T Helper 1 and T Helper 2 Cells Are Pathogenic in an Antigen-specific Model of Colitis

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Abstract

Dysregulated T cell responses to enteric bacteria have been implicated as a common mechanism underlying pathogenesis in rodent models of colitis. However, the bacterial species and T cell specificities that induce disease have been poorly defined. We have developed a model system in which target antigen, bacterial host, and corresponding T cell specificity are defined. OVA-specific T cells from DO11.RAG-2−/− TCR transgenic mice were transferred into RAG-2−/− recipients whose intestinal tracts were colonized with OVA-expressing or control Escherichia coli. Transfer of antigen-naive DO11.RAG-2−/− T cells into recipients colonized with OVA–E. coli resulted in enhanced intestinal recruitment and cell cycling of OVA-specific T cells; however, there was no development of disease. In contrast, transfer of polarized T helper (Th) 1 and Th2 populations resulted in severe wasting and colitis in recipients colonized with OVA–E. coli. The histopathologic features of disease induced by Th1 and Th2 transfers were distinct, but disease severity was comparable. Induction of disease by both Th1 and Th2 transfers was dependent on bacterially associated OVA. These results establish that a single bacterially associated antigen can drive the progression of colitis mediated by both Th1 and Th2 cells and provide a new model for understanding the immunoregulatory interactions between T cells responsive to gut floral antigens.

Key words: inflammatory bowel disease • E. coli • CD4 • cytokines • TCR transgenic

Introduction

Evidence from a growing number of rodent models of inflammatory bowel disease (IBD)* supports a central role for dysregulated CD4+ T cell responses to the enteric bacterial flora as a common disease mechanism (1). Targeted deletion or overexpression of a variety of genes important in immune regulation or mucosal barrier function, and disruption of normal thymic development have produced chronic inflammatory responses confined to the intestines (2–8). In addition, transfers of normal T cell subsets defined by differential expression of CD45RB into immunodeficient mice demonstrate that different T cell populations can promote or prevent colitis (9, 10). In the majority of these models, the pattern of cytokine expression in inflammatory lesions has implicated a Th1-dominated inflammatory disease, and neutralization of IL-12, IFN-γ, or TNF-α has been therapeutic (11–14). In a few models, a Th2 pattern of cytokine expression has been observed (15, 16), and neutralization of IL-4 or targeted deletion of IL-4-producing cells was protective (16, 17). In human IBD, there is evidence that Crohn’s disease is associated with a Th1 pattern of inflammation (18, 19). Elevation of some Th2 cytokines has been detected in ulcerative colitis (18), but whether the disease is associated with Th2 dominant responses is unclear.

Although many of the animal models of IBD have systemic disruptions of T cell regulatory mechanisms, inflammatory disease localizes primarily or exclusively to the large intestine. Because the cecum and colon of the large intestine contain the bulk of the enteric flora, it has been proposed that dysregulated T cell populations in these models are reactive to components of the normal intestinal flora (20, 21). This is supported by the abrogation of colitis development in germ-free HLA-B27 transgenic rats and IL-2−/− and IL-10−/− mice, and the rapid development of colitis upon reconstitution of the enteric flora (4, 22, 23). It has...
also been shown that T cells developing in the abnormal thymic microenvironment of Tg26 mice do not induce colitis in the absence of normal bacterial antigen (24). Similarly, depletion of the enteric flora by broad-spectrum antibiotic treatment has ameliorated disease in several murine models of colitis. Antibiotic manipulations in the HLA-B27 transgenic rats and DSS-treated mice have also suggested that complex interactions of aerobic and anaerobic commensal bacteria provide the stimulus for chronic inflammation in these models (25). More direct evidence implicating enteric bacterial antigens is found in data from studies in the spontaneously colitic C3H/HeJ/Bir mouse strain (26). CD4 T cells isolated from these mice are reactive to a mixture of cecal bacterial antigens and induce colitis upon transfer to immunodeficient hosts (27). These studies strongly suggest that the enteric bacterial flora provides the stimulus for development of colitis in the setting of altered immunoregulatory mechanisms.

The mechanism by which the bacterial flora stimulates colitis in susceptible models is unknown and is likely to be multifactorial. Bacterial components may act as immune adjuvants or may provide bacterial superantigens that nonspecifically activate an adaptive immune response. Alternatively, proinflammatory bacterial wall components may initiate or direct a pathologic inflammatory response independent of specific immunity. However, the fact that T cell regulatory dysfunction is involved in experimental colitis indicates that specific recognition of bacterial antigens is central to disease pathogenesis. An intriguing finding in this regard has been the relative selectivity of immune responses to a limited number of bacterial species in mouse and rat colitis models (22, 28, 29). Nevertheless, details regarding the types of enteric bacteria that are responsible for initiating and perpetuating a pathogenic T cell response and the specific bacterial antigens recognized remain poorly defined.

A significant impediment to advances in our understanding of homeostatic and pathogenic intestinal T cell responses is the lack of identified target antigens and their corresponding T cell receptor specificities. In none of the existing models are specific bacterial species and their antigens or the responsive T cell specificities defined. The goal of the current studies was the development of a model system for characterization of T cell responses to bacterially associated enteric antigens in which the target antigen, bacterial host, TCR specificity, and T cell developmental stage are each defined. To this end, we have adapted the well-characterized DO11.10 αβ TCR transgenic model to examine the inductive and regulatory effects stimulated by free or bacterially associated cognate antigen, OVA. Adoptive transfers of antigen-naive or polarized Th1 or Th2 cells derived from recombination activating gene (RAG)–deficient DO11.10 mice were made into congenic RAG–deficient recipients colonized with E. coli engineered to express OVA or a control antigen. Using this system, we have observed for the first time that precommitted Th1 and Th2 cells reactive to a single bacterially associated antigen can be colitogenic, whereas naive cells are not.

