INTRODUCTION

Toll-like receptors (TLRs) belong to a family of pattern-recognition receptors that detect conserved molecular products of microorganisms emerging as crucial elements in the activation of innate immunity as well as connectors between the innate and acquired immunity [1–4]. Although since their discovery, 11 mammalian TLRs have been identified in the bowel microenvironment, TLR2 and -4 represent the main sensors to recognize molecular products from gram-positive and gram-negative bacteria, respectively [5]. Under homeostatic conditions, the intestinal mucosal immune system protects the host against disease by neutralizing luminal infectious agents. In this sense, a novel function of TLRs as molecules involved in the homeostatic effector role of VIP involves a myriad of microcirculatory and physiological actions. A major challenge for the future is to understand the gut physiology and the interactions between VIP and TLRs in the context of inflammation.

Abstract: Toll-like receptor 2 (TLR2) and -4 mediate signals from a great variety of bacterial gut products, giving the host a panel of microbe-recognizing receptors. Under homeostatic conditions, TLRs act as protective receptors of the intestinal epithelium. When homeostasis is disrupted in diseases such as inflammatory bowel disease, TLR2 and -4 are deregulated. Our study demonstrates, by using a trinitrobenzene sulfonic acid-induced colitis model of Crohn’s disease, the constitutive expression and the up-regulation of TLR2 and -4 at messenger and protein levels in colon extracts, as well as in macrophages, dendritic cells, and lymphocytes from mesenteric lymphoid nodes. Vasactive intestinal peptide (VIP) treatment induced a decrease of TLR2 and -4 expressions approaching ethanol control levels. Our results suggest that VIP modulation of TLR2 and -4 could be explained by two possible mechanisms. The first one would be the secondary reduction of TLR2 and -4 caused by the VIP-mediated decrease of inflammatory mediators such as interleukin-1β and interferon-γ, which synergize with bacterial products, contributing to the amplification of TLR presence in the intestine. The other possible mechanism would involve a VIP-mediated decrease of nuclear factor-kB, which would cause a direct down-regulation of TLR expression. In summary, the resultant physiological effect is the decrease of TLR2 and -4 expressions to homeostatic levels. Our study describes for the first time the role of a peptide present in the gut microenvironment as an effective modulator of the initial steps of acute inflammation, acting at local and systemic levels and leading to the restoration of the homeostasis lost after an established inflammatory/autoimmune disease. J. Leukoc. Biol. 78: 491–502; 2005.

Key Words: immunomodulation · inflammation · homeostasis
could down-regulate, at least in part, the inflammatory response by modulating TLR expression. Thus, we chose an acute model of intestinal injury and inflammation based on the intrarectal administration of TNBS, which is known to induce an early production of inflammatory factors, followed by a Th1 response that resembles CD [15, 16]. The constitutive expression and the compartmentalization of TLR2 and TLR4 in the mouse gut as well as their up-regulation in dextran sulfate sodium (DSS)-induced colitis, as a model of human ulcerative colitis, have been reported recently [17]. However, there are no studies describing the time course of the expression of these receptors in the development of an experimental model of CD as well as the effect of a treatment with a therapeutic peptide on them.

Therefore, the aim of the present study is to know the possible variation of the expression of TLR2 and -4 receptors along the development of TNBS-induced colitis and to elucidate whether the beneficial effect of VIP involves the regulation of the expression of these receptors as important sensors of the gut homeostasis. Our results showed the constitutive expression and the up-regulation of TLR2 and -4 in a mice model of CD and the homeostatic effect of VIP approaching the expression of these receptors to control levels.

MATERIALS AND METHODS

Animals

Male BALB/c mice, obtained from Jackson Laboratory (Bar Harbor, ME), were kept in pathogen-free conditions. All mice used were between 6 and 5 weeks of age.

Induction of colitis and treatment protocol

Colitis was induced by rectal instillation of TNBS (Sigma Chemical Co., St. Louis, MO) as described previously [16]. Briefly, BALB/c mice were lightly anesthetized, and 120 μl of a mixture containing 3 mg TNBS dissolved in ethanol was administered through a catheter advanced 4 cm in the anus. The animals were then held by the tail during 30 s to ensure uniform contact with colonic mucosa. Following the same protocol, control animals were given 120 μl 50% ethanol. Each mouse received 1 nmol VIP (Neosystem, Strasbourg, France) intraperitoneally (i.p.) every other day after TNBS induction of colitis.

Preparation of colon membranes

Animals were then held by the tail during 30 s to ensure uniform contact with colonic mucosa. Following the same protocol, control animals were given 120 μl 50% ethanol. Each mouse received 1 nmol VIP (Neosystem, Strasbourg, France) intraperitoneally (i.p.) every other day after TNBS induction of colitis until the collection of samples.

