Title: V γ usage distinguishes pro- and anti-tumor intestinal $\gamma\delta$ T cell subsets

Authors: Bernardo S. Reis^{1†}, Patrick W. Darcy¹, Iasha Z. Khan¹, Olawale Eleso¹, Caixia Zhu^{1,2}, Marina Scherthanner¹, Ainsley Lockhart¹, Aubrey Reed¹, Juliana Bortolatto¹, Tiago B. R. Castro¹, Angelina M. Bilate¹, Sergei Grivennikov³, Daniel Mucida^{1,4,†}

Affiliations:

¹ Laboratory of Mucosal Immunology, The Rockefeller University, New York, NY, 10065, USA.

² Present address: Key Laboratory of Medical Molecular Virology, School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Shanghai, 200032 China.

³ Department of Medicine and Department of Biomedical Sciences, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA, 90048, USA.

⁴ Howard Hughes Medical Institute, The Rockefeller University, New York, NY, 10065, USA.

[†] Correspondence: breis@rockefeller.edu (B.S.R.), mucida@rockefeller.edu (D.M.)

Abstract: $\gamma\delta$ T cells physiologically scan the intestinal epithelium, representing a substantial fraction of infiltrating lymphocytes in colorectal cancer (CRC), albeit their role in CRC remains unclear. Using murine CRC models, we found that most $\gamma\delta$ T cells in pre- or non-tumor colon express $V\gamma 1^+$ or $V\gamma 7^+$ and exhibit a cytotoxic profile. Targeting these $\gamma\delta$ T cell subsets, as well as conditionally interfering with $\gamma\delta$ T cell function at early stages of tumorigenesis led to heightened tumor development, suggesting anti-CRC functions for $V\gamma 1^+$ and $V\gamma 7^+$ subsets. In contrast, $ROR\gamma t^+$ $\gamma\delta$ T cell subsets, including $V\gamma 4^+$ and microbiota-dependent $V\gamma 6^+$, accumulated during CRC progression. Conditional deletion of $ROR\gamma t$ or $V\gamma$ chains revealed redundant roles for IL-17–producing $V\gamma 4^+$ and $V\gamma 6^+$ $\gamma\delta$ T cells in promoting tumor growth. Our results uncover pro- and anti-tumor roles for $\gamma\delta$ T cell subsets.

Intestinal intraepithelial lymphocytes (IELs) comprise a large T cell population located at the critical interface between the core of the body and the intestinal lumen, which is constantly exposed to food, commensal microbes, and pathogens. Conversely, previous observations suggest an important role for IELs as a first line of immunity against pathogens in both mice and humans (1-4). Among the main IEL subsets in mice or humans, T cells harboring the $\gamma\delta$ T cell receptor (TCR) display epithelial surveillance and anti-pathogen activities that are modulated through crosstalk with the intestinal epithelium, highlighting that $\gamma\delta$ IELs are finely tuned to local epithelial signals (3, 5). In addition to their role in immune surveillance against enteric infections, sequencing analysis points to a $\gamma\delta$ T cell gene signature as the most favorable prognostic factor among tumor-infiltrating leukocytes across cancer types, including colorectal cancer (CRC) (6). CRC is the second most deadly cancer in the United States, affecting over 140,000 people each year, killing approximately 50,000 in the US (ACS Inc., 2020). The cumulative risk of IBD patients to develop CRC can reach 20%, although most CRCs develop in patients without underlying inflammation. In both cases, tumor-elicited inflammation triggered by epithelial disturbances and microbial invasion is essential for survival of transformed cells, and tumor growth (7-10). Here, we addressed whether epithelial surveillance by $\gamma\delta$ T cell subsets helps prevent damaged ECs from progressing into cancer and whether additional $\gamma\delta$ T cell subsets play a contrasting role, accelerating tumor progression.

To assess the role of $\gamma\delta$ T cells in CRC, we employed two distinct CRC-mouse models: the chemical AOM-DSS colitis-associated cancer (CAC) (11) and the genetically inducible APC deficiency (9, 12, 13) (Fig. 1A). Our initial characterization of $\gamma\delta$ T cells in naïve animals confirmed (14-16) a dominance by V γ 1 and V γ 7-expressing subsets, followed by V γ 4⁺ and V γ 6⁺ subsets at a low frequency, and changes in the expression of CD8 $\alpha\alpha$ homodimers according to proximal-distal and villus-crypt axes (fig. S1A to C). In colitis-associated CRC (AOM-DSS), we observed that tumor-infiltrating $\gamma\delta$ T cells adopt both anti- and pro-tumor phenotypes, with increased expression of CD107 α and IFN- γ [associated with anti-tumor responses (8, 17)] as well as IL-17 [associated with pro-tumorigenic function (9, 17)] (Fig. 1B). CD4⁺

TCR $\alpha\beta^+$ cells also display IFN- γ and IL-17-producing phenotypes, but in contrast to $\gamma\delta$ T cells, expression levels upon restimulation are consistent between tumor and non-tumor tissue sites (fig. S1D). In the CAC model, roughly 40% of IL-17-producing cells within the tumor are $\gamma\delta$ T cells, while about 30% are CD4 $^+$ T cells (Th17 cells), while in non-tumor areas they correspond to roughly 35% and 20%, respectively (fig. S1E). Similar to what was observed in naïve mice, $\gamma\delta$ T cells in healthy colon tissue display high expression of CD8 α , while a CD8 α^- population expressing the exhaustion marker PD-1 $^+$ is prominent among tumor-infiltrating $\gamma\delta$ T cells (Fig. 1C and 1D). PD-1 expression segregated cells with potential anti-tumor (PD-1 $^-$: IFN- γ^+ and CD107 $^+$) or pro-tumor phenotype (PD-1 $^+$: IL-17 $^+$, IFN- γ^- and CD107 $^-$) (Fig. 1E and 1F). These findings were replicated in the inducible APC loss-based CRC model, where $\gamma\delta$ T cells expressing IFN- γ and IL-17 also accumulate among tumor-infiltrating lymphocytes (Fig. 1G). As in the CAC model, tumor-infiltrating PD-1 $^+$ (CD8 α^-) preferentially express IL-17, while PD-1 $^-$ (CD8 α^+) preferentially secrete IFN- γ (Fig. 1H to K). CD4 $^+$ T cells again do not show significant differences in IL-17 and IFN- γ production between tumor or adjacent non-tumor areas (fig S1F). In the APC loss model, about 60% of IL-17-producing cells within the tumor are $\gamma\delta$ T cells, while Th17 cells constitute only 20%; outside tumor areas, almost 70% IL-17-producing cells are $\gamma\delta$ T cells and roughly 10% are Th17 (fig. S1G). To further characterize tumor-infiltrating $\gamma\delta$ T cells in the CAC model, we sorted these cells based on PD-1 expression and performed RNAseq analysis. PD-1 $^-$ $\gamma\delta$ T cells (blue) display increased expression of cytotoxic related genes (e.g. *Gzmk*, *Crtam*, *Klra5*), while PD-1 $^+$ $\gamma\delta$ T cells (red) show increased expression of genes associated with IL-17 and pro-tumorigenic responses (10) such as *Tmem176a* and *Il1r1* (Fig. 1L). These results point to opposing phenotypes between $\gamma\delta$ T cells found at steady state or in non-tumor areas, and $\gamma\delta$ T cells that accumulate during CRC.

Our bulk RNAseq analysis suggested divergent TCR usage between tumor-infiltrating PD-1 $^+$ enriched in *Tcr γ V6* transcripts, and PD-1 $^-$ $\gamma\delta$ T cells, enriched in *Tcr γ V7* transcripts (Fig. 1L). To directly investigate

the TCR repertoire of tumor-infiltrating $\gamma\delta$ T cells, we single-cell sorted $\gamma\delta$ T cells from tumor and adjacent non-tumor areas from mice subjected to AOM-DSS or APC loss CRC models. TCRseq analysis indicated that in the APC loss model, $\gamma\delta$ T cells from non-tumor areas are completely diverse while tumor-infiltrating $\gamma\delta$ T cells show noticeable clonal expansions (Fig. 1M). In the AOM-DSS model, some clonal expansion was seen in non-tumor areas likely due to a chronic inflammatory state (18), with slightly increased expansion observed in tumor-areas (Fig. 1M). Notably, the expanded clones found in tumor areas in both models were primarily $V\gamma 6^+V\delta 4^+$ clones rarely seen in non-tumor areas (Fig. 1M and fig. S1H and 1I). Flow cytometry analysis confirmed the relative increase of $V\gamma 6^+$ cells at the expense of decreased $V\gamma 7^+$ $\gamma\delta$ T cells in tumor areas (Fig. 1N and 1O). As suggested by the bulk RNAseq analysis, tumor-infiltrating $V\gamma 7^+$ T cells isolated from mice subjected to AOM-DSS are mostly PD-1⁻, while $V\gamma 6^+$ cells express PD-1 (Fig. 1P). Overall, tumor-infiltrating PD-1⁺ and IL-17⁺ $\gamma\delta$ T cells are composed of $V\gamma 6$ (85.7% $\pm$ 5.6 and 78.8% $\pm$ 6.4, respectively), and $V\gamma 4$ (6.5% $\pm$ 5.7 and 14.2% $\pm$ 6.4, respectively) while IFN- γ^+ $\gamma\delta$ T cells are composed of $V\gamma 1$ (44.9% $\pm$ 2.4) and $V\gamma 7$ (27.5% $\pm$ 9.6) in the CAC model (Fig. 1Q). Similar $V\gamma$ distribution was observed in tumor infiltrating TCR $\gamma\delta^+$ T cells isolated from mice subjected to the APC loss model (fig. S1J). $V\gamma 4^+$ and $V\gamma 6^+$ subsets were previously described to leave the thymus from E15.5 and E18.5 pre-committed to IL-17 production, accumulating in specific sites such as the lung, genital tract, and adipose tissue (19, 20). The analyses above suggest that tumor infiltrating $\gamma\delta$ T cells are composed of four major subsets based on $V\gamma$ -usage, divided into two functional groups: polyclonal $V\gamma 7^+$ and $V\gamma 1^+$ resembling colonic epithelium-resident $\gamma\delta$ T cell subsets seen at steady state and exhibiting an “anti-tumor” cytotoxic program; and clonally-expanded $V\gamma 6^+V\delta 4^+$ and at lower frequency, $V\gamma 4^+$ $\gamma\delta$ T cells, that express PD-1 and secrete IL-17.

