Bifunctional immune checkpoint-targeted antibody-ligand traps that simultaneously disable TGFβ enhance the efficacy of cancer immunotherapy

Rajani Ravi1, Kimberly A. Noonan2, Vui Pham1, Rishi Bedi3, Alex Zhavoronkov4, Ivan V. Ozerov4, Eugene Makarev4, Artem V. Artemov4, Piotr T. Wysocki1, Ranee Mehra5, Sridhar Nimmagadda6, Luigi Marchionni7, David Sidransky1, Ivan M. Borrello2, Evgeny Izumchenko1 & Atul Bedi1

A majority of cancers fail to respond to immunotherapy with antibodies targeting immune checkpoints, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) or programmed death-1 (PD-1)/PD-1 ligand (PD-L1). Cancers frequently express transforming growth factor-β (TGFβ), which drives immune dysfunction in the tumor microenvironment by inducing regulatory T cells (Tregs) and inhibiting CD8+ and TH1 cells. To address this therapeutic challenge, we invent bifunctional antibody-ligand traps (Y-traps) comprising an antibody targeting CTLA-4 or PD-L1 fused to a TGFβ receptor II ectodomain sequence that simultaneously disables autocrine/paracrine TGFβ in the target cell microenvironment (a-CTLA4-TGFβRIIecd and a-PDL1-TGFβRIIecd). a-CTLA4-TGFβRIIecd is more effective in reducing tumor-infiltrating Tregs and inhibiting tumor progression compared with CTLA-4 antibody (Ipilimumab). Likewise, a-PDL1-TGFβRIIecd exhibits superior antitumor efficacy compared with PD-L1 antibodies (Atezolizumab or Avelumab). Our data demonstrate that Y-traps counteract TGFβ-mediated differentiation of Tregs and immune tolerance, thereby providing a potentially more effective immunotherapeutic strategy against cancers that are resistant to current immune checkpoint inhibitors.
Genetic mutations accruing from the inherent genomic instability of tumor cells present neo-antigens that are recognized by the immune system. Cross-presentation of tumor antigens at the immune synapse between antigen-presenting dendritic cells and T lymphocytes can potentially activate an adaptive antitumor immune response that is mediated by CD4⁺ T-helper cells (T₄₁₁) and CD8⁺ cytotoxic effector cells, and sustained by tumor-reactive central memory T cells. However, tumors continuously evolve to counteract and ultimately defeat such immune surveillance by co-opting and amplifying mechanisms of immune tolerance to evade elimination by the immune system. This prerequisite for tumor progression is enabled by the ability of cancers to produce immunomodulatory factors that create a tolerogenic immune cell microenvironment.

Transforming growth factor-β (TGFβ) is a multifunctional cytokine that is overexpressed in a majority of cancers. The high-affinity binding of TGFβ to TGFβ receptor II (TGFβRII) recruits TGFβ receptor I into a heterotrimeric complex that initiates SMAD-mediated transcriptional activation or repression of several genes that control cell growth, differentiation, and migration. Besides promoting epithelial-to-mesenchymal transition, invasion, and metastases of tumor cells, TGFβ has a critical role in regulating the adaptive immune system. TGFβ suppresses the expression of interferon-γ (IFN-γ), restricts the differentiation of T₄₁₁ cells, attenuates the activation and cytotoxic function of CD8⁺ effector cells, and inhibits the development of central memory T cells. Most significantly, TGFβ induces the differentiation of regulatory T cells (Tregs), a sub-population of CD8⁺ effector cells, and induces the development of central memory T cells. TGFβ suppresses the expression of interferon-γ (IFN-γ), restricts the differentiation of T₄₁₁ cells, attenuates the activation and cytotoxic function of CD8⁺ effector cells, and inhibits the development of central memory T cells. Most significantly, TGFβ induces the differentiation of regulatory T cells (Tregs), a sub-population of CD8⁺ effector cells, and inhibits the development of central memory T cells. Among breast cancers, TGFβ⁻β is significantly more effective in reducing and counteracting tumor-infiltrating Tregs, activating antitumor immunity, and inhibiting tumor progression compared with the CTLA-4 antibody, Ipilimumab. Likewise, a-PD-L1-TGFβRIIeclcd exerts superior antitumor efficacy compared with PD-L1 antibodies (Atezolizumab or Avelumab). Our data demonstrate that Y-traps simultaneously disable immune checkpoints and counteract TGFβ-mediated differentiation of Tregs and immune tolerance, thereby providing a more effective immunotherapeutic strategy against cancers that fail to respond to current immune checkpoint inhibitors.

Results
TGFβ signaling correlates with FOXP3 expression in cancers. We used iPANDA, a bioinformatics software suite for qualitative analysis of intracellular signaling pathway activation based on transcriptomic data, to assess the level of TGFβ signaling in TCGA data sets of different types of cancer and investigate whether the TGFβ pathway activation in tumors is correlated with the level of expression of FOXP3, the signature transcription factor of the Treg lineage. Analysis of transcriptomic data from skin cutaneous melanoma (SKCM) data set (n = 472), using skin biopsy of healthy women (n = 122) as a reference, showed that upregulation in TGFβ signaling strongly correlated with increased messenger RNA expression levels of TGFβ1 and FOXP3 (Fig. 1a, b). The strong correlation between TGFβ pathway activation and FOXP3 expression was also noted in a TCGA breast cancer data set (n = 776), using normal breast tissue as a reference (Fig. 1c, d). Among breast cancers, TGFβ pathway activation and corresponding elevation of FOXP3 was especially striking in triple-negative breast cancer (TNBC) (Fig. 1c), an aggressive subtype that lacks expression of hormone receptors (estrogen receptor (ER)/progesterone receptor (PR)) and HER2/neu, and has a higher risk of metastases and death within 5 years of diagnosis. Although TGFβ and PD-L1 can cooperate to induce expression of FOXP3, expression of PD-L1 (CD274) mRNA did not exhibit a corresponding or consistent correlation with FOXP3 mRNA expression (Fig. 1d). The strong correlation of TGFβ activation with FOXP3 expression supports a crucial role of autocrine/paracrine TGFβ signaling in induction and maintenance of Tregs in diverse cancers.

Design and bifunctional target binding of a-CTLA4-TGFβRII. Anti-CTLA4-TGFβRIIeclcd (a-CTLA4-TGFβRII) was designed to simultaneously target both CTLA-4 and TGFβ by fusing the C terminus of the HC of a human anti-CTLA-4 antibody with a
ligand-binding sequence of the extracellular domain of TGFβRII via a flexible linker peptide (Fig. 2a, b). Protein identification of the purified antibody from CHO-K1 cell supernatants was performed by liquid chromatography Fourier transform tandem mass spectrometry (LC–MS/MS) to confirm the amino acid sequence of the HC of a-CTLA4-TGFβRII (Fig. 2b). SDS-polyacrylamide gel electrophoresis (PAGE) under reducing (R) and non-reducing (NR) conditions was used to compare the full-length (FL), HC, and light chain (LC) of a-CTLA4-TGFβRII and a-CTLA-4 antibody (Fig. 2c). MS analysis confirmed the expected higher molecular weight of the HC of a-CTLA4-TGFβRII (65,697 kDa) compared with the HC of a-CTLA-4 antibody (49,256 kDa). The bifunctional ability of a-CTLA4-TGFβRII to simultaneously bind CTLA-4 and TGFβ was confirmed by enzyme-linked immunosorbent assay (ELISA), where a-CTLA4-TGFβRII was added to coated plates, followed by recombinant human TGFβ (rhTGFβ1) that was detected by a biotinylated anti-human TGFβ1 antibody (Fig. 2d, e). Unlike a-CTLA-4, a-CTLA4-TGFβRII exhibited the additional ability to compete with a TGFβ capture antibody for binding to TGFβ1 (Fig. 2f).

