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Inflammation-induced desmoglein-2 ectodomain shedding compromises the mucosal barrier

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\textbf{ABSTRACT} Desmosomal cadherins mediate intercellular adhesion and control epithelial homeostasis. Recent studies show that proteinases play an important role in the pathobiology of cancer by targeting epithelial intercellular junction proteins such as cadherins. Here we describe the proinflammatory cytokine-induced activation of matrix metalloproteinase 9 and a disintegrin and metalloproteinase domain–containing protein 10, which promote the shedding of desmosomal cadherin desmoglein-2 (Dsg2) ectodomains in intestinal epithelial cells. Epithelial exposure to Dsg2 ectodomains compromises intercellular adhesion by promoting the relocalization of endogenous Dsg2 and E-cadherin from the plasma membrane while also promoting proliferation by activation of human epidermal growth factor receptor 2/3 signaling. Cadherin ectodomains were detected in the inflamed intestinal mucosa of mice with colitis and patients with ulcerative colitis. Taken together, our findings reveal a novel response pathway in which inflammation-induced modification of columnar epithelial cell cadherins decreases intercellular adhesion while enhancing cellular proliferation, which may serve as a compensatory mechanism to promote repair.

\textbf{INTRODUCTION} Epithelial cells form a selective barrier that separates the external environment from the underlying tissue compartments. In the intestine, where epithelial cell turnover occurs within 1 wk, barrier properties of the columnar epithelium are maintained by intercellular junctions, which include tight junctions, adherens junctions, and desmosomes. In addition to controlling adhesion, proteins in these intercellular junctions also regulate epithelial homeostasis through the processes of cellular proliferation, migration, differentiation, and apoptosis (Allen et al., 1996; Dusek et al., 2006; Dusek and Attardi, 2011; Getsios et al., 2009; Marchiando et al., 2010; Nava et al., 2013).

Along with E-cadherin in the adherens junction, the desmosomal cadherin family members desmoglein and desmocollin mediate cell–cell adhesion in the epithelium. Desmoglein and desmocollin constitute the adhesive core of desmosomes, anchoring intermediate filaments to sites of intercellular adhesion (Getsios et al., 2004; Green and Simpson, 2007). Both proteins are required for de novo desmosome formation in cells that lack cadherin-based junctions (Tselepis et al., 1998; Schlegel et al., 2010). In humans, four desmoglein isoforms (Dsg1–4) and three desmocollin isoforms (Dsc1–3) are expressed in a tissue-specific and differentiation-specific manner (Holthofer et al., 2007). Although stratified epithelia in the epidermis express multiple desmosomal cadherin isoforms, only desmoglein-2 (Dsg2) and desmocollin-2 (Dsc2) are expressed in the simple columnar epithelium of the human intestine (Holthofer et al., 2007).
Recent studies show that several proteinases target epithelial intercellular junction proteins during physiological maturation and in pathological states such as inflammation and cancer (Maretzky et al., 2008; Sewpaul et al., 2009; David and Rajasekaran, 2012). The cleavage products of these junction proteins have biological properties that influence the delicate balance of epithelial homeostasis, thereby contributing to disease pathogenesis (Brennan et al., 2007; Kolegoff et al., 2011a,b; Kamekura et al., 2014). Epithelial homeostasis is also perturbed by the proinflammatory cytokines interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), which are released into the epithelial milieu during mucosal inflammation, such as in inflammatory bowel disease (IBD; Rogler and Andus, 1998; Nava et al., 2010; Koch and Nusrat, 2012). A functional role for intercellular junction protein cleavage products in cancer progression has also been reported (Kuefer et al., 2003; Najy et al., 2008; Brouxhon et al., 2014a). However, the biological effect and mechanisms by which cadherin cleavage fragments influence intestinal epithelial homeostasis during mucosal inflammation remain unclear (Dusek et al., 2006; Nava et al., 2007; Steck et al., 2011).

