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Analysis of infiltrating γδ T cells in cutaneous melanoma: a hypothetic crosstalk for new immunotherapy

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Abstract

Introduction:

Melanoma is one of the most frequently occurring skin cancer and remains an important cause of mortality mainly in Caucasian populations. In clinical practice, the standard treatment for localized melanoma is surgical resection but for metastatic melanoma, few treatments are available, where chemo and radio-therapies are rarely indicated. Immunotherapy has revolutionized the management of metastatic melanoma that, as in others tumors, uses your body's own immune system to help fight cancer. Therapies targeting the immune checkpoint molecules have achieved objective responses in melanoma. Several receptors and/or ligands, such as PDL1, PD-1 and CTLA-4, have been identified as important regulatory molecules but these therapies are not effective for a large number of patients. The importance of the immune response in the clinical outcome was correlated with increased number of tumor-infiltrating lymphocytes (TILs) in melanoma and in several other tumors. Many studies underline the role of γδ T lymphocytes in the anti-tumor surveillance with a marked cytotoxic effect towards tumor cells. We investigated the expression of some novel immune checkpoint molecules, such as TIM3 (T-cell immunoglobulin and mucin-domain containing-3), TIGIT (Tcell Ig and ITIM domain) and LAG3 (lymphocyte activation gene-3) on $\boldsymbol{\gamma\delta}$ T lymphocytes.

Material and Methods:

From 2018 to 2021, 54 adult patients were enrolled in our study with clinical suspicion of melanoma, at the Plastic Surgery Department of the University Hospital in Palermo. A blood drawing (in a dry test tube) and a fragment (about 1 mm) of perilesional skin was taken before the tumoral excision. All bioptical samples were processed immediately through mechanical and enzymatic digestion. After the immunological cells were analysed by flow cytometry. We conducted also the immunofluorescence staining on tumoral samples and bioinformatics analysis through dataset online.

Results:

The analysis of the histological examination reports showed that in 75% of cases the diagnosis of suspected melanoma was confirmed. The infiltrating and circulating T$\boldsymbol{\gamma\delta}$ lymphocytes analysis showed that the cell percentage was higher in peripheral blood than in peritumoral tissues in particular

on melanoma's metastases. The study of the immunocheckpoint molecules expression (PD1, TIGIT, LAG3 and TIM3) showed higher values in the infiltrating compared to circulating $\boldsymbol{\gamma\delta}$ T cells. In addition, a detailed analysis of these data in correlation with pathological stage of melanoma, showed that, on perilesional infiltrating $\boldsymbol{\gamma\delta}$ T lymphocytes, PD-1 is more expressed in melanomas in situ, then decreasing from pt1a as pathological stage increases. TIM3, TIGIT and LAG3, were expressed in other stage. The circulating $\boldsymbol{\gamma\delta}$ T lymphocytes investigation showed variable values for pathological stage, except TIGIT, whose values were higher in the ptis and pt1a. In the mestastases, all immunocheckpoints were more expressed, expecially LAG3. Furthermore, the bioinformatic analysis showed that immunotherapy with checkpoint inhibitors (anti-PD1, anti-CTL4 and anti-PD1/CTL4) caused an increase in memory T lymphocytes, with a marked migratory phenotype and an increase in depleted γδ lymphocytes.

Conclusions:

In the literature, there are few data on the LAG3, TIM3 and TIGIT expression in melanoma, therefore, this study may be able to contribute in this regard. LAG 3 is the highest of all the immunocheckpoints analyzed. Further studies will be needed to fully understand the function of these molecules in order to develop new immunotherapy protocols. In particular, the impact of neutralizing LAG3 with certain inhibitors should be further studied to understand the functional role of LAG3 in anti-PD1 resistant patients.

Summary

The search for tumour-infiltrating lymphocyte cells belonging to the innate immunohistocompatibility system could be fundamental in the development of alternative therapies based on immunosurveillance as a mechanism of action against advanced malignant melanoma, when surgery does not improve the Overall survival (OS). As well as, the analysis of circulating cells and infiltrating of a specific tumour patient could provide valuable predictive information.

In this project, we evaluated the expression of some novel immune checkpoint molecules, such as LAG3 (lymphocyte activation gene-3), TIM3 (T-cell immunoglobulin and mucin-domain containing-3) and TIGIT (T-cell Ig and ITIM domain), potentially expressed on T lymphocytes. In particular, we isolated a specific subset of γδ cells, Vγ9/Vδ2, from tumour infiltrating immune cells derived from tumour samples. The Vγ9Vδ2 T cells are significantly correlated with the melanomas in the initial and advanced phase and open new horizons in immunotherapy field for those patients that actually do not benefit from any therapy. In this regard, a recent study of Cordova's group showed that the percentages of tumour-infiltrating Vγ9Vδ2 T cells were correlated negatively with stage of disease and positively with Relapse Free Survival (RFS) and Overall Survival (OS) of melanoma patients. In particular, then they highlighted the presence of Vγ9Vδ2 T cells in TILs of melanoma samples and the amount was correlated with early stage of melanoma (stage 0-I-II) and without metastasis. Moreover, patients who progressed to stage III or IV showed a decreased frequency of circulating Vγ9Vδ2 T cells compared to values at diagnosis. All these evidences lead to hypothesize the possibility of using combination of adoptive γδ T cell therapy with new immune checkpoint inhibitors (anti-LAG3, anti-TIM3 or anti-TIGIT) as a useful strategy to enhance the anti-tumour activity of these cells. Few data currently exist on the expression of these immunocheckpoint molecules in TILs isolated from melanomas and metastases samples.

CHAPTER 1

Introduction

Melanoma is an important cause of mortality mainly in Caucasian populations. Exposure to UV radiation and familial history are considered the most common risk factors. Melanoma is a leading cause of death due to skin cancer. The melanocytes are located at the basal layer (Stratum basale) of skin epidermis and their uncontrolled proliferation produce the tumor [1]. Though it accounts for less than 5% of all skin cancer types, it is the most aggressive type of skin cancer and nearly 80% of the skin cancer-related deaths are due to melanoma. It is characterized by an extremely high metastatic potential, leading to 5 year-survival rates of less than 5 % after the onset of metastatic spread [2]. In clinical practice, the standard treatment for localized melanoma is surgical resection but for metastatic melanoma, few treatments are available, with immunotherapies playing a central role, while chemo and radio-therapies are rarely indicated. Advanced melanoma in stages III and IV still display a poor prognosis, and further research is needed to provide new more effective therapeutic protocols [3], [4]. In the last decade numerous studies have focused on the efficacy of immunotherapy and on the TILS (tumor-infiltrating lymphocytes), in particular the number and histotype present according to the type of tumor and pathological stage. The goal is to effectively improve the immune system's response to the tumor.

Immunotherapy has revolutionized the management of metastatic melanoma that, as in others tumors, uses your body's own immune system to help fight cancer. Dacarbazine and high-dose IL-2 were the only FDA-approved drugs available for metastatic melanoma [5, 6]. In 2011 the use of vemurafenib and ipilimumab significantly changed the approach to the disease, these drugs increased the survival rates of patients and laid foundation for further research in targeted therapy and immunotherapy of melanoma respectively. The success of ipilimumab created the way for the next class of monoclonal

antibodies targeting programmed cell death protein 1 (PD-1) receptors on immune effector cells such as T cells and NK cells [7]. In the following months, their use was approved for other major cancer types such as non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), classic Hodgkin's lymphoma, and head and neck squamous cell carcinoma (HNSCC) [8–10].

Current research focuses on identifying new immune checkpoints to potentially use their inhibitors alone or in combination with current therapies such as T-cell immunoglobulin and ITIM domain (TIGIT), T-cell immunoglobulin-3 (TIM-3), and lymphocyte activation gene 3 (LAG-3) and developed monoclonal antibodies against the receptors; the antibodies are in clinical testing and are in advanced stages of approval (figure 1).

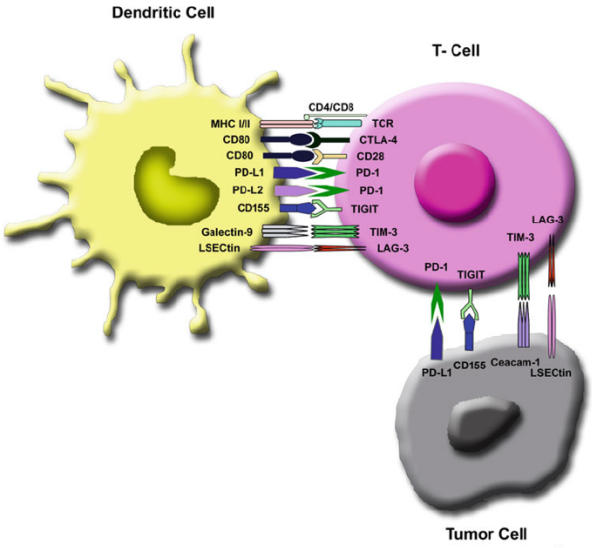

Figure.1: regulation of T cell activation. The outcome of T-cell priming in lymph node depends on the degree of TCR activation and on the levels of co-stimulatory/checkpoint receptor expression on T cells

1.0 Immonocheckpoints

1.1 Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4)

Structure and expression

CTLA-4 was discovered in 1987 by Brunet et al., and described as a 223-amino acid protein belonging to immunoglobulin (Ig) super family (Table 1). The protein was mainly found to be expressed in activated lymphocytes and was co-induced with T-cell mediated cytotoxicity [11]. Located on chromosome 2q33, human CTLA-4 gene encodes a type 1 transmembrane glycoprotein belonging to Ig super family that is composed of four domains including a single peptide, an extracellular ligand-binding domain, a transmembrane domain, and a short cytoplasmic tail [12, 13]. CTLA-4 expression is minimal in resting T cells; it is induced at both mRNA and protein level in response to TCR

activation, requires entry into cell cycle and peak expression is seen at 24–48 h post TCR stimulation [14, 15]. As listed in Table 5, CTLA-4 is generally expressed on activated T cells, however unlike other effector cells, regulatory T cells or TRegs constitutively express CTLA-4 owing to their high expression of forkhead transcription factor FoxP3, a known regulator of CTLA-4 expression [16].

Role in Immune Response

CTLA-4 binds to B7 ligands (B7-1 and B7-2) on APCs. Due to high degree of shape complementarity found in the binding interface of CTLA-4 and B7 ligands, the two are packed in a periodic arrangement in the crystal lattice where bivalent CTLA-4 homodimers bridge the bivalent B7-1 homodimers, forming stable signaling complexes at the T-cell surface [17]. During T-cell activation, CTLA-4 binds to B7 ligands with higher affinity and at a much lower surface density compared to CD28 and suppresses the activation by competing with CD28 for binding with B7 ligands expressed on APCs. Blockade of CD28-B7 ligand interaction prevents the T cell from receiving the second activation signal and induces anergy in T cells (Fig. 2) [18]. CTLA-4 sequesters B7-ligands, followed by trans-endocytosis and degradation of endocytosed ligands resulting in significant depletion of the B7 ligands from the surface of APCs and induction of a tolerogenic phenotype in APCs [19] (table 1) and (figure 2).

Target for Cancer Immunotherapy

The in vivo administration of anti-CTLA-4 antibodies was demonstrated the potential of CTLA-4 as a target for cancer immunotherapy. The study showed that mice treated with anti-CTLA-4 antibodies developed immune memory against tumors and resisted secondary exposure to tumor cells [20]. Three antibodies, ipilimumab (MDX-010; Yervoy™, Bristol-Myers Squibb), tremelimumab (CP-642206; formerly known as ticilimumab), and CP-642570 (parental antibody of tremelimumab) entered clinical trials. CP-642570 administration reportedly caused treatment-related thrombocytopenia and development was stopped [7]. Both ipilimumab and tremelimumab are fully human monoclonal antibodies but differ in their IgG backbone and their ability to initiate effector response. While ipilimumab is IgG1 monoclonal antibody with full effector functions (Table 3), tremelimumab is a IgG2 monoclonal antibody with no effector functions. Ipilimumab is approved for unresectable metastatic melanoma as well as adjuvant to surgery for “highrisk” melanoma. Tremelimumab is not yet approved for the treatment of cancer, but is in advanced stages of clinical development [7, 21] (table 2).

