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PHENOTYPIC AND FUNCTIONAL MODULATION OF REGULATORY T CELLS IN MELANOMA PATIENTS

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PART I

LIST OF PUBLICATIONS INCLUDED IN THIS THESIS

"LAG-3 expression defines a subset of CD4(+)CD25(high)Foxp3(+) regulatory T cells that are expanded at tumor sites"
Camisaschi C, Casati C, Rini F, Perego M, De Filippo A, Triebel F, Parmiani G, Belli F, Rivoltini L, Castelli C.
J Immunol. 2010 Jun 1;184:6545-51.

SUBMITTED: "Frequency and function of circulating regulatory T cells in melanoma patients receiving peptide-based vaccine"
Camisaschi C, Filipazzi P, Casati C, Beretta V, Maio M, Chiarion-Sileni V, Tregni G, Santimani M, Parmiani G, Rivoltini L and Castelli C.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

"Soluble Human LAG-3 Molecule Amplifies the In vitro Generation of Type 1 Tumor-Specific Immunity"
Chiara Casati, Chiara Camisaschi, Francesca Rini, Flavio Arienti, Licia Rivoltini, Frédéric Triebel, Giorgio Parmiani, Chiara Castelli.
Cancer Res. 2006 Apr 15; 66(8): 4450-60.

"Human lymphocyte activation gene-3 molecules expressed by activated T cells deliver costimulation signal for dendritic cell activation"
Chiara Casati, Chiara Camisaschi, Luisa Novellino, Arabella Mazzocchi, Frederic Triebel, Licia Rivoltini, Giorgio Parmiani, Chiara Castelli.
J Immunol. 2008 Mar 15; 180(6): 3782-8.

"Human plasmacytoid dendritic cells interact with gp96 via CD91 and regulate inflammatory responses"
AnnaMaria De Filippo, Robert J. Binder, Chiara Camisaschi, Valeria Beretta, Flavio Arienti, Antonello Villa, Pamela Della Mina,Giorgio Parmiani, Licia Rivoltini, Chiara Castelli.
J Immunol. 2008 Nov 1; 181(9): 6525-35.

ABSTRACT

Recent studies indicated that regulatory T cells (Tregs) are implicated in the suppression of the immune response against tumors. Accumulation of Tregs in peripheral blood and in tumor microenvironment has been described for patients with various types of cancer. The balance between T effector cells and Tregs, both at tumor site or at local draining lymph nodes is subverted thus limiting the immune response against cancer and restraining the effect of immunotherapy. As concern melanoma, data till now available confirm the presence of Tregs at tumor site, however no in deep analysis on phenotypic or functional characterization of these cells have been provided.

This thesis is aimed at evaluating the role of CD4+ Tregs in the immune response against melanoma either by *in vivo* or *in vitro* approaches.

Among molecules found in mice as expressed by Tregs, lymphocytes activation gene-3 (LAG-3) has been described to be expressed by activated Tregs and more in general as a molecule involved in the control of T cell expansion and homeostasis. In this thesis I explored the expression of LAG-3 in human CD4+ T cells and found that LAG-3 identifies a discrete subset of $CD4^+CD25^{high}Foxp3^+$ T cells. This $CD4^+CD25^{high}Foxp3^+LAG-3^+$ population is preferentially expanded in lymphocytes of tumor-invaded lymph nodes and in lymphocytes infiltrating visceral and *sub cute* metastasis of melanoma patients. *Ex-vivo* analysis showed that $CD4^+CD25^{high}Foxp3^+LAG-3^+$ T cells are functionally active cells that release the immunosuppressive cytokines interleukin-10 (IL-10) and transforming growth factor beta (TGF-β1). An *in vitro* suppression assay using $CD4^+CD25^{high}LAG-3^+$ T cells sorted from *in vitro* expanded $CD4^+CD25^{high}$ Tregs showed that this subset of cells is endowed with potent suppressor activity that requires cell-to-cell contact. All together, our data show that LAG-3 defines an active $CD4^+CD25^{high}$ Tregs subset in melanoma patients whose frequency is expanded at tumor sites.

My data showed that Tregs are accumulated in different immunological districts of patients with melanoma and they also stress the notion that in melanoma patients these Tregs are preferentially in an activation status.

However, the real impact of these cells on tumor progression has not been totally clarified.

To get insights on the relevance of Tregs in the immunological response to tumor, a phase II randomized trial of multipeptide vaccination in stage IIB-C/III melanoma patients has been designed to include the administration of low dose cyclophosphamide (CTX; 300mg/m^2) and low dose interleukin-2 (IL-2; 3x10^6). CTX has been described as limiting the expansion of Tregs, while IL-2 was given with the aim of expanding tumor-specific responses. The modulation that these drugs exert on different T cell compartment, namely Tregs and conventional T cells was evaluated for its impact on patients' immunological response.

Careful *ex-vivo* immunological monitoring has been performed aimed at assessing the status of vaccine-induced immune response and the levels of Tregs. Importantly, Treg frequency was defined combining physical and functional markers trying to take into account the plasticity of this population. We observed that CTX has a limited efficacy and a transient effect on Tregs modulation; frequency of Tregs identified by multiparametric fluorescence-activated cell sorting (FACS) analysis as CD4$^+$CD25^{high}Foxp3$^+$ dropped in peripheral blood mononuclear cells (PBMCs) collected 4-7 days after CTX administration, with 6 out of 13 patients displaying a reduction ranging from 20 to 65 %. IL-2 showed higher immunomodulatory effects, expanding both circulating conventional activated CD4+ T cells and Tregs; interestingly, a fraction of these Tregs displayed a Th-1 like phenotype, expressing *ex-vivo* T-bet (Th1 specific T-box transcription factor) and interferon-γ (INF-γ). Importantly, this enhanced frequency of Tregs does not significantly affect patients' immunization assessed *ex-vivo* by human leukocyte antigen (HLA)-A*0201/peptide multimer staining and IFN-γ ELISpot assays.

AIMS OF THE PROJECT

Aim of this thesis is to gain insights into the functional features of Tregs present at tumor sites, in tumor draining lymph nodes and in peripheral blood of melanoma patients. This study is mainly committed to evaluate two hypotheses:

- that LAG-3 molecule could be a marker for a subset of human Tregs. If so, to verify the hypothesis that LAG-3+ Tregs may represent a tumor-associated Treg population;

- that Tregs would exert an actual role on melanoma immunity, with the final goal of understanding whether their elimination or down-modulation by pharmacological treatments can be of any benefit to immunological therapies.

STATE OF THE ART

1. MELANOMA

1.1 Epidemiology

Cutaneous melanoma, also referred to as "malignant melanoma", represents 3-7% of all skin malignancies and is the most serious form of skin cancer because of its propensity for early invasion and widespread metastasis. With early detection and treatment, the cure rate of melanoma is about 95%, but advanced melanoma appears to be resistant to conventional therapies and, despite the overall advances in the fields of tumor biology and oncology therapies, prognosis for patients with widely metastatic disease remains poor, with a median survival rate of 6 months and 5-year survival rate of less than 5% (Cummins et al, 2006).

1.2 Pathological staging of melanoma

In 2001 the American Joint Committee on Cancer (AJCC) Tumor-Nodes-Metastasis (TNM) staging classification incorporated Breslow depth, Clark's level, ulceration and pathological micro-staging attributes. The TNM classification is widely used to describe many human cancers. In general T refers to the primary tumor size, N describes the involvement of regional lymph nodes, and M indicates the presence or absence of distant metastases. Paralleling the staging system for other cancers, melanoma patients with localized disease are characterized as stage I or II, those with regional metastases are considered stage III, and those with distant metastases are considered IV. To characterize the primary tumor, T in melanoma uses Breslow Depth (Breslow, 1970), Clark's level (Clark et al, 1969) and the presence or absence of ulceration.

Breslow depth is measured in millimeters and can be divided in T1 (<1.0mm), T2 (1.01 to 2.0mm), T3 (2.01 to 4.0mm), T4 (>4.0mm). Clark's level describes the depth of invasion and is useful for prognostic differentiation of T1 lesions. Level I is limited to the epidermis (in situ melanoma); Level II reaches the papillary dermis; Level III fills the papillary dermis; Level IV involves the reticular dermis; Level V penetrates within the subcutaneous fat. Tumors that have a Breslow depth

of less than 1mm and have a Clark's level of II or III without ulceration remain T1a melanomas; T1b melanomas are ulcerated or have a Clark's level IV or V. A final version of the 2009 AJCC staging and classification of melanoma included the mitotic index as a prognostic factor in stratifying T1 melanoma. Primary tumor mitotic rate was introduced as a major criterion for melanoma staging and prognosis that replaces the Clark's level invasion, and is now proven to be an important independent adverse predictor of survival (Balch et al., 2009).

The N category considers the status of regional lymph nodes. Stages I or II do not include any nodal or metastatic involvement. For Stage III melanoma, the number and dimension (micrometastasis versus macrometastasis) of nodal metastases or the presence of in-transit metastases (lesions that reside more than 5 cm from the primary tumor) or satellite lesions (lesions that reside within 5 cm from the primary tumor) are the most significant predictors of outcomes.

Distant metastases characterize Stage IV melanomas and can occur in skin and subcutaneous tissues, distant lymph nodes (M1a), lung (M1b), and other visceral organs (M1c) (Table 1).

In the absence of nodal or distant metastases, tumor thickness, mitotic rate and presence or absence of ulceration remain the best predictors of survival outcome (Balch et al, 2011).

Table 1. Pathological Staging of Melanoma (Adapted from Balch et al. 2011)

Pathologic Stage	TNM	Thickness (mm)	Ulceration	No Positive Nodes	Nodal Type	Distant Metastasis	Surv % 5Years	Surv % 10Years
IA	T1a	≤1	No	0	-	-	95.3	87.9
IB	T1b	≤1	Yes or Level IV,V	0	-	-	90.9	83.1
	T2a	1.01-2.0	No	0	-	-	89.0	79.2
IIA	T2b	1.01-2.0	Yes	0	-	-	77.4	64.4
	T3a	2.01-4.0	No	0	-	-	78.7	63.8
IIB	T3b	2.01-4.0	Yes	0	-	-	63.0	50.8
	T4a	>4.0	No	0	-	-	67.4	53.9
IIC	T4b	>4.0	Yes	0	-	-	45.1	32.3
IIIA	N1a	Any	No	1	Micro	-	69.5	63.0
	N2a	Any	No	2-3	Micro	-	63.3	56.9
IIIB	N1a	Any	Yes	1	Micro	-	52.8	37.8
	N2a	Any	Yes	2-3	Micro	-	49.6	35.9
	N1b	Any	No	1	Macro	-	59.0	47.7
	N2b	Any	No	2-3	Macro	-	46.3	39.2
IIIC	N1b	Any	Yes	1	Macro	-	29.0	24.4
	N2b	Any	Yes	2-3	Macro	-	24.0	15.0
	N3	Any	Any	4	Micro/Macro	-	26.7	18.4
IV	M1a	Any	Any	Any	Any	Skin/SQ	18.8	15.7
	M1b	Any	Any	Any	Any	Lung	6.7	2.5
	M1c	Any	any	any	any	Other visceral	9.5	6.0

1.3 Systemic therapy for melanoma

Surgical excision is the treatment of choice for melanoma, especially for early stages of disease, and systemic chemotherapy remains the most widely used treatment, especially when metastatic sites are not surgically accessible, but melanoma are less sensitive to standard treatment than other cancers.

1.3.1 Chemotherapy of melanoma

Traditional systemic chemotherapy has failed to significantly improve the survival of patients with non-resectable metastatic melanoma (Bajetta et al, 2002). Chemotherapy drugs more commonly used to treat melanoma include Dacarbazine (DTIC) and Temozolomide (TMZ). Response rate of DTIC as single agent ranges from 15% to 25%, but these responses have in general brief duration (3-6 months) and the complete response rate is only 5% (Lee et al., 1995). TMZ was shown to have a slightly

higher response rate (13.5% vs 12.1%) and median survival (7.7 vs 6.4 moths) than DITC, neither of which was statistically significant (Middleton et al., 2000). DTIC is also used with other chemotherapy drugs such as Carmustine (BCNU) and cisplatin. Cisplatin, vinblastine, and DTIC is another chemotherapy combination for treating melanoma. These chemotherapy drugs may also be combined with immunotherapy drugs, such as interferon alpha (IFN-α) and/or IL-2.

1.3.2 Cancer immunotherapy

Melanoma is one of the most immunogenic types of cancer, in fact several melanoma-specific antigens have been identified (Table 2) and large number of melanoma specific antibodies and functional lymphocytes are present in patients with melanoma (Boon et al., 2006); moreover, metastatic melanoma patients respond to immune-stimulating agents, such as interferons and IL-2 (Garbe et al., 2011) and spontaneous regression of melanoma with simultaneous onset of vitiligo has been reported (Speeckaert et al., 2011).

For this reason immunotherapy has been largely used in the last decades as experimental therapy to treat melanoma. Immunotherapeutic approaches, aimed at triggering and/or restoring host immune system against the tumor, can be divided in two main groups: adoptive immunotherapy and vaccination or active specific immunotherapy.

In adoptive immunotherapy, *in vitro* activated immune effector cells or specific antibodies are exogenously administered to patients.

Conversely, cancer active immunotherapy mainly consists in therapeutic vaccines aimed at the induction of antibodies or T cell-mediated tumor specific immune responses. Final goal of active immunization is the generation of a long lasting immunological memory.

Table 2. Human melanoma antigens classification (Adapted from Novellino et al., 2005)

Human melanoma antigens recognized by T cells		
Melanocyte lineage/differentiation antigens: shared between tumor and normal tissue, found in melanoma and melanocytes	Tyrosinase gp100 MC1R PSA MelanA-MART1	gp75 TRP-2 OA1 P Polypeptide
Oncofetal/cancer-testis antigens: normally expressed in testis and placenta, re-activated in tumor	MAGE family BAGE family GAGE family NY-ESO1	CAMEL NA88-A SSX-2 TRAG-3
Unique and shared tumor-specific antigens: point mutations or splicing aberration of normal genes, often associated with neoplastic transformation and/or progression	CDK-4 β-catenin MART-2 Myosin MUM family	BING-4 N-RAS TRP2 ARTC-1
Widely occurring overexpressed TAAs: universal antigens associated with tumorigenic phenotype, generally with lower expression in normal tissue	Survivin SOX10 707-AP HER-2/neu GnT-V Epha2-3 AIM-2 Annexin II	SART-2 hTERT p15 Adipophilin Livin PRAME RAGE RU1-2

1.3.3 Immune-based therapy in melanoma

The most important therapeutic agent for melanoma treatment has been the T-cell growth factor IL-2. IL-2 exerts its function through its ability to expand and activate T and natural killer (NK) cells. Studies reported overall response rate of 15-20% with durable responses (Atkins et al., 1999). In light of these findings IL-2 has been approved by the Food and Drug Administration (FDA) as treatment for metastatic melanoma, but the toxicities associated with high-dose IL-2 have been the main obstacle to its widespread use in the treatment of patients. In addition IL-2, extensively used in immunotherapy protocol to boost T effector cells

response, has been described to increase the frequency of circulating Tregs (Ahmadzadeh M et al., 2006).

Interferons (IFN) are a complex family of proteins with immunomodulatory and antiangiogenic properties that are produced in response to viral infection or after T-cell activation. IFN-α has been the most extensively studied in melanoma. It produces responses in about 16% of metastatic melanomas (Agarwala and Kirkwood, 1996). It has been used in combination with other agents, with higher response rates in the IFN-α containing regimens (Hwu et al, 2006). Currently, IFN-α is used for high risk melanoma patients to prevent recurrences (Ascierto and Kirkwood, 2008). However, the use of IFN-α is under debate since significant effects on overall survival are not confirmed and for its high toxicity and severe side effects.

New immune-based strategy for melanoma is based on the *in vivo* usage of immunomodulating antibodies directed against molecules crucially involved in the fine tuning of the immune-response. These antibodies include those receptors that suppress the host immune response (Cytotoxic T-lymphocyte antige-4, CTLA-4 and Programmed Death-1, PD-1) and those that activate receptors that amplify the immune response (CD40 on antigen presenting cells (APC), CD137 and OX40 on T cell).

Early clinical studies defined human anti-CTLA-4 antibodies (ticilimumab, iplimumab) pharmacokinetics and possibilities for combination therapeutic regimen. Phase II trials of ipilimumab in advanced melanoma showed objective responses and, in a phase III trial, ipilimumab was the first agent to demonstrate an improvement in overall survival (10.1 months) in patients with advanced melanoma. In 10-15% of the treated patient severe immune related adverse events occurred, but most this treatment-related toxicity was reversible with appropriate care (Hodi et al., 2010).

As previously discussed, active immunotherapy mainly includes therapeutic vaccine whose final aims is the generation of a long lasting, tumor-specific immune response. Vaccines for melanoma are designed to boost immune reactions against a malignancy that is already established, aimed at activating or boosting host immune responses possibly inducing an endogenous immunity. The selection of suitable antigens is the first

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decisive step in designing cancer vaccines; antigens should include molecules preferentially expressed by tumor tissues, in order to prevent cross-reaction with normal cells. Indeed, the majority of cancer antigens are represented by proteins preferentially expressed by tumor cells but still belonging to self and thus potentially subjected to tolerance (melanoma antigens classification is reported in Table 2). One major issue in vaccine design is therefore the selection of antigens which should induce tumor immunity without autoimmunity and antigen formulation is an additional crucial issue for achieving a successful immunization. Different vaccination modalities exist: cell-based vaccination using dendritic cells (DCs) loaded with antigens, protein/peptide vaccines, genetic vaccines (DNA or RNA encoding the antigen), viral vectors, or combination of these with or without adjuvant. Several studies have explored the efficacy of tumor-associated antigenic peptides with or without IL-2, DC or other adjuvant. However, despite the induction of tumor-specific immune responses induced by immunotherapy approaches can be observed in patients with melanoma, favourable clinical outcomes are scarce (Jandus et al., 2009).

The first evidence of clinical benefit was reported recently in a randomized phase III clinical study in which melanoma patients received gp100 and high dose of IL-2 (Schwartzentruber et al., 2009).

2. IMMUNOEDITING THEORY

The encounter between the immune system and a newly generated tumor develops through a process known as "immunoediting", in which three distinct phases can be defined (Dunn et al., 2002):

- eradication of cancer, in which cells of the innate and adaptive arms of the immune system are recruited following a "danger" signal generated from newly growing tumors (Gallucci and Matzinger, 2001);

- cancer equilibrium, a condition in which the host immune system and any tumor cell variant, survived the eradication phase, enter into a dynamic equilibrium: tumor cells are continuously eliminated, but the immune selection pressure induce the production of resistant tumor variants (Koebel et al., 2007). Equilibrium is probably the longest phase of cancer immunoediting, and it may last several years.

- tumor escape, that is the ability of tumor to growth despite activation of the immune system. This may be achieved through a variety of mechanisms that the tumor itself has developed to evade immune recognition and attack (Rivoltini et al., 2005).

2.1 Immune escape mechanisms

1) *Loss or down-regulation of MHC class I expression on tumor cells*

Down-regulation of major histocompatibility (MHC) class I proteins prevents the formation of MHC/peptide complexes necessary for cytotoxic T lymphocytes (CTL) recognition, therefore inhibiting cytotoxic T cell-mediated lysis (Chang and Ferrone, 2007).

2) *Impairment of antigen recognition*

Tumors may develop defective antigen processing pathways, thanks to mutations in genes coding for molecules crucial for antigen presentation or peptide transport to the endoplasmic reticulum, as the case of TAP (transporter associated with antigen presentation). When such mutations occur, tumor-associated antigens, although present, cannot be presented by MHC molecules, therefore allowing progression of the disease (Maeurer et al., 1996).

3) *Inactivation of final effector functions of immune cells*

Using different mechanisms, tumor cells may directly limit the function of the immune effector cells. Examples are the impaired binding of perforin molecules released by CTL to the surface of tumor cells, that became resistant to perforin-mediated killing (Lehmann et al., 2000); or down-regulation or mutations of Fas gene, leading to breakdown of Fas/FasL pathway (French and Tschopp, 2002).

 4) *Tolerance induction*

 Tumors can also activate mechanisms that directly impair immune responses and lead to tolerance. First, lack of costimulatory molecules (B7-1/CD80, B7-2/CD86, and CD40L) or activation of negative costimulatory signals (CTLA-4/B7, PD-1/PD-L1, Fas/FasL) can prevent activation of CTL, leading to clonal anergy of tumor-specific T cells (Gajewski, 2007). Moreover, pre-existing functionally activated CTL may become anergic in the absence of DC-activating signals (Staveley-O'Carroll et al., 1998) or in the presence of tolerogenic DC, such as plasmacytoid DC (pDC), which inhibit effector T cells proliferation by 2,3-dioxigenase (IDO) production (Puccetti and Grohmann, 2007). Tumors can also down-regulate the expression of endothelial adhesion molecules, thus reducing the homing of T cells at tumor site (Madhavan et al., 2002).

 5) *Active immunosuppression*

Apart from inducing tolerance, tumors can also actively suppress immune responses either systemically or in their microenvironment.