Here we report that IL-1β and TNF-α induce Dsg2 cleavage in intestinal epithelial cells (IECs), generating 25- and 100-kDa extra-cellular domain (EC) fragments. The use of epitope-mapped antibodies reveals the identity of the cleaved fragments as Dsg2 EC1 and Dsg2 EC1–4. Our results showed that proinflammatory cytokine-induced cleavage of Dsg2 ectodomains is mediated by matrix metalloproteinase 9 (MMP9) and a disintegrin and metalloproteinase domain–containing protein 10 (ADAM10). These Dsg2 ectodomains influence intercellular adhesion and epithelial proliferation by promoting relocation of cadherin from the plasma membrane. In addition, Dsg2 EC1 and EC1–4 enhance IEC proliferation by activating human epidermal growth factor receptor family members HER2 and 3, which activate downstream signaling through the Akt/mammalian target protein of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways. These findings reveal the biological mechanisms by which Dsg2 cleavage during mucosal inflammation influences intestinal epithelial homeostasis. Thus, interfering with the generation and/or function of these cleavage products may strengthen the epithelial barrier during mucosal inflammation.

RESULTS
Proinflammatory cytokines promote cleavage and shedding of cadherins in intestinal epithelial cells

Previous studies demonstrated that proinflammatory cytokines released during mucosal inflammation alter epithelial homeostasis and barrier function (Rogler and Andus, 1998; Bruewer et al., 2003; Maretzky et al., 2005, 2008; Simpson et al., 2010; Koch and Nusrat, 2012). To determine whether proinflammatory cytokines induce cadherin cleavage in IECs, we used epitope-mapped monoclonal antibodies that recognize extracellular domains of Dsg2 (AH112.2, EPR6767; Figure 1A), Dsc2 (7G6), and E-cadherin (HECD-1) to detect cadherin fragments in the supernatants of T84 human IECs treated with IFN-γ, IL-1β, and TNF-α. The specificity of Dsg2 antibodies was confirmed by immunoblotting (Supplemental Figure S1, A and B). Figure 1B shows that exposure to IL-1β and TNF-α promoted shedding of the soluble 25- and 100-kDa ectodomain fragments of Dsg2 (sDsg2), soluble 80-kDa fragments of Dsc2 (sDsc2), and soluble 80-kDa fragments of E-cadherin (sE-cadherin) into the culture medium. In addition, sDsc2 and sE-cadherin, but not sDsg2, were detected after IFN-γ treatment. We found that the shedding of sDsg2, sDsc2, and sE-cadherin increased with higher concentrations of IL-1β (Figure 1C). After treatment with 20 ng/ml IL-1β, cadherin ectodomain fragments were detected in the culture supernatants within 1 h, with maximum shedding observed 6 h after treatment (Figure 1D). Similar dose- and time-dependent shedding of Dsg2, Dsc2, and E-cadherin was observed after TNF-α treatment (Figure 1, E and F). To complement the in vitro studies, we analyzed the release of Dsg2, Dsc2, and E-cadherin fragments in ex vivo mouse colonic mucosa cultures and found that exposure to IL-1β and TNF-α increased sDsg2, sDsc2, and sE-cadherin by 2.4- to 7.0-fold in the culture supernatants, as assessed by densitometry (Figure 1G).

MMP9 and ADAM10 cleave cadherin ectodomains of intestinal epithelial cells

Proinflammatory cytokines have been reported to activate MMPs and ADAM proteins (Medina and Radomski, 2006; Santana et al., 2006; Puressmeyer et al., 2010), which cleave epithelial junction proteins in pathological states such as inflammation and cancer (Maretzky et al., 2008; Sewpaul et al., 2009; David and Rajasekaran, 2012). MMP activity and/or expression are increased in the intestinal mucosa of individuals with IBD and in mouse models of intestinal mucosal inflammation (Medina et al., 2003; Santana et al., 2006). Therefore we investigated the role of MMP and ADAM proteins in mediating the cleavage of IEC cadherins by pretreating IECs with TNF protease inhibitor 2 (TAPI-2), an inhibitor of MMPs and ADAM17, before incubation with IL-1β and TNF-α. Our results showed that TAPI-2 inhibited the shedding of sDsg2, sDsc2, and sE-cadherin induced by treatment with IL-1β, TNF-α, or an IL-1β/ TNF-α cocktail (Figure 2, A and B, and Supplemental Figure S1C).

