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Review

Tumor-infiltrating regulatory T cells as targets of cancer immunotherapy

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SUMMARY

Regulatory T cells (Tregs) are abundant in tumor tissues, raising a question of whether immunosuppressive tumor-infiltrating Tregs (TI-Tregs) can be selectively depleted or functionally attenuated to evoke effective anti-tumor immune responses by conventional T cells (Tconvs), without perturbing Treg-dependent immune homeostasis in healthy organs and causing autoimmunity. Here, we review current cancer immunotherapy strategies, including immune checkpoint blockade (ICB) antibodies against CTLA-4 and PD-1 and discuss their effects on TI-Tregs. We also discuss approaches that exploit differentially regulated molecules on the cell surface (e.g., CTLA-4) and intracellularly (e.g., T cell receptor signaling molecules) between TI-Tregs and Tconvs as well as their dependence on cytokines (e.g., IL-2) and metabolites (e.g., lactate). We envisage that targeting TI-Tregs could be effective as a monotherapy and/or when combined with ICB antibodies.

INTRODUCTION

Cancer immunology duly deserves credit for spawning several anti-tumor drugs and cell therapies that have improved and saved the lives of patients with terminal cancers. The most common treatment strategy is blockade of co-inhibitory molecules, also known as immune checkpoints (ICs), on cancer-killing T cells. Cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death 1 (PD-1) and its ligand PD-L1 are key IC targets. Their expression in T cells increase with activation. As T cells require T cell receptor (TCR) activation and CD28 co-activation to become effector T cells, CTLA-4 negatively regulates this process by competing with CD28 for the co-stimulatory ligands CD80 and CD86. PD-1 binds to its ligands, PD-L1 and PD-L2, to transmit signals that inhibit TCR signaling. These effects of CTLA-4 and PD-1 could compel cancer-killing T cells to withdraw into inactive states of dormancy or exhaustion.[1] Hence, antibodies that antagonize CTLA-4 and PD-1 are used to revive them and sustain their anti-tumor responses. These immune checkpoint blockade (ICB) antibodies have, thus far, achieved decent success. Unfortunately, however, 60%–70% of patients fail or only partially respond to these therapies.[2] Patients also find themselves with immune-related adverse events (irAEs), including autoimmune or immunopathological diseases.[3] Some organ-specific irAEs may be due to inadvertent activation of T cells that react with self-antigens (e.g., heart and skin)[4,5] or commensal microbes (e.g., colon).[6] It is, therefore, imperative to ascertain the reasons behind these shortcomings of ICBs and design novel therapies with better anti-tumor efficacies without over-exposing patients to irAEs.

$CD4^+$ regulatory T cells (Tregs), which constitutively express CD25 and CTLA-4 on the cell surface and the transcription factor Forkhead box P3 (Foxp3) in the nucleus, play key roles in preventing autoimmune and inflammatory diseases. However, their accumulation in tumors suppresses anti-tumor immunity. Animal studies have, indeed, demonstrated that systemic Treg depletion can promote anti-tumor immunity and bring about tumor rejection, but elicits various autoimmune diseases.[7] Mice deficient in CTLA-4 or PD-1 suffer autoimmunity; the effect of the former severe and fatal from young age, while the latter is relatively mild with late onset that affects only certain tissues depending on genetic background.[8–12] Hence, efforts have been devoted to determine the responses of Tregs and $Foxp3^-$ conventional T cells (Tconvs) to anti-CTLA-4 and anti-PD-1 antibodies as both populations highly express CTLA-4 and PD-1 in cancer tissues.[13–15] Given that CTLA-4 is key to Treg immunosuppressive function and PD-1 regulates Treg activity, CTLA-4 or PD-1 blockade on tumor-infiltrating Tregs (TI-Tregs) may have contrasting effects (i.e., tumor suppressing or promoting) that require further investigation.[14,16,17]

Many studies have attempted to selectively deplete only TI-Tregs in tumors without affecting Tregs in healthy tissues, in order to evoke only tumor immunity but not deleterious autoimmunity. Potential TI-Treg targets on the cell surface include cytokine and chemokine receptors, such as CD25 and CCR8, respectively. Intracellular molecules that govern TCR signaling and metabolic pathways are also viable candidates. We envision that combining TI-Treg depletion with ICB antibodies could induce potent anti-tumor immunity and negate prolonged treatments to minimize irAEs.

In this review, we first discuss the mechanisms of Treg-mediated suppression of anti-tumor immunity, particularly through CTLA-4 and PD-1/PD-L1, and the effects that ICB antibodies may have on these mechanisms. We then review recent progress and discuss future prospects of targeting TI-Tregs by exploiting differential properties between Tregs and Tconvs and characteristics unique to TI-Tregs.

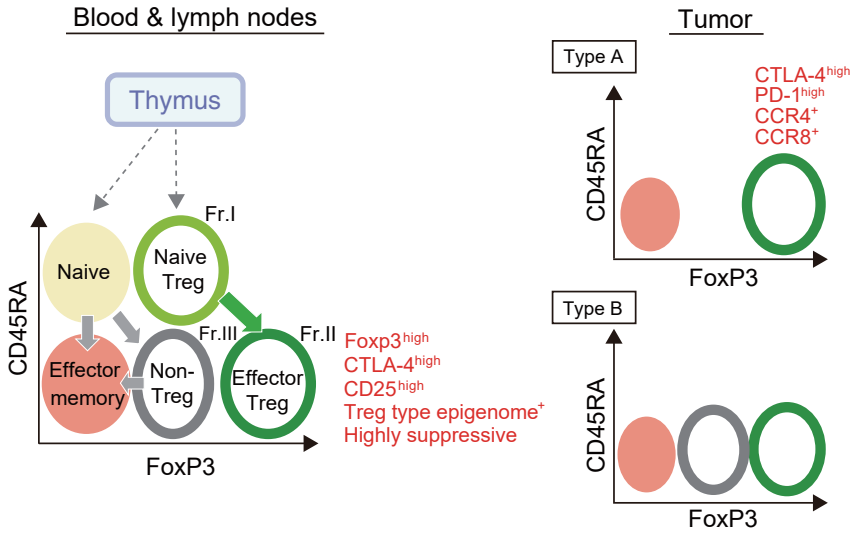

Figure 1. Functional classification of human Tregs in the blood and tumors

Among $CD4^+$ T cells in humans, $FoxP3^+$ T cells contain three functionally distinct fractions: naive Tregs (Fr. I) and effector Tregs (eTreg; Fr. II) cells with Treg-characteristic suppressive function and activated non-Tregs (Fr. III) without suppression function. The majority of cancers are infiltrated predominantly by effector Tregs (type A), whereas certain cancers are infiltrated by both effector Tregs and non-Tregs (type B). Tumor-infiltrating effector Tregs predominantly express various cell surface molecules including CTLA-4, CCR4, CCR8, and PD-1.

PROTECTIVE ROLE OF TREGS AGAINST AUTOIMMUNITY AND ITS IMPLICATIONS IN CANCER IMMUNITY

Treg development and infiltration into tumor tissues

Naturally occurring $FoxP3^+CD25^+CTLA\text{-}4^+CD4^+$ Tregs (nTregs) are indispensable for immunological self-tolerance. This is exemplified by spontaneous development of autoimmune diseases, allergy, and inflammatory bowel disease in humans and mice with congenital Treg deficiency due to FoxP3 gene mutations.[18–20] The majority of nTregs are produced in the thymus as a functionally distinct population (thymus-derived Tregs [tTregs]). Immunosuppressive Tregs can also differentiate in the periphery from Tconvs as peripherally derived Tregs (pTregs), especially in the intestinal mucosa where pTreg induction is crucial for immune tolerance to commensal microbes and food antigens.[21–24]

In the thymus, developing T cells with intermediate TCR affinity for self-peptide/MHC ligands commit to the Treg lineage, while T cells with low TCR affinity differentiate into naive Tconvs and those with high TCR affinity are deleted.[25] Tregs are thus able to recognize self-antigens, which may also be tumor-associated antigens.[26–28] Because of their self-reactive TCR repertoire, tTregs in the periphery may be under constant stimulation by self-antigens, which could account for their highly activated phenotype and proliferative behavior even during steady state.[29,30] Similarly, tTregs that recognize tumor-associated quasi-self-antigens may readily expand clonally and accumulate in tumors.

IL-2 is central to Treg and Tconv survival. In Tregs, Foxp3 has dual roles in controlling dependence on IL-2. On one hand, it prevents IL-2 transcription and, on the other, it promotes CD25 (IL-2Rα) expression.[31,32] Formation of the IL-2 receptor (IL-2R) composed of α, β, and γ chains enhances affinity for IL-2 by almost a hundred-fold compared with IL-2R with only β and γ chains, which is mostly the variant expressed by Tconvs.[33] With CD25, Tregs could thus outcompete Tconvs for IL-2. Without CD25, Tregs experience a developmental defect in the thymus and are prone to apoptosis in the periphery.[34] This explains the high reliance of tTregs on IL-2, as shown by transient IL-2 neutralization reducing only Treg, not Tconv, numbers.[35]

Tregs in the periphery are functionally adaptive. Tregs in an inflammation site may share a similar pattern of transcription factors and chemokine receptors as Tconvs at the site.[36] For example, Tregs in a type 1 inflammation site express T-bet and CXCR3, those in type 2 express GATA-3 and CCR4/CCR8, and those in type 3 express CCR6 and RORγt, consistent with Th1, Th2, and Th17 cells, respectively. They are also present in healthy tissues as tissue-resident effector Tregs that maintain local immune homeostasis.[37] Hence, TI-Tregs in tumors may have various origins, including circulating Tregs, tissue-resident Tregs, and pTregs generated *in situ* in tumors. To specifically deplete TI-Tegs, it is thus necessary to distinguish their phenotypes and functions from Tregs in healthy tissues.

Subpopulations of $Foxp3^+$ T cells in tumor tissues

nTregs in the periphery can be subdivided into naive and effector Tregs. In humans, naive or resting Tregs with the $CD45RA^+CD25^{lo}Foxp3^{lo}$ phenotype (designated Fr. I Tregs) upon antigenic stimulation differentiate into $CD45RA^-CD25^{hi}Foxp3^{hi}$ effector Tregs (Fr. II Tregs), which are $CTLA\text{-}4^{hi}$, proliferative, and strongly suppressive and possess Treg-specific epigenome (Figure 1).[38] Of note, some $CD45RA^-CD4^+$ T cells express low levels of Foxp3 and CD25 but barely exhibit suppressive activity and instead produce proinflammatory cytokines (Fr. III cells). A typical profile of $CD4^+$ T cells in tumors has elevated immunosuppressive Fr. II effector Tregs compared with $CD4^+$ T cells in the blood of both cancer patients and healthy individuals, suggesting that abundant $FoxP3^+$ T cells in tumors is a positive indicator of cancer progression.[13,14,39] It is perplexing, however, that $FoxP3^+$ T cell infiltration correlates with better prognosis in certain cancers such as colorectal and head and neck cancers.[40,41] To address this discrepancy, Saito et al.[41] divided colorectal cancer cases into two groups, one with Fr. II effector Tregs dominant (type A) and the other with Fr. III non-Treg $FoxP3^+$ cells dominant (type B; Figure 1). They then assessed the frequency of $FoxP3^+$ cells among $CD4^+$ T cells in each group and found that it correlated with poor prognosis in the former group and favorable prognosis in the latter. The expansion of Fr. III non-Tregs is facilitated, at least in part, by a particular species of colonic microbes.[41] Thus, proper fractionation of $CD4^+Foxp3^+$ T cells into Fr. I, Fr. II, and Fr. III and calculation of their relative proportions is a better

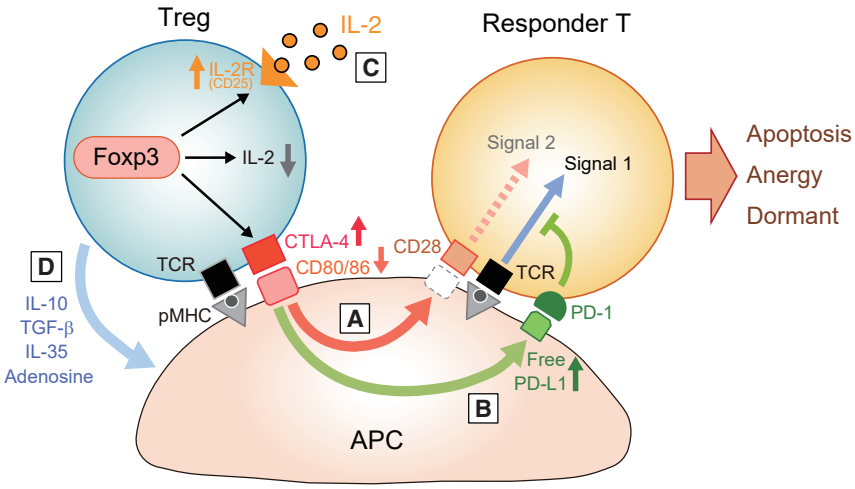

Figure 2. Major immunosuppressive functions of Tregs

(A and B) Cell contact-dependent mechanisms involving the (A) down-regulation of CD80 and CD86 co-stimulatory molecules to deprive Tconvs of CD28 signaling (signal 2) and (B) allowing more free PD-L1 (unbound to CD80) to suppress activated Tconvs through PD-1, which inhibits TCR signaling (signal 1).

(C) Sequestration of IL-2 by Treg-induced expression of CD25 limits the availability of IL-2 for Tconvs.

(D) Secretion of cytokines, such as IL-10, TGF-β, and IL-35, and production of adenosine to regulate APC activity.

way to gauge tumor dependence on TI-Tregs for cancer prognosis. It is also apt to account for changes in $CD8^+$ T cells in tumors that could ensue from TI-Treg suppression. In general, high TI-Treg/$CD8^+$ T cell ratios in tumors correlate with tumor progression and poor survival.[42,43]

Major immunosuppressive functions of Tregs that could suppress tumor immunity

Tregs have multiple immunosuppressive functions. One of the major functions is regulating Tconv access to CD80 and CD86 on APCs via CTLA-4.[16] Mice with Treg-specific deficiency of CTLA-4 had overt inflammatory disease as severe as mice with global deficiency of CTLA-4, indicating that CTLA-4 does not merely regulate CD28 co-stimulation in Tconvs.[8,9,16] Similarly, CTLA-4 haploinsufficiency in humans could cause immune dysregulation from defective Treg-dependent control of CD80 on APCs.[44,45] Constant cycling of CTLA-4 between the membrane and cytosol allows it to capture CD80/86 by trogocytosis and transendocytosis for internalization and degradation.[46,47] With higher levels of CTLA-4, higher rate of mobility and higher expression of the integrin, lymphocyte function-associated antigen-1 (LFA-1), Tregs congregate around APCs to dampen CD80/86 more actively than Tconvs.[48] The increase in CD80/86 on dendritic cells (DCs) in tumors depleted of TI-Tregs bears testament to such immunoregulation taking place within tumors.[49] In doing so, Tregs deprive Tconvs of CD28 co-stimulation and compel them to a hyporesponsive state known as anergy. This mode of Treg-mediated modulation of APCs, together with Treg-secreted immunosuppressive cytokines, may also exert "bystander suppression"; that is, Tregs suppress not only Tconvs recognizing the same antigen as Tregs but also Tconvs recognizing other antigens presented on the same APC or adjacent APCs.

