Gastroenterology 2016;■:1–17

Rectal Delivery of a DNAzyme That Specifically Blocks the Transcription Factor GATA3 and Reduces Colitis in Mice

Vanessa Popp,[1] Katharina Gerlach,[1] Stefanie Mott,[1] Agnieszka Turowska,[2] Holger Garn,[3] Raja Atreya,[1] Hans-Anton Lehr,[4] I-Cheng Ho,[5] Harald Renz,[3] Benno Weigmann,[1] and Markus F. Neurath[1]

[1]Department of Medicine, University of Erlangen-Nürnberg, Kussmaul Research Campus, Erlangen, Germany; [2]sterna biologicals GmbH Co & KG, Marburg, Germany; [3]Institute of Laboratory Medicine and Pathobiochemistry, Medical Faculty, Philipps University of Marburg, Marburg, Germany; [4]Institute of Pathology, Campus Bodensee, Friedrichshafen, Germany; and [5]Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts

BACKGROUND & AIMS: GATA3 is a transcription factor that regulates T-cell production of cytokines. We investigated the role of GATA3 in development of colitis in mice. **METHODS:** We performed quantitative polymerase chain reaction and immunofluorescence analyses of colon tissues from patients with Crohn's disease (n = 61) or ulcerative colitis (UC, n = 74) or from patients without inflammatory bowel diseases (n = 22), to measure levels of GATA3. Colitis was induced by administration of oxazolone or 2,4,6-trinitrobenzenesulfonic acid to control mice, mice with T-cell–specific deletion of GATA3, and mice with deletion of tumor necrosis factor receptor (TNFR) 1 and TNFR2 (TNFR double knockouts); some mice were given a GATA3-specific DNAzyme (hgd40) or a control DNAzyme via intrarectal administration, or systemic injections of an antibody to TNF before or during sensitization and challenge phase of colitis induction. Colon tissues were collected and immunofluorescence and histochemical analyses were performed. Lamina propria mononuclear cells and T cells were isolated and analyzed by flow cytometry or cytokine assays. Colonic distribution of labeled DNAzyme and inflammation were monitored by in vivo imaging (endoscopy) of mice. **RESULTS:** Levels of *GATA3* messenger RNA were higher in colon tissues from patients with UC, but not ileal Crohn's disease, than control tissues; levels of GATA3 correlated with levels of inflammatory cytokines (interleukin [IL] 9, IL17A, IL6, IL5, IL4, IL13, and TNF). We observed increased expression of GATA3 by lamina propria T cells from mice with colitis compared with controls. Mice with T-cell–specific deletion of GATA3 did not develop colitis and their colonic tissues did not produce inflammatory cytokines (IL6, IL9, or IL13). The DNAzyme hgd40 inhibited expression of *GATA3* messenger RNA by unstimulated and stimulated T cells, and distributed throughout the inflamed colons of mice with colitis. Colon tissues from mice given hgd40 had reduced expression of *GATA3* messenger RNA, compared with mice given a control DNAzyme. Mice given hgd40 did not develop colitis after administration of oxazolone or 2,4,6-trinitrobenzenesulfonic acid; lamina propria cells from these mice expressed lower levels of IL6, IL9, and IL13 than cells from mice given the control DNAzyme. Mini-endoscopic images revealed that hgd40 and anti-TNF reduced colon inflammation over 3 days; hgd40 reduced colitis in TNFR double-knockout mice. **CONCLUSIONS:** Levels of GATA3 are increased in patients with UC and correlate with production of inflammatory cytokines in mice and humans. A DNAzyme that prevents expression of GATA3 reduces colitis in mice, independently of TNF, and reduces levels of cytokines in the colon. This DNAzyme might be developed for treatment of patients with UC.

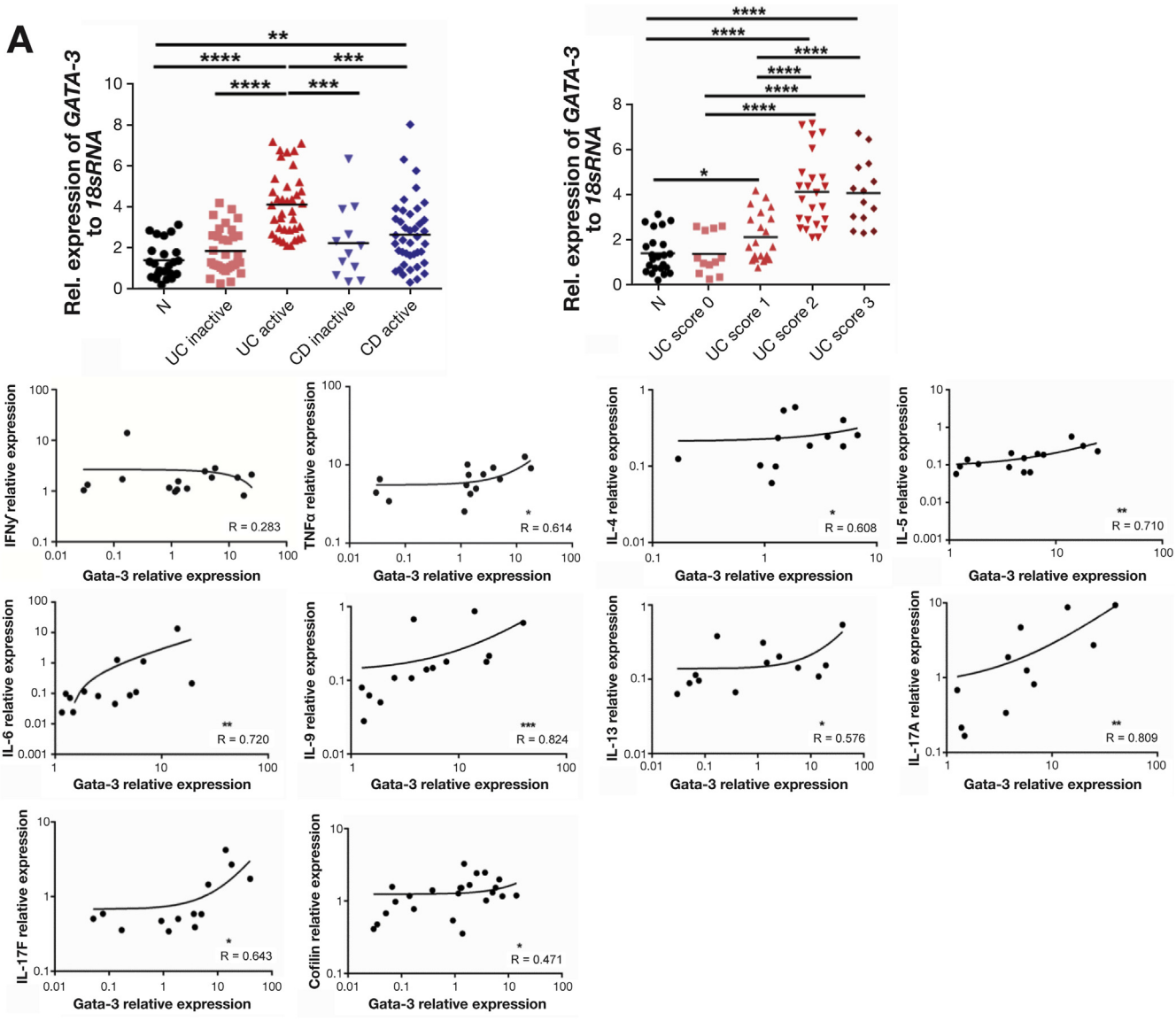
Keywords: Mouse Model; Gene Regulation; DNA Cleavage; Immune Response.

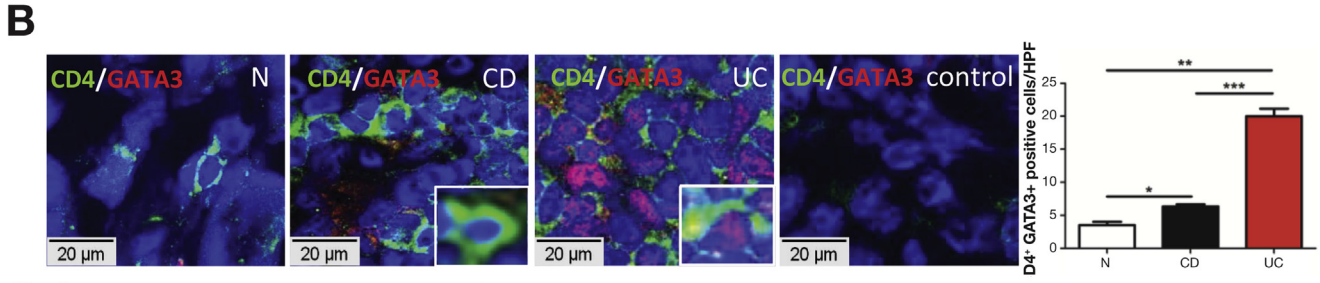
Inflammatory bowel disease (IBD) is composed of 2 major disorders: ulcerative colitis (UC) and Crohn's disease (CD).[1–3] Although the exact etiology of IBD is still unclear, studies have highlighted an important pathogenic role of both innate and adaptive immune systems.[4,5] Within the adaptive immune system, T-cell–derived cytokines have been characterized causing mucosal inflammation. However, T cells in CD have been found to produce augmented levels of Th1 cytokines, such as interferon (IFN) γ and tumor necrosis factor (TNF). In contrast, mucosal T cells in UC patients produced elevated amounts of the Th2 cytokines interleukin (IL) 5 and IL13.[6–8] Additionally, T cells in UC were shown to produce higher levels of IL9 than T cells in CD, suggesting the presence of Th9 cells in the former disease.[9,10] Finally, T cells in both CD and UC were found to produce IL6 and the Th17-associated cytokine IL17A.[4,11–13]

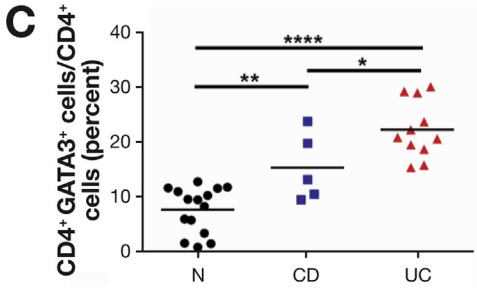
Cytokine production and gene transcription in T lymphocytes is controlled by regulatory transcription factors. While transcription factors, such as signal transducer and activator of transcription (STAT) 6, GATA3, c-Maf, STAT5a/b, JunB, and NF-ATc1, have been described to induce Th2 cytokine production by peripheral T cells,

Abbreviations used in this paper: **CD, Crohn's disease; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; mRNA, messenger RNA; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; Treg, regulatory T cell; UC, ulcerative colitis; WT, wild-type.**

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2016.09.005







BATF, STAT3, IRF4, and RORγt control Th17 cytokine gene transcription and STAT1, STAT4, RUNX3, and T-bet mediate Th1 cytokine production.[14–18] Additionally, various transcription factors, including PU.1, IRF4, and BATF have been identified as inducers of IL9 cytokine gene transcription in T cells.[19] In the mucosal immune system, several studies suggested the important roles of STAT4 and T-bet for Th1 cells in CD.[20,21] Additional studies demonstrated augmented levels of RORC expression in patients with IBD.[12] Furthermore, a recent study showed increased expression of both GATA3 and STAT4 messenger RNA (mRNA) in pediatric UC, suggesting that these factors may regulate cytokine production in the inflamed mucosa.[22] However, the role of GATA3 in mucosal T cells of adult IBD patients remains to be determined.

The initiating signals driving Th2-cell differentiation result in phosphorylation and activation of STAT6. STAT6 induces GATA3 via activation of the GATA3 promoters and an upstream conserved regulatory region.[23,24] GATA3 has been found to induce its own expression, either directly via autoregulatory loops or indirectly via the transcription factor Dec2, thereby stabilizing GATA3 expression in T cells.[25,26] Functionally, GATA3 was shown to inhibit STAT4 function and the production of IFN γ via suppression of RUNX3-mediated *Ifng* expression, thereby suppressing Th1-development.[27] Furthermore, GATA3 is both necessary and sufficient for Th2 cytokine gene transcription and expression in $CD4^+$ T cells by binding to the IL5 and IL13 gene promoter regions and to genomic Th2-cell specific DNase I hypersensitive sites. Moreover, GATA3 augments expression of c-Maf, a transcription factor that cooperates with JunB to enhance production of IL4. Finally, GATA3 can form a complex with the chromodomain helicase DNA-binding protein4 (CHD4) in Th2 cells, thereby favoring Th2 cytokine gene transcription.[28] Thus, GATA3 actively induces the chromatin remodeling machinery, activates additional Th2-promoting factors and transactivates Th2 gene promoters to orchestrate a 3-dimensional topography of Th2 cytokine gene-expression in T cells.[29]

In the present study, we analyzed the expression and function of GATA3 in IBD patients and experimental colitis. Our findings suggest that therapeutic approaches targeting transcription factors that control cytokine gene transcription will become options for clinical therapy of IBD.