Because previous studies showed that IL-1β and TNF-α, but not IFN-γ, increase the activity of MMP2 and 9 (Medina et al., 2003; Medina and Radomski, 2006; Santana et al., 2006), we investigated the role of these proteinases in the control of cadherin cleavage. The results of gelatin zymography revealed higher levels of MMP9 activity in the supernatants of IECs incubated with IL-1β and TNF-α but not in IECs incubated with IFN-γ. However, compared with MMP9, increase in MMP2 activity was observed only with IL-1β (Figure 2C).

We also analyzed ex vivo colonic mucosa cultures derived from healthy mice and mice with dextran sulfate sodium (DSS)–induced colitis. Consistent with results obtained using the in vitro cell cultures, MMP2 and 9 activities and protein levels were elevated in supernatants of colonic mucosal cultures derived from colitic mice (Figure 2D and Supplemental Figure S1D). To confirm the role of these proteinases in the cleavage of cadherin ectodomains, we treated T84 IEC cultures with pharmacologic inhibitors of MMP2 and 9. Immunoblot analysis of supernatants obtained from TNF-α–treated IECs revealed that inhibition of MMP9, but not inhibition of MMP2, decreased the release of sDsg2, sDsc2, and sE-cadherin (Figure 2E). Inhibition of both MMP2 and 9 did not produce a synergetic effect, suggesting the influence of an additional proteinase in this process (Figure 2E). A previous study showed that the membrane-bound metalloproteinase ADAM10 targets Dsg2 (Bech-Serra et al., 2006); therefore we examined whether ADAM10 contributes to cytokine-induced Dsg2 ectodomain cleavage in IECs. Figure 2F shows that the selective ADAM10 inhibitor GI254023X (Hundhausen et al., 2003) dose dependently inhibited the shedding of Dsg2, Dsc2, and E-cadherin ectodomains in the supernatants of TNF-α–treated IECs. GI254023X treatment also inhibited cadherin ectodomain shedding after IL-1β treatment (Supplemental Figure S1E).

Soluble Dsg2 decreases intercellular adhesion in intestinal epithelial cells

Given the role of desmosomes in controlling intercellular adhesion, we evaluated the effect of cadherin extracellular fragments on intestinal epithelial cell–cell adhesion using a dispase assay.
**FIGURE 1:** Proinflammatory cytokines promote cleavage and shedding of cadherins in intestinal epithelial cells. (A) Schematic representation of protein domain of Dsg2 and epitope mapping of AH12.2 and EPR6767 monoclonal antibodies against Dsg2. EC, extracellular subdomain; EA, extracellular anchor; TM, transmembrane domain; IA, intracellular anchor; ICS, intracellular cadherin-typical sequence; IPL, intracellular proline-rich linker domain; RUD, repeated-unit domain; DTD, desmoglein-specific terminal domain. (B) Immunoblot analysis showing cleavage products of epithelial cadherins in culture supernatants from T84 cells treated with 100 U/ml IFN-γ, 20 ng/ml IL-1β, or 50 ng/ml TNF-α for 24 h. Histograms represent the relative expression of Dsg2 (100 kDa), Dsc2, and E-cadherin cleavage products, as determined by densitometry analysis. Results are expressed as means ± SEM of five independent experiments; *p < 0.05, †p < 0.0001, ns, not significant, z test. (C–D) Immunoblot analysis showing soluble Dsg2 (sDsg2), Dsc2, and E-cadherin cleavage products, as determined by densitometry analysis. Results are expressed as means ± SEM of five independent experiments; *p < 0.05, †p < 0.0001, ns, not significant, z test. (E, F) Immunoblot analysis showing sDsg2, sDsc2, and sE-cadherin in culture supernatants from T84 cells treated with 1, 10, or 100 ng/ml IL-1β for 6 h (C) or 20 ng/ml IL-1β for up to 24 h (D). (E, F) Immunoblot analysis showing sDsg2, sDsc2, and sE-cadherin in culture supernatants from T84 cells treated with 1, 10, or 100 ng/ml TNF-α for 24 h (E) or 50 ng/ml TNF-α for up to 24 h (F). (G) Immunoblot analysis showing sDsg2, sDsc2, and sE-cadherin in culture supernatants from ex vivo mouse colonic mucosal cultures treated with 20 ng/ml IL-1β or 50 ng/ml TNF-α for 6 h. (B–G) Ponceau staining was used as a loading control. C, control (C–F).
associations. We first determined the localization of endogenous cadherins in epithelial cells cocultured with CHO cells secreting cadherin fragments by immunofluorescence labeling and confocal microscopy. Figure 3D shows that the localization of Dsg2, Dsc2, and E-cadherin in the lateral membrane was decreased in epithelial cells exposed to Dsg2 EC1–4 or E-cadherin EC1–5. However, the extent of Dsc2 relocalization from the lateral plasma membrane was not as prominent as observed for Dsg2 and E-cadherin. To determine whether the inhibition of intercellular adhesion mediated by soluble cadherin ectodomain fragments is dependent on interactions with endogenous cadherins, we performed coimmunoprecipitation experiments using T84 IECs exposed to Dsg2 EC1–4 or E-cadherin EC1–5. As shown in Figure 3E, Dsg2 EC1–4 and E-cadherin EC1–5 coimmunoprecipitated with endogenous Dsg2 and E-cadherin. Next we evaluated the association of recombinant ectodomain cadherin

