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Transcriptome analysis of tumor infiltrating T regulatory cells unveils specific coding and non coding gene signature

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SUMMARY

ABSTRACT

CD4+ Regulatory T cells (Treg cells) are a specialized subpopulation of T cells that act to suppress immune response, thereby maintaining homeostasis and self-tolerance. These cells play a critical role in preventing autoimmunity infections, and cancer. Treg cells can infiltrate tumor tissues where they suppress anti-tumor immune responses, contributing to the development of an immunosuppressive tumor microenvironment thus promoting immune evasion and cancer progression. Tumor infiltrating Treg cells can display function heterogeneity, depending on both the tumor type and the inflammatory milieu, therefore molecular characterization of Treg cells is crucial to understand how these cells can be modulated in the tumor to unleash effective anti-tumor T cell responses. To this aim, we investigated the transcriptional blueprints of Treg cells both in tumors and in the peripheral blood of healthy donors to define both coding and non-coding transcripts that best define the identity of these cells and might therefore represent novel prognostic markers or therapeutic targets. We performed a transcriptome analysis of both $CD4^+$ Treg cells and effector cells (Th1 and Th17) infiltrating two of the most frequent types of human cancer defining the molecular signatures of tumor-infiltrating Treg cells in these two cancer types. We found tumor-infiltrating Treg cells were highly suppressive, upregulated several immune-checkpoints, and expressed on the cell surfaces specific signature molecules such as interleukin-1 receptor 2 (IL1R2), programmed death (PD)-1 Ligand1, PD-1 Ligand2, and CCR8 chemokine enriched in tumor infiltrating Treg cells compared to both the peripheral blood of patients and healthy donors.

Given the high specificity of long non-coding RNA compared to coding sequences, we also performed bioinformatic analysis to assess the expression of known and novel non-coding transcripts. With this analysis we identified specific Treg cell non-coding transcript in proximity of CTLA4 locus. Since lncRNAs are by now considered as key regulatory elements in immune cell differentiation and maintenance of their identity, we characterized the identified lncRNAs in Treg cells in healthy donors, defining their epigenetic organization, expression level, localization and whether they contributed to the established Treg cell

suppressive activity. All these findings unveiled another layer of the complexity of Treg cells biology and warrants for more detailed functional studies that can fully explain the pathways and the cellular networks that are affected by the identified coding and non-coding transcripts.

INTRODUCTION

1. The immune system

The immune system has evolved to recognize and eliminate pathogens as well as reduce immune–mediated inflammation caused by commensal microorganisms, immune response against self and environmental antigens, and metabolic inflammatory disorders.

In this settings, innate and adaptive immunity, that represent the two main branches of immune response, play complementary roles: the innate immune system, also known as non-specific immune system, depends on physical epithelial barriers, macrophages, dendritic cells, granulocytes and natural killer cells which recognize conserved features of pathogens and become quickly activated to help fight pathogens directly at the site of invasion (Stuart E. Turvey, MB BS, DPhil and David H. Broide 2006); it initiates a primary response upon stimulation by damage (DAMPs, damage associated molecular signals) and pathogen signals (PAMPs, pathogen associated molecular patterns) (Kono et al, 2008). In contrast, the adaptive immune response is highly specific to different pathogens and is mediated by T and B-Lymphocytes that are able to recognize foreign antigens in peripheral lymphoid organs, carrying receptors with unlimited potential (Bonilla and Oettgen 2010).

The activation of adaptive immune cells allows affinity maturation and clonal expansion and comparing to innate immunity, adaptive immune system is characterized by a "memory response" through which immune system cells respond more rapidly and effectively to pathogens already encountered, providing long-lasting protection. Another important feature of adaptive immune response consists in the discrimination between self and non self, since lymphocyte receptor repertoire contains approximately 10^9-10^{15} specificities with potential to identify any epitope. This distinction is mediated by two separate mechanisms: central and peripheral tolerance. The former occurs in the thymus, where all T-lymphocytes carrying T cell receptors with high affinity to self proteins are deleted (Palmer 2003). The latter consists of different tolerance mechanisms in which T lymphocytes are deleted because of low levels or no accessibility to the antigen (Redmond and Sherman 2005).

2. Lymphocytes selection and differentiation

2.1 T cell thymic development: positive and negative selection

T lymphocytes progenitors arise from bone marrow deriving stem cells that migrate to the thymus. During their development in the thymus, thymocytes receive strict selection processes that only permits T lymphocytes functional TCRs to exit the thymus: less than 5% of thymocytes is selected and enable to move to periphery (Starr, Jameson, and Hogquist 2003) .

Differentiation is characterized by the temporally coordinated expression of cell surface proteins on the thymocyte, including CD4, CD8, CD44, and CD25; once into the thymus, the precursors lack expression of CD4 and CD8 and are called double negative (DN). They arrive in the cortico-medullary junction (CMJ), move through the cortex and sequentially pass different stages at the subcapsular zone (Lind et al. 2001) (Fig. 1):

- DN1 (CD44+/CD25-),
- DN2 (CD44+/CD25+)
- DN3 (CD44-/CD25+).

In this stage, the random recombination of V, D, J genes (or for the α chain, V and J only) generates TCR through the enzymatic complex composed of the recombination activating-genes RAG1 and RAG2 (Starr et al. 2003). The transition of thymocytes from a population of cells undergoing TCRβ chain genes to a population enriched in cells with productively rearranged TCRβ chain genes is known as "*beta selection*" (M. Michie et al., 2002). The rearranged TCRβ chain together with a pre-TCRα chain forms a pre-TCR complex and stimulates thymocyte proliferation, rearrangement of the TCRα chain locus and the induction of the two TCR co-receptors CD4 and CD8 (Hoffman et al., 1996).

Double Positive precursors expressing an MHC Class I– or Class II–restricted receptor migrate into the medulla and differentiate into mature T cells. This process is named "*positive selection*" and occurs several days to finalize, during which thymocytes receive continuous TCR signals with associated activation of the mitogen activated protein kinase (MAPK) pathway in order to pass this checkpoint in development (McNeil et al., 2005; Wilkinson et al., 1995).

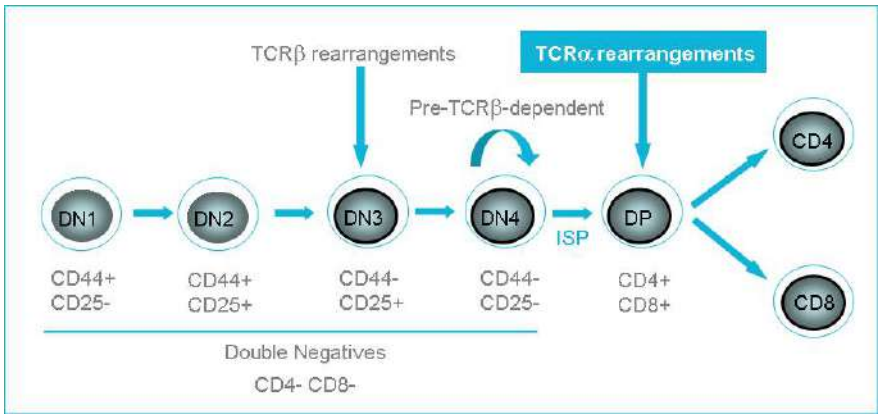


Figure 1: αβ chain T cell development. It is reported the different cell surface markers expressed at the different stages of T cell development.

The diversity of the TCRs is important for produce protection against an undefined number of pathogens, but the TCR rearrangement inevitably generates a certain number of T cells that recognize self-antigens. In this case, autoreactive T cells are negatively selected and eliminated in the thymic medulla, avoiding self-reactivity and autoimmunity phenomena. This negative selection in the thymus represents the major mechanism of central immune tolerance. It is also complemented by peripheral mechanisms that limit the expansion and reactivity of mature self-reactive cells, a phenomenon called *peripheral tolerance* (Mueller 2010).

In addition to these "passive" mechanism of controlling self reactive T cells, there is also a "dominant" control mechanism in which certain T cells actively downregulate activation/proliferation of self reactive T cells: regulatory T cells.

2.2 T helper cell subsets

In the secondary lymphoid organs, naive CD4+ lymphocytes circulate in searching the of antigen presenting APCs: if encountered in the context of co-stimulation and appropriate cytokines that get them the necessary stimuli for the differentiation in specialized lymphocytes. The major T helper lineages are characterized by T helper type1- Th1- and type 2 -Th2- which are involved in promoting cellular and humoral responses, respectively. The other CD4+ helper subsets identified during the time are Th9, Th17, T regulatory and T follicular helper (Tfh).

Th1 cell subset is responsible of cell-mediate immunity and it secretes interferon γ (INFγ), Interleukin-12 (IL-12) and tumor necrosis factor (TNF) as the main cytokines and it displays on its surface specific markers, including C-X-C Motif Chemokine Receptor 3, (CXCR3) and C-C chemokine receptor type 5 (CCR5). INFγ secreted by Th1 induces the activation of macrophages, allowing elimination of pathogens, such as viruses and intracellular bacteria. There are several transcription factors involved in the full differentiation of Th1 lymphocytes: T-box transcription factor (T-bet) is the master regulator and it has a major role in Th1 development by enhancing the production of INFγ and by inhibiting Interleukin 4 (IL-4) gene, GATA binding protein 3 (GATA3) and RORγt thus leading to the suppression of Th2 and Th17 lineages (Murphy and Reiner 2002).

Th2 are mainly involved in the humoral immunity response against extracellular parasites through the activation of eosinophils, basophils, mast cells and B cells. Th2 cells differentiate upon IL-4 stimulation and they display on their surface the chemokine receptor C-C chemokine receptor type 4 (CCR4), which interacts with chemokine ligands CCL17 and CCL22 and determines migration oh Th2 cells in inflamed skin (Luckheeram et al. 2012).

Th17 cells depict the third class of T helper cells that are implicated in the protection against extracellular bacteria and fungi infection and they can recruit also neutrophils. Th17 cells are characterized by the expression of CCR6 on their surface, which mediates migration to inflammatory sites through interaction with CCL20. Their differentiation is enhanced by IL-6, TGF-β, IL-23 and IL-21 and they produce IL-17, IL-6, IL-22 and TNF-α. The master regulator of Th17 is RORγt (Luckheeram et al. 2012).

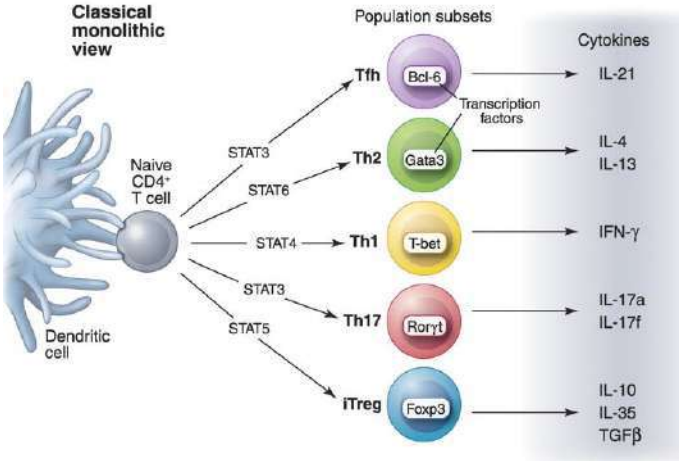


Figure 2: CD4+ T helper cell subsets. Naïve T cells are stimulated to expand after the interaction with APCs expressing MHC class II/peptide complexes. Depending on the type of APC and the cytokine milieu at the site of

antigen encounter, naïve T cells can differentiate into different subsets with different effector functions (O'Shea and Paul. 2010. *Science)*

3. CD4+ T Regulatory cells

3.1 Phenotype and heterogeneity of Treg population

Regulatory T cells are the primary mediators of "peripheral tolerance" and play out several sophisticated mechanisms responsible of the maintenance of immune homeostasis, prevention of autoimmunity and moderation of inflammation induced by pathogens and environmental insults (Vignali, Collison, and Workman 2009). They are naturally present in the immune system and their dysfunction causes fatal autoimmune disease, immunopathology and allergy (Sakaguchi, Yamaguchi, Nomura, & Ono, 2008 ; Gavin et al., 2006) .

They were first characterized by several groups in 2001 as CD4+CD25+ T cells expressing high amounts of IL2R- alpha chain (CD25) and possessing suppressor activity (Baecher-Allan et al. 2001; Dieckmann et al. 2001). An important advance in the study of Treg cells comes in 2003 with the identification of a key transcription factor, known as forkhead box P3 (FOXP3) that is required for their development, maintenance and function (Shohei Hori , Takashi Nomura and Shimon Sakaguchi). However, it has been shown that both CD25 and FOXP3 expression could be induced in naive CD4+ cells upon activation, obscuring the identification of FOXP3 as pure marker of Treg (Gavin et al. 2006). Other Treg cell markers were discovered over the time: the cytotoxic T lymphocyte antigen-4 (CTLA-4, CD152), the glucocorticoid-induced tumor necrosis factor receptor (GITR) and inducible T factor costimulator (ICOS) as well as programmed-death-1 (PD-1); they were found to be expressed on human Treg, but neither was specific for Treg (Fife and Bluestone 2008)(Strauss et al. 2008). Similarly, the chemokine receptors CCR4 (CD194) CCR6 (CD196) and CCR7 (CD197) which are expressed by human Treg are also found on other T cells (Mougiakakos et al. 2010). Treg are also characterized by the absence of certain surface markers that are expressed on Tconv such as IL-7-receptor, CD127 (Liu et al. 2006) and an integrin alpha subunit, CD49d (Kleinewietfeld et al. 2009).

Several discrepancies in the literature have emerged during the time regarding the phenotypic and functional characterization of Treg cells in healthy individuals and patients with

immunological disease, thus requiring new studies for reliable delineation of human Treg cells biology and phenotype.

3.2 Thymic and peripheral origins of treg cells

CD4+ CD25+ Foxp3+ Treg cells, which account for 10% of peripheral CD4+ cells, are subdivided into two subset: "natural" Treg (nTreg) and "induced" Treg (iTreg) cells. nTreg cells develop as a distinct lineage in the thymus during the course of positive and negative selection upon high-avidity interactions with peptide class II major histocompatibility complex, while iTreg cells arise from periphery upon Naive CD4+ T cell interaction with antigen-presenting cells (APC) (Quezada et al. , 2013).

A thymic origin for Treg cells was suggested by the neonatal thymectomy–induced autoimmunity models (Takahashi et al. 1998). Thymic development of Treg cells requires high-affinity interactions between their T cell receptor (TCR) and self-peptide-MHC complexes presented by thymic stromal cells (Hinterberger et al. 2010) These cells also provide co-stimulatory signals required for Treg cell lineage development as shown by the decrease in the number of Treg cells generated in the thymus following loss of CD40 or CD28 expression (Salomon et al. 2000). Another signal that is necessary for Treg cells differentiation is represented by IL-2: mice deficient in IL-2 or CD25 showed a 50% decrease in the proportion and absolute numbers of Foxp3+ cells (Fontenot et al. 2005). Regarding the role of IL-2 and TCR strength, a "two-step model" for thymic Treg cell differentiation has been proposed: high functional avidity TCR signals result in CD25 upregulation and a subsequent increase responsiveness of Treg precursors to IL-2 signals facilitate Foxp3 induction (Burchill et al. 2008). The peripheral pool of Treg cells not only includes those differentiated in the thymus but also Treg cells generated extra-thymically through the "conversion" of naïve precursors in the periphery (iTreg). Two main subsets of iTreg cells have been described based on the cytokines they produce and that their induction provoke: type 1 regulatory T cells (Tr1), which are induced by IL-10 (Vieira et al. 2004) and T helper 3 (Th3) cells, which are induced by TGF-β (Weiner 2001). Both subsets exert their suppressive activity trough secretion of the same cytokines that are responsible for their induction, IL-10 and TGF-β respectively. Since there are not phenotypic markers to distinguish peripherally-induced (iTreg) from thymically

derived natural Treg cells (nTreg), the question is whether nTreg and iTreg cells have the same or distinct functions. The finding that the requirements for the induction of Foxp3 in the thymus and periphery are distinct, suggests that the function of these two Treg cell subsets, nTreg and iTreg cells, are also distinct. iTreg cells are thought to be an important population in the gut mucosa to maintain tolerance to commensal flora and food antigens (Lathrop et al. 2011); they are also found within the tumor microenvironment and chronic inflammatory site and are important for the maintenance of immune tolerance at mucosal surfaces (Abdel-Gadir, Massoud, and Chatila 2018) (Fig. 2).

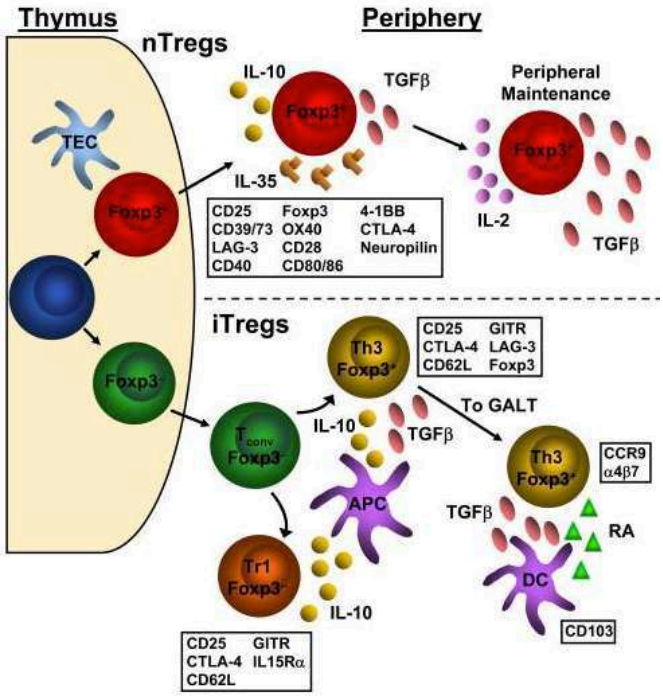


Figure 2: Development of nTreg and iTreg with their associated markers (C. Workman et al. 2009)

3.3 Mechanisms of Treg function

The definition of Treg cell function mechanism is crucial to provide insight into the control processes of peripheral tolerance and to indicate important therapeutic targets. *In vitro* studies have shown that Tregs cells require both TCR stimulation and costimulation to exert their suppressive activity, however, once activated, Treg population is non-specific in its suppression (Sakaguchi 2004).

From a functional perspective, the suppression mechanisms of Treg cells can be grouped in four different modes of action (Fig. 3):

- Suppression by inhibitory cytokines
- Suppression by cytolysis
- Suppression by metabolic disruption
- Suppression by modulation of dendridic cell (DC) maturation or function

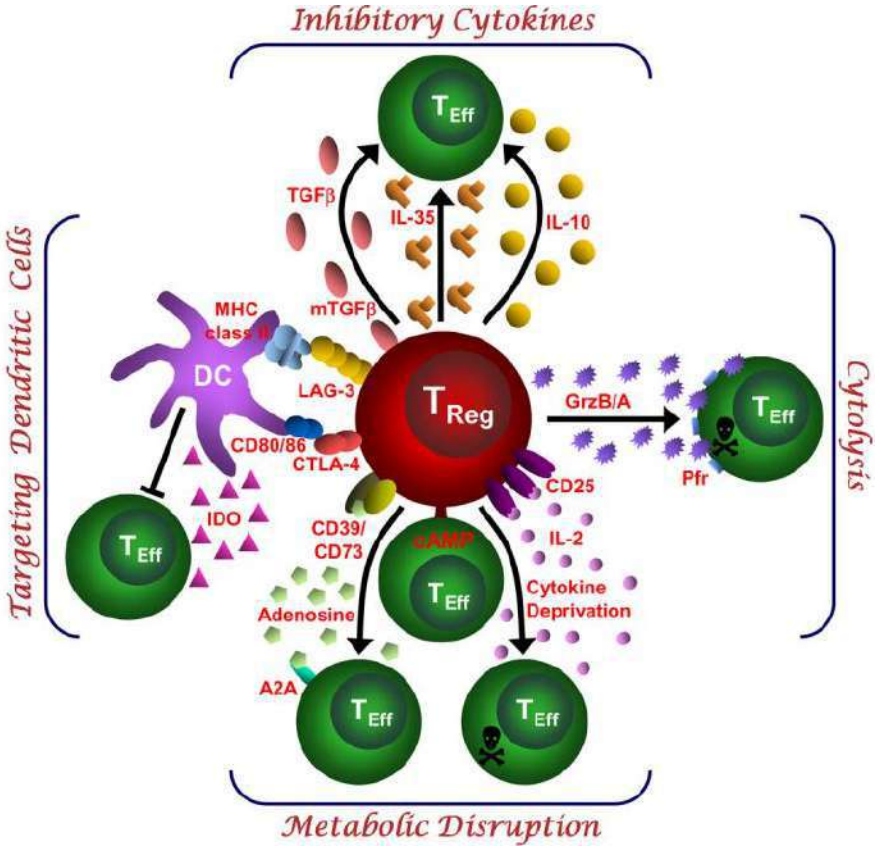


Figure 3: Mechanisms of Treg cells action arranged into four basic modes of action (C. J. Workman et al. 2009).

Suppression by inhibitory cytokines

The secretion of inhibitory cytokines such as IL-10 and TGFβ and IL-35 can inhibit the local immune response by acting on target cells such as APCs or T cells, and represent a cell-cell contact indipendent effector mechanism (Nakamura, Kitani, and Strober 2001). IL-10 has been described as the main immunomodulatory cytokine that additionally inhibits the production of

inflammatory cytokines such as IL-12, causing a decrease in the Th1 response and in INF-γ production and promoting the phagocytic activity (Loebbermann et al. 2012). One of the best known molecular mechanisms of IL-10 is the action on effector cells. The costimulatory molecule CD28 is involved in the interaction between effector cells and antigen-presenting cells. By binding its receptor, IL-10 inhibits tyrosine phosphorylation in CD28, inhibiting PI3K/AKT activation, which in turn inhibits the signaling cascade leading to NF-$\varkappa$B translocation (Taylor et al. 2006). TGFβ is able to regulating the effect of several immune cell types: it suppresses effector T cell differentiation; it promotes the differentiation of naïve T cells into regulatory T or Th17 cells; it inhibits T and B cell proliferation; it inhibits the activity of macrophages, dendritic cells, and NK (Ma et al. 2014). In Treg cells, the inhibitory activity of TGFβ is due to the high LAP (Latency-Associated Peptide, necessary for activation of TGFβ)-TGFβ expression in Treg membrane. Unlike T cells, monocytes and dendritic cells express TGFβ receptors, thus allowing for cell-to-cell interaction. Additionally, TGFβ regulates dendritic cell maturation and differentiation. TGFβ has been demonstrated as necessary to generate tolerogenic dendritic cells (DCs) by inducing IDO, an enzyme that inhibits T cell proliferation.

IL-35 is responsible of suppressing cell proliferation, blocking the shift to a Th1 profile, and mediating IL-10 production (Li et al. 2014).

Although the general importance of IL-10 and TGFβ as suppressive mediators is undisputed, their contribution to the function of thymus-derived nTreg cells is still a matter of debate (Shevach et al. 2006). This is partly due to the general perception that Treg cells work in a contact dependent manner (Takahashi et al. 1998) Indeed, *in vitro* studies using neutralizing antibodies or T cells that are unable to produce or respond to IL-10 and TGFβ suggested that these cytokines may not be essential for Treg -cell function (Jonuleit et al. 2001). However, this contrasts with data from *in vivo* studies in which, in allergy and asthma models, Treg cells are able to control disease in a manner IL-10 and TGFβ dependent (Hawrylowicz and O'Garra 2005): adoptive transfer of allergen-specific Treg cells induced significant IL-10 production by $CD4^+$ effector T cells in the lung following allergen challenge and this Treg cell-mediated control of disease was reversed by treatment with an IL-10-receptor-specific antibody (Kearley et al. 2005).

Suppression by cytolysis

Cytolysis mediated by secretion of granzimes had been considered the mileu stone of Natural killer cells (NK) and CD8+ cytotoxic T lymphocytes (CTLs)(Lieberman 2003). Indeed, Treg cells have been shown suppressive function through production of Granzime B and perforin (Grossman et al. 2004). Granzime B is a serine protease which allows Treg cells to induce apoptosis in effector T cells (Gondek et al. 2005). During the interaction between Treg cells and T effector cells, Treg cells produce granules to the extracellular space that contain granzymes and perforins. Once released from Treg cells, perforin molecules insert themselves into the lipid membrane of the target cell and polymerize in the presence of calcium ions to form a transmembrane cylinder, that, in turn, forms a pore through which granzymes enter the cell. Once within the target cell, Granzyme B could induce apoptosis by caspase-dependent or independent mechanisms (MacDonald et al. 1999).

Moreover, recent studies have suggested that activated Treg cells induce apoptosis of effector T cells through TRAIL-DR5 pathway (tumor necrosis factor related apoptosis inducing ligand-death receptor 5)(Ren et al. 2007) and upregulate galectin-1 (also known as LGALS1) which can induce T cell apoptosis (Garin et al. 2007). However, all these studies emphasize that more work is required to define the cytolytic mechanism that Treg cells use to mediate suppression.

Suppression by metabolic disruption

The 'metabolic disruption' is another mechanism described to suppress effector T-cells in which the high expression of CD25 empowers Treg cells to 'consume' local IL-2 and could starve actively dividing effector T cells by depleting the IL-2 they need to survive (de la Rosa et al. 2004). IL-2 receptor is expressed by T lymphocytes, NK cells, B cells, macrophages, and monocytes; however, only T lymphocytes are capable of producing this cytokine (Bayer, Pugliese, and Malek 2013). IL-2 receptor consists of three subunits: alpha chain (CD25), beta chain (CD122), and gamma chain (CD132). Alpha chain (IL-2Rα) has a very short cytoplasmic domain and does not participate in signal transduction but it is required to increase the affinity of IL-2 to its receptor. In contrast, beta (IL-2Rβ) and gamma (IL-2Rγ) chains are responsible of signal transduction (Manuscript and Nanobiomaterials 2013). Treg cells constitutively express high levels of IL-2 alpha chain, having thus a higher affinity to IL-2, and

compete for this growth factor with proliferating cells leaving them without a vital cytokine, causing metabolic interruption and cell death (Burchill et al., 2007).

Moreover, several evidences have shown another mechanism by which Treg cells suppress effector T cells: the release of adenosine nucleotides that inhibit IL-6 expression while promoting TGFβ secretion (Zarek et al. 2014). Treg cells were also shown to suppress effector T-cell function directly by transferring the potent inhibitory second messenger cyclic AMP into effector T cells via membrane gap junctions (Bopp et al. 2007).

Suppression by modulation of dendridic cell (DC) maturation or function

Studies *in vivo* have revealed direct interactions between Treg cells and Dendritic Cells in a process that involve CTLA4, which is constitutively expressed by Treg cells (Read, Malmström, and Powrie 2000). By using either CTLA4-specific blocking antibodies or CTLA4-deficient Treg cells it was shown that in the absence of functional CTLA4, Treg -cell-mediated suppression of effector T cells via DCs was compromised (Oderup et al. 2006). Importantly, it was also shown that Treg cells could condition DCs, through a mechanism dependent on interactions between CTLA4 and CD80 and/or CD86, to express indoleamine 2,3-dioxygenase (IDO), a potent regulatory molecule that induces the catabolism of tryptophan into pro-apoptotic metabolites that results in the suppression of effector T cells (Mellor and Munn 2004). There are also other studies suggesting that LAG3, lymphocyte activation gene 3, could block DC maturation. LAG is a CD4 homologue that binds MHC class II molecules with very high affinity and provokes immunoreceptor tyrosine-besed activation motif (ITAM) that suppresses DC maturation and their immunostimulatory capacity (Liang et al. 2008).

Cell-to-cell contact with DCs is one of the most important mechanisms for Treg cells mode of action. Depending on its phenotype, a DC can activate or control the immune response. When DC interacts with a Treg cell, it acquires a tolerogenic phenotype, which in turn promotes further Treg cell generation, providing a suppressor microenvironment. The competition for DC ligands between effector and Treg cells allows for an additional control mechanism of the immune response.

As discussed above, the mechanisms underlying Treg cells function are mediated by different factors but have the same objective: preserving homeostasis and thus guaranteeing the establishment of a fine equilibrium across all immune cells. In disease, and in particular in

cancer, Treg cell phenotype and function change, therefore this equilibrium is compromised, favoring the development of pathologic status.

4. Immunoediting

The immune surveillance theory was suggested more that 50 years ago by Burnet and Thomas and claims that the immune system is considered as a sentinel that recognizes and eliminates continuously arising, transformed cells (Burnet 1957). Over the time, this concept has evolved in a larger and more complex "cancer immunoediting" model, whereby the immune system not only protects against cancer development, but also it's responsible of the development of tumors (Dunn et al. 2002). Indeed, a central principle of cancer immunoediting is that T cell recognition of tumor antigens determines the immunological elimination or allows cancer development.

