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Supplementary Materials for

A tumor-specific mechanism of T_{reg} enrichment mediated by the integrin αvβ8

Robert I. Seed, Kenji Kobayashi, Saburo Ito, Naoki Takasaka, Anthony Cormier, Jillian M. Jespersen, Jean Publicover, Suprita Trilok, Alexis J. Combes, Nayvin W. Chew, Jocelyne Chapman, Matthew F. Krummel, Jianlong Lou, James Marks, Yifan Cheng, Jody L. Baron, Stephen L. Nishimura*

*Corresponding author. Email: stephen.nishimura@ucsf.edu

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Materials and Methods

Mice and orthotopic tumor models Tumor cells (TRAMP-C2, 5×10^6 and LLC, 1×10^6) were injected subcutaneously into the flanks of C57BL/6 mice and harvested when tumors reached volumes of 1,000–2,000 mm^3. Experiments were terminated when any mouse in any group reached endpoint. Tumor volumes were calculated using the formula (length × $width^2$)/2, which were previously validated in MC38 and LLC models where calculated volumes show a high degree of correlation with tumor weight (*21*). Isotype or anti-β8 (7 mg/kg I.P.) was given when β8-LLC tumors or TRAMP-C2 tumors reached 100 mm^3 (days 4, 7, and 11 for β8-LLC; days 18, 21, 24, and 27 for TRAMP-C2). Mice were treated (7 mg/kg I.P.) with anti-β8 (C6D4 Fab or isotype controls) after β8 LLC tumors were established (~50-100 mm^3). C6D4 Fab and isotype control was administered on day 5, 7, 9, 11 and 13 and C6D4 IgG2a was administered on day 5, 9 and 12. PC61.5.3 or (10mg/kg I.P.) or rat isotype control was given one day after tumor cell injection and then on days 4, 7 and 11.

Reagents Isotype control B5 and anti-SV5 (antibodies were grown and purified using FPLC, as previously described (*60*). Fragments of antigen binding (Fab) were produced as previously described (*29*). F9, is an antibody to the Psi domain of the β8 subunit engineered to be in a murine IgG2a backbone and optimized to bind to formalin-fixed paraffin embedded tissue and produced in CHO-K1 cells (*21*). Antibodies were tested for endotoxin to confirm levels <0.2 EU/μg as determined by LAL method (Genscript). Clone 1D11 (ATCC) is a pan-TGF-β isoform monoclonal antibody that cross-reacts with TGF-β1, -2, and -3 of human, mink, and mouse origin. Antibody 8322 which blocks function of human and mouse TGF-βR2 was a gift of A. Craig, (Queens University,

Kingston, ON, Canada) (*67*). Anti-GARP/L-TGF-β1 (MHG-8) was purchased (Creative Biolabs) (*51*). For sorting TMLC reporter lines anti-HA clone 5E11D8, (GenScript, Piscataway, NJ) or anti-LAP (R&D Systems, AF246) were used. For mouse immune cell staining: Fc receptor blocking antibody (2.4G2) was from BD Biosciences; CD25 (PC61), Gr1 (RB6-8C5), TCRβ (H57-597), CD4 (GK1.5), anti-LAP (TW7-16B4) were all from BioLegend; CD8α (5H10) was from Invitrogen.

For sorting TMLC reporter lines anti-HA clone 5E11D8, (GenScript, Piscataway, NJ) or anti-LAP (R&D Systems, AF246) were used. R-Phycoerythrin Goat anti-mouse conjugated secondary antibody (Jackson laboratories) was used for detection of C6D4 or anti-HA stained cells. For anti-LAP stained cells Streptavidin-APC was used (BioLegend 405207). For mouse Treg detection in tissues, rat anti-mouse FOXP3 was used (FJK-16S, Life Technologies/ eBiosciences).

For human samples: CD45 clone Hl30 (eBioscience 47–0459-42), CD3e clone OKT3 (eBioscience 46–0037-42), HLA-DR clone L243 (eBioscience 48–9952-42), CD56 clone CMSSB (eBioscience 46–0567-42), CD56 clone HCD65 (BioLegend 318304), CD19 clone H1B19 (eBioscience 46–0198-42 and 56–0199-42), CD4 clone RPA-T4 (BioLegend 300550), CD25 clone BC96 (BioLegend 302612), CD25 clone 2A3 (eBioscience 17–0259-42), CD44 clone G44-26 (BD Biosciences 564941), CD90 clone 5E10 (Biolegend 328110), CD127 (Biolegend A019D6), EpCam clone 9C4 (Biolegend 324226), Human FcX (BioLegend). For immunohistochemistry of human samples the following primary antibodies were used: rabbit anti-human β8 (clone F9) (*21*), rabbit anti-human FOXP3 (SP97, Abcam); rabbit anti-human CD4 (SP35, Ventana); rabbit anti-human CD8 (SP57, Ventana), with the following detection antibodies, DISCOVERY

anti-rabbit HQ (760-4815, Ventana) or, DISCOVERY UltraMap anti-Rabbit Alkaline Phosphatase (760-4314). The following human detection antibodies were used: Biotin-SP conjugated anti-mouse IgG or anti-rat (Jackson ImmunoResearch, West Grove, PA 20 712-065-153), labeled polymerase-HRP anti-mouse (Dako K4003). Puromycin, blasticidin and G418 were from Invitrogen.

