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DOWNREGULATION OF THE SYK SIGNALLING PATHWAY IN INTESTINAL DENDRITIC CELLS IS SUFFICIENT TO INDUCE DENDRITIC CELLS THAT INHIBIT COLITIS

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Abstract

Helminthic infections modulate host immunity and may protect people in less developed countries from developing immunological diseases. In a murine colitis model, the helminth *Heligmosomoides polygyrus bakeri* (*Hpb*) prevents colitis via induction of regulatory dendritic cells (DCs). The mechanism driving the development of these regulatory DCs is unexplored. There is decreased expression of the intracellular signaling pathway spleen tyrosine kinase (Syk) in intestinal DCs from *Hp*- infected mice. To explore the importance of this observation, it was shown that intestinal DCs from DC-specific Syk $^{-/-}$ mice were powerful inhibitors of murine colitis suggesting that loss of Syk was sufficient to convert these cells into their regulatory phenotype. DCs sense gut flora and damaged epithelium via expression of C-type lectin receptors many of which signal through the Syk signaling pathway. It was observed that gut DCs express mRNA encoding for CLEC7A, 9A, 12A and 4N. *Hpb* infection down modulated CLEC mRNA expression in these cells. Focusing on CLEC7A, which encodes for the dectin-1 receptor, flow analysis showed that *Hpb* decreases dectin-1 display on the intestinal DC subsets that drive Th1/Th17 development. DCs become unresponsive to the dectin-1 agonist curdlan and fail to

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phosphorylate Syk after agonist stimulation. Soluble worm products can block CLEC7A and Syk mRNA expression in gut DCs from uninfected mice after a brief *in vitro* exposure. Thus, down-modulation of Syk expression and phosphorylation in intestinal DCs could be an important mechanism through which helminths induce regulatory DCs that limit colitis.

Keywords

helminths; C-type lectin receptors; T cells; colitis; Syk

INTRODUCTION

Many diseases caused by immune dysregulation are frequent in developed nations, but uncommon in less developed countries. Helminths are worm-like parasitic organisms that frequently infect humans in geographic regions with low prevalence of these diseases. Helminthic infections are strong inducers of immune-regulatory circuits. This suggests that loss of helminthic infections in developed countries due to improved sanitation and a clean food supply could be one of the factors promoting the rise in immune-mediated diseases like inflammatory bowel disease (IBD). Several clinical and epidemiologic studies support this concept (1).

Animal models of colitis have shown that helminths modulate intestinal inflammation through enhancement of immune regulation. Cells implemented in this regulation include Tregs (2–4), macrophages (5–9) and dendritic cells (DCs) (10,11).

Alterations in DC function may be particularly important as demonstrated in a Rag/T cell transfer model of IBD. T and B cell-deficient Rag mice reconstituted with $IL10^{-/-}$ T cells develop colitis. Rag mice infected with *Heligmosomoides polygyrus bakeri (Hpb)* before $IL10^{-/-}$ T cell reconstitution are protected from the disease (10). The mechanism underlying this protection involves induction of regulatory DCs in the intestinal mucosa (10). Compared to DCs from uninfected animals, intestinal DCs isolated after *Hpb* infection only weakly support antigen-driven, IFNγ and IL17 secretion. Furthermore, DCs isolated from the intestines of *Hpb*-infected Rag mice transferred into colitis-susceptible mice block colitis (11). How these regulatory DCs quell colitis is only partly characterized (11).

DCs are the critical link between innate and adaptive immunity. They sample intestinal luminal contents and present antigens to T cells inducing their differentiation and proliferation, or perhaps rendering them inert (12,13).

Intestinal DCs sense threats coming from the gut through display of pattern recognition receptors. These are inherited, germ-line encoded receptors that instinctively engage classes of molecules common to many types of bacteria, fungi, viruses, helminths or host dead or dying cells. One family of such receptors is the C-type lectin receptors (CTLR). Many groups of CTLR are called CLECs, which are mostly transmembrane receptors that engage their ligands inducing intracellular signaling that alters cellular function (14). Many CLECs activate DCs via phosphorylation of the Syk signaling pathway (15).

In earlier experiments, microarray analysis was performed on intestinal DCs isolated from *Hpb*-infected and uninfected control mice to better understand how *Hpb* infection affects gene expression in gut DCs. The microarray data suggested that *Hpb* infection profoundly inhibited gene expression for nearly all of the CLECs expressed by the intestinal DCs and Syk (15).

Using rt-PCR, the present study confirmed the microarray data showing that DCs from the intestine express mRNA for Syk, and for CLEC 7A, 9A, 12A, and 4N whose levels of expression were substantially decreased by *Hpb* infection. $Syk^{-/-}$ DCs transferred into a murine model of inflammatory bowel disease inhibited colitis and induced regulatory DCs in the intestines that could block an antigen-induced, T cell response *in vitro*. Focusing on CLEC7A mRNA, which encodes for the receptor dectin-1, flow analysis showed a marked decrease in the display of this receptor on intestinal DC subsets after *Hpb* infection. Cells with diminished dectin-1 failed to respond to the dectin-1 agonist, curdlan, with Syk phosphorylation or enhanced antigen-induced T cell IFNγ/IL17 secretion. Moreover, *Hpb* secretes molecules that directly inhibit Syk and CLEC expression in these cells. Thus, it appears that down-modulation of the Syk signaling pathway is an important mechanism through which *Hpb* helps promote the development of regulatory DCs that control colitis.

MATERIALS AND METHODS

Mice

This study used Rag1, wild-type (WT), IL-$10^{-/-}$ and OT2 CD45.1 mice (Jackson Laboratory, Bar Harbor, ME) as well as $Syk^{f/f}$ and $Syk^{f/f}$ CD11c-cre mice (gift of Dr. Clifford A. Lowell, University of California, San Francisco, CA). The Syk mice were cross bred to get $Syk^{-/-}$ CD11c mice (16). All mice were on the C57BL6 background. Breeding colonies were maintained in SPF facilities at Tufts University. Animals were housed and handled following national guidelines and as approved by our Animal Review Committee.

Hpb infection

For some experiments, 5- to 6-wk-old mice were colonized with 125 *Hpb* third stage larvae by oral gavage, and infected mice were used after two weeks. Infective, ensheathed *Hpb* L3 (U.S. National Helminthological Collection no. 81930) were obtained from fecal cultures of eggs by the modified Baermann method and stored at 4°C.

Histological assessment of colitis severity

Stained histological sections of colon tissue were examined by two blind observers to assess the severity of inflammation. The process of tissue preparation and the 4-poimt scoring system are as previously described (17).

Dispersion of splenocytes and mesenteric lymph node (MLN) cells, and isolation of splenic T cells

Single cell suspensions of splenocytes and MLN cells were prepared by gentle teasing in RPMI 1640 medium (RPMI) (GIBCO, Grand Island, NY). The cells were washed three times in RPMI. Splenic T cells were isolated by negative selection using the EasySep mouse

T cell enrichment kit as outlined by the manufacturer (Stemcell Technologies, #19751, Vancouver, Canada). Viability was determined using exclusion of trypan blue dye.

Lamina propria mononuclear cells (LPMC) isolation and LP cell fractionation

Gut LPMCs were isolated from the terminal ileum as described (18). Cell viability was 90% as determined by trypan blue exclusion. Dendritic cells (DC) ($CD11c^+$) were isolated from dispersed LPMC by CD11c positive selection (Kit #18758, Stem Cell Technologies, Vancouver, Canada) according to kit directions. The beads used to isolate $CD11c^+$ cells from the gut recovered about 85% of these cells at about 95% purity as determined by FACS. The beads displayed equal efficiency at isolating both the $CD11c^{hi}$ and $CD11c^{lo}$ subsets.

Real-time PCR (rt-PCR)

Total RNA was isolated from individual samples using Quick-RNA Mini Prep (Zymo Research, Irvine, CA) as per manufacturer's instructions. RNA quality and quantity was determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was converted to cDNA using qScript cDNA SuperMix (Quanta Bioscience, Gaithersburg, MD). Rt-PCR was performed using the Eco rt-PCR System (Illumina, San Diego, CA). GAPDH levels were used to normalize the data. Taqman real time primers for CLEC7A, 9A, 12A, 4N; Syk, GADPH, HPRT and Reg3 were obtained from Applied Biosystems (Grand Island, NY).

Curdlan preparation

Curdlan (Invivogen cat # tlrl-cur, San Diego CA) (10 mg) is a linear beta-1.3-glucan that was dissolved in 1 ml of 0.1N NaOH. This was then diluted to 1 mg/ml in complete medium (see under "cell culture") and used in cultures at 100 ug/ml.

Production of *Hpb* excretory/secretory product (HES)(19)

HES was from Dr Mary Stevenson, McGill University, Canada. The *Hpb* were collected from the gut of infected mice and cultured in serum free RPMI with 2% glucose and antibiotics (Pen, Strep, Gentamycin, polymyxin). The supernatant was collected after 36 hrs of culture and concentrated using a 3KDa concentrator.

Sandwich ELISAs

ELISAs were performed using paired antibodies. To measure IFNγ, plates were coated with a mAb to IFNγ (HB170, ATCC) and incubated with supernatant. IFNγ was detected with polyclonal rabbit anti-IFNγ (gift from Dr. Mary Wilson, University of Iowa) followed by biotinylated goat anti-rabbit IgG (AXcell, Westbury, NY). IL17 ELISA was done using primary capture mAb and biotinylated anti-IL17A mAb (R & D Systems, Minneapolis, MN).

