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This information is current as of November 16, 2009

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J. Immunol. 2004;172;6528-6532

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Cutting Edge: Hemokinin Has Substance P-Like Function and Expression in Inflammation¹

Ahmed Metwali, Arthur M. Blum, David E. Elliott, Tommy Setiawan, and Joel V. Weinstock²

Substance P (SP) belongs to the tachykinin family of molecules. SP, cleaved from preprotachykinin A, is a neuropeptide and a proinflammatory leukocyte product. SP engages neurokinin 1 receptor (NK-1R) to stimulate cells. Hemokinin (HK) is another tachykinin that binds NK-1R. HK comes from preprotachykinin C, which is distinct from preprotachykinin A. We determined whether HK functions like SP at inflammatory sites. Preprotachykinin C mRNA was in murine schistosome granulomas and intestinal lamina propria mononuclear cells. Granuloma T cells and macrophages expressed preprotachykinin C mRNA. HK bound granuloma T cell NK-1R with high affinity. SP and HK stimulated IFN- γ production with equal potency. NK-1R antagonist blocked the effect of SP and HK on IFN- γ secretion. Thus, both HK and SP are expressed at sites of chronic inflammation and share cell origin, receptor, and immunoregulatory function. Two distinct but functionally overlapping tachykinins govern inflammation through NK-1R at sites of chronic inflammation. The Journal of Immunology, 2004, 172: 6528–6532.

The tachykinin 1 gene (*Tac1*) encodes a protein called preprotachykinin A, which is enzymatically processed to produce substance P (SP).³ SP stimulates cells via a high-affinity seven-transmembrane, G protein-coupled receptor named neurokinin 1 (NK-1R). Macrophages (1–3), dendritic cells (4), lymphocytes (5) (6), and/or other immune cell types (7) make SP locally in schistosome granulomas, at mucosal surfaces, and at other sites of inflammation.

Animal models of inflammation and human disease provide ample evidence that NK-1R, located on leukocytes and vascular endothelium, and presumably its natural ligand SP influence immune responses. For instance, *Trichinella spiralis* is a helminthic parasite that induces a Th2-type immune response in rat intestine. Intestinal colonization of rats with this organism induces a T cell-dependent increase in SP in the muscle-mesen-

teric plexus, and blocking NK-1R improves the intestinal inflammation (8). *Salmonella* is a bacterium that induces gastroenteritis. In a murine model of salmonellosis, treating mice with an NK-1R antagonist leaves them more susceptible to infection, and they display a diminished mucosal IFN- γ response (9). *Clostridium difficile* is a bacterium that can colonize the intestines and produce toxins, which induce colitis in humans. An animal model of this disease suggests that NK-1R helps mediate *C. difficile* toxin-induced mucosal injury (10).

Mice can harbor the human schistosome called *Schistosoma mansoni*. As in humans, ova lodge in host tissue inducing granulomatous inflammation. Although the inflammation of murine schistosomiasis is best characterized as a Th2 response, it also generates Th1-type cytokines like IFN- γ that are tightly constrained. NK-1R and possibly SP are important parts of this regulatory process (11). CD4⁺ T cells within the schistosome granulomas are the major producers of the IFN- γ (12). Mice with defective NK-1R expression develop schistosome granulomas with both impaired IFN- γ and IgG2a secretion (13). This observation attests to the importance of NK-1R in controlling IFN- γ circuitry. Additional in vitro and in vivo studies using highly selective NK-1R antagonists confirm the importance of NK-1R (14). SP regulates T cell IFN- γ production through interaction with the NK-1R expressed on these cells (15). Two T cell-selective, NK-1R expression models demonstrate that it is the T cell NK-1R which directly governs the IFN- γ response (15).

Humans can develop an immunologic disease associated with chronic, destructive inflammation of the intestines called inflammatory bowel disease (IBD). In human IBD, there is increased NK-1R mRNA expression in the tissue, which associates with mucosal T cells (16, 17).

Murine models of human IBD suggest that NK-1R helps regulate intestinal inflammation. Treatment with an NK-1R antagonist limits the inflammation in trinitro- or dinitrobenzene sulfonic acid-induced colitis (18, 19). The IL-10-deficient mouse spontaneously develops intestinal inflammation that

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Received for publication February 20, 2004. Accepted for publication April 5, 2004.

