Adaptive plasticity of IL-10+ and IL-35+ T_{reg} cells cooperatively promotes tumor T cell exhaustion

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Regulatory T cells (T_{reg} cells) maintain host self-tolerance but are a major barrier to effective cancer immunotherapy. T_{reg} cells subvert beneficial anti-tumor immunity by modulating inhibitory receptor expression on tumor-infiltrating lymphocytes (TILs); however, the underlying mediators and mechanisms have remained elusive. Here, we found that the cytokines IL-10 and IL-35 (Ebi3–IL-12x heterodimer) were divergently expressed by T_{reg} cell subpopulations in the tumor microenvironment (TME) and cooperatively promoted intratumoral T cell exhaustion by modulating several inhibitory receptor expression and exhaustion-associated transcriptomic signature of CD8 TILs. While expression of BLIMP1 (encoded by Prdm1) was a common target, IL-10 and IL-35 differentially affected effector T cell versus memory T cell fates, respectively, highlighting their differential, partially overlapping but non-redundant regulation of anti-tumor immunity. Our results reveal previously unappreciated cooperative roles for T_{reg} cell-derived IL-10 and IL-35 in promoting BLIMP1-dependent exhaustion of CD8 TILs that limits effective anti-tumor immunity.

Regulatory T cells (T_{reg} cells) are a specialized suppressive CD4+ T cell population capable of limiting deleterious immune responses to self and foreign antigens that underlie autoimmune and chronic inflammation. Conversely, T_{reg} cells suppress beneficial anti-tumor immunity and thereby pose an impediment to effective immunotherapies. Indeed, increased frequencies of T_{reg} cells and a reduced CD8+/T_{reg} cell ratio in tumors are linked to poor prognosis in many cancers. Although T_{reg} cell depletion dramatically reduces tumor burden, the ensuing autoimmune sequelae limit the use of this approach in the clinic. Hence, current trials are evaluating strategies targeting receptors preferentially enriched on intratumoral T_{reg} cells (CCR4, GITR, OX40, CTLA4). T_{reg} cells use a plethora of suppressive mechanisms—inhibitory cytokine secretion, metabolic disruption, modulation of antigen-presenting cell function and cytolytic effectors—often in a target cell and tissue-specific manner. While targeting suppressive mechanisms selectively used by intratumoral T_{reg} cells would be efficacious, the precise mechanisms and predominant suppressive mediators that promote T cell exhaustion and dominant suppression in the tumor microenvironment (TME) remain poorly defined.

One of the major contact-independent modes of T_{reg} cell suppression is via secretion of inhibitory cytokines (IL-10, IL-35 and TGF-β). The established suppressive cytokine duo (IL-10 and TGF-β) plays a critical role in steady-state immune homeostasis and taming exuberant responses at environmental interfaces. Perturbation of IL-10 and TGF-β signaling improved the function of exhausted T cells in chronic viral infections. Co-targeting T_{reg} cells or IL-10 along with the PD-1 immune checkpoint pathway resulted in a synergistic reversal of T cell exhaustion. IL-35 is required for maximal T_{reg} cell suppressor function and contributes to the regulatory milieu in numerous disease states. Previous work demonstrated enrichment of IL-35 expression on tumor-infiltrating T_{reg} cells in B16 tumors and a role of IL-35 in promoting inhibitory receptor expression (PD-1, TIM3, LAG3) on intratumoral T cells. Thus, while T_{reg} cells and suppressive cytokines have been linked to T cell exhaustion in chronic settings, the molecular mechanisms and the relative contribution of T_{reg} cell-derived suppressive cytokines underlying T_{reg} cell-induced exhaustion remain obscure.

In this study, we report a divergent and largely non-overlapping IL-10 and IL-35 expression pattern on T_{reg} cells infiltrating both murine tumors as well as patients with non-small cell lung cancer (NSCLC). We further demonstrate that these IL-10+ and Ebi3+ T_{reg} cell populations are not distinct lineages, but rather transitory states with concordant transcriptional and T cell receptor (TCR) profiles. This transition is inducible via TCR stimulation of purified T_{reg} cell subpopulations in vitro, potentially reflecting plasticity in inhibitory cytokine expression in chronic environments. While the two cytokines target a common BLIMP1 axis to promote the exhausted...
intratumoral T cell state, IL-10 and IL-35 differentially impacted effector versus memory generation, respectively. Our results uncover the previously unappreciated adaptive plasticity of IL-10 and IL-35 inhibitory cytokine expression by Treg cell subpopulations in the TME and how they cooperatively impinge on T cell function to promote exhaustion and limit anti-tumor immunity.

Results

Divergent IL-10 and IL-35 expression on Treg cells in murine tumors and NSCLC patients. Intratumoral Treg cells are more suppressive than their peripheral counterparts in both murine models and human tumors owing to their heightened activation status4,14. Thus, we reasoned that the major suppressive mediators might be co-expressed on intratumoral Foxp3+ Treg cells to maximize their functional impact. To address this hypothesis, we generated triple reporter mice (Il10Cre;Ebi3Tom;Foxp3Cre-YFP; Il10 for IL-10, Ebi3 for IL-35 and Foxp3 for Treg cells, respectively17,18; Fig. 1a and Supplementary Fig. 1a). We observed largely segregated expression of IL-10 and Ebi3 by Treg cells in various lymphoid and non-lymphoid organs, with minimal co-expression (Supplementary Fig. 1b), consistent with a previous report21. The only tissues with a notable (>5%) population of Treg cells co-expressing IL-10 and Ebi3 at steady state were skin and lamina propria, probably due to elevated IL-10+ Treg cells at these environmental interfaces (Supplementary Fig. 1b). Specifically, in the B16 TME, there was an approximately four- and two-fold intratumoral enrichment of IL-10+ (GFP+YFP+) and Ebi3+ (Tom+YFP+) Treg cells, respectively, compared to the periphery (Fig. 1a–c).

Furthermore, this divergent inhibitory cytokine distribution in Treg cells was not a characteristic limited to the T1p-polarized TME, as we noted a similarly divergent expression pattern in a T1p2-polarized fungal protease (Aspergillus oryzae) model of allergic airway disease (Fig. 1d–f)22. The enrichment of IL-10+ Treg cells was more pronounced in the allergic environment than the B16 TME, resulting in an approximately ten-fold expansion compared to the periphery, supporting the notion of adaptive plasticity in the relative abundance of IL-10+ and IL-35+ Treg cell subpopulations depending on the nature of the inflammatory microenvironment. Treg cell restricted deletion of Ebi3 in Il10Cre;Ebi3Tom;Foxp3Cre-YFP mice did not result in a compensatory change in the distribution of these Treg cell subpopulations in either T1p1- or T1p2-polarized inflammatory environment (Supplementary Fig. 1c–h). Human Treg cells from healthy donors and NSCLC patients also showed a similar segregated IL-10 and IL-35 expression pattern with a minimal percentage of double cytokine positive Treg cells (Fig. 1g–i). In this case, there was also an increased percentage of Ebi3+ intratumoral Treg cells, highlighting the translational importance of understanding the developmental and functional relationship between these intratumoral Treg cell subpopulations. Collectively, these data highlight preferential enrichment and divergent inhibitory cytokine expression pattern on mouse and human intratumoral Treg cells.

