Colitis Induced by Enteric Bacterial Antigen-Specific CD4+ T Cells Requires CD40-CD40 Ligand Interactions for a Sustained Increase in Mucosal IL-12

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*J Immunol* 2000; 165:2173-2182; doi: 10.4049/jimmunol.165.4.2173
http://www.jimmunol.org/content/165/4/2173
Colitis Induced by Enteric Bacterial Antigen-Specific CD4⁺ T Cells Requires CD40-CD40 Ligand Interactions for a Sustained Increase in Mucosal IL-12

Yingzi Cong,* Casey T. Weaver,† Audrey Lazenby,† and Charles O. Elson²*

C3H/HeJBir is a mouse substrain that is highly susceptible to colitis. Their CD4⁺ T cells react to Ags of the commensal enteric bacteria, and the latter can mediate colitis when activated by these Ags and transferred to histocompatible scid recipients. In this study, multiple long-term C3H/HeJBir CD4⁺ T cell (Bir) lines reactive to commensal enteric bacterial Ags have been generated. All these were Ag specific, pauciclonal, and Th1 predominant; most induced colitis uniformly after transfer to scid recipients. Lesions were focal and marked by increased expression of IL-12p40 and IFN-γ mRNA and protein. Pathogenic Bir T cell lines expressed CD40 ligand (CD40L) when cultured with Ag-pulsed APCs in vitro. Production of IL-12 was also increased in such cultures, an effect that was Ag- and T cell-dependent and required costimulation by CD40, but not by B7. The two Bir T cell lines that did not induce lesions after transfer failed to significantly express CD40L or increase IL-12 when cultured with Ag-pulsed APCs. Administration of anti-CD40L blocked disease expression induced by pathogenic T cells. We conclude that interactions in the colon mucosa between CD40L-expressing Bir Th1 cells with APCs endogenously loaded with commensal bacterial Ags are critical for sustained increases in local IL-12 production and progression to colitis.


In recent years many novel models of inflammatory bowel disease have been identified in rodents (1). Some of these have occurred in mice made deficient in one or another immune molecule by selective gene targeting, e.g., IL-2, IL-10, and TCRαβ (2–4). Although the deficiency of such molecules is global, inflammatory disease has been localized mainly to the colon, suggesting a role for the enteric bacterial flora in driving disease. Indeed, in multiple instances disease is abolished under germfree conditions (2, 5–8) or is ameliorated by antibiotics (9) or by limited bacterial colonization (10). The effector cell in most of these new animal models is the TCRαβ⁺, CD4⁺ T cell (11–14). In most cases these T cells produce high levels of IFN-γ. IL-12 has been shown in some studies to play an important role in the initiation and progression of disease (15–19). The exact mechanisms by which the flora triggers pathogenic responses by CD4⁺ T cells remains unknown in most models, i.e., whether by nonspecific or Ag-specific stimulation.

Some insights into the last question have come from studies in the C3H/HeJBir mouse, a new substrain that is highly susceptible to colitis (20). Under certain housing conditions, these mice can develop a spontaneous colitis that resolves over time. These mice have been previously shown to have increased Ab reactivity to commensal bacteria in the gut as well as increased CD4⁺ Th1 reactivity to enteric bacterial Ags (21, 22). CD4⁺ T cells from C3H/HeJBir mice stimulated with enteric bacteria-pulsed APCs induced colitis when transferred into histocompatible scid recipients. In this instance the stimulatory material in the enteric bacterial preparations was found to be conventional protein Ags of the commensal flora (22).

There are several mechanisms that direct the phenotype development of responding CD4⁺ T cells toward IL-2- and IFN-γ-producing Th1 cells or toward IL-4 and IL-10-producing Th2 cells. For example, low Ag concentration (23) and the presence of IL-4 (24) promote the development of Th2 cells. In contrast, the presence of IL-12, a heterodimeric cytokine produced by APCs, has been shown to greatly enhance Th1 cell development and IFN-γ production (25–28). An important role of IL-12 in experimental colitis has been demonstrated, e.g., treatment with anti-IL-12 mAb inhibited colitis in a trinitrobenzenesulfonic acid (TNBS)-induced model (16), in IL-10−/− mice, and in a CD4⁺ CD45RB⁺⁺ T cell scid transfer model (18, 19). Transfer of bone marrow cells from STAT-4-deficient mice, in which T cells do not respond to IL-12, into Tg26 mice induced milder disease compared with transfer of bone marrow cells from normal mice (18), but the precise mechanisms that regulate the production of IL-12 in enteric bacterial Ag-driven responses, and thus drive disease development, are not yet known. Several recent studies have demonstrated that the interaction of CD40 ligand (CD40L) on activated T cells with CD40 on APCs leads to the secretion of IL-12 by APC, including monocytes, dendritic cells, and macrophages, and that such interactions are critical for IL-12 production in Ag-driven responses (29–31).

