Commensal Gut Flora Drives the Expansion of Proinflammatory CD4 T Cells in the Colonic Lamina Propria under Normal and Inflammatory Conditions[1]

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We tested in B6 mice whether the local expansion of CD4 T cells producing proinflammatory cytokines including IL-17 (Th17 cells) in the colonic lamina propria (cLP) depends on the commensal microflora. High numbers of CD4 Th17 cells were found in the lamina propria of the ileum and colon but not the duodenum, jejunum, mesenteric lymph nodes, spleen, or liver of specific pathogen-free (SPF) mice. The microflora is required for the accumulation of cytokine (IL-17, IFN-γ, TNF-α, IL-10)-producing CD4 T cells in the cLP because only low numbers of cytokine-producing cLP CD4 T cells were found in syngeneic (age- and sex-matched) germfree mice. The fraction of cLP Th17 cells was higher in (type I and type II) IFN- but not IL-4- or IL-12p40-deficient SPF congenics. cLP CD4 Th17 cells produce IL-17 but not IFN-γ, TNF-α, IL-4, or IL-10. cLP CD4 Th17 cells accumulate locally in colitis induced by adoptive transfer of IFN-$\gamma^{+/+}$ or IFN-$\gamma^{-/-}$ CD4 T cells into congenic SPF (but not germfree) RAG$^{-/-}$ hosts. In this colitis model, cLP CD4 T cells that "spontaneously" produce IL-17 progressively increase in number in the inflamed cLP, and increasing serum IL-17 levels appear as the disease progresses. Commensal bacteria-driven, local expansion of cLP CD4 Th17 cells may contribute to the pathogenesis of this inflammatory bowel disease. ***The Journal of Immunology,*** **2008, 180: 559–568.**

The gastrointestinal immune system faces a continuous challenge of potent stimuli for the innate and the specific immune system from a large variety of commensal microorganisms (1, 2). Deregulated, local immune responses driven by constituents of commensal bacteria are a major factor in the pathogenesis of the two major forms of inflammatory bowel disease (IBD)[3] in humans, i.e., Crohn's disease and ulcerative colitis (3, 4). IBD (manifest often as a progressive colitis) can be readily elicited in many experimental models in mice reared under conventional or specific pathogen-free (SPF) conditions but not in syngeneic, germfree (GF) mice (reviewed in Ref. 5). Although GF mice and rats generate alloreactive T cell responses (6), the cellularity of their immune system is greatly reduced (7–9). Because the commensal flora is the major driving force of the homeostatic proliferation of naive T cells in the periphery (10, 11), the reduced cellularity in the immune system of GF mice may be the result of the deficient peripheral expansion of recent thymic T cell emigrants. In this study, we compared the numbers of CD4 CD3 T cells in the gut, mesenteric lymph nodes (MLN), liver, and spleen from GF and SPF mice and determined their cytokine expression profile.

Naive CD4 T cells have a substantial plasticity to develop into distinct effector or regulatory lineages in response to diverse signals. The development of (T-bet-dependent) Th1 and (GATA-3-dependent) Th2 effector cell lineages has been well-characterized in the last two decades (12). Recently, (RORγt-dependent) Th17 cells have been recognized as a distinct effector CD4 T cell lineage (reviewed in Ref. 13). Commitment of naive T cells to this lineage depends on TGF-β, IL-21, and IL-6 (14, 15), i.e., cytokines not involved in Th1 or Th2 development. Th17 cell development can be antagonized by products of T cells of the Th1 and/or Th2 lineages (16). Expansion of CD4 Th17 cells is facilitated by IL-23 (17). Local availability of this cytokine may regulate the selective clonal expansion of these T cells. Th17 cells play a role in experimental autoimmune diseases and bacterial infections. Pathogenic effector CD4 Th17 cells are found in collagen-induced arthritis, experimental allergic encephalitis, and chronic dermatitis (18–22). Th17 cells are prominent in murine responses to pathogenic *Citrobacter* (23), *Klebsiella* (24), or *Mycobacteria* species (25, 26). IL-17 is expressed in inflammatory mucosal lesions in patients with IBD (27). A colitogenic role of Th17 cells has been proposed in murine IBD models (28). These data stress the importance of Th17 cells in chronic inflammatory responses.

We tested whether Th17 cells expand preferentially in the gut in response to the endogenous commensal flora by comparing the numbers of IL-17-producing CD4 T cells in different tissues of SPF vs syngeneic (age- and sex-matched) GF mice. We furthermore followed the expansion of colonic lamina propria (cLP) CD4 Th17 cells in a transfer colitis model. The data indicate that the intestinal commensal flora is required for the local Th17 cell expansion under steady state conditions and that 5–10% of CD4 T cells recruited to the site of intestinal inflammation develop a Th17 phenotype.

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; SPF, specific pathogen free; GF, germfree; MLN, mesenteric lymph node; LP, lamina propria; cLP, colonic LP; cLPL, cLP lymphocyte; wt, wild type; NPC, nonparenchymal cell; FCM, flow cytometry; PP, Peyer's patch; DC, dendritic cell; KO, knockout.

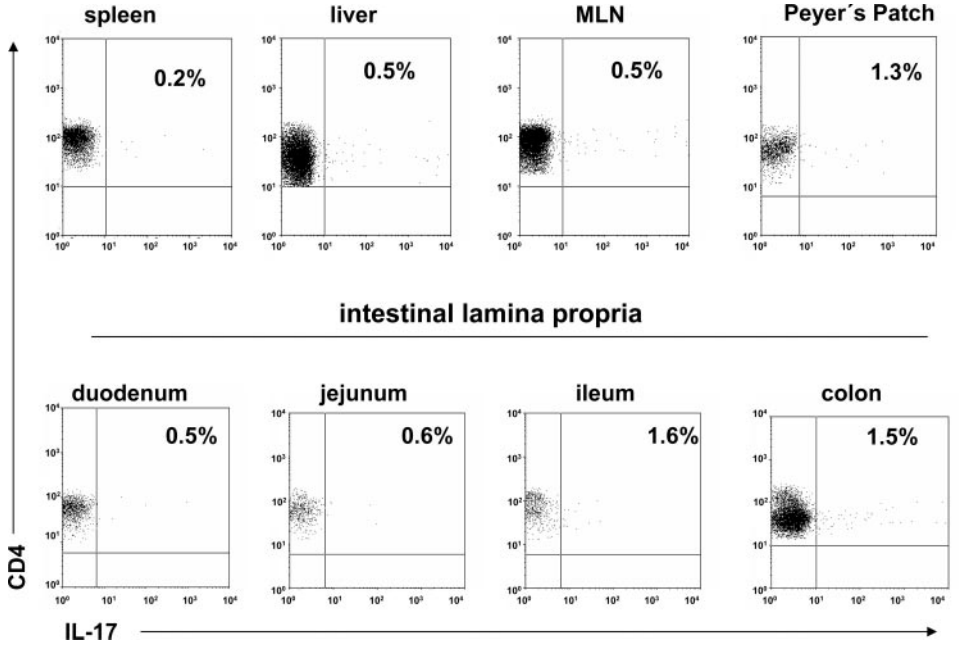

FIGURE 1. IL-17$^+$ CD4 T cells are increased in the cLP. CD4 T cells were isolated from spleen, liver, MLN, PP, as well as the LP of the duodenum, jejunum, ileum, and colon of an SPF B6 mouse. Cells were stimulated ex vivo with phorbol ester and ionomycin (in the presence of brefeldin A), surface stained for CD4, fixed, stained for intracellular IL-17A, and analyzed by flow cytometry. Numbers indicate percent of the CD4 T cells that express IL-17A. Data from one individual, representative mouse (of eight individual mice analyzed in two independent experiments) are shown.

