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Substance P Regulates Th1-Type Colitis in IL-10 Knockout Mice

Joel V. Weinstock,* Arthur Blum,* Ahmed Metwali,* David Elliott, Nigel Bunnett, † and Razvan Arsenescu*

Substance P (SP) is a proinflammatory molecule that interacts with a neurokinin 1 receptor (NK-1R), which is on T cells and helps control IFN-γ production. IL-10−/− mice given a nonsteroidal anti-inflammatory drug (NSAID) develop Th1 colitis. We studied the importance of SP and NK-1R in this colitis model. LP T cells were isolated to study their NK-1R expression. LP T cells from IL-10−/− mice expressed NK-1R and produced IFN-γ only after NSAID treatment and induction of colitis. LP T cells from NSAID-treated wild-type controls or from age-matched untreated IL-10−/− animals did not express NK-1R or produce IFN-γ. Experiments showed that IL-12 induced NK-1R transcription in CD4+ T cells cultured in vitro. However, T cells cultured with IL-12 and IL-10 did not express NK-1R. IL-10 also down-modulated ongoing NK-1R expression. Mice given NK-1R antagonist after NSAID induction of severe colitis showed nearly complete reversal of inflammation, and LP T cells ceased IFN-γ secretion. Thus, intestinal inflammation in IL-10−/− mice is associated with the appearance of NK-1R in mucosal T cells, and an interplay between IL-12 and IL-10 regulates T cell NK-1R transcription. NK-1R antagonist reverses ongoing intestinal inflammation attesting to the importance of SP and its receptor in mucosal inflammation. 


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3 Abbreviations used in this paper: SP, substance P; CD, Crohn’s disease; HPRT, hypoxanthine phosphoribosyltransferase; IBD, inflammatory bowel disease; LP, lamina propria; LPMC, lamina propria mononuclear cells; NEP, neutral endopeptidase; NK-1R, neurokinin 1 receptor; SM-SP, [Sar9,Met11]-substance P; WT, wild type; NSAID, nonsteroidal anti-inflammatory drug.

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Colitis induction and SR 140333 treatment

To induce colitis, mice 4–5 wk of age were given piroxicam (Sigma-Aldrich, St. Louis, MO) mixed into their feed (National Institute of Health-S31M) for 2 wk. They received 60 mg of piroxicam/250 g of food wk 1 and 80 mg piroxicam/250 g of food wk 2. Mice subsequently were placed on the normal rodent chow without piroxicam. The colitis was evaluated from 2–16 days after colitis induction.

In some experiments mice received the SP receptor antagonist SR 140333 (Tocris, Montpellier, France) at 1 mg/g/day for 2 wk starting 2 days after discontinuation of the piroxicam. The inhibitor was given by continuous SQ infusion using osmotic pumps (Alzet, Cupertino, CA). Control mice also had implantation of osmotic pumps releasing just control buffer.

Histologic analysis of colitis

Colons (from the ileocecal valve to the mid descending colon) were opened longitudinally and rolled up onto a glass rod. The tissue was fixed in 10% neutral buffered formalin, removed from the glass rods without unrolling the tissue, and processed for sectioning. Tissue was sliced to obtain longitudinal sections of colon that were 6 μm thick and then stained with H&E for light microscopic examination. The inflammation was scored from 0–4 using the following criteria: grade 0, no change from normal tissue; grade 1, patchy mononuclear cell infiltrates in the LP; grade 2, more uniform mononuclear cell inflammation involving both the epithelium and LP; this was accompanied by minimal epithelial hyperplasia and slight to no depletion of mucus from goblet cells; grade 3, some epithelial and muscle hyperplasia with patchy lymphocytic infiltrates extending into the muscle layers; there were mucus depletion and occasional crypt abscesses and epithelial erosions; and grade 4, lesions involved most of the intestinal layers; there were mucus depletion of both the epithelial and muscle layers. There was mucus depletion and more frequent crypt abscesses. Ulcerations were frequent.

Cell isolation and T cell enrichment

Single-cell suspensions of splenocytes were prepared from individual mouse spleens by gentle teasing in RPMI. The cells were resuspended briefly in distilled water to lyse RBC. The splenocytes then were washed three times in a large volume of RPMI.

