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Title: "Delineating the role of Bone Marrow T regulatory cells in tumor growth and anti-tumor immunity"

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Abstract

Over the past decade, regulatory T cells (Tregs) have received a tremendous amount of attention due to their ability to suppress the function of multiple types of immune cells restraining excessive immune responses and ensuring peripheral tolerance and overall maintenance of immune homeostasis. Recently, mounting evidence has revealed the presence of Treg cells in non-lymphoid tissues of both mice and humans where they control non immunological processes and contribute to a plethora of diseases. In the present study, we focused on Bone Marrow (BM) Tregs which until now remain poorly explored and their role in tumor development and anti-tumor immunity is really underestimated. By performing an extensive phenotyping and some functional experiments, we unraveled that BM Tregs exhibit a differential phenotypic and functional profile compared with the well-studied Tregs of lymph nodes upon tumor induction indicating that they constitute a special and distinct population of the Treg cell lineage which possibly affects the immune responses during tumor growth.

Περίληψη

Την τελευταία δεκαετία, τα Τ ρυθμιστικά κύτταρα βρίσκονται στο επίκεντρο της προσοχής της επιστημονικής κοινότητας λόγω της ικανότητας τους να καταστέλλουν τη λειτουργία πολλών κυττάρων του ανοσοποιητικού συστήματος και να περιορίζουν τις ανεξέλεγκτες ανοσολογικές απαντήσεις, εξασφαλίζοντας έτσι την περιφερική ανοχή και τη συνολική διατήρηση της ανοσολογικής ομοιόστασης. Τελευταία, όλο και περισσότερα στοιχεία που υποστηρίζουν την ύπαρξη Τ ρυθμιστικών κυττάρων σε μη λεμφικά όργανα τόσο στο ποντίκι όσο και στον άνθρωπο έρχονται στο φως και αναδεικνύουν τη συμβολή των κυττάρων αυτών στον έλεγχο μη ανοσολογικών διαδικασιών αλλά και σε πληθώρα ασθενειών. Η παρούσα μελέτη επικεντρώνεται στα Τ ρυθμιστικά κύτταρα που εδράζονται στο μυελό των οστών τα οποία μέχρι σήμερα παραμένουν ελάχιστα μελετημένα και ο ρόλος τους στην ανάπτυξη του όγκου και στην αντικαρκινική ανοσολογική απάντηση έχει υποτιμηθεί. Πραγματοποιώντας μια εκτενή φαινοτύπηση και κάποια λειτουργικά πειράματα, διαπιστώσαμε ότι τα Τ ρυθμιστικά κύτταρα του μυελού των οστών υιοθετούν ένα διαφορετικό φαινοτυπικό και λειτουργικό προφίλ σε σχέση με τα καλά μελετημένα Τ ρυθμιστικά κύτταρα των λεμφαδένων έπειτα από την επαγωγή του όγκου, υποδηλώνοντας ότι αποτελούν ένα ιδιαίτερο και διακριτό πληθυσμό της κυτταρικής γενεαλογίας των Τ ρυθμιστικών κυττάρων ο οποίος μάλιστα πιθανά επηρεάζει τις ανοσολογικές αποκρίσεις κατά την ανάπτυξη του όγκου.

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Introduction

Definition, incidence and hallmarks of cancer

Cancer is not a single disease. It is a group of more than 100 diseases which all share a common characteristic, the abnormal and uncontrolled growth of body's cells that have the potential to invade or spread almost anywhere in the body. Most types of cancer form a tumor, a lump or mass of cancerous cells with exception of leukemias, most types of lymphoma and myeloma. A tumor can be benign or malignant and the fundamental difference of these two categories is the ability of the latter to grow and metastasize to other parts of the body.

Although cancer is the second leading cause of death worldwide behind ischemic heart disease, it is estimated that in high-income countries, deaths from cancer are now more than those related to cardiovascular diseases. More specifically, it is expected that the higher a country's gross domestic product, the lower the incidence of deaths from cardiovascular disease compared with those from cancer, despite the public health initiatives in high-income countries such as cancer screening which have a positive effect on some cancers mortality rate. This could be possibly partially explained by the Western lifestyle choices such as the tobacco use, alcohol consumption, obesity, low-nutrient/ high-fat diet and lack of physical activity which seem to drive many of the trends seen in cancer incidence levels (1,2).

Hence, taking into consideration the high incidence and mortality rate of cancer, it is not surprising that scientists have been focused on studying this complex disease for decades now and try to delineate its generative causes as well as to find new more effective treatments. Indeed, all these years of intense research have made it clear that during the multistep process of tumorigenesis, cancer cells acquire some functional capabilities that allow them to survive, proliferate rapidly and disseminate. These functional capabilities include their self-sufficiency in growth signals, their insensitivity to anti-growth factors, their replicative immortality, their ability to sustain angiogenesis and metastasize as well as their ability to evade immune destruction and to reprogram their energy metabolism in order to meet their increased energy needs as a consequence of their continuous growth and division.

More analytically, one of the main characteristics of cancer cells is their ability to sustain chronic proliferation. In contrast to normal cells which require mitogenic growth signals from their microenvironment in order to activate their cell cycle and move from a quiescent state into mitosis, cancer cells are much less dependent on exogenous growth stimulation for starting their division and this acquired autonomy is responsible for the disruption of a critically important homeostatic mechanism that ensures the maintenance of normal tissue architecture and function. There are four common molecular strategies for achieving liberation from dependence on exogenously derived signals. Firstly, cancer cells may produce growth factor ligands themselves to which they are responsive via the expression of cognate receptors, creating in this way a positive feedback signaling loop often termed as autocrine proliferative stimulation. Alternatively, they can send signals to stimulate the adjacent normal cells within the supporting tumor-associated stroma to supply them with various growth factors (3). Additionally, they may deregulate receptor signaling

by elevating the levels of receptor proteins displayed on their cell surface in order to hyperresponse even to limiting amounts of growth factor ligands. The same outcome can be achieved from structural alterations in the receptor molecules that facilitate ligand-independent firing.

Moreover, cancer cells apart from their ability to sustain proliferation through their continuous activation by growth-stimulatory signals, they are also able to resist to antigrowth factors which like their positively activating counterparts are received by transmembrane cell surface receptors coupled to intracellular signaling circuits (4–7). Apart from that, cancer cells have also defects in negative feedback mechanisms which act as loops that normally dampen various types of signaling ensuring homeostatic regulation of the flux of signals coursing through the intracellular circuitry. A prominent example involves the PTEN phosphatase, which counteracts PI3-kinase by degrading its product, phosphatidylinositol (3,4,5) trisphosphate (PIP3). Loss-of-function mutations in PTEN amplify PI3K signaling and promote tumorigenesis in a variety of experimental models of cancer as well as in human tumors (8,9). Another mechanism that ensures normal tissue homeostasis is "contact inhibition". Contact inhibition is a process of arresting cell growth when normal cells that propagate in two dimensional culture, form a confluent-monolayer. This cell-to-cell inhibition of proliferation is lost when cells undergo malignant transformation, leading to their uncontrolled division and to the formation of solid tumors.

Additionally, the ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death or in other words apoptosis represents a major source of this attrition. The evidence is mounting, principally from studies in mouse models and cultured cells, as well as from descriptive analysis of biopsied stages in human carcinogenesis, that acquired resistance toward apoptosis is a hallmark of almost all types of cancer. Elucidation of the signaling circuitry governing the apoptotic program has revealed how apoptosis is triggered in response to various physiologic stresses that cancer cells experience during the course of tumorigenesis or as a result of anticancer therapy as well as how malignant cells manage to prolong their survival and evade apoptosis (10). More analytically, the resistance to apoptosis can be acquired through a variety of strategies. The most common among them, as it is seen in a greater than 50% of human cancers, is the loss of function mutation of p53 tumor suppressor gene which causes the inactivation of its product and results in the removal of a key component of the DNA damage sensing machinery that induces the apoptotic effector cascade when is needed (11). Moreover, apart from p53, the PI3K-AKT/PKB pathway which transmits antiapoptotic survival signals is likely involved in mitigating apoptosis in a substantial fraction of human tumors. This survival signaling circuit can be activated by extracellular factors such as IGF-1/2 or IL-3 (12) by intracellular signals emanating from Ras (13) or by the loss of PTEN tumor suppressor which has been already mentioned. Finally, another mechanism that enables evasion of apoptosis relies on the upregulation of a decoy receptor for FAS ligand which is observed in a high fraction of lung and colon carcinoma cell lines and abrogates the FAS death signal. Furthermore, by 2000, it was widely accepted that cancer cells require unlimited replicative potential in order to generate macroscopic tumors. This capability stands in marked contrast to the behavior of most normal cell lineages in the body, which are able to pass through only a limited number of successive cell divisions. More analytically, once a cell population has progressed through a certain number of doublings, it stops growing and enters into a viable but nonreplicative state called senescence. In

some cases, normal cells can circumvent senescence by disabling their pRb and p53 tumor suppressor proteins and thus they are able to continue multiplying for some additional generations until they enter into a second state termed crisis. This state is characterized by massive cell death and karyotypic disarray associated with end-to-end fusion of chromosomes (14). Multiple lines of evidence have defined the telomeres as the counting device for cell generation. Telomeres which are composed by multiple tandem hexanucleotide repeats, play undoubtedly a central role in the immortalization of cancer cells. To explain it further, replicative generations are counted by the 50-100bp loss of telomeric DNA from the ends of every chromosome each cell cycle. This progressive shortening has attributed to the inability of DNA polymerases to completely replicate the 3' ends of chromosomal DNA. The progressive erosion of telomeres through successive cycles of replication eventually results to their inability to protect the ends of chromosomal DNA. These unprotective parts of DNA form end-to-end fusions that inevitably lead to cell death (15). Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in nonimmortalized cells but expressed at functionally significant levels in the vast majority of human cancer cells. By extending telomeric DNA, telomerase is able to counter the progressive telomere erosion that would otherwise occur in its absence offering in this way resistance to the induction of both senescence and apoptosis to the malignant cells that express it.

Lastly, malignant cells similarly to their normal counterparts, require oxygen, nutrients, growth factors as well as an ability to evacuate their metabolic wastes and carbon dioxide. For this reason, they need to reside in close proximity to blood vessels to access the blood circulation system. The early observation that rabidly growing tumors were heavily vascularized, while dormant ones were not, led Judah Folkman to propose that initiation of tumor angiogenesis was required for tumor progression and dissemination to distant sites. A compelling body of evidence indicates that angiogenic switch which occurs almost always during neoplastic growth, is controlled by a complex biological rheostat that involves both the cancer cells and the associated stromal microenvironment (16). It is now clear that a repertoire of cell types originating from the bone marrow play crucial roles in pathological angiogenesis (17–19). This repertoire includes cells of the innate immune system notably macrophages, neutrophils, mast cells, and myeloid progenitors that infiltrate premalignant lesions and progressed tumors and help primarily to the activation of angiogenesis in previously quiescent tissues sustaining on this way the ongoing tumor growth and secondly, to the protection of the vasculature from the effects of drugs targeting endothelial cell signaling.

Tumor Microenvironment and anti-tumor immunity

Tumor mass constitutes a highly complex and heterogenous ecosystem also known as tumor microenvironment (TME). TME contains not only malignant cells but also endothelial cells, pericytes, stromal fibroblasts and a variety of immune cells that control tumor growth and invasion.

As far as the non-immune cells within the TME are concerned, recent evidence which highlights the clonal heterogeneity of many human tumors, has given rise to a new subclass of neoplastic cells named as cancer stem cells (CSCs). CSCs may represent a double-threat for the majority of cancer patients as they are more resistant to therapeutic killing and at the same time endowed with the ability to regenerate a tumor once therapy has been halted (20,21). Alarmingly, the origin of these

cells has not been clarified yet. However, two possible scenarios arise. According to the first hypothesis, in some tumors, normal tissue stem cells may serve as the cells of origin that undergo oncogenic transformation to yield CSCs. On the other hand, some scientists support that CSCs could originate from partially differentiated cells also termed progenitor cells, that may suffer the initial oncogenic transformation thereafter assuming a more stem-like character. Apart from CSCs, much of the cellular heterogeneity within tumors is found in their stromal compartment. Prominent among the stromal constituents are the endothelial cells which once been activated, they gradually start constructing new blood vessels that form the tumor-associated vasculature (22,23). In collaboration with endothelial cells, pericytes ,a specialized mesenchymal cell type, wrap around the endothelial tubing of blood vessels and help the vessel wall to withstand the hydrostatic pressure of blood flow. Indeed, their importance in supporting the tumor endothelium is pointed up by the pharmacological perturbation of their recruitment. Finally, cancer-associated fibroblasts are a group of activated fibroblasts with significant heterogeneity and plasticity in the TME that secrete a variety of active factors to regulate tumor occurrence, development, metastasis and therapeutic resistance, nevertheless the full spectrum of their functions remains to be elucidated (24).

As far as the immune compartment of the TME is concerned, a large variety of immune cells can infiltrate tumor tissues, and their composition and organization within them are tightly associated with the clinical outcome of cancer patients. More specifically, almost all types of immune cells can infiltrate the TME including macrophages, polymorphonuclear cells, mast cells, natural killer (NK) cells, dendritic cells (DCs), T and B lymphocytes(25). The role of these immune cell types in tumour evolution and growth is diverse and is tightly associated with their inherent functions and with the molecules they express and secrete.

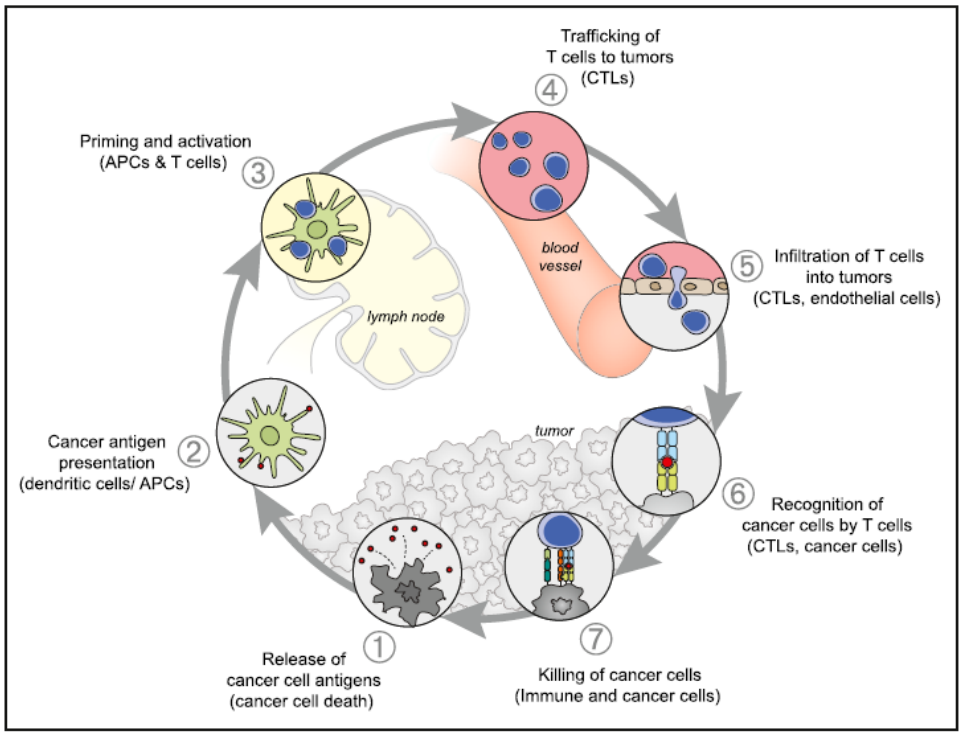


Figure 1a: The Cancer-Immunity Cycle, Daniel S. Chen and Ira Mellman, Immunity, 2013

Since cancer cells have accumulated a number of genetic alterations and have lost the normal cellular regulatory processes, they tend to express neoantigens, differentiation antigens or cancer testis antigens that are bound to major histocompatibility class I (MHCI) molecules on their surface distinguishing them from their normal counterparts. Boon et al. in 1994 showed for the first time that $CD8^+$ T cells are able to recognize these cancer-specific peptide - MHCI complexes and kill cancer cells (26). However, in order to a proper anti-tumor immune response take place, a series of events should precede. Firstly, neoantigens that are released by cancer cells should be captured and processed by DCs. Then, DCs present the captured antigens in complex with MHCI and MHCII molecules and this antigen presentation process in combination with the proinflammatory cytokines and factors that are released by dying tumor cells or by the gut microbiota are able to induce the priming and activation of naive T cells and elicit an effector T cell response. Although these interactions between naive T cells and mature DCs have traditionally been thought to take place in secondary lymphoid organs (i.e lymph nodes) it is now clear that they can also occur within, or adjacent to the tumoural tissue, in organized tertiary lymphoid structures (27). These specialized structures provide an area within the TME that is protected from the immunomodulatory effects of the tumour or stromal cells and is enriched with T cell activation cytokines. Finally, the activated effector T cells traffic to and infiltrate the TME, recognize and bind to tumour cells through the interaction between their T cell receptor (TCR) and its cognate antigen bound to MHCI and kill their target cancer cell (28). Apart from T cells, cytotoxic NK cells can also exert anti-tumor killing independently of any previous interaction with DCs, notably in case of a loss of MHCI from the surface of tumour cells.

However, although immune cells are able to recognize and kill malignant cells, it has been widely recognized that cancer cells are capable of escaping immune surveillance and antitumor immunity. There are several factors that contribute to tumor persistence despite a normal host immune system, including intrinsic mechanisms of carcinogenesis or extrinsic immunosuppressive characteristics of the TME. More analytically, as it has been already described, tumour cells must release immunogenic tumour antigens for the priming and activation of tumour specific T cells. Tumour-reactive T cells must then infiltrate tumor tissue and recognize cancer cells in the context of a peptide–MHC complex to induce cancer cell death. As a consequence, to evade immune-mediated elimination, tumours should develop strategies that disrupt this cycle. It is known that cancer cells can express a variety of non mutated and mutated antigens that have the potential to elicit tumor-specific immune responses (29). However, in order to avoid immune-mediated elimination, malignant cells may lose their antigenicity. Loss of antigenicity can arise due to the immune selection of cancer cells that lack or mutate immunogenic tumor antigens as well as through the acquisition of defects or deficiencies in antigen presentation. More specifically, even if a tumor expresses sufficient immunogenic antigens, immune detection is dependent on the capacity to present antigen in the context of a peptide–MHC complex. Thus, a more effective approach to address antigenicity is by assessing the capacity of malignant cells to present antigens. For example, tumours that lose MHC expression or acquire defects in antigen presentation machinery may escape immune-mediated elimination by tumor-specific T cells. To this end, downregulation of MHC class I molecules has been found in approximately 20% to 60% of common solid malignancies, including melanoma, lung, breast, renal, prostate, and bladder cancers (30).

In addition, although many tumors retain sufficient antigenicity for immune recognition, can escape elimination by decreasing their immunogenicity. For example, IFNγ produced by tumor-infiltrating lymphocytes (TILs) can induce the upregulation of the immunoinhibitory molecule PD-L1 on malignant cells (31) which once it binds on its receptor PD-1 on T cell surface, it inhibits T cell proliferation, survival and effector functions such as their cytotoxicity and release of effector cytokines (IFNγ, IL-2, TNFα), inducing their apoptosis. Interestingly, T cell dysfunction in cancer shares many features with the T cell exhaustion observed in chronic viral infections due to chronic antigen exposure on tumour cells and the unproductive interactions of TILs with DCs present in the TME. Similarly to PD-1/PD-L1 axis, other molecular pathways including those of LAG-3, TIM-3, VISTA, CTLA-4 and BTLA (32,33) can act to fine-tune the cellular fate of tumor-infiltrating T cells.

