Counterregulatory suppression by CD4$^+$ conventional T cells limits anti-tumor immunity driven by T_{reg} depletion

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Abbreviations: Chemokine (C-C motif) receptor 8 (CCR8), regulatory T (T_{reg}), conventional T (T_{conv}), T helper (Th), Diphtheria toxin (DTx), CellTrace Violet™ (CTV), antigen presenting cells (APCs), wildtype (WT), diphtheria toxin receptor (DTR), enhanced green fluorescent protein (EGFP)

Abstract

Regulatory T (T_{reg}) cells are essential for immune homeostasis but inhibit immune rejection of cancer. Strategies to disrupt T_{reg}-mediated cancer immunosuppression have been met with limited clinical success, but the underlying mechanisms for this failure are poorly understood. By modeling T_{reg}-targeted immunotherapy in mice, we find that a subset of $CD4^+$ $Foxp3^-$ conventional T (T_{conv}) cells with potent suppressive function undergoes activation and expansion upon depletion of $Foxp3^+$ T_{reg} cells and limits therapeutic efficacy. We noted that $Foxp3^-$ T_{conv} cells within tumors adopt a T_{reg}-like transcriptional profile upon T_{reg} depletion and acquire suppressive function. This is attributable to $CD4^+$ T_{conv} cells marked by expression of C-C motif receptor 8 (CCR8) and enriched in T_{reg}-associated transcripts. $CCR8^+$ T_{conv} cells are found in mouse and human tumors. Upon T_{reg} depletion, $CCR8^+$ T_{conv} cells undergo systemic and intratumoral activation and expansion, and mediate IL-10 dependent suppression of anti-tumor immunity. Consequently, conditional deletion of *Il10* within T cells augments anti-tumor immunity upon T_{reg}-depletion in mice, and antibody blockade of IL-10 signaling synergizes with T_{reg} depletion to overcome treatment resistance. These findings reveal a secondary layer of immunosuppression by T_{conv} cells released upon therapeutic T_{reg} depletion and suggest that broader consideration of suppressive function within the T cell lineage is required for development of effective T_{reg}-targeted therapies.

One sentence summary: $CCR8^+$ T_{conv} cells undergo expansion and activation upon T_{reg} depletion and limit anti-tumor immunity through production of IL-10.

Introduction

Immune checkpoint blockade therapies targeting the inhibitory receptors PD-1 and CTLA-4 on T_{conv} cells have revolutionized the treatment of advanced cancer (*1-5*). However, only a minority of patients with a subset of cancers respond to existing therapies (*6-8*), necessitating development of mechanistically distinct modes of immunotherapy. T_{reg} cells play a critical role in suppressing both endogenous and therapy-driven anti-tumor immunity (*9-11*). High relative ratios of T_{reg} cells to $CD4^+$ or $CD8^+$ T_{conv} cells within tumors are associated with poor prognosis in patients with a variety of cancers, including ovarian cancer (*12, 13*), breast cancer (*14*), non-small cell lung carcinoma (*15*), hepatocellular carcinoma (*16*), renal cell carcinoma (*17*), pancreatic cancer (*18*), gastric cancer (*19*), cervical cancer (*20*), intrahepatic cholangiocarcinoma (*21*) and colorectal carcinoma (*22*). $Foxp3^+$ T_{reg} cells also powerfully contribute to immunotherapy resistance, including to immune checkpoint inhibitor therapy (*15, 23-25*). There is intense medical interest in therapeutically depleting T_{reg} cells or modulating their immunosuppressive function in cancer patients.

Despite abundant experimental evidence of the immunosuppressive role of T_{reg} cells in cancer, T_{reg}-targeted therapies have had limited success in the clinic. Agents developed for depletion of T_{reg} cells in humans have included Daclizumab (Zenapax), a monoclonal antibody against CD25 which is expressed highly on the surface of most T_{reg} cells; Denikeukin Difitox (Ontak), an IL-2:diphtheria toxin fusion protein which targets T_{reg} cells through their ability to bind IL-2; and Mogamulizumab, a depleting monoclonal antibody against CCR4, which is expressed by high frequencies of tumor-infiltrating T_{reg} cells (*26*). Daclizumab therapy failed to enhance the efficacy of a dendritic cell vaccine in metastatic melanoma patients (*27*), and only modestly increased immune response parameters in patients with glioblastoma (*28*) and breast

cancer (*29*), while Denikeukin Difitox treatment failed to induce clinical responses in metastatic melanoma patients (*30*). Mogamulizumab therapy lacked anti-tumor efficacy in advanced cancer patients (*31*), likely attributable to concomitant depletion of activated $CD4^+$ and $CD8^+$ T_{conv} cells expressing CCR4 (*32*). Lack of clinical efficacy despite robust depletion of T_{reg} cells in many cases indicates a need to better understand the basis for treatment failure of T_{reg}-targeted therapies.

In this study, we sought to better understand mechanisms of treatment failure of T_{reg}-targeting cancer immunotherapies. We systematically evaluated the consequence of experimental T_{reg} cell ablation on T_{conv} cells within tumors. While $CD4^+$ and $CD8^+$ T_{conv} cells were markedly transcriptionally distinct from T_{reg} cells under steady-state conditions, T_{reg} cell ablation caused T_{conv} cells to adopt a T_{reg}-like transcriptional profile, upregulating expression of molecules associated with T_{reg} cell suppressive function. Consistent with acquisition of a T_{reg}-like transcriptional profile, $Foxp3^-$ T_{conv} cells from T_{reg}-depleted animals acquired a potent ability to suppress T_{conv} activation and proliferation *in vitro*, attributable to the expansion of a subset of T_{conv} cells marked by expression of CCR8. This subset of suppressive T_{conv} cells was found to be enriched in both murine and human tumors, and its suppressive function was dependent upon IL-10. Consequently, conditional deletion of *Il10* specifically within T cells, and blockade of IL-10 receptor (IL-10R) signaling during T_{reg} cell depleting immunotherapy reversed treatment failure and resulted in enhanced tumor clearance. These findings indicate that compensatory suppression by T_{conv} cells limits efficacy of T_{reg}-targeted therapy and suggests that broader consideration of suppressive activity within the T cell lineage will be required for development of more effective therapies.

Results

T_{reg} ablation causes T_{conv} cells to adopt a T_{reg}-like transcriptional profile

T_{reg} depletion has had limited success in clinical trials of cancer patients with advanced disease. To better understand mechanisms underlying treatment failure in the context of therapeutic T_{reg} depletion, we utilized *Foxp3*$^{EGFP\text{-}DTR}$ mice, which express human diphtheria toxin receptor (DTR) and enhanced green fluorescent protein (EGFP) under the transcriptional control of the endogenous *Foxp3* gene. Administration of diphtheria toxin (DTx) to *Foxp3*$^{EGFP\text{-}DTR}$ mice enables selective depletion of Foxp3$^+$ T_{reg} cells (*33*). We subcutaneously implanted syngeneic B16-F10 melanoma cells into *Foxp3*$^{EGFP\text{-}DTR}$ mice and ablated T_{reg} cells through administration of DTx. Early T_{reg} cell ablation resulted in incomplete rejection of primary tumors, whereas T_{reg} cell depletion in mice with established tumors had little discernible effect upon tumor growth (**Fig. 1A**), despite near complete ablation of Foxp3-expressing T_{reg} cells within the systemic and intratumoral compartments of DTx-treated mice (**Fig. 1B**).