One of the most frequent mechanisms adopted by tumors to actively suppress immunity relies on the secretion of a variety of soluble immunosuppressive factors, such as TGF-β (Teicher, 2007), IL-10 (Kawamura et al., 2002), nitric oxide (Zhang and Xu, 2001), galectin-1 (Danguy et al., 2002) and prostaglandin-E2 (PGE2). Tumors also induce accumulation of activated granulocytes (Schmielau and Finn, 2001), mast cells (Conti et al., 2007), tumor-associated macrophages (TAMs) (Mantovani et al., 2011) and the generation of a subset of inflammatory monocytes, so-called myeloid-derived suppressor cells (MDSC), which strongly inhibit CD8^{+} T cell responses, NK activity and DC maturation (Filipazzi et al., 2007). Moreover, the presence in the tumor microenvironment of tolerogenic subpopulations and the abundant

expression of cytokines and chemokines such as TGF-β, IL-10, chemokine (C-C motif) ligand 2 (CCL2), CCL17, CCL22, or IDO contribute to regulatory T cells (Tregs) accumulation and differentiation within the tumor mass and in the draining lymph nodes (LNs) (Curiel et al., 2004; Beyer and Shulze, 2006).Together with tolerogenic DC, Tregs inhibit the generation of effector T cells, resulting in the induction of tolerance against the tumor (Figure 1).
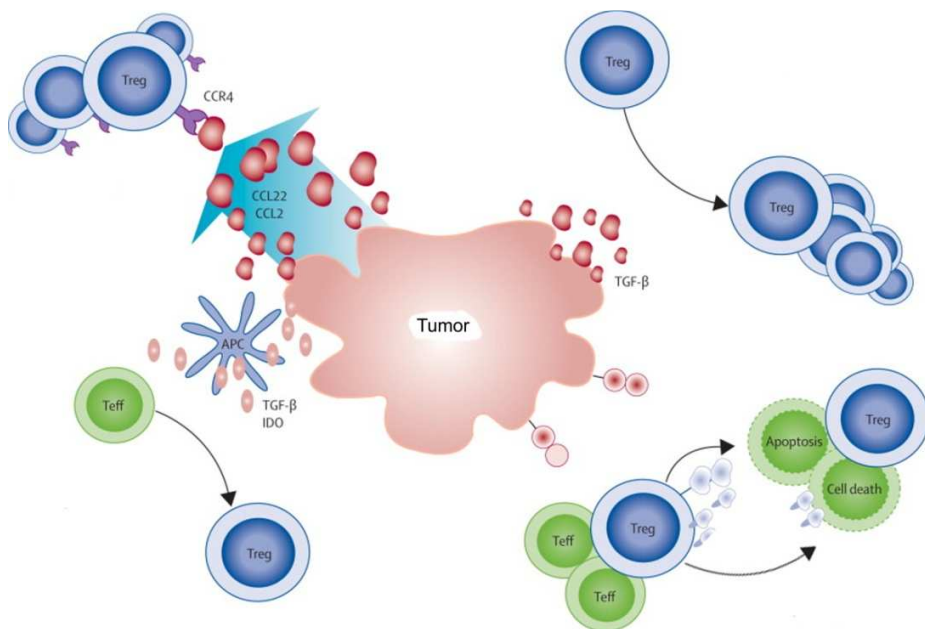

Figure 1. **Treg accumulation at tumor site.** Tregs expressing CCR4 are attracted at tumor site by the presence of chemokines as CCL2 and CCL22 (A). Factors released by tumor or tolerogenic APC in tumor microenvironment can induce Treg conversion and proliferation (B and C). Tregs induce effector T cells apoptosis and block their proliferation (D). *(Adapted from Jacobs et al., 2012).*

2.2 TREGs

Tregs, that comprise 5-10% of peripheral CD4+ T cells in humans, have an essential role in sustaining self-tolerance and immune homeostasis by suppressing many physiological and pathological immune-responses (Sakaguchi et al., 2008). Their development and function depends on the

nuclear transcription factor forkhead box P3 (Foxp3) and mutations in this gene cause fatal autoimmune diseases (Sakaguchi et al., 2008).

2.2.1 Treg subsets

Tregs consist of different subsets of cells that can be identified on the basis of their origin and mechanism of immune suppression (Roncarolo et al., 2000). Induced Tregs include Tr1 and Th3 CD4+ T cells. Tr1 cells arise from CD4+ T cells in a tolerogenic environment and secrete high level of IL-10; while Th3, which are induced by oral Ag administration, secrete high level of TGF-β. On the other hand, naturally occurring CD4+ Tregs, that constitutively express the interleukin-2 (IL-2) receptor alpha-chain (CD25) and Foxp3, are produced by the normal thymus as a functionally distinct and mature subpopulation of T cells (Jonuleit et al., 2003). Tregs can be also induced in the periphery from naïve T cells under certain condition (Akbar et al., 2007).

2.2.2 Mechanisms of suppression

The mechanisms by which Tregs mediate immune suppression are not completely elucidated, but are likely to include both cell-cell contact and cytokine secretion (Zou et al., 2006).

Natural Tregs, upon T cell receptor (TCR) engagement (Baecher-Allan et al., 2004) exert their suppressive functions in a contact-dependent fashion. However, Tregs act with different mechanisms and, based on their mode of action, Tregs can be further classified into discrete subsets that differently rely on cell to cell contact and on IL-10 secretion (Ito et al., 2008). Furthermore, the fine mechanism of action of TGF-β, an additional cytokine well accepted crucially involved in Treg suppression, has not been sort out completely.

Some Tregs are antigen specific (Tang et al., 2008), but once activated they can also suppress by an antigen-independent bystander mechanism (Tang et al., 2008).

In line with these observations, it has been clearly demonstrated that antigen-specific Tregs actively suppress proliferation of CD4+CD25- and CD8+ effector T cells in most human solid tumors as well as hematologic

malignancies, therefore limiting the immune response against cancer and contributing to tumor progression (Piersma et al., 2008).

2.2.3 Tregs in tumors

The significant role of CD4+ Tregs in suppressing anti-tumor immune response has been well documented for murine tumors (Sakaguchi et al., 2004). However, in humans the possible implication of CD4+ Tregs in down-modulating anti-tumor immunity is under intense investigation. A number of studies have reported an increase in circulating CD4+ Tregs in patients with various cancers (Viguier et al., 2004; Curiel et al., 2004; Woo et al., 2001). Furthermore, increased percentages of Tregs in the peripheral blood of gastric and esophageal cancer patients and in the tumor tissue correlated with poor prognosis and decreased survival (Curiel et al., 2004; Kono et al., 2006).

2.2.4 Tregs in melanoma

As compared with age matched healthy control, Tregs are overrepresented in peripheral blood of melanoma patients (Correll et al., 2010; Jandus et al., 2008; McCarter et al., 2007), in primary lesion (Ladanyi et al., 2010; Miracco et al., 2007), tumor invaded LNs (Jandus et al., 2008) and metastatic lesions (Mourmouras et al., 2007; Ahmadzadeh et al., 2008). Moreover, several studies reported a correlation between the extent of Tregs infiltration and prognosis in patients with melanoma, but results are contradictory in literature and often this correlation was not found (Ladanyi et al., 2010; Lagouros et al., 2009).

2.2.5 Treg depletion

Treg mediated immune-suppresion is one of the main obstacle for cancer immunotherapy. Depletion of Tregs in murine model of melanoma leads to enhanced induction of anti-tumor immunity and to an improvement in tumor clearance and survival (Nizar et al., 2010); so, Treg depletion is under intense investigation also in humans. There are different approaches aimed at depleting Tregs or blocking their activity. To date the main strategies adopted in clinical trials include the usage of

molecules targeting CD25 or the administration of Cyclophosphamide (N, N-bis (2-chloroethyl)-1, 3, 2- oxazaphosphinan -2 amine 2-oxide, the generic name for Cytoxan (CTX), Endoxan). Jacobs and colleagues achieved systemic depletion of CD25 positive cells with anti-CD25 antibody (daclizumab) in metastatic melanoma patients before the administration of a peptide-pulsed DC vaccine. This antibody treatment however did not lead to any increase in the extent of tumor-specific immune response and had no effect on progression-free survival (Jacobs et al., 2010). Conversely, depletion of CD25+ cells using the clinically approved IL-2 diphtheria toxin conjugated (Ontak) showed clinical responses when combined with vaccination in mice and colon cancer patients (Knutson et al., 2006; Dannull et al., 2005), but the same schedule applied to melanoma patients provided contradictory results (Mougiakakos et al., 2010; Attia et al., 2005). Of note, CD25 is also expressed on activated non-regulatory T cells and therefore administration of anti-CD25 antibodies could possibly limit also positive anti-tumor responses.

The alkylating agent CTX has a broad spectrum of action. It is used to treat vary types of diseases: solid and hematological malignancies, autoimmune disorder and transplantations. CTX reduces the number of circulating Tregs in animal models (Ghiringhelli et al., 2004; Lutsiak et al., 2005), but the same result in human has not been clearly demonstrated. The biological activities of CTX are dose dependent and the metronomic dosing, in combination with others therapies, has immunomodulatory and antiangiogenic attributes (Sistigu et al., 2011; Ghiringhelli et al., 2007).

Actually why CTX has these positive effects is not totally understood. CTX seems to have a complex role; in fact, it can act not only on proliferating tumor cells, but also on the different cell types composing the immune system. In addition, the study of this molecule is difficult due to its transient effect.

2.2.6 Treg heterogeneity and plasticity

The contradictory results about Treg monitoring are mainly due to the fact that most of the studies are retrospective and the exact definition of

Tregs and the techniques of Treg analyses greatly affect the result of Treg measurements.

Naturally occurring Tregs in mice are unequivocally defined as CD4$^+$CD25$^+$Foxp3$^+$ T cells. This same set of markers, together with low level expression of IL-7 receptor alpha (CD127) (Seddiki et al., 2006), identifies also human Tregs, but recent studies have clearly indicated that new hallmarks of Treg phenotype need to be identified, being CD4$^+$CD25$^+$Foxp3$^+$ expression shared also by effector T cells in humans. A more accurate analysis of human Tregs combining CD4, CD25, Foxp3 and CD45RA allows to identify different subsets of human Tregs and differentiate them from conventional activated T cells (Myiara et al., 2009). Furthermore, a part of molecules constitutively expressed by human Tregs such as CTLA-4, GITRL, CD27, CD28, OX40 and CD62L, the expression of molecules as MHC-class II, CCR7, CD147, CD39, GARP and co-stimulatory molecule as ICOS (Tosello V et al., 2008; Baecher-Allan C et al., 2006; Ito T et al., 2008; Solstad T et al., 2011; Borsellino et al., 2007; Tran et al., 2009) identifies different Treg subsets, highlighting the wide heterogeneity of these populations. In addition, recent studies provide evidence not only for functional heterogeneity, but also for high lineage plasticity of Tregs. They can indeed differentiate into IL-17 producing Th17, acquiring RORγt expression with or without losing Foxp3 expression (Bovenschen, et al., 2011; Raffin et al., 2011). Moreover, human Tregs cultured *in vitro* in the presence of IL-12 acquire a Th1 phenotype secreting IFNγ and expressing T-bet without the loss of Foxp3 expression (Dominguez-Villar M et al., 2011).

Different techniques to identify Tregs:
- immunohistochemistry: not the better way to identify Tregs since only a small number of Treg markers can be used, but it allows to visualize Tregs within the tissue contest and it can be useful for retrospective studies
- Treg quantification by methylation-specific quantitative PCR: it enables naturally occurring Tregs to be distinguished from activated T cells; in fact it has been recently demonstrated that Tregs possess a demethylated conserved region in the *FOXP3*

intron 1 that is completely methylated in other human cells of hematopoietic origin, even after activation (Zheng et al., 2010).

- multicolor flow cytometry: lacking an unique Treg specific marker to define Tregs, the combination of different markers are required to distinguish Tregs from activated T effector cells. Notably, this multiparametric Treg definition allows the combination of additional markers aimed at defining the functional properties of the different Treg subsets combining activation, homing and functional markers. It also allows Treg sorting, either to assess Treg suppressor activity *in vitro* or to further characterize them with microarray or mass spectrometry analysis.

3. LYMPHOCYTE ACTIVATION GENE-3 (LAG-3): A KEY REGULATOR OF THE IMMUNE RESPONSES

3.1 Basic concepts

Human LAG-3 was initially identified as a member of the immunoglobulin superfamily (IgSF) (Triebel et al., 1990) selectively transcribed in human activated T lymphocytes and NK cells. Early studies were focused on the analysis of gene sequence and protein structure and a close homology between LAG-3 and CD4 was found, thus suggesting the possibility that these two molecules did share common ligands. Now it is well known that LAG-3 is a physiological ligand of MHC class II and works as a key regulator of T cell homeostasis and also as a powerful activator of antigen presenting cells (Triebel, 2003).

3.2 Biology of LAG-3

3.2.1   Structure of human LAG-3 gene and protein

Human *LAG-3* gene is located on the distal part of the short arm of chromosome 12 (12p13.3), close to *CD4* gene (Barten et al., 2001). *LAG-3* gene is made up of eight exons that span 6 kb; the corresponding mRNA (a 2-kb message) encodes for a 498-amino acid protein (70 kDa weight) composed by 4 extracellular IgSF domains, a trans-membrane domain (TM) and a short cytoplasmic tail (Cyt) (Figure 2 A).

LAG-3 gene in mouse shares strong homology with its human counterpart. The cytoplasmic tail of the protein contains three different regions conserved between mouse and human LAG-3. The first motif is a potential serine phosphorylation site, resembling the protein kinase C binding site in CD4. The second is a conserved KIEELE motif with no homology to any other known protein. The third motif is an unusual glutamic acid-proline (EP) repetitive sequence.

In humans, an alternative splicing of *LAG-3* RNA just following exon 5 causes the generation of a truncated form of the protein, termed LAG-3V3 (for LAG-3 variant 3) (Figure 2 B); this 52 kDa variant, lacking both the trans-membrane and cytoplasmic domains, represents a natural soluble form of the protein and was indeed found in sera of healthy individuals after T cell activation (Annunziato et al., 1996). The natural

soluble form is present also in tuberculosis and breast cancer patients' sera at high level (Triebel et al., 2006).
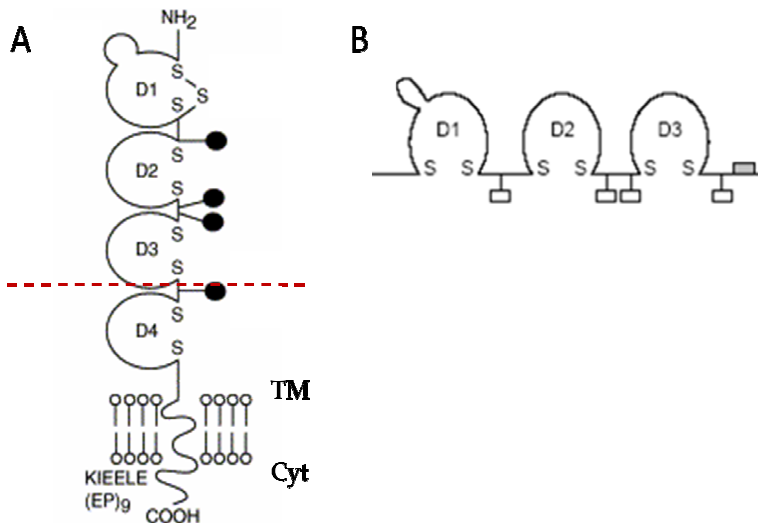

Figure 2. *Organization of LAG-3 protein.* Structure of LAG-3 protein: D1-D4, extracellular IgSF domains. The intra-cytoplasmic KIEELE and repeated EP motifs are indicated. Dashed line indicate the site of cleavage (A). Structure of natural soluble LAG-3V3 protein found in human serum. (B). Abbreviations: Trans-membrane domain (TM) and Cytoplasmic tail (Cyt). *(Adapted from Huard et al., 1994).*

3.2.2 Molecoular proprieties

CD4 and LAG-3 share common ligand, namely major histocompatibility complex (MHC) class II molecules (Huard et al., 1997) but, notably, LAG-3 binds MHC class II with higher affinity than CD4. The MHC class II binding site of LAG-3 is located in the N-terminal region and probably involves the 30-aa extra-loop sequence found in domain D1, the same domain contains a second binding site for another LAG-3 molecule, allowing the assembly of homodimers on the cell surface. Yet it is not clear if the oligomerization process is constitutively activated or induced after a transient interaction with MHC class II molecules, as in the case of CD4.

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MHC class II uses two alternative regions for its binding with LAG-3 and CD4, implying that LAG-3 and CD4 do not compete for their access to MHC class II (Huard et al., 1997).

Half the cellular content of LAG-3 is retained in intracellular compartments. This significant intracellular storage of LAG-3 and its close association with the microtubule organizing center and recycling endosomes appears to facilitate its rapid translocation to the cell surface following T-cell activation (Woo et al., 2010). LAG-3 cell surface expression is in fact tightly regulated by extracellular cleavage and two metalloproteases, namely ADAM10 and ADAM17, are responsible of the cleavage (figure 2).

3.2.3 Expression pattern and cellular localization of LAG-3 protein

Identification and first characterization of LAG-3 were performed in early 1990s. Human LAG-3 protein was found to be specifically expressed in activated CD4+ and CD8+ T lymphocytes and NK cells (Triebel et al., 1990). In humans rare LAG-3+ cells were detected in primary (adult thymus and bone marrow) and secondary (spleen and MALT) lymphoid organs, while no expression could be found in non lymphoid organs (Huard et al., 1994a); conversely, high levels of LAG-3 were found in inflamed tonsils and lymph nodes with follicular hyperplasia. LAG-3 expression was also observed in CD8+ tumor infiltrating lymphocytes (TIL) infiltrating various types of cancer (renal cell carcinoma, melanoma, lymphoma and ovarian cancer) (Demeure et al., 2001; Matsuzaki et al., 2010).

All together these data led to the definition of LAG-3 as a lymphocyte activation antigen broadly expressed and not restricted to a unique subset of cells.

3.3 LAG-3 functions

3.3.1 The enigmatic role of LAG-3 in the immune system: costimulatory or inhibitory molecule?

The physiological role of LAG-3 has remained elusive. However, recent evidences have now clearly revealed that this molecule plays a dual role

in the immune system, acting either as a negative regulator of activated T cells or as an activator of APC.

3.3.2 LAG-3 as a negative regulator of T cells

Studies on human activated T cells demonstrated that LAG-3 can act as a negative modulator of T cell functions. Thus, when LAG-3/MHC class II interactions were inhibited, a strong increase in T cell proliferation and cytokine secretion was induced, while its overexpression or the inhibition of its surface cleavage led to impaired antigen driven T cell proliferation without inducing T cell death (Li et al., 2007). When triggered by encounter with its nominal antigens, namely MHC-class II, LAG-3 induces down-modulation of TCR/CD3 and affects proximal TCR signaling, leading to T cell unresponsiveness and to the inhibition of TCR-induced calcium fluxes (Hannier et al., 1998), lower IL-2 production and reduced production of Th1 type cytokines (Huard et al., 1994).

LAG-3 signaling could induce in activated T cells a refractory state necessary to prevent undue activation without a proper TCR triggering; this inhibition of T cell expansion may be obtained by preventing the entry of T cells through the S-phase of cell cycle (Maçon-Lemaître and Triebel, 2005). LAG-3 might therefore act as a negative regulator by limiting the rate of cell division.

Recent studies in mouse model have highlighted the fact that exhausted CD8+ T cells following chronic viral infections express multiple inhibitory receptors such as PD-1. Among these inhibitory coreceptors, LAG-3 was observed to be expressed at high level during chronic viral infections; the simultaneous *in vivo* blockade of the PD-1 and LAG-3 inhibitory receptor pathways led to a strong reversal of T cell exhaustion and viral control indicating a synergy between this two negative co-receptors (Blackburn et al., 2009). In humans, coexpression of PD-1 and LAG-3 was observed in tumor infiltrating NY-ESO1 specific CD8+ T cells in patients with ovarian cancer, the two molecules indentify a subpopulation of T cells with diminished capacity to produce IFN-γ and tumor necrosis factor alpha (TNF-α); also in this case only the dual

blockade of LAG-3 and PD-1 increased T cell frequency and IFN-γ production (Matsuzaki et al., 2010).

Not surprisingly, being a molecule belonging to the inhibitory co-receptors group, such as CTLA-4 and PD-1, LAG-3 has been recently described in mice as a novel CD4 and CD8 Treg-associated marker that is directly involved in the control of T cell expansion and homeostasis (Huang et al., 2004; Grosso et al., 2007). Recent data in humans identified a new subset of CD8+ Tregs expressing LAG-3 that mediate immunosuppression via the secretion of CCL4 (Joosten et al., 2007). Moreover, there is evidence of its involvement in tumor-infiltrated CD4+ Treg function in Hodgkin's lymphoma (Ghandi et al., 2006). Importantly, LAG-3 expressed by human Tregs seems to play a functional role; in fact the interaction between LAG-3 and MHC class II molecules is involved in the functional inhibition of DC activation by Tregs (Bayry et al., 2007).

All together these findings strongly highlight the role of LAG-3 as molecule crucial in immune homeostasis and in the fine tuning of T cell activation.

3.3.3 LAG-3 as an APC activator

The majority of studies aimed at evaluating the effect of LAG-3 binding to MHC class II and its functional role on the transduction of signals into APC has been conducted by using a recombinant soluble form of the protein, termed LAG-3Ig. APC expressing high levels of MHC class II molecules tightly bind LAG-3Ig (Andreae et al., 2002) and this binding induces DC maturation, as detected by release of pro-inflammatory cytokines and chemokines able to direct their migration to secondary lymphoid organs (Andreae et al., 2002; Buisson and Triebel, 2003); in a physiological context, LAG-3 expressed on activated CD4+ T cells and able to bind class II-associated peptides may license DC to efficiently present class I-restricted peptides to CD8+ T cells (Machy et al., 2002). In this regard our laboratory showed that LAG-3 when expressed on activated CD4+ T cells functions as a costimulatory molecule and in association with CD40/CD40L induce a full functional activation of DC (Casati et al., 2006; Casati et al., 2008).