This process is constituted by three key events (Fig. 4):

- Elimination phase: elements of the immune response recognize and destroy tumors.
- Equilibrium phase: a balance is established between the tumor and the immune system. Tumors and immune cells are shaped reciprocally by each other.
- Escape phase: the immune system allows tumor variants to survive and to grow uncontrollably(Koebel et al. 2007).

Elimination

The elimination phase is constituted by the cooperation of innate and adaptive immune responses to transformed cells. The effector cells of the innate response, such as NK, NKT and gamma-delta T cells are activated by inflammatory cytokines released by growing tumor cells, macrophages and stromal cells located around the tumor. Perforin-, FasL- and TRAIL-mediated killing of tumor cells by Natural Killer cells releases tumor antigens (TAs), stimulating the adaptive immune response (Mori et al. 1997), in particular promoting the maturation of DCs and their migration to tumor draining lymph nodes (TDLNs), resulting in the enhancement of antigen presentation to naive T cells and the clonal expansion of Citotoxic T Lymphocytes (CTLs). They migrate to the primary tumor site and directly kill cancer cells

by producing INF-g, a cytokines that induces apoptosis (Wall et al. 2003), antiproliferative (Gollob et al. 2005) and anti-angiogenic effects (Qin et al. 2003).

Equilibrium

It is during the equilibrium phase that the interplay between immune system cells and the tumor will define the final outcome of the immune response. Tumor cells continuously produce variants that are less immunogenic and the immune selection pressure also favors the growth of tumor cell clones with a non-immunogenic phenotype that survive and grow in the tumor microenvironment. Since the equilibrium phase involves the continuous elimination of tumor cells and the production of resistant tumor variants by immune selection pressure, it is likely that equilibrium is the longest of the three processes in cancer immunoediting and may occur over a period of many years.

It is now clear that as tumors develop they can be infiltrated by different subsets of effector, helper and regulatory T cells (Treg) which, together with myeloid derived suppressor cells (MDSC), can shape the microenvironment into one less permissive for effector T cell function. Furthermore, transition through the equilibrium phase not only depends on the extrinsic control exerted by Treg cells and MDSC but also on the intrinsic regulation of T cell function by co-inhibitory and co-stimulatory receptor ligand pairs (Dunn et al. 2002). Understanding the key factors involved in maintaining the balance during the Equilibrium phase and recognizing ways to interfere with them will help us devise new therapeutic strategies capable of tilting this balance towards elimination instead of escape.

Escape

Tumor cells surviving the equilibrium phase get into the escape phase unstrained by immune pressure.

Progression from equilibrium to the escape phase can occur because the tumor cell population changes in response to the immune system's editing functions and/or because the host immune system changes in response to increased cancer-induced immunosuppression or immune system deterioration. Tumor cell escape can occur through many different mechanisms: at the tumor cell level, alterations such as a loss of antigens or increased resistance to the cytotoxic

effects of immunity, for example, through induction of anti-apoptotic mechanisms, promote tumor outgrowth.

Loss of tumor antigen expression is one of the best-studied escape mechanisms, and it can occur in at least three ways:

- through emergence of tumor cells that lack expression of strong rejection antigens,
- through loss of major histocompatibility complex (MHC) class I proteins that present these antigens to tumor-specific T cells,
- through loss of antigen processing function within the tumor cell that is needed to produce the antigenic peptide epitope and load it onto the MHC class molecule.

All of these alterations are probably driven by a combination of genetic instability inherent in all tumor cells and the process of immune-selection (Hung T. Khong and Nicholas P. Restifo, 2009). The final outcome is the generation of less immunogenic transformed cells that aren't recognized by immune system and thus acquire the capacity to grow uncontrollably.

Another escape mechanism consists in the establishment of an immunosuppressive state within the tumor microenvironment (Radoja et al. 2000): tumor cells can contribute to the development of such a state by producing immunosuppressive cytokines and by recruiting regulatory immune cells that are responsible of an immunosuppressive condition (Vesely et al. 2011).

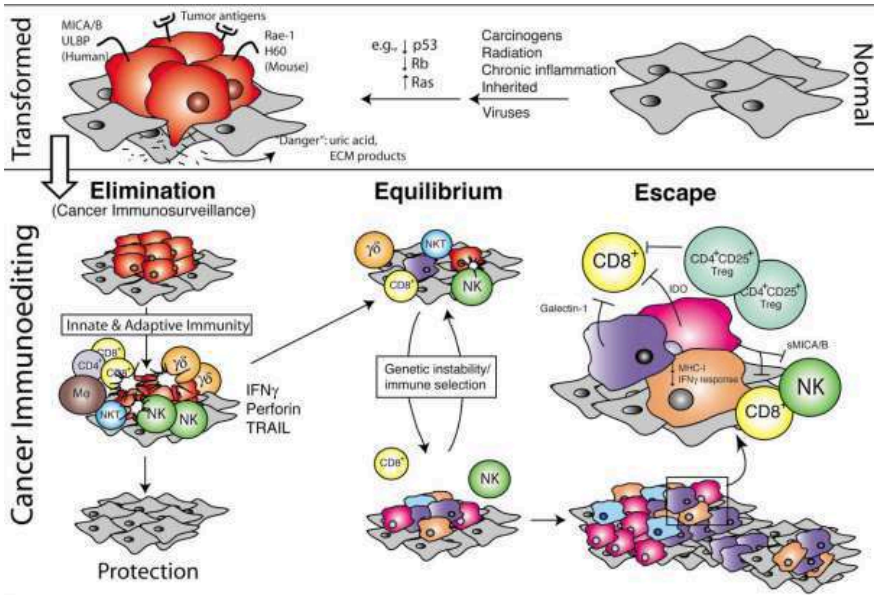


Figure 4: **The Three Phases of the Cancer Immunoediting Process**. In the first phase of elimination, cells and molecules of innate and adaptive immunity are able to eradicate the developing tumor and to protect the host from tumor formation. However, tumor cells get into the equilibrium phase where they are maintained chronically or

immunologically sculpted by immune "editors" to produce new tumor variants. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase (Dunn, Old, and Schreiber 2004)

4.1 Role of Treg cells in cancer immunity

Human tumors are immunogenic and are known to produce a broad variety of immunosuppressive factors such as adenosine (Hoskin et al. 2008), prostaglandine E2 (PGE2) (Wang and Dubois 2011), inhibitory cytokines including TGFb (Chen and Konkel 2010), gangliodide (Sa et al. 2009) and many others (Gabrilovich et al. 1998).

Immune cells, including cytotoxic T lymphocytes (CTL), are expected to recognize and eliminate tumor cells, indeed they are recruited to tumor site by chemokines and cytokines secreted by the various cells in the tumor milieu (Whiteside 2013) but, unfortunately, they are rendered dysfunctional (Kim et al. 2006).

In the tumor microenvironment Treg cells play a critical role in the evolution on tumor progression. They are found to infiltrate head and neck (Schaefer et al. 2005), lung (Wolf et al. 2003), liver (Ormandy et al. 2005), gastrointestinal tract (Sasada et al. 2003), breast and pancreas (Liyanage et al. 2002), ovary (Curiel et al. 2004) cancers and their infiltration is around 20-30% of total CD4+ cells (Zou 2006).

While an abundance of TIL (Tumor Infiltrating Lymphocytes) was considered initially a marker of good prognosis (Clemente et al. 1996), it is now accepted that it is rather the balance between Teff and Treg populations that determines the final outcome (Sato et al. 2005). Indeed, an incrementation of Treg cells despite Teff in tumors is correlated with poor prognosis in various types of cancers such as breast (Bates et al. 2006) and Non Small Cell Lung Cancer (Nguyen et al. 2016). However, there are many controversial debates about the role of Treg infiltration in ColoRectal Cancer, CRC: if on one hand, a low ratio between Teff:Treg is associated with reduced patient survival time (Sinicrope et al. 2009), on the other hand high concentration of Tregs within tumor is correlated with a more favorable clinical outcome (Hu, Li, and Wang 2017). Moreover, more recently, Saito et al., showed that CRC can be classified in two types by the degree of the additional infiltration of $FOXP3^{lo}$ non-suppressive T cells, which don't express Naive T cell marker CD45RA and are characterized by instability of FOXP3 and secrete inflammatory cytokines. Indeed, CRCs with abundant

infiltration of FOXP3^{lo} T cells showed significantly better prognosis than those with predominantly FOXP3^{hi} Treg cell infiltration (Saito et al., 2016).

4.2 Mechanisms through which Treg cells infiltrate within tumors

The processes that contribute to Treg accumulation in tumor can be divided into three modes of accumulation:

- *Increase trafficking*: Treg cells exhibit an enhanced capacity of infiltration and accumulation in comparison to Teff. Several chemokines and their receptors are implicated in this process: chemokine receptor CCR4 and CCR8 are expressed by Treg cells and their ligand CCL22 has been shown to be produced by ovarian (Curiel et al. 2004) and breast carcinoma (Gobert et al. 2009)and Tumor-infiltrating macrophages (Nizar et al. 2009). Blockade of CCL22 reduced Treg cells infiltration into ovarian tumors and induced tumor rejection in a murine xenograft model (Curiel et al. 2004). Another chemokine, CCL28 can be expressed by tumor during hypoxia, and it's reported to recruit preferentially Treg expressing CCR10 (Facciabene et al. 2011);
- *Preferential Treg expansion*: both *in situ* or in TDLNs, it has been reported that IL-2 produced by Teff within tumor stimulates the proliferation of Treg cells and Ki-65, a marker expressed by proliferative cells, has been observed in tumor infiltrating Treg cells (Quezada et al. 2011). Other evidences regarding preferential Treg cell proliferation come from TGF-b produced by DC that stimulate Treg expansion within TDLNs in rodent tumor models (Ghiringhelli et al. 2005);
- *De novo conversion of conventional CD4+ T cells into iTreg cells*: some studies suggest that the immunosuppressive potential of Tregs mostly derives from the conversion of conventional CD4$^+$ T cells into iTregs *in situ*, (Zhou and Levitsky 2007), but other studies suggested that the predominant mechanism of accumulation is due to nTreg (Elkord et al. 2011). Nonetheless, the contribution of iTreg versus nTreg is still controversial because no surface marker has been identified to distinguish nTreg from iTreg, so far. One possible way to gather information about the origin of Treg cells consists in the analysis of the T-cell receptor repertoire. One study based on the chemical carcinogen methylcholanthrene (MCA) has demonstrated that Tregs isolated from tumor

tissues present a TCR repertoire that is well distinct from that of naïve CD4+ T cells. The hypothesis underlying this study was that if Tregs in the tumor truly derived from conventional CD4+ T cells, then the overlap between the TCR repertoire of these two cell subsets would have been higher. They demonstrated that the tumor infiltrating Treg cells originate from nTreg cells because TCR repertoires of Treg and Tconv cells within tumor-infiltrating lymphocytes (TILs) are largely distinct and the conversion of Tconv cells does not contribute significantly to the accumulation of tumor-infiltrating Tregs; rather, Tconv and Treg cells arise from different populations with unique TCR repertoires (Hindley et al. 2011). However, results from another study analyzing the transgenic TCR repertoire in mice bearing B16 melanoma demonstrate that most intratumoral Tregs are generated by the conversion of Teffs (Kuczma et al. 2010). Thus, the relative contribution of these two Treg subsets to tumor immune escape is likely to depend, at least in part, to the features of the tumor microenvironment they are expose to.

5. Immunotherapy: targeting Treg cells in cancer

Immunotherapy represents a viable and powerful approach to cancer treatment that potentiates the anti-tumor immune-response rather than targeting cancer cells. Since Tregs are involved critically in promoting the tumor development by preventing optimal function of effector cells, Treg depletion or their suppressive functions manipulation have been increasingly investigated as therapeutic strategies. A lot of evidences in literature deal with various methods for Treg depletion (Colombo and Piconese 2007). Indeed, Treg cells express surface molecules that can be specifically targeted by antibodies or pharmacologic inhibitors such as CTLA-4, GITR, CCR4, PD-1, OX-40, and LAG3 that potentially could be targeted in order to deplete Treg cells or impair their suppressor functions.

For this purpose, there are several ways to target Treg cells in cancer:

- *Checkpoint blockade antibody with possible Treg-depleting effects*. In the case of T cells, the quality of the response, which is initiated through antigen recognition by the T cell receptor (TCR), depends on a balance between co-stimulatory and inhibitory signals, called immune checkpoints, which are dysregulated by tumors as an important immune

resistance mechanism (Pardoll, 2012); thus, blocking of immune checkpoints may improves the antitumor immune response that could be a primary goal of cancer treatment (Sharma and Allison, 2015; Zitvogel et al., 2013). The two most studied immune-checkpoint receptors in the context of clinical cancer immunotherapy are cytotoxic T-lymphocyte-associated antigen 4 (CTLA4; also known as CD152) and programmed cell death protein 1 (PD1; also known as CD279), which are both inhibitory receptors that are able to regulate immune responses at different levels and by different mechanisms (Fig. 5). CTLA-4 is expressed exclusively on T cells and contrasts the activity of the T cell co-stimulatory receptor CD28 in binding CD80 and CD86 molecules, delivering inhibitory signals to the T cell (Egen and Allison 2002). Even though CTLA4 is expressed by activated CD8+ effector T cells, the major physiological role of CTLA4 has distinct effects on the two major subsets of CD4+ T cells: down-modulation of helper T cell activity and enhancement of regulatory T (Treg) cell immunosuppressive activity (Peggs et al. 2009). Thus, in considering the mechanism of action for CTLA4 blockade, both enhancement of effector CD4+ T cell activity and inhibition of Treg cell-dependent immunosuppression are probably important factors, since Treg cell-specific CTLA4 knockout or down-modulation significantly inhibits their ability to regulate both autoimmunity and antitumor immunity (Kajsa, Onishi, and Prieto-Martin 2008). Anti-CTLA-4 monoclonal antibodies, such as Ipilimumab, (mAb) show remarkable success in metastatic melanoma, and more recently in non-small-cell lung cancer, prostate cancer, renal cell carcinoma, urothelial carcinoma and ovarian cancer. However, patients show no reduction in Treg numbers or in their suppressive capacity, speculating that the effect of anti-CTLA-4 treatment is due to increased activation of Teff (Maker, Attia, and Rosenberg 2006). Recent experimental evidence shows that anti-CTLA-4 mAb efficacy depends on FcγR mediated depletion of $CD4^+$ regulatory T cells within the tumor microenvironment (Peggs et al., 2009; Selby et al., 2013; Simpson et al., 2013; Twyman-Saint Victor et al., 2015).

- Programmed death 1 (PD-1) is another widely studied immune checkpoint receptor expressed by activated T cells, and it mediates immunosuppression. PD-1 functions primarily in peripheral tissues, where T cells may encounter the immunosuppressive PD-

1 ligands PD-L1 and PD-L2 which are expressed by tumor cells, stromal cells, or both (Freeman et al. 2000). Inhibition of the interaction between PD-1 and PD-L1 can enhance T-cell responses *in vitro* and mediate preclinical antitumor activity (Clinic 2002), but it remains to be elucidated whether anti-PD-1 antibody (Nivolumab) causes selective depletion of Treg cells in tumor tissues (Tanaka and Sakaguchi 2017).

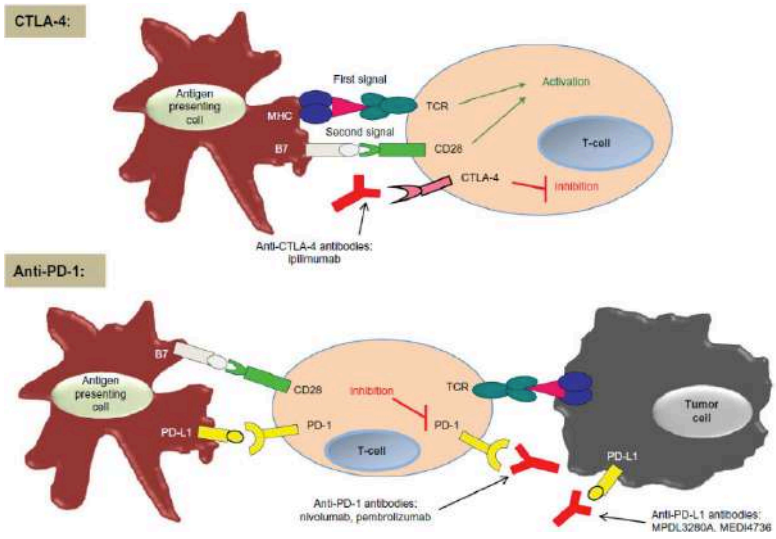


Figure 5: Simplified concept of CTLA-4 and PD-1 immune checkpoints. In the first phase, antigen-presenting cells present antigens to the T-cell. Two signals are essentials to initiate a T-cell response. CTLA-4 is upregulated after T-cell activation and inhibits the T-cell response. Anti-CTLA-4 antibodies bind to CTLA-4, turning off the 'inhibitory signal', thus resulting in an enhancement of T-cell function. In the effector phase, the PD-1 inhibitory receptor is expressed by the T-cell and, when it is engaged by its ligands PD-L1 and PD-L2, it is able to inhibit the T-cell response. Anti-PD-1 antibodies bind to PD-1, turning off the 'inhibitory signal' in the peripheral tissues and enhancing T-cell function (Dunn et al. 2004).

Cancer immunotherapies focusing on CTLA-4 and PD-1 blockade are frequently related to serious autoimmunity (Hodi et al. 2010). In general, the effectiveness of anti-tumor responses tends to correlate with the development of autoimmune diseases especially when a systemic Treg cell depletion approach occurs (Callewaert et al. 2012). However, these cell surface molecules are peculiar of an activated phenotype of effector Tcells, thus constituting an obstacle for specific depletion of Tregs. Therefore, the molecular characterization of Treg cells at different tumor sites should help to better define therapeutic targets through a better knowledge of their signature molecules and of the network that regulates Treg cell functions in the tumor microenvironment.

- *Depletion of effector Treg cells in tumor tissues*. In various cancers effector Treg cells are the most abundant cell type among FOXP3^{+}T cells. In order to distinguish and selectively deplete tumor-infiltrating Treg cells while preserving other Treg cells important for suppressing autoimmunity, one strategy is to specifically target effector Treg cells, which are highly activated and proliferative (Zou 2006). Since effector Treg cells are a substantial population in tumor tissues, depleting effector Treg cells would shift the balance in tumor microenvironment from immune suppression to immune activation against tumor cells. For this purpose, surface molecules expressed specifically or selectively on effector Treg cells are good targets. For example, CCR4 is predominantly expressed by effector Treg cells, not by naive Treg cells and Th2 cells which do not contribute significantly to tumor immunity regulation in humans (Sugiyama et al. 2013). As discussed above, one of the mechanism through which Treg cells infiltrate within the tumor consists of the expression of CCR4 ligands, CCL17 and CCL22, produced by tumor cells or infiltrating macrophages (Faget et al. 2011). *In vitro* depletion of CCR4+ T cells significantly reduced tumor-infiltrating Treg cells indicating that anti-CCR4 mAb treatment is able to selectively deplete effector Treg cells infiltrating into tumors (Sugiyama et al. 2013) (Fig. 6).

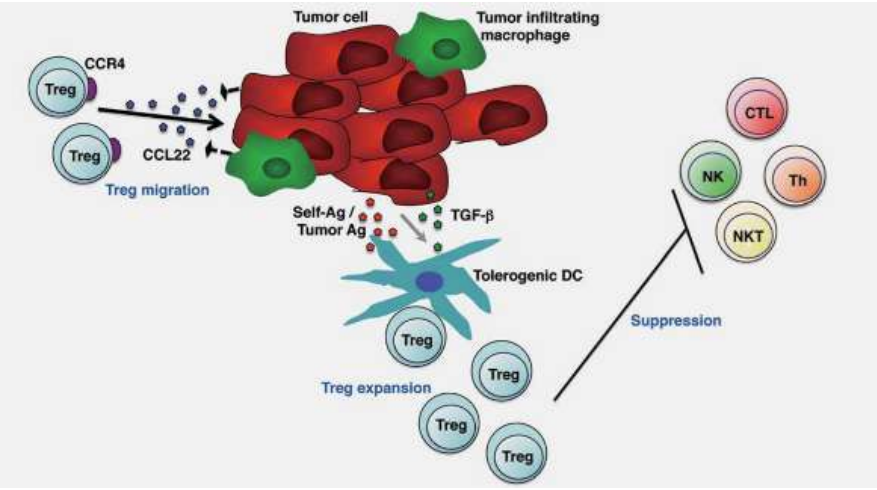


Figure 6: Treg cells suppress antitumor immune responses. CCL22 produced by tumors and tumor infiltrating macrophages recruit CCR4- expressing Treg cells (Treg migration). Treg cells accumulated via CCR4-CCL22 recognize tumor self-antigens (self-Ag) and proliferate (Treg expansion). Tolerogenic dendritic cells (DCs) induced by TGF-b derived from tumor cells further enhances Treg expansion. These Treg cells suppress antitumor effector cells (Nishikawa and Sakaguchi 2010) .

- *Agonistic antibody affecting Treg suppression*. GITR is another molecule that is expressed preferentially by Treg cells compared to Tconv cells. In tumor- bearing mice, it has been shown that agonistic anti-GITR antibody can affect suppressive function mediated by Treg cells and evoke potent anti-tumor immune response with an increase in the production of IFN-γ by CD8+ and CD4+ T cells, which are able to kill established tumors (Ko et al. 2005). The agonistic antibody for GITR is under clinical trials to test its efficacy in melanoma and other advanced solid tumor patients (Tanaka and Sakaguchi 2017). Agonistic antibodies specific for other TNFR super family molecules, such as OX40, are under clinical investigation too (Nishikawa and Sakaguchi 2014).
- *Small molecules for Treg depletion or functional modulation*. One example of small molecule targeting Treg cells is cyclophosphamide, an anti-neoplastic used frequently in traditional chemotherapy. It is an alkylating agent that interferes with DNA replication and it kills highly proliferating cells, and thus all T cells. However, when used at low doses over a long term, the drug has been shown to selectively reduce proliferating Treg cells including those in the tumor tissues, and enhance anti-tumor immune responses in humans and rodents (Motoyoshi et al. 2006; Ge et al. 2012).

  Another potential strategy to augment tumor immunity via depleting Treg cells is to target TCR signaling molecules, which are differentially controlled in Treg and Tconv cells. ZAP-70 (tyrosine kinase ζ-associated protein of 70 kDa), is a cytoplasmic protein tyrosine kinase, mainly expressed in T cells, that plays a critical role in the events involved in initiating T-cell responses by the antigen receptor. It is specifically repressed in Treg cells upon TCR activation (Ohkura et al. 2012). Targeting ZAP-70 could be responsible of selective reduction of TCR signaling, resulting in selective death of Treg cells, in particular effector Treg cells, due to signal deprivation-induced apoptosis (Tanaka and Sakaguchi 2017). Although these findings need to be confirmed in humans, the possibility to use TCR signaling molecules as therapeutic targets for selective depleting tumor infiltrating Treg cells is becoming a promising issue.

  To date, almost all studies about tumor associated Tregs have examined these cells as an homogenous population, with little, if any, characterization of Tregs heterogeneity. Thus, only an in-depth understanding of the functional features of tumor infiltrating Treg cell populations may lead to a comprehension of their role in tumor control and allow the identification of novel potential therapeutic targets for the effective modulation of these cells in cancer patients.

6. Treg cell plasticity: the role of long non coding RNAs

Treg cell identity is truly important for immune homeostasis (Ohkura, Kitagawa, and Sakaguchi 2013) and understanding the precise nature of Treg cell stability is crucial for therapeutic applications that use or modulate Treg cells to treat autoimmune diseases, allergies, graft rejection and tumors (Sakaguchi et al. 2013). Whether Treg cells are phenotypically and functionally stable is a controversial issue. To resolve this apparent controversy about the plasticity of Treg cells, a few models have been proposed. The first concerns the "heterogeneity model" proposed by Hori et al., in which Treg cells consist of heterogeneous populations with different degrees of commitment, including fully committed Treg cells that are stable and less committed Treg cells, unstable (Hori 2014). Another model is the "transient flexibility model" proposed by Piccirillo and coworkers, in which Treg cells are flexible in terms of their phenotype depending on the environment in which they are in (Bin Dhuban et al. 2014).

In this context, lncRNAs have a fundamental role in governing flexibility and plasticity or cell identity maintenance, together with lineage specific transcription factors. In particular, what is emerging from literature is that ncRNAs typically act as fine-tuners of fate choices and this seems to be true not only in the immune system. Nonetheless, in the case of CD4+ T cell subsets that are specified but not fully determined, subtle changes in extrinsic signals can reverberate through responsive ncRNAs inducing changes that alter cell phenotype (Pagani et al. 2013).

Usually the stability of lineage identity is achieved through the implementation and inheritance of epigenetic modification and lncRNAs can act directly on histone and DNA modifiers redefining this context. This condition allows them to be more flexible and sensitive to variations without disrupting the whole network integrity (Zhang et al. 2012). Several single-case or genome-wide studies on lncRNAs in the murine immune system are now available in literature, whereas only few studies have been conducted until now in the human context and their cumulative impact on immune functions remains largely unknown (Satpathy and Chang 2015).

6.1 Long non coding RNAs classification

The non coding RNAs are classified based on their size into small and long non coding (table1). Some of these ncRNAs have general housekeeping functions and include ribosome-associated RNA (rRNA), transfer RNA (tRNA) and small/nuclear RNA (sn/snoRNA). Different classes of short RNAs (miRNA, siRNA, piRNA) exhibit regulatory functions in various cellular processes, including cell identity (Ciaudo et al. 2009), cancer progression (Garg 2012) and immunity (Ha 2011). Long noncoding RNAs (lncRNAs) represent a large portion of noncoding regions across the genome (Derrien et al. 2012) and constitute a set of functional transcripts over 200 nucleotides on length with no potential to encode for functional proteins of more than 30 aminoacids (Li X et al., 2013).

ncRNA*	No. of known transcripts[†]	Transcript lengths (nucleotides; nt)[‡]	Functions
Precursors to short RNAs			
miRNA	1,756	>1,000	Precursors to short (21–23 nt) regulatory RNAs
snoRNA	1,521	>100	Precursors to short (60–300 nt) RNAs that help to chemically modify other RNAs
snRNA	1,944	1,000	Precursors to short (150 nt) RNAs that assist in RNA splicing
piRNA	89	Unknown	Precursors to short (25–33 nt) RNAs that repress retrotransposition of repeat elements
tRNA	497	>100	Precursors to short (73–93 nt) transfer RNAs
Long ncRNAs			
Antisense ncRNA	5,446	100–>1,000	Mostly unknown, but some are involved in gene regulation through RNA interference
Enhancer ncRNA (eRNA)[§]	>2,000	>1,000	Unknown
Enhancer ncRNA (meRNA)[‖]	Not fully documented	As variable as the length of mRNAs	Unknown, but they resemble alternative gene transcripts
Intergenic ncRNA	6,742	10^2–10^5	Mostly unknown, but some are involved in gene regulation
Pseudogene ncRNA	680	10^2–10^4	Mostly unknown, but some are involved in regulation of miRNA
3′ UTR ncRNA	12	>100	Unknown

*miRNA, microRNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; piRNA, piwi-interacting RNA; tRNA, transfer RNA; antisense ncRNA, transcripts mapping and overlapping coding and non-coding RNAs; enhancer ncRNA (eRNAs and meRNAs), transcripts that initiate within regions that regulate specific genes; intergenic ncRNA, transcripts that map to genome regions between annotated genes; pseudogene ncRNA, transcripts that come from processed or unprocessed pseudogenes; 3′ UTR ncRNA, 3′-untranslated regions of ncRNA.

Table 1 - Main classes and functions of mammalian ncRNAs.

LncRNAs are commonly classified in association with annotated protein-coding mRNA and comprise the long intergenic ncRNAs (lincRNA), intronic lncRNA, sense or antisense lncRNA, competitive endogenous RNAs (ceRNA) and enhancer RNA (eRNA). The more abundant category of ncRNAa are sense ncRNAa that overlap coding mRNA on the same strand and share some sequence to the latter, yet do not encode proteins (Kapranov et al. 2005). This class includes unspliced sense intronic RNAs (PINs) and spliced transcripts that combine exons from coding and non coding region of a gene (Djebali et al. 2012). The intronic lncRNAs emerge from the intron of a protein coding gene and these transcripts compose the majority (around 70%) of all non coding nuclear-encoded RNA. Some of these transcripts include circular ncRNAs (ciRNA) that are involved in the regulation of the expression of their

parent genes (Zhang et al. 2013). Another class of lncRNAs comprises antisense transcripts that overlap the opposite DNA strand of the associated protein coding genes (Medical 2005). Intergenic long ncRNAs are the most studied lncRNAs because they are independent transcriptional units and are more likely associated with an intrinsic function that being transcriptional noise (Fig. 7).