Materials and Methods

Mice

Inbred C57BL/6J (B6) mice and congenic RAG$^{-/-}$ (Rag1^{tm1Mom}), IFN-$\gamma^{-/-}$, IFN-$\beta^{-/-}$, IL-4$^{-/-}$, and IL-12p40$^{-/-}$ mice were bred and kept under SPF conditions in the animal facility of Ulm University (Ulm, Germany). GF B6 and RAG$^{-/-}$ B6 mice were generated and bred in the animal facility of Ulm University. GF mice were screened weekly for viral, bacterial, and fungal contamination. RAG$^{-/-}$ mice were transplanted with congenic CD4 T cells at 8–12 wk of age. All animal experiments were performed according to the guidelines of the local animal use and care committee and the National Animal Welfare Law.

CD4 T cells isolation and adoptive transfer

CD4 T cells were isolated from the spleen of wild-type (wt) or IFN-$\gamma^{-/-}$ B6 mice by MACS following the manufacturer's instructions (catalog no. 130-090-860; Miltenyi Biotec). The purity of the CD4 T cell populations used for transfer was >95% upon reanalyses. To isolate CD45RB^{high} and CD45RB^{low} CD4 T cell subsets, MACS-presorted CD4 T cells were stained with allophycocyanin-conjugated anti-CD4 mAb (catalog no. 553051; BD Biosciences) and biotin-conjugated anti-CD45RB mAb 23G2 (catalog no. 01532D; BD Biosciences). Cells were washed, suspended in PBS/0.3% w/v BSA and stained with PE-conjugated streptavidin (catalog no. 554061; BD Biosciences). CD45RB^{high} and CD45RB^{low} subsets were sorted using the FACSAria system (BD Biosciences). Sorted CD45RB^{high} and CD45RB^{low} subpopulations with a purity of >98% were injected i.p. into congenic RAG$^{-/-}$ B6 mice (3×10^5 cells/mouse). The weight of transplanted mice and their clinical condition were monitored semiweekly.

Lymphoid cell isolations

CD4 T cells were isolated from the intestinal lamina propria (LP), liver, spleen, and MLN of wt, IFN-$\gamma^{-/-}$, IFN-$\beta^{-/-}$, IL-4$^{-/-}$, and IL-12p40$^{-/-}$ B6 mice.

Intestinal LP. Segments of the murine gut were washed with PBS (to remove debris and mucous). The epithelium was removed by incubation at 37°C for 30 min under gentle shaking with 1 mM DTT and 1 mM EDTA in Ca^{2+}/Mg^{2+}-free PBS supplemented with 1% FCS. The remaining tissue was washed in PBS to remove residual epithelial cells and the supernatants were discarded. Denuded tissues were cut into 2- × 2-mm pieces and digested with 0.5 mg/ml collagenase type VIII (catalog no. C-2139; Sigma-Aldrich) and 5 U/ml DNase (catalog no. 1284932; Roche) for 2 h at 37°C in RPMI 1640/5% FCS. Supernatants were collected from which cLP lymphocytes (cLPL) were pelleted. cLPL were resuspended in RPMI 1640 medium containing 40% Percoll (density 1.124 g/dl; catalog no. L-6145; Biochrome). This cell suspension was overlaid onto 70% Percoll and centrifuged for 20 min at 750 × *g*. Viable cells at the 40/70% interface were collected for intracellular staining and flow cytometry analyses.

Nonparenchymal cells (NPC) of the liver. The abdomen of an anesthetized mouse was opened and a needle was inserted into the portal vein. The vena cava was ligated and punctured. The liver was perfused with liver perfusion medium (catalog no. 17701-038; Invitrogen Life Technologies). To obtain the NPC populations, the liver was perfused with liver digestion medium (catalog no. 17703-034; Invitrogen Life Technologies), removed, and gently pressed through a mesh. NPC were separated from parenchymal cells by centrifugation at 50 × *g* for 5 min. NPC were collected, washed in PBS, resuspended in 40% Percoll (catalog no. L6145; Biochrom), diluted in RPMI 1640 medium, overlaid onto 70% Percoll, and centrifuged at 750 × *g* for 20 min. NPC collected from the interface were washed twice in PBS and resuspended in medium and processed for flow cytometry.

Spleen and MLN cells. Single-cell suspensions were aseptically prepared from spleen and MLNs.

Intracellular cytokine staining

Cells (1×10^6/ml) from the liver, spleen, MLNs, and LP of the gut were stimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 2.5 μg/ml brefeldin A (catalog no. 15870; Sigma-Aldrich). Cells were harvested, washed and stained with allophycocyanin-conjugated anti-CD4 mAb (catalog no. 553051; BD Biosciences). Surface-stained cells were fixed (2% paraformaldehyde in PBS) and resuspended in permeabilization buffer (HBSS, 0.5% BSA, 0.5% saponin, 0.05% sodium azide). Permeabilized cells were incubated for 30 min at room temperature with FITC-conjugated anti-IFN-γ (catalog no. 554411; BD Biosciences), anti-TNF-α (catalog no. 554418; BD Biosciences), anti-IL-4 (catalog no. 557728; BD Biosciences), anti-IL-10 (catalog no. 554466; BD Biosciences), or PE-conjugated anti-IL-17 (catalog no. 559502; BD Biosciences) mAb. As isotype controls rat IgG2a,κ (catalog no. 553139; BD Biosciences) and rat IgG1 (catalog no. 553443; BD Biosciences) were used. Respective background stainings were <0.05%. Stained cells were washed twice in permeabilization buffer and resuspended in PBS supplemented with 0.3% w/v BSA and 0.1% w/v sodium azide. The number of cytokine-expressing CD4 T cells per 10^5 CD4 T cells was determined by flow cytometry.

Flow cytometry (FCM) analyses

Cells were washed twice in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide. Nonspecific binding of Abs to FcRs was blocked by preincubation of cells with mAb 2.4G2 (catalog no. 01241D; BD Biosciences) directed against the FcγRIII/II CD16/CD32 (0.5 μg of mAb/10^6 cells/100 μl). Cells were washed and incubated with 0.5 μg/10^6 cells of the relevant mAb for 30 min at 4°C, and washed again twice. In most experiments, cells were subsequently incubated with a second-step reagent for

10 min at 4°C. Four-color FCM analyses were performed using a FACS Calibur (BD Biosciences). The forward narrow angle light scatter was used as an additional parameter to facilitate exclusion of dead cells and aggregated cell clumps. Data were analyzed using the WinMDI software.

Histopathological examinations and immunofluorescence

Tissue samples for histopathological examination were taken from the large intestine, fixed in neutral-buffered formalin, embedded in paraffin, sectioned on a microtome, mounted on slides, and stained with H&E. Histology of the large intestine was categorized as normal (score 0); mild colitis (score 1) with few inflammatory cells in the LP, stroma edema, and a slight reduction of goblet cells; moderate colitis (score 2) with an intense inflammatory infiltration of the LP, hyperplasia of crypts, and a marked reduction of goblet cells; severe colitis (score 3) with a spillover of leukocytes beyond the mucosa into deeper layers of the colonic wall, complete loss of goblet cells, distortion of the mucosal architecture, erosions or ulcerations, and crypt abscesses as previously published (29).

Some samples were frozen in liquid nitrogen. For immunohistology, cryosections were used and fixed in acetone at 4°C for 30 min. The sections were incubated for 1 h with the Abs at an appropriate dilution, washed twice with PBS, and incubated with secondary Abs. Isotype-matched mAbs to an irrelevant target were applied at identical concentrations as negative control for the specific staining of the primary Abs. All incubation steps were conducted at room temperature in PBS. Stained sections were examined under an Axioskop microscope (Zeiss) and recorded as described (29). Anti-CD3ε mAb 500A2 (catalog no. 01511D; BD Biosciences) and anti-IL-17 mAb TC11-18H10.1 (catalog no. 559502; BD Biosciences) were used. Anti-CD3ε Ab was detected with a polyclonal Cy3-conjugated goat anti-Syrian hamster IgG (catalog no. 107-165-142; Dianova) and anti-IL-17 Ab was detected with a polyclonal Cy2-conjugated goat anti-rat IgG (catalog no. 112-227-003; Dianova).