Significant levels of LAG-3 protein have been found on the surface of lymphocytes infiltrating various types of tumors (Demeure et al., 2001); a positive direct role of LAG-3 expressed on these TIL in the engagement of class II molecules on APC has been described, thereby contributing to APC activation and Th1/Tc1 commitment.

So, LAG-3 is a two-way signaling molecule: when triggered it transduces an inhibitory signal inside the T cells, while promoting maturation and full activation of the APC bearing its natural ligand, MHC class II (figure 3).
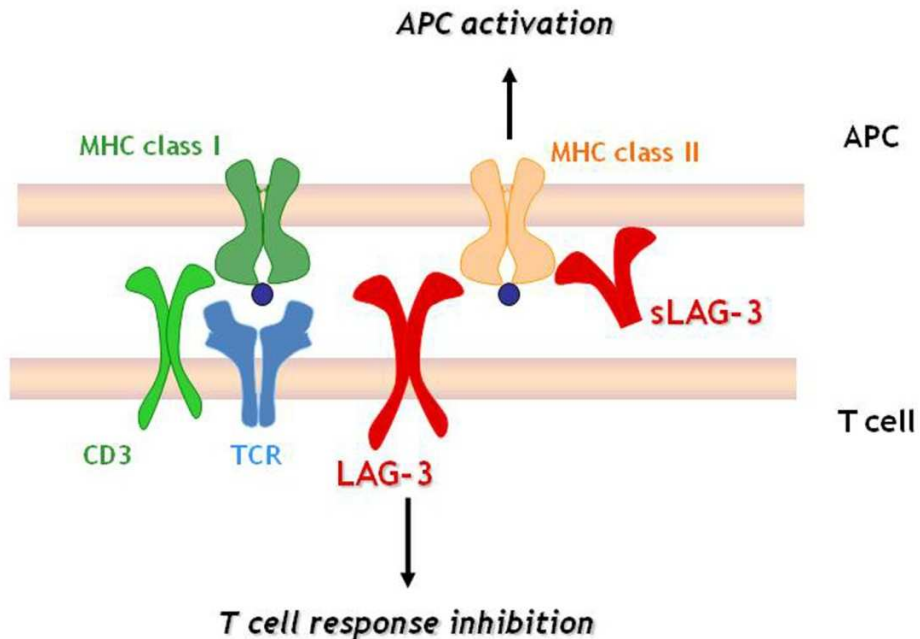
Figure 3. *Interactions between LAG-3 and MHC class II molecules.*
Schematic representation of different functional roles of LAG-3 protein through the interaction with MHC class II molecules (adapted from Triebel et al., 2003).

MAIN RESULTS

"Lymphocyte Activation Gene-3 Expression Defines a Subset of CD4$^+$CD25^{high}Foxp3$^+$ Regulatory T Cells That are Expanded at Tumor Sites". (Paper at page 64)

Studies in mice indicated that LAG-3 has a complex role in T cell homeostasis and is expressed by CD4+CD25+ Tregs. By the usage of multiparametric FACS analysis, I explored the distribution of LAG-3 in human CD4+ T cells. LAG-3 was found to define a discrete subset of CD4+CD25^{high}Foxp3+ T cells, indeed cells with a Treg phenotype **(figure 1)**.

To investigate whether this CD4+CD25^{high}Foxp3+LAG-3+ population were expanded in advanced melanoma patients, I analyzed their presence in PBMCs, LNs and visceral metastasis of patients with stage III and IV melanoma. I found that this Treg subset expressing LAG-3 is expanded in patients' PBMCs and this expansion was even more evident in lymphocytes of tumor-invaded as compared to tumor- free LNs and in lymphocytes infiltrating visceral and *sub cute* metastasis (TIL) **(figure 2)**. These cells were further characterized for the expression of CCR7 and CD45RA; as expected, both LAG-3- Tregs and LAG-3+ Tregs included effector memory T cells (CD45RA-CCR7-), while terminal effector T cells were preferentially found as part of the LAG-3+ Tregs **(figure 3)**.

The *ex-vivo* functional analysis performed by FACS and intracellular cytokine staining confirmed that CD4+CD25^{high}Foxp3+LAG-3+ were functionally active cells that released the immunosuppressive cytokines IL-10 and TGF-β1. These cytokines are involved in mediating Treg function. Of note CD4+CD25^{high}Foxp3+LAG-3+ T cells did not produce IL-2, excluding the possibility that CD4+CD25^{high}Foxp3+LAG-3+ cells were recently activated conventional effector T cells. Comparing LAG-3+ Tregs to LAG-3- Tregs, the association between LAG-3+ Tregs and the production of immunosuppressive cytokines was also observed in melanoma patients' PBMCs and tumor-invaded LNs **(figure 4)**.

In addition to produce cytokines, Tregs inhibited the proliferation of CD4+CD25- T cells by direct contact. Thus an *in vitro* suppression assay

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was set up. $CD4^+CD25^{high}LAG-3^+$ T cells were sorted from *in vitro* expanded $CD4^+CD25^{high}$ Tregs and they suppressive capacity assessed in an inhibition assay and compared with that of the LAG-3$^-$ counterpart. Interestingly, the LAG-3+ subset resulted more suppressive than the LAG-3$^{-/low}$ subset (**figure 5**). Suppression experiments were also performed in transwell plates and in the presence of anti-IL-10 and anti-TGF-β1 neutralizing mAbs; data showed that LAG-3+ Tregs exerted their suppressor activity mainly via direct cell-to-cell contact, because the prevention of a physical interaction between LAG-3+ Tregs and CD25-responder T cells completely restored their proliferative capacity (**figure 6**).

CONCLUSIONS

PHENOTYPICAL CARACHTERIZATION:

1) LAG-3 is preferentially expressed by regulatory $CD4^+CD25^{high}Foxp3^+$ T cells and defines a discrete subset of $CD4^+CD25^{high}Foxp3^+$ T cells in PBMCs of healthy donors **(figure 1)**

2) Cancer patients show increased frequency of $CD4^+CD25^{high}Foxp3^+LAG-3^+$ T cells in PBMCs, LNs and TIL of cancer patients **(figure 2)**

3) $CD4+CD25^{high}Foxp3^+LAG-3^+$ T cells display phenotypic features of effector-memory and terminal effector T cells **(figure 3)**

FUNCTIONAL CARACHTERIZATION:

4) $CD4^+CD25^{high}Foxp3^+LAG-3^+$ T cells produce IL-10 and TGF-β1 cytokines **(figure 4)**

5) $CD4^+CD25^+LAG-3^+$ T cells are highly suppressive *in vitro* **(figure 5)** and need cell-to-cell contact **(figure 6)**

All together, these data show that LAG-3 defines a functionally active $CD4^+CD25^{high}Foxp3^+$ regulatory T cell subset whose frequency is enhanced in melanoma patients, especially in tumor invaded LNs and at tumor sites.

A

B
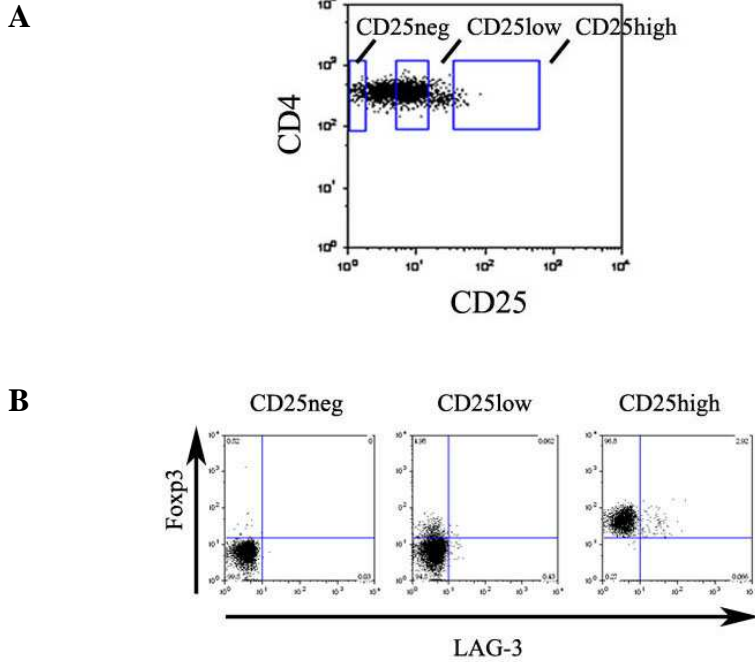
A) FACS gating strategy: Tregs belong to the CD4$^+$CD25^{high} subpopulation and are positive for Foxp3 expression.

B) In healthy donors' PBMCs, Foxp3 uniformly stained all the CD4$^+$CD25^{high} T cells, while LAG-3 expression defined a discrete subset of T cells inside the CD4$^+$CD25^{high}Foxp3+ compartment.

Figure 1

A

B
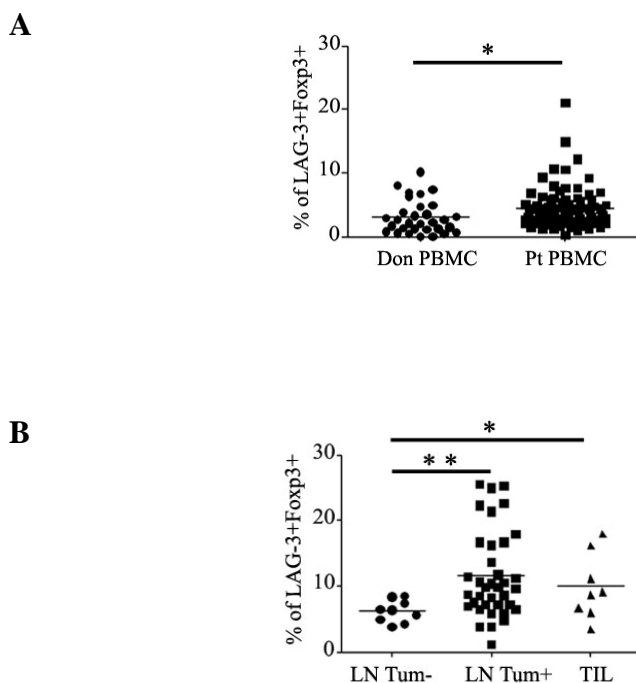
A) Percentage of LAG-3$^+$Foxp3$^+$ cells in CD4$^+$CD25^{high} T cells was significantly increased in melanoma patients' (Pt) PBMCs if compared to healthy donors (DON).
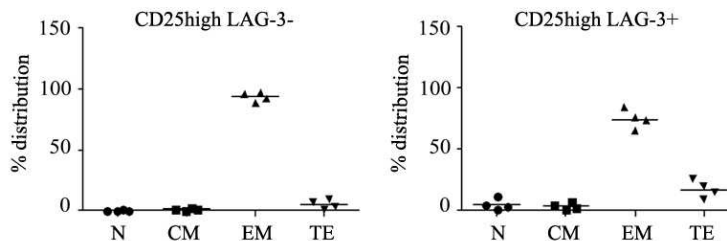
B) Frequency of LAG-3$^+$Foxp3$^+$ in CD4$^+$CD25^{high} T cells was significantly higher in lymphocytes from tumor-invaded lymph nodes (LN TUM$^+$) and tumor infiltrated lymphocytes (TIL) from visceral and *sub cute* metastases compared with those of tumor-free lymph nodes (LN TUM-).

Figure 2

A
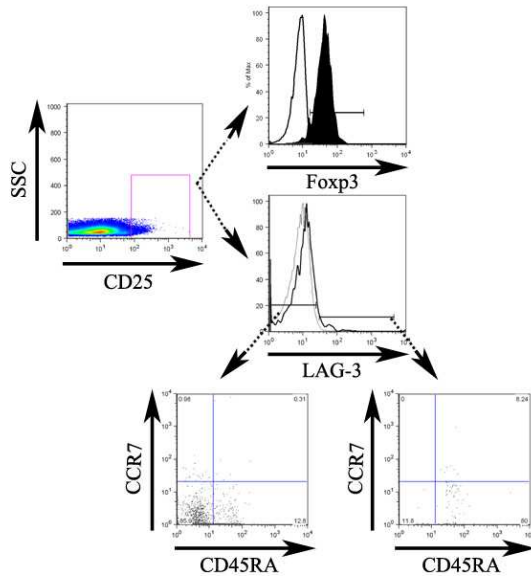
B

A) Gating strategy to define the *in vivo* differentiation stage of LAG-3- and LAG-3+ $CD4^+CD25^{high}Foxp3^+$ T cells based on their percentage distribution in naïve (N), central memory (CM), effector memory (EM) and terminal effector (TE).
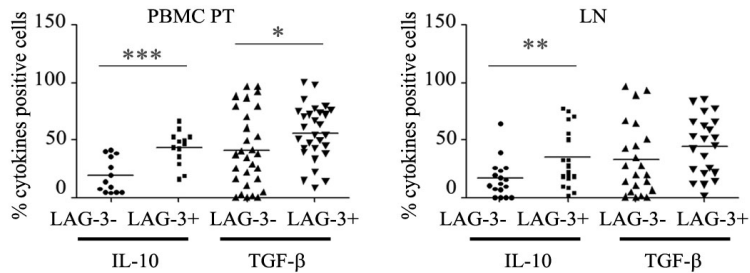
B) Melanoma patients' LAG-3- Tregs and LAG-3+ Tregs displayed features of effector memory cells and a small fraction of LAG-3+ Tregs showed also a terminal effector phenotype.

Figure 3

A
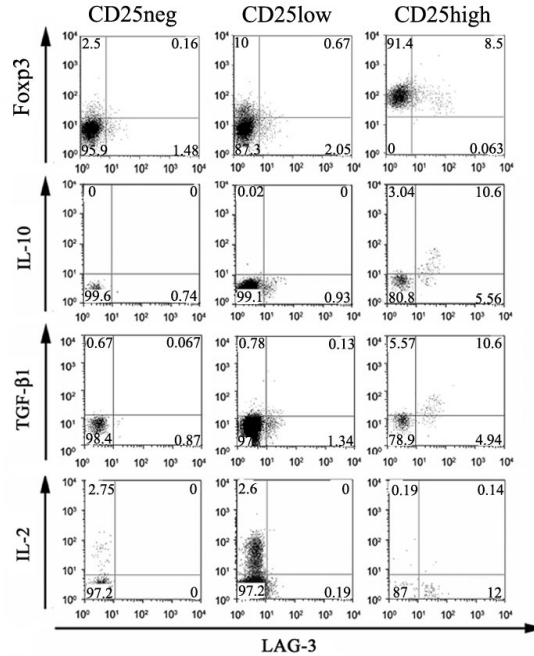
B

A) Healthy donors' PBMCs were activated with anti-CD3/CD28 beads and analyzed for intracellular production of the immunosuppressive cytokines IL-10 and TGF-β1. These cytokines were only present in the CD4$^+$CD25^{high}Foxp3$^+$ fraction and were mainly produced by LAG-3+ subset. Intracellular expression of IL-2 was detectable only in the CD4$^+$CD25^{low} compartment.

B) The same analysis comparing the intracellular cytokine production in patients' PBMCs (PT) and LNs showed that IL-10 and TGF-β1 production was increased in LAG-3+ Tregs.
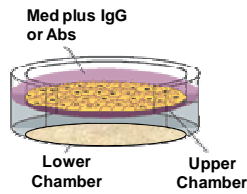
Figure 4

Inhibition assay
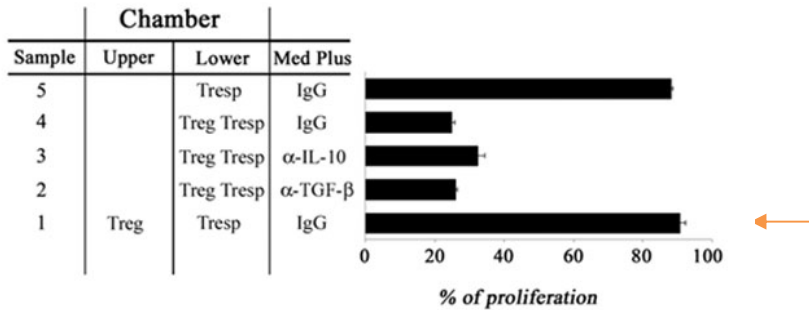

Sorted LAG-3⁺CD25⁺   **Sorted LAG-3⁻/ˡᵒʷCD25+**
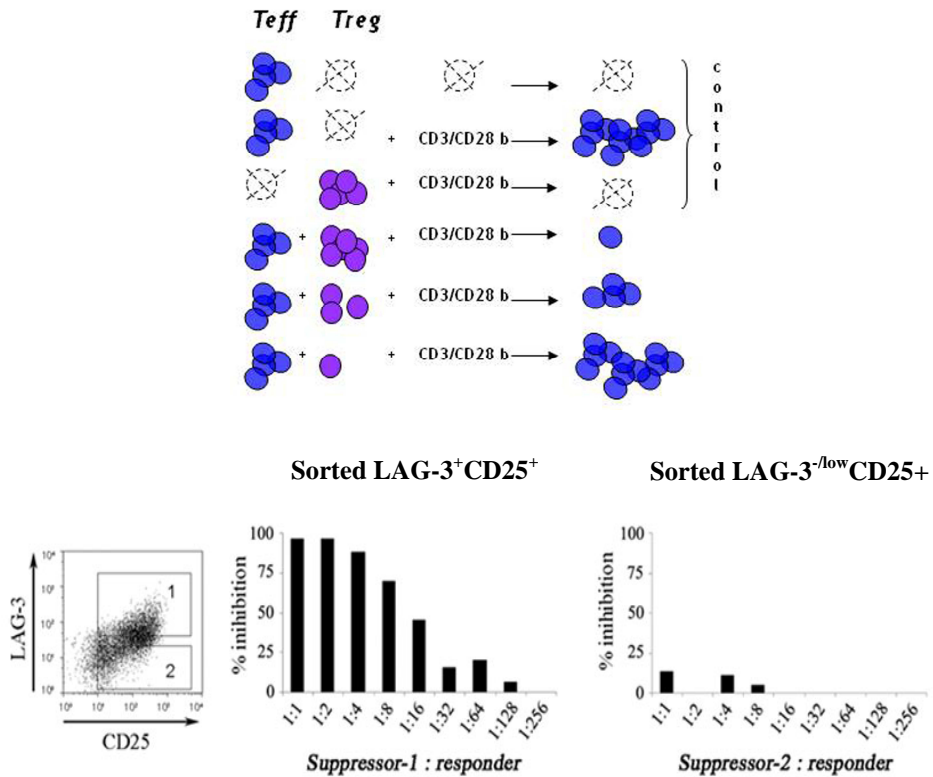
% proliferation of autologous CD25- T cells (Teff) was evaluated with [³H]thymidine incorporation assay or carboxyfluorescein succinimidyl ester (CFSE) labeling, after 3-5 days of co-culture with Tregs in the different conditions reported.

LAG-3⁺CD25⁺ and LAG-3⁻/ˡᵒʷCD25⁺ T cells were FACS sorted from *in vitro* expanded CD4⁺CD25⁺ lymphocytes and used as suppressor cells in a inhibition assay. The capacity to suppress proliferation of autologous CD25- T responder cells resulted markedly higher for LAG-3⁺ Tregs.

Figure 5

TRANSWELL PLATE

Med plus IgG or Abs

Lower Chamber

Upper Chamber

	Chamber		
Sample	Upper	Lower	Med Plus
5		Tresp	IgG
4		Treg Tresp	IgG
3		Treg Tresp	α-IL-10
2		Treg Tresp	α-TGF-β
1	Treg	Tresp	IgG

% of proliferation

Treg = CD4$^+$CD25$^+$LAG-3$^+$ sorted T cells
Tresp = CD4$^+$CD25$^-$ freshly isolated autologous T cells

Rato 1:1

LAG-3+ Tregs exert their suppressor activity mainly via a direct cell-to-cell contact, because prevention of physical interaction with transwell plate between Tregs and Tresp completely restored Tresp proliferation. The addition of anti-IL-10 and anti-TGF-β1 neutralizing mAbs did not affect the inhibitory activity of LAG-3+ Tregs.

Figure 6

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MAIN RESULTS

 "*Frequency and function of circulating regulatory T cells in melanoma patients receiving peptide-based vaccine*". (Manuscript at page 72)

Purpose: Tregs exert a detrimental effect on tumor immunity. My thesis evaluated the influence of low-dose cyclophosphamide (CTX), low-dose IL-2 and peptide vaccination on the frequency and functional behavior of Tregs of melanoma patients and the impact of Tregs on antigen-specific response.

Trial design: Phase II randomized study in HLA-A*0201 patients with melanoma (stage IIB/C-III): the study includes a vaccination and an observation arm. In the vaccination arm, patients received vaccination with HLA-A*0201 tumor peptides, low dose of CTX (300 mg/m^2 i.v.) as potential Treg-depleting agent and two injections of low dose IL-2 ($3x10^6$ IU) to boost vaccine induced immune-responses. Blood samples were serially collected at different time points from patients enrolled in the study (vaccination arm n=16, observation arm n=19) (**Figure 1**, Schematic representation of vaccine schedule). In the control arm, patients received no treatment beside surgery.