**Fig. 1** TGFβ pathway activation correlates with elevated TGFβ1 and FOXP3 mRNA expression in TCGA-SKCM and TCGA-BRCA data sets. iPANDA software suite for analysis of intracellular signaling pathway activation based on transcriptomic data was used to estimate the level of TGFβ signaling in melanoma or breast samples from TCGA-SKCM (n = 472) and TCGA-BRCA data set, respectively (n = 776). TCGA transcriptomic data from normal breast tissue (n = 114) or skin biopsy of healthy women (n = 122) was used as a reference after proper normalization. The TGFβ pathway activation scores for a 472 melanomas (metastatic and primary) and c 776 breast tumors (ER/PR positive, HER2 positive, and TNBC) are sorted from low to high. The corresponding expression of TGFβ1 gene, FOXP3, and CD274 is shown for each patient. The units on the color bar represent the pathway activation score or the fold change in the gene expression on the logarithmic scale in comparison with the average normal control level. Correlation matrix for melanoma (b) and breast cancer (d) are shown (numbers in plot indicate correlation coefficient value). Pairwise correlations between TGFβ pathway activation and message for TGFβ1, FOXP3, and CD274 were computed using the Pearson’s correlation coefficient. The p-values were calculated with the null hypothesis that the tested samples are unrelated. The p-values for all correlation <0.05

**a-CTLA4-TGFβRII counteracts Tregs and T<sub>H</sub>17 differentiation.** The FOXP3 transcription factor governs the differentiation and function of Tregs. The transcription factors SMAD3 and nuclear factor of activated T-cells (NFAT) are required for activation of a FOXP3 enhancer, and both factors are essential for induction of FOXP3 in primary T cells. TGFβ-activated SMAD-2/3 cooperates with interleukin (IL)-2-activated NFAT to induce FOXP3 expression and promote the conversion of naïve CD4<sup>+</sup> T cells to FOXP3-expressing Treg cells (induced Tregs or iTregs) that mediate immune tolerance<sup>15</sup> (Fig. 2a). Consistent with these observations, treatment with rhTGFβ1 induced the phosphorylation of SMAD-2/3 and increased expression of FOXP3 in human peripheral blood mononuclear cells (PBMCs) costimulated with anti-CD3/anti-CD28-coated beads and rhIL-2 (Fig. 3a, left panel).

a-CTLA4-TGFβRII is designed to exploit the FOXP3-mediated expression of CTLA-4 on Tregs to decorate the targeted cells with a decoy TGFβRII ectodomain that captures and disables TGFβ in their localized microenvironment (Fig. 2a, b). We examined the ability of a-CTLA4-TGFβRII to inhibit TGFβ-induced SMAD-2/3 phosphorylation and expression of FOXP3 in human T cells. Human PBMC were stimulated with rhIL-2 and anti-CD3/anti-CD28-coated beads in the presence of rhTGFβ1 with or without either a-CTLA4-TGFβRII or a-CTLA-4. Unlike a-CTLA-4, a-CTLA4-TGFβRII counteracted TGFβ-induced SMAD-2/3 phosphorylation and FOXP3 expression in co-stimulated T cells (Fig. 3a, right panel).

The in vivo effect of a-CTLA4-TGFβRII on Tregs was examined in human melanoma tumor-bearing NSG mice (NOD/Shi-scid IL-2<sup>−/−</sup> that were immune reconstituted with tumor-matched human leucocyte antigen (HLA) A2 + human CD3<sup>+</sup> bone marrow (BM) cells. The production of TGFβ by A375 tumor cells (842 pg per 10<sup>6</sup> cells per 24 h) and SK-MEL-5 tumor cells (513 pg per 10<sup>6</sup> cells per 24 h) was confirmed by ELISA assay. Tregs in BM and tumor-infiltrating T cells isolated from tumor-bearing mice treated with either a-CTLA4-TGFβRII or a-CTLA-4 and their untreated counterparts were measured by immunophenotype analysis. Compared with a-CTLA-4, treatment of tumor-bearing mice with a-CTLA4-TGFβRII resulted in a marked decline of FOXP3-expression in CD4<sup>+</sup> cells (Fig. 3b). A375 or patient-derived tumor xenograft (PDX)-bearing mice treated with a-CTLA4-TGFβRII exhibited a significant reduction of tumor-infiltrating Tregs (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>FOXP3<sup>+</sup> cells) compared to those treated with a-CTLA-4 (p < 0.001 for A375, p < 0.003 for PDX1, and p < 0.02 for PDX2; Student’s unpaired t-test) (Fig. 3c). These data show that a-CTLA4-TGFβRII counteracts FOXP3<sup>+</sup> Treg specification in a TGFβ-enriched tumor microenvironment.

As FOXP3 is instrumental for the suppressive function of Tregs, the relative ability of a-CTLA4-TGFβRII and a-CTLA-4 to counteract Treg-mediated suppression of tumor-reactive T cells was also examined using tumor-infiltrated BM from a patient. Anti-CD3/anti-CD28 and rhIL-2 activated CD3<sup>+</sup> marrow-
infiltrating lymphocytes (aMLs) were CFSE labeled and added to autologous BM that had been pulsed with either tumor cell lysate (tumor-specific antigen) or nonspecific antigen in the presence or absence of autologous CD4+CD25+ Tregs isolated from the same patient’s BM. Following culture of these cells for 3 days with or without either a-CTLA4-TGFβRII or a-CTLA4, tumor antigen-reactive T cells (CD3+CFSElow/IFNy+) were quantified by immunophenotype analyses (Fig. 3d). As expected, the addition of autologous Tregs suppressed the activation of tumor antigen-reactive T cells (CD3+CFSElow/IFNy+) in anti-CD3/anti-CD28-activated aMLs stimulated with tumor antigen-pulsed autologous BM. a-CTLA4-TGFβRII was far more effective than a-CTLA4 in counteracting Treg-mediated suppression and restoring activation of tumor antigen-specific T cells in the presence of autologous Tregs (Fig. 3d). These data demonstrate that a-CTLA4-TGFβRII is more effective than a-CTLA4 in counteracting Tregs in the tumor microenvironment.

The differentiation of CD4+ T cells into Th1, Th17, or iTreg cell lineages is determined by the cytokine milieu. IFN-γ drives Th1 differentiation, TGFβ is required for differentiation of both iTreg and Th17 cells. Although TGFβ cooperates with IL-2 to induce iTreg differentiation, TGFβ promotes Th17 differentiation in the presence of proinflammatory cytokines, such as IL-6. TIM-3 drives Th1 differentiation, TGFβ is required for differentiation of both iTreg and Th17 cells. Although TGFβ cooperates with IL-2 to induce iTreg differentiation, TGFβ promotes Th17 differentiation in the presence of proinflammatory cytokines, such as IL-6. In contrast to Th1 cells that are strongly associated with good clinical prognosis for all cancer types, Th17 cells are associated with tumor-promoting inflammation and autoimmune pathology. As a-CTLA4-TGFβRII can render the targeted T cells incapable of responding to TGFβ signals in their immediate milieu, we examined whether a-CTLA4-TGFβRII also skews the differentiation of CD4+ T cells away from Th17 cells toward an IFN-γ-expressing Th1 phenotype. Whereas the Th17 phenotype of CD4+ T cells costimulated with anti-CD3/anti-CD28 beads under Th17 skewing conditions was maintained in the presence of a-
CTLA-4, a-CTLA4-TGFβRII was able to abrogate the expression of IL-17 in CD4+ T cells and switch them to an IFN-γ-expressing T(reg)1 phenotype (Fig. 3e).

