Duodenal Helminth Infection Alters Barrier Function of the Colonic Epithelium via Adaptive Immune Activation[▽]

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Chronic infection with intestinal helminth parasites is a major public health problem, particularly in the developing world, and can have significant effects on host physiology and the immune response to other enteric infections and antigens. The mechanisms underlying these effects are not well understood. In the current study, we investigated the impact of infection with the murine nematode parasite *Heligmosomoides polygyrus*, which resides in the duodenum, on epithelial barrier function in the colon. We found that *H. polygyrus* infection produced a significant increase in colonic epithelial permeability, as evidenced by detection of elevated serum levels of the tracer horseradish peroxidase following rectal administration. This loss of normal barrier function was associated with clear ultrastructural changes in the tight junctions of colonic epithelial cells and an alteration in the expression and distribution of the junctional protein E-cadherin. These parasite-induced abnormalities were not observed in SCID mice but did occur in SCID mice that were adoptively transferred with wild-type T cells, indicating a requirement for adaptive immunity. Furthermore, the helminth-induced increase in gut permeability was not seen in STAT6 knockout (KO) mice. Taken together, the results demonstrate that one of the mechanisms by which helminths exert their effects involves the lymphocyte- and STAT6-dependent breakdown of the intestinal epithelial barrier. This increase in epithelial permeability may facilitate the movement of luminal contents across the mucosa, thus helping to explain how helminth infection can alter the immune response to enteric antigens.

The integrity of the intestinal epithelium is essential for maintenance of the dynamic barrier that regulates absorption of nutrients and water and at the same time restricts uptake of luminal bacteria and bacterial products (3, 40). The barrier function of the intestinal epithelium is maintained by the apical junctional (AJ) complex, which includes several distinct structures known as tight junctions (TJs), adherens junctions, desmosomes, and gap junctions. The TJ is the major paracellular barrier and functions as a “fence” separating apical and basolateral compartments. The adherens junction forms a continuous belt and is crucial for the maintenance of intercellular adhesion (9, 24, 28). The tight junction and adherens junction are regulated by (i) interactions between transmembrane TJ/AJ proteins on the opposing cell plasma membranes, (ii) interactions involving scaffolding proteins that cluster and stabilize transmembrane components of TJ and AJ proteins, and (iii) the interactions mediated by actin binding proteins such as those in the zonula occludin (ZO) family, catenin, etc., which link the AJ complex to actin microfilaments (9, 14, 24, 28). Major transmembrane proteins in the apical junctional complex include occludin, claudins, junction adhesion molecules, and E-cadherin (14). The list of proteins that constitute the intercellular junction complex has been expanding.

Limited information is available about the effects of intestinal helminth infection, and the Th2 response it induces, on the structure and function of intercellular junctional proteins in

the intestinal epithelium. The results from these few studies reveal a clear localized effect of helminth infection on intestinal epithelial function in the regions of the intestine that the parasites inhabit. Infection with the small intestinal nematode parasite *Trichinella spiralis* results in decreased expression of occludin in the tight junction, leading to increased paracellular permeability of the jejunum (25). Infection with the nematode *Nippostrongylus brasiliensis* results in altered expression of E-cadherin, leading to a loss of adhesion in epithelial cells (ECs) in the small intestine (21). A challenge infection of *Heligmosomoides polygyrus* resulted in increased mucosal permeability of the small intestine (30). While the Th2 cytokine interleukin 13 (IL-13) is able to alter epithelial tight junctions by increasing claudin 2 expression (with little effect on claudins 3 and 4) (19, 29), eosinophils, an important component of the helminth-induced Th2 response (37), induce the downregulation of the tight junctional molecule occludin through release of the eosinophil-granule proteins (major basic protein) (16). A recent report showed that E-cadherin is induced in alternatively activated macrophages by IL-4 and IL-13 in a JAK/STAT6-dependent manner (35). E-cadherin is a transmembrane protein, whose intracellular domain is associated with β -catenin, p120, and α -catenin (36). On the cellular level, E-cadherin is concentrated at the adherens junction and forms tight junctions between epithelial cells through homophilic interactions with E-cadherin in adjacent cells. Most of the studies to date have examined the effect of helminth infection on local epithelial permeability, and it is unclear whether and how a small intestinal helminth infection affects the barrier function of the colon, the location with the highest concentration of luminal bacteria *in vivo*.

Using our recently established coinfection mouse model, we

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have found that coinfection with the helminth parasite *Heligmosomoides polygyrus* (which resides in the duodenum) exacerbates the colonic inflammatory response to the enteric bacterial pathogen *Citrobacter rodentium* via a STAT6-dependent pathway (5, 6). Although *Citrobacter* infection is normally confined to the surface of the colonic epithelium, we found that coinfection with *H. polygyrus* resulted in translocation of the bacteria to deeper tissues (5). In the current investigation, we determined whether and how small bowel *H. polygyrus* infection may alter epithelial barrier function in the colon.