Moreover, several findings suggest that some tumours may retain sufficient antigenicity and immunogenicity for recognition by tumour-specific T cells but evade immune elimination by orchestrating a suppressive microenvironment. The ability of tumours to orchestrate an immunosuppressive microenvironment is dependent on reciprocal interactions between the cancer cells and host immune cells. Numerous populations of immune cells have been reported to have suppressive functions in the TME. Representative examples of those are the tumour-promoting M2 macrophages and immature granulocytic and monocytic myeloid-derived suppressor cells (MDSCs) which can favor tumour progression through the induction of stromal cell proliferation, vascularisation, extracellular matrix deposition (ECM), and cell migration (33–35) as wells as the T regulatory cells (Tregs) which constitute the prototypical immunosuppresive cells found in TME that directly secrete or facilitate the formation of immunosuppressive molecules (e.g., IL-10, TGF-β, IL-35, adenosine), modulate the APC function (e.g., via CTLA-4–CD80/86 interactions) and inhibit effector T cell functions.

Treg origin, characterization and stability

Focusing now on T regulatory cells (Tregs), over the last two decades, strong evidence has emerged for their dominant role in the regulation of immune homeostasis and tissue tolerance in both human and mice (36), although the concept of self/non-self discrimination and suppression mediated by T cells is nearly as old as the discovery of T cells as a separate lineage of lymphocytes. Already in the early 1970s, it was proposed that suppressor T cells would be capable of inhibiting other T cells and thereby mediate immunological tolerance(37,38). Suppressor T cells, which were characterized by the expression of the CD8 (Lyt-2) cell surface marker, have been the topic of more than 1000 scientific publications. However, the existence of suppressor T cells as a distinct lineage of T cells has been very controversial []. In fact, the concept of suppressor T cells was largely abandoned by the end of the 1980s, essentially because of the poor characterization of the cells and the lack of specific markers (39,40). In the mid-1990s, Tregs were identified as a new subpopulation of $CD4^+$ T cells and indeed hitherto, $CD4^+$ T cells are commonly divided into two distinct lineages: conventional T helper (Th) cells and Tregs which although they represent a large field of research, many central aspects of their cell biology remain obscure and hotly debated (41–48).

Tregs comprise approximately 5-10% of peripheral CD4+ T cells and can be divided into two major subtypes: the thymic or natural Tregs (nTregs) and the peripheral or induced Tregs (iTregs).The first subtype arises in thymus in early life during T cell development, while the second is generated later in the periphery upon exposure of naive $CD4^+$ T cells to specific tolerogenic stimuli (48,49). With recent advances in the ability to distinguish these two distinct subtypes based on the expression of unique cell surface markers and transcription factors (including Neuropilin-1 and Helios) (50), specific differences in gene expression, epigenetic modification, stability and function are starting to emerge. Indeed, nTregs and iTregs seem to exert different, yet complementary roles. To be more specific, nTregs seem to have a prominent role in recognizing self-antigens, while iTregs are implicated in establishing tolerance to non-self antigens (e.g. gut commensal bacteria and innocuous antigens present in food) (51,52).

Addressing now analytically the function of Tregs, their primary role was originally defined as prevention of autoimmune diseases and maintenance of tissue tolerance via inactivation of auto-reactive T-lymphocytes which have escape elimination during their development in thymus. Over the years, several additional functions have been suggested such as suppression of allergy and asthma (53–55), induction of tolerance against dietary antigens (56–58), induction of maternal tolerance to the fetus (59), suppression of pathogen-induced immunopathology (36-38), regulation of effector responses of other classes of immune cells (43,60), suppression of T-cell activation triggered by weak stimuli (61) as well as protection of commensal bacteria from elimination by the immune system (46).

Concerning whether the suppressive function of Tregs is antigen specific, several in vitro experiments have demonstrated that Tregs need to be first activated via the TCR to become suppressive(62,63), although this has been contested by others. This implies that Treg-cell activation is antigen-specific and that their suppressive activity is triggered in an antigen-specific fashion. The same requirement for antigen seems to apply for Treg functions in vivo, since the proliferation of Treg cells in lymph nodes was shown to be antigen dependent (64). Furthermore, in the experimental autoimmune encephalomyelitis (EAE) mouse model for multiple sclerosis, myelin basic protein (MBP)-specific Treg cells were detected and protection was associated with specificity for MBP (65). In the non-obese diabetic mouse model for type 1 diabetes, Treg cells specific for a pancreatic autoantigen were much more efficient at preventing diabetes than polyclonal Treg cells (64,66). It was further shown that pancreas-specific Tregs could only prevent diabetes when their specific antigen was present in vivo in the pancreas (67). Finally, destructive autoimmune gastritis could be prevented by transfer of stomach-specific Treg cells, but not with polyclonal Tregs(68) . Concerning the antigen specificity of the conventional Th cell that is suppressed by the Tregs, the key question here is whether the Treg and the Th cell need to recognize the same antigen or not. In vitro mixed-cultures experiments have demonstrated that Treg cells activated by their cognate antigen can suppress the proliferation of conventional Th cells with different antigen specificities (62,69). In vivo, there is also evidence that Treg cells may suppress Th cells with other antigen specificities (70,71). Therefore, taken all together, suppression mediated by Treg cells is clearly antigen-dependent. The activation of Tregs is antigen-specific, which implies that their suppressive activity is triggered in an antigen-specific fashion. Concerning the target cell, there is evidence that Tregs may suppress Th cells with different antigen specificities. However, it is possible that

suppression is more effective, and thereby physiologically more relevant, when the Treg cell and the suppressed Th cell have the same antigen specificity.

Similarly to any other subpopulation of immune cells, molecular markers are essential tools for the identification and analysis of Treg cells. The most widely used markers for defining Tregs are CD25 (72), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (73,74), glucocorticoid-induced tumour necrosis factor receptor family-related gene (GITR) (75), lymphocyte activation gene-3 (LAG-3) (75), CD127 (76,77) and forkhead ⁄ winged-helix transcription factor box P3 (Foxp3) (78–80). Unfortunately, accumulating evidence suggests that none of the above markers is strictly Treg-specific. Upon activation, all T cells express CD25, the a-chain of the interleukin-2 (IL-2) receptor (64,81,82) as IL-2 is a T-cell growth factor which is important for T-cell clonal expansion. Contrarily, CTLA-4 is a negative regulator of T-cell activation, which is upregulated on all $CD4^+$ and $CD8^+$ T cells, 2– 3 days following activation. Similarly, the expression of GITR (83) and LAG-3 (84,85) is induced in T cells upon activation. It has been suggested that CD127, the a chain of the IL-7 receptor, could be used to discriminate between CD127low Treg cells and CD127high conventional Th cells in humans. However, it has been recently reported that most $CD4^+$ T cells downregulate CD127 upon activation.

Importantly, the Foxp3 transcription factor is considered the most reliable marker for Treg cells as it represents the 'master regulator' or 'lineage-specification factor' for the development of this cell population (86). Continuous Foxp3 expression has been reported to be essential for maintenance of the developmentally established suppressive program in mature Treg cells in the periphery (87). It has been also suggested that expression of Foxp3 must be stabilized by epigenetic modification such as demethylation to allow the development of a permanent Treg cell lineage (88–90). Although Foxp3 is a transcription factor, its exact function remains largely unknown. It has been suggested that Foxp3 may act as a repressor of transcription with the function of regulating the amplitude of the response of $CD4^+$ T cells to activation (91). Moreover, it has been proposed that all human $CD4^+$ and $CD8^+$ T cells may upregulate Foxp3 and acquire suppressive properties upon activation (92), however, the main evidence supporting Foxp3 as a critical factor for Treg functions comes from experiments showing that naive T cells could be rendered suppressive by retroviral gene transfer of Foxp3 (78,79). Additionally, Foxp3 was considered an important factor for Treg functions because mutations of its gene were found to be the cause of two severe multiorgan autoimmune syndromes in humans, namely XLAAD (X-linked autoimmunity-allergic dysregulation syndrome) and IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (93–95). Similarly, mutant scurfy mice with a disrupted Foxp3 gene develop a fatal lymphoproliferative disorder and die within 4 weeks after birth (96). Thus, Foxp3 is clearly essential for Treg-cell functions and defective Foxp3 leads to lethal immune dysregulation.

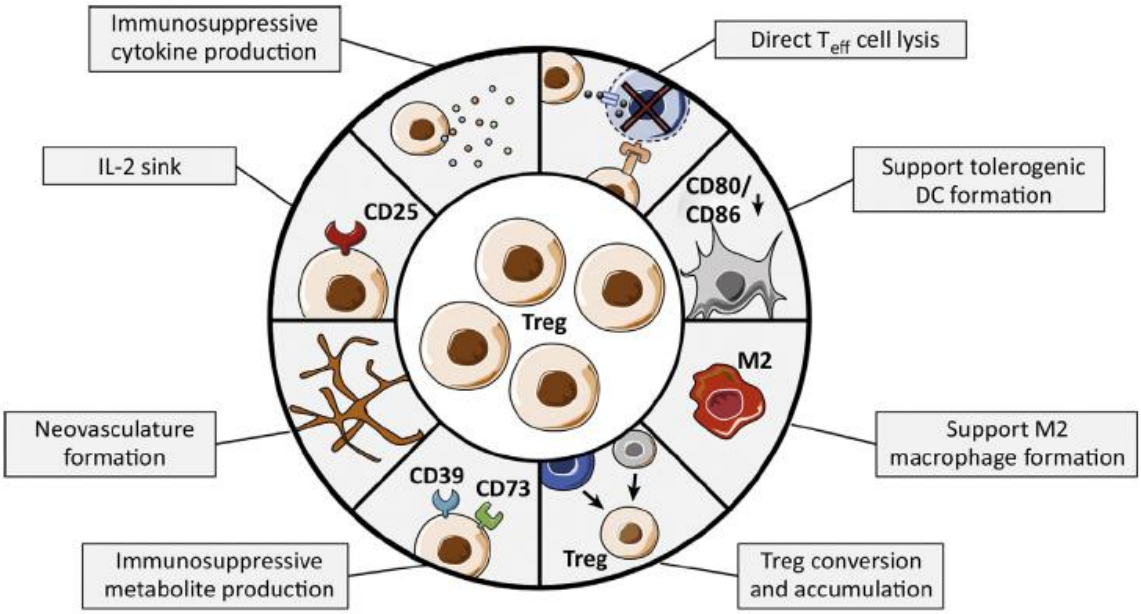


Figure 1b: Mechanisms of Treg-mediated suppression, Haiping Wang et al., Trends in Cancer, 2017

As far as the mechanisms of suppressive function of Tregs are concerned, they preserve the immune tolerance by a plethora of mechanisms which include both contact-dependent and contact-independent inhibition. More specifically, Tregs are able to suppress the effector functions of almost all cell types of the immune system including $CD4^+$ and $CD8^+$ T lymphocytes, NK cells as well as DCs via secretion of cytokines such as interleukin 10(IL-10), interleukin 35 (IL-35), and the transforming growth factor β (TGF-β) (97–99) , via metabolic disruption through CD39:CD73 adenosine production or interleukin 2 (IL-2) deprivation (100–102), via cytolysis through perforin and granzyme B or through the FasL-Fas signaling pathway upon direct cell-cell contact (103), and via modulation of DCs maturation and function. More analytically, Tregs modulate the activity of antigen presenting cells (APCs) by down-regulating the expression of co-stimulatory molecules on their surface (CD80, CD86, CD40) and by engaging their inhibitory receptors to their cognate ligands on DCs resulting in the attenuation or abolishment of signaling between APCs and T cells. Representative examples of this mechanism is the expression of lymphocyte activation gene 3 (LAG3) and cytotoxic T lymphocyte associated antigen 4 (CTLA-4) by Tregs (104,105) which indeed inhibits the interaction of DCs with the T effector cells and induces the expression of indoleamine 2,3-dioxygenase (IDO) that renders DCs tolerogenic respectively. Moreover, Tregs are able to inhibit the cytotoxic activities of immune effector cells by suppressing ,for instance ,the IFN-γ and TNF-α production in $CD8^+$ T cells. Collectively, through their interactions and their secreted mediators, Treg cells inhibit the proliferation and functions of immune effector cells, while they are also capable of killing both APCs and T cells. However, despite excessive experimental evidence regarding the biology and function of Tregs, the mechanisms and molecules dictating their contribution in the shaping of diseases, such as autoimmune syndromes, inflammatory disorders and cancer remain poorly determined.

Tregs and cancer

Studies in both mice and humans have shown that Tregs are abundantly recruited in the tumor mass, where they mediate the formation of a tolerogenic microenvironment that hampers the anti-tumor immunity, promotes immune evasion of cancer cells and favors tumor progression (106,107). Indeed, Tregs were found significantly increased within the TME of various tumor types in humans and mice (24,108). Higher Treg cell numbers in TME have been associated with reduced patient survival, increased likelihood for metastasis and advanced-stage disease in many types of cancer, including melanoma, pancreatic ductal adenocarcinoma (PDAC) ,non small-cell lung cancer, gastric and ovarian cancer (109,110). Treg levels have also been noted to be significantly elevated in the peripheral blood of patients with PDAC (111,112), gastrointestinal (113), eosophageal (114) and breast cancer (112), outlining their potential role as clinically relevant biomarkers of poor disease prognosis (113,114). However, the correlation of increased Treg cell numbers both in the tumor and in the periphery with the survival and disease stage of patients remains ambiguous, as other studies have demonstrated that a stronger Treg presence is correlated with favorable prognosis (115).

Experimentally, the role of Treg cells in tumor immunity was first demonstrated by an attempt to determine a common basis between tumor immunity and autoimmunity (116). Removal of Treg cells using cell-depleting anti-CD25 antibodies, either by in vivo antibody administration to mice or transfer of cell suspension depleted in vitro of CD25+ Treg cells into histocompatible T-cell-deficient mice, effectively eradicated a variety of inoculated syngeneic tumors (117). The mice showed an increase of tumor-infiltrating CD8+ T cells with strong tumor-specific killing activity, and upon re-challenge with the same tumor cells, exhibited more rapid rejection than the primary rejection, indicating the establishment of tumor-specific immunity (36). These studies have thus demonstrated that the removal of Treg cells is able to evoke effective anti-tumor immunity and have made it clear that intra-tumoral Tregs affect almost all immune cells present in the TME, besides T cells as for example, they impair presentation of tumor antigens by DCs, disrupting their recognition and consequently the elimination of cancer cells by immune effector cells.

The mechanisms that drive the accumulation of Tregs in the TME have not been fully elucidated yet, however, several lines of evidence associate intra-tumor Treg cell aggregation with the competition for extracellular nutrients and other challenges that the hostile TME poses. More analytically, it is known that cancer cells undergo a transition of OXPHOS to aerobic glycolysis, known as the Warburg effect (118). This metabolic shift in cancer cells leads to the consumption of environmental glucose and glutamine which is detrimental for conventional effector T cells that adopt unresponsive or functionally exhausted states but not for Treg cells (119). Treg cells have the metabolic advantage to respond in low-glucose and high-lactate TME (120) by reclaiming the fatty acid pathway in order to obtain energy supply. Thus, they have augmented survival capacity, which leads to their increased accumulation in the tumor masses. Additionally, metabolic factors other than nutrients also contribute to heightened Treg cell accumulation and function in the TME. Hypoxia and oxidative stress are common in some regions of tumor tissue due to lack of vascularity. Treg cells are relatively more sensitive to oxidative stress than conventional T cells and undergo potent apoptosis in the tumor microenvironment (121). Intriguingly, apoptotic Treg cells have been revealed to mediate superior immunosuppression through converting a large amount of ATP to

adenosine via CD39 and CD73 (121) , suggesting that tumor resident Treg cells sustain and amplify suppressive activity by inadvertent death via oxidative stress. These observations all underscore the importance of metabolic adaption of Treg cells in tumor microenvironment for their suppressive activity, although the precise metabolic status of tumor Treg cells needs to be determined by single cell metabolomics (122) or computational-based inference of metabolic gene expression in scRNA-seq data (123).

Aside from the metabolic causes, there are still many other triggers of the enriched Treg presence in the TME. Experimental evidence implicate the recruitment of Tregs in tumor masses through chemokines produced by either tumor or host cells such as CCL22, CCL5, CCL28, CCL2, and CXCL12 (109,124–129). For example, CCL22 is produced by infiltrating macrophages, DCs (124) and $CD8^+$ T cells in various tumors (130), while cancer associated fibroblasts (CAFs) produce CCL5 in experimental models of breast cancer (131). Furthermore, Tregs of cancer patients express CCR4, CCR1, CCR5, CCR10, and CXCR4 receptors, the cognate receptors of the above ligands and by blockading these chemokine receptors the migration of human Tregs in vitro is halted, while the recruitment of Tregs is impaired in solid tumor models (109,124). Besides their generation and accumulation in the tumor mass, the phenomenon of Treg induction is also quite expanded in the TME. A significant percentage of $CD4^+$ $Foxp3^-$ tumor-infiltrating T cells upregulates Foxp3 and is converted into Tregs, possibly due to molecules secreted in abundance by cells infiltrating the TME (132,133). For instance, cancer cells, DCs and stromal cells such as CAFs are a major source of TGFβ, a growth factor essential for the de novo formation of Tregs in the tumor stroma, while molecules facilitating iTreg differentiation such as interleukin 10 (IL-10), indoleamine 2,3-dioxygenase (IDO), cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) have been also found to be abundantly produced by cancer cells, immune cells (DCs and B cells) and other stromal cells in human tumor specimens.

Therefore, taking into consideration all the experimental and clinical evidence , it has been clear that intra-tumoral Tregs boost tumor growth and progression by suppressing the anti-tumor immune responses, promoting angiogenesis and stimulating metastasis. Thus, it is believed that by targeting Tregs in a clinical setting, either by depletion or functional modulation, shall prove to be an important therapeutic asset in the context of fighting cancer. For this reason, emerging approaches aim to define appropriate targets for selective interference with tumor-specific Tregs. Despite extensive analysis though, challenges on Tregs and their operation to cancer therapy are still undergoing.

Tregs and cancer therapy

Since Tregs impair T cell priming in secondary lymphoid organs and restrain antitumor immunity in the TME, it has been proposed that depleting Tregs would break this immune tolerance and unleash host antitumor immunity. Various therapeutic regimens currently used for the treatment of cancer have been shown to affect Tregs. First of all, low doses of cyclophosphamide eliminated Tregs in both mouse (134,135) and human studies (136) and also attenuated Treg function by

down-regulating FoxP3 and Glucocorticoid-induced TNFR-related protein (GITR) (137). In addition, fludarabine and gemcitabine, which inhibit DNA synthesis, have been shown to disrupt the proliferation, increase the apoptosis, and decrease the inhibitory functions of Tregs (138), while paclitaxel, a mitotic inhibitor, has been reported to induce their selective apoptosis (116). Finally, tyrosine-kinase inhibitors, such as imatinib, sorafenib, and sunitinib, attenuated their numbers and decreased CD69, CTLA-4, and FoxP3 expression on Tregs, allied with a decrease in IL-10 and TGF-β expression and secretion.