Figure 1. Profiling tumor-infiltrating $\gamma\delta$ T cells in CRC models reveals distinct subsets. (A) Representative images of colon containing tumors (arrows) from wild-type mice injected with 12.5mg/kg of Azoxymethane (AOM) 2 days prior to 3 rounds of: 1 week "on" and 2 weeks "off" 2% Dextran sodium sulfate (DSS) in the drinking water (top panel); or iCdx2^{AAPC} mice 5 weeks after 2 doses of 4mg/ml tamoxifen i.p. injection (lower panel). (B-F) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of mice subjected to the AOM-DSS model. (B) Frequency of CD107⁺, IFN- γ ⁺CD107⁺ double positive, and IL-17⁺ among TCR $\gamma\delta$ ⁺ cells. (C) Representative dot-plot and (D) frequency of CD8 α ⁺ and PD-1⁺ among TCR $\gamma\delta$ ⁺ cells. (E) Representative dot-plot and (F) frequency of IFN- γ ⁺ (left) or IL-17⁺ (right) among tumor-infiltrating PD-1⁺ or PD-1⁻ TCR $\gamma\delta$ ⁺ cells. (G-K) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of tamoxifen-treated iCdx2^{AAPC} mice. (G) Frequency of IFN- γ ⁺ (left) and IL-17⁺ (right) among TCR $\gamma\delta$ ⁺ cells. (H) Representative dot-plot and (I) frequency of CD8 α ⁺ and PD-1⁺ among TCR $\gamma\delta$ ⁺ cells. (J) Representative dot-plot and (K) frequency of IFN- γ ⁺ (left) or IL-17⁺ (right) among tumor-infiltrating PD-1⁺ or PD-1⁻ TCR $\gamma\delta$ ⁺ cells. (L) Volcano plot of differentially expressed genes from RNAseq analysis of sorted PD-1⁻ (blue) or PD-1⁺ (red) TCR $\gamma\delta$ ⁺ cells isolated from tumors of mice subjected to the AOM-DSS model. (M) Single-cell TCR sequencing of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of mice subjected to the AOM-DSS (top) and APC loss (bottom) models. Numbers in the center of pie charts represent number of clones per total cells sequenced. Expanded clones are fused. Clones are colored based on V γ usage. Purple clones represent expanded V γ 6V δ 4 cells. (N-Q) V γ usage by $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of mice subjected to the AOM-DSS model. Representative dot-plot (N) and frequency (O) of V γ 6⁺ and V γ 7⁺ among TCR $\gamma\delta$ ⁺ cells. (P) Frequency of V γ 6⁺ and V γ 7⁺ among PD-1⁺ or PD-1⁻ TCR $\gamma\delta$ ⁺ cells from tumor-containing colonic tissue. (Q) Frequency of V γ 1⁺, V γ 4⁺, V γ 6⁺ and V γ 7⁺ among tumor-infiltrating TCR $\gamma\delta$ ⁺ cells expressing PD-1, IL-17 or IFN- γ (AOM+DSS model). Representative data from 2 experiments with 3-4 animals per group. RNAseq and TCRseq data from pooled tumors. *P < 0.05, **P < 0.01, ***P < 0.001. (P, Q) one-way ANOVA with Dunnett's multiple comparison test; (B-O) two-tailed t-test. Error bars indicate SEM.

To address whether the anti-tumor phenotype of epithelium-resident TCR $\gamma\delta$ T cells plays a role in restricting CRC development, we first subjected *Trdc*^{-/-} knockout mice, which are deficient in $\gamma\delta$ T cells, to AOM-DSS (Fig. 2A to C). Because *Trdc*^{-/-} animals are more susceptible to the DSS regimen (21), we used a lower DSS concentration (1%), which does not result in noticeable inflammation or tumor development in wild-type control mice (Fig. 2B and fig. S2A to C). Following the 1% DSS regimen, *Trdc*^{-/-} mice displayed enhanced inflammation and significant increase in tumor development, again suggesting an anti-tumor, or anti-inflammatory role for intestinal epithelium-resident $\gamma\delta$ T cell populations (Fig. 2B and 2C). To extend these observations to a model which is not dependent on colitis, we lethally irradiated *iCdx2*^{ΔAPC} recipient mice and transferred bone marrow cells from *Trdc*^{-/-} or wild-type control donor mice. Eight weeks after bone-marrow transplant, recipient mice were treated with tamoxifen to induce tumor development and analyzed 10 days later (fig. S2D). Consistent with the observations made in the AOM-DSS, *iCdx2*^{ΔAPC} recipients of *Trdc*^{-/-} bone marrow show higher fecal lipocalin-2 and tumor incidence than mice recipient of wild-type bone marrow (fig. S2E and 2F). In these experiments, we opted to analyze tumor incidence instead of numbers given the difficulty in separating many joined tumors in mice recipient of *Trdc*^{-/-} bone marrow. V γ 6⁺ are canonical uterine $\gamma\delta$ T cells known to leave thymus early during ontogeny, hence do not reconstitute in these bone-marrow transfer settings (19, 22). However, we observed reconstitution of CD8 $\alpha\alpha$ ⁺ IFN- γ -producing populations (V γ 1⁺ and V γ 7⁺) and V γ 4⁺ $\gamma\delta$ T cells in mice recipient of wild-type bone marrow (fig. S2G and S2H). The above data reinforce the possibility of an anti-tumor role for epithelial-resident $\gamma\delta$ T cells.

Because total *Trdc*-deficiency also prevents the accumulation of $\gamma\delta$ cells during tumor progression, we next aimed to preferentially restrict epithelium-resident $\gamma\delta$ T cell function by inducible hemizygous or homozygous inactivation of *Slc2a1* [*iTrdc*^{Slc2a1f/+} or *iTrdc*^{ΔSlc2a1}] encoding the glucose transporter Glut1. We have previously reported that epithelium-resident $\gamma\delta$ T cells control early invasion by *Salmonella* Typhimurium via a metabolic switch towards glycolysis that is dependent on Glut1 expression (3), an

observation related to recent findings showing that IFN- γ -, but not IL-17-secreting $\gamma\delta$ T cells, are glycolytic and exert anti-tumor activity (23). We subjected $iTrdc^{\Delta Slc2a1}$, $iTrdc^{Slc2a1fl/+}$, and littermate control mice to the AOM-DSS model (Fig. 2D). Early targeting of Glut1 in $\gamma\delta$ T cells results in higher tumor number and load when compared to control animals, without affecting tumor size (Fig. 2E). Moreover, while Glut1 inactivation does not lead to changes in CD8 α , PD-1 or IL-17 expression by $\gamma\delta$ T cells, we detected a

Figure 2. Loss-of-function by epithelium-resident $\gamma\delta$ T cells results in increased tumor numbers. (A-C) $Trdc^{-/-}$ and littermate control mice were subjected to AOM-DSS model using low (1%) DSS dose and analyzed 12 weeks after initial AOM injection. (A) Percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (B) Fecal lipocalin-2 quantification at week 12. (C) Tumor number and size. (D-G) $iTrdc^{Slc2a1fl/+}$ or $iTrdc^{\Delta Slc2a1}$ and littermate control ($iTrdc^{Slc2a1fl/+}$) mice were subjected to AOM-DSS model, treated with tamoxifen for the first 3 weeks and analyzed 12 weeks after initial AOM injection. $iTrdc^{\Delta Slc2a1}$ were only used for tumor quantification. (D) Percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (E) Tumor number, size and load. Shaded area bounded by dashed lines indicates mean $\pm$ SEM of all control C57BL/6J mice analyzed in fig. S2B (AOM-DSS model). (F-G) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of $iTrdc^{Slc2a1fl/+}$ and littermate control ($iTrdc^{Slc2a1fl/+}$) mice subjected to AOM-DSS model. (F) Frequency of CD8 α^{+} (left) and PD-1 $^{+}$ (right), (G) IFN- γ^{+} (left) and IL-17 $^{+}$ (right) among TCR $\gamma\delta^{+}$ cells in tumor or non-tumor colonic tissue. V γ 7 $^{+}$ (yellow) vs V γ 7 $^{-}$ (blue) contribution to IFN- γ -producing $\gamma\delta$ T cells is also shown. $Trdc^{-/-}$ and $iTrdc^{Slc2a1fl/+}$ data are pooled from 3 experiments with 3-4 animals per group. $iTrdc^{\Delta Slc2a1}$ data are from 1 experiment with 4 animals per group. *P < 0.05, **P < 0.01, ***P < 0.001. (E-G) One-way ANOVA with Dunnett's multiple comparison test; (B, C) two-tailed t-test. Error bars indicate SEM.