Flow cytometry

LPMC were washed twice and adjusted to 1×10^7 cells/ml in the RPMI medium containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (complete

medium) (all from Life Technologies, Gaithersburg, MD) and stained with saturating amounts of conjugated mAb for 30 min at 4°C. Following staining, cells were washed twice and re-suspended in complete medium for analysis on the BDLSRii Flow cytometer using FACSDIVA V6.1.1 software (BD, Bioscience, Mountain View, CA). Before adding labeled mAb, each tube received 1 μg of anti-Fc mAb to block nonspecific binding of conjugated Abs to FcRs. The mAbs used for staining or cell sorting were anti-CD11c-FITC; anti-CD103-PE or APC; anti-Dectin-1-PE (all above from eBioscience, San Diego, CA); CD11b-APC-Cy7 (from BD Pharmingen).

Induction and modulation of colitis, and cell culture experiments

The first series of experiments determined if intestinal DCs from WT or $Syk^{-/-}$ mice could inhibit colitis in the Rag $IL10^{-/-}$ T cell-reconstituted/piroxicam-induced model of inflammatory bowel disease. Rag mice of similar age (5–6 wk old) were reconstituted i.p. with 4×10^6 $IL10^{-/-}$ splenic T cells. Some mice also received 4×10^4 TI DCs i.p. from WT or $Syk^{-/-}$ mice. After one week, mice were feed food with the non-steroidal anti-inflammatory drug called piroxicam for two weeks to induce colitis. Then, mice were sacrificed one week after stopping the piroxicam (11). The colons were removed, opened, rolled onto glass rods, fixed in formalin, section 4 um thick and stained with H&E for histological scoring of the intestinal of inflammation using a 4-point scale. Also, intestinal LPMC from $IL10^{-/-}$ T cell- or $IL10^{-/-}$ T cell/DC-reconstituted Rag mice were cultured (2×10^5 cells per well) for 48h in 96-well round-bottomed plates along with OT2 cells (10^5 cells/well). Cells were cultured in complete medium alone or with OVA (50 ug/ml) (Worthington Biochemicals #3054 Lakewood, NJ) to stimulate cytokine secretion. After culture, the supernatants were assayed for IFNγ and IL17A using ELISAs (described above). In some experiments, curdlan (100 ug/ml) and OVA were added to the cell cultures to determine if curdlan (CLEC7A agonist) would enhance cytokine release.

In other experiments, intestinal DCs isolated from piroxicam-treated Rag mice that had received WT or $Syk^{-/-}$ DC were mixed *in vitro* with LPMC isolated from colitic Rag mice that received no supplemental DCs to determine if these DCs could modulate the *in vitro* OVA response. Rag mice were reconstituted with $IL10^{-/-}$ T cells and either WT or $Syk^{-/-}$ DCs and then treated with piroxicam as described above. Then, intestinal CD11c DCs were isolated and cultured in complete medium with intestinal LPMC derived from Rag mice reconstituted just with $IL10^{-/-}$ T cells and treated with piroxicam. These cultures also contained OT2 cells, and some wells received OVA for the 48 h cultures before measuring the cytokines in the supernatants.

The final series of experiments involved culturing intestinal DCs isolated from Rag mice with or without HES to determine if HES could directly down-modulate Syk or CLEC7A expression in these cells. The DCs (2×10^5) were cultured for 2 hrs with or without HES (50 ug/ml). Then, RNA was extracted and analyzed for Syk and CLEC7A mRNA expression using rt-PCR.

Intracytoplasmic staining for phosphorylated Syk

CD11c^{+}103^{+} intestinal DCs from either *Hpb*-infected or uninfected Rag mice were isolated by FACS. DCs were placed in complete medium, and DCs (5×10^{4}) were cultured 5 min at 37°C in the presence or absence of the dectin-1 agonist, curdlan (100 ug/ml). The DCs then were fixed, permeabilized and intracellularly stained for phosphorylated Syk following the eBioscience intracellular staining protocol using anti-Syk-phosph-PE or APC (from eBioscience).

Treatment of mice with Syk inhibitor

Mice were treated with the Syk inhibitor, Cerdulatinib cat # S7634 (Selleckchem Houston, Texas) to determine if it can inhibit colitis in the Rag/IL10−/− T cell transfer model of colitis. The Inhibitor was dissolved in 5% DMSO and 95% corn oil as recommended by Selleckchem technical services. The inhibitor was started after the first week of piroxicam treatment and continued on weekdays for two weeks for a total of ten days treatment. The drug was dosed at 5mg per kg body weight. Mice were given the inhibitor dissolved in 0.1 ml via oral gavage once in the morning and once in the late afternoon. The last inhibitor treatment was given the day before sacrifice.

Statistical analysis

Data are means ±SE of multiple determinations. Difference between two groups was compared using Student's t-test. Multiple group comparisons used analysis of variation and Dunnett's t-test. P values <0.05 were considered significant.

RESULTS

Hpb infection leads to a down-modulation of Syk expression in intestinal DCs

Rag mice, after infection with *Hpb*, develop regulatory DCs in their intestines that can control colitis when transferred into a murine IL10$^{-/-}$ T cell model of IBD (20). Previous microarray analysis showed that *Hpb* infection caused a marked decreased in Syk expression in these gut DCs. This was confirmed by rt-PCR analysis of RNA extracted from intestinal DCs isolated from Rag mice infected with *Hpb* (Figure 1). *Hpb* infection also substantially reduced Syk expression in DCs isolated from MLN, but not spleens of infected mice.

DCs deficient in Syk are sufficient to inhibit colitis

The next series of experiments determined if the Syk signaling pathway was important for development of DCs that control colitis. These studies used intestinal DCs from uninfected WT mice and transgenic mice with a Syk deficiency limited to the CD11c+ DCs. Rag mice received IL10$^{-/-}$ T cells administered i.p. Some mice also received WT or Syk$^{-/-}$ DCs. After NSAID administration to induce colitis, the animals were sacrificed four weeks later to assess severity of colitis and the responsiveness of isolated LPMC to OVA stimulation after OT2 cell supplementation (Figure 2A, experimental design).

Figure 2B/C show, as expected, that severe colitis developed in Rag mice receiving no DCs. Mice receiving intestinal DCs from WT mice fared no better. Intestinal Syk$^{-/-}$ DC murine recipients displayed a reduction in the intensity of the inflammatory response.

LPMC isolated from Rag mice that received either no DCs or WT DCs produced substantial amounts of IFNγ and IL17 when cultured *in vitro* with OVA. However, LPMC from mice receiving $Syk^{-/-}$ DCs showed blunted responsiveness to OVA stimulation (Figure 2D).

The intestines of Rag mice protected from colitis by $Syk^{-/-}$ DC transfer contain regulatory DCs

The above experiments suggest that $Syk^{-/-}$ intestinal DCs from mice never infected with *Hpb* can protect mice from colitis. To further test the significance of this observation, it next was determined if animals protected from colitis by $Syk^{-/-}$ DC transfer have regulatory type DC within their intestines.

Rag mice were reconstituted with splenic $IL10^{-/-}$ T cells, and then treated with piroxicam to induce colitis. One week after stopping the piroxicam, the mice were sacrificed, and the intensity of the colitis was scored by examining histological tissue sections. Also, intestine was dissociated to isolate the LPMC, which were cultured with OT2 cells *in vitro* with or without OVA to stimulate IFNγ and IL17 release.

In parallel, a second group of age-matched Rag mice received similar $IL10^{-/-}$ T cells along with gut WT or $Syk^{-/-}$ DCs. They also were treated with piroxicam. One week after stopping the piroxicam, the mice were sacrificed and their colitis also was scored for intensity of inflammation by examining histological sections. In these mice, intestine was dissociated to isolate intestinal DCs, which were added to some of the cultures of LPMC from the severely colitic mice who received no supplement DCs to see if these DC would affect the OVA response. (Figure 3, experimental design)

Once again, $IL10^{-/-}$ T cell-reconstituted, Rag mice developed severe colitis after piroxicam treatment, unless they received $Syk^{-/-}$ DCs (data not shown). Isolated LPMC from the colitic mice produced IFNγ and IL17 after OVA stimulation (Figure 3B). Only intestinal DCs from the Rag mice protected from colitis by $Syk^{-/-}$ DC transfer diminished OVA-induced, cytokine secretion when they were added *in vitro* to the LPMC from the colitic animals. There was little or no IL4 or IL10 in the culture supernatants (≪100 pg). The amount of IL4 and IL10 did not increase with addition of DCs. We previously showed that DCs block colitis independently of IL10 (4).

Hpb infection decreases CLEC receptor expression in TI DCs

Microarray analysis suggested that intestinal DCs strongly express four types of transmembrane CLEC receptors (CLEC7A, 9A, 4N and 12A), all of which affect Syk signaling, whose expression was inhibited by *Hpb* infection. To confirm this observation, DCs were isolated from the intestines of *Hpb*-infected mice or their uninfected littermate controls. Their RNA was extracted, converted to cDNA and checked for CLEC mRNA expression using rt-PCR. Figure 4 shows that gut DCs express mRNA for CLEC7A, 9A, 4N and 12A. Moreover, *Hpb* infection substantially reduced the level of CLEC mRNA expression in gut DCs from *Hpb*-infected mice compared to that in DCs from uninfected control mice.

