UNIVERSIDAD DE CHILE – FACULTAD DE CIENCIAS - ESCUELA DE PREGRADO

“Tr1 cells reside within the tumor microenvironment: Comparison with conventional Foxp3+ T regulatory cells”

Seminario de Título entregado a la Universidad de Chile en cumplimiento parcial de los requisitos para optar al Título de Ingeniera en Biotecnología Molecular

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INFORME DE APROBACIÓN SEMINARIO DE TÍTULO

Se informa a la escuela de Pregrado de la Facultad de Ciencias de la Universidad de Chile que el Seminario de Título, presentado por la **Srta. Pamina Contreras Kallens**

"Tr1 cells contribute to the immunosuppressive tumor microenvironment by infiltrating better the tumor site than conventional Foxp3+ T regulatory cells"

Ha sido aprobado por la Comisión de Evaluación, en cumplimiento parcial de los requisitos para optar al Título de Ingeniera en Biotecnología Molecular.

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Santiago de Chile, ……

BIOGRAFÍA

Mi nombre es Pamina Contreras Kallens, nací el 3 de noviembre de 1994 en Santiago de Chile.

En 1999 entré a prekinder en el colegio Santa María de Lo Cañas en donde estuve hasta tercero básico. Luego, entré a cuarto básico en el colegio Pedro de Valdivia, en donde terminé mi enseñanza escolar.

El 2013 entré a Ingeniería en Biotecnología molecular en la Universidad de Chile egresando y obteniendo el grado de licenciada en Ingeniería en Biotecnología molecular el año 2018.

El 2016 entré al laboratorio de Inmunología de la Universidad de Los Andes, en donde realicé mi Unidad de Investigación y Seminario de Título bajo la guía de la Dra. Karina Pino-Lagos.

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LIST OF ABBREVIATIONS

AMP: Adenosine monophosphate

ANOVA: Analysis of variance

APC-Cy7: Allophycocyanin-Cy7

APC: Allophycocyanin

APCs: Antigen presenting cells

ATP: Adenosine triphosphate

BM: Bone marrow

CCL2: Chemokine (C-C motif) ligand 2

CCR2: C-C chemokine receptor type 2

CCR4: C-C chemokine receptor type 4

CCR5: C-C chemokine receptor type 5

CCR7: C-C chemokine receptor type 7

CD: Cluster of differentiation

cDNA: Complementary DNA

cTregs: Conventional T-regulatory cells

DCs: Dendritic cells

DNA: Deoxyribonucleic acid

EAE: Experimental autoimmune encephalomyelitis

EMC: Extracellular matrix

EMT: Epithelial to mesenchymal transition

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

Foxp3: Forkhead box P3 transcription factor

I.d.: Intradermal

I.p.: intraperitoneal

IBD: Inflammatory bowel disease

IFNγ: Interferon gamma

IFNγR1: Interferon gamma receptor 1

IFNγR2: Interferon gamma receptor 2

IL-10: Interleukin 10

IL-17A: Interleukin 17A

IL-2: Interleukin 2

IL-5: Interleukin 5

LAG-3: Lymphocyte-activation gene 3

LN: lymph nodes

MFI: median fluorescence intensity

MHC: major histocompatibility complex

MLN: mesenteric lymph nodes

nLN: naïve lymph nodes

non-Treg: non T-regulatory cells

Nrp1: Neuropilin-1

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PE-Cy7: Phycoerythrin-Cy7

PE: Phycoerythrin

PerCP-Cy5.5: Peridinin Chlorophyll-a Protein-Cy5.5

PerCP: Peridinin-Chlorophyll-protein

PLN: Peripheral lymph nodes

qPCR: Real-time polymerase chain reaction

Rab27α: Ras-related protein α

Rab27β: Ras-related protein β

RNA: Ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

RT: Room temperature

SP: Spleen

t-SNE: t-Distributed Stochastic Neighbor Embedding

TME: Tumor microenvironment

TAMs: Tumor-associated macrophages

TdLNs: Tumor-draining lymph nodes

Th1: T helper cell type 1

Th1: T helper cell type 2

TILs: Tumor-infiltrating lymphocytes

TNF-α: Tumor necrosis factor alpha

Tr1: Type 1 regulatory T cells

Tregs: regulatory T cells

VEGF: Vascular endothelial growth factor

1 RESUMEN

La función supresora de las células T reguladoras (Tregs) puede tener efectos negativos sobre la respuesta inmune antitumoral. Por lo tanto, es de gran importancia estudiar los factores que alteran la eficiencia de su inhibición, de tal forma de poder mejorar las terapias antitumorales. La alta heterogeneidad de las Tregs periféricas es uno de los problemas que deben ser abordados para mejorar y desarrollar nuevas terapias antitumorales. Dentro del subset de Tregs, nuevos marcadores superficiales para la población T reguladora tipo 1 (Tr1) no-clásica han sido reportados recientemente, permitiendo su identificación mediante la expresión de las moléculas CD49b y LAG-3 en su superficie. El efecto terapéutico de la población identificada mediante estos marcadores ya ha sido estudiado en modelos murinos de diabetes y de artritis inducida por colágeno, en los cuales mostraron un efecto protector. Sin embargo, su rol en el contexto tumoral ha sido poco estudiado. Es por esto por lo que buscamos caracterizar a la población Tr1, identificada mediante la expresión de CD49b, en un modelo murino de melanoma. Sorprendentemente, se encontró que su presencia parece estar fuertemente influenciada por el microambiente en el cual se encuentra. Mientras que en los linfonodos drenantes de tumor (TdLNs) este subset compone tan solo el 4% del total de células T CD4+, en el tumor alcanzan un 30% de las células T CD4+. Por otra parte, las Tregs convencionales Foxp3+ (cTregs), componen alrededor de un 15% de los linfocitos que infiltran el tumor (TILs) que expresan CD4, casi la mitad de lo observado para las Tr1. En cuanto a su fenotipo, se observó que, aunque en menores niveles que las cTregs, alrededor del 50% y 30% de las Tr1 expresan Nrp1, en los TdLNs y en el tumor, respectivamente. Esta

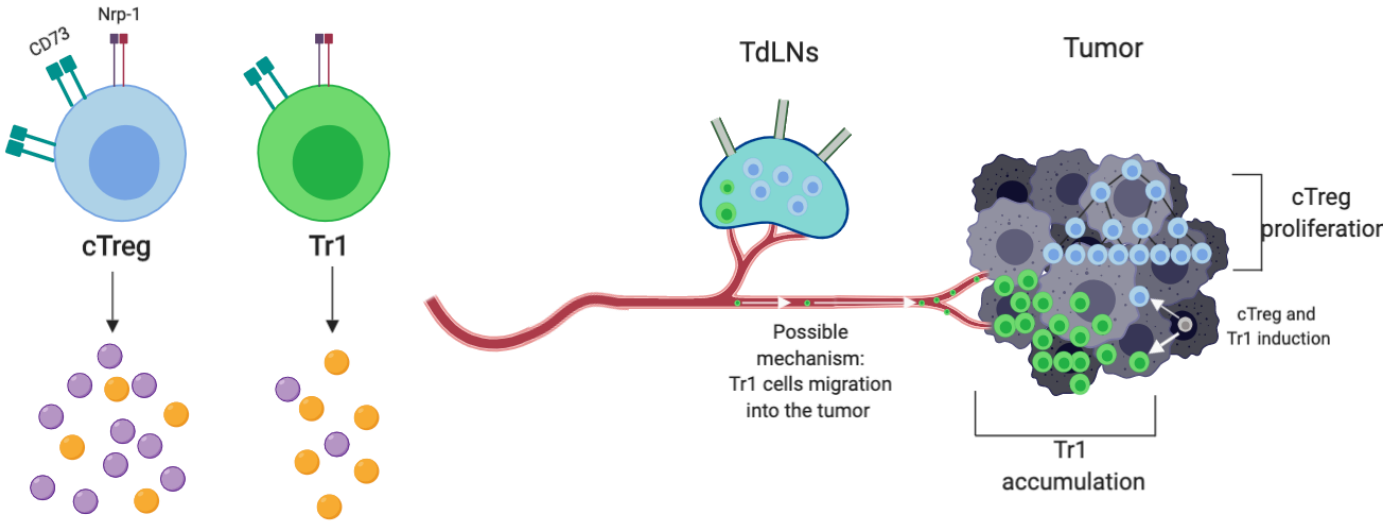
molécula es un co-receptor de VEGF, y se ha descrito que es esencial para la estabilidad y función del fenotipo supresor de las cTreg y para la progresión tumoral. Además, se observó que las Tr1 muestran un patrón diferencial de expresión de ciertas moléculas reguladoras, comparado con las cTregs: una mayor intensidad mediana de fluorescencia de la ectonucleotidasa CD73 en el tumor, contrario a lo que se observa en los TdLNs, y una menor producción de IL-10 en el tumor. Se encontró además que la capacidad proliferativa de las cTregs es significativamente mayor a la de las Tr1, tanto en el tumor como en los TdLNs. Así, nuestros resultados destacan las posibles diferencias entre los mecanismos de inmunosupresión de los subsets de Tregs, los cuales pueden variar dependiendo del microambiente (TdLNs versus tumor).

2 ABSTRACT

T regulatory cells (Tregs) suppressive function can have a detrimental effect on immune responses against tumor cells. Thus, in order to improve actual anti-tumor therapies, it is of great importance to study the factors altering their inhibition efficiency. The high heterogeneity of peripheral Tregs is one of the problems that have to be addressed to enhance and develop novel anti-tumoral therapies. Within the Tregs subsets, new surface markers for the non-classical type 1 regulatory T cells population (Tr1) have been recently reported, allowing their identification through the expression of the CD49b and LAG-3 molecules. Their therapeutic effect has already been studied in murine models of diabetes and collagen-induced-arthritis, in which they displayed a protective effect. Nevertheless, very few studies have focused on investigating their role in the tumoral context. Thus, we sought to investigate the function of the Tr1 cell subset, identified through the expression of CD49b, in a murine melanoma model. Surprisingly, we found that their presence seems to be strongly influenced by the microenvironment they encounter. Whereas in the tumor-draining lymph nodes (TdLNs) this subset composes only around 4% of total CD4+ T cells, in the tumor, they compose almost 30% of CD4+ T cells. On the other hand, conventional Fopx3+ Tregs (cTregs) compose around 15% of CD4+ tumor-infiltrating T cells, almost half of the percentage of Tr1 cells. Regarding their phenotype, we observed that, although in lower levels than cTregs, around 50 and 30% of Tr1 cells express Neuropilin-1 (Nrp1), in the TdLNs and in the tumor, respectively. Nrp1 is a VEGF co-

receptor, which has been described to be essential for the stability and function of cTregs suppressive phenotype and in tumor progression. Even more, we also observed that Tr1 cells show a differential pattern of expression of some regulatory molecules, compared to cTregs: a higher median fluorescence of the ectonucleotidase CD73 in the tumor microenvironment, contrary to what is seen in the TdLNs, and a lower production of IL-10 in the tumor. Furthermore, we found that the proliferative capacity of cTregs is significantly higher to Tr1 cells, both in the tumor and in the TdLNs. Thus, our results further highlight the possible differences between the immunosuppression mechanisms of the Tregs subsets depending on the microenvironment (TdLNs versus tumor site).

3 GRAPHICAL ABSTRACT



TdLNs	
%CD4	cTreg > Tr1
%Nrp-1+	cTreg > Tr1
%CD73+	cTreg~Tr1
CD73 MFI	cTreg> Tr1
%IL-10+	cTreg~Tr1
%IFNγ+	Tr1>cTreg
Proliferative capacity	cTreg> Tr1

Tumor	
%CD4	Tr1 > cTreg
%Nrp-1+	cTreg > Tr1
%CD73+	Tr1>cTreg
CD73 MFI	cTreg~Tr1
%IL-10+	cTreg>Tr1
%IFNγ+	Tr1~cTreg
Proliferative capacity	cTreg> Tr1

4 INTRODUCTION

Regulatory T cells (Tregs) are essential for modulating T cells activity and maintaining immunologic tolerance. However, their suppressive function can have detrimental effects on immune responses against tumor cells (Sakaguchi et al. 1995). Thus, it is of great importance to study the factors affecting their inhibition efficiency for improving anti-tumor therapies. To date, several subsets of Tregs have been described, which can be divided into two major groups depending on the expression of the transcription factor Foxp3: Foxp3+ Tregs, and Foxp3- Tregs (Fontenot et al. 2003; Groux et al. 1997). Although the population of Foxp3+ Tregs can be further subdivided in thymus- or periphery- generated Foxp3+ Tregs, we will refer to them as conventional Tregs (cTregs) based on the presence of Foxp3+ and not differentiating them by their origin.