LncRNAs are also classified according to their localization in the cell compartments: nuclear lncRNAs and cytoplasm lncRNAs. Nuclear lncRNAs have been found to be mainly implicated in the recruitment of chromatin modifiers in specific genomic loci (John L. Rinn 2013) and they can work as transcriptional activators through the recruitment of chromatin-modifying complexes and determine changes in three-dimensional chromatin conformation mediated by the activation of specific enhancer regions (Wang et al. 2011). It's possible to distinguish nuclear lncRNAs by their course of action: cis and trans-acting RNAs. The first one is involved in the control of the expression of genes located in the proximity of their transcription sites and sometimes can spread their action to long distance on the same chromosome (Fatica and Bozzoni 2014); the latters can both activate or repress the expression of genes located in independent loci (Guttman et al., 2009).

Cytoplasmatic lncRNAs mediated gene regulation mechanism: they can modulate the control of translation by base pairing recognition of the target and mRNA stability (Faghihi et al. 2010).

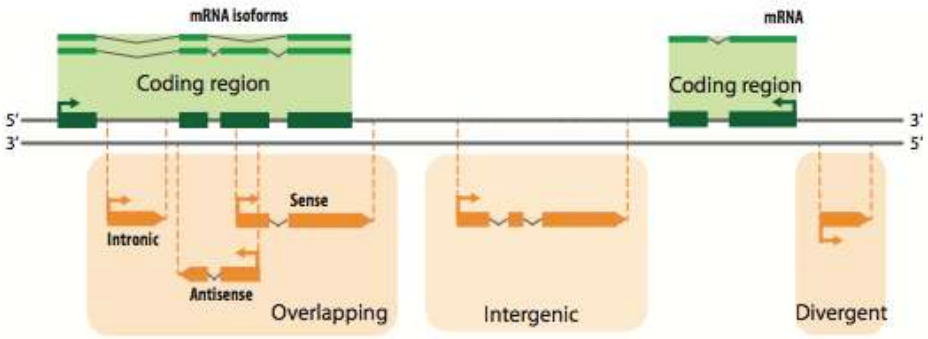


Figure 7 LncRNAs (orange) classification respect to neighbouring coding regions (green).

6.2 Involvement of lncRNAs in immune response

The development and activation of immune cells depend on the strictly integrated and dynamic gene expression mechanisms that are regulated by a series of transcriptional and post-transcriptional events. If the roles of coding genes, in the regulation of gene expression in the immune system have been elucidated, the regulatory roles of non-coding RNAs are still understood. So far, a large number of studies demonstrated the connection between lncRNAs and immune regulation such as immune responses and infectious diseases. The first evidence suggesting the potential role of lncRNAs in regulation of immune system comes from Guttman and colleagues reporting that CD11C+ bone-marrow-derived dendritic cells increase the expression of about 20 lincRNAs after being challenged by lipopolysaccharide (LPS), a specific agonist of the Toll-like receptor 4 (Guttman et al. 2009).

Some lncRNAs are dynamically expressed during T cell development, differentiation and activation in the adaptive immune responses. In mammalian CD8+ T cells, 1000 lncRNAs were identified and characterized, many of which displayed stage- or tissue-specific expression, neighbored protein-coding genes with well-characterized roles in $CD8^+$ T cells, and/or overlapped shorter functional RNAs (Pang et al. 2009).

Our group has generated a comprehensive atlas of transcriptome data from highly purified human lymphocyte subsets. This includes RNA-seq data for 13 human primary T and B lymphocyte subpopulations from peripheral blood of healthy donors, including CD4+naïve, Treg, T helper 1 (Th1), Th2, Th17, CD8+ naïve, central memory (TCM), effector memory (TEM), naïve B, memory B, and CD5+ B cells (Ranzani et al. 2015). Using *de novo* transcription reconstruction strategies, we have further discovered novel and previously unannotated noncoding transcripts, including intergenic non-coding RNAs (lincRNAs). Remarkably, analysis of lymphocyte cell-subset specificity revealed that lincRNAs display a more cell-specific expression pattern than coding genes (Fig. 8).

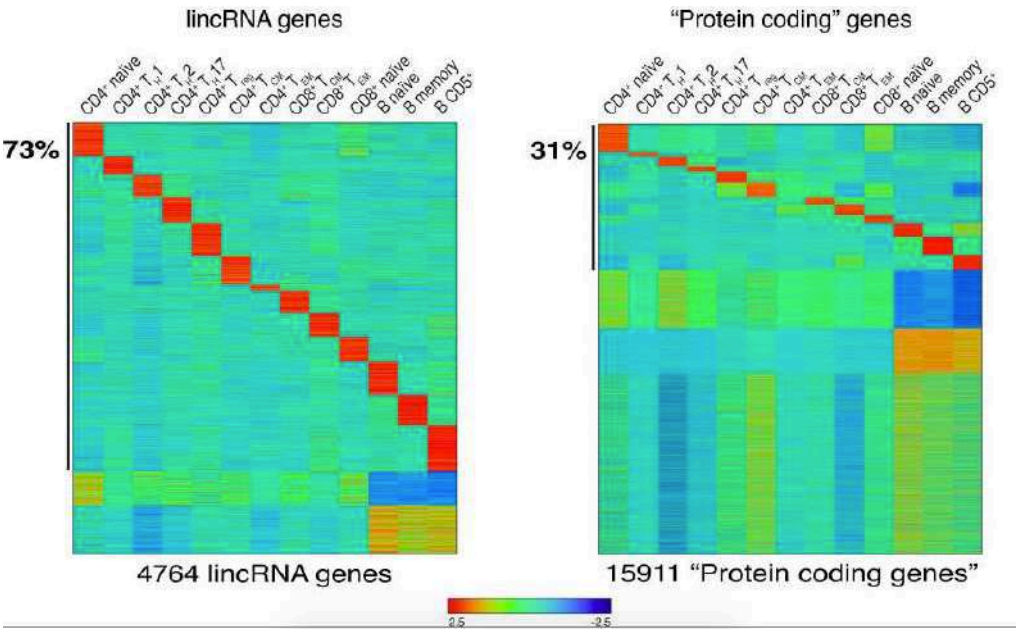


Figure 8: Unsupervised K-means clustering and specificity of lincRNAs (left) and coding genes (right) across 13 human lymphocyte subsets. Color intensity (key) indicates z-score log2-normalized raw FPKM counts.

One of the major advances of our studies was to identify previously un-annotated lincRNAs and to determine their lymphocyte cell-subset specificity. Interestingly, what emerged is the discovery of lincRNAs located within the ICOS- CTLA4-CD28 genomic region. lincRNAs were shown to be expressed very poorly not only in non-lymphoid but also in lymphoid tissues, suggesting that they are likely to exert a highly specific role in lymphocyte function.

These findings and the high cell specificity of lincRNAs suggest to consider lincRNAs as novel and highly specific molecular targets for the development of new therapies for diseases, such as autoimmunity, allergy and cancer in which altered $CD4^+$ T cell functions play a pathogenic role.

AIM OF THE PROJECT

One of the most peculiar features of CD4+ Treg cells consists on the phenotypic plasticity that they display adapting to different environment. Treg cells can accumulate in tumor tissues where they are generally associated with worsened prognosis. The molecular mechanisms, though, underlying Treg cell functional and phenotypic variation as well as their clinical significance at tumor sites are still largely unknown. For this reason, only a deep investigation of the molecular networks defining tumor infiltrating Treg cell populations may lead to a better comprehension of their role in tumor immune escape.

To achieve this goal we performed whole transcriptome analysis from Treg cells isolated from both healthy donors and from colorectal and non-small cell lung cancer patients, to identify the unique molecular features of Treg cells assessing the expression of both coding and non-coding transcripts that best define the identity of these cells and might therefore represent novel prognostic markers or therapeutic targets for functional modulation of these cells at tumor sites.

MATERIALS AND METHODS

Purification and colture of human lymphocytes subsets

Buffy-coated blood of healthy donors was obtained from the I.R.C.C.S. Istituto di Ricovero e Cura a Carattere Scientifico Policlinico Cà Granda in Milan and peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. All healthy donors were subjects with no history of autoimmune diseases ad malignant tumors. Lymphocyte subsets were purified >95% by sorting on a FACSAria (BD) by various combinations of surface markers: OP-CD4 (Miltenyi); FITC-CD25 (Miltenyi); PE-CD127 (Miltenyi); APC-CD45RO (Miltenyi); PECy5-CD62L (Miltenyi).

Isolated CD4+CD25 ^{high}CD127^{low} Treg cells and CD4+CD25 ^{low}CD127^{high} naïve Tcells were expanded *in vitro* according to Rapid Expansion Protocol in presence of RPMI 1600 medium, 10% fetal bovine serum (FBS), 100 unit/ml penicillin, IL-2 20 U/ml (Miltenyi), anti-human CD3 30 ng/ml (OKT3 clone, Biolegend), irradiated PMBC Feeders and Ebstein-Barr Virus immortalized B cell line (Rosi-EBV) (Dudley et al. 2003).

ShRNA oligonucleotides designing and cloning

Four shRNAs for each transcript were designed by using the GPP Web Portal (https://portals.broadinstitute.org/gpp/public/seq/search), an algorithm that ranks potential 21mer targets within each human and mouse Refseq transcript. Those "candidates" targeting transcripts at different position with the higher *intrinsic score* (a number from 0 to 15 predicting the knockdown successfulness) were selected (Table 1).

ShRNAs as well as shRNA-non targeting control, were cloned into pLKO.1 Vector.

Oligos containing a sense and an antisense sequence targeting mRNA, were annealed in presence of NEB Buffer2 New England Biolabs Restriction Endonuclease Reaction *Buffer (*50mMNaCl, 10mMTris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9@25°C). Once annealed, the dsDNA molecule obtained had at the 5' a sticky end compatible with an AgeI digested site, while at the 3' the end was suitable for ligation with an EcoRI-digested site. The sense and antisense sequences are connected by a spacer capable of forming a loop. The digested

fragments were purificated with Wizard® SV Gel and PCR Clean-Up System and ligated into pLKO.1 cloning vector by using T4 DNA ligase (Promega) and the ligation products was then transformed into competent STBL3 bacteria, following manufacturer's protocol and then plated on LB agar containing 100 ug/ml ampicillin.

Plasmids successfully ligated by restriction enzime digestion, were selected by sequencing and DNA plasmid was isolated using PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen).

ncRNA_up_1	SH A	5'- GGTCACATAATCCACTTATTT-3
	SH B	5'-CAGTCCTAATTGAAGTTATAA-3'
	SH C	5'-GACACGATCACAAAGTAATAA-3
	SH D	5'-CAGATAGCTCAGGTAAATATT-3'
	SH E	5'-TAGTGCGGTTATTCGAATTAT-3'

ncRNA_up_2	SH A	5'-CAGTCCTAATTGAAGTTATAA-3'
	SH B	5'-GTGGAACGTGGATTCAGATTT-3'
	SH C	5'-GAGCCTTAGCCCTAGTCATTA-3'
	SH D	5'-ATAGTCAGCCCATGGAGGATG-3'
	SH E	5'-GATGTGTCCACCAGTTCATTC-3'

Table1: shRNA design.

Lentiviral production and Titration

Twenty four hours prior to transfection, HEK293T were plated at 60% confluence in presence of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100unit/ml glutamine and incubated at 37°C with 5% CO2.

During transfection, 25 ug of shRNA-lentivirus-GFP were transfected with 16ug of viral packaging pS-PAX2 and 8.4ug viral envelope pMD2.G into HEK293T according to Clacium Phospate method. Twelve to sixteen hours after transfection, the medium was aspirated and replaced with 16 ml of pre-warmed fresh medium to the plate. Cell culture supernatant was collected 42 hours after transfection and ultra-centrifuged at 20000 rpm at 4°C for 2h (Beckman Coulter).

The viral particles were resuspended in RPMI medium and stored at -80°C. Concentrated viral particles were titrated at different dilutions and transduced into HEK 293T cells. The percentage of GFP positive cells was assessed two days after transduction by using FACS

CANTO FlowCytometer (BD).

Lentiviral transduction

Seven days post rapid expansion protocol, 300000 expanded *in vitro* Treg cells were transduced with either control lentiviral vector (LVV MOCK GFP) or ShRNA- lentiviral vectors (LVV shRNA GFP) at a multiplicity of infection of $1x10^8$ transducing units per ml in the presence of IL-2 20 U/ml (Miltenyi). At 7^{th} day post transduction, GFP positive cells were isolated by using a FACS Aria Flow Cytometer (BD), then they were used for RNA extraction as well as for suppression assay.

RNA isolation and RT-qPCR Analysis

Total RNA was isolated using mirVana Isolation Kit (Invitrogen) according to suggested protocol. Briefly, the lysates were extracted with Acid-Phenol Chloroform and further purified to yield total RNA. Extracted RNA was quantified with QuantiFluor RNA System (Promega) and then quality controlled for integrity with 2100 Bioanalyzer (Agilent Technologies). For reverse transcription, equal amounts of DNA-free RNA (70 ng) were reverse-transcribed with SuperScript III (LifeTechnologies) following the standard protocol. Diluted cDNA (3,5 ng) was then used for RT-qPCR to assess the following transcripts: ncRNA_up_1 (AR2W7P7), ncRNA_up_2 (AR47W2), 18s (Hs0392985_g1), B2M (Hs00174383_g1), and CTLA4 (Hs00175480_m1) gene expression levels with Inventoried TaqMan.

qPCR BioMark™

The time course analysis was performed exploiting the BioMark™ technology and using the 96x96 quantitative PCR (qPCR) DynamicArray microfluidic chips (Fluidigm). A 2.25 µl aliquot of amplified cDNA was mixed with 2.5 µl of TaqMan Fast Advanced Master Mix (Thermo Fisher) and 0.25µl of Fluidigm's "sample loading agent," then inserted into one of the chip "sample" inlets. A 2.5 µ l aliquot of each 20X TaqMan assay was mixed with 2.5 µl of Fluidigm's "assay loading agent" and individually inserted into one of the chip "assay" inlets. Samples and probes were loaded into 96 x96 chips using an IFC Controller HX (Fluidigm), then transferred to a BioMark real-time PCR reader (Fluidigm) following manufacturer's instructions.

Suppression assay

CFSE-labeled responders $CD4^+Naive^+$T cells from healthy donors were induced to proliferate in the presence of anti-CD3/CD28 dynabeads (Gibco) (1 bead : 20 cells) and co-cultured at different ratios (Naïve: Treg 1:1, 1: 0,5, 1:0,25) with unlabeled GFP+ transduced Treg cells from peripheral blood of healthy donors, using FACS Aria II (BD Biosciences).
Proliferation of CFSE-labeled cells was assessed after 96 hours, using FACS Canto flow cytometer (BD).

Fractionation

Cell fractionation was performed following the protocol published by Gagnon et al., (Gagnon et al. 2014).
For RNA analysis, $2*10^6$ expanded *in vitro* Treg cells were harvested at 500 *g* for 5 minutes at 4°C, resuspended in ice-cold HLB supplemented with 100U of SUPERase-In and leaved on ice for 10 minutes. The samples were centrifuged at 1000 *g* for 3 minutes at 4°C and then, the supernatant, the cytoplasmatic fraction, was resuspended in TRIzol Reagent® (Life Technologies) and stored at -20°C. The pellet representing the semipure nuclei was washed 3 times with ice-cold HLB and centrifuged at 200 g for 2 minutes at 4°C. The pellet was then further fractionated into nucleoplasmic and chromatin-associated RNA fractions by adding MWS buffer (10mM TrisHCl pH 7, 4 mM EDTA, 0.3 M NaCl, 1 M urea, 1% NP-40) supplemented with 100 units of SUPERase-in and leaved on ice for 5 minutes. Samples were centrifuged at 1000 g centrifugation for 3 minutes at 4°C, then the resulting supernatant rapresenting nucleoplasmic fraction was resuspended in TRIzol Reagent®. The resulting pellet constitutes the chromatin fraction and was washed 3 times in ice cold MWS, centrifuged at 500 g for 2 minutes at 4 °C and finally resuspended TRIzol Reagent®.

Chip

At 14^{th} day of Rapid Expansion Protocol, 10^7 *in vitro* expanded Treg cells from healthy donors were crosslinked in their medium with 1/10 of fresh formaldehyde solution (50 mM Hepes-KOH pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 11%formaldehyde) for 12 minutes. Then they were treated with 1/10 of 1.25 M glycine for 5 minutes and centrifuged at

1350 g for 5 minutes at 4°C. Cell membranes were lysated in LB1 (50 mM Hepes-KOH pH7.5, 10 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% Triton X-100 supplemented with Protease Inhibitor Cocktail Tablets cOmplete, EDTA-free (Roche) and Phenylmethanesulfonyl fluoride (Sigma) at 4°C.

Nuclei were pelletted at 1350 g for 5 minutes at 4°C and washed in LB2 (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1mM EDTA, 0.5 mM EGTA) supplemented with protease inhibitors.

Nuclei were again pelleted at 1350 g for 5 minutes at 4°C and resuspended with a syringe in 200ul LB3 (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% Nlauroylscarcosine) supplemented with protease inhibitors.

Chromatin was fragmented by ultrasound and cell debris were pelleted at 20000 g for 10 minutes at 4°C. Chromatin Immunoprecipitation was set up in LB3 supplemented with 1% Triton X-100, protease inhibitors and antibodies against H3K3Me1(Abcam), H3K4me3 (Abcam), H3K27me3 (Millipore), or no antibody (as negative control) o/n at 4°C. The day after Dynabeads® Protein G (Novex®) were added at left at 4°C rocking for 2 hours. Then the beads were washed twice with Low salt wash buffer (0.1% SDS, 2 mM EDTA, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 150 mM NaCl) and with High salt wash buffer (0.1% SDS, 2 mM EDTA, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 500 mM NaCl). Histones IPs were also washed with a LiCl solution (250 mM LiCl, 1% NP-40, 1 mM EDTA, 10 mM Tris-HCl pH 8.0). All samples were finally washed with 50 mM NaCl in 1X TE. Elution was performed o/n at 65°C in 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS. Samples were treated with 0.02 ug/ul RNase A (Sigma) for 2 hours at 37 °C and with 0.04 ug/ul proteinase K (Sigma) for 2 hours at 55°C. DNA was purified with phenol/chloroform extraction and quantified with QuantiFluor dsDNA System (Promega). qPCR was performed on diluted cDNA with PowerSyberGreen (LifeTechnologies) and specificity of the amplified products was monitored by performing melting curves at the end of each amplification reaction. ChIp quality was assessed by using primers designed on control genes (Table 2).

TIANII_IL2RA	FW	5'-AATGTGGAGGTAGGGAAAC-3'
TIANII_IL2RA	REV	5'-GTGTGCCAAGCAAATGAGT-3'
CTLA-4	FW	5'-GGCTTGGCTGGCTCTGTATTCC-3'
CTLA-4	REV	5'-CCCCTACTAAATACCTGGCGCTCT-3'
XOD11	FW	5'-TGACGATGTGACCAAGCAATGC-3'
XOD11	REV	5'-GGGGTGGGAGTTTTCTTCTTAGGT-3'
ACTB	FW	5'-AGACACCCACCTTGATCTTC-3'
ACTB	REV	5'-AAAGACCTGTACGCCAACAC-3'

Table 2. Primers designed on control genes.

ChIP-seq analysis was perfomed by bioinformatic team in our institute.

RESULTS

Molecular characterization of Treg cells was pursued by performing whole transcriptome analysis of tumor infiltrating Treg cells (results presented in Part I) and of cells isolated from the peripheral blood of healthy donors (results presented in Part II). Through the comparison of Treg cell transcriptome data with datasets of thirteen distinct human lymphocyte subsets we defined the profile of Treg specific coding and non-coding transcripts and then pursue the functional characterization of non-coding RNAs identified in proximity of CTLA4 gene whose prominent role in Treg cell biology is well established.

PART I

1. Tumor infiltrating Tregs cells upregulate immune checkpoints and are highly suppressive

Treg cells share with effector lymphocytes most of the molecules targeted for therapy, which can possible deplete also the tumor specific effector cells. Therefore, the molecular characterization of Treg cells at different tumor sites and the comparison with effector subsets should help to better define their signature molecules and of the network that regulates Treg cell functions in the tumor microenvironment.

To assess the gene expression landscape of tumor infiltrating $CD4^+$ T cells, we isolated different $CD4^+$ lymphocytes subsets from two different tumors, NSCLC and CRC, from the adjacent normal tissues, and from peripheral blood samples. From all these tissues, we purified by flow cytometry (Fig. 1) $CD4^+$ Treg (36 samples from 18 individuals), Th1 (30 samples from 21 individuals) and Th17 (22 samples from 14 individuals) cells (Table 1).

Tissue	Subset	Sorting Phenotype	Number of Samples	Mapped Reads (M)
NSCLC	CD4+ Treg	$CD4^+ CD127^- CD25^+$	8	587
	$CD4^+$ Th1	$CD4^+ CXCR3^+ CCR6^-$	8	409
	$CD4^+$ Th17	$CD4^+ CCR6^+ CXCR3^-$	6	206
CRC	$CD4^+$ Treg	$CD4^+ CD127^- CD25^+$	7	488
	$CD4^+$ Th1	$CD4^+ CXCR3^+ CCR6^-$	5	266
	$CD4^+$ Th17	$CD4^+ CCR6^+ CXCR3^-$	5	308
Lung (normal tissue)	$CD4^+$ Treg	$CD4^+ CD127^- CD25^+$	1 (pool of 6)	73
	$CD4^+$ Th1	$CD4^+ CXCR3^+ CCR6^-$	1 (pool of 6)	76
Colon (normal tissue)	$CD4^+$ Treg	$CD4^+ CD127^- CD25^+$	7	404
	$CD4^+$ Th1	$CD4^+ CXCR3^+ CCR6^-$	6	352
	$CD4^+$ Th17	$CD4^+ CCR6^+ CXCR3^-$	6	284
PB (healthy donor)	$CD4^+$ Treg	$CD4^+ CD127^- CD25^+$	8	259
	$CD4^+$ Th1	$CD4^+ CXCR3^+ CCR6^-$	5	70
	$CD4^+$ Th17	$CD4^+ CCR6^+ CXCR3^-$	5	77

Table 1: **Purification and RNA-Sequencing of Human Primary Lymphocyte Subsets.** For each cell subsets profiled by RNA-sequencing tissue of origin, surface marker combinations used for sorting, number of profiled samples, as well as number of mapped sequencing reads are indicated. M, million; CRC, colorectal cancer; NSCLC, non-small cell lung cancer; PB, peripheral blood.

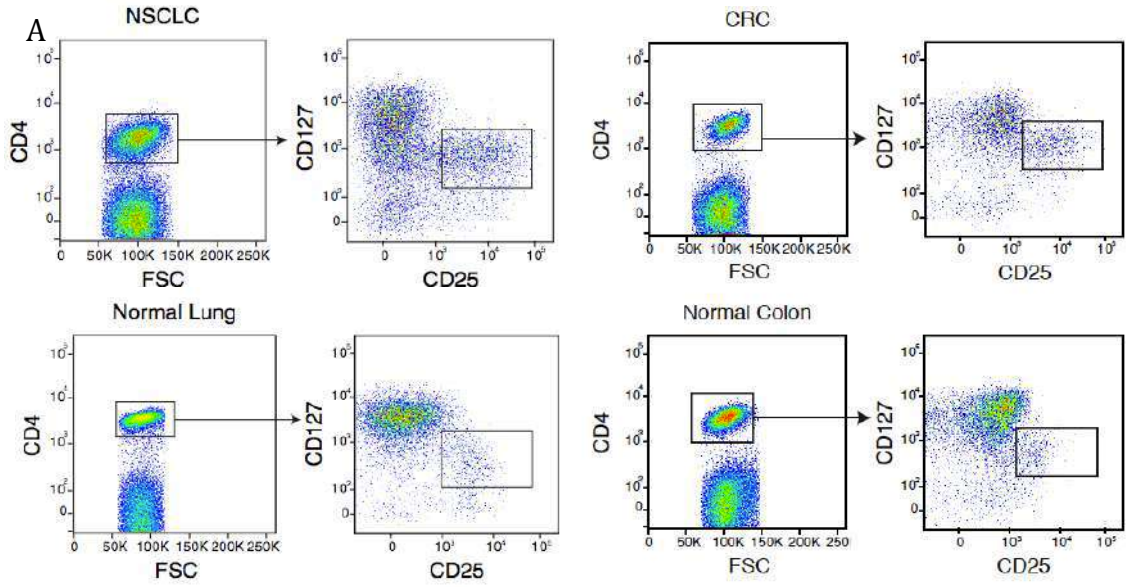


Figure 1: **Purification and isolation of tumor infiltrating lymphocytes**. Representation of sorting strategy of Treg cells infiltrating Non Small Cell Lung Cancer (NSCLC), Colorectal Cancer (CRC) and their respective normal tissues.

To assess Treg cell function we tested their suppressor activity and showed that Treg cells infiltrating either type of tumor tissues have a remarkably stronger suppressive activity *in vitro* compared to Treg cells isolated from the adjacent normal tissue and peripheral blood of the same patients (Fig. 2).

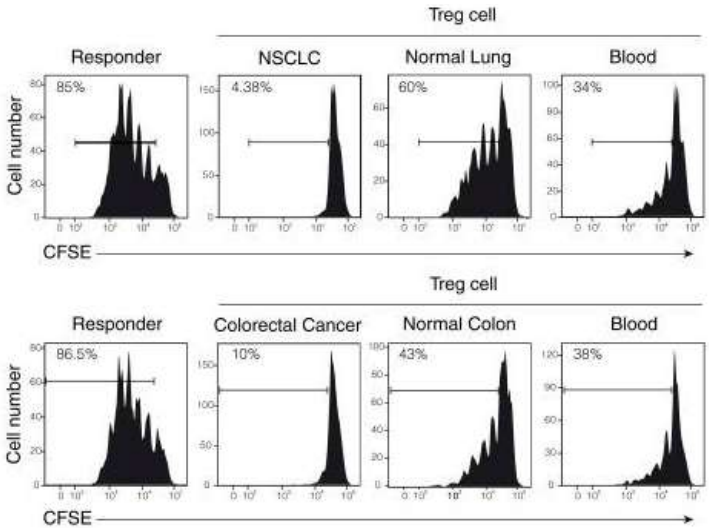
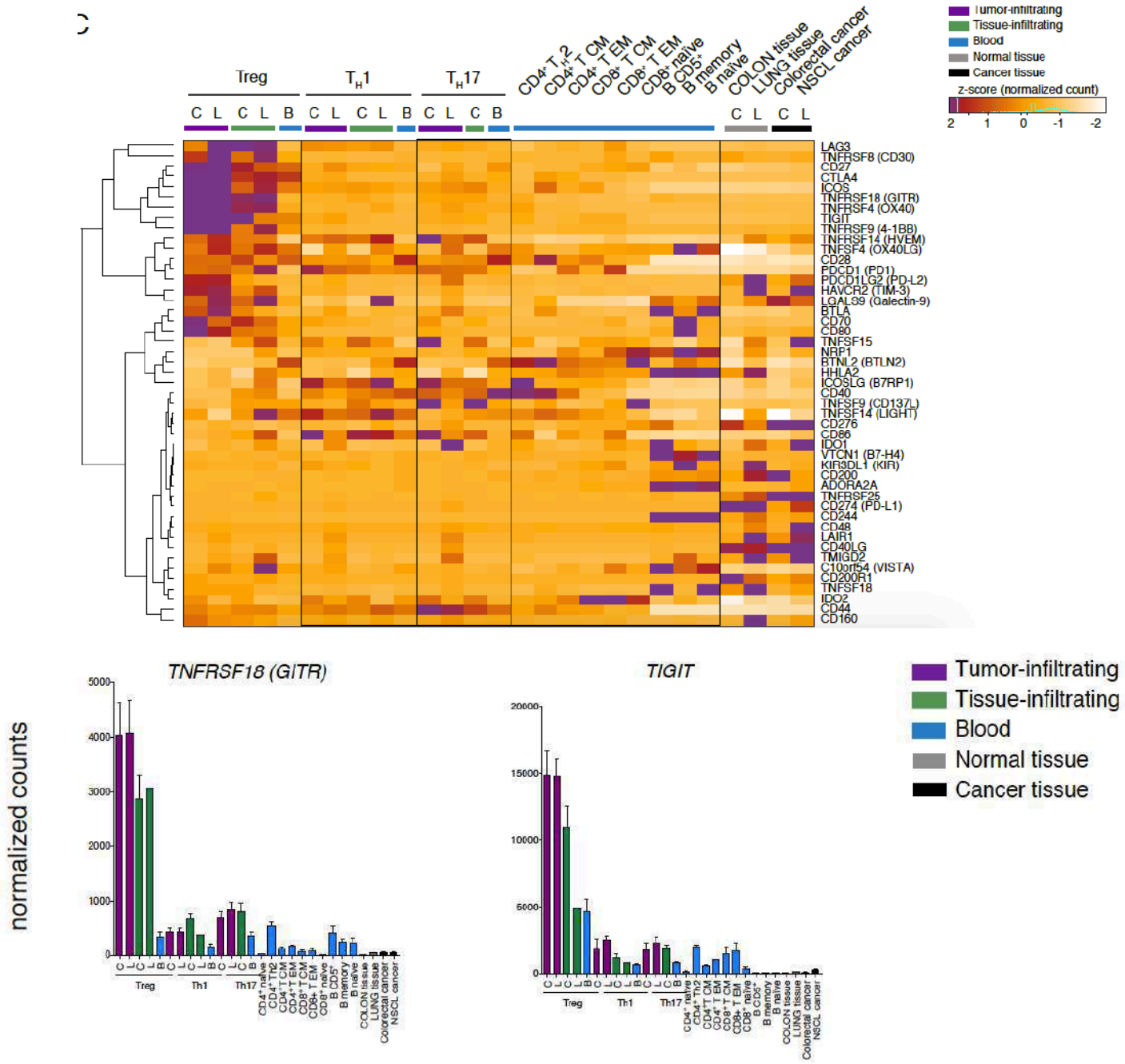


Figure 2: **Suppression activity of tumor infiltrating Treg cells and their respective healty tissues and peripheral blood.** Representative flow cytometry plots showing suppressive activity of Treg cells isolated from tumor (NSCLC or CRC), normal tissue and blood of the same patient. 4×10^5 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD4^+$ naive T cells from healthy donors were co-cultured with an equal number of Treg cells for 4 days with a CD3-specific mAb and $CD1c^+CD11c^+$ dendritic cells. Percentage of proliferating cells is indicated. Data are representative of three independent experiments.

