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### Authors

Seed, Robert I  
Kobayashi, Kenji  
Ito, Saburo  
[et al.](#)

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## A tumor-specific mechanism of T<sub>reg</sub> enrichment mediated by the integrin $\alpha\nu\beta 8$

Robert I. Seed<sup>1,\*</sup>, Kenji Kobayashi<sup>1,\*</sup>, Saburo Ito<sup>1</sup>, Naoki Takasaka<sup>1</sup>, Anthony Cormier<sup>1</sup>, Jillian M. Jespersen<sup>2</sup>, Jean Publicover<sup>2</sup>, Suprita Trilok<sup>2</sup>, Alexis J. Combes<sup>1,3,4</sup>, Nayvin W. Chew<sup>1,3,4</sup>, Jocelyne Chapman<sup>5</sup>, Matthew F. Krummel<sup>1,3</sup>, Jianlong Lou<sup>6</sup>, James Marks<sup>6</sup>, Yifan Cheng<sup>7,8</sup>, Jody L. Baron<sup>2,3</sup>, Stephen L. Nishimura<sup>1,3,†</sup>

<sup>1</sup>Department of Pathology, University of California, San Francisco, San Francisco, CA 94110, USA.

<sup>2</sup>Department of Medicine and Liver Center, University of California, San Francisco, San Francisco, CA 94143, USA.

<sup>3</sup>ImmunoX Initiative, University of California, San Francisco, San Francisco, CA 94143, USA.

<sup>4</sup>ImmunoX CoLabs, University of California San Francisco, San Francisco, CA 94143, USA.

<sup>5</sup>Department of Gynecology and Oncology, University of California, San Francisco San Francisco, CA 94110, USA.

<sup>6</sup>Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, CA 94110, USA.

<sup>7</sup>Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94158, USA.

<sup>8</sup>Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA 94158, USA.

<sup>†</sup>Corresponding author. [stephen.nishimura@ucsf.edu](mailto:stephen.nishimura@ucsf.edu).

\*These authors contributed equally to this work.

**Author contributions:** R.I.S., K.K., and S.L.N. established and developed the methodology and performed and analyzed in vivo and gene analysis experiments. A.C., A.J.C., S.I., J.L., J.M., and S.L.N. participated in antibody development, production, and characterization. Y.C., A.C., and S.I. participated in structural modeling. K.K., S.I., N.T., and S.L.N. participated in immunohistochemistry staining and analysis. R.I.S. and S.L.N. conceived and designed ex vivo lymphocyte cell culture assays. R.I.S., S.I., S.T., J.P., J.M.J., A.C., N.W.C., M.F.K., S.L.N., and J.L.B. performed flow cytometry experiments, RNAseq, and analysis. J.C. and S.L.N. performed human sample collection and processing. S.L.N. conceived, designed, and oversaw all experimentation. S.L.N., J.L.B., and R.I.S. wrote the manuscript. A.C. and N.W.C. provided biostatistical support.

### SUPPLEMENTARY MATERIALS

[immunology.sciencemag.org/cgi/content/full/6/57/eabf0558/DC1](https://immunology.sciencemag.org/cgi/content/full/6/57/eabf0558/DC1)

#### Materials and Methods

Fig. S1.  $\beta 8$  expression by tumor cells drives tumor growth, which is blocked by C6D4 Fab in vivo.

Fig. S2. Tumor T<sub>reg</sub> isolation and expression of select T<sub>reg</sub> genes by RNAseq in this report compared with single-cell RNAseq from splenic T<sub>reg</sub>.

Fig. S3. Non-T<sub>reg</sub> CD4<sup>+</sup> T cells express cell surface L-TGF- $\beta$ , are converted to T<sub>reg</sub> by contact with  $\beta 8$ -LLC cells, and are the source of suppressive iT<sub>reg</sub> generated on  $\alpha\nu\beta 8$ .

Fig. S4. Murine nT<sub>regs</sub> do not express detectable levels of cell-surface  $\alpha\nu\beta 8$ .

Fig. S5. *LRRC32* (*GARP*) is most highly expressed by T<sub>reg</sub> and stromal cells and *NRROS* by myeloid cells.

Table S1. Raw data table (Excel spreadsheet).

References (67–73)

[View/request a protocol for this paper from Bio-protocol.](#)

## Abstract

Regulatory T cells ( $T_{\text{regs}}$ ) that promote tumor immune evasion are enriched in certain tumors and correlate with poor prognosis. However, mechanisms for  $T_{\text{reg}}$  enrichment remain incompletely understood. We described a mechanism for  $T_{\text{reg}}$  enrichment in mouse and human tumors mediated by the  $\alpha\text{v}\beta 8$  integrin. Tumor cell  $\alpha\text{v}\beta 8$  bound to latent transforming growth factor- $\beta$  (L-TGF- $\beta$ ) presented on the surface of T cells, resulting in TGF- $\beta$  activation and immunosuppressive  $T_{\text{reg}}$  differentiation in vitro. In vivo, tumor cell  $\alpha\text{v}\beta 8$  expression correlated with  $T_{\text{reg}}$  enrichment, immunosuppressive  $T_{\text{reg}}$  gene expression, and increased tumor growth, which was reduced in mice by  $\alpha\text{v}\beta 8$  inhibition or  $T_{\text{reg}}$  depletion. Structural modeling and cell-based studies suggested a highly geometrically constrained complex forming between  $\alpha\text{v}\beta 8$ -expressing tumor cells and L-TGF- $\beta$ -expressing T cells, facilitating TGF- $\beta$  activation, independent of release and diffusion, and providing limited access to TGF- $\beta$  inhibitors. These findings suggest a highly localized tumor-specific mechanism for  $T_{\text{reg}}$  enrichment.

## INTRODUCTION

Regulatory T cells ( $T_{\text{regs}}$ ) are enriched in subsets of cancers and associated with poor clinical prognoses (1–3).  $T_{\text{regs}}$  suppress antitumor immune responses, with  $T_{\text{reg}}$  depletion promoting effector  $\text{CD}8^+$  T cell immunity (4–6). Tumor-specific  $T_{\text{reg}}$  enrichment likely contributes to resistance to current immunotherapies (7). How  $T_{\text{reg}}$  enrichment occurs in tumors is not well understood, and a better understanding of this may improve antitumor therapies.

$T_{\text{regs}}$  are induced by self-antigens in the thymus ( $tT_{\text{regs}}$ ) or foreign antigens in extrathymic peripheral tissues ( $pT_{\text{regs}}$ ) (8).  $tT_{\text{regs}}$  are recruited to tumors through chemokines specific to individual tumor types, whereas  $pT_{\text{regs}}$  are generated within tumors in response to signals generated within the tumor microenvironment (TME) (9, 10). The cytokine transforming growth factor- $\beta$  (TGF- $\beta$ ) may contribute to  $pT_{\text{reg}}$  enrichment in tumors (11). TGF- $\beta$  is critical for  $pT_{\text{reg}}$  generation because of its essential role in forkhead box P3 (FOXP3) expression during  $T_{\text{reg}}$  differentiation; the role of TGF- $\beta$  in  $tT_{\text{reg}}$  generation is less clear (9, 12). Elucidating the role of TGF- $\beta$  in  $pT_{\text{reg}}$  enrichment in tumors could help identify therapies specifically targeting the immunosuppressive effects of TGF- $\beta$  while minimizing toxicities associated with systemic inhibition of TGF- $\beta$  itself, TGF- $\beta$  receptors (TGF- $\beta$ Rs), or  $T_{\text{reg}}$  depletion (6, 13–15).

TGF- $\beta$  and its receptors are widely expressed in the TME (16, 17). TGF- $\beta$  is always expressed in an inactive form within a latent complex, latent TGF- $\beta$  (L-TGF- $\beta$ ), formed by noncovalent association of TGF- $\beta$  with its prodomain, latency-associated protein (LAP) (18). On  $T_{\text{regs}}$ , TGF- $\beta$  is presented at the cell surface through association with the scaffolding molecule glycoprotein A repetitions pre-dominant (GARP) (19). Within L-TGF- $\beta$ , mature TGF- $\beta$  cannot interact with its receptors unless undergoing “activation,” functionally defined as the acquired ability to initiate signaling through TGF- $\beta$ Rs.

TGF- $\beta$  is cleaved intracellularly by furin from LAP, which has led to the widespread assumption that mature TGF- $\beta$  must be physically released and diffused from the L-TGF- $\beta$

complex for activation and induction of signaling (20). However, why L-TGF- $\beta$  needs to be presented on the cell surface by GARP if TGF- $\beta$  diffusion gradients account for T<sub>reg</sub> enrichment in tumors is unclear. L-TGF- $\beta$  is present on the cell surface of CD4<sup>+</sup> T cells isolated from murine tumors (21), suggesting an alternative model where local regulation of TGF- $\beta$  activation only on specific L-TGF- $\beta$ -presenting CD4<sup>+</sup> T cells induces conversion to pT<sub>regs</sub>.

The integrin  $\alpha$ v $\beta$ 8 may be an important mediator of tumor-specific regulation of TGF- $\beta$  function (21).  $\alpha$ v $\beta$ 8 is highly expressed in multiple cancer types that have high numbers of T<sub>regs</sub> (6, 21, 22). The LAPs of L-TGF- $\beta$ 1 and L-TGF- $\beta$ 3 contain arginine-glycine-aspartate integrin-binding sites, which are recognized by several integrins, particularly  $\alpha$ v $\beta$ 8, which is critical for TGF- $\beta$  activation in immune cell function (23–26). L-TGF- $\beta$  is the only physiologically relevant ligand for  $\alpha$ v $\beta$ 8, and thus, targeting  $\alpha$ v $\beta$ 8 selectively inhibits TGF- $\beta$  function (27, 28). Anti- $\alpha$ v $\beta$ 8 inhibits tumor growth of  $\beta$ 8-expressing tumors, correlating with increased immune cell numbers and reversal of effector T cell exclusion; combination with anti-programmed cell death protein 1 (PD-1) improves these antitumor effects (21).

A structure-based model predicts that  $\alpha$ v $\beta$ 8 most efficiently activates L-TGF- $\beta$  when  $\alpha$ v $\beta$ 8 expressed by one cell binds to cell-surface L-TGF- $\beta$  presented by another. Within the cell-cell  $\alpha$ v $\beta$ 8/L-TGF- $\beta$  complex, active TGF- $\beta$  exclusively signals to the L-TGF- $\beta$ -presenting cell because active TGF- $\beta$  is not released and does not diffuse from the complex (29). Here, we found that a  $\alpha$ v $\beta$ 8/L-TGF- $\beta$  complex formed between  $\alpha$ v $\beta$ 8-expressing tumor cells and L-TGF- $\beta$ -presenting T cells was associated with T<sub>reg</sub> enrichment in tumors. The  $\alpha$ v $\beta$ 8/L-TGF- $\beta$  complex limited access to TGF- $\beta$  inhibitors that would have otherwise been free to bind if active TGF- $\beta$  was diffusible. These findings modify the conceptual framework for understanding how TGF- $\beta$  functions in immune differentiation and affects therapeutic approaches to effectively and selectively inhibit it.

## RESULTS

### T<sub>regs</sub> contributed to $\beta$ 8-dependent tumor growth

We investigated whether T<sub>regs</sub> were required for protumorigenic effects of tumor cell  $\alpha$ v $\beta$ 8 using the syngeneic orthotopic  $\beta$ 8-Lewis lung carcinoma (LLC) model (Fig. 1, A and B) (21).  $\beta$ 8-LLC tumors had significantly more T<sub>regs</sub> than non- $\beta$ 8-expressing mock-LLC tumors (Fig. 1, C and D). We depleted CD25<sup>+</sup> T cells with intraperitoneal injection of anti-CD25 (clone PC-61.5.3) 1 day after tumor implantation (30). PC-61.5.3 and anti- $\alpha$ v $\beta$ 8 (clone C6D4) both reduced FOXP3<sup>+</sup> cells from  $\beta$ 8-LLC tumors (Fig. 1, C and D). Neither antibody significantly affected FOXP3<sup>+</sup> cell numbers from mock-LLC tumors implanted on the contralateral side within the time frame in which euthanasia was required because of the size of the primary  $\beta$ 8-LLC tumor (Fig. 1, C and D).

$\beta$ 8-LLC cells formed tumors significantly faster than mock-LLC tumors grown on opposite flanks, which was reduced by PC-61.5.3 or C6D4 (Fig. 1, E to M). PC-61.5.3 or C6D4 did not affect mock-LLC tumor volume or mass at day 14 after tumor cell injection (Fig. 1, E and G), consistent with the inability of PC-61.5.3 or C6D4 to reduce FOXP3<sup>+</sup> cells from mock-LLC tumors (Fig. 1, C and D). We confirmed the protumorigenic effects of tumor

cell  $\alpha\text{v}\beta\text{8}$  in naturally  $\beta\text{8}$ -expressing TRAMP-C2 murine prostate carcinoma cells using a knockdown approach with *ITGB8* short hairpin RNA (shRNA) (fig. S1, A to H).

The mechanism of action of C6D4 could be from functional blockade of  $\alpha\text{v}\beta\text{8}$ -mediated TGF- $\beta$  activation or effector function (i.e., antibody-dependent cellular cytotoxicity). To test the role of antibody effector function, we performed  $\beta\text{8}$ -LLC tumorigenicity assays using a Fab of C6D4 lacking the Fc region required for effector function (31). C6D4-Fab blocked  $\beta\text{8}$ -LLC tumor growth, similar to intact C6D4, compared with isotype control (fig. S1, I to L). These results demonstrated that C6D4 functions in the TME by blocking  $\alpha\text{v}\beta\text{8}$  function, not by antibody effector function.

### Tumor cell $\alpha\text{v}\beta\text{8}$ expression caused $T_{\text{reg}}$ enrichment and an immunosuppressive $T_{\text{reg}}$ transcriptome

PC-61.5.3 and C6D4 specifically reduced  $T_{\text{reg}}$  numbers and growth of  $\beta\text{8}$ -LLC but not mock-LLC tumors, suggesting that  $T_{\text{regs}}$  in  $\beta\text{8}$ -LLC tumors were distinct from  $T_{\text{regs}}$  in mock-LLC tumors. We hypothesized that  $\alpha\text{v}\beta\text{8}$ -expressing tumor cells participated in the local conversion of  $\text{CD}25^+\text{FOXP}3^-$  T cells to  $\text{FOXP}3^+$   $pT_{\text{reg}}$ . The absence of an abscopal effect of  $T_{\text{regs}}$  generated in  $\beta\text{8}$ -LLC on contralateral mock-LLC tumor growth suggests that  $T_{\text{regs}}$  generated within  $\alpha\text{v}\beta\text{8}$ -expressing tumors are either retained or are phenotypically unstable outside the  $\alpha\text{v}\beta\text{8}$ -expressing TME because  $pT_{\text{reg}}$  may be less stable compared with  $tT_{\text{reg}}$  (32).