Materials and Methods

Mice. Specific pathogen-free BALB/cBJ, DO11.10 (30), BALB.RAG-2–/–, and DO11.10 TCR transgenic mice crossed with RAG-2–/– mice (DO11.RAG-2–/–) were bred and/or maintained in the Genetically Engineered Mouse facility at University of Alabama at Birmingham. DO11.10 and RAG-2–/– mice had been backcrossed onto the BALB/c background for at least 10 generations before intercrosses to generate mice homozygous for the RAG-2 targeted mutation and hemizygous for the DO11.10 transgene (DO11.RAG-2–/–). Male and female DO11.RAG-2–/– mice were used as donors for adoptive transfer studies; female BALB.RAG-2–/– mice were used as recipients. All experimental mice were 6–10 wk of age.

Generation of OVA-producing Esherichia coli. Esherichia coli was chosen as the bacterial host for these studies because of the relative ease with which it is genetically modified and its demonstrated role as an “immunodominant” organism in spontaneously colitic mice (28). The full coding sequence of OVA or TET-C (C fragment of tetanus toxoid, as a control) was expressed in E. coli strain DH5α under the control of an inducible bacterial promoter, nir15 (31). The nir15 promoter is an improved expression variant of the nirB promoter of the nitrite reductase gene of E. coli that is strongly induced by environmental nitrites or low oxygen tension (32). This promoter has been used to direct stable expression of heterologous antigens in vaccine strains of Salmonella (31, 33). The plasmid pTETnir15 (reference 31; referred to henceforth as pnr15.TET) expresses the C fragment of tetanus toxoid and was provided by Jerry McGhee and James Lillard, University of Alabama at Birmingham. To produce an OVA-expressing variant, pnr15.OVA, the TET coding sequence was excised by BglII/BamH1 digestion and replaced with a pcr-generated fragment that included an idealized Shine-Dalgarno sequence upstream of the entire OVA coding sequence (forward primer: 5′ CTTATAGATCTTAATCCACGAGATATTATTTATGAAAAACATCGGCGCAGGACGAAGCACCATGCC; reverse primer: 5′ GCTAGGGATCGCTTTAAGGGGAAACACATCGGCACCAAGCAAAGATGCAAATGGAA; reverse primer: 5′ GCTAGGGATCGCTTTAAGGGGAAACACATCGGCACCAAGCAAAGATGCAAATGGAA; reverse primer: 5′ GCTAGGGATCGCTTTAAGGGGAAACACATCGGCACCAAGCAAAGATGCAAATGGAA; reverse primer: 5′ GCTAGGGATCGCTTTAAGGGGAAACACATCGGCACCAAGCAAAGATGCAAATGGAA). The plasmid VR1020/OVA was used as a template for amplifying the OVA coding sequence (provided by William O. Rogers, Naval Medical Research Center, Bethesda, MD). DH5α E. coli were transformed by each plasmid (pnr15.OVA or pnr15.TET) by electroporation and selected for ampicillin resistance. Clones containing pnr15.OVA were isolated and sequenced to prove authenticity of the OVA coding sequence. Expression of OVA in E. coli was confirmed by Western blot analysis after overnight culture in media containing ampicillin, without shaking in tightly capped tubes at 37°C for anaerobic induction of nir15 promoter. Bacteria were lysed in 2× sample buffer (6% TRIS–Cl, pH 6.8, 20% glycerol, 4% SDS, 2% 2-ME), and heat denatured at 100°C for 5 min. Cell lysates (105 cell equivalents) and purified OVA (positive control) were resolved on a 10% SDS polyacrylamide gel and transferred to nylon membranes (Nynat Plus; Schleicher & Schuell). Membranes were incubated at room temperature with 1% blocking reagent (Boehringer Mannheim) for 1 h, a 1:500 dilution of murine anti-OVA mAb, OVA-3 (IgG2b, Brookwood Biomedical) for 2 h, and were detected with POD-conjugated anti-mouse IgG/anti–rabbit IgG (40 μg/ml; Boehringer Mannheim) using the chemiluminescent substrate luminol (Boehringer Mannheim).

Cell Purification and Flow Cytometry. CD4+ T cells were isolated from pooled spleens and peripheral lymph nodes of DO11.10 or DO11.RAG-2–/– mice using mouse CD4 (L3T4) Dynabeads® followed by CD4 DETACHaBEAD® per the manu-
facturer’s protocol (Dynal Biotech). The resulting CD4+ en-
riched cells were stained with FITC-conjugated KJ1–26 (anti-
DO11.10 TCR, reference 34) and PE-conjugated anti-CD4 and
analyzed by flow cytometry to determine purity of the popu-
tation. 10,000 total events were acquired on a Becton Dickinson
FACScan™, gating on cells with forward and side scatter proper-
ties of lymphocytes, and were analyzed using CELLQuest™ soft-
ware. Isolations routinely resulted in >92% purity of CD4+ cells
(DO11.10 donors) or CD4+KJ1–26+ cells (DO11.RAG-2–/–).

In Vitro and In Vivo Analyses of DO11.10 T Cell Response to
OA-A-expressing E. coli. For in vitro studies, splenic adherent
cells isolated from BALB/c mice were pulsed for 18 h with noth-
ing, 100 μg/ml whole OVA, or the indicated number of genta-
mycin-killed, anaerobically induced pnu15. TET or pnu15.OVA
E. coli. Gentamycin killing was done by incubating bacteria with
100 μg/ml gentamycin for 30 min. Pulsed splenic adherent cells
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100 μg/ml gentamycin for 30 min. Pulsed splenic adherent cells
were then irradiated (2,500 rads) before culture with CD4+ cells isolated from DO11.10 mice. Day 3 proliferative responses
were determined by [3H]-TdR incorporation as described previ-
ously (35). For in vivo studies, 5 × 10^6 CD4+KJ1–26+ T cells isolated from DO11.10 mice were transferred into BALB/c mice by tail vein injection. 1 d after the transfer, 10^6 bacteria (E. coli-
OVA or -TET) were injected IV and mice were killed at days 1,
3, 5, 7, and 9. Cells from spleen and lymph nodes were pooled and analyzed by FACS® for expression of CD4 and DO11.10 TCR and the total number of CD4+KJ1–26+ cells were calcu-
lated as described previously (36).