In 2004, Vieira et al. reported a group of T cells that in spite of the lack of Foxp3 expression showed a suppressive profile due to the elevated production of IL-10 (Vieira et al. 2004). This unique Treg cells subset was termed Tr1 and it has been isolated both in vitro and in vivo. In vitro, the generation of this subset occurs under conditions of high IL-10 and chronic antigen stimulation, while in vivo its biological relevance has been shown in several experimental models such as colitis, experimental autoimmune encephalomyelitis (EAE), diabetes, airway inflammation, infection, inflammatory bowel disease (IBD) and Islet transplants (Groux et al. 1997; Barrat et al. 2002; Battaglia et al. 2006; Roncarolo et al. 2006). Until recently, no unique marker had been reported for the

identification of this cell subset; however, in 2013, Gagliani et al. demonstrated that the co-expression of CD49b and LAG-3 was sufficient to identify Tr1 cells both in humans and in mice (Gagliani et al. 2013). This subset does not express the transcription factor Foxp3, which has been proposed as the master regulator of the development and function of cTreg cells (Vignali et al. 2008). Regarding their cytokine secretion profile, Tr1 cells are able to produce IL-5, IL-10, TGFβ and significant amounts of IFNγ, but produce low levels of IL-2, IL-4 and IL-17A (Groux et al. 1997; Vieira et al. 2004; Rahmoun et al. 2006; Gagliani et al. 2013). Among the mechanisms of suppression reported for Tr1 cells, we can find: inhibition of both naïve and memory T cell proliferation, suppression of Th2- and Th1- mediated immune response, cytotoxic activity through the secretion of granzyme B and perforin in human Tr1 cells (killing CD4+, CD8+ T cells, CD14+ monocytes and dendritic cells (DCs), and allogeneic tumor cell lines) (Vieira et al. 2004; Groux et al. 1997; Cottrez et al. 2000; Grossman et al. 2004; Roncarolo et al. 2006). Tr1 cells cytotoxic activity towards antigen presenting cells (APCs) has been demonstrated to occur specifically after the binding of CD226, an adhesion/signaling molecule, to CD155 and CD112, which are expressed on myeloid cells (Magnani et al. 2011; Gregori et al. 2012). The activation that occurs via CD226 leads to Tr1 cells activation and degranulation, causing the secretion of granzyme B and perforin (Magnani et al. 2011; Yan et al. 2017). Tr1-mediated suppression also includes the secretion of IL-10 and TGFβ since it was demonstrated that the neutralization of these molecules ablates its immunosuppressive effects, although not completely (Groux et al. 1997; Levings et al. 2001). Furthermore, as cTreg cells, Tr1 cells can modulate APCs by inhibiting their maturation and antigen

presentation or by down-regulating their expression of CD80/86 in a CTLA-4 and/or LAG-3 dependent manner (Roncarolo et al. 2014; Vignali et al. 2008).

By definition, the tumor microenvironment (TME) corresponds to a tolerogenic site composed of different types of cells interacting to prevent anti-tumor immunity and to promote angiogenesis and tumor growth (Becker et al. 2013). It has been reported that both CD4+ and CD8+ T cells can infiltrate tumors and mediate an antitumor effect. CD8+ T cells can kill tumor cells with cytotoxic molecules, such as granzymes and perforin, and can also secrete IFNγ, which increases the expression of MHC class I antigens by tumor cells, thus facilitating their targeting by CD8+ T cells (Tsukumo & Yasutomo 2018). On the other side, infiltrating CD4+ T cells can augment the accumulation of CD8+ T cells within the tumor and promote the expansion, trafficking, differentiation and cytotoxicity of tumor-specific CD8+ T cells through IL-2 stimulation, direct cell-cell interaction, and DCs licensing, thus allowing further CD8+ T cell activation (Brakeman et al. 1997; Lai et al. 2011). In addition to providing help for tumor-reactive CD8+ T cell responses, CD4+ T cells can also exert cytotoxic activity against tumor cells, mediate the up-regulation of MHC molecules expression on tumor cells, inhibit angiogenesis within the tumor and induce tumor dormancy, mainly through the secretion of IFNγ and TNFα (Lai et al. 2011). The infiltration of cTreg cells into the tumor and the activation of their immunosuppressive activities can dampen the effect of the aforementioned anti-tumor activities of T cells (Perrone et al. 2008; P. Yu et al. 2005). The mechanisms mediating the immunosuppressive effects of cTregs inside the tumor and various therapies targeting this subset have been already extensively studied and reviewed (Chaudhary & Elkord 2016b).

On the other side, Tr1 cells have already been researched in TME, however very few studies have used CD49b and LAG-3 as the markers for their identification; therefore, we aim to characterize and phenotype Tr1 cells identified as CD49b+Foxp3-CD4+ T cells in tumor-bearing animals

5 HYPOTHESIS

CD4+ T regulatory 1 cells, characterized by the lack of expression of Foxp3 and the expression of CD49b, are present in melanoma tumor and differ from conventional Foxp3+ T regulatory cells at the phenotypic level.

6 AIMS

6.1 General aim

To compare the frequencies of conventional Foxp3+ T regulatory cells (cTreg cells) and T regulatory 1 cells (Tr1 cells), and other cellular traits in melanoma-bearing mice.

6.2 Specific aims

5.2.1 To compare the phenotype of cTregs and Tr1 cell subsets in tumor-draining lymph nodes and tumor site of melanoma-bearing mice.

5.2.2 To describe the proliferative capacity of both cTregs and Tr1 cells in tumor-draining lymph nodes and tumor site of melanoma-bearing mice.

5.2.3 To determine the levels of expression of key characteristic genes associated with mechanisms of suppression related to cTregs and Tr1 cells obtained from tumor-draining lymph nodes and tumor masses harvested from melanoma-bearing mice.

7 MATERIALS AND METHODS

7.1 Animals

Wild type C57/BL6 and Foxp3/GFP reporter mice (male and female 6-8 weeks old) were housed at the animal facility located at the Universidad de los Andes and handled following the guidelines of Bioethical Committee of this Institution.

7.2 Cell cultures

B16 melanoma cell line (a gift from Dr. Daniela Sauma, Universidad de Chile) was cultured in RPMI 1640 medium (Sigma, Milano, Italy) supplemented with antibiotics and 10% fetal bovine serum at 37ºC in a humidified atmosphere containing 5% CO_2. When the culture reached 70% of confluence, the cells were detached by treatment with trypsin-EDTA (0.25%) (Gibco BRL, Paisley, United Kingdom) and a cell suspension was prepared for further cell number determination using trypan blue staining.

7.3 Determination of B16 cell dose for tumor induction

The optimal cell number necessary for tumor induction was determined by injecting 1 x 10^5 or 2 x 10^5 B16 cells in 100µl of sterile PBS into the right flank via intradermal injection (i.d). Tumor size was measured every other day using a digital caliper and the volume was calculated using the formula V = (L x W x W) / 2, where V is tumor volume, W is tumor width and L is tumor length in unit of mm^3.

7.4 Isolation of Tumor-infiltrating leukocytes (TILs)

Mice were euthanized around day 20 after tumor induction or when tumor volume reached ~2000 mm^3, and the tumor-draining lymph nodes (TdLNs) and tumor cells were harvested for analysis. Cell suspensions were prepared disrupting the tissue directly on 40 μm cell strainers (BD, Vienna, Austria). For the TILs preparation the solid tumor was first mechanically disrupted followed by enzymatic digestion for 40 min at 37ºC using a solution composed of DNase and Liberase (50 μg/ml and 250 μg/ml in plain RPMI, respectively) (both enzymes from Sigma, Milano, Italy). After that, the tumors were disrupted using 40 μm cell strainers and TILs were purified using a 40%/70% Percoll (GE Healthcare, IL, USA) gradient centrifugation as described previously (Newcomb et al. 2010). Briefly, after the enzymatic digestion, the cells were washed once in PBS and the pellet was resuspended in 4 ml of 40% isotonic Percoll and overlaid on 3 ml of 70% isotonic Percoll. Cells were then centrifuged at 800g for 40 min and TILs were collected from the 40% to 70% interface, washed once in PBS, and counted in a Neubauer Chamber.

7.5 Flow cytometry

Single cell suspensions were resuspended in PBS + 5% FBS and stained with fluorochrome-conjugated antibodies (as indicated in each figure) for 30 min at room temperature (RT). For intracellular cytokine staining, cells were first in vitro stimulated for 5 hours at 37ºC adding 50 ng/ml PMA (Sigma, Milano, Italy), 1 mg/ml ionomycin (Sigma, Milano, Italy), and 10 mg/ml Brefeldin A (eBioscience, CA, USA) to the culture

media. Subsequently, cells were stained for surface markers (for 30 min at RT). Cells were washed and then fixed using a permeabilization kit (Biolegend, CA, USA) according to the manufacturer's instructions, and subsequently stained for intracellular markers. The antibodies used were coupled to any of the following fluorochromes: FITC, PE, PerCP, APC, PerCP-Cy5.5, APC-Cy7 or PE-Cy7. The markers tested were: CD45, CD4, CD49b, CD73, IL-10, Foxp3, Nrp1, GATA-3 and IFNγ. Flow cytometric data was acquired using FACSCanto II (BD Immunocytometry System, CA, USA) and analyzed with FlowJo software (Treestar, OR, USA).

To perform visualization of complex flow cytometry data, we used the Cytobank computational tool viSNE (visualization of t-Stochastic Neighbor Embedding), that generates a two-dimensional map in which cell distance represents distance between cell parameters in high-dimensional space (Amir et al. 2013). Thus, cells that are phenotypically similar for the analyzed markers will be closer in a viSNE map (Becher et al. 2014; Leelatian et al. 2015). To generate viSNE maps, samples were uploaded to Cytobank, live single cells were gated based on cell size and length and negative to Zombie Dye viability staining and later gated in the CD4+ subset. Then, between 150,000 and 160,000 cells were subsampled from the data. After subsampling, viSNE was run at default parameters (1000 iterations, random seed, perplexity = 30, theta = 0.5). viSNE maps were visualized using Cytobank interface, which was used to generate figures (color coding by marker expression levels).

7.6 cTreg and Tr1 cells isolation

To fractionate CD4+ T cells into cTreg and Tr1 cells subpopulations, cell suspensions obtained from tumor-bearing mice (Foxp3/GFP reporter animals), both from TdLNs and the tumor, were stained with monoclonal antibodies against CD45, CD4 and CD49b (all from Biolegend, CA, USA) as described above. Cell sorting was performed using a BD FACSAria cytometer, identifying cTreg and Tr1 cells based on Foxp3/CD49b expression.

7.7 qRT-PCR

Total RNA was extracted from cTreg and Tr1 cells populations previously isolated either from TdLNs or tumor using the RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using the TopTaq Master Mix Kit (Qiagen, Hilden, Germany) in a thermal-cycler machine (Axygen, NY, USA). Gene expression levels were obtained by quantitative real-time RT-PCR (qPCR) using Stratagene Mx3000P I apparatus (Stratagene, CA, USA) and EvaGreen® qPCR Master Mix (Biotium, CA, USA). All primers (Table 1) were designed based on the coding sequences available on the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/Genbank Search.html). Expression of target gene was normalized to 18S as housekeeping gene expression levels within the same sample to determine ΔCq. The mean between technical replicates was then normalized to the expression of the target gene in the total TILs sample to find ΔΔCq expression.