The past decade, a revolution in cancer treatments has been taken place by moving away from drugs that target tumors broadly (for example, chemotherapy and radiation) toward the use of antibody-based immunotherapies that modulate immune responses against tumors. The first generation of antibody-based immunotherapy, so-called immune-checkpoint blockade (ICB), works by blocking the receptor and/or ligand interactions of co-inhibitory molecules that are involved in dulling T cell activation or function. Treatment with blocking antibodies for CTLA-4 (ipilimumab, tremelimumab), programmed death ligand 1 (PDL1) (durvalumab, avelumab, atezolizumab) and programmed death 1 (PD1) (nivolumab, pembrolizumab, pidilizumab, atezolizumab, nivolumab) are FDA approved and have created promising results in the therapy of melanoma, kidney cancer, colorectal cancer, head and neck malignancies and bladder cancer. However, although ICB therapies have shown significant clinical benefit for several cancer patients, who demonstrate durable responses, there is still a group of patients, who do not respond. Therefore, understanding the rationale of unresponsiveness and predicting responsiveness to ICB on the basis of high-resolution data on the character and quality of tumor immune infiltrates is a critical next step in improving the success of current ICB and developing next-generation immunotherapies.
New inhibitory pathways are already under investigation, and drugs blocking LAG-3, T cell immunoglobulin mucin 3 (TIM-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and V-domain Ig Suppressor of T cell Activation (VISTA) are being investigated. Similar to immune checkpoint molecules, agonistic antibodies for co-stimulatory pathways such as 4-1BB and OX40 that augment immunological responses against malignant cells are under clinical trials. Despite the promising clinical efficacy of the ICI the critical cellular targets for their function remain unknown. Tregs abundantly express both co-inhibitory (CTLA4, PD1, TIGIT,VISTA, TIM-3, LAG3) and co-stimulatory molecules (GITR, 4-1BB, ICOS, OX40) at levels that are dependent on the TME, indicating that antibodies targeting these proteins could affect their function. However a definite correlation between the therapeutic efficacy and Treg frequencies and function in cancer is yet to be determined.

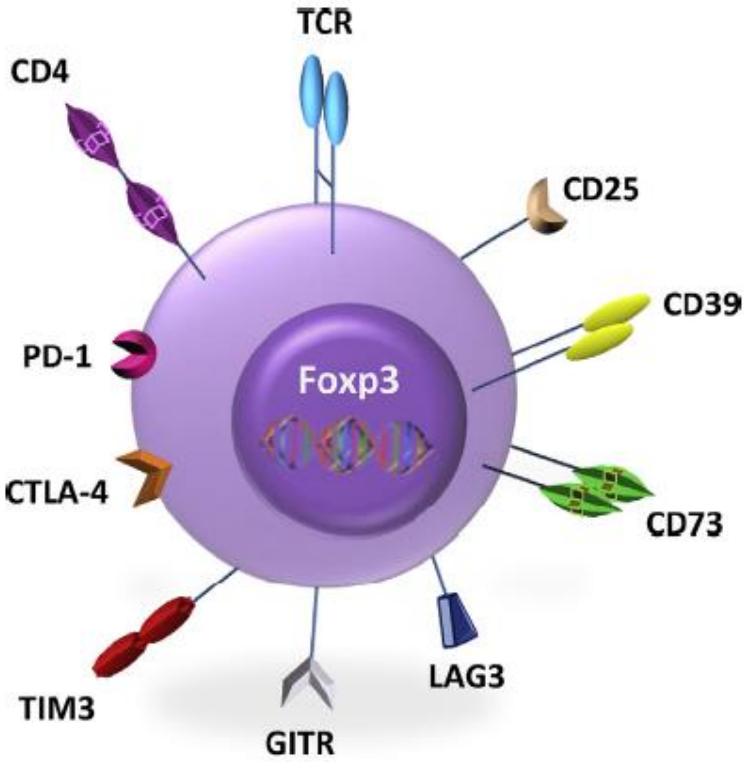


Figure 1c: Tregs express various checkpoint molecules that are targeted by ICB, T. Alissafi, et al., Journal of Autoimmunity, 2019

More analytically, as far as CTLA-4 is concerned, it is well established that it is highly expressed by Tregs and is generally considered to be a critical component for their suppressive function, as described previously. In this context, ipilimumab induces antibody-dependent cell-mediated cytotoxicity (ADCC) to decrease the Treg population (139). By contrast, tremelimumab does not have this activity but remains effective at inducing host antitumor immunity. In clinical trials, ipilimumab and tremelimumab induce similar patient outcomes, suggesting that Treg killing via ADCC is not required for tumor reduction. Importantly, although Tregs are the predominant immune population expressing CTLA-4 in tumors, the therapeutic effect of anti-CTLA-4 is not exclusive due to Treg deletion or inhibition. When only Tregs were targeted , tumor grew normally in a sophisticated set of experiments with melanoma-bearing immunodeficient Rag-/- mice (that lack mature T and B cells due to the deletion of recombination activating gene - Rag) reconstituted with different combinations of Tregs and T effector expressing the human or the murine CTLA-4 receiving GVAX vaccine and anti- CTLA-4 ICB. Thus , exclusive Treg targeting does not seem to account for the anti-tumor effect but anti-CTLA-4 effect on T effector cells is also needed (140). Intriguingly, conditional ablation of CTLA-4 expression in Tregs in adult mice has been recently reported to further strengthen their immunosuppressive functions (141). Furthermore, the results from in vitro suppression assays testing the capacity of peripheral Treg isolated from cancer patients to suppress NK and $CD8^+$ T cell killing as well as $CD4^+$ T cell proliferation are inconclusive. On the one hand, some studies support that anti-CTLA-4 did not alter the suppressive capacity of Tregs isolated from patient with renal carcinoma, progressive metastatic hormone refractory prostate cancer and melanoma (142,143) while others have shown that in vitro anti-CTLA-4 treatment results in diminished suppressive function and depletion of Tregs. Therefore, these

findings suggest that the biology of CTLA-4 in Tregs should be further examined to better harness the CTLA -4 targeting approach in cancer immunotherapy.

As far as PD-1 is concerned, it is well established that the interaction between PD-1 and its ligand PD-L1 inhibits antitumor T cell immunity by impairing effector T cell activation, and PD-1–PD-L1 blockade reactivates antitumor T cell response, resulting in inhibition of tumor growth (144). Importantly, apart from the activated and exhausted $CD4^+$ and $CD8^+$ T cells found inside the TME, PD-1 is also expressed by a fraction of Tregs, nevertheless, its exact function on them remains unclear. It is known that Tregs suppress the function of effector $CD8^+$ T cells by direct interactions of PD1 on Tregs with PD-L1 on $CD8^+$ cells. Thus, the disruption of this interaction with a blocking antibody is expected to abolish this suppressive function of Tregs (145). However, several lines of evidence have shown that PD1 can act as an inhibitor for T cell receptor (TCR) signaling. Thus, PD1 blockade may result in the reinforcement of Treg activation and suppressive function. Indeed, Tregs lacking PD1 presented increase suppressive capacity and rescued mice with autoimmune pancreatitis (146). Furthermore, several studies have highlighted the role of PD1 as a stabilizing signal for Tregs. Specifically, in melanoma tumor models PDL1 binding to PD1-expressing Tregs maintained Foxp3 expression and increased the numbers of induced Tregs. Indeed, in a clinical study with bladder cancer patients treated with two doses of anti-PD1 mAb before surgery, the percentages of peripheral $Foxp3^+$ Tregs increased after 3 weeks while decreased after 7 weeks following treatment (147). Furthermore, a recent study revealed that the treatment with monoclonal antibody to PD-1 reactivates $PD\text{-}1^+$ Treg cells found inside the TME by unleashing TCR and CD28 signals, which can explain the role of $PD\text{-}1^+$ Treg cells as a resistance mechanism to PD-1 blockade therapies. Thus, it is proposed that PD-1 expression by $CD8^+$ effector T cells relative to that of effector Treg cells in the TME can predict the efficacy of PD-1 blockade therapies (148) Therefore, taken all together, although several studies have been performed for the elucidation of the anti-PD1 effect on tumor infiltrating Tregs, the results remain inconclusive. On the one hand, anti-PD1 mAb enhances the suppressive capacity of effector Tregs in vitro, indicating that PD1 expressed on effector Tregs is a negative regulator of Treg cell-mediated immunosuppression (148) while on the other hand anti-PD1 treatment of peripheral blood mononuclear cells from melanoma patient's downregulates Tregs suppressive function by inhibiting Foxp3 expression (149,150).Thus, it is clear that further research is needed to understand the role of PD-1 receptor on Tregs as well as the effect of anti-PD1 mAb treatment on them.

Additionally, aside from ICB, it has been proposed that inhibiting Treg trafficking into the TME may promote cancer regression. $CXCR3^+$ Tregs accumulate in ovarian cancer, and tumor growth can be inhibited by blocking the interaction between CXCR3 and its ligands (CXCL4, CXCL9, CXCL10, and CXCL11) (151). Furthermore, CCR4-CCL17 and CCR4-CCL22 interactions can also promote Treg recruitment into tumor masses. Thus, clinical trials are currently underway to investigate the use of anti-CCR4 antibodies to interrupt the CCR4-CCL17 and CCR4-CCL22 pathways (152,153). Notably, targeting CCR4 using a monoclonal antibody can also induce ADCC in Tregs and cancer cells that express CCR4 (152) [186]. Moreover, the therapeutic benefits of targeting chemokine–chemokine receptor interactions to impair intratumoral Treg recruitment can be limited, since blocking these interactions may also suppress recruitment of effector helper and cytotoxic T cells into the TME and

other inflammatory tissues. Thus, abolishing general chemokine–receptor interactions could cause impairment of T cell-mediated immune responses.

Finally, neutralizing the immunosuppresive cytokines such as TGF-β, IL-10 and IL-35 produced by Tregs has been proposed to reestablish effective anti-tumor immunity. More specifically, blocking TGFβ signaling through injection of a TGFβ receptor recombinant protein linked to Fc also led to a reduction in metastasis in breast cancer models. However, blocking TGFβ alone may not be sufficient to promote tumor regression in established tumors (154). Moreover, it was recently reported that blocking IL-35, another immunosuppressive cytokine secreted by Tregs, can induce tumor regression in various engrafted tumor models. In contrast to directly targeting immunosuppressive molecules released by Tregs, targeting the GITR, an immunomodulatory receptor expressed by Tregs and activated T cells, has been suggested to abrogate Treg suppressive activities (155). Indeed, injection of an agonistic monoclonal antibody against GITR led to tumor regression by increasing T cell function and reducing the suppressive capacity of Tregs (156). Finally, combined treatment with an anti-CTLA4 antibody plus an anti-GITR antibody has been shown to induce a synergistic effect, eradicating advanced tumors in mouse models (156).

Tregs in non-lymphoid organs

It is widely accepted that Treg cells are primarily found in thymus, peripheral blood and secondary lymphoid organs such as the lymph nodes and spleen where they exert their well established functions which were previously described. Surprisingly, a growing body of evidence has recently come to appreciate populations of Tregs residing within non-lymphoid tissues such as visceral adipose tissue (VAT), muscle, intestin and bone marrow, collectively termed "tissue Tregs". The importance of these cells has been recognized not long ago, as apart from immunological processes, they also orchestrate tissue homeostasis and regulate non-immune or parenchymal cells.

Taking the tissues one by one, a unique population of Foxp3+CD4+ Tregs was discovered in VAT of lean mice (191) and serves as a paradigm for the tissue-Treg concept. Murine VAT Tregs and more specifically, those residing in the epididymal fat depot are distinct from their lymphoid counterparts by a number of criteria. First, Treg cells are unusually highly enriched within the VAT CD4+ T cell compartment of lean mice, in comparison with the 5–15% Treg representation typical of lymphoid organs such as the spleen and lymph nodes (157). This augmentation is first evident at 10–15 weeks of age in C57BL/6 mice, and a peak of 40–80% of CD4+ T cells is reached by 20–25 weeks. At even later ages, e.g., 40 weeks, VAT Treg levels unaccountably drop (158). Secondly, VAT Tregs display a distinct repertoire of antigen-specific receptors [T cell receptors (TCRs)]. Unlike what is seen in the corresponding lymphoid organs, the VAT Treg population of individual lean B6 mice exhibits clonal microexpansions (159). These two features suggest that VAT Tregs might be responding to a specific antigen or antigens in situ. Thirdly, Tregs in VAT have a transcriptome distinct from that of lymphoid organ Tregs. VAT Tregs, although they express the basic molecular markers of Treg lineage like Foxp3, CD25, and GITR, they transcribe only about 65% of the canonical Treg signature. Multiple classes of genes are differentially regulated in VAT, versus lymphoid organ Tregs, including those encoding transcription factors, chemokines and their receptors as well as a set of molecules

implicated in lipid metabolism (e.g., LDLR, Dgat). Remarkably, VAT Tregs uniquely express peroxisome proliferator-activated receptor γ (PPARγ) (160), a transcription factor usually restricted to adipocytes to drive adipose tissue development. Conditional knockout of PPARγ in Tregs results in a ~70% reduction of VAT Treg abundance, and the down-regulation of the VAT Treg-associated transcripts Ccr2, Gata3, Klrg1 and Cd69 (160). These findings indicate that PPARγ can act as a specific inducer and regulator of VAT Treg identity. Focusing now on their function, in vitro suppression assays of murine VAT Tregs showed that these cells are immunosuppressive (157) and are not functionally different from their counterparts found in typical lymphoid-organ (157) . However, their in vivo activities render them distinct. The initial suggestion that VAT Tregs might play a role in metabolic homeostasis came from correlative studies. First, Treg ablation by injection of diphtheria toxin into mice expressing the diphtheria toxin receptor (DTR) under the dictates of Foxp3 promoter/enhancer elements led to the induction of inflammatory mediators TNF-a, IL-6, RANTES and serum amyloid A-3 within VAT (157). Similarly, anti-CD25- mediated Treg depletion in diabetic leptin-deficient db/db mice elevated the pro-inflammatory cytokine transcripts, Ifng, Il6 and Tnfa, and down-regulated the VAT Treg signature markers Gata3, Ccr2, Klrg1 and Cd69. Additionally, the loss of Tregs resulted in pro-inflammatory macrophage and monocyte accumulation, without affecting anti-inflammatory monocytes, CD8+ T cells or B cells (160). Nevertheless, all of the above-mentioned studies entailed manipulation of Tregs systemically, so it was not possible to attribute the observed effects to adipose-tissue Tregs in particular. Generation of Foxp3cre PPARγfl/fl mice which specifically lack VAT Tregs solved this problem. In the lean state, these mice have a striking reduction in VAT, but not lymphoid-organ Tregs. As a consequence, VAT inflammation worsens and metabolic indices degrade. In addition, injection of PPARγ agonists, such as the thiazolidinedione drug, pioglitazone, into mice fed a high-fat diet expands the VAT Treg population and improves the local inflammatory tenor, as well as local and organismal metabolic health. Mice lacking PPARγ only within Tregs show a substantially muted metabolic response to pioglitazone treatment, arguing that an important component of the insulin-sensitizing effect of thiazolidinedione drugs, employed for many years as first-line agents in the treatment of type 2 diabetes, operates via VAT Tregs. Moreover, Bapat et al. by using the Foxp3cre PPARγfl/fl model, revealed that specific loss of VAT Tregs in lean, young (12-week-old) or high-fat diet-induced obese (24-week-old) mice does not impact glucose metabolism. In contrast, specific loss of VAT Tregs leads to improved fasting glucose and insulin levels in aged (36-week-old) mice suggesting an opposing role for VAT Tregs in age-associated insulin resistance (160). Nonetheless, there is an indisputable correlation between VAT Treg abundance and insulin sensitivity, and their essential role in maintaining metabolic homeostasis.

As far as skeletal muscles are concerned, a distinct population of CD4+ Foxp3+ T cells residing in acutely and chronically injured skeletal muscle was uncovered in 2013 (161), serving to extend the concept of tissue Tregs. Skeletal muscle Tregs can be distinguished from their lymphoid-organ counterparts by the same three criteria that set VAT Tregs apart from classical Tregs: their phenotype, TCR repertoire and transcriptome (161). A small population of Treg cells resides in normal muscle and it rapidly expands after mild cryoinjury or subsequent to the more severe damage induced by injecting cardiotoxin (ctx), reaching levels as high as 60% of the CD4+T cell compartment. An elevated muscle Treg fraction and number can be detected at least a month after acute injury. Tregs are located both within the inflammatory infiltrate of injured muscle and

between remote myofibers and it has been proven to potentiate muscle repair apart from maintaining tissue homeostasis. The transcriptome of skeletal muscle Tregs is readily distinguishable from that of lymphoid organ Tregs, although it is perhaps less distinct than that of VAT Tregs (161). Again, genes encoding transcription factors, chemokines and their receptors and cytokines and their receptors stand out as differential, certain of the same ones as seen for VAT versus lymphoid-organ Tregs, e.g., Ccr2 and Il10.

Focusing now on intestinal Tregs, both small intestinal and colonic Tregs contribute to 35% and 25% of residing CD4+ T cells respectively. The colonic Treg population includes two distinct components: one of them consists of thymus derived Tregs (tTregs) and is characterized by the expression of the transcription factors Gata3 and Helios, while the other differentiates from CD4+ Foxp3+ T cells in the periphery (pTregs) through the expression of the nuclear hormone receptor, RORγt, but little or no Helios [198]. The transcriptomes of RORγt+ and RORγt- Tregs residing in the large intestine differ by hundreds of transcripts (162). Indeed, given their clearly distinguishable transcriptomes, the $RORγt^{+}Helios^{lo/-}$ and $Gata3^{+}Helios^{+}$ Treg subpopulations residing in colon may have distinct functions. It has been hypothesized that the former controls local inflammatory responses whereas the latter participates in repair processes (198) given its induction by the alarmin cytokine IL-33 and the expression of the tissue-repair factor Areg (162). In general, intestinal Tregs are critical for oral tolerance, for tolerance to microbial mutualists and for controlling immune responses against enteric pathogens by regulating local APCs, CD4+ and CD8+ T cells as well as tissue resident B or plasma cells and their production of IgAs (163,164).

