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# **Endogenous control of inflammatory visceral pain by T cell-derived opioids in IL-10-deficient mice**

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**Running title:** Immune control of gut pain in IL-10<sup>-/-</sup> mice

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## **Abstract**

**Background:** The opioid-mediated analgesic activity of mucosal CD4<sup>+</sup> T lymphocytes in colitis has been reported in immunocompetent mice so far. Here, we investigated whether CD4<sup>+</sup> T lymphocytes alleviate from inflammation-induced abdominal pain in mice with defective immune regulation.

**Methods:** Endogenous control of visceral pain by opioids locally produced in inflamed mucosa was assessed in IL-10-deficient mice.

**Key Results:** CD4<sup>+</sup> T lymphocytes but not F4/80<sup>+</sup> macrophages isolated from the *lamina propria* of IL-10-deficient mice with colitis express enkephalin-containing opioid peptides as assessed by cytofluorometry. Colitis in IL-10<sup>-/-</sup> mice was not associated with abdominal pain. Intraperitoneal injection of naloxone-methiodide, a peripheral opioid receptor antagonist, induced abdominal hypersensitivity in IL-10<sup>-/-</sup> mice with colitis.

**Conclusion and inferences:** Opioid-mediated analgesic activity of mucosal T lymphocytes remains operating in IL-10<sup>-/-</sup> mice with impaired immune-regulation. The data suggest that endogenous T cell-derived opioids might reduce inflammation-induced abdominal pain in inflammatory bowel diseases associated with homozygous “loss of function mutations” in interleukin-10.

**Keywords:** Intestinal inflammation, Visceral pain, Opioids, T lymphocytes, IL10<sup>-/-</sup> mice

## Key Points

- ✓ Analgesia mediated by T lymphocyte-derived opioids is well documented in immunocompetent mice with colitis
- ✓ Here we show that the opioid-mediated analgesic effect of T lymphocytes persists in inflammatory bowel disease-like colitis in interleukin-10-deficient mice
- ✓ Our data suggest that the endogenous control of abdominal pain by colitogenic mucosal T lymphocytes could be generalized regardless of the origin of the chronic intestinal inflammation

## 1 | INTRODUCTION

Inflammatory bowel diseases (IBD) are characterized by an abnormal response of the mucosal immune system against microbiota. Etiology of IBD remains largely unknown, but genetic defects in immune regulation are predisposing to development of inflammatory bowel diseases including Crohn's disease<sup>1,2</sup>. Among more than 230 susceptibility loci reported so far, a number of genes are related to IL-10 biology including *IL10*, *IL10RA*, or *IL10RB* genes<sup>3-9</sup>. IL-10 is a key anti-inflammatory cytokine produced by both macrophages and regulatory T cells in the gut. Its role in the regulation of the inflammatory responses to microbes and in the maintenance of the regulatory T cell activity is fundamental for sustaining intestinal homeostasis<sup>10,11</sup>. In this context, IL-10-deficient mice, which develop colitis sharing physiopathological features with Crohn's disease including transmural lesions, represent a valuable animal model of IBD<sup>12</sup>. It has been shown that, piroxicam-induced alteration of epithelium integrity results in bacterial translocation which leads, in the absence of IL-10, to an excessive inflammatory immune response. Colitis which develops in IL-10<sup>-/-</sup> mice following piroxicam treatment is similar to the colitis that occurs spontaneously in untreated IL-10<sup>-/-</sup> mice after 3 to 6 month of age<sup>13,14</sup>. Colitis in IL-10<sup>-/-</sup> mice is characterized by an aberrant response of Th1 and Th17 subsets of CD4<sup>+</sup> T lymphocytes but also innate immune cells and, particularly macrophages<sup>15</sup>. Indeed, the inability of macrophages to produce IL-10 upon bacterial infection exacerbates innate immune response leading to fatal colitis<sup>16</sup>.

A number of previous studies have reported that colitogenic Th1 and Th17 lymphocytes produce endogenous opioids which inhibit inflammation-induced abdominal pain<sup>17-20</sup>. However, the analgesic effects of T cell-derived opioids were evidenced in intestinal inflammatory models mimicking an excessive immune response in which innate and adaptive immune cells display unaltered functional properties<sup>18,20-22</sup>.

In this study, we investigated whether the opioid-mediated analgesic properties of CD4<sup>+</sup> T lymphocytes still operate in a context of immunoregulatory deficiency with innate immune hyper-responsiveness.

## **2 | MATERIALS AND METHODS**

### **2.1 | Animals and ethics statement**

Ten to twelve week old *IL-10* gene-deficient male ( $IL-10^{-/-}$ ) mice on C57BL/6 genetic background were bred and raised in the animal care facility at Toulouse (INSERM US 006 ANEXPLO/CREFRE, Toulouse, France). Colitis was accelerated by adding 150 mg/Kg piroxicam, a non-steroidal anti-inflammatory drug, into standard chow diet for 10 days (SAFE, Scientific Animal Food & Engineering, Augy, FRANCE). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the European Council and were approved by the Animal Care and Ethics Committee of US006/CREFE (CEEA-122; application number APAFIS #7762- CE2016112509278235V2).

### **2.2 | Macroscopic assessment of colonic damage**

Macroscopic colonic tissue damage was evaluated using a scale ranging from 0 to 11 as follows: erythema (absent (0), length of the area less than 1 cm (1), more than 1 cm (2)), edema (absent (0), mild (1), severe (2)), strictures (absent (0), one (1), two (2), more than two (3)), ulceration (absent (0), present (1)), mucus (present (0), absent (1)), and adhesion (absent (0), moderate (1), severe (2)). Bowel wall thickness was measured with an electronic calliper in the distal part of the colon, at 0.5 cm below the cecum.

