Comprehensive analysis of current approaches to inhibit regulatory T cells in cancer

Helene Pere,1,2 Corinne Tanchot,1 Jagadeesh Bayry,3 Magali Terme,1 Julien Taieb,1 Cecile Badoual,1,4 Olivier Adotevi,5 Nathalie Merillon,1 Elie Marcheteau,1 Véronique Quillien,6 Claire Banissi,7 Alain Carpentier,7 Federico Sandoval,1 Mevyn Nizard,1 Françoise Quintin-Colonna,1,8 Guido Kroemer,9,13 Wolf H. Fridman,3,14 Laurence Zitvogel,15,16 Stéphane Oudard1,17 and Eric Tartour1,14,*

1INSERM U970 PARCC (Paris Cardiovascular Research Center); Université Paris Descartes; Sorbonne Paris Cité; Paris, France; 2Hôpital Européen Georges Pompidou; Service de Microbiologie; Paris, France; 3INSERM U872; Centre de Recherche des Cordeliers; Université Pierre et Marie Curie; Université Paris Descartes; Paris, France; 4Hôpital Européen Georges Pompidou; AP-HP; Service d'Anatomie Pathologique; Paris, France; 5CHU Besançon; UMR 645 INSERM; Université de Franche-Comté; Besançon, France; 6Centre Eugène Marquis; Département de Biologie Clinique; UMR 6061; Rennes, France; 7Laboratoire de Recherches Biochirurgicales; Université Paris Descartes; Paris, France; 8École Nationale Vétérinaire d'Alfort; Maisons Alfort, France; 9INSERM U848; Villejuif, France; 10Centre de Recherche des Cordeliers; Paris, France; 11Pôle de Biologie; Hôpital Européen Georges Pompidou; AP-HP; Paris, France; 12Université Paris Descartes; Sorbonne Paris Cité; Faculté de Médecine; Paris, France; 13Hôpital Européen Georges Pompidou; Service d’Immunologie Biologique; Paris, France; 14INSERM U1015; Institut Gustave Roussy; Villejuif, France; 15Center of Clinical Investigation CBTS05; Villejuif, France; 16Hôpital Européen Georges Pompidou; Service d’Oncologie Médicale; Paris, France

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Phenotype of Regulatory T Cells

In 1982, Sakaguchi and colleagues and, in the 1990s, Powrie and Mason identified two surface markers, CD5 (Lyt-1) and CD45RB, which are both expressed at low levels on suppressive T cells.3 In 1995, the α chain of IL-2R (CD25) was reported to be constitutively and highly expressed by suppressive CD4+ T cells.4 Suppressive CD4 T cells were renamed “regulatory T cells” (Treg) because of the skepticism in relation to the first “suppressive” T cell population described by Gershon.

The real evolution in the phenotype determination of Treg came in 2003 with the identification of a new gene called foxp3.3 This gene codes for a transcription factor expressed in the nucleus of Tregs, Foxp3 (forkhead box P3). In humans, absence of this gene is associated with a generalized autoimmune disorder called IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked), which comprises diabetes, eczema and food allergies. In a “scurfy” mouse model, a spontaneous mutation in foxp3 gene is linked to a very similar disorder.

Foxp3 is a specific marker of Treg in mice but, in humans, it can be transiently expressed by activated T cells.6 In humans, Foxp3+ regulatory and activated T cells can be distinguished by the differential expression of CD127 (which is present at high levels in activated T cells and at low levels in Treg) and by the methylation status of the transcription factor Foxp3 detected in the DNA encoding Foxp3 in Treg but not in activated T cells).7 Tregs also express effector surface molecules such as CTLA4, LAG3, CD39 or CD73 and co-stimulation molecules, CD28, CD80/86, CD40, OX40 or 4–1BB, which appear to be important for their peripheral maintenance and functions.9 Integrins and chemokine receptors such as CD62L, CCR4, CCR7 and CCR8 are responsible for Treg homing and migration to lymph...
nodes, skin and inflammatory sites and tumor tissues in response to various molecules or chemokines. Other regulatory CD4+ T cell (Tr1, TH3) and regulatory CD8+ T cell populations have also been described, but this review will only focus on the Foxp3+CD4+ Treg.

