CD4+ T cells from IL-10-deficient mice transfer susceptibility to NSAID-induced Rag colitis

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Submitted 18 December 2003; accepted in final form 23 January 2004

Blum, Arthur M., Ahmed Metwali, David E. Elliott, Daniel J. Berg, and Joel V. Weinstock. CD4+ T cells from IL-10-deficient mice transfer susceptibility to NSAID-induced Rag colitis. Am J Physiol Gastrointest Liver Physiol 287: G320–G325, 2004. 10.1152/ajpgi.00527.2003.—Products of arachidonic acid metabolism are important for mucosal homeostasis, because blockade of this pathway with an NSAID triggers rapid onset of severe colitis in the IL-10 knock out (IL-10−/−) model of IBD. Rag mice do not make T or B cells. This study determined whether reconstitution of Rag mice with T cells from IL-10−/− mice transferred NSAID colitis susceptibility. Rag mice were reconstituted by intraperitoneal injection with splenocytes from wild-type (WT) or IL-10−/− animals. Colitis was induced by using piroxicam and was graded histologically. Isolated lamina propria mononuclear cells (LPMC), lamina propria T cells, and LPMC depleted of T cells from reconstituted Rag mice were studied for cytokine production. Only animals reconstituted with IL-10−/− CD4+ T cells and administered piroxicam developed severe colitis. LPMC from these colitic animals made IFN-γ, whose production was dependent on T cells. Some IL-10 was produced but only from non-T cells. LPMC from the healthy Rag mice that were reconstituted with WT T cells and were piroxicam resistant made much more IL-10. This was mostly T cell dependent. In conclusion, only CD4+ T cells from IL-10−/− animals leave Rag mice susceptible to NSAID-induced, Th1 colitis. Lamina propria T cells normally make large quantities of IL-10, suggesting that IL-10 from T cells may be protective.

interleukin-10; nonsteroidal anti-inflammatory drugs; inflammatory bowel disease

PROSTAGLANDINS (PGs) modulate many physiological processes. The various PGs have both pro- and anti-inflammatory functions (27). In people with inflammatory bowel diseases (IBDs; e.g., ulcerative colitis or Crohn’s disease), use of NSAIDs that block endogenous PG production is discouraged because they may worsen disease activity (4, 10, 13). Animal models of IBD also suggest that blockade of PG production exacerbates or precipitates colitis (6, 14, 20, 25).

IL-10 has immunomodulatory properties (1, 18). Mice with targeted disruption of the IL-10 gene develop Th1-type colitis, which attests to the importance of IL-10 for maintenance of mucosal immune homeostasis (24). IL-12 drives the differentiation of T cells into the Th1 cell phenotype and promotes IFN-γ production. Development of intestinal inflammation in IL-10 knockout (IL-10−/−) mice is dependent on IFN-γ and IL-12 as well as CD4+ T cells (5, 8, 9). IL-10−/− colitis has a gradual onset (16).

IL-10−/− mice treated with an NSAID to inhibit PG production rapidly develop severe colitis that is histologically and immunologically similar to the spontaneous intestinal inflammation (6). The colitis persists when NSAID treatment is discontinued. Thus both IL-10 and PGs help maintain appropriate mucosal immunoreactivity.

To better understand mechanisms of NSAID-induced mucosal inflammation, we developed a Rag reconstitution model of NSAID-induced IL-10−/− colitis. We found that transfer of CD4+ T cells from IL-10−/− mice into Rag mice makes them susceptible to NSAID-induced colitis. Moreover, Rag mice that received wild-type (WT) CD4+ T cells do not develop colitis after NSAID treatment. Lamina propria T cells normally produce most of the IL-10 in the intestinal mucosa. This suggests that regional loss of T cell IL-10 may contribute to NSAID-induced colitis.

MATERIALS AND METHODS

Mice. Experiments used WT, IL-10−/−, and Rag-1 T and B cell-deficient mice all on the C57BL/6 background. Breeding colonies for the animals were maintained at the University of Iowa. The University of Iowa Animal Welfare Committee approved all studies.

Rag reconstitution and piroxicam treatment. WT splenocytes contained 25% Thy1.2+, 16% CD4+, and 9% CD8+ T cells. Each Rag mouse (~5 wk old) received either 2 × 107 unfractionated splenocytes or 5 × 106 purified Thy1.2+, 3.5 × 106 CD4+, or 2 × 106 CD8+ T cells by intraperitoneal injection. Two weeks later, animals received piroxicam (Sigma, St. Louis, MO) mixed in their food (NIH-31M) for 2 wk. They received 60 mg/250 g of food during week 1 and 80 mg/250 mg food during week 2. Mice subsequently were placed on the normal rodent chow without piroxicam. The colitis was evaluated usually 14 days after stopping the piroxicam.