Generation and Characterization of OVA-specific Th1 and Th2
Cells. Th1 and Th2 cells were generated in vitro from antigen-
naive CD4+ T cells as described previously (37). Briefly, purified
CD4+ T cells isolated from pooled spleens and peripheral lymph
nodes of DO11.RAG-2–/– were cultured at a ratio of 1:10 with irradiated BALB/c splenocytes and 5 μg/ml OVA peptide 323–339. The addition of 50 μg/ml IL-12 (R&D Systems) and 10 μg/ml anti–IL-4 (11B11; reference 38) was used to generate Th1 cells, while 1,000 μg/ml IL-4 (R&D Systems) and 10 μg/ml anti-
IL-12 (C17.8, reference 39) was used to generate Th2 cells (40). Recovered cells were washed and transferred on day 7 at 10^6
cells/RAG-2–/– recipient mouse. A separate aliquot of 5 × 10^6
cells was restimulated with 6 h with PMA (50 ng/ml) and ionom-
ycin (750 ng/ml; Sigma-Aldrich) for analysis of cytokine mRNA
expression by ribonuclease protection assay. Ribonuclease pro-
tection assays were performed with 1 μg of RNA using the tem-
plate set mCK1 (RiboQuant kit; BD Pharmingen).

Reconstitution of RAG2–/– Mice with CD4 Subsets, Colonization
with Bacteria, and Monitoring for Development of Colitis. Bacterial colonization was performed by gastric gavage of pnu15.OVA-E.
coli or pnu15.TET-E. coli (control) to RAG2–/– mice one day
before CD4 T cell transfer. Bacteria were grown in LB medium
overnight at 37°C under anaerobic conditions and 10^8 CFU in
100 μl PBS were administrated orally using a straight gavage
needle. Colonization was assessed by plating stool suspensions on LB plates containing ampicillin and was maintained at ~10^8 CFU/g of stool by biweekly gavage. 10^6 naive, Th1, or Th2 DO11.10 CD4+ T cells were transferred by tail vein injections into RAG-
2–/– mice a day after the first gavage. Mice were weighed weekly and monitored for appearance, and signs of loose stool and diar-
rhea. 0.8 mg/ml bromodeoxyuridine (Brdu) was added to the
drinking water of all groups for 9 d before killing and intestinal
tissues were evaluated histologically.

Histopathologic Assessment and Disease Scoring. At necropsy,
the small intestine, cecum, and colon were separated and Swiss
rolls of each prepared. Tissues were fixed in 10% buffered forma-
lin and paraffin 5-μm embedded sections were stained with
H&E. Additional sections were stained for identification of poly-
morphonuclear cells (Leder stain), eosinophils (cytolytic-resistant
eosinophil peroxidase stain; reference 41), T cells (anti-CD3), and/or BrdU as indicated. An immunohistochemical method for
coidentification of CD3 cells and BrdU-labeled cells was devel-
opned for these studies and will be described in detail elsewhere
(unpublished data). Briefly, 5-μm sections were digested with
pepsin, blocked with normal goat serum, and sequentially incu-
bated with polyclonal rabbit anti-human CD3 antibody (0.5 μg/
ml, 1:100 dilution, 30 min; Dako), biotinylated anti–rabbit IgG
(H+L) antibody (1.5 μg/ml, 1:100 dilution, 30 min), and 0.3% hydrogen peroxide (10 min). Bound antibody was then detected with a preformed avidin and biotinylated horseradish peroxidase
macromolecular complex (ABC; Vectastain Elite ABC kit; Vec-
tor Laboratories) and diaminobenzidine substrate was used for
development (Vector Laboratories). After development of anti-
CD3 staining, sections were rinsed with PBS followed by incuba-
tion in denaturing solution for 20 min (Brdu Staining kit; Zymed Laboratories). Slides were then sequentially incubated with biotinylated mouse anti-BrdU antibody for 30 min, alkaline
phosphatase labeled streptavidin for 20 min, and detected with
Fast Red TR chromogen for 30 min (BrdU Staining kit; Zymed
Laboratories). Slides were counterstained with hematoxylin and
coverslips mounted with crystal mount (Biomed). All incuba-
tions were performed at room temperature and each step was fol-
lowed by a rinse with PBS. Sections processed without primary
antibody served as negative controls.

Histological scoring was performed using a modification of the
scoring system reported previously by Sundberg et al. (42), and
was performed independently by two pathologists (A.J. Lazenby
and C.T. Weaver). Briefly, each section was scored for lesions
based on four criteria: severity of inflammation, hyperplasia of
mucosal epithelium, mucosal ulceration, and extent of involve-
ment. Severity of inflammation and hyperplasia were graded on
a score of 0–3, 0 being normal and 3 being severe. The extent of
disease involvement was determined for each and defined as: 0,
normal; 1, 1–33%; 2, 34–66%; and 3, 67–100%. The percent area
of involvement was determined separately for cecum, proximal
colon, middle colon, and distal colon/rectum. The average area
of involvement for the colon was calculated as the sum of the
three colonic segments divided by three and was used for calcu-
lation of the total colon score. The total score was assessed sepa-
ately for the cecum and colon by adding the scores of severity,
hyperplasia, area of involvement, and ulceration. The thickness of
mucosal hyperplasia was directly measured in the areas of most
severe disease using an ocular grid, but was not included as a
component of the disease score. All cell counts were performed
at original magnifications: 200X or 400X by point counting using
an ocular grid (36); a minimum of 1,000 total cells was counted
for each determination.

