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ORIGINAL PAPERS

Tumor-Infiltrating Regulatory T Cells: Phenotype, Role, Mechanism of Expansion In Situ and Clinical Significance

C. Tanchot · M. Terme · H. Pere · T. Tran · N. Benhamouda · M. Strioga · C. Banissi · L. Galluzzi · G. Kroemer · E. Tartour

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Abstract In immunocompetent individuals, the immune system initially eradicates potentially tumorigenic cells as they develop, a capacity that is progressively lost when malignant cells acquire alterations that sustain immunosubversion and/or immunoevasion. One of the major mechanisms whereby cancer cells block antitumor immune responses involves a specific class of immunosuppressive T cells that–in the vast majority of cases–express the Forkhead box P3 (FOXP3) transcription factor. Such FOXP3$^+$ regulatory T cells (Tregs) accumulate within neoplastic lesions as a result of several distinct mechanisms, including increased infiltration, local expansion, survival advantage and in situ development from conventional CD4$^+$ cells. The prognostic/predictive significance of tumor infiltration by Tregs remains a matter of debate. Indeed, high levels of intratumoral Tregs have been associated with poor disease outcome in cohorts of patients affected by multiple, but not all, tumor types. This apparent discrepancy may relate to the existence of functionally distinct Treg subsets, to the fact that Tregs near-to-invariably infiltrate neoplastic lesions together with other cells from the immune system, notably CD4$^+$ and CD8$^+$ T lymphocytes and/or to peculiar features of some oncogenic programs that involve a prominent pro-inflammatory component. In this review, we will discuss the phenotype, function and clinical significance of various Treg subsets.

Keywords CD4 · CD8 · Cytotoxic T-lymphocyte antigen 4 (CTLA-4) · Interleukin-10 (IL-10) · Interferon γ (IFN-γ) · Transforming growth factor β (TGF-β)

Introduction

Since the formulation of the "cancer immunosurveillance" hypothesis by Burnet and Thomas, multiple preclinical and clinical studies have demonstrated that tumors can elicit immune responses that–at least initially–exert antineoplastic

C. Tanchot (✉) · M. Terme · H. Pere · T. Tran · N. Benhamouda · C. Banissi · E. Tartour
INSERM U970, PARCC (Paris Cardiovascular Research Center), Paris, France
e-mail: corinne.tanchot@inserm.fr

C. Tanchot · M. Terme · H. Pere · T. Tran · N. Benhamouda · C. Banissi · L. Galluzzi · G. Kroemer · E. Tartour
Sorbonne Paris Cité, Université Paris Descartes, Paris, France

N. Benhamouda · M. Strioga · E. Tartour
Service d'immunologie biologique, Hôpital Européen Georges Pompidou, AP-HP, Paris, France

M. Strioga
Department of Immunology, Center of Oncosurgery, Institute of Oncology, Vilnius University, Vilnius, Lithuania

G. Kroemer
INSERM U848, Villejuif, France

G. Kroemer
Centre de Recherche des Cordeliers, Paris, France

G. Kroemer
Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, Paris, France

G. Kroemer
Metabolomics Platform, Institut Gustave Roussy, Villejuif, France

functions. However, as the malignant lesions grow, such immune responses virtually vanish, mostly due to the multipronged immunosuppressive network that develops in the tumor microenvironment [1, 2].

Immunosuppression as mediated by Forkhead box P3 ($FOXP3)^+$ regulatory T cells (Tregs) is a dominant mechanism whereby growing tumors escape immune responses and hence represents a major handicap for tumor immunotherapy. Whereas in healthy peripheral organs Tregs constitute approximately 10 % of total $CD4^+$ T cells, this proportion is consistently increased in the tumor microenvironment, where Tregs can account for 30–50 % of $CD4^+$ T cells, depending on tumor type [3]. The phenotype of intratumoral Tregs appears to differ from that of circulating Tregs, and the former have also been suggested to promote tumor angiogenesis, hence favoring tumor growth via immune-independent mechanisms. Various studies have reported a negative prognostic value for tumor infiltration by Tregs, yet this seems to be strongly influenced by other clinical and biological parameters including tumor type, location, stage as well as the presence or not of other immune effector cells, notably $CD8^+$ cytotoxic T lymphocytes (CTLs). Nowadays, an intense wave of investigation focuses on the development of novel strategies that combine Treg inhibitors with various immunostimulatory agents for the immunotherapy of various neoplasms [4, 5].

