CD4+ T Cells and *Lactobacillus casei* Control Relapsing Colitis Mediated by CD8+ T Cells

Feriel Hacini-Rachinel, Stephane Nancey, Gilles Boschetti, Fatima Sardi, Remi Doucet-Ladevèze, Pierre-Yves Durand, Bernard Flourié and Dominique Kaiserlian

*J Immunol* 2009; 183:5477-5486; doi: 10.4049/jimmunol.0804267

http://www.jimmunol.org/content/183/9/5477

---

**References**  This article **cites 42 articles**, 16 of which you can access for free at: http://www.jimmunol.org/content/183/9/5477.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD4⁺ T Cells and Lactobacillus casei Control Relapsing Colitis Mediated by CD8⁺ T Cells

Feriel Hacini-Rachinel,²§† Stephane Nancey,²§† Gilles Boschetti,⁎†‡ Fatima Sardi,⁎† Remi Doucet-Ladevèze,⁎† Pierre-Yves Durand,⁎† Bernard Flourie,⁎†‡ and Dominique Kaiserlian⁎†§

Evidence that CD4⁺ regulatory T cells can control Ag-specific CD8⁺ T cell-mediated colitis in immunocompetent mice is poorly documented. To examine the potential of CD4⁺ T cells to control colitis, we used our model of CD8⁺ T cell-mediated colitis induced by intracolonic sensitization followed by challenge with the hapten 2,4-dinitrobenzene sulfonic acid. The defect of CD4⁺ T cells in MHC class II-deficient (A/J⁻/⁻) mice allowed priming of 2,4-dinitrobenzene sulfonic acid-specific IFN-γ-producing CD8⁺ colitogenic effectors and development of colitis in the otherwise resistant C57BL/6 strain. Cotransfer experiments in RAG²⁻/⁻ mice and in vivo studies showed that CD4⁺ CD25⁺ T cells completely prevented CD8⁺ T cell-mediated colitis and controlled CD8⁺ T cell activation, respectively. In the susceptible BALB/c strain, Ab depletion revealed that lack of CD4⁺ regulatory T cells resulted in 1) acute colitis elicited by a suboptimal dose of hapten challenge and 2) more severe relapsing episodes of colitis induced by effector/memory CD8⁺ T cell-mediated colitis at an optimal dose of hapten challenge, even when CD4 depletion was performed just before the second challenge. Oral administration of the probiotic strain Lactobacillus casei DN-114 001 alleviated colitis and increased the suppressive function of Foxp3⁺ CD4⁺ regulatory T cells of colon lamina propria. These data demonstrate that CD4⁺ regulatory T cells exert a protective effect on colitis by controlling colitogenic effector/memory CD8⁺ T cells during the effector (symptomatic) phase of acute and relapsing colitis, respectively. Probiotics with natural adjuvant effects on mucosal regulatory T cells may represent a valuable approach to alleviate the colitogenic effect of Tc1-type CD8⁺ effectors. The Journal of Immunology, 2009, 183: 5477–5486.

Inflammatory bowel diseases (IBDs) are idiopathic chronic and relapsing inflammatory disorders of the gastrointestinal tract characterized by dysfunction of mucosal immune responses, abnormal cytokine production, and cellular inflammation, ultimately leading to mucosal damage (1–3). Circumstantial evidence supports a central role for dysregulated Th1 effector T cell responses to the normal enteric bacterial flora as a common disease mechanism. CD4⁺ T cells contain a naturally occurring subset of regulatory T cells (nTreg) with suppressive functions that control the outcome of various autoimmune and inflammatory disorders. Foxp3, a transcription factor of the fork-head-winged helix family, plays a crucial role in the development and suppressive function of nTreg (4, 5). That spontaneous onset of colitis occurs in rodents with deletion of gene coding for IL-2, IL-2R, IL-10, TGF-β, and Foxp3, which contribute to the differentiation, survival, or function of nTreg (reviewed in Ref. 6), supports the hypothesis that nTreg may play a key role in the control of IBD. This has been extensively documented in the CD4⁺ T cell transfer model of colitis in immunodeficient mice, essentially because colitis is believed to be a unique property of Th1 (7)- or Th2 (8)-type CD4⁺ T cells. Indeed, CD4⁺ CD25⁺ Foxp3⁺ nTreg prevented colitis by controlling colitogenic Th1-type CD4⁺ effectors (9–11). However, the inefficacy of anti-CD4 mAb therapy in patients with IBD (12) may be explained by the colitogenic function of T cells being distinct from CD4⁺ T cells and/or elimination of regulatory CD4⁺ T cells. The unanticipated role of CD8⁺ T cells in IBD (13, 14) and autoimmune enteropathy (15–17) is now emerging from studies using normal or TCR-transgenic immunocompetent mice as well as studies using SAMP1/Yit mutant or TNF⁻/⁻ mice (18, 19). These studies revealed that intestinal inflammation can be initiated by MHC class I-restricted CD8⁺ T cells in response to self or exogenous Ags present in the intestinal mucosa. In particular, we documented in BALB/c mice that acute colitis and its relapsing episodes result from an Ag-specific colonic delayed-type hypersensitivity (DTH) reaction that is initiated by CD8⁺ T cells without need for CD4⁺ T cell help (13). In this model, 2,4-dinitrobenzene sulfonic acid (DNBS)-specific IFN-γ-producing CD8⁺ T cells were generated in BALB/c mice by intracolonic DNBS immunization. Colitis developed within 24/48 h after intrarectal challenge with DNBS and was mediated by granzyme B⁺ CD8⁺ T cells recruited into the colon lamina propria (LP), which were cytolytic against hapten-modified epithelial cells. In this model, CD8⁺ T cells were the only effectors able to transfer colitis and CD4⁺ T cells were neither required for their generation nor for the development of intestinal inflammation. This model thus offers the...
unique opportunity to investigate the contribution of CD4+ T cells in the control of the outcome and severity of colitis mediated by CD8 effectors in immunocompetent mice.