To test whether  $\alpha\text{v}\beta\text{8}$ -expressing tumor cells locally mediated conversion of  $\text{CD}25^+\text{FOXP}3^-$  T cells to  $pT_{\text{regs}}$ , we determined the transcriptome of  $T_{\text{regs}}$  generated in  $\beta\text{8}$ -LLC compared with mock-LLC tumors.  $\beta\text{8}$ -LLC tumors had more  $T_{\text{regs}}$  than mock-LLC tumors, and anti- $\beta\text{8}$  reduced  $T_{\text{reg}}$  numbers in  $\beta\text{8}$ -LLC tumors, suggesting that tumor cell expression of  $\alpha\text{v}\beta\text{8}$  correlated with  $\text{CD}4^+\text{CD}25^+\text{FOXP}3^+$   $T_{\text{reg}}$  enrichment (Fig. 2, B and C). We next performed RNA sequencing (RNAseq), evaluating the transcriptome of sorted  $T_{\text{reg}}$  pools from mock-LLC or  $\beta\text{8}$ -LLC tumors from mice treated with isotype (SV5) or C6D4 (Fig. 2, A, D, and E, and fig. S2A). Similarly high read counts for *FoxP3* and *Ii2ra* (CD25) were seen in all  $T_{\text{reg}}$  pools, consistent with high  $T_{\text{reg}}$  purity (fig. S2B). Filtering of the dataset revealed 118 genes most highly and variably expressed across groups (Fig. 2, D and E).

$T_{\text{regs}}$  from  $\beta\text{8}$ -LLC tumors had distinct transcriptional profiles that could be blocked by C6D4 to resemble  $T_{\text{regs}}$  from mock-LLC tumors. Comparison of  $T_{\text{regs}}$  from C6D4-treated  $\beta\text{8}$ -LLC tumors with  $T_{\text{regs}}$  from isotype-treated  $\beta\text{8}$ -LLC tumors revealed 116 of the 118 most variably expressed genes changed in the same direction ( $R = 0.908$ ,  $P < 0.0001$ ; Fig. 2D). Therefore, systemic blockade of  $\alpha\text{v}\beta\text{8}$  with C6D4 had the same effect on the  $T_{\text{reg}}$  transcriptome as absence of  $\beta\text{8}$  expression by tumor cells, demonstrating the importance of tumor cell  $\alpha\text{v}\beta\text{8}$  to locally control  $T_{\text{reg}}$  gene expression. Hierarchical clustering of this gene set revealed tumor cell  $\beta\text{8}$ -dependent increases in expression of genes associated with  $T_{\text{reg}}$  immunosuppressive function and differentiation (i.e., *Ii10*, *Ctla4*, and *Icos*; Fig. 2E) (6, 33).  $T_{\text{regs}}$  from  $\beta\text{8}$ -LLC tumors treated with isotype clustered separately from  $T_{\text{regs}}$  from C6D4-treated  $\beta\text{8}$ -LLC tumors or mock-LLC tumors (Fig. 2E). In contrast,  $T_{\text{regs}}$  from mock or  $\beta\text{8}$ -LLC tumors treated with C6D4 clustered together and were indistinguishable

(Fig. 2E). These data are consistent with the idea that tumor cell  $\alpha\text{v}\beta 8$  contributed to the enrichment of a  $T_{\text{reg}}$  population distinct from those infiltrating mock-LLC tumors.

The mechanisms underlying  $T_{\text{reg}}$  enrichment in tumors were likely to involve recruitment of  $T_{\text{regs}}$  and local  $T_{\text{reg}}$  conversion (2, 34). Recruited  $T_{\text{regs}}$  have increased representation of  $tT_{\text{regs}}$ , which potentially allows them to be distinguished from  $pT_{\text{regs}}$  by high expression of two markers, Helios (*Ikzf2*) and neuropilin 1 (*Nrp1*) (9, 34–38). However, both  $\beta 8$ -LLC and mock-LLC tumor  $T_{\text{regs}}$  had low or un-detectable levels of *Ikzf2* and *Nrp1* in contrast to splenic  $T_{\text{regs}}$ , which consist of about 70%  $tT_{\text{regs}}$  and had high levels of these markers (fig. S2F) (36). Together, these data indicated that  $T_{\text{regs}}$  in  $\beta 8$ -LLC tumors were skewed toward  $pT_{\text{regs}}$  rather than  $tT_{\text{regs}}$ .

### L–TGF- $\beta$ was expressed on the surface of $T_{\text{reg}}$ and non- $T_{\text{reg}}$ CD4<sup>+</sup> T cells

We next determined whether non- $T_{\text{reg}}$  CD4<sup>+</sup> T cells expressed L–TGF- $\beta$  on their cell surface and were therefore “primed” for  $T_{\text{reg}}$  conversion. There is cell surface expression of L–TGF- $\beta$  on  $T_{\text{regs}}$  and activated non- $T_{\text{reg}}$  CD4<sup>+</sup> T cells; however, no function has been attributed to this expression (19, 39, 40). Activated non- $T_{\text{reg}}$  CD4<sup>+</sup> T cells [CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>–</sup> T cells from spleens of *FoxP3*-IRES-GFP mice (41)] expressed increased cell surface L–TGF- $\beta$  compared with nonactivated CD4<sup>+</sup> T cells (Fig. 3, A, B, and D, and fig. S3, A to H). Consistent with other reports, there were more L–TGF- $\beta$ <sup>+</sup> cells in the CD25<sup>+</sup>FOXP3<sup>+</sup>GFP<sup>+</sup> T cell population (~8 to 22%) (Fig. 3, C and D). Thus, significant fractions of both non- $T_{\text{reg}}$  CD4<sup>+</sup> T cells and  $T_{\text{regs}}$  express cell surface L–TGF- $\beta$ . The physiological relevance of these findings is highlighted by the detection of L–TGF- $\beta$  on the surface of both non- $T_{\text{reg}}$  CD4<sup>+</sup> T cells and  $T_{\text{regs}}$  isolated from  $\beta 8$ -LLC tumors (21).

### Tumoral $\alpha\text{v}\beta 8$ directly drove $T_{\text{reg}}$ differentiation in vitro

We next tested whether binding of L–TGF- $\beta$  on the surface of non- $T_{\text{reg}}$  CD4<sup>+</sup> T cells to  $\alpha\text{v}\beta 8$  expressed on an opposing cell surface was sufficient to drive induced  $T_{\text{reg}}$  ( $iT_{\text{reg}}$ ) conversion in vitro because  $iT_{\text{regs}}$  approximate many qualities of  $pT_{\text{regs}}$  generated in vivo (8). In this simplified system,  $iT_{\text{reg}}$  differentiation is mediated by binding to immobilized integrin  $\alpha\text{v}\beta 8$  independent of paracrine factors secreted by tumor cells, which could be influenced by  $\alpha\text{v}\beta 8$  ligand binding. The  $\alpha\text{v}\beta 8$  ectodomain is also free of cytoskeletal interactions, which modulate integrin conformational changes involved in force transduction and are important for TGF- $\beta$  activation by the closely related integrin  $\alpha\text{v}\beta 6$  but not  $\alpha\text{v}\beta 8$  (23, 29, 42, 43). Culturing activated non- $T_{\text{reg}}$  CD4<sup>+</sup> T cells on immobilized  $\alpha\text{v}\beta 8$  (Fig. 3, E to G), but not  $\alpha\text{v}\beta 3$  [which does not mediate activation of TGF- $\beta$  (Fig. 3, I to K) (29)], resulted in conversion to  $iT_{\text{regs}}$  as determined by increased FOXP3 expression and acquisition of suppressor function (fig. S3, I to L).  $T_{\text{reg}}$  conversion induced by  $\alpha\text{v}\beta 8$  was efficient (~60%) because it was similar to induction by a supraphysiologic concentration of recombinant TGF- $\beta$  (rTGF- $\beta$ ; Fig. 3, F and O). Effects of  $\alpha\text{v}\beta 8$  on  $iT_{\text{reg}}$  differentiation were not due to ligation of L–TGF- $\beta$ /GARP on activated T cells, or cell attachment, because no increase in  $iT_{\text{reg}}$  differentiation was seen when activated T cells expressing L–TGF- $\beta$  were plated on wells coated with anti-LAP (fig. S3, M and N). Anti- $\beta 8$  efficiently inhibited effects of immobilized  $\alpha\text{v}\beta 8$  on  $iT_{\text{reg}}$  differentiation (Fig. 3, G and H).  $\alpha\text{v}\beta 8$ -mediated conversion to  $iT_{\text{reg}}$  depended on TGF- $\beta$ , as demonstrated by blockade (~50%) with high

concentrations of a pan-TGF- $\beta$  isoform antibody, 1D11 (fig. S3, V and W). The source of the active TGF- $\beta$  mediating non-T<sub>reg</sub> CD4<sup>+</sup> T cell conversion to T<sub>reg</sub> on immobilized  $\alpha$ v $\beta$ 8 was not from secreted L-TGF- $\beta$  in the media or from stimulated non-T<sub>reg</sub> CD4<sup>+</sup> T cells (Fig. 3, Q and R). Last,  $\alpha$ v $\beta$ 8-mediated conversion to iT<sub>reg</sub> required contact of non-T<sub>reg</sub> CD4<sup>+</sup> T cells with  $\alpha$ v $\beta$ 8 (Fig. 3, S and T).

The immunosuppressive T<sub>reg</sub> phenotype induced by immobilized  $\alpha$ v $\beta$ 8 was confirmed by significant  $\beta$ 8-dependent induction of the immune checkpoint inhibitor *Ctla4* (Fig. 3L). *Ikzf2* and *nrp1* were barely or not detected, indicating that in vitro generated iT<sub>regs</sub> were similar to the pT<sub>reg</sub> phenotype seen in T<sub>regs</sub> isolated from murine LLC tumors (Figs. 2 and 3L). In addition, low levels of *Ikzf2* and *nrp1* in  $\alpha$ v $\beta$ 8-generated iT<sub>regs</sub> suggested that their origin was from non-T<sub>reg</sub> CD4<sup>+</sup> T cells, not from the small (~1%) population of CD25<sup>+</sup>CD4<sup>+</sup>FOXP3<sup>+</sup> T cells mostly consisting of tT<sub>reg</sub> (36). To directly address whether the iT<sub>reg</sub> in our system originated from the non-T<sub>reg</sub> CD4<sup>+</sup> T cell population, we sorted CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>-</sup> T cells, which were cultured on immobilized  $\alpha$ v $\beta$ 8 or  $\alpha$ v $\beta$ 3 as a control. Robust  $\alpha$ v $\beta$ 8-mediated conversion of CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>-</sup> to CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>+</sup> T cells was observed, demonstrating that the origin of iT<sub>reg</sub> in our system was CD4<sup>+</sup>CD25<sup>+</sup> GFP<sup>-</sup> T cells and not expansion of the small population of CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>+</sup> T cells (fig. S3, Q to W). In contrast, sorted CD4<sup>+</sup>CD25<sup>+</sup>GFP<sup>+</sup> T cells from CD4<sup>+</sup> splenocytes displayed minimal expansion when cultured on immobilized integrins or in response to rTGF- $\beta$  (fig. S3, R to T). These results indicated that iT<sub>regs</sub> in our system originated from non-T<sub>reg</sub> CD4<sup>+</sup> T cell populations rather than from expansion of existing T<sub>regs</sub>.

To reproduce these results with  $\alpha$ v $\beta$ 8-expressing tumor cells, we cocultured activated non-T<sub>reg</sub> CD4<sup>+</sup> T cells with  $\beta$ 8-LLC cells and measured iT<sub>reg</sub> conversion. Coculture with  $\beta$ 8-LLC significantly increased iT<sub>reg</sub> generation relative to mock-LLC cells (Fig. 3P and fig. S3, O and P). About 15 to 20% of non-T<sub>reg</sub> CD4<sup>+</sup> T cells were converted to iT<sub>regs</sub> in the presence of  $\beta$ 8-LLC cells (Fig. 3P), similar to the efficiency of conversion to iT<sub>reg</sub> on wells coated with  $\alpha$ v $\beta$ 8 at the same receptor density as  $\beta$ 8-LLC cells (Fig. 5E). These results suggest that  $\alpha$ v $\beta$ 8 binding to L-TGF- $\beta$ -presenting T cells, without any integrin cytoskeletal-mediated force transduction, was sufficient to mediate TGF- $\beta$  activation and conversion of non-T<sub>reg</sub> CD4<sup>+</sup> T cells to iT<sub>reg</sub>, consistent with the integrin force-independent mechanism of TGF- $\beta$  activation (29).

### **$\alpha$ v $\beta$ 8 expression by nontumor cells was not essential for conversion of T cells to T<sub>reg</sub>**

We were unable to detect cell-surface  $\alpha$ v $\beta$ 8 on T<sub>regs</sub> or conventional T cells (T<sub>convs</sub>) (fig. S4). These results mirror previous studies showing lack of  $\alpha$ v $\beta$ 8 expression on mouse CD4<sup>+</sup> and CD8<sup>+</sup> T cells, T<sub>regs</sub>, macrophages, and dendritic cells (21, 29). However, it remains possible that levels of cell-surface  $\alpha$ v $\beta$ 8 on T cells or T<sub>regs</sub> below the level of detection by cell surface staining are sufficient for T<sub>reg</sub> generation and function (44, 45). However, we could find no evidence of significant *itgb8* gene or functional  $\alpha$ v $\beta$ 8 surface expression by T cells or T<sub>regs</sub> in T<sub>reg</sub> generation, function, or immunosuppressive gene expression either in vitro or in vivo [Figs. 1 (C to E and G to I), 2 (C to E), and 3 (I to K and H) and fig. S2 (D and G)].

### ***ITGB8* was most highly expressed by tumor cells**

We next sought to translate our  $\alpha\text{v}\beta\text{8}$  cell-type expression data to the human TME by measuring the relative expression of *ITGB8* by various cell types in different human tumors. We performed bulk RNAseq of sorted immune (T cell,  $T_{\text{reg}}$ , or myeloid) and nonimmune (stromal or tumor) cells from human lung, gynecologic, and head and neck carcinomas (Fig. 4 and fig. S5, A to E). *ITGB8* was most highly and significantly expressed in tumor cells compared with immune cell populations in all three tumor types. *ITGB8* was also significantly expressed by stromal cells, albeit generally at a lower level than tumor cells. Rare samples with signal above background were seen in immune cells, likely representing technical noise, because such data points were identified as statistical outliers (Fig. 4, B to D). Accordingly, no significant expression of *ITGB8* was found in  $T_{\text{regs}}$  or other immune cell types known not to express *ITGB8*, such as  $\text{CD4}^+$  T cells and myeloid cells (Fig. 4, B to D) (21). Together, our mouse and human findings support our conclusion that tumor cells are a functionally important site for  $\alpha\text{v}\beta\text{8}$  expression in the TME.

### **L–TGF- $\beta$ /GARP was most highly expressed by immune cells**

Abundant evidence suggests that TGF- $\beta$  expression by T cells, not tumor cells, is important to maintain an immunosuppressive TME (46–48). T cell surface localization of TGF- $\beta$  may also be important in the TME because conditional deletion of *GARP* in  $T_{\text{reg}}$  inhibits growth of colitis-induced colon tumors (49). However, mechanisms of cell surface presentation of TGF- $\beta$  in the TME have not been comprehensively studied. Thus, we next sought to determine which cells in the TME expressed TGF- $\beta$ , *GARP*, or its functional homolog negative regulator of reactive oxygen species (*NRROS* or *LRRC33*) in the human TME.

