Corso di dottorato di ricerca in:

"Scienze biomediche e biotecnologiche"

Ciclo 31°

"Multiparametric immune profiling to predict the risk of cancer development in chronic immune suppressed solid organ transplant patients."

Dottorando
Michela Cangemi

Supervisore
Prof. Riccardo Dolcetti

Co-supervisore
Dr. Agostino Steffan

Anno 2019

Summary

Abstract……..……...…...pag.3

1.Introduction
1.1Solid organ transplantation and immunosuppressive regimen
1.1.1 Background…….....pag.4
1.1.2 Immunosuppressive therapy in solid organ transplantation……..……..pag.4
1.2 Clinical implication of immunosuppressive therapies
1.2.1 Viral infection/reactivation and cancer development……...……...…pag.6
1.2.2 Cancer onset and development……..……..pag.10
1.3 Immune system and immunosuppression……...……...……..pag.12
1.3.1 T lymphocytes and DCs……..…pag.13
1.3.2 NF-kB transcription factor…….…….....pag.18

2. Aims.....pag.19

3. Results
3.1 Patients characteristics and immunosuppressive regimen…….……..pag.20
3.2 Detection of viral and tumor epitope-specific T-cell responses in SOT patients and healthy donors……...……..……....pag.21
3.2.1 T cell responses against Tumor Associated Antigens (TAAs)……...……....pag.21
3.2.2. Association between TERTmRNA levels in the blood and tumor onset in SOTs……pag.23
3.2.3 T cell responses against Virus antigenic epitopes…….……pag.25
3.3 Phenotypic and functional analysis
3.3.1 Immunophenotypic characterization of T-cells from SOT patients…….……....pag.27
3.3.2 Evaluation of immune cells activation status through multispectral imaging flow cytometry ……...…pag.28
3.3.3 NF-kB nuclear translocation in circulating T lymphocytes…….…...pag.30
3.3.4 NF-kB nuclear translocation in monocyte-derived DCs…….....pag.32
3.3.5 Iatrogenic immunosuppression impairs maturation of DCs generated from healthy donors……....pag.34

4. Discussion……..……..pag.36

5. Material and Methods
5.1 Immunosurveillance protocol ……..……...pag.42
5.2 Patients, treatment and healthy donors…….……pag.44
5.3 Sample collection……...……..……pag.44
5.4 Antibodies and reagents……....…pag.45
5.5 Patients-derived dendritic cell generation……..….....pag.45
5.6 Multispectral imaging flow cytometry analyses
5.6.1 Lymphocytes staining……..……....…pag.45
5.6.2 Dendritic cells staining……...……...…….pag.46
5.7 ELISpot assay……....……..pag.46
5.8 Quantification of circulating TERT mRNA……....……pag.47
5.9 Dendritic cells from healthy donors culture generation protocol……...…..…pag.48
5.10 Flow cytometry analyses……..……....…pag.48
5.11 Statistical Analysis……...……..…....pag.49

References……...……..…….pag.50

Abstract

Solid organ transplantation (SOT) is currently recognized as the treatment of choice of patients with end-stage disease, but the length and type of pharmacologic immunosuppression strongly affect patients' immune system resulting in enhanced immune evasion strategies, oncogenic virus reactivation and malignant transformation. On these grounds, a more in depth characterization of functional defects of immune cells related to the different immunosuppressive regimens and the occurrence of relevant clinical events might be relevant to design novel and integrated cancer surveillance practices for immune suppressed transplant recipients. The main purpose of this study is to identify a reliable immunologic profile predictive of cancer development and progression in SOT patients through a multiparametric phenotypic and functional characterization of dendritic cells (DCs) and T lymphocytes in SOT patients treated with different immunosuppressive regimens. In particular, the activation status of these cells was assessed in serial samples through Multispectral imaging flow cytometry, whereas the extent of T-cell responses specific for pathogenic viruses and tumor-associated antigens was measured by ELIspot IFN-γ assay. Data obtained in SOT patients who developed a tumor (n=15) were compared with those from patients tumor-free during the follow-up (n=58). Interestingly, we observed that patients experiencing cancer onset during surveillance had lower numbers of activated $CD4^+$ and $CD8^+$ T lymphocytes and a decreased activation of monocyte-derived DCs as compared with the "no tumor" cohort and healthy donors. Moreover, we found that tumor onset patients display higher spontaneous T-cells responses against the "universal" tumor associated-antigens hTERT and Survivin if compared to "no tumor" patients and healthy controls. Globally, patients of the "tumor" cohort showed significantly higher levels of circulating hTERT mRNA as compared to those of the "no tumor" cohort, even before the diagnosis of cancer, suggesting that this biomarker could usefully complement the immunomonitoring of SOT patients and help identify those at risk of cancer. We also found that the levels of HPV-specific T-cell responses observed in both patient cohorts were significantly lower both at baseline and at follow up as compared to those of healthy donors, whereas no difference was detected with regard to T-cell responses specific for CMV and EBV. Given the critical role of severe immunosuppression that characterizes transplanted patients and the importance of immune surveillance integrity in the prevention and control of malignancies, the results of the present study provide a strong rationale to design and activate a prospective study in a larger cohort of SOT patients aimed at validating the role of the immune biomarkers identified as potential predictors of the risk of cancer in the highly heterogeneous setting of SOT patients.

1. Introduction

1.1 Solid organ transplantation and immunosuppressive regimen

1.1.1 Background

Solid organ transplantation (SOT) is currently recognized as the treatment of choice of patients with end-stage disease and the availability of potent anti-rejection drugs significantly reduced the occurrence of acute and chronic allograft rejections, even though long-term survival is still poor (1). Indeed, tumor onset, viral infections/reactivations and cardiovascular complications are among the major causes of morbidity and mortality in solid organ transplanted patients (OTRs) (2,3). Therefore, if on one side organ transplantation is the only treatment available for some end-stage diseases, on the other side the duration and type of immunosuppression can increase the risk of *de novo* malignancies and impair the control of viral infections and reactivation. In this respect, the unavoidable life-long immunosuppression plays a critical role in the increased incidence and risk of cancer development in OTRs. These patients have an overall 2- to 5-fold increased risk of developing tumors compared to the general population (4). Iatrogenic immunosuppression, in combination with lifestyle habits, age and concomitant comorbidities deriving from transplantation, play a central role in the pathogenesis of these complications, strongly affecting the efficacy of immunosurveillance in these patients. On these grounds, there is the pressing need to better characterize the immune dysfunctions related to the immunosuppressive treatment of these patients in order to tailor more precisely the therapeutic schedule and decrease the risk of life-threatening complications.

In this study, peripheral blood samples from 73 patients who underwent a several solid organ transplant were examined and analyzed. Patients enrolled since 2014 in an oncological surveillance study activated at the Centro di Riferimento Oncologico (CRO) in Aviano (PN), have been monotorized to prevent and/or diagnose the onset of *de novo* tumors. Of these, 15 patients developed *de novo* cancer during cancer surveillance. By comparing the two cohorts we tried to give a predictive, as well as pathogenetic meaning of the results obtained during viro-immunological immunomonitoring.

1.1.2 Immunosuppressive therapy in solid organ transplantation

Immunosuppression is a pharmacological treatment based on the administration of immunosuppressive drugs capable of inhibiting the immune system's response to non-self

antigens. Transplant recipients must take immunosuppressive drugs for the rest of their lives to prevent allograft rejection. Different types of immunosuppressive drugs are available, but in this thesis work we focused on the two main classes currently used in therapy: calcineurin inhibitors (CNI) and mTOR inhibitors (mTORi).

Calcineurin is a calcium/calmodulin-activated serine/threonine phosphatase that, once stimulated, de-phosphorylates and thereby activates members of the nuclear factor of activated T cells (NFAT) transcription factor family (5). Upon activation, NFAT family members migrate into the nucleus and activate transcription (6). NFAT includes a family of transcription factors involved in regulating immune response. Calcineurin dephosphorylates the cytoplasmic subunits of the NF-AT1 transcription complex (7), which are encoded by four genes (NF-ATc1-4) and which undergo nuclear translocation upon activation (8). CsA binds directly to the members of the NF-ATc family, dephosphorylates serine within SP (SP1 to SP3) repeats and the serine-rich region NF-ATc molecules, which then move into the nucleus where they are retained by the persistent elevation of intracellular $Ca2^{+}$ and continuous CsA activity (9–11). Calcineurin is also responsible for the transcription of the genes encoding for IL-2 and several other cytokines, including TNF-α and IFN-γ. CNIs, such as Cyclosporine A (CsA) (1970-80) and Tacrolimus (TAC) (1990), exert their immunosuppressive action through the inhibition of the Calcineurin pathway, inducing the activation of NFAT, which down-regulates IL-2 and INF-γ expression, and inhibits T-cell activation and proliferation in response to foreign antigen (12). CsA is a cyclic endecapeptide isolated in 1971 from the fungus *Tolypocladium inflatum* (13), commonly used to prevent rejection of liver, heart and kidney transplants. TAC also called FK-506, is a macrolide antibiotic isolated from *Streptomyces tsukubaensi.* The use of this drug was initially restricted to patients with liver transplantation, but, more recently, it was also extended to patients with heart, pancreas and kidney transplantation (14,15).

mTORi are a large class of drugs that inhibit mTOR, a serine-threonine kinase involved in cell growth, proliferation, protein synthesis and apoptosis (16,17). The PI3K/Akt/mTOR pathway is often up-regulated in various malignancies. mTOR is a catalytic subunit of two functionally distinct molecular complexes called mTORC1 and mTORC2. mTORC1 is composed of five proteins, mTOR, RAPTOR, mLST8, PRAS40 and FKBP38, and the complex performs its function by phosphorylating the p70S6 and 4E-BP1 kinases, thereby regulating the expression of proteins that promote cell proliferation and survival, such as c-Myc, cyclin D1, and STAT3. The mTORC2 complex includes RICTOR, MAPKAP1, PRR5/PRR5L, Mlst8 and Deptor. mTORC2 directly phosphorylates Akt and regulates the

organization of actin cytoskeleton by phosphorylating PKC-α. Unlike mTORC1, which is sensitive to acute treatment with rapamycin (RAPA), mTORC2 is less sensitive to this drug, although chronic treatments were shown to disrupt the integrity and function of this complex (18). The mTORi *Everolimus* (EVR) was derived from *Sirolimus* (SRL) and both compounds bind to the FK506-binding protein 12 (FKBP12) in the cytoplasm. mTORi have both immunosuppressant and anticancer activity, promoting T-cell anergy by compromising DCs maturation into antigen presenting cells (APCs) (19).

1.2 Clinical implication of immunosuppressive therapies

1.2.1. Viral infection/reactivation and cancer development

Since life-long chronic immunosuppression is necessary to preserve allograft function in transplanted patients, it is not surprising that opportunistic viral infections are among the most common side effects of immunosuppressants. Viral infection or reactivation can occur at any time after transplantation; in particular, CMV, Epstein-Barr virus (EBV), Polyomavirus BK (BKV) and Human papillomavirus (HPV) virus infections/reactivations occur more frequently within a few months after organ transplantation. CMV infection can be easily prevented by specific prophylaxis, while EBV or BKV reactivations can only be prevented by reducing immunosuppression. Some viral infections may result in post-transplant lymphoproliferative disorders (EBV), or Kaposi's sarcoma (human herpes simplex virus type 8/Kaposi sarcoma herpesvirus), or carcinoma of the skin and anogenital region (HPV). Therefore, it becomes essential to better understand the effects of immunosuppressive drugs on oncogenic and non-oncogenic virus infections and reactivations, to more precisely define the relative level of risk of cancer development in these patients.

Human CMV infection and reactivation are major causes of morbidity in transplanted recipients. CMV belongs to the β-herpesviruses family and is latently present in the majority of the general population, being detected in peripheral blood monocytes of 50-90% of healthy individuals worldwide. The first correlation between CMV subclinical infection and graft prognosis has been reported in a study of over 400 kidney transplants, which showed that, in the absence of antiviral treatments, CMV infection, even at subclinical level, was associated with increased risk of cardiovascular disease and death. The same study also demonstrated that subclinical presence of CMV infection in the first 100 days after transplantation increases the risk of subsequent organ rejection (20,21). Usually, CMV-related disease after liver transplantation appears as fever and bone marrow suppression and accounts for 60% of CMV

diseases in this setting. It may also occur as a gastrointestinal disease and accounts for more than 70% of invasive tissue CMV disease in patients with liver and other solid organ transplants (22). In a case-control study, Bond et al (23) demonstrated that increased serum levels of TAC were not associated with the detection of CMV DNA by PCR, treatment regimen, or death, whereas the elevated serum level of CsA significantly correlated with CMV-positive PCR, increasing the need for treatment, but without increasing the risk of death. This study is of particular relevance because it was carried out to better understand the possible pathogenic role of CMV infection in patients undergoing cardiac transplantation with the final goal to tailor the immunosuppressive treatment. The data obtained confirmed the results reported by a Spanish study carried out in 95 transplanted patients that showed lower rates of CMV infection in the group treated with TAC as compared to patients treated with CsA (24). The antiviral properties of mTORi have been ascribed to a variety of mechanisms, in particular involving memory T cells (25). In a meta-analysysis, Webster et al (26) examined a kidney transplant cohort treated with mTORi and showed that the treatment was related to a reduction of 51% of CMV infection compared to antimetabolite plus CNI. A recent study showed that patients receiving mTORi have a lower risk of CMV disease, and, interestingly, when these drugs were combined with Calcineurin, CMV infection was controlled more efficiently. Furthermore, Havenith et al (27) demonstrated that patients treated with EVR had a significant increase in CMV-specific $CD8^+$ and $CD4^+$ T cells compared with patients included in other treatment regimens. Moreover, the persistent stimulation of T cells by latent viruses, such as CMV, can determine the activation of effector mechanisms of memory T cells with progressive loss of co-stimulatory molecules (CD28) (28) and loss of replicative capacity (CD57 , PD-1, Tim3) (29). This process can culminate in the production of un-reactive and poorly functional T cell clones, facilitating evasion from non-self immune-surveillance (30,31).

EBV is a DNA virus belonging to the Gammaherpesvirinae family, responsible for infectious mononucleosis and involved in the genesis of lymphoproliferative disorders and some epithelial tumors (32). After EBV infection of B lymphocytes, the viral DNA persists in these cells in two forms, the *episomal* and, less frequently, the *integrated form.* Only in this latter case viral DNA is effectively incorporated in host's genome (33). Host immunity plays a crucial role in controlling EBV infection and the virus has evolved an elegant strategy that allows EBV to exploit B-cell differentiation to finally establish an asymptomatic latency in resting memory B lymphocytes. In post-transplant patients, impaired immunosurveillance against EBV may favor the onset of EBV-associated lymphoproliferative disorders, the so-

called post-transplant lymphoproliferative diseases (PTLDs). PTLDs is a heterogeneous clinical and pathologic group of lymphoid disorders that may represent an important cause of morbidity and mortality in the transplant setting. The first case of PTLD has been diagnosed in 1968 by Doak et al. in a kidney transplant (34). The incidence of PTLD in SOT recipients is increased 50-120 % compared to the general population (35). The risk of developing PTLD is lower in kidney (36) and liver (37) transplants than in other transplant types. Several factors play an important role in the development and malignant progression of these diseases, including viral infections, strength and duration of immunosuppression, age and even genetic factors (38). In adults, PTLDs represent the second more frequent group of post-transplant neoplasms after those of the skin, while they are the most common malignancy in organ transplanted pediatric patients. After transplantation, the EBV genome is found in 60-70% of B cells (39), the primary target of EBV infection. The immune response to EBV infection involves both innate and adaptive immunity mainly mediated by cytotoxic T lymphocytes ($CD8^+$) and NK cells (40). Symptomatic infection, mainly occurring during infectious mononucleosis, is associated with increased numbers of NK cells in peripheral blood, which may exert cytotoxicity against infected B cells and enhance the antigen-specific T-cell responses through the release of immunomodulatory cytokines (41). In immunosuppressed transplant patients, mainly pediatric, primary EBV infection is poorly controlled, finally resulting in the development of EBV-associated PTLDs. Considering that the ability to control EBV replication and expansion of EBV-infected B cells strongly depends on the number and the efficacy of EBV-specific T cells, it is clear that in SOT patients this response may be hampered by immunosuppressive regimens, increasing the risk of PTLD. Consistently, the risk of EBV-associated PTLD is increased when an EBV-seronegative patient receives a transplant from an EBV^+ donor (42) and this mainly occurs in pediatric patients. The most common malignant PTLD subtype is post-transplant large B-cell lymphoma (PT-DLBCL), followed by Burkitt lymphoma (PT-BL) and plasmablastic lymphoma (PT-PBL) (43), while Hodgkin lymphoma (HL) is a rare form of PTLD (44). The disease presentation is often nonspecific and may also involve the transplanted organ in about half of cases (45). Notably, early-onset PTLDs, usually occurring within the first year of transplantation, are almost always EBV^+ and treatable with a decrease in the dose of immunosuppressants (46). On the contrary, late-onset PTLDs in adult renal allograft recipients are more frequently EBV-negative and more difficult to treat. Pivotal studies, conducted by Tanner JE et al (47) showed that transplanted patients undergoing immunosuppressive therapy with CsA had an increased incidence of EBV-associated PTLDs.

Notably, these patients had increased serum levels of the B-cell growth promoting cytokine IL-6 probably due to the direct role of CsA in inducing EBV lytic cycle in infected B cells. Chen et al (48) also demonstrated that *in vitro* CsA is also able to promote the EBV-driven immortalization of primary B cells. Moreover, post-transplantation treatment with RAPA was shown to reduce the ability of B cells to undergo EBV lytic cycle replication (49), whereas the combination of mTORi and inhibitors of HSP90 (a dysregulated protein in EBV-related PTLDs) had a synergistic effect in inducing apoptosis and *in vitro* cytotoxicity of EBV-positive cells (50). Conversely, a reduction in the efficacy of EVR treatment has been shown when the mTORi are used in combination with TAC in EBV-positive cell lines (51).

Non-melanoma skin cancers are the most common malignancies observed after SOT, occurring in more than a half of recipients (52). The pathogenesis of these tumors is multifactorial being the result of the complex interplay of the effects of ultraviolet radiation, compromised immune surveillance, direct effects of immunosuppressive drugs and infection by oncogenic viruses. With regard to the potential risk conveyed by immunosuppressants, metabolites of AZA were shown to interact with UVA to generate oxygen species, causing DNA damage and protein oxidation, resulting in mutagenic effects in the skin (53,54). CNIs may also cooperate with UVA and UVB in increasing the levels of TGF-β and suppressing p53 expression through the induction of ATF3 (55). SOT recipients are 250 times more probably to develop non-melanoma skin cancer and treatment with mTORi has been associated with reduced cancer incidence (56). Cutaneous squamous cell carcinoma (SCC) is the predominant skin cancer in SOT patients and its occurrence is 65-250-fold higher compared to that of the general population (57). Squamous differentiation of tumor cells, increased mitotic rates and ulceration are frequent features of these carcinomas (58). Conversion from CNIs to SRL was shown to reduce the vascularization of SCC in SOT patients (59). Basal cell carcinoma (BCC) is the second most frequent cancer in these patients and, among the numerous histologic variants of this tumor, nodular BCC is the most common (60). Surgical excision is the first-line therapy for BCC and the occurrence of distant metastases is rare. Skin tumors in transplanted patients are often associated with the presence of oncogenic viruses, in particular HPV. Human papillomavirus is a double-stranded non-enveloped DNA virus which infects skin and mucosal keratinocytes (61). HPV infection is responsible for the development of viral warts that can cause significant morbidity in immunosuppressed individuals. In HPV-positive transplanted patients, lifelong immunosuppressive therapy with mTORi has been associated with a significant reduction in the incidence of *de novo* neoplasms (62). Available data show that the presence of HPV

infections, in particular by β-HPV, increases the risk for the development or progression of skin tumors up to 30%. HPV has a strong tropism for keratinocytes but the mechanism through which the virus infects these cells has not been clarified yet. The persistent presence of HPV predisposes mainly to BCC, although the virus is also detectable in SCC (63,64). Dharancy et al (65) reported the case of a young liver transplant patient in whom the conversion to SRL therapy was followed by a rapid regression of skin warts, suggesting that mTORi may be beneficial in immunosuppressed patients with HPV-induced relapsing warts.