In the present study, we demonstrate that CD4+ T cells account for the resistance of C57BL/6 mice to colitis and exert a protective effect on relapsing colitis in the susceptible BALB/c strain. We also document that preventive oral treatment with the probiotic bacterial strain Lactobacillus casei DN-114 001, which potentiates the suppressive function of Foxp3+ Treg in the colonic mucosa, also exerts a protective effect on colitis.

Materials and Methods

Mice
Male BALB/c and C57BL/6 mice were purchased from Charles River Laboratories and were used at 6–10 wk of age. MHC class-II deficient (A-Ki1/h-1) (a gift from D. Mathis and C. Benoist, Boston, MA (20)), Foxp3-IRES-eGFP knock-in (KI) mice (21) (provided by B. Malissen, CIML, Marseille, France), and RAG2-deficient mice (all on a C57BL/6 background) were bred in our animal facility (Plateau de Biologie Expérimentale de la Souris). All mice were fed with standard mice chow pellets ad libitum. All experimental procedures were previously approved by the Animal Care and Use Committee according to governmental guidelines.

DNBS-specific colonic DTH-mediated colitis
Mice were anesthetized using ketamine-xylazine and DNBS-specific colonic DTH was induced as previously described (13). Briefly, mice immunized by an intrarectal enema of 80 mg/kg of DNBS diluted in 50% ethanol (v/v) were challenged 5 days later with 40 mg/kg DNBS (unless otherwise stated). Mice were kept in an upside-down position for 30 s after each colon instillation.

Assessment and scoring of colitis
Mice were examined daily with respect to their general condition, body weight, and condition of stools. Forty-eight to 72 h after elicitation of DTH, macroscopic colonic lesions were analyzed in a double-blind fashion using the Wallace score taking into account hyperemia, thickening of the bowel wall, extent of colonic inflammation, and presence of mucosal ulcerations (22). Microscopic damage was assessed in a double-blind fashion on a segment of 1 cm of colon located 3 cm above the anal canal after fixation in 4% paraformaldehyde and H&E staining of paraffin-embedded sections (4 µm). Histological grading of colonic lesions, including thickness of the bowel wall, extent and severity of the inflammatory cell infiltration, and gland distortion was performed by using the Ameho score (23).

Purification of T cell subsets
Spleens and lymph nodes (mesenteric, inguinal, and axillary) were used to prepare single-cell suspensions. CD8+ T cells were isolated by MACS-positive selection using anti-CD8 mAb-coated microbeads (Miltenyi Biotechnology). CD4+ T cells were enriched by negative selection using a mixture of mAbs including anti-class II clone, anti-CD8, anti-CD19, anti-CD11b, and biotin-conjugated anti-CD25 mAb (7D4) and LS selection columns. Purity as assessed by flow cytometry was routinely >95%.

In vivo adoptive cell transfer experiments
Naive RAG2−/− recipient mice were injected i.v. with either 1 × 107 CD8+ T cells alone or along with 3 × 106 CD4+CD25+ T cells from pooled spleen and peripheral lymph nodes from naive C57BL/6 mice. One day after cell transfer, all mice were sensitized and challenged intra结itally 5 days apart with DNBS in 50% ethanol.

In vivo treatment with anti-CD4 and anti-CD8 mAb treatments
Mice were injected i.p. on days −1, 0, +1, and +3 (with respect to day 0 of DNBS immunization) with the anti-CD4 mAb (clone GK1.5) or with the anti-CD8 mAb or a control rat IgG mAb. In some experiments (relapsing colitis), anti-CD4 mAb was injected on days −1, 0, +1, and +3 and then every 3 days until mice were sacrificed. In some experiments, mice were injected with anti-CD4 mAb on days −4, −2, and −1 before the second challenge (day 0). Efficacy of CD4+ or CD8+ T cell depletion in peripheral blood, spleen, mesenteric lymph nodes (MLN), and colon LP was routinely >85–90%, as assessed by flow cytometry. CD8 mAb (clone H35.17.2) was provided by G. Milon (Institut Pasteur, Paris, France).

Probiotic treatment
L. casei DN-114 001 were prepared at a stationary phase of growth as previously described (24). Mice (five to seven per group) were fed daily by gavage with 200 μl of 106 CFU/ml L. casei DN 114 001 or 0.9% NaCl (control) from day 14 before colonic DNBS sensitization until the end of the experiments.

Proliferation assay
T cell proliferation assays were performed as previously described (25). Briefly, CD8+ T cells were purified from MLN and spleens of day 5 DNBS-sensitized mice by positive magnetic sorting using anti-CD8-coated microbeads and selection columns (Miltenyi Biotec). CD8 purity was routinely >95%. CD8+ T cells (2 × 106 cells/well) were cocultured at 37°C with 5 × 105 irradiated syngeneic spleen cells pulsed with 0.4 mM DNBS. Proliferation was assessed on day 3 of culture by [3H]thymidine incorporation during the last 12 h of culture. Radioactivity was counted using a TopCount NXT beta plate liquid scintillation counter (Packard Instrument).