*TGFB1* was most highly and significantly expressed in  $\text{CD4}^+$  T cells, with levels decreasing in rank order of  $T_{\text{regs}}$ , myeloid cells, and stromal and tumor cells (Fig. 4, E to G). *GARP* and *NRROS* were both expressed above background in different cell types;  $T_{\text{regs}}$  and stromal cells significantly expressed GARP, whereas myeloid,  $T_{\text{reg}}$ , and T cells significantly expressed *NRROS* (fig. S5). These data suggested multiple mechanisms for cell surface presentation of L–TGF- $\beta$ ;  $T_{\text{regs}}$  used both *GARP* and *NRROS*. Stromal cells used only GARP. Myeloid and T cells used *NRROS*, and tumor cells used neither (fig. S5). Our findings were consistent with previous identification of cell surface L–TGF- $\beta$  on murine T cells,  $T_{\text{reg}}$ , and myeloid cells but not tumor cells from orthotopic tumors (21). Together, our data supported that  $\text{CD4}^+$  T cells were a major source of cell surface L–TGF- $\beta$  in the TME of human tumors.

### **Formation of a localized tumor/T cell $\alpha\text{v}\beta\text{8}$ /L–TGF- $\beta$ signaling complex**

We next sought to test the physiological relevance of our structure-based model of TGF- $\beta$  activation (Fig. 5A) (29) by asking whether  $\alpha\text{v}\beta\text{8}$  expressed by tumor cell lines was sufficient to support TGF- $\beta$  activation without release and diffusion of TGF- $\beta$  from a cell-cell L–TGF- $\beta$ /GARP complex. The respective  $\alpha\text{v}\beta\text{8}$  cell surface receptor densities of  $\beta\text{8-LLC}$  (Fig. 5B), TRAMP-C2 (Fig. 5C), or human ovarian carcinoma (OVCAR-3; Fig. 5D) tumor cell lines were sufficient to efficiently support non- $T_{\text{reg}}$   $\text{CD4}^+$  T cell-to- $T_{\text{reg}}$  conversion (Fig. 5E). Accordingly, these  $\beta\text{8}$ -expressing lines efficiently increased TGF- $\beta$  reporter activity in L–TGF- $\beta$ /GARP– or L–TGF- $\beta$  (R249A)/GARP–expressing transformed



mink lung cell (TMLC) reporter cell lines, with respective TGF- $\beta$  activation efficiencies, correlating to  $\alpha\text{v}\beta 8$  surface receptor density (Fig. 5, E to H). These data demonstrated that  $\alpha\text{v}\beta 8$  expressed by tumor cells induced TGF- $\beta$  signaling in L-TGF- $\beta$ -presenting cells that they were in contact with.

### Therapeutic implications of the tumor: T cell $\alpha\text{v}\beta 8$ /L-TGF- $\beta$ complex

We developed a structural model of the  $\alpha\text{v}\beta 8$ /L-TGF- $\beta$ /GARP/TGF- $\beta$ R2 signaling complex (29), which predicted numerous geometric constraints affecting binding of TGF- $\beta$  protein inhibitors that have been developed to target freely diffusible mature TGF- $\beta$  (Fig. 5A). Such inhibitors include antibodies to TGF- $\beta$ , LAP, or GARP/L-TGF- $\beta$ , as well as TGF- $\beta$ R traps (29, 50–52). In contrast, the C6D4 antibody targets the  $\alpha\text{v}\beta 8$  ligand binding pocket and therefore would be predicted to efficiently prevent L-TGF- $\beta$  binding to  $\alpha\text{v}\beta 8$  (29).

We used our TGF- $\beta$  activation system to assess the relative ability of these inhibitors to block TGF- $\beta$  activation. As predicted, anti- $\beta 8$  (C6D4) efficiently inhibited TGF- $\beta$  activation in wild-type (WT) L-TGF- $\beta$ /GARP or L-TGF- $\beta$  (R249A)/GARP reporter cells when plated on immobilized  $\alpha\text{v}\beta 8$ , which, at low concentrations, was markedly more effective than antibody inhibitors to TGF- $\beta$ , GARP, TGF- $\beta$ R2, or TGF- $\beta$ R2 receptor traps (Fig. 5, I and J). Decreased efficacy of antibody inhibitors to TGF- $\beta$ , TGF- $\beta$ R2, or TGF- $\beta$ R2 receptor traps was due to the reduced ability to block mature TGF- $\beta$  within the L-TGF- $\beta$  complex because they were effective inhibitors of diffusible rTGF- $\beta$  (Fig. 5K). These results were consistent with the inaccessibility of target epitopes for TGF- $\beta$ , GARP, or TGF- $\beta$ R2 within the  $\alpha\text{v}\beta 8$ /L-TGF- $\beta$ /GARP complex.

We next extended these findings to T cells themselves by using activated murine CD4<sup>+</sup> T cells plated on immobilized  $\alpha\text{v}\beta 8$  versus control substrates. T<sub>reg</sub> generation on immobilized  $\alpha\text{v}\beta 8$  (Fig. 5L) was almost completely blocked by anti- $\beta 8$  but was inhibited significantly less by other TGF- $\beta$  protein inhibitors at the same concentration (Fig. 5, M to Q). The lack of efficacy of inhibitors preferentially targeting diffusible TGF- $\beta$  supported our structure-based hypothesis that  $\alpha\text{v}\beta 8$ -mediated T cell conversion to T<sub>reg</sub> was independent of diffusion of TGF- $\beta$ . This hypothesis was further tested using a Transwell filter assay (Fig. 5R), which demonstrated that non-T<sub>reg</sub> CD4<sup>+</sup> T cells binding to  $\alpha\text{v}\beta 8$  induced iT<sub>reg</sub> conversion only by non-T<sub>reg</sub> CD4<sup>+</sup> T cells in direct contact and not those separated by the filter (Fig. 5, S to V). Together, our structural model of the  $\alpha\text{v}\beta 8$ /L-TGF- $\beta$ /GARP/TGF- $\beta$ R2 signaling complex and comparative efficacy studies demonstrated a mechanism for T<sub>reg</sub> enrichment dependent on  $\alpha\text{v}\beta 8$ -mediated TGF- $\beta$  activation.

### $\beta 8$ expression in non-small cell lung cancer positively correlated with T<sub>reg</sub> density in the TME

Most of human cancers express  $\alpha\text{v}\beta 8$  in at least a fraction of tumor cells (21). We sought to test the hypothesis that a subpopulation of  $\beta 8$ -expressing tumor cells was sufficient to drive local immunosuppressive T<sub>reg</sub> differentiation. We correlated CD4<sup>+</sup>FOXP3<sup>+</sup> cell numbers with  $\beta 8$  tumor proportion scores (TPS), which estimates the percentage of tumor cells expressing  $\beta 8$ , in a cohort of non-small cell lung cancers (NSCLCs) distinct from the cohort used for RNAseq (Figs. 4 and 6). We assessed cells with dual staining

of CD4 and FOXP3 because most of these cells are T<sub>regs</sub> (53). We found significant increases in CD4<sup>+</sup>FOXP3<sup>+</sup> cell numbers with  $\beta$ 8-TPS and CD4<sup>+</sup>FOXP3<sup>+</sup> cell numbers and CD4<sup>+</sup>FOXP3<sup>+</sup>:CD4<sup>+</sup>FOXP3<sup>-</sup> cell ratios correlated significantly with  $\beta$ 8-TPS (Fig. 6, A to F).

To test the hypothesis that a limited proportion of  $\beta$ 8-expressing tumor cells were sufficient to drive local T<sub>reg</sub> differentiation,  $\beta$ 8-expressing LLC cells with mock-LLC cells were mixed in varying proportions. A significant trend for enrichment of FOXP3<sup>+</sup> cells with increasing proportions of  $\beta$ 8-expressing tumor cells was found (Fig. 6, G to J). Tumor growth correlated with FOXP3<sup>+</sup> cell proportions, consistent with T<sub>regs</sub> contributing to  $\beta$ 8-mediated tumor immune evasion (Fig. 6J). These results suggested that expression of  $\alpha$ v $\beta$ 8 in a small fraction of tumor cells was sufficient to drive T<sub>reg</sub> enrichment in human tumors.

## DISCUSSION

Here, we identified a mechanism of T<sub>reg</sub> enrichment in tumors, where  $\alpha$ v $\beta$ 8 expression on tumor cells caused T<sub>reg</sub> enrichment by increasing TGF- $\beta$  activation in the TME. Our in vitro data suggested that the T<sub>reg</sub> enrichment in  $\beta$ 8-expressing tumors occurred via contact of  $\alpha$ v $\beta$ 8-expressing tumor cells with L-TGF- $\beta$ -presenting non-T<sub>reg</sub> CD4<sup>+</sup> T cells, converting them to pT<sub>reg</sub>. We used cell-based assays to provide evidence that an intermolecular complex forms between  $\alpha$ v $\beta$ 8-expressing tumor cells and L-TGF- $\beta$ -presenting non-T<sub>reg</sub> CD4<sup>+</sup> T cell, where TGF- $\beta$  was activated without release and diffusion of TGF- $\beta$  and signaling was induced only on the L-TGF- $\beta$ -presenting non-T<sub>reg</sub> CD4<sup>+</sup> T cell.

Existing evidence supports two mechanisms of tumor T<sub>reg</sub> enrichment, either intratumoral non-T<sub>reg</sub> CD4<sup>+</sup> T cell conversion to pT<sub>reg</sub> or recruitment of preexisting tT<sub>reg</sub> to tumors (Fig. 7, A and B) (22, 24). While exogenous TGF- $\beta$  can lead to conversion of T cells to iT<sub>regs</sub>, no previous studies have addressed how TGF- $\beta$  is activated in the TME or whether this activation leads to conversion of T cells to pT<sub>regs</sub>, as seen in the murine colon (54). However, in human tumors, the relative importance of conversion of non-T<sub>reg</sub> CD4<sup>+</sup> T cells to pT<sub>reg</sub> is controversial because only a fraction of tumor T<sub>reg</sub> share common T cell receptor (TCR) clonotypes with non-T<sub>reg</sub> CD4<sup>+</sup> T cells (55, 56). Our analysis of human tumors does not allow discrimination of tT<sub>reg</sub> from pT<sub>reg</sub>; thus, it is unclear whether tumors with high  $\alpha$ v $\beta$ 8 expression have increased overlap between the TCR repertoires of non-T<sub>reg</sub> CD4<sup>+</sup> T cells and T<sub>regs</sub> compared with those from tumors with low  $\alpha$ v $\beta$ 8 expression.

Our study suggests that pT<sub>reg</sub> enriched in murine  $\alpha$ v $\beta$ 8-expressing tumors contributed to tumor immune evasion because both PC-61.5.3 and C6D4 reduced T<sub>regs</sub> in  $\beta$ 8-LLC. PC-61.5.3 depletes intratumoral CD4<sup>+</sup>CD25<sup>hi</sup> T cells when given after tumor cell injection but not CD4<sup>+</sup>CD25<sup>lo</sup>FoxP3<sup>+</sup> T cells (5, 30). Depleting T<sub>reg</sub> precursors (L-TGF- $\beta$ <sup>+</sup>CD4<sup>+</sup>CD25<sup>hi</sup>FoxP3<sup>-</sup>) in  $\beta$ 8-LLC tumors would be expected to have the same effect as blocking  $\alpha$ v $\beta$ 8 function on intratumoral conversion to pT<sub>regs</sub>. The mock-LLC T<sub>reg</sub> population likely consists of CD4<sup>+</sup>CD25<sup>lo</sup>FoxP3<sup>+</sup> T cells because PC-61.5.3 failed to deplete it. Reducing T<sub>reg</sub> by PC-61.5.3 or C6D4 decreased  $\beta$ 8-LLC tumor growth, suggesting the importance of T<sub>reg</sub>-induced immunosuppression in  $\alpha$ v $\beta$ 8-mediated tumor growth promotion. T<sub>regs</sub> inhibit expansion of effector CD8<sup>+</sup> T cells (57). Our current

and previous studies showed that C6D4 decreased the numbers of  $T_{\text{regs}}$  and increased the numbers of effector  $CD8^+$  T cells in  $\beta 8$ -LLC tumors (21).

RNAseq data supported that tumor cells were the major  $\alpha v\beta 8$ -expressing cell type, whereas T cells were the major TGF- $\beta 1$ -expressing cell in the human TME. Cell surface expression of L-TGF- $\beta$  in non- $T_{\text{reg}}$   $CD4^+$  T cells from murine tumors suggests that they are poised to differentiate to  $pT_{\text{regs}}$  through activation of cell surface L-TGF- $\beta$  (21). Our data supported a mechanism of  $T_{\text{reg}}$  enrichment where  $\alpha v\beta 8$  expression by tumor cells caused L-TGF- $\beta$ -presenting non- $T_{\text{reg}}$   $CD4^+$  T cells to undergo local conversion to  $pT_{\text{reg}}$ , rather than promoting recruitment of  $tT_{\text{reg}}$  (Fig. 7, A and B). This conclusion was supported by in vitro studies demonstrating that immobilized or tumor cell  $\alpha v\beta 8$  drove the conversion of activated T cells to immunosuppressive  $T_{\text{reg}}$  with low levels of  $tT_{\text{reg}}$  markers, *Helios* and *Nrp1*, similar to in vivo studies, demonstrating that tumor cell  $\alpha v\beta 8$  led to the enrichment of  $pT_{\text{regs}}$ .

Our inability to detect a functional role for  $\alpha v\beta 8$  expressed by  $T_{\text{regs}}$  contrasts with other reports proposing a cell-autonomous role for  $\alpha v\beta 8$  in  $T_{\text{reg}}$  function using  $T_{\text{regs}}$  from nontumor sources (44, 45, 58). In these reports, surface expression of  $\alpha v\beta 8$  is not assessed; rather, the  $\beta 8$  subunit (*itgb8*) was detected by quantitative polymerase chain reaction (qPCR) in murine  $tT_{\text{reg}}$  and effector  $T_{\text{reg}}$  populations, which is at very low levels relative to housekeeping genes (44, 45). Such low mRNA expression is consistent with our inability to detect cell-surface  $\alpha v\beta 8$  because surface expression of  $\alpha v\beta 8$  is regulated at the level of *itgb8* transcription (59). Thus, although it is possible that, in other tumor systems,  $\alpha v\beta 8$  can be expressed at functionally significant levels in  $T_{\text{regs}}$ , our current and past studies, which include surface expression surveys of both tumor and splenic  $T_{\text{reg}}$ , transcriptomic analysis, and in vitro and in vivo functional assays failed to produce evidence that expression of  $\alpha v\beta 8$  by T themselves played a role in non- $T_{\text{reg}}$   $CD4^+$   $T_{\text{reg}}$  cell-to- $T_{\text{reg}}$  conversion.

We propose a model (Fig. 7C) to explain why T cells require presentation of L-TGF- $\beta$  on their cell surface for  $T_{\text{reg}}$  conversion. In this model, the  $\alpha v\beta 8$  receptor and its ligand, L-TGF- $\beta$ /GARP, are concentrated on opposing cell surfaces, not on the same cell surface, and are specifically directed only to the L-TGF- $\beta$ /GARP-presenting T cell after binding TGF- $\beta$  signaling (29). This process is more efficient and context-specific than a TGF- $\beta$ R-bearing T cell encountering active TGF- $\beta$  diffusing through the extracellular space. Furthermore, this model predicts that if tumor cell  $\alpha v\beta 8$  binds to either secreted or matrix-bound L-TGF- $\beta$ , then a TGF- $\beta$ R-expressing T cell would have to find and orient its receptors to mature TGF- $\beta$  exposed within the latent complex (Fig. 7C) (29).

