Convergences and divergences of thymus- and peripherally derived regulatory T cells in cancer

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The expansion of regulatory T cells (Treg) is a common event characterizing the vast majority of human and experimental tumors and it is now well established that Treg represent a crucial hurdle for a successful immunotherapy. Treg are currently classified, according to their origin, into thymus-derived Treg (tTreg) or peripherally induced Treg (pTreg) cells. Controversy exists over the prevalent mechanism accounting for Treg expansion in tumors, since both tTreg proliferation and de novo pTreg differentiation may occur. Since tTreg and pTreg are believed as preferentially self-specific or broadly directed to non-self and tumor-specific antigens, respectively, the balance between tTreg and pTreg accumulation may impact on the repertoire of antigen specificities recognized by Treg in tumors. The prevalence of tTreg or pTreg may also affect the outcome of immunotherapies based on tumor-antigen vaccination or Treg depletion. The mechanisms dictating pTreg induction or Treg expansion/stability are a matter of intense investigation and the most recent results depict a complex landscape. Indeed, selected Treg subsets may display peculiar characteristics in terms of stability, suppressive function, and cytokine production, depending on microenvironmental signals. These features may be differentially distributed between pTreg and Treg and may significantly affect the possibility of manipulating Treg in cancer therapy. We propose here that innovative immunotherapeutic strategies may be directed at diverting unstable/uncommitted Treg, mostly enriched in the pTreg pool, into tumor-specific effectors, while preserving systemic immune tolerance ensured by self-specific Treg.

Keywords: Treg, development, heterogeneity, specialization, plasticity, epigenetic commitment, tumor antigens

Treg SUPPRESS PRO-TUMORAL INFLAMMATION OR ANTI-TUMOR RESPONSE

INTRODUCTION

Tumor onset is a very complex process, in which cells of both innate and adaptive immune system play crucial roles in inhibiting or fostering tumor development. The awareness that the immune system could act as an extrinsic tumor suppressor or as a tumor-sculpting player resulted in the cancer immunoeediting theory, which described the interaction between tumor and host as consisting of three different phases: elimination, equilibrium, and escape (1). During the last phase of this process, transformed cells acquire the ability to subvert the control exerted by immune cells thus originating the clinically evident pathology. The escape is due to different mechanisms, including reduced immunogenicity (low expression level of MHC class I and loss of antigen expression), acquired resistance to the cytotoxic functions of immune cells, and accumulation in the tumor microenvironment of immuno-suppressive mediators, like regulatory T cells (Treg) (1). The first marker to be identified as distinguishing Treg from the other CD4+ T lymphocytes was CD25 (2) and depletion of CD25-positive cells unveiled anti-tumor immunity in experimental models (3). Few years later, the transcription factor Forkhead Box P3 (Foxp3) was indicated as the master regulator of Treg (4, 5). In support of the crucial roles played by Foxp3 in Treg fate determination and immune homeostasis, Foxp3 mutations have been recognized as responsible for human Immune Dysfunction, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome (6, 7), and for the phenotype of scurfy mutant mice (8), both characterized by fatal autoimmune lymphoproliferation linked to severe defects in Treg development/functions. However, very recent data have demonstrated that the complete development of the Treg lineage requires the concomitant, Foxp3-independent, establishment of a Treg-specific pattern of DNA hypomethylation (9).

According to recently proposed recommendations (10), Treg are classified into two principal subsets based on their developmental origin: thymus-derived Treg (tTreg) develop in the thymus, while peripherally induced Treg (pTreg) develop in vivo in the periphery from conventional T cells (Tconv), through a process called "conversion" (11). Treg can also be induced in vitro (and are called iTreg) under TGF-β and/or retinoic acid exposure, but their complete commitment into fully differentiated Treg is still under debate (12). In physiological conditions, the pool of Treg, encompassing both iTreg and pTreg, which represents about the 5–10% of the circulating CD4+ T lymphocytes, assures peripheral self-tolerance and prevents exacerbated immune responses (7, 8). A huge amount of data now demonstrates that tumor onset and progression perturb Treg homeostasis and lead to increased Treg/Tconv and Treg/CD8 ratios both in peripheral blood and in the tumor

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MECHANISMS OF T\textsubscript{reg} SUPPRESSION IN TUMORS

It was recently demonstrated that T\textsubscript{reg} infiltrating different tissues have a specific gene signature (16), thus T\textsubscript{reg} may use peculiar suppression mechanisms in response to microenvironmental stimuli. This specialization may represent the basis for designing immune interventions targeting specific T\textsubscript{reg} functions in a given tissue while sparing systemic immune homeostasis. Even though a tumor T\textsubscript{reg}-specific gene signature has not been delineated yet, some mechanisms have been described to contribute to T\textsubscript{reg} suppression in tumors, which can be clustered in three main types: surface molecules, enzymatic activities, and cytokines (Figure 1).

Both human and mouse T\textsubscript{reg} constitutively express on their surface cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a coinhibitory member of the CD28/B7 family, endowed with strong immune-regulatory properties (17). The relevance of CTLA-4 in regulating T\textsubscript{reg} function was demonstrated in several settings, including autoimmune diseases and different tumor types (18).

A comparative gene expression profile between T\textsubscript{reg} and T\textsubscript{conv} revealed that T\textsubscript{reg} specifically up-modulate lymphocyte activation gene 3 (LAG-3) (19), a homolog of CD4, that binds to MHC class II on antigen-presenting cells (APCs). LAG-3 is upregulated in tumor-infiltrating T\textsubscript{reg} and experiments with anti-LAG-3 mAb demonstrated that functional LAG-3 is required for maximal T\textsubscript{reg} suppressive function (20, 21). T\textsubscript{reg}-DC interaction is also mediated by the transmembrane protein neuropilin-1 (Nrp-1), expressed on T\textsubscript{reg} membrane, which ensures the stability of T\textsubscript{reg}-DC interaction and allows T\textsubscript{reg} to efficaciously suppress DC (22). A study conducted on patients with early-stage cervical tumor showed that T\textsubscript{reg} infiltrating the tumor-draining lymph node of patients with metastasis have a higher expression of several immune-modulatory molecules, including Nrp-1 (23). The receptor activator of nuclear factor-kB ligand (RANKL), member of the tumor necrosis factor family, was found to be highly expressed on T\textsubscript{reg} isolated from tumor-bearing hosts, and substantial evidence indicates that RANKL expressed by T\textsubscript{reg} is involved in the onset of metastasis in both mammary (24) and prostate tumors (25).

Regulatory T cell suppression may be mediated by enzymatic activities, such as CD39/CD73 (26, 27), granzyme B, and perforin (28). CD39 and CD73 are two ecto-enzymes that dephosphorylate extracellular ATP and generate pericellular adenosine, which in turn exerts a strong pro-tumorigenic role modulating the function of numerous tumor-infiltrating immune cells. CD73-deficient mice develop a stronger anti-tumor immune response compared to CD73-sufficient mice (29). T\textsubscript{reg} are also able to control the proliferation and function of different immune cells via the Perforin pathway (30). In mouse models of melanoma, lymphoma, and acute myeloid leukemia it has been demonstrated that tumor-infiltrating T\textsubscript{reg}, but not naïve T\textsubscript{reg}, secrete high amounts of both perforin and granzyme B, which in turn induce NK and CD8\textsuperscript{+} T cell death (28).

