Protection against colitis by CD100-dependent modulation of intraepithelial γδ T lymphocyte function

TF Meehan1,6,8, DA Witherden1,8, C-H Kim1,7, K Sendaydiego1, I Ye1, O Garijo1, HK Komori1, A Kumanogoh2,4, H Kikutani3,4, L Eckmann5 and WL Havran1

Intraepithelial γδ T lymphocytes (γδ IEL) have important roles in repair of tissue damage at epithelial sites, such as skin and intestine. Molecules that orchestrate these γδ T-cell functions are not well defined. Recently, interaction of the semaphorin CD100 on skin γδ T cells with plexin B2 on keratinocytes was shown to be important for effective γδ T-cell function in the epidermis, which raised the possibility that CD100 may exert similar functions in the intestinal tract. In this study, we find that CD100 is expressed on all IEL, and plexin B2 is present on all epithelial cells of the mouse colon. Using the dextran sulfate sodium (DSS) mouse model of colitis, disease severity is significantly exacerbated in CD100−/− mice, with increased colon ulceration and mucosal infiltration with inflammatory cells. The severe colitis in CD100−/− mice is attributable to the failure of the colon epithelium to mount a proliferative response to damage. Unlike wild-type γδ IEL, γδ IEL from CD100−/− mice fail to produce keratinocyte growth factor-1 (KGF-1) in response to DSS treatment. Administration of recombinant KGF-1 to CD100−/− animals ameliorates disease and reverses colitis susceptibility. These results demonstrate that CD100-mediated signals are critical for effective activation of γδ IEL to produce growth factors, including KGF-1, that are required for healing of the colon epithelium during colitis.

INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by chronic inflammation of the intestine and is often accompanied by extensive epithelial ulcerations. Inflammation is thought to be driven by dysregulated cell-mediated immune responses to antigens derived from commensal bacteria in the intestinal lumen. Resolution of disease depends on re-establishment of the epithelial cell barrier to separate luminal antigens from the underlying immune cells in the inflamed tissue. Important in the healing process are intestinal intraepithelial lymphocytes (IEL) bearing the γδ form of the T-cell antigen receptor. These γδ IEL are in intimate contact with epithelial cells and respond to intestinal damage by secreting a number of cytokines, chemokines, and growth factors such as keratinocyte growth factor-1 (KGF-1) that promote tissue repair. Mice lacking γδ T cells (T-cell receptor δ−/− (TCRδ−/−)) have defects in enterocyte homeostasis and develop severe disease in different models of intestinal inflammation, including the dextran sulfate sodium (DSS) mouse model of colitis. This severe colitis is attributed to a lack of KGF-1 production by γδ IEL, a potent mitogen for epithelial cells that can stimulate proliferation and promote wound healing in the intestine. Exogenous KGF-1 ameliorates colitis when administered before DSS treatment. Conversely, mice unable to produce KGF-1 (KGF-1−/− mice) develop a severe form of DSS-induced colitis. These lines of evidence demonstrate that γδ IEL-promoted healing of the colon epithelium by production of KGF-1 is critical to recovery from colitis.
Although activation of γδ IEL is necessary for their production of KGF-1, the molecular events involved in this activation are poorly understood. By analogy with the skin, numerous interactions, in addition to those through the TCR, are likely to have a crucial role. The semaphorin CD100, which is expressed on the surface of skin γδ T cells, was shown recently to interact with plexin B2 on keratinocytes and to be necessary for the effector response of epidermal γδ T cells to stressed epithelial cells. Semaphorins are a large family of membrane bound and soluble proteins that are grouped into eight classes based on sequence similarity and shared structural domains. Semaphorins are mostly known for their ability to give directional cues to developing neurons by signaling through plexin family members, but recent findings indicate they have a broader physiological role in organogenesis, angiogenesis, and in metastasis of cancer cells. In addition, several semaphorins, including CD100, have roles in immune regulation. CD100 is a group IV transmembrane semaphorin (SEMA4D) that is expressed as a homodimer in a broad range of tissues, including cells of the immune system. CD100 has higher levels of expression on T cells compared with B cells, and expression is significantly enhanced on both cell types with cellular activation. Originally, a costimulatory role for CD100 was proposed for human T cells, because anti-human CD100 antibodies increased T-cell proliferation in the presence of suboptimal amounts of phorbol myristate acetate or anti-CD2 and anti-CD3 antibodies. However, CD100 can also transduce signals through other proteins that act as CD100 receptors with the best characterized receptor being plexin B1. When bound by CD100, plexin B1 acts as a GDPase-activating protein for r-Ras leading to inhibition of the phosphoinositide 3-kinase signaling pathway. This, and additional interactions of plexin B1 with other small GTP-bound proteins, leads to modifications of the cytoskeleton and subsequent changes in cell adhesion and migration. In the immune system, plexin B1 is expressed on subsets of dendritic cells and B cells. Interaction with CD100 inhibits migration and promotes survival of these immune cells. Dendritic cells and B cells also express CD72, a low affinity receptor for CD100. CD100 ligation results in dissociation of the tyrosine phosphatase SHP-1 from the intracellular domain of CD72 and turns off the inhibitory signals that CD72 delivers to B cells and dendritic cells.

