TCF-1 controls T_{reg} cell functions that regulate inflammation, CD8^{+} T cell cytotoxicity and severity of colon cancer

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The transcription factor TCF-1 is essential for the development and function of regulatory T (T_{reg}) cells; however, its function is poorly understood. Here, we show that TCF-1 primarily suppresses transcription of genes that are co-bound by FOXP3. Single-cell RNA-sequencing analysis identified effector memory T cells and central memory T_{reg} cells with differential expression of Klf2 and memory and activation markers. TCF-1 deficiency did not change the core T_{reg} cell transcriptional signature, but promoted alternative signaling pathways whereby T_{reg} cells became activated and gained gut-homing properties and characteristics of the T_{H}17 subset of helper T cells. TCF-1-deficient T_{reg} cells strongly suppressed T cell proliferation and cytotoxicity, but were compromised in controlling CD4^{+} T cell polarization and inflammation. In mice with polyposis, T_{reg} cell-specific TCF-1 deficiency promoted tumor growth. Consistently, tumor-infiltrating T_{reg} cells of patients with colorectal cancer showed lower TCF-1 expression and increased T_{H}17 expression signatures compared to adjacent normal tissue and circulating T cells. Thus, T_{reg} cell-specific TCF-1 expression differentially regulates T_{H}17-mediated inflammation and T cell cytotoxicity, and can determine colorectal cancer outcome.

T_{reg} cells are a heterogeneous population of thymic and extrathymic origins with diverse immunosuppressive functions. Expression of the lineage-determining transcription factor FOXP3 is essential for maintaining T_{reg} cell identity^{10}, but is not sufficient to account for the substantial functional diversity of T_{reg} cells^{4}. In addition to FOXP3, T_{reg} cells can express other transcription factors that are normally associated with helper T (T_{H}) cell functions, namely RORγT, GATA3 or TBET. More than half of gut-infiltrating T_{reg} cells in healthy mice express RORγT; RORγT^{+} T_{reg} cells are generated from naive CD4^{+} conventional T cells (T_{con}) upon stimulation by bacterial antigens, and suppress pathobiont-induced inflammation in an interleukin (IL)-10-dependent manner^{6}. GATA3-expressing T_{reg} cells express IKZF2 (encoding HELIOS) and IL1RL1 (encoding IL-33) and expand in response to IL-33 (ref. 4). These are mainly of thymic origin, although a subset that potentially originates from T_{con} cells can convert to RORγT^{+} T_{reg} cells^{4}. Both RORγT-expressing and GATA3-expressing T_{reg} cells accumulate in colon tumors, and have T cell-suppressive and tumor-promoting properties^{8,9}. Single-cell RNA-sequencing (scRNA-seq) studies of mouse and human cells have identified transcriptionally distinct subpopulations (clusters) of effector T_{reg} (eT_{reg}) cells and central memory T_{reg} (cT_{reg}) cells^{10}. However, the molecular underpinning of T_{reg} cell responses and adaptations at the single-cell level to their environment is still poorly understood.

In contrast to healthy mice, expansion of RORγT^{+} T_{reg} cells in human colorectal cancer (CRC) coincides with increased colon inflammation^{11}. In mouse models of polyposis T_{reg} cell-specific ablation of RORγT attenuates inflammation and tumor growth^{12}. Furthermore, the adoptive transfer of T_{reg} cells from healthy but not from tumor-bearing mice to polyposis-prone mice hinders polyposis^{13}. We found that T_{reg} cells in patients with CRC and mice with polyposis express elevated levels of β-catenin, which epigenetically programs the cells to gain proinflammatory properties^{14,15}. Our findings were corroborated by an independent report of elevated expression of β-catenin by proinflammatory T_{reg} cells in multiple sclerosis^{16}. These findings indicate cancer-related changes in T_{reg} cell functions.

TCF-1 is the T cell-specific DNA binding partner of β-catenin^{17}. Germline TCF-1 deficiency induces premature expression of FOXP3 in double-positive thymocytes^{18} and expands thymic T_{reg} cells^{19}, suggesting a role in T_{reg} cell specification. We and others have shown that TCF-1 and FOXP3 co-bind to overlapping regulatory sites of proinflammatory pathway genes^{19,20} and repress the MAF–RORγT–IL-17 axis^{14,21}. Here, we report that in the absence of TCF-1, FOXP3 fails to control these genes and T_{reg} cells gain proinflammatory and tumor-promoting properties, similarly to the T_{reg} cells that expand in human CRC and mouse polyposis. Moreover, TCF-1 is downregulated in CRC tumor-infiltrating T_{reg} cells. Therefore, TCF-1 differentially controls independent T_{reg} cell functions that are deregulated in CRC and contribute to tumor growth.

Results

TCF-1 negatively regulates gene expression in T_{reg} cells. To understand how TCF-1 regulates T_{reg} cell properties, we generated mice homozygous for the floxed exon4 Tcf7 (ref. 22) and the Foxp3^{−/−} alleles^{23} (Foxp3^{−/−}Tcf7^{geo}). Fluorescence-activated cell sorting (FACS) analysis of mesenteric lymph node (MLN) cells confirmed loss of
TCF-1 in Treg but not CD4+ Treg cells (Extended Data Fig. 1a,b,c). Bulk RNA-seq analysis revealed that deletion of Tcf7 upregulated 1,090 genes (fold change >1.5 and false discovery rate (FDR) <0.001), which included the core Treg cell signature genes Il2ra, Foxp3, Foxo1, Tgfbl1, Lef1, Rara and Gata3, and downregulated 422 genes including Cita4, Ifkz2 and Gzm (Fig. 1a). To identify pathways affected, we performed gene-set enrichment analysis (GSEA) on all KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways comparing transcriptomes of Treg cells from Foxp3<sup>Cre<sup>+/−</sup></sup> <sup>l2ra<sup>−/−</sup></sup> mice (FDR <0.25; Fig. 1b and Supplementary Table 1). The analysis indicated that the TCF-1-deficient Treg cells preserved the core Treg cell signature genes (Extended Data Fig. 1d), but were enriched in Wnt, MAPK, IL-17, transforming growth factor (TGF)-β, T cell antigen receptor (TCR) signaling and T<sub>j17</sub> differentiation pathways (Fig. 1b). The enhanced Wnt signature could result from reversal of TCF-1 inhibition of transcription<sup>11</sup>. The most significantly enriched genes within the leading edge for WNT signaling included Lef1, Lrp5, Gsk3β, Csnk1e, Csnk2a2a, Ep300 and Rac1; T<sub>j17</sub> differentiation genes included Tgfb1, Ile6a, Rara, Stat3, Ihf2r2, Gata3 and Tbx21 as well as genes downstream of the TCR; TGF-β signaling genes included Tgfb1, Smad3, Smad7 and Myc; and TCR signaling genes included Nfatc1, Nfatc2, Nfatc3, Rela, Fos, Jun, Pik3r1, Akt1, Nfkbl, Kras and Pleg1 (Fig. 1c). Earlier identified genes bound by TCF-1 as well as genes co-bound by TCF-1 and FOXP3 (ref. <sup>12</sup>) were highly upregulated in TCF-1-deficient Treg cells (Fig. 1d), suggesting dominant regulation by TCF-1. Altered gene expression coincided with opening of chromatin at gene regulatory sites, as determined by chromatin immunoprecipitation and sequencing (ChIP-seq), with key examples being Foxp3, Tgfb1, Stat3, Smad3 and Il2ra (Fig. 1e). Collectively, our data show that TCF-1 has a dominant role in its molecular regulation of Treg cells.

Using FACS analysis, we validated changes in expression of cell-surface proteins that mark T cell activation, including CD69, ICOS, PD-1 and CD44 and CD62L (Fig. 2a and Extended Data Fig. 2a,b,c,h). Loss of TCF-1 increased the Treg cell to CD4+ T cell ratios as well as the frequency and absolute numbers of Treg cells in secondary lymphoid organs (Fig. 2b and Extended Data Fig. 2e), but reduced the cell-surface expression of CD25 (Fig. 2b and Extended Data Fig. 2f). We confirmed earlier reports of activation of T<sub>cell</sub> genes, marked by changes in expression of CD69, ICOS, PD-1 and CD44 (ref. <sup>12</sup>; Fig. 2c and Extended Data Fig. 2a,b,c,d). The TCF-1-deficient T<sub>reg</sub> cells expressed higher levels of RORγT, TGF-βRI, TGF-βRII and phospho-SMAD2/3 (Fig. 2d,e and Extended Data Fig. 2g), phospho-STAT5 and phospho-S6 (a downstream target of mTORC1 that is highly active in T<sub>reg</sub> cells<sup>14</sup>; Fig. 2e). Collectively, these results show that TCF-1 deficiency enhances the activation and expression of core Treg cell signature genes causing the systemic expansion of RORγT<sup>+</sup> T<sub>reg</sub> cells.

