Inhibition of IL-25/IL-17RA improves immune-related adverse events of checkpoint inhibitors and reveals antitumor activity

Xizi Hu,1, Shoib M Bukhari,1 Carly Tymm,1 Kieran Adam,1 Shalom Lerrer,1 Brian S Henick2, Robert J Winchester1,3 Adam Mor1,3

ABSTRACT

Background Immune checkpoint inhibitors (ICIs) have improved outcomes and extended patient survival in several tumor types. However, ICIs often induce immune-related adverse events (irAEs) that warrant therapy cessation, thereby limiting the overall effectiveness of this class of therapeutic agents. Currently, available therapies used to treat irAEs might also blunt the antitumor activity of the ICI themselves. Therefore, there is an urgent need to identify treatments that have the potential to be administered alongside ICI to optimize their use.

Methods Using a translationally relevant murine model of anti-PD-1 and anti-CTLA-4 antibodies-induced irAEs, we compared the safety and efficacy of prednisolone, anti-IL-6, anti-TNFα, anti-IL-25 (IL-17E), and anti-IL-17RA (the receptor for IL-25) administration to prevent irAEs and to reduce tumor size.

Results While all interventions were adequate to inhibit the onset of irAEs pneumonitis and hepatitis, treatment with anti-IL-25 or anti-IL-17RA antibodies also exerted additional antitumor activity. Mechanistically, IL-25/IL-17RA blockade reduced the number of organ-infiltrating lymphocytes.

Conclusion These findings suggest that IL-25/IL-17RA may serve as an additional target when treating ICI-responsive tumors, allowing for better tumor control while suppressing immune-related toxicities.

INTRODUCTION

Immune checkpoint inhibitors (ICIs) increase the survival of patients with several kinds of malignancies. Blocking the inhibitory receptors CTLA-4, PD-1, and PD-L1 (the ligand for PD-1) as monotherapy or combined with other agents has improved cancer treatment responses.1-4 CTLA-4 and PD-1 are T cell surface inhibitory receptors that prevent excessive T cell responses. Tumor cells have developed mechanisms to usurp those inhibitory mechanisms to prevent T cell-mediated tumor killing. To do that, tumor cells express inhibitory ligands such as PD-L1, and in some cases also PD-L2 (the second ligand for PD-1), to prevent T cell recognition and activation.5 Consequently, the therapeutic blockade of these checkpoints with ICIs restores antitumor immunity. The PD-1-PD-L1 interaction directly inhibits tumor-specific T cell responses, promotes peripheral effector T cell exhaustion, and enhances the development of regulatory T-cell elements. The FDA (Food and Drug Administration) has approved 10 antibodies targeting inhibitory receptors for treating numerous cancers, including melanoma, lung adenocarcinoma, renal cell carcinoma, squamous cell carcinoma, and
Hodgkin lymphoma, to name a few. While ICI therapy has revolutionized cancer treatment, primary and secondary resistance is common. For example, ovarian cancer responds poorly to ICI therapy, with clinical trials reporting responses ranging from 6% to 22%.1–4

One of the reasons for anti-PD-1 treatment failure is the presence of additional inhibitory immune checkpoint pathways in mammals. PD-L1 is also the ligand of the receptor CD80 (B7-1), and their interaction inhibits T cell proliferation and cytokine production.10–14 PD-1 has a second ligand, PD-L2, expressed in various immunosuppressive stromal cells, endothelial cells, macrophages, and, in some cases, tumor cells.15–17 PD-L2 binds to PD-1 with a threefold stronger affinity compared with PD-L1. PD-L2 also interacts with repulsive guidance molecule b, an alternate receptor, and their interaction promotes respiratory immune tolerance by initiating immunoinhibitory signals.18 There are other mechanisms of tumor immune escape in ICI therapies, such as MHC (Major Histocompatibility Complex) downregulation, regulatory T cell deviation, and insufficient tumor-infiltrating lymphocytes (TILs). In murine models, we have shown that the presence of lymphocytes in different organs in mice treated with anti-CTLA-4 and anti-PD-1 antibodies varies and depends on multiple factors, including the genetic background of the mice.19