As far as the bone marrow (BM) is concerned, studies in both humans and mice have demonstrated that within the BM, the $CD4^{+}$ T cell compartment contains a substantially increased proportion of Tregs compared to that of other peripheral sites including peripheral blood (PB), spleen and lymph nodes (LN). Thus, within the BM, Tregs represent 20–60% of the $CD4^{+}$ T cell compartment (165), in contrast to the 5–15% typically found in the spleen (166). To date, however, whether BM Tregs represent pTreg or iTreg has not been elucidated. Although the majority of studies have focused on Treg form and function in the periphery (PB in humans and spleen and LN in mice), the limited number of studies which have investigated BM Tregs suggest that in addition to their enrichment, Tregs at this site exhibit features distinct from Tregs in the periphery. In this regard, comparison of the subset composition of Tregs in the BM, thymus and spleen of mice showed that 50% of BM Tregs express the co-inhibitory molecule TIGIT in contrast to 10% or 30% in thymus or spleen, respectively. TIGIT expression marks a highly suppressive activated/memory Treg population which is required for the control of autoimmune disease and contributes to anti-tumour immune responses (167). In further support of their activated/memory status, TIGIT+ Tregs express low levels of CD62L and high levels of CD44 and CXCR4 which is commonly upregulated on Tregs upon activation (165). Thus, it is supported that the BM appears to represent a niche for highly suppressive activated/memory Tregs. Through their enhanced CXCR4 expression, activated Tregs preferentially home to and are retained in the BM where stromal cells produce high levels of CXCL12/SDF-1 (165) and the disruption of this chemotaxis mobilizes Tregs into the periphery. Moreover, increasing evidence suggests that Tregs that reside within the BM are active and functionally relevant at this site. In elegant in vivo imaging studies, Fujisakie et al. demonstrated that Tregs co-localize with HSCs in the endosteal niche and are crucial for establishing an immune

privileged environment (168). In this study using non-irradiated recipients, transplanted purified syngeneic and allogeneic HSCs were engrafted and were maintained in the niche in similar numbers. However, Treg depletion resulted in a dramatic loss of allogeneic donor HSCs, which was associated with increased proinflammatory cytokine expression by BM CD4+ and CD8+ T cells. Mechanistically, IL-10 production by Tregs was shown to be critical for Treg-mediated protection of allogenic HSC within the niche. In line with this, recently, Hirata et al. identified a Treg cell population that localizes in the hematopoietic stem cell (HSC) niche of the BM that highly expresses the HSC marker CD150 and maintain HSC quiescence and immune privilege through adenosine. Furthermore, simirarly to Fujisakie et al. experiments, transfer of niche Tregs significantly improved allogeneic HSC engraftment (169). In addition, several studies report increased Treg frequencies in the BM of patients with metastatic prostate cancer compared to healthy donors. Thus, the observation that changes in the resident BM Treg population are associated with pathology further supports the importance of these cells in the regulation of disease. In the case of metastatic prostate cancer, BM Tregs were functional, highly proliferative and found to suppress osteoclast differentiation, in turn contributing to the osteoblastic bone lesions observed in patients with prostate cancer. Several studies support this finding, most notably Luo et al. demonstrated in vitro that Treg inhibit osteoclastogenesis and bone resorption through IL-10 and TGF-β secretion and that production of these cytokines was enhanced in the presence of estrogen (170).

Aim of the study

As it has been previously mentioned, several studies have revealed that within the BM, the $CD4^+$ T cell compartment contains substantially increased proportion of Treg cells compared to that of other peripheral sites. However, BM Tregs remain poorly studied and surprisingly almost all the studies that have been conducted until now have focused only on their role in the maintenance of HSC niche by acting as regulators of HSC quiescence, abundance and engraftment as well as controllers of graft-versus-host-disease (GVHD). Thus, it is obvious that many questions arise and remain unanswered as far as their phenotype, function and therapeutic potential in other pathologies such as cancer are concerned. Therefore, taking into consideration this gap of knowledge, the present study tries to delineate the role of BM Tregs in tumor development and anti-tumor immune response expanding the already established knowledge beyond the concept of stem cell transplantation.

Materials and Methods

Animals

C57BL/6J and FoxP3^{GFP+} transgenic mice (on a C57BL/6J background) were purchased from Jackson Laboratory and were maintained in the animal facility of IMBB. They were housed in cages under specific pathogen-free conditions, provided with standard food and UV purified water and maintained on a 12h light/12h dark cycle with lights on at 8:00$_{LT}$. The temperature was kept at 22^{o}C and the humidity at 45%. All animal procedures and experiments were in accordance with institutional guidelines and approved by the Institutional Committee of Protocol Evaluation together with the Directorate of Agriculture and Veterinary Policy. All in vivo experiments were performed by using female and age-matched mice aged between 8 and 12 weeks, unless otherwise stated. Tumor bearing mice were euthanized when tumor volumes calculated as the lenght*width^2*0.5 were >1.800mm^3 or at day 15 after tumor inoculation.

Cell lines

B16-F10, an adherent mouse skin melanoma cancer cell line, Lewis Lung Carcinoma (LLC), a mixed adherent and non-adherent mouse lung cancer cell line and MB49, an immunogenic semi-adherent mouse urothelial carcinoma cell line were kindly provided by A.Eliopoulos (School of Medicine, National and Kapodistrian University of Athens) and were negative for Mycoplasma spp., tested by PCR.

Cell Culture

B16-F10 were maintained in T-75 Corning flasks with 20ml RPMI Glutamax culture medium supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (p/s) and 0.1% β-mercaptoethanol at 37^{o}C, 5% CO_2, 95% humidity. Subculture was performed at a ratio of 1:10 when the flask reached its 90% confluency (every 2 days) by using 2ml Trypsin 0.25% in PBS which was incubated for 2-3 minutes at 37^{o}C and then was inactivated with 10ml fresh complete culture medium in order to cells be detached.

LLC, similarly to B16-F10, were maintained in RPMI Glutamax culture medium supplemented with 10% FBS, 1%p/s and 0.1% β-mercaptoethanol. Subculture was performed at a ratio of 1:4 when the flask reached its 90% confluency (every 2 days) by using 4ml Trypsin 0.25% diluted 1:1 in PBS sterile (2ml Trypsin 0.25% + 2ml PBS sterile for a T-75 corning flask) without incubation in order to detach cells. Culture supernatant with floated cells was collected and used for trypsin inactivation.

MB49 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% p/s and subculture was performed at a ratio of 1:5 when the flask reached its 90% confluency (every 2 days) and both floating clumps and adherent cells were collected by using 2ml Trypsin 0.25% in PBS similarly to B16-F10.
All washes were performed at 1650rpm for 6 minutes, RT.

Solid tumor induction

For the induction of solid tumors, cancer cells were collected at 2nd passage with Trypsin after a PBS wash. They were centrifuged at 1650rpm for 6 minutes, RT and then pellet was resuspended in 1ml PBS sterile. Cells were counted with diluted 1:5 Trypan blue in PBS with typical cell dilution 1:10 in Trypan blue. Mice were inoculated subcutaneously on the back at the base of their tail with $3x10^5$ B16-F10 melanoma cells or LLC cells or $7,5x10^5$ MB49 cells and each mouse received 100μl of cancer cell suspension. All cancer cells were kept at RT prior to injection.

Cell isolation from tumors and lymphoid organs

Single-cell suspensions from draining inguinal lymph nodes were obtained by smashing the tissues in 5% FBS in PBS and passing them through a 40-μm cell strainer. Bone marrow cells were isolated by flushing out with 5% FBS in PBS the 2 down legs in 15ml falcons, both femur and tibia bones. After a wash at 1800rpm at 4°C, erythrolysis was performed by using 2ml NH_4Cl/sample for 2min, RT. TILs were isolated by dissociating tumor tissue in the presence of collagenase D (1mg/ml, Roche) and DNAase I (0,25mg/ml, Sigma) in plain culture medium for 45 min in the waterbath at 37°C before passing through a 40-μm cell strainer. Up&down with a glass pasteur pipette every 15min during waterbath incubation is recommended to facilitate the process of tissue dissociation. All single cell suspensions were maintained at 4°C till their staining.

Flow cytometry

The antibodies that were used for extracellular staining were the following:

Antibodies	**Clone**	**Isotype**	**Source**	**Cat#**
CD45-PercPCy5.5	30-F11	Rat IgG2b, κ	Biolegend	103132
CD4-APC	GK1.5	Rat IgG2b, κ	Biolegend	100412
CD25-BV510	PC61	Rat IgG1, λ	Biolegend	102042
CD73-BV421	TY/11.8	Rat IgG1, κ	Biolegend	127217
CD39-PE	Duha59	Rat IgG2a, κ	Biolegend	143804
GITR (CD357)-PECy7	YGITR 765	Rat IgG2b, κ	Biolegend	120222
CTLA-4 (CD152)-PE	UC10-4B9	Armenian Hamster IgG	Biolegend	106306
PD-1 (CD279)-BV421	29F.1A12	Rat IgG2a, κ	Biolegend	135217
CD150 (SLAM)-PECy7	TC15-12F12.2	Rat IgG2a, λ	Biolegend	115914
CXCR4 (CD184)-PE	L276F12	Rat IgG2b, κ	Biolegend	146506
TIM-3 (CD366)-PECy7	RMT3-23	Rat IgG2a, κ	Biolegend	119716
LAG-3 (CD223)-BV421	C9B7W	Rat IgG1, κ	Biolegend	125221
Nrp1 (CD304)-PECy7	3E12	Rat IgG2a, κ	Biolegend	145212

For staining of extracellular markers, 50μl of each cell suspension was added in 1.5ml eppedorfs and incubated with 50μl of the appropriate antibody master mix for 20min at 4°C. Typical final antibody dilution in 5% FBS in PBS for extracellular staining was 1:200 (1:100 antibody dilution in each master mix)

The antibodies which were used for intracellular staining were the following:

Antibodies	Clone	Isotype	Source	Cat#
Helios-APC	22F6	Armenian Hamster IgG	Biolegend	137222
Foxp3-Alexa Fluor 488	FJK-16s	Rat / IgG2a, kappa	eBioscienc e	53-5773-82
phospho-mTOR (Ser2448)-PECy7	MRRBY	Mouse / IgG2a, kappa	eBioscience	25-9718-42
phospho-AKT1 (Ser473)-PE	SDRNR	Mouse / IgG2a, kappa	eBioscience	12-9715-42
phospho-4EBP1 (Thr36, Thr45)-PE	V3NTY24	Mouse / IgG2b, kappa	eBioscience	12-9107-42
phospho-S6 (Ser235, Ser236)-PECy7	cupk43k	Mouse / IgG1, kappa	eBioscience	25-9007-42

For intracellular staining, cells were stained for extracellular markers and then fixed and stained using the Foxp3 Staining Set (eBioscience) according to the vendor's instructions. Typical final dilution of Foxp3 and Helios antibodies in permabilization buffer was 1:50 while phospho-mTOR, phospho-AKT1, phospho-4EBP1 and phospho-S6 antibodies were used in 1:100 final dilution in permabilization buffer. All washes were performed in 1800rpm for 6min at 4^{o}C and BM, LN and Tumor cell pellets were typically resuspended in 400µl, 200µl and 400µl of 5% FBS in PBS respectively for acquisition. All samples were filtered before being analyzed using FACSCanto II (BD) and flow cytometry data were analyzed with FlowJo_V10.

Serum and supernatant isolation from tumors and bone marrow

Both naive and immunized mice were anesthetized and blood was collected by retro-orbital bleeding. Then blood samples rested at RT for at least 2 hours for blood starting to clot. Centrifugation at 2500rpm for 15min at 4^{o}C was performed and serums were isolated and stored at -80^{o}C in new 1,5ml eppedorfs till their use.

Tumors from immunized mice were isolated and weighed and tumor homogenates were generated in PBS containing a cocktail of protease inhibitors (PIs) (Roche) using a pestle. The volume of PIs in which the homogenization occurred was adjusted according to tumor weight. If tumor weight was lower than 0,25gr, homogenization was performed in 250µl of PIs as the minimum volume of homogenate which is required for ELISA is 200µl (each sample for ELISA is added in a 96-well plate in duplicates, 100µl/well). The homogenates were centrifuged at 13.000rpm for 10min, at 4^{o}C and the supernatants were collected in new 1,5ml eppedorfs and stored at -80^{o}C till their use.

For the generation of bone marrow supernatants, the 2 down legs of both naive and immunized mice were isolated and flush out of each femur bone was performed by using 300µl of PIs per femur bone (600µl PIs/mouse). Flush out was performed only once for each bone. All samples were centrifuged at 1800rpm for 10min at 4^{o}C and the supernatants were collected and stored at -80^{o}C in new 1,5ml eppedorfs till their use.

ELISA

Serums, tumor homogenates and BM supernatants were used for CXCL12/SDF-1 sandwich Elisa. In this assay, the detection of the cytokine of interest was a two-step process. The first day, the Elisa plate was coated with the capture antibody at the working concentration and overnight incubation at RT was occurred. The next day, samples were added undiluted in the appropriate wells followed

by the addition of the detection antibody. Each antibody which was used was specific for a different and non-overlapping region or epitope of CXCL12/SDF-1 and the whole assay procedure was performed according to the manufacturer's guidelines.

Suppression Assay

For the suppression assay, male FoxP3^{GFP} mice immunized with B16-F10 cancer cells were used. Mice were sacrificed at day 15 after tumor inoculation or when tumor volume was > 1800mm^3 and CD4$^+$FoxP3$^+$Treg cells and CD4$^+$FoxP3$^-$Teff cells were sorted from their Bone Marrow (BM) as well as from their draining inguinal lymph nodes (LNs). Then, the CD4$^+$FoxP3$^-$Teff cells were labeled with the division-tracking dye CellTrace Violet (CTV, Invitrogen, #C34557) according to the manufacture's protocol and co-cultured with beads coated with monoclonal antibody (mAb) to the invariant signaling protein CD3 plus mAb to CD28, which were prior washed with PBS sterile using a magnet, at a ratio 1 bead per 1 CD4$^+$FoxP3$^-$Teff cell as well as with CD4$^+$FoxP3$^+$Treg cells at different concentrations (ratio Teff/Tregs: 1:1, 2:1, 4:1). To be more specific, typically, 5*10^4 LN CD4$^+$FoxP3$^-$ Teff cells were plated in each well in a 96-well round bottom plate and cultured with 5*10^4, 2.5*10^4 and 1.25*10^4 BM and LN CD4$^+$FoxP3$^+$Treg cells respectively in the presence of the previously described T-Activator aCD3/aCD28 beads. As controls, CD4$^+$FoxP3$^-$CTV$^-$ Teff cells, CD4$^+$Foxp3$^-$CTV$^+$ Teff cells and CD4$^+$Foxp3$^-$CTV$^+$ Teff cells plus aCD3/aCD28 beads were used. For all experiments, cells were cultured in RPMI Glutamax supplemented with 10% FBS, 1% p/s, 0.1% β-mercaptoethanol. The plate was incubated at 37^{o}C, 5% CO_2 for 96h. After 96h, cells were collected, washed once with PBS and stained with anti-CD4 APC and anti-CD25 PE as indicator of activation and subjected to FACs.

Data analysis and statistics

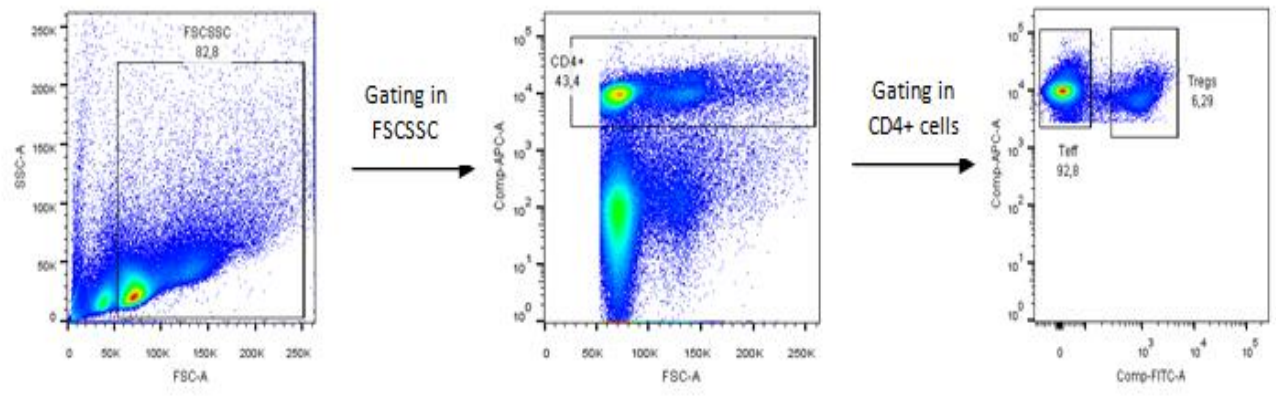
Results are presented as mean ± s.d or as frequencies of parent (frequency of a cell population in the previous gating). Data were analyzed using the two-tailed, Student’s t-test while multiple-group comparisons were performed using one-way analysis of variance (ANOVA test). All statistical analyses were performed on GraphPad Prism 7 and P values < 0.05 were considered to be statistically significant.

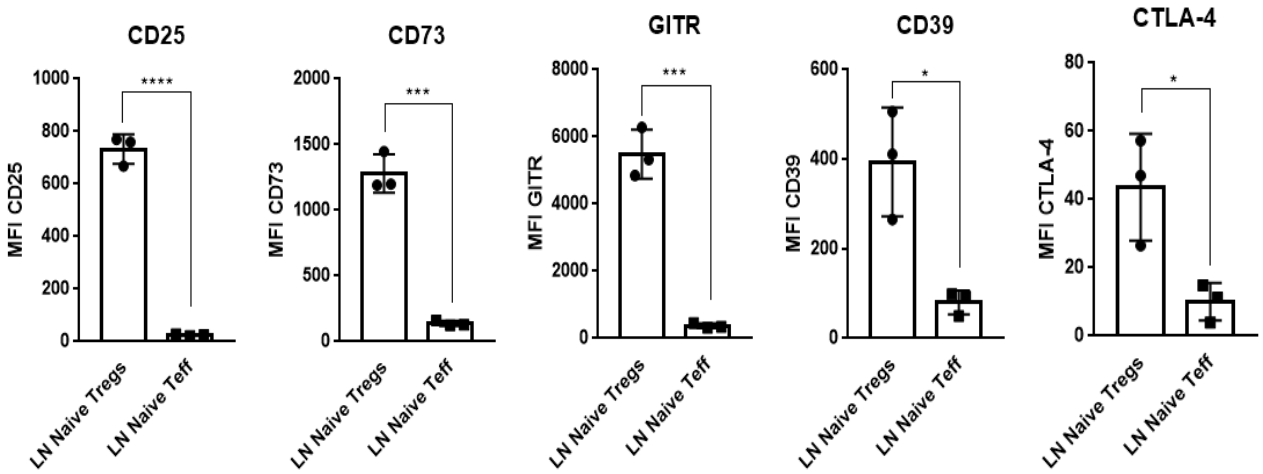
Results

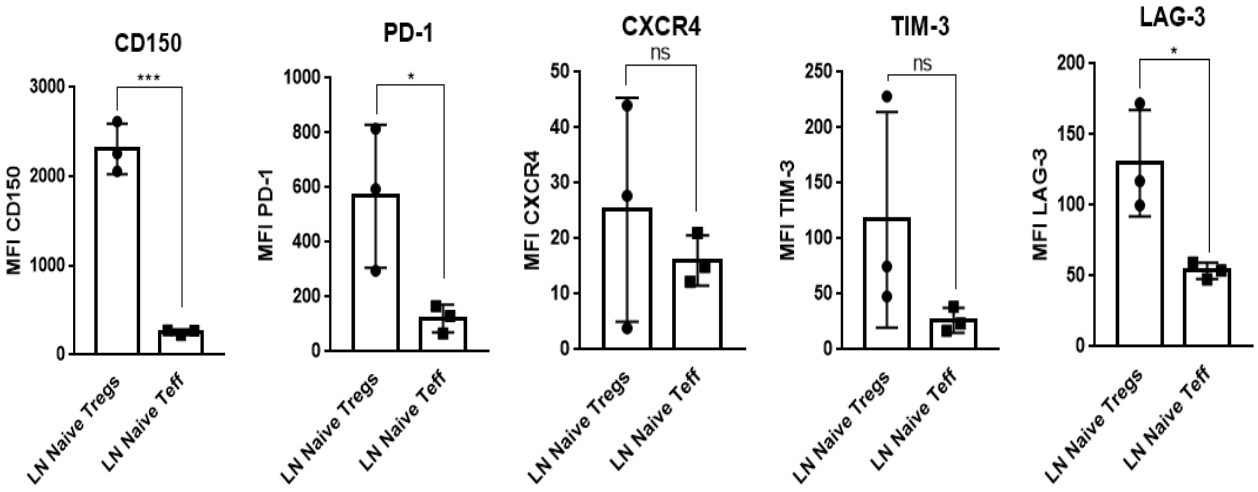
Although poorly studied, the experimental data that have been collected so far support that BM Tregs appear phenotypically and functionally unique to Tregs in the periphery. As a result, in order to better characterize them, we decided to compare them with the well studied Tregs of lymphoid organs (e.g LN Tregs).