Histological analysis of colitis. Colons (from the ileocecal valve to the mid descending colon) were opened longitudinally and rolled up onto a glass rod. The tissue was fixed in 4% neutral buffered formalin, removed from the glass rods without unrolling the tissue, and processed for sectioning. Tissue was sliced to obtain longitudinal sections of colon that were 6 μm thick and then stained with hematoxylin and eosin for light microscopic examination. The inflammation was scored from 0 to 4 by using the following criteria: grade 0, no change from normal tissue; grade 1, patchy mononuclear cell infiltrates in the lamina propria; grade 2, more uniform mononuclear cell inflammation involving both the epithelium and the lamina propria (accompanied by minimal epithelial hyperplasia and slight to no depletion of mucous from goblet cells); grade 3, some epithelial and muscle hypertrophy with patchy lymphocytic infiltrates extending into the muscle layers (mucus depletion and occasional crypt abscesses and epithelial erosions); grade 4, lesions involved most of the intestinal section. The inflammation, which was comprised mostly of lymphocytes and some neutrophils, was transmural and severe. There was prominent thickening of both the epithelial and muscle layers.
There was mucus depletion and more frequent crypt abscesses. Ulcerations were frequent.

**Cell isolation and T cell enrichment.** Single cell suspensions of splenocytes were prepared from individual mouse spleens by gentle teasing in RPMI. The cells were briefly resuspended in distilled water to lyse red blood cells. The splenocytes then were washed three times in RPMI.

 Gut lamina propria mononuclear cells (LPMC) were isolated as described below. Intestinal tissue (terminal ileum) was washed extensively with RPMI, and all visible Peyers patches were removed with a scissors. The intestine was opened longitudinally, cut into 5-mm pieces, and then incubated in 0.5 mM EDTA in calcium- and magnesium-free HBSS for 20 min at 37°C with shaking to release intraepithelial lymphocytes and epithelial cells. This was repeated after thorough washing. Tissue then was incubated 20 min at 37°C in 20 ml RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all GIBCO), and 1 mg/ml collagenase (catalog no. CO130; Sigma). At the end of the incubation, the tissue was subjected to further mechanical disruption with a 1-ml syringe. To remove debris, the LPMC preparations were washed through a dampened gauze layered in a funnel with RPMI. Then LPMC were sieved through a prewetted 2-μm nylon wool column gently packed into a 10-ml syringe. After being washed, cells (up to 2 × 10^7) were layered onto a column of Percoll with a 30:70% gradient. Cells were spun at 2,200 g at room temperature for 20 min. The LPMC collected from the 30:70 interface were washed and maintained on ice until being used. Cell viability was 90% as determined by eosin Y exclusion. Splenic T cells (Thy1.2^+^) or their CD4^+^ or CD8^+^ subsets were isolated by negative selection using the SpinSep enrichment procedure and antibody-coated, dense particles as described by the manufacturer (Stem Cell Technologies, Vancouver, BC, Canada).

 To further enrich splenic T cell subsets, cells (10^7^/ml) were incubated for 1 h at 4°C in RPMI containing anti-CD4 (GK1.5; ATCC) or anti-CD8 MAb (TIB112; ATCC) at an appropriate concentration. After incubation, the cells were washed in RPMI by centrifugation at 4°C, suspended in an equal volume of a 1/15 dilution of Low-Tox-M rabbit C (Cedarlane Laboratories, Hornby, ON, Canada), and incubated again for 1 h at 37°C in RPMI containing 25 mM HEPES and 3% BSA fraction V (Amresco, Solon, OH). After being washed, the cells were again treated with antibody + Low-Tox-M rabbit C as described above and washed in RPMI again. Deletion or enrichment of appropriate T cell subsets was confirmed by FACS.

 Lamina propria T cells (Thy1.2^+^) were isolated by using antibody-coated, paramagnetic beads as described by the manufacturer (Dynal, New Hyde Park, NY). Flow cytometry was used after each separation to ensure appropriate recovery and purity (>98%) of the Thy1.2^+^ T cell subsets. The Thy1.2^+^ cells contained all of the other expected leukocyte subsets and were thoroughly depleted of T cells (<1%).