Gut LPMC were isolated as described below. Intestinal tissue was washed vigorously with RPMI, and all visible Peyer’s patches were removed with a scissors. The intestine was opened longitudinally, cut into 5-mm pieces, and then incubated in 0.5 mM EDTA in calcium- and magnesium-free Hanks’ buffer for 20 min at 37°C with shaking to release intraepithelial lymphocytes and epithelial cells. This was repeated after thorough washing of the tissue. The tissue was incubated for 20 min at 37°C in 30 ml of RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM l-glutamine, 5 × 10^{-8} M 2-ME, 1 mM sodium pyruvate, 100 μM penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all from Life Technologies, CA). Control mice also had implantation of osmotic pumps releasing just control buffer.

Flow cytometric analysis

Spleen or LPMC were washed twice and adjusted to 10^7 cells/ml in FACS buffer (HBSS containing 20 mM HEPES, 10% FCS, and 0.02% sodium azide). The cell suspensions were then dispersed into microcentrifuge tubes, each containing 10^6 cells in 100 μl of FACS buffer and stained with saturating amounts of conjugated Abs for 30 min at 4°C. Following staining, cells were washed twice and resuspended for analysis on a FACS 440 flow cytometer (BD Biosciences, Mountain View, CA).

Before adding labeled Ab, each tube received 1 μg of 2-4G2 Ab (anti-Fcγ-RI) (American Type Culture Collection) to block nonspecific binding of conjugated Abs to FcRs. The other mAbs used for staining were anti-CD4-Cy5 (RM251; CalTag, Burlingame, CA), anti-CD8a-PE (53-6.7; Sigma-Aldrich), anti-Thy1.2-FITC (Sigma-Aldrich), and anti-CD19-FITC (D5; BD Pharmingen, San Diego, CA). Also used was [Sar9,Met11]SP (SM-SP) labeled with Alexa Fluor 594 (Molecular Probes, Eugene, OR) in the presence or the absence of NK-1R inhibitor SR 140333 to detect specific binding to NK-1R. Both were used at 10^-8 M. SM-SP is an analog of SP that only binds the NK-1R receptor. It will not bind with NK-2R or NK-3R. SR 140333 is an extremely selective NK-1R antagonist.

RNA extraction and PCR assay for NK-1R mRNA

For RNA analysis, total cellular RNA was extracted from freshy isolated splenocyte or LP T cell suspensions by homogenization in guanidine/acid-phenol. In some experiments isolated splenocytes (4 × 10^7/flash (T25 flask; Corning, Cambridge, MA) were cultured at 37°C in 6 ml of RPMI medium for 4 h with or without rIL-12 (1 ng/ml) and/or rIL-10 (1 ng/ml) before RNA extraction. Mucosal T cells (~10^7/well, 96-well microtiter plates; Corning) were cultured similarly, but for only 40 min. In other experiments splenocytes were incubated with rIL-12 for 8 h. However, rIL-10 was added to some of the cultures 4 h before harvest.

Cellular RNA (5 μg) was reverse transcribed with Moloney monkey leukemia virus (400 U) using an 18-mer of oligo(dT) (0.5 μg) as primer. The first-strand cDNA was diluted to 250 μl, and 15 μl (3 μg) RNA was added to PCR buffer containing 2 U Taq DNA polymerase, 1.4 μM MgCl_2, 50 mM KCl, and 100 mM Tris (pH 8.3) in a total volume of 50 μl. The sense primer to amplify NK-1R was 5’-CCA ACA CCT CCA AGA CTT CTG-3’, and the antisense primer was 5’-GCC ACA GCT GTC ATG GAG TAG AT-3’. The PCR consisted of 40 cycles at 93°C for 1 min, 63°C for 1.36 min, and 72°C for 1.14 min. Products of RT-PCR amplification were analyzed by agarose gel electrophoresis using 1.7% agarose GTG agarose (FMC Bioproducts, Rockland, ME) in 0.5 × TBE buffer. The authenticity of the 338-bp fragment was confirmed by sequencing.

Total RNA preparations contained equivalent 18- and 28S RNA bands. RNA extracts were quantified spectrophotometrically. Samples were compared for number of hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene transcripts to further confirm equivalent mRNA content and reverse transcription.