Table 1. Primers sequences used in real-time RT-PCR.

Gene	Forward (5'-3')	Reverse (3'-5')
18S	GCCCGAAGCGTTTACTTTGA	TTGCGCCGGTCCAAGAATTT
Granzyme B	ATGCTGCTAAAGCTGAAGAGT	TTCCCCAACCAGCCACATAG
Perforin	TTGGTGGGACTTCAGCTTTCC	TTCCCCAACCAGCCACATAG
IL10	TGGGTTGCCAAGCCTTATCG	AGAAATCGATGACAGCGCCTC
TGFβ	CAGTGGAAAGACCCCACATCTC	GACGCAGGCAGCAATTATCC
βActin	CTAAGAGGAGGATGGTCGCG	CTCAGACCTGGGCCATTCAG
Rab27a	AAGGGATAGAGCACAGCGAG	ATAAACTCAATCCGGTTTGGTGCTC
Rab27b	TGAAAATGAGCCTGCCACCA	TGCCTGCAGTTGACTCATCC

7.8 Assessment of cell proliferation by 5-bromodeoxyuridine (BrdU) labeling

In order to test leukocyte proliferation in TdLNs and tumor a solution of BrdU (100 μg/g in sterile PBS) was injected into the animals via intraperitoneal (i.p). BrdU is an analog of the thymidine nucleotide and is incorporated inside the DNA during the S phase of mitosis (Gratzner 1982). Two BrdU pulses were used in this procedure, the first one at 12 days after tumor inoculation and the second one at day 15. Then, at day 20 the animals were euthanized, and the TdLNs and tumor were harvested. In order to have a positive control

for cell proliferation, bone marrow cells were also recovered and analyzed. After obtaining cell suspensions of each sample, they were stained for cell surface markers (CD4, CD45, CD49b). Then, cells were fixed and permeabilized with 1% of Paraformaldehyde and 0.01% of Tween-20 solution overnight as described before (Curran 2001). After that, cells were treated with DNAse I buffer for 10 minutes at RT. Finally, cells were stained with anti-BrdU (Sigma, Milano, Italy) and anti-Foxp3 (Invitrogen, CA, USA) antibodies and analyzed by flow cytometry.

7.9 Statistical Tests

Significance was determined using the GraphPad Prism software and, depending on the data distribution, parametric or non-parametric tests were used to compare different groups. In the case of having more than two groups, an ANOVA test was used (one-way or two-way).

RESULTS

7.10 Distribution of cTreg and Tr1 cells in naïve animals

To better understand the dynamics of Treg cells in animals under normal or experimental conditions, we first determined the frequencies of conventional Treg (cTreg) and Tr1 cells in different organs/tissues from wild-type mice. To facilitate the identification of cTreg cells (Foxp3+) we used Foxp3/GFP reporter animals in the experiments (Lin et al. 2007; Haribhai et al. 2007). Peripheral blood, spleen (SP), peripheral (PLN) and mesenteric (MLN) lymph nodes were harvested, and single cell suspensions were obtained and stained for CD45, CD4 and CD49b and analyzed by flow cytometry. cTregs corresponded to CD4+ T cells expressing Foxp3/GFP, and Tr1 cells were identified as CD4+CD49b+ T cells lacking Foxp3/GFP. Thus, Figure 1A shows the expression of Foxp3/GFP and CD49b on previously gated CD4+ T cells from the indicated organs, and the bar graphs show pooled data, Figure 1B. We can see that the frequency of cTregs is higher than of Tr1 cells in the PLN, while in the blood samples Tr1 cells tend to be higher than cTregs. As expected, in the thymus, both cTreg and Tr1 frequencies are very low, 1% and 0,4% respectively.

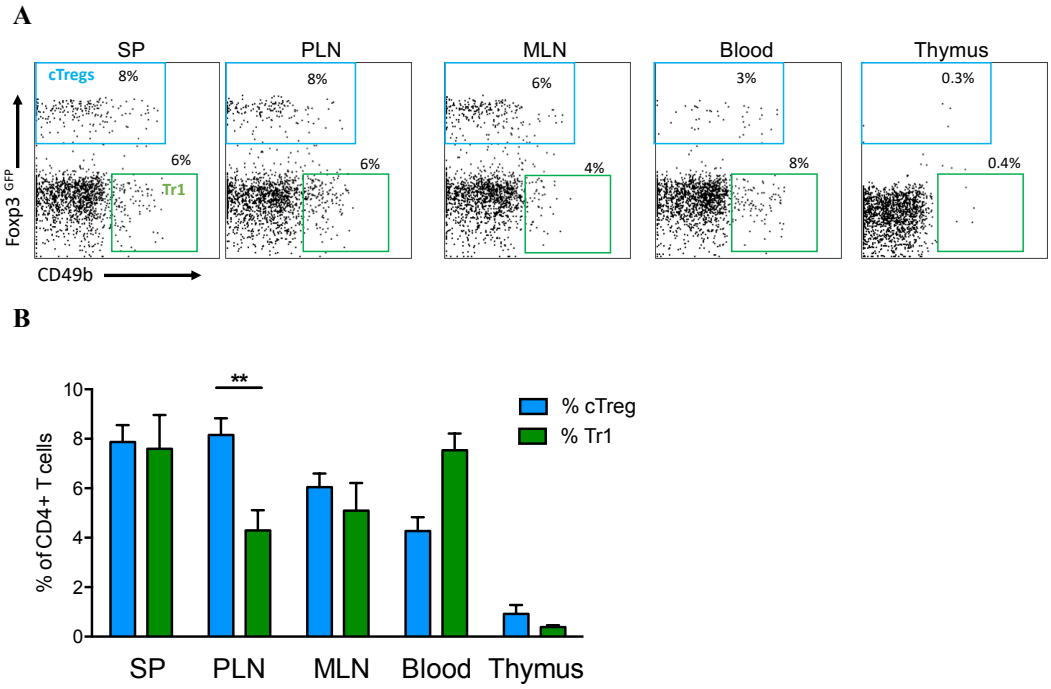


Figure 1. Distribution of cTreg and Tr1 cells among different tissues and organs. Spleen (SP), Peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN), blood and thymus were collected from naïve Foxp3/GFP reporter mice. Cell suspensions were obtained and stained for flow cytometry analysis. A. Dot plots show the expression of Foxp3/GFP and CD49b on previously gated CD4+ T cells. cTregs (indicated with a light blue square outline) were defined as CD4+Foxp3/GFP+ T cells while Tr1 cells (indicated with a green square outline) as CD4+Foxp3/GFP-CD49b+ T cells. B. The graph depicts the frequencies of both populations at the different organs/tissues. Frequencies are shown as mean ± s.e.m. n=6 animals. * p< 0,05; ** p< 0,01; *** p < 0,001; ns not significant according to according to Mann-Whitney Test.

7.2 B16 cell dose determination

As stated in the introduction section, Treg cells can infiltrate the tumor and block the effector function of other leukocytes resulting in inhibition of anti-tumor immunity (Facciabene et al. 2012). Thus, to study the dynamics of both cTreg and Tr1 cells in the tumor microenvironment we used a widely accepted tumor model in which recipient animals are inoculated with B16 melanoma cell line via an intradermal injection (i.d.) (Overwijk & Restifo 2001). In order to first determine the correct cell dose necessary for a homogeneous tumor growth between individuals, Foxp3/GFP reporter mice were administered with two different doses (1 and 2 x 10^5) of B16 cells in the right flank. Tumor growth was monitored and measured every other day. Tumor growth curves for both 1 and 2 x 10^5 inoculated cells are shown in Figure 2A and Figure 2B, respectively. As it can be seen in Figure 2A, injecting either 1 x 10^5 or 2 x 10^5 cells resulted in tumors being palpable at around day 13; however, a 2 x 10^5 cell dose generated a more consistent tumor growth between individuals and resulted in a higher tumor volume at day 20. Thus, it was determined that 2 x 10^5 cell per mice was an optimal dose for the tumor model.

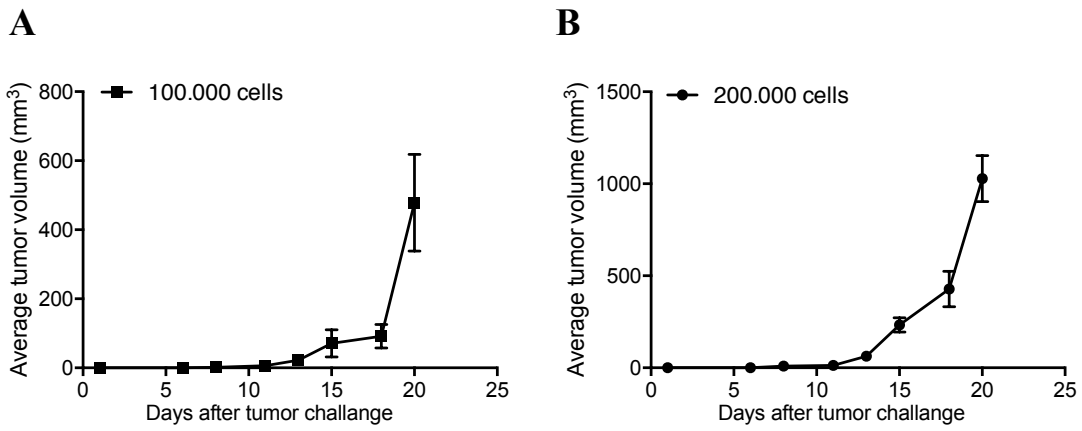


Figure 2. Tumor growth curves. A, B. 1 x 10^5 or 2 x 10^5 B16 cells were injected into the right flank of mice and tumor growth was monitored and measured using a digital caliper every other day from 2 to 20 days after tumor inoculation. Group mean values ± s.e.m. for these mice are shown at each point. n=4 animals for both groups.

7.11 Tr1 cells accumulate in the tumor

Once the optimal dose for tumor growth was determined, cell compartmentalization was investigated in tumor-bearing mice in both the TdLNs and at the tumor site. At day 20 (when the tumor reached ~1000 mm^3) mice were euthanized and both the TdLNs and the tumor were collected for cell subset analysis, focusing on the CD4+ T cell compartment. As shown in Figure 3A, there is no difference between the frequency of total CD4+ T cells in the lymph nodes (LN) from naïve (nLNs) or tumor-bearing mice (TdLNs) (both having ~30% of CD4+ T cells in the CD45+ cell compartment). As expected, the percentage of

total CD4+ T cells in the TILs is significantly lower than in the TdLNs. However, ~15% of total CD4+ T cells were found inside the tumor, suggesting dynamic recruitment of leukocytes to this location. Furthermore, we observed similar frequencies of cTreg cells in the LN from naïve or tumor-bearing mice, ~10% cells, however, cTreg cells are significantly increased in the TILs (~16%) compared to the TdLNs (~10%), Figure 3B. With respect to the Tr1 subset, its proportion reached ~30% in the TILs versus ~5% in the nLNs and TdLNs, Figure 3C. In accordance to the aforementioned results of increased frequencies of cTreg and Tr1 cells, CD4+ non-Treg cells frequencies (defined as CD4+Foxp3/GFP-CD49b- T cells) were much lower in the TILs (~50%) compared to the frequency in the TdLNs (~80%), Figure 3D. However, no significant differences of non-Treg cells frequencies were found between the nLNs and TdLNs.

