**Effector CD4 T cells with progenitor potential mediate chronic intestinal inflammation**

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Dysregulated CD4 T cell responses are causally linked to autoimmune and chronic inflammatory disorders, yet the cellular attributes responsible for maintaining the disease remain poorly understood. Herein, we identify a discrete population of effector CD4 T cells that is able to both sustain and confer intestinal inflammation. This subset of pathogenic CD4 T cells possesses a unique gene signature consistent with self-renewing T cells and hematopoietic progenitor cells, exhibits enhanced survival, and continually seeds the terminally differentiated IFNγ-producing cells in the inflamed intestine. Mechanistically, this population selectively expresses the glycosyltransferase ST6Gal-I, which is required for optimal expression of the stemness-associated molecule TCF1 by effector CD4 T cells. Our findings indicate that the chronicity of T cell–mediated inflammation is perpetuated by specific effector CD4 T cells with stem-like properties.

**Introduction**

CD4 T cells are central to the development of numerous chronic inflammatory disorders, and T helper 1 (Th1), Th2, and Th17 subsets of effector CD4 T cells have each been documented to be pathogenic in different disease settings (Maynard and Weaver, 2009; Leung et al., 2010; Nakayamada et al., 2012; Patel and Kuchroo, 2015). During inflammatory bowel disease (IBD), both Th1 and Th17 cells have been implicated in disease pathogenesis (Maynard and Weaver, 2009; Leung et al., 2010), yet the mechanisms by which these cells mediate pathology is unknown. IFNγ is the signature effector molecule secreted by Th1 cells, as well as plastic Th17 cells (Lee et al., 2009; O'Shea and Paul, 2010; Oestreich and Weinmann, 2012), and is one of the most abundant proinflammatory cytokines produced by mucosal CD4 T cells in IBD patients (MacDonald et al., 1990; Fuss et al., 1996; Hommes et al., 2006). Moreover, genome-wide association studies have identified polymorphisms in the IFNG gene that are associated with IBD (Gonsky et al., 2014). However, mouse models of colitis have demonstrated IFNγ to be both essential and dispensable for disease (Berg et al., 1996; Leach et al., 1996; Kullberg et al., 1998; Simpson et al., 1998), and these conflicting data illustrate the need for further investigation of the role of IFNγ during IBD.

Although much focus has been on the functional aspects of the effector CD4 T cell population that mediates chronic inflammation, less is known regarding how pathogenic CD4 T cells sustain disease. In the context of viral infection, effector CD8 T cells exist in a spectrum of differentiation states, and this correlates with their continued responsiveness (Kaech and Cui, 2012; Chang et al., 2014). Terminally differentiated effector CD8 T cells are characterized by the expression of specific transcription factors (ID2, Tbet, Blimp1, and ZEB2), diminished proliferative capacity, and high sensitivity to cell death (Joshi et al., 2007; Yang et al., 2011; Kaech and Cui, 2012; Dominguez et al., 2015). In contrast, recent studies demonstrate that there is a distinct subset of CD8 T cells that sustains the control of chronic viral infections and is responsive to anti–PD-1 therapy (Im et al., 2016). In addition to the specific cell surface phenotype, this unique cell population is distinguished by its stem-like qualities, including the capacity to self-renew, proliferate, and differentiate into effector cells (Im et al., 2016; Wu et al., 2016). Immune stemness is controlled at the molecular level by the transcription factors TCF1, LEF1, and KLF2 (Gattinoni et al., 2009, 2011, 2012; Utzschneider et al., 2016), and deletion of TCF1 results in the loss of stem-like CD8 T cells during chronic viral infection (Im et al., 2016). How the differentiation state of effector CD4 T cells during chronic inflammatory and autoimmune disorders affects the severity and maintenance of disease has yet to be examined.