Adaptive plasticity of cytokine expression and comparable transcriptionome of intratumoral IL-10+ and IL-35+ Treg cell subpopulations. To dissect the divergent cytokine expression pattern and the preferential generation of single inhibitory cytokine positive Treg cell subpopulations, we performed single-cell RNAseq (scRNAseq) comparing Treg cells isolated from naive, unchallenged lymph nodes or day 14 B16 tumors from Foxp3Cre-YFP mice (Fig. 2a). IL-10+ and Ebi3+ Treg cells exhibited comparable transcriptomic signatures (Fig. 2b,c). Although unsupervised K-means clustering identified six unique Treg cell clusters (Supplementary Fig. 2a), the distribution ratio of IL-10+ versus Ebi3+ Treg cell subpopulations among those clusters was comparable, especially for intratumoral Treg cells (Supplementary Fig. 2b). Consistent with the scRNAseq analyses, T cell receptor beta chain (TCRB)-sequencing of IL-10+ and Ebi3+ Treg cell subpopulations isolated from non-draining lymph nodes (NDLNts) and B16 tumors from Il10Cre;Ebi3Tom;Foxp3Cre-YFP mice showed a comparable clonal enrichment (Supplementary Fig. 2c). We did not observe any bias in TCR Vγ-gene usage or CDR3-length, which can play a role in regulating T cell development and differentiation via TCR affinity and signaling strength12,13 (Fig. 2d and Supplementary Fig. 2d). Furthermore, substantial TCR clonal overlap noted among intratumoral Treg cell subpopulations is consistent with the lack of distinct transcriptomic signatures (Supplementary Fig. 2e). TCR signaling plays a crucial role in the production of both IL-10 and IL-35 (ref. 21). We noted that TCR stimulation in vitro was sufficient to induce Il10 and Ebi3 expression in purified Treg cell subpopulations (Fig. 2e), inferring extensive developmental plasticity. There was a preferential upregulation of Ebi3 expression observed during in vitro fate mapping, which is consistent with a progressive enrichment of Ebi3+ (either IL-10+Ebi3+ or IL-10−Ebi3+) Treg cells revealed by diffusion pseudo-time analysis (Fig. 2f,g). Furthermore, while it has been reported that the development and function of Ebi3+ Treg cells are independent of BLIMP1 expression19, our results indicate that BLIMP1 may also play a key role in supporting the maintenance and function of Ebi3+ Treg cells as approximately 90% of TIL Treg cells express BLIMP1 and 30–50% of which are Ebi3+ (Supplementary Fig. 2f and Fig. 1a–c). These observations suggest that TCR signaling, and perhaps the suppressive tumor milieu, might drive this adaptive plasticity and lead to the generation of Treg cell subpopulations marked by transitory IL-10 and IL-35 expression.

We also performed bulk RNAseq-based transcriptome analysis on purified LN and tumor (TIL) Treg cell subpopulations defined by Il10 and Ebi3 reporter expression (Supplementary Fig. 3a), to further investigate the transcriptomic relationship between these Treg cell subpopulations with greater sequencing depth. Principal component analysis (PCA) of differentially regulated genes clearly separated lymph node versus TIL Treg cells, irrespective of cytokine expression pattern (Supplementary Fig. 3a). Consistent with the scRNAseq data, bulk profiling revealed no striking transcriptomic differences between intratumoral Treg cell subpopulations (Supplementary Fig. 3b). Modest differences were noted in expression patterns of genes encoding co-signaling molecules (Cd22, Tnfsf11, Cd2, Lag3, Cd27, Cd28), probably reflecting their relative suppressive capacity noted in vitro (Supplementary Fig. 3c–f). Collectively, the concordant transcriptional and TCR profiles highlight these IL-10+ and Ebi3+ Treg cell subpopulations as plastic transitory states, rather than distinct subsets.

Cooperative regulation of anti-tumor immunity by IL-10+ and IL-35+ Treg cells. It was previously shown that mice with a Treg cell-restricted Ebi3 deletion, and thus loss of IL-35 production, exhibit reduced tumor burden. To assess the functional impact of IL-10- and IL-35-producing Treg cell subpopulations on the TME, we compared the growth rate of three transplantable tumor models: B16 and BrafPten (clone 24) melanoma models and EL4 thymoma in mice with Treg cells that are unable to produce IL-10 (Il10L/L-Tom., Il10L/L-Tom., Cre-YFP) or both cytokines (Il10L/L-Tom., Cre-YFP) mice (Fig. 3a–d). These IL-10+ or Ebi3 resulted in a comparable reduction in tumor growth (Fig. 3a–c). Dual-deletion did not exhibit a significant additive or synergistic reduction in tumor burden in both the melanoma models and only a marginal effect on EL4, suggesting that Treg cell-derived IL-10 and IL-35 might regulate anti-tumor immunity through a common, cooperative pathway.

IL-35 can modulate inhibitory receptor expression on CD8+ and CD4+ TILs. A substantial fraction of CD8+ and CD4+ TILs in B16 tumor-bearing control Foxp3Cre-YFP mice were PD-1+ and co-expressed several inhibitory receptors (TIM3, LAG3, TIGIT and 2B4, referred to as PD-1+multi-inhibitory receptor+ TILs) (Fig. 3d–g).
Fig. 1  Reciprocal expression of IL-10 and IL-35 on both mouse and human Treg cells. 

**a.** Representative flow plots depicting the expression and distribution of IL-10+ and Ebi3+ cells within Foxp3+ YFP+ Treg cells, isolated from B16-tumor-bearing Il10GFP Ebi3Cre-YFP mice 14 days after tumor inoculation. The expression of IL-10 (GFP+) and IL-35 (Ebi3-Tomato+) assessed in the non-draining axillary and brachial lymph nodes (NDLN), draining inguinal lymph nodes (DLN) and B16 TILs. SPICE plots depicting the co-expression pattern of IL-10 and Ebi3 on Treg cells as in **a.** Scatter-bar graphs depicting the percent distribution of cytokine single- (SP) and double-positive (DP) Treg cells as in **a** (n = 11 mice). Bars represent mean values. Statistical significance was determined by a two-way ANOVA with Holm–Sidak multiple comparisons (****P < 0.0001 and other P values as indicated). 

**b.** Representative flow plots depicting the expression and distribution of IL-10 and IL-35 in human Treg cells (CD4+CD25+ Foxp3+) obtained from healthy donor (HD) PBMC (PBL), NSCLC PBL or NSCLC TILs. Cells were stimulated overnight with plate-bound anti-CD3 and anti-CD28. **c.** Representative flow plots depicting the co-expression pattern of IL-10 and Ebi3 on Treg cells as in **b.** SPICE plots depicting co-expression pattern of IL-10 and Ebi3 on Treg cells as in **b.** Scatter-bar graphs depicting the percent distribution of cytokine SP and DP Treg cells as described in **d** (n = 12 mice). Bars represent mean values. Statistical significance was determined by a two-way ANOVA with Holm–Sidak multiple comparisons (****P < 0.0001 and other P values as indicated). 

**d.** Representative flow plots depicting the expression and distribution of IL-10 and IL-35 in human Treg cells (CD4+CD25+ Foxp3+) obtained from healthy donor (HD) PBMC (PBL), NSCLC PBL or NSCLC TILs. Cells were stimulated overnight with plate-bound anti-CD3 and anti-CD28 in the presence of rhIL-2, followed by 4 h of stimulation with PMA-ionomycin before surface and intracellular (IC)-staining for IL-10 and IL-35 (EBI3) expression analysis. Isotype control (black) and intracellular-stained (red) are overlaid. Data representative of three independent experiments. 

**e.** SPICE plots depicting the co-expression pattern of IL-10 and Ebi3 on Treg cells as described in **g.** Scatter-bar graphs depicting the percent distribution of inhibitory cytokines as in **g.** HD PBL (n = 9), NSCLC PBL (n = 9) and NSCLC TIL (n = 16). Bars represent mean values. Statistical significance was determined by two-way ANOVA with Holm–Sidak multiple comparisons (****P < 0.0001). 

and Supplementary Fig. 4a,b). Consistent with previous observations, Ebi3Cre-Tom Foxp3Cre-YFP mice exhibited a drastic reduction of CD8+ PD-1+ multi-inhibitory receptor+ TILs on day 14. Interestingly, Il10Cre-Foxp3Cre-YFP mice also exhibited a reduction in the CD8+PD-1+ multi-inhibitory receptor+ TILs, albeit to a lesser extent. Mice with Treg cell-specific deletion of both Il10 and Ebi3 (Il10CreF, Ebi3Cre-Tom Foxp3Cre-YFP) demonstrated a near complete loss of the PD-1+ and multi-inhibitory receptor+ populations and significant enrichment of inhibitory receptor-negative CD8+ T cells (Fig. 3d,e and Supplementary Fig. 4a,b). B16 TIL analysis at a later time point (day 20) revealed a less extensive reduction of inhibitory receptor expression on CD8+ TILs from Il10Cre-Foxp3Cre-YFP mice, while CD8+ TILs from Ebi3Cre-Tom Foxp3Cre-YFP and Il10CreF, Ebi3Cre-Tom Foxp3Cre-YFP mice still exhibited diminished inhibitory receptor expression (Supplementary Fig. 4c,d). Although the overall extent of PD-1+ and multi-inhibitory receptor expression was not as prominent on CD4+ TILs, Treg cell-restricted deletion of Il10 and Ebi3 showed a comparable, substantial impact on PD-1+ multi-inhibitory receptor+ CD4+ TILs at both time points as their CD8+ counterparts (Fig. 3f,g and Supplementary Fig. 4e,f). These data suggest that while both Treg cell-derived IL-10 and IL-35 function cooperatively in driving inhibitory receptor induction on TILs, IL-35 may play a more dominant role.