Another major limitation of ICI therapy is associated with immune-related adverse events (irAEs).20–23 Since PD-1 is also expressed on non-tumor T cells, this on-target (PD-1) but off-tumor (irAEs organs) activation of exhausted autoimmune T cell clones is evident. Potentially, any organ can be injured as ICI disrupts self-tolerance to normal tissues. These irAEs range from mild to severe in various tissues, the most common of which include the skin, liver, lung, and gastrointestinal tract.24–26 Moderate irAEs require the temporary discontinuation of ICI and short-term use of corticosteroids with subsequent ICI treatment, limiting their efficacy. Severe irAEs often lead to the cessation of life-saving ICI therapy altogether. Other immunosuppressive drugs are usually required, such as high-dose prednisone, methotrexate, tocilizumab, vedolizumab, and infliximab. Additionally, prolonged immunosuppression may place the patients at risk of developing infections.

Given that similar immune mechanisms mediate both antitumor T cell responses and irAEs, there remains a concern that steroids or other immunosuppressive agents used to treat irAEs may impede tumor response. Indeed, worse outcomes have been reported in patients treated with immunotherapy while receiving corticosteroids.27–31 Additionally, steroid-refractory colitis, myocarditis, and pneumonitis have been described and are associated with high mortality, and their optimal management strategies remain unclear. Unfortunately, since management decisions are often based on expert opinion and not mechanistic studies, a recent publication32 suggests worse cancer outcomes in patients treated with anti-TNFα and anti-IL-6R agents. At the same time, other studies suggested that targeting IL-6R could be a practical approach to treat several cancers without hindering antitumor immunity. Additional controlled prospective trials and emerging pathophysiological insight are needed to evaluate these issues properly.

In this work, we tested the ability of several irAE treatments to inhibit irAEs inflammatory responses without dampening the much-needed antitumor immune response using an animal tumor model of ICI-induced irAEs and multiparameter flow cytometry.19

RESULTS

Anti-PD-1 and anti-CTLA-4 antibodies therapy induces irAEs in multiple organs

For replicating clinical irAEs, we used a pharmacological model where anti-PD-1 and anti-CTLA-4 antibodies are injected into tumor-inoculated B6/Lpr mice biweekly for 6 weeks (figure 1A). Immune checkpoint blockade with anti-PD-1 and anti-CTLA-4 antibodies suppressed tumor growth (figure 1Bi,Bii). However, compared with untreated mice, those who underwent ICI therapy experienced increased levels of immune cell infiltration in the liver, lung, heart, and colon (figure 1C).

To uncover differences in the compositions of the immune infiltrates of the irAEs organ and the tumor, we isolated immune cells from the livers and the tumors of the ICI-treated mice. We employed a 33-plex flow cytometry panel to generate a UMAP plot demonstrating clusters of CD45+ cells (figure 1Di), in which CD4+ and CD8+ T cells occupied different clusters (figure 1Dii,Diii). Remarkably, the immune cells isolated from the liver and from the tumor of the same mice differentially engaged other clusters (figure 1E). More specifically, we identified clusters enriched in the liver. In contrast, other clusters were increased in the tumor (figure 1F). While most cells in cluster 6 (CD4+CD8+FOXP3+) and cluster 1 (CD8+TCF1+CD2L+) were collected from the liver, cells in cluster 3 (CD8+CD69+CXCR3+) dominated the tumors. These data suggest that targeting specific T cell populations could inhibit irAEs inflammatory responses with minimal interference to the antitumor immune effect.