To understand mechanisms of treatment resistance, we first examined the consequence of T_{reg} cell ablation on the transcriptional profiles of CD4$^+$ and CD8$^+$ T_{conv} cells within tumors. Surprisingly, while intratumoral *Foxp3*$^{EGFP-}$ CD4$^+$ and CD8$^+$ T_{conv} cells were markedly transcriptionally distinct from *Foxp3*$^{EGFP+}$ T_{reg} cells under steady-state conditions, T_{reg} cell ablation caused T_{conv} cells to adopt a T_{reg}-like transcriptional profile. We noted that a large proportion of genes specifically enriched within tumor-associated T_{reg} cells compared with CD4$^+$ T_{conv} cells ($|FC| > 4$, $q < 0.05$) under steady-state conditions were induced at high levels within CD4$^+$ or CD8$^+$ T_{conv} cells upon T_{reg} cell ablation (**Fig. 1C** and **1D; Data file S1**). Clusters A and B comprised intratumoral T_{reg}-associated genes upregulated in both CD4$^+$ and CD8$^+$ T_{conv} cells upon T_{reg} cell ablation; Cluster C comprised intratumoral T_{reg}-associated genes whose expression was

upregulated exclusively in $CD8^+$ T_{conv} cells upon T_{reg} cell ablation; Cluster D comprised intratumoral T_{reg}-associated genes whose expression was upregulated exclusively in $CD4^+$ T_{conv} cells; Cluster E comprised a limited set of T_{reg}-specific transcripts that were not expressed at high relative levels in $CD4^+$ or $CD8^+$ T_{conv} cells upon T_{reg} cell ablation, including *Foxp3*, *Lrrc32*, *Ikzf2*, *Runx2,* and *Ctla4*. Similarly, a substantial fraction of transcripts highly expressed within intratumoral T_{conv} cells compared with T_{reg} cells were downregulated within T_{conv} cells upon T_{reg} cell ablation. (**Fig. S1; Data file S2**). Consistent with these observations, hierarchical clustering analysis of Pearson distances between global transcriptional profiles of samples revealed that intratumoral $CD4^+$ and $CD8^+$ T_{conv} cells from T_{reg}-depleted animals clustered more strongly with T_{reg} cells than T_{conv} cells from T_{reg}-sufficient animals (**Fig. 1E**). Moreover, differences in gene expression between $CD4^+$ T_{conv} cells in the absence versus presence of T_{reg} cells were significantly positively correlated with differences in gene expression between intratumoral T_{reg} cells and $CD4^+$ T_{conv} cells from T_{reg}-sufficient animals (**Fig. 1F**). Collectively, these results show that intratumoral T_{conv} cells adopt a T_{reg}-like transcriptional profile upon experimental ablation of T_{reg} cells *in vivo*.

Ablation of T_{reg} cells promotes the induction of T_{conv} cell suppression

T_{reg} cells suppress the proliferation of naïve T_{conv} cells when co-cultured *in vitro (34-36)*. Given their acquisition of a T_{reg}-like transcriptional profile, we asked whether T_{conv} cells develop suppressive function upon depletion of T_{reg} cells. To test this, we purified *Foxp3*$^{EGFP-}$ $CD4^+$ T_{conv} cells or *Foxp3*$^{EGFP+}$ $CD4^+$ T_{reg} cells by FACS from B16 tumor bearing *Foxp3*$^{EGFP\text{-}DTR}$ mice and incubated them with congenically distinct naïve $CD4^+$ T_{conv} cells *in vitro* (**Fig. 2A**). Strikingly, $CD4^+$ T_{conv} cells from the tumors of mice whose T_{reg} cells had been ablated by administration of DTx profoundly suppressed the proliferation of $CD4^+$ T_{conv} cell responders compared with $CD4^+$

T_{conv} cells from tumors of animals with intact T_{reg} cell populations (**Fig. 2B** and **C**). The level of suppression was only marginally less than the level of suppressive activity of a similar number of intratumoral Foxp3$^+$ T_{reg} cells. Splenic CD4$^+$ T_{conv} cells from DTx-treated mice were also more suppressive than CD4$^+$ T_{conv} from the spleens of PBS treated animals (**Fig. S2A-B**).In addition to suppressing T cell proliferation, T_{conv} cells from tumors of T_{reg}-depleted animals suppressed stimulation-driven induction of the activation marker CD44 on responder cells in contrast to T_{conv} cells from tumors of non-T_{reg}-depleted animals (**Fig. 2D** and **E**). T_{conv} cells from T_{reg}-depleted animals expressed similar levels of the co-inhibitory molecules TIGIT, TIM-3 and GITR to intratumoral T_{reg} cells (**Fig. 2F** and **G**). They also expressed higher levels of CTLA-4 compared to CD4$^+$ T_{conv} cells from tumors with intact T_{reg} populations.. Acquisition of suppressive function by T_{conv} cells was not an artefact of DTx treatment, since administration of DTx to *Foxp3*$^{EGFP\text{-}DTR}$ and control *Foxp3*^{EGFP} mice resulted in induction of suppressive activity only among CD4$^+$ T_{conv} cells from *Foxp3*$^{EGFP\text{-}DTR}$ animals, whose T_{reg} cells are sensitive to DTx treatment (**Fig. S2**). Thus, upon T_{reg} cell ablation, CD4$^+$ T_{conv} cells within tumors acquire transcriptional and functional characteristics of T_{reg} cells.

T_{reg} cell ablation results in induction of CCR8$^+$ T_{conv} cells within tumors

To understand whether the changes in the transcriptional and functional properties of bulk populations of T_{conv} cells in the absence of T_{reg} cells were driven by specific subpopulations, we performed scRNA-Seq of bulk T cell populations sorted by FACS from B16 melanoma tumors of DTx- and PBS-treated *Foxp3*$^{EGFP\text{-}DTR}$ animals at day 16 after tumor implantation. Single-cell gene expression data were clustered using Seurat and global transcriptional differences between cells were visualized in two-dimensional space using Uniform Manifold Approximation and Projection

(UMAP). *k*-means clustering revealed the presence of 8 transcriptionally distinct clusters of cells (**Fig. 3A**). Clusters 2 and 3 were enriched in control samples, whereas Clusters 0, 1, 5 and 7 were enriched among T cells from tumors of T_{reg}-depleted animals (**Fig. 3B** and **C**). Enrichment analysis was used to determine which clusters of cells are most responsible for induction of T_{reg}-like gene expression within bulk RNA-Seq profiles of T_{conv} cells from T_{reg}-depleted animals. This revealed that Cluster 0 (present at a ~3:1 ratio in the DTx treatment condition) was most enriched in genes specifically upregulated by $CD4^+$ T_{conv} cells upon DTx treatment (**Fig. 3D**). To define surface markers which would enable isolation of cells of Cluster 0, we performed an analysis of uniquely upregulated transcripts within each cluster. This analysis revealed that Cluster 0 cells express transcripts associated with T cell activation but which are also highly expressed by T_{reg} cells, including *Il2ra*, *Tigit* and *Tnfrsf4* (**Fig. 3E** and **Data file S3**). Strikingly, *Ccr8* mRNA expression was also upregulated in Cluster 0 cells upon depletion of T_{reg} cells, which was notable since we and others have shown that chemokine (C-C motif) receptor 8 (CCR8) marks highly suppressive T_{reg} cells under steady-state conditions within both murine and human tumors (*37-41*). A more focused analysis of the $CD4^+$ T cells in Cluster 0 revealed a population of cells (subcluster 5) enriched in expression of transcripts encoding molecules associated with Th2 differentiation, including *Ccr8, Gata3, Maf* and *Il10,* and suppressive/co-inhibitory function, including *Pdcd1*, *Tigit, Il10* and *Lag3* (**Fig. S3, Data file S4**) Accordingly, an analysis of the distribution of cells expressing *Ccr8, Il2ra*, *Tigit* and *Tnfrsf4* revealed that while in T_{reg}-replete animals, these markers are largely expressed by intratumoral of *Foxp3*$^{EGFP+}$ T_{reg} cells, they became expressed by a subset of *Foxp3*$^{EGFP-}$ $CD4^+$ T_{conv} cells upon T_{reg} depletion (**Fig. 3F** and **Fig. S4**).

We therefore analyzed the expression of CCR8 on the surface of T_{reg} and T_{conv} cells in tumors and lymphatics of *Foxp3*$^{EGFP\text{-}DTR}$ animals treated with PBS or DTx (**Fig. 3H** and **I**). We

found that the relative frequency of CCR8$^+$ cells among total CD4$^+$ *Foxp3*$^{EGFP–}$ T_{conv} cells increases within both the spleen and draining lymph nodes (dLN) of T_{reg}-depleted animals, but that their relative frequency within tumors was unchanged. However, since there is an expansion of CD4$^+$ T_{conv} cells within all tissues upon T_{reg} depletion, the absolute number of CCR8$^+$ T_{conv} cells within all tissues including tumors was increased.