The above paradigm may yet undergo a slight shift in light of recent findings on CD80 and PD-L1 jointly balancing immune activation and modulation. Since they were reported as binding partners, studies have found CD80 and PD-L1 existing as heterodimers on the same cells.[50–52] When *cis*-bound, CD80 is hindered from CTLA-4, but not CD28, and PD-L1 is concealed from PD-1.[53] CTLA-4-Ig disruption of CD80 increased unbound/free PD-L1 on APCs.[46] Furthermore, an anti-CD80 antibody that prevented formation of CD80:PD-L1 *cis*-duplexes was shown to ameliorate autoimmunity in mice by permitting increased PD-L1:PD-1 signaling in Tconvs.[54] It is thus conceivable that Treg-induced reduction of CD80 could accentuate PD-L1-mediated inhibition of $PD\text{-}1^+$-activated Tconvs, as suggested by the expansion of $CD80^{lo}$free-$PD\text{-}L1^{hi}$ APCs co-cultured with Tregs (Figure 2).[46]

Tregs have other immunosuppressive functions through the cell surface molecules, CD25, CD39 and CD73, and the cytokines IL-10, IL-35, and TGF-β. Tregs expressing CD25 can outcompete Tconvs for IL-2.[55] IL-2 sequestration by Tregs may be accompanied by deprivation of co-stimulation to effectively induce anergy in Tconvs.[56] Additionally, CD25 may serve as a "sensing" molecule to react to IL-2 production by Tconvs to keep them under control. Co-expression of CD39, which converts ATP to ADP and adenosine monophosphate (AMP), and CD73, which converts AMP to adenosine, allows Tregs to boost inhibitory cyclic adenosine monophosphate (cAMP) in Tconvs through adenosine receptors.[57,58] This may occur when Tregs and Tconvs release ATP through pannexin channels when they are activated in close proximity.[59] Treg-derived IL-10 and IL-35 are important in preventing tissue inflammation, particularly colitis.[60–62] IL-10- and IL-35-producing Tregs are, however, distinct populations. The differentiation of naive Tregs into $IL\text{-}10^+$ Tregs, but not $IL\text{-}35^+$ Tregs, is dependent on B-lymphocyte induced maturation protein-1 (Blimp-1).[63] Mice with Treg-specific deficiency of Blimp-1 had severe colitis but barely had systemic autoimmunity, consistent with mice with Treg-specific deficiency of IL-10.[60,61] TCR-stimulated Tregs express high latent TGF-β (L-TGF-β) bound to glycoprotein A repetitions predominant (Garp) on their cell surface.[64,65] Activated Tconvs also express L-TGF-β albeit at much lower levels. Interaction between L-TGF-β and integrins on cells and extracellular matrix can activate TGF-β in the L-TGF-β:Garp complex to engage TGF-β receptors.[66] Mice with T cell-specific deficiency of Garp, however, did not exhibit abnormal Treg and Tconv development; and Garp-deficient Tregs were as immunosuppressive as Garp-sufficient Tregs.[65] This was in contrast to human Tregs with Garp knockdown showing defective Foxp3 and immunosuppressive function.[64] To elucidate the functional significance of $Garp^+$ Tregs, it may be necessary to assess them in ongoing immunopathological conditions, such as in gut inflammation or even in tumors with high integrins, such

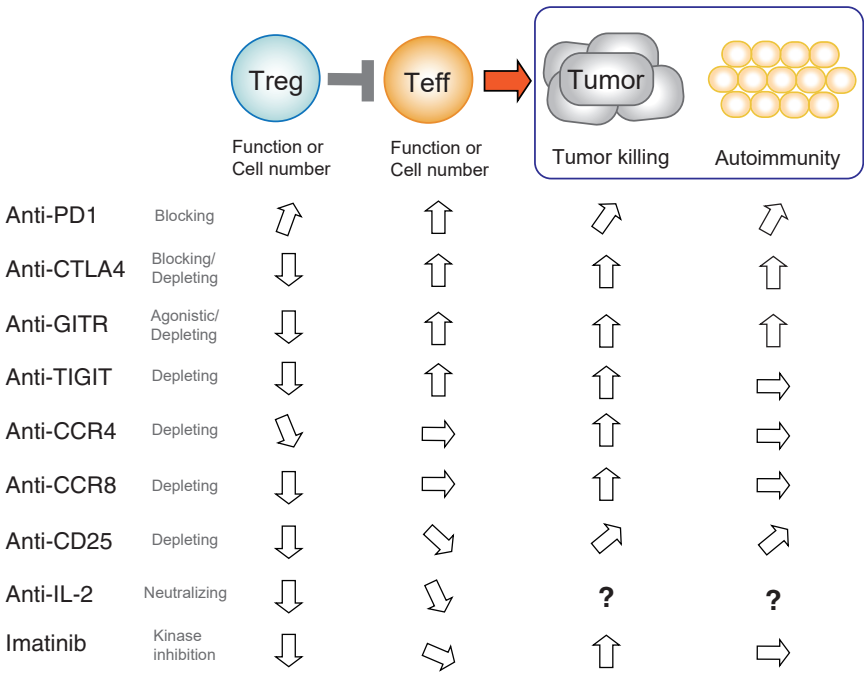

Figure 3. Overview of Treg-targeting antibody and small molecule compounds
Individual effects of indicated antibody and compound on Tregs and effector Tconvs and tumor immunity and autoimmunity. Arrows pointing down indicate negative effects, arrows pointing up indicate positive effects, and horizontal arrows indicate no or negligible effects.

as αvβ8, that may promote TI-Treg accumulation in tumor tissues.[67,68]

Further research is required to understand the complementary effects that each Treg function may have on another. One example is between cAMP and CD28 co-stimulation. Upon TCR stimulation in Tconvs, cAMP is generated to trigger a negative feedback through cAMP-dependent protein kinase A that inhibits TCR.[69,70] For TCR to be relieved from this autoinhibitory circuit, CD28 co-stimulation is essential for upregulating and recruiting phosphodiesterases (PDEs) to TCR to hydrolyze cAMP.[71] Shortage of PDEs may be a reason behind Tconvs becoming anergic from APCs lacking CD80/86. Tregs could enhance this process by transferring cAMP into Tconvs through gap junctions called connexin 43 (Cx43).[72] This may arise from the diffusion gradient between Tregs, typically containing higher cAMP, and Tconvs.[73] Cx43 expression is non-constitutive and strictly dependent on activation especially under Treg-inducing conditions (e.g., TGF-β).[74] There could be other molecules that do not have direct immunosuppressive effect but link and synergize Treg functions. These include chemokines and their receptors that spatially organize Tregs in tissues for cell-contact suppression.

POTENTIAL CONFLICT BETWEEN TREGS AND PD-1 BLOCKADE IMMUNOTHERAPY

Similar to its role in Tconvs, PD-1 also inhibits Treg activity as PD-1 blockade results in increased Treg activation.[14,75] Non-obese diabetic mice with Treg-specific deficiency of PD-1 were found to be better protected from autoimmune type 1 diabetes.[17] This may affect the outcome of anti-PD-1 immunotherapy, as increased Treg activity may compromise treatment efficacy (Figure 3).

We previously reported that PD-1$^+$ TI-Tregs in tumors may undermine PD-1 blockade immunotherapy. This was inferred from increased PD-1$^+$ TI-Tregs in tumors of patients who suffered from a fatal condition called hyper-progressive disease (HPD).[14] HPD is an accelerated growth of pre-existing tumors treated with ICB antibodies. Its incidence varies with cancer types, from 5.7% in non-small cell lung carcinoma (NSCLC) to 29% in head and neck squamous cell carcinoma.[76] HPD likely has multiple causes. Given that Tregs without PD-1 gene and Tregs treated with anti-PD-1 were more proliferative, were more immunosuppressive, and resulted in increased tumor growth in mice, we proposed that such an event may drive some HPD cases.[14] This may be pervasive in patients with tumors containing high PD-1$^+$ Treg/PD-1$^+$ CD8$^+$ T cell ratios. Indeed, PD-1 blockade in mice resulted in larger tumors that had high pre-existing TI-Treg/CD8$^+$ T cell ratios imposed by near infrared light that purged intra-tumoral CD8$^+$ T cells labeled with photosensitizer-conjugated anti-CD8β antibody.[77] As a corollary, low Treg/CD8$^+$ T cell ratios within tumors are associated with better clinical outcomes in patients treated with anti-PD-1.[78] Hence, measuring and tracking the said ratio with tumor growth could determine the suitability of patients for anti-PD-1 therapy.

Although still undocumented in humans, there may be cases in which PD-1 blockade reduces TI-Tregs and tumor development, as shown in mice with tamoxifen-dependent Treg-specific PD-1 deletion.[79] However, these effects came about only when PD-1 deficiency was induced before but not after tumor inoculation. Given the spontaneous increase in activation and proliferation of PD-1 deficient Tregs, prior induction of PD-1 deficiency in Tregs could lower and limit the frequency of Tregs available for *de novo* stimulation by tumor antigens and trafficking to tumors for site-specific immunosuppression. In addition, although excessive activation may destabilize and increase apoptosis in Tregs (discussed later), this likely varies with tumors; particularly as tumors resistant to anti-PD-1 therapy tend to have more TI-Tregs.[75,80] Stratification of tumors into anti-PD-1 responsive and non-responsive types may bring clarity on the tumor environment that disfavors TI-Tregs devoid of PD-1.

It may also be beneficial to identify the immunosuppressive effects that are augmented by anti-PD-1-induced activation of Tregs. Some clues recently surfaced from Treg-specific PD-1-deficient mice, which were shown to have increased levels of IL-10$^+$, CTLA-4$^+$, and LFA-1$^+$ Tregs.[81] Future studies also ought to assess less apparent immunosuppressive mechanisms such as the rate of adenosine production. Thus far, anti-CD73 has

been shown to enhance the anti-tumor efficacy of anti-PD-1 in mice.[82] This supports the plausibility of improving anti-PD-1 therapy by attenuating relevant Treg functions.

ANTI-CTLA-4 ANTIBODY AND ITS DEPLETING EFFECT ON TREGS

Anti-CTLA-4 has a broad range of effects through enhancing CD28 co-stimulation in Tconvs, blocking CTLA-4-mediated immunosuppressive function of Tregs and depleting Tregs by F_c-mediated antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) (Figure 3). To date, the relative contributions of these effects by anti-CTLA-4 to its overall anti-tumor efficacy are still under debate.[83,84] The rate of Treg depletion rests on the Fc IgG isotype of anti-CTLA-4 and FcγR polymorphism.[85] ADCC-mediated Treg depletion by anti-CTLA-4 has been widely documented in mice and humans and shown to be prevalent among TI-Tregs in tumors containing active natural killer (NK) cells.[86] In mice, though, ADCP by $CD11b^+$ macrophages could be the predominant mechanism since the absence of NK cells did not affect anti-CTLA-4-mediated depletion of TI-Tregs.[87] An unaddressed question is whether dormant/exhausted CTLA-4^{hi} Tconvs share a similar fate. Their reduced presence could blunt the efficacy of anti-CTLA-4. Nevertheless, a synergistic effect can be expected of combining anti-CTLA-4 and anti-PD-1, which has gained approval for treatment of certain aggressive cancers.[83] A major drawback lies in the high rate of irAEs, 55% for patients receiving combined therapy compared with 27.3% and 16.3% for patients receiving anti-CTLA-4 or anti-PD-1 monotherapy, respectively.[88] However, this may be alleviated by modifications to antibody structure. A new humanized anti-CTLA-4 variant that was designed to be smaller by excluding light chains and had constant regions of its heavy chains optimized for ADCC, was reported to permeate tumors more efficiently and evoked stronger tumor immunity compared with conventional anti-CTLA-4.[89] Moreover, its short systemic half-life could reduce irAEs. Hence, much hope remains for effective and safe anti-CTLA-4 and anti-PD-1 combination treatment against cancer.

BI-SPECIFIC ANTIBODIES AGAINST CTLA-4 AND PD-1

An intriguing advancement in dual blockade of CTLA-4 and PD-1 has recently been made with two bi-specific antibodies, MED15752 and MDG019.[90–92] They were constructed purely for blocking the IC molecules and did not affect Tregs *in vitro* and *in vivo*. Creation of these antibodies was spurred by the higher presence of CTLA-4^+PD-1^+ T cells in tumors compared with healthy tissues. Both MED15752 and MDG019 showed preferential binding to CTLA-4^+PD-1^+ over CTLA-4^+PD-1^- T cells. The same applied to their PD-1 binding properties, indicating that T cells in healthy tissues can be spared from inhibition since they are largely either CTLA-4 or PD-1 single positive. In mice expressing human CTLA-4 and PD-1, MED15752 localized mostly to tumors and was more effective in inhibiting tumors than the combination of mono-specific antibodies.[91] Similar results were obtained for MDG019 in monkeys, which had more circulating activated and memory $CD4^+$ T cells, whereas Tregs remained constant.[92] Clinical trials are currently under way, with early results showing promising tumor regression and irAEs that were considered moderate and tolerable.[90]

Although further tests on their mechanistic actions are required, MED15752 and MDG019 were found to be rapidly internalized and degraded along with their bound IC molecules, probably because of the perpetual membrane-cytosol cycling of CTLA-4.[91,92] Given that Tregs express high levels of CTLA-4 and PD-1, the effect of losing them in this context may be subtle on Tregs but profound on Tconvs, which could then be readily activated.

TRANSIENT TREG DEPLETION BY ANTI-CTLA-4 FOR TUMOR-REACTIVE TCONV ACTIVATION

High constitutive expression of CTLA-4 on Tregs and activation-dependent expression of CTLA-4 on Tconvs offer opportunities to first break tumor TI-Treg defenses then unleash Tconvs against tumor cells. This was unveiled as a practical approach in mice that had tumors regressed when infused with anti-CTLA-4 prior to tumor antigen vaccination that expanded tumor-reactive $CD8^+$ T cells.[13] Conversely, vaccination in tandem with infusion of anti-CTLA-4 caused a decrease in $CD8^+$ T cells in tumor tissues and did not change the $CD8^+$ T cell/Treg ratio, presumably as CTLA-4^+ cells in both populations were depleted in parallel. Tumors in this group did not regress like those in mice that were first treated with anti-CTLA-4 then vaccinated with tumor antigen. This may explain the failure of patients with late-stage melanoma to benefit from combining anti-CTLA-4 and glycoprotein 100 (gp100) vaccination.[93] Although Tregs and Tconvs were not assessed, it was possible that anti-CTLA-4 depleted CTLA-4^+ Tregs as well as Tconvs that upregulated CTLA-4 upon activation by gp100. Hence, coordination rather than mere combination of tumor antigen vaccination with anti-CTLA-4 could be key for such a therapeutic approach to be effective.

TARGETING OTHER DIFFERENTIALLY EXPRESSED CO-INHIBITORY/STIMULATORY SURFACE MOLECULES

Tregs and Tconvs share many activation and inhibitory molecules of which several are more highly expressed in Tregs than in Tconvs. Tconvs also tend to display delayed kinetics of expression (as described below). These differential properties may be exploited to re-balance the TI-Treg and Tconv rivalry to favor the latter in tumors.