**Molecularly distinct clusters of T<sub>reg</sub> cells.** To understand how TCF-1 regulates T<sub>reg</sub> cell gene expression and heterogeneity, we

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**Fig. 1 | TCF-1 deficiency selectively reprograms T<sub>reg</sub> cells without compromising their core signature.** a, Scatterplot comparing the expression of genes in TCF-1-deficient (Foxp3<sup>Cre<sup>−/−</sup></sup>) and TCF-1-sufficient (Foxp3<sup>Cre<sup>+/+</sup></sup>) T<sub>reg</sub> cells. Reads per kilobase of transcript per million mapped reads (RPKM) expression values were averaged from three biological replicates. Significantly upregulated and downregulated genes (fold change >1.5 and FDR <0.001) are shown in red and blue with exact numbers shown at the top and bottom corner, respectively. b, Significantly enriched KEGG pathways by GSEA induced in the transcriptomes of TCF-1-deficient versus sufficient Treg cells. Normalized enrichment scores (NESs) of all enriched KEGG pathways (FDR <25%) are shown. Select pathways are highlighted. See Supplementary Table 1 for the full list. c, The expression of all leading-edge genes from four indicated pathways. See Supplementary Table 1 for the raw expression levels of all genes. d, GSEA plots showing the enrichment of genes expressed more highly in TCF-1-deficient (Tcf7<sup>+/−</sup>/Foxp3<sup>+/+</sup>) versus TCF-1-sufficient (Foxp3<sup>+/+</sup>) T<sub>reg</sub> cells for genes that are bound by TCF-1 (upper) or co-bound by TCF-1 and FOXP3 (lower). e, TCF-1 ChIP-seq tracks in mouse T<sub>reg</sub> cells showing the Foxp3, Tgfbl1, Stat3, Smad3 and Il2ra gene loci. For simplicity, the input control signal was subtracted from visualized tracks using the Integrative Genomics Viewer tool. Detected TCF-1-bound sites against the input control are indicated with a blue arrow. Data in d and e are from GSE139960.
performed scRNA-seq of MACS-purified MLN Treg cells using the 10x Genomics platform (Extended Data Fig. 3a) for four types of mice: Foxp3<sup>Cre</sup>-Tcf7<sup>fl/fl</sup>, Foxp3<sup>Cre</sup>, the polyposis-prone APC<sup>Min</sup> and wild-type (WT) C57BL/6J mice. An unbiased integrative analysis across all four genotypes for representation of potential artifacts using the Seurat platform (Methods) resulted in 14,487 cells grouped into ten major subpopulations on a uniform manifold approximation and projection (UMAP) analysis (Fig. 3a, Supplementary Table 2 and Methods). These subpopulations were annotated according to the most salient identified cell markers (Fig. 3b). As expected, the exon4 deleted Tcf7 transcripts were still detected across the T<sub>reg</sub> cell clusters, although less intensely as compared to the WT Tcf7 transcripts in control Foxp3<sup>Cre</sup> T<sub>reg</sub> cells (Extended Data Fig. 3b).

We identified two eT<sub>reg</sub> cell clusters with activated/effector characteristics, and low expression of Kruppel-like factor 2 (Klf2) (Fig. 3b). These were annotated as Maf and Ikzf2 based on their high expression of the corresponding genes. The Maf cluster had the highest expression of Rorc, Icos and S100a4 (Fig. 3b,c). CMAF is essential for the generation of RORγ<sup>T</sup>-T<sub>reg</sub> cells and IgA response<sup>20</sup>, and is negatively regulated by TCF-1 (ref. 21). Expression of Rorc (encoding RORγT) by T<sub>reg</sub> cells is bacterial dependent<sup>2</sup>, suggesting that the Maf cluster represents peripherally induced T<sub>reg</sub> cells. The Ikzf2 cluster had the highest expression of Il17r, Rora and Gata3, and Klrg1, and the second-highest expression of Maf and Icos (Fig. 3b,c). Ikzf2 encodes HELIOS, a member of the IKAROS transcription factor family that regulates several T<sub>reg</sub> cell-suppressive functions<sup>22</sup>, and is preferentially but not exclusively expressed by thymus-derived naive/eT<sub>reg</sub> cells<sup>23</sup>. This cluster prominently expressed Gata3 and its downstream target gene Il1rl1, which encodes a subunit of the IL-33 receptor. Thymus-derived T<sub>reg</sub> cells, constitute a significant proportion of the GATA3<sup>+</sup> Il1rl1 expressing colonic T<sub>reg</sub> cells<sup>23</sup>, supporting the thymic origin of the Ikzf2 cluster. These two clusters were earlier described as the RORγ<sup>T</sup> and the HELIOS<sup>+</sup> subsets in mice<sup>24</sup>, or as nonlymphoid T cell-like (nLT) T<sub>reg</sub> cells in mice and peripherally induced T<sub>reg</sub> cells in humans<sup>25</sup>.

The macrophage migration inhibitory factor (Mif) cluster had high expression of Tgfbr1, Thf8sf9, Nkx61 and Nr4a1 (Fig. 3b and Extended Data Fig. 4). It also expressed Maf, Icos and Ikzf2 but less than the Maf and Ikzf2 clusters (Fig. 3b,c). Nr4a1 is an immediate-early activation gene downstream of the TCR that induces expression of Thf8sf9 and Ikzf2 (ref. 25). High expression of these genes together with Tgfbr1 is characteristic of early TGF-β-induced extrathymic T<sub>reg</sub> cells. Expression of Hif1α, a downstream target of β-catenin, was highest in the Mif and Maf clusters, suggesting TCR signaling<sup>26</sup> and a potential link between β-catenin signaling and activation of the Maf–RORγT axis<sup>13,24</sup>.

The remaining clusters expressed naive and central memory genes that identify the cT<sub>reg</sub> cells<sup>27</sup>, and varying levels of Klf2 (Fig. 3b).
and Extended Data Fig. 4), a nuclear factor that regulates migration of Treg cells. The Klf2\(^{++}\) cluster which had the highest expression of markers of early thymic emigrants (ETEs) and homing to secondary lymphoid organs, including Klf2, Slpr1 and Igfbp4 (Fig. 3b and Extended Data Fig. 4). The Klf2\(^{-}\) and Ncoa3 clusters had the lowest expression of these markers, suggesting that they contain more mature cells. The Ncoa3 cluster was outstanding in strong expression of Klf2 (Fig. 3b), a nuclear coactivator and partner of the arylhydrocarbone receptor and high expression of Notch2. Three other cTreg cell clusters, Klf2, Ifn and Vps8, expressed intermediate levels of ETE markers, and were together isolated from the main cluster pool (Fig. 3a,b). The Ifn cluster was conspicuous by its expression of multiple interferon-response genes including Stat1, Ifit1, Ifit3, Ifit1b1 and Ifit3b (Fig. 3b and Extended Data Fig. 4). The Vps8 cluster expressed Klf2 and Izumo1r, markers of cTreg cells, but was unique in showing strong expression of Vps8, a subunit of the CORVET complex that is involved in the formation of exosomes. The Cdh63 cluster had poor expression of Klf2 and Izumo1r (encoding FOLR4), expressed Ccl5 and was distant from the other clusters (Fig. 3a,b and Extended Data Fig. 4), hence it is likely not a Treg cell cluster. Overall, comparison of Klf2 and ETEs versus activation markers separated the Treg cell clusters into different stages of maturation.

To better define the Treg cell clusters, we performed Gene Ontology pathway analysis on the upregulated genes. The Maf and Ikkf2 clusters highlighted pathways that indicate terminal differentiation, such as lymphocyte activation, immune response, negative regulation of immune system processes, positive regulation of cytokine production and high apoptotic signaling. By contrast, the Mif cluster displayed regulation of the response to a cytokine stimulus but no other function, consistent with an intermediate stage of Treg cell specification/maturation (Fig. 3d). Because Klf2\(^{++}\) and Klf2\(^{-}\) were the two largest Treg cell clusters with the most extreme difference in Klf2 expression among the cTreg cells (Fig. 3a,b), we directly compared them using Metascape and identified the 20 most enriched pathways. The Klf2\(^{++}\) cluster was enriched for T cell migration and leukocyte cell–cell adhesion pathways, consistent with being less mature (Fig. 3c), while the Klf2\(^{-}\) cluster was enriched for Treg cell differentiation, IgA production and cytokine production (Fig. 3c), indicating a more mature state. Thus, expression of Klf2 appears to be correlated with the stage of maturity of Treg cells.

