Compromised Intestinal Epithelial Barrier Induces Adaptive Immune Compensation that Protects from Colitis

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SUMMARY

Mice lacking junctional adhesion molecule A (JAM-A, encoded by F11r) exhibit enhanced intestinal epithelial permeability, bacterial translocation, and elevated colonic lymphocyte numbers, yet do not develop colitis. To investigate the contribution of adaptive immune compensation in response to increased intestinal epithelial permeability, we examined the susceptibility of F11r−/− Rag1−/− mice to acute colitis. Although negligible contributions of adaptive immunity in F11r+/+Rag1−/− mice were observed, F11r−/−Rag1−/− mice exhibited increased microflora-dependent colitis. Elimination of T cell subsets and cytokine analyses revealed a protective role for TGF-β-producing CD4+ T cells in F11r−/− mice. Additionally, loss of JAM-A resulted in elevated mucosal and serum IgA that was dependent upon CD4+ T cells and TGF-β. Absence of IgA in F11r+/+Igha−/− mice did not affect disease, whereas F11r−/−Igha−/− mice displayed markedly increased susceptibility to acute injury-induced colitis. These data establish a role for adaptive immune-mediated protection from acute colitis under conditions of intestinal epithelial barrier compromise.

INTRODUCTION

The pathogenesis of many inflammatory conditions of mucosal surfaces involves the combined dysfunction of the mucosal barrier and local immune responses to gut lumenal contents. For example, compromised intestinal barrier function reported in people with inflammatory bowel disease (IBD) (Mankertz and Schulze, 2007; Welcker et al., 2004) has been linked to alterations in expression of barrier-forming tight junction molecules (Doğan et al., 1995; Gassler et al., 2001; Jankowski et al., 1998; Karayiannakis et al., 1998; Kucharzik et al., 2001). One transmembrane protein component of tight junctions that has been linked to regulation of intestinal permeability and IBD is junctional adhesion molecule A (JAM-A) (Laukoetter et al., 2007; Vetrano et al., 2008). JAM-A (encoded by F11r) has been implicated in a number of cellular functions, including regulation of paracellular permeability, cell migration, and proliferation (Corada et al., 2005; Liu et al., 2000; Mandell et al., 2005; Martin-Padura et al., 1998; Nava et al., 2011; Severson et al., 2008, 2009). JAM-A is expressed mainly at cell-cell junctions between epithelia and endothelia and is also expressed on the surface of certain immune cells including neutrophils and dendritic cells (Cera et al., 2004; Liu et al., 2000). We have previously shown that JAM-A deficiency in mice leads to a 10-fold increase in intestinal epithelial permeability (Laukoetter et al., 2007). Another unique feature of JAM-A-deficient mice that we previously observed is increased numbers of mucosal and submucosal isolated lymphoid aggregates in the colon, which predominantly contain B220+ cells.

Interestingly, despite a 10-fold increase in colonic permeability and lymphoid follicular hyperplasia in the colon, JAM-A-deficient mice do not develop spontaneous colitis. Although many reports have emphasized the pathogenic role of T and B lymphocytes in the development of colitis (Davidson et al., 1996; Mombaerts et al., 1993), subsets of T cells such as CD4+CD25+Foxp3+ regulatory (Treg) cells are clearly involved in limiting or suppressing intestinal inflammation (Barnes and Powrie, 2009). In addition to their direct role in maintaining intestinal homeostasis, T cells also play a crucial role in the induction of IgA-mediated mucosal immunity. The main function of luminal IgA antibodies is to neutralize and prevent bacteria from penetrating deeper into mucosal tissues and causing immune activation (Boullier et al., 2009; Fernandez et al., 2003; Robinson et al., 2001). Such adaptive immune protection may be critical in the face of increased intestinal permeability where there is enhanced exposure to lumenal microbial products, yet immune compensatory mechanisms in response to compromised intestinal permeability remain poorly understood.

In this study, we used JAM-A-deficient (F11r−/−) mice to investigate how adaptive immune pathways may compensate for a major increase in intestinal permeability to prevent...
spontaneous intestinal inflammation. Our results demonstrate that enhanced mucosal T and B cell responses, TGF-β production, and IgA secretion compensate for the leaky barrier and increased bacterial translocation in JAM-A-deficient mice. Elimination of individual adaptive immune components revealed a critical protective role for TGF-β-producing CD4+ T cells promoting IgA secretion in JAM-A-deficient mice. Altogether, our findings establish a role for adaptive immune responses in limiting severe, acute mucosal injury in the context of intestinal barrier compromise.

