Increased presence of effector lymphocytes during Helicobacter hepaticus-induced colitis

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Abstract

AIM: To identify and characterize drosophila mothers against decapentaplegic (SMAD)3-dependent changes in immune cell populations following infection with Helicobacter hepaticus (H. hepaticus).

METHODS: SMAD3+/+ (n = 19) and colitis-resistant SMAD3−/+ (n = 24) mice (8-10 wk of age) were infected with H. hepaticus and changes in immune cell populations [T lymphocytes, natural killer (NK) cells, T regulatory cells] were measured in the spleen and mesenteric lymph nodes (MsLNs) at 0 d, 3 d, 7 d and 28 d post-infection using flow cytometry. Genotype-dependent changes in T lymphocytes and granzyme B+ cells were also assessed after 28 d in proximal colon tissue using immunohistochemistry.

RESULTS: As previously observed, SMAD3+/+, but not SMAD3−/+ mice, developed colitis, peaking at 4 wk post-infection. No significant changes in T cell subsets were observed in the spleen or in the MsLNs between genotypes at any time point. However, CD4+ and CD8+CD62Llo cells, an effector T lymphocyte population, as well as NK cells (NKp46/DX5+) were significantly higher in the MsLNs of SMAD3−/− mice at 7 d and 28 d post-infection. In the colon, a higher number of CD3+ cells were present in SMAD3−/− compared to SMAD3+/− mice at baseline, which did not significantly change during infection. However, the number of granzyme B+ cells, a marker of cytolytic lymphocytes, significantly increased in SMAD3−/− mice 28 d post-infection compared to both SMAD3+/− mice and to baseline values. This was consistent with more severe colitis development in these animals.

CONCLUSION: Data suggest that defects in SMAD3 signaling increase susceptibility to H. hepaticus-induced colitis through aberrant activation and/or dysregulation of effector lymphocytes.

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Key words: Transforming growth factor-β; Colitis; Drosophila mothers against decapentaplegic; Colon cancer; T lymphocytes

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Individuals with inflammatory bowel disease (IBD), particularly ulcerative colitis (UC), are at a higher risk of developing colon cancer than the general population. A meta-analysis of 116 studies indicated that the prevalence of colon cancer in patients with UC is approximately 3.7% (95% CI: 3.2-4.2), with the cumulative probability reaching 18% by 30 years regardless of disease severity. Although the etiology of UC is poorly understood, there are indications that the immune system of individuals with UC reacts abnormally to bacteria in the digestive tract. This altered immune response leads to the inflammation-associated pathology of IBD.

Imbalances in both innate and adaptive immune cells, such as natural killer (NK) cells and T cell subsets, including CD4+ and CD8+ T cells and CD4/CD25/Foxp3+ T regulatory (Treg) cells, are associated with the pathogenesis of IBD. The inflammation and damage caused by increased secretion of inflammatory cytokines during an active disease state is thought to be triggered by cytotoxicity against the commensal bacteria. For example, levels of NK cytototoxicity in UC are related to the clinical stage of the disease. In active disease states, NK cells are present in normal numbers, but are functionally defective, whereas NK cells exhibit normal cytotoxic activity in an inactive disease state. Induction of inflammatory cytokines can also result from the disruption of the homeostatic balance between Treg and effector T helper (Th) cells. Elevated levels of pro-inflammatory CD4+ T cells lead to excess cytokine/chemokine production, thereby recruiting additional leukocytes and influencing the severity of the inflammatory response. CD8+ T cells are also important in the pathogenesis of UC in humans, as demonstrated by extensive CD8+ T cell infiltration within intestinal lesions contributing to mucosal damage.

Transforming growth factor (TGF)-β is a multifunctional cytokine that plays an important role in epithelial and immune cell homeostasis. TGF-β mediates many diverse biological functions on different cell types through receptor-mediated phosphorylation and activation of the drosophila mothers against decapentaplegic homolog (SMAD) family proteins, notably SMAD2 and SMAD3, which migrate to the nucleus and induce transcription of a targeted set of genes. Dysfunctions in one or more components of TGF-β signaling are commonly observed in human IBD and during colon cancer development. For example, loss of expression of the TGF receptor type II is observed in 90% of microsatellite unstable colon cancers, leading to loss of growth regulation in epithelial cells. Additionally, although the TGF-β isoform is overexpressed in the colon of individuals with IBD, nuclear signaling is impaired due to increased levels of SMAD7. SMAD7 normally inhibits TGF-β signaling by blocking activation of SMAD2/3 in response to receptor-ligand binding. Normalizing SMAD7 expression restores TGF-β signaling through SMAD3 and inhibits proinflammatory cytokine production by lamina propria mononuclear cells.