significant reduction in IFN- γ production (48.6%) by tumor-infiltrating $\gamma\delta$ T cells in *iTrdc*^{Sc12a1f/+} mice (Fig. 2F and 2G and fig. S2I). Within IFN- γ -producing $\gamma\delta$ cells, Glut1 inactivation had a preferential impact on V γ 7⁻ cells (primarily represented by V γ 1⁺) (Fig. 2G). Among total tumor-infiltrating $\gamma\delta$ T cells in tamoxifen-treated *iTrdc*^{Sc12a1f/+} mice we find a significant reduction in V γ 7 (fig. S2J), overall indicating that inactivation of Glut1 in $\gamma\delta$ T cells during early states of tumorigenesis mostly affects epithelium-resident V γ 1⁺ and V γ 7⁺ cells. In contrast, late Glut1 targeting (after the second DSS cycle) had no impact on tumor load or tumor-infiltrating $\gamma\delta$ T cells (fig. S2K to O). These results suggest that epithelium-resident $\gamma\delta$ T cells exert immunosurveillance against epithelial tumors and early impairment of Glut1-dependent $\gamma\delta$ T cells favors CRC development.

To directly address the possible role of epithelium-resident $\gamma\delta$ T cells in the regulation of tumor development, we generated V γ 7^{-/-} mice by CRISPR targeting of *Trgv7* gene. Fully backcrossed V γ 7^{-/-} mice were subjected to AOM-DSS and show no differences in tumor number, tumor size or colonic $\gamma\delta$ T cells (aside from a reduction in V γ 7⁺ and CD8 $\alpha\alpha$ ⁺ $\gamma\delta$ T cells) when compared to heterozygous littermate controls (fig. S3A to E). Although, overall CD4⁺ T cells responses were unaffected, V γ 7^{-/-} mice displayed a reduction in IL-17-secreting CD4⁺ T cells in non-tumor areas when compared to V γ 7^{+/-} controls (fig. S3F). While the ratio of $\gamma\delta/\alpha\beta$ T cells in non-tumor areas was significantly reduced, we observed near doubling of V γ 1⁺ cells in V γ 7^{-/-} mice when compared to V γ 7^{+/-} controls (fig. S3C), suggesting a compensatory expansion of remaining tissue-resident V γ 1⁺ T cells in the absence of V γ 7⁺ subset.

To address possible compensatory effects of tissue-resident V γ 1⁺ and V γ 7⁺ $\gamma\delta$ T cells, we treated wild-type, V γ 7^{-/-} and V γ 7^{+/-} littermate control mice with depleting anti-V γ 1 antibody (clone 2.11) starting one week prior to AOM injection until the second DSS cycle. In contrast to untreated V γ 7^{-/-} mice, V γ 1-depleted V γ 7^{-/-} and V γ 7^{+/-} mice developed significantly more tumors, resulting in higher tumor load (Fig. 3A). V γ 1-

depleted $V\gamma 7^{+/-}$ and particularly $V\gamma 1$ -depleted $V\gamma 7^{-/-}$ mice display a significant reduction in $\gamma\delta/\alpha\beta$ T cell ratio only in non-tumor areas, as well as in $CD8\alpha\alpha^{+}\gamma\delta$ T cells (Fig. 3B to D). We did not observe differences in IFN- γ or IL-17 production among the groups but noticed an increase in PD-1-expressing $\gamma\delta$ T cells in non-tumor areas, probably reflecting a relative increase in $V\gamma 4^{+}$ or 6^{+} subsets (Fig. 3D and 3E). Overall, these results reinforce the idea that epithelium-resident $V\gamma 1^{+}$ and $V\gamma 7^{+}$ T cells play redundant anti-tumor activity.

Figure 3. Tissue resident $V\gamma 1^{+}$ and $V\gamma 7^{+}$ $\gamma\delta$ T cells synergize to control colon cancer development. (A-E) Wild-type B6 (WT), $V\gamma 7^{-/-}$, and $V\gamma 7^{+/-}$ littermate control mice treated with were subjected to AOM-DSS model and analyzed 12 weeks after initial AOM injection. All groups were treated with anti- $V\gamma 1$ depleting antibody (2.11) twice a week, starting one week before AOM administration until the second DSS cycle. (A) Tumor number, size and load. (B) Ratio of TCR $\gamma\delta/\alpha\beta$ and (C) $V\gamma 1/TCR\alpha\beta$, $V\gamma 4/TCR\alpha\beta$, $V\gamma 6/TCR\alpha\beta$, $V\gamma 7/TCR\alpha\beta$ among $CD45^{+}$ cells from colonic tissue. (D) Frequency of $CD8\alpha\alpha^{+}$ (left) and IFN- γ^{+} (right) and (E) PD-1 $^{+}$ (left) and IL-17 $^{+}$ (right) among TCR $\gamma\delta^{+}$ cells. Data pooled from 2 experiments with 3-5 animals per group. * ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$) symbols represent statistical differences between groups.

While our observations point to an important anti-tumor function for epithelium-resident $\gamma\delta$ T cells, previous studies have also described that tumor microenvironment-, or microbiota-influenced $\gamma\delta$ T cell subsets could play an opposite role during tumor progression (3, 24). We therefore questioned whether the $V\gamma 4^+$ and clonally expanded $V\gamma 6^+$ PD-1⁺ IL-17 producing $\gamma\delta$ T cells that accumulate among tumor infiltrating lymphocytes contribute to CRC progression. We conditionally deleted the main transcription factor linked to IL-17 production in T cells, *Roryt* (25), using the same strategy described above. To specifically target $\gamma\delta$ T cells that accumulate during CRC progression, we treated *iTrdc* ^{Δ Rorc} and littermate control mice with tamoxifen after the second DSS cycle (Fig. 4A). While no differences in tumor numbers or load were noted, late *Roryt* deletion in $\gamma\delta$ T cells results in smaller tumors (Fig. 4B). Accordingly, this strategy leads to altered $V\gamma$ -usage among tumor infiltrating $\gamma\delta$ T cells: tamoxifen-treated *iTrdc* ^{Δ Rorc} mice display significantly decreased $V\gamma 6^+$ and $V\gamma 4^+$ populations when compared to littermate controls (Fig. 4C and fig. S4A). Tamoxifen-treated *iTrdc* ^{Δ Rorc} mice do not display changes in CD8 $\alpha\alpha$ -expressing $\gamma\delta$ T cells (Fig. 4D). However, late *Roryt* targeting results in reduced frequency of PD-1⁺ and IL-17-producing (71.2% and 39.08% suppression, respectively) tumor-infiltrating $\gamma\delta$ T cells, affecting both $V\gamma 6^+$ and $V\gamma 4^+$ populations (4E and fig. S4B). No difference was noted in IL-17 production among CD4⁺ T cells (fig. S4C). These data indicate that *Roryt* expression by $\gamma\delta$ T cells is an important factor for the accumulation of IL-17-producing $V\gamma 4^+$ and $V\gamma 6^+$ TCR $\gamma\delta^+$ cells and contributes to tumor growth.

Changes in resident bacterial communities have been associated to tumor burden in CRC models (9) and were shown to impact pro-tumorigenic T cells, including *Roryt*⁺ IL-17-producing $V\gamma 6^+$ $\gamma\delta$ T cells, in both lung and ovarian cancer models (26, 27). Due to complex changes in gut microbiota composition after cycles of DSS treatment (27), we focused on the impact of microbiota changes and antibiotic treatment in the APC loss model. 16S ribosomal RNA sequencing from feces of mice subjected to the APC loss model revealed a sharp decrease in microbial diversity as well as broad changes in bacterial composition that can

be detected as early as 2 weeks after tamoxifen administration and continue in the following timepoints (fig. S4D and 4E). Consistent with a role of gut microbiota in promoting tumor growth (9), subjecting tamoxifen-treated $iCdx2^{AAPC}$ mice to a broad-spectrum antibiotic cocktail (ABX; Ampicillin, Vancomycin, Metronidazole and Neomycin) during the last week of the CRC development results in smaller tumors while not impacting tumor number or load, resembling changes observed upon late Roryt targeting in the CAC model (Fig. 4F and 4G). Although microbiota depletion in tamoxifen-treated $iCdx2^{AAPC}$ mice leads to decreased intra-tumoral $V\gamma6^{+}$ $\gamma\delta$ T cells, it does not affect $V\gamma4^{+}$ cells (Fig. 4H). $CD8\alpha\alpha^{+}$ $\gamma\delta$ T cells, primarily represented by $V\gamma1^{+}$ and $V\gamma7^{+}$ cells, display increased frequency in both non-tumor and tumor sites in mice receiving ABX treatment (Fig. 4I). Moreover, ABX treatment results in reduced frequency of IL-17– and PD-1–expressing $\gamma\delta$ T cells (both over 75% suppression), primarily within the $V\gamma6^{+}$ subset (Fig. 4J and 4K), even though $V\gamma6^{-}$ $\gamma\delta$ T cells (mostly comprised of $V\gamma4^{+}$) also contribute to PD-1 and IL-17 expression in tumor sites (see fig. S1H).