GITR stimulation to induce cell death in GITR^{hi} Tregs and activation of GITR^{lo} Tconvs

Glucocorticoid-induced TNFR-related protein (GITR) is highly expressed in Tregs. Tconvs also upregulate GITR upon activation though to lower levels.[94] Increased levels of $CD4^+$GITR$^+$ Tregs and Tconvs in tumors is associated with poor prognosis in human gastric cancer.[95] GITR is an activation molecule with opposing effects on Tregs and Tconvs. This is evident from agonistic anti-GITR (DTA-1) increasing Treg and Tconv proliferation, only for the former to become unstable and less immunosuppressive and even differentiate into Th1-like cells with cytotoxic effects in tumors (Figure 3).[94,96–99] The ability of GITR stimulation to prevent Tconvs from becoming anergic

and the inability of GITR ligand-deficient APCs to expand Tregs suggest that GITR may be a source of co-stimulation.[94] High GITR expression on Tregs may thus subject them to over-stimulation by DTA-1, resulting in Treg instability and apoptosis.

Mice treated with DTA-1 developed smaller tumors that contained less Tregs and more effector Tconvs.[100,101] In murine glioblastoma, DTA-1 reduced tumor resistance to anti-PD-1.[99] When used simultaneously with cell-depleting antibodies, however, a pre-requisite is that the antibody-targeted molecules on Tconvs are not upregulated by DTA-1. For example, treating tumor-bearing mice with DTA-1 evoked strong tumor immunity and impeded tumor development, effects that were potentiated by non-cell-depleting anti-CTLA-4, but not by cell-depleting anti-CD25.[100] Combination of DTA-1 and anti-CD25 was noticeably worse than DTA-1 alone. This could be reasoned by anti-CD25 depleting both Tregs and Tconvs, the latter also expressing CD25 in tumors upon activation by DTA-1.

Unfortunately, anti-GITR has not shown promising efficacy in clinical trials despite reduction of TI-Tregs in tumor tissues. To resolve this, a fusion construct consisting of anti-PD-1 and GITR-Ligand (GITR-L) multimer was generated and found to be highly potent in preventing tumor growth in humanized mouse models.[102] PD-1 blockade by the anti-PD-1 portion was critical for GITR clustering that amplified downstream signals by GITR-L. There was also a marked reduction of TI-Tregs in tumors; and *in vitro* assay showed less Treg suppression brought about by anti-PD-1:GITR-L compared with combination of individual anti-PD-1 and GITR-L. Hence, GITR agonism could still be a viable therapeutic option. The key lies in converging its agonistic effect.

TIGIT modulation to selectively deplete Tregs and activate Tconvs

T cell immunoglobulin and ITIM domain (TIGIT) is a co-inhibitory receptor that is promoted by Foxp3, which binds to its locus to maintain a stable epigenetic state.[103] The baseline level of TIGIT is thus higher on Tregs than on Tconvs, further increasing on TI-Tregs.[104] In mice, anti-TIGIT with strong ADCC and anti-tumor efficacies was shown to deplete TI-Tregs without altering the numbers of Tconvs and extra-tumoral Tregs.[105] Similarly, anti-TIGIT human antibody preferentially depleted Tregs over Tconvs from the blood of cancer patients. The efficacy of ADCC correlated with the density of TIGIT expression per cell.[105] In another murine model of ovarian cancer, blocking TIGIT reduced the numbers and function of TI-Tregs in tumors and improved the survival of mice.[106]

TIGIT could be a good target for cancer immunotherapy in view of TIGIT knockout mice remaining healthy without spontaneous autoimmunity.[107] Upon binding to the ligands, CD155 and CD112, TIGIT transduces negative signals through its own inhibitory motifs.[108] This also prevents CD155 and CD112 from engaging the co-stimulatory molecules, CD226 and CD112R, respectively. Tregs have high TIGIT relative to CD226 and only upregulate TIGIT but not CD226 upon activation.[104] By contrast, Tconvs have low TIGIT/CD226 ratios and upregulate both molecules when activated. Although TIGIT signaling promotes Treg integrity and function, CD226 opposes, vice versa for Tconvs (Figure 3). Hence, differential control of Tregs and Tconvs may be possible through the reciprocal effects of TIGIT and CD226. Several clinical trials for anti-TIGIT in combination with ICB antibodies are in progress.[108]

TARGETING CHEMOKINE RECEPTORS SPECIFIC FOR TI-TREGS

TI-Tregs may possess specialized chemotactic features that allow them to populate tumors and create tumor immunosuppressive environments. These characteristics can be explored for future therapeutic applications that may be used in conjunction with ICB antibodies to treat cancer efficiently and safely.

CCR4:CCL17/22

This TI-Treg chemotactic axis came about from uncovering tumoral ovarian cells and macrophages as major producers of CCL22 and TI-Tregs with high CCR4 expression.[109] Indeed, CCL22 inhibition reduced Treg infiltration into tumors without affecting Tconvs. Since then, studies have pursued CCR4 as a TI-Treg-specific molecule (Figure 3). In a recent one, giving CCR4 antagonist to mice with Pan02 tumors that produced copious amounts of CCL17 and CCL22 blocked Treg infiltration into tumors and evoked strong tumor immunity.[110] More interestingly, tumors that were inherently low in CCL17 and CCL22 expression had both chemokines upregulated along with increased $CCR4^+$ TI-Tregs upon treatment with anti-CTLA-4. Although these tumors were partially reduced in size by anti-CTLA-4, their development was almost completely dismissed by joint blockade of CTLA-4 and CCR4.[110] Hence, it may be important to monitor for any rise in CCL17 and CCL22 levels in tumors that could be counterproductive during anti-CTLA-4 or other types of immunotherapy. A similar observation was reported with piperidinyl-azetidines that bind to a particular CCR4 motif, preventing recognition of CCL17 and CCL22.[111] They were effective in lowering TI-Tregs and enhancing anti-tumor efficacies of anti-CTLA-4 and anti-PD-L1.

Mogamulizumab, a humanized anti-CCR4 antibody used to treat adult T cell leukemia/lymphoma (ATLL), was previously found to deplete mostly activated Tregs from the blood and tumor infiltrates of melanoma patients, increasing tumor antigen-specific activation of Tconvs.[112] In recent clinical trials, good overall response rates (ORR) were attained for mogamulizumab against ATLL, peripheral T cell lymphoma and cutaneous T cell lymphoma with a pooled rate of 43%.[113] Toxic effects and irAEs were also within reasonable limits, the most common comprising lymphopenia, neutropenia, and skin rash. Skin reactions are particularly common among responders to mogamulizumab, which could be attributed to the depletion of $CCR4^+$ skin-resident Tregs as confirmed by immunohistological staining.[114] Similar results have also been obtained against advanced and recurrent solid tumors (40% ORR) treated with mogamulizumab alone and advanced solid tumors (hepatocellular carcinoma; 27%, NSCLC; 20% ORR) treated with combination of mogamulizumab and anti-PD-1.[114] Tumor biopsies in the latter group showed reduced TI-Tregs and increased $CD8^+$ T cells.

CCR8:CCL1

CCR8 is currently generating more excitement owing to its high specificity as a TI-Treg marker in several cancers (e.g., breast, colon, renal, pancreatic, gastric) (Figure 3).[49,95,115] Our group

and others recently found that CCR8$^+$ TI-Tregs account for 50%–60% of TI-Tregs and are highly immunosuppressive.[49,116–118] Remarkably, CCR8$^+$ TI-Treg depletion led to an almost complete remission of tumors and gave rise to strong resistance against secondary tumor challenge. These were attained without major loss of Tregs in other organs; and mice were in good health throughout treatment. Furthermore, a synergistic effect was obtained from combining anti-CCR8 and anti-PD-1.[49,116]

CCR8, however, is redundant to the migration and retention of TI-Tregs. These were reported by studies that found CCR8-deficient Tregs with similar rates of tumor infiltration as wild-type Tregs and CCR8 knockout mice without any tumor growth reduction.[119] Despite CCR8 not serving any immunosuppressive purpose, its engagement to CCL1 could promote Foxp3 transcription even in Foxp3$^+$ nTregs. This may be accompanied by increases in the Treg functional molecules CD39, IL-10, and granzyme B and decrease in PD-1.[120] Hence, CCR8 may enhance the stability of TI-Tregs and relieve them of PD-1 restraint. One downside of anti-CCR8 is while there are few CCR8$^+$ Tregs in healthy tissues, they could be increased in sites of inflammation or autoimmunity where they limit collateral damage.[49] Apolipoprotein E knockout (*ApoE*$^{-/-}$) mice infused with anti-CCR8 and *ApoE*$^{-/-}$*Ccl1*$^{-/-}$ mice had reduced Tregs in the aorta and more atherosclerosis.[121] Deletion of CCR8$^+$ Tregs in these regions could be detrimental especially when anti-CCR8 is combined with anti-PD-1.

Others: CCR5:CCL5 and CCR10:CCL28

Other less prominent chemotactic systems of TI-Tregs are CCR5:CCL5 and CCR10:CCL28. The former is pertinent to pancreatic and squamous cell carcinoma and hindering either receptor or ligand restricts Treg entry into tumors.[122,123] Mice that received such treatments had smaller tumors. CCR5 also makes a good biomarker with its higher expression in circulating Tregs compared with Tconvs becoming more pronounced during cancer.[122] CCR10$^+$ TI-Tregs are mobilized by hypoxia-induced CCL28 in ovarian cancer.[124] In tumors, CCR10$^+$ TI-Tregs can secrete vascular endothelial growth factor A (VEGFA) to expand VEGF receptor-2 (VEGFR-2)$^+$ Tregs to pack tumors with even more TI-Tregs.[124]

TARGETING IL-2/IL-2R AND IMMUNOSUPPRESSIVE MOLECULES OF TI-TREGS

Targeting IL-2/IL-2R to control the balance between TI-Tregs and Tconvs in tumors

Until recently, treating tumor-bearing mice with anti-CD25 had not shown significant reduction in tumors, mainly from insufficient reduction of TI-Tregs and interference of IL-2R signaling on Tconvs. These have since been rectified by F_c-optimized anti-CD25 of higher specificity (Figure 3).[125–127]

Alternatively, differential binding of IL-2 to the trimeric ($\alpha\beta\gamma$) and dimeric ($\beta\gamma$) forms of IL-2R can be leveraged to favor Tconvs over Tregs. The anti-IL-2 clone, S4B6, against murine IL-2, is one to model after (Figure 3). S4B6 obstructs IL-2 from IL-2Rα significantly more than IL-2Rβ. The slight steric hindrance of IL-2Rβ is compensated by a conformational change in IL-2 that strengthens its binding to IL-2Rβ.[33] Hence, S4B6 could abolish the advantage of expressing IL-2Rα, as attested in mice with more proliferating Tconvs than Tregs and bearing smaller tumors when given IL-2:S4B6 complex.[128,129]

In another approach, a fusion protein consisting of anti-PD-1 and low-affinity IL-2 was shown to drive tumor immunity through selective invigoration of PD-1$^+$CD8$^+$ T cells.[130] The greater expansion of CD8$^+$ T cells relative to TI-Tregs only occurred in tumors, not in peripheral tissues. This disparity between anatomical locations lends credence to IL-2 competition in tumors with high PD-1$^+$CD8$^+$ T cells. A second group made similar findings by fusing anti-PD-1 to a non-CD25 binding IL-2 variant.[131]

Sushi domain containing-2 (SUSD2) may also be a good candidate. SUSD2 is a membrane protein present on effector CD8$^+$ T cells, absent on Tregs.[132] It binds to the sushi domains on CD25, which is expressed at low levels on effector CD8$^+$ T cells, preventing IL-2 from engaging for stimulation. This was verified by SUSD2-deficient CD8$^+$ T cells ridding off tumor cells more robustly.[132] An IL-2 mutein that was generated for CD25 binding specifically in acidic environments, typical of tumors, may well displace SUSD2 to trigger tumor-reactive CD8$^+$ T cells.[133] However, this is likely to be effective only against tumors that do not have high TI-Treg/CD8$^+$ T cell ratio.

Blocking CD39 reduction of ATP and CD73 production of adenosine

CD39 and CD73 exist on cell surface membrane as well as on secreted soluble exosomes.[134] CD39 neutralizes the inflammatory threat by ATP through P2 purinergic receptors (e.g., P2XR and P2YR) and CD73 subdues immune cells through the cAMP-producing adenosine receptors, Adora2a (A2aR) and Adora2b (A2bR).[135–137] Tregs deficient in CD39, CD73, A2aR, or A2bR are dysfunctional to varying extent.[138–142] CD39-deficientand CD73-deficient Tregs have been found to give way to stronger anti-tumor immune responses in mice.[143,144] Tumor cells themselves use CD39 and CD73 to create an ATP-poor and adenosine-rich environment conducive to TI-Tregs.[145] To reverse this, inhibitors of CD39 and CD73 have to antagonize both their membrane and soluble forms. This was underscored by human membrane- and soluble-specific CD39 and CD73 antibodies inducing Tconv activation superior to the respective membrane only-specific antibodies.[146] Complete blockade may be vital given that TI-Tregs tend to apoptose from ATP stimulation of P2X7R and apoptotic TI-Tregs could in turn leak ATP to fuel CD73-mediated tumor immunosuppression.[147,148]

Targeting the immunosuppressive cytokines: IL-10, IL-35, and TGF-β

Within tumors, IL-10$^+$ Tregs are implicated in regulating tumor inflammation, while IL-35$^+$ Tregs may promote Tconv exhaustion.[149] Both IL-10$^+$ and IL-35$^+$ Tregs participate in creating immunosuppressive environments in tumors. This was seen in mice that lack both IL-10$^+$ and IL-35$^+$ Tregs bearing smaller tumors than mice without either Treg population.[149] Despite their negative roles in cancer, IL-10$^+$ and IL-35$^+$ Tregs do not yet appeal as therapeutic targets, because of their commitment to prevent peripheral tissue inflammation.

Tumor cells expressing $\alpha v\beta 8$ have been implicated to activate L-TGF-β on activated Tconvs, facilitating local conversion of activated Tconvs into pTregs in tumors.[68] Indeed, Foxp3$^+$ cells from $\alpha v\beta 8$-expressing and non-expressing tumors differ in their

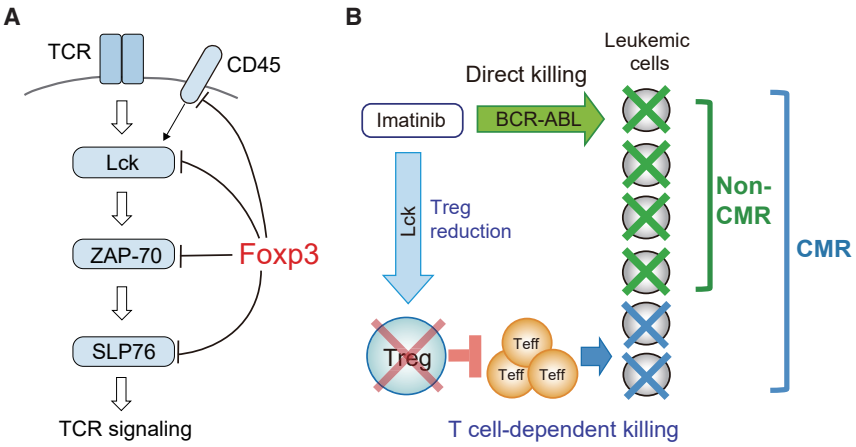

Figure 4. Treg-repressed TCR signaling molecules as targets for Treg reduction

(A) FoxP3-mediated repression of TCR signaling molecules intrinsic to Tregs.

