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"Characterization of human γδ T cells infiltrating prostate cancer"

Tesi di Dottorato

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Salmo 23 Il Buon Pastore

Il Signore è il mio pastore:
non manco di nulla;
su pascoli erbosi mi fa riposare
ad acque tranquille mi conduce.
Mi rinfranca, mi guida per il giusto cammino,
per amore del suo nome.

Se dovessi camminare in una valle oscura,
non temerei alcun male, perché tu sei con me.
Il tuo bastone e il tuo vincastro
mi danno sicurezza.

Felicità e grazia mi saranno compagne
tutti i giorni della mia vita,
e abiterò nella casa del Signore
per lunghissimi anni.

Index

Characterization of human infiltrating γδ T cells in prostate cancer.

Preface

Tumor-infiltrating lymphocytes are key mediators of tumor immune surveillance and are important prognostic indicators in cancer progression. Among the various lymphocyte subsets implicated in protection against cancer are γδ T lymphocytes, which can kill tumor cells and secrete potent antitumor cytokines. The role of γδT cells is controversial. They are potent mediators of antitumor cytotoxicity and have shown promising efficacy in early phase clinical trials when they were utilized in adoptive immunotherapy. Recent studies have demonstrated that cells with the Vδ1 chain and those with neither Vδ1 nor Vδ2 chains have properties which may make them more attractive anticancer effectors in adoptive immunotherapy [1].

By contrast, other reports have revealed an unexpected series of protumor functions of γδ T cells in mouse models

and human patients. In particular, specific γδ T-cell subsets are capable of recruiting immunosuppressive myeloid populations, inhibiting antitumor responses, and enhancing angiogenesis, thus promoting cancer progression. A common mediator of such functions appears to be the cytokine IL17, whose pathogenic effects can override the antitumor immune response orchestrated by IFNγ [2].

The implications for the manipulation of γδ T cells in cancer immunotherapy is an important field of study.

The first part of this work introduces general immunology aspects and afterwards describes T lymphocytes γδ T cells subpopulation, specifically their features and biological functions, their identification and activation methodologies.

More concretely, we have studied that population over prostatic tissue samples, taken from multiple biopsies coming from subjects who potentially have a prostate tumor on the basis of high serum PSA combined with digital rectal exploration of the prostate.

Based on this, the aim of this research has been to evaluate the frequency, the phenotype and the effector function of prostate cancer-infiltrating δ1 and δ2 T cells in prostatic biopsy samples and the correlation of these data with the

presence of cancer versus healthy tissues and, in cancer tissues, with Gleason pattern. The analysis has been performed in circulating γδ T cells of the same patients and as control in healthy subject who, after transrectal echo guided prostatic biopsy, did not receive cancer diagnosis. Given that γδ T cells mediate antigen-specific killing of tumor cells, we also studied the representation and the in vitro cytokine production, in particular interferon γ and interleukin 17.

Chapter 1

Prostate Cancer

1.1 Introduction

Prostate cancer has been the most common noncutaneous malignancy in U.S. men since 1984, now accounting for one quarter of all such cancers (American Cancer Society, 2008). The estimated lifetime risk of disease is 16.72%, with a lifetime risk of death at 2.57%. Prostate cancer incidence varies by race/ethnicity, with African-Americans at highest risk. The incidence of prostate cancer peaked in 1992 (approximately 5 years after introduction of the prostate-specific antigen [PSA] screening test).

Incidence rates show that prostate cancer is the fifth most common malignancy worldwide and the second most common in men [3]. Prostate cancer makes up 11.7% of new cancer cases overall, 19% in developed countries, and 5.3% in developing countries. Its incidence varies widely between countries and ethnic populations, with disease rates differing by more than 100-fold. The lowest yearly incidence

rates occur in Asia (1.9 cases per 100,000 in China) and the highest in North America and Scandinavia, especially in African-Americans (249 cases per 100,000).

Prostate cancer is rarely diagnosed in men younger than 50 years old, accounting for only 2% of all cases [4]. The median age at diagnosis is 68 years.

In addition to changes in prostate cancer incidence and mortality over the last several decades, there has been a substantial shift to more favorable stage at presentation in men with newly diagnosed disease. This clinical stage migration is largely if not exclusively accounted for by PSA screening Since the introduction of PSA testing, the incidence of local-regional disease has increased, whereas the incidence of metastatic disease has decreased Non palpable cancers (American Joint Committee on Cancer [AJCC] clinical stage T1c) now account for 60% to 75% of newly diagnosed disease Clinical stage migration has also been associated with improvements in 5- and 10-year disease-specific survival [5-7].

These observations have been consistent across the United States and Europe [8,9]

1.2 Risk Factors

Although the specific causes of prostate cancer initiation and progression are not yet known, considerable evidence suggests that **both genetics and environment play a role in the origin and evolution of this disease.** Traditional and molecular epidemiology have identified a number of potential risk factors associated with the development of prostate cancer.

1.3 Familial and genetic

Ample epidemiologic evidence suggests that prostate cancer has both a familial and genetic component.

Results of a meta-analysis demonstrate that relative risk increases according to the number of affected family members, their degree of relatedness, and the age at which they were affected [10].

Sporadic cancers account for about 85% of all prostate cancers and about 15% are familial and/or hereditary. Hereditary prostate cancer accounts for 43% of early onset disease (55 years of age or younger) but only 9% of all cancers that occur by 85 years of age [11].

1.4 Inflammation

Chronic inflammation leading to cellular hyperproliferation to replace damaged tissue contributes to the development of infection-associated cancers of the colon, esophagus, stomach, bladder, and liver [12]. Accumulating epidemiologic, histologic, and genetic evidence suggests

that a similar process may underlie the development of prostate cancer.

Inflammatory infiltrates and the histologic lesion called proliferative inflammatory atrophy (PIA) are frequent in clinical prostate specimens [13]. PIA is a spectrum of lesions characterized by epithelial atrophy, low apoptotic index, and an increased proliferative index usually associated with inflammatory infiltrates. Inflammation in PIA may include mononuclear infiltrates in the stroma and macrophages and/or neutrophils in the glandular lumen or epithelium. Macrophages activated by interferon-γ secrete proinflammatory cytokines and reactive nitrogen species (e.g., nitric oxide). Inducible nitric oxide synthase, which catalyzes the generation of nitric oxide, is overexpressed in macrophages in PIA but not in normal epithelium. PIA appears to be a regenerative lesion appearing as a consequence of infection or cell trauma resulting from oxidant damage, hypoxia, infection, or autoimmunity, and its hyperproliferative state may lead to cancer. PIA is often found adjacent to high-grade prostatic intraepithelial

neoplasia (HGPIN) or early cancer and there is an identifiable genetic pathway between PIA, HGPIN, and cancer [14-16], see figure 1.

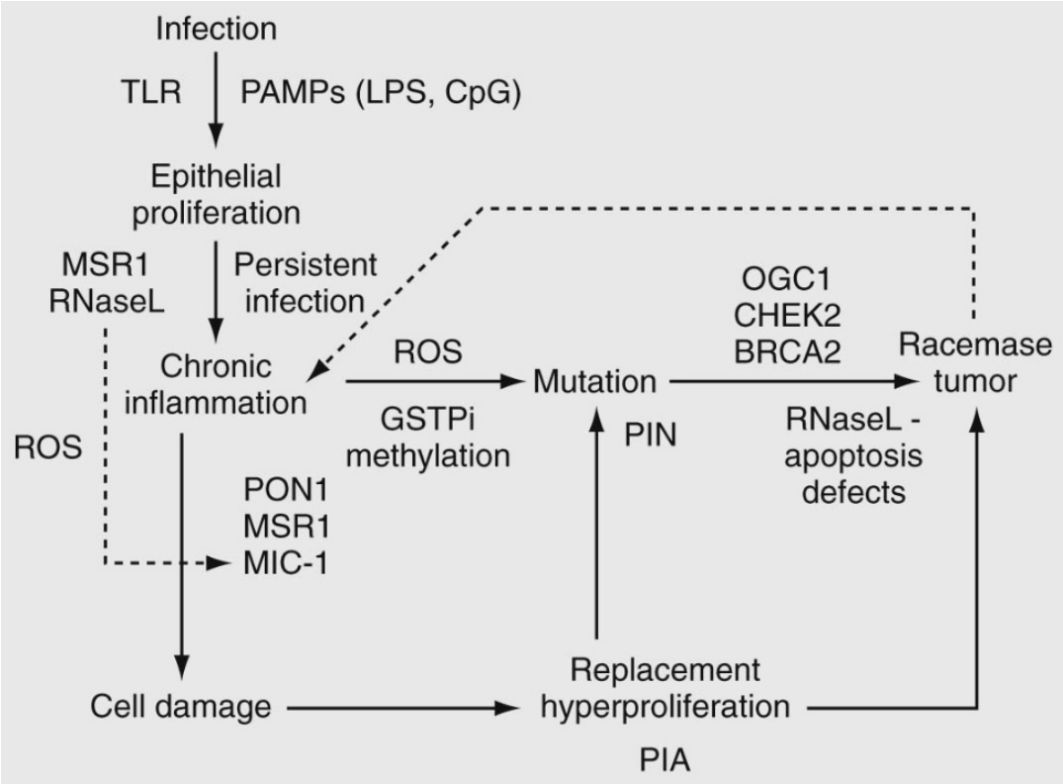

Figure 1 Genetic pathway

The previously described genetic and histologic observations in prostate cancer strongly suggest that compromised cellular defenses against inflammatory oxidants may initiate and/or perpetuate prostatic carcinogenesis.

1.5 Other factors

Androgens influence the development, maturation, and maintenance of the prostate, affecting both proliferation and differentiation of the luminal epithelium. There is little doubt that exposure of the prostate at key developmental times to androgens plays an important role in prostate carcinogenesis. Androgens are also important in the maintenance of established cancers, as supported by the historical observation that the majority of prostate cancers initially respond to androgen-deprivation therapy, and more recently by results of the Prostate Cancer Prevention Trial, which indicated that inhibition of the conversion of testosterone to the more potent dihydrotestosterone by finasteride reduces the incidence of prostate cancer by approximately 25% [17]. In addition, genetic polymorphisms of the androgen receptor (AR) [18, 19] the 5α-reductase type 2 isoenzyme [20], and the genes involved in biosynthesis of testosterone have also been implicated in prostate carcinogenesis [21].

Vitamin D (1, 25 dihydroxyvitamin D_3) is an essential vitamin that is a part of the steroid hormone superfamily. Human sources include both dietary intake and conversion from an inactive to active vitamin D in the skin through sunlight exposure. Interest in vitamin D as a determinant of prostate cancer risk comes from several epidemiologic observations [22]:

1. Men living in northern latitudes with less exposure to sunlight-derived ultraviolet exposure have a higher mortality rate from prostate cancer.
2. Prostate cancer occurs more frequently in older men, in whom vitamin D deficiency is more common both because of less ultraviolet exposure and age-related declines in the hydroxylases responsible for synthesis of active vitamin D.
3. African-Americans, whose skin melanin blocks ultraviolet radiation and inhibits activation of vitamin D, have the highest worldwide incidence and mortality rates for prostate cancer.

4. Dietary intake of dairy products rich in calcium, which depresses serum levels of vitamin D, is associated with a higher risk of prostate cancer.
5. Native Japanese, whose diet is rich in vitamin D derived from fish, have a low incidence of prostate cancer.

In addition, prostate cancer cells express the vitamin D receptor, and several studies have demonstrated an antiproliferative effect of vitamin D on prostate cancer cell lines by inducing cell cycle arrest [23].

Many studies show no or a weak association between vitamin D levels and prostate cancer risk [24-27]. The Cancer Prevention Study II Nutrition Cohort, a prospective cohort of 65,321 men demonstrated a modestly increased relative risk of 1.2 for total calcium intake (dietary and with supplements) and 1.6 for high dietary calcium intake alone (≥2000 vs. <700 mg/day), but not for dairy intake [28]. The results suggest that very high calcium intake, above daily recommendation, may modestly increase risk. These conflicting results regarding vitamin D, calcium, and

prostate cancer risk may be explained by variants in the vitamin D receptor (VDR). Polymorphisms resulting in a VDR with lower activity have been associated with increased risk for prostate cancer, as well as with increased risk of biochemical recurrence following radical prostatectomy [29-31].

1.6 Prostate cancer pathology

Most prostate cancers are adenocarcinomas (95%).

Although numerous grading systems exist for the evaluation of prostatic adenocarcinoma, the Gleason grading system is the most widely accepted [32]. The Gleason system is based on the glandular pattern of the tumor as identified at relatively low magnification Cytologic features play no role in the grade of the tumor. **Both the primary (predominant) and the secondary (second most prevalent) architectural patterns are identified and assigned a grade from 1 to 5, with 1 being the most differentiated and 5 being the least differentiated**. **Because both the primary and the**

secondary patterns are influential in predicting prognosis, there is a Gleason sum or score obtained by the addition of the primary and secondary grades. If a tumor has only one histologic pattern then for uniformity, the primary and secondary patterns are given the same grade.

Prostatic intraepithelial neoplasia **(PIN)** is characterized by cytologically abnormal cells lining existing prostatic ducts and acini, and is classified as either low- or high-grade (LGPIN, HGPIN). HGPIN most commonly occurs in the peripheral zone of the prostate (75 to 80% of cases) and rarely in the central zone (5% of cases). HGPIN is very common in specimens from radical prostatectomy (85 to 100% of cases). This finding, along with other evidence, suggests a strong link between HGPIN and carcinoma of the prostate.

