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IL-12 Induction of mRNA Encoding Substance P in Murine Macrophages from the Spleen and Sites of Inflammation

Razvan Arsenescu,² Arthur M. Blum, † Ahmed Metwali, † David E. Elliott, † and Joel V. Weinstock †

Substance P (SP), a neuropeptide, interacts with the neurokinin 1 receptor (NK-1R) on immune cells to help control IFN-γ production. In murine schistosomiasis mansoni, schistosome worms produce ova that incite focal Th2-type granulomatous inflammation within the liver and intestines. Normal gut is characterized by a controlled state of inflammation. IL-10 knockout mice develop chronic Th1-type colitis spontaneously. Both schistosome granulomas and gut mucosa display an SP immune regulatory circuit. However, the origin and regulation of SP production at these sites of inflammation are poorly understood. Macrophages are a potential source of SP. We therefore studied macrophages (F4/80⁺) from these models of inflammation. SP mRNA (preprotachykinin A (PPT A)) was detected within the schistosome granuloma, spleen, and lamina propria macrophages. Compared with those from wild-type mice, granuloma macrophages from STAT6⁻/⁻ mice had 10-fold higher PPT A mRNA expression, whereas in STAT4⁻/⁻ animals, PPT A mRNA expression was nearly abolished. IL-12 signals via STAT4 to induce Th1-type inflammation. It was demonstrated that IL-12, but not IL-18, induces SP mRNA expression in resting splenic macrophages from Schistosoma-infected mice and in wild-type lamina propria mononuclear cells. Thus, macrophages are a source for SP at these sites of chronic inflammation, and IL-12 and STAT4 are regulators of macrophage SP mRNA expression. The Journal of Immunology, 2005, 174: 3906–3911.

Macrophages help initiate and propagate the immune response through Ag presentation and cytokine secretion. They can be a source of both IL-12 and SP (11). However, the origin and regulation of SP production at sites of inflammation are poorly understood. Using two murine models of chronic inflammation, preprotachykinin (PPT) mRNA was detected within the macrophages of schistosome granulomas, spleen, and intestinal lamina propria. It was found that IL-12 and the STAT4 pathway drive PPT mRNA expression in these cells. These data and previous findings also suggest that SP, a regulator of IFN-γ production, is a component of the IL-12 immune regulatory circuit that promotes Th1 cell development and IFN-γ production.

Materials and Methods

Mice and infection

CBA/I and C57BL/6J wild-type (WT) mice (The Jackson Laboratory) were used throughout this study. Also used were C57BL/6J IL-10⁻/⁻ mice and BALB/c STAT4⁻/⁻ and STAT6⁻/⁻ mice that were bred and maintained at the University of Iowa. Some mice were infected s.c. with 35 cercariae of the Puerto Rican strain of the parasite S. mansoni (12). At 8 wk of infection, mice were killed to obtain splenocytes and liver granuloma cells.

Isolation and dispersal of splenocytes, granuloma cells, and LPMC

Spleens were dispersed by gently teasing the spleen tissue through a 100-μm pore size nylon cell strainer (BD Biosciences) using a rubber policeman and RPMI 1640 medium (Invitrogen Life Technologies). Splenocytes were spun down and resuspended in 5 ml of sterile distilled water for a few seconds to lyse RBC by hypotonic shock. Then, the spleen cells were washed twice in RPMI 1640 medium and resuspended in RPMI 1640 containing 10% FCS, 10 mM HEPES buffer, 2 mM L-glutamine, 100

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U/ml penicillin, and 100 μg/ml streptomycin (complete medium; Sigma-Aldrich). The cells were counted, and viability was determined using exclusion of trypan blue dye.

To isolate granulomas, livers from infected mice (usually three) were harvested and homogenized for 30 s at low speed in a Waring blender. The granulomas were collected by centrifugation at 500 × g and washed twice in RPMI 1640. The granulomas were dispersed by agitation in a shaking water bath at 37°C for 35 min in RPMI 1640 containing 5 mg/ml collagenase (type I from Clostridium histolyticum; Sigma-Aldrich). The granuloma cells then were dispersed further by repeated cycles of suction and expulsion through a 1-ml syringe, and the dispersed cells were passed through sterile gauze to filter out the nondispersed tissue fragments. The granuloma cells were washed twice in RPMI 1640 and resuspended in 20 ml of RPMI 1640 complete medium. The viability of these cells was determined using trypan blue dye.

