

Unique Role of Junctional Adhesion Molecule-A in Maintaining Mucosal Homeostasis in Inflammatory Bowel Disease

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Background & Aims: Junctional adhesion molecule-A (JAM-A) is localized at the tight junctions and controls leukocyte migration into the tissues. However, its functional role in inflammatory bowel disease (IBD) is unexplored. **Methods:** Control, Crohn's disease (CD), and ulcerative colitis (UC) tissue specimens were studied for JAM-A expression, as well as the colon of mice given dextran sodium sulfate (DSS). Wild-type and JAM-A^{-/-}, Tie-2-Cre-JAM-A^{-/-} (endothelial/hematopoietic-specific JAM inactivation) mice were studied for susceptibility to DSS. Disease activity and colonic inflammation were assessed using a disease activity index histology and endoscopy, and mucosal cytokines were measured by enzyme-linked immunosorbent assay. JAM-A function was investigated by RNA silencing in epithelial cells, and apoptosis was measured. **Results:** In both CD and UC, as well as in experimental colitis, there is a loss of epithelial but not endothelial JAM-A expression. Deletion of JAM-A results in a dramatic increase in susceptibility to DSS colitis, as assessed by weight loss, disease activity index, histologic and endoscopic severity, and strikingly high mortality rates. This is not caused by the absence of JAM-A in the endothelial or hematopoietic compartments because Tie-2-Cre-JAM-A^{-/-} mice are no more susceptible to DSS colitis than wild-type animals. JAM-A^{-/-} mice displayed increased intestinal permeability and inflammatory cytokine production, and marked epithelial apoptosis. Silencing of JAM-A in intestinal epithelial cells resulted in increased permeability in vitro. **Conclusions:** Our results show a nonredundant and novel role of JAM-A in controlling mucosal homeostasis by regulating the integrity and permeability of epithelial barrier function.

Junctional adhesion molecule-A (JAM-A) belongs to a family of cell adhesion molecules that localizes at intercellular junctions and mediates several different physiological processes, such as junction assembly and leu-

kocyte migration.^{1,2} JAM-A expression is particularly abundant at tight junctions of endothelial and epithelial cells, but also is present on leukocytes.³ At the endothelial sites, JAM-A has been shown to be crucially involved in mediating leukocyte transmigration through the paracellular pathway.^{1,4} Indeed, during vascular inflammation such as in atherosclerosis, JAM-A is up-regulated and controls leukocyte influx into the atherosclerotic lesion.^{5,6} In addition, JAM-A blockade by monoclonal antibody or genetic deletion in mice has been proven to reduce leukocyte infiltration in in vivo models of inflammation, such as experimental cytokine-induced meningitis, air-pouch model of skin inflammation, and in ischemia-reperfusion injury models in the liver and heart.⁷⁻¹⁰ At epithelial sites, JAM-A has been reported to regulate epithelial barrier function in the intestine and the cornea.¹¹⁻¹³ Indeed, JAM-A inhibition affects epithelial barrier recovery after an in vitro model of epithelial injury and plays an important role in epithelial tight junction assembly.¹³ However, the expression and functional roles in vivo of JAM-A have not been precisely characterized in intestinal inflammation.

Crohn's disease (CD) and ulcerative colitis (UC), the 2 major forms of inflammatory bowel disease (IBD), are chronic intestinal disorders. Although their etiology still is unknown, clear evidence points out that besides the widely recognized aberrant function of the immune cells, nonimmune cells such as those in the epithelium and endothelium actively participate in disease pathogenesis.¹⁴⁻¹⁶ Many molecules have been recognized as key effectors in both CD and UC pathogenesis, but the func-

Abbreviations used in this paper: DSS, dextran sodium sulfate; JAM-A, junctional adhesion molecule-A; KC, keratinocyte chemoattractant; KO, knockout; MIP, microphage inflammatory protein; RT, room temperature; TNF, tumor necrosis factor; wt, wild-type; ZO-1, zonula occludens 1.

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tional role of JAM-A in IBD pathogenesis has not been addressed fully.

Therefore, the aim of the present study was to analyze the expression of JAM-A in the normal and inflamed intestine, and to investigate the functional role of JAM-A in experimental colitis. Our results show that in both forms of human IBD and in experimental colitis, JAM-A is dramatically down-regulated at epithelial tight junctions. Furthermore, endothelial JAM-A is not involved in the regulation of intestinal inflammation, whereas epithelial JAM-A plays a key role in maintaining intestinal barrier function through the regulation of epithelial cell barrier integrity.

Materials and Methods

Reagents and Antibodies

All the tissue culture reagents were purchased from Life Technologies (Paisley, UK). Caco-2 cells were routinely cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, and 100 U/mL antibiotics (penicillin and streptomycin).

Mouse anti-human JAM-A (BV16) and rat anti-mouse JAM-A (BV12 and BV11) monoclonal antibodies were generated as previously reported⁷; rabbit anti-occludin and anti-zonula occludens 1 (ZO-1), and rat anti-E-cadherin were from Zymed-Invitrogen (San Francisco, CA); rabbit anti- β -catenin was from AbCam (Science Park Cambridge, UK); secondary Abs 488- or Cy3-conjugated were from Molecular Probes (San Francisco, CA) and Jackson ImmunoResearch Laboratories (Westgrove, PA), respectively. Alexa Fluor 488-conjugated goat anti-mouse, antirat, or anti rabbit immunoglobulin (Ig)G and/or Alexa Fluor 594-conjugated goat anti-mouse or antirat IgG antibodies were from Invitrogen. Antibody against murine pan-cytokeratin was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-claudins-1, -2, and -3 and cingulin were from Zymed.

Patients

Intestinal tissues were obtained from surgical specimens of patients with CD, UC, and as control from normal areas of the intestine of patients admitted for bowel resection because of colon cancer, polyps, or diverticulosis. Specimens were frozen in optimum cutting temperature tissue on dry ice and stored at -80°C . Human studies were approved by the ethical committee of the Istituto Clinico Humanitas.

Animals

Male JAM-A^{+/+}, JAM-A^{-/-}, and Tie-2 Cre JAM-A^{-/-} mice, generated and genotyped as described,⁸ were used in this study. These mice were on a C57BL/6J background, and wild-type littermates were used as controls

in all experiments. All mice were maintained under pathogen-free conditions. Procedures involving animals and their care conformed to institutional guidelines in agreement with national and international law, after approval by the ethical committee.

Induction of Colitis

Ten- to 12-week-old mice received 2% dextran sodium sulfate (DSS) (molecular mass, 40 kilodaltons; MP Biomedicals, Irvine, CA) ad libitum in filter-purified drinking water for 7 days. Colitis was scored daily using standard parameters that included body weight, diarrhea, presence of blood in the stools, and rectal prolapse, and a disease activity index was calculated as reported.^{17,18} Grading of intestinal inflammation was determined as previously described¹⁸ in a blinded fashion: no inflammation was scored as 0; modest numbers of infiltrating cells in the lamina propria was scored as 1; infiltration of mononuclear cells leading to separation of crypts and mild mucosal hyperplasia was scored as 2; massive infiltration with inflammatory cells accompanied by disrupted mucosal architecture, loss of goblet cells, and marked mucosal hyperplasia was scored as 3; and all of the earlier described plus crypt abscesses or ulceration were scored as 4, with a histologic score from 0 to 15.

