Ministero dell'Istruzione,
dell'Università e della Ricerca

Università degli Studi di Palermo

INTERNATIONAL PhD IN IMMUNOPHARMACOLOGY

CYCLE: XXIII

A.A. 2009-2010-2011

SSD Med/04

Induction of efficient killing of human colon cancer initiating cells by gamma delta T lymphocytes: therapeutic applications

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Chapter I

1.1 Introduction

Colon cancer is tumor of the large intestine, the lower part of digestive system; rectal cancer affects the last several inches of the colon. Together, they are often referred to as colorectal cancers (CRC). It represents the second highest cause of cancer-related death in the Western countries. The frequency of colorectal cancer varies around the world. It is common in the Western world, where the people have adopted western diets and it is rare in Asia and Africa. CRC is growths arising from the inner wall of the large intestine, beginning as small, noncancerous (benign) clumps of cells called adenomatous polyps (Figure 1).

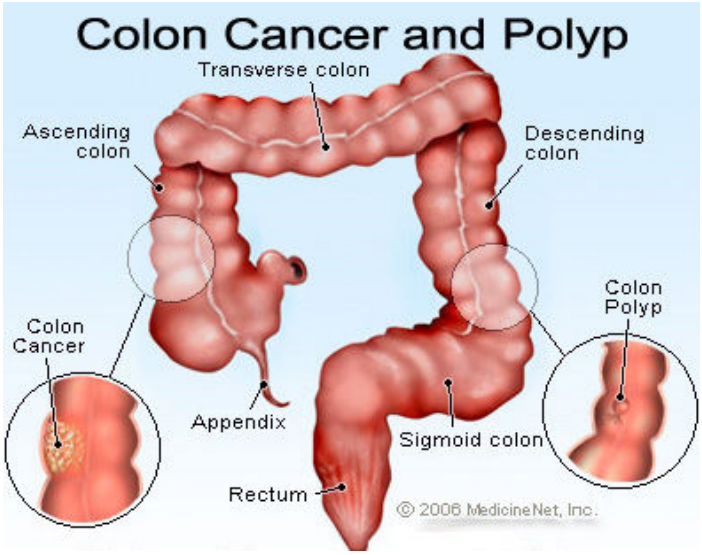

Figure 1. Schematic representation of early colon cancer stages

- age 50 or older;
- a family history of colon or rectal cancer;
- a history of polyps in the colon;
- hereditary condition, such as familial adenomatous polyposis and hereditary non polyposis colon cancer (HNPCC; Lynch Syndrome);
- a history of ulcerative colitis (ulcers in the lining of the large intestine) or Chron disease.

Colorectal cancer results from the accumulation of diverse structural and functional genomic aberrations, which develop over an extend time interval.Genetic alterations are generally random, but mutations may occur in a preferential order, correlating with histologic progression from the pre-invasive adenoma to carcinoma.

De-regulation of A*denomatous Polyposis Coli* (APC) gene represents one of the earliest alterations in the multistep process of colon carcinogenesis, proposed by Fearon and Vogelstein in 1990, causing early adenoma formation (1). In addition, mutations in BRAF and KRAS genes induce the intermediate adenoma stage, while mutations in Smad4 (*Small Mother against DPP homolog 4)*, CDC4 (C*ell Division Cycle 4*) , DCC (*Delected in Colorectal Cancer*) or MMR (mismatch repair) deficiency characterize late adenoma. Finally, p53 mutations degenerate in invasive cancer. Lastly, unknown factors lead to metastatic cancer (2,3). (Figure 2)

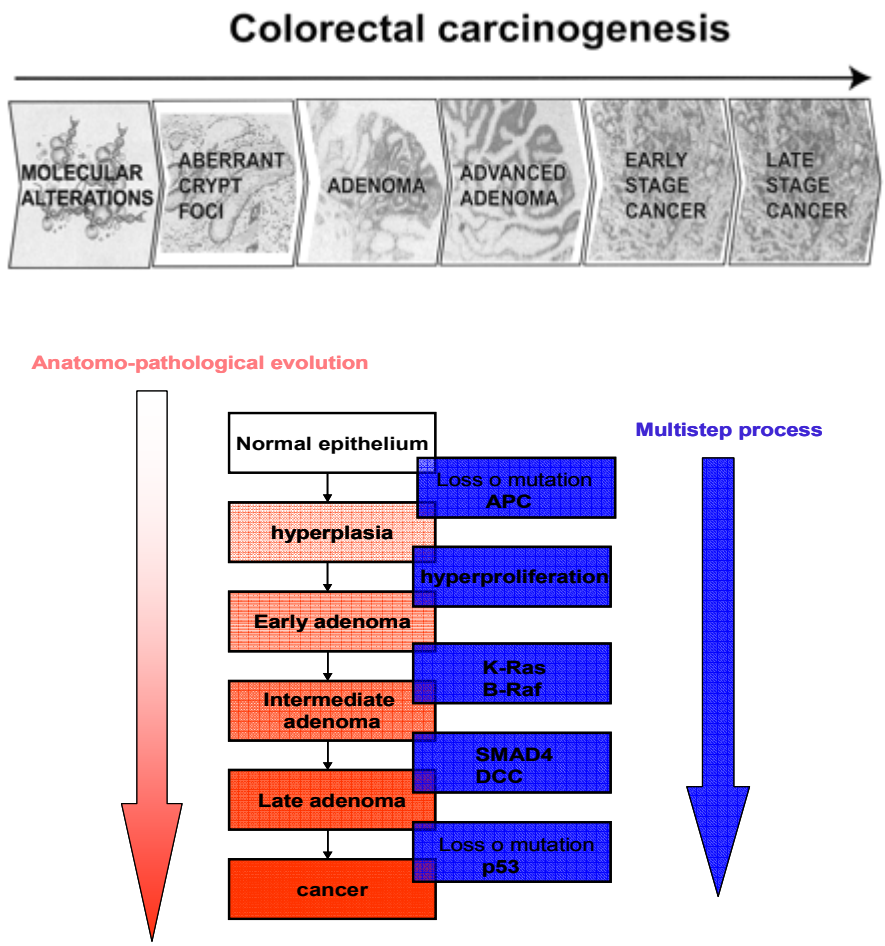

Figure 2. Anatomo-molecular correlation in colorectal cancer pathogenesis

1.2 Hystology and classification of colorectal cancer

Tumor histology will categorize the cancer into a particular type. It can be either adenocarcinoma (95 percent of all colon cancers), epidermoid carcinomas (0.52 to 0.29 percent) or other rarer types of cancer (e.g. sarcomas). The most frequent histological type of colon cancer is represented by adenocarcinoma which accounts for 90-95% among all large intestine tumors.

Colloidal or mucinous adenocarcinoma represent only 17% of colon cancer and these variants of adenocarcinoma are characterized by large amounts of extracellular mucin retained within the tumor. Furthermore, it is undifferentiated, thus it correlates with a worse prognosis.

Histological examination of the tumor also determines the grade level of the cancer. The prognosis in patients with colorectal cancer depends strongly on the degree of local tissue invasion, infiltration of adjacent organs and presence of metastases to lymph nodes or other organs. The two most common methods are the TNM classification and Dukes staging, which correlate closely with the profile prognosis and survival at 5 years. The Duke's classification, proposed in 1932, is based on tissue infiltration, lymph node involvement and the presence of distant metastases. In particular, a Dukes A colon cancer is confined to the muscular lining of the intestinal wall. A Dukes B cancer is one in which the cancer has grown beyond the muscular layer of the bowel wall, and a Dukes C colon cancer has spread to involve the lymph nodes.