Methods

Patients With Inflammatory Bowel Diseases

Patients are described in the Supplementary Material.

Isolation of Human and Murine Colonic Messenger RNA and Real-Time Polymerase Chain Reaction Analysis

Details are described in the Supplementary Material.

Immunofluorescence Staining of Human Colonic Tissues

Details are described in the Supplementary Material.

Animals

Mice (6–12 weeks old) were housed under specific pathogen-free conditions and experiments were performed in accordance with institutional guidelines. Mice with floxed *GATA3* allele were generated as described previously and crossed to *Cd4*-Cre lines to obtain mice with T-cell−specific ablation of *GATA3*.[30] TNF-R1/2 knockout mice were a kind gift from Frank Richter (University of Jena, Germany).

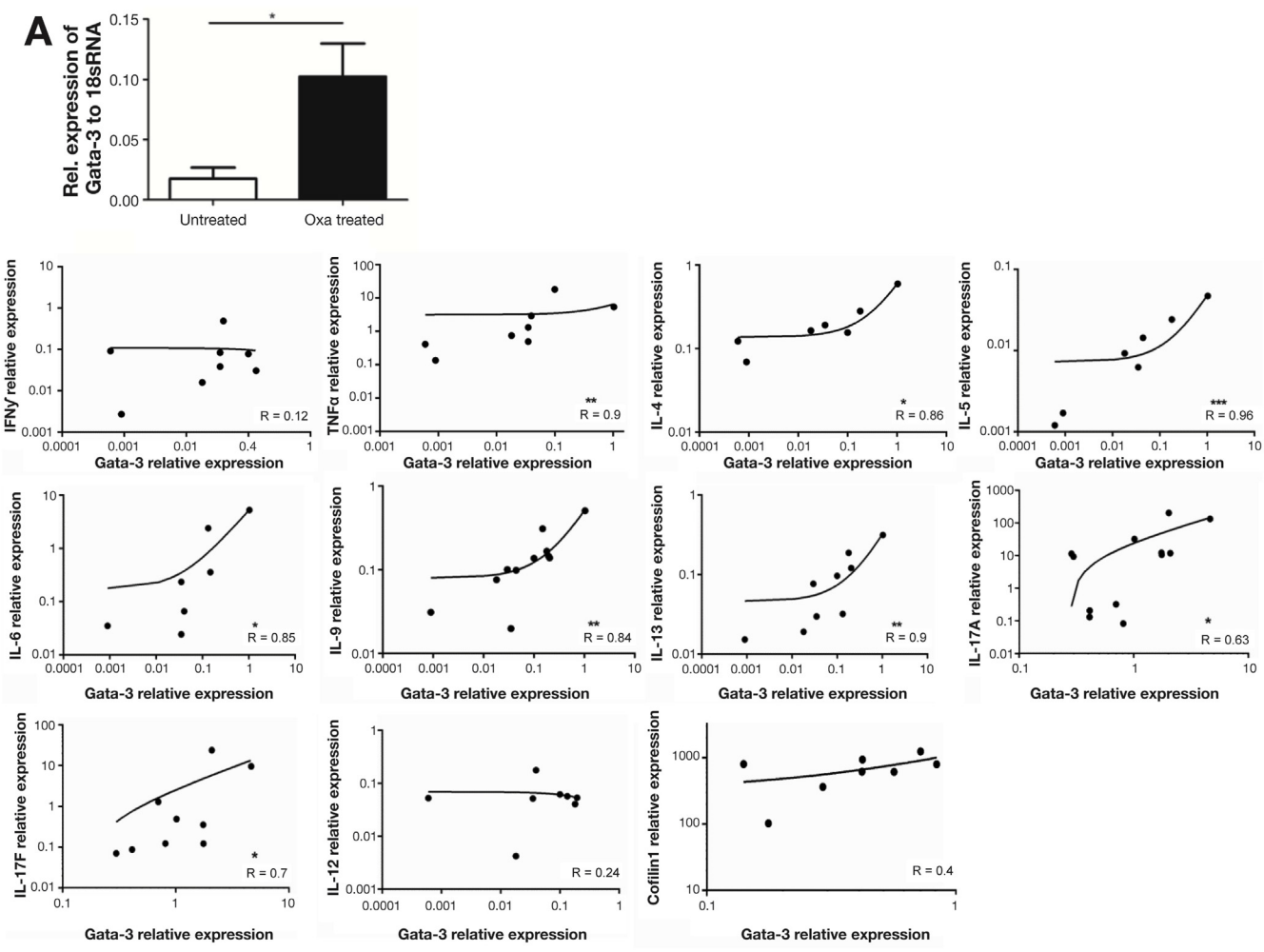
Oxazolone-induced Model of Intestinal Inflammation

Mice were treated as described previously and in the Supplementary Material.[31] For treatment of colitis, hgd40 (1000 μg) and control DNAzymes were intrarectally administered at indicated time points.

Immunofluorescence Staining in Murine Samples

Colonic cryosections were prepared for H&E staining. Immunofluorescence staining was done using rat-anti-mCD4 antibodies (BioLegend, San Diego, CA) and goat-anti-rat AlexaFluor488 antibody (Life Technologies, Carlsbad, CA). For GATA3 staining rabbit-anti-mGATA3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used together with goat-anti-rabbit AlexaFluor594 (Life Technologies). Negative control slides were incubated with secondary

Figure 1. Expression of GATA3 in IBD patients and correlation with levels of inflammatory cytokines. (*A*) Total mRNA from colonic biopsies was isolated and analyzed for *GATA3* mRNA expression in relation to *18S rRNA*. Healthy individuals, patients with active and inactive CD or UC were tested (*left upper panel*). Comparison of *GATA3* mRNA expression in UC patients with the inflammatory activity showed a correlation of GATA3 with the endoscopic Mayo activity score (*right upper panel*). Significant differences are indicated and the mean values are indicated by *horizontal lines*. *Lower panels*: The relative mRNA expression of GATA3 and IL9, IL6, IL13, IL4, IL5, TNFα, IFN γ, and cofilin was analyzed in active UC. The correlation coefficient between GATA3 and cytokine levels and significance levels are indicated. (*B*) Immunohistochemistry for GATA3- and CD4-expressing cells in colonic samples from IBD patients and controls. Negative controls showed no positive cells. Cells were counterstained with Hoechst-33342. Double-positive cells were counted per high-power field (HPF) (*right panel*). Significant differences are indicated. Data represent mean values ± SEM per HPF of 4 patients. (*C*) Fluorescence-activated cell sorting analysis of GATA3 expression in human colonic lamina propria mononuclear cells. The percentage of $GATA3^+CD4^+$ cells among $CD4^+$ cells is shown and significant differences are indicated. In the *dot graphs* in (*A*) and (*C*), each *dot* represents 1 patient.



antibodies. For myeloperoxidase staining, rabbit-anti-mouse myeloperoxidase antibody (Thermo Scientific, Logan, UT) was used, incubated with biotinylated donkey-anti-rabbit antibody (Immunoreagents, Raleigh, NC) and streptavidin-Dylight 549. For FOXP3 staining, cryosections were stained with rat-anti-mouse antibodies and goat-anti-rat AlexaFluor488 antibody. Nuclei were stained with Hoechst-33342.

High-Resolution Mini-Endoscopy and Histopathology

Colitis development was monitored with the Coloview System (Storz, Tuttlingen, Germany) and the MEICS (Modified Murine Endoscopic Index of Colitis Severity) scoring system.[32] In addition, colonic samples were analyzed by histopathology for grading of colitis activity in a blinded fashion.[33]

In Vivo Imaging of Inflammation

For in vivo imaging of murine colitis activity, the imaging system IVIS100 (Perkin-Elmer, Waltham, MA) was used. One hundred microliters of a sterile solution of 20 mmol luminol (Wako, Richmond, VA) was administered intraperitoneally.

DNAzymes

We used the GATA3 DNAzyme hgd40 as synthetic DNA antisense molecule (34 bases)[34,35] (5′-GTGGATGGAGGCTAGC TACAACGAGTCTTGGAG-3′), ATTO665-labeled hgd40 (coupled with fluorescence at the 5′ end) and scrambled control ODNg3 (5′-CCATGTGGAGGCTAGCTACAACGACTGGAATCA-3′) (BioSpring, Frankfurt am Main, Germany). All oligonucleotides were modified by the addition of an inverted thymidine at the 3′ end. The ODNg3 contained a random sequence of the binding arms and an intact catalytic domain sequence.

In Vivo and Ex Vivo Imaging of hgd40 Distribution in the Colon

Mice subjected to the oxazolone-induced model were treated with ATTO665-labeled hgd40. Pure ATTO665 dye was used as negative control. Subsequently, the gastrointestinal tract was analyzed with spectral fluorescence by using the Maestro In-vivo Imaging System (Cambridge Research & Instrumentation, Inc, Woburn, MA) with red filters. Staining of cryosections from the colon of hgd40-treated animals was performed by using confocal microscopy with Hoechst dye.

Isolation and Analysis of Spleen Mononuclear Cells and Lamina Propria Mononuclear Cells

Lamina propria mononuclear cells and splenic T cells were isolated with dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured in RPMI (10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine) and stimulated with anti-CD3 and anti-CD28 (4 μg/mL). For in vitro target regulation experiments, T cells were co-incubated with 1 mg/mL DNAzyme or controls for 48 hours.

Intracellular Fluorescence-Activated Cell Sorting Analysis of GATA3 Expression

Isolated cells were stained with rat-anti-mCD4^{PE} or mouse-anti-hCD4^{PE} and GATA3 labeled-AlexaFluor660 antibodies (eBioscience, San Diego, CA) together with permeabilization buffer. Cells were analyzed by fluorescence-activated cell sorting (BD, San Jose, CA) and CD4$^+$/GATA3$^+$ cells were determined in percentages.

Cytokine Analyses

Culture supernatants were taken 48 hours after stimulation. Cytokine concentrations were measured by using m13plex-FlowCytomix (eBioscience) or using enzyme-linked immunosorbent assay kits for IL5 (Biolegend), IL6 (eBioscience), IL9 (Cusabio), and IL13 (eBioscience).

Statistics

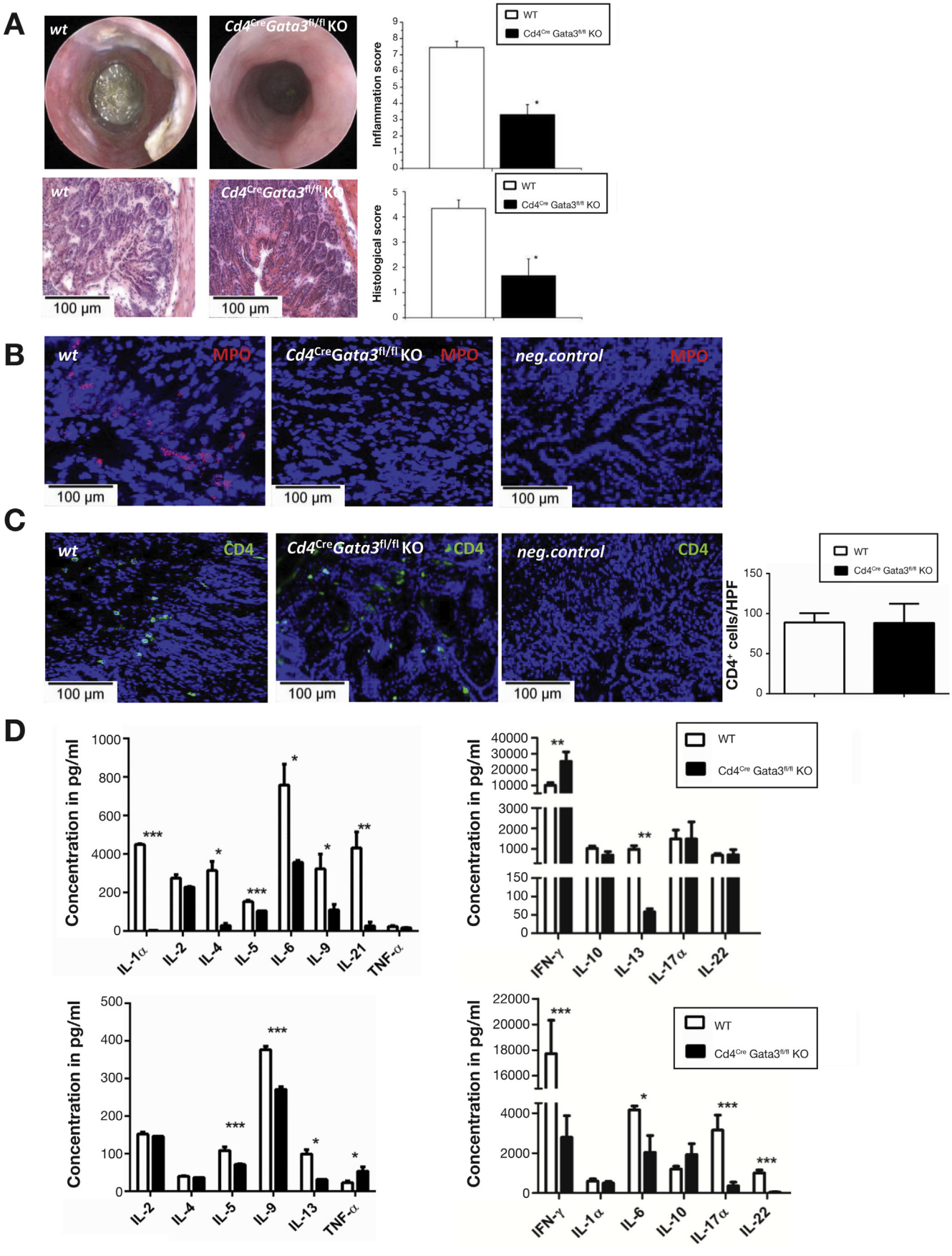
Refer to Supplementary Material for more information.