PIN can be recognized microscopically at low magnifications by three main characteristics: darker lining of ductal structures, thicker lining compared to ducts and acini in surrounding normal tissue, and complex intraluminal

growth. Three characteristics at high magnification are: nuclear enlargement and stratification, hyperchromasia, and prominence of nucleoli.

It appears that high-grade PIN is a precursor lesion to many peripheral intermediate- to high-grade adenocarcinomas of the prostate. However, PIN need not be present for carcinoma to arise.

1.7 Prostate biopsy

Early prostate cancer detection has benefited greatly from prostate-specific antigen (PSA) screening efforts, the introduction and refinement of systematic transrectal ultrasonography (TRUS)–guided prostate biopsy techniques, and increased public awareness about prostate cancer.

Prostate cancer's potential long natural clinical history is reflected in tumors found during autopsies carried out following other causes of death. The incidence of latent or autopsy cancer is much greater than cases of clinical cancer. The biopsy grade, clinical stage, PSA level and, when

available, the results of imaging studies can provide such prognostic information. Several treatments are available: radical prostatectomy and conformational radiotherapy for localized disease are potentially curative therapies; hormonal therapies (antiandrogenic) is the treatment of choice for advanced and metastatic disease. In asymptomatic, well selected and informed men, an active surveillance may be an option [7, 33]. Patients with androgen-indipendent prostate cancer have a median survival of only 20 months despite of the new chemotherapeutic therapies, docetaxel based.

The lack of effective and curative therapies for Hormon Refractory Prostate Cancer (HRPC) has fueled an intensive search for novel modalities, including immunotherapy [34,35].

The first step for the characterization of prostate cancer is the exact diagnosis and stadiation of the disease. The suspicious of prostate cancer derives from an elevated prostatic specific antigen (PSA) value and/or a suspected determination of the prostate during the digital rectal

examination. In case of suspected prostate cancer, the definitive exact diagnosis is made only by the prostate biopsy that is today performed under ultrasonographic transrectal guide.

Chapter 2

2.2 γδ T Cell

γδ T lymphocytes are important effector cells that may play a critical role in cancer immune surveillance [37] and recent clinical trials support their use as immunotherapeutic agents, either via the adoptive transfer of ex vivo expanded γδ T cells or following the activation of γδ T cells *in vivo*, by compounds such as phosphoantigens or aminobisphosphonates [38-39].

γδ T cells can be divided into three main populations based on δ chain expression, phenotypic and functional parameters: γδ T cells expressing the Vδ1 T-cell receptor (TCR) chain, which are mostly found in mucosal tissues, where they are involved in maintaining epithelial tissue integrity when facing damage, infection, or transformation, responding to stress antigens on epithelial cells and producing IL-10 but little or no IL-2, IL-4 or IFN γ. The second population of γδ T express Vδ2 TCR chain, products and represents the majority of circulating γδ T lymphocytes in healthy human adults, comprising up to 50%–90% of the

peripheral γδ T-cell population and secondary lymphoid organs [40]. The Vδ2 chain pairs almost exclusively with Vγ9 (also termed Vγ2). One attractive feature of Vδ2 T cells is that they can serve as professional antigen-presenting cells (APC) [41].

Activated Vδ2 T cells acquire characteristics of professional APC, such as the expression of antigen presenting, co stimulatory, and adhesion molecules, including MHC-II, CD_{80}, and CD_{86}. It is worthy of note that rapamycin or IL-18 treatment is reported to increase the expression of MHC-II, CD80, and CD86 on Vδ2 T cell lines [42]. The third population is Vδ3 T cells, which take up about 0.2% of circulating T cells including CD4+, CD8+, and CD4-CD8- subsets. They are expressed CD56, CD161, HLA-DR, and NKG2D but without NKG2A and NKG2C. The Vδ3 T cells are found only a little in blood but are rich in liver and in patients with leukemias and some chronic viral infections. Through mitogen stimulation with IL-2, Vδ3 T cells were expanded to recognize CD1d. Once activation, they can kill CD1d+ target cells, release

cytokines such as Th1, Th2, and Th17, and induce maturation of dendritic cells (DCs) into APCs [43].

It is currently believed that, due to their extremely limited diversity, Vδ1 T cells may not respond to a diversity of microbial antigens but rather to unique "stress antigens" that are markers of cell infection or transformation. Vδ1 T cells reside mainly within epithelial tissues such as the intestinal epithelium and epidermis, where they might provide a first line of immunosurveillance against malignancy by recognizing ligands such as MHC Class I-related molecules (i.e., MICA and MICB), whose expression can be induced in response to infection, injury or cellular transformation [44]. These molecules have no role in the presentation of peptide antigens, but may function themselves as tumor associated antigens. Vδ1 T cells can inhibit tumor cell growth and recruit other immune cells by releasing a number of cytokines, including tumor necrosis factor α and interferon γ. Because of Vδ1 T cells reside mainly within epithelial tissues, we consider prostate cancer an

appropriate model to study the antitumor properties of Vδ1 T cells [36].

T cells expressing the Vδ2 gene usually account for more than 90% of the circulating γδ T-cell pool (representing about 1–10% of human peripheral lymphocytes), while intraepithelial γδ T cells more commonly express the Vδ1 gene. Although no major differences exist relative to effector functions between T cells expressing the aß TCR and γδ T cells, the latter are capable of recognizing tumor-associated ligands that are neglected by conventional aß T cells in an MHC-independent manner [48]. Many preclinical studies and clinical trials have focused their attention on Vγ9Vδ2 T cells, as they can be easily isolated from the peripheral blood of most individuals and activated with conventional drugs such as synthetic phosphoantigens and aminobisphosphonates, see Fig.2.

The Vγ9Vδ2 displays a broad reactivity against microbial agents and tumors, recognize both microbial metabolites (intermediates of the non-mevalonate pathway of isoprenoid biosynthesis) and endogenous metabolites of the

mevalonate pathway, whose production is up-regulated upon cell stress.

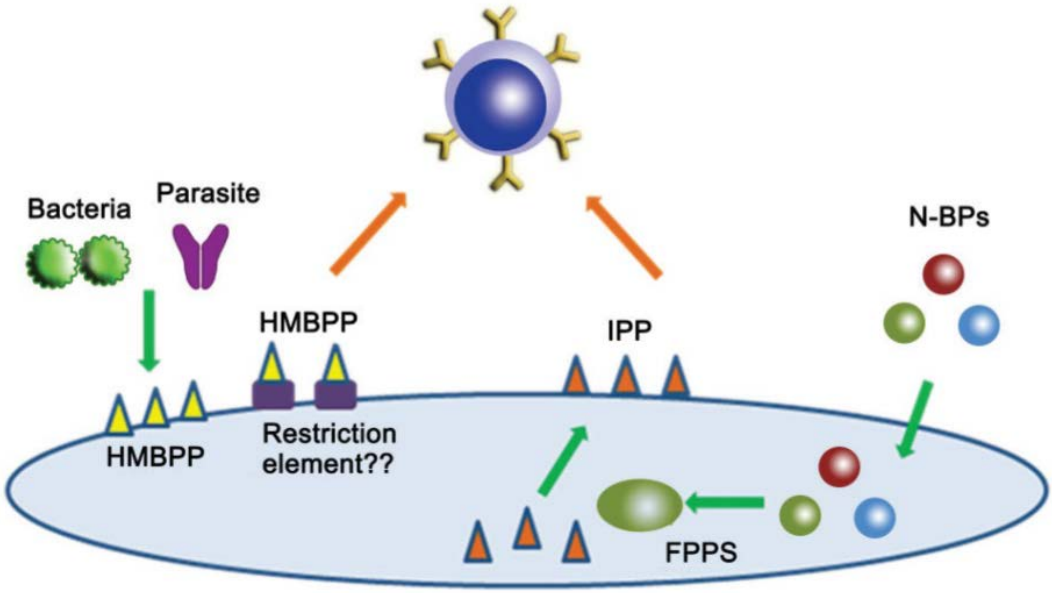

Figura 2 Nonpeptide antigens for γδ T cell stimulation.

2.2 γδ T cell receptor

Functional responses by γδ T cells can be stratified by the variable (V) region of the TCRδ chain. In humans, the TCRδ locus (TRD) lies within the TCRα locus (TRA). Three unique Vδ alleles, TRDV1, TRDV2, and TRDV3, code for TCRδ1, TCRδ2, and TCRδ3, respectively. Additionally, shared Vδ and Vα

variable regions exist in TRDV4/TRAV14, TRDV5/TRAV29, TRDV6/TRAV23, TRDV7/TRAV36, and TRDV8/TRAV38-2 loci.

Recombination of these shared V alleles with a TRA junction region (TRAJ) results in TCRα14, TCRα29, TCRα23, TCRα36, and TCRα38-2, respectively, but recombination of the shared V alleles with TRD junction(TRDJ) and diversity(TRDD) regions resultsinTCRδ4, TCRδ5, TCRδ6, TCRδ7, andTCRδ8, respectively (13). Expression of TCR γδ heterodimers on the T-cell surface in the thymus inhibits recombination of TCRb-chain locus during the CD4neg CD8neg stage there by committing the T cell to the γδ T-cell lineage [45]. This double negative status is typically maintained up on exit from the thymus, most likely because co-receptors are dispensable for functional TCR γδ binding to antigens [46]. However, the thymus is not required to complete all γδ T-cell development, as many γδ T cells directly take up residence in peripheral tissues following exit from the bone marrow and exhibit immediate effector functions against pathogens [47]. Human TCR γδ ligands are MHC/peptide complex-independent and are therefore conserved among stun

related individuals. Most of the known human ligands are specific for TCRδ1 orTCRδ2. TCRg1/TCRδ1 (alternativelytermedVγ1Vδ1) heterodimers have specificity for MHC Class-I chain-related A (MICA) [48], a molecule participating in evasion of immune surveillance following viral infection and expressed on tumor cells as it is involved in the cellular stress response [49]. In contrasttoVδ1 andVδ2 cells, very little is known about human γδ T cells expressing other TCR γδ alleles except for indirect evidence ofVδ3 cell's immunity against CMV and HIV [50]. Given the multivalent nature of γδ T cells, harnessing γδ T cells populations with polyclonal TCR repertoire is attractive for adoptive immunotherapy.

2.3 Classification and Funtions of γδ T cells

γδ T cells are distinguished from their αβ T cell counterparts by utilizing a distinct set of somatically rearranged variable (V), diversity (D), joining (J), and constant (C) genes. Compared to αβ T cells, available germ line repertoires of Vγ and Vδ genes are limited. γδ T cells contain fewer V, D, and J segments than αβ T cells. Regarding evolutionary forces that shape the V, D, and J gene segments, distinct forces were involved in the formation of the γ and δ loci. In fact, the primate Vγ and Jδ genes are highly conserved, whereas, the γ-locus is split: the Vγ9, Vγ10, and Vγ11 genes represent the conserved region of the Vγ gene locus, but the remaining Vγ genes have been evolving rapidly [51].

Similar to CD4 and CD8 T cells, Vγ9Vδ2 T lymphocytes are heterogeneous and comprise distinct populations that can be distinguished on the basis of surface marker expression and effector functions, such as cytokine secretion and cytotoxicity, [52]: naive (Tnaive) CD45RA+CD27+ and central

memory (TCM) CD45RA–CD27+ cells that express lymph node homing receptors, abound in lymph nodes and lack immediate effector functions, effector memory (TEM) CD45RA–CD27– and terminally differentiated (TEMRA) CD45RA+CD27– cells that express receptors for migration to inflamed tissues, are poorly represented in the lymph nodes while abounding at sites of inflammation, where they display immediate effector functions [53], as shown in Fig 3 .

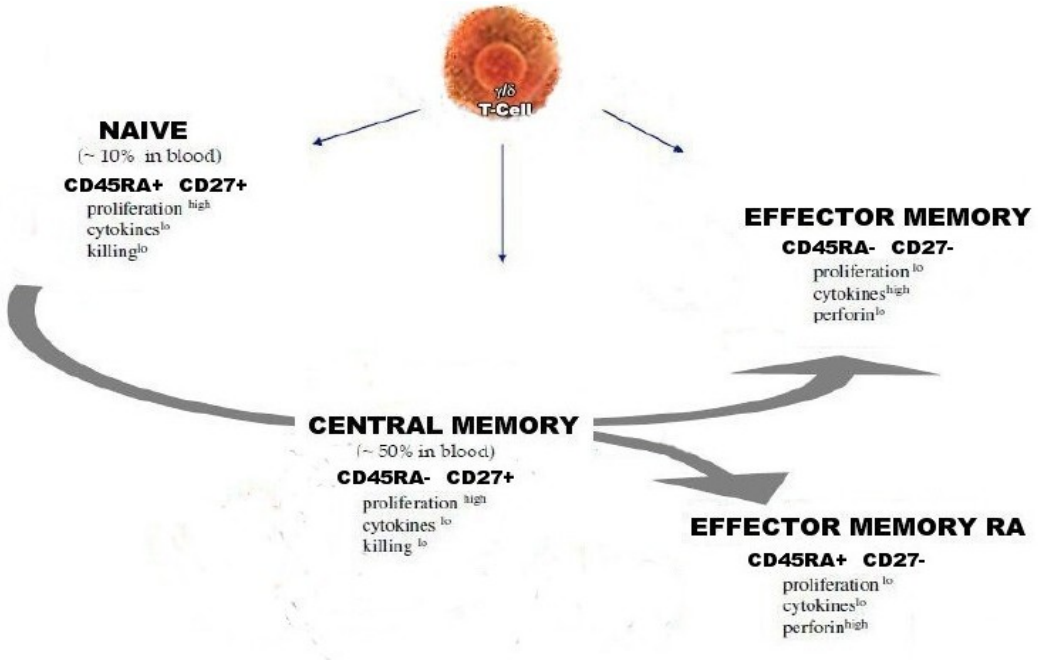

Figura 3 Phenotype of γδ T cells

The Vδ2 T cells are endowed with a variety of effector functions that contribute to antitumor immune responses.