 **Cell culture.** For cytokine analysis, cells were cultured for 48 h in 96-well microtiter plates (Corning, Cambridge, MA) with 200 μl of medium (5 × 10^5^ cells/well) at 37°C. The culture medium was RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5 × 10^-5^ M β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all GIBCO). The cells were cultured alone or with anti-CD3 (2C11; ATCC) and anti-CD28 MAb (PharMingen, San Diego, CA), each at 1 μg/ml. Isolated T cells were cultured in wells previously coated overnight with anti-CD3 (1D3) and CD28 MAb.

 **Flow cytometric analysis.** Spleen cells or LPMC were washed twice and adjusted to 10^7^ cells/ml in FACS buffer (HBSS containing 1% FCS and 0.02% sodium azide). The cell suspensions then were dispensed into microcentrifuge tubes each containing 10^6^ cells in 100-μl FACS buffer and stained with saturating amounts of conjugated antibodies for 30 min at 4°C. After being stained, cells were washed twice. Stained cells were analyzed on a FACS 440 flow cytometer (Becton Dickinson, Mountain View, CA).

Before adding labeled MAb, each tube received 1 μg 2.4G2 antibody (anti-FcyRII; ATCC) to block nonspecific binding of conjugated antibodies to Fc receptors. The other MoAbs used for staining were anti-CD4-Cy5 (Caltag, Burlingame, CA), anti-CD8a-PE (53–6.7; Sigma), anti-Thy1.2-FITC (Ts; Sigma) and anti-CD19-FITC (1D3; Pharmingen, San Diego, CA).

ELISAs. ELISA was used to measure various cytokines in the supernatants. To measure IFN-γ, plates were coated with a MAb to IFN-γ (HB170; ATCC) and incubated with supernatant. IFN-γ was detected with polyclonal rabbit anti-IFN-γ (gift from Dr. Mary Wilson, University of Iowa) followed by biotinylated goat anti-rabbit IgG (Accurate Chemical, Westbury, NY), streptavidin-horseradish peroxidase, and ABTS substrate (Zymed, San Francisco, CA). IL-4 was captured with 11B11 (HB191; DNAX Research Institute, Palo Alto, CA) and detected with biotinylated BV6D (provided by Kevin Moore and John Abrams; DNAX). IL-5 was captured with TRFK5 MAb and detected with biotinylated TRFK4 (Dr. Robert Coffman; DNAX) following by streptavidin-peroxidase conjugate. IL-10 was captured with anti-IL-10 MAb (catalog no. MAB417; R&D Systems, Minneapolis, MN) and detected with biotinylated MAb (catalog no. BAF417; R&D Systems). Sensitivities of the ELISAs were ~30 pg/ml.

MoAbs to IFN-γ (HB170), IL-4, and IL-5 were from cell lines maintained in our laboratory. These MoAbs were purified from culture supernatants by ammonium sulfate precipitation.

**Statistical analysis.** Data are means ± SE of multiple determinations. Each difference between two groups was compared by using the Student’s t-test. P values <0.05 were considered significant.

**RESULTS**

Transfer of IL-10^-/-^ splenocytes into Rag mice renders them susceptible to piroxicam-induced colitis. We previously showed that young IL-10^-/-^ mice reared in a specific pathogen-free (SPF) facility develop a severe and persistent Th1-type colitis after NSAID (piroxicam) treatment. WT mice are resistant to piroxicam-induced intestinal inflammation. We studied a Rag transfer model to determine the importance of T cell subsets and IL-10 in the NSAID-induced disease process.
The Rag mouse protocol involved transferring cells (splenocytes or T cell subsets) by intraperitoneal injection into 5-wk-old Rag mice. They were given piroxicam orally for 2 wk starting 2 wk after cell transfer. Colitis was assessed 2–4 wk after stopping the piroxicam (total of 6–8 wk).

Rag mice reconstituted with splenocytes from C57BL/6 IL-10−/− mice did not develop intestinal inflammation in the absence of piroxicam treatment when observed for up to 6 wk. However, piroxicam induced colitis in Rag mice reconstituted with splenocytes from IL-10−/− animals (Fig. 1). On visual inspection, the inflamed colons grossly appeared shortened, thickened, and leathery. Microscopically, the inflammation involved the colon diffusely, and there was involvement of the terminal ileum. Colitis in IL-10−/− mice frequently is patchy.