Hpb infection did not alter the relative proportion of LPMC expressing the DC marker CD11c (Figure 5A). CD11c+ DCs in the intestinal lamina propria can be subdivided into several functionally distinct subsets based on their differential expression of CD11b and CD103 (12,13). Further analysis of CD11c+ DCs from the TI showed no change in the relative proportion of these cells displaying CD11b, CD103 or both markers during *Hpb* infection (Figure 6A). Thus, the effect of *Hpb* infection on DC CLEC receptor expression did not simply result from the ingress of CLEC receptor negative DCs into the lamina propria diluting the resident DC population.

Hpb infection decreased dectin-1 expression on intestinal DC

CLEC7A encodes for a cell surface receptor called dectin-1. Availability of various reagents allowed the analysis of dectin-1. To further explore the importance of the above observation, experiments focused on the consequence of down-modulation of CLEC7A receptor mRNA expression with regards to dectin-1 display.

Flow analysis showed that intestinal DCs displayed dectin-1 and that fewer cells expressed this receptor after *Hpb* infection (Figure 5B). It next was determined which subsets of intestinal DCs expressed dectin-1. Flow analysis showed that dectin-1 was expressed on the CD11c+ CD103+CD11b+, the CD11c+ CD103+CD11b− and the CD11c+ CD103−CD11b+ DC subsets. *Hpb* infection substantially reduced dectin-1 expression only on DC subsets that were CD103+CD11b+ CD11c+ and CD103+CD11b− CD11c+ (Figure 6B). Also, the relative degree of fluorescence was lower on these dectin-1-positive DCs during *Hpb* infection suggesting lower density of receptor display.

DCs with diminished dectin-1display do not respond to curdlan

DCs are the major initiators and regulators of mucosal T cell responses. Engagement of CLECs by molecules derived from microorganisms or dying host cells often transmit molecular signals leading to DC activation and enhanced adaptive immune responses. Dectin-1 recognizes β-1,3-glucans often found in the cell walls of fungi (21). Curdlan, a β-1,3-glucan, is a dectin-1 agonist.

It was determined if this dectin-1 agonist would facilitate intestinal DC interactions with effector T cells and if *Hpb* infection altered this response. Splenic T cells from OT2 transgenic mice, which respond to ovalbumin antigen (OVA), were used in these experiments.

First, intestinal DCs from uninfected mice were mixed with splenic OT2 T cells and cultured with or without OVA in the present or absence of curdlan to see if this dectin-1 agonist would enhance IFNγ and IL17 secretion. We chose to measure these cytokines because IFNγ and IL17 drive colitis in most models of IBD. Also, CD11b+ CD103+ DCs are particularly noteworthy for their capacity to drive mucosal Th17 cell differentiation (22), whereas, CD11c+ CD103+CD11b− DCs can drive Th1 polarization and IFNγ production (23). OT2 cells mixed with intestinal DCs from uninfected mice produced IFNγ and IL17 in response to OVA (Figure 7). The cells stimulated with OVA secreted substantially more IFNγ and IL17 when curdlan was added to the culture mix. Cells stimulated with only curdlan did not release cytokines.

Similar experiments were performed using gut DCs isolated from *Hpb*-infected mice. When OT2 T cells were cultured with intestinal DCs from *Hpb*-infected mice, OVA still stimulated IFNγ and IL17 release; however curdlan failed to enhance cytokine production (Figure 7).

Engagement of dectin-1 with its ligand initiates intracellular signaling via phosphorylation of Syk. It was determined if curdlan, the dectin-1 agonist, induced Syk phosphorylation in intestinal DCs and if *Hpb* infection impeded this process. Intestinal DCs for either *Hpb*-infected or uninfected mice were briefly exposed to curdlan. Cells then were permeabilized and intracellularly stained with mAb to detect phosphorylated Syk using flow cytometry. It was interesting to note that baseline phosphorylation of Syk before curdlan stimulation was lower in DCs from *Hpb*-infected mice (Figure 8A). Figure 8B shows that curdlan stimulation induced Syk phosphorylation in these cells. However, curdlan failure to enhance Syk phosphorylation in DCs from *Hpb* infected mice.

Hpb secretes molecules that directly inhibit Syk and CLEC7A expression in intestinal DCs

Hpb cultured *in vitro* releases biologically active molecules into their culture medium. Preparations derived from these supernatants are called *Hpb* excretory/secretory product (HES). It was ascertained if HES could inhibit Syk or CLEC7A expression in intestinal DCs. DCs were isolated from the intestines of mice, which were cultured in medium alone or with HES for 2 hrs. Then, RNA was extracted from the cells and analyzed for Syk and CLEC7A expression using quantitative rt-PCR. Figure 9 shows that HES rapidly inhibits both Syk and CLEC7A expression in intestinal type lectin receptor and three housekeeping genes (actin, HPRT and GADPH). Their expression was not affected by HES (data not shown). We also transferred some of these 2-hr, HES exposed DCs into our rag colitis model of IBD. They did not block colitis perhaps because such a brief exposure was insufficient to maintain the regulatory phenotype.

Treatment mice with a Syk inhibitor blocks colitis

Using the Rag/IL10−/− T cell transfer model of colitis, mice were treated for two weeks with a Syk inhibitor to determine if it could inhibit colitis. The drug was started 2 wks after IL10−/− T cell transfer. Colons were assessed for colitis severity in stained histological sections using the usual 4-point scale. As expected, mice receiving no drug developed severe colitis (3.4±0.2 SE, two separate experiments using 12 mice). The colitis was substantially less in drug treated animals (1.5± 0.2 SE, two separate experiments, 8 mice) (treatment vs. control $p < 0.01$).

DISCUSSION

Previous studies showed that infection with *Hpb* induces changes in gut DCs enabling them to suppress intestinal DC-driven, antigen-specific T cell responses *in vitro* and to inhibit colitis *in vivo* in a murine model of IBD (11). The above experiments demonstrate that during *Hpb* infection the DCs located in the intestines express substantially less Syk, which is part of an important pro-inflammatory intracellular signaling pathway, and fewer CLECs capable of phosphorylating Syk. Using transgenic mice, it was shown that selective disruption of the Syk signaling pathway in intestinal DCs is sufficient to induce regulatory

DCs that can block colitis and inhibit antigen-induced, T cell responses in the gut. Thus, it appears that down-modulation of the Syk signaling pathway within gut DCs is an important mechanism leading to formation of these regulatory cells.

It is likely that *Hpb* infection down-modulates the activity of the Syk signaling pathway in resident intestinal DCs of infected mice. *Hpb* infection induced no inflammation in the distal intestines and did not change the relative number of DCs or DC subsets in the lamina propria of this region of the gut. Thus, the effect of *Hpb* infection on DC CLEC and Syk mRNA expression did not likely reflect ingress into the lamina propria of DCs simply displaying low levels of these mRNA diluting the resident DC population.

HES is the excretory/secretory product of *Hpb*. Exposing DCs isolated from the gut of uninfected mice to HES quickly and selectively inhibits Syk and CLEC expression further supporting the supposition that *Hpb* deactivates Syk signaling in gut DCs. Also, this latter observation suggests that *Hpb* produces one or more biologically active molecules that accomplish this task. It is tempting to speculate that *Hpb* has acquired the ability to regulate Syk signaling in host DCs to weaken immune responses injurious to the worm. Other investigators have shown that various immunoregulatory molecules like TGFβ (24), IL10 (25) (26), vitamin D3 (27) and dexamethasone (28) also can induce regulatory DCs.

Previously published studies have shown that helminths of various species produce factors that induce DCs which block inflammation in various animal models of immune-mediated disease and/or that enhance immune regulation *in vitro*. For instance, adoptive transfer of bone marrow-derived DCs exposed to excretory/secretory products from cultured *T. spiralis* muscle cyst larvae protects against EAE (29). Splenocytes from rats that received DCs previously exposed to this excretory/secretory product before EAE challenge have more Foxp3+ T cells. *Hymenolepis diminuta* antigen-pulsed DCs transferred into mice with dinitrobenzene sulfonic acid-induced colitis will block the inflammation in an IL10-dependent manner (30). Human PBMC-derived DCs exposed to soluble schistosome egg antigens and then co-cultured with autologous CD25− T cells increases T cell SMAD3 activation and Foxp3 expression (31). A 62Kd phosphocholine-containing glycoprotein (ES-62) isolated from *Acanthocheilonema viteae* prevents and inhibits established collagen-induced arthritis (32). Bone marrow-derived DCs stimulated with LPS and ES-62 make less TNFα, IL6 and IL23, and are less able to induce IL17 production by OT-II T cells *in vitro* (33). DCs exposed to HES from the helminth *Hpb* can induce differentiation of IL10 producing CD4+ Tregs, which suppress bystander T cell proliferation (34). Taenia crassiceps is a helminth used to study cysticercosis. Their excretory/secretory molecules impair DC responses to LPS and CpG. These worm-derived molecules bind mannose and galactose C-type lectin receptors triggering the cRAF intracellular signaling pathway. This blocks Th1 proinflammatory TLR signaling in DCs favoring Th2 polarization (35). *Fasciola hepatica*, a liver fluke, expresses a tegmental coat antigen that inhibits DC function. It inhibits LPS-induced NF-kB and MAPK pathways in these cells (36).