### TCF-1 regulates distinct Treg cell functions

To better understand the contribution of TCF-1 to Treg cell identity and function, we made a side-by-side comparison of the scRNA-seq data from Fovp3\(^{-}\)Tcf7\(^{+/}\) mice and control Fovp3\(^{+/}\) mice. Loss of TCF-1 did not alter the spatial distribution or the number of Treg cell clusters (Fig. 4a), but did suggest a possible increase in the frequency of cells in the Maf and Ikkf2 Treg cell clusters relative to the less differentiated clusters (Fig. 4a). There were significant changes in the expression of Maf, Ccr9 and Hsph1 across Treg cell clusters with the notable common exception of the Ifn cluster (Fig. 4b, Extended Data Fig. 5a,b and Supplementary Table 3). Accordingly, across the Treg cell clusters, we found changes in expression of MAF target genes and Treg17 pathway genes (Extended Data Fig. 5c), and corresponding increases in the Treg17 signaling pathway as revealed by GSEA against the Stubbington (Fig. 4c) or the KEGG (Extended Data Fig. 5d,e) datasets.

The Maf, Ikkf2, Klf2\(^{-}\) and Mif clusters showed the strongest upregulation of Ccr9, a gut-homing marker (Fig. 4b and Extended Data Fig. 5f). The Maf and Ikkf2 clusters had the strongest increase in expression of Hsph1 (Fig. 4b), a Treg cell activation marker. The Mif cluster also had the strongest increase in expression of the gut-associated integrin Igaec (encoding αE/ε-integrin; Fig. 4b), and together with the Ikkf2 cluster it showed the strongest increase in expression of fibrinogen-like protein 2 (Flg2)\(^{43}\), a downstream target of TGFβ (Fig. 4b). All TCF-1-deficient clusters had uniformly increased expression of Dnaaj1, which encodes a heat-shock protein co-chaperone\(^{44}\) (Fig. 4b), and Erdr1, which encodes a bacteria-sensitive secreted apoptotic factor\(^{47}\) (Extended Data Fig. 5g), but downregulated Igfbp4, an inhibitor of insulin-like growth factor receptor signaling\(^{48}\) (Fig. 4b and Extended Data Fig. 5h). The Ifn cluster was the only cluster that did not show significant changes with loss of TCF-1 (Fig. 4b and Extended Data Fig. 5b). The Vps8 cluster was unique in having high Treg1 and Treg17 signatures (Fig. 4c), raising speculation that this cluster may be a precursor to pathogenic Treg17 cells, which coexpress Treg1 and Treg17 cytokines\(^{49}\). These results highlight enhanced Treg17 cell activation, gut homing and Treg17 polarization with the loss of TCF-1.

Next, we determined how loss of TCF-1 affects the expression of genes that normally bind TCF-1, by integrating previously generated ChIP-seq analysis data\(^{41}\). Overall, these genes were upregulated with the loss of TCF-1, indicating negative regulation of gene expression by TCF-1; exceptions were the Ikkf2, Ncoa3 and Ifn clusters that remained unchanged (Fig. 4d,e). Importantly, expression of TCF-1 and FOXP3 co-bound genes also increased upon loss of TCF-1 (Fig. 4f,g), indicating that TCF-1 cooperates with FOXP3 in suppressing gene expression. Collectively these findings are consistent with TCF-1 functioning as a dominant regulator of FOXP3 in suppressing expression of Treg cell genes involved in Treg17 signaling, gut homing and bacterial response.

To better understand the intercluster relations of Treg cells, we overlaid RNA velocity vectors on the UMAP projection. RNA velocity uses scRNA-seq data of unspliced and spliced mRNAs to predict future states of transcriptionally distinct clusters of cells. Maf and Ikkf2 were identified as terminally differentiated Treg cell clusters, which derived from less mature clusters. While the Mif cluster exclusively gave rise to the Maf cluster, the Klf2\(^{-}\) and Ncoa3 clusters were immediate precursors to Ikkf2 (Fig. 4h). There was also some indication for interconversion of Ikkf2 to Maf, in agreement with an earlier report that HELIOS\(^{+}\) Treg cells can be induced to express RORγ\(^{+}\). The Ifn, Vps8 and Klf2\(^{-}\) clusters were isolated and less related to the other clusters, encouraging speculations that they may be intermediates to alternative fates, perhaps effector T cells. In total, the velocity analysis revealed stages of Treg cell specification and maturation, as well as potential differentiation to non-Treg cells.

### Polyposis causes activation and polarization of Treg cells

We next performed scRNA-seq analysis of Treg cells from the MLNs of WT and polyposis-ridden APC\(^{–}\) mice. The distribution and numbers of Treg cell clusters were similar to those from Fovp3\(^{-}\)Tcf7\(^{+/}\) and Fovp3\(^{+/}\) mice (Extended Data Fig. 6a and Supplementary Table 4). In both mice, expression of Tcf7 was lower in the terminally differentiated Maf and Ikkf2 cTreg cell clusters as compared with the less mature cTreg cell clusters (Extended Data Fig. 6b). Comparison of gene expression between WT and APC\(^{–}\) Treg cells revealed upregulation of Soc3s, JunD, Lag3 and Tgfb1 during polyposis (Extended Data Fig. 6c,d). Soc3s regulates IL-23-mediated STAT3 phosphorylation and polarization of CD4\(^{+}\) T cells to the Treg17 lineage\(^{41}\). JunD, encodes an AP1 transcription factor that is activated downstream of the TCR\(^{42}\). Lag3 mediates immune suppression by Treg cells\(^{43}\). Velocity analysis revealed conserved intercluster relations (Extended Data Fig. 6e). Collectively, these transcriptional changes are consistent with the activation and Treg17 polarization of Treg cells during polyposis.

### TCF-1 regulates Treg cell suppression of CD8\(^{+}\) T cells

Next, we related our molecular data to Treg cell function. Earlier we and others had reported that Treg cell suppression of CD8\(^{+}\) T cell cytotoxicity is TGF-β dependent\(^{44,45}\). Given their activated expression profile, preservation of the core Treg cell signature, and the enhanced TGF-β signature, we predicted that TCF-1-deficient Treg cells would...
Fig. 3 | Single-cell transcriptomics delineates distinct T<sub>H<sub>3<sub>2</sub> subpopulations in the mesenteric lymph nodes. a, Integrated UMAP showing ten major T<sub>H<sub>3<sub>2</sub> cell types isolated from the MLNs of mice used in this study. b, Expression of cell-defining features across all cell types. Color intensity is proportional to the average gene expression across cells in the indicated clusters. The size of circles is proportional to the percentage of cells expressing the indicated genes. c, mRNA expression of select indicated genes projected on the UMAP, focusing on features of the Maf and Ikzf2 T<sub>H<sub>3<sub>2</sub> cell clusters. d, Significantly enriched pathways by Metascape based on the top 200 genes upregulated in the indicated cell type as compared to all other cell types. See Supplementary Table 2 for the full list. e, The 20 most significantly enriched pathways by Metascape based on genes upregulated in Klf2<sup>−</sup> or Klf2<sup>++</sup> cell types directly compared to Klf2<sup>++</sup> or Klf2<sup>−</sup> cell types, respectively.
Fig. 4 | TCF-1-deficient and TCF-1-sufficient T<sub>reg</sub> cells show distinct effector functions. a, UMAP projection (left) and fraction of cells in each cell type (stacked bars; right) for TCF-1-sufficient (Foxp3<sup>Cre</sup>) and TCF-1-deficient (Foxp3<sup>CreTcf7<sup>fl/fl</sup></sup>) T<sub>reg</sub> cells. Data are from two replicates. b, Expression changes of the most differentially expressed genes between TCF-1-deficient and TCF-1-sufficient T<sub>reg</sub> cells. See Supplementary Table 3 for the full list. The fold change in expression intensities is color coded. The fold change in the percentage of cells expressing the indicated gene in each cell type is proportional to the circle size. c, GSEA analysis for the indicated gene lists comparing the transcriptomes of TCF-1-sufficient and TCF-1-deficient T<sub>reg</sub> cells across all cell types. NESs are color coded. −log<sub>10</sub> (FDR) values are proportional to the circle size. FDR > 15% are masked in gray. d, The UMAP projection of module scores for relative expression of TCF-1-bound genes and related violin plots. e, f, The UMAP projection of module scores for relative expression of TCF-1 and FOXP3 co-bound genes and related violin plots. g, h, UMAP and extrapolated future state of cells (overlaid arrows) based on RNA velocity for TCF-1-sufficient (Foxp3<sup>Cre</sup>) and TCF-1-deficient (Foxp3<sup>CreTcf7<sup>fl/fl</sup></sup>) T<sub>reg</sub> cells. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by one-sided (e) or two-sided (g) Wilcoxon test. NS, not significant.
efficiently suppress CD8+ T cells. To test this, we compared CD8 cytotoxic responses of Foxp3CreTcf7fl/fl and Foxp3Cre+ mice to acute infection with Thelier's murine encephalomyelitis virus (TMEV), using an in vivo kill assay. In an earlier study, we described an immunodominant virus-specific CD8+ T cell response to the viral VP2121–130 peptide that peaks on day 7 after infection3. We quantified this activity by adoptive transfer of an equal mix of TMEV–VP2121–130 peptide-pulsed and unpulsed splenocytes, at the peak of response to viral lysis. Lysis of the peptide-pulsed cells was significantly less effective in the Foxp3CreTcf7fl/fl than in the Foxp3Cre+ mice (~21% versus ~56% converted, P<0.0001, Student’s t-test) and treatment of mice with the small-molecule inhibitor of TGF-βR LY3200882 abrogated this difference (Fig. 5a). Using tetramers, we found that infection of Foxp3Cre+ mice triggered a nearly 14-fold expansion of VP2121–130-specific CD8+ T cells in the spleen, from 0.07% to almost 1% (P=0.0004) of total CD8+ T cells at the peak of response to TMEV.