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¹ This work was supported by grants from the National Institutes of Health (DK38327, DK58755, DK07663, DK25295) and the Crohn's and Colitis Foundation of America, Inc.

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³ Abbreviations used in this paper: SP, substance P; HK, hemokinin; IBD, inflammatory bowel disease; LPMC, lamina propria mononuclear cell; NK, neurokinin; NK-1R, NK-2R, NK-3R, NK 1, 2, or 3 receptor; PPT, preprotachykinin; SM-SP, [Sar⁹,Met¹¹]-SP; SEA, soluble egg Ag; HPRT, hypoxanthine phosphoribosyltransferase; WT, wild type.

worsens as the animal ages. NK-1R antagonists suppress intestinal IFN- γ production and inhibit the ongoing colitis (20).

Another recently discovered molecule belonging to the tachykinin family of neuropeptides called hemokinin (HK) 1 derives from a protein named preprotachykinin (PPT) C (21–23). It is a product of a gene (*Tac4*) distinct from that of SP. Murine SP and HK are both 11 aa long and share 55% homology. HK has similar pharmacological properties as SP with regard to receptor interaction. There is PPT C mRNA, as determined by RT-PCR, in mouse pre-B cells obtained from bone marrow, and it may help control B lymphopoiesis (22). We followed this lead to determine whether HK could have wider actions in the immune system. In this communication, we report that both HK and SP are expressed at sites of chronic inflammation. They share cell origin, receptor, and immune regulatory function. Thus, there are two distinct but functionally overlapping tachykinins that govern inflammation via high-affinity interaction with NK-1R.

Materials and Methods

Mice and infection

CBA/J and C57BL/6 wild-type (WT) mice (The Jackson Laboratory, Bar Harbor, ME) were used throughout this study. Also used were C57BL/6 IL-10^{-/-} mice that were bred and maintained at the University of Iowa. Some mice were infected s.c. with 35 cercariae of the parasite *S. mansoni* (24). Mice were sacrificed at 8 wk of infection.

Colitis induction

To induce colitis in IL-10^{-/-} mice, mice 4–5 wk of age were given piroxicam (Sigma-Aldrich, St. Louis, MO) mixed into their feed (National Institutes of Health-31M) for 2 wk. They received 60 mg of piroxicam/250 g of food in week 1 and 80 mg of piroxicam/250 g of food in week 2. Mice subsequently were placed on the normal rodent chow without piroxicam. Lamina propria mononuclear cells (LPMC) were isolated from the inflammation 14 days after colitis induction.

Isolation and dispersal of splenocytes, granuloma cells, and LPMC

Spleens were dispersed by gently teasing the spleen tissue through a 100- μ m nylon cell strainer (BD Labware, Franklin, NJ) and RPMI 1640 medium (Life Technologies, Grand Island, NY). RBC were lysed by hypotonic shock. Then the spleen cells were washed twice in RPMI 1640 and resuspended in RPMI 1640 containing 10% FCS, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (complete medium; Sigma-Aldrich). Viability was determined using exclusion of trypan blue dye.

To isolated granulomas, livers from infected mice (usually three) were harvested and homogenized for 30 s at low speed in a Waring blender. 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 dispersed cells were passed through sterile gauze to filter out the nondispersed tissue fragments. The granuloma cells then were washed twice in RPMI 1640 and resuspended in 20 ml of RPMI 1640 complete medium. Viability of these cells also was determined using trypan blue dye.

Gut LPMC were isolated as described below. Intestinal tissue (terminal ileum) was washed extensively with RPMI 1640, and 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 HBSS 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, 100 mg/ml streptomycin (all Life Technologies), and 1 mg/ml collagenase (Sigma-Aldrich no. co130). At the end of the incubation, the tissue was subjected to further mechanical disruption using a 1-ml syringe. After washing, cells (up to 2 \times 10⁷) were layered onto a column of Percoll with a 30:70% gradient. Cells were spun at 2200 \times g at room temperature for 20 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, macrophages, and B cells

T cells and B cells were isolated from dispersed granuloma cells using paramagnetic beads coated with Thy 1.2 or B220 mAb (Dynabeads M-450; Dynal, Lake Success, NY) as suggested by the manufacturer.