Recently, another member of the plexin B family, plexin B2, was described as having a high affinity interaction with CD100. The CD100/plexin B2 interaction was shown to be important for γδ T-cell responses to stressed keratinocytes in the skin. These responses mediated by the semaphorin/plexin interaction, including secretion of growth factors and morphological changes of the γδ T cell from a resting, dendritic shape to an activated, rounded shape, were defective in the absence of CD100. Taken together, current data indicate that CD100 ligation can mediate activation and function of a wide range of immune cells. Analysis of CD100-deficient mice further support this notion. Despite a wide distribution of CD100 in the central nervous system, the only known defects of CD100−/− mice are associated with immune cells. In addition to the defects in epidermal γδ T-cell function, these mice have deficient antigen-specific T-cell responses, reduced B-cell responses to T-cell-dependent antigens, and decreased numbers of B-1 B cells. In vitro assays using cells isolated from the CD100−/− mice reveal that expression of CD100 on T cells, but not dendritic cells, is essential for the T-cell response to antigen. The hypersensitivity of the immune system in CD100−/− mice leads to protection against experimental autoimmune encephalomyelitis and immune complex glomerulonephritis. As CD100-mediated signals are important for effective activation of γδ IEL, the function of these cells during colitis may be impaired in CD100−/− mice. Therefore, we hypothesized that DSS-induced colitis would be more severe in CD100−/− animals due to defects in γδ IEL-mediated healing of colon epithelium. In this study, we demonstrate that all IEL express CD100 and all colon epithelial cells express plexin B2 in wild-type (WT) mice. Upon induction of colitis, CD100−/− mice develop a much more severe form of the disease, with defects in proliferation of the colon epithelium. The γδ IEL from CD100−/− mice also have defects in proliferation and are unable to produce KGF-1, a mechanism which explains the protective role of CD100 during colitis. These results demonstrate the importance of the CD100/plexin B2 interaction in proper maintenance and repair of the colon epithelium by γδ IEL.

RESULTS

Plexin B2 is expressed on epithelial cells and CD100 on IEL in the colon

To determine whether plexin B2 is expressed in the normal murine colon, immunostaining studies were conducted with a monoclonal antibody specific for plexin B2. Strong constitutive expression of plexin B2 was found in the epithelium of the colon, while there was no expression by cells in the underlying lamina propria (Figure 1a,b). Flow cytometric analysis of isolated intestinal cells confirmed that epithelial cells expressed high levels of plexin B2 on their cell surface, while αβ and γδ IEL displayed little or no staining for plexin B2 (Figure 1c). Plexin B1 is also known to have a high affinity interaction with CD100. Real-time PCR was used to compare levels of colonic expression of plexin B1 and B2 mRNA. Although low levels of plexin B1 mRNA were detectable, expression of plexin B2 was markedly (10-fold) higher in RNA isolated from colon homogenates (Figure 1d). To examine the expression of CD100, a receptor for plexin B1 and B2, we developed a monoclonal antibody that recognizes the extracellular domain of CD100. CD100 was expressed on the surface of both αβ and γδ IEL but not on colon epithelial cells (Figure 1e). No expression of the low affinity-binding partner of CD100, CD72, was observed by flow cytometry or reverse transcriptase-PCR of the colon epithelium (data not shown). Constitutive expression of CD100 and plexin B2 in the normal colon supported the possibility that interactions between these molecules may mediate γδ IEL functions in...
the colon. To address this, expression levels of CD100 and its ligands were examined following treatment of mice with DSS to induce acute colitis. When 2.5% DSS is added to the drinking water for 5 days, followed by 3 days recovery on normal drinking water, C57Bl/6 mice develop acute colon inflammation that is characterized by crypt loss and mucosal infiltration with inflammatory cells.4 Plexin B2 expression was detected on epithelial cells adjacent to inflammatory lesions but was not evident on immune cells found within the inflammatory infiltrate (Figure 2a,b). Colonic plexin B1 mRNA expression was not induced in colitis (Figure 2c) nor was expression of the low affinity receptor to CD100, CD72, detectable by either flow cytometry or reverse transcriptase–PCR in colon epithelial cells under these conditions (data not shown). This suggests that plexin B2 is the most likely binding partner for CD100 in the colon epithelium. A role for plexin B2 in the DSS-induced colitis model is suggested by in vitro blocking of plexin B2 in isolated colon tissue following in vivo DSS treatment. In vitro culture of colon segments in the presence of blocking anti-plexin B2 antibodies reduced the interferon-γ (IFNγ) response of the colon to DSS-induced colitis (Supplementary Figure S1). As IFNγ is an important mediator in the pathogenesis of IBD,27 this suggests that interactions between plexin B2 and CD100 may be involved in the regulation of disease progression and/or tissue repair, similar to what has been seen for this receptor–ligand pair following epithelial damage in the skin.10