1.2.2 Cancer onset and development

The risk to develop a *de novo* neoplasm or a tumor relapse in solid OTRs is very high, about 2-5 times higher compared to the general population (66) and this is due not only to the establishment of oncogenic virus co-infections or reactivations, but also to the duration and potency of immunosuppressive drug treatment. The reduction of immune surveillance, together with patient lifestyle contributing factors, often lead to the development of aggressive tumors. Therefore, although pharmacologic immunosuppression has the power to inhibit the rejection of the transplanted organ, this treatment may have detrimental effects on the immune-surveillance efficacy of these patients. Immune-evasion plays a pivotal role in tumorigenesis in transplantation, because in immunocompetent individuals immune-surveillance controls the development of neoplasia, whereas, in immunocompromised individuals, chronic immunosuppression predisposes to a variety of infections and reactivations of oncogenic viruses and the control of developing cancers may be severely impaired. The mechanisms that drive the onset of *de novo* tumors in SOTs can be grouped into three major categories: 1) direct pro-oncogenic properties of select immunosuppressive drugs; 2) increased risk of oncogenic virus reactivations; 3) impaired immune-surveillance of tumor cells (67).

CsA and TAC were found to upregulate vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β), both implicated in cancer progression, angiogenesis, invasive phenotypes and uncontrolled cell growth (68–70). Furthermore, the use of CNIs can increase the frequency and severity of diseases related to viral infection/reactivation, such as those induced by EBV, HPV and CMV, exposing patients to increased risks to develop virus-related cancer. CsA can be correlated with cancer development, as suggested by Hojo et al, who showed that CsA induces TGF-β secretion through a cell-autonomous mechanism. In this study, adenocarcinoma cells treated with CsA showed marked morphological and functional alterations, including increased cell motility

and invasive growth (71). CNI were also shown to mediate Ras activation and promote renal cancer cell proliferation, thus highlighting an additional tumorigenic pathway by which CNI promotes oncogenesis. Some studies demonstrated that CsA treatment induces overexpression of VEGF in human cell renal carcinoma, an effect that is dependent on Ras activation (72,73). Wimmer et al reported that the risk of *de novo* malignancies is increased in TAC-treated patients compared to the CsA treatment (74). DNA repair is also inhibited by CsA, whereas TAC was also shown to inhibit DNA repair (75), another possible contributory factor to the increased risk of cancer in these patients. Furthermore, the levels of IL-6 were markedly increased in EBV-infected cells treated with CsA. This cytokine is capable to promote B cells activation and possibly immortalization (47,76). Indeed, the incidence of different types of tumors that have developed in transplant patients have been related to the use of CsA. A high incidence of cancer was found in kidney transplant recipients treated with high-dose CsA (77,78). In addition, the risk of skin cancer is higher in patients treated with CsA and exposed to ultraviolet-A light (79).

mTOR inhibitors were initially designed as anti-cancer drugs, as well as immunosuppressive agent, because of their ability to suppress the growth and proliferation of tumor cells in mice (80). One of the most important effects of mTORi is inhibition of angiogenesis and decreased VEGF synthesis (81). Dysregulation of cell cycle characterizes several types of tumors, and consequently, mTOR became an important therapeutic target for cancer patients. Loss of mTOR function leads to an arrest of G1 phase of the cell cycle and a severe reduction of protein synthesis (82), demonstrating that the mTOR pathway is crucial for cell survival and protein synthesis. In fact, the hyperactivation of the mTOR/PI3K/AKT pathway may be detectable in virtually all types of tumors. The most common mechanism resulting in aberrant PI3K signaling is somatic loss of PTEN, which is mutated or epigenetically inactivated in an large number of cancers (83). Akt is a cytosolic protein that phosphorylates different substrates, such as the proapoptotic factors Bad and procaspase-9, and is able to induce the expression of Fas ligand. Furthermore, Akt phosphorylation is involved in the induction of NF-kB transcriptional activity and in the decrease of p53 levels through the phosphorylation of the transcription factor Mdm2. Therefore, through the modulation of different biochemical pathways, Akt promotes cellular survival and resistance to apoptosis. Several studies have found Akt overexpression in gastric, ovarian, pancreas, breast and stomach cancer (84).

Noteworthy is the effect that RAPA has on the immunosuppressive regulatory T cells (Tregs). A study conducted in rats investigated the *in vivo* effects of RAPA, showing that

iDCs, treated with low-dose of RAPA and injected intravenously, are able to selectively expand $CD4^+CD25^+Foxp3^+$ Tregs, which play an indispensable role in inducing transplantation tolerance (85). Consistently, Strauss et al showed that RAPA promotes the expansion of $CD4^+CD25^{high}FOXP3^+$ Tregs compared to the $CD4^+CD25^{neg}FOXP3^+$ Treg subset (86). It has been aldo shown that $CD4^+CD25^{high}$ T cells are resistant to apoptosis and are dependent of IL-2 signaling for expansion and survival. In addition, RAPA may synergize with retinoic acid in the generation and activity of Tregs because retinoic acid promotes the expression of β7 integrin, a subunit of α4β7 integrin, while RAPA enhances the expression of CXCR4, a potent chemokine receptor for lymphocytes (87). Furthermore, a recent study has shown how the conversion from CNI to SRL in kidney transplants has reduced the incidence of skin tumors, one of the most frequent types of cancer (88).

1.3 Immune system and immunosuppression

In a normal state of immunocompetence, cells of the innate immune system, such as APCs, must differentiate from an immature to a mature state to allow the initiation of specific T-cell responses. DCs constitute a heterogeneous population of APCs that mediates critical connections between innate and adaptive immunity (89). These cells play a pivotal role in antigen processing and presentation resulting in the induction of effective immune responses against pathogens and tumor cells. Notably, these cells are the most powerful APCs, being able to orchestrate a primary immune response but also to induce immune tolerance (89). In particular, DCs play a central role in priming naive T and B cells, the first critical steps in the induction of an antigen-specific immune response. DCs develop from $CD34^+$ bone-marrow progenitor cells or from $CD14^+$ monocytes and differentiate into iDCs, which are functionally specialized to take up exogenous antigens. After recognition, exogenous antigens are internalized and the activated DCs migrate to the draining lymph node, where they can induce an adaptive immune response (90). This successful outcome requires the processing of a antigens are their loading in the form of small peptides on the main histocompatibility complex (MHC) molecules. Peptides loaded on MHC-II molecules may be recognized by antigen-specific $CD4^+$ T helper cells, while peptides loaded on MHC-I molecules may be recognized by antigen-specific $CD8^+$ T lymphocytes. The presentation of internalized antigens on MHC-I molecules is defined as cross-presentation, a crucial process in the induction of effective adaptive immune responses against tumors and viruses that do not infect DC directly and that may induce peripheral tolerance (91). The MHC-I pathway is normally used to present endogenous antigens and cross-presentation is particularly important

because it allows DCs to present through the MHC-I pathway also exogenous antigens, which are usually mainly presented by MHC-II molecules (92). The cross-presentation pathways of the antigens are essentially two: 1) the vacuolar pathway, in which the processing/loading takes place within the endo/lysosomal compartment and 2) the endosome pathway, in which the internalized antigens are transported from the endosome to the cytosol where they are degraded by the proteasome (93). The co-stimulatory CD40/CD40L axis along with the danger signal provided by an exogenous antigen are catalysts for DC licensing. Therefore, exogenous antigen cross presentation and the consequent activation of naive $CD8^+$ cytotoxic T cells, provides the immune system with an important mechanism for generating immunity to viruses while preserving tolerance to self (94).

The goal of immunosuppression is to avoid rejection of the transplanted organ, attenuating the immune response to the non-self, but as widely discussed earlier, this may result in the loss of functional immune surveillance in transplanted patients, thus facilitating the development of viral infections and the development of *de novo* tumors. Immunosuppressive therapies have an impact on all the compartments of the immune system, such as Natural Killer Cells (95,96), B lymphocytes (97,98) , Macrophages (99,100) and Myeloid-derived suppressor cells (101) In this thesis work, the attention is directed in particular to the impact of the immunosuppressive regimens on DCs and T cells.

1.3.1 T lymphocytes and DCs

T lymphocytes are cells derived from a pluripotent stem cell of the bone marrow and matured in the thymus and represent fundamental mediators of adaptive immune responses. T lymphocytes have a specific antigen receptor called T-cell receptor (TCR), associated to a set of molecules that form a structural unit, known as CD3 complex. T cells are dived in two major family: $CD4^+$ T cells and $CD8^+$ T cells. $CD4^+$ T cells perform multiple functions, ranging from the activation of cells of the innate immune system, B cells, cytotoxic T cells and suppression of the immune reaction. Several studies have continuously identified new subgroups of $CD4^+$ cells in addition to the classic T-helper cells 1 (Th1) and T-helper 2 (Th2): these include T-helper 17 (Th17), follicular helper T cells (Tfh), induced T-regulatory cells (iTreg), the regulatory type 1 (Tr1) cells and the potentially distinct T-helper 9 (Th9) subset. The differentiation of different lineages depends on the complex network of specific transcription signals mediated by cytokines and transcription factors followed by epigenetic modifications (102,103). $CD8^+$ T cells and are very important for immune defence against

intracellular pathogens, including viruses and bacteria, and for tumor surveillance. When a $CD8^+$ T cell recognises its cognate antigen and becomes activated, it has three major mechanisms to kill infected or malignant cells. The first is secretion of cytokines, primarily TNF-α and IFN-γ, which have anti-tumour and anti-viral microbial effects. The second mechanism is the production and relase of cytotoxic granules, wich perforin and granzyme, that induce the apoptosis of target cells. The last mechanism is the killing of target cells via Fas/FasL interaction, with induction of caspase cascade and apoptosis of target cells. (104,105)

Vadafari et al. showed that TAC inhibited the phosphorylation of NF-kB in $CD3^+$ T cells, $CD4^+$ T cells and $CD8^+$ cytotoxic T cells isolated from healthy donors. Similarly, the production of TNF-α was also suppressed in the presence of the drug. This work has shown that the immunosuppressive activity of TAC also influences the NF-kB signaling and the NFAT pathway as well (106). Interestingly, CsA and TAC were shown to prevent naive T-cell differentiation into cytokine-producing mature cells and inhibit cytokine-production by memory $CD4^+$ T cells (107). In human peripheral T cells costimulated with simultaneous engagement of T cell receptor (TCR)/CD3 and CD28 it has been demonstrated that 10 ng/ml CsA inhibited NF-AT and NF-kB migration into the nucleus followed by reduction of T-cell proliferation in response to the costimulation (108). Available data indicate that the conversion from TAC to SRL suppresses Th17 activity and up-regulates the percentage of Treg cells in kidney transplant recipients. SRL inhibited Ser705 phosphorylation of STAT3 in $CD4^+$ T cells, which promotes a differentiation switch towards Treg cells rather than to Th17 cells. A possible activation of STAT5 by SRL, which has an opposite effect to STAT3, was also suggested. The drug was also shown to induce a downregulation of IL-17 and an increased expression of Foxp3 in Th17 cells. Therefore, the conversion from TAC to SRL favorably regulates the Th17/Treg ratio (109). A recent study investigated the contribution of residual immune function in mediating the decreased incidence of SCC showed by renal transplant recipients switching CNI-based therapies to mTORi. While both RAPA and TAC enhanced the survival of OVA-expressing skin grafts, and inhibited short-term antigen-specific $CD8^+$ T cell responses, RAPA but not TAC induced a significant infiltration of $CD8^+$ effector memory T cells into UV-induced SCC lesions. Moreover, only RAPA was able to increase the number and enhance the function of $CD8^+$ effector and central memory T cells in a model of long-term contact hypersensitivity. These findings are consistent with the possibility that the lower risk of de novo SCC showed by patients switched to mTORi

regimens is probably due to enhanced $CD8^+$ memory T cell responses to new antigenic stimulations occurring in their skin (110).

Treatments with RAPA was shown to inhibit the differentiation of antigen-specific $CD8^+$ T cells *in vivo*, while increasing the number of $CD8^+$ memory T cells. In fact, RAPA promotes the generation of long-lived memory precursors by altering the process of development of short-lived precursors (111,112). These findings are consistent with the observation that the treatment of mice with RAPA following acute lymphocytic choriomeningitis virus infection improved not only the quantity but also the quality of virus-specific $CD8^+$ T cells and also improved the response of memory T cells in non-human primates after vaccination with a modified vaccinia virus-Ankara (25). Because of their strong immunosuppressive properties, Tregs have been intensely studied for their use in cell therapies, as they maintain tolerance against self-antigens and abort excessive immune responses. Treg cells are principally characterized by expression of CD25 and FoxP3 and represent 5-10% of all peripheral $CD4^+$ T cells(113). Tregs can be divided into resting Tregs ($CD45RA^+FoxP3^{low}$), effector Tregs ($CD45RA^-FoxP3^{high}$) and cytokine-producing Tregs, ($CD45RA^-FoxP3^{low}$)(114). Their mechanism of action involves immunosuppressive activities against other T cells, B cells, macrophages, DCs and NK cells and the release in the microenvironment of immunosuppressive cytokines such as IL-10, IL-35 and TGF-β to prevent T-cell proliferation and maturation of antigen presenting cells (115). Treg cells may also secrete granzymes and perforins (116) and express CTLA-4, which may de-regulate the activity of DCs (117). The pilot study by Levitsky et al. has shown that, in liver transplant patients, monotherapy with SRL resulted in a higher percentage of Tregs in peripheral blood compared to non-SRL monotherapy (118). In fact, the expression of FoxP3 requires IL-2, whose gene transcription is blocked by CNI but not by SRL. These data were confirmed in a subsequent work that showed that RAPA, but not CsA, does not inhibit the function of Treg cells but rather promotes their induction (119).

A recent study in kidney transplant recipients investigated the correlation between the use of immunosuppressants, sun exposed (SE) skin and the onset of skin tumors. In particular, the treatment with SRL significantly increases the absolute number of $CD4^+$ T cells, memory $CD8^+$ and CD4+ T cells, and Treg cells in the blood of patients with SE skin compared to those with non-SE skin (120). RAPA was also found to eliminate antigen-specific effectors $CD8^+$ T cells and expand Tregs. In fact, in mouse models used to test the efficacy of RAPA and CsA as a therapy for immune-mediate bone marrow failure (such as aplastic anemia) it

has been shown that that RAPA reduced the proportion of memory and effector T cells and maintained a pool of naïve T cells (121).

Immunosuppressive drugs employed in the management of allograft rejection in SOT patients can also influence the phenotypic and functional characteristics of DCs, suggesting that their impaired function may also play a role in the development of tumors or in promoting the reactivation of viral infections in immunosuppressed patients.

DCs exist in two differentiation states, each of which has distinct phenotypic, morphological and functional characteristics: iDCs and mature dendritic cells or mDCs. iDCs phenotypically show high expression of CCR1, CCR5, CCR6 and CD68, while the expression of CCR7, CD86, CD80, CD40 and CD83 is low. They are able to capture and process the antigens they encounter and subsequently evolve to mDCs, which are characterized by high expression levels of CD83, CD86, CD49, CD80, CCR7, MHC-II, CD1a and CD11c (122). Another classification of DCs is based on different DC subpopulations: classical DCs (cDCs), plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs) and Langherans cells (LCs).

The effects of CNIs on DCs are well recognized and several groups have investigated the impact of these drugs on mouse and human DCs. TAC and CsA were found to inhibit the allostimulatory capacity of *in vitro*-generated myeloid DCs without affecting DC maturation (123). On the contrary, the pioneering work of Lee et al has shown how the treatment with CsA impaired the allostimulatory capacity of *in vitro* generated mouse BM-derived DCs by downregulating CD40, CD80, and CD86 expression associated with reduced nuclear translocation of NF-κB, a transcription factor promoting DC maturation (124,125). In addition, CsA inhibited DC-dependent production of INF-γ, IL-2 and IL-4 by T cells, and IL-6, IL-12p40 and IL-12p70 by DCs (126). Recently, it was demonstrated that DCs generated in the presence of CsA lose their ability to induce Treg cell proliferation, with a strong reduction of IL-12 secretion and particularly of IL-2, which is necessary for the proliferation of $CD4^+CD25^+Foxp3^+$ T cells (127), thus having a negative impact on this population (128). Moreover, TAC was shown to inhibit the ability of DCs to stimulate T cells and to decrease the production CXCL10 and IL-12. Although IL-12 production by DC was impaired by TAC, these cells did not promote Th2 development as T cells stimulated by TAC-treated DCs produced less interferon IFN-γ, IL-4 and IL-10 (129).

In mouse models, RAPA strongly impacts DC maturation and function resulting in the induction of well-defined phenotypic characteristics of these cells. Data accumulated so far indicate that RAPA is able to impair maturation of DCs, reduce DC costimulatory molecule upregulation, decrease the production of pro-inflammatory cytokines, inhibit T cell

stimulatory capacity (130) and promote the selective development of Treg cells in animal models of solid organ transplantation (131). Mouse and human DCs treated with RAPA have an immature phenotype with low levels of CD80 and CD40 receptors and with a decreased expression of B7-H1, the PD-L1 ligand, a negative regulator of T cells activation and inducer of peripheral tolerance (132). In both mouse and human DCs, a reduced expression of CCR7 was observed after treatment with RAPA, an effect that may decrease the ability of DCs to migrate to draining lymph nodes. In an elegant study, Sordi et al. showed how the use of immunosuppressive drugs may interfere with the generation of effective immune responses by affecting DC function (133). In particular, these Authors investigated the functional relevance of chemokine receptors in the process of DC maturation both *in vitro* and *in vivo*. During the maturation process, DCs downregulate the CCR1 and CCR5 receptors and upregulate the expression of CCR7, which promotes the DCs migration from the peripheral tissues to the sites where they will encounter the naive T cells for their priming. CsA and TAC were shown to slightly modulate the expression of CCR7 but without affecting the function of this chemokine receptor. In contrast, RAPA increased the expression of CCR7 at the mRNA and protein level and enhanced the *in vitro* migration of human DCs to CCL19 and of mouse DCs to lymph nodes *in vivo*. Experiments carried out in LPS-maturing DCs suggested that these effects could be related to the ability of RAPA to inhibit autocrine IL-10 production. RAPA-treated alloantigen-pulsed DCs were shown to induce antigen-specific regulation and prolong experimental heart allograft survival. Taner et al. demonstrated that RAPA-exposed DC loaded with donor cell lysates and their adoptive infusion prior to experimental heart transplantation prolonged fully MHC mismatched murine heart allograft survival (134). Notably, RAPA-treated DCs were also shown to stimulate the generation of antigen-specific $Foxp3^+$ Treg cells thereby promoting organ transplant tolerance (135). The induction of tolerance by RAPA is further enhanced by the combination with Flt3L, which promotes the generation of tolerogenic, immunosuppressive DCs along with the production of $CD25^+Foxp3^+$ Treg cells and IL-10 secretion (136).

T cell responses are modulated by cytokines secreted by mDCs including pro-inflammatory cytokines, such as IL-12 and INF-α, and anti-inflammatory cytokines, such as IL-10. DCs treated with RAPA have a distinct cytokine secretion profile at different stages of maturation. In iDCs, RAPA reduced IL-10 and IL-12 production after LPS stimulation and increased apoptosis, while mDCs are resistant to RAPA-induced apoptosis, but they also show decreased production of IL-10 and TNF-α (137). A recent study demonstrated that relevant concentrations of RAPA (20 ng/ml) inhibit the ability of both TLR7- and TLR9-

activated pDCs to stimulate the production of IFN-γ and IL-10 by allogeneic T cells. On the contrary, RAPA-treated TLR7-activated pDCs were capable of stimulating the activation of naive and memory T cells, while also stimulating the generation and proliferation of $CD4^+$ $FoxP3^+$ Treg cells (138).