For these reasons, the role of IL-12 production in the C3H/HeJBir transfer model and the effects of CD40L-CD40 interactions were given particular emphasis in this study. To this end, eight enteric bacterial Ag-specific Bir T cell lines from colitic C3H/HeJBir mice were analyzed for their ability to express CD40L and IL-12 and to mediate disease in a scid recipient model. Results of these studies are described in this report.

²This work was supported by a grant from the National Institute of Diabetes, Digestive, and Kidney Diseases (DK44240).

3 Abbreviations used in this paper: CD40L, CD40 ligand; CBA, cecal bacterial Ag; RPA, RNase protection assay.

Received for publication August 4, 1999. Accepted for publication June 5, 2000.

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HeBir mice have been established. Six of the T cell lines expressed CD40L, stimulated IL-12 production by cecal bacterial Ag (CBA)-pulsed APCs in vitro, and induced colitis when transferred into scid recipients, lesions marked by increased IL-12 and IFN-γ production. The two Bir T cell lines that did not express CD40L did not stimulate IL-12 production and did not induce colitis in scid recipients despite their production of Th1 cytokines in vitro.

Materials and Methods

**Mice**

C3H/HeBir, C3H/HeJ, and C3H/HeSn x Pkrdc<sup>™/™</sup> (scid) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the University of Alabama animal facility. All studies were approved by the animal care and use committee of the University of Alabama at Birmingham.

**Reagents and Abs**

RPMI 1640, FBS, 2-ME, HEPES, L-glutamine, and sodium pyruvate were purchased from Life Technologies (Long Island, NY). The multiprobe RNase protection assay kit was purchased from PharMingen (San Diego, CA). mAbs to CD3, CD4, CD8, ICAM-1, β2, integrin, CD40L, B7.1, B7.2, CD44, L-selectin, TCRβ, TCRβ2, Vβ3, Vβ6, Vβ7, Vβ8, Vβ9, Vβ10, Vβ13, and Vβ14 were purchased from PharMingen. Anti-I-A<sup>β</sup> and anti-I-A<sup>α</sup> were purchased from American Type Culture Collection (Manassas, VA). Hamster anti-mouse CD40L mAb was purified on a protein A affinity column from culture supernatants of the hybridoma line MR1, which was a gift from Dr. Brian Kelsoe (National Institutes of Health, Bethesda, MD). Control hamster IgG of the same isotype was obtained from PharMingen.

**Preparation of enteric bacterial Ags**

Enteric bacterial Ags were prepared as previously described (21). Briefly, C3H/HeJ or C3H/HeSn x scid mice were sacrificed, and their ceca were removed. The ceca were opened and placed in 1 ml of PBS. Cecal bacteria were removed. After addition of DNase (10 μg/ml), 1 ml of this bacterial suspension was added to 1 ml of glass beads. The cells were disrupted at 5000 rpm in a Minin bead Beater (BioSpec Products, Bartlesville, OK) for 3 min and then placed on ice. The glass beads and unlysed cells were removed by centrifugation at 5000 × g for 5 min. The lysates were filtered sterilized through a 0.2-μm pore size syringe filter.

**Establishment and maintenance of enteric bacterial Ag-specific CD4<sup>+</sup> T cells**

C3H/HeBir spleen and mesenteric lymph nodes were removed from groups of four or five mice and placed into cell suspension by straining through a small mesh sieve as described previously (22). After two washes, the cells were passed through a nylon wool column to obtain T cells as described previously (22). After two washes, the cells were passed through a nylon wool column to obtain T cells as described previously (22). After two washes, the cells were passed through a nylon wool column to obtain T cells as described previously (22).

**Flow cytometric analysis**

Flow cytometric analysis of T cell lines was conducted as described previously (33). In brief, T cell lines were harvested from plates 8–10 days after restimulation, and dead cells were removed by centrifugation over Ficoll. After washing with PBS with 0.1% sodium azide plus 2% heat-inactivated fetal calf serum, the cells were incubated with various FITC- or PE-conjugated mAbs, washed, and fixed in 1% buffered paraformaldehyde. 10<sup>6</sup> stained cells were analyzed using a FACStar flow cytometer (Becton Dickinson, Mountain View, CA). An FITC- or PE-labeled mAb of the same isotype but irrelevant specificity was used as a negative control in all experiments.