We also analyzed the impact of inhibitory cytokine-driven multi-inhibitory receptor induction on CD8+ T cell differentiation as well as other cell types in the TME. Increased cellularity and cytokine production were noted in tumors of Il10Cre-Foxp3Cre-YFP mice (Fig. 4a,b and Supplementary Fig. 5a,b), while enhanced TCM differentiation was noted in both Ebi3Cre-Tom Foxp3Cre-YFP and Il10CreF, Ebi3Cre-Tom Foxp3Cre-YFP TMEs (Fig. 4c). We also examined myeloid
Fig. 2 | Adaptive plasticity of IL-10− and IL-35− Treg cells. a, scRNAseq tSNE plots of bulk Treg cells from naive lymph nodes or day 14 B16 tumors (TIL Treg cells) from Foxp3CreERTT mice. b, scRNAseq tSNE plot depicting the expression of Il10 and Ebi3 in individual Treg cells overlaid on the same tSNE plot as in a. c, Heat maps contrasting the top 30 genes selected on the basis of the differential expression analysis of Treg cells using the two-sided Negative Binomial = 3 cells). (TIL): IL-10−Ebi3− (n = 491 cells), IL-10+Ebi3− (n = 491 cells), IL-10−Ebi3+ (n = 88 cells), IL-10+Ebi3+ (n = 3 cells). (Top): naive Treg cells from LN and spleens were double-sort purified and stimulated with anti-CD3/CD28-coated beads in the presence of rhIL-2 and CD11c+ cells for 72 h, followed by fluorescence-activated cell sorting (FACS) analysis. (Bottom), stacked-bar graphs summarizing four independent experiments. Statistical significance was determined by two-way ANOVA with Holm-Sidak multiple comparisons (*P = 0.0017, **P = 0.0002, ***P < 0.0001). f, Diffusion pseudo-time analysis depicting the stochastic oscillation of IL-10 and IL-35 (Ebi3) expression on the basis of the transcriptomic features of sequenced Treg cells from the scRNAseq experiment as in b. g, Stacked bar graphs demonstrating the distribution of indicated Treg subpopulations along the pseudo-time projection as analyzed in f. The pseudo-time projection was evenly divided into ten fractions and the percent distribution of each Treg cell subpopulation was calculated.
populations and observed a comparable reduction of PD-1 expression and increase in T cell stimulatory molecules such as MHC-II and CD80 following the loss of Treg cell-derived IL-10 or IL-35 (Supplementary Fig. 5c–e). There was an increase in the M1-like tumor-associated macrophage population in Il10−/−, Foxp3Cre-YFP mice, which conversely was decreased in Ebi3Cre-LTom, Foxp3Cre-YFP and Il10−/−, Ebi3Cre-LTom, Foxp3Cre-YFP mice (Supplementary Fig. 5f) (34,35).

Collectively, these data point to the differential impact of Treg cell-restricted deletion of these two cytokines on the TME, with an apparent greater impact of IL-10 in limiting effector function and proliferation, whereas IL-35 seems to limit memory differentiation.

**Treg cell-derived IL-35 and IL-10 can directly induce inhibitory receptor expression on intratumoral CD8+ T cells.** We next investigated whether IL-10 and IL-35 were directly impacting CD8+ T cells in the TME or if this regulation required an intermediate or accessory cell. We created an experimental system in which only CD8+ T cells were unable to respond to IL-10 or IL-35 using a Rag1−/− reconstitution model (Fig. 5a). Reconstitution with IL-35 receptor-deficient (CD4Cre.L/L-Tom) or IL-10 receptor-deficient (Il10rb−/−) CD8+ T cells recapitulated the reduced B16 tumor growth observed in mice with Treg cell-deficient animals that cannot make IL-35 and IL-10, respectively (Fig. 5b). Although inhibitory receptor expression was substantively reduced on IL-35R−/− CD8+ T cells, there was only a partial reduction on IL-10R−/− CD8+ T cells assessed at both d14 and d18 time points (Fig. 5c,d, Supplementary Fig. 5g,h). These results in the Rag1−/− reconstitution model were consistent with our analysis of intact mice, showing a ‘weaker’ effect of Treg cell-derived IL-10 relative to IL-35 on inhibitory receptor expression (Fig. 5d,e and Supplementary Fig. 4c,d), limiting the possibility that an accessory cell is involved in this process.

These data further support the notion that both Treg cell-derived IL-10 and IL-35 can directly regulate multi-inhibitory receptor expression on intratumoral CD8+ T cells.

**Treg cell-derived IL-10 and IL-35 co-opt the BLIMP1-regulated exhaustion module to drive T cell dysfunction.** We next probed the mechanistic underpinnings of IL-10 and IL-35-driven multi-inhibitory receptor upregulation to assess if these Treg cell subpopulations used similar or distinct downstream mechanisms to drive T cell dysfunction. We performed RNAseq with CD8+ TIL subsets on the basis of the expression of PD-1 and TIM3: (1) PD-1−/−TIM3− DP, (2) PD-1+TIM3− SP, (3) PD-1−/− and (4) PD-1+ TIM3− T cell subsets (Fig. 6a and Supplementary Fig. 6a). PCA of these subsets from control Foxp3Cre-YFP mice segregated them into defined clusters on the basis of location (NDLN versus TIL) and state of exhaustion (NEG, INT, SP versus DP), highlighting the distinct transcriptional signatures specific to each subset (Fig. 6a,b). Differential gene expression analysis comparing the CD8+ TIL subsets from single and dual Treg cell cytokine-deficient mice to corresponding wild-type counterparts revealed that the alteration of the TME by Treg cell-restricted deletion of IL-10 and/or IL-35 influenced the transcriptome of all the CD8+ subsets (Supplementary Fig. 6b–d). In particular, gene set enrichment analysis (GSEA) confirmed significant downregulation of an exhaustion signature in SP and DP CD8+ subsets from Il10−/−, Foxp3Cre-YFP and Il10−/−, Ebi3Cre-LTom, Foxp3Cre-YFP mice, relative to control CD8+ T cells (Fig. 6c).

This exhaustion gene signature of CD8+ TIL subsets displayed strong congruence to the published data from LCMV infection (36,37), indicative of a core molecular program that drives PD-1 to PD-1h transition in chronic settings (Supplementary Fig. 6e). These results further support the ability of both IL-10+ and IL-35+ Treg cell subpopulations to drive the PD-1h to PD-1h transition thereby promoting the multi-inhibitory receptor− TIL state (Fig. 6c).

We next interrogated the transcription factors that were upregulated during the PD-1h to PD-1h transition in control CD8+ T cells and assessed their expression in mice lacking IL-10 and/or IL-35 in Treg cells. The transcription factor Prdm1 (encoding BLIMP1) was one of the top genes induced during this transition and was significantly reduced in SP and DP CD8+ T cells from Il10−/−, Foxp3Cre-YFP, Ebi3Cre-LTom, Foxp3Cre-YFP and Il10−/−, Ebi3Cre-LTom, Foxp3Cre-YFP mice (Fig. 6d). Reduced Prdm1 expression was accompanied by an upregulation of its target genes (many of which are characteristic of memory T cells: Tcf7, Id3, Ilt7) (Fig. 6d–g), which is in agreement with an increase in CD8+ central memory T cells (Tcm) (Fig. 4c). We also noted upregulation of a PD-1 blockade-responsive TCF-1+CXCR5+ memory gene signature in CD8+ T cells from Il10−/−, Foxp3Cre-YFP, Ebi3Cre-LTom, Foxp3Cre-YFP and Il10−/−, Ebi3Cre-LTom, Foxp3Cre-YFP mice (Supplementary Fig. 6f). Collectively, these data suggest common modulation of the BLIMP1 axis by the Treg cell-derived IL-10 and IL-35 for optimal induction of an exhaustion gene signature in intratumoral CD8+ T cells.