Macrophage/monocyte populations were isolated from dispersed cells also using the Dynabead method. Dispersed cells were exposed to rat IgG anti-F4/80 mAb (MCAP 497; Serotec, Raleigh, NC) at 1 μ g/10⁶ cells for 1 h on ice. The cells then were washed three times in complete medium, and magnetic beads (Dynal) coated with sheep anti-rat IgG were added for cell isolation. Flow analysis confirmed the adequacy of all separations (>95%).

Cell lines

D 1.1 (Th1 cell line) was from Dr. A. K. Abbas (Harvard University, Cambridge, MA). Also examined were Th2 cell line D10.G4.1, B cell lines 38C13 and CH12LX, and macrophage cell lines RAW 264.7 and TIB192 (American Tissue Culture Collection, Manassas, VA).

Cell culture

Cells were cultured for 24 h in 96-well microtiter plates (Corning, Cambridge, MA) with 200 μ l of medium (2 \times 10⁶ cells/well) at 37°C to measure IFN- γ secretion. The culture medium was RPMI 1640 containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5 \times 10⁻⁵ M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all Life Technologies). The cells were cultured alone or with soluble egg Ag (SEA) at 0.3 ng/ml. The SEA was made as described elsewhere (18). Also, some cultures contained SP (Sigma-Aldrich), HK (Bachem, Torrance, CA), and/or the NK-1R inhibitor SR 140333 (Sanofi Recherche, Montpellier, France).

Cytokine assay

IFN- γ was quantified using a two-sandwich ELISA. IFN- γ was captured with HB170 (American Type Culture Collection) and detected with a rabbit polyclonal anti-IFN- γ antiserum (Dr. M. Wilson, University of Iowa, Iowa City, IA) followed by application of biotinylated goat anti-rabbit mAb (Accurate Chemical and Scientific, Westbury, NY). The sensitivity of the IFN- γ ELISA was 30 pg/ml. The ELISA used streptavidin-peroxidase conjugate and ABTS substrate (Zymed Laboratories, San Francisco, CA).

Flow cytometric analysis

Granulomas or LPMC were washed twice and adjusted to 10⁷ cells/ml in FACS buffer (HBSS containing 20 mM HEPES, 10% FCS, and 0.02% sodium azide). The cell suspensions then were dispensed into microcentrifuge tubes each containing 10⁶ cells in 100 μ l of FACS buffer and stained with saturating amounts of conjugated mAb for 30 min at 4°C. Following staining, cells were washed twice and resuspended for analysis on a BD FACS 440 flow cytometer (BD Biosciences, Mountain View, CA).

Before adding labeled mAb, each tube received 1 μ g of 2.4G2 Ab (anti-Fc γ R; American Type Culture Collection) to block nonspecific binding of conjugated Abs to FcRs. The other mAbs used for staining were anti-CD4-Cy5 (RM2511; Caltag Laboratories, Burlingame, CA), anti-CD8a-PE (53-6.7; Sigma-Aldrich), anti-Thy 1.2-FITC (TS; Sigma-Aldrich), anti-CD19-FITC (1D3; BD PharMingen, San Diego, CA), and anti-F4/80-FITC (Caltag Laboratories).

For detection of NK-1R, we used [Sar⁹,Met¹¹]-substance P (SM-SP; Sigma-Aldrich) labeled with Alexa Fluor 594 (10⁻⁹M; Molecular Probes, Eugene, OR) with or without HK (10⁻⁸M) or NK-1R inhibitor SR 140333 (10⁻⁸M) to detect specific binding to NK-1R. SM-SP 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. The cell suspensions were handled and exposed to Alexa-SM-SP as per the procedure for conjugated mAb.