Receptor (synonyms)	Ligands	Cells expressing receptor	Cells expressing ligands
CTLA-4 (CD152)	CD80 and CD86	Effector T cells and TRegs	APCs
PD-1 (PDCD1 and CD279)	PD-L1 and PD-L2	Effector T cells, TRegs, NK cells, and macrophages	APCs, hematopoietic and nonhematopoietic cells and tumor cells
TIGIT (WUCAM)	PVR/CD155 and CD112	Effector T cells, memory T cells, TRegs, NK cells, and NKT cells	APCs, fibroblasts, endothelial cells, and tumor cells
TIM-3 (HAVCR2)	Ceacam-1, Galectin-9, HMGB-1, and phosphatidyl serine	Effector T cells, TRegs, DCs, NK cells, and monocytes	APCs and tumor cells
LAG-3 (CD223)	MHC II class LSECtin and galectin-3	Effector T cells, TRegs, and NK cells	APCs Liver cells and tumor cells

Table 1: commonly targeted checkpoints for the treatment of cancer

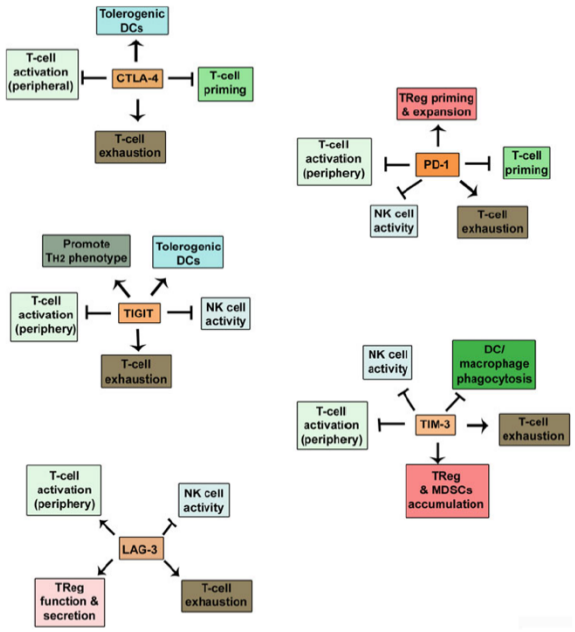

Figure 2: Effects of checkpoint inhibition on immune response. The distinct role of checkpoint receptors CTLA-4, PD-1, TIGIT, TIM-3 AND LAG-3 in regulation of T-cell responses are shown in the figure

1.2 Programmed Cell Death Protein 1 (PD-1)

Structure and Expression

PD-1 (called also PDCD1 and CD279) was first described by Honjo is a cell surface receptor expressed commonly on T cells [22]. Human PD-1 gene is located on chromosome 2q37.3; it encodes a type I transmembrane protein belonging to Ig super family (Table 1). PD-1 is composed of 288 amino acids with an extracellular N-terminal IgV-like domain, a transmembrane domain, and a cytoplasmic tail [23]. The extracellular domain of PD-1 is partly similar to CTLA-4 with 21–33% sequence homology, but it lacks the extracellular cysteine residue required for covalent dimerization and exists as a monomer on the cell surface as well as in solution, unlike CTLA-4, which is a dimer. Its cytoplasmic domain consists of two tyrosine residues including immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM). PD-1 expression is not detected on naıve T cells, whereas B cells have basal levels of PD-1 expression. Activation of TCR/BCR leads to induction in PD-1 expression and/or recruitment of PD-1 to the surface. In addition to activated Tcells and B cells, PD-1 expression is also seen on TRegs, natural killer T cells (NKT), natural killer (NK) cells, activated monocytes and on myeloid DCs (Table 1). Two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), have been identified to bind to PD-1 receptors. PD-L1 is the widely expressed ligand for PD-1; it is expressed on T cells, B cells, macrophages, DCs as well as non-hematopoietic cell types such as vascular endothelial cells, fibroblastic reticular cells, epithelia, pancreatic islet cells, astrocytes, and neurons. PD-L1 expression is also seen in cells at sites of immune privilege including trophoblasts in the placenta and retinal pigment epithelial cells and neurons in the eye. The second ligand for PD-1, PD-L2 is not as widely expressed as PD-L1 and is mainly seen on activated macrophages and DCs [24–26]. However, PD-L2 reportedly has higher binding affinity to PD-L1 and has ~three-fold lower Kd value compared to PD-L1 [23].

Role in Immune Response

PD-1 inhibit cell proliferation, cytokine secretion, and cytotoxic ability [27]. In addition, PD-1 signaling was also found to enhance FoxP3 expression and regulate the differentiation of induced TReg (iTReg) cells in murine models [28]. Like CTLA- 4, PD-1 receptors are critical regulators of immune response. The main purpose of PD-1: PD-L pathway is to control the extent of immune cell activation and prevent the damage to healthy neighboring tissues. PD-L1 expression is induced on the surface of the normal tissues in response to IFN-γ as a protective mechanism from T-cell-mediated damage. Tumor cells utilize this normal physiological mechanism and develop resistance to anti-tumor immune response by expressing PD-L1 [12]. Additionally, under conditions such as chronic

infections and tumors, the presence of increased levels of inflammatory cytokines and antigens results in T-cell exhaustion and dysfunction characterized by increased expression of PD-1 [29]. (figure 2)

Target for Cancer Immunotherapy

The PD-1: PD-L pathway plays an important roules for treatment of cancer. High level of expression of PD-1 can be seen by TILs and exhausted T cells, and PD-L1 in several cancer types including bladder, lung, colon, breast, kidney, ovary, cervix, melanoma, glioblastoma, multiple myeloma, and T-cell lymphoma. Targeting PD-1/PD-L1 to enhance anti-tumor immune response proved to be the most successful strategy to date; three antibodies including pembrolizumab (MK-3475; formerly known as lambrolizumab; anti-PD-1; Merck), nivolumab (BMS96558; anti-PD-1; Bristol-Myers Squibb), Atezolizumab (anti-PD-L1; Genentech/ Roche), Avelumab (anti-PD-L1; Pfizer), and Durvalumab (anti- PD-L1, AstraZeneca) are approved by US FDA for the treatment of different types of cancer [30]. Pembrolizumab and nivolumab were approved for the treatment of metastatic melanoma in 2014. Anti-PD-L1 antibodies, atezolizumab, avelumab, and durvalumab are yet to be approved for the treatment of melanoma. Atezolizumab is a humanized antibody and Avelumab and Durvalumab are fully human antibodies [31–34] (table 3).

Drug	Origin	IgG sub-type	Molecular weight (kDa)	Kd for target molecule	Affinity to activating FcγR	ADCC	Reference
Ipilimumab	Human	IgG1	148	5.24 nM	High	High	[46]
Nivolumamb	Human	IgG4	146	3.06 nM	Low	Low	[62, 63, 109]
Pembrolizumab	Humanized	IgG4	149	29 pM	Low-none	None	[62, 110]
Atezolizumab	Humanized	IgG1	145	0.433 nM	None (FcγR-binding region deleted)	None	[62]
Avelumab	Human	IgG1	147	0.667 nM	High	High	[64]
Durvalumab	Human	IgG1	146	0.0467 nM	None (FcγR-binding region deleted)	None	[64]

Table 3: details of origin, IgG subtype, Fcg binding, binding affinity to receptor, ADCC for monoclonal antibodies approved for treatment of cancer

1.3 T-Cell Immunoglobulin and ITIM Domain (TIGIT)

Structure and Expression

TIGIT was identified in 2008 on Tregs and memory T cells and it is a recently discovered inhibitory [35]. TIGIT gene is located on chromosome 3q13.31; the encoded 244-amino acid protein consists of single extracellular immunoglobulin domain, a type 1 transmembrane region and a single intracellular ITIM domain [35]. TIGIT is mainly expressed on resting CD4+CD25hi TReg cells, memory T cells, activated T cells, NKT cells, and NK cells, but it is not detected on naı ve CD4+ T cells. TIGIT expression is induced at mRNA levels upon activation of naıve CD4+ T cells, memory T cells and TRegs [35–37]. TIGIT expression has been reported to be a characteristic marker for exhausted CD8+ Tcells and TRegs in the tumor microenvironment and also in viral infections [38–40].

Role in Immune Response

TIGIT mainly acts by competing with the co-stimulatory receptors CD226 and CD96 expressed on T cells for PVR and PVRL2 ligands (also known as CD112 and nectin 2) (Table 1). TIGIT binds to PVR ligands with greater affinity and outcompetes CD226 and the pathway resembles the CD28, CTLA-4, and B7 ligand axis. TIGIT-PVR interaction inhibits the activation, proliferation, and differentiation of T cells. In addition to inhibition of proliferation, TIGIT engagement also activates the survival pathways and ensures the survival of inhibited T cells for long time. On the other hand, interaction of PVR on DCs with TIGIT skews the cytokine secretion profile of DCs with decreased IL-12 production and increased IL-10 production, thereby inducing a tolerogenic phenotype in DCs. Activation of TIGIT on NK cells results in decreased IFN-γ production, cytotoxic granule polarization, and NK cell cytotoxicity [41]. Finally, TIGIT engagement on TRegs results in shifting of the cytokine balance and promotes a Th2 phenotype while suppressing Th1 or Th17-phenotype [39].

Target for Cancer Immunotherapy

Upregulation of PVR and PVRL2 on tumor cell surface was identified even before the discovery of TIGIT. Expression of PVR and PVRL2 was detected on tumors from epithelial origin such as non-small cell lung cancer, colon cancer, and metastatic neuroblastoma as well as hematopoietic origin such as myeloid leukemia [81–83]. The development of anti-TIGIT antibodies for the treatment of cancer is currently in clinical trials. [10].

1.4 T-Cell Immunoglobulin-3 (TIM-3)

Structure and Expression

TIM-3 was identified by Kuchroo and associates in 2002, as a cell surface receptor expressed on differentiated CD4+ Th1 cells. Human TIM-3 gene is located on the chromosome 5q33.3 and its mouse counterpart is located on chromosome 11. Murine TIM-3 protein was reported as a type I membrane protein of 281 amino acids, with an extracellular domain consisting of immunoglobulin variable-region-like domain followed by a mucin-like domain and cytoplasmic region containing a tyrosine phosphorylation motif. Human TIM-3 protein is made of 301 amino acids and the sequence has 63% similarity to that of mouse Tim-3 and the cytoplasmic region shares 77% identity including the tyrosine phosphorylation motif [43]. Apart from Th1 cells, TIM-3 is also expressed on activated $CD8^+$ T cells, TRegs, and other innate immune cells such as DCs, NK cells, as well as monocytes [41, 43]. Three years after the identification of TIM-3, galectin- 9, a C-type lectin, expressed on APCs was identified as the ligand for TIM-3 [44]. Later other ligands including Ceacam-1, HMGB- 1, and phosphatidyl serine were identified to interact with TIM-3 [41]. Unlike the ligands for other checkpoints such as TIGIT, the ligands for TIM-3 have a broad expression profile and are expressed on a variety of cell types including cancer cells [43].

Role in Immune Response

TIM-3 suppress the immune responses of Th1 cells, inducing Th1 cell-apoptosis and CD8+ T-cell activity [41, 44, 45]. TIM-3 has also been implicated in accumulation of immunosuppressor cell types such as TRegs and MDSCs (Myeloid-derived suppressor cell), promotion of T-cell exhaustion, and the maintenance of peripheral tolerance by DCs and macrophages [46, 47]. Role of TIM-3 in development of tolerance was further shown through studies in Tim-3/mice, which were found to be refractory to induction of antigen-specific tolerance [47, 48].

Target for Cancer Immunotherapy

The potential for TIM-3-based cancer immunotherapy was illustrated by studies in tumor samples from patients with advanced melanoma, non-small cell lung cancer (NSCLC), and follicular B cell non-Hodgkin lymphoma, which found TIM-3 expression on exhausted T cells that correlated positively with cancer severity and prognosis [41]. Anti-TIM-3 antibodies are not yet approved for the treatment of cancer and are currently in clinical trials. [10].

