing tumor cells.

Our study focused on the design of a new protocol for TILs expansion that could allow the generation of a sufficient number of activated TILs to be re-infused into patients after careful characterization. This protocol also permits to isolate and expand autologous tumor cells (TC) as well, which could be used both as specific target for the evaluation of TILs potency and for studying a possible anti-cancer therapy whose target would $Ca2^+$ releasing pathways. Therefore, we had two main goals: one concerning the expansion and characterization of TILs obtained from surgery resection of primary or metastatic CRC and the other involving the study of $Ca2^+$ releasing pathways and their role in influencing TILs' cytotoxic activity and tumor cells growth. Based on the data available the aims of this research thesis were:

1. **Optimizing strategies for in vitro expansion of TIL.** TIL derived from tumor samples were activated and expanded using different cytokines combination. The first methodology, as described by Rosenberg and coworkers for rapid expansion of melanoma-derived TIL, include the use of high doses of IL-2 and IL-15 to sustain cultures (**Protocol A**). Since important prerequisites for successful anti-tumor ACT include the possibility to transfer a high numbers of tumor-reactive

cells, TIL obtained after one round of rapid expansion underwent a further round of rapid expansion using the same approach.

Recently it has been described that rapid expansion of TIL with a combination of cytokine (IL-15, IL-7 and IL-21), may favor the expansion of T cells endowed with high effector capacity. Based on this data we evaluated other strategies for TIL expansion utilizing this cocktail of cytokines in the presence of high-dose IL-2 **(Protocol B)** or without IL-2 **(Protocol C)**. See figure 6 (experimental design). A second round of expansion miming protocol A is currently under evaluation for TILs expanded with protocol B and protocol C methodologies.

Immunological characterization of TIL. TIL obtained with different experimental approaches were evaluated in terms of :

- expansion rate
- potency against autologous TC and other commercial long-term tumor cell lines usually resistant to T cell mediated lysis
- surface antigens, for the definition of memory T-cell compartment, activated/exhausted phenotype, presence of molecules involved in homing to tumor site.

2. **Evaluation of role of Ca2+ signaling pathways in TILs.** Due to the pivotal role played by SOCE in T cell functional activity and by Ca2+ signaling pathways in tumor cell growth and metastasis, we aim at assessing:
   - whether SOCE and Ca2+-dependent antitumor activity are upregulated in human CD8 TILs, thereby reducing their antitumor activity.
   - whether the submaximal inhibition of SOCE with specific drugs influences CD8 T cell cytotoxic activity.
   - whether intracellular Ca2+ entry affects growth, migration and invasion in CRC.

1.6 Experimental design

After signing an informed consent, 10 patients (> 18 years) affected by primary or metastatic CRC tumors who have undergone surgical intervention to remove or to biopsy the tumor were enrolled in this study. All biopsies were performed according to the guidelines prescribed for the treatment of these cancers, and no patient was subjected to unnecessary invasive procedures.

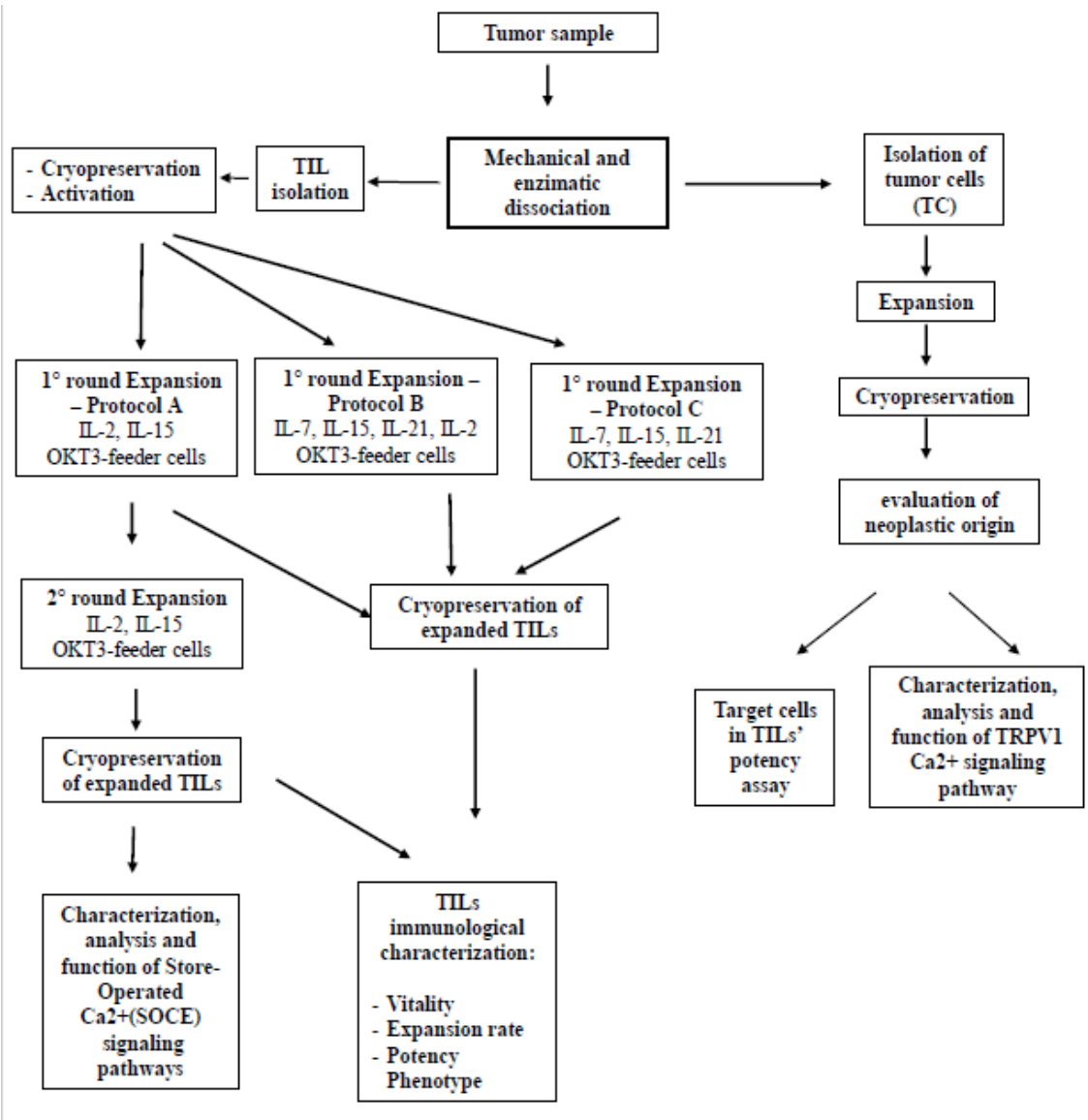

Figure 6: summary of the experimental design

From tumor specimens, lymphoid cells were separated from neoplastic cells by means of specific magnetic CD45 microbeads (typical of leukocytes). After separation, the two

distinct population (T cells and TC) were cultured in the presence of specific reagents and medium in order to expand them. TC, which growth as adherent cells, were expanded and several vials were cryopreserved after 3-5 *in vitro* passages, to be thawed and further expanded for further experiments. With this approach, it is possible to have in each experiment cells that are obtained after no more than 4-5 passages in culture. After in vitro expansion TC were evaluated in order to confirm their neoplastic origin.
The best approach for *in vitro* isolation and expansion of TILs from CRC tumor samples was optimized using different methodologies.
In particular, we have evaluated three protocols:

- **Protocol A**- TIL were rapidly expanded in antigen independent way, with IL-2 and IL-15 in the presence of OKT3 and irradiated allogeneic feeder cells. In addition, since the dose of TIL needed for ACT is crucial to obtain the best anti-tumor effect, we compared the feature of TIL after one or two rounds of antigen-independent expansion.
- **Protocol B**- TIL were rapidly expanded with a cocktail of cytokines including IL-7, IL-15, IL-21, and IL-2, in the presence of OKT3 and irradiated allogeneic feeder cells.
- **Protocol C**- TIL were rapidly expanded with a cocktail of cytokines including IL-7, IL-15, IL-21, in the presence of OKT3 and irradiated allogeneic feeder cells.

TIL expanded with different protocol and cryopreserved in several vials, were then analyzed for immunological features: vitality, expansion rate, potency and phenotype were evaluated. In particular, potency was evaluated by cytotoxicity assay using as target cell SW480 cell line and autologous TC. Immunophenotype analysis evaluated by cytofluorimetry assay using BD FACS Canto II, includes the analysis of surface antigens involved in cell proliferation, persistence and homing.
In TIL expanded with protocol A the characterization of SOCE was also evaluated.

1.7 References

1. de Palma, F. D. E. *et al.* The Molecular Hallmarks of the Serrated Pathway in Colorectal Cancer. *Cancers (Basel)* **11**, (2019).
2. Rusakiewicz, S. *et al.* Immune in filtrates are prognostic factors in localized gastrointestinal stromal tumors. *Cancer Res* **73**, 3499–3510 (2013).
3. Ternet, C. & Kiel, C. Signaling pathways in intestinal homeostasis and colorectal cancer: KRAS at centre stage. *Cell Commun Signal* **19**, 31 (2021).
4. Sawicki, T. *et al.* A Review of Colorectal Cancer in Terms of Epidemiology, Risk Factors, Development, Symptoms and Diagnosis. *Cancers (Basel)* **13**, (2021).
5. Vinay Kumar, Abul K. Abbas & Jon Aster. *Robbins Basic Pathology, 10th Edition*. (Elsevier, 2017).
6. Mlecnik, B. *et al.* Multicenter International Society for Immunotherapy of Cancer Study of the Consensus Immunoscore for the Prediction of Survival and Response to Chemotherapy in Stage III Colon Cancer. (2020) doi:10.1200/JCO.19.
7. Kunimasa, K. & Goto, T. Immunosurveillance and Immunoediting of Lung Cancer: Current Perspectives and Challenges. *Int J Mol Sci* **21**, (2020).
8. Cho, J. H. Immunotherapy for Non-small-cell Lung Cancer: Current Status and Future Obstacles. *Immune Netw* **17**, 378–391 (2017).
9. Riley, R. S., June, C. H., Langer, R. & Mitchell, M. J. Delivery technologies for cancer immunotherapy. *Nat Rev Drug Discov* **18**, 175–196 (2019).
10. Farhood, B., Najafi, M. & Mortezaee, K. CD8+ cytotoxic T lymphocytes in cancer immunotherapy: A review. *Journal of Cellular Physiology* vol. 234 8509–8521 Preprint at https://doi.org/10.1002/jcp.27782 (2019).
11. Anderson, N. M. & Simon, M. C. The tumor microenvironment. *Curr Biol* **30**, R921–R925 (2020).
12. Cervantes-Villagrana, R. D., Albores-García, D., Cervantes-Villagrana, A. R. & García-Acevez, S. J. Tumor-induced neurogenesis and immune evasion as targets of innovative anti-cancer therapies. *Signal Transduct Target Ther* **5**, 99 (2020).
13. Gros, A. *et al.* PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire infiltrating human tumors. *Journal of Clinical Investigation* **124**, 2246–2259 (2014).

14. Fernandez-Poma, S. M. *et al.* Expansion of tumor-infiltrating CD8+ T cells expressing PD-1 improves the efficacy of adoptive T-cell therapy. *Cancer Res* **77**, 3672–3684 (2017).
15. Rosenberg, S. A., Spiess, P. & Lafreniere, R. A New Approach to the Adoptive Immunotherapy of Cancer with Tumor-Infiltrating Lymphocytes. *Science (1979)* **233**, 1318–1321 (1986).
16. Lotze, M. T., Robinson, B. W. S., June, C. H. & Whiteside, T. L. 77 - Tumor immunotherapy. in *Clinical Immunology (Fourth Edition)* (eds. Rich, R. R. et al.) 935–945 (Elsevier, 2013). doi:https://doi.org/10.1016/B978-0-7234-3691-1.00091-X.
17. Morse, M. A., Gwin, W. R. & Mitchell, D. A. Vaccine Therapies for Cancer: Then and Now. *Target Oncol* **16**, 121–152 (2021).
18. Klebanoff, C. A., Acquavella, N., Yu, Z. & Restifo, N. P. Therapeutic cancer vaccines: are we there yet? *Immunol Rev* **239**, 27–44 (2011).
19. Galluzzi, L. *et al. Aled Clayton 23,24 , Mario P. Colombo 19 , Lisa Coussens 25 , Madhav V. Dhodapkar 26 , Alexander M. Eggermont 3 , Douglas T. Fearon 27 , Wolf H. Fridman 2,4,28,29 , Jitka Fučíková 6,30 , Dmitry I. Gabrilovich 31*. *François Ghiringhelli* vol. 5 www.impactjournals.com/oncotarget/.
20. Fu, C., Zhou, L., Mi, Q.-S. & Jiang, A. DC-Based Vaccines for Cancer Immunotherapy. *Vaccines (Basel)* **8**, 706 (2020).
21. Baxter, D. Active and passive immunization for cancer. *Human Vaccines and Immunotherapeutics* vol. 10 2123–2129 Preprint at https://doi.org/10.4161/hv.29604 (2014).
22. Mandriani, B. *et al.* Adoptive T-cell immunotherapy in digestive tract malignancies: Current challenges and future perspectives. *Cancer Treatment Reviews* vol. 100 Preprint at https://doi.org/10.1016/j.ctrv.2021.102288 (2021).
23. Nagasawa, D. T. *et al.* Passive Immunotherapeutic Strategies for the Treatment of Malignant Gliomas. *Neurosurgery Clinics of North America* vol. 23 481–495 Preprint at https://doi.org/10.1016/j.nec.2012.04.008 (2012).
24. Grimm, E. A. *Human lymphokine-activated killer cells (LAK cells) as a potential immunotherapeutic modality*. *Biochimica et Biophysica Acta* vol. 865 (1986).
25. Khayat, D., Weil, M., Soubrane, C. & Jacquillat, C. [LAK cells and immunotherapy of cancer]. *Bull Cancer* **75**, 3–7 (1988).

26. Introna, M. & Correnti, F. Innovative clinical perspectives for cik cells in cancer patients. *International Journal of Molecular Sciences* vol. 19 Preprint at https://doi.org/10.3390/ijms19020358 (2018).
27. Hughes, M. S. *et al.* Transfer of a TCR Gene Derived from a Patient with a Marked Antitumor Response Conveys Highly Active T-Cell Effector Functions. *Hum Gene Ther* **16**, 457–472 (2005).
28. Robbins, P. F. *et al.* Tumor Regression in Patients With Metastatic Synovial Cell Sarcoma and Melanoma Using Genetically Engineered Lymphocytes Reactive With NY-ESO-1. *Journal of Clinical Oncology* **29**, 917–924 (2011).
29. Turcotte, S. & Rosenberg, S. A. Immunotherapy for Metastatic Solid Cancers. *Advances in Surgery* vol. 45 341–360 Preprint at https://doi.org/10.1016/j.yasu.2011.04.003 (2011).
30. Kochenderfer, J. N., Yu, Z., Frasheri, D., Restifo, N. P. & Rosenberg, S. A. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. *Blood* **116**, 3875–3886 (2010).
31. Ma, S. *et al.* Current Progress in CAR-T Cell Therapy for Solid Tumors. *Int J Biol Sci* **15**, 2548–2560 (2019).
32. Martinez, M. & Moon, E. K. CAR T Cells for Solid Tumors: New Strategies for Finding, Infiltrating, and Surviving in the Tumor Microenvironment. *Front Immunol* **10**, (2019).
33. Marijt, W. A. E. *et al.* Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci U S A* **100**, 2742–7 (2003).
34. Clark, R. E. *et al.* Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. *Blood* **98**, 2887–93 (2001).
35. Goulmy, E. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. *Immunol Rev* **157**, 125–40 (1997).
36. Falkenburg, J. H. *et al.* Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. *Blood* **94**, 1201–8 (1999).

37. Gambacorti-Passerini, C. *et al.* Human CD4 lymphocytes specifically recognize a peptide representing the fusion region of the hybrid protein pml/RAR alpha present in acute promyelocytic leukemia cells. *Blood* **81**, 1369–75 (1993).
38. Montagna, D. *et al.* Ex vivo priming for long-term maintenance of antileukemia human cytotoxic T cells suggests a general procedure for adoptive immunotherapy. *Blood* **98**, 3359–66 (2001).
39. Montagna, D. *et al.* Generation and ex vivo expansion of cytotoxic T lymphocytes directed toward different types of leukemia or myelodysplastic cells using both HLA-matched and partially matched donors. *Exp Hematol* **31**, 1031–8 (2003).
40. Montagna, D. *et al.* Single-cell cloning of human, donor-derived antileukemia T-cell lines for in vitro separation of graft-versus-leukemia effect from graft-versus-host reaction. *Cancer Res* **66**, 7310–6 (2006).
41. Turin, I. *et al.* GMP production of anti-tumor cytotoxic T-cell lines for adoptive T-cell therapy in patients with solid neoplasia. *Cytotherapy* **9**, 499–507 (2007).
42. Montagna, D. *et al.* Feasibility and safety of adoptive immunotherapy with ex vivo-generated autologous, cytotoxic T lymphocytes in patients with solid tumor. *Cytotherapy* **14**, 80–90 (2012).
43. Haanen, J. B. A. G. Immunotherapy of melanoma. in *European Journal of Cancer, Supplement* vol. 11 97–105 (2013).
44. Rosenberg, S. A. *et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clinical Cancer Research* **17**, 4550–4557 (2011).
45. Tran, K. Q. *et al.* Minimally cultured tumor-infiltrating lymphocytes display optimal characteristics for adoptive cell therapy. *Journal of Immunotherapy* **31**, 742–751 (2008).
46. Dudley, M. E., Wunderlich, J. R., Shelton, T. E., Even, J. & Rosenberg, S. A. *Generation of Tumor-Infiltrating Lymphocyte Cultures for Use in Adoptive Transfer Therapy for Melanoma Patients NIH Public Access*. *J Immunother* vol. 26 (2003).
47. Morgan, R. A. *et al.* Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced With a Chimeric Antigen Receptor Recognizing ERBB2. *Molecular Therapy* **18**, 843–851 (2010).

48. Hege, K. M. *et al.* Safety, tumor trafficking and immunogenicity of chimeric antigen receptor (CAR)-T cells specific for TAG-72 in colorectal cancer. *J Immunother Cancer* **5**, 22 (2017).
49. Zhang, C. *et al.* Phase I Escalating-Dose Trial of CAR-T Therapy Targeting CEA+ Metastatic Colorectal Cancers. *Molecular Therapy* **25**, 1248–1258 (2017).
50. Parkhurst, M. R. *et al.* T Cells Targeting Carcinoembryonic Antigen Can Mediate Regression of Metastatic Colorectal Cancer but Induce Severe Transient Colitis. *Molecular Therapy* **19**, 620–626 (2011).
51. Füchsl, F. & Krackhardt, A. M. Paving the Way to Solid Tumors: Challenges and Strategies for Adoptively Transferred Transgenic T Cells in the Tumor Microenvironment. *Cancers* vol. 14 Preprint at https://doi.org/10.3390/cancers14174192 (2022).
52. Tran, E. *et al.* Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science (1979)* **350**, 1387–1390 (2015).
53. Tran, E. *et al.* T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. *New England Journal of Medicine* **375**, 2255–2262 (2016).
54. Turcotte, S. *et al.* Tumor-Reactive cd8+ tcells in metastatic gastrointestinal cancer refractory to chemotherapy. *Clinical Cancer Research* **20**, 331–343 (2014).
55. Zhao, Y. *et al.* Tumor Infiltrating Lymphocyte (TIL) Therapy for Solid Tumor Treatment: Progressions and Challenges. *Cancers* vol. 14 Preprint at https://doi.org/10.3390/cancers14174160 (2022).
56. Weidinger, C., Shaw, P. J. & Feske, S. <scp>STIM</scp> 1 and <scp>STIM</scp> 2-mediated Ca^{2+} influx regulates antitumour immunity by <scp>CD</scp> 8^{+} T cells. *EMBO Mol Med* **5**, 1311–1321 (2013).
57. Zhou, X. *et al.* A calcium optimum for cytotoxic T lymphocyte and natural killer cell cytotoxicity. *J Physiol* **596**, 2681–2698 (2018).