This expansion was reduced in the Foxp3CreTcf7fl/fl mice to the level of baseline uninfected Foxp3Cre+ mice (Fig. 5b). To independently validate this inhibition, we performed in vitro proliferation inhibition assays. FACS-purified CD4+CD25+YFP+ Treg cells were co-cultured with an equal number of naive CD4+CD25–CD62LhiCD44hi CD8+ T cells and then stimulated with allogeneic BALB/c CD11c+ dendritic cells (DCs) and αCD3. The TCF-1-deficient Treg cells exhibited stronger suppressive activity than TCF-1-sufficient Treg cells (P<0.05, Student’s t-test; Fig. 5c). Together, our data show that TCF-1 deficiency augments the ability of Treg cells to suppress CD8+ T cell cytotoxicity and T cell proliferation.

TCF-1 regulates Treg cell suppression of inflammation.

Inflammation requires CD4+ T cell help. Therefore, we compared TCF-1-deficient and TCF-1-sufficient Treg cells for their ability to suppress polarization of naive CD4+ T cells to the Th1 or Th17 lineage. For the in vitro assays, splenic CD4+ lymphocytes containing both Tconv and Treg cells were purified from Foxp3CreTcf7fl/fl mice and control Foxp3Cre+ mice, stimulated with αCD3 and αCD28 under Th1 or Th17 polarization conditions for 4 d. The polarized Treg cells from Foxp3CreTcf7fl/fl mice expressed significantly more interferon (IFN)-γ than the T cells from Foxp3Cre+ mice (~25% versus ~9% converted, P<0.0004; Fig. 6a). To standardize the CD4+ T cell to Treg cell ratios, we repeated the assay using sorted CD4+CD25–CD62LhiCD44hi naive T cells from WT CD45.1 mice mixed at an equal ratio with CD25+YFP+ Treg cells from Foxp3CreTcf7fl/fl mice or control Foxp3Cre+ mice. TCF-1-deficient Treg cells were consistently less effective in suppressing CD4+ T cell polarization to Th1 cells (Fig. 6b; ~16% versus ~8% converted, P<0.002). Similarly, the Th17 polarized T cells from Foxp3CreTcf7fl/fl mice expressed significantly more IL-17A than the T cells from Foxp3Cre+ mice (Fig. 6c; ~25% versus ~5% converted, P<0.0003), and this was confirmed when equal numbers of purified Tconv cells and Treg cells were mixed (Fig. 6d; ~11% versus ~5% converted, P<0.001). Thus, TCF-1 deficiency compromised the ability of Treg cells to suppress proinflammatory Th1 cell polarization, to Th1 or Th17.
We further validated our findings using well-established conditions that elicit T$_{\text{h}}$1 or T$_{\text{h}}$17 immunity in vivo. Mice were infected with TMEV and after 7 d mononuclear cells isolated from the spleen or MLNs were restimulated ex vivo with the phorbol ester PMA plus ionomycin and GolgiStop to measure intracellular IFN-γ. CD4$^+$ and CD8$^+$ T cells from Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/fl}}$ mice expressed significantly more IFN-γ than cells from Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/+}}$ mice (Fig. 6c,d). CD4: MLN 6% versus 3%, $P < 0.04$ and spleen 19% versus 12%, $P < 0.001$; CD8: MLN 26% versus 14%, $P < 0.003$ and spleen 41% versus 24%, $P < 0.001$). To measure T$_{\text{h}}$17 polarization, we followed an established protocol, injected the mice intraperitoneally (i.p.) with αCD3 and 4 d later quantified the expression of IL-17A by CD4$^+$ T cells in the small bowel by FACS. The Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/+}}$ mice generated significantly more IL-17-expressing CD4$^+$ T cells than the control Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/fl}}$ mice ($P < 0.01$; Fig. 6g). Collectively, these findings indicate that TCF-1-deficient T$_{\text{reg}}$ cells are compromised in suppressing T$_{\text{h}}$1 and T$_{\text{h}}$17 polarization in vitro and in vivo.

**TCF7 is downregulated in T$_{\text{reg}}$ cells of CRC tumors.** To determine the tumor-promoting properties of TCF-1-deficient T$_{\text{reg}}$ cells, we crossed the polyposis-prone APC$^{\text{Cre}}$ mice with Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/}}$ mice or Foxp3$^{\text{Cre}}$ mutants and aged the compound mutant mice to develop polyps. The TCF-1-deficient APC$^{\text{Cre}}$Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/}}$ mice had significantly more colon polyps than control APC$^{\text{Cre}}$Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/+}}$ mice (12% versus 4%, $P < 0.0001$; Fig. 7a), while tumor load in the small intestine did not change (Fig. 7b). Nuclear β-catenin staining revealed higher incidence of pre-invasive tumors in both the colon (Fig. 7c) and small bowel (Fig. 7d) of the APC$^{\text{Cre}}$Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/}}$ mice (Fig. 7e), compared with APC$^{\text{Cre}}$Foxp3$^{\text{Cre}}$ mice (Fig. 7f). The APC$^{\text{Cre}}$Foxp3$^{\text{Cre}}$Tcf7$^{\text{fl/}}$ colon tumors had high densities of Gr1$^+$ cells compared to APC$^{\text{Cre}}$Foxp3$^{\text{Cre}}$ mice (116 per field of view (FOV) versus 62 per FOV; $P < 0.0001$; Fig. 7g,h), as did the small-bowel tumors (13.5 per FOV) versus APC$^{\text{Cre}}$Foxp3$^{\text{Cre}}$ mice (8.5 per FOV; $P < 0.02$; Fig. 7i). The increase in Gr1$^+$ cells was also evident in the tumor-distant healthy tissue (colon: 1.2 versus 0.4 per FOV; $P < 0.009$ and small bowel: 2 versus 1 per FOV; $P < 0.01$; Fig. 7g,h,i). Based on these findings, we conclude that TCF-1-deficient T$_{\text{reg}}$ cells have enhanced tumor-promoting properties, which relates in part to their compromised suppression of inflammation.
available scRNA-seq data from 12 individuals with CRC, focusing on the T<sub>reg</sub> cells from paired peripheral blood mononuclear cells (PBMCs), tumor tissue and adjacent normal tissue. Tumor-infiltrating T<sub>reg</sub> cells had significantly lower expression of TCF7 compared to adjacent normal tissue and PBMCs (Fig. 8a). Moreover, genes that were highly expressed in tumor-infiltrating T<sub>reg</sub> cells were enriched in KEGG T<sub>h</sub>17 differentiation and IL-17-signaling pathways (Fig. 8b,c,d). These findings are consistent with our earlier observations in individuals with CRC. Furthermore, they establish the relevance of our findings with TCF-1 mice harboring deficient T<sub>reg</sub> cells to the immune pathology of CRC.