In this review, we will focus on tumor-infiltrating Tregs (TITregs), with a particular attention to their phenotype, the molecular and cellular mechanisms whereby they infiltrate neoplastic lesions, their role in tumor immune escape and their clinical value, both as a prognostic factor and as a predictive biomarker for response to therapy.

Regulatory T Cell Subsets

Two major populations of $FOXP3^+$ Tregs have been described so far: one "natural" (n) subset, which differentiates in the thymus during T cell ontogenesis and one "induced" (i) subset, developing in the periphery from conventional $CD4^+$ T cells [6]. The conversion of $CD4^+$ T cells into iTregs occurs as a response to multiple settings including, but not limited to, a suboptimal antigenic stimulation and/or inadequate costimulation, especially in the presence of transforming growth factor β (TGF-β) [7]. Tumor-infiltrating dendritic cells (DCs) that are blocked in an immature stage of differentiation due to the presence of specific mediators such as interleukuin (IL)-6, IL-10, vascular endothelial growth factor (VEGF) and prostaglandin E_2 (PGE_2) also stimulate the differentiation of Tregs.

In the vast majority of cases, Tregs express *FOXP3*, a gene that maps to the *p* arm of the X chromosome and codes for a member of the forkhead/winged-helix family of transcription factors. In humans, defects in *FOXP3* induce a generalized autoimmune disorder called immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX), a rare, early onset, disease affecting males and manifesting with severe enlargement of the secondary lymphoid organs, insulin dependent diabetes, eczema, food allergies, and infections. Mice bearing a spontaneous mutation in *Foxp3*, which are known as "scurfy", manifest a very similar disorder [1, 8]. Foxp3 is consider as a specific marker of Tregs in mice but, in humans, activated T cells can transiently expressed it. However, recent data indicate that a small fraction (about 10 %) of activated murine conventional T cells also upregulate FOXP3 expression which is unstable and does not confer immunosuppressive activity [9]. In this context, the differential expression of CD127 (the α subunit of the IL-7 receptor) may be instrumental for discriminating between Tregs and activated effector T cells (Teffs), being low in the former and very high in the latter [10]. However again, CD127 (i.e. absence of its expression) is not an ideal biomarker for the identification of Tregs, because a fraction of early-activated Teff cells are also $CD25^+FOXP3^+CD127^{low/-}$ and thus are indistinguishable from Tregs [11]. Conversely, it has been shown in mice that CD127 is also highly expressed on Tregs upon in vitro and in vivo activation and on fraction of Tregs located in tissues [12].

To date, several phenotypically and functionally distinct iTreg subsets of both the CD4 and CD8 lineage have been described [6]. Among the best known of such populations are Th3 cells, which are often associated with oral tolerance and $IL\text{-}10^+$ T regulatory 1 (Tr1) cells. The Tr1 subset was initially described in vitro, arising in the presence of high IL-10 levels and chronic antigenic stimulation [13]. Multiple clinical studies have shown that increased frequencies of IL-10-producing $CD4^+CD25^{high}$ Tregs can be found in the peripheral blood, tumor stroma, draining lymph nodes, and ascitic fluids of gastro-esophageal cancer patients, and are robustly associated with disease stage [14, 15]. More recently, a new Treg subtype has been identified to arise and expand in mice bearing orthotopic melanomas, liver and lung tumors [16]. This subset expresses the early activation marker CD69 (a transmembrane C-type lectin), membrane-bound TGF-β1, which inhibits T-cell proliferation, as well as the β chain of the IL-2 receptor, but not the α chain of the IL-2 receptor (CD25) and FOXP3. In addition, these cells secrete a wide array of cytokines including IL-2, IL-10, soluble TGF-β1 and interferon γ (IFN-γ). Although these results suggest that another subset of Tregs can suppress antitumor immune responses, the role of these cells in cancer patients has not yet been fully elucidated. Moreover, it remains to determine whether different subsets of Tregs really belong to distinct lineages or whether they only reflect the plasticity of the Treg population. In support of the latter interpretation, it has been shown that Tregs can convert into