CCR8 expression marks highly suppressive T_{conv} cells within tumors

To better understand the identity of CCR8$^+$ T_{conv} cells, we purified CCR8$^+$ and CCR8$^–$ CD4$^+$ T_{conv} cells by FACS from B16-F10 tumors of DTX-treated *Foxp3*$^{EGFP\text{-}DTR}$ mice and subjected them to bulk RNA-Seq. CCR8$^+$ T_{conv} cells were enriched in transcripts encoding molecules associated with both T cell activation such as *Tnfrsf9* (encoding 4-1BB) and T_{reg} cell suppressive function, including *Il2ra, Areg,* and *Il10* (**Fig. 4A** and **Data file S5**). Interestingly, CCR8$^+$ T_{conv} cells were not enriched for transcripts indicative of the suppressive type 1 regulatory T cell (Tr1) subset such as *Eomes, Gzmk, Itga2* (encoding CD49b), *Ccr5*, or *Cd226*, suggesting that they are distinct from Tr1 cells. Gene set enrichment analysis (GSEA) of global gene expression differences between CCR8$^+$ and CCR8$^–$ T_{conv} cells revealed a negative enrichment of genes upregulated in *Foxp3*$^–$ T_{conv} cells *vs Foxp3*$^+$ T_{reg} cells among CCR8$^+$ T_{conv} cells compared with CCR8$^–$ T_{conv} cells (**Fig. 4B)**. Consistently, we observed that global differences in gene expression between CCR8$^+$ and CCR8$^–$ T_{conv} cells were positively correlated with global differences in gene expression between intratumoral T_{reg} and T_{conv} cells (**Fig. 4C**), further suggesting that CCR8$^+$ *Foxp3*$^–$ T_{conv} cells possess a T_{reg}-like transcriptional profile.

We compared the phenotype of CCR8$^–$ and CCR8$^+$ CD4$^+$ T_{conv} cells from tumors of mice whose T_{reg} cells had been depleted by DTx with that of T_{reg} cells. Like T_{reg} cells, we found that

$CCR8^+$ $CD4^+$ T_{conv} cells expressed high levels of CD25 (**Fig. 4D** and **E**). Similar to T_{reg} cells, $CCR8^+$ $CD4^+$ T_{conv} cells also exhibited increased expression of OX40, GITR, TIGIT and LAG-3 compared to $CCR8^-$ $CD4^+$ T_{conv} cells (**Fig. 4D** and **E**). $CCR8^+$ T_{conv} cells also expressed increased levels of the transcription factor GATA3, suggesting that they possess a Th2-like differentiation state. To test whether the accumulation of $CCR8^+$ cells within intratumoral T_{conv} cell populations following T_{reg} cell ablation accounts for their increased suppressive activity, we separately sorted $CCR8^+$ and $CCR8^-$ *Foxp3*$^{EGFP-}$ T_{conv} cells from the tumors of DTx-treated *Foxp3*$^{EGFP-DTR}$ mice and assessed their ability to suppress naïve T_{conv} cell proliferation *in vitro*. Notably, suppressive function was enriched within the $CCR8^+$ T_{conv} cell fraction, which were more capable of restricting proliferation of responder cells compared to the $CCR8^-$ T_{conv} cell fraction (**Fig. 4F** and **G**). Taken together, these results suggest that CCR8 expression marks a subset of highly activated and suppressive T_{conv} cells which accumulate systemically and within tumors upon T_{reg} cell depletion.

$CD4^+$ $FOXP3^-$ $CCR8^+$ T_{conv} cells are found within tumors of NSCLC patients

In order to determine if $CCR8^+$ $FOXP3^-$ T_{conv} cells are enriched in human cancer, we analyzed $CD4^+$ T cells from 48 patents with non-small cell lung carcinoma (NSCLC) by flow cytometry (**Fig. S5**). Similar to our observations in mouse, $CCR8^+$ $FOXP3^-$ T_{conv} cells expressed high levels of CD25 and were significantly enriched in tumor tissue compared to healthy adjacent tissue and blood from the same patients (**Fig. 5A** and **5B**). Moreover, the frequency of $CCR8^+$ $CD25^+$ $FOXP3^-$ T_{conv} cells was inversely correlated with the frequency of cytotoxic $CD8^+$ T cells within tumors (**Fig. 5C**), suggesting that they possess suppressive function, and co-expressed the inhibitory receptors PD-1, TIGIT and TIM-3 (**Fig. 5D** and **E**). $CD4^+$ $FOXP3^-$ $CCR8^+$ T cells displayed increased expression of the tissue-residency marker CXCR6 and co-stimulatory receptor

CD27 compared to CCR8$^-$ cells and their phenotype was largely overlapping with that of FOXP3$^+$ T_{reg} cells (**Fig. 5E** and **Fig 5F**). CD4$^+$ FOXP3$^-$ CCR8$^+$ T cells lacked expression of EOMES and granzyme K, providing further evidence that this cell type is distinct from Tr1 cells (**Fig. 5E**). The presence of CCR8$^+$ FOXP3$^-$ T_{conv} cells in human tumors under steady-state conditions and without T_{reg} depletion is consistent with our observations within murine tumors (**Fig. 3H** and **I**), which contain a population of CCR8$^+$ CD4$^+$ T_{conv} cells under basal conditions that undergo substantial expansion upon T_{reg} cell ablation.

T_{conv} cell suppression is dependent on IL-10 signaling

We sought to understand mechanisms by which intratumoral CD4$^+$ T_{conv} cells exert their suppressive function. Informed by the results of our transcriptional analyses, we screened for the involvement of suppressive mechanisms by which T_{conv} cells from T_{reg}-depleted animals suppress T cell activation and proliferation *in vitro*. We tested whether blocking antibodies directed against CD25, CTLA-4, IL-10 receptor (IL-10R) and CCR8, neutralizing antibodies specific for transforming growth factor (TGF)-β, or pharmacological inhibition of steroid biosynthesis preferentially produced by Th2 cells using aminoglutethimide (AG) (*42*), are able to reverse the suppressive activity of intratumoral T_{conv} cells from T_{reg}-depleted animals. While the proliferation of naïve CD4$^+$ T cells was suppressed by Foxp3$^-$ T_{conv} cells, these differences induced by the presence of suppressive Foxp3$^-$ T_{conv} cells were abolished upon treatment of cells with anti-IL-10R (**Fig. 6A** and **B**).

Given these observations, we asked whether CCR8$^+$ T_{conv} cells are the primary producers of *Il10* mRNA upon depletion of T_{reg} cells. We first examined whether the expression of *Il10* mRNA is co-correlated with *Ccr8*, and therefore co-expressed by cells, using scRNA-Seq of T

cells from tumors of T_{reg}-replete and -depleted animals. Since IL-10 is known to be produced by $CD4^+$ T_R1 cells which express LAG3 and CD49b (*43-46*), $Eomes^+$ $CD4^+$ T_{conv} cells (*47-49*), exhausted $CD8^+$ T cells which express PD-1 (*50*) and $CD4^+$ Th2 cells which express GATA3, IL-4 and IL-13 (*51, 52*), we included the genes encoding these and other markers in our co-correlation analysis. We found that under steady-state (T_{reg}-replete) conditions *Il10* formed a predominant co-correlation cluster with *Cd8a, Pdcd1, Ifng, Tnf,* and *Eomes* but also a smaller cluster containing *Gata3* and *Foxp3*, suggesting that $CD8^+$ T cells in differential states of exhaustion, and Th2-like T_{reg} cells are a predominant source of IL-10 under steady-state conditions (**Fig. 6C**). However, we found that T_{reg} depletion results in a striking change in the co-correlation relationship of *Il10* mRNA with the other genes examined, forming a predominant cluster of co-correlated genes containing *Cd4, Ccr8, Il13, Il14* and *Gata3*. These striking results suggested that upon T_{reg} ablation, the source of *Il10* shifts to the previously identified $CD4^+$ $CCR8^+$ T_{conv} cell subset with Th2-like characteristics. To confirm this, we sorted $CCR8^–$ and $CCR8^+$ T_{conv} cells from tumors of T_{reg}-replete and -depleted animals and subjected them to qRT-PCR. We found that *Il10* mRNA expression was enriched among $CCR8^+$ T_{conv} from T_{reg}-depleted animals compared with both $CCR8^–$ cells from T_{reg}-depleted animals and $CCR8^+$ or $CCR8^–$ cells from T_{reg}-replete animals (**Fig. 6D**). These findings supported the hypothesis that $CCR8^+$ T_{conv} cells become the predominant source of T cell-expressed IL-10 upon T_{reg} depletion.