CD100−/− mice display increased susceptibility to colitis
Extensive histological and flow cytometric characterization of untreated WT and CD100−/− mice revealed normal architecture and cellular composition of the colon (Figure 3a,b), including normal numbers and localization of both αβ and γδ T cells (Supplementary Figure S2a and data not shown), suggesting that CD100 was not critical for maintaining mucosal structure or having a major role in the development of the intestinal tract. In addition, mucin secretion (Supplementary Figure S2b) and antimicrobial peptide secretion (data not shown).
To elucidate the function of CD100 during acute inflammation of the colon, colitis was induced in CD100−/− mice through administration of DSS for 5 days followed by a return to normal drinking water for either 3 days or 14 days to allow for recovery. Three days after removal of DSS from the drinking water, focal areas of the colon mucosa in WT mice had the characteristic appearance of colitis, including crypt dropout, mucosal infiltration with mononuclear cells, submucosal edema, and hyperproliferation and Goblet cell loss in the epithelium (Figure 3c). By comparison, CD100−/− mice displayed much more severe signs of colitis, including large areas of crypt loss and inflammation, and extensive ulcers in all the animals examined (Figure 3d). It was previously shown that 14 days after removal of DSS, the epithelium is almost completely healed in C57Bl/6 WT mice, but defects persist in TCRδ−/− and KGF-1−/− mice, indicating a role for γδ IEL and KGF-1 in the recovery process. Fourteen days after removal of DSS, ulcers and inflammation were still evident in one-third of the CD100−/− mice, whereas the colon of WT mice had returned to a normal appearance (Figure 3e,f). These results suggest that CD100 has a protective or healing role in DSS-induced colitis.

Assessment of body weight, a sensitive clinical parameter of disease progression, revealed that CD100−/− mice lost significantly more weight than WT mice in the course of the colitis (Figure 3g). Furthermore, using a previously described histological scoring system (summarized in Supplementary Figure S3a) based on epithelial damage and cellular infiltration in the mucosa, submucosa, and the muscularis, we found that histological scores were significantly higher in the CD100−/− mice 3 days after removal of DSS (Supplementary Figure S3b). Ulcers were present in all of the CD100−/− mice, but in only 36% of the WT mice at that time. Histological scores 2 weeks after discontinuation of DSS treatment indicated that CD100−/− mice continued to have more severe disease than their WT controls (Supplementary Figure S3c). An assessment of epithelial damage using a second approach, measuring the total length of ulcers in colons 3 days after

![Figure 3](image-url)
removal of DSS treated water, revealed striking differences between WT and CD100\(^{-/-}\) animals. Lesion lengths in WT mice ranged from none (64% of the mice) to 0.84 mm in length in the entire colon, with a mean of 0.20 mm (Figure 3h,i). CD100\(^{-/-}\) mice had on average 10-fold greater ulcerations than WT controls, with lesions ranging from 0.42 to 8.70 mm in length (Figure 3h,i). Thus, by all the parameters measured, CD100\(^{-/-}\) animals developed a substantially more severe DSS-induced colitis than their WT counterparts.

**Altered IEL response in CD100\(^{-/-}\) mice**

In response to epithelial damage, \(\gamma\delta\) IEL in the colon are activated and produce cytokines and growth factors that are critical for proper healing of the mucosal layer.\(^{4,6,8}\) As CD100 has been shown to have an important role in \(\gamma\delta\) T-cell activation in the skin,\(^9\) the increased colon epithelial damage observed in the CD100\(^{-/-}\) mice could be the result of improper activation or function of \(\gamma\delta\) IEL. IEL activation was assessed by cytokine production, proliferation, and growth factor production. A population of \(\gamma\delta\) IEL isolated from the colons of both WT and CD100\(^{-/-}\) animals produced tumor necrosis factor \(\alpha\) at day 5+2 following DSS treatment (Supplementary Figure S4), indicating that early cytokine production by \(\gamma\delta\) IEL is unaffected by a deficiency in CD100–plexin B2 interactions. An analysis of proliferation, as assessed by in vivo bromodeoxyuridine (BrdU) incorporation, showed that \(\gamma\delta\) IEL from CD100\(^{-/-}\) mice were defective in their proliferative response to colitis (Figure 4a). Both \(\gamma\delta\) and \(\alpha\beta\) IEL from WT animals increased their proliferation following DSS treatment, however no increase in proliferation was seen in \(\gamma\delta\) or \(\alpha\beta\) IEL isolated from CD100\(^{-/-}\) animals (Figure 4a). Although \(\gamma\delta\) IEL from CD100\(^{-/-}\) animals had a somewhat higher baseline proliferation than their WT counterparts (Supplementary Figure S5a,b), this was not reflected in a general activation of these cells under homeostatic conditions (Supplementary Figure S6). Similar results were observed for \(\alpha\beta\) IEL from CD100\(^{-/-}\) mice (Supplementary Figure S5c,d), whereas other responses, such as myeloperoxidase activity (Supplementary Figure S7a), appeared comparable in WT and CD100\(^{-/-}\) animals both early and late in the response, suggesting a selective defect in the T-cell response. These results indicate that IEL from CD100\(^{-/-}\) mice do not show a normal response to colitis induction. This is most likely due to an impaired response of the \(\gamma\delta\) T cells to epithelial damage, resulting in deficient healing of the colon epithelium in colitis.

