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Intratumoral regulatory T cells: markers, subsets and their impact on anti-tumor immunity

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Summary

Regulatory T (Treg) cells play a crucial role in maintaining self-tolerance and resolution of immune responses by employing multifaceted immunoregulatory mechanisms. However, Treg cells readily infiltrate into the tumor microenvironment (TME) and dampen anti-tumor immune responses, thereby becoming a barrier to effective cancer immunotherapy. There has been a substantial expansion in the development of novel immunotherapies targeting various inhibitory receptors (IRs), such as CTLA4, PD1 and LAG3, but these approaches have mechanistically focused on the elicitation of anti-tumor responses. However, enhanced inflammation in the TME could also play a detrimental role by facilitating the recruitment, stability and function of Treg cells by up-regulating chemokines that promote Treg cell migration, and/or increasing inhibitory cytokine production. Furthermore, IR blockade may enhance Treg cell function and survival, thereby serving as a resistance mechanism against effective immunotherapy. Given that Treg cells are comprised of functionally and phenotypically heterogeneous sub-populations that may alter their characteristics in a context-dependent manner, it is critical to identify unique molecular pathways that are preferentially used by intratumoral Treg cells. In this review, we discuss markers that serve to identify certain Treg cell subsets, distinguished by chemokine receptors, IRs and cytokines that facilitate their migration, stability and function in the TME. We also discuss how these Treg cell subsets correlate with the clinical outcome of patients with various types of cancer and how they may serve as potential TME-specific targets for novel cancer immunotherapies.

Keywords: chemokine/chemokine receptors; cytokines; inhibitory/activating receptors; regulatory T cells; tumor immunology.

Introduction

Regulatory T (Treg) cells are a subset of $CD4^+$ T cells characterized by their expression of a key transcription factor forkhead box P3 (FoxP3).[1] Treg cells play a crucial role in the maintenance of self-tolerance and resolution of inflammation.[2] Mutations within the *Foxp3* gene result in defective Treg cell development, leading to lethal systemic auto-immune diseases in both humans[3] and mice.[4] Treg cells regulate immune responses through four major mechanisms: metabolic regulation, direct cytolysis, regulation of antigen-presenting cells, and secretion of inhibitory cytokines.[2]

However, Treg cells play a detrimental role in the context of cancer. Treg cells readily infiltrate into the tumor microenvironment (TME) and play a significant role in suppressing anti-tumor immune responses,[5–8] making them a barrier to effective cancer immunotherapy. Indeed, an increase in intratumoral Treg cells has been correlated with poor patient prognosis in many cancer types, including ovarian carcinoma.[5] However, there have been reports

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suggesting that the infiltration of FoxP3$^+$ Treg cells can be a favorable prognostic marker for certain types of cancer, such as colorectal cancer,[9] although this may also be an indirect consequence of enhanced overall T-cell infiltration. Importantly, while Foxp3 expression is a faithful marker to identify Treg cells in mice, human FoxP3$^+$ CD4$^+$ T cells are not necessarily a homogeneously immunosuppressive population. Human FoxP3$^+$ CD4$^+$ T cells can be stratified into three subsets: CD45RA$^+$ FoxP3^{lo} (resting Treg cells), CD45RA$^-$ FoxP3^{hi} (activated Treg cells) and CD45RA$^-$ FoxP3^{lo} subsets,[10] with the latter representing recently activated effector T cells with up-regulated expression of pro-inflammatory cytokines.[11] Indeed, enrichment of the CD45RA$^-$ FoxP3^{lo} subset in the TME has been associated with long-term disease-free survival of patients with colorectal cancer,[6] suggesting that previously reported beneficial prognostic correlation with intratumoral FoxP3$^+$ T cells may have been due to a CD45RA$^-$ FoxP3^{lo} effector subset. Hence, activated Treg cell infiltration may be detrimental across all types of cancer.

Treg cells are functionally and phenotypically heterogeneous, altering their 'flavor' in a context-dependent manner,[11] and it is unclear which suppressive mechanism(s) plays a dominant role in the TME. Furthermore, it remains elusive whether distinct subsets of Treg cells exist, or if there is phenotypic plasticity that is modulated based on the microenvironment. It is also unclear if the same or different subpopulations differentially use these regulatory mechanisms. In this review, we focus on key cell surface markers or secreted proteins that have a key impact on the identity and function of different Treg cell subsets, facilitating their infiltration, stability and/or regulatory functions in the TME. We will also discuss correlations between these Treg cell subsets and patient clinical outcome, as well as the development of therapeutic approaches targeting these key cell surface markers or secreted proteins.

Chemokine receptors

Although Treg cells prevent catastrophic systemic autoimmunity,[4] their migratory capacity is a key factor impacting their ability to regulate tissue-restricted inflammation. Targeting chemokine receptors that are preferentially used by tumor-infiltrating Treg cells may therefore be an attractive approach to elicit beneficial anti-tumor immune responses in patients. In this section, we review Treg cell subsets characterized by selective upregulation of C-C chemokine receptors and potential therapeutic opportunities to target these Treg cell subsets (Fig. 1).

C-C chemokine receptor 2

C-C chemokine receptor 2 (CCR2) plays a critical role in the migration of Ly6C$^+$ inflammatory monocytes through interaction with its ligands C-C motif chemokine ligand 2 (CCL2) and CCL7.[12] However, recent studies have demonstrated a chemotactic role for CCR2 in T cells during inflammation.[13] Interestingly, a subset of CCR2$^+$ Treg cells was enriched in both tumor and draining lymph nodes of mice bearing transplantable OVA-expressing murine sarcoma (MCA-OVA), but CCR2-deficient Treg cells failed to infiltrate the TME.[14] Furthermore, CCR2-deficient Treg cells resulted in reduced CD25 expression, rendering them less suppressive,[15] suggesting an alternative non-chemotactic role for CCR2 in Treg cells. CCR2 expression has also been positively correlated with increased expression of inhibitory cytokine interleukin-10 (IL-10) in Treg cells.[16] These observations suggest that CCR2 may play a dual role in tissue-infiltrating Treg cells by facilitating their migration to the inflammatory site and promoting their functional fitness to maintain tissue homeostasis.

The importance of the CCL2–CCR2 axis in tumor development and progression has been reported in various cancer types, such as clear-cell renal cell carcinoma[17] in which high CCL2 and/or CCR2 expression was strongly correlated with poor patient prognosis. These observations suggest that targeting CCR2 may be a practical therapeutic approach to prevent Treg cell infiltration and intrinsically impair their suppressive function in the TME (Table 1).

C-C chemokine receptor 4

C-C chemokine receptor 4 (CCR4) is a high-affinity receptor for CCL17 and CCL22 that is elevated in inflamed tissues and plays a robust chemotactic role on activated T cells.[18] Although only a small fraction of naive Treg cells express CCR4, activated effector Treg cells residing in non-lymphoid tissues, such as skin and lungs, or peripheral activated effector Treg cells show enhanced expression of CCR4,[19] suggesting that CCR4 plays a dual role in directing activated effector T cells while recruiting Treg cells to the site of inflammation to maintain immune homeostasis. Indeed, CCR4-deficient Treg cells were unable to infiltrate localized tissue inflammation and failed to control immune responses in various models of inflammatory disease.[13,19]

Consistent with these observations, infiltration of CCR4$^+$ T cells in the TME has been reported in various types of cancer including lung adenocarcinoma[20] in which increased CCR4$^+$ tumor-infiltrating lymphocytes (TILs) was correlated with poor patient prognosis,[20] suggesting a pro-tumor role of CCR4$^+$ TILs. Administration of an afucosylated humanized anti-CCR4 monoclonal antibody (mAb) (Mogamulizumab; Table 1), which has enhanced capacity for antibody-dependent cellular cytotoxicity due to removal of *N*-glucan attachment sites in the Fc region, in patients with NY-ESO-1-positive adult T-cell leukemia-lymphoma selectively depleted CD4$^+$ FOXP3^{hi} CD45RA$^-$