RNA extraction and RT-PCR assay

The total cellular RNA was extracted using guanidinium/acid-phenol (25). The RNA was quantified spectrophotometrically and checked for intact 18S and 28S bands by gel electrophoresis. Also, samples were compared for content of hypoxanthine phosphoribosyltransferase (HPRT) to further confirm equivalent mRNA content and reverse transcription. Reverse transcriptase reactions were performed for 2 h at 42°C using 5 μ g of RNA, 400 U of Moloney murine leukemia virus reverse transcriptase, and 0.5 μ g of 18-mer oligo(dT) for random priming all in a total volume of 40 μ l.

PCR was performed using a Robocycler 40 (Stratagene, Menasha, WI) in a total volume of 50 μ l using 3 U of *Taq*DNA polymerase and a primer pair specific for a 330-bp fragment of mouse PPT C mRNA that spanned over intron 1–3 of mouse *Tac4*. The primers for HK1 PCR were: sense, 5'-AACTG GCTTTTGGTGACAGAG-3' and anti-sense, 5'-AGTGCTACACGTTGCT GGTG-3'. PCR was performed at 1.2 nM Mg²⁺. Temperature conditions

were denaturing at 93°C for 60 s, annealing at 65°C for 85 s, and extension at 72°C for 72 s. Each tube contained 5 μ l each of 2 mM dNTP, 1.5 mM Mg²⁺ in 10 \times PCR buffer, 1.5 U *Taq* DNA polymerase, and 10 pM of both primers in a total volume of 50 μ l. The PCR sequence was 93°C for 60 s to denaturing, 60°C for 80 s to anneal, and 72°C for 80 s to extend.

HPRT primers were: sense, 5'-GTTGGATACAGGCCAGACTTTGT TG-3' and antisense, 5'-GAGGGTAGGCTGGGCTATGGCT-3'. The PCR cycling sequence was 93°C for 60 s to denaturing, 60°C for 80 s to anneal, and 72°C for 80 s to extend. All PCR were repeated for 40 cycles.

Statistical analysis

Data are means \pm SE of multiple determinations. Difference between two groups was compared using Student's *t* test. Values of *p* < 0.05 were considered to be significant.

Results and Discussion

PPT C mRNA is expressed at sites of inflammation

PPT A mRNA encodes for SP, whereas PPT C encodes for HK. LPMC from the intestine of WT mice or LPMC from IL-10^{-/-} animals with or without colitis express PPT A transcripts. Also, granulomas from mice with schistosomiasis contain PPT A mRNA (6). Experiments determined whether LPMC or granuloma cells expressed PPT C mRNA. Also studied were dispersed bone marrow cells, which are a known source of PPT C transcripts (22).

RNA was extracted from freshly dispersed LPMC, liver granulomas, and bone marrow cells. A PCR assay readily detected PPT C transcripts in LPMC RNA from either WT or IL-10^{-/-} animals and in RNA from dispersed schistosome liver granulomas (Fig. 1*A*). The control bone marrow preparation was positive also. The authenticity of the PCR product derived from LPMC and granuloma was confirmed by sequencing (data not shown).

Granulomas are complex inflammatory responses composed of various inflammatory cell types. Previous studies showed that granuloma T cells and macrophages express PPT A mRNA. Therefore, T cells, B cells, and macrophages were isolated from dispersed granulomas to localize the source of the granuloma PPT C transcripts. The cellular preparations were >98% pure as determined by flow analysis. PPT C mRNA was detected in both the T cells and macrophages. B cells were negative. All

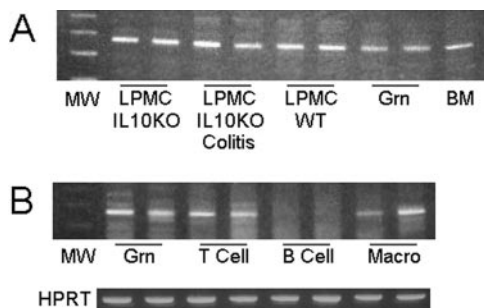


FIGURE 1. *A*, LPMC and granuloma cells express PPT C mRNA. Cellular RNA was extracted from unfractionated LPMC from WT (LPMC WT) or IL-10^{-/-} (LPMC IL-10KO) mice. Some of the IL-10^{-/-} mice had colitis (LPMC IL-10KO colitis). Also extracted was RNA from dispersed granuloma cells (Grn) from livers of schistosome-infected animals. *B*, Granuloma T cell, B cell, and macrophage (Macro) subsets were isolated using paramagnetic beads as described in *Materials and Methods*. Cellular RNA was extracted from these cells for PCR analysis. All samples contained comparable amounts of HPRT housekeeping gene transcripts. Both *A* and *B* show results from two separate experiments. Also shown is a positive bone marrow control (BM) and molecular weight standards (MW).