2. Ex vivo isolation and expansion of tumor infiltrating lymphocytes (TIL) derived from metastatic colorectal carcinoma m(CRC) patients for adoptive immunotherapy approaches.

2.1 Introduction

Adoptive cell therapy (ACT) has started to establish itself as a new pillar in the treatment of solid tumors refractory to conventional therapies. ACT is a highly personalized strategy that involves administration of immune cells with direct anticancer activity.

LAK cells, PBMC activated *ex-vivo* with high doses of IL-2, were the first attempt at ACT, however they had to be abandoned for the high levels of toxicity due to the administration of high doses of IL-2[1]. CIKs are a sub-population of immune cells with a a T/NK phenotype that have been tested for the treatment of some gastric tumors, particularly in Asian countries, and their clinical value is still under evaluation.[2] Engineered T cells – such as CAR-Ts – gave excellent results in the treatment of hematological malignancies, such as B cell lymphoma[3] but at present there are major obstacles to translate their use for the treatment of patients with solid tumors[4].

One important strategy for the treatment of solid tumors refractory to conventional therapies, is the *ex vivo* selection, expansion and re-infusion of TILs. The presence of an immune infiltrate in the TME, confirms the existence of a highly tumor-specific T cell population that may bring to the generation of a highly-personalized anti-tumor treatment for each patient.

TILs were a successful clinical strategy for metastatic melanoma after a non-myeloablative lymphodepletion or total body irradiation [5], bringing to the complete regression of disease in part of them. TIL therapy has been tested on other solid tumors as well – such as pancreatic cancer[6], non-small cell lung cancer[7], breast cancer [8] and colorectal carcinoma[9], giving some clinical results on solid tumors which are considered weakly immunogenic. Tran and colleagues[10] showed that the mutated antigens in a non-melanoma epithelial cancers could give rise to immune responses, despite the low number of mutations present. Thus, proving that tumors' low immunogenicity is not a determining feature for TILs as they are still able to specifically recognize tumor-antigens, however weakly expressed.

The success of ACT based on the infusion of *ex vivo* manipulated cells is influenced by several tumor-intrinsic factors including tumor mutational burden, neoantigen burden, HLA type and expression, DNA damage repair capacity, and programmed death ligand-1 expression [11], however T cell intrinsic factors must be taken in consideration as well for immunotherapy response. A subset of self-renewing stem-cell-like TILs has been

reported to exist in unique intra-tumoral structures and is characterized by the lack of cell surface inhibitory markers such as CD39 or TIM3. Analysis of tumor-reactive populations documented that anti-tumor neoantigen-specific TILs were enriched in subsets defined by the expression of PD-1[12,13] or CD39 [14]. However, Krishna et all. [15] reported $CD39^{neg}CD69^{neg}$ tumor-reactive TIL might result in superior tumor control and that $CD39^{+}$ TILs were likely terminated TILs with lack of proliferative potential and with no efficiency in mediating tumor control. Other studies in literature reported that subset of $CD8^{+}CD137^{+}$ TILs appeared to be tumor-reactive T cells with good proliferation quality. [16,17]

Therefore, there is a lack of consensus regarding the tumor-reactive TIL subset that is directly responsible for the success of ACT, since the phenotypic fitness landscape of tumor-reactive TILs associated with ACT response against human cancer is still unclear. Our study focused on the expansion of TILs deriving from mCRC. We point out a protocol for TILs' isolation consisting of a mechanical and enzymatical dissociation of freshly resected tumor to recover both TILs and autologous tumor cells (TCs). Different strategies for TILs' isolation and expansion after tumor dissociation were developed to evaluate the best approach in terms of cellular recovery and phenotypic and functional characteristic of expanded cells[18].

Phenotypical analysis of TILs include the evaluation of expression of immune-checkpoint molecules, such as PD-1,which might be a marker determining activated TILs, TIM-3, GITR and LAG-3; activation molecules, such as CD28 and CD69; the controversial CD39; CD137, also known as 41BB-1 , which some consider a marker of tumor-reactive TILs [7]; CCR7 to determine activated TILs' and analysis of co-expression of CCR7 and CD45-RA to highlight a memory-like phenotype or an exhausted phenotype.

2.2 Materials and methods

10 patients (age>18 years) affected by primary or metastatic CRC tumors who have undergone surgical intervention to remove the tumor were enrolled in this study (table 1) .All patients signed an informed consent. All biopsies were performed according to the guidelines for the treatment of these cancers, and no patient was subjected to unnecessary procedures.

2.2.1 Tumor dissociation

After tumor resection, the sample was delivered to the laboratory in a sterile container with physiological solution; the sample was moved in tissue storage solution (Miltenyi Biotech, , Bergisch Gladbach, Germany) and kept at 2°-8° C. To recover cell suspension, mechanical and enzymatical dissociations were performed.

The tumor bulk was then cut using a scalpel to generate small fragments of 1-2 that were added in a dissociation tube (Miltenyi Biotech) containing a solution made with RPMI (Euroclone, Milan, Italy) without animal- or human-deriving protein and a cocktail of enzymes from the Tumor Dissociation Kit (Miltenyi Biotech). The mechanical dissociation consisted in adding the tube to the GentleMACS Dissociator (Miltenyi Biotech) and having it spin around for 30 seconds at a determined speed selected by the manufacturer, according to the type of tumor. Dissociation tubes contain a spiral which help split the tumor fragments during the spin. Tubes were incubated for 30 minutes at 37 °C and 7% of CO_2 for enzymatical dissociation and then put under continuous rotation. A second round of mechanical dissociation was performed, followed by 30 minutes at 37 °C and 7% of CO_2. After the last round of mechanical dissociation, the solution containing the tumor suspension was filtered using 70 micrometers filters (Falcon, Corning Costar, NY, USA) to ensure only cellular material went through. Cells suspension was then centrifuged at 1200 rpm for 10 minutes, counted and tested for cell recovery and viability.

UPN	Gender	Type of Tumor	TCs' expansion	TIL protocol A 1st round	TIL protocol A 2nd round	TIL protocol B 1st round	TIL protocol C 1st round
BNZ008	M	Liver mCRC	Yes	Yes	Yes	Yes	Yes
CMP019	M	Liver mCRC	Yes	Yes	Yes	Yes	Yes
CTT007	F	Liver mCRC	Yes	Yes	Yes	n/a	n/a
FRV010	F	Liver mCRC	Yes	Yes	Yes	Yes	Yes
MRT025	M	Liver mCRC	ND *	Yes	ND *	Yes	Yes
MRN014	F	Liver mCRC	ND *	Yes	Yes	n/a	n/a
PSC031	F	Liver mCRC	Yes	Yes	ND *	Yes	Yes
VCC028	M	Liver mCRC	ND *	Yes	Yes	n/a	n/a
GRN017	F	Liver mCRC	Yes	Yes	Yes	n/a	n/a
DMR027	M	Liver mCRC	Yes	Yes	Yes	n/a	n/a

Table 1: Characteristics of patients included in the study. All tumor samples derived from liver metastases.
* ND= not done

As reported in table 1, a sufficient number of TCs were obtained in 7 out of 10 tumor samples. In three patients, despite TCs obtained after the first passages were enough for analysis of the tumor's morphology, after some passages in culture, they stopped growing and there wasn't a sufficient number of cells for cryopreservation. 1st round of expansion with protocol A was performed on TIL derived from 10 patients. 2nd round of expansion with protocol A was performed on 8/10 patients. Protocols B and C were performed on 5/10 patients .

2.2.2 Isolation of TIL and TC

The cell suspension obtained after dissociation contains at least two different type of cells: TILs and tumor cells (TCs). Considering that CD45 is a marker for leukocytes, a positive selection using magnetic microbeads anti-CD45 (Miltenyi Biotech) was performed to isolate TILs from TCs, which do not express CD45. After labelling, cell-suspension went

through separation columns (Miltenyi Biotech) to collect positive-CD45 selection (TILs) and negative-CD45 selection (TCs).

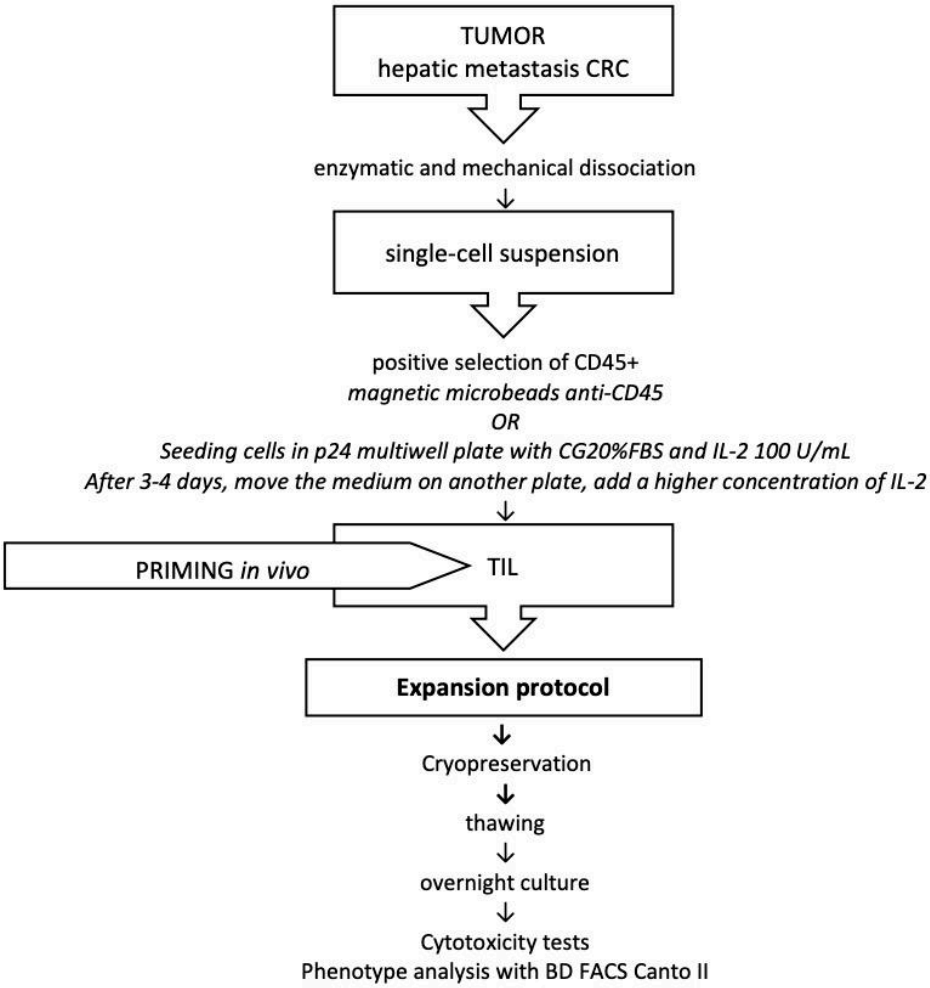

Figure 9: Steps for TILs' isolation and expansion

It is to underline that the cell recovery after dissociation cannot be standardized, as each tumor is patient-dependent and unique for tumor-infiltrate quality/quantity. The labelling of an insufficient number of cells for CD45+ positive selection could determine the loss of positive cells; therefore, we decided to develop a second protocol based on cells' features. It is known that TC grown adherent to the plate, while T cells grown in suspension. Based on these characteristics, the whole cell-suspension was seeded in a 24-multiwell plate (Corning Costar, NY, USA) containg CellGro (Cell Genix, New Hampshire, USA) with 20% FBS (Euroclone) and low doses of IL-2 (Novartis, Basilea, Switzerland). Cells were incubated at 37°C and 7% of CO_2 for 3-4 days.

After this period the whole cell suspension was collected, while adherent cells, containing TC were further supplemented with medium and maintained as described below.

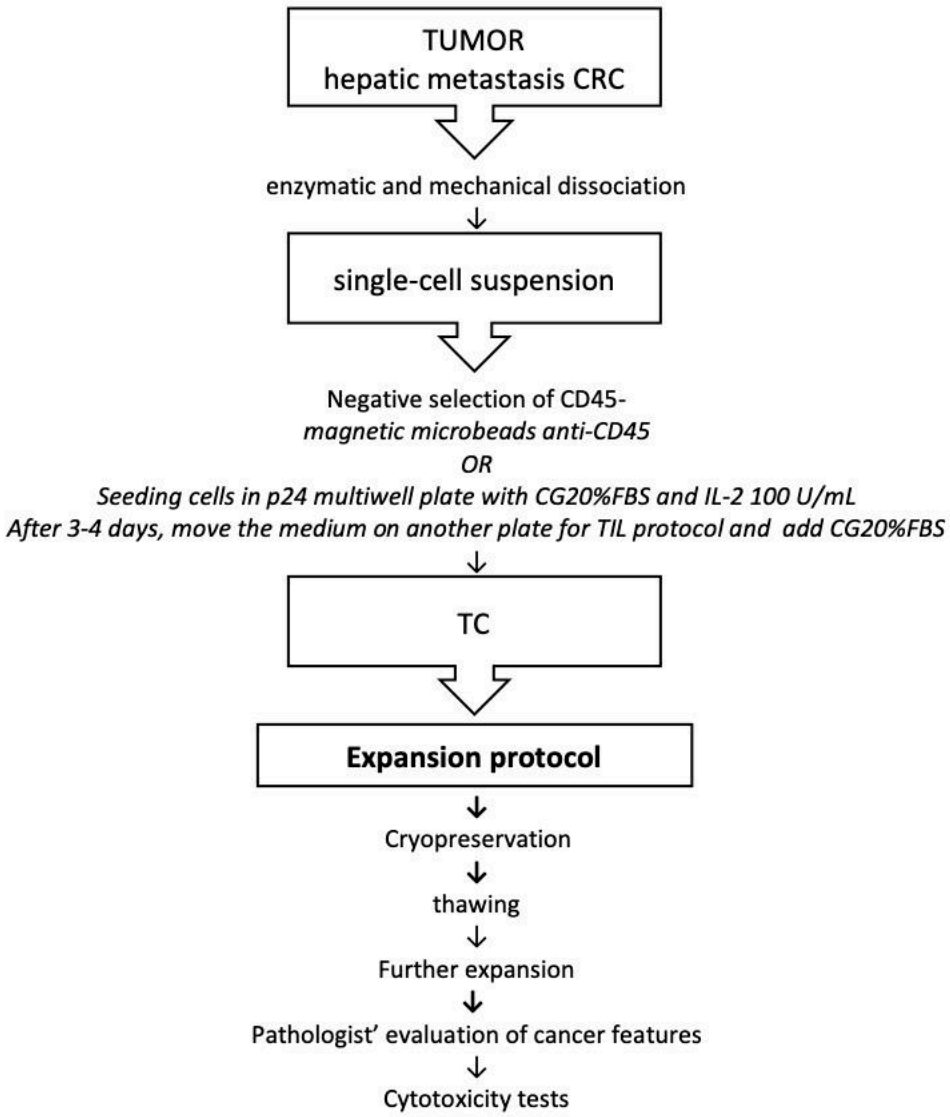

Figure 10: TCs isolation, expansion and functional analysis

Medium is CellGro 20% FBS (Euroclone) with 0,1% gentamycin (Gibco, Massachusetts, USA). Once cells reach confluence, they are splitted using trypsine (Euroclone), centrifuged and counted. The cell suspension obtained is moved to a 12 multiwell (Corning) plate for further expansion using the same tumor medium described above.

Depending on cell recovery, some aliquots are cryopreserved and a cytospin is performed to allow the pathologist to analyze tumor features. Briefly, 3-5x10^4 cells are centrifuged on a glass ideal for staining (cytospin), dried at room temperature (RT) and frozen at -20°C.

2.2.3 TILs' expansion protocols

2.2.3.1 Protocol A

After activation in culture, TILs are collected and counted, if enough, part of the cells were cryopreserved, while part of them is expanded.
The expansion protocol consists in culturing activated TILs with irradiated (30 Gy) feeder cells at a 1:50 ratio along with CG 10% FBS, 0,1% gentamycin, OKT3 28 ng/mL, IL-2 3000 U/mL and IL-15 2 ng/mL in a 12 multiwell plate. At day 4, 100 µL of medium containing IL-2 3000 U/ml was added. At day 7 cells were counted and splitted in a 6 multiwell plate (Corning), keeping cells concentration 5-7x10^5/mL with medium containing IL-2 and IL-15. At day 10± , cells were counted and if necessary, medium was added or they were further splitted to have at cell concentration of 7-8x10^5/mL. At day 14, cells were collected, counted and cryopreserved. A further round of expansion with the same conditions was performed on thawed cells.

2.2.3.2 Protocol B

Activated TILs are expanded with irradiated feeders cells at a 1:50 ratio in a 12 multiwell plate containing CG 10% FBS, 0,1% gentamycin, OKT3 (30 ng/mL), IL-15, IL-7, IL-21 (10 ng/ml) (Miltenyi Biotech) and IL-2 (1000 U/ml). At day 7± cells were counted and moved into a 6 multiwell plate, keeping cells concentration between 5-7x10^5/mL. The same cytokines at the same initial concentrations were added to the medium. Cells were counted at day 14±; a further round of splitting was performed if necessary, always maintaining cell concentration between 5-7x10^5/mL and always adding the same cytokines at the same concentration. At day 21, cells are collected, counted and cryopreserved. A second round of expansion with IL-2 and IL-15 is currently under evaluation.

2.2.3.3 Protocol C

Protocol C is identical to protocol B, without IL-2.

2.2.4 Vitality after thawing

The vitality was evaluated according to the trypan blue exclusion assay: cells were labeled with trypan blue to discriminate living (unlabeled) and unliving (labeled) cells. Cells were thawed in physiological saline solution (BBraun) supplemented by 4% of human albumin (Kedrion Biopharma, Lucca, Italy).

2.2.5 Cytotoxicity assay

TILs expanded according to previously described protocols were tested for potency in a ^{51}Cr-release cytotoxicity assay. Target cells include SW480, an immortalized human colorectal carcinoma cell line with a mutation on the twelfth codon of *KRAS*, and autologous TC.

Briefly, target cells (TCs and SW480) were labeled with ^{51}Cr and left ON at 37°C and 7% of CO_2. The next day, cells were washed three times with RPMI 1% Glutammine (Gibco) and 10% FBS to eliminate excessive chromium. Detection of ^{51}Cr embed by the cells was determined by using a Gamma-counter (Perkin-Elmer, Massachusetts, USA); the quality of labelling is necessary to determine the number of target cells – T - and effector cells – E - to use to determine if efficient cytolysis was performed. Cells were plated on a 96-well round plate and E:T were plated at 50:1, 25:1, 12,5:1 and so on ratios. Three wells to determine total release of the label – by killing all the targets through energic mechanical re-suspension – and six wells to determine spontaneous release of the label were plated as well. After 5 hours for SW480 and 8 hours for TCs, the cytolytic effect of TILs can be determined by calculating the quantity of label released.

Supernatants are collected and moved to a 96-well round plate (Lumaplate, Perkin Elmer), which is left to dry ON.
The following day, the released label can be determined by using a MicroBeta Counter (Perkin Elmer).

2.2.6 Phenotype analysis

Phenotypical analysis of TILs included the use of CD3 (clone UCHT1), CD4 (clone: SK3) and CD8 (clone: SK1), (Beston, Dickinson and company, BD Bioscience, New Jersey, USA) monoclonal antibodies for basic identification of T cells. The presence of an activated/exhausted phenotype was also taken in consideration through phenotypical analysis: the expression of TIM-3 (clone: 344823), GITR (clone:110416) (R&D Systems. Minnesota, USA), PD1 (clone: PD1.3)(Beckman Coulter, California, USA), CD39 (clone: TU66), CD69 (clone: FN50), CD28 (clone: CD28.2), LAG-3 (clone: T47-530), CD137 (clone: 4B4-1), CCR7 (clone: 150503) and CD45-RA (clone: HI100)(BD Biosciences) was evaluated on expanded TILs.