They can produce different kinds of cytokines and chemokines such as interferon-γ (IFN-γ) and tumor necrosis factor- α (TNF-α), and growth factors such as IGF-1. Besides, they can regulate immune responses via interactions with other cells, for example, they can provide help for B cells and present antigens for αβ T cells.

Human γδ T cells induced DC maturation [54] via TCR-CD1 [55] and Fas–FasL interactions [56]. Because DCs are uniquely resistant to Fas-induced cell death, Fas-FasL interaction can transduce maturation signaling. When human Vγ9Vδ2 T cells were co-cultured with iDCs, they induced increased expression of CD86 and MHC class I on iDCs [57], mainly via γδ T cell-derived TNF-α and IFN-γ. DC maturation induced by LPS was further enhanced by γδ T cells [58]. Fourthly, γδ T cells are involved in the macrophage recruitment.

γδ T cells produce growth factors that maintain epidermal integrity. γδ T cells in psoriasis, an immune mediated disease associated with hyperproliferation of keratinocytes and

angiogenesis, have been showed to produce growth factors like IGF-1, VEGF and FGF-2 [59].

The Vδ2 T cells are endowed with a variety of effector functions that contribute to antitumor immune responses. In particular, γδ T cells can mediated cytotoxic activity against cancer cells, produce Th1- associated cytokines and cross-talk with dendritic cells, macrophages and B cells. Under appropriate conditions, γδ T cells may divert from their typical Th1-like phenotype and polarize toward Th2, Th17, and regulatory T cells (Tregs), all of which (at least potentially) inhibit antitumor immune responses [53,60].

Activated γδ T cells exhibit broad cytotoxic activity against a wide variety of tumor cells, in which they utilize death receptor/ligand (e.g. Fas/Fas-ligand)-dependent and perforin/granzyme or granulysin-dependent pathways. γδ T cells secrete various cytokines [61] and chemokines including proinflammatory Th1-like cytokines such as IFN-γ and TNF-α, and contribute to reduced survival of autologous tumor cells [62], Fig.4-5. Besides, they also produce Th2

cytokines such as IL-4 in the broncho alveolar lavage fluid of patients with allergic asthma [63-64],

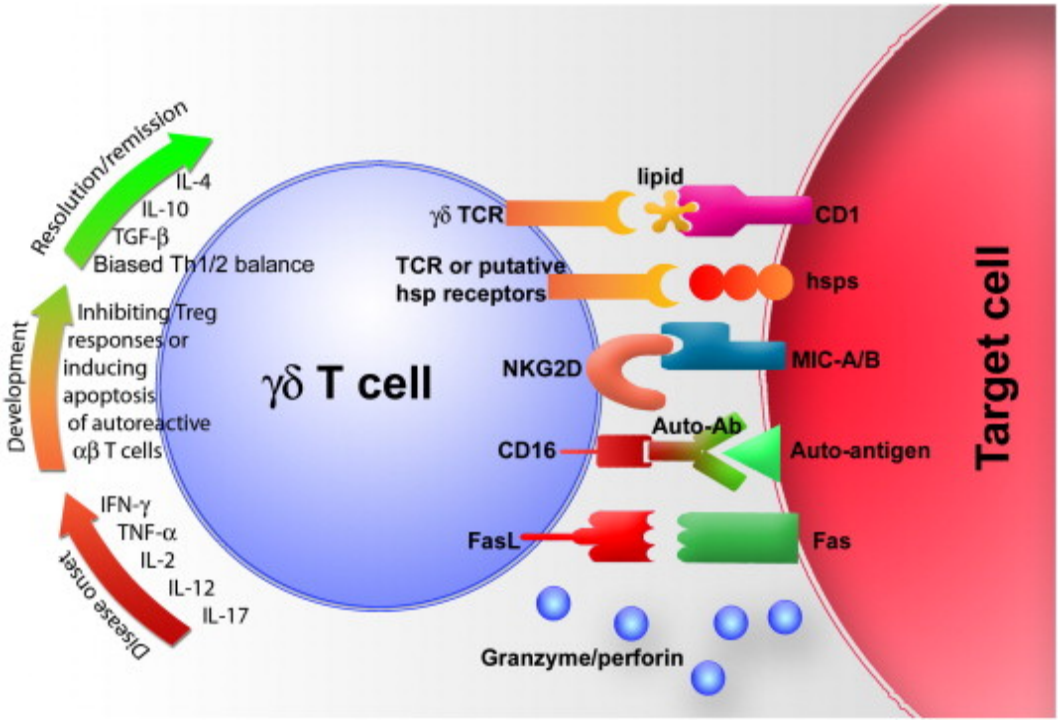

Figura 4 Receptors of γδ T cells

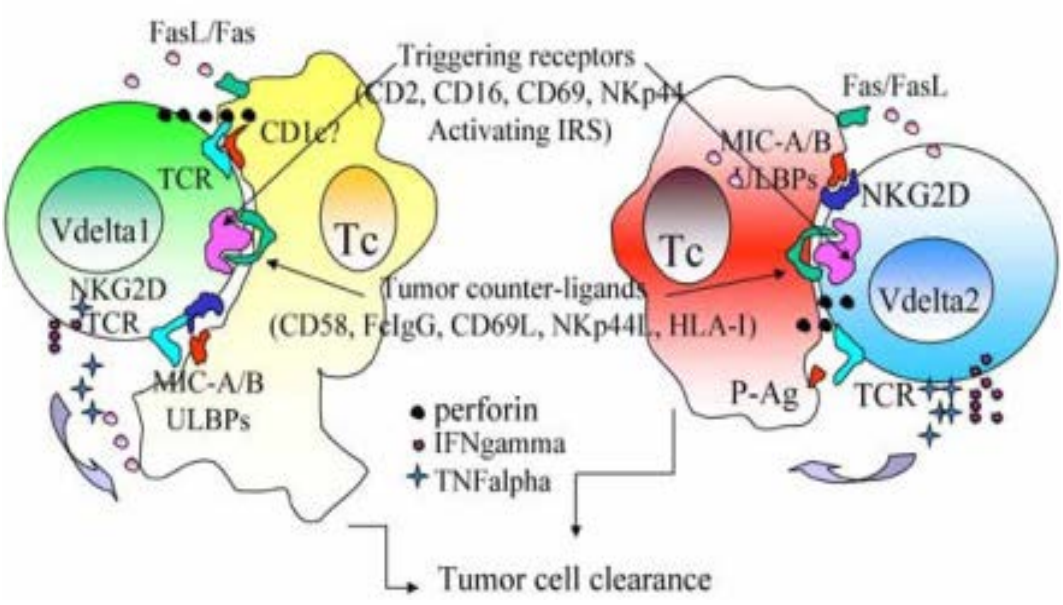

Figura 5 Schematic Funtions of γδ T cells

γδ T cells have been reported to secrete interleukin-10 (IL-10) [65], controlling CD8+ T cell expansion and regulating TNF-α secretion by activated CD8+ T cells, and IL-17 [66], regulating the expansion and recruitment of neutrophils and monocytes to initiate inflammatory responses. The role of IL-17-producing γδ T cells has been evaluated in various models of infection [67] and autoimmunity. Secretion of multiple cytokines and chemokines by activated γδ cells and their physiological roles are shown in Fig. 6

Recent studies have revealed that a high frequency of γδ T cells infiltrating renal cell carcinoma lesions failed to correlate with prognostic features, and in a group of breast cancer patients the amount of intratumoral γδ T cells correlated positively with advanced tumor stages and lymph node metastasis [68], but inversely with both relapse-free and overall survival.

Such functions are attractive weapons for immunotherapy strategies, and there are promising results from recent, relatively small-scale applications of Vγ9Vδ2 T cells to hematologic [69] and solid-tissue malignancies, including prostate cancer [70] and advanced renal cell carcinoma [71].

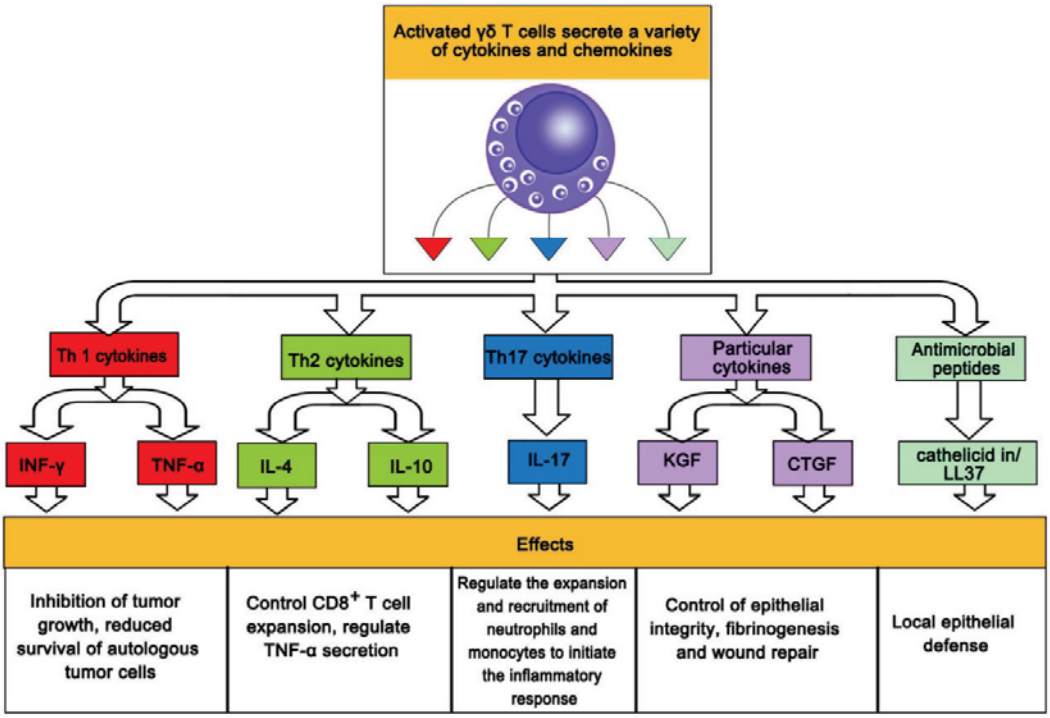

Figura 6 Secretion by activated γδ cells of multiple cytokines and their physiological roles.

2.4 Immune surveillance

In the 1950s, Frank MacFarlane Burnet an Lewis Thomas formulated the 'immune surveillance' hypothesis, in which cells of the immune systems would detect and destroy tumor cells. Since then, it has become clear than that relationship between the immune system and cancer is considerably more complex, and this hypothesis has been modified to consider three phase of tumor growth. The first

is the '**elimination phase**,' in which the immune system recognizes and destroys potential tumor cells- the phenomenon previously called immune surveillance. If elimination phase is not completely successful, what follows is an '**equilibrium phase**,' in which tumor cells undergo changes or mutations that aid their survival as a result of the selection pressure imposed by the immune system. During the equilibrium phase, a process known as "cancer immunoediting" continuously shapes the properties of the tumor cells that survive. In the final '**escape phase**,' tumor cells that have accumulated sufficient mutations elude the attentions of the immune system and grow unimpeded to become clinically detectable. According to the immunoediting hypothesis, those tumor cells that survive the equilibrium phase have acquired additional mutations that prevent their elimination by the immune system. In an immunocompetent individual, the equilibrium immune response continually removed tumor cells, delaying tumor growth; if the immune system is compromised, the equilibrium phase quirkily turns into escape, as no tumor

cells at all are removed. Another situation in which the breakdown of immune surveillance can lead is tumor development in post-transplant lymphoproliferative disorder, which can occur when patients are immunosuppressed after, for example, solid organ transplantation. It usually takes form of a B-cell expansion driven by Epstein-Barr virus (EBV) in which the B cells can undergo mutations and become malignant. Immune surveillance therefore seems to be critical for the control of virus-associated tumors [72].