Th17 cells in the presence of TGF-β and IL-6, a cytokine that is strongly produced during inflammatory reactions [17, 18]. However, Miyao et al. provide solid evidence that peripherally-induced conventional (non-regulatory) $CD25^{-}FOXP3^{+}$ T cells give rise to highly proliferative $CD25^{-}FOXP3^{-}$ T cells with a robust pro-inflammatory potential, whereas *bona fide* Tregs represent a stable cell lineage which is committed to immunosuppressive function both under steady state and in a changing microenvironmental conditions, including those that have previously been claimed to induce complete Treg cell reprogramming [9].

Is it Possible to Distinguish Between nTreg and iTreg Subsets?

Although both nTregs and iTregs have been shown to promote tumor immune escape, it is difficult to clearly discriminate these two cell populations based on surface markers. Numerous molecules are expressed at the surface of Tregs including the tumor necrosis factor receptor (TNFR) superfamily members GITR and OX40, the CD28 family members cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed death 1 (PD1), receptors for different chemo- and cytokines as well as Toll-like receptors (TLRs). However these markers do not allow for the discrimination between Tregs and Teffs nor for distinguishing nTregs from iTregs.

Some studies have attempted to differentiate nTregs from iTregs based on molecular biology approaches. For instance, it has been shown that while nTregs exhibit a completely demethylated *FOXP3* locus, iTregs show an incomplete demethylation that is associated with unstable FOXP3 expression [19]. However others studies showed that a fraction of nTregs could convert to an effector phenotype following transfert into lymphopenic recipients suggesting that Tregs retain some plasticity [20, 21]. Recent results from microarrays studies indicate that Helios, a member of the Ikaros family of transcription factors, is expressed to high levels by Tregs. Thornton et al. reported that Helios is exclusively expressed by nTregs and not by iTregs both in vitro and in vivo [22]. Conversely, Vergahen et al. showed that the method of activating Tregs in vitro, rather than the Treg subset, determines the expression of Helios [23]. Finally, it has been proposed that Helios expression can be initiated during T-cell activation and proliferation in functionally distinct T-cell populations, encompassing Tregs and Teffs [24]. Hence, it appears that Helios expression also doesn't allow for the discrimination between nTregs and iTregs, in particular in vivo, where iTregs are found in several different microenvironments. In summary, no study performed up to date has identified a gene or a gene signature that is exclusively expressed by nTregs or iTregs. This remains a critical point and will surely be extensively investigated in the future. Determining the real contribution of nTregs versus iTregs in tumor immune escape is indeed fundamental for the development of targeted and efficient immunotherapeutic interventions.

Mechanisms of Immunosuppression by Tregs

Tregs use different mechanisms to inhibit antitumor immune responses. First, both iTregs and nTregs populations are capable of secreting immunosuppressive mediators including cytokines like IL-10, TGF-β and IL-35 [6], as well as small molecules like adenosine [7]. Second, at least in some instances, Tregs can induce Teffs to undergo apoptosis, either as they release granzyme A and B or as they promote a status of metabolic disruption secondary to the deprivation of IL-2 [25]. Third, nTregs engage in contact-dependant mechanisms of immunosuppression. Thus, nTregs are able to inhibit DC maturation following the interaction of CTLA-4 with CD80/CD86 on DCs, which can deliver a negative signal that inhibits the priming of antitumor immune responses, perhaps involving the upregulation of the immunosuppressive enzyme indoleamine 2,3 dioxygenase (IDO). Of note, Tregs devoid of CTLA-4 have been shown to lose their immunosuppressive activity [26]. Fourth, other molecules expressed on the surface of Tregs might contribute to their immunosuppressive activity, at least in a few settings [6]. These include, though perhaps are not limited to, LAG-3 (an immunoglobulin-like transmembrane protein), CD39 (an ATP-degrading enzyme operating in the pericellular microenvironment), neuropilin 1 (a protein originally characterized for its functions in the central nervous system) and galectin 1 (a β-galactoside-binding protein involved in cell-to-cell and cell-to-matrix interactions).