**Origin of Foxp3+Treg**

Two main populations of Foxp3+Treg have been described: a “natural” (n) population, which differentiates within the thymus during T cell ontogenesis, and another “induced” (i) population, which arises in the periphery from conventional CD4+ T cells. Conversion of CD4+ T cells into iTreg occurs in response to various mechanisms, for example, suboptimal antigenic stimulation in the presence of TGFβ. Dendritic cells (DC) blocked at an immature stage in the cancer microenvironment, secondary to the presence of inhibitors (IL-6, IL-10, VEGF, PGE2…) express membrane TGFβ and promote Treg differentiation.

iTreg differentiation from peripheral naïve CD4+ T cells in periphery was initially described to be strictly opposed to Th1, Th2 or Th17 differentiation. Nevertheless, recent studies report that differentiation to a particular phenotype is not definitive and that iTreg present a real plasticity. For example, it has been shown that, regulatory T cells can be converted to Th17 cells in presence of IL-6 or IL-21 and TGFβ.

**Mechanisms of Action of Treg**

iTreg and nTreg share various ways to inhibit immune response (Fig. 1). Both populations use cytokine-dependent mechanisms and are able to secrete immunosuppressive cytokines (IL-10, TGFβ) or IL-35 (at least in mice), but also immunosuppressive metabolites such as adenosine.

Treg may also lyse effector cells by means of granzyme A and B or disrupt the metabolism of effector cells by causing their IL-2 deprivation.

nTreg also use contact-dependent mechanisms. They are able to inhibit DC maturation by means of the interaction of CTLA-4 with CD80/CD86 on DC, which delivers a negative signal to DC preventing priming of anti-tumor responses. Induction of an immunosuppressive enzyme, IDO (indoleamine 2,3 dioxygenase), by CTLA-4 may also participate in inhibition of effector T cells. Other surface molecules (Lag3, CD39, Nrp, galectin…) expressed by Treg may also contribute to their suppressive activity.

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**Figure 1.** Mechanisms of regulatory T cell inhibition (A) Secretion of immunosuppressive cytokines (IL-10, IL-35 and TGFβ) inhibiting effector T cells. (B) Cytolysis of effector T cells by production of Granzyme A and/or B. (C) Metabolic disruption of effector T cells by IL-2 deprivation. IL-2 is captured by CD25 expressed by Treg. (D) Inhibition of DC maturation by contact-dependent mechanisms (CTLA-4, CD80-CD86 interaction, Lag3/CMHII interaction) and effector function by IDO secretion.
Given these immunosuppressive properties, Treg are therefore important for peripheral tolerance and confer protection against autoimmunity and inflammation.

**Treg and Cancer**

Most tumor-associated antigens are self-proteins, which elicit weak natural or induced T cell responses after immunotherapy. It has been demonstrated that Treg are able to recognize tumor-associated self-antigens and control T cell responses against various cancer antigens, which may explain the failure of many cancer vaccines. For example, tyrosinase and NY-ESO1-specific CD4+ T cells can expand and become detectable by in vitro antigenic stimulation of peripheral CD4+ T cells only after depletion of Treg. In addition, therapeutic cancer vaccines could induce tumor-specific Treg that blunt the expansion and function of anti-tumor T cells. In line with these results, Treg depletion or blockade has been shown to enhance tumor immunity elicited by vaccination.

Treg are recruited to the tumor bed mainly but not exclusively via chemokine gradients, mainly the CCL22/CCR4 axis, as many tumor cells or myeloid intratumor cells produce CCL22. Hypoxia also attracts Treg into the tumor, mostly through induction of the CCL28 chemokine. However, Treg are not always “tumor bodyguards.” Treg infiltration has also been correlated with good prognosis in hematological malignancies and in some solid tumors such as head and neck or colon cancer that are often associated with chronic inflammation. This may be explained by the fact that Treg negatively control inflammation and that inflammation plays an important role in disease progression in these cancers.