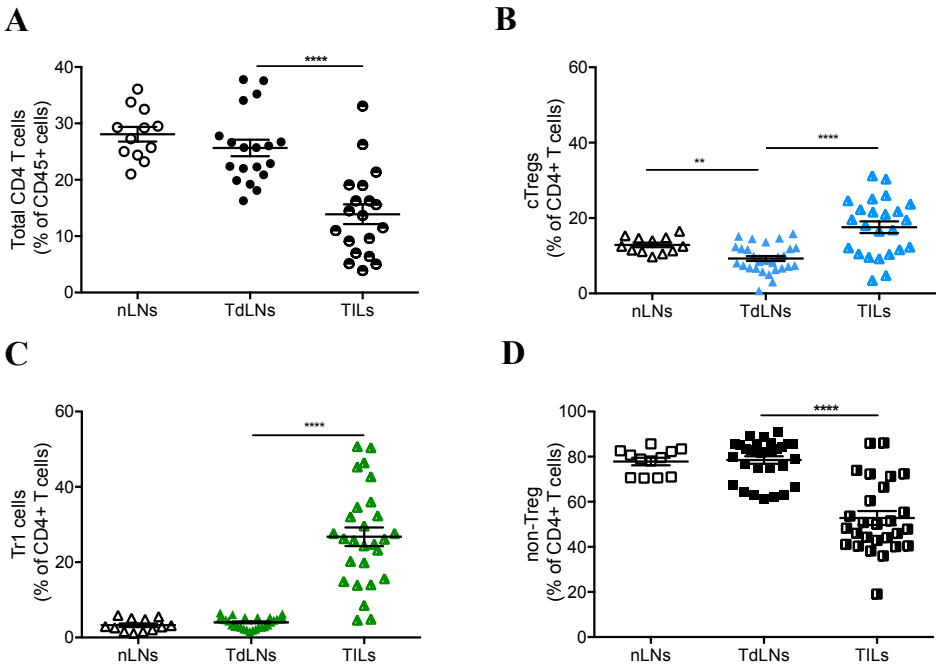


Figure 3. Compartmentalization of CD4+ T cell subsets in tumor-bearing mice.

A; B; C; D; Frequencies of total CD4+ T cells, conventional Treg cells (cTreg), type 1 regulatory T (Tr1), and nonazds-Treg CD4+ T cells subpopulations in naïve inguinal lymph nodes (nLNs), tumor draining lymph nodes (TdLNs) and in the tumor site (TILs). Foxp3/GFP reporter mice were injected in the flank via i.d. with 2 x 10^5 cells. Mice were euthanized at day 20 or when the tumor reached ~1000 mm^3. LN from naïve animals, the TdLNs and the tumor mass were removed for flow cytometry analysis. Data are shown as the mean ± s.e.m. n=10-30 animals. * $p< 0,05$; ** $p< 0,01$; *** $p < 0,001$; ns not significant according to according to Mann-Whitney Test.

7.12 Molecular characterization of Tr1 cells

Neuropilin-1

In order to validate the "regulatory" signature of Tr1 cells, we included in our study the expression of other markers characteristics for Treg cells. The expression levels of the type I transmembrane protein Neuropilin 1 (Nrp1) was assessed. This molecule acts as a receptor for the vascular endothelial growth factor (VEGF) regulating angiogenesis and migration towards a VEGF gradient thus bearing an important role in both pre and post-natal vascular development and in the migration of motor neurons (Hansen et al. 2012). Additionally, this molecule is highly expressed in cTreg cells, in contrast to non-Treg cells, and its deletion in this subset impairs tumor progression due to reduced infiltration of cTreg cells into the tumor site, as demonstrated in a melanoma model (Hansen et al. 2012; Bruder et al. 2004). We found that the frequencies of Nrp1+ Tr1 cells are significantly lower than those of Nrp1+ cTregs in all of the analyzed organs, Figure 4A. Additionally, the percentage of Nrp1+ in both Treg subsets was found significantly higher than in non-Treg cells, Figure 4A. Furthermore, we can see that the percentage of Nrp1+ cells in the non-Treg subset is higher in the TILs fraction, than in the TdLNs and nLNs, and also is higher in the TdLNs than in the latter (Figure 4B). On the other hand, both cTreg and Tr1 cells have a lower percentage of Nrp1+ cells in the TILs fraction, compared to the TdLNs fractions, while there is no difference between nLNs and TdLNs (Figure 4B)

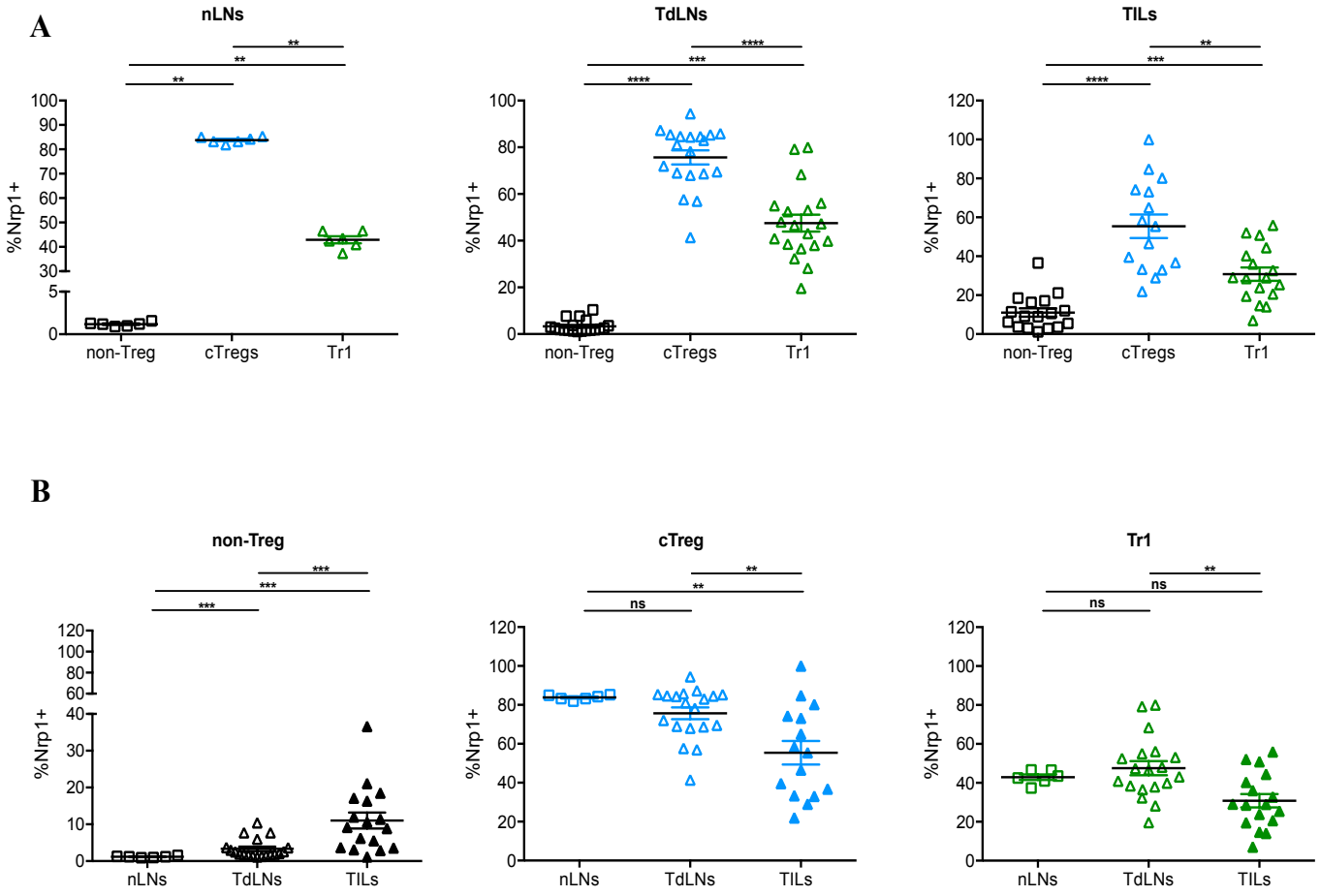


Figure 4. Frequencies of Nrp1+ cells in CD4+ T cell subsets in the nLNs, TdLNs, and TILs. Frequencies of Nrp1+ cells on non-Treg, cTreg and Tr1 subsets isolated from nLNs, obtained from naïve mice, TdLNs, and TILs obtained from Foxp3/GFP tumor-bearing mice after 20 days of tumor injection or when the tumor reached ~1000 mm^3. Nrp1 expression on the subsets was measured through flow cytometry. **A.** The frequencies of Nrp1+ cells is compared between the different CD4+ T cell subsets in the nLNs, TdLNs and TILs. **B.** The frequencies of Nrp1+ cells in non-Treg, cTreg and Tr1 are compared between the organs from which they were isolated (nLNs, TdLNs, and TILs).. Frequencies are shown as the mean ± s.e.m. n=6-19 animals. *< 0,05; ** p< 0,01; *** p < 0,001; ns not significant according to according to Mann-Whitney Test.

Ecto-5´-nucleotidase CD73

Ecto-5'-nucleotidase or CD73 is an ectoenzyme that acts in concert with CD39 to convert ATP into adenosine (Beavis et al. 2012). While CD39 catalyzes the conversion of ATP to AMP, CD73 dephosphorylates the latter producing adenosine which, contrary to the immune-activating ATP, is a potently immunosuppressive nucleoside that acts on both CD4+ and CD8+T cell, inhibiting their proliferation, cytotoxicity and pro-inflammatory cytokine production (Becker et al. 2013; Beavis et al. 2012). Accordingly to the elevated levels of adenosine found at the TME, CD39, and CD73 are highly expressed by human and murine cTreg cells present in tumor models (Beavis et al. 2012; Ohta et al. 2006). Particularly on Tr1 cells, an in vitro study that identified these cells by their capacity to produce IL-10 , described the expression of both enzymes on Tr1 cells surface and highlighted their importance in the Tr1 cells suppressive activity (Mandapathil & Whiteside 2011; Mandapathil et al. 2010). However, the expression of these ectonucleotidases has not been described on Tr1 cells using CD49b and LAG-3 as markers.

In the current study, we evaluated the expression of CD73 on cTreg and Tr1 cells (which were identified as CD4+ T cells that expressed CD49b but did not express Foxp3), and demonstrated that both subsets express this protein in higher levels than non-Treg CD4+ T cells. Nonetheless, we found that cTreg cells express higher levels of CD73 than Tr1 cells, having values of around 3000 of median fluorescence intensity (MFI), in the lymph nodes of both naïve and tumor-bearing animals, while Tr1 cells showed values of around 2000 MFI in the same organs, Figure 5A. In the TME, no significant differences were found between the two Treg subsets in terms of the measured MFI for CD73; however,

Tr1 cells tend to have a higher expression of this molecule than cTregs in this microenvironment. Specifically, in the TME, Tr1 cells show similar values of MFI to those observed in the TdLNs, while cTreg cells seem to have lower levels than in the TdLNs, Figure 5A. Furthermore, non-Treg cells have a higher MFI values in the TILs fraction than in the TdLNs, and also in the latter compared to the values found nLNs. cTregs cells, on the other hand, have lower CD73 MFI values in cells obtained from the TILs fraction than from the TdLNs, while Tr1 cells have higher values in the TILs fraction than in the TdLNs, and in the latter compared to nLNs (Figure 5B).