Results: Tregs defined as $CD4^+CD25^{high}Foxp3^+$ were analyzed by FACS in PBMCs of vaccinated melanoma patients and compared to Tregs in control patients. As compared to pre-treatment, frequency of $CD4^+CD25^{high}Foxp3^+$ Tregs shows a slight decrease in PBMCs collected 4-7 days after CTX administration (P1 versus P0). However, this reduction was transient and although patients received additional CTX, the frequency of Tregs regained the original pre-treatment level (P4). Instead, low dose IL-2 induced a significant expansion of $CD4^+CD25^{high}Foxp3^+$ Tregs (P6) (**figure 2**).

To evaluate whether Tregs expanded by IL-2 administration were endowed with suppressive activity, an *in vitro* suppression assay was assessed. $CD4^+CD25^{high}$ T cells isolated from PBMCs of a vaccinated patient at P6 of the vaccination were coltured in the presence of autologous $CD4^+CD25^-$ T cells obtained at P0. Data indicate that low dose of IL-2 expanded Tregs functionally active, since proliferation of conventional $CD4^+CD25^-$ cells was strongly inhibited (**figure 3**).

To further characterize these cells, the CD45RA marker was added in the multiparametric FACS analysis; in fact, in combination with CD25, it enables to discriminate between activated CD4+ Tregs (CD25^{high}Foxp3^{+}CD45RA^{-}) and conventional activated CD4+ T cells (CD25^{int}Foxp3^{int}CD45RA^{-}). Data confirmed that IL-2 boosted activated Tregs (Fr.b), but at the same time also conventional activated CD4+ T cells were augmented (Fr.a) **(Figure 4)**. Activated T cells and Tregs pre and post IL-2 administration were characterized *ex-vivo* for the expression of two Th1 markers: T-bet (a transcription factor crucial for CD4+ Th1 commitment) and IFN-γ (the hallmark of Th1 cytokine). In activated conventional T cells, increase in T-bet positive cells paralleled the increase in the percentage of T cells producing IFN-γ. Thus these data support the conclusion that IL-2 may also expand activated CD4+ T cells producing Th1 related cytokines. Surprising enough T-bet and IFN-γ were also present and boosted after IL-2 administration in the fraction of activated Tregs, indicating a strong plasticity of Tregs that probably may acquire a Th1 phenotype in a pro-inflammatory environment **(figure 4)**.

To investigate the impact that Tregs exerted during the vaccination on antigen-specific CD8+ T cells, monitoring of peptide-specific, vaccine-induced T cells was performed *ex-vivo* by HLA-A*0201/peptide pentamer staining. Results indicated that, the percentage of CD8+ pentamer positive cells increased after the second vaccination and was further boosted after IL-2 administration. Thus the raise in Tregs induced by IL-2 was not limiting the expansion of antigen-specific T cells **(figure 5)**. Moreover, in order to assess whether Tregs were negatively affecting the functional activity of antigen-specific CD8+ T cells, an IFN-γ ELISpot assay was performed. Data reported for two patients indicated that although IL-2 administration led to strong increase in Treg frequency, the number of CD8+ T cells producing IFN-γ in response to antigenic peptide stimulation remained stable or was further increased **(figure 6)**.

The same experiments were done in parallel with control patients' PBMCs; Treg modulation during the vaccine course was never observed in these patients.

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CONCLUSIONS

Effect of CTX:

1) Low dose CTX has a limited efficacy in modulating Treg frequency in vaccinated patients' PBMCs and Tregs regain the original pre-treatment level in few weeks **(figure 2)**

Effect of IL-2:

2) Low dose of IL-2 strongly boosts the number of circulating $CD4^+CD25^{high}Foxp3^+$ Tregs **(figure 2)**
3) Low dose IL-2 directly affects Treg homeostasis by inducing an expansion of suppressive Tregs **(figure 3)**
4) Low dose IL-2 expands both circulating conventional activated CD4+ T cells and Tregs; a fraction of these Tregs displays a Th-1 like phenotype, expressing *ex-vivo* T-bet and INF–γ **(figure 4)**
5) IL-2 induced Treg boosting is not hampering the vaccine induced immunity **(figure 5)**

The results of this study indicate that CTX has only a limited efficacy in modulating Tregs. Instead there are clear evidences that low dose of IL-2 given in a vaccination setting, although expanding Tregs, does not produce detrimental effect on the expansion and functional activities of antigen-specific CD8+ T cells induced by vaccination. Probably the balance among the different functional T cell subsets amplified by IL-2 is most likely influencing the immunological and clinical outcome of the vaccinated patients.

VACCINE SCHEDULE:
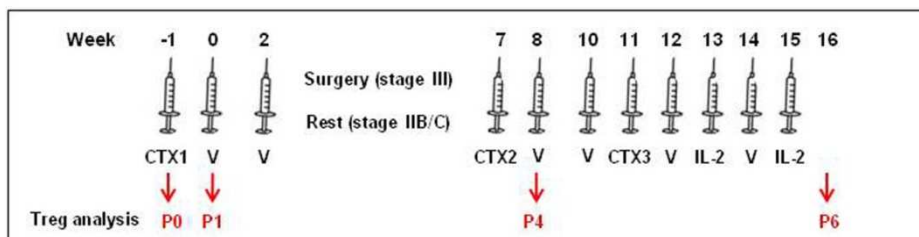
V= peptide vaccine made of HLA-A*0201-restricted (Melan-A/MART-1[27L], gp100[210M], NY-ESO-1[165V], and Survivin[97M]) 250 μg each, emulsified in Montanide ISA-51

CTX= Cyclophosphamide i.v. 300mg/m^2

IL-2 s.c. $3x10^6$ IU/day for three daily injections

P= blood samples collected (P0 = pre treatment; P1= after 1 dose of CTX; P4 = after 2 doses of vaccine and CTX; P6 = after 6 vaccinations, 3 doses of CTX, 2 of IL-2)

Figure 1
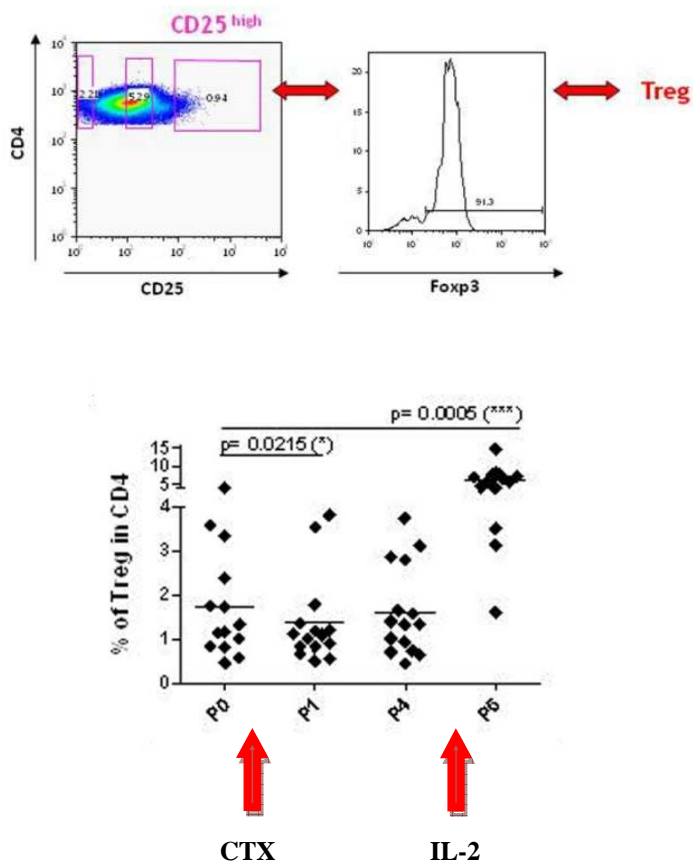
Tregs identified in vaccinated patients' PBMCs as CD4$^+$CD25^{high}Foxp3$^+$ T cells were analyzed at different time points of the vaccination.

Tregs in P1 (after one dose of CTX) showed a slight decrease if compared to those in P0. Their frequency regained the original level after two weeks (P4). After two doses of IL-2 (P6) a strong boost in Treg frequency was observed (P6 versus P0).

Figure 2
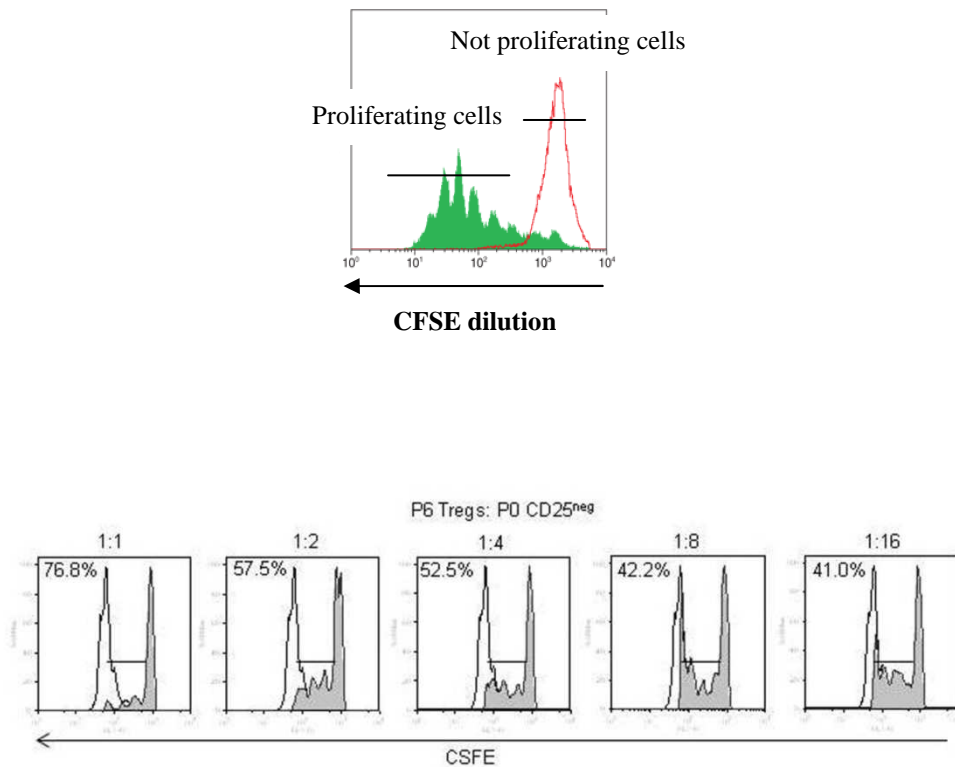
CD4$^+$CD25$^-$ Tresp cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and their proliferation in the presence of autologous Tregs were measured by FACS analysis as CFSE dilution after 72h of *in vitro* activation with anti-CD3/CD28 microbeads.

Upon each cell division CFSE^{high} cells lose half of their CFSE label resulting in populations of CFSE^{low} daughter cells which can be visualized by FACS.

Figure 3

45
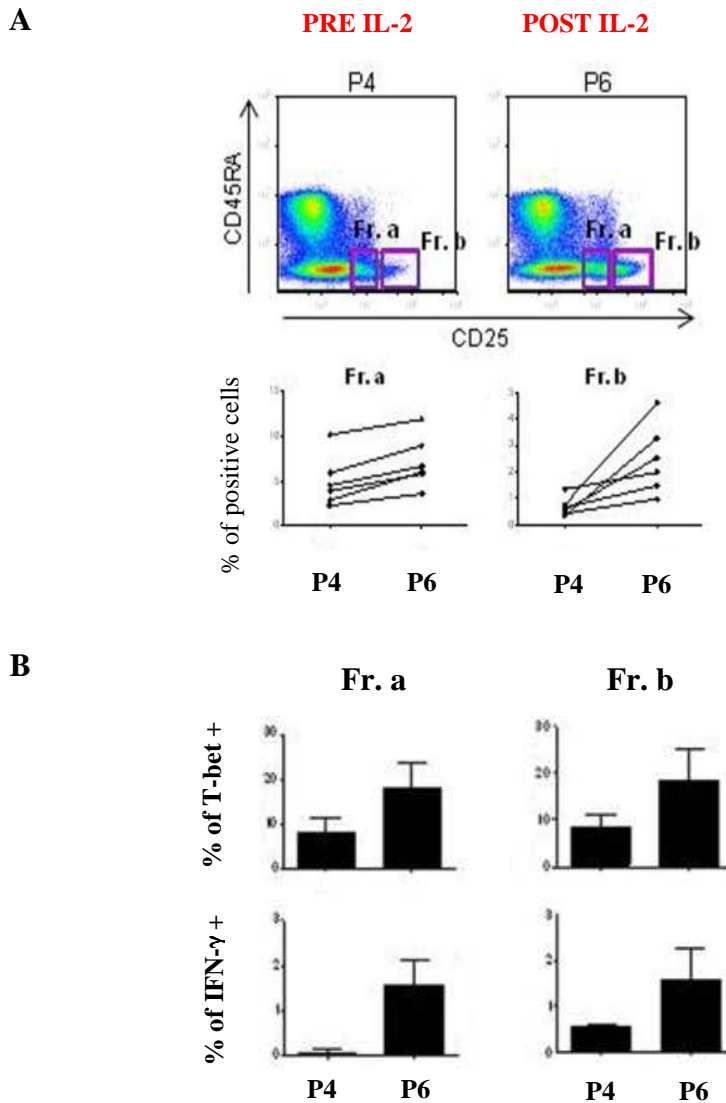
A) In all six patients analyzed IL-2 boosted both activated Tregs (Fr. b) and conventional T cells (Fr. a).

B) Th1 markers, T-bet and IFN-γ, augmented after IL-2 administration (P6 versus P4) in conventional activated T cells. A fraction of Tregs displayed a Th-1 like phenotype, expressing *ex-vivo* T-bet and INF-γ, boosted after IL-2 administration.

Figure 4

PEPTIDES:

a = gp100

b = melanA/MART1

c = NY-ESO1

d = SVV-1
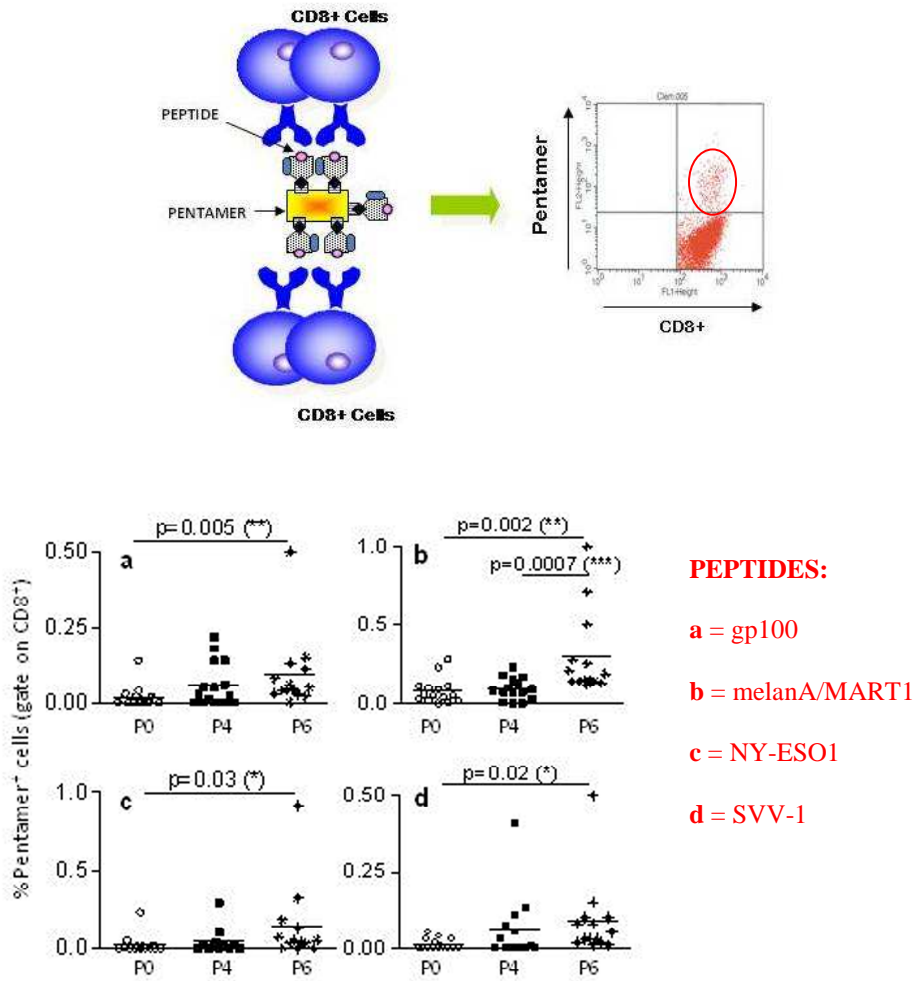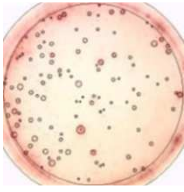
Antigen-positive T cells induced by the vaccination were evaluated as percentage of pentamer positive CD8+ T cells.

For all the four peptides composing the vaccine, the percentage of pentamer positive CD8+ T cells increased after the second vaccination (P4), even if to a different extent, and it was further boosted after IL-2 administration (P6).

Figure 5

The frequency of CD8+ T cells responding to each peptide used in the vaccine was evaluated by IFN-γ ELISpot assay as the number of CD8+ T cells releasing IFN-γ after antigen stimulation at different time points during the vaccine course.
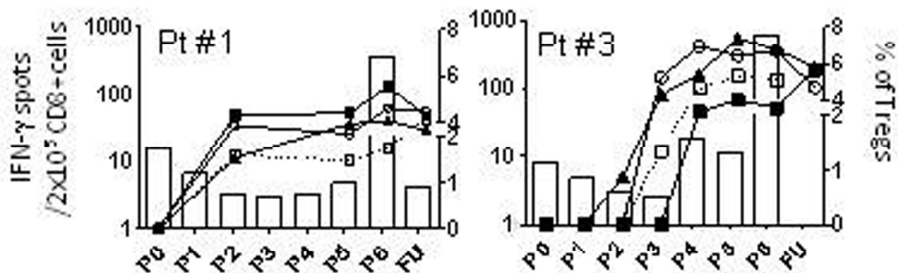
■ = Survivin

□ = gp100

▲ = NY-ESO-1

o = MelanA/MART-1

The kinetic of IFN-γ releasing peptide-specific CD8+ T cells reported together with the percentage of $CD4^+CD25^{high}Foxp3^+$ T cells in PBMCs of two vaccinated patients during the vaccine course, showed that despite the presence and the boost of Tregs, there was an increase in the frequency of antigen-specific CD8+ T cells in both patients.

Figure 6

ONGOING RESULTS

Stage III patients enrolled in the study underwent surgical LNs dissection; patients in the vaccination arm received one CTX treatment and two vaccinations before surgery (see for the vaccination schedule page 43), while patients in the control arm received neither CTX nor vaccine. FACS analysis of $CD4^+CD25^{high}Foxp3^+$ activated Tregs performed *ex-vivo* on LNs cell suspension showed that LNs of vaccinated patients were more homogeneous in their Treg content and displayed a lower Treg frequency as compared to LNs of patients not receiving any treatment but surgery. Of note, difference in LNs Treg number between vaccinated and control group was more evident when comparison was restricted to tumor invaded LNs (**figure 1**). This data may suggest that CTX is potentially working in counteracting the generation of tumor-induced Tregs and thus its administration reduced the level of Tregs frequency in tumor-invaded LNs.

Studies are now directed toward the definition of the immunological status of LNs of vaccinated as well as those of control patients.
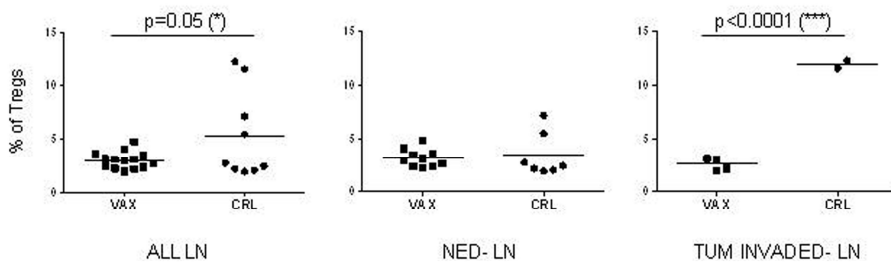
Figure 1. Tregs in LNs of vaccinated patients are reduced and more homogeneous if compared to that of control patients. Lymphocytes obtained from patients' LNs were stained with anti-CD4, -CD25 and -Foxp3 mAbs and analyzed by FACS. Comparison between Treg frequency ($CD25^{high}Foxp3^+$ cells in CD4+ lymphocytes) in control patients' LNs and vaccinated patients' LNs is reported for all the LNs examined (ALL LN), for LNs not tumor invade (NED-LN) and for tumor invaded LNs (TUM INVADED-LN). Significant p values were calculated with Student's t Test.

CONCLUSION AND FUTURE PROSPECTS

Role of LAG-3 in Treg biology

Since we observed that LAG-3+ Tregs are functionally different from LAG-3- counterpart and expanded in melanoma patients, future studies will be aimed at evaluating the functional role of LAG-3 molecule expressed on melanoma patients' Tregs and the role of melanoma in increasing Treg frequency.

Specific objectives of future research will be the following:

1. The ability of tumor cells or of cells composing the tumor stroma (tumor activated fibroblast and monocytes/macrophages or DCs) to selectively attract this discrete subset of activated LAG-3+ Tregs or their capacity to selectively induce LAG-3 expression in Tregs, will be studied.