MATERIALS AND METHODS

Mice. Six- to eight-week-old female BALB/c ByJ and STAT6 knockout (KO) (on a BALB/c background) mice were purchased from the Jackson Laboratory (Bar Harbor, ME), and SCID mice were obtained from Massachusetts General Hospital. All mice were fed autoclaved food and water and maintained in a specific-pathogen-free facility. All animal experiments were approved by the institutional Subcommittee on Research Animal Care.

***H. polygyrus* infection.** *H. polygyrus* was propagated as previously described and stored at 4°C until use (5). Mice were inoculated orally with 200 third-stage larvae.

Analysis of colonic permeability. Colonic permeability was determined *in vivo* by measuring the appearance in blood of rectally administered horseradish peroxidase (HRP). At 7 days after *H. polygyrus* infection, mice were deprived of food for 12 h. They were then anesthetized with Avertin (200 mg/kg of body weight; Avertin consists of 1 g of 3-bromoethanol and 0.62 ml of 2-methyl-2-butanol dissolved in 79 ml of deionized, distilled sterile water [27]), after which 50 μ l of phosphate-buffered saline (PBS) containing 0.6 mg of HRP (Sigma) was rectally administered with a feeding needle. Blood samples were then collected from the tail vein at 0, 45, and 120 min after HRP administration, and serum HRP concentrations were measured by an enzyme-linked immunosorbent assay (ELISA).

Transmission EM analysis. Small pieces of colon tissues from *H. polygyrus*-infected BALB/c mice or uninfected controls were collected and fixed in 2.0% glutaraldehyde in 0.1 M sodium cacodylate (Electron Microscopy Sciences, Hatfield, PA), pH 7.4. Ultrathin sections were prepared on a Reichert Ultracut E ultramicrotome. The ultrastructure of the tissues was examined using a JEOL 1011 transmission electron microscope (EM) at an acceleration voltage of 80 kV.

Immunofluorescence microscopy. Colon tissues were frozen in Tissue-Tek embedding medium as described previously (6). Sections (5 μ m) were cut on a cryostat and fixed in ice-cold acetone. The sections were then washed with PBS and blocked with PBS-1% bovine serum albumin (BSA) and avidin/biotin blocking agent (Vector Laboratories). The tissue sections were incubated with primary antibodies (Abs), including those recognizing the tight junction protein ZO-1 and adherens junction protein E-cadherin (Zymed). After overnight incubation at 4°C, the slides were incubated with fluorochrome-conjugated secondary antibodies and examined by immunofluorescence microscopy.

Lymphocyte isolation and adoptive transfer. Spleens and peripheral and mesenteric lymph nodes (MLN) from normal BALB/c mice were collected aseptically into complete Dulbecco's modified Eagle's medium (DMEM) (containing 10% fetal calf serum [FCS], 10 mM HEPES, 2 mM L-glutamine, 100 U penicillin/ml, 100 μ g of streptomycin/ml, 50 μ M 2-mercaptoethanol [2-ME], 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate; Invitrogen Life Technologies) as previously discussed (5). Lymphocyte suspensions were prepared from the MLN and spleens by pressing the cells through a 70- μ m nylon cell strainer (Falcon; BD Labware) in complete DMEM. Red blood cells were lysed. After being washed, total lymphocytes were resuspended at 5×10^7 cells/ml and adoptively transferred into SCID recipient mice (10^7 cells/mouse) via tail vein injection. The SCID recipient mice were infected with *H. polygyrus* on the same day. Helminth-infected SCID mice that did not receive any cells served as controls. For CD4⁺ T-cell adoptive transfer experiments, total lymphocytes were isolated from the spleens and MLN of normal BALB/c mice, and CD4⁺ T cells were purified using a magnetic cell separation system (MACS) (6). The purity of CD4⁺ T-cell preparation was over 90% as determined by fluorescence-activated cell sorting (FACS). Each SCID recipient mouse was injected with 2×10^6 to 5×10^6 CD4⁺ T cells via the tail vein.

Lymphocyte isolation and *in vitro* restimulation. SCID recipient mice (helminth infected with or without lymphocyte transfer) were sacrificed 7 days after helminth infection. Lymphocyte suspensions were prepared from the MLN and spleens as described above. Cells (5×10^6 cells/ml) were cultured in 24-well

plates in the presence or absence of surface-bound anti-CD-3 monoclonal antibody (MAB; 10 μ g/ml), and culture supernatants were collected 72 h later and stored at -20°C until they were assayed for cytokine production.