We therefore asked whether T_{reg} cell depletion triggers induction of IL-10-dependent suppressive activity among $Foxp3^–$ T_{conv} cells, limiting efficacy of T_{reg}-depletion *in vivo*. To test whether IL-10 production by T_{conv} cells is responsible for resistance to T_{reg}-depleting therapy *in vivo*, we generated *Il10*$^{flox/flox}$ *Cd4*^{Cre} *Foxp3*$^{EGFP\text{-}DTR}$ and littermate *Cd4*^{Cre} *Foxp3*$^{EGFP\text{-}DTR}$ (IL-10 proficient) control mice. This allowed us to examine the effect of T_{reg} depletion in animals whose

T cells can or cannot produce IL-10. Strikingly, we found that conditional deletion of IL-10 within T cells resulted in loss of resistance to T_{reg} ablation, as indicated by reduced tumor growth when T_{reg} cells were ablated in animals lacking T cell-restricted IL-10 expression, but not when either condition was present alone (**Fig. 6D**). Similar to results with IL-10R blockade, T_{reg} cell ablation in animals bearing a conditional deletion of *Il10* within T cells was associated with increased frequencies of $CD8^+$ T cells and $CD4^+$ T_{conv} cells expressing the cytokines IFN-γ and TNF (**Fig. 6E-F**). Furthermore, there was an increase in expression of the activation marker CD44 on $CD4^+$ T_{conv} cells and $CD8^+$ T cells from animals bearing a conditional deletion of *Il10* and whose T_{reg} cells had been ablated (**Fig. 6G**). These results demonstrate a critical role for T cell-produced IL-10 in resistance to T_{reg}-depletion.

We therefore asked whether antibody blockade of IL-10 signaling synergizes with T_{reg} depletion *in vivo.* We had observed that T_{reg} depletion was ineffective at significantly reducing growth of established B16 tumors, while T_{reg} depletion during early disease delayed tumor growth but was ineffective at inducing complete responses (**Fig. 1A**). We first tested whether blockade of IL-10R using anti-IL-10R antibodies reverses resistance to T_{reg}-depleting therapy. We found that late T_{reg} cell depletion or IL-10R blockade alone failed to drive significant reduction in tumor growth, whereas their combination resulted in potent tumor regression (**Fig. 7A)**. Moreover, IL-10 blockade synergized with early T_{reg} cell ablation to induce complete responses in a proportion of animals receiving combined therapy (**Fig. S6**). We found that IL-10R blockade both alone and in combination with T_{reg} depletion increased the ratio (percentage) of $CD8^+$ T cells expressing IFN-γ and TNF within tumors, but the absolute number of IFN-γ- and TNF-expressing $CD8^+$ T cells was markedly increased upon combined T_{reg} ablation and IL-10R blockade, reflecting a combination of increased T cell infiltration and cytokine production (**Fig. 7B**). Similarly, the

combination of T_{reg} depletion and IL-10R blockade resulted in an increase in the frequency and absolute number of IFN-γ- and TNF-expressing $CD4^+$ T cells (**Fig. 7C** and **D**). These findings demonstrate that T_{conv} cells within tumors adopt IL-10 dependent suppressive activity upon therapeutic elimination of T_{reg} cells, contributing to treatment failure of T_{reg} cell depleting immunotherapies and that combined targeting of T_{reg} cells and counterregulatory IL-10-dependent suppression invoked upon their depletion may enhance therapy.

Discussion

T_{conv} cells and T_{reg} cells share components of their activation programs to meet similar metabolic, proliferative and migratory requirements as they transition from quiescent states to activated states (*53-55*). A substantial effort is now underway to develop therapies which specifically target molecules that distinguish T_{reg} cells within tumors from their T_{conv} cell counterparts. These efforts have been informed by comparative analyses of the molecular profiles of T_{reg} cells and T_{conv} cells within tumors under steady-state conditions (*56, 57*). We compared the transcriptional profiles of T_{reg} cells with T_{conv} cells not only under steady-state conditions, but upon immune activation provoked by experimental T_{reg} cell ablation. This analysis revealed that T_{conv} cells adopt a highly similar transcriptional profile to T_{reg} cells upon T_{reg} cell depletion. The extent of this reprograming goes beyond what would be expected as a result of the shared properties of T_{reg} cell and T_{conv} cell activation programs and reveals that a subset of T_{conv} cells marked by CCR8 expression undergo activation and take on counterregulatory IL-10-dependent suppressive function when T_{reg} cells are eliminated.

Acquisition of a T_{reg}-like transcriptional profile by T_{conv} cells upon T_{reg} cell ablation suggests that in practice there are very few molecules whose targeting will enable highly specific depletion of T_{reg} cells within tumors. Nevertheless, a small cluster of genes was identified in our analyses which has an expression profile limited to T_{reg} cells compared with T_{conv} cells under both steady-state conditions and upon T_{reg} cell depletion. While this cluster of genes may contain targets for specific depletion of T_{reg} cells within tumors, a question raised by this study is whether specific depletion of T_{reg} cells is indeed desirable, given induction of counterregulatory suppressive function by subsets of intratumoral T_{conv} cells.

It is known that CCR8 marks highly suppressive T_{reg} cells found in both mouse and human tumors (*37-41, 58*). There is significant interest in the development of therapies which deplete $CCR8^+$ Treg cells within tumors. Given our observation that CCR8 also marks T_{conv} cells whose suppressive function is induced upon experimental T_{reg} cell ablation *in vivo*, it is reasonable to postulate that depletion of CCR8-expressing cells is a superior approach to T_{reg} cell depletion using other Treg-expressed markers for induction of anti-tumor immunity, since CCR8-depleting therapies would target both T_{reg} cells and suppressive T_{conv} cells for destruction. It will be important to consider effects of CCR8-depleting therapies on both Treg cells and $CCR8^+$ Tconv cells within tumors, both in pre-clinical investigations (*39, 58*), and once robust intatumoral depletion of $CCR8^+$ cells in the human clinical context has been achieved. Alternatively, our data suggests that combining T_{reg}-targeted immunotherapies with blockade of IL-10 signaling will overcome counter-regulatory suppression by T_{conv} cells.

It is interesting that the $CCR8^+$ T_{conv} cell subset observed in human tumors did not phenotypically overlap with previously described Tr1 cells within tumors. Suppressive Tr1 cells have been characterized in several human tumors including head neck and squamous cell carcinoma (HNSCC) (*59*), colorectal cancer (*49, 60*), hepatocellular carcinoma (*46*), Hodgkin lymphoma (*61*), metastatic melanoma (*62*) and non-small-cell lung cancer (*49*), and their presence is often associated with tumor progression. The suppressive subset of $CCR8^+$ T_{conv} cells we observe does not express EOMES or granzyme K, markers previously reported to be indicative of Tr1 cells. Recent studies have described a contribution of tumor-infiltrating follicular helper T (T_{FH}) cells and follicular regulatory T (T_{FR}) cells to anti-tumor immunity (*63, 64*). However, CXCR5 was not expressed by $CCR8^+$ T_{conv} cells suggesting their distinction from T_{FH} cells. We did however observe high levels of GATA3 and *Il10* expression among $CCR8^+$ T_{conv} cells, suggesting that they