I) LN Tregs express abundantly the molecular markers that characterize the Treg cell lineage

First of all, we investigated the expression of 8 immunomodulatory receptors characteristic of Treg cell lineage (CD25, CD73, CD39, GITR, CTLA-4, PD-1, TIM-3, LAG-3), of CD150 hematopoietic marker as well as of CXCR4 chemokine receptor by LN $CD4^+$ $Foxp3^+$ Tregs at their steady state and we compared it with the corresponding expression of these molecular markers by LN $CD4^+$ $Foxp3^-$ Teff cells. In particular, we found that LN Tregs express more abundantly all the studied immune checkpoint mediators (CD73, CD39, CTLA-4, PD-1, TIM-3, LAG-3) as well as the activation markers CD25 and GITR compared with Teff cells [**Figure 1**]indicating that Tregs that reside in lymph nodes exhibit all the well established phenotypic traits of Treg cell lineage. Interestingly, although the difference is not statistically significant, LN Tregs seem to also express higher levels of CD150 and CXCR4 compared with LN Teffs [**Figure 1**], which possibly denotes that CXCR4 receptor is more important for Treg than Teff cell homing to the lymph nodes.



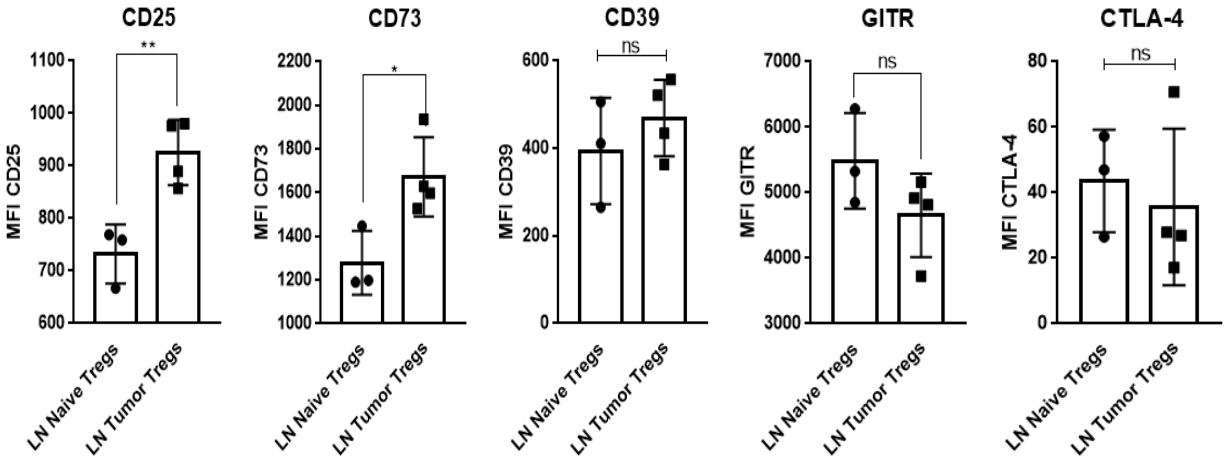




[Figure 1](): LN Tregs exhibit the phenotypic signature of Treg cell lineage. LN gating strategy and flow cytometry analysis of CD25, CD73, CD39, GITR, PD-1, CTLA-4, TIM-3, LAG-3, CD150, CXCR4 expression levels. MFI, mean fluorescence intensity. These results were reproducible in two independent experiments (total 7 mice). A representative figure from one experiment (3 mice) is shown here. Data are presented as mean ± SD and analyzed by t-test. p value<0.05 is considered statistically significant.

II) The expression profile of LN Tregs remains almost unmodified after tumor induction

Next, we examined whether the expression of the above molecules by LN Tregs is changed after tumor induction. In order to do that, we compared the expression profile of LN Tregs from naive animals with that of Tregs isolated from the tumor-draining lymph nodes (tdLNs) of B16-F10 melanoma cell inoculated mice. Of note, the expression of CD73 and CD25 was significantly enhanced while no important differences were observed in the expression levels of the other molecular markers after tumor inoculation [**Figure 2**]. The augmented expression of CD25 by LN Tregs isolated from tumor bearing mice may partially explain their activated state upon recognition of cancer-associated antigens.



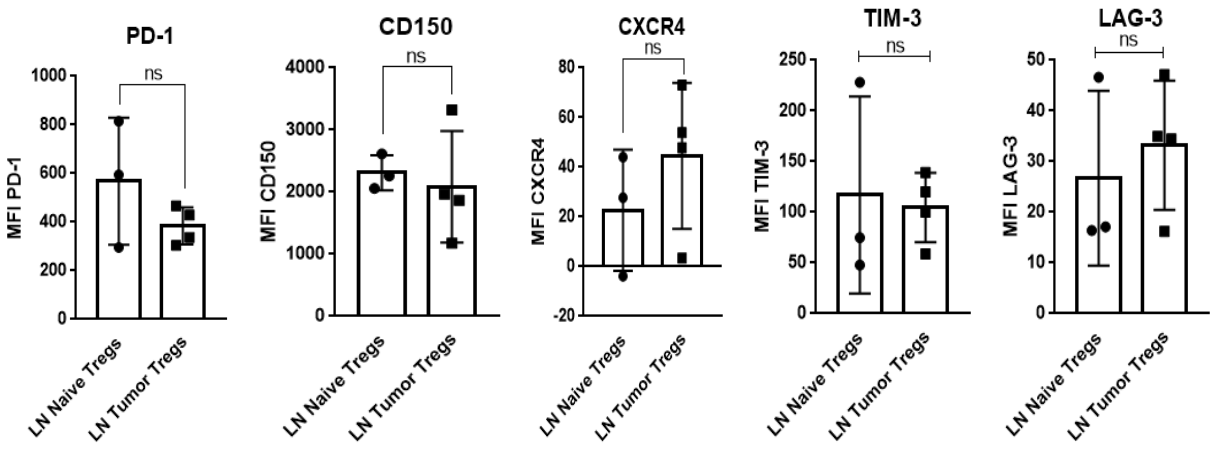


Figure 2: Tumor induction does not impact significantly LN Treg phenotype. Flow cytometry analysis of CD25, CD73, CD39, GITR, PD-1, CTLA-4, TIM-3, LAG-3, CD150, CXCR4 expression levels. MFI, mean fluorescence intensity. The results for CD25, CD73, CD39, GITR, CTLA-4 ,PD-1 and CD150 were reproducible in two independent experiments (total 14 mice). The data for CXCR4, TIM-3 and LAG-3 are representative of 1 experiment due to variation, therefore further investigation is required for conclusive results. A representative figure from one experiment (7 mice) is shown here. Data are presented as mean ± SD and analyzed by t-test. p value<0.05 is considered statistically significant.

III) BM Tregs express higher levels of all the studied molecular markers compared with LN Tregs both at steady state and during tumor development

Focusing now on BM Tregs, as it has been already mentioned, the precise phenotype of these cells remains ill defined. For this reason, firstly, we examined whether this distinctive Treg population shares the same phenotypic signature with its well defined counterparts found in lymph nodes at its steady state. Notably, we found that BM Tregs not only express all the above molecular markers but also they express almost all of them at higher levels that LN Tregs. No important differences were observed at the expression levels of CD150 and PD-1 [**Figure 3**]. Additionally, consistent with what Hirata et al. had demonstrated, BM Tregs express significantly elevated levels of CD73 and CD39 [**Figure 3**], the two cell-surface ectoenzymes that are required for the generation of extracellular adenosine, which according to their experiments , mediates the allo-HSC engraftment and maintains HSC quiescence and number in the hematopoietic niche **[Hirata et al]**.

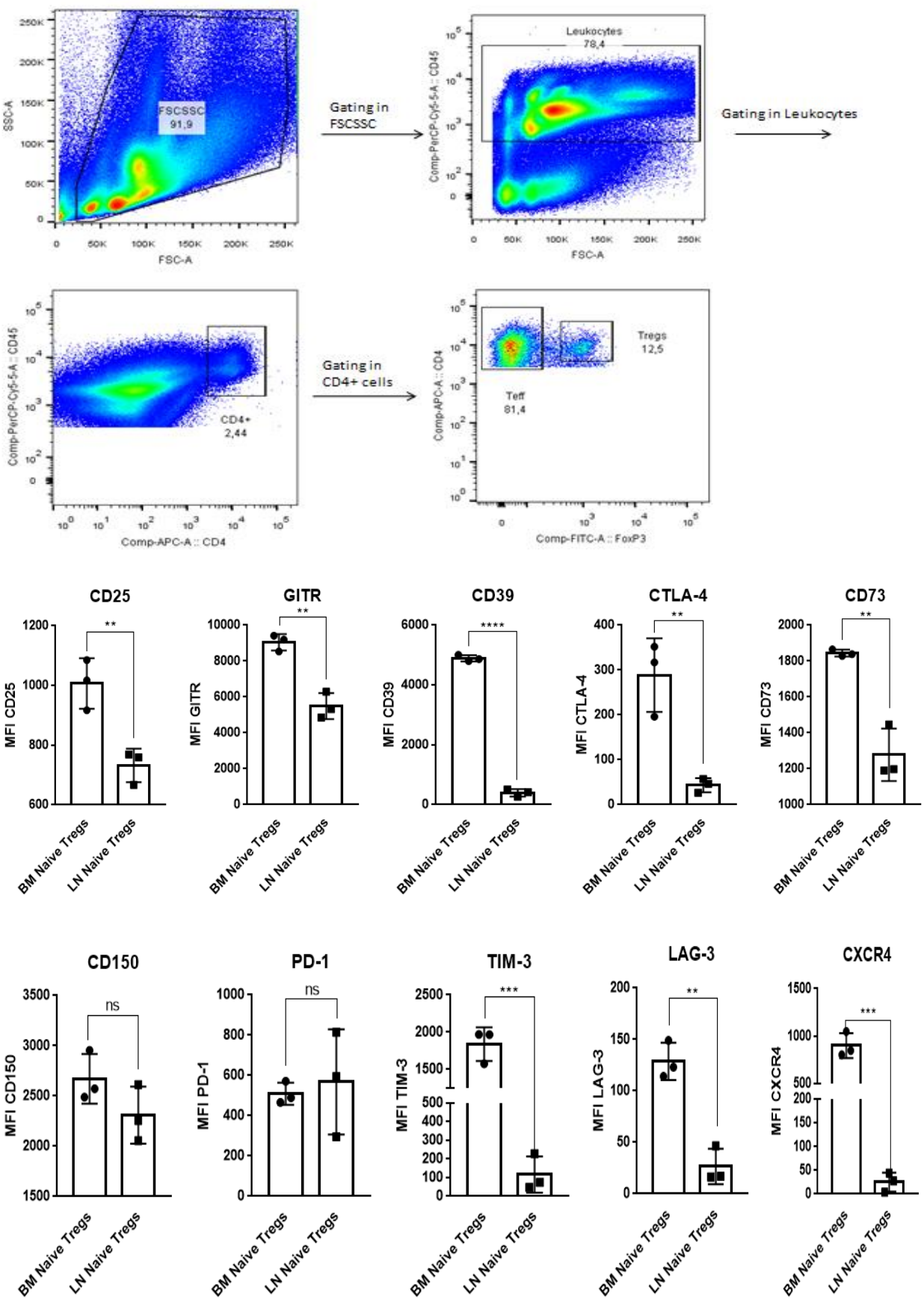


Figure 3: BM Tregs express intensively at steady state all the molecular markers that characterize the Treg cell lineage. BM gating strategy and flow cytometry analysis of CD25, CD73, CD39, GITR, PD-1, CTLA-4, TIM-3, LAG-3, CD150, CXCR4 expression levels. MFI, mean fluorescence intensity. The results for CD73, CD39, CTLA-4, CD150, CXCR4, TIM-3 and LAG-3 were reproducible in two independent experiments (total 7 mice).

The data for CD25, GITR and PD1 are representative of 1 experiment therefore at least one more revision of the experiment is required for conclusive results. A representative figure from one experiment (3 mice) is shown here. Data are presented as mean ± SD and analyzed by t-test. p value<0.05 is considered statistically significant.

To assess now the expression profile of the above molecular markers in the context of tumor induction, we compared the BM Tregs with LN Tregs isolated from B16-F10 melanoma bearing mice. Surprisingly, we observed again that even in the case of cancer, BM Tregs express markedly increased levels of almost all the above surface molecules compared with LN Tregs [**Figure 4**]. This finding indicates that upon tumor development, the suppressive function of BM Tregs may relies more on the utilization of the co-inhibitory receptors CTLA-4, PD-1, TIM-3 and LAG-3 as well as on the metabolic perturbation through CD39 and CD73 ectoenzymes compared with their counterparts found in lymph nodes [**Figure 4**]. In fact, although CD25 and CD150 did not demonstrate any significant difference, there is a trend of increasing expression toward BM Tregs [**Figure 4**].

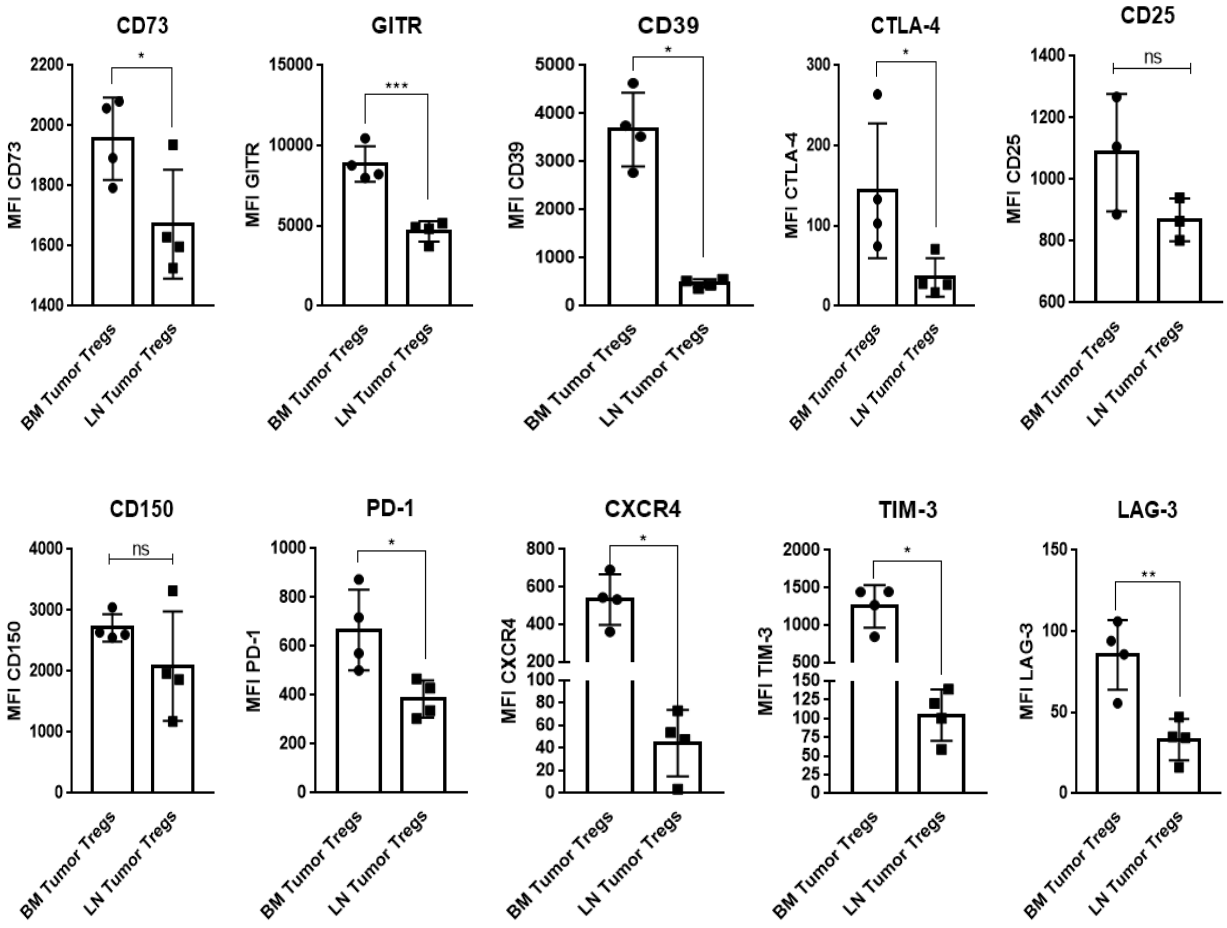


Figure 4: BM Tregs exhibit a similar but also a more enhanced phenotypic profile compared with LN Tregs during tumor development. Flow cytometry analysis of CD25, CD73, CD39, GITR, PD-1, CTLA-4, TIM-3, LAG-3, CD150, CXCR4 expression levels. MFI, mean fluorescence intensity. These results were reproducible in two independent experiments (total 7 mice). A representative figure from one experiment (4 mice) is shown here. Data are presented as mean ± SD and analyzed by t-test. p value<0.05 is considered statistically significant.

IV) The phenotypic traits of BM Tregs remain almost the same before and after tumor induction

Next we sought to unravel whether BM Tregs alter the expression profile of the studied molecular markers during tumor development. In order to do that, we compared the expression of each molecule by BM Tregs isolated from naive mice with its corresponding expression by BM Tregs isolated from B16-F10 melanoma bearing mice. Interestingly, the expression of cell surface molecules LAG-3 and TIM-3 is markedly reduced in tumor inoculated mice [**Figure 5**] indicating that the suppressive function of BM Tregs is possibly mediated mainly by other mechanisms apart from these two co-inhibitory receptors. A similar reduction is observed for CXCR4 receptor which is known to play a critical role for Treg homing to the BM but not to the spleen and lymph nodes [**Figure 5**]. The other studied markers do not demonstrate any notable alteration after tumor induction [**Figure 5**].