**Direct regulation of BLIMP1 locus by Treg cell-derived IL-10 and IL-35.** BLIMP1 is a well-characterized regulator of terminal differentiation for both T and B cell lineages, and it has been reported to drive inhibitory receptor expression and exhaustion of CD8+ T cells in chronic viral infection (38). However, its role in intratumoral T cell exhaustion and whether it links Treg cell-derived cytokines with inhibitory receptor expression is unknown. Analysis of B16 tumor-bearing Prdm1YFP reporter mice revealed that BLIMP1 expression was strongly correlated with PD-1h and multi-inhibitory receptor+ CD8+ TILs (Supplementary Fig. 7a–c) and BLIMP1 protein...
BLIMP1 expression in B16-OVA tumor-bearing mice was significantly reduced in the TME that lacks T reg cell-derived IL-10 or IL-35 (Supplementary Fig. 7d). Adoptively transferred OT-1 Prdm1 Treg transgenic CD8+ T cells failed to upregulate high BLIMP1 expression in B16-OVA tumor-bearing II10+/L.Ebi3Cre-YFP/Ebi3+/L-Tom. Foxp3Cre-YFP and II10+/L.Ebi3Cre-YFP/Foxp3Cre-YFP mice compared to Foxp3Cre-YFP counterparts, further corroborating the role of both T reg cell-derived IL-10 and IL-35 to cooperatively promote the intratumoral multi-inhibitory receptor+ TIL state via BLIMP1 induction (Fig. 7a–c).

We next assessed inhibitory receptor expression in mice with a CD8+ T cell-restricted Prdm1 deletion (Prdm1L/L.E8ICreGFP) to validate whether BLIMP1 has a cell-intrinsic role in inhibitory receptor-regulation in tumors. Bi-allelic deletion of BLIMP1 in CD8+ T cells resulted in a substantial loss of multi-inhibitory receptor+
CD8+ TILs, mirroring our observations in Treg cell-mutant mice, while heterozygous Prdm1L/L-E8I-CreGFP mice exhibited a substantial but intermediate inhibitory receptor reduction (Fig. 7d and Supplementary Fig. 7e). Consistent with the Treg cell cytokine-deficient mice, expression of memory-associated genes (IL-7R and TCF-1) were also upregulated in Prdm1L/L-E8I-CreGFP mice (Fig. 7e). It has been reported previously that bi-allelic deletion of BLIMP1 results in a loss of effector functions in CD8+ T cells in the context of chronic viral infection despite diminished inhibitory receptor expression32. Indeed, no loss of tumor growth was observed in Prdm1L/L-E8I-CreGFP mice (Fig. 7f). Induced BLIMP1 haploinsufficiency32 or double-deletion of BLIMP1 and c-Maf34 in CD8+ T cells was required to reinvigorate the cytotoxic effector function and memory-potential, as c-Maf is highly upregulated in the absence of BLIMP1 and is capable of regulating a largely overlapping network of genes, maintaining the exhausted phenotype31. Consistent with these observations, heterozygous Prdm1L/L-E8I-CreGFP mice exhibited improved B16 tumor control comparable to Treg cell cytokine-deficient mice (Fig. 7f).

Finally, ChIP–quantitative PCR (qPCR) revealed that IL-10-induced STAT3 (ref. 34) and IL-35-induced STAT1 and STAT4 (ref. 26) were differentially enriched at STAT-binding sites inside the Prdm1 gene locus (Fig. 7g,h), suggesting that IL-10 and IL-35 may act directly on CD8+ T cells to modulate the BLIMP1-inhibitory receptor axis. Overall, these data confirm an intrinsic role of BLIMP1 in regulating inhibitory receptor expression of T cells in tumors and validate BLIMP1 as a direct downstream target of both Treg cell-derived cytokines, IL-10 and IL-35, in their cooperative promotion of multi-inhibitory receptor expression on TILs (Supplementary Fig. 7f).

Discussion

Collectively, our data support a model in which different subpopulations of intratumoral Treg cells produce IL-10 and IL-35 while exhibiting adaptive plasticity in their cytokine production, which seems to favor single rather than double inhibitory cytokine-secreting states. Although these two inhibitory cytokines cooperatively regulate the BLIMP1-inhibitory receptor axis in CD4+ and CD8+ TILs and exhibit largely overlapping functions such as induction of an inhibitory receptor module (including PD-1, LAG3, TIM3, TIGIT, 2B4), IL-35 appears to play a greater role in inhibitory receptor induction and limiting Treg differentiation, whereas IL-10 plays a greater role in regulating cytokine production and effector function. Cooperatively, they have a substantive impact on anti-tumor
immunity. Loss of Treg cell-derived IL-10 and IL-35 also results in (1) significant downregulation of the exhaustion gene signature, (2) upregulation of a memory-associated transcriptional profile and (3) development of a CXCR5+ signature in CD8+ TILs that is observed following PD-1 checkpoint blockade. These data suggest that IL-10- or IL-35-targeted immunotherapy may have a broader therapeutic impact than previously appreciated.