RESULTS

Loss of T and B Cells in JAM-A-Deficient Mice Increases Susceptibility to Acute Colitis

Given our previous observations that JAM-A-deficient mice display a 10-fold increase in intestinal epithelial permeability and greatly increased numbers of colonic lymphoid aggregates, we investigated whether adaptive immune cells play a compensatory role in preventing JAM-A-deficient mice from developing spontaneous colitis. We first evaluated the lymphocyte composition in the large intestine of F11r+/+ and F11r−/− (JAM-A-deficient) mice. As shown in Figure 1A, F11r−/− mice exhibited a significant increase in T and B cells. The lamina propria of F11r−/− mice displayed a 2.4 ± 0.8-fold increase in TCRb+ cells and a 5.2 ± 1.0-fold increase in B220+ cells compared to F11r+/+ mice. Additionally, we observed a 4.5 ± 1.3-fold increase in CD4+IL-17A+ T cells in F11r−/− mice compared to F11r+/+ controls (Figure 1B). In contrast, no significant differences were observed in the numbers of CD4+IL-4+ or CD4+IFN-γ+ T cells between F11r−/− and F11r+/+ mice. Notably, CD3+CD4+, CD3+CD8+ T cells, and B220+ cells did not express cell surface JAM-A (data not shown), excluding the possibility that absence of JAM-A expression on lymphocytes in F11r−/− mice could directly alter their accumulation and/or function. We then generated F11r−/− mice deficient in T and B cells by crossing F11r−/− mice with Rag1−/− mice. F11r−/−Rag1−/− mice did not develop spontaneous colitis; however, ~15% of the animals developed spontaneous severe mucocutaneous infections from commensal Pasteurella microorganisms, requiring sacrifice. All experiments presented here were performed using healthy mice that had no sign of infection.

In order to evaluate whether adaptive immunity played a role in the response to acute mucosal injury, we treated F11r−/−Rag1−/− mice with DSS and monitored disease activity. F11r−/−Rag1−/− mice were far more susceptible to DSS-induced colitis when compared to F11r+/− mice, characterized by significant body weight loss (4.8% ± 0.07% of initial body weight) and presence of blood in their stools as early as day 3 of DSS treatment (Figures 1C and 1D). By day 5, disease activity in the F11r−/−Rag1−/− mice was so severe that sacrifice was necessary, with animals consistently losing more than 20% of the initial body weight (23% ± 0.2%) and displaying severe diarrhea and macroscopic signs of bleeding. Histological analyses at day 5 revealed extensive colonic injury in F11r−/−Rag1−/− mice compared to F11r+/−, Rag1+/−, or F11r+/+ control mice (Figures 1E and 1F). The extent of mucosal injury characterized by crypt loss, epithelial damage, and ulceration was significantly less in other groups at the same time point. Colonic inflammation was mainly restricted to the mucosa and submucosa; however, focal areas of transmural inflammation were also observed in F11r−/−Rag1−/− mice. Analysis of several proinflammatory cytokines in colonic homogenates from F11r−/−Rag1−/− mice revealed increased levels of IL-1β, IL-6, and TNF-α (Figure 1G).

Next, we investigated whether the enhanced susceptibility to DSS-mediated acute colitis in F11r−/−Rag1−/− mice was driven by the local increased exposure to the intestinal microflora. F11r−/−Rag1−/− mice were administered a mixture of broad-spectrum antibiotics for 7 days prior to DSS treatment to limit the gut flora. Following DSS treatment, we found that antibiotic-treated F11r−/−Rag1−/− mice were significantly less susceptible to DSS compared with mice not treated with antibiotics. The reduced susceptibility to DSS was evidenced by a general decrease in body weight loss (Figure 1H) and disease activity index (Figure 1I). The same trend was observed in F11r−/− mice where there was a decrease in disease activity index in antibiotic-treated mice compared to untreated controls (data not shown). Altogether, these results demonstrate that adaptive immunity is a critical compensatory component that limits bacterial-driven acute colitis in JAM-A-deficient mice.

CD4+ T Cells Are a Key Component in Protecting JAM-A-Deficient Mice from Acute Mucosal Injury