To address whether $V\gamma6^{+}$ cells accumulate during CRC progression due to increased recruitment or *in situ* proliferation, we performed *in vivo* EdU labelling. We observe higher proliferation rates in $V\gamma6^{+}$ *versus* $V\gamma6^{-}$ $\gamma\delta$ T cells, which are reduced (56.7%) upon ABX treatment; $V\gamma4^{+}$ proliferation does not appear to be impacted by ABX treatment (Fig. 4L and 4M). These observations point to distinct susceptibility to microbiota manipulations by two main subsets of tumor-accumulating $\gamma\delta$ T cells: $V\gamma6^{+}$, but not $V\gamma4^{+}$ cells, proliferate in response to microbiota and depend on microbiota signals to sustain PD-1 and IL-17 expression, as well as to boost tumor growth.

Our findings so far indicate that IL-17–producing, or PD-1–expressing tumor–infiltrating $\gamma\delta$ T cells are composed ~75-80% of microbiota-dependent $V\gamma6^{+}$, and ~20-25% of $V\gamma4^{+}$ cells. To directly assess the roles of $V\gamma4^{+}$ and $V\gamma6^{+}$ $\gamma\delta$ T cells in CRC progression, we generated $V\gamma4^{-/-}$ and $V\gamma6^{-/-}$ mice by CRISPR targeting

of *Trgv4* and *Trgv6* genes, respectively. Fully backcrossed $V\gamma 4^{-/-}$ mice were subjected to AOM-DSS and show no differences in tumor number, tumor size or tumor-infiltrating $\gamma\delta$ T cells (aside from $V\gamma 4$) when compared to heterozygous littermate controls (fig. S5A-G). The ratio of $\gamma\delta/\alpha\beta$ T cells among of tumor-infiltrating lymphocytes in $V\gamma 4^{-/-}$ mice remains the same as the ratio observed in $V\gamma 4^{+/+}$ controls (fig. S5B), suggesting a compensatory expansion of remaining $\gamma\delta$ T cells, including $V\gamma 6^{+}$ T cells, in the absence of $V\gamma 4^{+}$ subset.

Figure 4. Loss-of-function by tumor-infiltrating IL-17⁺ $\gamma\delta$ T cells results in smaller tumors. (A-D) *iTrdc^{Rorcfl/+}* and littermate control (*iTrdc^{RorcΔRorc}*) mice were subjected to AOM-DSS model, treated with tamoxifen for the last 6 weeks of the experiment and analyzed 12 weeks after initial AOM injection. (A) Percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (B) Tumor number, size and load. Shaded area bounded by dashed lines indicates mean $\pm$ SEM of all control C57BL/6/J mice analyzed in fig. S2B (AOM-DSS model). (C-E) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue. (C) Frequency of $V\gamma 6^{+}$ among TCR $\gamma\delta^{+}$ cells. (D) Frequency of CD8 α^{+} and (E) PD-1⁺ (left), and IL-17⁺ (right) cells among TCR $\gamma\delta^{+}$ cells. $V\gamma 6^{+}$ (purple) vs $V\gamma 6^{-}$ (orange) contribution to PD-1⁺ and IL-17-producing $\gamma\delta$ T cells is also shown. (F-M) *iCdx2^{ΔAPC}* mice were treated with 2 i.p. injections of 0.8mg tamoxifen and analyzed 5 weeks after. Mice were treated with antibiotic mix (ABX) and EdU in the drinking water or only EdU (control) for the last week of the experiment. (F) Protocol. (G) Tumor number, size and load. (H-M) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue. (H) Frequency of $V\gamma 6^{+}$ (left) or $V\gamma 4^{+}$ (right) among TCR $\gamma\delta^{+}$ cells. (I) Frequency of CD8 α^{+} cells among TCR $\gamma\delta^{+}$ cells. (J) Frequency of PD-1⁺ and (K) IL-17⁺ among TCR $\gamma\delta^{+}$ cells. $V\gamma 6^{+}$ (purple) vs $V\gamma 6^{-}$ (orange) contribution to PD-1⁺ and IL-17-producing $\gamma\delta$ T cells is also shown. (L) Frequency of EdU incorporation by $V\gamma 6^{-}$ or $V\gamma 6^{+}$ TCR $\gamma\delta^{+}$ cells in tumor-containing colonic tissue isolated from control *iCdx2^{ΔAPC}* mice. (M) Frequency of EdU incorporation by $V\gamma 6^{+}$ and $V\gamma 4^{+}$ TCR $\gamma\delta^{+}$ cells in tumor-containing colonic tissue. *iTrdc^{RorcΔRorc}* tumor data are pooled from 3 experiments with 3-6 animals per group. Data from *iCdx2^{ΔAPC}* are representative from 2 independent experiments with 3-4 animals per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C-E and I-M) One-way ANOVA with Dunnett's multiple comparison test; (B, G, H) two-tailed t-test. Error bars indicate SEM.

We next analyzed backcrossed naïve $V\gamma 6^{-/-}$ mice. Like $V\gamma 4^{-/-}$ mice, $V\gamma 6^{-/-}$ mice subjected to AOM-DSS display similar CRC development, progression (Fig. 5A) and parameters of tumor-infiltrating $\gamma\delta$ T cells to $V\gamma 6^{+/-}$ littermate controls (Fig. 5B to F). The absence of an otherwise large intra-tumor $V\gamma 6^{+}$ population did not result in altered $\gamma\delta/\alpha\beta$ T cell ratio (Fig. 5B), explained by a compensatory increase in other $\gamma\delta$ T cells, particularly $V\gamma 4^{+}$ cells (Fig. 5C). We did not observe differences in tumor-infiltrating $CD4^{+}$ T cells between $V\gamma 6^{-/-}$ and $V\gamma 6^{+/-}$ mice (fig. S5H). The overall similar tumor development and composition of tumor-infiltrating $\gamma\delta$ T cells in $V\gamma 4^{-/-}$ and $V\gamma 6^{-/-}$ mice raise the possibility that tumor-infiltrating, PD-1⁺ IL-17–producing $V\gamma 4^{+}$ and $V\gamma 6^{+}$ populations play redundant roles in promoting CRC growth.

To address possible compensatory, and redundant, roles between tumor-infiltrating $V\gamma 4^{+}$ and $V\gamma 6^{+}$ $\gamma\delta$ T cells, we treated $V\gamma 6^{-/-}$ and $V\gamma 6^{+/-}$ littermate control mice with depleting anti- $V\gamma 4$ antibody (UC3-10A6) starting after the second DSS cycle until analysis. In contrast to untreated $V\gamma 6^{-/-}$ mice, $V\gamma 4$ –depleted $V\gamma 6^{-/-}$ mice developed significantly smaller tumors than $V\gamma 4$ –depleted $V\gamma 6^{+/-}$ littermate control mice, while no significant changes in tumor numbers or load were noted (Fig. 5G). In contrast to previous strategies, $V\gamma 4$ –depleted $V\gamma 6^{-/-}$ mice display a roughly 50% reduction in tumor infiltrating $\gamma\delta/\alpha\beta$ T cell ratio (Fig. 5H). $V\gamma 4$ –depleted $V\gamma 6^{-/-}$ mice also display enhanced intra-tumoral $CD8\alpha^{+}$ $\gamma\delta$ T cells when compared to $V\gamma 4$ –depleted $V\gamma 6^{+/-}$ mice (Fig. 5J), similar to what was observed in ABX-treated mice in the APC loss model. Additionally, and consistent with a functional redundancy between $V\gamma 4^{+}$ and $V\gamma 6^{+}$ $\gamma\delta$ T cells, $V\gamma 4$ –depleted $V\gamma 6^{-/-}$ mice show about 80% reduction in the frequency of PD-1⁺ and IL-17–producing $\gamma\delta$ T cells within the tumor (Fig. 5J to L). No changes were observed in IFN- γ production, or in IL-17 production by $CD4^{+}$ T cells, when compared to $V\gamma 4$ –depleted $V\gamma 6^{+/-}$ mice (fig. S5I). Hence, in sharp contrast to an anti-tumor role by epithelium-resident subsets, dominated by $V\gamma 1^{+}$ and $V\gamma 7^{+}$ $\gamma\delta$ T cells, these data suggest redundant roles for tumor-infiltrating $V\gamma 4^{+}$ and $V\gamma 6^{+}$ $\gamma\delta$ T cells in promoting CRC progression.