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**FIGURE 2:** MMP9 and ADAM10 cleave cadherin ectodomains in intestinal epithelial cells. (A, B) Immunoblot analysis of soluble Dsg2, Dsc2, and E-cadherin in culture supernatants from T84 cells treated with 20 ng/ml IL-1β or 50 ng/ml TNF-α for 24 h (B) in the presence or absence of 20 μM TAPI-2. Histograms represent the relative expression of Dsg2 (100 kDa), Dsc2, and E-cadherin cleavage products, as determined by densitometric analysis. Results are expressed as means ± SEM of five independent experiments; *p < 0.05, †p < 0.0001, z test. (C) Zymographic analysis of MMP2 and 9 activities in culture supernatants from T84 cells treated with 100 U/ml IFN-γ, 20 ng/ml IL-1β, or 50 ng/ml TNF-α for 24 h. (D) Zymographic analysis of MMP2 and 9 activities in the supernatant of ex vivo mouse colonic mucosa cultures derived from wild-type control mice (WT) and mice with DSS-induced colitis (DSS). The colonic mucosa from control and colitic mice was incubated with culture medium for 6 h. The top and bottom images were obtained from gels loaded with a small amount (10 μg/lane) and a large amount (15 μg/lane) of protein in the gels, respectively. (E) Immunoblot analysis of sDsg2, sDsc2, and sE-cadherin in culture supernatants from T84 cells treated with 50 ng/ml TNF-α in the presence or absence of 5 μM MMP2 inhibitor, 100 μM MMP9 inhibitor, or 2.5 μM MMP2/9 inhibitor for 24 h. (F) Immunoblot analysis showing sDsg2, sDsc2, and sE-cadherin in culture supernatants from T84 cells treated with 50 ng/ml TNF-α in the presence or absence of GI254023X (1, 3, or 10 μM) for 24 h. (A, B, E, F) Ponceau staining was used as a loading control. C, control (A, B).
and suggest that the binding of sDsg2 and endogenous Dsg2 likely interferes with the trans endogenous Dsg2–Dsg2 association between epithelial cells, thereby inhibiting intercellular adhesion and contributing to the relocalization of cell surface cadherin.

Dsg2 extracellular fragments regulate HER2/3-Akt, mTOR, and MAPK signaling pathways and regulate epithelial cell proliferation

Disruption of intercellular adhesion by cleaved junctional proteins has been linked to increased cell proliferation (Lynch et al., 2010).
FIGURE 4: Dsg2 extracellular fragments regulate HER2/3-Akt, mTOR, and MAPK signaling pathways and regulate epithelial cell proliferation. (A) T84 cells were cocultured with CHO cells secreting sDsg2 or sE-cadherin, and cell proliferation was evaluated by measuring EdU incorporation into newly synthesized DNA. Images were obtained by confocal microscopy, and EdU-positive nuclei were counted. ***p < 0.001, ****p < 0.0001 vs. control, t test.

