7-2004

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A Novel Triterpenoid Induces Transforming Growth Factor β Production by Intraepithelial Lymphocytes to Prevent Ileitis

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Background & Aims: **The loss of homeostasis is a hallmark of inflammatory bowel disease. Oral infection of susceptible mice with *Toxoplasma gondii* results in an acute lethal ileitis characterized by increased interferon γ, tumor necrosis factor α, and inducible nitric oxide synthase; homeostasis results from transforming growth factor β production by intraepithelial lymphocytes. The synthetic oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is a potent anti-inflammatory molecule previously shown in vitro to suppress the de novo synthesis of inducible nitric oxide synthase and to induce the transcription and activation of genes from the transforming growth factor β signaling pathway.** ***Methods:*** **We evaluated the immune response in the small intestine and by intraepithelial lymphocytes after a single intraperitoneal dose of CDDO at the time of *T. gondii* oral infection. We abrogated the homeostatic effects of CDDO by blocking transforming growth factor β in vivo.** ***Results:*** **CDDO acid prevented ileitis development through the global down-regulation of inflammatory cytokines and chemokines. Total transforming growth factor β_1 production by the intraepithelial lymphocytes increased, as did Smad2 expression. Blocking transforming growth factor β reversed CDDO–induced protection and prevented the up-regulation of Smad2 in the small intestine.** ***Conclusions:*** **CDDO acid is a novel anti-inflammatory molecule capable of preventing ileitis by activating the transforming growth factor β signaling pathway in a pathogen-driven ileitis model. This could represent a new treatment of inflammatory bowel disease.**

The synthetic oleanane triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is a potent multifunctional molecule. It is a powerful agent for the induction of differentiation in both malignant and nonmalignant cells.[1] It is active as an inhibitor of proliferation of many malignant or premalignant cells and induces apoptosis by a caspase-8–dependent mechanism.[2] Furthermore, it suppresses the abilities of various inflammatory cytokines—such as interferon (IFN)-γ, interleukin 1, and tumor necrosis factor (TNF)-α—to induce de novo formation of the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 in mouse peritoneal macrophages, rat brain microglia, and colon fibroblasts.[1,3]

CDDO was recently shown to modulate the transforming growth factor (TGF)-β signaling pathway by using an in vitro transfection model. CDDO increases the expression of TGF-β–dependent genes, such as the type II TGF-β receptor and plasminogen activator protein-1.[4] Furthermore, CDDO modulates the Smad signaling pathway through its ability to prolong the activation of Smad2 induced by TGF-β, increases the binding of Smad3 to the Smad-binding element, and reverses the inhibitory effects of Smad7.[4]

Recent studies with human inflammatory bowel disease (IBD) implicate a role of the TGF-β signaling pathway in the prevention of T-helper 1 (Th1)-type IBD. Crohn's disease is an example of a type I inflammatory condition characterized by increased levels of iNOS, IFN-γ, and TNF-α and controlled in part by TGF-β.[5] Enhancing the TGF-β signaling pathway has been proposed as a useful therapeutic target for chronic IBD.[6] We have previously shown that homeostatic imbalance between the synthesis of proinflammatory factors and their down-regulation leads to severe hyper-IBD in certain strains of mice after oral infection with *Toxoplasma gondii*.[7] Without any genetic or chemical manipulation, this model has remarkable similarities to human ileitis with regard to disease localization, histological findings, and immunologic profile.[7–14] Orally infected mice have discontinuous areas of transmural intestinal inflammation, specifically scattered in the ileum. Histological examination shows mononuclear and polymorphonuclear cell infiltrates in the lamina propria, submucosa, and muscle layers.[7,9,10] There is villous blunting and hemorrhage in

Abbreviations used in this paper: CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; DMSO, dimethyl sulfoxide; IEL, intraepithelial lymphocyte; iNOS, inducible nitric oxide synthase; PCR, polymerase chain reaction; RPA, ribonuclease protection assay; TGF, transforming growth factor; Th, T-helper cell; TNF, tumor necrosis factor.

0016-5085/04/$30.00
doi:10.1053/j.gastro.2004.03.053

inflamed small-intestinal mucosa. Moreover, Th1-type T cells seem to be involved in this pathogen-driven ileitis. Neutralization of either IFN-γ or $CD4^+$ T cells during oral *T. gondii* infection prevents severe necrosis of the ileum and acute mortality.[9,15] Production of IFN-γ and TNF-α by $CD4^+$ T cells isolated from the lamina propria of mice infected with parasites is thought to mediate this ileitis.[15]

Different regulatory mechanisms maintain homeostasis in the infected host. TGF-β seems to be an essential component in the control of the hyperinflammatory process in the intestine after oral infection with *T. gondii*.[12] This inflammatory response can also be reversed if the mice are treated with an inhibitor (such as aminoguanidine) of iNOS.