**Immunoscope analysis**

Details of the method have been published (34, 35). Briefly, total RNA was isolated from viable cells of the Bir cell lines using RNeasy miniprep columns according to the manufacturer’s instructions (Qiagen, Santa Clarita, CA), CDNA was synthesized from 1 μg of total RNA using (dT)<sub>15</sub> primer and 10 U of avian myeloblastosis virus (AMV) reverse transcriptase (Roche, Indianapolis, IN) in the presence of 25 U of RNasin (Promega, Madison, WI). The TCRβC- and TCRβV- specific primers used in the PCR were previously described (36). All PCRs were performed using 25 U/ml Taq polymerase, dNTPs (0.2 μM each), primers (0.5 μM each), and 10 μl of reverse transcribed RNA in a 25-μl final volume. Forty cycles of PCR amplification were performed, each consisting of 45 s at 94°C, 45 s at 60°C, and 45 s at 72°C, following a hot start of 30 s at 94°C. An aliquot of the resulting PCR products was subject to a run-off reaction in which one to five cycles of further amplification were performed under similar conditions as the PCR, but substituting a single TCRβC dye-labeled primer at nucleotide 311 (34). The run-off products were loaded on a 6% polyacrylamide sequencing gel and electrophoresed on an ABI-373 DNA sequencer (Perkin-Elmer, Emeryville, CA) together with size standards. The data were analyzed with Immunoscope software developed by C. Pannetier (35).

**Ex vivo organ culture**

Ex vivo organ culture fragments of colon and small intestine were performed by removing and longitudinally opening small and large intestines. After washing with cold RPMI containing 20 μg/ml of gentamicin three times, three 3-mm circular full-thickness pieces of small intestine and colon were obtained using a 3-mm dental punch (Baker-Cummings, Miami, FL). Each fragment was then placed in 0.5 ml of complete medium in separate wells of a 48-well plate and incubated for 24 h at 37°C in 5% CO₂ humid air. Culture supernatant from each biopsy was collected and stored at −20°C before analysis for cytokine content.

**Cytokine assays**

T cell lines were cultured with Ag-pulsed APCs as described above. Culture supernatants were collected at different times and pooled for assay. The supernatants collected after 24 h of culture were used for IL-2 assay, and the supernatants collected at 48–72 h of culture were used for IL-4, IL-10, and IFN-γ assays. For IL-12 production, C3H/HeSn x scid spleen APC were cultured with T cell lines under different conditions as described in text, and the supernatant was collected at 48 h of culture.

The cytokine content in supernatants was determined by ELISA. Briefly, 96-well polystyrene microtiter plates (Dynatech, Rockville, MD) were coated with primary anti-cytokine capture mAb (2 μg/ml) overnight. After an incubation with sample supernatant or cytokine standards, the wells were incubated with biotinylated detection anti-cytokine mAb, followed by an incubation with HRP-avidin. Finally, 50 μl of tetramethylbenzidine was added to each well, and the color development after incubation at room temperature was measured at OD<sub>490</sub> in an EL 312e ELISA reader (Bio-Tek Instruments, Winooski, VT). A standard curve was constructed for each assay, and the values of the samples were interpolated using the DeltaSoft III program (BioMetallics, Princeton, NJ). All mAb pairs were purchased from PharMingen and are listed by capture/biotinylated detection mAb as follows: IL-2, JES5-1A12/JES6-5H4; IL-4, BV4D-1B1/BV6D-24G2; IL-10, JES5-2A5/SXC-1; IFN-γ, R4-6A2/XMG12; and IL-12, C17.8/C15.6.

**Flow cytometric analysis**

Flow cytometric analysis of T cell lines was conducted as described previously (33). In brief, T cell lines were harvested from plates 8–10 days after restimulation, and dead cells were removed by centrifugation over Ficoll. After washing with PBS with 0.1% sodium azide plus 2% heat-inactivated fetal calf serum, the cells were incubated with various FITC- or PE-conjugated mAbs, washed, and fixed in 1% buffered paraformaldehyde. 10<sup>6</sup> stained cells were analyzed using a FACStar flow cytometer (Becton Dickinson, Mountain View, CA). An FITC- or PE-labeled mAb of the same isotype but irrelevant specificity was used as a negative control in all experiments.
mRNA hybrids were extracted with phenol-chloroform, precipitated with ethanol, dissolved in loading buffer, and electrophoresed on a 5% polyacrylamide-urea sequencing gel. The wet gel was placed on photographic film overnight at 80°C, then the film was developed.

Cell transfer and evaluation of colitis

For control CD4+ T cell transfers, 2 × 10^6 freshly prepared or anti-CD3-activated CD4+ T cells from spleen and mesenteric lymph nodes of C3H/HeJ or C3H/HeBir mice were transferred i.v. into C3H/HeSnJ scid recipients. For CD4+ Bir T cell line transfers, the T cell lines were harvested 5 days after restimulation with CBA-pulsed APCs, and dead cells were removed by centrifugation over lymphocyte-M (Cedarlane, Hornby, Canada). Then, 2 × 10^6 viable T cells were transferred i.v. into C3H/HeSnJ scid recipients. Three months later, the recipients were killed, and the cecum and the proximal, middle, and distal portions of the colon were fixed in formalin. If animals looked ill and had lost >20% of initial body weight, they were killed at that point. In that instance one mouse from the control group was also sacrificed at the same time to make the comparison equivalent. Fixed tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin for histologic examination. All slides were read by an experienced pathologist (A.L.) without knowledge of their origin.