This said, which of these mechanisms predominantly execute Treg-mediated immunosuppression in specific tumor settings, and hence which constitute best target for immunotherapeutic interventions, remains to be understood.

Phenotype of Tumor-Infiltrating Tregs

nTregs developing in the thymus exhibit a $CD25^{high}CD62L^{+}$ $CCR7^{+}$ surface phenotype (similar to naïve conventional T cells) and preferentially migrate to secondary lymphoid organs. Conversely, TITregs harbor an effector memory phenotype, i.e., they express high levels of CD44 (a cell-surface glycoprotein involved in cell-to-cell interactions,) but fail to express the selectin CD62L (also known as L-selectin) and CCR7 (the receptor for chemokines CCL19 and CCL21), being $CD44^{high}CD62L^{-}CCR7^{-}$ [6]. Activated nTregs express additional chemokine receptors, allowing them to migrate to

the tumor site, as well as high levels of CTLA-4 and other inhibitory molecules, which confer them a robust regulatory activity [27, 28]. Gobert et al. have shown that peripheral Tregs are recruited to lymphoid aggregates located in the tumor bed through a CCR4/CCL22 chemokine gradient. Surprisingly, Tregs isolated from malignant ovarian ascites express high levels of CCR4, whereas Tregs found within breast carcinoma express very low levels of CCR4 as compared to their circulating counterparts [29, 30]. It has been suggested that high levels of CCL22 may be responsible for the down-regulation of CCR4 expression at the plasma membrane [31].

TITregs expressing high level of inducible costimulator (ICOS) represent activated Tregs and are capable of strongly suppressing $CD4^+$ conventional T-cell responses [30]. Tr1 cells found within human head and neck squamous cell carcinoma lesions have been shown to co-express CD39, CD73 (another enzyme participating in the degradation of extracellular nucleotides) and cyclooxygenase 2 (COX2), resulting in robust immunosuppressive activity due to abundant production of adenosine and PGE_2 [32, 33]. T-cell immunoglobulin mucin-3 (TIM-3) has been shown to be upregulated in both $CD4^+$ and $CD8^+$ lymphocytes infiltrating human non-small cell lung cancer (NSCLC) lesions. In this setting, 60 % of $FOXP3^+$ tumor-infiltrating leukocytes (TILs) were $TIM\text{-}3^+$ and TIM-3 expression correlated with poor clinical and pathological parameters. Although the mechanism of TIM-3-mediated immunosuppression remains to be fully eludicated, these findings suggest that TIM-3 plays a role in the pro-tumorigenic functions of TITregs [34].

Generally, Treg markers associated with activation, including–but not limited to–CD39, CD79, ICOS, TIM-3, TNFR2, GARP and chemokines receptors such as CCR4 and CXCR4, characterize the most potent immunosuppressive Tregs within the tumor microenvironment. Specifically targeting these highly immunosuppressive Treg subsets may constituted the most efficient approach for blocking the pro-tumorigenic activity of Treg in the context of anticancer immunotherapy [35, 36].

Mechanisms of Intratumoral Treg Accumulation

The consistent accumulation of Tregs in the tumor microenvironment may result from non-mutually exclusive mechanisms involving infiltration as promoted by multiple chemotactic factors, increased local proliferation, survival advantage and/or the generation of iTregs in situ from naïve $CD4^+$ T cells.