Results

Transcription Factor GATA3 Is Highly Expressed in Lamina Propria CD4$^+$ T Cells in Ulcerative Colitis

We found that *GATA3* mRNA expression is significantly elevated in patients with active UC compared to control

Figure 2. Regulatory role of GATA3 in CD4$^+$ T cells in the experimental oxazolone-induced colitis model. (*A*) *Upper panel*: quantitative polymerase chain reaction analysis of *GATA3* mRNA expression in relation to *18S* rRNA mRNA in colon tissue of oxazolone-induced colitis and untreated mice (5–6 mice per group). Significant differences are indicated. *Lower panels*: relative mRNA expression of *GATA3*, cofilin mRNA, and cytokine mRNA expression was investigated in oxazolone-induced colitis. The correlation coefficient is indicated. (*B*) Analysis of GATA3-producing cells in oxazolone-treated and untreated control mice was done by double-staining with anti-GATA3 and anti-CD4. Cell nuclei were counterstained with Hoechst-33342. *Inserts* show higher magnifications of stained nuclei. *Arrows* highlight GATA3$^+$ T cells in oxazolone colitis (nuclear GATA3 and membrane CD4). Additionally, GATA3-expressing CD4$^+$ cells were quantified in oxazolone-treated and control animals. Data are representative of 7–9 samples per group. (*C*) Fluorescence-activated cell-sorting analysis of GATA3 expression in murine lamina propria mononuclear cells was performed. The percentage of GATA3$^+$CD4$^+$ cells among CD4$^+$ cells is shown in healthy and oxazolone-treated colitic mice (n = 5–7). Representative images are shown. Significant differences are indicated.



BASIC AND TRANSLATIONAL AT

print & web 4C/FPO

patients (Figure 1*A*). In contrast, mean GATA3 levels were not augmented in patients with active CD, although higher levels were observed in colonic compared to ileal CD, as well as in non-stricturing and non-penetrating compared to stricturing or penetrating CD (Supplementary Figure 1*A*). In UC patients, GATA3 expression levels correlated with the activity of the disease. As GATA3 is a key transcription factor controlling production of various T-cell–derived cytokines simultaneously,[24,30] we next correlated GATA3 expression with the levels of inflammatory cytokines in UC (Figure 1*A*). Highest correlations were seen between *GATA3* mRNA expression and levels of *IL9*, *IL17A*, *IL6*, and *IL5*. Additional correlations were detected with *IL4*, *IL13,* and *TNFα* but not with IFN γ and cofilin. These observations were consistent with the hypothesis that GATA3 is a key regulator of inflammatory Th2 and Th9 cytokine production by lamina propria $CD4^+$ T cells in UC. To analyze GATA3 expression in lamina propria T cells in IBD, we then performed CD4/GATA3 staining analysis of colon cryosections from UC, CD patients (Figure 1*B*), and controls, as well as flow cytometric analysis (Figure 1*C*). These studies revealed a significantly increased number of mucosal $CD4^+$/$GATA3^+$ cells in UC patients compared to control and CD patients. Additional staining revealed GATA3 expression in some $EpCAM^+$ epithelial cells, but little or no GATA3 expression in B cells, dendritic cells, and macrophages in UC (Supplementary Figure 1*B*).

Augmented Expression of GATA3 in Oxazolone-Induced Colitis

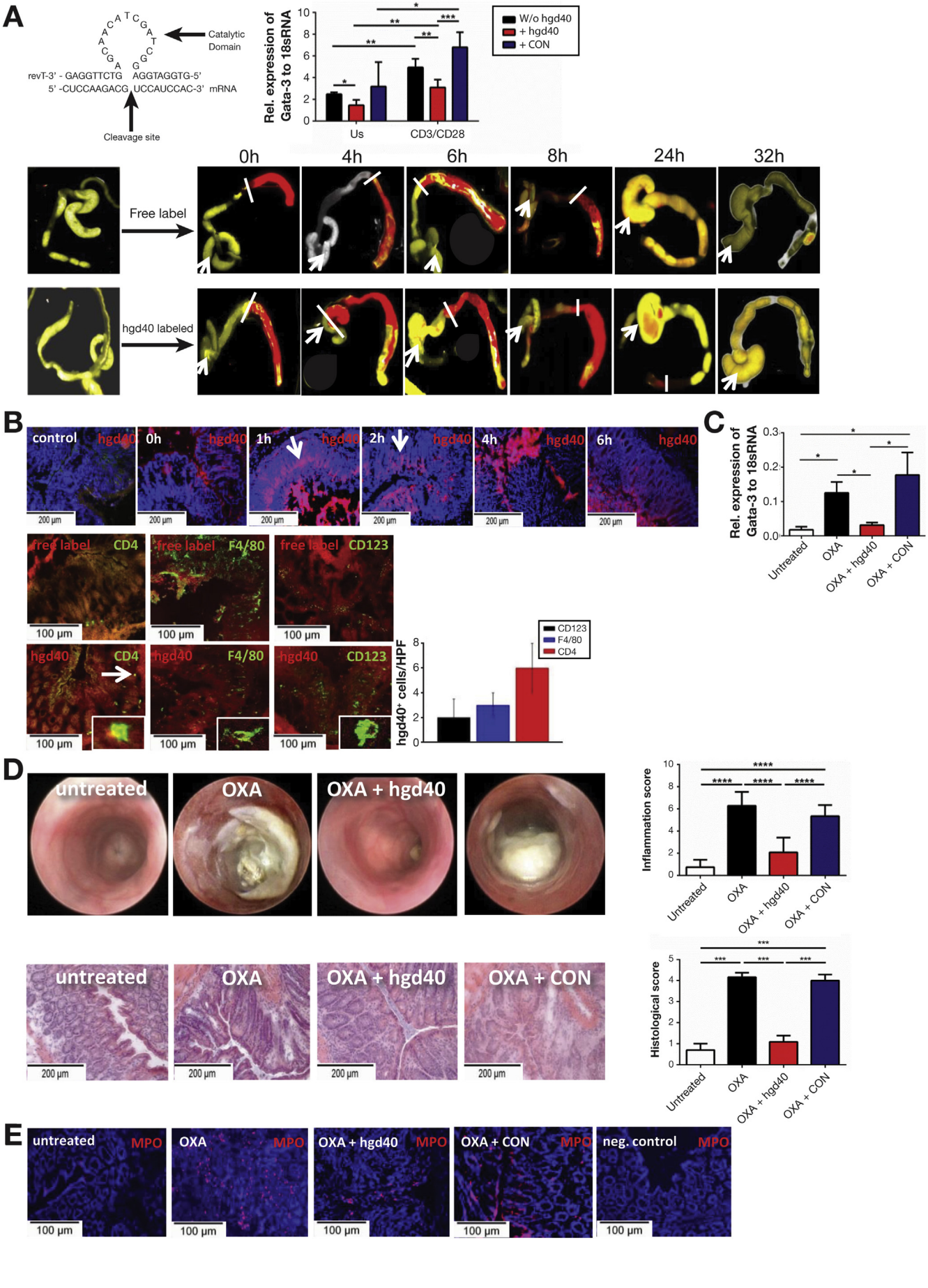
We next determined the expression of GATA3 in experimental oxazolone colitis.[36,37] These studies revealed an up-regulation of *gata3* mRNA expression levels in colonic tissue from oxazolone-treated mice compared to untreated control mice (Figure 2*A*). *Gata3* mRNA levels showed highest correlation with mucosal cytokine expression levels of *Il4*, *Il5*, *Il6*, *Il9*, *Il13*, *Ifna*, and *Il17a/f* (Figure 2*A*). In contrast, low correlation levels were noted between *Gata3* and *Ifng* and *Il12* mRNA and cofilin expression. To analyze GATA3 expression in lamina propria T cells, we then performed CD4/GATA3 staining analysis in untreated and oxazolone-treated murine colons. A significantly increased number of double-positive T cells was seen in oxazolone-treated compared to untreated tissue (Figure 2*B*). In addition, GATA3 expression was observed in some $EpCAM^+$ epithelial cells, but not in B cells, dendritic cells, and macrophages (Supplementary Figure 2). Additionally, intracellular staining with flow cytometry for the expression of GATA3 showed increased numbers of GATA3-expressing $CD4^+$ T cells from colonic tissue in oxazolone-treated mice compared to untreated controls (Figure 2*C*).

Conditional Targeting of GATA3 in T Cells Suppresses Oxazolone-Induced Colitis

To determine the functional role of GATA3 in experimental colitis, we generated T-cell–specific *GATA3*-deficient mice ($Cd4^{Cre}GATA3^{fl/fl}$) and subjected these mice to oxazolone-induced colitis. $Cd4^{Cre}GATA3^{fl/fl}$ mice were protected from experimental colitis compared to controls. Scoring of colitis activity by mini-endoscopy demonstrated a significantly higher activity of mucosal inflammation in wild-type mice compared to $Cd4^{Cre}GATA3^{fl/fl}$ mice (Figure 3*A*). Furthermore, histopathologic assessment confirmed a significant suppression of oxazolone-induced colitis in the absence of GATA3 in T cells. Consistently, the staining of myeloperoxidase in colonic tissue samples revealed more pronounced inflammation in wild-type mice compared to $Cd4^{Cre}GATA3^{fl/fl}$ animals (Figure 3*B*). To determine numbers of $CD4^+$ T cells in $Cd4^{Cre}GATA3^{fl/fl}$ mice, we then performed analysis of colon cryosections from mice with an antibody against CD4. Similar numbers of $CD4^+$ T cells could be observed in wild-type and $Cd4^{Cre}GATA3^{fl/fl}$ mice (Figure 3*C*), indicating that GATA3 does not directly control T-cell accumulation in the inflamed intestine.

To elucidate potential mechanisms of protection of GATA3 deficiency in colitis, cytokine production by purified splenic T cells and lamina propria mononuclear cells was determined. Cytokine levels of IL4, IL6, IL9, IL13, IL1α, IL5, and IL21 were significantly reduced in supernatants of splenic $Cd4^{Cre}GATA3^{fl/fl}$ T cells compared to wild-type mice in oxazolone-mediated colitis. Furthermore, a significant reduction of IFN γ, IL22, IL17A, IL5, IL9, IL13, and IL6 production by mucosal cells was noted in

Figure 3. Conditional GATA3 deficiency in T cells protects animals from experimental colitis. (*A*) Wild-type and *Cd4-Cre-GATA3*$^{fl/fl}$ mice were treated with oxazolone. The inflammation was monitored by mini-endoscopy and scoring of colitis activity (*upper left panels*). Histopathologic analysis was performed using H&E staining of colon specimens (*lower left panels*). Quantitative endoscopic (*upper right panel*) and histopathologic (*lower right panel*) assessment of colitis activity in both groups is shown (3 independent experiments with 3–6 animals per group). (*B*) Myeloperoxidase (MPO) immunostaining of colonic tissue was performed by using MPO antibody in both groups treated with oxazolone. Negative controls showed no positive cells. Representative results of 3 independent experiments (n = 3–6) are shown. (*C*) CD4 immunostaining of colonic tissue from both knockout and wild-type animals was performed. Negative controls showed no positive cells. In addition, $CD4^+$ cells were counted per high-power field (HPF) (*right panel*). Representative results of 2 independent experiments (n = 3–4) are shown. (*D*) Cytokine production in experimental oxazolone colitis was measured. Cells were isolated from wild-type and $Cd4^{Cre}GATA3^{fl/fl}$ mice, followed by anti-CD3/anti-CD28 stimulation. Analysis of supernatants from splenic T cells or lamina propria mononuclear cells was done in $Cd4^{Cre}GATA3^{fl/fl}$ mice compared to wild-type controls. The data represent results of 3–6 mice per group.