Measurement of adoptively transferred T-cell cytokine production. Th1 (gamma interferon [IFN- γ]) and Th2 (IL-4) cytokines were assayed using ELISA as previously described (5). ELISA capture (BVD4-1D11, IL-4; R4-6A2, IFN- γ) and biotinylated second antibodies (Abs) (BVD6-24G2, IL-4; XMG1.2, IFN- γ) were purchased from BD Pharmingen. Standard curves were obtained using recombinant murine IFN- γ and IL-4 (Genzyme).

Epithelial cell isolation. Mice were euthanized, and each colon was removed and flushed for 5 min with 37°C Hanks' buffer containing 30 mM EDTA, as described previously (7). The tissue was then slit longitudinally and rinsed in calcium- and magnesium-free Hanks balanced salt solution (HBSS) containing 2% fetal calf serum (FCS) (CMF2%), cut into 1-cm pieces, and placed into ice-cold CMF2%. Following vigorous shaking, the tissue was then placed into calcium- and magnesium-free HBSS (containing 10% FCS, 1 mM EDTA, 1 mM dithiothreitol, 100 units/ml penicillin, and 100 μ g/ml streptomycin) (CMF10%) and incubated at 37°C for 30 min. After shaking, the supernatant was recovered and passed through a 100- μ m cell strainer (Becton Dickinson) and centrifuged at 3,000 rpm (4°C) for 10 min. The pellet was washed with 20 ml of ice-cold Hanks' buffer twice and dissolved in cell lysis buffer (8, 26).

SDS-PAGE and Western blotting. Colonic epithelial cellular lysates were prepared, and protein content was determined using a bicinchoninic acid (BCA) protein assay (Bio-Rad Laboratories). Cellular lysates were mixed with XT sample buffer (Bio-Rad, Hercules, CA). Proteins were separated through a Criterion XT precast gel (10% Bis-Tris) (Bio-Rad) and transferred to nitrocellulose. Immunoblot analyses were performed using rat anti-E-cadherin antibody (Zymed). Blots were developed with the appropriate HRP-conjugated secondary antibody and chemiluminescence (Super Signal ECL kit; Pierce). Each blot was analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein expression as an internal loading control, using a specific rabbit anti-mouse GAPDH antibody (Santa Cruz Biotechnology). The density of the E-cadherin band was normalized to that of GAPDH in each case.

Statistical analysis. All results were expressed as the mean $\pm$ standard error of the mean (SEM). *n* refers to the number of mice used. Statistical differences were determined using a two-tailed Student *t* test with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). A *P* value of <0.05 was considered significant.

RESULTS

***H. polygyrus* infection results in a significant increase in colonic epithelial permeability.** It has been demonstrated that *H. polygyrus* infection induces functional alterations in small intestinal mucosa (30). Since we have shown previously that *H. polygyrus* influences the progression and severity of bacterium-mediated colonic inflammation (5), we decided to examine the impact of helminth infection on epithelial barrier function in the colon. We infected BALB/c mice with *H. polygyrus* and then introduced HRP as a tracer rectally into the lumen of the colon. Serum HRP level was determined at different time points after rectal administration. As shown in Fig. 1, control mice that were not infected with helminth showed a very limited amount of HRP in their sera. In sharp contrast, serum HRP levels were significantly higher in helminth-infected mice (starting 45 min after administration of HRP). These results, therefore, suggest that infection with the small intestinal parasite *H. polygyrus* results in a significant increase in colonic epithelial permeability (Fig. 1).

Helminth-infected mice have clear abnormalities of intercellular junctions in the colon. To directly determine the effect of *H. polygyrus* infection on the colonic epithelium, we collected colon tissues from normal and *H. polygyrus*-infected BALB/c mice and examined the ultrastructural pathology using transmission electron microscopy. Our EM analysis showed that helminth-infected mice had clear abnormalities of intercellular junctions in the colon (Fig. 2). These abnormalities

FIG. 1. Helminth infection results in an increase in colonic epithelial permeability in BALB/c mice. Control mice or animals infected 7 days earlier with *H. polygyrus* (H.p.) were subjected to intrarectal administration of HRP. The appearance of HRP in serum was measured 0, 45, and 120 min later. Data shown represent the time course of change in serum HRP concentration. *, $P < 0.05$; $n = 3$ to 6/group. The data shown here are from one of two experiments showing similar results.

included structural alterations in the apical junctional complex and widening and distortion of the paracellular space. These results, therefore, support the idea that *H. polygyrus* infection compromises the mechanical integrity of the colonic epithelial

monolayer at a location distinct from where the parasite resides in the small intestine.