1.3.2 NF-kB transcription factor

NF-kB is a protein complex that functions as a transcription factor and has a critical role in the regulation of the immune response during infections and inflammatory processes. NF-kB represents a group of structurally related and evolutionarily conserved proteins that belong to the Rel family and are regulated via shuttling from the cytoplasm to the nucleus in response to cell stimulation (139). In the absence of stimulation, the NF-kB complex composed of *Rel* and *p50* protein subunits is in an inactive state in the cytosol, due to the binding with inhibitory proteins *IkBa*, *IkBβ* and *IkBε.* Different signals favor the activation of NF-Kb with consequent phosphorylation of IkB inhibitors from κB kinase (IKK) complex, composed of the kinases IKKα, IKKβ and the regulatory subunit NEMO (IKKγ). Activation of the IKK complex requires K63-ubiquitination of the regulatory subunit NEMO/IKKγ (140). When NF-kB is freed from this complex is made available for translocation into the nucleus where it may activate the transcription of several immune-related genes (141). One of the main roles of NF-kB is to antagonize apoptosis, promoting cell survival by inducing the expression of pro-survival genes (Bcl-2, Bcl-xL, IEX1) and repressing the pro-apoptotic genes (Bax and Bim) (142,143). Since the OTRs have an altered immunological function, the evaluation of the transitional state of the transcription factor NF-kB in T cells and in DCs could represent an index of functional intregrity of the immune system of these patients. The effects of CNI and mTORi against the nuclear translocation of NF-kB are known. In fact, as already described above, the pioneering works of Vadafari et al (106) and Lee et al (124) have shown how CNI reduced nuclear NF-kB translocation in T cells and DCs, respectively. NF-kB persistence in the cytoplasm in phosphorilated form may prevent the transcription of pro-inflammatory cytokines, such as IL-12 and IFN-γ, and the inhibition of specific T-cell responses (106). Furthermore, the inhibition of mTOR regulates negatively signaling in moDCs, potentially leading to the differential phenotypes observed in moDCs (132) . In addiction, in human myeloid moDCs, the inhibition of NF-kB during differentiation and activation has been shown to be associated with apoptosis due to caspase 3 induction and loss of mitochondrial transmembrane potential, demonstrating that this pathway is essential for the development and function of moDCs (144).

2. Aims

The final goal of this study is to improve the current strategies of cancer surveillance for long-term immunosuppressed patients with the identification of an immunologic profile potentially able to more reliably estimate the risk of *de novo* malignancy in OTRs. This is highly relevant considering the central role of immunosurveillance in the control of nascent tumours and the increasing numbers of OTRs living and aging after transplantation.
The specific aims of this project are:

1) To assess whether the occurrence of oncogenic viral reactivation or *de novo* cancer development correlates with measurable changes in the levels of T-cell responses specific for oncogenic viruses and/or tumor-associated antigens.
2) To assess the impact of chronic immunosuppression on the impairment of anti-tumor immune responses through functional analyses of immune cell activation status carried out during the clinical follow-up.
3) To identify an immunologic profile predictive of increased risk of tumor development and viral reactivation in SOTs.

In particular, the study aims to identify and validate, through multiparametric investigations, the potential predictive role of immunological biomarkers, such as CD4 and CD8 T-cell specific immune responses against viral and tumor-associated antigens; furthermore, the activation status of effector cells and DCs and the levels of TERTmRNA in the plasma samples will be also investigated as additional markers of the level of retained immune proficiency in SOT recipients.
The study takes advantage of the immunosurveillance protocol for SOT recipients activated at the Centro di Riferimento Oncologico (CRO) in Aviano (PN), which includes a clinical surveillance program focused on the most frequent and diagnosable *De Novo* tumors with standardized screening (skin, lung, kidney, rectum, cervix and pharynx carcinomas) with integrated immune-virological and clinical follow-up.

3. Results

3.1 Patients characteristics and immunosuppressive regimen

Globally, since 2014, 111 patients were enrolled in our immune-monitoring protocol, of whom 73 were considered evaluable for the study, on the basis of the availability of at least two serial samples for analysis. 57 patients had received kidney transplantation, 8 heart transplantation, 4 liver transplantation, 3 kidney+kidney transplantation and 1 heart+kidney transplantation. Among these patients, 2 were treated with AZA, 55 with CNI, 7 with mTORi and 9 with CNI+mTORi. According to the guidelines of the surveillance protocol, patients at high risk (HR) of tumor onset have had intensive surveillance every2 to 6 months based on the target organ. For low risk (LR) patients, a non-intensive surveillance is performed annually, with a program screening similar to that of the general population. During surveillance, 15 patients were diagnosed with a *de novo* tumor: 9 BCC, 4 SCC, 1 in situ melanoma and 1 kidney tumor. This group include 12 kidney transplants, 1 heart transplant and 2 double transplants. "Tumor" patients had an average of 69 years and 14 years of immunosuppression. 73% of these patients were treated with CNI, 20% with CNI+mTOR and 7% with mTORi. Their clinical characteristics are summarized in Table 1.

Tumor-free patients had an average age of 60 years and 16 years of immunosuppression, and include 43 kidney transplants, 7 heart transplant, 4 liver transplants and two kidney+kidney double transplants. 74% of these patients were treated with CNI, 10% with mTORi and CNI + mTORi respectively and and the remaining 17% with AZA.

Table 1. Clinical characteristics of SOT tumor cohort

Patient	Age	Age of transplant	Years of IS	Transplantated organ	Type of IS	Type of tumor	HR	LR
SOT 04	82	55 and 73	27	Heart+kidney	CNI+mTOR	BCC	yes	no
SOT 07	69	62	7	Kidney	CNI	BCC	yes	no
SOT 08	60	29	31	Kidney	CNI	BCC	yes	no
SOT 11	64	58	6	Kidney	CNI	Kidney	yes	no
SOT 14	67	60	7	Kidney	CNI+mTOR	SCC	yes	no
SOT 34	70	45	25	Kidney	CNI	Melanoma in situ	yes	no
SOT 35	57	41	16	Kidney	CNI	BCC	yes	no
SOT 37	73	65	8	Kidney	CNI	SCC	yes	no
SOT 40	62	57	5	Kidney+kidney	CNI+mTOR	BCC	yes	no

SOT 43	63	49	14	Kidney	mTOR	BCC	yes	no
SOT 49	70	55	15	Heart	CNI	BCC	yes	no
SOT 80	82	68	20	Kidney	CNI	BCC	yes	no
SOT 83	59	50	9	Kidney	CNI	SCC	yes	no
SOT 89	76	62	14	Kidney	CNI	SCC	yes	no
SOT 98	80	73	7	Kidney	CNI	BCC	yes	no

3.2 Detection of viral and tumor epitope-specific T-cell responses in SOT patients and healthy donors

Tumor and viral antigen-specific T-cell responses were comparatively assessed in two cohorts: "tumor" cohort with a *de novo* tumor occurred during surveillance (n=15 patients), and a "no tumor" cohort (58 patients). For all these patients, samples taken at baseline and during follow-up/diagnosis were available. For the first group, we chose as reference the sample taken as close as possible prior to the time of the diagnosis of cancer (± 4 months from the diagnosis). T-cell responses against pools of peptides were assessed by IFN-γ ELISpot assay (interferon-γ-Enzime linked immunosorbent Spot) in samples from all 73 patients and from 9 healthy donors.

3.2.1 T cell responses against Tumor Associated Antigens (TAAs)

T-cell responses to 2 "universal" TAA-derived peptides mix (Survivin and hTERT) were evaluated in un-stimulated PBMCs by IFN-γ ELISpot assay in 9 healthy donors as control and in 65 patients at baseline and at one follow up time point. To investigate the correlation between the extent of spontaneous T-cell responses against TAAs and the tumor onset, we divided our patients into two cohorts: 15 patients with cancer occurred during surveillance and 50 tumor-free patients. As shown in Figure 1A, the levels of T-responses specific for hTERT detected at baseline (prior to tumor development) in the "tumor" cohort were significantly lower compared to those observed at the time of the diagnosis of cancer. The level of hTERT-specific T-cell responses was higher at the time of diagnosis of cancer in the "tumor" cohort compared to the levels observed at the baseline in the "no tumor" cohort and in healthy donors. No significant differences were observed between to the values observed at baseline and follow up in the "no tumor" cohort and as compared to those detected in healthy donors (Figure 1A). Similarly, the levels of T-cell responses against Survivin at the time of

diagnosis of cancer in the "tumor" cohort were significantly higher as compared to those observed before the onset of the tumor in the same group. In addition, also the levels of Survivin-specific T-cell responses observed at the time of follow up in "no tumor" cohort were significantly higher than those of healthy donors. We also detected significantly higher levels of Survivin-specific T-cell responses at follow up in the "no tumor" cohort as compared with those of healthy donors (Figure 1B). Table 2 summarizes the mean and median values of results obtained in both cohort of patients at baseline and follow up.

Table 2. Spontaneous T cell responses against TAAs in SOTs

		"Tumor" cohort		"No Tumor" cohort		Donors
		Baseline	Diagnosis	Baseline	Follow up	
hTERT	**Mean**	89	90	19.5	41.74	9
	SD	306	138	39	88	5
	Median	9	20	9.5	15	8
Survivin	**Mean**	44	210	53.2	61.94	7
	SD	131	358	158	167	4
	Median	10	11	8	15	7
TOT pts		15		50		9

	B Tc vs D Tc	B Tc vs B NTc	B Tc vs F NTc	B Tc vs d	D Tc vs B NTc	D Tc vs F NTc	D Tc vs d	B NTc vs F NTc	B NTc vs d	F NTc vs d
P-values hTERT	**0.026***	0.960	0.238	0.834	**0.006***	0.854	**0.010***	0.066	0.984	0.194
P-values Survivin	0.142	0.837	0.153	0.509	0.434	0.535	**0.049***	0.057	0.674	**0.036***

B: Baseline; D: Diagnosis F: Follow up; Tc: "Tumor" cohort; NTc: No "Tumor" cohort; d: donors *$p < 0.05$

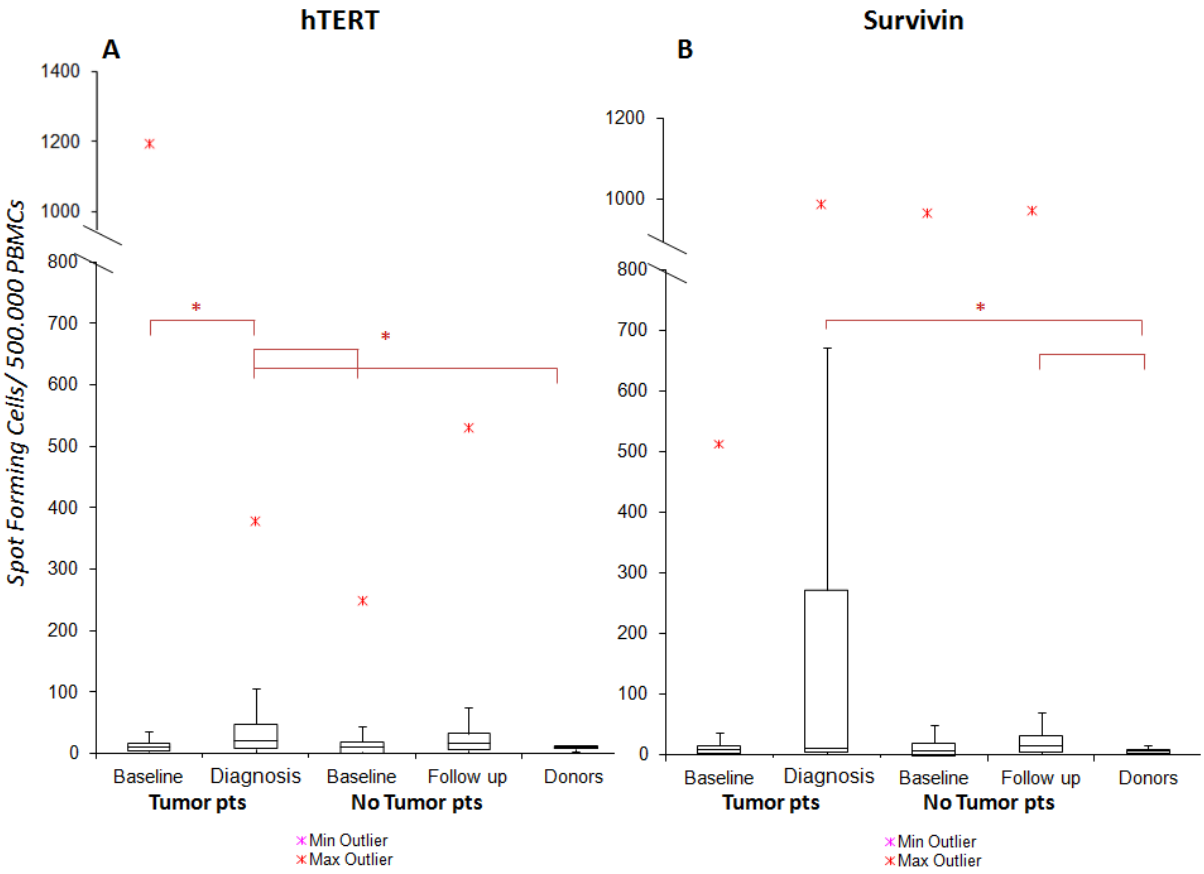

Figure 1. Spontaneous T cell responses against TAAs in SOTs. All tests were performed using PBMCs from patients at different time point. The number (enumerated as SFC, spot forming cells) of TAA-specific T cells responses was investigated in tumor and no tumor cohort of patients. (*p < 0.05, Mann-Whitney test).

3.2.2. Association between TERTmRNA levels in the blood and tumor onset in SOTs

Telomerase is responsible for telomere maintenance in the majority of human cancers, but its activity is absent in normal cells. Telomerase consists of two subunits: a functional catalytic protein subunit, the human telomerase reverse transcriptase (h*TERT*) encoded by the *TERT* gene, and a RNA component called human telomerase RNA component (h*TERC* or h*TR*), encoded by the *TERC* gene. This specialized DNA polymerase plays a pivotal role in carcinogenesis through the maintenance of telomeres (145). Recent evidence indicates that quantification of hTERT mRNA in the blood may be a reliable tumor biomarker that may correlate with clinicopathological parameters and treatment efficacy. Considering that SOT patients who developed a tumor showed increased levels of T-cell responses against hTERT, we investigated whether the levels of TERT mRNA in the blood could constitute an early marker of tumor development in these patients. On these grounds, circulating cell-free TERT mRNA was quantified by real-time PCR (146) in 30 patients at baseline and follow up,

respectively. Patients were stratified into two groups: 15 patients with a tumor event and 15 cancer-free patients during surveillance. In the "tumor cohort" at baseline, the median of TERT levels was 61 (0-576, mix and max value respectively; mean=116) copies/ml, while at follow up/diagnosis the median value was 56 (0-421, mix and max value respectively; mean=102) copies/ ml (p=0.773, not significant). Globally, the levels of circulating TERT mRNA were significantly higher in patients who developed a tumor as compared with the tumor-free cohort. In particular, in the "no tumor" cohort at baseline, the median of TERT levels was 0 (0-88, mix and max value respectively; mean=24) copies/ml and at follow up the median was 0 (0-92, mix and max value respectively; mean=25) copies per ml (P=0.918, not significant). The level of circulating cell-free TERT mRNA at baseline in the "tumor" cohort was significantly higher if compared with circulating cell-free TERT mRNA at baseline of "no tumor" cohort (P=0.033). Further, the level of circulating cell-free TERT mRNA at follow up of "tumor" cohort was significantly higher if compared with circulating cell-free TERT mRNA at follow up of "no tumor" cohort (P=0.022). (Figure 2).

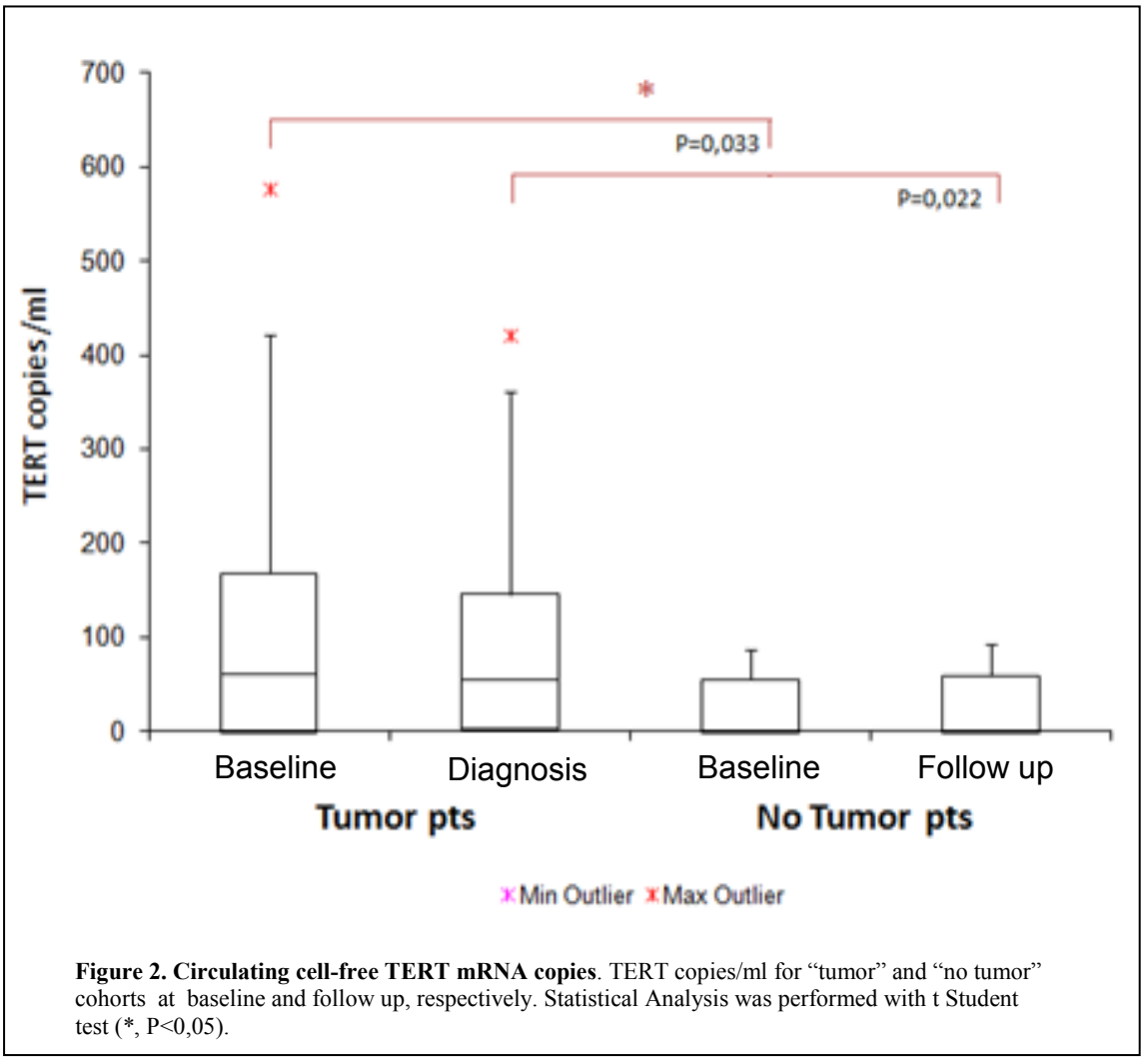

Figure 2. Circulating cell-free TERT mRNA copies. TERT copies/ml for "tumor" and "no tumor" cohorts at baseline and follow up, respectively. Statistical Analysis was performed with t Student test (*, P<0,05).

3.2.3 T cell responses against Virus antigenic epitopes

T-cell responses in un-stimulated PBMCs to CMV, EBV and HPV peptide mix were investigated by IFN-γ ELISpot assay in 5 healthy donors and in 73 patients at baseline and at one follow up time point. To evaluate the correlation between tumor onset and viral reactivation/infection in patients with cancer, we considered two groups: 15 patients of the "tumor" cohort and 58 patients of the "no tumor" cohort. No significant differences were detected for both CMV- (Figure 3A) and EBV-specific (Figure 3B) T-cell responses by comparing the two patient cohorts and each cohort with controls. The levels of T-cells responses against HPV before and at the time of the diagnosis of cancer in the "tumor" cohort were significantly lower if compared with those detected in healthy donors. Significantly lower levels of HPV-specific T-cells responses were also observed between both baseline and follow up in the "no tumor" cohort and in healthy donors (Figure 4). Table 3 summarizes the mean and median values obtained in both cohort of patients at baseline and follow up.