In this study, we investigated the role of IFNγ-producing effector CD4 T cells in propagating chronic intestinal inflammation. Using IFNγ reporter mice, we find that IFNγ-producing CD4 T cells are not able to confer colitis upon adoptive transfer, nor are these cells required to sustain disease. Instead, the pathogenic CD4 T cells capable of eliciting and maintaining...
Results and discussion

**IFNγ-producing CD4 T cells are prevalent during intestinal inflammation; however, the IFNγ-nonproducing CD4 T cell population mediates disease**

IFNγ production by effector CD4 T cells has been extensively investigated during chronic inflammation; nevertheless, the contribution of these cells to IBD remains controversial. To resolve this, we used IFNγ-Thy1.1 knock-in (KI) reporter mice that up-regulate the cell surface Thy1.1 molecule when IFNγ is produced (Harrington et al., 2008), in conjunction with a CD4 T cell–dependent model of colitis (Leach et al., 1996). CD45RB^hi^ CD25^-^ CD4 T cells from IFNγ KI mice were adoptively transferred into Rag1^−/−^ mice to induce disease. We observed that ~25–40% of CD4 T cells up-regulated IFNγ as denoted by Thy1.1 staining, and the frequency of IFNγ^+^ CD4 T cells was significantly elevated in the large intestine (Fig. 1, A and B). IFNγ secretion by CD4 T cells was associated with expression of proinflammatory cytokines, including TNFα, GM-CSF, and IL-17A (Fig. S1 A). To test the pathogenic potential of the IFNγ^+^ CD4 T cells, we isolated IFNγ^+^ (Thy1.1^+^) and IFNγ^-^ (Thy1.1^-^) CD4 T cells from the spleen and LNs of colitic mice and transferred these cells into disease-free hosts. Paradoxical to the known proinflammatory actions of IFNγ, the IFNγ^+^ effector CD4 T cells caused only minimal pathology upon secondary transfer. In contrast, the IFNγ^-^ effector CD4 T cells induced severe epithelial damage and intestinal inflammation (Fig. 1, C and D).

These findings show that IFNγ^+^ effector CD4 T cells alone are not capable of conferring colitis in disease-free hosts; however, it is possible that these cells impact the severity and/or maintenance of intestinal inflammation. To evaluate this, we depleted IFNγ^+^ (Thy1.1^+^) CD4 T cells from recipient mice throughout the course of disease using an anti-Thy1.1 depletion mAb. Interestingly, the removal of IFNγ^+^ CD4 T cells did not alter the severity or maintenance of colitis (Fig. S1, B–D). Moreover, the IFNγ^-^ nonproducing CD4 T cells were able to sustain the intestinal inflammation, which is consistent with the adoptive transfer experiments. Together, these findings demonstrate that IFNγ^-^ CD4 T cells are dispensable for the initiation and maintenance of colitis and, more importantly, that within the IFNγ^-^ nonproducing effector CD4 T cell subset exists a population of cells capable of sustaining chronic intestinal inflammation.
Transcriptional profiling of pathogenic effector CD4 T cells identifies a progenitor-like gene signature

To understand the molecular mechanisms that confer differential pathogenicity, IFNγ⁺ (Thy1.1⁺) and IFNγ⁻ (Thy1.1⁻) CD4⁺CD44hiCD4 T cells were isolated 4 wk after the initiation of colitis and subjected to RNA sequencing. A total of 942 and 1,091 genes were significantly increased in the IFNγ⁺ and IFNγ⁻ effector CD4 T cells, respectively (Fig. 2 A). The IFNγ⁺ CD4 T cells that do not confer inflammation expressed several effector molecules including Ifng, Fasl, Prf1, Tnf, and Csf1 (Fig. 2 D). These data are consistent with our earlier intracellular staining analyses (Fig. S1 A) and indicate that the inability to mediate disease was not caused by the lack of proinflammatory functions. GSEA of the data revealed that the IFNγ⁺ CD4 T cells present during colitis express genes associated with Th1 cytotoxicity and KLRG1hi and effector CD8 T cells, as well as apoptosis (Figs. 2 B and S2 A; Kaech et al., 2002; Wherry et al., 2007; Sarkar et al., 2008; Hale et al., 2013). Interestingly, these IFNγ⁺ CD4 T cells that exist during chronic intestinal inflammation mirror the phenotype of terminally differentiated effector CD8 T cells, as denoted by up-regulation of the genes Id2, Prdm1, Tbx21, Klrg1, Havcr2, and Zeb2 (Fig. 2, B and D; Kaech et al., 2002; Joshi et al., 2007; Yang et al., 2011; Kaech and Cui, 2012; Chang et al., 2014; Dominguez et al., 2015). This terminally differentiated state may explain why these cells are unable to sustain inflammation over time.