Immunosuppressive cytokines, like TGF-β and IL-10, are critical players in T\textsubscript{reg} biology, being involved in both their differentiation and suppressive potential, especially in tumors. T\textsubscript{reg}-derived TGF-β was found relevant in suppression of anti-tumor T cell response in both mouse (31) and human (32, 33) tumors. IL-10 is a well-known immunosuppressive mediator, and several pieces of evidence highlight the relevance of T\textsubscript{reg}-derived IL-10 in controlling inflammation at the mucosal interfaces such as gut and lung (34, 35). Despite these data, little information is available...
about the roles of T\textsubscript{reg}-derived IL-10 in tumor microenvironments. We have recently demonstrated that tumor-associated T\textsubscript{reg} secrete high amounts of IL-10, which in turn impairs DC migration to the draining lymph nodes and the mounting of a specific anti-tumor immune response. This phenotype could be reverted by stimulating the receptor OX40 on the surface of intratumoral T\textsubscript{reg}. Indeed, OX40-triggered T\textsubscript{reg} showed reduced secretion of IL-10 as a consequence of the down-modulation of the interferon regulatory factor 1 (IRF1), a transcription factor active in the IL-10 promoter (36). Another cytokine recently described as critical for the full T\textsubscript{reg} suppressive function is IL-35, formed by Epstein–Barr-virus-induced gene 3 (EBI3) and IL-12α (p35) (37). In two different mouse transplantable tumor models (melanoma and colon carcinoma), it was observed that T\textsubscript{reg} secrete abundant IL-35, thus promoting the differentiation of induced IL-35-secreting T\textsubscript{reg} (37). It is well known that tumor growth is associated with a consistent process of new angiogenesis in response to hypoxia. A circuit involving tumor hypoxia, T\textsubscript{reg} recruitment, and angiogenesis has been recently discovered (38). In the hypoxic tumor microenvironment, the chemokine axis CCL28–CCR10 plays a determinant role in the recruitment of T\textsubscript{reg} which secrete huge amounts of vascular endothelial growth factor (VEGF), further stimulating the new angiogenesis process and the establishment of a tolerogenic microenvironment (38).

**DOUBLE EFFECTS OF T\textsubscript{reg} ON PROGNOSIS**

Since their discovery, T\textsubscript{reg} were considered one of the main obstacles for tumor clearance, according to their tolerogenic properties and their accumulation along tumor progression. In this view, several anti-tumor immune-therapies focus on T\textsubscript{reg} depletition or inhibition, in order to “contrasuppress” T\textsubscript{reg} inhibitory functions and to block the conversion of non-regulatory cells (non-T\textsubscript{reg}) into regulatory cells (15). Reduced T\textsubscript{conv}/T\textsubscript{reg} ratio was observed in patients with pancreatic tumor (39, 40), breast cancer (39), ovarian cancer (41), Hodgkin lymphoma (42), and melanoma (43). Increased T\textsubscript{reg} frequency is generally associated to advanced tumor stage and poor prognosis, as recently demonstrated in a study on ovarian cancer (44). In the ascites of patients with advanced tumor, the percentage of T\textsubscript{reg} was increased compared to the ascites of patients with early-stage tumor. Same results were obtained with the mouse WF-3 transplantable ovarian tumor model, showing augmented percentages of T\textsubscript{reg} in both spleen and tumor-associated cells of mice with advanced tumors, compared to naïve or mice with early lesions. In addition, the treatment of tumor-bearing mice with the T\textsubscript{reg}-depleting mAb PC61 (αCD25) reduced tumor growth and prolonged mice survival (44). Similarly, it has been demonstrated that T\textsubscript{reg} number inversely correlated with the therapy outcome in melanoma patients treated with non-myeloablative chemotherapy, in combination or not with total body irradiation, followed by adoptive T cell transfer (45). Respondent patients had a lower frequency of T\textsubscript{reg} in peripheral blood compared to non-respondent patients (45). A study conducted on patients affected by invasive ductal carcinoma showed a positive correlation among T\textsubscript{reg}, Th17, and tumor aggressiveness. These data imply that T\textsubscript{reg} and Th17 cells may concomitantly expand during tumor progression, with T\textsubscript{reg} mainly suppressing protective effector T cell proliferation while sparing Th17 proangiogenic activities, fostering cooperatively tumor progression, and the metastatic process (46).

Nevertheless, recent data, in particular tumor systems, point out that T\textsubscript{reg} may exert a protective role for the host (13, 47, 48). The connection between tumor and inflammation is a well-assessed process (49), but now it is clearly emerging that the type of tumor-associated inflammation imprint the behavior of T\textsubscript{reg} becoming detrimental or beneficial for the host. Type-1 inflammation, characterized by high concentration of IFN-γ and IL-12 and fully active cytotoxic cells, represents efficient anti-tumor immunity (49). In this setting, the inhibitory properties of T\textsubscript{reg} may promote tumor escape and aggressiveness (47). On the contrary, immune responses dominated by cytokines like TNF-α, IL-1β, IL-6, IL-23, and IL-17 act as pro-tumoral mediators (47). In this environment T\textsubscript{reg} may suppress a pro-tumoral inflammatory status, thus playing a protective role for the host (47).

These unexpected anti-tumoral T\textsubscript{reg} Properties were observed in patients with colorectal cancer (CRC) (50–52). In these patients, with different tumor stages, a better prognosis and an increased overall survival were associated with higher infiltration of FOXP3+ T cells compared to patients with a poor tumor outcome. These data suggested the hypothesis that FOXP3+ T cells could be considered as an independent prognostic factor for CRC. Following a strong activation, both conventional CD4\textsuperscript{+} (53) and CD8\textsuperscript{+} (54) lymphocytes up-regulate Foxp3 expression in colonic tissue. These observations indicated that the CRC prognosis positively correlated with non- regulatory FOXP3+ cells rather than to T\textsubscript{reg}. However, in vitro suppression assays demonstrated that FOXP3+ cells, isolated from CRC tissues, were endowed with suppressive functions, confirming their nature as regulatory cells (55). In a recent study conducted on 65 patients with different stages of CRC, FOXP3 expression was systematically evaluated in both tumor-infiltrating lymphocytes and neoplastic cells, and was correlated to tumor progression and clinical-pathological features (56). From this study the notion emerged that high FOXP3 expression in tumor cells correlated with poor tumor outcome, compared to tumors poorly expressing FOXP3; on the contrary, no correlation was observed between CRC prognosis and FOXP3 expression by T cells (56).

A protective role of T\textsubscript{reg} was also found in head and neck squamous cell carcinoma (HNSCC) (57). Univariate and multivariate analysis demonstrated that the locoregional control of the tumor was positively associated with CD4\textsuperscript{+} FOXP3\textsuperscript{+} regulatory cell infiltration (57). However, also for this type of neoplasia, there are some discordant data regarding the role of T\textsubscript{reg} in tumor progression. Indeed, another study showed that T\textsubscript{reg} frequency and suppressive function were higher in the peripheral blood of tumor-bearing patients than in healthy volunteers (58).

The discrepancies observed in these studies may be due to the number of patients included, different strategies of analysis and non-homogeneity of tumor samples (stage, metastasis, etiology). Certainly, to properly define the role of T\textsubscript{reg} in tumor outcome, the new studies should take into account the tumor stage and the related inflammatory features, depending on the anatomical localization. In general, those tumors arising from chronic inflammation, almost at their initial stage, can benefit from the suppressive properties of T\textsubscript{reg}. In fact, during the inflammatory...
Many efforts have been recently addressed to the identification of phenotypic, molecular, and functional features distinguishing tT$_\text{reg}$ and pT$_\text{reg}$, besides their site of origin (11). Some markers have been proposed to distinguish pT$_\text{reg}$ and tT$_\text{reg}$, even though with some limitations: the initial enthusiasm for the suggestion of Helios as able to identify tT$_\text{reg}$ (59) has been soon moldered by the observation of Helios expression in pT$_\text{reg}$ (60); the recent finding of the Nrp-1 as a marker of tT$_\text{reg}$ (61, 62) has application limited to murine cells, being not expressed on human T$_\text{reg}$ (63).