In this study, we show marked antiinflammatory ability of CDDO in the *T. gondii*–infected animal model. CDDO exerts significant protection against the development of lethal inflammatory disease as determined histologically but does not result in a toxic effect on the parasite, because the parasite burden does not change with CDDO treatment. After CDDO treatment, IFN-γ and TNF-α messenger RNA (mRNA) levels decrease, whereas TGF-β_1 mRNA increases. Total TGF-β_1 secretion from the small intestine increases with CDDO treatment. Messages for the proinflammatory chemokines CCL2, CXCL1, and CXCL10 also decrease.

Intestinal intraepithelial lymphocytes (IELs) isolated from CDDO-treated mice produce increased amounts of TGF-β_1 and have increased Smad2 expression. The protective effect of CDDO is abrogated by pretreatment of mice with antibodies to TGF-β, further suggesting that CDDO functions by modulating the TGF-β and, specifically, the Smad signaling pathway. Blocking TGF-β also prevents the up-regulation of Smad2 mRNA observed in CDDO-treated mice. Triterpenoids such as CDDO represent a novel class of therapeutic agents that may act to prevent human inflammatory diseases such as Crohn's disease.

Materials and Methods

Mice and Parasites

Female 8–10-week-old C57BL/6 mice obtained from the Jackson Laboratories (Bar Harbor, ME) were housed under approved conditions of the Animal Research Facility at Dartmouth Medical School. Mice were infected orally by intragastric gavage with 35 cysts from the 76K statin of *T. gondii* maintained through passage in CBA/J mice. All experiments were performed with 4–6 mice per group and were repeated a minimum of 3 times unless otherwise specified.

CDDO Treatment

Mice received 50 μg of CDDO (provided by Michael B. Sporn, Dartmouth Medical School) in 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) 0.10 mL intraperitoneally, injected 2 minutes before infection with the parasite. Control mice received 10% DMSO in PBS alone. After preliminary testing of doses of 25, 50, and 100 μg of CDDO, 50 μg was found to be optimal, with minimal side effects, and this dose was chosen for all subsequent experiments.

Histology

Intestines were immediately fixed in 10% formalin overnight, embedded in paraffin, and sectioned. Sections were stained with H&E and photographed. A histological inflammatory score from 0 to 4 was applied in a blinded fashion as previously described[13]: 0, no inflammation; 1, slight infiltrating lymphocytes in the lamina propria with focal acute infiltration; 2, mild infiltrating cells in the lamina propria with increased blood flow and mild edema; 3, diffuse and massive infiltrating cells leading to disturbed mucosal architecture; and 4, crypt abscesses and superficial necrosis of the intestinal villi.

Ribonuclease Protection Assay

Intestines were washed with PBS and placed immediately in ice-cold Trizol reagent according to the manufacturer's protocols (Life Technology, Grand Island, NY). Specific mRNA expression from whole small intestines was evaluated with a RiboQuant Multi-probe Ribonuclease Protection Assay (RPA) System Kit (BD Pharmingen, San Diego, CA) as described by the manufacturer. A total of 10 μg of RNA from the intestine was analyzed. Bands were quantified by densitometric analysis with NIH Image (National Institutes of Health, Bethesda, MD) and normalized to L32 and glyceraldehyde phosphate dehydrogenase gene expression.

Transforming Growth Factor-β_1 Enzyme-Linked Immunosorbent Assay

On day 7 after infection and treatment, intestines were removed and washed 2 times in PBS; 1-cm pieces of ileum were removed and cultured for 18 hours in RPMI 1640 (Invitrogen, Carlsbad, CA), 10% fetal calf serum (Hyclone, Logan, UT), and 1% penicillin/streptomycin with 1% amphotericin B (Fungizone; Invitrogen). Cell supernatants were harvested and assayed for total TGF-β_1 secretion by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Purification of Intraepithelial Lymphocytes

IELs were purified as previously described.[7] Isolated IELs ($\sim 2 \times 10^6$ cells per mouse) were then obtained by Ficoll centrifugation with Histopaque (d = 1.077; Sigma). IELs were approximately 80% $CD8^+$ as determined by fluorescence-activated cell-sorting analysis. Purified IELs were plated in

48-well plates at 3×10^5 cells per 0.5 mL of RPMI 1640 (Invitrogen) with 10% fetal calf serum (Hyclone), 1% penicillin/streptomycin, and 1% amphotericin B. Cell supernatants were collected 14 hours after infection and assayed for TGF-β_1 secretion by enzyme-linked immunosorbent assay (R&D Systems).