Syk is a non-receptor tyrosine kinase. It is rapidly phosphorylated when receptors linked to Syk are engaged. Syk–mediated downstream signaling can lead to activation of proinflammatory pathways like NF-kB, NFAT and CARD9 (37). Syk has a role in cell

adhesion being important for several integrin and selectin-mediated functions. Many pattern recognition receptors that recognize potential pathogens or dead/dying host cells use the Syk signaling pathway to promote adaptive immunity and strong Th1/Th17 responses (15). This includes a number of CLECs (15) as well as TLR5 (38). The latter recognizes flagellin, which is the primary protein component of bacterial flagella. Amply demonstrated in our study was that engagement of dectin-1, the receptor encoded by CLEC7A, induces Syk phosphorylation in intestinal DCs and enhances an antigen-induced, T cell-dependent IFNγ and IL17 response. Syk also is reported to induce the production of other cytokines like IL-1β (39) and several chemokines.

It remains unknown how inactivation of the Syk signaling pathway in intestinal DCs leads to development of powerful regulatory DCs in the intestine that block colitis and mucosal antigen-induced, T cell responses. Syk signaling in DCs appears to be important for Th1/Th17 responses (40). In many murine models of IBD, poorly regulated effector T cells located in the gut respond to luminal antigens and release pro-inflammatory molecules like IFNγ and IL17 that drive the colitis (41). These two cytokines commonly are expressed at high levels in many patients with either ulcerative colitis or Crohn's disease (42) and appear to have a role in the disease process. Thus, down-modulation of Syk may decrease Th1/Th17 responsiveness leading to inhibition of IBD.

Other mechanisms also must be considered. Blocking of Syk in WT DCs *in vitro* reduces their display of co-stimulatory markers (CD80, CD86) and MHC class II as well as secretion of pro-inflammatory cytokines (e.g. IL-12p40) (43). Regulatory DCs from the gut of *Hpb*-infected mice have a similar phenotype (10) (44). A known effect of regulatory DCs with impaired MHC complex expression is induction of T cell anergy due to delivery of defective costimulation to CD8+ and CD4+ T cells (45). They also restrain NK cell IFNγ production (46). Thus, induction of T cell anergy could be one of the mechanisms through which they work to limit the T cell responses that induce colitis.

This study also characterized the CLEC subsets highly expressed in intestinal DCs from Rag mice. CLEC7A, 9A, 4N and 12A were expressed at high levels in gut DCs of uninfected healthy Rag mice (44). *Hpb* infection strongly inhibited expression of these four CLECs without inducing the display of other membrane bound CLEC subtypes. CLEC7A, 9A and 4N encode for Syk-coupled C-type lectin receptors.

The various membrane-bound CLECs, expressed on a number of different cell types, affect immune responses following recognition of their various ligands. Studies using other model systems show that CLECs affect DC behavior. CLEC7A encodes for the receptor dectin-1, which engages β-1,3-glucans present on several fungal species and at least another unidentified ligand on mycobacteria. Activation of this receptor promotes Th1 and Th17 T cell development (47). In this paper, this was demonstrated for the first time using intestinal DCs mixed with OT2 cells that were stimulated with an antigen and a dectin-1 agonist. After *Hpb* infection, intestinal DCs no long responded to CLEC7A agonist. This loss of response is assumed to be the consequence of the diminution of dectin-1 on the surface of these cells as was demonstrated using FACS. Also shown was that a dectin-1 agonist no longer could induce Syk phosphorylation in gut DCs after *Hpb* infection. Syk is the critical intracellular

signaling pathway for many CLECs. Dectin-1 also can collaborate with MyD88-coupled TLRs to modulate their function (48,49).

CLEC9A encodes for DNGR1, which is displayed on plasmacytoid DCs and CD8a+DCs. This receptor recognizes F-actin coming from dead or dying cells, and it helps prime cytotoxic T cells (50). Loss of this receptor and the Syk signaling pathway could conceivably affect how the mucosal immune system responds to damaged mucosal epithelium or virally infected cells (51).

CLEC4N (dectin-2) binds α-mannan and zymosan expressed by potentially many different pathogens (52). It also participates in Th2 (53,54) and Th17 (55,56) cell development.

CLEC12A (MICL) may be an inhibitory CTLR (14). Yet, it may synergize with signals from other receptors to facilitate DC migration and their capacity to stimulate T cells (57). CLEC12A is a receptor that detects dead cells, and it binds uric acid crystals and perhaps other ligands. In neutrophils, uric acid crystals activate Syk, and engagement of CLEC12A inhibits this activation (58).

Rag intestinal DCs expressed two additional CLECs (CLEC2 and 2d) whose level of expression was not affected by *Hpb* infection (44). In conjunction with LPS, ligation of CLEC2 on murine bone marrow-derived DCs augments IL10 production (59). CLEC2d encodes for lectin-like transcript-1, which binds NKR-P1A inhibiting NK cell cytotoxicity (60). It is notable that the infection did not modulate gene expression for these two CLECs that have immune inhibitory functions.

In intestinal DCs, blocking the expression of Syk-coupled C-type lectin receptors and Syk following a helminthic infection could have major implications for host immunity. These changes could lead to marked alterations in DC responses to intestinal flora and gut helminthic infections. The latter is of note since helminths have ligands for host CLECs (61–64). Since Syk promotes T cell differentiation and/or activation, it is conceivable that the functional loss of this signaling pathway will render DCs less able to support T cell-mediated intestinal pathology such as that seen in IBD. Targeting selective Syk inhibitors (65) to the intestinal mucosa could prove useful for control of these intestinal diseases. Also, shown in this study is that *Hpb* infection decreases Syk expression in DCs located in the MLNs. Thus, it is conceivable that the effect of *Hpb* on Syk expression spreads beyond the gastrointestinal tract. Selective blockade of Syk expression could be an effective way to control inflammatory bowel disease.

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Abbreviations

APC	Antigen presenting cells
Curd	Curdlan

CLEC	C-type lectin receptor
DC	Dendritic cells
Hpb	*Heligmosomoides polygyrus bakeri*
HES	*Hpb* excretory/secretory product
IBD	Inflammatory bowel disease
LP	Lamina propria
LPMC	Lamina propria mononuclear cells
MLN	Mesenteric lymph node
OVA	Ovalbumin
RPMI	RPMI 1640 medium
Syk	Spleen tyrosine kinase
TI	Terminal ileum

Reference List

1. Weinstock JV, Elliott DE. Translatability of helminth therapy in inflammatory bowel diseases. Int J Parasitol. 2013; 43:245–251. [PubMed: 23178819]
2. Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF Jr, Weinstock JV. Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice. Eur J Immunol. 2004; 34:2690–2698. [PubMed: 15368285]
3. Grainger JR, Smith KA, Hewitson JP, McSorley HJ, Harcus Y, Filbey KJ, Finney CA, Greenwood EJ, Knox DP, Wilson MS, Belkaid Y, Rudensky AY, Maizels RM. Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. J Exp Med. 2010; 207:2331–2341. [PubMed: 20876311]
4. Hang L, Blum AM, Urban J, Stoyanoff K, Weinstock JV. *Heligmosomoides bakeri* infection activates colonic Foxp3+ T cells enhancing their capacity to prevent colitis. J Immunol. 2013; 191:1927–1934. [PubMed: 23851695]
5. Smith P, Mangan NEWCM, Fallon RE, McKenzie ANJ, van Rooijen N, Fallon PG. Infection with a helminth parasite prevents experimental colitis via a macrophage-mediated mechanism. J Immunol. 2007; 178:4557–4566. [PubMed: 17372014]
6. Schnoeller C, Rausch S, Pillai S, Avagyan A, Wittig BM, Loddenkemper C, Hamann A, Hamelmann E, Lucius R, Hartmann S. A helminth immunomodulator reduces allergic and inflammatory responses by induction of IL-10-producing macrophages. J Immunol. 2008; 180(6): 4265–72. [PubMed: 18322239]
7. Johnston MJ, Wang A, Catarino ME, Ball L, Phan VC, MacDonald JA, McKay DM. Extracts of the rat tapeworm, Hymenolepis diminuta, suppress macrophage activation in vitro and alleviate chemically induced colitis in mice. Inf Immun. 2010; 78:1364–1375.
8. Hunter MM, Wang A, Parhar KS, Johnston MJ, van RN, Beck PL, McKay DM. In vitro-derived alternatively activated macrophages reduce colonic inflammation in mice. Gastroenterology. 2010; 138:1395–1405. [PubMed: 20044996]
9. Leung G, Wang A, Fernando M, Phan VC, McKay DM. Bone marrow-derived alternatively activated macrophages reduce colitis without promoting fibrosis: participation of IL-10. Am J Physiol - Gastrointestinal & Liver Physiology. 2013; 304:G781–G792.