Isolation and staining of tumor and immune cells. For mouse tumor immune cell isolation, single cell suspensions were prepared from tumors by mincing and digesting with Collagenase NB8 (1 mg/ml; Crescent Chemical Co. Inc.), 0.1% hyaluronidase and 30 µg/ml DNAse I (MilliporeSigma) in RPMI 1640 for 90 minutes in a shaking 25°C incubator. After passing through a 70-µM nylon cell strainer (BD Biosciences), live tumor cells were negatively selected by magnetic beads (030-110-187, Miltenyi Biotec) or infiltrating lymphoid cells were enriched by density gradient centrifugation in Percoll (GE Heathcare) and harvested at the 40%/80% interface.

Fresh patient tumor samples were dissected in aseptic conditions and immediately placed in a sterile container on saline soaked gauze (or submerged in PBS) and transported on ice to the laboratory for evaluation. Samples were minced, transferred to a GentleMACs™ C Tube (Miltenyi Biotec) with 5mg/mL Liberase TL (Roche) and 10mg/mL DNase (Roche) at a volume of 3 ml/gram of tissue, dissociated in the GentleMACS™ Octo Dissociator (Miltenyi Biotec) following the manufacturer's dissociation protocol (Miltenyi Biotec Tumor Dissociation Kit). 10 ml of sort buffer (PBS + 2% fetal calf Serum (FCS) + 2mM EDTA) was added to samples and filtered through a 100 µm filter, spun down, and red blood cells lysed with 175 mM ammonium chloride

followed by neutralization with 10mL PBS. Cell suspension was then counted and stained with the LIVE/DEAD™Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific, L34966) and washed. Cells were then stained with Human TruStain FcX (Biolegend 422302) and an extracellular antibody cocktail. After washing, cells were resuspended in sort buffer and sorted.

Fluorescent activated cells sorting FOXP3+ cells were sorted following Fc receptor blocking (10 µg/ml), followed by staining with fluorochrome-labeled antibodies as described (*21*). For human tumor cell sorting, samples were incubated with Fc receptor blocking solution (Human: BioLegend Human FcX) and stained with antibodies in PBS + 2% FCS + 2 mM EDTA for 30 min on ice. Viability was assessed by staining with Zombie Aqua fixable viability dye (BioLegend, 423102) or Zombie NIR fixable viability dye (BioLegend 423106). All human samples were fixed with BD Cytofix prior to analysis following the manufacturer's protocol. Cell sorting was performed on a BD Fortessa or Aria Fusion flow cytometers available through UCSF Core Immunology Laboratories. Analysis of flow cytometry data was done using FlowJo (Treestar) software. Human tumor immune cells, stromal or tumor cells were isolated and defined as: CD4+Tcells are CD45+, CD4+, CD19-, CD20-, CD56-/CD3e+, and out of that, Treg are CD25+, CD127lo (*68*). Myeloid are CD19-, CD20-, CD56-/CD3e-, and HLADR+. Stromal cells are CD45-, CD44+, CD90+. Tumor cells are EpCam+, CD45-, CD44-, CD90-.

Flow cytometry Flow cytometry acquisition was performed on a LSR II Flow Cytometer or Accuri C6 (BD Biosciences) and cell sorting by FACSAria (BD Biosciences).

RNAseq and qPCR The quality of the input RNA was confirmed by agarose gel electrophoresis and confirmed using the Agilent 2100 Bioanalyzer system. cDNA synthesis and amplification was performed using the SMART-Seq Ultra® v4 Ultra® kit (TakaRa Bio USA, Inc.). The cDNA amplicons were quantified using Qubit (1X dsDNA High Sensitivity Assay) and analyzed with Agilent 2100 Bioanalyzer (High Sensitivity DNA Kit) for quality control. Sequencing libraries were constructed using the Nextera XT DNA Library Preparation Kit (Illumina, Inc.) from the cDNA. The libraries were quantified by Qubit and analyzed for size distribution by Agilent 2100 Bioanalyzer. The libraries were pooled according to its effective concentration and expected data volume, and were sequenced on HiSeq 4000 with 150 bp paired-end reads. Sequences were read after adapter trimming and data quality assessed using FastQC (version 0.11.8) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The computational pipeline for expression quantification was as described (*69*), primarily based on STAR aligner (*70*) and RSEM (*71)*. Reads from RNA-seq were subjected to quality trimming using Trim_Galore (version 0.5.0) and aligned to mouse transcriptome (GRCm38.91) using STAR (version 2.6.1a) (*70*). Duplicated reads were discovered using Picard tools and removed. Gene annotations (version GRCm38.91) were obtained from Ensembl. TPM and FPKM values of genes were estimated using RSEM (version1.3.1) (*71*). FPKM values across the transcriptome and all samples were quantile normalized using the R package "preprocessCore" (https://www.bioconductor.org/packages/release/bioc/html/preprocessCore.html). The quantile normalized FPKM values were used in downstream analyses. Heatmap and unsupervised clustering of all samples using the top 118 most variably transcribed

genes across all samples were calculated based on FPKM cut-off or 50 and a 30% increase or decrease in expression relative to the reference sample (Treg from β8-LLC) using Heatmapper.