Superior antitumor immunity and efficacy of a-CTLA4-TGFβRII. As a-CTLA4-TGFβRII effectively counteracted tumor-infiltrating Tregs in vivo, we examined its ability to increase tumor-reactive IFNγ expression in T cells and inhibit tumor growth in human melanoma tumor-bearing NSG mice that were immune reconstituted with HLA A2+ human BM CD34+ cells. Treatment with a-CTLA4-TGFβRII was significantly more effective at inhibiting the growth of A375 tumors compared with a-CTLA4- (p < 0.004, Student’s unpaired t-test) or untreated controls (p < 0.0001, Student’s unpaired t-test) (Fig. 4a, b). Consistent with its superior antitumor efficacy, treatment of A375-tumor-bearing mice with a-CTLA4-TGFβRII resulted in a greater elevation in tumor-reactive IFN-γ-expressing CD8+ cells compared with treatment with a-CTLA4 (Fig. 4c). We next evaluated the comparative antitumor efficacy of a-CTLA4-TGFβRII, nonspecific IgG-TGFβRII, and the combination of a-CTLA4 and IgG-TGFβRII using the same model. Treatment of tumor-bearing mice with a-CTLA4-TGFβRII was significantly more effective at inhibiting tumor progression (p < 0.02, Student’s unpaired t-test) (Fig. 4d), reducing FOXP3+ expressing Tregs (p < 0.001, Student’s unpaired t-test) (Fig. 4e, left) and elevating tumor reactive IFN-γ-expressing CD8+ cells (p < 0.01, Student’s unpaired t-test) (Fig. 4e, right) compared with the a-CTLA4-alone, IgG-TGFβRII-alone, and even the combination of a-CTLA4 and nonspecific IgG-TGFβRII (Fig. 4d, e). Mice treated with a-CTLA4-TGFβRII maintained serum hepatic enzymes within a normal range of liver function (mean ± SEM) (alanine aminotransferase (ALT): 52±2 U/L, aspartate aminotransferase (AST): 64±2 U/L, alkaline phosphatase (ALP): 58±4 U/L, γ-glutamyl transpeptidase (GGT): 27±6 U/L, bilirubin (Direct): 0.3±0.02 mg/dL, bilirubin (Indirect): 0.9±0.1 mg/dL, total protein: 6.7±0.2 g/dL, albumin: 3.4±0.1 g/dL, creatinine: 0.7±0.1 mg/dL, total cholesterol: 138±8 mg/dL, triglycerides: 113±17 mg/dL). These data suggest that a-CTLA4-TGFβRII is a potent anti-tumor agent that can effectively inhibit tumor growth and reactivate Tumor-infiltrating Tregs in vivo.
Mice engrafted with human CD3+ cells were inoculated subcutaneously with either human melanoma tumor cells (A375) or patient-derived melanoma bearing mice: (mean ± SEM of 12 mice in each indicated group). a Representative images and in vivo tumor growth curves in A375 tumor-bearing mice: (mean ± SEM of 12 mice in each indicated group). b Representative images and mass of tumors at the end of treatment in A375 tumor-bearing mice: (seven mice in each indicated group). c Flow cytometric analysis of the comparative in vivo effect of the indicated treatments on tumor-reactive IFN-γ expression in CD8+ T cells in A375 tumor-bearing mice. Representative flow data is shown (mean ± SEM). d, e Comparative antitumor efficacy of α-CTLA4-TGFβRII, α-CTLA-4, non-specific IgG-TGFβRII, and the combination of α-CTLA-4 and IgG-TGFβRII in A375 tumor-bearing mice. d Tumor growth curves (mean ± SEM of five mice). The p-value (p > 0.02, Student’s unpaired t-test) denotes significant difference between α-CTLA4-TGFβRII and each other treatment group. e Flow cytometric analysis of infiltrating FOXP3-expressing Tregs (left) and tumor-reactive IFN-γ expressing CD8+ T cells (right). f Flow cytometric analysis of infiltrating central memory T cells (%CD45ROhighCD62Lhigh) in PDX-bearing mice: (mean ± SEM of five mice). g Representative flow data of in vivo effect of α-CTLA4-TGFβRII and α-CTLA-4 on tumor-specific IFN-γ expression in CD8+ T cells in PDX-bearing mice. h Flow cytometric analysis of the comparative in vivo effect of α-CTLA4-TGFβRII and α-CTLA-4 on tumor-infiltrating central memory T cells (CD45ROhighCD62Lhigh) in PDX-bearing mice.
transaminase \((14 \pm 3 \text{ U l}^{-1})\), aspartate transaminase \((67 \pm 4 \text{ U l}^{-1})\), alkaline phosphatase \((68.5 \pm 6 \text{ U l}^{-1})\), total bilirubin \((0.25 \pm 0.1 \text{ mg ml}^{-1})\) and demonstrated no loss of body weight during the course of experiment. The superior antitumor efficacy of \(\alpha\)-CTL4A-TGFβRII compared with \(\alpha\)-CTL4-A was further confirmed in immune-reconstituted NSG mice bearing primary PDX (Fig. 4f). Accordingly, treatment with \(\alpha\)-CTL4A-TGFβRII also resulted in higher tumor-reactive IFN-γ-expressing CD8\(^{+}\) cells compared to treatment with \(\alpha\)-CTL4-A in mice bearing human melanoma PDX (Fig. 4g). The comparative in vivo effect of \(\alpha\)-CTL4A-TGFβRII and \(\alpha\)-CTL4-A on the differentiation of tumor-infiltrating T cells into central memory T cells (CD45RO\(^{hi}\)/CD62L\(^{lo}\)) was also evaluated in tumors collected from PDX-bearing immune-reconstituted mice. Treatment with \(\alpha\)-CTL4A-TGFβRII was more effective than \(\alpha\)-CTL4-A in increasing the percentage of CD4\(^{+}\) and CD8\(^{+}\) T cells with a central memory phenotype (Fig. 4h). These results demonstrate that TGFβ in the tumor microenvironment reduces tumor-reactive IFN-γ-expressing CD8\(^{+}\) cells and tumor-infiltrating central memory T cells, and that \(\alpha\)-CTL4A-TGFβRII is required to effectively counteract these effects by rendering the targeted T cells incapable of responding to autocrine/paracrine TGFβ signals in their immediate milieu.

\(\alpha\)-CTL4A-TGFβRII is more effective than \(\alpha\)-CTL4-A and \(\alpha\)-PD1. Besides CTL4-A, engagement of PD-1 by PD-L1 expressed on tumor cells or T cells also inhibits antitumor T cells. Although monoclonal antibodies (mAbs) against PD-1, such as Nivolumab or Pembrozulmab, are effective in some patients, the vast majority of cancers fail to respond to either PD-1 blockade or even dual checkpoint inhibition with \(\alpha\)-CTL4-A and \(\alpha\)-PD-1. Therefore, we investigated the ability of \(\alpha\)-CTL4A-TGFβRII to elicit antitumor immunity and inhibit the growth and metastases of cancers that are refractory to current checkpoint inhibitors, such as TNBC. Approximately 15–25\% of patients with breast cancer have TNBC, an aggressive type that does not respond to hormonal agents or targeted therapy and has an increased risk of metastases. As TNBC is representative of a tumor type that exhibits a TGFβ-mediated immune tolerance (Fig. 1c), we used human immune reconstituted NSG mice bearing the bioluminescent human MDA-MB-231-luc (D3H2LN) TNBC cell line that expresses TGFβ (1D11); \(\alpha\)-PD1 (Pembrozulmab); (five to six mice per group). 