For treatment studies, JAM-A^{+/+} mice undergoing DSS treatment were injected intraperitoneally at day -3, 0, 3, and 6 with a dose of 100 μg /mouse of monoclonal antibody BV11 in a pyrogen-free saline. In some experiments, mice underwent a combination of ciprofloxacin (50 mg/kg/day) and metronidazole (100 mg/kg/day) for 4 weeks before DSS treatment and during DSS treatment.

Endoscopic Damage Assessment

Damage to the colonic mucosa during treatment with DSS was followed up in vivo using the Coloview (Karl Storz, Tuttlingen, Germany) system experimental endoscopy set-up, as previously described.¹⁹ This endoscopic procedure was performed under anesthesia. The endoscopic score of colitis severity (murine endoscopic intestinal colitis score: range, 0–15 points) was based on the evaluation of colon translucency (0–3 points), presence of fibrin attached to the bowel wall (0–3 points), granular aspect of the mucosa (0–3 points), morphology of the vascular pattern (0–3 points), and the presence of loose stools (0–3 points).

Colon Organ Culture and Enzyme-Linked Immunosorbent Assay

Colons from all mice were excised, opened, and cut longitudinally into 3 parts. One of all 3 parts was washed in cold phosphate-buffered saline (PBS) supplemented with penicillin, streptomycin, and amphotericin B (Cambrex; BioWhittaker, Walkersville, MD) and incubated in serum-free RPMI 1640 medium with 0.1% fetal

bovine serum, penicillin, streptomycin, and amphotericin B at 37°C in 5% CO₂. After 24 hours, supernatant fluid was collected, centrifuged, and stored at -20°C. Supernatants were analyzed for tumor necrosis factor α (TNF- α), macrophage inflammatory protein (MIP)-1- α , MIP-2, and keratinocyte chemoattractant (KC) content in duplicate using commercially available enzyme-linked immunosorbent assay kits, as previously reported (R&D Systems, Minneapolis, MN).¹⁵ In some experiments, serum was obtained by centrifugation, stored at -80°C, and then cytokine concentration was measured.

Western Blot Analysis

Western blot analysis was performed as previously reported.²⁰

Immunofluorescence Staining

Caco-2 cells (40,000 cells/cm²) were seeded on fibronectin-coated (5 μ g/mL in PBS; Sigma, St Louis, MO) glass coverslips (13-mm diameter; Carolina Biological Supply Company, Burlington, NY) in 1 mL of complete medium. Cells were fixed on ice with prechilled methanol for 5 minutes and stained with ZO-1 (Zymed) 1:125 or β -catenin (AbCam). Alternatively, for the occludin staining (Zymed), the cells were permeabilized with 0.2% Triton X-100 (2 min on ice), fixed with PFA 3.7% at room temperature (RT) for 30 minutes, and then permeabilized with 0.05% Triton X-100 (5 min on ice). E-cadherin-stained cells (Zymed) were fixed with prechilled ethanol 95% for 30 minutes at 4°C and then with prechilled acetone for 1 minute at RT. Blocking was performed in 2% bovine serum albumin (Sigma) for 1 hour at RT. After incubation with the primary antibody (2–5 μ g/mL in PBS 2% bovine serum albumin) for 1 hour at RT, cells were washed 3 times with PBS 0.2% bovine serum albumin and labeled with the appropriate fluorescently conjugated secondary antibody for 45 minutes at RT.

Frozen sections (4- μ m) of colonic mucosa from human and murine tissue were fixed in cold acetone (10 minutes at -20°C). The sections then were blocked with PBS containing 2% bovine serum albumin for 60 minutes at RT and incubated with primary antibodies: anti-JAM-A (1:50), E-cadherin (1:50), or ZO-1 (1:125) for 1 hour at RT. Alexa Fluor 488-conjugated goat-antimouse, anti-rat, or anti-rabbit IgG, and/or Alexa Fluor 594-conjugated goat-antimouse or anti-rat IgG antibodies were used as secondary antibodies (1:1000, 30 minutes, RT), followed by incubation with 1 μ g/mL Hoechst 33258 (5 minutes, RT), as previously described.^{18,21}

Sections were mounted with FluorSave reagent (Calbiochem, San Diego, CA) and analyzed with a laser scanning confocal microscope (Fluoview FV1000; Olympus, Tokyo, Japan). Images (1024 $\times$ 1024 pixels) were acquired with an oil immersion objective (100 $\times$ 1.4 NA Plan-Apochromat; Olympus). Quantification of JAM-A expres-

sion was performed in a blinded manner on stained sections by a quantitative method that assessed the fluorescence intensity in pixels (FV1000 FlowView; Olympus).^{18,22,23} Fluorescence intensity in pixels was quantified in triplicate from each tissue section.

Assessment of Apoptosis and Down-Regulation of JAM-A Expression

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay was performed on cryostatic sections using the in situ cell death detection kit (Fluorescein; Roche, Mannheim, Germany) according to the manufacturer's recommendations. For detection of epithelial cells (E-cadherin) and nuclei (Hoechst 33258), immunofluorescence staining were performed in parallel to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling, as previously reported.²⁴ Apoptosis was quantified as the percentage of apoptotic epithelial cells/field.

In vitro apoptosis was studied by flow cytometry and by acridine orange and ethidium bromide staining.²⁵

Down-regulation of JAM-A expression was performed as previously described.²⁶

Epithelial Transepithelial Resistance

Short hairpin RNA (shRNA)-infected or control Caco-2 cells (4×10^5) (see earlier) were seeded in the upper chamber of a transwell filter (pore diameter, 3 μ m; Costar, Milan, Italy) for 10 days. The transepithelial resistance (TER) was measured every other day until day 7 and then daily until day 10 with a Voltmeter (Millicels; Millipore, Bedford, MA), in the presence or absence of 50 nmol/L of the caspase inhibitor Z-VAD (R&D Systems).

Statistical Analysis

Data were analyzed by Graphpad software (San Diego, CA) and expressed as mean $\pm$ SEM. The Kruskal-Wallis test was used for global comparisons within multiple groups, and the Mann-Whitney *U* test was used for comparison between 2 groups. Statistical significance was set at a *P* value of less than .05.

Results

Evidence for Down-Regulation of JAM-A in the IBD Epithelium

To investigate the expression levels of JAM-A in normal and IBD-involved intestine, we performed confocal fluorescence microscopy of 11 histologically control and 13 active CD- and 15 UC-involved mucosa derived from surgical specimens. Normal colonic mucosa showed strong epithelial and vascular immunostaining of JAM-A. The specificity of JAM-A at the epithelial and endothelial junction sites was confirmed by co-staining with the tight junctions marker ZO-1 (Figure 1).