Cytokine detection by ELISA

IL-17 in the serum of diseased mice was detected by a conventional double-sandwich ELISA (catalog no. 555068; BD Biosciences). Extinction was measured at 405/490 nm on a Tecan microplate-ELISA reader using EasyWin software (Tecan).

Statistical analyses

A t test for two unequal variances was used. A value of $p < 0.05$ was considered statistically significant.

Results

Many Th17 CD4 T cells are found in the cLP of SPF mice

We determined the fraction of cells that produce IL-17 in cell populations from spleen, liver, MLN, Peyer's patches (PP), and LP of the small intestine (duodenum, jejunum, and ileum) as well as the large intestine from normal SPF B6 mice. Cells were isolated from these tissues, stimulated for 4 h ex vivo with phorbol ester and ionomycin (in the presence of brefeldin A), surface stained for CD4, fixed, stained for intracellular IL-17A, and analyzed by FCM. CD40 stimulation has been reported to activate IL-17-producing $CD11c^+CD4^+$ dendritic cells (DC) in $RAG^{-/-}$ mice (30). We therefore stained cells in control experiments with anti-CD4 and anti-CD11c mAb followed by intracellular staining with anti-IL-17 mAb to exclude the presence of IL-17-producing $CD11c^+$ $CD4^+$ DC in the analyzed cell populations. We found $CD11c^-CD4^+$ T cells but not $CD11c^+CD4^+$ IL-17-producing DC (data not shown). A fraction of 10–20% of the IL-17-producing cells was $CD4^-$ but these cells were not further analyzed.

Few CD4 Th17 cells were found in spleen (0.2%), liver (0.5%), and MLNs (0.5%) (Fig. 1). Increased numbers of CD4 Th17 cells were found in Peyer's patches (1.3%) (Fig. 1). Although the percentage of the CD4 T cells that can produce IL-17 was low (0.5–0.6%) in the LP of the upper small intestine (duodenum and jejunum), the fraction of $IL\text{-}17^+$ CD4 T cells increased 3- to 4-fold in the LP of the ileum and colon where they represented 1–2% of all CD4 T cells (Fig. 1). The numbers of intestinal CD4 Th17 cells thus correlate with the bacterial load of the gut segment suggesting

Table I. *Cytokine-producing CD4 T cells in different organs/tissues of GF and SPF B6 mice*[a]

	CD4 T Cells per Organ ($\times 10^4 \pm$ SEM)													
	GF						SPF							
Tissue	Total[b]	IL-17+ [c]	IFN-γ+ [c]	TNF-α+ [c]	IL-10+ [c]	IL-4+ [c]	Total[b]	IL-17+ [c]	IFN-γ+ [c]	TNF-α+ [c]	IL-10+ [c]	IL-4+ [c]
cLP	**2.9 ± 0.3**	**0.01 ± 0.005 (0.5%)**	**0.007 ± 0.002 (0.2%)**	**0.005 ± 0.001 (0.2%)**	**0.005 ± 0.003 (0.2%)**	**0.04 ± 0.01 (1.1%)**	**46 ± 15**	**0.8 ± 0.4 (1.7%)**	**0.7 ± 0.3 (1.7%)**	**0.7 ± 0.3 (1.5%)**	**1.1 ± 0.6 (2.2%)**	**0.1 ± 0.06 (0.3%)**
S	**1048 ± 137**	**2.5 ± 0.5 (0.2%)**	**1.9 ± 1.2 (0.2%)**	**1.9 ± 9.4 (0.2%)**	0.6 ± 0.5 (0.06%)	**12.6 ± 6.1 (1.2%)**	**3823 ± 293**	**12.3 ± 4.0 (0.4%)**	**20 ± 5.9** (0.5%)	**17.7 ± 4.5 (0.5%)**	2.5 ± 2.3 (0.07%)	**2.6 ± 1.9 (0.07%)**
L	46 ± 17	**0.08 ± 0.03 (0.2%)**	**0.1 ± 0.08 (0.2%)**	0.2 ± 0.2 (0.6%)	0.02 ± 0.01 (0.03%)	0.4 ± 0.4 (1%)	119 ± 5.2	**0.7 ± 0.2 (0.6%)**	**1.0 ± 1.0 (0.8%)**	0.9 ± 0.6 (0.7%)	0.4 ± 0.4 (0.3%)	0.5 ± 0.2 (0.4%)
MLN	**45 ± 19**	**0.02 ± 0.005 (0.04%)**	**0.005 ± 0.002 (0.02%)**	**0.13 ± 0.06 (0.3%)**	0.01 ± 0.005 (0.02%)	**0.07 ± 0.03 (0.2%)**	**193 ± 42**	**1.4 ± 0.2 (0.8%)**	**0.4 ± 0.1 (0.2%)**	**0.7 ± 0.2 (0.4%)**	0.9 ± 0.7 (0.6%)	**0.8 ± 0.4 (0.4%)**

[a] Mean numbers of four individual mice (±SEM) per group are shown. Bold numbers indicate significant differences between the corresponding groups from GF vs SPF mice. Significant differences were detected using the two-tailed Student t test; a p value <0.05 was considered statistically significant. This experiment was repeated once giving comparable results.

[b] Total numbers (mean ± SEM) of CD4 T cells isolated from the indicated organ/tissue are shown (S, spleen; L, liver).

[c] Total numbers (mean ± SEM) of CD4 T cells producing the indicated cytokine after a brief ex vivo stimulation are shown. The mean percentage (±SEM) of cells in the CD4 T cell population from the indicated organ/tissue producing the respective cytokine is shown in parentheses.

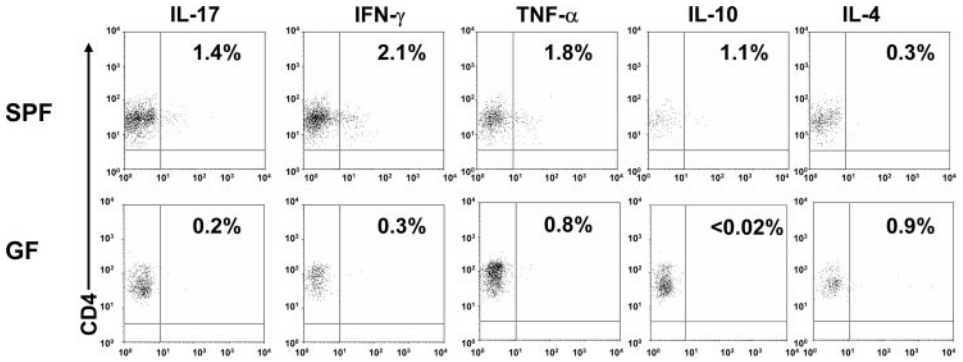

FIGURE 2. IL-17$^+$ CD4 T cells accumulate in the cLP challenged by commensal bacteria. CD4 T cells were isolated from the cLP of individual, SPF, or (age- and sex-matched) GF B6 mice. cLP CD4 T cells were stimulated ex vivo with phorbol ester and ionomycin (in the presence of brefeldin A), surface stained for CD4, fixed, stained for intracellular IL-17A, IFN-γ, TNF-α, IL-10, and IL-4 and analyzed by flow cytometry. The percent of cells in the cLP CD4 T cell population that express the indicated cytokine is shown. Data from two individual, representative mice (of 8 individual GF and 10 individual SPF mice analyzed in independent experiments) are shown.

that the commensal microflora of the gut drives the local development of mucosal Th17 cells. To test this assumption we compared syngeneic GF and SPF B6 mice.