Current dogma suggests that actin cytoskeletal force generation through the  $\beta$ -integrin cytoplasmic domain is required to induce conformational changes through the integrin for force generation to L-TGF- $\beta$  to release TGF- $\beta$  (42). However, our structural studies show that  $\alpha v\beta 8$  does not undergo major conformational rearrangements, always remaining in a single extended-closed conformation poised for ligand binding but not force transduction (43, 60). Hence,  $\alpha v\beta 8$  on cell surfaces is always available for binding to an L-TGF- $\beta$ -presenting cell, and this binding creates an anchor point to focus the inherent flexibility of L-TGF- $\beta$  to allow active TGF- $\beta$  to be sufficiently exposed to bind to its receptors but only on the cell-presenting surface L-TGF- $\beta$ /GARP (29). Here, we tested this model in vitro

using isolated T cells and showed that T cell TGF- $\beta$  activation did not require cytoskeletal force transduction from the  $\alpha\text{v}\beta\text{8}$  integrin because the  $\alpha\text{v}\beta\text{8}$  ectodomain immobilized on a solid substrate was freely capable of inducing conversion of contacting non-T<sub>reg</sub> CD4<sup>+</sup> T cells to T<sub>regs</sub>. This conversion occurred without release and diffusion of TGF- $\beta$ . The identification of a TGF- $\beta$  activation mechanism that is diffusion-independent has biologic implications because any L-TGF- $\beta$ -presenting cell type could potentially increase its TGF- $\beta$  signaling when contacting an  $\alpha\text{v}\beta\text{8}$ -expressing cell (21, 60). Our model also has therapeutic implications because protein-based therapies directed at TGF- $\beta$  currently in clinical trials are conceptually designed to inhibit diffusible TGF- $\beta$  (61). Our data indicated that such therapies would poorly target  $\alpha\text{v}\beta\text{8}$ -mediated TGF- $\beta$  activation, while still exposing patients to considerable risk.

Overall, this study highlights a mechanism of T<sub>reg</sub> enrichment regulated by TGF- $\beta$  activation in specific tumors that express sufficient levels of  $\alpha\text{v}\beta\text{8}$ . We propose that targeting  $\alpha\text{v}\beta\text{8}$  to prevent TGF- $\beta$  activation will provide a highly effective and more selective approach to overcome T<sub>reg</sub>-mediated tumor immune evasion in patients with  $\alpha\text{v}\beta\text{8}^+$  tumors.

## MATERIALS AND METHODS

### Study design

This study tested the hypothesis that T<sub>regs</sub> were required for integrin  $\alpha\text{v}\beta\text{8}$ -mediated tumor growth promotion using cell-based assays, in vivo tumor models, and correlative human studies. In vivo models used randomization and blinding maintained until end points were reached and data analysis was completed. Sample sizes for in vitro and in vivo experiments were estimated using power calculations with predetermined effect sizes and variances based on experience.

### Mice and orthotopic tumor models

Syngeneic bilateral tumor models were performed in C57BL/6 mice expressing *foxp3*-IRES-GFP [B6.Cg-*FOXP3*<sup>tm2(EGFP)</sup>Tch/J, Jax] (41) and WT C57BL/6 mice (all female except for TRAMP-C2 experiments using male), 8 to 10 weeks of age, were purchased (the Jackson laboratory), as described (21). See Supplementary Materials and Methods for more information.

### Human subjects

Patients were consented for tissue collection under University of California San Francisco Institutional Review Board (UCSF IRB)-approved protocols (UCSF CHR 10-04727, 14-15342, 11-06107, and 10-03413). The study enrollment period started from January 2015 to present, and the sample size was determined by the availability of specimens throughout this period. Samples were selected without regard to prior treatment. Two separate cohorts were developed: the first for cell sorting and RNAseq including samples from gynecologic ( $n = 53$ ), lung ( $n = 41$ ), and head and neck squamous cell carcinoma ( $n = 38$ ) and the second for immunohistochemistry from patients undergoing resection for NSCLC ( $n = 32$ ).

## Study approval

Human tissues were obtained with full approval of the UCSF IRB in full accordance with Declaration of Helsinki principles. Written informed consent was received from participants before inclusion in the study. All animal studies have been approved by the UCSF Institutional Animal Care and Use Committee.

## Cell lines

LL/2 (LLC1) [American Type Culture Collection (ATCC), CRL-1642] and TRAMP-C2 (gift from L. Fong, UCSF, San Francisco, CA, USA) were used in TGF- $\beta$  reporter assays and syngeneic tumor models. OVCAR-3 (UCSF Cell and Genome Engineering Core) and transformed mink lung TGF- $\beta$  reporter cells (TMLC) (62) were a gift from J. Munger (New York University Medical Center, New York, NY, USA) and were stably transfected with L-TGF- $\beta$  WT or L-TGF- $\beta$  (R249A), with or without GARP, as previously described (29). LLC cells were stably transfected to overexpress vector only (mock-LLC) or  $\beta$ 8 ( $\beta$ 8-LLC) as previously described (21). CHOlec3.2.8.1 (gift from P. Stanley, Albert Einstein College of Medicine, New York, NY, USA), CHO-K1 (ATCC, CCL-61), and human embryonic kidney (HEK) 293 cells (ATCC, CRL-1573) were used for recombinant integrin expression and immunoglobulin G2a (IgG2a) production. All cell lines were maintained in the appropriate media with selection agents and antibiotics, as previously described (21, 29, 43).

## Reagents

Anti-CD25 (clone PC-61.5.3) and rat isotype control (HRPN, BP0088) were obtained from Bio X Cell. Anti- $\beta$ 8 (C6D4) is a highly specific engineered recombinant antibody to the specificity determining loop 2 (SDL2) domain of  $\alpha$ v $\beta$ 8 consisting of humanized V genes and CH1 domains, with murine linker and CH2/3 domains in an IgG2a format and is produced in CHO-K1 cells (21, 29). The  $\alpha$ v $\beta$ 8 and  $\alpha$ v $\beta$ 3 ecto-domains were expressed and purified as previously described (43, 60). For all other reagents, see Supplementary Materials and Methods.

## DNA constructs

The following PCR products were produced using the following primers and templates: 5'-GATTGTGGGCCCTCTGGGCTCGTCC-GGATTGCTGGTGTATATTCTTCTGAG-3' and 5'-CT-GTGGACGCGTATCGCC-3', human *TGFBR2* expression vector (Sino Biological, HG10358-ACG) and CTCAGAAGAATATAACACCAGCAATCCGGACGAGCCCAGAGGGCCCAATC and AACGGATCCTCATTACCCGGAG, mouse IgG2a expression vector. The products were joined by splice overlap extension PCR and cloned into AbVec 2.0 (Addgene, plasmid no. 80795), as described (60). The resulting plasmid was transiently transfected into HEK-293 cells and protein purified as described (21).

## Lentiviral transduction

For *itgb8* knockdown, TRAMP-C2 cells were stably transfected with *itgb8*-specific shRNA or nonmammalian control shRNA via lentiviral transduction [Sigma-Aldrich MISSION

lentiviral transduction particles TRCN0000067303 (*itgb8*) or SHC002V (nonmammalian control)].

### Isolation, staining, and RNAseq of mouse tumor and immune cells

Mouse tumor immune cell isolation, RNA isolation, and sequencing were performed as described (21). Briefly, mouse tumors were digested; live tumor cells were negatively selected by magnetic beads, or infiltrating lymphoid cells were enriched by density gradient centrifugation. FOXP3<sup>+</sup> cells were then sorted from the enriched lymphoid cells by staining with fluorochrome-labeled antibodies as described (21). Total RNA was isolated using kits as described (21), after which cDNA synthesis and amplification were performed. For specific details including library construction and analysis, see Supplementary Materials and Methods.

### Isolation, staining, and RNAseq of human tumor and immune cells

Fresh patient tumor samples were dissociated, and the resulting cell suspension was enumerated and stained with the LIVE/DEAD stain and an extracellular antibody cocktail before cell sorting. RNA isolation, cDNA synthesis, library construction, sequencing, and analysis are described in Supplementary Materials and Methods.

### T<sub>reg</sub> maturation assays

Briefly, CD4<sup>+</sup> T cells from *foxp3*-IRES-GFP mice were purified and plated onto tissue culture plates coated with  $\alpha$ v $\beta$ 8tr or control substrate [ $\alpha$ v $\beta$ 3tr or bovine serum albumin (BSA)] under T cell stimulation conditions. CD4<sup>+</sup> T cells were incubated for 72 hours before phenotyping via flow cytometric analysis. For Transwell diffusion assays, cells were plated into each chamber of the Transwell culture wells containing a 0.4- $\mu$ m filter using the same conditions as above with further details provided in Supplementary Materials and Methods.

### Lymphocyte suppression assays

Cells were cultured as above to create T<sub>reg</sub> pools and labeled with carboxyfluorescein diacetate succinimidyl ester fluorescent tracking dye exactly as described (63). Labeled cells were stimulated using anti-CD3/CD28 Dynabeads and plated in round-bottom 96-well culture plates. Labeled CD4<sup>+</sup> cells were then cocultured with  $\alpha$ v $\beta$ 8-generated T<sub>regs</sub> or control cells at ratios ranging between 1:1 and 8:1 (labeled CD4<sup>+</sup> T cells:T<sub>regs</sub>). T<sub>conv</sub> proliferation was measured using flow cytometry after 4 days. For specific details, see Supplementary Materials and Methods.

### TGF- $\beta$ bioassays

TGF- $\beta$  bioassays were performed as previously described (29) using the cell-intrinsic TGF- $\beta$  activation reporter system described in (64). Reporter cells were seeded onto culture wells coated with  $\alpha$ v $\beta$ 8tr or control substrate ( $\alpha$ v $\beta$ 3tr or BSA). Cells were incubated for 18 hours before cell lysis and assessment of luciferase activity. For some experiments mock-LLC,  $\beta$ 8-LLC, TRAMP-C2, or OVCAR-3 ( $3 \times 10^4$ ) cells were used in place of immobilized substrates.

### Determination of receptor density

CHO WT or CHO transfected with human  $\alpha\text{v}\beta\text{8}$  were plated onto culture plates over a concentration range of  $5 \times 10^3$  to  $30 \times 10^3$  cells per well, and recombinant  $\alpha\text{v}\beta\text{8}$  ectodomain [phosphate-buffered saline (10 to 5000 ng/ml)] were coated onto separate wells on the same plate. After cell attachment, cells and coated receptor wells were fixed with 4% paraformaldehyde. Cell-associated or recombinant  $\alpha\text{v}\beta\text{8}$  was detected with clone F9, followed by anti-mouse horseradish peroxidase and colorimetric detection, and cell surface receptor density was determined on the basis of standard curve of estimated recombinant  $\alpha\text{v}\beta\text{8}$  receptor coating efficiency, as described in Supplementary Materials and Methods.

### Immunohistochemical analysis of murine and human tumors

Immunohistochemistry was performed as previously described (21). Briefly, prepared formalin-fixed paraffin-embedded (FFPE) sections were stained with anti-mouse  $\beta\text{8}$  (clone F9), B5 (anti-human  $\beta\text{8}$ , which does not work in FFPE immunostaining and thus used as isotype control for F9), anti-CD4, anti-CD8, or anti-FOXP3, followed by appropriate detection reagents. For multiplex immunostaining, the Ventana Discovery platform was used as described in Supplementary Materials and Methods.

### Quantification and statistical analysis

All data are reported as means  $\pm$  SEM unless otherwise specified. Comparisons between two different groups were determined using two-tailed Student's *t* test. One-way analysis of variance (ANOVA) was used for multiple comparisons, and Tukey's, Dunnett's, or Sidak's post hoc tests were used to test for statistical significance. Outliers were included unless stated otherwise. Significance was defined as  $P < 0.05$ . All statistical analyses, including outlier identification ( $R_{\text{out}}$ ), were performed using the software package Prism 7.0b (GraphPad Software).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Competing interests:

S.L.N. and J.M. serve on the Scientific Advisory Board of Venn Therapeutics. S.L.N. is a consultant for Fleet Therapeutics. J.L. is a recipient of sponsored research funds from Venn Therapeutics. The antibodies C6D4 and F9 are included in U.S. Patent application no. 16/331,902 (S.L.N., Y.C., J.M., J.L., J.L.B., and N.T.).

## Data and materials availability:

Antibodies and cell lines used in this manuscript will be available upon execution of a material transfer agreement. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. RNAseq datasets are deposited in the Gene Expression Omnibus (GSE158031).

## REFERENCES AND NOTES

1. Fridman WH, Pagès F, Sautès-Fridman C, Galon J, The immune contexture in human tumours: Impact on clinical outcome. *Nat. Rev. Cancer*12, 298–306 (2012). [PubMed: 22419253]
2. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W, Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med*10, 942–949 (2004). [PubMed: 15322536]
3. Curiel TJ, Tregs and rethinking cancer immunotherapy. *J. Clin. Invest*117, 1167–1174 (2007). [PubMed: 17476346]
4. Shimizu J, Yamazaki S, Sakaguchi S, Induction of tumor immunity by removing CD25<sup>+</sup>CD4<sup>+</sup> T cells: A common basis between tumor immunity and autoimmunity. *J. Immunol*163, 5211–5218 (1999). [PubMed: 10553041]
5. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E, Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor  $\alpha$ ) monoclonal antibody. *Cancer Res.* 59, 3128–3133 (1999). [PubMed: 10397255]
6. Tanaka A, Sakaguchi S, Targeting Treg cells in cancer immunotherapy. *Eur. J. Immunol*49, 1140–1146 (2019). [PubMed: 31257581]
7. Chen DS, Mellman I, Oncology meets immunology: The cancer-immunity cycle. *Immunity*39, 1–10 (2013). [PubMed: 23890059]
8. Yadav M, Stephan S, Bluestone JA, Peripherally induced Tregs—Role in immune homeostasis and autoimmunity. *Front. Immunol*4, 232 (2013). [PubMed: 23966994]
9. Valzasina B, Piconese S, Guiducci C, Colombo MP, Tumor-induced expansion of regulatory T cells by conversion of CD4<sup>+</sup>CD25<sup>-</sup> lymphocytes is thymus and proliferation independent. *Cancer Res.* 66, 4488–4495 (2006). [PubMed: 16618776]
10. Paluskiewicz CM, Cao X, Abdi R, Zheng P, Liu Y, Bromberg JS, T regulatory cells and priming the suppressive tumor microenvironment. *Front. Immunol*10, 2453 (2019). [PubMed: 31681327]
11. Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B, Zitvogel L, Tumor cells convert immature myeloid dendritic cells into TGF- $\beta$ -secreting cells inducing CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell proliferation. *J. Exp. Med*202, 919–929 (2005). [PubMed: 16186184]
12. Klein L, Robey EA, Hsieh CS, Central CD4<sup>+</sup> T cell tolerance: Deletion versus regulatory T cell differentiation. *Nat. Rev. Immunol*19, 7–18 (2019). [PubMed: 30420705]
13. Anderton MJ, Mellor HR, Bell A, Sadler C, Pass M, Powell S, Steele SJ, Roberts RRA, Heier A, Induction of heart valve lesions by small-molecule ALK5 inhibitors. *Toxicol. Pathol*39, 916–924 (2011). [PubMed: 21859884]
14. Vitsky A, Waire J, Pawliuk R, Bond A, Matthews D, LaCasse E, Hawes ML, Nelson C, Richards S, Piepenhagen PA, Garman RD, Andrews L, Thurberg BL, Lonning S, Ledbetter S, Ruzek MC, Homeostatic role of transforming growth factor-beta in the oral cavity and esophagus of mice and its expression by mast cells in these tissues. *Am. J. Pathol*174, 2137–2149 (2009). [PubMed: 19406991]
15. Tolcher AW, Berlin JD, Cosaert J, Kauh J, Chan E, Piha-Paul SA, Amaya A, Tang S, Driscoll K, Kimbung R, Kambhampati SRP, Gueorguieva I, Hong DS, A phase I study of anti-TGF $\beta$  receptor type-II monoclonal antibody LY3022859 in patients with advanced solid tumors. *Cancer Chemother. Pharmacol*79, 673–680 (2017). [PubMed: 28280971]