Discussion
We have provided evidence that TCF-1 differentially controls independent T<sub>reg</sub> cell–suppressive mechanisms. TCF-1-deficient T<sub>reg</sub> cells gained a ‘split personality’ similar to T<sub>reg</sub> cells in CRC, failing to suppress inflammation but becoming more active in suppressing T cell proliferation and cytotoxicity. In a mouse model of spontaneous polyposis, these changes fueled tumor growth by promoting inflammation while blocking antigen-specific CD8<sup>+</sup> T cell responses. We demonstrated the relevance of these findings to CRC in humans by meta-analysis of publicly available data, which showed that tumor-infiltrating T<sub>reg</sub> cells had reduced TCF-1 expression and increased of T<sub>h</sub>17 and IL-17 signaling.

TCF-1-deficient T<sub>reg</sub> cells strongly expressed the core T<sub>reg</sub> cell signature genes, along with Wnt, T<sub>h</sub>17, MAPK and TCR signaling. scRNA-seq identified two cT<sub>reg</sub> clusters, marked by high expression of cMaf or Ikzf2, and assigned several cT<sub>reg</sub> cell clusters to different stages of maturation based on their expression of Klf2, ETEs and activation markers, as well as their spatial distribution in the UMAP. This classification was confirmed by pathway analysis. Gene expression data strongly suggested peripheral and thymic origins of the Maf and Ikzf2 clusters, respectively. Our UMAP superimposed velocity analysis suggested intercluster relations, indicating that the Maf and Ikzf2 cT<sub>reg</sub> cell clusters might originate from two cT<sub>reg</sub> cell clusters with low Klf2 expression, namely Klf2<sup>-</sup> and Ncoa3<sup>-</sup>, while the Mif cluster exclusively led to the Maf cluster. Among the cT<sub>reg</sub> cell clusters, Ifn, Klf2 and Vps8 were the most isolated, based on velocity analysis, and could represent transitions to effector T cells.

Changes in gene expression caused by loss of TCF-1 occurred within conserved T<sub>reg</sub> cell clusters. Side-by-side comparison of scRNA-seq data from TCF-1-deficient and TCF-1-sufficient T<sub>reg</sub> cells revealed changes in activation, T<sub>h</sub>17 signaling and gut homing. Ablation of TCF-1 broadly enhanced expression of genes that are normally bound by TCF-1 and FOXP3 with few exceptions, such as the Ikzf2/HELIOS cluster. Expression of cMaf and T<sub>h</sub>17 signaling signature was increased across T<sub>reg</sub> cell clusters, again with little change in the Ikzf2/HELIOS cluster. These findings agree with our earlier finding that in individuals with inflammatory bowel disease and dysplasia expression of proinflammatory...
cytokines (IL-17, IFN-γ and TNF) by Treg cells is mostly limited to the RORγT+HELIOS+ Treg cells. Using ex vivo and in vivo assays, we demonstrated that TCF-1-deficient Treg cells strongly suppressed T cell proliferation and antigen-specific T cell cytotoxicity of CD8+ T cells; however, they were compromised in inhibiting the polarization of CD4+ T cell to the proinflammatory Th17 or Te17 lineages and failed to suppress inflammation in polyposis. Notably, pharmacologic inhibition of TGF-βR1 signaling blocked the suppression of CD8+ T cell cytotoxicity by TCF-1-deficient Treg cells, in line with active TGF-β signaling in the absence of TCF-1 and the essential role of this pathway in Treg cell suppression of CD8+ T cells. The combined proinflammatory and T cell suppressive action of TCF-1-deficient Treg cells increased tumor load and tumor aggression in polyposis, demonstrating relevance to CRC. These findings demonstrate a bifurcation of Treg cell–suppressive activities upon loss of TCF-1, which favors tumor growth.

Polyposis in mice upregulated Treg cells gene associated with activation, inflammation and immune suppression, similarly to TCF-1-deficient Treg cells. At the single-cell level, TCF-1 expression was lower in the most differentiated relative to the less mature Treg cell clusters. We found these findings to be relevant to human CRC. Reanalysis of publicly available data showed reduced TCF-1 expression in tumor-infiltrating Treg cells in CRC. These observations are in line with the tumor dependence Treg cell proinflammatory properties in patients with CRC and in polyposis mice.
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Methods

Mice. Mouse strains described below were housed and bred at the Mayo Clinic animal facility. Tgβ8Cre (European Mouse Mutant Archive) were crossed to Fopp3ΔΔ mice (designated as Fopp3ΔΔ mice) to generate mice with Tcf7 cell specific deletion of Tcf7. Fopp3ΔΔ mice were crossed to ApcCre mice to generate the polyposis-prone compound mutant APcFopp3ΔΔ/Tcf7ΔΔ mice. Animal experiments were approved by the Animal Ethics Committee of the institutes responsible for housing the mice. Unless otherwise specified, all experimental procedures were performed on 5.5-month-old laboratory mice.

Viral infections. Mice were infected with TMEV at day 0. For acute viral infection, 2.5-5.0x10^5 plaque-forming units were used. Virus was prepared in plain DMEM and i.p. injected.

In vivo cytotoxicity assay. In vivo cytotoxic T lymphocyte assays followed established protocols44,55. Briefly, splenocytes from naive WT CD45.1 mice on the B6 background were prepared as single-cell suspensions to 1x10^6 per ml in Ca/Mg-free Hank's balanced salt solution (GE Healthcare). The specific target population (half of the cells) was pulsed with 1μM ml^-1 VP2121–130 peptide and the negative control target population was not pulsed with peptide. Cells were incubated for 60 min at 37°C and then were washed twice in complete medium and brought up in Ca/Mg-free Hank's balanced salt solution for labeling with CFSE (79898, BioLegend). Peptide-pulsed cells were incubated with 10μM CFSE (CFSE^p) or non-pulsed with 1μM CFSE (CFSE^-). Concentrations for 10 min in a 37°C water bath, and then quenched by addition of complete medium. Cells were washed three times, then viable cells counted and mixed in a 1:1 ratio before injection into recipient mice. A total of 15 million cells per 200 μl Ca/Mg-free PBS (Lenza; at room temperature) were transferred into mice on day 7 after TMEV, by i.v. injection into the tail.Recipient mice were euthanized 4 h later, and the collected MLNs and splenocytes were analyzed by Flow cytometry to determine the percentage of CFSE^p and CFSE^- cells. The percentage of VP2121–130-specific cytotoxicity was calculated as follows:

% specific lysis = 1 - (naive reinfected / 100) * (CFSE^p cells / CFSE^- cells)

In some experiments, mice were gavaged twice a day with LY3200882 (Eli Lilly) at 105 mg per kilogram body weight or 1% hydroxyethylcellulose (90368; Sigma) as vehicle from the day of infection until day 7 after infection. Then the cytotoxicity was measured as described above.

Dissection of mesenteric lymph nodes and spleen. A single-cell suspension was obtained from MLNs and splenocytes after physical dissociation with a 40-μm mesh ( Falcon). Red blood cell lysis on splenocytes was performed using 1 ml of Sigma) buffer. The specific target population (half of the cells) was then washed twice in complete medium. Then viable cells counted and mixed in a 1:1 ratio before injection into recipient mice. A total of 15 million cells per 200 μl Ca/Mg-free PBS (Lenza; at room temperature) were transferred into mice on day 7 after TMEV, by i.v. injection into the tail. Recipient mice were euthanized 4 h later, and the collected MLNs and splenocytes were analyzed by Flow cytometry to determine the percentage of CFSE^p and CFSE^- cells. The percentage of VP2121–130-specific cytotoxicity was calculated as follows:

% specific lysis = 1 - (naive reinfected / 100) * (CFSE^p cells / CFSE^- cells)

In vitro T cell polarization assay. Total CD4^+ T cells from the spleens of Fopp3ΔΔ mice and Fopp3ΔΔ/Tcf7ΔΔ mice were negatively isolated through the use of a mouse CD4^+ T cell isolation kit (100-104-453, Miltenyi). Then, 1 x 10^6 CD4^+ T cells were seeded with 1 x 10^6 irradiated antigen-presenting cells, 0.75 μg ml^-1 anti-CD3 (2C11, BioLegend) and 1.5 μg ml^-1 anti-CD28 (37.51, BioLegend) in a coated plate. For T1 polarization, cells were supplemented with 5 μg ml^-1 of anti-IL-4 (11B1, BD Biosciences), 10 ng ml^-1 IFN-γ (485-M1-100, R&D Systems) and 10 ng ml^-1 IL-12 (1419-M, R&D Systems). For T17 polarization, cells were treated with 5 μg ml^-1 anti-IL-4, 5 μg ml^-1 anti-IFN-γ (XMG1.2, eBioscience), 10 μg ml^-1 anti-IL-2 (JES6-51H4, Bio Cell), 30 μg ml^-1 IL-6 (406-ML-R, R&D Systems) and 1.5 ng ml^-1 TGF-I (PHG9204, Thermo Fisher). After 65h, cells were removed from the TCR signaling and recultured in a non-coated plate. Four days after activation, cells were restimulated with PMA/ionomycin and GolginStop for 5h, followed by IFN-γ and IL-17A staining.