Chapter 3

3.1 Immunotherapy

Immunotherapy is the treatment of cancer or inflammatory/autoimmune disease by inducing, enhancing or suppressing an immune responce. Immunotherapy can be nonspecific or (antigen) specific. Nonspecific Immunotherapy aims to enhance the overall host immune response, whereas specific immunotherapy targets the immune system against a particular tumor or increases tolerance towards a specific allergen. There are four immunotherapy: adoptive immunotherapy, antibody-based immunotherapy, cancer vaccine therapy and allergen-specific immunotherapy. From these, adoptive and antibody based immunotherapies are passive approaches, whereas cancer vaccine therapy and allergen-specific immunotherapy are active approaches. Despite advances in oncological research, cancer remain a leading cause of death throughout the developed world. Nonspecific approaches to cancer treatment, such as surgery, radiotherapy and

generalized chemotherapy, have been successful in the management of a distinct group of leukemias and slow growing solid cancer. Immunotherapy is an emerging alternative area of cancer treatment. Cancer immunotherapy includes both passive and active strategies. Passive immunotherapy involves the ex vivo creation of established tumor-immune elements (antibodies, immune cells) that are administered to patients to mediate anti-tumor activity directly or indirectly, and which do not stimulate the host immune system. In contrast active immunotherapy induces a tumor-specific immune response in the patients, leading to the production of specific immune effectors (antibodies and T-cells).

3.2 Nonspecific Immunotherapy

1) Bacillus Calmette-Guerin (BCG) is the most effective intravesical nonspecific immunotherapeutic agent, and is used for the prevention and treatment of superficial bladder cancer. The proposed anti-tumor mechanism of BCG involves

activation of the immune system and the promotion of a local acute nonspecific inflammation in the bladder lumen. Immune cells activation in response to BCG is mediated by a family of transmembrane recognition receptors called Toll Like receptors

2) Cytokine are low-molecular-weight, soluble proteins that regulate the innate and adoptive immune system. The anti-tumor activity of cytokines is mediated by one of two general mechanism: first, a direct anti-tumor effect, and second, indirect enhancement of the anti-tumor immune response. It has been hypothesized that both the cytokine-activated lymphocytes and their secretory products such as interferon gamma and tumor necrosis factor-beta may contribute to the lisys of tumor cells in vivo. [73-74]

3.3 Specific immunotherapy

1) Adoptive Immunotherapy involves the infusion immunologically- component, ex vivo-expanded, donor-derived lymphocytes, which specifically destroy tumor cells

by graft-versus leukemia or graft-versus tumor effect. In addition, peripheral blood-derived lymphokine activated killer cells and tumor-infiltrating lymphocytes derived from tumor section have proven to be effective anti-tumor agents To address MHC and exogenous cytokine-indipendent activation of anti-tumor effector function, T cells can be engineered of a recognition unit and an intracytoplasmic signaling molecule. Such receptors can be used to target various type of effectot cells including cytotoxic T-cells towards any tumor-associated antigen for which there is a suitable antibody.

2) Antibody based immunotherapy exploits the higly specific binding between antibodies and their corresponding tumor-associated antigens, resulting in some significant clinical response. Tumor-associated antigens are structures presented predominantly by tumor cells, thereby allowing antibodies to distinguish tumors from non-malignant tissue. Therapeutic monoclonal antibodies can destroy tumor cells directly by inducing apoptosis or indirectly through immunological mechanism such as antibody-dependent

cell-mediated cytotoxicity and or complement- dependent cytotoxicity. In addition, the natural function of antibodies can be enhanced by conjugating them to toxins, radionucleotides, liposomes and cytotoxix drugs. Host immune responces can be enhanced through the induction of anti-idiotypic antibodies or through the use of bispecific antibodies containing arms with different specificities.

3) Cancer vaccine therapy represents an active, systemic, tumor-specific immune response of host origin. It is used either to treat existing cancers or to prevent cancer development. There are several types of cancer vaccine: isolated whole cell cancer vaccines or tumor cell lysates, protein- or peptide-containing vaccines, viral vector vaccines and anti-idiotype vaccines. Following the administration of a vaccine-antigen that resembles a specific target, the patient's humoral and T-cells-specific immune response induces defence mechanism to combat the target in vivo

4) Cytokine Receptor as the Target for immunotherapy and Immunotoxin Therapy

Cancer immunotherapy attempts to stimulate the immune system to reject and destroy tumors. For example, administration to interferon can activate the systemic immunity. Cancer vaccines take advantage of the fact that certain molecules on the surface of cancer are either unique or more abundant than those found on normal or noncancerous cells. These molecules act as antigens, stimulating the immune system to evoke a specific immune response. Immunotoxin are protein toxin connected to a cell binding ligand or antibody. Classically, immunotoxins were created by chemically conjugating an antibody to a whole protein toxin, or, for more selective activity, by using a protein toxin devoid of its natural binding domain. Targeted cancer therapy such us immunotoxin therapy which target tumor-specific cells surface receptors is one of the most effective strategies against cancer. The targeted agents require a threshold level of receptor expression on the cancer cells to achieve their antitumor activity [73-74].

Chapter 4

4.1 How can tumor-infiltrating lymphocytes kill tumours?

The treatment of patients with cell populations that have been expanded ex vivo is called adoptive cell transfer (ACT). Cells that are infused back into a patient after ex vivo expansion can traffic to the tumour and mediate its destruction. 'Preparative lymphodepletion' the temporary ablation of the immune system in a patient with cancer can be accomplished using chemotherapy alone or in combination with total-body irradiation, and the addition of this step is associated with enhanced persistence of the transferred T cells.

During the process of expanding TIL populations in culture, tumour cells die or are killed by activated natural killer (NK) cells or newly expanding T cell populations. The TILs are dissociated from immunosuppressive cell

populations such as myeloid-derived suppressor cells (MDSCs) and possibly exposed to lower levels of immunosuppressive cytokines during this early period in culture. The ex vivo expansion of TIL populations to more favourable numbers followed by the transfer of these cells back into the host can trigger the death and complete eradication of the tumour, leading to the durable and complete remission and even the 'cure' of established cancers [52]. MHC class I-restricted T cells that are specific for tumours (either naturally or as a result of gene engineering) are, theoretically, capable of directly recognizing many types of tumour cells, because virtually every nucleated cell in the body expresses MHC class I molecules. However, neoplastic cells are notoriously unstable targets, as these cells often downregulate their expression of MHC class I molecules [68,75, 76]. Professional APCs process exogenous antigens and present them to T cells in the context of MHC class II molecules, or in the context of MHC class I molecules through a mechanism known as cross-priming. Such indirect recognition of tumour-associated antigens by T cells might

provide an effective means of targeting tumour masses that have lost MHC expression by triggering innate immunity and vascular collapse.

Immune surveillance might eliminate many microscopic tumours before they become evident and, based on experiments in mice, some investigators have proposed that tumors experience immunoediting. However, tumor masses that grow uncontrollably and ultimately kill their hosts demonstrably express immunologically targetable antigens. Thus, not only do tumor masses 'escape' recognition by eliminating antigenic targets that they express, but they also coopt or render insufficient the adaptive immune system of the host [77,78]. After transformation, tumor cells generally continue to express antigens that are characteristic of their tissue site of origin. Tissue-differentiation antigens can be excellent targets for ACT-based therapy, but only for tissues that are not essential for life. By sharp contrast, populations of TILs can be given usually without causing evidence of eye or ear toxicity, despite having TCRs specific for the same non-mutated self antigens. The induction of long-term,

durable immune responses, which can kill all of the tumor cells, can usually be accomplished by the transfer of TILs without causing the toxicity attributable to T cells. This suggests that the naturally occurring TILs in prostate cancer might respond to antigens other than non-mutated self antigens. Immunotherapy based on the adoptive transfer of tumor-specific lymphocytes has a rich history that dates back several decades [79]. It is important to distinguish these ACT-based treatments from other immunotherapies, such as therapeutic cancer vaccines, which are aimed at boosting immune reactivity in the tumor-bearing host. Therapeutic vaccines are attractive because they are easy to use and have shown low toxicities in preclinical and clinical trials at the US National Cancer Institute and elsewhere [80,81]. Vaccines can be augmented using prime–boost regimens, MHC anchor modifications, cytokines and co-stimulation [82,83]. However, broad reviews of clinical trials show that cancer vaccines only induce objective tumour regression in less than 4% of patients treated [84,85]. Immunotherapies based on the adoptive transfer of TILs are the best available

treatment for patients with metastatic melanoma. However, these therapies have limitations. Not all patients can be entered into trials, as they are still limited to patients with good performance status who are capable of withstanding the rigours of the lymphodepletion and IL-2 based treatments that are currently used. Chimeric antigen receptors (CARs) are another means for providing specificity to transduced T cells and can originate from antibodies. The variable regions from antibody genes can be engineered to encode single-chain structures fused to TCR intracellular domains that are capable of activating T cells. Recombinant retroviruses can then be used to transduce T cells with the CAR, which has antibody-like specificity. Thus, CARs recognize MHC-nonrestricted structures on the surfaces. of target cells, whereas TCRs recognize mainly intracellular antigens that have been processed and presented as peptide complexes with MHC molecules. Finally, it is worth noting that, rather than engineering the T cells themselves, another approach that can be used to promote T cell recognition of tumors is to engineer bispecific antibodies

[86,87]. One subgroup of these, known as bispecific T cell engagers (BITEs), can promote tumour elimination by linking T cells to tumors, even in the absence of cognate TCR–MHC interactions.

In the previous paragraphs, we have elaborated on the mechanism of ligand recognition, activation, cytokine secretion, and applications of γδ T cells. To be understood better, we summarized the above-mentioned contents in Fig 7 and 8.

4.2 γδ T Cells and Their Potential for Immunotherapy

There is good evidence that tumor can naturally be controlled by the immune system, and most immunotherapy strategies aim to induce adoptive, tumor-specific responses of B cells and MHC- restricted αβ T cells, particularly CD8 T cells [37, 75, 76, 88]. The regressing tumor lesions are often markedly infiltrated by mononuclear cells

and the presence of T lymphocytes has been associated with improved prognosis in patients affected by different types of cancer [37] γδ T cells are a natural component of resistance to cutaneus carcinogenesis in mice [76] and in humans display potent MHC-unrestricted cytotoxic activity in vitro against varius tumors, including prostate cancer cell line [96]. Human Vγ9Vδ2 T cells expanded *ex vivo* and then adoptively transferred to severe combined immunodeficient mice xenografted with tumor cells showed efficacy against B-cell lymphoma, renal and pancreatic carcinoma [89]. Bulding on this, the potential of human γδ T cells for tumor immunotherapy has been analyzed and was recently expertly reviewed [90].

γδ T cells possess a combination of innate and adaptive immune cell qualities rendering them attractive for immunotherapy [106–108]. They can produce inflammatory cytokines, directly lyse infected or malignant cells, and establish a memory response to attack pathogens upon re-exposure.

The most potent antitumor cytolytic mediators are γδ T cells, innate-like lymphocytes that recognize their targets independently of major histocompatibility complex (MHC)–mediated antigen presentation [71,91]. Human γδ T cells kill a vast repertoire of tumor cell lines and primary samples in vitro, including leukemia and lymphoma, melanoma, neuroblastoma, and multiple types of carcinoma [92-96]. More recently, in vitro–activated γδ T cells have also been shown to target a small population of colon cancer stem cells, responsible for tumor resistance to conventional therapies [96], and to kill chemotherapy and imatinib–resistant chronic myelogenous leukemia lines [93]. Moreover, γδ T cells have been frequently isolated from tumor-infiltrating lymphocytes and shown to react in vitro to tumors but not to healthy cells [71]. The activated human γδ T cells produce large amounts of interferon- γ [97], a central cytokine in antitumor immune responses.

Hematopoiesis is regulated by several cytokines with pleiotropic activity. Several evidences have clearly demonstrated that these molecules, formerly regarded as

specific for the hematopoietic system, also affect certain endothelial cell functions and that hematopoietic factors clearly influence angiogenesis. IL-17 is secreted by activated CD4 T cells and T cells [98]. IL-17 promotes angiogenesis via the induction of prostaglandins E1 and E2 (PGE1 and PGE2), VEGF, MCP-2 and NO by fibroblasts and tumor cells [99,100]. Moreover, IL-17 increased the secretion of angiogenic CXC chemokines, including CXCL1, CXCL5, CXCL6, and CXCL8 by tumor cell lines [101] and induced EC migration and tube formation via the PI3K/AKT1 pathway [102].

γδ T cells have recently gathered significant attention following the discovery that they produce IL-17 in various mouse models of infection and autoimmune disease. In contrast, the secretion of large amounts of IFN-gamma by γδ T cells has long been known, and has been tightly linked to their anti-tumor function. A study unexpectedly about lymphoid T cells that infiltrate tumor foci induced in the mouse skin produce very little IFN-gamma, but abundant IL-17. In fact, these γδ T cells are the major source of IL-17 within the tumor microenvironment, where they appear to

promote angiogenesis, and thus tumor growth. This Commentary discusses the relevance of these interesting findings in the context of the currently paradoxical pro- versus anti-tumor roles of IL-17 in cancer immunology [103], see figure 6.