A
Catalytic Domain
revT-3' - GAGGTTCTG AGGTAGGTG-5'
5' -CUCCAAGACG UCCAUCCAC-3' mRNA
Cleavage site
Rel. expression of Gata-3 to 18sRNA
W/o hgd40
+ hgd40
+ CON
Us
CD3/CD28
0h
4h
6h
8h
24h
32h
Free label
hgd40 labeled
B
control
hgd40
0h
1h
2h
4h
6h
free label CD4
free label F4/80
free label CD123
hgd40 CD4
hgd40 F4/80
hgd40 CD123
100 µm
200 µm
hgd40+ cells/HPF
CD123
F4/80
CD4
C
Rel. expression of Gata-3 to 18sRNA
Untreated
OXA
OXA + hgd40
OXA + CON
D
untreated
OXA
OXA + hgd40
OXA + CON
Inflammation score
Histological score
E
untreated MPO
OXA MPO
OXA + hgd40 MPO
OXA + CON MPO
neg. control MPO

oxazolone-treated $Cd4^{Cre}GATA3^{fl/fl}$ mice compared to controls (Figure 3*D*). Although we assumed that the regulation of IFN- γ levels in the knockout mice was caused by long-term deficiency of GATA3 in knockout T cells rather than by direct short-term effects of GATA3 on IFN- γ production, we performed additional studies with IFN- γ knockout mice and excluded the possibility that the reduced IFN- γ production protects mice from oxazolone colitis (Supplementary Figure 3). Taken together, these findings highlighted a crucial role of GATA3 in T lymphocytes for the development of colitis.

A Specific DNAzyme Targeting GATA3 Suppresses Expression of GATA3 Messenger RNA in T Cells and Can Be Administered Topically to the Inflamed Colon

Next, we aimed at targeting of GATA3 expression by using a GATA3-specific DNAzyme to inhibit mRNA expression (Figure 4*A*). In initial studies, we tested the effects of the GATA3 DNAzyme, denoted hgd40, in T-cell cultures in vitro. T cells were stimulated with anti-CD3/CD28 in the presence or absence of hgd40 and ODNg3/controls, followed by quantitative analysis of the relative expression of *GATA3* mRNA compared to *18S rRNA*. We found that hgd40 is able to inhibit *GATA3* mRNA expression in unstimulated as well as in stimulated T cells (Figure 4*A*).

In subsequent experiments, we determined the potential uptake and distribution of hgd40 in the inflamed colonic tissue after topical intrarectal application. Thus, red fluorescence–labeled hgd40 was applied once after challenge with oxazolone and distribution of labeled hgd40 was then determined with an in vivo imaging system. The labeled hgd40 was detectable directly after application and showed distribution throughout the murine inflamed colon after 4 hours, while pure dye was localized in the rectum mainly and in the sigmoid colon (Figure 4*A*). After 24 hours, hgd40 was still visible in the rectum and cecum, although with low intensity, suggesting degradation. After 32 hours, the fluorescently labeled hgd40 could no longer be detected.

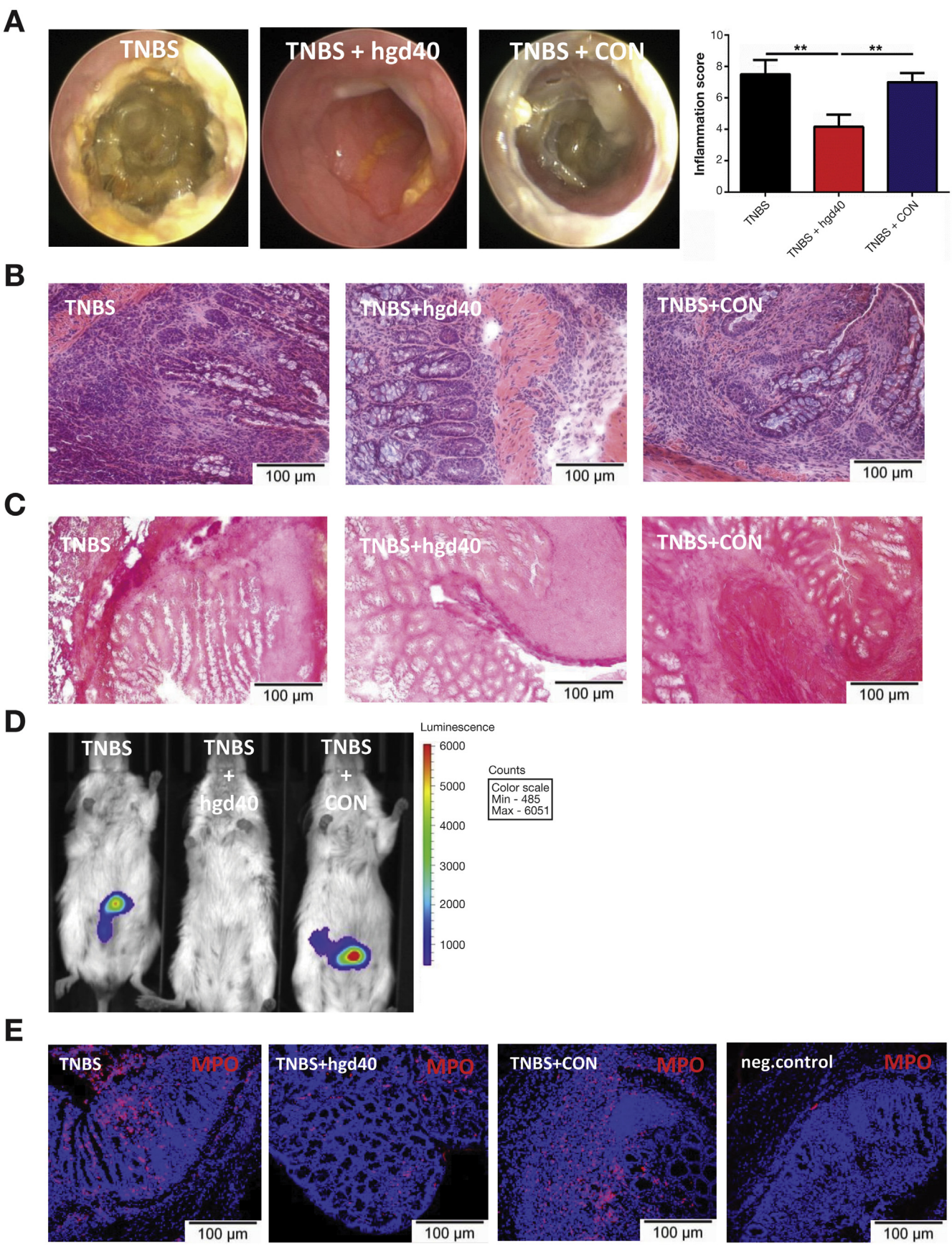
The uptake of hgd40 could already be observed 1 hour after administration and after 2 hours, the DNAzyme was almost selectively localized in the mucosa (Figure 4*B*). Furthermore, we analyzed which cells showed uptake of labeled hgd40 by immunostaining. These studies identified mucosal $CD4^+$ T cells rather than macrophages and dendritic cells as key targets of hgd40 (Figure 4*B*). Collectively, these findings demonstrated that local administration of labeled hgd40 resulted in T-cell uptake in the inflamed colon.

Intrarectal Administration of hgd40 Suppresses Experimental Colitis

Then, we investigated *GATA3* mRNA expression in the colon of colitic mice in the presence or absence of hgd40 or control DNAzyme administration compared to control mice. The expression of *GATA3* mRNA was up-regulated in inflamed tissue in oxazolone-mediated colitis compared to untreated mice (Figure 4*C*). Furthermore, local administration of hgd40 led to significant suppression of *GATA3* mRNA compared to control-treated and untreated mice, suggesting that hgd40 can effectively suppress GATA3 expression in the inflamed colon in vivo.

Mini-endoscopic images from the colon demonstrated marked inflammation in oxazolone-treated mice compared to untreated control mice (Figure 4*D*). Whereas hgd40 administration caused a significant suppression of endoscopic signs of colitis compared to untreated mice with oxazolone-induced colitis, control DNAzyme

Figure 4. Distribution and efficacy of hgd40 in experimental colitis model. (*A*) *Left upper panel*: Sequence and idealized structure of the GATA3-specific DNAzyme hgd40. The localization of the catalytic domain and the cleavage site are indicated. *Right upper panel*: Quantitative polymerase chain reaction analysis of *gata3* mRNA expression in stimulated or unstimulated murine spleen $CD4^+$ T cells with and without (w/o) hgd40 or ODNg3 incubation (1 mg/mL) for 48 hours. Data represent results of 4 independent experiments. *Lower panels*: kinetics of hgd40 distribution in colitic mice was investigated by in vivo imaging. Mice were treated with oxazolone and ATTO665-labeled hgd40 (*red*) was administered intrarectally the day after oxazolone challenge. Analyses were performed before (*left*) and at indicated time points after hgd40 administration (*right*). As control pure dye was used. In vivo imaging was used to display the distribution of ATTO665-labeled hgd40 in colon. Representative images from 2 independent experiments are shown. *White lines* indicate the most proximal area of the murine colon where positive staining was detected and *white arrows* indicate the cecum. (*B*) *Upper panels*: cryosections from colonic tissue of mice after administration of ATTO665-hgd40 were analyzed immediately before and 1, 2, 4, and 6 hours after hgd40 administration. Samples from untreated mice served as negative control. The uptake of hgd40 (*red*) into the mucosa after 1 and 2 hours is highlighted by *arrows*. After 4 and 6 hours, the DNAzyme was mainly detected in the gut lumen. *Lower panels*: images of $CD4^+$ T cells, $F4/80^+$ macrophages, and $CD123^+$ dendritic cells in colonic sections 2 hours after hgd40 administration in oxazolone colitis. Double-positive CD4 T cells containing ATTO665-hgd40 could be detected in colonic mucosa as *yellow-colored cells* and counted per high-power field (HPF). One representative experiment is shown. (*C*) Quantitative polymerase chain reaction analysis of *gata3* mRNA expression in colonic tissue of mice given oxazolone (OXA) that were treated with hgd40 or ODNg3 (as a control DNAzyme, denoted as CON) in relation to 18S rRNA. The data represent results of 3 independent experiments. (*D*) *Upper panels*: mini-endoscopic analysis of mucosal inflammation in mice given oxazolone that were treated with hgd40 or ODNg3. *Lower panels*: histopathologic analysis of murine mucosal inflammation given oxazolone that were treated with hgd40 or ODNg3. The data represent results of 3 independent experiments ($n = 5$). (*E*) Myeloperoxidase (MPO) immunostaining of murine colonic cryosections with oxazolone colitis in the presence or absence of hgd40 and ODNg3. Representative staining from 3 independent experiments ($n = 5$) are shown.


A
TNBS
TNBS + hgd40
TNBS + CON
Inflammation score
10
8
6
4
2
0
**
**
TNBS
TNBS + hgd40
TNBS + CON
B
TNBS
TNBS+hgd40
TNBS+CON
100 µm
C
TNBS
TNBS+hgd40
TNBS+CON
100 µm
D
TNBS
TNBS + hgd40
TNBS + CON
Luminescence
6000
5000
4000
3000
2000
1000
Counts
Color scale
Min - 485
Max - 6051
E
TNBS MPO
TNBS+hgd40 MPO
TNBS+CON MPO
neg.control MPO
100 µm

application had no significant effects. Similarly, histopathologic assessment of colonic inflammation revealed significant reduction of colitis activity upon application of hgd40, but not control DNAzyme. Furthermore, the staining of myeloperoxidase in colonic tissue samples showed that hgd40 administration leads to marked reduction of neutrophil infiltrations in oxazolone-mediated colitis compared to control treated and untreated mice (Figure 4*E*). Finally, we analyzed mucosal $FOXP3^+$ cells as a marker for regulatory T cells by immunostaining in colonic tissue samples, but no difference in the number of $FOXP3^+$ cells was noted (Supplementary Figure 4).

To verify the beneficial effects of hgd40 in a second, independent model of colitis, we addressed its effects in chronic 2,4,6-trinitrobenzenesulfonic acid–mediated colitis. Mice that had been given hgd40 had significantly reduced inflammation and fibrosis compared to control DNAzyme-treated mice (Figure 5*E*), indicating therapeutic efficacy of hgd40 administration.