Several attempts have been conducted to identify genetic (64–66) and/or epigenetic signatures distinguishing pT$_\text{reg}$ and tT$_\text{reg}$. The T$_\text{reg}$-specific demethylated region (TSDR) is involved in the stable commitment of the T$_\text{reg}$ lineage, and controversy still remains on whether tT$_\text{reg}$ or pT$_\text{reg}$ can efficiently demethylate this region and become fully committed T$_\text{reg}$ (66–69). Despite this growing amount of information, distinguishing the relative contribution of pT$_\text{reg}$ and tT$_\text{reg}$ to immune suppression in physiological and pathological conditions remains hard. However, some pieces of evidence have accumulated in the last years that speak in favor of tT$_\text{reg}$ or pT$_\text{reg}$ prevalence or concomitance in tumors.

**EVIDENCE FOR pT$_\text{reg}$ OR tT$_\text{reg}$ ACCUMULATION IN TUMORS**

**DISTINGUISHING FEATURES OF pT$_\text{reg}$ AND tT$_\text{reg}$**

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**EVIDENCE FOR tT$_\text{reg}$ ACCUMULATION IN CANCER**

One of the first attempts to distinguish between pT$_\text{reg}$ conversion and tT$_\text{reg}$ expansion in cancer was pursued by Bui and colleagues who adoptively transferred CD4$^+$CD25$^+$ cells, mixed at 1:10 ratio with CD25-depleted Thy1.1-congenic splenocytes, into immunodeficient mice bearing a progressive sarcoma (70). The analysis performed 10 days after tumor injection showed that the vast majority (around 80%) of tumor-infiltrating CD4$^+$CD25$^+$ cells derived from expansion/recruitment of the transferred T$_\text{reg}$, rather than from conversion of non-T$_\text{reg}$. This and other reports, appeared in the “pre-Foxp3” era, were biased by the idea that CD25 was the most stringent T$_\text{reg}$ marker and that CD25-depleted cells, mixed at high levels with multiple myeloma showed that the TREC content in naive cells was significantly lower in T$_\text{reg}$ (identified as CD4$^+$CD25$^{\text{high}}$ cells) than CD4$^+$CD25$^-$ or CD25$^{\text{low}}$, suggesting that the T$_\text{reg}$ pool mainly derived from peripheral expansion rather than recent thymic emigration (75). However, the observation of high T$_\text{reg}$ proliferation at tumor sites cannot be considered as an unequivocal proof of tT$_\text{reg}$ prevalence over pT$_\text{reg}$, since both subsets could be endowed with the same proliferative potential in vivo. Indeed, several pieces of evidence indicate that conversion and proliferation may represent uncoupled and independent events (see pT$_\text{reg}$ Development and tT$_\text{reg}$ Expansion as Independent Processes).

**EVIDENCE FOR pT$_\text{reg}$ INDUCTION IN CANCER**

Some studies support the idea that pT$_\text{reg}$ conversion actually occurs in tumor-bearing hosts at higher efficiency than in physiological conditions, even if controversy still exists on whether pT$_\text{reg}$ may prevail numerically over tT$_\text{reg}$ at the tumor site. We have in the past demonstrated that thymectomized and CD25-depleted mice, subsequently transplanted with carcinoma cells, showed a significantly higher T$_\text{reg}$ recovery in tumor-draining than in contralateral lymph nodes, suggesting that in tumor-bearing mice the T$_\text{reg}$ pool might be replenished mostly by newly derived pT$_\text{reg}$ than by proliferation of residual T$_\text{reg}$. To prove this possibility, CD25-depleted CD4$^+$CD25$^-$ cells were transferred into immunocompetent, Thy1.1-congenic, CT26 tumor-bearing mice. In this setting, we could show that the transferred cells acquired CD25 and Foxp3 at significantly higher levels in draining lymph nodes, compared to contralateral lymph nodes of tumor-bearing mice, or to the lymph nodes of tumor-free mice (76). This result clearly showed that tumor progression actively promoted the conversion of non- regulatory precursors into pT$_\text{reg}$. Some tumor-derived molecular signals were found to be involved in tumor-associated conversion.

For instance, in different mouse models, tumor cells have been shown to induce in vivo T$_\text{reg}$ conversion through TGF-$\beta$ and TGF-$\beta$ neutralization abrogated T$_\text{reg}$ accumulation at the tumor site (77). Human leukemic cells converted in vitro non-T$_\text{reg}$ into T$_\text{reg}$ through the tumor cell-restricted IDO activity, and IDO blockade prevented T$_\text{reg}$ induction in vivo in a leukemia mouse model (78). A confirmation of extensive pT$_\text{reg}$ infiltration in murine tumors has been recently obtained thanks to the recent discovery of Nrp-1 as a T$_\text{reg}$-restricted marker (61, 62). The analysis of Nrp-1 expression has indeed revealed that Nrp-1-negative bona fide pT$_\text{reg}$ cells were significantly enriched at tumor site compared to spleen, ranging around 40–90% of total tumor-infiltrating T$_\text{reg}$ depending on the tumor type (61). These Nrp-1-negative cells also presented a TREC content in naive cells was significantly lower in T$_\text{reg}$ (identified as CD4$^+$CD25$^{\text{high}}$ cells) than CD4$^+$CD25$^-$ or CD25$^{\text{low}}$, suggesting that the T$_\text{reg}$ pool mainly derived from peripheral expansion rather than recent thymic emigration (75). However, the observation of high T$_\text{reg}$ proliferation at tumor sites cannot be considered as an unequivocal proof of tT$_\text{reg}$ prevalence over pT$_\text{reg}$, since both subsets could be endowed with the same proliferative potential in vivo. Indeed, several pieces of evidence indicate that conversion and proliferation may represent uncoupled and independent events (see pT$_\text{reg}$ Development and tT$_\text{reg}$ Expansion as Independent Processes).

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DEVELOPMENTAL AND FUNCTIONAL RELATIONS BETWEEN PT\textsubscript{reg} AND TT\textsubscript{reg} IN CANCER

PT\textsubscript{reg} DEVELOPMENT AND TT\textsubscript{reg} EXPANSION AS INDEPENDENT PROCESSES

Many attempts have been made to understand whether TT\textsubscript{reg} accumulation and PT\textsubscript{reg} development are mutually exclusive or rather cooperative in establishing immune suppression. The evidence that TT\textsubscript{reg} may "educate" T\textsubscript{conv} to convert into T\textsubscript{reg} through the secretion of cytokines, such as TGF-\beta and IL-10 (79), may support the latter possibility. This event would generate a cascade of suppressive function transmitted from T\textsubscript{reg} to bystander cells, establishing a loop of immunosuppression, reminiscent of a phenomenon called as "infectious tolerance" (80). Zhou and coworkers have addressed this issue in the tumor setting, and have demonstrated that tumor-antigen-specific PT\textsubscript{reg} could indeed arise from T\textsubscript{reg}-depleted cells (adoptively transferred in mice carrying the cognate antigen-expressing tumor), but that the extent of PT\textsubscript{reg} induction was not affected by the concomitant presence of TT\textsubscript{reg}, either exogenous (adoptively co transferred) or endogenous (pre-existing in the host) (81, 82). This result indicated that TT\textsubscript{reg} and PT\textsubscript{reg} accumulate in tumors in a reciprocally independent fashion and that "infectious tolerance" may play minor roles in shaping the tumor-associated T\textsubscript{reg} pool.

