UNIVERSITÀ DEGLI STUDI DI PALERMO

INTERNATIONAL PhD PROGRAM IN IMMUNOPHARMACOLOGY
Dipartimento di Biopatologia e Biotecnologie Mediche (MED 04)

CHARACTERIZATION OF CIRCULATING AND INFILTRATING γδ AND REGULATORY T LYMPHOCYTES IN NMSC

PhD CANDIDATE
Elena Lo Presti

PhD COORDINATOR
Prof. Francesco Dieli

TUTOR
Prof. Francesco Dieli

Co-TUTOR
Dott. Serena Meraviglia

XXV CYCLE
ACCADEMIC YEAR 2015

Dedicated to my parents who, with great love, have lead me towars this goal!

I'm greatful to all my friends and colleagues for their support. A special thanks to my sisters for their patience with me, and finally but not the least, to Professor F. Dieli and Dr S. Meraviglia because they trusted in me since the very first moment we met.

1 Introduction

1.1 Incidence and epidemiology of nonmelanoma skin cancer (NMSC)

Epithelial surfaces serve as selective barriers and together with immune system defend from tissue-damaging radiation, toxins, mutagens and microorganisms. Reliance on the layer involved in the mutations it is possible distinct different type of tumors such as melanoma (keratinocytes layer) or SCC (spinosa layer of epithelia). Commonly Melanoma and nonmelanoma skin cancer (NMSC mainly basal and squamous cell carcinoma – BCC and SCC respectively) (Figure 1) are known as the most common types of cancer in white populations. Both tumor entities show an increasing incidence rate worldwide. (1). Incidence rates of NMSC vary vastly by geographical area: Australia, New Zealand and United States in which 200,000 new cases of SCC were diagnosed in 2000 (2). In Europe, the incidence rate of NMSC was reported to be 129.3 in men and 90.8 in women (European standard), in northern Germany (Cancer Registry of Schlewig Holstein) the crude incidence rate was 119.3/100,000 in men and 113.8/100,000 in women between 1998 and 2001 (3).

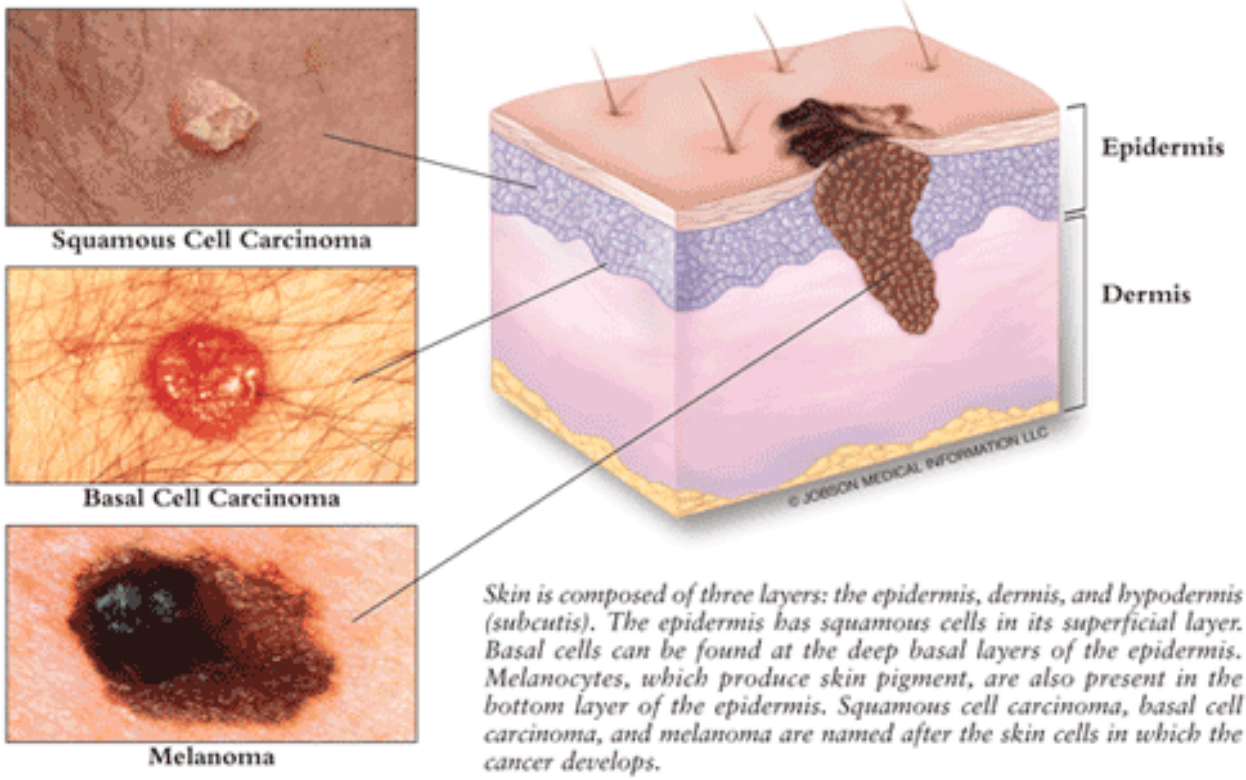
Nonmelanoma skin cancer generally occurs in persons older than 50 years and in this age group, its incidence is increasing rapidly. Squamous cell carcinoma (SCC) is an uncontrolled growth of abnormal cells arising in the squamous cells, which compose most of the skin's upper layers (the epidermis). The increasing incidence rates of NMSC are probably caused by a combination of increased sun exposure or exposure to ultraviolet

(UV) light, increased outdoor activities, changes in clothing style, increased longevity, ozone depletion, genetics and in some cases, immune suppression. The incidence of NMSC (BCC and SCC) is 18/20 times higher than that of malignant melanoma. (4). The annual increase varies between populations but in general has been estimated to be between 3 and 7%, with mortality rates increasing less quickly. These estimates suggest a doubling of rates every 10/20 years.

The anatomic pattern of increase in BCC and SCC incidence was consistent with an effect of greater sunlight exposure. Over 80% of NMSCs occur on areas of the body that are frequently exposed to sunlight, such as the head, neck and back of the hands (5). While in melanoma the anatomic site varies according to gender. In men most of the tumors are localized on the trunk, in women the preferred site is lower extremity (6).
The immunoregulatory events leading to NMSC is not clear and it remains yet not clear how it develop in aggressive and metastatic shape.
Among the hypothesis of the events involved there were: infections with human papilloma virus HPV5 and HPV8 may represent an increased risk for SCC development in transplant recipients (7). The mechanisms by which these viruses may contribute to skin cancer development still remain unclear. In organ transplant recipients a highly increased risk for nonmelanoma skin cancer was found in several studies. This cancer risk associated with transplantation is higher for sun exposed than for nonsun-exposed epithelial tissues, even among populations living in regions with low insolation (8-9).
There is strong evidence to suggest that the role of ultraviolet (UV) radiation in the development of skin cancer is multifold: (10) Most UV induced damage to the cellular DNA is repaired, however, mutations may

occur as a result of base mispairing of the cellular DNA. The genes involved in the repair process are also potential UV targets. p53 is a nucleoprotein encoded by a tumour suppressor gene. Mutations of the tumour suppressor gene p53 are implicated in the genesis of a wide variety of human neoplasia including NMSC. These mutations were reported to be present in 50% to 90% of SCCs and approximately 55% of BCCs including very small lesions (11,12). It induces a state of relative cutaneous immune suppression that might prevent tumour rejection and might allow the persistent infection with Human Papilloma Viruses (HPV) as shown in immune suppressed patients (7).

Figure 1. Types of Skin Cancer



Skin is composed of three layers: the epidermis, dermis, and hypodermis (subcutis). The epidermis has squamous cells in its superficial layer. Basal cells can be found at the deep basal layers of the epidermis. Melanocytes, which produce skin pigment, are also present in the bottom layer of the epidermis. Squamous cell carcinoma, basal cell carcinoma, and melanoma are named after the skin cells in which the cancer develops.

Source: National Cancer Institute. NCI Visuals Online. http://visualsonline.cancer.gov. Accessed October 9, 2012.

Figure 1: Characterization of types of skin cancer. NMSC is named reliance on the skin cells in which the cancer development.

2 Tumor-infiltrating γδ T lymphocytes

In this chapter we review a studies of tumor-infiltrating γδ T lymphocytes from patients with different types of cancer, and we will discuss their clinical relevance (13).

2.1 γδ T lymphocytes: antigen recognition and effector functions

T cells carrying the T cell receptor (TCR) are important effector cells that may play a role in the anti-tumor immune response. ãä cells are not a homogeneous population of cells with a single physiological role. Instead, ever increasing complexity in both phenotype and function is being ascribed to ãä cell subsets from various tissues and locations, both in mice and humans.

γδ T cells account for 1% to 5% of CD3+ T cells in the peripheral blood, but constitute a major subset in other anatomic sites, such as the intestine or the skin (here, however, only in the murine but not in human skin [1]). In the blood of most healthy individuals, T cells expressing the Vδ2 gene paired with one particular Vγ9 chain (referred to as Vγ9Vδ2 T cells) account for 50% to > 90% of the γδ T cell population. In contrast, intestinal intraepithelial γδ T cells frequently express the Vä1 gene, which can associate with different Vγ elements [1,2]. Vδ1 γδ T cells recognize the MHC class I-related molecules MICA, MICB and ULBPs which are expressed on epithelial cells by heat shock or oxidative stress and are constitutively expressed to variable levels on many epithelial and hematopoietic tumor cells [3,4]. It has been debated whether

MICA/MICB and ULBPs are directly recognized by the Vδ1 TCR or, indirectly activate Vδ1 T cells upon binding to the stimulatory natural killer (NK) receptor, NKG2D, which is also expressed by the vast majority of γδ T cells

Vγ9Vδ2T cells recognize phosphoantigens (PAgs) without requirement for antigen processing and presentation, and MHC restriction. PAgs are pyrophosphates derived from the microbial non-mevalonate isoprenoid byosynthesis pathway [5,6]. Structurally related pyrophosphates are generated in eukaryotic cells through the mevalonate pathway. Micromolar concentrations of endogenous pyrophosphates are requied for Vã9Vä2 T cell activation and such concentrations are achieved after cellular stress and trasformation [7]. Given the cross-reactivity between microbial and self PAgs, there is a great interest in elucidating how TCR signaling can be induced by such small molecules. PAgs can directly activate Vã9Vä2 T cells, but such activation is greatly enhanced by monocytes and/or dendritic cells (DCs). Hence, either PAgs are presented as cargo to the reactive ãä TCR or their cellular processing somehow sensitizes cell recognition through the engagement of the Vγ9Vδ2 TCR by stabilizing surface expression of a TCR-binding molecules [8]. A candidate molecule involved in intracellular PAg processing is the F1-ATPase, which directly binds the Vγ9Vδ2TCR and interacts with ApppI, an adenosine derivative of IPP [9]. Moreover, it has been recently found that PAg-induced Vã9Vä2 T cell activation requires butyrophilin 3A1 (BTN3A1)[10]. Therefore, production of exogenous PAgs or up-regulation of endogenous PAgs in human cells in response to either infections or tumor transformation provokes Vγ9Vδ2 T cell reactivity, albeit at substantially different sensitivity. Intracellular levels of PAgs can

be manipulated by drugs. Aminobisphosphonates, such as zoledronic acid, which are in clinical use for the treatment of osteoporosis and bone metastasis, are potent inhibitors of the downstream enzyme of the mevalonate pathway farnesyl pyrophosphate synthase, thereby leading to the intracellular accumulation of upstream metabolites as IPP and in consequence to Vγ9Vδ2 T cell activation [11,12]. On the contrary, statins block the upstream enzyme hydroxy-methylglutaryl-CoA reductase and inhibits IPP production, inhibiting Vã9Vä2 T cell activation [12].

2.2 γδ T cells for tumor immunotherapy

The major goal of tumor immunotherapy is the induction of adaptive responses of B cells and MHC-restricted αβ T cells, particularly CD8 cytotoxic T cells. Nonetheless, despite major advances in this area, durable responses are rare and immunotherapy is not yet an established modality to treat tumors. Furthermore, tumors frequently develop strategies to escape immune responses [25, 26]. In contrast to αβ T cells, γδ T cells have unique features (see Table 1) which makes them good candidates for effective tumor immunotherapy. For instance, they lack MHC restriction, and do not require co-stimulation. Therefore, common tumor antigens without MHC restriction provide broader applicability of γδ T cells across a wide range of tumors and patients with diverse MHC alleles. Moreover, γδ T cells display potent cytotoxic and anti-tumor activity in vitro [27-31] and in xenograft models in vivo [32,33]. Cytotoxicity of γδ T cells against tumor cells is associated with increased production of PAgs [34], which is, at least, partly due to the increased expression of hydroxy-methylglutaryl-CoA reductase, the rate limiting

enzyme of the mevalonate pathway [34]. Moreover, intracellular levels of IPP can be manipulated by aminobisphosphonates [11-13, 35-37], thereby leading to the intracellular accumulation of IPP and in consequence to activation of Vγ9Vδ2 T cells [34]. As above discussed, in addition to the binding of the antigenic molecules to the reactive TCR, Vγ9Vδ2 T cells express NK cell activating receptors such as NKG2D, which recognizes target cells expressing MICA, MICB, and ULBPs [3,4,38,39]. These interactions may prove crucial in Vγ9Vδ2 T cell recognition and killing of tumors of hematopoietic origin. In fact, the expression levels of ULBP1 determine lymphoma susceptibility to Vγ9Vδ2 T cell-mediated cytolysis, highlighting a thus far unique physiologic relevance for tumor recognition by Vγ9Vδ2 T cells [40,41].

After recognizing target cells via the TCR, or NKG2D, or both, Vγ9Vδ2 T cells preferentially use the perforin/granzyme [42] and/or TRAIL [43] pathways, as well as the Fas/FasL killing signal [44], for cytotoxicity against target cells like tumor cells. In addition, activated Vγ9Vδ2 T cells secrete IFN-γ and TNF-α, which have cytotoxic activity against tumor cells directly and indirectly via stimulating macrophages and dendritic cells [45].

Overall, the potent antitumor activity of Vγ9Vδ2 T cells and their wide reactivity to several tumor cell types has led to the exploration of their therapeutic potential. Two strategies have been developed to apply the anti-tumor activities of Vγ9Vδ2 T cells to cancer immunotherapy: 1) in vivo administration of compounds that activate Vγ9Vδ2 T cells and 2) adoptive transfer of ex vivo expanded Vγ9Vδ2 T cells. Several small-sized clinical trials have tested the efficacy of any of these two strategies in patients with various tumor types and a recent meta-analysis based on

data from 13 clinical trials including a total of 204 patients has demonstrated that Vγ9Vδ2 T cell-based immunotherapy improves overall survival and, in view of its low toxicity grade [45], provides a proof of principle for its utilization as adjuvant to conventional therapies."

2.3 Tumor infiltrating gd T cells and their correlation to cancer outcome

Tumor-infiltrating leukocytes are an heterogeneous population of immune cells that have been found in a wide variety of solid tumors [47] and the extent of leukocyte infiltration has been often associated with improved prognosis [48]. However, there is a limited number of studies regarding the contribution of individual leukocyte subsets to survival. Tumor-infiltrating leukocytes include cells of the myeloid lineage (granulocytes, macrophages, myeloid-derived suppressor cells) and several different lymphocyte subsets (T, B, NK), each with different impact on tumor progression. Results of mouse tumor models and human cohort studies have suggested that any individual leukocyte population may correlate with poor or better prognostic factors, such as tumor stage/grade, presence of metastasis and disease-free/overall survival. In general, infiltration by myeloid cells has been associated with tumor progression, while the presence of abundant T cells (particularly of the CD8 subset) is associated with tumor regression and improved prognosis. However, the limits of the immunohistochemical techniques largely used in retrospective clinical studies, have so far prevented a detailed descriptions of different tumor-infiltrating leukocyte populations as well as evaluation of their functional

properties in the tumor microenvironment. For instance, tumor-infiltrating T lymphocytes may be endowed with regulatory function and hence promote tumor progression.

Several studies have shown that γδ T cells are present among TILs from patients affected by different types of cancer, but their clinical relevance remains still obscure because of conflicting results obtained. In detail, there have been 5 relatively recent large studies which have correlated tumor infiltrating γδ T cells with several different clinical features:

Bialasiewicz et al. [49] evaluated by immunohistochemical analysis TILs in 113 specimens from patients with necrotising choroidal melanoma. They detected TILs in 76% of samples and γδ T cells, mainly of the Vδ1 subset were present in 52% of samples. Most notably, the presence of γδ T cells in tumors positively correlated with patient's survival, indicating that tumor-infiltrating γδ T cells are a prognostically favorable factor.

Inman et al, [50] assessed by immunohistochemical analysis total γδ T cells in 248 renal cancer specimens and correlated these values with clinicopathologic prognostic factors and cancer outcome. They found that percentages of intratumoral γδ T cells were usually very low (<1% of the CD3+ population) in nearly all tested tumor specimens and did not correlate with any examined prognostic factor or even with survival. Authors concluded that the role of γδ T cells in renal cancer is questionable.