Measurement of myeloperoxidase (MPO) activity in colon extracts

Total RNA (5 μg) was added to a RT mixture: 3 mM MgCl₂, 75 mM KCl, 50 mM Tris- HCl, pH 8.3, 10 mM dithiothreitol, 0.5 mM 2′-deoxy nucleoside-5′-triphosphates, 100 U Superscript® II H RNase H’ RT (Invitrogen, Carlsbad, CA), 25 pmol oligo d(T)₁₆, and 40 U RNase out (Invitrogen). The resulting mixture was incubated for 50 min at 42°C, and the sample was heated to 70°C for 15 min on a GeneAmp® PCR System 2700 (Applied Biosystems) and maintained at 4°C until PCR amplification. To control genomic DNA contamination, the reaction was performed regularly in the absence of RT. cDNA was then amplified using the specific primers for TLR2, TLR4, and actin as a control, the same used for real-time RT-PCR. RT-cDNA (2 μl) was amplified in PCR mixtures (total volume 25 μl) containing 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 U Taq DNA polymerase (Invitrogen), 1.5 mM MgCl₂, 0.2 mM deoxy- unspecified nucleoside 5′-triphosphates, and 1 μM primers. PCR conditions were: denaturation at 94°C for 3 min, followed by 25 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, then a final cycle for 10 min at 72°C. PCR products were analyzed in 2% agarose gels.

Preparation of colon membranes and Western blot

For Western blot of TLR2 and TLR4, colons were removed at the indicated times, washed with PBS, and cut in small pieces. Samples were homogenized in 0.1 M Tris-HCl, pH 7.4, 0.3 M NaCl, 0.25 M sucrose, 2 mM EDTA, and protease inhibitor cocktail tablets (Roche, Mannheim, Germany) using a Polytron (Kinematica) and then centrifuged at 1600 × g for 15 min at 4°C to remove debris. Supernatants were centrifuged at 30,000 × g for 30 min at 4°C to isolate the membrane fraction and then stored at −80°C until assay. Protein concentration was estimated by using the Bradford method (Bio-Rad, Hercules, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed loading 100 μg proteins per lane, followed by transfer to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked for 2 h in Tris-buffered saline/Tween 20 (TBST; 2 mM Tris-HCl, pH 7.6, 13.7 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and then incubated at 4°C overnight with goat anti-mouse TLR2 or TLR4 polyclonal antibodies (1:250; Santa Cruz Biotechnology, CA) in TBST containing 3% nonfat dry milk.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Quantitative RT-PCR analysis was performed using the SYBR® Green PCR Master Mix and RT-PCR kit (Applied Biosystems, Foster City, CA), as suggested by the manufacturer. Briefly, reactions were performed in 20 μl with 20 ng RNA, 10 μl 2× SYBR Green PCR Master Mix, 6.25 U MultiScribe RT, 10 μl RNAse inhibitor, and 0.1 μM primers. The sequences of primers used were: mouse β-actin sense 5’-AGAGGGAAATCGTGCGTGAC-3’, antisense 5’-TTTCTTTCAGGGACAGCCTGTT-3’; mouse TLR2 sense 5’-GGGACTTGTCATGATGCC-3’, antisense 5’-AAAGGCAGGGTGACTGTC-3’; mouse TLR4 sense 5’-GGTTGCAGAAAATGGAACGATG-3’, antisense 5’- CAGG GATTCGACGGTTCGTCGCT-3’; mouse interleukin (IL)-1β sense 5’-CTTCTCGCCAGGCGATAGTCT-3’, antisense 5’-TTTCTTTACGGGACGCTGT-3’; and mouse interferon-γ (IFN-γ) sense 5’-TGCTGATGGGAGGAGATGTCT-3’, antisense 5’-GAAAGGGGGAGAGGAGATGTCT-3’. The GenBank accession numbers and numbers for the 5′ and 3′ ends of the nucleotides for the PCR products are: β-actin, NM_001039, 604–519; TLR2, NM_021297, 4–104; IL-1β, NM_15131, 914–1014; and IFN-γ, K00083, 929–1029. Amplification conditions were 30 min at 48°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min.

For relative quantification, we used a method that compared the amount of target normalized to an endogenous reference. The formula used was 2^ΔΔCt, representing the n-fold differential expression of a specific gene in a treated sample compared with the control sample, where Ct is the mean of threshold cycle (cycle at which the amplification of the PCR product is initially detected). ΔCt is the difference in the Ct values for the target gene and the reference gene, β-actin (in each sample assayed), and ΔΔCt represents the difference between the Ct from the control and each datum. Before using this method, we performed a validation experiment comparing the standard curve of the target and the reference to demonstrate that efficiencies were approximately equal [19]. The correct size of the amplified products was checked by electrophoresis.
Horse-radish peroxidase-conjugated donkey anti-goat immunoglobulin G (IgG; 1:10,000, Santa Cruz Biotechnology) was used for detection. Proteins were visualized using an enhanced chemiluminescence Western blotting analysis system (Santa Cruz Biotechnology). Incubation with mouse anti-β-actin antibody (1:10,000, Oncogene, Cambridge, MA) was used as a loading control. Densitometry analysis of proteins bands from scanned-ray films were performed using Scion Image software (Scion Corp., Frederick, MD), and the values were normalized against the intensity of β-actin.