In terms of frequencies, both Tr1 cells and cTregs had higher percentages of CD73+ cells compared to non-Treg cells, in all analyzed organs (Figure 6A). Furthermore, Tr1 cells had higher frequencies of CD73+ cells, compared to the cTreg fraction, in lymph nodes obtained both naïve mice and in the TILs fraction, while no difference was found between TdLNs and TILs (Figure 6A). Additionally, we can see that the frequency of CD73+ cells in the non-Treg fraction is higher in the TdLNs, compared to the nLNs, and also in the TILs, compared to the TdLNs (Figure 6B). However, no difference was found between the CD73+ cTregs frequencies in naïve lymph nodes and in TdLNs, although the percentage of CD73+ cTreg cells is significantly lower in the TILs fraction than in the TdLNs (Figure 6B). In the case of Tr1 cellswe can see that the percentage of CD73+ cells is significantly higher in the TdLNs than in the nLN, while it is also higher in the TILs than in the TdLNs (Figure 6B)

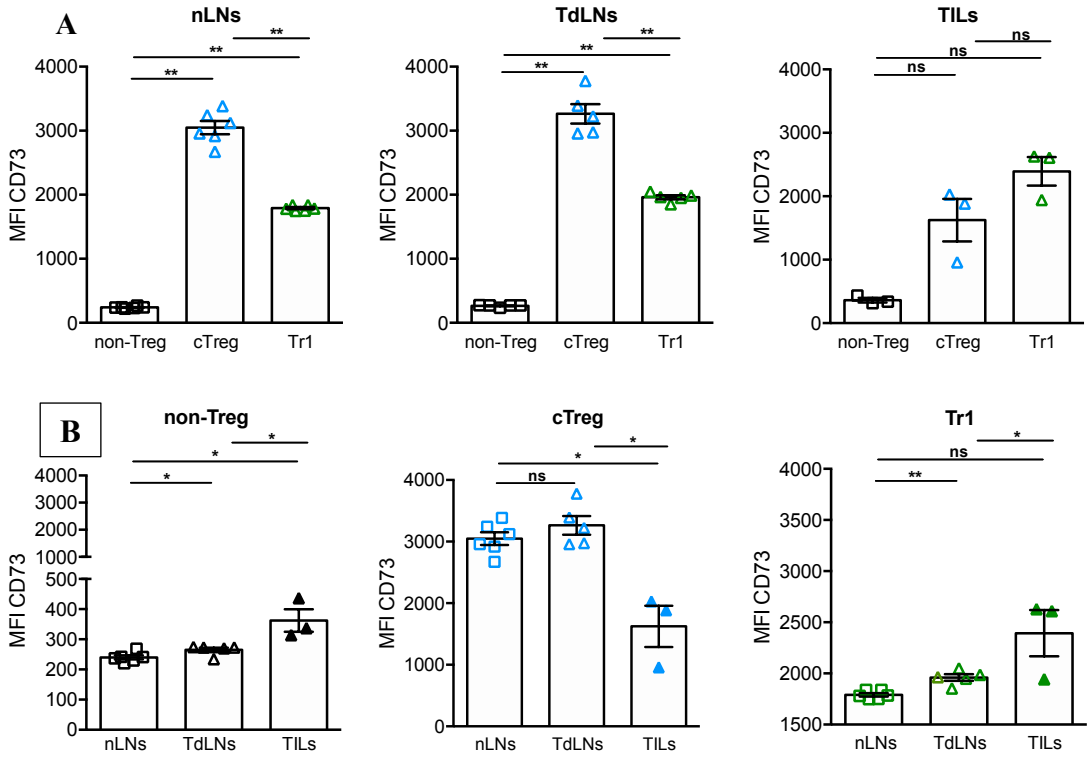


Figure 5. CD73 expression levels in CD4+ T cell subsets in the nLNs, TdLNs and TILs. Mean fluorescence intensities (MFI) of CD73 on non-Treg, cTreg, and Tr1 cells isolated from nLNs, TdLNs, and TILs obtained from Foxp3/GFP tumor-bearing mice after 20 days of tumor injection or when the tumor reached ~1000 mm^3 measured through flow cytometry. **A.** The MFI of CD73 is compared between the different CD4+ T cell subsets in the nLNs, TdLNs, and TILs. **B.** The MFI of CD73 in non-Treg, cTreg and Tr1 are compared between the organs from which they were isolated (nLNs, TdLNs, and TILs). Values are shown as the mean ± s.e.m. n=3-6 animals. p*< 0,05; ** p< 0,01; *** p < 0,001; ns not significant according to according to Mann-Whitney Test.

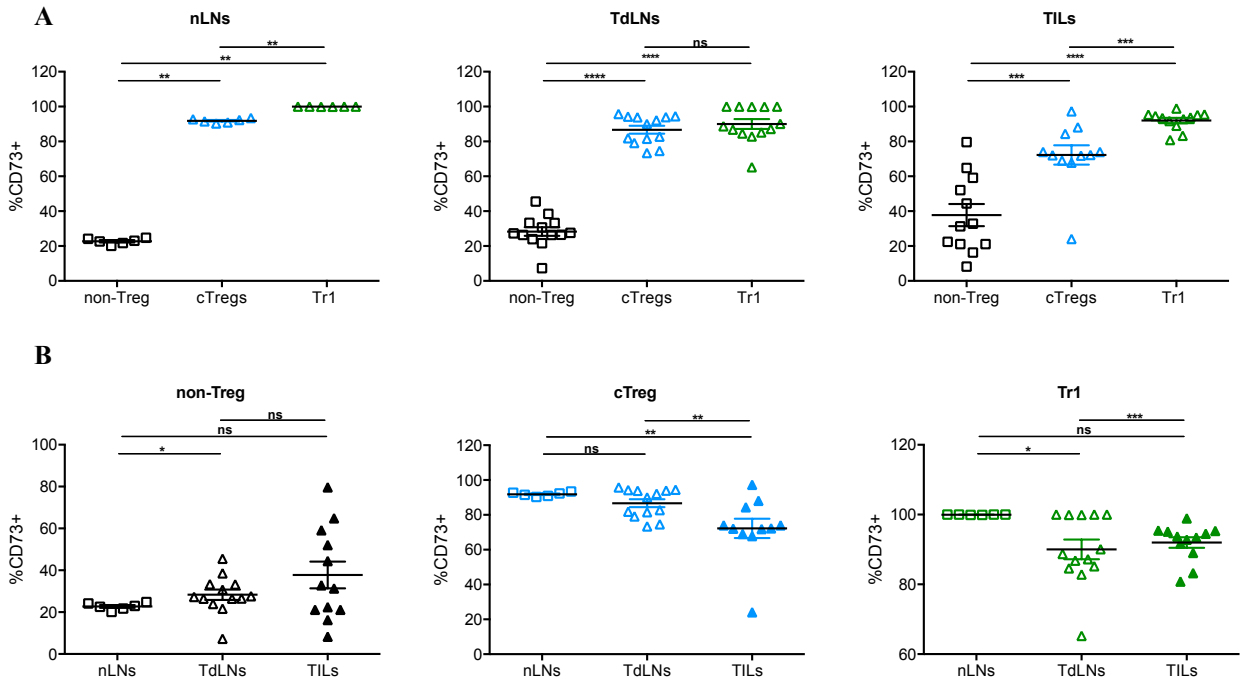


Figure 6. Frequencies of CD73+ cells in CD4+ T cell subsets from nLNs, TdLNs and TILs. Frequencies of CD73+ cells on non-Treg, cTreg and Tr1 subsets harvested from nLNs, TdLNs and TILs obtained from Foxp3/GFP tumor-bearing mice after 20 days of tumor injection or when the tumor reached ~1000 mm^3. **A.** The frequencies of CD73+ cells is compared between the different CD4+ T cell subsets in the nLNs, TdLNs and, TILs. **B.** The frequencies of CD73+ cells in non-Treg, cTreg, and, Tr1 are compared between the organs from which they were isolated (nLNs, TdLNs, and, TILs) The frequencies were calculated with the positive fraction of CD73 in every subset using flow cytometry. Frequencies are shown as the mean ± s.e.m. n=6-13 animals. p*< 0,05; ** p< 0,01; *** p < 0,001; ns not significant according to according to Mann-Whitney Test.

7.13 Tr1 cells cytokine production

Interleukin-10

As it was stated in the introduction, Tr1 cells are characterized by secreting high levels of IL-10 and TGFβ. Additionally, they secrete significant amounts of IL-5 and IFNγ, and low levels of IL-2, IL-17, and IL-4 (Groux et al. 1997; Roncarolo et al. 2006). Specifically, IL-10 production is important for Tr1 cells function and, together with TGFβ, it inhibits T cell responses by suppressing IL-2, and IFNγ and by preventing T cell proliferation (Vieira et al. 2004). This cytokine also modulates APCs, down-regulating their expression of co-stimulatory molecules and production of pro-inflammatory cytokines, and B cells by promoting isotype switching (Gregori et al. 2012; de Waal Malefyt et al. 1991; Meiler et al. 2008; Satoguina et al. 2005). Since IL-10 is also produced by cTreg cells, and a variety of other T cell populations and leukocytes, we determined the levels of production of this cytokine by intracellular staining (Kole & Maloy 2014; Roncarolo et al. 2014). No significant difference was found between the percentage of IL-10 secreting cTregs and Tr1 in the TdLNs, where both subpopulations reached 10% of the total, Figure 7. However, in the TME, the percentage of cTregs that secrete IL-10 (~20%) is significantly higher than the percentage of Tr1 (~6%). Additionally, we found that while cTregs IL-10 production inside the tumor is significantly higher than in the TdLNs, Tr1 IL-10 production is significantly lower, Figure 7.

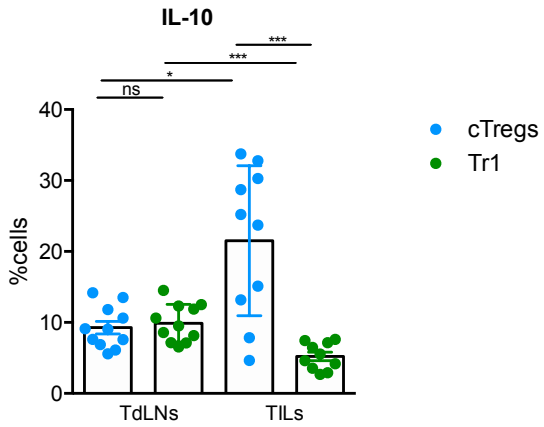


Figure 7. Frequencies of IL-10-secreting cells in CD4+ T cell subsets from TdLNs and TILs. The frequencies of IL-10+ cells in cTregs (light blue dots) and Tr1 (green dots) cell subsets were determined in TdLNs and TILs cell suspensions from Foxp3/GFP tumor-bearing mice after 20 days of tumor injection or when the tumor reached ~1000 mm^3through flow cytometry. Samples were stimulated for 4 hours with PMA/ionomycin

Interferon-γ

IFNγ has been described as one of the cytokines secreted by Tr1 cells and, although the specific role of this cytokine in Tr1 cells function has not been researched yet, it has been shown that this cytokine is relevant for cTregs suppressive function in vivo, in a graft-versus-host disease (GVHD) model (Daniel et al. 2014). Furthermore, in 2006, Wood et al. suggested that the early production of IFNγ by induced Tregs during an immune response could directly inhibit the activation and proliferation of IFNγR1- and IFNγR2-bearing T cells. Also, Tregs-derived IL-10 may prevent further activation of T cells by affecting the function of APCs (Wood & Sawitzki 2006).

In this study, we found that the TdLNs contained ~7% of IFNγ+ Tr1 cells, which was significantly higher than the ~3% of IFNγ+ cTreg cells, Figure 8. At the tumor site, we

found similar frequencies of IFNγ+ cells in these populations, reaching ~7% of IFNγ+ of cTregs and Tr1 cells, Figure 8. Furthermore, the frequency of IFNγ cTreg inside the tumor was significantly higher than in the TdLNs, but no differences were observed between Tr1 cells isolated from the TdLNs and the TILs fraction.

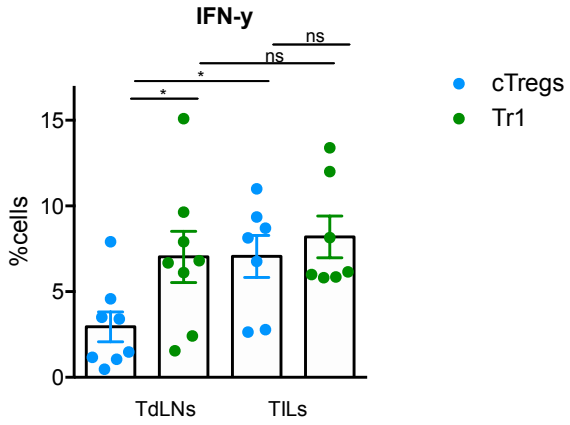


Figure 8. **Frequencies of IFNγ secreting cells in CD4+ T cell subsets from TdLNs and TILs.** The frequencies of IFNγ+ cells within cTreg (light blue dots) and Tr1 cells (green dots) subsets were determined in the TdLNs and TILs cell suspensions from Foxp3/GFP tumor-bearing mice after 20 days of tumor injection or when the tumor reached ~1000 mm^3. Cells were stimulated for 4 hours with PMA/ionomycin plus Brefeldin-A, followed by IFNγ intracellular staining. The production of the IFNγ was then measured using flow cytometry. Data is shown as the mean ± s.e.m. n=10-14 animals. * $p < 0.05$; ** $p< 0.01$; *** $p < 0.001$; ns not significant according to Mann-Whitney Test.