### **2.3 | Histological assessment of colonic damage**

Colonic tissue specimens were excised 2 cm proximal to the anus and immediately transferred into 10 % formol to be further embedded in paraffin. Five-micrometer colonic sections were then stained with haematoxylin-eosin (H & E). Damage scoring was evaluated on a scale ranging from 0 to 12. Inflammatory cell infiltration, epithelial/mucosal alteration (including vasculitis, goblet cell depletion and crypt abscesses), mucosal architecture alteration (including ulceration and crypt loss) and submucosal edema were graded from 0 to 3 (absent, mild, moderate and severe).

### **2.4 | Immunofluorescent staining of lymphocytes in colon tissue biopsy**

Colonic sections (5 $\mu$ m) were saturated with PBS 1% BSA and then incubated with rabbit anti-CD3 (Clone SP7, Diagnostic BioSystems, Pleasanton, CA) monoclonal antibodies (mAb) for 1 hour at room temperature. After washing with PBS, bound antibodies were revealed with Alexa Fluor 555-labeled goat anti-rabbit IgG antibodies (Invitrogen). Slides were mounted and nuclei were stained with 4',6-Diamidino-2-Phenylindole (DAPI) fluorescent mounting medium (VECTASHIELD<sup>®</sup>, Vector laboratories Inc., Burlingame, CA). Fluorescence images were taken using confocal laser scanning microscope LEICA TCS SP8 (LEICA microsystems, Nanterre, France) with x 20 objective.

## **2.5 | Isolation of lamina propria mononuclear cells**

Intestine was longitudinally opened, cut into small pieces, washed and incubated twice with RPMI 5% FCS 5 mM EDTA at 37° C for 15 minutes. After washing, colonic tissues were digested with 0.02 % collagenase VIII (Sigma, St Louis, MO, USA) for 1 hour at 37°C. Supernatant was then passed through a 70 µm cell strainer and centrifuged. Mononuclear cells were then isolated upon Percoll gradient <sup>17</sup>.

## **2.6 | Isolation and activation of CD4<sup>+</sup> T lymphocytes**

CD4<sup>+</sup> T lymphocytes were isolated from both splenocytes and lymph node cells using cell negative isolation kit according to manufacturer's instructions (Invitrogen Dynal AS, Oslo, Norway). Twenty-four-well cell culture plates (Corning, Life Sciences, Amsterdam, Netherlands) coated with 2.5 µg mL<sup>-1</sup> of both anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) mAbs (BD Biosciences, San Jose, CA) were seeded with 5 × 10<sup>5</sup> purified naive CD4<sup>+</sup> T cells (more than 88 % pure) in RPMI-1640 medium (GIBCO Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (GIBCO Life Technologies), 1% non-essential amino-acids, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 IU mL<sup>-1</sup> penicillin, 100 µg mL<sup>-1</sup> streptomycin (GIBCO-BRL), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ) and 2 × 10<sup>-5</sup> M 2-β-Mercapto-ethanol. Proliferation of the cells was monitored using the CellTrace™ Violet Cell Proliferation Kit (Invitrogen, Carlsbad, CA).

## 2.7 | Cytofluorometric analysis

Mononuclear cells purified from *lamina propria* or CD4<sup>+</sup> T lymphocytes were first incubated with blocking buffer (PBS with 1% FCS, 3% normal mouse serum, 3% normal rat serum, 5 mM EDTA, 0.1% NaN<sub>3</sub>) containing 5 µg mL<sup>-1</sup> rat anti-CD16/CD32 mAb (mouse Fc block™, clone 2.4G2, BD Biosciences) for 15 min at room temperature. Cells were then incubated with Viability Dye eFluor™ 780 (e-bioscience). CD4<sup>+</sup> T lymphocytes were stained with both BV510-conjugated rat anti-mouse CD4 (clone RM4-5) and PE-Cy7-conjugated hamster anti-mouse TCR β chain (clone H57-597) mAbs and, to monitor activation status of the cells, PE-conjugated rat anti-mouse CD25 (clone PC61) together with FITC-conjugated Hamster anti-mouse CD69 (clone H1.2F3) mAbs. Macrophages were stained with APC-labelled rat anti-mouse F4/80 (clone BM8) mAb. All antibodies were 1:400 diluted in FACS buffer (PBS 1 % FCS, 5 mM EDTA, 0.1% NaN<sub>3</sub>) for 30 min on ice. To assess the content in Met-enkephalin-containing peptides in CD4<sup>+</sup> T lymphocytes and macrophages, cells were incubated with the anti-CD16/CD32 mAb prior adding both anti-CD4 and anti-F4/80 mAbs for 30 minutes at 4°C. After washing, cells were fixed, permeabilized and then stained with either rabbit anti-Met-enkephalin polyclonal IgG antibodies (Merck-Millipore-Chemicon International, Temecula, CA) or control rabbit non-immune serum IgG (Jackson Immunoresearch Lab, Baltimore, PA) as previously described<sup>23</sup>. Data were acquired for each individual sample on 100,000 cells by forward and side scatter intensity on a Fortessa (BD Biosciences) and further analyzed using the FlowJo software (Tree Star, Ashland, OR).

