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SerpinB2 is Critical to Th2 Immunity against Enteric Nematode Infection

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SerpinB2, a member of the serine protease inhibitor family, is expressed by macrophages and is significantly upregulated by inflammation. Recent studies implicated a role for SerpinB2 in the control of Th1 and Th2 immune responses, but the mechanisms of these effects are unknown. In this study, we used mice deficient in SerpinB2 (SerpinB2<sup>−/−</sup>) to investigate its role in the host response to the enteric nematode, Heligmosomoides bakeri. Nematode infection induced a STAT6-dependent increase in intestinal SerpinB2 expression. The <i>H. bakeri</i>-induced upregulation of IL-4 and IL-13 expression was attenuated in SerpinB2<sup>−/−</sup> mice coincident with an impaired worm clearance. In addition, lack of SerpinB2 in mice resulted in a loss of the <i>H. bakeri</i>-induced smooth muscle hypercontractility and a significant delay in infection-induced increase in mucosal permeability. Th2 immunity is generally linked to a CCL2-mediated increase in the infiltration of macrophages that develop into the alternatively activated phenotype (M2). In <i>H. bakeri</i>-infected SerpinB2<sup>−/−</sup> mice, there was an impaired infiltration and alternative activation of macrophages accompanied by a decrease in the intestinal CCL2 expression. Studies in macrophages isolated from SerpinB2<sup>−/−</sup> mice showed a reduced CCL2 expression, but normal M2 development, in response to stimulation of Th2 cytokines. These data demonstrate that the immune regulation of SerpinB2 expression plays a critical role in the development of Th2-mediated protective immunity against nematode infection by a mechanism involving CCL2 production and macrophage infiltration.  

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mediated regulation of SerpinB2 expression during nematode infection; 2) the contribution of SerpinB2 in host Th2 protective immunity against *H. bakeri*; 3) the role of SerpinB2 in the *H. bakeri* infection–induced alterations in intestinal smooth muscle and epithelial cell function; and 4) the mechanisms by which SerpinB2 regulates host protective immunity against a gastrointestinal nematode infection. This study showed that SerpinB2 plays a critical role in the host protective immunity against nematode infection via a control of monocyte recruitment that impacts Th2 cytokine responses.

**Materials and Methods**

**Mice**

C57BL/6 wild-type (WT) mice were purchased from the Small Animal Division of the National Cancer Institute or The Jackson Laboratory (Bar Harbor, ME). Mice deficient in SerpinB2 (SerpinB2<sup>−/−</sup>) backcrossed six times onto a C57BL/6 background were obtained from Dr. David Ginsburg (University of Michigan Medical School, Ann Arbor, MI) and were then further backcrossed for these studies a total of 12 times onto the C57BL/6 background. Mice deficient in STAT6 (STAT6<sup>−/−</sup>) were obtained from The Jackson Laboratory. These studies were conducted in accordance with principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Health and Human Services Publication (National Institutes of Health 85-23, revised 1996), Beltsville Area Animal Care and Use Committee (#07-003), and of University of Michigan School of Medicine Institutional Animal Care and Use Committee.

**Enteric nematode infection and worm expulsion**

*H. bakeri* infection with third-stage larvae (L<sub>3</sub>; specimens on file at the U.S. National Parasite Collection, U.S. National Helminthological Collection, Collection 81930, Beltsville, MD) was described previously (4, 13). Infective L<sub>3</sub> of *Nippostrongylus brasiliensis* were propagated and stored at room temperature in fecal/charcoal/peat moss culture plates until use (4). Groups of mice were inoculated s.c. with 500 L<sub>3</sub> and studied 10 d later. The timing of the studies postinfection (p.i.) with *N. brasiliensis* correlated with the time of the maximal effects on gut function and coincided with worm expulsion (4). Appropriate age-matched controls were performed for each infection. Adult worms were detected quantitatively by scanning the intestinal sections for the presence of worms (4). Appropriate age-matched controls were performed for each infection. Adult worms were detected quantitatively by scanning the intestinal sections for the presence of worms (4).