1.5 Lymphocyte Activation Gene 3 (LAG-3)

Structure and Expression

LAG-3 (also known as CD223) was detected, three decades ago, on activated T cells as well as NK cells [49]. LAG-3 expression is activated on CD4+ T cells, CD8+ T cells, and NK cells and also on both natural and induced TRegs [41]. LAG-3 gene is located on chromosome 12p13.32 adjacent to the CD4 gene and the protein shares 20% similarity in amino-acid sequence to that of CD4 [41, 50]. LAG-3 protein is composed of 498-amino acids with four extracellular IgSF domains, including a V-SET domain, which consists of an extra loop in the middle of the domain and an unusual intrachain disulfide bridge and three C2-SET domains [49]. The cytoplasmic tail of LAG-3 has three regions: a serine phosphorylation site containing region, a KIEELE motif containing region, and a glutamic acid-proline repeat-containing region [51]. Owing to its structural resemblance to CD4 co-receptor, LAG-3 binds to MHC class II molecules with a higher affinity than CD4 [41]. The presence of additional ligands for LAG-3 was speculated from the fact that LAG-3 regulated the functions of CD8+ T cells and NK cells, which do not interact with MHC class II molecules. LSECtin, a member of DC-SIGN family of molecules, expressed in liver as well as several tumor subtypes has been suggested as a LAG-3 ligand in CD8+ T cells and NK cells [52].

Role in Immune Response

LAG-3 inhibits the effector cell responses and promotes suppressive activity of TRegs. The receptor is thought to mainly function in coordination with other checkpoints such as PD-1 and promote the development of tolerance [41]. Further, LAG-3 also plays a key role in suppressor functions and the IL-10 secretion capacity of Tregs (29). However, the inhibitory effects of LAG-3 are considered to be comparatively mild, as the LAG-3-deficient mice do not develop autoimmune disorders directly. Only when LAG-3 deficiency and administration of anti-LAG-3 antibodies are tested in mice with a permissive genetic background such as NOD mice, its negative effects are demonstrated as acceleration of type I diabetes development in the mice [53]. Similarly, increased susceptibility to Hg-induced autoimmunity is seen in LAG-3-deficient mice from B6.SJL background [54].

Target for Cancer Immunotherapy

Increased expression of LAG-3 along with other checkpoints such as PD-1 is a characteristic feature of T-cell exhaustion and studies in tumor samples from lung, ovarian, and colorectal cancer patients showed a positive correlation between LAG-3 expression on tumor infiltrating lymphocytes and tumor progression [55–59]. Actually many pharmaceutical companies have shown interest in

targeting LAG-3 for treatment of cancer and are separately developing monoclonal antibodies against LAG-3 receptors [10].

1.6 Tumor-infiltrating lymphocytes (TILs)

The importance of the immune response in the clinical outcome was also correlated with increased number of tumor-infiltrating lymphocytes (TILs) in melanoma and in several other tumors [60-62]. The predominant types of lymphocytes within TILs are NK, T cells, such as CD4+ and CD8+, T-regs but recently γδ T lymphocytes have been detected too [63], [64]. Many studies underline the role of γδ T lymphocytes in the anti-tumor surveillance [65] with a marked cytotoxic effect towards tumor cells. A study of Cordova et al. demonstrated that γδ T lymphocytes are well represented amongst TILs in cutaneous melanomas; they produce proinflammatory cytokines such as TNF-α and IFN-γ and exert a strong cytotoxic activity against melanoma cells in vitro [66].

There is a strong relationship between the number of γδ T lymphocytes and the stage of disease. In particular, it has been shown that a high number of infiltrating T γδ is associated with early stages of the disease and favorable prognosis, while in the advanced stages of the disease a reduced number of T γδ lymphocytes is observed. This suggests the importance of cytotoxic activity of these immune cells and their role as immune surveillance.

In a future the concentration of infiltrating T γδ cell could be use as a prognostic marker in melanoma [67].

The number of circulating γδ T cell assessed at the time of diagnosis doesn't undersure significant variations between the pre and post operative values, and doesn't correlation was found between these values and patient outcomes. However, a decrease in circulating γδ T lymphocytes has invariably been observed at the time of disease progression [68].

1.7 γδ T lymphocytes

γδ T lymphocytes are important effector cells of the immune system that may play a role in the anti-tumor surveillance in the periphery [69]. Human γδ T cells can be divided into two main populations based upon d chain expression [70]: γδ T cells expressing the Vd1 chain are most often found in mucosal tissues, where they are involved in maintaining epithelial tissue integrity in the face of damage, infection, or tumor transformation, while γδ T cells expressing the Vd2 chain predominate in the peripheral blood and secondary lymphoid organs [71]. While the ligand(s) recognized by Vδ 1 $^{+}$ cells remain unknown, Vδ2 $^{+}$ cells recognize non peptidic antigens by a MHC unrestricted mechanism, an important feature which distinguishes them from ab T cells [70]. Specifically, Vδ2 T cells recognize phosphoantigens that are produced through the isoprenoid biosynthesis pathways [72–

74]. Phosphoantigens are not stimulatory at physiologic levels, but transformed and infected cells, produce increased levels of metabolic intermediates that are able to activate Vδ2 T cells [75–76]. Accordingly, Vδ2 T cells can also be activated, through an indirect mechanism, by aminobisphosphonates, a class of drugs used to treat certain bone diseases, that inhibit farnesyl pyrophosphate synthase, and cause accumulation of endogenous upstream metabolites such as isopentenylpyrophosphate (IPP) [77]. γδ T cells may indirectly contribute to the immune defense against cancer cells, by producing cytokines typical of Th1, Th2 or Th17 cells [78–80], or cross-talking with dendritic cells [81], macrophages [82] and B cells [83, 84]. Additionally, cd T cells perform cytotoxic activity toward cancer cells, which is mediated in much the same manner as for ab T cells, through perforin/ granzyme, Fas/FasL, TNF/TNF-R and TRAIL-TRAIL-R pathways [71]. Moreover, the localization of γδ T cells within epithelia suggests that these cells may contribute to the surveillance of malignancies, including melanoma [69].

Literature review revealed that strong preclinical and limited clinical evidence supports the role of $\gamma\delta$ T cells in immunotherapeutic strategies for advanced melanoma. Clinical data on $\gamma\delta$–based immunotherapy in melanoma is limited, with only 3 clinical studies on a total of 24 patients evaluating different immunotherapeutic protocols for advanced melanoma [85–87] and most studies being performed on melanoma cell lines. Investigated strategies include in vivo stimulation, or an ex vivo adoptive therapy or vaccination. Some authors propose direct stimulation through nitrogen-containing bisphosphonate ± IL-2, IL-36, IL-15, or intralesional-BCG, while others proposed a vaccination strategy through dendritic cells or dendritic cell–derived exosomes; adoptive cell therapeutic approach includes RNAtransfection of $\gamma\delta$ T cells with a chimeric antigen receptor or an $\alpha\beta$ T cell receptor (Figure 3). Future prospective on the development of $\gamma\delta$-based immunotherapy for melanoma will be discussed on the basis of selected studies and other relevant literature—mainly descriptive—on $\gamma\delta$ T cells and melanoma. Furthermore, all $\gamma\delta$ T cells share characteristics of both adaptive and innate immunity, posing tricking questions still debated by immunologists about their belonging to one branch or the other of immunity. Nevertheless, new discoveries are regularly published which describe features of $\gamma\delta$ T cells belonging to both branches of immune system [88, 89]. Notably $\gamma\delta$ T cells have also the perforin, and there are some hints about phagocytosis too [90]. Furthermore, they are activated in a MHC-unrestricted way, whose expression is typically lost in cancer cells that try to escape to immune-surveillance. Then, their killing ability with the freedom from the MHC system makes $\gamma\delta$ T cells a very interesting candidate for immunotherapies. Several studies have shown that $\gamma\delta$ T cells are an important component of tumor-infiltrating lymphocytes and have a positive prognostic role in patients affected by different types of cancer; a recent analysis of ∼18,000 transcriptomes from 39 human tumors identified tumor-infiltrating $\gamma\delta$ T cells as the most

significant favorable cancer-wide prognostic signature [91–93]. In melanomas, $\gamma\delta$ T cells, expressing either Vγ9/Vδ2 or Vδ1 TCRs, have been found within tumor infiltrating lymphocytes (TILs) [94–96]. Previous studies from our group showed that $\gamma\delta$ T cells are the most represented subset of melanoma-infiltrating lymphocytes, displaying an activated phenotype and a strong in vitro cytotoxic activity toward melanoma cells. We showed a correlation among $\gamma\delta$ TILs and melanoma stage, and an inverse correlation of peripheral $\gamma\delta$ T with mortality and relapse rates in metastatic melanoma [67].Wistuba-Hamprecht K. et al. also showed an interesting correlation between V$\delta2^+$ high frequencies and V$\delta1^+$ low frequencies with favorable overall survival of melanoma patients treated with ipilimumab [97].

While $\gamma\delta$-based immunotherapy is already a clinical reality for other solid tumors, research on $\gamma\delta$ T cells and melanoma is mostly at a preclinical descriptive stage, and their immunotherapeutic potential has not been deeply investigated. Γδ immunotherapeutic strategies have already proved their efficacy in several preclinical and clinical studies on other solid and nonsolid tumors. (yd t cell-based immunotherapy in melanoma [98].

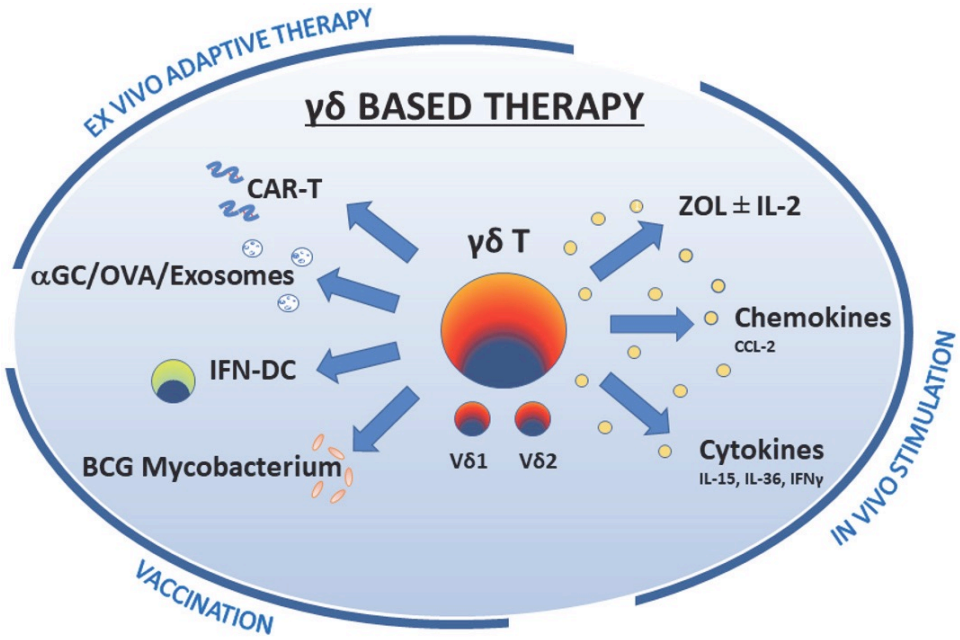

Figure 3: Schematic representation of the main preclinical and clinical research lines on $\gamma\delta$ T cell-based immunotherapy in melanoma.

CHAPTER 2

Materials and Methods

2.1 Patients

The study was approved by the Ethical Committee of the University Hospital, Palermo, where the patients were recruited. The study was performed in accordance to the principles of the Helsinki declaration and those of the "Good Clinical Practices", and all individuals gave written informed consent to participate. 57 adult patients were enrolled in our study with clinical suspicion of melanoma. The patients were treated surgically at the Plastic Surgery Department of the University Hospital in Palermo, from 2018 to 2021 and all were followed post operatively at the oncological Department for the development of clinical outcomes such as metastasis and death. The median age was 51 (21-88 years). A blood drawing (in a dry test tube) was taken before the surgical excision and a fragment (about 1 mm) of perilesional skin was taken before the tumoral excision. The histological diagnosis of the samples was classified in: 9 melanoma in situ, 14 melanoma pt1a, 2 melanoma pt1b, 3 melanoma pt2a, 2 melanoma pt2b, 2 melanoma pt3b, 1 lentigo maligna melanoma pt1a, 6 metastases from melanoma (of which 1 satellites), 3 basal cell carcinoma, 1 squamous cell carcinoma in situ, 5 congenital nevi, 4 dysplastic nevi, 1 reed nevus, 1 lentigo simplex.