Clinically used treatments for irAEs counteract ICI’s antitumor effect

Prednisone is the first line of treatment for irAEs in the clinic. We simulated this care in vivo by providing prednisolone to irAEs mice induced by treatment with anti-PD-1 and anti-CTLA-4 antibodies (figure 2A). Through H&E analysis of multiorgans collected at the endpoint of the experiment, we observed that prednisolone decreased immune-cell infiltration in the liver, lung, heart, and colon (figure 2B). However, prednisolone’s anti-inflammatory properties counteract the antitumor effects of anti-PD-1 and anti-CTLA-4 antibodies and led to a notable increase in tumor growth and a decrease in survival probability (figure 2Gi,Gii,D). In search of a specific druggable target that could be neutralized to treat irAEs, we performed a
Luminex assay of serum samples of irAEs-treated mice. As shown, prednisolone decreased IL-22, IL-9, and CCL3 levels. Yet, the decrease in the levels of the inflammatory cytokines TNFα, IL-25, IL-5, and IL-1β was not notable (figure 2E). This suggested to us that neutralizing TNFα, as other groups, have previously studied,33 or IL-25 could potentially lower excessive off-tumor immune response through a pathway different from prednisolone treatment and be less detrimental to ICI’s antitumor response.

We next examined the effects of anti-TNFα antibodies in the irAEs mice model compared with anti-IL6 antibodies (figure 2F), another agent used in irAEs clinical
Figure 2  Treatments for irAEs counteract ICI’s antitumor effect. (A) Experiment design for examining the effect of prednisolone on tumor and immune-related adverse event development. Prednisolone was given daily via oral gavage from day 8 to day 12 (5 doses total) at 20µg/dose in addition to regular aPD-1 and aCTLA-4 administration. (B) Immune infiltration gradings of H&E-stained organs harvested on mouse euthanasia reveal levels of organ-specific immune infiltration. (Ci) Each curve represents a treatment group illustrating tumor growth. (Cii) Average tumor volume on day 18. (D) Kaplan-Meier (KM) plot shows survival estimate of untreated mice and mice receiving aPD-1+aCTLA-4 with and without prednisolone. (E) Luminex detects levels of cytokines in peripheral blood serum collected at in vivo endpoints. (F) Experiment design for examining the effect of anti-IL-6 and anti-TNFα antibodies on tumor and immune-related adverse event development. Either aIL-6 or aTNFα was given biweekly starting day two at 200 µg per dose through intraperitoneal injections for 2 weeks on top of the regular dosage of aPD-1 and aCTLA-4. (G) Immune infiltration gradings of H&E-stained liver and lung harvested at the in vivo endpoint. (Hi) Tumor growth curves of different treatment groups. (Hii) Average tumor volumes on day 18. (I) KM plot of estimated survival probabilities of aPD-1+aCTLA-4 treated mice, aPD-1+aCTLA-4+aIL-6 treated mice, and aPD-1+aCTLA-4+aTNFα treated mice within the 40 days treatment period. ICI, immune checkpoint inhibitor; irAEs, immune-related adverse events.
trials. The experiments demonstrated decreased immune cells infiltrating the liver (figure 2G). However, the anti-inflammatory effects of TNFα and IL-6 blocking antibodies were not confined to the liver and lung. Also, they affected the tumor, clashing with the desired anti-tumor immune activation of the ICI (figure 2Hi,Hi). Anti-TNFα and anti-IL-6 antibody treatments lowered mice’s survival by accelerating tumor growth compared with anti-PD-1 and anti-CTLA-4 antibody therapy alone (figure 2If).

Neutralizing IL-25 prevents irAEs while promoting tumor regression
With the reasoning described earlier, we examined the effect of neutralizing IL-25 for preventing multiorgan irAEs. In our experimental model, mice received a single intraperitoneal administration of anti-IL-25 antibody at either a high dose (400 µg) or a low dose (200 µg) (figure 3A). H&E stains of the liver, lung, and colon of anti-IL-25 antibody-treated mice showed a significant decrease in levels of infiltrating immune cells compared with that of mice receiving no anti-IL-25 antibody treatment (figure 3B). Tumor recordings illustrated additional control over tumor growth and multiple cases of tumor regression associated with high doses of anti-IL-25 antibodies (figure 3Ci,Cii). We also observed prolonged survival in tumor-bearing mice treated with high-dose anti-IL-25 antibodies (figure 3D). Moreover, the anti-IL-25 antibody acted dose-dependently as an effective irAEs-reducing treatment.