Since we observed that lack of adaptive immune cells in JAM-A-deficient mice promotes intestinal inflammation during acute DSS-mediated colitis, we examined the specific role of T cells in controlling intestinal inflammation in JAM-A-deficient mice using an antibody-based depletion approach. To this end, we used CD4 and CD8α antibodies to deplete the respective T cell subsets in vivo and tested whether they were critical in preventing excessive intestinal inflammation in F11r−/− mice following injury-induced acute colitis. F11r−/− mice treated with anti-CD4 were significantly more susceptible to DSS than untreated F11r−/− mice, losing 6.1% of their initial body weight starting at day 3 and 21.0% by day 6 (Figure 2A). Anti-CD4 treated F11r−/− mice exhibited disease symptoms as early as day 4, similar to what was observed for F11r−/−Rag1−/− mice, including loose stool and presence of macroscopic, fecal blood. The disease was more severe at days 5 and 6, with signs of diarrhea and further body weight loss (Figure 2B). Histological analyses of the large intestine at day 6 revealed severe mucosal ulceration reminiscent of the histopathology associated with F11r−/−Rag1−/− mice (Figures 2C and 2D). In contrast, treatment of F11r−/− mice with anti-CD8 did not have any effect on the susceptibility to DSS, with anti-CD8-treated F11r−/− mice displaying disease activity indices and histopathology scores similar to untreated F11r−/− mice. Importantly, T cell subset depletion in the spleens and colons was confirmed by flow cytometry (data not shown). Furthermore, depleting antibodies did not affect DSS-induced colitis in F11r−/− mice whether administered in combination or individually (data not shown).

To further investigate if particular CD4+ T cell subsets were responsible for limiting excessive intestinal inflammation in JAM-A-deficient mice during acute mucosal injury, we investigated the role of CD4+CD25+Foxp3+ Treg cells in compensating for defective barrier function. We first determined the numbers of CD4+CD25+Foxp3+ Treg cells in F11r−/− lamina propria cell preparations using flow cytometry. F11r−/− mice...
had a 2.5- ± 0.6-fold increase in the numbers of CD4+CD25+ Foxp3+ Treg cells in the lamina propria compared to F11r+/+ mice (Figure 2E). We then treated F11r−/− mice with anti-CD25 following a previously published protocol to neutralize intestinal CD4+CD25+ Treg cells (Cong et al., 2009), and disease activity was monitored after DSS administration. There were no significant differences between anti-CD25 -treated F11r−/− mice and untreated F11r−/− controls (Figures 2F and 2G). Weight loss during DSS-induced acute colitis in anti-CD25-treated F11r−/− mice followed a pattern similar to that of untreated F11r−/− control mice. Also, no differences were observed in terms of stool consistency and presence of occult blood.
Depletion of CD4+ Foxp3+ Treg cells in the intestinal lamina propria was incomplete (~30% depletion), thus limiting definitive assessment of the role of Foxp3+ Treg cells in this protective response. Altogether, these results highlight an important role of CD4+ T cells in adaptive immune compensation to acute injury under conditions of chronic barrier compromise.

**CD4-Dependent TGF-β Production Suppresses Acute Colitis in JAM-A-Deficient Mice**

In addition to CD4+ Treg cells, TGF-β-producing CD4+ T cells are also well appreciated to regulate intestinal immune responses (Thorstenson and Khoruts, 2001; Zhang et al., 2001). Interestingly, we observed that baseline TGF-β mRNA and protein levels in colonic homogenates were more than 2-fold higher in F11r−/− mice when compared to F11r+/+ mice (Figures 3A and 3B). To investigate the role for enhanced TGF-β expression in F11r−/− mice, animals were treated with neutralizing TGF-β antibodies at day −5 and day −2 prior to DSS treatment. Antibody-treated F11r−/− mice were far more susceptible to DSS-induced acute colitis than untreated F11r−/− mice, beginning to lose weight from day 3 post-DSS treatment (6.6% ± 1.1% weight loss) and losing close to 20% of their initial body weight by day 6 (20.6% ± 4.4% weight loss) (Figure 3C). In addition, anti-TGF-β-treated F11r−/− mice also developed diarrhea at a much earlier...
time point (day 4 post-DSS treatment) than their untreated counterparts, which contributed to a significantly higher disease activity index (3.2 ± 0.1 versus 2.1 ± 0.1) (Figure 3D). Histologic analyses revealed extensive ulceration, mucosal injury, and inflammation in the anti-TGF-β-treated $\text{F11r}^{-/-}$ mice that were similar to that observed with $\text{F11r}^{-/-}$ mice treated with DSS (Figures 3E and 3F). In contrast, the increased susceptibility to DSS following anti-TGF-β administration was not observed in $\text{F11r}^{+/+}$ control mice. In addition, treatment of $\text{F11r}^{-/-}$ mice with an IgG1 isotype control antibody did not have any effect on the course of the disease (data not shown).