The most recent TNM classification, gives more attention to the degree of tissue infiltration, discriminating among the invasion of only the mucosa, muscle layer or serosa. Staging of colon cancers is useful in predicting the probability of the cancer recurring after surgical removal. It also helps in determining whether chemotherapy may be helpful in preventing or decreasing the likelihood of a cancer recurrence. Stage I cancers have a survival rate of 85-90 percent. Stage II tumors have survival rates ranging from 55 to 80 percent. A stage III colon cancer has about a 40 percent chance of cure and a patient with a stage IV tumor has only a 5 percent chance of a cure (2,4) (Table 1).

Dukes	TNM	Numerical classification	Anatomopathological description	5 years survival
A	T1 N0 M0	I	It affects the mucosa and submucosa	>90%
B1	T2 N0 M0	I	It extends to the muscular layer	85%
B2	T3 N0 M0	II	It extends to the serosa	55-80%
C	Tx N1 M0	III	Involvement of regional lymph nodes	35-65%
D	Tx Nx M1	IV	Distant metastases	5%

Tabella 1-summary classification of colorectal cancer

Another important parameter is the degree of aggressiveness of the tumor or its cellular differentiation degree (G) raging from 1 to 3 :

- G1, well-differentiated carcinoma (25%)
- G2, moderately differentiated carcinoma (60%)
- G3, poorly differentiated carcinoma (15%)

The grading affects the prognosis that worsens progressively from G1 to G3.

Despite the prognostic power of TNM staging system, determining the outcome for patients is imprecise. Tumor cells live a complex of cellular components comprising fibroblasts, endothelial cells, and immune cells.

Solid tumors are commonly infiltrated by immune cells (e.g., T and B lymphocytes, natural killer cells, dendritic cells, macrophages, neutrophils, eosinophils, and mast cells). All of them are variably scattered within the tumor and loaded with an assorted array of cytokines, chemokines, and inflammatory and cytotoxic mediators. This complex network reflects the diversity in tumor biology and tumor-host interactions.

Recently, data collected from large cohorts of human cancers demonstrated that the immune contexture of the primary tumors is an essential prognostic factor for patients' s disease-free and overall survival.

According, recent studies argues that cancer development is strongly influenced by the host's immune system (5,6). This underlines the importance of the systemic and local immunological markers that even at the level of clinically apparent tumors should be evaluated in predicting the outcome. Moreover, this immune score approach is superior in predicting the disease outcome as compared to clinical parameters (e.g.TNM staging) (7).

In conclusion, a new classification of colorectal cancer patients could be based on the immune score.

1.3 Cancer Stem Cell hypothesis

Over the last 50 years, researchers have shown that genetic and/or epigenetic events, that can hit any somatic cells within the tissue, induce activation or inhibition of genes involved in cells proliferation (oncogenes and tumor suppressor genes respectively). Accumulation of the above reported alterations generate cells with uncontrolled proliferative potential that supports the onset and growth of tumor. According to this "clonal evolution" hypothesis (or stochastic model) described by Nowell in 1976 (7), tumor is heterogeneous and every cell within the neoplasm would have the same capacity to proliferate extensively and thus the same potential to sustain tumour growth (from an experimental point of view, each colon cancer cell can be potentially able to recapitulate the original tumour in animal models) (8,9).

However, this old concept could not explain why the current anti-cancer strategies show limited or no effects on cancer cure. Furthermore during the development, tumor tissue accumulates number of alterations over years or decades, but during the self-renewal of tissues, most of the cells are lost or eliminated within a short time and thereby losing all acquired mutations until then. These setbacks mostly reflect the need to propose a new concept of cancer that could explain the tumorigenesis. In the last few years, the cancer biology has radically changed the view of tumorigenesis; accumulating evidences have suggested that the capacity of initiating a tumour,

including colon carcinoma, is rather a unique feature of a small long-term population (residing in tissues) with stem-like properties, called “Cancer Stem Cells” (CSCs) or “tumour-initiating cells” CSCs have the exclusive ability to self-renewal property and generate a progeny of non-tumorigenic transit-amplifying cells (TAC) which give rise the non-tumorigenic differentiated population representing majority of tumor (bulk) (10,11).

Although solid evidence is lacking to date, CSCs are thought to derive from self-renewing normal stem cells (SCs) that acquire epigenetic and/or genetic changes required for tumorigenicity, or from proliferative progenitors (PCs) that reprogram themselves acquiring self-renewal capacity (12).

CSCs, as the SCs, maintain multipotentiality giving rise to a hierarchical organization of cell populations that go to aberrant organogenesis. Moreover, similarly to the SCs, the CSCs show a low cicling rate (compared to an high proliferative potential) that spares by current chemotherapy treatments which target highly proliferative cells. Chemoresistance, characterizing CSCs, is also due to high expression of proteins belonging to the family of ABC membrane transporters (which favor the cellular efflux of drugs), the presence of cytoprotective factors, such as telomerase activity and high expression of anti-apoptotic factors (Bcl-2, Bcl-xL, FLIP and PED) (13, 14).

Several experimental data from *in vitro* and *in vivo* experiments have shown that autocrine production of IL-4 by cancer cells from breast, thyroid, colon, and lung is an important negative regulator of apoptosis, conferring resistance to death receptors and chemotherapy-induced cell death (15). Moreover, it has been demonstrated that death-resistant colon CSCs also release IL-4, which installs a death-resistant phenotype.

CSCs were originally identified in acute leukemias (Bonnet et al., 1997), later in breast cancer and other solid tumors. The first identification of the CSCs within a solid tumor dates back to 2003, Al-Hajj and colleagues showed that 200 cells $CD44^{+}/CD24^{-}/lin^{low/-}$ in *fat pad* of NOD/SCID mice generated a breast cancer with the same phenotypic characteristics of original tumor (10).

1.4 Colorectal Stem Cells

The outer layer of the colon wall (mucosa), is surrounded by an absorptive and secretory epithelium, folded several times to form a set (about 14,000) of invaginations. Each unit presents about 2000 cells, named colonic crypt consisting of different cell populations: the columnar cells (also called colonocytes), abundant muco-secreting cells also known as globet cells, and a small fraction (about 1%) of entero-endocrine cells (16).

The crypt's cells possesses a high turnover rate, are costantly renewed about every 6 days in normal conditions increasing their turnover after tissue damaged. Normal SCs reside in specialized regions defined "niches"(include fibroblasts, endothelia and inflammatory cells), which provide a microenvironment designed to ensure their survival and proliferation (17). SCs removal from the niche induces the loss of their properties, while niche survives (18). It has been suggested that intestinal subepithelial myofibroblasts (ISEMFs) play a critical role in the regulation of a correct balance between SCs self-renewal and differentiation, by paracrine secretion of growth factors and cytokines (2,19). The colorectal SCs localized near or at the bottom of the crypts (Figure 3) surrounded by ISEMFs, allow this high frequency of tissue renewal, dividing asymmetrically, to generate a cell identical to itself (SC) and a PC fated to differentiate in more mature cells of colonic epithelium.

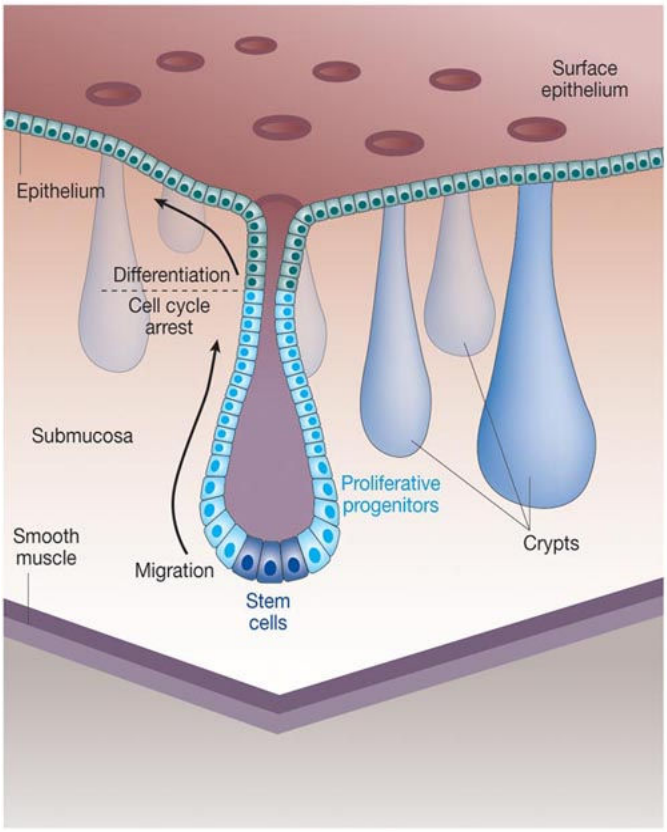

Figure 3. Schematic representation of the intestinal crypt with hypothetical stem cells localization.