**Immunohistochemistry**

For immunohistochemistry studies, monoclonal antibodies (mAb) against TLR2 and TLR4 were used (rat anti-mouse TLR2 or TLR4 mAb, eBioscience, San Diego, CA). With this purpose, colons were frozen, and 7 μm-thick sections were obtained and fixed in cold acetone for 20 min. To avoid unspecific labeling, sections were incubated with DAKO® protein block serum-free (Dako Corp., Carpinteria, CA) at room temperature for 30 min, and an avidin/biotin-blocking kit (Vector Laboratories, Burlingame, CA) was used. Subsequently, sections were incubated at 4°C overnight with primary antibody at 1:100 dilution in PBS + 0.1% bovine serum albumin and then with 0.36% β-glucose, 0.01% glucose oxidase, and 0.013% sodium azide in PBS for 1 h at room temperature to block endogenous peroxidase activity. Biotinylated mouse anti-rat polyclonal antibody (eBioscience) was used as secondary antibody at 1:100 dilution for 1 h at room temperature, followed by an incubation with 0.03 mg/ml avidin-peroxidase labeled for 1 h at room temperature. A solution of 0.05% 3,3′-diaminobenzidine and 0.015% H2O2 was used to stain. Toluidine blue was used for counterstaining. As negative controls, sections were treated with rat serum instead of primary antibody.

**Fluorescein-activated cell sorter (FACS) analysis**

Mesenteric lymph node cell suspensions were prepared by mechanical dissociation using a stainless-steel screen (60 mesh, Sigma Chemical Co.). Cells (3×10⁶) were labeled with phycoerythrin (PE)-conjugated rat anti-mouse TLR4/MD2 (clone MTS510, eBioscience) or PE-conjugated rat anti-mouse TLR2 (clone 6C2, eBioscience). Double labeling was also performed by combining the TLR2 and TLR4 antibodies with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD11b (clone M1/70.15, Caltag Laboratories, S. San Francisco, CA) and FITC-conjugated rat anti-mouse CD11c (clone N418, Caltag Laboratories). Lymphocytes were distinguished by their different forward-scatter (FSC) versus side-scatter (SSC) profiles and were electronically gated and scored. Incubation with the antibodies was performed at 4°C for 30 min. Aliquots of lymph node cells were incubated with a single fluorochrome-conjugated antibody or isotype-matched control antibodies to compensate for fluorescence emission overlap and nonspecific fluorescence, respectively. Cells were fixed with 0.3% paraformaldehyde and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using an appropriate scatter gate to exclude dead cells, cellular debris, and residual erythrocytes.

**Statistical analysis**

All values are expressed as the mean ± SEM of data obtained from at least three mice. Comparison between groups was made using the Student’s t-test, Mann-Whitney W test, and ANOVA test, and P < 0.05 was the significance level.

**RESULTS**

**Time-course quantitative analysis of TLR2 and TLR4 mRNA expression in colon extracts in TNBS-induced colitis and their decrease after VIP treatment**

In the experimental model of CD, mice treated with TNBS in 50% ethanol, which haptenates autologous colonic proteins with trinitrophenol, developed severe bloody diarrhea and extensive wasting disease. VIP was administered i.p. 12 h after the induction of colitis, and this treatment was maintained on alternate days. As shown in **Figure 1A**, the macroscopic analysis of colons obtained 3 days after administration of TNBS showed necrosis, hyperemia, and inflammation compared with controls. Treatment with VIP prevented hyperemia and inflammation in the studied colons (Fig. 1A). This model of IBD induces, at histological level, infiltration of neutrophils and macrophages (MØ) into the colonic mucosa and submucosa associated with a thickening of the colon wall, loss of goblet cells, and finally, the disruption of the epithelial barrier [15]. Administration of VIP improved these signs, restoring the histological appearance of the mucosa and submucosa. As a quantitative measure of the development of the disease and the anti-inflammatory effect of VIP, we evaluated neutrophil infiltration by determining MPO activity in colon extracts. Our results showed that colon MPO activity in the TNBS animals was increased significantly compared with untreated and ethanol-treated control mice, whereas VIP treatment significantly inhibited this activity (Fig. 1B).