Because JAM-A-deficient mice were observed to have increased mucosal TGF-β production, and anti-CD4 as well as anti-TGF-β administration led to enhanced susceptibility to DSS, we investigated whether CD4+ T cells were the source of TGF-β. Real-time PCR data from lamina propria lymphocyte fractions, which are highly enriched in CD4+ T cells, indeed revealed that lymphocytes are a major source of TGF-β (Figure 3G). Furthermore, to evaluate whether CD4+ T cells contribute to the elevated TGF-β amounts observed in $\text{F11r}^{-/-}$ mice treated with anti-CD4-depleting antibodies (GK1.5) at day −2 and day −1 with 500 μg of antibody per mouse per injection, or left untreated, followed by 6 days of DSS treatment. Data are shown as the mean ± SEM (n = 3) percent reduction in TGF-β mRNA levels after CD4 T cell depletion. Expression levels were normalized to the endogenous control $\text{gapdh}$. *p < 0.05; **p < 0.01.

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- **Figure 3. TGF-β Signaling Protects from Acute Colonic Inflammation JAM-A-Deficient Mice**
  - (A) TGF-β mRNA levels in $\text{F11r}^{+/+}$ and $\text{F11r}^{-/-}$ (JAM-A-deficient) mice were evaluated by real-time PCR using total RNA from colon as described in the Experimental Procedures. Data are presented as relative expression following normalization with respect to $\text{gapdh}$.
  - (B) TGF-β protein levels in $\text{F11r}^{+/+}$ and $\text{F11r}^{-/-}$ mice were determined by ELISA using colonic tissue homogenates.
  - (C and D) Body weight changes (C) and disease activity index scores (DAI) (D) from $\text{F11r}^{+/+}$ and $\text{F11r}^{-/-}$ mice treated with 100 μg anti-TGF-β neutralizing antibodies at day −5 and day −2 prior to DSS treatment, or left untreated. Data are represented as means ± SEM and are pooled from three independent experiments (n = 10).
  - (E) Photomicrographs of representative hematoxylin and eosin-stained colon sections from $\text{F11r}^{+/+}$ and $\text{F11r}^{-/-}$ mice treated with anti-TGF-β neutralizing antibodies or untreated control groups at day 5 following 2% DSS treatment. Images show sections of colon highlighting predominant histological findings in each of the experimental groups. Scale represents 100 μm.
  - (F) Histologic damage index scores from Swiss roll mounts of whole mouse colons collected after 5 days of DSS treatment. Data are from three mice per group and represented as mean ± SD. *p < 0.05; **p < 0.01.
  - (G) TGF-β mRNA levels were measured in colonic intestinal epithelial cells (IEC) and lamina propria lymphocyte (LPL) samples by real-time PCR. Data are normalized to the endogenous control $\text{gapdh}$.
  - (H) TGF-β mRNA levels in colons of $\text{F11r}^{+/+}$ and $\text{F11r}^{-/-}$ mice treated with anti-CD4-depleting antibodies (GK1.5) at day −2 and day −1 with 500 μg of antibody per mouse per injection, or left untreated, followed by 6 days of DSS treatment. Data are shown as the mean ± SEM (n = 3) percent reduction in TGF-β mRNA levels after CD4 T cell depletion. Expression levels were normalized to the endogenous control $\text{gapdh}$. *p < 0.05; **p < 0.01.

Increased IgA and Bacterial Translocation in JAM-A-Deficient Mice

We previously demonstrated that JAM-A-deficient mice have increased numbers of isolated lymphoid follicles in the large intestine when compared to wild-type mice (Laukoetter et al., 2012).
Figure 4. Increased IgA Levels and Bacterial Translocation in JAM-A-Deficient Mice

(A and B) Immunofluorescence labeling of IgA in F11r+/+ and F11r−/− (JAM-A-deficient) colonic mucosa. Frozen sections (7 μm thickness) were stained with a monoclonal anti-IgA antibody directly conjugated with PE (red). Tissues were counterstained with the nuclei stain TO-PRO-3 (blue). Scale represents 50 μm.

(C) Quantification of IgA staining was performed in F11r+/+ and F11r−/− colonic mucosa on ten different fields per section (n = 3) as described in the Experimental Procedures.

(D) Representative images of colonic mucosa from F11r+/+ and F11r−/− mice showing CD138/IgA double-positive cells. Insets show CD138 staining (green, inset) and IgA staining (red, inset) on representative double-positive cells indicated by arrows. Tissues were counterstained with TO-PRO-3 (blue). Scale bar is 10 mm. Lower bar graph represents the mean ± SD of the number of CD138/IgA double-positive cells per high-power field (40×) in the colonic lamina propria from 40 determinations (two mice per group). **p < 0.005.

(E) Serum IgA levels as determined by ELISA assays on serum samples from F11r+/+ and F11r−/− mice samples. Negative controls include serum samples from Igha−/− mice (ND, none detected). *p < 0.05.