Impairments in one or more components of the TGF-β signaling pathway are implicated in intestinal inflammation in rodent models. For example, homologous knockout of the TGF-β1 gene in mice causes an excessive inflammatory response in multiple organs, including the heart, lungs, and intestinal tract leading to premature death. Additionally, Maggio-Price et al have demonstrated that disruption of the transcription factor SMAD3 modulates colitis susceptibility following infection with certain Helicobacter spp. Among these, Helicobacter hepaticus (H. hepaticus) is a Gram-negative spiral bacterium that colonizes the lower intestine and the hepatobiliary tract of mice. Although generally asymptomatic, infection can lead to hepatic and intestinal inflammation in certain strains of immunodeficient mice. In the complete absence of SMAD3 signaling, H. hepaticus induces a moderate inflammatory response in the cecum and colon, eventually leading to mucinous adenocarcinoma formation after 15-30 wk. It is generally accepted that chronic low levels of inflammation lead to cancer promotion and progression, therefore, the SMAD3 mouse model is very similar to the development of specific human cancers where pathogen-induced inflammation is necessary (but not sufficient) to cause dysplasia and tumor formation.

Using this model, the focus of the current study was to investigate the effect of SMAD3 deficiency on changes in local and systemic immune cell populations following infection with H. hepaticus. We hypothesized that colitis susceptibility in SMAD3−/− mice induced by H. hepaticus is associated with altered immune cell populations compared to colitis resistant SMAD3+/− mice. The aims of this study were to: (1) characterize the colitis induced by H. hepaticus in colitis-sensitive SMAD3−/− vs resistant SMAD3+/− mice; (2) compare the immune cell population changes in the spleen and mesenteric lymph nodes (MsLN); and (3) compare local immune cell changes by immunohistochemistry in the colon.

**MATERIALS AND METHODS**

**Murine model**

SMAD3+/− and SMAD3−/− (129-Smad3+/−) mice were bred in-house. Homozygous males and heterozygous females were mated to obtain both SMAD3+/− and SMAD3−/− pups. Genotypes were confirmed by polymerase chain reaction (PCR). Animals were housed under specific pathogen-free (SPF) conditions in 60 square
inch plastic cages (maximum of five adult mice per cage) with microisolator lids in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at Michigan State University. SPF conditions were assured through quarterly serology testing by Charles Rivers (Wilmington, MA, United States) and in-house testing for ectoparasites, endoparasites and fecal Helicobacter species (PCR). Full necropsies (including culture and sensitivity) were performed at least yearly on rodent breeding colonies. Animal rooms were maintained at 23.3 ± 2.2 °C with a 12-h light/dark cycle. Mice were fed Harlan Teklad 7913 rodent chow and sterile water ad libitum. Animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

**Bacterial culture and infection**

The wild-type *H. hepaticus* strain 3B1 (ATCC 51488) was utilized for these experiments. Isolates were aseptically streaked onto sheep blood agar and incubated at 37 °C for 24-48 h inside GasPak™ gas generating pouch systems (BD Diagnostic Systems, Sparks, MD, United States). Mice were infected as previously described[19]. Briefly, bacteria were collected and resuspended in tryptic soy broth at a concentration of 10⁶-10⁷ CFU/mL. Animals were then gavaged with 0.3 mL doses of fresh bacterial suspension on two consecutive days. Previously, Maggio-Price et al[20] have shown that *Helicobacter* infection is localized primarily in the cecum and proximal colon, and that bacterial DNA is still present in the tissue and luminal contents of the cecum at 12 wk post-infection. Bacterial presence was confirmed in the current study via DNA isolation at 3 d post-infection using a commercial kit (QIAGEN tissue kit; Valencia, CA, United States) as previously described[20].