Tumor-infiltrating lymphocytes are essential components of anti-tumor responses and represent major targets for immunotherapies (28-31). Here we observed that γδ T cell subsets play contrasting roles in CRC: epithelial surveillance by steady-state IFN-γ-producing cytotoxic γδ populations help prevent tumor initiation, while accumulating intra-tumor γδ T cells support tumor progression. Specialized “dendritic

epidermal T cells” (DETC) $\gamma\delta$ T cells in the skin, mostly comprised of $V\gamma 5^+$ cells have been shown to suppress tumor development via an NKG2D–dependent cytotoxic mechanism (2, 32, 33), while dermal $\gamma\delta$ T cells, pre-committed to IL-17 production and expressing $V\gamma 4$ or $V\gamma 6$, were shown to promote tumor growth (31). While these observations parallel our findings, whether such anatomical segregation can also be observed in the intestinal epithelium *versus* lamina propria in specific conditions (16), remain to be determined. Our observations in the intestine indicate that $\gamma\delta$ IELs in pre- or non-tumor areas harbor a diverse TCR repertoire while sharing primarily $V\gamma 1$ or 7 segments, particularly in the APC loss model. The accumulation of $V\gamma 7^+$ IELs was previously linked to binding of the germline $V\gamma 7$ chain to Btln proteins; a tissue-specific selection not associated with TCR $\gamma\delta$ CDR3 region (16). Analogous to changes we observed in $\gamma\delta$ T cells during CRC progression, previous studies described an irreversible expansion and repertoire reshaping of $\gamma\delta$ T cells in chronically inflamed conditions such as in Celiac patients (33). Another important parallel with this study is their observation that $\gamma\delta$ T cells from patients with active disease acquire a pro-inflammatory cytokine profile in contrast to $\gamma\delta$ T cells isolated from patients in remission, which are primarily cytotoxic (33). Additionally, a recent study uncovered a metabolism-driven dichotomy in $\gamma\delta$ T cell function: IL-17-producing $\gamma\delta$ T cells are found to be dependent on oxidative phosphorylation, thriving in lipid-rich environments such as tumors; IFN- γ –producing $\gamma\delta$ T cells required glycolysis for their energy expenditure (23). Conversely, previous observations suggested a role for clonally–restricted IL-17–producing T cells, including $\gamma\delta$ cells, in tumor-progression (9, 26, 27). These observations are in line with our observations that, like their response to invading bacteria (3), IFN- γ –producing $\gamma\delta$ T cells, particularly the $V\gamma 7^+$ subset, display anti-tumor activity dependent on Glut1 expression, while IL-17–producing $\gamma\delta$ T cells, particularly the $V\gamma 6V\delta 4$ clone, were highly expanded in tumor areas. Our results, and previous literature (9, 26, 31, 34–36), point to a tumor-progression role for intra-tumoral IL-17. Whether downstream mechanisms induced by IL-17, such as neutrophil activity, or whether specific localization of these cells are necessary for tumor growth remain to be defined. Because IL-17 production by $\gamma\delta$ T cells in the gut has

also been linked to tissue repair (37), it remains possible that in addition to IL-17, other factors or molecules expressed by $V\gamma 4^+$ or $V\gamma 6^+$ cells aid tumor growth in the balance with tissue repair mechanisms. It would be relevant to define whether high PD-1 expression, specific localization within the tumor, or other factors, distinguish a tumor progression role for $\gamma\delta$ T cells *versus* other IL-17-secreting cells, such as Th17 cells. Additionally, future studies are required to define the mechanisms by which the tumor microenvironment mediates the accumulation of microbiota-dependent $V\gamma 6^+$ and microbiota-independent $V\gamma 4^+$ subsets that aid tumor growth; it is tempting to speculate that analogous mechanisms to the Btl-1-dependent $V\gamma 7$ selection (15, 16, 22, 38), are utilized. The results presented here caution against broad targeting of $\gamma\delta$ T cells in future immune-therapy strategies, but they open possibilities of specific targeting of $\gamma\delta$ T subsets based on $V\gamma$ -usage, pending confirmation of whether human CRC (30) parallels $\gamma\delta$ T cell dynamics observed here.

References

1. J. Ettersperger *et al.*, Interleukin-15-Dependent T-Cell-like Innate Intraepithelial Lymphocytes Develop in the Intestine and Transform into Lymphomas in Celiac Disease. *Immunity* **45**, 610-625 (2016).
2. T. Sujino *et al.*, Tissue adaptation of regulatory and intraepithelial CD4(+) T cells controls gut inflammation. *Science* **352**, 1581-1586 (2016).
3. D. P. Hoytema van Konijnenburg *et al.*, Intestinal Epithelial and Intraepithelial T Cell Crosstalk Mediates a Dynamic Response to Infection. *Cell* **171**, 783-794 e713 (2017).
4. B. D. McDonald, B. Jabri, A. Bendelac, Diverse developmental pathways of intestinal intraepithelial lymphocytes. *Nature reviews. Immunology* **18**, 514-525 (2018).
5. K. L. Edelblum *et al.*, gammadelta Intraepithelial Lymphocyte Migration Limits Transepithelial Pathogen Invasion and Systemic Disease in Mice. *Gastroenterology* **148**, 1417-1426 (2015).
6. A. J. Gentles *et al.*, The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nature medicine* **21**, 938-945 (2015).
7. S. Grivnikov *et al.*, IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* **15**, 103-113 (2009).

8. S. I. Grivennikov, F. R. Greten, M. Karin, Immunity, inflammation, and cancer. *Cell* **140**, 883-899 (2010).
9. S. I. Grivennikov *et al.*, Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* **491**, 254-258 (2012).
10. O. Dmitrieva-Posocco *et al.*, Cell-Type-Specific Responses to Interleukin-1 Control Microbial Invasion and Tumor-Elicited Inflammation in Colorectal Cancer. *Immunity* **50**, 166-180 e167 (2019).
11. I. Okayasu, T. Ohkusa, K. Kajiura, J. Kanno, S. Sakamoto, Promotion of colorectal neoplasia in experimental murine ulcerative colitis. *Gut* **39**, 87-92 (1996).
12. Y. Feng *et al.*, Sox9 induction, ectopic Paneth cells, and mitotic spindle axis defects in mouse colon adenomatous epithelium arising from conditional biallelic Apc inactivation. *Am J Pathol* **183**, 493-503 (2013).
13. T. Hinoi *et al.*, Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res* **67**, 9721-9730 (2007).
14. R. Di Marco Barros *et al.*, Epithelia Use Butyrophilin-like Molecules to Shape Organ-Specific gammadelta T Cell Compartments. *Cell* **167**, 203-218 e217 (2016).
15. A. Jandke *et al.*, Butyrophilin-like proteins display combinatorial diversity in selecting and maintaining signature intraepithelial gammadelta T cell compartments. *Nature communications* **11**, 3769 (2020).
16. D. Melandri *et al.*, The gammadeltaTCR combines innate immunity with adaptive immunity by utilizing spatially distinct regions for agonist selection and antigen responsiveness. *Nature immunology* **19**, 1352-1365 (2018).
17. F. R. Greten, S. I. Grivennikov, Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity* **51**, 27-41 (2019).
18. M. Yassin *et al.*, Upregulation of PD-1 follows tumour development in the AOM/DSS model of inflammation-induced colorectal cancer in mice. *Immunology* **158**, 35-46 (2019).
19. J. D. Haas *et al.*, Development of interleukin-17-producing gammadelta T cells is restricted to a functional embryonic wave. *Immunity* **37**, 48-59 (2012).
20. A. C. Kohlgruber *et al.*, gammadelta T cells producing interleukin-17A regulate adipose regulatory T cell homeostasis and thermogenesis. *Nature immunology* **19**, 464-474 (2018).
21. Y. Chen, K. Chou, E. Fuchs, W. L. Havran, R. Boismenu, Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 14338-14343 (2002).
22. A. C. Hayday, gammadelta T Cell Update: Adaptate Orchestrators of Immune Surveillance. *Journal of immunology* **203**, 311-320 (2019).