## 2.8 | Real-time quantitative PCR analysis

Colonic tissue samples were homogenized in 500  $\mu$ L TRIzol™ Reagent (Sigma). Total RNA was then isolated by using GenElute™ Mammalian total RNA miniprep Kit following the manufacturer's instructions (Sigma). RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using random hexamers for priming. Transcripts encoding hypoxanthine phosphoribosyl transferase (HPRT), proenkephalin (PENK), proopiomelanocortin (POMC), prodynorphin (PDYN), TNF $\alpha$ , IFN $\gamma$ , IL-1 $\beta$ , IL-17, IL-22 were quantified by real-time using the following forward and reverse primers: 5'-GTTCTTTGCTGACCTGCTGGAT-3' and 5'-CCCCGTTGACTGATCATTACAG-3' for HPRT, 5'-CGACATCAATTCCTGGCGT-3' and 5'-AGATCCTTGCAGGTCTCCCA-3' for PENK, 5'-TGGCCCTCCTGCTTCAGAC-3' and 5'-CAGCGAGAGGTCGAGTTTGC-3' for POMC, 5'-TGTGTGCAGTGAGGATTCAGG-3' and 5'-AGACCGTCAGGGTGAGAAAAGA-3' for PDYN<sup>24</sup>, 5'-CCACGCTCTTCTGTCTACTGAAC-3' and 5'-GGTCTGGGCCATAGAACTGATG-3' for TNF $\alpha$ , 5'-CAGCAACAGCAAGGCGAAA-3' and 5'-AGCTCATTGAATGCTTGGCG-3' for IFN $\gamma$ , 5'-ACCTTCCAGGATGAGGACATGAG-3' and 5'-CATCCCATGAGTCACAGAGGATG-3' for IL-1 $\beta$ , 5'-TCCAGAAGGCCCTCAGACTA-3' and 5'-CAGGATCTCTTGCTGGATG-3' for IL-17 and 5'-ACCGCTGATGTGACAGGAGC-3' and 5'-AGGTGGTGCCTTTCCTGACC-3' for IL-22. The target gene expression was normalized to the HPRT mRNA and quantified relative to a standard cDNA (calibrator sample) prepared either from mouse inflamed colon (for inflammatory cytokines) or mouse brain (for opioid precursors) using the  $2^{-\Delta\Delta C_T}$  method, where  $\Delta\Delta C_T = \Delta C_{T \text{ sample}} - \Delta C_{T \text{ calibrator}}$ <sup>25</sup>.

## 2.9 | Colorectal distension and electromyography recording

Three days before colorectal distension, 2 electrodes (Bioflex insulated wire AS631; Cooner Wire, Chatsworth, CA) were implanted into the abdominal external oblique musculature of mice previously anaesthetized with xylazine and ketamine. Electrodes were exteriorized at the back of the neck and protected by a plastic tube attached to the skin. When indicated, mice were intraperitoneally injected with 200  $\mu\text{L}$  of either PBS or naloxone methiodide (NLX-meth 10 mg  $\text{mL}^{-1}$ )<sup>18</sup>. Thirty minutes after injection, electrodes were connected to a Bio Amp (ADInstruments, Inc., Colorado Springs, CO), which was connected to an electromyogram acquisition system (PowerLab, ADInstruments). A 10.5 mm diameter balloon catheter (Fogarty catheter for arterial embolectomy, 4F; Edwards Lifesciences, Nijmegen, Netherlands) was gently inserted into the colon at 5 mm from the anus and progressively inflated in a stepwise of 15 mm Hg. Ten-second distensions were performed at pressures of 15, 30, 45 and 60 mm Hg with 5 minutes rest intervals. Electromyography activity of abdominal muscles was recorded and visceromotor responses were calculated using Chart 5 software (ADInstruments)<sup>26</sup>.

## 2.10 | Statistics

Data are expressed as means  $\pm$  SEM. Comparison between two groups of mice was performed using Mann-Whitney U test. The statistical analysis of the loss body weight induced by piroxicam treatment was performed using Wilcoxon matched pairs test. Comparison of visceral sensitivity in response to increasing distension pressures between two groups of animals were estimated with two-way repeated measurements analysis of variance (ANOVA). Due to the skewed distribution of the response; non-parametric tests were applied

on longitudinal data using a ranked-based approach on factorial experiments. The abdominal responses over the different pressures were explained in the statistical model by: treatment groups, an interaction term between treatment groups and distension pressures as fixed effects and a random effect to take into account the repeated measures of distension pressure on mice. Post hoc analysis using a Mann-Whitney-Wilcoxon test was performed at each distension pressure.  $P < 0.05$  was considered as significant <sup>21</sup>.

## 3 | RESULTS

### 3.1 | Piroxicam treatment accelerates development of colitis in IL-10<sup>-/-</sup> mice

Addition of piroxicam in standard chow diet for 10 days triggered development of colitis in IL-10<sup>-/-</sup> mice as shown by significant body weight loss (Figure 1A) and severe colonic injuries. Both macroscopic and microscopic colonic lesions in IL-10<sup>-/-</sup> mice treated with piroxicam were more severe than those in untreated IL-10<sup>-/-</sup> mice (Figure 1B-E and Figure 3D). Development of colitis in piroxicam treated-IL10<sup>-/-</sup> mice was associated respectively with a 5-, 18-, 32- and 37-fold increase in TNF $\alpha$ , IL-17, IL-1 $\beta$ , IFN $\gamma$  and IL-22 mRNA expression levels (P < 0.001). By contrast, the expression level of proenkephalin (PENK) mRNA was significantly reduced (Figure 2). mRNA encoding for the dynorphin and  $\beta$ -endorphin precursors, respectively prodynorphin and proopioidmelanocortin were undetectable.

### 3.2 | CD4<sup>+</sup> T lymphocytes within *lamina propria* of IL-10<sup>-/-</sup> mice with colitis express endogenous opioids

*Lamina propria* mononuclear cells were isolated from the inflamed mucosa of piroxicam-treated IL10<sup>-/-</sup> mice developing colitis. The frequency of F4/80<sup>+</sup> macrophages in *lamina propria* of colitis IL10<sup>-/-</sup> mice was higher than that of CD4<sup>+</sup> T lymphocytes (Figure 3A and 3B). The average number of F4/80<sup>+</sup> macrophages was almost 3.5 fold higher than that of CD4<sup>+</sup> T lymphocytes (Figure 3A). Although, their number was heterogeneous between individuals, mucosal F4/80<sup>+</sup> macrophages were predominant relative to CD4<sup>+</sup> T lymphocytes

in almost 90% of the animals (Figure 3B) (ratio F4/80<sup>+</sup> macrophages versus CD4<sup>+</sup> T lymphocytes was  $3.6 \pm 0.7$  (average  $\pm$  SEM, n = 15 for each group of mice).