For human samples, 50,000 live cells from each sample were directly sorted into lysis buffer by fluorescence activated cell sorting (FACS) to isolate RNA. RNA was isolated using the Ambion Dynabeads mRNA DIRECT purification kit (ThermoFisher Scientific 61012) following the manufacturer's suggested protocol. Isolated RNA was converted into amplified cDNA using the Nugen Ovation RNA-Seq System V2 kit (NuGen 7102-A01) and cDNA was converted into RNAseq libraries using the Illumina Nextera XT DNA Library Prep kit (Illumina FC-131–1096), following the manufacturer's suggested protocol. Libraries are then pooled and sequenced via a single-read 65bp MiniSeq (Illumina) run and libraries containing >10% of reads aligned to coding regions and >1000 unique reads in the total library were selected for further sequencing. RNAseq libraries that met the previous quality control criteria were submitted to the University of California, San Francisco Center for Advance Technology for paired-end, 150bp (PE150) sequencing on the Novaseq6000 (Illumina). Using the Burrows-Wheeler Aligner, sequenced RNAseq library reads were then aligned against a reference of ribosomal RNA (rRNA) and mitochondrial RNA (mtRNA) to deplete the dataset of rRNA and mtRNA. The remaining reads were then aligned to the Ensembl GRCh38.85 transcriptome build and genome using Spliced Transcripts Alignment to a Reference (STAR). Picard duplication metrics were obtained using MarkDuplicates. Alignment statistics were obtained using samtools flagstats. Alignment metrics were obtained using Picard CollectRnaSeqMetrics. QC plots were then generated and transcripts per

million (TPM) were calculated by normalizing read counts to gene length (RPK), then scaling RPK by a scaling factor of 1 million to get TPM. TPM for individual genes were used for analyses and in all cases TPMs were normalized by z-score allowing comparison between samples sequenced at different times. RNAseq libraries with $>1\times10^6$ protein-coding, non-mtRNA read counts and expression levels of at least 7 of the 10 Eisenberg housekeeping (EHK) genes above minimum threshold were used to analyse RNA expression. qPCR was performed as previously described, with the exception that a gene specific primer was used for *ikzf2* cDNA preparation (*21*).

Treg maturation assays: Splenocytes from *FOXP3*-IRES-GFP mice were harvested aseptically, and subject to red cell lysis as per standard protocols and bead purified (*21)*. Wells of 24-well tissue culture wells plates were coated with 1 μg/ml αvβ8tr or control substrate (1 μg/ml αvβ3tr or BSA) in PBS supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+} for 2 hrs at room temperature. Purified CD4+ lymphocytes were then plated into culture wells at a density of $1x10^6$ per ml in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum, 1% non-essential amino acids, 1% penicillin/streptomycin 135 U/ml recombinant murine IL-2 2 mM all-trans retinoic acid and 55 μM 2-mercaptoethanol. CD4+T-cells were incubated for 72 hours under standard culture conditions (37°C, 5% CO_2) before phenotyping via flow cytometric analysis. For transwell diffusion assays, cells were plated in to each chamber of the transwell culture wells containing a 0.4 μm filter (Corning Transwell 3413) using the same conditions as above. rTGF-β was plated into the lower chamber to demonstrate that the inserts permitted diffusion of TGF-β into the upper chamber. To test whether TGFβ was released following αvβ8 mediated TGF-β activation αvβ8 was coated onto the lower or

upper surface and Treg phenotype was measured to assess Treg differentiation of cells in contact with αvβ8 versus cells separated by the diffusible barrier.

Lymphocyte suppression assays 8 x 10^5 freshly labelled cells were stimulated using anti-CD3/CD28 dynabeads at a ratio of 1:1(beads:Cells) and plated in round-bottom 96-well culture plates in RPMI-1640 supplemented with 10% heat inactivated serum, 1% non-essential amino acids, 1% penicillin/streptomycin. Labelled CD4+ cells were then co-cultured with αvβ8 generated Tregs or control cells at ratios ranging between 1:1 and 8:1 (labelled CD4+T-cells: Tregs). Tconv proliferation was measured using flow cytometry after 4 days. Proliferating cells were identified by gating out non-viable cells (Sytox red positive) and excluding doublets.

TGF-β bioassays.We used a cell intrinsic TGF-β activation system based on stable transfection of TMLC (Amaxa) with a vector containing either a WT human TGF-β1 IRES GFP, or a human TGF-β1 (R249A) IRES GFP cassette with puromycin resistance to obtain TMLC cells expressing WTL-TGF-β1 either capable of dissociating into LAP and mature TGF-β or not, due to the R249A mutation that normally allows furin cleavage of LAP from mature TGF-β (*64*). Human TGF-β1 IRES GFP or human TGF-β1 (R249A) IRES GFP TMLC cells were sorted for equal expression using GFP fluorescence and do not present any L-TGF-β on their cell surface (*29*). After stable transfection of these lines with a HA-GARP construct with a blastacidin resistance cassette followed by selection and sorting resulted in high surface expression of TGF-β1/GARP or TGF-β1 (R249A)/GARP, as measured by anti-HA or anti LAP (*29*).

Immunohistochemical analysis of murine and human tumors Formalin-fixed paraffin-embedded (FFPE) tissue sections were deparaffinized, and antigen retrieval

was performed using either 0.4% pepsin (#P-7125, Sigma) at 37°C for 5 min in a water bath, or a Tris-EDTA buffer, pH 9.0 in a pressure cooker (97°C for 20 min), Reveal Declocker (Biocare Medical, 10 Pacheco, CA) in a pressure cooker (95°C for 20 min), or a combination of pepsin and Reveal Decloaker followed by treatment with peroxidase blocker (K4007, Dako). Non-specific binding was blocked with a protein blocking solution (X0909, Dako) and Avidin/Biotin Blocking kit (004303, Thermo Fisher Scientific). Sections were stained with anti-mouse β8 (clone F9), B5 (anti-human β8 which does not work in FFPE immunostaining and thus used as isotype control for C6D4), anti-CD4, anti-CD8 or anti-FOXP3, followed by detection with Biotin-SP conjugated anti-mouse IgG or anti-rat (Jackson ImmunoResearch, West Grove, PA 20 712-065-153), streptavidin-HRP, for 1 hr at RT, washed, and detected with DAB, or for β8 detection, DAB was omitted and slides were treated with labeled polymerase-HRP anti-mouse (Dako K4003) followed by Tyramide signal amplification solution A at a dilution of 1:100 (Life Technologies) prior to DAB detection, as described (*21*). For multiplex immunostaining the Ventana Discovery platform was used with the following detection agents (all Ventana Discovery): Purple HQ (760-229), Teal HQ (760-247), or Yellow AP (760-239).