In *vitro smooth muscle and epithelial cell function*

In *vitro* smooth muscle contractility was measured as described previously (4). Smooth muscle responses to electric field stimulation (20 Hz, 100V) or acetylcholine (10 nM to 0.1 mM) and the amplitude of spontaneous contractions were determined. Tension was expressed as force per cross-sectional area (14). For in *vitro* epithelial cell ion transport in *Ussing* chambers, muscle-free segments of small intestine were mounted in *Ussing* chambers as described previously (15). After a 15-min period, concentration-dependent changes in short-circuit current were determined for the cumulative addition of acetylcholine to the serosal side or glucose to the mucosal side. Responses from all tissue segments exposed to acetylcholine from an individual animal were averaged to yield a mean response per animal.

**Microsnap well assay for mucosal transepithelial electrical resistance**

The modified microsnap well system is a miniaturized version of the standard *Ussing* chamber that has been engineered to measure mucosal transepithelial electrical resistance (TEER) (16) where a decrease in TEER reflects increased tissue permeability. In brief, segments of mouse intestine taken from control or *H. bakeri*-infected WT and SerpinB2<sup>−/−</sup> mice were stripped of both muscle and serosal layers, and placed in the microsnap well system. A total of 250 μl MEM medium (Thermo) pre-equilibrated at 37°C was collected overnight in alpha MEM containing 10% FBS and 1% penicillin/streptomycin in humidified incubator at 37°C with 5% CO<sub>2</sub>. The nonadherent cells were collected by centrifugation after lysis of RBCs using lysis buffer (Sigma), and mononuclear cells were counted. Mature macrophages were generated by differentiating the isolated mononuclear cells with 20 ng/ml R-ML-C57 (R&D Systems, Minneapolis, MN) for 7 d. Cells were then treated with IL-4, IL-13, or LPS for 24 h to determine the ability of macrophages to differentiate into classically (M1) or alternatively activated (M2) phenotype.

**CCL2 elicited peritoneal monocyte recruitment and preparation of peritoneal exudate cells**

Mice were injected i.p. with 10 μg CCL2 in 200 μl NaCl (0.9%, LPB-free) and euthanized at 18 h after injection as described previously (18). The peritoneal cavity was washed twice with 8 ml PBS, and the number of peritoneal exudate cells (PECs) was counted from the total lavage pooled from individual mice. The percentage of macrophages in PECs was determined by counting the macrophages on PEC smear prepared by cytopsin and stained with Giemsa.

**RNA extraction, cDNA synthesis, and real-time quantitative PCR**

Total RNA was extracted from whole intestinal tissue or bone marrow–derived macrophages (BMDMs) as described previously (19). RNA samples (2 μg) were reverse-transcribed to cDNA using random hexamer primers and RevertAid reverse transcriptase. Real-time quantitative PCR (qPCR) was performed using an iCycler detection system (Bio-Rad, Hercules, CA) as described previously (20). Primers for qPCR were designed using Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA) and synthesized by Sigma. The primer sequences are SerpinB2: 5'-ACTTAA-TGGGCTTATCTCTTCC-3' (sense), 5'-TGCGCTCCTATGATGTTCTAC-3' (antisense), and CCL2: 5'-TTTGAATGTGAAGTTGACCCGTAAATC-3' (sense), 5'-GAAGTGTCTGTTGAGTGGTTGG-3' (antisense). Primer sequences for other genes have been described previously (5).

**Immunofluorescence staining**

Frozen blocks of midjejunum were prepared using the Swiss-roll technique and stored at −80°C. Tissue sections (4 μm) were cut from frozen blocks using an HM505E cryostat (Richard-Allan Scientific, Kalamazoo, MI). For immunofluorescence staining, tissue slides were fixed in cold acetone for 30 min and blocked with 5% normal donkey serum in PBS for 1 h at room temperature. The slides were then incubated with rat anti-F4/80 Ab (1:50; BioLegend, San Diego, CA) and goat anti-mouse CCL2 (1:40; BioLegend, San Diego, CA) overnight at 4°C. After being washed, the slides were stained with Dylight 488-Donkey anti-rat IgG and Dylight 649-Donkey anti-goat IgG (1:400; Jackson ImmunoResearch, West Grove, PA) for 2 h and then digitally photographed with a Nikon TE 2000-E microscope (Melville, NY) using MetaVue version 6.1 software (Universal Imaging Corporation, Downingtown, PA). The images were taken by establishing settings for the samples from individual vehicle groups and using the same conditions to evaluate the samples from infected groups. Comparisons were made only among slides prepared on the same day. Smooth muscle thickness was determined using Giemsa-stained sections for each treatment group.