2. To fully explore the biological features of LAG-3+ Tregs, LAG-3- and LAG-3+ Tregs will be sorted and subjected to microarray gene expression profile. Genes differentially expressed by cells and/or pathways activated in LAG-3+ Treg could became selectively targets for intervention aimed at limiting the functional activities of these cells.

Evaluation of Treg down-modulation and their impact in melanoma patients treated with immunotherapy

Goal of my thesis was to assess the actual impact that Tregs exert on tumor immunity and disease progression in order to understand whether Treg elimination or down/modulation by pharmacological treatments can be of any benefit to cancer therapies.

My thesis demonstrated that low-dose CTX administered during vaccination reduces peripheral blood Tregs only in a minority of patients

and this down-modulation is transient. Thus new efforts will be done to design new clinical trials that will include different CTX dose and administration timing potentially more effective in modulating Tregs.

Moreover, we observed that Tregs are present in LNs of patients who underwent surgery, so we will also analyze Tregs modulation due to CTX administration comparing lymphocytes obtained from LNs of patients belonging to control arm versus vaccinated arm. Treg phenotype and histological localization will be also taken in account with the aim of defining their influence on the priming of active anti-tumor responses.

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PART II

LAG-3 Expression Defines a Subset of CD4$^+$CD25^{high}Foxp3$^+$ Regulatory T Cells That Are Expanded at Tumor Sites

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Licia Rivoltini,* **and Chiara Castelli***

Human natural regulatory CD4$^+$ T cells comprise 5–10% of peripheral CD4$^+$T cells. They constitutively express the IL-2Rα− chain (CD25) and the nuclear transcription Foxp3. These cells are heterogeneous and contain discrete subsets with distinct phenotypes and functions. Studies in mice report that LAG-3 has a complex role in T cell homeostasis and is expressed in CD4$^+$CD25$^+$ T regulatory cells. In this study, we explored the expression of LAG-3 in human CD4$^+$ T cells and found that LAG-3 identifies a discrete subset of CD4$^+$CD25^{high}Foxp3$^+$ T cells. This CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ population is preferentially expanded in the PBMCs of patients with cancer, in lymphocytes of tumor-invaded lymph nodes and in lymphocytes infiltrating visceral metastasis. Ex vivo analysis showed that CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ T cells are functionally active cells that release the immunosuppressive cytokines IL-10 and TGF-β1, but not IL-2. An in vitro suppression assay using CD4$^+$CD25^{high}LAG-3$^+$ T cells sorted from in vitro expanded CD4$^+$CD25^{high} regulatory T cells showed that this subset of cells is endowed with potent suppressor activity that requires cell-to-cell contact. Our data show that LAG-3 defines an active CD4$^+$CD25^{high}Foxp3$^+$ regulatory T cell subset whose frequency is enhanced in the PBMCs of patients with cancer and is expanded at tumor sites. *The Journal of Immunology*, 2010, 184: 6545–6551.

I n the immune system, several mechanisms are in operation to finely tune the immune response. An active role in preventing autoimmune diseases and maintaining immune homeostasis is played by the so-called T regulatory (Treg) cells. Human Treg cells comprise two main groups of CD4$^+$ T cells with different origins: natural occurring Treg cells generated in the thymus, and adaptive or induced Treg cells. Induced Treg cells are not present in the thymus, but differentiate in the periphery from naive CD4$^+$ T cells upon polyclonal or Ag-specific activation in the presence of specialized immunoregulatory cytokines. Two subsets of induced Treg cells have been described and are shown to also exist in humans: Tr1 cells, which secrete high levels of IL-10 and arise from CD4$^+$ T cells upon encountering Ags in a tolerogenic environment (1), and Th3 cells, which are induced by oral Ag administration and secrete high levels of TGF-β1 (2). Moreover, recent experimental evidence demonstrated that in humans, adaptive Treg cells may also emerge from the memory CD4$^+$CD25$^-$CD45RO$^+$ T cell pool in the presence of antigenic stimulation. These converted Treg cells, once generated, are highly susceptible to apoptotic death, thus representing a highly dynamic T cell pool that mirrors its responsive counterpart (3–5).

Naturally occurring Treg cells in mice are unequivocally defined as CD4$^+$CD25$^+$Foxp3$^+$ T cells. This same set of markers, together with a low expression level of CD127 (6, 7), also identifies human natural Treg cells. However, unlike in mice, human Foxp3$^+$ T cells are heterogeneous. For example, studies have reported that TCR-mediated activation of CD4$^+$CD25$^-$ T cells induces the transient expression of Foxp3 without conferring suppressive activity (8, 9). Similarly, it was shown that CD4$^+$CD25^{low}Foxp3^{low} T cells, which display limited to no suppressive capacity, are present in human PBMCs (10).

Further phenotypic studies revealed that other subclasses of human natural Treg cells could be identified based on the expression of molecules, such as MHC-II, CD45RO/RA, CCR7, and costimulatory molecules, such as ICOS (11–14). The expression of CD45RO/RA and CCR7 identifies populations that are at different in vivo differentiation stages, whereas their combined expression defines the presence of the natural naive, effector memory, or central memory Treg cells.

Natural Treg cells, upon TCR engagement (15, 16), exert their immunosuppressive functions in a contact-dependent fashion. However, it has been shown that the expression of MHC-II and ICOS characterizes natural Treg cells that are functionally different (13, 14). These latest data suggest that Treg cells can be further differentiated into discrete subsets that differently rely on cell-to-cell contact and on IL-10 or TGF-β production for their functional activities.

Recent studies have also shown that CD4$^+$ Treg cells are implicated in the suppression of the immune response against tumors (17–19). There is accumulating evidence that demonstrates a significant increase in the number of Treg cells in the peripheral blood and tumor microenvironment of patients with various types

of cancer (20, 21). Moreover, although data remain controversial and depend on the type of tumor studied (21), a higher accumulation of Treg cells is often associated with advanced disease stages and is inversely correlated with favorable prognosis and overall survival (22, 23). It has been clearly demonstrated that in most human solid tumors as well as hematologic malignancies, Ag-specific Treg cells actively suppress the proliferation of CD4$^+$CD25$^-$ and CD8$^+$ effector T cells, thereby limiting the immune response against cancer and contributing to tumor growth (24).

LAG-3 has been recently described in mice as a novel Treg-associated marker that is directly involved in the control of T cell expansion and homeostasis (25, 26). There is increasing evidence on the role of LAG-3 in the downregulation of T cell responses (27) and on its involvement in tumor-infiltrated Treg function in Hodgkin's lymphoma (28). Similarly, an Ag-specific LAG-3$^+$CD25$^+$Foxp3$^+$ induced regulatory CD8$^+$ T cell subset has been identified in a series of patients with tuberculosis (29).

In the current study, we show that inside the suppressor CD4$^+$CD25^{high}Foxp3$^+$ T cell population, LAG-3 expression identifies a discrete subset of cells that displays an effector-memory/terminal-effector phenotype. This subset of Treg cells is expanded in peripheral blood and tumor sites of cancer patients, suggesting that discrete compartments of Treg cells are selectively modulated in cancer patients.

Materials and Methods

Blood and tissue samples

Upon obtaining informed written consent, blood samples were collected from healthy donors or patients with melanoma or colorectal cancer at different stages of disease. PBMCs were isolated by Ficoll/PaqueTM PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation as previously described (30). Lymphocytes were also obtained from tumor-free or tumor-invaded lymph nodes, whereas tumor-infiltrated lymphocytes were collected from visceral and sub cute metastases of advanced cancer patients who underwent curative resection at the Melanoma and Sarcoma Unit or at the Colorectal Surgery Unit of our institute.

Abs and flow cytometry

The following Abs were used: allophycocyanin-conjugated anti-CD4, PE-Cy7–conjugated anti-CD25, FITC- or PE-conjugated anti-CD45RA and -CCR7, FITC-conjugated Ki67, and as an isotype control, fluorochrome-conjugated mouse IgG (BD Biosciences, San Jose, CA); FITC-conjugated or biotin-conjugated anti–LAG-3 (clone 17B4; Apotech, Epalinges, Switzerland); and PE- or allophycocyanin-conjugated anti-Foxp3 (eBioscience, San Diego, CA). Intracellular staining for human Foxp3 was performed according to the manufacturer's protocol (eBioscience). Ki67 FITC staining was performed using eBioscience fixation and permeabilization buffers.

Intracellular staining for IL-10, IL-2, and TGF-β1 was performed as follows: lymphocytes that were freshly isolated or activated overnight with anti-CD3/CD28 beads (DynaBeads CD3/CD28 T cell Expander; Invitrogen Dynal AS, Oslo, Norway) or with 1 μl/ml Golgi Plug (BD Biosciences), were stained for cell surface markers CD4, CD25, and LAG-3, washed, fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), and stained with PE-labeled anti–IL-10, anti–IL-2 (BD Biosciences) or PE-labeled anti–TGF-β1 (IQ Products, Groningen, The Netherlands) mAb. The fluorescence intensity was evaluated using a BD FACSCalibur flow cytometer and analyzed using BD CellQuest software (BD Biosciences) or FlowJo Cytometry Analysis software (Tree Star, Ashland, OR).

Isolation of CD4$^+$ and CD4$^+$CD25$^+$ T cells

CD4$^+$ T cells were purified from freshly isolated PBMCs or from lymph nodes (LNs) by immunomagnetic depletion of non-CD4$^+$ T cells using the human CD4$^+$ T Cell Isolation Kit II following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated cells was checked by surface staining with an anti-CD4 mAb.

CD4$^+$CD25$^+$ T cells were purified from freshly isolated PBMCs by immunomagnetic sorting using human the CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit and following the manufacturer's instructions (Miltenyi Biotec). The purity of the isolated cells was checked by surface staining with anti-CD4 and anti-CD25 mAb.

Expansion of peripheral Treg cells was performed following a previously published protocol (31). CD4$^+$CD25$^+$ T cells (2 × 10^5 per milliliter) were stimulated with 1 μg/ml OKT3 in the presence of 10^6 per milliliter allogeneic gamma-irradiated (10,000 cGy) feeder PBMCs and 10^5 per milliliter allogeneic gamma-irradiated lymphoblastoid cell line. Cell cultures were maintained in X-VIVO-15 medium (Lonza, Basel, Switzerland) supplemented with 10% FCS (Lonza), 1% heat-inactivated human serum, 2 mM glutamine (Lonza), and antibiotics. Recombinant IL-2 (Proleukin-Chiron, Amsterdam, The Netherlands) was added 3 d after activation at 40 U Cetus/ml. Isolated CD4$^+$CD25$^+$ T cells, activated in vitro as described above, were stained with FITC-conjugated anti–LAG-3 mAb and sorted using a FACS-Vantage DiVa (BD Biosciences).

Suppression assay

In vitro suppression assays were performed in 96-well round-bottom plates or HST Transwell-96 plates (Corning Glass, Corning, NY). The responder CD4$^+$CD25$^-$ T cells were stimulated using anti-CD3/CD28 beads and incubated alone or with increasing numbers of freshly isolated autologous CD4$^+$CD25$^+$ LAG-3$^+$ T cells. The proliferation of the responder T cells was evaluated 72 h after the incubation of T suppressor cells with [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ). [^{3}H]Thymidine was then added at 1 μCi per well for an additional 18 h. For some experiments, the proliferation of responder cells was evaluated by CSFE labeling (see *Materials and Methods, CFSE proliferation assay*). For certain experiments performed in transwell
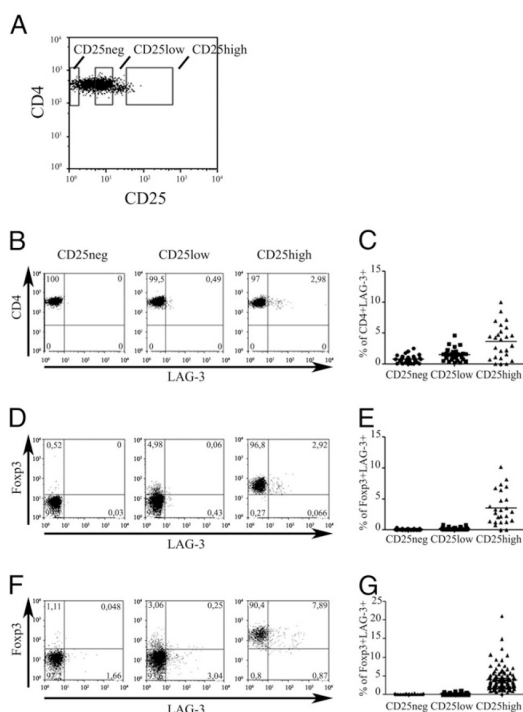

FIGURE 1. LAG-3 is preferentially expressed by regulatory CD4$^+$CD25^{high}Foxp3$^+$ T cells and defines a discrete subset of CD4$^+$CD25^{high}Foxp3$^+$ T cells in PBMCs of both healthy donors and cancer patients. PBMCs from healthy donors or melanoma and colorectal cancer patients were stained with anti-CD4, -CD25, –LAG-3 and -Foxp3 mAb and analyzed by FACS. The gating strategy is shown (*A*). Dot plots showing examples of LAG-3 (*B*) and LAG-3/Foxp3 (*D, F*) expression in CD4$^+$CD25^{neg}, CD4$^+$CD25^{low}, and CD4$^+$CD25^{high} gated populations of PBMCs from healthy donors (*B, D*) and patients with melanoma (*F*) are reported. Cumulative data of the percentage of double positive cells in the gated T cell subsets of PBMCs from healthy donors (*C, E*) or patients with cancer (*G*) are illustrated by the dot graphs. PBMCs analyzed: healthy donors, *n* = 25; cancer, *n* = 85 (melanoma, *n* = 74; colorectal cancer, *n* = 11). *C, E,* and *G,* Absolute numbers of LAG-3+ cells have been reported in Supplemental Table I.
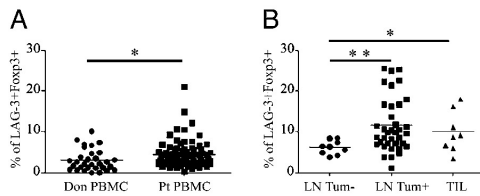
FIGURE 2. Cancer patients show increased frequency of CD4⁺CD25^high Foxp3⁺LAG-3⁺ T cells in PBMCs, LNs, and TILs of patients with cancer. Cumulative data of the percentage of LAG-3⁺Foxp3⁺ cells in the gated CD4⁺ CD25^high subpopulation of healthy donors' and cancer patients' PBMCs are reported. Don PBMC = PBMCs of healthy donors ($n = 31$); Pt PBMC = PBMCs of patients with melanoma ($n = 74$) and colorectal cancer ($n = 11$) (A). The same analysis performed in PBMCs and described in Fig. 1 was also conducted on lymphocytes from tumor-free lymph nodes (LN TUM-, melanoma, $n = 9$), tumor-invaded lymph nodes (melanoma, LN TUM⁺, $n = 29$; colorectal cancer, $n = 8$) and TILs from visceral and sub cute metastases (melanoma, $n = 8$) (B). Individual data and mean values are shown. Statistically significant differences are indicated as determined by Student t test. Absolute numbers of LAG-3+ cells in A and B have been reported in Supplemental Tables I and II, respectively. TIL, tumor infiltrating lymphocyte.

plates, anti–IL-10 (10 μg/ml) or anti–TGF-β1 (10 μg/ml; R&D, Minneapolis, MN) was added to the samples. The whole human IgG molecule (10 μg/ml; Chemicon International, Temecula, CA) was used as a control. All experiments were performed in triplicate. The percentage of inhibition was calculated as follows: % of inhibition = 100 × [1 − (mean proliferation [responder and suppressor]/mean proliferation [responder only with beads])]

CSFE proliferation assay

For in vitro CFSE proliferation assays, purified CD4⁺CD25⁻ T cells were labeled by incubation with 5 μM CFSE (Invitrogen, Carlsbad, CA) in RPMI 1640 medium containing 10% FCS for 15 min at 37°C. This re-

action was blocked by the addition of cold FCS, followed by washing with RPMI 1640 medium supplemented with 10% FCS. The cells were subsequently prepared for a suppression assay (see *Materials and Methods, Suppression assay*).

Statistical analysis

The differences between groups were assessed using the two-tailed Student t test. Differences were regarded as significant at $p \leq 0.05$ (*$p \leq$ 0.05; **$p \leq 0.02$; ***$p \leq 0.005$).

Results

LAG-3 is preferentially expressed by CD4⁺CD25^high T cells and defines a discrete subset of CD4⁺CD25^high Foxp3⁺ T cells

Studies in mice indicated that LAG-3 has a complex role in T cell homeostasis and is expressed by CD4⁺CD25⁺ Treg cells. To explore the distribution of LAG-3 in human CD4⁺ T cells, the expression of the LAG-3 molecule was evaluated inside the CD4⁺CD25^neg, CD4⁺ CD25^low and CD4⁺CD25^high gated populations of PBMCs from healthy donors. We consistently observed the LAG-3 molecule within the CD4⁺CD25^high compartment (Figure 1A–C).

Human natural Treg cells constitutively express a high level of CD25 (15, 31–34) and the nuclear transcription factor Foxp3 (10, 35, 36). Thus, the expression of Foxp3 and LAG-3 inside the CD4⁺ CD25^high gated cells was evaluated by multiparametric flow cytometry. As expected, Foxp3 uniformly stained all the CD4⁺CD25^high T cells. Conversely, LAG-3 expression defined a discrete subset of T cells inside the CD4⁺CD25^highFoxp3⁺ compartment (Figure 1D–G).

The frequency of CD4⁺CD25^highFoxp3⁺LAG-3⁺ T lymphocytes is increased in PBMCs of tumor-bearing patients and at tumor sites

To investigate whether CD4⁺CD25^highFoxp3⁺LAG-3⁺ T cells were expanded in cancer patients and could thus possibly represent
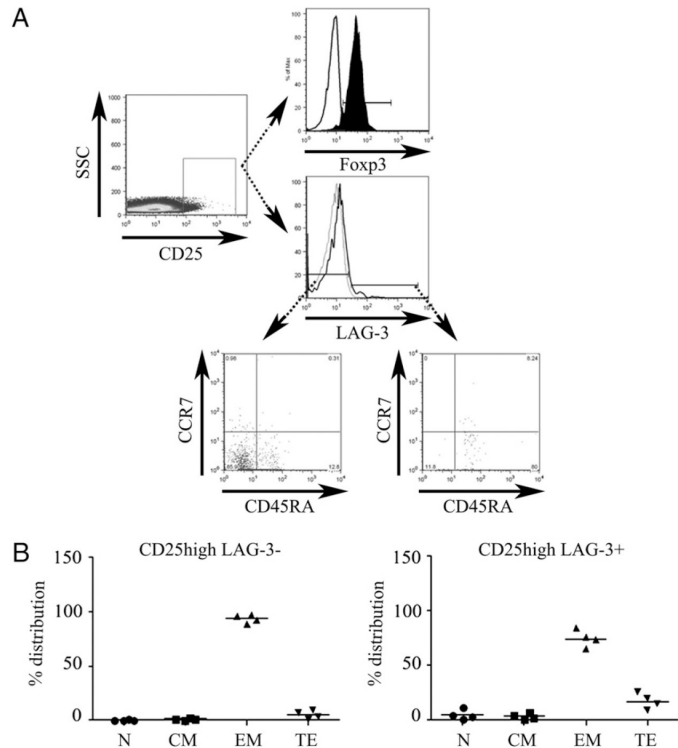
FIGURE 3. In vivo differentiation stages of CD4⁺ CD25^highFoxp3⁺LAG-3⁺ lymphocytes. CD4⁺ T cells from melanoma patients, negatively purified by magnetic sorting (purity >95%), were analyzed for expression of CD25, Foxp3, LAG-3, CD45RA, and CCR7. Gating strategy is shown (A). Dot plots represent the percentage distribution in N (CD45RA⁺CCR7⁺), CM (CD45RA⁻CCR7⁺), EM (CD45RA⁻CCR7⁻), and TE (CD45RA⁺CCR7⁻) of CD4⁺CD25^highLAG-3⁻ and CD4⁺CD25^highLAG-3⁺ gated lymphocytes. The narrow bar represents the mean percentage of expression (B). Lymphocytes from PBMCs of patients with melanoma ($n = 2$) and from melanoma-invaded LNs ($n = 2$) were analyzed. Absolute numbers of LAG-3+ cells in B have been reported in Supplemental Table III. CM, central memory, EM, effector memory; N, naive; TE, terminal effector.

a subset of tumor-induced Treg cells, LAG-3 expression was analyzed in PBMCs of patients with advanced tumor-bearing stage III and IV melanoma and colorectal cancer. Similar to what we found in healthy donors, LAG-3$^+$Foxp3$^+$ cells were mainly confined to the CD4$^+$CD25^{high} compartment (Fig. 1F, 1G). However, the frequency of LAG-3$^+$Foxp3$^+$ cells among CD4$^+$CD25^{high} T cells was significantly increased in the PBMCs of patients with cancer, compared with those of healthy donors (Fig. 2A).

The effect of a tumor on the percentage of CD4$^+$CD25^{high} Foxp3$^+$LAG-3$^+$ T cells became more evident in the analysis of T lymphocytes derived from tumor-positive or tumor-negative lymph nodes of stage III melanoma and colorectal cancer patients as well as lymphocytes infiltrating s.c. or visceral stage IV melanoma metastasis. Results reported in Fig. 2B show that lymphocytes of tumor-invaded lymph nodes and tumor-infiltrated lymphocytes were significantly enriched in CD4$^+$CD25^{high}Foxp3$^+$ LAG-3$^+$ T cells.

CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ T cells display phenotypic features of effector-memory T cells

CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ T cells of cancer patients were then characterized for expression of CCR7, CD45RA and Ki67. CD45RA and CCR7 expression analysis indicated that both LAG-3$^-$ and LAG-3$^+$ CD25^{high}Foxp3$^+$ T cells mainly displayed an effector-memory phenotype. However, a small percentage of cells that reacquired CD45RA and displayed a terminal effector phenotype was detectable in the LAG-3$^+$ compartment (Fig. 3).

As previously reported (5), we found that a high percentage of CD4$^+$CD25^{high}Foxp3$^+$ T cells express the proliferation marker Ki67 (Fig. 4B). However, analysis in regard to the expression of LAG-3 revealed that among CD4$^+$CD25^{high}Foxp3$^+$ T cells, the proliferating compartment was mainly represented by LAG-3– negative cells (Fig. 4B), and only a small percentage of Ki67$^+$ LAG-3$^+$ cells was found.

CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ T cells from patients with cancer produce immunosuppressive cytokines

To assess whether LAG-3$^+$ Treg cells from cancer patients were endowed with functional activities, we evaluated the production of the immunosuppressive cytokines IL-10 and TGF-β1, which are known to play a key role in the generation and function of natural Treg cells and Tr1 cells (14, 37–39), ex vivo via intracellular staining, and FACS. IL-10 and TGF-β1 were exclusively found in

the CD4$^+$CD25^{high} Treg cells. Moreover, these cytokines were mainly produced by the LAG-3$^+$ subset (Fig. 5A). Conversely, IL-2 was detectable only in CD4$^+$CD25^{low} gated cells, while the CD4$^+$ CD25^{high} compartment remained negative for IL-2 (Fig. 5A). These results exclude the possibility that CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ cells were recently activated conventional effector T cells. Cumulative data reporting on the percentage of cytokine-secreting cells inside the CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ and CD4$^+$CD25^{high} Foxp3$^+$LAG-3$^-$ gated populations of PBMCs from patients with

FIGURE 5. CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ T cells produce IL-10 and TGF-β1 cytokines, but not IL-2. Upon activation with anti-CD3/CD28 beads, PBMCs from patients with melanoma and lymphocytes from tumor-invaded LNs were stained with anti-CD25, CD4, Foxp3, and LAG-3 mAb. Production of IL-10, TGF-β1, and IL-2 was evaluated by intracellular staining. The FACS analysis of PBMCs from a representative melanoma patient (A). The strategy for gating and analysis (B). The percentage of IL-10– or TGF-β1– producing cells in the CD4$^+$CD25^{high}LAG-3$^+$ or LAG-3$^-$ gated population is shown in the graphics (C). Individual data and mean values are shown. Statistically significant differences are indicated as determined by unpaired Student t test. PBMCs: IL-10, n = 13; TGF-β1, n = 28. Lymphocytes from melanoma-invaded LNs: IL-10, n = 17; TGF-β1, n = 22. Absolute numbers of LAG-3+ cells in C are reported in Supplemental Table V.
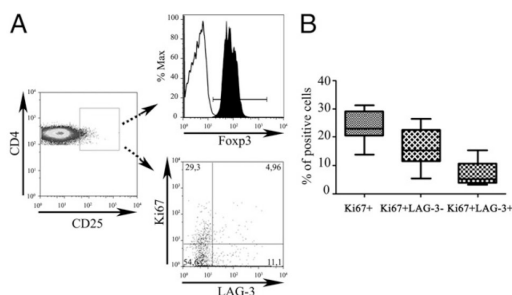

FIGURE 4. CD4$^+$CD25^{high}Foxp3$^+$LAG-3$^+$ T cells proliferate less than their LAG-3$^-$ counterparts. PBMCs from patients with melanoma (n = 4) and lymphocytes from tumor-invaded LNs (melanoma n = 1; colorectal cancer n = 4) were stained with anti-CD25, -CD4, -Foxp3, –LAG-3 and anti-Ki67 mAb and analyzed by flow cytometry as shown (A). Percentage of total Ki67$^+$, Ki67$^+$/LAG-3$^-$, and Ki67$^+$/LAG-3$^+$ cells in the CD4$^+$ CD25^{high}Foxp3$^+$ subpopulation is reported in the graphic (B). Absolute numbers of LAG-3$^+$ cells in B are reported in Supplemental Table IV.
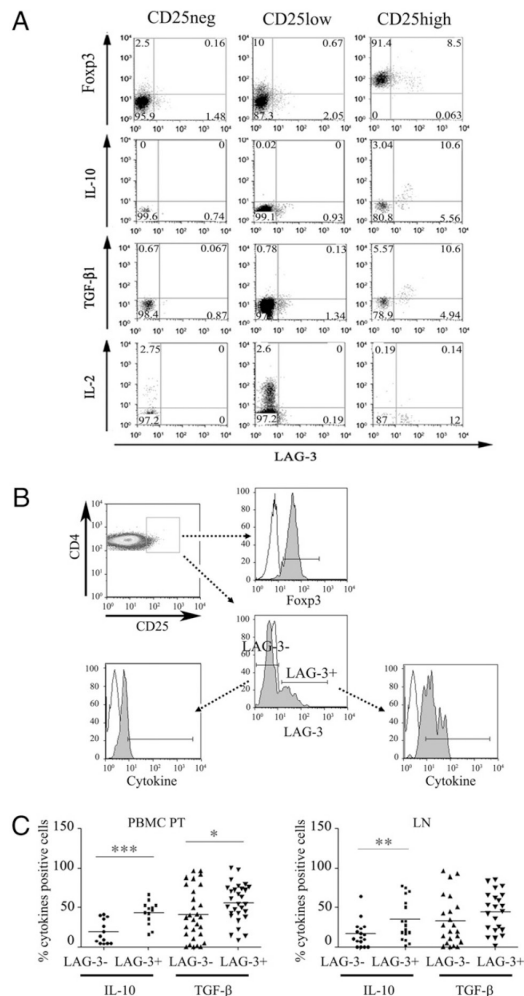
cancer and lymphocytes of tumor-invaded LNs confirmed the preferential association between LAG-3$^+$ Treg cells and the production of suppressive cytokines. However, this broad analysis indicated that TGF-β1 displays a less restricted distribution; therefore, a high proportion of LAG-3$^-$ Treg cells also produces this immunosuppressive cytokine (Fig. 5C).

Suppressor activity of LAG-3$^+$ Treg cells

Our analysis showed that inside the CD4$^+$CD25^{high}Foxp3$^+$ T cell compartment, LAG-3 positive cells display a regulatory phenotype and produce suppressive cytokines that are involved in mediating Treg cell function. Thus, we investigated the suppressive ability of these cells. To this end, Treg cells from PBMCs of healthy donors were magnetically isolated (purity >98%) and shortly expanded in vitro according to a published protocol (31). Cells were collected and analyzed for CD25 and LAG-3 surface expression. All cells were CD25-positive and displayed varying percentages of LAG-3 positivity (Fig. 6A, 6B). Cells were sorted into LAG-3$^+$ (Fig. 6A, 6B, indicated as suppressor 1) and LAG-3$^{-/low}$ fractions (Fig. 6A, 6B, indicated as suppressor 2); their ability to inhibit the proliferation of autologous freshly isolated CD4$^+$CD25$^-$ T cells was evaluated in a [^{3}H]thymidine incorporation assay. As shown in Fig. 6A and 6B, LAG-3$^+$ T cells displayed enhanced suppressor activity as compared with their LAG-3$^{-/low}$ counterpart.

Suppression experiments were also performed in transwell plates in which sorted LAG-3$^+$ Treg cells and responder T cells were separated by a membrane permeable to soluble molecules, but that prevented direct cell-to-cell contact. As shown in Fig. 6C, LAG-3$^+$ Treg cells exerted their suppressor activity mainly via a direct cell-to-cell interaction, because the prevention of a physical interaction between LAG-3$^+$ Treg and responder T cells permitted responder T cells to completely reacquire their proliferation activity. The addition of anti–IL-10 or anti–TGF-β1 mAb did not affect the inhibitory activity of LAG-3$^+$ Treg cells. These data indicate that LAG-3$^+$ Treg cells exert their suppressor activities in a contact-dependent fashion, in line with what is known for CD4$^+$CD25^{high} Foxp3$^+$ Treg cells. The possible in vivo contribution of IL-10 and TGF-β1 still remains to be investigated.

Discussion

The LAG-3 molecule has been defined as an activation marker that is expressed by human and mouse CD4$^+$ T cells. LAG-3 has been shown to play a key role in T cell homeostasis and has been associated with murine CD4$^+$ and CD8$^+$ Treg cells (25, 40). Moreover, recent data in humans identified a new subset of CD8$^+$ Treg cells expressing LAG-3 that mediate immunosuppression via the secretion of CCL4 (29). Based on these observations, we analyzed the expression of this marker in human CD4$^+$ T cells to
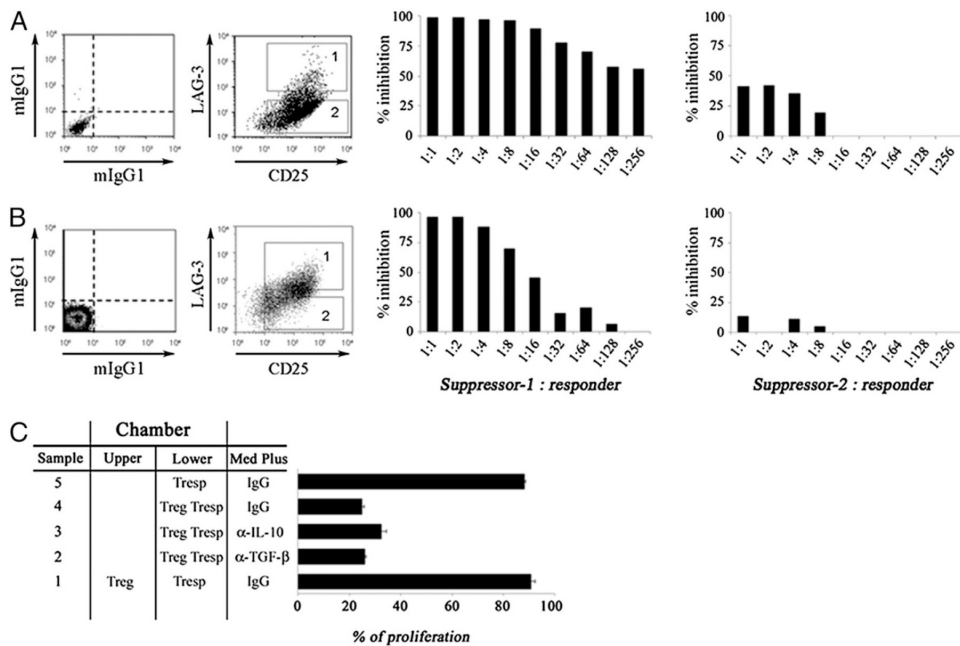

FIGURE 6. In vitro suppression of autologous CD4$^+$CD25$^-$ lymphocytes by sorted CD4$^+$CD25$^+$LAG-3$^+$ T cells. CD4$^+$CD25^{high} Treg cells purified by immunomagnetic sorting from two donors' PBMCs (A, Donor A; B, Donor B; CD25 and CD4 purity >97%) were expanded in vitro and then FACS-sorted into CD4$^+$CD25$^+$LAG-3$^+$ (box 1) and CD4$^+$CD25$^+$LAG-3$^{-/low}$ (box 2) lymphocytes. Dot plots show isotype controls (left) and expression of CD25 and LAG-3 (right) in the CD4$^+$ gated population. Suppressive function of CD4$^+$CD25$^+$LAG-3$^+$ (suppressor 1) and CD4$^+$CD25$^+$LAG-3$^{-/low}$ (suppressor 2) sorted T cells was evaluated in a [^{3}H]thymidine incorporation assay as the ability to inhibit proliferation of freshly isolated autologous CD4$^+$CD25$^-$ T cells in response to stimulation with anti-CD3/CD28 beads. Histograms report percentage of inhibition; ratios of suppressor to responder cells are indicated. The suppression mechanism of sorted CD4$^+$CD25$^+$LAG-3$^+$ T cells (Treg) was evaluated in a proliferation assay using CFSE-labeled CD4$^+$CD25$^-$ autologous Tresps. Tresps were cocultured with suppressor cells at a 1:1 ratio, and proliferation was evaluated by flow cytometry. Cultures were prepared in transwell plates where Tresps were allocated into the lower chamber and Treg cells in the upper chamber, or the cells were cultured together as indicated. Both chambers received anti-CD3/CD28 beads. Cultures were supplemented with either neutralizing anti–IL-10 or anti–TGF-β1 mAb or IgG as controls, as indicated. Percentage of proliferation of Tresps at day 6 in each culture condition is reported (C). Tresp, T cell responder.

explore its distribution in relation to CD25 and its involvement in human Treg cells.

In this study, we found that the expression of LAG-3 identifies a discrete population of $CD4^+CD25^{high}Foxp3^+$ Treg cells in human peripheral blood. Treg cells are no longer considered a homogenous group of cells, but are instead known to contain subclasses of T cells that can be differentiated by their expression of molecules, such as CD45RO/RA, CCR7, MHC-II, and the costimulatory molecule ICOS (13, 14). Our data on LAG-3 add to the complexity to the world of human Treg cells.

Furthermore, we show that this subset of LAG-3$^+$ Treg cells is expanded in PBMCs of advanced tumor-bearing patients, as well as in CD4$^+$ T cells found at tumor sites. Importantly, in patients with melanoma or colorectal cancer, LAG-3 expression defines a subpopulation of Treg cells that produce the suppressive cytokines IL-10 and TGF-β1. These findings, together with the observation that $CD4^+CD25^{high}LAG-3^+$ cells display an effector-memory/terminal-effector phenotype and lack a significant proliferation capacity, suggest that $CD4^+CD25^{high}LAG-3^+$ cells could be considered as activated or differentiated Treg cells.

By sorting LAG-3$^+$ cells from expanded Treg cells, we found that the expression of LAG-3 is associated with strong functional activity. Specifically, we found that LAG-3$^+$ Treg cells inhibit the proliferation of $CD4^+CD25^-$ autologous T cells by contact-dependent mechanisms as expected for $CD4^+CD25^{high}Foxp3^+$ natural Treg cells. Whether IL-10 and TGF-β1 contribute to the suppressor activity of this cell subset in vivo still remains to be assessed. These cytokines might play a role in Treg/dendritic cell interactions at tumor sites as reported for the $CD4^+CD25^+ICOS^+$ Treg cells, which suppress T cell proliferation through a contact-dependent mechanism, but also produce IL-10 to counteract dendritic cell function and maturation (14). It is yet to be determined in vivo whether multifunctional Treg cells simultaneously use all of these mechanisms to suppress and restrain the immune response (41). To keep the immune system in check, Treg cells adapt their suppression mechanisms in response to the local environment. In addition, it is likely that each mechanism plays a specific role in a given inflammatory tissue setting. It has been shown, for example, that Treg-derived IL-10 was mainly necessary for limitation of inflammation in the colon, lung, and skin (42), whereas recent data indicate that, at tumor sites, suppression is also due to the action of a subset of $CD4^+CD25^+$ Foxp3$^+$ T cells that release IL-10 and TGF-β1 (37, 43).

Our data define phenotypic features that distinguish tumor-related Treg cells. In fact, we showed that $CD4^+CD25^{high}Foxp3^+$ LAG-3$^+$ Treg cells were not only selectively expanded in lymphocytes infiltrating visceral or cutaneous metastasis, but they were also selectively enriched in tumor-invaded LNs as compared with loco-regional tumor-free LNs. Our data are in agreement with previous findings, indicating that melanoma-infiltrating lymphocytes are enriched in a subpopulation of strongly suppressive Treg cells expressing ICOS (44). Like ICOS, LAG-3 becomes expressed by T cells as a consequence of a TCR-mediated activation (45, 46). Interestingly, patients with advanced tumor-bearing cancer display in their peripheral blood an enhanced percentage of $CD4^+CD25^{high}Foxp3^+LAG-3^+$ cells, suggesting the presence of activated Treg cells similar to those found at tumor site.

Our observations raise the question of whether this tumor-associated subset of LAG-3$^+$ Treg cells should be considered as tumor-induced/adaptive Treg cells. They certainly differ from classical Tr1 because they express high levels of CD25, are Foxp3$^+$, and exert their suppressive function by direct cell-to-cell contact. In addition, recent data in a human setting seem to stress the functional and numerical relevance of adaptive Treg cells, which are likely constantly produced in the periphery from a differentiation stage that is equipped with suppressor functions (3–5).

Nevertheless, our data are in agreement with previous findings in mice in which the LAG-3 molecule was shown to be expressed both by induced Treg cells and by natural Treg cells upon TCR activation. In mice, evidence is also provided indicating a functional involvement of LAG-3 in mediating suppression (25). In our hands, LAG-3–specific 17B4 Ab, used in vitro in different experimental settings, did not revert the suppressor activity of LAG-3+ Treg cells (data not shown). However, because these results are negative and with no clear evidence of the antagonistic activity of this Ab, the direct contribution of LAG-3 molecule in mediating suppression in humans still remains to be fully addressed, although initial evidence on the role of the Treg-expressed LAG-3 in inhibiting dendritic cell maturation has been recently reported (47).

Our data shed new light on the phenotypic heterogeneity of human Treg cells and provide evidence for a qualitative difference between Treg cells of healthy donors and those found in patients with cancer, showing that LAG-3$^+$ Treg cells are selectively expanded in the PBMCs of cancer patients and in the tumor microenvironment.

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Disclosures

The authors have no financial conflicts of interest.

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Manuscript

Title: "Frequency and function of circulating regulatory T cells in melanoma patients receiving peptide-based vaccine"

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Running title: Melanoma vaccine and Treg modulation

Research article: Clinical Cancer Therapy

ABSTRACT

Purpose: Regulatory T cells (Tregs) exert a detrimental effect on tumor immunity. We evaluated the influence of low-dose cyclophosphamide (CTX), low-dose interleukin-2 (IL-2) and peptide vaccination on the frequency and functional behavior of Tregs of melanoma patients. Impact of Tregs on antigen-specific response was also investigated.

Experimental Design: Tregs were analyzed by multiparametric fluorescence-activated cell sorting (FACS) in peripheral blood mononuclear cells (PBMCs) of melanoma patients vaccinated with HLA-A*0201 tumor peptides and compared to Tregs in control patients not receiving any treatment but surgery. Vaccinated patients received CTX (300 mg/m^2 i.v.) before the first, the third and the fifth vaccination and two injections of low dose IL-2 ($3x10^6$ IU).

Results: Frequency of Tregs dropped in PBMCs collected 4-7 days after CTX administration, with 6 out of 13 patients displaying a reduction ranging from 20 to 65 %. Tregs regained the original pre-treatment level in few weeks. Low dose IL-2 expanded both circulating conventional activated CD4+ T cells and Tregs; a fraction of these Tregs displayed a Th-1 like phenotype, expressing *ex-vivo* T-bet and INF-γ. No Treg modulation was detectable in control patients. *Ex-vivo* HLA-A*0201/peptide multimer (HLA-multimer) staining and IFN-γ ELISpot assays indicated that peptide-specific T cells induced in vaccinated patients were further boosted following IL-2 administration.

Conclusions: Low dose CTX has a limited efficacy and its effect is transient. In a vaccination setting, Tregs boosted by low dose IL-2 do not limit the vaccine induced immune-responses. CD4+ cells boosted by IL-2 include conventional activated T cells, suppressive Tregs and Tregs that in the pro-inflammatory environment created by the vaccine display functional plasticity acquiring a Th-1 like phenotype.

TRANSLATIONAL RELEVANCE

Drugs depleting Tregs may favor the efficacy of immune-based therapy of cancer. Conversely IL-2 mediated boosting of Tregs may limit the expansion of tumor-specific T cells. Our data indicate that CTX reduces Tregs only in a minority of the patients and this down-modulation is transient, thus underscoring the need to further explore a more effective schedule of CTX administration. CD4+ T cells boosted by low dose IL-2 include conventional activated T cells, suppressive Tregs and Tregs acquiring a Th-1 like phenotype likely induced by the pro-inflammatory environment created by the vaccine. Our study provides definitive evidence that low dose of IL-2 given in a vaccination setting, although expanding Tregs, does not produce detrimental effect on the expansion and functional activities of antigen-specific CD8+ T cells induced by vaccination. The balance among the different functional T cell subsets amplified by IL-2 is most likely influencing the immunological and clinical outcome of the vaccinated patients.

INTRODUCTION

Tregs are essential for the maintenance of self tolerance and immune homeostasis; their archetypal feature is the ability to suppress, mostly in a contact-dependent fashion, the activation, proliferation and effector functions of a wide variety of immune cells (1).