Immunohistochemical scoring: All samples were blinded as to group, isotype, or test antibody. Digital images of blinded tumor groups were taken (Spot Imaging) and assessed for staining. For human samples, digital images of isotype or F9, CD4, CD8 or FOXP3 were taken (5-10 per tumor at 200x) and assessed for positivity using PD-L1 scoring criteria, as previously described (*21*). Briefly, tumor cells with membrane staining were counted and compared to numbers of tumor cells without membrane

staining and tumor proportion score (TPS) determined as stained cells per total tumor cells (*21*). Ambiguous staining results (i.e. faint membrane staining, or heavy background) were resolved by comparison to isotype stained slides. For mouse tumors, FOXP3+ cells were assessed from 10 random digital images (Spot Imaging) as described (*21*). The mean immune cell count was determined from cell number/mm^2, and total FOXP3+ cells / tumor cells calculated based on tumor volume, as described (*21*).

Determination of receptor density CHO WT or CHO transfected with human $\alpha v\beta 8$ were plated onto 96 well cell culture plates in triplicate in DMEM with 10% FCS over a concentration range of (5-30 x 10^3 cells/well), and recombinant $\alpha v\beta 8$ ectodomain (10-5000 ng/ml PBS) added to wells on the same plate, and coated wells blocked with BSA, as above. After cells were fully attached (incubation at 37°C in a humidified incubator, 5% CO_2), the medium was removed, the cells washed in PBS and cells fixed with 4% paraformaldehyde, washed and non-specific binding sites blocked with BSA. Cell associated or recombinant $\alpha v\beta 8$ was detected with clone F9 (2 μg/ml), followed by anti-mouse HRP and colorimetric detection. Recombinant receptor concentration was calculated based on the following assumptions, binding capacity of 200 ng/cm^2, and 50% coating efficiency (*72*). Cell surface receptor density/cell was estimated based on the recombinant $\alpha v\beta 8$ standard curve. $\alpha v\beta 8$ receptor density/cell was determined for $\beta 8$-LLC, TRAMP-C2 and OVCAR3 cells by calibration of $\alpha v\beta 8$ mean fluorescence intensity measured by staining with C6D4-PE.