represent a Th2-like subset expressing high levels of markers associated with T cell activation, including CD25, expanded systemically and within tumors upon T_{reg} depletion. Prior work are consistent with these data, showing systemic expansion of Th2 cells expressing either GATA3 or Th2 cytokines upon experimental T_{reg} ablation (*65, 66*), and the intratumoral presence of CD4+ Tconv cells expressing CCR8(*37-41, 58*). We found that the frequency of $CCR8^+$ $CD25^+$ $FOXP3^-$ T_{conv} cells inversely correlates with the frequency of tumor-infiltrating $CD8^+$ T cells, suggesting that they play an inhibitory role in human tumor immunity. The observation that $CCR8^+$ T_{conv} cells expand and undergo activation to express IL-10 following depletion of T_{reg} cells provides an explanation of how bulk T_{conv} cells from tumors of T_{reg}-depleted animals acquire IL-10-dependent suppressive function despite lack of a change in the relative frequency of $CCR8^+$ cells within the tumor $CD4^+$ T_{conv} compartment. While our pre-clinical data from mouse models suggests that $CCR8^+$ T_{conv} cells expand numerically within tumors upon experimental T_{reg} cell ablation, this is difficult to formally assess in humans due to the lack of specific markers that are differentially expressed in comparison with $FOXP3^+$ T_{reg} cells, enabling their isolation *ex vivo*, and of clinically approved T_{reg}-depleting therapies in widespread use. However, a number of T_{reg}-targeted therapies are under development and it will be an important topic of future investigation to determine their effect upon $CCR8^+$ T_{conv} cells in humans. It will also be important to better define how $CCR8^+$ Tconv cells contribute to immune regulation in other contexts including infection and inflammation, as clones of $Foxp3^-$ $CD4^+$ T cells expressing CCR8, CD25 and IL-10 have previously been described in mice with experimental pulmonary granulomata (*67*), while $CD4^+$ Tconv cells expressing CCR8 are observed upon experimental allergic lung inflammation in mice (*68*), and infiltrating human skin (*69*).

Our findings show that in the context of T_{reg} cell depleting immunotherapies, IL-10 production by T_{conv} cells reveals a secondary layer of immune suppression responsible for driving immunotherapy resistance. It is essential that this immunosuppressive function of IL-10 in limiting the efficacy of T_{reg} cell targeted therapies is appreciated, especially since in other contexts, IL-10 has been shown to possess immunostimulatory activity both in pre-clinical models and in clinical trials (*70-72*). Our findings suggest that IL-10 blocking antibodies may be used as a synergistic therapy with T_{reg}-targeted immunotherapies to improve patient outcomes.

Preclinical studies suggest that T_{reg} cell depletion can reinvigorate T_{conv} cell responses, however clinical trials of T_{reg} cell depleting therapies have thus far been met with limited clinical efficacy (*32, 73*). While our analysis of human tumor-infiltrating $Foxp3^{-}$ T_{conv} cells revealed a fraction of $CCR8^{+}$ T_{conv} cells under steady-state conditions, it will be valuable to examine whether such cells are expanded upon immunotherapy with either novel T_{reg}-depleting therapies, or anti-CTLA-4 therapy, the therapeutic efficacy of which is postulated to in part depend upon depletion or blockade of the suppressive function of T_{reg} cells (*74, 75*). Indeed, the prognostic significance of such cells in determining the outcome of the immunotherapy responses will reveal insights into their contribution to immunotherapy resistance.

Materials and Methods

Study design

The objective of this study was to understand how T_{reg} depletion affects the function and immunoregulatory capacity of the T cell lineage in the context of tumor immunity. We used a syngeneic B16-F10 melanoma heterotopic tumor implantation model and modeling therapeutic T_{reg} ablation by experimentally depleting T_{reg} cells using the well-established *Foxp3*$^{EGFP\text{-}DTR}$ model. We examined the consequences of T_{reg} depletion for tumor progression, as measured by blinded serial caliper measurements and tumor immunity, as assessed by scRNA-Seq and flow cytometry. We found that T_{conv} cells acquire T_{reg}-like suppressive functions upon depletion of T_{reg} cells. Using transcriptional profiling and *in vitro* suppression assays to better understand the nature of this suppressive activity, we found that this suppressive function was enriched among a Th2-like T_{conv} subset marked by expression of CCR8. Moreover, using antibody blockade and conditional *Il10* deletion experiments, we found that the suppressive activity induced upon T_{reg} depletion was dependent upon IL-10. The sample size for each experiment is specified in the figure legends. The number of independent experiments performed is stated in the figure legends. Age-matched male and female mice were randomly assigned to each group.

Mice

Foxp3$^{EGFP\text{-}DTR}$ mice were originally described by Kim et al. (*33*), *Foxp3*$^{IRES\text{-}EGFP}$, *Ptprc*^{a} (CD45.1), and *Rag2*$^{-/-}$ mice were obtained from the Jackson Laboratory. *Il10*$^{flox/flox}$ *and Cd4*^{Cre} mice were obtained from Jean Langhorne (Francis Crick Institute) and crossed with *Foxp3*$^{EGFP\text{-}DTR}$ mice to generate *Il10*$^{flox/flox}$ *Cd4*^{Cre} *Foxp3*$^{EGFP\text{-}DTR}$ animals. Experiments were performed using 8-14 week old mice, with male and female mice equally distributed between experimental and control groups. Mice were housed at the University of Cambridge University Biomedical Services (UBS) Gurdon

Institute Facility and Babraham Institute Biological Services Unit (BSU). Experiments were conducted in accordance with UK Home Office guidelines and were approved by University of Cambridge Animal Welfare and Ethics Review Board or by the Babraham Research Campus Animal Welfare and Ethics Review Board.

Human primary tissues

Primary tumors and adjacent healthy tissue were acquired from 48 NSCLC patients. Patients gave consent to be included in the study which was approved by the institutional review board of Humanitas Research Hospital (protocols no. 2578). Patients did not receive chemotherapy, radiotherapy or palliative surgery before samples were obtained. Samples were processed using the gentleMACS Dissociator (Miltenyi Biotec) into single cell suspensions as previously described (*76*), resuspended in dimethylsulfoxide (DMSO) with 10% Fetal Bovine Serum (FBS) and stored in liquid nitrogen.

High-dimensional flow cytometry analysis of human samples and computational processing of flow cytometric data.

Samples were prepared for flow cytometry as previously described (*76*). Panels were developed according to an established protocol (*77*). Briefly, Flow Cytometry Standard (FCS) 3.0 files were analysed by standard gating in FlowJo version 9 to remove dead cells and cell aggregates, and identify $CD4^+$ $FOXP3^-$ T cells. 5,000 $CD4^+$ $FOXP3^-$ T cells per tumor sample (n = 48) were subsequently imported into FlowJo (version 10), biexponentially transformed, and exported in order to be analyzed by a custom-made publicly available pipeline of PhenoGraph (https://github.com/luglilab/Cytophenograph). All samples were converted into comma separated value (CSV) files and concatenated in a single matrix by using the merge function of pandas

package. The K value, indicating the number of nearest neighbors identified in the first iteration of the algorithm, was set at 500. Uniform Manifold Approximation and Projection (UMAP) was obtained by UMAP Python package.

Tumor challenge and treatment

Mice were injected subcutaneously in the left flank with 1.25×10^5 B16-F10 melanoma cells (ATCC). Tumors were measured at serial time points following implantation using digital calipers and tumor area was calculated as the length × width. Tumor measurements were completed by an independent investigator who was not aware of treatment groups or genotypes. When tumors reached a size of ~25mm^2, mice were injected intraperitoneally (i.p) with 1 μg of diphtheria toxin (DTx) to achieve transient depletion of T_{reg} cells in *Foxp3*$^{EGFP\text{-}DTR}$ mice every other day for a total of four injections. Anti-IL-10R (clone 1B1.3A) was used *in vivo* and obtained from BioXcell. Mice received 250 μg of anti-IL-10R daily for 10 days starting when tumors reached a size of 25mm^2. DTx from *Corynebacterium diphtheriae* (Sigma-Aldrich) was obtained in lyophilized powder form and reconstituted in sterile double-distilled water according to the manufacturer's instructions.