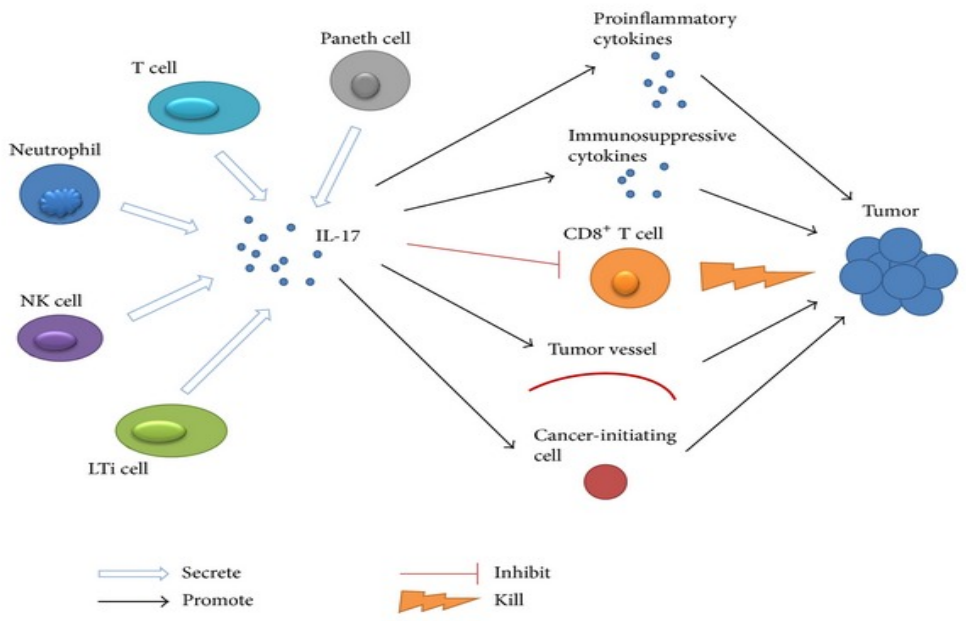

Figure 7 This is an illustration about the protumor activity of IL-17 in CRC microenvironment. Blue colored arrow shows cells producing IL-17. Black arrow shows the latter stimulated by the former. T-shaped arrow shows process inhibited by IL-17 and lightning arrow shows attack on tumor cells.

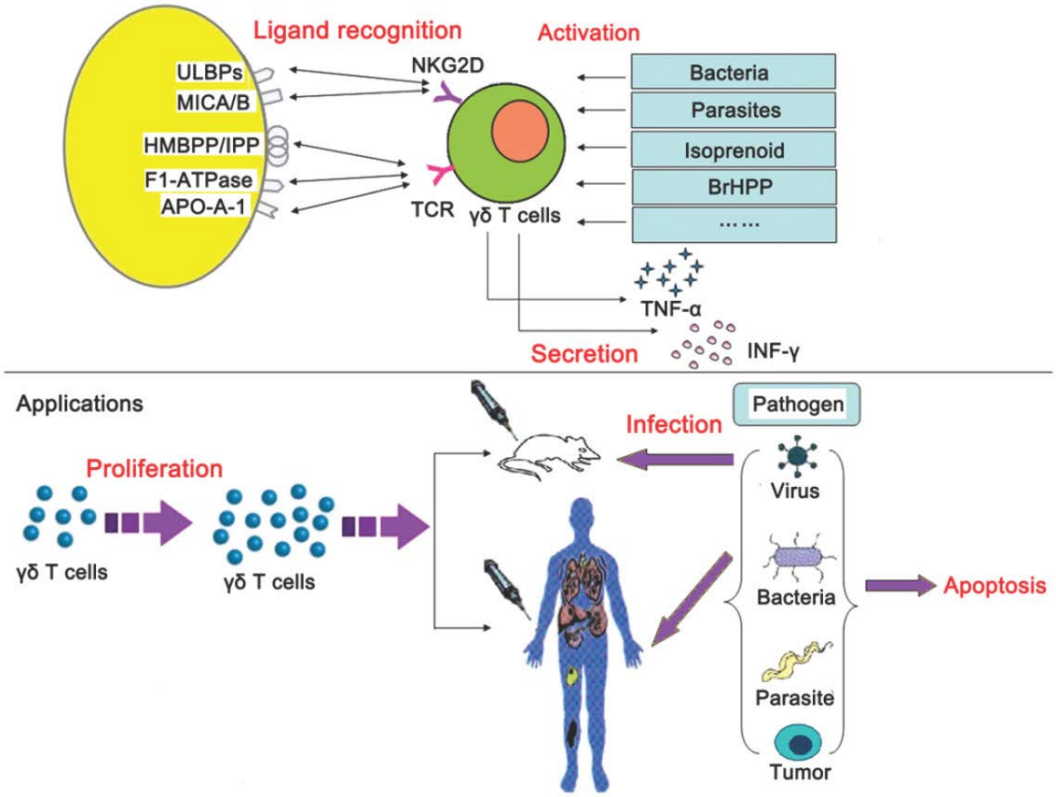

Figure 8 Mechanism underlying γδ T cell recognition of nonpeptide antigens and clinical applications

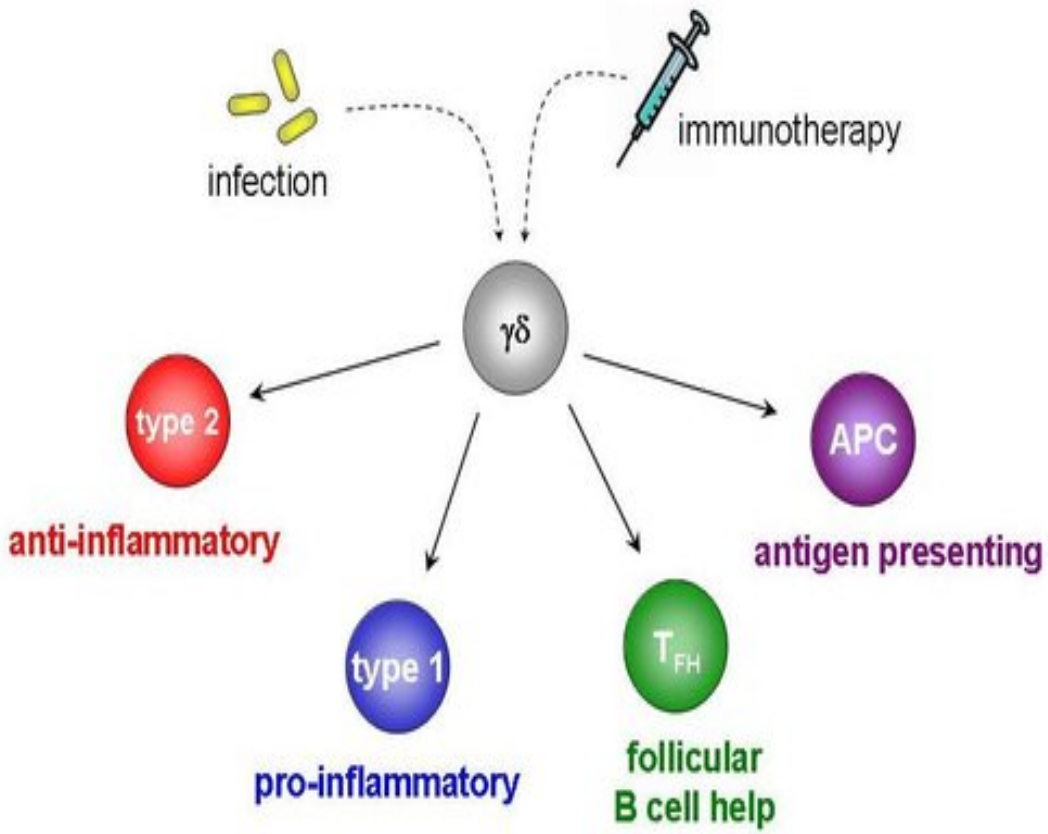

Figura 9 Different lineage of γδ T cells

Chapter 5

Aims

The aims of this research were to evaluate the frequency, the phenotype and the effector function of prostate cancer-infiltrating δ1 and δ2 T cells in prostatic biopsy samples and the correlation of these data with the presence of cancer versus healthy tissues and, in cancer tissues, with Gleason pattern. The analysis has been performed in circulating γδ T cells of the same patients and as control in healthy subject who, after transrectal echo guided prostatic biopsy, did not receive cancer diagnosis.

We also studied the representation and the in vitro intra cellular concentration of some cytokine such as IL-17 and INFγ and, in particular how distributions these in the tumor infiltrating γδ T from 43 subjects.

The cytokines response induced by activating Vγ9Vδ2 T cells using BrHPP has been also determined.

Another aim has been to study the phenotypic characteristics of γδ T cell in the blood and into the core samples in order to evaluate whether reproducible

phenotypic changes in the γδ T cell compartment could be induced by the tumor microenvironmenent.

Chapter 6

Materials and Methods

6.1 Patients:

We identified a consecutive series of outpatients who made transrectal echo guided prostatic biopsy.

Prostate tissues, neoplastic and not, were obtained from Urological Department of the University Hospital of Palermo, from 2013 to 2015.

Until now, we enrolled 43 patients undergoing a transrectal Echo guided prostatic biopsy using 16 Gauge needle.

During the biopsy after a standard 12 core procedure was followed, taking two cores from the margin of right and left side of the prostate gland and for each patient peripheral blood was taken. All individuals gave written informed consent to participate. According to Italian rules (art. 13, DLgs n. 196/03), this study did not require authorisation by the local ethical committee.

After medical report we divided the individuals in three groups. The first group consists of 21 subject with prostate cancer (Mean Age 70,04 range 62 to 80; Mean PSA 10,11 range 5,5 to 16,7); the second group consists of 4 subject with prostatic intraepithelial neoplasia (Mean Age 71 range 65 to 77 Mean PSA 5,88 range 4,2 to 7,92) and the third groups consists of 17 healthy individual (Mean Age 64,20 range 43 to 80 Mean PSA 4,46 range 2,06 to 7,05).

The principal characteristics of the patients are summarized in table 1 A,B,C:

Table 1 A Patients with Prostate Cancer

Number of patient	Age	PSA (ng/ml)	Histological Examination	Gleason Score
1	66	15,05	Acinar adenocarcinoma	6 (3+3)
2	62	12,33	Acinar adenocarcinoma	6 (3+3)
3	78	7,86	Acinar adenocarcinoma	6 (3+3)
4	67	7,95	Acinar adenocarcinoma	6 (3+3)

5	76	11	Acinar adenocarcinoma	7 (3+4)
6	71	13	Acinar adenocarcinoma	7(3+4)
7	65	10,79	Acinar adenocarcinoma	8 (4+4)
8	65	7,76	Acinar adenocarcinoma	8 (3+5)
9	80	8,79	Acinar adenocarcinoma	8 (3+5)
10	67	10,5	Acinar adenocarcinoma	6 (3+3)
11	63	5,5	Acinar adenocarcinoma	6 (3+3)
12	65	7,11	Acinar adenocarcinoma	6 (3+3)
13	69	6,43	Adenocarcinoma	6 (3+3)
14	70	5,96	Acinar adenocarcinoma	6 (3+3)
15	78	9,02	Acinar adenocarcinoma	6 (3+3)
16	78	14,65	Acinar adenocarcinoma	6 (3+3)
17	68	6,5	Acinar adenocarcinoma	7 (3+4)

18	73	11	Acinar adenocarcinoma	7(3+4)
19	70	9,98	Acinar adenocarcinoma	7 (3+4)
20	71	15,06	Acinar adenocarcinoma	6 (3+3)
21	69	16,07	Acinar adenocarcinoma	6 (3+3)

Table 1 B Patients with Prostatic Intraepithelial Neoplasia

Number of patient	Age	PSA ng/ml	Histological Examination
1	65	5,01	Prostatic intraepithelial neoplasia Pin High Grade
2	66	4,2	Prostatic intraepithelial neoplasia Pin High Grade
3	76	7,92	Prostatic intraepithelial neoplasia Pin High Grade
4	77	6,4	Prostatic intraepithelial neoplasia Pin High Grade

Table 1 C Healthy Subject

Number of patients	Age	PSA ng/ml	Histological Examination
1	65	4,65	not observed sign of tumor lesion
2	73	2,45	not observed sign of tumor lesion
3	69	3,05	not observed sign of tumor lesion
4	59	5,35	not observed sign of tumor lesion
5	63	5,84	not observed sign of tumor lesion
6	43	7,05	not observed sign of tumor lesion
7	80	2,06	not observed sign of tumor lesion
8	58	3,50	not observed sign of tumor lesion
9	68	3,2	not observed sign of tumor lesion
10	68	5,1	not observed sign of tumor lesion
11	48	4,6	not observed sign of tumor lesion
12	62	5,67	not observed sign of tumor lesion
13	59	4,87	not observed sign of tumor lesion
14	71	5,9	not observed sign of tumor lesion

15	67	4,03	not observed sign of tumor lesion
16	65	4,6	not observed sign of tumor lesion
17	72	4,5	not observed sign of tumor lesion
18	66	3,9	not observed sign of tumor lesion

6.2 Isolation and FACS analysis of Tumor-infiltrating Immune Cells (TILs) and Peripheral blood mononuclear cells (PBMC)

Tissue was obtained fresh and immediately transported to the laboratory in sterile saline for processing. Tissue was first minced into small pieces followed by digestion with collagenase type IV, Hyaluronidase and DNAase (Sigma, St. Louis, MO) for one hour at 37°C. After digestion, the cells extracted was washed twice in RPMI 1640 medium.

Whole blood samples were obtained from the same patients recruited for the collection of tissue specimens and used for the comparative analysis between immunological status in peripheral blood and in the cancer or inflamed tissue. The peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). PBMC were used for the staining with the same mAbs used for the study of the TILs, acquired and analyzed by flow cytometry on an FACSCalibur.

The fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-,PE-Cy5- or allophycocyanin (APC)- conjugated monoclonal antibodies (mAbs) used to characterized the entire population and cytokine were the following: anti-CD3, anti-Vδ1, anti-Vδ2, anti-CD27 and anti-CD45RA, anti IFNγ and anti IL17 all purchased from BD Biosciences (Mountain View, CA). Expression of surface markers was determined by flow cytometry on an FACSCalibur. Flow cytometry analysis was used to identify and characterize the γδ T cell populations and IL 17 and IFNγ using the following conjugated

monoclonal antibodies (mAbs): anti-Vδ1FITC, anti-Vδ2PE, anti-CD27APC and anti-CD45RA PECy5, antiCD3, anti-Il 17 PerCp/Cy5.5 and anti-IFNγ APC.

The gating strategy involved progressively measuring total cells; viable cells only; lymphomonocytes and specific cell types. For every sample 100.000 nucleated cells were acquired and values are expressed as percentage of viable lymphomonocytes, as gated by forward and side scatter.

6.3. Stimulation of γδ T cells in vitro and Cytokine Production

Purified γδ T cells (500000/1000 µl) were cultured in 24-well round-bottom plates,in complete RPMI medium with 10% Fetal Bovin Serum in absence or presence of 10 µg/ml of BrHPP;1µg/ml of Ionomicina and 15 µg PMA for 1 h, at 37°C in 5% CO2.

The cytokine production capacity of Vδ1 and Vδ2 T cell derived both cancer tissues and healthy tissues were determined after stimulation with PMA (BD Biosciences, 15 µg /ml) and ionomycin (BD Biosciences, 1 mM) and 10 µg/ml of BrHPP for 1 hrs at a cell concentration of 500000/ml.

For the intracellular staining for IFN-γ and IL-17, we taken the TILs and we stimulated in the presence of monensin for 3 h at 37°C in 5% CO2 were harvested, washed, and stained with anti-Vδ2 and anti-Vδ1 mAbs in incubation buffer (PBS containing 1% FCS and 0.1% Na azide) for 30 min at 4°C. Cells were then washed twice in PBS with 1% FCS and fixed with PBS containing 4% paraformaldehyde overnight at 4°C. Fixation was followed by permeabilization with PBS containing 1% FCS, 0.3% saponin, and 0.1% Na azide for 15 min at 4°C, and fixed permeabilized cells were stained with an anti–IFN-γ and IL-17 antibodies. After two more washes in PBS containing 1% FCS, the cells were analyzed by FACSCalibur. Lymphocytes were gated by forward and side scatter and analysis done on 100,000 acquired events for each sample. The detection of IFNγ and IL17 were obtained

from the following conjugated monoclonal antibodies (mAbs): anti-Il 17 PerCp/Cy5.5 and anti-IFNγ APC, BD Pharmingen and performed according to the manufacturer's instructions.

6.4 Statistical analysis

Statistical significance of obtained results were evaluated by (T-StudenT) ($p \leq 0.05$). All experiments were performed at least three times and representative cytograms are presented.

Chapter 7

7.1 Results

The γδ T cells analysis, performed on prostatic samples using common bioptic technique, has been always feasible.

The γδ T cells present in the prostatic tissue disclose wide variability in all the samples and in the samples of the three groups: healthy, prostatic cancer and prostatic intraepithelial neoplasia.

γδ T cells variability has been also observed in PBMC samples.

The Percentage of Vδ1 and Vδ2 T cells in PBMCs from healthy and prostatic cancer is showed in the **figure 10**. As shown in the figure the percentage of δ2 T cells was higher in PBMC of cancer patients while δ1 T cells in PBMC of healthy patients. Number of enrolled patients = 39 (21 with cancer,18 healthy), these data were statistically significance (P**< 0,05).** When we compared Vδ2 T cells from cancer

patients and healthy subject the data were a statistically significant P<0,05.

The percentage of infiltrating γδT cells in the cores prostatic from patients with prostate cancer, prostatic intraepithelial neoplasia and healthy tissue is displayed in the **figure 11.** Both δ1 and δ2 T cells are more expressed in prostate cancer patients, respectively 6,15 % and 8,49 % than in tissues with PIN, 2 % and 5,98 %. and healthy patients 5,71 %, and 5,23 % P >0,05. The percentage of tumor-infiltrating γδT cells in adenocarcinoma in according to Gleason score is showed in the **figure 12.** This figure shows the association between the rate of γδ T cells and the clinical features in patients with prostate cancer and grading according to Gleason classification. The distribution of infiltrating δ2 T cell in the cancer with Gleason pattern 4-5 decreases than pattern with Gleason 3. Cancers with a higher Gleason score are more aggressive and have a worse prognosis. The data showed that more aggressive tumors have a lower percentage of Vδ2 and Vδ1 T cells, these results were statistically significant P<0,05 for Vδ2 T subset.

To understand the phenotypes of the tumor-infiltrating Vδ2^{+}T cells was due to the tumor microenvironment or simply reflected an overall bias in prostate cancer patients, we studied the phenotype of γδ T cells in the peripheral blood and tissue as shown in **Figure 13A and 13B.** Vδ2+T cells obtained from peripheral blood of cancer subjects showed a predominant TCM phenotype (38,51%) P<0,05.

While a different pattern was found for Vδ2+ γδ T cells in the prostate cancer-infiltrating where the dominant phenotype (40,28 %.) was the TEM phenotype P<0,05. These results clearly demonstrate that the cells committed to effector activities at the tumor site are phenotypically different respect to circulating Vδ2 T cell.

The phenotype of the Vδ1 T cells in PBMCs of patients with prostate cancer and healthy individuals and in the infiltrating Vδ1 T cells of healthy tissues and cancer tissues, is showed in **figure 14 A-B.** The most prevalent phenotype in PBMCs δ1 T cells of patients with prostate cancer was TEMRA (40.73%.) P>0,05 while the most prevalent phenotype in TILS was TEM phenotype (38.31%) P<0,05. The most prevalent phenotype in PBMCs of healthy subject was TNaive.

The data, produced so far, show that in the patients with prostate cancer the main subset infiltrating lymphocyte is represented by Vδ2 T-Cells had a phenotype TEM. Human γδ

T cells include those with naive or central-memory phenotypes that home to secondary lymphoid organs, but that lack immediate effect function, and those with effector-memory and terminally-differentiated phenotypes that home to sites of inflammation and that display immediate effector functions as cytokine production and cytotoxic activity.

The majority of tumor-infiltrating Vδ2 T cells shows a TEM phenotype, indicative for cells that home to sites of inflammation and that display immediate effector functions as cytokine production or cytotoxic activity.

The percentages of Vδ1 and Vδ2 T cells expressing intracellular cytokines IL17 and IFNγ from TILS derivate from Prostate cancer tissues and healthy is showed in **figure 15 - 16 -17**. The flow cytometry quantitative assay shows an amount of IL17 and IFN γ in the control sample (RPMI). Vδ1 and Vδ2 T cells IFN γ positive were higher in prostate cancer tissues than in healthy tissues. After stimulation with Ionomycina and BrHpp the samples induced an increase of IFN γ in healthy tissues but were not able to induce an increase of IFN γ in cancer tissues, these data were statistically significance for Vδ2 T cells and not significance

for Vδ1 T cells P<0,05 (when we used Ionomycin the P was 0,0456 and P was 0,0364 when we stimulated with BrHpp).

The percentage of cytokine IL-17 in cancer tissues did not show an increase after Ionomycin togheter PMA and BrHpp stimulation in both Vδ1 and Vδ2 T cells. Only Vδ2 T cells in healthy tissues showed an increase of cytokine IL-17 percentage after Ionomycin and BrHpp stimulation, the results were statistically significance P<0,005.

Chapter 8

8.1 Discussion

Substantial evidence indicates that the immune system participates in tumorigenesis and may contribute to either disease progression or inhibition of tumor growth, for

decrease tumor lesion to be markedly infiltrated by mononuclear cells and the presence of γδT cells has been associated with improved prognosis in patients with different types of carcinoma [70].

Given that γδ cells mediate antigen-specific killing of tumor cells, we studied their distribution and the intracellular cytokine expression in tumor infiltrating γδ T cells from patients with prostate cancer and healthy subject. We found that γδ cells are largely present in infiltrating prostate cancer, and both Vγ1 + and Vγ2+ cells are involved.

The majority of adenocarcinoma prostatic infiltrating γδ cells showed effector memory and terminally-differentiated phenotypes and, accordingly γδT obtained from tumor-

infiltrating immune cells produced IFN γ and IL17. Our study showed the presence of higher rate of γδT isolation, particularly Vδ2 T cells than healthy tissue in prostate cancer with Gleason Pattern 3, while a decrease in prostate cancer with Gleason Pattern 4 and 5.

Given that Vγ2+ T cells are absent from normal skin [88], their presence in the context of prostate cancer is highly suggestive of a migratory process from the blood or lymphoid tissues. Accordingly, the majority of prostate cancer-infiltrating Vγ2+ T cells have a TEM and TEMRA phenotype, characteristic for cells that home to sites of inflammation and that display immediate effector function as cytokine production. Conversely, circulating Vγ2+ T from the same patients have a predominant TCM phenotype, similarly to that occurring in healthy subjects, and that identifies cells that home to secondary lymphoid organs, but that lack immediate effector function.

Specific γδ T-cell subsets are capable of recruiting immunosuppressive myeloid populations, inhibiting antitumor responses, and enhancing angiogenesis, thus

promoting cancer progression. A common mediator of such functions appears to be the cytokine IL17, whose pathogenic effects can override the antitumor immune response orchestrated by IFNγ [2].

These results strongly indicate that while Vγ1+ T cells may be activated locally in the tumor microenvironment, Vγ2+ T are recruited from the blood or secondary lymphoid organs by virtue of a migratory process orchestrated by cytokines.

Altogether, our results suggest that a natural immune response mediated by γδ T lymphocytes may contribute to the immunosurveillance of prostate cancer.

The localization of γδ T cells, as Vδ2 T cells, within epithelia suggests that these cells may contribute to the surveillance of malignancies, including prostate cancer. Vδ1 T cells are the prevalent γδT cells population in human tissues including intestinal epithelium and skin [88].

Vδ2 T cells are absent in normal skin and their presence in the context of prostate cancer, more than in healthy prostate tissue, as we have observed in our study, is highly

suggestive of a migratory process from the blood or lymphoid tissues .

IL-17 expression is elevated in several human tumors, such as ovarian, cervical cancer, breast cancer, hepatocellular carcinoma, esophageal cancer, gastric cancer and colorectal cancer [103] and accumulative evidence reveals that IL-17 can promote tumor initiation and progression in most cancer but the exact mechanism of IL17 in tumor initiation and progression is not yet clear. Some researchers propose that IL 17 promotes tumor initiation and progression through suppressing antitumor immune response.

Thus, some attempts for cancer immunotherapy targeting IL-17, such as neutralizing antibody, and approaches or decreasing IL-17 production or blocking the downstream signaling pathways would come true with the deepening of IL-17 related research in the future. In our study we observed that IL-17 γδT cells both Vδ1 and Vδ2, enhanced after Ionomycin and BrHpp stimulation only in healthy tissues but not in prostate cancer tissues.

IFNγ positive Vδ1 and Vδ2 T cells are probably those more implicated in the immunosurveillance against tumour and we observed an increase of IFNγ positive Vδ2 T cells in prostate cancer tissues that did not show an increase after Ionomycin and BrHpp stimulation.

The analysis of IFNγ positive Vδ2 T cells in prostate cancer tissue could have a clinical prognostic significance and could guide the different therapeutic options, for example active surveillance versus radical prostatectomy. A prostate cancer with abundant IFNγ positive Vδ2 T cells could be a sign of a more favourable prognosis in add to the other known prognostic information like Gleason score and clinical stage. This is particularly important because this analysis is feasible in prostate tissues derived by prostatic biopsy, as we showed in our study.

Although great progress has been made in γδ T cell-based immunothearpies, many aspects need to be improved in future clinical trials.

The identification of biomarkers to predict clinical outcome is crucial for patient selection. A recent study, for example, has identified a panel of 10 genes which encode cell surface proteins that segregated "γδ-susceptible" from "γδ-resistant" hematologic tumors [104]. Equivalent markers could be promptly characterized in multiple cancer types, and their predictive value should be accessed in γδ T cell-based clinical trials. The combination of "susceptible" tumor profiles with improved strategies for γδ T -cell activation in vivo may be the way forward for γδ T cell-based cancer immunotherapy. γδ T cells are attractive targets for cellular immunotherapy, but protocols for their therapeutic use need to be optimized. In addition, it is necessary to explore better antigens which help us stimulate γδ T cell expansion in vitro for the preparation of a large number of cells for adoptive cell transfer. Future studies should focus on the possible advantages of combining γδ T cell–based immunotherapy with conventional chemotherapy or other therapeutic approaches, such as antiangiogenic drugs. Future trials should harness biphosphonate activated γδ T

cells in combination with chemotherapy or monoclonal antibodies for treatment of solid tumors and haematolic malignancies.