IFN-γ ELISA
IFN-γ secretion in day 3 supernatant was titrated by ELISA, as described previously (25), using the anti-mouse IFN-γ mAb (R46A2; BD Pharmingen) as capture Ab and biotinylated anti-mouse IFN-γ mAb (XGM1.2; BD Pharmingen) for detection. The reaction was developed using o-phenylendiamine substrate and H2O2 and stopped with 12.5% H2SO4. OD at 490 and 650 nm was determined using an ELISA reader. The results are expressed as pg/ml IFN-γ with reference to recombinant mouse IFN-γ (BD Pharmingen) as standard.

IFN-γ ELISPOT
The frequency of hapten-specific IFN-γ-producing T cells was determined as described elsewhere (13). The number of IFN-γ spot-forming cells (SFC) was counted and the results are expressed as the number of IFN-γ SFC per 106 cells.

Isolation of colon LP leukocytes
Colon fragments were incubated with 5 mM EDTA in PBS to remove epithelial cells and LP leukocytes were extracted from the remaining tissue by a 45-min incubation at 37°C in 35 µg/ml Liberase (Roche Diagnostics) and 10 U/ml DNase (Roche Diagnostics) as previously described (13). Leukocytes were enriched by centrifugation over 40% Percoll (GE Healthcare). The resulted cell suspension contained >90% viable cells.

Flow cytometry analysis
Cells were incubated for 15 min at 4°C with the Fc-specific mAb 2.4G2 and then stained using the following mAbs, all from BD Pharmingen: allophycocyanin Anti-CD45 (clone 30-F11), PerCP-Cy5.5-anti-CD4 (clone RM4-5), FITC-anti-CD25 (clone 7D4), or with relevant isotype-matched conjugates. Rat anti-mouse Foxp3 (clone FJK-16s) staining was performed according to the manufacturer’s instructions (eBioscience). Staining was analyzed using a FACSCanto and CellQuest software (BD Biosciences).

Regulatory T cell (Treg) suppression assay
CD4+CD25+ Treg were purified by depletion of non-CD4+ T cells. Then CD25+ cells were positively selected from the enriched CD4+ T cell fraction using anti-CD25 mAb-coated microbeads and magnetic columns (Miltenyi Biotec). Purity was routinely >80%. CD8+ T cells from day 5 hapten-skin-sensitized mice were stimulated by culture with irradiated lymph node CD11c+ cells as APC, previously purified using a mouse CD11c kit (Miltenyi Biotec), at a ratio of 1 APC/10 CD8+ T cells in the presence of plate-bound anti-mouse CD3 mAb (BD Pharmingen). CD4+CD25+ Treg purified from MLN of day 5 DNBS-sensitized B6 mice were added to the culture at 1:1–1:81 ratios of Treg/CD8+ T cells. In other experiments, CD4+CD25+eGFP (nTreg) and CD4+CD25+ eGFP (conventional T cells (Tcon)) were purified from MLN of Foxp3-IRESGFP transgenic mice by FACS sorting (FACSAria; BD Biosciences) after staining with PerCP-Cy5.5-anti-CD4 (clone RM4-5) and PE-anti-CD25 (clone 7D4). CD4+CD25+ eGFP Treg were FACs sorted from colon LP cells stained with allophycocyanin-anti-CD45 (clone 30-F11). Treg purity was routinely >98%. Tconv were stimulated with culture with irradiated lymph node CD11c+ cells as APC at a ratio of 1 APC/10 Tconv in the presence of soluble anti-mouse CD3 mAb. mTreg were added to the culture at 1:1–1:32 ratios of Treg/Tconv. Proliferation (cpm) was determined on day 5 of the culture by [3H]thymidine (μCi) incorporation over the last 12 h of culture.

Downloaded from http://www.jimmunol.org/ on April 12, 2017
Statistics

The nonparametric Mann-Whitney U test (for two experimental groups) and the one-way ANOVA (for three or more experimental groups) were used to compare the experimental groups. The Bonferroni multiple comparison test was used to generate p values for selected pairwise comparisons, and p < 0.05 were considered significant. GraphPad Prism 4.0 software was used for statistical analysis.

Results

CD4+ T cells protect C57BL/6 mice from CD8+ T cell-mediated colitis

We previously observed that unlike BALB/c mice, C57BL/6 mice did not develop colitis upon colonic sensitization and challenge 5 days apart with DNBS, even at high doses of hapten allowing priming of hapten-specific IFN-γ-producing CD8+ T cells (our unpublished data). To determine whether CD4+ T cells contributed to protection from colitis in C57BL/6 (B6) mice, we compared the outcome and severity of colitis in BALB/c and syngeneic MHC class II-deficient mice (Aβ°/°), which are deficient in class II-restricted CD4+ T cells. In contrast to wild-type B6 mice, Aβ°/° mice sensitized and challenged with 80 and 40 mg/kg DNBS, respectively, developed obvious clinical signs of colitis within 48 h, manifested by body weight loss (Fig. 1A) and colon inflammation (Fig. 1, B and C). Aβ°/° and BALB/c mice exhibited comparable body weight loss and histological (Ameho) score of colitis. Histological colonic lesions were characterized by edema, dense transmural lymphocytic inflammatory infiltrate, and mucosal erosions with a median Ameho score of colitis of 3 (n = 12), 4 (n = 11) in BALB/c and Aβ°/° mice, respectively, compared with 0 (n = 12) in B6 mice (Fig. 1C). Analysis of the hapten-specific T cell response by IFN-γ ELISPOT assay on day 5 after colonic sensitization revealed that, while no hapten-specific T cells were primed in wild-type B6 mice, DNBS-sensitized Aβ°/° mice exhibited a high frequency of IFN-γ-producing DNBS-specific CD8+ T cells in colon-draining lymph nodes and at a lower level in the spleen that were severalfold higher than those in BALB/c mice (Fig. 1D). Thus, the defect of CD4+ T cells in Aβ°/° mice allowed for in vivo priming and differentiation of CD8 effectors mediating colitis of similar clinical severity than that in the susceptible BALB/c mice.