Intestinal CD4 Th17 cell development depends on the gut microflora

We isolated cLPL from SPF and syngeneic (age-and sex-matched) GF B6 mice. CD4 T cell numbers in GF B6 mice were 4- to 15-fold reduced in the cLP, MLN, and spleen when compared with syngeneic, age-, and sex-matched SPF B6 mice (Table I).

Compared with SPF mice the numbers of IL-17-producing CD4 T cells in cLP CD4 T cell populations from GF mice were >25-fold reduced (Table I). This was also evident when the fractions of cells within the cLP CD4 T cell populations from GF vs SPF mice that showed inducible IL-17 expression were compared: a 10-fold lower fraction of cLP CD4 T cells could be induced to produce IL-17 in GF than SPF mice (Fig. 2, Table I). Low frequencies (and low numbers) of CD4 Th17 cells were found in CD4 T cell populations from MLN, spleen, and liver of GF and SPF mice (Table I). Hence, stimulation by the commensal flora in the ileum and colon of SPF mice correlates with increased numbers and frequencies of CD4 Th17 cells in local CD4 T cell populations.

Similarly, we found only low numbers of IFN-γ- and/or TNF-α-producing cLP CD4 T cells in GF mice (Fig. 2; Table I). Compared with age- and sex-matched syngeneic SPF mice, the numbers of IFN-γ- and/or TNF-α-producing CD4 T cells in cLP CD4 T cell populations from GF mice were >100-fold lower. The reduction of IL-17-producing CD4 T cells in the cLP of GF mice is thus not selective for this cytokine but also observed for CD4 T cells producing the two proinflammatory cytokines IFN-γ or TNF-α.

Many cLP CD4 T cells can be induced to produce IL-10 by repeated treatment with anti-CD3 Ab (31). In the cLP CD4 T cell population of GF mice, we found virtually no IL-10-producing cell while 2% of the cLP CD4 T cell from SPF mice produced IL-10 (Fig. 2, Table I). Hence, the commensal flora drives not only local

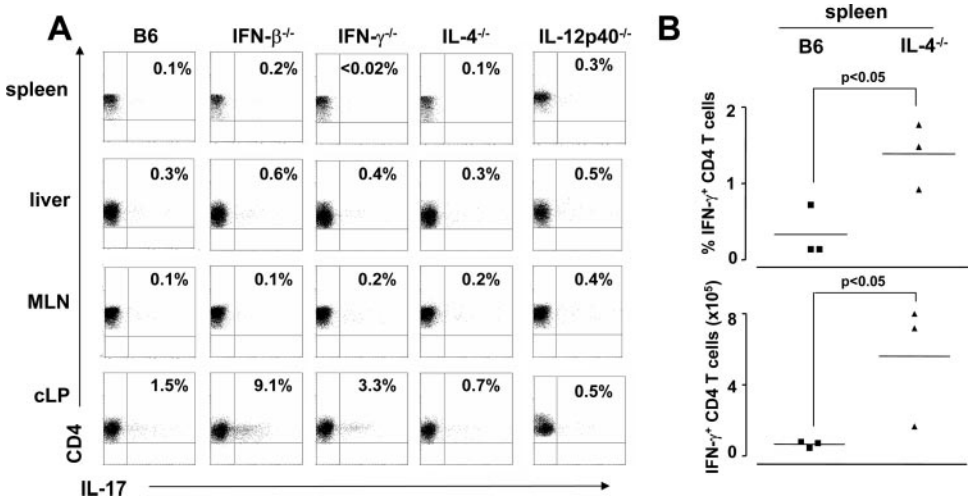

FIGURE 3. Cytokines can modulate the local CD4 Th17 cell prevalence in the cLP of SPF mice. *A*, CD4 T cells were isolated from spleen, liver, MLN, and cLP of wt B6 mice or congenic IFN-$\beta^{-/-}$, IFN-$\gamma^{-/-}$, IL-$4^{-/-}$, or IL12p$40^{-/-}$ KO B6 mice. Cells were stimulated ex vivo with phorbol ester and ionomycin (in the presence of brefeldin A), surface stained for CD4, fixed, stained for intracellular IL-17A, and analyzed by flow cytometry. Numbers indicate the percent of CD4 T cells that express IL-17A. Data from an individual, representative mouse per group (of four to seven individual mice analyzed per group in three independent experiments) are shown. *B*, Percent and total number of IFN-γ-secreting CD4 T cells in the splenic CD4 population from wt B6 or congenic IL-$4^{-/-}$ KO B6 mice. Data from three individual mice from one (of two independent) experiment(s) performed are shown. A p value <0.05 in the two-tailed Student t test is considered statistically significant.

Table II. *IL-17-producing CD4 T cells isolated from the cLP of wt or congenic (sex-/age-matched) cytokine KO B6 mice*[a]

B6 Line	Total Numbers of CD4 T Cells in the cLP per Mouse ($\times 10^4 \pm$ SEM)	IL-17$^+$ CD4 T Cells in the cLP: Total Number of IL-17$^+$ CD4 Cells per Mouse ($\times 10^4$)	IL-17$^+$ CD4 T Cells in the cLP: % CD4 T Cells Producing IL-17
B6	45 ± 21	0.8 ± 0.6	**1.6 ± 0.4**
IFN-$\beta^{-/-}$	57 ± 31	4.1 ± 1.2	**6.9 ± 2.0**
IFN-$\gamma^{-/-}$	29 ± 12	1.2 ± 0.5	**4.1 ± 1.3**
IL-4$^{-/-}$	32 ± 16	0.3 ± 0.1	**0.4 ± 0.3**
IL-12p40$^{-/-}$	24 ± 6	0.07 ± 0.04	**0.1 ± 0.08**

[a] Mean numbers (±SEM) of four individual mice per group are shown. CD4 T cells were isolated from the cLP of B6 mice and congenic (age-/sex-matched) IFN-$\beta^{-/-}$, IFN-$\gamma^{-/-}$, IL-4$^{-/-}$, or IL-12p40$^{-/-}$ KO B6 mice. Bold numbers indicate significant differences between the corresponding groups from wt vs KO mice. Significant differences were determined by the two-tailed Student *t* test; a p value <0.05 was considered statistically significant. The experiment was repeated twice with comparable results.

expansion of proinflammatory Th1 and Th17 CD4 T cells but also of immunoregulatory (IL-10-producing) CD4 T cells.

More IL-4-producing CD4 T cells were found in the cLP and MLNs of SPF than GF mice (Table I) although the fraction (percent) of CD4 T cells producing this cytokine was higher in the cLP of GF mice (Table I). In contrast, ~3-fold more IL-4-producing CD4 T cells were found in the spleen of GF as compared with SPF mice (Table I). All tissues tested in GF mice hence contained strikingly lower numbers of CD4 T cells but the fraction of cells within this population that could be induced to produce one of the cytokines tested varied between GF and SPF mice.