2.2 Tissues dissociation and Flow cytometry

All bioptical samples were collected in phosphate buffered saline (PBS) and processed immediately. They first subjected to mechanical dissociation in a petri dish, cutting in small pieces with surgical scalpel. Then, for enzymatic digestion, pieces were transferred into a falcon tube containing RPMI 1640 culture media with following enzyme cocktail: collagenase IV (Life-technologies 1.5 mg/ml), hyaluronidase (Sigma 20 µg/ml) and DNAse (Sigma 50 µg/ml). Biopsies were incubated at 37°C (CO_2 5%) for 2 hours, after which digested tissues were sieved through 40□m cell strainer, in order

to remove the unwanted debris and recover TILs. After, the filtrate was centrifuged at 1600 rpm for 5 min and the final pellet was resuspended in flow cytometry buffer. The cells were stained with following anti-human monoclonal antibodies (Miltenyi Biotec): TIM3 FITC (F38-2E2), CD223 (LAG3) VioBright B515 (REA351), CD279 (PD1) APC (PD1.3.1.3), TIGIT APC (REA1004), CD45 APC-Vio 770 (REA747), CD3 PerCP-Vio 700 (REA613), TCR□□□PE (11F2).

The cells were analyzed by FACS Canto II (BD Biosciences) on FACS Diva software (BD Biosciences). Viable lymphocytes were gated by forward and side scatter, and analysis was performed for each sample by using FlowJo (Tree Star, version 10.5.3).

2.3 Immunofluorescence staining

Immunohistochemical analysis was performed on 5 mm-thick paraffin-embedded sections of melanoma samples. Slides were heated for antigen retrieval in 10 mM sodium citrate (pH 6.0). After rinsing in dH_2O, inhibition of endogenous peroxidase was performed with 3% H_2O_2 (5 min). After two washes in TBS, slides were incubated with 10% human serum for 20 min to clock unspecific staining. Following elimination of excess serum, sections were then exposed to specific antibodies against anti-human TCR □□ (purified mouse anti-human cd TCR, IgG1, BD Pharmingen), immunocheckpoint molecules (PD-1, CTL4, TIGIT, LAG 3, TIM 3) and their respective ligands (PD-L1, CD86, CD112, GAL3 and GAL9) overnight at 4°C. Then sections were washed in TBS and incubated with secondary antibody for 60 min at room temperature. The nuclei were counterstained with Hoechst for 10 min at RT and the immunofluorescence analysis was conducted with confocal microscope.

2.4 Bioinformatics and statistical analysis

GEPIA 2 (Gene Expression Profiling Interactive Analysis) web tool (http://gepia2.cancer-pku.cn) analyze the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects. We conducted gene expression correlation analysis between immune checkpoint receptor genes (PDCD1, TIM3, LAG3) and TCR γδ gene expression signature (TRDC, TRGC1 and TRGC2 genes). The correlation coefficient was determined by Spearman's method.

The significance of difference between peritumoral and circulating data were determined by Student's t test.

CHAPTER 3

Results

3.1 Analysis of histological samples

The total number of samples collected, with strong clinical suspicion of melanoma, during the research project was 54 samples. The histological reports were: 9 melanomas in situ, 14 melanomas pt1a, 2 melanomas pt1b, 3 melanomas pt2a, 2 melanomas pt2b, 2 melanomas pt3b, 1 lentigo maligna melanoma pt1a, 6 metastases from melanoma (of which 1 satellitosis). 15 samples did not confirm the diagnosis of suspicion: 3 basal cell carcinomas, 1 squamous cell carcinoma in situ, 5 congenital nevi, 4 dysplastic nevi, 1 reed nevus, 1 lentigo simplex. The analysis of the histological examination reports has showed that in 75% of cases the diagnosis of suspected melanoma was confirmed, demonstrating the clinical feasibility of our study protocol. The thin melanomas were the most frequent 45.8% (in situ and pt1a) with a greater incidence on the trunk (this also applies to the thickness), finally comparing the incidence of the diagnosis in relation to 'age we have noted that the most recorded cases were between 40 and 80 years (confirming the data collected in the literature) (figure 4).

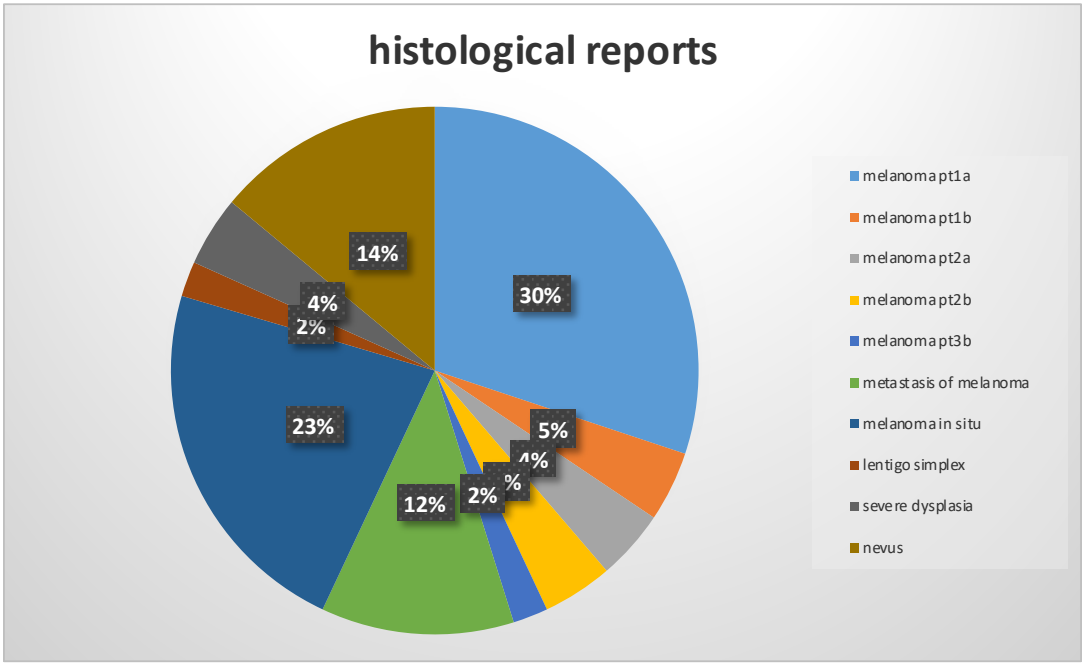

Figure 4: Histological reports

3.2 Analysis of the digested samples

In 33% of cases, a suitable cell quantity was obtained for subsequent analyzes, in 30% of cases the samples were frozen because the size of the peritumoral fragment was considered insufficient, and finally in 37% of the digested samples, a sufficient cell quantity was not obtained for subsequent analyzes. In detail, in 33% of the samples for which it was possible to carry out the subsequent analyzes, 68% were melanomas while 32% were the other histotypes (basal cell carcinoma, lentigo, congenital and dysplastic nevi) (Figure 5).

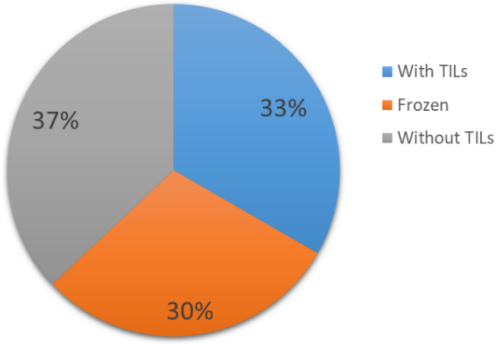

Figure 5: analysis of the digested samples

3.3 Analysis of the $\gamma\delta$ T lymphocytes

After digestion of the samples, a subpopulation of T$\gamma\delta$ lymphocytes (Vγ9 / Vδ2) was isolated in the peri-tumor fragments (infiltrating) and in the peripheral blood (circulating) of the same patient. The percentage of this population was slightly higher in peripheral blood than in peri-tumor tissue even if wasn't statistical significant (Figure 6).

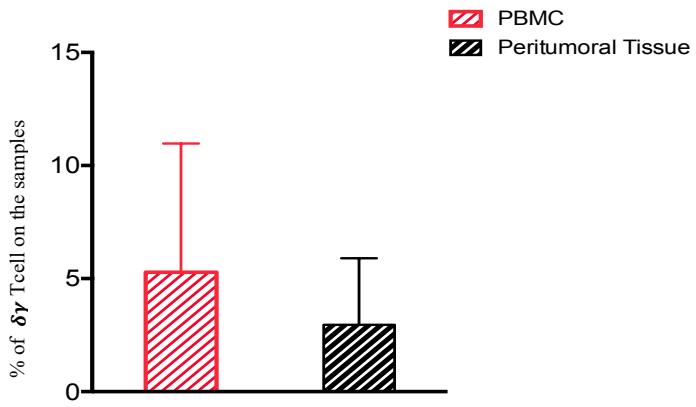

Figure 6: Analysis of T $\gamma\delta$ lymphocytes in PBMCs and TILs

The analysis concerned the most representative histotype (nevus, melanoma and metastasis of melanoma) excluding basal cell carcinomas and lentigos and others.

The percentage of infiltrating $\gamma\delta$ T cell was higher in primary melanoma samples, slightly lower in melanoma's metastases and lower still in nevi (Figure 7).

In the peripheral blood, the percentage of circulating $\gamma\delta$ T cell was greater in metastases from melanoma than in primary melanomas and nevi.

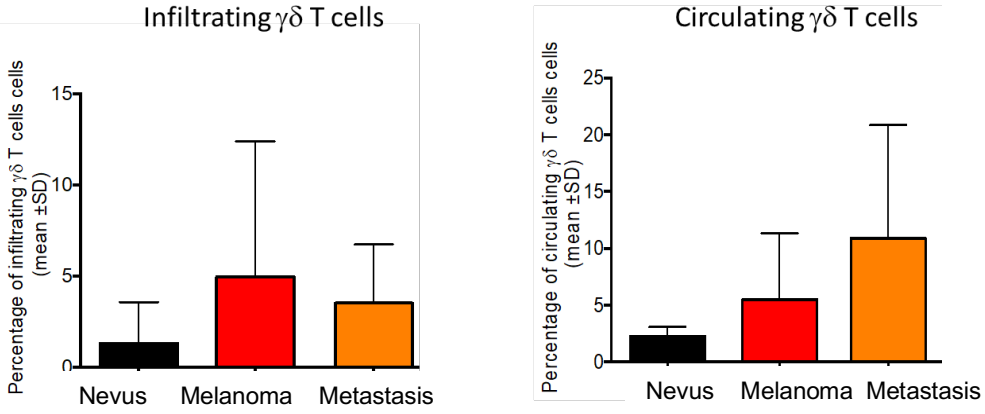

Figure 7: Analysis of infiltrating and circulating T lymphocytes

3.4 Analysis of immunocheckpoints expression

The expression analysis of the immunocheckpoint molecules, PD1, TIGIT, LAG3 and TIM3 was carried out on the isolated cells and it can be seen that their levels of expression in infiltrating $\gamma\delta$ rather than circulating ones are clearly higher (Figure 8).