![Fig. 1](image-url) Development of TNBS-induced colitis and effect of VIP. Colitis was induced by rectal instillation of TNBS in 50% ethanol, and 1 nmol VIP was injected i.p. on alternate days. Controls received PBS or 50% ethanol. (A) Macroscopic analysis of colons obtained 3 days after administration of TNBS showed necrosis, hyperemia, and inflammation compared with control colons. Treatment with VIP prevented hyperemia and inflammation. (B) Colons were removed and homogenized on day 5 to obtain protein extracts, and MPO activity was determined as described in Materials and Methods. Error bars represent ± SEM of eight mice per group. **P < 0.01, versus TNBS-treated animals.
As it is known that TNBS-induced colitis evolves along the time, we chose several time-points for the next experiments, as representative of the early acute (1 and 3 days) and late inflammatory (5 and 7 days) phases of the disease. First, our results showed the constitutive expression of TLR2 and -4 mRNA in colon extracts from control mice (Fig. 2, A and B). VIP treatment in normal mice did not affect the constitutive expression of TLR2 and -4 (data not shown). After TNBS treatment, TLR2 and -4 mRNA expression was up-regulated in comparison with the ethanol control that showed a mild inflammation on days 5 and 7, probably as a result of the ethanol-mediated breakage of epithelial barrier (Fig. 2, A and B). The up-regulation was gradual and significantly higher in the TNBS group from days 1 to 5, and the highest levels were on day 5, decreasing more drastically for TLR4 on day 7 (Fig. 2, A and B). VIP treatment on alternate days decreased TNBS-induced TLR2 and -4 mRNA expressions significantly (Fig. 2, A and B). Thus, VIP reduced TLR2 mRNA expression on days 3, 5, and 7 after TNBS-induced colitis (Fig. 2A). The inhibition of TLR4 mRNA expression was constant and significant from days 1 to 5 and was more evident on day 5 (Fig. 2B), thus, at the early and late inflammatory phases of the disease.

VIP approaches TLR2 and TLR4 proteins to steady-state levels in TNBS-induced colitis

Next, we addressed the question of whether mRNA of TLRs studied was translated into protein. Western blot analysis of controls using specific antibodies against TLR2 and -4 showed reactive protein bands at the size of TLR2 (86 kDa; Fig. 3A) and TLR4 (94 kDa; Fig. 3B), confirming the constitutive expression of both receptors. In TNBS-induced colitis, protein levels of TLR2 were increased from days 1 to 5, and the strongest signal was on day 5, decreasing significantly at day 7 (Fig. 3A). TLR2 mRNA and protein levels were time-correlated. TLR4 protein expression in colon extracts also increased gradually until day 5, decreasing significantly on day 7 (Fig. 3B), in correlation with the transcripts. Thus, protein and mRNA of TLR2 and TLR4 increase gradually during TNBS-induced colitis and are maximal on day 5. TLR2 and -4 proteins are also down-regulated after VIP treatment during TNBS-induced colitis development, as Western blots of both proteins showed a decreased signal in the reactive protein bands with equal time-course pattern from days 3 to 7 and were significant on days 3 and 5 (Fig. 3, A and B).

Once we studied the evolution and the effect of VIP treatment in the expression of TLRs in the colon microenvironment, the next step was to determine which cells in the colon are involved in this regulation. In this sense, we performed immunohistochemical analysis using specific antibodies against TLR2 and TLR4. Our results showed two different TLR2- and TLR4-positive cell types in the colon mucosa of control animals: epithelial cells from the intestinal crypts and mononuclear cells within the lamina propria (Fig. 4. A, D, and G). A stronger expression was noted in both cell types in animals with colitis (Fig. 4. A, D, and G). A stronger expression was noted in both cell types in animals with colitis.

![Fig. 2. Real-time RT-PCR analysis of TLR2 and TLR4 mRNA expression in colon extracts in TNBS-induced colitis and their down-regulation after VIP treatment.](http://www.jleukbio.org)
treated with TNBS, corroborating the fact that TNBS inflammation induces TLR2 and -4 protein expression on day 5 (Fig. 4, B, E, and H). The histological analysis confirmed the VIP reduction of TLR2 and -4 proteins after TNBS-induced colitis in epithelial cells from the gut crypts (Fig. 4, F and I) as well as in lamina propria mononuclear cells (Fig. 4C, data not shown).

Cell-surface expression of TLR2 and TLR4 in mesenteric lymph nodes from TNBS-induced and VIP-treated colitis

To assess whether VIP is not only acting locally in the colon but also systemically, we evaluated TLR expression in specific immune cell subpopulations from mesenteric lymph nodes, which act as checkpoints of their traffic to/from the inflamed colon. With this aim, we evaluated the proportion of cells bearing TLR2 or -4 by FACS analysis. Total lymph node cells were stained with isotype control antibodies or antibodies to TLR2 (IgG2b; Fig. 5, A and B) and TLR4 (IgG2a; Fig. 6, A and B). Our results indicate that 10–20% of total cells from mesenteric lymph nodes of control animals showed TLR2 and TLR4 immunoreactivity, respectively (Figs. 5B and 6B). To determine how TLR2 and -4 expressions could be modulated during TNBS-induced colitis, cell suspensions were processed at different time-points (1, 3, 5, and 7 days). We found a significant increase in the proportion of TLR2- and -4-positive cells on days 3–7, reaching 30–40% of total lymph node cells (Figs. 5B and 6B). Treatment with VIP reduced the percentage of TLR-labeled cells significantly from days 3 to 7, and a maximal inhibitory effect was on day 5 (Figs. 5B and 6B). The regulatory effect of VIP on TLRs could be a result of a modulation of the number of recruited cells from different subpopulations into mesenteric lymph nodes and/or a down-regulation of TLR surface expression.