Helminth infection induces abnormalities of E-cadherin expression and distribution in the colon epithelium. To elucidate the cellular and molecular basis for the *H. polygyrus*-induced disruption of colonic epithelial barrier function, we examined the expression and distribution of junctional proteins using immunofluorescence microscopy. Frozen colonic sections from uninfected and *H. polygyrus*-infected mice were prepared and stained by immunofluorescence with antibodies against the junctional proteins ZO-1 (a tight junction protein located on the cytoplasmic membrane surfaces of intercellular tight junctions) and E-cadherin (a key molecule in the establishment and stabilization of cellular junctions in epithelial cells) (36). The tight junction protein ZO-1 was found to be expressed and localized similarly in helminth-uninfected and -infected colons (Fig. 3A and B). In contrast, helminth infection induced a clear alteration in the distribution of the adhesion molecule E-cadherin: in the colon from uninfected mice, this protein was localized predominantly on the cell membrane (Fig. 3C), whereas in the tissues from infected animals, the membrane localization was perturbed and much of the protein appeared

FIG. 2. Infection with *H. polygyrus*, a small intestinal parasite, induces alterations in colonic epithelial cell-cell junctions. Colon tissues were collected from individual uninfected control (a and b) and *H. polygyrus*-infected (7 days after infection, c and d) BALB/c mice, and their morphology was examined by EM. Arrows point to cell-cell junction. (a) Tight junction; (b) adherens junction; (c) desmosome; (d) gap junction. An asterisk indicates a disrupted cell junction.

FIG. 3. Helminth infection induces disorganized distribution and reduced expression of E-cadherin in colonic tissues. BALB/c mice were infected with *H. polygyrus* for 7 days. Colon tissues were collected in OCT. Frozen sections were prepared and stained by immunofluorescence with anti-ZO-1 or anti-E-cadherin antibodies with Cy3 (A and B)- or FITC (C and D)-conjugated secondary antibodies. The distribution of ZO-1 and E-cadherin from three to five individual mice in each group was examined. Representative figures from each group are shown in panels A to D. (A and C) Tissues from uninfected mice. (B and D) Tissue sections from *H. polygyrus*-infected mice. (E) Colonic epithelial cells were isolated, and cellular lysates were prepared. Colonic cellular proteins were separated by SDS-PAGE and transferred to nitrocellulose. Immunoblots were performed using anti-E-cadherin and anti-GAPDH antibodies. Each lane represents the colonic epithelial cell sample from an individual mouse. (F) Densitometry on E-cadherin showed a decrease of E-cadherin expression after normalization with GAPDH. *, $P < 0.05$. The data represent the means $\pm$ SEM for three mice in each group.

FIG. 4. Helminth-induced changes in colonic epithelial permeability in SCID mice. (A) Reconstitution of helminth-infected SCID mice with total lymphocytes or purified CD4⁺ T cells results in increased colonic epithelial permeability. Unfractionated lymphocytes or CD4⁺ T cells were prepared from spleen and lymph nodes of normal BALB/c mice and adoptively transferred into SCID mice, which were infected with *H. polygyrus* or left uninfected. Seven days later, HRP was administered rectally to the lumen of the colon, and blood HRP levels were measured at 0 and 45 min after HRP inoculation. *, $P < 0.05$. $n = 3$ to 5/group. The data shown here are from one of two experiments showing similar results. (B and C) Helminth infection fails to induce alteration in distribution of E-cadherin in colon tissues of SCID mice. SCID mice were infected with *H. polygyrus* for 7 days. Colon sections were stained by anti-E-cadherin or anti ZO-1 with FITC- or Cy3-conjugated secondary antibodies. (D) Adoptive transfer of total lymphocytes into helminth-infected SCID mice results in alteration in distribution of E-cadherin in colon tissues. SCID mice that were infected with helminth and adoptively transferred with lymphocytes (on the same day; 5×10^6 cells/mouse) were sacrificed at 7 days postinfection. Colon sections were stained by anti-E-cadherin or anti ZO-1 with FITC- or Cy3-conjugated secondary antibodies. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (in blue). Representative images display the distribution of E-cadherin and ZO-1 in colonic sections for one mouse from each of the treatment groups. There were 3 to 5 mice examined in each group, showing similar results. (B) Normal SCID mice. (C) *H. polygyrus*-infected SCID mice. (D) *H. polygyrus*-infected, cell transferred SCID mice (Hp + cells). Green, E-cadherin; red, ZO-1. Magnification, $\times 200$.

to be in the cytoplasm (Fig. 3D). These observations provide evidence to suggest that *H. polygyrus* infection induces alterations in the distribution of specific junctional proteins in colonic epithelium. In addition, Western blot analysis revealed a clear reduction in the expression levels of E-cadherin in colonic epithelial cells that were purified from *H. polygyrus*-infected mice compared to the levels in epithelial cells from uninfected mice (Fig. 3E and F). These observations indicate that *H. polygyrus* infection alters the amount and distribution of E-cadherin in colonic epithelial cells, abnormalities that could contribute to the opening of the tight junction and an increase of paracellular permeability.