PE	FITC	PerCP-Cy5.5	APC	APC-H7	V450
/	/	7AAD	/	/	/
PD1	TIM-3	7AAD	CD3	CD8	CD4
CD39	CD69	7AAD	CD3	CD8	CD4
CD28	LAG-3	7AAD	CD3	CD8	CD4
GITR	CD137	7AAD	CD3	CD8	CD4
CCR7	CD45RA	7AAD	CD3	CD8	CD4

Table 2: Phenotypical analysis through BD FACS Canto II. 6 vials were prepared: 5 contained mAbs for T cell identification subsets (CD3, CD4 and CD8), 7AAD for live/dead gating and mAbs for activated/exhausted markers. A control sample was prepared containing only 7AAD for background exclusion.

T cells were washed and resuspended at a 3-5x10^5 in 50 µL of buffer made of PBS (Euroclone) with 0,5% Human serum (Carlo Erba, Milan, Italy) before mAbs labelling. Cells were incubated for 30 minutes at 2-8 °C according to manufacturer instructions, washed with buffer and resuspended in 500 µL of physiological solution (B.Braun, Melsungen, Germany); 7-AAD (BD Bioscience) was used for live/dead gating. Cells

were analyzed using a BD FACS Canto II and results were analyzed using Kaluza (Beckman Coulter).

2.3 Results

2.3.1 Expansion of Tumor Cells

Tumor cells were isolated and cultured after the dissociation protocol described above. After at least 4/5 passages in culture and after determination of their neoplastic origin, cells were cryopreserved in several vials for further experiments .[18]
TC derived from patients MRN014, MRT025 and VCC028 after few *in vitro* passages stopped to growth and it was not possible to cryopreserve the amount of cells necessary to assess functional assays (table 1)

2.3.2 Evaluation of TIL cell recovery and expansion rate

Recovery and expansion rate of TIL obtained with the different protocols, after one or two rounds, when performed, of rapid expansion were evaluated.
The number of cells obtained after dissociation of the tumor sample was variable (range: $7x10^4$-$2,6x10^6$). Based on the cell recovery, when possible, part of the cells was cryopreserved in different aliquots, while a part was expanded according to the Protocol A. As described in the experimental design, not all the cells obtained on day 0 after dissociation were all expanded, as the rapid expansion method is very expensive both from an economic point of view, as it requires of high doses of recombinant cytokines, than cellular, as large numbers of irradiated feeder cells derived from healthy donors are required. For this reason, only a small number of cells were rapidly expanded.
TILs were expanded with protocol A from 10 patients. All TIL, except DMR027, demonstrated good proliferative capacity, with the expansion rate ranging from 1:11 to 1:300 (Table 3). Based on the expansion rate documented for each patient after 1 round of rapid expansion, we then calculated what would have been the cell recovery if all the cells obtained after dissociation were expanded (Table 3). For patients CMP019,

MRN014, GRN017, and VCC028, given the small cell recovery, all the cells obtained after dissociation were rapidly expanded.

UPN	Post-dissociation cell suspension	Expanded cells at day 0	Recovery after 1st round of expansion	Expansion rate	Estimated recovery *
BNZ008	4,5x10e5	1,3x10e5	4,4x10e6	1:33	15x10e6
CMP019	4,5x10e5	4,5x10e5	33x10e6	1:73	33x10e6
CTT007	3x10e5	1,9x10e5	72x10e6	1:378	114x10e6
FRV010	6,5x10e5	1,3x10e5	20x10e6	1:154	100x10e6
MRT025	1x10e6	2,6x10e5	25x10e6	1:96	96x10e6
MRN014	3x10e5	3x10e5	90x10e6	1:300	90x10e6
PSC031	2,6x10e6	7x10e5	28x10e6	1:40	104x10e6
VCC028	7x10e4	7x10e4	12x10e6	1:171	12x10e6
GRN017	1,5x10e5	1,5x10e5	14x10e6	1:93	14x10e6
DMR027	1,12x10e5	1,12x10e5	1,3x10e6	1:11	1,3x10e6

Table 3: Recovery of TIL after one round of rapid expansion with protocol A. *In this column recovery of TIL calculated based on expansion rate, if all cells obtained after dissociation were expanded, is reported.

After one round of rapid expansion, the cells were collected and cryopreserved in several vials. One vial was then thawed and subjected to a 2° round of rapid expansion using the same protocol. The second round of rapid expansion, with high doses of IL-2, was performed on TIL derived from 8 of 10 patients. The results show good levels of cellular recovery with an expansion rate ranging from 1:24 to 1:93 (table 4). The expansion rates were lower than those observed after the first round, but they are much more homogeneous. The greater homogeneity is probably because the quality of the cells obtained after the 1° round of expansion is comparable between the TILs derived from the different patients, while the cells obtained fresh after dissociation can have a different quality due to several variables. Furthermore, it is known that already activated cells, such as in our case the TILs after 1° round expansion, generally have a lower proliferative capacity than non-stimulated cells.

Based on the expansion rate we calculated how many total cells we would get if all cells estimated after 1° round were expanded (Table 4).

UPN	Estimated recovery after 1st round	Expanded cells at day 0	Recovery after 2nd round of expansion	Expansion rate	Estimated total recovery *
BNZ008	15x10e6	1,2x10e6	111x10e6	1:93	1,4x10e9
CMP019	33x10e6	2x10e6	145x10e6	1:72	2,4x10e9
CTT007	114x10e6	1x10e6	52,3x10e6	1:52	5,9x10e9
FRV010	100x10e6	6x10e5	34x10e6	1:57	5,7x10e9
MRT025	96x10e6	ND **	ND **	ND **	ND **
MRN014	90x10e6	1,8x10e6	123x10e6	1:68	6.1x10e9
PSC031	104x10e6	ND **	ND **	ND **	ND **
VCC028	12x10e6	1,8x10e6	14,1x10e6	1:8	96x10e6
GRN017	14x10e6	1,5x10e6	36x10e6	1:24	336x10e6
DMR027	1,3x10e6	9,6x10e5	22x10e6	1:23	30x10e6

Table 4: Cell Recovery of TIL after two rounds of rapid expansion. * In this column the total estimated cell recovery of TILs based on estimated recovery after the first round of expansion is reported ; ** ND= not done

2.3.2.1 TILs expanded with protocol B and C had a better expansion rate compared to protocol A expanded TILs

TIL derived from 5 patients (see table 1) cryopreserved after dissociation at day 0, were expanded with Protocol B and C, and simultaneously also with protocol A.

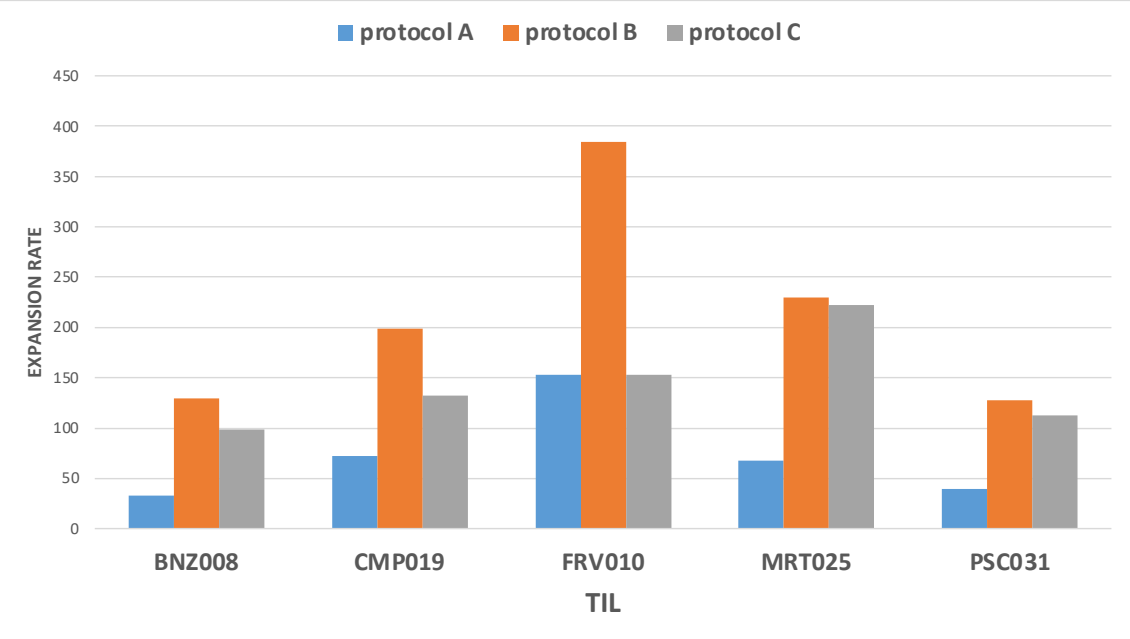

Figure 12: expansion rate of TILs derived from five patients expanded with different protocols.

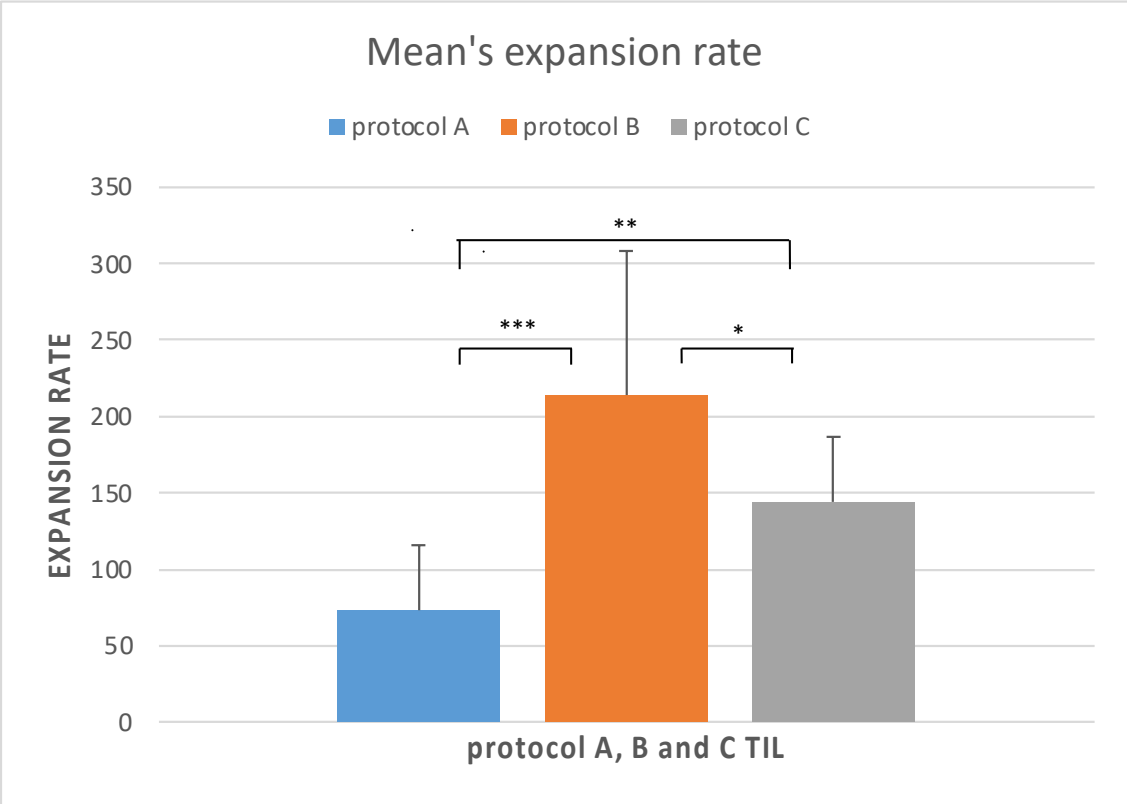

Figure 13: Mean and SD of expansion rates between TILs expanded with different protocols; *p>0,05, ** p≤0,05, *** p≤0,01. Statistical analysis was performed using Student t test.

Results reported in figure 12 documented that all TIL expanded with Protocol B have a higher levels of expansion compared with protocol A, also the expansion rate of TIL expanded with protocol C are higher than that observed with protocol A in 4 out of 5 patients. In particular the levels of expansions were significantly higher in TIL obtained with Protocol B vs A (p=0,002) and with protocol C vs A (p=0,01), whilst no significant difference was shown between protocol B and C (p=0,08) (figure 13).

UPN	Cell recovery after thawing	Expanded cells at day 0	Recovery	Expansion rate	Estimated recovery
BNZ008	4,5x10e5	1,3x10e5	4,4x10e6	1:33	15x10e6
CMP019	9x10e5	4,5x10e5	33x10e6	1:73	66x10e6
FRV010	6,5x10e5	1,3x10e5	20x10e6	1:154	100x10e6
MRT025	1x10e6	2,6x10e5	18x10e6	1:69	69x10e6
PSC031	2,5x10e6	7,2x10e5	28x10e6	1:39	98x10e6

Table 5: recovery of TILs expanded with Protocol A.

UPN	Cell recovery after thawing	Expanded cells at day 0	Recovery	Expansion rate	Estimated recovery
BNZ008	4,5x10e5	1,3x10e5	17x10e6	1:131	59x10e6
CMP019	9x10e5	1,5x10e5	30x10e6	1:200	180x10e6
FRV010	6,5x10e5	2,6x10e5	100x10e6	1:384	250x10e6
MRT025	1x10e6	3,9x10e6	90x10e6	1:230	230x10e6
PSC031	2,5x10e6	9x10e5	116x10e6	1:128	320x10e6

Table 6: recovery of TILs expanded with protocol B.

UPN	Cell recovery after thawing	Expanded cells at day 0	Recovery	Expansion rate	Estimated recovery
BNZ008	4,5x10e5	1,3x10e5	13x10e6	1:100	45x10e6
CMP019	9x10e5	3x10e5	40x10e6	1:133	120x10e6
FRV010	6,5x10e5	2,6x10e5	40x10e6	1:154	100x10e6
MRT025	1x10e6	3,9x10e5	87x10e6	1:223	223x10e6
PSC031	2,5x10e6	9x10e5	103x10e6	1:114	320x10e6

Table 7: recovery of TIL expanded with protocol C

2.3.3 Evaluation of TILs' potency

TILs expanded with three protocols were tested in a cytotoxicity assay for their ability to *in vitro* recognize and lyse SW480 cell line and autologous TC, when available. Many *in vitro* studies use long-term stabilized cell lines, such as SW480, which are derived from a patient with CRC. Long-term commercial cell lines represent a good opportunity to evaluate antitumor activity when the patient's tumor cells are not available, but they are not always representative of the characteristics of autologous TC. For this reason, in this study, we also isolated and expanded in vitro TC from patients according to a methodology previously developed in our laboratory. This approach allows us to expand tumor cells from about 80% of the tumor pieces. The cells were expanded in vitro no later than 4-5 passages, in order to maintain the characteristics of the primary tumor as much as possible. After confirmation of their neoplastic nature, they are cryopreserved in different vials and used as targets in the cytotoxicity test, together with SW480.

TIL obtained with protocol A after one round of rapid expansion showed high levels of cytotoxicity against SW480 cells, as reported in figure 14, with a mean of specific lysis of 66% at E:T ratio of 50:1. Cytotoxicity activity is maintained even at lower concentration of E:T ratios (figure 14).

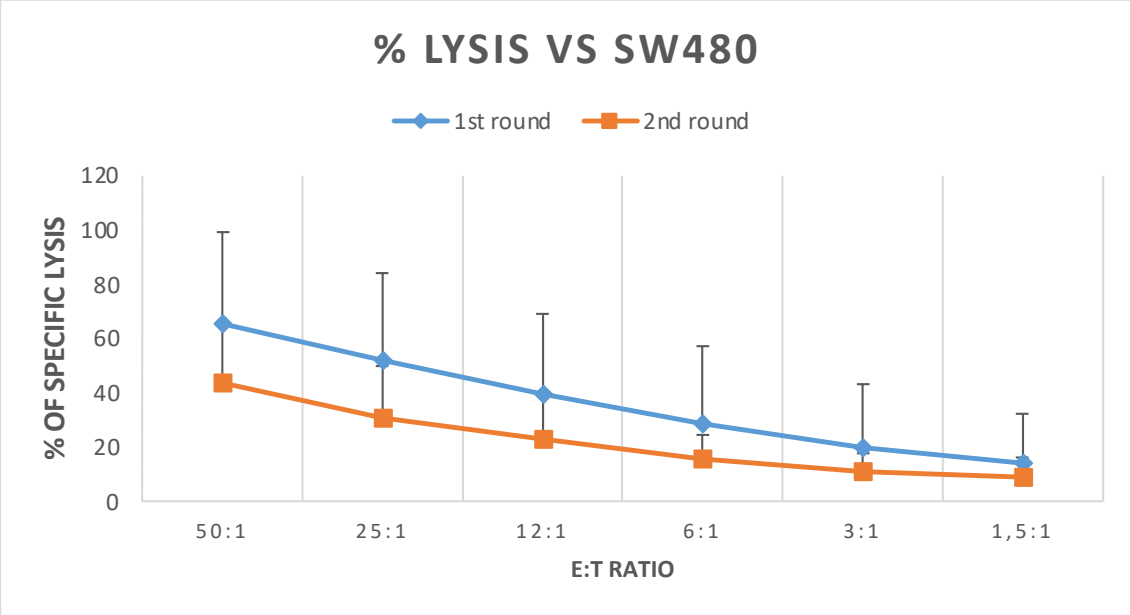

Figure 14: Cytotoxic activity of TILs' against SW480, after one (blue line) and two rounds (orange line) of expansion. In the figure M and SD of specific lysis obtained at E:T ratio ranging from 50:1 to 1.5:1, are reported

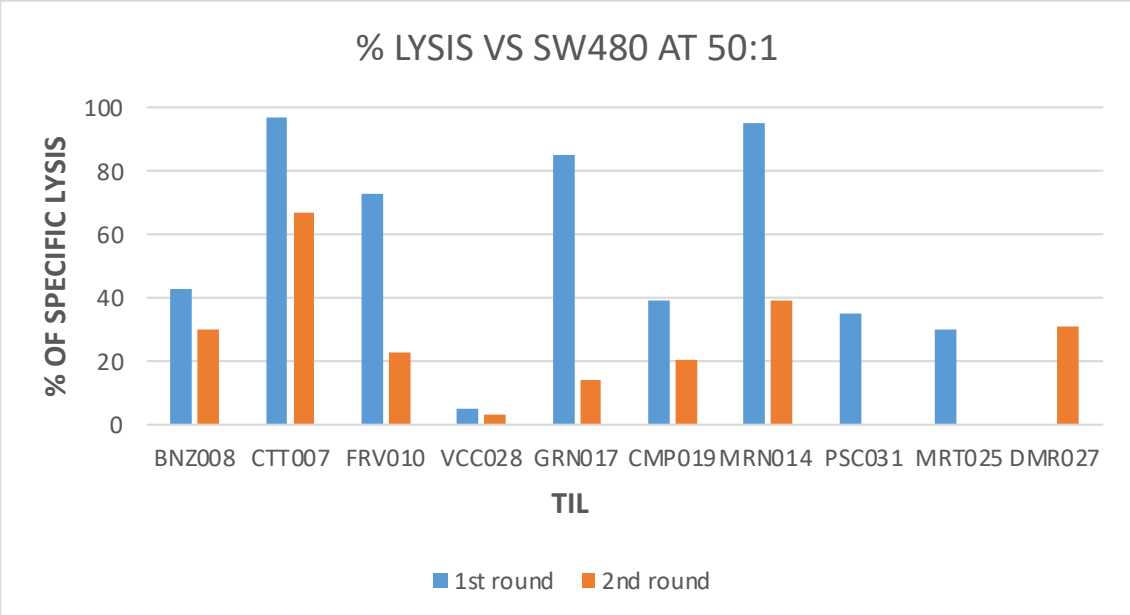

Figure 15: Cytotoxic activity of TILs' against SW480 after one (blue) and two rounds (orange) of expansion. In the figure, the percentage of specific lysis of TIL derived from each patients at E:T ratio of 50:1 is reported