To further solidify the anti-IL-25 antibody’s dose dependency, we conducted similar experiments where low dosing of anti-IL-25 antibody was administered weekly for four consecutive weeks (figure 3E). In these experiments, we observed that unlike a single low-dose injection (figure 3Ci,Cii), repeated doses of low-dose anti-IL-25 antibodies exerted additional tumor-control when added to anti-PD-1 and anti-CTLA-4 antibody treatments (figure 3Fi,Fii). The mice were euthanized 3.5 weeks into the experiment. Even at this midpoint for irAEs development, we observed a tendency for anti-IL-25 to reduce off-target immune infiltration in multiorgans (figure 3G). The observation was validated by CD3 Immunohistochemistry staining and quantification; images marked up with Halo software show fewer CD3 T cells in the lungs of the anti-IL-25 antibody-treated mice than those treated with anti-PD-1 and anti-CTLA-4 antibodies alone (figure 3Hi,Hi). Remarkably, treatment with a different anti-IL-25 clone inhibited checkpoint inhibitor-induced pneumonitis and the progression of two tumor models (online supplemental figure 1).

IL-17RA is an alternative and better target than IL-25
Illustrated in figure 4A is the IL-17 ligand and receptor family. It has been previously reported that blocking IL-17A enhanced the antitumor response of anti-PD-1 therapy. Combined with our data on neutralizing IL-25, we aim to achieve higher efficacy by targeting IL-17RA, a receptor for both IL-17A and IL-25 (IL-17E). To test this hypothesis, we performed an in vivo experiment using an anti-IL-17RA neutralizing antibody (figure 4B). Tumor growth curves show enhanced antitumor response in mice treated with anti-IL-17RA antibody in addition to anti-PD-1 and anti-CTLA-4 combination of antibodies (figure 4Ci). Additionally, compared with mice that received the same dose of anti-IL-25 antibody, mice treated with anti-IL-17RA had smaller tumors (figure 4Gi,Gii). Immune infiltration grade on H&E stained tissue slides collected at a 3.5-week in vivo endpoint reveal that anti-IL-17RA treatment decreased irAEs in the liver and lung (figure 4D). Altogether, IL-17RA neutralization promotes MC38 tumor regression while preventing ICI-induced hepatitis and pneumonitis in B6/lpr mice.

Neutralizing IL-17RA leads to more T cell activation and less T cell exhaustion
To characterize B6/lpr mice, we performed flow cytometry on splenocytes collected from B6/lpr mice. We observed that 73% of the CD4 T cells and 97% of the CD8 T cells were positive for IL-17RA (figure 4E). Since there is no IL-17RA negative population in CD8 T cells, we focused our IL17RA expression association analysis on CD4 T cells. In the CD4 T cells, we discovered that IL-17RA negative cells expressed more CD69 (activation marker) than IL-17RA positive cells (figure 4F). Moreover, among the CD4+ positive T cells, effector memory cells had lower expression of IL-17RA than central memory cells (figure 4F). Flow cytometry of splenocytes collected from the previously described in vivo experiment reveals that therapeutic neutralization of IL-17RA offers protection against CD4 T cell exhaustion (figure 4Gi,Gii).

T cell IL-17RA gene expression correlates with worse patient outcomes
We extended our study of IL-17RA to human subjects. Analysis of anti-PD-1 immunotherapy patient survival data shows no significant survival difference between overall IL-17RA high and IL-17RA low patients; differences occur when looking at IL-17RA at the T cell compartment (figure 5A). In fact, both IL-17RA in CD4 and IL-17RA in CD8 T cells inversely correlate with anti-PD-1 immunotherapy patient survival (figure 5B). We next look at RNA-seq and gene chip data of IL-17RA in CD4 T cells and patient survival in multiple cancer types. Data consistently reveal a higher probability of survival in patients with lower IL-17RA in CD4 T cells across head-neck squamous cell carcinoma, sarcoma, liver hepatocellular carcinoma, gastric cancer, colorectal cancer, and breast cancer (figure 5Ci,Cii). On the other hand, IL-17RB, being the coreceptor for IL-25, has also been reported to be associated with poor prognosis in malignancies such as oral cancer. However, we did not observe enhanced survival probability for colorectal or breast cancer patients with lower IL-17RB expression in CD4 T cells (figure 5D). Altogether, retrospective patient survival data suggest that patients with cancer could benefit from IL-17RA neutralization but not IL-17RB neutralization.