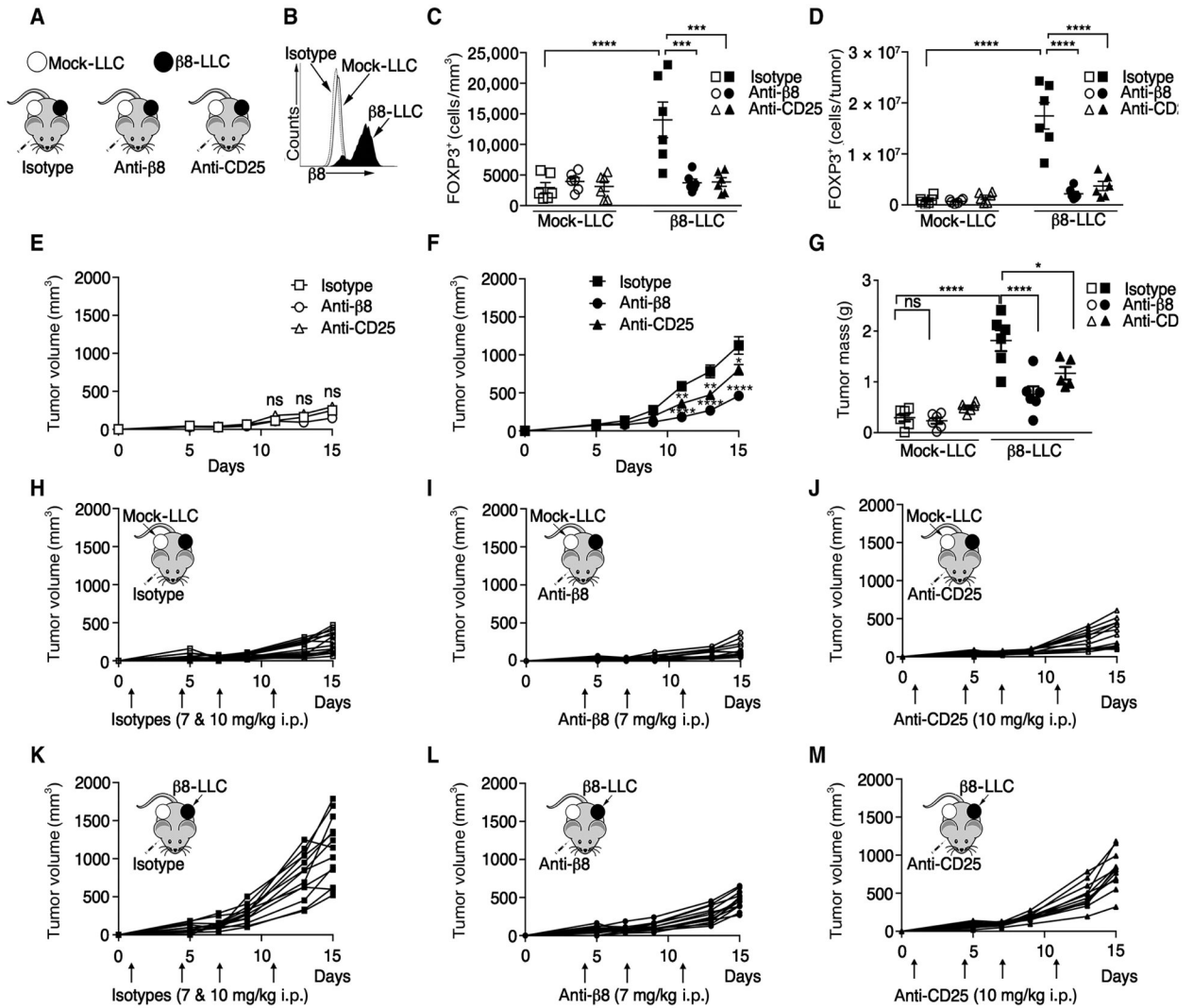
16. Elliott RL, Blobe GC, Role of transforming growth factor Beta in human cancer. *J. Clin. Oncol*23, 2078–2093 (2005). [PubMed: 15774796]
17. Massagué J, TGF $\beta$  in cancer. *Cell*134, 215–230 (2008). [PubMed: 18662538]
18. Nishimura SL, Integrin-mediated transforming growth factor- $\beta$  activation, a potential therapeutic target in fibrogenic disorders. *Am. J. Pathol*175, 1362–1370 (2009). [PubMed: 19729474]
19. Edwards JP, Fujii H, Zhou AX, Creemers J, Unutmaz D, Shevach EM, Regulation of the expression of GARP/latent TGF- $\beta$ 1 complexes on mouse T cells and their role in regulatory T cell and Th17 differentiation. *J. Immunol*190, 5506–5515 (2013). [PubMed: 23645881]
20. Batlle E, Massagué J, Transforming growth factor- $\beta$  signaling in immunity and cancer. *Immunity*50, 924–940 (2019). [PubMed: 30995507]
21. Takasaka N, Seed RI, Cormier A, Bondesson AJ, Lou J, Elattma A, Ito S, Yanagisawa H, Hashimoto M, Ma R, Levine MD, Publicover J, Potts R, Jespersen JM, Campbell MG, Conrad F, Marks JD, Cheng Y, Baron JL, Nishimura SL, Integrin  $\alpha$ v $\beta$ 8-expressing tumor cells evade host immunity by regulating TGF- $\beta$  activation in immune cells. *JCI Insight*3, (2018).
22. Stockis J, Roychoudhuri R, Halim TYF, Regulation of regulatory T cells in cancer. *Immunology*157, 219–231 (2019). [PubMed: 31032905]
23. Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, Broaddus VC, Nishimura SL, The integrin  $\alpha$ v $\beta$ 8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF- $\beta$ 1. *J. Cell Biol*157, 493–507 (2002). [PubMed: 11970960]
24. Tanaka A, Sakaguchi S, Regulatory T cells in cancer immunotherapy. *Cell Res.* 27, 109–118 (2017). [PubMed: 27995907]
25. Aluwihare P, Mu Z, Zhao Z, Yu D, Weinreb PH, Horan GS, Violette SM, Munger JS, Mice that lack activity of  $\alpha$ v $\beta$ 6- and  $\alpha$ v $\beta$ 8-integrins reproduce the abnormalities of *Tgfb1*- and *Tgfb3*-null mice. *J. Cell Sci*122, 227–232 (2009). [PubMed: 19118215]
26. Yang Z, Mu Z, Dabovic B, Jurukovski V, Yu D, Sung J, Xiong X, Munger JS, Absence of integrin-mediated TGF $\beta$ 1 activation in vivo recapitulates the phenotype of TGF $\beta$ 1-null mice. *J. Cell Biol*176, 787–793 (2007). [PubMed: 17353357]
27. Ozawa A, Sato Y, Imabayashi T, Uemura T, Takagi J, Sekiguchi K, Molecular basis of the ligand binding specificity of  $\alpha$ v $\beta$ 8 integrin. *J. Biol. Chem*291, 11551–11565 (2016). [PubMed: 27033701]
28. Kitamura H, Cambier S, Somanath S, Barker T, Minagawa S, Markovics J, Goodsell A, Publicover J, Reichardt L, Jablons D, Wolters P, Hill A, Marks JD, Lou J, Pittet JF, Gauldie J, Baron JL, Nishimura SL, Mouse and human lung fibroblasts regulate dendritic cell trafficking, airway inflammation, and fibrosis through integrin  $\alpha$ v $\beta$ 8-mediated activation of TGF- $\beta$ . *J. Clin. Invest*121, 2863–2875 (2011). [PubMed: 21646718]
29. Campbell MG, Cormier A, Ito S, Seed RI, Bondesson AJ, Lou J, Marks JD, Baron JL, Cheng Y, Nishimura SL, Cryo-EM reveals integrin-mediated TGF- $\beta$  activation without release from latent TGF- $\beta$ . *Cell*180, 490–501.e16 (2020). [PubMed: 31955848]
30. Arce Vargas F, Furness AJS, Solomon I, Joshi K, Mekkaoui L, Lesko MH, Miranda Rota E, Dahan R, Georgiou A, Sledzinska A, Ben Aissa A, Franz D, Werner Sunderland M, Wong YNS, Henry JY, O'Brien T, Nicol D, Challacombe B, Beers SA, Turajlic S, Gore M, Larkin J, Swanton C, Chester KA, Pule M, Ravetch JV, Marafioti T, Peggs KS, Quezada SA, Spain L, Wotherspoon A, Francis N, Smith M, Strauss D, Hayes A, Soultati A, Stares M, Spain L, Lynch J, Fotiadis N, Fernando A, Hazell S, Chandra A, Pickering L, Rudman S, Chowdhury S, Swanton C, Jamal-Hanjani M, Veeriah S, Shafi S, Czyzewska-Khan J, Johnson D, Laycock J, Bosshard-Carter L, Goh G, Rosenthal R, Gorman P, Murugaesu N, Hynds RE, Wilson G, Birkbak NJ, Watkins TBK, McGranahan N, Horswell S, Mitter R, Escudero M, Stewart A, van Loo P, Rowan A, Xu H, Turajlic S, Hiley C, Abbosh C, Goldman J, Stone RK, Denner T, Matthews N, Elgar G, Ward S, Biggs J, Costa M, Begum S, Phillimore B, Chambers B, Nye E, Graca S, al Bakir M, Hartley JA, Lowe HL, Herrero J, Lawrence D, Hayward M, Panagiotopoulos N, Kolvekar S, Falzon M, Borg E, Simeon C, Hector G, Smith A, Aranda M, Novelli M, Oukrif D, Janes SM, Thakrar R, Forster M, Ahmad T, Lee SM, Papadatos-Pastos D, Carnell D, Mendes R, George J, Navani N, Ahmed A, Taylor M, Choudhary J, Summers Y, Califano R, Taylor P, Shah R, Krysiak P, Rammohan K, Fontaine E, Booton R, Evison M, Crosbie P, Moss S, Idries F, Joseph L, Bishop P, Chaturved A, Quinn AM, Doran H, Leek A, Harrison P, Moore K, Waddington R, Novasio J,

Blackhall F, Rogan J, Smith E, Dive C, Tugwood J, Brady G, Rothwell DG, Chemi F, Pierce J, Gulati S, Naidu B, Langman G, Trotter S, Bellamy M, Bancroft H, Kerr A, Kadiri S, Webb J, Middleton G, Djearaman M, Fennell D, Shaw JA, le Quesne J, Moore D, Nakas A, Rathinam S, Monteiro W, Marshall H, Nelson L, Bennett J, Riley J, Primrose L, Martinson L, Anand G, Khan S, Amadi A, Nicolson M, Kerr K, Palmer S, Remmen H, Miller J, Buchan K, Chetty M, Gomersall L, Lester J, Edwards A, Morgan F, Adams H, Davies H, Kornaszewska M, Attanoos R, Lock S, Verjee A, MacKenzie M, Wilcox M, Bell H, Iles N, Hackshaw A, Ngai Y, Smith S, Gower N, Ottensmeier C, Chee S, Johnson B, Alzetani A, Shaw E, Lim E, de Sousa P, Barbosa MT, Bowman A, Jorda S, Rice A, Raubenheimer H, Proli C, Cufari ME, Ronquillo JC, Kwayie A, Bhayani H, Hamilton M, Bakar Y, Mensah N, Ambrose L, Devaraj A, Buderer S, Finch J, Azcarate L, Chavan H, Green S, Mashinga H, Nicholson AG, Lau K, Sheaff M, Schmid P, Conibeare J, Ezhil V, Ismail B, Irvin-sellers M, Prakash V, Russell P, Light T, Horey T, Danson S, Bury J, Edwards J, Hill J, Matthews S, Kitsanta Y, Suvarna K, Fisher P, Keerio AD, Shackcloth M, Gosney J, Postmus P, Feeney S, Asante-Siaw J, Fc-optimized anti-CD25 depletes tumor-infiltrating regulatory T cells and synergizes with PD-1 blockade to eradicate established tumors. *Immunity*46, 577–586 (2017). [PubMed: 28410988]

31. Pincetic A, Bournazos S, DiLillo DJ, Maamary J, Wang TT, Dahan R, Fiebiger BM, Ravetch JV, Type I and type II Fc receptors regulate innate and adaptive immunity. *Nat. Immunol*15, 707–716 (2014). [PubMed: 25045879]
32. Thornton AM, Lu J, Korty PE, Kim YC, Martens C, Sun PD, Shevach EM, Helios<sup>+</sup> and Helios<sup>-</sup> Treg subpopulations are phenotypically and functionally distinct and express dissimilar TCR repertoires. *Eur. J. Immunol*49, 398–412 (2019). [PubMed: 30620397]
33. Landuyt AE, Klocke BJ, Colvin TB, Schoeb TR, Maynard CL, Cutting edge: ICOS-deficient regulatory T cells display normal induction of *Ill10* but readily downregulate expression of Foxp3. *J. Immunol*202, 1039–1044 (2019). [PubMed: 30642977]
34. Ondondo B, Jones E, Godkin A, Gallimore A, Home sweet home: The tumor microenvironment as a haven for regulatory T cells. *Front. Immunol*4, 197 (2013). [PubMed: 23874342]
35. Bruder D, Probst-Kepper M, Westendorf AM, Geffers R, Beissert S, Loser K, von Boehmer H, Buer J, Hansen W, Neuropilin-1: A surface marker of regulatory T cells. *Eur. J. Immunol*34, 623–630 (2004). [PubMed: 14991591]
36. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM, Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3<sup>+</sup> T regulatory cells. *J. Immunol*184, 3433–3441 (2010). [PubMed: 20181882]
37. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, Xiong H, Dolpady J, Frey AB, Ruocco MG, Yang Y, Floess S, Huehn J, Oh S, Li MO, Niec RE, Rudensky AY, Dustin ML, Littman DR, Lafaille JJ, Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3<sup>+</sup> T reg cells. *J. Exp. Med*209, 1723–1742 (2012). [PubMed: 22966001]
38. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S, Anthony BA, Sverdrup FM, Head R, Kuster DJ, Ruminski P, Weiss D, Schack DV, Bluestone JA, Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J. Exp. Med*209, 1713–1722 (2012). [PubMed: 22966003]
39. Oida T, Weiner HL, TGF- $\beta$  induces surface LAP expression on murine CD4 T cells independent of Foxp3 induction. *PLOS ONE*5, e15523 (2010). [PubMed: 21124798]
40. Nakamura K, Kitani A, Strober W, Cell contact-dependent immunosuppression by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells is mediated by cell surface-bound transforming growth factor  $\beta$ . *J. Exp. Med*194, 629–644 (2001). [PubMed: 11535631]
41. Haribhai D, Lin W, Relland LM, Truong N, Williams CB, Chatila TA, Regulatory T cells dynamically control the primary immune response to foreign antigen. *J. Immunol*178, 2961–2972 (2007). [PubMed: 17312141]
42. Dong X, Zhao B, Iacob RE, Zhu J, Koksals AC, Lu C, Engen JR, Springer TA, Force interacts with macromolecular structure in activation of TGF- $\beta$ . *Nature*542, 55–59 (2017). [PubMed: 28117447]