Ma et al. [51] examined by immunohistochemistry total γδ T cells infiltrating breast cancer in specimens of 46 patients. γδ T cells were detected in nearly all cancer patients (93%), but only in 3% of normal breast specimen. Authors did not quantify the percentages of

intratumoral γδ T cells, but when an arbitrary cutoff of 9 γδ T cells per high magnification microscopic field was used to define TIL-high (> 9) and TIL-low (< 9) groups, authors found that γδ T cell numbers were positively correlated with advanced tumor stages, HER2 expression status and high lymph node metastasis, but inversely correlated with relapse- free survival and overall survival of patients. Multivariate and univariate analysis of tumor- infiltrating γδ T cells and other prognostic factors further suggested that intratumoral γδ T cells represented the most significant independent prognostic factor for assessing severity of breast cancer compared with the other known factors. Authors concluded that tumor- infiltrating γδ T cells play a crucial role in breast cancer progression and pathogenesis and may serve as a valuable and independent prognostic biomarker for human breast cancer.

Cordova et al. [52] studied the representation of tumor infiltrating γδ T cells from 74 patients with primary melanoma. γδ T cells were the major subset among CD3+ T lymphocytes and comprised equal percentages of Vδ1 and Vδ2 T cells with effector memory (TEM) or terminally differentiated effector memory (TEMRA) phenotypes. In this study, the presence of γδ T cells, and in particular the Vδ2 subset, among TILs significantly correlated with early stage melanoma, while percentages of infiltrating Vδ1 T cells did not correlate with any examined prognostic factor of melanoma.

Finally, Wu et al. [53] demonstrated that γδ T cells (γδ17) are the major source of IL-17 in human colon cancer, with the majority (80%) of the IL-17+ γδ T cells expressing Vδ1 and 20% expressing Vδ2. Importantly, analysing 117 colon cancer samples, authors found that γδ17 cell infiltration positively correlated with tumor stages and other

clinicopathological factors (tumor size, tumor invasion, lymphatic and vascular invasion, lymph node metastasis, and serum CEA levels), indicating that tumor-infiltrating γδ17 T cells are associated with tumor invasiveness and progression and may thus represent a prognostic factor in human colon cancer.

2.4 Tumor-infiltrating γδ T cells: what are they and what do they do?

The above discussed findings that tumor-infiltrating γδ T cells correlate with tumor remission, or with tumor progression or even fail to correlate with any prognostic feature strongly suggest that γδ T cells in the tumor microenvironment may play substantially different functions; hence positive or negative correlation with prognosis may depend on the specific γδ T cell subset/function recruited at the tumor site. Furthermore, the net biologic effects of γδ T cells may depend on the tumor type and the tumor site, perhaps reflecting microenvironmental differences: for instance TGF-β, which is abundantly secreted at the tumor site by tumor-infiltrating macrophages or by tumor cells themselves, may favor the differentiation of γδ cells with Treg-like properties, which in turn inhibit anti- tumor immune responses.

Initial studies on the functional properties of tumor-infiltrating γδ T cells were performed using polyclonal γδ T cell lines generated in vitro upon long term culture with mitogen/antigen and IL-2: this approach was mainly due to the very low number of γδ T cells recovered from tumor specimen and to the lack of suitable techniques which allowed precise detection of functional markers. These studies have unequivocally demonstrated that ex vivo expanded γδ T cell lines and clones from renal,

breast, lung, ovarian, colon and pancreatic cancer efficiently kill stabilized tumor cell lines and freshly isolated tumor cells and generally Vδ1 T cell lines had the higher cytotoxic activity compared to Vδ2 T cell lines [54-59]. Accordingly, Cordova et al. [52] confirmed these results using polyclonal γδ T cell lines derived from melanoma; both Vδ1 and Vδ2 T cell lines produced equal amounts of TNF-α and IFN-γ, but while the majority (75%) of Vδ1 T cell lines exerted potent cytotoxic activity against melanoma cell line in vitro, only 25% of the Vδ2 T cell lines showed appreciable lytic activity. Therefore, based on their cytolytic activity and production of cytokines with proven anti-tumor effect, tumor-infiltrating γδ T cells have been long regarded as important players of the anti-tumor immune response. However, both the failure to consistently detect a positive correlation between the presence of γδ T cells in the tumor microenvironment and the patient's prognosis, as well as the improvement of immunological techniques to detect functional signatures even in very small tissue samples have subverted the concept that γδ T cells are simply an important component of resistance to cancer and suggested that their function may be extremely pleiotropic and including either effector or suppressive potential.

In 2007, Peng and colleagues [60] unexpectedly identified a Vδ1+ population (which comprised over 95% of the total γδ T cells population) among breast cancer-infiltrating lymphocytes capable to suppress immune responses. In particular, Vδ1 cells inhibited CD4 and CD8 T cell activation and impaired dendritic cell maturation and function. Although the mechanisms responsible for the regulatory activity of tumor-infiltrating Vδ1 cells was not investigated in that paper, it seems to involve TLR8 signaling pathway, as suppression was reversed by TLR8

ligands. Later on, the same group reported [61] that breast cancer infiltrating Vδ1 cells induced both T cell and DC senescence and the senescent T cells and DCs in turn became regulatory cells, thus determining amplification of the immunosuppressive process. Interestingly, and surprisingly, accumulation of regulatory Vδ1 cells in the context of breast cancer (where they account for approximately 30% of the total lymphocyte population) is not due to proliferation of resident Vδ1 cells but to their recruitment mediated by IP-10 secreted by breast cancer cells.

In addition to the above quoted studies on tumor-infiltrating human regulatory γδ T cells, four recent reports, three in mice and one in humans, have shed light on the regulatory role played by IL-17-producing γδ T cells (γδ17) and have also defined the underlying mechanisms.

Using a transplantable tumor mouse model, Wakita et al. [62] observed that γδ T cells accounted for 25% of all tumor-infiltrating lymphocytes and selectively produced IL-17 but not IFN-γ. Importantly, absence of IL-17 caused inhibition of tumor growth which correlated with a reduced number of blood vessels within the tumor and reduced expression levels of VEGF and Ang-2 in tumor cells. This indicates that tumor-infiltrating γδ17 T cells promote angiogenesis, and thus tumor growth.

A similar detrimental effect of IL-17 has been reported by Ma and colleagues [63] in an hepatocellular carcinoma mouse model. Similarly to the findings of Wakita et al, γδ T cells were the major source of IL-17 amongst lymphocytes infiltrating hepatocellular carcinoma. In this model, absence of IL-17 reduced tumor growth, while its administration promoted the growth of hepatocellular carcinoma. However, the mechanism

responsible for the anti- tumor activity of IL-17 was different from that reported by Wakita and involved a reciprocal activatory interaction between the γδ17 cells and MDSC which was mediated by cancer cells: in detail, γδ T cell-derived IL-17 induced CXCL5 production by tumor cells which in turn recruited MDSC to the tumor sites via CXCL5/CXCR2-interaction. Once at the tumor site, IL-17 induced production of IL-1β and IL-23 in MDSC which amplify differentiation of γδ17 cells. This positive feedback between γδ17 cells and MDSC sustains immunosuppression and promotes tumor growth.

The third mouse study by Silva Santos and colleagues [64] used a transplantable peritoneal/ovarian cancer, and confirmed the crucial role of γδ17 in promoting cancer growth. γδ17 accumulated in the peritoneal cavity and were the main source of IL-17 also in this model. γδ17 caused the recruitment at the tumor site of an unconventional population of small macrophages that expressed IL-17 receptor and a number of pro- tumor and pro-angiogenic molecules amongst which VEGF and TGF-β, which promoted cancer cell proliferation and tumor growth.

The fourth study on the participation of γδ17 cells in cancer was performed by Wu et al. [53] in human colorectal cancer. In that study, tumor-infiltrating γδ T cell were the main source of IL-17 and 80% of the γδ17 cells expressed Vδ1. Of note however, γδ17 constituted approximately 25% of all tumor-infiltrating Vδ1 cells and co-produced TNF-α, IL-8 and GM-CSF. All these cytokines, in different combinations, caused recruitment (IL-8 and GM-CSF) and survival, activation and proliferation (TNF-α, IL-8 and IL-17) of MDSC that in turn mediate immunosuppression and promote tumor growth.

Altogether these results clearly demonstrate that γδ17 cells are key mediators of tumor- associated immunosuppression thereby influencing tumor progression.

2.5 Hypothesis: tumor microenvironment as the critical determinant of tumor-infiltrating γδ T cell fate

The conditions under which γδ T cells can contribute to tumor control versus immune suppression need to be defined. There are several theoretical possibilities to answer the fundamental question of the molecular mechanisms that explain these two γδ T cell phenotypes.

First, it is possible that genetic differences in tumor cells influence the host response, through the involvement of different pathways that are mutated or activated in a heterogeneous fashion and that regulate the expression of immune system regulatory genes. For instance, tumor cells with STAT3 activation show impaired production of chemokines and cytokines, but increased production of immunosuppressive factors and thus escape immune recognition [65].

Second, it is possible that polymorphism of regulatory genes might influence lymphocyte activation at the tumor site. For instance, IRF5 polymorphism is associated with clinical response to adoptively transferred TILs in melanoma patients [66].

Third, it is likely that exposure to certain pathogens or even the intestinal microbiome could change the frequency, phenotype and functions of TILs. For instance Wu et al. [53] showed that in colon cancer patients, distruction of the epithelial barrier caused by tumor development results in

tumor invasion by commensal bacteria (E. Coli) and release of bacterial product which promote IL-23 production by DCs and γδ17 cell polarization in situ. Forth, it is likely (and this is the possibility we favor) that tumor microenvironment plays a key role. By definition, tumor microenvironment is a complex network of different cell types, soluble factors, signaling molecules and extracellular matrix components, which orchestrate the fate of tumor progression [67]. In fact, in addition to the tumor cells and to the several lymphoid and myeloid cell types that infiltrate tumors, classical cellular components of the solid tumor stromal microenvironment also influence the host immune response. The tumor stroma consists of fibroblasts, macrophages and vascular endothelial cells, with variable amounts of extracellular matrix, all of which contribute not only as a support structure for tumor growth, but can also impair host immune responses and likely contribute to the quality of immune cell infiltration [67]. We hypothesize (Figure 1) that, at early stages of tumor development γδ T cells of the γδ1 type producing cytokines with proven anti-tumor activity (IFN-γ and TNF-α) and equipped with cytotoxic potential either expand locally (Vδ1) or are recruited at the tumor site from peripheral blood (Vδ2) and may exert anti-tumor activity; however, with tumor progression, factors produced in the microenvironment cause polarization of γδ cells from γδ1 to γδ17 and γδreg which instead promote tumor progression. A plethora of cell types present in the tumor microenvironment may actually provide the source of such γδ cells polarizing factors.

For instance, colon cancer stem cells and tumor-associated macrophages and fibroblasts produce huge amounts of TGF-β [68] which, in combination with other cytokines present in the microenvironment,

contribute to polarization of γδ T cells to γδ17 and γδreg. Macrophages, DCs and other myeloid cells, which are typically found in the solid tumor microenvironment, produce IL-15 which in combination with TGF-β determines γδreg polarization [24], and IL-1β, IL-6, IL-23 and TGF-β, which in different combination promote γδ17 polarization [20].

Once activated, γδreg and γδ17 amplify the immunoregulatory process in different ways (Figure 2): IL-17 promotes VEGF production by cancer cells and macrophages, and CXCL5 production by tumor cells which, in turn, recruited MDSC. An activatory cross-talk is then established at the tumor site between MDSC and γδ17, by which IL-17 induces IL- 1β and IL-23 production by MDSC and these cytokines promote further differentiation and activation of γδ17 T cells. Finally, γδreg produce IL-10 and TGF-β which act on several cellular targets to promote immunosuppression at the tumor site and favor tumor progression.

3 What about the immune response in SCC? Studies and news

This study starts with the consideration that T cells isolated from SCCs of both normal and immunocompromised patients had greatly increased numbers of FOXP3+ Treg cells when compared with the population found in normal skin. Indeed we have found more publications about Treg than gd T cells in NMSC. Because of the bad correlation between Treg and progression of the tumor vary studies focused the attention on the suppression activity of Treg in different tumors. The object of the suppression may be multiple and, in general regard to the effector cells such as NK, CD8, CD4 Th1 and other subpopulations (89-94). A lot authors have analysed the immunosurveillance activity of γδ T cells in mouse and thanks to the clinical trial in human (using Vδ2 as anti-tumoral agent) there was an increased interested also in cancer disease. On the other hand, an emergent new hypothesis of pro-tumoral activity of the gd T cells has increased the interesting (75). Moreover the tumors selected for the research are usually related to the incidence and aggressiveness. For the above cited we try to describe some related works about γδ T cells and Treg mainly in skin and NMSC. Given that there aren't study, until now, on the relationship between Treg and γδ T cells in NMSC, we also present a brief on the studies about the some possible cellular relation in human.

3.1 SCC and γδ T cells

Whereas the majority of T cells express a T-cell receptor (TCR) composed

of αβ heterdimers, a smaller population expresses a γδ TCR with less TCR diversity than αβ T cells. In 2005 Havran's (95) murine model summarized the relevant role of epithelia infiltrating γδ T cells (termed DETC) expressing Vγ5Vδ1 T-cell receptor (TCR) only found into skin in the wound-healing (14). They are derived from fetal thymic precursor cells and are in constant contact with neighbouring epidermal cells and they played unique roles in tissue homeostasis. Indeed, during tissue repair, epithermal γδ T cells produced in loco distinct growth factors such as keratinocyte growth factors that induced epithelial cells to produce hyaluronic and insulin-like growth factor-1(IGF-1). In healthy human skin γδ T cells are rare and until now mouse DETC doesn't exist. Vδ1 T cells are considered to be the only primary γδ T cells subset in skin playing the role in skin cancer immune surveillance and wound healing. Studies in mice and humans have improved the understanding of responces of T cells and in particular their relationship to other lymphoid cells. Girardi in 2006 summerized the relative contributions of αβ and γδ T cells to coetaneous tumor immunosurvellance in mice (considering the cutaneous functions of γδ T cells compare to αβ T cells). Actually, mouse model permits to elucidate three different pathway in which gd T cells can carry out in skin: direct killing of transformed T cells, IFN–γ production and finally immunoregulatory mechanism. In particular Girardi et al made tumor rapidly in γδ-deficient mice after injection of SCC or melanoma tumor cell lines revealing that γδ T cells not only inhibit the early stage of the tumor development but also limit progression to carcinoma probably thanks to more different antitumor mechanisms (96). Anyway, the excessive activities of lymphoid cells may result in autoimmune and inflammatory disease and within this context there is the crucial roles played by Treg with their immune regualtory function to maintain the immunologic

balance. Interesting data provided by studies in TCRb-/- mice reveal suggest that gd T cells may downregulate potentially pro-tumoral activity by ab T cells (97). On the other hand, the selective repopulation of TCRd-/- mice with Vγ5 DETC by neonatal transfer with fetal thymic DETC precursors abrogate the augmented dermatitis, thus demonstrating that resident gd T cells may provide a local T regulatory function (98).

Recently, Nestle and colleagues (99) investigated whether other subsets of γδ T cells perform a similar lymphoid stress-surveillance role as their murine counterpart. They found it in Vγ9Vδ2 T cells subset that in general are found in peripheral blood. In psoriasis patient blood, a common chronic inflammatory skin disease with genetic origin, they found a decreased numbers of circulating Vγ9Vδ2 T cells compare with healthy control and increased presence of Vγ9Vδ2 T cells in psoriatic skin. They used flow cytometry analysis of fresh PBMC to assess the skin homing phenotype of circulating Vγ9Vδ2 T cells in psoriasis patients and healthy volunteers. The frequency of Vγ9Vδ2 T cells expressing CLA^+ (marker for skin homing T) are significantly decreased in patients than healthy and the skin-associated chemokine receptors such as CCR6 it was increased.

Expression of CLA on peripheral Vγ9Vδ2 T cells suggested the existence of a specialized skin homing possibly recruited to skin under conditions of skin perturbation. Making in vivo perturbed human skin, Nestle et al , showed the migration of CLA+ Vγ9Vδ2 T cells to skin. Moreover this subsets were preferentially located in psoriasis skin lesions but no in normal skin such as demonstrated in immunohistochemical staining for Vγ9+ and CLA. Finally the underlined a correlation between severity of psoriatic disease and decreased percentage of circulating Vγ9Vδ2 T cells while infiltrating skin Vγ9Vδ2 T cells was increased. These data strongly

highlight that Vγ9Vδ2 T cells may redistribute from blood to skin during the inflammation (99).

γδ T cells were analysed in necrotising choroid melanoma by Bialasiewicz et al. (100) evaluated through immunohistochemical analysis TILs in specimens from patients with. They detected TILs in 76% of samples and γδ T cells, mainly of the Vδ1 subset were present in 52% of samples. Most notably, the presence of γδ T cells in tumors positively correlated with patient's survival, indicating that tumor-infiltrating γδ T cells are a prognostically favourable factor. For the other hand Cordova et al. [65] studied the representation of tumor infiltrating γδ T cells from patients with primary melanoma and γδ T cells showed effector memory (TEM) or terminally differentiated effector memory (TEMRA) phenotypes. Surprisly, in this study, the presence of γδ T cells, and in particular the Vδ2 subset, among TILs significantly correlated with early stage melanoma, while percentages of infiltrating Vδ1 T cells did not correlate with any examined prognostic factor of melanoma.

It is clear the role of γδ T cells in the immune response into the perturbate skin displayed a significantly different role of the subsets. Even if, don't in direct way in skin, Vδ1 T cells can take on a different role than Vδ2 T cells and this certain in Breast cancer and ovaric cancer (respectively human study and mouse) in which don't have a Th1 function but regulatory function. This last aspect is a critical point of the studies on γδ Tcells. In 2006, Hoffmann and Whiteside (101) published a research on circulating γδ T cells in SCC patients. They underline the correlation with the metachronous secondary primary and the high level of γδ T cells. After surgical removed and chemotherapy, the frequency was always higher than before treatment. They concluded than this increment was

related to the host answer to the tumor and the therapeutic treatment may influence the gd T cells anti-tumoral activity.