10. Hang L, Setiawan T, Blum AM, Urban J, Stoyanoff K, Arihiro S, Reinecker HC, Weinstock JV. Heligmosomoides polygyrus infection can inhibit colitis through direct interaction with innate immunity. J Immunol. 2010; 185:3184–3189. [PubMed: 20702728]
11. Blum A, Hang L, Setiawan T, Urban J, Stoyanoff K, Leung J, Weinstock JV. *Heligmosomoides bakeri* induces tolerogenic dendritic cells that block colitis and prevent antigen-specific gut T cell responses. J Immunol. 2012; 189:2512–2520. [PubMed: 22844110]
12. Farache J, Zigmond E, Shakhar G, Jung S. Contributions of dendritic cells and macrophages to intestinal homeostasis and immune defense. Immunology & Cell Biology. 2013; 91:232–239. [PubMed: 23399695]
13. Persson EK, Scott CL, Mowat AM, Agace WW. Dendritic cell subsets in the intestinal lamina propria: ontogeny and function. Eur J Immunol. 2013; 43:3098–3107. [PubMed: 23966272]
14. Plato A, Willment JA, Brown GD. C-type lectin-like receptors of the dectin-1 cluster: ligands and signaling pathways. Int Rev Immunol. 2013; 32:134–156. [PubMed: 23570314]
15. Kerrigan AM, Brown GD. Syk-coupled C-type lectins in immunity. Trends Immunol. 2011; 32:151–156. [PubMed: 21334257]
16. Saijo K, Schmedt C, Su IH, Karasuyama H, Lowell CA, Reth M, Adachi T, Patke A, Santana A, Tarakhovsky A. Essential role of Src-family protein tyrosine kinases in NF-kappaB activation during B cell development. Nature Immunol. 2003; 4:274–279. [PubMed: 12563261]
17. Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, Lynch RG. Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology. 2002; 123:1527–1542. [PubMed: 12404228]
18. Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF Jr, Weinstock JV. *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. Eur J Immunol. 2004; 34:2690–2698. [PubMed: 15368285]
19. Valanparambil RM, Segura M, Tam M, Jardim A, Geary TG, Stevenson MM. Production and analysis of immunomodulatory excretory-secretory products from the mouse gastrointerstinal nemtode Heligmosomoides polyygrus bakeri. Nature Protoc. 2014; 12:2740–2754. [PubMed: 25375989]
20. Blum AM, Hang L, Setiawan T, Urban JP Jr, Stoyanoff KM, Leung J, Weinstock JV. Heligmosomoides polygyrus bakeri induces tolerogenic dendritic cells that block colitis and prevent antigen-specific gut T cell responses. J Immunol. 2012; 189:2512–2520. [PubMed: 22844110]
21. Brown GD, Gordon S. Immune recognition. A new receptor for beta-glucans. Nature. 2001; 413:36–37. [PubMed: 11544516]
22. Persson EK, Uronen-Hansson H, Semmrich M, Rivollier A, Hagerbrand K, Marsal J, Gudjonsson S, Hakansson U, Reizis B, Kotarsky K, Agace WW. IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. Immunity. 2013; 38:958–969. [PubMed: 23664832]
23. Fujimoto K, Karuppuchamy T, Takemura N, Shimohigoshi M, Machida T, Haseda Y, Aoshi T, Ishii KJ, Akira S, Uematsu S. A new subset of CD103+CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity. J Immunol. 2011; 186:6287–6295. [PubMed: 21525388]
24. Belladonna ML, Volpi C, Bianchi R, Vacca C, Orabona C, Pallotta MT, Boon L, Gizzi S, Fioretti MC, Grohmann U, Puccetti P. Cutting edge: Autocrine TGF-beta sustains default tolerogenesis by IDO-competent dendritic cells. J Immunol. 2008; 181:5194–5198. [PubMed: 18832670]
25. Steinbrink K, Jonuleit H, Muller G, Schuler G, Knop J, Enk AH. Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. Blood. 1999; 93:1634–1642. [PubMed: 10029592]
26. Boks MA, Kager-Groenland JR, Haasjes MS, Zwaginga JJ, van Ham SM, ten BA. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction--a comparative study of human clinical-applicable DC. Clin Immunol. 2012; 142:332–342. [PubMed: 22225835]
27. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. Eur J Immunol. 2009; 39:3147–3159. [PubMed: 19688742]

28. Bosma BM, Metselaar HJ, Nagtzaam NM, de HR, Mancham S, van der Laan LJ, Kuipers EJ, Kwekkeboom J. Dexamethasone transforms lipopolysaccharide-stimulated human blood myeloid dendritic cells into myeloid dendritic cells that prime interleukin-10 production in T cells. Immunology. 2008; 125:91–100. [PubMed: 18312359]
29. Sofronic-Milosavljevic LJ, Radovic I, Ilic N, Majstorovic I, Cvetkovic J, Gruden-Movsesijan A. Application of dendritic cells stimulated with Trichinella spiralis excretory-secretory antigens alleviates experimental autoimmune encephalomyelitis. Medical Microbiology & Immunology. 2013; 202:239–249. [PubMed: 23307236]
30. Matisz CE, Leung G, Reyes JL, Wang A, Sharkey KA, McKay. Adoptive transfer of helminth antigen-pulsed dendritic cells protects against the development of expreimental colitis in mice. Eur J Imunol. 2015; 45:3126–3139.
31. Correale J, Farez MF. Parasite infections in multiple sclerosis modulate immune responses through a retinoic acid-dependent pathway. J Immunol. 2013; 191:3827–3837. [PubMed: 23975865]
32. McInnes IB, Leung BP, Harnett M, Gracie JA, Liew FY, Harnett W. A novel therapeutic approach targeting articular inflammation using the filarial nematode-derived phosphorylcholine-containing glycoprotein ES-62. J Immunol. 2003; 171(4):2127–33. [PubMed: 12902519]
33. Pineda MA, McGrath MA, Smith PC, Al-Riyami L, Rzepecka J, Gracie JA, Harnett W, Harnett MM. The parasitic helminth product ES-62 suppresses pathogenesis in collagen-induced arthritis by targeting the interleukin-17-producing cellular network at multiple sites. Arthritis & Rheumatism. 2012; 64:3168–3178. [PubMed: 22729944]
34. Segura M, Su Z, Piccirillo C, Stevenson MM. Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. Eur J Immunol. 2007; 37:1887–1904. [PubMed: 17563917]
35. Terrazas CA, Alcantara-Hernandez M, Bonifaz L, Terrazas LI, Satoskar AR. Helminth-excreted/secreted products are recognized by multiple receptors on DCs to block the TLR response and bias Th2 polarization in a cRAF dependent pathway. FASEB J. 2013; 27:4547–4560. [PubMed: 23907435]
36. Vukman KV, Adams PN, O'Neill SM. Fasciola hepatica tegumental coat antigen suppresses MAPK signalling in dendritic cells and up-regulates the expression of SOCS3. Parasite Immunol. 2013; 35:234–238. [PubMed: 23495757]
37. Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. Nature Rev Immunol. 2010; 10:387–402. [PubMed: 20467426]
38. Atif SM, Lee SJ, Li LX, Uematsu S, Akira S, Gorjestani S, Lin X, Schweighoffer E, Tybulewicz VL, McSorley SJ. Rapid CD4+ T-cell responses to bacterial flagellin require dendritic cell expression of Syk and CARD9. Eur J Immunol. 2015; 45:513–524. [PubMed: 25430631]
39. Yasukawa S, Miyazaki Y, Yoshii C, Nakaya M, Ozaki N, Toda S, Kuroda E, Ishibashi K, Yasuda T, Natsuaki Y, Mi-ichi F, Iizasa E, Nakahara T, Yamazaki M, Kabashima K, Iwakura Y, Takai T, Saito T, Kurosaki T, Malissen B, Ohno N, Furue M, Yoshida H, Hara H. An ITAM-Syk-CARD9 signalling axis triggers contact hypersensitivity by stimulating IL-1 production in dendritic cells. Nature Communications. 2014; 5:3755.
40. Leibundgut-Landmann S, Gross O, Robinson MJ, Osorio F, Slack EC, Tsoni SV, Schweighoffer E, Tybulewicz V, Brown GD, Ruland J, Reis e Sousa. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nature Immunol. 2007; 8:630–638. [PubMed: 17450144]
41. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature. 2011; 474:298–306. [PubMed: 21677746]
42. Kobayashi T, Okamoto S, Hisamatsu T, Kamada N, Chinen H, Saito R, Kitazume MT, Nakazawa A, Sugita A, Koganei K, Isobe K, Hibi T. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. Gut. 2008; 57(12):1682–9. [PubMed: 18653729]
43. Morrison VL, James MJ, Grzes K, Cook P, Glass DG, Savinko T, Lek HS, Gawden-Bone C, Watts C, Millington OR, MacDonald AS, Fagerholm SC. Loss of beta2-integrin-mediated cytoskeletal linkage reprogrammes dendritic cells to a mature migratory phenotype. Nature Communications. 2014; 5:5359.