Isolation and Cytokine Phenotyping of Intestinal Lamina Propria T
Cells. Colon and small intestine were washed thoroughly with
PBS to remove debris, opened longitudinally, and then cut into
0.5 cm pieces. The epithelium was removed by incubation with
2 mM DTT (Sigma-Aldrich) and 1 mM EDTA (Sigma-Aldrich) in
RPMI 1640 medium supplemented with 2% FCS at 37°C for 30
min with gentle shaking. Tissue was collected, further cut into
8-10 mm pieces. The epithelium was removed by incubation with
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RPMI 1640 medium supplemented with 2% FCS at 37°C for 30
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min with gentle shaking.
beads® and mouse CD4 DETACHaBEAD® as described previously (Dynal). Recovered cells were cultured in 96-well plates (Nunc) that were precoated with anti-CD3 mAb (145–2C11; reference 43; 10 μg/ml) in 100 μl PBS at 37°C for 4 h and washed with PBS three times to remove unbound Ab. LP CD4+ T cells (5 × 10^6 cells per milliliter) were incubated in the presence of coated anti-CD3 and soluble anti-CD28 (37.51, reference 44; final concentration, 1 μg/ml) at 37°C. After 72 h of culture, supernatants were harvested and assayed for IFN-γ, IL-4, and IL-10 by capture ELISA using commercially available ELISA kits (R&D Systems).

Statistical Analyses. Parametric data were analyzed by Student’s t test. Nonparametric data were analyzed by the Mann-Whitney test. P values < 0.05 were considered significant.

Results

Generation and Characterization of OVA-producing E. coli. To generate bacteria that express the antigen recognized by the DO11.10 TCR, a plasmid encoding the full-length OVA cDNA (pnir15.OVA) was introduced into DH5α E. coli (Fig. 1 A). E. coli was chosen as the bacterial host because it is a normal commensal and because it is an immune stimulatory organism in spontaneously colitic mice (28). Western blot analyses of lysates of DH5α E. coli transformed with the pnir15.OVA plasmid indicate that this construct directs excellent cytosolic expression of OVA protein under anaerobic conditions (Fig. 1 B). E. coli transformed with a plasmid in which the coding sequence of OVA was replaced with that of the C fragment of tetanus toxoid (pnir15.TET-E. coli) served as a negative control. Based on densitometric comparisons between immunoblots performed with bacterial lysates of pnir15.OVA-E. coli and purified OVA, the anaerobically induced plasmid expressed ~1 μg OVA per 10^6 bacteria under in vitro conditions. The immunogenicity of pnir15.OVA-E. coli was determined in vitro by T cell proliferation assays (Fig. 1 C). Splenic adherent cells pulsed with anaerobically induced, gentamycin-killed pnir15.OVA-E. coli–stimulated proliferation of naive DO11.10 T cells, whereas splenic adherent cells pulsed with pnir15.TET-E. coli did not. The dose of bacteria that stimulated a maximal response (10^7 organisms) expressed ~10 μg OVA; the dose of free OVA that was required for a maximal response was 100 μg/ml. Thus, induced pnir15.OVA-E. coli are a potent immunogen for stimulation of DO11.10 cells in vitro.

The immunogenicity of live pnir15.OVA-E. coli was assessed in vivo using a DO11.10 adoptive transfer system (45). Normal BALB/c mice that received transfers of 5 × 10^6...
10^6 DO11.10 CD4+ T cells (DO11.10/BALB mice) were inoculated intravenously with 10^6 pnir15.OVA-E. coli or pnir15.TET-E. coli, and expansion of the clonotypic T cell population in the spleens of recipient mice was monitored by flow cytometric analysis (Fig. 2). In agreement with a previous study by Chen et al. (46), intravenous administration of the OVA-producing E. coli stimulated a robust clonal expansion in the spleens and lymph nodes of DO11.10 adoptive transfer recipients. Significant clonal expansion (approximately fivefold) was induced by challenge with pnir15.OVA-E. coli, but not the pnir15.TET-E. coli control. The peak response measured was on day five, with a rapid decline to baseline by day nine. Induction of a comparable peak response with free OVA required intraperitoneal administration of a ~100-fold higher dose of OVA (100 µg) in complete Freund's or a block copolymer adjuvant (data not shown), indicating that the bacterially associated OVA is more efficient at stimulating an in vivo response. Collectively, these data indicate that the pnir15.OVA plasmid produces high-level expression of OVA that is potently immunogenic for DO11.10 T cells in vitro and in vivo.

Rag-2^-/- Mice Reconstituted with Th1- or Th2-polarized DO11.RAG-2^-/- T Cells Develop Severe Wasting Disease that is Ova-E. coli-dependent; Mice Reconstituted with Antigen-Naive DO11.10 T Cells Do Not Develop Wasting Disease. To evaluate the in vivo responses of antigen-specific T cells to a floral, bacterially associated antigen, OVA-specific T cells were transferred into congenic, immunodeficient recipients whose intestinal tracts were colonized with E. coli that expressed either OVA (pnir15.OVA-E. coli) or a control antigen, TET (pnir15.TET-E. coli). Three T cell populations were examined: naive CD4+ T cells or Th1 and Th2 cells derived by short-term in vitro culture under selective cytokine conditions. A 1 wk protocol was used for Th1/Th2 derivation because it induced excellent polarization of cytokine phenotype (Fig. 3 A), but did not significantly affect T cell migration to the secondary lymphoid organs compared with Th1 and Th2 cells maintained in vitro for longer periods of time (unpublished data). In previous studies, we found that a fraction of CD4+ T cells that expressed the transgenic TCR in DO11.10 mice coexpressed a second, endogenous TCR that recognized bacterial antigens in the normal flora (35).

![Figure 2](image-url)  
**Figure 2.** OVA-expressing E. coli induce clonal expansion of DO11.10 T cells in vivo. DO11.10/BALB/c adoptive transfer recipients were challenged with a single intravenous injection of 10^6 viable pnir15.OVA-E. coli or pnir15.TET-E. coli. Two-color flow cytometric analysis was performed on splenic or pooled peripheral lymph node lymphocytes isolated at the indicated times after immunization as described in Materials and Methods. The percentages of KJ1–26CD4+ cells in the lymphocyte gate were determined and used to calculate the total number of splenic KJ1–26 CD4+ as described previously (reference 36). (A) Representative flow cytometric profiles for lymph node lymphocytes isolated 5 d after challenge with pnir15.TET-E. coli (left) or pnir15.OVA-E. coli (right). The percentages of total gated cells in each quadrant are indicated. (B) Kinetics of clonal expansion of total splenic KJ1–26CD4+ T cells induced by pnir15.OVA-E. coli versus control pnir15.TET-E. coli. Data points are the mean ± SD of 3–4 animals per group.