The asymmetrical division generates a pool of immortal SCs. This long lived stem cells are the source of cells with mutations and epigenetic changes. According to the CSC hypothesis, such mutations would be passed on to the progeny, allowing for progression towards cancer over time and ultimately resulting in a pool of CSCs that feed neoplastic formation.

1.5 Identification of Colon Cancer Stem Cells

Recent experimental evidences suggest that a combination of several putative stemness markers could allow SCs isolation by cell sorting.

The mammalian RNA binding protein Musashi1 (Msi-1), was proposed as the first marker of colon SCs. Initially it was identified in neural stem cells and involved in their self- renewal, was also confirmed in human colon SCs (20) located at the base of the crypt of small murine and human intestine.

Barker *et al*., suggest a Wnt regulation target, Lgr5 (Leucine-rich-repeat-containing G-protein-coupled Receptor 5) protein, as elective colorectal stem marker. It's highly expressed by murine crypt base columnar cells, defined as adult stem cells, due to their ability to generate all epithelial lineages. Musashi1 (Msi-1), was proposed as the first marker of colon SCs.

Many research groups have proposed the surface molecule CD133 (Prominin-1) as a marker associated with colon cancer stemness (21). The subpopulation of $CD133^{+}$ colon tumor cells show characteristics of CSCs, these cells are highly tumorigenic and metastatic cells (22, 23).

Convincing experiments recently conducted by two independent groups showed that only the CD133+ small fraction of cells within a colon carcinoma is capable of initiating tumor outgrowth . In line with these findings, it has been recently showed that a high number of CD133-depleted cells ($2x10^{6}$ CD133 cells) from colon cancer specimens fails to generate heterotopic tumors in mouse models, whereas as few as $2.5x10^{3}$ CD133+ cells are sufficient to engraft and serially reproduce the original human tumor phenotype Although the role of CD133 in colon CSCs is still under debate, these contradictory data indicate that the use of a single marker is not always appropriate for the correct identification of stem cell population and that standardization of technical approaches for the validation of SCs markers is necessary.

1.6 Colorectal CSC: clinical implications for innovative therapies

The discovery of CSCs in a variety of tumors has changed the view of carcinogenesis and therapeutic strategies.

A combination of 5-FU, oxaliplatin and leucovorin (referred to as FOLFOX) and a combination of 5-FU, oxaliplatin and irinotecan (referred to as FOLFIRI) are the current therapy for colon cancer patients. Actually, the therapeutic approach for colorectal cancer includes anti-VEGF or EGFR monoclonal antibodies which improve positive outcomes in patients suffering from metastatic colon cancer and severe hepatic dysfunction (2). However, none of these anticancer therapies is curative in most patients with metastatic disease due to failure to eradicate the CSCs compartment. An effective therapeutic approach against cancers would eliminate the pool of CSCs which are quiescent or slowly replicating and thus more resistant to apoptosis induced by current cytotoxic regiments (Figure 4).

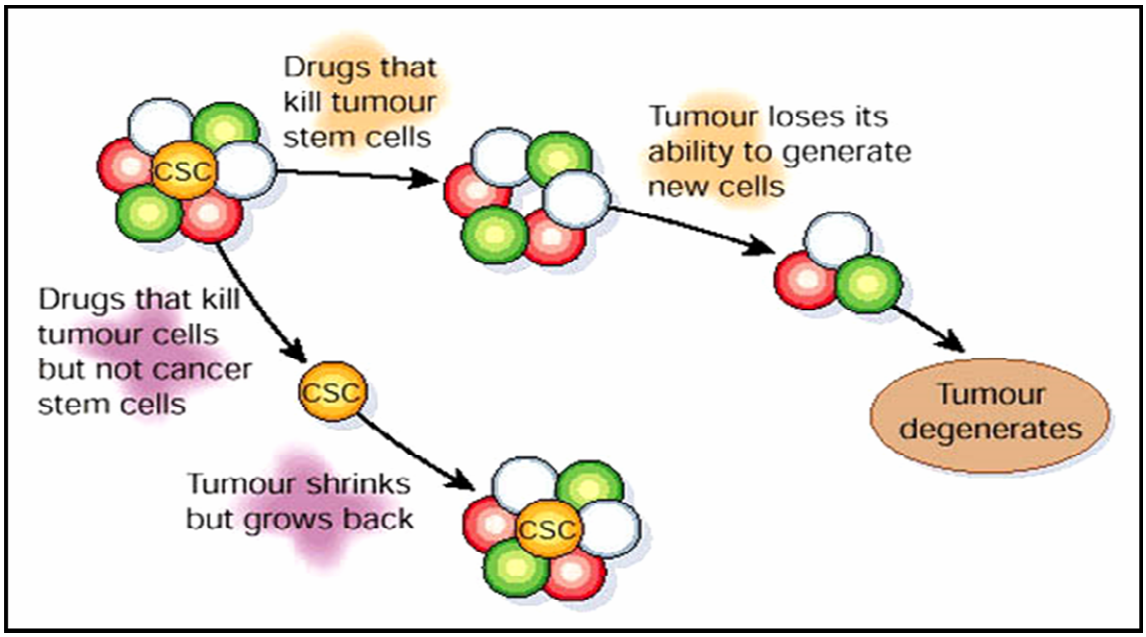

Figure 4. Resistance of CSCs to conventional therapies

The CSCs hypothesis has exciting clinical implications in colorectal cancer: it could explain therapy failure and relapses as the result of CSCs resistance to death stimuli. Indeed, retaining the biological hallmarks of tissue stem cells, such as quiescence, self-renewal ability and multidrug resistance. CSCs constitute the population intrinsically refractoriness to therapies developed to eradicate the rapidly dividing cells that constitute the majority of the non stem cell component within the tumor.

Future tumour diagnosis and treatments must be turned from eliminating the bulk of rapidly dividing but terminally differentiated components of the tumor and to be refocused on the minority stem cell population that fuels tumor growth.

It is therefore evident that a therapeutic approach to selectively target CSCs pool bypassing their chemoresistance could be more effective to eradicate bulk tumor. Recent clinical trials show that new therapeutic strategies support chemotherapy to obtain complete tumor eradication, they use:

- inhibitors of survival pathways (24)
- differentiation-inducing agents (25)
- immunotherapy

1.7 The immunotherapy

The immunotherapeutical approach is based on the stimulation of the adaptive immune response; in fact, the discovery of tumor antigens recognized by T lymphocytes has stimulated the development of a variety of cancer treatment protocols aimed at enhancing antitumor-specific T cell responses.

αβ $CD8_+$ T lymphocytes recognize CT antigens (Cancer/testis antigen also known as cancer/germline or shared tumor-specific antigens), which are particularly interesting targets because they are predicted to be expressed specifically by the tumor cells and not by the adjacent normal epithelial cells.

CT antigens are expressed in melanoma cell lines grown in embryonic stem cell media (26).