44. Weinstock JV. Do we still need worms to promote immune health? Clinical Reviews in Allergy and Immunology. 2015; 47:227–231. [PubMed: 25326880]
45. Anderson AE, Sayers BL, Haniffa MA, Swan DJ, Diboll J, Wang XN, Isaacs JD, Hilkens CM. Differential regulation of naive and memory CD4+ T cells by alternatively activated dendritic cells. J Leukoc Biol. 2008; 84:124–133. [PubMed: 18430785]
46. Spallanzani RG, Torres NI, Avila DE, Ziblat A, Raffo Iraolagoitia XL, Rossi LE, Domaica CI, Fuertes MB, Rabinovich GA, Zwirner NW. Regulatory dendritic cells restrain NK cells IFNg production through mechanisms involving NKp46, IL-10, and MHC Class I-specific inhibitory receptors. J Immunol. 2015; 195:2141–2148. [PubMed: 26232426]
47. Agrawal S, Gupta S, Agrawal A. Human dendritic cells activated via dectin-1 are efficient at priming Th17, cytotoxic CD8 T and B cell responses. PLoS ONE [Electronic Resource]. 2010; 5:e13418.
48. Underhill DM. Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. Immunol Rev. 2007; 219:75–87. [PubMed: 17850483]
49. Eberle ME, Dalpke AH. Dectin-1 stimulation induces suppressor of cytokine signaling 1, thereby modulating TLR signaling and T cell responses. J Immunol. 2012; 188:5644–5654. [PubMed: 22547696]
50. Zelenay S, Keller AM, Whitney PG, Schraml BU, Deddouche S, Rogers NC, Schulz O, Sancho D, Reis e Sousa. The dendritic cell receptor DNGR-1 controls endocytic handling of necrotic cell antigens to favor cross-priming of CTLs in virus-infected mice. J Clin Invest. 2012; 122:1615–1627. [PubMed: 22505458]
51. Iborra S, Izquierdo HM, Martinez-Lopez M, Blanco-Menendez N, Reis e Sousa, Sancho D. The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice. J Clin Invest. 2012; 122:1628–1643. [PubMed: 22505455]
52. Graham LM, Brown GD. The Dectin-2 family of C-type lectins in immunity and homeostasis. Cytokine. 2009; 48:148–155. [PubMed: 19665392]
53. Parsons MW, Li L, Wallace AM, Lee MJ, Katz HR, Fernandez JM, Saijo S, Iwakura Y, Austen KF, Kanaoka Y, Barrett NA. Dectin-2 regulates the effector phase of house dust mite-elicited pulmonary inflammation independently from its role in sensitization. J Immunol. 2014; 192:1361–1371. [PubMed: 24453247]
54. Barrett NA, Rahman OM, Fernandez JM, Parsons MW, Xing W, Austen KF, Kanaoka Y. Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. J Exp Med. 2011; 208:593–604. [PubMed: 21357742]
55. Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya K, Yamamoto N, Kawakami K, Yamasaki S, Saito T, Akira S, Iwakura Y. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. Immunity. 2010; 32:681–691. [PubMed: 20493731]
56. Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, Verbeek JS, Ruland J, Tybulewicz V, Brown GD, Moita LF, Taylor PR, Reis e Sousa. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. J Exp Med. 2009; 206:2037–2051. [PubMed: 19703985]
57. Chen CH, Floyd H, Olson NE, Magaletti D, Li C, Draves K, Clark EA. Dendritic-cell-associated C-type lectin 2 (DCAL-2) alters dendritic-cell maturation and cytokine production. Blood. 2006; 107:1459–1467. [PubMed: 16239426]
58. Neumann K, Castineiras-Vilarino M, Hockendorf U, Hannesschlager N, Lemeer S, Kupka D, Meyermann S, Lech M, Anders HJ, Kuster B, Busch DH, Gewies A, Naumann R, Gros O, Ruland J. Clec12a is an inhibitory receptor for uric acid crystals that regulates inflammation in response to cell death. Immunity. 2014; 40:389–399. [PubMed: 24631154]
59. Mourao-Sa D, Robinson MJ, Zelenay S, Sancho D, Chakravarty P, Larsen R, Plantinga M, van RN, Soares MP, Lambrecht B, Reis e Sousa. CLEC-2 signaling via Syk in myeloid cells can regulate inflammatory responses. Eur J Immunol. 2011; 41:3040–3053. [PubMed: 21728173]

60. Rosen DB, Bettadapura J, Alsharifi M, Mathew PA, Warren HS, Lanier LL. Cutting edge: lectin-like transcript-1 is a ligand for the inhibitory human NKR-P1A receptor. J Immunol. 2005; 175:7796–7799. [PubMed: 16339513]

61. Ritter M, Gross O, Kays S, Ruland J, Nimmerjahn F, Saijo S, Tschopp J, Layland LE, Prazeres da CC. Schistosoma mansoni triggers Dectin-2, which activates the Nlrp3 inflammasome and alters adaptive immune responses. Proc Natl Acad Sci U S A. 2010; 107:20459–20464. [PubMed: 21059925]

62. Klaver EJ, Kuijk LM, Laan LC, Kringel H, van Vliet SJ, Bouma G, Cummings RD, Kraal G, van DI. Trichuris suis-induced modulation of human dendritic cell function is glycan-mediated. Int J Parasitol. 2013; 43:191–200. [PubMed: 23220043]

63. Vazquez-Mendoza A, Carrero JC, Rodriguez-Sosa M. Parasitic infections: a role for C-type lectins receptors. BioMed Research International. 2013; 2013:456352. [PubMed: 23509724]

64. Meevissen MH, Driessen NN, Smits HH, Versteegh R, van Vliet SJ, van KY, Schramm G, Deelder AM, Haas H, Yazdanbakhsh M, Hokke CH. Specific glycan elements determine differential binding of individual egg glycoproteins of the human parasite Schistosoma mansoni by host C-type lectin receptors. Int J Parasitol. 2012; 42:269–277. [PubMed: 22673410]

65. Liao C, Hsu J, Kim Y, Hu DQ, Xu D, Zhang J, Pashine A, Menke J, Whittard T, Romero N, Truitt T, Slade M, Lukacs C, Hermann J, Zhou M, Lucas M, Narula S, DeMartino J, Tan SL. Selective inhibition of spleen tyrosine kinase (SYK) with a novel orally bioavailable small molecule inhibitor, RO9021, impinges on various innate and adaptive immune responses: implications for SYK inhibitors in autoimmune disease therapy. Arthritis Research & Therapy. 2013; 15:R146. [PubMed: 24286216]

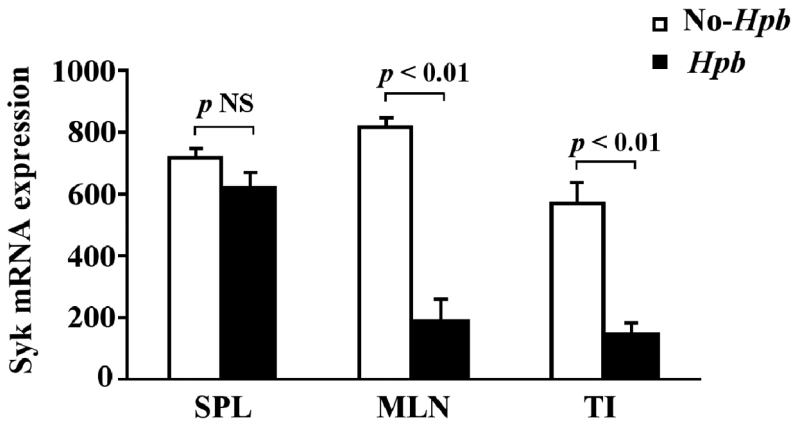


Figure 1. *Hpb* infection inhibits Syk mRNA expression in intestinal DCs

Mice were infected with *Hpb* (*Hpb*). Control mice of similar age were not infected (No *Hpb*). Two wks late, DCs were isolated from the TI, MLN or spleen (SPL) of groups of 3–4 mice. Then, RNA was extracted and converted to cDNA. Quantitative rt-PCR was used to assess the levels of Syk mRNA expression. Data show that Syk mRNA expression in intestinal DC from *Hpb* infected mouse was lower than that in DC from uninfected mice ($p < 0.01$). Data were normalized relative to the expression of the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Data are means ± SE from 3 separate experiments.

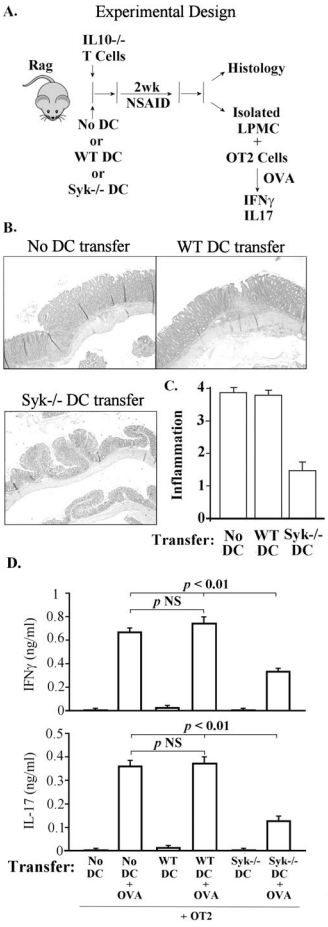


Figure 2. Syk$^{-/-}$ DC transferred reduced colonic inflammation

The experimental design is outlined in the **panel (A)**. DCs were isolated from the TI of WT or Syk$^{-/-}$ mice, and the DCs were transferred (4×10^4/mouse) into Rag mice that received splenic IL10$^{-/-}$ splenic T cells (4×10^6) i.p. injection. A third group of Rag mice was reconstituted with IL10$^{-/-}$ T cells, but received no DC. Animals then were treated with piroxicam as described to induce colitis.

At the end of the experiment, colonic tissue was examined microscopically to score the severity of the colitis using a 4-point scale. The data in **panels B)** and **C)** show the severity of the colitis in mice receiving no DCs (No DC), DCs from WT mice (WT DC) or DCs from Syk$^{-/-}$ mice (Syk$^{-/-}$ DC). **Panel B)** Colonic tissue was sectioned and stained with H&E, and then photographed at × 40. The intense lymphocytic infiltration in the mucosa of no DC and WT DC recipients was not seen in mice receiving Syk$^{-/-}$ DC. **Panel C)** Colitis was scored for severity of inflammation on a 4-point scale in stained histology sections again showing that Syk$^{-/-}$ DC transfer inhibited the intensity of the colitis. Data are means ± SE from 3 separate experiments each containing 4–5 mice/group. No DCs or WT DCs transferred vs. Syk$^{-/-}$ DC transferred, p<0.01.