Gut LPMC were isolated as described below. Intestinal tissue (terminal ileum or colon) was washed extensively with RPMI 1640, and all visible Peyer’s patches were removed with scissors. The intestine was opened longitudinally, cut into 5-mm pieces and then incubated in 0.5 mM EDTA in calcium- and magnesium-free Hank’s buffer for 20 min at 37°C with shaking to release intraepithelial lymphocytes and epithelial cells. This was repeated after thorough washing. Tissue then was incubated for 20 min at 37°C in 20 ml of RPMI 1640 containing 5% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 μg/ml streptomycin (all from Invitrogen Life Technologies) and 1 mg/ml collagenase (col130; Sigma-Aldrich). At the end of the incubation, the tissue was subjected to further mechanical disruption using a 1-ml syringe. To remove debris, the LPMC preparations were washed through damp gauze layers at the top of a Percoll gradient (RPMI 1640). Then LPMC were sieved through a pre-wet 2-cm nylon wool column gently packed into a 10-ml syringe with a 30:70% gradient. Cells were spun at 2200 × g at room temperature for 10 min. The LPMC collected from the 30:70% interface were washed and maintained on ice until used. Cell viability was 90% as determined by eosin Y exclusion.

Isolation of T cells and macrophages/monocytes

T cells were isolated from dispersed LPMC using paramagnetic beads coated with Thy.1.2 mAb (Dynabeads M-450; Dynal Biotech). LPMC from CBA/J or C57BL/6 mice were exposed directly to these beads, and the T cells were separated as suggested by the manufacturer.

Macrophage/monocyte populations from dispersed splenic or granuloma cells were isolated using the Dynabead method. Dispersed cells were exposed to a rat IgG, anti-F4/80 mAb (MCAP 497; Serotec), at 1 μg/10⁶ cells for 15 min on ice. The cells were then washed three times in complete medium, and magnetic beads (Dynal Biotech) coated with sheep anti-rat IgG were added at 4 times the number of the targeted cells. Cells were incubated at 4°C with slow rocking for 20 min. At the end of this incubation, F4/80⁺ cells were separated using a magnet, as suggested by the manufacturer. Flow analysis confirmed the adequacy of all separations, which was always >95%.

Cell culture

In some experiments, dispersed LPMC or splenocytes (5 × 10⁶ cells/flask) were incubated for 4 h in 10 ml of RPMI 1640 complete medium at 37°C in T25 flasks. Some cultures contained mouse rIL-12 (1 ng/ml; PeproTech) and granuloma or splenic macrophages. Constitutive expression also was noted in the granuloma is an excellent model to assess the leukocyte origins of SP. CBA/J mice were killed 6–8 wk after initiation of infection with S. mansoni. Macrophages from Schistosoma-infected mice were obtained from dispersed liver granulomas using rat anti-F4/80 mAb and anti-rat IgG-coated paramagnetic beads. Cellular RNA was extracted from both F4/80⁺ and F4/80⁻ subsets. Qualitative RT-PCR analysis (Fig. 1) showed that PPT A mRNA is expressed in F4/80⁺ granuloma macrophages. Constitutive expression also was noted in the F4/80⁻ fraction, which consisted mostly of eosinophils, T cells, and B cells. The purity of each preparation was ~99%. Using a

RNA extraction, RT-PCR, and competitive PCR assay for PPT A

The total cellular RNA was extracted as described previously (13). Briefly, spleen or granuloma cells (~5 × 10⁶) were washed twice in RPMI 1640, and the pelleted cells were homogenized in guanidinium-phenol to extract the RNA. The RNA was quantitated spectrophotometrically and checked for intact 18S and 28S bands by gel electrophoresis. Samples were compared for hypoxanthine phosphoribosyltransferase (HPRT) content to confirm equivalent mRNA content and RT.

RT reactions were performed for 2 h at 42°C using 5 μg of RNA. 400 U of Moloney murine leukemia virus reverse transcriptase, and 0.5 μg of 18-mer oligo(dT) for random priming in a total volume of 40 μl. The first-strand cDNA was diluted to 250 μl, and 25 μl of the product was used in each PCR. PCR was performed using a Robocycler 40 (Stratagene) in a total volume of 50 μl using 3 U of Taq DNA polymerase and a primer pair specific for a 182-bp fragment of exon VII of mouse α, β, and γ prepro-tachykinin A mRNA. The sequences of the primers were 5'-GCCAATG CAGAACTACGAAA and 5'-GCTTGAGACGTCCTCTCTAC. Each tube contained 5 μl each of 2 mM dNTP, 1.6 mM Mg²⁺, 1.5 U of Taq DNA polymerase, and 10 pM of both primers. The PCR sequence was 93°C for 70 s to melt, 56°C for 80 s to anneal, and 72°C for 70 s to extend. The PCR was repeated for 40 cycles.