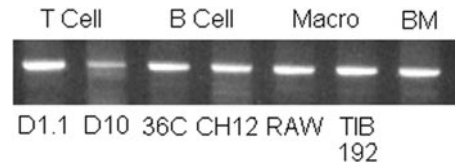


FIGURE 2. Various cell lines express PPT C mRNA. RNA was extracted from the indicated T cell, B cell, and macrophage cell lines and unfractionated bone marrow cells (BM) and analyzed for PPT C transcripts using PCR.

RNA preparations had comparable quantities of the HPRT housekeeping gene, suggesting that the mature B cells expressed little or no PPT C transcripts compared with the other cell types (Fig. 1*B*). Several well-characterized murine T cell and macrophage cell lines readily expressed PPT C mRNA (Fig. 2). Both the D1.1 Th1 cell line and the D10 Th2 cell line tested positive. Immature B cell lines also contained PPT C transcripts. Isolated splenic B cells tested positive also.

HK binds to the granuloma T cell NK-1R

SP binds to granuloma T cells via engagement of NK-1R (15). Cells that express NK-1R can be identified by flow cytometry using fluorescently labeled SM-SP (Alexa-SM-SP). Flow analysis of dispersed granuloma cells showed that ~10% of both the CD4⁺ and CD8⁺ T cells bound Alexa-SM-SP (Fig. 3). SR 140333 is a potent and highly selective inhibitor of NK-1R. This inhibitor displaced Alexa-SM-SP from the T cells showing that the binding was specific for NK-1R (Fig. 3). HK used at equimolar concentration to that of the inhibitor also displaced Alexa-SM-SP with comparable efficiency. This suggested that HK, like SP, binds to the granuloma NK-1R with high affinity.

SP and HK induce IFN- γ production

Soluble Ags from the schistosome egg (SEA) stimulate splenic T cells to produce IFN- γ . SP enhances T cell IFN- γ production

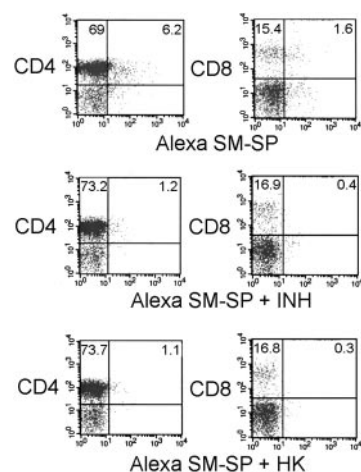


FIGURE 3. HK binds to the granuloma T cell NK-1R. Using flow analysis, Alexa 594 conjugated to the highly selective NK-1R agonist SM-SP (Alexa-SM-SP; 10⁻⁹ M) detects NK-1R on CD4⁺ and CD8⁺ granuloma T cells (*top panels*). *Middle panels* show that the NK-1R inhibitor SR 140333 (10⁻⁸ M) blocks Alexa-SM-SP binding. The *bottom panels* demonstrate that HK (10⁻⁸ M) also prevents Alexa-SM-SP binding. This experiment was repeated three times, yielding similar results. Numbers are the percentage of T cells in the lymphoid gate expressing either CD4⁺ or CD8⁺ and which are either positive or negative for Alexa-SM-SP staining.