Helminth infection fails to induce alterations in intestinal epithelial permeability in SCID mice. Intestinal helminth infection induces profound immune activation in the host. To further elucidate the potential mechanism underlying the *H. polygyrus*-induced alteration in colonic epithelial barrier func-

tion, we determined whether the adaptive immune system is required for the small intestinal helminth to exert its effects on colonic epithelial physiology. We infected SCID mice (which lack both T and B lymphocyte populations) with *H. polygyrus* and measured serum HRP levels at different time points after rectal administration as described above. In contrast to the results in BALB/c mice (Fig. 1), the serum HRP levels did not differ significantly between *H. polygyrus*-infected and uninfected SCID mice (Fig. 4), which indicates a requirement for lymphocytes in the induction of increased colonic permeability by *H. polygyrus*. This idea was further supported by results from immunofluorescence microscopic studies, which showed that *H. polygyrus* failed to induce an alteration in the distribution of the junctional protein E-cadherin in the colonic tissues of SCID mice (Fig. 4B and C). Similar to what was seen in BALB/c mice, the tight junctional protein ZO-1 was not affected by helminth infection in SCID mice (Fig. 4). These

FIG. 5. T-cell cytokine profile in SCID mice with lymphocyte reconstitution and *H. polygyrus* infection. SCID mice were adoptively transferred with lymphocytes isolated from normal BALB/c mice and infected with or without *H. polygyrus*. SCID recipient mice were sacrificed 7 days after helminth infection. Lymphocyte suspensions were prepared from MLN and restimulated with anti-CD3 MAb (10 μ g/ml). Th2 cytokine production (left panel, IL-4; right panel, IL-10) was measured using ELISA. The data shown are from one of two experiments performed showing similar results ($n = 3$ to 5 mice per group).

results, therefore, suggest a role for adaptive immune activation in the alteration of intestinal epithelial barrier function by *H. polygyrus*.

Reconstitution of *H. polygyrus*-infected SCID mice with lymphocytes results in increased colonic epithelial permeability and redistribution of E-cadherin. *H. polygyrus* has been shown to induce a vigorous, Th2-type polarized immune response (12), which may alter the host response to other pathogens or antigens (13, 20, 31, 32, 34). To directly demonstrate the role of the adaptive immune system in the immune modulation of colonic epithelial barrier function during intestinal helminth infection, we next adoptively transferred unfractionated lymphocytes isolated from the spleens and lymph nodes of normal BALB/c mice into SCID mice, which were infected with 200 third-stage *H. polygyrus* larvae or left uninfected. Seven days later, we introduced HRP rectally to the lumen of the colon and measured serum HRP levels at different time points thereafter. We found that adoptive transfer of lymphocytes in helminth-infected SCID mice resulted in a significant increase in colonic epithelial permeability in these mice, as evidenced by detection of increased circulating HRP levels (Fig. 4A). Similar results were also obtained from SCID mice that were adoptively transferred with purified CD4⁺ T cells (Fig. 4A). In contrast, the serum HRP levels for helminth-infected SCID mice without lymphocyte transfer did not differ from the levels for uninfected control mice (Fig. 4A). The clear increase in circulating HRP levels in helminth-infected SCID mice that received CD4⁺ T lymphocytes indicates that the helminth-induced alteration in intestinal permeability is T cell dependent.

To further elucidate the mechanism responsible for the *H. polygyrus*-induced alterations in the colonic epithelial barrier function, we collected colon tissues from helminth-infected and uninfected SCID mice that were adoptively transferred with or without lymphocytes and examined the expression and distribution of tight junction proteins (ZO-1 and E-cadherin). Immunofluorescence microscopic analysis revealed that the expression and localization of tight junction protein ZO-1 were similar in uninfected and infected colons with or without lymphocyte transfer (Fig. 4B to D). In contrast, adoptive transfer of lymphocytes to helminth-infected SCID mice induced a clear alteration in the distribution of E-cadherin (Fig. 4D): in the colons from uninfected mice or helminth-infected SCID mice (without lymphocyte reconstitution), this protein was lo-

calized predominantly to the cell membrane (Fig. 4B and C), whereas in the tissues from infected animals that received lymphocytes, the membrane localization was perturbed and much of the protein appeared to be in the cytoplasm (Fig. 4D). This demonstrates that adoptive transfer of lymphocytes in helminth-infected SCID mice results in alterations in the distribution of junctional proteins (E-cadherin), which is associated with an increased permeability in colonic epithelium (Fig. 4A). These observations provide strong evidence for the role of lymphocytes in the alteration of colonic epithelial barrier function during intestinal helminth infection.