The polyadenylated RNA fraction extracted from the sorted $CD4^+$ Treg, Th1 and Th17 cells was then analyzed by paired-end RNA sequencing obtaining about 4 billion of mapped 'reads' (Table1). First, we interrogated RNA-sequencing data of $CD4^+$ T cells infiltrating both CRC and NSCLC and their matched normal tissues, to quantitate mRNA expression of known immune checkpoints and their ligands. Second, we analyzed RNA-seq data of CRC and NSCLC, as well as of normal colon and lung sample. We found that several immune checkpoints and their ligands transcripts were strikingly upregulated in tumor infiltrating Treg cells compared to both normal tissue and peripheral blood-derived Treg cells as well as to T and B lymphocyte subsets purified from peripheral blood mononuclear cells (PBMCs) (Fig. 3A and 3B).


Tumor-infiltrating
Tissue-infiltrating
Blood
Normal tissue
Cancer tissue
z-score (normalized count)
2 1 0 -1 -2
Treg
T_H1
T_H17
$CD4^+$ T_H2
$CD4^+$ T CM
$CD4^+$ T EM
$CD8^+$ T CM
$CD8^+$ T EM
$CD8^+$ naive
B $CD5^+$
B memory
B naive
COLON tissue
LUNG tissue
Colorectal cancer
NSCL cancer
C L C L B C L C L B C L C B C L C L
LAG3
TNFRSF8 (CD30)
CD27
CTLA4
ICOS
TNFRSF18 (GITR)
TNFRSF4 (OX40)
TIGIT
TNFRSF9 (4-1BB)
TNFRSF14 (HVEM)
TNFSF4 (OX40LG)
CD28
PDCD1 (PD1)
PDCD1LG2 (PD-L2)
HAVCR2 (TIM-3)
LGALS9 (Galectin-9)
BTLA
CD70
CD80
TNFSF15
NRP1
BTNL2 (BTLN2)
HHLA2
ICOSLG (B7RP1)
CD40
TNFSF9 (CD137L)
TNFSF14 (LIGHT)
CD276
CD86
IDO1
VTCN1 (B7-H4)
KIR3DL1 (KIR)
CD200
ADORA2A
TNFRSF25
CD274 (PD-L1)
CD244
CD48
LAIR1
CD40LG
TMIGD2
C10orf54 (VISTA)
CD200R1
TNFSF18
IDO2
CD44
CD160
TNFRSF18 (GITR)
TIGIT
normalized counts
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4000
3000
2000
1000
0
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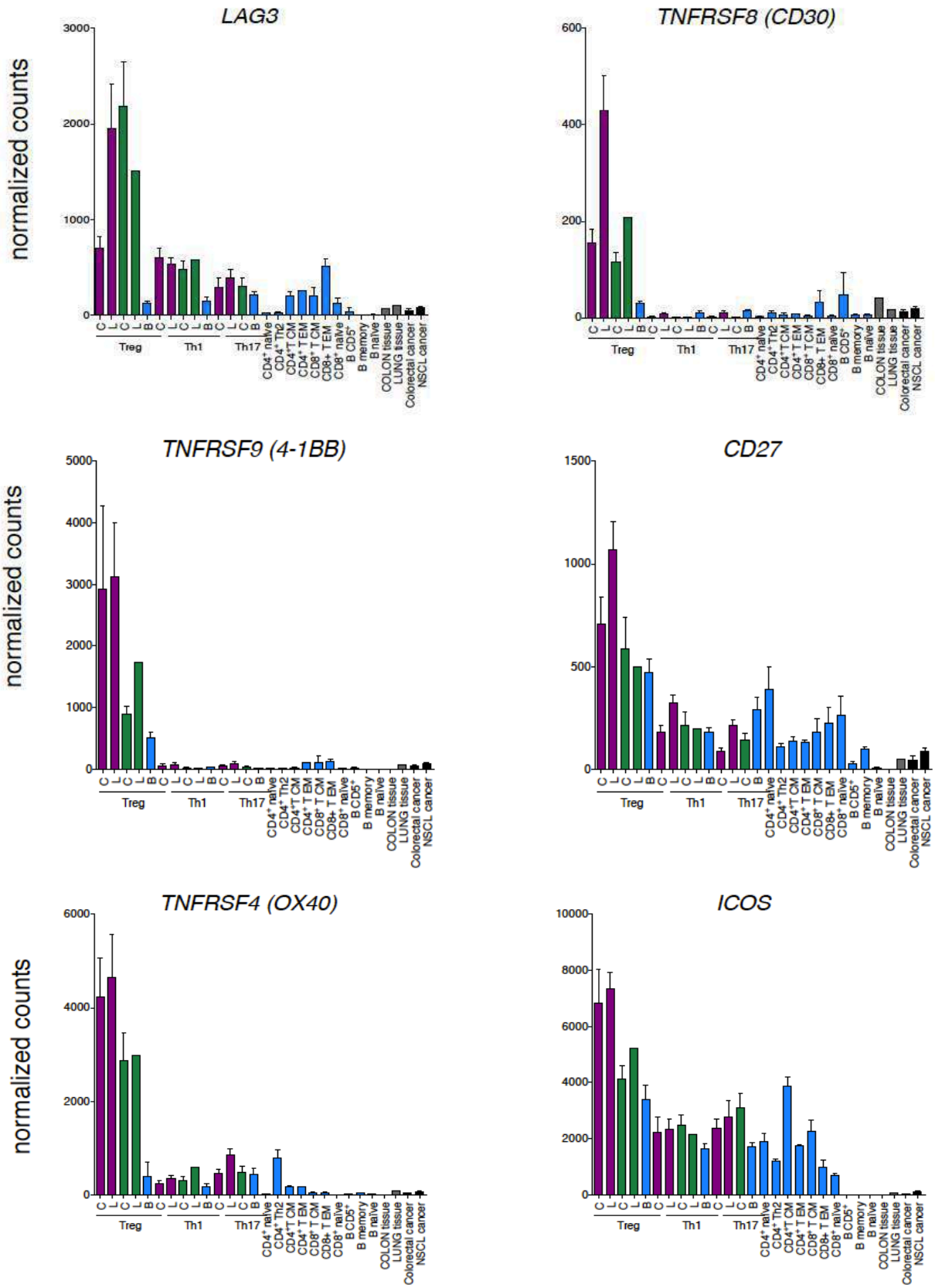
15000
10000
5000
Tumor-infiltrating
Tissue-infiltrating
Blood
Normal tissue
Cancer tissue


LAG3
TNFRSF8 (CD30)
TNFRSF9 (4-1BB)
CD27
TNFRSF4 (OX40)
ICOS
normalized counts
Treg
Th1
Th17
CD4+ naive
CD4+ Th2
CD4+ TCM
CD4+ TEM
CD8+ TCM
CD8+ TEM
CD8+ naive
B CD5+
B memory
B naive
COLON tissue
LUNG tissue
Colorectal cancer
NSCL cancer

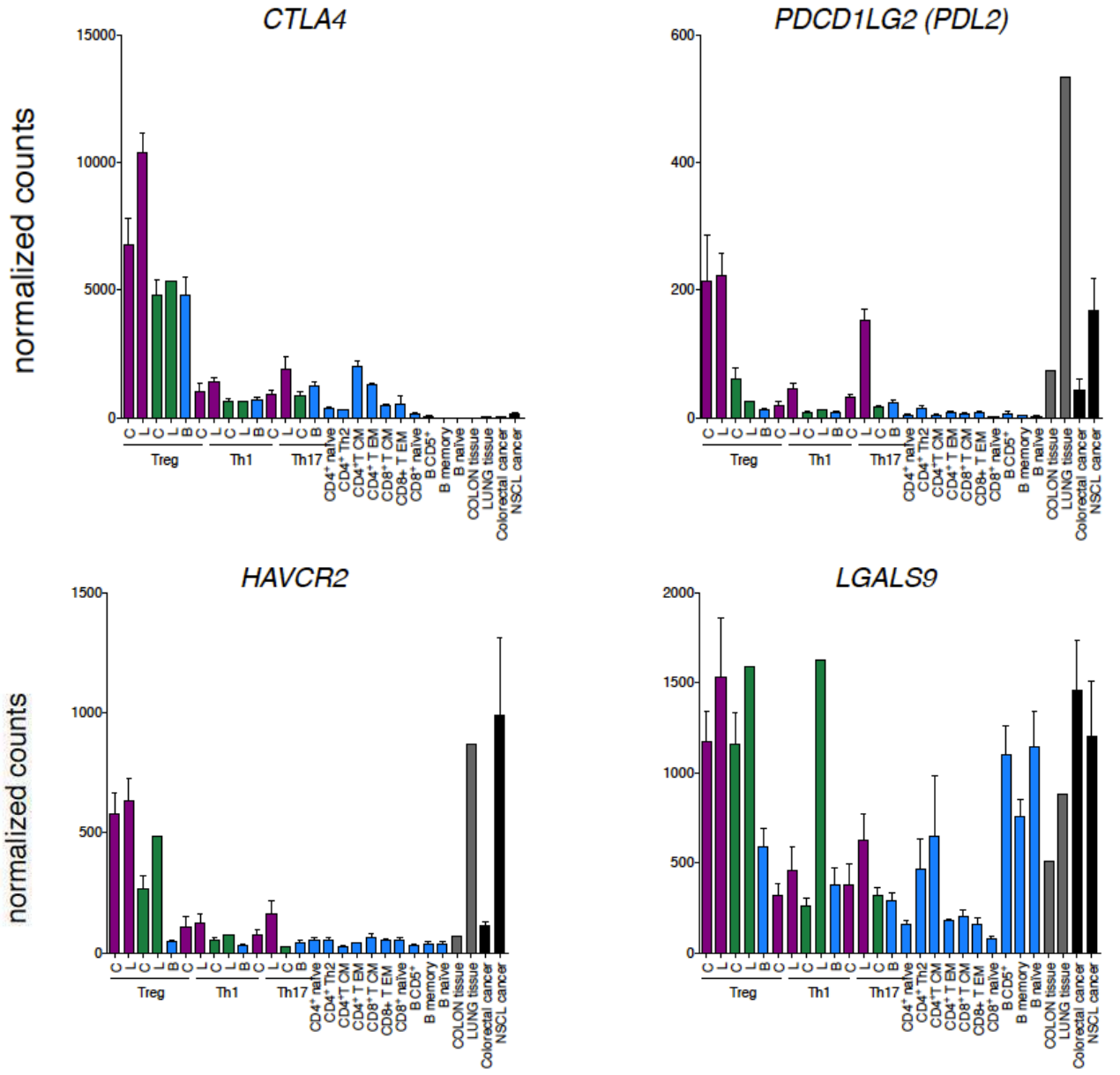


Figure 3: Transcriptome analysis of Tumor infiltrating Lymphocytes.

A) Z-score normalized RNA-seq expression values of immune checkpoints genes are represented as a heatmap. Cell populations are reported as a color code in the upper part of the graph, while gene names have been assigned to heatmap rows. Hierarchical clustering results are shown as a dendrogram drawn on the left side of the matrix. Colon tissues are indicated as C, lung tissues as L, and peripheral blood as B.

B) RNA-seq normalized counts data for selected immune checkpoints and their ligands are shown as histogram plot. Cell population names are reported in the lower part of each graph, while gene names are shown in the upper part. To distinguish the origin of the different populations a color code has been assigned (upper right part of the figure).

Our findings highlight the specific expression patterns of immune checkpoints and their ligands in tumor infiltrating Treg and effector cells and suggest that their functional relevance should be investigated directly at tumor sites.

2. Tumor-infiltrating Treg cells express a specific gene signature

We then asked whether tumor infiltrating Treg cells could be defined by specific gene expression patterns. Firstly, in order to capture the overall similarity between the tumor infiltrating lymphocytes we performed a Principal Components Analysis (PCA) on the whole transcriptomes. Tumor infiltrating Treg cells purified from CRC and NSCLC tissues clustered together, and were clearly separated from Th1 and Th17 cells purified from CRC and NSCLC tissues (Fig. 4A). PCA showed a distinct grouping of Treg cells purified from different sites; in fact, separation along the first principal component (PC1) clearly divided peripheral blood Treg cells from tissue infiltrating Treg cells (Figure 4B), whereas normal-tissue and tumor-tissue infiltrating Treg cells are mostly divided by the second component (PC2). These findings indicate that tumor-infiltrating Treg cells have specific expression patterns compared not only to other $CD4^+$ T cell subsets but also compared to Treg cells isolated from normal tissues.

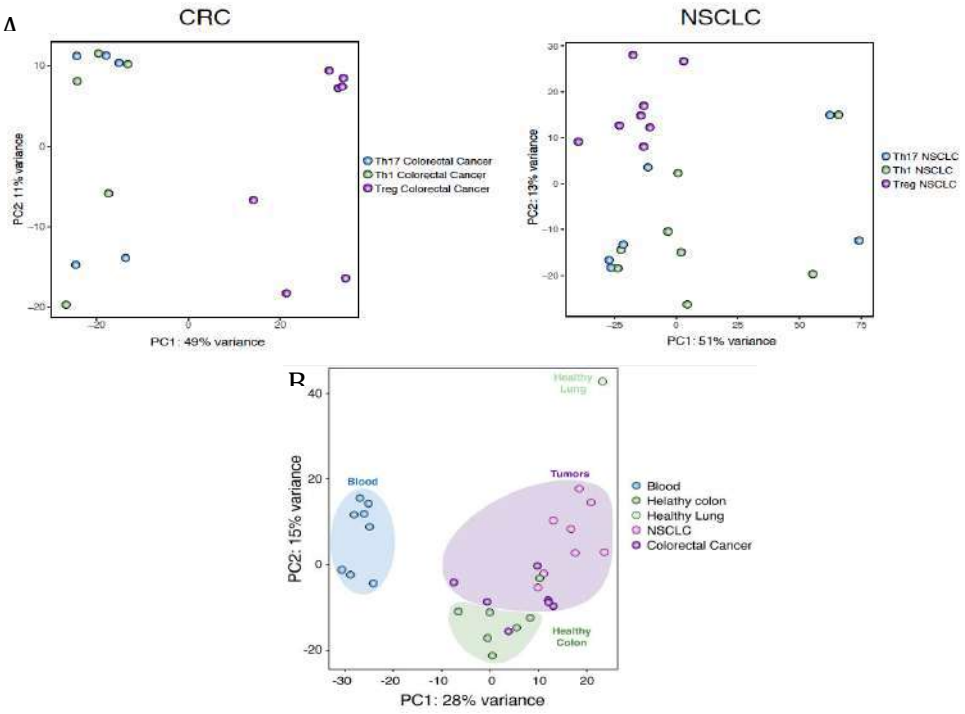


Figure 4: Principal Components Analysis (PCA) on the whole transcriptomes.
A) Principal Component Analysis (PCA) has been performed on rlog-normalized (DESeq2) counts for tumor infiltrating CD4+ Treg, Th1 and Th17 cells RNA-seq data in CRC and NSCLC samples. B) PCA has been performed on rlog-normalized counts for all T regulatory cell RNA-seq samples (36 samples from 18 individuals).

To identify signature transcripts of tumor-infiltrating Treg cells, we included in the expression pattern analyses the transcriptome dataset previously obtained from different T and B lymphocyte subsets purified from PBMCs (Ranzani et al., 2015). In so doing, we obtained a signature of 309 transcripts whose expression is higher in tumor infiltrating Treg cells compared to the other lymphocyte subsets purified from non-tumoral tissues and from PBMCs of healthy or neoplastic patients (Fig. 5).

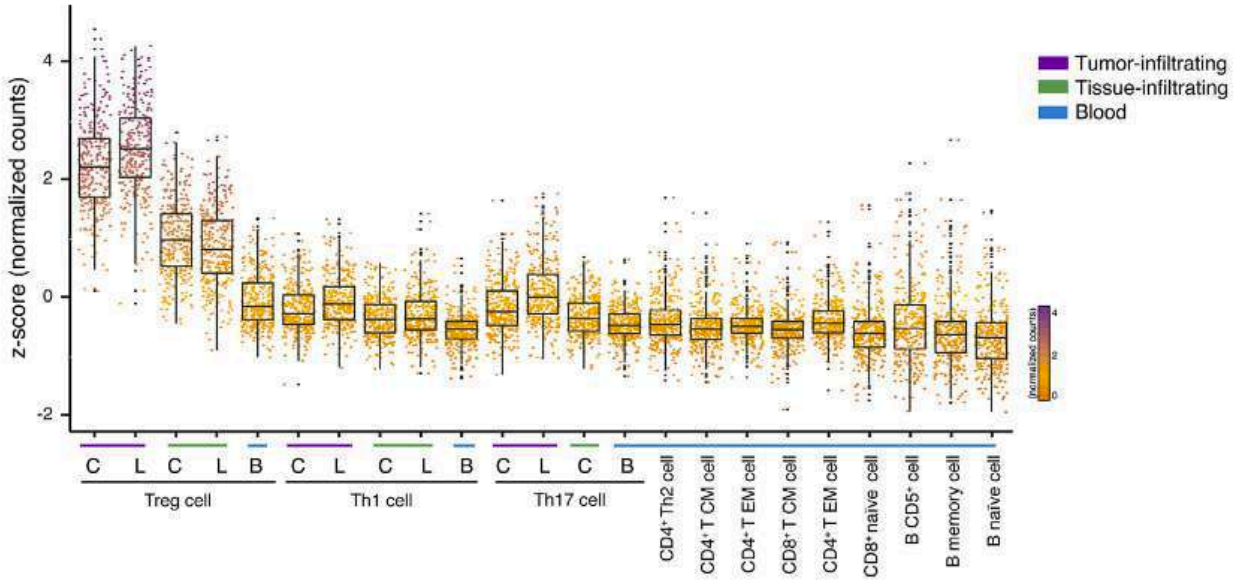
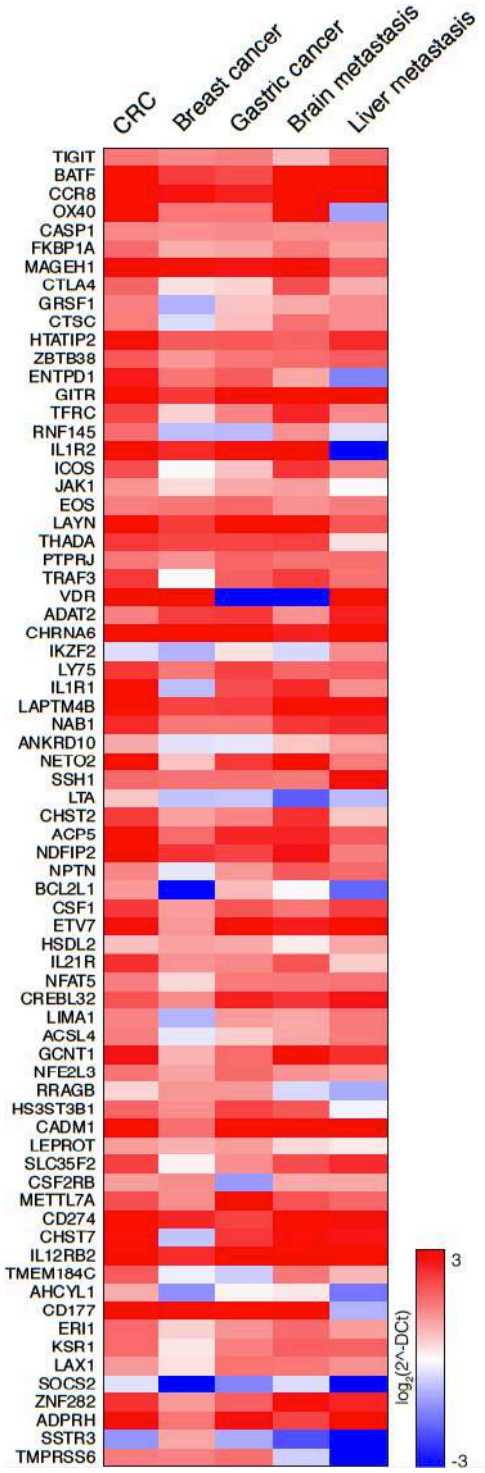


Figure 5: **Tumor-infiltrating Treg cells express a specific gene signature.** *Z*-score normalized expression values of genes that are preferentially expressed in tumor-infiltrating Treg cells (Wilcoxon Mann Whitney test $p < 2.2 \times 10–16$) over the listed cell subsets are represented as boxed plots. Colon tissues are indicated as C, lung tissues as L, and peripheral blood as B.

Altogether, the data show that Treg cells display the most pronounced differences in transcripts expression among $CD4^+$ T cell subsets infiltrating normal and tumor tissues. We defined a subset of signature genes that describe the specific gene expression profile of tumor infiltrating Treg cells.

3. Tumor infiltrating Treg signatures genes are upregulated in other tumors

The overlap between the signature genes in the CRC and NSCLC infiltrating Treg cells (Fig. 5) prompted us to assess whether this signature were also enriched in Treg cells infiltrating other tumors. RNA was thus extracted from Treg cells infiltrating breast cancer, gastric cancer, brain metastasis of NSCLC, and liver metastasis of CRC. We found by RT-qPCR that tumor infiltrating Treg signatures genes were mostly upregulated also in these tumors (Fig. 6).



Overall these data show that the tumor-infiltrating Treg cell signature genes are co-expressed at single cell level with *FOXP3* and *IL2RA and* that several primary and metastatic human tumors express the tumor-infiltrating Treg cell signature.

Figure 6: **Gene-expression analysis of tumor Treg signature genes in different tumor types.** Expression values are expressed as log2 (2^-DCt).

4. Gene signature of tumor infiltrating Treg cells is translated in protein signature

We then assessed at the single cell level by flow cytometry the protein expression of ten representative signature genes present in CRC and NSCLC infiltrating Treg cells, adjacent normal tissues, and patients PBMCs. Of the ten proteins, two were proteins (OX40 and TIGIT) whose relevance for Treg cells biology has been demonstrated (Joller et al., 2014; Voo et al., 2013), seven are proteins (BATF, CCR8, CD30, IL-1R2, IL-21R, PDL-1 and PDL-2) whose expression has never been described in tumor-infiltrating Treg cells, and one protein, 4-1BB, is a co-stimulatory receptor expressed on several hematopoietic cells, whose expression on Treg cells has been shown to mark antigen-activated cells (Schoenbrunn et al., 2012). Our findings showed that all these proteins were upregulated (Fig 7A), to different extent, in tumor infiltrating Treg cells compared to the Treg cells resident in normal tissues. Given the increasing interest in the PD1 - PDLs axis as targets for tumor immunotherapy, we assessed the effect of antibodies against PDL-1 and PDL-2 on the suppressive function of Tumor-infiltrating Treg cells toward

effector CD4$^+$ T cell proliferation *in vitro*. We found that pre-incubation of tumor infiltrating Treg cells with monoclonal antibodies against PDL-1 or PDL-2 reduced their suppressive activity as demonstrated by the increased proliferation of effector CD4$^+$ T cells (Fig 7B).

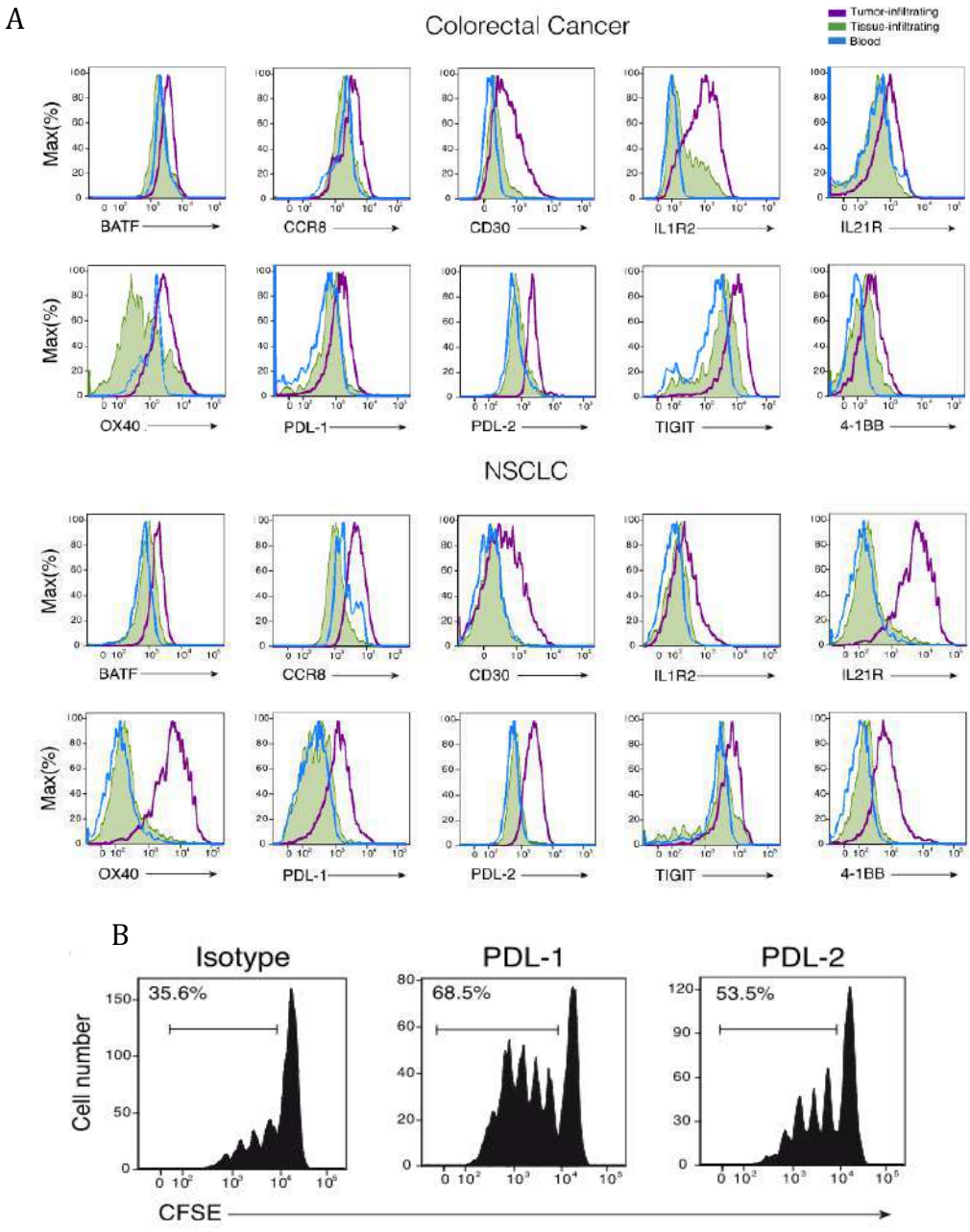


Figure 7: **Expression of Tumor-Infiltrating Treg Cells Protein Signatures in CRC and NSCLC Samples.**
A) Representative flow cytometry plots for tumor (purple line) normal (green area) tissue infiltrating Treg cells and peripheral blood Treg cells (blue line) analyzed for the expression of the indicated proteins.
B) Flow cytometry plots representative of four independent experiments showing suppressive activity of CRC infiltrating Treg cells on proliferation (shown as CFSE dilution) of CD4$^+$ effector T cells. First panel shows the

inhibitory effect of Treg cells on the effector T cell proliferation in the presence of an isotype control antibody. The other panels show the inhibitory effect of Treg cells that have been pre-incubated with anti PD-L1 or PD-L2 antibodies. Percentage of proliferating cells is indicated. The calculated division index is 0.26 in the presence of the control antibody; 0.57 in the presence of anti-PDL-1 and 0.39 in the presence of anti-PDL-2. Data are representative of four independent experiments.