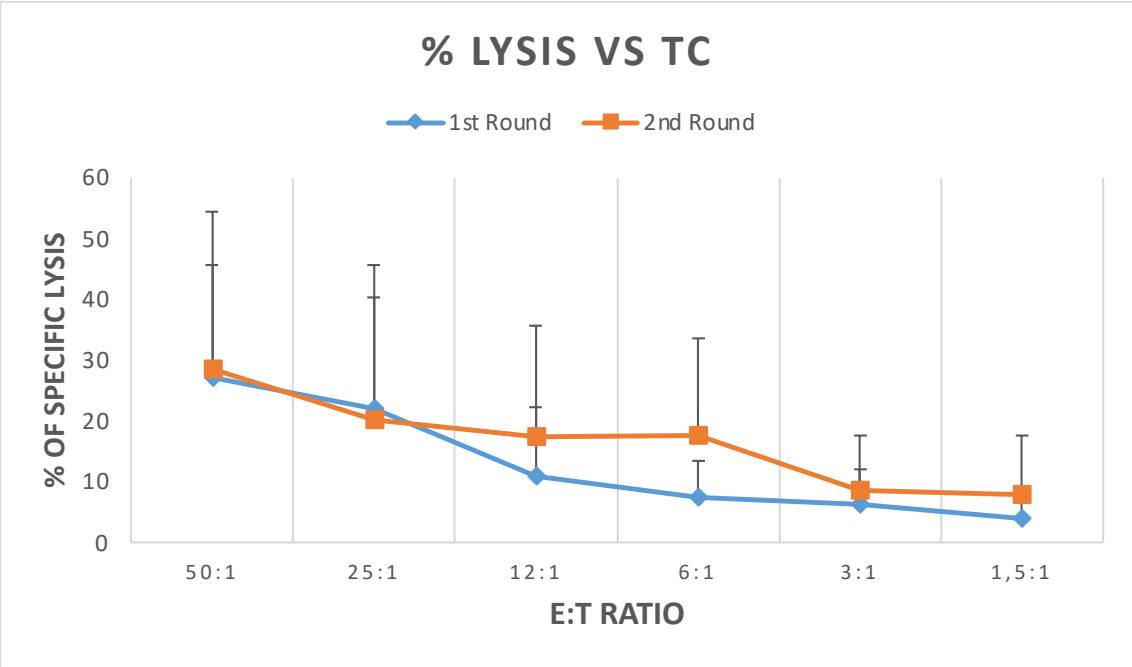

Figure 16:Cytotoxic activity of TILs' against autologous TC, after one (blue line) and two rounds (orange line) of expansion. In the figure M and SD of specific lysis obtained at E:T ratio ranging from 50:1 to 1.5:1, are reported

Although the cytotoxicity levels against TC are lower than that observed against SW480, these data indicate that TILs are able to recognize and kill autologous cancer cells *in vitro* as well. The lower levels of cytotoxicity observed are probably because cells deriving from solid neoplasia have a lower susceptibility to lysis than immortalized cell lines and therefore require more time to be lysed. Unfortunately, the *in vitro* cytotoxicity test has some limitations, in that it is not possible to extend the duration of the experiment beyond 24 hours, as the TC undergo spontaneous death in culture and therefore it is no possible to determine the tumor-specific lysis levels.

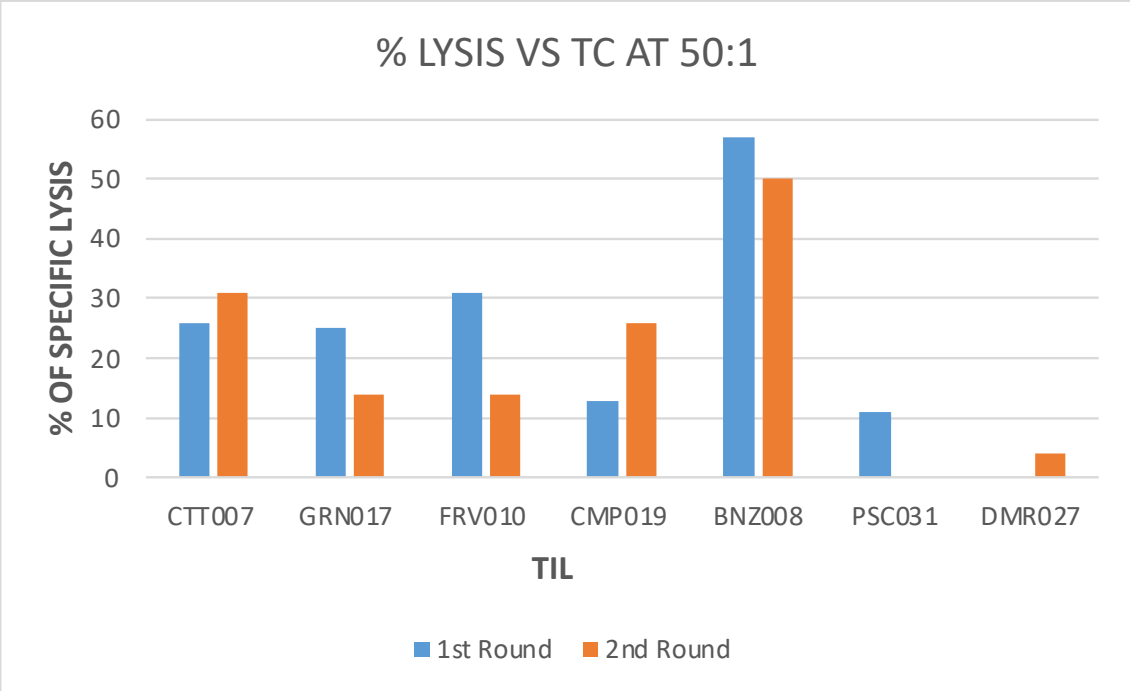

Figure 17: Cytotoxic activity of TILs' against autologous TC after one (blue) and two rounds (orange) of expansion (when available). In the figure the percentage of specific lysis of TIL derived from each patients at E:T ratio of 50:1 is reported

Analysis of TIL obtained after two rounds of rapid expansion documented that they maintained the *in vitro* ability to efficiently autologous TC, while the levels of cytotoxicity against SW480 are reduced, even though they remain sizeable. (Figure 14 and 16).

Evaluation of TIL obtained with different protocol documented that TIL derived with Protocol B and C displayed levels of specific lysis against SW480 superior even that not significantly higher than those obtained with Protocol A at all E:T ratio.

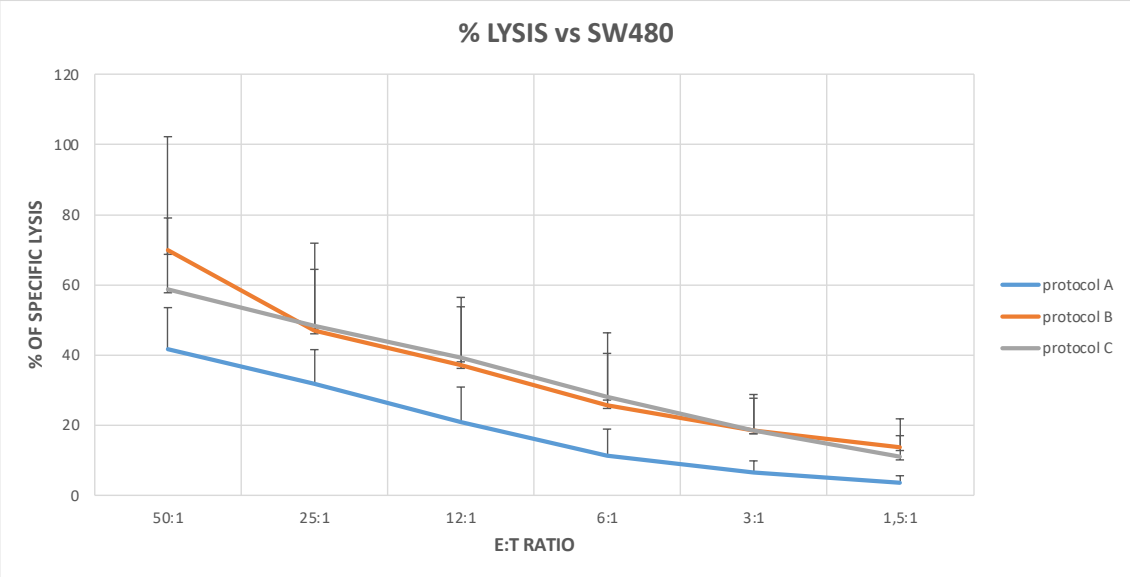

Figure 18: comparing cryopreserved, protocol A-, B- and C- expanded TILs's cytotoxicity versus SW480.

2.3.4 TILs' phenotype analysis

TILs' phenotypical evaluation was designed considering three criteria:

1. The presence of T cell lymphocyte marker CD3
2. The distinction between CD4+ T cells and CD8+ T cells
3. The presence of ICs such as PD-1 and TIM-3, activation markers such as CD28, memory like markers such as CCR7 and CD45-RA and the controversial CD39.

2.3.4.1 Phenotype analysis of protocol A TIL

In TIL expanded with protocol A similar percentages of the two subpopulation of CD3+/CD4+ and CD3+/CD8+ were documented (Figure 19). In particular, the mean of % CD3+ cells was 90% (±12%), while the mean of % of CD3+CD4+ cells was 44% (± 32%) and 48% for CD3+/CD8+ (± 28%)- , respectively (Figure 19).

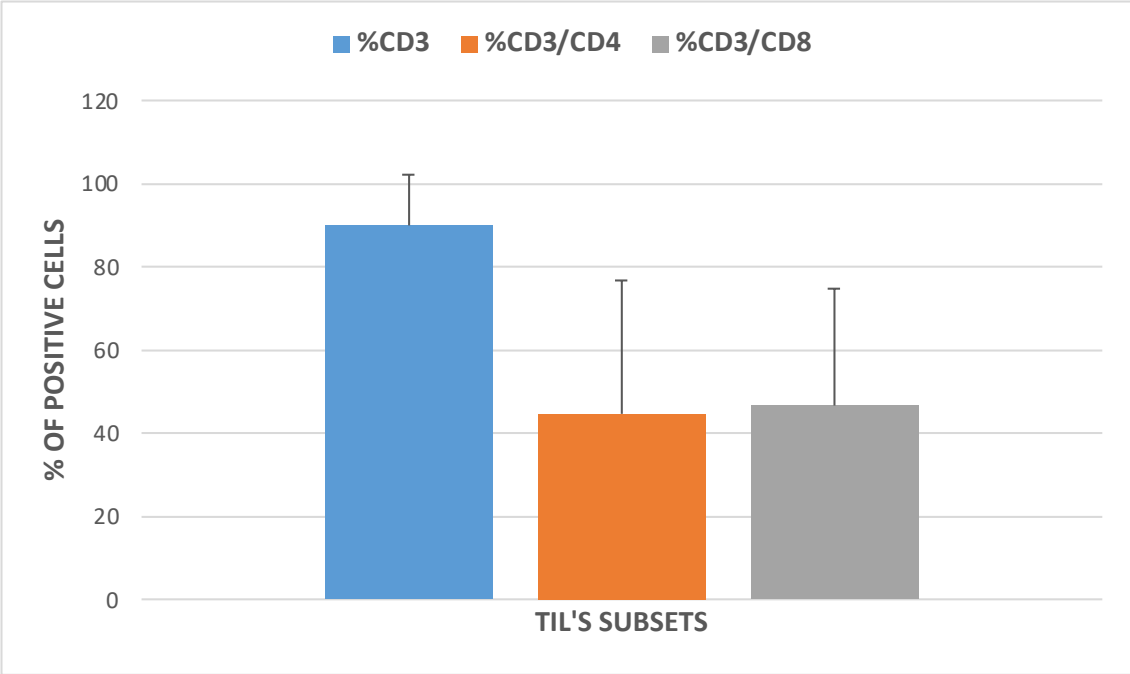

Figure 19: Mean and SD deviation of the % of the populations of CD3+/CD4+ and CD8+ cells in TIL expanded with protocol A.

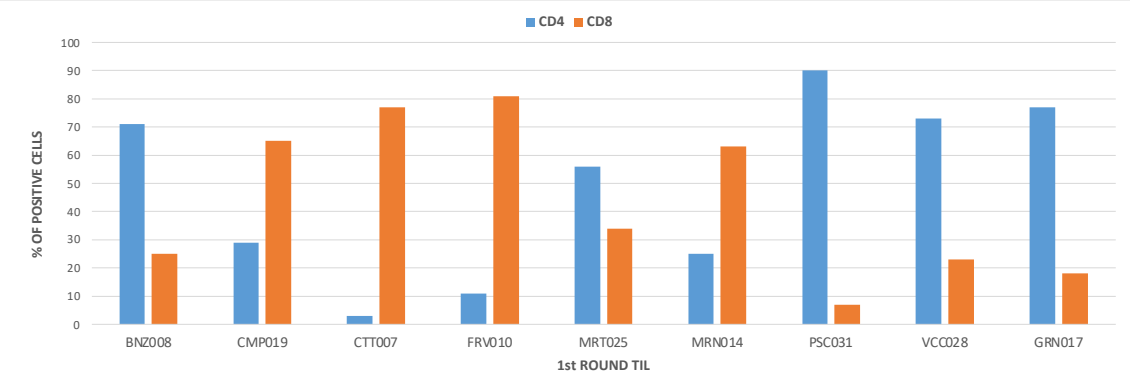

Figure 20: % of CD3+/CD4+ (blue) and CD3+CD8+ (orange) for 1st round protocol A TILs

However, analysis of CD3+/CD4+ and CD3+CD8+ populations in TIL derived from each patient documented a great variability of expression. TIL from patients CTT007 e FRV010 showed a prevalent expansion of CD3+CD8+ cells, while TIL from patients BNZ008, PSC031, VCC028 and GRN017, after one round of expansion, were predominantly composed of CD3/CD4+ cells. Interestingly, we documented that, with the exception of TIL derived from VCC028, TIL with a predominant expansion of

CD3+/CD4+ cells displayed sizeable levels of cytotoxic activity against both SW480 or autologous TC, when available, comparable to that displayed by TIL in which there were an expansion of CD3+/CD8+ cells (figure 15 and figure 17).

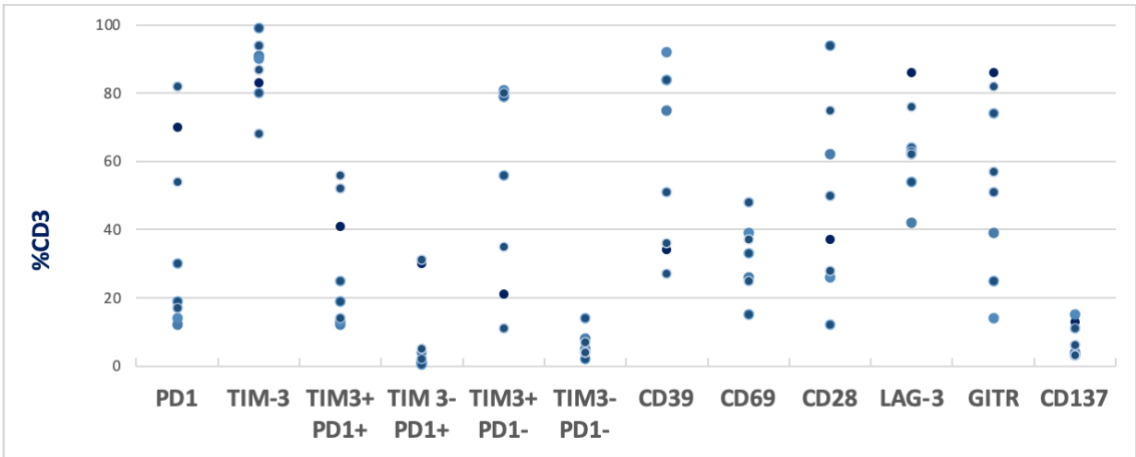

Figure 21: % of surface markers expressed on 1st round CD3+

The levels of expression of surface markers involved in activation/exhaustion status between patients are highly heterogeneous. (Figure 21. 22 and 23).

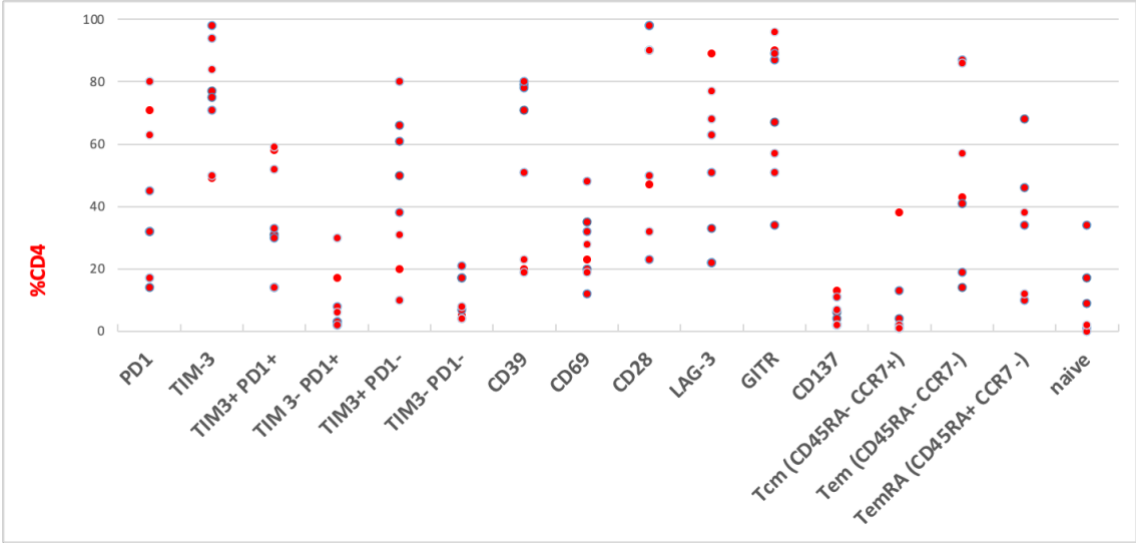

Figure 22: difference in expression markers between one round CD4+ TILs

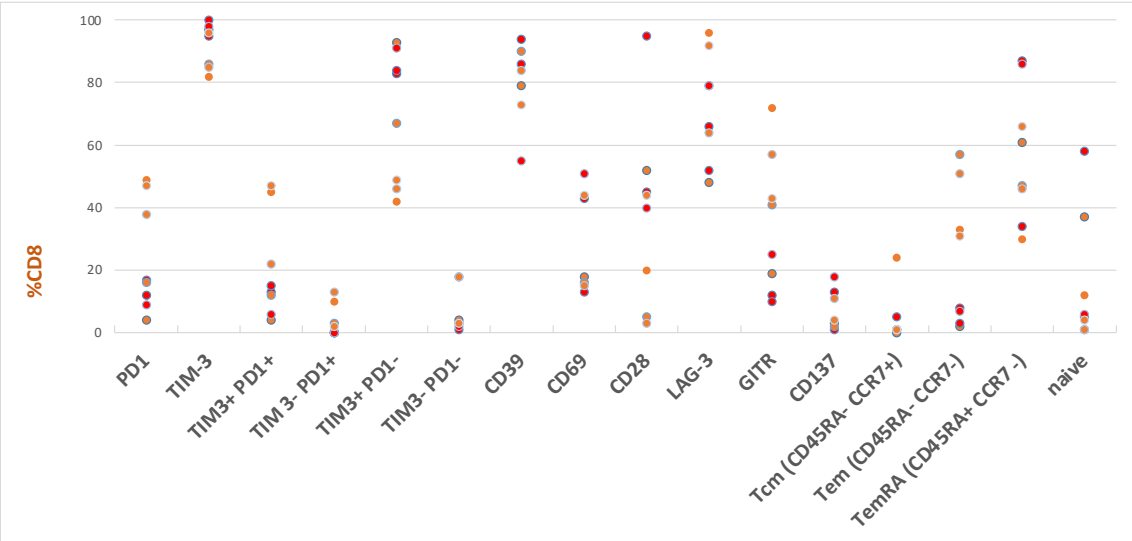

Figure 23: difference in expression markers between one round CD8+ TILs

Evaluation of surface antigens expressed by CD3+/CD4+ or CD3+/CD8+ populations documented that PD1, whose presence on the cell surface is associated with a state of exhaustion, is less expressed in CD3+/CD8+ population, equally the % of Tim-3+PD-1+ TILs, usually considered the most severe exhausted cells are variable, in general lower in CD3+/CD8+ population. ON the contrary, CD39 surface antigen associated to cell exhaustion is higher in CD8 population.