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Cancer remains the second-leading cause of death in the USA, accounting for 25% of all deaths nationwide. ICI bolsters immune cells’ ability to target cancer cells and has improved cancer treatments immensely. Anti-PD-1 and CTLA-4 antibodies provide excellent clinical...
efficacy, as evidenced by tumor regression and increased overall patient survival. While these therapies are highly successful in some patients, they fail or cause irAEs in others. More than 50% of the patients who receive ICI develop irAEs characterized by multiple organ inflammation with T cell infiltrates. irAEs may occur at any time during ICI treatment but are most commonly observed within the first 3 months. These clinically observed irAEs share similarities with primary autoimmune diseases. The contribution PD-1 to peripheral tolerance is a significant mechanism for protection against the expansion of self-reactive T cell clones and autoimmune disease.

Several studies highlight cytokine dysregulation in irAE-affected tissues. Rahma et al analyzed the levels
of 34 cytokines in 52 melanoma patients receiving ICI who developed irAEs. There were no differences in cytokine levels between patients with grades 1–2 vs grades 3–4 irAEs. Patients with irAEs dermatitis had higher baseline Ang-1 and CD40L, and patients with pneumonitis had more elevated baseline IL-17. They observed a fold-change increase in the levels of many cytokines in patients who developed irAEs without receiving steroids. Multiple clinical studies evaluated the efficacy of anti-IL-6 (tocilizumab) treatment for managing irAEs. Clinical improvement or benefit was demonstrated in most patients without apparent adverse effects on tumor progression. However, the conclusions on cancer progression are based on retrospective data and limited statistical analysis. Also, the cancer types of the reported anti-IL-6 studies are limited to mainly melanoma and NSCLC. To properly assess the impact of anti-IL-6 therapy on cancer outcomes, prospective trials on different tumor types are required. TNFα inhibitors are commonly used to treat inflammatory diseases and have also been successfully adopted as second-line agents to treat irAEs refractory to steroids without reported interference with ICI’s antitumor efficacy. However, retrospective analysis of previous studies is limited, and whether TNFα inhibition can be safely used to treat irAEs without promoting cancer progression, either by compromising ICI therapy efficacy or via another route, remains an open question.

The most critical challenge in the field of irAEs remains their management. Despite the heterogeneity in presentation, the management of irAEs relies on

Figure 5  T cell IL-17RA gene expression correlates with worse patient outcomes. (A) Survival probability of patients receiving aPD-1 immunotherapy predicted by IL-17RA expression and IL-17RA/CD3 gene expression ratio. (B) aPD-1 immunotherapy patient survival predicted by IL-17RA/CD4 and IL-17RA/CD8 gene expression ratio. (C, Cii) Probability of survival predicted by IL-17RA/CD4 mRNA expression ratio in multiple malignancies. (D) Colorectal and breast cancer patient survival predicted by the IL-17RB/CD4 mRNA expression ratio.
broad, non-specific immunosuppression with glucocorticoids. Using our translationally relevant murine model of antibodies-induced irAEs, we compared the efficacy of anti-IL-6, anti-TNFα, and anti-IL-25 administration to prevent irAEs and their effect on colon adenocarcinoma progression. While all interventions inhibited irAEs pneumonitis and hepatitis, only treatment with anti-IL-25 exerted significant antitumor activity. Mechanistically, anti-IL-25 increased the TILs’ numbers and cytotoxic activity. These findings suggest that IL-25 may serve a dual role in treating tumors responsive to ICI, allowing for extended ICI therapy by suppressing autoimmune and irAEs. We also found that in the anti-IL-25 treated mice, IL-4, IL-5, IL-6, IL-25, and IL-12p70 levels were elevated while TNFα levels were decreased.