We then assessed whether CD4<sup>+</sup> T lymphocytes and/or macrophages in piroxicam-treated IL-10<sup>-/-</sup> mice were able to produce endogenous opioids. As depicted in Figure 3C, CD4<sup>+</sup> T lymphocytes isolated from *lamina propria* of IL10<sup>-/-</sup> mice treated with piroxicam, express enkephalin-containing peptides. By contrast, cytoplasmic accumulation of enkephalin-containing peptides was not found in mucosal F4/80<sup>+</sup> macrophages as assessed by cytofluorometry (Figure 3C). The transmural injuries, 10-fold more frequent in IL10<sup>-/-</sup> mice treated with piroxicam, displayed T lymphocytes in the muscularis (Figure 3 D and 3E).

### **3.3 | Colitis is not associated with intestinal hypersensitivity in IL-10<sup>-/-</sup> mice**

Since CD4<sup>+</sup> T lymphocytes in IL10<sup>-/-</sup> mice treated with piroxicam were able to produce endogenous opioids, we assessed their potency to induce analgesia. As shown in Figure 4A, the visceral sensitivity of piroxicam-treated IL10<sup>-/-</sup> mice which develop colitis was superimposable to that of untreated IL10<sup>-/-</sup> mice, as assessed by colorectal distension. The injection of naloxone-methiodide, an antagonist of the opioid receptors unable to cross the brain-blood barrier, in piroxicam-treated IL10<sup>-/-</sup> mice induced abdominal pain (Figure 4B). Given that naloxone-methiodide antagonizes only peripheral opioid receptors, the data indicate that the inhibition of abdominal pain is dependent on local release of opioids within the inflamed intestine. The induction of abdominal pain was not due to the inhibition of opioid-mediated anti-inflammatory effects, since, as already reported, the injection of naloxone-methiodide thirty minutes before visceral sensitivity assessment did not worsen inflammation-induced tissue damage (Figure 4C).

### **3.4 | The production of enkephalins by CD4<sup>+</sup> T lymphocytes is lower in IL-10<sup>-/-</sup> mice**

The ability of CD4<sup>+</sup> T lymphocytes to synthesize PENK mRNA in response to TCR triggering was compared between wild-type (IL-10<sup>+/+</sup>) and IL-10-deficient (IL-10<sup>-/-</sup>) mice. Purified naïve CD4<sup>+</sup> T lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies under non-polarizing conditions for 6 days *in vitro*. At the third day following the stimulation, all CD4<sup>+</sup> T lymphocytes were activated as shown by the up-regulation of CD25 and/or CD69 markers. CD4<sup>+</sup> T lymphocyte was distributed as non-cycling (M1), intermediately (M2) and highly proliferative (M3) T lymphocytes depending on Celltrace violet fluorescence intensity which gradually decreases as cells divide (Figure 5A-D). As shown in Figure 5 E, although the proliferative response of CD4<sup>+</sup> T lymphocytes originating from IL-10<sup>-/-</sup> mice was higher than that of CD4<sup>+</sup> T lymphocytes from wild-type mice, their ability to synthesize PENK mRNA was lower.

## 4. Discussion

Opioids peptides are constitutively produced by mucosal immune cells in steady-state conditions but their production is not sufficient to inhibit abdominal pain upon acute colitis<sup>18,21,27-30</sup>. By contrast, chronic colitis is associated with a significant reduction of abdominal pain which correlates with the migration of opioid-producing CD4<sup>+</sup> T lymphocytes within the inflamed colon<sup>17,18,21,31-33</sup>. As a matter of fact, in the model of colitis induced by passive transfer of naïve CD4<sup>+</sup>CD45RB<sup>high</sup> T lymphocytes into lymphodeficient mice, the expansion of Th1 and Th17 lymphocytes results in severe colonic tissue injuries, but paradoxically, the local release of their opioids content prevents abdominal pain<sup>17,18</sup>. The peripheral opioid-mediated analgesic activity of T lymphocytes is, however, not so efficient in all intestinal inflammatory models. Indeed, 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced colitis results in visceral hypersensitivity which persist more than 14 days in mice<sup>34</sup> and at least 28 days<sup>35</sup> in rats, a period of time at which it could be assumed that effector T lymphocytes (generated in response to hapten-modified antigens) accumulate within the inflamed mucosa. By contrast, the visceral hypersensitivity associated with the mild inflammation induced by instillation of 50 % ethanol (vehicle) reduces spontaneously on day 7 and is resolved in day 14<sup>35</sup>. The absence of T cell-mediated analgesia in TNBS-induced colitis may be related to central sensitization of spinal dorsal horn neurons and/or the significant loss of extrinsic afferent innervation in mucosal layer<sup>34,36,37</sup>.

Here, we examined the endogenous control of visceral hypersensitivity in a model of colitis in mice with impaired IL10-dependent immune regulation in which CD4<sup>+</sup> T lymphocytes produce less PENK mRNA upon activation (Figure 5). In this model where the number of macrophages is 3-4 times higher than that of CD4<sup>+</sup> T lymphocytes, we show that

the opioid-mediated analgesia, mostly due to CD4<sup>+</sup> T lymphocytes, still operates. However, the efficacy of the T cell-mediated analgesia in the IL10-deficient mouse colitis model is weaker than in the T cell-induced colitis model<sup>18</sup>. The visceral sensitivity is similar between IL10<sup>-/-</sup> mice with (piroxicam-treated) or without (untreated) colitis while it was significantly reduced in mice with T cell-induced colitis as compared to control mice without colitis (immunodeficient mice non-transferred or transferred with both naïve and regulatory T lymphocytes)<sup>18</sup>. In line with studies reporting that colitis in IL-10-deficient mice is mostly dependent on the inflammatory activity of macrophages, we found that the endogenous production of enkephalins is significantly decreased in inflamed colon<sup>13,38</sup>. Thus, although the intensity of abdominal pain may differ depending on genetic background of the mouse strains, we speculate that the reduced efficacy of the peripheral immune-mediated analgesia may be dependent on both the lower ability of lymphocytes to produce enkephalins and the ratio between opioid-producing CD4<sup>+</sup> T lymphocytes and pro-nociceptive F4/80<sup>+</sup> macrophages within inflamed mucosa.