To this extent, we investigated, along TNBS-induced colitis, the effect of VIP on cellular traffic of dendritic cells (DC), MØ, as the main cellular type expressing TLR2 and -4, and lymphocytes, as the predominant cell type in the lymph node.

Fig. 3. Western blot analysis of TLR2 and TLR4 in colon extracts in TNBS-induced colitis down-regulation after VIP treatment. Colon extracts from control and ethanol-, TNBS-, and TNBS/VIP-treated animals were prepared and electrophoresed as described in Materials and Methods. Specific bands for TLR2 (A) or TLR4 (B) were quantified by densitometry and rectified with respect to β-actin protein levels in each case. Each result is the mean ± SEM of at least four mice. +, P < 0.05; ++, P < 0.01; ++++, P < 0.001, versus control animals, and *, P < 0.05; **, P < 0.01; ***, P < 0.001, versus TNBS-treated animals. A representative experiment of three others is shown in the upper panels. O.D. = optical density.

Fig. 4. Immunohistochemical analysis of TLR2 and -4 at day 5 in gut sections. Immunohistochemical study was performed to study TLR2 and TLR4 expressions in colon mucosa as described in Materials and Methods. Mononuclear cells positive to antibody against TLR2 within the lamina propria in control (untreated animals; A), TNBS (B), and TNBS + VIP-treated animals (C; original ×400). Gut crypts showing positive staining for TLR2 and TLR4 in control (untreated animals; D and G), TNBS (E and H), and TNBS + VIP-treated animals (F and I). Original magnification, ×200. Arrows indicate the positive staining. VIP reduced the TNBS-induced expression of TLR2 and TLR4 in epithelial cells from intestinal crypts and mononuclear cells.

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CD11c is a cell-surface molecule predominantly expressed on DC and thus, is being used as a marker of this cell type, although it can also be found on natural killer (NK) cells, granulocytes, and MΦ. Besides, CD11b is frequently used to distinguish MΦ, although it’s also expressed on NK, granulocytes, and DC. Therefore, we used two rat anti-mouse CD11c and CD11b mAb to label mainly DC and MΦ, whereas lymphocytes were identified by their FSC versus SSC profiles, as the FSC/SSC plot shows (Figs. 5E and 6E). The results are summarized in Table 1. First, we detected a significant decrease of absolute cell numbers on days 3 and 5 after TNBS treatment, more drastic on day 5. Administration of VIP restored cell count, suggesting that this peptide could modulate the cellular traffic. Next, we analyzed the different populations into mesenteric lymph nodes during TNBS colitis development. VIP treatment significantly reduces the TNBS increased proportion of CD11c- and CD11b-positive cells on days 5 and 7, restoring the cellular number to values of control animals. In addition, TNBS treatment significantly reduces the percentage of lymphocytes at these time-points, whereas VIP counterbalances this effect.

Afterwards, we investigated the effect of VIP on the percentage of TLR2- and TLR4-positive cells from specific subpopulations. Our results show that approximately 70% and 77% of CD11c and 55% and 65% of CD11b cells in untreated mice are positive for TLR2 and TLR4, respectively. The time-course profiles for TLRs were similar in CD11b- and CD11c-positive cells, showing a significant up-regulation of TLR-positive cells between days 3 and 7 after TNBS treatment, and maximal percentages were on day 5, near almost cells positive for TLR2 or TLR4 (Figs. 5, C and D, and 6, C and D). VIP treatment resulted in a significant reduction at these points, restoring the percentages of TLR-positive cells to control levels. Regarding lymphocytes, only approximately 8% and 10% express TLR2 and TLR4 constitutively. TNBS treatment resulted in a significant increase of positive lymphocytes for TLR2 or TLR4 only on day 5 and was restored after VIP administration (Figs. 5F and 6F). Figure 7 shows a representative example of the expressions of TLR2 and -4 in CD11c- and CD11b-positive cells and lymphocytes in TNBS colitis and their reduction in the number of positive cells and in the mean fluorescence intensity after VIP treatment (day 5). Taken together, our results demonstrate an effect of VIP through the modulation of cellular traffic and an approach to the steady-state levels of the number of TLR2- and TLR4-positive MΦ, DC, and lymphocytes in mesenteric lymph nodes.
Time-course analysis of IL-1β and IFN-γ expression in colon extracts in TNBS-induced colitis and their decrease after VIP treatment