For this reason this study try to analyse, for the first one, the frequency of both subset of γδ T cells (Vδ1 and Vδ2 T cells) in NMSC (focusing the attention on SCC) comparing it with the peripheral blood from the same patient.

Given that it doesn't clear their role (that it may be different and distinctive) to improving our understanding we also retain necessary to evaluate Treg cells.

3.2 SCC and Tregs

Given that exist different subsets of Treg: tr1, tr10, natural and induced Treg, we focus our attention on resident Treg and induced Treg capable to suppress the immune response.

The role of CD4+ T cells is complex indeed subsets of CD4+ T cells are involved in initiating and maintaining anticancer immune responses and down-regulate the antitumor response such as CD8+ T lymphocytes. For long time is known that Treg frequency is increased in the peripheral circulating and their accumulate in the tumor is related with reducing survival. Whiteside's group (102) described an enrichment of Treg in tumor-infiltrating and circulating in SCC and two years after suggested that the oncologic therapy might contribute to expansion of active Treg perhaps using a selective mechanism. For this reason the studies of interactions of Treg with different immune cell subsets are urgently required to improve the immunotherapeutic strategies to overcome tumor

immune escape.

Moreover, data obtained from Harvard Skin Disease Research Center by Kupper's lab evidenced that blood vessels in SCCs did not express E-selectin and this evade the immune response at least in part. This factor has effect in the immunesurveillance because tumor infiltrating lymphocytes contained few cutaneous lymphocyte antigen (CLA)+ CD8+ T cells and about 50% of these T cells infiltrating untreated SCCs were FOXP3+ regulatory T (Treg) cells. More than these cells were recruitment from the blood and accumulated within SCCs. Probably the mechanism to accumulate is caused by the chemokines attraction that permit to migrate efficiently to tumor skin. In addition, human skin also contains a resident population of regulatory T cells (Treg) that represent between 5 and 10% of the total resident skin T cells (103,104).

According to Clark (105) these cells accumulated over time and they keep memory to antigens encountered through skin and can proliferate in inflamed tissue even in an antigen-independent manner (103).

Another point of view to look at Treg from skin it is to analysed the composition of normal human skin that contains also distinct populations of dendritic cells (DC): Langerhans Cells (LC) (106), and dermal resident DC (DDC) expressing CD1c, which is also known as blood dendritic cell antigen (BDCA1) (107,108).
Murine LC are more known than human. LC has been found to polarize of naïve CD4 T cells into Th1,Th2 and Th17, Th22 but, until now, there is substantial controversy regarding the physiologic role of LC with regard to immunoregulation versus immunostimulation (109). Seneshal et al in 2012 (110) demonstrated that LC induced the proliferation of a subset of

autologous skin resident memory T cells in vitro. These proliferating T cells were exclusively CD4+ CD25+ FoxP3+ CD127− skin resident Treg cells, and their function was demonstrated by suppression of proliferation of autologous skin resident T effector memory cells.

Finally, we wanted to cite a work by Gasparoto et al (111) that, in the same way had underlined Strauss in 2007 (112), compare the percentage of infiltrating and circulating Treg in oral SCC. Suppression assay was used to demonstrate an inhibitor activity on effector cells to produce cytokine or proliferate in presence of Treg obtained from SCC patients.

3.3 Relationship Treg vs gd T cells:

Many studies have demonstrated that CD4+ CD25+ Foxp3+ Treg suppression of human CD4+ T cells is contact dependent and independent of IL-10 or surface molecules such as CTLA4, PDL1, GITR (52 Mahan Ref) and also various immune cell populations such as conventional CD8 T cells, (113) natural killer (NK) T cells, (114) B cells, (115) dendritic cells, (116) monocytes/macrophages,(117) neutrophils (118), and mast cells (119).

In TB patients have higher numbers of CD4+ CD25+ Foxp3+ T cells both in peripheral blood and at sites of infection (120-122). Starting with these studies grew the first suspicious about a direct interplay between Treg and γδ T cells given that γδ T cells during Mycobacterium tuberculosis infection have been found to expand to high levels and represent the majority of circulating T cells. Guangming Gong et al (123), demostrated a counting effect of γδ T cells on Tregs under treatment in vitro with

Picostim and IL-2 in TB patients. Indeed activation of Vγ2Vδ2 T cells by adding phosphoantigen Picostim to the IL-2 treatment regimen down-regulated IL-2–induced expansion of CD4+CD25+Foxp3+ T cells. Picostim (Innate Pharma) was another phosphoantigen compound that shares chemical structure and bioactivity with HMBPP except that only one atom is different linking the carbon chain and the phosphate moiety (oxygen or carbon).

On the other hand and in the same period Mahan et colleagues (124) and Li Li et colleagues (125), belonging to different research group, demonstrated that depletion of Tregs from peripheral blood mononuclear cells increased Vδ2+ T-cell expansion in response to M. tuberculosis (H37Ra) in tuberculin-skin-test-positive donors. Moreover they evidenced that mycobacterial antigen-reactive Tregs suppress antigen-specific V $\delta 2^+$ T-cell responses finding in this a possible explanation that Vδ2+ T-cell deficits in TB.

Several reports have demonstrated the crucial contributions of γδ T cells to the reservoirs of such cytokines in the context of anti-tumor immunity (126). Since activation of Vγ9Vδ2T cells by phosphoantigen/IL-2 treatment regimen itself has been shown to confer anticancer therapy and this investigation was continued with the others study from patients with different types of cancer by Kunzmann.

Kunzmann (127) was the first one to investigate the role of Tregs in hampering γδ T-cell function. In this study he provided the evidence that Tregs potently suppress phosphoantigen-induced γδ T-cell proliferation via a novel cell–cell contact-independent mechanism. He used PBMC

from patients with different types of cancer to isolate Tregs. BrHPP (Innate Pharma) was added to activate and expand in vitro γδ T cells from the same patients. He performed several experiment to show the suppression activity of Treg on γδ T cells. The requirement was that phosphoantigen-induced proliferation of γδ T cells in vitro is frequently impaired in patients with lymphoid malignancies (128) and there is also an increased frequencies of Tregs (characterized as $CD4^+$ $CD25^+$ $FoxP3^+$ T cells) in patients with haematological malignancies (129) as compared with healthy individuals. He demonstrated the capacity of Tregs to inhibit γδ T-cell proliferation in vitro via a cell–cell contact-independent mechanism.

To complete this overview and the possible influence of Treg on γδ T cells cytokine production, Clark's lab in 2009, discussed about the repristinated IFNγ production by effector T cells in SCC after treatment with Imiquimod (Toll-like receptor agonist). Honestly he didn't affirm γδ T cells directly produce IFNγ even if several reports have demonstrated the crucial contributions of gd T cells to the reservoirs of such cytokines (in particular IFNγ and TNFα) in the context of anti-tumor immunity (128). Nonetheless others data support the view that effective anti-tumor activities are an aggregate of CD8 and IFNγ-producing CD4 cells (130).

Recently, Sakaguchi's group (131) demonstrated that human Tregs can be divided into three functionally distinct subsets on the basis of CD45RA, Foxp3 and CD25 expression: CD45RA+Foxp3low Tregs (resting Tregs), which are CD25++, CD45RA–Foxp3high Tregs (activated Tregs), which are CD25+++, and CD45RA–Foxp3lowCD4+ T cells (cytokine-secreting non-suppressive T cells), which are $CD25^{++}$. Thus correctly functional

study on Treg revealed that $CD45RA^{-}CD25^{+++}$ Tregs significantly inhibit the proliferation of CD4+CD25− T cells and importantly, the frequency of $CD45RA^{-}Foxp3^{high}$ Tregs positively correlate with tumor stage (132).

Taken together all affirmations confirm that the ab T cell response to SCC contains within it an activity that can promote tumor progression (126) and therefore, effective clinical manipulation of the anti-tumor immune response will be required to improve the understanding of αβ T cell activities on tumor growth.

Moreover in human the effectly relation between Treg and γδ T cells in SCC has not been studied thoroughly. The suspicious that increased Treg:gd T-cell ratios may contribute to the frequently impaired γδ T-cell proliferative response in cancer patients push us to speculate about an inverse correlation between Treg and γδ T cells in NMSC.

We have to also add that not always γδ t cells is seen to perform a positive role in tumor. Silva Santos et al (133) found that nTregs isolated from lymph node or spleen suppress mouse gd T cells in a contact-dependent manner and independently of IL-10 or TGFβ. Indeed we have a clear evidence about the interplay between both cells in mouse. He demonstrated that this results in an anergic state of γδ T cells and can be partially abrogated by manipulating GITR signals. In contrast, recent studies also showed that γδT17 cells could facilitate tumor growth via promoting angiogenesis in mice (133) and more recently, Wu et al, suggested that γδT17 cells might be key players in human CRC progression. Thus if Treg would suppress gd T cells it could be a good manner to damage the progress of the tumor. Unfortunately it wasn't clear the role of gd t cells in tumor and also if this possible relatioship between

gd Tcells and Treg could damage or advantage the prognosis of the patients. Moreover we can say that the only direct relationship between Treg and gd T cells isn't realistic and, such as suggested by Tae Jung Jang in 2008 (131), Dendritic cells (DCs) inducing cutaneous immune response are involved in immunosuppression and so there is a prevalence of Tregs and DCs infiltration in cutaneous premalignant and malignant squamous lesions.

3.4 Aim of the research

The major goal of this research project is to analyse the frequencies, phenotypes and functions of tumor-infiltrating and circulating gd T cells (Vdelta1 and Vdelta2) and Treg T cells from patients affected by Non Melanoma Skin Cancer (NMSC). Next,
we characterised the relation between Treg and γδ T cells in ex-vivo using the data obtained from biopsy and blood from the patients at different stage of the tumor. Finally we tried also to correlate it with the stage of the tumor in term to define if the characterization of the immune contexture is a major prognostic factor for survival and may thus represent a target for innovative cancer therapy. Moreover, given that a subset of dermal DC in mice is not observed in human skin and also many resident T cells in murine skin are TCRγδ+ and have a limited antigenic repertoire, while T cells resident to human skin are predominantly TCRαβ and have great TCR diversity (104), this studies propose to understand the relationship between Treg and γδ Tcells and hypothesis an correlation. This approach takes in consideration the great variance of each analysed samples and the internal variability of the tumor skin regarding to the stage and the general

state of the patient. On the other hand, this analysis tries to answer to these questions:

Could γδ T cells have an immunoregulatory role in SCC or could Tregs inhibits the activity of effector γδ T cells in the SCC tumor microenvironment?

Actually the first point that emerges from all finding studies it was that Vδ1 resident role could be related to the skin state (alterated conditions compared to normal state) while about circulating Vδ2 is more certain the antitumoral role with, in addition, the professional antigenic-presenting cells capable to stimulate both CD4+ and CD8+ subsets in the same way. The problem is to understand which role γδ T cells assume in this tumor (SCC) in which the skin is perturbed. For this reason it will be interested also to compare it with the trend of Treg in the same patients. Moreover, we just knew that isolated HCC-infiltrating CD4+CD25+ regulatory T cells (Treg cells) directly suppressed the cytotoxic function and IFN-γ secretion of γδ T cells in a TGFβ- and IL-10-dependent manner (134).

4 Materials and Methods

4.1 Clinicopathologic characteristics of patients with NMSC

Non Melanoma Skin Cancer tissues were obtained in accordance with the ethical standards of the institutional committee of human experimentation from 47 patients undergoing a skin resection for SCC and BCC in Surgical Department of Policlinico "P.Giaccone" Hospital, University of Palermo. 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) were purified from peripheral blood using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden).
According to Italian rules (art. 13, DLgs n. 196/03), this study did not require authorisation by the local ethical committee. The study was performed in accordance to the principles of the Helsinki declaration and all individuals gave written informed consent to participate.

We had a set of samples composed of 47 samples. We distinguished two main histology subgroups: SCC (74,4%) and BCC (12,7%) while the rest wasn't determinated. All of these included 78,7% male (37/47) and 21,7% (10/47) female. The median age of the cohort samples at diagnosis was 75 years in men and 74 in women (range of 62–79 years). After surgical removal about 89% (42/47) of the samples occurred most frequently head-neck and only 5 of the NMSC samples showed the tumor in the limbs. Among the oral cavity we found that 15/20 samples showed the tongue site (75%). Tumor-infiltrating immune cells were isolated only in the 76,6% of the samples set and them were studied focused our attention in Vd1, Vd2 and Treg populations of T cells population while about the others we found

nothing or poor infiltrating cells. The predominant tumor stage was T2 (45,7%) on the staged samples and only 25% were in T3 stage. The major samples had a moderate differentiation G2 (68%) compared with the total determinated samples (undeterminated grade was 31,9%). For each samples we had the biopsy and the PBMC and compare and analyse the frequencies, phenotypes (in ex-vivo) and functions (in vitro) of tumor-infiltrating and circulating gamma-delta T cells (Vd1 and Vd2) and Treg T cells. Moreover it was evident than continuous to be the same inverse proportion between Treg and Vd2 not only in the tumor site but also in the blood at the same stage or grade.

4.2 Isolation of Tumor-infiltrating Immune Cells and PBMCs and FACS Analysis

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 1 hr at 37°C. After digestion, the cells extracted were washed twice in RPMI 1640 medium (Gibco, Grand Island, NY, USA). Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden)

Both PBMC and tumor-infiltrating cells were stained for live/dead discrimination using Invitrogen LIVE/DEAD fixable violet dead cell stain kit (Invitrogen, Carlsbad, CA). Fc receptor blocking was performed with human immunoglobulin (Sigma, 3 mg/ml final concentration) followed by surface staining with different fluorochrome-conjugated antibodies to study the composition of the different subpopulations.

The fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-Cy5- or PE-

Cy7, allophycocyanin (APC), and APC-Cy7- conjugated monoclonal antibodies (mAbs) used to characterized the entire population were the following: anti-CD3, anti-CD4, anti-CD25, FoxP3,CD127, anti-CD14, anti-CD19, anti-CD45, anti-pan γδ TCR, anti-Vδ1, anti-Vδ2, anti-CD27 and anti-CD45RA, all purchased from BD Biosciences (Mountain View, CA) and Thermofisher. Moreover, Treg were determinated following the instructions by Miltenyi Kit. Expression of surface markers was determined by flow cytometry on an FACSCalibur or FACSCanto II Flow Cytometer with the use of FACS DIVA and analysed by FlowJo software (BD Biosciences). The gating strategy involved progressively measuring total cells; viable cells only; lymphomonocytes, single cells 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. Negative control (background) values were not subtracted, as the median backgrounds for isotype- matched mAbs was 0.0028% (range, 0.000%–0.0063%). Samples were considered positive if the number of cells was equal to or greater than 0.01% and at least 10 clustered events were apparent. This empiric cut-off value was predicted to be .90% different from background, at an a of 0.05 [136].

4.3 Generation of Polyclonal Vd1+ and Vd2+ T and Treg Cell Lines from PBMC

Polyclonal Vδ1+ and Vδ2+ T cell lines were generated after cell sorting of Treg through a FACSAria (BD Biosciences) with specific mAbs (CD4, CD25, CD127). γδ Tcells were then cultured into each well of round-bottom, 96-well plates containing IPP 30mM/ml and 200 U/ml recombinant interleukin 2 (Proleukin; Novartis Pharma, Basel, Switzerland). Growing lines were expanded in 200 U/ml IL-2 and restimulated every 2 weeks. Usually, cells

were collected after 2 weeks of culture to be used for functional assays in vitro. After control of the purity, sorted Treg were activated with the beads for 1 day and used the day after for the suppression assay.

4.4 Cytokine Production by Vd2 T cells

The cytokine production capacity of polyclonal Vd2+ T cell lines was determined after stimulation with PMA (BD Biosciences, 20 ng/ml) and ionomycin (BD Biosciences, 1 mM) and Zometa (Novartis) for 24 h at a cell concentration of 10^6/ml. Flow cytometry was used to determinate the cells producing IFNg, TNFa and IL17A. Moreover as a control and to evaluate the capacity to responde to the stimulations, we had the cells without stimulations.

4.5 Suppression assay

To perform the suppression assay we had collected the effector cells (γδ T cells) after expansion and stained with carboxyfluorescein succinimidyl ester (CFSE)-labelled. γδ T-cell line was incubated with medium alone or co-cultured with autologous purified Tregs at a ratio of 1 : 1; 1: 2; 1:4 (Treg: γδ). On day 4, cells were analysed by flow cytometry. γδ T-cell receptor (TCR)/CD3 double-positive T cells were gated, and loss of CFSE represented proliferation of gated cells. The ratio with IgG indicated the Tregs ON cultured in IgG precoacted plate and kept with IL2.

4.6 Statistical analysis

We have done a correlation between two specific group of data creating a

matrix in which we have infiltrating or circulating Treg and gd T cells. We have computed our data using a Pearson's coefficient. We calculted the correlation coefficient for each pair of variables. This coefficient is based on the assumption that both X and Y values are sampled from populations that follow a Gaussian distribution, at least approximately. The significance of difference between groups was determined by Student's t test.

We used ANOVA unparametric test and we set two tailed and unpaired test to compare the effect of cancer stage on the recovery rate and percentage of γδ T cells. Differences between subgroups with a probability of ,0.05 were regarded as significant. All p-values and Pearson's coefficient were performed using PRISM software package.