After the 2° round of rapid expansion no significant differences were observed, compared to 1° round in the % of CD3+/CD4+ and CD3+/CD8+ populations. (figure 24)

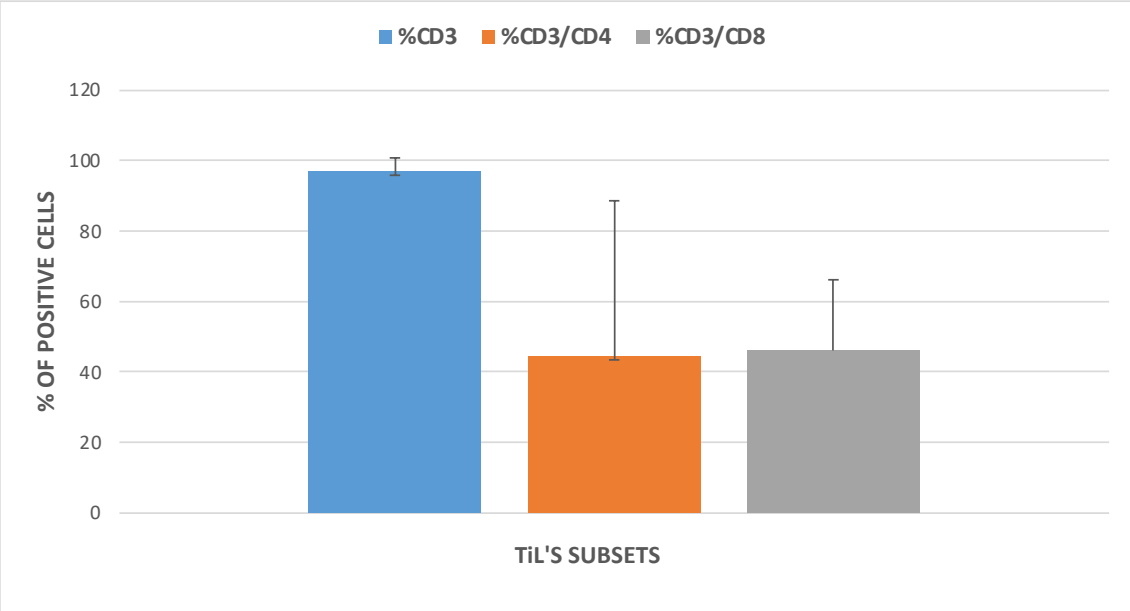

Figure 24: Mean and SD deviation of the % of the populations of CD3+/CD4+ and CD8+ cells in 2nd round TILs expanded with protocol A

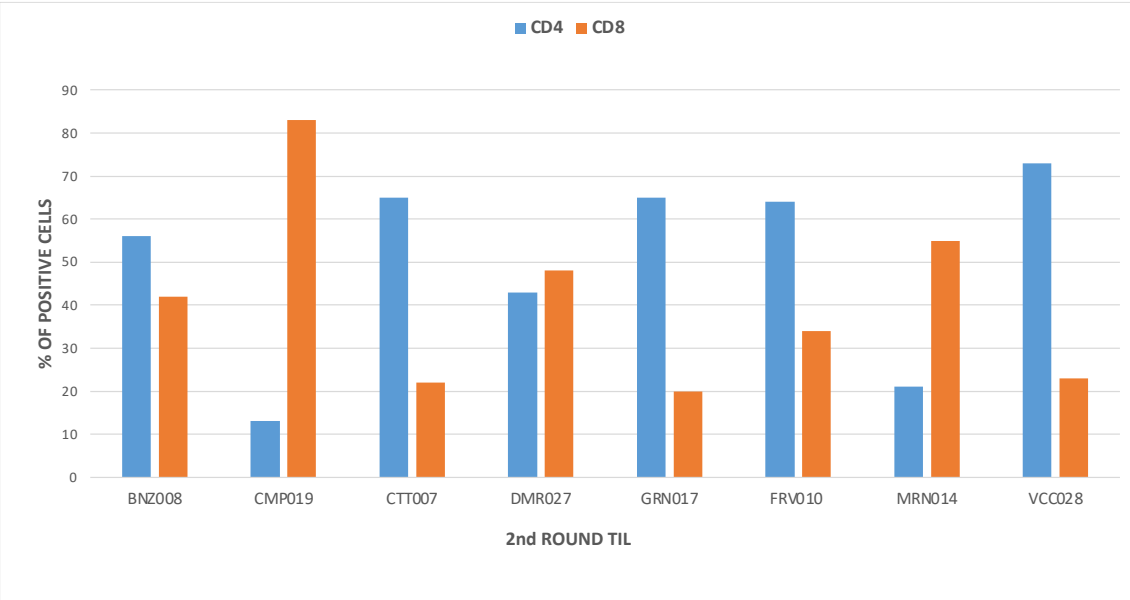

Figure 25: % of CD3+/CD4+ (blue) and CD3+CD8+ (orange)in TIL after 2nd round of rapid expansion.

Analysis of expression of these populations in each TIL after 2° round of rapid expansion, confirms the high variability observed after one round of expansion. Whilst CMP019, maintained a high% of CD3+/CD8+,cells in 2nd round TILs, in CTT007 and FRV010, CD3+/CD4+ population showed a sizeable increasement. An increase in expansion for CD3+/CD4+ can be observed in 2nd round BNZ008 and GRN017 TILs as well.

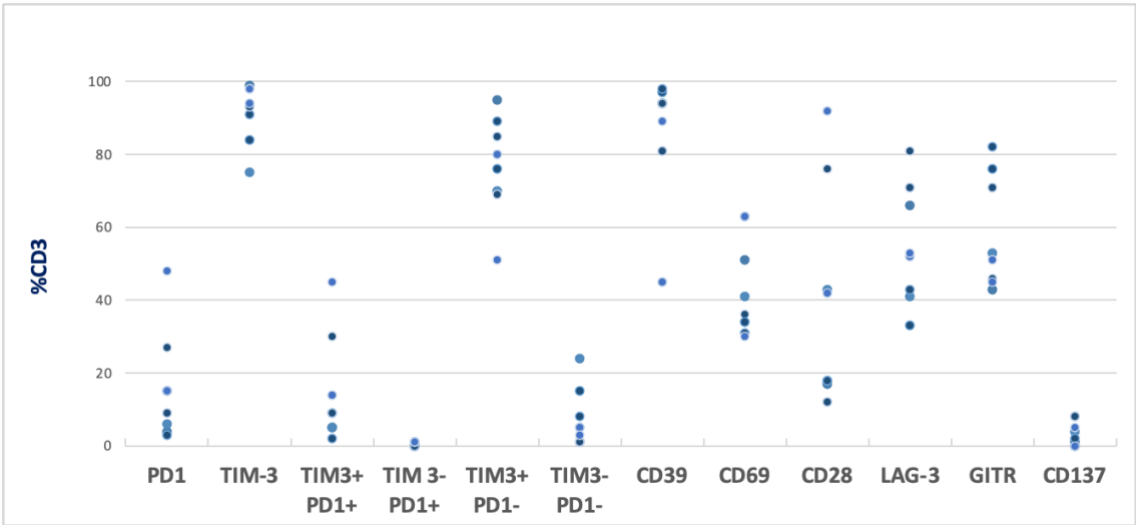

Figure 26: distribution of surface markers expression in CD3+ cells of TIL derived after 2° round of expansion. 2nd round TILs

Despite expression markers appear to be fairly similar to 1st expanded TILs – with a distribution peculiar for each patient, as we can observe on figure 26, 27 and 28 -, with some exception: TIM-3 and CD39 are far more heterogenous on 2nd round CD3+CD4+ cells compared to 1st round (figure 27 and figure 22). Whilst PD1's expression appears as heterogenous as in 1st round TILs (figure 27), it can still be noted how it dropped compared to 1st round CD3+/CD4+ (figure 30). What appears to be particularly striking between the two sets is the difference in CD39 and CD69 expression on CD4+ cells (figure 30).

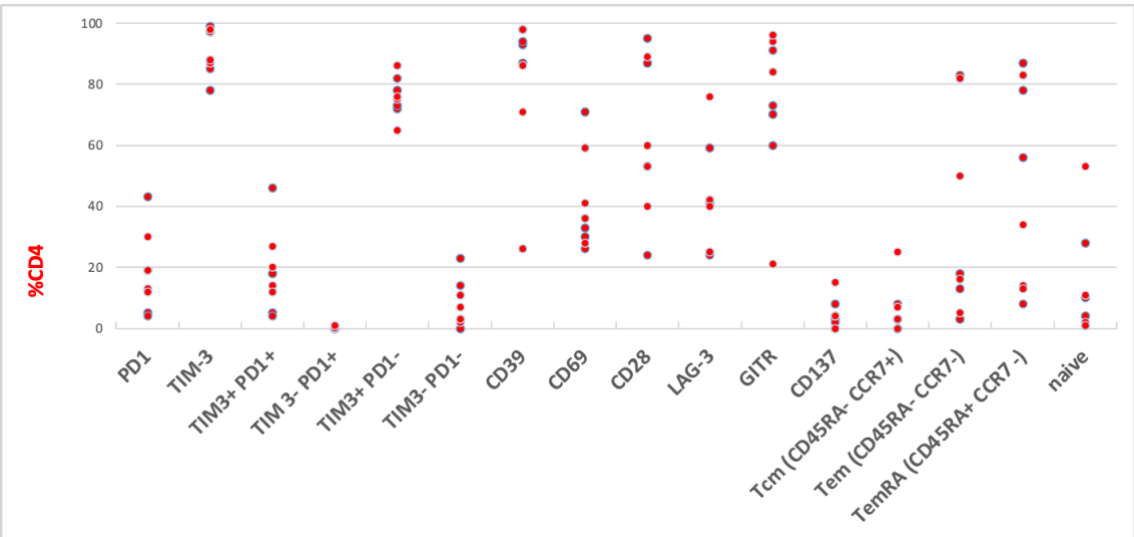

Figure 27: distribution of surface markers expression in 2nd round CD3+/CD4 + TILs

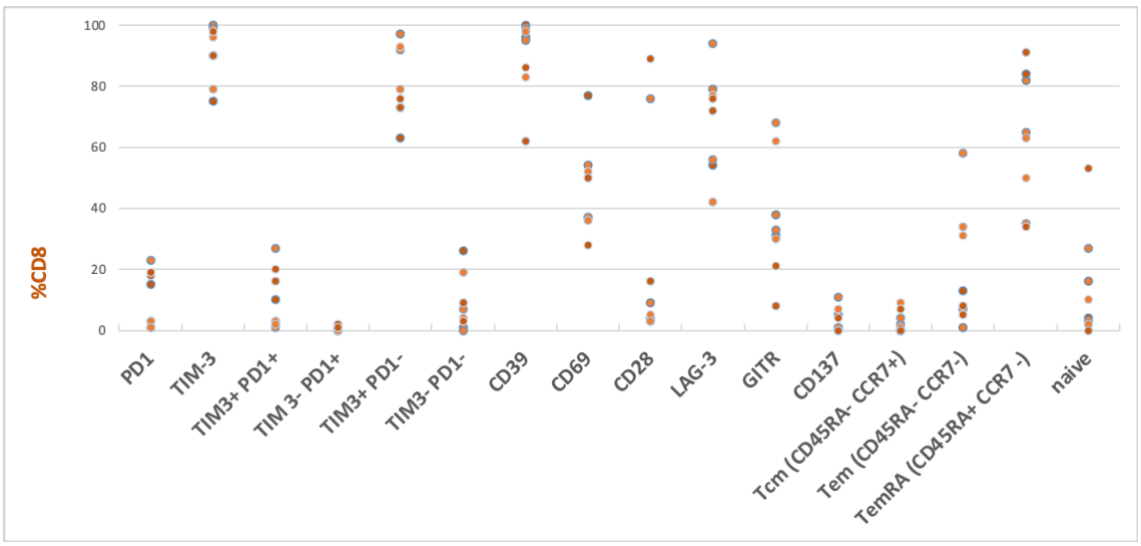

Figure 28:distribution of surface markers expression in 2nd round CD3+/CD8 + TILs

CD3+/CD8+ appear to be fairly similar to 1st expansion CD3+/CD8, although even here it can be observed a drop in PD1 expression. The same was observed for CD28. TIM3 and CD39 are fairly similar to 1st round CD3+/CD8+. An augmented expression, however, can be observed for CD69.

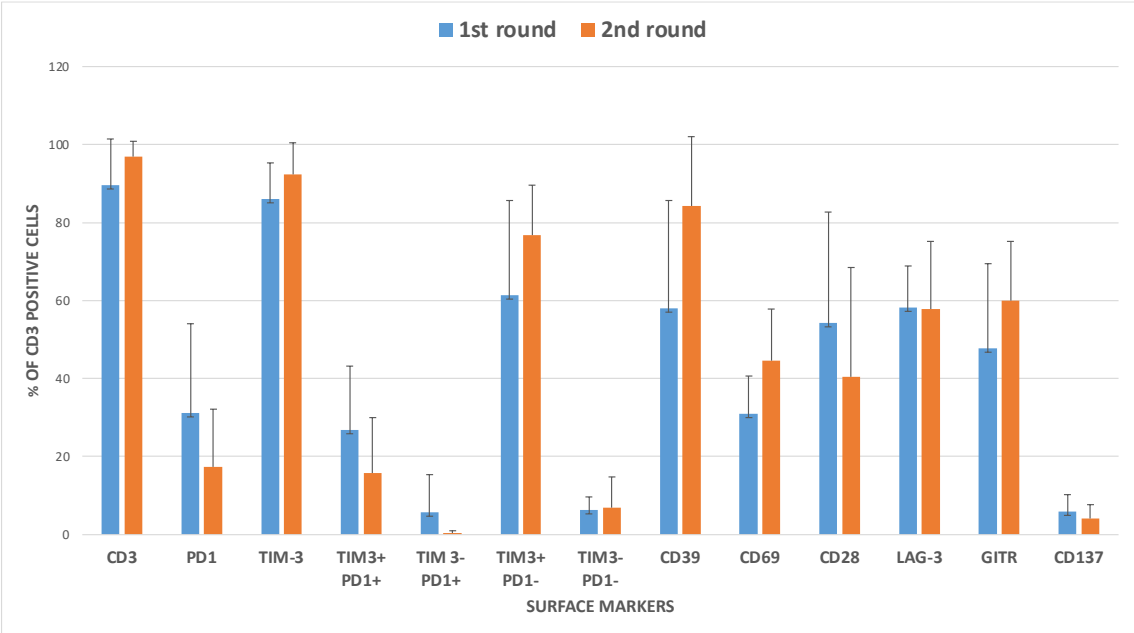

Figure 29: mean and SD of phenotypical markes on 1st and 2nd round CD3+ TILs

No differences were documented in surface antigens expressed by the whole CD3+ population of TIL obtained with protocol A after one or two rounds of rapid expansion,

with the exception of CD39,CD69, CD28 and PD1 (figure 29) and comparable results were documented analyzing the CD3+/CD4+ and CD3+/CD8+ populations.

Statistical analysis showed a highly significant difference between 1st round CD4+CD39+ and 2nd round CD4+CD39+ (p=0,03) and 1st and 2nd round CD4+CD69+ TILs (p=0,009) (figure 30A).

No significant difference was documented between 1st and 2nd round CD8+CD39+ TILs (p=0,06), whilst CD8+CD69+ from 1st and 2nd round expanded TILs showed significant difference (p=0,03)(figure 31A).

Krishna and colleagues[15] reported in their study on a CD8 TILs population which determined cancer regression; in depth-data showed how patients which obtained regression had mainly a CD39-CD69- CD8 population.

Another feature showed by our data is the lowered expression of CD28 – an activation marker – and PD1, which some have linked to an activated TIL population, [12,13] on both CD3+/CD4+ and CD3+/CD8+ (figure 30A and figure 31A). A highly significant difference was reported between 1st and 2nd round CD4+PD1+ TILs (p=0,01) which was not repeated for CD8+PD1+ TILs (p=0,05). CD4+CD28+ and CD8+CD28+ TILs had no significant difference after two round of expansion (p=0,2 for both subsets).

Evaluation of memory T cell compartment documented that the majority of CD3+/CD4+ TIL obtained with protocol A, both at 1st and 2nd round of expansion, are effector memory T cells (T_{EM}) or terminal memory (T_{emRA}), with a measurable percentage of central memory T cells as well (T_{CM}) (figure 30B).

In CD3+/CD8+ TILs the majority of cells, instead, are T_{emRA} in both 1st round and 2nd round expanded TILs (figure 31B).

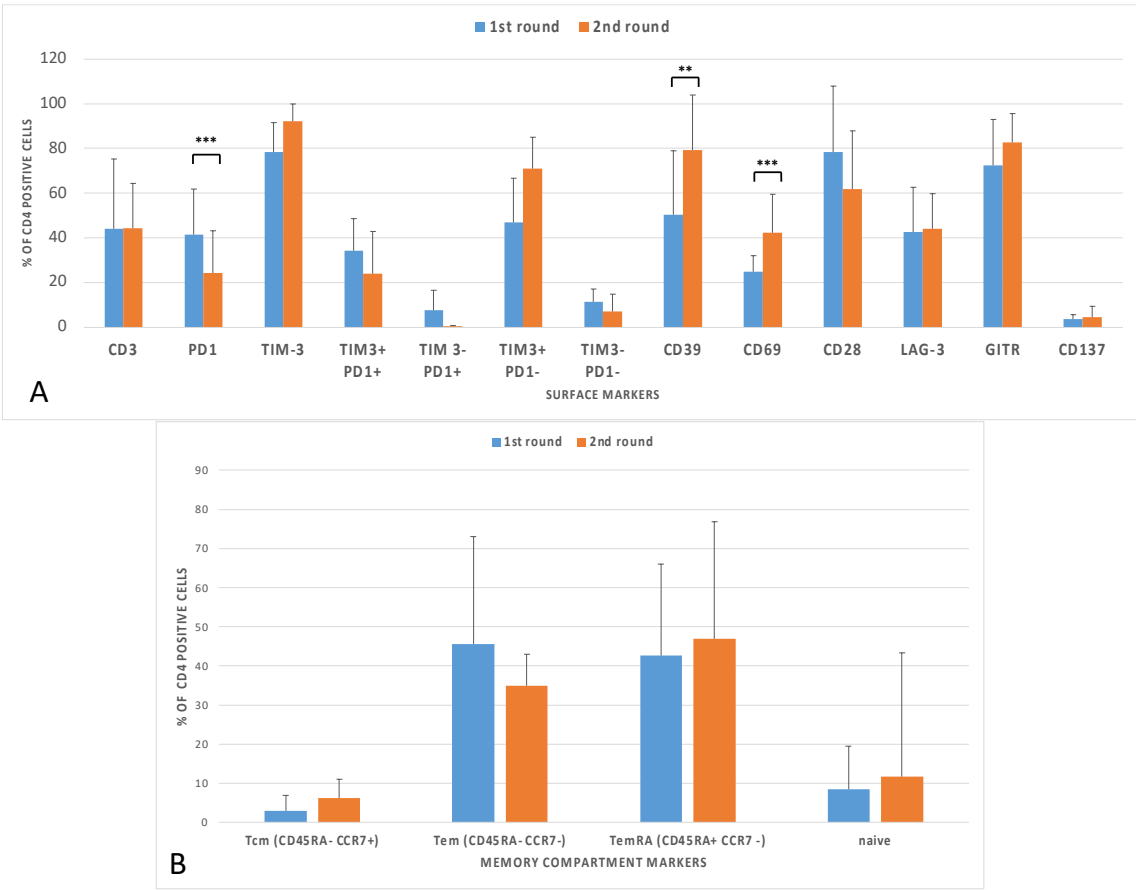

Figure 30: Mean and SD of surface markes (A) and of memory T cell compartment markers (B) on 1st and 2nd round CD3+/CD4+ TILs (**p<0,05, *** p≤0,01)