We further probed our RNA sequencing dataset to ascertain the properties of the IFNγ⁺ CD4 T cells that contribute to their pathogenicity. In marked contrast to the IFNγ⁺ CD4 T cells, GSEA
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of the colitogenic, IFNγ− CD4 T cells identified a transcriptional profile associated with self-renewal and stemness; these cells were enriched transcriptionally for pathways of naive and memory CD8 T cells and hematopoietic progenitor cells (Figs. 2 B and S2 B; Kaech et al., 2002; Park et al., 2002; Sarkar et al., 2008; Ramirez et al., 2012). The CD44hi IFNγ− pathogenic subset of CD4 T cells also showed increased expression of Tcf7, Lef1, Id3, and Kit (Fig. 2, D and E), molecules important for the maintenance of self-renewing cell populations (Joshi et al., 2007; Yang et al., 2011; Gattinoni et al., 2012; Im et al., 2016; Wu et al., 2016; Nish et al., 2017). This stemness transcriptional signature, as well as the terminal differentiation program, were validated at the protein level (Fig. S2, C and D). Hence, the ability of these cells to continually maintain intestinal inflammation may be provided by the stem-like profile they possess.

Recent publications have described a subset of CD8 T cells during chronic viral infection that provide effective immunity upon anti-PD-1 treatment. The anti-PD-1 responsive CD8 T cells express CXCR5, as well as the transcription factor TCF1, and exhibit a stem cell–like gene signature (Im et al., 2016). Additional GSEA of the pathogenic IFNγ− CD4 T cell population from colitis mice with the CXCR5hi stem-like CD8 T cells from chronic infection revealed a strikingly strong positive association, whereas the IFNγ+ CD4 T cells more closely resembled the Tim3+ CD8 T cell population (Fig. 2 C). Taken together, the gene expression analyses highlight that CD4 T cells present during intestinal inflammation not only are functionally heterogeneous, but also adopt distinct differentiation states that directly correlate to their pathogenicity.

IFNγ-nonproducing effector CD4 T cells during colitis are capable of self-renewal, are less susceptible to cell death, and show enhanced mitochondrial reserve capacity (RC)

The observation that pathogenic CD4 T cells during chronic intestinal inflammation possess a gene signature associated with stemness propelled us to biologically test these characteristics. Consistent with the self-renewing gene profile, significantly higher numbers of the pathogenic IFNγ− CD4 T cells were recovered at 7 d and 8 wk after secondary transfer (Fig. 3, A and B). We posited that this was the result of differential proliferation and/or susceptibility to apoptosis. Both the IFNγ+ and IFNγ− CD4 T cells divided robustly after cell transfer (Fig. 3 C); however, a greater percentage of the IFNγ+ CD4 T cells succumbed to apoptosis (Fig. 3 D). This implies that the stem-like gene profile of colitogenic IFNγ− CD4 T cells promotes a cell intrinsic program that favors cell survival by preventing apoptosis.

Previous studies have demonstrated that T cell stemness and long-term survival are supported by enhanced mitochondrial RC, which is the difference between basal and maximal oxygen consumption rates (OCRs) in the cells (Hill et al., 2012; van der Windt et al., 2012; Sukumar et al., 2016). Therefore, we examined the OCR in the IFNγ+ and IFNγ− effector CD4 T cells isolated from mice with colitis. The RC exhibited by the IFNγ− CD4 T cells was comparable to that of CD4 T cells derived from

Figure 3. Colitogenic IFNγ-nonproducing CD4 T cells have increased survival and enhanced mitochondrial RC. (A–C) 4 wk after colitis induction, sorted Thy1.1+ and Thy1.1− CD4 T cells were transferred into Rag−/− mice. (A) Representative plots show the frequency of live cells 7 d later. (n = 11–13, three experiments). (B) Total CD4 T cell numbers enumerated 8 wk after secondary transfer (n = 10–17, three to four experiments). (C) Representative plots and graph show in vivo proliferation of the cells 7 d after transfer. (n = 11–13, three experiments). (D) Annexin V staining of Thy1.1+ and Thy1.1− CD4 T cells (n = 8–10 mice were pooled for each experiment, three experiments). (E) Representative OCR graphs of Thy1.1+ and Thy1.1− CD4 T cells 8 wk after primary transfer. (n = 8–12 mice per experiment, two experiments). Graphs show the mean ± SD; unpaired t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
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The pathogenic IFNγ− CD4 T cells, in contrast, had greater RC than both control CD4 T cells and IFNγ+ CD4 T cells (Fig. 3 E). These data, together with the cell survival findings, are consistent with the stem-like gene signature of this CD4 T cell subset and further support the concept that the degree of effector T cell differentiation correlates with the capability of cells to survive and promote disease during chronic inflammation.