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mice were reconstituted with splenic T cells from IL-10 mediated this susceptibility to piroxicam-induced colitis. Rag inflammation at point, because in Subsequent experiments used the 2-wk postpiroxicam time
reconstituted with WT T cells. Cell preparations were 99% Thy1.2 mice or with splenocytes thoroughly depleted of T cells. The T appeared typical of IL-10 and does not involve the terminal ileum. Otherwise, the colitis appeared typical of IL-10−/− intestinal inflammation showing thickening of the epithelial and muscle layers with lymphocytic infiltration extending from the lamina propria deep into the muscle layers and serosa (Fig. 2). Most mice had focal mucosal ulcerations. Rag mice reconstituted with WT splenocytes and observed over the same interval (up to 4 wk) were resistant to piroxicam-induced injury (Fig. 1). Also, there was no colitis in Rag mice receiving IL-10−/− splenocytes but no piroxicam. Subsequent experiments used the 2-wk postpiroxicam time point, because inflammation at weeks 2 and 4 appeared similar. It did not resolve.

Experiments then determined whether IL-10−/− T cells mediated this susceptibility to piroxicam-induced colitis. Rag mice were reconstituted with splenic T cells from IL-10−/− mice or with splenocytes thoroughly depleted of T cells. The T cell preparations were 99% Thy1.2 as determined by flow analysis. The T cell-depleted spleen cell preparations contained <1% T cells. Piroxicam only induced colitis in mice reconstituted with IL-10−/− T cells. Rag recipients of unfractionated T cells from WT mice were not susceptible to piroxicam-induced colitis (Fig. 3). Rag recipients of equal numbers of IL-10−/− and WT T cells also were resistant to piroxicam injury.

Next, IL-10−/− CD4+ and CD8+ T cell subsets were isolated by using a rigorous purification protocol. The CD4+ T cell preparations contained no detectable CD8, whereas the CD8 preparation had ~1% CD4. These were adoptively transferred into Rag mice. Following piroxicam treatment, CD4+ T cell-reconstituted mice had severe colitis, whereas those reconstituted with CD8+ T cells showed little inflammation (Fig. 4). Once again, there was no colitis unless mice were treated with piroxicam. As revealed by flow analysis, isolated LPMC from colitic CD4+ T cell-reconstituted Rag mice contained ~20% CD4+ Thy1.2+ T cells and <1% CD8. The LPMC from CD8-reconstituted mice contained ~10% CD8+ Thy1.2+ T cells but also had ~5% CD4. Thus even partial depletion of the CD4+ T cell compartment in Rag animals appeared to afford protection.

Origin of cytokines in LPMC from Rag mice. Also examined was cytokine secretion from LPMC isolated from piroxicam-treated, IL-10−/− T cell-reconstituted colitic Rag mice. Isolated LPMC contained about equal numbers of CD4+ Thy1.2+ and CD8+ Thy1.2+ T cells (30% each) as determined by flow analysis. LPMC cultured in vitro released IFN-γ (Fig. 5). The lamina propria T cells isolated by using paramagnetic beads produced IFN-γ and made substantially more with anti-CD3 and anti-CD28 MAb stimulation. However, LPMC devoid of T cells made none. There was no IL-4 or IL-5 in the culture supernatants even after T cell stimulation. These data suggested that the inflammation was a Th1 response and that IFN-γ production in the mucosa was strictly T cell dependent.

Also examined was IL-10 production in LPMC from Rag mice reconstituted with T cells. LPMC from Rag mice reconstituted with WT T cells, as opposed to LPMC from mice that received IL-10−/− T cells, produced large amounts of IL-10 (Fig. 6). Only lamina propria T cells from Rag mice reconstituted with WT T cells secreted IL-10 (Fig. 7). T cell-depleted LPMC from Rag animals reconstituted with either IL-10−/− or WT T cells made small amounts of IL-10 at similar rates (Fig. 7).
**DISCUSSION**

*Rag* mice do not produce T cells or B lymphocytes. However, it is possible to reconstitute their T cell compartment through adoptive transfer of T cells from T cell-competent animals. Experiments presented here show that *Rag* mice reconstituted with IL-10−/− T cells are highly susceptible to piroxicam-induced colitis and terminal ileitis. However, *Rag* mice receiving T cells from WT control mice are resistant to disease. Previous studies showed that blockade of prostanoid synthesis with the NSAID piroxicam can induce a severe and persistent Th1-type colitis in young IL-10−/− mice (6). The piroxicam-induced colitis in reconstituted *Rag* mice is similar to piroxicam-induced colitis in IL-10−/− animals (6) and to the spontaneous colitis in IL-10−/− mice that comes on naturally (5). They all exhibit mononuclear infiltration in the lamina propria, which frequently extends transmurally. This is associated with epithelial hyperplasia, muscular thickening, and focal mucosal ulceration. The mucosal inflammation is most severe on the right side of the colon. In the *Rag* piroxicam model, the colitis is more diffuse and involves the terminal ileum. In all three models, the inflammation is Th1, producing IFN-γ and IL-12.