Table 3. T-cell responses against virus mix derived-peptides in SOTs

		"Tumor" cohort		"No Tumor" cohort		Donors
		Baseline	Diagnosis	Baseline	Follow up	
CMV	**Mean**	753	548	558	906	269
	SD	886	331	788	1177	289
	Median	410	459	337	487	95
EBV	**Mean**	217	271	212	307	134
	SD	350	417	322	425	120
	Median	84	58	101	143	80
HPV	**Mean**	18	121	81	171	235
	SD	36	239	268	495	173
	Median	7	12	10	21	280
TOT pts		15		58		5

	B Tc vs D Tc	B Tc vs B NTc	B Tc vs F NTc	B Tc vs d	D Tc vs B NTc	D Tc vs F NTc	D Tc vs d	B NTc vs F NTc	B NTc vs d	F NTc vs d
p-values CMV	0.834	0.472	0.849	0.222	0.267	0.741	0.190	0.112	0.407	0.159
p-values EBV	0.787	0.857	0.322	0.897	0.810	0.298	0.928	0.142	0.772	0.358
P-values HPV	0.280	0.575	**0.012**	**0.002***	0.575	0.303	**0.037***	**0.030***	**0.005***	**0.021***

B: Baseline; D: Diagnosis F: Follow up; Tc: "Tumor" cohort; NTc: No "Tumor" cohort; d: donors *$p < 0.05$

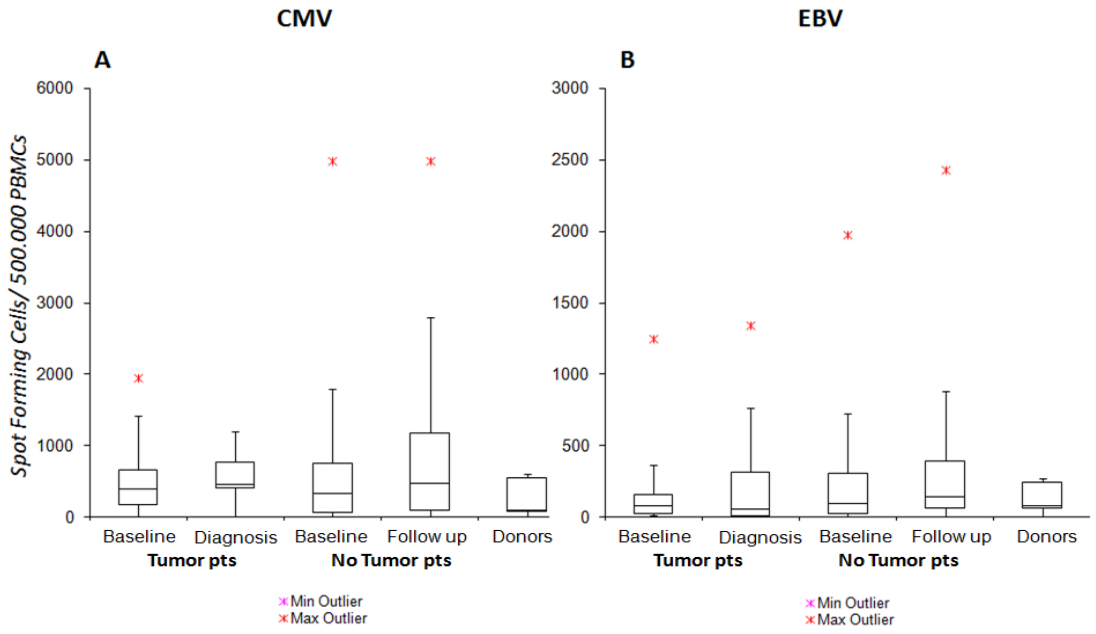

Figure 3. Spontaneous T cell responses against CMV and EBV mix peptides. All tests were performed using PBMCs from patients at different time point. The number (enumerated as SFC, spot forming cells) of TAA-specific T cells responses was investigated in tumor and no tumor cohort of patients. ((*p < 0.05, Mann-Whitney test).

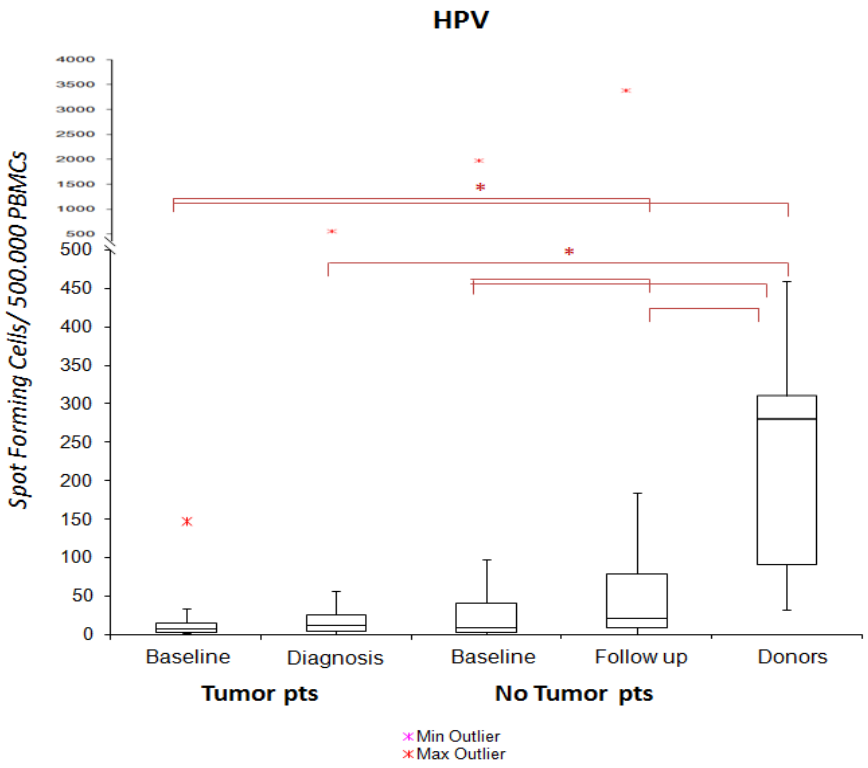

Figure 4. Spontaneous T cell responses against HPV mix peptides. All tests were performed using PBMCs from patients at different time point. The number (enumerated as SFC, spot forming cells) of TAA-specific T cells responses was investigated in tumor and no tumor cohort of patients. (*p < 0.05, Mann-Whitney test).

3.3 Phenotypic and Functional analyses

3.3.1 Immunophenotypic characterization of T-cells from SOT patients.

Globally, peripheral T lymphocytes from 27 patients were investigated for the main markers of senescence, exhaustion and activation (Table 4). In parallel, samples from the same patients were analyzed for the activation status of T-cells and DCs through multispectral imaging flow cytometry. Patients were stratified for the onset (n=6 patients) or absence (n=21 patients) of tumor during surveillance. Patients' whole blood was labeled with CD3-FITC, CD4-APC-H7, HLA-DR-PerPC-Cy5.5, CD38-PE-Cy7, Tim-3-APC antibodies and with CD3-PerPC-Cy5.5, CD4-APC, CD8-APC-Cy7, CD28- PE-Cy7, CD57- FITC, PD-1-PE antibodies.

13 samples from healthy donors were stained for the same antibodies. The control cohort of healthy donors had an average age of 55 years. Table 4 summarizes the average numbers of the different subpopulations investigated in the $CD4^+$ and $CD8^+$ compartments, respectively, at a single time point. The percentages of the investigated subpopulations were stable in the serial samples investigated for each patient. No significant differences were observed between the two cohorts for any of the subpopulations considered and also compared to healthy donors. Globally, SOT patients showed a higher percentage of both $CD4^+$ and $CD8^+$ T cells as compared with donors. In addition, both SOT cohorts showed a significant increase in the percentage of $CD4^+CD28^-CD57^+$ and $CD8^+CD28^-CD57^+$ terminally differentiated senescent T-cells, and a decrease in the percentage of $CD8^+PD\text{-}1^+$ T lymphocytes, probably exhausted.

Table 4. Immunophenotype of senescent and activate $CD4^+$ and $CD8^+$ T-cells of SOT patients

	"Tumor"cohort		"No tumor" cohort		Donors	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
% tot						
CD3+CD4+	41.93%	8.78	46.12%	10.83	44.58%	6.01
CD3+CD8+	27.13%	10.89	22.15%	9.41	19.57%	6.15
Senescence markers						
$CD4^+CD28^-$ $CD57^+$	8.15%	13.79	5.94%	9.62	2.98%	4.65
$CD8^+CD28^-$ $CD57^+$	20.70%	19.05	28.28%	17.79	21.12%	15.84
Exhaustion markers						
$CD4^+PD\text{-}1^+$	28.50%	7.97	28.81%	13.15	27.52%	7.41
$CD8^+PD\text{-}1^+$	26.95%	19.28	25.84%	15.73	33.73%	8.54
$CD4^+Tim\text{-}3^+$	1.16%	1,34	0.86%	0.74	1.14%	0.71
$CD8^+Tim\text{-}3^+$	3.58%	4.08	2.38%	2.05	3.47%	3.57
Activation markers						
$CD4^+CD38^+DR^+$	3.03%	3.62	1.27%	0.94	NA	NA
$CD8^+CD38^+DR^+$	4.75%	3.43	3.27%	2.64	NA	NA

*$p < 0.05$, Mann-Whitney test

3.3.2 Evaluation of immune cells activation status through multispectral imaging flow cytometry

The activation status of the immune cells involved in antigen-presentation (DCs) and effector functions (T-cells) was investigated by analyzing a functional marker in an assay based on multispectral imaging flow cytometry. Multispectral imaging flow cytometry allows a precise enumeration of cells carrying a nuclear translocation of the NF-κB p65 protein, as a functional marker of NF-κB activation. A recent study carried out in our laboratory in oligometastatic breast cancer patients treated with stereotactic body radiotherapy, the number of circulating NK cells showing NF-kB nuclear translocation correlated with an increase in perforin production and NK cell-mediated cytotoxicity (147). We have also previously demonstrated that the number of circulating NK cells showing NF-kB nuclear translocation

correlated with the induction of a pathologic complete responses in HER2^{+} breast cancer patients treated with a neo-adjuvant chemio-immunotherapy schedule (148)

Patients' PBMCs were stained for anti-NF-κB/p65-PE and DRAQ5 for nuclear imaging. Cells were run on the ImageStreamX cytometer using the INSPIRE software and imaged. Cells that were in focus were positive for both DRAQ5 and NF-kB/p65 and images were analyzed using the IDEAS software. The extent of internalization was calculated with an algorithm of the IDEAS software that measures the similarity score between DRAQ5 and NF-kB staining. A similarity score is determined for every cell based on a pixel by pixel correlation of the nuclear image to the NF-kB image (Figure 5). The threshold of similarity score to consider translocated NF-kB was defined using as negative and positive control ML-1 cell line untreated or treated with TNF-α, respectively (Figure 6).

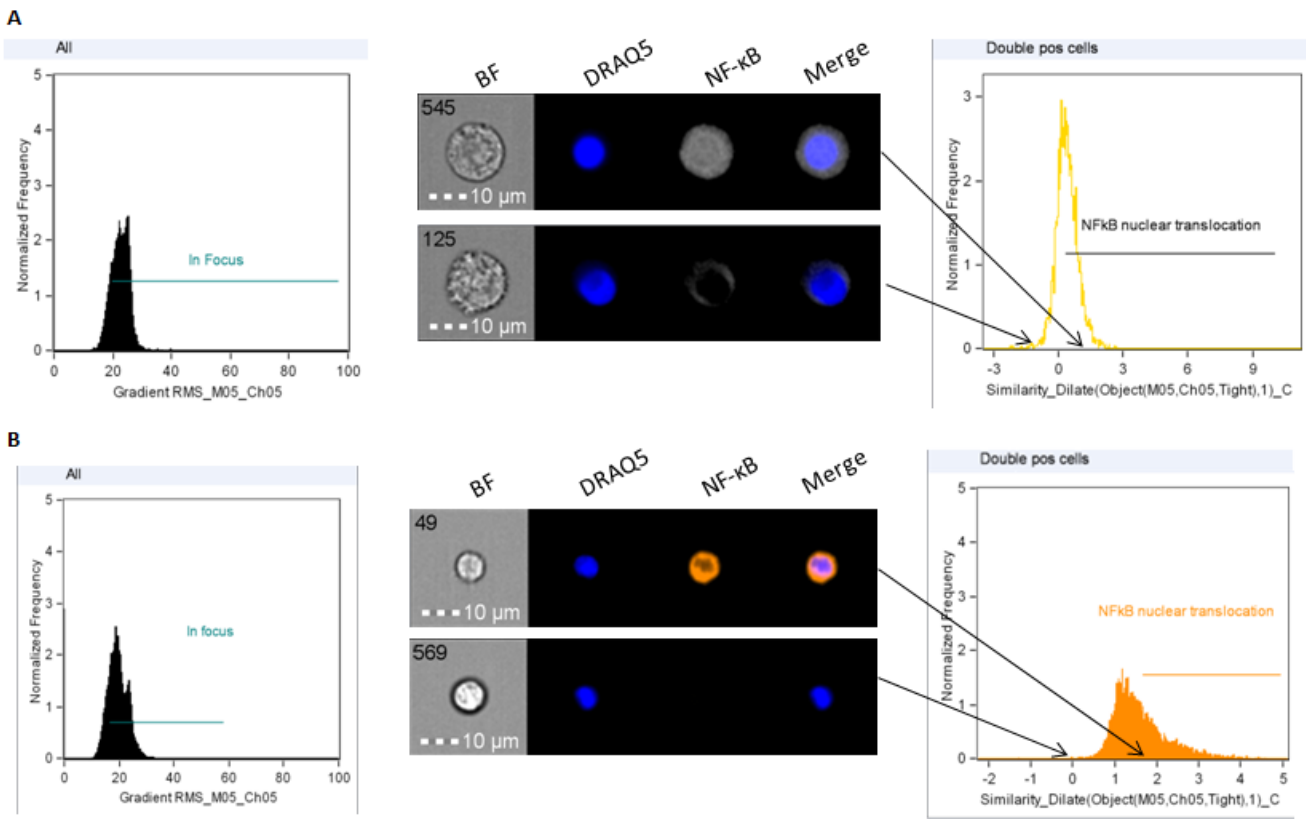

Figure 5. NF-kB nuclear translocation. A) Representative figure of NF-kB translocation in INF-γ DCs. The positivity of acquired events was established through correlation between similarity score of DRAQ5 and NF-kB staining. For dendritic cells the score was set at 0.5. **B)** Representative figure of NF-kB translocation in CD4^{+} T cells. The positivity of acquired events was established through correlation between similarity score of DRAQ5 and NF-kB staining. For T cells the score was set at 1.5.

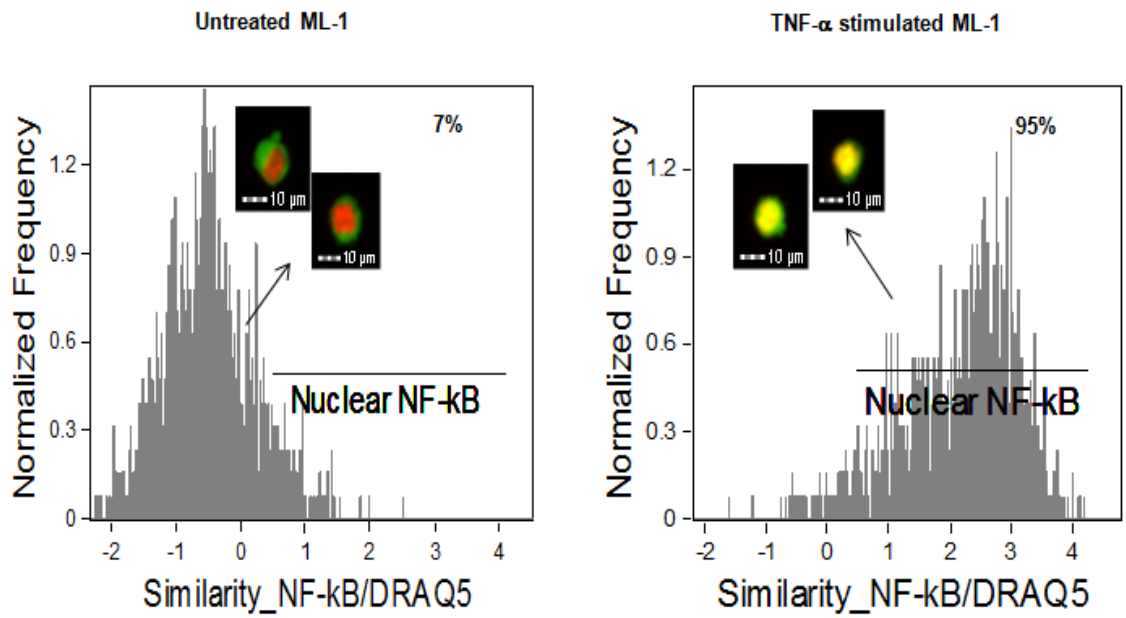

Figure 6. ML-1 cell line untreated or treated with TNF-α was used as a negative and positive control, respectively, for defining the threshold of SS (Similarity Score) to considered translocated NF-kB.

3.3.3 NF-kB nuclear translocation in circulating T lymphocytes

To evaluate the activation status of patient T lymphocytes, PBMCs were labeled with CD4-FITC and CD8-PC7 antibodies. After permeabilization, T-cells were labeled with NF-kB/p65-PE antibody and DRAQ5. Cells were run on ImageStreamX cytometer using 488nm and 785nm laser excitation and 30000 events were acquired for each sample. Patients were stratified for the onset (n=6 patients) or absence (n=21 patients) of tumor during surveillance. T cells from healthy donors (n=4) were stained with the same protocol as control. In the "tumor" cohort, significantly lower numbers of activated $CD4^+$ T cells were observed after tumor onset as compared to the baseline. The numbers of activated $CD4^+$ T cells detected in the "tumor" cohort after tumor development were significantly lower than those observed at baseline in the "no tumor" cohort or in healthy donors (Figure 7A). The activation status of $CD8^+$ T cells at diagnosis was significantly decreased if compared with the activation status of the same cells in healthy controls. On the contrary, the group of "no tumor" patients retained a nearly normal level of $CD8^+$ T-cell activation at both baseline and follow-up (Figure 7B). Table 5 summarizes the average and median numbers of $CD4^+$ and $CD8^+$ T cells showing NF-kB nuclear translocation at baseline and at follow up in both cohorts.