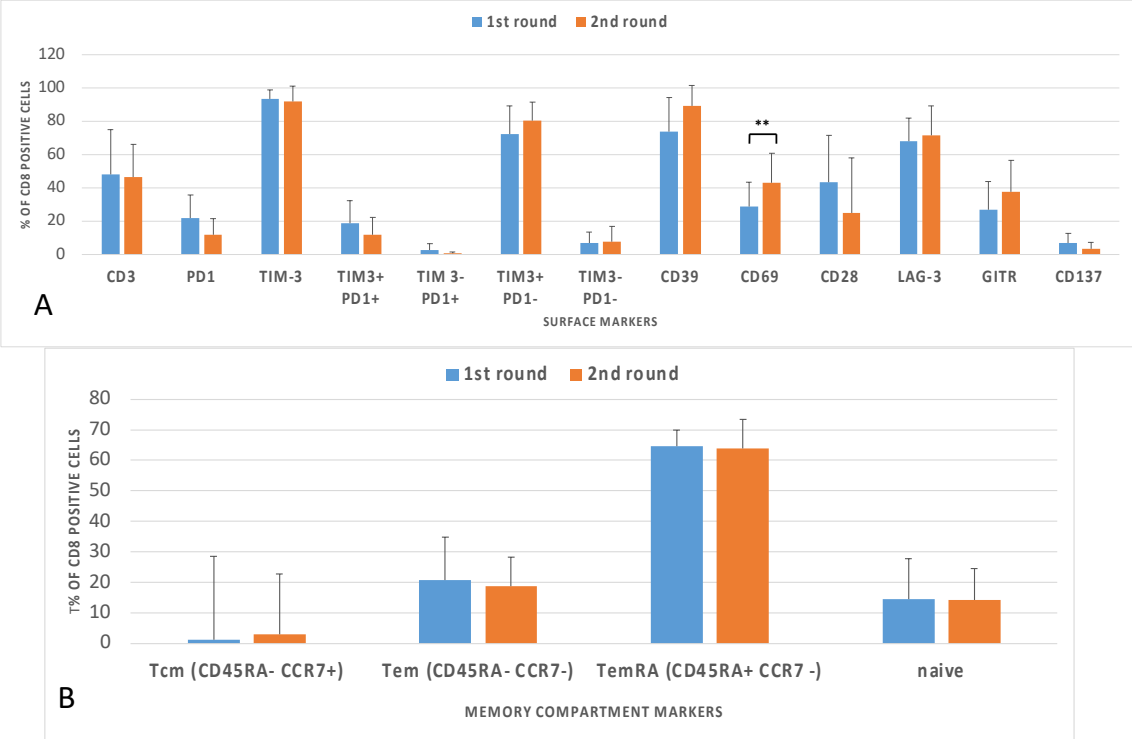

Figure 31: mean and SD of surface markes (A) and of memory T cell compartment (B) on 1st and 2nd round CD3+/CD8+ TILs **p<0,05

2.3.4.2 Phenotypical analysis on protocol A, B and C TILs

There was no significant difference between A, B and C in the % of CD3+ cells (p=0,1). CD3+/CD4 + population had mean 54% (SD: ±28%) for protocol A, 45% (SD: ±24%) for protocol B and 37% (SD: ±22%) for protocol C. Statistical analysis showed these different expression of CD3+CD4+ to be highly significant (p=0,04 for A vs B TILs, p=0,005 for A vs C TILs and p=0,006 for B vs C TILs). CD3+/CD8+ had mean 45% (SD: ±27%) for protocol A, 50% (SD: ±24%) for protocol B and 57% (SD: ±22%) for protocol C. No significant difference was noted between CD3+CD8+ A TILs compared to the same subset in B TILs (p=0,2), however a highly significant difference was noted between A and C CD3+CD8+ (p=0,02) and B and C CD3+CD8+ (p=0,01).

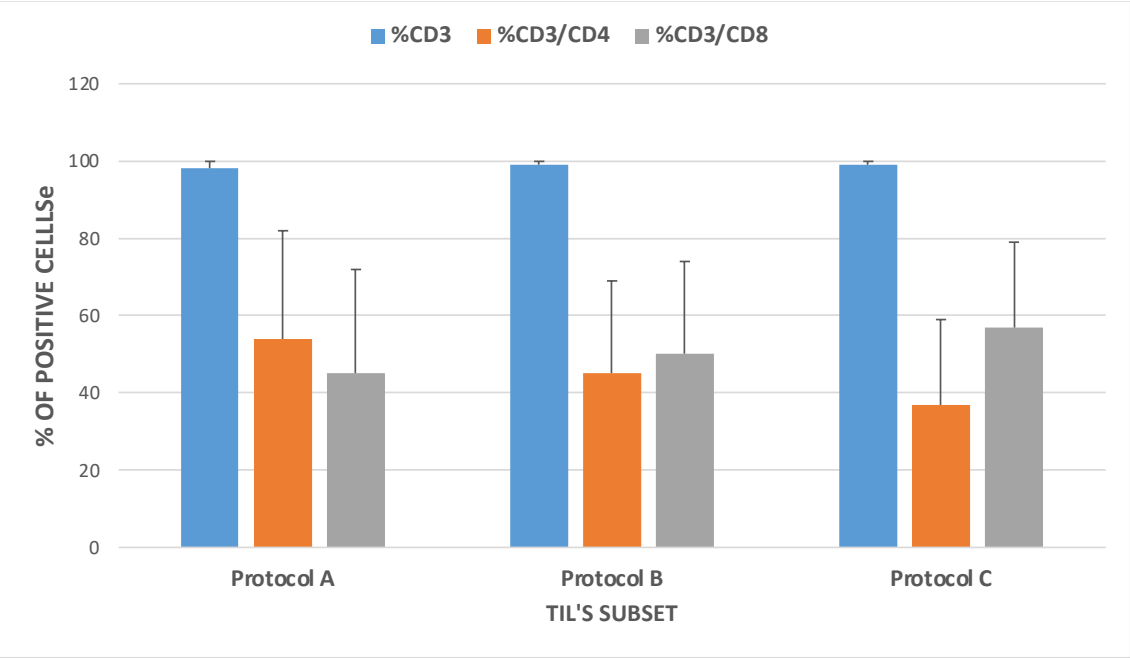

Figure 32: mean and SD of CD3+, CD3+/CD4+ and CD3+/CD8+ in all three protocols

Our data showed how the mean expression of CD28 was superior on protocol B- and C-CD4+ cells compared to protocol A TILs, however the difference was not significant (p=0,07 for protocol A vs protocol B, p=0,05 for protocol A vs protocol C and p=0,2 for protocol B vs protocol C, as shown in figure 33). Expression of CD39 was higher on CD4 protocol A TILs and the difference was significant compared to protocol B (p=0,02) and protocol C (p=0,02) and between protocol B and C (p=0,01). CD69 was equally expressed on all CD4+. Protocol C (mean: 67%; SD: ±14%) CD4+ showed a higher expression of PD-1 compared to protocol A (mean: 54%; SD: ±21%)and protocol B (mean: 54% SD: ±19%), and this difference appeared to be highly significant(p=0,03). CD4+ TILs expanded with each protocol were mostly $T_{EM,}$ whilst T_{CM} cells seem to decrese in TILs expanded with protocol B and C.(see figure 34A and 34B).

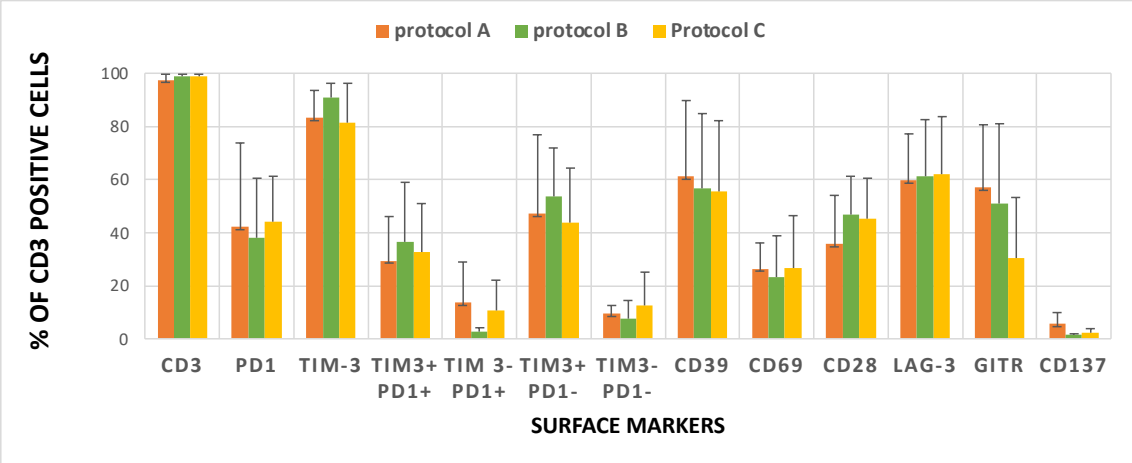

Figure 33: phenotypical analysis on CD3+ of all three protocols

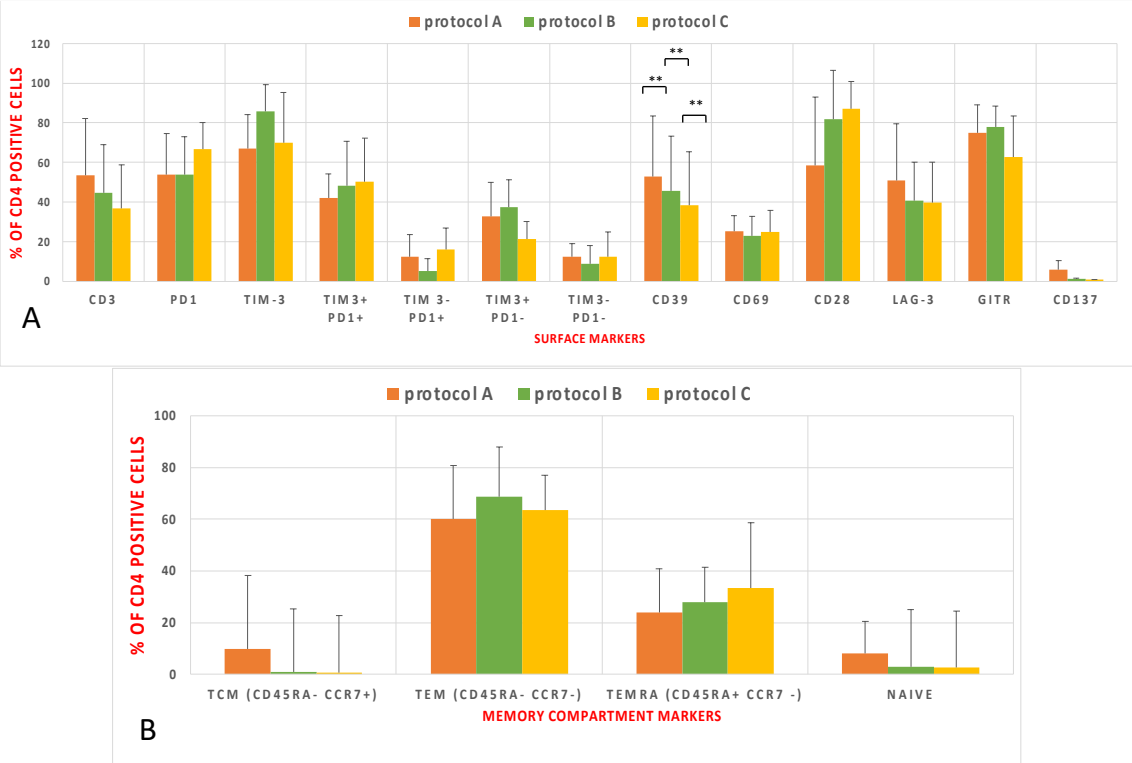

Figure 34: mean and SD for surface markers for CD3+/CD4+ expanded TILs (A) and memory T cell compartment (B) for all protocols **p<0,05

Protocol B and protocol C CD8+ cells had a slightly higher CD28 expression compared to protocol A, however the difference is not significant (p=0,3). Tim-3 was highly expressed on all three protocols. CD8+PD-1+ cells were 22% for protocol A (SD: ±16%), 26% for protocol B (SD: ±15%) and 29% for protocol C (SD: ±11%). No significant difference was reported between protocol A and B (p=0,2), A and C (p=0,1) and B and C ((p=0,2).

CD8+ had a higher expression for CD39 compared to CD4+ on all three subsets. (see figure 34A and 35A). A significant difference was noted between protocol A and C CD4+CD39+ TILs (p=0,04), which is lost between protocol A and B (p=0,1) and protocol B and C (p=0,1).

All CD3+/CD8+ TILs appeared to share the T_{emRA} and T_{EM} in all three protocols; even in CD8+ TILs, T_{CM} appear to decrease in protocol B and C TILs.

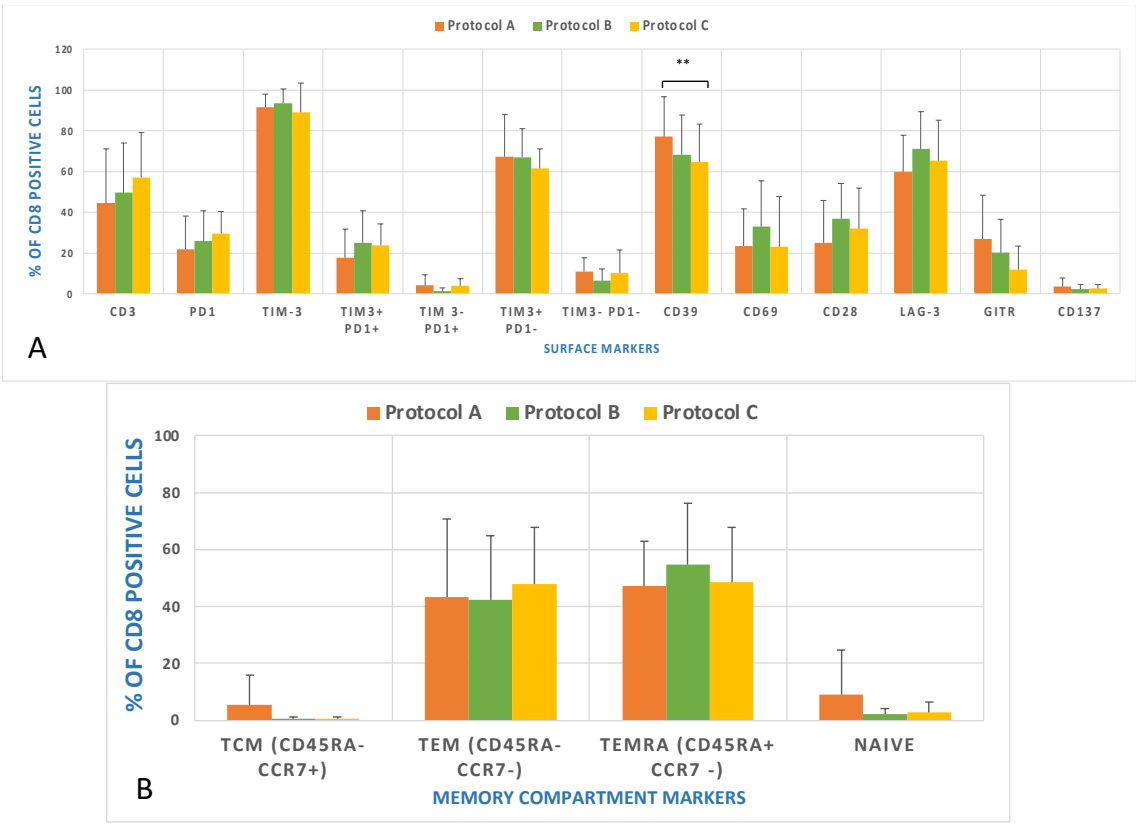

Figure 35: mean and SD of surface markers for CD3+/CD8+ expanded TILs (A) and memory T cell compartment (B) for all protocols **p<0,05