Recent evidence show that CD133+ melanoma cells have increased expression of NY-ESO-1 cancer/testis (CT) antigens and can be targeted by specific cytolytic T lymphocytes (CTLs) (27).

Furthemore, treatment of ovarian cancer cell lines with 5-aza-2-deoxycytidine upregulates CT antigen expression. (28)

However, the modern immunotherapy protocols have not been achieved yet positive clinical response. The failure of these protocols can be explained by different tumor escape mechanisms that have been defined in various types of malignancy. The best analyzed mechanism is the loss or downregulation of MHC class I antigens in tumor cells and it often renders tumor cells resistant to CD8 T-cell-mediated cytotoxicity (29). The role of other T-cell subsets, such as **γδ T** and NK cells, exhibits potent cytolytic MHC-indipendent activity against different tumor cells *in vitro*, suggesting their potential utility as an anticancer therapy (30).

1.8 γδ T cells

T cells expressing γ and δ T cell receptor (TCR) chains with limited usage represent only a small subset (2%–3%) within the total T cell population.

Of the two major subsets of human γδ T cells, Vγ9Vδ2 T cells (referred as Vδ2 T cells) predominate in the peripheral blood and they constitute an abundant effector cell reservoir for anticancer immunotherapies (31) or anti-infectious vaccines by nonpeptidic (bacterial or tumor) phosphoantigens, alkylamines, and aminobiphosphonates recognition.

The other major subset, Vδ1 T cells, also called intraepithelial lymphocytes (IELs), comprises 70%–90% of the γδ T cells in epithelial tissues, such as in the intestine and

skin (32), and could respond to stress-induced MHC class I-related chain A or B (MICA or MICB) on epithelial and tumor cells (33).

γδ T cells include different subsets that are phenotypically and functionally comparable to those in the αβ compartment: immature ("naïve") T cells and their mature counterparts.

Naive (T_{Naive}, CD45RA+/CD27+) T lymphocytes are non effector cells and numerous in the cord blood (34). On the other hand, experienced T cells comprise "central memory" (T_{CM}, CD45RA-/CD27+), "effector memory" (T_{EM}, CD45RA-/CD27-), and "effector memory" cells with terminally differentiated phenotype that re-express CD45RA (T_{EMRA}, CD45RA+/CD27-). T_{EMRA} subset exercises the effector function by potent cytolytic activity perforin release-mediated (34-37). T_{CM} Vδ2 cells are abundant in lymph nodes and lack immediate effector functions. Conversely, T_{EM} and T_{EMRA} Vδ2 effector cells are poorly represented in lymph nodes but home in peripheral tissue where they display immediate effector functions. (38).

γδ T cells have been consistently identified and isolated from tumor-infiltrating lymphocytes (TIL) in various types of cancer including prostate, colorectal, breast, ovarian, and renal carcinoma (39-43); conversely, low percentage of γδ T cells was found in melanoma derived TILs. (44)

Vδ2 T cells have been detected in the majority of colon cancer TIL populations, and the response of this T-cell subpopulation to colon cancer cells suggests that a natural immune response mediated by these lymphocytes contributes to the immunosurveillance of these tumors.

Human Vγ9Vδ2 T cells response can be tuned by a variety of synthetic phosphoantigens (BrHPP) and nitrogen-containing bisphosphonates (N-BPs), such as zoledronate, probably via accumulation of phosphoantigens inside N-BP–treated cells (45) Furthemore N-BPs exhibit direct antitumor activity by both inhibiting proliferation and inducing apoptosis in tumor cells (46).

1.9 Immunotherapy using γδ T cells

In tumor immunotherapy *in vivo* and/or *in vitro* strategies are presently being envisaged i.e., the adoptive cell transfer of *in vitro* expanded γδ T cells and the *in vivo* therapeutic administration of γδ-stimulating phosphoantigens or aminobisphosphonates (zoledronate) together with low dose IL-2. Trials with aminobisphosphonates plus IL-2 are under consideration in colon tumor and pancreatic adenocarcinoma (47).

Todaro *et al.* demonstrated that the pretreatment of chemotherapy-sensitive or resistant chronic myelogenous leukemia (CML) SCs with zoledronate induces the activation of Vγ9Vδ2 T cells in terms of proliferation, Th1 cytokines such as IFN-γ and TNF-α and cytotoxic molecules release. Zoledronate-treated CSCs were more susceptible to Vγ9Vδ2 T cell cytotoxicity and they were almost exclusively killed by perforin release rather than death receptor/ligand interactions with TRAIL and FasL. Results consistent with previous findings of perforin/granzyme-dominated killing (48,49). Furthemore, Vγ9Vδ2 T cells recognition and cytotoxicity of zoledronate-treated CML target cells was TCR-mediated, instead NKG2D and other NK ligands/receptors do not appear to contribute.

Similar results were obtained in colon cancer (50), thus intentional activation of Vγ9Vδ2 T cells by zoledronate could represent a novel strategy for colorectal CSCs adjuvant immunotherapy.

Chapter II

2.1 Introduction

Several experiments in mice models suggest that immune system plays an important role in controlling the progression of solid tumors. As, the latter are commonly infiltrated by immune cells (e.g. T and B lymphocytes, natural killer, mast cells, macrophages etc.) together with different cytochines, chemokines and cytotoxic and inflammatory mediators. This complex network reflects the diversity in tumor biology, explaining the variability of response in different patients with the same tumor. For this reason, in the last few years the tumor immune microenvironment has been extensively studied, specifically in colorectal carcinoma.

Galon et al considered the importance of TIL for tumor progression and correlation with clinical outcome. These authors assert that the ***type, density*** and ***location*** of immune cells within human colorectal tumors are *"prognostic factors superior and independent then those of UICC-TNM classification"*.

In particular they found a positive correlation between high density of cytotoxic (CD8+/GZMB+), memory (CD45RO+) T cells and a low incidence of colorectal tumor recurrence (7).

Furthermore, they showed an inverse correlation between immune cell density (immune scores) and tumor stage in fact high tumor infiltrating CD8+ / GZMB+ cytotoxic T cells were *in situ* or T1 stage tumors; in contrast, the most T4 stage tumors were low T cell infiltrated.

The current study also demostrated that in colorectal cancer patients with heterogeneous densities of CD8+ T cells (intermediate outcome), the pro-inflammatory cytokine IL17+ could be used as interesting prognostic tool to discriminate between better (low level of IL17+ cells) and worse prognosis (high level of IL17+ cells). According to, experimental evidence have recently highlighted (revealed) high percentages of

CD4+ Th17 cell population in TILs from ovarian (51), melanoma, breast and colon cancer patients suggesting that IL 17 plays an important role in tumor microenvironment (52).

In colorectal, unlike melanoma (44), tumor derived-TIL it has been consistently identified and isolated γδ T cells.

Upon recognition of non-peptidic phosphoantigens, Vγ9Vδ2 T cells exert antitumor cytotoxic action by Perforin release. Intentional zoledronate activation of Vγ9Vδ2 T cells increases activity against colorectal CSCs targets and it could represent a novel strategy for colon cancer immunotherapy (50).

As demonstrated by recent evidence, there is a prevalence of infiltrating Vδ 1 T cells in breast and prostate cancer. In breast tumor derived-TIL it was identified a dominant VγVδ 1T cell population with a potent immunosuppressive function: ability to suppress naive and effector T cell responses and to block the maturation and function of dendritic cells (44).

Although several studies have shown the presence of different type immune cells in some human cancers,. little is known regarding their generation and regulation and their functional role within the tumor microenvironment (53-55).

2.2 Aim of the study

As demonstrated in recent efforts (56) tumor immune microenvironment is related with clinical outcomes in colorectal carcinoma. Furthermore, γδ T cells populate colorectal tumor TILs and constitute an important reservoir of antitumoral effectors MHC-unrestricted.