Adoptively transferred T cells in helminth-infected SCID mice display a Th2-biased cytokine profile. *H. polygyrus* is known to elicit a Th2-biased immune response. To characterize the immune phenotype of adoptively transferred lymphocytes in SCID recipient mice that were infected with the helminth parasite, we collected MLN lymphocytes from SCID recipient mice and restimulated the cells *in vitro* with plastic surface bound anti-CD3. Cytokine secretion of the cells was determined by ELISA (5). As shown in Fig. 5, a Th2-biased cytokine production profile (IL-4 and IL-10) was detected in cells isolated from helminth-infected SCID mice that were adoptively transferred with total lymphocytes. We also observed undistinguishable low levels of IFN- γ and IL-17 production in cells from helminth-infected SCID recipient and helminth-infected BALB/c mice (data not shown). These results indicate that *H. polygyrus* infection in SCID mice that were reconstituted with lymphocytes stimulates the differentiation and development of a Th2 response.

Helminth infection results in unchanged colonic permeability in STAT6 KO mice. Helminths induce Th2 polarization of helper T cells that is characterized by upregulation of Th2 cytokines, elevated serum IgE levels, eosinophilia, and increased numbers of mucosal mast cells (12). We next tested the hypothesis that the observed alterations in colon epithelial permeability in helminth infected mice may be mediated via the helminth-induced Th2 immune response by utilizing Th2-deficient STAT6^{-/-} mice. We repeated the experiments described in the legend to Fig. 1 and measured the level of rectally administered HRP that appeared in the sera of helminth-infected or uninfected wild-type and STAT6 KO mice. In contrast to the observations from BALB/c mice showing a marked increase in serum HRP levels after infection (Fig. 1), serum HRP levels were found to be similar in helminth-in-

FIG. 6. *H. polygyrus* infection results in unchanged colonic epithelial permeability in STAT6 KO mice. (A) Uninfected STAT6 KO or STAT6 KO mice infected 7 days earlier with *H. polygyrus* were subjected to intrarectal administration of HRP. The appearance of HRP in serum was measured 0, 45, and 120 min later. Data shown represent the time course of change in serum HRP concentration. $n = 3$ to 6/group. Significant differences were detected in serum HRP levels between uninfected and infected BALB/c mice. *, $P < 0.05$. Such differences were not seen in STAT6 KO mice. The data shown here are from one of two experiments showing similar results. (B to E) Distribution of E-cadherin was examined using methods described in the legend to Fig. 3. (B) Tissue from uninfected STAT6 KO mice. (C) Tissue section from *H. polygyrus*-infected STAT6 KO mice. (D) Colonic tissue section from normal BALB/c mice. (E) Tissue section from *H. polygyrus*-infected BALB/c mice. (F and G) Helminth infection induces disorganized distribution and reduced expression of E-cadherin in colonic tissues of BALB/c mice but not in STAT6 KO mice. *, $P < 0.05$; $n = 3$ /group. Data for BALB/c mice shown in panels F and G represent the same results as data presented in Fig. 3E and F.

ected and uninfected STAT6 KO mice (Fig. 6A). In addition, confocal immunofluorescence microscopy revealed that the expression and localization of junctional protein E-cadherin, which was found to be localized predominantly to the cell membrane, were similar in uninfected and infected colons of STAT6 KO mice (Fig. 6B, C, F, and G). These observations suggest that the helminth-induced increase in gut permeability is STAT6 dependent. Western blot analysis revealed that the amount of E-cadherin detected in STAT6 KO mice with *H. polygyrus* infection appeared to be smaller than that detected in uninfected STAT6 KO mice. The differences, however, were not statistically significant. Interestingly, we also observed an elevated basal level of serum HRP in uninfected STAT6 KO mice after rectal administration compared to the level in uninfected BALB/c mice (Fig. 6A). The elevated serum HRP levels in uninfected STAT6 KO mice correlated with a reduction in basal expression of E-cadherin in colonic epithelial cells (Fig. 6G).