CD4+CD25+ Treg protect from CD8+ T cell-mediated colitis

To further demonstrate that Treg control DNBS-induced colitis, we examined whether CD4+CD25+ T cell transfer could prevent colitis induced in RAG2°/° mice reconstituted with naive CD8+ T cells. We thus set up an adoptive cotransfer model of CD4+CD25+ Treg and CD8+ T cells into RAG2°/° mice. MACS-sorted CD4+CD25+ T cells harvested from naive C57BL/6 donor mice were cotransferred with CD8+ T cells from naive C57BL/6 into syngeneic RAG2°/° recipients 1 day before colonic sensitization. The colitis response to DNBS was examined after colonic challenge with the hapten (Fig. 2A). Whereas no evidence of colitis was observed in untransferred RAG2°/° mice, CD8+ T cells alone transferred in DNBS-sensitized recipient mice induced at 24–48 h after DNBS challenge a severe colitis, which was manifested by body weight loss (Fig. 2B), macroscopic signs of colitis (Fig. 2C), and histopathological lesions of the colon (Fig. 2, D and E). In contrast, cotransfer of CD4+CD25+ T cells protected mice from CD8+ T cell-mediated colitis, as demonstrated by prevention of body weight loss, normal macroscopic appearance of the colon, and dramatic reduction of the inflammatory cell infiltrate in mucosa (Fig. 2, B–E).

FIGURE 1. CD4+ T cells prevent CD8+ T cell-mediated colitis in C57BL/6 mice. C57BL/6 ( ), Aβ°/° (C57BL/6 background, ), and BALB/c ( ) mice (five to seven mice per group) were sensitized with 80 mg/kg DNBS on day 0 and challenged on day 5 with 40 mg/kg DNBS. A, Colitis was assessed by body weight change. Box-and-whisker plot represents median, quartiles, and the smallest and greatest values of two experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. *, p < 0.05; nonsignificant (n.s.), p > 0.05. B, Histological H&E staining of distal colon sections at 72 h after challenge. C, Histological (Ameho) score of colitis. Statistical analysis was performed on day 5 after sensitization by ELISPOT assay. The data represent the mean value ± SD of IFN-γ SFC per 106 cells in individual mice. Data are representative of two experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. ***, p < 0.001 and ns, p < 0.05.

CD4+CD25+ T cells inhibit CD8+ effector T cell response in vitro

We next examined whether CD4+CD25+ T cells from colon-sensitized B6 mice could directly inhibit CD8+ effector T cell responses. Given the lack of DNP-specific CD8 T cells in B6 mice after intrarectal DNBS immunization (Fig. 2F), CD8+ T cells were purified from spleen and draining lymph nodes from skin-painted
**FIGURE 2.** CD4⁺CD25⁺ T cells alleviate CD8⁺ T cell-mediated colitis in RAG2<sup>−/−</sup> recipient mice. **A**, RAG2<sup>−/−</sup> mice (three per group) were either untransferred or transferred i.v. with naive CD8⁺ T cells (10⁶) alone or along with naive CD4⁺CD25⁺ T cells (3 × 10⁶) from C57BL/6 mice. All recipient mice were sensitized and challenged by an intrarectal administration of DNBS. **B**, Body weight change was recorded at various time after colonic challenge in recipient mice either untransferred (○), transferred with CD8⁺ T cells alone (■), or transferred with CD8⁺ plus CD4⁺CD25⁺ T cells (□). **C**, Macroscopic (Wallace) scores of colitis at 48 h after challenge. Results represent values for individual mice and the median of two experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. **D**, Histological H&E staining of distal colon sections at 48 h after challenge. **E**, Histological (Ameho) scores of colitis. Results represent values for individual mice and the median are obtained from two experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest. **F**, The ability of CD4⁺CD25⁺ Treg to control CD8⁺ effector T cells was assessed using an in vitro suppressive assay. Day 6 hapten-sensitized CD8 T cells (10⁵) were cultured alone (□) or along with graded numbers of CD4⁺CD25⁺ T cells (■) from MLN of day 5 intrarectally sensitized B6 mice in the presence of CD11c⁺ cells (10⁴) as APC and immobilized anti-CD3 mAb. Proliferation was determined after 3 days by [³H]thymidine uptake. Background proliferation in culture of Treg with APC and anti-CD3 mAb was < 2 × 10³ cpm. Positive control cultures of CD8⁺ T cells with APC and Con A (5 μg/ml) was 120 × 10³ ± 2 × 10³ cpm. Results from two pooled experiments are expressed as mean ± SD of triplicate wells. Statistical analysis was performed using one-way ANOVA with Bonferroni posttest by comparing CD8⁺ T cells cultured with CD4⁺CD25⁺ T cells to CD8⁺ T cells cultured alone. ***, p < 0.001 and *p < 0.05.**
B6 mice as a source of DNP-specific CD8 effectors (26, 27). CD8<sup>+</sup> effector T cells were restimulated in vitro with anti-CD3 mAb in the presence of graded numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells from MLN isolated from day 6 DNBS colon-sensitized B6 mice. As expected, CD8<sup>+</sup> T cells, but not CD4<sup>+</sup>CD25<sup>+</sup> T cells, proliferated in response to in vitro stimulation with anti-CD3 mAb in response to in vitro stimulation with anti-CD3 mAb (data not shown). As shown in Fig. 2F, CD4<sup>+</sup>CD25<sup>+</sup> T cells from hapten-sensitized mice suppressed CD8<sup>+</sup> T cell proliferation (Fig. 2F) and IFN-γ production (data not shown) in a dose-dependent manner with >70% inhibition when used at a 1:1 ratio with effector CD8<sup>+</sup> T cells. Thus, CD4<sup>+</sup>CD25<sup>+</sup> cells from MLN of colon DNBS-sensitized mice can control CD8<sup>+</sup> effector T cell proliferation.