**Experimental design**

In study 1, SMAD3+/− mice (*n* = 30) at 8-10 wk of age were infected with *H. hepaticus* to determine onset and duration of colitis. At the time of necropsy, mice were asphyxiated with CO₂ and exsanguinated via cardiac puncture. Intestinal tissue was collected and processed for histopathology at 2-8 wk post-infection. In study 2, SMAD3+/− (*n* = 24) and SMAD3−/− mice (*n* = 19) at 8-10 wk of age were infected with *H. hepaticus* once per day for two consecutive days. At select time points after infection (0, 3, 7 and 28 d), the spleen and MsLNs were collected and processed for lymphocyte isolation as described below. Colon and cecum tissue was collected, fixed, and processed for immunohistochemistry.

**Histopathology**

The colon and cecum were removed and flushed with phosphate-buffered saline (PBS). Tissues were fixed in 10% formalin overnight, embedded in paraffin, then sectioned and stained with hematoxylin and eosin (HE). Longitudinal sections were graded for inflammation and epithelial dysplasia/neoplasia by a pathologist using a blinded scoring system adapted from Maggio-Price et al[20]. Cecum and colons were scored on a 1 to 4 scale both for inflammation (1, no inflammation; 2, mild inflammation; 3, moderate inflammation; 4, marked inflammation) and dysplasia (1, no dysplasia; 2, low-grade dysplasia; 3, high-grade dysplasia; 4, high-grade dysplasia with invasion/adenocarcinoma). The two scores for colon and two scores for cecum tissue in each animal were combined such that a score of 4 indicated no inflammation or dysplasia and a score of 16 reflected maximal inflammation and neoplasia.

Immunohistochemistry was performed on paraffin-embedded colon sections. Antibodies specific for CD3 and granzyme B were purchased from Abcam (Cambridge, MA, United States). Colon sections were sectioned at 5 μm, mounted on coated slides, deparaffinized in xylene, and rehydrated through graded ethanol-water baths. Antigen retrieval was performed using citrate buffer (10 mmol/L, pH 6.0) and a vegetable steamer. Tissues were incubated in 3% hydrogen peroxide to block endogenous peroxidase activity and then incubated overnight at 4 °C in primary antibody. On the following day, tissues were washed in Tris-buffered saline containing Tween-20 (0.05%), then incubated with biotinylated secondary antibodies followed by streptavidin horseradish peroxidase for 45 min each at room temperature (Dako, Carpentaria, CA, United States). After extensive washing, antigen-bound horseradish peroxidase was detected using the chromagen 3,3’-diaminobenzidine (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO, United States) dissolved in PBS (10 mmol/L, pH 7.2). Identification of cellular infiltrate in the colons of mice was performed by a pathologist. CD3+ and granzyme B+ cells were identified under a light microscope using a 20 × objective. The occurrence of positively stained cells was scored in proximal colons of mice in five fields using a 1-cm² grid reticle as follows: 0 = average of 0 cells/grid, 1 = average of 1-10 cells/grid, 2 = average of 11-20 cells/grid, 3 = average of 21-30 cells/grid, 4 = average of > 31 cells/grid. Final values represent mean ± SE per group (*n* = 3-5/group).

**Lymphocyte isolation**

Spleens and MsLNs were removed and placed in ice-cold RPMI medium at the time of necropsy. Spleens were processed with a dounce homogenizer, pelleted, and washed in RPMI. Cells were resuspended in ACK lysing buffer (Invitrogen, Carlsbad, CA, United States) and washed twice in RPMI. MsLNs were treated with 5 mL enzymatic digest [5% fetal bovine serum (FBS), 0.5 mg/mL collagenase, 0.05 mg/mL DNase I] for 30 min at 37 °C. Cells were passed through 70-μm filters and washed with RPMI. Cell counts were performed with a hemocytometer using trypan blue exclusion and resuspended to a concentration of one million cells per milliliter of medium.

**Flow cytometry**

Lymphocytes were resuspended in fluorescence-activated cell sorting (FACS) buffer (0.1% sodium azide, 1% FBS, in dPBS) blocked with anti-Fc receptor R II/III (CD16/
CD32 (purified from clone 2.4G2 hybridoma; ATCC, Manassas, VA, United States) for 10 min on ice, and subsequently incubated with combinations of the following fluorochrome-conjugated antibodies (E-bioscience, San Diego, CA, United States; or BD Bioscience, San José, CA, United States) at concentrations ranging from 1:100 to 1:300 in FACS buffer: CD3 (PerCP-Cy5.5), CD4 (eFluor450), CD8 (PE-Cy7), CD25 (PE), FoxP3 (FTTC or Alexa Fluor488), CD62 (APC), Nkp46 (FTTC) and DX5 (APC). Cells were incubated in staining cocktails (one million cells per cocktail) on ice in the dark for 30 min. Intracellular staining was performed using FoxP3 staining buffer set as per the manufacturer’s instructions (E-bioscience). Briefly, after surface staining, cells were washed twice in FACS buffer, fixed in 4% paraformaldehyde for 25 min, and permeabilized for 30 min. Permeabilization was followed by incubation for 30 min with the appropriate antibodies diluted in permeabilization diluent. Samples were then acquired on a LSR II (BD Bioscience) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, United States). The number of cells in each population of interest was determined by multiplying cell percentages by the total cell number.