43. Cormier A, Campbell MG, Ito S, Wu S, Lou J, Marks J, Baron JL, Nishimura SL, Cheng Y, Cryo-EM structure of the  $\alpha\text{V}\beta\text{8}$  integrin reveals a mechanism for stabilizing integrin extension. *Nat. Struct. Mol. Biol.* 25, 698–704 (2018). [PubMed: 30061598]
44. Edwards JP, Thornton AM, Shevach EM, Release of active TGF- $\beta$ 1 from the latent TGF- $\beta$ 1/GARP complex on T regulatory cells is mediated by integrin  $\beta$ 8. *J. Immunol* 193, 2843–2849 (2014). [PubMed: 25127859]
45. Worthington JJ, Kelly A, Smedley C, Bauché D, Campbell S, Marie JC, Travis MA, Integrin  $\alpha\text{V}\beta\text{8}$ -mediated TGF- $\beta$  activation by effector regulatory T cells is essential for suppression of T-cell-mediated inflammation. *Immunity* 42, 903–915 (2015). [PubMed: 25979421]
46. Azhar M, Yin M, Bommireddy R, Duffy JJ, Yang J, Pawlowski SA, Boivin GP, Engle SJ, Sanford LP, Grisham C, Singh RR, Babcock GF, Doetschman T, Generation of mice with a conditional allele for transforming growth factor beta 1 gene. *Genesis* 47, 423–431 (2009). [PubMed: 19415629]
47. Thomas DA, Massagué J, TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* 8, 369–380 (2005). [PubMed: 16286245]
48. Donkor MK, Sarkar A, Li MO, Tgf- $\beta$ 1 produced by activated CD4<sup>+</sup>T cells antagonizes T cell surveillance of tumor development. *Onco. Targets. Ther* 1, 162–171 (2012).
49. Salem M, Wallace C, Velegraki M, Li A, Ansa-Addo E, Metelli A, Kwon H, Riesenberger B, Wu B, Zhang Y, Guglietta S, Sun S, Liu B, Li Z, GARP dampens cancer immunity by sustaining function and accumulation of regulatory T cells in the colon. *Cancer Res.* 79, 1178–1190 (2019). [PubMed: 30674536]
50. Moulin A, Mathieu M, Lawrence C, Bigelow R, Levine M, Hamel C, Marquette JP, le Parc J, Loux C, Ferrari P, Capdevila C, Dumas J, Dumas B, Rak A, Bird J, Qiu H, Pan CQ, Edmunds T, Wei RR, Structures of a pan-specific antagonist antibody complexed to different isoforms of TGF $\beta$  reveal structural plasticity of antibody-antigen interactions. *Protein Sci.* 23, 1698–1707 (2014). [PubMed: 25209176]
51. Lienart S, Merceron R, Vanderaa C, Lambert F, Colau D, Stockis J, van der Woning B, De Haard H, Saunders M, Coulie PG, Savvides SN, Lucas S, Structural basis of latent TGF- $\beta$ 1 presentation and activation by GARP on human regulatory T cells. *Science* 362, 952–956 (2018). [PubMed: 30361387]
52. Gabrieli G, da Cunha AP, Rezende RM, Kenyon B, Madi A, Vandeventer T, Skillin N, Rubino S, Garo L, Mazzola MA, Kolypetri P, Lanser AJ, Moreira T, Faria AMC, Lassmann H, Kuchroo V, Murugaiyan G, Weiner HL, Targeting latency-associated peptide promotes antitumor immunity. *Sci. Immunol* 2, eaaj1738 (2017). [PubMed: 28763794]
53. Gavin MA, Torgerson TR, Houston E, deRoos P, Ho WY, Stray-Pedersen A, Ocheltree EL, Greenberg PD, Ochs HD, Rudensky AY, Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc. Natl. Acad. Sci. U.S.A* 103, 6659–6664 (2006). [PubMed: 16617117]
54. Pratama A, Schnell A, Mathis D, Benoist C, Developmental and cellular age direct conversion of CD4<sup>+</sup> T cells into ROR $\gamma$ <sup>+</sup> or Helios<sup>+</sup> colon Treg cells. *J. Exp. Med* 217, e20190428 (2020). [PubMed: 31685531]
55. Ahmadzadeh M, Pasetto A, Jia L, Deniger DC, Stevanovi S, Robbins PF, Rosenberg SA, Tumor-infiltrating human CD4<sup>+</sup> regulatory T cells display a distinct TCR repertoire and exhibit tumor and neoantigen reactivity. *Sci. Immunol* 4, eaao4310 (2019). [PubMed: 30635355]
56. Plitas G, Konopacki C, Wu K, Bos PD, Morrow M, Putintseva EV, Chudakov DM, Rudensky AY, Regulatory T cells exhibit distinct features in human breast cancer. *Immunity* 45, 1122–1134 (2016). [PubMed: 27851913]
57. Kastenmuller W, Gasteiger G, Subramanian N, Sparwasser T, Busch DH, Belkaid Y, Drexler I, Germain RN, Regulatory T cells selectively control CD8<sup>+</sup> T cell effector pool size via IL-2 restriction. *J. Immunol* 187, 3186–3197 (2011). [PubMed: 21849683]
58. Stockis J, Liénart S, Colau D, Collignon A, Nishimura SL, Sheppard D, Coulie PG, Lucas S, Blocking immunosuppression by human Tregs in vivo with antibodies targeting integrin  $\alpha\text{V}\beta\text{8}$ . *Proc. Natl. Acad. Sci. U.S.A* 114, E10161–E10168 (2017). [PubMed: 29109269]

59. Markovics JA, Araya J, Cambier S, Jablons D, Hill A, Wolters PJ, Nishimura SL, Transcription of the transforming growth factor beta activating integrin beta8 subunit is regulated by SP3, AP-1, and the p38 pathway. *J. Biol. Chem*285, 24695–24706 (2010). [PubMed: 20519498]
60. Minagawa S, Lou J, Seed RI, Cormier A, Wu S, Cheng Y, Murray L, Tsui P, Connor J, Herbst R, Govaerts C, Barker T, Cambier S, Yanagisawa H, Goodsell A, Hashimoto M, Brand OJ, Cheng R, Ma R, Knelly KJM, Wen W, Hill A, Jablons D, Wolters P, Kitamura H, Araya J, Barczak AJ, Erle DJ, Reichardt LF, Marks JD, Baron JL, Nishimura SL, Selective targeting of TGF- $\beta$  activation to treat fibroinflammatory airway disease. *Sci. Transl. Med*6, 241ra79 (2014).
61. Derynck R, Turley SJ, Akhurst RJ, TGF $\beta$  biology in cancer progression and immunotherapy. *Nat. Rev. Clin. Oncol*18, 9–34 (2021). [PubMed: 32710082]
62. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB, An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal. Biochem*216, 276–284 (1994). [PubMed: 8179182]
63. Lim JF, Berger H, Su IH, Isolation and activation of murine lymphocytes. *J. Vis. Exp*, 54596 (2016).
64. Shi M, Zhu J, Wang R, Chen X, Mi L, Walz T, Springer TA, Latent TGF- $\beta$  structure and activation. *Nature*474, 343–349 (2011). [PubMed: 21677751]
65. Khan SA, Joyce J, Tsuda T, Quantification of active and total transforming growth factor- $\beta$  levels in serum and solid organ tissues by bioassay. *BMC. Res. Notes*5, 636 (2012). [PubMed: 23151377]
66. Radaev S, Zou Z, Huang T, Lafer EM, Hinck AP, Sun PD, Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily. *J. Biol. Chem*285, 14806–14814 (2010). [PubMed: 20207738]
67. Newsted D, Banerjee S, Watt K, Nersesian S, Truesdell P, Blazer LL, Cardarelli L, Adams JJ, Sidhu SS, Craig AW, Blockade of TGF- $\beta$  signaling with novel synthetic antibodies limits immune exclusion and improves chemotherapy response in metastatic ovarian cancer models. *Oncotargets. Ther*8, e1539613 (2018).
68. Hartigan-O'Connor DJ, Poon C, Sinclair E, McCune JM, Human CD4+ regulatory T cells express lower levels of the IL-7 receptor alpha chain (CD127), allowing consistent identification and sorting of live cells. *J. Immunol. Methods*319, 41–52 (2007). [PubMed: 17173927]
69. Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szczepaniak MW, Gaffney DJ, Elo LL, Zhang X, Mortazavi A, A survey of best practices for RNA-seq data analysis. *Genome Biol.* 17, 13 (2016). [PubMed: 26813401]
70. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR, STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*29, 15–21 (2013). [PubMed: 23104886]
71. Li B, Dewey CN, RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*12, 323 (2011). [PubMed: 21816040]
72. Sorensen K, Brodbeck U, Assessment of coating-efficiency in ELISA plates by direct protein determination. *J. Immunol. Methods*95, 291–293 (1986). [PubMed: 3794350]
73. Zemmour D, Zilionis R, Kiner E, Klein AM, Mathis D, Benoist C, Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. *Nat. Immunol*19, 291–301 (2018). [PubMed: 29434354]



**Fig. 1.  $T_{reg}$  depletion specifically inhibited  $\beta 8$ -LLC but not mock-LLC tumor outgrowth.**

(A) Cartoon of tumor model. (B) Representative surface staining: anti- $\beta 8$  (C6D4) or isotype control of mock-LLC or  $\beta 8$ -LLC cells. (C to M) Mock-LLC and  $\beta 8$ -LLC tumors were established on opposing flanks of C57BL/6 mice. (C to G, I, and L) Mice were treated [7 mg/kg, intraperitoneally (i.p.)] with anti- $\beta 8$  (C6D4) after tumor establishment. (J and M)  $T_{regs}$  were depleted with anti-CD25 (clone PC-61.5.3) starting 1 day after tumor cell injection. (H and K) Isotype controls. (C and D) Intratumoral  $T_{reg}$  numbers (outliers removed) confirmed by immunohistochemistry of FOXP3 of mock (open) or  $\beta 8$ -LLC (filled) tumors shown by FOXP3<sup>+</sup> cells/tumor surface area I or FOXP3<sup>+</sup> cells/tumor (D). Average LLC tumor volumes for mock-LLC (E) and  $\beta 8$ -LLC (F), with day 15 tumor weights (G). Corresponding spider plots for mock-LLC (H to J) treated with isotype (H), anti- $\beta 8$ , C6D4 (I), anti-CD25 (J), or  $\beta 8$ -LLC treated with isotype (K), anti- $\beta 8$ , C6D4 (L), or anti-CD25 (M). Arrows indicate antibody injection days. (H to M) Cartoons show tumor type (arrow) in accompanying plots. One-way ANOVA was used for multiple comparisons followed by Tukey's post-test. Student's unpaired *t* test was used for comparing two

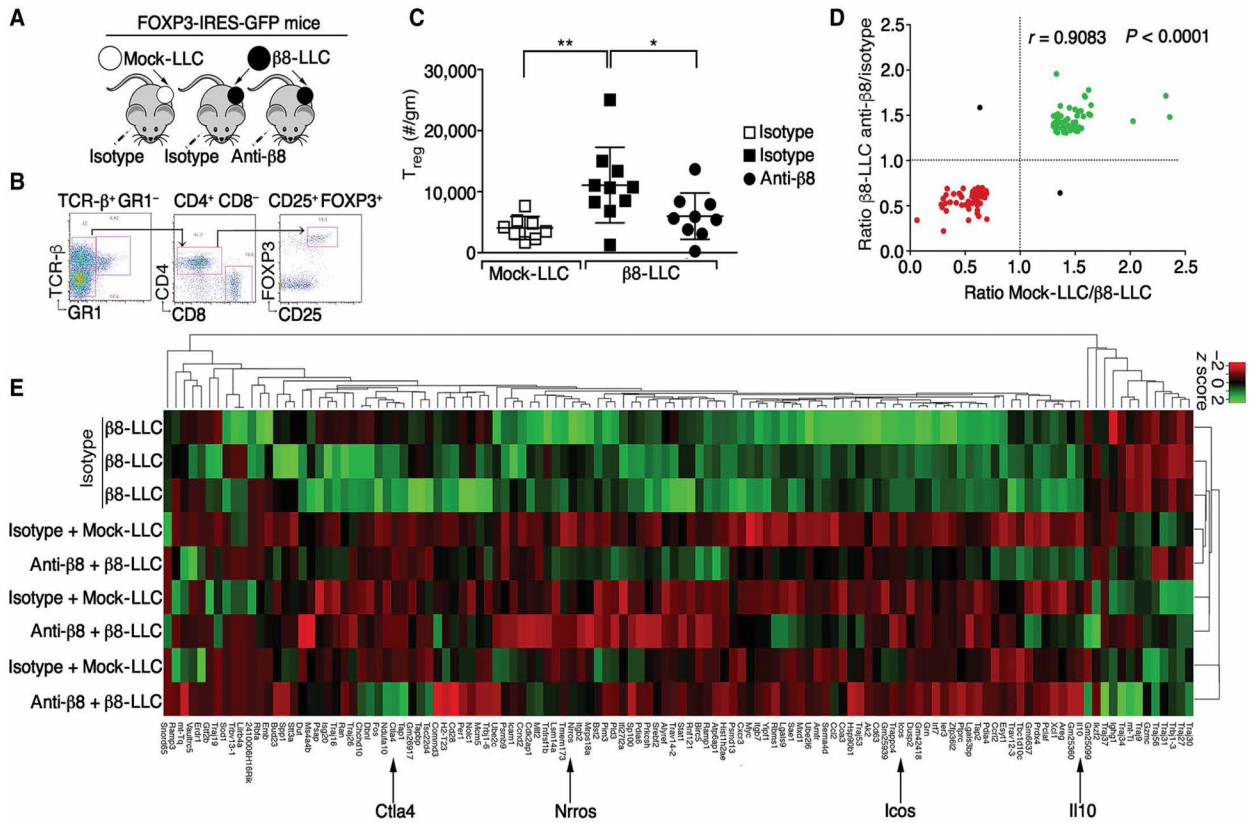
datasets. Shown is SE, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . ns, not significant.