**Fig. 5** \(\alpha\)-CTL4A-TGFβRII inhibits tumor growth more effectively than the combination of \(\alpha\)-CTL4-A and \(\alpha\)-PD1. NSG mice immune reconstituted with tumor-matched HLA A2\(^{+}\) human CD34\(^{+}\) BM cells and bearing MDA-MB231-Luc human TNBC tumor xenografts were treated (5 mg kg\(^{-1}\) i.p. weekly) with vehicle alone (untreated control) or the following antibodies (either alone or in combination), as indicated: \(\alpha\)-CTL4A-TGFβRII; \(\alpha\)-CTL4-A; \(\alpha\)-TGFβ (1D11); \(\alpha\)-PD1 (Pembrozulmab); (five to six mice per group). 

**Legend:** a In vivo tumor growth curves \((\text{mean} \pm \text{SEM})\). \(p\)-values were derived using unpaired, two-sided \(t\)-test. b Bioluminescence assay of primary tumors in untreated controls or the indicated treatment groups at 10 and 25 days after tumor cell inoculation. c Bioluminescence assay of lung metastases in untreated controls or the indicated treatment groups at 40 days post tumor inoculation. d Immunophenotype analysis of the effect of the indicated treatments on the percentage of Tregs \((\text{CD4}^{+}/\text{CD25}^{hi}/\text{CD127}^{li}/\text{FOXP3}^{+})\), tumor-reactive IFN-γ-expressing CD8\(^{+}\) T cells, and central memory CD4\(^{+}\) and CD8\(^{+}\) T cells (CD45RO\(^{hi}\)/CD62L\(^{hi}\)). Bars represent mean ± SEM of three animals per treatment group. Asterisks above each bar denote the statistical significance of the difference between the indicated group and \(\alpha\)-CTL4A-TGFβRII (blue bar)
animals (Fig. 5a–c). In contrast, treatment with a-CTLA4-TGFβRII was significantly more effective at inhibiting the progression of MDA-MB-231-luc tumors compared with untreated controls (p < 0.0001, Student’s unpaired t-test), or animals treated with either a-CTLA-4 alone (p < 0.001, Student’s unpaired t-test) or the combination of a-CTLA-4 and a-PD-1 mAbs (p < 0.0001, Student’s unpaired t-test) (Fig. 5a, b). In addition, a-CTLA4-TGFβRII exhibited significantly better antitumor efficacy compared with either a-TGFβp (p < 0.001, Student’s unpaired t-test) or a combination of a-CTLA-4 and a-TGFβp (p < 0.04, Student’s unpaired t-test) (Fig. 5a, b), and was more effective in inhibiting lung metastases (Fig. 5c). Consistent with its superior antitumor efficacy, a-CTLA4-TGFβRII was more effective in reducing Tregs, elevating tumor-reactive IFN-γ-expressing CD8+ cells and increasing the CD4+ and CD8+ central memory T cells compared with the combination of a-CTLA-4 and a-PD-1 mAbs (Fig. 5d).

**Design and bifunctional target binding of a-PD1-TGFβRII.**

PD-L1 is overexpressed on tumor cells as well as tumor-infiltrating T cells, where it cooperates with TGFβ to inhibit T cell activation and induce and maintain immunosuppressive Treg cells. Although TGFβ and PD-L1 can cooperate to induce FOXP3, our analysis of both TCGA data sets showed that the correlation of TGFβ pathway activation with FOXP3 expression was substantially stronger than its correlation with PD-L1 (CD274) mRNA (Fig. 1). These data suggest that PD-L1/PD-1 checkpoint and TGFβ signaling exercise independent, yet cooperative mechanisms of immune tolerance, thereby supporting a therapeutically rationale for simultaneously counteracting both axes in the tumor immune microenvironment.

Anti-PD1-TGFβRIIedcd (a-PD1-TGFβRII) is a bifunctional antibody–ligand trap that was designed to target PD-L1 and simultaneously inactivate TGFβ by fusion of an extracellular domain sequence of TGFβRII to the C terminus of the HC of anti-PD-L1 antibody via a flexible linker sequence, (GGGGS)3 (Fig. 6a, b). SDS-PAGE of two different anti-PD-L1 antibodies (Atezolizumab and Avelumab) and their corresponding anti-PD1-TGFβRII products (Ab1 and Ab2) under R and NR conditions showed the expected higher molecular weight of the HC of anti-PD1-TGFβRII. Size exclusion-high-performance liquid chromatography (SEC-HPLC) analysis showed a single peak corresponding to purified a-PD1-TGFβRII with no aggregation (Fig. 6c).

The comparative ability of a-PD1-TGFβRII and a-PD1-L to bind PD-L1 was evaluated by ELISA assay, wherein biotinylated recombinant human PD-L1 (rh B7-H1-biotin) was added to plates coated with a-PD1-TGFβRII or a-PD1-L antibody, and detected by horseradish peroxidase (HRP)-Avidin. Plates coated with nonspecific IgG-TGFβRII showed no binding to PD-L1 and served as a negative control. Each a-PD1-TGFβRII (Ab1 and Ab2) exhibited specific binding to rhPD-L1 with an efficiency that was similar to the respective a-PD-L1 (Fig. 6d). The comparative ability of a-PD1-TGFβRII and a-PD1-L to bind TGFβ was evaluated by ELISA assay, wherein rhTGFβ1 was added to plates coated with a-PD1-TGFβRII or a-PD1-L and then detected by biotinylated anti-TGFβ1 and HRP-Avidin. Plates coated with nonspecific IgG-TGFβRII and rhTGFβRII-Fc served as positive controls to analyze the binding ability of the test samples to TGFβ. In contrast to the respective a-PD-L1 that failed to bind TGFβ, each corresponding a-PD1-TGFβRII exhibited binding to TGFβ with an efficiency that was similar to the positive controls (Fig. 6e). The ability of a-PD1-TGFβRII to simultaneously bind both PD-L1 and TGFβ was also evaluated by a bispecific ELISA assay, wherein a-PD1-TGFβRII or a-PD-L1 was added to PD-L1-Fc-coated plates, followed by rhTGFβ1 that was detected by a biotinylated anti-human TGFβ1 antibody. In contrast to the respective a-PD-L1, each corresponding a-PD1-TGFβRII (Ab1 and Ab2) exhibited simultaneous binding to PD-L1 and TGFβ (Fig. 6f).

**a-PD1-TGFβRII is more effective than a-PD-L1 antibodies.**

The comparative antitumor efficacy of a-PD1-TGFβRII, a-PD-L1, nonspecific IgG-TGFβRII, and the combination of a-PD-L1 and IgG-TGFβRII against human cancers expressing both PD-L1 and TGFβ was evaluated in either A375 (Fig. 7a, b) or MDA-MB-231-Luc (Fig. 7c, d) bearing NSG mice reconstituted with human CD34+ hematopoietic stem cells (HSCs). Moreover, in the TNBC model, independent experiments were conducted to compare two different a-PD1-TGFβRII antibody-ligand traps with their respective a-PD-L1 antibodies (Atezolizumab and Avelumab) (Fig. 7c).

In vivo tumor growth curves (mean ± SEM) in both tumor models demonstrated that treatment of tumor-bearing mice with a-PD1-TGFβRII was significantly more effective at inhibiting the progression of A375 (p < 0.01, Student’s unpaired t-test) (Fig. 7a) or MDA-MB-231-Luc (p < 0.004, Student’s unpaired t-test) (Fig. 7c) tumors compared with the respective a-PD-L1 alone, IgG-TGFβRII alone, and the combination of a-PD-L1 and nonspecific IgG-TGFβRII. Consistent with its superior antitumor efficacy, treatment with a-PD1-TGFβRII resulted in significant inhibition of FOXP3+ expressing Tregs (p < 0.05, Student’s unpaired t-test) (Fig. 7b, d: left) and a greater elevation in percentage of tumor-reactive IFNγ-expressing CD8+ cells (p < 0.01, Student’s unpaired t-test) (Fig. 7b, d: right) compared with treatment with the a-PD-L1 alone, IgG-TGFβRII alone, and even their combination. Mice treated with a-PD1-TGFβRII maintained serum hepatic enzymes within a normal range of liver function and demonstrated no loss of body weight during the course of experiment.