Cytokine-dependent expansion of the cLP CD4 Th17 cell population

Type I and type II IFNs, IL-27, or IL-4 negatively regulate Th17 development (16) while IL-23 supports clonal expansion of CD4 Th17 cells (17). We used (age- and sex-matched) congenic IFN-$\gamma^{-/-}$, IFN-$\beta^{-/-}$, IL-4$^{-/-}$, or IL-12p40$^{-/-}$ B6 mice raised under similar SPF condition as wt control B6 mice to test the influence of these cytokines on the local expansion of CD4 Th17 cells especially in the cLP. T cells were isolated from spleen, liver, MLNs, and cLP of wt and knockout (KO) B6 mice and the frequencies of CD4 Th17 cells was determined in these cell populations by FCM. High frequencies of CD4 Th17 cells were found in the cLP (but not spleen or liver) of B6 mice deficient for IFN (IFN-β or IFN-γ) (Fig. 3*A*, Table II). Type I

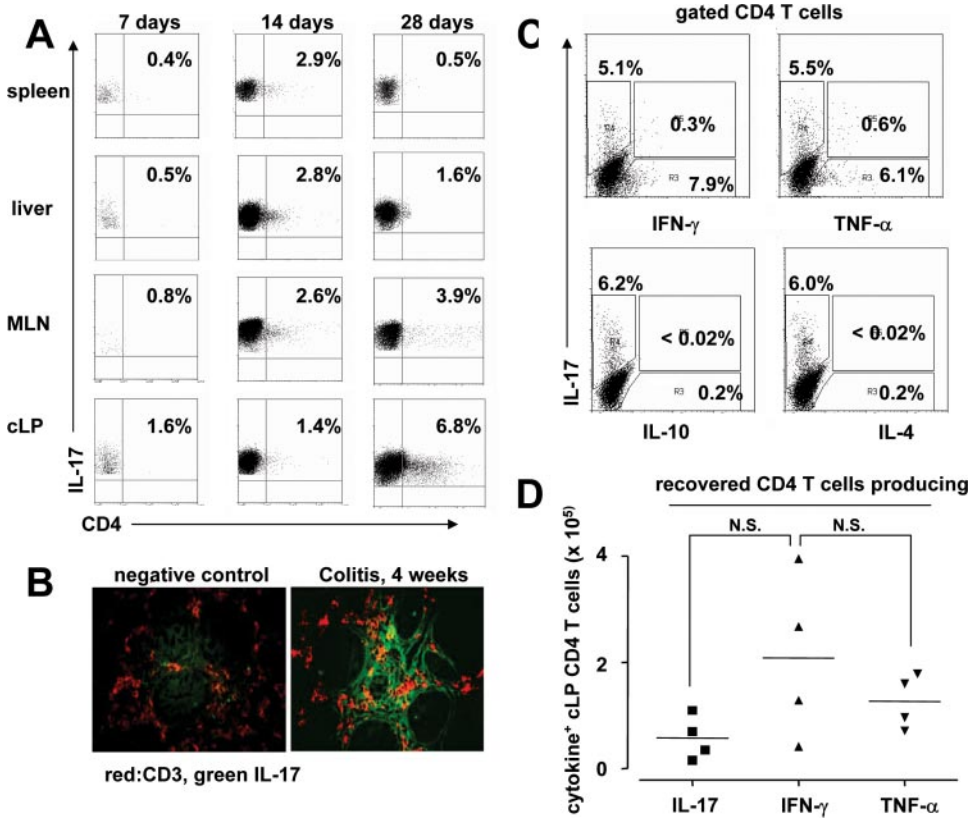

FIGURE 4. Th17 cells accumulate in the cLP of mice with transfer colitis. *A*, cLP CD4 T cells were isolated from spleen, liver, MLN, and cLP of transplanted RAG$^{-/-}$ mice at 7, 14, or 28 days after transfer of nonfractionated, immunocompetent CD4 T cells. The percent of IL-17-producing CD4 T cells were determined. Data from three individual, representative mice (of four individual mice per group analyzed) are shown. *B*, Intestinal tissues obtained from transplanted RAG$^{-/-}$ hosts 28 days after transfer of nonfractionated CD4 T cells from wt donors were cryopreserved and acetone fixed. Tissue sections were stained for CD3ε (red) and IL-17A (green). In the negative control, the anti-IL-17A primary Ab was replaced by an isotype-matched control Ab to stain the same tissue. *C*, cLP cells isolated from transplanted RAG$^{-/-}$ hosts 28 days after adoptive transfer were stimulated with phorbol ester and ionomycin (in the presence of brefeldin A). Cells were surface stained for CD4, fixed, stained for intracellular IL-17A and IFN-γ, TNF-α, IL-10, or IL-4. Double expression of two cytokines was analyzed on gated CD4 T cells by flow cytometry. Data from an individual, representative mice (of four individual mice analyzed) are shown. *D*, cLP CD4 T cells were isolated 28 days after transfer of nonfractionated CD4 T cells from wt donor mice into congenic RAG$^{-/-}$ host mice. The total number of cLP CD4 T cells secreting IL-17, IFN-γ, or TNF-α recovered from the cLP of four individual, diseased mice is shown. (NS, not significant). The data are from one representative of two independent experiment(s) performed.

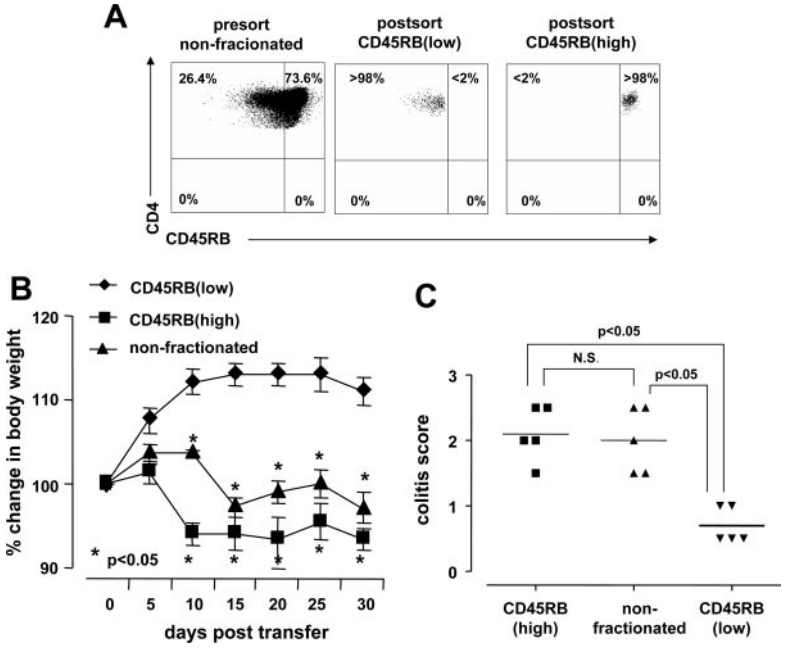

FIGURE 5. Nonfractionated CD4 T cells (that have not been separated into CD45RB^{high} and CD45RB^{low} subsets) induce colitis after transfer into immunocompromised RAG$^{-/-}$ hosts. *A*, CD4 T cells isolated from the spleen of B6 donors by MACS were electronically sorted into a CD45RB^{high} and a CD45RB^{low} subset. Reanalysis by flow cytometry showed that the respective subsets had a purity of >98%. *B*, Purified CD45RB^{high} or CD45RB^{low} CD4 T cells, or nonfractionated CD4 T cells (that have not been separated into CD45RB^{high} and CD45RB^{low} cells) were adoptively transferred into immunocompromised RAG$^{-/-}$ hosts (five hosts per group). The body weight was monitored semiweekly, and the mean percent of loss of body weight (± SEM) of five individual mice per group was plotted. *, Significant differences between the indicated RAG$^{-/-}$ recipients vs RAG$^{-/-}$ hosts transplanted with CD45RB^{low} cells. *C*, Biopsies from the upper large intestine were taken from five individual mice per group, and the colitis score was determined by a blinded pathologist. NS, Not significant. The experiment was repeated twice and gave comparable results.

and II IFNs thus negatively and selectively regulate local expansion of CD4 Th17 cells in the cLP. In contrast, IL-4$^{-/-}$ and IL-12 p40$^{-/-}$ B6 mice showed a reduced fraction of CD4 Th17 cells in the cLP (Fig. 3*A*, Table II). The number and frequency of IFN-γ-producing CD4 T cells in the spleen but not in the cLP was higher in IL-4$^{-/-}$ than wt B6 mice (Fig. 3*B*, data not shown). The reduced cLP Th17 cell numbers in IL-4$^{-/-}$ mice may thus result from increased numbers of IFN-γ-producing CD4 T cells. Reduced Th17 cell numbers in IL-12 p40$^{-/-}$ KO B6 mice (deficient in IL-12 and IL-23) may indicate that IL-23 supports local clonal expansion of CD4 Th17 cells in the cLP challenged by commensal bacteria.