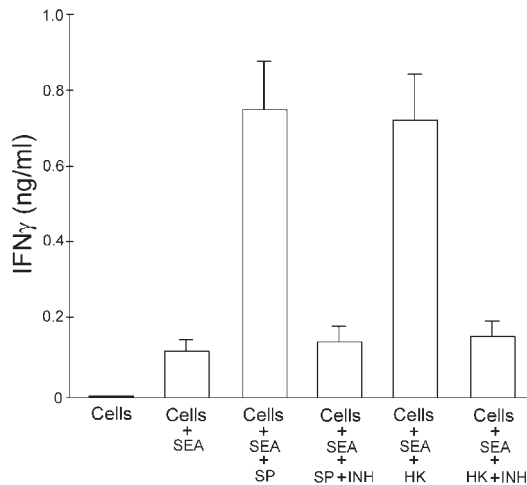


FIGURE 4. Both SP and HK induce IFN- γ secretion from spleen cells. Spleen cells (Cells; 2×10^6 /well) from schistosome-infected mice were cultured in vitro in 200 μ l of medium for 24 h at 37°C in microtiter plates. Some wells contained SEA (0.3 μ g/ml). Also, some cultures contained SP (10^{-9} M) or HK (10^{-9} M) with or without the NK-1R inhibitor SR 140333 (10^{-8} M; INH). IFN- γ was measured in the culture supernatants after the incubation. The data show that SP and HK induce IFN- γ secretion comparably and that SR 140333 blocks this stimulation. Data are means of multiple determinations from three separate experiments \pm SE.

(15). We thus examined the effect of SP and HK on the production of this T cell product. Spleen cells cultured in vitro released no measurable IFN- γ spontaneously. The cells produced small quantities of IFN- γ after SEA stimulation. In the presence of SP or HK, spleen cells released substantially more IFN- γ (>5-fold increase; Fig. 4). HK and SP used concomitantly were not synergistic or antagonistic.

SP engages a T cell NK-1R to regulate IFN- γ secretion (15). The NK-1R inhibitor SR 140333 was used to determine whether HK-induced IFN- γ production was NK-1R dependent. SR 140333 completely blocked the effects of SP and HK on IFN- γ secretion (Fig. 4). The inhibitor did not alter splenic IFN- γ synthesis in the absence of SP or HK. These data suggest that modulation of IFN- γ production by HK is mediated through NK-1R.

The major mammalian tachykinins include SP, HK, neurokinin (NK) A and NKB. There are three distinct tachykinin receptors termed NK-1, NK-2, and NK-3. NKA is the natural ligand for NK-2R, while NKB binds NK-3R with highest affinity. Studies using transfected Chinese hamster ovary cells showed that murine HK is only a weak agonist of NK-2R and NK-3R (26). Therefore, it is not likely that HK used at 10^{-9} M binds NK-2R or NK-3R to affect IFN- γ synthesis in our experimental system. Moreover, neither the granulomas nor the splenocytes have NK-2 or NK-3R mRNA (27). In murine schistosomiasis, NKA and NKB only stimulate IFN- γ production in vitro at 10^{-6} M or higher concentrations (28), and NK-1R but not NK-2R or NK-3R antagonists block this stimulation. Thus, HK regulates T cell IFN- γ production by interaction with NK-1R.

Zhang et al. (22) first described murine PPT C and HK (22). Human PPT C encodes for a peptide with partial homology to murine HK and also for a truncated version (4–11, 26). PCR analysis detected PPT C transcripts in murine bone marrow (22) and in other tissues (i.e., skin, heart) (26), suggesting that it may have several functions in humans and rodents.

In the bone marrow, expression was localized to B cells that were in their early stage of development (22). We detected HK in two immature B cell lines (38C-13 and CH12.LX) and splenic B cells, but not in granuloma B cells. Other studies suggested that HK was an autocrine or paracrine factor, which supports survival and proliferation of early B cells. However, NK-1R $^{-/-}$ mice are not lymphopenic (29), which suggests that HK is not essential for B cell growth. Granuloma B cells are mature and end-stage differentiated. This may explain why HK was not detected in these cells.

In summary, we have examined HK expression and function at a site of chronic inflammation in murine schistosomiasis. Our results show that HK and SP likely have similar origins and functions in the granulomatous response. This expands the immunoregulatory function of HK from a pre-B cell growth factor to a cytokine involved in the control of chronic inflammation. The presence of HK mRNA in intestinal LPMC from healthy and colitic mice suggests that HK also has immune regulatory functions in other inflammatory states.

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