In other experiments, CD4^+CD25^+CD62L^+CD44^- naive T cells were FACS sorted from magnetic-activated cell sorting (MACS)-pre-purified naive CD4^+ T cells (130-104-453, Miltenyi) isolated from the spleens of WT CD4^+ mice and labeled with 4 μM CellTrace Violet (C34557, Thermo Fisher). CD25^+ FACS CD4^+ T cells were FACS sorted from MACS-pre-purified CD4^+ T cells (130-104-453, Miltenyi) isolated from the spleens of Fopp3ΔΔ mice and Fopp3ΔΔ/Tcf7ΔΔ mice. Cells in equal number were stimulated under TH1 or TH17 polarized conditions for 72 h. Allogeneic DCs from BALB/c mice were used as antigen-presenting cells. All cultures were performed in a volume of 200 μl in 96-well U-bottomed plates.

T cell proliferation suppression assay. CD25^+ FACS CD4^+ T cells as suppressor cells were FACS sorted from MACS-pre-purified CD4^+ T cells (130-104-453, Miltenyi) isolated from the spleen of Fopp3ΔΔ mice and Fopp3ΔΔ/Tcf7ΔΔ mice. Cells in equal number were stimulated under TH1 or TH17 polarized conditions for 72 h. Allogeneic DCs from BALB/c mice were used as antigen-presenting cells.
by incubation with MACS microbeads coated with anti-CD11c mAb (130-104-453; Miltenyi Biotech) and irradiated at 3,000 rad. Cells were activated with anti-CD3 (0.5 μg ml⁻¹) by coating 96-well round-bottomed plates for 2 h at 37 °C.

**Histology and immune staining.** Gut tissues were collected, opened longitudinally and fixed using 10% formalin for 12–18 h, and routinely paraffin embedded and processed. For immune staining, 5-μm tissue sections were deparaffinized in xylene and rehydrated in ethanol. Following rehydration, slides were immersed in target retrieval solution (G8096; Dako), and heat-induced epitope retrieval was performed in a Decloaking Chamber (BioCare Medical). Following antigen retrieval, tissues were washed with PBS and nonspecific background staining was blocked using dual endogenous enzyme block (S2003; Dako), Fc-block (2.6G2, Antibody Hybridoma Core, Mayo Clinic; kindly provided by T. Beito) and Background Sniper (BS9601; BioCare Medical). Nonspecific avidin/biotin was blocked when needed (SP-1000; Vector Laboratories), and the samples were incubated in antibody dilution solution (S0809; Dako) and incubated overnight at 4 °C. For β-catenin staining, anti-β-catenin (dilution: 1:200; 14C-5/β-catenin (ROU), 610154, BD Biosciences) as a primary antibody and Envision+ System-HRP-labeled polymer anti-mouse (K4001; Dako) as a secondary antibody for 45 min. For Gr1 staining, anti-Gr1 (dilution: 1:50; NIMP R14, NB600-1387, Novus Biologicals) as primary antibodies and biotinylated rabbit anti-rat (BA-4001, Vector Laboratories) as secondary antibodies were applied for 45 min, followed by streptavidin (HRP conjugate, 016-030-084, Jackson Laboratories) for 30 min. Counterstaining was performed using Chromogen DAB+ Substrate (K3468, Dako) followed by hematoxylin counterstain. A Leica light microscope mounted with a Zeiss Axiocam 503 camera was used for imaging of IHC staining.

**mRNA isolation for RNA sequencing.** Around 2–4 × 10⁵ CD25⁺Foxp3⁻ Treg cells were FACs sorted from MACS-pre-purified CD4⁺ T cells (mouse CD4⁺ T cell Isolation Kit, Miltenyi) isolated from MLNs of Foxp3⁺ and Foxp3− T cells (mouse CD4⁺ T cell Isolation Kit, Miltenyi) isolated from MLNs of Foxp3⁺ and Foxp3− T cells (mouse CD4⁺ T cell Isolation Kit, Miltenyi). Total RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus) following the manufacturer’s instructions. Libraries were generated and sequenced by the University of Chicago Genomics Facility.

**Single-cell RNA sequencing.** CD25⁺Foxp3⁻ Treg cells were MACS purified (mouse CD4⁺ CD25⁺Foxp3⁻ cell Isolation Kit, Miltenyi) from the MLNs of Foxp3⁺ and Foxp3⁻ T cells (mouse CD4⁺ T cell Isolation Kit, Miltenyi) and immediately submitted to the Genomics Facility. The cells were first counted and measured for viability using the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter), as well as a basic hemocytometer with light microscopy. The barcoded gel beads were thawed from −80 °C and the reverse transcription master mix was prepared according to the manufacturer’s instructions for Chromium Single Cell 3′ v2 library kit (10x Genomics). Based on the desired number of cells to be captured for each sample, a volume of live cells was mixed with the master mix. The cell suspension/master mix, thawed gel beads and partitioning oil were added to a Chromium Single Cell 3′ chip. The filled chip was loaded onto the chromium controller, where each sample was processed and the individual cells within the sample were captured into uniquely labeled (gel beads-in-emulsion) GEMs. The GEMs were collected from the chip and taken to the bench for reverse transcription, GEM dissolution and cDNA cleanup. Resulting cDNA was a pool of uniquely barcoded molecules. Single-cell libraries were created from the cleaned and measured, pooled cDNA. During library construction, standard Illumina sequencing primers and unique i7 sample indices were added to each cDNA pool. Each sample was uniquely indexed.

All cDNA pools and resulting libraries were measured using Qubit High Sensitivity assays (Thermo Fisher Scientific), Agilent Bioanalyzer High Sensitivity chips (Agilent) and Kapa DNA Quantification reagents (Kapa Biosystems). Libraries were sequenced at 50,000 fragment reads per cell following Illumina’s standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit. The flow cells were sequenced as 100 × 2 paired-end reads on an Illumina HiSeq 10000 PE sequencing kit and HCS v3.5.2.4 sequencing software. Base calling was performed using Illumina’s RTA v2.7.3.

**Single-cell RNA-sequencing data analysis.** The 10x Genomics Cell Ranger (v2.0.2) mkfastq was applied to demultiplex the Illumina BCL output into FASTQ files. The Cell Ranger count was then applied to each FASTQ file to align reads to the mm10 reference genome and generate barcode and unique molecular identifier counts. We followed the Seurat¹¹ (v3.2.2) integrated analysis and comparative analysis workflows to do all scRNA-seq analyses. Genes expressed in <3 cells and cells with <200 genes or >15% mitochondrial genes were excluded for downstream analysis in each dataset. Cell cycle score for each cell was calculated with the CellCycleScoring function from Seurat using mouse cell cycle genes. The SC3F score function was invoked to normalize the dataset (using default parameters), regress out mitochondrial (percent.MT) and cell cycle (%) and G2M contents and identify variable genes.

The datasets were integrated based on ‘anchors’ identified between datasets (nfeatures = 2000, normalization.method = ‘SC’), before performing linear dimensional reduction by principal-component (PC) analysis. The top 25 PCs were included in a LMAP dimensionality reduction. After obtaining the top 25 PCs, we computed the shared nearest-neighbor graph and we identified clusters in the network using the Louvain algorithm. Differential gene expression was determined by the ‘findMarkers’ function with the default Wilcoxon’s rank-sum test either as one versus the rest or as a direct comparison with parameters min.pct = 0.1 and logfc.threshold = 0. For Metascape analysis, the 200 upregulated genes were then determined based on reported adjusted P values. For GSEA analysis, a preranked gene list was created based on sorted scores defined by −log2 (reported P value) × sign (reported average log fold change). The cell annotation was based on the top differentially expressed genes.