**Western blot and ELISA**

Tissue lysates were prepared by homogenizing the intestine strips in T-PER (Thermo Scientific Pierce, Rockford, IL) with protease inhibitor mixture. Proteins were separated on 4–12% Bis-Tris NuPage gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk in PBST and then incubated with primary Abs overnight. Affinity purified rabbit anti-mouse SerpinB2 Ab was prepared after immunization with a purified recombinant GST-murine SerpinB2 fusion protein produced in *Escherichia coli* as described previously (21). The Western blot was reprobed with anti-GAPDH (Cell Signaling Technology) as a loading control. Immunoreactive bands were detected by HRP-conjugated secondary Abs using standard techniques. In situ intestinal production of IL-13 was analyzed by ELISA in tissue lysates per manufacturer’s instruction (eBioscience, San Diego, CA).

**Data analysis**

Agnostic responses were fitted to sigmoid curves (GraphPad, San Diego, CA). Statistical analysis was performed using one-way ANOVA followed...
by Bonferroni tests to compare the responses and gene expression among the different treatment groups.

**Results**

*Enteric nematode infection upregulates SerpinB2 expression in the intestine*

SerpinB2 expression is upregulated in response to microbe infection or inflammation, and is implicated in regulation of the Th1 cytokine response (9–11). To determine whether SerpinB2 also plays a role in Th2 immunity, we used enteric nematode infection in mice, a well-characterized model featuring a highly polarized Th2 cytokine response that is essential for host defense. We first infected groups of mice with *H. bakeri* to analyze the kinetics of SerpinB2 gene expression in the small intestine. *H. bakeri* is a strictly enteral infection with larvae developing first in the submucosa of the duodenum followed by release of adult worms into the lumen around day 8 after inoculation. This infection significantly upregulated SerpinB2 mRNA expression p.i., with the highest levels observed at day 4 (Fig. 1A), a time when the L3 encyst in the intestinal mucosa and persistent high levels of SerpinB2 are present during the entire course of infection. The infection-induced increase in SerpinB2 expression was further confirmed by Western blot, as the specific 46-kDa SerpinB2 protein band was significantly stronger in samples from infected versus uninfected WT mice and was not seen in samples from SerpinB2^−/−^ mice (Fig. 1B). Notably, *H. bakeri* infection did not affect SerpinB2 expression in mice with STAT6 deficiency (Fig. 1A), demonstrating that the increased expression of SerpinB2 in WT mice is dependent on IL-4/IL-13. Moreover, this infection-induced upregulation of SerpinB2 was not specific to *H. bakeri* because *N. brasiliensis*, a nematode that migrates parenterally before adults develop in the intestinal lumen, also increased the expression of SerpinB2 in the small intestine of WT, but not STAT6^−/−^, mice (Fig. 1C). The infection-induced elevation of SerpinB2 expression was independent of the genetic background of mouse because similar effects were detected in both BALB/c and C57BL/6 mice (data not shown).

**Worm expulsion was impaired in mice with SerpinB2 deficiency**

As described earlier, *H. bakeri* infection induced upregulation of SerpinB2 expression early in the p.i. period and proceeded the peak of Th2 cytokine response, implicating a role of SerpinB2 in host protective immunity against the infection. To explore this further, we monitored worm expulsion during the memory response to a secondary infection with *H. bakeri*. In general, C56BL/6 WT mice cleared adult worms by day 14 after a secondary inoculation of *H. bakeri*. At 10 d p.i. (DPI), WT and SerpinB2^−/−^ mice harbored a significant, yet comparable number of adult worms in the intestinal lumen (data not shown), indicating that SerpinB2 deficiency did not alter the development of larvae into adult worms. There were no adult worms present in WT mice at 14 DPI; however, there were significant numbers of worms remaining in the small intestine of SerpinB2^−/−^ mice consistent with impaired worm expulsion (Fig. 2A).