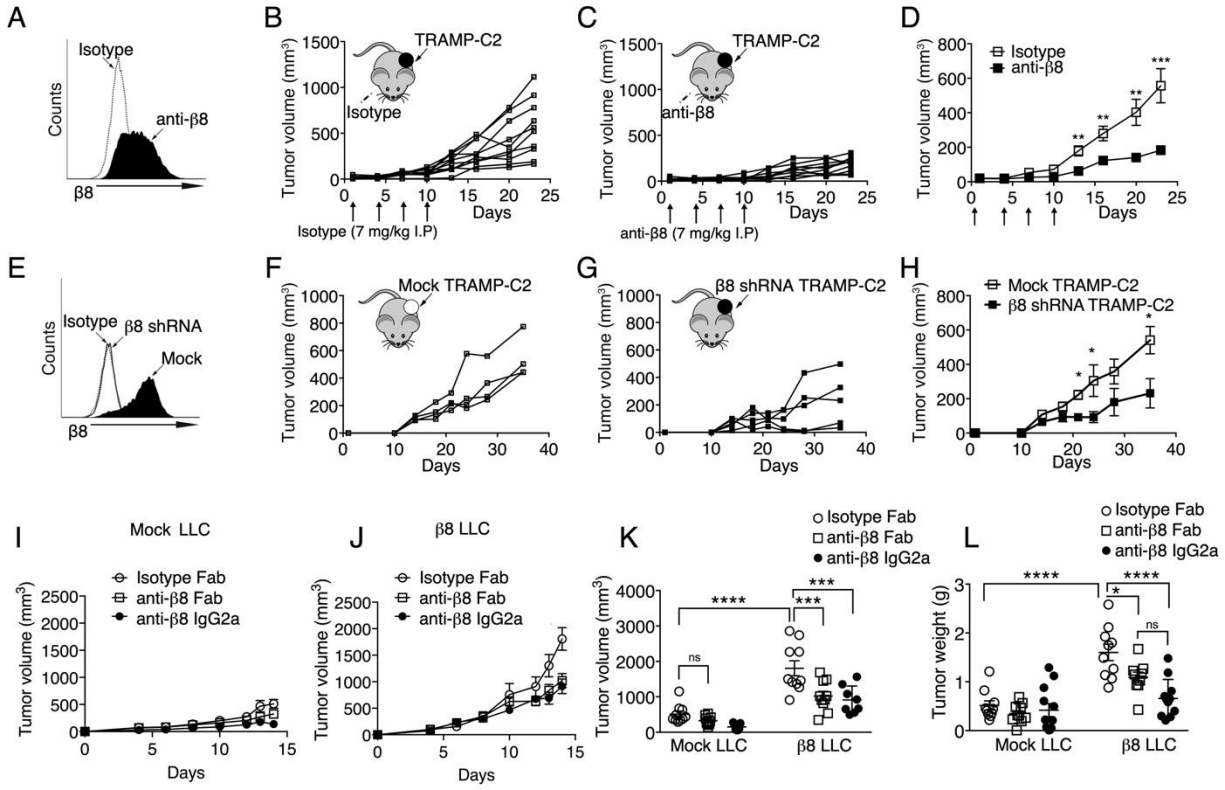

Fig. S1 β8 expression by tumor cells drives tumor growth, which is blocked by C6D4 Fab in vivo. A) Representative histogram plots depicting the endogenous expression levels of αvβ8 on the cell surface of TRAMP-C2 cells stained with isotype (open) or anti-β8, C6D4 (filled). **B-D**) Growth of TRAMP-C2 tumors shown in spider plots (**B, C**) from mice treated with isotype control (**B**), or C6D4 (**C**) or shown as averages of tumor volume (**D**). Antibodies were injected (7 mg/kg I.P.) on the days indicated by arrows. Cartoons depict the model and location of the tumor. Tumor growth was followed until the first tumor reached endpoint (day 24). **E**) Representative histogram plots showing the effects of stable shRNA knockdown of *itgb8* compared to non-mammalian shRNA controls (mock) compared to staining with isotype control. **F-H**) Growth of mock shRNA TRAMP-C2 tumors (**F**) or β8 shRNA (**G**) shown in spider plots or shown as averages of tumor volume (**H**). Cartoons depict the model and location of the tumor. Tumor growth was followed until the first tumor reached endpoint (day 37). Unpaired Student's t-test was used to compare two groups. *p<0.05, **p<0.01, ***p<001 **I**-**L**) mock LLC or β8 LLC cells (1 x 10^6) were injected S.C. into opposing flanks of C57Bl/6 mice and tumor outgrowth measured 3

times /week until a tumor in any group reached endpoint, at which point mice were euthanized and tumors weighed.

Mice were treated (7 mg/kg I.P.) with anti-β8 (C6D4 Fab or isotype controls) after β8 LLC tumors were established (~50-100 mm^3). C6D4 Fab and isotype control was administered on day 5, 7, 9, 11 and 13 and C6D4 IgG2a was administered on day 5, 9 and 12. Average LLC tumor volume curves are shown for mock LLC (**I**) and β8 LLC (**J**) and average β8 LLC tumor volume on day 14 are presented in **K**. Tumor weights at day 14 are shown in **L**. One-way ANOVA was used for multiple comparisons and Tukey's post-test used to determine where the differences lay. Student's unpaired t-test was used for comparison of two data sets. Error bars represent standard error, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