5 Results and Discussion

This study starts with the consideration that T cells isolated from SCCs of both normal and immunocompromised patients had greatly increased numbers of FOXP3+ T reg cells when compared with the population found in normal skin and several distinct mechanisms identified are responsible for the escape of NMSC from immune recognition. γδ T cells participate in immune responses in infectious and neoplastic diseases and still more knowledges are necessary on the role of γδ cells in cancer-patients and in particular in NMSC in which not enough data are available.

We analysed a set of 47 samples. We distinguished two main histology subgroups: SCC (74,4%) and BCC (12,7%) while the rest wasn't determinate. All the samples included 78,7% male (37/47) and 21,7% (10/47) female. The median age of the cohort samples at diagnosis was 75 years in men and 74 in women (range of 62–79 years). After surgical removal about 89% (42/47) of the samples occurred most frequently in head-neck and only 5 of the NMSC samples showed the tumor in the limbs. Among the oral cavity we found that 15/20 samples showed in tongue site (75%) (Table 1).

5.1 Frequency of T cells in the peripheral blood and biopsy of NMSC patients.

Tumor-infiltrating immune cells were isolated only in the 76,6% of the samples set and them were studied focused our attention in Vδ1, Vδ2 and

Treg populations of T cells population while about the others we found nothing or poor infiltrating cells. For each sample we had the biopsy and the PBMC. For each sample we compare and analyse the frequencies, phenotypes (in ex-vivo) and functions (in vitro) of tumor-infiltrating and circulating gamma-delta T cells (Vδ1 and Vδ2) and Treg T cells. We used FACS analysis (CANTO II) to detect the respective percentages. In the present study we found higher proportion of infiltrating Treg compared to PBMC (6,1± 1,4% for SCC vs 2,1 ± 0.4% for PBMC, p< 0.001) (Figure 1). We hypothese that this significative difference percentage may be related to the recruitment of Treg from the periphery to the tumor site to represtinate the homeostatic immune response in tumor site. In the same way we evaluated the respective proportion for Vδ1 and Vδ2 in PBMC from SCC patents. As expected Vδ2 subpopulation was the major population represented of γδ T cells in PBMC (Vδ1 that represented only the 0,5 % ± 0,1) while Vδ2 about 2,5% (with SEM ± 0,3) of CD3+ T cells. Moreover, the percentage of Vδ2 T cells was significantly more than healthy donor PBMC (p<0,05) (Figure 6). We considerated also the percentage of Vδ2 T cells obtained from biopsy of lesion skin. Surprisly, when we analysed in *ex-vivo* a consistent frequency of infiltrating γδ T cells population was represented by Vδ1 3% respect to Vδ2 2,0± 0,3 (Figure 3 and 4).

Actually, with great statistical significant value, SCC infiltrating Vδ2 were more than Vδ2 from healthy skin. Thus this data, push us to continue to analyse it for all samples in the tumor microenvironment too (Figure 5).

5.2 Phenotypic analysis of the peripheral and infiltrating gamma-delta T cells fraction.

To evote the increased doubts about this finding, we started to compare the phenotype of both subpopulation of γδ T cells not only from PBMC but also from tumor infiltrating (Figure 7). We found that infiltrating Vδ1 had predominant Effector memory phenotype (EM) while only the naïve phenotype was considered the second one represented phenotype. About infiltrating Vδ2 , we found the similar effector memory phenotype but in different way to Vδ1, the second population represented was the Central memory phenotype (CM). To esclude a possible contamination by blood during the infiltrating cells extraction we analysed the phenotype in blood from the same patients studied. The phenotype of circulating Vδ2 appeared different and there were no population predominant. The results could confirm nothing contamination and also it may make to think about a migration process of Vδ2 in the tumor microenvironment with consequent modification of the phenotype in activated phenotype.

To have a clear understanding about the infiltrating lymphocytes in the SCC microenvironment, we compared instantly the percentage of both population of γδ T cells and Treg obtained from infiltrating normal skin and SCC. We found that Vδ2 were only about 0,3 % represented in normal skin while the average value from Vδ1 was about the 1,5 %. These data have to see considering all variations related to the samples. Tregs was better represented than γδ T cells with about 4% of the frequency. When we compare infiltrating skin from HD and SCC we instantly

observed an increased level of Vδ2 in SCC microenvironment. On the other hand, it was more evident the enhanced number of Treg compared Vδ1 from HD and SCC and showed major percentage than γδ T cells. Furthermore, we assessed whether the difference between SCC infiltrating Treg and Vd2 was significant. With great surprise, the statistical analysis with Sidak's multiple comparisons test gave us a significant relation between them ($p<0,001$). Notwithstanding the latter, there weren't a strong significant link with Vδ1 from SCC (data don't showed) and Treg SCC.

5.3 Cytokine Production of NMSC-infiltrating gd T Cells

All this finding encouraged us to continue to focus our attention on Vδ2 T cells subsets.Given the increaed interested for Vd2 with good candidate for the immunotherapy and in the tentative to demostrate an effector function of Vδ2 infiltrating SCC, we prepared a set of experiment in which we stimulated the cells for short period (24h) at 37°C , 5%of $C0_2$. We treated the cells with IONO/PMA for the same time and as negative control we hold the cells with only RPMI. We want to underline that we found some impediments to make this experiment using the infiltrating lymophocyes. The reason was found in the poor number of infiltrating cells to put in culture. For this reason a selective number of samples were analysed for the cytokine production. The results showed that spontanusly Vδ2 produced the IL17, IFNγ and TNFa but major increased level of the Vδ2 T cells producing them compared under treated with IONO/PMA (Figure 8). The zometa treatment showed overt defect in the cytokine production. The reason is explained when we consider the time of the

incubation with the drug. More studies have demostrated that Zometa treatment need long period (about 3 days) to reach a complete activation. Such as declared by Bruno Silva Santos, the population CD27- γδ T cells are the major producer of IL17 than CD27+ that produce IFNγ. Unfortunately, we didn't analyse both the different subpopulation of γδ T cells (CD27+ and CD27- producing cytokine). Actually, we may speculate that, given that we have both EM and CM as predominant phenotype in infiltrating Vd2 and also we found an enhanced level of IL17 than IFNγ produced by Vd2 infiltrating SCC (IL17 and IFN γ respectively produced by CD27- and CD27+ γδ T cells subsets and by the way EM cells are CD27-CD45RA- and CM are CD27+ CD45RA-), probably we can image an presence of both of subsets in situ.

A different situation we found in PBMC in which the percentage of γδ the cells producing the cytokine were decreased. This data it was aspected whether we considered the phenotype of circulating Vδ2. The cytokine such as IL17 and IFNγ were producted but no one of them was more than others them.

To demostrate the different between circolating and infiltrating Vδ2 T cells in SCC and to underline the effect of Vδ2 in this tumor, we analysed the perforine production. Infiltrating Vδ2 accumulated significally perforine better than circulating ($p<0,04$) (Figure9).

5.4 Ex-vivo analysis of the correlation between gd T cells and Tregs in tumor infiltration and PBMC

Next, we examined the correlation between various subpopulations of TILs (Vδ1SCC/TregSCC, Vδ1HD/Treg HD, Vδ2SCC/TregSCC). A significant indirect correlation was found between Vδ1SCC/TregSCC. The Pearson coefficient index was -6 and R-square 0,3 with the confident interval 99% ($p<0,01$). Even if the last studies about infiltrating γδ T cells underlined a significant regulatory role in tumor microenvironment as it was confermed not only in mouse (Silva Santos 2014) in which was underlined that IL17 by γδ T cells have a relevant role in the progression of the tumor but also in BC (Ma et al demostrated in human a regulatory role related to the bad prognosis of the patients and poor surveval), we continued to find an inverse correlation of Vδ1 T cells with Treg. This finding may demostrate that Vδ1 continue to perform a anti-tumoral action that Treg can put down. Thus the dipendence of both variables may demostrate in contrast an enhanced immune defence balanced by the increased level of Treg. Indeed to verify whether this correlation was indipendent to the skin lesion, we evaluated the correlation index between the same type of the cells from healthy skin. Surprisly we obtained a Pearson coefficient 0,8 and R-square 0,6 (data doesn't showed). This data underlined that there is a different tissue microenvironmnet. In normal condition there is a positive balance between type cells ($p<0,01$). On the other hand this latter confirm the data that infiltrating Vδ1 and Treg were in not till clear way related.

To continue to characterize Vδ2 infiltrating SCC, we tried to evaluate the Vδ2/Treg from tumor site. With great surprise, we found an positive linear

regression with Pearson index 0,55 and r-squared 0,3. Moreover this data appear significant with the interval confidence at 99% (p=0,003). Moreover, when we tried to evaluate the Vδ2 SCC/Treg SCC from PBMC, we found nothing strong correlation. Indeed the pearson index was too few strong (r= -0,3) and the p value demostrated a significant lacking relation (p<0,08) (Figure 11 A, B and C). Surprisly these trends could open at new point of view. Indeed it could demostrate a significant role of the tumor microenvironmnet on the recruitment and role of the circulating T cells. Until now the precise mean wasn't clear. This data doens't permit to say what role Vd2 explain in tumor. We could only suppose an similar role of Vδ2 and Treg in the progress of the tumor and it could mean a possible shift of the role (unpubblicated results) and in contrast in PBMC, Vδ2 could mantain their anti-tumoral role (it could explained by the inverse correlation with Treg). In term of clarify the concept we may mention huge review about the immunotherapy using Vd2 expanded and re-infused in the same patients took. In the same way we can remind the very proof study on Treg and the pro-tumor behavior when it permits the immunoescape of the tumor cells. The latter implies that in the presence of Treg and the humor composition of tumor microenvironmnet could revert the function of the migrated T cells. Furthermore, no studied until now have explain a possible influence of Treg on Vδ2 in the tumor and this constitutes the first report on the relationship between Vδ2/Treg infiltrating NMSC.

We would image also that Treg have a different realtion with both subpopulation of gd T cells. We have found a research about the relation with Treg and CD8 in SCC (Watanabe 2010). CD8+ and Treg have a significant relation only from the follow-up patients analysed such as we

had found in our study. Notwithstanding there are more studies on Treg in SCC, very few was focused on the relation between Treg in the SCC tissue. Although the primary role of Tregs in the immune system is to prevent autoimmunity, Tregs have also been shown to suppress immune responses against persistent pathogens thus, the first report on the suppression of phosphoantigen-reactive Vd2+ T cells by antigen-specific Tregs it was found but mycobacterium tubercolosis system and this study cames from Mahan et al in 2008. Kunzumann talked about the possible inverse relation in ematoncology Vd2 and Treg but only in blood.

5.5 Suppression of circulating gd T cells by antigen-specific Tregs

Remain to clarify if in tumor tissue Vδ1 and Vδ2 were inhibited by Treg . To be honest, it really hart to study it because of not cospicous number of the cells obtained in *ex-vivo*. For this reason we present some data on suppression activity by Treg on gd T cells in PBMC. To perform it we collaborated with the colleagues at King's College London at Guy's Hospital in the Hayday's lab. The assay was made in term of to check the suppression assay for further studies. We had used an expanded protocol for Vd1 T cells from PBMC. Vδ1 usually rappresented about 0,5% of the circulating CD3+ T cells. After the expansion we obtained the 7% of the expansion more. To check the efficiency method we also used in parallel the Vd2 T cells expandend contemporary with Vd1 and in the end we had 80%.

Thus we are the first one that report on the suppression on activated Vd1 T cells by Treg. We had used a CFSE staining that permit us to evaluate the

proliferation of the target cells. The experiment was conducted for 3 days in culture at 37°C with the completed medium and very few IL2 concentration. This choice was took to prevent an eventually false positive and to evote the enhanced mortality for starvation of IL2. Moreover Vd1 and Vd2 were activated because they came from expanded process (for 12 days in culture).

We activated ON sorted Treg in two different way to be sure about the result and to have an extra-control. The ratio 1:1 gdT cell/Treg inhibit Vd1 and Vd2 in the same way and in the both different type of activated Treg. At 1:2 and 1:4 ratio (it mains that the gd T cells was diluted 2 fold or 4fold than Treg) both effector cells showed a better proliferation compared the formed condition. (Figure 10 A, B and C)

The internal control implies the use of Treg activated with IgG with the same long time and used at the same condition to inhibit gd T cells.

5.6 The frequency of Infiltrating and circulating T cells vs clinical variables.

Histological examination of the tumor also determines the grade level of the cancer. The prognosis in patients with solid tumot depends strongly on the degree of local tissue invasion, infiltration of adjacent organs and presence of metastases to lymph nodes or other organs.
We introduce 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 [135].

From all samples analysed, the predominant tumor stage was T2 (45,7%) on the staged samples and only 25% were in T3 stage. The major samples had a moderate differentiation G2 (68%) compared with the total determinated samples (undeterminated grade was 31,9%) (**Table 1)**.
To demonstrate the hypothesis in which Vd1 and Vd2 could advantage the tumor progression like Treg or in contrast to demonstrate anti-tumor behaviour, we tried to compare respectively the percentages obtained ex-vivo from tumor biopsy with tumor stage of the same samples analysed. Even with all pains, this evaluation was hard to do. In deed not all samples were evaluated for all infiltrating subpopulation because of a lot of variables that influence the analysis such as the age of the patients, the sex and others feature of the clinical story of the patients that we can kept.

Anyway the data showed that Treg was more represented than Vδ1 and Vδ2 (respectively 5,27 ± 2,4; 2,7 ± 0,5; 2,5 ± 0,79) and the latter were in equal proportion in T1N0M0. In T2 we found a significant increased level of Treg (10,8 ± 3,5) compared to Vδ1 and Vδ2. Actually Vδ1 appears few more of Vd2 (2,4 ±0,7 and 1,66 ±0,4) in the same proportion. Interesting, in T3 Vd2 was more than Vδ1 and surprisly, than Treg (8,6±4,2; 1,58 ± 0,9; 7,6 ±5). Vδ1 was few than Treg. In opposite, when we analysed the complex view of different single population with the stage byself, we

observed that Treg was the best population represented in T2 among the all three stages and only moderating represented in T1 and T3. Vδ1 showed a progressive decreased frequencies during the growing of the tumor while, a different behaviour, was manifested for Vδ2. Indeed Vδ2 was the best γδ T cells subpopulation represented in T3 and in T2 appears in opposite with Treg.

About the blood unaspectly, Vδ2 for each stage was more than Treg. This data may fit with the concept described above, in which circulating Vδ2 depend with Treg but in inverse way. This finding not only was significant but also push us to proof the studies on Vδ2 and Treg. The final aim is to find a way to evote the influence of Treg on Vδ2. Indeed, until now circulating Vδ2 is consider the best candidate for the immunotherapy. In contrast, the information about the possible migration of Vδ2 in the tissue (clearly demonstrated by Nestle's group in psoriasis) make more easy to consider an direct anti-tumoral role of circulating Vδ2. Thus, the hypothetic suppressor role by Treg on infiltrating Vδ2 was an interesting and motional idea. Remain to evaluate *in vitro* the suppression activity of infiltrating Treg and Vδ2 or Vd1 T cells again.

Unfortunately, technical problems don't permit to make it and until now the main question remain unsolved.

Different trend we had when we correlated the grade of the tumor with the frequencies of respectively T cells. Unfortunately, the major samples showed a moderate differentiation G2 (68%) compared with the total samples analysed for it (undetermined grade was 31,9%). In this condition, infiltrating Vd2 was few than Treg but showed an overt

changing of the trend in periphery where Vδ2 was more than Treg (Figure 12 C and D). In the Table from 3 to 6 to showed the statistical significant of our studies .

6 Conclusions

The mutual and interdependent interaction between tumor and its microenvironment is a crucial topic in cancer research and therapy, as recently demonstrated by the finding that targeting stromal factors could improve efficacies of current therapeutics and prevent metastasis. For instance, combinatorial therapy with an agonistic mAb against CD40 and standard gemcitabine chemotherapy, proved unexpectedly efficient in pancreatic cancer [82]. In this system, the anti-CD40 mAb caused massive recruitment of macrophages at the tumor site which caused severe disruption of the tumor stroma thus allowing increased concentrations of gemcitabine to accumulate to the tumor site [82]. Similarly, the identification of defined immunosuppressive pathways in the tumor microenvironment has pointed toward therapeutic targets that are amenable to clinical intervention: these include, for instance, mAbs to PD-1 or PD-L, CTLA-4, CD25 and small-molecule inhibitors that block IDO enzymatic activity (reviewed in [83]). These novel strategies must be kept in mind when designing γδ T cell-based therapy. Moreover radiation therapy, low dose traditional chemotherapeutic drugs and aminobishosphonates not only sensitize tumor cells to immune recognition and killing, but also modulate the tumor microenvironment and contribute to the therapeutic effect [84-86]: for instance, zoledronic acid at clinically achievable doses repolarizes tumor-associated macrophages to a M1 phenotype and reduces the number of MDSC [87] [88]. Therefore, combination regimens consisting of γδ T cell-based therapies and strategies targeting the tumor microenvironment may prove efficacious and achieve clinical benefit in patients with different types of tumor.

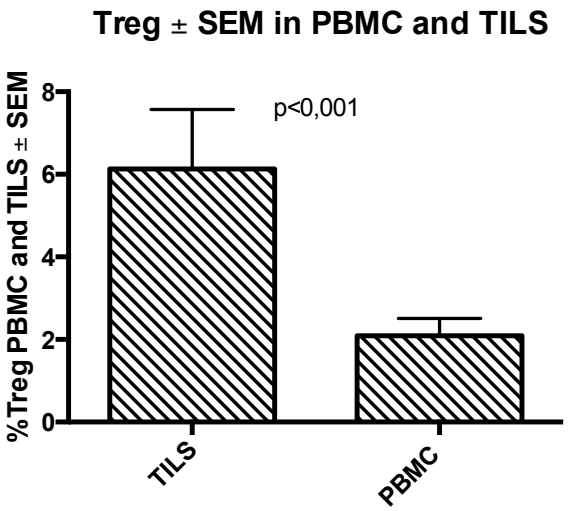


Figure 1: Percentages of Treg in TILs and PBMCs of patients with NMSC. Tregs were obtained as described under Materials and Methods and were stained with anti-CD45, anti-CD3, anti-CD4, anti-CD25, anti-FoxP3 mAbs. Cells were determined by FACS analysis using CANTO II (BD). Shown are mean with SEM.