7.14 viSNE visualization of the data

In order to have a better overview of the two populations of Treg cells, the data was visualized using viSNE, a visualization tool for high-dimensional single-cell data based on the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm. viSNE preserves the main structure in the data while reducing a high- dimensional data description to a 2D representation in which the position of cells reflects their proximity in high-dimensional space. Color is used as a third dimension by which we can visualize the features of the analyzed cells (Amir et al. 2013). This type of visualization allows a more objective and unbiased visualization of the cell populations and the markers (Saeys et al. 2016; Laurens van der Maaten 2014). In Figure 9A, we can see that in the TdLNs and in the tumor, cTregs and Tr1 cells conform two separate distinct populations inside the CD4+ T cell subset, which can be identified by their Foxp3/GFP and CD49b expression. In Figure 9B, we can observe that IL-10 is mainly secreted by these two populations in the TdLNs, while in the tumor there seem to be more IL-10-secreting cell populations apart from cTregs and Tr1 cells. In addition, we can see that Tr1 cells seem to produce higher levels of IFNγ than cTreg cells in the TdLNs, while the opposite is observed at the tumor site.

The expression of the canonical Th2 transcription factor GATA-3 was also analyzed since it has been shown that its expression can control Treg polarization to an effector phenotype, and may enhance Treg accumulation at inflamed sites (Wohlfert et al. 2011). As depicted in Figure 9B, GATA-3 is expressed by cTreg and Tr1 cells, in addition to other cell populations. Interestingly, both populations increase their GATA-3 expression inside the tumor, compared to the expression levels found in the TdLNs.

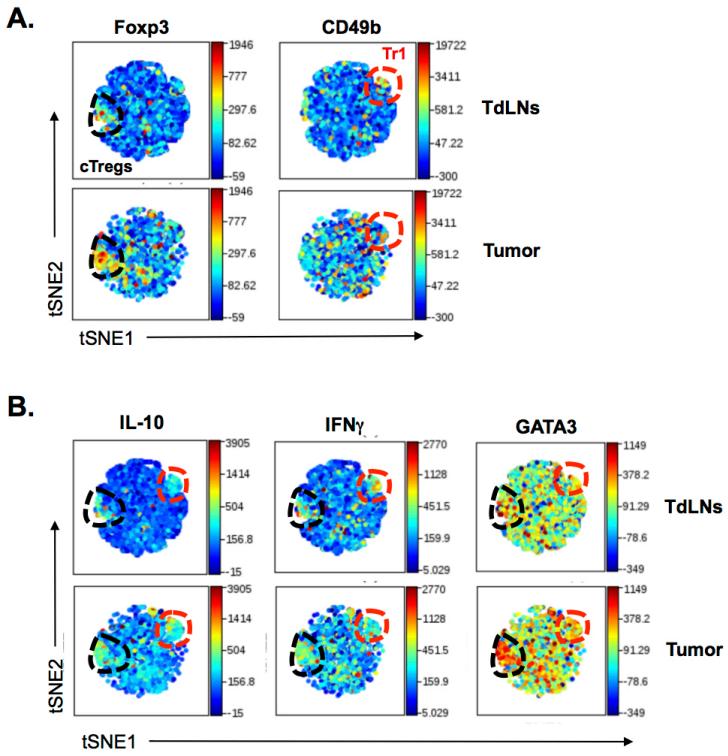


Figure 9. Phenotypic identity of cTreg and Tr1 cells. Lymphocytes were obtained from TdLNs and TILs of tumor-bearing mice and stained with antibodies against the indicated molecules. Cells were gated on the CD4+ subset and subjected to viSNE dimensionality reduction. Automatic clustering was performed using k-means, and clusters plotted into the t-SNE maps (**A** and **B**). **A**. Plots show a representative viSNE map of analyzed color-coded T regulatory cell subtypes, as identified by the expression of Foxp3/GFP and CD49b. The cTreg subset is marked in the map with a black dashed circular outline and the Tr1 cell subset with a red circular outline. **B**. Indicated markers are color mapped from blue (low expression) to red (high expression) into the t-SNE map. The position of the Treg subsets in the map is shown with a black dashed circular outline (cTreg subset) and a red circular outline (Tr1 cell subset). For phenotype analysis, antibodies against the indicated molecules were used.

7.15 Gene expression of cTreg and Tr1 cells obtained from tumors

In order to investigate other suppression mechanisms already described for Treg cells, such as the killing of effector cells in a perforin-dependent or granzyme-B-dependent manner, and the secretion of regulatory molecules via release of extracellular vesicles, we measured the expression of key genes related to these mechanisms as perforin and granzyme B (killing activity) and Rab27α plus Rab27β (release of extracellular vesicles), using quantitative polymerase chain reaction (qPCR) (Cao et al. 2007; Grossman et al. 2004; Ostrowski et al. 2010). Additionally, we also measured the expression of another anti-inflammatory cytokine (TGFβ) and the expression of CD39 (ATP metabolization) (Borsellino et al. 2007; Vignali 2012).

To accomplish this, we inoculated B16 tumor cells into Foxp3/GFP reporter animals. At day 20, both conventional Treg and Tr1 cells were FACS cell-sorted from the TdLNs and TILs following the gating strategy shown in Figure 10A. Next, we obtained the total RNA from the sorted populations, which was used to obtain cDNA in order to perform a qPCR assay. In Figure 10B we can see that, as seen in the flow cytometry experiments, IL-10 expression in cTregs tends to be increased inside the tumor compared to the expression in the TdLNs. Besides, cTregs expression of this cytokine inside the TME seems higher than in Tr1 cells. In the case of TGFβ, its expression tends to increase in both subsets inside the tumor compared to the TdLNs. Additionally, TGFβ expression by Tr1 cells tends to be higher than in cTregs both in the TdLNs and TILs. Similar results were found for granzyme B, which expression in both subsets also tends to increase inside the tumor

compared to the expression in the TdLNs. However, its expression seems to be similar between both subsets in the TdLNs and TILs. In the case of perforin, which acts together with granzyme B inducing apoptosis in target cells, its expression in Tr1 cells tends to be lower inside the tumor, compared to the expression in the TdLNs (Chowdhury & Lieberman 2008). Additionally, its expression inside the tumor seems to be similar between both cell subsets, while Tr1 cells expression of this enzyme seems to be higher than in cTregs in the TdLNs.

The expression of Rab27α and Rab27β, which are implicated in exosome secretion, was also analyzed, and in the case of Rab27α, we can see that its expression is similar between both subsets inside the tumor, while in the TdLNs its expression seems higher in cTregs than in Tr1 cells (Ostrowski et al. 2010). For Rab27β, we can see that its expression tends to be higher in Tr1 cells than in cTregs inside the tumor, while in the TdLNs its expression is similar in both subsets. Additionally, the expression of both Rab27α and Rab27β in Tr1 cells seems to be higher in the tumor than in TdLNs.

In the case of CD39, we can observe that its expression seems to be higher in the cTreg subset than in Tr1 cells in the tumor, while in the TdLNs the opposite was observed.

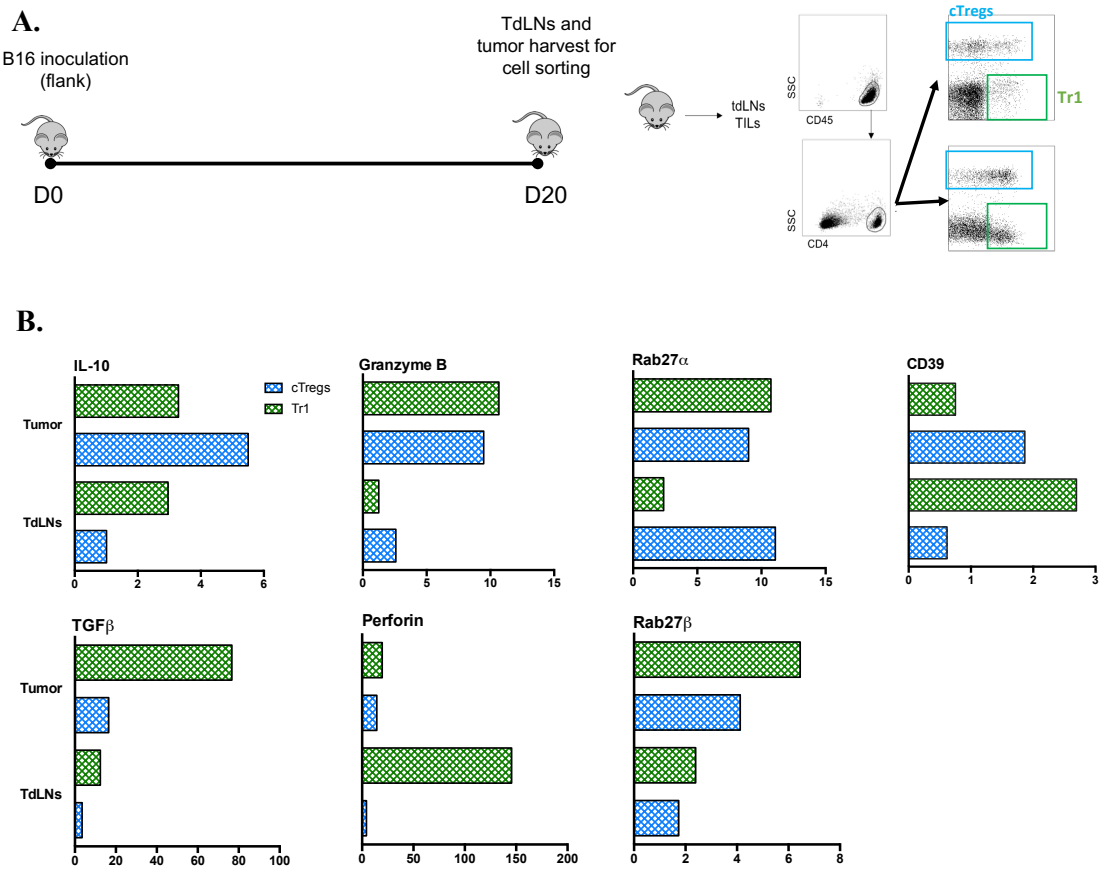


Figure 10. Molecular characterization of ex-vivo Tr1 cells. A. Schematic of the experimental design in which Foxp3/GFP reporter mice were injected in the flank via i.d. with 2 x 10^5 cells. Mice were euthanized at day 20 or when the tumor reached 1000 mm^3. The TdLNs and tumor mass were removed for single cell suspension preparation. Tr1 cells and cTregs were cell sorted using the indicated gates. **B.** Immediately after sorting, mRNA was obtained and converted to cDNA, which was used for measuring the expression of the indicated genes by qPCR. Results were normalized to 18S as housekeeping gene. The mean between technical replicates was then normalized to the expression of the target gene in the total TILs sample to find ΔΔCq Expression. n=1.

7.16 Comparison between the proliferative capacity of cTreg and Tr1 cells in vivo

It has been well documented that cTreg cells can infiltrate the tumor site and, due to the immune regulatory signals received within the tumor, they can also differentiate from non-suppressive CD4+CD25- T cells to suppressive CD4+CD25+Foxp3+ T cells (Tanaka & Sakaguchi 2017; Facciabene et al. 2012; Chaudhary & Elkord 2016a; Liu et al. 2007; Valzasina et al. 2006). Apart from cTreg cells being able to migrate and be induced inside the tumor, it has been reported that these cells bear enhanced proliferation capacity once at the TME (Bui et al. 2006; Wainwright et al. 2011). In contrast, the dynamics of Tr1 cells are less understood. Therefore, we designed an experiment to evaluate the proliferative status of cTreg and Tr1 cells in tumor-bearing animals. Mice were inoculated with B16 cells as before, and at day 12 and 15 post-tumor inoculation, a solution of BrdU (100 μg/g) was administered via i.p. to the mice. At day 20, the TdLNs and tumors were harvested and stained for flow cytometry analysis. As a control, we also analyzed LNs and bone marrow (BM) of naïve mice injected with BrdU (nLN+ and BM+) and with no treatment (nLN- and BM-). Our results indicate that the tumor contains a significantly higher frequency of total BrdU+ cells (~7%) compared to TdLNs (~3%), Figure 11C. Additionally, in the TdLNs there is a significantly higher percentage of BrdU+ cells compared with nLN+ (~1.5%). With respect to the proliferative capacity of cTreg and Tr1 cells, we found that nearly 40% of cTreg cells in nLNs+, TdLNs and tumor site were BrdU+. However, less than 10% of Tr1 cells in all samples were BrdU+, Figure 11D. These results suggest that cell proliferation and/or accumulation is taking place

preferentially at the tumor site versus TdLNs and that among Treg cell populations, cTreg cells seem more highly proliferative in comparison with Tr1 cells.