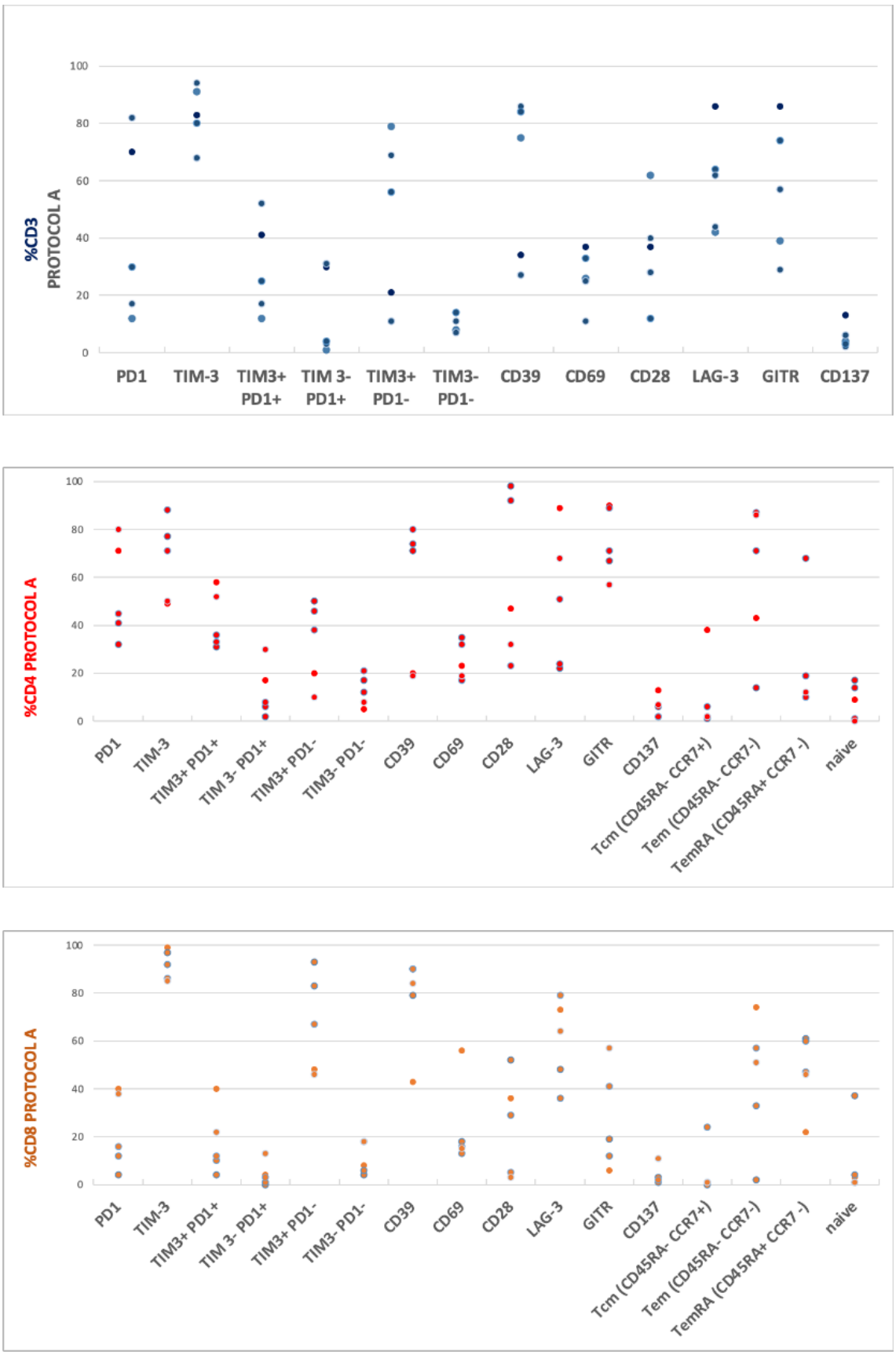

Figure 43: distribution of surface markers for protocol A TILs

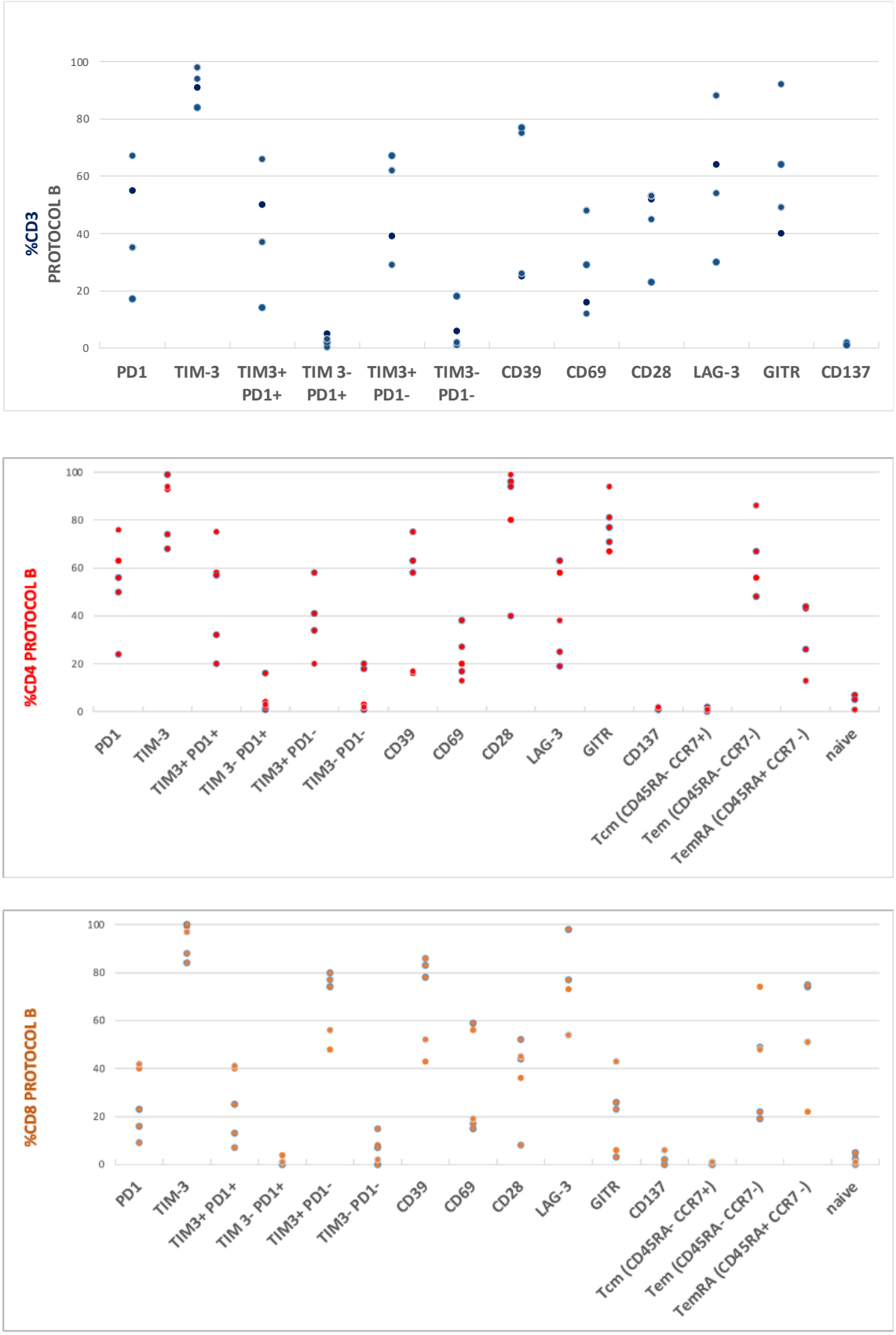

Figure 44: distribution of surface markers for protocol B TILs

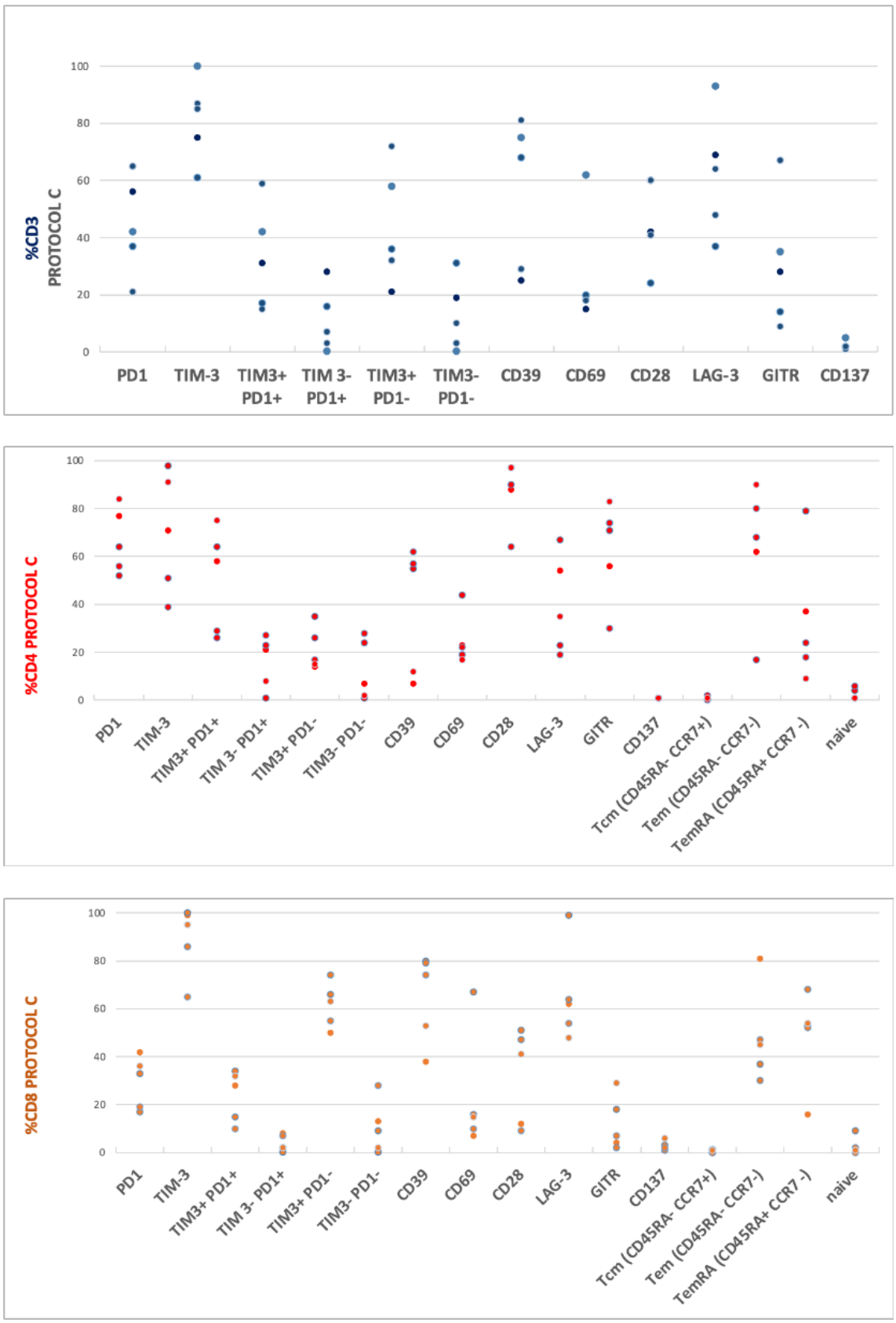

Figure 45: distribution of surface markers for protocol C TILs

2.4 Discussion

Recent advances in molecular targeted therapies and the refinement of surgical procedures have improved the life expectancy of patients with mCRC. Nevertheless, most patients will eventually die of their disease, and new therapeutic options are needed, especially for those patients with molecular profiles that preclude the use of biological agents. Adoptive cell therapy with *ex vivo* expanded TIL derived from metastatic samples might be a way to improve the outcome of mCRC patients refractory to conventional therapies. The success of cell therapy based on the transfer of cells manipulated and expanded *in vitro* depends on many factors, including the ability to obtain large quantities of cells capable of maintaining their ability to recognize and eliminate tumor cells and to persist *in vivo*. For these reasons, the first aim of our study was to optimize a protocol for *ex vivo* expansion of TIL after their isolation from tumor samples. Based on data from Rosenberg and coworkers in melanoma patients, high quantity of TIL were expanded in vitro using high doses of IL-2 . However, translation of this methodology in tumor other than melanoma did not give the expected results, probably due to the different characteristics of the TILs obtained at the tumor site.

We demonstrated that the use of a cocktail of cytokines, including IL-15, IL-7 and IL-21, results in a significant higher expansion rate of TIL while preserving their cytotoxic activity against tumor cells. This is an important data, in that the amounts of TIL obtained after isolation from tumor samples is highly variable and does not always depend on the size of the tumor piece, but on its intrinsic characteristics such as the presence of connective tissue, fat or other. In particular, our results documented that with only two cycles of independent antigen expansion, it is possible to generate a high number of TILs even when the number of cells obtained after dissociation is very low. These data are very important, as Rosenberg and collaborators on melanoma have clearly demonstrated that for effective cell therapy based on the infusion of TILs expanded from tumor samples, large quantities of cells are required. *In vivo* persistence is favored when the infused TILs are "young", i.e. not subjected to too many expansion cycles, which lead to an exhaustion of their potential both to expand and to mediate effective antitumor activity. [19]

Good levels of expansion rate were also documented after 2° round of rapid expansion, even that lower than those observed after the first round. This finding is not unexpected as it is well known that already activated cells, generally have a lower proliferative capacity than non-stimulated cells. It is important to underline that the phenotypic and functional characteristics of TIL after two rounds of antigen independent expansion are quite similar. Expansion rate also appeared more homogenous between patients compared to that documented 1st round TILs, probably because the quality of the cells obtained after one round of stimulation is comparable between the TILs derived from the different patients, while the cells obtained fresh after dissociation can have a different quality due to a number of variables.

Our results also showed how TILs showed good capacity to in vitro recognize and kill TC in all protocols evaluated . We documented how an increase in expansion rate is not associated with loss of potency against tumor cells, as cells with a high proliferative capacity may also show a loss in potency as they may switch to an exhausted phenotype. Despite the potency appeared to be reduced after a second round of expansion against SW480, TILs' ability to kill tumor cells remained sizeable and no significant difference was seen against autologous tumor cells between 1st and 2nd round of expansion.

It has been described that CD3+/CD8+ are in general responsible for direct killing of tumor cells. , [20]s [20], Our results documented that also CD3+/CD4+ cells are endowed with sizeable levels of tumor-specific cytotoxic activity. CD8+ T cells likely play a major role in specific tumor killing; however, it has been previously described by our and other groups that also CD4+ T cells are involved in anti-tumor effector activity. [21–23] Our results further support the role played by CD4+ T cells not only in providing help for optimal priming and expansion of tumor-reactive CD8+ T cells, but also as active effectors of the immune response. Previous studies have shown the importance of CD4+ T cells for the in vivo persistence of the immune response against viral and tumor antigens. [24] The transfer of CD4+ cells able not only to furnish help for activating and sustaining tumor-specific CD8+ cells, but also endowed with anti-tumor activity, might improve the efficacy of anti-tumor immunotherapy.

Antigen-specific CD4+ cells recognizing mutations have previously been identified in melanoma.[25] Tran and colleagues[10] demonstrated how a subpopulation of antigen-specific CD3+/CD4+ determined regression of lesions in metastatic epithelial cancer[10] and gastrointestinal cancer.[26]

Identification of surface markers which may determine the selection of an active TIL population appeared particularly difficult, as their expression is very heterogenous and peculiar for each patient. In particular, no significant difference was observed in 1st round TILs between surface markers, determining how expansion rate was not due to a higher or lower expression of a particular marker.

What we noted is the constant high expression of TIM3 in all sets of expanded TILs. Since TIM3 is considered a de-activation and exhausted phenotype marker[27], our results showed how a highly expressed TIM3 was not linked to a low potency or low expansion in TILs. CD137's expression was homogenous in all subsets just like TIM3, with the latest being lowly expressed.

Results showed PD1, CD39 and CD69 all showed peculiar features for each TILs subset: whilst CD3+/CD4+/PD1+ and CD3+/CD4+/CD39+ appeared to have a wide range of expression, it was far tighter in CD3+/CD8+ TILs. These markers also showed a significant difference in CD3+/CD4+ cells between 1st and 2nd round of expansion, as PD1 expression appeared to be lowered after 2nd round whilst CD39 and CD69 showed a higher expression.

Memory T cell compartment appeared fairly homogenous between all expanded TILs.

One chance to determine the active population, in future, could be analyzing the outcomes of patients which received infusion of TILs and cross the data between patients which showed regression and patients which had no results, as showed by Krishna[15] for melanoma.

2.5 Conclusion

To design approaches of adoptive cell transfer-based immunotherapy in patients who have a small fraction of tumor reactive T cells, it is mandatory to improve the selection, enrichment and in vitro expansion of TILs in order to obtain amount of cells compatible with ACT, able to maintain the ability to recognize and kill cancer cells. The results, albeit preliminary in this study, demonstrate that it is possible to isolate cells with TIL characteristics from the tumor samples of mCRC. These cells can be cultured in vitro with two rounds of independent antigen rapid expansion to reach quantities of TILs, starting from liver metastasis samples of CRC, even when the number of cells obtained after tumor dissociation is very low.

Evaluation of different methodological approaches to expand them in vitro demonstrated that the rapid expansion using a cocktail of homeostatic cytokines including IL-12, IL-15, IL-7 and Il-2 allow the generation of high amounts of TIL able to in vitro efficiently kill not only, long-term commercial line SW480, but also autologous TC. This approach is superior in term of final recovery of cells compared with classical protocol point out by Rosenberg and collaborators in the setting of melanoma, without losing in cytotoxic potential.

Evaluation of the expression of surface antigens demonstrates that both CD3/CD4 populations and CD3/CD8 populations are present in TILs in variable percentages depending on the patients.

Analysis of cell activation and exhaustion markers expressed by TIL in both CD3+/CD4+ and CD3+/CD8+ population documented document a high heterogeneity of expression between TIL, with little difference between the different expansion protocols.

Evaluation of memory T-cell compartment documented that the majority of TIL are T_{EM} or T_{EMRA} cells. In TIL obtained with protocol A also a measurable percentage of T_{CM} cells were documented, while this subpopulation decrease in TIL obtained with protocol B and C in both CD3+/CD4+ and CD3+/CD8+ cells.

Finally we can conclude that TILs, isolated from tumor tissue and subsequently expanded in culture, potentially possess some of the characteristics necessary to perform an anti-tumor activity in vivo. Although these results are promising, it is necessary to further expand the patient cohort to confirm the results obtained and evaluate the cellular and molecular mechanisms underlying cytotoxicity and the pathways involved in the

recognition of autologous tumor cells in order to possibly manipulate the response pharmacologically and increase the susceptibility of TCs to the action of TILs.

2.6 References

1. Khayat, D., Weil, M., Soubrane, C. & Jacquillat, C. [LAK cells and immunotherapy of cancer]. *Bull Cancer* **75**, 3–7 (1988).
2. Mandriani, B. *et al.* Adoptive T-cell immunotherapy in digestive tract malignancies: Current challenges and future perspectives. *Cancer Treatment Reviews* vol. 100 Preprint at https://doi.org/10.1016/j.ctrv.2021.102288 (2021).
3. Neelapu, S. S. *et al.* Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *New England Journal of Medicine* **377**, 2531–2544 (2017).
4. Morgan, R. A. *et al.* Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced With a Chimeric Antigen Receptor Recognizing ERBB2. *Molecular Therapy* **18**, 843–851 (2010).
5. Rosenberg, S. A., Spiess, P. & Lafreniere, R. A New Approach to the Adoptive Immunotherapy of Cancer with Tumor-Infiltrating Lymphocytes. *Science (1979)* **233**, 1318–1321 (1986).
6. Hall, M. L. *et al.* Expansion of tumor-infiltrating lymphocytes (TIL) from human pancreatic tumors. *J Immunother Cancer* **4**, (2016).
7. Creelan, B. C. *et al.* Tumor-infiltrating lymphocyte treatment for anti-PD-1-resistant metastatic lung cancer: a phase 1 trial. *Nat Med* **27**, 1410–1418 (2021).
8. Zacharakis, N. *et al.* Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med* **24**, 724–730 (2018).
9. Tran, E. *et al.* T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. *New England Journal of Medicine* **375**, 2255–2262 (2016).
10. Tran, E. *et al.* Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science (1979)* **344**, 641–645 (2014).
11. Rosenberg, S. A. & Restifo, N. P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* vol. 348 62–68 Preprint at https://doi.org/10.1126/science.aaa4967 (2015).
12. Gros, A. *et al.* PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire infiltrating human tumors. *Journal of Clinical Investigation* **124**, 2246–2259 (2014).
13. Fernandez-Poma, S. M. *et al.* Expansion of tumor-infiltrating CD8+ T cells expressing PD-1 improves the efficacy of adoptive T-cell therapy. *Cancer Res* **77**, 3672–3684 (2017).

14. Duhen, T. *et al.* Co-expression of CD39 and CD103 identifies tumor-reactive CD8 T cells in human solid tumors. *Nat Commun* **9**, 2724 (2018).
15. Krishna, S. *et al.* Stem-like CD8 T cells mediate response of adoptive cell immunotherapy against human cancer. *Science (1979)* **370**, 1328–1334 (2020).
16. Parkhurst, M. *et al.* Isolation of T-Cell Receptors Specifically Reactive with Mutated Tumor-Associated Antigens from Tumor-Infiltrating Lymphocytes Based on CD137 Expression. *Clin Cancer Res* **23**, 2491–2505 (2017).
17. Draghi, A. *et al.* Rapid Identification of the Tumor-Specific Reactive TIL Repertoire via Combined Detection of CD137, TNF, and IFNγ, Following Recognition of Autologous Tumor-Antigens. *Front Immunol* **12**, 705422 (2021).
18. Cantero-Cid, R. *et al.* PD-L1/PD-1 crosstalk in colorectal cancer: Are we targeting the right cells? 11 Medical and Health Sciences 1107 Immunology 11 Medical and Health Sciences 1112 Oncology and Carcinogenesis. *BMC Cancer* **18**, (2018).
19. Tran, K. Q. *et al.* Minimally cultured tumor-infiltrating lymphocytes display optimal characteristics for adoptive cell therapy. *Journal of Immunotherapy* **31**, 742–751 (2008).
20. Montagna, D., Maccario, R. & Locatelli, F. Expansion of antileukaemia CTL lines and clones for adoptive cell therapy in paediatric patients given allogeneic haematopoietic stem cell transplantation. *Int J Immunogenet* **35**, 389–393 (2008).
21. Montagna, D. *et al.* Ex vivo priming for long-term maintenance of antileukemia human cytotoxic T cells suggests a general procedure for adoptive immunotherapy. *Blood* **98**, 3359–66 (2001).
22. Pardoll, D. M. & Topalian, S. L. The role of CD4+ T cell responses in antitumor immunity. *Curr Opin Immunol* **10**, 588–94 (1998).
23. Guo, Y. *et al.* Direct recognition and lysis of leukemia cells by WT1-specific CD4+ T lymphocytes in an HLA class II-restricted manner. *Blood* **106**, 1415–8 (2005).
24. Marzo, A. L. *et al.* Tumor-specific CD4+ T cells have a major 'post-licensing' role in CTL mediated anti-tumor immunity. *J Immunol* **165**, 6047–55 (2000).
25. Veatch, J. R. *et al.* Tumor-infiltrating BRAFV600E-specific CD4+ T cells correlated with complete clinical response in melanoma. *Journal of Clinical Investigation* **128**, 1563–1568 (2018).
26. Tran, E. *et al.* Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science (1979)* **350**, 1387–1390 (2015).

27. Das, M., Zhu, C. & Kuchroo, V. K. Tim-3 and its role in regulating anti-tumor immunity. *Immunological Reviews* vol. 276 97–111 Preprint at https://doi.org/10.1111/imr.12520 (2017).