A number of colon biopsies from IL-10<sup>-/-</sup> colitis mice exhibit transmural damage with an inflammation of muscularis. The muscle layer and mucosa both innervated by spinal afferents, that can be inhibited by opioids<sup>28,39-41</sup>, are infiltrated by lymphocytes. Based on previous studies reporting that T lymphocytes may specifically migrate towards afferent nerve endings innervating the inflammatory site including mucosa as well as muscularis, it could be speculated that T cell-mediated pain relief is still observed because of an optimal topographic distribution of opioid-producing CD4<sup>+</sup> T lymphocytes at the vicinity of sensory nerves<sup>42</sup>. The observation of an endogenous opioid-mediated analgesic effect in IL-10<sup>-/-</sup> colitis mice also suggests that, in contrast to the TNBS-induced colitis, most of the primary afferents remains still functioning.

Taken together our results show that the opioid-mediated analgesic activity of T lymphocytes remains operating in IL-10<sup>-/-</sup> mice with impaired immune-regulatory function. Thus, our study suggests that even in severe infantile inflammatory bowel diseases associated with homozygous “loss of function mutations” in interleukin-10, the endogenous opioid tone may modulate the intensity of the inflammation-induced abdominal pain through opioid receptors expressed on nociceptors innervating the gut <sup>40</sup>. However, this endogenous opioid-mediated immune control of abdominal pain may last for a limited period of time that still remains to define.

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**Competing Interests:** the authors have no competing interests.

## REFERENCES

1. Hilsden RJ, Meddings JB, Sutherland LR. Intestinal permeability changes in response to acetylsalicylic acid in relatives of patients with Crohn's disease. *Gastroenterology* 1996;110:1395-1403.
2. Zamora SA, Hilsden RJ, Meddings JB, Butzner JD, Scott RB, Sutherland LR. Intestinal permeability before and after ibuprofen in families of children with Crohn's disease. *Can J Gastroenterol* 1999;13:31-36.
3. Begue B, Verdier J, Rieux-Laucat F, Goulet O, Morali A, Canioni D, Hugot JP, Daussy C, Verkarre V, Pigneur B, Fischer A, Klein C, Cerf-Bensussan N, Ruemmele FM. Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease. *Am J Gastroenterol* 2011;106:1544-1555.
4. Franke A, Balschun T, Karlsen TH, Sventoraityte J, Nikolaus S, Mayr G, Domingues FS, Albrecht M, Nothnagel M, Ellinghaus D, Sina C, Onnie CM, Weersma RK, Stokkers PC, Wijmenga C, Gazouli M, Strachan D, McArdle WL, Vermeire S, Rutgeerts P, Rosenstiel P, Krawczak M, Vatn MH, Mathew CG, Schreiber S. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet* 2008;40:1319-1323.
5. Glocker EO, Frede N, Perro M, Sebire N, Elawad M, Shah N, Grimbacher B. Infant colitis--it's in the genes. *Lancet* 2010;376:1272.

6. Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schaffer AA, Noyan F, Perro M, Diestelhorst J, Allroth A, Murugan D, Hatscher N, Pfeifer D, Sykora KW, Sauer M, Kreipe H, Lacher M, Nustede R, Woellner C, Baumann U, Salzer U, Koletzko S, Shah N, Segal AW, Sauerbrey A, Buderus S, Snapper SB, Grimbacher B, Klein C. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 2009;361:2033-2045.
7. Kotlarz D, Beier R, Murugan D, Diestelhorst J, Jensen O, Boztug K, Pfeifer D, Kreipe H, Pfister ED, Baumann U, Puchalka J, Bohne J, Egritas O, Dalgic B, Kolho KL, Sauerbrey A, Buderus S, Gungor T, Enninger A, Koda YK, Guariso G, Weiss B, Corbacioglu S, Socha P, Uslu N, Metin A, Wahbeh GT, Husain K, Ramadan D, Al-Herz W, Grimbacher B, Sauer M, Sykora KW, Koletzko S, Klein C. Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy. *Gastroenterology* 2012;143:347-355.
8. Moran CJ, Walters TD, Guo CH, Kugathasan S, Klein C, Turner D, Wolters VM, Bandsma RH, Mouzaki M, Zachos M, Langer JC, Cutz E, Benseler SM, Roifman CM, Silverberg MS, Griffiths AM, Snapper SB, Muise AM. IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis* 2013;19:115-123.
9. Pigneur B, Escher J, Elawad M, Lima R, Buderus S, Kierkus J, Guariso G, Canioni D, Lambot K, Talbotec C, Shah N, Begue B, Rieux-Laucat F, Goulet O, Cerf-Bensussan N, Neven B, Ruemmele FM. Phenotypic characterization of very early-onset IBD due to mutations in the IL10, IL10 receptor alpha or beta gene: a survey of the Genius Working Group. *Inflamm Bowel Dis* 2013;19:2820-2828.

10. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999;190:995-1004.
11. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, Kronenberg M. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* 2009;10:1178-1184.
12. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;75:263-274.
13. Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, Lynch RG. Rapid development of colitis in NSAID-treated IL-10-deficient mice. *Gastroenterology* 2002;123:1527-1542.
14. Hale LP, Gottfried MR, Swidsinski A. Piroxicam treatment of IL-10-deficient mice enhances colonic epithelial apoptosis and mucosal exposure to intestinal bacteria. *Inflamm Bowel Dis* 2005;11:1060-1069.
15. Ueda Y, Kayama H, Jeon SG, Kusu T, Isaka Y, Rakugi H, Yamamoto M, Takeda K. Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. *Int Immunol* 2010;22:953-962.
16. Krause P, Morris V, Greenbaum JA, Park Y, Bjoerheden U, Mikulski Z, Muffley T, Shui JW, Kim G, Cheroutre H, Liu YC, Peters B, Kronenberg M, Murai M. IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. *Nat Commun* 2015;6:7055.
17. Basso L, Garnier L, Bessac A, Boue J, Blanpied C, Cenac N, Laffont S, Dietrich G. T-lymphocyte-derived enkephalins reduce Th1/Th17 colitis and associated pain in mice. *J Gastroenterol* 2018;53:215-226.