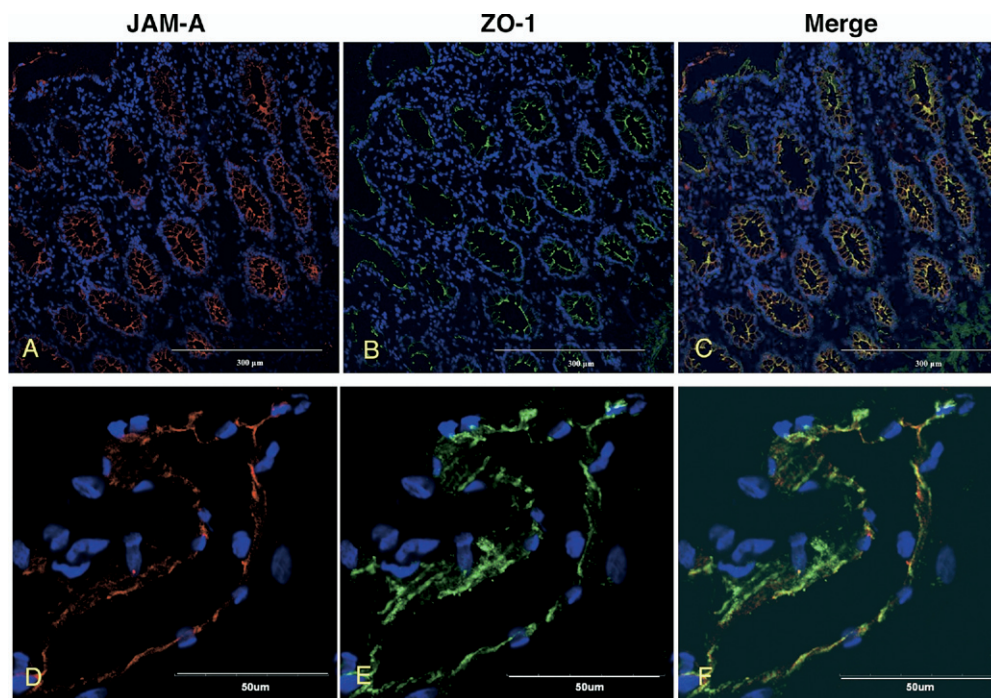


Figure 1. Expression of epithelial and endothelial JAM-A in human colons of control subjects. Fluorescence micrographs of colonic mucosa epithelial and endothelial cells in normal mucosa, stained for (A and D) JAM-A (red), (B and E) ZO-1 (green), (C and F) nuclei (blue) and merge (yellow). The panels are representative of 11 controls (magnification, 10 $\times$).

In contrast to control intestine (1755 ± 66 fluorescence intensity of pixels), in the mucosa of actively inflamed CD (728 ± 70 fluorescence intensity of pixels) or UC (650 ± 65 fluorescence intensity of pixels) patients, epithelial JAM-A was down-regulated significantly (both $P < .01$) without any apparent difference between the 2 forms of IBD (Figure 2A), whereas its endothelial expression was similar between controls and IBD patients (not shown). When normal mucosal tissues were compared with uninvolved CD and UC mucosa (1645 ± 70 and 1745 ± 85 fluorescence intensity of pixels, respectively), no significant differences were observed in JAM-A expression (Figure 2A). When assessed by Western blot analysis, JAM-A levels in inflamed mucosa also were significantly lower ($P < .05$) in mucosal extracts from CD- (0.17 ± 0.01) and UC- (0.28 ± 0.02) involved tissues compared with control tissue (0.67 ± 0.02) (Figure 2B).

We next investigated JAM-A expression in murine intestine using DSS to induce colitis, a widely used model of experimental IBD. As assessed by confocal microscopy, the murine JAM-A expression pattern was essentially similar to that of human specimens. JAM-A was expressed mainly by epithelial and endothelial cells, as determined by its colocalization with E-cadherin (not shown). However, when compared with the normal epithelium where JAM-A was expressed abundantly, there was a dramatic down-regulation in DSS colitis (Figure 3). No differences were observed in JAM-A expression levels between the control and colitic microvasculature of the murine intestine (not shown).

Effect of Genetic Deletion of JAM-A on Experimental Colitis

Because JAM-A is expressed by both intestinal endothelial and epithelial cells, we investigated the effect of JAM-A deletion in mice administered DSS. We used both mice with a selective endothelial JAM-A deletion (mice Tie-2 Cre JAM-A^{-/-}) and total JAM-A knockout (KO). Of note, Tie-2 also is active in the hematopoietic lineage, leading to Cre expression and deletion of loxP flanked genes in this compartment.²⁷ Compared with wild-type mice, Tie-2 Cre JAM-A^{-/-} showed no changes in their susceptibility to DSS-induced colitis over a period of up to 10 days (Figure 4A). On the contrary, mice with a total KO of JAM-A showed a dramatically increased susceptibility ($P < .05$) to DSS-induced colitis as early as at day 4 compared with the wild-type (wt) mice, as shown by a weight loss and colitic disease activity index (Figure 4A and B). Strikingly, 8 days after 2% DSS treatment, 100% of JAM-A total KO mice died compared with 0% of wt mice (Figure 4C). In addition, mice with a total KO of JAM-A displayed significantly ($P < .05$) more severe endoscopic damage and a very high histologic inflammation score (Figure 5). No differences in endoscopic and histologic inflammation scores were observed between control wt and JAM-A^{-/-} mice before DSS treatment (Figure 5). Wild-type mice administered DSS and treated with the BV11 monoclonal antibody to block JAM-A displayed a similar phenotype to JAM-A^{-/-} mice, as assessed by weight loss (supplementary Figure 1A; see supplementary material online at www.gastrojournal.org), disease activity index, and histologic scores (not shown).