It has been reported that transfer of 129/SvEv IL-10−/− splenic CD4+ T cells into *Rag*2-deficient mice can spontaneously lead to moderately severe colitis 8–12 wk later (7). However, in this study T cell-reconstitution of C57BL/6 *Rag* mice did not lead to colitis over the 6- to 8-wk observation period unless the animals were reconstituted with IL-10−/− T cells and treated with piroxicam.

LPMC from *Rag* mice reconstituted with WT T cells produce large amounts of IL-10 derived to a great extent from lamina propria T cells. They do not make IL-4 or IL-5 and make little IFN-γ. *Rag* mice reconstituted with IL-10−/− T cells demonstrated a marked impairment in mucosal IL-10 production due to the loss of intestinal IL-10-producing T cells. *Rag* mice reconstituted with CD4+ CD45RBhigh T cells can develop severe colitis, which can be prevented by cotransfer of CD4+ CD45RBlow T cells (2). Transforming growth factor-β (TGF-β) (22) and IL-10 (3) are required for protection, suggesting a role for these cytokines in the regulatory process. It is tempting to speculate that intestinal IL-10-producing T cells are critically important for the protection from piroxicam-induced colitis.

There currently is great interest in regulatory T cells, which can induce peripheral tolerance and limit mucosal reactivity (17). In various animal models, several regulatory T cell phenotypes have been reported. Some express CD4, whereas others express CD8 (11). In some systems, they are distinguished through differential expression of surface molecules like CD25 (26), CD45RB (2), and CTLA-4 (23). The pattern of expression suggests that they may be in a primed effector or memory state. These regulatory cells may mediate some of their effects through production of IL-10 and TGF-β. An anergic regulatory T cell that produces high levels of IL-10 (Tr1) has been described (17). Another cell called Th3 suppresses induction of experimental autoimmune encephalitis primarily through production of TGF-β. Still others are not dependent on soluble IL-10 or TGF-β but instead express on their surface latency-associated peptide, which is the amino-terminal domain of the TGF-β precursor peptide (21). This cell surface-bound TGF-β complex can induce suppression via cell-cell contact. It remains unknown if IL-10 produced by regulatory-type cells mediates protection from piroxicam-induced colitis.

Arachidonic acid released from cell membranes is transformed by cyclooxygenase enzymes (COX-1 and COX-2) into PGH₂, which subsequently is changed into other PGs (12). COX-1 activity is constitutively expressed in various cell types, whereas COX-2 is upregulated at sites of inflammation. A previous study showed that COX-1- and COX-2-derived PGs are important regulators of the mucosal immune response in IL-10−/− mice and that inhibition of PG production is central to the development of colitis in piroxicam-treated animals (6). PGs help to maintain the mucosal barrier and promote epithelial repair. Importantly, PGE₂ also can inhibit TNF-α (15) and IL-12 production (28) and affects dendritic cell functions (19). These immunoregulatory effects overlap with that of IL-10. Thus the simultaneous loss of T cell IL-10 and PGs like PGE₂ could be particularly harmful.

Piroxicam-induced chronic colitis in IL-10−/− T cell-reconstituted *Rag* mice will be a useful model for dissecting various aspects of mucosal immunoregulation. Unlike the variable and often delayed expression of IBD in the CD45RBhigh *Rag* transfer model, piroxicam rapidly induces a uniformly severe and persistent colitis and terminal ileitis in the *Rag* IL-10−/− T cell reconstitution model. Also, once IBD is induced and the drug is discontinued, the severe inflammation lasts at least 4 wk and probably indefinitely. Also, this *Rag* reconstitution model only requires intraperitoneal transfer of IL-10−/− splenocytes or T cells.

It is concluded that CD4+ T cells from IL-10−/− but not WT mice transfer susceptibility to NSAID-induced colitis in *Rag* mice. Lamina propria T cells normally can make large quantities of IL-10 that are absent from IL-10−/− mice. CD45RB+ T cells demonstrate a marked impairment in mucosal IL-10 production due to the loss of intestinal IL-10-producing T cells. *Rag* mice reconstituted with CD4+ CD45RBlow T cells can develop severe colitis, which can be prevented by cotransfer of CD4+ CD45RBlow T cells (2). Transforming growth factor-β (TGF-β) (22) and IL-10 (3) are required for protection, suggesting a role for these cytokines in the regulatory process. It is tempting to speculate that intestinal IL-10-producing T cells are critically important for the protection from piroxicam-induced colitis.

**REFERENCES**