DISCUSSION

The results from our current investigation provide evidence to demonstrate that the small intestinal Th2-stimulating helminth parasite *H. polygyrus* compromises mucosal barrier function in the colonic epithelial monolayer as evidenced by an increase in colonic epithelial permeability (Fig. 1), abnormalities of intercellular junctions (Fig. 2), and alterations in the distribution of specific junctional proteins (E-cadherin) in the colon (Fig. 3) in wild-type BALB/c mice but not in lymphocyte-deficient SCID or Th2-deficient STAT6^{-/-} mice. Reconstitution of helminth-infected SCID mice with lymphocytes resulted in recapitulation of helminth-induced morphological and functional alterations that were seen in helminth-infected wild-type mice with Th2 polarization of the transferred T cells. These results indicate that one of the mechanisms by which helminths exert their effects involves the T lymphocyte- and STAT6-dependent alteration of colonic epithelial barrier function.

The impacts of intestinal helminth infection and helminth-induced Th2 cytokine responses on intestinal epithelial function have been studied using both *in vivo* and *in vitro* approaches. The previous studies have shown a clear localized effect of helminth infection on intestinal epithelial function in the regions of the intestine where the worms reside. For example, infection with *N. brasiliensis* induced a loss of adhesion in epithelial cells in the small intestine (21), and *T. spiralis* infection resulted in increased paracellular permeability of the jejunum (25). A secondary challenge infection of *H. polygyrus* resulted in an increased mucosal permeability of the small intestine (30). The *H. polygyrus*-induced, IL-4/IL-13-mediated effects on small intestinal epithelial cell function have been suggested to be mediated through direct effects on epithelial cells and through indirect, enteric nerve-mediated prosecretory effects (30). One of the major observations from the current study is that infection with *H. polygyrus*, a nematode parasite that resides mostly in the duodenum of the small intestine (2), impairs epithelial barrier function in the colon. Through assessment of mucosal-to-serosal flux of the macromolecular probe HRP, a technique that has been used extensively for measuring transcellular transport through intestinal epithelial cells (33), we showed that *H. polygyrus* infection has a marked impact on the transcellular pathway of epithelial cells. Normally, soluble luminal antigens are transported across the intestinal epithelium via one of two routes, the transcellular pathway or the paracellular pathway. Transcellular permeability is important for appropriate transport of macromolecules across the epithelium, which helps to regulate immune activation. The paracellular pathway occurs between ECs, which is restricted by intercellular tight junctions that limit the passage of macromolecules (1). Our results from electron microscopic (Fig. 2) and immunofluorescence (Fig. 3) analysis provide evidence to suggest that *H. polygyrus* infection also induces alterations in the paracellular pathway, suggesting the impact of helminth infection on both paracellular and transcellular pathways, contributing to increased epithelial permeability. These observations are supported by the results showing that intestinal helminth infection increases the intestinal translocation/absorption of bacterial products such as lipopolysaccharide (LPS) into the portal circulation by altering barrier function (10) and that IL-4 treatment of keratinocytes significantly enhanced the permeability to high-molecular-mass material (40-kDa fluorescein isothiocyanate [FITC]-dextran) through modification of intercellular adhesion molecules (23). In contrast, a recent study that used an Ussing chamber approach measured mucosal permeability and secretion *ex vivo* and showed that *H. polygyrus* infection increased colonic mucosal resistance (34). The differences in helminth-induced colonic permeability between this report (34) and our current study may be due to the differences in the experimental approaches used. The Ussing chamber-based approach used in the previous study (34) measures the short-circuit current as an indicator of net ion transport taking place across the epithelium. In the current study, an *in vivo* approach was utilized by direct administration of the soluble protein HRP rectally in mice. The transepithelial transport of HRP, which can be mediated by both transcellular and paracellular pathways (1), is shown to be affected by *H. polygyrus* infection.

The permeability of the intestinal epithelium depends on the

regulation of the intercellular tight junctions (11) by both immunologic and pathophysiologic stimuli. In the current study, we demonstrate for the first time that a small intestinal helminth infection compromises the mechanical integrity of the colonic epithelial monolayer at a location distinct from where it resides in the small intestine and alters the localization patterns of the intercellular junctional protein E cadherin in colonic epithelium, resulting in impaired epithelial barrier function *in vivo*. These results may offer a mechanistic explanation for the observed increased permeability in colonic tissue in helminth-infected mice. Our data are supported by the results obtained by Hyoh et al., who used another small intestine-dwelling nematode, *N. brasiliensis*, and showed that infection with this intestinal nematode resulted in altered expression of E-cadherin in the small intestine, leading to a loss of adhesion in epithelial cells (21). Other studies have shown that Th2 cytokines IL-4 and IL-13 downregulate E-cadherin expression in colon cancer cell lines (22) and in keratinocytes (15). In line with these observations, our immunoblotting experiments showed that helminth infection resulted in a reduction in E-cadherin expression at the protein level in colon tissues (Fig. 3E). Our findings indicate that the abundance as well as the distribution of E-cadherin protein in colon tissue can be affected by *H. polygyrus* infection. It was suggested that high levels of E-cadherin may allow the cells to form homotypic clusters and interact with epithelial cells (35). As E-cadherin can act as an orchestrator of epithelial cell biology (35), a dysregulation in E-cadherin expression on the cell surface observed in the colonic epithelium of helminth-infected mice (Fig. 3D and 4D) may have significant consequences, contributing to disrupted junctional interaction between colonic epithelial cells (Fig. 2) and leading to increased permeability. It has also been suggested that disruption of E-cadherin-mediated junctions between epithelial cells may result in specific Th2 cell recruitment and promotion of Th2-type inflammation (18). It is clear that junctional abnormalities and functional alterations of colonic epithelial cells that are associated with helminth infection and/or helminth-induced immune responses can contribute significantly to the increased colonic epithelial permeability in infected hosts, promoting immune activation as a result of increased movement of luminal antigens and/or microorganisms across the epithelial monolayer.