Kinetics of hgd40 Effects in Comparison to Anti-Tumor Necrosis Factor Treatment in Oxazolone-Induced Colitis

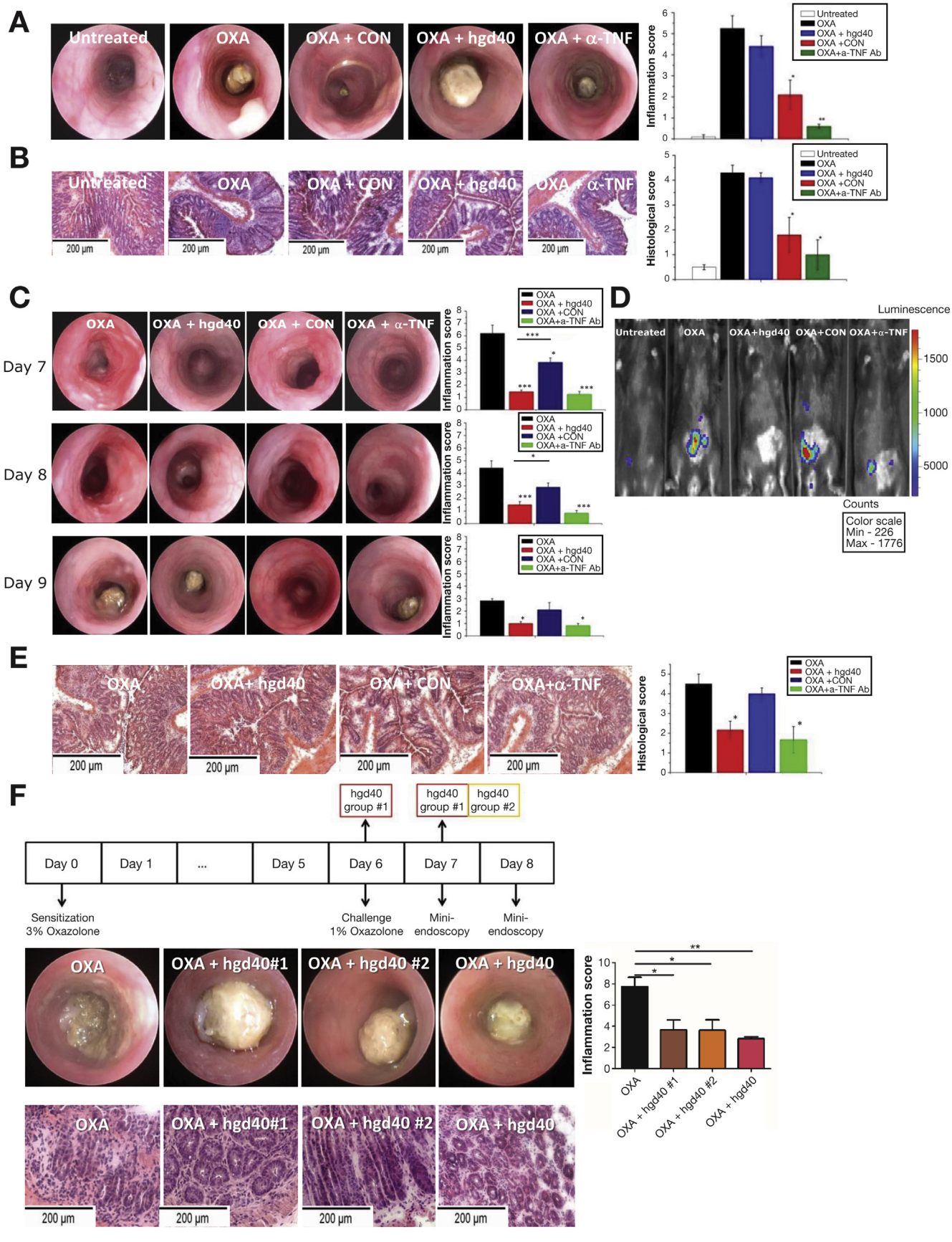
Anti-TNFα has been shown to be effective for limiting experimental colitis and human IBD.[1,38–41] We therefore studied the therapeutic effects of hgd40 in comparison to anti-TNF treatment. Accordingly, mice with oxazolone-mediated colitis were given hgd40 or control DNAzyme intrarectally or anti-TNF systemically. Mini-endoscopic images demonstrated significant mucosal inflammation in oxazolone-treated and control DNAzyme-treated mice, whereas mice receiving hgd40 intrarectally or anti-TNF systemically showed significant suppression of colitis activity (Figure 6*A*). Similar findings were obtained by histopathologic assessment of colitis activity (Figure 6*B*). No significant differences between anti-TNF and hgd40 application were noted, suggesting that both types of therapy are suitable for effective suppression of intestinal inflammation.

Next, we analyzed the effects of hgd40 and anti-TNF application in oxazolone-mediated colitis in a time-dependent manner. Accordingly, mice with oxazolone colitis were monitored by mini-endoscopy over 3 consecutive days after hgd40, control DNAzyme administration, or anti-TNF application. Mini-endoscopic images of the untreated control group with oxazolone-mediated colitis displayed marked inflammation over the entire period (Figure 6*C*). While control DNAzyme administration led to a small decrease of colitis activity on days 7 and 8, hgd40 and anti-TNF application caused a highly significant suppression of colitis activity over 3 days. In vivo imaging demonstrated signs of inflammation in oxazolone-treated mice and the control DNAzyme-treated group rather than in the hgd40- or anti-TNF–treated groups (Figure 6*D*). Similarly, histopathologic scoring showed significant suppression of gut inflammation in hgd40 and anti-TNF–treated groups (Figure 6*E*). Collectively, these findings indicated that the efficacy of hgd40 administration is preserved over a 3-day time period. In a final series of studies, we determined the efficacy of hgd40 application in mice with established oxazolone-mediated colitis. Accordingly, mice were given hgd40 at day 6 or 7 after administration of oxazolone, while control mice were left without application (Figure 6*F*). Mini-endoscopic images of these mice at day 8 showed clear inflammation in untreated mice with oxazolone-mediated colitis, whereas administration of hgd40 led to significantly less inflammation comparably to mice receiving hgd40 given preventively. Additionally, histopathologic assessment of colonic inflammation revealed significant reduction of colitis activity in mice with established oxazolone-mediated colitis upon hgd40 administration (Figure 6*F*).

Intrarectal Administration of hgd40 Suppresses Tumor Necrosis Factor Receptor-Independent Oxazolone-Induced Colitis

The fact that hgd40 administration suppresses several inflammatory cytokines suggested that such application might be effective in TNF-independent or TNF-refractory situations. To test this concept, we analyzed the capacity of hgd40 to block TNF-independent colitis by using TNFR1/2 double-knockout mice. TNFR1/2 knockout mice were subjected to oxazolone-induced colitis followed by administration of hgd40, control DNAzyme, or phosphate-buffered saline. While mice that were given hgd40 showed significant

Figure 5. Efficacy of topical hgd40 treatment in the chronic 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model. (*A*) Mice with chronic TNBS colitis (8 cycles) were treated with hgd40 or ODNg3 as control. Untreated mice with TNBS colitis served as controls. Mini-endoscopic analysis and endoscopic scoring were performed in all groups. Data represent results of 2 independent experiments (n = 5). (*B*) Histopathologic analysis of colon from mice with chronic TNBS-colitis model in the presence or absence of hgd40 or ODNg3 treatment, as indicated. (*C*) Histopathologic analysis of collagen with colon cryosections from chronic TNBS-induced colitis in mice in the presence or absence of hgd40 or ODNg3 treatment, as indicated. *Sirius red staining* showed less collagen in hgd40-treated animals. (*D*) In vivo imaging of treated mice by using luminol for detection of inflammation was performed in all groups. Higher levels of inflammation were detected in chronic TNBS-induced colitis and ODNg3 control-treated mice compared to hgd40-treated animals. No inflammatory signs were observed in hgd40-treated mice. Representative images are shown. (*E*) Myeloperoxidase (MPO) immunostaining of murine colonic cryosections with chronic TNBS colitis in the presence or absence of hgd40 and ODNg3. Representative staining are shown. Fewer MPO^+ cells were found in hgd40-treated animals.



protection from colitis activity, control DNAzyme or phosphate-buffered saline treatment did not result in amelioration of colitis activity (Figure 7*A*). Consistent results were observed by histologic scoring (Figure 7*B*). In addition, hgd40 administration led to reduction of myeloperoxidase-expressing neutrophils in experimental colitis (Figure 7*C*).

Next, we studied cytokine production by lamina propria mononuclear cells in TNFR1/2-deficient animals with oxazolone-mediated colitis. A significant reduction of IL5, IL6, IL9, and IL13 production was noted, as well as lower mean IL17α, IFN γ, and TNFα levels in lamina propria mononuclear cells from oxazolone-treated animals upon hgd40 administration compared to untreated animals (Figure 7*D*). In contrast, no significant changes were noted in control animals given phosphate-buffered saline or control DNAzyme, suggesting that hgd40 mainly suppresses Th2 and Th9 cytokine production in TNF-independent oxazolone-induced colitis.

Discussion

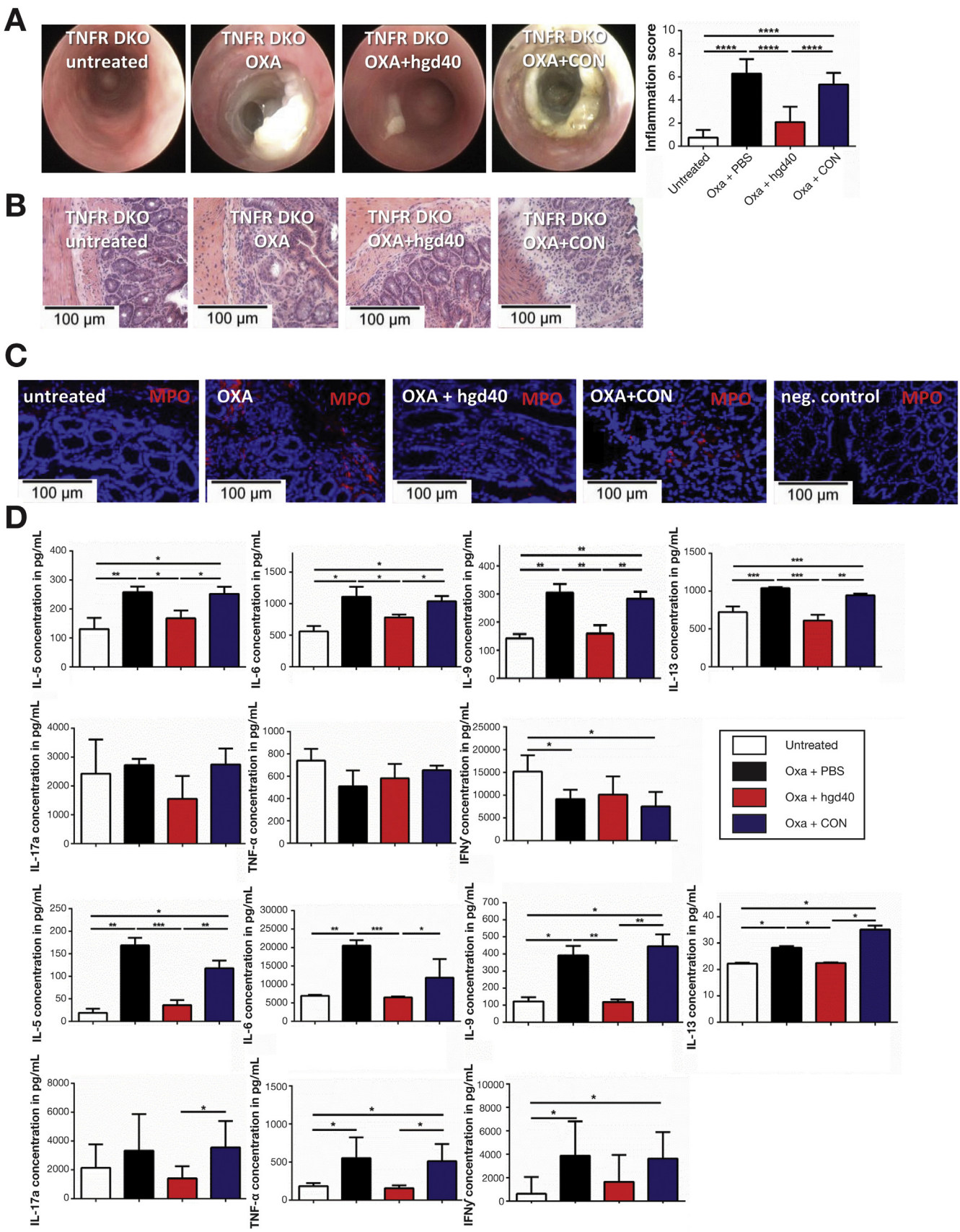
Here, we observed increased expression of GATA3[24,42] in mucosal T lymphocytes from patients with active UC that correlated with mucosal Th2 and Th9 cytokine expression. In addition, conditional GATA3 deficiency in T cells prevented experimental oxazolone-induced colitis; a murine model of UC associated with augmented IL9 and IL13 production.[9,36] Finally, intrarectal administration of GATA3-specific DNAzyme suppressed Th2 and Th9 cytokine production by mucosal T cells and ameliorated oxazolone-derived colitis. Taken together, these findings suggest a key regulatory role of GATA3 in experimental colitis and identify GATA3 as an attractive target to block the activity of various inflammatory cytokines simultaneously.