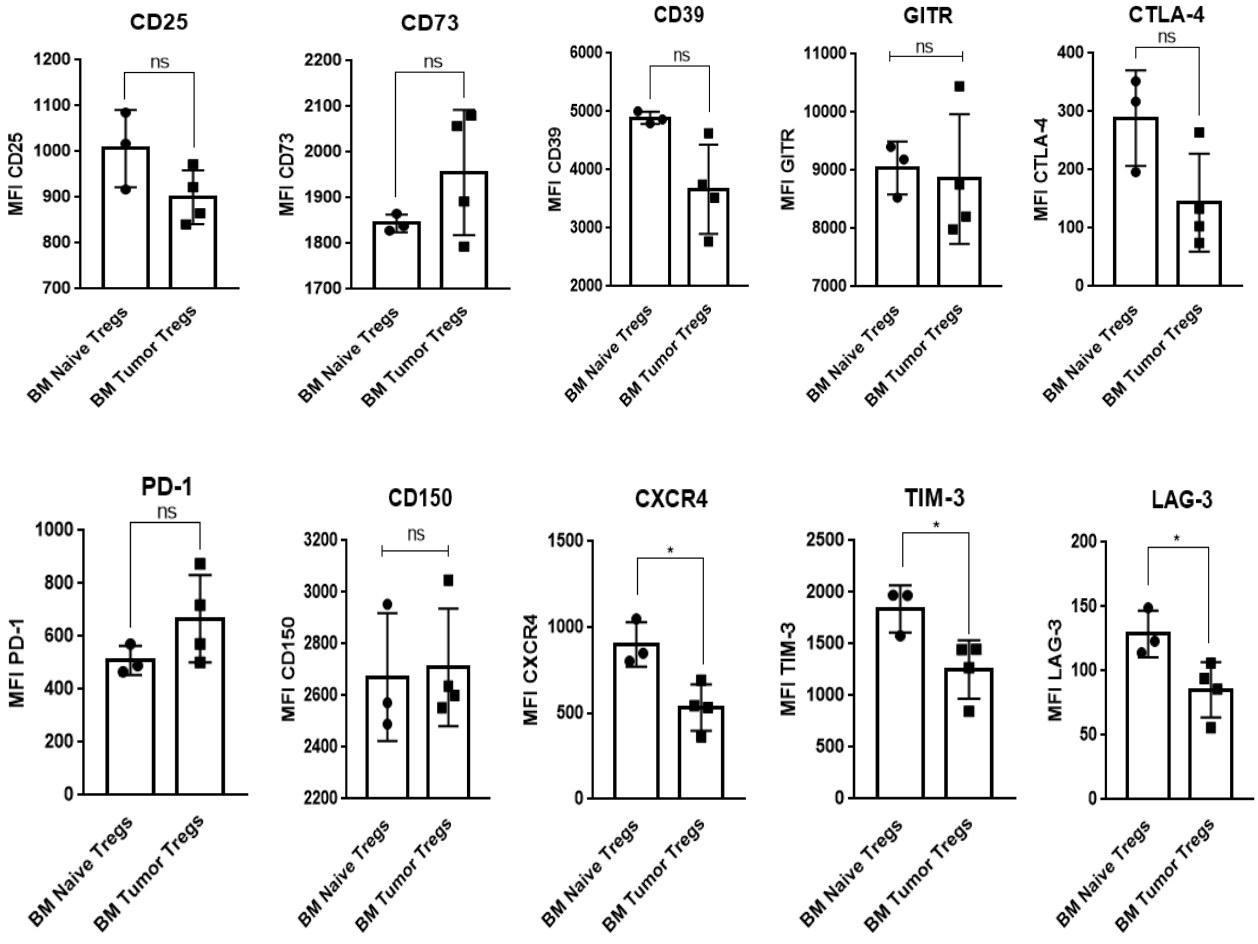
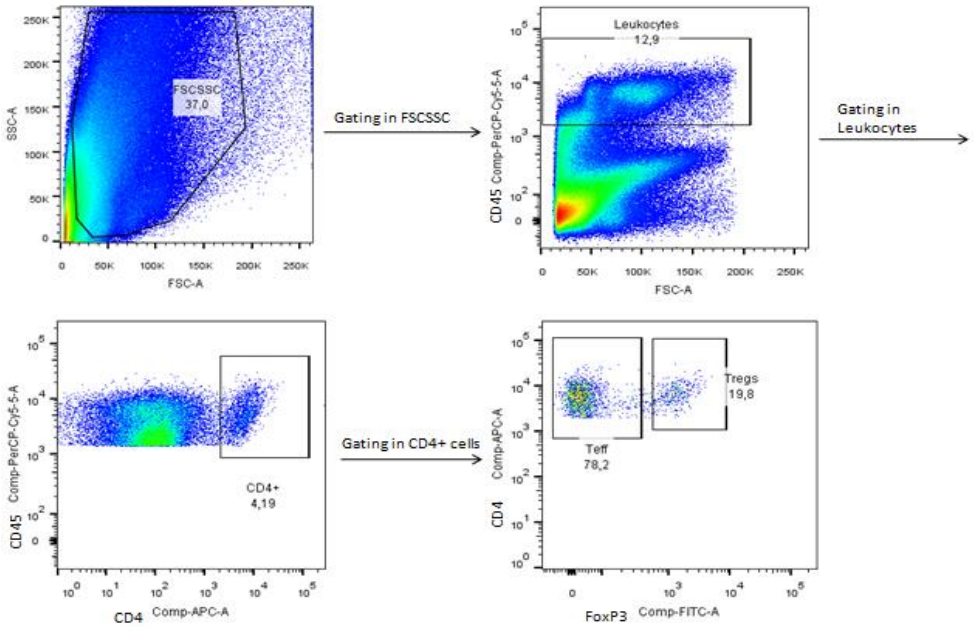
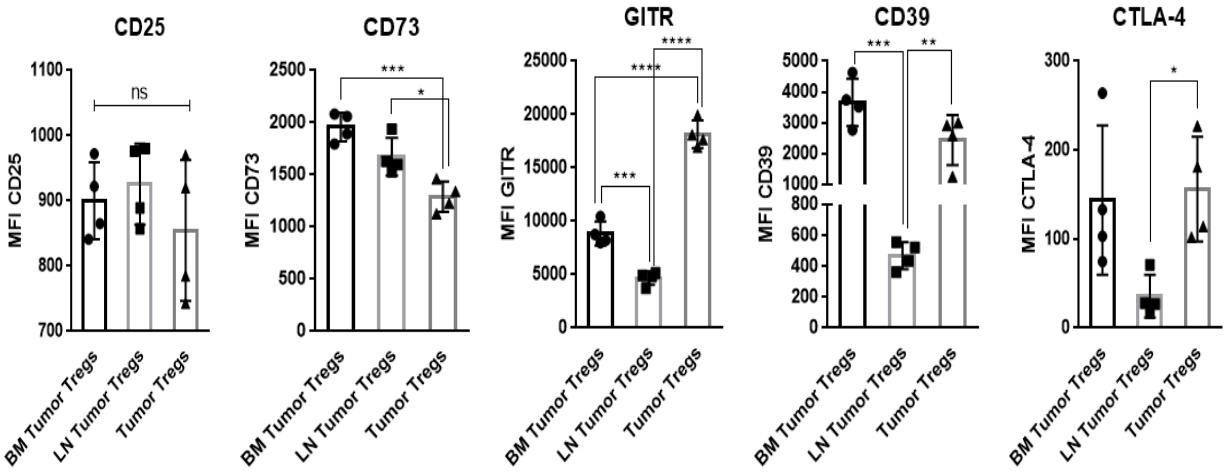


Figure 5: Tumor induction does not change extremely the phenotypic traits of BM Tregs. Flow cytometry analysis of CD25, CD73, CD39, GITR, PD-1, CTLA-4, TIM-3, LAG-3, CD150, CXCR4 expression levels. MFI, mean fluorescence intensity. The results for CD73, CD39, CXCR4 and LAG-3 were reproducible in two independent experiments (total 14 mice). The data for CD25, GITR, CTLA-4, PD1, CD150 and TIM-3 are representative of 1 experiment therefore at least one more revision of the experiment is required for conclusive results. A representative figure from one experiment (7 mice) is shown here. Data are presented as mean ± SD and analyzed by t-test. p value<0.05 is considered statistically significant.

V) BM Tregs phenotypically more closely resemble intratumoral than LN Tregs during tumor development

To further elucidate the phenotypic traits of BM Tregs after tumor induction, we compared them with the activated and cancer-antigen specific intratumoral Tregs. More specifically, we compared the expression profile of BM Tregs with that of LN Tregs as well as of intratumoral Tregs all isolated from B16-F10 melanoma bearing mice. As it is shown in the figure below, BM Tregs express almost similar levels of the activation marker CD25 and of the immune checkpoint molecules CTLA-4, PD-1, TIM-3 and LAG-3 with the intratumoral Tregs [**Figure 6**] indicating that possibly the previous mentioned co-inhibitory receptors represent a common immunosuppressive mechanism that BM and Tumor resident Tregs share during tumor growth. Additionally, BM Tregs on the one hand demonstrate an enhanced expression of CD73 while on the other hand exhibit a downregulation of the co-stimulatory receptor GITR compared with the intratumoral Tregs [**Figure 6**].





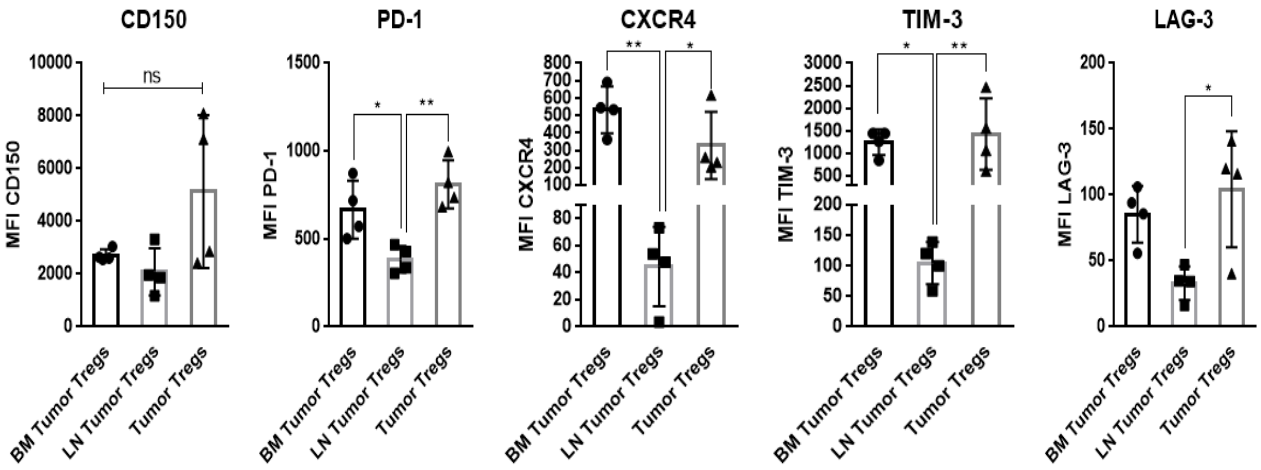
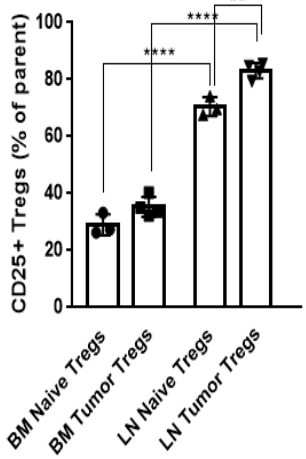


Figure 6: BM Tregs share more common phenotypic traits with intratumor Tregs than with LN Tregs during tumor development. Tumor gating strategy and flow cytometry analysis of CD25, CD73, CD39, GITR, PD-1, CTLA-4, TIM-3, LAG-3, CD150, CXCR4 expression levels. MFI, mean fluorescence intensity. The results for CD73, CD39, CTLA-4, PD-1,CXCR4 and LAG-3 were reproducible in two independent experiments (total 7 mice). The data for CD25, GITR, CD150 and TIM-3 are representative of 1 experiment therefore further investigation is required for conclusive results. A representative figure from one experiment (4 mice) is shown here. Data are presented as mean ± SD and analyzed by one-way ANOVA. p value<0.05 is considered statistically significant.

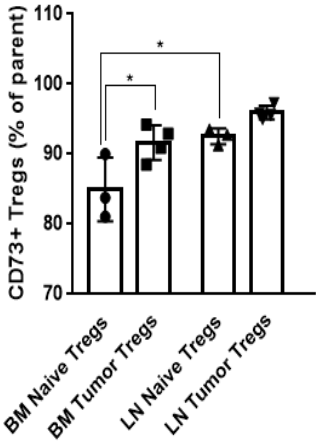
VI) The relative frequencies of several BM Treg subpopulations differ compared with their corresponding of LN Tregs.

Additionally, we assessed apart from the intensity of expression, the relative frequency of Tregs that express each marker separately in the total population of BM Tregs both at steady state and after tumor inoculation and we compared it with its corresponding frequency of Tregs found in lymph nodes. Interestingly, the frequencies of $CD25^+$ and $GITR^+$ Tregs in the total Treg population are significantly reduced in the BM compared with the LNs of both naive and tumor bearing mice [**Figure 7**] indicating that based on these two activation markers, BM compartment may contains a remarkably smaller proportion of activated Tregs at both states. In contrast, an increase in $CD150^+$, $GITR^+$ and $CD73^+$ Tregs is observed in the BM of mice after tumor induction [**Figure 7**]. Similarly, BM is more enriched in $CXCR4^+$ Tregs at both steady and activated state of mice compared with LNs [**Figure 7**] denoting probably that the $CXCR4^+$ Treg subpopulation preferentially home in BM than in LNs . Finally, the frequency of $TIM\text{-}3^+$ Tregs in the BM of tumor inoculated mice is markedly reduced compared with that of naive BM while mice at their steady state have more $LAG\text{-}3^+$ Tregs in their BM compared with their LNs [**Figure 7**]. This finding reveals that in the BM, Tregs not only express higher levels of LAG-3 [**Figure 3**] but also a larger number of the total BM resident Treg cells expresses this co-inhibitory receptor [**Figure 7**]indicating that LAG-3 may play an important role in the regulatory function of this distinct Treg population.

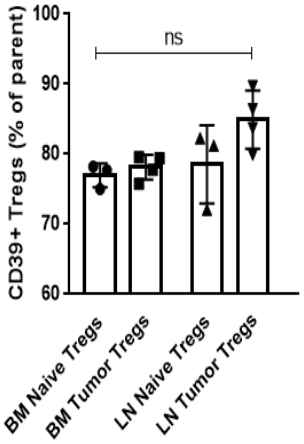
CD25+ Tregs (% frequency in Tregs)



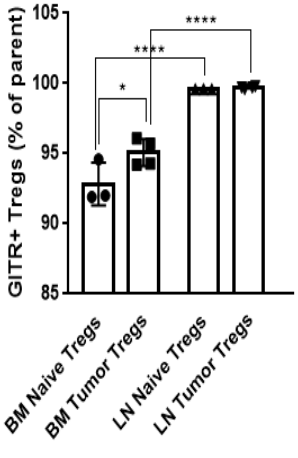
CD73+ Tregs (% frequency in Tregs)



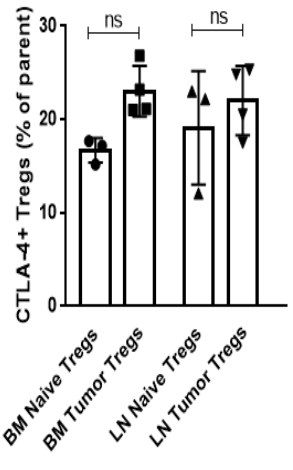
CD39+ Tregs (% frequency in Tregs)



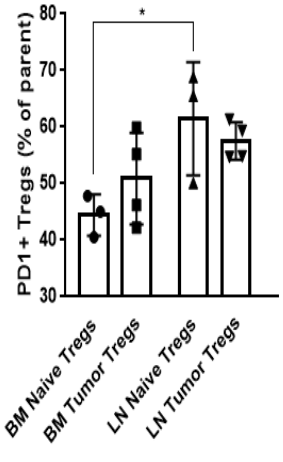
GITR+ Tregs (% frequency in Tregs)



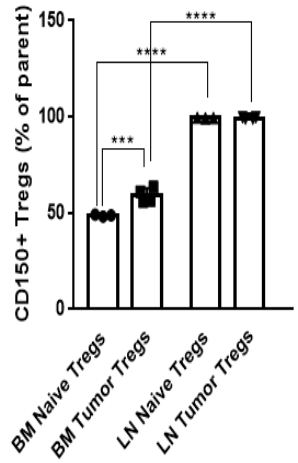
CTLA-4+ Tregs (% frequency in Tregs)



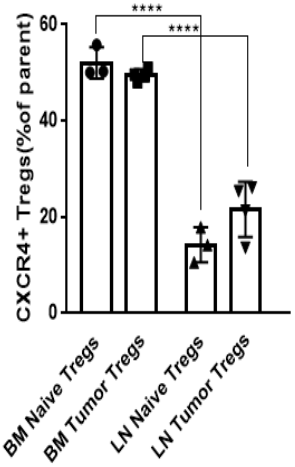
PD-1+ Tregs (% frequency in Tregs)



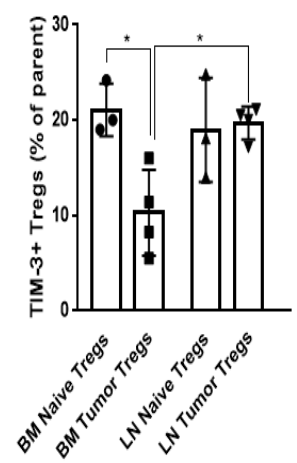
CD150+ Tregs (% frequency in Tregs)



CXCR4+ Tregs (% frequency in Tregs)



TIM-3+ Tregs (% frequency in Tregs)



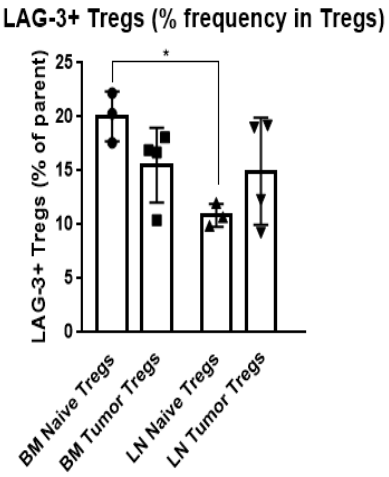


Figure 7: BM Tregs are differentially distributed to distinct subpopulations compared with LN Tregs at both steady state and after tumor induction. Frequencies of CD25+, CD73+, CD39+ GITR+, CTLA-4+, PD-1+,CD150+, CXCR4+, TIM-3+ and LAG-3+ Tregs in total Tregs. The results for CD25+, GITR+, CTLA-4+, CD150+, CXCR4+, TIM-3+ and LAG-3+ Tregs were reproducible in two independent experiments (total 14 mice). The data for CD73+, CD39+ and PD-1+ Tregs are representative of 1 experiment therefore further investigation is required for conclusive results. A representative figure from one experiment (7 mice) is shown here. Data are presented as mean ± SD and analyzed by one-way ANOVA. p value<0.05 is considered statistically significant.

Finally, we sought to determine how the tumor inoculation influences the frequencies of BM Tregs in the total population of $CD4^+$ T cells. Of note, a remarkable increase in BM Tregs was observed in B16-F10 melanoma bearing compared with naive mice [**Figure 8**] which indicates that either the endogenous BM Tregs proliferate or Tregs migrate from other sites of the periphery and home the BM after tumor induction. Interestingly, whatever happens, this finding denotes that BM Tregs may play a role in tumor development and anti-tumor immunity.