**Statistical analysis**

Data for the colitis and immunohistochemistry scores were analyzed using the nonparametric Kruskal-Wallis test and Dunn's post-test for specific comparisons. Flow cytometric data was analyzed using a two-way analysis of variance in GraphPad Prism (GraphPad Software, La Jolla, CA, United States). When statistical differences were detected, Tukey’s multiple comparison test was used to determine differences between the two groups. \( P < 0.05 \) was considered significant. All data are represented as mean ± SE.

**RESULTS**

**SMAD3-deficient mice are susceptible to colitis 4 wk post-infection**

Colitis severity in SMAD3\(^{-/-}\) mice (Figure 1A) peaked at 4 wk post-infection, with an average colitis score of 7.8 ± 0.4. This value was significant compared to samples taken at all other time points (\( P < 0.05 \)). Colitis resolved to baseline levels in SMAD3\(^{-/-}\) mice by 8 wk post-infection. In comparison, SMAD3\(^{-/-}\) mice were resistant to colitis development at all time points (data not shown).

There was no statistically significant change in colitis scores in SMAD3\(^{-/-}\) mice compared to baseline at any time point post-infection. Representative HE images from SMAD3\(^{+/+}\) and SMAD3\(^{-/-}\) mice prior to and 4 wk following infection are presented in Figure 1B.

**SMAD3-dependent changes in lymphocyte populations following H. hepaticus infection**

We next evaluated genotype- and time-dependent changes in lymphocyte populations in the spleen and MsLNs using flow cytometry. There were no significant changes in total CD3\(^{+}\), CD4\(^{+}\) or CD8\(^{+}\) lymphocytes in the spleen at baseline or at any time point following infection (Figure 2A-C). Tregs (FoxP3\(^{+}/\)CD25\(^{+}/\)CD4\(^{+}\)) and NK cells (Nkp46\(^{+}/\)DX5\(^{+}\)) increased in both genotypes following infection but returned to baseline by 28 d (Figure 2D and E).

In the MsLNs, CD3\(^{+}\), CD4\(^{+}\) and Treg cells were significantly higher in both genotypes at 7 d and 28 d post-infection (Figure 3A, C, and D), whereas there were no significant changes in CD8\(^{+}\) cells at any time point examined (Figure 3B). NK cells increased in SMAD3-deficient mice by 7 d post-infection, and were significantly different from baseline values at 28 d (Figure 3E). Comparably, NK cells were not significantly altered at any time point in SMAD3\(^{+/+}\) mice (Figure 3E).

To determine activation status of the different T lymphocyte populations, we next evaluated surface expression of CD62L. L-Selectin (CD62L) is an adhesion marker expressed at high levels in naïve T cells and is cleaved from the surface (CD62L\(^{lo}\)) in activated and/or in memory T cells. There were no statistically significant changes or observable trends in the proportion or total number of activated T cells in the spleen at any time point after infection (data not shown). However, the proportion of CD3\(^{+}\), CD8\(^{+}\) CD62L\(^{lo}\) and CD3\(^{+}\), CD4\(^{+}\) CD62L\(^{lo}\) cells was significantly higher in SMAD3\(^{-/-}\) mice at 7 d and 28 d compared to baseline values and to SMAD3\(^{+/+}\) mice (Figure 4A and D). Effector Treg cells increased in both strains at 7 d and 28 d compared to baseline values (Figure 4G). CD62L expression became dimmer at later time points in the SMAD3\(^{-/-}\) mice for both CD8\(^{+}\) and CD4\(^{+}\) populations (Figure 4C and F) in the MsLNs, however, the intensity of CD62L expression was maintained consistently in SMAD3\(^{+/+}\) mice through all time points (Figure 4B and E). No significant differences were observed in the percentage of Treg cells expressing reduced levels of CD62L between genotypes at any time point (Figure 4H and I).