**Defective Th2 cytokine response in mice deficient in SerpinB2 during nematode infection**

Th2 cytokines, especially IL-4 and IL-13, are the major effector molecules in host protective immunity against nematode infection. To dissect the underlying mechanisms by which SerpinB2 deficiency impairs worm expulsion, we analyzed the kinetics of Th2 cytokine expression in the intestine post-*H. bakeri* infection. The mRNA expression of IL-13 or IL-4 in WT mice was elevated significantly at 4 DPI, peaked at 10 DPI, and remained elevated at 14 DPI even after worm expulsion (Fig. 2B, 2C). The level of IL-13 expression in infected SerpinB2^−/−^ mice was significantly lower than that in infected WT mice at all time points examined, whereas the expression of IL-4 was increased transiently on day 7 and was significantly lower than WT at all other time points. Correspondingly, in situ intestinal IL-13 production in infected SerpinB2^−/−^ mice was considerably lower than that of WT mice at 7, 10, and 14 DPI (Fig. 2D). Increased levels of IL-13 upregulate expression of IL-13Rα2, the decoy receptor for IL-13, through its binding to IL-13Rα1 that, with the IL-4Rα, is part of the type II IL-4R that activates STAT6 (19). Expression of IL-

![FIGURE 1.](http://www.jimmunol.org/Downloadedfrom)
were relative to the WT-vehicle (WT-VEH) groups after normalization to 18S rRNA. (D) with *H. bakeri* mRNA expression of (E) SerpinB2 compared smooth muscle and epithelial cell function in WT and nematode infection and facilitate worm clearance; therefore, we Changes in intestinal function are a hallmark feature of enteric infection in mice deficient in SerpinB2. WT or SerpinB2−/− mice were infected with H. bakeri. (A) Numbers of adult worms in the lumen of small intestine of C57BL/6 mice (WT) mice at 14 DPI. qPCR was performed to measure the mRNA expression of (B) IL-13, (C) IL-4, (E) IL-13Rα2, (F) IL-25, and (G) IFN-γ in the small intestine of mice at 4, 7, 10, and 14 DPI. The fold changes were relative to the WT-vehicle (WT-VEH) groups after normalization to 18S rRNA. (D) ELISA analysis of the in situ IL-13 production of the intestines.

### SerpinB2 AND TH2 IMMUNITY

**FIGURE 2.** Impaired Th2 protective immunity against H. bakeri (Hb) infection in mice deficient in SerpinB2. WT or SerpinB2−/− mice were infected with H. bakeri. (A) Numbers of adult worms in the lumen of small intestine of C57BL/6 mice (WT) mice at 14 DPI. qPCR was performed to measure the mRNA expression of (B) IL-13, (C) IL-4, (E) IL-13Rα2, (F) IL-25, and (G) IFN-γ in the small intestine of mice at 4, 7, 10, and 14 DPI. The fold changes were relative to the WT-vehicle (WT-VEH) groups after normalization to 18S rRNA. (D) ELISA analysis of the in situ IL-13 production of the intestines.

13Rα2 was increased significantly by infection in both WT and SerpinB2−/− mice, but levels were reduced significantly in SerpinB2−/− mice at all times points p.i. corresponding to the decreased IL-13 expression (Fig. 2E). IL-25 is an epithelial-derived cytokine that plays a key role in Th2 immunity. We showed previously that upregulation of IL-25 in nematode infection is dependent on IL-13 and STAT6 (20). Of interest is that *H. bakeri* infection upregulated IL-25 mRNA expression in WT, but not in SerpinB2−/− mice, indicating a role for SerpinB2 in the IL-13–driven epithelial cell response to nematode infection (Fig. 2F). Taken together, these data support a role for SerpinB2 in the full development of a Th2 immune response.

A defect in Th2 cytokine response may lead to an exaggerated Th1 cytokine response as described previously in STAT6−/− mice infected with N. brasiliensis (5). To determine whether this occurred during *H. bakeri* infection in SerpinB2−/− mice, we further analyzed the expression of Th1 and Th17 cytokines in the intestine. IFN-γ was slightly, but significantly, upregulated at day 14 post–H. bakeri infection in both WT and SerpinB2−/− mice (Fig. 2G), but other major Th1/Th17 cytokines including IL-17A, IL-1β, IL-12a, IL-12b, IL-23a, and TNF-α were not significantly altered by infection in either strain (data not shown). These data indicated that the impaired Th2 immune response due to SerpinB2 deficiency did not result in an elevated proinflammatory Th1 or Th17 response.