Two-Step SYBR Green Quantitative Real-Time Polymerase Chain Reaction

A total of 0.5 to 2.0 μg (within each experiment, the same quantity of mRNA was used) of deoxyribonuclease-treated (Ambion, Austin, TX) mRNA was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen). A total of 100 ng of complementary DNA was amplified with the SYBR green core reagents (AP Biosystems, Foster City, CA) on a Bio-Rad (Hercules, CA) iCycler. Plasmid controls of all amplicons were constructed by using the TA cloning kit (Invitrogen) and were run in parallel with experimental samples. Relative expression was expressed by using the ΔCT method, where $CT = 2^{-(\exp-\beta\text{-actin})\times 1000}$. Previously published primers were as follows—iNOS: 5′-CATTGGAAGTGAAGCGTTTCG-3′, 5′-CAGCTGGGCGCGACAAACCT-3′[16]; IFN-γ: 5′-GCATTCATGAGTATTGCCAAG-3′, 5′-GGTGGACCACTCGGATGA-3′; TNF-α: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, 5′-TGGGAGTAGACAAGGTACAACCC-3′; β-actin: 5′-AGAAAGGGTCGTGCGTGAC-3′, 5′-CAATAGTGATGACCTGGCCGT-3′[17]; and Smad2: 5′-GAAACCTGCATTCTGGTGTT-3′, 5′-CGAGTTTGATGGGTCTGTGA-3′.

Immunoblots

Purified IELs were prepared for protein analysis by using standard techniques from 4–6 mice per group, repeated 2 times. A total of 50 μg of protein was loaded per lane. Gels were transferred onto Bio-Rad ImmunoBlot Nitrocellulose membranes. Transferred blots were stained with ponceau red to ensure equal loading of lanes as well as efficient transfer. Immunoblots were probed with Smad2 antibody (Zymed, South San Francisco, CA) and β-actin (Sigma). Anti-mouse (Sigma) and anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies conjugated to horseradish peroxidase were visualized by using ECL-Plus chemiluminescence (Amersham Biosciences, Piscataway, NJ).

In Vivo Blocking Studies

A total of 1 mg of TGF-β–blocking antibody (1d11; provided by Genzyme, Boston, MA) was administered intraperitoneally on days −1, 0, 2, 4, and 6 (200 μg/100 μL per mouse; 6 mice per group). MOPC21 isotype control antibody (Sigma) was given in parallel as a negative control. Antibody-treated and control mice that achieved the animal welfare–approved end point for termination at day 6 after infection were scored for histological evidence of inflammation.

Statistical Analyses

Groups were compared by using Prism (GraphPad, San Diego, CA) statistical software's unpaired *t* test; significance was expressed as $P < 0.05$, $P < 0.01$, or $P < 0.001$.

Results

Histological Analysis of CDDO Protection

Because CDDO was previously shown to alter the expression of key proinflammatory and anti-inflammatory cytokines, we evaluated the effects of varying doses of CDDO for the prevention of an acute pathogen-driven ileitis by examining the histology of the mouse intestine after oral infection with *T. gondii*. Mice orally infected with a lethal dose of 35 cysts followed 2 minutes later with an intraperitoneal treatment of 50 μg of CDDO or 10% DMSO alone, the vehicle control, were assayed for inflammation. Histological analysis performed 7 days after infection showed the characteristic histopathology of shortened villi, swollen lamina propria packed with inflammatory cells, necrosis, and hemorrhage evident at the tip of the villi (Figure 1*A*). In contrast, mice treated with 50 μg of CDDO displayed a normal intestinal mucosa; some intestines showed a mild infiltration of lymphocytes, but there was no evidence of major inflammation and necrosis (Figure 1*A*). Histological scoring showed significant protection with CDDO treatment ($P < 0.001$; Figure 1*B*). This protection was observed only when treatment was delivered at the time of infection, because CDDO did not protect when administered 24, 48, or 72 hours after infection (data not shown). Similarly, multiple doses of CDDO delivered throughout the infection did not provide any further protection against the development of this inflammatory disease (data not shown). Treatment with CDDO or DMSO in the absence of infection had no effect on the integrity of the intestinal mucosal surface (Figure 1*A*). A single dose of 50 μg of CDDO was the most effective and had no observable side effects; 25 μg was not effective at preventing ileitis, and 100 μg had a side effect of swelling of the small bowel and adipocyte accumulation along the basal layer of the mucosal surface but had no evidence of inflammation or necrosis (data not shown). Daily treatment with 50 μg of CDDO at days −2, −1, and 0 resulted in the same outcome as a single dose of 50 μg of CDDO; in contrast, treatment of mice with 50 μg of CDDO 1 and 2 days after oral infection with *T. gondii* did not protect mice against ileitis (100% of mice succumbed to ileitis). Because 50 μg of CDDO just before infection was the most effective dose at preventing ileitis, this dose was chosen for the remainder of the study.