**Discussion**

Cancer immunotherapy is currently focused on targeting immune inhibitory checkpoints that control T cell activation, such as CTLA-4 and PD-1. Monoclonal antibodies that block these immune checkpoints can unleash antitumor immunity and produce durable clinical responses in a subset of patients with advanced cancers, such as melanoma and non-small-cell lung cancer. However, these immunotherapeutics are currently constrained by their inability to induce clinical responses in the vast majority of patients. A key limitation of checkpoint inhibitors is that they narrowly focus on modulating the immune synapse but do not address the key molecular determinants that are primarily responsible for immune dysfunction in the tumor microenvironment. Our data indicate that elevated expression of TGFβ is a root cause of such T-cell dysfunction in the tumor microenvironment. We find that autocrine and paracrine TGFβ signaling fundamentally affects tumor-infiltrating T cells by skewing the differentiation of Treg1 cells toward a Treg phenotype, attenuating the activation of CD8+ effector cells and limiting the development of central memory cells. As Tregs express and employ TGFβ to maintain their own phenotype and function, counteracting these deleterious cells and restoring beneficial Treg1 cells is contingent upon making them impervious to such autocrine signaling. This poses the therapeutic challenge of specifically breaking this TGFβ-driven autocrine loop in tumor-infiltrating Tregs. Systemic TGFβ antagonists fall short of interrupting autocrine signaling in Tregs, as they lack preferential localization to T cells and fail to efficiently compete with the native TGFβ receptor for binding TGFβ.
Sequestration of TGFβ by TGFβRIIe

Fig. 6 Design and bifunctional target binding ability of anti-PDL1-TGFβRII. a Schematic representation of PD-L1 and TGFβ entrained independent and cooperative mechanisms of immune tolerance. Schematic structure and targets of α-PDL1-TGFβRII are shown. α-PDL1-TGFβRII was designed by fusing the C terminus of the heavy chain of a human α-PD-L1 antibody with a ligand-binding sequence of the extracellular domain of TGFβ Receptor II (TGFβRII ECD) via a flexible linker peptide, (GGGGS)₃. b SDS-PAGE under non-reducing (NR) and reducing (R) conditions was used to compare the full-length, heavy chain and light chain of α-PDL1-TGFβRII and α-PD-L1 antibody. Figure shows the results of SDS-PAGE analyses of each of two separate α-PDL1-TGFβRII Y-traps (Ab1 and Ab2) and their respective human α-PD-L1 antibody (atezolizumab and avelumab). c SEC-HPLC analysis of purified α-PDL1-TGFβRII. d ELISA showing the comparative ability of α-PDL1-TGFβRII and α-PD-L1 antibody to bind PD-L1. Biotinylated recombinant human PD-L1 (rhB7-H1-biotin; 0–100 ng ml⁻¹) was added to plates coated with α-PDL1-TGFβRII or α-PD-L1 antibody (1 μg ml⁻¹), followed by detection with HRP-Avidin. Plates coated with nonspecific IgG-TGFβRII showed no binding to PD-L1 and served as a negative control to analyze the binding ability of the test samples. e ELISA showing the comparative ability of α-PDL1-TGFβRII and α-PD-L1 antibody to bind TGFβ. Recombinant human TGFβ (rhTGFβ; 0–2,000 pg ml⁻¹) was added to plates coated with α-PDL1-TGFβRII or α-PD-L1 antibody (1 μg ml⁻¹), which was detected by biotinylated α-TGFβ and HRP-Avidin. Plates coated with nonspecific IgG-TGFβRII and rhTGFβRII-Fc served as positive controls to analyze the binding ability of the test samples to TGFβ. f ELISA showing the ability of α-PDL1-TGFβRII to simultaneously bind both PD-L1 and TGFβ. Anti-PDL1-TGFβRII or α-PD-L1 antibody (0–100 ng ml⁻¹) was added to PD-L1-Fc coated plates (1 μg ml⁻¹) that was detected by a biotinylated anti-human TGFβ antibody. For d–f, the data show the optical density (OD) values (mean of three replicate wells for each assay condition) from a representative of two independent experiments.
CTLA-4 antibodies, such as ipilimumab, can target FOXP3-expressing Tregs and counteract CTLA-4-mediated inhibition of B7-CD28 interaction. However, a-CTLA-4 fails to counteract autocrine/paracrine TGF-β signaling, thereby resulting in NFAT/SMAD3-mediated upregulation of FOXP3 and a paradoxical increase in tumor-infiltrating Tregs in the TGF-β-enriched immune microenvironment found in the majority of cancers. Our data demonstrate that a-CTLA4-TGFβRII effectively addresses this challenge by exploiting the FOXP3-driven expression of CTLA-4 to not only disable the CTLA-4 checkpoint, but also decorate the targeted Tregs with a decoy TGFβRII ectodomain that traps TGFβ at the surface of the T cell, thereby rendering them virtually unresponsive to autocrine or paracrine TGFβ in their immediate milieu. As a result, a-CTLA4-TGFβRII counteracts autocrine/paracrine TGFβ/SMAD3-dependent expression of FOXP3, thereby reducing the differentiation and suppressive activity of Tregs. By skewing CD4+ T cells away from FOXP3+ Tregs or Th17 cells to a Th1 helper phenotype, a-CTLA4-TGFβRII enables effective activation of antitumor CD8+ effector T cells. An especially attractive feature of a-CTLA4-TGFβRII is its targeted ability to trap TGFβ at the surface of the T cell in a CTLA-4-directed manner, thereby interrupting the TGFβ-autocrine loop that drives FOXP3-mediated expression of CTLA-4. This distinguishing feature allows a-CTLA4-TGFβRII to enjoy a better therapeutic index compared with non-targeted TGFβ antagonists or even combinatorial therapy with a PD-L1 antibody and a systemic TGFβ antagonist that is not directed to the T cell microenvironment. This unique ability to counteract Tregs and correct immune tolerance in a TGFβ-enriched tumor immune microenvironment enables a-CTLA4-TGFβRII to be significantly more effective in activating antitumor immunity and inhibiting tumor progression compared with a CTLA-4 antibody, a PD-L1 antibody, or even the combination of both mAbs. Interestingly, a-CTLA4-TGFβRII was able to exhibit superior single agent activity against PD-L1-expressing tumors and the addition of a-PD1 antibody did not significantly enhance its antitumor efficacy in the breast cancer model. Although the highly effective counteraction of Tregs and immune tolerance by a-CTLA4-TGFβRII was sufficient to inhibit tumor growth, this might have obscured any potential value of combination therapy with a-PD1 during the course of the experiment. As IFN-γ-mediated upregulation of PD-L1 has been shown to be a mechanism of adaptive immune tolerance, it remains possible that PD-1/PD-L1 blockade could potentially enhance the antitumor activity of a-CTLA4-TGFβRII over a more extended treatment period or in other tumor models.

Whereas CTLA-4 is highly expressed on Tregs, PD-L1 is overexpressed on tumor cells as well as tumor-infiltrating T cells, where it cooperates with TGFβ to inhibit T-cell activation and induce and maintain Tregs. As PD-L1 and TGFβ entrain independent but cooperative mechanisms of immune tolerance, autocrine and paracrine TGFβ signaling in the tumor immune microenvironment may also limit the therapeutic efficacy of PD-1/PD-L1 antagonists. Consistent with this notion, our data demonstrate that a-PD-L1-TGFβRII is significantly more effective in inhibiting tumor progression compared with the corresponding a-PD-L1 antibody due to its bifunctional ability to not only block PD-L1/PD-1 interaction, but simultaneously interrupt autocrine/paracrine TGFβ signaling in the localized microenvironment of PD-L1 expressing tumor-infiltrating immune cells and tumor cells.