**CD4<sup>+</sup> T cells prevent CD8<sup>+</sup> T cell-mediated colitis at a suboptimal dose of Ag challenge in susceptible BALB/c mice**

In susceptible BALB/c mice, development of a DNBS-specific colonic DTH response in intrarectally immunized mice depended on the dose of hapten used for challenge, i.e., colitis developed after challenge with 40 mg/kg but not 13 mg/kg DNBS (data not shown). We thus tested the outcome of CD4<sup>+</sup> T cell depletion on colitis induced by challenge with the infra-optimal dose of 13 mg/kg DNBS in previously sensitized BALB/c mice. Efficacy of CD4 depletion in MLN was >95% as illustrated in Fig. 3A. As expected, control IgG-injected DNBS-sensitized mice developed a hapten-specific CD8<sup>+</sup> T cell response with cell proliferation (Fig. 3B) and IFN-γ production (Fig. 3C) in MLN and spleen, but did not develop colitis upon challenge with the suboptimal dose of 13 mg/kg DNBS (Fig. 3, D–F). In contrast, anti-CD4<sup>+</sup> mAb treatment resulted in enhanced priming of specific CD8<sup>+</sup> effector T cells (Fig. 3, B and C) and induction of colitis with significant body weight loss (Fig. 3D) and severe colon inflammation at 48 h after challenge, as revealed by the Ameho score (Fig. 3, E and F). Injection of anti-CD25 mAb to deplete Treg before sensitization resulted in wasting disease with weight loss upon challenge and death of mice either before or after the challenge (data not shown).

**CD4<sup>+</sup> T cells control the severity of colitis relapse in BALB/c mice**

We previously documented that mice recover from acute DNBS colitis and undergo a relapse upon a second intrarectal challenge performed after a 30-day remission period (13). We thus tested the effect of anti-CD4 mAb treatment conducted throughout the experimental protocol on acute and relapsing phases of colitis. This regimen led to depletion of >95% of CD4<sup>+</sup> T cells in MLN and spleen as assessed by flow cytometric analysis (Fig. 3A). As expected, control IgG mAb-injected DNBS-sensitized mice developed an acute and relapsing colitis after challenge with the optimal dose of 40 mg/kg DNBS on days 5 and 35 after DNBS sensitization, respectively (Fig. 4). After the first challenge, CD4-depleted and undepleted mice exhibited similar kinetics and severity of acute colitis as shown by body weight loss (Fig. 4A, left panel).

Despite the modest increase in intestinal inflammation detected in some anti-CD4 mAb-treated mice (Fig. 4B), the Ameho score was not significantly increased (Fig. 4C, left panel). Alternatively, after a rest of 30 days, a more severe relapse of colitis that appeared as early as 24 h after the second challenge and persisted for at least 3 days was observed in anti-CD4 mAb-treated mice (Fig. 4A, right panel). This was revealed by the more dramatic body weight loss at 48 h after DNBS challenge (5.7 ± 1.8% in anti-CD4-treated vs 3.6 ± 1.2% in control IgG-treated mice, p < 0.05; Fig. 4A, right panel). Histological examination also showed more severe colonic lesions in CD4<sup>+</sup> T cell-depleted mice with erosions, massive infiltration of colonic mucosa by inflammatory cells and wasting disease with weight loss upon challenge and death of mice either before or after the challenge (data not shown).

**FIGURE 3.** CD4 depletion increases the susceptibility of BALB/c mice to colitis. Mice (five to seven per group) injected with anti-CD4 or isotype control IgG mAbs were sensitized intrarectally with DNBS (A). In some experiments (B and C), CD8<sup>+</sup> T cells from spleen and MLN of day 5 DNBS-sensitized mice were restimulated in vitro with or without DNBS-pulsed or -unpulsed APC. In other experiments (D–F), colitis was induced by intrarectal challenge of day 5 sensitized mice with 13 mg/kg DNBS. A, Dot plot FACS analysis of CD4<sup>+</sup> T cells in MLN from control IgG or anti-CD4 mAb-injected mice. B, CD8<sup>+</sup> T cells proliferation on day 3 of culture. Results are expressed as cpm (mean ± SD) obtained from two independent experiments. Statistical analysis was performed using twotailed Mann-Whitney U test between anti-CD4 mAb-treated (■) and control IgG-treated (□) mice. * p < 0.05. C, IFN-γ production in day 3 culture supernatants. Results are expressed as pg/ml (mean ± SD) obtained from two independent experiments. Statistical analysis was performed using two-tailed Mann-Whitney U test between anti-CD4 mAb-treated and control IgG-treated mice. * p < 0.05. D, Colitis was assessed by body weight change. Results represent mean ± SD of two experiments. Statistical analysis was performed using a two-tailed Mann-Whitney U test between anti-CD4 mAb-treated (●) and control IgG-treated (○) groups. * p < 0.05. E, Histological (Ameho) scores of colitis. Results represent median, quartiles, and the smallest and greatest values. Data are representative of two experiments. Statistical analysis was performed using two-tailed Mann-Whitney U test between anti-CD4 mAb-treated (●) and control IgG-treated (○) groups. * p < 0.05. F, H&E staining of distal colon were evaluated at 72 h after challenge.
To further determine whether CD4+ T cells could control the symptomatic phase of the colitis in previously sensitized mice, we tested the outcome of injecting anti-CD4 or anti-CD8 mAb just before the second intrarectal challenge (Fig. 5). Efficacy of depletion of CD4+ and CD8+ T cells in spleen, MLN (data not shown), and colon LP (Fig. 5A) was >85%. Whereas CD8 depletion performed just before the second challenge completely abrogated the relapse (Fig. 5, B–E, gray boxes), CD4-depleted mice (hatched boxes) exhibited more severe colitis as shown by body weight loss, macroscopic, and histological scores (Fig. 5, B–E) than undepleted mice (Fig. 5, B–E, white boxes).