**Immunohistochemical analysis of colon sections 28 d post-infection**

We next evaluated local changes in CD3\(^{+}\) cells and the serine protease, granzyme B, in the proximal colons of SMAD3\(^{-/-}\) and SMAD3\(^{+/+}\) mice 4 wk post-infection. The lamina propria in SMAD3\(^{-/-}\) mice was moderately expanded by lymphocytic cells. Based on morphology and immunohistochemistry, these cells consisted primarily of CD3\(^{+}\) lymphocytes (Figure 5A). Additionally, numerous granzyme B\(^{+}\) cells were noted in the intestine of SMAD3\(^{-/-}\) infected mice, primarily within the villous epithelium, but sometimes also within the lamina propria (Figure 5B).

**DISCUSSION**

Functional TGF-β signaling is crucial for maintaining immune cell homeostasis\[^{[30]}\]. In the present study, we evaluated changes in local and systemic immune cell populations in colitis resistant SMAD3\(^{+/+}\) and sensitive
SMAD3–/– mice during the course of infection with the enteric pathogen, \textit{H. hepaticus}. A major finding of this study was a significantly higher number of CD4 and CD8 effector cell populations in the mesenteric lymph nodes of SMAD3–/– mice at 7 d and 28 d post-infection compared to baseline values and to SMAD3+/− mice. The number of granzyme B+ cells, a marker of cytolytic lymphocytes, was also higher in proximal colon tissue at 28 d post-infection, consistent with colitis development in these animals. Our findings suggest loss of TGF-β signaling through SMAD3 leads to aberrant activation of colitogenic T cell subsets in response to \textit{H. hepaticus}, whereas changes in specific T cell numbers were unaffected by genotype. These data are consistent with Maggio-Price \textit{et al.} \cite{19}, who reported no significant T cell response to infection with \textit{Helicobacter in vitro}, although it is important to note that in that study only splenic lymphocytes were assessed, and that both \textit{H. hepaticus} and \textit{Helicobacter bilis} were used for infection. Additionally, Yang \textit{et al.} \cite{31} reported no differences between SMAD3–/– and wild-type controls on development of T and B lymphocytes and NK cells, but found increased activated phenotype of T lymphocytes in SMAD3–/– mice that were resistant to TGF-β1 inhibition \textit{in vitro}. The inflammation associated with \textit{H. hepaticus} infection in susceptible strains leads to a dysregulated Th1-type immune response, characterized by increased expression of interleukin (IL)-12 and interferon (IFN)–γ \cite{19,32,33} as well as the proinflammatory cytokines IL-1α, IL-1β, IL-6 and tumor necrosis factor-α \cite{34}. Treg cells normally function to control the inflammatory response by suppressing proliferation and activation of CD4+ and CD8+ cells.

Figure 1 Drosophila mothers against decapentaplegic 3−/− mice are more susceptible to colitis following infection with \textit{Helicobacter hepaticus}. A: Inflammation and dysplasia scores in drosophila mothers against decapentaplegic (SMAD)3−/− mice post-infection (wk). The colon and cecum from each animal were given a separate score for inflammation and dysplasia (n = 30 animals/trt). Each animal received a total of four numerical scores for each of these criteria. The figure displays the average total of these scores with a lowest possible score of 4 and a highest possible of 16. *P < 0.05, **P < 0.001 vs control animals. There was no change in colitis scores among SMAD3+/− mice throughout the course of infection (data not shown); B: Hematoxylin and eosin-stained sections from the cecum and colon of SMAD3−/− (upper panel) and SMAD3+/− mice (lower panel) comparing uninfected and 4 wk after infection with \textit{Helicobacter hepaticus} (\textit{H. hepaticus}). Four weeks following infection, the number of inflammatory cells and primarily lymphocytes in the lamina propria was slightly increased in both tissues, consistent with mild inflammation (arrows denote infiltrate).
Figure 2 Changes in T lymphocyte populations and natural killer cells in the spleen of drosophila mothers against decapentaplegic 3+/- and drosophila mothers against decapentaplegic 3-/- mice following infection with Helicobacter hepaticus. Flow cytometric analysis of lymphocyte populations at days 0, 3, 7 and 28 post-infection. Gates were drawn on viable cells using forward scatter vs side scatter parameters. A: Total CD3+ lymphocytes gated on forward scatter vs CD3; B: Total CD8+ lymphocytes gated on CD3+ lymphocytes; C: Total CD4+ lymphocytes gated on CD3+ lymphocytes; D: Total CD25+/FOXP3+ Treg cells gated on CD3+/CD4+ lymphocytes; E: Total natural killer (NK)p46+/DX5+ NK cells in spleen tissue (n = 4-6 animals per time point). *P < 0.05 vs baseline values (7 d vs 0 d); **P < 0.05 denotes significance between genotypes [drosophila mothers against decapentaplegic (SMAD)3+/- vs SMAD3-/-].