A comprehensive scenario of T\textsubscript{reg} accumulation in tumors should take into account, beside de novo conversion, the active proliferation of not only TT\textsubscript{reg} but also PT\textsubscript{reg}. Proliferation plays opposite roles in the differentiation of T\textsubscript{conv} into PT\textsubscript{reg} versus the expansion of already differentiated PT\textsubscript{reg}. Regarding the former aspect, we have demonstrated that T\textsubscript{conv} proliferation was not required for their conversion into PT\textsubscript{reg}, since CD25\textsuperscript{low}Foxp3\textsuperscript{+} cells could develop in tumor-bearing mice from CD25-depleted cells treated with an anti-proliferative agent (76). A seminal study by Kretschmer and colleagues showed that T\textsubscript{conv} proliferation was not only dispensable but also detrimental to conversion: indeed, low levels of T cell proliferation, in conditions of suboptimal antigen presentation, lack of co-stimulation, and IL-2 paucity, favored TGF-\beta-mediated PT\textsubscript{reg} induction, thus suggesting that an inverse relationship might exist between T\textsubscript{conv} proliferation and conversion into PT\textsubscript{reg} (83). However, once developed, PT\textsubscript{reg} promptly proliferated in response to experimental antigens (83) and, more importantly, in response to tumor antigens (81, 82). Experiments performed in CNS1-mutated mice, which are genetically unable to generate PT\textsubscript{reg}, have shown that PT\textsubscript{reg} and TT\textsubscript{reg} may occupy distinct "niches": indeed, the efficiency of PT\textsubscript{reg} differentiation from T\textsubscript{conv} was higher when those T\textsubscript{conv} were co-transferred, in lymphopenic recipients, with a CNS1-deficient (non-containing PT\textsubscript{reg}) compared to a CNS1-sufficient (containing PT\textsubscript{reg}) counterpart, suggesting that not only the PT\textsubscript{reg} pool, but also the PT\textsubscript{reg} niche, may be controlled by autonomous homeostatic mechanisms (84).

DIVISION OF LABOR BETWEEN TT\textsubscript{reg} AND PT\textsubscript{reg} IN CANCER

Both TT\textsubscript{reg} and PT\textsubscript{reg} have been generally recognized as immune suppressive cells in a variety of in vivo and in vitro experimental settings (12). However, whether the two subsets are endowed with peculiar activities remains unclear and is a matter of intense investigation.

Gene expression profiling revealed that the PT\textsubscript{reg} and TT\textsubscript{reg} signatures were mostly overlapping but also presented some differentially expressed genes, encoding for proteins involved in T\textsubscript{reg} suppressive function, suggesting that PT\textsubscript{reg} may preferentially exploit different molecules and related mechanisms to exert suppression (64–66). The Nrp-1 itself is not only a marker discriminating murine TT\textsubscript{reg} from PT\textsubscript{reg}, but also plays a role in T\textsubscript{reg} suppression: since this molecule prolongs T\textsubscript{reg} interactions with immature dendritic cells, TT\textsubscript{reg} may benefit from this pathway in preferentially modulating dendritic cell and cognate T cell activation (22). Many data suggest that PT\textsubscript{reg} may be specialized suppressors of immune responses at interfaces with external environments, such as airways, gut, and maternal-fetal interface (64, 84–87). Of note, a peculiar T\textsubscript{reg} suppressive molecule, IL-10, plays crucial roles at environmental interfaces, therefore PT\textsubscript{reg} may perform their specialized activity through IL-10 secretion (34, 88). IL-10 is critically involved in the establishment of tumor-associated immune suppression, and we have clearly demonstrated IL-10 production by around 40% of tumor-infiltrating T\textsubscript{reg} in murine transplanted tumors (36). It would be interesting to understand whether the fraction of IL-10-producing TT\textsubscript{reg} is enriched in PT\textsubscript{reg}, rather than TT\textsubscript{reg}, in tumors. One study has directly addressed the issue of induced T\textsubscript{reg} functional specialization in tumors, by generating in vitro tumor-specific TT\textsubscript{reg} and co-culturing these cells, or TT\textsubscript{reg} as control, with NK cells: these authors found that TT\textsubscript{reg} and PT\textsubscript{reg} equally suppressed IL-2-induced NK activation, but only TT\textsubscript{reg} were endowed with the surprising ability not to suppress, but to enhance, NK cytotoxicity induced by tumor target cell contact (89). This observation may speak in favor of differential roles played by TT\textsubscript{reg} and PT\textsubscript{reg} in cancer, with the former more involved in preventing target cell-independent, and possibly self-directed, unwanted immune responses, and the latter concurrently enhancing tumor-specific immunity.

This division of labor may result in the progressive shaping of the immune response toward an effective anti-tumor immunity with minimal side effects. Such dichotomy is also reminiscent of the double role played by T\textsubscript{reg} in different cancers, according to the hypothesis that high T\textsubscript{reg} frequency is associated to poor or good prognosis in non-inflammatory or inflammatory cancer onset, respectively (13, 47). In the former case, i.e., non-inflammatory cancers in which protective type-1 responses are suppressed by high T\textsubscript{reg} infiltration, T\textsubscript{reg} may mainly recognize tumor-associated self-antigens, and mostly include TT\textsubscript{reg}; conversely, in the case of inflammatory cancers, related to chronic low-dose type-17 cytokines, which are typical of mucosal tissues, high numbers of PT\textsubscript{reg} may suppress pro-tumoral inflammation through IL-10, relevantly produced by PT\textsubscript{reg} at those sites. We are tempting to speculate that TT\textsubscript{reg} may dominate in suppressing anti-tumor type-1 responses, while PT\textsubscript{reg} may prevail in shaping pro-tumor type-2 and type-17 inflammatory responses. Notably, the prototypical example of an inflammation-related tumor in which T\textsubscript{reg} accumulation associates to good prognosis is CRC (50), developing in the gastrointestinal mucosa, in which immune tolerance is under the control of PT\textsubscript{reg} (84).
Antigen presentation in the tumor context may favor T cells. In different tumor models, TCR-transgenic T cells are generally believed to recognize self-antigens encountered during thymic selection. On the other side, pTreg, deriving from Tconv, are thought to display the same TCR repertoire of Tconv and thus to mainly recognize foreign antigens. Indeed, only a small overlap exists between TCR repertoires of pTreg and tTreg, and pTreg are believed to recognize non-self-antigens such as commensal microbiota, allergens, and fetal alloantigens.

Tumor cells can express a variety of antigens that can be broadly classified into: (i) self-antigens physiologically expressed as in the tissue of origin, (ii) self-antigens aberrantly expressed, in terms of expression level, developmental stage, or histotype (called tumor-associated antigens or TAAs), and (iii) neo-antigens uniquely expressed by tumor cells, mostly derived from oncogenic mutations (named tumor-specific antigens or TSAs). Based on the above considerations, self-antigens and TSA should be recognized by tTreg while TSA would induce and activate pTreg. However, a complex picture arises from studies analyzing the TCR specificity of tumor-associated Treg.