The physiological requirement for inducible, transitional, divergent intratumoral Treg cell subpopulations that shift between IL-10 and IL-35 single inhibitory cytokine expressing states remains unclear. Adaptive plasticity in Treg cell function has been reported in a number of inflammatory scenarios. While expression of Foxp3 and TCR signaling drive the core suppressive module, Treg cells retain developmental plasticity to adapt to their microenvironment leading to their acquisition of additional suppressive modules characterized by expression of transcription factors, miRNAs, chemokine receptors and suppressive mediators. The current study exemplifies this plasticity in Treg cell fates in the context of the tumor microenvironment, wherein priming in response to tumor antigens and/or the suppressive tumor milieu leads to the generation of IL-10+ and Ebi3+ Treg cells for optimal tumor-induced immune suppression. Reciprocal IL-10 and IL-35 expression on Treg cells has also been reported to play a role in the maintenance of immune tolerance. In this context, differential TCR signal strength led to the generation of distinct effector IL-10+ and IL-35+ Treg cell subsets that function in a complimentary fashion in the control of autoimmunity. In fact, such heterogeneity in inhibitory cytokine expression expands beyond the Treg cell lineage and has also been noted for regulatory plasma cells. During Salmonella enterica Typhimurium infection, different subsets of CD138+ plasma cells, depending on their maturation level, expressed either IL-10 or IL-35 and very few co-transcribed Il10, Eb13 and Il12a together. This functional segregation may represent a unifying theme of regulatory cell subsets for maximizing immunoregulation offering a ‘last-resort’ to subvert beneficial anti-tumor or detrimental autoreactive T cell responses while limiting collateral tissue damage. While the mechanism that limits the accumulation of dual inhibitory cytokine expressing Treg cell subpopulations will require further analysis, it is possible that IL-10 and IL-35 cooperatively limit Treg cell development and expansion in a cell-intrinsic manner limiting the abundance of IL-35/IL-10-dual expressing Treg cells.
Fig. 6 | T<sub>reg</sub> cell-derived IL-10 and IL-35 regulate CD8<sup>+</sup> TILs through BLIMP1-inhibitory receptor axis. **a–e** Data averaged from five independent RNAseq experiments: Foxp3<sup>Cre-YFP</sup> (n = 3), Il10<sup>L/L</sup>-Foxp3<sup>Cre-YFP</sup> (n = 2), Ebi3<sup>L/L</sup>-Foxp3<sup>Cre-YFP</sup> (n = 3) and Il10<sup>L/L</sup>-Ebi3<sup>L/L</sup>-Foxp3<sup>Cre-YFP</sup> CDBs (n = 4). **a**, The gating strategy for double-sorted CD8<sup>+</sup> subsets for RNAseq (PD-1<sup>+</sup>Tim3<sup>+</sup>; NEG; PD-1<sup>+</sup>Tim3<sup>+</sup>; INT; PD-1<sup>+</sup>Tim3<sup>+</sup>; SP and PD-1<sup>+</sup>Tim3<sup>+</sup>; DP). PCA plot depicting the NDLN and CD8<sup>+</sup> TIL subsets from Foxp3<sup>Cre-YFP</sup> mice. Each symbol represents an independently sequenced replicate. **b**, Heat map of the tumor-exhaustion gene signature derived from SP (left) and DP (right) subsets of Foxp3<sup>Cre-YFP</sup> mice; The y axis represents differentially expressed transcription factors in SP subsets. The x axis represents differentially expressed genes in the PD-1<sup>+</sup> (SP and DP) subsets relative to NEG subsets from control Foxp3<sup>Cre-YFP</sup> mice; The y axis represents differentially expressed genes in the T<sub>reg</sub> cell cytokine-deficient environments relative to control Foxp3<sup>Cre-YFP</sup> counterparts. Only significantly changed transcription factors are listed. We only considered transcription factors that were differentially expressed in exhausted cells (PD-1<sup>+</sup>, DP and SP) with a minimum log<sub>2</sub> fold change of 1.5, which was further filtered for ones that were differentially expressed across genotypes in the SP subset with the same cutoffs. **e**, GSEA of the BLIMP1 associated gene signature in SP (left) and DP (right) subsets of Il10<sup>L/L</sup>-Foxp3<sup>Cre-YFP</sup> mice; P values as indicated. **c, e**, Statistical significance was determined by the ‘RankSumTestWithCorrelation’ test in the limma R package with correction for loss in degrees of freedom due to correlation among genes. All P values are two sided. No correction for multiple hypotheses was performed. **f**, Representative histograms for validation of BLIMP1 and its associated gene (TCF-1 and IL-7R) at the protein level in the CD8<sup>+</sup> TILs from B16 tumor-bearing Foxp3<sup>Cre-YFP</sup> (n = 6), Il10<sup>L/L</sup>-Foxp3<sup>Cre-YFP</sup> (n = 4), Ebi3<sup>L/L</sup>-Foxp3<sup>Cre-YFP</sup> (n = 5) and Il10<sup>L/L</sup>-Ebi3<sup>L/L</sup>-Foxp3<sup>Cre-YFP</sup> (n = 6) mice from two independent experiments. **g**, Scatter-bar plots tabulating the expression levels of BLIMP1, IL-7R and TCF-1 as in **f**, Statistical significance was determined by one-way ANOVA with Holm–Sidak multiple comparisons (**P < 0.0001 and other P values as indicated).
Despite the differential inhibitory cytokine expression pattern, comprehensive profiling of IL-10+ and Ebi3+ Treg cells did not reveal striking transcriptional and TCR differences between these subpopulations. These results differ from previous observations where differences were noted in transcription factor dependency, chemokine receptor expression and activation status of IL-10+ and...
IL-35+ Treg cells isolated from secondary lymphoid organs. In this study, BLIMP1 expression marked the IL-10+ Treg cell subset while IL-35+ Treg cells were predominantly BLIMP1-independent. In contrast, we noted that over 90% of Treg cells express BLIMP1 in B16 tumors, indicating that BLIMP1 may also play a role in regulating the development and function of IL-35+ Treg cells as approximately 30–50% of these intratumoral Treg cells express IL-35. It is conceivable that following priming in response to tumor antigens, the Treg cell repertoire that migrates to the tumor is fixed in its core transcriptional identity, while still retaining plasticity in inhibitory cytokine expression. Treg cell-restricted IL-35 depletion did not lead to a compensatory increase in IL-10+ Treg cells. While we were unable to assess the effect of Treg cell-restricted IL-10 loss on IL-35+ Treg cells, it has been suggested that mice with BLIMP1-deficient Treg cells (unable to produce IL-10) exhibited a two-fold enrichment of IL-35+ and IL-10-/- Treg cells, respectively, and (3) lack of synergy or additivity in B16 tumor control in IL10/-/-, Ebi3/-/-, Foxp3/-/- mice, recapitulating BLIMP1 haploinsufficiency and translating to increased effector responses and decreased memory generation. Although BLIMP1 is well-established as a driver of inhibitory receptor expression, a recent report demonstrated that another transcription factor, c-MAF, sufficiently promoted inhibitory receptor expression in the absence of BLIMP1, by impacting overlapping transcriptional networks. However, our RNAseq dataset did not reveal significant modulation of Maf expression in CD8+ TILs from Treg cell cytokine-deficient mice relative to Foxp3cre-VEP counterparts, highlighting specific modulation of the BLIMP1 axis by IL-35 and IL-10 in cooperative regulation of T cell exhaustion. In summary, the adaptive plasticity and cooperative regulation of anti-tumor immunity by IL-10+ and IL-35+ Treg cells pose another tumor-immune evasive strategy and potential resistance mechanism to immunotherapy. Understanding this complex Treg cell-driven regulatory circuitry in the TME may inform the rational design of combinatorial modalities targeting Treg cells and their downstream mediators that promote T cell exhaustion to maximize responsiveness to checkpoint blockade and other immunotherapies while limiting adverse events and the risk of inflammatory or autoimmune complications.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0346-9.

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Author contributions
D.A.A.V. conceived, directed and obtained funding for the project. D.V.S., H.Y. and D.A.A.V. conceptualized, designed, analyzed the experiments and wrote the manuscript. D.V.S. and H.Y. performed the experiments with help from Q.Z. for Treg cell TCRseq and Treg cell subpopulation analysis with multiple organs. M.C. analyzed RNAseq data. C.L., T.T. and R.L. performed single-cell RNAseq experiments and contributed critical reagents and experiment design. Z.S., T.S. and W.C. analyzed single-cell RNAseq data. A.C.P. contributed critical reagents and helped with experimental design and analysis with ChIP–qPCR experiments. A.P. and J.D.L. obtained the NSCLC lung specimens. M.L. and T.C.B. processed and analyzed human healthy donor samples and NSCLC lung specimens. A.P. contributed critical reagents and helped with experimental design and analysis with the NSCLC lung specimens. D.V.S. and D.I.C. performed the allergic airway model experiments. D.B.C. contributed critical reagents and helped with experimental design and analysis for the allergic airway model experiments. C.J.W. contributed to experimental design, analysis and developing mouse strains. All authors provided feedback and approved the manuscript.

Competing interests
D.A.A.V. and C.J.W. have submitted patents covering IL-35 that are pending and are entitled to a share in net income generated from licensing of these patent rights for commercial development.

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Methods

Mice. Unless otherwise specified, all experimental procedures were performed on 5–8-week-old laboratory mice housed in Helicobacter/MNV free SPF facilities at the University of Pittsburgh in accordance with the current National Institutes of Health guidelines and with the approval and guidance of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Female mice were used for tumor growth and RNA sequencing experiments, while both male and female mice were used for TIL flow cytometry analysis.

**IL10**CreEbi3**YFP** and **Il6st**−/− mice were crossed to **Prdm1L/L** and **Prdm1YFP**. OT-I mice were purchased from Jackson Laboratory. IL-35R–/– (CD4Cre.1, Invitrogen, clone: CD28.2) and 200 U ml−1 murine IL-2 were used according to the manufacturer’s protocol.

Human specimen collection and processing. All specimens were acquired under University of Pittsburgh approved Institutional Review Board protocol, and informed consent was obtained from all patients. All lung tumors were processed to single-cell suspension by a combination of mechanical and enzymatic digestion. Dithiothreitol digestion (Sigma Aldrich) was used at 50 μg ml−1 for 15 minutes at 37°C in Roswell Park Memorial Institute (RPMI) medium (Lonza) to release TIL. For healthy donor peripheral blood samples, Ficoll Paque Plus (GE Healthcare) was used to separate peripheral blood leukocytes (PBL) and lysis buffer (BD Biosciences) was used to remove red blood cells when necessary.

Staining for EB1 and IL-10 in human specimens. Lung TIL and healthy donor PBL were incubated in complete RPMI (10% FBS (Atlanta Biologicals), non-essential amino acids (Sigma Aldrich), L-glutamine (Lonza) and penicillin/streptomycin (Corning) and sodium pyruvate (Sigma Aldrich)) with penicillin/streptomycin (Corning) and sodium pyruvate (Sigma Aldrich) was used at 50 μg ml−1 and 200 U ml−1, respectively. Red blood cells were lysed with Gey’s solution when appropriate. Digested cell suspension was then processed through a 70 μm cell-strainer and washed with cDMEM. Isolated cells were then used in various assays. Red blood cells were lysed with Gey’s solution when appropriate.