NK-1R competitive PCR assay

A plasmid containing an elongated mimic NK-1R sequence of 606 bp was cloned and quantified as previously described (12). Various quantities of mimic plasmid DNA containing double-stranded elongated NK1 cDNA were added to a series of PCR reactions containing sample cDNA. The concentration of the unknown mRNA was determined through competition with known concentrations of this engineered plasmid by localization of bands of equivalence. The sensitivity of this assay is <100 NK1 transcript/μg total RNA.

In some experiments a mimic plasmid was used to quantify the HPRT housekeeping mRNA (13). This was to assure that reactions containing no detectable NK-1R mRNA transcripts had appropriate mRNA content.

ELISAs

ELISA was used to measure various cytokines in the culture supernatants. To measure IFN-γ, plates were coated with an mAb to IFN-γ (HB170; American Type Culture Collection) and incubated with supernatant. IFN-γ was detected by polyclonal rabbit anti-IFN-γ (gift from Dr. M. Wilson, University of Iowa, Ames, IA), followed by biotinylated goat anti-rabbit IgG (Accurate Chemical Co., Westbury, NY), streptavidin-HRP, and ABTS substrate (Zymed, San Francisco, CA). IL-4 was captured with 1B11 (HB191; DNAX Research Institute, Palo Alto, CA) and detected with biotinylated BV6 (provided by K. Moore and J. Abrams, DNAX). IL-5 was captured with TRFK5 and detected with biotinylated TRFK4.
NK-1R is an inducible TCR. SP is a Th1-type cytokine that can regulate T cell IFN-γ production through engagement of this receptor. It first was determined whether T cells in the LP of IL-10−/− or WT mice without colitis expressed NK-1R mRNA. T cell RNA from dispersed LPMC isolated from the terminal ileum of 7-to 8-wk-old IL-10−/− or WT mice raised in a specific pathogen-free facility expressed no NK-1R transcripts. NK-1R transcripts were measured using an NK-1R quantitative PCR assay sensitive down to 100 transcripts/µg RNA (Fig. 1).

Four- to 5-wk-old IL-10−/− mice then were given piroxicam for 2 wk to induce colitis. After induction, LP T cells were isolated from the mucosa. These cells expressed NK-1R mRNA strongly (Fig. 2). The level of expression was 45,036 ± 1,158 transcripts/µg RNA (±SE; n = 4). LP T cells from age-matched IL-10−/− controls that received no piroxicam had little or no NK-1R transcripts. Piroxicam-treated, WT animals had no colitis, and their LP T cells did not express NK-1R mRNA (data not shown).

In the IL-10−/− mice with colitis, flow analysis using AlexaSM-SP showed that ~17% of LP CD4+ T cells bound SP. AlexaSM-SP was displaced by the NK-1R inhibitor SR 140333 showing that the binding was specific (Fig. 3). Mucosal NK-1R+ CD4+ T cells were CD25+, and ~90% of these cells were CD45RBlow. A small subset of CD8+ cells was positive also (data not shown). No specific binding was seen on T cells from IL-10−/− mice without colitis.

IL-10 inhibits NK-1R expression

IL-10 is an important regulator of T cell function. Splenic T cells from young and healthy wild-type mice maintained specific pathogen-free did not respond to IL-12 with NK-1R induction. However, IL-12 readily induces NK-1R expression on splenic CD4+ T cells in murine schistosomiasis. Thus, experiments determined whether IL-10 regulates IL-12-dependent, NK-1R mRNA induction using splenocytes from schistosome-infected animals. Splenic CD4+ T cells were exposed to rIL-12 in the presence or the absence of rIL-10. Fig. 4A shows that IL-12 induced nearly 50 × 103 NK-1R transcripts/µg splenic T cell RNA after just a 4-h exposure. IL-10 totally prevented this expression (<100 transcripts/µg of total RNA). IL-12-induced, T cell NK-1R expression persisted for at least 48 h in vitro. In other experiments cells were exposed to IL-10 only after IL-12 induced NK-1R transcripts. IL-10 reversed NK-1R mRNA expression by ~80% after just a 4-h exposure (Fig. 4B). IL-10 suppressed NK-1R mRNA expression with comparable efficiency even after T cells were maintained in culture for 24 h (data not shown).