Table 5. “Tumor” patients

	$CD4^+$ T cells baseline	$CD4^+$ T cells diagnosis	Donors $CD4^+$ T cells	$CD8^+$ T cells baseline	$CD8^+$ T cells diagnosis	Donors $CD8^+$ T cells
% cells gated	46.59%	50.69%	40.2%	19.30%	12.61%	21.65
Mean NF-kB	38.90%	21.68%	44.65%	35.80%	22.81%	42.96%
SD	11.64	13.56	2.73	12.42	16.45	2.30
Median NF-kB	41.485	20.835	44.64	39.009	26.4	42.95

Table 5. “No tumor” patients

	$CD4^+$ T cells baseline	$CD4^+$ T cells follow-up	Donors $CD4^+$ T cells	$CD8^+$ T cells baseline	$CD8^+$ T cells follow-up	Donors $CD8^+$ T cells
% cells gated	51.40%	76.46%	40.2%	20.05%	17.23%	21.65
Mean NF-kB	38.16%	37.17%	44.65%	38.45%	38.10%	42.96%
SD	14.10	14.14	2.73	14.10	15.04	2.30
Median NF-kB	40.76	40.88	44.64	43.4	41.88	42.95

	p values $CD4^+$ T-cells	p values $CD8^+$ T-cells
Baseline Tc vs Diagnosis Tc	**0.040***	0.156
Baseline Tc vs Baseline NTc	0.899	0.665
Baseline Tc vs Follow up NTc	0.767	0.711
Baseline Tc vs Donors	0.294	0.224
Diagnosis Tc vs Baseline NTc	**0.030***	0.071
Diagnosis Tc vs Follow up NTc	**0.039***	0.077
Diagnosis Tc vs Donors	**0.008***	**0.029***
Baseline NTc vs Follow up NTc	0.822	0.938
Baseline NTc vs Donors	0.074	0.192
Follow up NTc vs Donors	**0.042***	0.184

Tc: “Tumor” cohort; NTc: No “Tumor” cohort; *$p < 0.05$

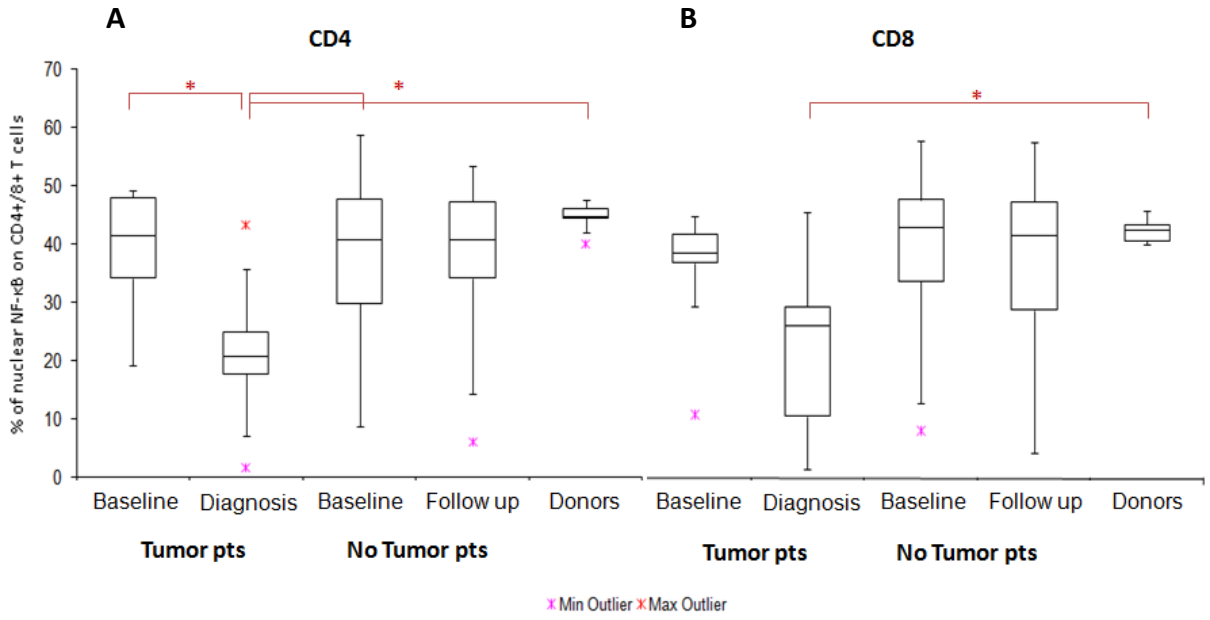

Figure 7. Effects of IS treatment on NF-kB nuclear translocation in T cells. 2x10^6 PBMCs of each sample were strained with CD4-FICT, CD8-PEPC7, NF-kB-PE and DRAQ5 antibodies. Cells were run on ImageStreamX cytometer using 488nm and 785nm laser excitation and were collected 30000 events for each samples. **A)** Graphs represent the % of nuclear NF-kB nuclear translocation in $CD4^+$ T cells of each group of samples, stratified for tumor and no tumor event, both at baseline and follow up (*p < 0.05, t Student test). NF-kB nuclear translocation was evaluated by multispectral imaging flow cytometry through the Similarity Score (SS) algorithm. NF-kB/DRAQ5 SS indicates the percentage of T cells with NF-kB translocated into the nucleus. **B)** Graphs represent the % of nuclear NF-kB nuclear translocation in $CD8^+$ T cells of each group of samples, stratified for tumor and no tumor event, both at baseline and follow up (*p < 0.05, t Student test). NF-kB nuclear translocation was evaluated by multispectral imaging flow cytometry through the Similarity Score (SS) algorithm. NF-kB/DRAQ5 SS indicates the percentage of T cells with NF-kB translocated into the nucleus.

3.3.4 NF-kB nuclear translocation in monocyte-derived DCs

Monocytes from patients' PBMCs were cultured in duplicate for 1 week with GM-CSF and IL-4 for differentiation in iDCs. At day 6 of culture, LPS + IFN-γ were added O/N as maturation stimulus, and mDCs were harvested at day 7 of culture. Subsequently, DCs were labeled with CD3-FITC and CD14-ECD antibodies to exclude contaminating lymphocytes and myeloid cells from the analysis. After permeabilization, DCs were labeled with the NF-kB/p65-PE antibody and DRAQ5. Cells were run on ImageStreamX cytometer using 488nm and 785nm laser excitation and 20000 events were collected for each sample. Patients were stratified for the onset (n=6 patients) or absence of cancer (n=20 patients) occurred during surveillance. DCs from healthy donors (n=4) were cultured and stained with the same protocol. The number of INF-γ DCs with activated nuclear NF-kB was significantly lower at diagnosis in the "tumor" cohort. Activation status of INF-γ DCs at diagnosis was also significantly lower if compared with activation status at both baseline and follow up in the "no tumor" cohort. Activation status of IFN-γ DCs of both cohorts at baseline and follow up was significantly lower if compared with that of DCs generated from healthy donors (Figure

8). Table 6 summarizes the average and median numbers of IFN-γ DCs showing NF-kB nuclear translocation at baseline and at follow up in both cohorts.

Table 6. Tumor patients

	IFN- γ DCs baseline	IFN- γ DCs Diagnosis	IFN- γ DCs Donors
Mean NF-kB	16.53%	13.19%	29.25%
SD	5.77	4.94	2.86
Median NF-kB	17.34	13.9	29.25

Table 6. No tumor patients

	IFN- γ DCs baseline	IFN- γ DCs follow-up	IFN- γ DCs Donors
Mean NF-kB	20.79%	20.72%	29.25%
SD	12.82	13.51	2.86
Median NF-kB	26.43	26.125	29.25

	p values IFN-γ DCs
Baseline Tc vs Diagnosis Tc	0.308
Baseline Tc vs Baseline NTc	0.265
Baseline Tc vs Follow up NTc	0.287
Baseline Tc vs Donors	**0.002***
Diagnosis Tc vs Baseline NTc	**0.041***
Diagnosis Tc vs Follow up NTc	**0.050***
Diagnosis Tc vs Donors	**0.000***
Baseline NTc vs Follow up NTc	0.987
Baseline NTc vs Donors	**0.019***
Follow up NTc vs Donors	**0.022***

Tc: "Tumor" cohort; NTc: No "Tumor" cohort; *$p < 0.05$

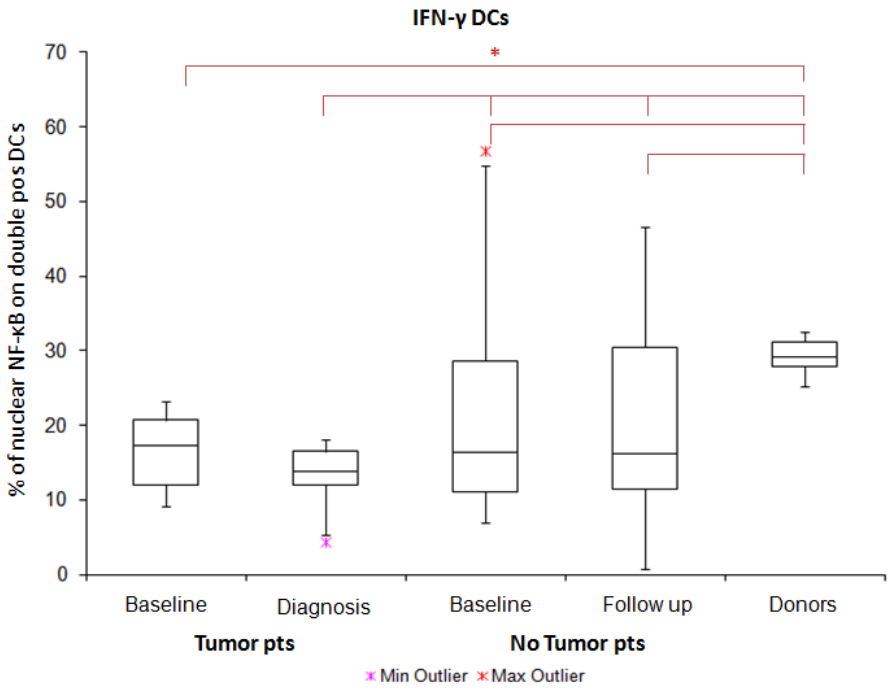

Figure 8. Effects of IS treatment on NF-kB nuclear translocation in INF-γ DCs. 1x106 DCs of each sample were strained with CD4NF-kB-PE and DRAQ5 antibodies. Cells were run on ImageStreamX cytometer using 488nm and 785nm laser excitation and were collected 20000 events for each samples. NF-kB nuclear translocation was evaluated by multispectral imaging flow cytometry through the Similarity Score (SS) algorithm. NF-kB/DRAQ5 SS indicates the percentage of IFN-γ DCs with NF-kB translocated into the nucleus. (*p < 0.05, t Student test).

3.3.5 Iatrogenic immunosuppression impairs maturation of DCs generated from healthy donors

To more directly investigate the functional impact of immunosuppressive drugs on DCs, we have also characterized by flow cytometry the phenotypic profile of DCs generated from healthy donors and treated with Tacrolimus or Everolimus *in vitro*. DCs were isolated and matured from purified $CD14^+$ cells obtained from 4 healthy donors and cultured with the same protocol adopted for patient-derived DCs. DCs derived from healthy donors were treated with Tacrolimus (TAC) or Everolimus (EVR) at different concentrations (Figure 9).

mDCs were labeled for the main lineage markers, HLA-DR-PeCy7 and CD11c-PerCP-Cy5.5 antibodies, and for the main maturation markers, CD86-BV421 and CD83-FITC antibodies. The results obtained showed that immunosuppressive treatment with CNI or mTORi at different concentrations inhibited iDCs maturation ability. The expression of HLA-DR and CD11c in mDCs from healthy donors treated with CIN and mTORi was significantly lower if compared to untreated mDCs (NT). Interestingly, the significant downregulation of CD11c

was observed at both concentrations of EVR. Both concentrations of EVR significantly down-regulated CD86 expression as well as did the highest concentration of TAC. For CD83, however, we did not observe significant changes in cells treted with TAC, although a trend to a lower expression of these markers was detected. On the contrary, both concentrations of EVR significantly decreased CD86 expression.

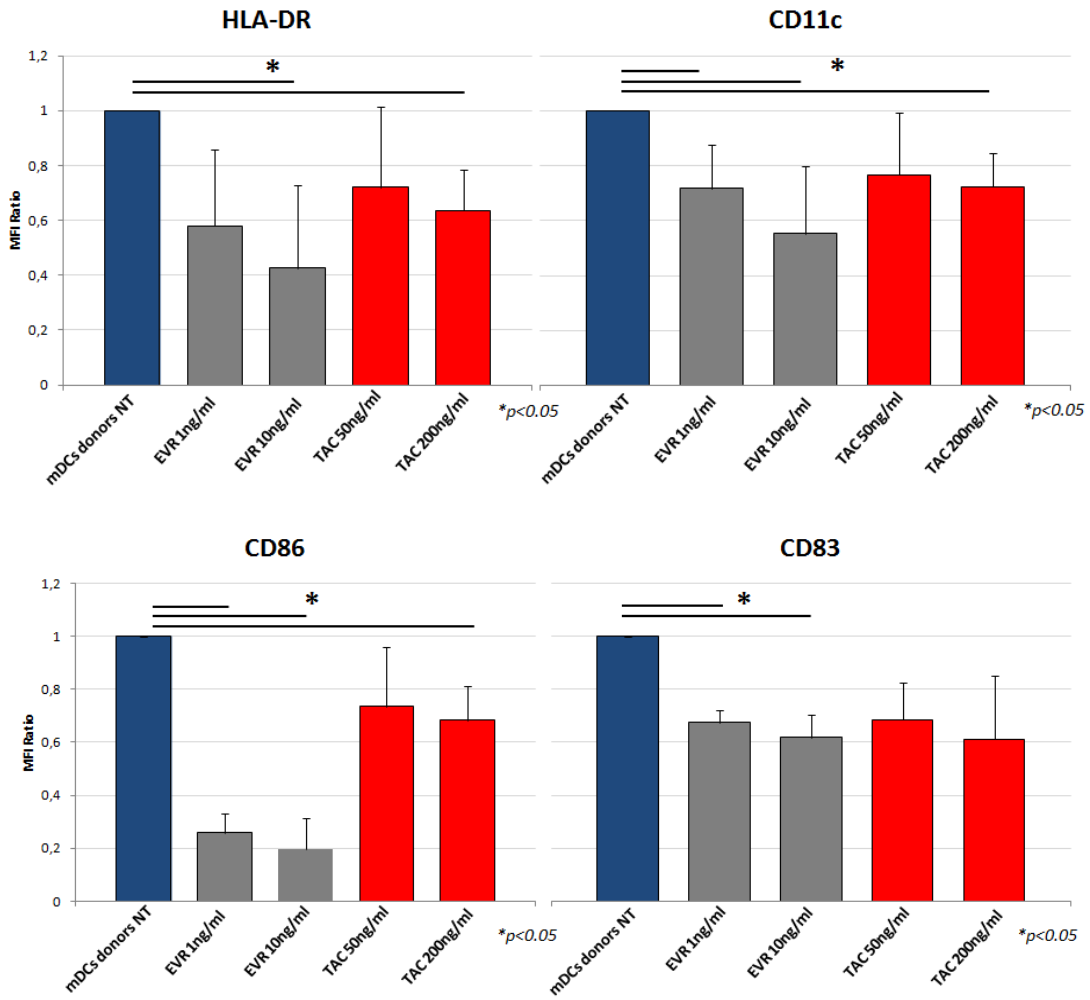

Figure 9. Flow cytometry evaluation of lineage and maturation markers. Immunophenotyping of DCs from healthy donors was performed to through the analysis of the expression of the main line and maturation markers. The differentiation of IFN-γ DCs from healthy donors was investigate after a week of culture in the presence of two different concentration of TAC and EVR. Statistical Analysis was performed with t Student test (*, P<0,5).

4. Discussion

Although pharmacologic immunosuppression is essential to prevent allograft rejection in SOTs, it may be variably associated with an increased risk of tumor development and reduced control of viral infections by the immune system. Despite the availability of new immunosuppressive drugs, such as mTORi, the possibility of developing a cancer 10 years after transplantation remains high in these patients. Various concomitant factors play a fundamental role in this scenario, such as the duration of the immunosuppressive regimen, the age of transplantation, physiological aging and especially the persistence of opportunistic infections by oncogenic virus.

Available data clearly indicate that SOT patients have a 2-5-fold higher risk to develop a *De Novo* neoplasm compared to the general population (4). In particular, the increased risk correlates with the duration of iatrogenic immunosuppression, which may markedly reduce the efficacy of host immune immune-surveillance. This may result in uncontrolled proliferation of tumor cells and sustained reactivation/replication of oncogenic viruses, particularly HPV and EBV, which are pathogenically associated with non-melanoma skin cancer and lymphoproliferative diseases, respectively. Non-oncogenic latent viruses may also play a role in tumor development in SOTs, due to their ability to inhibit T-cell functions, as in the case of CMV, whose infection/reactivation may result in loss of the replicative capacity of immune effector cells.

The mechanisms of immune-surveillance involve different cell populations of the immune system, which are able to capture, process and eliminate the non-self. In particular, APCs are the main orchestrators of the initiation of the immune response against non-self viral and tumor antigens. T-cell activation is driven by the simultaneous triggering of the TCR/CD3 receptor and co-stimulatory molecules such as CD28 by the APCs, necessary for the optimal activation of T cells, resulting in migration of the NF-kB factor into the nucleus and massive production of IL-2 and other relevant cytokines (149,150).

As discussed above, some immunosuppressive drugs such as CNIs may have a direct role in promoting neoplastic transformation, as they increase the expression of TGF-β and VEGF, which may promote local immunosuppression and tumor cell proliferation. In addition, administration of CNIs would seem to reduce the extent of NF-kB nuclear translocation in both T cells and DCs, respectively (106,124), consistent with decreased activation levels of these immune cells. Other drugs, such as mTORi, exert powerful anti-proliferative effects, being able to inhibit neoangiogenesis and direct growth-inhibitory action in neoplastic cells, by blocking the mTOR pathway, a serine-threonine kinase involved in cell

growth, proliferation, protein synthesis and apoptosis. Despite these anti-tumor effects, however, mTORi may also have a negative impact on the activation and function of immune cells in the SOT patients (132).

Considering that the long-term iatrogenic immunosuppression of transplanted patients may lead to the development of virus-unrelated and oncogenic virus-driven tumors, the identification of biomarkers predictive of the risk of impending tumor development constitutes an unmet clinical need in this setting. To address this clinically relevant issue, we have taken advantage of the program of clinical and laboratory surveillance of SOT patients recently activated at the CRO-IRCCs Aviano. The routine workup of these patients was implemented with a panel of phenotypic and functional immunoassays aiming to identify and validate, through multiparametric investigations, the potential predictive role of immunological biomarkers, such as CD4 and CD8 T-cell specific immune responses against viral and TAAs and the activation status of effector cells and DCs.

Our results concerning the activation status of patient-derived DCs showed that, in the "tumor" cohort, the activation status of DCs at the time of diagnosis was significantly lower than that at baseline and that observed in the “no tumor cohort”. Due to the limited number of patients who have so far developed a tumor, it was not possible to make any informative stratification according to the type of treatment. With the exception of one patient who received a CNI+mTORi combined therapy, all patients who developed a tumor were treated with a CNI-based immunosuppressive regimen. Despite the limited size of our cohort, we observed a significant decrease in the level of NF-kB activation in IFN-γ DCs generated from these patients, consistently with previous findings on the inhibitory effects exerted by CNIs on immune cells (124,125). In the cohort of SOT patients who did not develop tumor (CNI=15; mTORi=2; CNI +mTORi=3), we observed that IFN-γ DCs were significantly less activated as compared to healthy donors, both at baseline and during follow-up. Globally, the activation status of IFN-γ DCs in the “tumor” cohort was lower than that observed in SOT patients of the “no tumor” cohort, suggesting that DC activation could constitute a potential predictive marker. Nevertheless, the interpatient variability of the data observed together with the limited sample size of this pilot study did not allow the identification of a cut-off value of potential applicative relevance. These findings may also have pathogenic implications, suggesting that a more profound impairment in DC activation could lead to suboptimal generation of anti-tumor T-cell immunity, thus engancing the risk to develop a tumour in chronically immunosuppressed SOT recipients.

The analysis of NF-kB nuclear translocation gave similar results with regard to the activation status of T cells. Globally, activation of $CD4^+$ and $CD8^+$ T-cells in the "tumor" cohort before the diagnosis of cancer, was comparable to that of tumor-free SOT patients and healthy donors. These findings are consistent with a retained ability of these T-cell subpopulations to be activated at near-physiological levels despite the treatment with immunosuppressive drugs. Notably, the activation status of both $CD4^+$ and $CD8^+$ T lymphocytes at the time of diagnosis of cancer was significantly lower than that observed in the same patients prior to tumor onset.

Although the number of patients analyzed in the present study is too limited to draw definite conclusions, our results suggest that inclusion of the analysis of the activation profile of T cells and DCs in a larger prospective study could validate the potential role of these biomarkers as predictive of the cancer onset in SOT patients.

A larger number of patients were analyzed by ELISpot assay to monitor the number of IFN-γ-releasing T-cells against viral and TTA epitopes. Of note, significantly higher numbers of T-cells specific for hTERT and Survivin were detected at the time of diagnosis of cancer in the blood of SOT patients although with a certain degree of variability among patients. These findings indicate that, despite iatrogenic immunosuppression, SOT patients developing a tumor are able to mount IFN-γ T-cell responses against "universal" TAAs, such as hTERT and Survivin. These findings are of particular relevance in the light of the possible role of T-cell responses specific for hTERT and Survivin as potential biomarkers of impending tumor development in these patients. Globally, IFN-γ T-cell responses against hTERT and Survivin in the "no tumor" cohort were on average lower than those of the "tumor" cohort, with the only exception of the specific T-cell responses detected against Survivin in the follow up of the "no tumor" cohort. Notably, in these cases, Survivin-specific T-cells responses were significantly higher compared to healthy donors. Survivin is an inhibitor of apoptosis that interferes with post-mitochondrial events including activation of caspases The detection of increased T-cell responses to Survivin during the follow-up in the cohort of cancer-free patients is intriguing and deserves further investigation to better understand its biological meaning and particularly whether it may help predicting the risk of cancer in this setting. These findings provide the rationale to prospectively analyze T-cell responses to universal TAAs in a large series of SOT patients to better define the potential contribution of these analyses to the definition of their risk of cancer.