CD4 Th17 cells accumulate in the inflamed colonic mucosa in transfer colitis

We used the transfer colitis model to test whether Th17 cells accumulate in the cLP during the development of this CD4 T cell-induced mucosal inflammation. Purified, nonfractionated CD4 T cells were adoptively transferred into SPF RAG$^{-/-}$ B6 hosts (3×10^5 cells/mouse). All transplanted animals developed severe, histologically verified colitis by day 28 posttransfer (data not shown) and showed a striking increase in the numbers of Th17 cells in the cLP (Fig. 4*A*), with 5–10% of all cLP CD4 T cells producing this cytokine. Accumulation of IL-17-producing CD4 T cells in situ in colitis-associated cell infiltrations was verified by immunohistology (Fig. 4*B*). The large majority of these cLP Th17 cells produced IL-17 directly ex vivo, i.e., did not require PMA/iono stimulation to reveal their cytokine production potential. The increase in the number of Th17 cells was selective for the inflamed cLP but not seen in liver or spleen of the diseased, adoptive host (Fig. 4*A*). In addition to IL-17-producing CD4$^+$ CD3$^+$ T cells, we also found CD3$^-$ IL-17-producing cells that may be granulocytes or macrophages. The disease-associated Th17 cells engrafted into the adoptive RAG$^{-/-}$ host were specialized to produce IL-17 but not IFN-γ, IL-4, IL-10, or TNF-α (Fig. 4*C*). The total numbers of IFN-γ$^+$ or TNF-α$^+$ cLP CD4 T cells exceeded the numbers of IL-17$^+$ cLP CD4 T cells (Fig. 4*D*), suggesting that an interplay of different proinflammatory cytokines drives the disease process locally.

In the studies described above, we adoptively transferred nonfractionated CD4 T cells (i.e., CD4 T cells that had not been separated into CD45RB^{high} and CD45RB^{low} cells) into congenic RAG$^{-/-}$ hosts (29, 32). In the following experiments, we compared the induction of colitis by transfer of equal numbers of sorted CD45RB^{high} CD4 T cells, nonfractionated CD4 T cells (that contain CD45RB^{high} and CD45RB^{low} cells) or sorted CD45RB^{low} CD4 T cells (Fig. 5*A*). RAG$^{-/-}$ hosts that received CD45RB^{high} cells or nonfractionated CD4 T cells showed a similar course of disease development (Fig. 5*B*). In contrast, RAG$^{-/-}$ hosts that received CD45RB^{low} CD4 T cells showed a delayed and attenuated course of the disease (Fig. 5*B*). No differences were apparent in the histological disease scores between RAG$^{-/-}$ hosts that received CD45RB^{high} or nonfractionated CD4 T cells (Fig. 5*C*). To avoid unnecessary manipulations of the T cell graft (that may affect Th17 precursor cell numbers), we chose a transfer model in which splenic CD4 T cells (that had not been separated into CD45RB^{high} and CD45RB^{low} cells) were transferred into RAG$^{-/-}$ hosts.

Th17 cell accumulation in transfer colitis is independent of IFN-γ

We tested whether IFN-γ has an influence on the accumulation of Th17 cells in the cLP and/or on the severity of the transfer colitis by transferring wt (IFN-γ$^{+/+}$) or IFN-γ$^{-/-}$ CD4 T cells into RAG$^{-/-}$ hosts. We monitored the clinical course of the disease, the percent loss of body weight (Fig. 6*A*) and the histological severity of colitis (Fig. 6*B*) at day 28 posttransfer. We confirmed that IFN-γ$^{-/-}$ CD4 T cells induce IBD in congenic RAG$^{-/-}$ hosts (33). Transfer of CD4 T cells from IFN-γ$^{-/-}$ donor mice induced a more severe colitis in the immunodeficient host than transfer of wt (IFN-γ$^{+/+}$) CD4 T cells. This was apparent by accelerated body weight loss (Fig. 6*A*), more severe histopathology (Fig. 6*B*) and early lethality. Expanded fractions and higher numbers of Th17 cells were detected in cLP CD4 T cell populations of adoptive RAG$^{-/-}$ hosts after transfer of CD4 T cells from IFN-γ$^{-/-}$ as compared with wt donor mice (Fig. 6*C*, Table III). Furthermore, high IL-17 serum levels were detected in diseased mice transplanted with IFN-γ$^{-/-}$ CD4 T

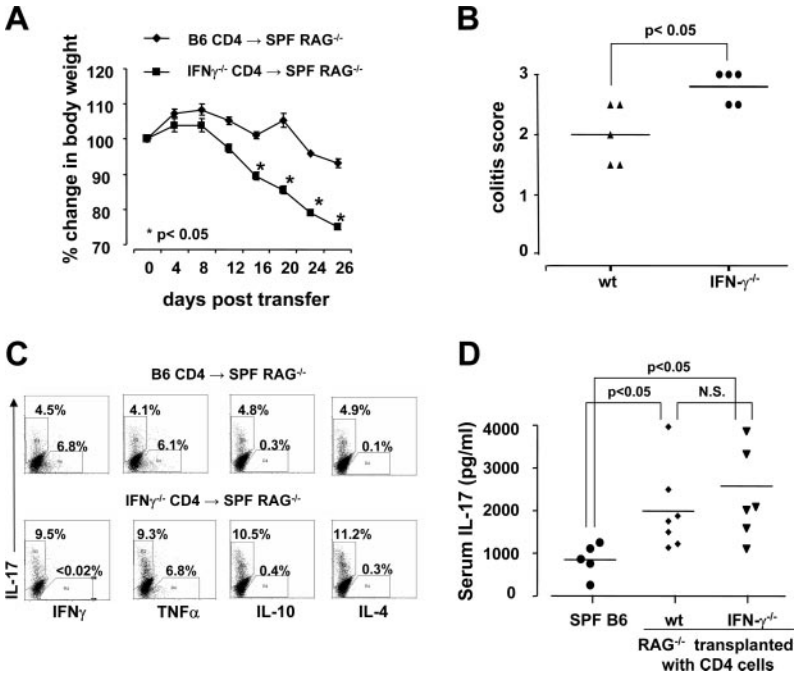

FIGURE 6. The adoptive transfer of IFN-$\gamma^{-/-}$ CD4 T cells into RAG$^{-/-}$ mice induces a severe colitis. RAG$^{-/-}$ mice were transplanted with equal numbers (3×10^5 cells/mouse) of splenic CD4 T cells derived from either IFN-$\gamma^{+/+}$ or IFN-$\gamma^{-/-}$ donor B6 mice. *A*, The body weight was monitored semiweekly. The mean percent loss of body weight (± SEM) of five mice per group for 25 days posttransfer is plotted. ∗, Significance of differences between RAG$^{-/-}$ recipients receiving B6 CD4 T cells and hosts receiving IFN-$\gamma^{-/-}$ CD4 T cells. *B*, Biopsies were taken from the upper large intestine of individual mice, and the colitis score was determined by a blinded pathologist. *C*, cLP CD4 T cells were isolated from RAG$^{-/-}$ mice transplanted with CD4 T cells from either wt (IFN-$\gamma^{+/+}$), or IFN-$\gamma^{-/-}$ donor B6 mice. These CD4 T cells were stimulated ex vivo with phorbol ester and ionomycin (in the presence of brefeldin A), surface stained for CD4, fixed, stained for intracellular IL-17A, IFN-γ, TNF-α, IL-10, and IL-4, and analyzed by flow cytometry. The double expression of two cytokines in dot blots gated on all CD4 T cells is shown. Representative data from one individual mouse per group (of five mice analyzed) are presented. *D*, IL-17A serum concentrations from wt B6 or congenic RAG$^{-/-}$ mice transplanted with CD4 T cells from either wt (IFN-$\gamma^{+/+}$) or IFN-$\gamma^{-/-}$ donor mice was determined by conventional double-sandwich ELISA. Individual values and the mean for each group are shown. A p value <0.05 in the two-tailed Student t test was considered statistically significant (NS, not significant). This experiment was repeated once with similar results.