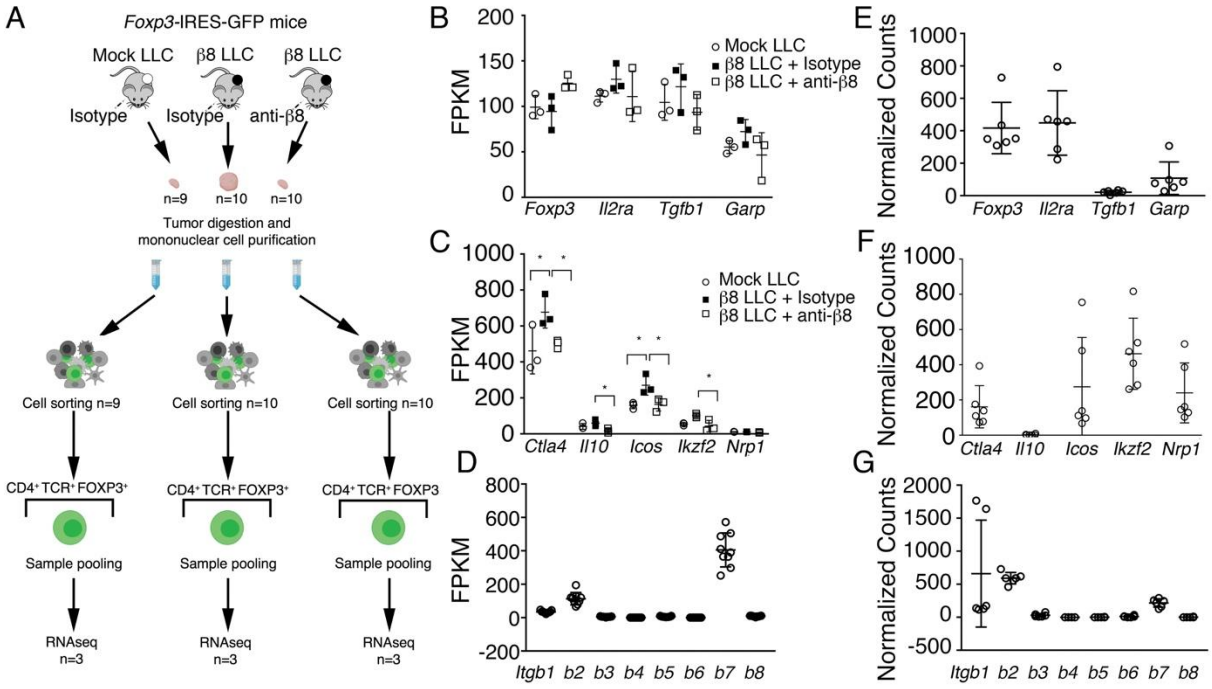

Fig. S2 Tumor T_{reg} isolation and expression of select T_{reg} genes by RNAseq in this report compared with single-cell RNAseq from splenic T_{reg}, refers to Fig. 2. A) Cartoon depicting the sorting scheme to isolate CD4+ TCRβ+ FOXP3+ T-cells from LLC tumors for RNAseq analysis. Sorted samples were pooled into groups of three before RNA isolation and RNAseq, as depicted. **B-D**) Scatter plots depicting the relative expression of key genes associated with Treg differentiation, **B)** *FOXP3*, *Il2ra* (CD25), *Tgfb1* and *Garp* (*Irrc32*), **C**) *Ctla4*, *Il10*, *Icos*, *Ikzf2* (helios), and *Nrp1*, comparing Treg from mock LLC (open circles), β8 LLC + isotype (filled squares), or β8 LLC +anti-β8, C6D4 (open squares). **D**) Integrin β-subunit expression (*Itgb1-8*) by Treg in each group were similar and are grouped together in a single column (n=9). Gene expression is shown as FPKM. **E-G**) Expression of the same genes shown in **B-D** in murine splenic Tregs from a publicly available single cell RNAseq dataset (*73*), where each data point in scatterplots represents average normalized transcript counts from each of 6 discrete Treg clusters. For multiple comparisons one-way ANOVA was used followed by Dunnett's post-test to identify differences. *p<0.05

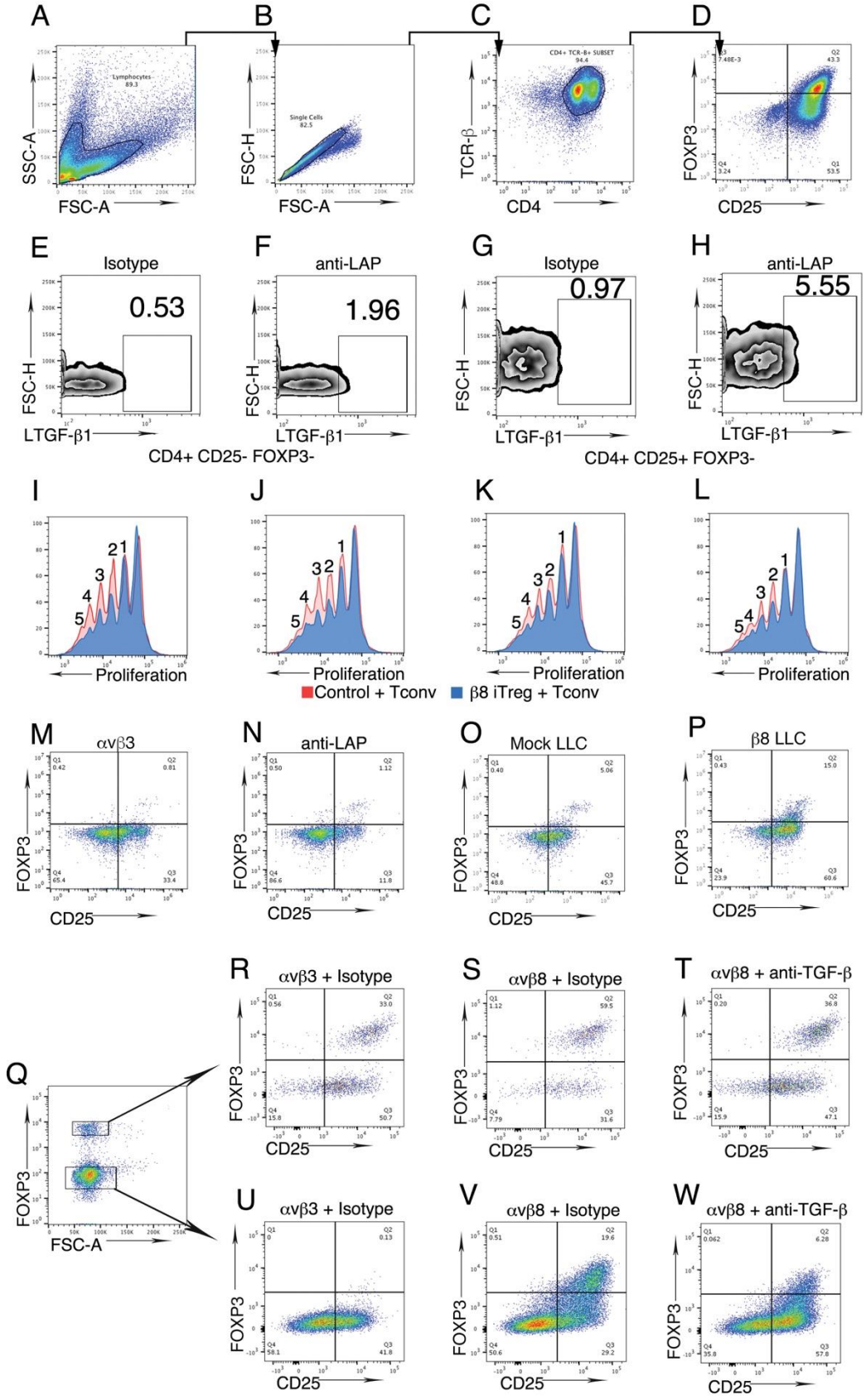
A
B
C
D
SSC-A
FSC-A
Lymphocytes
89.3
FSC-H
FSC-A
Single Cells
82.5
TCR-β
CD4
CD4+ TCR-B+ SUBSET
94.4
FOXP3
CD25
E
Isotype
0.53
F
anti-LAP
1.96
G
Isotype
0.97
H
anti-LAP
5.55
FSC-H
LTGF-β1
CD4+ CD25- FOXP3-
CD4+ CD25+ FOXP3-
I
J
K
L
Proliferation
Control + Tconv
β8 iTreg + Tconv
M
αvβ3
N
anti-LAP
O
Mock LLC
P
β8 LLC
FOXP3
CD25
Q
FSC-A
R
αvβ3 + Isotype
S
αvβ8 + Isotype
T
αvβ8 + anti-TGF-β
U
αvβ3 + Isotype
V
αvβ8 + Isotype
W
αvβ8 + anti-TGF-β