In some experiments a quantitative RT-PCR assay was performed to measure the number of PPT A mRNA transcripts in total cellular RNA preparations. To construct a competitor plasmid for this assay, the 182-bp PCR PPT A PCR product was cloned into PgemTez (Promega), then cut with BstEII. A 117-bp fragment of A DNA was ligated into the BsvEII site of the PPT A sequence to create an elongated mimic sequence of 282 bp. Its authenticity was confirmed by nucleotide sequencing. The mimic plasmid was selected, expanded, purified, then quantified by UV spectrophotometry. Various quantities of mimic plasmid DNA containing double-stranded elongated PPT A cDNA were added to a series of PCRs containing sample cDNA. The concentration of the unknown mRNA was determined by competition with known concentrations of this engineered plasmid by localization of bands of equivalence.

Statistical analysis

Student’s t test was used to compare the means of two populations for their significant difference.

Results

PPT A mRNA is expressed in granuloma macrophages

SP can be a product of both neuronal and immune cells (14). Because granulomas are devoid of nerves (9), the granuloma is an excellent model to assess the leukocyte origins of SP. CBA/J mice were killed 6–8 wk after initiation of infection with S. mansoni. Macrophages from Schistosoma-infected mice were obtained from dispersed liver granulomas using rat anti-F4/80 mAb and anti-rat IgG-coated paramagnetic beads. Cellular RNA was extracted from both F4/80⁺ and F4/80⁻ subsets. Qualitative RT-PCR analysis (Fig. 1) showed that PPT A mRNA is expressed in F4/80⁺ granuloma macrophages.
Quantitative PCR assay to measure PPT A mRNA transcript number, macrophages contained ~20,000 PPT A mRNA transcripts/μg RNA, T cells contained 2,000 PPT A mRNA transcripts/μg RNA, and B cells contained 28,000 mRNA transcripts/μg RNA. Granulomas contain 30% macrophages and 5% B cells. Thus, macrophages are a major, although not the exclusive, source for PPT A mRNA in granulomas.

PPT A mRNA expression is STAT4/6 dependent

Inflammatory processes can be polarized into either Th1 or Th2 responses. IL-12 and IL-4 are the major cytokines responsible for this immunologic polarization. IL-12 signals through STAT4, and IL-4 signals through STAT6. Blocking STAT4 or STAT6 pathways substantially alters the immunological characteristics of these predominantly Th2-type granulomas (15). Thus, it was conceivable that changes in STAT4/6 signals would significantly alter these predominantly Th2-type granulomas (15). Thus, it was hypothesized that IL-12, via STAT4 signaling, could be a major regulator of PPT A transcription. To test this hypothesis, unfractionated splenocytes from schistosome-infected mice were incubated for 4 h in vitro with and without IL-12 (1 ng/ml). Cellular mRNA was then extracted and analyzed for PPT A mRNA using RT-PCR. The constitutive expression of PPT A mRNA was negligible, whereas IL-12 induced robust expression (Fig. 3). F4/80+ splenic macrophages were isolated using paramagnetic beads. The cells then were cultured for 4 h with and without rIL-12. PPT A mRNA was analyzed as described above. Resting macrophages displayed weak expression of PPT A mRNA, whereas IL-12 strongly induced its expression (Fig. 3A). Separate experiments using IFN-γ, LPS, IL-18, IL-10, and TGFB showed lack of induction. This induction was dose dependent. IL-12 could induce PPT A mRNA at concentrations as low as 10 pg/ml (Fig. 4B).

IL-12 induces macrophage PPT A mRNA expression

PPT A mRNA expression was severely impaired in STAT4−/− macrophages. Thus, it was hypothesized that IL-12, via STAT4 signaling, could be a major regulator of PPT A transcription. To test this hypothesis, unfractionated splenocytes from schistosome-infected mice were incubated for 4 h in vitro with or without rIL-12 (1 ng/ml). After the incubation, cellular RNA was extracted and analyzed for PPT A mRNA expression using PCR. Shown are the results from two individual experiments. All samples contained comparable amounts of the HPRT housekeeping gene transcripts.

LP macrophages express PPT A mRNA and SP

The gut is continuously exposed to bacteria. Consequently, a controlled state of inflammation is present under normal conditions (16). Thus, in addition to granulomas, this physiological source of inflammatory cells was examined for PPT mRNA expression. The figure shows the results of such an experiment.
IL-10 KO mouse develops chronic intestinal inflammation in an age-dependent fashion, and SP has a role in regulating the inflammatory response (17). Nerves are the classic source of SP in the gut. It was not known whether murine LPMC, specifically the LP macrophages, express PPT A mRNA and SP.