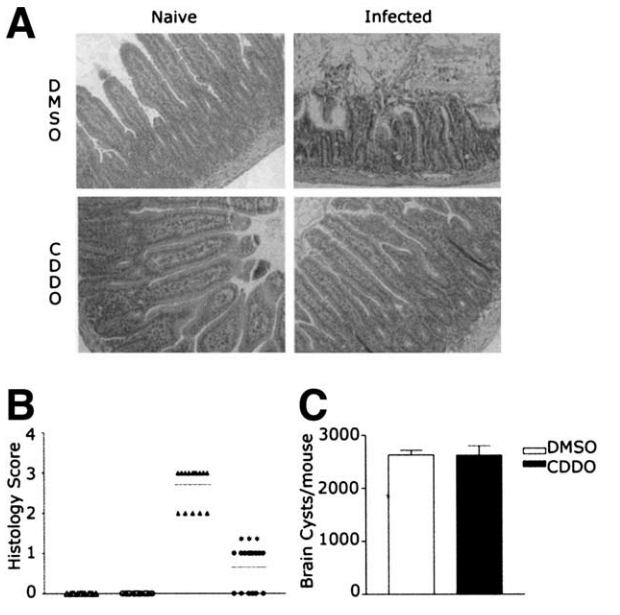

Figure 1. CDDO prevents acute ileitis. (*A*) Ileum sections treated with either CDDO or DMSO and obtained from naive and day 7 infected mice showed a decrease in inflammation (magnification, 200×). (*B*) CDDO treatment with infection (●) significantly reduced the inflammation score compared with DMSO with infection (◆) ($P < 0.001$); neither DMSO alone (△) nor CDDO alone (○) caused inflammation. Histological scoring of ileum sections—where 0 indicates no inflammation; 1 indicates slight infiltrating lymphocytes in the lamina propria with focal acute infiltration; 2 indicates mild infiltrating cells in the lamina propria with increased blood flow and mild edema; 3 indicates diffuse and massive infiltrating cells leading to disturbed mucosal architecture; and 4 indicates crypt abscesses and superficial necrosis of the intestinal villi[13]—showed statistically significant protection against inflammation and necrosis with CDDO treatment; the *line* represents the mean (***$P < 0.001$). (*C*) The parasite burden, as attested to by the brain cyst load ± SEM after a sublethal dose of *T. gondii,* showed no statistical difference between infected groups that received CDDO or DMSO. Parasite burden data are from 1 representative experiment.

Parasite Viability

Parasite viability ruled out a possible direct toxic effect of CDDO on the microorganism. After challenge with a sublethal dose (25 parasites per mouse), there was no statistical difference in the brain cyst load between mice treated with CDDO and those treated with DMSO (Figure 1*C*). Additionally, polymerase chain reaction (PCR) amplification of the B1 gene specific to *T. gondii* at day 7 after infection showed no difference in parasite burden between intestines obtained from mice that had received CDDO or DMSO (data not shown).

Chemokine Expression

After *T. gondii* infection, $CD4^+$ T cells from the periphery migrate to the lamina propria and synergize with infected epithelial cells to induce the secretion of several inflammatory chemokines, including CCL1, CCL2, CCL3, CXCL1, and CXCL10.[15] To determine whether CDDO treatment could alter the chemokine profile in the small intestine, RNA from day 7 treated/infected mice was analyzed by RPA. There was a significant down-regulation of CCL2, CXCL1, and CXCL10 ($P < 0.01$; Figure 2). In the absence of infection, CDDO treatment did not affect chemokine expression in the small intestine (data not shown). The differences between chemokine mRNA levels peaked at day 7 after infection, as previously described,[18] with very low levels of chemokine secretion at earlier time points (days 1, 3, and 5; data not shown).

Cytokine Expression

The overproduction of Th1-type cytokines is a hallmark of human IBD and *T. gondii*–induced ileitis in mice.[9,15,19–21] CDDO was previously shown in vitro to suppress the ability of inflammatory cytokines, including IFN-γ, interleukin 1, and TNF-α, to induce de novo formation of iNOS in mouse peritoneal macrophages.[1,3] At day 7 after infection, CDDO-treated and infected mice produced significantly less mRNA for both IFN-γ and TNF-α compared with DMSO-treated and infected mice, as determined by an RPA (Figure 3*A*). We confirmed a significant decrease in IFN-γ mRNA with SYBR green quantitative real-time PCR, a more sensitive method for mRNA detection (Figure 3*B*). DMSO and CDDO alone did not induce cytokine production (data not shown). Increases in cytokine profiles from