In certain experiments pathogenic Bir T cells were transferred into C3H/HeSnJ scid recipients. The recipients received 200 μg/ml of CBA, food, epithelial Ag, or OVA overnight. Bir T cells (2 × 10^6) were cultured with 2 × 10^6 APCs, [3H]TdR (0.5 μCi) was added to the wells for the last 18 h of a 4-day culture. Results are expressed as mean cpm ± SD of triplicate cultures.

Statistics

The results were expressed as the mean ± SD. The significance of the difference between means was determined by Student’s t test.

Results

Ag specificity of Bir T cell lines

Our previous studies indicated that C3H/HeBir mice, a substrain of C3H/HeJ that can develop colitis spontaneously, have a strong CD4+ T cell response to Ags of the commensal bacterial flora (22). To further characterize such enteric bacterial Ag-specific T cells, T cell lines specific for enteric bacterial Ags were established by repeated stimulation of purified CD4+ T cells from spleen and mesenteric lymph nodes of C3H/HeBir mice with APCs pulsed with lysates of enteric bacteria obtained from the cecum. Eight T cell lines were generated at different times over a 1-yr interval, and each was maintained in continuous culture with CBA-pulsed APCs for over 6 mo. To determine the Ag specificity of CBA-specific Bir T cell lines, we assessed their ability to proliferate when stimulated with CBA, food, and epithelial or unrelated Ags. None responded to CBA in the absence of APC or in the presence of unpulsed APC. All strongly responded to CBA-pulsed APCs pulsed with epithelial cell, food, or unrelated protein Ags such as OVA (Table I).

Because our previous studies found that the C3H/HeBir CD4+ T cell response to enteric bacterial Ags was MHC class II restricted (22), mAbs to MHC class II I-A^k and I-A^b molecules were added to cultures of Bir T cell lines. Addition of anti-I-A^k mAb, but not irrelevant control anti-I-A^b mAb, blocked T cell proliferation in response to CBA-pulsed APCs (data not shown), confirming the MHC dependence of the CBA-specific response of these Bir T cell lines.

Table 1. Proliferative responses of Bir T cell lines to stimulation with cecal bacterial and other Ags*  

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>APC alone</th>
<th>CBA-APC</th>
<th>Food Ag-APC</th>
<th>Epithelial Ag-APC</th>
<th>OVA-APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC alone</td>
<td>658 ± 99</td>
<td>843 ± 268</td>
<td>1,321 ± 444</td>
<td>1,203 ± 196</td>
<td>1,209 ± 268</td>
</tr>
<tr>
<td>CBA-APC</td>
<td>698 ± 101</td>
<td>981 ± 166</td>
<td>1,321 ± 454</td>
<td>1,222 ± 356</td>
<td>1,321 ± 343</td>
</tr>
<tr>
<td>Food Ag-APC</td>
<td>891 ± 233</td>
<td>1,321 ± 454</td>
<td>1,321 ± 454</td>
<td>1,222 ± 356</td>
<td>1,321 ± 343</td>
</tr>
<tr>
<td>Epithelial Ag-APC</td>
<td>1,024 ± 314</td>
<td>1,019 ± 356</td>
<td>1,321 ± 343</td>
<td>1,321 ± 343</td>
<td>1,321 ± 343</td>
</tr>
<tr>
<td>OVA-APC</td>
<td>1,187 ± 255</td>
<td>1,366 ± 587</td>
<td>1,397 ± 571</td>
<td>1,200 ± 300</td>
<td>1,333 ± 320</td>
</tr>
</tbody>
</table>

* Splenic APCs from C3H/HeJ mice were incubated with medium alone or 200 μg/ml of CBA, food, epithelial Ag, or OVA overnight. Bir T cells (2 × 10^6) were cultured with 2 × 10^6 APCs. [3H]TdR (0.5 μCi) was added to the wells for the last 18 h of a 4-day culture. Results are expressed as mean cpm ± SD of triplicate cultures.
Phenotypic characterization of Bir T cell lines

To define the phenotype of the CBA-specific T cell lines, Bir T cells were harvested 8–10 days after restimulation with CBA-pulsed APCs, stained with mAb to various surface membrane molecules, and analyzed by flow cytometry. All T cell lines were CD4+ TCR Vβ6, TCRββ and expressed high levels of CD44 and ICAM-1 and low levels of L-selectin and CD69. They also expressed CD25, the high affinity IL-2R. Interestingly, even after long-term maintenance in culture, a significant fraction of the cells expressed high levels of CD45RB Ag. β2 integrin was expressed by 30–60% of cells in the different T cell lines, compatible with cells able to home to mucosal sites. Fig. 1 shows the flow cytometric profile of Bir 3 T cells as a representative example.

