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Endogenous Regulation of Visceral Pain via Production of Opioids by Colitogenic CD4$^+$ T Cells in Mice

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BACKGROUND & AIMS: A dysregulated response of CD4$^+$ T cells against the microbiota contributes to the development of inflammatory bowel disease. Effector CD4$^+$ T cells, generated in response to microbe-derived antigens, can reduce somatic inflammatory pain through the local release of opioids. We investigated whether colitogenic CD4$^+$ T cells that accumulate in the inflamed colon also produce opioids and are able to counteract inflammation-induced visceral pain in mice. **METHODS:** Colitis was induced via transfer of naive CD4$^+$CD45RB^{high} T cells to immune-deficient mice or by administration of dextran sulfate sodium. Mice without colitis were used as controls. Samples of colon tissue were collected, and production of opioids by immune cells from inflamed intestine was assessed by quantitative polymerase chain reaction and cytofluorometry analyses. The role of intestinal opioid tone in inflammation-induced visceral hypersensitivity was assessed by colorectal distention. **RESULTS:** In mice with T cell– or dextran sulfate sodium–induced colitis, colitogenic CD4$^+$ T cells (T-helper 1 and Th17 cells) accumulated in the inflamed intestine and expressed a high level of endogenous opioids. In contrast, macrophages and epithelial cells did not express opioids; opioid synthesis in the myenteric plexus was not altered on induction of inflammation. In mice with colitis, the local release of opioids by colitogenic CD4$^+$ T cells led to significant reduction of inflammation-associated visceral hypersensitivity. **CONCLUSIONS:** In mice, colitogenic Th1 and Th17 cells promote intestinal inflammation and colonic tissue damage but have simultaneous opioid-mediated analgesic activity, thereby reducing abdominal pain.

Keywords: Mouse Model; UC; Visceral Sensitivity; Mucosal Immunity.

Microbe infection commonly results in the production of a number of inflammatory mediators that stimulate sensory neurons and elicit pain. This stimulation of primary afferent neurons is modulated by endogenous opioids locally released by immune cells. Both neutrophils and monocytes that enter the inflammatory site within the first hours after inflammation produce opioids to some extent. However, the spontaneous release of opioids by innate immune cells is not sufficient to relieve inflammatory pain.[1] The inflammatory response to microbes is also a pivotal step in the initiation of adaptive immunity. Inflammatory dendritic cells, mainly originating from the differentiation of monocytes emigrating from blood to inflamed tissue, ingest and process pathogen-derived antigens. Antigen-loaded dendritic cells migrate into draining lymph nodes and stimulate cognate naïve CD4$^+$ T lymphocytes. The antigen priming of CD4$^+$ T lymphocytes results in an up-regulation of opioid synthesis.[2] Then, antigen-experienced effector CD4$^+$ T lymphocytes move back to the inflammatory site; after a new stimulation by the antigen for which they are specific, they release opioids locally.[3] In contrast with innate immune cells, effector T cells that accumulate in the inflammatory site produce sufficient amounts of endogenous opioids to relieve pain.[2,3]

Inflammatory bowel diseases (IBDs) are characterized by an uncontrolled inflammatory response to colonic luminal content involving both innate and adaptive immunity. As a matter of fact, aberrant responses of CD4$^+$ T-helper (Th) 1 and CD4$^+$ Th17 lymphocytes against microbiota highly contribute to the development of IBD.[4] Given that the production of opioids is up-regulated on T-cell activation, we investigated whether colitogenic CD4$^+$ Th1 and Th17 lymphocytes, which accumulate in inflamed intestinal mucosa, also produce opioids and thereby potentially display analgesic properties.

In 2 models of colitis, a T lymphocyte–mediated model and a mucosal damage–based model, we showed that proinflammatory colitogenic T lymphocytes exhibit a pernicious opioid-mediated analgesic effect, rendering intestinal inflammation less painful.

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Abbreviations used in the paper: CBA, Cytometric Bead Array; DAPI, 4′,6-diamidino-2-phenylindole; DSS, dextran sulfate sodium; FCS, fetal calf serum; HPRT, hypoxanthine phosphoribosyltransferase; IBD, inflammatory bowel disease; IFN, interferon; Ig, immunoglobulin; IL, interleukin; mAb, monoclonal antibody; mRNA, messenger RNA; OVA, ovalbumin; PBS, phosphate-buffered saline; PDYN, prodynorphin; PENK, proenkephalin; POMC, proopiomelanocortin; Th, T-helper.

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Materials and Methods

Differentiation of Th1 and Th17 Subsets of CD4$^+$ T Lymphocytes In Vitro

CD4$^+$ T lymphocytes were isolated from splenocytes and lymph node cells originating from T-cell receptor–transgenic DO11.10 BALB/c mice in which more than 80% of CD4$^+$ T lymphocytes are specific for ovalbumin (OVA) peptide 323–339. Isolation of CD4$^+$ T cells was performed using cell negative isolation kits according to the manufacturer's instructions (Invitrogen Dynal AS, Oslo, Norway). Purified CD4$^+$ T cells (5×10^5 cells) were stimulated with irradiated BALB/c spleen cells in the presence of 0.3 μg/mL OVA 323–339 peptide for 2 weeks. Th1 skewing was fulfilled by adding to cell culture both 5 ng/mL interleukin (IL)-12 (PeproTech Inc, Rocky Hill, NJ) and 10 μg/mL anti–IL-4 monoclonal antibody (mAb) (clone 11B11; eBioscience, San Diego, CA).[5] Th17 skewing was fulfilled by culturing the cells in the presence of anti–IL-4 and anti–interferon (IFN)-γ mAbs (10 and 50 μg/mL, respectively) together with 20 ng/mL IL-6 and 3 ng/mL transforming growth factor β. On day 3 of Th17 differentiation, IL-23 (3 ng/mL) was added to the cell culture. On day 7, T cells were recovered and stimulated in the same conditions for one more week. At the end of the differentiation procedure, the cells were collected, washed, and incubated with anti-CD3 (clone 145-2C11; 2.5 μg/mL) and anti-CD28 (clone 37.51; 2.5 μg/mL) mAbs (BD Biosciences, San Jose, CA) for 24 hours.