**Attenuated intestinal smooth muscle and epithelial function in response to H. bakeri infection in mice deficient in SerpinB2**

Changes in intestinal function are a hallmark feature of enteric nematode infection and facilitate worm clearance; therefore, we compared smooth muscle and epithelial cell function in WT and SerpinB2−/− mice. Based on the results from time-course experiments, we selected 14 DPI as the time point for comparison when infection-induced smooth muscle hypercontractility is maximal in WT mice. As expected, *H. bakeri* infection in WT mice induced a characteristic smooth muscle hypercontractility of the intestine including elevated responses to acetylcholine (Fig. 3A) and electric field stimulation (Fig. 3B), as well as an increase in the amplitude of spontaneous contractions (Fig. 3C). Notably, this smooth muscle hypercontractile response was absent in the infected SerpinB2−/− mice (Fig. 3A–C). Intestinal smooth muscle hypertrophy/hyperplasia is often associated with enteric nematode infection and is attributed to the increased production of macrophage-derived growth factors. There was a significant increase in smooth muscle thickness in *H. bakeri*–infected WT mice, but not in infected SerpinB2−/− mice (Fig. 3D).

Other characteristics of the intestinal functional response to nematode infection are decreased glucose absorption, hyposecretion to acetylcholine, decreased net ion flux, and increased mucosal permeability (3, 15, 22). There were no differences in the basal short-circuit current across the tissue or in the intestinal mucosal responses of WT or SerpinB2−/− mice to glucose or acetylcholine (data not shown). In contrast, during the course of *H. bakeri* infection, there was a gradual decrease in TEER at both 7 and 10 DPI in WT mice, but not in SerpinB2−/− mice (Fig. 3E). By 14 DPI, however, TEER was similarly low in both strains of mice. These data indicate a specific role for SerpinB2 in the initiation of infection-induced changes in mucosal permeability.

**Nematode infection failed to recruit monocytes to the intestine of mice with SerpinB2 deficiency**

During enteric nematode infection, monocytes are recruited to the intestine and differentiated into macrophages that play a crucial role in host protective immunity (2, 9). To determine whether the impaired host immunity to nematode infection in SerpinB2−/− mice is associated with changes in the number/function of macrophages, we monitored various macrophage markers in whole intestinal tissue. The constitutive expression level of F4/80,
a general marker for macrophages, was not different in WT and SerpinB2−/− intestine, suggesting that the number of resident macrophages were similar in the two strains of mice. The mRNA expression of F4/80 in WT intestine was upregulated transiently at day 4 PI, but declined thereafter (Fig. 4A). H. Bakeri infection, however, did not induce significant changes in F4/80 expression in SerpinB2−/− mice, suggesting a failure to recruit monocytes to the site of infection. This defect in monocyte recruitment was further confirmed by immunofluorescence staining of intestinal sections (Fig. 4B). Consistent with our previous results (5), H. Bakeri infection increased the number of M2 macrophages in WT mice, as evidenced by the significant upregulation of arginase-1 and CD206 (Fig. 4C, 4D). SerpinB2 deficiency resulted in a severely attenuated expression of arginase-1 and CD206 (Fig. 4C, 4D) consistent with impaired infiltration. The reduced M2 development in the intestine did not result in a more pronounced M1 response because the expression of NO synthase 2 (NOS-2), a marker for M1, did not differ between the infected SerpinB2−/− and WT mice (data not shown).

CCL2 is the major chemoattractant protein responsible for monocyte recruitment during nematode infections (23). H. Bakeri significantly upregulated CCL2 expression in WT intestine at 4 DPI, and this increase was maintained through 14 DPI (Fig. 5A). In contrast, CCL2 expression was increased significantly in SerpinB2−/− mice only at 7 DPI, and levels were significantly lower than WT at all other time points (Fig. 5A). Reduced CCL2 production in SerpinB2−/− mice was further confirmed by immunofluorescence staining of intestinal sections from mice at 4 DPI (Fig. 5B). These data suggested that an insufficient production of CCL2 might be one of the factors responsible for the impaired monocyte recruitment to the intestine of SerpinB2−/− mice during H. Bakeri infection. Because CCL2 signals the recruitment of monocytes to the site of infection by binding the receptor, CCR2, we compared CCR2 expression levels in BMDMs and macrophage-enriched PECs derived from WT and SerpinB2−/− mice. qPCR showed no significant differences in the CCR2 expression in either BMDMs or PECs taken from WT and SerpinB2−/− mice. qPCR showed no significant differences in the CCR2 expression in either BMDMs or PECs taken from WT and SerpinB2−/− mice (Fig. 5B). In response to i.p. injection of CCL2, however, the number of monocytes recruited to the peritoneal cavity and differentiated into macrophages in SerpinB2−/− mice was significantly less than that in WT mice (Fig. 5C), indicating an intrinsic defect in the macrophage response to CCL2 stimulation caused by SerpinB2 deficiency.