Altogether, our data show there is a molecular signature of tumor infiltrating Treg cells, which can be detected both at the mRNA and at the protein levels.

5. Expression of Tumor Treg signature genes is negatively correlated with patient survival

In an attempt to correlate our findings with clinical outcome, we asked whether the expression of the tumor-Treg signature transcripts correlated with disease prognosis in CRC and NSCLC patients. We therefore interrogated for expression of Treg signature genes transcriptomic datasets obtained from resected tumor tissues of a cohort of 177 CRC patients (GSE17536 (Smith et al., 2010) and of a cohort of 263 NSCLC patients (GSE41271 - (Sato et al., 2013), and correlated high and low gene expression with the 5-years survival data. Among those genes whose expression is highly enriched in tumor infiltrating Treg cells, we selected *LAYN*, *MAGEH1* and *CCR8* that are the three genes more selectively expressed (Fig. 8A).

To normalize for differences in T cell densities within the resected tumor tissues, we used the ratio between expression of the selected signature genes and *CD3G*. We found that high expression of the three signature genes is in all cases correlated with a significantly reduced survival (Fig. 8B). We also observed that expressions of the three signature genes increased with tumor staging of CRC patients (Fig. 8C).

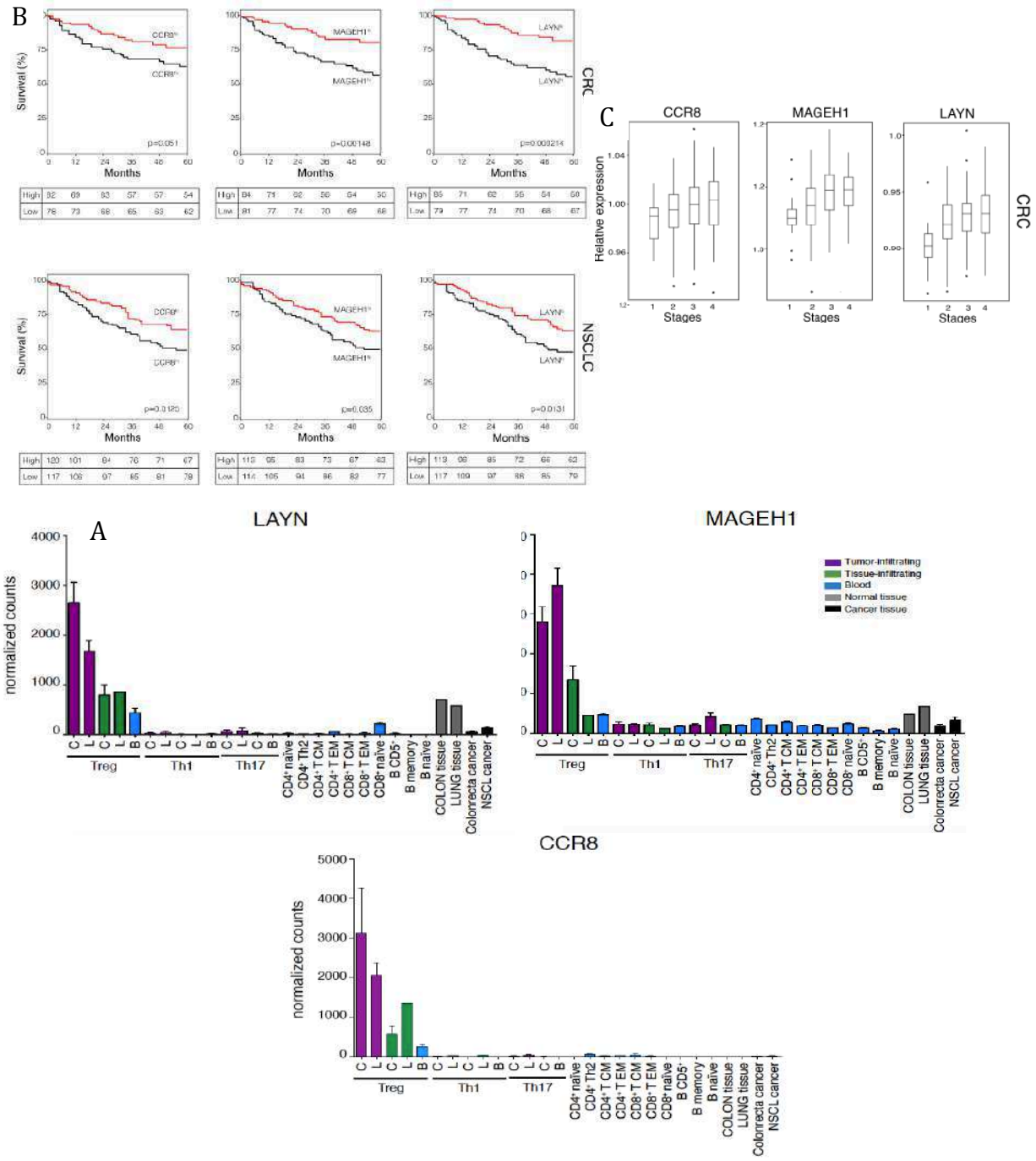


Figure 8: Prognostic Value of Signature Transcripts of Tumor Infiltrating Treg Cells. A) Kaplan-Meier survival curve comparing the high and low expression of the tumor Treg signature transcripts (*CCR8*, *MAGEH1*, *LAYN*) normalized to the *CD3G* for the CRC (n = 177) and NSCLC (n = 263) studies. Univariate analysis confirmed a significant difference in overall survival curve comparing patients with high and low expression. Statistical significance was determined by the log-rank test. (CRC: p = 0.05 for *CCR8*, p = 1.48×10^{-3} for *MAGEH1*, p = 2.1×10^{-4} for *LAYN*; NSCLC: p = 0.0125 for *CCR8*, p = 0.035 for *MAGEH1*, p = 0.0131 for *LAYN*.) Each table depicts the Kaplan-Meier estimates at the specified time points. B) Expression distributions of *CCR8*, *MAGEH1*, and *LAYN* according to tumor staging at the time of surgery in the cohort of CRC patients.

In conclusion, high expression in the whole tumor samples of three genes (*LAYN*, *MAGEH1* and *CCR8*) that are specifically and highly expressed in tumor infiltrating Treg cells, correlates with a poor prognosis in both NSCLC and CRC patients.

PART II

6. Identification of putative Treg specific long-non coding RNA

Besides unique coding genes that characterize Treg cells, we also investigated the expression and specificity of non-coding transcripts whose regulatory role is by now well established in many cellular contexts. To assess the landscape of long-non coding RNA in human immune cell subsets, we generated a comprehensive transcription analysis from 13 human primary T and B subpopulations from peripheral blood of healthy donors, including CD4+ naive, Treg, Thelper 1 (Th1), Th2, Th17, TCM, TEM, CD8+ naive, TCM, naive B, memory B, and CD5+ B cells (Figure 9a) (Ranzani et al. 2015)

To discover novel specific non-coding transcripts, we performed *de novo* transcriptome reconstruction, identifying 4021 previously not-annotated non coding transcripts, including long intergenic non coding RNAs (lincRNAs) (Ranzani et al. 2015). LincRNAs were identified by selecting transcripts longer than 200 nucleotides, multiexonic, and which did not overlap with protein coding genes. Furthermore we excluded transcripts that contain:

- a conserved protein-coding region and transcripts with putative ORFs that contain protein domains catalogued in Pfam (protein family database) (Finn et al. 2014)
- a comparative genomics method that efficiently predict transcript coding potential exploiting PhyloCSL.

Among all these novel non-coding RNAs, we found 4 different transcripts codified by 2 genes located in proximity of CTLA4 gene: INGMG_001499 in the upstream region of CTLA4 gene and INGMG_001500, downstream CTLA4 gene. Each of them is transcribed into two specific primary transcripts that give rise to two different isoforms for each: ncRNA_up_1 and ncRNA_up_2 for INGMG_001499, ncRNA_down_1 and ncRNA_down_2 regarding

INGMG_001500 (Fig 9b). Given the importance of CTLA4 in Treg cell biology we focused our attention on these specific transcripts and investigated their role in Treg cell biology.

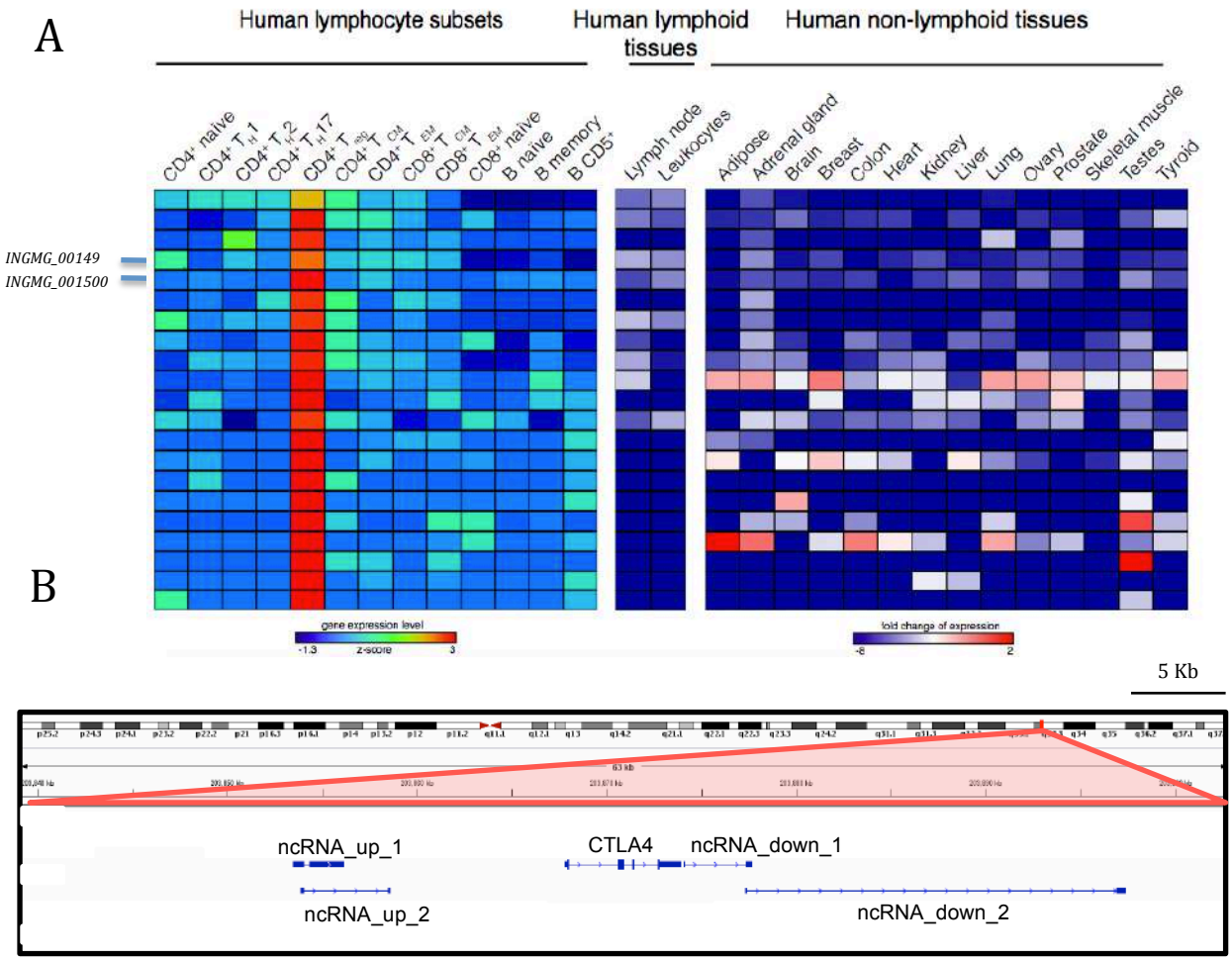


Figure 9: **Identification of new Treg specific lincRNAs**. A) Heatmap of normalized expression values of lymphocytes signature lincRNAs selected on the basis of fold change (>2.5 with respect to all the other subsets), intrapopulation consistency (expressed in at least 3 out of 5 samples) and non parametric Kruskal-Wallis test ($P < 0.05$). B) Localization of ncRNA_up_1, ncRNA_up_2, ncRNA_down_1 and ncRNA_down_2 within CTLA4 genomic region on chromosome 2q33.

We then compared the expression levels of these non-coding transcripts in RNA-seq data of CRC infiltrating Treg cells as well as Treg cells isolated by peripheral blood of healthy donors. Interestingly, we found that the expression levels of these new non-coding transcripts were higher in tumor infiltrating Treg cells compared to healthy donors peripheral blood (Fig. 10).

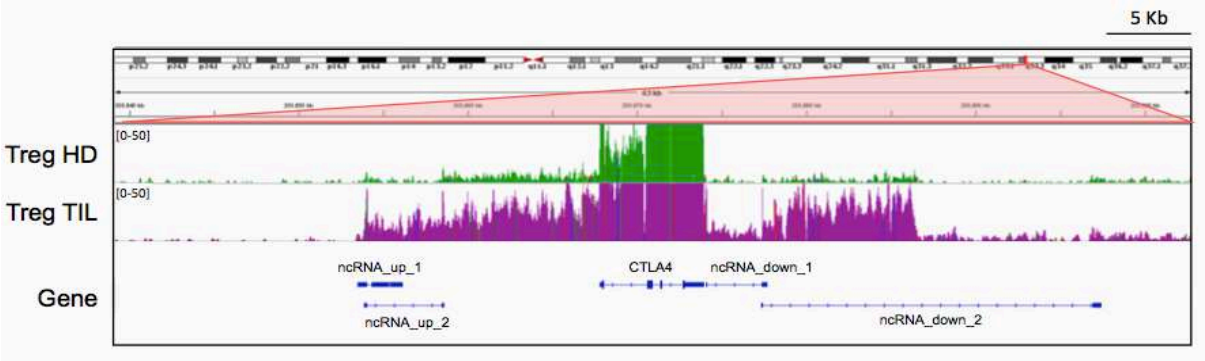


Figure 10: RNA-seq data in the CTLA4 genomic locus. The position of the novel Treg-specific lincRNAs in relation to the co-receptor gene is shown. Reads mapping CTLA4 locus regions in Treg isolated from healthy donor peripheral blood (Treg HD) as well as tumor infiltrating Treg cells (Treg TIL) are displayed.

Moreover, gene expression analysis confirmed that INGMG_001499 and INGMG_001500 gene expression is correlated with CTLA4 only in Treg cells isolated from peripheral blood compared to other cell subsets analyzed (Fig. 11)

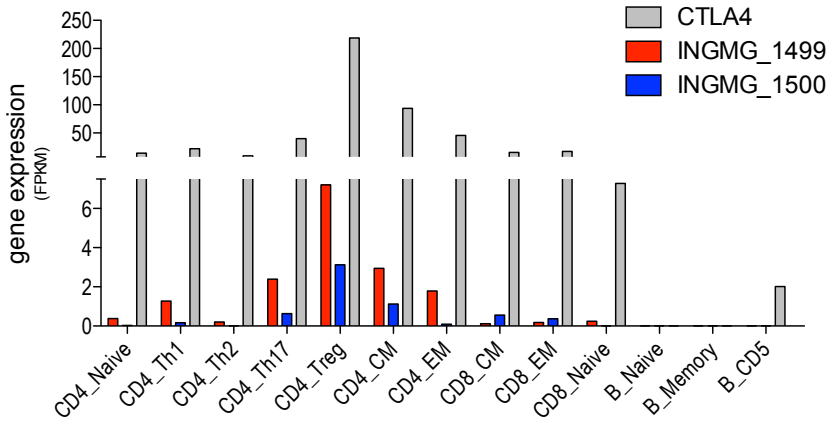


Figure 11: Gene expression analysis on INGMG_1499, INGM_1500 and CTLA4 among different cell subsets.

The data obtained *in silico* were also confirmed *in vitro* through qRT-PCR performed on total RNA isolated from CD4+ naïve T cells and Treg cells of healthy donor's peripheral blood (Fig.12).

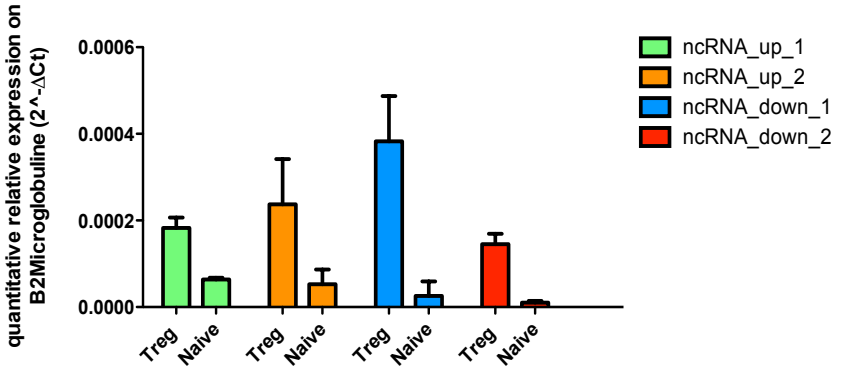


Figure 12: **Relative expression levels of non coding transcripts in Treg and naive T cell**. The qPCR was performed on three different biological replicates.

Finally, we investigated the gene expression changes of CTLA4 and these novel non coding RNAs under investigation in a time course analysis on expanded *in vitro* Treg cells with *anti-CD3-CD28* beads. In these conditions, we observed the transient expression of CTLA4 upon activation, that is not associated with the up-regulation of ncRNAs, which, in contrast, are characterized by stable expression pattern over the time. These results suggested that CTLA4, INGMG_001499 and INGMG_001500 genes are independent transcriptional units (Fig. 13).

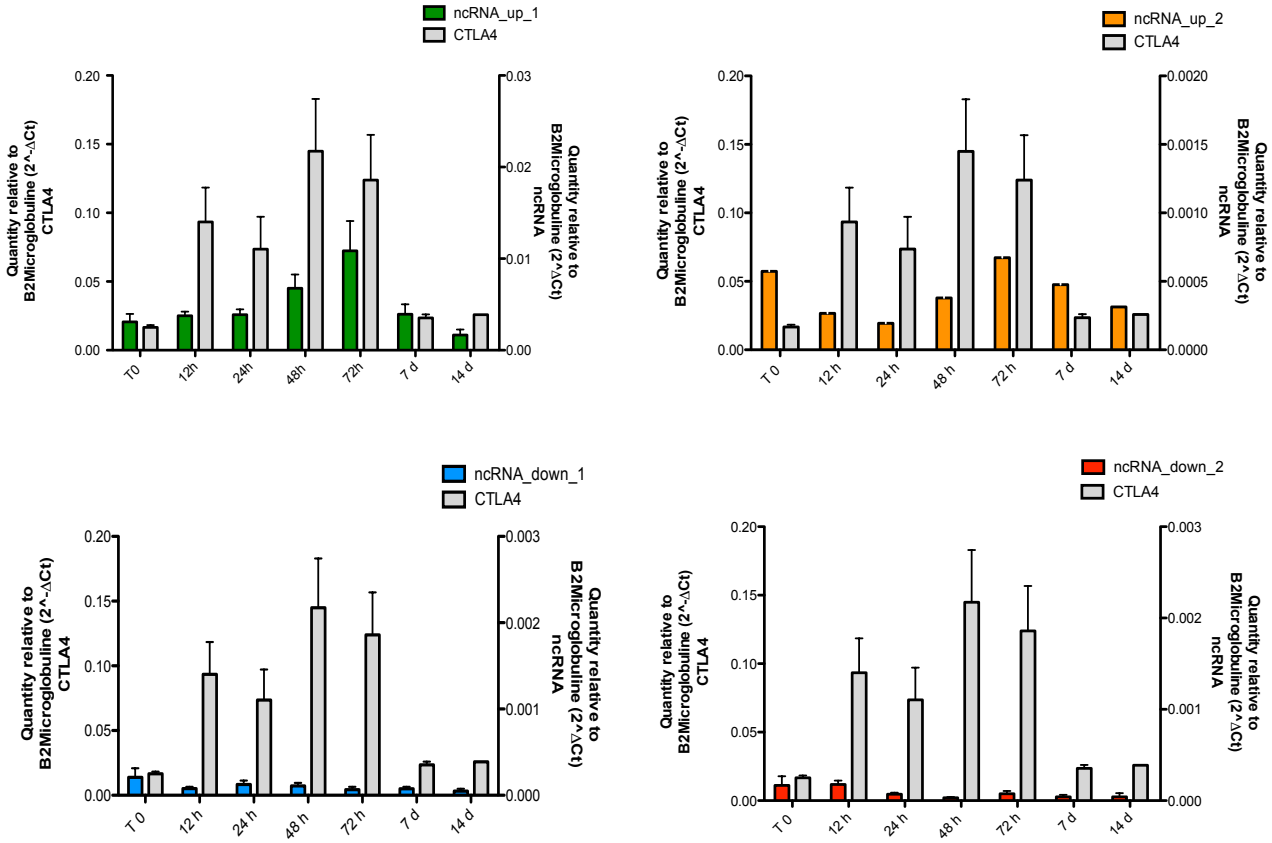


Figure 13: **Relative expression levels of CTLA4 and ncRNAs transcripts**. Treg cells were stimulated with anti-CD3/CD28 beads and mRNA was isolated after 12h, 24h, 72h, 7days and 14 days activation *stimulus*. Results are representative of three independent experiments.

7. Treg specific long-non coding RNAs are chromatin bound

Hints on the long non-coding RNA functions might derive from information on their subcellular localization. We therefore investigated the cellular compartmentalization of the identified non-coding transcripts by performing biochemical cell fractionation. Thereby, we

obtained three different fractions, including cytoplasm, nucleoplasm and chromatin. To evaluate the quality of the isolated sub-cellular fractions we tested subcellular enrichment of three known RNAs: RNU2.1 (RNA, U2 small nuclear 1) is RNA component of the U2 snRNP that interacts with 3' region of the intron during splicing events and for this reason it is localized in the chromatin fraction; Malat1 is a long non coding RNA retained in the nucleus that is thought to form molecular scaffolds for ribonucleoprotein complexes and it may also act as a transcriptional regulator for numerous genes; Linc00339 is a long intergenic non coding RNA mainly localized in the cytoplasm, as shown in Fig. 17. Interestingly, both upstream and downstream CTLA4 non coding transcripts are mainly enriched in chromatin fraction (Fig. 14).

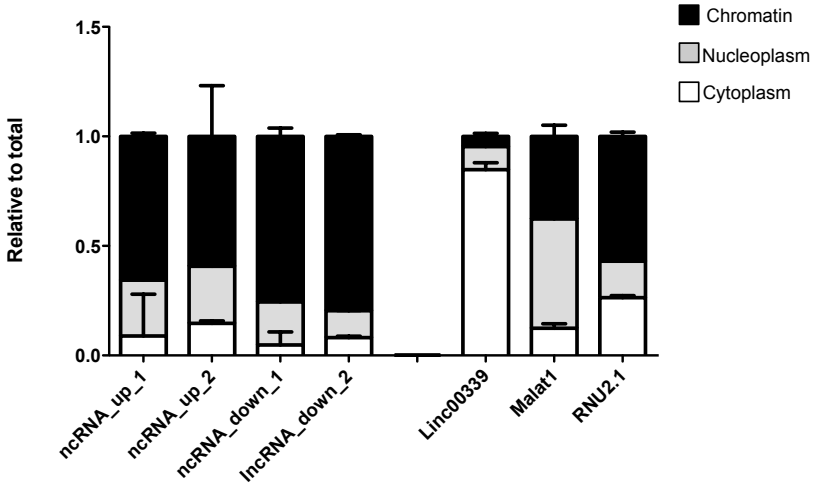


Figure 14: **Biochemical fractionation of novel lncRNAs and controls**. The results represent the average of three different experiments.

8. Epigenetic analysis of Upstream and Downstream CTLA4 regions

Due to the prominent chromatin localization of these lncRNAs we performed a more detailed analysis of the epigenetic modifications to assess whether these genes are long non-coding RNA or enhancer RNAs. To address this issue, we characterized the epigenetic modifications upstream and downstream CTLA4 regions by chromatin immunoprecipitation seq (ChIP-seq). We profiled genomic occupancy of H3K4me3, associated with active promoters, H3K27me3 that marks repressive chromatin, H3K27ac, associated with active promoters and enhancers

(Creyghton et al. 2010) and H3K4me1, related to regulatory regions including enhancers (Heintzman et al. 2007).

We first tested the histone modifications enrichments of control genes: IL2RA (CD25), that should display epigenetic modification associated to active transcription and HOXD11 that we know to be switched off in Treg cells. As reported in Fig.15, we obtained expected results.

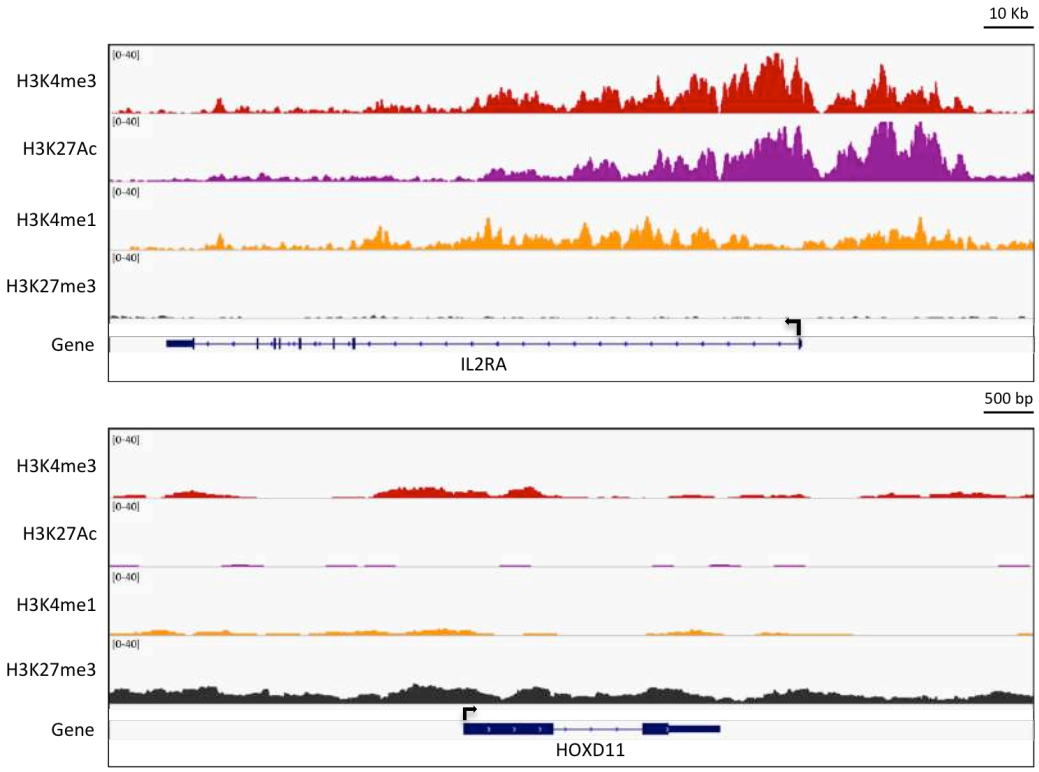


Figure 15: **Epigenomic tracks of control genes, IL2RA (CD25) and HOXD11**. H3K4me3, H3K27Ac and H3K27me3 enrichments are displayed. The arrows reflect the transcriptional direction.

We then generated genome-wide H3K4me3, H3K27Ac and H3K4me1 maps of the non-coding regions of our interest. As shown in Fig. 16, CTLA4 locus regions are defined by marks associated to active chromatin. H3K4me3 and H3K27Ac enrichments are preponderant both in upstream and dowstream regions of CTLA4 gene. Intriguingly, we observed a different H3K4Me1 enrichment between upstream and downstream CTLA4 regions. It is by now established that H3K4Me1^{hgh}/H3K4Me3^{low} correlates with active enhancers in various systems while H3K4Me1^{low}/H3K4Me3^{high} is associated with active promoters (Natoli and Andrau 2012). While the upstream regions presented a low ratio between H3K4Me1 and H3K4Me, which define them as long non-coding RNAs, the downstream ones are characterized by a

stronger enrichment of H3K4Me1 compared to H3K4Me3, suggesting that they are indeed more similar to eRNAs (Fig. 16).