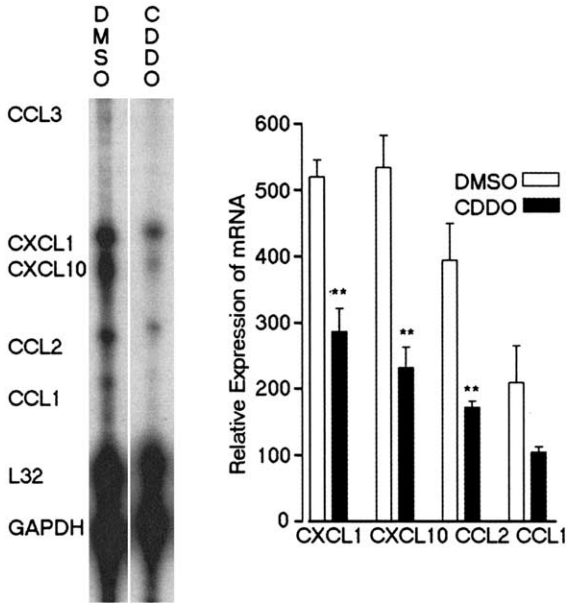

Figure 2. Modification of chemokine expression in the small intestine by CDDO. Ribonuclease protection assay of ileum samples showed a significant down-regulation in mRNA expression of inflammatory chemokines with CDDO treatment compared with DMSO; 1 representative experiment is shown. The *error bars* are ± SEM; **$P < 0.01$; ***$P < 0.001$.

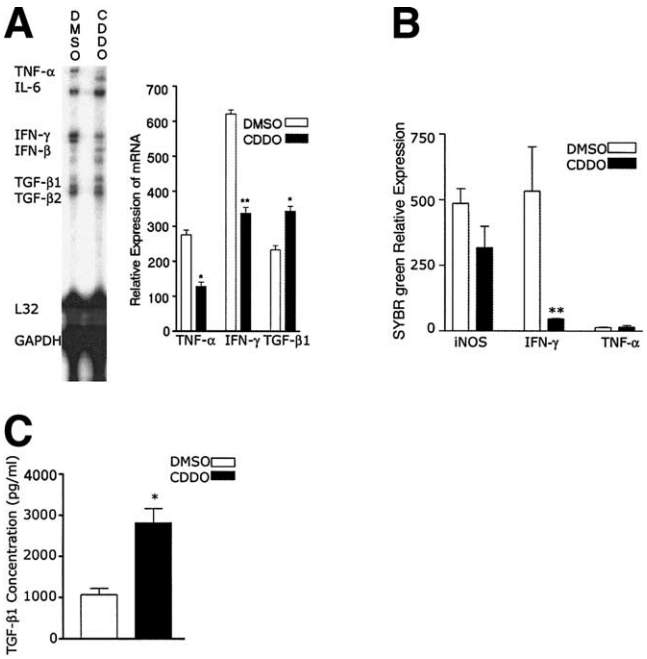

Figure 3. Modification of cytokine expression in the small intestine by CDDO. Ribonuclease protection assay showed that CDDO treatment resulted in a statistically significant decrease in mRNA for TNF-α and IFN-γ (*A*). TGF-β_1 levels significantly increased compared with DMSO; (*B*) SYBR green quantitative real-time PCR (QRTPCR) resulted in a statistically significant decrease of IFN-γ production with CDDO treatment compared with DMSO. One representative experiment is shown (mean ± SEM). (*C*) TGF-β_1 secretion of ileum pieces by enzyme-linked immunosorbent assay showed a significant increase in total TGF-β_1 in intestines from CDDO-treated mice on day 7 after infection (mean ± SEM; n = 3 mice per group repeated 2 times). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

infected mice were optimal at day 7 after infection; earlier time points (days 1, 3, and 5) showed no detectable up-regulation in cytokine levels in the infected control groups (data not shown). Because mRNA for TGF-β_1 increased, as determined by an RPA (Figure 3*A*), infected small intestines from mice treated with CDDO were cultured, and the supernatants were measured for total TGF-β_1 secretion by enzyme-linked immunosorbent assay. CDDO treatment significantly increased the total TGF-β_1 secretion at day 7 after infection (Figure 3*C*).