Taken together, these data demonstrate that CD4+ T cells control the severity of colitis relapse induced by colonic reexposure to the hapten in mice that recovered from acute colitis, supporting the hypothesis that CD4+ T cells control the function of pathogenic effector/memory CD8+ T cells responsible for colitis.

**Oral L. casei treatment alleviates colitis**

We previously documented that fermented milk, including the probiotic *L. casei* strain DN-114 001, could significantly reduce the
respectively (L. casei enhances the suppressive function of Foxp3

L. casei susceptible BALB/c strain. /H11005

H11005

colitis, with a mean body weight loss at 48 h after challenge of

before sensitization until sacrifice greatly reduced the severity of

protocol could alleviate acute DNBS-specific CD8 colitis in

sections in NaCl-treated and L. casei-treated mice at 48 h after challenge.

Statistical analysis was performed using a two-tailed Mann-Whitney

represent values for individual mice and the median of two experiments.

C, Histological (Ameho) score of colitis at 48 h after challenge. Results

represent values for individual mice and the median of two experiments.

Statistical analysis was performed using a two-tailed Mann-Whitney U test

between L. casei-treated and control NaCl-treated group. *, p < 0.05. B, Histological staining of colon sections in NaCl-treated and L. casei-treated mice at 48 h after challenge.

severity of CD8 T cell-mediated skin contact hypersensitivity to

2,4-dinitro-1-fluorobenzene via a mechanism that required CD4+ T cells (24). We thus asked whether the same L. casei treatment protocol could alleviate acute DNBS-specific CD8 colitis in BALB/c mice. L. casei treatment performed daily from day 14 before sensitization until sacrifice greatly reduced the severity of colitis, with a mean body weight loss at 48 h after challenge of

2.6 ± 3.2% compared with 11.5 ± 5.6% for NaCl-treated mice

( p < 0.05; Fig. 6A) and a median Ameho score of colitis of 2

(n = 10) and 3 (n = 8) in L. casei-treated and NaCl-treated mice, respectively ( p < 0.05; Fig. 6, B and C). These data show that oral administration of live L. casei can alleviate acute colitis in the susceptible BALB/c strain.

L. casei enhances the suppressive function of Foxp3+ Treg of colon LP

To examine the outcome of L. casei treatment on mucosal Treg, phenotypic and functional analysis of Foxp3+ Treg was conducted using Foxp3-eGFP KI mice. In naive untreated mice MLN, eGFP+ (Foxp3+) T reg represented 10% of MLN CD4+ T cells and ~70% of them expressed CD25 (Fig. 7A, upper panel). Likewise, ~12% of CD4+ T cells in the colon LP expressed Foxp3, among which 50% were CD25 positive (Fig. 7A, lower panel). Functional analysis was then conducted using FACS-sorted CD4+CD25+ eGFP+ Tconv from MLN and colon LP. Treg from MLN displayed a dose-dependent suppression of conventional MLN CD4+CD25+ eGFP+ T cell (Tconv) proliferation in response to anti-CD3 stimulation that was still detected at a ratio of 1:1 LP Treg:Tconv (Fig. 7B). Colon LP Treg exhibited a weaker suppressive activity compared with Treg from MLN, since a ratio of 1:4 LP Treg:Tconv was necessary to inhibit T cell proliferation and no inhibition was induced at a ratio of 1:1 Treg:T conv (Fig. 7B). This indicated that resident Treg are present in the colonic LP and have a weaker suppressive property compared with MLN Treg.
Interestingly, after 14 days of oral treatment with live *L. casei*, Foxp3-eGFP KI mice displayed a weak but significant increase in CD4⁺ eGFP⁺ T cells in the colon LP that affected both the CD25⁺ and CD25⁻ subsets (Fig. 8A), without detectable changes in MLN or spleen (data not shown). Functional studies were conducted to test whether the probiotic treatment affected the suppressive function of these mucosal Treg and/or the sensitivity of Tconv to the suppressive effect of Treg. CD4⁺ CD25⁺ eGFP⁺ Tconv isolated from MLN of either NaCl-treated control or *L. casei*-treated Foxp3-eGFP KI mice were equally sensitive to the suppressive effect of CD4⁺ CD25⁺ eGFP⁺ Treg from MLN (data not shown). Likewise, *L. casei* treatment did not affect the suppressive efficacy of MLN Treg (Fig. 8B). Alternatively, *L. casei* enhanced the suppressive function of CD4⁺ CD25⁺ eGFP⁺ cells from colon LP as shown by the 50% inhibition of Tconv proliferation at a ratio of 1 Treg:4 Tconv (Fig. 8C). Thus, oral treatment with *L. casei* induces the expansion and/or differentiation of functional Treg in colon LP.