Cytokine production by Bir T cell lines

To measure the cytokine production of Bir T cell lines stimulated with CBA-pulsed APCs, culture supernatants were collected at different times after Ag restimulation, and IL-2, IL-4, IL-10, and IFN-γ production was measured by cytokine-specific ELISA (see Materials and Methods). As shown in Table II, although different T cell lines produced varying amounts of cytokines in response to CBA stimulation, all T cell lines produced relatively high levels of IL-2 (97–222 U/ml) and IFN-γ (82–282 U/ml) and relatively small amounts of IL-4 (11–32 U/ml) and IL-10 (11–31 U/ml), indicating that all eight Bir T cell lines were Th1 predominant.

TCR Vβ usage and CDR3 length analysis of Bir T cell lines

To determine the TCR Vβ usage by the Bir T cell lines, cells were stained with different anti-TCR Vβ Abs and analyzed by flow cytometry. In addition, TCR repertoire was assessed by Immunoscope, a PCR-based CDR3 length display analysis. At the time of these analyses, the cell lines had been in culture for at least 6 mo. Compared with fresh splenic CD4+ T cells, Bir T cell lines used a restricted set of TCR Vβ families (Fig. 2). Six Bir T cell lines (Bir 3, Bir 4, Bir 5, Bir 6, Bir 7, and Bir 9) used predominantly TCR Vβ6, -8, and -10. Bir 1 and Bir 2 lines used mostly TCR Vβ10 (>95% of the total cells). These lines thus use the same TCR Vβs that are predominant in the C3H/HeJ Bir colon lamina propria (Fig. 2). Using Immunoscope, fresh splenic CD4+ T cells demonstrated a polyclonal TCRVβ CDR3 pattern as expected (Fig. 3). In contrast, the Bir 3 and Bir 4 cell lines had a pauciclonal TCRVβ CDR3 phenotype (Fig. 3) despite being stimulated with an unselected mixture of thousands of enteric bacterial Ags. Other Bir lines had a pauciclonal phenotype as well, consistent with a response to a restricted number of immunodominant Ags.

Transfer of colitis by Bir T cell lines

To investigate the ability of these CBA-specific Bir T cell lines to induce colitis, 2 × 10⁶ cells from different Bir T cell lines were separately transferred into groups of four histocompatible C3H/HeSnJ scid recipients 5 days after restimulation with CBA-pulsed APCs. Three months after cell transfer, the recipients were sacrificed, and the histopathology of cecum and proximal, middle, and distal portions of colon and small intestine was examined. All recipients of six T cell lines, Bir 3, Bir 4, Bir 5, Bir 6, Bir 7, and Bir 9, developed colitis (Table II and Fig. 4). The lesions in the recipients were focal and localized to the cecum and proximal colon, similar to the pattern seen in the donor C3H/HeJ Bir mice. The small intestines of these recipients were not inflamed. Transfer of Bir 1 and Bir 2 cells did not induce colitis, although both lines reconstituted gut lymphoid tissue based on histological analysis. Transfer to scid mice of either fresh C3H/HeJ Bir CD4+ T cells or C3H/HeJ Bir CD4- T cells activated in vitro with anti-CD3 mAb did not result in colitis in any recipient (Table II and data not shown). Cotransfer of Bir 1 and Bir 2 with pathogenic Bir T cell lines did not inhibit disease caused by the latter (data not shown).

Increased IL-12 and IFN-γ expression in colon lesions

To investigate cytokine production in the colonic lesions of Bir T cell recipients, cytokine mRNA expression was measured by a multiprobe RPA. Representative data are shown in Fig. 5. IL-12p40 and IFN-γ mRNA expression was increased, as were IL-1α, IL-1β, and IL-6 mRNA in the colon of mice receiving pathogenic Bir T cell lines. In contrast, none of these cytokine mRNA was increased in the colon of the recipients of Bir 1 T cells, Bir 2 T cells, and either fresh or anti-CD3-activated C3H/HeJ Bir CD4+ T cells, none of which developed colitis (Fig. 5 and data not shown).

To determine the IL-12 protein levels produced locally in the lesions, ex vivo organ cultures of small and large intestines of scid recipients of control anti-CD3-activated CD4+ T cells or of pathogenic Bir 3 T cells were incubated in medium for 24 h, the supernatants were collected, and IL-12 and IFN-γ levels were measured by ELISA. Colon from Bir 3 T cell recipients produced high levels of IL-12p40. In contrast, colon from recipients of anti-CD3-activated CD4+ T cells did not produce detectable IL-12p40, nor did colon from scid mice that received no T cells (Fig. 6 and data not shown). Colon of Bir 3 T cell recipients, but not that of recipients of anti-CD3-activated CD4+ T cells or of naive scid mice, also produced detectable IFN-γ (Fig. 6 and data not shown). No small intestinal cultures for any group produced detectable IL-12 or IFN-γ after overnight culture.