Suppression assays

The suppressive capacity of tumor T_{conv} and T_{reg} cell was measured *in vitro* as previously described (*78*). Briefly, GFP$^+$ CD4$^+$ T_{reg} cells were isolated from B16-F10 tumors of *Foxp3*$^{EGFP\text{-}DTR}$ mice treated with PBS using florescence-activated cell sorting (FACS) 16 days post implantation. GFP$^-$ CD4$^+$ T_{conv} cells were isolated from B16-F10 tumors of *Foxp3*$^{EGFP\text{-}DTR}$ mice treated with PBS or DTx by florescence-activated cell sorting (FACS) at day 16 post-implantation. Naïve CD4$^+$ T_{conv} cells (CD25$^-$ CD44$^-$ CD62L$^+$) were purified from the spleens of WT CD45.1 mice by FACS and

stained with CellTrace Violet™ (CTV) according to the manufacturer's protocol (Thermo Fisher Scientific). $2.5x10^4$ or $1.25x10^4$ (as indicated) suppressor $CD4^+$ T_{reg} or T_{conv} cells were co-cultured with $1x10^5$ T_{resp} cells in the presence of anti-CD3 (BioLegend 1 µg/mL) and $5.0x10^4$ *Rag2*$^{-/-}$ antigen presenting cells (APC). Naïve $CD4^+$ T_{conv} cells cultured without tumor T_{reg} cells or T_{conv} cells were used as a control. Cell division was evaluated by flow cytometry after 4 days of culture.

Flow cytometry of murine samples

Tumor samples were digested using collagenase and DNase for 30 minutes at 37 ºC. Percoll was used to isolate lymphocytes from tumors. Tumors and spleens were mechanically dissociated over a 40 µm cell strainer. Red blood cells were lysed using ACK Lysing Buffer (Gibco). Cells were stained with the Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific) to discriminate between live and dead cells and then incubated with the following surface antibodies for 30 minutes on ice:, anti-TCRβ PE (H57-597), anti-CD8 PE-Cy7 (53-6.7), anti-CD25 APC (PC61.5), anti-CD44 PerCP-Cyanine5.5 (IM7), anti-CD45.1 APC (A20), anti-GITR (DTA-1), anti-LAG-3 (C9B7W) anti-OX40 (OX-86) anti-TIGIT (GIGD7), anti-TIM-3 (RMT3-23) from eBioscience anti-CD4 BUV395 (GK1.5), anti-CD62L BUV-737 (MEL-14), from BD Biosciences and anti-CCR8 BV421 (SA214G2), anti-Thy1.2 BV605 (clone 53-2.1), from BioLegend. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin and blocked with brefeldin A (BFA) for 4 hours in RPMI 1640 complete medium. The intracellular antibodies anti-Foxp3 APC (FJK-16S), anti-CTLA-4 (UC10-4B9) anti-IFN-γ FITC (XMG1.2), anti-GATA3 (TWAJ) and anti-TNF PE-Cy7 (MP6-XT22) were purchased from eBioscience and used with the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. For determination of CCR8 expression by

Foxp3^{-} T_{conv} cells, cells from tumor-bearing *Foxp3*$^{EGFP\text{-}DTR}$ animals were surface-stained and analyzed unfixed using flow cytometry, with EGFP expression used to discriminate T_{reg} and T_{conv} cells. Samples were analyzed using BD Fortessa and Beckman Coulter CytoFLEX analyzers. After analysis, data were analyzed using FlowJo software (Tree Star, Inc.).

scRNA Sequencing and analysis

Single cell suspensions of T cells were purified by total pan T cell enrichment, and live TCR-β^{+} cells were sorted from B16 tumors by FACS 16 days post implantation. RNA libraries were prepared for single cell RNA sequencing (scRNA-Seq) using the Chromium Single Cell 5' Library & Gel Bead Kit v2 (10x Genomics) processed with Chromium (10x Genomics), and sequenced using the HiSeq 4000 System (Illumnia). Raw 10x sequencing data were processed as previously described and mapped to mm10. We confirmed that cells were sequenced to saturation. Data were merged with cell ranger aggr (cellranger-v5.0.0). Merged data were transferred to the R statistical environment for analysis primarily using the package Seurat (v3.2.2) in R v4.0.3. The analysis included only cells expressing between 200 and 2,500 genes, <5% mitochondrial-associated transcripts, and genes expressed in at least three cells. The data were then log-normalized and scaled per cell, and variable genes were detected using the FindVariableFeatures function in Seurat, as per default settings, using 2000 features and further processed as per the ScaleData function. Principal component analysis (PCA) was run on the variable genes, and the first six principal components (PCs) were selected for further analyses, based on the standard deviation of the PCs, as determined by an "elbow plot" in Seurat. Cells were clustered using the FindClusters function in Seurat with default settings, resolution = 0.5, and six PCs. UMAP was calculated using six PCs (RunUMAP function). For broadly defining the transcriptional features of each cluster,

the FindAllMarkers function (only.pos = FALSE, min.pct = 0.1, thresh.use = 0.2, test.use = "MAST") was used, and the associated heatmap was generated using the DoHeatmap function using up to the top 10 transcripts identified per cluster as defined by FindAllMarkers. The transcriptomic score of a particular cluster was calculated using the AddModuleScore function with default settings. Further visualizations of exported normalized data were generated using the Seurat RidgePlot functions and custom R scripts.

RNA Sequencing and analysis

Single-cell suspensions were purified by FACS, as described above 16 days post tumor implantation, and stored in 40 µl RNA*later*™ Stabilization Solution at -80 °C. RNA was extracted from samples using the RNeasy Plus Mini Kit (Qiagen) with optional QIAshredder step according to the manufacturer's protocol. RNA-Sequencing (RNA-Seq) analyses were performed using ≥ 2 biological replicates. RNA-Seq was performed and analyzed as described previously (*54*). RNA Libraries were prepared using the Clontech SMARTer Ultra Low-input RNA kit (Takara) and sequenced on an Illumina HiSeq 2500 instrument using Illumina TruSeq v4.0 chemistry. The resulting FastQ files underwent quality control with FastQC, adaptor trimming with Cutadapt and alignment to the NCBIM37 *Mus musculus* genome annotation with hisat2 using ClusterFlow pipelines. Uniquely mapped reads were used to calculate gene expression and FPM values normalized to total library size with intergenic read normalization were calculated. Differential expression and statistical significance were calculated using the Wald test with adjustment for multiple testing using the Benjamini-Hochberg method using DESeq2 (*79*). Differentially expressed genes were further analyzed using R. PCA was performed using R *plotPCA* with count data transformed using variance stabilizing transformation (VST) from fitted dispersion-mean

relationships generated using DESeq2 *vst*. Expression heatmaps were generated using FPM values normalized to row maxima using the R *pheatmap* package. Hierarchical clustering was performed using the Ward method. Dendrograms were cut at levels sufficient to allow 3-5 clusters to be discriminated.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism software. Two-tailed Student's *t* tests or one-way ordinary ANOVAs were used as indicated to calculate statistical significance of the difference in sample means. *P* values of less than 0.05 were considered statistically significant. Statistical tests used are specified in the figure legends. In all figures, data represent the mean ± the standard error of the mean (SEM). *P* values correlate with symbols as follows: ns = not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Supplementary Materials

Figure S1. A majority of transcripts highly expressed within intratumoral T_{conv} cells compared with T_{reg} cells are downregulated within T_{conv} cells upon T_{reg} cell ablation.

Figure S2. Isolation of $CD4^+$ $FOXP3^-$ T cells from tumors of NSCLC patients.

Figure S3. IL-10 blockade synergized with early T_{reg} cell ablation to induce complete responses in a proportion of animals receiving combined therapy.

Data file S1. Expression of T_{reg}-associated transcripts (q<0.05; |FC|>3) in indicated cell types isolated from B16 tumors 18 days following tumor implantation in *Foxp3*$^{EGFP\text{-}DTR}$ animals administered with either PBS or DTx.

Data file S2. Expression of transcripts upregulated in tumor T_{conv} cells versus T_{reg} cells under steady-state conditions (q<0.05; |FC|>3) within indicated cell types isolated from B16 tumors of *Foxp3*$^{EGFP\text{-}DTR}$ animals administered with either PBS or DTx.

Data file S3. Differentially expressed genes significantly up-/down-regulated within clusters identified using scRNA-Seq of T cells isolated from B16 tumors 18 days following tumor implantation in *Foxp3*$^{EGFP\text{-}DTR}$ animals administered either PBS or DTx.