Treg can recognize TAA and TSA in tumors
In different tumor models, TCR-transgenic Treg have been shown to promptly proliferate in response to the cognate antigen specifically expressed by tumor cells, suggesting that Treg can undergo tumor-antigen-driven activation and expansion. Antigen presentation in the tumor context may favor Treg expansion: in a mouse model of spontaneous prostate cancer, an efficient Treg induction/expansion occurred only when TCR-transgenic, antigen-specific CD4 T cells encountered the cognate antigen expressed in the context of prostate cancer cells, rather than non-transformed cells or viral vector-infected cells. In this model, TAA-specific Treg were recognized as pTreg induced in vivo in a TGF-β-independent fashion.

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Repertoire analysis as an estimation of pTreg/tTreg balance
The direct comparison between the repertoires of tumor-associated Treg and Tconv may help understanding the processes underlying Treg enrichment in cancer. Some authors have reported that the analysis of TCR diversity (performed with the immunoscope technology) showed that Treg infiltrating murine transplanted tumors displayed a biased TCR repertoire toward “public” CDR3 sequences (i.e., shared by different mice), suggesting Treg intra-tumor clonal expansion driven by the recognition of dominant antigens. Also tumor-infiltrating activated Tconv showed a biased TCR repertoire, but it was distinct from the Treg spectrum, and the minimal overlap between the two subsets was mainly confined to “private” specificities. By using a similar approach, others have reported distinct and not overlapping TCR repertoires of Treg and Tconv infiltrating prostate tumors in a genetically engineered mouse model of spontaneous prostate carcinogenesis. Lack of overlap between Tconv and Treg repertoires was also found in tumors and tumor-draining lymph nodes in a mouse model of chemical carcinogenesis.
the lack of overlap between T_reg and T_conv has been interpreted in many cases as the result of negligible pT_reg conversion at the tumor site; however, pT_reg and T_reg may share more specificities than expected, since T_reg-associated antigens may preferentially drive T_conv fate decision toward the conversion into pT_reg rather than toward the conventional activation; moreover, already established pT_reg may then undergo intra-tumor clonal expansion together with T_reg in response to the same antigens. Indeed, in one study performed in advanced melanoma patients, TAA-specific TCRs, expressed by tumor T_reg clones, could be detected in both T_reg and T_conv populations, demonstrating that TAA-specific T_reg may be comprised of pT_reg derived from the conversion of T_conv (95).

Indirect data support the notion that TAA-specific T_reg may contain pT_reg. TAA-specific T_reg clones, obtained from patients with advanced melanoma, suppressed in vitro the cognate antigen-specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines (95), and showed low FOXP3 expression and TSDR specific T-cell response, but produced high levels of Th1 and/or Th2 cytokines.

HETEROGENEITY AND PLASTICITY OF T_reg AND P_T_REG

T_reg HETEROGENEITY IN CANCER: RELATIONS WITH THE P_T_REG/T_REG DICHTOMY

During the latest years, it has become increasingly clear that T_reg, meant as Foxp3-positive cells, are not a homogeneous lineage, but rather represent a mixture of subpopulations. Indeed, beside the T_reg/pT_reg distinction based on their developmental origin, diverse T_reg subsets can be identified endowed with peculiar features in terms of suppression, proliferation, and stability, even though not properly classifiable as developmentally distinct lineages (Figure 2). Tumor microenvironmental signals may differentially affect these subsets, thus shaping T_reg heterogeneity to the advantage of tumor progression.

T_reg stability and epigenetic commitment in cancer

Foxp3 inherent stability, rather than Foxp3 expression in a given moment and tissue, is intimately linked to an actual commitment to the T_reg lineage and therefore to the maintenance of immune suppression. Pioneer studies have demonstrated that Foxp3 stability is strictly related to an epigenetic imprinting of CpG demethylation in the TSDR region of the Foxp3 locus (67, 86, 108). TSDR demethylation was then recognized as the mechanism featuring the distinction between committed (demethylated) and uncommitted (methylated) T_reg, irrespective of Foxp3 expression (9). Controversy exists on whether pT_reg show complete or partial TSDR demethylation and can then be considered as committed T_reg. Many studies show that iT_reg have a partially or completely methylated TSDR (9, 67–69), while pT_reg have been described as TSDR-demethylated (68), TSDR-methylated (66), or as a mixed population of stable and unstable cells, characterized by CD25 high or low expression respectively (69).

Few data exist on the extent of TSDR demethylation in tumor-associated T_reg. The frequency of TSDR-demethylated cells is higher in peripheral and intratumoral leukocytes of lung, colon, prostate, or breast cancer patients, in relation to a higher T_reg
frequency as determined by flow cytometry or immunohistochemistry (109). Of note, the extent of TSDR demethylation in CRC patients was only slightly higher than in healthy volunteers, in contrast to the significantly increased Treg frequency in these samples shown by previous studies (110, 111). This discrepancy may be explained with the peculiar nature of this inflammation-related and mucosal tissue-located cancer, in which the inflammatory response may specifically involve pTreg, possibly containing more uncommitted (TSDR-methylated) cells than Treg.

The evaluation of TSDR demethylation has been used as a reliable analytical tool for the estimation of committed Treg in some tumor conditions and therapies. An increased frequency of epigenetically committed (TSDR-demethylated) Treg has been determined in tumor-infiltrating cells of ovarian, colorectal, and bronchial cancers compared to non-tumoral tissue counterparts (112). TSDR demethylation was decreased in the peripheral blood of metastatic renal carcinoma patients receiving tumor vaccination (113), and increased in patients treated with dendritic cell vaccination and cytokine therapy (114).

**Treg functional dynamics in cancer**

The idea of Foxp3 as the master transcription factor of Treg lineage has been challenged by the observation that some Treg features are Foxp3-independent, and that Foxp3 plays Treg-unrelated functions (115). This is especially true for human FOXP3-positive cells, since human activated Tconv can transiently express this transcription factor that acts as an intrinsic T cell regulator (116). The concomitant analysis of CD45RA and FOXP3 in human Treg in both physiological and pathological contexts allowed delineating a classification into three subsets: CD45RA-FOXP3low/resting Treg (rTreg), CD45RA-FOXP3low non-Treg and CD45RA-FOXP3high (CD45RO+) activated Treg (aTreg), endowed with different potentials of proliferation, suppression, stability, and plasticity (117). Whether each subset mainly contains Treg or pTreg is unclear. While rTreg were recognized as CD31+ recent thymic emigrants, thus belonging to the aTreg pool, aTreg can be considered as a mixed population of activated Treg (derived from rTreg) and pTreg (derived from non-Treg or Tconv). The CD45RA-FOXP3low non-Treg subset may represent a mixture of activated Tconv (promiscuously and unstably expressing FOXP3), latent Treg (transiently downregulating FOXP3), and recently converted pTreg (117).

The three human Treg subsets can be differentially expanded in distinct pathologies. In conditions characterized by exacerbated immune responses, such as autoimmune diseases, rTreg and non-Treg are expanded; conversely, in diseases associated to immune unresponsiveness, such as sarcoidosis, the aTreg subset is instead enriched in the peripheral blood (117). The tumor context, which conceivably belongs to the latter category, should be characterized by aTreg expansion. In line with this hypothesis, CD45RO+FOXP3high aTreg were found significantly expanded in the peripheral blood, and much more at the tumor site, in patients with malignant melanoma (118). Also the non-Treg and the Treg fractions were increased, but only in the peripheral blood, in cancer patients compared to healthy controls, and both subsets positively correlated with tumor progression (118). The non-Treg pool produced some IFN-γ and its frequency returned to normal levels after tumor removal, thus probably representing aberrantly activated Tconv, or Treg with attenuated FOXP3 activity (118). A much deeper knowledge on Treg dynamics in cancer is needed to better understand the role played by each specialized component in suppressing anti-tumor type-I, or pro-tumor inflammatory, responses.