Fungal protease-induced allergic airway disease. Mice received seven intranasal challenges with a mixture comprised of a fungal protease derived from *Aspergillus oryzae* and Ovalbumin. Every alternate day for 2 weeks. On day 14, mice were intubated, tracheas were cannulated and flushed with PBS (twice) for isolation of BAL fluid, before isolation of lungs and the draining (mediastinal) and non-draining (inguinal) lymph nodes. Lungs were perfused with 10 ml PBS injected through the right ventricle of the heart using a 20 gauge needle immediately after BAL fluid collection. Harvested lungs were minced into small pieces and digested in 5 ml capped polystyrene tube with 1 mg ml−1 Collagenase D (Roche) in 2 ml PBS for 45 minutes at 37°C. Digested cell suspension was then processed through a 70μm cell-strainer and washed with cDMEM. Isolated cells were then used in flow cytometry analysis. Red blood cells were lysed with Gey’s solution when appropriate.

Flow cytometry. Single-cell suspensions from mouse or human specimens were stained with live/dead exclusion dye, followed by florescence-conjugated antibodies in the presence of 5% normal mouse serum. TruNuclear transcription factor staining kit (Biolegend) was used for intracellular staining. Flow cytometry data were acquired on BD Fortessa and analyzed by FlowJo (Treestar, Inc.). Pie charts were created using the SPICE program.

Processing and analysis of scRNAseq data. We used Cell Ranger (v2.0) (10X Genomics) to analyze sequencing data generated from Chromium Single Cell 3’ RNASeq libraries. We first ran ‘celranger mkkfastq’ on the Illumina BCL output folder to generate fastq files. We next generated the UMI count matrix with ‘celranger count’ for each library. The data were normalized with R package cellrangerRKit and visualized via r-distributed stochastic neighbor embedding (t-SNE). The diffusion pseudo-time analysis was run with R package Destiny.

Briefly, we built a transition matrix for all cells on the basis of their adjacency using a locally scaled Gaussian kernel and determined the diffusion components according to the eigenvectors of the matrix. Then, a new matrix M was generated by removing the first eigenvalue of the original transition matrix and the diffusion properties were calculated as distance metric between the rows of M. Finally, we plotted the trajectory lines, which were fitted by the locally weighted scatter-plot smoothing (LOESS) regression using the previously calculated values. For the differential expression analyses between the IL-10 and Ebi3 gene-expressing cells in lymph node and tumor Treg cells, we used the ‘seq’ method from the standard R package cellrangerRKit. Additionally, the differential expression analysis were determined by performing the two-sided Negative Binomial Exact test and the P values were adjusted to control the FDR.

RNAseq profiling and data analysis. Treg cell fractions and NDLN Foxp3−/− T cell control were FACSorted from day 14 B16 tumor-bearing IL10**CreEbi3**YFP and **Il6st**−/− mice. CD4+ T cell subsets were FACSorted from day 14 B16-bearng wild-type control and Treg cell cytokine-deficient mice.

Each cell fraction was double-sorted to ensure high purity (>95%) and directly lysed using the Clontech SMART-Seq v4 (Tunel cells) or v3 (CD8) kit for complimentary DNA synthesis. Libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina). Libraries were normalized at 2 nm using Tris-HEC 10 (pH 8.5) with 0.1% Tween20, diluted and denatured to a final concentration of 1.8 nM. Cluster generation and 75 base pair paired-end dual-indexed sequencing were performed on Illumina NextSeq 500 system.

RNAseq data were aligned to the mm10 genome using the STAR aligner and quantified against the Refseq gene models using featureCounts. The number of uniquely aligned reads ranged from 10 to 12 million. The raw data counts were processed for differential expression using the ‘voom’ function in the limma R package with the robust model option. Gene set enrichment testing was performed using the ‘RankSumTestWithCorrelation’ function in the limma R package, which automatically corrects enrichment statistic inflation due to correlation among genes. We used the GSEA-style enrichment score for visualization of pathway enrichment results.

We defined the tumor-specific exhaustion signature to be the set of genes that are overexpressed in the PD-1 hi state (both DP and SP) when compared to PD-1 neg state in Foxp3−/− mice at a fold change of four and q value FDR of 0.05. Tumor exhaustion signature was aligned with the chronic LCMV exhaustion profile dataset (Gene Expression Omnibus (GEO) accession number: GSE96150). The CXCGR5 PD 1-responsive CD8 + T cell signature was derived from GEO accession number: GSE84115 (ref. 50).

In vitro microsuspension assay. Sorted Treg cell subpopulations from NDLN and B16 tumors were co-cultured with CellTrace Violet (Life Technologies)-labeled CD4+Foxp3+ responder T cells (Tresponder) in the presence of mitomycin-C-treated TCRβ-depleted splenocytes and anti-CD3ε (1 μg ml−1) (clone: 2C11, Biolegend) for 72 h at 57°C as previously described. Briefly, 4×105 CellTrace Violet-labeled CD4+Foxp3+ Tresponder were stimulated with soluble anti-CD3ε in the presence of 1×10^5 TCRβ-depleted splenocytes to induce proliferation. To assess the function of Treg cells, sorted Treg cells were added at Treg cells:Tresponder ratio ranging from 1:2 to 1:64 and cultured for 72 h.

TCRseq and data analysis. Treg cell fractions and IL-10 Ebi3 Foxp3−/− YFP+ CD4+ T cells were purified by FACSorted from day 14 B16-bearing IL10**CreEbi3**YFP+ YFP− mice. DNA was purified using a QIAamp DNA Micro Kit (QIAGEN), and TCRbeta-enriched library was generated with TCRbeta immunoSeq (Adaptive Biotechnologies), following the manufacturer’s protocol. Cluster generation and sequencing were performed on the Illumina high output NextSeq 500 system. The data were analyzed using an immunoSeq Analyzer (Adaptive Biotechnologies).

Adoptive transfer experiments. BLIMP1YFPOT−1 transfer experiment. Recipient mice received intravenous injections of 0.5 × 10^6 BLIMP1YFP CD8+ T cells isolated by negative selection from spleen and lymph nodes of Prdm1YFPOT−1: Thy1.1 mice, followed by i.d. injection of 2.5 × 10^6 B16-OVA cells 2 days following adoptive transfer. Tumors and NDLN were harvested at day 14 post-tumor inoculation for assessment of BLIMP1YFP induction.

Rag1 knockout reconstitution experiment. Rag1 knockout recipient mice received intravenous injections of CD8+ depleted splenocytes isolated from Foxp3−/−, Thy1.1 mice containing 1 × 10^6 Treg cells day −8, followed by injection of 6 × 10^6.
wild type, IL-10R−/− or IL-35R−/− CD8+ T cells on day −1 and i.d. B16 tumor inoculation (1.25×10^6 cells per mouse) on day 0. Tumor size was measured every 3 days until day 18 post-tumor inoculation. Tumors were harvested on day 14 or day 18 for end-point experimental analysis.

ChIP-qPCR. Naïve CD8+ T cells were purified and activated for 2 days with plate-bound anti-CD3 (3 μg ml−1) and anti-CD28 (5 μg ml−1) supplemented with 50 U ml−1 rIL-2, followed by expansion for 4 days in rIL-2-containing cRPMI. Following serum deprivation for 3 hours, cells were pulsed with recombinant IL-10 or IL-35 for 30 mins before fixation with 1% formaldehyde. 15×10^6 cells per sample were sonicated with Bioruptor Pico in shearing buffer. ChIP was performed overnight for STAT1 (D1K9Y), STAT3 (124H6), STAT4 (C46B10) and IgG (Cell Signaling Technology) using Protein A Dynabeads (Thermo Fisher). EvaGreen-based qPCR was performed using primers previously described39.

Statistical analysis. Except for RNAseq and scRNAseq data analysis, GraphPad Prism software was used to determine the statistical significance. Group means were compared with two-tailed Student’s t-test when only two experimental groups were involved. Tumor growth was analyzed using two-way ANOVA with multiple comparison correction and sequential time point measurements. For other analyses, one- or two-way ANOVA with multiple comparison correction were used. All P values were two-sided and statistical significance was assessed at or below 0.05.

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Data availability
Bulk RNAseq datasets of CD8+ T cells and Treg cell subpopulations have been deposited in the GEO with the accession code GSE127735. Single-cell RNAseq dataset has been deposited in the GEO with the accession code GSE126184. The RNAseq data sets reported by other studies used to cross-examine with our sequencing data in this study were obtained from GSE9650 and GSE84105. The main data supporting the findings of this study are available in the article and its Supplementary Figures. Data are available from the corresponding authors upon appropriate and reasonable request.

Code availability
Computational and mathematical codes used in the RNAseq analyses supporting the findings of this study are available in the article. Additional information is available from corresponding author on reasonable and appropriate request.