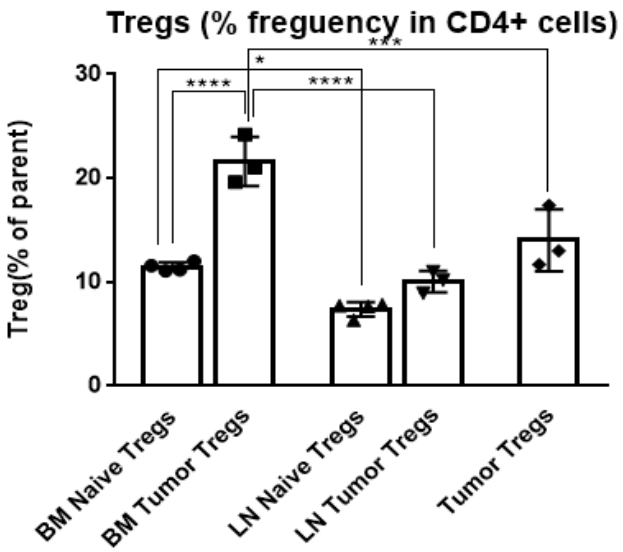
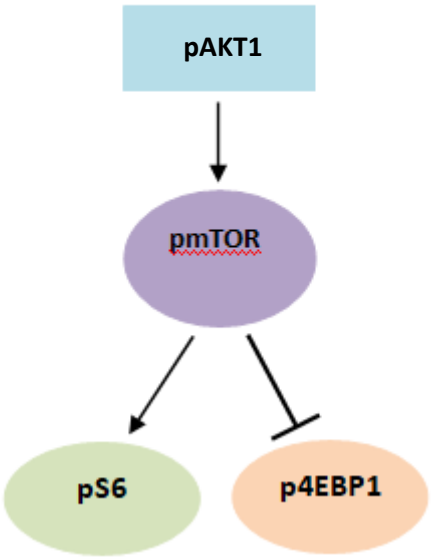


Figure 8: Treg compartment of BM is more enriched after tumor induction than at naive state. Frequencies of Tregs in $CD4^+$ T cells. These results were reproducible in two independent experiments (total 14 mice). A

representative figure from one experiment (7 mice) is shown here. Data are presented as mean ± SD and analyzed by one-way ANOVA. p value<0.05 is considered statistically significant.

VII)BM Tregs are less depended on mTOR metabolic pathway compared with LN Tregs during tumor development

It is known that Treg cells adopt the mechanistic target of rapamicin (mTOR) signalling pathway to link immunological signals from T-cell receptor (TCR) and IL-2 to metabolic activity and functional fitness, rendering this pathway a fundamental "rheostat" that programs Treg-cell suppressive activity through a non-conventional mechanism **[210]**. Thus, having that in mind, we assessed the metabolic profile of BM Tregs of tumor inoculated mice as far as the utilization of the mTOR pathway is concerned by measuring the expression of its main downstream components [phosporylated AKT1, mTOR, S6 and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1)]. Importantly, we found that BM Tregs demonstrate a significant downregulation of all the components of the mTOR pathway compared with the LN Tregs of tumor bearing mice [**Figure 9**], indicating that these cells are metabolically less depended on this pathway during tumor development for meeting their energy needs. Thus, taking into consideration that mTOR is the master regulator of glycolysis, we could hypothesize that BM Tregs may rely more on lipid metabolism and oxidative phosphorylation for their functional fitness and stability during tumor growth.



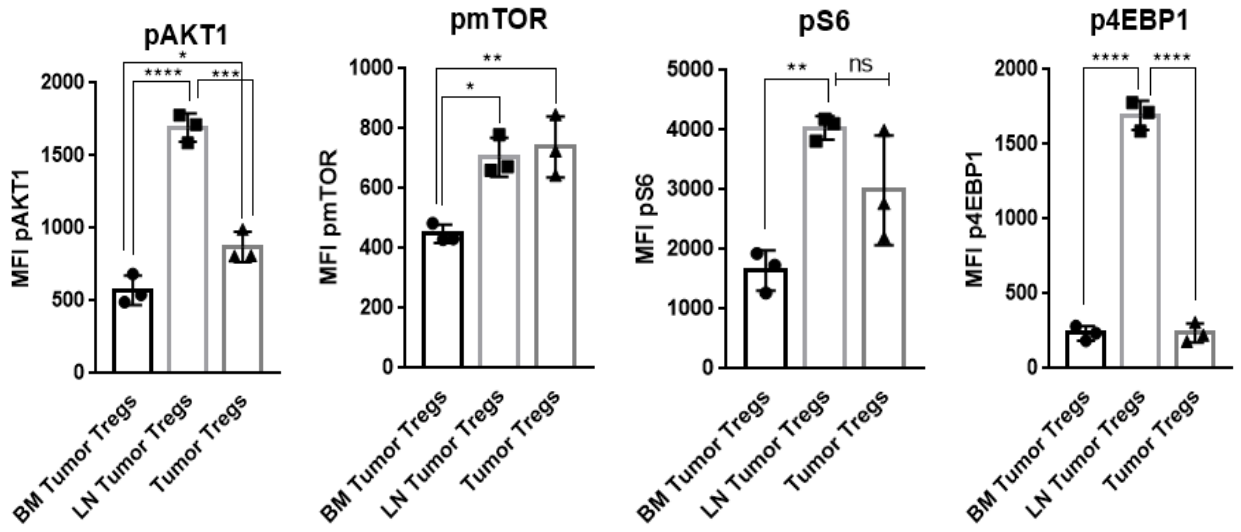
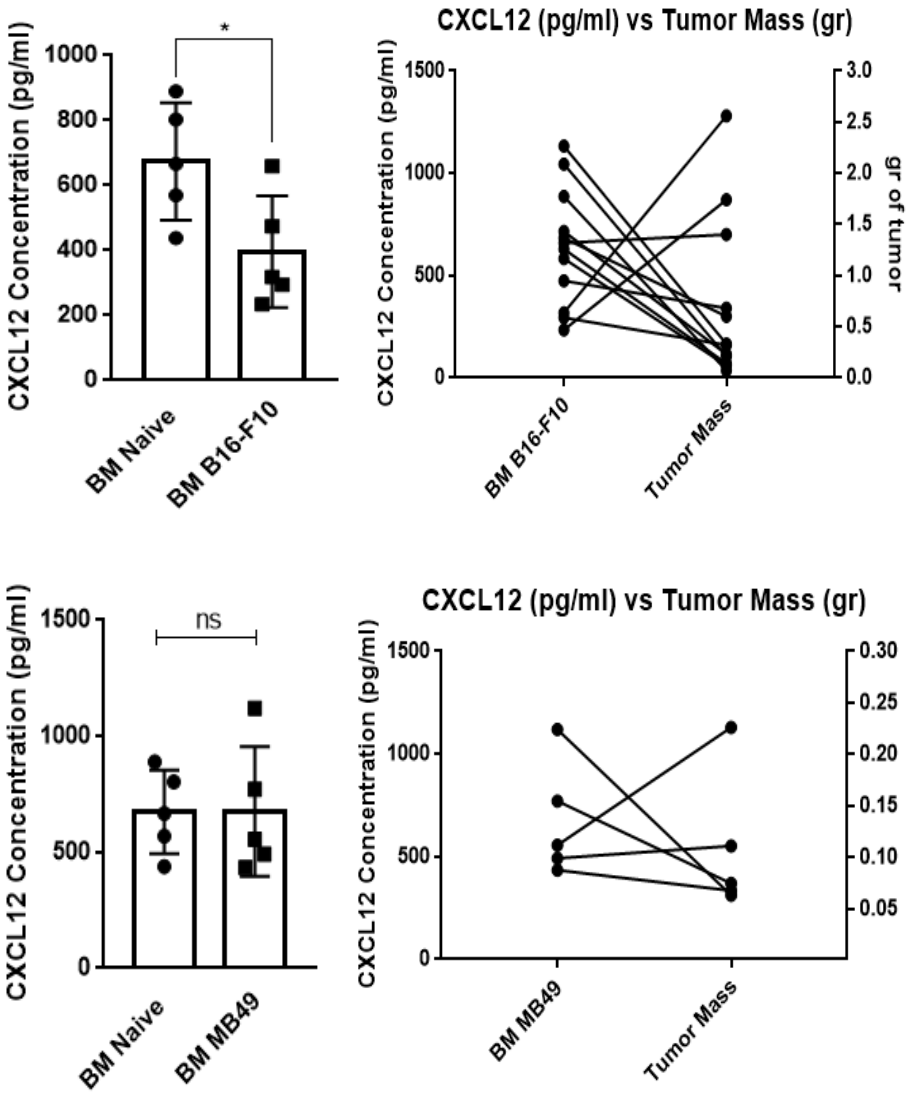


Figure 9: BM Tregs utilize less intensively the mTOR metabolic pathway compared with LN Tregs during tumor development. Flow cytometry analysis of pAKT1, pmTOR, pS6, p4EBP1 expression levels. MFI, mean fluorescence intensity. These results were reproducible in two independent experiments (total 6 mice). A representative figure from one experiment (3 mice) is shown here. Data are presented as mean ± SD and analyzed by one-way ANOVA. p value<0.05 is considered statistically significant.

VIII) Tumor induction reduces the CXCL12 levels in the BM of B16-F10 melanoma bearing mice while tumors with different immunogenicity afford different CXCL12 levels in their TME.

As it has been already mentioned the CXCR4-CXCL12 axis is critical for Treg homing to the BM [**Zou et al. 2004**]. In 2018, Hirata et al. showed that the deletion of CXCR4 receptor in Tregs reduced the Treg frequencies and numbers in the BM but not in the spleen and LNs indicating that the disruption of the above mentioned axis affects selectively the Treg homing in the BM [**Hirata et al. 2018**]. Paradoxically, until now, it is not known whether alterations of CXCL12 levels, the chemokine ligand of CXCR4 receptor, could affect the homing of Tregs in different tissues. For this reason, first of all, we sought to assess whether tumor induction alters the CXCL12 levels in the BM. In order to do that, we compared the BM CXCL12 levels between naive and B16-F10 melanoma bearing mice. Interestingly we found that CXCL12 concentration is significantly reduced in the BM of B16-F10 melanoma inoculated mice and a negative correlation between the tumor volume and CXCL12 levels exist. In particular, we observed that the higher the tumor mass, the lower the CXCL12 concentration and vice versa [**Figure 10**]. The effect of tumor inoculation on BM CXCL12 levels was also examined by using two additional ectopic tumor models, the less immunogenic Lewis Lung Carcinoma (LLC) model and the more immunogenic Mouse Bladder Carcinoma (MB49) model. In the case of LLC bearing mice, we observed the same trend, although is not statistically significant, with that of B16-F10 melanoma bearing mice, however the previously mentioned correlation is not confirmed [**Figure 10**]. Notably, no differences in CXCL12 levels were observed in the BM of mice following their inoculation with MB49 cancer cells, yet the same correlation between tumor mass and CXCL12 concentration with B16-F10 melanoma bearing mice is observed [**Figure 10**]. Therefore, taken all together, these findings indicate that possibly the tumor induction alters the levels of CXCL12 in the BM which in turn may affect the Treg homing in this tissue. Next, having in mind that

CXCL12 chemokine attracts Tregs to different tissues, we examined whether the different immunogenicity of the above mentioned tumor models is accompanied by different concentrations of CXCL12 in the TME. Indeed, we found that the more immunogenic cell line among the three (MB49) affords the higher levels of CXCL12 in the tumor mass [**Figure 10**] indicating that MB49 tumors may attract more intensively Tregs in the tumor mass compared with the other two.



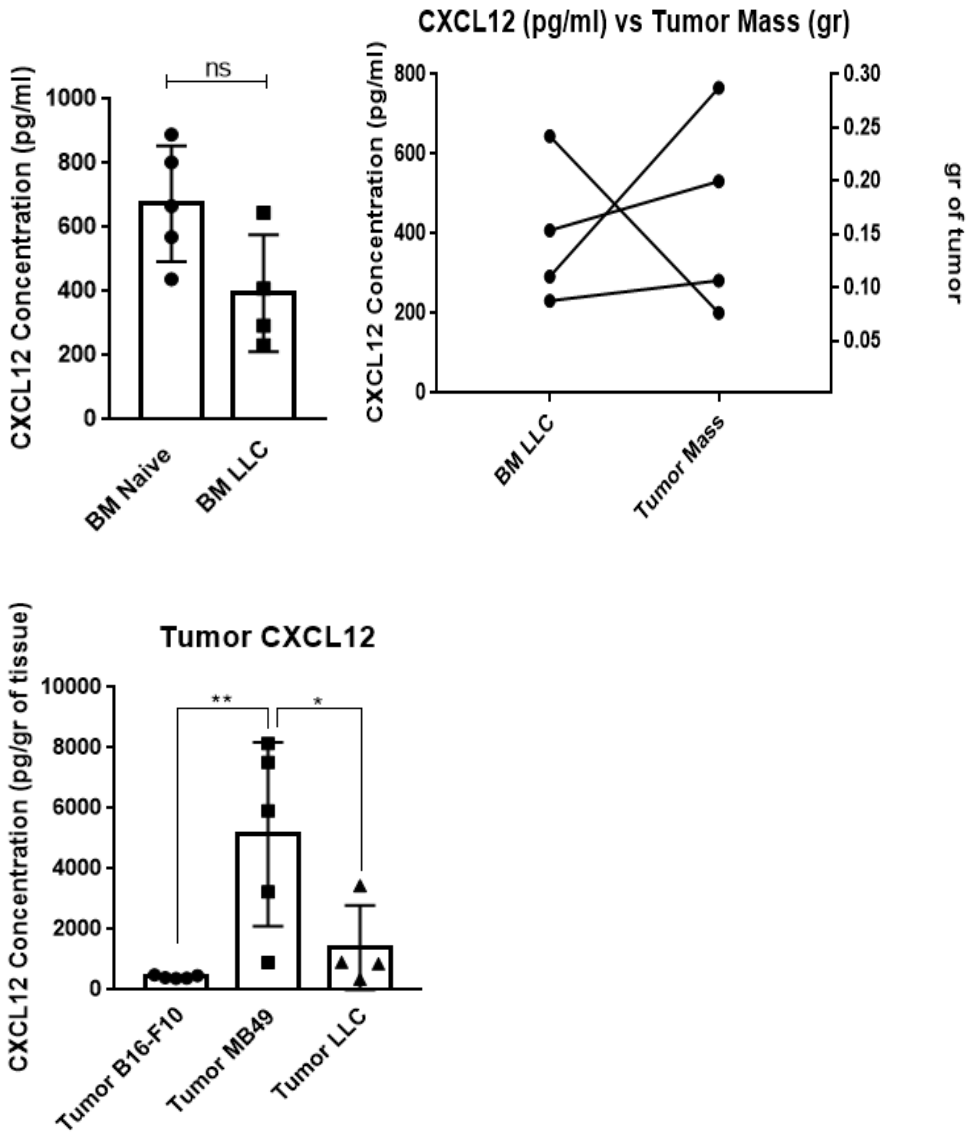
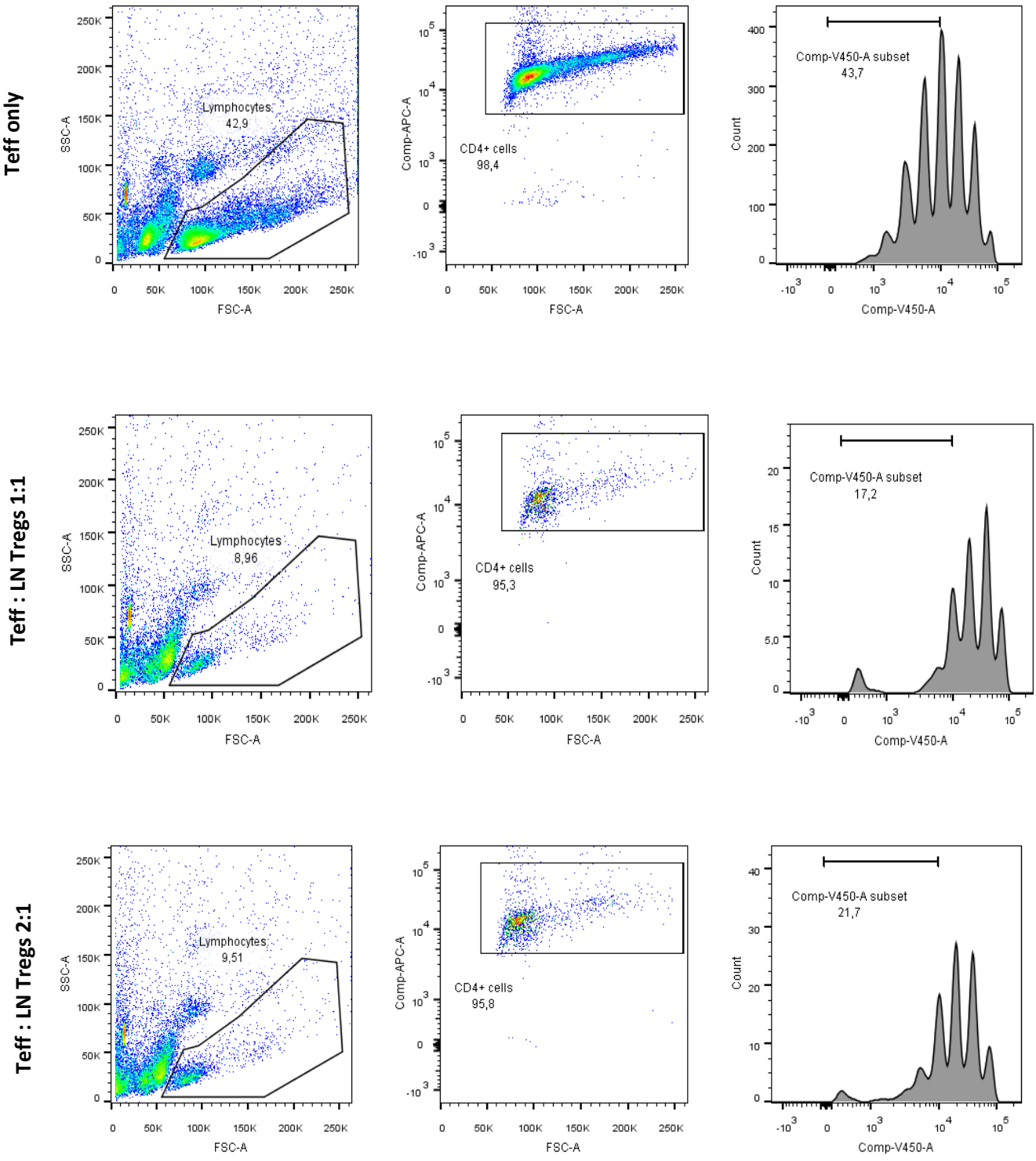


Figure 10: Tumor induction affects the BM CXCL12 chemokine levels of B16-F10 and LLC tumor bearing mice and MB49 tumors afford the higher levels of CXCL12 in their TME compared with the other two. CXCL12 levels (pg*ml^{-1}) in BM and tumor homogenates from B16-F10, MB49 and LLC inoculated mice. These data are representative of one experiment. Two independent experiments with B16-F10 inoculated mice (total 8 mice) have been performed, however due to conflicting results further investigation is required. Experiments using LLC and MB49 tumor bearing mice were performed once and thus at least one more revision is obligatory. Data are presented as mean ± SD. pvalue<0.05 is considered statistically significant.