(F) Bacterial cfu counts from spleen, mLN, liver, and colonic lamina propria (LP) samples from F11r+/+ and F11r−/− mice (n = 3). Organs were collected and tissue homogenates were prepared as described in the Experimental Procedures. Serial dilutions were plated onto blood agar medium and the numbers of bacterial cfu were enumerated. No bacterial growth was observed for F11r−/− spleen, mLN, and liver samples (ND). *p < 0.05.

Since these lymphoid aggregates are mainly composed of B220 staining B cells, we examined whether this could be associated with increased IgA production. By immunofluorescence staining, we found elevated amounts of IgA in colonic mucosa from F11r−/− mice compared to F11r+/+ control mice (Figures 4A–4C). Consistent with the elevated levels of IgA in the colonic mucosa, there were abundant collections of lamina propria CD138+IgA+ plasma cells in F11r−/− mice (Figure 4D). Furthermore, quantification of CD138+IgA+ plasma cells revealed a significant increase in F11r−/− mice compared to F11r+/+ control mice (Figure 4D). These elevated local amounts of IgA were also associated with higher amounts of systemic IgA, as demonstrated by ELISA of serum samples from F11r−/− mice (Figure 4E). The increased amounts of IgA were isotype specific since there was no significant difference in serum IgG or IgM. Furthermore, no difference in IgM staining was observed between F11r+/+ and F11r−/− colonic mucosa (data not shown).

Because JAM-A-deficient mice have increased colonic permeability, we examined whether the increased IgA levels correlated with enhanced translocation of luminal bacteria into the colonic LP. Bacterial counts were determined by plating serial dilutions and enumerating colony-forming units (cfu) in colonic LP tissue homogenates derived from washed mucosa that was processed to remove the epithelium. We found a significantly higher number of bacteria in the colonic LP from F11r−/− mice compared to F11r+/+ control mice with a two-log difference in the number of cfu (Figure 4F). In addition, bacterial growth was also detected in the spleen, mesenteric lymph nodes, and liver from F11r−/− mice, but not from F11r+/+ control mice, suggesting that in F11r−/− mice bacteria that translocated into the lamina propria further disseminated into secondary lymphoid organs and peripheral tissues.

IgA Production Limits Acute Colitis in JAM-A-Deficient Mice

Given the observation that CD4+ T cell-mediated responses are critical to limit the susceptibility of JAM-A-deficient mice to DSS-induced colitis and that IgA levels are significantly increased, we crossed F11r−/− mice with IgA-deficient (Igha−/−) mice and measured their susceptibility to acute injury-induced colitis. F11r−/−Igha−/− mice were far more susceptible to DSS-induced acute colitis than F11r−/− mice or Igha−/− mice. F11r−/−Igha−/− mice weighed only 83.5% ± 1.0% of their initial body weights compared to 92.1% ± 1.7% for F11r−/− mice at day 6 (Figure 5A) and had significantly higher disease activity indices than F11r−/− mice beginning at day 4 of DSS treatment (Figure 5B). Histologic analyses of colonic mucosa confirmed significantly increased disease in the F11r−/−Igha−/− mice as compared to F11r−/− control mice (Figure 5C). These findings suggest that IgA plays a critical role in limiting the susceptibility of JAM-A-deficient mice to DSS-induced colitis.
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Evident by extensive mucosal injury and ulceration (Figures 5C and 5D). Given that TGF-β is important for IgA class switch recombination and plasma cell differentiation, we explored whether TGF-β impacted IgA production in F11r+/− mice. First, we examined IgA in DSS-treated F11r+/− mice that were depleted of CD4+ T cells, since these cells are the predominant producers of TGF-β. We observed a significant decrease in IgA protein levels in CD4+ T cell-depleted JAM-A-deficient mice compared to untreated F11r+/− mice (Figure 5E). Moreover, TGF-β neutralization also resulted in a significant reduction of IgA protein levels in anti-TGF-β-treated F11r+/− mice compared with untreated F11r+/− mice (Figure 5F). Altogether, these data demonstrate that increased production of IgA is a unifying protective mechanism promoted by TGF-β-producing CD4+ T cells that compensates for increased colonic permeability under conditions of acute mucosal injury.

Since TGF-β was shown to induce IgA and limit colonic disease induced by DSS, we also investigated the role of IL-10, another cytokine involved in maintaining intestinal homeostasis. Blockade of IL-10 signaling in F11r+/− mice using IL-10 receptor antibodies resulted in a significant increase in disease susceptibility compared to untreated F11r+/− mice, albeit to a lesser extent compared to anti-TGF-β treatment (Figures 5G and 5H). However, treatment of F11r+/− mice with anti-IL-10R antibodies did not have any significant effects on colonic IgA levels as determined by ELISA (Figure 5I), suggesting that the protective effects of IL-10 in F11r+/− mice are independent of IgA.