IFNγ-nonproducing CD4 T cells serve as progenitor cells to terminally differentiated IFNγ-producing CD4 T cells

Our data demonstrate that IFNγ production by CD4 T cells reflects distinct states of differentiation; hence, we hypothesized that IFNγ− CD4 T cells act as progenitors to the more differentiated subsets, whereas terminally differentiated IFNγ− CD4 T cells maintain their phenotype. To test this, we isolated IFNγ− and IFNγ− CD4 T cells at 4 wk after colitis induction and subsequently transferred equal numbers of these cells into separate cohorts of disease-free Rag1−/− mice. 8 wk later, the few surviving IFNγ+ CD4 T cells retained their phenotype, whereas more than 50% of the previously IFNγ− CD4 T cells up-regulated IFNγ (Fig. 4, A and B). Importantly, similarly results were observed when the IFNγ− CD4 T cells were transferred into disease-matched hosts (Rag1−/− mice that received CD45.1 CD45RBhi CD25− CD4 T cells 4 wk previously; Fig. 4 C), indicating that the environment of the empty host or the lack of intestinal inflammation does not cause the differentiation of IFNγ− into IFNγ+ CD4 T cells. Moreover, this suggests that the progenitor capacity of a fraction of effector CD4 T cells is constantly replenishing the terminally differentiated effector T cell pool.

Next, we examined whether the ability of the IFNγ+ CD4 T cells to produce IFNγ after further differentiation contributes to the intestinal pathology. To test this, Rag1−/− mice that received IFNγ+ effector CD4 T cells were treated with anti-Thy1.1 mAb to deplete the emerging IFNγ+ cells. After 8 wk, no histological differences were observed in the recipient mice with subsequent...
Thy1.1 depletion or control treatment (Fig. 4, D and E). This indicates that the IFNγ+ CD4 T cells derived from the progenitor IFNγ− CD4 T cells are not necessary to elicit chronic intestinal inflammation, and further suggests that these cells propagate disease via an IFNγ-independent process.

The glycosyltransferase ST6Gal-I is selectively expressed by the IFNγ+ effector CD4 T cell population during colitis

The evidence that effector CD4 T cells with progenitor-like properties are critical for maintaining chronic inflammation prompted us to delineate the mechanism that promotes and/or sustains this cell population. To ascertain potential molecules necessary for the stemness phenotype, we dissected the RNA sequencing gene set for IFNγ− CD4 T cells with strong enrichment toward naive T cells and multipotent progenitor cells. Strikingly, superimposition of these datasets resulted in only two shared genes: St6gal1 and Fam101b (Fig. 5 A). The gene St6gal1 encodes β-galactoside α-2,6-sialyltransferase 1 (ST6Gal-I), which catalyzes the addition of α-2,6–linked sialic acids to cell surface N-glycans, including CD45, and this has been shown to impact signaling events that regulate CD4 T cell gene expression (Martin et al., 2002; Clark and Baum, 2012). ST6Gal-I was of particular interest because it is up-regulated in both cancer and human pluripotent stem cells, confers stem cell-like properties in tumor cells and human induced pluripotent stem cells (Wang et al., 2015; Schultz et al., 2016), and prevents apoptosis by inhibiting the activity of the Fas and TNFR1 death receptors (Toscano et al., 2007; Liu et al., 2011; Swindall and Bellis, 2011).

To verify the ST6Gal-I mediated sialylation in the effector CD4 T cell populations, we analyzed Sambucus nigra agglutinin (SNA) binding, which specifically interacts with the product of this glycosyltransferase, α-2,6–sialylated glycans on the cell surface. The SNA binding assay confirmed in all tissues analyzed that a unique subset of effector CD4 T cells from colitic mice possess α-2,6–sialylated glycans compared with the IFNγ+ CD4 T cells (Fig. 5, B and C).