| Table II. Bir T cell line cytokine production and ability to transfer colitis |
|------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Cell Line | IL-2 (U/ml) | IFN-γ (U/ml) | IL-4 (U/ml) | IL-10 (U/ml) | Transfer of Colitis to scid Recipients |
| Bir 1 | 121 ± 23 | 189 ± 38 | 32 ± 5 | 26 ± 4 | 1/4 |
| Bir 2 | 98 ± 15 | 68 ± 21 | 25 ± 6 | 31 ± 3 | 0/20 |
| Bir 3 | 186 ± 39 | 225 ± 44 | 16 ± 5 | 19 ± 5 | 0/20 |
| Bir 4 | 222 ± 36 | 98 ± 18 | 20 ± 2 | 13 ± 4 | 0/8 |
| Bir 5 | 156 ± 29 | 165 ± 27 | 11 ± 5 | 21 ± 11 | 4/4 |
| Bir 6 | 139 ± 14 | 111 ± 18 | 19 ± 5 | 11 ± 4 | 4/4 |
| Bir 7 | 97 ± 16 | 82 ± 11 | 24 ± 7 | 12 ± 3 | 4/4 |
| Bir 9 | 209 ± 43 | 282 ± 45 | 32 ± 6 | 28 ± 5 | 4/4 |

* Bir T cells were stimulated with CBA-pulsed APCs, and the supernatants were collected at day 1 for measuring IL-2 and day 3 for measuring IL-4, IL-10, and IFN-γ by ELISA.

* Bir T cells (2 × 10⁶) were transferred into scid recipients, and 12 wk later the mice were killed and the histopathology assessed.

* One recipient had a mild lesion.
IL-12 production by Ag-pulsed APCs in the presence of Bir T cell lines

To investigate the mechanism of the increased IL-12 production driven by Bir T cells, IL-12p40 production by APCs interacting with pathogenic Bir T cell lines was assessed in vitro. C3H/HeSnJ scid spleen APCs were incubated overnight with or without CBA, then cultured with various Bir T cells. Culture supernatants were collected after 48 h, and IL-12p40 production was measured by ELISA. As shown in Fig. 7A, high levels of IL-12p40 were produced in cocultures containing CBA-pulsed APCs, and each of the six pathogenic Bir T cell lines (Bir 3, Bir 4, Bir 5, Bir 6, Bir 7, and Bir 9). No detectable IL-12p40 was produced by APCs in the absence of Bir T cells regardless of whether these APCs were pulsed with CBA (Fig. 7B), indicating that APC IL-12p40 production is both Ag and T cell dependent. In contrast, CBA-pulsed APCs cocultured with nonpathogenic Bir 1 or Bir 2 T cell lines produced very low levels of IL-12p40 compared with the pathogenic Bir T cell lines under the same conditions (Fig. 7A).

Bir T cell stimulation of APC IL-12 production required CD40 costimulation

To determine whether CD40-CD40L interactions between CBA-pulsed APC and Bir T cells were involved in APC IL-12p40 production, anti-CD40L mAb MR1 was added to the cultures. As shown in Fig. 7B, addition of anti-CD40L inhibited IL-12p40 production by 80–90%. Anti-CD40L also inhibited IFN-γ production by Bir 3 T cells (data not shown). To examine whether the anti-CD40L mAb inhibition of IL-12p40 production was mediated by...
decreased IFN-γ production by Bir T cells, anti-IFN-γ mAb was added to cultures of Bir 3 T cells and CBA-pulsed APCs. Addition of anti-IFN-γ mAb inhibited IL-12p40 production by about 30–40% (data not shown), but never reached the degree of inhibition seen with anti-CD40L, indicating that CD40-CD40L interactions were directly involved in the induction of IL-12p40 production. Addition of anti-B7.1 (CD80) and anti-B7.2 (CD86) mAbs, which block B7-CD28 costimulation, had no effect on IL-12 production, but did inhibit the Bir 3 T cell proliferation stimulated by CBA-pulsed APCs (Fig. 7B). Interestingly, addition of anti-CD40L had no effect on Bir 3 T cell proliferation (Fig. 7B).

Because the nonpathogenic Bir 1 and Bir 2 T cell lines stimulated only low levels of IL-12p40 in vitro, we examined their expression of CD40L after CBA-pulsed/APC activation using flow cytometry. Eight hours after Ag stimulation, 31% of Bir 3 T cells expressed CD40L on the surface (Fig. 8A), whereas only 2–3% of the nonpathogenic Bir 1 or Bir 2 T cells expressed CD40L (Fig. 8B). To determine whether the low level of CD40L expression on Bir 1 and Bir 2 T cells was due to differences in the kinetics of expression, CD40L expression on Bir 1 and Bir 2 T cells was measured at 4, 8, 12, 24, and 48 h after Ag stimulation. Bir 1 and Bir 2 T cells expressed low levels of CD40L at all these time points.

The data are plotted as fluorescence intensity in arbitrary units on the abscissa, and CDR3 size is shown in amino acids on the ordinate. The latter was deduced from the size of the PCR fragments.
points; these cell lines were also deficient in CD40L expression after activation by αCD3 mAb (data not shown).