23. N. Lopes *et al.*, Distinct metabolic programs established in the thymus control effector functions of gammadelta T cell subsets in tumor microenvironments. *Nature immunology* **22**, 179-192 (2021).
24. S. Curio *et al.*, NKG2D signaling regulates IL-17A-producing $\gamma\delta$ T cells to promote cancer progression. *bioRxiv*, 2021.2008.2026.457761 (2021).
25. Ivanov, II *et al.*, The Orphan Nuclear Receptor RORgammat Directs the Differentiation Program of Proinflammatory IL-17(+) T Helper Cells. *Cell* **126**, 1121-1133 (2006).
26. C. Jin *et al.*, Commensal Microbiota Promote Lung Cancer Development via gammadelta T Cells. *Cell* **176**, 998-1013 e1016 (2019).
27. M. Rei *et al.*, Murine CD27(-) Vgamma6(+) gammadelta T cells producing IL-17A promote ovarian cancer growth via mobilization of protumor small peritoneal macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E3562-3570 (2014).
28. M. Girardi *et al.*, The distinct contributions of murine T cell receptor (TCR)gammadelta+ and TCRalphabeta+ T cells to different stages of chemically induced skin cancer. *J Exp Med* **198**, 747-755 (2003).
29. Y. Wu *et al.*, An innate-like Vdelta1(+) gammadelta T cell compartment in the human breast is associated with remission in triple-negative breast cancer. *Sci Transl Med* **11**, (2019).
30. J. Mikulak *et al.*, NKp46-expressing human gut-resident intraepithelial Vdelta1 T cell subpopulation exhibits high antitumor activity against colorectal cancer. *JCI Insight* **4**, (2019).
31. B. Silva-Santos, S. Mensurado, S. B. Coffelt, gammadelta T cells: pleiotropic immune effectors with therapeutic potential in cancer. *Nat Rev Cancer* **19**, 392-404 (2019).
32. H. Cheroutre, F. Lambolez, D. Mucida, The light and dark sides of intestinal intraepithelial lymphocytes. *Nature reviews. Immunology* **11**, 445-456 (2011).
33. T. Mayassi *et al.*, Chronic Inflammation Permanently Reshapes Tissue-Resident Immunity in Celiac Disease. *Cell* **176**, 967-981 e919 (2019).
34. F. McAllister *et al.*, Oncogenic Kras activates a hematopoietic-to-epithelial IL-17 signaling axis in preinvasive pancreatic neoplasia. *Cancer Cell* **25**, 621-637 (2014).
35. S. Ma *et al.*, IL-17A produced by gammadelta T cells promotes tumor growth in hepatocellular carcinoma. *Cancer Res* **74**, 1969-1982 (2014).
36. S. B. Coffelt *et al.*, IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* **522**, 345-348 (2015).
37. J. S. Lee *et al.*, Interleukin-23-Independent IL-17 Production Regulates Intestinal Epithelial Permeability. *Immunity* **43**, 727-738 (2015).
38. S. P. Fahl *et al.*, Role of a selecting ligand in shaping the murine gammadelta-TCR repertoire. *Proceedings of the National Academy of Sciences of the United States of America* **115**, 1889-1894 (2018).

39. B. S. Reis, A. Rogoz, F. A. Costa-Pinto, I. Taniuchi, D. Mucida, Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4(+) T cell immunity. *Nature immunology* **14**, 271-280 (2013).
40. J. J. Trombetta *et al.*, Preparation of Single-Cell RNA-Seq Libraries for Next Generation Sequencing. *Current protocols in molecular biology* **107**, 4 22 21-17 (2014).
41. N. L. Bray, H. Pimentel, P. Melsted, L. Pachter, Near-optimal probabilistic RNA-seq quantification. *Nature biotechnology* **34**, 525-527 (2016).
42. H. Pimentel, N. L. Bray, S. Puente, P. Melsted, L. Pachter, Differential analysis of RNA-seq incorporating quantification uncertainty. *Nature methods* **14**, 687-690 (2017).
43. M. E. Ritchie *et al.*, limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic acids research* **43**, e47 (2015).
44. A. Alexa, J. Rahnenfuhrer, topGO: Enrichment Analysis for Gene Ontology. *R package version 2.24.0.*, (2019).
45. Carlson M, GO.db: A set of annotation maps describing the entire Gene Ontology. *R package version 3.8.2*, (2019).
46. A. Han, J. Glanville, L. Hansmann, M. M. Davis, Linking T-cell receptor sequence to functional phenotype at the single-cell level. *Nature biotechnology* **32**, 684-692 (2014).
47. A. P. Masella, A. K. Bartram, J. M. Truszkowski, D. G. Brown, J. D. Neufeld, PANDAsseq: PAired-eND Assembler for Illumina sequences. *Bmc Bioinformatics* **13**, (2012).
48. X. Brochet, M. P. Lefranc, V. Giudicelli, IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic acids research* **36**, W503-508 (2008).

Acknowledgements: We thank all Mucida Lab members and Rockefeller University employees for their continuous assistance; A. Rogoz and S. Gonzalez for the maintenance of mice. We thank Rebecca O'Brien for the anti-V γ 7 hybridoma, and M. Constantinides & Y. Belkaid for anti-V γ 6 hybridoma. We also thank Victora and Lafaille labs for fruitful discussions. This work was supported by NIH R01CA218133 (S.G.), R01DK093674, R01DK113375, Food Allergy FARE/FASI Consortium, Mathers Foundation and Pershing Square Sohn Cancer Research Alliance (D.M.).

Author Contributions: B.S.R. conceived, initiated, designed, performed, and analyzed experiments, and wrote the manuscript. P.D., A.L. and A. B. designed and performed experiments. I.Z.K., O.E., C.Z. and A. R. performed experiments. J.B. generated CRISPR mutant mice. T.B.R.C performed RNAseq and scTCRseq analysis. S.G. helped initiating, designing, and analyzing experiments. D.M. conceived, initiated, designed, supervised the research, and wrote the manuscript. All authors revised and edited the manuscript and figures.

Competing interests: The authors declare no competing financial interests.

Supplementary Materials

Materials and Methods

Figures S1-S5

Methods

Mice. C57BL/6J control (000664), *Trdc*^{GFP} (016941), *Trdc*^{-/-} (002120), *Trdc*^{CreER} (031679), *Tbx21*^{fl/fl} (022741), *Scl2a1*^{fl/fl} (031871), *Rorc*^{fl/fl} (008771), *Cdx2*^{CreER} (022390), Rosa26^{dTomato} (007914) were purchased from Jackson Laboratories and kept in our facility. APC^{fl/fl} mice were provided by S. Grivennikov (C. Sinai) (9, 12, 13). E81^{CRE} mice were provided by Ichiro Taniuchi (RIKEN, Japan). Vγ4^{-/-}, Vγ6^{-/-}, and Vγ7^{-/-} mice were developed by CRISPR-Cas9 gene editing targeting *Trgv4*, *Trgv6*, and *Trgv7* genes (see below). Heterozygous or wild-type homozygous littermates were used as controls. Mice were bred within our facility to obtain strains described and were 7-12 weeks of age for all experiments. To minimize microbiome fluctuations in the AOM-DSS model, genetically modified and wild-type control mice were co-housed from birth. Male and female mice were used in all experiments, in similar distribution, unless otherwise stated in the figure legends. Estrous cycle was not controlled for female mice. Animal care and experimentation were consistent with NIH guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University. All animals were kept in specific pathogen free (SPF) conditions.

Generation of Vγ-specific knockout strains by CRISPR-Cas9 gene editing. gRNA targeting specific *Trvg* genes were designed and injected into one cell stage fertilized C57BL/6J embryos together with Cas9 protein. Embryos were implanted into pseudo-pregnant foster Swiss mice and mutant offspring were selected by specific PCR genotyping; mutations were determined by sanger DNA sequencing. Mutant mice were backcrossed to wild-type C57BL/6J animals for 5-10 generations. For *Trvg4* gene targeting sgRNA sequence GUGUAACCAUACACUGGUACCGG was used. For *Trvg6* gene targeting, sgRNA AGCCCGAUGCAUACAACACUGG and for *Trvg7* gene targeting sgRNA sequence

ACUGGUACCGAUUCCAGAAAGGG was used. Mice were kept in our facility under the Rockefeller University IACUC protocol.

Colorectal cancer (CRC) models

AOM-DSS model. Mice were injected with 12.5mg/kg of Azoxymethane (AOM) 1 day prior to 3 consecutive oral exposures of dextran sodium sulfate (DSS, 2% in drinking water) for 1 week with 2 weeks interval between exposures. Animals were sacrificed 12 weeks after initial AOM injection. Tumors were counted and measured at the time of experiment termination.

APC model. *Cdx2^{CreER}·APC^{fl/fl} (iCdx2^{ΔAPC})* mice were injected twice with 0.8 mg of tamoxifen (Sigma) 4mg/ml in corn oil (Sigma), two days apart. Four weeks after the first injection, mice were sacrificed, and tumors were measured and counted. Animals were monitored for weight loss and sickness behavior and the experiment was terminated when weight loss was greater than 20% of initial weight.

Tamoxifen treatment regimen in AOM-DSS model. Tamoxifen (Sigma) was administrated by intra-peritoneal injections, twice a week, of 0.8mg of tamoxifen diluted 4 mg/mL in corn oil (Sigma). For early gene depletion, tamoxifen injections started 1 week before AOM administration and continued until week 3 of AOM-DSS CRC model. For late gene targeting, tamoxifen injections started 1 week after second round of DSS and continued until the week 12 of the AOM-DSS CRC model.

Antibiotic and EdU administration. *iCdx2^{ΔAPC}* mice were treated for one week with broad spectrum antibiotics consisting of 1mg/ml ampicillin, 1mg/ml neomycin, 0.5mg/ml vancomycin, 0.5mg/ml metronidazole and 10g/ml sucralose in the drinking water during the final week of the experiment. EdU was also added to the antibiotic containing water at 1mg/ml. As a control, 10mg/ml sucralose solution containing 1mg/ml EdU was used. EdU detection was performed using the Click-iT™ Plus EdU Flow Cytometry Assay kit (Thermo Fisher Scientific, C10632), according to manufacturer's instructions.