\(\gamma\delta\) IEL promote healing of the colon epithelium by secreting the potent epithelial mitogen KGF-1.\(^4\) In the absence of \(\gamma\delta\) T cells, KGF-1 production is greatly reduced,\(^4\) resulting in reduced proliferation of the colon epithelium in response to colitis and exacerbation of disease. To assess the baseline proliferative rates of colon epithelial cells in WT and CD100\(^{-/-}\) mice, in vivo BrdU incorporation was determined by immunohistochemistry on colon sections. The number of BrdU-positive epithelial cells per crypt was counted under a microscope, and IEL were excluded based on cell morphology. Similar to the findings with IEL, CD100\(^{-/-}\) mice had increased baseline proliferation of epithelial cells (Figure 4b,d). BrdU incorporation was then measured in mice 3 days after discontinuation of DSS treatment. Epithelial cells from WT mice exhibited greatly increased proliferative rates in response to colitis (Figure 4c,d), similar to previously published results.\(^4\) By contrast, there was no significant increase in proliferation of colon epithelial cells in
CD100<sup>−/−</sup> mice in response to DSS treatment (Figure 4c,d). No difference in epithelial apoptosis was found between WT and CD100<sup>−/−</sup> animals either under homeostatic conditions or upon colitis induction (Supplementary Figure S7b).

The defective proliferation of colon epithelial cells in TCR<sup>δ−/−</sup> animals has been attributed to a lack of production of KGF-1. To assess the ability of CD100<sup>−/−</sup> IEL to produce KGF-1 in response to epithelial damage, real-time PCR was performed on cDNA from isolated IEL. Positive control = mRNA from the spleen of KGF<sup>−/−</sup> mice (<sup>n</sup> = 3, mean ± s.d., *<sup>P</sup> < 0.05 compared with WT untreated, **<sup>P</sup> < 0.01 compared with wild-type (WT) γδ IEL treated with dextran sulfate sodium). (b, c) Photomicrographs of hematoxylin and eosin (H&E)-stained colon from CD100<sup>−/−</sup> mice that had been treated with phosphate-buffered saline (PBS) or recombinant KGF-1. Lines represent areas of ulceration. (d) Total length of ulcers in the epithelial layer of PBS- and KGF-1-treated mice (<sup>n</sup> = 4 per group, bar = mean).

Figure 5a shows the relative expression of epithelial apoptosis between WT and CD100<sup>−/−</sup> mice upon colitis induction (Figure 5b, c). Figure 5d, e, f illustrate the localization of γδ IEL to sites of epithelial cell damage where they express mRNA for CD100 and plexin B2. Strikingly, KGF-1 mRNA was undetectable in γδ IEL isolated from CD100<sup>−/−</sup> animals, suggesting that exogenous KGF-1 could indeed bypass the requirement for CD100-mediated signals in γδ IEL.

DISCUSSION

The semaphorin CD100 has a central role in lymphocyte function and has recently been described to have a fundamental role in the γδ T-cell response to keratinocyte damage in the skin through interaction with a plexin ligand, plexin B2. Here we show defective γδ IEL activation in the colon of mice lacking CD100. The absence of signals through CD100 abolished KGF-1 production by γδ IEL, resulting in extensive damage to, and delayed repair of, the colon epithelium. These results demonstrate a central role for CD100–plexin B2 interactions in the γδ IEL response to DSS-induced damage of the colon epithelium and provide a molecular mechanism for KGF-1 production by γδ IEL.

The vital role played by γδ IEL in the repair of the colon epithelium has been known for a number of years. In response to DSS-induced damage, large numbers of γδ IEL localize to sites of epithelial cell damage where they express mRNA for KGF-1 and plexin B2. In TCR<sup>δ−/−</sup> animals, the absence of γδ T-cells results in more severe mucosal injury and slower repair of the damaged epithelium than seen in WT animals. However, little is known about the molecular interactions between γδ IEL and the neighboring epithelia that mediate the critical responses of γδ IEL. It is likely that multiple interactions, in concert with those through the TCR, are important for effective γδ IEL activation, as has recently been found for γδ T-cells resident in the epidermal layer of the skin. The observation here that CD100<sup>−/−</sup> mice develop a severe form of colitis similar to the colitis seen in TCR<sup>δ−/−</sup> mice led us to speculate that CD100 has a vital role in the function of γδ IEL. This notion is in agreement with several lines of evidence pointing to CD100 being critical in B-cell and γδ T-cell function as well as skin γδ T-cell responses to tissue damage.