SerpinB2 deficiency did not alter the abilities of macrophages to become M1 or M2 in response to the respective stimuli in vitro

SerpinB2 has been linked to monocyte proliferation and differentiation, and prevention of macrophage apoptosis induced by
inflammatory stimuli such as LPS and TNF-α (9, 11). It is possible, therefore, that the attenuated monocyte recruitment and development of the intestinal M2 macrophages in SerpinB2−/− mice resulted from a defect in macrophage survival or the ability of macrophages from SerpinB2−/− mice to differentiate into M1 or M2 phenotype. However, stimulation of BMDMs from WT or SerpinB2−/− mice with IL-4 or IL-13 resulted in a similar up-regulation of arginase-1 (Fig. 6A) and CD206 by qPCR. The fold changes are relative to the WT-vehicle (WT-VEH) groups after normalization to 18s rRNA. (B) Tissue sections of intestine were cut from frozen blocks collected at 4 DPI, and stained with anti-F4/80 (blue) and anti-CCL2 (red) for visualizing macrophages and CCL2 production, respectively. The images are representative from five mice per group. Original magnification ×100. *p < 0.05 versus the respective vehicle (VEH), **p < 0.05 versus the respective WT (n ≥ 5 for each group).

Discussion
In this study, we demonstrated that SerpinB2 plays a critical role in host protective immunity against enteric nematode infection. The impaired worm expulsion after inoculation with H. bakeri in SerpinB2−/− mice was associated with a reduced Th2 cytokine response and a defect in monocyte recruitment and activation in the intestine. In addition, the infection-induced intestinal smooth muscle hypercontractility and mucosal permeability, functions that contribute to worm expulsion (4, 5), were absent or attenuated in SerpinB2−/− mice. Although SerpinB2 deficiency did not affect the ability of macrophages to differentiate into M1 or M2 phenotype in vitro, IL-4-stimulated CCL2 expression was significantly less in SerpinB2−/− macrophages and may contribute to the failure of monocyte recruitment in SerpinB2−/− mice infected with H. bakeri. Moreover, SerpinB2 deficiency resulted in a defective monocyte recruitment elicited by CCL2. These results revealed a previously unrecognized role for SerpinB2 in the
duction and maintenance of Th2 immunity against enteric nematode infection.

SerpinB2 is a single-chain protein with 415 aa and exists predominantly as a 47-kDa nonglycosylated intracellular form. There is also a small portion of SerpinB2 that can enter the secretory pathway and be released as a 60-kDa glycosylated protein (reviewed in Ref. 24). Although upregulation of the uPA system is associated generally with the transition from mucosa to adenoma in colorectal cancers (25), increased levels of SerpinB2 are associated also with prolonged survival and decreased tumor growth and metastasis (25, 26). The biological activities of the extracellular form of SerpinB2 are linked to inhibition of the plasminogen activators uPA and tPA; however, the functional role of intracellular SerpinB2 remains unclear and is thought not to be related to these protease inhibitory activities. Expression of SerpinB2 in the healthy gastrointestinal tract is considered to be limited to macrophages, although there is evidence for induction of SerpinB2 in response to H. pylori infection in chief and mucous cells, as well as in metastatic tissue and transformed intestinal cancer cell lines (27).

As a highly regulated gene, SerpinB2 expression is modulated at multiple levels by different factors ranging from cytokines, hormones, growth factors, toxins, and bacterial products. In this study, enteric nematode infection induced a significant STAT6-dependent upregulation of SerpinB2 expression in the small intestine of WT mice. Thus, SerpinB2 can be added to the list of IL-4/IL-13–dependent genes that play a key role in the Th2 immune response. Abnormal expression of SerpinB2 is associated with a number of Th1 dominant inflammatory pathologies including diabetes mellitus (28) and colorectal cancer (26), as well as in Th2-dominant diseases such as asthma (29). Its role in inflammation in the gastrointestinal tract, however, is relatively unexplored. SerpinB2−/− mice showed an exaggerated Th1 cytokine response after immunization with OVA in CFA, suggesting that a general function of SerpinB2 is the control of proinflammatory Th1-mediated immune responses (11). In addition, infection of SerpinB2−/− mice with the