GATA3 expression was detected in gut T cells and some epithelial cells rather than in macrophages, B cells, and dendritic cells in experimental colitis and IBD. In particular, studies in patients with IBD revealed an increased expression of *GATA3* in mucosal T cells from patients with UC compared to CD and control patients. The reasons for these differences are unclear, but it should be noted that increased levels were also found in subgroups of UC-like CD patients with colonic inflammation and non-stricturing and non-penetrating disease behavior. However, elevated *GATA3* expression has been previously reported in other chronic inflammatory disorders, most notably atopy and allergic asthma, where GATA3 levels were correlated with augmented production of Th2 cytokines, such as IL5 and IL13.[35,36,43] Similarly, we noted a positive correlation between GATA3 and mucosal IL5/IL13 levels in UC patients. However, the highest correlation was observed between GATA3 and the Th9 cytokine IL9. Indeed, GATA3 has been found to bind to the IL9 promoter region[44] and GATA3-deficient T cells have been shown to fail to produce IL9 when cultured under Th9 cell skewing conditions,[45] suggesting that GATA3 may control expression of this cytokine in T cells. Therefore, our findings were consistent with the idea in which T-cell–derived GATA3 drives Th2 and Th9 cytokine production in UC.

A recent study indicated that overexpression of *GATA3* in T cells from transgenic mice augments dextran sulfate sodium–induced colitis.[46] However, the effects of *GATA3* inactivation in experimental colitis have not been studied. Here, we identified elevated *GATA3* expression in murine T cells in oxazolone-induced colitis, where Gata3 levels were correlated with mucosal Th2 and Th9 cytokine levels. The functional relevance was highlighted by studies in $Cd4^{Cre}GATA3^{fl/fl}$ mice. These mice were protected from experimental colitis and had reduced inflammatory cytokine production. Thus, these findings suggested that GATA3 may represent a promising target for therapeutic intervention in colitis.

Because *Gata3* is expressed only intracellularly, we next tested the therapeutic effects of a GATA3-specific DNAzyme with in vivo cell-penetrating capabilities that allow cleavage of *GATA3* mRNA and subsequent degradation by endogenous, intracellular enzymes.[34] DNAzymes of the 10–23 family are single-stranded DNA molecules that are characterized by their capability to specifically cleave RNA molecules after appropriate binding.[47] Thus, they directly exert RNA endonuclease

Figure 6. Efficacy of topical hgd40 treatment in comparison to systemic anti–TNF-antibody treatment in the oxazolone-induced colitis model. (*A*) Mice with oxazolone colitis were treated with hgd40, ODNg3, or anti-TNF. Untreated mice with oxazolone colitis served as controls. Mini-endoscopic analysis and endoscopic scoring were performed in all groups. Data represent results of 2 independent experiments (n = 5). (*B*) Histopathologic analysis and scoring were performed in mice with oxazolone colitis in the presence or absence of hgd40 and ODNg3 or anti-TNFα, as indicated. Data represent results of 2 independent experiments (n = 5). (*C*) Kinetic analysis of the effects of DNAzyme and anti-TNFα administration in experimental oxazolone colitis. Mice were given hgd40, ODNg3, or anti-TNFα. Mucosal inflammation was determined by mini-endoscopy at days 7, 8, and 9. (*D*) In vivo imaging of treated mice by using luminol for detection of inflammation was performed in all groups. Higher levels of inflammation were detected in oxazolone colitis and control-treated mice compared to hgd40- and anti-TNFα–treated animals. Representative images from mice are shown. (*E*) Histology score was performed in all groups of mice. Data represent results of 2 independent experiments (n = 5). (*F*) Treatment of mice with established oxazolone colitis. Oxazolone-treated mice were given hgd40 starting at days 6 and 7 (OXA+hgd40 group #1) or day 7 (OXA+hgd40 group #2). Untreated mice with oxazolone colitis (OXA group) and colitic mice given hgd40 before oxazolone challenge (OXA+hgd40 group) served as controls. Mini-endoscopy was performed at days 7 and 8 and representative images at day 8 for all groups are shown. In addition, histopathologic analysis and scoring were performed in all groups.



activity after Watson-Crick base-pairing of their binding domains to their corresponding sequence in the target mRNA and thus activation of their catalytic domain. Such DNAzymes represent a particular class of antisense molecules combining the superior specificity of antisense molecules with an inherent catalytic activity that makes them an attractive tool for the blockade of GATA3.[48] In our studies on Gata3-specific DNAzyme hgd40, we used local delivery of the drug directly to the gut and noted efficient distribution throughout the colon in oxazolone colitis. Subsequent studies with fluorescent hgd40 demonstrated penetration through the inflamed gut epithelium and identified intracellular uptake of hgd40 by mucosal T cells within 2 hours after administration. Uptake of DNAzyme led to significant suppression of colitis activity associated with marked suppression of Th2 cytokine production. These observations are consistent with previous studies suggesting that Gata3 expression is essential to maintain a Th2 phenotype in T cells.[42,49] Additionally, production of IL9, a Th9 cytokine-driving activity of oxazolone-induced colitis,[9] was significantly suppressed by hgd40 administration. Taken together, our findings in the $Cd4^{Cre}GATA3^{fl/fl}$ mice and with the GATA3 DNAzyme consistently show that GATA3 deficiency has profound effects on Th2 and Th9 cytokine production by mucosal T cells in colitis. The differences between some cytokines, such as IFN γ in knockout mice vs DNAzyme-receiving mice, are most likely due to the long-term effects of GATA3 deficiency in the knockout mice vs short-term GATA3 suppression in hgd40-treated mice, and highlight the preferential rapid regulation of mucosal Th2 and Th9 cytokines by GATA3 compared to additional, later-occurring effects on other proinflammatory cytokines. It is tempting to speculate that long-term treatment of IBD patients with GATA3 blockers, such as hgd40, may not only block Th2 and Th9 cytokines, but also other proinflammatory cytokines, such as IFN γ, over time, and may therefore exhibit cumulative beneficial effects.

In contrast to effector T-cell function, a recent report showed that function of regulatory T cells is not affected by selective GATA3 inactivation.[50] Furthermore, Gata3-deficient and wild-type regulatory T cells (Tregs) were comparable in their suppressive function of effector T-cell proliferation, as well as their capacity to survive and proliferate.[51] However, GATA3 has been found to control Treg accumulation in inflamed tissues in vivo,[51] suggesting that long-standing suppression of Gata3 expression in T cells may interfere with Treg function. However, we did not obtain evidence for impaired Treg accumulation under our experimental conditions.

Anti-TNF is routinely used for clinical therapy with active UC.[3] However, 30%−50% of the patients fail to respond to anti-TNF therapy due to the presence of TNF-independent mucosal inflammation. We therefore considered the possibility that hgd40 might be effective in TNF-independent colitis by blocking various inflammatory cytokines. Accordingly, we used TNFR1/2 double-knockout mice[52] to induce TNF-independent oxazolone-mediated colitis and analyzed the effects of hgd40 application. We noted efficient suppression of colitis activity and significant impairment of production of various inflammatory cytokines by mucosal T cells indicating that hgd40 suppresses TNF-independent experimental colitis. Additionally, we found that hgd40 application was effective in another model of colitis mediated by chronic 2,4,6-trinitrobenzenesulfonic acid administration.

In summary, we have targeted expression and function of the transcription factor GATA3 by genetic ablation strategies and local administration of a GATA3-specific DNAzyme in experimental colitis. GATA3 blockade ameliorated colitis activity and was associated with suppression of local production of multiple inflammatory Th2 and Th9 cytokines in experimental colitis. The local rather than systemic targeting of GATA3 may prevent potential side effects caused by systemic blockade of multiple biologically relevant cytokines. Interestingly, hgd40 was effective in protecting mice in 2 independent colitis models, as well as in TNF-independent colitis consistent, with the concept that blockade of GATA3 function may be broadly applicable for blockade of colitis. As a recent clinical study indicated the potential benefit of local DNAzyme-mediated GATA3 blockade in patients with allergic asthma,[34] hgd40 emerges as a novel approach for therapy in human UC.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2016.09.005.

Figure 7. Intrarectal administration of hgd40 in TNFR double-knockout mice during oxazolone colitis. (*A*) TNFR1/2 double-knockout mice with oxazolone colitis were treated with hgd40 or ODNg3. Untreated and oxazolone-treated knockout mice served as controls. The inflammation of the mucosa was measured by mini-endoscopic scoring at day 8. Representative images are shown. Data represent results of 2 independent experiments (n = 5). (*B*) Representative histologic images from all groups are shown. A marked reduction of colitis activity was noted in the hgd40 group compared to the oxazolone group. (*C*) Myeloperoxidase immunostaining of colonic cryosections in TNFR1/2 knockout mice with oxazolone colitis in the presence or absence of treatment with hgd40 and ODNg3. Representative staining from 2 independent experiments are shown. (*D*) Cytokine production in experimental colitis in TNFR1/2 knockout mice. Cells were isolated from knockout mice followed by anti-CD3/anti-CD28 stimulation. Analysis of supernatants from spleen T cells or lamina propria mononuclear cells demonstrated significant suppression of IL5, IL6, IL9, and IL13 production by cells in mice given hgd40 compared to controls. The data represent results of 3–5 mice per group.

References

1. Baumgart DC, Sandborn WJ. Crohn's disease. Lancet 2012;380:1590–1605.
2. Beaugerie L, Seksik P, Nion-Larmurier I, et al. Predictors of Crohn's disease. Gastroenterology 2006; 130:650–656.
3. Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med 2011;365:1713–1725.
4. Monteleone I, Sarra M, Pallone F, et al. Th17-related cytokines in inflammatory bowel diseases: friends or foes? Curr Mol Med 2012;12:592–597.
5. Neurath MF. Cytokines in inflammatory bowel disease. Nat Rev Immunol 2014;14:329–342.
6. Fuss IJ, Neurath M, Boirivant M, et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol 1996;157:1261–1270.
7. Fuss IJ, Heller F, Boirivant M, et al. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. J Clin Invest 2004;113:1490–1497.
8. Breese E, Braegger CP, Corrigan CJ, et al. Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. Immunology 1993; 78:127–131.
9. Gerlach K, Hwang Y, Nikolaev A, et al. TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. Nat Immunol 2014;15:676–686.
10. Nalleweg N, Chiriac MT, Podstawa E, et al. IL-9 and its receptor are predominantly involved in the pathogenesis of UC. Gut 2015;64:743–755.
11. Atreya R, Mudter J, Finotto S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat Med 2000;6:583–588.
12. Pariente B, Mocan I, Camus M, et al. Activation of the receptor NKG2D leads to production of Th17 cytokines in CD4+ T cells of patients with Crohn's disease. Gastroenterology 2011;141:217–226; 226 e1−e2.
13. Kleinschek MA, Boniface K, Sadekova S, et al. Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. J Exp Med 2009; 206:525–534.
14. Neurath MF, Finotto S, Glimcher LH. The role of Th1/Th2 polarization in mucosal immunity. Nat Med 2002; 8:567–573.
15. Ivanov II, Atarashi K, Manel N, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 2009;139:485–498.
16. Schraml BU, Hildner K, Ise W, et al. The AP-1 transcription factor Batf controls T(H)17 differentiation. Nature 2009;460:405–409.
17. Szabo SJ, Kim ST, Costa GL, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000; 100:655–669.
18. Ho IC, Tai TS, Pai SY. GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation. Nat Rev Immunol 2009;9:125–135.
19. Kaplan MH, Hufford MM, Olson MR. The development and in vivo function of T helper 9 cells. Nat Rev Immunol 2015;15:295–307.
20. Parrello T, Monteleone G, Cucchiara S, et al. Up-regulation of the IL-12 receptor beta 2 chain in Crohn's disease. J Immunol 2000;165:7234–7239.
21. Neurath MF, Weigmann B, Finotto S, et al. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. J Exp Med 2002;195:1129–1143.
22. Ohtani K, Ohtsuka Y, Ikuse T, et al. Increased mucosal expression of GATA-3 and STAT-4 in pediatric ulcerative colitis. Pediatr Int 2010;52:584–589.
23. Scheinman EJ, Avni O. Transcriptional regulation of GATA3 in T helper cells by the integrated activities of transcription factors downstream of the interleukin-4 receptor and T cell receptor. J Biol Chem 2009;284: 3037–3048.
24. Tindemans I, Serafini N, Di Santo JP, et al. GATA-3 function in innate and adaptive immunity. Immunity 2014; 41:191–206.
25. Ouyang W, Lohning M, Gao Z, et al. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity 2000;12:27–37.
26. Yang XO, Angkasekwinai P, Zhu J, et al. Requirement for the basic helix-loop-helix transcription factor Dec2 in initial TH2 lineage commitment. Nat Immunol 2009; 10:1260–1266.
27. Usui T, Nishikomori R, Kitani A, et al. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. Immunity 2003;18:415–428.
28. Hosokawa H, Tanaka T, Suzuki Y, et al. Functionally distinct Gata3/Chd4 complexes coordinately establish T helper 2 (Th2) cell identity. Proc Natl Acad Sci U S A 2013;110:4691–4696.
29. Lee GR, Kim ST, Spilianakis CG, et al. T helper cell differentiation: regulation by cis elements and epigenetics. Immunity 2006;24:369–379.
30. Pai SY, Truitt ML, Ting CN, et al. Critical roles for transcription factor GATA-3 in thymocyte development. Immunity 2003;19:863–875.
31. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. Nat Protoc 2007;2:541–546.
32. Becker C, Fantini MC, Neurath MF. High resolution colonoscopy in live mice. Nat Protoc 2006;1:2900–2904.
33. Weigmann B, Lehr HA, Yancopoulos G, et al. The transcription factor NFATc2 controls IL-6-dependent T cell activation in experimental colitis. J Exp Med 2008; 205:2099–2110.
34. Krug N, Hohlfeld JM, Kirsten AM, et al. Allergen-induced asthmatic responses modified by a GATA3-specific DNAzyme. N Engl J Med 2015;372:1987–1995.
35. Homburg U, Renz H, Timmer W, et al. Safety and tolerability of a novel inhaled GATA3 mRNA targeting