Although the intestinal epithelium appeared functionally intact in untreated CD100<sup>−/−</sup> animals, increased homeostatic proliferation of the colon epithelium was evident. Epithelial γδ T-cells are required for maintenance of epithelial homeostasis, however the high baseline proliferation rate in CD100<sup>−/−</sup> mice seemed unlikely to be a result of defective γδ IEL function as mice lacking γδ T-cells have decreased, rather than increased,
rates of epithelial homeostatic proliferation. Instead, it may well be that dysregulated WNT signaling accounts for the increased epithelial proliferation. WNT signaling is critical in maintaining the proper epithelial homeostasis of the gut. CD100 signaling through another plexin ligand, plexin B1, has been shown to increase levels of activated glycogen synthase kinase-3β, a potent inhibitor of the canonical WNT signaling pathway. As signals through plexin B1 and plexin B2 likely use similar pathways, the absence of CD100-plexin B2 signals may also result in altered regulation of WNT signaling and subsequent altered epithelial homeostasis.

In contrast to the high baseline proliferation, the colon epithelium of the CD100−/− mice failed to increase proliferation rates in response to DSS treatment. In WT animals, γδ IEL-derived KGF-1 stimulates epithelial cell proliferation, a vital component of the epithelial repair process. The crucial role of KGF-1 in effective epithelial repair during colitis is evident in both rodents and humans. Increased levels of KGF-1 are observed in IBD patients, with expression corresponding to sites of inflammation. In mice, it has been demonstrated that defective proliferation of colon epithelial cells in TCRδ−/− mice is due to a lack of production of KGF-1. Here we found that CD100 deficiency resulted in ablation of KGF-1 production by γδ IEL. The lack of KGF-1 in CD100−/− animals thus likely accounts for the reduced epithelial cell proliferation seen in these animals in response to DSS. This notion is further supported by the observed restoration of epithelial repair to CD100−/− animals upon administration of exogenous KGF-1. Together, these findings highlight the importance of CD100-mediated signals in γδ IEL functional integrity. Despite its importance, the signaling pathways involved in KGF-1 production by γδ IEL are poorly understood. Here we provide a link between cell-surface receptor signaling, namely through CD100–plexin B2 interactions, and production of KGF-1. Although we were unable to directly induce KGF-1 through CD100 ligation in isolated cells, the identification of CD100 as a critical component for downstream KGF-1 production by γδ IEL could lead to therapeutic strategies to increase localized production of this protective factor at the site of epithelial damage.

Through interaction with neighboring epithelial cells, γδ IEL produce innate antimicrobial factors that protect against invasion of intestinal tissues by resident bacteria. In the DSS colitis model, a blunted γδ IEL response to damaged epithelium leading to defects in healing would likely increase the bacterial exposure of cells in the underlying lamina propria and thereby exacerbate the inflammatory response. It should be noted that CD100−/− animals do have reduced numbers of B1 B cells, and subsequently, reduced immunoglobulin A levels, which may also alter the mucosal immune system. However, this is unlikely to be responsible for the increased severity of colitis in CD100−/− mice as immunoglobulin A deficiency does not result in a more severe course of DSS-induced colitis.

In addition, while the lack of CD100 on γδ T IEL may alter their function, a perturbed γδ T IEL response is not likely to affect healing of the colon epithelium following DSS treatment, as γδ IEL are neither known to regulate intestinal epithelial proliferation during homeostasis nor to produce KGF-1 in response to colon mucosal damage. In addition, mice lacking γδ T cells have no significant differences from WT mice in disease severity or epithelial repair in the DSS-induced colitis model. Although a role for γδ IEL cannot be completely ruled out, restoration of colon epithelial integrity after injury clearly relies on a functional γδ IEL population.

In conclusion, the interaction between CD100 on γδ IEL and plexin B2 on colon epithelial cells appears to represent a vital component of the γδ IEL activation pathway and subsequent function of these IEL in mediating healing of the colon epithelium during colitis. The absence of CD100 on intestinal γδ IEL leads to an impaired growth factor response to damaged epithelium. With plexin B2 having a broad tissue distribution, this novel interaction may represent a general mechanism by which γδ T cells regulate inflammation and wound healing.

**METHODS**

**Antibodies.** Anti-TCR γδ (PE-GL3), anti-TCR αβ (FITC- or APC-H75-597), and anti-BrdU (3D4) were purchased from BD Biosciences (San Diego, CA). Anti-mouse plexin B2 (3E7) and anti-mouse plexin B1 (H57-597), and anti-BrdU (3D4) were purchased from BD Biosciences (San Diego, CA). Anti-mouse plexin B2 (3E7) and anti-mouse KGF-1 (A43) were purchased from R&D Systems (Minneapolis, MN). Anti-mouse CD100 (1D7) was purchased from BioLegend (San Diego, CA). Anti-mouse plexin B2 (3E7) and anti-mouse KGF-1 (A43) were purchased from R&D Systems (Minneapolis, MN). Anti-mouse CD100 (1D7) was purchased from BioLegend (San Diego, CA).