![Figure 3](image-url)  
**Figure 3.** Th1 and Th2 cells derived from DO11.RAG-2^-/- mice induce chronic wasting in RAG-2^-/- recipients colonized with pnir15.OVA-E. coli. (A) Cytokine analysis of naive and Th1- or Th2-polarized OVA-specific T cells. Th1 and Th2 cells were generated in vitro under polarizing cytokine conditions as described in Materials and Methods. Briefly, CD4+ T cells isolated from DO11.RAG-2^-/- were cultured for 7 d with OVA323-339 peptide, irradiated BALB/c splenocytes, and either IL-12 plus anti-IL-4 (Th1) or IL-4 plus anti-IL-12 (Th2). To confirm Th1/Th2 polarization, aliquots were restimulated for 6 h with PMA and ionomycin, and analyzed for cytokine mRNA expression by ribonuclease protection assay (RPA; mCK-1 RiboQuant kit; BD PharMingen) in comparison with naive T cell isolates. (B) Weight changes of BALB.RAG-2^-/- mice that were colonized with 10^6 CFU pnir15.OVA-E. coli (black symbols) or pnir15.TET-E. coli (white symbols), reconstituted with 10^6 naive (○), Th1 (△), or Th2 (□) cells from DO11.RAG-2^-/- donors and monitored for weight loss (B). Data are the mean weekly weights and SD (n = 5–6 mice per group).
Hence, the DO11.10 donors used for these experiments were crossed onto a BALB.RAG-2−/− background (DO11.RAG-2−/−) to obviate possible complications due to dual TCR specificities. In preliminary colonization studies, it was found that delivery of 10^10 anaerobically induced organisms by gastric gavage resulted in a transient colonization of the colon (half-life of 3–4 d; data not shown). Biweekly gavage maintained the floral colony count at a minimum of 10^6 CFU per gram of colonic contents and was used in experimental protocols.

1 d after gavage with anaerobically induced pnir15. OVA-E. coli or pnir15.TET-E. coli, RAG-2−/− mice received a transfer of 10^6 naive, Th1, or Th2 DO11.RAG-2−/− CD4+ T cells by tail vein injection. Bacterial colonization was maintained with biweekly gavage (10^10 induced organisms per dose), and mice were weighed and monitored for clinical signs of disease (Fig. 3 B). Significant weight loss was evident at week 6 in animals from groups that were colonized with pnir15.OVA-E. coli and reconstituted with either Th1 or Th2 cell transfers. Mice that received naive T cells and were colonized with pnir15. OVA-E. coli did not show weight loss or clinical signs of disease. Similarly, mice that received Th1 or Th2 cells and were colonized with control pnir15.TET-E. coli also showed no clinical evidence of disease. All mice were administered 0.8 mg/ml BrdU in their drinking water for 9 d before sacrifice at week 8 and tissue was processed for histological analyses.

At necropsy, macroscopic evaluation of animals from Th1 and Th2 groups colonized with pnir15.OVA-E. coli was remarkable for short, thickened large intestines. Focal superficial erythema and hemorrhages were present on cecal and colonic mucosal surfaces. The pnir15.TET-E. coli–colonized Th1 and Th2 groups were grossly normal, as were naive T cell recipients colonized with either pnir15.OVA-E. coli or pnir15.TET-E. coli. On microscopic evaluation, the large intestines from Th1 and Th2 groups colonized with pnir15.OVA-E. coli had moderate to severe disease characterized by extensive inflammatory cell infiltrates, marked epithelial cell hyperplasia and depletion of epithelial cell mucin (Fig. 4). As shown in results from a representative experiment (Table I), the incidence and severity of disease was comparable in Th1 and Th2 groups. The distribution of involvement was also similar in Th1- and Th2-mediated disease (Fig. 5), and was patchy along the length of the cecum and colon. Disease severity was modestly increased in colon compared with cecum; the rectum was spared. Although there was good population of the small intestinal LP with clonotypic T cells in all groups, no pathologic abnormalities were identified (data not shown). Similarly, there was no inflammation in other organs.

The histologic features of disease in Th1 and Th2 recipient mice colonized with pnir15.OVA-E. coli were distinct. The lesions in mice that received Th1 cells were characterized by predominantly mononuclear cell infiltrates composed of lymphocytes and monocytes/macrophages that expanded the LP and distorted the crypt architecture (Fig.


6 and Table II). Focally, there was extension of the inflammation into the underlying muscularis and serosa. Scattered, small granulomas were evident, but were not a dominant feature. Frequent crypt abscesses were evident, but T cells invaded the epithelium. Neutrophils were present in the areas of most severe epithelial injury, particularly bordering and infiltrating damaged crypts. Eosinophils were rarely seen.

While the disease severity was comparable in pnir15. OVA-E. coli-colonized Th2 animals, the histological features were different (Fig. 6 and Table I). In contrast to Th1-mediated disease, the inflammatory infiltrates in Th2 mice included a predominance of polymorphonuclear cells, including a significant fraction of eosinophils (~40% of total PMNs), with lesser numbers of lymphocytes and even fewer monocytes/macrophages. The crypt architecture was less tortuous, but crypts were markedly elongated and hyperplastic. Indeed, the mean crypt length in Th2 lesions was generally greater than in Th1 lesions (Th2, 0.73 mm, or 3.65 times nondiseased; Th1, 0.58 mm, or 2.64 times nondiseased), consistent with a more hyperplastic epithelial response in Th2 lesions. Compared with Th1-mediated disease, there was less epithelial cell injury and necrosis, and few crypt abscesses were found. There were significantly fewer intraepithelial T cells in the Th2-diseased mucosa. No granulomas were identified.