Considering that elevated hTERT mRNA in peripheral blood is closely related with clinicopathological parameters, treatment efficacy and other tumor biomarkers of cancer

patients, we implemented our immune profiling with the quantification of circulating hTERT mRNA in our SOT cohorts. The rationale for this investigation was also supported by the finding of increased T-cell responses specific for hTERT in SOT patients developing a tumor. Globally, patients of the "tumor" cohort showed significantly higher levels of circulating hTERT mRNA as compared to those of the "no tumor" cohort. These findings are consistent with available data indicating that hTERT expression is specific of the transformed phenotype (151–153). In fact, several studies have been demonstrated that circulating TERT mRNA is an independent prognostic marker in different types of tumors. Circulating TERT mRNA was found in plasma samples of patients with gastric cancer but was not detected in plasma samples of healthy volunteers (154). Plasma hTERT mRNA levels were also detected in patients with elevated PSA levels and associated with poor prognosis, potentially behaving as a factor indicative of biochemical recurrence (155). In addition, hTERT mRNA levels in plasma samples of patients with colorectal (146) or lung cancer were identified as possible promising non-invasive tumor biomarker in clinical practice (156). Our findings are consistent with the available data mentioned above, indicating that hTERT expression is specific of the transformed phenotype (151–153). Our observation that the levels of TERT mRNA were significantly higher even before the diagnosis of cancer in the "tumor" cohort is intriguing and strongly suggests the potential clinical relevance of the inclusion of circulating hTERT mRNA among the biomarkers to investigate in a prospective study in this setting.

The analysis of T-cell responses against CMV and EBV antigens did not disclose any significant difference between the two cohorts of SOT patients and between baseline and follow up samples, although the numbers of antigen-specific T cells were on average higher than those detected in healthy controls. Infections with these viruses are highly prevalent in the general population, and the immunosuppressive regimen can trigger viral reactivation or facilitate post-transplantation infection, thus accounting for the maintaining of proficient memory responses to these viruses. The fact that we did not observe significantly increased levels of T-cell responses to EBV is consistent with the observation that, in our series, no patient developed a EBV-related cancer thus preventing the possibility to assess the predictive value of this analysis in our cohort of patients. In a recent retrospective study, 197 solid organ transplant recipients were examined to study the reactivity of EBV-specific lymphocytes using the ELISpot assay. The results obtained, combined with the EBV viremia data, we can see how this approach can be a tool to guide the modulation of immunosuppression in patients with active EBV replication, since the values of the viremia tended to be higher in the first year after transplantation in the group of patients with a positive positive immune response

specific for positive EBV (157). With regard to CMV infection in solid organ transplant recipients, post-transplant CMV immune monitoring can guide the duration of antiviral prophylaxis, identify recipients at risk of CMV disease and predict recurrent CMV reactivation (158). Banas B et al have demonstrated the validity of the use of the ELISpot assay in the monitoring of CMV infection, as the specific response to CMV has decreased as a result of immunosuppressive treatment and is increased in patients with graftrejection, indicating the ability of the ELISpot test to monitor the status immunosuppressive treatment of patients (159).

Intriguingly, the levels of HPV-specific T-cell responses observed in both patient cohorts were significantly lower both at baseline and at follow up as compared to those of healthy donors. These findings markedly contrast with the almost preserved level of T-cell responses against the herpesviruses EBV and CMV. This difference could be due to the fact that herpesvirues may undergo spontaneous reactivations over time, thereby maintaining the pool of memory T cells specific for these viruses. The iatrogenic immunosuppression characterizing SOT patients may also promote EBV and CMV reactivation. Although our knowledge of memory immune responses specific for HPV is limited, this virus has a completely different lifecycle and is probably less prone to reactivation as compared to herpesviruses. It is worth considering however, that 60% of patients of the "tumor" cohort have developed a non-melanoma skin cancer, which are often associated with HPV-β 5 and 7. The HPV peptide mix used in our ELISpot assays is composed primarily of peptides associated with the high-risk HPV strains 16 and 18, associated with tumors of genital tract, and this may at least in part explain the low responses detected, although the possibility of a cross-reactivity can not be ruled out. Further studies using a mix of peptides belonging to the β-HPV 5 and 8 strains are also required to assess the potential predictive value of T-cell responses against these beta HPV. However, HPV can be expected to play a role in the initiation processes of oncogenesis, acting as a co-carcinogen with some drugs, such as CNI (71, 153). Notably, all patients of this cohort were treated with a CNI-based immunosuppressive regimen. These findings are consistent with the hypothesis that the lower levels of HPV-specific T-cell responses coupled with the oncogenic effects of CNI treatment may act synergistically to increase the risk of non-melanoma skin cancer in SOT patients.

Our results, even if preliminary and on a small cohort, show that, in SOT patients, it would be clinically relevant to implement a specific program of oncological (immune) monitoring, which takes into account the different variables present in such complex patients. Monitoring programs should also be integrated with various investigative strategies that can

identify and prospectively validate markers predictive of *de novo* tumors, to be combined with already established approaches that help identify high-risk patients. To the best of our knowledge, this is the first comprehensive immunomonitoring study in a prospective cohort of SOT patients aimed at identifying such biomarkers. The results obtained in this pilot series, although not conclusive, are consistent with the hypothesis that the identification of phenotypic and/or functional changes in immune cells, coupled with the detection of early tumor markers such as TERT, may indeed provide a more precise estimate of the risk of cancer in SOT patients. In perspective, we plan to implement our work by performing phenptypic and functional analysis in T-cells and DCs and monitoring the levels of spontaneous T-cells responses in a larger prospective series, which may allow patient stratification according to for the type of immunosuppressive therapy administered. We also plan to evaluate the cytokine and chemokine secretion profile in the supernatants of all cultures performed in this study, in order to provide a more complete immune profile of SOT patients and identify correlations of potential clinical relevance. In addition, given that CNIs but also of mTORi, may modulate the ability of the NF-kB transcription factor to migrate into the nucleus, it is likely that these same drugs may also interfere with the intracellular trafficking of other transcription factors or with the activation of other signal transduction pathways. In particular, though a trascriptome analysis and differential gene expressions profiling, we plan to e indentify a predictive DC immune signature that we hypothesize could be modulated by different immunosuppressive regimens The interesting preliminary data that will be obtained on DCs derived from healthy donors treated with different drugs, can then be validated and confirmed on material derived from patients, always with the prospect of identifying an immune signature that can, together with the other markers, provide a complete profile predictive of the onset of *de novo* tumors in these patients.

In conclusion, the results of the present study provide a strong rationale to design and activate a prospective study in a larger cohort of SOT patientsto validate the encouraging results obtained in a pilot cohort with the final goal to identify reliable biomarkers able to better estimate the risk of cancer in the highly heterogeneous setting of SOT patients.

5. Material and Methods

5.1 Immunosurveillance protocol

The immunosurveillance protocol for SOT recipients activated at the Centro di Riferimento Oncologico (CRO) in Aviano (PN) proposes a surveillance program focused on the most frequent and diagnosable *de novo* tumors with standardized screening (skin, lung, kidney, rectum, cervix and pharynx carcinomas) with integrated immune-virological and clinical follow-up. The study includes a sub-project of translational research to identify candidate biomarkers predictive of the development of *de novo* tumors in OTRs.
Patients are stratified according to the type of transplant (kidney, heart, liver) and, within each category, the high and low risk of tumor development during surveillance is determined.

The group of high risk (HR) includes transplant recipients with at least one of these risk factors:

- Duration of immunosuppression ≥ 10 years
- Age of transplant ≥ 50
- Multiple non-synchronous transplants
- Abuse of smoking, tobacco, alcohol.
- HIV infection

The group of low risk (LR) includes transplant recipients with at least one of these risk factors:

- Duration of immunosuppression < 10 years
- Age of transplant < 50
- Single or multiple synchronous transplants
- Absence of smoking, tobacco, alcohol.
- Absence of HIV infection

Eligibility criteria:

a) Time from transplantation ≥ 1 year
b) Age ≥ 18
c) Performance status ECOG: 0-2
d) Life expectancy ≥ 6 months

e) Compliance of regular follow up
f) Informed consent

Exclusion criteria:

a) Pre-transplanted tumors with exclusion of non-melanoma skin cancer, Tis cervix and hepatocellular carcinoma in liver transplant recipients.
b) Post-transplant and pre-enrollment tumors of active disease or complete remission < 3 years, with exclusion of non-melanoma skin cancers and Tis cervix.
c) severe co-morbidities at enrollment or in the previous year: heart failure, myocardial infarction, stroke, severe hepatic and / or renal failure, tuberculosis, psychiatric pathology.
d) acute rejection at the time of enrollment
e) Pregnancy

Removal criteria from the study:

a) Development of tumors requiring chemo and / or radiotherapy
b) Development of tumors treated with root surgery, cutaneous carcinomas are excised
c) Myocardial infarction, heart failure, stroke, infections require long-term therapy (eg tuberculosis), severe hepatic and / or renal insufficiency, psychiatric pathology.
d) Retreat of informed consent
e) Serious non-compliance with the surveillance program.

HR patients have an intensive surveillance focused on more frequent de novo tumors and diagnosed with standardized screening, such as carcinoma of the skin, lung, kidney, HCC, colorectal carcinomas, cervix, head-neck and esophagus. LR patients follow the general population guidelines (161) Breast and prostate cancer screening follows the general population guidelines in both groups.

General clinical evaluation includes “surveillance oncology visit” and laboratory tests of organ functionality. The general assessment is performed every 6 months for HR patients and annually for LR patients. Standard laboratory tests are performed regularly every 3 months in the patient's transplant centers.

Immuno-virological screening comprise:

- Serology for HBV, HCV and HHV8.
- Viremia for EBV, HHV8, CMV e BKV in patients with documented viruria
- Basic immunological evaluation, focused on the quantitative evaluation of lymphocyte subpopulations: B lymphocytes (CD19), T helper (CD4), cytotoxic T (CD8) and Natural Killer (CD16)
- **Immuno-phenotype of senescent and activate CD4 and CD8 T cells.**
- **Maturation and differentiation status of DCs**
- **Antigen-specific functional investigations: T cell responses against TAAs (tumor associated-antigens) and viral antigens (EBV, CMV, HPV) through ELIspot IFN-γ assays.**
- **Activation status and nuclear NF-kB translocation on T cells and DCs through multispectral imaging flow cytometry (ImageStreamX).**

5.2 Patients, treatment and healthy donors

The enrollment protocol to the program of oncologic immune-surveillance of *De Novo* tumor in SOT patients was approved recently at Centro di Riferimento Oncologico (CRO) in Aviano. Our laboratory enrolled 111 patients who received kidney (87), heart (10), liver (7) and double transplant (7, of which: kidney+kidney, heart+kidney, kidney+pancreas). Patients who had organ rejection or return to dialysis were excluded of surveillance. We stratified our patients according to the main currently used immunosuppressive drugs: 2 patients treated with AZA, 88 patients treated with CNI, 10 patients treated with mTORi and 11 patients treated with both drugs.. Healthy donors used were collected in our lab from 2012.

5.3 Sample collection

Blood samples were collected from all patients at the moment of the first visit and during the follow-up. Fresh heparinized peripheral blood was centrifuged at 800 rpm for 10 minutes within 4 hours of withdrawal. The plasma fraction was further centrifuged at 2100 rpm for 15 minutes, aliquoted in two vials and frozen at -80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from patients by Ficoll-Hypaque gradient. Cells were washed once in PBS and counted by ADAM Cell Counter (DigitalBio). PBMCs were resuspended in 1ml of FCS containing 10% DMSO and were stored viably to perform functional analysis and ELISpot assays.

5.4 Antibodies and reagents

CD3-FITC (mouse IgG1, clone UCHT1) was purchased from Dako. CD4-APC (mouse IgG1, clone RPA-T4), CD4-APC-H7 (mouse IgG1, clone RPA-T4), CD8-PE (Mouse BALB/c IgG1, clone SK1), CD8-APC-Cy7 (mouse IgG1, clone SK1), CD28- PerCP-Cy7 (Mouse C3H x BALB/c IgG1, clone CD28.2), CD38-PE-Cy7 (mouse IgG1, CLONE HB7), CD57-FITC (mouse IgM, clone HNK-1), PD-1-PE (mouse IgG1, eh12.1), CD8-PE-Cy7 (mouse IgG1, clone RPA-T), CD11c-PerCP-Cy5.5 (BALB/c mice IgG1, clone X63), CD83-FITC (mouse IgG1, clone HB15e), CD86-BV421 (mouse BALB/c IgG1, clone 2331 (FUN-1)), HLA-DR-PE-Cy7 (mouse IgG2a, clone G46-6) αCD3 and αCD28 were purchased from BD Biosciences (Becton Dickinson). CD3- FITC (mouse IgG1,clone UCHT1), CD4-FITC (mouse IgG1, clone 13B8.2) and CD14-ECD (mouse IgG2a, clone RMO52) were purchased from BC (Beckman Coulter). NF-kB-PE (rabbit IgG, clone D14E12) was purchased from Cell Signaling Technology. HLA-DR- PerCP-Cy5.5 (mouse IgG2a, clone L243) was purchased by BioLegend. Human TIM-3-APC (Rat IgG2a, clone 344823) was purchased by R&D System. CD14 human Microbeads and LS columns were purchased from MiltenyiBiotec. Lysing solution was purchased from BD Biosciences (Becton Dickinson). ProMix CMV, EBV, HPV, Survivin and hTERT peptides pools were purchased from ProImmune. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit was purchased from ThermoFisher Scientific. Vital nuclear dye DRAQ5 (DR50200) was purchased from Alexis Biochemicals. GM-CSF, IL-4 and IFN-γ were purchased from Promokine supplied by Bio-Connect.

5.5 Patients-derived dendritic cell generation

Briefly, PBMCs were thawed, washed once in PBS and placed in 2 well for each patient in 24 well plate in 2 ml RMPI medium serum free for 2 hour. Subsequently, lymphocytes in suspension were collected and washed in PBS. Adherent monocytes were cultured for 1 week in cellGRO medium with GM-CSF 50ng/ml and IL-4 25ng/ml. On days 2 and 5 of culture, fresh medium containing the same cytokine amount as on day 0 was added. At the 6th day were added O/N maturative stimulus, 1.5 ug/ml LPS + 20 ng/ml IFN-γ, and dendritic cells were harvested on day 7 of culture.

5.6 Multispectral imaging flow cytometry analyses

5.6.1 Lymphocytes staining

T cells staining was performed using CD4-FITC and CD8-PE-Cy7 antibodies for 2x10^6 cells for 20 minutes in ice. T cells were washed twice with 5ml of PBS-BSA 5%, fixed in 1ml

of complete RPMI containing PFA 2% and permeabilized with 600ul methanol 90% for 10 minutes in ice. Cells were washed once with PBS-BSA 5% and incubated with NF-kB/p65-PE antibody for 45 minutes in ice. After incubation cells were washed once with PBS-BSA 5% and resuspended in 70ul of PBS containing PFA 1% and DRAQ5. Cells were run on ImageStreamX cytometer using the INSPIRE software and images were analyzed using the IDEAS software (Amnis). Cells were excited with a 488-nm laser (50 mW intensity) and 785-nm laser (0.50 mW intensity). Brightfield, side scatter, and fluorescent cell images were acquired at ×40 magnification. For each samples 30000 cells were collected . The extent of internalization was calculated with an algorithm of the IDEAS software that measures the similarity score between DRAQ5 and NF-kB staining. A Similarity score is determined for every cell based on a pixel by pixel correlation of the nuclear image to the NF-kB image. The threshold of similarity score to consider translocated NF-kB was defined using as n egative and positive control ML-1 cell line untreated or treated with TNF-α, respectively.

5.6.2 Dendritic cells staining

After stimulus incubation O/N immature (no stimulus) and mature dendritic cells were collected, washed in PBS, fixed in complete RPMI containing PFA 2% and permeabilized with methanol 90% for 10 minutes. After that DCs were washed and incubated with NF-kB/p65-PE antibody (for 45 minutes in ice. Cells were washed and resuspended in 70ul of PBS containing PFA 1% and DRAQ5 Cells were run on ImageStreamX cytometer using the INSPIRE software and images were analyzed using the IDEAS software (Amnis). Cells were excited with a 488-nm laser (50 mW intensity) and 785-nm laser (0.50 mW intensity). Brightfield, side scatter, and fluorescent cell images were acquired at ×40 magnification. Were collected 20000 events for each samples. The extent of internalization was calculated with an algorithm of the IDEAS software that measures the similarity score as described in the previous paragraph.

5.7 ELISpot assay

Spontaneous T cell responses were quantified by ELISpot-IFN-γ assay commercial kit (Cellular Technology Limited (CTL), Human IFN gamma IMMUNOSPOT; OH, USA) . Ninety-six-well plates were pre-coated with anti-human interferon (IFN)-γ Capture antibody (2 μg/ml) O/N at 4°C. Next day PBMCs were tawed and washed once in RPMI 1640 serum free, counted and resuspended in CTL-test Medium in a concentration of 10.5x10^6 for each samples. Unspecif stimimuli (0.5 mg/ml αCD3/αCD28) and peptide mix (0.2 ng/ml of each

peptide mix) were resuspended in CTL-test Medium, placed (in triplicate) and incubated for 10-20 minutes. Patients's PBMCs were tawhed, washed and were placed (500,000 cell/well) in co-culture with viral (CMV, EBV and HPV) and tumor associated antigens (TAAs) (Survivin and hTERT) and incubated O/N. At day 3 spots were detected with anti-human IFN-γ (biotin), streptavidinalkaline phosphatase, and Blue Developer Solution. Spots were counted and analyzed by ImmunoSpot plate scanning and analysis service (Bonn, Germany).

5.8 Quantification of circulating TERT mRNA

The expression of TERT transcripts in the plasma samples was quantified by real-time PCR. Primers AT1 (5′-CGGAAGAGTGTCTGGAGCAA-3′) and AT2b (5′-CGCAGCTGCACCCTCTTCA-3′) were designed on exon 3 and 4, respectively; they bind to nucleotide sequences located upstream of the RT motif 1 on the *TERT* gene allowing amplification of all *TERT* transcripts producing an amplified product of 68 bp. The fluorogenic probe AT (FAM 5′-TTGCAAAGCATTGGAATCAGACAGCAC-3′ TAMRA) recognises the sequence located inside the product amplified by AT1/AT2b. The PCR was performed using an ABI prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) in 50 µl of mixture containing 25 µl 2X TaqMan Universal (PE Applied Biosystems), 100 nM of fluorogenic probe, 600 nM of primer AT1, 900 nM of primer AT2b and 10 µl of cDNA sample. After 2 min at 50 °C to allow the uracil *N*-glycosylase to act and a denaturation step lasting 10 min at 95 °C, 50 cycles were run, each consisting of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Each sample was run in triplicate and the mean Ct values were plotted against the standard TERT reference curve, which was generated with serial fivefold dilutions of the TERT amplicon, as previously described. TERT values were estimated per ml according to the X 8 X 2 conversion factor and then expressed as TERT copies per ml. Ten microlitres cDNA from each sample were also amplified for the housekeeping hypoxantin-guanine phosphoribosyl transferase 1 (*HPRT1*) gene. The forward primer HPRT1for (5′-TCAGGCAGTATAATCCAAAGATGGT-3′) was designed between exons 4 and 5, and the reverse primer HPRT1rev (5′-CTTCGTGGGGTCCTTTTCAC-3′) was designed on exon 6, generating a cDNA amplified product of 64 bp. The fluorogenic probe (VIC 5′-AAGGTCGCAAGCTT-3′ MGBNFQ) recognises the sequence located within the products amplified by HPRT1for/HPRT1rev. PCR was performed using an ABI prism 7900 Sequence Detection System (PE Applied Biosystems) in 50 µl of mixture containing 25 µl 2 × TaqMan universal master mix (PE Applied Biosystems), 200 nM of fluorogenic probe, 900 nM of primer HPRT1for and HPRT1rev and 10 µl of cDNA sample. The thermal cycler

profile consisted of 2 min at 50 °C, 10 min at 95 °C and then 45 cycles were run, each consisting of 30 s at 95 °C, 30 s at 60 °C and 30 s at and 72 °C. Each sample was run in triplicate and the mean Ct values were plotted against the standard HPRT1 reference curve, prepared using serial dilutions of the HPRT1 amplicon, as previously described. TERT values were expressed as TERT copies per 10^3 HPRT1 copies (146).