**Mice and colitis induction.** Eight-to-twelve-week-old female C57Bl/6 WT, CD100−/−, and KGF−/− mice on the C57Bl/6 background were bred at the Scripps Research Institute (La Jolla, CA) and housed under specific pathogen-free conditions. To induce colitis, mice were given drinking water containing 2.5% (w/v) DSS ad libitum. Drinking bottles were weighed before and after administration to measure consumption. After 5 days of treatment, mice were returned to normal drinking water for an additional 1–14 days. Mice were weighed daily. In some experiments, mice were administered recombinant KGF-1 intraperitoneally at 3 mg kg−1 daily for 3 days, beginning at day 5 of DSS treatment. In other experiments, colons were removed 5 days after DSS treatment and small segments cultured in vitro in complete DMEM (Dulbecco’s modified Eagle’s medium) + 10% fetal calf serum for 48 h, as described, in the presence or absence of 20 μg ml−1 anti-plexin B2 mAb, 3E7. Four to six mice were included per group and experiments were repeated three times. All animal studies were approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

**Histological grading of colitis.** Paraffin-embedded sections of the colon were prepared and stained with hematoxylin/eosin as described. To assess the severity of DSS-induced colitis, the most severe lesion present in each colon section was graded blindly by two independent observers using a published scoring scheme that is summarized in Figure S2. Epithelial ulcerations were measured at ×100 magnification using Axiovision AC Software (Zeiss, Thornwood, NY) with a digital camera mounted on a Nikon Eclipse E800 microscope (Nikon, Melville, NY).

**Monoclonal antibody production for CD100.** A female Lewis Rat was immunized once with CD100-transfected CHOK1 cells (CHOK1-CD100) in complete Freund’s adjuvant, four times with CHOK1-CD100 in Ribi adjuvant, and twice with CD100-Fc fusion protein in Ribi adjuvant and once with CD100-Fc in PBS. The spleen from the immunized rat was fused with SP3 myeloma cells. Following selection, clones were screened by flow cytometry for binding to CHOK1-CD100.
cells but not to CHOK1 cells. A single positive clone (8G5) was found and subcloned twice. 8G5 was subsequently purified on Protein G sepharose beads.

**Flow cytometry.** Colon epithelial cells were isolated essentially as described. Expression of surface markers was examined by staining cells with 0.5 μg ml⁻¹ Ab in PBS containing 2% fetal calf serum and 0.2% NaN₃ (fluorescence-activated cell sorting (FACS) buffer) for 20 min at 4°C. Cells were washed with FACS buffer, and then secondary antibodies were added at 0.5 μg ml⁻¹. Cells were washed in FACS buffer, and data were collected on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences) and analyzed using Flowjo software (Treestar, Ashland, OR). In some experiments, IEL were pooled from 3–4 mice and then were stained and sorted based on TCR expression on a FACS Vantage DiVa instrument (BD Biosciences). For intracellular flow cytometry, isolated colon cells were incubated for 4 h in 5 μg ml⁻¹ Brefeldin A, stained for surface marker expression followed by intracellular staining for cytokines, using the Cytofix/Cytoperm kit (BD Biosciences) as per the manufacturer’s instructions. For analysis of apoptosis, colon epithelial cells were stained with Annexin V and propidium iodide using an Annexin V Apoptosis Detection kit according to manufacturer’s instructions (BD Biosciences). IEL were identified by expression of the γδ or αβ TCR and epithelial cells identified by EpCAM (epithelial cell adhesion molecule) staining.

**Immunohistochemistry.** Biotinylated antibody to plexin B2 (10 μg ml⁻¹ in PBS containing 2% fetal bovine serum) was deposited onto acetone-fixed, avidin-biotin-blocked (Vector Laboratories, Burlingame, CA) frozen colon sections essentially as described, with the exception that 0.3% H₂O₂ in cold (4°C) methanol was used to block endogenous peroxidase. Specificity of the antibody was confirmed by a lack of staining with a species-matched negative control antibody.