The relevance of CD4+ Tregs in suppressing anti-tumor immune response is well accepted for murine tumors (2). In humans, several studies reported an increase in circulating CD4+ Tregs in patients with various types of cancers and accumulation of Tregs is generally associated to tumor progression and bad prognosis (3-5). The influence of Tregs in down-modulating anti-tumor immunity in cancer patients is under intense investigation and several strategies have been employed to block or to eliminate Tregs (6-8). With the aim of reducing Treg number, cyclophosphamide (N,N-bis (2-chloroethyl)-1, 3, 2- oxazaphosphinan-2 amine 2-oxide, the generic name for Cytoxan (CTX), Endoxan) is used to treat a variety of diseases: solid and hematological malignancies, autoimmune disorder and transplantations. CTX limits the number of circulating Tregs in murine models (9, 10). The biological activities of CTX are dose dependent and metronomic dosing has been shown to possess strong capacity in limiting Treg expansion in advanced cancer patients (11, 12). However, when administered at low dose, biological effects of CTX are less clear and conflicting results have been reported probably due to the different schedule of administration as well as to the different methods used to detect Tregs (13-15).

In humans, Tregs represent less than 10% of the whole CD4+ T cells. They constitutively express IL-2 receptor α-chain (CD25) and Foxp3, together with low level of CD127 (16, 17). Naturally occurring Tregs, ready to exert their suppressive activities, namely activated Tregs, are defined as CD4+CD25^{hi}Foxp3+ T cells (18).

The discovery of a CpG dinucleotides sequence in the first intron of *FOXP3* gene specifically demethylated in Tregs and detected by a methylation-specific qPCR assay has been recently proposed as a more precise method to detect population of human Tregs (19, 20). However, this method of Treg definition cannot be used for a direct quantification of Tregs inside tumor samples (21) and, most importantly, it cannot be associated to any *ex-vivo* functional studies. Conversely, the multiparametric Treg definition using anti -CD4, -CD25 and anti-Foxp3 mAbs allows the combination of additional markers aimed at defining *ex-vivo* the functional properties of the identified T cell subsets. This

implementation is crucial in view of the functional heterogeneity and high lineage plasticity of Tregs (1, 22-24).

In this study, taking advantage of a phase II randomized trial of stage IIB-C/III HLA-A*0201 melanoma patients (peptide vaccination versus observation), we assessed the *in vivo* effect of CTX and IL-2 on Treg modulation. Tregs was investigated combining phenotypic and functional markers aimed at dissecting their plasticity in the context of vaccination. Extensive immunological monitoring of the vaccine–induced T cell responses was performed to understand whether the modulation of the frequency and functional behavior of Tregs impacted patients' immune-response to the vaccine.

MATERIALS AND METHODS

Study design and patients' eligibility criteria

This was a phase II randomized study involving HLA-A*0201 patients with melanoma (stage IIB/C-III). The study included a vaccination and an observation arm.

The peptides used in the present study were synthesized under GMP conditions by Merck Biosciences AG Clinalfa (>95% purity). The vaccine included four altered peptide ligands (APLs): HLA-A*0201-restricted (Melan-A/MART-1[27L], gp100[210M], NY-ESO-1[165V], and Survivin[97M]); 250 µg of each peptide was emulsified in Montanide-ISA51 (0.5 ml, Seppic, Franklin Lakes, NJ) and injected subcutaneously (s.c.) close to the inguinal/axillary LN in combination with CTX (ENDOXAN) (300 mg/m^2) and IL-2 (PROLEUKIN) ($3x10^6$ IU). Schedule is detailed in Figure 1S.

Patients were selected after primary tumor removal and were enrolled at the Istituto Nazionale Tumori of Milan (N=37), Azienda Ospedaliera Universitaria Senese (N=4), and Istituto Oncologico Veneto of Padova (N=2). The protocol was conducted in compliance with the Declaration of Helsinki and was approved by the ethical committees of each institution. Written informed consent was obtained. Patients with histologically confirmed (American Joint Committee on Cancer) stage IIB/C-III resectable melanoma (ECOG score 0–1) and normal hematopoietic, liver and renal function were eligible. Patients were checked for HLA-A*0201 expression using an OLERUP SSPTM HLA kit (Qiagen S.p.A, Milan, Italy). Of the 43 patients enrolled, 22 were randomized into vaccination arm and 21 into observation arm (receiving

neither vaccination nor IL-2 and cyclophosphamide). Two patients belonging to the vaccination arm did not complete the treatment.

Blood and tissue samples
Upon written informed consent, blood samples were collected from healthy donors and patients. PBMCs were isolated by Ficoll/Paque[TM] PLUS density gradient centrifugation as previously described (25). Donors' and patients' sera was obtained from blood cells by high speed centrifugations and stored at -30° C.

Treg analysis
Thawed PBMCs were analyzed by FACS after staining with the following mAbs: APCH7 or APC-conjugated anti-CD4, PE-Cy7-conjugated anti-CD25, FITC-conjugated anti-CD45RA and, as an isotype control, fluorochrome-conjugated mouse IgG (all from BD Biosciences, San Josè, CA); PE- or APC-conjugated anti-Foxp3 as intracellular staining using eBioscience buffers according to manufaturer's protocol (eBioscience, San Diego, CA).
Intracellular staining was performed as follows: lymphocytes freshly isolated or activated overnight with anti-CD3/CD28 beads (DynaBeads® CD3/CD28 T cell Expander, Invitrogen Dynal AS, Oslo, Norway) in the presence of 1 µl/ml Golgi Plug (BD Biosciences), were stained for cell surface markers CD4 and CD25, washed, fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) and then stained with PE-labeled anti-IFNγ or FITC-labeled anti-Ki67 (all from BD Biosciences), PE-labeled anti-T-bet (eBioscience) or PE-labeled anti-TGF-β1 (IQ Products, Groningen, The Netherlands). If combined with anti-Foxp3, staining was performed using eBioscience Fixation/Permeabilization buffers. The fluorescence intensity was evaluated using a BD FACSCalibur® or Navios[TM] (Beckman Coulter, Brea, CA) flow cytometer and analyzed using FlowJo® Cytometry Analysis software (Tree Star Inc, Ashland, OR).

Isolation of CD4+CD25+ T cells
CD4+CD25+ T cells were purified from freshly isolated PBMCs by immunomagnetic sorting using human CD4+CD25+ Regulatory T Cell Isolation Kit following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated cells (> 95%) was checked by surface staining with anti-CD4 and anti-CD25 mAbs.

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Suppression assay

In vitro suppression assays were performed in 96-well round-bottom plates and evaluated by Carboxyfluorescein Succinimidyl ester (CFSE) proliferation assay as previously described (26). The proliferation of the responder T cells was evaluated after 72 hours and the percentage of proliferation's inhibition calculated as follows:

% of inhibition = 100 x [1- (mean proliferation (responder and suppressor) / mean proliferation (responder only with beads)]

Cytokines flow cytomix

Sera IL-12p70 levels and IFNγ release in SN of anti-CD3/CD28 beads activated PBMCs were measured by FlowCytomix^{TM} multiple analyte detection kit (eBioscience) according to the manufacturer's protocol. Cytokines production was evaluated as fluorescence intensity with a BD FACSCalibur® flow cytomiter and quantified by the Pro 2.4 FlowCytomix^{TM} software.

ELISpot

Immunomonitoring was performed by *ex-vivo* IFNγ-Enzyme-Linked Immunospot (ELISpot) assay (1-D1K, MabTech AB, Nacka, Sweden) after incubating thawed PBMCs overnight at 37°C in culture medium, as previously described (27). Data were evaluated by a computer-assisted ELISpot reader A.EL.VIS. (Thema Ricerca, Bologna, IT). Results are presented as the number of APL-reactive cells/2×10^{5} CD8+ cells.

Multimer staining

HLA-A*0201-multimers, including the irrelevant HLA-A*0201/HIV-p24 peptide (HIVgag24; TLNAWVKVV), were provided by Proimmune (Ltd, Oxford, UK) and used as previously described (25). Fluorescence intensity was evaluated using a BD FACSCalibur® flow cytometer and analyzed using FlowJo® Cytometry Analysis software (Tree Star Inc, Ashland, OR). A phycoerythrin-conjugated HLA-A*0201-negative multimer was used as a control.

Statistics

Student's t Test and two-tailed Wilcoxon's matched pairs test (Confidence Interval [Cl] 95%) were used as indicated in graph's legends. Statistical calculations were carried out using Prism5 software (GraphPad Software, La Jolla, CA, USA). p-values ≤0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Modulation of Tregs in melanoma vaccinated patients

HLA-A*0201 stage IIB/C-III melanoma patients were enrolled in the study and they underwent vaccination or observation. As potential Treg-depleting agent, patients in the vaccination arm received low-dose CTX (300 mg/m^2 i.v.) 1 week before and 7 and 11 weeks after the initiation of vaccination. To boost vaccine-induced immune-responses, they also received at week 13 and 15 three daily injections of low dose IL-2 ($3x10^6$ IU). In the control arm, patients received no treatment beside surgery (observation only) (Supplementary Figure 1S).

Melanoma patients enrolled in the study displayed a baseline frequency of circulating Tregs slightly enriched as compared to that of age and sex matched healthy donors either if Tregs were identified as CD4+CD25^{hi}Foxp3+ activated Tregs or broadly defined as T cells positive for CD4+CD25+Foxp3+ (Supplementary Figure 2S).

Blood samples were serially collected at different time points (Supplementary Figure 1S) from patients enrolled in the vaccination (n=16) and observation (n=19) arms and frequency of Tregs monitored by FACS analysis. As compared to pre-treatment, frequency of CD4+CD25^{hi}Foxp3+ Tregs dropped in PBMCs collected 4-7 days after CTX administration (P1 versus P0, Figure 1A). Six out of 13 patients for whom paired pre-post CTX blood samples were available displayed a reduction of Tregs frequency ranging from 20 to 65 % (P1 versus P0, Figure 1B). Of note at this stage patients only received CTX, since vaccination started after week 1. However, this reduction was transient and although patients received additional CTX, the frequency of Tregs at week 8 (P4, Figure 1A) regained the original pre-treatment level.

Low dose IL-2 induced a significant expansion of the CD4+ T cells compartment (P6, Supplementary Figure 3S) and strongly boosted the number of circulating CD4+CD25^{hi}Foxp3+ Tregs (P6, Figure 1A). Two to 9 fold increase in the percentage of CD4+CD25^{hi}Foxp3+ Tregs was detected in those patients receiving IL-2 for whom blood sample at day 7 after IL-2 administration was available (P6 versus P4, Figure 1C). Three patients, namely Pt # 9, 11 and 28 received only the first cycle of IL-2 and PBMCs collected at week 16, 22 days after the first IL-2 administration, displayed a lower increase in Tregs frequency (Figure 1C) suggesting the transient nature of the IL-2 induced Treg boosting. Treg frequency profile for Pt # 1, for whom PBMCs collected at additional

time points during the vaccination course were available for analysis, confirmed that CD4+CD25^{hi}Foxp3+ Treg boost occurring at day 7 after IL-2 administration is transitory and no longer detectable after 15 days (Supplementary Figure 4S). No significant variation in Tregs was detectable in the cohort of patients enrolled in the control arm (Figure 1D, E, F). Frequency of CD4+CD25+Foxp3+ T cells displayed a similar modulated profile as that of CD4+CD25^{hi}Foxp3+ activated Tregs in vaccinated and control patients (Supplemental Figure 5S A, B).

In Tregs, stable *FOXP3* expression is ensured by a selective demethylation of a conserved region in the *FOXP3* intron 1. This specific demethylation is unique for Tregs, thus, an alternative method to detect Tregs relies on methylation specific quantitative PCR assays (MS-qPCR). MS-qPCR assay, performed on a set of patients' PBMCs, confirmed the CTX and IL-2 mediated effect on Tregs. Down-modulation of Tregs frequency was detected in 5 out of 6 patients for whom DNA of PBMCs in P0 (day -7) and P1 (day 0), pre-post CTX, was available. Boost of Tregs in P6 (day 112) induced by IL-2 was found in all the patients analyzed by MS-qPCR (n=9) (28).

Circulating Tregs exerted suppressive activity

TGF-β is a key cytokine mediating Treg suppressive functions (29, 30). It regulates many aspects of immunological networks and it directly impairs both NK and T cell functions (31, 32). Moreover, variation in the frequency of TGF-β producing Tregs may impact clinical outcome as shown in a phase II dendritic-based vaccination trial in which vaccine-induced reduction of circulating TGF-β+ Tregs in patients' PBMCs was associated to a prolong survival and disease stability (33).

In our study, we evaluated the functional status of circulating CD4+CD25^{hi}Foxp3+ Tregs by analyzing *ex-vivo* their capacity to produce TGF-β1 upon TCR stimulation. As reported in figure 2A, few TGF-β1+ CD25^{hi} T cells were detectable in the CD4+ T cell compartment at the baseline both in vaccinated and in control patients and thus no significant decrease could be evidenced after CTX treatments. For three patients, namely Pt # 8, 9 and 28, displaying at baseline a higher number of TGF-β1+ Tregs, a slight reduction was detected in PBMCs collected 4 days after CTX (Supplementary Figure 6S). Conversely, a significant increase in the frequency of TGF-β1 producing Tregs was detected in PBMCs of vaccinated patients after IL-2 administration (P6, Figure 2A).

No down-modulation was observed in PBMCs of patients enrolled in the observation arm (Figure 2B and Supplementary Figure 6S).

We assessed whether Tregs found in PBMCs after IL-2 administration were endowed with the capacity to inhibit the proliferation of conventional CD4+CD25- T cells; for Pt # 4 sufficient material was available to sort CD4+CD25^{hi} Tregs from PBMCs pre-vaccine (P0, week -1) and post IL-2 treatment (P6, week 11) to assess their suppressive capacity *in vitro*. *In vitro* suppression assays were set up using as responder CD4+CD25- T cells isolated form autologous pre-vaccine PBMCs. Data reported in Figure 3 clearly show that Tregs isolated after IL-2 administration displayed contact-dependent inhibitory activity further supporting the notion that IL-2 indeed boost a population of functional Tregs.

Collectively these data are in line with previous reports showing that high dose of IL-2 expands T cells with regulatory phenotype *in vivo* (34). Here we extend this notion by showing that also low dose IL-2, as those administrated in our clinical study during an on-going peptide vaccination, directly affects Treg homeostasis by inducing an expansion of suppressive-competent Tregs.

Conventional CD4+T cells were also boosted *in vivo* by IL-2

In cancer patients, increase frequency of circulating Tregs and their accumulation at tumor site is associated with unfavorable prognosis and limited overall survival (4, 35). Tumor-specific Tregs actively suppress the proliferation of CD4+CD25- and CD8+ effector T cells, thereby limiting the immune response against cancer and contributing to tumor growth (36, 37). These immune suppressive functions and the ability of IL-2 to boost *in vivo* Tregs may provide indication for discontinuing the *in vivo* usage of this cytokine in therapeutic vaccination of cancer patients. Conversely, several independent clinical trials have consistently shown that administration of IL-2 into immune competent patients results in 15% to 20% objective clinical responses with 7% complete long-term responders (38, 39). Few explanations may account for the finding that, although increasing Tregs, IL-2 is nevertheless inducing a positive clinical outcome. One of the mechanisms may include a better homing of T lymphocytes at tumor site.

In our study we investigated the hypothesis that, in addition to boost Tregs, IL-2 favors the expansion of conventional CD4+ T cells potentially including Th1 anti-tumor T cells.

Thus to assess whether IL-2 was indeed able to modulate conventional activated CD4+ T cells, PBMCs pre and post IL-2 of 6 vaccinated patients were stained with CD4, Foxp3, CD25 and CD45RA markers. The addition of CD45 marker allows to phenotypically define conventional activated CD4+ T cells that have intermediate levels of CD25 molecules and transiently express Foxp3 (40,41), but are negative for CD45 (Figure 4A, Fr. a: $CD4^+CD25^{int}Foxp3^{int}CD45RA^{neg}$) (18). $CD4+CD25^{hi}Foxp3^+$ activated Tregs are gated in Fr.b (Figure 4A). With this gating strategy, the analysis performed for 6 vaccinated patients and reported in figure 4B confirms that IL-2 indeed boosted Tregs and it also shows that, in all the 6 analyzed patients, IL-2 led to a variable although consistent enhancement of conventional activated CD4+ T cells.

Activated T cells and Tregs in PBMCs pre and post IL-2 of 3 representative patients were characterized *ex-vivo* for their proliferation capacity evaluated as positivity for Ki67, a nuclear protein expressed by proliferating cells in all phases of the active cell cycle (42) and for the expression of T-bet, a Tbox transcription factor crucial for effector Th1 CD4+ T cells commitment and so far the most specific marker for this T-cell subset (43) (Figure 4C-D). Activated conventional CD4+ T cells included T-bet positive cells, whose percentage increased in PBMCs post IL-2 (Figure 4C). Since the T-box transcription factor T-bet controls the expression of IFN-γ, the hallmark of Th1 cytokine (44), CD4+ T cells in PBMCs of vaccinated patients were evaluated for their capacity to produce *ex-vivo* this Th1 cytokine upon TCR triggering. In activated conventional cells increase in T-bet positive cells paralleled the increase in the percentage of T cells producing IFN-γ (Figure 4C). Thus these data support the conclusion that, in addition to functionally suppressive Tregs, IL-2 may also expand activated CD4+ T cells producing Th1 related cytokines.

Functional plasticity of Tregs in vaccinated patients

Surprising enough, after IL-2 treatment expression of T-bet was present and boosted also in the fraction of cells containing activated Tregs (Figure 4D) and the percentage of Tregs functionally competent in IFN-γ production was strongly increased in PBMCs post IL-2 (Figure 4D and

4E). No variation occurred in PBMCs of patients in the control arm (Figure 4F).

Tregs found in PBMCs post IL-2, at the end of the vaccination cycle, displayed a phenotype similar to the recently described T helper type 1-like Foxp3+ regulatory T cells. Indeed, natural Tregs are endowed with extensive functional plasticity and their conversion in Th-1 like Tregs producing INF-γ has been recently described in the context of autoimmune diseases and in a vaccination setting (45-47). IL-12 was shown to be the key cytokine inducing such a phenotypic shift (45). Monitoring of IL-12p70 confirmed that, also in our vaccinated patients, an increase in the levels of this cytokine was detectable in sera collected post vaccine as compared to pre-treatment sera, to sera of patients enrolled in the control arm or to sera of healthy donors (Supplementary Figure 7S). These data support the hypothesis that, in a vaccination setting, favoring a pro-inflammatory environment characterized by enhanced level of IL-12, Tregs may undergo to functional plasticity and acquire Th-1 like phenotype. These functionally modulated Tregs can be then expanded in number by the local availability of IL-2.

IL-2 induced Treg boosting was not hampering the vaccine induced immunity

Extensive analysis was performed assessing the immunological status of patients enrolled in the study either in the vaccine or in the control arm; monitoring of peptide specific, vaccine-induced T cells was performed *ex-vivo* by HLA-A*0201/peptide multimer (HLA-multimer) staining and by IFN-γ ELISpot assays at different time points during vaccination.

As reported in Figure 5A, the percentage of tetramer positive cells, increased after the 2nd vaccination, was further boosted at week 16, at the end of the 2nd cycle of vaccination and after IL-2 administration. These data imply that the raise in Tregs induced by IL-2 was not limiting the expansion of antigen-specific T cells evaluated as percentage of pentamer positive CD8+ T cells. In order to assess whether Tregs were negatively shaping the functional activation of antigen-specific CD8+ T cells, the frequency of CD8+T responding to each peptide used as vaccine was evaluated by IFN-γ ELISpot assays at different time points during the vaccination course. Figure 5B reports the kinetic of peptide-specific CD8+ T cell induction for two vaccinated patients, namely Pt#1 and Pt#3. Of note, for both patients, frequency of peptide specific CD8+ T

cells became detectable after the 2nd vaccination and although IL-2 administration led to strong increase in the Treg frequency (Figure 5B, bars in the histograms), the number of CD8+ T cells producing IFN-γ in response to the antigenic peptide stimulation remained stable or was further increased. This trend was confirmed in all the analyzed patients (Filipazzi et al., manuscript submitted).

Thus circulating Tregs boosted by IL-2 administered in a vaccination setting are not hampering the immune response of these patients to the vaccine. Our results are in agreement with recent findings showing that in a multicentre trial of gp100 peptide vaccine plus IL-2, boost of Tregs defined as CD4+CD25+Foxp3+ not only was not detrimental to immune response, but indeed patients who displayed a higher increase in their circulating Tregs had better clinical response (48).

In light of the results of our phenotypic and functional analysis, we can conclude that IL-2 boosted CD4+CD25+Foxp3+ T cells include conventional activated T cells, suppressive Tregs and Tregs that in the pro-inflammatory environment created by the vaccine display functional plasticity. The balance among these different functional T cell subsets is likely influencing the immunological and clinical outcome of the treated patients.

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LEGENDS

Figure 1. Tregs are modulated after CTX and IL-2 administration.
Patients' PBMCs collected at different time points during vaccination (A)
or observation period (D) (see Supplementary Figure 1S for details) were
stained with anti-CD4, -CD25 and -Foxp3 mAbs (Treg markers) and
analyzed by FACS. Graphs show the percentage distribution of
CD4+CD25^{hi}Foxp3+ Tregs in gated CD4+ T subset at different time
points. Histograms (B) and (E) indicate the percentage reduction of
CD4+CD25^{hi}Foxp3+ Tregs after CTX administration (P1 compared to
P0) in vaccine and control arm respectively. Histograms (C) and (F) repot
the fold increase of CD4+CD25^{hi}Foxp3+ Tregs after IL-2 administration
(P6 compared to P4) in vaccinated and control patients respectively. P
values calculated with Wilcoxon statistical test.