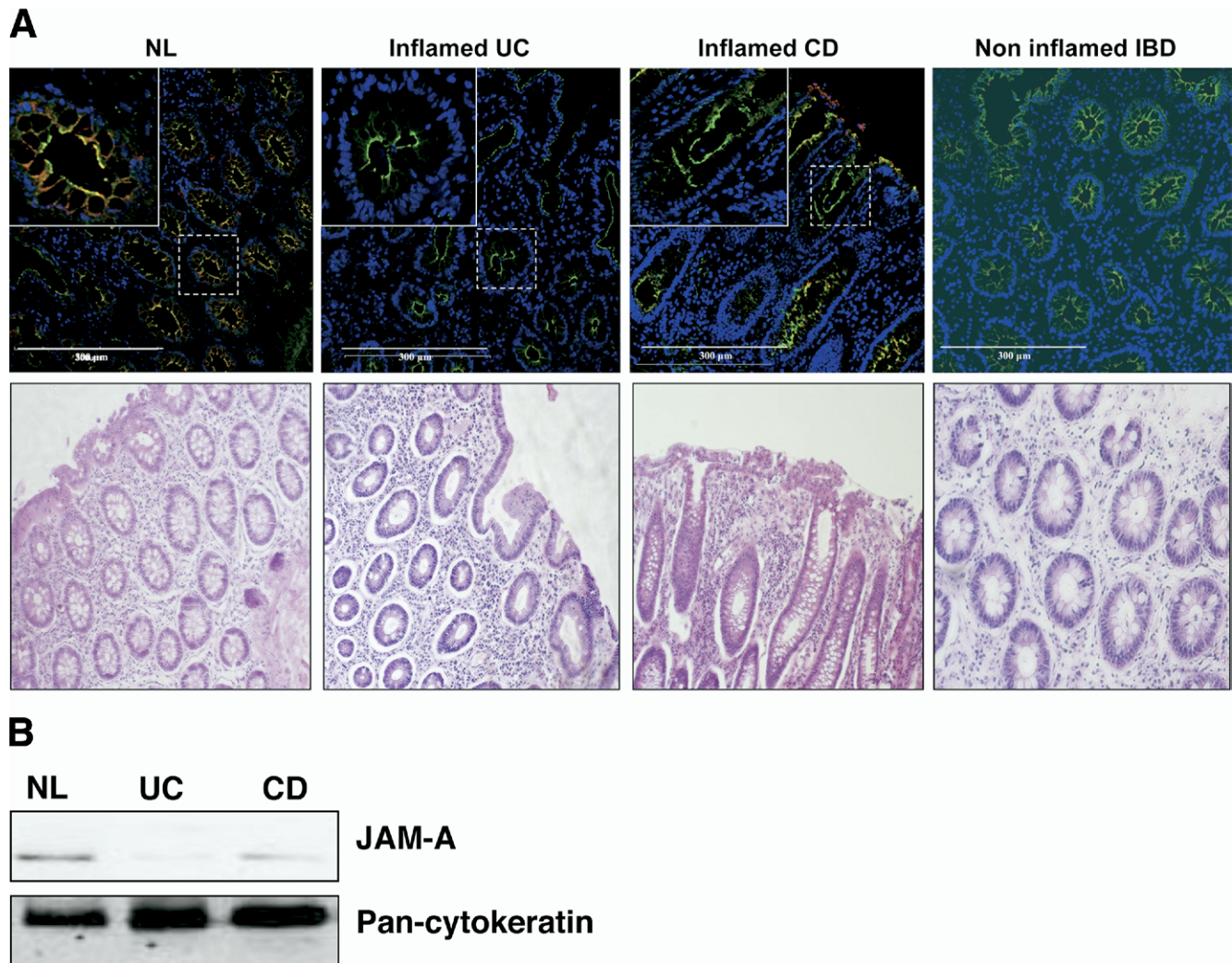


Figure 2. JAM-A expression in IBD mucosa. (A) Fluorescence micrographs of colonic mucosa in (a) normal and actively inflamed (b) UC and (c) CD, and (d) inactive IBD mucosa, and (f–h) their relative H&E stainings. The staining represents the yellow merge of the staining for JAM-A (red), ZO-1 (green), and the nuclei (blue). The panels are representative of 11 controls, 15 UC, and 13 CD samples (magnification, 10×). (B) Western blot for JAM-A in mucosal extracts derived from normal and UC and CD subjects. The blot is representative of 6 controls, 8 UC, and 7 CD samples.

To rule out the possible involvement of the intestinal flora in the increased susceptibility to intestinal inflammation, experiments were run in parallel in JAM-A^{-/-} and wt mice administered antibiotics. No differences were found in the susceptibility to DSS colitis regardless of antibiotic treatment (not shown). Altogether, these data suggest that the absence or reduced expression of epithelial JAM-A results in an increased colitic score and mortality rate in DSS colitis.

Effect of JAM-A Genetic Deletion on Colonic Cytokine Levels and Intestinal Permeability

Intestinal inflammation is associated with local increases in cytokines and chemokines. To test whether the severe colitis we observed in the JAM-A^{-/-} mice was associated with increased cytokine and chemokine production in the intestinal mucosa, the levels of TNF-α, MIP-1-α, MIP-2, and KC were measured in a colonic

organ culture system. Before DSS treatment, no differences in the production of the inflammatory mediators were found between JAM-A^{-/-} and wt mice (Figure 6). However, after colitis induction, the levels of TNF-α, MIP-1-α, MIP-2, and KC increased dramatically ($P < .05$) in colons from colitic JAM-A^{-/-} mice compared with wt animals at day 7 (Figure 6). Indeed, the increased cytokine production already was evident at days 3 and 5, including in the serum of the JAM-A^{-/-} mice. At 3 days after treatment with DSS, levels of TNF-α were not significantly greater in the JAM-A^{-/-} mice than in wt mice; however, by day 5 there was a significant ($P < .05$) increase in both the mucosa and serum of the JAM-A^{-/-} mice compared with wt mice (0.43 ± 0.08 pg/μg vs 0.16 ± 0.03 pg/μg in the mucosa, and 1731 ± 18 pg/mL vs 896 ± 184 pg/mL in the serum). On the other hand, the increase in the levels of KC in JAM-A^{-/-} vs wt mice already

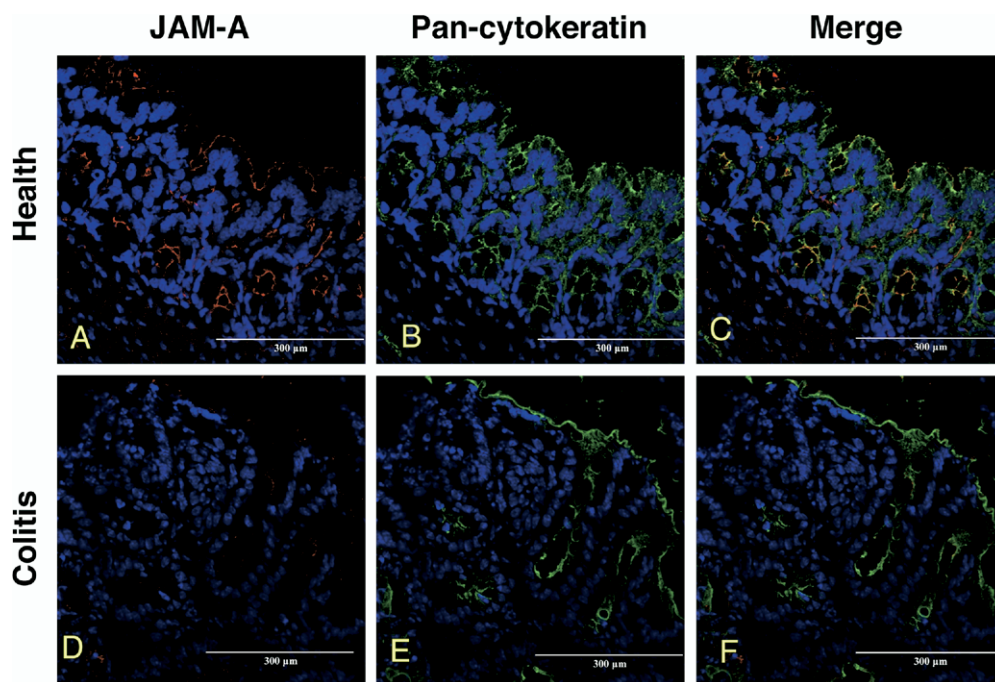


Figure 3. JAM-A expression in control and colitic colons in mice. Fluorescence micrographs of murine colonic mucosa in normal and colitic mucosa. Fluorescence micrographs of colons in normal and colitic epithelium, stained for (A and D) JAM-A (red), (B and E) E-cadherin (green), (C and F) nuclei (blue) and merge (yellow). The panels are representative of 7 controls and 8 colitic mice (magnification, 10 $\times$).

had reached significance ($P < .05$) by day 3 in the mucosa (18.6 ± 0.4 pg/ μ g vs 8.6 ± 1.7 pg/ μ g in the mucosa), and further had increased significantly (both $P < .05$) at day 5 in both mucosa and serum (28 ± 2.3 pg/ μ g vs 11.6 ± 0.3 pg/ μ g in the mucosa, and 2445 ± 258 vs 1220 ± 186 pg/mL in the serum).

Similarly, production of TNF- α also was increased in DSS-treated wt mice that were administered the BV11 monoclonal antibody. At 7 days after administration of DSS, mice that had been treated with BV11 produced significantly ($P < .05$) greater mucosal levels of TNF- α than DSS-treated mice that had not received BV11 (0.63 ± 0.08 pg/ μ g vs 0.2 ± 0.01 pg/ μ g, respectively). Expression of TNF- α was not increased above baseline in mice that were administered the BV11 monoclonal antibody at days -3, 0, 3, and 6 in the absence of administration of DSS (not shown).