Migration and Retention of Tregs Numerous studies have demonstrated that Tregs selectively migrate to the tumor site following chemotactic gradients that are sensed and transduced into a biological response by chemokines/chemokine receptor and integrins/integrin receptor interactions. For instance, CCL22, which is secreted by ovarian cancer cells as well as by intratumoral macrophages, induces the selective migration of Tregs, which constitutively express high levels of CCR4, the receptor for CCL22 [29, 37]. Several other studies have shown that CCL22 mediates the trafficking of $CCR4^{high}$ Tregs to many different tumors [30, 38]. Our group has also recently demonstrated the crucial role of CCR4 expression by Tregs in the development of immune tolerance to spontaneous mammary tumors [35]. Inhibition of the CCR4-expressing Treg population by means of a CCR4 antagonist was sufficient to break immune tolerance, suggesting a major role for CCR4 in the immunosuppressive activity of Tregs [39]. Other chemokine receptors such as CCR5, CCR8 and CXCR4 have also been suggested to promote Treg migration. For instance, the CCL5/CCR5 interaction appears to be crucial for the infiltration of Tregs in pancreatic adenocarcinomas [40]. Tregs have been observed to exhibit increased CXCR4 levels following the administration of IL-2 to ovarian carcinoma patients [41]. CXCR4 is the receptor for CXCL12, which is strongly involved in the regulation of metastasis in various cancers [42]. Accordingly the expression level of CXCL12 in cervical carcinoma tissues has been found to positively correlate with tumor infiltration by $FOXP3^+$ Tregs and disease progression [43]. Finally, it has recently been demonstrated that TITregs express high levels of CCR8 and CXCR4, while lacking CD62L and CCR7. Importantly antigen priming appears to be required for the induction of this Treg phenotype as well as for the efficient migration of Tregs into tumors [44].

Intratumoral Expansion of Tregs Besides recruiting nTregs via chemotactic gradients, the tumor microenvironment promotes the expansion of nTregs as well as the generation of iTregs in situ, due the abundance of mediators such as IL-10, TGF-β and adenosine, which are produced from both tumor cells and tumor-infiltrating myeloid-derived suppressor cells (MDSCs) [45]. MDSCs, which are often enriched within neoplastic lesions, play an important role in the recruitment and proliferation of Tregs, at least in part as they express the IL-4 receptor and the co-stimulatory receptor CD40 [46, 47]. An increased proportion of $Ki67^+$ Tregs has been detected in multiple types of tumors, demonstrating their highly proliferative potential [30, 48]. Finally, the upregulation of IDO in melanoma lymph node metastases has been associated with increased amounts of TITregs [49]. Accordingly, IDO expression by antigen-presenting cells (APCs) has been reported to directly activate Tregs and promote their proliferation [50, 51].

Survival Advantage A survival advantage of Tregs over Teffs results when the former kill the latter either by releasing cytotoxic mediators such as perforin and granzyme or as

they engage Teff cytotoxic receptors via FASL and PD-L1 [52]. Moreover, the tumor microenvironment is often rich of reactive oxygen species (ROS), which are known to exert a detrimental effect on Teffs [53]. It has recently been demonstrated that nTregs are more resistant to oxidative stress than conventional $CD4^+$ T cells, resulting in an increased abundance of the former over the latter in the tumor milieu [54].