The distinct patterns of inflammation in diseased colons of Th1- and Th2-reconstituted mice strongly suggested that either effector T cell population could induce a pathogenic response. However, given the short-term in vitro polarizations before transfer, and evidence for some heterogeneity of cytokine profiles of the starting T cell populations (see RPA analysis, Fig. 3), it was possible that a shift in cytokine phenotype might have occurred in vivo. To determine if the T cells present in colitic lesions retained a similar cytokine phenotype to those of the original T cell populations transferred, cytokine analyses were performed on recovered T cells (Fig. 7). CD4+ T cells isolated from the intestinal LP of pnir15.OVA-E. coli-colonized recipients reconstituted with Th1- or Th2-polarized DO11.

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<tr>
<th>T cells</th>
<th>Intestinal colonization</th>
<th>Colitis†</th>
<th>Severity</th>
<th>Hyperplasia</th>
<th>Extent of involvement</th>
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<td>Naive</td>
<td>pnir15.TET-E. coli</td>
<td>0/10</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>pnir15.OVA-E. coli</td>
<td>1/10</td>
<td>0.1 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>0.3 ± 1.0</td>
</tr>
<tr>
<td>Th1</td>
<td>pnir15.TET-E. coli</td>
<td>0/8</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>pnir15.OVA-E. coli</td>
<td>7/7</td>
<td>2.5 ± 0.6</td>
<td>2.3 ± 0.5</td>
<td>2.5 ± 0.6</td>
<td>7.8 ± 1.0</td>
</tr>
<tr>
<td>Th2</td>
<td>pnir15.TET-E. coli</td>
<td>0/7</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>pnir15.OVA-E. coli</td>
<td>6/6</td>
<td>2.3 ± 0.6</td>
<td>2.7 ± 0.5</td>
<td>2.3 ± 0.3</td>
<td>7.3 ± 0.6</td>
</tr>
</tbody>
</table>

RAG-2−/− recipients were reconstituted with 106 CD4+ T cells of the indicated phenotypes that were isolated or generated from DO11.RAG-2−/− donors. 1 d before T cell reconstitution, recipients were colonized with 1010 CFU of the indicated bacteria and were maintained with biweekly administration of an additional dose of 1010 CFU bacteria. 8 wk after reconstitution, mice were killed and necropsied.

†See Materials and Methods for details of scoring system.

‡All data are the means ± SEM of two independent pathological evaluations of the indicated numbers of mice pooled from three separate experiments. Scores that differed significantly from control (P > 0.05) are indicated in bold.

Bacterially Associated Antigen Is Required for Colitis Development in Th1- and Th2-reconstituted RAG-2−/− Mice. Previous studies have shown that continuous oral delivery of free OVA at a dose of 0.1 μg/ml could activate DO11.10-
Figure 6. Histological analyses of colonic tissues from mice with Th1- or Th2-mediated colitis. Paraffin-embedded sections of colon from the indicated experimental groups were processed and stained as described in Materials and Methods. (A) Th1- and Th2-reconstituted/pnr15.0VA- E. coli–colonized, serial sections of proximal colon stained with anti-CD3 to identify T cells (left, top, and bottom) or Leder stain to identify neutrophils and eosinophils (right, top, and bottom). Arrows identify intraepithelial Th1 cells in diseased proximal colon. (B) Proximal colon from naive-reconstituted RAG-2−/− mice colonized with pnr15.TET-E. coli (top) or pnr15.0VA-E. coli mice (bottom) stained with anti-CD3. (C) Low and high power images of Th1-reconstituted/pnr15.0VA- E. coli–colonized middle colon double-labeled with anti-CD3 (brown) and anti-BrdU (red). Arrowheads identify CD3+ DO11.RAG-2−/− T cells that have incorporated BrdU after 9 d in vivo administration. Note also the incorporation of BrdU by colonic crypt epithelial cells.
associated OVA was required. RAG-2 sufficient to cause intestinal disease, or whether bacterially
E. coli
Th1 pnir15.TET- E. coli
ND, not determined.

cipients colonized with pnir15.OVA-
Th2 phenotype. Intestinal tissues from Th1 and Th2 adoptive transfer re-
T cells recovered from colitic mice retain a polarized Th1 or
Th2 phenotype. Intestinal tissues from Th1 and Th2 adoptive transfer re-

Table II. Histological Characterization of Inflammatory Lesions in DO11.RAG-2-/-/RAG-2-/- Recipients Colonized with E. coli-OVA versus E. coli-TET

<table>
<thead>
<tr>
<th>T cells</th>
<th>Intestinal colonization</th>
<th>CD3+/mm² total</th>
<th>CD3+/mm² IEL</th>
<th>PMN/mm² total</th>
<th>Percentage of eosinophils (PMNs)</th>
<th>Percentage of BrdU⁺ (CD3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>pnir15.TET- E. coli</td>
<td>77.4 ± 44.2b</td>
<td>5.5 ± 5.0</td>
<td>4.0 ± 3.7</td>
<td>ND</td>
<td>1.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>pnir15.OVA- E. coli</td>
<td>192.3 ± 73.5</td>
<td>7.3 ± 3.6</td>
<td>8.5 ± 3.8</td>
<td>2.4 ± 2.9</td>
<td>24.0 ± 5.2</td>
</tr>
<tr>
<td>Th1</td>
<td>pnir15.TET- E. coli</td>
<td>203.7 ± 55.8</td>
<td>17.9 ± 8.4</td>
<td>17.9 ± 8.4</td>
<td>ND</td>
<td>2.5 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>pnir15.OVA- E. coli</td>
<td>796.1 ± 190.3</td>
<td>66.5 ± 29.3</td>
<td>246.0 ± 97.3</td>
<td>5.7 ± 4.8</td>
<td>34.8 ± 7.6</td>
</tr>
<tr>
<td>Th1</td>
<td>pnir15.OVA- E. coli</td>
<td>140.1 ± 50.0</td>
<td>13.0 ± 8.3</td>
<td>0.5 ± 1.0</td>
<td>ND</td>
<td>7.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>pnir15.OVA- E. coli</td>
<td>385 ± 96.2</td>
<td>33.0 ± 5.2</td>
<td>496.7 ± 44.7</td>
<td>39.6 ± 8.9</td>
<td>38.8 ± 9.6</td>
</tr>
</tbody>
</table>