(B) Schematic representation of Dsg2 and E-cadherin ectodomain constructs used in this study. EC, extracellular subdomain; EA, extracellular anchor; TM, transmembrane domain; IA, intracellular anchor; ICS, intracellular cadherin-typical sequence; IPL, intracellular proline-rich linker domain; RUD, repeated-unit domain; DTD, desmoglein-specific.
Therefore we investigated the influence of sDsg2 and sE-cadherin on IEC proliferation using a noncontact coculture system of T84 IECs cocultured with CHO cells secreting Dsg2 EC1–4, Dsg2 EC1, or E-cadherin EC1–5 (Figure 3, A and B). As shown in Figure 4A, the percentage of 5-ethyl-2′-deoxyuridine (EdU)-positive nuclei in IECs exposed to Dsg2 EC1–4, Dsg2 EC1, or E-cadherin EC1–5 (Figure 4B) was significantly higher than that of IECs cocultured with control CHO cells transfected with an empty expression vector. We sought to confirm these findings by treating IECs with purified Dsg2 EC1–4, Dsg2 EC1, E-cadherin EC1–5, or signal regulatory protein α (SIRPα), an unrelated protein that served as a control (Figure 4C). Purified proteins were analyzed by Coomassie staining of gels and immunoblotting (Supplemental Figure S3). Our results showed increased proliferation of IECs exposed to sDsg2 (Dsg2 EC1–4 or Dsg2 EC1) compared with that of IECs exposed to SIRPα, as assessed by EdU incorporation into newly synthesized DNA (Figure 4C). Consistent with results for other cells (Najy et al., 2008; Lynch et al., 2010; Brouxhon et al., 2014a), increased proliferation was also observed for IECs exposed to SIRPα (Figure 4C). To verify results obtained using the IEC cell line, we investigated the influence of sDsg2 on proliferation of primary mouse IECs grown as enteroid cultures (Fuller et al., 2012) and observed increased proliferation in cultures exposed to purified Dsg2 EC1 or E-cadherin EC1–5 (Figure 4D). Taken together, the results suggest that Dsg2 EC1 and E-cadherin EC1–5 fragments promote proliferation of IECs. We next investigated the mechanism by which Dsg2 EC1 promotes IEC proliferation. These analyses focused on sDsg2 because the effects of sE-cadherin have been previously described (Najy et al., 2008; Brouxhon et al., 2014a). Shed ectodomains of epithelial cadherins are believed to promote cell proliferation by activating tyrosine receptor kinases such as human epidermal growth factor receptor (EGFR, also known as HER1; Najy et al., 2008; Klessner et al., 2009; Brouxhon et al., 2014a). We therefore examined EGFR activation in IECs incubated with Dsg2 EC1. Of interest, EGFR phosphorylation was not altered in IECs exposed to either Dsg2 EC1 or SIRPα (negative control; Figure 4E). Therefore we investigated the effect of sDsg2 on the phosphorylation and activation of other HER family members (Citri and Yarden, 2006). Figure 4E shows that exposure to sDsg2, but not to SIRPα, increased phosphorylation of HER2 (Tyr-1196) and HER3 (Tyr-1289), indicating the activation of these receptors; however, HER2 and HER3 protein levels were unchanged after Dsg2 EC1 treatment (Figure 4E). Dimerization and activation of HER2 and HER3 promote tumor growth through MAPK and phosphoinositide 3-kinase/Akt/mTOR signaling (Baselga and Swain, 2009); therefore, we investigated whether Dsg2 EC1 can activate these signaling cascades. Indeed, results of densitometric analysis showed increased phosphorylation of Akt Thr-308 (pAkt T308), Akt Ser-473 (pAkt S473), extracellular signal-regulated protein kinase 1/2 (pERK1/2, Tyr-204 (pERK1/2)), and S6 ribosomal protein (pS6) (2.6-, 3.1-, 1.7-, and 2.7-fold increases, respectively) in IECs exposed to sDsg2 EC1; however, total protein levels of Akt, Erk, and S6 in sDsg2-treated IECs did not differ from those of controls (Figure 4F). Exposure to Dsg2 EC1–4 also activated HER2 and HER3 signaling in IECs (Supplemental Figure S4, A and B). To confirm the role of Akt/mTOR and MAPK signaling in mediating sDsg2-induced cellular proliferation, we treated controls with pharmacologic inhibitors specific for these signaling proteins. As shown in Figure 4, G–J, and Supplemental Figure S4, C–F, inhibition of HER2 (Arvy-380), Akt (Akt inhibitor VIII), mTOR complex 1 (mTORC1, rapamycin), and MAPK kinase (MEK, PD98059) inhibited Dsg2 EC1-induced IEC proliferation. Akt inhibition also suppressed proliferation of IECs exposed to Dsg2 EC1–4 (Supplemental Figure S4G). Taken together, these results indicate that sDsg2 activates HER2 and HER3 signaling and IEC proliferation.