Chapter 9

Graphics

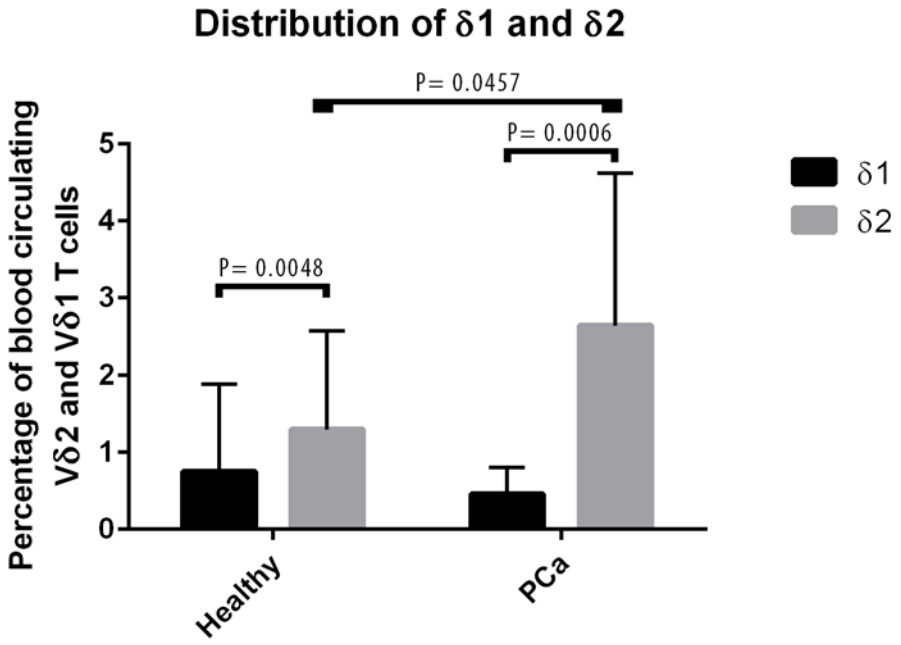

Figura 10 Percentage of Vδ1 and Vδ2 T cells in PBMCs of healthy patients and PBMCs of prostate cancer PCa and percentage of Vδ1 and Vδ2 T cells in TILS-PCa and Healthy tissue. Number of enrolled patients = 39 (21 with cancer, 18 Healthy subject) . (P=0,0048 DS δ1 1,62 DS δ2 =0,91) P=0,0457 DS δ healthy 1,29 δ2 cancer DS 1,96) P=0,0006 DS δ21,96 DSδ1 0,34

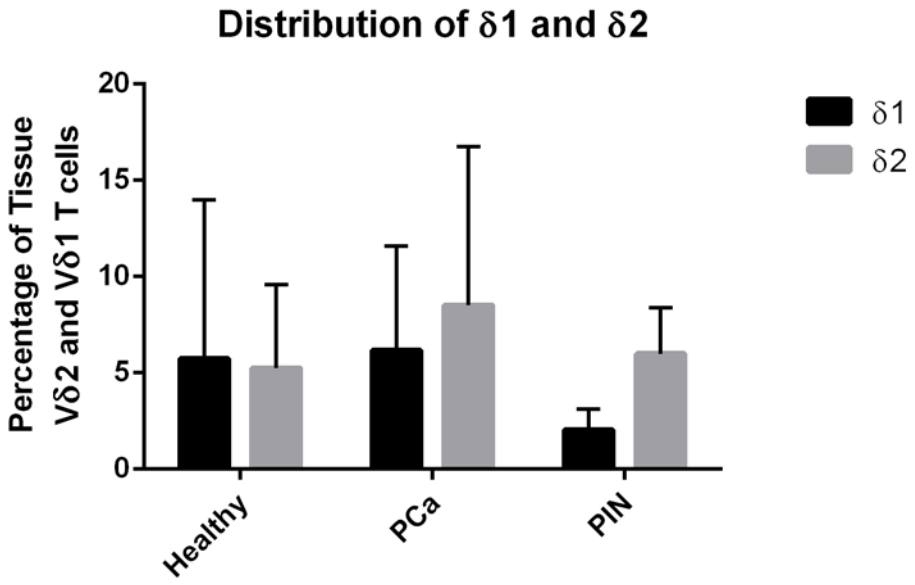

Figura 11 Percentage of Vδ1 and Vδ2 T cells in TILS, in prostatic intraepithelial neoplasia (PIN) tissue and healthy tissue. Number of enrolled patients = 43 (21 with cancer, 18 with healthy , 4 with PIN) (P>0,05).

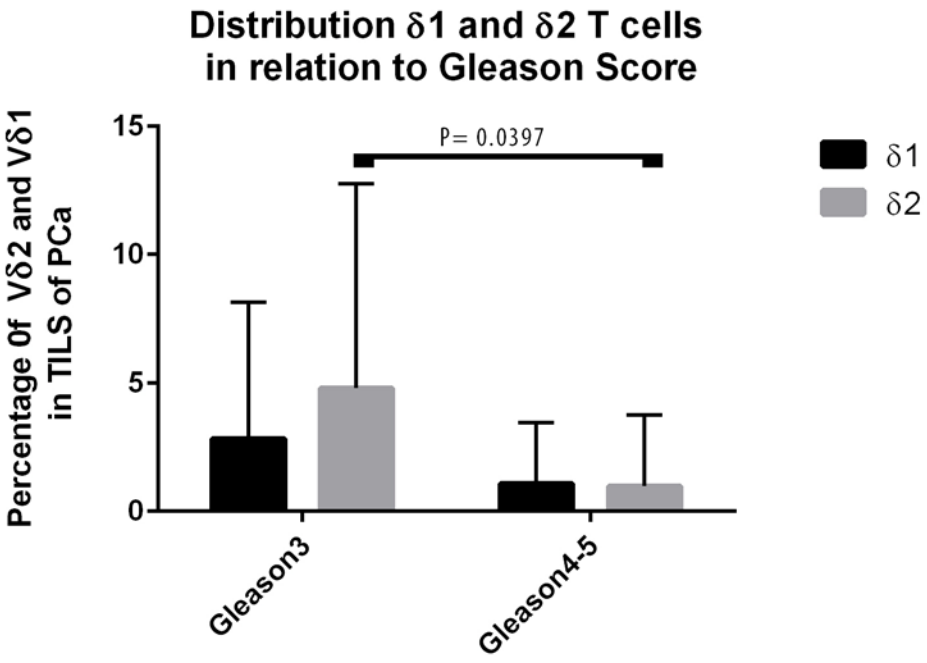

Figure 12 Percentage of Vδ2 and Vδ1 T cells in TILs from patients with prostatic cancer with Gleason pattern 3 and 4-5. Total patients 21 (13 patients of them have a Gleason score with pattern 3 and 8 of them have a pattern 4-5) (P<0,03 Vδ2 T in relation to Gleason score pattern 3 and score 4-5). The subset Vδ1 was P>0,05 Gleason 3 correlation with pattern 4-5. DS gleason 3 δ2=7,97 DS gleason 4-5 δ2=2,80

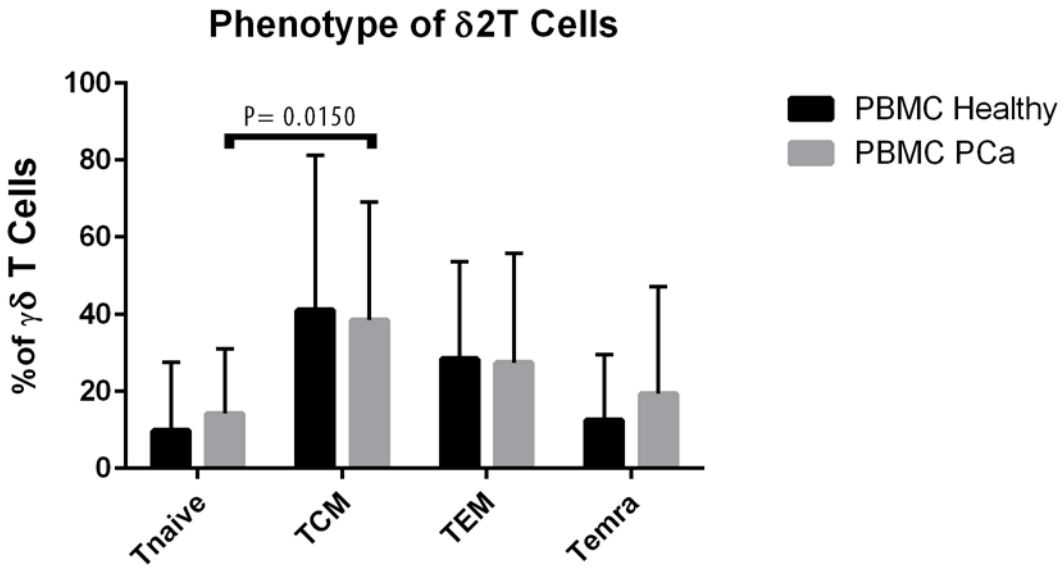

Figura 13 A- **Comparision of Vδ2 T cells subset in PBMCs form patients with prostate cancer and healthy individuals.** PBMCs from patients with cancer PBMC-PCa and healthy subjects were obtained as described under Materials and Methods and were stained with anti-Vδ2 T, anti-CD45RA and CD27 mAbs. Percentage of T_{Naive} ($CD45RA^{+}$ $CD27^{+}$)T_{CM}($CD45^{-}CD27^{+}$) T_{EM} ($CD45RA^{-}$ CD27) were obtained by FACS analysis. DS TN=17,29 DS TEM=33,63

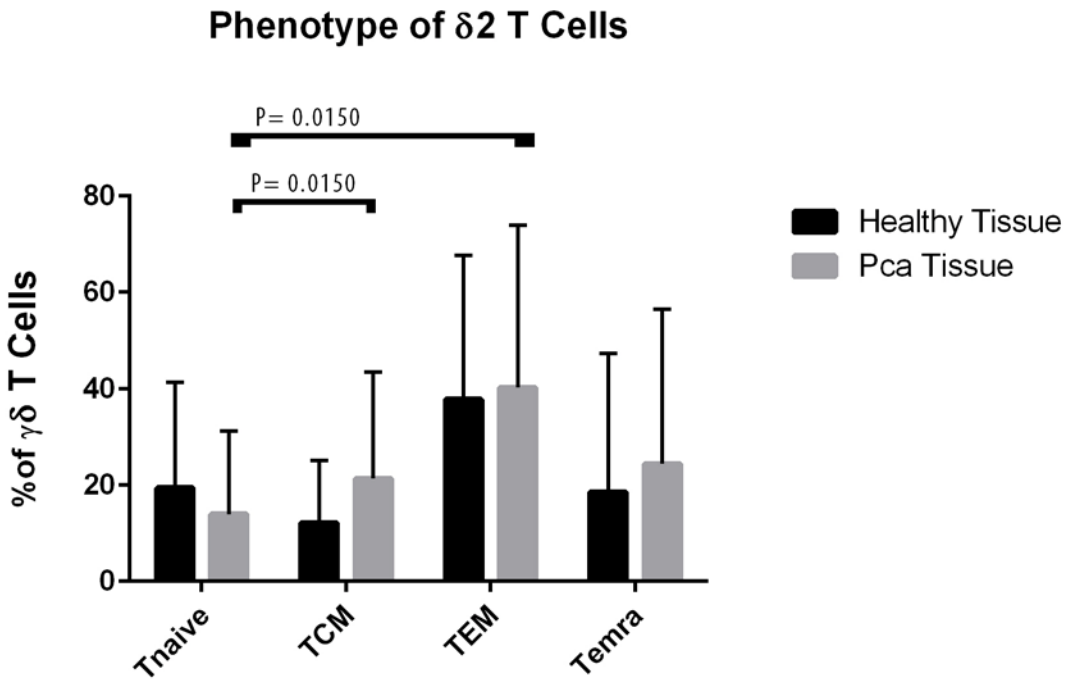

Figura 13 B- Comparision of Vδ2 T cells subset in TILS form patients with prostate cancer and healthy individuals. TILS from patients with cancer PCa and healthy tissue were obtained as described under Materials and Methods and were stained with anti-Vδ2 T, anti-CD45RA and CD27 mAbs. Percentage of T_{Naive} ($CD45RA^{+}$ $CD27^{+}$)T_{CM}($CD45^{-}CD27^{+}$) T_{EM} ($CD45RA^{-}$ CD27) were obtained by FACS analysis. The Phenotype distribution of the infiltrating Vδ2 T cells in healthy tissues and cancer tissues the P<0,0150 in cancer tissues TN-TCM and TN-TEM**).** DS TN 16,91 DS TCM 30,53) TN DS 17,29 TEM DS 33,63