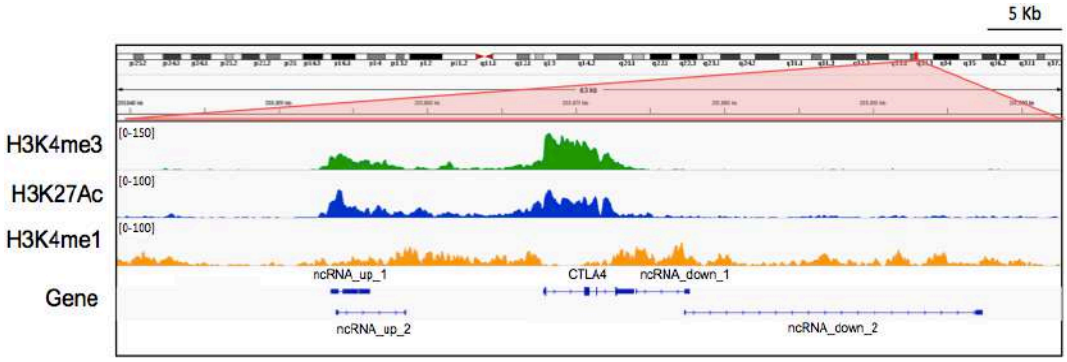


Figure 16: **Epigenetic tracks of CTLA4 locus**. The position of the novel Treg-specific lincRNAs in relation to the co-receptor genes is shown. H3K4me3, H3K27Ac, H3K4me1 peaks for Treg isolated from peripheral blood of healthy donors are displayed.

9. shRNAs downregulate at least 50% lincRNAs ncRNA_up_1 and ncRNA_up_2

Since CTLA4 is one of the key transcript for Treg cell biology we wondered whether long non-coding RNA upstream of CTLA4 region might have a role in regulating Treg cell function as well. Indeed there are many examples in the literature of chromatin bound long non-coding RNAs that recruit activating or repressive histone modifiers on specific genomic region (Joh, RI, Palmieri, CM, Hill, IT, Motamedi 2015).

Therefore, in order to study the putative effects of these long non-coding RNAs, we carried out knockdown experiments via shRNA to determine if their modulation could have an effect on the Treg suppressive function.

We designed 5 shRNAs for each transcript and we assessed the knockdown efficiency, selecting shRNAs that decreased transcript levels by at least 50%.

Expanded *in vitro* Treg cells were transduced with either control lentiviral vector (shRNA GFP) or lncRNA shRNA. At day 7 post transduction, GFP positive cells were isolated by FACS sorting, and downregulation effect was assessed by qRT-PCR. As shown in Fig. 17, compared to mock control, three different shRNAs downregulated efficiently ncRNA_up_1 (shB 60%, shD 55%, shE 80%) as well as ncRNA_up_2 (shB 85%, shC 75%, shD 80%). We

therefore selected these shRNAs to further perform functional experiments and investigated their role in Treg cell suppressive activity.

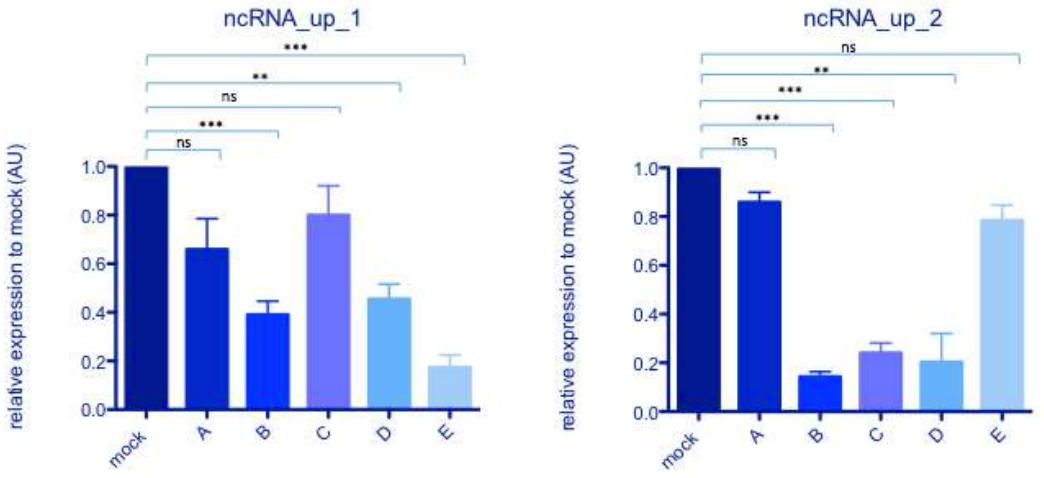


Figure 17: Downregulation effects on ncRNAup_1 and ncRNAup_ 2. Relative mRNA expression of ncRNA_up_1 and ncRNA_up_2 normalized to mock control are reported. Two-tailed paired t-test was performed (ns – not significant; ** p<0.01; *** p<0.001). Results are representative of three different experiments.

10. Suppressive activity of Treg is compromised by downregulating ncRNA_up_1 and ncRNA_up_2

Treg cells are characterized by their ability to suppress proliferation of effector T cells, which can be assessed with *in vitro* co-culture experiments. To test the effect of ncRNA_up_1 and ncRNA_up_2 down regulation on Treg cells suppressive function, CFSE-labeled responder naïve cells were put in culture in the presence of different ratios to Treg cells (1:1; 1: 0,5; 1: 0,25). We first showed that Treg transduced with scrambled shRNA control vector (MOCK) and untransduced Treg cells (UT), had a remarkably strong suppressive activity *in vitro*. Fig. 23 shows that, at different ratios between Naïve and Treg cells, MOCK and UT Treg cells remarkably reduce Naïve proliferation, which goes to 7%-15% and 1-5% respectively (Fig.18)

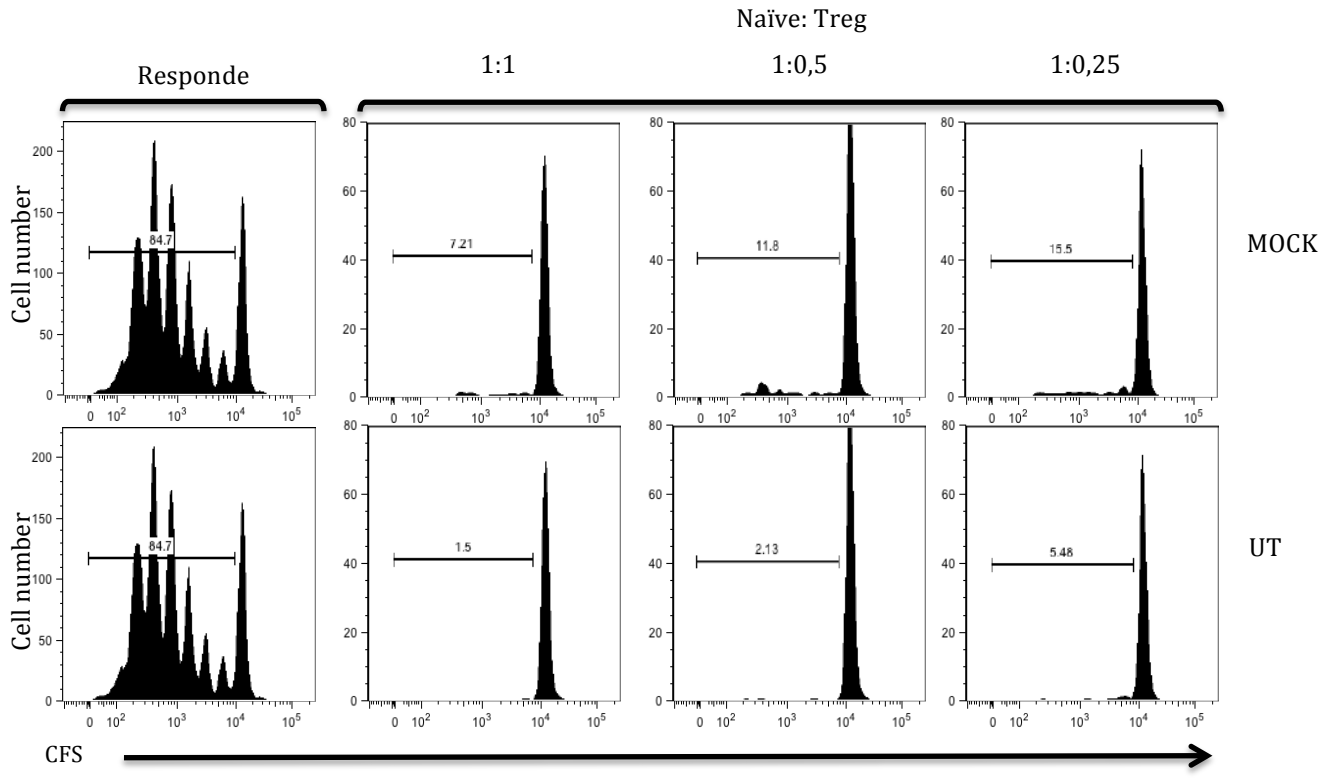


Figure 18: Representative flow cytometry plots showing suppressive activity of Treg cells isolated from peripheral blood of healthy donors. 2×10^5 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD4^+$ naive T cells from healthy donors were cocultured alone (Responder) or with different ratios of Treg cells (1:1-1:0,5 –1,025) for 4 days in presence of anti CD3/CD28 stimulus. Percentage of proliferating cells is indicated. Data are representative of three independent experiments.

In contrast, Treg cells transduced with shRNA against ncRNA_up_1 and ncRNA_up_2 displayed a significant reduction of suppressive activity. Fig. 19 and 20 show that, naive cells co-cultured in presence of Treg cells transduced with shRNAs against ncRNA_up_1 and ncRNA_up_2, respectively, exhibit a remarkably increase of proliferation compared to MOCK and UT samples. These findings suggest that modulation of non-coding RNAs upstream of CTLA4 gene impairs Treg cell function, even if the molecular mechanisms underlying such modulation remain to be identified.

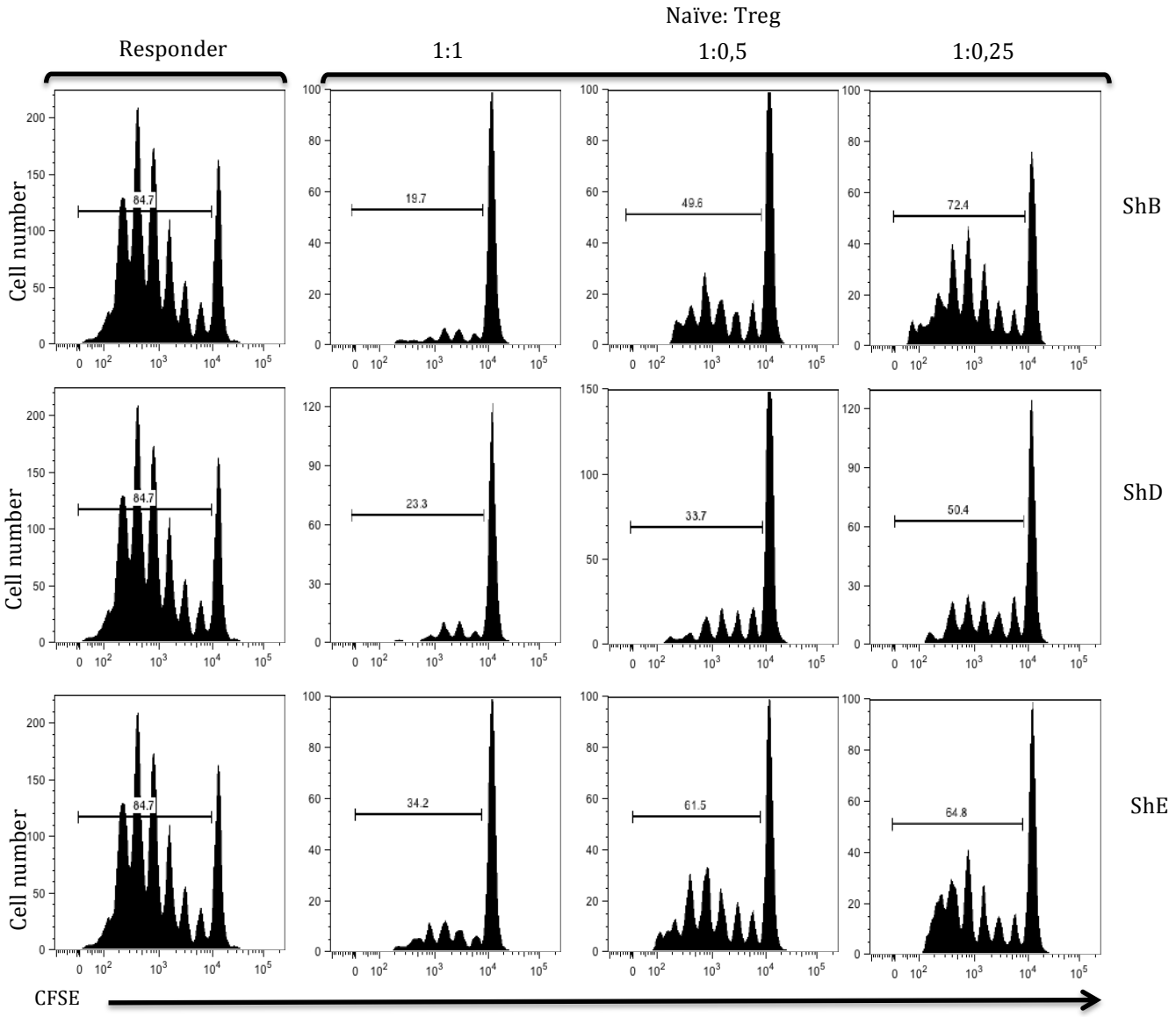


Figure 19: Representative flow cytometry plots: 2×10^5 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD4^+$ naive T cells (Responder) from healthy donors were cocultured alone or with different ratios of Treg cells transduced with three different shRNAs against ncRNA_up_1 transcript (sh B, sh D, sh E). They were put in culture for 4 days with anti CD3/CD28 beads. Percentage of proliferating cells is indicated. Data are representative of three independent experiments.

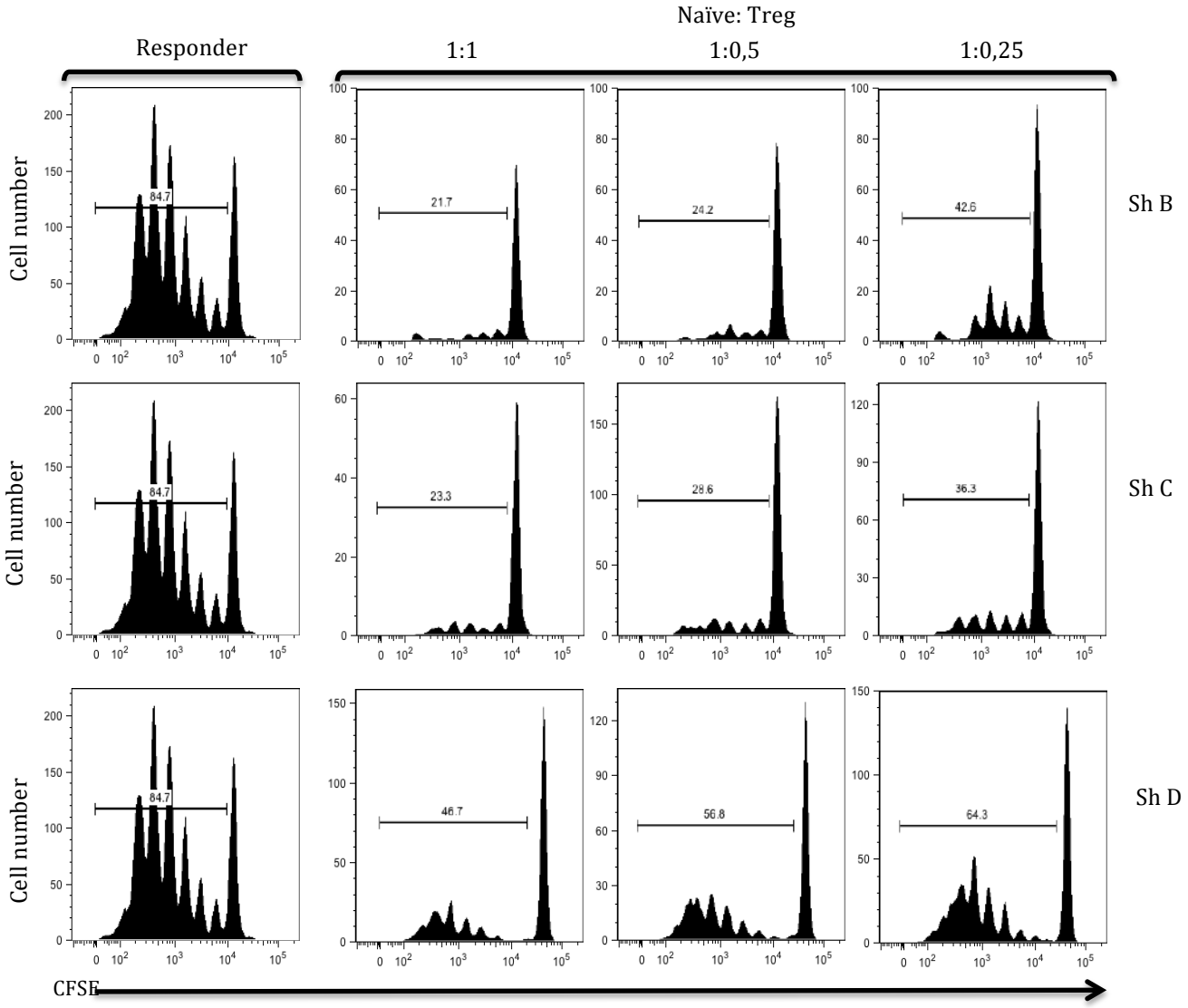


Figure 20: **Representative flow cytometry plots**: 2×10^5 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD4^+$ naive T cells (Responder) from healthy donors were cocultured alone or with different ratios of Treg cells transduced with three different shRNAs against ncRNA_up_2 transcript (sh B, sh C, sh D). They were put in culture for 4 days with anti CD3/CD28 beads. Percentage of proliferating cells is indicated. Data are representative of three independent experiments.

11. Single Nucleotides Polymorphisms (SNPs) overlap with discovered lincRNAs

Another aspect that is worthwhile investigating is the presence within the identified lincRNAs of disease-associated polymorphisms reported in genome-wide association studies (GWAS).

Single nucleotide polymorphism (SNPs) in the CTLA4 locus have been associated with several autoimmune diseases including rheumatoid arthritis, type 1 diabetes, autoimmune thyroiditis, and multiple sclerosis (Welter et al. 2014). Beside SNPs identified in coding regions there are several SNPs that lie in non-coding regions and whose relevance has been neglected so far. We have thus intersected the lincRNAs with CTLA4-CD28-ICOS polymorphisms identified from publicly available GWAS catalogues (Li et al. 2016), or from fine-mapping studies based on probabilistic identification of causal SNPs (PICS)(Farh et al. 2015). Notably, lincRNAs exons from novel transcripts overlap with PICS as well as linkage disequilibrium (LD) blocks containing GWAS SNPs and their highly correlated proxy (r^2 >0.8). This is in accordance with increasing evidence that disease-associated variants can affect regulatory elements whilst leaving their target protein coding genes intact. (Farh et al. 2015)(Hon et al. 2017). Figure 21 displays the novel lincRNAs within CTLA4 locus, the position of SNPs as well as the distribution of promoter and enhancer-associated histone marks (H3K4me3, H3K27Ac, H3K4me1) in peripheral blood Treg cells.

Maybe the SNPs falling within these regions might affect lncRNAs function in turn be relevant for Treg cell biology, such as in suppressive activity.

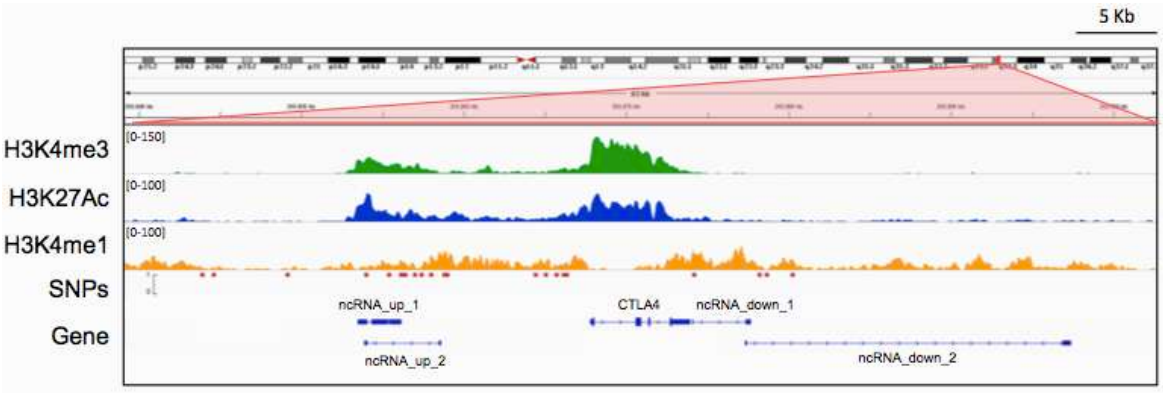


Figure 21: **Genetic, transcriptional and epigenetic data in the CTLA4 genomic locus**. The position of the novel Treg-specific lincRNAs in relation to the co-receptor gene is shown. Candidate causal SNPs are displayed along with H3K4me3, H3K27Ac, H3K4me1 peaks for Treg isolated from peripheral blood of healthy donors.

DISCUSSION AND FUTURE PERSPECTIVES

Innate and adaptive immune responses can play a role in many aspects of tumor biology. On the one hand, effector T-lymphocytes can suppress tumor growth by destroying cancer cells or inhibiting their growth. On the other hand, they can influence tumor progression either by selecting for tumor cells that are more fit to survive or by contributing to inflammation that facilitates tumor growth (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). Tumor specific CD8+ cytotoxic T cells have been for a long time considered as the ultimate anti-cancer effector cells of the immune system, since they can directly kill target cancer cells. Recently, a more direct anti-tumor role of effector CD4+ T lymphocytes has been documented as "helper cells" for CD8+ T-cell responses: tumor specific CD8+ and CD4+ effector T cells traffic to the tumor site, where CD8+ mediated killing of tumor cells thanks to the augmented cytokines released by CD4+ Th1 and Th17. These effector cells can be inhibited by different cell types contributing to the immunosuppressive environment at the tumor site. Among these cells, tumor infiltrating CD4+ T regulatory cells represent the most controversial but at the same time the most fascinating cell population. Indeed, we decided to focus in particular on Treg cells because their role as inhibitors of antitumor immune response has been fully recognized: they allow tumors to escape from immune surveillance, thus impairing immunotherapy (Frey, 2013; Perrone et al., 2008). Despite promising clinical trials directed to deplete Treg cells in tumor microenvironment, the prognostic significance of tumor infiltration by Treg cells remains a matter of debate: while high levels of intra-tumoral Treg cells have been associated with poor prognosis in most human tumors, including lung, breast and liver, (Wilke, Wu, Zhao, Wang, & Zou, 2010), Treg cells infiltration of colorectal and gastric cancers has been associated with favorable outcomes (Ladoire, Martin, & Ghiringhelli, 2011). It is possible that the discrepancy about the role of Treg cells in different types of cancer depends on the heterogeneity of this cell population. Besides the classical division in natural-Tregs generated in the thymus and in induced-Treg cells generated in the periphery, Treg cells have recently been found to respond to varying contextual cues exhibiting substantial versatility. Treg cells adapt their transcriptional program to the various cytokines to which they are exposed in the inflammatory milieu, so resulting in various "effector-Treg cell populations" with different

migratory properties and effector functions (Cretney et al., 2011). This functional diversity illustrates the importance of studying Treg cells contextually to appreciate the multifaceted role of tumor-infiltrating Treg cells may play. To date, almost all studies about tumor associated Treg cells have examined these cells as an homogenous population, with little, if any, characterization of Treg cells heterogeneity.

It is the rationale of this thesis: only in-depth understanding of the functional features of tumor infiltrating Treg cell populations may lead to a comprehension of their role in tumor control and allow the identification of novel potential therapeutic targets for cancer treatment. We first demonstrated that CRC- and NSCLC- infiltrating T cells are different compared to peripheral blood and normal tissue infiltrating Treg cells, suggesting that the tumor microenvironment determines specific gene signature in Treg cells. Moreover, the number of genes highly expressed in tumor infiltrating cells was significantly higher in Treg than in Th17 and Th1 cells, indicating that Treg cells are also a more plastic cell population than other T cell subsets, and their phenotype is influenced by tumor microenvironment they are exposed to. Among *signature* genes, we found also that immune checkpoints, such as GITR, OX40, TIGIT, LAG-3 and TIM-3 as well as some of their ligands, such as OX40LG, Galectin-9, CD70, are upregulated in tumor infiltrating Treg cells, suggesting that they could be taken into account as new therapeutic targets in cancer. Moreover, there were a number of interesting and less expected genes who were validated at the protein level and most of them have never been characterized in Treg cells such as PD-L1 and PD-L2. We also reported that tumor-infiltrating Treg cells express high amounts of 4-1BB (CD137) a marker of TcR-mediated activation (Schoenbrunn et al., 2012) and have shown an highly suppressive function on effector T cell proliferation, suggesting that expression of the signature genes correlated with the enhanced suppressive ability and so contributed to the development of a strong immunosuppressive environment at tumor sites.

Another issue we provided, concerns the relationship between increased number of Treg cells in the tumor and the worst clinical outcome. In fact, *LAYN*, *MAGEH1* and *CCR8*, which represent three of the most enriched genes in tumor infiltrating Treg cells, are highly detected in whole tumor samples and their detection is correlated with a significant worsening of the 5 years survival of both CRC and NSCLC patients. Since LAYN, MAGEH1 and CCR8 have never been characterized in Treg cells, it could be very interesting investigating more in detail

their functional roles in Treg cells in order to develop new therapeutic approaches for cancer treatment.

So far, there are several therapeutic approaches based on the modulation of specific genes expressed in Treg cells within the tumor environment, such as CTLA4. Compelling evidences come from a study by Simpson et al. which described the mode of action of a monoclonal antibody specific for cytotoxic T lymphocyte– associated antigen 4 (CTLA-4) (Simpson et al., 2013), Anti-CTLA-4 treatment, that has demonstrated significant antitumor activity in clinical trials for metastatic melanoma (Dixon et al. 2003), has been shown to enhance intratumoral effector T cells activity by selective depletion of Treg cells in the tumor microenvironment. CTLA4 is an inhibitory costimulatory molecule constitutively expressed in FoxP3+ Treg cells. In contrast to CD28 signaling that promotes T cell activation, CTLA-4 serves an immunoregulatory function, suppressing the T cell response.

RNA-seq data analysis performed in our laboratory revealed the identification of novel long non coding RNA signature, specifically expressed in CD4+ T regulatory cells. In particular, we focused our attention on those located within CTLA4 locus and we found that these lincRNAs, in addition to being Treg cell specific, are correlated with CTLA4 gene expression. The chromatin localization of these lncRNAs suggests a possible role in the functional modulation of CTLA4, but also of CD28 and ICOS genes that are localized near CTLA4 locus and that are pivotal genes for Treg cell identity. Indeed we demonstrated that modulation of CTLA4 lincRNAs expression impair suppressive function of Treg cells. Nonetheless the underlying molecular mechanisms remain to be characterized. These aspects will be investigated with experiments that assess whole transcriptome changes upon lincRNAs modulation.

Given the chromatin localization of CTLA4 lincRNAs, it is possible to postulate a role for these transcripts in inducing changes in the chromatin organization of this region which could be investigated with chromatin conformation capture experiments.

Finally, our study reveals that mutations and dysregulations of these lincRNAs are associated with the development and progression of various complex human diseases including rheumatoid arthritis, type 1 diabetes, autoimmune thyroiditis, and multiple sclerosis. These findings suggest that some of these genetic mutations located within lincRNA might be pathogenic if they compromise the functions of these molecules by changes in their folding pattern, secondary structure expression, and stability (Mirza, Kaur, Brorsson, & Pociot, 2014).

All these findings demonstrate that Treg cells phenotype and function is the result of a fine equilibrium between coding and non coding genes and the dysregulation of both these elements may contribute to the development of diseases (Fig.1). Understanding the mechanisms of function of both coding genes and non-coding transcripts in Treg cells will provide us with important leads for translational studies and will drive the identification of novel and more specific therapeutic targets for immune-related diseases.

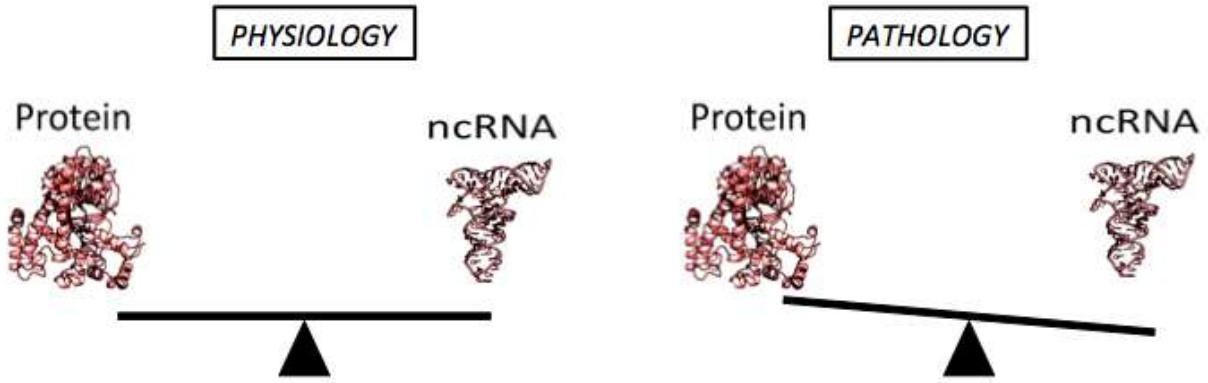


Figure 1: Schematic representation of physiologic and pathologic conditions due to the equilibrium/disequilibrium state between coding and non-coding elements.