cells (Fig. 6*D*). The adoptive transfer of IFN-γ-deficient CD4 T cells did not result in changes in the frequency or numbers of TNF-α-, IL-10-, or IL-4-producing CD4 T cells in the inflamed cLP (Fig. 6*C*, Table III). Type II IFN thus seems to attenuate local CD4 Th17 cell accumulation during colitis. These data support the notion that IL-17 contributes to the local immunopathology of transfer colitis.

Colitis induction in congenic, immunodeficient hosts requires CD4 T cells and a commensal gut microflora

The commensal gut flora is required for the induction of transfer colitis (3, 4). This was confirmed when we adoptively transferred CD4 T cells (from SPF donor mice) into SPF or GF RAG$^{-/-}$ hosts. Transfer of CD4 T cells into congenic SPF RAG$^{-/-}$ hosts resulted in the repopulation of the host with CD4 T cells, the progressive loss of body weight (Fig. 7*A*), histopathological signs of colitis (Fig. 7*B*), accumulation of Th17 cells in the inflamed cLP (Fig. 4), and the appearance of increasing serum levels of IL-17 (Fig. 7*C*). In contrast, adoptive CD4 T cell transfer into GF RAG$^{-/-}$ hosts did not lead to the repopulation of the cLP of the host with CD4 T cells, did not induce loss of body weight (Fig. 7*A*), did not induce histopathological signs of colitis (Fig. 7*B*), and did not trigger the rise in IL-17 serum levels (Fig. 7*C*). Hence, manifestation of colitis and local accumulation of cLP Th17 cells

Table III. *CD4 T cells recovered from the cLP of RAG$^{-/-}$ mice with colitis transplanted with IFN-γ-producing or IFN-γ-deficient, congenic CD4 T cells*[a]

	Recovered CD4 T Cells ($\times 10^4$ ± SEM)					
Transfers	Total[b]	IL-17^{+}[c]	IFN-γ^{+}[c]	TNF-α^{+}[c]	IL-10^{+}[c]	IL-4^{+}[c]
B6 CD4→SPF RAG-1$^{-/-}$	251 ± 107	**5.8 ± 2.9 (2.5 ± 0.7%)**	20.8 ± 11 (8.7 ± 2.9%)	17.2 ± 5.6 (7.1 ± 3.1%)	0.6 ± 0.2 (0.2 ± 0.07%)	0.7 ± 0.06 (0.3 ± 0.05%)
IFN-$\gamma^{-/-}$ CD4→ SPF RAG1$^{-/-}$	195 ± 55	**16.4 ± 6.6 (8.6 ± 2.3%)**	ND	18.4 ± 6.3 (9.4 ± 1.9%)	0.8 ± 0.3 (0.4 ± 0.07%)	0.5 ± 0.04 (0.2 ± 0.01%)

[a] Mean numbers of five individual mice (±SEM) per group are shown. Bold numbers indicate significant differences in the number and the percentage of IL-17-producing cLP CD4 T cells between the two groups as determined by the two-tailed Student t test. A p value of <0.05 was considered statistically significant. Differences between all other groups were statistically not significant. ND, not detected. The experiment was repeated twice with comparable results.

[b] Total CD4 T cells recovered from the cLP of RAG$^{-/-}$ mice transplanted with 3×10^5 B6 or IFN-$\gamma^{-/-}$ CD4 T cells.

[c] Total numbers of cLP CD4 T cells producing the indicated cytokine after a brief ex vivo stimulation that were recovered from the RAG$^{-/-}$ hosts with colitis are shown. The percentage of cells within the isolated cLP CD4 T cell population producing the indicated cytokine is shown in parentheses.

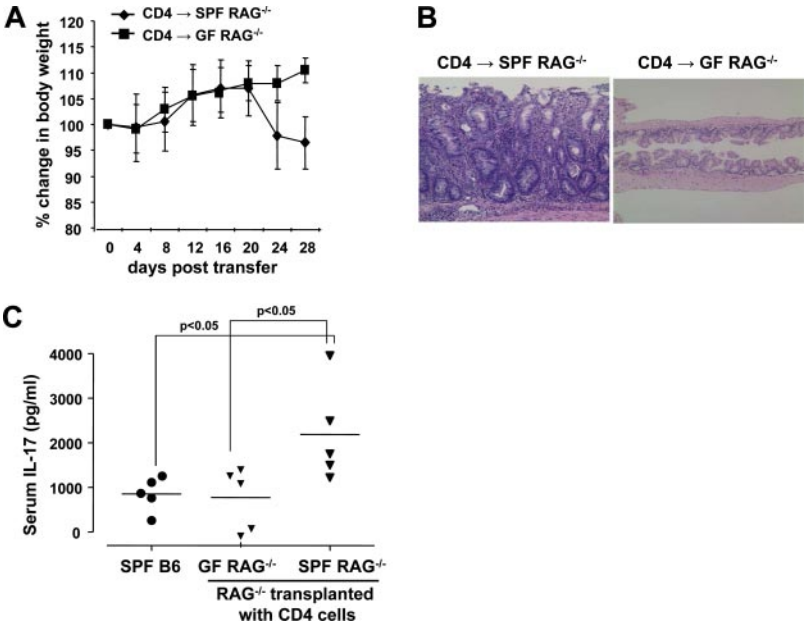

FIGURE 7. The endogenous microflora is required for local Th17 T cell accumulation in colitis. Nonfractionated CD4 T cells from B6 mice were adoptively transferred into congenic $RAG^{-/-}$ host mice reared under either SPF or GF conditions. *A*, The body weight was monitored semiweekly. The mean percent loss of body weight (±SEM) of five individual mice per group is shown. *B*, Large intestinal tissue samples were taken from all individual mice of the two groups, fixed in paraffin, and stained with H&E. Representative images from one individual mouse per group (from five individual mice per group analyzed) are shown. *C*, IL-17 serum concentrations of transplanted $RAG^{-/-}$ mice kept under either SPF or GF conditions were determined by conventional double-sandwich ELISA. The mean and the five individual values per group are shown. A p value <0.05 in the two-tailed Student t test was considered statistically significant.

was observed in diseased SPF but not healthy GF $RAG^{-/-}$ hosts. Deregulated commensal-driven, local expansion of cLP Th17 cells seems to be a key event in the pathogenesis of colitis.