In vivo antibody administration. For depletion of Vγ4⁺ T cells, 200μg of anti-Vγ4 antibody UC3-10A6 (Bio X Cell) was injected intra peritoneally twice a week for the final 6 weeks of AOM-DSS model. For

antibody depletion of V γ 1⁺ T cells, 200 μ g of anti-V γ 1 antibody 2.11 (Bio X Cell) was injected as described for UC3-10A6.

Isolation of intestinal and tumor infiltrating cells. Intraepithelial lymphocytes were isolated as previously described (39). Briefly, large intestines were harvested and washed in PBS and 1mM dithiothreitol (DTT) followed by 30 mM EDTA. Intraepithelial cells were recovered from the supernatant of DTT and EDTA washes. Tumor infiltrating cells were obtained after collagenase digestion of the tissue. Dissected tumor was minced and incubated with 50mg/ml of collagenase VIII, 200mg/ml of DNase in RPMI for 30 minutes. Mononuclear cells were isolated by gradient centrifugation using Percoll. Single-cell suspensions were then stained with fluorescently labeled antibodies for 25min at 4°C prior to flow cytometry analysis and/or cell sorting.

Flow cytometry. Cells were stained for anti-CD45 (BD 561487 clone 30-F11), anti-TCR β (BioLegend 109220 clone H57-597) anti-TCR $\gamma\delta$ (eBioscience 11-5711-85 clone GL-3), anti-CD8 α , (eBioscience 56-0081-82 clone 53-6.7), anti-CD4 (BioLegend 100552 clone RM4-5), anti-PD-1 (BioLegend 135219 clone 29F.1A12), anti-V γ 1 (BioLegend 141109 clone 2.11), anti-V γ 4 (eBioscience 25-5828-82 clone UC3-10A6), anti-V γ 6 (clone 17D1, kindly donated by Yasmin Belkaid, Ph.D.), anti-V γ 7 (clone GL1.7, kindly donated by Rebecca O'Brien, Ph.D.), anti-CD107 (eBioscience 46-1071-82 clone 1D4B), anti-IFN- γ (eBioscience 45-7311-82 clone XMGI.2), anti-IL-17a (BD 559502 clone TC11-18H10), anti-CD11b (eBioscience 47-0112-82 clone M1/70). For anti-V γ 6 staining, cells were first stained with anti-TCR $\gamma\delta$ (clone GL-3) for 30 minutes prior anti-V γ 6 17D1 antibody staining. For cytokine profiling, tissue isolated cells were stimulated ex-vivo with 1mg/ml of phorbol myristate acetate (PMA) and 1mg/ml of Ionomycin in the presence of 10mg/ml of Brefeldin for 4 hours prior staining.

Bulk mRNA-Seq Library Preparation. Sorted cells (300-800 cells) were lysed in a guanidine thiocyanate buffer (TCL buffer, QIAGEN) supplemented with 1% β -mercaptoethanol. RNA was isolated by solid-phase reversible immobilization bead cleanup using RNAClean XP beads (Agentcourt, A63987), reverse transcribed, and amplified as described (40). Uniquely barcoded libraries were prepared using Nextera XT kit (Illumina) following manufacturer's instructions. Sequencing was performed on an Illumina NextSeq500 for a total yield of 400M reads.

Bulk RNA-Seq Analysis. Raw fastq files were processed by using the mouse transcriptome (gencode M23) with the kallisto (v0.46) software (41). Analysis of transcript quantification was performed at the gene level by using the sleuth (v0.30) package for R (42). Shortly, we modeled batch effect and our experimental design using the sleuth_fit function and detected differentially expressed genes between all groups by the likelihood ratio test (LRT). To spot statistically significantly enriched genes between group pairs, we used the wald-test function. Genes with false discovery rate less than 0.05 and 1 log2 fold-change were used in downstream analysis. The batch effect was removed from the expression matrix by using the *removebatcheffect* function available in the limma package (43). Gene set enrichment analysis (GSEA) was performed by using signature gene sets in gmt format and a pre-ranked gene list by log2 fold-change between two groups as input for the fgsea. Gene ontology (GO) analysis was executed by comparing all detected genes as our background and the lists of differentially expressed genes against the biological processes gene sets by using topGO (44, 45).

Single-Cell TCR Sequencing. Single cells were index-sorted using a FACS Aria into 96-well plates containing 5 μ L of lysis buffer (TCL buffer, QIAGEN 1031576) supplemented with 1% β -mercaptoethanol and frozen in -80°C prior to RT-PCR. RNA and RT-PCRs for TCR γ and TCR δ were prepared using specific primers (supplemental table 1). PCR products for TCR γ and TCR δ were multiplexed with barcodes and submitted for MiSeq sequencing (46) using True Seq Nano kit (Illumina). Fastq files were de-

multiplexed and paired-end reads were assembled at their overlapping region using PANDASEQ (47) and FASTAX toolkit. De-multiplexed and collapsed reads were assigned to wells according to barcodes. Fasta files were aligned and analyzed on IMGT (<http://imgt.org/HighV-QUEST>) (48). Cells with identical TCR γ and TCR δ CDR3 nucleotide sequences were considered as the same clones. Clonality was assigned based on paired TCR $\gamma\delta$ per mouse. Only in frame junction sequences of TCR $\gamma\delta$ were included in the analysis.

FocusClear and cell localization on villus-crypt axis. Tissue clearing was performed according to FocusClear manufacturer's (Celexplorer Labs. Co.) instructions. Briefly, E8_I (*Cd8a* enhancer I)^{Cre}:*Rosa26*^{fsf-dTomato} (E8_I^{dTomato}):*Trdc*^{GFP} double-reporter mice were injected with Hoechst dye (blue) for visualization of epithelial cell nuclei. After 15-30 min, mice were sacrificed by cervical dislocation and segments of the small intestine were removed, washed (intact) and fixed in 4% PFA at gentle agitation for 2h at RT. After fixation, samples were washed in DPBS and placed in FocusClear solution for approximately 15 min. at room temperature. Once visual confirmation of clearance was obtained, samples were mounted in 3D printed slides with MountClear, sealed and imaged using an inverted LSM 880 NLO laser scanning confocal and multiphoton microscope (Zeiss). As this protocol maintains native GFP and dTomato, and introduced Hoechst fluorescence, no antibody staining was necessary. Cell X and Y coordinates were obtained using IMARIS (Bitplane) software, as described before (3). Multiple technical replicates per sample (segment) were obtained.

Statistical Analyses. Statistical analyses were carried out using GraphPad Prism v.9. Flow cytometry analyses were carried out using FlowJo software. Data in graphs show mean $\pm$ SEM and P values < 0.05 were considered significant. Statistical analyses were performed using ordinary one-way ANOVA test with Dunnett's multiple comparison test, two-tail t-test or Chi-square test, as indicated in the figure legends. *P < 0.05, **P < 0.01, ***P < 0.001. GraphPadPrism v.9 was used for graphs and statistical analysis, and Adobe Illustrator 2020 was used to assemble and edit figures.

Supplementary Figures

Figure S1. Supporting data to Figure 1. (A, C) Flow cytometry analysis of naïve C57BL/6 mice. (A) Frequency of $CD8\alpha\alpha^+$ and $CD8\alpha\alpha^-$ among $TCR\gamma\delta^+$ cells from duodenum D, jejunum J, ileum I and colon C. (B) Whole-mount imaging of tissue-cleared ileum from $E8_{Tomo}^{Tomo}Trdc^{GFP}$ reporter mouse at steady-state. Image shows $TCR\gamma\delta^+CD8\alpha\alpha^+$ (Tomato⁺GFP⁺) and $TCR\gamma\delta^+CD8\alpha\alpha^-$ (Tomato⁺GFP⁻) cells along the villus-crypt axis. Scale bar = 40 μ m. (C) Frequency of $V\gamma$ usage by colonic $TCR\gamma\delta^+$ cells at steady state. (D, E) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of C57BL/6 mice subjected to AOM-DSS model. (D) Frequency of $IFN\gamma^+$ (left), $CD107^+$ (center) and $IL-17^+$ (right) among $TCR\beta^+CD4^+$ cells. (E) Representative dot-plot (left) and frequency (right) of $CD4^+$ and $TCR\gamma\delta^+$ cells among $IL-17^+$ cells. (F, G) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of tamoxifen-treated $iCdx2^{\Delta APC}$. (F) Frequency of $IFN\gamma^+$ (left) and $IL-17^+$ (right) among $TCR\beta^+CD4^+$ cells from colonic tumor. (G) Representative dot-plot (left) and frequency (right) of $CD4^+$ and $TCR\gamma\delta^+$ cells among $IL-17^+$ cells. (H, I) Single-cell TCR sequencing of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue of mice subjected to the AOM-DSS (left, center) and APC loss (right) models. (H) Numbers in the center of pie charts represent number of clones per total cells sequenced. Expanded clones are fused. Clones are colored based on $V\gamma$ usage. Purple clones represent expanded $V\gamma 6V\delta 4$ cells. Each pie chart represents an individual mouse. (I) Amino acid sequence used by $V\gamma 6V\delta 4$ expanded clones (purple). Font size represents frequency of usage. (J) Frequency of $V\gamma 1^+$, $V\gamma 4^+$, $V\gamma 6^+$ and $V\gamma 7^+$ among tumor-infiltrating $\gamma\delta$ T cells expressing PD-1, IL-17 or IFN- γ (APC loss model). Data representative from 2 experiments with 3-5 animals per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A, C, D, F) One-way ANOVA test with Dunnett's multiple comparison test; (E, G, J) two-tailed t-test. Error bars indicate SEM.