Although humanized NSG mice used in this study exhibit a functionally validated surrogate human immune system, this model supports the growth of human cancer cell line and PDXs...
even when they are not specifically HLA matched to the human CD34+ HSC used for immune reconstitution2,3. The absence of tumor rejection or inhibition of tumor progression in this model demonstrates that there is no spontaneous anti-allogeneic or tumor-specific immune response against such xenografts. However, as HLA-A*02 is the most highly prevalent HLA-A allele in patients with melanoma and breast cancer (including tumors cells used in this study), NSG mice were reconstituted with HLA-A*02 CD34+ HSCs. This was designed to ensure that HLA-A2-restricted TILs recognize HLA-A2-expressing xenografts, enabling generation of HLA-A2-restricted cytotoxic T cells. As such, our models were used to assess the comparative ability α-CTLA4-TGFβRII or α-PDL1-TGFβRII and their respective parent antibodies (α-CTLA-4 and α-PD-L1) to counteract immune tolerance in the TME and activate antitumor immune responses. Although these include HLA-A2-restricted responses against tumor antigens, they could exclude HLA-Arestricted T-cell responses to antigens presented by other class I or II HLA loci that may not be matched. The elicited immune responses may not be restricted to tumor antigens, but may potentially encompass anti-allogeneic responses. As such, our tumor models stringently compare T-cell-mediated antitumor immune responses between treatment groups and controls under the same conditions, rather than estimate the absolute efficacy of each independent treatment.

Our preclinical studies indicate that both antibody-ligand traps (α-CTLA4-TGFβRII and α-PDL1-TGFβRII) have a superior therapeutic index compared with their parent immune checkpoint inhibitors that are currently in clinical use. Although no adverse events were observed in mice treated with either α-CTLA4-TGFβRII or α-PDL1-TGFβRII, any novel immunotherapeutic strategy that seeks to counteract Treg cells and unleash antitumor immunity carries a potential risk of autoimmune sequelae in patients. As such, the clinical translation of this approach requires well-designed phase I dose-escalation trials to carefully evaluate the safety of each novel agent, determine the maximum tolerated dose, and identify the optimal therapeutic dose and schedule that can elicit an antitumor immune response without prohibitive immune-related adverse events. As elevated TGFβ is an especially common denominator of immune dysfunction in many types of cancer, these Y-traps may provide an effective immunotherapeutic strategy against cancers that fail to respond to current immune checkpoint inhibitors by simultaneously disabling immune checkpoints and counteracting TGFβ-mediated immune tolerance.

Methods

Correlative analysis of TGFβ pathway and FOXP3 expression. RNA sequencing (RNA-Seq) data for 472 melanomas and 776 breast tumors were retrieved from DEseq package from Bioconductor. To adjust for the possible batch and processing preprocessing and normalization steps were performed in R version 3.1.0 using the Mass Spectrometry and Proteomics Facility, Johns Hopkins University School of Medicine). SDS-PAGE under R and ND conditions was used to compare the FL, HC, and LC of α-CTLA4-TGFβRII with α-CTLA-4 and α-PDL1-TGFβRII with α-PD-L1.

Bifunctional target-binding ability of fusion antibodies. The ability of α-CTLA4-TGFβRII antibody to simultaneously bind both CTLA-4 and TGFβ was evaluated by a ‘double-sandwich’ ELISA, wherein anti-CTLA4-TGFβRII or anti-CTLA-4 antibody (1 μg ml\(^{-1}\)) was added to CTLA-4-Fc-coated plates, followed by rhTGFβ1 (0–2,000 pg ml\(^{-1}\)) that was detected by a biotinylated anti-human TGFβ antibody (R&D Systems). The positive standard curve (TGFβRII-Fc coated plate) was used to analyze the binding ability of the test samples. The ability of α-CTLA4-TGFβRII to bind TGFβ1 was also evaluated by competition ELISA. The ELISA plate was coated with the capture antibody (α-TGFβ, 1 μg ml\(^{-1}\)), followed by rhTGFβ1 in the presence of either α-CTLA4-TGFβRII or α-CTLA-4 (Antibody: TGFβ1 ratio 1 : 1 to 100 : 1) for 1 h at room temperature. Each experiment was performed twice, with triplicated wells. The comparative ability of anti-PDL1-TGFβRII and anti-PD-L1 antibody to bind PD-L1 was evaluated by ELISA, wherein biotinylated recombinant human PD-L1 (1 ng/ml; R&D Systems) was added to plates coated with anti-PDL1-TGFβRII or anti-PD-L1 antibody (1 μg ml\(^{-1}\)), followed by detection with HRP-Avidin. Plates coated with nonspecific IgG-TGFβRII served as a negative control to analyze the binding ability of the test samples. The comparative ability of α-PDL1-TGFβRII and α-PD-L1 antibody to bind TGFβ was evaluated by ELISA, wherein rhTGFβ1 (0–2,000 pg ml\(^{-1}\)) was added to plates coated with α-PDL1-TGFβRII or α-PD-L1 antibody (1 μg ml\(^{-1}\)), followed by detection with HRP-Avidin. Plates coated with nonspecific IgG-TGFβRII and rhTGFβRII-Fc served as positive controls to analyze the binding ability of the test samples to TGFβ. The ability of α-PDL1-TGFβRII to simultaneously bind both PD-L1 and TGFβ was evaluated by a bispecific ELISA, wherein α-PDL1-TGFβRII or α-PD-L1 antibody (1 ng/ml), which was added to PD-L1-Fc-coated plates (1 μg ml\(^{-1}\)), followed by rhTGFβ1 (1,000 pg ml\(^{-1}\)) that was detected by a biotinylated anti-human TGFβ antibody (R&D Systems).

Design of α-CTLA4-TGFβRII and α-PDL1-TGFβRII. Anti-CTLA4-TGFβRII was designed by fusing the C terminus of the HC of a human anti-CTLA-4 antibody (Ipilimumab) with a ligand-binding sequence of the extracellular domain of TGFβRII (TGFβRII ECD) via a flexible linker peptide, (GGGGS\(_5\)). Anti-PDL1-TGFβRII was designed to simultaneously target both PD-L1 and TGFβ by fusing the C terminus of the HC of human anti-PD-L1 antibody (Atezolizumab and Avelumab) with a ligand-binding sequence of the extracellular domain of TGFβRII (TGFβRII ECD) via a flexible linker peptide, (GGGGS\(_5\)). The amino acid sequences were codon optimized with GeneOptimizer (Life Technologies). Anti-gp120-TGFβRII antibody was used as a non-specific IgG-TGFβRII control. Amino acid sequences of all fusion antibodies used in this study are provided as Supplementary Material.

The complementary DNA for the antibody HC and the cDNA for the antibody LC were gene synthesized and subsequently cloned into separate plasmids (pEV3; evitria AG, Switzerland) under the control of a mammalian promoter and polyadenylation signal. Plasmid DNA was amplified in Escherichia coli and DNA was purified using anion exchange kits for low endotoxin plasmid DNA preparation. The plasmid DNAs for the antibody LC and the cDNA for the antibody HC were subsequently co-transfected into CHO K1 cells with eviFect (evitria AG, Switzerland), and the CHO cells were cultured in eviMake (evitria AG, Switzerland), a serum-free, animal-component-free medium. Production was terminated once viability reached 75%, which was determined by flow cytometry 8 days after transformation. Supernatant was then harvested and antibody was purified at 20 °C by Protein A affinity chromatography on a Bio-Rad BioLogic FuoFlow FPLC machine with subsequent gel filtration as polishing and re-charging step. The purified antibody was re-buffered into phosphate-buffered saline, sterile-filtered, aliquoted, and frozen at −80 °C. Protein identification of the purified antibody from CHO cell supernatants was performed by LC−MS/MS to confirm the amino acid sequence and size of the HC of α-CTLA4-TGFβRII and α-PDL1-TGFβRII (Mass Spectrometry and Proteomics Facility, Johns Hopkins University School of Medicine). SDS-PAGE under R and ND conditions was used to compare the FL, HC, and LC of α-CTLA4-TGFβRII with α-CTLA-4, and α-PDL1-TGFβRII with α-PD-L1.