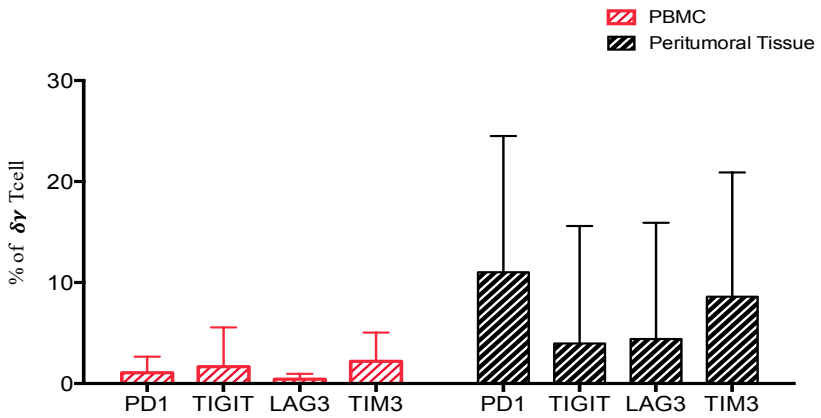

Figure 8: Analysis of the expression of new immunocheckpoints on PBMCs and TILs

In particular the same analysis was conducted on main tumor histotype (nevi, melanoma and metastasis) in circulating and infiltrating $\gamma\delta$ T cells. In the group of infiltrating samples, it wasn't able to isolate $\gamma\delta$ T cells from nevi samples. The expression of LAG3 appears to be the same in the circulating $\gamma\delta$ T cells of nevi and primary melanoma samples, it is down-regulated in metastatic melanoma samples; whereas in infiltrating $\gamma\delta$ T cells the data were opposite: higher in metastatic than in primary melanoma. PD1 expression in circulating $\gamma\delta$ T cells was higher in nevi and primary melanoma and lower in metastatic; while in infiltrating cells, it remains constant among the samples analyzed. The expression levels of TIM3 in circulating blood were very low in nevi and increase in metastatic and primary melanoma; while in the infiltrating cells, they are almost kept constant and with higher values. Finally, TIGIT was up-regulated in circulating $\gamma\delta$ T cells of primary melanomas compared to metastatic melanoma and in nevi. On the other hand, the expression levels of TIGIT decrease drastically in the infiltrating T cells (Figure 9).

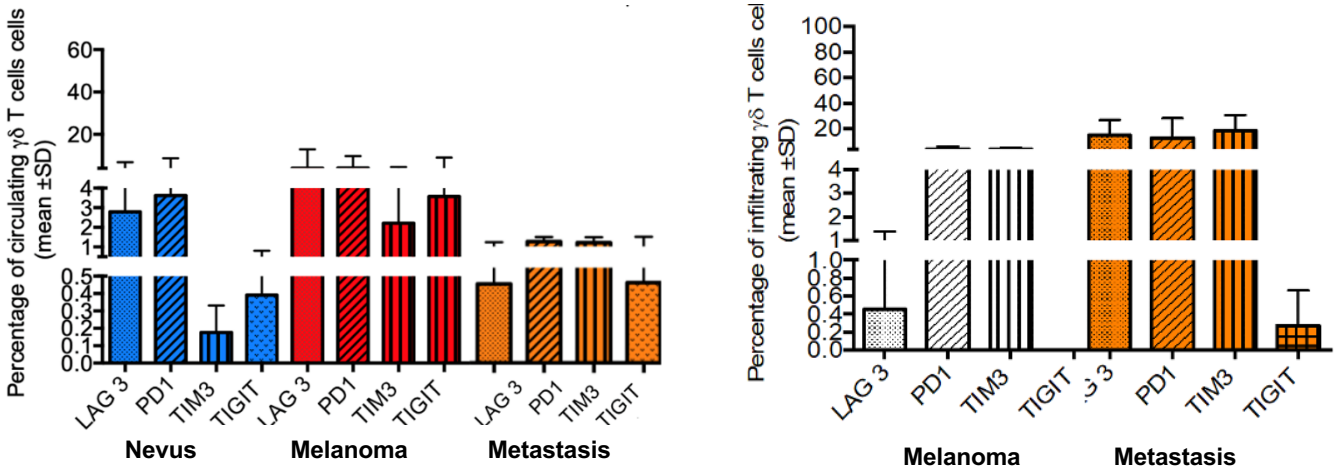

Figure 9: Analysis of expression immunocheckpoints on infiltrating and circulating T lymphocytes in the melanoma samples

3.5 Immunofluorescence investigations

Immunofluorescence investigations of the analyzed samples confirmed the expression of the PDL1, GAL3, CD112 and GAL9 (data not shown) ligands on primary melanoma tumor cells. As well as the expression of PD1, LAG3, TIGIT and TIM3 on the same sections. As well as, the expression of the TCR receptor, typical of γδ T lymphocytes, was also evident in the intratumoral area (figure 10).

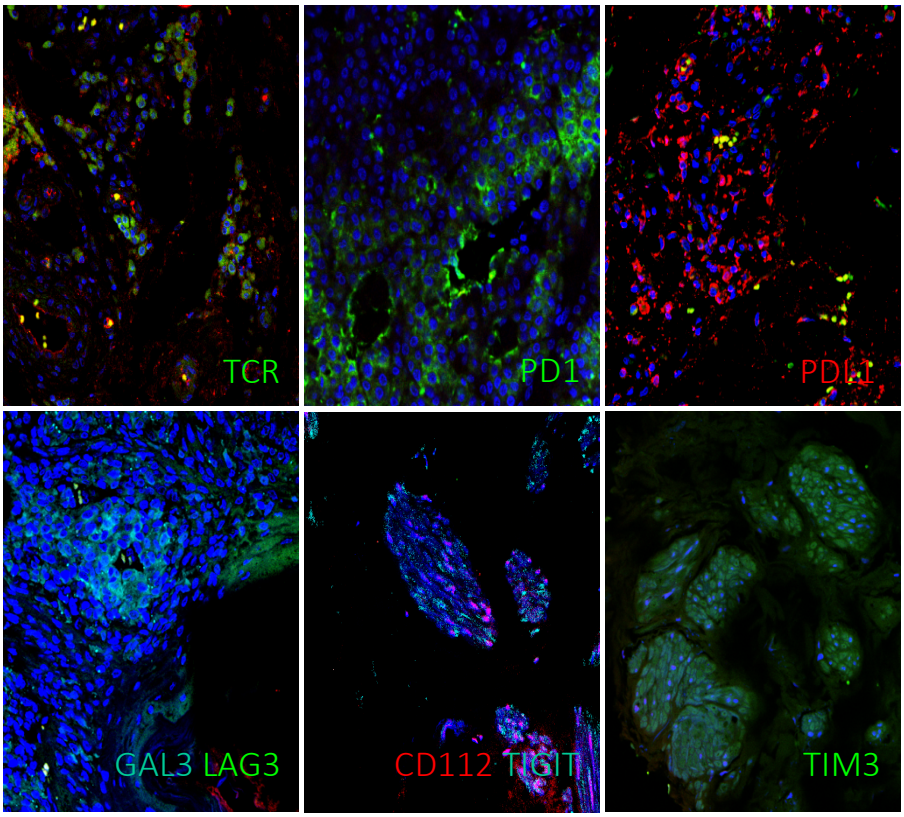

Figure 10: Representative images of TCR and immunocheckpoints receptors and ligands.

3.6 Histological correlation analysis

We performed an analysis of correlation between the histotype of our samples and the expression of γδ T cells and immunocheckpoints molecules valued by flow cytometry in circulating and infiltrating samples.

Primary melanoma:

The melanoma samples were divided according to the pathological stages into ptis, pt1a, pt1b, etc. The percentages of γδ lymphocytes, on peritumoral samples, showed a particular trend: 1.94% on ptis, 1.53% on pt1a, 8.3% on pt1b, 1.23% on pt2b, nd on pt3b and 1.3% on ptx.

On peripheral blood, the average values obtained were: 4.08% ptis, 2.41% pt1a, nd pt1b, 1.34%pt2b, nd pt3b and 3.65% ptx.

The immunocheckpoint expression's percentage was:

• on peritumoral samples, LAG3 and TIGIT were not expressed in situ stage, whereas highly detectable in pt1a (LAG3 21.86% and TIGIT 12.26%). In the advanced stages, only LAG3 was expressed in γδ lymphocytes (1.85% in pt1b, 15.8% in pt2b and 3.8 in ptx). TIGIT wasn't expressed in these others histotype. PD1 and TIM3 were expressed at high levels in the early stages (pt1s 13.45% / 6.2%, pt1a 15.21% / 26.4%) and were subsequently no longer detectable in the pt1b, pt2b and pt3b stages (only PD1 1.49% in pt1b).

• On peripheral blood samples, LAG3 and TIGIT were expressed 1.85% and 5.13% respectively in ptis, in pt1a LAG3 was not detectable (0.36%), the TIGIT's mean values was 10.41 %. As the pathological state increases, TIGIT is never present while LAG3 has variable mean values with a peak of 4.61% in pt2b. PD1 and TIM3 were well represented in ptis with values of 2.13% and 6.20%, they tend to decrease in pt1a (PD1 1.65%, TIM3 0.99%) and subsequently increased as the stage increases (PD1 8.18% and TIM3 14%).

MELANOMA						
PERITUMORAL SAMPLES n° 18						
	Ptis n° 5	Pt1a n° 8	Pt1b n° 1	Pt2b n° 2	Pt3b n° 1	Ptx n° 1
% γδ T cells	1.94	1.53	8.3	1.23	nd	1.3
% LAG3	0.2	21.86	nd	15.8	nd	3.8
% TIGIT	0	12.26	0	0	nd	0
% PD1	15.21	13.45	1.49	0	nd	6.67
% TIM3	6.2	16.4	0	0	nd	3.93
BLOOD SAMPLES n° 16						
	Ptis n° 5	Pt1a n° 9	Pt1b -	Pt2b n° 1	Pt3b -	Ptx n° 1
% γδ T cells	4.08	2.41	-	1.34	-	3.65
% LAG3	1.85	0.36	-	4.61	-	0.24
% TIGIT	5.13	10.41	-	0	-	0
% PD1	2.13	1.65	-	8.18	-	0.38
% TIM3	6.2	0.99	-	14	-	0

Metastatic melanoma:

The percentage of γδ lymphocytes was of 2.68% on peritumoral tissue samples and of 4.54% on peripheral blood samples

Instead, the analysis of immunocheckpoints expression showed very high values both on tissue samples and on peripheral blood as shown in the table.

METASTASIS n° of samples=3	Tissue (%)	PBMCs (%)
% γδ T cells	2.68	4.54
LAG3	22.58	4.42
TIGIT	3.6	5.02
PD1	7.22	7.22
TIM3	3.8	17.2

3.7 γδ T cell signature correlates with immune-checkpoints gene expression in melanoma

A bioinformatic analysis was performed on a web tool (GEPIA2.0) by comparing the expression of some immunological checkpoint receptors in primary melanoma, of which in particular PD1 (PDCD1), TIM3 and LAG3 showed a positive Spearman correlation and statistically significant with the genetic signature characteristic of γδ T lymphocytes (i.e. TRGC1 TRGC2 AND TRD, genes that code for the γδ receptor) (Figure 11).

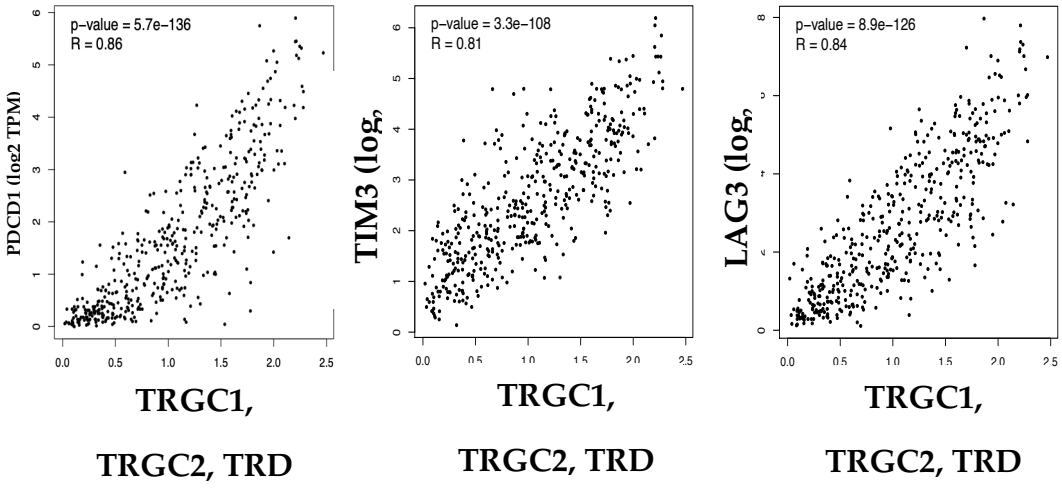

Figure 11: γδ T cell signature in melanoma

3.8 Immune-checkpoint blockade therapy modifies γδ T cell gene expression

In addition, a bioinformatic analysis of the GSE120575 dataset containing transcriptomic data of single cell RNA 16291 immune cells from 48 samples obtained from patients with melanoma treated with inhibitors of the checkpoints CTLA4, PDL1 and CTLA4 + PDL1 was performed, in order to

define the status function of γδ T lymphocytes and the impact on it of immunotherapy with immune checkpoint inhibitors (Figure 12).