Also studied was the effect of DC transfer on mucosal cytokine production **(panel D)**. LPMC were isolated from the TI at the time of sacrifice for histological analysis. These cells were cultured *in vitro* at 2×10^5 cells per well in RPMI complete medium for 48 hrs with

OT2 cells (ratio 2:1). Some wells contained OVA (10 ug/ml) to stimulate cytokine secretion. Cell culture supernatants were assayed for IFNγ and IL17 using ELISAs after the 48 h culture period. LPMC from Rag mice reconstituted with $Syk^{-/-}$ secrete less cytokines as compared to LPMC form Rag mice that receive no DCs or DCs from WT mice. Data are means ± SE of 3 independent experiments, each comprised of triplicate determinations. LPMC from No DC- or WT DC-transferred mice +OVA vs. LPMC from $Syk^{-/-}$ DC-transferred mice+ OVA, $p<0.01$

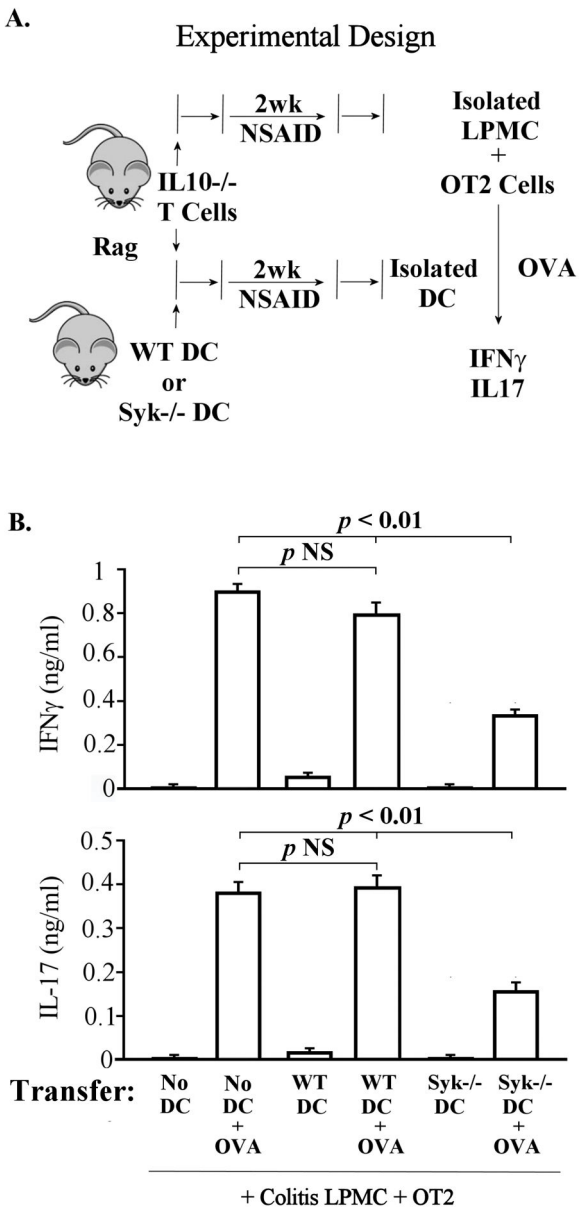


Figure 3. Syk$^{-/-}$ DCs transferred Rag DCs regulated the inflammation cytokines *in vitro*

A. In these experiments, Rag mice were reconstituted with 4×10^6 splenic IL10$^{-/-}$ T cells given i.p. and then given piroxicam for 2 wks to induce colitis as described in the methods section. One wk after stopping the piroxicam, LPMC were isolated from the TI. A second group of Rag mice received intestinal DCs from WT or Syk$^{-/-}$ mice along with IL10$^{-/-}$ splenic T cells before administration of piroxicam. One week after stopping the piroxicam treatment, DCs were isolated from their TI. To study the effect of these DCs on cytokine secretion from the LPMC isolated from the mice with colitis who received no DCs, DCs (4×10^4) were added to some of the LPMC (2×10^5) along with OT2 T cells (10^5) and cultured for 48 hrs. Some wells also contained OVA (10 ug/ml) to stimulate IFNγ and IL17 release, which was measure in the culture supernatants at the end of the incubation.

B. OVA induced cytokine release (IFNγ and IL17) from these cell cultures. Only intestinal DCs from the Rag mice reconstituted with $Syk^{-/-}$ DCs inhibited the *in vitro* LPMC OVA-induced cytokine response. Data are means ± SE from 3 independent experiments each performed in triplicate. LPMC + OVA or LPMC + WT DC + OVA vs. LPMC + $Syk^{-/-}$ DCs + OVA, $p<0.01$

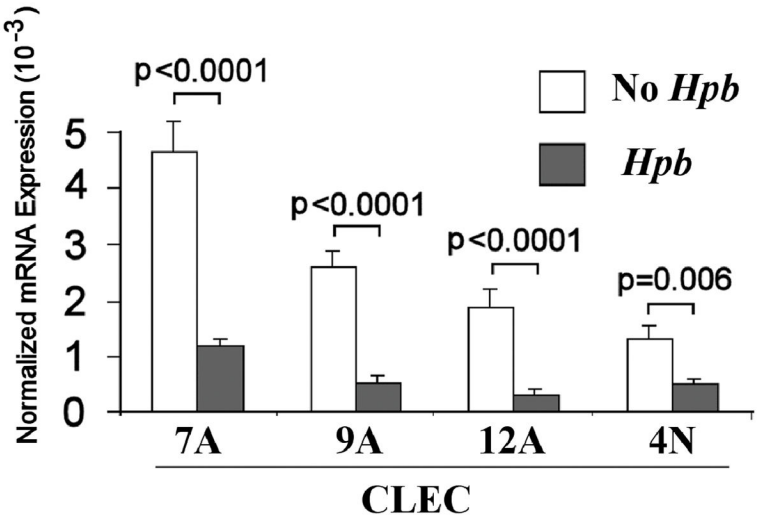
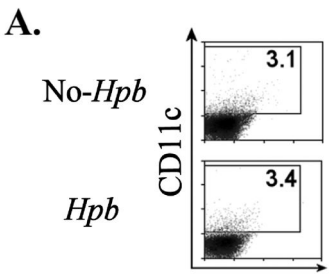
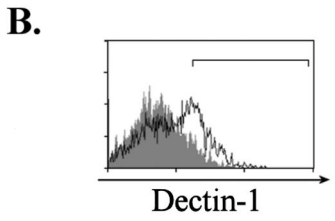


Figure 4. *Hpb* infection inhibits CLEC expression in intestinal DCs

Mice were infected with *Hpb* (*Hpb*). Control mice of similar age were not infected (no *Hpb*). Two wks later, DCs were isolated from the TI of groups of 3–4 mice. The RNA was extracted and converted to cDNA. Quantitative rt-PCR was used to assess the level of CLEC7A, CLEC9A, CLEC12A and CLEC4N mRNA expression. Data were normalized relative to expression of the housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). DCs from *Hpb*-infected mice expressed less CLEC mRNA compared to DCs from uninfected mice, $p < 0.01$. Data are means +/− SE from 4 independent experiments each performed in triplicate.



	DC (%)
No *Hpb*	3.05 ± 0.31
Hpb	3.65 ± 0.84
p	NS



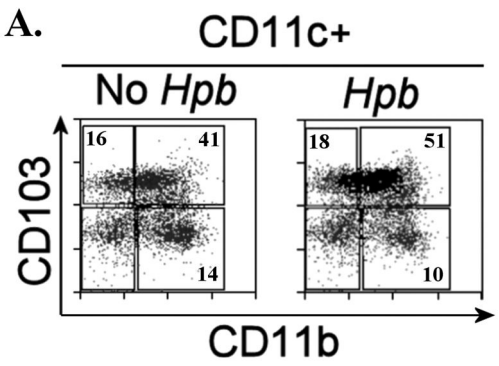
	(%)Dectin-1+
No *Hpb*	22.34 ± 3.22
Hpb	12.18 ± 2.83
p	<0.05

Figure 5. *Hpb* infection decreases dectin-1 display on intestinal DCs

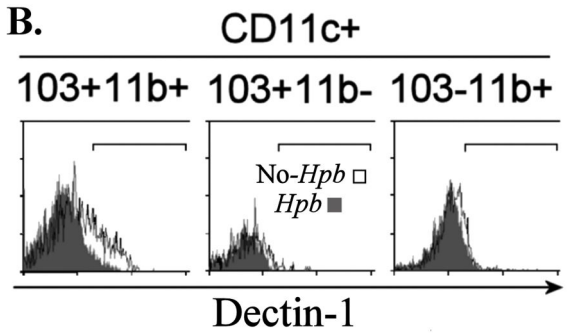
A. The upper two panels are representative FACS plots showing the percentage of LPMCs isolated from the TI that express CD11c. The table shows that the percentage of LPMCs expressing CD11c in the TI were similar in *Hpb*-infected (*Hpb*) and uninfected control mice (No *Hpb*).

B. The figure is an overlapping flow cytometry histogram gated on the $CD11c^+$ DCs in LPMCs isolated from the TI of *Hpb*-infected (*Hpb*) (gray area) and uninfected (no *Hpb*) control (white area) mice. The bracket in the histogram indicates the dectin-1 positive cell gate. The figures and the table show that the intestine contains fewer dectin-1 positive DCs after *Hpb* infection.

For both table A and B, the numbers are mean percentages ± SE from eight independent experiments containing 3–4 mice per group.