Gene-list module scores were calculated with Seurat function AddModuleScore(). This calculates the average scaled expression levels of each gene list, subtracted by the expression of control feature sets. To compare the single-marker expression between cell types, Wilcoxon’s test was used.

To calculate the RNA velocity, the loom files were generated from the bam files by Velocyto⁴⁰ the RNA velocity was then calculated using the RunVelocity function in Velocytor R package. The velocity for each sample was shown by showvelocity. R package.

**Quantification and statistical analysis.** Except for deep-sequencing data, statistical significance was calculated with GraphPad Prism. Error bars in graphs indicate the s.e.m. and statistical comparisons were performed by unpaired Student’s t-test. P values ≤0.05 were considered statistically significant.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The bulk RNA-seq and scRNA-seq datasets are deposited in the Gene Expression Omnibus under accession GSE163084. Source data are provided with this paper.

**Code availability**

The codes used for bulk RNA-seq and scRNA-seq analysis followed typical pipelines from public R packages (DESeq2 and Seurat). All codes are available upon request.

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Extended Data Fig. 1 | TCF-1 deficiency selectively reprograms $T_{reg}$-cells without compromising their core signature. $T_{reg}$-cells were isolated from the mesenteric lymph nodes of Foxp3$^{cre}$ and Tcf7$^{fl/fl}$Foxp3$^{cre}$ mice. (a) Representative FACS histograms of MLN purified cells from Foxp3$^{cre}$ Tcf7$^{fl/fl}$ and control Foxp3$^{cre}$ showing selective loss of TCF-1 from $T_{reg}$-cells in Foxp3$^{cre}$ Tcf7$^{fl/fl}$ mice. (b and c) Histogram plots showing the cumulative data of the same. (b: $n = 4$; $p < 0.0001$ & c: $n = 5$) Data are representative of two independent experiments and $n$ represents biologically independent replicate mice; means ± SEM; two-sided, unpaired $t$-test. (d) GSEA plot comparing the enrichment of genes expressed more strongly in Foxp3$^{cre}$ versus Foxp3$^{cre}$ Tcf7$^{fl/fl}$ $T_{reg}$-cells.
Extended Data Fig. 2 | Representative FACS plots of lymphocyte surface markers expressed by T<sub>reg</sub>-cells. T<sub>reg</sub>-cells were isolated from the mesenteric lymph nodes of Foxp3<sup>Cre</sup> and Tcf7<sup>fl/flFoxp3Cre</sup> mice. See cumulative data presented in Fig. 2. (a-c) CD4<sup>+</sup> cells were pre-gated and frequency of CD69<sup>+</sup>, ICOS<sup>+</sup>, and PD1<sup>+</sup> cells among CD4<sup>+</sup>FOXP3<sup>−</sup>T<sub>con</sub> or CD4<sup>+</sup>FOXP3<sup>+</sup>T<sub>reg</sub>-cells was measured, as indicated. (d) CD4<sup>+</sup> cells were pre-gated and frequency of CD44<sup>+</sup>CD62L<sup>−</sup> cells among CD4<sup>+</sup>FOXP3<sup>−</sup>T<sub>con</sub> cells was measured. (e) CD4<sup>+</sup> cells were pre-gated and frequency of CD4<sup>+</sup>FOXP3<sup>+</sup>T<sub>reg</sub>-cells was measured. (f) CD4<sup>+</sup> cells were pre-gated and frequency of FOXP3<sup>+</sup>CD25<sup>+</sup>T<sub>reg</sub>-cells was measured. (g) CD4<sup>+</sup>FOXP3<sup>+</sup>T<sub>reg</sub>-cells were pre-gated and frequency of RORγT<sup>−</sup>HELIOS<sup>−</sup> or RORγT<sup>+</sup>HELIOS<sup>+</sup> was measured. (h) CD4<sup>+</sup>FOXP3<sup>+</sup>T<sub>reg</sub>-cells and frequency of CD44<sup>+</sup>CD62L<sup>−</sup> cells among T<sub>reg</sub>-cells was measured. Numbers inside quadrants indicate percent cells in the respective quadrants.
Extended Data Fig. 3 | T<sub>reg</sub> purification. T<sub>reg</sub>-cells were isolated from the mesenteric lymph nodes of Foxp3<sup>Cre</sup> and Tcf7<sup>fl/flFoxp3<sup>Cre</sup> mice. (a) Schematic representation of purification of T<sub>reg</sub>-cells, and FACS analysis showing over 90% purity. (b) Expression changes of the Tcf7 transcripts between TCF-1-deficient and TCF-1-sufficient T<sub>reg</sub>-cells. The color intensity is proportional to the average gene expression across cells in the indicated T<sub>reg</sub> cluster. The size of circles is proportional to percentage of cells expressing indicated genes.
Extended Data Fig. 4 | Single-cell RNAseq reveals distinct T<sub>reg</sub> populations. mRNA expression of select indicated genes projected on the UMAP. Note varied expression of *Klf2* but broad and uniform expression of *Izumo1r* by T<sub>reg</sub> clusters, high expression of *Mif*, *Vps8*, and *Ifit1* in the respective *Mif* (cluster 3), *Vps8* (cluster 8), *Ifn* (cluster 9). Expression of *Ccl5* is prominent in the *Cd63* (cluster 7), which is likely not T<sub>reg</sub>-cells.
Extended Data Fig. 5 | See next page for caption.
**Extended Data Fig. 5 | TCF-1-deficient and sufficient T<sub>reg</sub>-cells show distinct effector functions.** T<sub>reg</sub>-cells were isolated from the mesenteric lymph nodes of Foxp3<sup>Cre</sup> and Tcf7<sup>fl/fl</sup>Foxp3<sup>Cre</sup> mice. (a) mRNA expression of Maf projected on the UMAP, comparing T<sub>reg</sub>-cells derived from Foxp3<sup>Cre</sup> to Tcf7<sup>fl/fl</sup> Foxp3<sup>Cre</sup> mice. (b) Violin plots showing expression of Maf in individual T<sub>reg</sub> clusters. (c) GSEA of MAF downregulated genes and T<sub>17</sub> pathway defined by Stubbington. (d) Kegg IL17 signaling pathway projected on UMAP, comparing TCF-1-sufficient and TCF-1-deficient T<sub>reg</sub>-cells (e) GSEA analysis for the Kegg IL17 signaling pathway comparing transcriptomes of TCF-1-sufficient and TCF-1-deficient T<sub>reg</sub>-cells across all cell types. Normalized enrichment scores (NES) are color coded. -log<sub>10</sub> (FDR) values are proportional to the circle size. FDR > 15% are masked with gray color. (fgh) mRNA expression of Ccr9, Erdr1 and Igfbp4 projected on the UMAP, comparing TCF-1-sufficient and TCF-1-deficient Klf2<sup>−/−</sup> cells for the Kegg IL17 pathway.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | T_{reg}-cells are activated and polarized during polyposis. T_{reg}-cells were isolated from the mesenteric lymph nodes of WT and APC^{Δ486} mice. (a) UMAP projection (left panel) and fraction of cells in each cell type (stack bars; right panel) for APC^{Δ486} and control B6 T_{reg}-cells. Data are from two replicates. (b) Dot plot showing the expression of Tcf7 across all cell types in Apc^{Δ486} and control B6 T_{reg}-cells. Color and size of the dots are proportional to the expression level and percent of cells expressing Tcf7 in each indicated cluster. (c) Expression of Socs3, Jund, Lag3 and Maf between APC^{Δ486} and B6 cells projected on the UMAP. See TableS4 for the full list. The fold change in percent of cells expressing the indicated gene in each cell type is proportional to the circle size. Adjusted-p-values > 0.01 are masked with gray color. (d) Expression changes of the most differentially expressed genes between APC^{Δ486} and control B6 T_{reg}-cells. See TableS4 for the full list. The fold change in expression intensities is color-coded. (e) RNA velocity vectors overlaid on UMAP for B6 (left) and APC^{Δ486} (right) T_{reg}-cells.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

The codes used for bulk and single-cell RNA-seq analysis followed typical pipelines from public R packages and software. All codes are available upon request.