**Treg subsets specified by functional/developmental markers**

Several surface or intracellular markers have been suggested to identify Treg subsets endowed with peculiar abilities other than suppressive functions. A portion of human Treg with an effector/memory-like phenotype (26, 119, 120) expresses CD39, which has been proposed as a target to enrich human suppressive Treg (119). CD39 was found overrepresented in peripheral and tumor-infiltrating Treg from HNSCC, and was further increased in patients with advanced-stage disease or after radiochemotherapy (120, 121). CD39 is also expressed by a Tconv subset, which produces lower levels of pro-inflammatory cytokines than the bulk Tconv population, and is more prone to convert, at least in vitro, into Treg (120). Both CD39+ Treg and CD39– Tconv were enriched in peripheral blood, and further increased at tumor site, in HNSCC patients, and a positive correlation existed between frequencies of these two populations (120). Therefore, these data suggest that tumor-associated CD39+ Tconv may represent a reservoir of CD39+ Treg precursors. As a consequence, it could be suggested that CD39+ Treg may include both Treg and pTreg, and that both Treg subsets can exploit the CD39-mediated suppressive mechanisms of ATP degradation and adenosine generation.

Not only the functional arms of suppression, but also the activation requirements may differ in Treg and pTreg: for instance, TNFR2 expression is needed to activate Treg but not pTreg suppressive ability in experimental colitis (122). Of note, TNFR2-positive Treg have been found enriched in murine tumors, in association with a higher suppressive ability, ex vivo, in the standard suppression assay (123). In a mouse model of metastatic melanoma, TNF-α caused enhanced tumor progression through the TNFR2-mediated Treg expansion at the site of metastasis (124). These data suggest that TNFR2 expression may label tumor-infiltrating Treg of thymic origin, and that TNF-α at the tumors site may preferentially expand and activate Treg. Supporting the idea that Treg may represent more stable cells, TNFR2 was found to be involved in the maintenance of Foxp3 stability in mouse models of inflammation (125). Also in human peripheral blood, CD25 and TNFR2 co-expression identifies cells highly expressing FOXP3, showing an effector/memory phenotype and strong suppression, ex vivo (126). The TNF-α/TNFR2 pathway may amplify Treg activation also through the induction of a NF-kB-driven transcriptional program enriched for other members of TNF superfamily, such as 4-1BB, FAS, and OX40 (127).

The early idea that Helios could differentiate Treg from pTreg (59, 128) prompted the use of this marker in delineating Treg accumulation in cancer. The vast majority of tumor-infiltrating Treg were found to express Helios in a mouse model of glioblastoma (129), in glioblastoma multiforme patients (129), and renal cell carcinoma patients (130). However, the value of Helios as univocal marker of Treg has been questioned by other studies that showed Helios also expressed in pTreg (60, 131), and in association to Treg suppression (128, 131) and commitment. Indeed,
Helios<sup>−</sup>FOXP3<sup>+</sup> cells freshly isolated from healthy donors or autoimmune disease patients showed decreased TSDR demethylation compared to Helios<sup>+</sup>FOXP3<sup>+</sup> cells (132, 133), and also displayed a higher plasticity in terms of cytokine production (133). In a murine transplanted tumor model, tumor-infiltrating T<sub>reg</sub> were enriched in Helios<sup>+</sup> cells, representing the subset with the highest proliferative potential (as shown by Ki67 staining) (131). In summary, the well-recognized enrichment of Helios<sup>+</sup> T<sub>reg</sub> in several human and mouse tumors may be attributed, rather than to preferential attraction and expansion of T<sub>reg</sub> to the tumor-driven local activation and/or commitment of both T<sub>reg</sub> and pT<sub>reg</sub>.

**SPECIALIZATION AND PLASTICITY OF T<sub>reg</sub>/pT<sub>reg</sub> IN CANCER**

It is now well established that T<sub>reg</sub> (or better, their specific subsets) adapt their molecular programs to optimize their *in vivo* suppressive function in distinct inflammatory milieus, which may be alternatively dominated by Th1, Th2, Th17, or T<sub>FH</sub> responses. Strikingly, these T<sub>reg</sub> specialized programs are orchestrated by the same transcription factors that drive the polarization of the targeted T-helper subset: therefore, T-bet, IRF4, Stat3, and Bcl6 expression are respectively and selectively required for the T<sub>reg</sub> specialized suppression of Th1 (134, 135), Th2 (136), Th17 (137), and T<sub>FH</sub> (138, 139) responses. Indeed, by acquiring master T-helper genes, T<sub>reg</sub> may gain the expression of chemokine receptors driving the recruitment of specialized T<sub>reg</sub> into inflamed tissues. However, in some contexts, T<sub>reg</sub> (or, again, some T<sub>reg</sub> subsets) can express not only T-helper-related transcription factors and migratory molecules, but also cytokines such as IFN-γ or IL-17, thus turning from specialized suppressors into so-called Th1-like or Th17-like T<sub>reg</sub> that may rather contribute to inflammation (140). Some data, mostly from mouse experimental models, suggest that such T<sub>reg</sub> plasticity is not a lineage reprogramming of committed T<sub>reg</sub>, which appear instead quite stable; rather, Th1-like or Th17-like T<sub>reg</sub> may derive from uncommitted cells expanded in inflammatory conditions (69, 141). However, other studies have shown that in both mouse and human pathological T<sub>reg</sub> can produce relevant amounts of type-I and type-17 cytokines even though preserving Foxp3 expression (142–146).

**Th17-like T<sub>reg</sub> in cancer**

Regulatory T cells may shift to a Th17-like phenotype in inflamed microenvironments dominated by type-17 cytokines, thus favoring, rather than suppressing, pro-inflammatory mechanisms of chronic inflammation. According to this idea, human T<sub>reg</sub> have been found to spontaneously secrete IL-17 in the intestine of patients carrying inflammatory bowel disease (145, 147) and colon carcinoma (147). In epithelial ovarian cancer, a malignancy associated to chronic inflammation, T<sub>conv</sub> were found to secrete high levels of IL-17 (and other cytokines) when cultured *ex vivo* with IL-2; under similar conditions, tumor-infiltrating T<sub>reg</sub> were prone to FOXP3 down-regulation, attenuation of suppressive function, and prompt IL-17 production (148). In human lung adenocarcinoma, FOXP3 message amounts correlated with Th17-related transcripts enriched at the tumor site, where IL-17 antagonized the development of the anti-tumor, T-bet-dependent, T<sub>helper</sub> response (149). Myeloid antigen-presenting cells and cytokines such as IL-2, TGF-β, IL-1, IL-23, and IL-6 may initiate T<sub>reg</sub> polarization into Th17-like cells in these tumor contexts (147–149). In a mouse model of hereditary colon polyposis, as well as in human colon cancer, the Th17-like T<sub>reg</sub> co-expressed the Th17-related transcription factor ROR-γt, and fostered tumor progression, also through the promotion of mast cell local expansion (150, 151). This study clearly demonstrated that microenvironmental signals could direct T<sub>reg</sub> plasticity toward pro-inflammatory and pro-tumoral activities.

One group has demonstrated that Th17-like T<sub>reg</sub> can also arise in experimental tumors as an outcome of vaccination strategies (152). In this study, vaccination with antigen plus TLR-9 ligand induced T<sub>reg</sub> reprogramming into polyfunctional T-helper-like cells, producing a wide array of cytokines including IL-2, TNF-α, and IL-17, and expressing cell-surface CD40L, thus providing efficient T cell help for tumor-antigen cross-presentation and development of anti-tumor response (152). The IDO immunosuppressive enzymatic activity was responsible for preventing this anti-tumor T<sub>reg</sub> polarization, which was instead enhanced using an IDO blocker (152).