TGFβ-binding ability of a-CTFL and IgG-TGFβRII. The ability of a-CTFL (1D11) and nonspecific IgG-TGFβRII (anti-gp120-TGFβRII) antibody to equally bind TGFβ in vitro was evaluated by a standard ELISA assay (Supplementary Figure 1). rhTGFβ1 (0–2,000 pg ml\(^{-1}\)) was added to the plates coated with either TGFβRII-Fc (R&D Systems), α-CTFL (Biochrom), or IgG-TGFβRII (Biochrom) (1 μg ml\(^{-1}\) each), and binding to rhTGFβ1 was detected by a biotinylated anti-human TGFβ antibody (R&D Systems). TGFβRII-Fc coated plates were used as a TGFβ binding positive control (Supplementary Figure 1). To demonstrate that both agents were administered at doses sufficient to saturate systemic TGFβ in vivo, sequestration of serum TGFβ was assessed in mice treated with α-CTFL or IgG-TGFβRII (5 mg kg\(^{-1}\) per week) for 4 weeks (Supplementary Figure 2). At the endpoint, serum was collected from tail bleed and levels of TGFβ were detected using the TGFβ1 Human ELISA Kit (ThermoFisher Scientific) following the manufacturer’s protocol.

The comparative ability of anti-PDL1-TGFβRII and anti-PD-L1 antibody to bind PD-L1 was evaluated by ELISA, wherein biotinylated recombinant human PD-L1 (1 ng/ml; R&D Systems) was added to plates coated with anti-PDL1-TGFβRII or anti-PD-L1 antibody (1 μg ml\(^{-1}\)), followed by detection with HRP-Avidin. Plates coated with nonspecific IgG-TGFβRII served as a negative control to analyze the binding ability of the test samples. The comparative ability of α-PDL1-TGFβRII and α-PD-L1 antibody to bind TGFβ was evaluated by ELISA, wherein rhTGFβ1 (0–2,000 pg ml\(^{-1}\)) was added to plates coated with α-PDL1-TGFβRII or α-PD-L1 antibody (1 μg ml\(^{-1}\)), which was detected by biotinylated anti-human TGFβ antibody (R&D Systems). Plates coated with nonspecific IgG-TGFβRII and rhTGFβRII-Fc served as positive controls to analyze the binding ability of the test samples to TGFβ. The ability of α-PDL1-TGFβRII to simultaneously bind both PD-L1 and TGFβ was evaluated by a bispecific ELISA, wherein α-PDL1-TGFβRII or α-PD-L1 antibody (0–100 ng ml\(^{-1}\)) was added to PD-L1-Fc-coated plates (1 μg ml\(^{-1}\)), followed by rhTGFβ1 (1,000 pg ml\(^{-1}\)) that was detected by a biotinylated anti-human TGFβ antibody (R&D Systems).

TGFβ-induced FOXP3 expression and Treg differentiation. Human PBMCs (ALLCELLS) were stained with anti-CD3 and Glycophorin A to enumerate T cells. Cells were cultured with rhIL-2 (100 IU ml\(^{-1}\), cultured with rhIL-2 (100 IU ml\(^{-1}\) and rhIL-15 (10 ng ml\(^{-1}\)); Dynabeads (Life Technologies) at a ratio of 1:3 (cell : bead) in the presence or absence of 2.5 ng ml\(^{-1}\) rhTGFβ1 with or without either α-CTLA4-TGFβRII or α-
CTLA-4 antibody (5 μg ml⁻¹). Following culture for 24–48 h, cells were lysed and subjected to immunoblot analyses with the following primary antibodies: FOXP3, SMAD-2/3 (D18G7), or phospho-SMAD-2/3, and β-actin (Cell Signaling Technologies). On day 5, anti-CD3/anti-CD28 beads were magnetically removed and the number of Tregs (CD4⁻/CD25high/CD127low/FOXP3⁺) were enumerated by flow cytometry. Cells were stained extracellularly with anti-human CD4-PE, anti-human CD25-APC, and anti-human CD62L-FITC, and analyzed by flow cytometry to quantify T cells with a central memory phenotype (CD45R0⁺CD62L⁺). To evaluate tumor-specific IFN-γ expression in CD3⁺/CD8⁺ T cells, BM cells were plated in 96-well plates (2 x 10⁶ cells per well) in the presence of tumor cell lysate, nonspecific control peptide, or medium alone. Cells were cultured for 72 h followed by incubation with Golgi stop for 4 h. Cells were stained extracellularly with anti-CD3-FITC and anti-CD8-APC antibodies, permeabilized, stained intracellularly with anti-IFN-γ-PE or its corresponding isotype control, and then analyzed by flow cytometry. All the antibodies were from BD Biosciences.

### Analysis of T cell suppressor function

Patient-derived tumor-infiltrated BM (myeloma-BM) was stained with anti-CD3 and Glycophorin A to enumerate T cells. Following activation for 7 days with rhIL-2 and anti-CD3/anti-CD28 beads, the cells were magnetically separated and stained with anti-CD3. Concurrently, autologous Tregs were isolated from the same patient’s peripheral blood lymphocytes using anti-CD4/anti-CD25 beads (Miltenyi Biotechnology). The activated T cells were CFSE labeled (Life Technologies) and added to autologous BM that had been pulsed for 30 min in medium with or without tumor-specific antigen (tumor cell lysate) or nonspecific antigen (nonspecific cell lysate) and plated in the presence or absence of the selected autologous Tregs. Following culture for 3 days with or without either a CTLA-4-TagFIIIR or a CTLA-4-antibody (5 μg ml⁻¹), the cells were stained with anti-CD3 and anti-IFN-γ. Tumor antigen-specific T cells were considered as CD3⁺/CFSFlow/IFNγ⁺.

### Analysis of T,17 cell differentiation

PBMCs were co-incubated with anti-CD3/CD28 beads (1 : 3 cell to bead ratio) in AIM-V medium (Invitrogen) in the presence of IL-17-skewing cytokines (10 ng ml⁻¹ IL-6, 5 ng ml⁻¹ TGFβ, 10 ng ml⁻¹ anti-IL-1Ra and anti-IL-4 (R&D Systems)) with either a CTLA4-TGFβRII or a CTLA-4 antibody (5 μg ml⁻¹). Following incubation for 2 days, cells were collected and stained extracellularly with anti-human CD4 (BD Biosciences), permeabilized (BD Cytofix/Cytoperm Kit), and stained intracellularly with IL-17 and IFN-γ antibodies (eBioscience). The stained cells were washed twice with FACS buffer, run on the Gallios Flow Cytometer, and analyzed utilizing Kaluza Software (Beckman Coulter).

### Tumor cells lines and treatments

A375 and SK-MEL-5 human melanoma cell lines were purchased from ATCC and maintained according to ATCC guidelines. MDA-MB-231 is a metastatic human TNBC cell line with mesenchymal-like morphology (Basal B-like). MDA-MB-231-Luc (DH2L2N) is a TNBC subline with enhanced primary tumor growth and lung metastases that was derived from a metastatic deposit of bioluminescent MDA-MB-231 cells stably expressing firefly luciferase. MDA-MB-231-Luc cells were maintained in Dulbecco’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cell lines were periodically monitored for mycoplasma at Johns Hopkins Genetic Resources Core Facility using the MycoDetect kit (Greiner Bio-One) and authenticated using genetic fingerprinting (Identifiler, Applied Biosystems) before use.