Bacterial products from colon stimulate TLR2 and -4, mediating a cascade that finally induces the expression of proinflammatory cytokines, as well as the induction of cytokines related to an acquired immune response of Th1 type. To evaluate the relevance of TLR2 and -4 in the development of TNBS-induced colitis, we compared their expression with the kinetics of two crucial cytokines in the development of an inflammatory/autoimmune disease, such as IL-1β and IFN-γ in colon extracts. In correlation with TLR2 and -4 expressions, TNBS treatment induces an increase of IL-1β and IFN-γ mRNA expression, peaking on day 5 and decreasing more markedly than TLRs on day 7. Ethanol-treated animals showed only a mild increase (Fig. 8A).

To date, the beneficial effect of VIP is mediated by the down-regulation of proinflammatory and Th1 cytokines. To elucidate the possible involvement of this down-regulation in the complex in vivo cytokines/TLRs/VIP system, we have studied the time-course effect of VIP treatment in the expression of both cytokines. After VIP treatment, IL-1β kinetics correlated to TLR2 on days 3 and 5 and to TLR4 from days 1 to 5 (Fig. 8A). Regarding IFN-γ kinetics, it correlated to TLR2 expression on days 5 and 7 but to TLR4 expression only on day 5 (Fig. 8).

DISCUSSION

TLR2 and -4 mediate signals from a great variety of bacterial gut products, giving the host a panel of microbe-recognizing receptors. The constitutive expression and the inflammation-induced up-regulation of TLR2 and TLR4 suggest them as sensors for normal and pathogen luminal intestinal flora. In the present study, we demonstrated the constitutive expression of both receptors in vivo, as well as their inflammation-induced up-regulation in a mice model of CD. Several authors have described the constitutive expression of TLR2 and -4 in human and murine intestinal epithelial cells [8, 9, 20–22], as well as their modulation after gut inflammation. Data obtained in vivo about the behavior of TLR human expression in mucosal...
TABLE 1. Effect of VIP on Cellular Traffic in Mesenteric Lymph Nodes of TNBS-Treated Animals

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Control</th>
<th>Ethanol</th>
<th>TNBS</th>
<th>TNBS + VIP</th>
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<tr>
<td></td>
<td>16.0 ± 2.3</td>
<td>19.5 ± 1.6</td>
<td>21.5 ± 1.6</td>
<td>14.0 ± 1</td>
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Absolute numbers of lymph node cells (×10⁶)

Control 1.6 2.3 1.5 1.6 1.6 1.6
Ethanol 1.6 2.3 1.9 ± 4.5 1.5 ± 5.7 2.39 ± 2.2
TNBS 1.9 ± 2 10.4 ± 1.4 6.1 ± 1.1 8.2 ± 0.4
TNBS + VIP 1.4 ± 1 1.7 ± 2.9 24.4 ± 5.5 27 ± 4.6

CD11c, %

Control 4.4 ± 0.3 4.4 ± 0.3 4.4 ± 0.3 4.4 ± 0.3
Ethanol 4.1 ± 0.4 5.3 ± 0.2 4.1 ± 0.5 4.3 ± 0.3
TNBS 4.9 ± 1.2 3.5 ± 0.6 15.0 ± 0.8 6.7 ± 0.4
TNBS + VIP 4.8 ± 1.2 5.1 ± 0.7 4.7 ± 0.3 5.4 ± 0.7

CD11b, %

Control 6.6 ± 0.3 6.6 ± 0.3 6.6 ± 0.3 6.6 ± 0.3
Ethanol 6.9 ± 1.3 5.8 ± 0.3 5.7 ± 0.3 6.7 ± 0.9
TNBS 6.5 ± 0.3 3.8 ± 1.4 23.0 ± 5.1 15.8 ± 0.7
TNBS + VIP 6.3 ± 0.6 7.1 ± 0.5 7.6 ± 0.4 9.3 ± 1.4

Lymphocytes, %

Control 83.0 ± 2.1 83.0 ± 2.1 83.0 ± 2.1 83.0 ± 2.1
Ethanol 81.5 ± 0.6 87.2 ± 0.6 81.1 ± 5.5 80.0 ± 2.8
TNBS 84.3 ± 2.1 86.6 ± 1.4 61.4 ± 2.3 68.7 ± 3.2
TNBS + VIP 83.3 ± 0.6 86.6 ± 0.5 73.3 ± 0.4 84.5 ± 4.1

Cell suspensions were prepared from four mesenteric lymph nodes as described in Materials and Methods, and absolute numbers of cells were monitored. Next, cells suspensions were selected for CD11c or CD11b phenotypes and then subjected to flow cytometry. Lymphocytes were determined by electronic gating. Numbers indicate the percentage of the selected subpopulation into the cell suspension. Each result is the mean ± SEM of n = 6 from two separated experiments (three mice/group/experiment). ††† P < 0.01; †‡‡ P < 0.001 (ANOVA) with respect to control animals; * P < 0.05; ** P < 0.001 (ANOVA) with respect to TNBS-treated mice; *** P < 0.01.