*Anti-CD40L blocked disease expression in vivo*

To directly test the role of CD40L-CD40 interactions in the pathogenesis of colitis in vivo, anti-CD40L was administered to C3H/HeSnJ scid recipients of a pathogenic Bir T cell line. A control group received polyclonal IgG. All four recipients in the control group developed colitis at 10 wk after transfer (Fig. 9). None of the four recipients of anti-CD40L mAb developed colitis, although CD3+ T cells were clearly present in the colon based on immunohistochemical stain.

**Figure 6.** IL-12 and IFN-γ production in colon explant cultures of recipients of pathogenic Bir 3 T cells vs control anti-CD3-activated CD4+ T cells. Ten weeks after cell transfer, ex vivo organ fragment cultures of colon were performed as described in Materials and Methods. Culture supernatants were collected, and IL-12p40 (A) and IFN-γ (B) were measured by ELISA.

**Figure 7.** Pathogenic Bir T cell stimulation of IL-12 production by CBA-pulsed APCs. Bir 3 T cells (2 x 10^5) were cultured with splenic APCs from anti-scid mice in the absence or the presence of anti-CD40L, anti-B7.1, or anti-B7.2. A, IL-12p40 production by CBA-pulsed APCs reactive with different Bir T cell lines. B, IL-12p40 production (left) and proliferation of Bir T cells (right) as measured by [3H]TdR incorporation assay.

**Figure 8.** CD40L expression by Bir T cells stimulated with CBA-pulsed APCs. Bir 3 T cells (A) and Bir 1 T cells (B) were cocultured with CBA-pulsed APCs for 8 h, then incubated with mAb against CD40L (solid line) and analyzed by FACS. Cells were incubated with mAb of same isotype but irrelevant specificity as a negative control (dotted line). Data are representative of two similar experiments.
mice. All Bir T cell lines maintain a Th1-predominant cytokine phenotype in long-term cultures in vitro, similar to the cytokine phenotype identified previously in freshly obtained C3H/HeJBir CD4⁺ T cells activated by CBA-pulsed APCs (22).

In a previous study of the Ab response to enteric bacterial Ags of the C3H/HeJBir mouse, we found the response to be highly selective, targeting a very small number of the large numbers of protein Ags present in the enteric bacteria (21). Because the Bir T cell lines are stimulated by freshly obtained enteric bacterial Ags, which contain a myriad of different proteins, a polyclonal T cell response would be expected. We used the Immunoscope technique, which provides a more sensitive analysis of the TCR repertoire than is possible by flow cytometry. All the Bir T cell lines were pauciclonal rather than polyclonal. Most of the TCR peaks were individual to the different cell lines (compare Bir 3 and Bir 4 in Fig. 4); however, there were some TCR that were shared among lines, suggesting the existence of public specificities in the T cell antibacterial response (38). The most dramatic example of such a public specificity came from analysis of the Bir 1 and Bir 2 cell lines, which both use a Vβ10, Jβ2.7 chain with a CDR3 length of eight residues. Sequencing of the CDR3 region of these two lines has demonstrated that they use the same TCR β-chain even though they were generated from different mice at different times. This fits the definition of a public specificity TCR response and is consistent with a T cell response to the same immunodominant enteric bacterial Ag. The pauciclonal Bir T cell response is consistent with the highly selected Ab response identified in the previous study (21). Indeed, the same immunodominant Ags are probably triggering both B cells and T cells, because depletion of CBA preparations by immunoprecipitation with serum Ab reduces the CD4⁺ T cell response by 70% (data not shown).

The transfer of six of the Bir T cell lines to histocompatible scid mice induced colitis in all recipients (Table II). The resulting colitis was focal and transmural, similar to that previously shown after transfer of C3H/HeJBir CD4⁺ T cells activated by CBA-pulsed APCs in short term cultures. The reasons for the focality of lesions are not yet known, but may relate to the pauciclonal TCR used by the lines and/or the presence of relevant bacterial Ags in high concentrations in certain niches in the colon. Most lesions are found in the cecum and proximal colon, which is where the bulk of the bacterial Ag is present. There were two notable exceptions to the uniform induction of colitis after transfer of these Th1-predominant cell lines. Transfer of the Bir 1 or Bir 2 cell lines did not cause colitis in scid recipients, although they clearly repopulated the colon lamina propria as seen on histology. These lines did not inhibit colitis when cotransferred with one of the pathogenic Bir T cell lines. As mentioned above, the Bir 1 and Bir 2 cell lines were nearly clonal and shared the same TCR β-chain, but otherwise they were indistinguishable from the pathogenic cell lines, e.g., producing substantial amounts of IFN-γ in vitro. Although these two lines had a much more limited TCRBV expression, the Ags triggering them were presumably immunodominant, and thus the reasons for their lack of pathogenicity remained puzzling.