Because sE-cadherin fragments have been shown to associate with EGFR, we hypothesized that sDsg2 binds to HER2 or HER3, promoting phosphorylation/activation. Indeed, our results showed that Dsg2 EC1 coimmunoprecipitated with HER2 and HER3 (Figure 4K), and interactions of Dsg2 EC1–4 and sE-cadherin with HER2 and HER3 were observed (Supplemental Figure S4, H and I). In summary, the results suggest that sDsg2 promotes cellular proliferation through activation of HER2 and HER3 and the downstream effectors Akt/mTOR and MAPK.

Increased levels of Dsg2 extracellular fragments in inflamed intestinal mucosa

Increased proinflammatory cytokine and protease activities have been reported in the inflamed intestinal mucosa of patients with IBD and in animal models of colitis (Rogler and Andus, 1998; Baugh et al., 1999). For that reason, we investigated Dsg2 cleavage in the intestinal mucosa from patients with active ulcerative colitis that showed neutrophil infiltration and epithelial erosions. Inactive ulcerative colitis (intact epithelium) mucosa was used as a control. As shown in Figure 5A, increased levels of Dsg2 extracellular cleavage fragments (∼25 and 50 kDa) were detected in inflamed intestinal mucosa. To confirm cleavage of Dsg2 and E-cadherin in the mucosa during active inflammation, we analyzed colonic mucosa of mice injected with IL-1β or TNF-α. As shown in Figure 5, B and C, cleavage products of Dsg2 and E-cadherin were increased in the mucosa of mice treated with proinflammatory cytokines. Immunoblots detected 100- and 80-kDa fragments corresponding to the ectodomains of Dsg2 and E-cadherin, respectively. Next we confirmed Dsg2 cleavage during inflammation by analyzing the presence of sDsg2 in the mucosa of mice with DSS-induced colitis. DSS terminal domain. (C) Proliferation of T84 cells treated with sDsg2 (EC1–4 or EC1), sE-cadherin (EC1–5), or SIRPα (negative control) was determined by EdU incorporation. ****p < 0.0001 vs. vehicle control; ns, not significant; t test. Scale bar, 50 μm. (D) Proliferation of enteroids treated with sDsg2, sE-cadherin, or bovine serum albumin (BSA, negative control) was determined by EdU incorporation. ****p < 0.0001 vs. vehicle control; ns, not significant; t test. Scale bar, 50 μm. (E) Immunoblot analysis of pEGFR (Y1068), EGFR, pHER2 (Y1196), HER2, pHER3 (Y1289), and HER3 in T84 cells exposed to Dsg2 EC1 or SIRPα (negative control) for 1 h. (F) Immunoblot analysis of EGFR-HER signaling in T84 cells treated with Dsg2 EC1 or SIRPα (negative control) for 1 h. Cell lysates were analyzed by immunoblotting against pAkt (T308, S473), pan Akt, pS6 (S235/S236), S6, pErk1/2 (T202/Y204), and Erk1/2. GAPDH was used as a loading control. (G–J) EdU incorporation showing proliferation of T84 cells treated with Dsg2 EC1 and 100 nM Arvy-380 (HER2 inhibitor), 2.12 μM Akt inhibitor VIII, 2 nM rapamycin (mTORC1 inhibitor), or 20 μM PD98059 (MEK inhibitor). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not significant; t test. (K) Results of coimmunoprecipitation demonstrating interactions between Dsg2 EC1 and HER2 or HER3 in T84 cells. IP, immunoprecipitation; IB, immunoblotting. (C, D) EdU, green; TO-PRO-3 iodide (nuclei), blue. (A, C, D, G–J) Histograms represent the percentage of nuclei that were EdU-positive. Results are expressed as means ± SEM of at least 10 different fields.
FIGURE 5: Increased levels of Dsg2 extracellular fragments in inflamed intestinal mucosa. (A) Immunoblot analysis of Dsg2 (AH12.2) in noninflamed and inflamed human colonic tissues from patients with ulcerative colitis (UC) and healthy controls (Normal). (B, C) Immunoblot analysis of Dsg2 (EPR6767) and E-cadherin (DEMA-1) in the colonic mucosa of mice that received intraperitoneal injections of IL-1β (B) or TNF-α (C). (D) Immunoblot analysis of Dsg2 protein in the colonic mucosa of mice with DSS-induced colitis (DSS) and wild-type controls (WT). The samples were analyzed using antibodies against Dsg2 clone ERP6767. (A–D) GAPDH was used as a loading control. FL, full-length. C, control (B, C).