Figure 3 Changes in T lymphocyte populations and natural killer cells in the mesenteric lymph nodes of drosophila mothers against decapentaplegic 3+/- and drosophila mothers against decapentaplegic 3-/- mice following infection with Helicobacter hepaticus. Flow cytometric analysis of lymphocyte populations at days 0, 3, 7 and 28 post-infection. Gates were drawn on viable cells using forward scatter vs side scatter parameters. A: Total CD3+ lymphocytes gated on forward scatter vs CD3; B: Total CD8+ lymphocytes gated on CD3+ lymphocytes; C: Total CD4+ lymphocytes gated on CD3+ lymphocytes; D: Total CD25+/FOXP3+ Treg cells gated on CD3+/CD4+ lymphocytes; E: Total natural killer (NK)p46+/DX5+ NK cells in mesenteric lymph nodes (n = 4-6 animals per time point). *P < 0.05 vs baseline values; **P < 0.05 denotes significant interaction between genotypes [drosophila mothers against decapentaplegic (SMAD)3+/- vs SMAD3-/-].
lymphocytes, inhibiting production of the cytokines IL-2 and IFN-γ, as well as producing the anti-inflammatory cytokine IL-10[34,35]. Transgenic mice lacking T and B lymphocytes, including scid and rag-2-deficient mice exhibit a more severe colitis that can be partially alleviated by adoptive transfer of IL-10-producing Treg cells[36-40]. Additionally, adoptive transfer of wild-type Treg cells into ragg-2-deficient mice inhibits H. hepaticus-induced colon cancer development[41,42], further establishing an important role for this cell type in suppressing inflammatory signaling.

Importantly, Treg cell development is intricately dependent on TGF-β signaling, whereas Treg cells themselves are a major source of this cytokine, deriving much of their suppressive function from TGF-β production as well as IL-10. Given the importance of this cell type in suppressing colitis in other models, we next evaluated whether SMAD3-deficiency impaired the development and/or activation of CD4+/CD25+/Foxp3+ T regulatory cells. We found no significant difference between genotypes at baseline, suggesting normal development of this cell type in the absence of SMAD3 signaling. Following infection, Treg cells increased proportionally in both genotypes. Thus,

Figure 4 Changes in effector T lymphocyte populations in the mesenteric lymph nodes of drosophila mothers against decapentaplegic 3+/− and drosophila mothers against decapentaplegic 3−/− mice following infection with Helicobacter hepaticus. Flow cytometric analysis of lymphocyte populations at days 0, 3, 7 and 28 post-infection. Gates were drawn on viable cells using forward scatter vs side scatter parameters. Histograms represent CD62L expression at 0 d (solid grey), 3 d (dotted black), 7 d (dashed black) and 28 d (solid black) post-infection. Y-axis represents relative cell frequency. X-axis is CD62L expression. Brackets indicate differences vs baseline values; a, c P < 0.01, b, d, e, f, g, h P < 0.05. A: Total CD8+/CD62L+ lymphocytes gated on CD3+/CD8+ lymphocytes; B: CD62L expression in drosophila mothers against decapentaplegic (SMAD3+/−) CD8+ lymphocytes; C: CD62L expression in SMAD3+/CD8+ lymphocytes; D: Total CD4+/CD62L+ lymphocytes gated on CD3+/CD4+ lymphocytes; E: CD62L expression in SMAD3+/CD4+ lymphocytes; F: CD62L expression in SMAD3+CD4+ lymphocytes; G: Total CD25+/Foxp3+ CD62L− Treg cells gated on CD3+/CD4+ lymphocytes (n = 4-6 animals per time point); H: CD62L expression in SMAD3+/− CD25+/Foxp3+ lymphocytes; I: CD62L expression in SMAD3−/CD25+/Foxp3+ lymphocytes. *P < 0.05 vs baseline values; †P < 0.01 denotes significant interaction between genotypes (SMAD3+/− vs SMAD3−/−).
our findings suggest that SMAD3 deficiency does not influence Treg cell numbers in peripheral lymphoid tissue; however, this does not rule out the possibility that the suppressive effect of this cell type is influenced in a SMAD3-dependent manner. This is further supported by recent findings of Fantini et al[31] who have reported that overexpression of SMAD7 in CD4 T lymphocytes, which blocks TGF-β-mediated activation of SMAD2/3, impairs the ability of Treg cells to suppress T cell proliferation and proinflammatory cytokine expression both in vitro and in vivo.