3 MOLECULAR ASSESSMENT OF TILs AND TCs: THE ROLE OF CA^{2+} SIGNALING PATHWAYS

3.1 Introduction

We have previously described the role of Ca2+ signaling pathways in modulating tumors' lysis and functions, in particular if they may condition their ability to grow and disseminate and how they might be a potential target for pharmacological anti-cancer therapy. We have particularly focused in the role of intracellular calcium concentration ($[Ca^{2+}]_i$) and if a modulation of such might turn into a pharmacological anti-cancer treatment. The protocol used in this study and the results obtained have been described in a previously published manuscript named "Hydrogen Sulfide-Evoked Intracellular Ca2+ signals in primary cultures of metastatic colorectal cancer cells", which was published on Cancers in 2020 (Volume 12, issue 11)[1] and are briefly summarized below.
The role of Ca2+ signaling pathways has been assessed on T cells as well, since data on TILs expanded *ex-vivo* remains limited. One of the main aims of this study was to determine if Store Operated Calcium Entry (SOCE) is up-regulated in TILs, determining a reduction in anti-tumor activity, and if the submission of determined drugs blocking SOCE may enhance TILs' anti-tumor activity. Results have been described in a published manuscript named "Store-Operated Ca2+ Entry Is Up-Regulated in Tumour- Infiltrating Lymphocytes from Metastatic Colorectal Cancer Patients" [2] of which I shared the authorship. The manuscript was published on "Cancers" in 2022 (Volume 14, issue 14) and are briefly summarized below.

3.1.1 Administration of hydrogen sulfide's donors block tumor's proliferation by increasing ($[Ca^{2+}]_i$)

We have decided to test the exogenous administration of hydrogen sulfide (H_2S) on mCRC cells, since it's been emerging as an alternative anticancer treatment. H_2S is endogenously generated from the precursor L-cysteine by pyridoxal-5' phosphate-dependent (PLP) enzymes [3,4] and its produced in response to appropriate cell stimulation. It also regulates vascular tone and blood flow, angiogenesis, synaptic transmission, cellular stress, inflammation, apoptosis and energy metabolism. [5–9] Considering its

polyfunctional role, it is of no surprise how disruption of this compound has been implicated in multiple disorders[5], whilst high cellular levels of H_2S result in severe cytototoxic effects. [10] H_2S is a double edged sword in cancer, since endogenous production determines tumor growth and metastasis by stimulating mitochondrial bioenergetics and determining an enhancement in proliferation, migration and invasion. [11–13] However, exogenous administration of H_2S through H_2A-releaseing compounds determined a strong anti-cancer effect or by inducing apoptosis or by halting cancer proliferation.[11,13,14] The mechanism whereby exogenous H_2S affects cancer proliferation could involve an increase in $[Ca^{2+}]I$, since it has been known how H_2S is able to elevate intracellular Ca2+ concentration [15] by inducing Ca2+ released from the endoplasmic reticulum [7] or my promoting extracellular Ca2+ entry by several pathways, including Transient Receptor Potential Vanilloid 1, or TRPV1. [16]

TRPV1 is a polymodal non-selective cation channel, which is gated by multiple stimuli and is regarded among the main mediators of H_2S-induced extracellular Ca^{2+} entry in healthy cells. [16,17] Notably, exogenously delivered H_2S may suppress cell proliferation by promoting TRPV1-mediated extracellular Ca^{2+} entry, since capsaicin-induced TRPV1 activation exerts an anticancer effect in CRC. [18] Therefore, the present investigation aimed at assessing for the first time whether and how exogenously added H_2S exerts an anticancer effect in primary cultures of mCRC cells. Our findings demonstrated that H_2S was able to trigger extracellular Ca2+ entry in mCRC cells by activating TRPV1 and the reverse (i.e., Ca2+ entry) mode of the Na+/Ca2+ exchanger (NCX). H_2S-induced Ca2+ entry was in turn able to suppress mCRC proliferation by arresting the cell cycle in the S-phase, thereby confirming that exogenous delivery of H_2S may represent a reliable strategy to treat metastatic CRC patients. To do so, we employed sodium hydrosulfide (NaHS), one of the most widely employed H_2S donors and provided the evidence that NaHS induced extracellular Ca^{2+} entry in mCRC cells by activating the Ca^{2+}-permeable channel Transient Receptor Potential Vanilloid 1 (TRPV1) followed by the Na^{+}-dependent recruitment of the reverse-mode of the Na^{+}/Ca^{2+} (NCX) exchanger. In agreement with these observations, TRPV1 protein was expressed and capsaicin, a selective TRPV1 agonist, induced Ca^{2+} influx by engaging both TRPV1 and NCX in mCRC cells. Finally, NaHS reduced mCRC cell proliferation, but did not promote apoptosis or aberrant mitochondrial depolarization. These data support the notion that

exogenous administration of H_2S may prevent mCRC cell proliferation through an increase in $[Ca^{2+}]_i$, which is triggered by TRPV1.

3.1.2 SOCE is enhanced in *ex-vivo* expanded TILs

Store-operated Ca2+ entry (SOCE) has long been known to regulate the differentiation and effector functions of T cells as well as to be instrumental to the ability of cytotoxic T lymphocytes to target cancer cells. At the moment, informations regarding the expression and function of SOCE in TILs that have been expanded in vitro for adoptive cell therapy (ACT) are limited. The determination of using TILs to effectively target mCRC passes through in analysis of the Ca^{2+} intracellular signaling pathways, as it is necessary to know if such signals, recruited downstream of T-cell receptors, are deranged. An increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is of paramount importance to promote the differentiation and effector functions of T cells by driving processes that are key to the immune response, including proliferation, differentiation, cytokine secretion and cytotoxicity. [19,20] TCR engagement results in the activation of phospholipase C- 1γ1 (PLCγ1) that cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ binds to and gates the ionotropic $InsP_3$ receptors ($InsP_3Rs$), which mediate Ca^{2+} release from the endoplasmic reticulum (ER), the largest endogenous Ca^{2+} reservoir in T cells. The following reduction in ER Ca^{2+} concentration ($[Ca^{2+}]_{ER}$) is detected by STIM1, which serves as sensor of $[Ca^{2+}]_{ER}$ and is thereafter prompted to translocate from perinuclear to peripheral ER cisternae, where STIM1 traps and activates the Ca^{2+}-selective Orai1 channels at the plasma membrane. [19,20] The following influx of Ca^{2+} , known as SOCE, induces the nuclear translocation of the Ca^{2+}-dependent nuclear factor of activated T cells c2 (NFATc2 or NFAT1), cytokine production and cytotoxicity. [19–21] Therefore, *ex vivo*-expanded TILs are predicted to impinge on a functional SOCE to be therapeutically effective against cancer cells. In accord, the pharmacological or genetic deletion of SOCE impairs the cytotoxic activity of human CTLs,[21] therefore it favours the engraftment of

cancer cells and promotes tumor growth. [22] Surprisingly, although neoplastic transformation may induce a dramatic derangement in the expression and/or function of the SOCE machinery,[23–25] it is still unknown whether a functional SOCE is expressed in TILs that are generated for ACT. In this work, we addressed this compelling issue by focusing on *ex vivo*-expanded TILs isolated from the liver metastases of mCRC patients. Peripheral blood T (PBT) cells from healthy donors (hPBTs) and mCRC patients (cPBTs) were used as controls, as previously suggested. [26,27]

Our results showed how SOCE amplitude is enhanced in TILs compared to hPBTs and cPBTs, but the STIM1 protein is only up-regulated in TILs. Pharmacological manipulation showed that the increase in SOCE mainly depends on tonic modulation by diacylglycerol kinase, which prevents the protein kinase C-dependent inhibition of SOCE activity. The larger SOCE caused a stronger Ca2+ response to T-cell receptor stimulation by autologous mCRC cells. Reducing Ca2+ influx with BTP-2 (a selective Orai1 inhibitor) during target cell killing significantly increases cytotoxic activity at low target:effector ratios. This preliminary evidence might suggest an alternative pharmacological strategy to improve the therapeutic outcome of ACT in mCRC patients and is thoroughly described here. [2]

3.2 References

1. Faris, P. *et al.* Hydrogen sulfide-evoked intracellular ca^{2+} signals in primary cultures of metastatic colorectal cancer cells. *Cancers (Basel)* **12**, (2020).
2. Faris, P. *et al.* Store-Operated Ca2+ Entry Is Up-Regulated in Tumour-Infiltrating Lymphocytes from Metastatic Colorectal Cancer Patients. *Cancers (Basel)* **14**, (2022).
3. Li, L., Rose, P. & Moore, P. K. Hydrogen Sulfide and Cell Signaling. *Annu Rev Pharmacol Toxicol* **51**, 169–187 (2011).
4. Paul, B. D. & Snyder, S. H. H2S signalling through protein sulfhydration and beyond. *Nat Rev Mol Cell Biol* **13**, 499–507 (2012).
5. Wang, R. Physiological Implications of Hydrogen Sulfide: A Whiff Exploration That Blossomed. *Physiol Rev* **92**, 791–896 (2012).
6. Austgen, J. R., Hermann, G. E., Dantzler, H. A., Rogers, R. C. & Kline, D. D. Hydrogen sulfide augments synaptic neurotransmission in the nucleus of the solitary tract. *J Neurophysiol* **106**, 1822–1832 (2011).
7. Potenza, D. M. *et al.* Hydrogen sulphide triggers VEGF-induced intracellular Ca2+ signals in human endothelial cells but not in their immature progenitors. *Cell Calcium* **56**, 225–234 (2014).
8. Yang, G. *et al.* H2S as a Physiologic Vasorelaxant: Hypertension in Mice with Deletion of Cystathionine γ-Lyase. *Science (1979)* **322**, 587–590 (2008).
9. Altaany, Z., Moccia, F., Munaron, L., Mancardi, D. & Wang, R. Hydrogen Sulfide and Endothelial Dysfunction: Relationship with Nitric Oxide. *Curr Med Chem* **21**, 3646–3661 (2014).
10. Jiang, J. *et al.* Hydrogen Sulfide--Mechanisms of Toxicity and Development of an Antidote. *Sci Rep* **6**, 20831 (2016).
11. Szabo, C. Gasotransmitters in cancer: from pathophysiology to experimental therapy. *Nat Rev Drug Discov* **15**, 185–203 (2016).
12. Katsouda, A., Bibli, S.-I., Pyriochou, A., Szabo, C. & Papapetropoulos, A. Regulation and role of endogenously produced hydrogen sulfide in angiogenesis. *Pharmacol Res* **113**, 175–185 (2016).
13. Hellmich, M. R. & Szabo, C. Hydrogen Sulfide and Cancer. in 233–241 (2015). doi:10.1007/978-3-319-18144-8_12.

14. Cao, X. *et al.* A Review of Hydrogen Sulfide Synthesis, Metabolism, and Measurement: Is Modulation of Hydrogen Sulfide a Novel Therapeutic for Cancer? *Antioxid Redox Signal* **31**, 1–38 (2019).
15. Szadvari, I. *et al.* Sodium/calcium exchanger is involved in apoptosis induced by H2S in tumor cells through decreased levels of intracellular pH. *Nitric Oxide* **87**, 1–9 (2019).
16. Munaron, L., Avanzato, D., Moccia, F. & Mancardi, D. Hydrogen sulfide as a regulator of calcium channels. *Cell Calcium* **53**, 77–84 (2013).
17. Yang, R. *et al.* Hydrogen sulfide maintains dental pulp stem cell function via TRPV1-mediated calcium influx. *Cell Death Discov* **4**, 69 (2018).
18. Hou, N. *et al.* TRPV1 Induced Apoptosis of Colorectal Cancer Cells by Activating Calcineurin-NFAT2-p53 Signaling Pathway. *Biomed Res Int* **2019**, 1–8 (2019).
19. Vaeth, M., Kahlfuss, S. & Feske, S. CRAC Channels and Calcium Signaling in T Cell-Mediated Immunity. *Trends Immunol* **41**, 878–901 (2020).
20. Trebak, M. & Kinet, J.-P. Calcium signalling in T cells. *Nat Rev Immunol* **19**, 154–169 (2019).
21. Feske, S., Skolnik, E. Y. & Prakriya, M. Ion channels and transporters in lymphocyte function and immunity. *Nat Rev Immunol* **12**, 532–547 (2012).
22. Weidinger, C., Shaw, P. J. & Feske, S. <scp>STIM</scp> 1 and <scp>STIM</scp> 2-mediated Ca $^{2+}$ influx regulates antitumour immunity by <scp>CD</scp> 8 $^{+}$ T cells. *EMBO Mol Med* **5**, 1311–1321 (2013).
23. Zuccolo, E. *et al.* Stim and Orai mediate constitutive Ca2+ entry and control endoplasmic reticulum Ca2+ refilling in primary cultures of colorectal carcinoma cells. *Oncotarget* **9**, 31098–31119 (2018).
24. Moccia, F. *et al.* Targeting Stim and Orai Proteins as an Alternative Approach in Anticancer Therapy. *Curr Med Chem* **23**, 3450–3480 (2016).
25. Dragoni, S. *et al.* Enhanced expression of Stim, Orai, and TRPC transcripts and proteins in endothelial progenitor cells isolated from patients with primary myelofibrosis. *PLoS One* **9**, e91099 (2014).
26. Wang, S.-F. *et al.* Early T Cell Signalling Is Reversibly Altered in PD-1+ T Lymphocytes Infiltrating Human Tumors. *PLoS One* **6**, e17621 (2011).
27. Chimote, A. A. *et al.* Kv1.3 Channels Mark Functionally Competent CD8+ Tumor-Infiltrating Lymphocytes in Head and Neck Cancer. *Cancer Res* **77**, 53–61 (2017).

ACKNOWLEDGEMENTS

Al termine di questo elaborato, ho piacere di ringraziare tutti coloro mi hanno offerto il loro supporto, professionalmente e moralmente, in questo percorso di dottorato.

Ringrazio il professor Lorenzo Cobianchi per la sua pazienza e disponibilità.

Ringrazio di cuore la professoressa Montagna per aver creduto in me e avermi dato la possibilità di partecipare a questo lavoro. Grazie a lei ho avuto la possibilità di crescere professionalmente con un progetto stimolante, originale e di grande utilità. Non ho parole per dirle quanto ha cambiato la mia vita.

Grazie al professor Moccia e alla dottoressa Faris per la loro collaborazione che mi ha permesso di vedere, in una chiave del tutto inaspettata, i progressi del nostro lavoro.

Vorrei ringraziare i miei colleghi, Enrica e Matteo, per tutto ciò che hanno fatto per me negli ultimi anni: Enrica è la definizione vivente di rigore scientifico e le sarò per sempre grata per tutta la conoscenza che mi ha trasmesso negli ultimi anni e per avermi aiutato ad avere più fiducia in me stessa. Matteo è una delle persone più brillanti che conosca e che, con pazienza ed ironia, mi ha insegnato a ragionare con il citofluorimetro.

Un ringraziamento particolare va a tutti i colleghi del laboratorio di immunologia e dei trapianti per avermi sopportato in questi ultimi anni e per avermi lasciato usare la strumentazione di laboratorio quando necessaria. Dedico di cuore il mio pensiero e il mio affetto a Stefania, Elisa e Massimo.

Tutto questo non sarebbe stato possibile senza l'aiuto della persona più importante nella mia vita: la mia mamma. Mamma, è tutto merito tuo e dei tuoi sacrifici se sono la persona che sono adesso. Grazie per la tua educazione, il tuo garbo e la tua eleganza. Grazie per la tua pazienza e per avermi impedito di mollare. Grazie per non avermi mai lasciato sola. Tu sapevi. Tutto questo è solo merito tuo, devo tutto a te. Andrò avanti solo per renderti felice, sapendo che tu ora hai la serenità che aspettavi da tanto tempo.

Ringrazio il mio caro papà per avermi insegnato l'essenza stessa dell'affetto, per tutti i suoi sacrifici, per avermi trasmesso la passione per la lettura, per la natura e per la Juventus. Per avermi fatto capire cosa è importante. So che saresti stato fiero di me. Avrei voluto leggere Carlo Levi, Fenoglio e Dostoevskji insieme a te.

Grazie a mio nonno Vittorio e mia nonna Agnese: i vostri sacrifici sono arrivati fino a vostra nipote e mi hanno permesso di studiare con serenità. Mi mancate tanto.

Ai miei amici del collegio ONAOSI: ad Antonello, Amedeo, Jay, Ilaria, Eva, Antonio, Elettra, Lorenza, Mirta, Claudia ed Edoardo. Anche se siamo lontani, ognuno per la sua strada, questo percorso è anche merito vostro. Grazie per il vostro amore, il periodo passato insieme mi ha cambiato completamente. Se persone meravigliose come voi mi vogliono bene, significa che anche io non sono poi così male.

Grazie ad Angela e Francesco, alle piccole Giulia e Alessandra e ai miei nonni adottivi Pietro, Marianna, Franco e Caterina: grazie per non avermi mai lasciata sola. Anche la mamma vi sarà eternamente grata.

Grazie al grande, immenso, immortale clan dei Rumolo: a Vittorio, Nicola, Margherita, Andrea, Caterina, Pina, Veronica, Angelo, Antonio, Gherard, Anthony ed Angelo. Siete un esempio per me, una costante, un tornado. Siete invincibili. Un ringraziamento in particolare va alla dolce Angelica e alla sua immensa forza. Vi voglio bene.

Grazie di cuore a Mattia, Angelina e al piccolo Marco per tutto l'amore che mi dimostrate ogni giorno. Grazie a Rina e Nicola, a Pina e Giannino per il vostro costante pensiero ed impegno, siete sempre nel mio cuore. Il mio pensiero e il mio affetto va anche ai "piccoli" Nicola, Carmen e Rosario.

Grazie di cuore allo zio Francesco per il suo immenso affetto e alla zia Enza per il suo costante pensiero. Grazie di cuore per il sostegno che mi dimostrate ogni giorno. State tranquilli, io vado avanti. Vi voglio bene.

Grazie allo zio Michi, alla zia Terry e alle mie cuginette Claudia ed Alessia per avermi sempre accolto in casa loro, per avermi abbracciato e sostenuto nel momento del bisogno

e per l'immensa pazienza che mi hanno sempre dimostrato. Grazie alla zia Marisa per farmi sempre ridere, anche se siamo lontane. Vi voglio bene.

Grazie a Carmen e Giuseppe per la vostra pazienza, per il vostro amore incondizionato e per essere una delle colonne portanti della mia vita. Grazie per la vostra delicatezza e per avermi sempre fatta sentire a casa.

Alla zia Anna, allo zio Donato e alla Zia Stella, e a Fabrizio, allo zio Luigi e allo zio Rocco. Ai miei cugini, Francesco e Giuseppe: grazie. Grazie per fare parte della mia vita, per avermi amato e sostenuto, per tutte le risate che ci siamo fatti e che faremo ancora in futuro. Grazie per i ricordi che custodiamo insieme della mamma e della zia Rosetta.

A Franco e Margherita Procaccio va un immenso grazie per tutto l'amore con cui mi hanno travolto negli ultimi anni: avete cambiato la mia vita, grazie a voi è più colorata e divertente. Vi voglio bene.

Grazie di cuore a Carlo, Emma e Lorenzo per tutto l'affetto con il quale mi travolgono ogni volta, sorprendendomi sempre di più. Siete una vera forza della natura!

Grazie a Carmine per tutto il sostegno e l'affetto che mi hai dato negli ultimi anni. Sei travolgente!

Grazie allo zio Mario per aver sempre creduto in me e per non avermi mai abbandonato, osservandomi discretamente da lontano, e alla piccola, grande Isabella che diventerà la più brava di tutti.

Grazie a Pietro Marino, Marta, Gina e Jessica: anche se siamo stati poco insieme, il vostro affetto ha fatto breccia nel mio cuore.

Grazie di cuore ad Enza e ai suoi Mauro e Stefano: grazie per i tuoi consigli, per il tuo affetto e per la tua presenza. Vorrei aver fatto di più per te. Ti voglio bene.

Alle dolci Patrizia e Teresa e a tutto quello che hai fatto per me e per la mamma: grazie.

Infine, uno speciale ringraziamento va al meraviglioso gatto Romeo.