**RNA extraction and real-time reverse transcriptase–PCR analysis.** Total RNA from tissue or from a confluent flask of 3T3 fibroblast cells was isolated using Trizol reagent (Invitrogen Life Technologies, Grand Island, NY), treated with RQ1 DNase (Promega, Madison, WI) and cDNA derived from 2 μg of RNA using Superscript Reverse Transcriptase III and oligo dT primer (Invitrogen). RNA from sorted IEL was isolated and DNase treated using the RNAqueous Micro-isolation Kit (Ambion Life Technologies, Grand Island, NY) and cDNA generated using the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA). Real-time PCR was performed using the Quantitect SYBR Green PCR kit (Qiagen) on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems Life Technologies, Grand Island, NY). cDNA was incubated at 95°C for 15 min, and then cycled (45 times) at 95°C for 15 s, 59°C for 30 s, and 72°C for 30 s. Relative mRNA levels were determined by the comparative threshold cycle (2⁻^∆CT) method and data are presented as relative to β-actin values for each sample. mRNA from 3T3 cells served as a positive control, and RNA from the spleen of a KGF⁻/⁻ mouse was used as a negative control. All data shown result from at least three independent experiments. Sequences of all primers used in this study are the following: plexin B1 forward, 5’-GGACCAAGGTGTTGCTGACATT-3’; plexin B1 reverse, 5’-ACCCCTTGATGCTCCAGAAT-3’; plexin B2 forward, 5’-AGTCCGATGAGCAAGCTAATAA-3’; plexin B2 reverse, 5’-TAGGGCCGTGAACGCTAAGA-3’; KGF-1 forward, 5’-CGGAGCAAAGGACTCATCGTACTCCTG-3’; KGF-1 reverse, 5’-CCCTCTCCTCCTGATGCTAATCTC-3’; IFN-γ forward, 5’-CCTTTTGGGACCCTC-3’; IFN-γ reverse, 5’-AGCGAAGATGCAAG-3’; β-actin forward, 5’-TCTGCGCTCTGAGCACCATGAGA-3’; and β-actin reverse, 5’-GGGACTCATCTGCTACTGCTTGG-3’.

**Cellular proliferation analysis.** Cells undergoing DNA replication were labeled in vivo with BrdU (55 mg kg⁻¹ of body weight) from a stock solution (5 μg ml⁻¹) dissolved in PBS. BrdU was administered by intraperitoneal injection. Twenty-four hours later, mice were killed and the colon isolated. For FACS analysis, the BD Pharmingen FITC BrdU Flow Kit (BD Biosciences) was used according to the manufacturer’s instructions. For immunohistochemistry, sections (5–8 μm) from paraffin-embedded tissue were air dried, fixed with 70% ethanol, treated sequentially with 2 N HCl for 20 min and 0.1 M Borax for 2 min, followed by washes in PBS, 1% Tween-20 in PBS, and 2% fetal bovine serum in PBS. Slides were then incubated for 1 h with anti-BrdU antibody followed by further incubations with biotinylated monoclonal donkey anti-mouse IgG antibody peroxidase-conjugated streptavidin and metal-enhanced diaminobenzidine (Pierce, Rockford, IL). For untreated mice, the total number of cells and the number of BrdU-positive cells present in 10 crypts were counted for each animal. For mice treated with DSS, only crypts adjacent to sites of inflammation were counted for each animal.

**Myeloperoxidase activity analysis.** Mice were either untreated or treated with DSS for 5 days and then returned to normal drinking water for 0, 7, or 21 days. Colonos were isolated and homogenized in 0.05% hexadecyltrimethylammonium bromide in phosphate buffer (HTAB) and centrifuged at 18,000 g for 30 min at 4°C. Supernatants were transferred to fresh tubes and 10 μl added in triplicate to wells of a 96-well flat-bottomed plate. Ninety microtiter o-dianisidine dihydrochloride in HTAB (DDC) was added to the supernatants. A hundred microliters of 0.0005% H₂O₂ in DDC was added to each well, and myeloperoxidase activity was measured by absorbance at 450 nm.

**Data analysis.** Statistical analysis was performed using Student’s t-test, except for analysis of ulcer sizes where the Wilcoxon’s rank sum test was used. A probability level of P<0.05 was considered statistically significant.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at http://www.nature.com/mi

**ACKNOWLEDGEMENTS**

We would like to thank Kelly Martin and Courtney St. Clair for technical help and Charlie Surh for critical reading of the manuscript. Supported by NIH grants AI52257 (to W.L.H.), AI36964 (to W.L.H.), T32AI07244 (to T.F.M.), RR17030 (to L.E.), DK35108 (to L.E.), DK80506 (to L.E.), a NSF fellowship (to H.K.K.), and a fellowship from the Crohn’s and Colitis Foundation of America (to T.F.M.).

**DISCLOSURE**

The authors declare no conflict of interest.

© 2014 Society for Mucosal Immunology

**REFERENCES**

1. Vora, P., Shih, D.O., McGovern, D.P. & Targan, S.R. Current concepts on the immunopathogenesis of inflammatory bowel disease. Front. Biosci. 4, 1451–1477 (2012).

2. Dignass, A.U. Mechanisms and modulation of intestinal epithelial repair. Inflamm. Bowel Dis. 7, 68–77 (2001).

3. Sturm, A. & Dignass, A.U. Epithelial restitution and wound healing in inflammatory bowel disease. World J. Gastroenterol. 14, 348–353 (2008).