Resource

Immunity

Transcriptional Landscape of Human Tissue Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells

Highlights

- Transcriptome analysis performed on tumor-resident CD4$^+$ Th1, Th17, and Treg cells
- Tumor-infiltrating Treg cells are defined by the expression of signature genes
- Treg-specific signature genes correlate with patients' survival in both CRC and NSCLC

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In Brief

Tumor-infiltrating regulatory T cells can suppress effector T cells specific for tumor antigens. De Simone et al. (2016) demonstrate that tumor-infiltrating Treg cells display specific gene signatures that were also validated at the single-cell level. These data can contribute to dissect the molecular networks underlying the biology of tumor-infiltrating Treg cells. As part of the IHEC consortium, this study integrates genetic, epigenetic, and transcriptomic profiling in three immune cell types from nearly 200 people to characterize the distinct and cooperative contributions of diverse genomic inputs to transcriptional variation. Explore the Cell Press IHEC webportal at www.cell.com/consortium/IHEC.

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GSE50760
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GSE17536
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Immunity
Resource

Transcriptional Landscape of Human Tissue Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells

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SUMMARY

Tumor-infiltrating regulatory T lymphocytes (Treg) can suppress effector T cells specific for tumor antigens. Deeper molecular definitions of tumor-infiltrating-lymphocytes could thus offer therapeutic opportunities. Transcriptomes of T helper 1 (Th1), Th17, and Treg cells infiltrating colorectal or non-small-cell lung cancers were compared to transcriptomes of the same subsets from normal tissues and validated at the single-cell level. We found that tumor-infiltrating Treg cells were highly suppressive, upregulated several immune-checkpoints, and expressed on the cell surfaces specific signature molecules such as interleukin-1 receptor 2 (IL1R2), programmed death (PD)-1 Ligand1, PD-1 Ligand2, and CCR8 chemokine, which were not previously described on Treg cells. Remarkably, high expression in whole-tumor samples of Treg cell signature genes, such as *LAYN*, *MAGEH1*, or *CCR8*, correlated with poor prognosis. Our findings provide insights into the molecular identity and functions of human tumor-infiltrating Treg cells and define potential targets for tumor immunotherapy.

INTRODUCTION

The combination of genetic mutations and epigenetic modifications that are peculiar to all tumors generate antigens that T and B lymphocytes can use to specifically recognize tumor cells (Jamal-Hanjani et al., 2013). It is increasingly clear that T lymphocytes recognizing tumor-derived peptides presented by major histocompatibility complex (MHC) molecules play a central role in immunotherapy and in conventional chemo-radiotherapy of cancer (Galluzzi et al., 2015). In fact, anti-tumor T cell responses arise in cancer patients but are disabled upon tumor progression by suppressive mechanisms triggered by the interplay between malignant cells and the tumor microenvironment (Munn and Bronte, 2016). The tumor-dependent immunosuppressive mechanisms depend on the integrated action of infiltrating leukocytes and lymphocytes that upregulate a range of modulatory molecules, collectively called immune checkpoints, whose function is only partially characterized (Pardoll, 2012). Therefore, the search for agonists of co-stimulatory complexes or antagonists of inhibitory molecules to potentiate antigen-specific T cell responses is a primary goal of current anti-tumor research (Sharma and Allison, 2015; Zitvogel et al., 2013). Indeed, clinical trials have unequivocally shown that the blockade of immune checkpoints unleashes the spontaneous anti-tumor immune responses in such a powerful way that it has created a paradigm shift in cancer therapy (Sledzińska et al., 2015; Topalian et al., 2015).

Among the immune checkpoints targeted by blocking strategies, CTLA-4 has been one of the first to be translated into therapeutic applications.

Anti-CTLA-4 monoclonal antibodies (mAb) show remarkable success in metastatic melanoma, and more recently in non-small-cell lung cancer, prostate cancer, renal cell carcinoma, urothelial carcinoma, and ovarian cancer (Carthon et al., 2010;

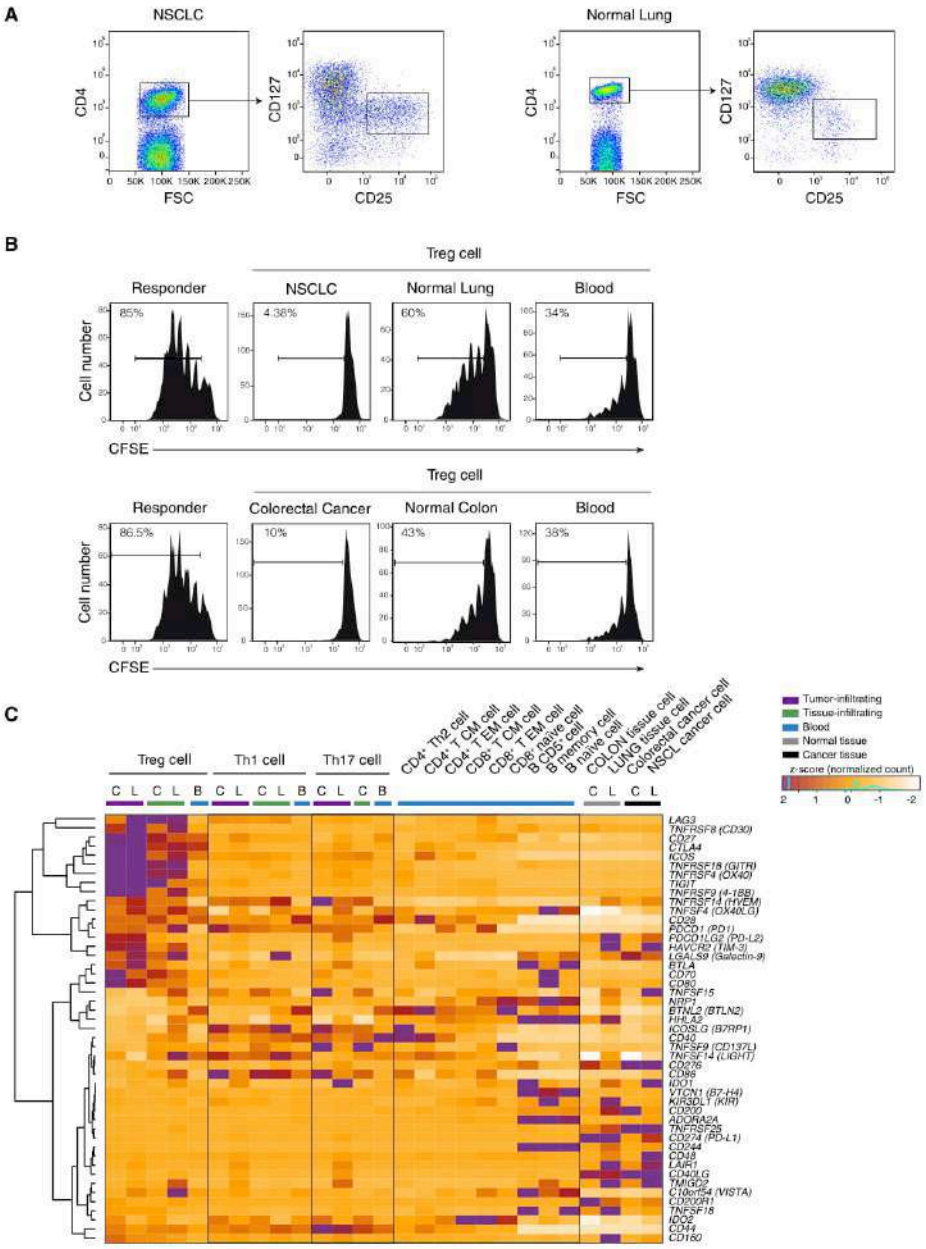

Figure 1. Purification, Functional Characterization, and Expression of Immune Checkpoints in Tumor Infiltrating Cells

(A) Representation of the sorting strategy of Treg cells infiltrating tumor or normal tissue.

(B) Representative flow cytometry plots showing suppressive activity of Treg cells isolated from tumor (NSCLC or CRC), normal tissue and blood of the same patient. 4×10^5 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD4^+$ naive T cells from healthy donors were cocultured with an equal number of Treg cells for 4 days with a CD3-specific mAb and $CD1c^+CD11c^+$ dendritic cells. Percentage of proliferating cells is indicated. Data are representative of three independent experiments.

(legend continued on next page)

Hodi et al., 2010; van den Eertwegh et al., 2012; Yang et al., 2007). However, the fraction of patients that do not respond remains high, prompting a deeper investigation of the mechanisms underpinning the modulation of immune responses by tumors. Recent experimental evidence shows that anti-CTLA-4 mAb efficacy depends on FcγR-mediated depletion of $CD4^+$ regulatory T cells (Treg cells) within the tumor microenvironment (Peggs et al., 2009; Selby et al., 2013; Simpson et al., 2013; Twyman-Saint Victor et al., 2015).

Treg cells, which are physiologically engaged in the maintenance of immunological self-tolerance and immune homeostasis (Josefowicz et al., 2012; Sakaguchi et al., 2008), are potent suppressors of effector cells and are found at high frequencies in various types of cancers (Fridman et al., 2012; Nishikawa and Sakaguchi, 2010). Treg cells adapt their transcriptional program to the various cytokines to which they are exposed in the inflammatory milieu (Campbell and Koch, 2011). This versatility is controlled by transcription factors generally associated with the differentiation of other effector $CD4^+$ T cell subsets, resulting in various Treg cell populations with unique features and immunomodulatory functions (Duhen et al., 2012; Geginat et al., 2014). Moreover, Treg cells infiltrating non-lymphoid tissues are reported to exhibit unique phenotypes and transcriptional signatures, because they can display functions beyond their well-established suppressive roles, such as metabolic modulation in adipose tissue (Cipolletta et al., 2012) or regulation of tissue repair in skeletal muscle (Burzyn et al., 2013) and in lung tissue (Arpaia et al., 2015).

Treg cell depletion has been reported to increase anti-tumor specific immune responses and to reduce tumor burden (Marabelle et al., 2013; Teng et al., 2010; Walter et al., 2012). Although promising clinical results have been achieved with Treg cell depleting strategies, some relevant issues are to be addressed, for a safer, more effective, and wider clinical application of these therapies. First, severe autoimmunity can occur following systemic Treg cells depletion (Nishikawa and Sakaguchi, 2010), which could be avoided if selective depletion of tumor infiltrating Treg cells were feasible. A second issue concerns the specificity of targeting. Indeed, Treg cells share with effector lymphocytes most of the molecules targeted for therapy, which can possibly deplete also the tumor-specific effector cells. Therefore, the molecular characterization of Treg cells at different tumor sites should help to better define therapeutic targets through a better description of their signature molecules and of the network that regulates Treg cell functions in the tumor microenvironment.

Non-small-cell lung cancer (NSCLC) and colorectal cancer (CRC) are the two most frequent cancers in both genders (Torre et al., 2015). NSCLC has the worst prognosis due to its high mortality rate even in early stages. Although CRC survival rate is highly dependent on the tumor stage at diagnosis, about 50% of patients will progress to metastatic cancer (Gonzalez-Pons and Cruz-Correa, 2015). Both tumors have been targeted with therapies based on monoclonal antibodies to checkpoint inhibitors, but the outcomes have been different. While remarkable clinical success has been obtained in NSCLC, evidence of durable response in CRC is scarce with the exception of mismatch repair-deficient CRC lesions (Jacobs et al., 2015; Kroemer et al., 2015; Le et al., 2015).

Here we provide a comprehensive transcriptome analysis of human $CD4^+$ Treg cells and effector cells (Th1 and Th17) infiltrating NSCLC or CRC and their matched normal tissues. We defined molecular signatures of tumor-infiltrating Treg cells in these two cancer types and confirmed the relevance of these signatures by single-cell analyses. These data could help a better understanding of Treg functional role at tumor sites and pave the way to the identification of therapeutic targets for more specific and safer modulation of Treg cells in cancer therapy.

RESULTS

Tumor Infiltrating Treg Cells Upregulate Immune Checkpoints and Are Highly Suppressive

To assess the gene expression landscape of tumor infiltrating $CD4^+$ T cells, we isolated different $CD4^+$ lymphocytes subsets from two different tumors, NSCLC and CRC, from the adjacent normal tissues, and from peripheral blood samples. From all these tissues, we purified by flow cytometry (Figure 1A and S1A and S1B) $CD4^+$ Treg (36 samples from 18 individuals), Th1 (30 samples from 21 individuals), and Th17 (22 samples from 14 individuals) cells (Table 1 and Table S1). To assess Treg cell function, we tested their suppressor activity and showed that Treg cells infiltrating either type of tumor tissues have a remarkably stronger suppressive activity in vitro compared to Treg cells isolated from the adjacent normal tissue and peripheral blood of the same patients (Figure 1B).

The polyadenylated RNA fraction extracted from the sorted $CD4^+$ Treg, Th1, and Th17 cells was then analyzed by paired-end RNA sequencing obtaining about 4 billion mapped "reads" (Table 1). First, we interrogated RNA-sequencing data of $CD4^+$ T cells infiltrating both CRC and NSCLC and their matched normal tissues, to quantitate mRNA expression of known immune checkpoints and their ligands. Second, we analyzed RNA-seq data of CRC and NSCLC, as well as of normal colon and lung samples. We found that several immune checkpoints and their ligands transcripts were strikingly upregulated in tumor infiltrating Treg cells compared to both normal tissue and peripheral blood-derived Treg cells, as well as to T and B lymphocyte subsets purified from peripheral blood mononuclear cells (PBMCs) (Figures 1C and S1C and Table S5). Our findings highlight the specific expression patterns of immune checkpoints and their ligands in tumor infiltrating Treg and effector cells and suggest that their functional relevance should be investigated directly at tumor sites.

Tumor-Infiltrating Treg Cells Express a Specific Gene Signature

We then asked whether tumor infiltrating Treg cells could be defined by specific gene-expression patterns. First, in order to

(C) Z-score normalized RNA-seq expression values of immune checkpoints genes are represented as a heatmap. Cell populations are reported as a color code in the upper part of the graph, while gene names have been assigned to heatmap rows. Hierarchical clustering results are shown as a dendrogram drawn on the left side of the matrix. Colon tissues are indicated as C, lung tissues as L, and peripheral blood as B.
See also Figure S1.

Table 1. Purification and RNA-Sequencing of Human Primary Lymphocyte Subsets

Tissue	Subset	Sorting Phenotype	Number of Samples	Mapped Reads (M)
NSCLC	CD4+ Treg	$CD4^+$ $CD127^-$ $CD25^+$	8	587
	$CD4^+$ Th1	$CD4^+$ $CXCR3^+$ $CCR6^-$	8	409
	$CD4^+$ Th17	$CD4^+$ $CCR6^+$ $CXCR3^-$	6	206
CRC	$CD4^+$ Treg	$CD4^+$ $CD127^-$ $CD25^+$	7	488
	$CD4^+$ Th1	$CD4^+$ $CXCR3^+$ $CCR6^-$	5	266
	$CD4^+$ Th17	$CD4^+$ $CCR6^+$ $CXCR3^-$	5	308
Lung (normal tissue)	$CD4^+$ Treg	$CD4^+$ $CD127^-$ $CD25^+$	1 (pool of 6)	73
	$CD4^+$ Th1	$CD4^+$ $CXCR3^+$ $CCR6^-$	1 (pool of 6)	76
Colon (normal tissue)	$CD4^+$ Treg	$CD4^+$ $CD127^-$ $CD25^+$	7	404
	$CD4^+$ Th1	$CD4^+$ $CXCR3^+$ $CCR6^-$	6	352
	$CD4^+$ Th17	$CD4^+$ $CCR6^+$ $CXCR3^-$	6	284
PB (healthy donor)	$CD4^+$ Treg	$CD4^+$ $CD127^-$ $CD25^+$	8	259
	$CD4^+$ Th1	$CD4^+$ $CXCR3^+$ $CCR6^-$	5	70
	$CD4^+$ Th17	$CD4^+$ $CCR6^+$ $CXCR3^-$	5	77

For each cell subsets profiled by RNA-sequencing tissue of origin, surface marker combinations used for sorting, number of profiled samples, as well as number of mapped sequencing reads are indicated. M, million; CRC, colorectal cancer; NSCLC, non-small cell lung cancer; PB, peripheral blood.
See also Table S1.

capture the overall similarity between the tumor infiltrating lymphocytes, we performed a principal components analysis (PCA) on the whole transcriptomes. Tumor-infiltrating Treg cells purified from CRC and NSCLC tissues clustered together and were clearly separated from Th1 and Th17 cells purified from CRC and NSCLC tissues (Figures S2A and S2B). PCA showed a distinct grouping of Treg cells purified from different sites; in fact, separation along the first principal component (PC1) clearly divided peripheral blood Treg cells from tissue infiltrating Treg cells (Figure 2A), whereas normal-tissue and tumor-tissue infiltrating Treg cells are mostly divided by the second component (PC2). These findings indicate that tumor-infiltrating Treg cells have specific expression patterns compared not only to other $CD4^+$ T cell subsets but also compared to Treg cells isolated from normal tissues.

In order to identify genes that are preferentially expressed in tumor-infiltrating lymphocytes, we performed self-organizing maps (SOM) analyses that provide a powerful way to define coordinated gene-expression patterns that are visualized in spatial proximity in a 2D mosaic grid heatmap (Wirth et al., 2012). In this way, we analyzed 7,763 genes that were differentially expressed between the different $CD4^+$ T cell subsets purified from PBMCs and tumor tissues (DESeq2 package; FDR < 0.05). Among the different $CD4^+$ T cell subsets (Th1, Th17, and Treg) assessed with SOM, only the tumor-infiltrating Treg cells displayed peculiar gene-expression patterns that were similar between NSCLC and CRC samples (Figures 2B and S2C), thus allowing the identification (FDR < 0.1) of transcripts upregulated in both CRC and NSCLC infiltrating Treg cells (Figure 2C and Table S2). Gene-ontology (GO) analyses of those genes upregulated in tumor infiltrating Treg cells showed significant enrichment for terms related to lymphocytes activation (Figure 2C and Table S3).

To identify signature transcripts of tumor-infiltrating Treg cells, we included in the expression pattern analyses the transcriptome datasets we previously obtained from different T and B lymphocyte subsets purified from PBMCs (Ranzani et al., 2015). In so doing, we obtained a signature of 309 transcripts whose expression is higher in tumor infiltrating Treg cells (Wilcoxon Mann Whitney test $p < 2.2 \times 10–16$) (Figures 2D and S2D and Table S4) compared to the other lymphocyte subsets purified from non-tumoral tissues and from PBMCs of healthy or neoplastic patients.

Altogether, the data show that Treg cells display the most pronounced differences in transcripts expression among $CD4^+$ T cell subsets infiltrating normal and tumor tissues. We defined a subset of signature genes that describe the specific gene-expression profile of tumor infiltrating Treg cells.

Gene Signature of Tumor-Infiltrating Treg Cells Is Present in Primary and Metastatic Human Tumors

We then look at the single cell level for the differential expression profile of signature genes of tumor infiltrating Treg cells. We isolated $CD4^+$ T cells from 5 CRC and 5 NSCLC tumor samples, as well as from 5 PBMCs of healthy individuals (Table S1), purified Treg cells, and using an automated microfluidic system (C1 Fluidigm) captured single cells (a total of 858 Treg cells: 320 from CRC and 286 from NSCLC; 252 from PBMCs of healthy individuals). We then assessed by high throughput RT-qPCR (Biomark HD, Fluidigm) the expression of 79 genes selected among the highly expressed (> 10 FKPM) tumor Treg cell signature genes (Figures 3A, S3A and S3B).

Notably, we found that the vast majority (75 over 79; 95%) of the tumor-infiltrating Treg cell signatures were co-expressed with bona fide Treg cell markers (i.e., *FOXP3*$^+$ and *IL2RA*) (Figure 3B). The percentage of co-expression between these Treg cell markers and the 79 genes selected among the tumor-infiltrating-Treg-cell signature genes ranged between 81% of *TIGIT* and 0.59% of *CGA* (Figure 3B). The expression of Treg signature genes in the RNA-seq of the whole Treg cell population correlated with the percentage of single cells expressing the different genes (Figure 3C). In order to reduce the "drop-out" effect of the single cell data (i.e., events in which a transcript is detected in one cell but not in another one because the transcript is "missed" during the reverse-transcription step) (Kharchenko et al., 2014), we defined a threshold (median value t = 8.4%) based on the expression distribution for each transcript and

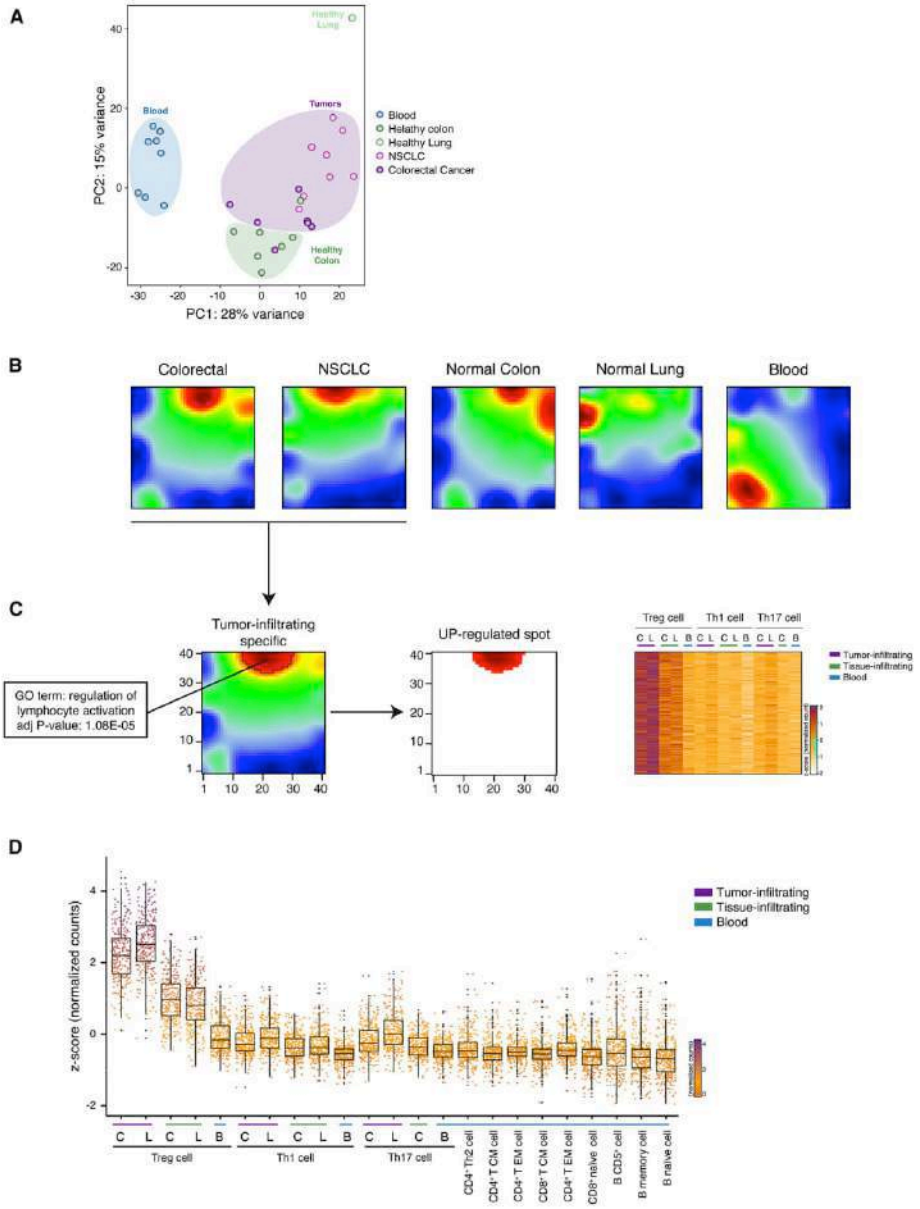

Figure 2. SOM Analysis Identifies Co-regulated Genes in Tumor Infiltrating Treg Cells

(A) PCA has been performed on rlog-normalized (DESeq2) counts for all T regulatory cell RNA-seq samples (36 samples from 18 individuals).

(B) Self-organizing maps analysis has been performed on the RNA-seq dataset comprising Treg, Th1, and Th17 cell subsets. Bidimensional SOM profiles are reported for Treg cells.

(C) Group-centered analysis for the identification of upregulated spot (FDR < 0.1) in Treg cells infiltrating both NSCLC and CRC is described as 2D heatmap. Heatmap representing *Z*-score normalized expression values of genes selected from the upregulated spot is shown on the right side of the figure. Top enriched

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discarded genes below this threshold (see the Supplemental Experimental Procedures). The forty-five signature transcripts of tumor infiltrating Treg cells detected above this threshold were in most cases significantly overexpressed in Treg cells from both tumors (39 over 45, 87%; Wilcoxon Mann Whitney test $p < 0.05$) or in one tumor type (43 over 45, 96%; Figure 3D). Homogeneity of the purified tissue infiltrating Treg cells can be affected by the carry-over of cells from other lymphocyte subsets. To quantitate this possible contamination, the single cell RT-qPCR analyses of Treg cells was performed including markers specific for other lymphocytes subsets (i.e., Th1, Th2, Th17, Tfh, CD8 T cells, B cells) (Figure S3C). Our data showed that only a very low fraction of the purified single cells displayed markers of lymphocytes subsets different from Treg cells (Figure S3C).

The overlap between the signature genes in the CRC and NSCLC infiltrating Treg cells (Figure 2D) prompted us to assess whether this signature were also enriched in Treg cells infiltrating other tumors. RNA was thus extracted from Treg cells infiltrating breast cancer, gastric cancer, brain metastasis of NSCLC, and liver metastasis of CRC. We found by RT-qPCR that tumor infiltrating Treg signatures genes were mostly upregulated also in these tumors (Figure 3E).

Overall these data show that the tumor-infiltrating Treg cell signature genes are co-expressed at single cell level with *FOXP3* and *IL2RA and* that several primary and metastatic human tumors express the tumor-infiltrating Treg cell signature.

Gene Signature of Tumor Infiltrating Treg Cells Is Translated in a Protein Signature

We then assessed at the single cell level by flow cytometry the protein expression of ten representative signature genes present in CRC and NSCLC infiltrating Treg cells, adjacent normal tissues, and patients PBMCs. Of the ten proteins, two were proteins (OX40 and TIGIT) whose relevance for Treg cells biology has been demonstrated (Joller et al., 2014; Voo et al., 2013), seven are proteins (BATF, CCR8, CD30, IL-1R2, IL-21R, PDL-1, and PDL-2) whose expression has never been described in tumor-infiltrating Treg cells, and one protein, 4-1BB, is a co-stimulatory receptor expressed on several hematopoietic cells, whose expression on Treg cells has been shown to mark antigen-activated cells (Schoenbrunn et al., 2012). Our findings showed that all these proteins were upregulated (Figure 4A), to different extent, in tumor infiltrating Treg cells compared to the Treg cells resident in normal tissues. Given the increasing interest in the PD1 - PDLs axis as targets for tumor immunotherapy, we assessed the effect of antibodies against PDL-1 and PDL-2 on the suppressive function of tumor-infiltrating Treg cells toward effector $CD4^+$ T cell proliferation in vitro. We found that preincubation of tumor infiltrating Treg cells with monoclonal antibodies against PDL-1 or PDL-2 reduced their suppressive activity as demonstrated by the increased proliferation of effector $CD4^+$ T cells (Figure 4B).

Altogether, our data show there is a molecular signature of tumor infiltrating Treg cells, which can be detected both at the mRNA and at the protein levels.

Expression of Tumor Treg Signature Genes Is Negatively Correlated with Patient Survival

In an attempt to correlate our findings with clinical outcome, we asked whether the expression of the tumor-Treg signature transcripts correlated with disease prognosis in CRC and NSCLC patients. We therefore interrogated for expression of Treg signature genes transcriptomic datasets obtained from resected tumor tissues of a cohort of 177 CRC patients (GSE17536; Smith et al., 2010) and of a cohort of 263 NSCLC patients (GSE41271; Sato et al., 2013) and correlated high and low gene expression with the 5-year survival data. Among those genes whose expression is highly enriched in tumor-infiltrating Treg cells, we selected *LAYN*, *MAGEH1*, and *CCR8* that are the three genes more selectively expressed (Figure S5A). To normalize for differences in T cell densities within the resected tumor tissues, we used the ratio between expression of the selected signature genes and *CD3G*. We found that high expression of the three signature genes is in all cases correlated with a significantly reduced survival (Figure 5A). We also observed that expressions of the three signature genes increased with tumor staging of CRC patients (Figure 5B).