(B) A schematic diagram showing imatinib's dual effects on leukemic cells. Direct killing of leukemic cells by BCR-ABL inhibition or T cell-dependent killing of leukemic cells by Lck-inhibition mediated Treg reduction for achieving complete molecular remission (CMR) in chronic myelogenous leukemia.

transcriptional profiles. Unfortunately, there were no clear gene signatures suggesting that the former and latter groups were dominated by pTregs and tTregs, respectively.[68] A reasonable speculation is that both may increase under the influence of αvβ8-mediated activation of TGF-β. Given the clear reductions of TI-Tregs and tumor growth after treating αvβ8-expressing, but not non-expressing, tumors with anti-αvβ8, a role of αvβ8 in promoting TI-Treg accumulation in tumors is likely. This also implies that blocking αvβ8 could be more effective than blocking soluble active TGF-β for treating cancer.

INHIBITING FOXP3-REPRESSED TCR-RELATED MOLECULES TO REDUCE TI-TREGS

Tregs and Tconvs differ in TCR signaling upon TCR stimulation and also at basal state due to Foxp3-mediated downregulation of certain TCR signaling components, including lymphocyte-specific protein tyrosine kinase (Lck), zeta-chain-associated protein kinase 70 (ZAP-70), SLP76, and CD45 (Figure 4A).[150] Foxp3-induced downregulation of these molecules may enable Tregs to better survive and elude activation-induced cell death in an inflammation site to suppress Tconvs. Hence, TCR-related molecules, such as Lck, can be felicitous targets for selective control of Tregs. Indeed, imatinib, a tyrosine kinase inhibitor (TKI) for oncogenic BCR-ABL fusion kinase in chronic myelogenous leukemia (CML) and with off-target effects on Lck, was found to preferentially deplete effector Tregs, allowing expansion of antigen-specific $CD8^+$ T cells in healthy individuals and in mice (Figure 4B).[151] As the amount of Lck expressed by effector Tregs is much lower compared with $CD8^+$ T cells, therapeutic doses of imatinib could affect Tregs more than $CD8^+$ T cells. In mice bearing imatinib-insensitive tumors, imatinib treatment reduced effector Tregs in the periphery and in tumors and reduced tumor growth.[150] Other small molecule inhibitors of Lck (e.g., dasatinib, AMG-47a) were also found to selectively reduce Tregs.

In CML, imatinib-treated patients can be segregated into two groups; complete molecular remission (CMR) and non-CMR. Analysis of peripheral blood cells showed close association between CMR and reduction of effector Tregs coupled with marked increase in effector/memory $CD8^+$ T cells. This suggests that imatinib may not only mediate direct killing of leukemic cells by BCR-ABL inhibition but also by T cell mediated immunity (Figure 4B).[151] Another benefit of imatinib is the rare occurrence of irAEs. This encourages the development of second generation TKIs, such as dasatinib with higher Lck specificity and less variable in BCR-ABL inhibition and effector Treg depletion.[152] Inhibitors of other Foxp3-repressed TCR-related molecules can also be tested and optimized for therapeutic doses that reduce TI-Tregs, but not $CD8^+$ T cells, to promote tumor immunity.

METABOLIC ADAPTATION OF TI-TREGS IN TUMORS THAT MAY BE THERAPEUTICALLY TARGETED

Tumors with low glucose, high lactate, and high lipid content may be the most accommodating to TI-Tregs as discussed below. Changing the levels of one or more of these nutrients or blocking their metabolism in TI-Tregs may prime the tumor immune landscape for ICB therapies to be more effective.

Glycolysis and oxidative phosphorylation

Tumors rely primarily on glycolysis to generate ATP. This imposes a limitation on Tconvs which depend on glycolysis for effector responses.[153] In contrast, not only do TI-Tregs have higher glucose transporter-1 for consuming glucose, they also have the benefit of Foxp3-induced oxidative phosphorylation (OXPHOS) to produce more ATP per glucose input.[154,155] The readiness of Tregs to turn on OXPHOS can be attributed to Foxp3-induced inhibition of c-Myc.[155] Moreover, pyruvate dehydrogenase may be less inhibited as protein kinase B (Akt) signaling wanes from increased cAMP-PKA during Treg activation. This could permit the conversion of pyruvate to acetyl-CoA for OXPHOS. PD-1 deficient Tregs are ideal examples as they have increased proliferation, decreased phosphoinositide 3-kinase (PI3kinase)-Akt transduction and increased OXPHOS.[17]

Lactate metabolism

Highly glycolytic tumors are amassed with its by-product, lactate. After oxidizing lactate, Tconvs may be short of nicotinamide adenine dinucleotide (NAD^+) required for glycolysis.[155] This is not so for Tregs as they can replenish NAD^+ through OXPHOS. Hence, high lactate in tumors may incapacitate Tconvs, while TI-Tregs may scavenge lactate through uptake by monocarboxylate transporter-1 (MCT1) and lactate dehydrogenase, which converts lactate to pyruvate for OXPHOS.[156,157] This is underscored by MCT1-deficient Tregs that are able to sustain immune tolerance in healthy tissues but unable to

suppress tumor immunity.[156] More compelling evidence came to light when a positive correlation was found between lactate concentration in tumors and frequency of PD-1$^+$ TI-Tregs.[157] Phosphoenol pyruvate from lactate metabolism was found to increase PD-1 expression in TI-Tregs. Genetic ablation or chemical blockade of MCT1 in TI-Tregs greatly improved anti-PD-1 efficacy, indicating that co-blockade of MCT1 and PD-1 could be an effective therapeutic strategy.[157]

Curcumin, a component of turmeric used in food, has gained a reputation for its anti-tumor properties, which may be related to defective glycolysis in Tregs and decreased lactate production by tumor cells.[158] GO-Y030, a curcumin analog, displayed strong propensity for these effects and also rendered TI-Tregs unstable.[159] Its synergy with anti-PD-1 coincides with anti-CTLA-4 establishing immune memory only against moderately glycolytic tumors.[160] Within these tumors, Tconv activation is not constrained by glucose and Tregs are less poised to suppress Tconvs because of reduced lactate.

Lipid metabolism

Tumors may have an abundance of lipids that TI-Tregs feast on to complement their energy needs.[161] Gastric cancers with *RHOA* Y42 mutations are at risk for this circumstance and do not respond well to anti-PD-1 unless co-treated with PI3K inhibitor to prevent tumors from releasing free fatty acids (FFAs) and so reduce TI-Tregs.[162] In untreated tumors, signs of TI-Tregs profiting from FFAs are presented by increases in lipid absorption, CD36 scavenger receptor and carnitine palmitoyltransferase 1A (CPT1A). Blocking CD36 decreased TI-Treg and increased Tconv numbers. Although this was inadequate to counter tumor growth, it did pave the way for anti-PD-1 to do so.[162]

FFAs captured by CD36 are guided to the mitochondria for fatty acid oxidation (FAO).[163] Long-chain FFAs (Lc-FAs) are transferred across the inner mitochondrial membrane by CPT1A.[163] Short- and medium-chain FFAs can enter freely, and this may suffice for most Tregs as deduced from the unaltered Treg numbers in mice with Treg-specific deficiency of CPT1A.[164,165] However, the roles of Lc-FAs and CPT1A in TI-Tregs are hitherto unknown. Given the increase in CPT1A in TI-Tregs and Lc-FAs inducing CD8$^+$ T cell exhaustion, tumor immune evasion may abate when Lc-FAs are reduced in tumors.[154,162,166] This may be possible as shown by the blockade of fatty acid synthesis through sterol-regulatory element-binding proteins (SREBPs), which are governed by SREBP cleavage-activating protein (SCAP), in Tregs. Mice with Treg-specific deficiency of SCAP had no appreciable tumor growth nor immune dysregulation except for minor pancreatic inflammation.[167] Interestingly, SREBP signals were found to reinforce TCR signaling, suggesting that SCAP inhibitors could be potential alternatives to TKIs for TI-Treg depletion.

Anti-lipid peroxidation

A drawback that TI-Tregs have from lipid metabolism is lipid peroxidation, a major precursor to ferroptosis. TI-Tregs are able to cope and resist ferroptosis by expressing high amounts of the anti-oxidative enzyme, glutathione peroxidase 4 (Gpx4).[168] Like SREBPs, Gpx4 is required for the survival of TI-Tregs more than other Tregs. Mice with Gpx4-deficient Tregs had smaller tumors and were still capable of maintaining immune homeostasis in healthy organs except for a slight increase in Th17 cells in lymphoid organs.[168] Hence, blocking Gpx4 may facilitate cell death of only TI-Tregs under high oxidative stress in tumor tissues but not Tregs in healthy tissues.

BLOCKING TUMOR ANGIOGENIC FACTORS ALSO REDUCES TI-TREGS IN TUMORS

Angiogenesis is instrumental to the supply of nutrients and drainage of waste in tumors. The two main pro-angiogenic factors induced by hypoxia in tumors are angiopoietin-2 (Ang-2) and VEGF.[169] Ang-2 can also stimulate IL-10 production by resident monocytes and macrophages.[170,171] This was shown to enhance suppression of Tconvs and expansion of Tregs.[171] Similarly, VEGF can suppress tumor immunity by blocking the maturation of DCs and monocytes, inducing CD8$^+$ T cell exhaustion and expanding VEGFR-2$^+$ TI-Tregs in tumors.[172–176] VEGFR-2$^+$ Tregs have higher Foxp3 expression and are more immunosuppressive than VEGFR-2$^-$ Tregs.[177] Neuropilin-1, a Treg signature molecule, confers an added advantage by acting as a co-receptor that stabilizes VEGF interaction with VEGFR-2.[178] TI-Tregs are sources of VEGF themselves. In comparison with Tconvs, Tregs express higher VEGF under hypoxia and Treg-conditioned media generated more capillaries *in vitro* and endothelial cells on Matrigel plugs *in vivo*, effects abrogated by anti-VEGF.[124] Accordingly, depletion of VEGF-producing TI-Tregs reduced VEGF and angiogenesis in tumors and hampered tumor growth.[124]

The immunosuppressive roles of Ang-2 and VEGF may result in resistance to ICB antibodies. Indeed, high serum Ang-2 level associates with poor clinical response to anti-CTLA-4 and anti-PD-1.[179] A bi-specific antibody against Ang-2 and VEGF, which reduced tumor vasculature and restored antigen presentation by tumor-resident APCs, was reported to be highly efficacious in blocking tumor development in synergy with anti-PD-1.[180] Besides, several treatment regimens combining ICB antibodies and TKIs are undergoing evaluation. At low doses, TKIs promote orderly, rather than aberrant, vascularization in tumors with increased adhesion molecules for lymphocyte infiltration.[181] This could maximize effectiveness of ICB antibodies, as shown with combined VEGF and PD-1 blockade.[182,183] As mentioned above, some TKIs (e.g., imatinib) can also attenuate Tregs, further curbing tumor development.[151,184] Research on merging anti-angiogenic and anti-Treg or ICB therapies is still in its infancy. In time, these combinations may be added to the list of cancer treatment options.

FUTURE DIRECTIONS

The successes of cancer immunotherapies rest not only on their effectiveness against cancer but also safety standards. Therefore, the strategy that we propose is one led by molecular targets that are differentially expressed between TI-Tregs and Tconvs in terms of specificity, density, kinetics, and activation dependency (Figure 3). CTLA-4 and PD-1 rank high on the list of such targets. However, although CTLA-4 on Tregs is a convenient target for anti-CTLA-4, PD-1 on Tregs is not, because its blockade enhances the activation of Tregs. To circumvent this, PD-1$^+$ Tregs have to be kept to a minimum relative to Tconvs before and

during anti-PD-1 therapy. Although anti-CTLA-4-mediated Treg depletion may alleviate this unwanted effect of anti-PD-1, this combination could lead to increased incidence of irAEs. This calls for calibration of immune responses to evoke tumor immunity with less autoimmunity.

Another approach to adopt is the blockade of lactate metabolism, which prevents upregulation of PD-1 particularly in TI-Tregs within highly glycolytic tumors.[157] This could lessen the frequency of PD-1^{hi} TI-Tregs and tip the balance in favor of PD-1^{hi} dormant/exhausted tumor-reactive Tconvs for re-activation by anti-PD-1.

Besides targeting activation and inhibitory surface molecules, intracellular molecules involved in TI-Treg lipid metabolism, like CPT1A and SCAP, can be considered for inhibition by small-molecule drugs. On the basis of evidence that the mevalonate pathway supports TCR signaling in TI-Tregs, and that TI-Tregs have a fragile TCR machinery inflicted by Foxp3, inhibiting these molecules may complement Lck inhibitors (e.g., imatinib) in depleting TI-Tregs with higher specificity and efficiency.[150,167]

Finally, with the advent of novel antibodies, the membrane recycling property of CTLA-4 could be exploited by bi- or even tri-specific antibodies against CTLA-4 and other TI-Treg-specific molecules, such as CD25 and CCR4/CCR8. These multi-specific antibodies may be internalized once bound to CTLA-4 along with their target markers; for example, CD25, which could then render TI-Tregs less competitive for IL-2, allowing Tconvs to become activated. The same may be done to other cell surface molecules critical for TI-Treg survival and expansion such as MCT1 (lactate uptake), CD36 (Lc-FA uptake), and VEGFR-2 (VEGF ligation). This approach is not likely to cause drastic or overt changes in the immune system and may only affect TI-Tregs in tumor tissues, but not Tregs in healthy tissues. On a cautionary note, the riddance of TI-Tregs in established tumors may not necessarily lift the barrier completely for tumor-reactive Tconvs to attack tumor cells. A second hurdle lies in the persistent lack of glucose for glycolysis-dependent effector Tconv response. This may be one of the prevailing reasons behind the low efficacies of ICBs, which leave much food for thought.

CONCLUSION

Tackling cancers that exploit Tregs for their immunosuppressive finesse requires finesse on our part. This can come only from learning more about the factors that delineate Tregs and Tconvs. It is also becoming increasingly clear that combination therapies that deal with both Tregs and Tconvs, whether sequentially or simultaneously, produce better outcomes than monotherapies targeting either of them. Even chimeric antigen receptor (CAR) T cell therapy may require interventions against CAR Tregs, which may be present at the time of generating CAR T cells or differentiate and expand from CAR Tconvs post-infusion.[185,186] In all, we strongly believe that removing the Treg roadblock in tumors is necessary to treat cancer, but this is only one of the many roadblocks that need to be removed before we can defeat cancer.

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AUTHOR CONTRIBUTIONS

C.T., A.T., and S.S. wrote and designed figures for this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Ribas, A., and Wolchok, J.D. (2018). Cancer immunotherapy using checkpoint blockade. Science *359*, 1350–1355. https://doi.org/10.1126/science.aar4060.

2. Bagchi, S., Yuan, R., and Engleman, E.G. (2021). Immune checkpoint inhibitors for the treatment of cancer: clinical impact and mechanisms of response and resistance. Annu. Rev. Pathol. *16*, 223–249. https://doi.org/10.1146/annurev-pathol-042020-042741.

