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

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 knockout (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 and administered piroxicam make more IL-10. This may worsen disease activity (4, 10, 13). Animal models of IBD (e.g., ulcerative colitis or Crohn’s disease), use of NSAIDs that target 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 × 10⁵ 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 g 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 mucus 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.

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There was mucus depletion and more frequent crypt abscesses. Ulcers 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 was then 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 layer 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 × 10⁹) 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 (10⁶/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 cells. 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 × 10⁵ 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 with anti-CD3 and -CD28 MAb.

 Flow cytometric analysis. Spleen cells or LPMC were washed twice and adjusted to 10⁶ 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 10⁶ cells in 100-μl FACS buffer and stained with saturating amounts of conjugated antibodies for 30 min at 4°C. After being washed, 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-FcγR; ATCC) to block nonspecific binding of conjugated antibodies to Fc receptors. The other MAbs 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 BVD6 (provided by Kevin Moore and John Abrams; DNAX). IL-5 was captured with TRFK5 MAb and detected with biotinylated TRFK4 (Dr. Robert Coffman; DNAX) followed 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. BA147; R&D Systems). Sensitivities of the ELISAs were ~30 pg/ml.

 MAb to IFN-γ (HB170), IL-4, and IL-5 were from cell lines maintained in our laboratory. These MAb 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.

 Fig. 1. Piroxicam induces colitis in Rag mice reconstituted with IL-10-deficient (IL-10⁻⁻) splenocytes. Rag mice received splenocytes (Spl; 2 × 10⁶ cells/mouse) from either IL-10⁻⁻ or age-matched wild-type (WT) mice (control). Some animals received piroxicam (pirox) in their food for 2 wk starting 2 wk after the cell transfer as described in MATERIALS AND METHODS. The colitis was scored histologically on a 0–4 point scale 2 wk after the piroxicam was stopped. Data are means ± SE from 15 animals/group studied in 3 separate experiments.

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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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mediated this susceptibility to piroxicam-induced colitis. 

Subsequent experiments used the 2-wk postpiroxicam time

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

CD4+ cells from IL-10 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).

Fig. 7. Only the non-T cell element of the mucosa makes IL-10 in Rag reconstituted with IL-10−/− T cells. Rag mice were reconstituted with splenic T cells from IL-10−/− (A) or WT (B) mice. Then 2 wk later, lamina propria T cells (T cells) or LPMC depleted of T cells (non-T) (2.5 × 10^6 cells/well) were cultured for 48 h with or without anti-CD3 and anti-CD28 MAb (αCD3). Culture supernatants were assayed for IL-10 after the incubation. Data are means ± SE of 6 separate determinations from 2 independent experiments.
**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{sup +} T cells into Rag2-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{sup +} CD45RB{sup high} T cells can develop severe colitis, which can be prevented by cotransfer of CD4{sup +} CD45RB{sup low} 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 aminoterminal 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 PGs, 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 CD45RB{sup high} 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{sup +} 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^{-/-} T cell-reconstituted Rag mice, leading us to speculate that mucosal T cell IL-10 is part of the protective process.

**GRANTS**

This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-38327, DK-58755, DK-02428, DK-25295) and the Crohn’s and Colitis Foundation of America.

**REFERENCES**