18. Boue J, Basso L, Cenac N, Blanpied C, Rolli-Derkinderen M, Neunlist M, Vergnolle N, Dietrich G. Endogenous regulation of visceral pain via production of opioids by colitogenic CD4(+) T cells in mice. *Gastroenterology* 2014;146:166-175.
19. Boue J, Blanpied C, Djata-Cabral M, Pelletier L, Vergnolle N, Dietrich G. Immune conditions associated with CD4+ T effector-induced opioid release and analgesia. *Pain* 2012;153:485-493.
20. Valdez-Morales E, Guerrero-Alba R, Ochoa-Cortes F, Benson J, Spreadbury I, Hurlbut D, Miranda-Morales M, Lomax AE, Vanner S. Release of endogenous opioids during a chronic IBD model suppresses the excitability of colonic DRG neurons. *Neurogastroenterol Motil* 2013;25:39-46 e34.
21. Basso L, Boue J, Auge C, Deraison C, Blanpied C, Cenac N, Lluel P, Vergnolle N, Dietrich G. Mobilization of CD4+ T lymphocytes in inflamed mucosa reduces pain in colitis mice: toward a vaccinal strategy to alleviate inflammatory visceral pain. *Pain* 2018.
22. Philippe D, Dubuquoy L, Groux H, Brun V, Chuoi-Mariot MT, Gaveriaux-Ruff C, Colombel JF, Kieffer BL, Desreumaux P. Anti-inflammatory properties of the mu opioid receptor support its use in the treatment of colon inflammation. *J Clin Invest* 2003;111:1329-1338.
23. Basso L, Boue J, Mahiddine K, Blanpied C, Robiou-du-Pont S, Vergnolle N, Deraison C, Dietrich G. Endogenous analgesia mediated by CD4(+) T lymphocytes is dependent on enkephalins in mice. *J Neuroinflammation* 2016;13:132.
24. Martin L, Auge C, Boue J, Buresi MC, Chapman K, Asfaha S, Andrade-Gordon P, Steinhoff M, Cenac N, Dietrich G, Vergnolle N. Thrombin receptor: An endogenous inhibitor of inflammatory pain, activating opioid pathways. *Pain* 2009;146:121-129.

25. Benard A, Cavailles P, Boue J, Chapey E, Bayry J, Blanpied C, Meyer N, Lamant L, Kaveri SV, Brousset P, Dietrich G. mu-Opioid receptor is induced by IL-13 within lymph nodes from patients with Sezary syndrome. *J Invest Dermatol* 2010;130:1337-1344.
26. Perez-Berezo T, Pujo J, Martin P, Le Faouder P, Galano JM, Guy A, Knauf C, Tabet JC, Tronnet S, Barreau F, Heuillet M, Dietrich G, Bertrand-Michel J, Durand T, Oswald E, Cenac N. Identification of an analgesic lipopeptide produced by the probiotic *Escherichia coli* strain Nissle 1917. *Nat Commun* 2017;8:1314.
27. Verma-Gandhu M, Bercik P, Motomura Y, Verdu EF, Khan WI, Blennerhassett PA, Wang L, El-Sharkawy RT, Collins SM. CD4<sup>+</sup> T-cell modulation of visceral nociception in mice. *Gastroenterology* 2006;130:1721-1728.
28. Hughes PA, Harrington AM, Castro J, Liebrechts T, Adam B, Grasby DJ, Isaacs NJ, Maldeniya L, Martin CM, Persson J, Andrews JM, Holtmann G, Blackshaw LA, Brierley SM. Sensory neuro-immune interactions differ between irritable bowel syndrome subtypes. *Gut* 2013;62:1456-1465.
29. Hughes PA, Moretta M, Lim A, Grasby DJ, Bird D, Brierley SM, Liebrechts T, Adam B, Blackshaw LA, Holtmann G, Bampton P, Hoffmann P, Andrews JM, Zola H, Krumbiegel D. Immune derived opioidergic inhibition of viscerosensory afferents is decreased in Irritable Bowel Syndrome patients. *Brain Behav Immun* 2014;42:191-203.
30. Reiss D, Ceredig RA, Secher T, Boue J, Barreau F, Dietrich G, Gaveriaux-Ruff C. Mu and delta opioid receptor knockout mice show increased colonic sensitivity. *Eur J Pain* 2017;21:623-634.

31. Guerrero-Alba R, Valdez-Morales EE, Jimenez-Vargas NN, Lopez-Lopez C, Jaramillo-Polanco J, Okamoto T, Nasser Y, Bunnett NW, Lomax AE, Vanner SJ. Stress activates pronociceptive endogenous opioid signalling in DRG neurons during chronic colitis. *Gut* 2016.
32. Valdez-Morales E, Guerrero-Alba R, Ochoa-Cortes F, Benson J, Spreadbury I, Hurlbut D, Miranda-Morales M, Lomax AE, Vanner S. Release of endogenous opioids during a chronic IBD model suppresses the excitability of colonic DRG neurons. *Neurogastroenterol Motil* 2013;25:39-46.
33. Verma-Gandhu M, Verdu EF, Bercik P, Blennerhassett PA, Al-Mutawaly N, Ghia JE, Collins SM. Visceral pain perception is determined by the duration of colitis and associated neuropeptide expression in the mouse. *Gut* 2007;56:358-364.
34. Feng B, La JH, Tanaka T, Schwartz ES, McMurray TP, Gebhart GF. Altered colorectal afferent function associated with TNBS-induced visceral hypersensitivity in mice. *Am J Physiol Gastrointest Liver Physiol* 2012;303: G817-824.
35. Zhou Q, Price DD, Caudle RM, Verne GN. Visceral and somatic hypersensitivity in TNBS-induced colitis in rats. *Dig Dis Sci* 2008;53: 429-435.
36. Miampamba M, Sharkey KA. Distribution of calcitonin gene-related peptide, somatostatin, substance P and vasoactive intestinal polypeptide in experimental colitis in rats. *Neurogastroenterol Motil* 1998;10: 315-329.
37. Basso L, Lapointe TK, Iftinca M, Marsters C, Hollenberg MD, Kurrasch DM, Altier C. Granulocyte-colony-stimulating factor (G-CSF) signaling in spinal microglia drives visceral sensitization following colitis. *Proc Natl Acad Sci U S A* 2017;114: 11235-11240.