3. Wojtukiewicz, M.Z., Rek, M.M., Karpowicz, K., Górska, M., Polityńska, B., Wojtukiewicz, A.M., Moniuszko, M., Radziwon, P., Tucker, S.C., and Honn, K.V. (2021). Inhibitors of immune checkpoints-PD-1, PD-L1, CTLA-4-new opportunities for cancer patients and a new challenge for internists and general practitioners. Cancer Metastasis Rev. *40*, 949–982. https://doi.org/10.1007/s10555-021-09976-0.

4. Johnson, D.B., Balko, J.M., Compton, M.L., Chalkias, S., Gorham, J., Xu, Y., Hicks, M., Puzanov, I., Alexander, M.R., Bloomer, T.L., et al. (2016). Fulminant myocarditis with combination immune checkpoint blockade. N. Engl. J. Med. *375*, 1749–1755. https://doi.org/10.1056/NEJMoa1609214.

5. Berner, F., Bomze, D., Diem, S., Ali, O.H., Fässler, M., Ring, S., Niederer, R., Ackermann, C.J., Baumgaertner, P., Pikor, N., et al. (2019). Association of checkpoint inhibitor-induced toxic effects with shared cancer and tissue antigens in non-small cell lung cancer. JAMA Oncol. *5*, 1043–1047. https://doi.org/10.1001/jamaoncol.2019.0402.

6. Chaput, N., Lepage, P., Coutzac, C., Soularue, E., Le Roux, K., Monot, C., Boselli, L., Routier, E., Cassard, L., Collins, M., et al. (2017). Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. Ann. Oncol. *28*, 1368–1379. https://doi.org/10.1093/annonc/mdx108.

7. Shimizu, J., Yamazaki, S., and Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25^{+}CD4^{+} T cells: a common basis between tumor immunity and autoimmunity. J. Immunol. *163*, 5211–5218.

8. Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science *270*, 985–988.

9. Tivol, E.A., Borriello, F., Schweitzer, A.N., Lynch, W.P., Bluestone, J.A., and Sharpe, A.H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity *3*, 541–547.

10. Nishimura, H., Nose, M., Hiai, H., Minato, N., and Honjo, T. (1999). Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity *11*, 141–151.

11. Nishimura, H., Okazaki, T., Tanaka, Y., Nakatani, K., Hara, M., Matsumori, A., Sasayama, S., Mizoguchi, A., Hiai, H., Minato, N., and Honjo, T. (2001). Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science *291*, 319–322. https://doi.org/10.1126/science.291.5502.319.

12. Sakurai, Y., Usui, Y., Hattori, T., Takeuchi, M., Takayama, K., Karasawa, Y., Nishio, Y., Yamakawa, N., Saitoh, D., Goto, H., and Ito, M. (2021). Programmed cell death-1 pathway deficiency enhances autoimmunity leading to dacryoadenitis of mice. Am. J. Pathol. *191*, 1077–1093. https://doi.org/10.1016/j.ajpath.2021.02.014.

13. Ha, D., Tanaka, A., Kibayashi, T., Tanemura, A., Sugiyama, D., Wing, J.B., Lim, E.L., Teng, K.W.W., Adeegbe, D., Newell, E.W., et al. (2019). Differential control of human Treg and effector T cells in tumor immunity by Fc-engineered anti-CTLA-4 antibody. Proc. Natl. Acad. Sci. USA *116*, 609–618. https://doi.org/10.1073/pnas.1812186116.

14. Kamada, T., Togashi, Y., Tay, C., Ha, D., Sasaki, A., Nakamura, Y., Sato, E., Fukuoka, S., Tada, Y., Tanaka, A., et al. (2019). PD-1^{+} regulatory T-cells amplified by PD-1 blockade promote hyperprogression of cancer. Proc. Natl. Acad. Sci. USA *116*, 9999–10008. https://doi.org/10.1073/pnas.1822001116.

15. Suzuki, S., Ogawa, T., Sano, R., Takahara, T., Inukai, D., Akira, S., Tsuchida, H., Yoshikawa, K., Ueda, R., and Tsuzuki, T. (2020). Immune-checkpoint molecules on regulatory T-cells as a potential therapeutic target in head and neck squamous cell cancers. Cancer Sci. *111*, 1943–1957. https://doi.org/10.1111/cas.14422.

16. Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3^{+} regulatory T cell function. Science *322*, 271–275. https://doi.org/10.1126/science.1160062.

17. Tan, C.L., Kuchroo, J.R., Sage, P.T., Liang, D., Francisco, L.M., Buck, J., Thaker, Y.R., Zhang, Q., McArdel, S.L., Juneja, V.R., et al. (2021). PD-1 restraint of regulatory T cell suppressive activity is critical for immune tolerance. J. Exp. Med. *218*, e20182232. https://doi.org/10.1084/jem.20182232.

18. Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057–1061. https://doi.org/10.1126/science.1079490.

19. Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. *4*, 330–336. https://doi.org/10.1038/ni904.

20. Khattri, R., Cox, T., Yasayko, S.A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat. Immunol. *4*, 337–342. https://doi.org/10.1038/ni909.

21. Tanoue, T., and Honda, K. (2012). Induction of Treg cells in the mouse colonic mucosa: a central mechanism to maintain host-microbiota homeostasis. Semin. Immunol. *24*, 50–57. https://doi.org/10.1016/j.smim.2011.11.009.

22. Josefowicz, S.Z., Niec, R.E., Kim, H.Y., Treuting, P., Chinen, T., Zheng, Y., Umetsu, D.T., and Rudensky, A.Y. (2012). Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature *482*, 395–399. https://doi.org/10.1038/nature10772.

23. Kim, K.S., Hong, S.W., Han, D., Yi, J., Jung, J., Yang, B.G., Lee, J.Y., Lee, M., and Surh, C.D. (2016). Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. Science *351*, 858–863. https://doi.org/10.1126/science.aac5560.

24. Campbell, C., Dikiy, S., Bhattarai, S.K., Chinen, T., Matheis, F., Calafiore, M., Hoyos, B., Hanash, A., Mucida, D., Bucci, V., and Rudensky, A.Y. (2018). Extrathymically generated regulatory T cells establish a niche for intestinal border-dwelling bacteria and affect physiologic metabolite balance. Immunity *48*, 1245–1257.e9. https://doi.org/10.1016/j.immuni.2018.04.013.

25. Klein, L., Kyewski, B., Allen, P.M., and Hogquist, K.A. (2014). Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat. Rev. Immunol. *14*, 377–391. https://doi.org/10.1038/nri3667.

26. Legoux, F.P., Lim, J.B., Cauley, A.W., Dikiy, S., Ertelt, J., Mariani, T.J., Sparwasser, T., Way, S.S., and Moon, J.J. (2015). CD4+ T cell tolerance to tissue-restricted self antigens is mediated by antigen-specific regulatory T cells rather than deletion. Immunity *43*, 896–908. https://doi.org/10.1016/j.immuni.2015.10.011.

27. Nishikawa, H., Kato, T., Tawara, I., Saito, K., Ikeda, H., Kuribayashi, K., Allen, P.M., Schreiber, R.D., Sakaguchi, S., Old, L.J., and Shiku, H. (2005). Definition of target antigens for naturally occurring CD4(+) CD25(+) regulatory T cells. J. Exp. Med. *201*, 681–686. https://doi.org/10.1084/jem.20041959.

28. Malchow, S., Leventhal, D.S., Nishi, S., Fischer, B.I., Shen, L., Paner, G.P., Amit, A.S., Kang, C., Geddes, J.E., Allison, J.P., et al. (2013). Aire-dependent thymic development of tumor-associated regulatory T cells. Science *339*, 1219–1224. https://doi.org/10.1126/science.1233913.

29. Fisson, S., Darrasse-Jèze, G., Litvinova, E., Septier, F., Klatzmann, D., Liblau, R., and Salomon, B.L. (2003). Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. J. Exp. Med. *198*, 737–746. https://doi.org/10.1084/jem.20030686.

30. Wyss, L., Stadinski, B.D., King, C.G., Schallenberg, S., McCarthy, N.I., Lee, J.Y., Kretschmer, K., Terracciano, L.M., Anderson, G., Surh, C.D., et al. (2016). Affinity for self antigen selects Treg cells with distinct functional properties. Nat. Immunol. *17*, 1093–1101. https://doi.org/10.1038/ni.3522.

31. Schubert, L.A., Jeffery, E., Zhang, Y., Ramsdell, F., and Ziegler, S.F. (2001). Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J. Biol. Chem. *276*, 37672–37679. https://doi.org/10.1074/jbc.M104521200.

32. Ohkura, N., Hamaguchi, M., Morikawa, H., Sugimura, K., Tanaka, A., Ito, Y., Osaki, M., Tanaka, Y., Yamashita, R., Nakano, N., et al. (2012). T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. Immunity *37*, 785–799. https://doi.org/10.1016/j.immuni.2012.09.010.

33. Spangler, J.B., Tomala, J., Luca, V.C., Jude, K.M., Dong, S., Ring, A.M., Votavova, P., Pepper, M., Kovar, M., and Garcia, K.C. (2015). Antibodies to interleukin-2 elicit selective T cell subset potentiation through distinct conformational mechanisms. Immunity *42*, 815–825. https://doi.org/10.1016/j.immuni.2015.04.015.

34. Toomer, K.H., Lui, J.B., Altman, N.H., Ban, Y., Chen, X., and Malek, T.R. (2019). Essential and non-overlapping IL-2Rα-dependent processes for thymic development and peripheral homeostasis of regulatory T cells. Nat. Commun. *10*, 1037. https://doi.org/10.1038/s41467-019-08960-1.

35. Setoguchi, R., Hori, S., Takahashi, T., and Sakaguchi, S. (2005). Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J. Exp. Med. *201*, 723–735. https://doi.org/10.1084/jem.20041982.

36. Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F., and Campbell, D.J. (2012). Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. Blood *119*, 4430–4440. https://doi.org/10.1182/blood-2011-11-392324.

37. Sharma, A., and Rudra, D. (2018). Emerging functions of regulatory T cells in tissue homeostasis. Front. Immunol. *9*, 883. https://doi.org/10.3389/fimmu.2018.00883.

38. Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., et al. (2009). Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity *30*, 899–911. https://doi.org/10.1016/j.immuni.2009.03.019.

39. Ohue, Y., and Nishikawa, H. (2019). Regulatory T (Treg) cells in cancer: can Treg cells be a new therapeutic target? Cancer Sci. *110*, 2080–2089. https://doi.org/10.1111/cas.14069.

40. Shang, B., Liu, Y., Jiang, S.J., and Liu, Y. (2015). Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. Sci. Rep. *5*, 15179. https://doi.org/10.1038/srep15179.

41. Saito, T., Nishikawa, H., Wada, H., Nagano, Y., Sugiyama, D., Atarashi, K., Maeda, Y., Hamaguchi, M., Ohkura, N., Sato, E., et al. (2016). Two FOXP3(+)CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. Nat. Med. *22*, 679–684. https://doi.org/10.1038/nm.4086.

42. Sato, E., Olson, S.H., Ahn, J., Bundy, B., Nishikawa, H., Qian, F., Jungbluth, A.A., Frosina, D., Gnjatic, S., Ambrosone, C., et al. (2005). Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc. Natl. Acad. Sci. USA *102*, 18538–18543. https://doi.org/10.1073/pnas.0509182102.

43. Saleh, R., and Elkord, E. (2020). FoxP3+ T regulatory cells in cancer: prognostic biomarkers and therapeutic targets. Cancer Lett. *490*, 174–185. https://doi.org/10.1016/j.canlet.2020.07.022.

44. Schubert, D., Bode, C., Kenefeck, R., Hou, T.Z., Wing, J.B., Kennedy, A., Bulashevska, A., Petersen, B.S., Schäffer, A.A., Grüning, B.A., et al. (2014). Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. Nat. Med. *20*, 1410–1416. https://doi.org/10.1038/nm.3746.

45. Kuehn, H.S., Ouyang, W., Lo, B., Deenick, E.K., Niemela, J.E., Avery, D.T., Schickel, J.N., Tran, D.Q., Stoddard, J., Zhang, Y., et al. (2014). Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. Science *345*, 1623–1627. https://doi.org/10.1126/science.1255904.

46. Tekguc, M., Wing, J.B., Osaki, M., Long, J., and Sakaguchi, S. (2021). Treg-expressed CTLA-4 depletes CD80/CD86 by trogocytosis, releasing free PD-L1 on antigen-presenting cells. Proc. Natl. Acad. Sci. USA *118*, e2023739. https://doi.org/10.1073/pnas.2023739118.

47. Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E.M., Baker, J., Jeffery, L.E., Kaur, S., Briggs, Z., et al. (2011). Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science *332*, 600–603. https://doi.org/10.1126/science.1202947.

48. Onishi, Y., Fehervari, Z., Yamaguchi, T., and Sakaguchi, S. (2008). Foxp3(+) natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc. Natl. Acad. Sci. USA *105*, 10113–10118. https://doi.org/10.1073/Pnas.0711106105.

49. Kidani, Y., Nogami, W., Yasumizu, Y., Kawashima, A., Tanaka, A., Sonoda, Y., Tona, Y., Nashiki, K., Matsumoto, R., Hagiwara, M., et al. (2022). CCR8-targeted specific depletion of clonally expanded Treg cells in tumor tissues evokes potent tumor immunity with long-lasting memory. Proc. Natl. Acad. Sci. USA *119*. e2114282119. https://doi.org/10.1073/pnas.2114282119.

50. Butte, M.J., Keir, M.E., Phamduy, T.B., Sharpe, A.H., and Freeman, G.J. (2007). Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity *27*, 111–122. https://doi.org/10.1016/j.immuni.2007.05.016.

51. Chaudhri, A., Xiao, Y., Klee, A.N., Wang, X., Zhu, B., and Freeman, G.J. (2018). PD-L1 Binds to B7-1 Only in cis on the same cell surface. Cancer Immunol. Res. *6*, 921–929. https://doi.org/10.1158/2326-6066.CIR-17-0316.

52. Sugiura, D., Maruhashi, T., Okazaki, I.M., Shimizu, K., Maeda, T.K., Takemoto, T., and Okazaki, T. (2019). Restriction of PD-1 function by by cis-PD-L1/CD80 interactions is required for optimal T cell responses. Science *364*, 558–566. https://doi.org/10.1126/science.aav7062.

53. Zhao, Y., Lee, C.K., Lin, C.H., Gassen, R.B., Xu, X., Huang, Z., Xiao, C., Bonorino, C., Lu, L.F., Bui, J.D., and Hui, E. (2019). PD-L1:CD80 Cis-Heterodimer triggers the Co-stimulatory receptor CD28 while repressing the inhibitory PD-1 and CTLA-4 pathways. Immunity *51*, 1059–1073.e9. https://doi.org/10.1016/j.immuni.2019.11.003.

54. Sugiura, D., Okazaki, I.M., Maeda, T.K., Maruhashi, T., Shimizu, K., Arakaki, R., Takemoto, T., Ishimaru, N., and Okazaki, T. (2022). PD-1 agonism by anti-CD80 inhibits T cell activation and alleviates autoimmunity. Nat. Immunol. *23*, 399–410. https://doi.org/10.1038/s41590-021-01125-7.

55. Pol, J.G., Caudana, P., Paillet, J., Piaggio, E., and Kroemer, G. (2020). Effects of interleukin-2 in immunostimulation and immunosuppression. J. Exp. Med. *217*, e20191247. https://doi.org/10.1084/jem.20191247.