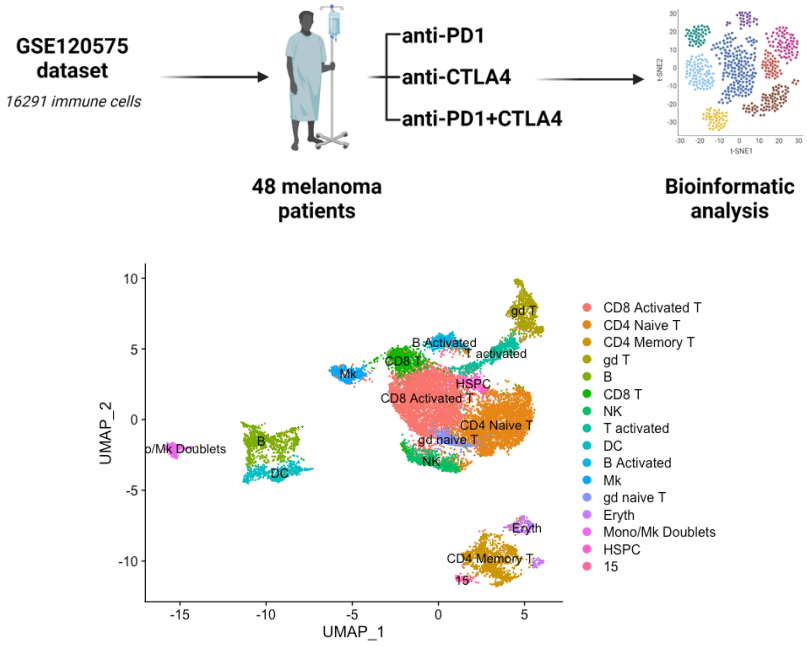

Figure 12: UMAP plot of the cellular clusters in melanoma

In order to define the functional status of γδ T cells and the impact of therapy with checkpoint inhibitors, the expression of cell cycle (A), migration (B), cytotoxicity (C) and exhaustion genes (D) was analyzed. The results showed that checkpoint inhibitor therapy induces a greater expression of the cytotoxicity genes, particularly NKG7 and PRF1. The expression of the migration genes as CXCR3, CXCR4 and CCR5 is particularly increased after therapy with checkpoint inhibitors, especially CXCR4. On the other hand, the expression of genes of various phases of the cell cycle showed an increase in the expression of CD27 and ITGAL, and a slight decrease in the expression of IL7R, SELL and CCR7, genes particularly expressed in naive T lymphocytes located in the thymus and in periphery.

Finally, we evaluated the expression of genes related to cell function depletion, HAVCR2 (TIM3), PDCD1, TOX, TIGIT and CTLA4. These results showed that immunotherapy with checkpoint inhibitors causes an increase in memory T lymphocytes, with a marked migratory phenotype and an increase in depleted γδ lymphocytes. The analysis suggests that immunotherapy with checkpoint inhibitors induces the upregulation of genes associated with the cytotoxic (NKG7 and PRFI), memory

(CD27 and ITGAL) and migratory (CXCR3, CXCR4 and CCRS) phenotype, and clonal depletion (HAVCR2, PDCD1, TOX, TIGIT and CTLA4) in γδ T lymphocytes (figure 13).

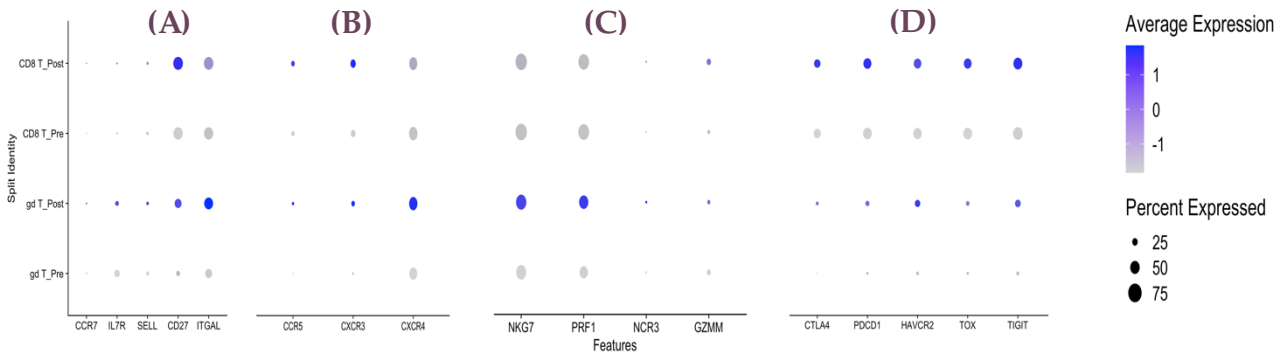

Figure 13: the functional status of γδ T cells

CHAPTER 4

Discussion

Melanoma is one of the most sensitive tumours to immune modulation and currently immunotherapy is the major challenge for melanoma patients' survival. Particularly, immune checkpoint inhibitors therapy with anti-CTLA4 and anti-PD-1 antibodies induces regression and long-term durable cancer control in nearly 50% of melanoma patients [99], moreover despite the absence of powered trial data, combined anti-CTLA4 and anti-PD1 checkpoint inhibition has the highest 5-year overall survival rate of all therapies in advanced melanoma. Several checkpoint inhibitors, such as ipilimumab, pembrolizumab, nivolumab, and others, are currently FDA approved in the treatment of several malignancies including metastatic melanoma. Populations of patients, primary nonresponders or with secondary resistance, have been the majority of cases, even in melanoma. Approximately 45%-60% of untreated metastatic melanoma patients treated with PD-1 inhibitors have an initial response, but by three years 43% of patients will have developed resistance and relapsed with fatal results. Over 80% of melanoma patients treated with CTLA-4 inhibitor monotherapy have primary resistance. Mechanisms of resistance include those inherent to the tumor microenvironment, the tumor cells themselves, and the function of the patient's native immune cells [100].

In addition to CTLA-4 and PD-1, several other immune checkpoint receptors have been identified including lymphocyte activation gene 3 (LAG-3), T-cell immunoglobulin and mucin 3 (TIM-3), V-domain Ig-containing suppressor of T-cell activation (VISTA), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) that are expressed by T-cells and negatively regulate immune responses [101].

In view of their well-known and potent MHC-unrestricted antitumoral activity against a broad variety

of tumours including melanoma, γδ T cells are considered optimal candidates for cellular immunotherapy [66]. Literature review revealed that strong preclinical and limited clinical evidence supports the role of $\gamma\delta$ T cells in immunotherapeutic strategies for advanced melanoma.

Thus, our aim was to evaluate the percentage of melanoma-infiltrating γδ T cells and the expression of immunocheckpoint molecules, in order to hypothesize a new combined immunotherapeutic approach.

In this study, we showed that γδ T cells are present among melanoma infiltrating lymphocytes, with higher percentage than healthy skin. In particular, the concentration of infiltrating T $\boldsymbol{\gamma\delta}$ lymphocytes in the peritumor tissue undergoes a reduction correlating with the stage of the disease progresses (except pt1b although it is only one sample). Data on circulating $\boldsymbol{\gamma\delta}$ T lymphocytes also decrease with increasing disease severity. This correlation must be seen as a reduction in the immune response to the tumor, and therefore a worse prognosis for the patient, that it could also have an important predictive value in response to immunotherapy.

The other extremely important aspect that we have evaluated is that relating to the expression of immunocheckpoints. As we would have expected, the overall evaluation in melanoma samples confirms that the expression of immunocheckpoints is considerably more represented in TIL's than in circulating ones. In particular, LAG3 increases from melanoma to metastasis samples.

In the literature, a recent study of Lee et al. showed that LAG-3 and TIGIT expression on tumor-infiltrating lymphocytes were significantly correlated with PD-1 expression and associated with negative prognostic factors: deeper Breslow thickness, lymph node involvement, and advanced stage of disease. High expression of LAG-3 or TIGIT was associated with worse survival and in an analysis of Breslow thickness, they have seen that both immunocheckpoint molecules have prognostic significance regardless of tumor thickness. They found that LAG-3 and TIGIT were expressed in TILs and correlated with the density of TILs. They suggested that a combination of LAG-3 or TIGIT antibodies with PD-1 antibody might have important implications for immune checkpoint therapy in advanced cutaneous melanoma [102]. According to this study, we analysed the immunocheckpoints expression of the infiltrating or circulating $\boldsymbol{\gamma\delta}$ T lymphocytes, in correlation with histological features of the samples. A detailed analysis of these data, on perilesional infiltrating $\boldsymbol{\gamma\delta}$ T lymphocytes, showed that PD-1 is more expressed in melanomas in situ, and then, as the stage increases, its value decreases from pT1a. On the other hand, the immunocheckpoints analysis, TIM3, TIGIT and above all LAG3, show a considerable increase, suggesting that the latter plays a fundamental role in the loss of control by the immune system over tumour progression. The data on the expression of the immunocheckpoints analyzed in metastases samples are indeed conflicting, although LAG 3 is again the highest of all the immunocheckpoints.

On the other hand, the results of immunocheckpoint expression on circulating $\boldsymbol{\gamma\delta}$ T lymphocytes showed highly variable values in the analysis for pathological stage, except TIGIT, whose values are high in the ptis and pt1a. While in the mestastases samples, all immunocheckpoints were more expressed.

The limit of this study is the small number of samples from which it was possible to isolate a sufficient amount of $\boldsymbol{\gamma\delta}$ T lymphocytes for cytofluorimetric analysis, in fact, in some cases we had one or two samples. Furthermore, we obtained less peritumour tissue samples from advanced melanoma than from thin melanoma (PT1s and pt1a), according to the epidemiological data of the disease.

The detection of γδ T lymphocytes classically require access to fresh tumour samples that are much difficult to obtain due to the immediate need for diagnosis. However, recent developments in bioinformatics analysis, expecially scRNA sequencing, have proved instrumental in providing us with the best level of resolution for gene expression patterns of large sets of cells and allowing finer aspects of the cells and their classification from human tissues [103]. Therefore, our bioinformatics analysis of checkpoint ligands expression in melanoma showed higher levels of LGALS3, LGALS9 and CD274 compared to healthy tissue, even though the relative expression of the latter was lower than others. Moreover, Spearman correlation analysis between immune checkpoint ligand genes and γδ T cells gene signature was significantly positive only for CD274 and LGALS9 whilst LGALS3 expression was negatively correlated.

In summary, these promising preliminary results showed that:

- $\boldsymbol{\gamma\delta}$ T lymphocytes were mostly present in peripheral blood than in peri-tumor tissue;
- immunocheckpoints molecules were more expressed in peritumoral samples and increased in metastases ones, supporting the idea that it was related to a decreased immune response to the tumor and thus a worse prognosis for the patient;
- Immunotherapy with checkpoint inhibitors brings about qualitative changes in γδ T lymphocytes and in their gene expression pattern: in particular, there was an increase in lymphocytes with cytotoxic activity, memory and migratory phenotype, and in γδ T lymphocytes with characteristic phenotype of clonal exhaustion.
- Of all the immunochekpoints analyzed, LAG3 was the most highly expressed in both primary and metastatic melanoma samples. Therefore, in the future the impact of neutralizing LAG3 with some inhibitors should be further investigated to understand the functional role of LAG3 in anti-PD1 resistant patients.

Taken together, these results provide useful information in relation to possible prognostic and therapeutic role of melanoma-infiltrating γδ T cells, and support the possibility to combine immune checkpoint inhibitors and γδ T cell based therapy in melanoma.