38. Holgersen K, Kvist PH, Markholst H, Hansen AK, Holm TL. Characterisation of enterocolitis in the piroxicam-accelerated interleukin-10 knock out mouse--a model mimicking inflammatory bowel disease. *J Crohns Colitis* 2014;8: 147-160.
39. Grundy L, Erickson A, Brierley SM. Visceral Pain. *Annu Rev Physiol* 2019;81: 261-284.
40. Guerrero-Alba R, Valdez-Morales EE, Jimenez-Vargas NN, Bron R, Poole D, Reed D, Castro J, Campaniello M, Hughes PA, Brierley SM, Bunnett N, Lomax AE, Vanner S. Co-expression of mu and delta opioid receptors by mouse colonic nociceptors. *Br J Pharmacol* 2018;175: 2622-2634.
41. Hockley JRF, Taylor TS, Callejo G, Wilbrey AL, Gutteridge A, Bach K, Winchester WJ, Bulmer DC, McMurray G, Smith ESJ. Single-cell RNAseq reveals seven classes of colonic sensory neuron. *Gut* 2019;68 :633-644.
42. Hua S, Hermanussen S, Tang L, Monteith GR, Cabot PJ. The neural cell adhesion molecule antibody blocks cold water swim stress-induced analgesia and cell adhesion between lymphocytes and cultured dorsal root ganglion neurons. *Anesth Analg* 2006;103:1558-1564.

## FIGURE LEGENDS

**FIGURE 1** Piroxicam treatment induces (accelerates) colitis in IL-10<sup>-/-</sup> mice. IL-10<sup>-/-</sup> mice were fed with standard chow diet without (white symbols) or with piroxicam (black symbols) for 10 days (n = 26 for each group of mice). The severity of the disease was assessed by body weight loss (A, body weight before (white circle) and after piroxicam treatment (black circle), each symbol represent 1 mouse) and by comparing wall thickness (B), and both macroscopic (C) and histological (D) colonic tissue damage between age-matched mice fed or not with piroxicam. (E) Representative histopathological analysis performed on H&E-stained colon sections. IL-10<sup>-/-</sup> mice fed without piroxicam display a low cellular infiltration, a submucosal edemas and a muscle thickening (upper panel). IL-10<sup>-/-</sup> mice fed with piroxicam display a strong cellular infiltration, a muscle thickening, a massive epithelial disruption, and a loss of epithelial architecture (lower panel). Scale 50  $\mu$ m. In panel A, statistical analysis was performed using Wilcoxon matched pairs test. In panels B to D, data are expressed as means  $\pm$  SEM; statistical analyses were performed using Mann-Whitney U test. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**FIGURE 2** Piroxicam treatment increases inflammatory cytokine mRNA levels in colon of IL-10<sup>-/-</sup> mice. mRNA encoding for pro-inflammatory cytokines IL-1 $\beta$ , TNF $\alpha$ , IFN $\gamma$ , IL-17 and IL-22 as well as for endogenous opioid precursors were quantified by real-time PCR in colonic biopsies from IL-10<sup>-/-</sup> mice fed with standard chow diet containing (black histogram) or not (white histogram) piroxicam (n = 15 for each group of mice). mRNA content was normalized to the HPRT mRNA and quantified relative to standard cDNA prepared from referential mouse inflamed colonic tissue for cytokines or from normal mouse brain for PENK

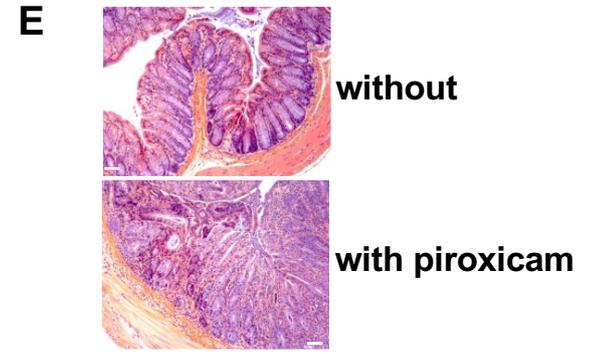
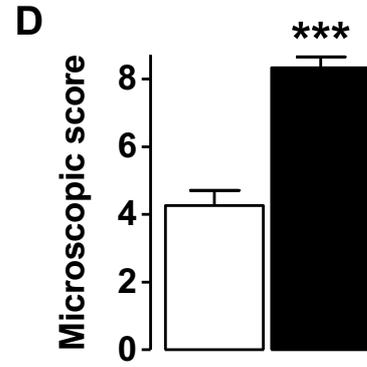
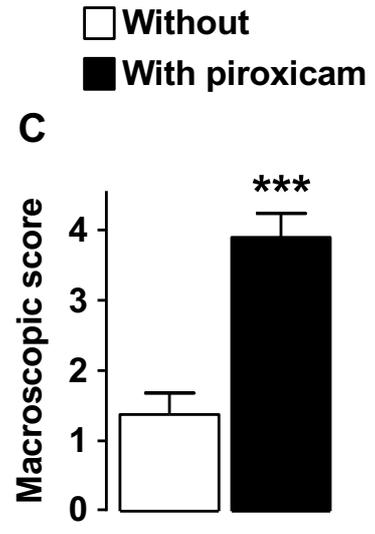
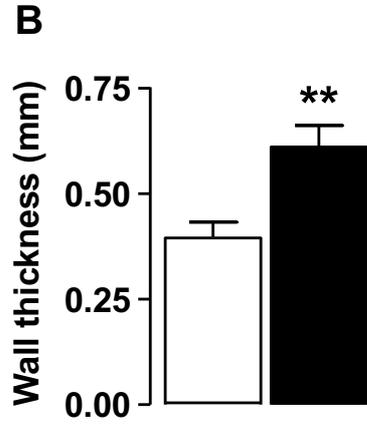
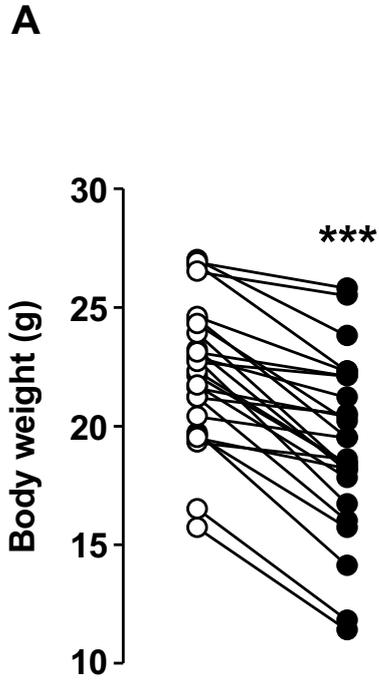
(calibrator samples). For each sample, mRNA level was expressed relative to the average of mRNA levels in untreated IL10<sup>-/-</sup> mice. Data are expressed as means ± SEM. Statistical analysis was performed using Mann-Whitney U test. \*\**p* < 0.01, \*\*\**p* < 0.001.