De Novo Conversion of Conventional $CD4^+$ T Cells into iTregs While several mechanisms have been described accounting for the conversion of conventional $CD4^+$ T cells into iTregs, the relative contribution of iTregs versus nTregs in tumor escape is still controversial [8]. Some studies suggest indeed that the immunosuppressive potential of Tregs mostly derives from the conversion of conventional $CD4^+$ T cells into iTregs in situ [55–59], while other reports suggest that the infiltration and local expansion of nTregs would constitute the predominant mechanism [44, 60–62]. As mentioned above, it is difficult to differentially track nTregs and iTregs in vivo because no truly population-specific surface marker has been identified so far. Thus, most of the knowledge on iTregs has been generated upon the conversion of conventional $CD4^+$ T cells into iTregs in vitro (through TCR stimulation in the presence of TGF-β and IL-2) and subsequent adoptive transfer into mice [8]. In vitro findings indicate that mouse prostate cancer cells can promote FOXP3 expression by $CD4^+CD25^-$ T cells, mainly achieved through TGF-β signaling. Importantly, the neutralization of TGF-β in vivo reduces tumor burden in mice. Hence, it seems that tumors promote the differentiation of iTregs due to a milieu rich in TGF-β [56]. However TGF-β also can expand pre-existing nTregs [61]. Zhou et al. studied the contribution of nTregs versus iTregs in vivo, in a model involving influenza hemagglutinin (HA)-expressing tumor cells and HA-specific TCR transgenic mice. In this context, naïve HA $CD25^-GITR^-$ $CD4^+$ T cells and nTregs were co-injected into A20HA tumor-bearing mice. A subset of mice was then vaccinated 2 weeks later with a vaccinia virus encoding HA. The authors reported that both nTregs and iTregs expanded upon vaccination and contributed to the tumor immune escape. However, the expansion of nTregs largely exceeded the conversion and expansion of iTregs [59]. Results from two additional studies indicate that $FOXP3^+$ Tregs that expand in the tumor microenvironment express Helios, suggesting that they derive from nTregs [44, 60]. Still, this interpretation may have to be revised in view of the fact that Helios may represent a marker of activated Tregs rather than an nTreg-specific biomarker [24].

The analysis of the T-cell repertoire might also give indirect hints on the origin of Tregs. One study based on the chemical carcinogen methylcholanthrene (MCA) has demonstrated that Tregs isolated from tumor tissues present a TCR repertoire that is well distinct from that of naïve $CD4^+$ T cells. The hypothesis of the authors of this study was that if Tregs in the tumor truly derived from conventional $CD4^+$ T cells, then the overlap between the TCR repertoire of these two cell subsets would have been higher. Thus, they concluded that TITregs originated from nTregs [62]. In another study, the immunoscope technology was employed to analyze the TCR repertoire of $CD4^+$ TILs in mice bearing TC-1 solid neoplasms. This work demonstrated that Teffs and Tregs exhibit an altered distribution of CDR3 length, which is characteristic of clonal expansion. Moreover, the TCRs of Tregs were found to be skewed toward public sequences that were not shared by tumor infiltrating Teffs. These findings support the notion that nTregs, rather than the conversion of naive $CD4^+$ T cells into iTregs, account for the enrichment of Tregs within tumors [63]. Results from another study analyzing the transgenic TCR repertoire in mice bearing B16 melanoma, however, indicate that most intratumoral Tregs are generated by the conversion of Teffs [55].

In conclusion, the controversy regarding the role of nTregs and iTregs in tumor immune escape still persists and will not be easily solved until the identification of reliable population-specific surface marker. At least theoretically, however, both populations participate in the establishment of an immunosuppressive microenvironment that exerts pro-tumorigenic functions. The relative contribution of these two Treg subsets to tumor immune escape is likely to depend, at least in part, to the features of the tumor microenvironment as developed by each particular type of cancer.

Tregs and Angiogenesis

Until recently, Tregs were believed to support tumor progression only as a consequence of their immunosuppressive functions. Now, a link between Tregs, hypoxia and angiogenesis has been unveiled, suggesting that Tregs can exert non-immune pro-tumorigenic functions [64]. Facciabene et al. have indeed shown that hypoxia promotes the secretion of CCL28 in ovarian cancer cells, in turn leading to the recruitment of $CD4^+CD25^+FOXP3^+$ Tregs upon ligation of the cognate receptor CCR10 [65]. Hypoxia-exposed Tregs are more effective at suppressing the proliferation of Teffs than their counterparts in normoxic microenvironments [66]. By inhibiting DC maturation, VEGF–whose production is upregulated in response to hypoxia–may also favor the differentiation of Tregs in the tumor microenvironment [67, 68]. A subset of human Tregs expresses VEGFR2 [69], and our group has recently shown that VEGF may provide Tregs with a direct, VEGFR2-dependent, co-stimulatory signal that increases proliferation *(Terme* et al. *Submitted)*.