**DISCUSSION**

Intestinal immune cells are constantly challenged to maintain homeostasis in the face of stimulation by gut microbiota. In a situation of defective epithelial barrier function as encountered in JAM-A-deficient mice, one would predict that spontaneous disease might develop due to increased bacterial translocation across the epithelium. Interestingly, despite defects in barrier function, JAM-A-deficient mice do not develop clinical signs of spontaneous colitis, which suggests that protective mechanisms are in place to suppress inflammatory cues and prevent spontaneous disease. In the present study, we provide evidence that adaptive immunity, which plays a negligible role in protecting from acute intestinal inflammation in the context of a normal epithelial barrier, is indeed essential in protecting from...
acute, injury-induced colitis in the context of increased intestinal permeability. In particular, this adaptive immunity-based “protective” effect is dependent on TGF-β-producing CD4+ T cells that serve to promote IgA secretion in JAM-A-deficient mice. Altogether, these observations establish a role for adaptive immune responses in limiting severe, acute mucosal injury in the context of intestinal barrier compromise.

Our findings that acute intestinal inflammation is markedly enhanced in JAM-A-deficient mice lacking CD4+ T cells are in accordance with the well-established role of immunoregulatory T cells in suppressing colitis (Groux et al., 1997; Kim et al., 2007; Powrie et al., 1996; Rubtsov et al., 2008; Sakaguchi et al., 1985; Zhang et al., 2001). Thus, it would be tempting to speculate that the enhanced susceptibility of JAM-A-deficient mice to DSS-induced acute colitis is due to the absence of functional CD4+CD25+ Treg cells. A critical role for CD4+CD25+ Treg cells in mediating IgA production in response to commensal flora has been reported (Cong et al., 2009). Here we used anti-CD25 to attempt to eliminate CD4+Foxp3+ Treg cells but only achieved partial depletion and observed no increase in susceptibility to colitis. While our findings preclude definitive assessment of the role of Foxp3+ Treg cells in regulating the adaptive immune compensation observed here, they are consistent with those reported by Cong et al. (Cong et al., 2009) and highlight a critical role for intestinal permeability in regulating TGF-β-producing Treg cell-mediated IgA production.

We also evaluated the relative contribution of TGF-β, which is well appreciated for its role in mediating Treg cell immunosuppressive functions (Li et al., 2007; Marie et al., 2005). Neutralization of TGF-β in JAM-A-deficient mice led to the striking finding that compensation for intestinal barrier defects also depends on TGF-β signaling. It is possible that the increased TGF-β production in JAM-A-deficient mice compared with wild-type controls represents a secondary response to increased bacterial translocation and subsequent immune activation. Given the fact that TGF-β amounts in JAM-A/Rag1-deficient mice are greatly reduced compared to JAM-A-deficient mice (data not shown), we demonstrated that TGF-β-producing T cells are a major source of TGF-β in JAM-A-deficient mice. We further showed that increased colonic TGF-β production in JAM-A-deficient mice during acute mucosal injury-induced colitis is CD4+ T cell dependent. These findings suggest that TGF-β-secreting cells (Weiner, 2001) may be a critical component of immune-mediated protection during increased intestinal permeability.

The role of T cells in promoting TGF-β-mediated immune regulation of intestinal inflammation has been previously described in a model of CD4+CD45RBhi-induced colitis. T cells that express dnTGF-βRII escape immunoregulation by Treg cells, indicating that TGF-β-mediated control of inflammation is T cell dependent (Fahien et al., 2005). Thus, in JAM-A-deficient mice, TGF-β-producing CD4+ T cells may regulate pathogenic CD4+ T cells to limit intestinal inflammation. However, it is also conceivable that TGF-β exerts its effect via T cell-indepen dent mechanisms. Our data are also consistent with TGF-β-producing CD4+ T cells playing a critical role in IgA isotype class-switch recombination in B cells, thereby promoting IgA-mediated immune responses and control of intestinal inflammation in the gut induced by antigen exposure (Borsutzky et al., 2004; Cazac and Roes, 2000; Cerutti, 2008).