Effects of CDDO on Cytokine Expression by Intestinal Intraepithelial Lymphocytes

TGF-β_1 production is essential for maintaining intestinal homeostasis in human IBD and in the *T. gondii*–induced acute ileitis model.[6,12,22–24] TGF-β_1 mRNA and total TGF-β_1 protein were up-regulated in the intestine and peaked at day 7 in mice treated with CDDO and infected (Figure 3*A* and *C*). IELs were previously shown to be essential for maintaining gut homeostasis after *T. gondii* infection. The principal mechanism for this protective effect is via the production of TGF-β_1.[12,22] Culture supernatants of IELs purified from day 7 infected and CDDO-treated mice produced significantly more total TGF-β_1 than those from infected and DMSO mice ($P < 0.001$; Figure 4*A*). CDDO treatment of uninfected control mice did not alter the TGF-β production compared with DMSO-treated mice; the difference in TGF-β production was observed early after infection but peaked at day 7 after infection/treatment compared with earlier time points (day 1: DMSO plus infection, 48.75 ± 1.02 pg/mL; CDDO plus infection, 62.29 ± 1.05 pg/mL; day 4 after infection: DMSO plus infection, 59.51 ± 1.05 pg/mL; CDDO plus infection, 49.3 ± 1.04 pg/mL). There was no difference in the production of IFN-γ and TNF-α by the IELs isolated from the CDDO- or DMSO-treated and infected IELs at day 7 after infection (Figure 4*B*).

CDDO Modulates Smad2 Expression by the Small Intestine and by the Intestinal Intraepithelial Lymphocytes

TGF-β_1 induced by CDDO was previously shown to modulate the Smad signaling pathway in vitro.[4] To determine whether CDDO could alter Smad2, 3, 4, and 7 expression early in the whole intestine, we performed RNA analysis. Of the Smads tested, only Smad2 analysis showed a significant difference in mRNA expression; Smad2 mRNA increased at day 1 after infection (Figure 5*A*) and continued to increase at day 7 after infection (Figure 5*B*). No statistical difference in Smad3 or Smad4 expression was observed among the CDDO-treated and DMSO-treated groups (data not shown). Moreover, IELs isolated from infected and CDDO-treated mice expressed high levels of Smad2 protein 1 day after infection (Figure 6); CDDO alone did not increase Smad2 protein expression (data not shown).

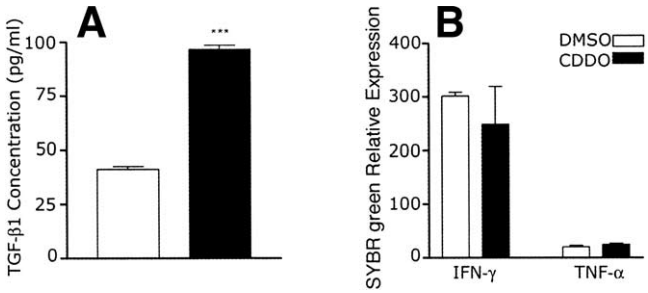

Figure 4. Cytokine expression from IELs after CDDO treatment. (*A*) Supernatants from ex vivo–cultured CDDO-treated IELs contained increased levels of total TGF-β_1 compared with DMSO; data are representative of 2 independent experiments (mean ± SEM). (*B*) SYBR green quantitative real-time PCR showed that IELs from CDDO-treated and infected mice did not express reduced mRNA for IFN-γ and TNF-α compared with IELs from DMSO-treated mice; 1 representative experiment is shown (mean ± SEM).

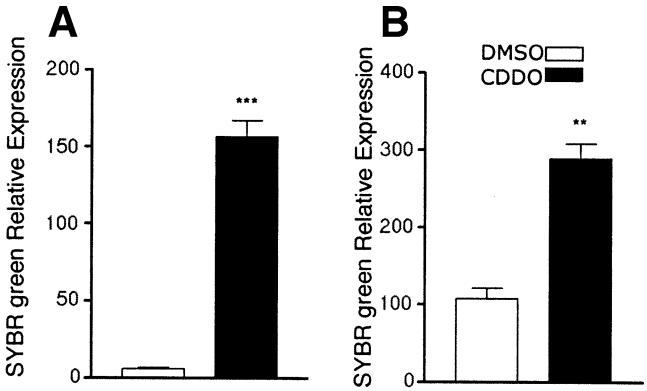

Figure 5. Messenger RNA expression of Smad2 from the small intestine from CDDO-treated mice. (*A*) SYBR green quantitative real-time PCR of Smad2 mRNA in the intestine increased after CDDO treatment on day 1 after treatment/infection compared with DMSO; data are from 1 representative experiment (mean ± SEM). $***P < 0.0001$. (*B*) Quantitative real-time PCR for Smad2 in the intestine showed an increase of Smad2 mRNA at day 7 after infection; data are from 1 representative experiment (mean ± SEM; $**P < 0.001$).