Data file S4. Differentially expressed genes significantly up-/down-regulated within subclusters in cluster 0 identified using scRNA-Seq of T cells isolated from B16 tumors 18 days following tumor implantation in *Foxp3*$^{EGFP\text{-}DTR}$ animals administered either PBS or DTx.

Data file S5. Differentially expressed genes between $CCR8^-$ and $CCR8^+$ $CD4^+$ *Foxp3*$^{EGFP-}$ T_{conv} cells isolated from B16 tumors 18 days following tumor implantation in *Foxp3*$^{EGFP\text{-}DTR}$ animals.

Data file S6. Raw data file.

Reproducibility checklist

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Competing interests

R.R. holds or has held paid consultancies with Lyell Immunopharma, Achilles Therapeutics and Enhanc3D Genomics; and is a principal investigator of research projects funded by FStar Therapeutics and AstraZeneca on unrelated topics that do not constitute competing interests. E.L. served as a consltant for BD Biosciences on a topic unrelated to this work. All other authors declare no competing interests.

Data and materials availability

All bulk RNA-seq and scRNA-seq data will be made publicly available under NCBI Gene Expression Omnibus (GEO) accession number GSE236825. All other data needed to support the conclusions of the paper are present in the paper or the Supplementary Materials.

Figure Legends

Figure 1. T_{reg} ablation results in acquisition of T_{reg} cell transcriptional features by T_{conv} cells. (**A**) Tumor growth of B16-F10 tumors subcutaneously implanted into *Foxp3*$^{EGFP\text{-}DTR}$ mice. Gray shading indicates time period over which PBS or DTx was administered (day 7-13 post-implantation, early disease; day 10-16 post-implantation, established). Dashed lines indicate individual mice. Solid line indicates average tumor area over time. Data representative of 3 individually repeated experiments, n > 8 $^{**}P < 0.01$; ordinary one-way ANOVA, Tukey's multiple comparisons.. (**B**) Representative frequency of Foxp3$^+$ T_{reg} cells among total CD4$^+$ T cells within spleens (top) or tumors (bottom) of *Foxp3*$^{EGFP\text{-}DTR}$ mice with established tumors administered PBS or DTx. (**C**) Heatmap showing the relative expression of transcripts upregulated in intratumoral T_{reg} cells compared with CD4$^+$ T_{conv} cells (q<0.05; FC>3) in the indicated T cell subsets isolated at day 16 after implantation of B16-F10 tumors in *Foxp3*$^{EGFP\text{-}DTR}$ animals administered PBS or DTx. Colors indicate expression normalized to row maxima. *x*-axis hierarchical clustering of intratumoral T_{reg}-expressed transcripts identifies 5 clusters of genes with distinct expression patterns. Gray bars to right of heatmap indicate expression greater than a third of the expression of given transcripts in intratumoral T_{reg} cells. (**D**) Average expression of genes within the 5 clusters identified in each T cell subset. **(E)** Heatmap showing pairwise Pearson distances between the global gene expression profiles of the indicated T cell subsets from B16-F10 tumor-bearing *Foxp3*$^{EGFP\text{-}DTR}$ animals administered PBS or DTx. (**F**) Scatterplot comparing the global differences in gene expression between intratumoral T_{reg} cells and T_{conv} cells with transcriptional differences between CD4$^+$ T_{conv} cells isolated from DTx versus PBS-treated animals. A highly significant correlation is observed indicating transcriptional convergence of intratumoral T_{reg} cells with T_{conv}

cells in absence of T_{reg} cells. Data from 2-4 biological replicates isolated on independent days (C-F).

Figure 2. T_{reg} ablation promotes induction of T_{conv} cell suppression. (**A**) Experimental schema. B16-F10 cells were subcutaneously implanted into *Foxp3*$^{EGFP\text{-}DTR}$ CD45.2$^+$ mice and administered PBS or DTx on day 7, 9, 11, and 13. Cells were harvested from tumors of PBS- and DTx-treated animals at day 16 post-implantation. (**B** and **C**) CD45.2$^+$ TCRβ$^+$ CD4$^+$ GFP$^-$ T_{conv} and TCRβ$^+$ CD4$^+$ GFP$^+$ T_{reg} suppressor cells were sorted by FACS from tumors and co-cultured with CD45.1$^+$ CTV-labeled naïve CD4$^+$ T_{conv} cell responders at a ratio of 1:4, with 2.5x10^4 suppressor CD4$^+$ T_{reg} or T_{conv} cells co-cultured with 1x10^5 T_{resp} cells in the presence of 5.0x10^4 antigen-presenting cells (APC). TCRβ$^+$ CD4$^+$ GFP$^+$ T_{reg} suppressor cells were sorted by FACS from mice treated with PBS only and used as controls. Representative histograms and replicate measurements of dividing CD45.1$^+$ responder cells incubated with intratumoral T_{reg} or T_{conv} cells. Naïve splenic T_{conv} cells without intratumor T_{reg} or T_{conv} cells were used as a control. Data are representative of >4 independently repeated experiments, n > 5 per group. *P < 0.05 ****, P < 0.0001; ns, not significant; ordinary one-way ANOVA, Tukey's multiple comparisons. Bars and error show mean and s.e.m.(**D** and **E**) Representative histograms and replicate measurements of CD44^{hi} CD45.1$^+$ responder cells incubated with indicated suppressor cell populations. Naïve T_{conv} cells without tumor T_{conv} cells were used as control. (**F**) Representative flow cytometry and (**G**) replicate measurements of the expression of the indicated proteins from intratumoral T_{reg} cells and CD4$^+$ T_{conv} cells isolated at day 16 after implantation of B16-F10 tumors in *Foxp3*$^{EGFP\text{-}DTR}$ mice treated with PBS or DTx. Data are representative of > 3 independently repeated experiments, n > 7 per

group. ****P <0.0001; ns, not significant; ordinary one-way ANOVA, Tukey's multiple comparisons. Bars and error show mean and s.e.m.

Figure 3. T_{reg} ablation promotes the expansion of tumor-infiltrating CCR8$^+$ T_{conv} cells. (**A**) Uniform manifold approximation and projection (UMAP) of scRNA-seq of TCRβ$^+$ cells isolated at day 16 after implantation of B16-F10 tumors in *Foxp3*$^{EGFP\text{-}DTR}$ mice treated with PBS or DTx on days 7, 9, 11 and 13. (**B**) Density plots showing change in distribution of cells in PBS- and DTx-treated animals. (**C**) Relative frequency of cells within each cluster normalized to their average ratio among PBS replicates. (**D**) Average enrichment of expression of the genes in Cluster D from Fig. 1C across scRNA-Seq clusters, (n=3, unpaired two-tailed Student's *t*-test, *P < 0.05, **P < 0.01). (**E**) Heatmap showing the expression of differentially upregulated genes in each cluster identified. (**F**) UMAP plots showing expression of indicated genes within T cells of tumors from tumor-bearing PBS- or DTx-treated *Foxp3*$^{EGFP\text{-}DTR}$ animals. (**G**) Representative flow cytometry and (**H**) replicate measurements of the frequency and total counts of CCR8$^+$ of CD4$^+$ T_{conv} cells from spleens, draining lymph nodes (dLN) and tumors of B16-F10 tumor-bearing *Foxp3*$^{EGFP\text{-}DTR}$ mice treated with PBS or DTx. Data are representative of 3 independently repeated experiments (**G** and **H**). Numbers in gates show percentages. n > 11. ordinary one-way ANOVA, Tukey's multiple comparisons. **P <0.01, ***P < 0.001, ****P < 0.0001; . Bars and error show mean and s.e.m.