Thus, in this work we studied density as well as phenotypic and functional characterization of γδ (and their major subtypes) colorectal tumor-derived TIL cells related to tumor grading . We also investigated the effects of colorectal tumor derived TIL Vγ9Vδ2 T cell enhancement by phosphoantigen BrHPP or Zoledronate in terms of cytokines production and cytotoxic molecules content.

Taken together, our studies could have a prognostic value and serve as intriguing target for novel immunotherapeutic approaches.

2.3 Materials and Methods

Human colorectal samples

Colorectal cancer tissues were obtained in accordance with the ethical standards of the institutional committee of human experimentation from 60 patients undergoing a colon resection for colon adenocarcinoma in Surgical Department of Policlinico "P.Giaccone" Hospital, University of Palermo, (32 women and 28 men, in a range of 53 and 87 years). Histological diagnosis was based on microscopic features of carcinoma cells determining the histological type and grade.

Peripheral blood mononuclear cells (PBMCs) (where it was possible) isolated from cancer patients were purified from peripheral blood by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and cultured in RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS), antibiotics, L-glutamine and 2-mercaptethanol.

Generation of TILs

Tumor tissue-infiltrating lymphocytes were generated from different CRC tumors (60 patients) as described previously (44).

Briefly, CRC tissues were minced into small pieces followed by digestion with two enzymes mixture containing collagenase type IV, hyaluronidase and DNAase for 1 hr at 37°C. After digestion, the cells were washed twice in RPMI 1640 and cultured in RPMI 1640 containing 10% fetal calf serum (FCS) supplemented with and 50 U/ml IL-2 for the generation of T cells.

Freshly isolated PBMCs and TILs were stimulating with BrHPP 100 nM or Ionomycin 1mg/ml and PMA 10 mg/ml for 5 hrs at 37°C. After stimulation, the cells were washed twice in RPMI 1640 and followed for labelling by monoclonal antibodies**.**

Flow cytometry analysis

The phenotipical and functional analysis of colorectal TIL γδ T cells were performed by FACS analysis after surface or intracellular staining with anti-human-specific mAbs conjugated with either different fluorochromes. These human Abs included anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD56, anti-TCR γδ, anti-Vδ2, anti-CD27 and anti-CD45RA, anti-IL17, anti-IFN-γ, anti-TNF-α, anti-IL10, anti-perforin and anti-granzyme B, all purchased from Becton Dickinson, Mountain View, CA, following manufacturer's recommendations. Cells were analyzed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). For every sample 10,000 viable nucleated cells were acquired. The results were analyzed using CellQuest software.

Immunofluorescence analysis

Immunofluorescence stainings were performed on 5-µm-thick paraffin-embedded sections of human CRC tissues. For cytoplasmatic epitopes detection, samples were permeabilized with 0.2% TritonX-100 in PBS for 10 min, blocked with 3% BSA for 30 min and exposed overnight at 4°C to antibodies against γδ T cell-receptor (GL3, mouse,

BD Biosciences Pharmingen) or isotype-matched controls at appropriate dilutions. Then, cells were treated with FITC anti-mouse (Molecular Probes, Inc.) plus RNase (200 ng/ml, Sigma) and counterstained nuclei using Toto-3 iodide (642/660, Molecular Probes). Confocal analysis was used to acquire fluorescence stainings.

Ex vivo expansion of Vγ9 Vδ1 and Vγ9 Vδ2 T cells

To expand human Vδ1 and Vδ2 T cells, PBMCs or TILs isolated from patients with colorectal cancer by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics in the presence of 100 nM BrHPP or 0.5 mM zoledronate (Novartis Pharma) and Ionomycin 1mg/ml and PMA 10 mg/ml, added at day 0. After 48 h, recombinant human IL-2 (Novartis Pharma; 50 IU/ml final concentration) was added to the cultures, and the cells were supplemented with IL-2 every 3 days. Following 12–15 day culture, 90% of the cells expressed the Vδ1 and Vδ2 TCR, as determined by flow cytometry (FACS) analysis. The cells were harvested, and purified populations of Vδ1 and Vδ2 T cells were obtained by positive selection using magnetic microbeads conjugated to antihuman Vδ1 and Vδ2 mAb (mouse IgG1k, clone B6; Beckman-Coulter-Immunotech, Marseille, France) and immunomagnetic sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells consisted of 98% Vγ9 Vδ2 T cells, as determined by FACS analysis, and cell viability exceeded 95% as determined by trypan blue exclusion.

Statistical analysis

Values of p were derived from two-tailed ANOVA tests. Values of $p < 0.05$ were considered significant

2.4 Results

TIL characterization in colorectal tumors.

As suggested by Galon et al., it exists a correlation between ***type*** and ***density*** of T infiltrating immune cells in human colorectal tumors and clinical outcome. (57,7).
We investigated the immune cellular profiles of lymphocytes infiltrating colon cancer by flow cytometry on 60 freshly resected primary tumors, using anti-CD3, anti-CD4, anti-CD8, anti-CD56, anti-CD16, anti-CD19 and anti-γδ TCR mAbs conjugated with different fluorocromes.

Flow cytometry data show that TILs consist of a heterogeneous population: the most represented population consisted of CD3+ T cells, both cytotoxic (CD8+) and helper (CD4+) T cells; also NK and B cells were represented. On the contrary, γδ T cells were in low percentage in agreement with immune cellular profile where T cells expressing γ and δ TCR chains represent only a small subset (around 2% –3%) within the total T cell population (Figure 5).

In addition, there was significant variability in the number of colorectal TILs among cancer patients, in fact we found CD3+ TIL number oscillating between 5% and 80%.

Reason of this variability remains enigmatic but it could be related to tumor type and stage, general condition and age of the patient and chemio-treatment or untreatment.

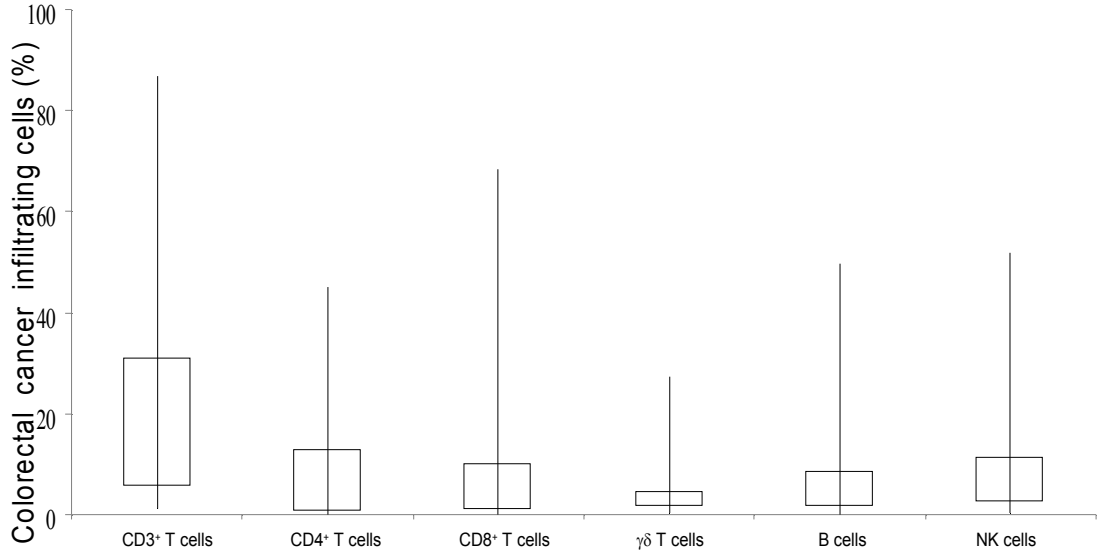

Figure 5. Analysis of colorectal tumor TILs by flow-cytometric analysis

Different location of γδ T cells

Recent bibliographic data (57) emphasize the importance of adaptive immune cell localization, within distinct tumor regions, to the risk of tumor recurrence. In particular it has been observed that patients with high infiltration of CD8+ and CD45RO+ cells in the centre and invasive margin of the tumor had a lower recurrence rate (< 10%) than patients with low infiltration of these cells in the same regions (80%).