Little data exist on the precursors of Th17-like T<sub>reg</sub> in cancer. In the peripheral blood of healthy subjects, the CD45RA<sup>−</sup>FOXP3<sup>low</sup> non-T<sub>reg</sub> subset was found enriched in Th17-related transcripts and in cells actively secreting IL-17, even at higher levels than naïve or memory T<sub>conv</sub>, a data suggesting that this population contains Th17 or Th17-like precursors (117). It would be interesting to understand whether the Th17 potential resides, within the non-T<sub>reg</sub> gate, in activated T<sub>conv</sub>, in latent T<sub>reg</sub> and/or in recently induced pT<sub>reg</sub>, possibly co-expressing FOXP3 and RORγt and thus pre-committed to the Th17 lineage.

**Th1-like T<sub>reg</sub> in cancer**

Pioneer studies from Koch and colleagues demonstrated that, following exposure to IFN-γ in Th1-dominated microenvironments, a subset of T<sub>reg</sub> can up-regulate the Th1-related transcription factor T-bet, which drives T<sub>reg</sub> expansion, migration (CXCR3-mediated), and function specifically during type-1 inflammation (134). Further experiments have shed light on the developmental requirements and the alternative fates of murine T-bet<sup>+</sup> T<sub>reg</sub>; following IFN-γ stimulation, T<sub>reg</sub> could gain T-bet expression but failed to fully polarize into IFN-γ-producing Th1-like T<sub>reg</sub> due to an impaired T<sub>reg</sub> susceptibility to IL-12. Indeed, IL-12 receptor β2, which is inducible in T<sub>conv</sub>, in an IFN-γ- and T-bet-dependent fashion, is epigenetically inaccessible in T<sub>reg</sub> (135). Only long-lasting IFN-γ pre-conditioning could unlock IL-12 responsiveness, thus allowing the complete T<sub>reg</sub> polarization into Th1-like cells (135). Presumably, in contexts characterized by chronic IFN-γ and IL-12 abundance, such as autoimmunity, inflammatory, and viral diseases, T<sub>reg</sub> will be oriented to a full reprogramming into Th1-like cells. Supporting this idea, IFN-γ-producing T<sub>reg</sub> have been reported in mouse models of graft-versus-host disease (153), viral (154) or parasitic (142) infection, in human multiple sclerosis (144), and diabetes (146, 155). In one of these systems, IFN-γ-producing T<sub>reg</sub> were recognized to be specific for a foreign (viral) antigen (154). Whether such Th1-like T<sub>reg</sub> can be yet considered as classical regulatory cells is still debated. One study has shown that *in vitro* polarized Th1-like T<sub>reg</sub> were less suppressive than conventional T<sub>reg</sub> in the standard *in vitro* suppression assay, and that suppression could be partially rescued with concomitant IL-10.
and IFN-γ neutralization (144). Another study has proven that IFN-γ-producing human tTreg were equally functional as natural Treg in suppressing both proliferation and cytokine production of responder T cells (156). In a mouse model of graft-versus-host disease, IFN-γ-producing cells by stable (TSDR-demethylated) Treg was shown to be even required for Treg protective effect (153), suggesting that IFN-γ-releasing Treg can display in vivo unexpected functions depending on the context.

Conversely, it could be envisaged that, in the tumor context, the low levels of IFN-γ derived from Tconv, NK, and CD8 cells, and the paucity of IL-12 production by tumor-associated APCs, may concur to induce a pool of Treg expressing T-bet but not secreting IFN-γ, thus specialized in suppressing antitumor type-I immunity. In line with this possibility, TAA-specific Tconv, but not TAA-specific Treg, produced IFN-γ in patients with epithelial ovarian cancer (99). In both healthy subjects (117) and malignant melanoma patients (118), IFN-γ-producing cells were enriched within the circulating CD45RA-γδ T cells, highly expressing T-bet but poorly producing IFN-γ, and strongly suppressing Th1 response ex vivo (157). Tumor-associated CXCR3+ Treg were mostly Helios-positive, and T-bet+ Treg could be generated in vitro by culturing CD45RA+CCR7+ rtTreg (mostly containing tTreg) under Th1-polarizing conditions (157), suggesting their derivation from committed Treg. This finding was in accordance to Koch et al. who showed that T-bet+ Treg derived from T-bet- Treg, rather than from activated Tconv (134). These data support the idea that tTreg, rather than pTreg, may contain the precursors for Th1-specialized suppressors, thus playing critical roles in suppressing protective responses in tumors whose high Treg frequency correlates with poor prognosis (13).

Some therapeutic interventions can force tumor-associated Treg toward a fully differentiated Th1-like phenotype. For instance, circulating Treg from melanoma patients showed significantly higher IFN-γ secretion following a protocol of tumor peptide vaccination plus IL-2 and cyclophosphamide, in line with enhanced serum IL-12 (158). On the whole, these data suggest that, especially in the human system, the transition from T-bet+ Treg, specialized Th1 suppressors, into T-bet+ IFN-γ+ Treg, Th1-like plastic cells, may not only depend on the availability and the responsiveness to exogenous stimuli, but may differentially occur in distinct Treg precursors: on the one side, tTreg, enriched in committed and self-specific cells, may be forced to arrest to the specialization (T-bet+) endpoint; on the other side, pTreg, containing less committed and foreign antigens-specific cells, may be more prone to the complete reprogramming into pro-inflammatory (T-bet+ IFN-γ+) cells. Future studies will elucidate the mechanisms by which different growing tumors may favor the expansion of protumoral specialized Th1 suppressors or the induction of Th1-like plastic Treg.

**IMPLICATIONS FOR CANCER IMMUNOTHERAPY**

The initial enthusiasm on the use of therapeutic cancer vaccines has been soon disappointed by the observation of a low response rate in many trials (159). After the discovery of Treg as potent immune suppressive cells hampering the establishment of antitumor immunity, it soon became clear that anti-tumor vaccination might fail to elicit an effective immune response and to achieve successful tumor eradication, because of the immune suppressive barrier created by Treg. In addition, since Treg may recognize TAA and TSA at higher frequency than Tconv, tumor-antigen-based vaccines may expand/induce Treg rather than effector cells, thus inhibiting rather than boosting the anti-tumor response. Indeed, Zhou et al. first demonstrated that TCR-transgenic CD4 T cells specific for a TAA, adaptively transferred into mice bearing TAA-expressing tumor cells, proliferated extensively after administration of a therapeutic tumor vaccine (in the form of a recombinant vaccinia virus encoding the antigen), but tumor-antigen-experienced cells were mostly regulatory cells, ex vivo suppressive, and anergic to subsequent stimulation (81).

In cancer patients receiving tumor-antigen vaccination, the expansion of antigen-specific Treg has been documented. Circulating NY-ESO-1-specific Treg spontaneously develop in late-stage melanoma patients and are expanded following immunization with NY-ESO-1 protein supplemented with adjuvants (96). Therapeutic vaccination with an HPV synthetic long peptide vaccine, administered to patients with HPV-positive cervical carcinoma, induced both CD8 and CD4 T cell immunity, but also enhanced the HPV-specific Treg pool (160). The pool of vaccine-specific Treg may derive not only from the expansion of pre-existing tumor-antigen-specific clones, but also from de novo generation of vaccine-specific pTreg. This is suggested by results obtained vaccinating melanoma patients with an HLA-DP4-restricted MAGE-A3 peptide: in this setting, a subset of vaccine-specific Treg becomes detectable only after vaccination (161). Vaccine-elicited Treg showed some degree of heterogeneity: out of five CD25+ regulatory clones isolated from vaccinated patients, four expressed high FOXP3 mRNA levels, produced TGF-β, and showed demethylated TSDR; one clone expressed less FOXP3, had methylated TSDR and produced some Th2 cytokines (161). These data suggest that antigen-specific Treg, induced in the periphery following antigen exposure and thus recognizable as pTreg, can contain both committed and uncropped cells.