NK cells are generally acknowledged to be important for cell-mediated immunity, and play an important role in the control of cellular infections as well as in antitumor immunity[44]. For example, NK cells can directly lyse infected/dysplastic cells through perforin-granzyme-dependent mechanisms and induce apoptosis[45-51]. Additionally, NK cells activate other effector immune cells through local production of cytokines[52]. Fort et al[53] have demonstrated that NK cells exert a protective effect on colitis by controlling the responses of effector CD4 T cells through perforin-dependent mechanisms[53]. Other studies have provided evidence that NK cells are in fact an innate source of IL-22 in the colon; a cytokine that has proinflammatory properties but is also proposed to protect tissues during inflammation[54-56].

Yang et al[57] previously have found no effect of SMAD3 deficiency on development of NK cells in the spleen or MsLN s. In the current study, we determined whether SMAD3 deficiency would influence NK cells in peripheral lymphoid tissue in response to infection with H. hepaticus. Surprisingly, we found higher numbers of NK cells (NKp46+/DX5+) in SMAD3−/− mice both at 7 d and 28 d post-infection in the MsLN s, whereas no corresponding changes in population numbers were observed in SMAD3+/+ mice. Our findings of increased NK cell populations are somewhat inconsistent with the previously established protective role of this cell type[43]; however, it is possible that SMAD3 signaling mediates the balance of NK cell subsets in response to infection and/or cytotoxicity of NK cells. For example, significant enrichment of lamina propria NK cells of the CD56+CD16− cytotoxic subset in individuals with IBD has been reported[57]. Additionally, individuals with Crohn's disease have been reported to have a higher proportion of NKp46− compared to NKp44+ NK cells in the intestinal mucosa, which is suggested to mediate pathogenesis through increased production of IFN-γ[58]. Importantly, TGF-β inhibits INF-γ production by NK cells[59,60], suggesting the possibility of altered balance of NK cell subsets in our model.

Our findings highlight that mice infected with H. hepaticus and deficient in SMAD3 signaling have elevated levels of effector lymphocyte subsets in the MsLN s and in the colon likely contributing to increased colitis severity. Although no genotype differences in numbers of natural Treg cells were found following infection, it is possible that defective TGF-β signaling through SMAD3 may impair suppressive function. Alternatively, the latent presence of effector T cells may indicate continuous antigen presenting cell stimulation which was not addressed in these studies. Given the pleiotropic role of TGF-β signaling in immune cell homeostasis, further evaluation of cytokine production by activated T cells
derived from infected SMAD3-deficient mice would lead to a more thorough understanding of SMAD3 in colitis susceptibility. Additionally, very little is known about the role of SMAD3 in NK cell function, however, the higher presence of NKP46+/DX5+ NK cells in the MsLN.s of SMAD3-deficient mice might indicate altered NK subsets present in the MsLN.s due to different chemokines being released throughout the course of the infection. The signaling pathways involved in initiating the inflammatory response to H. hepaticus in susceptible mouse strains has also not been well characterized. H. hepaticus activates nuclear factor-κB and extracellular signal-regulated kinase signaling in bone-marrow-derived macrophages[33,36,60], which can induce both pro- and anti-inflammatory pathways[33,60,61]. Given the importance of TGF-β signaling in both IBD and colon cancer development in humans, further identifying innate targets involved in initiating the SMAD3-dependent inflammatory response to pathogenic stimuli would prove highly useful in understanding the pathogenesis of IBD as well as for designing interventions that may alter immune cell populations and/or activation. Future studies addressing some of these possibilities are currently under investigation.

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