Intratumoral Tregs secrete high levels of VEGF, suggesting that they may directly contribute to angiogenesis and tumor growth [65, 70]. In addition, Tregs might promote angiogenesis via an indirect cellular circuitry as they inhibit Th1 effector T cells, which normally produce antiangiogenic cytokines (IFN-γ) and chemokines (CXCL9, CXCL11) [64]. In line with this notion, biomarker of angiogenesis (VEGF, CD31) in the tumor microenvironment and the intratumoral accumulation of Tregs have been shown to positively correlate [71, 72]. Hence, a bidirectional link seems to exist between Tregs and tumor angiogenesis.

The role of VEGF in the expansion of Tregs within the tumor microenvironment may explain the observation that some antiangiogenic molecules decrease the number of both circularing and intratumoral Tregs in cancer patients [73–76]. Intriguingly, although all these antiangiogenic molecules efficiently inhibit the VEGF/VEGFR axis, sunitinib appears to be more potent at decreasing the number of Tregs than many other agents including sorafenib, whose Treg-modulatory potential remains matter of debate [75, 77–80]. Intriguingly, it has been shown that Tregs infiltrating breast carcinoma lesions are capable of promoting the metastatic dissemination of mammary carcinoma cells as they express on their surface the pro-metastatic TNF superfamily member RANKL [40].

Specificity of Tregs

The actual specificity of Tregs involved in antitumor responses remains poorly unknown. As most cancer antigens are self-antigens, antigen-specific Tregs are likely to exist. However, a few antigen-specific Tregs have been identified so far, most likely due to hitherto inadequate technical tools (e.g., MHC class II tetramer assays). Studies in mice have demonstrated that antigen-specific Tregs show a superior immunosuppressive activity as compared to non-specific Tregs [81, 82]. Wang et al. were the first to isolate human tumor-associated antigen (TAA)-specific Tregs, in particular Tregs that were specific for the cancer/testis antigen LAGE1 among the TILs of melanoma patients [83]. One year later, the same authors identified Tregs that specifically recognized the ARTC-1 peptide [84]. Another group has successfully detected–again in melanoma patients–circulating Tregs that were specific for multiple distinct melanoma-associated antigens including the glycoprotein 100 (gp100), tyrosinase-related protein 1 (TRP1), the cancer/testis antigen NY-ESO-1 and the anti-apoptotic protein survivin [85]. A study performed in colorectal carcinoma patients has demonstrated the presence of TAA-specific Tregs and suggests that these cells may exert immunosuppressive activity over Teffs of the same specificity [86]. In line with this model, in human papillomavirus $(HPV)^+$ cervical carcinoma patients, Tregs specific for the HPV proteins E6 and E7 have been detected.

Interestingly, anticancer vaccines have also been shown to promote the development of antigen-specific Tregs. In mice, it has been reported that the expansion of iTregs upon vaccination dampens antitumor Th1 responses [87]. In human, the group of Peter Van der Bruggen has demonstrated that the $CD4^+$ T-cell response of melanoma patients to a MAGE-A3-derived peptide vaccine involves regulatory T cells [88]. In individuals vaccinated with E6/E7-derived long peptides, immunosuppressive cells has been shown to expand [89], correlating to the development of resistance against an anti-HPV therapeutic vaccine [90]. A better identification of the specificity of Tregs in cancer patients will help to design TAA-based vaccine that induce optimal Teff responses while failing to activate Tregs to significant extents.