Analysis of the local cytokine production in the colon of scid recipients of pathogenic Bir cell lines demonstrated a pattern consistent with a Th1-mediated disease. Both IFN-γ and IL-12 were increased, as measured by mRNA expression determined by RPA and by cytokine protein levels found in ex vivo organ explant cultures of affected colon. In addition, IL-1β, IL-1α, and IL-6 were elevated. IL-12 has been implicated as playing a central role in colitis induction and progression in several other animal models, including trinitrobenzene sulfonic acid-ethanol-induced colitis (16), IL-2-deficient mice (17), IL-10-deficient mice (19), and bone marrow-reconstituted Tg26 mice (18). Recent data indicate that
IL-12 may play a role in progression of disease independently of IFN-γ by mechanisms that remain obscure (19). The results from the present study demonstrate an important role for IL-12 in the C3H/HeJ/Bir mouse as well. Recent reports indicate that IL-12 production by APCs requires CD40-CD40L interactions (29–31). Accordingly, we tested the interactions between pathogenic Bir cell lines with enteric bacterial Ag-pulsed APCs in vitro, specifically asking what costimulatory signals were required in this interaction. Interestingly, different costimulatory signals were involved in IL-12 production compared with T cell expansion. Blocking CD40-CD40L interaction substantially inhibited IL-12 production by APCs, but had no effect on Bir CD4+ T cell proliferation, whereas blocking CD28-B7 interactions had no effect on IL-12 production, but substantially inhibited Bir CD4+ T cell proliferation. In regard to the latter, anti-B7.1 had more effect than did anti-B7.2, but the combination of the two was consistently superior to either alone, suggesting a role for both B7.1 and B7.2 in the expansion of pathogenic Bir cell lines both in vitro and in vivo. Although these studies were performed in vitro, the local increase in IL-12 mRNA and production the colon of scid recipients of pathogenic Bir T cell lines argues strongly that the same interactions occur in vivo between transferred Bir CD4+ T cells and local APCs in the colon mucosa. Because no IL-12 or IFN-γ is produced in the absence of Ag, i.e., in cultures of CD4+ T cells with naïve APCs, it follows that disease following transfer with pathogenic Bir T cells requires interaction with APCs in the colon that are primed naturally with enteric bacterial Ags (39, 40). Further support for this idea comes from additional studies performed on the nonpathogenic, but Th1-predominant, Bir 1 and Bir 2 cell lines. Analysis of the interactions of these cell lines with Ag-pulsed APCs in vitro revealed that these lines only weakly expressed CD40L and stimulated little or no IL-12 production by APCs. The former was not due to insufficient activation by Ag, in that the same result occurred after anti-CD3 activation of the Bir 1 and Bir 2 cell lines. Moreover, the Bir 1 and Bir 2 T cell proliferative responses to enteric Ag-pulsed APCs are comparable to those of the pathogenic T cell lines. The Bir 1 and Bir 2 T cell lines also fail to increase IL-12 or IFN-γ mRNA in the colon after transfer to scid recipients. The inability to up-regulate CD40L by the Bir 1 and Bir 2 cell lines explains their lack of pathogenicity in vivo. If so, then blocking CD40L-CD40 interactions between a pathogenic Bir T cell line and endogenous APCs in vivo should prevent the development of colitis. Indeed, anti-CD40L treatment of recipients of the pathogenic Bir 3 cell line did prevent lesions from developing. These results indicate that CD40L-up-regulation and stimulation of IL-12 production in the mucosa are pivotal events in the pathogenesis of colitis mediated by bacteria-reactive CD4+ T cells and support previous evidence of this in a hapten-mediated system (41). These studies clearly demonstrate that CD4+ T cells directed at commensal bacterial Ags can cause chronic inflammatory bowel disease. Significantly, these CD4+ Bir T cell lines are derived from C3H/HeJ/Bir mice at 3 mo of age, at a time when the mice themselves have recovered from colitis. The reason for the lack of disease despite the presence of potentially pathogenic T cells in vivo is probably due to the activity of regulatory T cells that prevent expression of the pathogenic T cell program in the bowel. The identity of such regulatory T cells is unclear, but candidates include Th2, Th3, and Tr1 cells. The lack of colonic disease in normal mice despite the huge antigenic load in the colon indicates a tight regulation of the mucosal immune response to these Ags (1). Perturbation of this regulation is the likely explanation for the colitis developing in a number of gene-targeted knockout mice. Indeed, a state of tolerance to Ags of the enteric bacterial flora may exist in normal hosts (42, 43), but how such tolerance to living organisms is induced and maintained remains unknown. However, the present studies give support to this idea by demonstrating the detrimental consequences of nontolerized, unregulated CD4+ effector T cells in the colon mucosa.

Acknowledgments

We thank P. Pierre-Antoine Cazenave and Philippe Kourilsky of Pasteur Institute (Paris France) for their support of the Immunoscope analysis performed in this study, and Dr. John P. Sundberg for critical review of the manuscript.

References