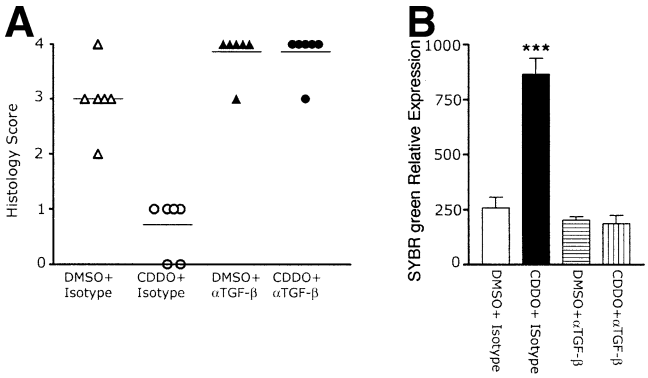

Figure 7. Histological analysis after blocking TGF-β. Blocking TGF-β in vivo abrogated CDDO's protection against *T. gondii*–induced inflammation, as determined by histological score, compared with DMSO. DMSO and isotype control (△), CDDO and isotype control (○), DMSO and anti–TGF-β (▲), CDDO and anti–TGF-β (●). Results are from 6 mice per group; lines represent means; $***P < 0.001$ (*A*). Blocking TGF-β prevents the ability of CDDO to up-regulate Smad2 mRNA at day 7 after infection/treatment. $***P < 0.0001$ between DMSO plus isotype control and CDDO plus isotype control (*B*); data are from the mice used in (*A*) (mean ± SEM).

CDDO Protection Is Dependent on TGF-β

To determine whether CDDO's protection against inflammation required TGF-β, mice were treated in vivo with TGF-β–blocking antibody (Genzyme 1d11). Treatment with 1 mg of anti–TGF-β–blocking antibody abrogated the protection mediated by CDDO. The intestines of mice treated with anti–TGF-β and CDDO developed severe necrosis, a loss of villi architecture, and increased inflammatory infiltrates and hemorrhages. Quantification of the inflammation showed no significant protection against ileitis after blocking TGF-β (Figure 7*A*). To confirm that Smad2 mRNA up-regulation by CDDO was essential for the protection observed in this model of inflammation, mRNA analysis of Smad2 was performed from the small intestines with TGF-β blocked. As expected, Smad2 mRNA from infected mice treated with CDDO and the isotype control showed a significant increase in Smad2 mRNA compared with the DMSO and isotype control ($P < 0.001$). Blocking TGF-β prevented this increase in Smad2 mRNA in CDDO-treated mice (Figure 7*B*).

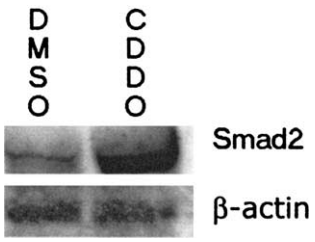

Figure 6. Smad2 protein expression by IEL from CDDO-treated mice. CDDO treatment and infection increases Smad2 protein expression as determined by Western blot analysis compared with DMSO and infection; data are from 1 representative experiment.

Discussion

In this study, we show the novel in vivo effect of CDDO at suppressing pathogen-driven intestinal inflammation. Treatment of *T. gondii*–infected mice with a single intraperitoneal injection of CDDO at the time of infection impairs the development of an acute lethal ileitis. CDDO has no toxic effect on either parasite multiplication or entry into the host cells.

CDDO's ability to prevent this pathogen-driven acute ileitis is dependent on TGF-β production. Previous studies implicate TGF-β as an essential component in the control of the hyperinflammatory process in the gut after oral infection with this parasite.[12] The protective effect of CDDO is abolished by blocking TGF-β. We show that IELs, which comprise a major compartment of the gut-associated lymphoid tissue, respond to CDDO exposure and up-regulate the production of TGF-β. Treatment of CBA/J mice (although these mice can become infected with the parasite, they do not succumb to the same inflammation as C57BL/6 mice) with antibody to TGF-β turns their resistant phenotype into a susceptible one.[12] In susceptible C57BL/6 mice, adoptive transfer of antigen-primed IELs that produce TGF-β prevents the inflammatory process.[12] Blocking TGF-β abolishes adoptive protection in this model of pathogen-driven ileitis. In this study, treatment with anti–TGF-β worsened the inflammation. However, we showed that the CDDO could not overcome the absence of TGF-β. Additionally,

our data show that TGF-β and Smad2 are up-regulated in response to CDDO. Taken together, it is reasonable to anticipate that if the homeostatic balance in the gut is disturbed by the blocking antibody and if CDDO cannot rescue these mice, then CDDO is working through TGF-β.

Synthesis of TGF-β by antigen-primed IELs inhibits IFN-γ production by lamina propria $CD4^+$ T cells,[15] the principal cell type responsible for the production of TNF-α and IFN-γ in the intestine. When IELs were assayed for a possible reduction in IFN-γ, TNF-α, and iNOS mRNA, there was no significant reduction, further suggesting that the $CD4^+$ T cells from the lamina propria could be the cell type targeted by the increase in TGF-β_1 production.