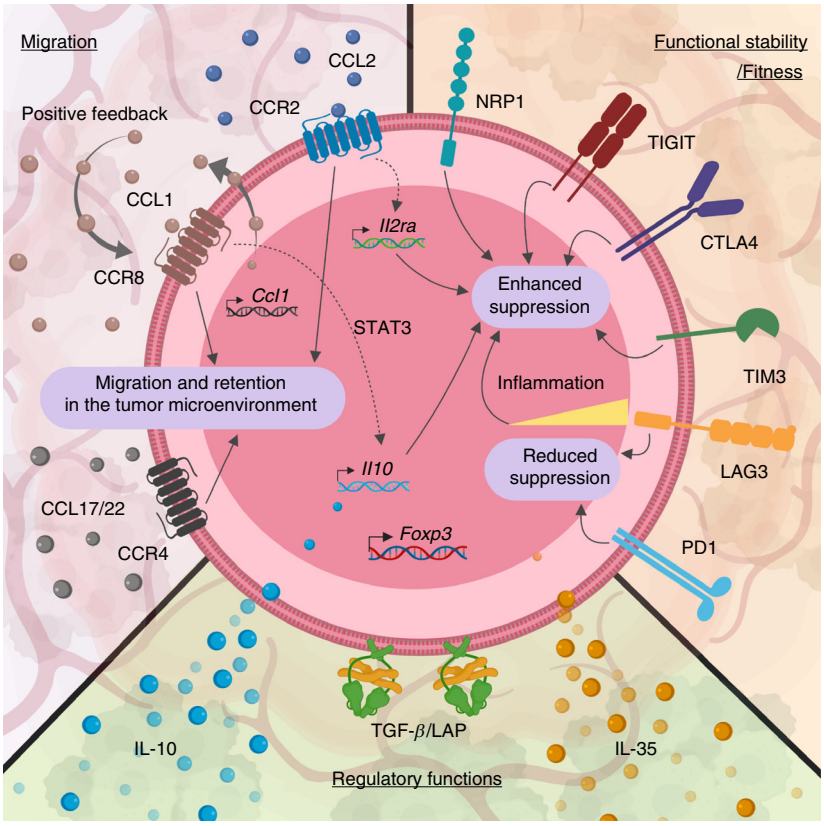

Figure 1. Subset stratification of intratumoral regulatory T (Treg) cells. Heterogeneous intratumoral Treg cells can be characterized based on their expression pattern on functional surface molecules or secretion of inhibitory cytokines. Activated Treg cells up-regulate various chemokine receptors in a context-dependent manner to home to the site of inflammation. Some chemokine receptors, such as CCR8, have been shown to also support Treg function and stability in addition to providing chemotactic navigation to guide Treg cells to the tumor microenvironment (TME). Furthermore, Treg cells also up-regulate numerous inhibitory receptors (IRs), including PD1 and LAG3. Although many of these IRs have been associated with dysfunctional, exhausted $CD8^+$ tumor-infiltrating lymphocytes (TILs), the exact cell-intrinsic role(s) of IRs in intratumoral Treg cells have not been fully elucidated. Some IRs, such as TIGIT, maintain and promote the suppressive function of Treg cells, whereas other IRs, including PD1 and LAG3, have been associated with a reduced suppressive activity of Treg cells. Lastly, there are divergent subpopulations of intratumoral Treg cells secreting different inhibitory cytokines, such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10) and IL-35.

activated Treg-subset and subsequently increased interferon-γ (IFN-γ)/tumor necrosis factor-α production by NY-ESO-1-responsive $CD8^+$ T cells.[21] In addition, given that CCR4 is also highly up-regulated on tumor cells,[22] the mechanism underlying CCR4-targeting clinical efficacy may be through dual-depletion of $CCR4^+$ tumor cells and $CCR4^+$ TILs including Treg cells.

C-C chemokine receptor 8

Early studies identified C-C chemokine receptor 8 (CCR8) as a marker of $CD4^+$ type 2 helper T (Th2) cells.[23] However, CCR8 was later found to be expressed on human peripheral Treg cells, and its ligand CCL1 was able to induce their migration *in vitro*.[24] CCR8-deficient Treg cells showed increased susceptibility to cell death upon allogeneic adoptive transfer and were unable to prevent T cell-induced graft-versus-host disease in lungs and colon,[25] indicating an essential role of CCR8 in preserving long-term fitness and functionality of Treg cells in non-lymphoid organs. Indeed, recent studies have shown that intratumoral Treg cells or normal adjacent tissue-resident Treg cells selectively up-regulate CCR8 expression compared with their peripheral counterparts or other T-cell subsets.[26] In addition, the CCR8 expression within $CD45^+$ intratumoral immune cells was almost exclusively on Treg cells in breast cancer;[26] and the enrichment of CCR8 expression has been correlated with worse prognosis in patients with various types of cancer including breast cancer and melanoma.[26]

Interestingly, stimulation with its cognate ligand CCL1, but not other CCR8 ligands such as CCL8, CCL16 and CCL18, enhanced suppressive capacity of human Treg cells *in vitro* in a signal transducer and activator of

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Table 1. Clinical studies of cancer immunotherapy targeting markers associated with tumor-infiltrating Treg cells

Target molecule	Drug	Manufacturer	Description	Phases of trials actively recruiting	Types of cancer tested in clinical trial
CCR2	BMS-813160	Bristol-Myers Squibb	CCR2/5 small molecule inhibitor	Phase I, II	Metastatic colorectal cancer (CRC), pancreatic cancer
CCR4	Mogamulizumab (KW-0761)	Kyowa Kirin Pharmaceutical Development	Monoclonal anti-human CCR4 (afucosylated humanized IgG1)	Phase I, II	Metastatic triple negative breast cancer (TNBC), non-small-cell lung cancer (NSCLC), gastric cancers, locally advanced hepatocellular carcinoma (HCC), relapsed or refractory non-Hodgkin's (NHL), Hodgkin's (HL), diffuse large B-cell lymphomas (DLBCL), esophageal squamous cell cancer, renal cell carcinoma (RCC), oral squamous cell carcinomas
CCR8	No CCR8-targeted agents are currently investigated in cancer immunotherapy clinical trial.			N/A	
CTLA4	Ipilimumab	Bristol-Myers Squibb	Monoclonal anti-human CTLA4 (human IgG1)	Phase I, II, III	Melanoma, salivary gland cancer, head and neck squamous cell carcinoma (HNSCC), brain and hepatic metastasis, DLBCL, bladder cancer, RCC, clear cell renal carcinoma (CCRCC), pancreatic cancer, prostate cancer, CRC, NSCLC, TNBC, Merkel cell carcinoma, HCC, gastric, stomach, esophageal, gastroesophageal, gastroesophageal junction (GEJ) cancer, HL, NHL, acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CML), and other advanced malignancies
	Tremelimumab	MedImmune (Pfizer)	Monoclonal anti-human CTLA4 (human IgG2)	Phase I, II, III	Melanoma, RCC, CCRCC, CRC, HCC, NSCLC, HNSCC, Merkel cell carcinoma, cutaneous T-cell lymphoma (CTCL), glioblastoma, breast cancers, germ cell tumor, non-seminomatous germ cell tumor, seminoma, germinomatous germ cell tumor, dysgerminoma, pineal germ cell tumor, bladder cancer, thyroid cancer, and other advanced malignancies
	AGEN1884	Agenus	Monoclonal anti-human CTLA4 (human IgG1)	Phase I, II	Advanced NSCLC, cervical cancer, advanced solid tumors and lymphomas
	BCD-145	Biocad	Monoclonal anti-human CTLA4 (IgG information unavailable)	Phase I	Melanoma
	REGN4659	Regeneron Pharmaceuticals	Monoclonal anti-human CTLA4 (human IgG1)	Phase I	NSCLC
	ADU-1604	Aduro Biotech	Monoclonal anti-human CTLA4 (humanized IgG1)	Phase I	Metastatic melanoma
	CS1002	CStone Pharmaceuticals	Monoclonal anti-human CTLA4 (human IgG1)	Phase I	Therapy refractory metastatic solid tumors
	MGD019	MacroGenics	Dual-affinity re-targeting (DART) anti-CTLA4/PD1 bearing hinge-stabilized human IgG4 Fc	Phase I	Unresectable, locally advanced or metastatic solid tumors
	MEDI5752	MedImmune	Bispecific monovalent anti-CTLA4/PD1 (Fc-engineered human IgG1)	Phase I	Advanced solid tumors

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Table 1. *(Continued)*

Target molecule	Drug	Manufacturer	Description	Phases of trials actively recruiting	Types of cancer tested in clinical trial
PD1	Pembrolizumab (MK-3475, SCH 900475)	Merck Sharp & Dohme	Monoclonal anti-human PD1 (human IgG4)	Phase I, II, III	Melanoma, TNBC, RCC, CRC, NSCLC, HNSCC, HCC, NHL, small-cell lung carcinoma (SCLC), CTCL, peripheral T-cell lymphoma (PTCL), Merkel cell carcinoma, breast cancers, glioblastoma, pancreatic cancer, mesothelioma, peripheral nerve sheath tumor (MPNST), and other advanced malignancies
	Nivolumab (BMS-936558, MDX-1106, ONO-4538)	Bristol-Myers Squibb	Monoclonal anti-human PD1 (human IgG4)	Phase I, II, III	Melanoma, brain metastasis, HCC, PTCL, HL, NHL, RCC, CCRCC, SCLC, NSCLC, HNSCC, MM, CLL, AML, CRC, DLBCL, bladder cancer, cervical, ovarian, primary peritoneal, Fallopian tube cancers, Merkel cell carcinoma, breast cancers, salivary gland cancer, urinary bladder cancer, biliary tract cancer, pancreatic cancer, thyroid cancer, and other advanced malignancies
	Camrelizumab (SHR-1210)	Incyte Biosciences (Jiangsu Hengrui Medicine)	Monoclonal anti-human PD1 (humanized IgG1)	Phase I, II, III	Melanoma, HL, NSCLC, CRC, gastric, esophageal, gastroesophageal cancers, esophageal squamous cell carcinoma, biliary tract cancer, cholangiocarcinoma, cervical, ovarian, endometrial cancers, HCC, nasopharyngeal carcinoma, breast cancer, primary mediastinal large B-cell lymphoma, RCC, urothelial carcinoma
	Tislelizumab (BGB-A317)	Celgene (BeiGene)	Monoclonal anti-human PD1 (humanized IgG4)	Phase I, II, III	NSCLC, high-level microsatellite instability (MSI-H) or mismatch repair-deficient (dMMR) solid tumors, locally advanced or metastatic solid tumors, gastric cancer, GEJ adenocarcinoma, esophageal squamous cell carcinoma (ESCC)
	BAT1306	Bio-Thera Solutions	Monoclonal anti-human PD1 (humanized IgG information unavailable)	Phase II	MSI-H/dMMR or high TMB CRC
	Toripalimab (JS001, TAB001)	Shanghai Junshi Biosciences	Monoclonal anti-human PD1 (humanized IgG4)	Phase I, II, III	Gastric cancer, ESCC, nasopharyngeal carcinoma, HNSCC, NSCLC, melanoma, RCC, HCC, neuroendocrine tumors, bladder urothelial carcinoma
	JTX-4014	Celgene (Jounce Therapeutics)	Monoclonal anti-human PD1 (human IgG4)	Phase I	Histologically or cytologically confirmed extracranial solid malignancies
	Dostarlimab (TSR-042)	Tesaro (AnaptysBi)	Monoclonal anti-human PD1 (humanized IgG4)	Phase I, II, III	Ovarian cancer, NSCLC, endometrial cancers, MSI-H solid tumors, advanced or metastatic solid tumors
	Cemiplimab-rwlc (REGN2810)	Regeneron Pharmaceuticals (Sanofi)	Monoclonal anti-human PD1 (human IgG4)	Phase I, II, III	Cervical cancer, advanced cutaneous squamous cell carcinoma, HL, NHL, NSCLC, prostate cancer, RCC, glioblastoma, HNSCC, basal cell carcinoma, plasma cell myeloma, and other advanced malignancies with no alternative therapeutic options
	Sintilimab (IBI308)	Eli Lilly (Innovent Biologics)	Monoclonal anti-human PD1 (human IgG4)	Phase I, II, III	Gastric cancer, NSCLC, HCC, and other advanced solid malignancies
	RO7121661	Roche	Bispecific anti-TIM3/PD1 antibody	Phase I	Melanoma, NSCLC, advanced solid malignancies