Importantly, loss of IgA appears to be a unifying mechanism by which depletion of CD4+ T cells and neutralization of TGF-β increases susceptibility to acute mucosal damage in the context of a leaky epithelial barrier. Based on our data, it is clear that IgA-associated responses are critical for immune mediated-compensation during enhanced intestinal permeability. Murine studies have demonstrated an important role for IgA antibodies in immune exclusion. For example, mice deficient in the polymeric immunoglobulin receptor, which are unable to secrete IgA into the gut lumen, display increased mucosal leakiness and antigen uptake (Johansen et al., 1999; Sait et al., 2007). The current study highlights the observations that: (1) IgA production is augmented in mice with a leaky gut barrier, most likely representing a humoral compensatory mechanism in response to the increased antigen uptake across the epithelium, and (2) that regulation of intestinal inflammation in the specific context of increased epithelial permeability and acute mucosal injury relies on IgA-mediated immunity. The exact mechanisms by which IgA antibodies participate in dampening tissue inflammation in JAM-A-deficient mice remain unclear, but may involve increased immune exclusion at the interface between lumen and epithelial cells and/or increased transport of antigens across the epithelium and subsequent recognition and phagocytosis by antigen-presenting cells.

It is important to note that the development of intestinal inflammation during DSS-induced acute mucosal injury is a lymphocyte-independent process in wild-type mice (Dielemann et al., 1994). However, in JAM-A-deficient mice, which have enhanced colonic permeability, severity of colitis depends on adaptive immune cells, as demonstrated by the increased susceptibility to DSS in JAM-A/Rag1-deficient mice compared to JAM-A-deficient mice. This finding implicates a crucial role of adaptive immunity under conditions of existing defects in barrier function.

In summary, our findings provide new insights into the regulation of mucosal homeostasis in the context of impaired intestinal epithelial barrier function. JAM-A-deficient mice represent an important tool to study immune mechanisms implicated in the containment of acute inflammatory responses triggered by enhanced exposure to luminal antigens and immune activation. We have identified an essential role for TGF-β-producing CD4+ T cells promoting IgA secretion in controlling intestinal inflammation in the presence of a compromised intestinal epithelial barrier.

**EXPERIMENTAL PROCEDURES**

**Mice**

JAM-A-deficient mice (F11r−/−) and littermate controls were bred on site. Rag1−/−Mm/− (Rag1−/−) mice on a C57BL/6 background were purchased from The Jackson Laboratories (Bar Harbor, ME). IgA-deficient mice (Igha−/−) (Harriman et al., 1999) were a kind gift from Dr. L. Eckmann. Rag1−/− and Igha−/− mice were crossed with JAM-A-deficient mice to generate F11r−/−Rag1−/− and F11r−/−Igha−/− mice. Mice were maintained under specific pathogen-free conditions at Emory University. All animal procedures were reviewed and approved by the Emory University Institutional Animal Care and Use Committee.

**Genotyping**

PCRf were performed on tail genomic DNA using the following primers: F11r forward 5’-TCTTTTCCACCATCGGAACG-3’ and reverse 5’-CGGCAT

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TAATCCCCAGAAGGT-3'; Rag1 forward common 5'-CCGCAACAGGTTTTTCA TCCT-3', reverse wild-type allele 5'-GAGGTTCCGCTACGACTCTG-3', and reverse mutant allele 5'-TGAGATGGAATGTGTCGAG-3'.

**DSS-Induced Colitis**

Mice were provided 2% (wt/vol) DSS (molecular mass = 36–50 kDa) (MP Biomedicals) for 5–7 days, and then colons were assessed for weight, length, and histology. Daily clinical assessment of DSS-treated animals included evaluation of stool consistency, detection of blood in stool, and body weight loss measurement. An individual score (ranging from 0 to 4) was attributed for each one of these parameters, and a disease activity index (DAI) ranging from 0 to 4 was calculated by combining all three scores (Laukoetter et al., 2007).

**Histology**

Histological examination was performed on whole colons of experimental and control mice. In addition, colonic tissue samples were embedded in OCT for immunofluorescence staining and stored at −80 °C. Histological parameters were quantified in a blinded fashion by two experienced gastrointestinal pathologists using parameters as previously described (Nava et al., 2010) with some modification. Briefly, hematoxylin and eosin sections of Swiss roll mounts of entire mouse colons were assessed for the percentage of the mucosa containing ulceration or crypt epithelial injury/damage/active inflammation comprising at least 1/3 of the thickness of the mucosa. The ulceration and injury/damage values were added and reported as a histologic damage index.

**Flow Cytometry**

Lamina propria lymphocyte isolation was performed as previously described (Benning et al., 2011; Medina-Contreras et al., 2011). Intestinal cell preparations included lymphoid follicles. Cells were stained with PerCP-conjugated anti-CD45 (30-F11), FITC-conjugated anti-CD19 (ID3), allophycocyanin-conjugated TCRβ (H57-597), eFlour 450-conjugated anti-CD4 (L3T4) (eBioscience), and PE/Texas Red-conjugated anti-CD8α (5H10) (Invitrogen) monoclonal antibodies. Data were acquired using a LSR II flow cytometer (Becton Dickinson) and analyzed using Flowjo software (TreeStar). For intracellular cytokine detection, cells were restimulated with PMA and ionomycin ex vivo in the presence of GolgiPlug (BD Biosciences).