**Why and When Do We Need to Inhibit Treg in Cancer?**

Tumor-associated antigens are often self-antigens, which elicit weak immune responses partly due to the presence of antigen-specific Treg. This may explain why elimination of Treg improves induction of CD8+ T cells response for priming of anti-tumor T cells.
In addition, it has been observed that some immunostimulatory molecules such as IL-2 and IFNγ used in immunotherapy may be linked to immunosuppressive activities partly due to the concomitant induction of Treg via Stat5 activation.\textsuperscript{31,32} Helper peptides derived from tumor antigens may also increase Treg, which partially inhibit anti-tumor CD8\textsuperscript{+} T cell induction.\textsuperscript{33} Anticancer immunotherapies could therefore be improved by concomitant Treg blockade. Importantly, transient depletion of Treg during priming of anti-tumor immunity rather than chronic depletion of Treg should be considered to avoid the development of autoimmune side effects.

Many strategies are currently used to manipulate Treg, including Treg depletion, inhibition of Treg function or blockade of Treg trafficking into lymph nodes or tumors. We will briefly describe these approaches by emphasizing their potential limitations.

**Strategies Currently Used to Block or Inhibit Treg**

**Treg depletion.** Chemotherapy. Some chemotherapies may lead to immunogenic cell death resulting in activation of DC and priming of anti-tumor immune responses.\textsuperscript{34} This promotion of DC maturation might also explain the capacity of some chemotherapies to reduce Treg. In addition, as a higher frequency of proliferating cells is observed in Treg compared with the non-Treg compartment, chemotherapy, which mostly destroys proliferating cells, may tilt the balance from Treg toward effector T cells. Cyclophosphamide (CTX) is the leading product of this therapeutic class. Reversal of immunological tolerance by CTX via inhibition of suppressor cells was reported more than 35 years ago.\textsuperscript{35} Selective depletion of Treg induced by CTX or other chemotherapeutic drugs such as paclitaxel requires the use of these agents at low, so-called metronomic doses.\textsuperscript{36,37} Some studies in humans have shown improvement of T cell effector function associated with a reduction in Treg numbers after low dose CTX administration.\textsuperscript{38} However, “clinical benefit/toxicity (deletion of effector T cells)” therapeutic index for this kind of drug is low and no consensus has been reached concerning the use of metronomic doses.\textsuperscript{38} CD25 antibody and Denileukin diftitox (ONTAK). The implication of Treg in tumor immunity was initially studied by systemic depletion of CD25\textsuperscript{+} T cells. Studies in mice demonstrated that in vivo administration of CD25-specific antibody (PC61) suppressed growth of progressively growing tumors.\textsuperscript{39} However CD25 is also expressed by activated effector T cells, complicating the CD25-based Treg targeting strategy. In this regard, it was shown that while administration of anti-CD25 mAb before tumor inoculation triggered effective antitumor responses, anti-CD25 mAb treatment after tumor inoculation was much less effective to eradicate tumors.\textsuperscript{39}

In humans, an anti-CD25 mAb, daclizumab, has been used to deplete Treg with contradictory results.\textsuperscript{38,40} More recently, the recombinant IL-2 diphtheria toxin conjugate called denileukin diftitox (ONTAK) was developed to target T cells with high CD25 expression. Upon internalization, diphtheria toxin irreversibly inhibits protein synthesis, ultimately triggering cell death. Diftitox administration combined with vaccine has demonstrated some efficacy in renal cell carcinoma (RCC) and melanoma patients.\textsuperscript{41} However, recent studies performed in melanoma patients reported neither a reduction in peripheral Treg numbers nor any favorable clinical improvement after diftitox treatment.\textsuperscript{8} This therapeutic failure might be explained by the presence of CD25\textsuperscript{+}Foxp3\textsuperscript{+} T cells, which cannot be depleted by diftitox.

**Targeting Treg function.** One possible strategy to avoid Treg depletion is to use antibodies that target molecules constitutively expressed by Treg leading to their functional inhibition.