Fig. S3 Non-T_{reg} $CD4^+$ T cells express cell surface L–TGF-β, are converted to T_{reg} by contact with β8-LLC cells, and are the source of suppressive iT_{reg} generated on αvβ8, supports Fig. 3. A-D) Representative scatter plots depicting the gating strategy used to enumerate Treg in Fig. 3. **E-H**) Representative scatter plots showing gating strategy used to identify L-TGF-β1 expression on the surface of conventional T-cells (Tconv) using anti-LAP. **I-L**) Histogram plots depicting Tconv proliferation measured in co-culture with differing ratios of Treg generated on αvβ8 (blue peaks) or control non-Treg CD4+ T-cells (red peaks) or. Ratios of Treg:Tconv are **I**) 1:1, **J**) 1:2, **K**) 1:4, **L**) 1:8. Cell doubling corresponds to distinct peaks of decreasing CFSE fluorescence intensity (number of cell divisions are labeled above the peaks). **M-N**) Representative scatter plots showing that stimulated (anti-CD3, rIL-2) CD4+ T-cells plated on immobilized **M**) αvβ3 or **N**) anti-LAP do not show increased Treg differentiation, which illustrates that ligation of the cell surface L-TGF-β/GARP complex is not sufficient to induce Treg differentiation. **O, P**) Representative scatterplots show increased numbers of CD25+ FOXP3+ Tregs when co-cultured with **P**) β8 LLC, compared to **O**) mock LLC as shown in Fig. 3P. **Q**) Representative scatter plot showing the sorting strategy used to enrich FOXP3+ cells from Tconv. **R-T)** FOXP3+ nTregs or **U-W)** FOXP3- Tconv were cultured under T-cell stimulatory conditions in the presence of immobilized **R, U)** αvβ3 or **S,T V,W)** αvβ8 treated with **S,V)** isotype control or **T,W)** the pan TGF-β blocking antibody 1D11 (anti-TGF-β).

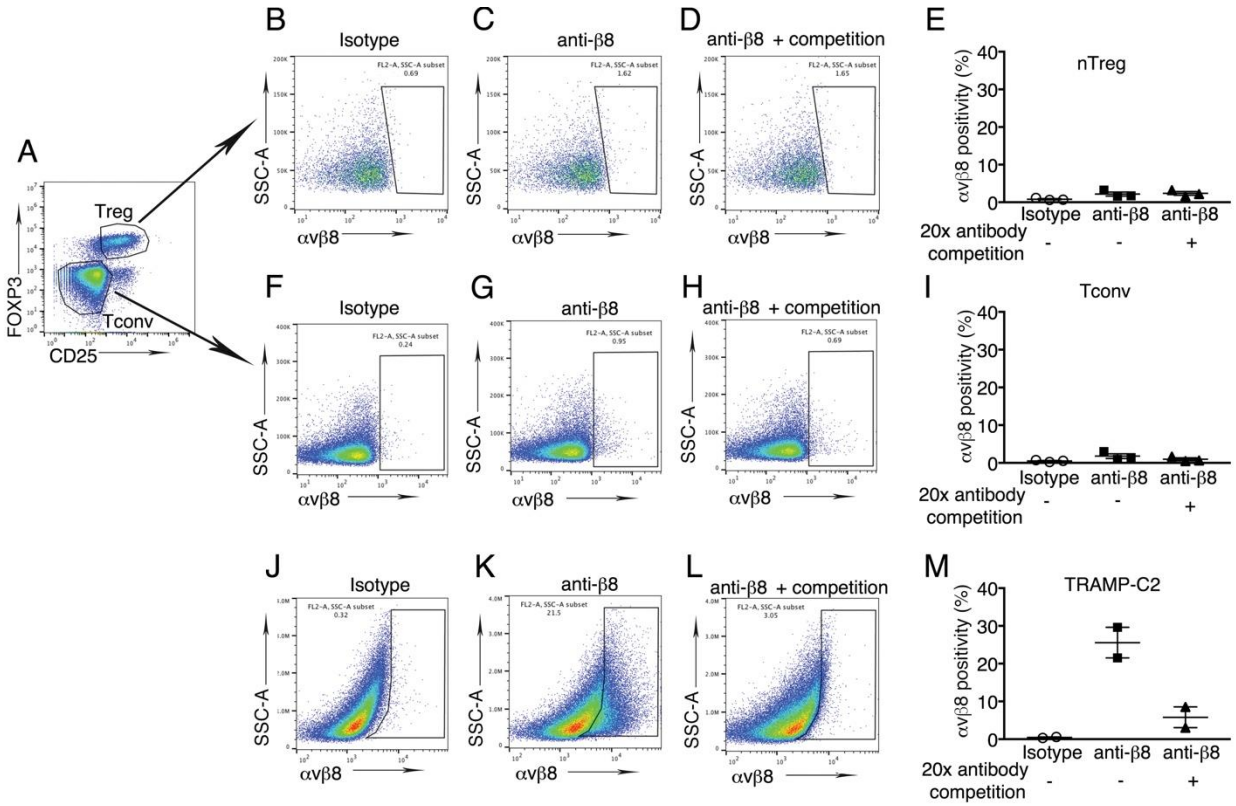

Fig. S4. Murine nT$_{regs}$ do not express detectable levels of cell-surface αvβ8. Supports Fig. 3 **A**) murine ntregs or Tconv were phenotyped based on FOXP3 and CD25 expression as depicted. **B,C**) nTregs or **F,G**) Tconv were stained with **B,F**) isotype or **C,G**) C6D4-PE (5 μg/ml) to assess αvβ8 surface expression. **D,H**) To confirm staining specificity **D**) ntreg or **H**) Tconv were also stained with C6D4-PE in the presence of a 20-fold molar excess of unlabeled C6D4. **E,I**) summary of αvβ8 staining (n=3). **J-M**) Murine prostate cancer cells (TRAMP-C2) were stained, as above, with **K**) C6D4-PE or **L**) C6D4-PE in the presence of 20-fold molar excess of unlabeled C6D4 compared to **J**) isotype matched control antibody. **M**) summary of αvβ8 staining (n = 2).