**FIGURE 3** Macrophages are more abundant than opioid-producing CD4<sup>+</sup> T lymphocytes within inflamed colonic mucosa of piroxicam-treated IL-10<sup>-/-</sup> mice. The relative frequency of CD4<sup>+</sup> T lymphocytes and F4/80<sup>+</sup> macrophages within the colonic *lamina propria* of piroxicam-treated IL-10<sup>-/-</sup> mice was determined by cytofluorometric analysis. A representative dot-plot of CD4<sup>+</sup> T lymphocytes and F4/80<sup>+</sup> macrophages is shown in panel A. Their respective frequency in *lamina propria* mononuclear cells expressed as means ± SEM (n = 15) is indicated on the picture. The frequency of F4/80<sup>+</sup> macrophages (white circle) and CD4<sup>+</sup> T lymphocytes (black circle) in *lamina propria*, expressed as percentage of *lamina propria* mononuclear cells (LPMC) isolated from inflamed mucosa of piroxicam-treated mice (each symbol represent 1 mouse), is depicted in B. Intra-cytoplasmic accumulation of Met-enkephalin-containing peptides in F4/80<sup>+</sup> macrophages (C, left panel) and CD4<sup>+</sup> T lymphocytes (C, right panel) was assessed by cytofluorometry. Cells were incubated with either control rabbit IgG (white histogram) or rabbit anti-Met-enkephalin IgG antibodies (grey histogram). The figure shows one representative experiment. The percentage of transmural bowel injuries in IL10<sup>-/-</sup> mice treated (black histogram) or not (white histogram) with piroxicam (n = 23 for each group of mice) is shown in (D). A representative H&E-stained colon section exhibiting longitudinal muscle thickening and massive inflammatory cells infiltration in the mucosal, submucosal and in the muscular layers (upper panel) and the corresponding anti-CD3 immunofluorescence staining of T lymphocytes within the muscular layer stained with DAPI (lower panel) is depicted in E. Scale 20 μm.

**FIGURE 4** Local production of endogenous opioids in inflamed intestine of piroxicam-treated IL10<sup>-/-</sup> mice inhibits abdominal pain. (A) Colonic sensitivity of IL-10<sup>-/-</sup> mice fed with standard chow diet containing (black circles) or not (white circles) piroxicam was measured by colorectal distension. Abdominal muscle contraction was recorded in response to distension pressure of 15, 30, 45 and 60 mmHg. (B) Piroxicam-treated IL10<sup>-/-</sup> mice were injected with PBS (white circles) or naloxone-methiodide (NLX-meth) (black circles) 30 min before pain assessment. Data are expressed as means ± SEM (n = 10-14 animals). Statistical analysis was performed using repeated-measures two-way ANOVA and subsequent post hoc tests as described in Materials and Methods. \*p < 0.05. (C) Colitis severity assessed by wall thickness (left panel) and macroscopic tissue damage (right panel) 30 min after intra-peritoneal administration of either PBS (white histogram) or NLX-meth (black histogram) (same groups of animals as above). Data are expressed as means ± SEM. Statistical analysis was performed using Mann-Whitney U test.

**FIGURE 5** CD4<sup>+</sup> T lymphocytes from IL-10<sup>-/-</sup> mice activated under non-polarizing conditions produce lesser amounts of PENK mRNA than those from wild-type. Naïve CD4<sup>+</sup> T lymphocytes isolated from either wild-type (IL-10<sup>+/+</sup>) or IL-10<sup>-/-</sup> mice were activated with a cocktail of anti-CD3 and anti-CD28 antibodies for 6 days. Proliferation was monitored by analyzing CellTrace Violet dispersion in live CD4<sup>+</sup> T cells before (A) and, on days 3 (B) and 6 (C) following activation (left panels). Cells were distributed as non-proliferative cells (M1), intermediately (M2) and highly proliferative (M3) cells. Percentage of cells in M1, M2 and M3 sections expressed as means ± SEM from four independent experiments is shown in panel D. The activation status was also estimated by the up-regulation of CD69 and CD25 on CD4-gated cells (right panels A, B, C). Left and right panels of A, B and C depict one representative experiment out of 4 performed. PENK mRNA expression in CD4<sup>+</sup> T

lymphocytes originating from wild-type (IL-10<sup>+/+</sup>, white histogram) or IL-10<sup>-/-</sup> (black histogram) mice (n = 4) was quantified by real-time PCR each day from day 0 to day 6 following stimulation. mRNA content was normalized to the HPRT mRNA and quantified relative to standard mouse brain cDNA. Data are expressed as means  $\pm$  SEM. Statistical analysis was performed using Mann-Whitney U test. \* $p < 0.05$ .



□ Without  
■ With piroxicam