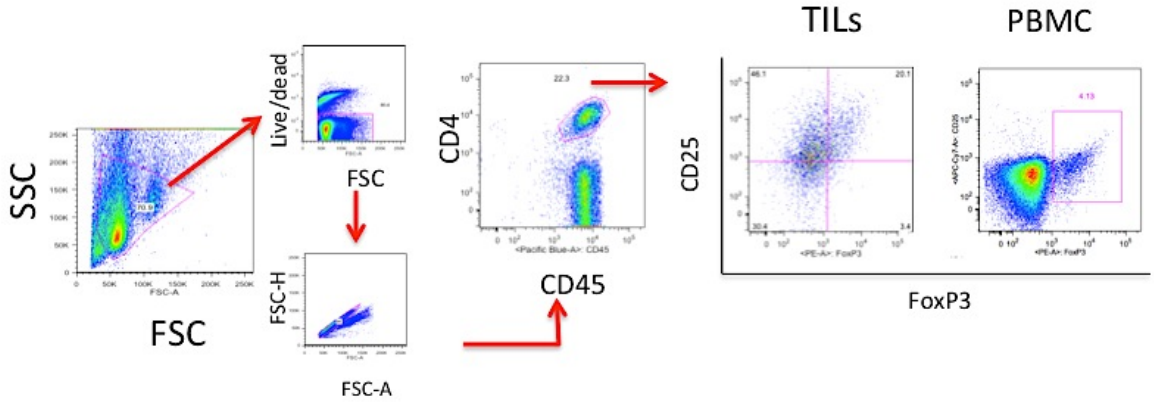


Figure 2: Gating strategy used to analysed Infiltrating and ccirculating Treg of patients with NMSC. Tregs were obtained as described under Materials and Methods and were stained to descriminate Live and Dead cells and from them the single cells were stained with anti-CD45, anti-CD3, anti-CD4, anti-CD25, anti-FoxP3 mAbs. The analysis were performed by FACS

analysis using CANTO II (BD).

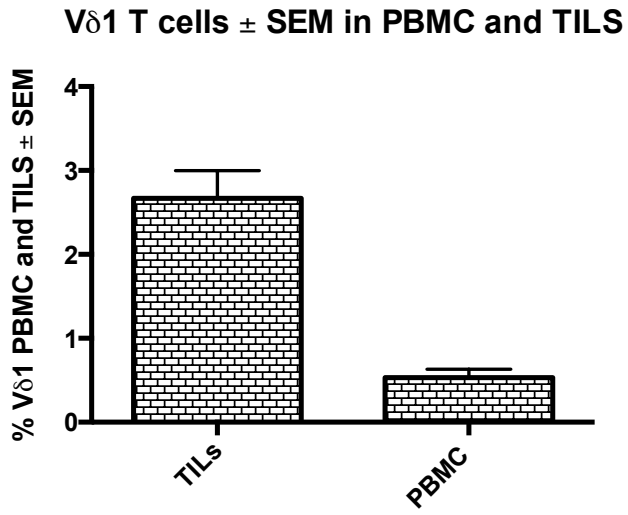


Figure 3: Percentages of Vδ1 subsets of γδ Tcells in TILs and PBMCs of patients with NMSC. Vd1 were obtained as described under Materials and Methods and were stained with anti-CD45, anti-CD3, anti-Vδ1 mAbs. Cells were determined by FACS analysis using CANTO II (BD). Shown are mean with SEM.

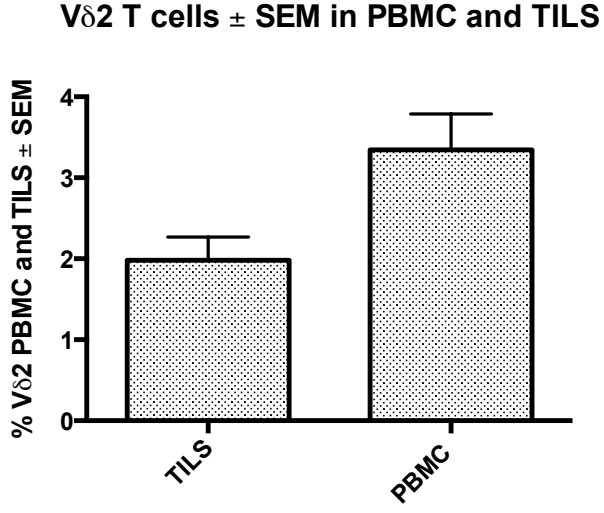


Figure 4: Percentages of Vδ2 subsets of γδ Tcells in TILs and PBMCs of patients with NMSC. Vd2 were obtained as described under Materials and Methods and were stained with anti-CD45, anti-CD3, anti-Vd2 mAbs. Cells were determined by FACS analysis using CANTO II (BD).

Shown are mean with SEM.

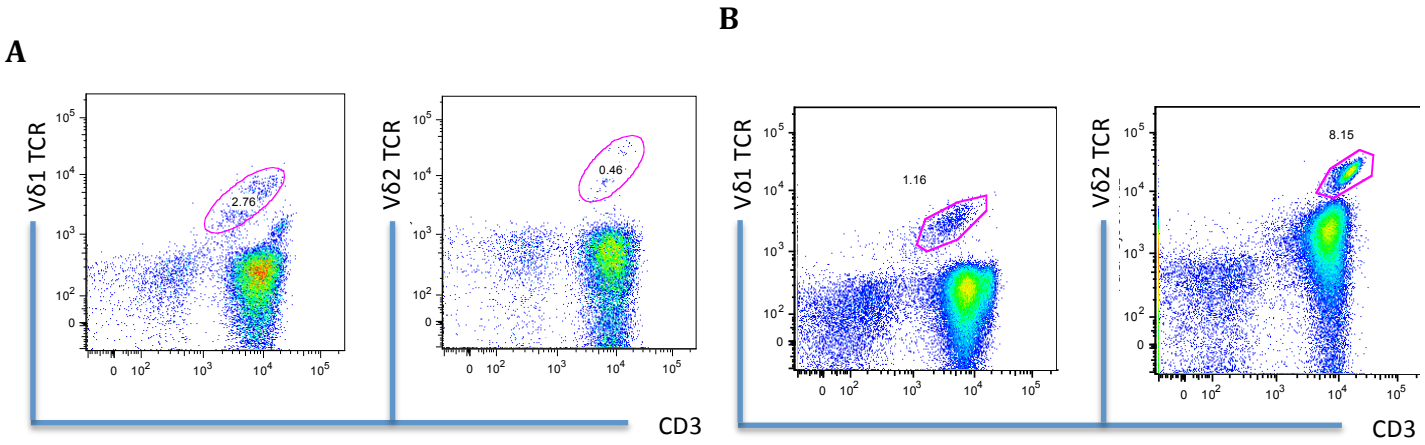


Figure 4 A and B: Rappresentative dot-plots from patients with NMSC , upon gating on Vδ1 and Vδ2 subsets of γδ Tcells of patients with NMSC. A) Vδ1 and Vδ2 subsets obtained from TILs as described under Materials and Methods. B) Vδ1 and Vδ2 subsets obtained from PBMC as described under Materials and Methods.

Table 2 Mean with SEM of Treg, Vδ1 and Vδ2 of γδ T cells infiltrating and circulating NMSC

Treg cells ± SEM	
TILS	6,1 ± 1,4
PBMC	2,1 ± 0,4
Vδ2 T cells ± SEM	
TILS	2,0 ± 0,3
PBMC	3,3 ± 0,4
Vδ1 T cells ± SEM	
TILS	2,7 ± 0,3
PBMC	0,5 ± 0,1

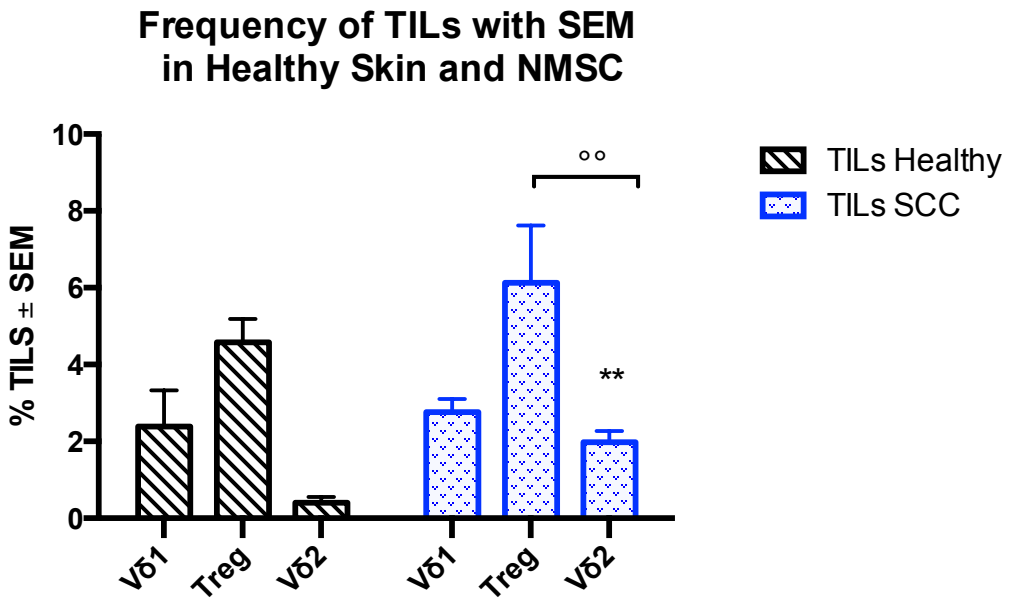


Figure 5: Normal skin infiltrating lymphocytes and skin from patients with Squamous Cell Carcinoma (SCC a subtype of NMSC) were obtained as described under Materials and Methods and were stained with anti- anti-CD45, CD3, antiVδ1, anti-Vδ2, anti-CD4, anti-CD25, anti-FoxP3 mAbs CD45, anti-CD3, anti-Vδ2 mAbs. Mean of percentages of infiltrating skin were determined by FACS analysis using CANTO II (BD). Shows mean data with SEM. °°p<0,001 when compared Treg with Vδ2 both obtained from SCC and **p<0,001 compared Vδ2 infiltrating SCC with Vδ2 from normal skin.

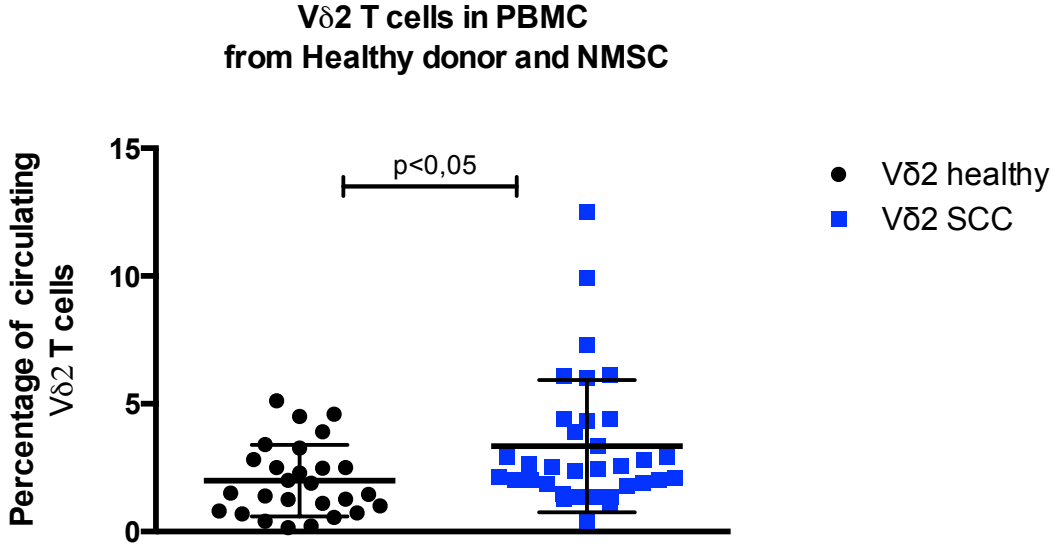


Figure 6: Circulating lymphocytes from HD and patients with Squamous Cell Carcinoma (SCC a subtype of NMSC) were obtained as described under Materials and Methods and were stained to descremenate Live/dead, single cells and anti- CD3, anti-Vδ2 mAbs. Mean of percentages of circulating cells were determined by FACS analysis using CANTO II (BD). Shows mean data. p<0,05 when compared Vδ2 from HD blood and SCC blood.

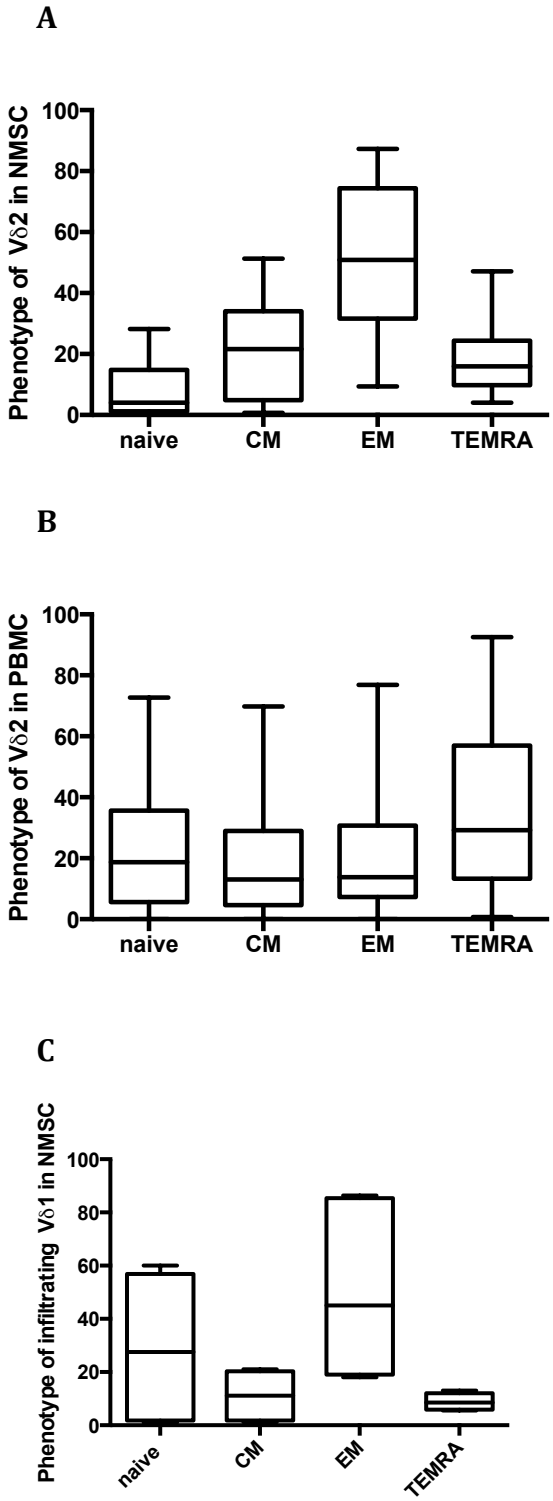


Figure 7: Comparison of Vd2 T cell subset distribution in TILS and PBMCs form patients with NMSC. TILs and PBMC from patients with NMSC were obtained as described under Materials and Methods and were stained with anti-CD3, anti-Vδ2, anti-CD45RA and CD27 mAbs.

Percentages of T_{naive} ($CD45RA^{+}CD27^{+}$), T_{CM} ($CD45RA^{-}CD27^{+}$), T_{EM} ($CD45RA^{2}CD27^{2}$) and T_{EMRA} ($CD45RA^{+}CD27^{-}$) cells were determined by FACS analysis. (A) Shows cumulative data for infiltratingVδ2 T cells and (B) shows representative flow cytometry panels from circulating Vδ2 T cells and (C) shows data for infiltrating Vδ1 T cells with NMSC, upon gating on $Vd2^{+}$ and $Vd1^{+}$ cells respectively and staining with CD27 and CD45RA.