**Anti-CTLA-4.** CTLA-4 is expressed on both regulatory and activated T cells. Early studies have reported that CTLA4 blockade resulted in improved tumor immunity and tumor regression. However, a recent study elegantly demonstrated that blockade of CTLA-4 specifically on Treg failed to enhance anti tumor responses.\textsuperscript{42} In contrast, concomitant blockade on both effector T cells and Treg led to a synergistic effect with maximal anti-tumor activity.\textsuperscript{42} Surprisingly, anti-CTLA-4 recently approved for metastatic melanoma patients, induced activated effector T cells, Foxp3\textsuperscript{+} Treg as well as IL-10-producing Treg.\textsuperscript{43} Despite this Treg expansion, anti-CTLA-4 mAb administration resulted in severe autoimmunity. This might be explained by the fact that conventional T cells become resistant to the inhibitory effects of Treg during therapy with anti-CTLA-4 mAb.\textsuperscript{44}

**Anti-GITR.** Like CTLA-4, GITR is constitutively expressed by Treg, but it is also detected, albeit at lower levels, on CD4\textsuperscript{+} and CD8\textsuperscript{+} effector T cells. Stimulation by agonistic antibodies to either GITR or GITR ligand has a dual effect leading to suppression of Treg activity (at least in mice) and enhanced proliferation of effector T cells and possible resistance to Treg-mediated suppression. Administration of GITR mAb protected mice from B16 tumor challenge,\textsuperscript{45} and induced tumor regression in mice bearing methylcholanthrene-induced fibrosarcoma.\textsuperscript{46} The tumors were infiltrated by large numbers of effector T cells, and an increase in INFγ was observed. Importantly, anti-GITR mAb therapy was more effective in mice with established tumors than in prophylactic settings.\textsuperscript{46} A study performed on GITR-knockout mice revealed that reversal of suppression by GITR signaling may be attributed to the costimulatory activity of GITR on responder CD4\textsuperscript{+}CD25\textsuperscript{+} T cells, which made them resistant to Treg suppression.\textsuperscript{47} This indicates that anti-GITR stimulation enhances the activity and expansion of antigen-primed effector T cells rather than their generation. Altogether, a direct role of GITR mAb on Treg cell functions remains elusive.

**Anti-OX40.** OX40, a costimulatory molecule of the TNF receptor family, is constitutively expressed on Treg and transiently expressed on activated T cells. An early study showed that activation of OX40 signaling by an agonistic anti-OX40 mAb was able to inhibit the suppressive activity of Treg.\textsuperscript{48} A recent murine study demonstrated that intratumoral injection of anti-OX40 mAb induced strong inhibition of tumor growth.\textsuperscript{49} The authors demonstrated that activation of OX40 signaling has a dual role, inhibiting Treg suppression while enhancing effector T cells functions. Further studies are required before translating agonistic anti-OX40 mAb strategies to patients especially in combination with conventional therapies.\textsuperscript{50}
Taken together, targeting CTLA-4, GITR or OX40 may have a huge therapeutic potential, as recently demonstrated for the anti-CTLA4 mAb (ipilimumab). However, discrepancies exist in the literature as to whether Treg functions are indeed affected by such regimens. Moreover, non-specific co-stimulatory effects of these mAbs on effectors CD4 and CD8 T cells may lead to severe systemic inflammation (induction of a cytokine storm) and multi organ-specific autoimmunity.\(^5\)

TLR ligands, adenosine inhibitors and peptide inhibitors of Foxp3. Treg express various TLRs and notably high levels of TLR4, TLR5, TLR7 and TLR8;\(^2\) TLR 8 activation by its natural or synthetic ligands has been shown to inhibit Treg function and enhances in vivo tumor immunity.\(^53\) Appropriate TLR stimulation might therefore be an important tool for vaccination strategies, since it could inhibit Treg-mediated tolerance.\(^64\) However, this area of research requires further investigation as many counteracting effects might emerge due to the expression of TLRs on almost all murine and human normal and tumor cells.\(^55\) In addition, TLR ligands could also induce IL-10-producing Treg. This unfavorable bystander effect could be reversed by blocking p38 MAPK signaling.\(^56\)