DNAzyme in patients with T2-driven asthma. J Allergy Clin Immunol 2015;136:797–800.
36. Heller F, Fuss IJ, Nieuwenhuis EE, et al. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. Immunity 2002; 17:629–638.
37. Boirivant M, Fuss IJ, Chu A, et al. Oxazolone colitis: A murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. J Exp Med 1998; 188:1929–1939.
38. Danese S, Colombel JF, Peyrin-Biroulet L, et al. Review article: the role of anti-TNF in the management of ulcerative colitis—past, present and future. Aliment Pharmacol Ther 2013;37:855–866.
39. Powrie F, Leach MW, Mauze S, et al. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity 1994;1:553–562.
40. Atreya R, Zimmer M, Bartsch B, et al. Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal CD14(+) macrophages. Gastroenterology 2011;141:2026–2038.
41. Neurath MF. Cytokines in inflammatory bowel disease. Nat Rev Immunol 2014;14:329–342.
42. Zhou M, Ouyang W. The function role of GATA-3 in Th1 and Th2 differentiation. Immunol Res 2003;28:25–37.
43. Finotto S, De Sanctis GT, Lehr HA, et al. Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression. J Exp Med 2001;193:1247–1260.
44. Nakatsukasa H, Zhang D, Maruyama T, et al. The DNA-binding inhibitor Id3 regulates IL-9 production in CD4(+) T cells. Nat Immunol 2015;16:1077–1084.
45. Dardalhon V, Awasthi A, Kwon H, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. Nat Immunol 2008;9:1347–1355.
46. Okamura M, Yoh K, Ojima M, et al. Overexpression of GATA-3 in T cells accelerates dextran sulfate sodium-induced colitis. Exp Anim 2014;63:133–140.
47. Chan CW, Khachigian LM. DNAzyme delivery approaches in biological settings. Curr Med Chem 2013; 20:3448–3455.
48. Khachigian LM. Catalytic DNAs as potential therapeutic agents and sequence-specific molecular tools to dissect biological function. J Clin Invest 2000;106:1189–1195.
49. Pai SY, Truitt ML, Ho IC. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. Proc Natl Acad Sci U S A 2004;101:1993–1998.
50. Yu F, Sharma S, Edwards J, et al. Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. Nat Immunol 2015; 16:197–206.
51. Wohlfert EA, Grainger JR, Bouladoux N, et al. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. J Clin Invest 2011;121:4503–4515.
52. Suzuki J, Cole SE, Batirel S, et al. Tumor necrosis factor receptor -1 and -2 double deficiency reduces graft arterial disease in murine cardiac allografts. Am J Transplant 2003;3:968–976.

Author names in bold designate shared co-first authorship. Q15

Received November 13, 2015. Accepted September 6, 2016.

Reprint requests
Address requests for reprints to: Markus F. Neurath, MD, Department of Medicine 1, Kussmaul Campus for Medical Research, University Erlangen-Nürnberg, Ulmenweg 18, 91054, Nürnberg Germany. e-mail: markus.neurath@uk-erlangen.de; fax: ■■■. Q3 Q4

Acknowledgments
The authors thank A. von Berg, L. Sologub, A. Diener, and S. Fiedler for excellent technical assistance and F. Richter and U. Schleicher for the generous gift of knockout mice.

Author contributions: B.W., V.P., K.G., and S.M. performed experiments. A.T., B.W., V.P., K.G., S.M., and H.-A.L. analyzed the data. A.T., H.G., and H.R. advised on data evaluation. R.A. collected human samples, I.-C.H. provided material and discussed data. H.R. and M.F.N. designed and supervised the project. B.W. and M.F.N. wrote the manuscript.

Benno Weigmann and Markus F. Neurath contributed equally to this work. Q14

Conflicts of interest
These authors disclose the following: Markus F. Neurath is consultant for MSD, Boehringer, Janssen, AbbVie, Giuliani Pharma, and Pentax. Harald Renz and Holger Garn are co-founders and advisors for sterna biologicals. Agnieszka Turowska is an employee of sterna biologicals. The remaining authors disclose no conflicts. Q5

Funding
Katharina Gerlach was supported by the Interdisciplinary Center for Clinical Research Erlangen (J50). Benno Weigmann was supported by Deutsche Forschungsgemeinschaft grant WE4656/2-2 and SFB 1181/B02. Markus F. Neurath and Raja Atreya were funded by Clinical Research Unit 257. This study on hgd40 was supported by sterna biologicals. Q6 Q7

Supplementary Material and Methods

Patients With Inflammatory Bowel Diseases

To determine the expression of GATA3 mRNA in humans, samples from IBD patients and healthy controls were analyzed. Specimens from patients with CD (n = 61) and UC (n = 74) were compared with control samples (n = 22). To determine expression of GATA3 in CD patients, samples were determined after Montreal classification[1] and analyzed. Characteristics of the patient samples are described in Supplementary Table 1. The collection of samples was approved by the ethical committee of University Erlangen-Nürnberg.

Animals

Mice (6–12 weeks old) were housed under specific pathogen-free conditions and experiments were performed in accordance with institutional guidelines. IFN- γ knockout mice were a kind gift from Ulrike Schleicher (University of Erlangen, Germany).

Isolation of Human and Murine Colonic Messenger RNA and Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from colonic tissue with the RNA-Micro kit (Machery-Nagel, Bethlehem, PA). Complementary DNA was subsequently generated with iScript (Bio-Rad, Hercules, CA). Quantitative reverse transcription polymerase chain reaction was performed with SensiFAST-SYBR (Bioline, Taunton, MA) and specific primers (Qiagen, Valencia, CA). Using 18S rRNA, the relative expression level of cytokine mRNA was calculated with the formula: relative cytokine mRNA expression = $2^{\wedge}[c_t(\text{mRNA of interest}) - c_t(\text{mRNA 18S rRNA})]$, where c_t is the number of the cycle in which emission exceeds an arbitrarily defined threshold.

Immunofluorescence Staining of Human Colonic Tissues

Colonic cryosections from healthy and UC patients were stained with mouse-anti-hCD4 antibody (BioLegend). Afterward, slides were incubated with goat-anti-mouse AlexaFluor488, goat-anti-rat AlexaFluor488, or goat-anti-rabbit AlexaFluor594 secondary antibody (Life Technologies). Nuclei were counterstained with Hoechst-33342 (Invitrogen). Double staining for CD4, EpCAM (clone 9C4), CD14 (clone RA3-6B2), CD45R (clone 63D3), CD123 (clone 6H6), and rabbit-anti-h/mGATA3 antibodies (Santa Cruz Biotechnology) was performed together with permeabilization buffer (eBioscience). For detection goat-anti-rabbit AlexaFluor594 antibodies (Life Technologies) were applied. Negative control slides were incubated with secondary antibodies.

Immunofluorescence Staining in Murine Samples

Murine colonic cryosections were fixed with methanol and immunofluorescence staining was done using rat-anti-mEpCAM, -mB220, -mF4/80, and -mCD123 antibodies (BioLegend). Afterwards, sections were incubated with goat-anti-rat AlexaFluor488 antibody (Life Technologies). For GATA3 staining, rabbit-anti-mGATA3 antibodies (Santa Cruz Biotechnology) were used together with permeabilization buffer and goat-anti-rabbit AlexaFluor594 (Life Technologies). Negative control slides were incubated with secondary antibodies. Nuclei were stained with Hoechst-33342. For FOXP3 staining, colonic cryosections were acetone-fixed and staining was done using rat-anti-mouse antibodies. Then, slides were incubated with goat-anti-rat AlexaFluor488 antibody. Nuclei were stained with Hoechst-33342.

Collagen Staining in Murine Samples

Cryosections of murine colon were prepared and first incubated with hematoxylin, then in Pico-Sirius red solution for 1 hour before mounting.

Oxazolone-Induced Model of Intestinal Inflammation

Oxazolone-induced colitis was performed as follows: mice were sensitized by epicutaneous application of 2.5% oxazolone (Sigma-Aldrich, St Louis, MO) at a 4:1 dilution in acetone/oil mixture on day 0, followed by intrarectal administration of 1% oxazolone in 50% ethanol, as described previously. Monitoring of inflammation and endoscopy was performed as described previously.

Chronic 2,4,6-Trinitrobenzenesulfonic Acid–Induced Model of Intestinal Inflammation

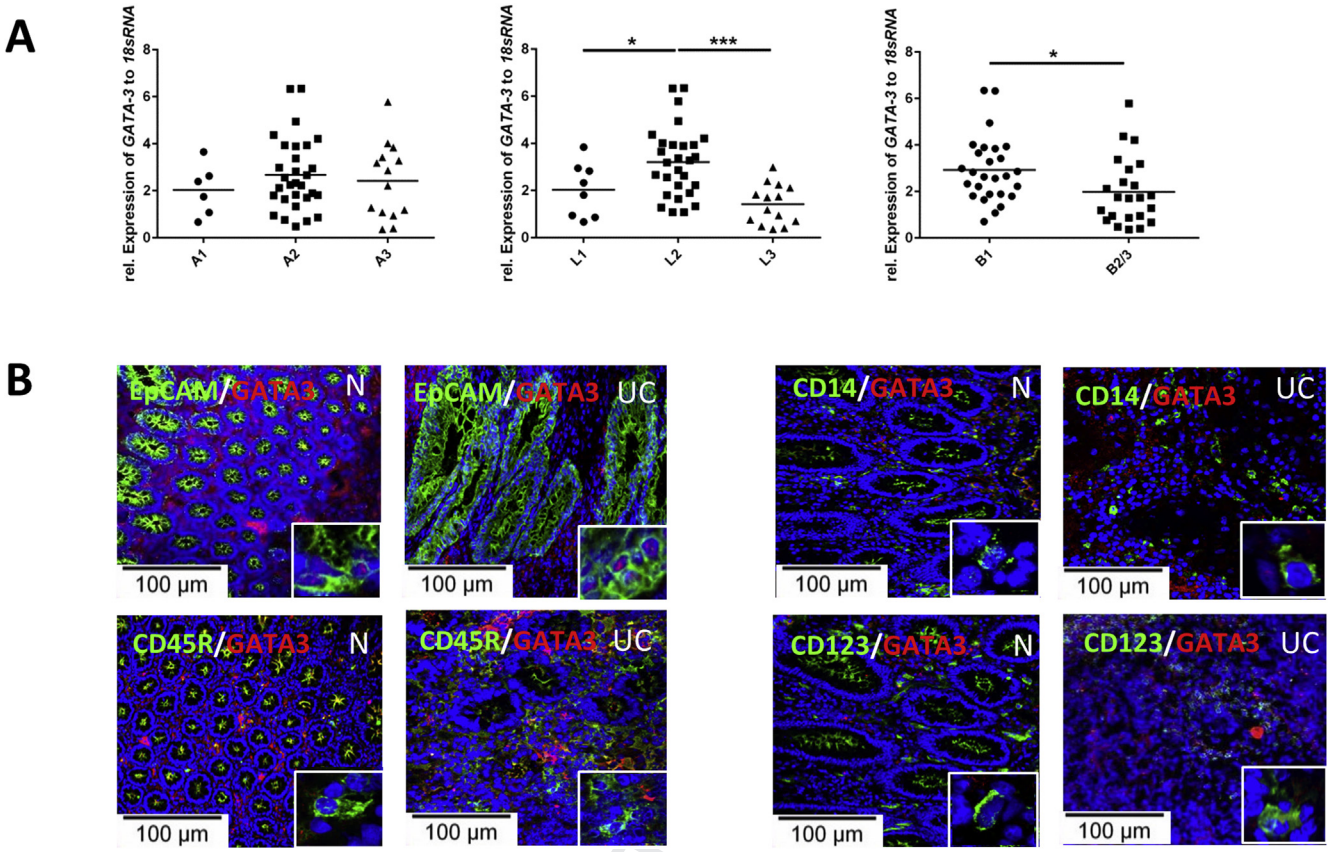
2,4,6-Trinitrobenzenesulfonic acid–induced colitis was described previously by Fichtner-Feigl et al[2] and performed as follows: mice were sensitized by epicutaneous application of 1% 2,4,6-trinitrobenzenesulfonic acid (Sigma-Aldrich) at a 4:1 dilution in acetone/oil mixture on day 0, followed by intrarectal administration of 1%–2.5% 2,4,6-trinitrobenzenesulfonic acid in 50% ethanol for 8 cycles. For treatment of experimental colitis hgd40 (1000 μg) and control DNAzymes (1000 μg) were intrarectally administered at beginning of each cycle.