Although TLRS are mainly expressed on myelomonocytic cells, relatively little is known about TLRS expression on lymphocytes. Recent studies using real-time PCR have demonstrated that murine mRNA TLR2 and mRNA TLR4 are expressed in mouse T cells [25] and T and B cells from human peripheral blood mononuclear cells [26]. However, the modulation of TLR2 and TLR4 cell-surface expression after an inflammatory stimulus has not been well-characterized to date. Our data showed that approximately 10% of lymphocytes constitutively expressed TLRs and their up-regulation after TNBS treatment. Thus, this inflammatory agent causes the up-regulation of TLR-positive cells of innate and acquired immunity.

Medzhitov and his group [6] have recently postulated a new meaning of TLR presence in the gut mucosa, suggesting their protective role in the intestinal homeostasis through two possible mechanisms: the steady-state induction of protective factors, via the constitutive detection of TLR ligands on commensals by TLRS expressed on colon epithelium, and/or the induction by commensal-derived TLR ligands of the production of protective factors upon epithelial damage.

As the inflammatory disorders may break intestinal homeostasis up-regulating TLR expression, a close control of the initial proinflammatory response is crucial to restore the host homeostasis.

In the last 5 years, VIP has emerged as an endogenous agent for the treatment of inflammatory/autoimmune diseases as septic shock, RA, and CD, inhibiting the expression and release of proinflammatory cytokines and chemokines, enhancing the production of anti-inflammatory cytokines, and regulating the Th1/Th2 cytokine profile [11–13]. Moreover, VIP effects are mediated by the presence of pituitary adenylyl cyclase type 1, VIP type 1 (VPAC1), and VPAC2 receptors on lymphocytes and MØ. The therapeutic effect of VIP in TNBS-induced colitis was associated to the down-regulation of inflammatory and bowel disease, TNBS-induced colitis, characterized by a Th1-driven autoimmune component. The same pattern and behavior were found for TLR2 expression. Moreover, we evaluated for the first time the expression of these receptors through the development of the disease. Both receptors showed the highest levels of messenger and protein expression on day 5, when most of the inflammatory parameters of the disease peak, and the late phase of the disease starts, whereas a decrease was seen on day 7, when the chronic phase starts. In addition, we demonstrated the constitutive and the TNBS-induced TLR2 and -4 expressions in immune cells from mesenteric lymph nodes. These secondary lymphoid organs play an important role as checkpoints controlling the traffic of antigen-presenting cells and lymphocytes and constitute organized centers, where the antigen presentation and lymphocyte activation occur. Our data showed that after TNBS treatment, there is a significant increase in TLR-positive cells from days 3 to 5, reaching the 30–40% of total lymph node cells. It is well known that MØ and DC are important players on innate immunity, and DC have evolved specifically to translate innate recognition into acquired immunity. Our results demonstrate that approximately 60% of MØ and 70% of DC, characterized by CD11c and CD11b surface expression, expressed TLR2 and TLR4 in untreated mice, increasing the percentages after TNBS treatment.
Flow cytometry analysis of CD11c and CD11b cells and lymphocytes from mesenteric lymph nodes of control and treated animals. Cell suspensions were processed for double immunofluorescence staining for TLR2 and TLR4 and a subpopulation marker (CD11c and CD11b). Lymphocytes were identified by a FSC versus SSC gate and subjected to TLR2 and TLR4 immunofluorescence staining. Cell suspensions were incubated with isotype control antibodies or antibodies to TLR2 (IgG2b) or TLR4 (IgG2a), as described. The values shown in right quadrants and histograms indicate the percentage of CD11c/CD11b or lymphocytes and TLR2/4-positive cells. The results presented are representative of two independent experiments using three mice per group.
Th1-driven, autoimmune responses, including tumor necrosis factor α (TNF-α), IL-1β, and IL-6 in colon extracts and serum as well as IFN-γ by splenic and lamina propria CD4 T cells [13].