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Table 1. *(Continued)*

Target molecule	Drug	Manufacturer	Description	Phases of trials actively recruiting	Types of cancer tested in clinical trial
	Cetrelimab (JNJ-63723283)	Janssen Research & Development	Monoclonal anti-human PD1 (human IgG4)	Phase I, II	Prostate cancer, urothelial carcinoma, SCLC, NSCLC, melanoma, RCC, bladder cancer, gastric, esophageal cancers, and high-level MSI-H/dMMR CRC, and other advanced solid malignancies
	INCMGA00012 (MGA012)	MacroGenics	Monoclonal anti-human PD1 (human IgG4)	Phase I, II	Advanced solid malignancies
	AK105	Akeso Biopharma	Monoclonal anti-human PD1 (IgG information unavailable)	Phase I, II, III	HL, NSCLC, HNSCC, advanced solid malignancies
	HX008	Taizhou Hanzhong Pharmaceuticals	Monoclonal anti-human PD1 (human IgG information unavailable)	Phase II	dMMR or MSI-H advanced solid malignancies
	SCT-I10A	Sinocelltech	Monoclonal anti-human PD1 (humanized IgG information unavailable)	Phase I	Advanced solid tumors or lymphomas
	HLX10	Henlix Biotech	Monoclonal anti-human PD1 (humanized IgG information unavailable)	Phase I	Advanced or metastatic malignancies refractory to standard therapy
	Sym021	Symphogen	Monoclonal anti-human PD1 (human IgG information unavailable)	Phase I	Locally metastatic malignancies that are refractory to available therapy
	Spartalizumab (PDR001)	Novartis	Monoclonal anti-human PD1 (humanized IgG4)	Phase I, II	Melanoma, TNBC, NSCLC, RCC, pancreatic cancer, urothelial cancer, HNSCC, DLBCL, MSS-CRC, HCC, endometrial cancer, MM, poorly-differentiated gastroenteropancreatic neuroendocrine carcinoma, ovarian cancer, AML
	Genolimzumab (GB226)	CBT Pharmaceutical	Monoclonal anti-human PD1 (humanized IgG4)	Phase I, II	Alveolar soft part sarcoma (ASPS), RTCL, NHL
	CS1003	CStone Pharmaceuticals	Monoclonal anti-human PD1 (humanized IgG4)	Phase I	Refractory advanced or metastatic solid malignancies, unresectable lymphomas
	MGD019	MacroGenics	Dual-affinity re-targeting (DART) anti-CTLA4/PD1 bearing hinge-stabilized human IgG4 Fc	Phase I	Unresectable, locally advanced or metastatic solid malignancies
	MEDI5752	MedImmune	Bispecific monovalent anti-CTLA4/PD1 (Fc-engineered human IgG1)	Phase I	Advanced solid malignancies

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Table 1. *(Continued)*

Target molecule	Drug	Manufacturer	Description	Phases of trials actively recruiting	Types of cancer tested in clinical trial
LAG3	Sym022	Symphogen	Monoclonal anti-human LAG3 (recombinant human Fc-inert)	Phase I	Lymphoma, metastatic solid malignancies
	Relatlimab (BMS-986016)	Bristol-Myers Squibb	Monoclonal anti-human LAG3 (human IgG4)	Phase I, II, III	Melanoma, CRC, MSS-CRC, gastric, esophageal, gastroesophageal cancers, GEJ cancer, cervical, ovarian, bladder, cancer, HNSCC, HCC, NSCLC, RCC, CLL, HL, NHL, MM, DLBCL
	REGN3767	Regeneron Pharmaceuticals	Monoclonal anti-human LAG3 (human hinge-stabilized IgG4)	Phase I	PD-1/PD-L1 inhibitor treatment-naive malignancies
	TSR-033	Tesaro (AnaptysBi)	Monoclonal anti-human LAG3 (humanized high affinity IgG4 kappa chain)	Phase I	Advanced, unresectable solid malignancies
	MGD013	MacroGenics	Dual-affinity re-targeting (DART) anti-PD1/LAG3 bearing human IgG4 Fc	Phase I	Advanced, unresectable solid tumors and hematological malignancies
	IMP321	Immutep	Human LAG3-Fc fusion protein	Phase I, II	Advanced estrogen receptor-positive (ER+) and progesterone receptor-positive (PR+) breast cancers, and other locally advanced or metastatic solid malignancies
	FS118	F-star	Bispecific anti-LAG3/PDL1 antibody composed of anti-human LAG3 binding Fc (Fcab) structurally incorporated into the Fc-region of anti-human PDL1 IgG1 monoclonal antibody	Phase I	Locally advanced, unresectable or metastatic malignancies that progressed on or after PD-1/PD-L1 containing therapy
	INCAGN02385	Agenus (Incyte Corporation)	Monoclonal anti-human LAG3 (Fc-engineered immunoglobulin G1-kappa (IgG1κ))	Phase I	Melanoma, cervical cancer, MSI-high endometrial cancer, gastric cancer, GEJ cancer, esophageal cancer, HCC, Merkel cell carcinoma, mesothelioma, MSI-high CRC, SCLC, NSCLC, ovarian cancer, HNSCC, RCC, TNBC, urothelial carcinoma, DLBCL
	LAG525 (IMP701)	Novartis (Immutep)	Monoclonal anti-human LAG3 (humanized IgG4)	Phase I, II	Advanced TNBC, melanoma
	MK4280	Merck Sharp & Dohme	Monoclonal anti-human LAG3 (humanized IgG4)	Phase I, II	Classical Hodgkin's lymphoma (CHL), DLBCL, indolent non-Hodgkin's lymphoma (iNHL), and other metastatic solid malignancies
TIGIT	MK-7684	Merck Sharp & Dohme	Monoclonal anti-human TIGIT (humanized IgG1)	Phase I	Advanced solid tumors, inoperable adenocarcinoma of stomach and/or GEJ cancers
	AB154	Arcus Biosciences	Monoclonal anti-human TIGIT (humanized IgG1)	Phase I	Advanced malignancies, NSCLC, HNSCC, RCC, breast cancer, CRC, melanoma, bladder, ovarian, endometrial gastrointestinal cancers, Merkel cell carcinoma