DC subsets (%)	103+11b+	103+11b-	103-11b+
No *Hpb*	37.4 ± 4.7	20.3 ± 1.6	10.5 ± 2.3
Hpb	47.4 ± 2.6	22.6 ± 3.9	8.3 ± 1.1
p	NS	NS	NS



Dectin-1+ (%)	103+11b+	103+11b-	103-11b+
No *Hpb*	25.9 ± 2.5	12.1 ± 1.6	31.3 ± 5.1
Hpb	11.2 ± 1.0	3.7 ± 0.7	27.9 ± 3.8
p	<0.01	<0.01	NS

Figure 6. *Hpb* infection decreases dectin-1 display on specific intestinal DC subsets

A. The upper two panels depict representative FACS plots showing the percentage of CD11c + DCs expressing CD103 and/or CD11b in LPMC isolated from the TI of *Hpb*-infected (*Hpb*) or uninfected (No *Hpb*) control mice. The table shows that the intestinal DCs from *Hpb*-infected and uninfected mice contain similar percentages of the three indicated DC subsets.

B. The three figures are representative overlapping flow cytometry histograms gated on $CD11c^+$ DC subsets in isolated LPMC from the TI of *Hpb*-infected (gray area) and uninfected control (white area) mice. The bracket in each histogram indicates the dectin-1-

positive cell gate. The table shows that the percentage of DCs displaying dectin-1 drops in the CD103+ CD11b+ CD11c+ and the CD103+CD11b− CD11c+ DC subsets after *Hpb* infection, $p < 0.05$.

For both **A)** and **B)**, the data in the tables are means ± SE derived from four independent experiments each using three mice/group.

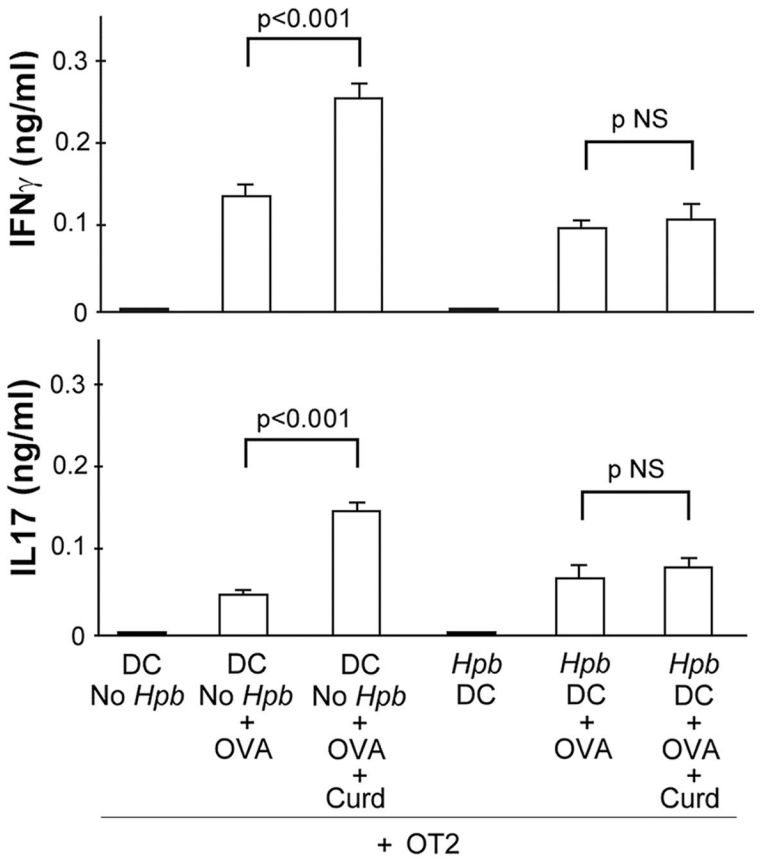
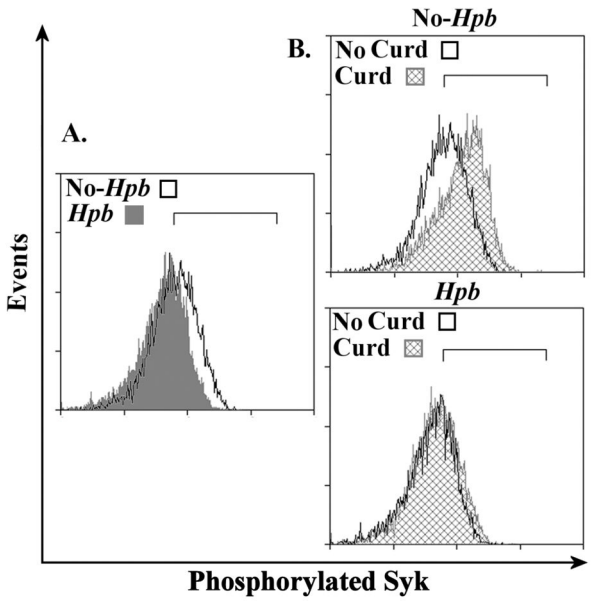


Figure 7. The dectin-1 agonist, curdlan, promotes cytokine secretion only from intestinal DCs isolated from uninfected mice

DCs isolated from the TI of uninfected mice (No *Hpb*) or from *Hpb*-infected mice (*Hpb*) were mixed with OT2 T-cells (OT2) and cultured 48h with or without OVA (50 ug/ml) in the present or absence of curdlan (100 ug/ml)(curd) as outlined in the methods section. After the incubations, cytokine concentrations in the supernatants were assayed with ELISAs. OVA stimulated IFNγ and IL17 secretion in cultures containing OT2 T cells and DCs from either source. However, curdlan enhanced cytokine secretion only in the presence of DCs from uninfected mice, $p < 0.001$. Data are means ± SE from 3 independent experiments for which each condition was cultured in triplicate. DCs were isolated from the TI of 3–4 mice for each experimental group.



A.

	p-Syk (%)
No-*Hpb*	53 ± 1.9
Hpb	33 ± 2.5
p	< 0.05

B.

p-Syk (%)	No-*Hpb*	*Hpb*
No Curd	52 ± 2.1	34 ± 1.6
Curd	68 ± 2.4	36 ± 1.8
p	< 0.05	NS

Figure 8. *Hpb* infection decreases curdlan-induced Syk phosphorylation

$CD11c^{+}103^{+}$ DC were isolated by FACS from dispersed LPMC derived from the TI of Rag mice. The DCs then were subject to intracellular staining (see material and methods) to determine the level of Syk phosphorylation in each cell. The three figures are representative overlapping flow cytometry histograms showing the degree of Syk phosphorylation in these cells. The bracket in each FACS plot indicates the window of phosphorated Syk-specific staining. Figure (A) shows that intestinal DCs from *Hpb*-infected mice (*Hpb*, gray area) have lower constitutive expression of phosphorylated Syk than do DC from uninfected mice (No *Hpb*, white area). Figure (B) shows that the dectin-1 agonist, curdlan, induces Syk phosphorylation in DCs from uninfected mice (No *Hpb*) (upper panel), while it fails to do so in DCs from infected mice (*Hpb*) (lower panel). The table shows the means of three individual experiments.

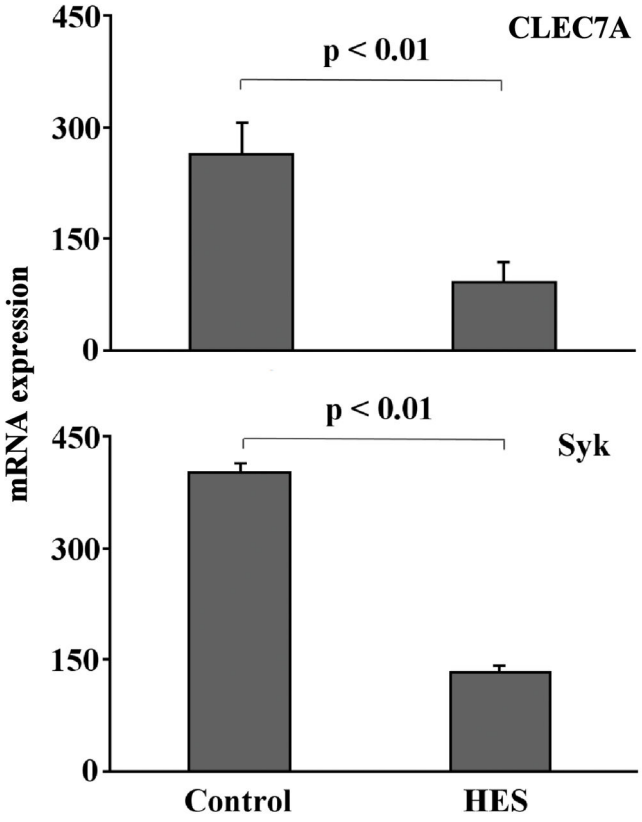


Figure 9. *Hpb* ESP (HES) inhibits Syk and CLEC7A mRNA expression in intestinal DCs

DCs isolated from the TI of Rag mice were culture *in vitro* for 2 hrs in the presence or absence of HES (50 ug/ml). DC RNA then was extracted and converted to cDNA. Quantitative rt-PCR was used to assess the levels of Syk and CLEC7A mRNA expression in these samples. Data were normalized relative to expression of the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Data are means ± SE from 3 separate experiments, and each experiment was performed in duplicate. The decreases in Syk and CLEC7A mRNA expression after HES exposure were both $p < 0.01$.