5.9 Dendritic cells from healthy donors culture generation protocol

Dendritic cells from healthy donors were isolated from PBMCs by CD14 positive separation according to the protocol. Briefly, PBMCs were tawed and washed once in RPMI 1640 serum free and once in PBS. For each 10^7 cells were added 20ul CD14 human MicroBeads and 80ul of MACS Buffer (PBS+BSA 0.5%+ 2mM EDTA) and stained 20 min in ice. After that cells were washed with 30ml of MACS Buffer. After the purification with anti-CD14-coated micro beads, $CD14^+$ monocytes were sorted with a magnetic device in LS columns and washed once in PBS, counted and plated 1x10^6 for condition. On days 2 and 5 of culture, fresh medium containing the same cytokine amount as on day 0 was added and cells were treated with two different concentration of drugs (NT, 50ngLml and 200ng/ml for TAC; NT, 1ng/ml and 10ng/ml for EVR).

5.10 Flow cytometry analyses

For the immunophenotypic analyses of DCs $1x10^6$ cells were stained with the antibodies listed below, anti-CD11c-PerCPCy-5.5, anti-CD83-FITC, anti-CD86-BV421, anti-HLA-DR-PECy7, AquaLive). DCs were harvested at day 7 of culture, washed once in FACS Buffer at 1500 rpm for six minutes at +4°C and stained with 50ul antibodies for 20 min at 4°C in plate. After that DCs were washed twice with 150ul of FACS Buffer and resuspended in 300ul of FACS Buffer. Cells were run on LSR-Fortessa (Becton Dickinson) and photomultiplier voltages and compensation with the CompBeads Set Anti-Mouse Ig or Anti-Rat Ig, k Sets (BD Biosciences). Flow cytometry data were analyzed with FlowJo (Tree Star, Ashland, OR, USA) software.

Patients' whole blood, to characterized the T lymphocytes phenotype, were stained for the following antibodies in two different tubes: the first was labeled CD3-FITC, CD4-APC-H7, HLA-DR-PerPC-Cy5.5, CD38-PE-Cy7,Tim-3-APC antibodies and the second was labeled with CD3-PerPC-Cy5.5, CD4-APC, CD8-APC-Cy7, CD28- PE-Cy7, CD57- FITC, PD-1-PE antibodies. Briefly, 100ul of whole blood for each tube were labeled with the different combination of 6 antibodies for 30 minutes at room temperature. Subsequently, the whole

blood was lysed with 1.5 ml of lysis solution for 15 minutes and vortexed. The samples were washed once with PBS at 1200rpm for 7 minutes, to stopped the lysis. Samples were lysed a second time with lysis solution for 15 minutes and washed once with PBS at 1200rpm for 7 minutes. After that, the supernatant was discarded and the pellet resuspended in 250ul of PBS. Cells were run on FACScanto (Becton Dickinson) and photomultiplier voltages and compensation were set with unstained and stained cells. Flow cytometry data were analyzed with BD FACS DIVA v7.0.

5.11 Statistical Analysis

Data obtained from multiple independent experiments were expressed as median for ELISpot assay and functional analysis. R software was used to determine the homogeneity of data distributions. The Student's *t*-test for two-tailed distributions and paired data was used for statistical analysis of nuclear NF-kB translocation data in T cells and DCs-derived patients, obtained by multispectral imaging flow cytometry. The Student's *t*-test was also employed to compare immunophenotypic data regarding of DCs from healthy donors culture. The Mann–Whitney test was employed to compare spontaneous T cells responses against viral and TAAs mix peptides detected by ELISpot IFN-γ assay. In all cases, statistical significance was considered for $p < 0.05$.

References

1. Watson CJE, Dark JH. Organ transplantation: historical perspective and current practice. Br J Anaesth. gennaio 2012;108 Suppl 1:i29-42.

2. Schrem H, Kurok M, Kaltenborn A, Vogel A, Walter U, Zachau L, et al. Incidence and long-term risk of de novo malignancies after liver transplantation with implications for prevention and detection. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. novembre 2013;19(11):1252–61.

3. Hall EC, Pfeiffer RM, Segev DL, Engels EA. Cumulative Incidence of Cancer After Solid Organ Transplantation. Cancer. 15 giugno 2013;119(12):2300–8.

4. Chapman JR, Webster AC, Wong G. Cancer in the transplant recipient. Cold Spring Harb Perspect Med. 1 luglio 2013;3(7).

5. Radu RG, Fujimoto S, Mukai E, Takehiro M, Shimono D, Nabe K, et al. Tacrolimus suppresses glucose-induced insulin release from pancreatic islets by reducing glucokinase activity. Am J Physiol Endocrinol Metab. febbraio 2005;288(2):E365-371.

6. Northrop JP, Ho SN, Chen L, Thomas DJ, Timmerman LA, Nolan GP, et al. NF-AT components define a family of transcription factors targeted in T-cell activation. Nature. 9 giugno 1994;369(6480):497–502.

7. Loh C, Shaw KT, Carew J, Viola JP, Luo C, Perrino BA, et al. Calcineurin binds the transcription factor NFAT1 and reversibly regulates its activity. J Biol Chem. 3 maggio 1996;271(18):10884–91.

8. Crabtree GR. Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. Cell. 5 marzo 1999;96(5):611–4.

9. Timmerman LA, Clipstone NA, Ho SN, Northrop JP, Crabtree GR. Rapid shuttling of NF-AT in discrimination of Ca2+ signals and immunosuppression. Nature. 31 ottobre 1996;383(6603):837–40.

10. Beals CR, Clipstone NA, Ho SN, Crabtree GR. Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes Dev. 1 aprile 1997;11(7):824–34.

11. Crabtree GR. Calcium, calcineurin, and the control of transcription. J Biol Chem. 26 gennaio 2001;276(4):2313–6.

12. Liu J, Farmer JD, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell. 23 agosto 1991;66(4):807–15.

13. Borel JF, Feurer C, Gubler HU, Stähelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Actions. luglio 1976;6(4):468–75.

14. Kapturczak MH, Meier-Kriesche HU, Kaplan B. Pharmacology of calcineurin antagonists. Transplant Proc. marzo 2004;36(2 Suppl):25S-32S.

15. Reichenspurner H. Overview of tacrolimus-based immunosuppression after heart or lung transplantation. J Heart Lung Transplant Off Publ Int Soc Heart Transplant. febbraio 2005;24(2):119–30.

16. Jacinto E, Hall MN. Tor signalling in bugs, brain and brawn. Nat Rev Mol Cell Biol. febbraio 2003;4(2):117–26.

17. Granata S, Dalla Gassa A, Carraro A, Brunelli M, Stallone G, Lupo A, et al. Sirolimus and Everolimus Pathway: Reviewing Candidate Genes Influencing Their Intracellular Effects. Int J Mol Sci. 14 maggio 2016;17(5).

18. Yang H, Rudge DG, Koos JD, Vaidialingam B, Yang HJ, Pavletich NP. mTOR kinase structure, mechanism and regulation. Nature. 9 maggio 2013;497(7448):217–23.

19. Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. Nat Rev Immunol. maggio 2009;9(5):324–37.

20. Sagedal S, Nordal KP, Hartmann A, Sund S, Scott H, Degré M, et al. The impact of cytomegalovirus infection and disease on rejection episodes in renal allograft recipients. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. ottobre 2002;2(9):850–6.

21. Sagedal S, Hartmann A, Nordal KP, Osnes K, Leivestad T, Foss A, et al. Impact of early cytomegalovirus infection and disease on long-term recipient and kidney graft survival. Kidney Int. luglio 2004;66(1):329–37.

22. Fica A, Cervera C, Pérez N, Marcos MA, Ramírez J, Linares L, et al. Immunohistochemically proven cytomegalovirus end-organ disease in solid organ transplant patients: clinical features and usefulness of conventional diagnostic tests. Transpl Infect Dis Off J Transplant Soc. settembre 2007;9(3):203–10.

23. Bond MMK, Bond MMK, Sehn A, Dias VH, Said TL, Dos Santos CC, et al. Cyclosporine Versus Tacrolimus: Which Calcineurin Inhibitor Has Influence on Cytomegalovirus Infection in Cardiac Transplantation? Transplant Proc. aprile 2018;50(3):809–14.

24. Rodríguez-Serrano M, Sánchez-Lázaro I, Almenar-Bonet L, Martínez-Dolz L, Portolés-Sanz M, Rivera-Otero M, et al. Does the calcineurin inhibitor have influence on cytomegalovirus infection in heart transplantation? Clin Transplant. gennaio 2014;28(1):88–95.

25. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, et al. mTOR regulates memory CD8 T-cell differentiation. Nature. 2 luglio 2009;460(7251):108–12.

26. Webster AC, Lee VWS, Chapman JR, Craig JC. Target of rapamycin inhibitors (sirolimus and everolimus) for primary immunosuppression of kidney transplant recipients: a systematic review and meta-analysis of randomized trials. Transplantation. 15 maggio 2006;81(9):1234–48.

27. Havenith SHC, Yong SL, van Donselaar-van der Pant KAMI, van Lier RAW, ten Berge IJM, Bemelman FJ. Everolimus-treated renal transplant recipients have a more robust CMV-specific CD8+ T-cell response compared with cyclosporine- or mycophenolate-treated patients. Transplantation. 15 gennaio 2013;95(1):184–91.

28. Strioga M, Pasukoniene V, Characiejus D. CD8+ CD28- and CD8+ CD57+ T cells and their role in health and disease. Immunology. settembre 2011;134(1):17–32.

29. Jin H-T, Anderson AC, Tan WG, West EE, Ha S-J, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc Natl Acad Sci U S A. 17 agosto 2010;107(33):14733–8.

30. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. Curr Opin Immunol. aprile 2013;25(2):214–21.

31. Thorp EB, Stehlik C, Ansari MJ. T-cell exhaustion in allograft rejection and tolerance. Curr Opin Organ Transplant. febbraio 2015;20(1):37–42.

32. Ali AS, Al-Shraim M, Al-Hakami AM, Jones IM. Epstein- Barr Virus: Clinical and Epidemiological Revisits and Genetic Basis of Oncogenesis. Open Virol J. 2015;9:7–28.

33. Ohshima K, Suzumiya J, Kanda M, Kato A, Kikuchi M. Integrated and episomal forms of Epstein-Barr virus (EBV) in EBV associated disease. Cancer Lett. 9 gennaio 1998;122(1–2):43–50.

34. Doak PB, Montgomerie JZ, North JD, Smith F. Reticulum cell sarcoma after renal homotransplantation and azathioprine and prednisone therapy. Br Med J. 21 dicembre 1968;4(5633):746–8.

35. Opelz G, Henderson R. Incidence of non-Hodgkin lymphoma in kidney and heart transplant recipients. Lancet Lond Engl. 18 dicembre 1993;342(8886–8887):1514–6.

36. Caillard S, Lelong C, Pessione F, Moulin B, French PTLD Working Group. Post-transplant lymphoproliferative disorders occurring after renal transplantation in adults: report of 230 cases from the French Registry. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. novembre 2006;6(11):2735–42.

37. Hartmann C, Schuchmann M, Zimmermann T. Posttransplant lymphoproliferative disease in liver transplant patients. Curr Infect Dis Rep. febbraio 2011;13(1):53–9.

38. Shroff R, Rees L. The post-transplant lymphoproliferative disorder-a literature review. Pediatr Nephrol Berl Ger. aprile 2004;19(4):369–77.

39. Cockfield SM. Identifying the patient at risk for post-transplant lymphoproliferative disorder. Transpl Infect Dis Off J Transplant Soc. giugno 2001;3(2):70–8.

40. Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD. The immunology of Epstein-Barr virus-induced disease. Annu Rev Immunol. 2015;33:787–821.

41. Azzi T, Lünemann A, Murer A, Ueda S, Béziat V, Malmberg K-J, et al. Role for early-differentiated natural killer cells in infectious mononucleosis. Blood. 16 ottobre 2014;124(16):2533–43.

42. Nourse JP, Jones K, Gandhi MK. Epstein-Barr Virus-related post-transplant lymphoproliferative disorders: pathogenetic insights for targeted therapy. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. maggio 2011;11(5):888–95.

43. Swerdlow SH. Lymphoma classification and the tools of our trade: an introduction to the 2012 USCAP Long Course. Mod Pathol Off J U S Can Acad Pathol Inc. gennaio 2013;26 Suppl 1:S1–14.

44. Jagadeesh D, Woda BA, Draper J, Evens AM. Post transplant lymphoproliferative disorders: risk, classification, and therapeutic recommendations. Curr Treat Options Oncol. marzo 2012;13(1):122–36.

45. Al-Mansour Z, Nelson BP, Evens AM. Post-transplant lymphoproliferative disease (PTLD): risk factors, diagnosis, and current treatment strategies. Curr Hematol Malig Rep. settembre 2013;8(3):173–83.

46. Nelson BP, Wolniak KL, Evens A, Chenn A, Maddalozzo J, Proytcheva M. Early Posttransplant Lymphoproliferative DiseaseClinicopathologic Features and Correlation With mTOR Signaling Pathway Activation. Am J Clin Pathol. 1 ottobre 2012;138(4):568–78.

47. Tanner JE, Menezes J. Interleukin-6 and Epstein-Barr virus induction by cyclosporine A: potential role in lymphoproliferative disease. Blood. 1 dicembre 1994;84(11):3956–64.

48. Chen C, Johnston TD, Jeon H, Gedaly R, McHugh P, Ranjan D. Cyclosporine promotes epstein-barr virus-infected human B-cell transformation assayed by three correlated assay methods. Transplant Proc. febbraio 2009;41(1):366–70.

49. Adamson AL, Le BT, Siedenburg BD. Inhibition of mTORC1 inhibits lytic replication of Epstein-Barr virus in a cell-type specific manner. Virol J. 11 giugno 2014;11:110.

50. Alsayed Y, Leleu X, Leontovich A, Oton AB, Melhem M, George D, et al. Proteomics analysis in post-transplant lymphoproliferative disorders. Eur J Haematol. ottobre 2008;81(4):298–303.

51. Wowro SJ, Schmitt KRL, Tong G, Berger F, Schubert S. Effects of mTOR and calcineurin inhibitors combined therapy in Epstein-Barr virus positive and negative Burkitt lymphoma cells. Int Immunopharmacol. gennaio 2016;30:9–17.

52. Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. N Engl J Med. 24 aprile 2003;348(17):1681–91.

53. O'Donovan P, Perrett CM, Zhang X, Montaner B, Xu Y-Z, Harwood CA, et al. Azathioprine and UVA light generate mutagenic oxidative DNA damage. Science. 16 settembre 2005;309(5742):1871–4.

54. Perrett CM, Walker SL, O'Donovan P, Warwick J, Harwood CA, Karran P, et al. Azathioprine treatment photosensitizes human skin to ultraviolet A radiation. Br J Dermatol. luglio 2008;159(1):198–204.

55. Dziunycz PJ, Lefort K, Wu X, Freiberger SN, Neu J, Djerbi N, et al. The oncogene ATF3 is potentiated by cyclosporine A and ultraviolet light A. J Invest Dermatol. luglio 2014;134(7):1998–2004.

56. Knoll GA, Kokolo MB, Mallick R, Beck A, Buenaventura CD, Ducharme R, et al. Effect of sirolimus on malignancy and survival after kidney transplantation: systematic review and meta-analysis of individual patient data. BMJ. 24 novembre 2014;349:g6679.

57. Lindelöf B, Sigurgeirsson B, Gäbel H, Stern RS. Incidence of skin cancer in 5356 patients following organ transplantation. Br J Dermatol. settembre 2000;143(3):513–9.

58. Harwood CA, McGregor JM, Swale VJ, Proby CM, Leigh IM, Newton R, et al. High frequency and diversity of cutaneous appendageal tumors in organ transplant recipients. J Am Acad Dermatol. marzo 2003;48(3):401–8.

59. Rival-Tringali A-L, Euvrard S, Decullier E, Claudy A, Faure M, Kanitakis J. Conversion from calcineurin inhibitors to sirolimus reduces vascularization and thickness of post-transplant cutaneous squamous cell carcinomas. Anticancer Res. giugno 2009;29(6):1927–32.

60. Saldanha G, Fletcher A, Slater DN. Basal cell carcinoma: a dermatopathological and molecular biological update. Br J Dermatol. febbraio 2003;148(2):195–202.

61. Gupta S, Kumar P, Das BC. HPV: Molecular pathways and targets. Curr Probl Cancer. aprile 2018;42(2):161–74.

62. Kauffman HM, Cherikh WS, Cheng Y, Hanto DW, Kahan BD. Maintenance immunosuppression with target-of-rapamycin inhibitors is associated with a reduced incidence of de novo malignancies. Transplantation. 15 ottobre 2005;80(7):883–9.

63. Arron ST, Jennings L, Nindl I, Rosl F, Bouwes Bavinck JN, Seçkin D, et al. Viral oncogenesis and its role in nonmelanoma skin cancer. Br J Dermatol. giugno 2011;164(6):1201–13.

64. Howley PM, Pfister HJ. Beta genus papillomaviruses and skin cancer. Virology. maggio 2015;479–480:290–6.

65. Dharancy S, Catteau B, Mortier L, Boleslawski E, Declerck N, Canva V, et al. Conversion to sirolimus: a useful strategy for recalcitrant cutaneous viral warts in liver transplant recipient. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. dicembre 2006;12(12):1883–7.

66. Vajdic CM, van Leeuwen MT. Cancer incidence and risk factors after solid organ transplantation. Int J Cancer. 15 ottobre 2009;125(8):1747–54.

67. Sherston SN, Carroll RP, Harden PN, Wood KJ. Predictors of cancer risk in the long-term solid-organ transplant recipient. Transplantation. 27 marzo 2014;97(6):605–11.

68. Billups K, Neal J, Salyer J. Immunosuppressant-driven de novo malignant neoplasms after solid-organ transplant. Prog Transplant Aliso Viejo Calif. giugno 2015;25(2):182–8.

69. Gutierrez-Dalmau A, Campistol JM. Immunosuppressive therapy and malignancy in organ transplant recipients: a systematic review. Drugs. 2007;67(8):1167–98.

70. Engels EA, Jennings L, Kemp TJ, Chaturvedi AK, Pinto LA, Pfeiffer RM, et al. Circulating TGF-β1 and VEGF and risk of cancer among liver transplant recipients. Cancer Med. agosto 2015;4(8):1252–7.

71. Hojo M, Morimoto T, Maluccio M, Asano T, Morimoto K, Lagman M, et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature. 11 febbraio 1999;397(6719):530–4.

72. Shihab FS, Bennett WM, Yi H, Andoh TF. Effect of cyclosporine and sirolimus on the expression of connective tissue growth factor in rat experimental chronic nephrotoxicity. Am J Nephrol. 2006;26(4):400–7.

73. Datta D, Contreras AG, Basu A, Dormond O, Flynn E, Briscoe DM, et al. Calcineurin inhibitors activate the proto-oncogene Ras and promote protumorigenic signals in renal cancer cells. Cancer Res. 1 dicembre 2009;69(23):8902–9.

74. Wimmer CD, Angele MK, Schwarz B, Pratschke S, Rentsch M, Khandoga A, et al. Impact of cyclosporine versus tacrolimus on the incidence of de novo malignancy following liver transplantation: a single center experience with 609 patients. Transpl Int Off J Eur Soc Organ Transplant. ottobre 2013;26(10):999–1006.

75. Ori Y, Herman-Edelstein M, Zingerman B, Rozen-Zvi B, Gafter U, Malachi T, et al. Effect of immunosuppressive drugs on spontaneous DNA repair in human peripheral blood mononuclear cells. Biomed Pharmacother Biomedecine Pharmacother. settembre 2012;66(6):409–13.