56. Sakaguchi, S., Mikami, N., Wing, J.B., Tanaka, A., Ichiyama, K., and Ohkura, N. (2020). Regulatory T cells and human disease. Annu. Rev. Immunol. *38*, 541–566. https://doi.org/10.1146/annurev-immunol-042718-041717.

57. Schuler, P.J., Saze, Z., Hong, C.S., Muller, L., Gillespie, D.G., Cheng, D., Harasymczuk, M., Mandapathil, M., Lang, S., Jackson, E.K., and Whiteside, T.L. (2014). Human CD4+ CD39+ regulatory T cells produce adenosine upon co-expression of surface CD73 or contact with CD73+ exosomes or CD73+ cells. Clin. Exp. Immunol. *177*, 531–543. https://doi.org/10.1111/cei.12354.

58. Allard, B., Longhi, M.S., Robson, S.C., and Stagg, J. (2017). The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. Immunol. Rev. *276*, 121–144. https://doi.org/10.1111/imr.12528.

59. Medina, C.B., Chiu, Y.H., Stremska, M.E., Lucas, C.D., Poon, I., Tung, K.S., Elliott, M.R., Desai, B., Lorenz, U.M., Bayliss, D.A., and Ravichandran, K.S. (2021). Pannexin 1 channels facilitate communication between T cells to restrict the severity of airway inflammation. Immunity *54*, 1715–1727.e7. https://doi.org/10.1016/j.immuni.2021.06.014.

60. Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., et al. (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity *28*, 546–558. https://doi.org/10.1016/j.immuni.2008.02.017.

61. Cretney, E., Xin, A., Shi, W., Minnich, M., Masson, F., Miasari, M., Belz, G.T., Smyth, G.K., Busslinger, M., Nutt, S.L., and Kallies, A. (2011). The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. Nat. Immunol. *12*, 304–311. https://doi.org/10.1038/ni.2006.

62. Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S., and Vignali, D.A.A. (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature *450*, 566–569. https://doi.org/10.1038/nature06306.

63. Wei, X., Zhang, J., Gu, Q., Huang, M., Zhang, W., Guo, J., and Zhou, X. (2017). Reciprocal expression of IL-35 and IL-10 defines two distinct effector Treg subsets that are required for maintenance of immune tolerance. Cell Rep. *21*, 1853–1869. https://doi.org/10.1016/j.celrep.2017.10.090.

64. Probst-Kepper, M., Geffers, R., Kröger, A., Viegas, N., Erck, C., Hecht, H.J., Lünsdorf, H., Roubin, R., Moharregh-Khiabani, D., Wagner, K., et al. (2009). GARP: a key receptor controlling FOXP3 in human regulatory T cells. J. Cell Mol. Med. *13*, 3343–3357. https://doi.org/10.1111/j.1582-4934.2009.00782.x.

65. Edwards, J.P., Fujii, H., Zhou, A.X., Creemers, J., Unutmaz, D., and Shevach, E.M. (2013). Regulation of the expression of GARP/latent TGF-β1 complexes on mouse T cells and their role in regulatory T cell and Th17 differentiation. J. Immunol. *190*, 5506–5515. https://doi.org/10.4049/jimmunol.1300199.

66. Campbell, M.G., Cormier, A., Ito, S., Seed, R.I., Bondesson, A.J., Lou, J., Marks, J.D., Baron, J.L., Cheng, Y., and Nishimura, S.L. (2020). Cryo-EM reveals integrin-mediated TGF-β activation without release from latent TGF-β. Cell *180*, 490–501.e16. https://doi.org/10.1016/j.cell.2019.12.030.

67. Eschborn, M., Weigmann, B., Reissig, S., Waisman, A., Saloga, J., and Bellinghausen, I. (2015). Activated glycoprotein A repetitions predominant (GARP)-expressing regulatory T cells inhibit allergen-induced intestinal inflammation in humanized mice. J. Allergy Clin. Immunol. *136*, 159–168. https://doi.org/10.1016/j.jaci.2015.04.020.

68. Seed, R.I., Kobayashi, K., Ito, S., Takasaka, N., Cormier, A., Jespersen, J.M., Publicover, J., Trilok, S., Combes, A.J., Chew, N.W., et al. (2021). A tumor-specific mechanism of Treg enrichment mediated by the integrin avB8. Sci. Immunol. *6*, eabf0558. https://doi.org/10.1126/sciimmunol.abf0558.

69. Vang, T., Torgersen, K.M., Sundvold, V., Saxena, M., Levy, F.O., Skålhegg, B.S., Hansson, V., Mustelin, T., and Taskén, K. (2001). Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. J. Exp. Med. *193*, 497–507. https://doi.org/10.1084/jem.193.4.497.

70. Tasken, K., and Ruppelt, A. (2006). Negative regulation of T-cell receptor activation by the cAMP-PKA-Csk signalling pathway in T-cell lipid rafts. Front. Biosci. *11*, 2929–2939. https://doi.org/10.2741/2022.

71. Abrahamsen, H., Baillie, G., Ngai, J., Vang, T., Nika, K., Ruppelt, A., Mustelin, T., Zaccolo, M., Houslay, M., and Taskén, K. (2004). TCR- and CD28-mediated recruitment of phosphodiesterase 4 to lipid rafts potentiates TCR signaling. J. Immunol. *173*, 4847–4858. https://doi.org/10.4049/jimmunol.173.8.4847.

72. Bopp, T., Becker, C., Klein, M., Klein-Hessling, S., Palmetshofer, A., Serfling, E., Heib, V., Becker, M., Kubach, J., Schmitt, S., et al. (2007). Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. J. Exp. Med. *204*, 1303–1310. https://doi.org/10.1084/jem.20062129.

73. Klein, M., and Bopp, T. (2016). Cyclic AMP represents a crucial component of Treg cell-mediated immune regulation. Front. Immunol. *7*, 315. https://doi.org/10.3389/fimmu.2016.00315.

74. Kuczma, M., Lee, J.R., and Kraj, P. (2011). Connexin 43 signaling enhances the generation of Foxp3+ regulatory T cells. J. Immunol. *187*, 248–257. https://doi.org/10.4049/jimmunol.1003785.

75. Vick, S.C., Kolupaev, O.V., Perou, C.M., and Serody, J.S. (2021). Anti-PD-1 checkpoint therapy can promote the function and survival of regulatory T cells. J. Immunol. *207*, 2598–2607. https://doi.org/10.4049/jimmunol.2001334.

76. Wei, Z., and Zhang, Y. (2022). Immune cells in hyperprogressive disease under immune checkpoint-based immunotherapy. Cells *11*, e111758. https://doi.org/10.3390/cells11111758.

77. Wakiyama, H., Kato, T., Furusawa, A., Okada, R., Inagaki, F., Furumoto, H., Fukushima, H., Okuyama, S., Choyke, P.L., and Kobayashi, H. (2022). Treg-dominant tumor microenvironment is responsible for hyperprogressive disease after PD-1 blockade therapy. Cancer Immunol. Res. *10*, 1386–1397. https://doi.org/10.1158/2326-6066.CIR-22-0041.

78. Kumagai, S., Togashi, Y., Kamada, T., Sugiyama, E., Nishinakamura, H., Takeuchi, Y., Vitaly, K., Itahashi, K., Maeda, Y., Matsui, S., et al. (2020). The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. Nat. Immunol. *21*, 1346–1358. https://doi.org/10.1038/s41590-020-0769-3.

79. Kim, M.J., Kim, K., Park, H.J., Kim, G.R., Hong, K.H., Oh, J.H., Son, J., Park, D.J., Kim, D., Choi, J.M., et al. (2023). Deletion of PD-1 destabilizes the lineage identity and metabolic fitness of tumor-infiltrating regulatory T cells. Nat. Immunol. *24*, 148–161. https://doi.org/10.1038/s41590-022-01373-1.

80. Dodagatta-Marri, E., Meyer, D.S., Reeves, M.Q., Paniagua, R., To, M.D., Binnewies, M., Broz, M.L., Mori, H., Wu, D., Adoumie, M., et al. (2019). α-PD-1 therapy elevates Treg/Th balance and increases tumor cell pSmad3 that are both targeted by α-TGFβ antibody to promote durable rejection and immunity in squamous cell carcinomas. J. Immunother. Cancer *7*, 62. https://doi.org/10.1186/s40425-018-0493-9.

81. Perry, J.A., Shallberg, L., Clark, J.T., Gullicksrud, J.A., DeLong, J.H., Douglas, B.B., Hart, A.P., Lanzar, Z., O'Dea, K., Konradt, C., et al. (2022). PD-L1-PD-1 interactions limit effector regulatory T cell populations at homeostasis and during infection. Nat. Immunol. *23*, 743–756. https://doi.org/10.1038/s41590-022-01170-w.

82. Allard, B., Pommey, S., Smyth, M.J., and Stagg, J. (2013). Targeting CD73 enhances the antitumor activity of anti-PD-1 and anti-CTLA-4 mAbs. Clin. Cancer Res. *19*, 5626–5635. https://doi.org/10.1158/1078-0432.CCR-13-0545.

83. González-Navajas, J.M., Fan, D.D., Yang, S., Yang, F.M., Lozano-Ruiz, B., Shen, L., and Lee, J. (2021). The impact of Tregs on the anticancer immunity and the efficacy of immune checkpoint inhibitor therapies. Front. Immunol. *12*, 625783. https://doi.org/10.3389/fimmu.2021.625783.

84. Sato, Y., Casson, C.N., Matsuda, A., Kim, J.I., Shi, J.Q., Iwasaki, S., Chen, S., Modrell, B., Chan, C., Tavares, D., et al. (2022). Fc-independent functions of anti-CTLA-4 antibodies contribute to anti-tumor efficacy. Cancer Immunol. Immunother. *71*, 2421–2431. https://doi.org/10.1007/s00262-022-03170-z.

85. Arce Vargas, F., Furness, A.J.S., Litchfield, K., Joshi, K., Rosenthal, R., Ghorani, E., Solomon, I., Lesko, M.H., Ruef, N., Roddie, C., et al. (2018). Fc effector function contributes to the activity of human anti-CTLA-4 antibodies. Cancer Cell *33*, 649–663.e4. https://doi.org/10.1016/j.ccell.2018.02.010.

86. Sanseviero, E., O'Brien, E.M., Karras, J.R., Shabaneh, T.B., Aksoy, B.A., Xu, W., Zheng, C., Yin, X., Xu, X., Karakousis, G.C., et al. (2019). Anti-CTLA-4 activates intratumoral NK cells and combined with IL15/IL15Rα complexes enhances tumor control. Cancer Immunol. Res. *7*, 1371–1380. https://doi.org/10.1158/2326-6066.CIR-18-0386.

87. Simpson, T.R., Li, F., Montalvo-Ortiz, W., Sepulveda, M.A., Bergerhoff, K., Arce, F., Roddie, C., Henry, J.Y., Yagita, H., Wolchok, J.D., et al. (2013). Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J. Exp. Med. *210*, 1695–1710. https://doi.org/10.1084/jem.20130579.

88. Seidel, J.A., Otsuka, A., and Kabashima, K. (2018). Anti-PD-1 and anti-CTLA-4 therapies in cancer: mechanisms of action, efficacy, and limitations. Front. Oncol. *8*, 86. https://doi.org/10.3389/fonc.2018.00086.

89. Gan, X., Shan, Q., Li, H., Janssens, R., Shen, Y., He, Y., Chen, F., van Haperen, R., Drabek, D., Li, J., et al. (2022). An anti-CTLA-4 heavy chain-only antibody with enhanced Treg depletion shows excellent preclinical efficacy and safety profile. Proc. Natl. Acad. Sci. USA *119*. e2200879119. https://doi.org/10.1073/pnas.2200879119.

90. Burton, E.M., and Tawbi, H.A. (2021). Bispecific antibodies to PD-1 and CTLA4: doubling down on T cells to decouple efficacy from toxicity. Cancer Discov. *11*, 1008–1010. https://doi.org/10.1158/2159-8290.CD-21-0257.

91. Dovedi, S.J., Elder, M.J., Yang, C., Sitnikova, S.I., Irving, L., Hansen, A., Hair, J., Jones, D.C., Hasani, S., Wang, B., et al. (2021). Design and efficacy of a monovalent bispecific PD-1/CTLA4 antibody that enhances CTLA4 blockade on PD-1+ activated T-cells. Cancer Discov. *11*, 1100–1117. https://doi.org/10.1158/2159-8290.CD-20-1445.

92. Berezhnoy, A., Sumrow, B.J., Stahl, K., Shah, K., Liu, D., Li, J., Hao, S.S., De Costa, A., Kaul, S., Bendell, J., et al. (2020). Development and preliminary clinical activity of PD-1-guided CTLA-4 blocking bispecific DART molecule. Cell Rep. Med. *1*, 100163. https://doi.org/10.1016/j.xcrm.2020.100163.

93. Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C., et al. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. N. Engl. J. Med. *363*, 711–723. https://doi.org/10.1056/NEJMoa1003466.

94. Tian, J., Zhang, B., Rui, K., and Wang, S. (2020). The role of GITR/GITRL interaction in autoimmune diseases. Front. Immunol. *11*, 588682. https://doi.org/10.3389/fimmu.2020.588682.

95. Ke, S., Xie, F., Guo, Y., Chen, J., Wang, Z., Yu, Y., Geng, H., Xu, D., Liu, X., Xia, X., et al. (2022). High-level of intratumoral GITR+ CD4 T cells associate with poor prognosis in gastric cancer. iScience *25*, 105529. https://doi.org/10.1016/j.isci.2022.105529.

96. Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y., and Sakaguchi, S. (2002). Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat. Immunol. *3*, 135–142. https://doi.org/10.1038/ni759.

97. Ephrem, A., Epstein, A.L., Stephens, G.L., Thornton, A.M., Glass, D., and Shevach, E.M. (2013). Modulation of Treg cells/T effector function by GITR signaling is context-dependent. Eur. J. Immunol. *43*, 2421–2429. https://doi.org/10.1002/eji.201343451.

98. Mahne, A.E., Mauze, S., Joyce-Shaikh, B., Xia, J., Bowman, E.P., Beebe, A.M., Cua, D.J., and Jain, R. (2017). Dual roles for regulatory T-cell depletion and costimulatory signaling in agonistic GITR targeting for tumor immunotherapy. Cancer Res. *77*, 1108–1118. https://doi.org/10.1158/0008-5472.CAN-16-0797.

99. Amoozgar, Z., Kloepper, J., Ren, J., Tay, R.E., Kazer, S.W., Kiner, E., Krishnan, S., Posada, J.M., Ghosh, M., Mamessier, E., et al. (2021). Targeting Treg cells with GITR activation alleviates resistance to immunotherapy in murine glioblastomas. Nat. Commun. *12*, 2582. https://doi.org/10.1038/s41467-021-22885-8.

100. Ko, K., Yamazaki, S., Nakamura, K., Nishioka, T., Hirota, K., Yamaguchi, T., Shimizu, J., Nomura, T., Chiba, T., and Sakaguchi, S. (2005). Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells. J. Exp. Med. *202*, 885–891. https://doi.org/10.1084/jem.20050940.

101. Cohen, A.D., Schaer, D.A., Liu, C., Li, Y., Hirschhorn-Cymmerman, D., Kim, S.C., Diab, A., Rizzuto, G., Duan, F., Perales, M.A., et al. (2010). Agonist anti-GITR monoclonal antibody induces melanoma tumor immunity in mice by altering regulatory T cell stability and intra-tumor accumulation. PLoS One *5*, e10436. https://doi.org/10.1371/journal.pone.0010436.