IX) BM Tregs do not suppress the proliferation of Teff cells upon stimulation

Finally, we sought to investigate the suppressive function of BM Tregs isolated from B16-F10 tumor bearing mice by addressing their effect on the proliferation of $CD4^+$ $Foxp3^-$ Teff cells. Thus, we performed in vitro T cell suppression assays by co-culturing CellTrace Violet (CTV) labeled Teff cells which were activated by monoclonal antibodies to CD3 and CD28, with different ratios (1:1 or 2:1) of either LN or BM Tregs. Interestingly, we observed that when Teff cells were co-cultured with different ratios of BM Tregs, their proliferation was much less strongly inhibited compared with the corresponding ratios of LN Tregs where Teff proliferation was almost abolished. Therefore, this

finding indicates that BM Tregs exhibit a diminished immunosuppressive activity compared with LN Tregs.



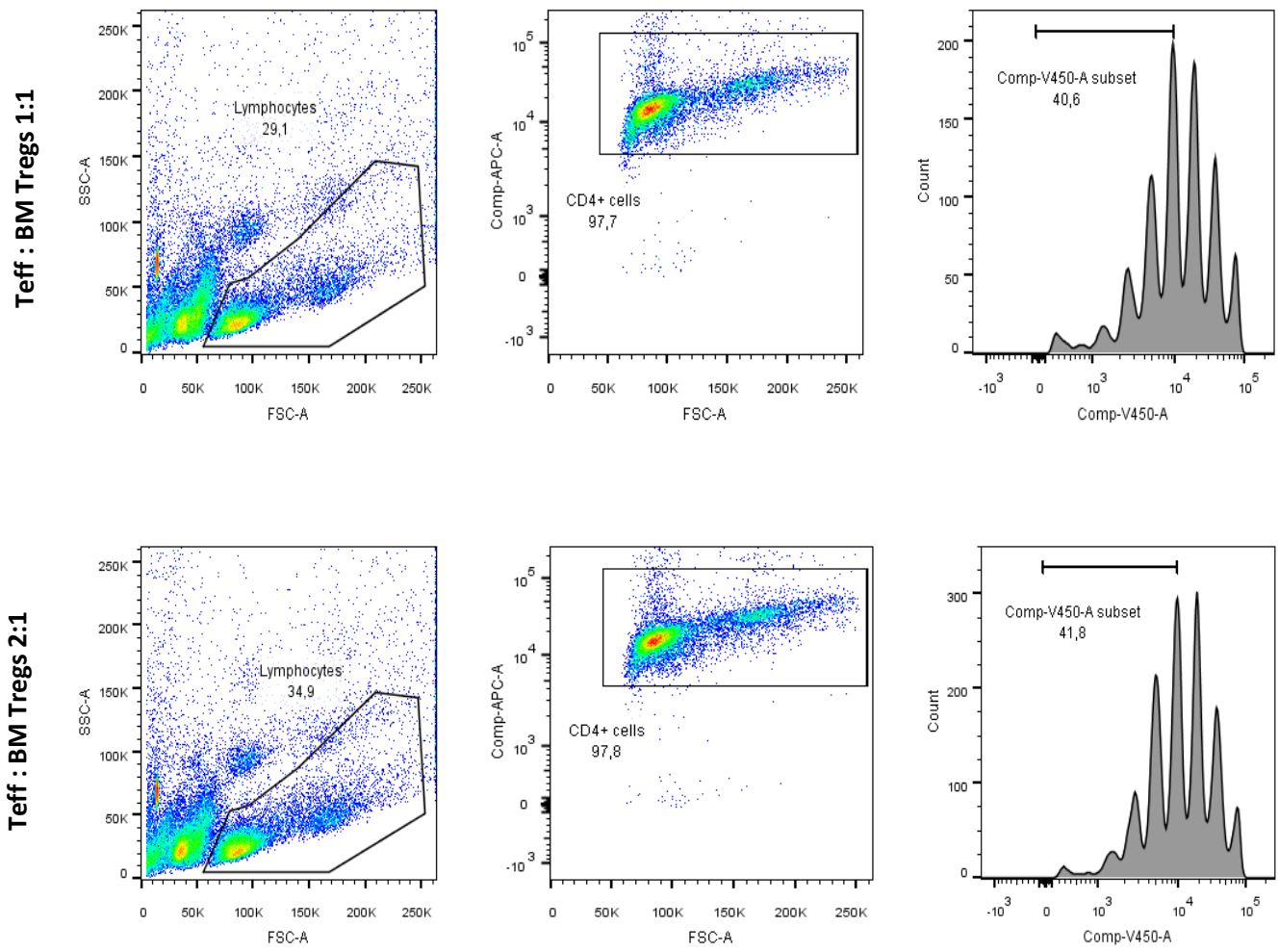


Figure 11: LN Tregs suppress the proliferation of Teff cells much stronger than BM Tregs. Representative flow cytometry gatings and histograms of CTV dilution for CTV-labeled Teff cells co-cultured with different ratios of LN and BM Tregs in the presence of anti-CD3/anti-CD28 beads. These results were reproducible in two independent experiments (total 3 B16-F10 tumor bearing mice). A representative figure from one experiment is shown here.

Discussion

The majority of the published Treg cell studies from the past decade described Treg cells residing in the spleen and lymph nodes. Only recently, the presence of Treg cells has been documented in various non-lymphoid tissues of both mice and humans such as adipose tissue, atherosclerotic plaques, injured muscle, intestinal mucosa, skin, lung, liver and bone marrow (171). These discoveries broadened the realms of the Treg family and highlighted some insightful biological implications concerning their functional relationships with local tissues. Recent evidence suggests that environmental signals found in peripheral non-lymphoid tissues are responsible for the development of tissue-specific Treg cell subsets, nevertheless their exact proportion within tissues is difficult to be determined due to differences between inflammatory and steady state conditions as well as the presence of both long-term resident and short-term migratory Treg cells. In addition, mounting evidence has revealed that tissue-resident Tregs regulate also non-immunological processes, thereby expanding the scope of Treg-related functions. Furthermore, recent reports have shown that tissue resident Tregs may possess unique tissue-specific characteristics in terms of phenotype and function compared with their counterparts in lymphoid organs.

The present study is focused on BM Tregs which until now remain poorly explored and their role in tumor development and anti-tumor immunity is really underestimated. By performing an extensive phenotyping, we revealed that BM Tregs, similarly to skin and liver Tregs (172,173), exhibit a unique surface expression profile compared with the well-studied Tregs of lymph nodes as they express higher levels of all the examined markers (CD25, GITR, CD39, CD73, CTLA-4, PD-1, TIM-3, LAG-3, CXCR4, CD150) at both steady state and during tumor development indicating that they constitute a unique more activated (based on CD25 and GITR expression) Treg subpopulation which utilizes all the "typical" Treg cell immunomodulatory receptors (CD39, CD73, CTLA-4, PD-1, TIM-3, LAG-3) for its suppressive function. Indeed, this enhanced expression of CTLA-4, PD-1, TIM-3 and LAG-3 co-inhibitory receptors indicates that BM Tregs represent a direct target of the best described and FDA-approved immune checkpoint inhibitors (ICIs) (CTLA-4 and PD-1) which have become a cornerstone in the management of malignancies but also of the upcoming new generation of immune checkpoint blockade (TIM-3, LAG-3), however the effect of their targeting, their role in the efficacy of ICI treatment as well as their implication in the "on target-off tumor" toxicity remains largely unknown, highlighting the necessity of further research in the field.

Next, having in mind that mTOR is a major sensor of environmental cue including immune signals and nutrient availability as well as a major orchestrator of the glycolytic-lipogenic switch required for the transition of Tregs from a hyporesponsive into a proliferative status and for the support of their suppressive function via mitochondrial activation, we assessed the metabolic profile of BM Tregs as far as the utilization of the mTOR pathway is concerned. Interestingly, we observed that BM Tregs metabolically rely less on this pathway for meeting their energy needs after tumor induction compared with the Tregs found in the lymph nodes. Thus, we could speculate that on the one hand BM Tregs are less dependent on the mTOR-mediated glycolysis-lipogenesis and on the other hand they probably activate other metabolic pathways such as the oxidative phosphorylation for meeting the needs of their distinct activities during tumor growth. Hence, this finding constitutes a first indication of the distinct metabolic signature that BM Tregs probably adopt for their homeostasis and function in the context of cancer. This metabolic differentiation of a Treg cell

subpopulation is not observed for the first time. There are for example scientific evidence which demonstrate that Treg cells isolated from tumors are often in an activated state with metabolic signature that is distinct from lymphoid tissue Treg cells while their transcriptome shares high similarities with that of Tregs which reside in other non-lymphoid tissues (174). Therefore, it remains to be determined whether this altered metabolic profile is indicative of their further differentiation in their tissues of residence as well as whether is representative of the additional functions that they may serve there.

Afterwards, taking into consideration that CXCL12 chemokine attracts Tregs to different tissues, we investigated whether tumor induction affects its concentration in the BM of mice which were inoculated with 3 distinct cancer cell lines that differ in their immunogenicity (B16-F10-partially immunogenic, LLC-poorly immunogenic, MB49-highly immunogenic). Interestingly, we found that the CXCL12 concentration in the BM of B16-F10 melanoma and LLC tumor bearing mice was reduced compared with that of naive mice while the levels of the BM CXCL12 remained unaltered after inoculation with MB49 cancer cells. Next, we assessed whether the different immunogenicity of the above mentioned tumor models is associated with differences in the CXCL12 concentration inside the tumor mass. Notably, we observed that the more immunogenic tumor (MB49) among the three affords the higher levels of CXCL12 in its TME indicating that the more immunogenic tumors may attract more strongly the Treg cells in their tumor mass creating a highly immunosuppressive microenvironment that dampens anti-tumor immunity and favors tumor growth. Until now, several studies have shown that the highly immunogenic tumors show significantly greater presence of T-cell co-stimulatory molecules compared with the less immunogenic, that facilitate the effector T cell cytotoxic function (175).Thus, we could speculate that the enhanced Treg chemotaxis that they exhibit, may represent a desperate effort to escape the immune-mediated eradication as an effective anti-tumor immune response requires overcoming the inhibitory effects of Tregs.

Lastly, we investigated the suppressive potential of BM Tregs of tumor inoculated mice and surprisingly we found that BM Tregs were unable to suppress the proliferation of Teff cells upon anti-CD3/anti-CD28 stimulation. This finding constitutes a first indication of the fragile phenotype that BM Tregs possibly adopt during tumor development. Importantly, several studies have correlated Treg fragility which is characterized by retention of Foxp3 expression but also by loss of suppressive function and increased production of IFNγ, with the responsiveness to immune checkpoint blockade, as patients who respond to immunotherapy exhibit a more fragile intratumoral Treg phenotype. Notably, an increasing body of evidence highlights that Treg fragility possibly serves as a prerequisite of response to anti–PD-1 treatment in murine tumor models. More specifically, in an adenocarcinoma mouse model that is sensitive to PD-1 blockade, treatment of WT mice with anti–PD-1 led to the upregulation of IFNγ^{+} Tregs, consistent with an increased fragile phenotype. When Tregs were insensitive to IFNγ through the conditional deletion of their IFNγ receptor (Foxp3^{YFPCre}IFNγR$^{fl/fl}$ mouse model), mice were completely resistant to PD-1 blockade in comparison with 40% response in WT mice, suggesting a role of Treg fragility in responsiveness to immunotherapy. Therefore, we could speculate that the loss of BM Treg suppressive function may affect directly or indirectly the effectiveness of ICI treatment. However, it remains to be determined whether this impaired BM Treg functionality is accompanied with the other characteristics of

fragility such as the increased production of IFNγ and reduced secretion of IL-10 as well as whether BM Tregs play indeed a role in the responsiveness to immunotherapy.

Future Directions

Taken all together, the data that have been collected so far, have given us some first indications about the phenotype and the suppressive potential of BM Tregs in the context of cancer. In short, we have shown that BM Tregs exhibit a differential phenotypic and functional profile compared with LN Tregs under the condition of tumor induction indicating that they constitute a special and distinct population of the Treg cell lineage. However, it is obvious that more experiments are required in order to delineate their exact role in tumor development and anti-tumor immunity. Below, some representative examples of them are presented.

I)How do BM Tregs affect tumor growth?

First of all, in order to assess how BM Tregs affect tumor growth, we could use the mouse model that Hirata et al. used for the first time which is characterized by specific reduction of BM Tregs due to the conditional deletion of CXCR4 receptor in Tregs (FoxP3^{Cre}CXCR4$^{fl/fl}$ mice). The comparison of tumor volume as well as of the frequencies of immune cell types (CD4^{+}, CD8^{+} T cells, NK cells, DCs, Tregs, MDSCs etc) found inside the TME, between the Foxp3^{YFPCre} CXCR4$^{fl/fl}$ and Foxp3^{YFPCre} tumor bearing mice could give us an idea about how the presence or absence of Tregs from the BM affects the tumor development and the immune cell infiltrates in the TME which will be indicative about whether the tumor mass is turning more "hot" or "cold". In addition to that, in order to investigate whether BM Tregs directly impact tumor development, we could create an experimental system in which only BM Tregs would be present and able to regulate the anti-tumor immune response by using a Rag -/- reconstitution model. More specifically, tumor bearing Rag-/- mice on a Thy1.1 background which lack mature B and T cells could be reconstituted with either WT Thy1.2 splenocytes containing splenic Tregs (Tregs from a lymphoid organ) or with WT Treg-depleted Thy1.2^{+} splenocytes supplemented with BM Tregs. The measurement of tumor volume as well as the assessment of BM Treg distribution in BM and TME would show us whether BM Tregs directly affect tumor growth. The measurement of frequencies of immune cell populations of myeloid lineage in BM and TME would give us a first indication about how myelopoiesis is affected.

II)How do BM Tregs affect hematopoiesis during tumor development? Which is the mechanism through which they regulate HSCs maturation and differentiation?

Based on Hirata et al. experiments which show that Tregs in the BM are in close proximity with HSCs maintaining their quiescence and number during steady state, we could speculate that BM Tregs may also interact with HSCs during tumor development and play a role in regulating their differentiation and maturation affecting in this way indirectly the anti-tumor immune response. In order to examine whether this hypothesis is true, we could compare the frequencies of myeloid and lymphoid progenitors in the BM, in the presence or absence of BM Tregs under the condition of tumor induction by using Foxp3^{YFPCre} CXCR4$^{fl/fl}$ and Foxp3^{YFPCre} tumor bearing mice. Additionally, we

could also sort HSCs from the above mentioned immunized mouse models on a CD45.1 background and transplant them to NBSGW host mice on a CD45.2 background which are highly immunodeficient, carry a c-kit mutation and represent an appropriate model for studying BM transplantation. The comparison of the generation and maturation of lymphocytic, myeloid and granulocytic lineages between the two groups of recipient mice could give us an answer about whether the presence or absence of Tregs from the BM affects the process of hematopoiesis during tumor growth. If we observe that indeed BM Tregs regulate hematopoiesis during tumor development by promoting for example the differentiation and maturation of HSCs towards the generation of pro-inflammatory myeloid cells, we would also try to delineate the mechanism that controls this effect. Thus, having in mind that BM Tregs abundantly express PD-1 and CTLA-4 while HSCs constitutively express low levels of PD-L1 and CD86, the ligands of PD-1 and CTLA-4 receptor respectively, we could hypothesize that the interaction of these molecules may be responsible for the above mentioned phenomenon. In order to elucidate whether this hypothesis is true, we should disrupt this interaction by using anti-PD1 or anti-PD-L1 or anti-CTLA-4 Abs. More specifically, we could use tumor bearing Foxp3^{YFRCre} mice treated either with anti-PD1 or anti-CTLA-4 Abs and the study of frequencies of lymphoid, myeloid and granulocytic progenitors in the BM, would give us a first indication about the role of PD1-PDL1 or CTLA-4-CD86 axis in the regulation of HSCs differentiation and maturation during tumor growth. Moreover, in order to assess directly the effect of BM Tregs on the process of hematopoiesis in the context of cancer, we could sort Tregs from the BM of tumor bearing Foxp3^{GFP} mice and co-culture them in vitro with naive HSCs in the presence or absence of anti-PD1 or anti-CTLA-4 Abs. The comparison of the colony forming potential of HSCs would give us the answer about whether BM Tregs regulate HSCs through PD1 or CTLA-4 receptor.

III) How do BM Tregs influence the responsiveness to ICI treatment?

It is well established that anti-PD1 and anti-CTLA-4 Abs, also known as immune checkpoint inhibitors (ICI), constitute a real breakthrough in the field of immunotherapy. Thus, taking into consideration that Treg cells represent a direct target of ICI immunotherapy, it would be nice to estimate how BM Tregs are affected by this therapy as well as how their presence or absence impacts the responsiveness to it. More analytically, in order to assess how ICI treatment affects BM Treg cells, we could perform an extensive phenotyping of Tregs that reside in the BM of Foxp3^{GFP} tumor inoculated mice which would be treated either with ICI or simply injected with PBS. The alteration of the phenotypic profile of these cells would be indicative of their targeting by this type of treatment and the upregulation of specific immunomodulatory receptors may represent a compensatory mechanism that these cells would activate in order to maintain their suppressive function upon the inhibition of their PD-1 and CTLA-4 receptors. Finally, to elucidate how the presence or absence of BM Tregs influence the responsiveness to ICI treatment, we could compare the tumor volumes of Foxp3^{YFPCre} CXCR4$^{fl/fl}$ and Foxp3^{YFPCre} tumor bearing mice both treated with ICI. If the tumor volume of Foxp3^{YFPCre} CXCR4$^{fl/fl}$ mice is diminished compared with that of Foxp3^{YFPCre} mice, we could speculate that BM Tregs impede the effectiveness of ICI treatment.

IV) Do BM Tregs exhibit a "fragile" phenotype?

We have already revealed that BM Tregs exhibit an impaired suppressive potential during tumor growth. It is known that the loss of suppressive function is a characteristic of fragile Tregs. However fragility is not identified only by diminished suppressive activity but also by increased IFNγ production and reduced secretion of anti-infammatory cytokines without loss of Foxp3 expression. Therefore in order to determine whether BM Tregs indeed adopt a fragile phenotype upon tumor induction, we could measure and compare the levels of the secreted IFNγ and IL-10 in the supernatants isolated from the co-culture of BM and LN Tregs with Teff cells of the in vitro suppression assay which has been already performed. An increase in the production of IFNγ and a reduction in the secretion of IL-10 by BM Tregs compared with LN Tregs both isolated from tumor bearing mice would be indicative of their fragility. Next, in order to determine whether fragility is an inherent or tumor-induced characteristic of BM Tregs, we could perform the same in vitro suppression and ELISA assay and the only difference would lie in that BM and LN Tregs would be isolated from naive mice. If BM Tregs of naive mice exhibit the same functional profile with those isolated from tumor inoculated mice , we could support that fragility is an inherent trait of BM Tregs. Of note, the fact that Treg fragility is a key component that determines the response to immunotherapy, underlines the necessity of understanding how fragile BM Tregs contribute to the effectiveness of immunotherapy as this knowledge may open new doors in the evaluation of BM Treg frequencies as predictors of patient responsiveness to ICI.

To conclude, all the above experiments represent only a small sample of those that should be performed in order to further elucidate the characteristics and function of Tregs that reside in the hematopoietic niche during tumor development. In vivo experiments in combination with high-throughput sequencing technologies will give answers about the mechanisms that BM Tregs utilize for regulating the immune response during tumor development and will also elucidate their potential utility as new therapeutic targets in the context of cancer.

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