Because the mucosal epithelium controls intestinal permeability and because JAM-A is located at the junctional sites of epithelial cells, we measured intestinal permeability in wt and JAM-A $^{-/-}$ mice. As assessed by the Evans blue permeability test, we detected a significant ($P < .05$) increase in intestinal permeability in JAM-A KO compared with wt animals, even without the administration of DSS (Figure 7A). Upon induction of DSS colitis, there was an approximately 3-fold increase in intestinal permeability at days 4 and 7. These results also were confirmed by the measurement of TER in Ussing chambers (Figure 7B).

Because the increase of permeability observed in JAM-A $^{-/-}$ mice could be owing to an altered expression of the tight junctions, we investigated claudin-1, -2, and -3, and

cingulin. The expression levels of these proteins did not differ between wt and JAM-A $^{-/-}$ mice (supplementary Figure 2; see supplementary material online at www.gastrojournal.org), as well as ZO-1 (not shown). Interestingly, healthy wt and JAM-A $^{-/-}$ mice displayed different expression levels of claudin-10 and -15 because increased ($P < .01$) expression was detected in JAM-A $^{-/-}$ vs wt mice (supplementary Figure 3; see supplementary material online at www.gastrojournal.org).

Permeability also was investigated in mice in which JAM-A function was neutralized by the BV11 monoclonal antibodies, which led also to an increased ($P < .05$) permeability similar to JAM-A $^{-/-}$ mice, as assessed both by Evans blue and TER (supplementary Figure 1B and C; see supplementary material online at www.gastrojournal.org).

Effect of Genetic Deletion of JAM-A on Epithelial Permeability and Apoptosis

As a potential cause of JAM-A $^{-/-}$ mice increased permeability, we also investigated epithelial apoptosis because of its involvement in regulating the intestinal mucosal barrier. When such process is altered, pathologic changes may occur, leading to a breakdown of the intestinal barrier resulting in IBD.¹⁹ To investigate whether increased epithelial apoptosis was present in JAM-A $^{-/-}$ mice, we performed a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay. After induction of colitis, JAM-A $^{-/-}$ mice displayed a significantly higher number of apoptotic epithelial cells compared with wt mice (Figure 8A and B).

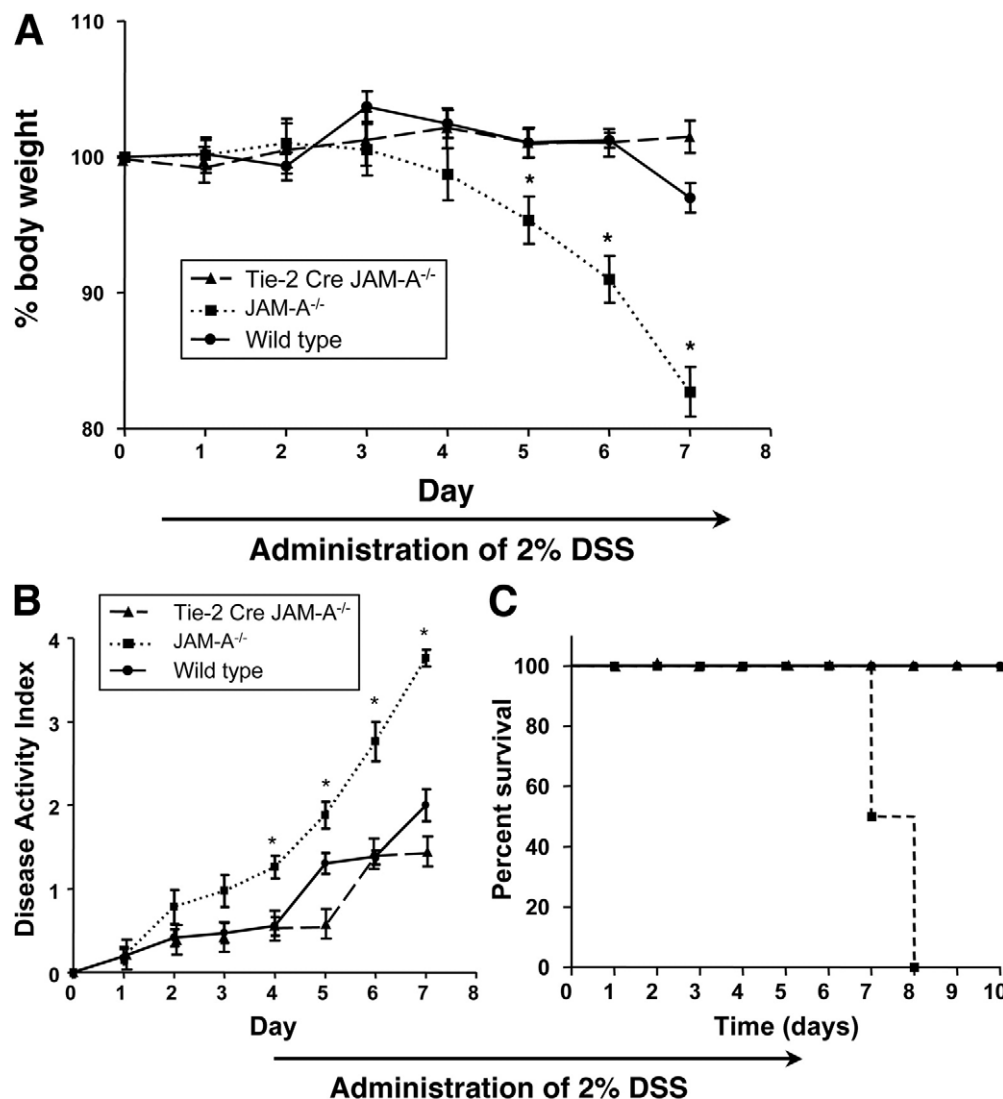


Figure 4. (A) Susceptibility to DSS-induced colitis in Tie-2 Cre JAM-A^{-/-} and JAM-A^{-/-} mice. Wt (●, n = 11), Tie-2 Cre JAM-A^{-/-} (▲, n = 12), and JAM-A^{-/-} (■, n = 14) animals underwent DSS treatment for 7 days. Weight was monitored daily. After 7 days, mice were killed and colons were assessed for histologic colitis. **P* < .05 for wild-type vs JAM-A^{-/-} mice. (B and C) Susceptibility to DSS-induced colitis in JAM-A^{-/-} mice. Wt (n = 11) or JAM-A^{-/-} (n = 14) animals underwent DSS treatment for 10 days. (B) Clinical disease activity index was calculated daily, and (C) survival was monitored. **P* < .05 for wt vs JAM-A^{-/-} mice.

No differences in epithelial cell apoptosis were found in JAM-A^{-/-} and wt mice before DSS treatment (Figure 8B).

Apoptosis also was investigated in vitro in Caco-2 cells where JAM-A was silenced. When apoptosis was measured in cells in which JAM-A had been silenced, we detected an increase in the apoptosis rate of epithelial cells as measured by annexin-V/propidium iodine staining (Figure 8C).