102. Chan, S., Belmar, N., Ho, S., Rogers, B., Stickler, M., Graham, M., Lee, E., Tran, N., Zhang, D., Gupta, P., et al. (2022). An anti-PD-1-GITR-L bispecific agonist induces GITR clustering-mediated T cell activation for cancer immunotherapy. Nat. Can. (Que.) *3*, 337–354. https://doi.org/10.1038/s43018-022-00334-9.

103. Zhang, Y., Maksimovic, J., Naselli, G., Qian, J., Chopin, M., Blewitt, M.E., Oshlack, A., and Harrison, L.C. (2013). Genome-wide DNA methylation analysis identifies hypomethylated genes regulated by FOXP3 in human regulatory T cells. Blood *122*, 2823–2836. https://doi.org/10.1182/blood-2013-02-481788.

104. Fourcade, J., Sun, Z., Chauvin, J.M., Ka, M., Davar, D., Pagliano, O., Wang, H., Saada, S., Menna, C., Amin, R., et al. (2018). CD226 opposes TIGIT to disrupt Tregs in melanoma. JCI Insight *3*, e121157. https://doi.org/10.1172/jci.insight.121157.

105. Preillon, J., Cuende, J., Rabolli, V., Garnero, L., Mercier, M., Wald, N., Pappalardo, A., Denies, S., Jamart, D., Michaux, A.C., et al. (2021). Restoration of T-cell effector function, depletion of Tregs, and direct killing of tumor cells: the multiple mechanisms of action of a-TIGIT antagonist antibodies. Mol. Cancer Therapeut. *20*, 121–131. https://doi.org/10.1158/1535-7163.MCT-20-0464.

106. Chen, F., Xu, Y., Chen, Y., and Shan, S. (2020). TIGIT enhances CD4+ regulatory T-cell response and mediates immune suppression in a murine ovarian cancer model. Cancer Med. *9*, 3584–3591. https://doi.org/10.1002/cam4.2976.

107. Levin, S.D., Taft, D.W., Brandt, C.S., Bucher, C., Howard, E.D., Chadwick, E.M., Johnston, J., Hammond, A., Bontadelli, K., Ardourel, D., et al. (2011). Vstm3 is a member of the CD28 family and an important modulator of T-cell function. Eur. J. Immunol. *41*, 902–915. https://doi.org/10.1002/eji.201041136.

108. Ge, Z., Peppelenbosch, M.P., Sprengers, D., and Kwekkeboom, J. (2021). TIGIT, the next step towards successful combination immune checkpoint therapy in cancer. Front. Immunol. *12*, 699895. https://doi.org/10.3389/fimmu.2021.699895.

109. Curiel, T.J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-Garcia, J.R., Zhang, L., Burow, M., et al. (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat. Med. *10*, 942–949. https://doi.org/10.1038/nm1093.

110. Marshall, L.A., Marubayashi, S., Jorapur, A., Jacobson, S., Zibinsky, M., Robles, O., Hu, D.X., Jackson, J.J., Pookot, D., Sanchez, J., et al. (2020). Tumors establish resistance to immunotherapy by regulating Treg recruitment via CCR4. J. Immunother. Cancer *8*, e000764. https://doi.org/10.1136/jitc-2020-000764.

111. Robles, O., Jackson, J.J., Marshall, L., Talay, O., Chian, D., Cutler, G., Diokno, R., Hu, D.X., Jacobson, S., Karbarz, E., et al. (2020). Novel piperidinyl-azetidines as potent and selective CCR4 antagonists elicit antitumor response as a single agent and in combination with checkpoint inhibitors. J. Med. Chem. *63*, 8584–8607. https://doi.org/10.1021/acs.jmedchem.0c00988.

112. Sugiyama, D., Nishikawa, H., Maeda, Y., Nishioka, M., Tanemura, A., Katayama, I., Ezoe, S., Kanakura, Y., Sato, E., Fukumori, Y., et al. (2013). Anti-CCR4 mAb selectively depletes effector-type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans. Proc. Natl. Acad. Sci. USA *110*, 17945–17950. https://doi.org/10.1073/pnas.1316796110.

113. Zhang, T., Sun, J., Li, J., Zhao, Y., Zhang, T., Yang, R., and Ma, X. (2021). Safety and efficacy profile of mogamulizumab (Poteligeo) in the treatment of cancers: an update evidence from 14 studies. BMC Cancer *21*, 618. https://doi.org/10.1186/s12885-021-08363-w.

114. Yoshie, O. (2021). CCR4 as a therapeutic target for cancer immunotherapy. Cancers *13*, 5542. https://doi.org/10.3390/cancers13215542.

115. Yi, G., Guo, S., Liu, W., Wang, H., Liu, R., Tsun, A., Jin, G., and Li, B. (2018). Identification and functional analysis of heterogeneous FOXP3+ Treg cell subpopulations in human pancreatic ductal adenocarcinoma. Sci. Bull. *63*, 972–981. https://doi.org/10.1016/j.scib.2018.05.028.

116. Van Damme, H., Dombrecht, B., Kiss, M., Roose, H., Allen, E., Van Overmeire, E., Kancheva, D., Martens, L., Murgaski, A., Bardet, P.M.R., et al. (2021). Therapeutic depletion of CCR8+ tumor-infiltrating regulatory T cells elicits anti tumor immunity and synergizes with anti-PD-1 therapy. J. Immunother. Cancer *9*, e001749. https://doi.org/10.1136/jitc-2020-001749.

117. Campbell, J.R., McDonald, B.R., Mesko, P.B., Siemers, N.O., Singh, P.B., Selby, M., Sproul, T.W., Korman, A.J., Vlach, L.M., Houser, J., et al. (2021). Fc-optimized anti-CCR8 antibody depletes regulatory T cells in human tumor models. Cancer Res. *81*, 2983–2994. https://doi.org/10.1158/0008-5472.CAN-20-3585.

118. Weaver, J.D., Stack, E.C., Buggé, J.A., Hu, C., McGrath, L., Mueller, A., Wong, M., Klebanov, B., Rahman, T., Kaufman, R., et al. (2022). Differential expression of CCR8 in tumors versus normal tissue allows specific depletion of tumor-infiltrating T regulatory cells by GS-1811, a novel Fc-optimized anti-CCR8 antibody. OncoImmunology *11*, 2141007. https://doi.org/10.1080/2162402X.2022.2141007.

119. Whiteside, S.K., Grant, F.M., Gyori, D.S., Conti, A.G., Imianowski, C.J., Kuo, P., Nasrallah, R., Sadiyah, F., Lira, S.A., Tacke, F., et al. (2021). CCR8 marks highly suppressive Treg cells within tumours but is dispensable for their accumulation and suppressive function. Immunology *163*, 512–520. https://doi.org/10.1111/imm.13337.

120. Barsheshet, Y., Wildbaum, G., Levy, E., Vitenshtein, A., Akinseye, C., Griggs, J., Lira, S.A., and Karin, N. (2017). CCR8+Foxp3+ Treg cells as master drivers of immune regulation. Proc. Natl. Acad. Sci. USA *114*, 6086–6091. https://doi.org/10.1073/pnas.1621280114.

121. Vila-Caballer, M., González-Granado, J.M., Zorita, V., Abu Nabah, Y.N., Silvestre-Roig, C., Del Monte-Monge, A., Molina-Sánchez, P., Ait-Oufella, H., Andrés-Manzano, M.J., Sanz, M.J., et al. (2019). Disruption of the CCL1-CCR8 axis inhibits vascular Treg recruitment and function and promotes atherosclerosis in mice. J. Mol. Cell. Cardiol. *132*, 154–163. https://doi.org/10.1016/j.yjmcc.2019.05.009.

122. Tan, M.C.B., Goedegebuure, P.S., Belt, B.A., Flaherty, B., Sankpal, N., Gillanders, W.E., Eberlein, T.J., Hsieh, C.S., and Linehan, D.C. (2009). Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer. J. Immunol. *182*, 1746–1755. https://doi.org/10.4049/jimmunol.182.3.1746.

123. de Oliveira, C.E., Gasparoto, T.H., Pinheiro, C.R., Amôr, N.G., Nogueira, M.R.S., Kaneno, R., Garlet, G.P., Lara, V.S., Silva, J.S., Cavassani, K.A., and Campanelli, A.P. (2017). CCR5-Dependent homing of T regulatory cells to the tumor microenvironment contributes to skin squamous cell carcinoma development. Mol. Cancer Therapeut. *16*, 2871–2880. https://doi.org/10.1158/1535-7163.MCT-17-0341.

124. Facciabene, A., Peng, X., Hagemann, I.S., Balint, K., Barchetti, A., Wang, L.P., Gimotty, P.A., Gilks, C.B., Lal, P., Zhang, L., and Coukos, G. (2011). Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. Nature *475*, 226–230. https://doi.org/10.1038/nature10169.

125. Arce Vargas, F., Furness, A.J.S., Solomon, I., Joshi, K., Mekkaoui, L., Lesko, M.H., Miranda Rota, E., Dahan, R., Georgiou, A., Sledzinska, A., et al. (2017). Fc-optimized anti-CD25 depletes tumor-infiltrating regulatory T cells and synergizes with PD-1 blockade to Eradicate established tumors. Immunity *46*, 577–586. https://doi.org/10.1016/j.immuni.2017.03.013.

126. Solomon, I., Amann, M., Goubier, A., Arce Vargas, F., Zervas, D., Qing, C., Henry, J.Y., Ghorani, E., Akarca, A.U., Marafioti, T., et al. (2020). CD25-Treg-depleting antibodies preserving IL-2 signaling on effector T-cells enhance effector activation and antitumor immunity. Nat. Can. (Que.) *1*, 1153–1166. https://doi.org/10.1038/s43018-020-00133-0.

127. van Elsas, M.J., van der Schoot, J.M.S., Bartels, A., Steuten, K., van Dalen, D., Wijfjes, Z., Figdor, C.G., van Hall, T., van der Burg, S.H., Verdoes, M., and Scheeren, F.A. (2022). Regulatory T cell depletion using a CRISPR fc-optimized CD25 antibody. Int. J. Mol. Sci. *23*, 8707. https://doi.org/10.3390/ijms23158707.

128. Jin, G.H., Hirano, T., and Murakami, M. (2008). Combination treatment with IL-2 and anti-IL-2 mAbs reduces tumor metastasis via NK cell activation. Int. Immunol. *20*, 783–789. https://doi.org/10.1093/intimm/dxn036.

129. Krieg, C., Létourneau, S., Pantaleo, G., and Boyman, O. (2010). Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells. Proc. Natl. Acad. Sci. USA *107*, 11906–11911. https://doi.org/10.1073/pnas.1002569107.

130. Ren, Z., Zhang, A., Sun, Z., Liang, Y., Ye, J., Qiao, J., Li, B., and Fu, Y.X. (2022). Selective delivery of low-affinity IL-2 to PD-1+ T cells rejuvenates antitumor immunity with reduced toxicity. J. Clin. Invest. *132*, e153604. https://doi.org/10.1172/JCI153604.

131. Codarri Deak, L., Nicolini, V., Hashimoto, M., Karagianni, M., Schwalie, P.C., Lauener, L., Varypataki, E.M., Richard, M., Bommer, E., Sam, J., et al. (2022). PD-1-cis IL-2R agonism yields better effectors from stem-like CD8+ T-cells. Nature *610*, 161–172. https://doi.org/10.1038/s41586-022-05192-0.

132. Zhao, B., Gong, W., Ma, A., Chen, J., Velegraki, M., Dong, H., Liu, Z., Wang, L., Okimoto, T., Jones, D.M., et al. (2022). SUSD2 suppresses CD8+ T-cell antitumor immunity by targeting IL-2R signaling. Nat. Immunol. *23*, 1588–1599. https://doi.org/10.1038/s41590-022-01326-8.

133. Gaggero, S., Martinez-Fabregas, J., Cozzani, A., Fyfe, P.K., Leprohon, M., Yang, J., Thomasen, F.E., Winkelmann, H., Magnez, R., Conti, A.G., et al. (2022). IL-2 is inactivated by the acidic pH environment of tumors enabling engineering of a pH-selective mutein. Sci. Immunol. *7*, eade5686. https://doi.org/10.1126/sciimmunol.ade5686.

134. Lin, C., Guo, J., and Jia, R. (2022). Roles of regulatory T cell-derived extracellular vesicles in human diseases. Int. J. Mol. Sci. *23*, 11206. https://doi.org/10.3390/ijms231911206.

135. Schenk, U., Frascoli, M., Proietti, M., Geffers, R., Traggiai, E., Buer, J., Ricordi, C., Westendorf, A.M., and Grassi, F. (2011). ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. Sci. Signal. *4*, ra12. https://doi.org/10.1126/scisignal.2001270.

136. Ohta, A., and Sitkovsky, M. (2001). Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature *414*, 916–920. https://doi.org/10.1038/414916a.

137. Rueda, C.M., Jackson, C.M., and Chougnet, C.A. (2016). Regulatory T-cell-mediated suppression of conventional T-cells and dendritic cells by different cAMP intracellular pathways. Front. Immunol. *7*, 216. https://doi.org/10.3389/fimmu.2016.00216.

138. Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., et al. (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J. Exp. Med. *204*, 1257–1265. https://doi.org/10.1084/jem.20062512.

139. Wang, L., Fan, J., Chen, S., Zhang, Y., Curiel, T.J., and Zhang, B. (2013). Graft-versus-host disease is enhanced by selective CD73 blockade in mice. PLoS One *8*, e58397. https://doi.org/10.1371/journal.pone.0058397.

140. Kinsey, G.R., Huang, L., Jaworska, K., Khutsishvili, K., Becker, D.A., Ye, H., Lobo, P.I., and Okusa, M.D. (2012). Autocrine adenosine signaling promotes regulatory T cell-mediated renal protection. J. Am. Soc. Nephrol. *23*, 1528–1537. https://doi.org/10.1681/ASN.2012010070.

141. Lee, D.J., and Taylor, A.W. (2013). Both MC5r and A2Ar are required for protective regulatory immunity in the spleen of post-experimental autoimmune uveitis in mice. J. Immunol. *191*, 4103–4111. https://doi.org/10.4049/jimmunol.1300182.

142. Ehrentraut, H., Westrich, J.A., Eltzschig, H.K., and Clambey, E.T. (2012). Adora2b adenosine receptor engagement enhances regulatory T cell abundance during endotoxin-induced pulmonary inflammation. PLoS One *7*, e32416. https://doi.org/10.1371/journal.pone.0032416.

143. Sun, X., Wu, Y., Gao, W., Enjyoji, K., Csizmadia, E., Müller, C.E., Murakami, T., and Robson, S.C. (2010). CD39/ENTPD1 expression by CD4+Foxp3+ regulatory T cells promotes hepatic metastatic tumor growth in mice. Gastroenterology *139*, 1030–1040. https://doi.org/10.1053/j.gastro.2010.05.007.

144. Stagg, J., Divisekera, U., Duret, H., Sparwasser, T., Teng, M.W.L., Darcy, P.K., and Smyth, M.J. (2011). CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. Cancer Res. *71*, 2892–2900. https://doi.org/10.1158/0008-5472.CAN-10-4246.