**Figure 2. TGF-β1 producing Tregs show a significant increase after
IL-2 administration.** Staining for Treg markers and intracellular staining
for TGF-β1 were performed in patients' PBMCs collected at different
time points upon *ex-vivo* activation with anti-CD3/CD28 beads. The
percentage of TGF-β1+ Tregs in CD4 lymphocytes is shown in graphs;
vaccinated (A) and control (B) patients. P value calculated with
Wilcoxon statistical test.

Figure 3. Tregs expanded by IL-2 exert suppressive activity.
Proliferation of CFSE-labeled CD4^{+}CD25^{-} peripheral blood T cells
stimulated by anti-CD3/CD28 beads was assessed after 72h in the
absence (unshaded histograms) or in the presence (shaded histograms) of
autologous Tregs at 1:1 ratio (A) or at decreasing ratio of Tregs (B).
CD4^{+}CD25^{-} responder T cells (P0 CD25^{neg}) were isolated from PBMCs
of patient # 4 at week -1 before the initiation of vaccination (see
supplementary Figure 1S); Tregs were purified from PBMCs collected at
week -1 (P0 Tregs) of the same patient or after IL-2 administration (P6
Tregs). Proliferation is shown as FACS data analysis and inhibition of the
proliferation is reported as mean percentage of two technical replicates.

**Figure 4. IL-2 increases both Treg and conventional CD4+ T cell
frequency.** CD4+ T cell subsets, defined by the FACS expression of
CD45RA and CD25 according to Miyara et al. (18), in PBMCs pre- and
post-IL-2 administration (P4 versus P6) of patient #33 are represented in
Dot Plots. Fr. a= CD45RA^{neg}CD25^{low} (T conv) and Fr. b=

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CD45RA^{neg}CD25^{hi} (Tregs), (A). Comparison between percentage distributions pre- and post-IL-2 (P4 versus P6) of these three subpopulations are shown for 6 vaccinated patients (B). Mean percentage expression of Ki67, T-bet and IFN-γ pre- and post-IL-2 (P4 versus P6) is reported as histogram in Fr. a (C) and Fr. b (D) (n=3 vaccinated patients; error bar represents SEM). Percentage distribution of IFN-γ+/activated Tregs in CD4+ lymphocytes of PBMCs collected at different time points is shown for vaccinated (left panel) and control (right panel) patients; p value calculated with Wilcoxon statistical test (E).

Figure 5. CD8+ T cell response to the vaccine peptides is not affected by Treg expansion. PBMCs of vaccinated patients were analyzed *ex-vivo* by the indicated A2/peptide pentamer staining (a = gp100; b = melanA/MART1; c = NY-ESO-1; d = SVV-1) at the indicated time points; percentage of pentamer positive cells is calculated in CD8+ gate; p values were calculated with Wilcoxon test (A). For each time point, CD8+ T-cell response to the vaccine peptides, measured by IFN-γ-ELISpot assay is reported together with the percentage of CD4+CD25^{hi}Foxp3+Tregs in PBMCs of patients Pt #1 and Pt #3.

Figure 1S. Schematic representation of the vaccination schedule. V= peptide vaccine made of HLA-A*0201-restricted (Melan-A/MART-1[27L], gp100[210M], NY-ESO-1[165V], and Survivin[97M]) 250 μg each, emulsified in Montanide ISA-51. CTX= Cyclophosphamide i.v. 300mg/m^2. IL-2 s.c. $3x10^6$ IU/day for 3 days. P= blood samples collected.

Figure 2S. Frequency of Tregs in melanoma patients enrolled in the trial is enriched as compare to that of healthy donors. PBMCs of healthy donors (HD) and PBMCs of melanoma patients (PTs) collected 1 week before being enrolled in the study were stained with anti-CD4, -CD25 and -Foxp3 mAbs and analyzed by FACS. Graphs show the percentage distribution of CD25^{hi}Foxp3+ (A) and CD25+Foxp3+ T cells (B) in gated CD4+ T cells. P values were calculated with Student's t-Test.

Figure 3S. IL-2 induces the expansion of the CD4+ compartment. PBMCs of melanoma patients were analyzed by FACS for CD4 expression in the lymphocyte region (G1) at different time points during vaccination (A) or observation (B). Significant p values were calculated with Wilcoxon test.

Figure 4S. Modulation of CD4+CD25+Foxp3+ T cells. Panel A and B show the frequency of CD25+Foxp3+ T cells in CD4 region in patients PBMCs collected at different time points; patients enrolled in the vaccination (A) or in the control arm (B). P value calculated with Wilcoxon statistical test.

Figure 5S. IL-2 mediated Treg boost is transient. Graph shows the frequency profile of CD25^{hi}Foxp3+ T cells in gated CD4 lymphocytes in PBMCs of Pt # 1 collected at different time points during vaccination (A); vaccination schedule and points of PBMCs collection is reported (B).

Figure 6S. Modulation of TGF-β+ Tregs after CTX administration. Histograms show the frequency of CD25^{hi}Foxp3+ T cells in gated CD4 lymphocytes, while points and connection lines indicate the frequency of TGF-β+ Tregs in CD4 cells pre- and post-CTX treatment (P0-P1) in three vaccinated patients (Pt # 8, Pt # 9, Pt # 28); data of two control patients (Pt # 17, Pt # 18), at the same time course in the vaccination schedule, are reported.

Figure 7S. Concentration of IL-12p70 in patients' sera increases after vaccination. Histograms represent the IL-12p70 release, indicated as pg/ml, in the sera of healthy donors (HD, n=10), control patients (CRL, n=6) and vaccinated patients (Vax, n=8) before (pre) and after (post) vaccination. Error bar represents SEM.

Fig. 1

Fig. 2

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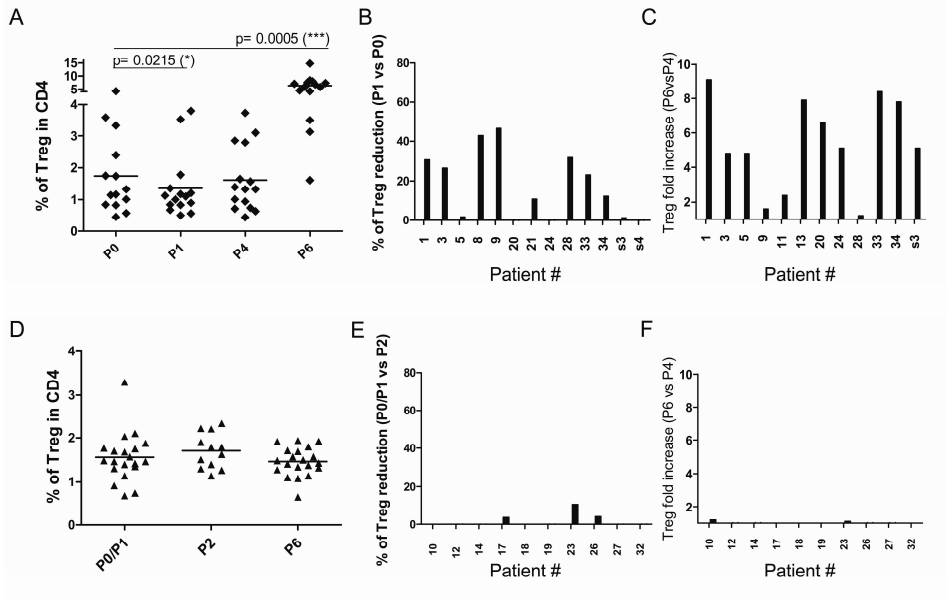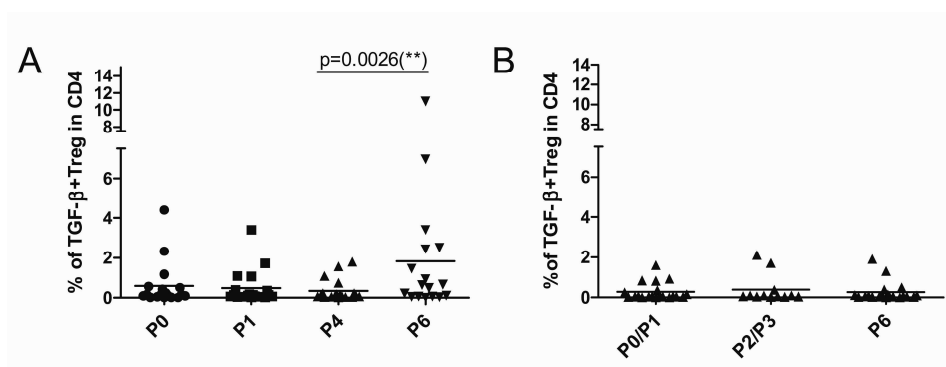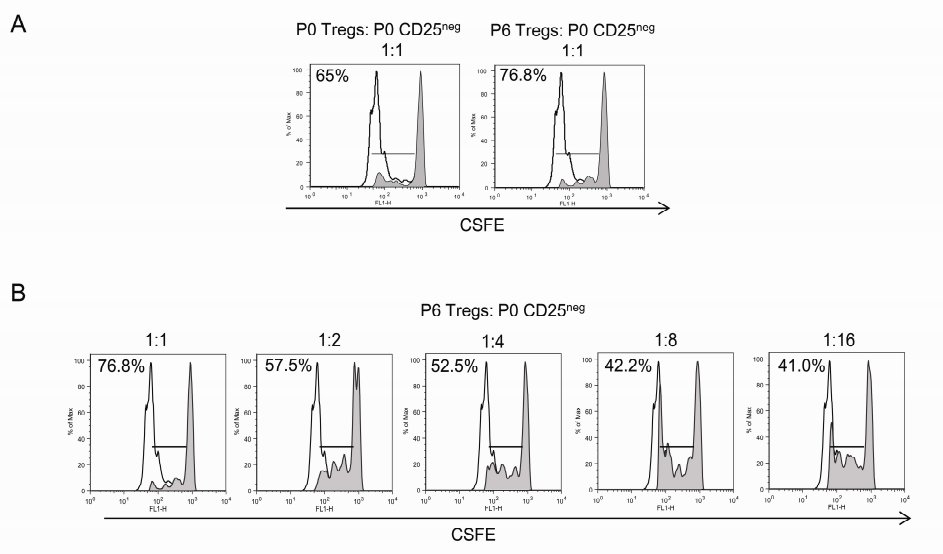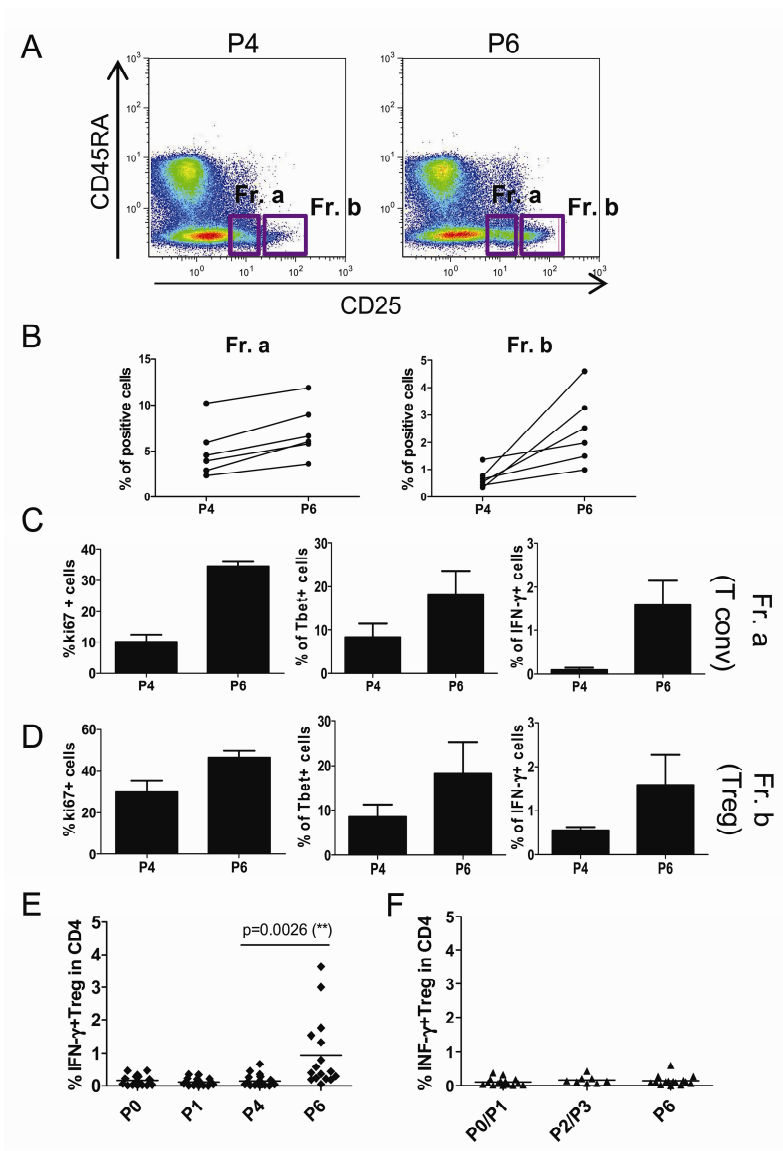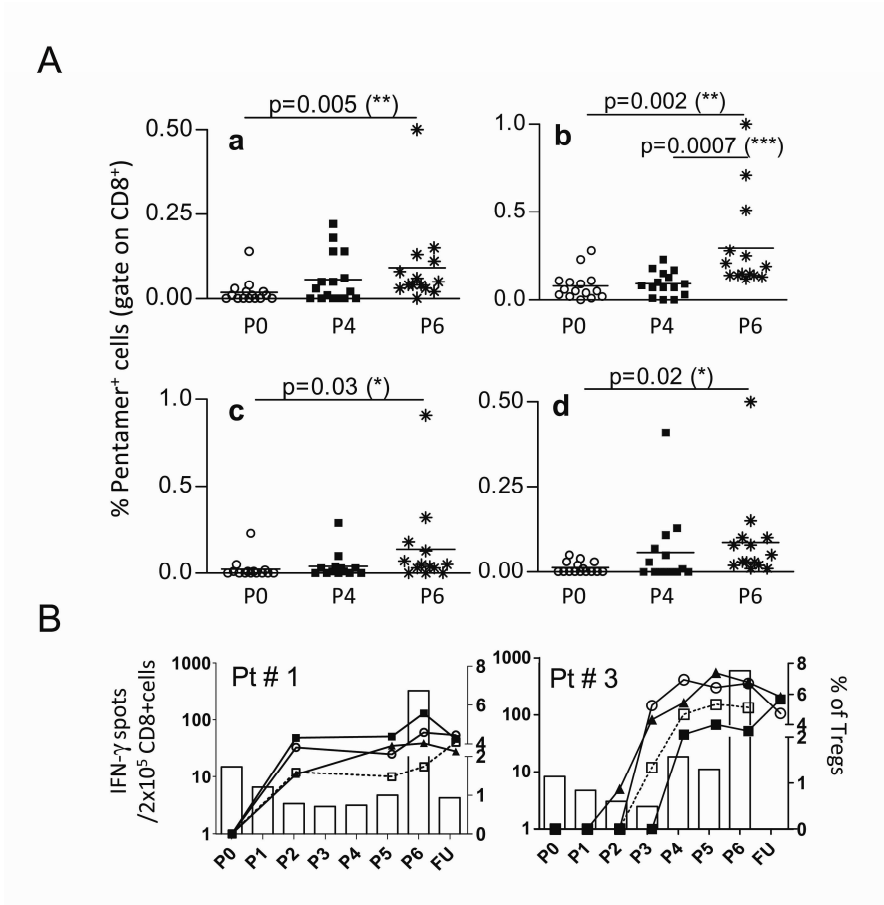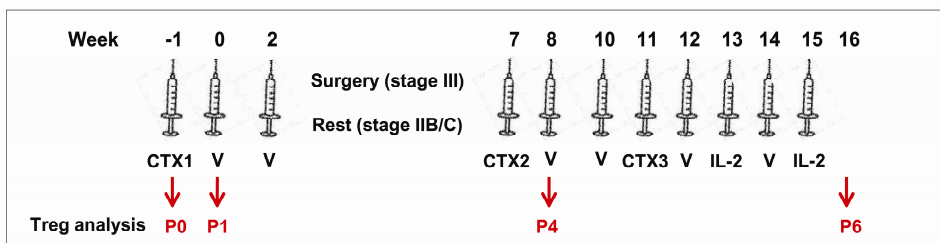
Fig. 3

Fig. 4

Fig. 5

Fig. 1S
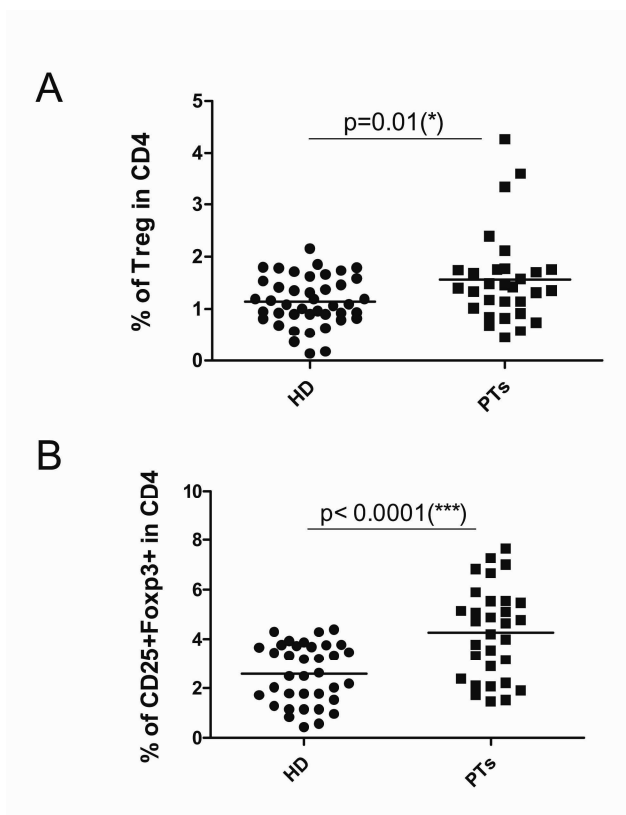
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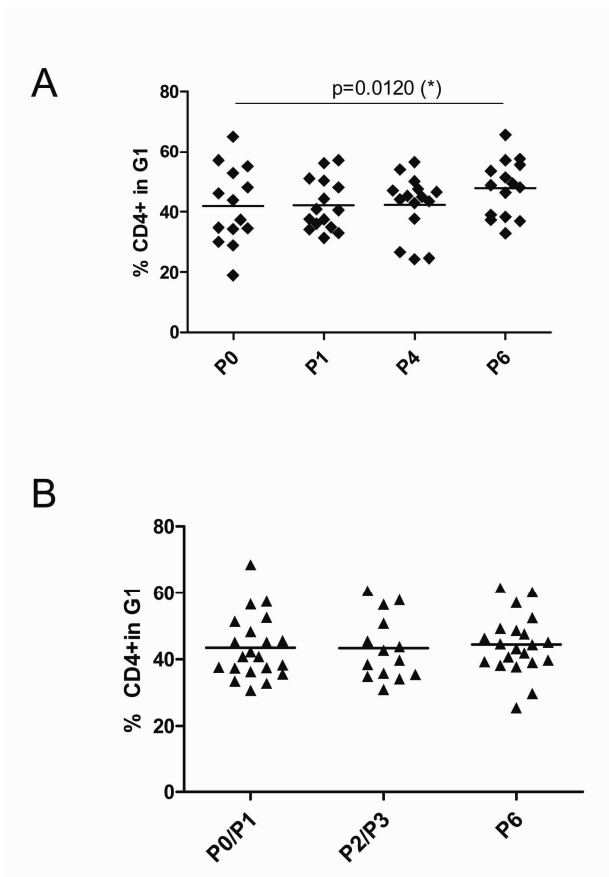
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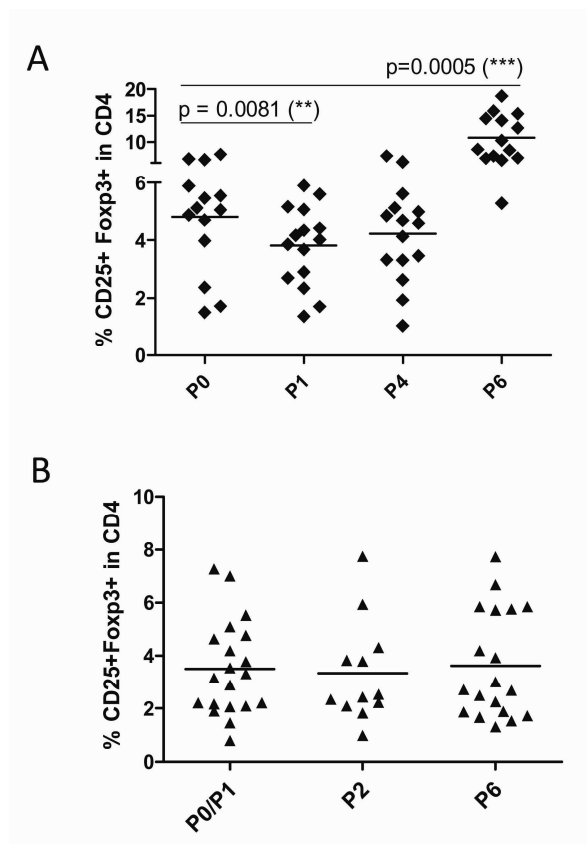
Fig. 4S

A

B

Fig. 5S

Fig. 6S

Fig. 7S