Treg produce adenosine via catabolism of adenosine nucleotides (ATP, ADP and AMP) by extracellular ectonucleotidases, CD39 and CD73. Adenosine is a major immunosuppressive factor that may participate in the immunosuppressive activity of Foxp3\(^+\) T cells.\(^16\) Low molecular weight inhibitors and adenosine receptor antagonists, some of which are already used in clinic settings for other indications, are available to block adenosine-mediated immune suppression.\(^57\) Inhibition of CD39 with enzymatic inhibitors blocks Treg function and improve the effects of chemotherapy.\(^58\)

A peptide inhibitor of Foxp3 (P60) impairs Treg activity and improves vaccine efficacy in mice.\(^59\) P60 administration to newborn mice but not in adult mice induced a lymphoproliferative autoimmune syndrome resembling the reported pathology in scurfy mice lacking functional Foxp3.\(^59\)

Disrupting lymph node and tumor homing of Treg. Another strategy to control Treg function is to target chemokine/chemokine receptor molecules (i.e. CCL17/CCL22-CCR4 axis) that are involved in Treg trafficking. It has been shown that Treg preferentially express CCR4 compared with conventional T cells, both in mice and humans.\(^10,60\) CCR4-expressing Treg mainly represent activated Treg with potent suppressive activity. The binding of CCL17 and CCL22 produced by DC in the lymph node to their CCR4 receptor guides CCR4-expressing Treg toward DC. This interaction can suppress DC-mediated immune responses by inhibiting DC maturation and expression of costimulatory molecules required for effector T cell activation, as well as by inhibiting stable contact between DC and effector cells.\(^61\) The role of CCR4 in the migration of Treg toward lymph nodes is also reinforced by studies showing that CCR4-deficient Tregs fail to traffic to lymph nodes to inhibit pathogenic T cells.\(^52\)

Tumor cells and their microenvironment also attract Treg by the secretion of CCL22,\(^22\) and a correlation has been reported between the presence of tumor-infiltrating Treg and CCL22 in breast cancer.\(^23\) In a murine model, it has been shown that monoclonal antibodies specific for CCL22 significantly reduce the migration of Treg into ovarian tumors.\(^22\)

Recently, small molecule antagonists to CCR4, designed in silico, have been shown to prevent the interaction of CCL22/CCL17 with their receptor. In vitro experiments in human showed that these CCR4 antagonists inhibit the recruitment of Treg mediated by CCL22 and CCL17. Preclinical studies showed that, when administered in combination with vaccines, CCR4 antagonists increased CD4\(^+\) T cell and humoral responses directed against foreign antigens.\(^63,64\) We found that immunization of mice against relevant tumor-associated self antigens (Her2/neu, gp100) failed to reverse the tolerance controlled by Treg. In contrast, the same vaccines combined with a CCR4 antagonist led to the induction of effector CD8\(^+\) T cells and partial tumor protection.\(^21\) The CCR4 antagonist was more efficient than CTX to elicit anti-self CD8\(^+\) T cells. One of the main advantages of this CCR4 antagonist is its short lifespan (~24 h)\(^65\) allowing transient inhibition of Treg only during the priming phase and avoiding the potential autoimmune complications caused by long-term blockade or depletion of Treg by mAbs (e.g., anti-CD25, anti-OX40, anti-GITR) with longer half-lives (2–3 weeks).\(^65\) We found that administration of the CCR4 antagonist did not lead to induction of biological markers of autoimmunity.\(^21\) In addition, it appears that Treg expressing CCR4 mainly represent activated Treg with potent suppressive activity.

Other chemokine receptors such as CCR7 and CCR5 may also play a role in Treg migration. For example, the CCL5/CCR5 interaction has been shown to be crucial for Treg attraction in pancreatic adenocarcinoma.\(^66\) Disrupting this interaction by systemic administration of a CCR5 inhibitor reduced Treg migration into the tumor and led to significant tumor reduction.\(^66\) In some models, CCL5 blockade improved the efficacy of immunochemothotherapy.\(^57\) However, it should be noted that, chemokine/chemokine receptor molecules especially CCL5/CCR5 and CCL20-CCL21/CCR7 may also be involved in trafficking of effector T cells, and disruption of these pathways might therefore be deleterious to killing of tumor cells.