It has been previously reported that blocking IL-17A enhanced the antitumor response of anti-PD-1 therapy. Combined with our data on neutralizing IL-25 (IL-17E), we envisioned achieving higher efficacy by targeting IL-17RA, the receptor for both IL-17A and IL-25. Indeed, we showed that inhibition of IL-17RA ameliorated ICI-induced pneumonitis and promoted antitumor activity. Tumor growth inhibition and prevention of irAEs were also demonstrated in the EO771 (online supplemental figure 1) syngeneic tumor model. Remarkably, analysis of data from the TCGA revealed that high expression of IL-17RA is associated with worse prognosis and shorter survival in patients having multiple types of cancers. These data suggest that neutralizing IL-17RA might be beneficial in treating patients with cancer and irAEs.

Our work is significant for several reasons. Current treatments of irAEs are based on protocols used to treat phenotypically similar conditions, with corticosteroids being the first-line intervention. These non-specific treatments are associated with toxicities, and increasing evidence suggests that they also interfere with the antitumor immune receptors induced by the ICI. Our work defined the role of IL-25 and IL-17RA in irAEs and has great significance because of the need to develop therapeutic approaches that exploit differences between antitumor immunity and the processes resulting in irAEs. Our research will lead to a better understanding of irAE mechanisms.

Limitations of our study include non-comprehensive irAEs type observed in our murine model for irAEs. For example, our mice do not develop arthritis and dermatitis. Also, blockage of other PD-1-associated cytokine targets as irAEs treatment has yet to be tested due to the unavailability of clinical grade/in vivo grade antibodies. Moreover, our anti-IL-6 and anti-TNFα studies are limited to one tumor type. It would be beneficial to explore in vivo the effect of anti-IL-6 and anti-TNFα on other tumor types besides melanoma and NSCLC (Non Small Cell Lung Cancer), which most clinical trials cover, to predict clinical outcomes in unstudied patient populations better. Nonetheless, our work provides direction for developing therapeutic agents for irAEs and cancer.

There remain several open questions. Our data showed that neutralizing IL-17RA with antibodies inhibited tumor growth and resolved irAEs. However, IL-17RA tissue tropism and the mechanism of action associated with these effects must be clarified. Also, the contribution of IL-17RA to human tumor growth needs to be defined and further studied. To summarize, we show that anti-IL-6, anti-TNFα, anti-IL-25, and anti-IL-17RA administration ameliorate irAEs, but only the latter reduced tumor size. These findings suggest that IL-25 may serve a dual role in treating tumors responsive to ICI, allowing for extended ICI therapy by suppressing immune-related toxicities.

**STAR METHOD**

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METHODS

Cell culture
The murine colon adenocarcinoma cancer cell line MC38 (Kerafast) was cultured in DMEM medium (Corning) with 10% FBS (Gibco) and 1% Pen-Strep (Corning). The murine breast cancer cell line EO771 (Gift from Robert F. Schwabe) was cultured in DMEM medium (Corning) with 10% FBS (Fetal Bovine Serum) (Gibco), 1% Pen-Strep (Corning), and 20 mM HEPES (Corning). Cells were grown in a 37°C incubator and routinely examined for mycoplasma using mycoplasma detection kit (InvivoGen).

Mice breeding
B6/Lpr mice purchased from JAX (Cat. 000482) were housed in the Columbia Institute of Comparative Medicine animal facility under protocol AAABO7553. Breeding cages were set up with two females and one male. Litters are routinely genotyped through PCR using Fas gene primers—oLMR1678 (5’-GTA AAT AAT TGT GCT TCG TCA G-3’) as standard primer, oLMR1679 (5’-TAG AAA GGT CGG GTG TG-3’) for Faslmutant, and oLMR1680 (5’-CAA ATC TAG GCA TTA ACA GTG-3’) for FasWT. Mutant mice with homozygote alleles were recruited as new breeders or used for experiments. B6 WT mice were purchased from JAX (Cat. 000664) and used directly.