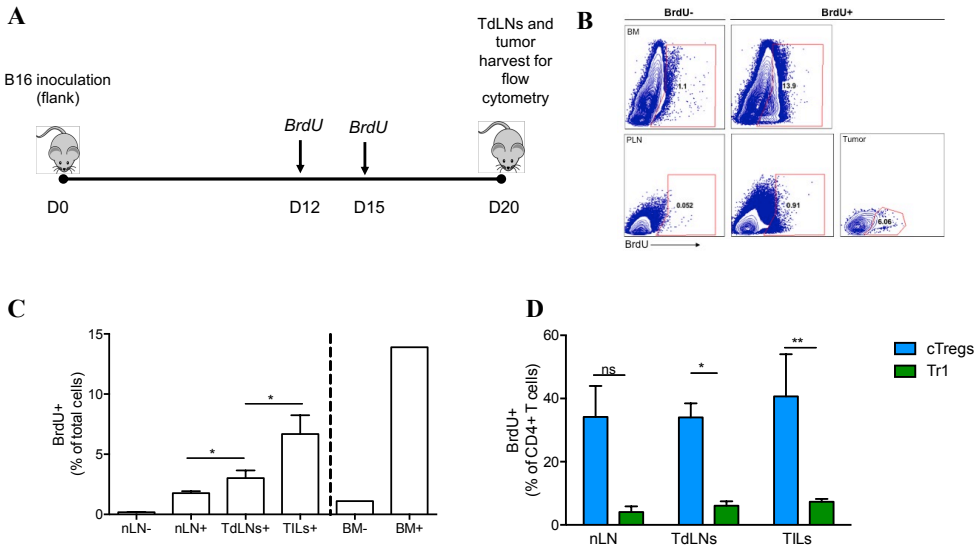


Figure 11. **Comparison of proliferative capacity between cTreg and Tr1 cells in tumor-bearing animals. A.** Schematic diagram of the experiment in which cell proliferation was determined using BrdU incorporation/staining. BrdU was administered via i.p. at days 12 and 15 after tumor induction. Organs were harvested at day 20, and BM was used as positive control. Naïve mice that did not receive BrdU injection were used as negative control. **B.** Representative plots of BrdU proliferation measurement. **C.** Total cell proliferation as measured in terms of BrdU+ cells in different samples: naïve lymph nodes of untreated animals (nLN-, negative control), naïve lymph nodes of mice injected with BrdU (nLN+), tumor-draining lymph nodes of mice injected with BrdU (TdLNs+), tumor sample of mice injected with BrdU (TILs+), bone marrow of mice injected or not with BrdU (BM+, positive control, and BM- respectively). **D.** cTreg and Tr1 cells proliferation in nLN+, TdLNs+ and within the tumor. All results are shown as mean ± s.e.m. n=2-5 animals. *$p < 0.05$; ** $p< 0.01$; *** $p < 0.001$; ns not significant according to Mann-Whitney Test.

To complement our previous findings, we then assessed whether the relative abundance of both populations changed in time. For this purpose, we harvested the TdLNs and tumors on day 7 and 21 after tumor challenge, Figure 12A. In this experiment, we observed that, while cTreg cells increase their frequencies inside the tumor over time, Tr1 cells do not, Figure 12 B-C.

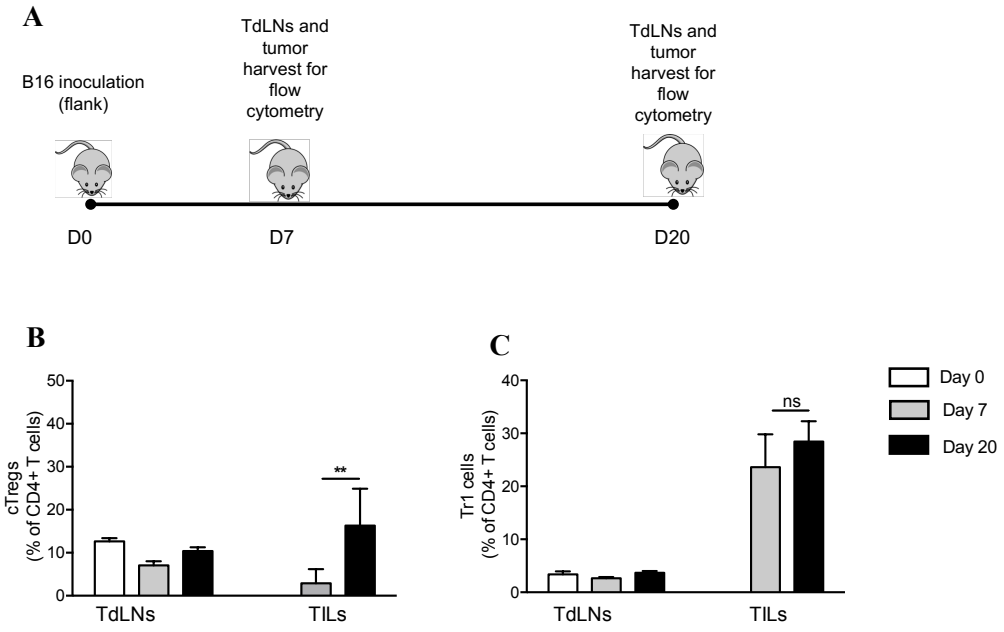


Figure 12. Relative abundance of cTreg and Tr1 cells in tumor-bearing mice. A. Schematic depiction of the experiment in which relative abundance of cTreg and Tr1 cells was measured at day 0, 6 and 20 in the TdLNs and tumor. Inguinal/tumor-draining lymph nodes (TdLNs) were harvested at day 0, 7 and 20 from control naïve and tumor-bearing animals. **B.** Relative abundance of cTreg cells among total CD4+ T cell subset in TdLNs at days 0, 6 and 20. **C.** Relative abundance of Tr1 cells among CD4+ T cell subset in TdLNs at days 0, 6 and 20. All results are shown as mean ± s.e.m. n=5-19 animals. *$p < 0.05$; ** $p< 0.01$; *** $p < 0.001$; ns not significant according to Mann-Whitney Test.

8 DISCUSSION

T regulatory cells are essential for establishing tolerance to self and non-self-antigens and thus maintaining immune homeostasis by suppressing the activation and differentiation of CD4+ T helper cells and CD8+ cytotoxic T cells reactive to autologous, environmental or tumor-expressed antigens (Chao & Savage 2018). Due to their immunosuppressive activity, these cells are capable of promoting an immunosuppressive microenvironment, causing a detriment to the anti-tumor immune response, thus promoting tumor growth. Numerous studies have focused on the relevance of either CD25+CD4+ T cells or Foxp3+CD4+ T cells in the tumor microenvironment, in which it has been found that the presence of these cTregs in a multitude of cancers, such as ovarian, breast, colorectal, lung, pancreatic cancers and melanoma, is predictive of poor clinical outcome (Zhou et al. 2017; DeLeeuw et al. 2012; Zou 2006). However, in other types of tumor, such as colorectal carcinoma, these cells are able to suppress tumor-promoting inflammation against gut microbes, thus constituting a marker for a favorable clinical outcome (Chao & Savage 2018). Unlike conventional Treg cells, the involvement of Tr1 cells in tumors has not received as much attention. This partly because until recently there was not a defined cell surface signature and could therefore only be characterized by their production of IL-10 and TGFβ, variable levels of IFNγ and the absence of IL-4 and Foxp3 (Gregori et al. 2012). The lack of a reliable surface marker for the identification of these cells hindered the further investigation of this cell subset in in vivo models; however, a small number of

studies have focused on these cells in the tumor context. For instance, it was found that ex vivo generated CD4+CD25−Foxp3^{low}IL-10+TGFβ+ T cells induced from CD4+CD25- T cells isolated from the TILs fraction of head and neck carcinoma patients had a higher suppressive capacity than those isolated from PBMCs (Bergmann et al. 2008). Similar results were found in samples from colorectal cancer, where Tr1 cells, identified by their lack of expression of Foxp3 and the expression of LAP, were found to have a higher suppressive activity than cTregs (Scurr et al. 2014). Tr1 cells, defined as CD4+IL-10+ T cells, were also found elevated in lymph nodes of patients with Hodgkin's lymphoma, (Marshall et al. 2004). However, the variability of the markers used in these previous studies and the fact that it was necessary using a cytokine profile to distinguish this type of cell undermines the reliance of these studies in this subject since these cells are not the only T cell subset that secretes IL-10 and TGFβ, and expresses LAP. The recent description of co-expression of CD49b and LAG-3 as markers that can specifically identify this subset has allowed new insights into the role of this population, and it has already been found that cells identified by these markers are elevated in human samples of liver tumors (Pedroza-Gonzalez et al. 2015). However, the specificity of these two markers is still questioned by some researchers. In 2016, White and Wraith reported that, while 50% of antigen-specific CD4+IL-10+ T cells obtained ex vivo from EAE mice were positive for CD49b (in contrast to 6-9% of the IL-10- subset), LAG-3 was found in the majority of CD4+ T cells, regardless of their IL-10 production (White & Wraith 2016). This same study reported that only a small proportion of in vitro-induced CD4+IL-10+ T

cells was positive for both CD49b and LAG-3, and while almost 60% of IL-10+ T cells were CD49b+, only 10% were LAG-3+ (White & Wraith 2016). Furthermore, it has been reported that murine CD49b+ T cells show a suppressive phenotype and can be used to treat arthritis when injected in vivo (Vicente et al. 2016). The inefficiency of using LAG-3 as a marker for identifying Tr1 cells has been suggested to be related with the dynamic recruitment of LAG-3 to the cell surface which, together with others inhibitory receptors, is stored intracellularly (White & Wraith 2016; Bae et al. 2014). These facts, together with the accessibility of various fluorochrome-linked antibodies, lead us to only use CD49b as a marker for Tr1 cells. Furthermore, since CD49b and LAG-3 were reported to be co-expressed in other cell subsets beside Tr1, such as IL-10-producing cTregs, CD8+ T cells, and Natural Killer cells, a precise gating was used, which ruled-out both CD4- and Foxp3+ T cells (Arase et al. 2001; Roncarolo et al. 2018).

After establishing that CD4+Foxp3-CD49b+ T cells were going to be considered as Tr1 cells in our study, we described its presence in various organs, where we found that its relative frequency was similar in all of the analyzed sites except in the PLNs, where cTregs were found to be significantly higher than Tr1 cells. This result could be related to the phenotypic differences between these two subsets, that renders their differential presence in different organs. For example, both subsets can express different chemokine receptors on their surface, which in turn causes differential migratory patterns into different organs/tissues (Yuan et al. 2014). The presence of different Treg subsets helps maintain the homeostasis in different tissues and organs, and these cells often also have functional differences, besides their migratory patterns (Yuan et al. 2014).

Once we established the relative abundance of cTregs and Tr1 cells in different organs of naïve mice, we aimed to study the possible differences between the subsets in the tumor context. For this purpose, we used a melanoma model, where we found that Tr1 cells are significantly elevated inside the tumor, comprising almost 30% of the CD4+ T cell subset. On the contrary, Tr1 cells comprise less than 4% of the CD4+ T cells in the TdLNs. This result is coherent with the ones found in human samples of liver tumors, where Tr1 cells, identified using CD49b and LAG-3 as markers, were found to be elevated compared to the frequencies found in the blood and samples from the tumor-free area of the liver (Pedroza-Gonzalez et al. 2015). This finding led us to continue researching the mechanisms underlying the significant presence of Tr1 cells in the tumor site.