Because annexin-V only detects early phases of apoptosis, we also performed acridine orange and ethidium bromide staining to verify that apoptosis actually is executed. Analysis of the nuclear morphology of JAM-A-silenced Caco-2 cells revealed that more cells with condensed nuclei and perinuclear chromatin condensation (yellow/bright green) or red (late apoptosis stage) were

present compared with the scrambled short interfering RNA (siRNA) (Figure 8D). To test if apoptosis was responsible for the increased permeability detected in vivo in the JAM-A^{-/-} mice, we investigated permeability and the effects of apoptosis inhibition in JAM-A-silenced cells. In Caco-2 cells where we stably silenced the expression of JAM-A using short hairpin RNA interference (shRNAi) delivered by lentiviral vectors, shRNAi inhibited the expression of JAM by 80% (Figure 8E). In addition, in cells deficient for JAM-A, TER was decreased significantly (*P* < .05) in JAM-A-interfered vs control cells (Figure 8F). When JAM-A-silenced cells were treated with the pancaspase inhibitors Z-VAD, which blocks apoptotic events, still a significant (*P* < .05) difference in TER between control and JAM-A-silenced cells was detectable, suggest-

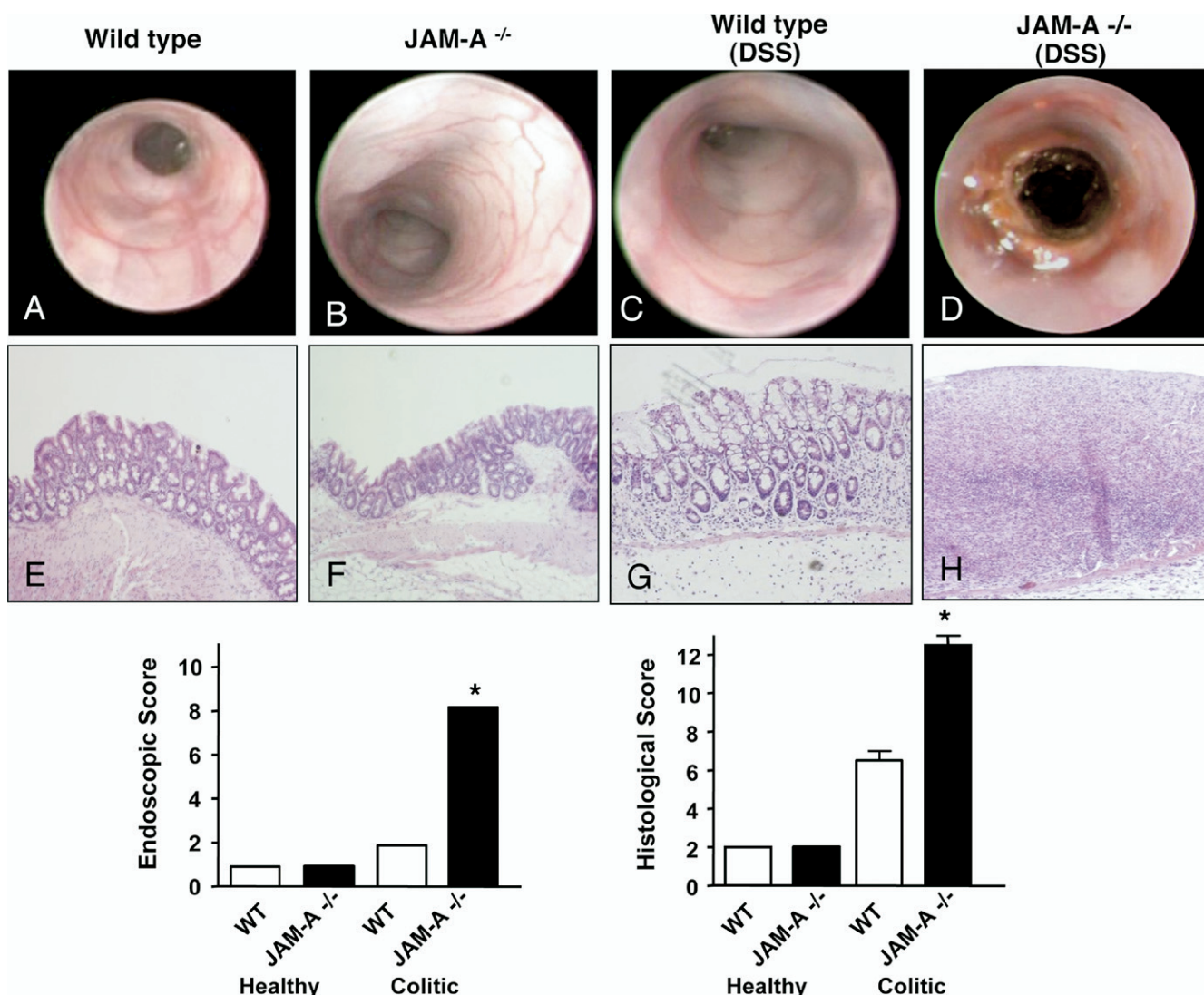


Figure 5. Endoscopic and histologic colitis in JAM-A^{-/-} mice. Wt (n = 11) or JAM-A^{-/-} (n = 14) animals underwent DSS treatment for 7 days. An endoscopy was performed at the beginning and after 7 days of DSS in (A and C) wt and (B and D) JAM-A^{-/-} and an endoscopic score was calculated. After endoscopy, wt and JAM-A^{-/-} mice were sacrificed. An histologic colitis score was calculated at the beginning and after 7 days of DSS in wt (E and G) and JAM-A^{-/-} (F and H). *P < .05 for wt vs JAM-A^{-/-} mice.

ing that apoptosis is not implicated in regulating epithelial permeability in JAM-A-silenced cells.

Discussion

The results of the present study show that JAM-A plays a fundamental role in maintaining mucosal integrity in the intestine by controlling epithelial barrier integrity. To investigate the functional role of JAM-A in IBD pathogenesis, we first investigated its expression levels in normal and inflamed tissues. A strong expression level was observed in the endothelial microvasculature. However, unexpectedly, although JAM-A is up-regulated in other forms of vascular inflammation such as atherosclerosis, we did not observe any modulation of JAM-A in the microcirculation of control individuals and patients with active IBD. On the contrary, our data

showed a strong reduction of epithelial expression at the epithelial tight junction in both inflamed CD and UC tissues, as colocalized with ZO-1. Interestingly, we did not observe such down-regulation in the noninflamed epithelium in uninvolved IBD tissues, suggesting that inflammation is responsible for this process. Indeed, our in vivo data fit well with previous in vitro data, where it was reported that inflammatory cytokines such as interferon- γ or TNF- α affect the expression of JAM-A in epithelial cell lines.^{11,28}

Because of the very similar expression pattern observed in experimental and human IBD, genetic manipulation of JAM-A allowed us to investigate its functional role in IBD pathogenesis. Because JAM-A crucially regulates leukocyte migration across the endothelium, we took advantage of the Tie-2 Cre JAM-A^{-/-} mice. Surprisingly, selective