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- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

For flow cytometry data collection, we utilized BD LSRIIFORTESSA with DIVA software, and exported the data in fcs3.0 version. RNAseq data collection was performed according to the instruction of illumina on NextSeq500 system. For single cell RNAseq, cDNA library preparation was performed using and following the instruction of the 10X Genomics platform and the sequencing data was collected on NextSeq500 system.

Data analysis

For flow cytometry data analysis, we utilized FlowJo (version 10 (most recently updated to 10.5.3 for Mac)) and numerical data exported to Excel sheet were further analyzed by using GraphPad Prism (version 7 and 8 for Mac) to generate graphical representation and for ANOVA statistical analysis. Furthermore, comma separated value (csv) files exported from Excel sheet upon multi-parameter flow cytometry analysis was import to SPICE program (NIH-NIAID) to generate SPICE plots to visually represent the expression level and co-expression combination of multiple inhibitory receptors analyzed. For Treg in vitro suppression assay, FlowJo legacy version 9 for Mac was used in order to utilize the proliferation analysis module.

For single cell RNAseq data analysis, we used Cell Ranger (v2.0) (10X Genomics) to analyze sequencing data generated from Chromium Single Cell 3’ RNA-seq libraries40. We first ran “cellranger mkfastq” on the Illumina BCL output folder to generate fastq files. We next generated the UMI count matrix with “cellranger count” for each library. The data were normalized with R package cellrangerRkit and visualized via t-distributed stochastic neighbor embedding (t-SNE). The diffusion pseudo-time analysis was run with R package Destiny41,42. Briefly, we built a transition matrix for all cells based on their adjacency using a locally-scaled Gaussian kernel and determined the diffusion components according to the eigenvectors of the matrix. Then, a new matrix M was generated by removing the first eigenvalue of the original transition matrix and the diffusion pseudo-time was calculated as a distance metric between the rows of
Lastly, we plotted the trajectory lines, which were fitted by the locally weighted scatterplot smoothing (LOESS) regression using the previously calculated values. For the differential expression analyses between the IL10 and Ebi3 gene-expressing cells in LN and Tumor Tregs, we used the "ssqe" method from the standard R package CellrangerRkit.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Bulk RNAseq and single cell RNAseq data will be deposited in the Gene Expression Omnibus (GEO), and the accession codes will be provided. Other data are available from the corresponding authors upon appropriate and reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

For all studies, the numbers of animals in experiments are determined by formal statistical procedures based on preliminary or internal pilot data. For example, the mean growth rate of the Foxp3Cre-YFP mice (considered WT in this manuscript) were estimated by applying a mixed-effects linear model to the log-transformed tumor volumes; this is similar to (but not exactly the same as) fitting a line to each animal’s log (tumor volume) versus day data, and treating the per-animal slopes as the analytic variable. From the mixed model, we determined that the mean growth rate of the Foxp3Cre-YFP animals was 0.100, and that the standard error of that estimate was 0.007 (on 250 degrees of freedom). We also determined that the estimate of the standard error was consistent across the other experimental groups. From this, we can determine the number of animals required to test the null hypothesis of no difference between growth rates of different experimental groups when the true difference in growth rates is any given percentage (α=0.05, 80% power).

Data exclusions
No data were excluded from the analyses.

Replication
Replications were successful once the experimental conditions were first optimized by pilot experiments.

Randomization
For all the experiments used in the study, we prepared minimum of 4-6 breeder pairs from which we took litters randomly that were age matched within 1 week between the WT and other mutant groups. For flow cytometry and tumor growth curve experiments, we analyzed each of individual mice separately so that any variation that may exist within a group can be truthfully reported in an unbiased manner. For both bulk and single cell RNAseq experiments, we pooled tumors and lymph nodes within groups for each experimental replicate, and the cells of interest were purify by using BD Aria sorter.

Blinding
For tumor growth curves, the measurement was conducted by a blinded, second personnel for the duration of each experiment.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |
| ☑ | Clinical data |

Methods

| n/a | Involved in the study |
| --- | --- |
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
B16F10 cell line (referred to as B16 throughout the manuscript) and EL4 T cell lymphoma (referred to as EL4 in the manuscript) were purchased from ATCC (Manassas, Virginia). B16-OVA cell line and BrafPten (clone24) cell line were kindly provided by Greg Delgoffe (University of Pittsburgh). BrafPten (clone 24) was originally generated by A.V. Menk and G.M. Delgoffe by cloning a cell line from a tumor harvested from BrAfCA.Pten/L/L.TyrCreERT2 mouse induced by 4-OHT administration.

Authentication
Those cell lines are not subsequently authenticated by ourselves.

Mycoplasma contamination
The cell lines used in this study have been tested for and are free of mycoplasma contamination.

Commonly misidentified lines
(See iCLAC register) No commonly misidentified cell lines were used in this study.

Validation
All the antibodies used in our study are well-validated antibodies that are commercially readily available. In addition to commercial vendors' staining validation data contrasting to the isotype IgG stained counterparts, we have also carefully monitored non-specific binding of those antibodies by examining cells that do not express the antigen.

Antibodies

| Antibodies used | Specificity | Dilution Factor |
|-----------------|-------------|-----------------|
| CD90.1 BV650 (OX-7, Biologend 202533, lot# B212183, dilution factor = 1:1500) | CD90.1 | 1:1500 |
| CD90.2 APC/Cy7 (30-H12, Biolegend 105328, lot# B253519, dilution factor = 1:400) | CD90.2 | 1:400 |
| TCRb BV60S (H57-597, Biologend 109241, lot# B256848, dilution factor = 1:500) | TCRb | 1:500 |
| CD4 BUv395 (GK1.5, BD Biosciences 563790, lot# B213920, dilution factor = 1:500) | CD4 | 1:500 |
| CD8a BUv737 (53-6-7, BD Biosciences 564297, lot# B203281, dilution factor = 1:500) | CD8a | 1:500 |
| PD-1 (RMP1-30, Biologend 109110, lot# B235159, dilution factor = 1:400) | PD-1 | 1:400 |
| TIM3 APC (RM73-23, eBioscience 17-5870-82, lot# 4342179, dilution factor = 1:300) | TIM3 | 1:300 |
| LA63 eFluor450 (eBioC987W, eBioscience 48-2231-82, lot# 4295329, dilution factor = 1:300) | LA63 | 1:300 |
| TiGIt PerCp-eFluor710 (GIGD7, eBioscience 46-9501-82, lot# 4274784, dilution factor = 1:100) | TiGIt | 1:100 |
| 2B4/2CD244.2 APC-Vio770 (REA388, Miltenyri 130-105-991, lot# 5180219484, dilution factor = 1:100) | 2B4/2 | 1:100 |
| Foxp3 FITC (FJK-16s, eBioscience 11-5773-82, lot# 4306781, dilution factor = 1:200) | Foxp3 | 1:200 |
| CD19 Alexa Fluor647 (SE7, Biologend 150004, lot# B222216, dilution factor = 1:50) | CD19 | 1:50 |
| IL7R/CD127 PE-Cy5 (A7R34, eBioscience 25-1271-82, lot# 47599-1635, dilution factor = 1:250) | IL7R/CD127 | 1:250 |
| CD90.1 BV650 (OX-7, Biolegend 202533, lot# B212183, dilution factor = 1:1500) | CD90.1 | 1:1500 |
| CD90.2 APC/Cy7 (30-H12, Biolegend 105328, lot# B253519, dilution factor = 1:400) | CD90.2 | 1:400 |
| TCRb BV60S (H57-597, Biologend 109241, lot# B256848, dilution factor = 1:500) | TCRb | 1:500 |
| CD4 BUv395 (GK1.5, BD Biosciences 563790, lot# B213920, dilution factor = 1:500) | CD4 | 1:500 |
| CD8a BUv737 (53-6-7, BD Biosciences 564297, lot# B203281, dilution factor = 1:500) | CD8a | 1:500 |
| PD-1 (RMP1-30, Biologend 109110, lot# B235159, dilution factor = 1:400) | PD-1 | 1:400 |
| TIM3 APC (RM73-23, eBioscience 17-5870-82, lot# 4342179, dilution factor = 1:300) | TIM3 | 1:300 |
| LA63 eFluor450 (eBioC987W, eBioscience 48-2231-82, lot# 4295329, dilution factor = 1:300) | LA63 | 1:300 |
| TiGIt PerCp-eFluor710 (GIGD7, eBioscience 46-9501-82, lot# 4274784, dilution factor = 1:100) | TiGIt | 1:100 |
| 2B4/2CD244.2 APC-Vio770 (REA388, Miltenyri 130-105-991, lot# 5180219484, dilution factor = 1:100) | 2B4/2 | 1:100 |
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| TCRb BV60S (H57-597, Biologend 109241, lot# B256848, dilution factor = 1:500) | TCRb | 1:500 |
| CD4 BUv395 (GK1.5, BD Biosciences 563790, lot# B213920, dilution factor = 1:500) | CD4 | 1:500 |
| CD8a BUv737 (53-6-7, BD Biosciences 564297, lot# B203281, dilution factor = 1:500) | CD8a | 1:500 |
| PD-1 (RMP1-30, Biologend 109110, lot# B235159, dilution factor = 1:400) | PD-1 | 1:400 |
| TIM3 APC (RM73-23, eBioscience 17-5870-82, lot# 4342179, dilution factor = 1:300) | TIM3 | 1:300 |