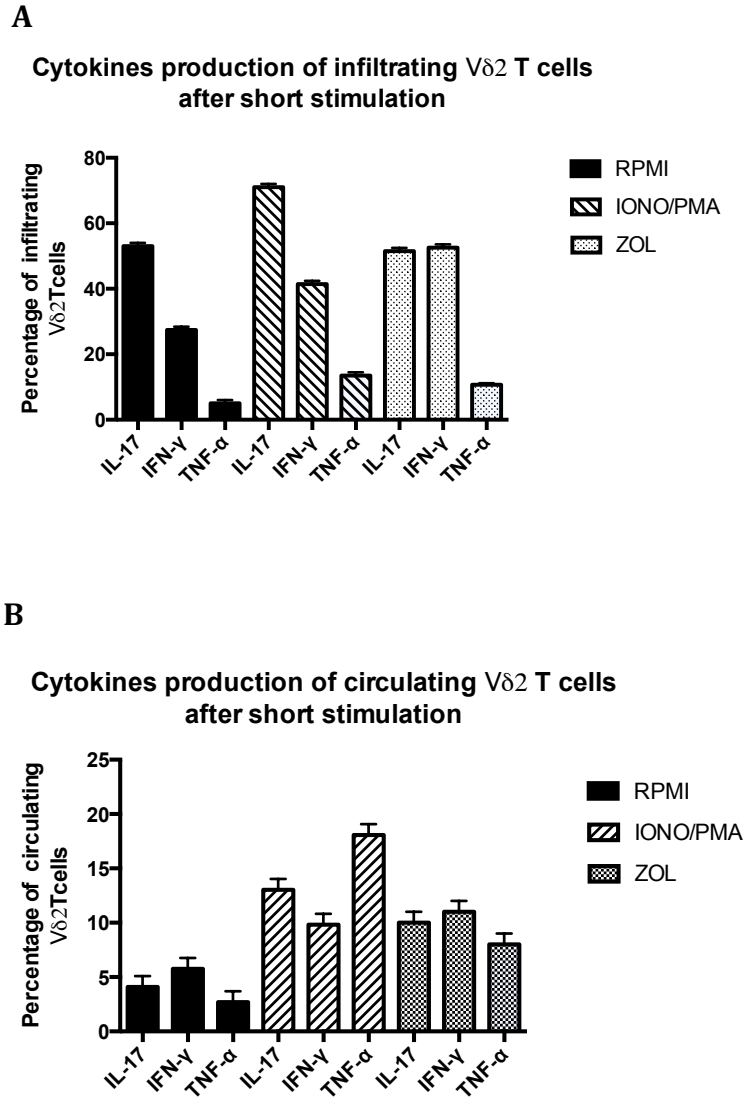


Figure 8: Cytokine production capacity of *ex-vivo* SCC-derived γδ T. (A) Cytokine production by SCC- derived Vδ2 and (B) by PBMC derived Vδ2 of the patients with NMSC was assessed by flow cytometry analysis after short stimulation *in vitro* with PMA and ionomycin or Zoledronate Acid and after the first hour were inclused of monencyn. Percentages of Vδ2 T cells producing cytokines were stained with anti- CD3, anti-Vδ2 mAbs and using BD kit to fix and permeabilize the cells to detect IL17A, IFN-γ and TNF-α.

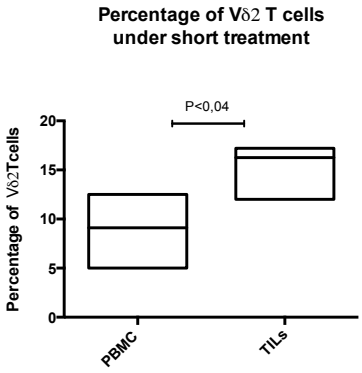
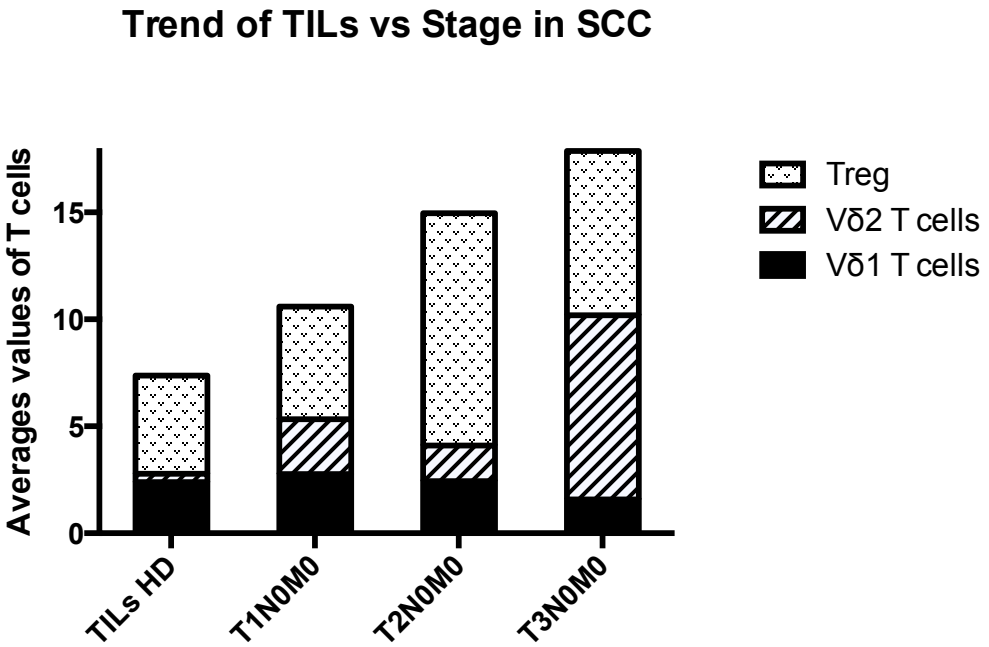
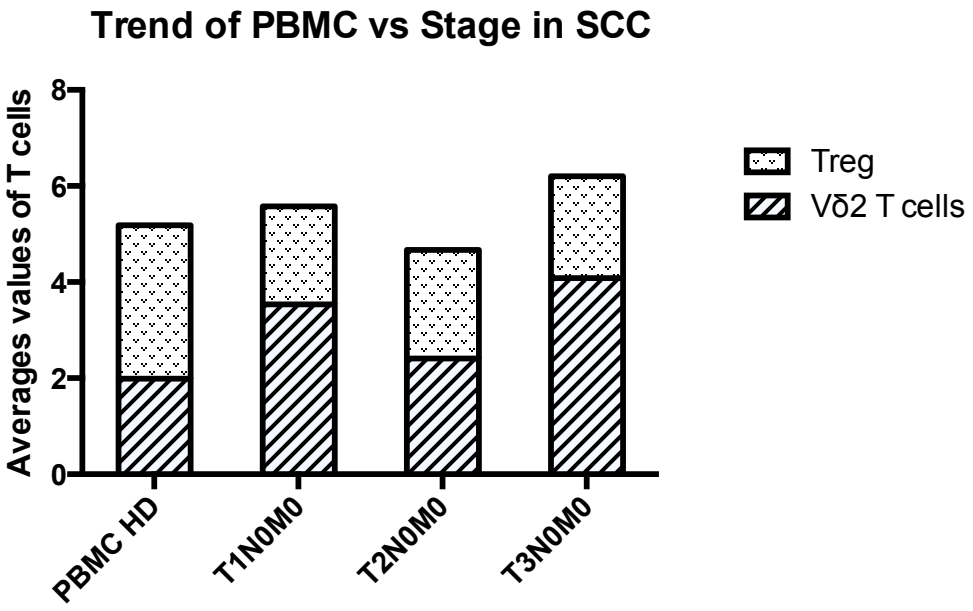


Figure 9: T cells were short time stimulated with PMA and ionomycin and after the first hour were inclused of monencyn. The cells were stained for surface marker expression, fixed, permeabilized, and stained with anti- perforin and examined by flow cytometry. p<0,04 when compared circulating and infiltrating Vd2 producing perforin.

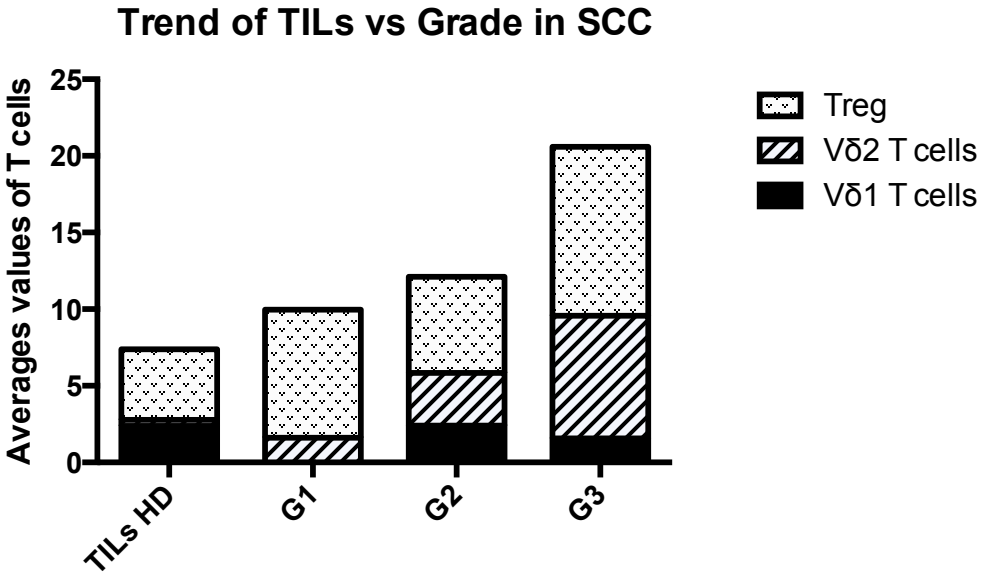
A



B


Trend of PBMC vs Stage in SCC
Averages values of T cells
8
6
4
2
0
PBMC HD
T1N0M0
T2N0M0
T3N0M0
Treg
Vδ2 T cells

C


Trend of TILs vs Grade in SCC
Averages values of T cells
25
20
15
10
5
0
TILs HD
G1
G2
G3
Treg
Vδ2 T cells
Vδ1 T cells

D

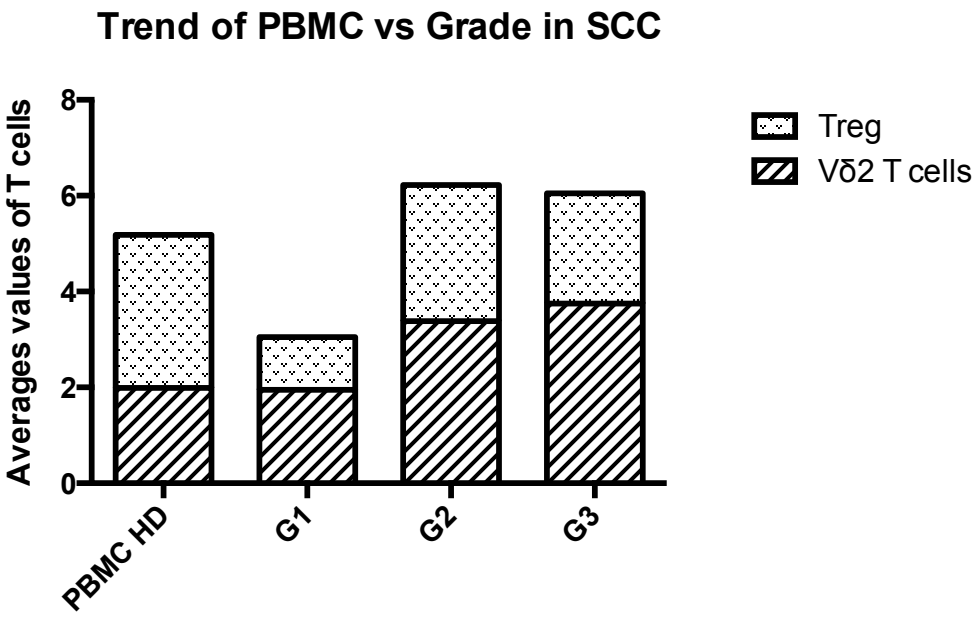
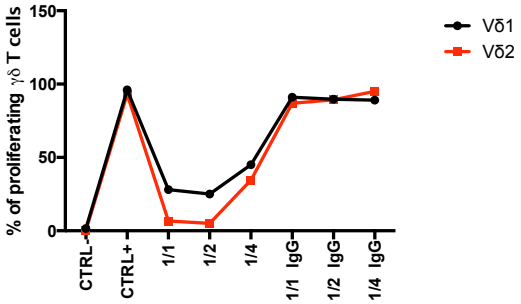
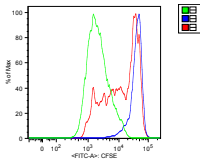


Figure 12: Association between rate of γδ T cells and clinical features in patients with SCC.

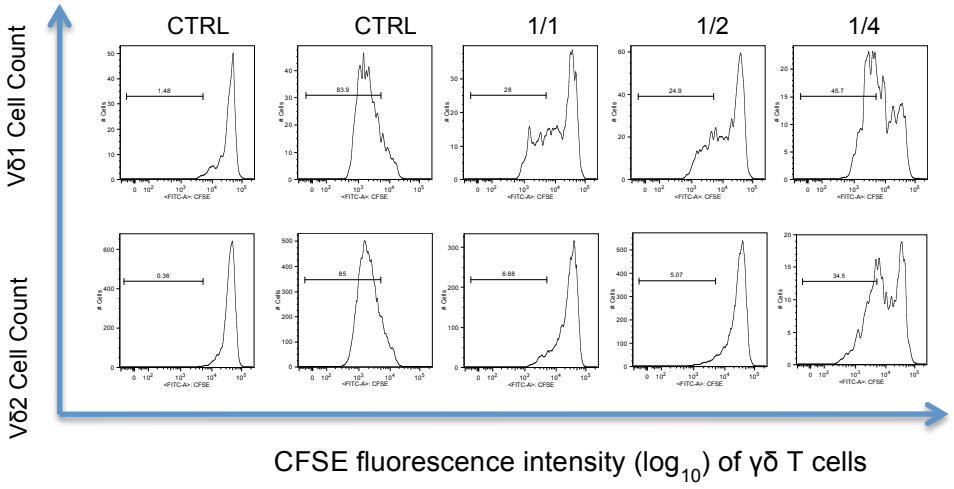
A



B



Ratio Treg:γδ



Activated Treg on plate

Activated Treg on plate

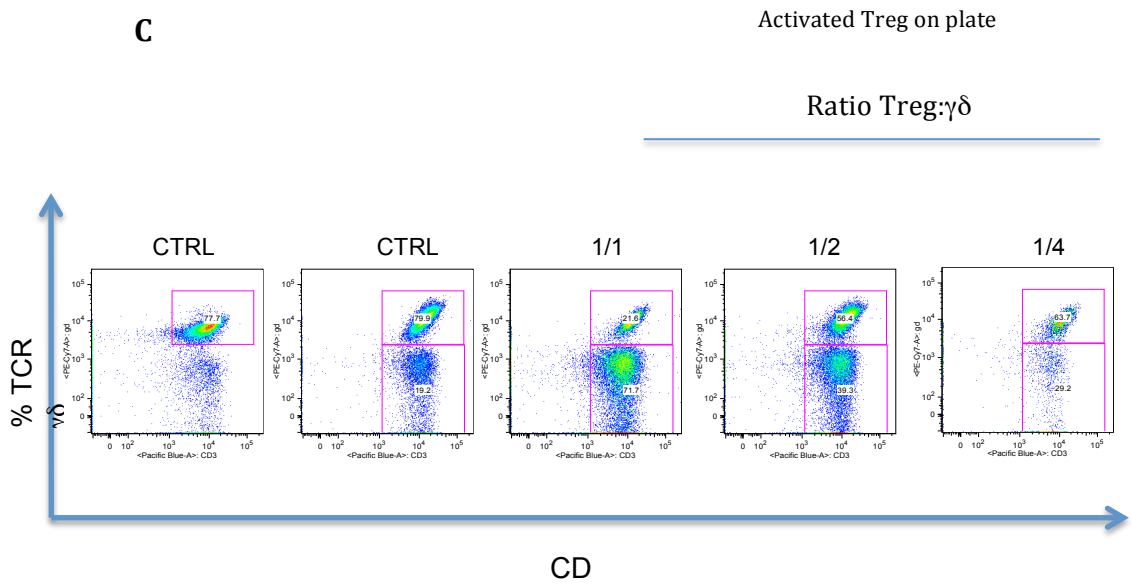
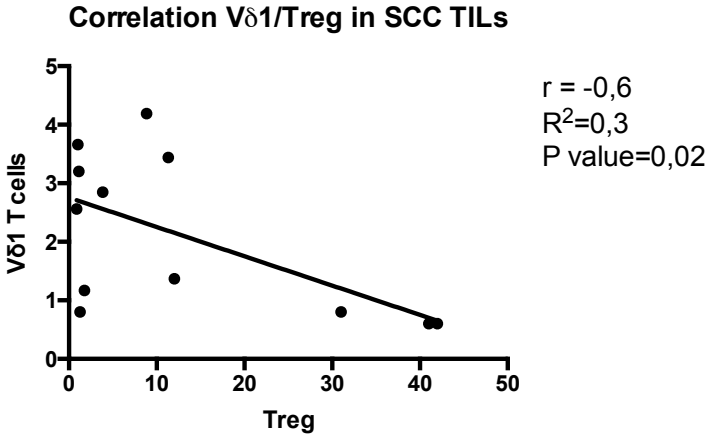


Figure 10: Inhibitory effects of purified regulatory T cells on induced γδ T cells proliferation. CD4+CD25+ cells were isolated from PBMC by using FACS Aria cell sorter cytometer. After sorting the cells were activated ON using Pre-coacted plate and IL2. γδ T cells were expanded from whole PBMC fro 10 days. Carboxyfluorescein succinimidyl ester (CFSE) –labelled gd Tc cell line were incubated with medium alone or co-cultured with autolougous purified Treg at ratio 1:1, 1:2, 1:4 (Treg: γδ T cells). On day 4, cells were analysed by flow cytometry. γδ T cells receptor TCR /CD3 double –positive were gated and loss of CFSE represented proliferation of gated cells. The ratio IgG indicated the Treg ON cultured in IgG pre-coacted plate and kept with IL2. A) Grapht showed the inhibition by Treg on γδ T cells. B) The histograms rappresented the cells proliferating at different ratio and the Control + or – and the internal control assay with IgG. C) Panel representative flow cytometric analysis of data showing the percentages of γδ T cells at different ratio with Treg during the suppression assay