Statistics

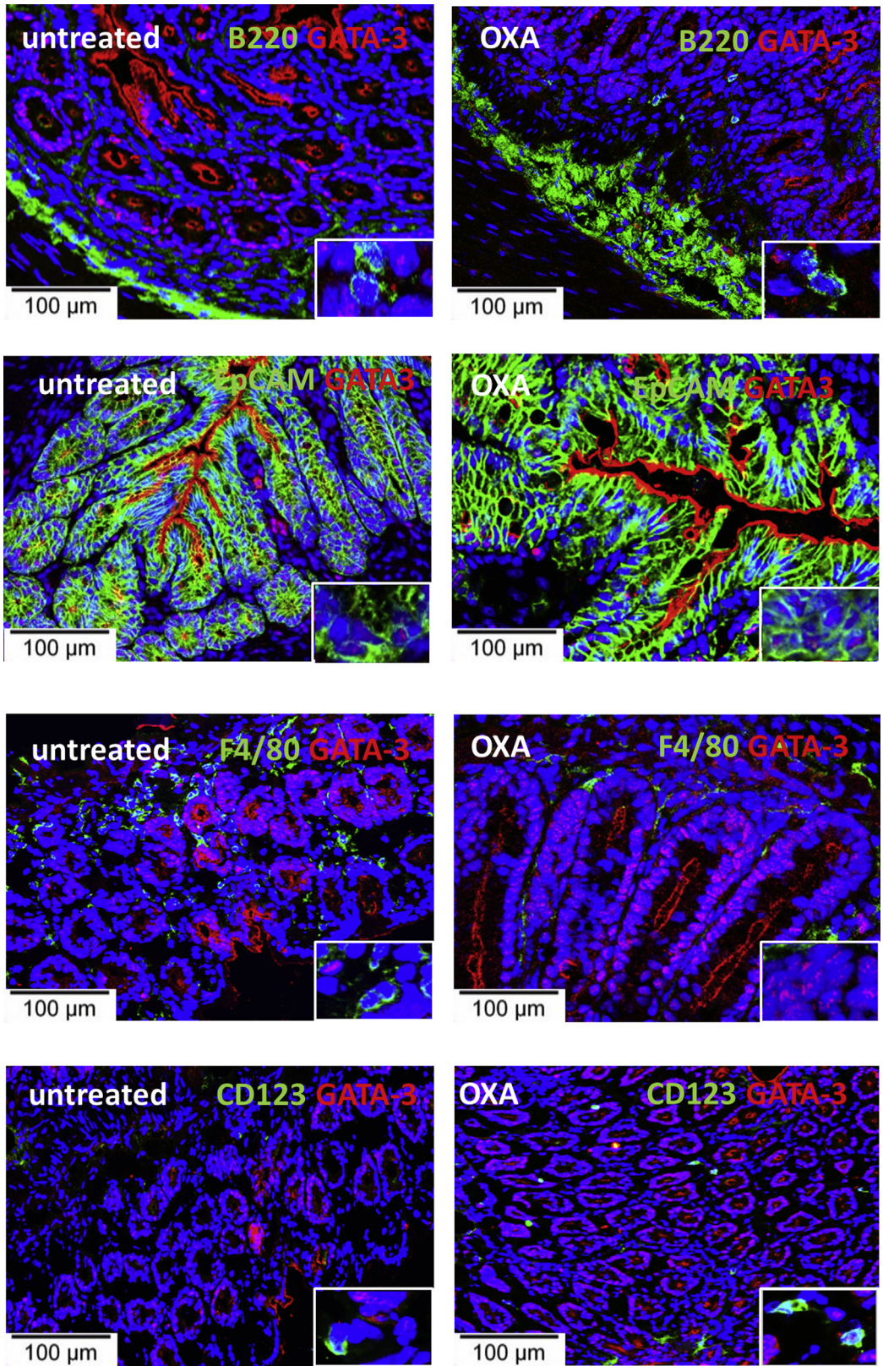
Statistical differences between groups were determined by using analysis of variance or Student t tests. Correlation studies were performed by Spearmen's ρ. P values $<.05$ were considered statistically significant ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$). Results are expressed as mean values with SEM.

Supplementary References

1. Silverberg MS, Satsangi J, Ahmad T, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a working party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol 2005;19:5–36.
2. Fichtner-Feigl S, Fuss IJ, Young CA, et al. Induction of IL-13 triggers TGF-beta1-dependent tissue fibrosis in chronic 2,4,6-trinitrobenzene sulfonic acid colitis. J Immunol 2007;178:5859–5870.

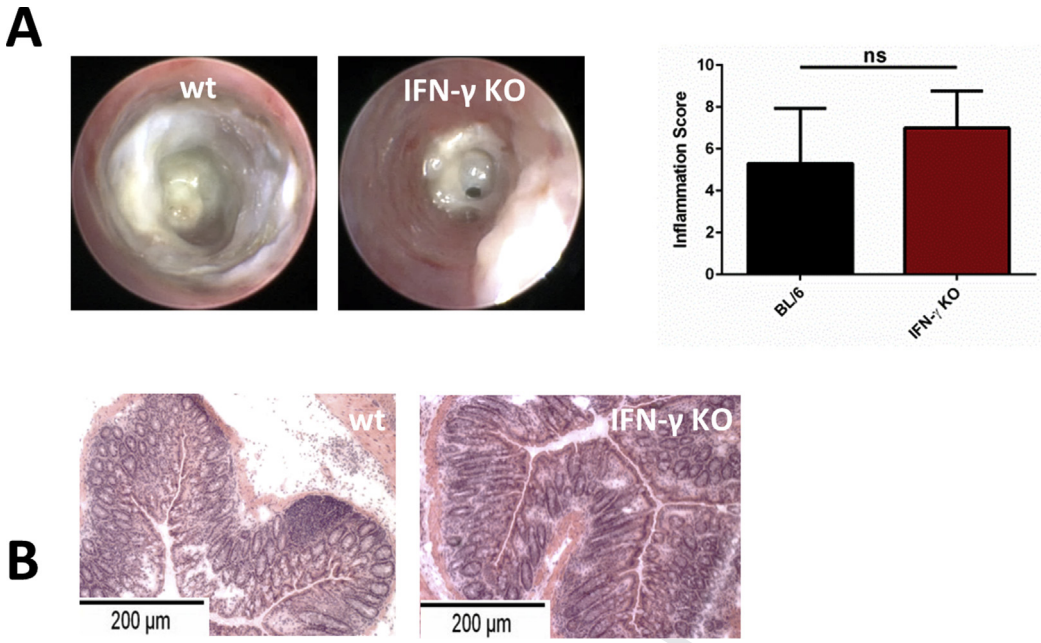


Supplementary Figure 1. Expression of GATA3 in CD patients and immunohistochemical staining of GATA3 in UC patients. (*A*) Total mRNA from biopsies of CD patients was isolated and analyzed for *GATA3*-mRNA expression in relation to *18S rRNA*. Patients with active and inactive CD were tested for *GATA3* mRNA expression level. CD samples were categorized according to the Montreal classification into different subgroups, dependent on age of sample taking (*A1–3*), behavior (*B1–3*), and localization (*L1–3*). Data are representative of 7–35 patients per group. (*B*) Immunohistochemistry for GATA3 and EpCAM, CD45R, CD14, and CD123 expressing cells in colonic samples from UC patients and controls. Cells were counterstained with Hoechst-33342. *Inserts* show higher magnifications of stained nuclei. Whereas few or no GATA3-expressing cells could be observed together with CD45R, CD14, and CD123 cells, few GATA3/EpCAM-positive cells were found in healthy controls and UC patients. Images are representative of 2–3 samples per group.

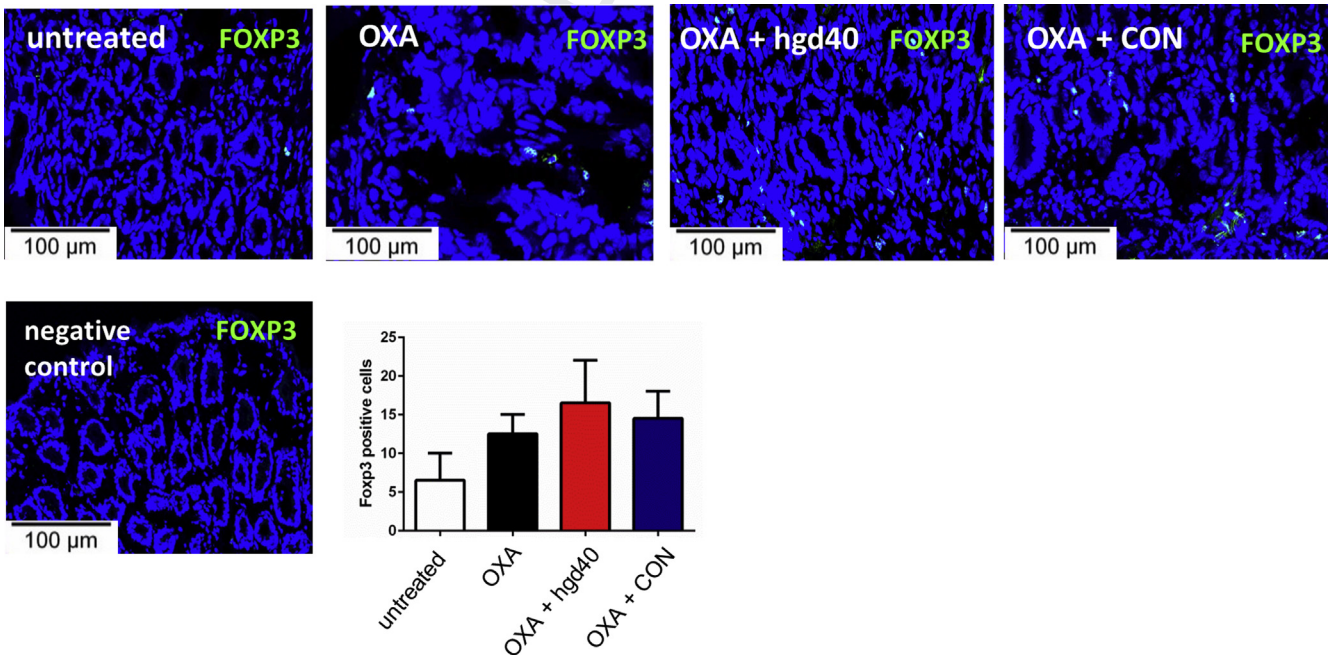


Supplementary Figure 2. Immunohistochemical staining of GATA3 expression in inflamed murine colonic cells. Analysis of GATA3-producing cells in oxazolone-treated and untreated control mice was done by double staining with anti-GATA3 and anti-B220, anti-EpCAM, anti-F4/80, and anti-CD123 antibodies. Cell nuclei were counterstained with Hoechst-33342. *Inserts* show higher magnifications of stained nuclei. While some GATA3-expressing epithelial cells were noted, little or no macrophages and dendritic cells or B cells expressed GATA3. No differences between oxazolone colitis and control samples with regard to the GATA3-expressing cells were noted. Data are representative of 3 samples per group.

web 4C/FPO



Supplementary Figure 3. IFN- γ deficiency did not protect animals from experimental colitis. (*A*) Wild-type and IFN- γ knockout mice (n = 5) were treated with oxazolone. The inflammation was monitored by mini-endoscopy and scoring of colitis activity (*upper left panels*). No significant differences were noted. (*B*) Histopathologic analysis was performed using H&E staining of colon specimens.



Supplementary Figure 4. Immunohistochemical staining of FOXP3 expression in inflamed murine colonic cells. FOXP3 immunostaining of murine colonic cryosections with oxazolone colitis and untreated controls in the presence or absence of hgd40 and ODNg3 was done. FOXP3$^+$ cells were counted per high-power field (HPF). Representative staining from 3 independent experiments (n = 5) are shown. No significant differences in numbers of FOXP3$^+$ cells were observed.

Supplementary Table 1. Patient Characteristics

Characteristics	Data
n	157
Control, n	22
CD, n	61
UC, n	74
Age, *y*, range (XX)	10–79 (37, 78)
Female, n (%)	40 (13)