Discussion

Increased numbers of CD4 Th17 cells were found in the LP of the ileum and colon (with a high load of bacteria) of SPF mice but not in the LP of the duodenum and jejunum (with a low load of bacteria). In contrast, syngeneic (age- and sex-matched) GF mice (with no bacteria in the gut) have only low numbers of CD4 Th17 cells in the intestinal LP. The commensal bacterial flora hence stimulates the local accumulation of CD4 Th17 cells in the mucosa. This may be regulated indirectly through bacteria-elicited, local cytokine effects because (type I and type II) IFNs suppress while IL-4 and IL-23 enhance the accumulation of CD4 Th17 cells in the cLP. Adoptive transfer of CD4 T cells induces colitis in congenic, immunodeficient SPF, but not GF, hosts. The CD4 T cell-induced colitis in SPF mice is associated with extensive local accumulation of CD4 Th17 cells in the inflamed cLP. This suggests that the deregulated, local, commensal bacteria-driven CD4 Th17 cell response contributes to the colitogenic potential of the mucosal immune response that drives inflammatory bowel disease.

The IL-17 family comprises six members (IL-17A-F). IL-17A is the best characterized member of this family and seems to be responsible for most of the known IL-17-mediated effects because mice with a targeted deletion of the IL-17A-encoding gene alone have a phenotype that is not compensated by intact IL-17F (34). In this study, we investigated mucosal IL-17A-producing CD4 T cells. Th17 lineage development has been expertly reviewed (13, 35–40). There is consensus that the Th17 lineage is distinct from other effector or regulatory CD4 T cell development programs (16, 41). Differentiation of CD4 Th17 cells by IL-6, IL-21, and TGF-β is amplified by IL-1β and TNF-α, and requires the costimulatory molecules CD28 or ICOS (41). Development of Th17 cells from naive precursor cells is potently inhibited by IFN-γ and IL-4 while committed Th17 cells are resistant to suppression by these Th1 or Th2 cytokines (16). Clonal expansion of Th17 cells (but not their lineage commitment) is driven by IL-23 produced by activated DCs (14, 42). IL-23-deficient mice phenotypically resemble IL-17-deficient animals (43). IL-27 limits the intensity and duration of Th17 immune responses (37). IL-27R-deficient mice generate more IL-17-producing T cells, and IL-27 directly suppresses the development of IL-17-producing T cells (37, 44, 45). Th17 cells hence represent a novel and unique lineage that is induced and regulated by distinct sets of cytokines.

In SPF mice, we found most CD4 Th17 cells in the cLP. In contrast to other reports (46), we found only few Th17 cells in MLNs and spleen. Our data confirm that the Th1 cytokine IFN-γ negatively regulates cLP Th17 cell development. The low numbers of cLP CD4 Th17 cells in SPF B6 mice deficient for IL-23 (IL-12 $p40^{-/-}$ KO mice) are expected because IL-23 supports clonal expansion of Th17 cells. Interpretation of these data is complicated by the absence of IL-12 in these animals that results in cLP Th17 cell development facilitated by IFN-γ deficiency. Little is known about the regulation of Th17 cell commitment or expansion by type I IFNs. The enhanced cLP Th17 cell numbers in SPF IFN-$\beta^{-/-}$ KO B6 mice were more striking than the enhanced cLP Th17 cell numbers in SPF IFN-$\gamma^{-/-}$ KO B6 mice. This observation is under further investigation. Interestingly, treatment of naive primary T cells with IL-27 suppresses the development Th17 cells (induced by IL-6/TGF-β) and this is dependent on STAT1 signaling (45). Potent STAT1 activation by type I IFNs may interfere with Th17 cell generation. This may underlie the effectiveness of IFN-β therapy in experimental allergic encephalitis (44, 47), an almost exclusive Th17 pathology. IFN-β treatment of IBD may hence be of interest to elucidate the colitogenic role of Th17 cells.

The IL-17RA receptor is ubiquitously expressed. Binding of T cell-derived IL-17A to its receptor triggers release of IL-6, IL-8, PGE_2, MCP-1, G-CSF, and TNF-α, e.g., by fibroblasts, keratinocytes, epithelial and endothelial cells (36, 42). IL-17 induces growth, differentiation, mobilization, recruitment, and activation of neutrophils and their progenitors. Injection of IL-17 into mice induces IL-6-dependent neutrophilia. Although neutrophil infiltrations are a hallmark of Th17 cell activation, neutrophils are not prominent in the cLP lesions of transfer colitis in which Th17 cells accumulate. Th17 cells can control extracellular bacteria (48). IL-17 plays a role particularly in chronic, T cell-mediated immune responses to microorganisms or autoantigens and is associated with tissue damage in the joints, brain, heart, lungs, and intestine. *Cryptococcus neoformans* infection of IL-23 $p19^{-/-}$ mice (with deficient IL-17 production) shows reduced survival and delayed fungal clearance in the liver (with fewer hepatic granulomata)

pointing to a role of IL-17 in antifungal immunity. IL-17-producing T cell populations significantly contribute to the severe immunopathology in schistosomiasis (49). The synovial fluid of *Borrelia burgdorferi*-infected patients with Lyme arthritis contain many Th17 cells, and *Borrelia*-derived products induce Th17 cells in vitro (50). In transfer colitis, the cellular immune response to commensal bacteria apparently triggered an increasingly severe, local immunopathology that was correlated with the increasing local accumulation of Th17 cells. These recently emerging data from murine models and chronic clinical conditions point to the Th17 subset as a key mediator in chronic inflammation stimulated by microbial and/or self Ags.

Th17 cells are constitutively present in different compartments of the intestine. The key transcription factor required to induce the Th17 lineage is the orphan nuclear receptor RORγt (51). RORγt is expressed by intestinal intraepithelial $\alpha\beta$ T cells (52). We found high numbers of Th17 in the LP of the murine gut. The local abundance of LP Th17 cells correlated with the bacterial load of the gut segment being highest in the cLP. Commensal bacteria may locally stimulate generation, recruitment, and/or expansion of these T cells. Th17 cells may preferentially stay and/or expand in the gut because only low numbers of Th17 cells were found in MLN, spleen, or liver. IL-23 (but not IL-12) was required for the experimental induction of chronic intestinal inflammation stimulated by CD40 ligation; depletion of IL-23 was associated with decreased proinflammatory responses in the intestine (53). In the murine *Helicobacter hepaticus*-triggered, T cell-dependent colitis model, IL-23 (but not IL-12) is essential for the development of intestinal disease (54). *Helicobacter*-infected patients show extensive Th17 cell infiltrations in the gastric LP while *Helicobacter* eradication is associated with a marked down-regulation of local IL-17 expression (55). The colitis developing in IL-10-deficient mice is IL-23 dependent and mediated by Th17 cells (17). A genome-wide association study revealed a highly significant association between Crohn's disease and the *IL-23R* gene (56). Although IL-23 stimulates the expansion and differentiation of Th17 cells, it may well trigger IL-17-independent effector functions in chronic local inflammation (57). Ab-mediated neutralization of IL-17 enhances the mucosal expression of TNF-α, IFN-γ, IL-6, RANTES, and IFN-γ-inducible protein-10 and aggravates the course of dextran sulfate-induced colitis pointing to an inhibitory or protective role of IL-17 in the development of this experimental colitis (58). IL-17 enhances TNF-α-induced IL-6 and IL-8 secretion but inhibits TNF-α-induced RANTES secretion by human colonic subepithelial myofibroblasts (59). IL-17 thus modulates the proinflammatory effects of TNF-α that play a key role in IBD. It is unlikely that a single cytokine regulates a vital process like tissue damage. It will be a constellation of cytokines, tuned in concert, that ultimately produces a complex phenotype like "tissue damage" or "recovery from tissue damage" (39). Although we found a striking local commensal-dependent accumulation of Th17 in the inflamed cLP in the transfer colitis model, many T cells that produced TNF-α and/or IFN-γ were also present in the lesions. It may be the sequential or simultaneous interplay between immune cells of different lineages that will ultimately be most informative on the disease process and reveal novel targets for therapeutic intervention.

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Disclosures

The authors have no financial conflict of interest.

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