We studied γδ T cell location within tumor, by immunofluorescence analysis on paraffin-embedded sections of human colorectal cancers. As shown in Figure 6, the positivity was detected in intra-tumor and interstitial region. Actually, we don't know the reasons of this different location, but further investigations may highlight their different response to treatments making it useful in clinical practice.

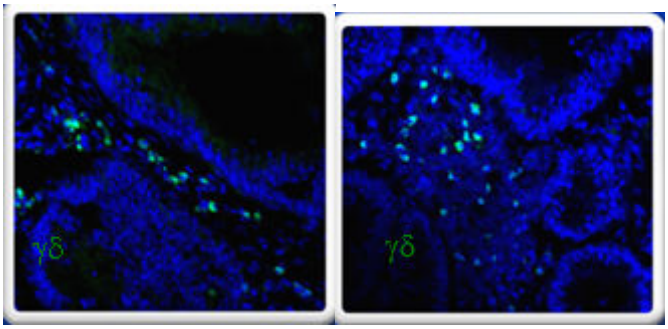

Figure 6. Different location of γδ T cells in the context of tumor, analysed by immunofluorescence analysis

Positive correlation between the increase of γδ T cells population and lower histological grade of tumors

We investigated the possible correlation between colorectal-tumor derived TIL density, in particular γδ T cells, and histological grade of human colorectal tumors.

To this goal, in the last 3 years we collected a group of 60 patients Human CRC specimens were obtained from these patients undergoing to colorectal resection, in accordance with the ethical standards of the institutional committee.

We tested the histological type and grade (G1, G2, G3) of colorectal tumor specimens by histological diagnosis based on morphological microscopic features of carcinoma cells and conduced by Phatological Anatomy Department, policlinico "P. Giaccone", Palermo.

As shown in Figure 7, the most of the analyzed samples were moderately differentiated (G2), while 11% of specimens were characterized by a poorly differentiated histotype (G3).Due to the worse prognosis related to mucinous adenocarcinoma histotype, some authors prefer to group them with the most undifferentiated tumors, we found that a good percentage (16%) of samples correspond to this inauspicious histotype and only a small percentage (6%) belong to soft tissue tumors (sarcomas) which are very rare.

After, we found a inverse correlation between infiltrating γδ T cell density and tumor grade (Table2), because G3 tumors (associated with poor prognosis) were characterized by lower tumor-infiltrating T cell number than G2 and G1 samples .

Taken together, these data suggest that a strong intratumoral immune response is associated with improved clinical outcome and survival in colorectal cancer patients according to recent experimental evidence (57).

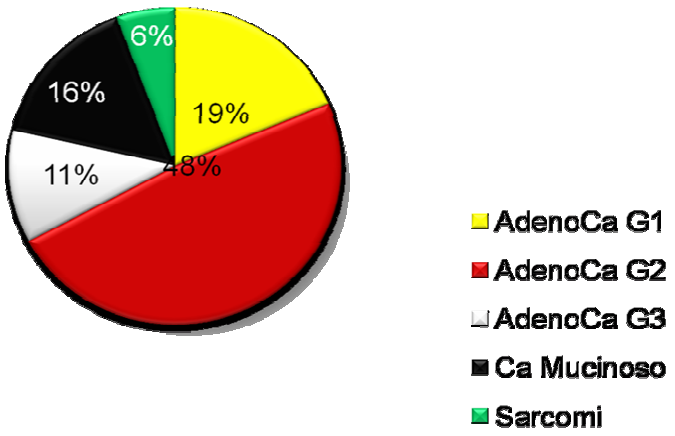

Figure 7. Schematic representation of histological characterization in cancer samples analyzed

tumor grade	γδ T cell density (%)
AdenoCa G1	3.79%
AdenoCa G2	3.1%
AdenoCa G3	2.92%
Ca mucinoso	2.91%
sarcomi	2.19%

Tab 2. Correlation between infiltrating γδ T cell density and tumor grade.

T_{EM} and T_{EMRA} are most represented phenotypes in infiltrating Vδ1 and Vδ2 T cells

In order to characterize the phenotype of different Vδ1 and Vδ2 T cell subsets we performed a polychromatic flow cytometry on purified and *ex vivo* expanded human Vδ T lymphocytes from colorectal TIL.

As shown in Figure 8 and Figure 9, infiltrating human Vδ1 and Vδ2 T lymphocytes comprise heterogeneous population of T_{Naive} and T_{CM} (non effector) cells together with 2 effector memory subsets, T_{EM} and T_{EMRA} , that are the most represented phenotypes according to their intratumoral location where effector subsets of Vδ1 and Vδ2 T lymphocytes predominate, compared to non-effector T cells, to exert antitumor action.

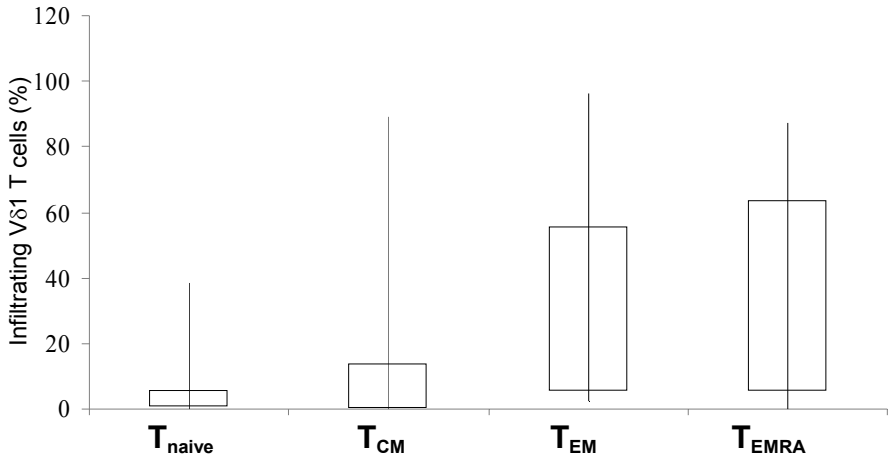

Fig 8. Phenotypic characterization of Vδ1 T cell subset by flow cytometry

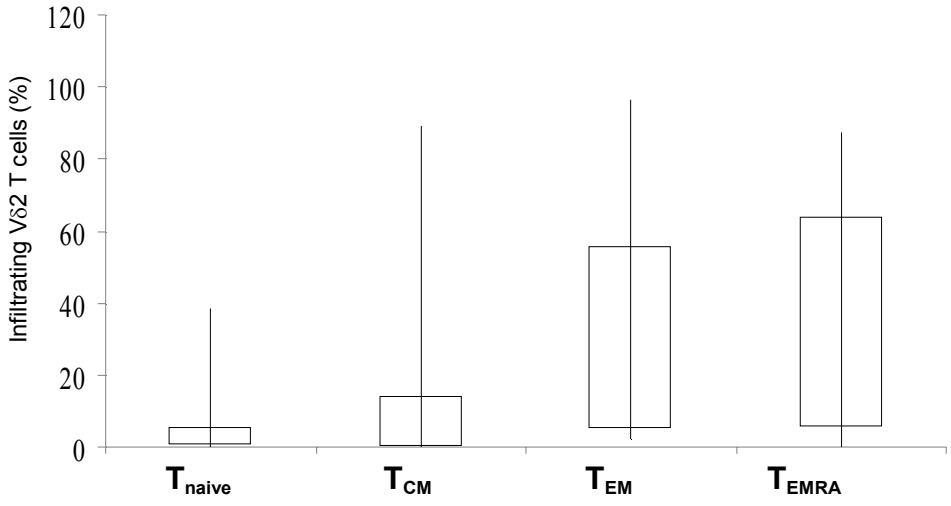

Fig 9. Phenotypic characterization of Vδ2 T cell subset by flow cytometry

Then we compared Vδ2 T cell subset phenotypes of freshly collected PBMCs with ones of colorectal TIL in each cancer patient.