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Table 1. *(Continued)*

Target molecule	Drug	Manufacturer	Description	Phases of trials actively recruiting	Types of cancer tested in clinical trial
	MTIG7192A	Genentech	Monoclonal anti-human TIGIT (human IgG1)	Phase I	Advanced, metastatic malignancies, NSCLC
	BMS-986207	Bristol-Myers Squibb	Monoclonal anti-human TIGIT (human IgG1)	Phase I, II	Advanced solid malignancies
	ASP8374 (PTZ-201)	Astellas Pharma (Potenza)	Monoclonal anti-human TIGIT (human IgG4)	Phase I	Advanced solid malignancies
TIM3	Sym023	Symphogen	Monoclonal anti-human TIM3 (human)	Phase I	Refractory lymphomas, locally advanced, unresectable or metastatic solid malignancies,
	Cobolimab (TSR-022)	Tesaro (AnaptysBi)	Monoclonal anti-human TIM3 (humanized IgG4)	Phase I	Metastatic solid malignancies, HCC, NSCLC
	RO7121661	Roche	Bispecific anti-TIM3/PD1 antibody	Phase I	Metastatic solid malignancies, melanoma, NSCLC
	LY3321367	Eli Lilly	Monoclonal anti-human TIM3 (human IgG1κ, Fc-null)	Phase I	Advanced solid malignancies
	BGB-A425	BeiGene	Monoclonal anti-human TIM3 (humanized IgG1)	Phase I, II	Locally advanced or unresectable, metastatic solid malignancies
	LY3415244	Eli Lilly	Bispecific anti-TIM3/PDL1 antibody	Phase I	Advanced solid malignancies
	INCAGN02390	Agenus (Incyte Corporation)	Monoclonal anti-human TIM3 (human IgG1, Fc-silent)	Phase I	Cervical, gastric, stomach, GEJ, esophageal, ovarian cancers, melanoma, HCC, Merkel cell carcinoma, mesothelioma, SCLC, NSCLC, HNSCC, RCC, TNBC, urothelial carcinoma, MSI cancers
	MBG453	Novartis	Monoclonal anti-human TIM3 (human IgG4)	Phase I, II	Advanced or metastatic solid malignancies, AML
	BMS-986258	Bristol-Myers Squibb	Monoclonal anti-human TIM3	Phase I, II	Advanced malignancies
NRP1	ASP1948 (PTZ-329)	Astellas Pharma (Potenza)	Monoclonal anti-human NRP1 (human IgG4)	Phase I	Advanced solid malignancies, NSCLC
TGF-β	Galunisertib (LY2157299)	Eli Lilly	TGF-βRI small molecule inhibitor	Phase I, II	CRC, uterine, ovarian, fallopian tube, peritoneal cancers, prostate cancer, rectal adenocarcinoma (Stage IIA-IIIC or AJCC Stage IV), advanced (Stage IV) metastatic AR negative TNBC
	M7824 (MSB0011359C)	Merck	Anti-PDL1/TGF-β TRAP bifunctional fusion protein composed of avelumab (anti-PDL1) fused to soluble extracellular domain of human TGF-βRII	Phase I, II	Breast cancer, NSCLC, HPV associated malignancies, anal, vulvar, vaginal, penile, squamous cell rectal and neuroendocrine cervical cancers, CRC, SCLC, TNBC, prostate cancer, locally advanced solid malignancies

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Table 1. *(Continued)*

Target molecule	Drug	Manufacturer	Description	Phases of trials actively recruiting	Types of cancer tested in clinical trial
	AVID200	Forbius (Formation Biologics)	TGF-β TRAP composed of TGF-β receptor ectodomains fused to human Fc	Phase I	Any locally advanced or metastatic solid malignancies
	Fresolimumab (GC1008)	Cambridge Antibody Technology (Genzyme Corporation)	Pan-specific monoclonal anti-human TGF-βRI, II, and III (fully human IgG4)	Phase I, II	Newly diagnosed early NSCLC
	LY3200882	Eli Lilly	TGF-β small molecule inhibitor	Phase I	Any solid malignancies
	PF-06952229	Pfizer	TGF-βR1 inhibitor	Phase I	Metastatic and standard therapy-resistant solid malignancies, breast cancer, prostate cancer
IL-10	AM0010	Merck	PEGylated recombinant human IL-10	Phase II, III	Metastatic pancreatic adenocarcinoma, NSCLC
IL-35	No IL-35-targeted agents are currently investigated in cancer immunotherapy clinical trial			N/A	

Table represents a list of drugs found on ClinicalTrials.gov (as of February 2019) that are currently in clinical trials actively recruiting patients to investigate the safety and efficacy in various types of cancer as indicated. (Search terms: 'CCR2', 'CCL2', 'CCR4', 'CCR8', 'CCL1', 'CTLA4', 'PD1', 'LAG3', 'TIGIT', 'TIM3', 'Neuropilin 1', 'NRP1', 'TGF', 'TGFβ', 'IL-10', 'IL-35'. There are no current clinical trials investigating drugs targeting CCL2, CCR8, CCL1, NRP1, and IL-35.)

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transcription 3 (STAT3)-dependent manner.[27] Moreover, CCR8$^+$ Treg cells up-regulate CCL1 expression, thereby possibly promoting a positive paracrine feedback loop to sustain their suppressive potential *in situ*.[27] Targeting CCR8$^+$ Treg cells through either anti-CCR8 mAb or anti-CCL1 neutralizing mAb drastically reduced tumor-infiltrating Treg cells while robustly enhancing the anti-tumor immune response against murine tumor models such as colorectal adenocarcinoma.[28] Although there are currently no known CCR8-targeted therapeutics in clinical trials (Table 1), targeting CCR8 may be a highly selective therapeutic strategy sparing the peripheral Treg cells that do not express CCR8.

Inhibitory receptors

Inhibitory receptors (IRs), such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4, CD152) and programmed cell death protein 1 (PD1, CD279), have been extensively investigated in the context of effector T-cell exhaustion,[29] but their impact on Treg cells is less well defined despite their up-regulation in the TME.[10] In this section, we review the impact of IRs on intratumoral Treg cells and their contribution to regulating anti-tumor immunity (Fig. 1).

Cytotoxic T-lymphocyte-associated protein 4

Regulatory T cells constitutively express CTLA4 as its expression is controlled by Foxp3.[1] Although CTLA4 is often retained intracellularly in circulating Treg cells, a subset of Treg cells up-regulates surface CTLA4 expression in the TME.[30] CTLA4 binds to and blocks CD80/CD86 with a significantly higher affinity than its co-stimulatory counterpart CD28.[10] Strikingly, CTLA4 can also physically remove CD80/CD86 from the surface of antigen-presenting cells by trans-endocytosis.[31] In addition, dendritic cells up-regulate indoleamine-2,3-dioxygenase (IDO) upon CTLA4-binding and convert tryptophan to kynurenine in the local microenvironment.[32] A recent study demonstrated that kynurenine induces T-cell receptor (TCR)-independent up-regulation of PD1 in CD8$^+$ TILs through activation of aryl hydrocarbon receptor, leading to CD8$^+$ T-cell exhaustion.[33] It is possible that Treg cells regulate anti-tumor immune responses via a CTLA4–IDO–kynurenine axis.

Consistent with these observations, the enrichment of CTLA4$^+$ TILs or Treg cells was associated with poor prognosis in patients with various types of cancer including non-small cell lung cancer.[34] Furthermore, the finding that administration of anti-CTLA4 blocking antibody resulted in effective anti-tumor immunity and protection against a secondary tumor challenge in murine cancer models[35] led to the development of two mAbs against human CTLA4, ipilimumab (MDX-010)[36] and tremelimumab (CP-675206)[37] (Table 1). After successful clinical trials demonstrating an improved overall survival rate (20% after 4 years),[38] ipilimumab was approved by the US Food and Drug Administration for treating patients with unresectable or metastatic melanoma in 2011, with other CTLA4-targeted therapeutics in clinical trials (Table 1). In addition, a recent study has shown that blocking CTLA4 on both effector T cells and Treg cells was required for maximal enhancement of anti-tumor immunity.[39] Hence, CTLA4 blockade not only inhibits CTLA4$^+$ Treg-mediated inhibition of T-cell activation but it also improves effector T-cell activity in a cell-intrinsic manner.

Programmed cell death protein 1

Although effector T cells up-regulate expression of PD1 upon TCR stimulation, PD1 is constitutively expressed on a small proportion of peripheral Treg cells,[40] which is further up-regulated in the TME.[41] However, the cell-intrinsic impact of PD1 expression on intratumoral Treg cells has not been fully elucidated. Despite the excitement around the success of anti-PD1 checkpoint blockade for cancer immunotherapy [Nivolumab[42] and Pembrolizumab,[43] with many others in clinical trials (Table 1)], there remains a large proportion of patients who do not respond or who develop resistance overtime.[44] It is therefore crucial to understand the potential impact of PD1 blockade on other intratumoral immune cells.

PD1 plays a crucial role in Treg cell homeostasis and survival as IL-2 stimulation with PD1 blockade or genetic deletion of PD1 resulted in an overt proliferation of Treg cells followed by a rapid contraction due to increased apoptosis.[40] A recent study reported that apoptotic intratumoral Treg cells express a low level of PD1 (PD1^{lo}) whereas viable intratumoral Treg cells showed enhanced PD1 expression (PD1^{hi}). Interestingly, apoptotic PD1^{lo} Treg cells displayed superior suppressive capacity in an adenosine/A_{2A}-dependent manner through sustained expression of CD39 and CD73,[45] indicating that PD1 expression on Treg cells may not positively correlate with their functionality. Consistently, PD1^{hi} Treg cells isolated from blood or tumor of patients with glioblastoma multiforme have been characterized as a dysfunctional, effector T-cell-like population with inferior suppressive capacity.[41] Further investigation is warranted to understand whether and how PD1^{hi} and PD1^{lo} Treg cells may be involved in the development of resistance to immunotherapy.

Lymphocyte-activation gene 3

Lymphocyte-activation gene 3 (LAG3), like other IRs, is transiently expressed on effector T cells upon TCR stimulation and cell-intrinsically regulates proliferation and survival.[46,47] LAG3 is highly up-regulated on exhausted

$CD8^+$ T cells,[29] and increased expression of LAG3 on TILs has been associated with poor patient survival in various cancer types including non-small cell lung cancer.[48] Currently, there are at least 10 LAG3-targeted therapeutics in clinical trials (Table 1).[49] However, the impact of LAG3 blockade on $LAG3^+$ intratumoral Treg cells has not been fully elucidated.