α-2,6-Sialylation correlates with stem-like properties of IFNγ-nonproducing CD4 T cells and promotes TCF1 expression

The selective expression of ST6Gal-I by the colitogenic IFNγ− CD4 T cells, together with the known functions of ST6Gal-I in nonimmune cells, led us to postulate that this glycosyltransferase supports the progenitor characteristics of these cells. To test this, we stratified the effector CD4 T cells during colitis by the expression of IFNγ (Thy1.1) and ST6Gal-I mediated sialylation (SNA; Fig. 5 D) and examined the susceptibility to apoptosis and the expression of stemness-associated transcription factor TCF1. Interestingly, the survival of IFNγ+ CD4 T cells, but not the IFNγ− cells, strongly correlated with SNA binding: SNA+ IFNγ− CD4 T cells showed significantly less annexin V binding than SNA− IFNγ− CD4 T cells, whereas both SNA+ IFNγ+ and SNA− IFNγ− CD4 T cells stained annexin V positive (Fig. 5 E). Moreover, the IFNγ+ CD4 T cells with robust SNA binding activity expressed the highest levels of the stemness-associated transcription factor TCF1 and minimal amounts of the terminal differentiation marker KLRG1, whereas the SNA− IFNγ− CD4 T cells displayed the opposite phenotype (Fig. 5, F and G).

The combination of the RNA sequencing analyses, SNA binding, cell survival data, and TCF1 expression pattern imply a causal relationship between ST6Gal-I mediated sialylation and effector CD4 T cell stemness during chronic intestinal inflammation. To address this directly, we induced colitis with WT and ST6Gal-I−/− CD4 T cells and analyzed the differentiation state of the cells. In comparison with the WT effector CD4 T cells, the frequency of ST6Gal-I−/− effector CD4 T cells that expressed TCF1 was significantly reduced (Fig. 5, H and I). In contrast, the percentage of terminally differentiated KLRG1+ CD4 T cells was not affected by the deletion of ST6Gal-I. Thus ST6Gal-I mediated sialylation not only identifies the unique effector CD4 T cell population with progenitor potential, but it also functions to promote the expression of the stemness-associated transcription factor TCF1 in these cells during chronic intestinal inflammation.

In conclusion, we show that during chronic intestinal inflammation, effector CD4 T cells exist in a spectrum of differentiation states, and the pathogenic potential of the cells is directly linked to the differentiation status. Importantly, we demonstrate that a unique subset of effector CD4 T cells with progenitor-like properties promotes the chronicity of inflammation during IBD. These effector CD4 T cells possess a transcriptional profile that is consistent with other self-renewing cell populations, exhibit enhanced survival, preferentially express the glycosyltransferase ST6Gal-I as well as the stemness associated transcription factor TCF1, and mediate colitis upon adoptive transfer into disease-free hosts. Concomitant with the presence of these cells, we also find effector CD4 T cells at the opposite end of the spectrum that display a terminally differentiated phenotype. In contrast to the progenitor-like effector CD4 T cells, these terminally differentiated cells do not persist long term and cannot provoke inflammation in vivo. In the future, it will be important to determine whether similar effector CD4 T cell diversity is present in other T cell–mediated chronic inflammatory disorders such as type 1 diabetes and rheumatoid arthritis and if a causal relationship exists between the progenitor-like CD4 T cells and the disease pathogenesis.

Materials and methods

Mice

C57BL/6J (WT), B6.129S7-Ragfimim/MmJ (Rag1−/−), B6.SJL-Ptpcrp Pecp−/BoyJ (CD45.1), B6.129-St6gal1m2/xm/fJ (ST6Gal-Ifl/fl), and Tg(CAG-cre/Esrt1)xSAmc (CAGG-creERT2) mice were purchased from the Jackson Laboratory and bred at the University of Alabama at Birmingham. B6.Ifng/Thy1.1 KI (IFNγ KI) mice were described previously (Harrington et al., 2008). All animals were bred and maintained under specific pathogen–free conditions at the University of Alabama at Birmingham according to Institutional Animal Care and Use Committee regulations. The University of Alabama at Birmingham Institutional Animal Care and Use Committee approved all experimental procedures with mice in accordance with National Institutes of Health (NIH) guidelines.

Adoptive transfer model of colitis

For the induction of primary colitis, IFNγ KI CD45RB1−/− CD25− CD4 T cells from spleen and LNs were FACS sorted, and 5 × 10⁶ cells were...
injected in Rag1-/- hosts intraperitoneally. To obtain ST6Gal-I-/- CD4 T cells, ST6Gal-Ifl/fl mice were bred to CAGG-CreERT2 mice, and the progeny ST6Gal-Ifl/flxCAGG-CreERT2 mice were treated with tamoxifen (3 mg/20 g body weight) for three consecutive days.