LPMC from ileum and colon of WT or IL-10 KO mice were isolated. Total mRNA was extracted from the freshly isolated cells and analyzed by RT-PCR for PPT A mRNA. Both WT and IL-10 KO LPMC constitutively expressed PPT A mRNA (Fig. 5).

Using paramagnetic beads, the LPMC from the terminal ileum of IL-10 KO mice were fractionated in T cell-enriched and T cell-depleted fractions (non-T cell fraction). Four separate experiments showed that the non-T fraction was responsible for all the PPT A mRNA expressed in the dispersed LPMC (Fig. 6). Similar results were obtained using WT terminal ileal LPMC (data not shown).

We then assessed the potential origin of SP in the LPMC using IHC. Double-staining IHC was performed on freshly isolated LPMC from WT and IL-10 KO C57BL/J mice. FITC-labeled, rat anti-mouse F4/80 mAb (green fluorescence) and biotinylated rabbit anti-mouse SP Ab in a biotin-avidin system with Alexa 594 fluorochrome (red fluorescence) were used. SP localized only to the F4/80 + LPMC (Fig. 7).

IL-12 induces PPT A mRNA expression in LPMC from WT animals

We showed that splenic macrophages from Schistosoma-infected mice express PPT A mRNA and that IL-12 induces it. Therefore, we investigated whether IL-12 can enhance PPT A expression in LPMC from WT C57BL/J mice. Freshly isolated LPMC were incubated for 30 min with and without rIL-12 (1 ng/ml). Total RNA was extracted and analyzed for PPT A mRNA. IL-12 caused a 3-fold augmentation of PPT A mRNA (Fig. 8).

Discussion

SP is a product of human, rat, and mouse leukocytes that can be released at sites of inflammation to modulate immune responses (3, 18, 19). In mice colonized with S. mansoni, a helminthic parasite, eggs localize in the liver and intestinal wall. A focal, chronic granulomatous response develops around the eggs. The granulomas comprise ~50% eosinophils, 30% macrophages, 10% T cells, and <1% mast cells (20). They are a rich source of SP and PPT A mRNA. Nerves can be a source of SP. Schistosome granulomas in the liver do not contain nerves. Thus, all the SP and PPT A mRNA

FIGURE 5. LPMC from colon or ileum of WT or IL-10 KO mice express PPT A mRNA. RNA was extracted from the freshly isolated ileal or colonic LPMC obtained from either WT or IL-10 KO mice (IL10KO). RNA was analyzed for PPT A mRNA expression using PCR. Shown are the results from two separate LPMC extractions for each tissue.

FIGURE 6. LPMC PPT A mRNA localized to the non-T cell subset. LPMC isolated from the terminal ileum of IL-10 mutant mice were fractionated into T cell-enriched and T cell-depleted subpopulations using paramagnetic beads. Cellular RNA was extracted and analyzed for PPT A mRNA expression using PCR. Shown are results from four separate experiments.

FIGURE 7. Immunoreactive SP localized to LP macrophages. IHC with double staining was carried out on freshly isolated, unfractionated LPMC from either WT or IL-10 KO mice. FITC-F4/80 rat anti-mouse mAb and SP rabbit anti-mouse biotinylated Ab in a strepavidin system with Alexa 594 fluorochrome were used. Green fluorescence identifies F4/80 + cells. Red fluorescence denotes intracellular SP. Colocalization made staining appear orange.

FIGURE 8. IL-12 enhanced PPT A expression in LPMC. LPMC from WT mice were cultured for 30 min in vitro with or without rIL-12 (1 ng/ml). After the incubation, cellular RNA was extracted and analyzed for PPT A mRNA expression using quantitative RT-PCR. Shown are the mean ± SE of four determinations from two separate experiments.
comes from the granuloma leukocytes. Our laboratory showed that eosinophils and T cells from schistosome granulomas make SP (21, 22).

In the present study we showed that macrophages (F4/80<sup>+</sup> cells) isolated from schistosome-infected mice express PPT A mRNA. Thus, F4/80<sup>+</sup> cells from granulomas could also be a source of SP. Because macrophages are the second most common cell subset in schistosome granulomas, they may provide a significant contribution to SP production and, hence, modulation of inflammation. Although splenocytes contain macrophages, no constitutive PPT A mRNA expression was noted. Thus, it was hypothesized that SP mRNA expression in granulomas is induced.