Unlike effector T cells, a subset of peripheral Treg cells constitutively expresses a low level of LAG3, which is further up-regulated upon activation.[47] $LAG3^+$ Treg cells are highly enriched in the TME as well as in the circulation of individuals with cancer.[50] Early studies have suggested that the expression of LAG3 is required for the maximal suppressive activity of Treg cells, as an antibody-mediated blockade or genetic deletion of LAG3 severely impaired their function both *in vitro* and *in vivo*.[46,47] Moreover, recent studies demonstrated that human Treg cells isolated from individuals with head and neck squamous cell carcinomas (HNSCC) showed enhanced suppressive function compared with Treg cells from matched patient peripheral blood mononuclear cells or healthy donors.[50] Although the exact role of LAG3 on intratumoral Treg cells remains unclear, a recent study using a mouse model of autoimmune diabetes has shown that LAG3 intrinsically limits Treg cell function and survival, while LAG3-deficient Treg cells substantially delayed the disease onset.[51] Hence, it is possible that LAG3 blockade may limit or augment anti-tumor immunity depending on the ratio of $LAG3^+$ intratumoral Treg cells versus T effector cells as well as the severity of inflammation in the microenvironment.

T-cell immunoreceptor with immunoglobulin and ITIM domains

T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is a recently discovered IR that belongs to the poliovirus receptor (PVR) family.[52] The expression of TIGIT is highly restricted to the lymphocyte compartment, such as T cells and natural killer cells.[53] Although TIGIT expression is up-regulated upon TCR stimulation,[52] a relatively large proportion of human Treg cells constitutively expresses TIGIT, which is further enhanced in the TME.[53] TIGIT regulates effector T-cell activation by competitively binding to its receptor PVR on antigen-presenting cells with approximately 100-fold higher affinity than its co-stimulatory counterpart CD226.[52] TIGIT can bind to both PVR and CD226, and prevention of CD226-dimerization on the T-cell surface is one of the key cell-intrinsic regulatory mechanisms of TIGIT.[52,53] In addition, TIGIT is co-expressed with PD1 on exhausted CD8 T cells,[29] and $TIGIT^+$ $CD8^+$ T cells present a severely dysfunctional state with diminished cytokine production and proliferation.[54,55] Preclinical studies with $Tigit^{-/-}$ mice or anti-TIGIT mAb treatment have been shown to greatly improve anti-tumor immune responses in a $CD8^+$ T-cell-dependent manner.[54] Hence, TIGIT has been implicated as one of the potential cancer immunotherapeutic targets to elicit effective anti-tumor immunity (Table 1).

However, recent studies have revealed previously unappreciated intrinsic roles of TIGIT in Treg cells. Despite previous observations that TIGIT expression marks a highly suppressive population of Treg cells,[55,56] the exact underlying mechanisms and the potential roles of intrinsic TIGIT-signaling in Treg cells have not been fully elucidated. Whereas effector T cells up-regulate both CD226 and TIGIT upon activation, Treg cells preferentially up-regulate TIGIT over CD226.[56] CD226-signaling detrimentally impacts Treg cell stability and function, and TIGIT–PVR interaction was required to maintain the suppressive function of Treg cells.[56] Furthermore, a recent study has demonstrated that TIGIT-signaling repressed the PI3K-Akt axis in an inositol polyphosphate-5-phosphatase D-dependent manner leading to sustained nuclear localization of Foxo1, which was required to rescue Treg cells from an IFN-γ-secreting effector Th1-like Treg phenotype induced by IL-12 in a highly inflammatory environment, such as multiple sclerosis.[57] These observations suggest that unlike LAG3, TIGIT is a selective IR that represses effector T-cell function while enhancing Treg cell stability and function. Consistently, increased infiltration of $TIGIT^+$ Treg cells has been correlated with poor prognosis of patients with melanoma.[56] Interestingly, $CD8^+$ T-cell-restricted TIGIT deletion did not improve anti-tumor response in a $Rag1^{-/-}$ adoptive transfer system,[55] suggesting that preclinical efficacy observed with TIGIT blockade and $Tigit^{-/-}$ mice may have been due to functional destabilization of intratumoral Treg cells.

T-cell immunoglobulin and mucin-domain containing-3

T-cell immunoglobulin and mucin-domain containing-3 (TIM3) was first discovered and characterized as a marker for Th1 cells and type 1 cytotoxic $CD8^+$ T cells.[58] Interestingly, chronic T-cell activation is required for sustained TIM3 expression on Th1-polarized $CD4^+$ T cells, implying a role for TIM3 during late-stage T-cell differentiation.[58] TIM3 was subsequently characterized as one of the key markers associated with exhausted $CD4^+$ and $CD8^+$ T cells in the context of both chronic viral infection[59] and cancer.[60] However, TIM3 lacks known inhibitory signaling motifs (ITIM or ITSM) around cytoplasmic tyrosine residues,[59] so the TIM3-signaling pathway has not been fully understood.

Similar to PD1, TIM3 is expressed by a small fraction of peripheral Treg cells, whereas a large proportion of intratumoral Treg cells express TIM3.[50,61] However, unlike $PD1^{+/hi}$ Treg cells, $TIM3^+$ intratumoral Treg cells showed enhanced suppressive capacity due to increased

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expression of CTLA4 and CD39.[50,61] Increased infiltration of TIM3$^+$ CD4$^+$ T cells or TIM3$^+$ Treg cells is associated with poor prognosis of patients with various malignancies including non-small cell lung cancer.[62] Given the preclinical observations that TIM3-blocking mAbs could reinvigorate anti-tumor immunity,[63] several clinical trials are actively examining the safety and efficacy of TIM3 blockade therapy in both solid tumors and lymphomas (Table 1). However, as with PD1- and LAG3-targeted therapies, further investigation of the impact of TIM3 on intratumoral Treg cells is warranted.

Markers for stability and enhanced function

Neuropilin 1

Neuropilin 1 (NRP1) is a type 1 transmembrane protein, first characterized as a receptor for a neural chemorepellent Semaphorin 3a (Sema3a).[64] However, an early study demonstrated the role of NRP1 in priming T-cell activation through a T cell–dendritic cell interaction-dependent mechanism.[65] Furthermore, NRP1 is constitutively expressed on murine Treg cells[66] and has been defined as a discriminatory marker between thymically derived Treg cells and peripherally induced Treg cells.[67,68]

Our recent study demonstrated that NRP1 expressed on the surface of murine Treg cells is constitutively associated with phosphatase and tensin homolog (PTEN) and Sema4a-mediated NRP1 signaling is required to potentiate the immunoregulatory function of Treg cells by nuclear retention of Foxo3a through the PTEN–Akt axis at the immunological synapse (Fig. 1).[69] Mice with a Treg-restricted deletion of NRP1 exhibited an enhanced anti-tumor response comparable to the Treg-depletion model without succumbing to autoimmunity.[69] These findings suggest that the NRP1–PTEN–Akt–Foxo3 axis is required for the functional stability of Treg cells in an inflammatory environment. Although conventional Treg instability is marked by a loss of Foxp3 expression,[70] Treg cells with functional instability induced through the loss of NRP1 maintain the expression of Foxp3 while ectopically up-regulating effector T-cell-like gene signature such as IFN-γ;[8,69] hence, this unique state is referred to as Treg fragility.[8] Strikingly, Treg fragility was required for the effective PD1-blockade immunotherapy on established transplantable mouse adenocarcinoma (MC38) in an IFN-γ-dependent manner as Treg-restricted deletion of IFN-γR resulted in the diminished therapeutic efficacy of anti-PD1 treatment.[8]

Unlike murine Treg cells, human Treg cells do not constitutively express NRP1. Instead, NRP1 is induced upon TCR stimulation, and perhaps other factors.[71] Some studies have reported that a subset of Treg cells up-regulate NRP1 expression in various types of cancer such as melanoma,[8] consistent with the notion that the physiological role of NRP1 in Treg cells is restricted to inflammatory sites. In addition, increased infiltration of NRP1$^+$ Treg cells in the TME has been associated with poor prognosis in patients with melanoma and HNSCC.[8] Furthermore, tumor-derived vascular endothelial growth factor (VEGF) has been shown to promote Treg cell infiltration into the TME in an NRP1-dependent manner,[72] suggesting a migratory role of NRP1 in Treg cells. The therapeutic targeting of NRP1 should provide insight into the impact of NRP1 blockade on the fragility and infiltration of human intratumoral Treg cells (Table 1).

Inhibitory cytokines

Secretion of inhibitory cytokines is one of the primary mechanisms used by Treg cells to regulate immune responses.[2] Increased intratumoral expression of inhibitory cytokines is associated with poor prognosis in various cancer types.[73–75] In this section, we discuss our current understanding of the role played by transforming growth factor (TGF)-β^+, IL-10$^+$ and IL-35$^+$ Treg subsets in the TME (Fig. 1).