CHAPTER 5

Conclusion

This study once again demonstrates the correlation between the disease stage and the immune response, as well as the importance of studying the molecules expressed on TIL's. Other studies with larger case series are needed to confirm the results we have obtained, however this preliminary study opens the way to the evaluation of new possible molecular targets, in particular LAG3, in the immunotherapeutic treatment of melanoma, as an alternative to the more well-known PD-1 and CTLA-4.

CHAPTER 6

References

1. Miller AJ, Mihm MC Jr (2006) Melanoma. N Engl J Med 355(1):51–65. https://doi.org/ 10.1056/NEJMra052166

2. Cummins, D.L., Cummins, J.M., Pantle, H., Silverman, M.A., Leonard, A.L., and Chanmugam, A. (2006) Cutaneous malignant melanoma. Mayo Clin Proc 81, 500-507.

3. Bhatia, S., Tykodi, S.S., and Thompson, J.A. (2009) Treatment of metastatic melanoma: an overview. Oncology (Williston Park) 23, 488-496.

4. Becker, J.C., K mpgen, E., and Br cker, E. (2000) Classical chemotherapy for metastatic melanoma. Clin Exp Dermatol 25, 503-508.

5. Rotte A, Bhandaru M, Zhou Y, McElwee KJ (2015) Immunotherapy of melanoma: present options and future promises. Cancer Metastasis Rev 34(1):115–128. https://doi. org/10.1007/s10555-014-9542-0

6. Rotte A, Bhandaru M (2016) Melanoma – treatment (immunotherapy of melanoma). Springer, Cham

7. Rotte A, Bhandaru M (2016) Ipilimumab (immunotherapy of melanoma). Springer, Cham

8. Rotte A, Bhandaru M (2016) Nivolumab (immunotherapy of melanoma). Springer, Cham

9. Rotte A, BhandaruM(2016) Pembrolizumab (immunotherapy of melanoma). Springer, Cham

10. Rotte A, Jin JY, Lemaire V (2018) Mechanistic overview of immune checkpoints to support the rational design of their combinations in cancer immunotherapy. Ann Oncol 29 (1):71–83. https://doi.org/10.1093/ annonc/mdx686

11. Brunet JF, Denizot F, Luciani MF, Roux- Dosseto M, Suzan M, Mattei MG et al (1987) A new member of the immunoglobulin superfamily—CTLA-4. Nature 328 (6127):267–270. https://doi.org/10.1038/ 328267a0

12. Dariavach P, Mattei MG, Golstein P, Lefranc MP (1988) Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains. Eur J Immunol 18(12):1901–1905. https://doi. org/10.1002/eji.1830181206

13. Metzler WJ, Bajorath J, Fenderson W, Shaw SY, Constantine KL, Naemura J et al (1997) Solution structure of human CTLA-4 and delineation of a CD80/CD86 binding site conserved in CD28. Nat Struct Biol 4 (7):527–531

14. Perkins D, Wang Z, Donovan C, He H, Mark D, Guan G et al (1996) Regulation of CTLA-4 expression during T cell activation. J Immunol 156(11):4154–4159

15. Alegre ML, Noel PJ, Eisfelder BJ, Chuang E, Clark MR, Reiner SL et al (1996) Regulation of surface and intracellular expression of CTLA4 on mouse T cells. J Immunol 157 (11):4762–4770

16. Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12(4):252–264. https://doi. org/10.1038/nrc3239

17. Stamper CC, Zhang Y, Tobin JF, Erbe DV, Ikemizu S, Davis SJ et al (2001) Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. Nature 410 (6828):608–611. https://doi.org/10.1038/35069118

18. Fife BT, Bluestone JA (2008) Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol Rev 224:166–182. https://doi.org/10. 1111/j.1600-065X.2008.00662.x

19. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z et al (2008) CTLA-4 control over Foxp3+ regulatory T cell function. Science 322 (5899):271–275. https://doi.org/10.1126/ science.1160062

20. Leach DR, Krummel MF, Allison JP (1996) Enhancement of antitumor immunity by CTLA-4 blockade. Science 271 (5256):1734–1736

21. CHMP (2011) Assessment report for Yervoy (Ipilimumab): procedure no.: EMEA/H/C/ 002213 (2011). In: C.f.M.P.f.H.U. (CHMP) (Ed.). EMA, London

22. Ishida Y, Agata Y, Shibahara K, Honjo T (1992) Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J 11(11):3887–3895

23. Shinohara T, Taniwaki M, Ishida Y, Kawaichi M, Honjo T (1994) Structure and chromosomal localization of the human PD-1 gene (PDCD1). Genomics 23(3):704–706. https://doi.org/10.1006/geno.1994.1562

24. Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26:677–704. https://doi.org/10.1146/annurev.immunol. 26.021607.090331

25. Francisco LM, Sage PT, Sharpe AH (2010) The PD-1 pathway in tolerance and autoimmunity. Immunol Rev 236(1):219–242. https://doi.org/10.1111/j.1600-065X. 2010.00923.x

26. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I et al (2001) PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2(3):261–268. https://doi.org/10.1038/ 85330

27. Carreno BM, Bennett F, Chau TA, Ling V, Luxenberg D, Jussif J et al (2000) CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. J Immunol 165 (3):1352–1356

28. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK et al (2009) PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 206 (13):3015–3029. https://doi.org/10.1084/ jem.20090847

29. Cheng X, Veverka V, Radhakrishnan A, Waters LC, Muskett FW, Morgan SH et al (2013) Structure and interactions of the human programmed cell death 1 receptor. J Biol Chem 288(17):11771–11785. https:// doi.org/10.1074/jbc.M112.448126

30. Bhandaru M, Rotte A (2017) Blockade of programmed cell death protein-1 pathway for the treatment of melanoma [short communication]. J Dermatol Res Ther 1(3):1–11. https://doi.org/10.14302/issn.2471-2175. jdrt-17-1760

31. Wang C, Thudium KB, Han M, Wang XT, Huang H, Feingersh D et al (2014) In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. Cancer Immunol Res 2(9):846–856. https://doi. org/10.1158/2326-6066.CIR-14-0040

32. Mahoney KM, Rennert PD, Freeman GJ (2015) Combination cancer immunotherapy and new immunomodulatory targets. Nat Rev Drug Discov 14(8):561–584. https://doi. org/10.1038/nrd4591

33. Tan S, Zhang H, Chai Y, Song H, Tong Z, Wang Q et al (2017) An unexpected N-terminal loop in PD-1 dominates binding by nivolumab. Nat Commun 8:14369. https://doi.org/10.1038/ncomms14369

34. Tan S, Liu K, Chai Y, Zhang CW, Gao S, Gao GF et al (2018) Distinct PD-L1 binding characteristics of therapeutic monoclonal antibody durvalumab. Protein Cell 9(1):135–139. https://doi.org/10.1007/s13238-017- 0412-8

35. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B et al (2009) The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatorydendritic cells. Nat Immunol 10 (1):48–57. https://doi.org/10.1038/ni. 1674

36. Boles KS, Vermi W, Facchetti F, Fuchs A, Wilson TJ, Diacovo TG et al (2009) A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. Eur J Immunol 39(3):695–703. https://doi.org/ 10.1002/eji.200839116

37. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A et al (2009) The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. Proc Natl Acad Sci U S A 106(42):17858–17863. https:// doi.org/10.1073/pnas.0903474106

38. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y et al (2014) The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. Cancer Cell 26(6):923–937. https://doi. org/10.1016/j.ccell.2014.10.018

39. Kurtulus S, Sakuishi K, Ngiow SF, Joller N, Tan DJ, Teng MW et al (2015) TIGIT predominantly regulates the immune response via regulatory T cells. J Clin Invest 125 (11):4053–4062. https://doi.org/10.1172/ JCI81187

40. Chew GM, Fujita T, Webb GM, Burwitz BJ, Wu HL, Reed JS et al (2016) TIGIT marks exhausted T cells, correlates with disease progression, and serves as a target for immune restoration in HIV and SIV infection. PLoS Pathog 12(1):e1005349. https://doi.org/ 10.1371/journal.ppat.1005349

41. Anderson AC, Joller N, Kuchroo VK (2016) Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. Immunity 44 (5):989–1004. https://doi.org/10.1016/j.immuni.2016.05.001

42. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T et al (2002) Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 415 (6871):536–541. https://doi.org/10.1038/ 415536a

43. Gorman JV, Colgan JD (2014) Regulation of T cell responses by the receptor molecule Tim-3. Immunol Res 59(1–3):56–65. https://doi.org/10.1007/s12026-014- 8524-1

44. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ et al (2005) The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 6 (12):1245–1252. https://doi.org/10.1038/ ni1271

45. Anderson AC (2014) Tim-3: an emerging target in the cancer immunotherapy landscape. Cancer Immunol Res 2 (5):393–398. https://doi.org/10.1158/ 2326-6066.CIR-14-0039

46. Sehrawat S, Suryawanshi A, Hirashima M, Rouse BT (2009) Role of Tim-3/galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators. J Immunol 182(5):3191–3201. https://doi.org/10.4049/jimmunol. 0803673

47. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sanchez-Fueyo A, Zheng XX et al (2003) Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nat Immunol 4(11):1102–1110. https://doi.org/10.1038/ni988

48. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA et al (2003) Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat Immunol 4(11):1093–1101. https://doi. org/10.1038/ni987

49. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E et al (1990) LAG-3, a novel lymphocyte activation gene closely related to CD4. J Exp Med171(5):1393–1405

50. He Y, Rivard CJ, Rozeboom L, Yu H, Ellison K, Kowalewski A et al (2016) Lymphocyte-activation gene-3, an important immune checkpoint in cancer. Cancer Sci 107 (9):1193–1197. https://doi.org/10.1111/ cas.12986

51. Workman CJ, Dugger KJ, Vignali DA (2002) Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. J Immunol 169(10):5392–5395

52. Xu F, Liu J, Liu D, Liu B,Wang M, Hu Z et al (2014) LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. Cancer Res 74 (13):3418–3428. https://doi.org/10.1158/0008-5472.CAN-13-2690

53. Bettini M, Szymczak-Workman AL, Forbes K, Castellaw AH, Selby M, Pan X et al (2011) Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. J Immunol 187 (7):3493–3498. https://doi.org/10.4049/ jimmunol.1100714

54. Jha V, Workman CJ, McGaha TL, Li L, Vas J, Vignali DA et al (2014) Lymphocyte activation gene-3 (LAG-3) negatively regulates environmentally-induced autoimmunity. PLoS One 9(8):e104484. https://doi.org/ 10.1371/journal.pone.0104484

55. Thommen DS, Schreiner J, Muller P, Herzig P, Roller A, Belousov A et al (2015) Progression of lung cancer is associated with increased dysfunction of T cells defined by coexpression of multiple inhibitory receptors. Cancer Immunol Res 3(12):1344–1355. https://doi.org/10.1158/2326-6066.CIR- 15-0097

56. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T et al (2010) Tumorinfiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc Natl Acad SciU S A 107(17):7875–7880. https://doi.org/ 10.1073/pnas.1003345107

57. Chen J, Chen Z (2014) The effect of immune microenvironment on the progression and prognosis of colorectal cancer. Med Oncol 31(8):82. https://doi.org/10.1007/s12032-014-0082-9

58. Lee HT, Lee JY, Lim H, Lee SH, Moon YJ, Pyo HJ et al (2017) Molecular mechanism of PD-1/PD-L1 blockade via anti-PD-L1 antibodies atezolizumab and durvalumab. Sci Rep 7(1):5532. https://doi.org/10.1038/ s4159801706002-8

59. Madorsky Rowdo FP, Baron A, Urrutia M, Mordoh J (2015) Immunotherapy in cancer: a combat between tumors and the immune system; you win some, you lose some. Front Immunol 6:127. https://doi.org/10.3389/ fimmu.2015.00127

60. Sasada, T., and Suekane, S. (2011) Variation of tumor-infiltrating lymphocytes in human cancers: controversy on clinical significance. Immunotherapy 3, 1235-1251.

61. Gooden, M.J., de Bock, G.H., Leffers, N., Daemen, T., and Nijman, H.W. (2011) The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer 105, 93-103.