IL-12 induces and IL-10 regulates NK-1R expression in mucosal T cells

LP T cells were isolated from 4- to 5-wk-old, colitis-free, IL-10−/− mice. These cells were cultured for 40 min in the presence or the absence of IL-12. Some cultures contained IL-10 also. Fig. 5 shows that IL-12 induces NK-1R transcripts in resting mucosal T cells, and that IL-10 prevents this induction. Similar experiments using LP T cells from 4- to 5-wk-old WT control animals yielded similar results (data not shown).

NK-1R blockade reverses mucosal inflammation

Mice received piroxicam to induce colitis. Previous studies showed that this induces severe colitis that persists at least 4 wk after stopping piroxicam. After induction of the inflammation, the

FIGURE 1. LP T cells from WT or IL-10−/− mice, 7–8 wk of age, without colitis contained no NK-1R mRNA, as determined by quantitative PCR. Shown are the results from two separate experiments. The positive band is HPRT, which is the housekeeping gene.

FIGURE 2. LP T cells from IL-10−/− mice with colitis express NK-1R transcripts strongly. Animals received piroxicam for 2 wk as described in Materials and Methods. The LP T cells were isolated 2 wk after discontinuing piroxicam. Data are from four independent experiments.

FIGURE 3. Detection of NK-1R on mucosal CD4+ T cells from IL-10−/− mice with colitis using Alexa-labeled SM-SP. The upper right panel in the top figure shows SM-SP binding. The lower figure shows that AlexaSM-SP does not bind these T cells in the presence of the SP inhibitor SR 140333 (10−7 M). Thus, the binding is specific.
animals were treated with the NK-1R antagonist SR140333 for 2 wk or an appropriate control. The colons then were examined for colitis. Control mice had severe colitis with ulcerations, muscle thickening, and epithelial hypertrophy. Mice treated with NK-1R antagonist had only mild inflammation, limited to the LP. There was no muscle or epithelial hypertrophy (Fig. 6). However, T cells from the LP of these treated mice continued to express NK-1R transcripts (data not shown).

Also examined was LPMC cytokine secretion from the SR 140333-treated and control colitic mice. Isolated LPMC from either the control or NK-1R antagonist treatment groups were composed of 40% T cells, 40% B cells, and 20% macrophage as determined by flow analysis. Isolated LPMC from control mice cultured in vitro released IFN-γ (Fig. 7). The LP T cells isolated using paramagnetic beads produced IFN-γ, whereas LPMC devoid of T cells made none. LPMC isolated from mice receiving SR 140333 made no IFN-γ even after anti-CD3 and anti-CD28 stimulation. There was no IL-4 or IL-5 detected in the culture supernatants from either group.

**FIGURE 5.** IL-12 can induce NK-1R mRNA in mucosal T cells, and IL-10 can inhibit this induction. LP T cells from ~5-wk-old IL-10−/− mice without colitis were cultured 40 min with or without rIL-12 (1 ng/ml). Some of these cultures also contained rIL-10 (1 ng/ml). Data are the means of four separate determinations ± SE.

**FIGURE 4.** IL-10 inhibits NK-1R mRNA expression in T cells. A, In some experiments splenic T cells from schistosome-infected mice were cultured 4 h with or without rIL-12 (1 ng/ml). Some of these cultures also received rIL-10 (1 ng/ml). B, In other experiments splenocytes were cultured for 8 h with or without rIL-12. IL-10 was added to the cultures 4 h into the IL-12 exposure. A, IL-10 can block IL-12-induction of NK-1R mRNA in splenic T cells. B, IL-10 can inhibit ongoing NK-1R mRNA expression. Data are the means of four separate experiments ± SE.

**FIGURE 6.** The NK1 receptor antagonist SR 140333 inhibits colitis. Animals received either the inhibitor by continuous sc infusion at 1 mg/kg/day or a buffer control using osmotic pumps. The infusion began 2 days after stopping piroxicam, and colons were removed for histological examination 2 wk later. The inflammation was scored on a 0–4 point scale. Data are the mean ± SE from 10 or 11 animals studied in two separate experiments. A and B, Typical inflammation induced by piroxicam. There was deep lymphocytic infiltration of the colonic wall with ulceration, muscle thickening, and epithelial hypertrophy. C and D, Improvement in colitis after receiving SR 140333. Magnification, ×4; H&E staining.