Our results showed that in acute inflammatory conditions, as TNBS-induced colitis, VIP treatment exerts a time-course inhibition of TLR2 and -4 expressions at mRNA and protein levels from days 1 to 7, producing a general down-regulation at the colon local microenvironmental level with the involvement of epithelial and mononuclear cells. Furthermore, VIP exerts its action at a systemic level in lymph nodes, regulating the cellular traffic, reducing significantly the TNBS-increased proportion of DC and MØ, and restoring the number of cells at the level of control animals. The arrival of MØ and DC from inflamed tissues to regional lymph nodes has been described to perform antigen processing and presentation to T cells, which results in a migration of lymphocytes to sites of inflammation. TNBS-induced inflammation is characterized by a massive infiltration of neutrophils and MØ in the early stages of the disease, producing high levels of proinflammatory cytokines; these events are followed by an infiltration of T cells producing high levels of IFN-γ [13]. Our results reveal that VIP could selectively modulate cell recruitment into lymph nodes, which could prevent the pathological effects of TNBS. In addition, VIP exerts its effect by modulating the number of TLR2- and TLR4-positive DC, MØ, and lymphocytes. Although further studies are needed to deepen a potential, direct action of VIP on TLR expression, we do not rule out this hypothesis.

The stimulation of TLR2 and -4 mediates a cascade that finally leads to activation of nuclear factor (NF)-κB, contributing to the expression of inflammation-related genes such as TNF-α or IL-1β [2]. In this sense, TLR4 activation activates two cascades: the myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the early phase of NF-κB activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway activates the late phase of NF-κB activation and IFN regulatory factor 3 (IRF3), which lead to the production and expression of IFN-inducible genes [5].

The VIP-mediated down-regulation of TLR2 and -4 could be explained through two possible ways that are not mutually exclusive. The first one would be a primary through the VIP-mediated decrease of NF-κB, which would cause a down-regulation of TLR expression, as a binding site for NF-κB in the murine TLR2 has been described [27]. In this sense, it has been shown that VIP decreases NF-κB binding in vitro and in vivo. In vitro, a cyclic adenosine monophosphate-independent pathway associated with the VPAC1 receptor leads to the inhibition of inhibitor of κB (IkB) phosphorylation [28]. In vivo, data from our laboratory in a murine model of RA (manuscript in preparation) revealed that the beneficial effect of VIP treatment is mediated by the inhibition of IkBα phosphorylation/degradation. Thus, VIP prevents NF-κB nuclear translocation and inhibits the transcription of several inflammatory factors [29]. Furthermore, we have described VIP-mediated inhibition of IFN-γ at the transcriptional level [30], and the promoter for TLR4 was shown to contain an IRF-binding site that regulates expression of TLR4 in myeloid cells [31].

Conversely, different studies have suggested that inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ could act synergistically, together with gram-positive and gram-negative bacterial components, up-regulating TLR2 and -4 expressions [20, 21, 32, 33]. Thus, TLR2 and -4 would be activated by molecular products of bacteria, leading to the production of proinflammatory cytokines, and many of these cytokines would be able to amplify TLR expression, establishing a positive feedback that may lead to the perpetuation of inflammatory conditions, favoring the chronic phase of the disease. Therefore, the other possible mechanism to explain VIP-induced TLR2 and -4 modulations is the secondary reduction of the Toll expression caused by the decrease of inflammatory mediators such as TNF-α, IL-1β, and IFN-γ, which amplify TLR signaling. In this sense, we have previously demonstrated in this model of colitis that VIP treatment down-regulates the protein levels of different proinflammatory cytokines [13]. In this study, we report that the expressions of IL-1β and IFN-γ are time-correlated to TLR2 and TLR4 in TNBS-nontreated and

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**Fig. 8.** Real-time RT-PCR analysis of IL-1β and IFN-γ mRNA expression in colon extracts in TNBS-induced colitis and their regulation after VIP treatment. mRNA was extracted from colons at days 1, 3, 5, and 7. IL-1β (A) and IFN-γ (B) mRNA expressions were measured by real-time RT-PCR, values were rectified by mRNA expression of β-actin for each sample, and arbitrary units were calculated with respect to control expression, being 1 (2−ΔΔCt). Each result is the mean ± SEM of at least three mice performed by duplicate. +++, P < 0.001, versus control animals, and *, P < 0.05; ***, P < 0.01; ****, P < 0.001, versus TNBS-treated animals.

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VIP-treated animals. Thus, through the reduction of IL-1β and to a minor extent, of IFN-γ VIP could break the positive feedback that perpetuates inflammation, reducing the presence and availability of both TLRs, maintaining their homeostatic levels. Through a VIP primary reduction of NF-κB, which causes a decrease of TLR expression, or through a secondary, VIP-mediated decrease of the proinflammatory cytokines, which synergize with bacterial products to up-regulate TLR expression or acting in both ways, the resultant physiological effect is the approach of TLR2 and -4 expressions to constitutive and thus, homeostatic levels (Fig. 9).

Although additional studies are required to fully dissect the signaling VIP pathways involved in this decrease of TLR expression, our study describes for the first time the possible involvment of a peptide present in the gut microenvironment, in the effective temporal control of the first step of acute inflammation, as well as the restoration of the lost homeostasis in an established inflammatory/autoimmune disease.

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