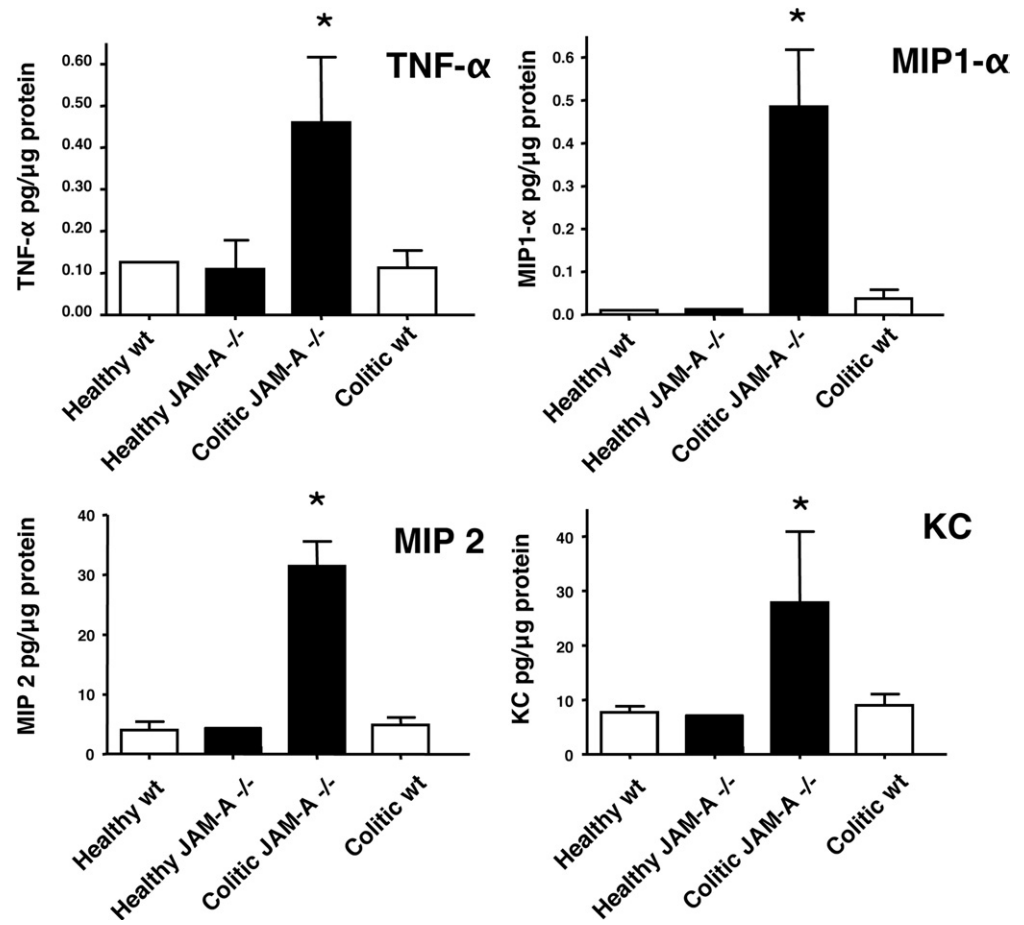


Figure 6. JAM-A^{-/-} mice show an exacerbated mucosal cytokine production. Wt (n = 11) or JAM-A^{-/-} (n = 14) animals underwent DSS treatment. After 7 days, animals were killed, their colons removed and organ-cultured, and supernatants were collected for cytokine and chemokine content by enzyme-linked immunosorbent assay. *P < .05 for wt vs JAM-A^{-/-} mice.

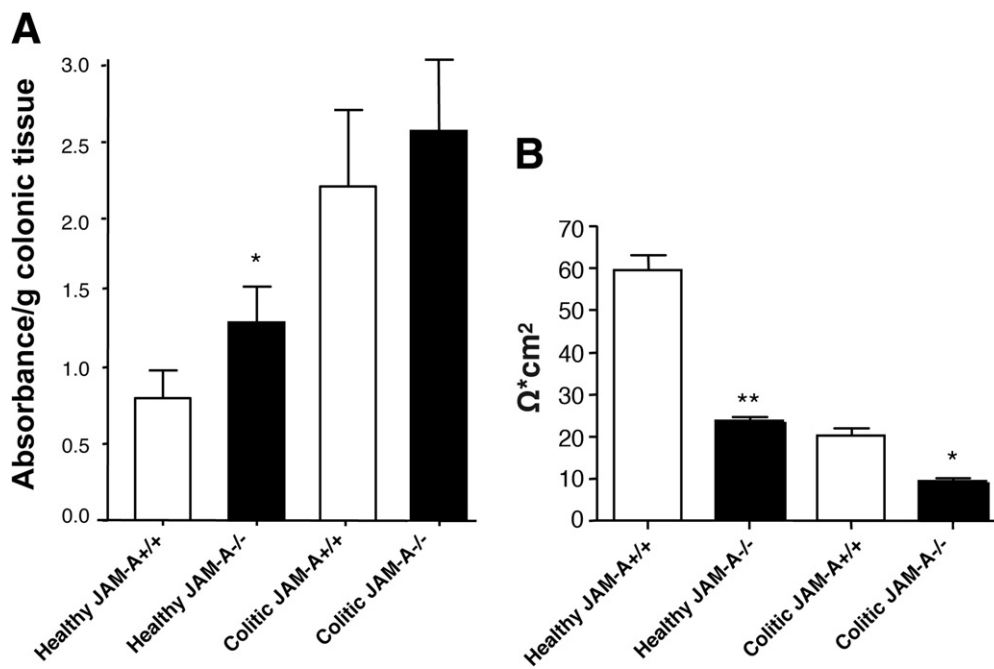
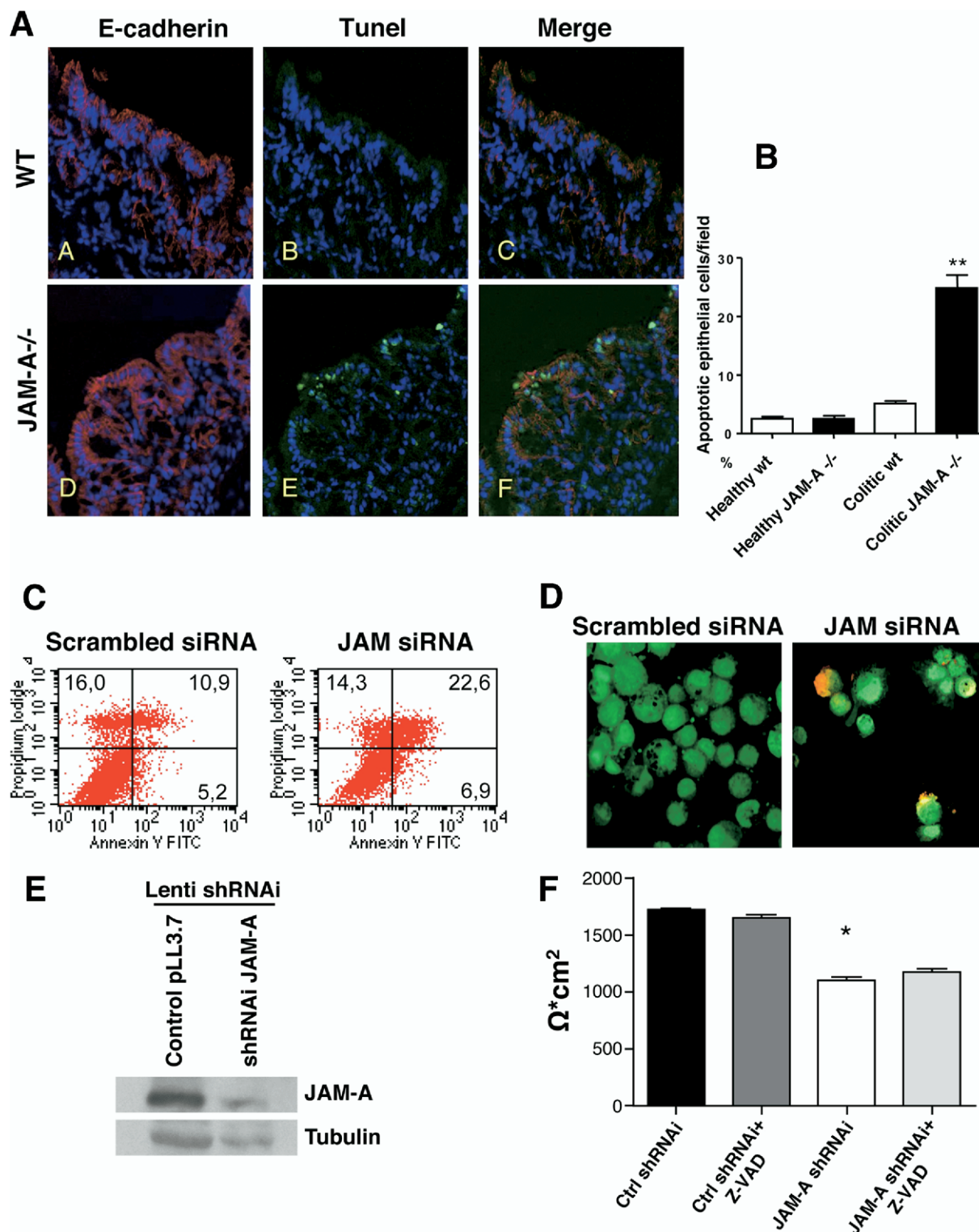