**FIGURE 7.** LPMC make IFN-γ in piroxicam-induced colitis, but not after NK-1R antagonist treatment (SR 140333). As described in Fig. 5, animals with piroxicam-induced colitis received SR 140333 or control buffer for 2 wk. After the treatment, LPMC, LP T cells (T cells), or LPMC depleted of T cells (non-T cells; ~5 × 10^5 cells/well) were cultured 48 h with adherent anti-CD3 and anti-CD28 mAbs. Culture supernatants were assayed for IFN-γ after the incubation. Data are the mean ± SE of six separate determinations from three independent experiments.
Discussion

This study examined the expression and regulation of T cell NK-1R in the intestinal mucosa. Also explored was the role of this receptor and, by inference, SP in the regulation of a Th1-type chronic colitis.

We addressed these questions using an animal model of IBD that is most suggestive of human CD. The model chosen was piroxicam-induced, IL-10−−/− colitis (14). IL-10−−/− mice develop chronic colitis that gradually worsens as the animals age. However, young animals rapidly develop severe inflammation of the colon and terminal ileum when they are given a nonsteroidal anti-inflammatory medication (piroxicam). The colitis persists following withdrawal of the medication. Arachidonic acid blockade by piroxicam triggers a lymphocyte-rich, intestinal Th1-type inflammation that generates IFN-γ and IL-12. There is little or no IL-4 or IL-5 produced. As postulated for human IBD, IL-10−−/− colitis results from a dysregulated immune response to constituents of normal intestinal flora (15).

This study showed that mucosal LP T cells from young IL-10−−/− mice raised in an exceedingly clean, specific pathogen-free facility express NK-1R transcripts. However, mucosal T cells strongly express NK-1R mRNA and NK-1R protein after induction of colitis. WT, age-matched, specific pathogen-free mice do not develop colonic inflammation following identical piroxicam administration, and their mucosal T cells do not express NK-1R transcripts.

We used Alexa-labeled SM-SP at 10−8 M and flow analysis to estimate the number of T cells expressing NK-1R protein. This was an extremely sensitive and highly specific method for demonstrating NK-1R on mucosal T cells. SM-SP is a highly selective ligand of NK-1R. It does not bind the other tachykinin receptors, NK-2 or NK-3. Also, the NK-1R-selective inhibitor SR 140333 displaced the binding, which further suggests that this assay was NK-1 receptor specific. Approximately 17% of the CD4+ T cells displayed NK-1R after the onset of colitis. Mucosal NK-1R− CD4+ T cells were CD25−, and 90% of these cells were CD45RBlow. Thus, they expressed the activation/memory phenotype. Our analysis could have underestimated NK-1R expression, since the gut is an environment rich in SP, and surface NK-1R internalizes once engaged with its ligand (16, 17).

NK-1R is an inducible receptor on CD4+ splenic and liver granuloma T cells in murine schistosomiasis (12). TCR activation by Ag or IL-12 stimulates its expression. The experiments presented here revealed that IL-12 can induce NK-1R expression in resident LP T cells isolated from the mucosa of young IL-10−−/− mice before the onset of their colitis.

IL-12 has an important physiologic role in IL-10−−/− colitis. The intestinal inflammation generates IL-12. Also, blocking IL-12 can delay the onset of disease and even partially reverse the ongoing inflammation (18). Thus, it is reasonable to conclude that NK-1R on LP T cells is inducible through the action of endogenously produced IL-12.

IL-10 is critically important for limiting immunoreactivity in the intestines. This was shown using both the IL-10−−/− mouse that develops spontaneous colitis (19) and the SCID T cell transfer model of IBD (20). IL-10 from T cells is particularly important. IL-10 inhibits both macrophage and dendritic cell function. It also promotes T cell tolerance by blocking the CD28 costimulatory pathway. Regulatory T cells may function partly through the production of IL-10 (21, 22). However, it remains uncertain how it controls intestinal inflammation.

A major new observation of this investigation was that IL-10 can prevent IL-12-mediated, NK-1R induction in splenic and LP T cells. Also, it can suppress ongoing NK-1R expression. Several animal models support a physiological role for SP and its receptor in liver and intestinal inflammation (1–6). In many cases NK-1R functions as an enhancer of Th1 pathways. It can stimulate IL-12 (23) and IFN-γ production (24) and inhibit TGF-β synthesis (25). It is tempting to speculate that IL-10 helps limit Th1 reactivity in the intestine through the control of mucosal T cell NK-1R expression.