In conclusion, high expression in the whole-tumor samples of three genes (*LAYN*, *MAGEH1*, and *CCR8*) that are specifically and highly expressed in tumor infiltrating Treg cells correlates with a poor prognosis in both NSCLC and CRC patients.

DISCUSSION

Diversity of tumor-infiltrating Treg cells should be fully elucidated to understand their functional relevance and prognostic significance in different types of cancer and to possibly improve the therapeutic efficacy of Treg cell modulation through the selective depletion of tumor infiltrating Treg cells. The transcriptome analysis we performed on CRC- and NSCLC-infiltrating T cells showed that tumor-infiltrating Treg cells are different from both circulating and normal tissue-infiltrating Tregs, suggesting that the tumor microenvironment influences specific gene expression in Treg cells. Our findings further support the view that Treg cells from different tissues are instructed by environmental factors to display different gene-expression profiles (Panduro et al., 2016). Indeed the list of signature genes includes a number of molecules that are consistently upregulated in tumor-infiltrating Treg cells isolated from different tumor types, and these signature genes would have not been identified if we had not profiled specifically tumor infiltrating Treg cells.

The number of genes highly expressed in tumor infiltrating cells, as defined by differential expression and SOM analyses, was significantly higher in Treg than in Th17 and Th1 cells, suggesting that Treg cells are more susceptible than other

GO term (DAVID) for genes assigned to upregulated spot is reported with the corresponding significance p value. Colon tissues are indicated as C, lung tissues as L, and peripheral blood as B.
(D) *Z*-score normalized expression values of genes that are preferentially expressed in tumor-infiltrating Treg cells (Wilcoxon Mann Whitney test $p < 2.2 \times 10–16$) over the listed cell subsets are represented as boxed plots. Colon tissues are indicated as C, lung tissues as L, and peripheral blood as B.
See also Figure S2.

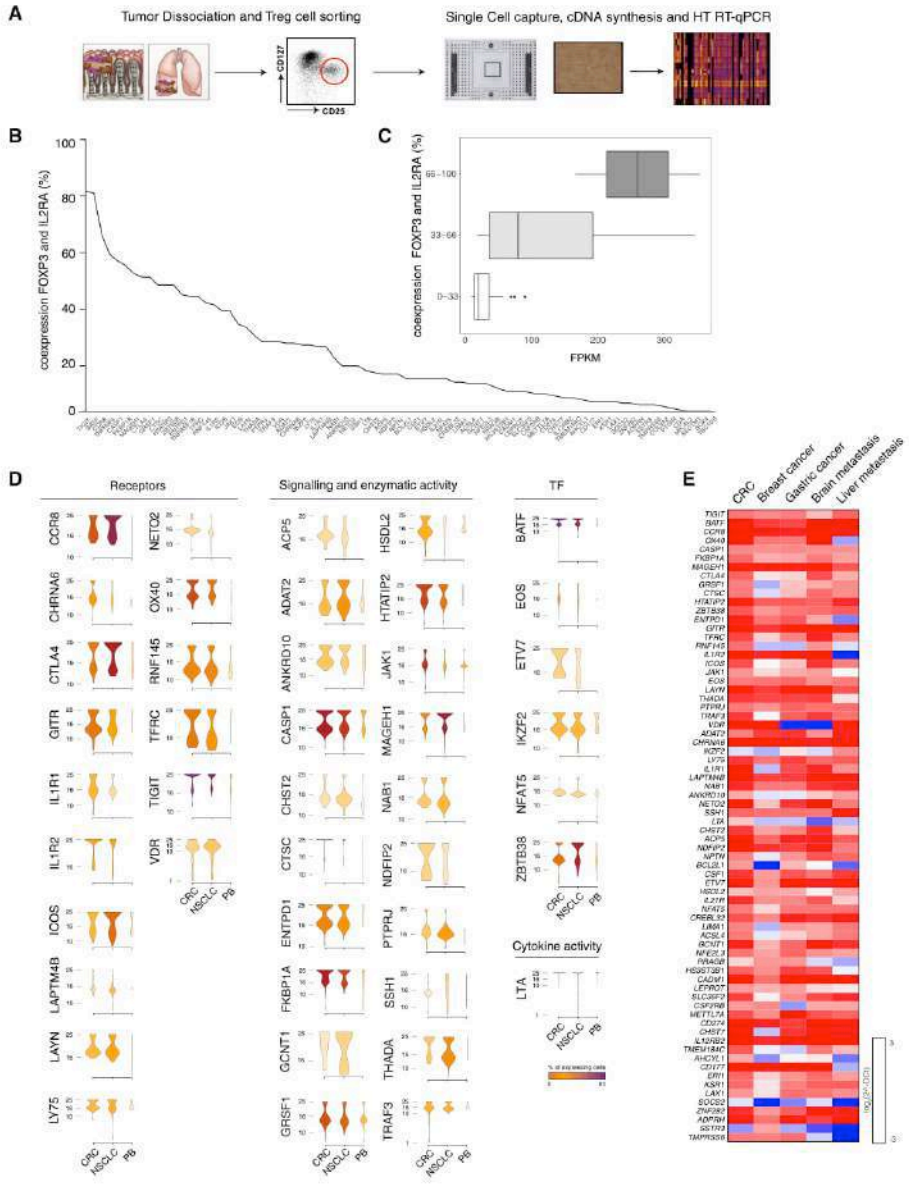


Figure 3. Single Cell Analysis of Tumor Infiltrating Treg Cells

(A) Schematic representation of the experimental workflow. Experiments were performed on Treg cells infiltrating CRC, NSCLC, or isolated from peripheral blood of healthy donors (PB); five samples were collected for each tissue.

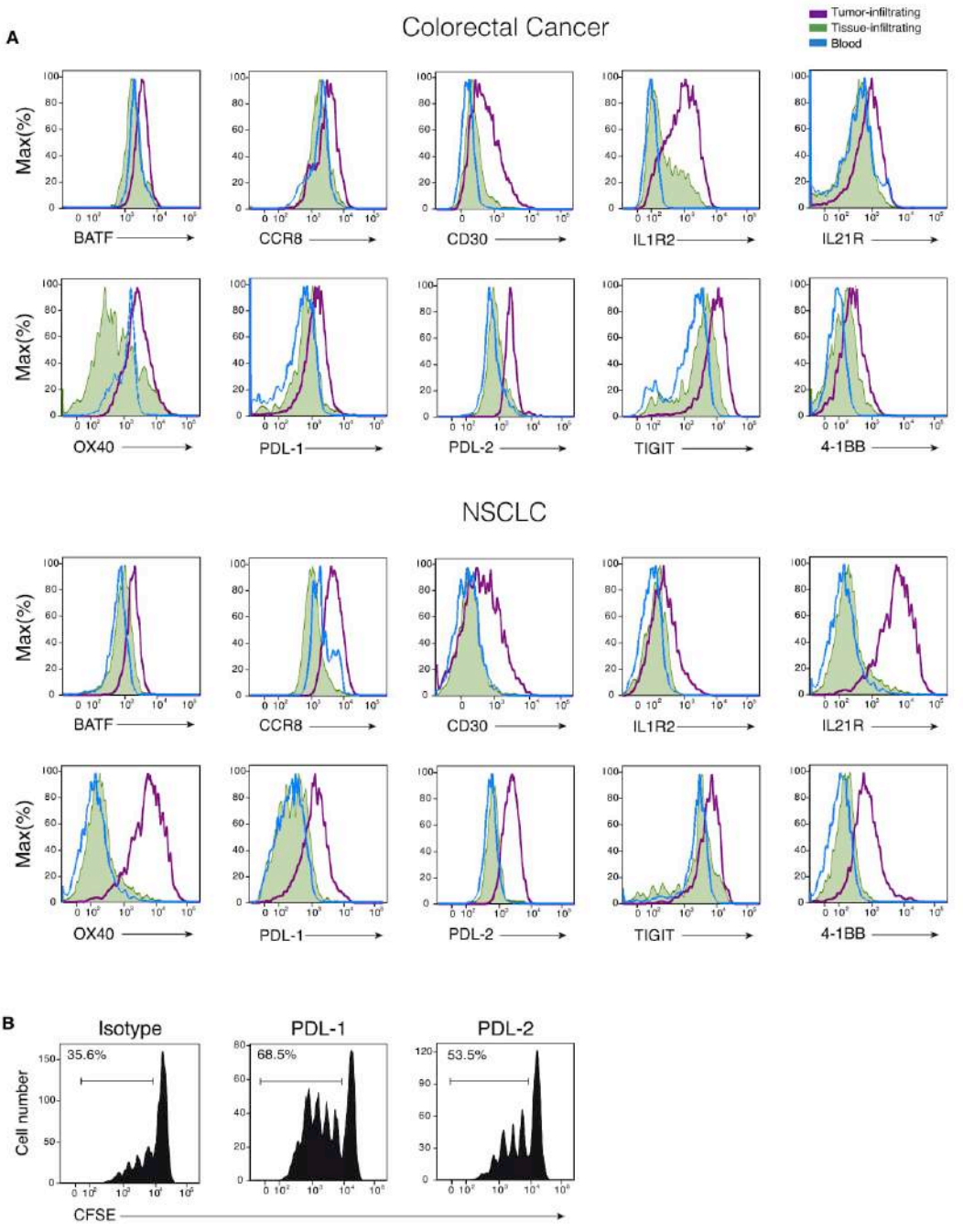
(B) Percentage of co-expression of signature genes with *FOXP3* and *IL2RA* is depicted.

(C) Expression levels of the signature genes classified by the percentage of co-expression are represented as boxplot.

(D) Expression distribution (violin plots) in Treg cells infiltrating CRC, NSCLC, or PB. Plots representing the ontology classes of receptors, signaling and enzymatic activity, cytokine activity, and transcription factors are shown (Wilcoxon Mann Whitney test $p < 0.05$). Color gradient indicates the percentage of cells expressing each gene in Treg cells isolated from the three tissues.

(E) Gene-expression analysis of tumor Treg signature genes in different tumor types. Expression values are expressed as log2 (2^-DCt).

See also Figure S3.



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T cell subsets to external cues they are exposed to in tumor tissues. We found that tumor-infiltrating-Treg signature genes are not only largely shared between CRC- and NSCLC-infiltrating cells but are also conserved in breast and gastric cancers, as well as in CRC and NSCLC metastatic tumors (in liver and brain, respectively) suggesting that expression of these genes is a common feature of tumor infiltrating Treg cells that might correlate with Treg cell-specific function within the tumor microenvironment.

Although our knowledge on the function of immune checkpoints on lymphocytes is still incomplete, agonist or antagonist monoclonal antibodies targeting checkpoints are in clinical development. We have found that some of these checkpoints (such as GITR, OX40, TIGIT, LAG-3, and TIM-3) and some of their ligands (such as OX40LG, Galectin-9, CD70) are upregulated also in tumor-infiltrating Treg cells, and this fact should be taken into account in interpreting clinical results with checkpoint inhibitors. Indeed, it is likely that assessment of the expression of checkpoints and of their ligands on the various subsets of tumor infiltrating lymphocytes will help to elucidate conflicting results and provide the rationale for combination therapies. Therefore, expression pattern of checkpoints should be evaluated both in tumor-infiltrating lymphocytes and in tumor cells.

Single-cell analysis on selected tumor Treg signature genes confirmed the whole transcriptomic data and provided information on the expression frequency of these genes. Tumor-infiltrating Treg cells express with high frequency genes that are associated with increased suppressor activity, such as the well characterized *OX40*, *CTLA4*, and *GITR*. Moreover, there were a number of interesting and less expected genes the specific expression of which was validated also at the protein level. For example, IL-1R2 upregulation could be another mechanism that tumor resident Treg cells employ to dampen anti-tumor immune responses through the neutralization of IL-1β function on effector cells. PD-L1 and PD-L2 expression has been recently reported on activated T cells or APCs (Boussiotis et al., 2014; Lesterhuis et al., 2011; Messal et al., 2011) but, to the best of our knowledge, neither PD-L2 nor PD-L1 expression has ever been reported in Treg cells, and our finding that they are overexpressed in tumor infiltrating Treg cells adds an additional level of complexity to the PD1 - PD-Ls immunomodulatory axis within the tumor microenvironment. BATF is a transcription factor that has been mainly associated to Th17 development and $CD8^+$ T cells differentiation (Murphy et al., 2013). Our findings revealed that *BATF* transcript is upregulated in tumor-infiltrating Treg cells more than in tumor infiltrating Th17 cells (Figure S4). Expression of BATF in $CD8^+$ T cells is induced by IL-21 (Xin et al., 2015), and we found that IL21R is highly expressed in tumor-infiltrating Treg cells (Figure 4).

We showed that tumor-infiltrating Treg cells express high amounts of 4-1BB (CD137) a marker of TcR-mediated activation (Schoenbrunn et al., 2012) and have shown they display very high suppressor function on effector T cell proliferation. It could be that expression of the signature genes correlated with the enhanced suppressive ability and so contributed to the establishment of a strong immunosuppressive environment at tumor sites.

A corollary to our findings would have that increased number of Treg cells in the tumor environment should associate with a worst clinical outcome. In fact, when *LAYN*, *MAGEH1*, and *CCR8* (which represent three of the most enriched genes in tumor-infiltrating Treg cells) are highly detected in whole-tumor samples there is a significant worsening of the 5-year survival of both CRC and NSCLC patients. Although, the functional roles in Treg cells of LAYN, a transmembrane protein with homology to c-type lectin (Borowsky and Hynes, 1998), and of MAGEH1, a member of the melanoma antigen gene family (Weon and Potts, 2015), are unknown, the high expression of the chemokine receptor CCR8 is instead intriguing. Indeed, CCL18, the ligand of CCR8 (Islam et al., 2013), is highly expressed in different tumors including NSCLC (Chen et al., 2011; Schutyser et al., 2005). The high specificity of CCR8 expression on tumor-infiltrating Treg cells suggests it could be an interesting therapeutic target to inhibit Treg cells trafficking to tumor sites, without disturbing recruitment of other effector T cells that do not express CCR8.

Considerable efforts have been recently put in the development of sophisticated bioinformatics approaches that exploit lymphocyte gene-expression data to understand the immune-modulatory networks at tumor sites, to predict clinical responses to immune-therapies, and to define therapeutic targets (Bindea et al., 2013a; Bindea et al., 2013b; Gentles et al., 2015). The data we present here represent a comprehensive RNA-sequencing analysis performed on tumor-infiltrating human $CD4^+$ Treg, Th1, and Th17 cells. Our findings highlight the relevance of assessing gene-expression patterns of lymphocyte at tumor-sites and suggest that generation of more transcriptomic data of tumor-infiltrating lymphocyte subsets purified from different cancer types might contribute to a better understanding of the dynamics underlying immune modulation in the tumor microenvironment. Moreover, our data represent a resource to generate and validate hypotheses that will increase our knowledge on tumor-infiltrating Treg cell biology and should lead to the identification of therapeutic targets.

EXPERIMENTAL PROCEDURES

Human Primary Tissues

Primary human lung or colorectal tumors and non-neoplastic counterparts were obtained from 15 and 14 patients, respectively. Patients' records

Figure 4. Expression of Tumor-Infiltrating Treg Cells Protein Signatures in CRC and NSCLC Samples

(A) Representative flow cytometry plots for tumor (purple line) normal (green area) tissue infiltrating Treg cells and peripheral blood Treg cells (blue line) analyzed for the expression of the indicated proteins.

(B) Flow cytometry plots representative of four independent experiments showing suppressive activity of CRC infiltrating Treg cells on proliferation (shown as CFSE dilution) of $CD4^+$ effector T cells. First panel shows the inhibitory effect of Treg cells on the effector T cell proliferation in the presence of an isotype control antibody. The other panels show the inhibitory effect of Treg cells that have been preincubated with anti PD-L1 or PD-L2 antibodies. Percentage of proliferating cells are indicated. The calculated division index is 0.26 in the presence of the control antibody; 0.57 in the presence of anti-PDL-1 and 0.39 in the presence of anti-PDL-2. Data are representative of four independent experiments.

See also Figure S4.

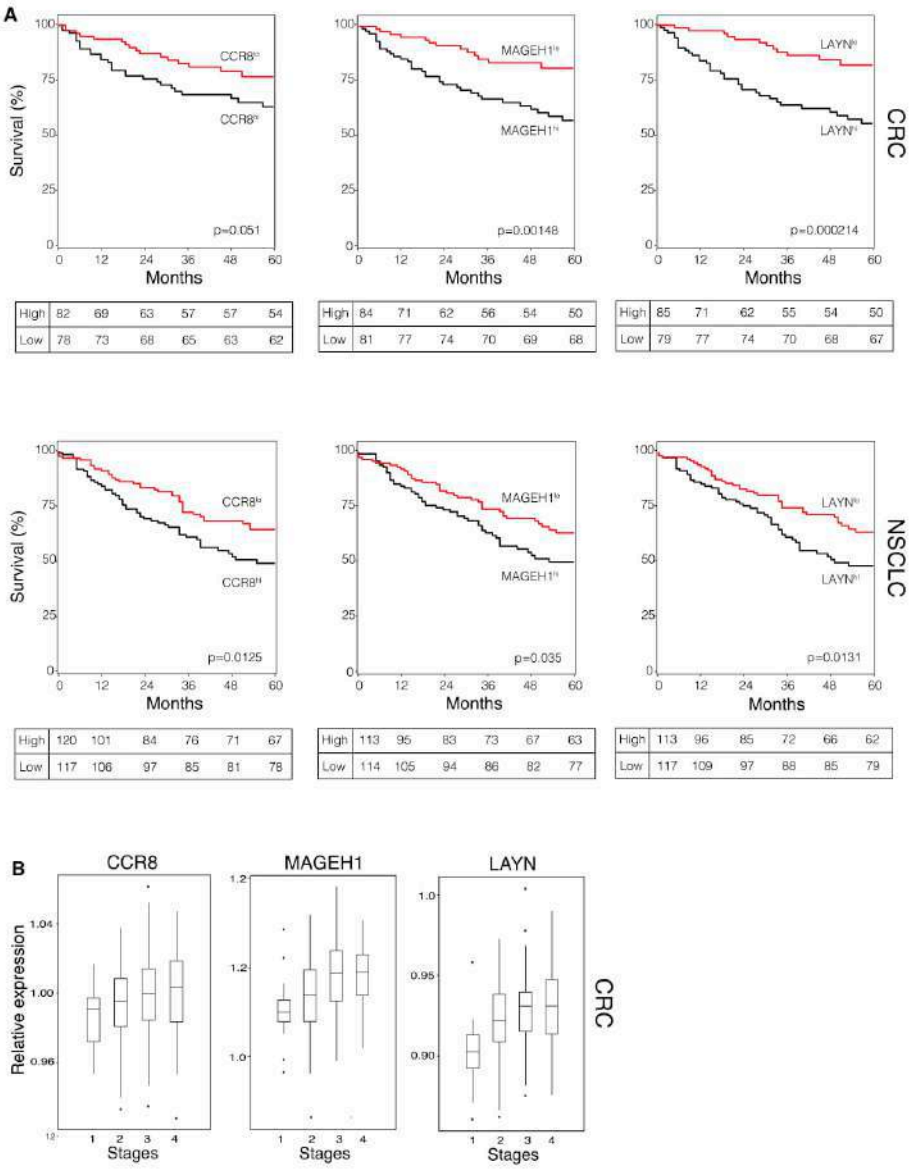

Figure 5. Prognostic Value of Signature Transcripts of Tumor Infiltrating Treg Cells

(A) Kaplan-Meier survival curve comparing the high and low expression of the tumor Treg signature transcripts (*CCR8*, *MAGEH1*, *LAYN*) normalized to the *CD3G* for the CRC (n = 177) and NSCLC (n = 263) studies. Univariate analysis confirmed a significant difference in overall survival curve comparing patients with high and low expression. Statistical significance was determined by the log-rank test. (CRC: p = 0.05 for *CCR8*, p = 1.48 × 10^{-3} for *MAGEH1*, p = 2.1 × 10^{-4} for *LAYN*; NSCLC: p = 0.0125 for *CCR8*, p = 0.035 for *MAGEH1*, p = 0.0131 for *LAYN*.) Each table depicts the Kaplan-Meier estimates at the specified time points.

(B) Expression distributions of *CCR8*, *MAGEH1*, and *LAYN* according to tumor staging at the time of surgery in the cohort of CRC patients.

See also Figure S5.

clinicopathological staging, tumor histotype, and grade are listed in Table S1. Informed consent was obtained from all patients, and the study was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda (approval n.30/2014). No patient received palliative surgery or neo-adjuvant chemo- and/or radiotherapy. NSCLC specimens were cut into pieces and single-cell suspensions were prepared by using the Tumor Dissociation Kit, human and the gentleMACS Dissociator (Miltenyi Biotech cat. 130-095-929). Cell suspensions were than isolated by ficoll-hypaque density-gradient centrifugation (Amersham Bioscience). CRC specimens were cut into pieces, incubated in 1 mM EDTA (Sigma-Aldrich) for 50 min at 37°C, and then incubated in type D collagenase solution 0.5 mg/mL (Roche Diagnostic) for 4 hr at 37°C. T cell fractions were recovered after fractionation on a four-step gradient consisting of 100%, 60%, 40%, and 30% Percoll solutions (Pharmacia). See also Supplemental Experimental Procedures.

$CD4^+$ T cell subsets were purified by flow cytomtery sorting using the following fluorochrome conjugated antibodies: anti-CD4 APC/Cy7 (clone OKT4), anti-CD27 Pacific Blue (clone M-T271), anti-IL7R PE (clone MB15-18C9), anti-CD25 PE/Cy7 (clone BC96), anti-CXCR3 PE/Cy5 (clone 1C6/CXCR3), anti-CCR6 APC (clone G034E3), and anti-CCR5 FITC (clone j418F1) using a FACSAria II (BD).

RNA Isolation and RNA Sequencing

RNA from tumor-infiltrating lymphocytes was isolated using mirVana Isolation Kit. Libraries for Illumina sequencing were constructed from 50 ng of total RNA with the Illumina TruSeq RNA Sample Preparation Kit v2. Paired-end sequencing (2 × 125) was then performed on an Illumina HiSeq 2500. See also Supplemental Experimental Procedures.

RNA-Seq Data Analysis, Mapping, and Quantification

Raw.fastq files were analyzed using FastQC v0.11.3, and adaptor removal was performed using cutadapt 1.8. Trimming was performed on raw reads using Trimmomatic: standard parameters for phred33 encoding were used. Reads mapping to the reference genome (GRCh38) was performed on quality-checked and trimmed reads using STAR 2.4.1c. The reference annotation is Ensembl v80. The overlap of reads with annotation features found in the reference.gtf was calculated using HT-seq v0.6.1. The output computed for each sample (raw read counts) was then used as input for DESeq2 analysis. Raw counts were normalized using DESeq2's function "rlog," and normalized counts were used to perform and visualize principal component analysis (PCA) results (using DESeq2's "plotPCA" function). See also Supplemental Experimental Procedures.

Differential Expression Analysis

Differential expression analyses of tumor-infiltrating $CD4^+$ Treg, Th1, and Th17 subsets versus $CD4^+$ Treg, Th1, and Th17 from PBMC were performed using DESeq2. Regulated genes were selected for subsequent analyses if their expression values were found to exceed the threshold of 0.05 FDR (Benjamini-Hochberg correction).

SOM Analysis

SOM analyses were carried out using the R package oposSOM using default parameters. Expression values of genes selected in the previous differential expression step were *Z*-score normalized and supplied in input to the automated pipeline for SOM training and analysis. Genes from regulated spots in the bidimensional output space were selected according to FDR threshold (< 0.1) at group-level. Expression values of genes assigned to regulated spots extracted from the oposSOM output were subject to correlation analysis using model vectors to further refine the results and genes having expression profiles with $p < 0.05$ were discarded from further analysis and signature definition. See also Supplemental Experimental Procedures.

GO Analysis

A GO enrichment analysis was performed for biological process terms associated with genes assigned to upregulated spots in the SOM bidimensional space using DAVID. Adjusted p (< 0.05) has been used for terms ranking and selection.

Capturing of Single Cells, cDNA Preparation, and Single-Cell PCR

Treg cells from CRC and NSCLC were isolated as previously described (see also Table S1). Single cells were captured on a microfluidic chip on the C1 System (Fluidigm) and whole-transcriptome amplified cDNA was prepared on chip using the SMARTer Ultra Low RNA kit (Clontech). For qPCR experiments, harvested cDNA from single cells was pre-amplified using the same pool of TaqMan gene expression assays to be used for qPCR. Single-cell gene expression experiments were performed using the 96 × 96 quantitative PCR (qPCR) DynamicArray microfluidic chips (Fluidigm) on a BioMark real-time PCR reader following manufacturer's instructions. A list of the 78 TaqMan assays used in this study is provided in Supplemental Experimental Procedures.

Single-Cell Data Analysis

Raw Ct data have been converted to Log2Exp. Co-expression analysis has been performed by considering both CRC and NSCLC samples and genes for which co-expression with *FOXP3* and *IL2RA* was null were discarded for the subsequent analysis. Gene expression was depicted as violin plots after log2 scale transformation. The violin color gradient represents the percentage of cells that are expressing the gene of interest. A non-parametric test (Mann-Whitney $p < 0.05$) has been performed on the selected genes by comparing tumor versus peripheral blood samples (see also Supplemental Experimental Procedures).

Flow Cytometry Analysis

Surface markers were directly stained with the following fluorochrome-conjugated antibodies and analyzed by flow cytometry: anti-CD4 (OKT4), anti-PD1-LG2 (CL24F.10C12), anti-CD127 (clone RDR5), anti-CD25 (clone 4E3), anti-4-1BB (clone 4B4), anti-CCR8 (Biolegend clone L263G8), anti CD30 (eBioscience, clone Ber-H2), anti-PD-L1 (Biolegend clone 29E.2A3), anti-TIGIT (eBioscience, clone MBSA43), anti-IL1R2 (R and D clone 34141), IL21R (Biolegend clone 2G1-K12), and anti-OX40 (Biolegend clone Ber-ACT35). FOXP3 and BATF intracellular staining was performed with anti-FOXP3 antibody (clone 236A/E7), anti-BATF (clone MBM7C7), and expression analyzed by flow cytometry. See also Supplemental Experimental Procedures.

Suppression Assay

(CFSE)-labeled responders $CD4^+$ $Naive^+$ T cells from healthy donors were cocultured with different effector to target (E/T) ratios with unlabeled $CD127^-CD25^{low}CD4^+$ T cells sorted from TILs or PBMCs of patients with CRC or NSCLC, using FACS Aria II (BD Biosciences), in the presence of $CD11c^+$ $CD1c^+$dentritic cells as antigen-presenting cells and anti-CD3 (OKT3) mAb. Proliferation of CFSE-labeled cells was assessed after 96 hr. Some suppression assays were also performed with tumor Treg cells that were preincubated with the following antibodies (at a final concentration of 20 μg/ml): anti-human PD-L1 (Biolegend clone 29E.2 A 3), anti-human PD-L2 (Biolegend clone MIH18), and anti-human Functional Grade as isotype control (eBioscience clone MBSA43).

Kaplan-Meier Analysis

The Kaplan-Meier analysis (KM) was used to compare the high and low expression of the tumor-Treg signature transcripts either CRC (GSE17536, n = 177) and NSCLC (GSE41271, n = 263) patients. See also Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession numbers for the data in this paper are as follows: ENA: PRJEB11844 for RNA-seq tumor and tissue infiltrating lymphocytes; ArrayExpress: E-MTAB-2319 for RNA-seq human lymphocytes datasets; ArrayExpress: E-MTAB-513 for Illumina Human BodyMap 2.0 project; GEO: GSE50760 for RNA-seq datasets CRC; GEO: GSE40419 for RNA-seq datasets NSCLC; GEO: GSE17536 for CRC expression profiling by array; and GEO: GSE41271 for NSCLC expression profiling by array.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.10.021.

AUTHOR CONTRIBUTION

M.D., A.A., G.R., and P.G. designed and performed the main experiments, analyzed the data, and contributed to the preparation of the manuscript. V.R. and R.J.P.B. set up all the bioinformatics pipelines, performed the bioinformatics analyses, and contributed to the preparation of the manuscript. S.M., M.M., M.C., E.P., C.P., M.L.S., I.P., and V.V. performed experiments and analyzed the data. S.B., V.V., N.Z., and G.B. coordinated pathology analyses. A.P., L.S., M.T., N.M., P.C.A., O.E., and L.G. coordinated clinical contributions. R.A.P., G.C., R.D.F., H.G.S., and J.G. discussed results, provided advice, and commented on the manuscript. M.D., A.A., G.R., S.A., and M.P. wrote the manuscript. S.A. and M.P. designed the study and supervised research. All authors discussed and interpreted the results.

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