Transforming growth factor-β

Transforming growth factor-β plays a pleiotropic role in the immune system and is also involved in thymic development of all T-cell subsets. The absence of TGF-β-signaling results in defective thymic Treg cell development during the first 3–5 days of murine development.[76] In addition, TGF-β promotes the differentiation of induced Treg cells *in vitro*,[2,76] demonstrating its broad immunoregulatory functions in controlling inflammation. Furthermore, Treg-derived TGF-β plays a crucial role in regulating immune responses. Recent studies have reported the enriched presence of TGF-β-producing intratumoral Treg cells in various types of cancer, such as HNSCC,[77] and their enhanced suppressive potential compared with peripheral Treg cells,[78] indicating that TGF-β is one of the major regulatory mechanisms that Treg cells employ in the TME. Indeed, elevated TGF-β has been correlated with poor prognosis in patients with pancreatic cancer[79] and breast cancer.[74] However, TGF-β-signaling blockade had no impact on intratumoral Treg cell accumulation or epigenetic status in a murine mammary gland tumor model.[80] In addition, intratumoral effector T cells and Treg cells showed minimal TCR repertoire overlap,[81] suggesting that thymically derived Treg cells may be the dominant intratumoral population and not intratumorally converted peripherally induced Treg cells.

These observations led to an increasing interest in targeting TGF-β as a therapeutic approach (Table 1).[82] For instance, a preclinical study using a mouse model of transplantable lung cancer (AG104L^d) demonstrated that blocking TGF-β with a neutralizing mAb (clone A411) achieved tumor rejection comparable to transient Treg cell depletion with anti-CD25 mAb (clone PC61) administration.[83]

In addition to its impact on the immune infiltrate, TGF-β also directly supports tumorigenesis by promoting (i) angiogenesis in concert with VEGF, (ii) fibrosis and (iii) metastasis by promoting cancer cell motility and epithelial-to-mesenchymal transition.[82] Further investigation is warranted to understand how many of these pro-tumor factors are directly contributed by Treg-derived TGF-β in order to rationally design effective therapy against individual cancers that may present varying degrees of TGF-β-mediated pathophysiology in the TME.

Interleukin-10

Interleukin-10 was initially characterized as a Th1-regulating factor produced by Th2 cells.[84] Later studies demonstrated that its predominant suppressive mechanism is the regulation of the immunostimulatory potential of antigen-presenting cells, resulting in impaired production of the pro-inflammatory cytokine IL-12, as well as expression of major histocompatibility complex class II and CD86.[85,86] Although many different cell types produce IL-10,[87] deletion in Treg cells was sufficient to induce spontaneous colitis,[88] highlighting the physiological importance of Treg-derived IL-10. Although TCR stimulation is sufficient to induce the secretion of IL-10 by Treg cells, co-culturing in the presence of other immune cells, such as effector T cells, further enhanced the production of IL-10 *in vitro*.[89] A large proportion of intratumoral Treg cells show up-regulation of IL-10 in both humans and mice,[90,91] and in some tumor models, Treg cells are the predominant source of IL-10.[87] In addition, the enriched IL-10 expression in the TME has been associated with poor prognosis in patients with HNSCC.[75] We have recently demonstrated that intratumoral Treg-derived IL-10 directly modulates the BLIMP1 expression in $CD8^+$ TILs, which in turn further promotes IR expression and T-cell exhaustion.[91] Treg-restricted deletion of IL-10 resulted in an altered myeloid compartment in the TME by upregulating T cell stimulatory molecules, such as major histocompatibility complex class II and CD80, on intratumoral dendritic cells, suggesting that Treg-derived IL-10 alters the TME, which can indirectly provide additional regulation of T-cell-mediated anti-tumor immune responses.[91]

However, there has been increasing evidence that IL-10 may also play an anti-tumor role.[92] For instance, early administration of IL-10 impaired the dendritic cell vaccine-mediated anti-tumor response,[93] consistent with the conventional inhibitory function of IL-10. However, IL-10 administration at a later time-point, 7 days post-vaccination, resulted in tumor regression as well as the expansion of antigen-specific memory $CD8^+$ T cells.[93] Consistent with these observations, a recent study demonstrated that Treg-derived IL-10 was required during the resolution phase of inflammation to promote $CD8^+$ T-cell memory development by modulating the maturation status of dendritic cells.[94] Furthermore, PEGylated IL-10, which has enhanced *in vivo* stability, elicited increased activation of intratumoral $CD8^+$ T cells with heightened IFN-γ production, resulting in a remarkable rate of tumor regression and survival of mice with established large tumor burdens.[95] However, given the enrichment of IL-10^+ Treg cells in progressively growing and established tumors,[90] it appears that the outcome of anti-tumor responses depends on the balance between immunostimulatory and immunoregulatory roles of Treg-derived IL-10. Further investigation is warranted to determine potential biomarkers that help to identify patients with cancer who may benefit from IL-10 blockade or exogenous IL-10 administration, especially given that PEGylated IL-10 is in clinical trials (Table 1).

Interleukin-35

Interleukin-35 is a member of the IL-12 cytokine family and is composed of p35 (encoded by *Il12a*) and Ebi3 (encoded by *Ebi3*).[96] Although IL-35 was initially reported to be preferentially expressed by activated Treg cells,[97] two studies have shown that regulatory B cells can also express IL-35.[98,99] The IL-35 receptor (IL-35R) on T cells consists of two shared subunits, IL-12Rβ2 (encoded by *Il12rb2*) and gp130 (encoded by *Il6st*), which can be expressed as a heterodimer or homodimers of either chain. However, it has been suggested that the receptor on B cells may differ and consist of an IL-12Rβ2 (encoded by *Il12rb2*)/WSX1 (encoded by *Il27ra*) heterodimer,[98] highlighting the variability and promiscuity of the IL-35R.[96]

Interestingly, the up-regulation of IL-35 expression in Treg cells required activation in the presence of cell–cell contact with effector T cells.[89] This observation suggested that effector T cells provide positive feedback to enhance Treg cell functions, leading to the discovery of the NRP1–Sema4a axis[69] as discussed above.

We have previously demonstrated that IL-35^+ Treg cells were highly enriched in the TME, comprising approximately 50% of intratumoral Treg cells in B16F10 tumor model[7] and they promoted the expression of multiple IRs on $CD4^+$ and $CD8^+$ TILs.[7] We have recently reported that one of the underlying mechanisms of Treg-derived IL-35-mediated regulation of anti-tumor responses was through direct modulation of BLIMP1 expression through IL-35R-signaling in $CD8^+$ T cells, which in turn promoted IR expression and limited differentiation of central memory $CD8^+$ T cells.[91] Interestingly, Treg-restricted single-deletion of IL-35 or double-deletion of both IL-10 and IL-35 resulted in a comparable reduction of tumor burden and enhanced central memory T-cell differentiation.[91] These observations suggest that Treg-derived IL-35 may play a dominant immunoregulatory role over other inhibitory cytokines. Furthermore, enhanced expression of IL-35 in the TME, by use of an IL-35–B16F10 transfectant,

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accelerated tumor growth by enhancing the accumulation of myeloid-derived suppressor cells and promoting angiogenesis.[100] These observations indicate that Treg-derived IL-35 actively contributes to the immunosuppressive TME. There are currently no IL-35-targeted therapeutics in the clinic (Table 1), but systemic neutralization of IL-35 has resulted in increased proliferation and inflammatory cytokine production by $CD8^+$ TILs and reduced tumor growth in a preclinical murine tumor model.[7] This may be a potent immunotherapeutic approach that promotes the anti-tumor response of effector T cells while preventing IL-35-mediated pro-tumor tissue remodeling.

Conclusion

Targeting immunoregulatory mechanisms, such as CTLA4 and PD1, have successfully provoked long-term anti-tumor immune responses in patients with advanced cancer, such as unresectable metastatic melanoma.[38,42,43] This has led to an exponential growth of clinical trials investigating the efficacy of new cancer immunotherapies targeting additional immunoregulatory mechanisms. However, there remains a large proportion of cancer patients who do not benefit from checkpoint-blockade cancer immunotherapy. Although these therapeutic approaches have been focused on the elicitation of inflammatory responses against cancer, enhanced inflammation could also play a detrimental role by facilitating the recruitment of Treg cells through chemokines such as CCL1 and CCL22, resulting in a dampening of the anti-tumor responses. In addition, as demonstrated by the paradoxical functions of Treg-derived IL-10, the timing of therapeutic administration may also be critical.

Furthermore, although there is a largely overlapping list of effector molecules that Treg cells up-regulate in the TME, intratumoral Treg cells may be highly heterogeneous, using distinct transcriptional programs to support their survival and functions. In addition, it is still unclear whether these Treg subsets represent distinct and stable lineages. For instance, intratumoral Treg cells seem to preferentially express IL-10 or IL-35, rarely both.[91] It has been suggested that $IL\text{-}10^+$ or $IL\text{-}35^+$ Treg cells represent stable subsets,[88] but we have found that the expression pattern of IL-10 and IL-35 can be altered upon TCR stimulation *in vitro*, indicating that this may instead represent transitional states of activated Treg cells in the TME.[91] To effectively target intratumoral Treg cells, further investigation is warranted to fully understand the phenotypic and functional plasticity of Treg cell subsets that may potentially play a role in resistance to immunotherapy. Hence, to rationally design effective cancer immunotherapies, the next generation of cancer immunotherapies must consider: (i) appropriate combination of targets that augment effector responses, (ii) block Treg cell infiltration or function specifically in the TME, and (iii) determine the correct sequence of therapeutic administration to maximize beneficial impact, thereby also minimizing detrimental adverse effects.

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Disclosures

The authors declare competing financial interests. DAAV and CJW have submitted patents covering LAG3 and IL-35, and DAAV has submitted patents covering NRP1 that are pending or approved and are entitled to a share in net income generated from licensing of these patent rights for commercial development.