145. Clayton, A., Al-Taei, S., Webber, J., Mason, M.D., and Tabi, Z. (2011). Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. J. Immunol. *187*, 676–683. https://doi.org/10.4049/jimmunol.1003884.

146. Perrot, I., Michaud, H.A., Giraudon-Paoli, M., Augier, S., Docquier, A., Gros, L., Courtois, R., Déjou, C., Jecko, D., Becquart, O., et al. (2019). Blocking antibodies targeting the CD39/CD73 immunosuppressive pathway unleash immune responses in combination cancer therapies. Cell Rep. *27*, 2411–2425.e9. https://doi.org/10.1016/j.celrep.2019.04.091.

147. Aswad, F., Kawamura, H., and Dennert, G. (2005). High sensitivity of CD4+CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. J. Immunol. *175*, 3075–3083. https://doi.org/10.4049/jimmunol.175.5.3075.

148. Maj, T., Wang, W., Crespo, J., Zhang, H., Wang, W., Wei, S., Zhao, L., Vatan, L., Shao, I., Szeliga, W., et al. (2017). Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. Nat. Immunol. *18*, 1332–1341. https://doi.org/10.1038/ni.3868.

149. Sawant, D.V., Yano, H., Chikina, M., Zhang, Q., Liao, M., Liu, C., Callahan, D.J., Sun, Z., Sun, T., Tabib, T., et al. (2019). Adaptive plasticity of IL-10 and IL-35 Treg cells cooperatively promotes tumor T cell exhaustion. Nat. Immunol. *20*, 724–735. https://doi.org/10.1038/s41590-019-0346-9.

150. Tanaka, A., Maeda, S., Nomura, T., Llamas-Covarrubias, M.A., Tanaka, S., Jin, L., Lim, E.L., Morikawa, H., Kitagawa, Y., Akizuki, S., et al. (2023). Construction of a T cell receptor signaling range for spontaneous development of autoimmune disease. J. Exp. Med. *220*, e20220386. https://doi.org/10.1084/jem.20220386.

151. Tanaka, A., Nishikawa, H., Noguchi, S., Sugiyama, D., Morikawa, H., Takeuchi, Y., Ha, D., Shigeta, N., Kitawaki, T., Maeda, Y., et al. (2020). Tyrosine kinase inhibitor imatinib augments tumor immunity by depleting effector regulatory T cells. J. Exp. Med. *217*, e20191009. https://doi.org/10.1084/jem.20191009.

152. Kantarjian, H.M., Shah, N.P., Cortes, J.E., Baccarani, M., Agarwal, M.B., Undurraga, M.S., Wang, J., Ipiña, J.J.K., Kim, D.W., Ogura, M., et al. (2012). Dasatinib or imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: 2-year follow-up from a randomized phase 3 trial (DASISION). Blood *119*, 1123–1129. https://doi.org/10.1182/blood-2011-08-376087.

153. Chang, C.H., Qiu, J., O'Sullivan, D., Buck, M.D., Noguchi, T., Curtis, J.D., Chen, Q., Gindin, M., Gubin, M.M., van der Windt, G.J.W., et al. (2015). Metabolic competition in the tumor microenvironment is a driver of cancer progression. Cell *162*, 1229–1241. https://doi.org/10.1016/j.cell.2015.08.016.

154. Pacella, I., Procaccini, C., Focaccetti, C., Miacci, S., Timperi, E., Faicchia, D., Severa, M., Rizzo, F., Coccia, E.M., Bonacina, F., et al. (2018). Fatty acid metabolism complements glycolysis in the selective regulatory T cell expansion during tumor growth. Proc. Natl. Acad. Sci. USA *115*, E6546–E6555. https://doi.org/10.1073/pnas.1720113115.

155. Angelin, A., Gil-de-Gómez, L., Dahiya, S., Jiao, J., Guo, L., Levine, M.H., Wang, Z., Quinn, W.J., Kopinski, P.K., Wang, L., et al. (2017). Foxp3 reprograms T cell metabolism to function in low-glucose, high-lactate environments. Cell Metabol. *25*, 1282–1293.e7. https://doi.org/10.1016/j.cmet.2016.12.018.

156. Watson, M.J., Vignali, P.D.A., Mullett, S.J., Overacre-Delgoffe, A.E., Peralta, R.M., Grebinoski, S., Menk, A.V., Rittenhouse, N.L., DePeaux, K., Whetstone, R.D., et al. (2021). Metabolic support of tumour-infiltrating regulatory T cells by lactic acid. Nature *591*, 645–651. https://doi.org/10.1038/s41586-020-03045-2.

157. Kumagai, S., Koyama, S., Itahashi, K., Tanegashima, T., Lin, Y.T., Togashi, Y., Kamada, T., Irie, T., Okumura, G., Kono, H., et al. (2022). Lactic acid promotes PD-1 expression in regulatory T cells in highly glycolytic tumor microenvironments. Cancer Cell *40*, 201–218.e9. https://doi.org/10.1016/j.ccell.2022.01.001.

158. Bhattacharyya, S., Md Sakib Hossain, D., Mohanty, S., Sankar Sen, G., Chattopadhyay, S., Banerjee, S., Chakraborty, J., Das, K., Sarkar, D., Das, T., and Sa, G. (2010). Curcumin reverses T cell-mediated adaptive immune dysfunctions in tumor-bearing hosts. Cell. Mol. Immunol. *7*, 306–315. https://doi.org/10.1038/cmi.2010.11.

159. MaruYama, T., Kobayashi, S., Nakatsukasa, H., Moritoki, Y., Taguchi, D., Sunagawa, Y., Morimoto, T., Asao, A., Jin, W., Owada, Y., et al. (2021). The curcumin analog GO-Y030 controls the generation and stability of regulatory T cells. Front. Immunol. *12*, 687669. https://doi.org/10.3389/fimmu.2021.687669.

160. Zappasodi, R., Serganova, I., Cohen, I.J., Maeda, M., Shindo, M., Senbabaoglu, Y., Watson, M.J., Leftin, A., Maniyar, R., Verma, S., et al. (2021). CTLA-4 blockade drives loss of Treg stability in glycolysis-low tumors. Nature *591*, 652–658. https://doi.org/10.1038/s41586-021-03326-4.

161. Wang, H., Franco, F., Tsui, Y.C., Xie, X., Trefny, M.P., Zappasodi, R., Mohmood, S.R., Fernández-García, J., Tsai, C.H., Schulze, I., et al. (2020). CD36-mediated metabolic adaptation supports regulatory T cell survival and function in tumors. Nat. Immunol. *21*, 298–308. https://doi.org/10.1038/s41590-019-0589-5.

162. Kumagai, S., Togashi, Y., Sakai, C., Kawazoe, A., Kawazu, M., Ueno, T., Sato, E., Kuwata, T., Kinoshita, T., Yamamoto, M., et al. (2020). An oncogenic alteration creates a microenvironment that promotes tumor progression by conferring a metabolic advantage to regulatory T cells. Immunity *53*, 187–203.e8. https://doi.org/10.1016/j.immuni.2020.06.016.

163. Nagarajan, S.R., Butler, L.M., and Hoy, A.J. (2021). The diversity and breadth of cancer cell fatty acid metabolism. Cancer Metabol. *9*, 2. https://doi.org/10.1186/s40170-020-00237-2.

164. Saravia, J., Zeng, H., Dhungana, Y., Bastardo Blanco, D., Nguyen, T.L.M., Chapman, N.M., Wang, Y., Kanneganti, A., Liu, S., Raynor, J.L., et al. (2020). Homeostasis and transitional activation of regulatory T cells require c-Myc. Sci. Adv. *6*, eaaw6443. https://doi.org/10.1126/sciadv.aaw6443.

165. Raud, B., Roy, D.G., Divakaruni, A.S., Tarasenko, T.N., Franke, R., Ma, E.H., Samborska, B., Hsieh, W.Y., Wong, A.H., Stüve, P., et al. (2018). Etomoxir actions on regulatory and memory T cells are independent of cpt1a-mediated fatty acid oxidation. Cell Metabol. *28*, 504–515.e7. https://doi.org/10.1016/j.cmet.2018.06.002.

166. Manzo, T., Prentice, B.M., Anderson, K.G., Raman, A., Schalck, A., Codreanu, G.S., Nava Lauson, C.B., Tiberti, S., Raimondi, A., Jones, M.A., et al. (2020). Accumulation of long-chain fatty acids in the tumor microenvironment drives dysfunction in intrapancreatic CD8+ T cells. J. Exp. Med. *217*, e20191920. https://doi.org/10.1084/jem.20191920.

167. Lim, S.A., Wei, J., Nguyen, T.L.M., Shi, H., Su, W., Palacios, G., Dhungana, Y., Chapman, N.M., Long, L., Saravia, J., et al. (2021). Lipid signalling enforces functional specialization of Treg cells in tumors. Nature *591*, 306–311. https://doi.org/10.1038/s41586-021-03235-6.

168. Xu, C., Sun, S., Johnson, T., Qi, R., Zhang, S., Zhang, J., and Yang, K. (2021). The glutathione peroxidase Gpx4 prevents lipid peroxidation and ferroptosis to sustain Treg cell activation and suppression of antitumor immunity. Cell Rep. *35*, 109235. https://doi.org/10.1016/j.celrep.2021.109235.

169. Rahma, O.E., and Hodi, F.S. (2019). The intersection between tumor angiogenesis and immune suppression. Clin. Cancer Res. *25*, 5449–5457. https://doi.org/10.1158/1078-0432.CCR-18-1543.

170. Coffelt, S.B., Tal, A.O., Scholz, A., De Palma, M., Patel, S., Urbich, C., Biswas, S.K., Murdoch, C., Plate, K.H., Reiss, Y., and Lewis, C.E. (2010). Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. Cancer Res. *70*, 5270–5280. https://doi.org/10.1158/0008-5472.CAN-10-0012.

171. Coffelt, S.B., Chen, Y.Y., Muthana, M., Welford, A.F., Tal, A.O., Scholz, A., Plate, K.H., Reiss, Y., Murdoch, C., De Palma, M., and Lewis, C.E. (2011). Angiopoietin 2 stimulates TIE2-expressing monocytes to suppress T cell activation and to promote regulatory T cell expansion. J. Immunol. *186*, 4183–4190. https://doi.org/10.4049/jimmunol.1002802.

172. Han, Z., Dong, Y., Lu, J., Yang, F., Zheng, Y., and Yang, H. (2021). Role of hypoxia in inhibiting dendritic cells by VEGF signaling in tumor microenvironments: mechanism and application. Am. J. Cancer Res. *11*, 3777–3793.

173. Bourhis, M., Palle, J., Galy-Fauroux, I., and Terme, M. (2021). Direct and indirect modulation of T cells by VEGF-A counteracted by anti-angiogenic treatment. Front. Immunol. *12*, 616837. https://doi.org/10.3389/fimmu.2021.616837.

174. Kim, C.G., Jang, M., Kim, Y., Leem, G., Kim, K.H., Lee, H., Kim, T.S., Choi, S.J., Kim, H.D., Han, J.W., et al. (2019). VEGF-A drives TOX-dependent T cell exhaustion in anti-PD-1-resistant microsatellite stable colorectal cancers. Sci. Immunol. *4*, eaay0555. https://doi.org/10.1126/sciimmunol.aay0555.

175. Terme, M., Pernot, S., Marcheteau, E., Sandoval, F., Benhamouda, N., Colussi, O., Dubreuil, O., Carpentier, A.F., Tartour, E., and Taieb, J. (2013). VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. Cancer Res. *73*, 539–549. https://doi.org/10.1158/0008-5472.CAN-12-2325.

176. Tada, Y., Togashi, Y., Kotani, D., Kuwata, T., Sato, E., Kawazoe, A., Doi, T., Wada, H., Nishikawa, H., and Shitara, K. (2018). Targeting VEGFR2 with Ramucirumab strongly impacts effector/activated regulatory T cells and CD8. J. Immunother. Cancer *6*, 106. https://doi.org/10.1186/s40425-018-0403-1.

177. Suzuki, H., Onishi, H., Wada, J., Yamasaki, A., Tanaka, H., Nakano, K., Morisaki, T., and Katano, M. (2010). VEGFR2 is selectively expressed by FOXP3high CD4+ Treg. Eur. J. Immunol. *40*, 197–203. https://doi.org/10.1002/eji.200939887.

178. Djordjevic, S., and Driscoll, P.C. (2013). Targeting VEGF signalling via the neuropilin co-receptor. Drug Discov. Today *18*, 447–455. https://doi.org/10.1016/j.drudis.2012.11.013.

179. Wu, X., Giobbie-Hurder, A., Liao, X., Connelly, C., Connolly, E.M., Li, J., Manos, M.P., Lawrence, D., McDermott, D., Severgnini, M., et al. (2017). Angiopoietin-2 as a biomarker and target for immune checkpoint therapy. Cancer Immunol. Res. *5*, 17–28. https://doi.org/10.1158/2326-6066.CIR-16-0206.

180. Schmittnaegel, M., Rigamonti, N., Kadioglu, E., Cassará, A., Wyser Rmili, C., Kiialainen, A., Kienast, Y., Mueller, H.J., Ooi, C.H., Laoui, D., and De Palma, M. (2017). Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. Sci. Transl. Med. *9*, eaak9670. https://doi.org/10.1126/scitranslmed.aak9670.

181. Fukumura, D., Kloepper, J., Amoozgar, Z., Duda, D.G., and Jain, R.K. (2018). Enhancing cancer immunotherapy using antiangiogenics: opportunities and challenges. Nat. Rev. Clin. Oncol. *15*, 325–340. https://doi.org/10.1038/nrclinonc.2018.29.

182. Kudo, M. (2020). Scientific rationale for combined immunotherapy with PD-1/PD-L1 antibodies and VEGF inhibitors in advanced hepatocellular carcinoma. Cancers *12*, 1089. https://doi.org/10.3390/cancers12051089.

183. Ishikura, N., Sugimoto, M., Yorozu, K., Kurasawa, M., and Kondoh, O. (2022). Anti-VEGF antibody triggers the effect of anti-PD-L1 antibody in PD-L1 and immune desert-like mouse tumors. Oncol. Rep. *47*, 36. https://doi.org/10.3892/or.2021.8247.

184. Kwilas, A.R., Ardiani, A., Donahue, R.N., Aftab, D.T., and Hodge, J.W. (2014). Dual effects of a targeted small-molecule inhibitor (cabozantinib) on immune-mediated killing of tumor cells and immune tumor microenvironment permissiveness when combined with a cancer vaccine. J. Transl. Med. *12*, 294. https://doi.org/10.1186/s12967-014-0294-y.

185. Haradhvala, N.J., Leick, M.B., Maurer, K., Gohil, S.H., Larson, R.C., Yao, N., Gallagher, K.M.E., Katsis, K., Frigault, M.J., Southard, J., et al. (2022). Distinct cellular dynamics associated with response to CAR-T therapy for refractory B cell lymphoma. Nat. Med. *28*, 1848–1859. https://doi.org/10.1038/s41591-022-01959-0.

186. Good, Z., Spiegel, J.Y., Sahaf, B., Malipatlolla, M.B., Ehlinger, Z.J., Kurra, S., Desai, M.H., Reynolds, W.D., Wong Lin, A., Vandris, P., et al. (2022). Post-infusion CAR Treg cells identify patients resistant to CD19-CAR therapy. Nat. Med. *28*, 1860–1871. https://doi.org/10.1038/s41591-022-01960-7.