**Discussion**

This study demonstrates, using our model of relapsing colitis mediated by DNBS-specific CD8⁺ T cells (13), that CD4⁺ T cells negatively control the outcome and severity of colitis in immunocompetent mice. These data extend our previous findings by showing that in this model: 1) CD4⁺ T cell help is required neither for the priming nor for the colitogenic function of effector/memory CD8⁺ T cells; 2) negative control of CD8⁺ T cells by CD4⁺ T cells decreases the severity of colitis relapses, prevents development of colitis at a suboptimal challenge dose in susceptible BALB/c mice, and accounts for lack of colitis in the refractory C57BL/6 mouse strain; and 3) oral *L. casei* treatment alleviates colitis and is able to enhance the function of mucosal regulatory Foxp3⁺ CD4⁺ T cells in colon LP.

We have documented that CD4⁺ T cells control DNBS-specific CD8 colitis in three distinct settings: 1) acute colitis at a suboptimal dose of hapten challenge in susceptible BALB/c mice, 2) relapsing colitis induced in this mouse strain by a second challenge at an optimal dose of hapten, and 3) lack of colitis at an optimal dose of hapten challenge in the resistant C57BL/6 strain. These data indicate that Treg suppressive function plays an important role in the development and severity of inflammatory bowel disorders in an immunocompetent host. Treg could be involved in 2) limiting the severity of flares and chronicity of gut inflammation, 2) modulating the sensitivity of mucosal T cells to stress and adjuvant signals from the gut microenvironment, and 3) genetically determined mechanisms of intestinal homeostasis.

In BALB/c mice, lack of Treg by Ab depletion induced the outcome of an acute colitis elicited at a suboptimal dose of challenge, but did not aggravate colitis elicited with an optimal hapten dose. The most likely explanation is that a low dose of hapten challenge is insufficient to reactivate specific CD8 effectors and/or promote their recruitment into the LP, but could still activate Treg, that could then control extravasation of CD8 effectors into inflamed tissues (28). Alternatively, the fact that Treg depletion did not aggravate acute colitis elicited at a high hapten dose could be related to the local inflammatory response elicited by the challenge, impairing Treg suppressive function or susceptibility of CD8 effectors to their suppressive effect. The surprising finding that a more severe relapse of colitis occurs after a second rectal challenge in the absence of Treg (Fig. 5) underscores that Treg can regulate relapsing colitis induced by memory CD8⁺ T cells and suggests that colitogenic effector and memory CD8⁺ T cells could have differential sensitivity to Treg.

The observation that colitis can be induced in MHC class II-deficient *Aβ°/°* mice that are genetically deficient in CD4⁺ T cells but not in wild-type syngeneic B6 mice indicates that resistance to colitis depends on the presence of class II-restricted Treg and/or other MHC class II-dependent suppressive mechanisms. We cannot formally exclude that CD8⁺ T cells from *Aβ°/°* have a greater ability to differentiate into colitogenic effectors, as suggested by the higher frequency of hapten-specific IFN-γ-producing cells generated after colonic sensitization in *Aβ°/°* compared with wild-type B6 mice. However, we have provided evidence from both in vivo cotransfer experiments and in vitro studies that CD4⁺ Treg can regulate colitis by controlling the response of CD8 effectors. CD4⁺ T cells may exert their regulatory effect on the CD8⁺ T cell response at two temporally distinct stages of the colonic DTH...
response. During the afferent (i.e., the asymptomatic) phase induced by intrarectal immunization, class II-restricted CD4+ T cells control the priming of specific CD8+ T cells, as demonstrated by the enhanced frequency of DNBS-specific IFN-γ-producing CD8+ T cells in MLN of Aβ2/ mice and anti-CD4 mAb-treated BALB/c mice. In addition, CD4+ T cells also control the effector (i.e., symptomatic) phase of colitis elicited by hapten challenge, during which primed CD8+ effector T cells are recruited into colon LP as granzyme B-expressing colitogenic effectors (13). This is illustrated by the observation that, despite the high frequency of specific IFN-γ-producing CD8+ T cells at day 5 after sensitization, BALB/c mice fail to develop colitis when challenged with an infra-optimal dose of hapten, unless CD4+ T cells or CD4+CD25+ T cells were lacking. This reflects the possibility that challenge with hapten at this low dose may be sufficient to activate Treg but not to recruit sufficient numbers of pathogenic CD8+ effectors in the colon LP. In addition, although efficient depletion of CD4+ T cells only moderately affected acute colitis, it clearly enhanced the severity of relapsing colitis induced by a second challenge performed after a 30-day remission period. This indicated that CD4+ T cells are capable of regulating a secondary DTH response mediated by effector/memory CD8+ T cells. This is in line with recent reports using double-transgenic mice with TCR-αβ-transgenic CD8+ T cells specific for a neo self-Ag expressed in the intestine, showing that lack of control by CD4+ Treg results in a chronic autoimmune enteropathy and colitis driven by autoreactive CD8+ T cells (15–17). It may thus be proposed that recurrence of intestinal inflammation results from inappropriate Treg control of colitogenic CD8+ T cells.