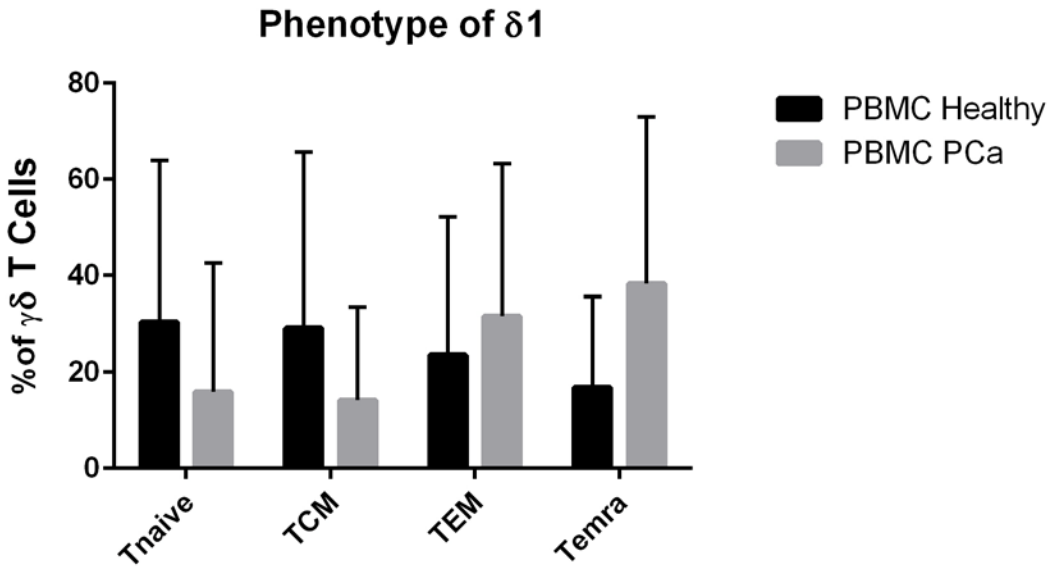

Figura 14 A- Comparision of Vδ1 T cells subset in PBMCs form patients with prostate cancer and healthy individuals. PBMCs from patients with cancer PBMC-PCa and healthy subjects were obtained as described under Materials and Methods and were stained with anti-Vδ2 T, anti-CD45RA and CD27 mAbs. Percentage of T_{Naive} ($CD45RA^{+}$ $CD27^{+}$)T_{CM}($CD45^{-}CD27^{+}$) T_{EM} ($CD45RA^{-}$ CD27) were obtained by FACS analysis. P>0,05

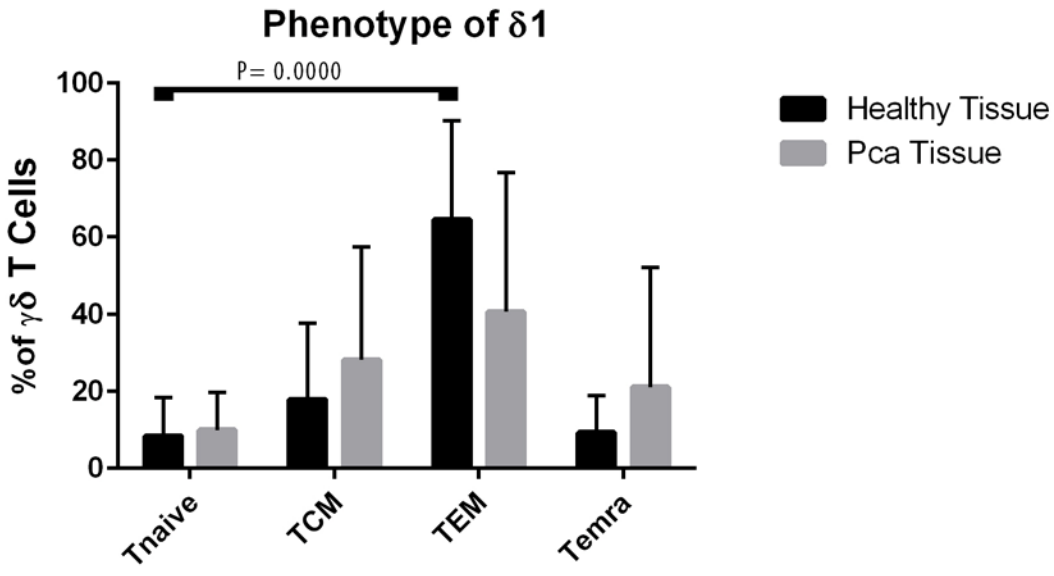

Figura 14 B- Comparision of Vδ1 T cells subset in PBMCs form patients with prostate cancer and healthy individuals. PBMCs from patients with cancer PBMC-PCa and healthy subjects were obtained as described under Materials and Methods and were stained with anti-Vδ2 T, anti-CD45RA and CD27 mAbs. Percentage of T_{Naive} ($CD45RA^{+}$ $CD27^{+}$)T_{CM}($CD45^{-}CD27^{+}$) T_{EM} ($CD45RA^{-}$ CD27) were obtained by FACS analysis. The Phenotype distribution of the infiltrating Vδ1 T cells in healthy tissues and cancer tissues the was P=0,0000 in cancer tissues TN- TEM**). TN DS 9,78 TEM 35,94**

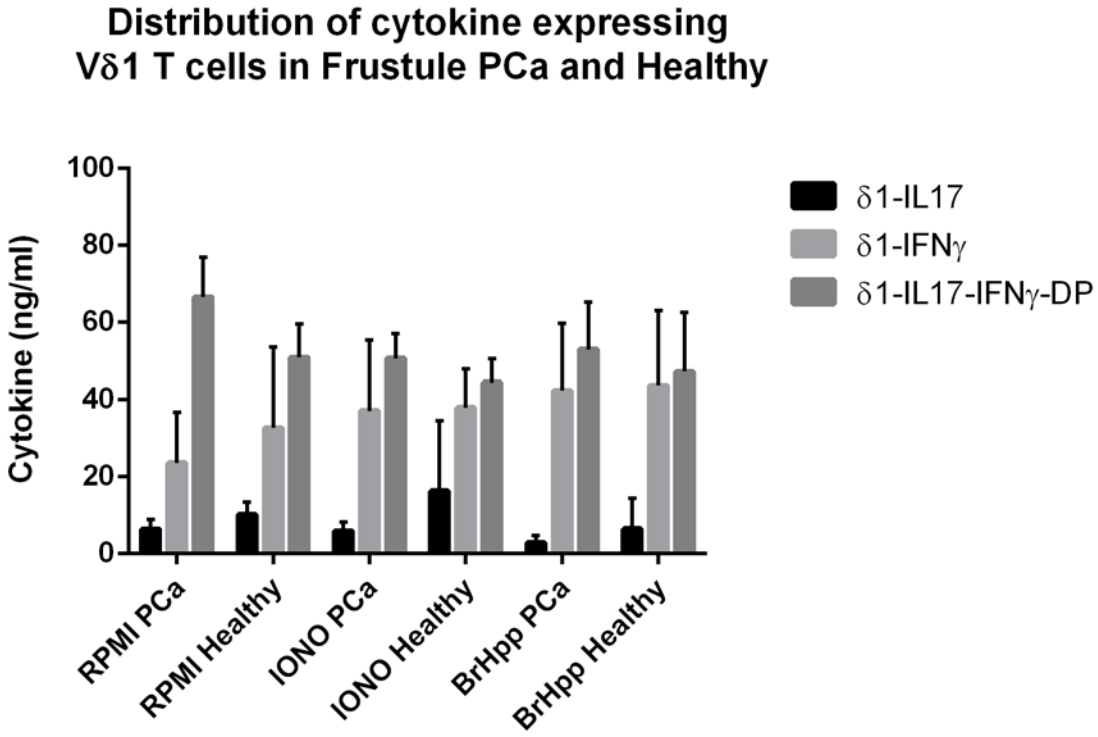

Figura 15 Cytokine production capacity of Prostate cancer PCa-derived γδ T cells TILS and Healthy Tissue. Cytokine production by Vδ1 T from cancer and healthy tissue were determined by FACS analysis of supernatants with only RPMI (negative control without stimulation) and after 4 hrs stimulation with PMA and ionomycin (Positive control) and stimulation with BrHpp (Antigen Specific Control) . P>0,05

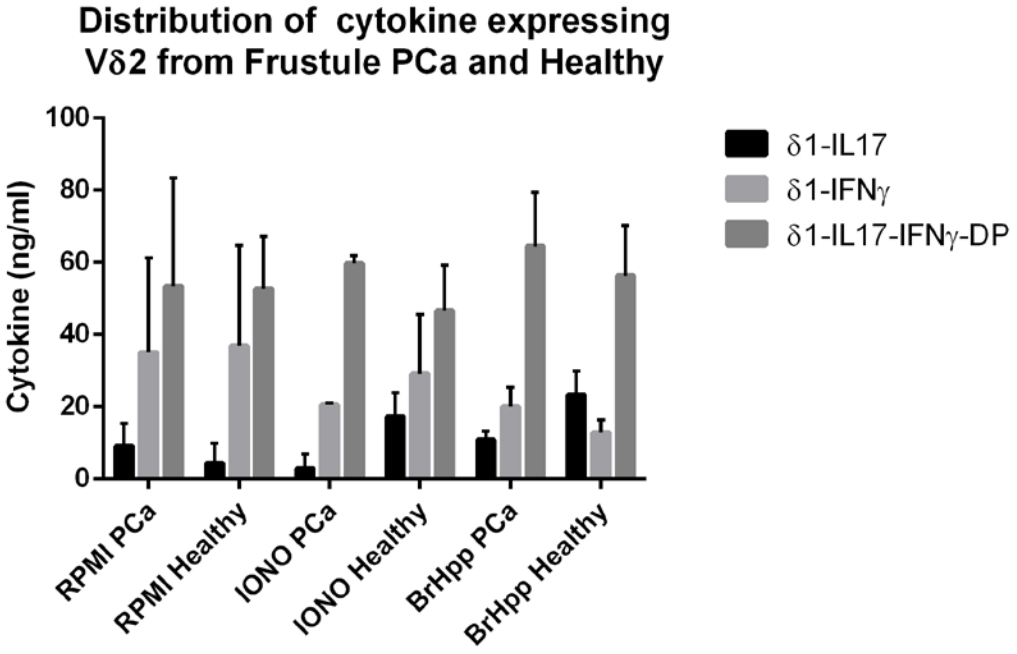

Figura 16 Percentages of Cytokines Vδ2 T from CaP and Healthy tissues. Cytokine production capacity of Prostate cancer PCa-derived γδ T cells TILS and Healthy Tissue. Cytokine production by Vδ2 T from cancer and healthy tissue were determined by FACS analysis of supernatants with only RPMI (negative control without stimulation) and after 4 hrs stimulation with PMA and ionomycin (Positive control) and stimulation with BrHpp (Antigen Specific Control) . P>0,05

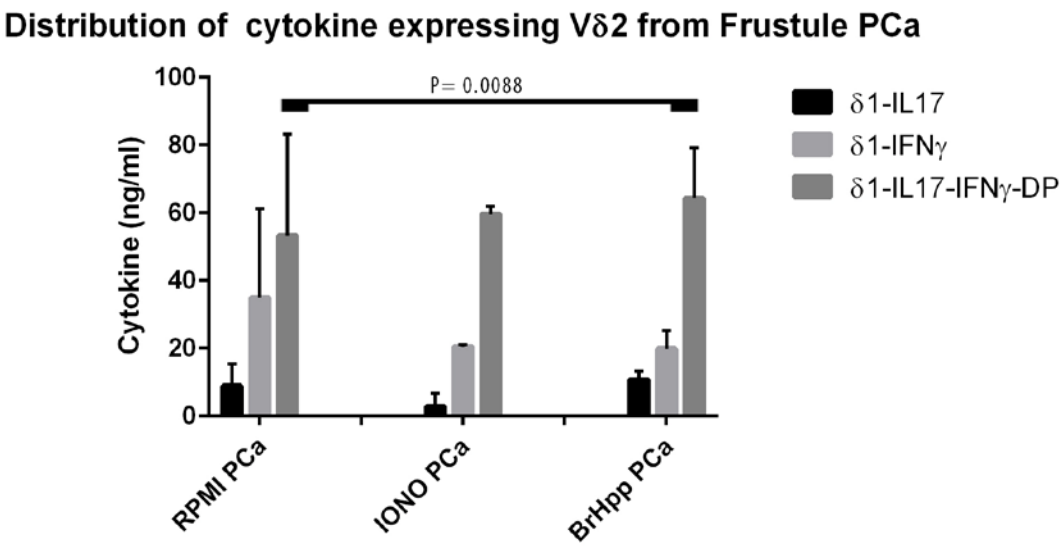

Figura 17 Cytokine production capacity of Prostate cancer PCa-derived γδ T cells TILS. Cytokine production by Vδ2 T from TILS were determined by FACS analysis of supernatants with only RPMI (negative control without stimulation) and after 4 hrs stimulation with PMA and ionomycin (Positive control) and stimulation with BrHpp (Antigen Specific Control) . P<0,05 DS 6,36 DS 2,27

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CURRICULUM VITAE

Daniela Coniglio was born on 19th February 1980 in Rheinfelden, Germany.

In 2000 she got a Agricultural Diploma.

In December 2008 she got a certificate in "Expert in Proteomic and Genomic applications", C.N.R and Clinic Maddalena (DOSAC) of Palermo.

In 2010 she graduated in Biotechnology for Industrial and Scientific Research at the University of Palermo with full mark.

She was exchange student in Biochemistry Department, University of Leicester, United of Kingdom, under the supervisor Professor Barlev M. from March 2011 to October 2011.

In 2011 she qualified also as a professional Biologist with full mark.

In January 2011, she started her doctorate under the supervision of Prof. Serena Meraviglia purchasing advanced specialized studies in Immunology with particular attention on human infiltrating γδ T cells in cancer.

She has attended the Department of Immunology at the Faculty of Biology of the University of Warsawa from 1 May to 9 December 2014, under the supervision of Prof. Drela N.