Although our study focused on the IEL population because they have been previously shown to be important regulators of intestinal homeostasis, IELs may not be the unique cell type targeted by CDDO treatment. Our unpublished in vitro observation suggests that CDDO may be able to induce TGF-β signaling in another TGF-β–producing cell, the enterocytes. It is possible that these other cellular players may increase the protective effect of CDDO by further inducing TGF-β production.

Previously published data with CDDO further implicate the importance CDDO's ability to target TGF-β_1 production and specifically the Smad system, because CDDO increases the binding of Smad3 to its CAGA binding element and also increases the phosphorylation of Smad2.[4] We showed that CDDO increases the expression of Smad2 in the whole intestine and more specifically among the IEL population but that the up-regulation of Smad2 is early after the induction of inflammation, further suggesting that CDDO's primary effects are early after treatment. We also show that the up-regulation of Smad2 mRNA by the small intestine can be observed at day 7, a time when the ileitis score is high among the untreated groups. An inability to induce the effects of TGF-β through a defect in the Smad signaling pathway is crucial in the development of human IBD such as Crohn's disease.[6,23] Smad2- and Smad4-deficient mice are embryonic lethal, further implicating these proteins as essential for normal immune function.[25–27]

CDDO has a demonstrably fast half-life of 30 minutes, and this suggests that the action of this compound is immediate and perhaps alters the early immune response in the mucosa. Treatment with CDDO after *T. gondii* infection does not protect against ileitis, presumably because the homeostatic balance in the intestine has already been altered because of the infection. CDDO treatment just before infection could maintain the homeostasis in the intestine, as indicated by a decrease in inflammation and a decrease in chemokine expression. TGF-β induction has been previously shown to decrease the chemokine expression after *T. gondii* infection.[10,12] Because CDDO treatment alters the chemokine expression in the intestine, this effect could be explained by the early induction of TGF-β by CDDO. Early secreted chemokines play an essential role in monocyte and neutrophil attraction and activation. A lack of chemokine secretion, as observed with CDDO treatments, could lead to a defect in lymphocyte migration and activation to the site of injury, as seen in the histology.

In addition to a decrease in chemokine secretion, TGF-β induction by CDDO could explain the decrease in inflammatory cytokines in the intestine. In this and other models of inflammation, TGF-β production by T cells can shift the homeostatic balance away from the production of proinflammatory cytokines such as IFN-γ, which, in turn, begin the inflammatory cascade.[12,14,15,28] CDDO prevents overproduction of the Th1-related inflammatory molecules and cytokines such as nitric oxide and IFN-γ. The ability of CDDO to down-regulate iNOS was evaluated in a series of in vitro experiments that used tissue cultured cells (data not shown); however, in vivo, CDDO did not down-regulate iNOS mRNA. These studies are the first in vivo reports of the actions of this novel drug; therefore, it is not surprising that CDDO may act differently in vivo than in vitro.

CDDO and DMSO alone did not induce mRNA for Th1-type cytokines. Furthermore, there may be a discrepancy between the amount of IFN-γ required to control the parasite and the amount required to induce ileitis; this may explain why the parasite burden was not affected by the CDDO treatment. The decreased production of those inflammatory molecules correlated with a significant increase in TGF-β mRNA and protein by cells in the small intestine, including IELs, which have been shown to be important regulators of this pathogen-driven inflammatory pathology.[12] Thus, the enhancement of TGF-β levels in mice treated with CDDO might explain the decrease in observed intestinal IFN-γ and TNF-α.[6,12,14,15,23,28] CDDO did not significantly reduce TNF-α mRNA in the small intestine as measured by quantitative real time PCR, even though with the RPA there was a decrease in mRNA. This difference in mRNA is presumably due to the decreased sensitivity of quantitative real-time PCR, because all PCR primers were previously published and control plasmids were run in parallel with all PCR reactions.

In summary, CDDO seems to induce TGF-β secretion in the intestine, at least by the IELs. This early induction of TGF-β seems to decrease cytokines and chemokines though a downstream effect of TGF-β. Thus, novel therapeutics such as CDDO that target this pathway may represent a new approach for the treatment of human inflammatory diseases such as Crohn's disease.[6]

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Received September 12, 2003. Accepted March 18, 2004.

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Supported by National Institutes of Health Institutional Training grant T32 AI007363 (to L.A.M.) and by National Institutes of Health grants H AI19613 and AI30000 (to D.B.-G. and L.H.K.).

The authors thank Michael B. Sporn and Nanjoo Suh for providing materials and insight; James D. Gorham, Joseph Schwartzman, and Toni Bauman for their discussions; and Il-Kyoo Park and Danielle Adams for their participation in the preparation of the manuscript.