References

1 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**:1057–61.
2 Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008; **8**:523–32.
3 Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L *et al.* The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001; **27**:20–1.
4 Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* 2007; **8**:191–7.
5 Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004; **10**:942–9.
6 Saito T, Nishikawa H, Wada H, Nagano Y, Sugiyama D, Atarashi K *et al.* Two $FOXP3^+CD4^+$ T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat Med* 2016; **22**:679–84.
7 Turnis ME, Sawant DV, Szymczak-Workman AL, Andrews LP, Delgoffe GM, Yano H *et al.* Interleukin-35 limits anti-tumor immunity. *Immunity* 2016; **44**:316–29.
8 Overacre-Delgoffe AE, Chikina M, Dadey RE, Yano H, Brunazzi EA, Shayan G *et al.* Interferon-γ drives Treg fragility to promote anti-tumor immunity. *Cell* 2017; **169**:1130–1141.e1111.
9 Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D *et al.* Tumor-infiltrating $FOXP3^+$ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009; **27**:186–92.
10 Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res* 2017; **27**:109–18.
11 Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A *et al.* Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. *Immunity* 2009; **30**:899–911.
12 Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* 2011; **11**:762–74.
13 Zhang N, Schröppel B, Lal G, Jakubzick C, Mao X, Chen D *et al.* Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity* 2009; **30**:458–69.
14 Loyher PL, Rochefort J, Baudesson de Chanville C, Hamon P, Lescaille G, Bertolus C *et al.* CCR14 Influences T regulatory cell migration to tumors and serves as a biomarker of cyclophosphamide sensitivity. *Cancer Res* 2016; **76**:6483–94.
15 Zhan Y, Wang N, Vasanthakumar A, Zhang Y, Chopin M, Nutt SL *et al.* CCR2 enhances CD25 expression by $FoxP3^+$ regulatory T cells and regulates their abundance independently of chemotaxis and $CCR2^+$ myeloid cells. *Cell Mol Immunol* 2018. [Epub ahead of print]. https://doi.org/10.1038/s41423-018-0187-8
16 Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A *et al.* Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* 2009; **15**:930–9.

17 Wang Z, Xie H, Zhou L, Liu Z, Fu H, Zhu Y *et al.* CCL2/CCR17 axis is associated with postoperative survival and recurrence of patients with non-metastatic clear-cell renal cell carcinoma. *Oncotarget* 2016; **7**:51525–34.

18 Scheu S, Ali S, Ruland C, Arolt V, Alferink J. The C-C chemokines CCL17 and CCL22 and their receptor CCR18 in CNS autoimmunity. *Int J Mol Sci* 2017; **18**:2306–23.

19 Sather BD, Treuting P, Perdue N, Miazgowicz M, Fontenot JD, Rudensky AY *et al.* Altering the distribution of Foxp3$^+$ regulatory T cells results in tissue-specific inflammatory disease. *J Exp Med* 2007; **204**:1335–47.

20 Karasaki T, Qiang G, Anraku M, Sun Y, Shinozaki-Ushiku A, Sato E *et al.* High CCR20 expression in the tumor microenvironment is a poor prognostic indicator in lung adenocarcinoma. *J Thorac Dis* 2018; **10**:4741–50.

21 Sugiyama D, Nishikawa H, Maeda Y, Nishioka M, Tanemura A, Katayama I *et al.* Anti-CCR21 mAb selectively depletes effector-type FoxP3$^+$ CD4$^+$ regulatory T cells, evoking antitumor immune responses in humans. *Proc Natl Acad Sci USA* 2013; **110**:17945–50.

22 Kumai T, Nagato T, Kobayashi H, Komabayashi Y, Ueda S, Kishibe K *et al.* CCL17 and CCL22/CCR22 signaling is a strong candidate for novel targeted therapy against nasal natural killer/T-cell lymphoma. *Cancer Immunol Immunother* 2015; **64**:697–705.

23 Zingoni A, Soto H, Hedrick JA, Stoppacciaro A, Storlazzi CT, Sinigaglia F *et al.* The chemokine receptor CCR23 is preferentially expressed in Th2 but not Th1 cells. *J Immunol* 1998; **161**:547–51.

24 Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F *et al.* Unique chemotactic response profile and specific expression of chemokine receptors CCR24 and CCR24 by CD4$^+$CD25$^+$ regulatory T cells. *J Exp Med* 2001; **194**:847–53.

25 Coghill JM, Fowler KA, West ML, Fulton LM, van Deventer H, McKinnon KP *et al.* CC chemokine receptor 8 potentiates donor Treg survival and is critical for the prevention of murine graft-versus-host disease. *Blood* 2013; **122**:825–36.

26 Plitas G, Konopacki C, Wu K, Bos PD, Morrow M, Putintseva EV *et al.* Regulatory T cells exhibit distinct features in human breast cancer. *Immunity* 2016; **45**:1122–34.

27 Barsheshet Y, Wildbaum G, Levy E, Vitenshtein A, Akinseye C, Griggs J *et al.* CCR27$^+$ FOXp3$^+$ Treg cells as master drivers of immune regulation. *Proc Natl Acad Sci USA* 2017; **114**:6086–91.

28 Villarreal DO, L'Huillier A, Armington S, Mottershead C, Filippova EV, Coder BD *et al.* Targeting CCR28 induces protective antitumor immunity and enhances vaccine-induced responses in colon cancer. *Cancer Res* 2018; **78**:5340–8.

29 Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol* 2015; **15**:486–99.

30 Kwiecien I, Stelmaszczyk-Emmel A, Polubiec-Kownacka M, Dziedzic D, Domagala-Kulawik J. Elevated regulatory T cells, surface and intracellular CTLA-4 expression and interleukin-17 in the lung cancer microenvironment in humans. *Cancer Immunol Immunother* 2017; **66**:161–70.

31 Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM *et al.* Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 2011; **332**:600–3.

32 Grohmann U, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A *et al.* CTLA-4-Ig regulates tryptophan catabolism *in vivo*. *Nat Immunol* 2002; **3**:1097–101.

33 Liu Y, Liang X, Dong W, Fang Y, Lv J, Zhang T *et al.* Tumor-repopulating cells induce PD-1 expression in CD8$^+$ T cells by transferring kynurenine and AhR activation. *Cancer Cell* 2018; **33**:480–494.e487.

34 Paulsen EE, Kilvaer TK, Rakaee M, Richardsen E, Hald SM, Andersen S *et al.* CTLA-4 expression in the non-small cell lung cancer patient tumor microenvironment: diverging prognostic impact in primary tumors and lymph node metastases. *Cancer Immunol Immunother* 2017; **66**:1449–61.

35 Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 1996; **271**:1734–6.

36 Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ *et al.* Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci USA* 2003; **100**:8372–7.

37 Ribas A, Camacho LH, Lopez-Berestein G, Pavlov D, Bulanhagui CA, Millham R *et al.* Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675206. *J Clin Oncol* 2005; **23**:8968–77.

38 Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB *et al.* Improved survival with Ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**:711–23.

39 Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *J Exp Med* 2009; **206**:1717–25.

40 Asano T, Meguri Y, Yoshioka T, Kishi Y, Iwamoto M, Nakamura M *et al.* PD-1 modulates regulatory T-cell homeostasis during low-dose interleukin-2 therapy. *Blood* 2017; **129**:2186–97.

41 Lowther DE, Goods BA, Lucca LE, Lerner BA, Raddassi K, van Dijk D *et al.* PD-1 marks dysfunctional regulatory T cells in malignant gliomas. *JCI Insight* 2016; **1**: e85935:1–15.

42 Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; **366**:2443–54.

43 Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R *et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* 2013; **369**:134–44.

44 Bifulco CB, Urba WJ. Unmasking PD-1 resistance by next-generation sequencing. *N Engl J Med* 2016; **375**:888–9.

45 Maj T, Wang W, Crespo J, Zhang H, Wang W, Wei S *et al.* Oxidative stress controls regulatory T cell apoptosis and suppressor activity and PD-L1-blockade resistance in tumor. *Nat Immunol* 2017; **18**:1332–41.

46 Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G *et al.* Role of LAG-3 in regulatory T cells. *Immunity* 2004; **21**:503–13.

47 Workman CJ, Vignali DA. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol* 2005; **174**:688–95.

48 He Y, Yu H, Rozeboom L, Rivard CJ, Ellison K, Dziadziuszko R *et al.* LAG-3 protein expression in non-small cell lung cancer and its relationship with PD-1/PD-L1 and tumor-infiltrating lymphocytes. *J Thorac Oncol* 2017; **12**:814–23.

49 Andrews LP, Marciscano AE, Drake CG, Vignali DA. LAG3 (CD223) as a cancer immunotherapy target. *Immunol Rev* 2017; **276**:80–96.

50 Jie HB, Gildener-Leapman N, Li J, Srivastava RM, Gibson SP, Whiteside TL *et al.* Intratumoral regulatory T cells upregulate immunosuppressive molecules in head and neck cancer patients. *Br J Cancer* 2013; **109**:2629–35.

51 Zhang Q, Chikina M, Szymczak-Workman AL, Horne W, Kolls JK, Vignali KM *et al.* LAG3 limits regulatory T cell proliferation and function in autoimmune diabetes. *Sci Immunol* 2017; **2**:eaah4569:1–10.