In vivo irAEs model
A total of 2×10⁵ MC38 cells were suspended in cold, sterile PBS (Corning) and implanted subcutaneously into the right flank of 7–12 weeks B6 Lpr mice. Anti-PD-1 and anti-CTLA-4 ICI treatments are initiated once the tumor is visible. 200 µg anti-PD-1 (BioXCell) and 200 µg anti-CTLA-4 (BioXCell) antibodies are administered biweekly through intraperitoneal injections. Tumor width and length are measured daily using a digital caliper. Tumor volume is calculated using the formula volume=(shorter dimension)²×(larger dimension)/2. In addition, prednisolone (Sigma), anti-IL-6 (BioXCell; MP5-20F3), anti-TNFα (BioXCell; XT3.11), anti-IL-25 (Lanier; LNR125), anti-IL-25-μ (Lanier; LNR125.38), and anti-IL-17RA (R&D; 657603) were given using varying methods, amounts, and schedules for different experimental conditions. For the breast cancer tumor study, 2×10⁵ EO771 cells were suspended in cold, sterile PBS (Phosphate Buffered Saline) (Corning) and implanted subcutaneously into the right flank of 8-week-old wild-type B6 mice. Tumor volumes were tracked. Once the tumor reached 20–50 mm³, all-25-μ treatment was initiated. The mice were injected with 200 µg clone aIL-25-μ biweekly for 2 weeks.

Luminex assay and analysis
Peripheral blood was collected from the heart of the mice posteuthanizing. Blood serum was isolated by centrifugation at 10,000×g for 10 min. Isolated serum was stored at −80°C before the Luminex assay. Columbia Biomarkers Core Laboratory performed Luminex magnetic bead assay using 36-plex mouse panel (Invitrogen) and IL-25 simplex (Invitrogen) kits. Each sample was run in duplicates. The coefficient of variation (CV) between duplicated samples was calculated. Repeated samples with CV>20% were eliminated. Models with cytokine/chemokine levels below the lower level of detection (LLOD) were assigned a value equal to LLOD/√2. Samples with cytokine/chemokine levels above the upper level of detection (ULOD) were given the value of ULOD. Cytokines/chemokines with more than 40% samples of undetectable values were discarded. Cytokine/chemokine values underwent a natural log transformation for normal distributions. Samples outside of their treatment groups’ respective 95% CI were eliminated. The data presented only show stratified data of interest.

Materials

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**Histology study**

At the in vivo endpoint, mice were transcardially perfused with 10 mL saline to clear blood. Heart, liver, lung, colon, pancreas, and tumor were collected and washed in 10 mL PBS before being transferred to 10 mL 10% formalin (Thermo Scientific). After >24 hours of fixation in formalin, the tissues were transferred to 70% histology-grade anhydrous ethanol (Fisher Bioreagents). Samples were then sent to Columbia Molecular Pathology Shared Resource for slicing, H&E stain, and paraffin embedding. H&E slides were viewed under a light microscope. Immune cell infiltration severity scores were graded by two trained experts on a scale of 0–3.19

**Immunohistochemistry**

Immune-blank slides are made from paraffin-embedded blocks and stained with anti-CD3 (GeneTex) by HistoWiz. Scanned images of IHC slides are processed using HALO (Indica Labs) for artificial intelligence CD3+ T cell labeling.50