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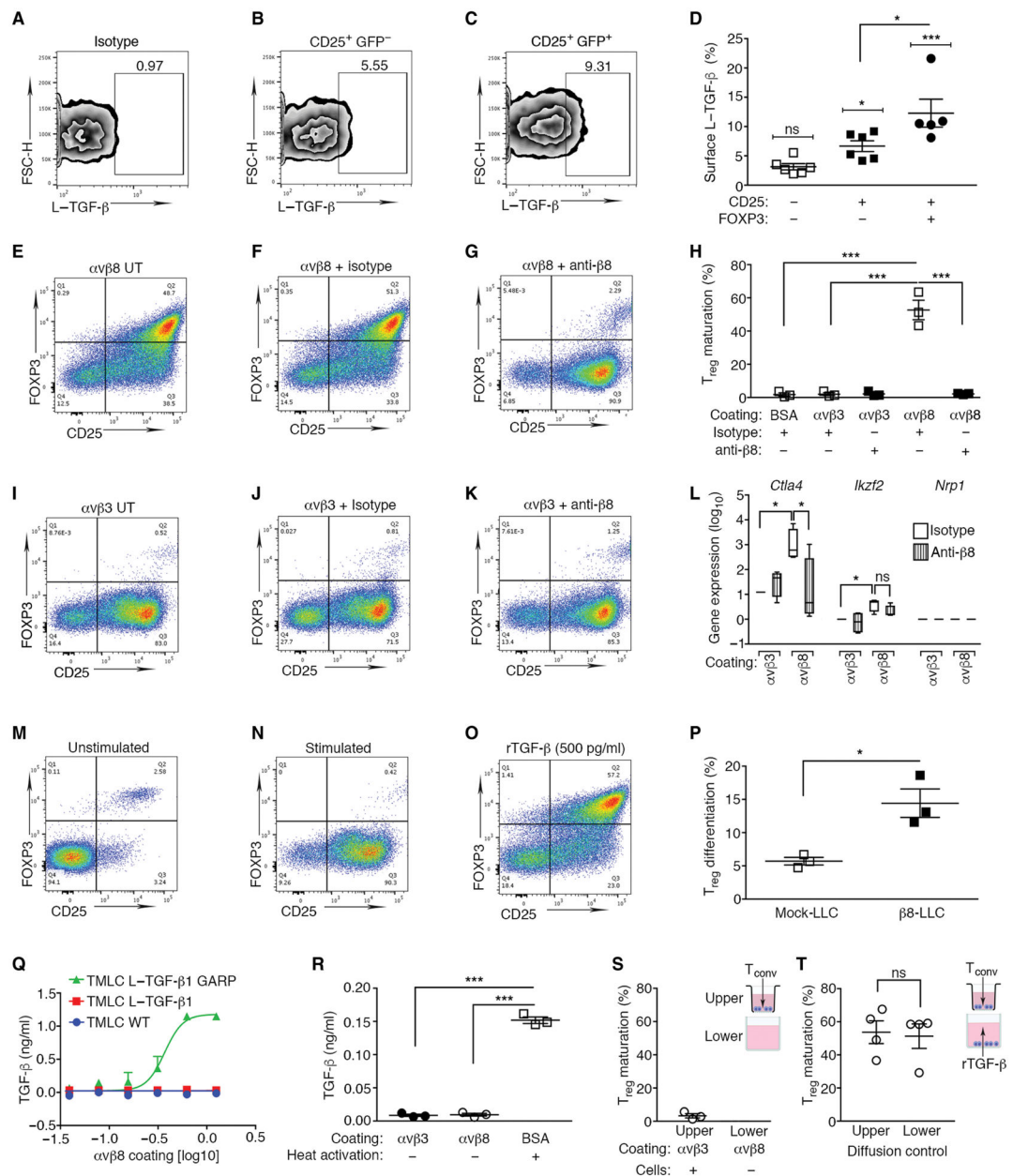
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**Fig. 2. Tumor cell expression of  $\alpha v \beta 8$  drove a distinct immunosuppressive  $T_{reg}$  transcriptome.** (A) Cartoon of model. (B) Gating strategy for  $FOXP3^+CD25^+$  cells, enumerated in (C) as  $FOXP3^+CD25^+$  cells/g (outliers removed) of mock (open boxes) or  $\beta 8$ -LLC tumors treated with isotype (filled boxes) or anti- $\beta 8$  (C6D4, filled circles). (D) Bulk RNAseq of sorted pools (9 to 10 mice per group in three pools) of  $CD4^+GFP^+$  cells. Differential expression plot of 118 most highly expressed genes [ $>50$  average fragments per kilobase million (FKPM)] increased (green) or decreased (red) in expression by at least 30% in  $T_{reg}$  groups treated with anti- $\beta 8$  compared with isotype control or mock-LLC compared with  $\beta 8$ -LLC, with Pearson  $R$  and two tailed  $P$  value. (E) Hierarchical clustering and heatmap of 118 most highly and variably expressed genes shown in (D). Note that  $T_{regs}$  from  $\beta 8$ -LLC isotype-treated tumors are distinct (top three rows) from mock or  $\beta 8$ -LLC  $T_{reg}$  treated with anti- $\beta 8$  (C6D4). Arrows indicate key genes. For multiple comparisons, one-way ANOVA was used followed by Tukey's post-test. \* $P < 0.05$  and \*\* $P < 0.01$ .

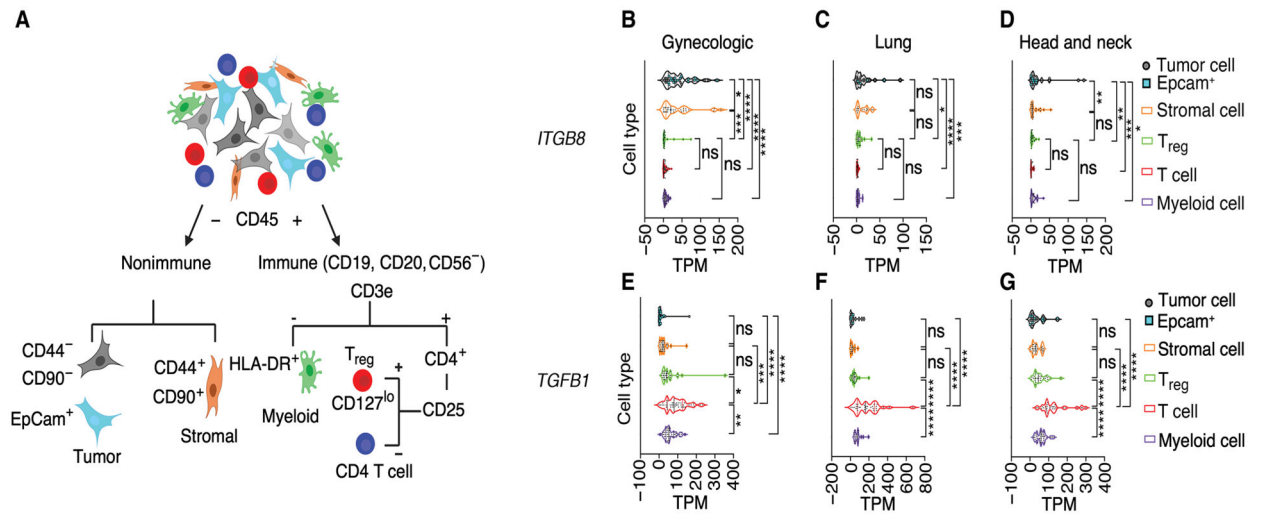


**Fig. 3. Contact of L-TGF-β-presenting non-T<sub>reg</sub> CD4<sup>+</sup> T cells with αvβ8 drove iT<sub>reg</sub> differentiation.**

(A to C) CD4<sup>+</sup> mouse splenocytes express L-TGF-β1 on the cell surface. (A) Isotype-matched negative control for (B) CD4<sup>+</sup>CD25<sup>+</sup> FOXP3<sup>-</sup> T cells and (C) CD4<sup>+</sup>CD25<sup>+</sup> FOXP3<sup>+</sup> T<sub>reg</sub> stained with anti-LAP. (D) L-TGF-β1 surface staining (outlier removed) in CD4<sup>+</sup>CD25<sup>-</sup>FOXP3<sup>-</sup>, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup>, and CD4<sup>+</sup>CD25<sup>+</sup> FOXP3<sup>+</sup> T cell subsets (brackets above each column indicate comparisons relative to isotype control). (E to G) CD4<sup>+</sup> mouse splenocytes undergo iT<sub>reg</sub> differentiation when cultured on immobilized αvβ8, but not on (I to K) integrin αvβ3, or on (M and N) BSA. (E to K and N) CD4<sup>+</sup> splenocytes from *FOXP3*-IRES-GFP mice were activated [anti-CD3 and interleukin (IL-2)] or (M) not stimulated. (O) As positive control, stimulated CD4<sup>+</sup> T cells were treated

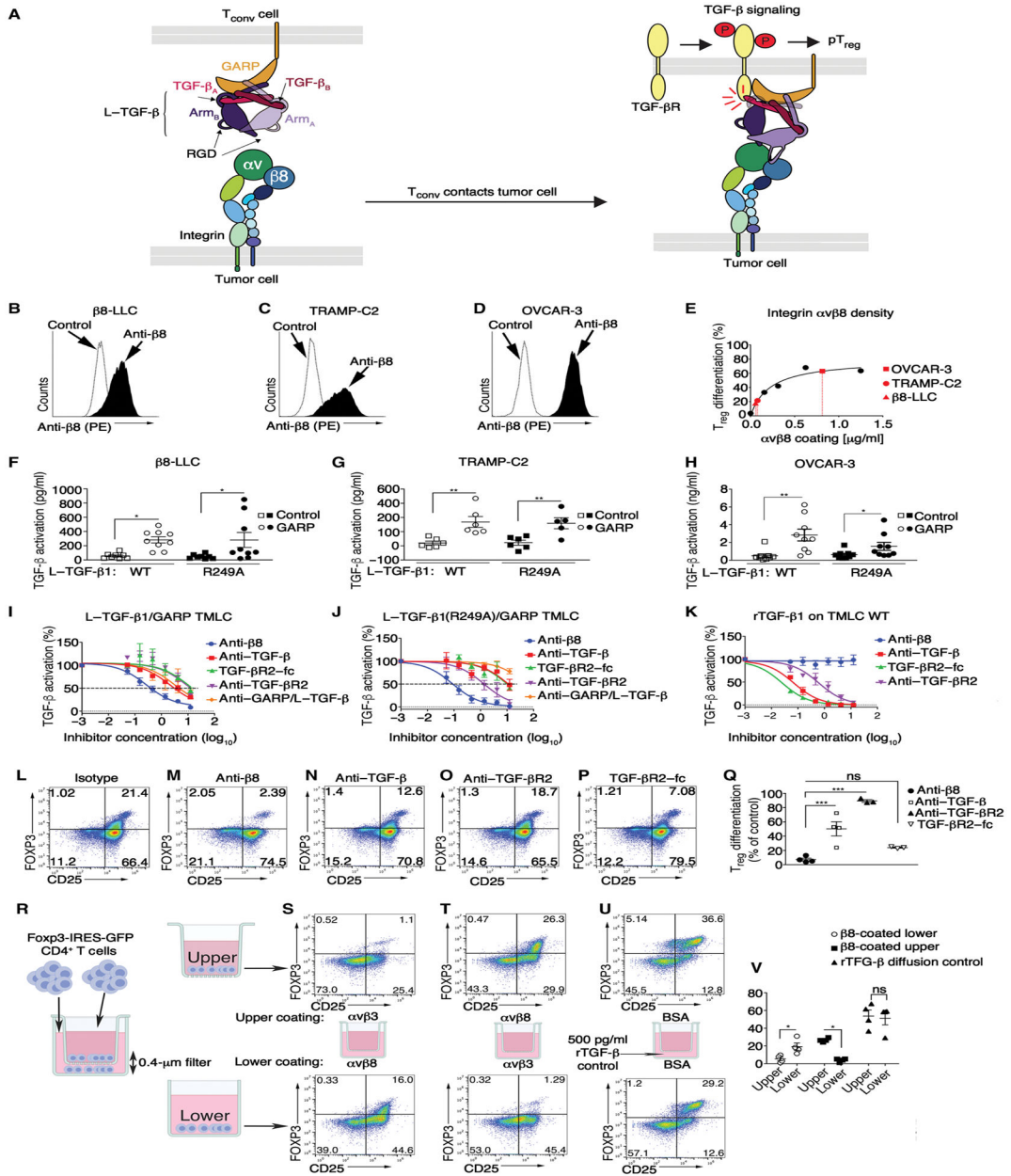


with supraphysiologic levels of rTGF- $\beta$ 1 (500 pg/ml) (65). Representative experiment ( $n = 3$ ) depicts CD4<sup>+</sup> gated T cells stained with anti-CD25 ( $x$  axis) with FOXP3 expression determined by green fluorescent protein (GFP;  $y$  axis). T<sub>reg</sub> (CD4<sup>+</sup>CD25<sup>+</sup> GFP<sup>+</sup>, upper right quadrant). Gating strategy is shown in fig. S3 (A to D). (E and I) Individual wells were untreated (UT), treated with isotype (F and J), or anti- $\beta$ 8 and C6D4 (1  $\mu$ g/ml) (G and K). (H) Lower column ( $n = 3$ ) coating substrate indicated as BSA,  $\alpha$ v $\beta$ 3, or  $\alpha$ v $\beta$ 8. (L) *Ctla4*, *Ikzf2*, or *Nrp1* expression determined by qPCR demonstrates  $\alpha$ v $\beta$ 8-dependent T<sub>reg</sub> differentiation under identical culture conditions as in (F, G, J, and K). Results ( $\log_{10}$ ) normalized to  $\alpha$ v $\beta$ 3 controls. Treatment with isotype (open) or C6D4 (vertically striped) are indicated. (P) Activated T cells cocultured with mock or  $\beta$ 8-LLC cells significantly increased T<sub>reg</sub> differentiation compared with coculture with mock-LLC controls. (Q) TGF- $\beta$  activation over range of  $\alpha$ v $\beta$ 8 coating concentrations reported by WT TMLC (blue), L-TGF- $\beta$ 1-transfected TMLC (red), or L-TGF- $\beta$ 1/GARP-transfected TMLC cells (green). (R) L-TGF- $\beta$  within conditioned media of T<sub>conv</sub> cultured under stimulatory conditions for 48 hours identical to conditions in (N). Reporter cells plated on control substrate  $\alpha$ v $\beta$ 3 or  $\alpha$ v $\beta$ 8 with secreted L-TGF- $\beta$ . Heat (80°C) activation of conditioned media showed total amount of L-TGF- $\beta$  present. (S) Transwell assay determined the importance of cell contact in  $\alpha$ v $\beta$ 8-mediated T<sub>reg</sub> differentiation.  $\alpha$ v $\beta$ 8 was coated on the lower chambers. CD4<sup>+</sup> T cells were plated only into the upper chambers under stimulating conditions (IL-2 and anti-CD3). The upper chamber Transwell surface contains 0.4- $\mu$ m pores, allowing diffusion of soluble mediators from the lower chamber, but not cells. (T) Active rTGF- $\beta$  added to the medium in the lower chamber demonstrates that TGF- $\beta$  freely diffuses from the lower to the upper chamber to induce CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>reg</sub> differentiation. \* $P < 0.05$  and \*\*\* $P < 0.001$  by one-way ANOVA for multiple comparisons followed by Sidak's post-test or unpaired Student's  $t$  test to compare two populations.