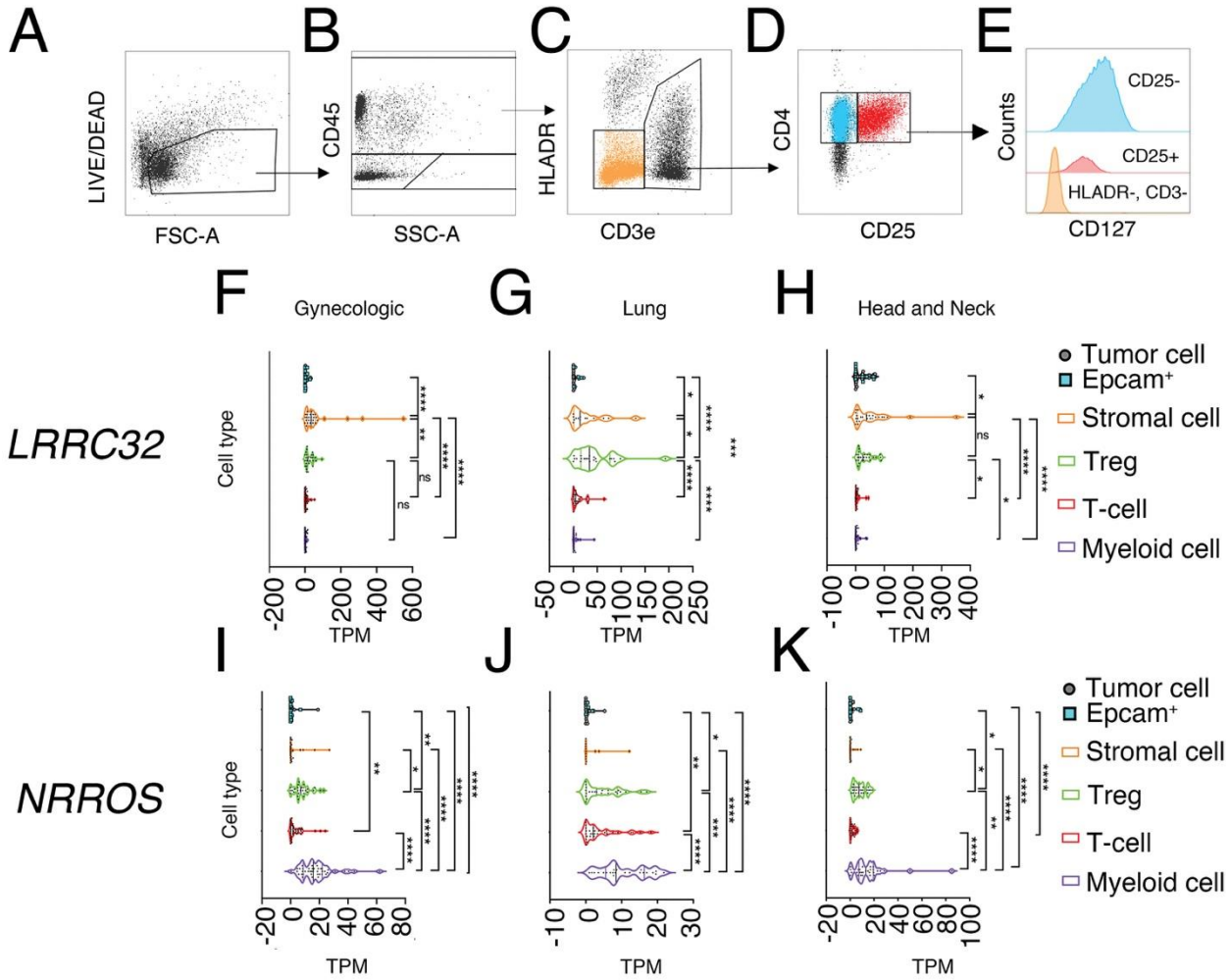

Fig. S5 ***LRRC32*** **(*GARP)* is most highly expressed by T_{reg} and stromal cells and *NRROS* by myeloid cells, related to Fig. 4.** **A-E**) Gating strategy used to sort CD127lo, CD25+ FOXP3+ human Treg. **F-K**). Bulk RNAseq was performed on sorted cell populations from a cohort of **F, I**) human gynecologic (n = 47), **G,** J) non-small cell lung carcinoma (n = 38), or **H, K)** head and neck cancer specimens (n = 23). Shown are bar scatterplots of normalized TPM of **F-H**) *GARP* and **I-K**) *NRROS* of CD44- CD90- tumor cells (grey circles) some of which stain with anti-EpCam (blue filled squares), CD44+ CD90+ stromal cells (orange), MCD4+ CD25+ Treg (green), or CD4+ T-cells (red) or MHC II + (HLA-DR+) myeloid cells (purple). Shown is S.D. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one-way ANOVA for multiple comparisons followed by Dunnett's post-test to find where the differences lay.