Cytofluorometric Analysis

Cells were incubated with anti-CD16/CD32 (mouse Fc block, clone 2.4G2; BD Biosciences) before adding optimal concentrations of anti-CD4 (clone RM4-5) and anti-F4/80 (clone BM8) mAbs or goat anti–cytokeratin 18 (Santa Cruz Biotechnology, Inc, Dallas, TX) antibodies for 30 minutes at 4°C in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) and 2 mmol/L EDTA. After washing, cells were fixed and permeabilized with BD Cytofix/Cytoperm solution before staining with anti–IL-17A mAb (clone TC11-18H10), anti–IFN-γ mAb (clone XMG1.2), and rabbit anti–Met-enkephalin polyclonal immunoglobulin (Ig) G antibodies (Chemicon International, Temecula, CA).

Immunocytochemistry

Cells were centrifuged onto glass coverslips and fixed in 4% paraformaldehyde for 15 minutes at room temperature. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 and then incubated with PBS containing 5% FCS for 15 minutes at room temperature. Rat anti-CD3 (clone CD3-12; AbD Serotec, Raleigh, NC) mAb or rat anti-F4/80 (clone CI:A3-1) mAb was then added with rabbit anti–Met-enkephalin polyclonal IgG antibodies for 90 minutes at room temperature. After washing with PBS containing 5% FCS, cells were incubated with Alexa Fluor 555–labeled goat anti-rat IgG antibodies and Alexa Fluor 488–labeled donkey anti-rabbit Fcγ-specific antibodies for 30 minutes at room temperature. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images were taken using an upright laser scanner confocal Zeiss 710 microscope (Carl Zeiss MicroImaging GmbH, Germany) with 63× oil immersion objective.

Immunohistochemistry

Colonic tissue samples were snap frozen in OCT (Dako, Glostrup, Denmark). Five-micrometer tissue sections were incubated with anti-CD3, anti-F4/80, or anti–Ly-6B.2 mAbs for 1 hour at room temperature. After washing with PBS, cells were incubated with Alexa Fluor 555–labeled goat anti-rat IgG antibodies for 30 minutes at room temperature. Slides were mounted and nuclei were stained with DAPI fluorescent mounting medium.

Polymerase Chain Reaction Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen). RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase using random hexamers for priming. Transcripts encoding hypoxanthine phosphoribosyltransferase (HPRT), proenkephalin (PENK), proopiomelanocortin (POMC), and prodynorphin (PDYN) were quantified by real-time polymerase chain reaction using the following forward and reverse primers: 5′-GTTCTTTGCTGACCTGCTGGAT-3′ and 5′-CCCCGTTGACTGATCATTACAG-3′ for HPRT, 5′-CGACATCAATTTCCTGGCGT-3′ and 5′-AGATCCTTGCAGGT CTCCCA-3′ for PENK, 5′-TGGCCCTCCTGCTTCAGAC-3′ and 5′-CAGCGAGAGGTCGAGTTTGC-3′ for POMC, and 5′-TGTGTGCAGT GAGGATTCAGG-3′ and 5′-AGACCGTCAGGGTGAGAAAAGA-3′ for PDYN as previously described.[6] The target gene expression was normalized to the HPRT messenger RNA (mRNA) and quantified relative to a standard complementary DNA (calibrator sample) prepared from mouse brain using the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_{\mathrm{T}} = \Delta C_{\mathrm{T\ sample}} - \Delta C_{\mathrm{T\ calibrator}}$.[7]

Induction of T Cell–Dependent Colitis

Spleen cells isolated from BALB/c mice (Janvier, Le Genest Saint Isle, France) were stained with PercP-Cy5.5–conjugated anti-CD4, APC-conjugated anti-CD25 (clone PC61.5), and fluorescein isothiocyanate–conjugated anti-CD45RB (clone C363.16A) mAbs. CD4$^+$CD25$^-$CD45RB^{high} and CD4$^+$CD25$^+$CD45RB^{low} T lymphocytes were then separated by fluorescent cell sorting. Colitis was induced in 6-week-old SCID mice by intraperitoneal injection of 400,000 naive CD4$^+$CD25$^-$CD45RB^{high} T lymphocytes in 100 μL PBS. SCID mice injected with both CD4$^+$CD25$^-$CD45RB^{high} and CD4$^+$CD25$^+$CD45RB^{low} T lymphocytes were used as controls.

Dextran Sulfate Sodium–Induced Colitis

Colitis was induced by adding 3% (weight/volume) dextran sulfate sodium (DSS) to the drinking water for 5 days. Then, from day 5 to day 10, animals received only water.

Macroscopic Assessment of Inflammation-Associated Colon Damage

Macroscopic colonic tissue damage was evaluated using a scale ranging from 0 to 11 as follows: erythema (0, absent; 1, length of the area <1 cm; 2, length of the area >1 cm), edema (0, absent; 1, mild; 2, severe), strictures (0, absent; 1, one; 2, two; 3, more than two), ulceration (0, absent; 1, present), mucus (0, absent; 1, present), and adhesion (0, absent; 1, moderate; 2, severe). Colon wall thickness was measured with an electronic caliper.