Clinical Significance of Intratumoral Tregs

Tumor infiltration by Tregs, near-to-invariably detected by the immunohistochemical detection of $FOXP3^+$ lymphocytes, has been associated with poor prognosis in cohorts of patients affected by multiple distinct neoplasms, including Hodgkin lymphoma, melanoma as well as breast, gastric and ovarian carcinoma [29, 91–94]. In line with these results, it has been shown that clinical response to chemotherapy is often associated with a reduction in TITregs and the recruitment of intratumoral $CD8^+$ T cells [95]. Moreover, imatinib mesylate (a small agent initially developed as a BCR-ABL-specific inhibitor) has been demonstrated to potentiate antitumor T-cell responses in patients affected by gastrointestinal stromal tumors by inhibiting IDO expression (by tumor cells) and hence promoting the apoptotic demise of Tregs [96].

On the contrary, we were the first to report that intratumoral Treg infiltration in head and neck cancer lesions also correlates in multivariate analyses with a better locoregional control of the tumor [91, 97]. Such a good prognostic value of Tregs in head and neck cancer has subsequently been confirmed by others [98, 99] and extended to additional solid neoplasms including colorectal carcinoma [100, 101] and bladder cancer [102]. In some tumor types including hepatocellular carcinoma and breast cancer, high levels of intratumoral Tregs have been clearly associated with bad prognosis, whereas in other types such as colorectal cancer, head and neck carcinoma and lymphoma, robust tumor infiltration by Tregs frequently correlates with improved disease outcome [103, 104].

Various factors may explain these seemingly contradictory observations. First, intratumoral Tregs have been shown to display a consistent degree of functional heterogeneity, which may obviously influence prognosis and/or

response to therapy. For instance, a subpopulation of Tregs expressing IL-17 has recently been detected within colorectal cancer lesions, and these cells have been shown to potently suppress T-cell activation while promoting the production of pro-inflammatory cytokines [105]. Second, distinct subsets of Tregs appear to specifically control limited subsets of Teffs. Thus, a subpopulation of intratumoral Tregs that express the transcription factor T-bet (a critical regulator of the Th1 differentiation program) and (consequently) CXCR3, has been shown to preferentially inhibit $CXCR3^+$ Th1 effector cells [106]. Third, specific subsets of Tregs, in particular those expressing activation markers such as CCR4, may exert more robust immunosuppressive functions, and hence be more closely associated with prognosis, than the entire population of Tregs [107]. Fourth, some oncogenic programs, such as those underlying (at least some instances of) lymphoma [108], head and neck cancer [109, 110], gastric cancer [111] and colorectal carcinoma [112], involve a prominent pro-inflammatory component. In this setting, Treg-mediated immunosuppression may exert *bona fide* antitumor functions. Finally, the intratumoral accumulation of Tregs is often associated with that of other immune cells, and hence may reflect the overall level of tumor infiltration by immune cells, including Teffs [113, 114]. In line with this notion, the ratio between tumor-infiltrating $CD8^+$ Teff and intratumoral Tregs appears to convey a more robust prognostic/predictive information than either parameter alone. In particular, a high $CD8^+$ Teff/Treg ratio has been associated with favorable disease outcome in ovarian and hepatocellular carcinoma patients [93, 115] and with a poor prognosis in subjects affected by head and neck cancer [116]. Of note, while in some studies, the prognostic value of Tregs seems to be influenced by tumor stage [29, 117], results from a recent meta-analysis indicate that tumor stage does not account for the variations of FOXP3 prognostic performance [104].

Concluding Remarks

Tregs are a major component of the tumor stroma and appear to influence not only immune cells but also malignant cells and other stromal cells, as they promote angiogenesis. Various subpopulations of TITregs have been shown to exert relatively distinct functions and hence to be associated with different clinical significance. A more refined phenotypic and functional definition of the Treg subsets as well as a better understanding of their role in the regulation of immune responses is a major challenge for future years. Since in some clinical scenarios TITregs appear to be associated with an improved disease outcome, current strategies aimed at unspecifically inhibiting Tregs will have to be substituted with more selective approaches, involving (i) the targeting of specific Treg subsets and/or (ii) the inhibition of Tregs in selected subgroups of cancer patients.

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