As first step, we stained purified and *ex vivo* expanded human Vδ2 T lymphocytes of freshly collected PBMCs (where it was possible) and bulk colorectal TIL with CD27, CD45RA and Vδ2 antibody.

FACS analysis gating on $V\delta2^+$ T cell population revealed high density of T_{EM} (CD45RA-/CD27-) cells in the total Vγ9 Vδ2 T cell population isolated from PBMCs conversely T_{EMRA} (CD45RA+/CD27-) phenotype was the most represented in TIL-derived Vδ2 T cells (Figure 10) reflecting that upon recognition of specific tumor antigen, such as non peptidic phosphorylated metabolites, tumor infiltrating Vδ2 T cells differentiate into cytotoxic effector phenotypes i.e. T_{EMRA} in order to hit cancer targets.

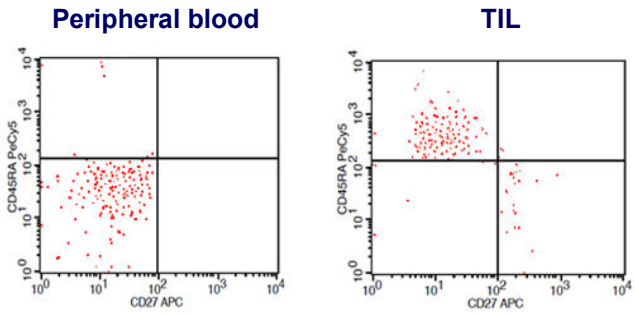

Figure 10. Comparison of Vδ2 T cell subset phenotypes between freshly collected PBMCs and colorectal TILs

Activated γδ1 T and γδ2 T cells secrete mainly cytokines such as IFN-γ and IL17.

It has been proposed that human γδ T cells have distinct patterns of activation and responses for killing tumor cells (58). These pathways include release of proinflammatory cytokines and proapoptotic molecules (Th1 cytokines as IFN-γ and TNF-α) or cell contact-dependent lysis through an NK-like or TCR-recognition.

To further elucidate activation and response mechanisms of stimulated tumor-infiltrating Vδ2 (*in vitro* stimulation with zoledronate or BrHPP) and (*in vitro* stimulation with PMA and ionomycin) Vδ1 T cells, we assayed levels of proinflammatory and proapoptotic cytokines by intracellular staining for IL-10, IL-17, IFN-γ and TNF-α by flow cytometry analysis.

We found that stimulated human colorectal infiltrating Vδ1 and Vδ2 T lymphocytes are activated as detected by multiple cytokine secretion (Fig. 11-12) including the prototypical effector cytokine, IFN-γ which is expressed at high levels (30% and 20% IFN-γ+ Vδ1 and Vδ2 T cells respectively) as well as IL 17 (35% and 45% IL17+ Vδ1 and Vδ2 T cells respectively). In addition, unstimolated Vδ1 and Vδ2 T cells produce mainly IFN-γ and IL 17 cytokines but with lower levels than stimulated T cells.

As suggested by Angelini *et al*. (34) *ex vivo* data, intracellular production of IFN- γ and TNF-α is induced by BrHPP stimulation in V δ2 T_{EM} h but not in V δ2 T_{EMRA}, thus in this study activated Vδ2 and Vδ1T subset is realistically T_{EM} rather than $T_{EMRA.}$

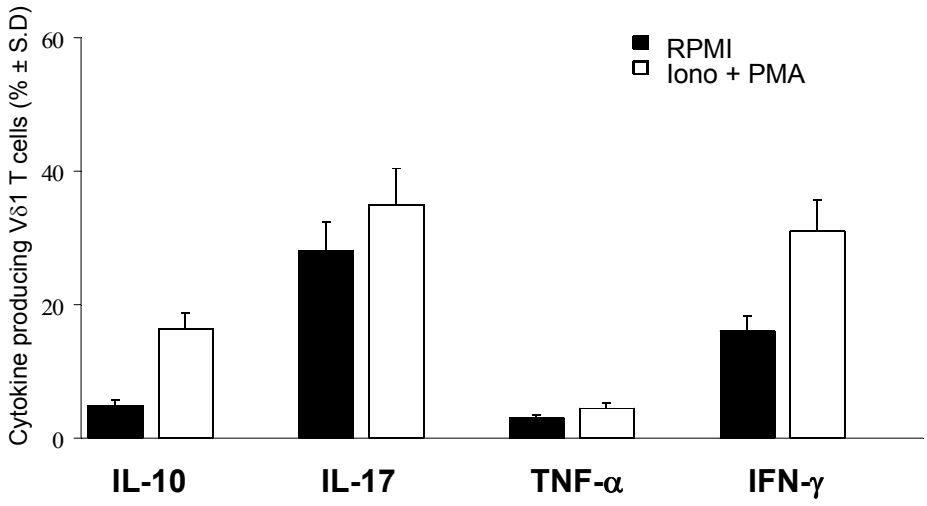

Figure 11. Cytokine production in activated tumor-infiltrating Vδ1 T cells

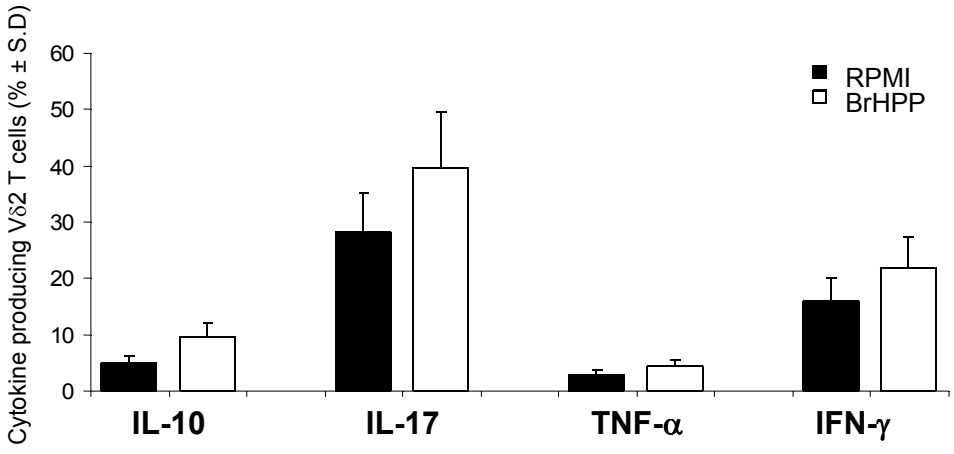

Figure 12. Cytokine production in activated tumor-infiltrating Vδ2 T cells

Activated Vγ9Vδ1 and Vγ9Vδ2 T cells induce cytotoxic Perforin/Granzyme B release

It is known that δ2 T cells kill tumor targets via a number of mechanisms including death receptor/ligand interactions with TRAIL and FasL and Perforin/Granzymes release. (59)

In order to investigate cytotoxic pathway, TIL-derived Vγ9Vδ1 and Vγ9Vδ2 T were cultured with PMA/ionomycin and phosphoantigen BrHPP or zoledronate, respectively, and upon stimulation, perforin and granzime B content were analysed by flow cytometry analysis.