### Treatment of NSG mice reconstituted with human CD34⁺ cells

Female immune-deficient NSG mice (NOD/Shi-scid IL-2rg⁻/⁻)(8–8 week old) were irradiated at 200 cGy, followed by adoptive transfer of human BM CD34⁺ cells (7 x 10⁶ per mouse) from a normal donor (HLA-matched to the tumor) (ALLCELLS). Mice were tested for engraftment of human CD3⁺ T cells in peripheral blood obtained via tail-bleed at 7–8 weeks following injection of CD34⁺ cells. The cells were stained with anti-CD3-APC and anti-CD19-FITC antibodies. Mice demonstrating engraftment of human CD3⁺ cells were inoculated with either human melanoma tumor cells (A375 or SK-MEL-5) (2 x 10⁶ cells subcutaneously), PDOX (subcutaneously), or human TNBC cells (MDA-MB-231-Luc) (2 x 10⁶ in Matrigel; mammary fat pad). At 10d following tumor inoculation, mice were allocated to groups using blinded block randomization and treated (5 mg kg⁻¹ intraperitoneally (i.p.) with either vehicle alone (untreated control) or the following antibodies: a-CTLA4-TGFβRII; a-CTLA-4 (ipiilimumab); nonspecific IgG-TGFβRII (a-CTLA4-TGFβRII; a-TGFβRII (D111); a-PD-1 (pembrolizumab); combination of a-CTLA-4 and IgG-TGFβRII; combination of a-CTLA-4 and a-TGFβRII; combination of a-CTLA-4 and a-TGFβRII; combination of a-CTLA4 and a-PD-1; combination of a-CTLA4-TGFβRII and a-PD-1; a-PD-L1-Fc (ipilimumab) and avelumab (avelumab) and anti-PD-1 + IgG-TGFβRII; and Vehicle alone (Control). Tumor size was measured weekly blinded to the treated group and tumor volume was calculated using the formula (length x width x height).
5. Massagué, J. TGFbeta signalling in context. Nat. Rev. Mol. Cell. Biol. 13, 615–630 (2012).
6. Pickup, M., Novitskyy, S. & Moses, H. L. The roles of TGF[beta] in the tumour microenvironment. Nat. Rev. Cancer 13, 788–799 (2013).
7. Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. & Flavell, R. A. Transforming growth factor-beta regulation of immune responses. Annu. Rev. Immunol. 24, 99–146 (2006).
8. Li, M. O. & Flavell, R. A. TGF-beta: a master of all T cell trades. Nat. Rev. Immunol. 16, 275–300 (2016).
9. Takai, S., Schöm, J., Tucker, I., Tsang, K. Y. & Greiner, J. W. Inhibition of TGFbeta1 signaling promotes central memory T cell differentiation. J. Immunol 191, 2299–2307 (2013).
10. Chen, L. C. Regulatory T cells and treatment of cancer. Nat. Rev. Cancer 13, 441–452 (2013).
11. Curiel, T. J. Regulatory T cells and treatment of cancer. Nat. Rev. Cancer 7, 95–106 (2007).
12. Pardoll, D. M. The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer 12, 252–264 (2012).
13. Topalian, S. L., Drake, C. G. & Pardoll, D. M. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell 27, 450–461 (2015).
14. Liu, V. C. et al. Tumor evasion of the immune system by converting CD4+ T cells into regulatory T cells. Nat. Rev. Immunol. 12, 307–318 (2012).
15. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T cells and immune tolerance. Cell 133, 775–787 (2008).
16. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T cells and immune tolerance. Cell 133, 775–787 (2008).
17. Liu, Z., Kim, J. H., Falo, L. D. Jr. & You, Z. Tumor regulatory T cells potently abrogate antitumor immunity. J. Immunol. 182, 6160–6167 (2009).
18. Chen, M. L. et al. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. Proc. Natl Acad. Sci. USA 102, 419–424 (2005).
19. Tone, Y. et al. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat. Immunol. 9, 194–202 (2008).
20. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. 4, 330–336 (2003).
21. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. Science 299, 1057–1061 (2003).
22. von Boehmer, H. Mechanisms of suppression by suppressor T cells. Nat. Rev. Immunol. 6, 334–344 (2006).
23. Shevach, E. M. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity 30, 636–645 (2009).
24. Zheng, Y. et al. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 445, 936–940 (2007).
25. Wing, K. et al. CTLA-4 control over Foxp3 regulatory T cell function. Science 320, 271–275 (2008).
26. Oida, T., Xu, L., Weiner, H. L., Kitani, A. & Strober, W. TGF-beta-mediated suppression by CD4+CD25+ T cells is facilitated by CTLA-4 signaling. J. Immunol. 177, 2331–2339 (2006).
27. Tang, Q. et al. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. Eur. J. Immunol. 34, 2996–3005 (2004).
28. Zhu, C. et al. The Tim-3 ligand galectin 9 negatively regulates T helper type 1 immunity. Nat. Immunol. 6, 1245–1252 (2005).
29. Wu, C. et al. Galectin-9/Tim-3 interaction enhances stability and function of adaptive regulatory T cells. Immunity 41, 270–282 (2014).
30. Fridman, W. H., Pages, F., Sautès-Fridman, C. & Galon, J. The immunomodulatory role of immune cells in tumour inflammation: implications for cancer therapy. Nat. Rev. Cancer 12, 298–306 (2012).
31. Dranoff, G. The therapeutic implications of intratumoral regulatory T cells. Clin. Cancer Res. 11, 8226–8229 (2005).
32. Yamaguchi, T. & Sakaguchi, S. Regulatory T cells in immune surveillance and treatment of cancer. Semin. Cancer Biol. 16, 115–123 (2006).
33. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. Nat. Rev. Cancer 12, 295–306 (2012).
34. Curiel, T. J. Regulatory T cells and treatment of cancer. Curr. Opin. Immunol. 20, 241–246 (2008).
35. Tosolini, M. et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer. Cancer Res. 71, 1263–1271 (2011).
36. Chen, L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat. Rev. Immunol. 4, 336–347 (2004).
Acknowledgements
This work was supported by NIH grants R01 CA184199 and SPORE P50 DE019032, and a Maryland Innovation Initiative (MII) award from the Maryland Technology Development Corporation (TEDCO) to Atul Bedi and Rajani Ravi.

Author contributions
A.B., R.R., K.N., E.I., I.B., and D.S. planned and designed the work. A.B., R.R., R.B., and V.P. performed analysis and interpretation of data. A.B., R.R., K.N., I.B., D.S., E.I., A.Z., I.O., A.A., E.M., S.N., R.M., P.W., and V.P. contributed to the development of methodology. R.R., K.N., R.B., E.I., A.Z., I.O., A.A., E.M., S.N., R.M., P.W., and V.P. contributed to the manuscript preparation. A.B. and R.R. conceived and supervised the project.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-017-02696-6.

Competing interests: A.B. and R.R. are inventors on patents covering antibody–ligand traps assigned to Johns Hopkins University (United States Patent Number 8,993,524 and United States Patent Number 9,850,306; European Patent Number EP2542590; Japanese Patent Number 6066732; PCT/US2011/027317; WO2011/109789) and licensed to Y-Trap Inc. A.B., R.R., E.L., I.B., and K.N. are founders and stockholders of Y-Trap Inc. The terms of this arrangement are managed by Johns Hopkins University in accordance with its conflict of interest policies. The remaining authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.