Figure S2. Supporting data to Figure 2. (A-C) C57BL/6 mice were injected with 12.5mg/kg of Azoxymethane (AOM) 2 days prior to three rounds of 1 week "on" and 2 weeks "off" of 1% vs 2% Dextran sodium sulfate (DSS) in the drinking water. (A) Percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (B) Tumor number, size and load. (C) Fecal lipocalin-2 quantification. (D-H) iCdx2Δ^{APC} mice were lethally irradiated (900 rad) and transferred with bone marrow from Trdc^{-/-} or control wild-type B6 animals. Eight weeks after bone marrow transfer, iCdx2Δ^{APC} recipient mice were injected with a single dose of 0.8 mg tamoxifen and analyzed 10 days later. (D) Protocol. (E) Fecal lipocalin-2 quantification. (F) Quantification of tumors in the colon. (G, H) Flow cytometry analysis of γδ T cells from non-tumor colonic tissue of control (WT.B6) bone marrow reconstituted iCdx2Δ^{APC}. (G) Frequency of CD8α⁺ and CD8α⁻. (H) Vγ usage among TCRγδ⁺ cells. (I, J; K-O) iTrdc^{Slc2a1}^{fl/+} and iTrdc^{Slc2a1}^{fl/fl} littermate control mice were subjected to AOM-DSS model, treated with tamoxifen for the first 3 weeks (I, J) or the last 6 weeks (K-O) and analyzed 12 weeks after initial AOM injection. (I, J) Flow cytometry analysis of intra-tumor γδ T cells in colon. (I) Ratio of IL-17⁺ to IFN-γ⁺ cells among TCRγδ⁺ cells. (J) Frequency of Vγ usage by TCRγδ⁺ cells. (K) Percentage of body weight changes during AOM-DSS treatment. Gray bars represent DSS treatment. (L) Tumor number, size and load. Shaded area bounded by dashed lines indicates mean ± SEM of all control C57BL/6/J mice analyzed in fig. S2B (AOM+DSS model). (M-O) Flow cytometry analysis of γδ T cells from tumor or non-tumor colonic tissue. (M) Frequency of PD-1⁺ cells among TCRγδ⁺ cells. (N) Frequency of IFN-γ⁺ (left) and IL-17⁺ (right) among TCRγδ⁺ cells. (O) Frequency of Vγ usage by tumor infiltrating TCRγδ⁺ cells. iCdx2Δ^{APC} bone marrow transfer and iTrdc^{Slc2a1}^{fl/+} data representative from 2 experiments with 3-5 animals per group. 1% vs 2% DSS data are pooled from 2 experiments with 2-5 animals per group. *P < 0.05, **P < 0.01, ***P < 0.001. (M, N) One-way ANOVA test with Dunnett's multiple comparison test; (B-E; G-J; O) Two-tailed t-test; (F) two-tailed Chi-test. Error bars indicate SEM.

Figure S3. Supporting data to Figure 3. (A-G) Wild-type B6 (WT), $V\gamma 7^{-/-}$, and $V\gamma 7^{+/-}$ littermate control mice treated with were subjected to AOM-DSS model and analyzed 12 weeks after initial AOM injection. (A) Tumor number, size and load. (B) TCR $\gamma\delta/\alpha\beta$ ratio and (C) frequency of $V\gamma 1$, $V\gamma 4$, $V\gamma 6$, $V\gamma 7$ among TCR $\gamma\delta^{+}$ cells from colonic tissue. (D) Frequency of $CD8\alpha^{+}$ (left) and $IFN-\gamma^{+}$ (right) and (E) $PD-1^{+}$ (left) and $IL-17^{+}$ (right) among TCR $\gamma\delta^{+}$ cells. (F) Frequency of $IFN-\gamma^{+}$ and $IL-17^{+}$ among $CD4^{+}$ T cells. (G) All groups were treated with anti- $V\gamma 1$ depleting antibody (2.11) twice a week, starting one week before AOM administration until the second DSS cycle. Frequency of $IFN-\gamma^{+}$ (left) and $IL-17^{+}$ (right) among $CD4^{+}$ T cells. Data pooled from 3 (A-F) and 2 (G) experiments with 3-5 animals per group. * ($p>0.05$) ** ($p>0.01$) *** ($p>0.001$) symbols represent statistical differences between groups.

Figure S4. Supporting data to Figure 4. (A-C) *iTrdc Δ Rorc* and *iTrdc^{Rorclfl/+}* littermate control mice were subjected to AOM-DSS model, treated with tamoxifen for the last 6 weeks of the experiment and analyzed 12 weeks after initial AOM injection. Flow cytometry analysis of $\gamma\delta$ T cells from tumor and non-tumor colonic tissue. (A) Frequency of V γ usage among TCR $\gamma\delta^+$ cells isolated from tumor colonic tissue. (B) Frequency of V γ 6 $^+$ vs V γ 6 $^-$ among IL-17-producing TCR $\gamma\delta^+$ cells isolated from tumor colonic tissue. (C) Frequency of IL-17 $^+$ among TCR $\alpha\beta^+$ CD4 $^+$ cells. (D, E) Bacterial 16S ribosomal RNA amplicon (16S rRNA) sequencing from fecal pellets of tamoxifen-treated *iCdx2 Δ APC* and *APC^{fl/fl}* littermate control mice. (D) Shannon alpha diversity plot from *iCdx2 Δ APC* (*Cre $^+$* blue) and *APC^{fl/fl}* control animals (*Cre $^-$* red), four weeks after tamoxifen administration. (E) Bi-dimensional principal coordinate analysis plot from *iCdx2 Δ APC* (*Cre $^+$* blue) and *APC^{fl/fl}* control animals (*Cre $^-$* red) before (week 0), 2, 3 and 4 weeks after tamoxifen administration. 16S rRNA sequencing data representative of 2 experiments with 2-4 animals per group. *iCdx2 Δ APC* and *APC^{fl/fl}* control mice were co-housed. Fecal samples were collected from mice housed in different cages. (A) two-tailed t-test (P values are written in the figure); (B, C) One-way ANOVA test with Dunnett's multiple comparison test. Error bars indicate SEM.

Figure S5. Supporting data to Figure 5. (A-G) Female $V\gamma 4^{-/-}$ and $V\gamma 4^{+/-}$ littermate control, (H, I) $V\gamma 6^{-/-}$ and $V\gamma 6^{+/-}$ littermate control mice were subjected to the AOM-DSS model and analyzed 12 weeks after initial AOM injection. In panel I, mice received injections of α - $V\gamma 4$ depleting antibody (UC3-10A6) antibody twice a week starting one week after the 2nd DSS cycle (last six weeks of experiment). (A) Tumor number, size and load. Shaded area bounded by dashed lines indicates mean $\pm$ SEM of all control C57BL/6J mice analyzed in fig. S2B (AOM+DSS model). (B-F) Flow cytometry analysis of $\gamma\delta$ T cells from tumor or non-tumor colonic tissue. (B) TCR $\gamma\delta$ / $\alpha\beta$ ratio within CD45 $^{+}$ cells from tumor colonic tissue. (C) Frequency of $V\gamma 6^{+}$ (left) and $V\gamma 4^{+}$ (right), and (D) CD8 $\alpha\alpha^{+}$ (left) and PD1 $^{+}$ (right) among TCR $\gamma\delta^{+}$ cells. (E) Frequency of IFN γ^{+} (left) and IL-17 $^{+}$ (right) among TCR $\gamma\delta^{+}$ cells from tumor colonic tissue. (F) Frequency of $V\gamma 6^{+}$ and $V\gamma 4^{+}$ among IL-17-producing $\gamma\delta$ T cells in tumor colonic tissue. (G-I) Frequency of IFN γ^{+} (left) and IL-17 $^{+}$ (right) among TCR $\alpha\beta$ CD4 $^{+}$ cells from tumor or non-tumor colonic tissue of $V\gamma 4^{-/-}$ (G), $V\gamma 6^{-/-}$ (H) or $V\gamma 4$ -depleted $V\gamma 6^{-/-}$ (I) and littermate heterozygous control mice. $V\gamma 4^{-/-}$ data pooled from 2 experiments with 3-5 animals per group. *P < 0.05, **P < 0.01, ***P < 0.001. (C, D; F-I) One-way ANOVA test with Dunnett's multiple comparison test; (A, B, E) two-tailed t-test. Error bars indicate SEM.