The concomitant and detrimental Treg expansion in antitumor vaccination can be avoided by using CD8 T cell-targeted approaches. A melanoma vaccination protocol based on an MHI CIrestricted Melan-A peptide significantly decreased the frequency of melan-A-specific Treg, in association with an improved and more diverse Th1 response (97).

Some attempts have also been made to combine active immunotherapy with Treg depletion or functional blockade. Several studies showed that depletion of CD25+ cells in vivo in cancer patients could enhance the tumor-specific T cell responses induced by cancer vaccines (15). However, CD25-directed strategies may fail to achieve sustained results, since activated effector cells may be concomitantly eliminated and pTreg may replenish the Treg pool after depletion (15). Interestingly, a recent study has demonstrated that different Treg-depleting agents, either CD25-targeted (IL-2/diphtheria toxin fusion protein, or anti-CD25 antibody) or not (low-dose cyclophosphamide), failed to consistently eliminate more than 50% of committed Treg, as identified by TSDR demethylation (162).
Therefore, alternative strategies are needed to counteract the “hard core” of immune suppression that is represented by epigenetically committed T_{reg}. We have proposed in the past that T_{reg} functional inactivation, rather than depletion, may represent a successful strategy to prevent massive pT_{reg} induction and concomitantly block T_{reg} suppression (15). This idea may be corroborated by the observation that markers associated to T_{reg} suppressive functions, and therapeutically targetable, may show enriched or restricted expression in epigenetically committed T_{reg}. For instance, GITR stimulation has been shown to attenuate T_{reg} suppression and favor the rejection of experimental tumors (163).

A recent study has demonstrated that GITR engagement in vivo led to the downregulation of Foxp3 expression in intratumoral T_{reg} (164). Of note, GITR^{+} T_{reg} were found enriched in Helios^{+} cells, representing highly committed T_{reg} (131), thus GITR targeting may preferentially block the strongest suppressors among the T_{reg} pool. A similar possibility could be envisaged for therapeutic strategies aimed at TNF-α/TNFR2 blockade, since this axis may be mainly involved in the activation of more committed and stable cells (122–126). Committed T_{reg} may also be targeted by virtue of their high proliferative potential: indeed, high proliferation rates, in terms of Ki67 positivity, were detected in healthy subjects within the aT_{reg} subset, enriched in stable and committed T_{reg} (117), and also in murine tumor-infiltrating Helios^{+} T_{reg} (131). Therefore, treatments based on the depletion of proliferating cells, such as low-dose cyclophosphamide, may efficiently target committed T_{reg}.

An innovative way to improve immunotherapy would be to reprogram tumor-associated T_{reg} into fully armed effector cells, which would then become “exT_{reg}.” Different from other vaccine-based approaches, T_{reg} reprogramming is expected to trigger anti-tumor response very rapidly, since T_{reg} are already located at the tumor site and already tumor-antigen-experienced, thus not requiring a de novo T cell priming. Therefore, exT_{reg} may function in an “innate-like” manner, promptly providing co-stimulatory and pro-inflammatory signals when adequately modulated, before a novel adaptive anti-tumor response develops (140). An example of this approach has been proposed by Sharma et al. who demonstrated that reprogramming of mature pre-existing tumor-associated T_{reg} into CD40L-expressing helper effector cells was needed to achieve tumor regression in a model of immunotherapy combining antigen vaccination, TLR-9 stimulation, and IDO blockade (152).

The above-discussed data overall indicate that tT_{reg} and pT_{reg} may not be equally susceptible to functional reprogramming, but this dichotomy may turn into a benefit for the efficacy and safety of the evoked response. Indeed, on the one hand, tT_{reg}, predominantly self-specific, highly committed, and hard to be reprogrammed into T-helper-like cells, would be preserved, thus ensuring immune tolerance to self-antigens and maintaining systemic immune homeostasis. On the other hand, pT_{reg}, mainly representing tumor-specific and uncommitted cells, may be more easily converted into exT_{reg}, thus mounting an immediate helper and/or effector response in a mostly tumor-antigen-specific fashion.

Reprogramming into exT_{reg} may be achieved by immunotherapies aimed at subverting the immune suppression mechanisms established by innate cells in tumor microenvironments. For instance, in the above-reported model of tumor vaccination, CD40L upregulation by T_{reg} following TLR-9 stimulation was strictly dependent on host-derived MyD88 and IL-6 signals (152). In melanoma patients, tumor peptide antigen vaccination combined with low-dose cyclophosphamide and low-dose IL-2 evoked Th1-like T_{reg} accumulation, in line with a less tolerogenic microenvironment and with enhanced IL-12 availability (158). Of note, in this system, depletion of proliferating (conceivably committed and thymus-derived) T_{reg} by means of cyclophosphamide allowed the functional reshuffling of innate cells that in turn unveiled the emergence of Th1-like exT_{reg}.

However, it is arguable that microenvironmental rearrangements would better accomplish full T_{reg} reprogramming with the concomitant direct modulation of T_{reg} activities, aimed especially at enhancing T_{reg} susceptibility to external signals. For instance, expression of IL-12 receptor, which is epigenetically regulated in T_{reg} (135), could be artificially boosted by pharmacological approaches. Also, targeting with monoclonal antibodies some receptors expressed on T_{reg} surface and correlated with T_{reg} stability (such as TNFR2 and GITR) could result in enhancing T_{reg} propensity to reprogramming. In line with this idea, treatment of murine melanomas with a GITR agonistic antibody resulted in the accumulation of exT_{reg} at the tumor site (164). Suppressor of cytokine signaling (SOCS) 1 and 2, which maintain Foxp3 stability and prevent T_{reg} polarization into effector cells (165, 166), may be pharmacologically inhibited to unlock T_{reg} responsiveness to pro-inflammatory microenvironmental cytokines.

CONCLUSION

Even though discrimination between pT_{reg} and tT_{reg} by simple surface phenotyping is not yet possible many pieces of evidence indicate that both subsets contribute to the T_{reg} pool conditioning the tumor microenvironment. Nevertheless, the development/expansion of pT_{reg} and tT_{reg} are independent processes, possibly resulting from disparate antigens and signals, and their activities seem characterized by very peculiar features in terms of specificity, stability, and specialization. On the one side, tT_{reg} may expand at tumor site in response to self-antigens expressed by tumor cells, mostly included committed (TSDR-demethylated) Helios- and TNFR2-expressing cells, and contain the precursors of specialized T-bet^{+} Th1-suppressing cells, thus representing not only the guardians of systemic immune homeostasis but also the “hard core” of tumor immune escape. On the other side, pT_{reg} may mostly develop following local encounter with TAA or TSA antigens, possibly represent a mixed population of committed (TSDR-demethylated) and uncommitted (TSDR-methylated) cells, and are more prone to be reprogrammed into Th1-like or Th17-like effector cells. We envisage that future successful immunotherapies may not only target committed T_{reg} but also favor “recycling” uncommitted T_{reg} into prompt anti-tumor effectors.

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