**Cytokine and IgA Assays**

For TGF-β1 protein level analyses, proximal colonic tissues were homogenized in sterile 1× PBS supplemented with 1% Triton X-100 and protease and phosphatase inhibitor cocktails (Sigma) and centrifuged for 20 min at maximum speed at 4°C. Supernatants were used to measure TGF-β1, IL-6, IL-1β, and TNF-α protein levels using ELISA kits (eBioscience). IgA protein levels were evaluated on colonic homogenate supernatants or serum samples using an ELISA kit (ImmunoLogic Consultants Laboratory, Inc.).

**Broad-Spectrum Antibiotic Treatment**

Mice were treated using a modified protocol described elsewhere (Rakoff-Nahoum et al., 2004).

**Bacterial Translocation**

Organs were weighed and colon length was measured. For the spleen and mLN, the whole organ was homogenized in 500 μl sterile PBS. For the liver, a piece (200 mg) of the right lobe was collected and homogenized. For colonic bacterial counts, fecal contents were removed by rinsing longitudinally opened colons with sterile PBS. Colons were then cut into 1.5 cm segments, and epithelial cells were removed by vigorous horizontal shaking at 37°C for 20 min in HBSS containing 5% FBS, 10 mM HEPES, and 2 mM EDTA (pH 8.0). This procedure was repeated two times. Samples were subsequently homogenized in 500 μl sterile PBS using an Omni-Prep homogenizer. Serial dilutions were then plated onto blood agar plates and incubated at 37°C for 36 hr, and cfu were enumerated.

**Antibody-Mediated Depletion**

Anti-CD4 (clone GK1.5) and anti-CD8α (YTS169.4) neutralizing antibodies (Bio X Cell) were used. For each antibody, two 500 μg injections were given i.p. at day −2 and day −1 prior to DSS treatment. CD25 depletion was performed as previously described (Cong et al., 2009). To assess depletion efficiency, leukocytes were isolated from large intestine, small intestine, spleen, and mLN and subjected to flow cytometric analyses. TGF-β1 depletion prior to DSS treatment was carried out by performing two 100 μg injections i.p. at day −5 and day −2 prior to DSS treatment using anti-TGF-β1 (1D11.16.8) monoclonal antibody (Bio X Cell). For IgA protein level measurement, chronic TGF-β1 depletion was conducted by injecting 1-week-old mice with 100 μg anti-TGF-β1 neutralizing antibodies twice weekly for the first 2 weeks followed by 100 μg once every week for a total of 7 weeks.

**Real-Time PCR Analysis**

Total RNA was extracted from proximal colons of F11r+/- and F11r+ mice using RNeasy Mini kit (Invitrogen), and genomic DNA contamination was removed following DNaseI treatment (Qiagen). Real-time PCR reactions were set up using the iScript One Step RT-PCR kit with SYBR Green (Bio-Rad Laboratories) and the following primers: forward tgfβ1 5'-ACCATGCC AACCTCTGTCTG-3' and reverse tgfβ1 5'-CGGTTGTTGTTGTTGTAAG-3'; forward gapdh 5'-TCATTGACCTCAACTACATGGTCTA-3' and reverse gapdh 5'-ACACCTAGACTCCACGACATACT-3'. For the preparations of IECs and lamina propria-derived lymphocytes, cell suspensions were processed through a Percoll gradient and individual fractions were collected and used for RNA extraction. Reactions were run on a MyiQ Single Color Real-Time PCR Detection system (Bio-Rad Laboratories), and Cq values were used to calculate relative expression.

**Confocal Microscopy**

Frozen tissue sections (7 μm thickness) from large intestines were fixed in cold ethanol for 20 min at −20°C. Sections were then blocked with 3% BSA prior to incubation with PE-conjugated rat anti-mouse IgA (eBioscience) for 20 min. Plasma cells were stained using anti-mouse CD138 (eBioscience). Tissues were counterstained with TO-PRO-3 for 3 min and mounted on covergrips. Images were acquired with a LSM 510 confocal microscope (Zeiss) and analyzed using ImageJ software (National Institutes of Health).

**Data Analysis**

Results are expressed as means ± SEM unless otherwise indicated. p values were calculated using one-way ANOVA and unpaired two-tailed Student’s t test. p < 0.05 was considered statistically significant.

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