76. Walz G, Zanker B, Melton LB, Suthanthiran M, Strom TB. Possible association of the immunosuppressive and B cell lymphoma-promoting properties of cyclosporine. Transplantation. gennaio 1990;49(1):191–4.

77. Herman M, Weinstein T, Korzets A, Chagnac A, Ori Y, Zevin D, et al. Effect of cyclosporin A on DNA repair and cancer incidence in kidney transplant recipients. J Lab Clin Med. gennaio 2001;137(1):14–20.

78. Dantal J, Hourmant M, Cantarovich D, Giral M, Blancho G, Dreno B, et al. Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens. Lancet Lond Engl. 28 febbraio 1998;351(9103):623–8.

79. Marcil I, Stern RS. Squamous-cell cancer of the skin in patients given PUVA and ciclosporin: nested cohort crossover study. Lancet Lond Engl. 29 settembre 2001;358(9287):1042–5.

80. Eng CP, Sehgal SN, Vézina C. Activity of rapamycin (AY-22,989) against transplanted tumors. J Antibiot (Tokyo). ottobre 1984;37(10):1231–7.

81. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med. febbraio 2002;8(2):128–35.

82. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 13 aprile 2012;149(2):274–93.

83. Chiang GG, Abraham RT. Targeting the mTOR signaling network in cancer. Trends Mol Med. ottobre 2007;13(10):433–42.

84. Vara JÁF, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev. 1 aprile 2004;30(2):193–204.

85. Wang G-Y, Zhang Q, Yang Y, Chen W-J, Liu W, Jiang N, et al. Rapamycin combined with allogenic immature dendritic cells selectively expands CD4+CD25+Foxp3+ regulatory T cells in rats. Hepatobiliary Pancreat Dis Int HBPD INT. aprile 2012;11(2):203–8.

86. Strauss L, Czystowska M, Szajnik M, Mandapathil M, Whiteside TL. Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. PloS One. 22 giugno 2009;4(6):e5994.

87. Candia E, Reyes P, Covian C, Rodriguez F, Wainstein N, Morales J, et al. Single and combined effect of retinoic acid and rapamycin modulate the generation, activity and homing potential of induced human regulatory T cells. PloS One. 2017;12(7):e0182009.

88. Smith A, Niu W, Desai A. The Effect of Conversion from a Calcineurin Inhibitor to Sirolimus on Skin Cancer Reduction in Post-renal Transplantation Patients. Cureus. 13 agosto 2017;9(8):e1564.

89. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol. 2000;18:767–811.

90. Ardavín C, Amigorena S, Reis e Sousa C. Dendritic cells: immunobiology and cancer immunotherapy. Immunity. gennaio 2004;20(1):17–23.

91. Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Bone marrow-derived cells present MHC class I-restricted tumour antigens in priming of antitumour immune responses. Ciba Found Symp. 1994;187:229–40; discussion 240-244.

92. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nat Rev Immunol. 13 luglio 2012;12(8):557–69.

93. Embgenbroich M, Burgdorf S. Current Concepts of Antigen Cross-Presentation. Front Immunol [Internet]. 16 luglio 2018 [citato 1 settembre 2018];9. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6054923/

94. Heath WR, Carbone FR. Cross-Presentation, Dendritic Cells, Tolerance and Immunity. Annu Rev Immunol. 2001;19(1):47–64.

95. Meehan AC, Mifsud NA, Nguyen THO, Levvey BJ, Snell GI, Kotsimbos TC, et al. Impact of commonly used transplant immunosuppressive drugs on human NK cell function is dependent upon stimulation condition. PloS One. 2013;8(3):e60144.

96. Wai L-E, Fujiki M, Takeda S, Martinez OM, Krams SM. Rapamycin, but not cyclosporine or FK506, alters natural killer cell function. Transplantation. 15 gennaio 2008;85(1):145–9.

97. De Bruyne R, Bogaert D, De Ruyck N, Lambrecht BN, Van Winckel M, Gevaert P, et al. Calcineurin inhibitors dampen humoral immunity by acting directly on naive B cells. Clin Exp Immunol. giugno 2015;180(3):542–50.

98. Traitanon O, Mathew JM, La Monica G, Xu L, Mas V, Gallon L. Differential Effects of Tacrolimus versus Sirolimus on the Proliferation, Activation and Differentiation of Human B Cells. PloS One. 2015;10(6):e0129658.

99. Tourneur E, Ben Mkaddem S, Chassin C, Bens M, Goujon J-M, Charles N, et al. Cyclosporine A impairs nucleotide binding oligomerization domain (Nod1)-mediated innate antibacterial renal defenses in mice and human transplant recipients. PLoS Pathog. gennaio 2013;9(1):e1003152.

100. Mercalli A, Calavita I, Dugnani E, Citro A, Cantarelli E, Nano R, et al. Rapamycin unbalances the polarization of human macrophages to M1. Immunology. ottobre 2013;140(2):179–90.

101. Nakamura T, Nakao T, Yoshimura N, Ashihara E. Rapamycin Prolongs Cardiac Allograft Survival in a Mouse Model by Inducing Myeloid-Derived Suppressor Cells. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. settembre 2015;15(9):2364–77.

102. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. Nat Rev Immunol. febbraio 2006;6(2):127–35.

103. Luckheeram RV, Zhou R, Verma AD, Xia B. $CD4^+$T cells: differentiation and functions. Clin Dev Immunol. 2012;2012:925135.

104. Obar JJ, Lefrançois L. Memory CD8+ T cell differentiation. Ann N Y Acad Sci. gennaio 2010;1183:251–66.

105. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nat Rev Immunol. novembre 2012;12(11):749–61.

106. Vafadari R, Kraaijeveld R, Weimar W, Baan CC. Tacrolimus inhibits NF-κB activation in peripheral human T cells. PloS One. 2013;8(4):e60784.

107. Tsuda K, Yamanaka K, Kitagawa H, Akeda T, Naka M, Niwa K, et al. Calcineurin inhibitors suppress cytokine production from memory T cells and differentiation of naïve T cells into cytokine-producing mature T cells. PloS One. 2012;7(2):e31465.

108. Nishiyama S, Manabe N, Kubota Y, Ohnishi H, Kitanaka A, Tokuda M, et al. Cyclosporin A inhibits the early phase of NF-kappaB/RelA activation induced by CD28 costimulatory signaling to reduce the IL-2 expression in human peripheral T cells. Int Immunopharmacol. aprile 2005;5(4):699–710.

109. Kim KW, Chung BH, Kim B-M, Cho M-L, Yang CW. The effect of mammalian target of rapamycin inhibition on T helper type 17 and regulatory T cell differentiation in vitro and in vivo in kidney transplant recipients. Immunology. gennaio 2015;144(1):68–78.

110. Jung J-W, Veitch M, Bridge JA, Overgaard NH, Cruz JL, Linedale R, et al. Clinically-Relevant Rapamycin Treatment Regimens Enhance CD8+ Effector Memory T Cell Function In The Skin and Allow their Infiltration into Cutaneous Squamous Cell Carcinoma. Oncoimmunology. 2018;7(9):e1479627.

111. Araki K, Youngblood B, Ahmed R. The role of mTOR in memory CD8 T-cell differentiation. Immunol Rev. maggio 2010;235(1):234–43.

112. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang L-S, et al. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature. 2 luglio 2009;460(7251):103–7.

113. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med. 4 giugno 2001;193(11):1285–94.

114. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. 19 giugno 2009;30(6):899–911.

115. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich J-M, et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity. 22 aprile 2011;34(4):566–78.

116. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. luglio 2008;8(7):523–32.

117. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science. 29 aprile 2011;332(6029):600–3.

118. Levitsky J, Miller J, Wang E, Rosen A, Flaa C, Abecassis M, et al. Immunoregulatory profiles in liver transplant recipients on different immunosuppressive agents. Hum Immunol. marzo 2009;70(3):146–50.

119. Bocian K, Borysowski J, Wierzbicki P, Wyzgal J, Klosowska D, Bialoszewska A, et al. Rapamycin, unlike cyclosporine A, enhances suppressive functions of in vitro-induced CD4+CD25+ Tregs. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. marzo 2010;25(3):710–7.

120. Burke MT, Sambira Nahum LC, Isbel NM, Carroll RP, Soyer HP, Francis R, et al. Sirolimus Increases T-Cell Abundance in the Sun Exposed Skin of Kidney Transplant Recipients. Transplant Direct. luglio 2017;3(7):e171.

121. Feng X, Lin Z, Sun W, Hollinger MK, Desierto MJ, Keyvanfar K, et al. Rapamycin is highly effective in murine models of immune-mediated bone marrow failure. Haematologica. 2017;102(10):1691–703.

122. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 19 marzo 1998;392(6673):245–52.

123. Szabo G, Gavala C, Mandrekar P. Tacrolimus and cyclosporine A inhibit allostimulatory capacity and cytokine production of human myeloid dendritic cells. J Investig Med Off Publ Am Fed Clin Res. settembre 2001;49(5):442–9.

124. Lee JI, Ganster RW, Geller DA, Burckart GJ, Thomson AW, Lu L. Cyclosporine A inhibits the expression of costimulatory molecules on in vitro-generated dendritic cells: association with reduced nuclear translocation of nuclear factor kappa B. Transplantation. 15 novembre 1999;68(9):1255–63.

125. Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P. Dendritic cell survival and maturation are regulated by different signaling pathways. J Exp Med. 7 dicembre 1998;188(11):2175–80.

126. Matsue H, Yang C, Matsue K, Edelbaum D, Mummert M, Takashima A. Contrasting impacts of immunosuppressive agents (rapamycin, FK506, cyclosporin A, and dexamethasone) on bidirectional dendritic cell-T cell interaction during antigen presentation. J Immunol Baltim Md 1950. 1 ottobre 2002;169(7):3555–64.

127. Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Pénit C. Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. Int Immunol. aprile 1998;10(4):371–8.

128. Pino-Lagos K, Michea P, Sauma D, Alba A, Morales J, Bono MR, et al. Cyclosporin A-treated dendritic cells may affect the outcome of organ transplantation by decreasing CD4+CD25+ regulatory T cell proliferation. Biol Res. 2010;43(3):333–7.

129. Tiefenthaler M, Hofer S, Ebner S, Ivarsson L, Neyer S, Herold M, et al. In vitro treatment of dendritic cells with tacrolimus: impaired T-cell activation and IP-10 expression. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. marzo 2004;19(3):553–60.

130. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A, et al. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. Blood. 1 giugno 2003;101(11):4457–63.

131. Battaglia M, Stabilini A, Roncarolo M-G. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood. 15 giugno 2005;105(12):4743–8.

132. Haidinger M, Poglitsch M, Geyeregger R, Kasturi S, Zeyda M, Zlabinger GJ, et al. A versatile role of mammalian target of rapamycin in human dendritic cell function and differentiation. J Immunol Baltim Md 1950. 1 ottobre 2010;185(7):3919–31.

133. Sordi V, Bianchi G, Buracchi C, Mercalli A, Marchesi F, D'Amico G, et al. Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells: selective upregulation by rapamycin. Transplantation. 27 settembre 2006;82(6):826–34.

134. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce ag-specific T cell regulation and prolong graft survival. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. febbraio 2005;5(2):228–36.

135. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. J Immunol Baltim Md 1950. 1 giugno 2007;178(11):7018–31.

136. Xiong A, Duan L, Chen J, Fan Z, Zheng F, Tan Z, et al. Flt3L combined with rapamycin promotes cardiac allograft tolerance by inducing regulatory dendritic cells and allograft autophagy in mice. PloS One. 2012;7(10):e46230.

137. Wang G-Y, Chen G-H, Li H, Huang Y, Wang G-S, Jiang N, et al. Rapamycin-treated mature dendritic cells have a unique cytokine secretion profile and impaired allostimulatory capacity. Transpl Int Off J Eur Soc Organ Transplant. ottobre 2009;22(10):1005–16.

138. Boor PPC, Metselaar HJ, Mancham S, van der Laan LJW, Kwekkeboom J. Rapamycin has suppressive and stimulatory effects on human plasmacytoid dendritic cell functions. Clin Exp Immunol. dicembre 2013;174(3):389–401.

139. Birbach A, Gold P, Binder BR, Hofer E, de Martin R, Schmid JA. Signaling molecules of the NF-kappa B pathway shuttle constitutively between cytoplasm and nucleus. J Biol Chem. 29 marzo 2002;277(13):10842–51.

140. Zhou H, Wertz I, O'Rourke K, Ultsch M, Seshagiri S, Eby M, et al. Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO. Nature. 8 gennaio 2004;427(6970):167–71.

141. Gilmore TD. The Rel/NF-kappaB signal transduction pathway: introduction. Oncogene. 22 novembre 1999;18(49):6842–4.

142. Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene. 1 novembre 2001;20(50):7342–51.

143. Wu MX, Ao Z, Prasad KV, Wu R, Schlossman SF. IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. Science. 14 agosto 1998;281(5379):998–1001.

144. van de Laar L, van den Bosch A, van der Kooij SW, Janssen HLA, Coffer PJ, van Kooten C, et al. A nonredundant role for canonical NF-κB in human myeloid dendritic cell development and function. J Immunol Baltim Md 1950. 15 dicembre 2010;185(12):7252–61.

145. Leão R, Apolónio JD, Lee D, Figueiredo A, Tabori U, Castelo-Branco P. Mechanisms of human telomerase reverse transcriptase (hTERT) regulation: clinical impacts in cancer. J Biomed Sci

[Internet]. 12 marzo 2018 [citato 19 ottobre 2018];25. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5846307/

146. Rampazzo E, Del Bianco P, Bertorelle R, Boso C, Perin A, Spiro G, et al. The predictive and prognostic potential of plasma telomerase reverse transcriptase (TERT) RNA in rectal cancer patients. Br J Cancer. 20 marzo 2018;118(6):878–86.

147. Muraro E, Furlan C, Avanzo M, Martorelli D, Comaro E, Rizzo A, et al. Local High-Dose Radiotherapy Induces Systemic Immunomodulating Effects of Potential Therapeutic Relevance in Oligometastatic Breast Cancer. Front Immunol [Internet]. 6 novembre 2017 [citato 3 ottobre 2018];8. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5681493/

148. Muraro E, Comaro E, Talamini R, Turchet E, Miolo G, Scalone S, et al. Improved Natural Killer cell activity and retained anti-tumor CD8+ T cell responses contribute to the induction of a pathological complete response in HER2-positive breast cancer patients undergoing neoadjuvant chemotherapy. J Transl Med [Internet]. 27 giugno 2015 [citato 4 ottobre 2018];13. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4483222/

149. Wang D, Matsumoto R, You Y, Che T, Lin X-Y, Gaffen SL, et al. CD3/CD28 costimulation-induced NF-kappaB activation is mediated by recruitment of protein kinase C-theta, Bcl10, and IkappaB kinase beta to the immunological synapse through CARMA1. Mol Cell Biol. gennaio 2004;24(1):164–71.

150. Kane LP, Lin J, Weiss A. It's all Rel-ative: NF-κB and CD28 costimulation of T-cell activation. Trends Immunol. 1 agosto 2002;23(8):413–20.

151. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 4 marzo 2011;144(5):646–74.

152. Terrin L, Trentin L, Degan M, Corradini I, Bertorelle R, Carli P, et al. Telomerase expression in B-cell chronic lymphocytic leukemia predicts survival and delineates subgroups of patients with the same igVH mutation status and different outcome. Leukemia. maggio 2007;21(5):965–72.

153. Bertorelle R, Briarava M, Rampazzo E, Biasini L, Agostini M, Maretto I, et al. Telomerase is an independent prognostic marker of overall survival in patients with colorectal cancer. Br J Cancer. 5 febbraio 2013;108(2):278–84.

154. Tani N, Ichikawa D, Ikoma D, Tomita H, Sai S, Ikoma H, et al. Circulating cell-free mRNA in plasma as a tumor marker for patients with primary and recurrent gastric cancer. Anticancer Res. aprile 2007;27(2):1207–12.

155. March-Villalba JA, Martínez-Jabaloyas JM, Herrero MJ, Santamaria J, Aliño SF, Dasí F. Cell-free circulating plasma hTERT mRNA is a useful marker for prostate cancer diagnosis and is associated with poor prognosis tumor characteristics. PloS One. 2012;7(8):e43470.

156. Miura N, Nakamura H, Sato R, Tsukamoto T, Harada T, Takahashi S, et al. Clinical usefulness of serum telomerase reverse transcriptase (hTERT) mRNA and epidermal growth factor receptor (EGFR) mRNA as a novel tumor marker for lung cancer. Cancer Sci. 1 dicembre 2006;97(12):1366–73.

157. Rittà M, Costa C, Sinesi F, Sidoti F, Di Nauta A, Mantovani S, et al. Evaluation of Epstein-Barr Virus–Specific Immunologic Response in Solid Organ Transplant Recipients With an Enzyme-Linked ImmunoSpot Assay. Transplant Proc. 1 settembre 2013;45(7):2754–7.

158. Azevedo* LS, Pierrotti LC, Abdala E, Costa SF, Strabelli TMV, Campos SV, et al. Cytomegalovirus infection in transplant recipients. Clinics. luglio 2015;70(7):515–23.

159. Banas B, Steubl D, Renders L, Chittka D, Banas MC, Wekerle T, et al. Clinical validation of a novel enzyme-linked immunosorbent spot assay-based in vitro diagnostic assay to monitor cytomegalovirus-specific cell-mediated immunity in kidney transplant recipients: a multicenter, longitudinal, prospective, observational study. Transpl Int Off J Eur Soc Organ Transplant. 2018;31(4):436–50.

160. Jensen P, Hansen S, Møller B, Leivestad T, Pfeffer P, Geiran O, et al. Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens. J Am Acad Dermatol. febbraio 1999;40(2 Pt 1):177–86.

161. Smith RA, Andrews K, Brooks D, DeSantis CE, Fedewa SA, Lortet-Tieulent J, et al. Cancer screening in the United States, 2016: A review of current American Cancer Society guidelines and current issues in cancer screening. CA Cancer J Clin. aprile 2016;66(2):96–114.

- **Published Articles during 3 years of the program**

Fighting Viral Infections and Virus-Driven Tumors with Cytotoxic $CD4^+$ T Cells.
Muraro E, Merlo A, Martorelli D, Cangemi M, Dalla Santa S, Dolcetti R, Rosato A.
Front Immunol. 2017 Feb 27;8:197. doi: 10.3389/fimmu.2017.00197. eCollection 2017.

Abstract
$CD4^+$ T cells have been and are still largely regarded as the orchestrators of immune responses, being able to differentiate into distinct T helper cell populations based on differentiation signals, transcription factor expression, cytokine secretion, and specific functions. Nonetheless, a growing body of evidence indicates that $CD4^+$ T cells can also exert a direct effector activity, which depends on intrinsic cytotoxic properties acquired and carried out along with the evolution of several pathogenic infections. The relevant role of $CD4^+$ T cell lytic features in the control of such infectious conditions also leads to their exploitation as a new immunotherapeutic approach. This review aims at summarizing currently available data about functional and therapeutic relevance of cytotoxic $CD4^+$ T cells in the context of viral infections and virus-driven tumors.

- **Submitted Articles during 3 years of the program**
  (review submitted in September 2018)

Dissecting the multiplicity of immune effects of immunosuppressive drugs to better predict the risk of *de novo* malignancies in solid organ transplant patients.
Cangemi M, Montico B, Faè DA, Steffan A, Dolcetti R.
Frontiers in Oncology

Acknowledgements

This work was possible thank to the valuable contributions of Dr. Emanuela Vaccher (responsible researcher), Dr. Ferdinado Martellotta, Dr. Ornella Schioppa, Dr. Stefania Zanussi, Dr. Chiara Pratesi (Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, 33081 Aviano, Italia), Dr. Anita De Rossi and Dr. Enrica Rampazzo (IOV, Istituto Oncologico Veneto, IRCCS).

I would like my supervisor Prof. Riccardo Dolcetti for the support and scientific advices, my co-supervisor Dr. Agostino Steffan and all members of my research group, Dr. Elena Muraro, Dr. Barbara Montico, Dr. Antonia Damiana Faè, Dr. Katy Mastorci, Dr. Elisabetta Fratta and MLT Elisa Comaro for their support and scientific contribution.