RAG-2-/- recipients were reconstituted with 10⁶ CD4⁺ T cells of the indicated phenotypes that were isolated/generated from DO11.RAG-2-/- donors. 1 d before T cell reconstitution, recipients were colonized with 10¹⁰ CFU of the indicated bacteria and maintained with biweekly administration of an additional dose of 10¹⁰ CFU bacteria. 8 wk after reconstitution, mice were killed and necropsied.

aThe total number of CD3⁺ T cells per mm² of colonic mucosa, including LP and IEL compartments (see Material and Methods for details).

bData are the means ± SEM from 3–6 animals per group. Values that differed significantly from control (P > 0.05) are indicated in bold font. ND, not determined.

derived Tr1 regulatory cells to control colitis development in adoptively transferred mice (47). We sought to determine if free OVA delivered to RAG-2-/- mice reconstituted with DO11.RAG-2-/- Th1 and Th2 cells would be sufficient to cause intestinal disease, or whether bacterially associated OVA was required. RAG-2-/- mice were reconstituted with DO11.RAG-2-/- Th1 and Th2 cells and were challenged with free versus bacterially associated OVA. Free OVA was delivered continuously in the drinking water over an 8-wk course, with or without pnir15.TET-E. coli colonization to control for potential nonspecific adjuvanticity of the E. coli organisms used for colonization (Fig. 8). Control animals received no OVA or pnir15.OVA-E. coli.

In contrast to Th1 and Th2 recipients colonized with pnir15.OVA-E. coli, animals challenged with free OVA did not develop significant disease, irrespective of whether they were also colonized with pnir15.TET-E. coli control organisms (Fig. 8). To determine if this was due to an absence of antigenic exposure of colonic T cells in animals fed OVA, the recruitment and/or proliferation of T cells in the colonic LP were assessed. Th1 recipients challenged with low dose OVA (0.1 μg/ml) did not have appreciably increased densities of T cells in the LP compared with no antigen controls (92.5 vs. 78.0 CD3⁺ cells/mm²), but a significantly greater fraction of T cells present had incorporated BrdU (4.2 vs. 0.8%). At the higher dose of free OVA (1,000 μg/ml), both the T cell density and frequency of BrdU-labeled cells were increased compared with no antigen controls, albeit less so than in animals colonized with pnir15.OVA-E. coli. Interestingly, Th2-reconstituted mice treated with both low and high doses of free OVA had increases in T cell densities in the colonic LP that were comparable to pnir15.OVA-E. coli-colonized controls. However, although frequencies of BrdU-positive cells were greater than the no antigen controls in both groups treated with free OVA, the frequencies were significantly lower than in the pnir15.OVA-E. coli-colonized group. This suggests that there might be more rapid turnover of colonic T cells stimulated with bacterially associated OVA than free OVA. Nevertheless, the absence of colitis in Th1 and Th2 recipient mice challenged with free OVA was not due to the absence of antigen recognition by

Figure 7. T cells recovered from colitic mice retain a polarized Th1 or Th2 phenotype. Intestinal tissues from Th1 and Th2 adoptive transfer recipients colonized with pnir15.OVA-E. coli were recovered and processed for isolation of LP lymphocytes 8 wk after transfer (Materials and Methods). CD4⁺ T cells were purified by magnetic sorting and stimulated with plate-bound anti-CD3 and anti-CD28 for 72 h. Culture supernatants were assayed for IFN-γ, IL-4, and IL-10 by ELISA. Data are the means and SE of triplicate determinations for T cells pooled from 6–10 mice per group. The detection limits of the respective ELISA assays are indicated for those values that were not greater than background (P < 0.01).
Discussion

This paper establishes that a single bacterially associated antigen is both necessary and sufficient to cause colitis mediated by a defined, monoclonal T cell population. In this model, transfer of both Th1 and Th2 cells derived from OVA-specific DO11.RAG-2−/− donors was associated with colitis that was dependent on the colonization of RAG-2−/− recipient mice with E. coli that expressed OVA. Importantly, transfer of antigen-naive T cells of identical specificity did not induce appreciable disease, despite in vivo responsiveness to bacterially associated antigen. Further, in vivo recognition of free antigen by either Th1 or Th2 effectors was insufficient to induce colitis. Taken together, these results suggest that both committed effector T cells and bacterially associated antigen are required for disease pathogenesis in this model, and emphasize the fact that unopposed Th1 and Th2 responses to enteric bacterial antigens can be equally deleterious to the host.

In each of the rodent models of colitis studied to date, disease development has been associated with the presence of the enteric flora. However, in none of these models has the relevant bacterial antigens been identified, and it has been difficult to define contributions from distinct bacterial species or specific antigens. The lack of identification of either the antigenic targets or T cell specificities responsible for disease has compromised efforts to understand the pathogenic contribution of the enteric flora. Studies of spontaneously colitic C3H/HeJBlir mice have demonstrated a surprisingly selective immune reactivity to the enteric flora, and have suggested that facultative anaerobes such as E. coli and Salmonella species were immune stimulatory organisms, despite comprising <1% of the total bacterial flora (28). Similarly, Bacteroides spp. was a common component of bacterial cocktails that induced colitis in germ-free HLA-B27 transgenic rats colonized with defined flora (22). Helicobacter hepaticus has been proposed as a requisite component of the enteric flora in the CD45RB transfer and IL-10−/− colitis models (48, 49), although this has been controversial (50). However, the diversity and specificities of the T cell populations responsive to these bacteria are yet to be defined in these or other IBD models. Advantages of the DO11.10 OVA colitis model described here include the ability to track a specific T cell population that is responsive to a defined bacterially associated antigen in vivo (45, 46), and the ability to control the developmental stage and effector function of the T cell population. This model also offers the potential to vary the bacterial host and the bacterial compartment in which the target antigen, OVA, is expressed.