FIGURE 5. Attenuated upregulation of CCL2 expression in the intestines in response to H. bakeri infection and a defect of monocyte recruitment in response to CCL2 stimulation in mice with SerpinB2 deficiency. (A) Segments of small intestine were collected at days 4, 7, 10, and 14 post-H. bakeri infection (DPI) for analyzing mRNA expression of CCL2 by qPCR. The fold changes were relative to the WT-vehicle (WT-VEH) groups after normalization to 18s rRNA. *p < 0.05 versus the respective VEH; †p < 0.05 versus the respective WT (n ≥ 5 for each group). (B) BMDMs or enriched PECs were prepared from WT or SerpinB2−/− mice and analyzed for CCR2 expression by qPCR. (C) WT or SerpinB2−/− mice were injected with CCL2, and peritoneal lavages were collected at 18 h after injection for macrophage counting. n = 4 per group; *p < 0.05.

FIGURE 6. In vitro macrophage production of CCL2, but not the development of M1 or M2 phenotype, was impaired by SerpinB2 deficiency. Macrophages were generated from bone marrow mononuclear (BMDM) cells of WT or SerpinB2−/− mice. Cells were treated with IL-4, IL-13, or LPS for 24 h and analyzed for mRNA expression of (A) arginase-1, (B) NOS-2, or (C) CCL2 by qPCR. The fold changes are calculated relative to the WT-BMDM-VEH after normalization to 18S rRNA. *p < 0.05, **p < 0.01 versus the respective VEH; †p < 0.05 versus the respective WT-BMDM. Data shown in bar graphs are the mean ± SEM and are representative of two independent experiments performed in triplicate.
Th2-inducing trematode, *Schistosoma japonicum*, resulted in diminished hepatic Th2 cytokine responses associated with low levels of IL-13 and arginase-1 expression, but increased levels of the Th1 cytokines IL-6, TNF-α, and NOS-2 (10). Our studies in *H. bakeri*-infected mice indicated that SerpinB2 is important for host protective immunity evidenced by delayed worm expulsion and a significantly attenuated Th2 cytokine response. This occurred in the absence of compensatory changes in the expression of proinflammatory Th1 or Th17 cytokines observed in other systems (10) and may be because of differences either in the pathogen (*Spiraea japonica* versus *H. bakeri*) or in the affected region (liver versus small intestine).

Nematode infection induces stereotypic STAT6-dependent changes in epithelial function leading to increased intraluminal fluid, as well as smooth muscle hypercontractility, all of which promote worm expulsion (3, 4). The infection-induced increase in permeability observed in WT mice was delayed significantly in SerpinB2-/- mice, yet TEER was similar in both strains at 14 DPI, the time of worm expulsion. Changes in permeability are linked to both worm-generated products and STAT6-dependent gene transcription (12). The deferred increase in barrier function in the absence of SerpinB2, therefore, was likely attributed to decreased Th2 cytokines and STAT6 gene transcription. Surprisingly, there were no differences in the infection-induced inhibition of epithelial secretion or glucose absorption between WT and SerpinB2-/- mice, suggesting that SerpinB2 lacks a direct effect on epithelial cell function. In contrast, the infection-induced increase in smooth muscle hypercontractility was absent in SerpinB2-/- mice, indicating a major role for SerpinB2. We showed previously that p.i. hypercontractility was due to a direct effect of IL-13/STAT6-dependent transcription of genes involved in smooth muscle function, as well as an indirect effect mediated by macrophages (4, 5, 19, 30, 31). The absence of the smooth muscle responses to adult *H. bakeri* in SerpinB2-/- mice may be because of impaired monocyte recruitment, function, or both.

We found that *H. bakeri* infection resulted in an early increase in CCL2 expression (4 DPI) that is important for recruitment of monocytes to the site of infection and also coincides with the early elevation in tissue macrophage expression of F4/80. These recruited monocytes develop into the M2 macrophages that are essential to Th2 host protective immunity. Because macrophages are the primary cells that express SerpinB2 in the intestine, it is likely that the infection-induced upregulation of SerpinB2 is attributed to newly recruited and differentiated macrophages. M2 macrophages are characterized by a STAT6-dependent increase in the expression of arginase-1 and CD206 (5, 32), and are emerging as an important source of IL-13 during infection (33). The M2 phenotype is also critical for the smooth muscle hypercontractility and hypertrophy mediated by arginase-1 activity, as well as growth factors such as IGF-1 (5). In SerpinB2-/- mice, *H. bakeri* infection failed to increase the number of macrophages and, therefore, resulted in fewer M2 macrophages in the SerpinB2-/- intestine. Consequently, these mice were unable to develop the changes in smooth muscle morphology and function required for efficient worm expulsion.