Figure 7. JAM-A^{-/-} mice exhibit increased intestinal permeability. Wt (n = 4) or JAM-A^{-/-} (n = 4) animals underwent the (A) Evans blue (EB) permeability test or investigation of TER (B) by an Ussing chamber, and intestinal permeability was measured. Data are expressed as absorbance of EB/g of tissue or Ω/cm². *P < .05 for wt vs JAM-A^{-/-} mice.

deletion at the endothelial or hematopoietic compartments of JAM-A had no effect on the susceptibility of mice to DSS colitis, thus indicating that endothelial/hematopoietic JAM-A is not functionally implicated in intestinal inflammation. However, when JAM-A was deleted ubiquitously, a dramatic increase in DSS-induced

intestinal inflammation was observed, as characterized by accelerated colitis, massive leukocyte infiltration, exacerbated endoscopic inflammation, and excessive production of inflammatory cytokines and chemokines in the gut, and strikingly high mortality rates. Similar results also were obtained by blocking JAM-A with the mono-



clonal antibody BV11, which induces in wt mice a similar phenotype to that of JAM-A^{-/-} mice during DSS treatment. We also found in colons and serum of colitic mice increased levels of inflammatory cytokines and chemokines such as TNF- α , MIP-1- α , MIP-2, and KC. These cytokines could recruit inflammatory leukocytes, including granulocytes and macrophages, which could participate in tissue destruction and exacerbation of the disease.

Taken together, these results would suggest that the presence of the epithelial JAM-A, but not the endothelial/hematopoietic JAM-A, is fundamental for intestinal homeostasis. It is difficult to assess whether the down-regulation of JAM-A is a causative or a correlative effect in human IBD, but the experiments in mice would favor the second option. What is clear is that the absence of JAM-A in the epithelium drastically exacerbates the development of colitis.

What drives the down-regulation of JAM-A in IBD patients remains to be identified. Having shown this dramatic phenotype in the JAM-A^{-/-} mice, we investigated the capacity of JAM-A to control intestinal permeability in vivo. Compatible with its function of junction gate keeper, JAM-A^{-/-} mice displayed an increased permeability compared with wt mice, suggesting that in vivo JAM-A plays an important role in controlling intestinal permeability. A recent report by Laukoetter et al²⁹ showed that JAM-A regulates intestinal permeability. They reported that JAM-A^{-/-} mice display an overexpression of claudin-10 and -15, and speculate that such up-regulation might be responsible for the increased permeability and susceptibility to DSS in JAM-A^{-/-} mice. Our data support and expand these observations. We report that JAM-A and wt mice have identical expression levels of claudin-1, -2, -3, cingulin, and ZO-1, but not claudin-10 and -15. In fact, our results confirm an increase of the expression levels of both claudins, suggesting a major role in mediating epithelial permeability. In addition, by using mice-Tie-2 Cre JAM-A^{-/-}, we could better dissect in vivo the cell type responsible for the increased DSS susceptibility, leading us to identify that epithelial but not endothelial/hematopoietic JAM-A regulates intestinal permeability and homeostasis.

Even though wt mice may lose JAM-A expression with colitis induction, JAM-A KO mice show increased perme-

ability even in the absence of any stimuli, and performed poorly as soon as DSS was introduced. This is likely owing to a leakier epithelial barrier whose increased permeability would allow more DSS to penetrate and alter the mucosa, thereby inducing a more severe inflammatory phenotype with increased cytokine production and epithelial apoptosis. Apoptosis was increased in vivo only when inflammation was established, suggesting that increased epithelial cell apoptosis is not the cause for increased permeability. In agreement, although we found increased apoptosis in vitro in JAM-A-silenced Caco-2 cells, this was not the cause of the augmented permeability because the caspase inhibitor Z-VAD had no impact on the observed difference in TER. The latter still was decreased in JAM-A-silenced as compared with wt Caco-2 monolayers, suggesting that apoptosis is not implicated in regulating epithelial permeability, in agreement with previous work.³⁰

A direct link between defects in epithelial barrier integrity and colitis has been proposed recently in several mouse models. Mice lacking MyD88, the adaptor protein of interleukin-1R and Toll-like receptors, or Toll-like receptor 4, show increased susceptibility to experimental colitis and decreased protection against gut injury by DSS,^{31,32} presumably via reduced cyclooxygenase-2 activation and prostaglandin-E2 release.³³ Mice deficient for NF- κ B essential modulator 1, which is essential for nuclear factor- κ B activation, display increased epithelial cell apoptosis and permeability that leads to severe chronic spontaneous colitis.¹⁹ In conclusion, our study shows a nonredundant and unique role of JAM-A in the intestine in controlling epithelial integrity and maintaining an intact barrier function. Down-regulation of JAM-A leads to a leaky epithelial barrier that contributes to the increased intestinal permeability observed in IBD. Therefore, therapeutic strategies aimed at restoring the epithelial gatekeeper capacity through up-regulation may harbor a new approach to decreasing the inflammation observed in patients with either CD or UC.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gas-*

Figure 8. Effects of JAM-A genetic deletion on apoptosis and permeability. (A) Fluorescence micrographs of murine colonic mucosa in JAM-A^{-/-} and wt mice after DSS treatment. Fluorescence micrographs of colons in wt and JAM-A^{-/-} mice, stained for (a and d) E-cadherin (red), (b and e) terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (green), (c and f) nuclei (blue) and merge. The panels are representative of 5 wt and 6 JAM-A^{-/-} colitic mice (magnification, 10 $\times$). (B) Apoptotic cells were quantified by counting the percentage of apoptotic cells/field. ***P* < .01 for wt vs JAM-A^{-/-} mice. (C) Spontaneous apoptosis was measured by annexin-V/propidium iodide staining 72 hours after oligo transfection. Annexin-V single positive events represent cells in early apoptosis; annexin-V/propidium iodide double positive events represent late apoptotic cells; propidium iodide single positive events represent dead cells. (D) Ethidium bromide and acridine orange shows increased early (bright green/yellow) and late apoptotic cells (red) in JAM-A-interfered cells compared with control cells. (E) Western blot showing the effects of JAM-A down-regulation by use of shRNA lentivirus. (F) JAM-A interfered (JAM shRNA) or not (CTRL) cells were seeded on transwells. TER was measured at day 10 in the presence or absence of 50 nmol/L of Z-VAD. Data show the average of TER measures of 6 transwells $\pm$ SD. The difference observed at day 10 in TER was statistically significant (**P* < .05).

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