SP drives IFN-γ expression within the granuloma (8). It was determined whether alteration of the cytokine profile within the inflammation would affect PPT A expression. In keeping with Th1/Th2 polarization, two opposing scenarios were considered: STAT4<sup>−/−</sup> (IL-12/IL-23-impaired) and STAT6<sup>−/−</sup> (IL-4/IL-13-impaired). Compared with WT granuloma macrophages, PPT A mRNA expression was nearly abolished or was 10-fold increased, respectively. The data suggested that perhaps STAT4 signaling and, by inference, IL-12, which signals through the STAT4 pathway, were required for optimal expression of PPT A mRNA by macrophages. The stimulatory effect of IL-12 was confirmed in subsequent experiments. IL-12 induced PPT A mRNA in a dose-dependent manner. Concentrations as low as 10 pg/ml were able to drive this expression. Given the importance of IL-12 for induction of the Th1 pathway, other related cytokines and nonspecific activators of T cells were assessed for effects on PPT A mRNA expression. Neither IL-18 (5 ng/ml) nor anti-CD3/CD28 (1 μg/ml) could induce splenocytes to express Tac1 transcripts. TGFB-β and IL-10 (5 ng/ml), both inhibitors of Th1 responses, had no effect. IL-12 induction of PPT A transcripts was not secondary to stimulation of IFN-γ production, because rIFN-γ could not induce PPT A in splenic F4/80<sup>+</sup> cells. IL-23 is a cytokine that shares some IL-12 functions with respect to IFN-γ induction and Th1 T cell differentiation (23). Signaling through the IL-23R activates STAT4 and thus may play a role in PPT A expression.

IL-12 is important for IFN-γ expression in schistosomiasis (24). Both in vitro and in vivo evidence suggests that SP, via the T cell NK1-R, regulates the expression of the critical Th1 cytokine IFN-γ (25). RAG-1 mice reconstituted with T cells from NK-1R KO animals had an impairment in IFN-γ circuitry (25). Because IL-12 induces T cells to express NK-1R, SP can modulate the IL-12-dependent IFN-γ production.

Recently, we showed that IL-12 can up-regulate NK-1R expression through the NF-κB intracellular signaling pathway (26). We demonstrate in this study that STAT4 is a critical signaling pathway for PPT A mRNA expression, thus uncovering the complex regulation of the SP/NK-1R system.

We also explored whether SP regulation by IL-12 applies to another model of chronic inflammation. LPMC from a Th1 model of chronic colitis (IL-10<sup>−/−</sup>) were assessed for effects on PPT A mRNA expression. Although both WT and IL-10<sup>−/−</sup> murine LPMC constitutively expressed PPT A mRNA, this expression was further augmented by rIL-12. The non-T (macrophage-enriched) fraction was responsible for the constitutive expression. IHC provided evidence that LP macrophages were the major storage site for SP, supporting the idea that these cells are a major source of SP in the mucosa. Macrophages can express NK-1R (27, 28). It is theoretically possible that the immunoreactive SP detected within the cells by IHC staining was actually made by other cell types and subsequently taken up by macrophages through engagement with their NK-1R. However, this scenario is not likely, because internalized SP/NK-1R complex is processed within endosomes, and the cytoplasm would not have been expected to stain profusely for immunoreactive SP.

We recently studied the importance of SP and NK-1R in the IL-10 KO colitis model used in this investigation (10). In this model, IL-10 mutant animals were fed a nonsteroid anti-inflammatory drug (NSAID) to induce the intestinal inflammation. LP T cells from IL-10<sup>−/−</sup> mice expressed NK-1R and produced IFN-γ only after NSAID treatment. LP T cells from NSAID-treated WT controls or from age-matched, untreated IL-10<sup>−/−</sup> animals did not express NK-1R or produce IFN-γ. IL-12 is important for driving IFN-γ expression in IL-10 KO inflammatory bowel disease. Experiments showed that IL-12, but not IFN-γ, induces NK-1R transcription in CD4<sup>+</sup> T cells cultured in vitro, suggesting that IL-12 is important for NK-1R induction on T cells in inflammatory bowel disease. However, T cells cultured with IL-12 and IL-10 do not express NK-1R. IL-10 also down-modulates ongoing NK-1R expression. Thus, intestinal inflammation in IL-10<sup>−/−</sup> mice is associated with the appearance of NK-1R on mucosal T cells, and an interplay between IL-12 and IL-10 appears to regulate T cell NK-1R transcription. Thus, the absence of IL-10 in these mutant mice with subsequent failure to down-regulate NK-1R expression could be contributing to the disease process. Treatment with an NK-1R antagonist reverses the ongoing intestinal inflammation, attesting to the importance of SP and its receptor in the mucosal inflammation.