The lowered infiltration of macrophages in the small intestine in SerpinB2-/- mice could be because of impaired generation of chemokines or a defect in the macrophage itself. Resident intestinal macrophages have a specific nonreactive tolerogenic phenotype. In response to nematode infection, recruitment of monocytes relies on the chemoattractant proteins produced by intestinal mucosal epithelial cells or lamina propria immune cells, mostly resident macrophages. CCL2 is the major chemokine for increased monocyte infiltration in mice during infection, and elevated expression inhibits macrophage differentiation into nonreactive tissue macrophages. These data suggest that during infection, SerpinB2 functions to maintain CCL2 expression, thereby preventing the development of the newly recruited monocytes into the tolerogenic macrophage phenotype (34) and allowing them to become M2. Indeed, mice with CCL2 deficiency had fewer macrophages in the intestine and mesenteric lymph nodes in response to infection with *Trichuris muris*, a cecal and proximal colon dwelling nematode, and failed to expel these worms (23). In this study, *H. bakeri*-induced upregulation of CCL2 was reduced markedly in SerpinB2-/- mice, implicating insufficient production of CCL2 as one of the mechanisms for the impaired protective immune response in these mice. Interestingly, the majority of the CCL2 staining was not colocalized with F4/80 staining cells. As a secreted protein, CCL2 is released rapidly from the producing cells soon after they sense the danger signal (*H. bakeri* infection in this case), which may explain why much of the CCL2 staining was located in the intracellular space. The proximity of CCL2 staining to F4/80+ cells, however, suggests that it is released from macrophages. It is also true that cells other than macrophages produce CCL2 as indicated by the staining in submucosal area and myenteric plexus. The ability of SerpinB2 deficiency to impact CCL2 production in cells other than macrophages or during other types of infection remains to be elucidated. Additional experiments demonstrated that a significantly lower number of monocytes were recruited to peritoneal cavity of SerpinB2-/- relative to WT mice after administration of CCL2. Notably, loss of SerpinB2 function did not alter the CCR2 expression in macrophages. It remains to be determined whether SerpinB2 is involved in regulating the intracellular signaling of CCL2 that might contribute to the intrinsic defect of macrophage response to CCL2.

An alternate explanation for the decreased number of macrophages in SerpinB2-/- mice may be the loss of the protective effects of SerpinB2 on macrophage survival (9). SerpinB2 deficiency renders macrophages more susceptible to apoptosis induced by LPS and TNF-α (9, 11). In addition, the attenuated Th2 cytokine production in response to *H. bakeri* in SerpinB2-/- mice similarly impacts M2 development. In vitro experiments, however, revealed that SerpinB2-/- macrophages have normal development into M1 in response to LPS or into M2 in response to IL-4 or IL-13. Of interest was that IL-4-stimulated CCL2 expression was significantly less in SerpinB2-/- macrophages than in WT macrophages. These data indicate that SerpinB2 is important for macrophage production of CCL2, and this likely contributes to the decreased recruitment of monocytes to the small intestine in response to *H. bakeri* infection in vivo.

In conclusion, mice deficient in SerpinB2 exhibit an attenuated upregulation of Th2 cytokines in response to *H. bakeri* infection. This effect was associated with an absence of macrophage infiltration that can be attributed to a decreased expression of CCL2 demonstrated both in vivo and in vitro, as well as a defect in CCL2-elicited monocyte recruitment. There is an emerging role for macrophages as a source of IL-4/IL-13 cytokines (33). SerpinB2-/- mice exhibited a reduced expression of intestinal M2 markers in vivo, and this is a likely mechanism for the lack of smooth muscle responses to infection. The lack of M2 markers in SerpinB2-/- mice in vivo was not an inherent defect in the macrophage because isolated BMDMs from WT and SerpinB2-/- mice had a similar upregulation of arginase-1 expression in response to IL-4 or IL-13 and NOS-2 expression in response to LPS. These studies show that the immune regulation of SerpinB2 expression plays an important role in the development of the Th2 protective host immunity against gastrointestinal parasitic nematodes.
Disclosures
The authors have no financial conflicts of interest.

References