62. Cipponi, A., Wieers, G., van Baren, N., and Coulie, P.G. (2011) Tumor-infiltrating lymphocytes: apparently good for melanoma patients. But why? Cancer Immunol Immunother 60, 1153-1160.

63. Itoh, K., Tilden, A.B., and Balch, C.M. (1986) Interleukin 2 activation of cytotoxic T-lymphocytes infiltrating into human metastatic melanomas. Cancer Res 46, 3011-3017.

64. Maus, M.V., Fraietta, J.A., Levine, B.L., Kalos, M., Zhao, Y., and June, C.H. (2014) Adoptive immunotherapy for cancer or viruses. Annu Rev Immunol 32, 189-225.

65. Hayday, A.C. (2009) Gammadelta T cells and the lymphoid stress-surveillance response. Immunity 31, 184-196.

66. Cordova, A., Toia, F., La Mendola, C., Orlando, V., Meraviglia, S., Rinaldi, G., Todaro, M., Cicero, G., Zichichi, L., Donni, P.L., Caccamo, N., Stassi, G., Dieli, F., and Moschella, F. (2012) Characterization of human γδ T lymphocytes infiltrating primary malignant melanomas. PLoS One 7, e49878.

67. Toia F, Buccheri S, Anfosso A, Moschella F, Dieli F, Meraviglia S, et al. (2016) Skewed Differentiation of Circulating Vγ9Vδ2 T Lymphocytes in Melanoma and Impact on Clinical Outcome. PLoS ONE 11(2): e0149570. doi:10.1371/journal. pone.0149570

68. Hayday AC (2009) cd T cells and the lymphoid stress-surveillance response. Immunity 31: 184–196.

69. Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, et al. (1989) Human lymphocytes bearing T cell receptor c/d are phenotypically diverse and evenly distributed throughout the lymphoid system. J Exp Med 169: 1277–1294.

70. Bonneville M, O'Brien RL, Born WK (2010) cd T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol 10: 467–478.

71. Constant P, Davodeau F, Peyrat MA, Poquet Y, Puzo G, et al. (1994) Stimulation of human cd T cells by nonpeptidic mycobacterial ligands. Science 264: 267–270.

72. Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, et al (2003) Microbial isoprenoid biosynthesis and human cd T cell activation. FEBS Lett 544: 4–10.

73. Tanaka Y, Morita CT, Nieves E, Brenner MB, Bloom BR (1995) Natural and synthetic non-peptide antigens recognized by human cd T cells. Nature 375: 155–158.

74. Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, et al (2003) Human T cell receptor cd cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 197: 163–168.

75. Kistowska M, Rossy E, Sansano S, Gober HJ, Landmann R, et al. (2008) Dysregulation of the host mevalonate pathway during early bacterial infection activates human cd TCR cells. Eur J Immunol 38: 2200–220.

76. Sireci G, Espinosa E, Di Sano C, Salerno A, Fournie JJ, et al. (2001) Differential activation of human cd T cells by nonpeptide phosphoantigens. Eur J Immunol 31: 1628–1634.

77. Guo RT, Cao R, Liang PH, Ko TP, Chang TH, et al. (2007) Bisphosphonates target multiple sites in both cis- and trans-prenyltransferases. Proc Natl Acad Sci USA 104: 10022–10027.

78. Vermijlen D, Ellis P, Langford C, Klein A, Engel R, et al. (2007) Distinct cytokine-driven responses of activated blood cd T cells: insights into unconventional T cell pleiotropy. J Immunol 178: 4304–4314.

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

80. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, et al. (2011) Differentiation, phenotype and function of interleukin-17-producing human Vc9Vd2 T cells. Blood 118: 129–138.

81. Meraviglia S, Caccamo N, Salerno A, Sireci G, Dieli F (2010) Partial and ineffective activation of Vc9Vd2 T cells by Mycobacterium tuberculosis-infected dendritic cells. J Immunol 185: 1770–1776.

82. Eberl M, Roberts GW, Meuter S, Williams JD, Topley N, et al. (2009) A rapid crosstalk of human cd T cells and monocytes drives the acute inflammation in bacterial infections. PLoS Pathog 5: e1000308.

83. Caccamo N, Battistini L, Bonneville M, Poccia F, Fournie´ JJ, et al. (2006) CXCR5 identifies a subset of Vc9Vd2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. J Immunol 177: 5290–5295.

84. Bansal RR, Mackay CR, Moser B, Eberl M (2012) IL-21 enhances the potential of human cd T cells to provide B-cell help. Eur J Immunol 42: 110–119.

85. Yang, M.j., Jones S., Ramos R. I., Chan A. A., Lee A. F., and Lee D. J., "Insights into local tumor microenvironment immune factors associated with regression of cutaneous melanoma metastases by mycobacterium bovis bacille calmette–gu´erin," Frontiers in Oncology, vol. 7, pp. 1–13, 2017.

87. Nicol,H. Tokuyama, S. R.Mattarollo et al., "Clinical evaluation of autologous gamma delta T cell-based immunotherapy formetastatic solid tumours," British Journal of Cancer, vol. 105, no. 6, pp. 778–786, 2011.

88. Conlon K. C., Lugli E., Welles H. C. et al., "Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer," Journal of Clinical Oncology, vol. 33, no. 1, pp. 74–82, 2015.

89. Melandri D., Zlatareva I., Chaleil R. A. et al., "The γδTCR combines innate immunity with adaptive immunity by utilizing spatially distinct regions for agonist selection and antigen responsiveness," Nature Immunology, vol. 19, no. 12, pp. 1352– 1365, 2018.

90. Davey M. S., Willcox C. R., Hunter S. et al., "The human Vδ2+ T-cell compartment comprises distinct innate-like Vγ9+ and adaptive Vγ9- subsets," Nature Communications, vol. 9, no. 1, 2018.

91. Wu Y., Wu W., Wong W.M. et al., "Human T Cells: A Lymphoid Lineage Cell Capable of Professional Phagocytosis," e Journal of Immunology, vol. 183, no. 9, pp. 5622–5629, 2009.

92. Tosolini M., Pont F., Poupot M. et al., "Assessment of tumor infiltrating TCRVγ9Vδ2 γδ lymphocyte abundance by deconvolution of human cancers microarrays," OncoImmunology, vol. 6, no. 3, 2017.

93. Gentles A. J., Newman A. M., and Liu C. L., "The prognostic landscape of genes and infiltrating immune cells across human cancers," Nature Medicine, vol. 21, no. 8, pp. 938–945, 2015.

94. Meraviglia S., Lo Presti E., Tosolini M. et al., "Distinctive features of tumor-infiltrating γδ T lymphocytes in human colorectal cancer," OncoImmunology, vol. 6, no. 10, p. e1347742, 2017.

95. Donia M., Ellebaek E., Andersen M. H., Straten P. T., and Svane I. M., "Analysis of Vδ1 T cells in clinical grade melanoma infiltrating lymphocytes," OncoImmunology, vol. 1, no. 8, pp. 1297–1304, 2012.

96. Bachelez H., Flageul B., Degos L., Boumsell L., and Bensussan A., "TCR γδ bearing T lymphocytes infiltrating human primary cutaneousmelanomas," Journal of Investigative Dermatology, vol. 98, no. 3, pp. 369–374, 1992

97. Wistuba-Hamprecht K., Di Benedetto S., Schilling B. et al., "Phenotypic characterization and prognostic impact of circulating cd and ab T-cells in metastatic malignant melanoma," International Journal of Cancer, vol. 704, pp. 698–704, 2016.

98. Toia F., Di Stefano A.B., Meraviglia S., Lo Presti E., Pirrello R., Rinaldi G., Fulfaro F., Dieli F. and Cordova A.. ΓδT Cell-Based Immunotherapy in Melanoma: State of the Art. Volume 2019, Article ID 9014607, 8 pages.

*99. Hogan, S.A., Levesque, M.P., and Cheng, P.F. (2018) Melanoma Immunotherapy: Next-Generation Biomarkers. Front Oncol **8**, 178.*

100. Wang X, Zhang H, Chen X. Drug resistance and combating drug resistance in cancer. Cancer Drug Resist. 2019;2:141-160. doi: 10.20517/cdr.2019.10. Epub 2019 Jun 19. PMID: 34322663;

*101. William H. Ward, MD and Jeffrey M. Farma, MD. **Cutaneous Melanoma: Etiology and Therapy. 170 page** ISBN: 978-0-9944381-4-0. DOI: http://dx.doi.org/10.15586/codon.cutaneousmelanoma.2017*

102. Lee WJ, Lee YJ, Choi ME, Yun KA, Won CH, Lee MW, Choi JH, Chang SE. Expression of lymphocyte-activating gene 3 and T-cell immunoreceptor with immunoglobulin and ITIM domains in cutaneous melanoma and their correlation with programmed cell death 1 expression in tumor-infiltrating lymphocytes. J Am Acad Dermatol. 2019 Jul;81(1):219-227. doi: 10.1016/j.jaad.2019.03.012. Epub 2019 Mar 14. PMID: 30880064

103. Pizzolato G, Kaminski H, Tosolini M, Franchini DM, Pont F, Martins F, Valle C, Labourdette D, Cadot S, Quillet-Mary A, Poupot M, Laurent C, Ysebaert L, Meraviglia S, Dieli F, Merville P, Milpied P, Déchanet-Merville J, Fournié JJ. Single-cell RNA sequencing unveils the shared and the distinct cytotoxic hallmarks of human TCRVδ1 and TCRVδ2 γδ T lymphocytes. Proc Natl Acad Sci U S A. 2019 Jun 11;116(24):11906-11915. doi: 10.1073/pnas.1818488116. Epub 2019 May 22. PMID: 31118283;

Scientific Productions

1. Di Stefano, A., Grisafi, F., Castiglia, M., Perez, A., **Montesano, L.,** Gulino, A., et al. (2018). Spheroids from adipose-derived stem cells exhibit an miRNA profile of highly undifferentiated cells. JOURNAL OF CELLULAR PHYSIOLOGY, 2018 Nov;233(11):8778-8789. doi: 10.1002/jcp.26785. Epub 2018 May 24. PMID: 29797571.

2. Di Stefano AB, Massihnia D, Grisafi F, Castiglia M, Toia F, **Montesano L**, Russo A, Moschella F, Cordova A. Adipose tissue, angiogenesis and angio-MIR under physiological and pathological conditions. Eur J Cell Biol. 2019 Jun;98(2-4):53-64. doi: 10.1016/j.ejcb.2018.11.005. Epub 2018 Nov 30.

3. Pappalardo M, **Montesano L**, Toia F, Russo A, Di Lorenzo S, Dieli F, Moschella F, Leto Barone AA, Meraviglia S, Di Stefano AB. Immunomodulation in Vascularized Composite Allotransplantation: What Is the Role for Adipose-Derived Stem Cells? Ann Plast Surg. 2019 Feb;82(2):245-251. doi: 10.1097/SAP.0000000000001763. PMID: 30628936.

4. **Montesano L**, Di Stefano AB., Belmonte B, Gulino A, Gagliardo C, Florena AM, Moschella F, Cordova A, Leto Barone AA, Toia F. Human Spheroids from Adipose-derived Stem Cells (S-ASCs) Induce Calvarial Bone Production in a Xenogeneic Rabbit Model. Annal Plastic surgery, 2021 Jun 1;86(6):714-720. doi: 10.1097/SAP.0000000000002579. PMID: 33346554.

5. EMERGING IMMUNOLOGICAL CHECKPOINTS PUT THE BRAKES ON MELANOMA INFILTRATING gd T CELLS Marta Di Simone, Anna Barbara Di Stefano, Anna Maria Corsale, **Luigi Montesano**, Elena Lo Presti, Francesca Toia, Adriana Cordova, Francesco Dieli, Serena Meraviglia. Abstract Siica 2021.

6. Correlation between Tissue-harvesting Method and Donor-Site with the Yield of Spheroids from Adipose-derived Stem Cells. Anna Barbara Di Stefano , Emanuele Cammarata, Marco Trapani , Roberto Pirrello, **Luigi Montesano**, Francesco Moschella , Adriana Cordova and Francesca Toia. 2022 Short communication JPRAS