As shown in Figures 13 and 14, Vγ9Vδ1 and Vγ9Vδ2 T express cytotoxic perforin and cytolytic granzime B molecules, suggesting that activated Vδ1 and Vδ2 T cells include effector subsets with cytotoxicity mediated by granule exocytosis-pathway.

Furthemore, Galon et al. evidence show that in colon tumor a strong intratumoral immune response, such as high densities of CD8+ and GZMB+ cytotoxic T cells, protects against tumor progression and disease relapse (disease recurrence); for these patients, surgery may be curative. In this light, Perforin/Granzime B could have prognostic value in colorectal cancer and could indicate whether in some patients the surgery may be curative or not.

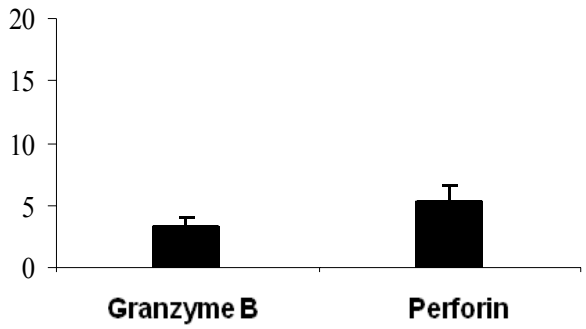

Figure 13. Perforin and Granzime B levels in activated tumor-infiltrating Vγ9Vδ1 T cells

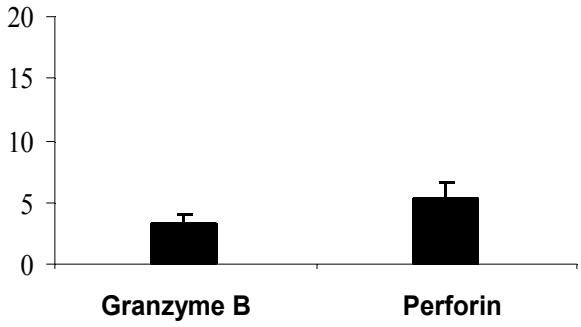

Figure 14. Perforin and Granzime B levels in activated tumor-infiltrating Vγ9Vδ2 T cells

2.5 Discussion

In colorectal cancer patients, the mean survival to 5 years is 43-47%. High mortality due to inability of actual therapy to eliminate the resistant cell clone; one of the major impediments to this goal is the chemoresistance of CSCs.

Recent clinical trials show that the immunological therapy supports chemotherapy to obtain better results; the immunotherapeutical approach is based on the stimulation of the adaptive immune response.

The γδ T-cell receptor (TCR) lymphocytes are particularly attractive for anticancer immunotherapies (31) as well as anti-infectious vaccines because this unconventional lymphoid population, contrary to "conventional" αβ TCR T lymphocytes, exhibits MHC class I–independent recognition. In tumor cells, loss or downregulation of MHC class I antigens makes γδ T-cells an important reservoir of antitumoral effectors.

Vδ2 T-cells are microbial and tumoral non peptide phosphoantigens responsive, conversely V δ1 T-cells fail to recognize these phosphoantigens (60).

Thus, it is crucial to define precisely how intentional activation by synthetic phosphoantigens (zoledronate) may be exploited to tune Vδ2 T-cells response against tumor progression. Todaro et al. (50) suggests Zoledronate treatment as a novel strategy for colon CSCs adjuvant immunotherapy.

γδ T cells have been consistently identified and isolated from several tumor-derived TIL.

As suggested by Galon et al. there is a close correlation between ***type, density, location*** **of TIL** and clinical outcome (57).

Thus, in this work the study of density as well as phenotypic and functional characterization of γδ colorectal-tumor-derived TIL cells could have a prognostic value and serve as targets for novel immunotherapeutic approaches.

Here we show that colorectal TILs consist of a heterogeneous population ($CD3^+$ T, $CD4^+$T, $CD8^+$T, γδ T, B and NK cells) with lower percentage of T cells in agreement with immune γδ cellular profile (2%–3%) within the total T cell population**.** In addition, we found significant variability in the number of colorectal TILs among cancer patient group, such as CD3+ TIL number oscillating between 5% and 80%.

We speculate that this variability could be correlated to tumor type and stage, general condition and age of the patient and chemio-treatment or untreatment raising the possibility that an assessment of CD3+ TIL may prove a useful prognostic tool for the evaluation of colorectal tumors.

We then found a inverse correlation between infiltrating γδ T cell number and histological tumor grade, because immune-cell density is lower with increasing tumor grade in agreement with to Galan's study that show a strong intratumoral immune response (CD8+ and GZMB+ cytotoxic T cells) associated to low tumor (T) stage and disease-free survival. Thus, we speculate that enhancement of natural immune response (immunosurveillance) mediated by γδ T lymphocytes could represent a novel adjuvant immunotherapy for colorectal cancer especially in cancer patients with poor prognosis (high tumor grade).

In tumor immunotherapy *in vivo* and/or *in vitro* strategies are presently being envisaged i.e., the adoptive cell transfer of *in vitro* expanded γδT cells and the *in vivo* therapeutic administration of γδ-stimulating phosphoantigens or aminobisphosphonates (zoledronate) together with low dose IL-2. Trials with aminobisphosphonates plus IL-2 are under consideration in colon tumor and pancreatic adenocarcinoma (47) .

Our findings indicate a different γδ T cell localization: intra-tumor and interstitial, however reason of different localization remains enigmatic.

We focused our studies on the two major human γδ T cell subsets: γδ 1 T cells and γδ 2 T cells. In this study we showed that, by phenotypic and functional analogy with differentiation stages of αβ T lymphocytes (61), human colorectal infiltrating Vδ1 and Vδ2 T lymphocytes comprise variable frequencies of T_{Naive} and T_{CM} non effector cells, together with 2 effector memory subsets, T_{EM} and $T_{EMRA,}$ which are the most represented phenotypes suggesting that upon recognition of specific tumor antigen, infiltrating Vδ1 and Vδ2 T cells differentiate into effector phenotypes to exert antitumor action.

In addition, we compared Vδ2 T cell subset phenotypes of freshly collected PBMCs with ones of colorectal TIL in each cancer patient and we found high density of T_{EM} (CD45RA-/CD27-) cells in the total Vγ9 Vδ2 T cell population isolated from PBMCs

conversely T_{EMRA} (CD45RA+/CD27-) phenotype was the most represented in TIL-derived Vδ2 T cells reflecting that upon recognition of specific tumor antigen, such as non peptidic phosphorylated metabolites, tumor infiltrating Vδ2 T cells differentiate into cytotoxic effector phenotypes (T_{EMRA}) in order to kill cancer targets.

By functional characterization we demonstrated that stimulated Vδ1 T cells (PMA and ionomycin) and Vδ2 T cells (BrHPP or zoledronate) are activated as detected by high expression levels of prototypical effector cytokine IFN-γ and proinflammatory cytokine IL17 (minor IL10 and TNFα levels). Recent experimental data demonstrate that colorectal cancer patients with high expression of the IL17 had a poor prognosis (7, 56).

Finally, in this work we showed that activated TIL-derived Vδ1 and Vδ2 T effector subsets have cytotoxic action mediated by Perforin/Granzime B release.

Taken together, our data show that Th1 or cytotoxic cell markers such as Perforin/Granzime B or IL17 could represent not only a prognostic marker but, even more importantly, a novel immunotherapy target.

Furthermore, recent evidence dimostrate that NK-like Vδ2 T_{EMRA} subset is highly active against tumoral target cells and responds to activation via CD16 (but not phosphoantigens) suggesting that cytokines environment is crucial in the development of distinct subsets rather than antigen recognition (34). An intriguing future goal could be to investigate the tumor microenvironment conditions that induce selective differentiation into human Vδ2 T_{EMRA} lymphocytes generating cytotoxic effectors for anticancer immunotherapy purposes.

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