**Fig. 4. *ITGB8* was highly expressed in tumor cells, and *TGFBI* was highly expressed in immune cells.**

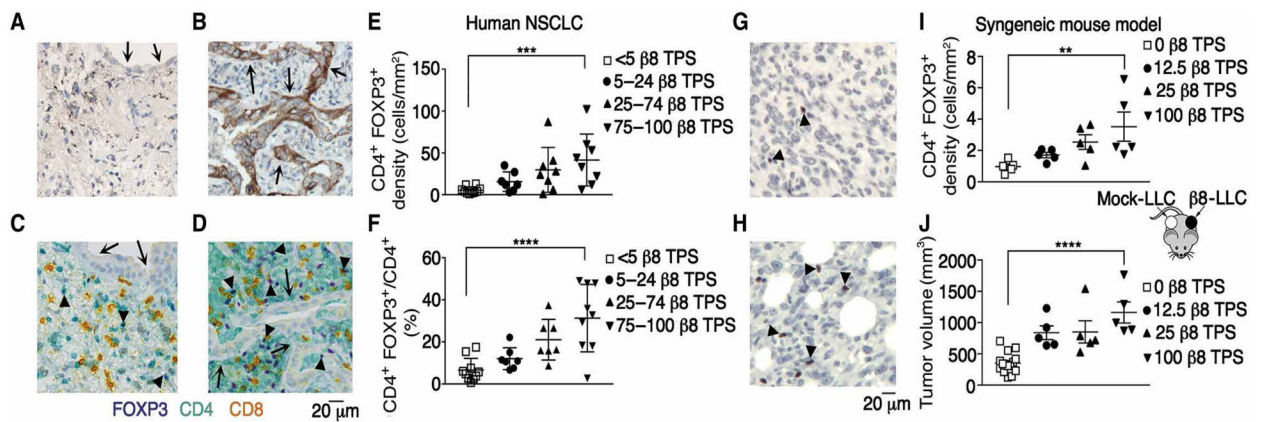
(A) Schematic of sorting strategy to purify tumor, stromal, myeloid, CD4<sup>+</sup> T cell, and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> T<sub>reg</sub> populations from disaggregated human tumors. (B to G). Bulk RNAseq performed on sorted cell populations from cohorts of human gynecologic ( $n = 53$ ), non-small cell lung carcinoma ( $n = 41$ ), or head and neck cancer specimens ( $n = 38$ ) represented as transcript per million (TPM) (normalized read counts to gene length and scaling  $1 \times 10^6$ ). Violin plots of normalized TPM of (B to D) *ITGB8* and (E to G) *TGFBI* of CD44<sup>-</sup>CD90<sup>-</sup> tumor cells (gray circles), some of which stain with anti-epithelial cell adhesion molecule (EpCam) (blue filled squares), CD44<sup>+</sup>CD90<sup>+</sup> stromal cells (filled circles), CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> (green) or CD4<sup>+</sup> T cells (red), or major histocompatibility complex class II (MHCII<sup>+</sup>) (human leukocyte antigen DR isotype, HLA-DR<sup>+</sup>) myeloid cells (purple). All data points were included in the analysis without outlier exclusion and were analyzed for significance by one-way ANOVA for multiple comparisons followed by Dunnett's post-test; means  $\pm$  SD. ns,  $P > 0.05$ ; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .



**Fig. 5. Formation of a localized tumor/T cell  $\alpha v\beta 8$ /L-TGF- $\beta$  signaling complex.**

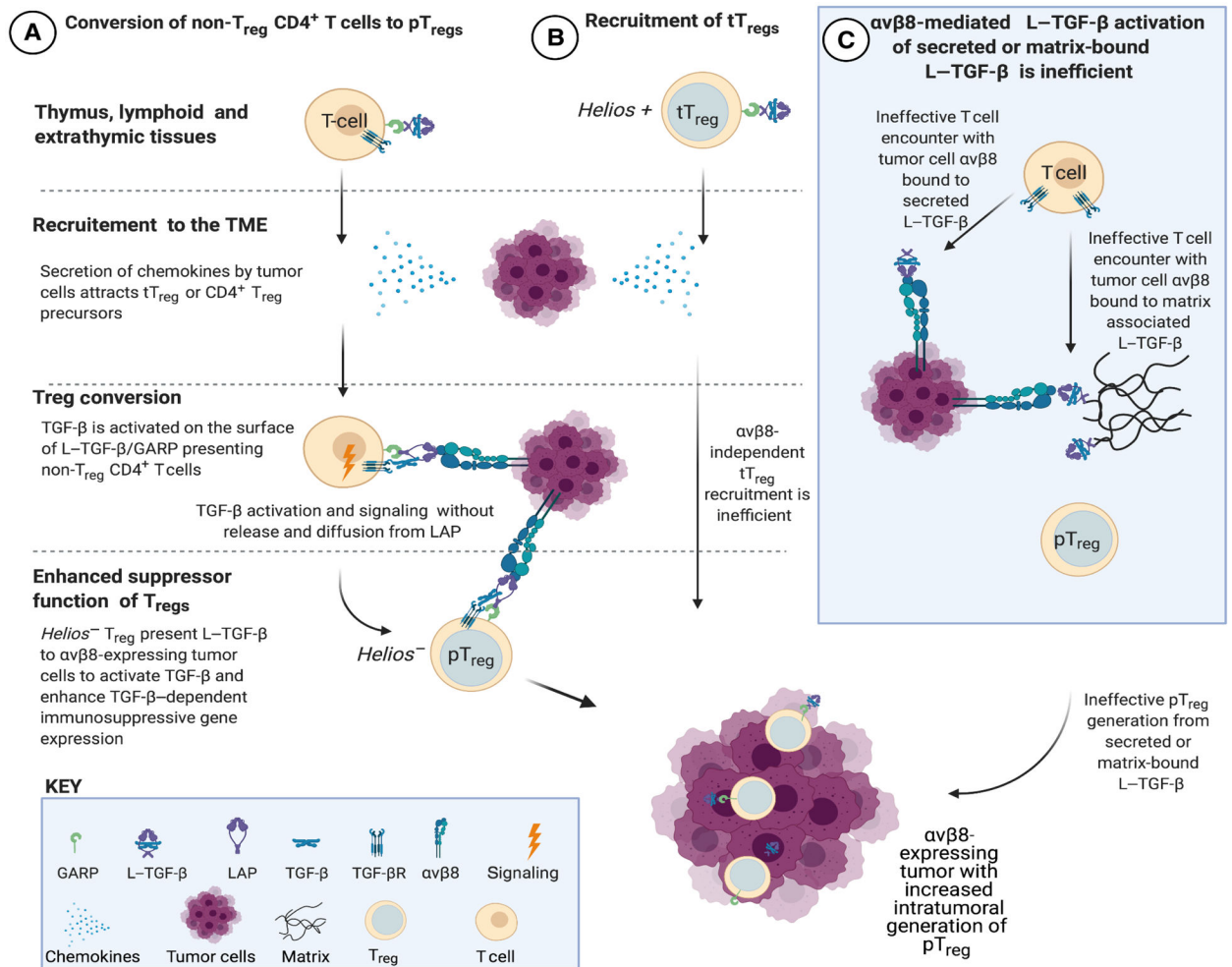
(A) Cartoon of structure-based model of  $\alpha v\beta 8$ -mediated TGF- $\beta$  activation and signaling based on structures of  $\alpha v\beta 8$ /L-TGF- $\beta$  (29, 43), L-TGF- $\beta$ /GARP (51), and TGF- $\beta$ R2/TGF- $\beta$ 1 (66). Integrin  $\alpha v$  and  $\beta 8$  subunits, latency associated peptide (LAP) of dimeric L-TGF- $\beta$  (subunit A + B), dimeric TGF- $\beta$  (subunit A + B), TGF- $\beta$ R2, and GARP color-coded matching annotations. Integrin and GARP/TGF- $\beta$ R2 trans-membrane domains span tumor or T<sub>reg</sub> lipid bilayers, respectively. (B)  $\alpha v\beta 8$  surface expression in  $\beta 8$ -LLC, (C) TRAMP-C2, and (D) OVCAR-3 stained with C6D4 (1  $\mu$ g/ml) compared with iso-type. (E) T<sub>reg</sub> differentiation over a range of  $\alpha v\beta 8$  coating concentrations. Superimposed in red are  $\beta 8$ -LLC (red triangle), TRAMP-C2 (red circle), and OVCAR-3 (red square) according to calculated  $\alpha v\beta 8$  cell surface receptor density. (F to H) WT human L-TGF- $\beta$ 1 or mutant

incapable of producing diffusible TGF- $\beta$ 1 [L-TGF- $\beta$ 1(R249A)] expressed alone (square symbols) or coexpressed with human GARP (circles) in TGF- $\beta$  reporter cells (TMLC) and cocultured with (F)  $\beta$ 8-LLC, (G) TRAMP-C2, or (H) OVCAR-3. Outliers ( $R_{out}$ ) were removed from (F and G). Shown is TGF- $\beta$  activation (means  $\pm$  SEM) determined using rTGF- $\beta$  standard curve of each TMLC line ( $n = 6$ ). **(I to K)** Inhibition curves of anti- $\beta$ 8 (C6D4, blue line) compared with anti-pan-TGF- $\beta$  (1D11, red line), TGF- $\beta$ 2-Fc receptor trap (green line), anti-human GARP/L-TGF- $\beta$  (MHG-8, purple line), or anti-human/mouse TGF- $\beta$ 2 (clone 8322, orange line) generated using (I) WT human L-TGF- $\beta$ 1/human GARP TMLC, (J) human L-TGF- $\beta$ 1(R249A)/human GARP TMLC or control, and (K) WT TMLC cells with 500 pg of rTGF- $\beta$ 1. Shown is percent inhibition relative to no antibody control. Inhibitor concentrations are shown in  $\mu$ g/ml ( $\log_{10}$ ). **(L to P)** iT<sub>reg</sub> differentiation of activated CD4<sup>+</sup> T cells from *foxp3*-IRES-GFP splenocytes on immobilized  $\alpha$ v $\beta$ 8 in the presence of (L) isotype, (M) anti- $\beta$ 8 (C6D4), (N) anti-TGF- $\beta$ 1 (1D11), (O) anti-TGF- $\beta$ 2 (clone 8322), (P) or TGF- $\beta$ 2-Fc. **(Q)** Results enumerated in scatterplots ( $n = 3$ ). **(R)** Schematic of Transwell assay showing that diffusible TGF- $\beta$  has no role in  $\alpha$ v $\beta$ 8-mediated iT<sub>reg</sub> differentiation. CD4<sup>+</sup> T cells plated into upper and lower chambers under stimulating conditions. **(S)**  $\alpha$ v $\beta$ 8 coated on lower,  $\alpha$ v $\beta$ 3 control on upper, or **(T)** vice versa. **(U)** Active rTGF- $\beta$  added to lower chamber media demonstrating diffusion of rTGF- $\beta$  into the upper chamber inducing conversion of non-T<sub>reg</sub> CD4<sup>+</sup> T cells ( $T_{conv}$ ) to CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>reg</sub>. **(V)** Scatterplots ( $n = 4$ ) show gated CD4<sup>+</sup> T cells stained with anti-CD25 ( $x$  axis) with FOXP3 expression determined by GFP ( $y$  axis). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  by one-way ANOVA for multiple comparisons followed by Sidak's post-test.



**Fig. 6. Proportion of  $\beta 8$ -expressing tumor cells correlated with  $CD4^+ FOXP3^+$  T cell number in human and murine lung cancer.**

Representative images of immunohistochemical staining of human ( $n = 32$ ) (A to D) or murine ( $n = 30$ ) tumors (G and H). Immunohistochemical localization of integrin  $\beta 8$  in an independent cohort of human NSCLC with a  $\beta 8$  TPS of (A) <10% compared with (B) a high >50% TPS. Arrows in (B) indicate positively staining tumor cells. (C and D) Multiplex immunohistochemical staining of the same samples shown in (A and B) with anti-CD4 (teal), anti-CD8 (yellow), and anti-FOXP3 (purple). Arrows indicate tumor cells, and arrowheads indicate  $CD4^+ FOXP3^+$  cells. Scale bar, 20  $\mu m$ . (E)  $CD4^+ FOXP3^+$  density according to TPS cutoffs <5%, 5 to 24%, 25 to 74%, and 75 to 100%. (F) Ratio of  $CD4^+ FOXP3^+$  to all  $CD4^+$  cells grouped according to the same cutoffs as in (E) ( $n = 36$ ). (G and H) Immunohistochemical localization of FOXP3<sup>+</sup> cells in (G) mock-LLC compared with (H)  $\beta 8$ -LLC tumors. Arrowheads point to examples of stained nuclei. Scale bar, 20  $\mu m$ . (I)  $T_{reg}$  density depends on proportion of  $\beta 8$ -expressing tumor cells.  $\beta 8$ -LLC cells were mixed with mock-LLC cells in proportions of 1:0 (filled inverted triangles), 1:4 (filled upright triangles), and 1:8 (filled circles) and injected on the left flanks of mice. Mock-LLC (open squares) was injected on the right flank (see cartoon schematic). Shown are  $T_{reg}$  (I) surface density (in mm<sup>2</sup>) and (J) tumor volume (in mm<sup>3</sup>) in mock-LLC tumors compared with tumors with various ratios of  $\beta 8$ -LLC to mock-LLC. For multiple comparisons, one-way ANOVA and  $P$  test for trend were used. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , and \*\*\*\*  $P < 0.0001$ .



**Fig. 7. Proposed mechanisms of  $T_{reg}$  enrichment and differentiation in  $\alpha v\beta 8$ -expressing tumors.** (A) Non- $T_{reg}$   $CD4^+$  T cells expressing L-TGF- $\beta$ /GARP infiltrate tumors in response to chemokines in the TME (21). TGF- $\beta$  cannot interact with TGF- $\beta$ R unless it undergoes activation. L-TGF- $\beta$ /GARP-expressing non- $T_{reg}$   $CD4^+$  T cells undergo  $T_{reg}$  conversion to  $Helios^-$   $pT_{reg}$  after binding to integrin  $\alpha v\beta 8$  expressed by tumor cells. The mechanism of TGF- $\beta$  activation does not require the release and diffusion of TGF- $\beta$ , ensuring that only T cells in contact with  $\alpha v\beta 8$ -expressing tumor cells are converted to  $pT_{reg}$  (29). (B) Thymically derived  $Helios^+$   $T_{reg}$  ( $tT_{reg}$ ) can potentially be recruited to the TME, but this is not evident in  $\alpha v\beta 8$ -expressing tumors. (C) When L-TGF- $\beta$  is soluble or matrix bound, TGF- $\beta$ R are not positioned on the same surface as L-TGF- $\beta$ . Thus, if active TGF- $\beta$  is not released from L-TGF- $\beta$  when exposed to  $\alpha v\beta 8$  bearing tumor cells, then TGF- $\beta$ R-expressing T cells need to find, orient, and overcome steric hindrance to bind to TGF- $\beta$  exposed within the L-TGF- $\beta$  complex. This activation process is less efficient than when L-TGF- $\beta$  and TGF- $\beta$ R are on the same surface (29). Therefore, soluble or matrix-bound L-TGF- $\beta$  is less likely to significantly contribute to  $\alpha v\beta 8$ -mediated  $pT_{reg}$  conversion in the TME. Created in BioRender.