**Flow cytometry and gating strategy**

Livers and tumors were harvested after perfusion at the endpoint of in vivo experiments. Livers were smashed through 45 μM filters using syringe plungers and collected in 20 mL of FACS buffer (2% FBS in PBS) in a 50 mL centrifuge tube. Liver cells pellet after centrifugation at 50×g for 2 min (brake off). The supernatant was collected and washed with PBS. Tumors were diced into small pieces using a scalpel blade and transferred to a 5 mL digestion mixture (500 mL PBS+500 mg collagenase D+25 mL FCS+10 mg DNase) in 15 mL conical tubes. The samples were incubated in a 37°C water bath for 30 min. Digested tissue samples were vortexed vigorously before being smashed through 45 μM filters and collected using 5 mL RPMI medium (Corning) containing 10% FBS and 1% Pen-Strep. Following centrifugation and collection, the cells were washed once with PBS. Liver and tumor lymphocytes were isolated using Lymphoprep (Stem Cell Technologies) following Lymphoprep’s standard protocol. Isolated lymphocytes were washed with PBS and used immediately for flow cytometry. Lymphocytes isolated from the liver and tumor were stained for 34-plex flow cytometry using a protocol consisting of four parts: (1) live/dead staining, (2) surface staining, (3) fixation/permeabilization, and (4) intracellular staining. Live/dead stain was performed with a live/dead fixable blue dead cell stain kit (Invitrogen). Following live/dead colors, Fc receptors, and monocytes were blocked using TrueStain FcX PLUS (BioLegend) and TrueStain Monocyte Blocker (BioLegend). Then, the cells were stained for cell surface proteins using fluorophore conjugated antibodies: TCR-B BV395, CD103 BV496, CD44 BV563, PD-1 BV615, Nrp1 BV661, CD4 BV805, CD39 BV421, IA-IE PacBlue, ST2 BV480, CD8 PacOrange, CD62L BV570, CD11c BV605, ICOS BV624, CCR3 BV711, KLRG1 BV750, PD-L1 BV785, CD45 A532, Sca-1 PerCP, Ly6C PerCP-Cy5.5, CD206 PerCPeF710, NK1.1 PE-Cy5, B220 PE/Fire 810, CD69 SN685, CD11b A700, F4/80 APC-Fire750, and CD38 APC-Fire810. Following cell surface staining, the cells were fixed/permeabilized using a Fixation/Permeabilization Buffer Set (BioLegend). Post-fix and perm, the cells were stained for intracellular proteins using fluorophore-conjugated antibodies: Ki67 BV737, iNOS FITC, TOX PE, Ly6G SYG593, Helios PE-Dazzle594, FoxP3 PE-Cy7, and TCF-1 APC. Data were acquired using Cytex 5L Aurora and analyzed using FlowJo and Python UMAP. Spleen was harvested at the endpoint of in vivo experiments. Spleen tissue was smashed through 70 μM filters, washed twice in FACs, resuspended in ACK lysis buffer, and washed twice in FACs. Spleen-mixed white blood cells were used immediately for flow cytometry or frozen with 20% DMSO (Fisher Bioreagents) in FBS (Gibco). Dead cells in the mixed white blood cells isolated from the spleens were stained using Zombie UV Fixable Viability Kit, while the Fc receptors were blocked using TrueStain FcX (BioLegend). Cell surface proteins were stained with C fluorophore-conjugated-antibodies, CD3 AF488 (BioLegend), CD4 BV510 (BioLegend), CD8 PerCp/Cy5.5 (BioLegend), CD44 BV421 (BioLegend), CD62L BV711 (BioLegend), PD-1 PE-Cy7 (BioLegend), CD69 APC, and IL-17RA PE, for CD4+/CD8+ T cell identification, T_{Naive}/T_{cm}/T_{em}/TEMRA subset differentiation, activation/exhaustion observation, and IL-17RA expression assessment. Data were recorded using Cytex 5L Aurora and analyzed with FlowJo.

**Kaplan-Meier plots**

KM plotter was used to investigate IL-17RA’s function in human subjects with cancer. IL-17RA/CD4 mRNA expression ratio versus survival was mapped for breast and colon cancer using gene chip data (60-month follow-up threshold). IL-17RA/CD4 mRNA expression ratio versus survival was mapped for liver hepatocellular carcinoma, liver cancer, head-neck squamous cell carcinoma, and sarcoma using RNA sequencing data (60-month follow-up threshold). IL-17RA/CD4 and IL-17RA/CD8 (control) gene expression ratio vs survival was mapped for patients only receiving PD-1 immunotherapy regardless of which aPD1 drug (KM plotter).

**Statistical analysis**

Data were analyzed using Prism V9 (GraphPad) and presented as mean or means±SEM. For comparison analysis of immune infiltration/cytokine level/tumor volume on day 18 bar graphs, an unpaired two-tailed t-test was performed. Additionally, one-way analysis of variance was performed on tumor data in the nonlinear fitted tumor curves. Survival probabilities were analyzed using the KM estimate.

**Twitter** Brian S Henick @BHenickMD

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Patient consent for publication Not applicable.

Ethics approval Mice breeding and experiment protocols have been carefully examined and meet the ethics approval standard of the Columbia Institute of Comparative Medicine. The approved protocols are AC-AA907553 and AC-AABP3559.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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ORCID iDs
Xizi Hu http://orcid.org/0009-0000-2192-6185
Brian S Henick http://orcid.org/0000-0003-2881-0805

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