The capacity of SP to function as an inflammatory mediator is governed at several levels. As shown in this report, one point of control is through transcriptional regulation of the biosynthesis of its natural ligand NK-1R (12, 26). Also, engagement of SP with its receptor at the cell surface results in temporary receptor desensitization and loss of bioavailability. This results from transient disruption in receptor signal transduction, and internalization of the receptor (16, 17).

Neutral endopeptidase (NEP) is an enzyme expressed on the surface of many cells that degrades SP. The bioactivity of SP in extracellular fluid is regulated partly through the local expression of this enzyme. Inhibition or loss of NEP makes mice more susceptible to intestinal inflammation (27, 28). NEP−−/− mice are more sensitive to dinitrobenzene sulfonic acid-induced colitis. Pharmacological blockade of NK-1R prevents this heightened inflammation (27). These models suggest that NEP, perhaps through the failure to degrade SP, also helps govern mucosal immune responses.

Our experiments using IL-10−−/− mice showed that a NK-1R antagonist used in vivo can suppress ongoing colitis and intestinal IFN-γ production. Di Sebastiano et al. (29) reported that the same NK-1R inhibitor helped prevent inflammation in rat trinitrobenzene-induced colitis. Thus, these models of intestinal inflammation collectively support the concept that SP, the major endogenous ligand for NK-1R, has an important role in intestinal inflammation.

The point of action of the inhibitor was not determined in this current investigation. NK-1R are on vascular endothelial cells (30), epithelial cells (31), smooth muscle cells, neurons, lymphocytes (32, 33), macrophages (23, 34), dendritic cells, and other cell types. The vascular NK-1R can promote egress of leukocytes from blood vessels. Macrophage NK-1R activation can stimulate IL-12 (23) and reduce TGF-β secretion (25). The T cell NK-1R enhances IFN-γ production (24). The colitis of IL-10−−/− mice is dependent on IL-12 and IFN-γ, since blockade of IL-12 and, to a lesser extent, IFN-γ reduces the intensity of the ongoing inflammation (18). It is most likely that the NK-1R antagonist affected the colitis through disruption of several of these important immunoregulatory pathways.

SP belongs to the tachykinin family of hormones. It derives from a precursor protein called preprotachykinin A, which is encoded by the tachykinin 1 gene. Preprotachykinin A also can be processed to produce neukolin A and two N-terminal elongated forms of neukolin A, called neuropeptide K and neuropeptide γ. Another tachykinin, neukolin B, comes from preprotachykinin B, which is encoded by a different gene (murine tachykinin 2 or human tachykinin 3). Yet another tachykinin, neukolin 4, produces preprotachykinin c and a novel tachykinin called hemokin (35).

Three distinct tachykinin receptors, designated NK-1, NK-2, and NK-3, mediate the biological function of tachykinins. SP and hemokin bind NK-1R with high affinity (36). However, hemokin may signal somewhat differently from SP and may have some separate functions (35). Most reports suggest that the other tachykinins bind either NK-2R or NK-3R with high affinity and are low affinity agonists of NK-1R.
The expression of NK-1R on intestinal leukocytes could imply an important role for SP in the regulation of mucosal inflammation. While SP is abundant in the intestines, there are somewhat conflicting data regarding the concentration of SP in the intestinal mucosa of patients with IBD (37–39). Some studies report high levels that correlate with the activity of the disease (38–40). Others also report high levels of SP in intestinal nerve fibers (41–43). It currently is unknown whether the other natural agonist of NK-1R, hemokin, is expressed in the gut.

In summary, these are the first data showing that NK-1R is important for the ongoing intestinal immune response in a spontaneous murine model of IBD. Also revealed was the importance of IL-12 and IL-10 for the control of mucosal T cell NK-1R expression. This may be physiologically important, since NK-1R is heavily expressed on mucosal T cells in human IBD (10). The piroxicam-IL-10 model of IBD will allow further exploration into the roles of tachykinins and their receptors in the control of intestinal inflammation.

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