For the secondary transfer colitis, effector CD4 T cells were isolated from spleen and LNs of colitic recipient Rag1-/- mice at 4 wk after primary transfer. CD44+ Thy1.2+ CD4 T cells were FACS sorted, and 5 x 10^5 cells were intraperitoneally injected into disease-free Rag1-/- littermate mice. For all colitis experiments, weight loss of recipient mice was monitored weekly. Large intestine and cecum samples were prepared by paraformaldehyde fixation followed by hematoxylin and eosin staining. The images were made with a 10× objective. A veterinary pathologist scored slides for disease in a blinded manner.

In vivo Thy1.1 depletion
For the anti-Thy1.1 depletion antibody treatment, recipient mice were intraperitoneally injected with 100 µg anti-Thy1.1 depleting

Figure 5. ST6Gal-1 mediated sialylation correlates with pathogenic, stem-like CD4 T cells during chronic inflammation and promotes TCF1 expression. (A) Area-proportional Venn diagrams illustrate superimposition of enriched gene sets derived from GSEA of Thy1.1- CD4 T cells with the indicated published datasets. The number of genes is reported. (B and C) Representative histograms and the percentage of SNA+ CD4 T cells 4 wk after colitis induction (n = 35, three experiments). (D and E) Sorting strategy and annexin V staining 4 wk after colitis induction (n = 3–7 mice pooled per experiment, three experiments). (F and G) Representative plots gated on the indicated cell populations and the frequency of TCF1+ or KLRG1+ cells is shown (n = 13, three experiments). (H and I) Live ST6Gal-I+/+ or ST6Gal-I-/- CD44hi CD4 T cells from the mLNs were analyzed 4–7 wk after colitis induction. Naive CD4 T cells are from disease-free IFNγ KI mLNs (n = 10–13, three experiments). Graphs show the mean ± SD; one-way ANOVA (E and G) or unpaired t test (C and I): *, P < 0.05; **, P < 0.01; ***, P < 0.001.
mAb (clone 19e1.2; UAB hybridoma facility) every 4 d for 6–8 wk starting on day 0 of the colitis induction.

**RNA purification, RNA sequencing, and analysis**

For RNA sequencing, total RNA was isolated from FACS-sorted CD44+ Thy1.1+ and CD44+ Thy1.1− CD4 T cells using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s protocols and submitted to GENEWIZ. Total RNA was sequenced on Illumina HiSeq 2500 (2 × 100 base pair, paired-end reads) and aligned using mouse mm10 reference genome. The R project software along with DESeq2 software was used for differential expression analysis. RNA sequencing data have been submitted to the Gene Expression Omnibus (GEO) Repository (GSE114346).

For GSEA, RNA sequencing data were preranked according to an adjusted P value and the sign of differential expression. The gene sets from the immunological signature collection (C7) and the hallmark gene sets (H) of the Molecular Signatures Database (MSigDB) were used for computing enrichment. The normalized enrichment score, nominal P value, and false detection rate q-value were assessed using GSEA software from Broad Institute by running in preranked list mode with 1,000 permutations. To perform GSEA with chronic CD8 T cell gene signature, the microarray data from GEO database (GSE84105) was used. Briefly, differential expression between CXCR5+ and Tim3+ CD8 T cells was analyzed using GEO2R, and the gene sets associated to the Gene Expression Omnibus (GEO) Repository (GSE114346).

**ORC assessment**

To determine ORC, CD44+ CD4 T cell progenitor cells versus CD4 T cells (GEO accession no. GSE37301). Briefly, differential expression between CXCR5+ and Tim3+ CD8 T cells was analyzed using GEO2R, and the gene sets associated with each cell type were determined by adjusted P value <0.01 and fold-change >2.

The area-proportional Venn diagram was constructed using the microarray data from GEO database (GSE84105) was used. The q-values were assessed using GSEA software from Broad Institute by running in preranked list mode with 1,000 permutations.

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**Lymphocyte preparation**

Single-cell suspensions of spleen and mesenteric LNs (mLNs) were prepared as previously described (Yeh et al., 2011) and resuspended in R10 medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1× nonessential amino acids, 1 µM sodium pyruvate, and 50 µM β-mercaptoethanol). Large intestine lamina propria lymphocytes were isolated using the Lamina Propria Dissociation Kit (Miltenyi Biotec) according to the manufacturer’s instructions. Cells were then isolated by centrifugation over a Percoll gradient, washed, and resuspended in R10 media.