Figure 4. CCR8 marks highly suppressive T_{conv} cells. (**A**) Heatmap showing the relative expression of differentially expressed genes between intratumoral CCR8$^+$ and CCR8$^-$ CD4$^+$ T_{conv}

cells (q<0.05; |FC|>3) isolated at day 16 after subcutaneous implantation of B16-F10 tumors in *Foxp3*$^{EGFP\text{-}DTR}$ animals treated with DTx at days 7, 9, 11 and 13. Data from 4 biological replicates isolated on independent days. (**B**) Gene-set enrichment analysis (GSEA) demonstrating a negative enrichment of genes upregulated in *Foxp3*$^{-}$ T_{conv} cells *vs* *Foxp3*$^{+}$ T_{reg} cells among CCR8^{+} T_{conv} cells compared with CCR8^{-} T_{conv} cells isolated from tumors of DTx-treated *Foxp3*$^{EGFP\text{-}DTR}$ mice. (**C**) Scatterplot comparing global changes in gene expression between intratumoral T_{reg} and T_{conv} cells with transcriptional differences between CCR8^{+} and CCR8^{-} CD4^{+} T_{conv} cells. (**D**) Representative flow cytometry and (**E**) replicate measurements of the expression of the indicated proteins from intratumoral T_{reg} cells and CCR8^{+} and CCR8^{-} CD4^{+} T_{conv} cells from tumors of B16-F10 tumor-bearing *Foxp3*$^{EGFP\text{-}DTR}$ mice treated with PBS or DTx. Data are representative of 3 independently repeated experiments n > 4. ordinary one-way ANOVA, Tukey's multiple comparisons. **P <0.01, ***P < 0.001, ****P < 0.0001, ns, not significant. (**F**) Representative histograms and (**G**) replicate measurements of CTV dilution within gated CD45.1^{+} responder cells incubated with intratumoral CD45.2^{+} TCRβ^{+} CD4^{+} GFP^{-} CCR8^{-} or CD45.2^{+} TCRβ^{+} CD4^{+} GFP^{-} CCR8^{+} suppressor T_{conv} cells isolated at day 16 after implantation of B16-F10 tumors in *Foxp3*$^{EGFP\text{-}DTR}$ mice. Suppressor cells were incubated with responders at a ratio of 1:8, with $1.25x10^{4}$ suppressor CD4^{+} T_{conv} cells co-cultured with $1x10^{5}$ T_{resp} cells in the presence of $5x10^{4}$APC. Data are representative of 2 independently repeated experiments n > 3. ordinary one-way ANOVA, Tukey's multiple comparisons. *P < 0.05; ***P < 0.001, ****P < 0.0001; Bars and error show mean and s.e.m.

Figure 5. CCR8^{+} T_{conv} cells expressing high levels of CD25 are found within the tumors of NSCLC patients. (**A**) CCR8 and CD25 expression among FOXP3^{-} CD4^{+} T cells from

representative samples from NSCLC patients (n=48). (**B**) Frequency of FOXP3^{-} CCR8^{+} CD25^{bright} cells among CD4^{+} T cells from the indicated patients' samples. Lines indicate paired samples. (**C**) Correlation of the frequency of CD8^{+} T cells (out of CD3^{+} T cells) with FOXP3^{-} CCR8^{+} CD25^{bright} cells (out of CD4^{+} T cells) in tumors from patient samples. (**D**) UMAP analysis of concatenated CD4^{+} FOXP3^{-} T_{conv} cells. Colors depict cell clusters identified by Phenograph (k=500). Separate UMAP plots of relative marker expression by concatenated CD4^{+} T cells from tumors. (**E**) Representative frequency and (**F**) replicate measurements of indicated markers from patient samples. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; two-tailed Mann−Whitney *U*-test.

Figure 6. IL-10 production by CD4^{+} T_{conv} cells limits anti-tumor efficacy of T_{reg} depletion. (**A**) CD4^{+} *Foxp3*$^{EGFP-}$ T_{conv} cells (suppressors) isolated at day 16 from B16-F10 tumors of DTx-treated *Foxp3*$^{EGFP-DTR}$ animals. DTx was administered to mice on days 7, 9, 11, and 13. Cells were cultured alone (gray) or with suppressors (red) and with indicated reagents. Cell proliferation of responder cells was analyzed after 4 days. CD45.2^{+} TCRβ^{+} CD4^{+} GFP^{-} T_{conv} suppressor cells were sorted by FACS from tumors and co-cultured with CD45.1^{+} CTV-labeled naïve CD4^{+} T_{conv} cell responders at a ratio of 1:8, with $1.25x10^{4}$ suppressor CD4^{+} T_{conv} cells co-cultured with $1x10^{5}$ T_{resp} cells in the presence of $5.0x10^{4}$ antigen-presenting cells (APC). Data are representative of 2 independently repeated experiments n > 4. *P* values show significance of difference between no suppressor and suppressor (Student *t* test) and are Benjamini-Hochberg corrected. $^{*}P < 0.05$; $^{****}P < 0.0001$, ns, not significant. (**B**) Representative frequency of dividing CD45.1^{+} responder cells incubated with tumor CD4^{+} GFP^{-} T_{conv} cells in the presence of anti-IL-10R antibodies or vehicle control. Naïve CD4^{+} T_{conv} cells without tumor T_{conv} cells were used as a control. (**C**) Co-correlation between the expression of indicated genes within single cell gene expression profiles

of T cells from tumors of PBS- or DTx-treated B16 tumor-bearing *Foxp3*$^{EGFP\text{-}DTR}$ animals. Pearson correlation co-efficient values indicated by color scale and genes are hierarchically clustered to identify clusters of co-expressed transcripts within T cell populations. scRNA-Seq data are representative of 3 biological replicates per group. (**D)** Measurement of *Il10* mRNA expression within CCR8^{-} and CCR8^{+} T_{conv} cells from PBS- and DTx-treated animals. Data representative of 4 biological replicates per group. . ordinary one-way ANOVA, Tukey's multiple comparisons. **P* < 0.05; ****P* < 0.001, *****P* < 0.0001; ns, not significant. (**E**) Tumor area of heterotopic B16-F10 melanoma tumors at indicated time-points following implantation into *Il10*$^{flox/flox}$ *Cd4*^{Cre} *Foxp3*$^{EGFP\text{-}DTR}$ or *Il10*$^{+/+}$ *Cd4*^{Cre} *Foxp3*$^{EGFP\text{-}DTR}$ control mice administered DTx or PBS from day 10-16 post-implantation. Data are representative of 2 independently repeated experiments n > 14. ordinary one-way ANOVA, Tukey's multiple comparisons. ***P* < 0.01; ****P* < 0.001, ****P* < 0.0001 (**F**) Representative histograms (top) and replicate measurements (bottom) of the frequency of CD8^{+} CD44^{+} T cells and Foxp3^{-} CD4^{+} CD44^{+} T_{conv} cells from tumors of animals within indicated treatment groups. (**G-H**) Representative frequency (**G**) and replicate measurements (**H**) of the frequency and total counts of CD8^{+} IFN-γ^{+} TNF^{+} T cells within tumors. Data are representative of 2 independently repeated experiments, . n >5, ordinary one-way ANOVA, Tukey's multiple comparisons. ***P* < 0.01; ****P* < 0.001, ****P* < 0.0001 (**E-H**) **P* < 0.05, ***P* <0.01; ****p* <0.001, *****p* <0.0001; Ordinary one-way ANOVA, Tukey's multiple comparisons. Bars and error show mean and s.e.m.

Figure 7. Blockade of IL-10 signaling synergizes with T_{reg}-depletion to drive potent anti-tumor immune responses. (**A**) Tumor area of B16-F10 melanoma tumors at indicated time-points following implantation into *Foxp3*$^{EGFP\text{-}DTR}$ animals administered indicated combinations of DTx and anti-IL-10R or control reagents from days 10-16 after tumor implantation. Data are

representative of 2 independently repeated experiments. n > 6, ordinary one-way ANOVA, Tukey's multiple comparisons. $^{**}P < 0.01$; $^{***}P < 0.001$, $^{***}P < 0.0001$ (**B**) Representative frequency and (**C**) replicate measurements of the frequency and total counts of $CD8^+$ IFN-γ^+ TNF^+ T cells from tumors. n > 4, ordinary one-way ANOVA, Tukey's multiple comparisons, $^{*}P < 0.05$; $^{**}P < 0.01$.(**D**) Representative frequency and (**E**) replicate measurements of the frequency and total counts of $Foxp3^-$ $CD4^+$ IFN-γ^+ TNF^+ T cells from tumors. Data from >2 independent biological replicates. n > 4, ordinary one-way ANOVA, Tukey's multiple comparisons, $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.; ns, not significant. Bars and error show mean and s.e.m.