4. Chen, Y., Zhou, B., Fuchs, E., Havran, W.L. & Boismenu, R. Protection of the intestinal mucosa by intraepithelial yh T cells. Proc. Natl. Acad. Sci. USA 99, 14338–14343 (2002).

5. Boismenu, R., Feng, L., Xia, Y.Y., Chang, J.C. & Havran, W.L. Chemokine expression by intraepithelial yh T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. J. Immunol. 157, 985–992 (1996).

6. Yang, H., Antony, P.A., Wildhaber, B.E. & Teitelbaum, D.H. Intestinal intraepithelial lymphocyte yh-T cell-derived keratinocyte growth factor modulates epithelial growth in the mouse. J. Immunol. 172, 4151–4158 (2004).
7. Egger, B., Procaccino, F., Sarosi, I., Tolmers, J., Buchler, M.W. & Eyssettel, V.E. Keratinocyte growth factor ameliorates dextran sodium sulfate colitis in mice. *Dig. Dis. Sci.* **44**, 836–844 (1999).

8. Boismenu, R. & Havran, W.L. Modulation of epithelial cell growth by intraepithelial γδ T cells. *Science* **266**, 1253–1255 (1994).

9. Withen, D.A. et al. The functional adhesion molecule JAM-L is a costimulatory receptor for epithelial γδ T cell activation. *Science* **329**, 1206–1210 (2010).

10. Witherden, D.A. et al. The CD100 receptor interacts with its plexin B2 ligand to regulate epidermal γδ T cell function. *Immunity* **37**, 314–325 (2012).

11. Yazdani, U. & Terman, J.R. The semaphorins. *Genome Biol.* **62**, 1027–1034 (2003).

12. Takamatsu, H. & Kumanogoh, A. Diverse roles for semaphorin-plexin signaling in the immune system. *Trends Immunol.* **33**, 127–135 (2012).

13. Takegahara, N., Kumanogoh, A. & Kikutani, H. Semaphorins: a new class of immunoregulatory molecules. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **360**, 1673–1680 (2005).

14. Herold, C., Bismuth, G., Bensussan, A. & Boumsell, L. Activation signals are delivered through two distinct epitopes of CD100, a unique 150 kDa human lymphocyte surface structure previously defined by BB18 mAb. *Int. Immunol.* **7**, 1–8 (1995).

15. Ito, Y., Oinuma, I., Katoh, H., Kaibuchi, K. & Negishi, M. Semaphorin 4D/plexin-B1 activates GSK-3β through R-Ras GAP activity, inducing growth cone collapse. *Exp. Cell Res.* **295**, 621–631 (2004).

16. Yuan, J., Pricolo, V., Wu, A. & Finkelstein, S.D. Increased expression of keratinocyte growth factor messenger RNA associated with inflammatory bowel disease. *J. Gastroenterol. Hepatol.* **21**, 1372–1380 (2006).

17. Shui, J.W. et al. HVEM signalling at mucosal barriers provides host defence against pathogenic bacteria. *Nature* **488**, 222–225 (2012).

18. Oinuma, I., Katoh, H. & Negishi, M. Semaphorin 4D/Plexin-B1-mediated cell activation leading to pathogenetic humoral responses and immune complex glomerulonephritis. *J. Immunol.* **177**, 3406–3412 (2006).

19. Xavier, R.J. & Podolsky, D.K. Unravelling the pathogenesis of inflammatory bowel disease. *Immunity* **13**, 427–434 (2007).

20. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

21. Masuda, K., Furuyama, T., Takahara, M., Fujoka, S., Kurinami, H. & Inagaki, S. Sema4D stimulates axonal outgrowth of embryonic DRG sensory neurones. *Genes Cells* **9**, 821–829 (2010).

22. Kumanogoh, A. et al. Requirement for the lymphocyte semaphorin, CD100, in the induction of antigen-specific T cells and the maturation of dendritic cells. *J. Immunol.* **169**, 1175–1181 (2002).

23. Shi, W. et al. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. *Immunol. Rev.* **182**, 633–642 (2000).

24. Li, M. et al. CD100 enhances dendritic cell and CD4+ cell activation leading to pathogenetic humoral responses and immune complex glomerulonephritis. *J. Immunol.* **177**, 3406–3412 (2006).

25. Xavier, R.J. & Podolsky, D.K. Unravelling the pathogenesis of inflammatory bowel disease. *Immunity* **13**, 427–434 (2007).

26. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

27. Murthy, A.K., Dubose, C.N., Banas, J.A., Coalston, J.J. & Arulanandam, B.P. Contribution of polymeric immunoglobulin receptor to regulation of intestinal inflammation in dextran sulfate sodium-induced colitis. *J. Gastroenterol. Hepatol.* **21**, 1372–1380 (2006).

28. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

29. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

30. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

31. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

32. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

33. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).

34. Vert et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity* **13**, 621–631 (2000).