**Flow cytometry**

Cell surface and intracellular staining was performed as previously described (Yeh et al., 2011). For the analysis of cytokine production, single-cell suspensions were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 750 ng/ml ionomycin (Calbiochem) in the presence of GolgiPlug (BD Biosciences) for 4 h according to the manufacturer’s instructions and then stained using the Foxp3 Permeabilization/Fixation kit (eBioscience). A viability dye (Life Technologies, Aqua) was applied to exclude dead cells. Samples were acquired using LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo version 10 (Tree Star).

The following antibodies were used in this study (all eBioscience except where noted otherwise: anti-CD3ε PerCP-Cy5.5/PE-Cy7/APC (clone 145-2C11); anti-CD4 PerCP-Cy5.5/eFluor 450/PE-Cy7/APC/APC70 (clone RM4-5); anti-CD25 PerCP-Cy5.5 (clone PC61.5); anti-CD39 PE-Cy7 (clone 24DS1); anti-CD44 FITC/PE-Cy7/PerCP-Cy5.5 (clone IM7); anti-CD45.1 FITC (clone A20); anti-CD45.2 PerCP-Cy5.5/APC (clone 104); anti-CD45RB FITC (clone C63.61A); anti-CD127 FITC (clone A7R34); anti–CXCR5+ biotin (BD Biosciences, clone 2G8); anti-KLRG1 APC (clone 2F1); anti–Thy1.1 PE (clone OX-7; BD Biosciences); anti-CD11c PerCP/Cy7 (clone HIS51); anti-Thy1.2 APC (clone 53-2.1); anti-Foxp3 FITC/eFluor 450 (clone FJ16a); anti–GM-CSF PE (clone MPI-22E9; BD Biosciences); anti–IFNγ PerCP/eFluor 450 (clone XM11.2); anti–IL-2 PE-Cy7/APC (clone JES6-5H4); anti–IL-17A PE (clone TC11-18H10; BD Biosciences); anti–Thet PB (clone 4B10; BioLegend); anti–TCF1 PE (clone S33-966; BD Biosciences); anti–TNFα FITC/Alexa Fluor 647 (clone MP6-XT22); and streptavidin APC.

**cDNA synthesis and real-time PCR**

cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) in accordance with manufacturers’ instructions. Real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad), and reactions were run on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). For the housekeeping gene, β2-microglobulin was used. Relative gene expression was calculated according to the ΔΔ threshold cycle (ΔCt) method. Primers used for indicated genes are as follows: TcPd forward 5’-GATGCTGGGATCTGTGTTGAC-3’, reverse 5’-CTCCTTTAGTCGTGCACCTCAG-3’, Sell forward 5’-GGGCTGAGAACAATCCTGAAG-3’, reverse 5’-TGCAGAAGTTCTGAGTCCC-3’, Kdx2 forward 5’-GCAAAGCCTACACCAAGACG-3’, reverse 5’-CTGTGACCTGTGTTGTCCTG-3’, Left forward 5’-TCATCACCTACAGGGACAGG-3’, reverse 5’-GGGTAGAAGTGGGAGATTTCC-3’; and Stğall forward 5’-TCTTCCAGAGAAGATATAAGTGTTGAGC-3’, Stğall reverse 5’-GACCTATTGAGAAGATGAG-3’. In vivo proliferation CD44+ Thy1.1+ and CD44+ Thy1.1− CD4 T cells were FACS sorted from spleen and mLNs of colitic mice 4 wk after disease induction and labeled with eFluor450 proliferation dye (eBioscience) in accordance with the manufacturer’s protocols. 2 × 10^5 labeled cells were transferred into Rag1^−/− mice and analyzed 7 d later by flow cytometry.
Statistics

Unpaired *t* test was used to calculate statistical significance for individual groups. The ratio paired *t* test was performed to analyze statistical significance of real-time PCR data. To compare the mean of three or more groups, one-way ANOVA with Tukey's multiple comparisons test was used; *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001. All statistical analyses (excluding RNA-seq, described above) were performed using Prism 6.0c software (GraphPad).

Online supplemental material

Fig. S1 shows that IFNγ-producing CD4 T cells secrete multiple colitis-associated cytokines, but IFNγ-producing CD4 T cells are dispensable to induce intestinal inflammation. Fig. S2 demonstrates that IFNγ-nonproducing CD4 T cells show a progenitor-like profile, whereas IFNγ-producing CD4 T cells are terminally differentiated.

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