Data analysis

Graphpad Prism V7 and V5, FlowJo V10.6.2, Microsoft Office 2016, IX Genomics Cellranger (v2.0.2), Uniform Manifold Approximation and Projection (UMAP), Seurat function AddModuleScore, Velocyto.R package, Cuffdiff2, EdgeR, DESeq2, Galaxy, Homer, ngs.plot, Genepattern, GSEA V4 were used.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All described RNA-seq, and scRNA-seq data were submitted under the GEO accession number: GSE163084 (It has been made public); The colorectal cancer (CRC) patient data is sourced from GSE108989.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- **Sample size**: Sample size was chosen according to standard practices in the field. RNA-seq and scRNA-seq samples were prepared in triplicates and duplicates, respectively. All biochemical experiments were performed 3+ times.

- **Data exclusions**: None excluded

- **Replication**: All in vivo, in vitro, and flow experiments were performed at least 2+ times

- **Randomization**: No clinical studies

- **Blinding**: IHC scoring was done blinded. FACS and molecular analyses by nature required grouping of data and could not use blinding.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑️  | Antibodies             |
| ☑️  | Eukaryotic cell lines  |
| ☑️  | Palaeontology and archaeology |
| ☐️  | Animals and other organisms |
| ☐️  | Human research participants |
| ☑️  | Clinical data          |
| ☑️  | Dual use research of concern |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑️  | ChiP-seq              |
| ☑️  | Flow cytometry        |
| ☑️  | MRI-based neuroimaging |

### Antibodies

**Antibodies used**
- Invitrogen LIVE/DEAD Fixable Blue Stain (dilution: 1/750; L34962)
- BioLegend anti-CD4-PerCP/Cyanine5.5 (dilution: 1/300; clone: RM4-5; Cat: 116012)
- anti-CD4-AF700 (dilution: 1/200; clone: RM4-5; Cat: 116022)
- anti-CD25-Brilliant Violet 650 (dilution: 1/200; clone: PC61; Cat: 102038)
- anti-CD44-Brilliant Violet 785 (dilution: 1/500; clone: IM7; Cat: 103059)
- anti-CD278 (ICOS)-PE-Cy7 (dilution: 1/200; clone: C398.4A; Cat: 315218)
- anti-CD45.1-PE/Cy7 (dilution: 1/500; clone: A20; Cat: 110730)
- anti-Helios-Brilliant Violet 421 (dilution: 1/100; clone: 22F6; Cat: 137234)
- anti-Helios-PerCP/Cyanine5.5 (dilution: 1/200; clone: 22F6; Cat: 137230)
- anti-CD45.2-PE (dilution: 1/500; clone: 104; Cat: 155194)
- anti-F4/80-PE (dilution: 1/200; clone: M1/70; Cat: 123456)
- anti-CD68-Brilliant Violet 785 (dilution: 1/200; clone: H1.2F3; Cat: 564683)
- anti-ROSA26-Brilliant Violet 421 (dilution: 1/200; clone: Q31-378; Cat: 562894)
- anti-ROSA26-PE (dilution: 1/200; clone: Q31-378; Cat: 562894)
- anti-IL-17A-PE, (clone: TC11-18H10; Cat: 559502)
- Purified mouse β-catenin (dilution: 1/200; clone: 14/ β-catenin (RUO); Cat: 610154)
- National Institutes of Health Tetramer Core Facility
- 50 μl of a 1:50 dilution of APC-conjugated Db:VP2121-130 tetramer
- R & D Systems TGFβ RI-PE (dilution: 10μl/test; Cat: FAB5871P)
- Rat IgG2A-PE (dilution: 10μl/test; IC006P)
TGFβ RII-PE (10μl/test; cat: FAB532P)
Goat IgG-PE (dilution: 10μl/test; IC108P) eBioscience
anti-Foxp3-APC or anti-Foxp3-FITC (dilution: 1/200; clone: FJK-16s Cat: 17-5773-82)
anti-IFNγ-APC (dilution: 1/200; clone: XMG1.2; cat: 17-7311-82)
FITC-conjugated Stats phosphorylated at Tyr694 (dilution: 1μg/test; clone: SRBCZ; cat: 11-9010-42)
Mouse IgG kappa-FITC (dilution: 1μg/test; clone: P3.6.2.8.1; cat: 11-4714-81)
Cell Signaling Technology
anti-TCF1/TCF7-Alexa Fluor 647 (dilution: 1/300; clone: C63D9; cat: 6709S)
PE-conjugated S6 phosphorylated at Ser235 and Ser236 (dilution: 1/100; clone: D57.2.2E; cat: 5316S)
rabbit IgG-PE (dilution: 1/100; clone: DA1E; cat: 5742S).
Novus Biologicals
anti-Gr-1 (dilution: 1/50; clone: cat: NIMP-R14; NB600-1387)
Invitrogen
LIVE/DEAD Fixable Blue Stain
BioLegend
Anti-CD4-PerCP/Cyanine5.5
Reactivity: Mouse
Application: Flow Cytometry
Anti-CD4-AF700
Reactivity: Mouse
Application: Flow Cytometry
Anti-CD25-Brilliant Violet 650
Reactivity: Mouse
Application: Flow Cytometry
Anti-CD44-Brilliant Violet 785
Reactivity: Mouse, Human
Application: Flow Cytometry
Anti-CD278 (PD-1)-Brilliant Violet 421
Reactivity: Mouse
Application: Flow Cytometry
Anti-RORγT-Brilliant Violet 421 or Anti-RORγT-PE
Mouse (QC Testing)
Application: Intracellular staining (flow cytometry) (Routinely Tested)
Anti-IL-17A-PE
Mouse (QC Testing)
Application: Intracellular staining (flow cytometry) (Routinely Tested)
Purified mouse β-catenin
Reactivity: Human (QC Testing), Mouse, Rat, Dog, Chicken (Tested in Development)
Application: Western blot (Routinely Tested), Immunofluorescence, Immunohistochemistry, Immunoprecipitation (Tested During Development)
National Institutes of Health Tetramer Core Facility
50 μl of a 1:50 dilution of APC-conjugated Db:VP2121-130 tetramer
R & D Systems
TGFβ RI-PE
Specificity: Detect mouse TGF-beta RI/ALK-5 in flow cytometry.
Application: Flow Cytometry
Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

| Animals | Notes |
|----------|-------|
| C57BL/6 mice, male unless otherwise stated, up to 5 mice per cage, 12/12 light dark cycle, tumor bearing mice and controls were at 5.5 months of age, otherwise ages as indicated |

**Wild animals**

| Animals | Notes |
|----------|-------|
| none |

**Field-collected samples**

| Animals | Notes |
|----------|-------|
| none |

**Ethics oversight**

| Ethics | Notes |
|--------|-------|
| Protocol #A73113, approved by the Mayo Clinic Institutional Animal Care and Use Committee |

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

Confirm that:

☑️ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑️ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☑️ All plots are contour plots with outliers or pseudocolor plots.
☑️ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Mesenteric lymph nodes, and spleens were resected. Single cell suspensions were generated by mashing the organs through 40-100 μm cells strainers while flushing the strainers with ice-cold 1 x PBS. Erythrocytes in the spleen single cell suspensions were lysed by resuspending the cell pellets in 1 ml ACK buffer (Lonza), for 1 min on ice. The reaction was stopped by adding 15 ml of PBS + 2% FCS, cells were spun down immediately after and washed in 10 ml ice cold PBS. Dead cells were removed by filtering the lysed cells suspensions through 40 μm cell strainers.

Instrument

BD Fortessa X20, BD LSR II, BD FACSAriall

Software

BD Diva, FlowJo Version10.6.2

Cell population abundance

90-97 % purity

Gating strategy

MLNs and spleen cells were gated for lymphocytes by FSC,SSC, doublets were discriminated, and live cells were analyzed for CD4+ expression. CD4+ T cells were further analyzed for Foxp3+ expression. Foxp3+ and Foxp3-CD4+ were further divided into RORyt+, CD69+, ICOS+, PD-1+ and CD25 subpopulations. Foxp3+ were also divided into RORyt+ and Helios- subpopulations. Mononuclear cells from gut tissues were gated for lymphocytes by FSC,SSC, doublets were discriminated, and live cells were analyzed for CD3+ CD4+expression. CD3+CD4+ T cells were further analyzed as described above.

☑️ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.