After identifying Tr1 cells in the tumor site, we aimed to describe the expression of certain molecules related to mechanisms of suppression. We analyzed the expression of Nrp1, which is highly expressed in cTreg cells and has been associated with tumor progression (Hansen et al. 2012; Bruder et al. 2004). We found that this molecule is not as highly expressed in Tr1 cells as in cTregs, although its expression is higher than in non-Treg cells. This result is similar to the one observed by Yao et al. who highlights the fact that the expression of this marker is not restricted to Foxp3+Treg cells and cannot be used as a marker to identify this subset, as it had been proposed before (Yao et al. 2015; Bruder et al. 2004). The expression of Nrp1 in Tr1 cells could be related to the stability of the Treg cells phenotype in inflammatory microenvironments such as the tumor site, as it has been previously shown for the cTreg subset (Delgoffe et al. 2013).

However, we also found that both subsets downregulated Nrp1 expression inside the tumor, compared to lymph nodes. This finding can be related to the increased production

of IFNγ by both subsets that we found inside the tumor since it has been proposed that Nrp1-deficient Treg cells produce more IFNγ (Overacre-Delgoffe et al. 2017). Tr1 cells, on the other side, have been described to produce variable amounts of IFNγ (Gregori & Roncarolo 2018). On this matter, we found that Tr1 cells produced high levels of IFNγ both in the TdLNs and in the tumors. Although IFNγ has been described to enhance tumor cell immunogenicity, thus helping tumor clearance, the overall results of these therapies are not conclusive (Zaidi & Merlino 2011). It has been shown that the presence of this cytokine can also increase tumor aggressiveness by enhancing its metastatic capacity and resistance to NK cells in various in vitro and in vivo experiments (Zaidi & Merlino 2011). In addition, it has been described that IFNγ can induce cTreg cells and other immunosuppressive cells, such as myeloid-derived suppressor cell (MDSC) (Wang et al. 2006). Thus, it is possible that the production of IFNγ by these suppressive cells inside the tumor could have a pro-tumorigenic effect rather than enhancing tumor clearance.

In the case of IL-10, we found that its production changed in both Treg subsets: cTregs increased IL-10 production inside the tumor while Tr1 cells decreased it, compared to the production measured in the TdLNs. This change in the cytokine production by Tregs is not surprising since it is well known that immune cells change its cytokine secretion pattern depending on the signals they receive, which in turn depends on the microenvironment (Karnoub et al. 2007).

Although IL-10 is normally rendered as an immunosuppressive cytokine, it has been proposed that its effect is context-dependent since it has been found that under certain

conditions, its presence can induce the proliferation of CD8+ cytotoxic T cells and increase NK cytolytic activity (Mannino et al. 2015).

In the case of CD73, we observed that its levels seem to be higher in the cTreg cells subset than in the Tr1 subset both in nLNs and TdLNs and that the expression on both subsets was higher than in non-Treg cells. However, in the tumor microenvironment, cTregs and Tr1 cells seem to express similar levels of this ectonucleotidase, and it seems that while Tr1 cells maintain their expression levels, cTregs decrease it, compared to the TdLNs. In terms of percentage, no significant difference was found between both subsets in the TdLNs; however, we did observe that a significantly higher percentage of Tr1 cells expressed CD73 on their surface compared to cTregs. Additionally, while cTregs significantly decrease the percentage of CD73+ cells in the tumor, compared to the TdLNs, Tr1 cells maintain the percentage of CD73+ cells. As it was stated before, adenosine metabolism is relevant in TME since it has anti-inflammatory properties and the ability to suppress various types of effector immune cells (Beavis et al. 2012). Its expression has been well documented both in murine and human cTregs and Tr1 cells, and can contribute to the accumulation of adenosine inside the TME (10-20 fold increase in tumor versus normal tissues) (Deaglio et al. 2007; Borsellino et al. 2007; Blay et al. 1997; Gregori & Roncarolo 2018). Adenosine results from the conversion of extracellular ATP previously released from tissue disruption and dying tumor cells, thus preventing tumor cells from dying from ATP-associated toxicity, and thus promoting tumor growth (Mandapathil & Whiteside 2011; Spychala 2000). Another relevant evidence of the importance of adenosine metabolism in the TME is that tumor progression is reduced in CD73-deficient mice (Yegutkin et al. 2011). Therefore, our findings of elevated

expression of CD73 in the TME on the Tr1 cell subsets suggests that these cells could be suppressing other immune cells through this mechanism. Even more, the fact that Tr1 cells maintain their CD73 expression in the tumor, contrary to what is observed in the case of cTregs, further supports that Tr1 cell subsets could be a key player in maintaining an immunosuppressed environment inside the tumor. It is interesting, however, that when we measured CD39 expression on Tr1 cells by qPCR it tended to be diminished inside the TME, compared to the TdLNs since it has been reported that the released ATP from B16 melanoma lines can up-regulate the expression of this ectonuclease on the surface of Tregs (Burnstock & Di Virgilio 2013). However, we did observe that CD39 expression tended to be higher in the TME than in the TdLNs in the case of cTregs, and it is possible that only this subset does so. Nevertheless, further investigation using a higher number of samples and using other techniques to measure CD39 is needed to have conclusive results about the expression of this molecule on the surface of Tr1 cells.

We also measured the expression of other genes through qPCR, and though the number of samples was not enough to conclude if the differences were significant, we will briefly discuss what was found. In the case of TGFβ, as it was stated before, is one of the main cytokines, together with IL-10, released by Tr1 cells to exert their immunosuppressive function (Bacchetta et al. 2005). It was found that its gene expression in the tumor seems to be highly upregulated, compared to the TdLNs, and also compared with the gene expression found in cTregs cells both in the TdLNs and in the tumor. As IL-10, TGFβ acts through the suppression of various immune effector cells at the tumor site, thus favoring tumor escape and promoting tumor growth (Wan & Flavell 2007). However, it can also promote invasiveness, and metastasis by regulating the extracellular matrix (ECM)

composition and degradation that plays complex roles in tumor invasion and metastasis and by indirectly inducing epithelial to mesenchymal transition (EMT) (Busse & Keilholz 2011). Finally, it can also promote tumor angiogenesis, which is correlated to a poor prognosis (Busse & Keilholz 2011). Altogether this suggests that the secretion of this cytokine could be a key role in the mechanisms by which Tr1 cells could be affecting the tumor growth. However, further studies that verify that this cytokine is being secreted to the media are key, since measuring the gene expression is not enough evidence. The same can be said about the expression of other genes that we measured. In the case of the granzyme B and perforin, which act together to induce apoptosis, our results seem contradictory since in the case of granzyme B we found that the expression of this gene tends to be higher in the tumor, compared to the expression in the TdLNs, in both subsets, which could be suggesting a higher activity of the perforin/granzyme B apoptosis pathway. However, in the case of perforin, its expression tends to be higher in the tumor compared to the expression in the TdLNs only in the case of cTregs, while in the case of Tr1 the expression tends to be much higher in the TdLNs. As it was stated in the introduction, the perforin/granzyme B pathway has been proposed as a mechanism by which Tr1 cells could be inhibiting tumor growth by killing TAMs, however, this pathway has also been proposed as a key mechanism by which cTregs promote tumor growth (Cao et al. 2007; Yan et al. 2017). In this sense, both Tregs subsets could be targeting different cell subsets inside the tumor with the same mechanisms and thus have a different overall effect on tumor growth. However, further studies that research the role of this pathway and its effect in the TME are needed in order to fully clarify how Tr1 cells could be affecting the tumor growth. In the case of Rab27a and Rab27b, as it was stated before,

they participate in the exosome release pathway, and exosomes contribute to the recruitment and reprogramming of constituents associated with TME (Kahlert & Kalluri 2013). Although exosome research in the tumor context is generally focused into cancer cells derived exosomes, it has been reported that exosome production by cTregs appears to be quantitatively greater than by other murine T cells (Agarwal et al. 2014). Furthermore, its release seems to contribute significantly to its immune suppressing activity since inhibiting the release of exosomes reversed these cells suppressive capabilities (Agarwal et al. 2014). Specifically, in the tumor context, it was reported that CD4+ T cell-derived exosomes could inhibit CD8+ cytotoxic T-lymphocyte responses and antitumor immunity in vivo in a B16 melanoma model (Zhang et al. 2011). Although our data is not enough to have conclusive results, we can see that the expression of both enzymes tends to increase in the tumor compared to the TdLNs in the case of Tr1, which could suggest that exosome release could be a mechanism of this subset to regulate tumor progression. It would be interesting to continue researching if the exosome secretion is a relevant mechanism of suppression by Tr1 cells.

Regarding the abundance of Tr1 cells in the tumor site, since we found such a high percentage of these cells in the TME (~30% of CD4+ T cells) we decided to do a BrdU assay to measure the proliferative capacity of this cell subset. Surprisingly, we found that Tr1 cells bear a low proliferative capacity compared to cTregs both in the TdLNs and in the tumor. Thus, this high percentage of Tr1 cells in the tumor cannot be explained by the proliferation of these cells in situ. A possible explanation could be that these cells are being induced in the TME by the chronic activation of CD4+ T cells in the presence of IL-10, which is naturally secreted by melanomas (Zeng et al. 2015; Jacobs et al. 2012).

Another explanation could be that these cells are migrating towards the tumor site at a high rate because of the expression of various chemokine receptors and integrins on the cell surface. This has been observed in the case of cTregs that accumulate in the TME of ovarian cancer, which releases CCL2 in large quantities, that is recognized by the CCR4 receptor on the surface of cTregs (Jacobs et al. 2012). Another relevant CCL2 chemokine receptor is CCR2, which has been implicated in the migration of cTregs into the tumor site from the TdLNs in a breast cancer model(Loyher et al. 2016).

Specifically, it has been reported that intestinal Tr1 cells, identified by their IL-10 secretion, express CCR4, CCR5, and CCR7 on their surface and are able to migrate to the periphery to suppress diabetogenic T cells (H. Yu et al. 2017). Thus, further research that takes into account the expression of relevant chemokine receptors that could be relevant to the trafficking of Tr1, such as CCR4 and CCR2, are needed to better understand the role of this subset in the tumor context.

Altogether, these data suggest that the local tissue environment (lymph node, tumor) influences Treg cells frequency and probably their activity and more research is needed to fully understand the role of Tr1 cells in the tumor growth, its mechanisms and its interactions with other cell subtypes in the TME.

9 CONCLUSIONS

In this study, we have provided a characterization of Tr1 cell subset (CD49b+Foxp3-CD4+ T cells) comparing and underscoring their similarities with the cTreg cell subset. We found that a high percentage of the CD4+ T cell subset in the TME is comprised of the Tr1 cell subset, compared to the percentage found in the TdLNs.

In order to demonstrate that this subset bore an immunosuppressive phenotype, we determined the expression of surface markers such as CD73 and Nrp-1 on their surface and the production of the IL-10 cytokine that are related to a Treg phenotype. The expression of these Treg markers supports the idea that this highly abundant subset present in the TME has a suppressive phenotype and might be promoting the tumor growth. We also determined that the expression of some of these markers changed depending on the microenvironment (TdLNs or tumor). However, further studies are required to demonstrate that this subset is able to suppress immunity and to promote tumor growth.

We also determined that this subset has a low proliferative capacity, compared to the cTreg subset, both in the TME and in the TdLNs. We hypothesize that Tr1 cells are either being induced in situ or are migrating from the TdLNs to the tumor. However, further research is required to answer this question.

Overall, our results support the idea that the TME is comprised of a variety of different cell types with different functions and that, besides cTregs, Tr1 cells are also present and have a suppressive activity. Furthermore, our findings highlight the clinical importance of this subset in the development of new antitumor therapie

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