Due to the differences in the site of the intestinal localization of *H. polygyrus* (mostly in the duodenum) and its observed effects on the colonic epithelial barrier function, it is unlikely that *H. polygyrus* directly contributes to the alteration of colonic barrier functions. In the current study, we tested the hypothesis that the small intestinal helminths may affect intestinal epithelial barrier function indirectly via helminth-induced adaptive immune activation by utilizing both the T- and B-cell-deficient SCID and the Th2-deficient STAT6 KO mice. Although a recent study showed that in SCID mice, *H. polygyrus* infection resulted in a significant reduction in the villus length and a significant increase in the crypt length compared to the levels for WT mice (17), the results from the present study show that the intestinal epithelial permeability was not altered by *H. polygyrus* infection in SCID (as well as STAT6 KO) mice. However, lymphocyte reconstitution of helminth-infected SCID mice resulted in a significant increase in colonic epithelial permeability and a clear alteration in the distribution of

E-cadherin (Fig. 4) in the colon tissues of these recipients. Analysis of the responses of the transferred T cells in helminth-infected SCID recipient mice revealed a Th2-biased cytokine response (Fig. 5) that was very similar to that observed for infected BALB/c mice. These results, therefore, provide strong evidence to demonstrate a role for activated lymphocytes, and Th2 cells in particular, in the alteration of intestinal barrier function during intestinal helminth infection.

The Th2 cytokines IL-4 and IL-13 can affect their target cells by activation of STAT6 or phosphatidylinositol 3-kinase (PI3K) pathways. Previously, Ceponis et al. examined the signal transduction events involved in IL-4 and IL-13 regulation of epithelial paracellular permeability using T84 cells and model human colonic epithelial cells and provided evidence for PI3K as the major proximal signaling event in IL-4 and IL-13 regulation of transepithelial resistance (TER) (4). A more recent study, however, provided evidence to suggest that Th2 (IL-13)-dependent barrier regulation does not require PI3K activity but may involve STAT6, as inhibition of STAT6, but not inhibition of PI3K, prevented IL-13-induced TER loss (38). In line with the latter observations, results from the current study showing that *H. polygyrus* infection failed to alter the permeability of colonic epithelium in STAT6 KO mice (Fig. 6) support a role for STAT6 in regulating intestinal epithelial barrier function during helminth infection. Moreover, our study provides evidence to suggest that the basal level of permeability of colonic epithelial cells to HRP is higher in STAT6 KO mice (than in their WT BALB/c controls). The enhanced basal colonic permeability in uninfected STAT6 KO mice was found to be associated with reduced expression of E-cadherin in colonic epithelial cells. These observations further support a role for E-cadherin in the maintenance of gut permeability and suggest the potential involvement of the STAT6 signaling pathway and/or Th2 immune responses in regulating the function of E-cadherin in the gut. However, the mechanism responsible for this is unclear and is currently being actively investigated in our laboratory.

In conclusion, our investigation provides evidence that one of the potential mechanisms by which helminths exert their effects on intestinal mucosa involves the helminth-induced and lymphocyte-dependent alterations of the integrity of the colonic epithelium and intestinal epithelial barrier functions. The results from our prior studies (5, 6, 39) together with the current investigation provide evidence to indicate that the modulation of the intestinal mucosal immune system by helminth parasites involves multiple mechanisms, including regulatory as well as effector cells in both the innate and the adaptive immune systems. A better understanding of the immunomodulatory effects of helminths will yield information not only for establishing novel and more-effective treatments for immune-mediated diseases but also for the design of effective intestinal vaccines for the prevention and treatment of microbial diseases in areas where chronic intestinal helminth infection is a significant problem.

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