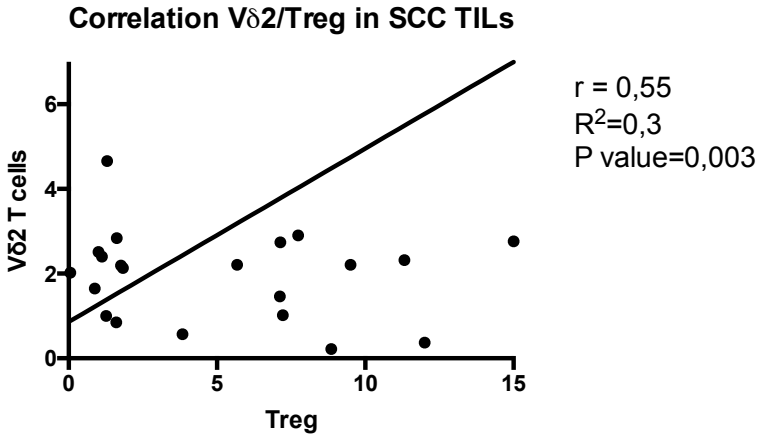
Table 1:Clinicapathologic characteristics of patients with NMSC who donated Biopsy and PBMC for this study

Sex		
Male		37
Female		10
Total		47
Age (Median)		
Range		39-94
Male		75
Female		74
Tumor Site		
Head-Neck		42
Tongue	15	
Lips	3	
Oral cavity	5	
Face	14	
Hair	5	
Limbs		5
Tumor Differentiation		
G1 (Poor)		3
G2 (Moderate)		23
G3 (Well)		6
Non determined		15
Tumor Stage		
T1		10
T2		16
T3		9
Unstaged		12
Histology		
SCC		35
BCC		6
Unclassified		6

A



B



C

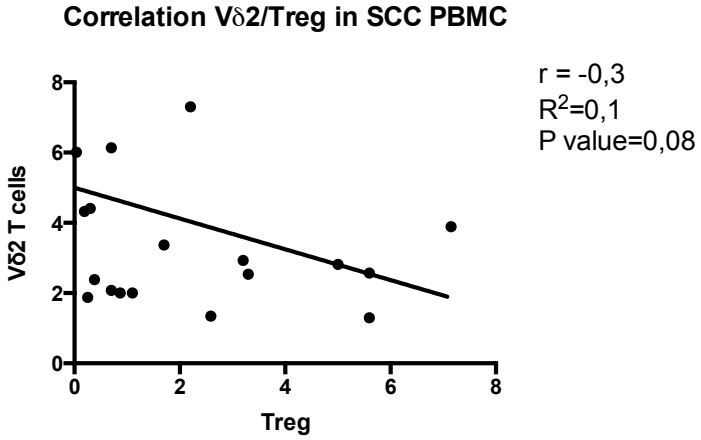


Figure 11: Comparison Correlations between various TIL populations. A) SCC Vδ1 T cells versus SCC Treg. B) SCC Vδ2 T cells T cells versus SCC Tregs. C) PBMC Vδ2 T cells versus

PBMC Tregs from SCC patients. The diagrams on the left of the respective graphts show a Pearson's coefficient (r) and R- squared that quantity the coefficient of determination. The significant correlation in all of these combinations of TIL populations are showed.

Table 2

Stage of the Tumor					
T- test					
Two-tailed / nn paramet /Unpaired					
Infiltrating	**Vδ1**	**Vδ2**		**Treg**	
HD vs T1	NA	p<0,01	**	NA	
HD vs T2	NA	p<0,05	*	p<0,05	*
HD vs T3	NA	p<0,01	**	NA	
T1 vs T2	NA	NA		NA	
T1 vs T3	NA	NA		NA	
T2 vs T3	NA	p<0,05	*	p<0,05	*

Table 3

Stage of the Tumor					
T- test					
Two-tailed / nn paramet /Unpaired					
Circulating	**Vδ1**	**Vδ2**		**Treg**	
HD vs T1	/	p<0,01	***	NA	
HD vs T2	/	NA		NA	
HD vs T3	/	p<0,01	**	p<0,05	*
T1 vs T2	/	p<0,05	*	NA	
T1 vs T3	/	NA		NA	
T2 vs T3	/	p=0,05		NA	

Table 4

Grade of the Tumor					
T- test					
Two-tailed / nn paramet /Unpaired					
Infiltrating	**Vδ1**	**Vδ2**		**Treg**	
HD vs G1	/	p<0,05	*	p<0,05	*
HD vs G2	NA	p<0,01	**	no	
HD vs G3	NA	p<0,01	**	no	
G1 vs G2	/	NA		NA	
G1 vs G3	/	NA		NA	
G2 vs G3	NA	NA		NA	

Table 5

Grade of the Tumor				
T- test				
Two-tailed / nn paramet /Unpaired				
Circulating	**Vδ1**	**Vδ2**		**Treg**
HD vs G1	/	NA		/
HD vs G2	/	p<0,01	**	NA
HD vs G3	/	p<0,05	*	NA
G1 vs G2	/	NA		NA
G1 vs G3	/	NA		NA
G2 vs G3	/	NA		NA

7 Reference:

[1]: Chen J, Ruczinski I, Jorgensen TJ, Yenokyan G,et al. Nonmelanoma skin cancer and risk for subsequent malignancy. J Natl Cancer Inst. 2008 Sep 3;100(17):1215-22. doi: 10.1093/jnci/djn260. Epub 2008 Aug 26. PubMed PMID: 18728282; PubMed Central PMCID: PMC2720713.

[2]: Rigel DS, Carucci JA. Malignant melanoma: prevention, early detection and treatment in the 21st century. CA Cancer J Clin 2000; 50:215□36.

[3]: Bosetti C, La Vecchia C, Naldi L et al. Mortality from cutaneous malignant melanoma in Europe. Has the epidemic levelled off? Melanoma Res 2004; 14:301□9.

[4]: Staples M, Marks R, Giles G. Trends in the incidence of nonmelanocytic skin cancer (NMSC) treated in Australia 1985□1995: are primary prevention programs starting to have an effect? Int J Cancer 1998; 78:144□8.

[5]: Green A, MacLennan R, Youl P et al. Site distribution of cutaneous melanoma in Queensland. Int J Cancer 1993; 53:232□6.

[6]: Garbe C, Buttner P, Bertz J et al. Primary cutaneous melanoma. Prognostic classification of anatomic location. Cancer 1995; 75:2492□8.

[7]: de Villiers EM. Human papillomavirus infections in skin cancers. Biomed Pharmacother 1998; 52:26:33.

[8]: Ulrich C, Schmook T, Nindl I et al. Cutaneous precancers in organ transplant recipients: an old enemy in a new surrounding. Br J Dermatol 2003; 149 Suppl 66:40-2.

[9]: Ulrich C, Schmook T, Sachse MM et al. Comparative epidemiology and pathogenic factors for nonmelanoma skin cancer in organ transplant patients. Dermatol Surg 2004; 30:622-7.

[10]: Holme SA, Malinovszky K, Roberts DL. Changing trends in nonmelanoma skin cancer in South Wales, 1988-98. Br J Dermatol 2000; 143:1224-9.

[11]: Ziegler A, Leffell DJ, Kunala S et al. Mutation hotspots due to sunlight in the p53 gene of non- melanoma skin cancers. Proc Natl Acad Sci USA 1993; 90:4216-20.

[12: Zhang H, Ping XL, Lee PK et al. Role of PTCH and p53 genes in early-onset basal cell carcinoma. Am J Pathol 2001; 158:381-5.

[13]: Lo Presti E, Dieli F, Meraviglia S, Tumor-infiltrating γδ T lymphocytes: pathogenic role, clinical significance and differential programming in the tumor microenvironment. Frontiers of Immunology 2014;

[14] Hayday AC. γδ T cells: A right time and a right place for a conserved third way of protection. Annu Rev Immunol (2000) 18: 975-1026.

[15] Bonneville M, O'Brien RL, Born WK. γδ T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol (2010) 10: 467-78. doi: 10.1038/nri2781. doi: 10.1038/nri2781.

[16] Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial γδ T cells. Science (1998) 279: 1737-40.

[17] Poggi A, Venturino C, Catellani S, Clavio M, Miglino M, Gobbi M, et al. Vδ1 Tlymphocytes from B-CLL patients recognize ULBP3 expressed on leukemic B cells and up-regulated by trans-retinoic acid. Cancer Res (2004) 64: 9172-9.

[18] Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human γδ T cells. Nature (1995) 75: 155-8.

[19] Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, Jomaa H. Microbial isoprenoid biosynthesis and human γδ T cell activation. FEBS Lett (2003) 544: 4-10.

[20] Sireci G, Espinosa E, Di Sano C, Dieli F, Fournié JJ, Salerno A. Differential activation of human γδ cells by nonpeptide phosphoantigens. Eur J Immunol (2001) 31: 1628-35.

[21] Vantourout P, Hayday A. Six-of-the-best: unique contributions of γδ T cells to immunology. Nat Rev Immunol (2013) 13: 88-100. doi: 10.1038/nri3384.

[22] Scotet E, Martinez LO, Grant E, Barbaras R, Jeno P, Guiraud M, et al. Tumor recognition following Vγ9Vδ2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. Immunity (2005) 22: 71-80.

[23] Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human γδ T cells. Nat Immunol (2013) 14: 908-16. doi: 10.1038/ni.2665.

[24] Roelofs AJ, Jauhiainen M, Monkkonen H, Rogers MJ, Monkkonen J, Thompson K. Peripheral blood monocytes are responsible for γδ T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. Br J Haematol (2009) 144: 245-50. doi: 10.1111/j.1365-2141.2008.07435.x.

[25] Li J, Herold MJ, Kimmel B, Muller I, Rincon-Orozco B, Kunzmann V, et al. Reduced expression of the mevalonate pathway enzyme farnesyl pyrophosphate synthase unveils recognition of tumor cells by Vγ9Vδ2 T cells. J Immunol (2009) 182: 8118-24. doi: 10.4049/jimmunol.0900101.

[26] Gober HJ, Kistowska M, Angman L, Jeno P, Mori L,De Libero G. Human T cell receptor γδ T cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med (2003) 197: 163-8.

[27] Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, et al. Differentiation of effector/memory Vδ2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med (2003) 198:391-7.

[28] Caccamo N, Meraviglia S, Ferlazzo V, Angelini D, Borsellino G, Poccia F, et al. Differential requirements for antigen orhomeostatic cytokines for proliferation and differentiation of human Vγ9Vδ2 naive, memory and effector T cell subsets. Eur J Immunol (2005) 35:1764-72.

[29] Caccamo N, Todaro M, Sireci G, Meraviglia S, Stassi G, Dieli F. Mechanisms underlying lineage commitment and plasticity of human γδ T cells. Cell Mol Immunol. (2013) 10: 30-4. doi: 10.1038/cmi.2012.42.

[30] Sireci G, Champagne E, Fournié JJ, Dieli F, Salerno A. Patterns of phosphoantigen stimulation of human Vγ9Vδ2 T cell clones include Th0 cytokines. Hum Immunol (1997) 58: 70-82.

[31] Wesch D, Glatzel A, Kabelitz D. Differentiation of resting human peripheral blood γδ T cells toward Th1- or Th2-phenotype. Cell Immunol (2001) 212: 110-117.

[32] Ness-Schwickerath KJ, Jin C, Morita CT. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vγ9Vδ2 T cells. J Immunol (2010) 184: 7268-80. doi: 10.4049/jimmunol.1000600.

[33] Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G et al. Differentiation, phenotype and function of interleukin-17-producing human Vγ9Vδ2 T cells. Blood (2011) 118: 129-138."doi: 10.1182/blood-2011-01-331298.

[34] Fenoglio D, Poggi A, Catellani S, Battaglia F, Ferrera A, Setti M et al. Vδ1 T lymphocytes producing IFN-γ and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. Blood (2009) 113: 6611-6618. doi: 10.1182/blood-2009-01- 198028.

[35] Bansal RR, Mackay CR, Moser B, Eberl M. IL-21 enhances the potential of human γδ T cells to provide B-cell help. Eur J Immunol (2012) 42: 110-119. doi: 10.1002/eji.201142017.

[36] Caccamo N, Todaro M, La Manna MP, Sireci G, Stassi G, Dieli F. IL-21 regulates the differentiation of a human γδ T cell subset equipped with B cell helper activity. PLoSONE (2012) 7:e41940. doi: 10.1371/journal.pone.0041940.

[37] Casetti R, Agrati C, Wallace M, Sacchi A, Martini F, Martino A et al. TGF-β1 and IL-15 Induce $FOXP3^{+}$ γδ regulatory T cells in the presence of antigen stimulation. J Immunol (2009) 183: 3574-3577. doi: 10.4049/jimmunol.0901334.

[38] Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: The roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Adv Immunol (2006) 90: 1-50.

[39] Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol (2006) 6: 383-93.

[40] Kunzmann V, Bauer E, Feurle J, Weihinger F, Torny HP, Wilhelm M. Stimulation of γδ T cells by aminobisphosphonates and induction of anti plasma cell activity in multiple myeloma. Blood (2000) 86: 384-92.

[41] Corvaisier M, Moreau-Aubry A, Diez E, Bennouna J, Mosnier JF, Scotet E, *et al*. Vγ9Vδ2 T cell response to colon carcinoma cells. J Immunol (2005) 175: 5481-8.

[42] Belmant C, Decise D, Fournie JJ. Phosphoantigens and aminobisphosphonates: new leads targeting γδ T lymphocytes for cancer immunotherapy. Drug Discov Today Ther Strateg (2006) 3: 17-23.

[43] Liu Z, Guo BL, Gehrs BC, Nan L, Lopez RD. *Ex vivo* expanded human Vγ9Vδ2 $\gamma\delta^{+}$ T cells mediate innate antitumor activity against human prostate cancer cells *in vitro*. J Urol (2005) 173: 1552-6.

[44] Viey E, Fromont G, Escudier B, Morel Y, Da Rocha S, Chouaib S, *et al*. Phosphostim activated γδ T cells kill autologous metastatic renal cell carcinoma. J Immunol (2005) 174: 1338-47.

[45] Kabelitz D, Wesch D, Pitters E, Zoller M Characterization of tumor reactivity of human Vγ9Vδ2 γδT cells *in vitro* and in SCID mice *in vivo*. J Immunol (2004) 173: 6767-76.

[46] D'Asaro M, La Mendola C, Di Liberto D, Orlando V, Todaro M, Spina M, *et al*. Vγ9Vδ2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells. J Immunol (2010) 184: 3260-8. doi: 10.4049/jimmunol.0903454.

[47] Harwood HJ Jr, Alvarez IM, Noyes WD, Stacpoole PW. *In vivo* regulation of human leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase: increased enzyme protein concentration and catalytic efficiency in human leukemia and lymphoma. J Lipid Res (1991) 32: 1237-52.

[48] Kunzmann V, Bauer E, Wilhelm M. γδ T-cell stimulation by pamidronate. N Engl J Med (1999) 340: 737-8.

[49] Girlanda S, Fortis C, Belloni D, Ferrero E, Ticozzi P, Sciorati C, *et al*. MICA expressed by multiple myeloma and monoclonal gammopathy of undetermined significance plasma cells co-stimulates pamidronate-activated γδ lymphocytes. Cancer Res (2005) 65: 7502-8.

[50] Dieli F, Gebbia N, Poccia F, Caccamo N, Montesano C, Fulfaro F, *et al*. Induction of γδ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients *in vivo*. Blood (2003) 102: 2310-1.

[51] Das H, Groh V, Kuijl C, Sugita M, Morita CT, Spies T, *et al*. MICA engagement

by human Vγ9Vδ2 T cells enhances their antigen dependent effector function. Immunity (2001) 15: 83-93.

[52] Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T. Activation of Vγ9Vδ2 T cells by NKG2D. J Immunol (2005) 175: 2144-51.

[53] Lanca T, Correia DV, Moita CF, Raquel H, Neves-Costa A, Ferreira C, *et al*. The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to γδ T-cell cytotoxicity. Blood (2010) 115: 2407-11. doi: 10.1182/blood-2009- 08-237123.

[54] Gomes AQ, Correia DV, Grosso AR, Lança T, Ferreira C, Lacerda JF, *et al*. Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood γδ T cells. Haematologica (2010) 95: 1397-404. doi: 10.3324/haematol.2009.020602.

[55] Dieli F, Troye-Blomberg M, Ivanyi J, Fournie M, Bonneville MA, Peyrat G, *et al*. Granulysin dependent killing of intracellular and extracellular *Mycobacterium tuberculosis* by Vγ9Vδ2 T lymphocytes. J Infect Dis (2001) 184: 1082-5.

[56] Vermijlen D, Ellis P, Langford C, Klein A, Engel R, Willimann K, *et al*. Distinct cytokine driven responses of activated blood γδ T cells: insights into unconventional T cell pleiotropy. J Immunol (2007) 178: 4304-14.

[57] Dalton JE, Howell G, Pearson J, Scott P, Carding SR. Fas-Fas ligand interactions are essential for the binding to and killing of activated macrophages by γδ. J Immunol (2004) 173: 3660-7.

[58] Ismaili J, Olislagers V, Poupot R, Fournie JJ, Goldman M. Human γδ T cells induce dendritic cell maturation. Clin Immunol (2002) 103: 296-302.

[59] Buccheri S, Guggino G, Caccamo N, Li Donni P, Dieli F. Efficacy and safety of γδ T cell-based tumor immunotherapy: a meta-analysis. J Biol Regul Homeost Agents. (2014) 28: 81-90.

[60] Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer (2011) 105: 93-103. doi: 10.1038/bjc.2011.189.