Anti-angiogenic molecules and tyrosine kinase inhibitors. Accumulating evidence strongly suggests that angiogenesis inhibition overcomes various immunosuppressive networks including Treg.\(^68\) In mice and human, it has been demonstrated that sunitinib, an inhibitor of tyrosine kinases involved in angiogenesis (VEGF-R, PDGF-R, FGF-R\(\ldots\)), reduced the percentage and absolute number of Treg.\(^69-71\) which have been shown to be increased in many tumors.\(^25,27\) We more thoroughly analyzed this decrease in Foxp3\(^+\) Tregs in humans and observed a progressive reduction in circulating Foxp3\(^+\) Tregs after each cycle of sunitinib therapy. This reduction became statistically significant after the second cycle of therapy. A significant (at least 20%) reduction in the absolute number of Foxp3\(^+\) Treg occurred in 32% and 40% of patients after the first and second cycle respectively, and in 70% of patients after the third cycle. We found a correlation between the number of Foxp3\(^+\) Treg at baseline and the changes in this population during sunitinib-based therapy. Patients with baseline Treg levels above the median value were more likely to experience a decrease in this population
after the second or third cycles of sunitinib-based therapy than patients with low baseline Treg levels. Sunitinib appears to have an indirect effect on Treg, as sunitinib did not inhibit in vitro Treg expansion even over a 14 d coculture period. The impact of anti-angiogenic molecules on Treg has been mainly demonstrated with sunitinib. The ability of other molecules (sorafenib, bevacizumab…) to mimic this effect is currently debated.27,73

Two mechanisms have been proposed to explain the impact of anti-angiogenic molecules on Treg (1) at an immature stage, DC in the presence of TGFβ are able to induce Foxp3+/CD4+/CD25hi Treg. Pioneering studies showed that VEGF inhibits DC maturation via a nuclear factor κB (NFκB)-dependent pathway mediated by VEGFR-1 signaling37. (2) Since Treg express VEGFR2, a modulation of Treg activity by VEGF could also be hypothesized.76

Other immunosuppressive cell populations, especially MDSC (myeloid-derived suppressor cells), could also be blocked by anti-angiogenic therapy.68

This role of anti-angiogenic molecules on reversal of immunosuppression in cancer may explain the synergy observed in preclinical models between anti-angiogenic molecules and immunotherapy. Studies are ongoing to address the ability of these molecules to potentiate immunotherapy in human clinical trials.77

Other tyrosine kinase inhibitors (imatinib mesylate, dasatinib, temozolomide) have demonstrated an impact on the decrease of Treg number or functions.78,79 An elegant study in a mouse model of gastrointestinal sarcoma (GIST) and in human GISTs showed that imatinib mesylate induced Treg Suppression (T(reg) cell) apoptosis within the tumor by reducing tumor-cell expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase.80 Reduction of Treg numbers unleashes NK cell functions and contribute to NKP30-dependent antitumor effects in GIST.80

Conclusions and Perspectives

Treg and activated T cells share several common features and surface markers, which explain the weak selectivity of several drug candidates to specifically inhibit Treg. New strategies are designed to inhibit Treg function rather than eliminate Treg, in order to improve their specificity. This field has recently benefited from the finding that many drugs (chemotherapies, anti-angiogenic molecules, tyrosine kinase inhibitors…) exhibit off-target effects and inhibit Treg, thereby accelerating their evaluation in clinical trials either as monotherapies or in combination with immunotherapy. In terms of the clinical indications of these molecules, elimination or inhibition of Treg might be particularly useful in the context of therapeutic vaccination against tumor-associated antigens. For this kind of indication, transient inhibition of Treg during the short window of immune priming (few days) rather than long-term blockade might be particularly appropriate to minimize organ-specific or generalized autoimmunity side effects.

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