The subset of CD4+ T cells which controls colitis in our model most likely includes CD4+CD25+ Treg inasmuch as anti-CD4 or anti-CD25 mAb treatments induced colitis in response to an infra-optimal dose of Ag challenge. Moreover, cotransfer of CD4+CD25+ Treg with CD8+ T cells is sufficient to prevent DNBS-induced colitis in Rag2−/− mice. In a closely related mouse model of skin DTH to 2,4-dinitro-1-fluorobenzene mediated by DNBS-specific CD8+ Tc1-type cells and regulated by MHC class II-restricted CD4+ T, we and others documented that skin inflammation is controlled by CD4+CD25+Foxp3+ Treg, which regulate 1) the priming of hapten-specific CD8+ effectors in draining lymphoid organs, 2) their influx in the challenged skin cells (28), and 3) the resolution of skin inflammation after challenge (25, 26). Likewise, studies in the T cell transfer model of colitis in SCID mice showed that CD4+CD25+ Treg were able to control the activation and function of colitogenic effectors (9, 11). Along these lines, we found that functional Foxp3+ Treg reside in the colon LP of naive mice and are increased following colonic exposure to DNBS (data not shown), suggesting that they may be either expanded locally in response to DNBS and/or recruited into the site of inflammation as previously documented (29). In addition, Foxp3+ Treg can differentiate from Foxp3− CD4+ T cells in the MLN (30) and small intestine (31) in the presence of TGF-β and retinoic acid. However, whether similar a mechanism operates in the large intestine remains to be demonstrated.

How MLN Foxp3+ Treg with a greater suppressive capacity compared with their counterparts in the colon LP can control a disease process that occurs in the intestinal mucosa remains to be fully elucidated. Indeed, we have provided evidence that Treg are able to control gut inflammation by acting during the symptomatic phase of colitis, leading to colon lesions. Several types of Treg including natural resident Foxp3+CD4− T cells, inducible Foxp3+ or Foxp3− Treg, as well as de novo-converted Foxp3− Treg from MLN or LP may all play a role. These Treg might act by suppressing the reactivation and/or migratory potential of pathogenic CD8 effectors generated in MLN. We propose from our data that resident Foxp3+ LP Treg, with a lower suppressive capacity compared with MLN Treg, could be less potent at controlling CD8 effectors that have already migrated into the colonic mucosa, unless local inflammatory signals are tempered by probiotic treatment, which, at the same time enhances their frequency and more importantly their suppressive function.

In this respect, some probiotics including L. casei can polarize CD4+ T cells into Treg in vitro (32) (33, 34) and when administered orally negatively regulate systemic (24) as well as intestinal (35, 36) T cell-mediated inflammation, suggesting that they may act as Treg adjuvants. The probiotic mixture VSL#3, administered during the remission phase of recurrent trinitrobenzene sulfonic acid-induced colitis, was shown to attenuate intestinal inflammation via induction of IL-10-dependent TGF-β-bearing cells in the small intestine (35) that may represent Ag-induced Treg. Our finding that L. casei alleviates acute colitis mediated by CD8+ T cells is reminiscent of the ability of L. casei-fermented milk to control hapten-specific CD8+ T cell-mediated skin DTH via a mechanism dependent on MHC class-II-restricted CD4+ T cells (24). In this study, we document the ability of oral L. casei treatment to selectively increase the suppressive function of CD4+Foxp3+ nTreg residing in colon LP (Fig. 8) without affecting Treg in MLN or systemic lymphoid organs (data not shown). The precise mechanism by which L. casei potentiates Treg in the LP is as yet unknown. However, several features of the biology of the bacteria itself as well as of its effects on innate and adaptive immunity could explain the observed beneficial effect on colitis. Indeed, L. casei DN-114 001 is able to initiate protein synthesis during its transit via the gut lumen (37) and release anti-inflammatory mediators, which counteract invasive enteropathogenic bacteria and prevent their adhesion and invasion of intestinal epithelial cells (38). Although L. casei does not colonize the gut after oral administration (37), components of the bacteria translocating the gut epithelium during its transit could, by acting via the gut microenvironment, condition mucosal dendritic cells and favor Treg differentiation, survival, or function. An attractive hypothesis could be that conditioning of intestinal bacteria may induce the activation of preexisting nTreg or their local conversion from CD4+Foxp3− precursor Treg. However, using an in vivo model of Foxp3+ Treg neocconversion by transferring sorted eGFP+ T cells from Foxp3−eGFP mice (31) into Rag2−/− recipients, we found that Foxp3+ Treg conversion (either in lymphoid organs or intestinal LP) was unaffected by daily oral L. casei treatment of the Rag2−/− recipients for up to 2 mo (data not shown). It is possible that L. casei enhances the survival of preexisting Treg and might protect from colitis by conferring resistance of resident Treg to the negative influence of the local proinflammatory milieu. An alternative hypothesis is that L. casei conditioning of mucosal dendritic cells could promote survival and/or function of preexisting Treg or could polarize Tconv into Treg. This is supported by recent in vitro data documenting that human myeloid dendritic cells cultured with L. casei DN-114 001 induce the polarization of Tconv into Treg in vitro (39). Whether L. casei could operate in vivo via professional APC, epithelial cells, or stromal intestinal cells remains to be fully explored.

Patients with active IBD exhibit accumulation of functional Treg in MLN and increased Foxp3+ CD4+ T cells in the gut LP that correlates with disease activity (40–42), suggesting that intestinal inflammation does not merely result from a Treg defect nor ability to migrate to the inflamed site. Alternatively, it has been proposed that the suppressive efficacy of Treg may be impaired within the inflamed mucosal microenvironment (29). Thus, the ability of probiotics such as L. casei to act as natural adjuvants for mucosal Foxp3+ nTreg and also to attenuate innate inflammatory signals may provide more efficient regulation of the mucosal inflammatory response in IBD.
Acknowledgments

We thank the staff of the Plateau de Biologie Experime~ nale de la Souris of the Ecole Normale Superieure de Lyon for the maintenance of mouse colonies and Chantal Bella from the flow cytometry facility of Institut Fede~ rat de Recher~ che-128 Biosciences Gerland-Lyon Sud for FACS sorting.

Disclosures

The authors have no financial conflict of interest.

References