[61] Sasada T, Suekane S Variation of tumor-infiltrating lymphocytes in human cancers: controversy on clinical significance. Immunotherapy (2011) 3: 1235-1251. doi: 10.2217/imt.11.106.

[62] Bialasiewicz AA, Ma JX, Richard G. αβ- and γδ TCR^{+} lymphocyte infiltration in necrotising choroidal melanomas. Br J Ophthalmol (1999) 83:1069-73.

[63] Inman BA, Frigola X, Harris KJ, Kuntz SM, Lohse CM, Leibovich BC, et al. Questionable relevance of γδ T lymphocytes in renal cell carcinoma. J Immunol (2008) 180: 3578-84.

[64] Ma C, Zhang Q, Ye J, Wang F, Zhang Y, Wevers E, et al. Tumor-infiltrating γδ T lymphocytes predict clinical outcome in human breast cancer. J Immunol (2012) 189: 5029-36. doi: 10.4049/jimmunol.1201892.

[65] Cordova A, Toia F, La Mendola C, Orlando V, Meraviglia S, Rinaldi G, et al. (2012) Characterization of Human γδ T Lymphocytes Infiltrating Primary Malignant Melanomas. PLoS ONE 7: e49878. doi:10.1371/journal.pone.0049878.

[66] Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, et al. γδ T17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. Immunity (2014) 40:785-800. doi: 10.1016/j.immuni.2014.03.013.

[67] Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived γδ T cells of MICA and MICB. Proc Natl Acad Sci U S A. (1999) 96: 6879-84.

[68] Chen J, Niu H, He W, Ba D. Antitumor activity of expanded human tumor-infiltrating γδ T lymphocytes. Int Arch Allergy Immunol (2001) 125: 256-63.

[69] Choudhary A, Davodeau F, Moreau A, Peyrat MA, Bonneville M, Jotereau F. Selective lysis of autologous tumor cells by recurrent γδ tumor-infiltrating lymphocytes from renal carcinoma. J Immunol (1995) 154: 3932-40.

[70] Ferrarini M, Heltai S, Pupa SM, Mernard S, Zocchi R. Killing of laminin receptor-positive human lung cancers by tumor infiltrating lymphocytes bearing $\gamma\delta^+$ T-cell receptors. J Natl Cancer Inst (1996) 88: 436-41.

[71] Maeurer MJ, Martin D, Walter W, Liu K, Zitvogel L, Halusczcak K, et al. Human intestinal $V\delta1^+$ lymphocytes recognize tumor cells of epithelial origin. J Exp Med (1996) 183: 1681-96.

[72] Kitayama J, Atomi Y, Nagawa H, Kuroda A, Mutoh T, Minami M, Juji T. Functional analysis of TCR $\gamma\delta^+$ T cells in tumour-infiltrating lymphocytes (TIL) of human pancreatic cancer. Clin Exp Immunol (1993) 93: 442-7.

[73] Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-infiltrating γδ T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. Immunity (2007) 27: 334-48.

[74] Ye J, Ma C, Hsueh EC, Eickhoff CS, Zhang Y, Varvares MA, et al. Tumor-derived γδ regulatory T cells suppress innate and adaptive immunity through the induction of immunosenescence. J Immunol (2013) 190: 2403-14. doi:10.4049/jimmunol.1202369.

[75] Ma S, Cheng Q, Cai Y, Gong H, Wu Y, Yu X, et al. IL-17A produced by γδ T cells promotes tumor growth in hepatocellular carcinoma. Cancer Res (2014) 74: 1969-82. doi: 10.1158/0008-5472.CAN-13-2534.

[76] Wakita D, Sumida K, Iwakura Y, Nishikawa H, Ohkuri T, Chamoto K, et al.

Tumor- infiltrating IL-17-producing γδ T cells support the progression of tumor by promoting angiogenesis. Eur J Immunol (2010) 40: 1927-37. doi: 10.1002/eji.200940157.

[77] Rei M, Gonçalves-Sousa N, Lança T, Thompson RG, Mensurado S, Balkwill FR, et al. Murine $CD27^{-}$ $V\gamma6^{+}$ γδ T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages. Proc Natl Acad Sci USA (2014) 111: E3562-70. doi: 10.1073/pnas.1403424111.

[78] Burdelya L, Kujawski M, Niu G, Zhong B, Wang T, Zhang S, et al. STAT3 activity in melanoma cells affects migration of immune effector cells and nitric oxide-mediated antitumor effects. J. Immunol (2005) 174: 3925-31

[79] Ugurel S, Schrama D, Keller G, Schadendorf D, Bröcker EB, Houben R, et al. Impact of the CCR5 gene polymorphism on the survival of metastatic melanoma patients receiving immunotherapy. Cancer Immunol Immunother (2008) 57: 685-91.

[80] Catalano V, Turdo A, Di Franco S, Dieli F, Todaro M, Stassi G. Tumor and its microenvironment: a synergistic interplay. Semin Cancer Biol (2013) 23: 522-32. doi: 10.1016/j.semcancer.2013.08.007.

[81] Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, et al. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. Cell Stem Cell (2014) 14: 342-56. doi: 10.1016/j.stem.2014.01.009.

[82] Beatty GL, Chiorean EG, Fishman MP, Saboury B, Teitelbaum UR, Sun W,et al. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. Science (2011) 331: 1612-6. doi: 10.1126/science.1198443.

[83] Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol (2013) 14: 1014-22. doi: 10.1038/ni.2703.

[84] Burnette B, Fu YX, Weichselbaum RR. The confluence of radiotherapy and immunotherapy. Front Oncol (2012) 2: 143. doi: 10.3389/fonc.2012.00143.

[85] Todaro M, Meraviglia S, Caccamo N, Stassi G, Dieli F. Combining conventional chemotherapy and γδ T cell-based immunotherapy to target cancer-initiating cells. Oncoimmunology (2013) 2: e25821.

[86] Todaro M, D'Asaro M, Caccamo N, Iovino F, Francipane MG, Meraviglia S, et al. Efficient killing of human colon cancer stem cells by γδ T lymphocytes. J Immunol (2009) 182: 7287-96. doi: 10.4049/jimmunol.0804288.

[87] Coscia M, Quaglino E, Iezzi M, Curcio C, Pantaleoni F, Riganti C, et al. Zoledronic acid repolarizes tumor-associated macrophages and inhibits mammary carcinogenesis by targeting the mevalonate pathway. J Cell Mol Med (2010) 14: 2803-15. doi: 10.1111/j.1582-4934.2009.00926.x.

[88] Santolaria T, Robard M, Léger A, Catros V, Bonneville M, Scotet E. Repeated

systemic administrations of both aminobisphosphonates and human Vγ9Vδ2 T cells efficiently control tumor development in vivo. J Immunol (2013) 191: 1993-2000. doi: 10.4049/jimmunol.1300255.

[89] Haeryfar SM, DiPaolo RJ, Tscharke DC, Bennink JR, Yewdell JW. Regulatory T cells suppress CD8□ T cell responses induced by direct priming and cross-priming and moderate immunodomi- nance disparities. J Immunol. 2005;174:3344- 3351.

[90] Ralainirina N, Poli A, Michel T, et al. Control of natural killer (NK) cell functions by CD4□CD25□ regulatory T cells. J Leukoc Biol. 2007;81:144- 153.

[91] Lim HW, Hillsamer P, Banham AH, Kim CH. Cut- ting edge: direct suppression of B cells by CD4□ CD25□ regulatory T cells. J Immunol. 2005;175: 4180-4183.

[92] Houot R, Perrot I, Garcia E, Durand I, Lebecque S. Human CD4□CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. J Immunol. 2006;176:5293-5298.

[93] Taams LS, van Amelsfort JM, Tiemessen MM, et al. Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells. Hum Immunol. 2005;66:222-230.

[94] Lewkowicz P, Lewkowicz N, Sasiak A, Tchorzewski H. Lipopolysaccharide-activated CD4+CD25+ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. J Immunol. 2006;177:7155-7163

[95] Sharp LL, Jameson JM, Witherden DA, Komori HK, Havran WL. Dendritic epidermal T-cell activa- tion. Crit Rev Immunol. 2005;25:1-18

[96] Girardi M,Immunosurveillance and Immunoregulation by γδ T Cells.Journal of Investigative Dermatology (2006) 126, 25–31. doi:10.1038/sj.jid.5700003

[97] Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, et al. (2001) Regulation of cutaneous malignancy by γδ T cells. Science 294:605–609

[98] Van Damme N, De Keyser F, Demetter P, Baeten D, Mielants H, Verbruggen G, et al. (2001) The proportion of Th1 cells, which prevail in gut mucosa, is decreased in inflammatory bowel syndrome. Clin Exp Immunol 125:383–390

[99] Laggner U, Di Meglio P, K. Perera G, Hundhausen C et al and O. Nestle Identification of a Novel Proinflammatory Human Skin-Homing V γ9Vδ2 T Cell Subset with a Potential Role in Psoriasis.J. of Immunology 2011;10.4049/1100804

[100] Bialasiewicz AA, Ma JX, Richard G: Alpha/beta- and gamma/delta TCR(þ) lymphocyte in¢ltration in necrotising choroidal melanomas. Br J Ophthalmol 83:1069^1073, 1999

[101] Murat Bas a, Henning Bier a, Kerstin Schirlau a, Ulrike Friebe-Hoffmann b, Kathrin Scheckenbach a, Vera Balz a, Theresa L. Whiteside c,Thomas K. Hoffmann Gamma–delta T-cells in patients with squamous cell carcinoma of the head and

neck. Oral Oncology (2006) 42, 691–697

[102] Christoph Bergmann, Laura Strauss, Yun Wang, et al. Clin Cancer Res 2008;14:3706-3715. Published online June 16, 2008.

[103] Clark, R.A., and T.S. Kupper. 2007. IL-15 and dermal fibroblasts in- duce proliferation of natural regulatory T cells isolated from human skin. Blood. 109:194–202.

[104] Clark R A, Huang S J, Murphy G F, Mollet I G and Kupper Human squamous cell carcinomas evade the immune response by down-regulation of vascular E-selectin and recruitment of regulatory T cells. J. Exp. Med. Vol. 205 No. 10 2221-2234

[105] Clark RA. Skin-resident T cells: the ups and downs of on site immunity. J Invest Dermatol. 2010; 130:362–370.

[106] Valladeau J, Ravel O, Dezutter-Dambuyant C, Moore K, Kleijmeer M, Liu Y, Duvert-Frances V, Vincent C, Schmitt D, Davoust J, et al. Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. Immunity. 2000; 12:71–81.

[107] Zaba LC, Fuentes-Duculan J, Steinman RM, Krueger JG, Lowes MA. Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163+FXIIIA+ macrophages. J Clin Invest. 2007; 117:2517–2525.

[108] Zaba LC, Krueger JG, Lowes MA. Resident and “inflammatory” dendritic cells in human skin. J Invest Dermatol. 2009; 129:302–308.

[109] Stoitzner P. The Langerhans cell controversy: are they immunostimulatory or immunoregulatory cells of the skin immune system? Immunol Cell Biol. 2010; 88:348–350.

[110] Julien Seneschal,Rachael A. Clark1, Ahmed Gehad, Clare M. Baecher-Allan, and Thomas S. Kupper. Human Epidermal Langerhans Cells Maintain Immune Homeostasis in Skin by Activating Skin Resident Regulatory T Cells mmunity. 2012 May 25; 36(5): 873 doi:10.1016/j.immuni.2012.03.018.

[111] Gasparoto E, Salles de Souza Malaspina T , Benevides L, et al.Patients with oral squamous cell carcinoma are characterized by increased frequency of suppressive regulatory T cells in the blood and tumor microenvironment.Cancer Immunol Immunother (2010) 59:819–828

[112] Laura Strauss, Christoph Bergmann, William Gooding, et al.The Frequency and Suppressor Function of CD4+CD25high Foxp3+ T Cells in the Circulation of Patients with Squamous Cell Carcinoma of the Head and Neck. Clin Cancer Res 2007;13:6301-6311.

[113] Haeryfar SM, DiPaolo RJ, Tscharke DC, Bennink JR, Yewdell JW. Regulatory T cells suppress CD8□ T cell responses induced by direct priming and cross-priming and moderate immunodomi- nance disparities. J Immunol. 2005;174:3344- 3351.

[114] Ralainirina N, Poli A, Michel T, et al. Control of natural killer (NK) cell functions by CD4□CD25□ regulatory T cells. J Leukoc Biol. 2007;81:144- 153.

[115] Lim HW, Hillsamer P, Banham AH, Kim CH. Cut- ting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. J Immunol. 2005;175: 4180-4183.

[116] Houot R, Perrot I, Garcia E, Durand I, Lebecque S. Human CD4+CD25^{high} regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. J Immunol. 2006;176:5293-5298.

[117] Taams LS, van Amelsfort JM, Tiemessen MM, et al. Modulation of monocyte/macrophage function by human CD4□CD25□ regulatory T cells. Hum Immunol. 2005;66:222-230.

[118] Lewkowicz P, Lewkowicz N, Sasiak A,chorzewski H. Lipopolysaccharide-activated CD4+CD25+ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. J Immunol. 2006;177:7155-7163.

[119] Kashyap M, Thornton AM, Norton SK, et al. Cut- ting edge: CD4 T cell-mast cell interactions alter IgE receptor expression and signaling. J Immu- nol. 2008;180:2039-2043.

[120] Ribeiro-Rodrigues R, Resende Co T, Rojas R, Toossi Z, Dietze R, Boom WH, Maciel E, Hirsch CS. A role for $CD4^+$ $CD25^+$ T cells in regulation of the immune response during human tuberculosis. Clin Exp Immunol 2006; 144:25–34.

[121] Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. Reg- ulatory T cells are expanded in blood and disease sites in patients with tuberculosis. Am J Respir Crit Care Med 2006; 173:803–10.

[122] Chen X, Zhou B, Li M et al. CD4(+)CD25(+)FoxP3(+) regula- tory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease. Clin Immunol 2007; 123:50–9.

[123] Guangming Gong, Lingyun Shao, Yunqi Wang, Crystal Y. Chen, Dan Huang, Shuyu Yao, Ximei Zhan, Helene Sicard, Richard Wang, and Zheng W. Chen Phosphoantigen-activated Vγ2Vδ2 T cells antagonize IL-2–induced CD4+CD25+Foxp3+ T regulatory cells in mycobacterial infection.November 3, 2008; DOI 10.1182/blood-2008-06- 162792.

[124] C. Scott Mahan, Jeremy J. Thomas, W. Henry Boom and Roxana E. Rojas3. CD4+ CD25high Foxp3+ regulatory T cells downregulate human Vd2+ T-lymphocyte function triggered by anti-CD3 or phosphoantigendo.Immunology,2008:10.1111/j.1365-2567.2008.02982.x

[125] Li Li and Chang-You Wu CD4+CD25+ Treg cells inhibit human memory γδ T cells to produce IFN-γ in response to M tuberculosis antigen ESAT-6April 3, 2008; DOI 10.1182/blood-2008-02-139899.

[126] Gao, Y., Yang, W., Pan, M., Scully, E., Girardi, M., Augenlicht, L. H., Craft, J. and Yin, Z., gd T cells provide an early source of interferon-g in tumor immunity. J. Exp. Med. 2003. 198: 433–442.

[127] Volker Kunzmann, Brigitte Kimmel, Thomas Herrmann, Hermann Einsele and Martin Wilhelm3.Inhibition of phosphoantigen-mediated cd T-cell proliferation by CD4+ CD25+ FoxP3+ regulatory T cells.Immunity 2008.doi:10.1111/j.1365-2567.2008.02894.x.

[128] Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony HP. cd T cells for immune therapy of patients with lymphoid malignancies. Blood 2003; 102:200–6.

[129] Beyer M, Schultze JL. Regulatory T cells in cancer. Blood 2006; 108:804–11.

[130] Clark R, Huang S J, Murphy G F and Kupper et al. Human squamous cell carcinomas evade the immune response by down-regulation of vascular E-selectin and recruitment of regulatory T cells.J. Exp. Med. Vol. 205 No. 10 2221-2234.

[131] Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C,Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochov G, Sakaguchi S: Functional delineation and differentiation dynamics of human CD4+ T cells expressing the Foxp3 transcription factor. Immunity 2009, 30:899–911.

[132]Wei Sun, Wei-Jin Li, Chang-You Wu, Hua Zhong and Wei-Ping Wen. CD45RA–Foxp3high but not CD45RA+Foxp3low suppressive T regulatory cells increased in the peripheral circulation of patients with head and neck squamous cell carcinoma and correlated with tumor progression.Journal of Experimental & Clinical Cancer Research 2014, 33:35

[133] Julie C Ribot, Ana deBarros, Dick John Pang, Joana F Neves, Victor Peperzak, Scott J Roberts, Michael Girardi, Jannie Borst, Adrian C Hayday, Daniel J Pennington, and Bruno Silva-Santos, CD27 is a thymic determinant of the balance between interferonγ- and interleukin 17–producing γδ T cell subsets.Nat Immunol. 2009 April; 10(4): 427–436. doi:10.1038/ni.1717.

[134] Yong Yi et al; Isolated HCC-infiltrating CD4+CD25+ regulatory T cells (Treg cells) directly suppressed the cytotoxic function and IFN-γ secretion of γδ T cells in a TGFβ- and IL-10-dependent manner.J.Hepatology 2013.

[135] Greene, F.L., Staging of colon and rectal cancer: from endoscopy to molecular markers. Surg Endosc.2006. 20 Suppl 2: p. S475-8.

[136] Motulsky H (1995) Choosing an appropriate sample size. Intuitive Biostatistics 199–200. Oxford Univ. Press, New York.