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
We utilized the following mouse models for the study:

Unless otherwise specified, all experimental procedures were performed on 5-8 week old laboratory mice housed in Helicobacter and MNV free SPF facilities at University of Pittsburgh in accordance with guideline of National Institute of Health (NIH) under the approved protocol and guideline from the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Female mice were used for tumor growth and RNA sequencing experiments, while both male and female mice were used for tumor-infiltrating lymphocyte flow cytometry analysis.

II10GFP.Ebi3Tom/Foxp3Cre-YFP mice were generated by crossing II10GFP (Vert-X; Jackson Laboratory) to Ebi3Tom/Foxp3Cre-YFP (developed in our laboratory). Ebi3L/L-Tom mice were developed in our laboratory and have been previously described. II10L/L (provided by Werner Muller (Germany)) and Ebi3L/L-Tom were crossed to Foxp3Cre-YFP mice to generate Treg cell-specific cytokine deletion strains. Prdm1L/YFP and Prdm1L/L strains were provided by Amanda Poholek at University of Pittsburgh. EB1Cre mice (provided by Dan R. Littman, New York University; previously described) and OT-I transgenic mice (obtained from Jackson Laboratory) were crossed to Prdm1L/L and Prdm1L/YFP strains, respectively, to generate EB1Cre.Prdm1L/L and Prdm1L/YFP.OT-I mice. Rag1–/– and IL-10R–/– (Il10rb–/–) mice were purchased from Jackson Laboratory. IL-35R–/– (CD4Cre.IIfsS/Y.LIL12b2–/–) mice were generated as described previously.
Human research participants

Policy information about studies involving human research participants

Population characteristics

- All samples were from non-small cell lung cancer patients (age range of 45-80) with either adeno or squamous cell carcinoma (early stage—Ⅰ and Ⅱ) and no prior immunotherapy.
- Solid primary tumors were collected via surgical resection and were placed into sterile collection media (RPMI+ 10%FBS+ 1%Penstrep) and kept on ice until further dissection (1-2 hours post-surgery).
- Peripheral blood (PBL) was drawn pre-operatively into EDTA tubes and was kept on a rocker at room temperature until processing (1-2 hours post collection).
- Tumor-infiltrating lymphocytes and peripheral blood mononuclear cells were collected via Ficoll gradient and cryopreserved in FBS containing 10% DMSO.

Recruitment

- Subjects were first approached by their treating surgeon, radiologist, pulmonologist or medical oncologist who determined their willingness to participate in research studies. If a patient was willing to be a subject, a complete explanation of the research was given to them by a research coordinator and informed consent was obtained and documented. The goal of the research study and its methods was discussed and all pages of the informed consent document was reviewed with the subject and any questions answered. Once there was a thorough understanding by the subject of the contents of the consent form and a reiteration of the nature of the research study, the patient was asked to sign and date the form. A signed and dated copy, including the signature of the person obtaining the consent was given to the patient at the time consent was obtained, and the subject was again informed that participation would not influence medical care and that withdrawal was possible at any time. There were no self-selection bias or other biases that would have impacted results as the research group received all patient samples in a de-identified manner.

Ethics oversight

- All specimens were acquired under University of Pittsburgh approved Institutional Review Board (IRB) protocol, and informed consent was obtained from all patients.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

- For mouse specimen processing:
  - To obtain single cell suspension samples for flow cytometry analysis, the samples were processed as following:
  - Spleens or lymph nodes were processed through 40μm cell strainers to obtain single suspension. Tumor samples were first mechanically minced into small pieces with scissors, followed by enzymatic digestion with Collagenase IV and Dispase in complete RPMI1640 medium for 30 minutes. Digested tumors were then processed through 70μm cell strainer. Red blood cells in spleens and tumors were lysed with ammonium chloride solution. All the samples were first stained with live/dead discrimination dye in PBS prior to antigen staining in FACS buffer containing 5% FBS and 5% normal mouse serum.
  - For human specimen processing:
  - All specimens were acquired under a University of Pittsburgh approved and exempt Institutional Review Board (IRB) protocol with a non human subjects designation as all specimens were collected in a blinded fashion, and informed consent was obtained from all patients and healthy donors.
  - We collected peripheral blood from healthy donor to obtain PBL samples, and human tumor samples were collected from patients with non-small cell lung carcinoma (NSCLC).
  - For enzymatic digestion, Liberase (Sigma Aldrich) was used at 50 μg/mL for 15 minutes at 37°C in RPMI medium (Lonza) to release tumor infiltrating lymphocytes (TIL). For healthy donor peripheral blood samples, Ficoll Paque Plus (GE Healthcare) was used to separate peripheral blood leukocytes (PBL) and lysis buffer (BD biosciences) was used to remove red blood cells when necessary.
  - Lung TIL and HD PBL were incubated in complete RPMI medium (10% FBS (Atlanta biologicals), non-essential amino acids (Sigma Aldrich), L-glutamine (Lonza) and penicillin/streptomyycin (Corning) and sodium pyruvate (Sigma Aldrich)) with TCR stimulation:
  - plate-bound anti-CD3 (0.5 μg/mL, Invitrogen, clone: OKT3) and soluble anti-CD28 (1 μg/mL Invitrogen, clone: CD28.2) and 200U/ml rhIL-2 overnight followed by four hours of stimulation with PMA/ionomycin and GolgiStop. The cells were then harvested and stained with surface antibodies (CD3, CD4, CD8, CD25) and viability dye. A fixation/permeabilization kit (eBiosciences) was used
according to manufacturer’s protocol, followed by intracellular staining.

**Instrument**
BD LSRFORTESSA for flow cytometry analysis and BD Aria sorter for cell purification.

**Software**
FlowJo was used to gate the target populations, and Microsoft Excel, Graphpad Prism, and SPICE were used for further analyses.

**Cell population abundance**
For RNAseq experiments where cells were purified by FACS-sorting, we ensured the purity of the sorted samples by sequential double-sorting. The purity was determined by re-analyzing the double-sorted samples on the same instrument (BD FACSAria). All the fractionated subpopulations used for RNAseq experiments were uniformly 500 cells per sample, which was directly sorted into 96-well plate containing lysis buffer for cDNA synthesis purpose.
For adoptive transfer experiments, we purified the target cells by streptavidin-magnetic bead negative selection. The purity of the isolated cells was determined on BD LSRFORTESSA prior to transfer.

**Gating strategy**
For all the experiments involving flow cytometry (either for analyzing or FACS-sorting), the following gating strategy was applied to eliminate non-specifically stained cells. First, lymphocyte gate was applied on the SSC-A/FSC-A window. The gating size was determined by comparing the lymphocyte cluster from lymph node samples to the matched tumor samples in order to adjust for the blasted tumor infiltrating lymphocyte cell size. Subsequently, sequential singlet/doublet discrimination was applied via SSC-W/SSC-H and FSC-W/FSC-H. From the selected singlets, dead cells were discriminated out by gating on live/dead dye negative population.
For our study is focused on tumor infiltrating T cells, we have subsequently gated on TCRb+CD4+CD8- or TCRb+CD8+CD4- for further analysis.
In order to set the gating for positive populations, we used unstained samples and also stained lymph node samples for inhibitory receptor staining as lymph node counterparts do not express them compared to tumor infiltrating lymphocytes.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.