A technical impediment confronted at the outset of these studies was difficulty in the development of bacterial strains that could stably express OVA in vivo and could establish colonization of a murine host. Poor retention of plasmids and/or silencing of plasmid promoters have hampered efforts to express heterologous antigens in bacterial vectors in vivo (45). It has also been difficult to establish long-term colonization of laboratory strains of bacteria introduced into the normal flora (33). Adoption of the pnir15 promoter has extended expression of OVA in vivo, and we have been able to document stable plasmid retention and expression of OVA up to 5 wk after a single inoculation of pnir15-OVA-E. coli strain used in these studies without repeated bacterial inoculation. More recent efforts using E. coli strains derived from...
from the normal flora of mice in our colony have resulted in prolonged colonization (unpublished data) and are being characterized for future studies. Nevertheless, it is clear from our results that the protocol developed for in vivo delivery of bacterially associated OVA is effective.

This study establishes that a single antigenic specificity can drive the development of colitis by both Th1 and Th2 effectors, but does not prove that specific bacterial antigens are responsible for pathogenic T cell responses in other colitis models or in human disease. Indeed, pioneering studies by Braun et al. has identified by representational difference analysis a novel bacterial superantigen, I2, which is associated with inflammatory lesions in patients with Crohn’s disease (51). While a causal relationship between this molecule and Crohn’s disease is not yet established, this study raises the possibility that bacterial superantigens rather than specific bacterial antigens are important contributors to a pathogenic T cell response. However, TCR repertoire analyses in monozygotic twins with Crohn’s disease do not support a major role for superantigens (52). Further, recent studies of the TCR-β repertoire of T cells from colitic animals in the CD45RB and C3H/HeJ Bir colitis models favor an oligoclonal T cell response to specific bacterial antigens rather than a superantigen (27, 53, 54). In the present model, colonization of adoptive transfer recipients with E. coli that express a control antigen (TET) did not develop disease, and there was no evidence of reactivity of either naive or effector DO11.10 T cells to control E. coli or the endogenous enteric flora of RAG-2−/− hosts. Hence, this model does not involve a contribution by bacterial superantigens, and demonstrates that superantigens are not required for colitis development.

The induction of colitis by both Th1 and Th2 effector cells in this model was somewhat unanticipated. Although elevated Th2 cytokines have been demonstrated in the TCR-α−/− and oxazolone colitis models (15, 16), the majority of the rodent models that have been described demonstrate characteristics of a Th1-mediated disease. In the well-characterized CD45RB colitis model, CD4 T cells recovered from animals with active disease produced Th1 cytokines and disease was attenuated by specific bacterial antigens rather than a superantigen (27, 53, 54). In the present model, colonization of adoptive transfer recipients with E. coli that express a control antigen (TET) did not develop disease, and there was no evidence of reactivity of either naive or effector DO11.10 T cells to control E. coli or the endogenous enteric flora of RAG-2−/− hosts. Hence, this model does not involve a contribution by bacterial superantigens, and demonstrates that superantigens are not required for colitis development.

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A curious feature of the current model concerns the tempo of disease development mediated by Th1 and Th2 transfers into OVA-E. coli−colonized mice. Similar to each of the spontaneous colitis models that have been characterized, development of clinically apparent disease after Th1 or Th2 transfers evolved over a period of several weeks to months. In previous models, where the precursor frequency of pathogenic or protective T cells was not defined, it was plausible that this relatively slow development of disease was due to a requirement for clonal expansion of a pathogenic T cell population that was initially limiting in the repertoire. Given the monoclonality of the pathogenic T cells in the current model, it is surprising that disease development is not accelerated relative to other models. It is also provocative that Th1 and Th2 disease development progressed at similar rates. This suggests that the rate-limiting step for disease induction and progression in this model may not be the availability of pathogenic T cells. Rather, the immunogenicity of the relevant bacteria or the rate at which translocations of the relevant enteric bacteria occur might be more critical. Because the OVA-E. coli used in this study are quite potently immunogenic (Figs. 1 and 2), we favor a dominant role for the latter. The rate of bacte-
rial translocation is typically low for commensal organisms, and is related to the density of a given bacterial species in the enteric flora and its representation relative to other bacterial species that comprise the flora (61). In our studies, reduced colonization by OVA-E. coli to levels below ~10^7 CFU/g of luminal contents resulted in a marked decrease in disease severity and incidence despite transfers of the same number of Th1 or Th2 cells (data not shown). This suggests that the rate at which bacteria are transported across the mucosa may be limiting, and might explain the focal nature of lesions in this and other colitis models, as well as the relatively slow progression of disease. Since the specific bacterial species responsible for disease is identified in the DO11.10/OVA-E. coli model, it will be possible to examine this directly. It would be predicted that higher colonization rates or delivery of OVA via more invasive organisms would accelerate disease development, and studies to directly quantitate this are in progress.

The basis for the lack of colitis development in recipients of antigen-naive DO11.RAG-2/− T cells colonized with OVA-E. coli is not yet known, but it is not due to antigenic ignorance or neglect, nor defective trafficking of reactive T cells to the colonic mucosa. Since in previous models of colitis the differentiation state of the T cell population at the onset of experimental protocols could not be defined, it has been unclear whether a pathogenic population was present in the T cell repertoire or developed in response to stimulation by enteric bacteria. In the CD45RB colitis model, for example, it has not been possible to determine whether the pathogenic Th1 response has been due to the development of a Th1 effector population from uncommitted CD45RBhigh precursors in the absence of CD45RBlow regulatory cells, or whether it was due to the release of committed, memory CD45RBhigh Th1 cells from regulatory control (20). Our data are inconsistent with the former mechanism, since recipients of a uniformly naive (and CD45RBhigh) population of T cells did not develop colitis, despite the absence of a CD45RBlow regulatory population. This suggests that a committed Th1 or Th2 population may be required for disease development and/or may recruit the differentiation of naive precursors activated by bacterial antigens. Alternatively, naive CD4 T cells responsive to bacterially associated antigen in the absence of either pathogenic or regulatory effector cell populations may reestablish a homeostatic balance of both pathogenic and regulatory T cells. Experiments to distinguish these alternatives can be addressed in our model, and are in progress.

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84 Both Th1 and Th2 Are Pathogenic in Colitis