52 Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B *et al.* The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* 2009; **10**:48–57.

53 Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y *et al.* The immunoreceptor TIGIT regulates antitumor and antiviral CD8$^+$ T cell effector function. *Cancer Cell* 2014; **26**:923–37.

54 Guillerey C, Harjunpää H, Carrié N, Kassem S, Teo T, Miles K *et al.* TIGIT immune checkpoint blockade restores CD8$^+$ T-cell immunity against multiple myeloma. *Blood* 2018; **132**:1689–94.

55 Kurtulus S, Sakuishi K, Ngiow SF, Joller N, Tan DJ, Teng MW *et al.* TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest* 2015; **125**:4053–62.

56 Fourcade J, Sun Z, Chauvin JM, Ka M, Davar D, Pagliano O *et al.* CD226 opposes TIGIT to disrupt Tregs in melanoma. *JCI Insight* 2018; **3**:e121157:1–13.

57 Lucca LE, Axisa PP, Singer ER, Nolan NM, Dominguez-Villar M, Hafler DA. TIGIT signaling restores suppressor function of Th1 Tregs. *JCI Insight* 2019; **4**:e124427:1–13.

58 Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T *et al.* Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* 2002; **415**:536–41.

59 Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR *et al.* Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 2008; **205**:2763–79.

60 Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 2010; **207**:2187–94.

61 Liu Z, McMichael EL, Shayan G, Li J, Chen K, Srivastava R *et al.* Novel effector phenotype of Tim-3$^+$ regulatory T cells leads to enhanced suppressive function in head and neck cancer patients. *Clin Cancer Res* 2018; **24**:4529–38.

62 Du W, Yang M, Turner A, Xu C, Ferris RL, Huang J *et al.* TIM-3 as a target for cancer immunotherapy and mechanisms of action. *Int J Mol Sci* 2017; **18**:645–57.

63 Ngiow SF, von Scheidt B, Akiba H, Yagita H, Teng MW, Smyth MJ. Anti-TIM3 antibody promotes T cell IFN-γ-mediated antitumor immunity and suppresses established tumors. *Cancer Res* 2011; **71**:3540–51.

64 Kawakami A, Kitsukawa T, Takagi S, Fujisawa H. Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. *J Neurobiol* 1996; **29**:1–17.

65 Tordjman R, Lepelletier Y, Lemarchandel V, Cambot M, Gaulard P, Hermine O *et al.* A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. *Nat Immunol* 2002; **3**:477–82.

66 Bruder D, Probst-Kepper M, Westendorf AM, Geffers R, Beissert S, Loser K *et al.* Neuropilin-1: a surface marker of regulatory T cells. *Eur J Immunol* 2004; **34**:623–30.

67 Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S *et al.* Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets *in vivo*. *J Exp Med* 2012; **209**(1713–1722):s1711–9.

68 Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S *et al.* Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol* 2013; **14**:307–8.
69 Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE *et al.* Stability and function of regulatory T cells is maintained by a neuropilin-1–semaphorin-4a axis. *Nature* 2013; **501**:252–6.
70 Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martínez-Llordella M, Ashby M *et al.* Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells *in vivo*. *Nat Immunol* 2009; **10**:1000–7.
71 Milpied P, Renand A, Bruneau J, Mendes-da-Cruz DA, Jacquelin S, Asnafi V *et al.* Neuropilin-1 is not a marker of human Foxp3$^+$ Treg. *Eur J Immunol* 2009; **39**:1466–71.
72 Hansen W. Neuropilin 1 guides regulatory T cells into VEGF-producing melanoma. *Oncoimmunology* 2013; **2**:e23039.
73 Zhao Z, Chen X, Hao S, Jia R, Wang N, Chen S *et al.* Increased interleukin-35 expression in tumor-infiltrating lymphocytes correlates with poor prognosis in patients with breast cancer. *Cytokine* 2017; **89**:76–81.
74 de Kruijf EM, Dekker TJ, Hawinkels LJ, Putter H, Smit VT, Kroep JR *et al.* The prognostic role of TGF-β signaling pathway in breast cancer patients. *Ann Oncol* 2013; **24**:384–90.
75 Bornstein S, Schmidt M, Choonoo G, Levin T, Gray J, Thomas CR Jr *et al.* IL-10 and integrin signaling pathways are associated with head and neck cancer progression. *BMC Genom* 2016; **17**:38.
76 Li MO, Flavell RA. TGF-β: a master of all T cell trades. *Cell* 2008; **134**:392–404.
77 Strauss L, Bergmann C, Szczepanski M, Gooding W, Johnson JT, Whiteside TL. A unique subset of CD4$^+$ CD25^{high} Foxp3$^+$ T cells secreting interleukin-10 and transforming growth factor-β1 mediates suppression in the tumor microenvironment. *Clin Cancer Res* 2007; **13**:4345–54.
78 Hilchey SP, De A, Rimsza LM, Bankert RB, Bernstein SH. Follicular lymphoma intratumoral CD4$^+$ CD25$^+$ GITR$^+$ regulatory T cells potently suppress CD3/CD28-costimulated autologous and allogeneic CD8$^+$ CD25$^-$ and CD4$^+$ CD25$^-$ T cells. *J Immunol* 2007; **178**:4051–61.
79 Javle M, Li Y, Tan D, Dong X, Chang P, Kar S *et al.* Biomarkers of TGF-β signaling pathway and prognosis of pancreatic cancer. *PLoS ONE* 2014; **9**:e85942.
80 Waight JD, Takai S, Marelli B, Qin G, Hance KW, Zhang D *et al.* Cutting edge: epigenetic regulation of Foxp3 defines a stable population of CD4$^+$ regulatory T cells in tumors from mice and humans. *J Immunol* 2015; **194**:878–82.
81 Hindley JP, Ferreira C, Jones E, Lauder SN, Ladell K, Wynn KK *et al.* Analysis of the T-cell receptor repertoires of tumor-infiltrating conventional and regulatory T cells reveals no evidence for conversion in carcinogen-induced tumors. *Cancer Res* 2011; **71**:736–46.
82 Neuzillet C, Tijeras-Raballand A, Cohen R, Cros J, Faivre S, Raymond E *et al.* Targeting the TGFβ pathway for cancer therapy. *Pharmacol Ther* 2015; **147**:22–31.
83 Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H *et al.* Intratumor depletion of CD4$^+$ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 2005; **201**:779–91.
84 Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 1989; **170**:2081–95.
85 D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 1993; **178**:1041–8.
86 Buelens C, Willems F, Delvaux A, Piérard G, Delville JP, Velu T *et al.* Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells. *Eur J Immunol* 1995; **25**:2668–72.
87 Stewart CA, Metheny H, Iida N, Smith L, Hanson M, Steinhagen F *et al.* Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation. *J Clin Invest* 2013; **123**:4859–74.
88 Wei X, Zhang J, Gu Q, Huang M, Zhang W, Guo J *et al.* Reciprocal expression of IL-35 and IL-10 defines two distinct effector Treg subsets that are required for maintenance of immune tolerance. *Cell Rep* 2017; **21**:1853–69.
89 Collison LW, Pillai MR, Chaturvedi V, Vignali DA. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *J Immunol* 2009; **182**:6121–8.
90 Amedei A, Niccolai E, Benagiano M, Della Bella C, Cianchi F, Bechi P *et al.* *Ex vivo* analysis of pancreatic cancer-infiltrating T lymphocytes reveals that ENO-specific Tregs accumulate in tumor tissue and inhibit Th1/Th17 effector cell functions. *Cancer Immunol Immunother* 2013; **62**:1249–60.
91 Sawant DV, Yano H, Chikina M, Zhang Q, Liao M, Liu C *et al.* Adaptive plasticity of IL-10$^+$ and IL-35$^+$ Treg cells cooperatively promotes tumor T cell exhaustion. *Nat Immunol* 2019; **20**:724–35.
92 Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The paradoxical role of IL-10 in immunity and cancer. *Cancer Lett* 2015; **367**:103–7.
93 Fujii S, Shimizu K, Shimizu T, Lotze MT. Interleukin-10 promotes the maintenance of antitumor CD8$^+$ T-cell effector function *in situ*. *Blood* 2001; **98**:2143–51.
94 Laidlaw BJ, Cui W, Amezquita RA, Gray SM, Guan T, Lu Y *et al.* Production of IL-10 by CD4$^+$ regulatory T cells during the resolution of infection promotes the maturation of memory CD8$^+$ T cells. *Nat Immunol* 2015; **16**:871–9.
95 Mumm JB, Emmerich J, Zhang X, Chan I, Wu L, Mauze S *et al.* IL-10 elicits IFNγ-dependent tumor immune surveillance. *Cancer Cell* 2011; **20**:781–96.
96 Vignali DA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. *Nat Immunol* 2012; **13**:722–8.
97 Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007; **450**:566–9.
98 Wang RX, Yu CR, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev YV *et al.* Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* 2014; **20**:633–41.
99 Shen P, Roch T, Lampropoulou V, O'Connor RA, Stervbo U, Hilgenberg E *et al.* IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* 2014; **507**:366–70.
100 Wang Z, Liu JQ, Liu Z, Shen R, Zhang G, Xu J *et al.* Tumor-derived IL-35 promotes tumor growth by enhancing myeloid cell accumulation and angiogenesis. *J Immunol* 2013; **190**:2415–23.