Treatment was restricted to 4 d to avoid widespread erosions and ulcerations in the colonic mucosa. Figure 5D shows an increase in Dsg2 cleavage (100 kDa) in the mucosa of colitic mice. Taken together, the results indicate that Dsg2 is increased in the colonic mucosa during inflammation.

DISCUSSION

Desmosomes function as spot welds below the adherens junction in simple epithelia, such as intestinal epithelia, to mediate adhesive strength (Tsuchita et al., 2008). Our results suggest that proinflammatory cytokines promote ectodomain cleavage of cadherins in columnar IECs by activating the extracellular proteinases MMP9 and ADAM10. Proinflammatory cytokines have been reported to up-regulate such extracellular proteinases (Medina and Radomski, 2006; Santana et al., 2006; Pruessemeyer et al., 2010). Both MMP and ADAM proteinases cleave classic cadherins (E-, V-, VE-cadherin), as well as desmosomal cadherin (Dsg2) ectodomains (Lochter et al., 1997; Noe et al., 2001; Maretzky et al., 2005; Bech-Serra et al., 2006; Nava et al., 2013). MMP and ADAM activities are up-regulated not only during inflammation, but also in other pathological conditions, such as cancers, vascular disorders, and autoimmune diseases (Rocks et al., 2008; Gialeli et al., 2011). Cadherin shedding mediated by MMPs and/or ADAMS has been reported in other epithelia, such as the complex skin epithelium, where the shed fragments inhibit intercellular adhesion (Lorch et al., 2004; Maretzky et al., 2005; Reiss et al., 2005; Klessner et al., 2009). In addition, cleavage of the Dsg1 ectodomain in keratinocytes by Staphylococcus aureus exfoliative toxin A/B disrupts intercellular adhesion, contributing to the pathogenesis of blistering skin diseases (Amagai et al., 2002). The soluble 80-kDa N-terminal E-cadherin fragment also disrupts adherens junctions (Wheelock et al., 1987; Noe et al., 1999) and has been implicated in the progression of breast, prostate, and skin cancer (Kuefer et al., 2003; Najy et al., 2008; Brouxhon et al., 2014b). Soluble E-cadherin has been detected in the serum of cancer patients, and activation of EGFR and MAPK signaling by sE-cadherin appears to play a role in the growth and survival of the tumors (Wilmanns et al., 2004; Najy et al., 2008; Gogali et al., 2010; Brouxhon et al., 2014a). However, the generation of cadherin fragments and their contribution to epithelial barrier compromise in mucosal inflammatory disorders is not well understood. One of the proposed mechanisms links cadherin ectodomain shedding and decreased intercellular adhesion to the endocytic trafficking of cadherins (Bryant and Stow, 2004), which modifies epithelial adhesion during remodeling in development and cancer cell metastasis (Canel et al., 2013). In this study, we observed that mucosal inflammation also promotes Dsg2 and E-cadherin ectodomain shedding. During inflammation, proinflammatory cytokines induce activation of epithelial-derived MMPs and MMPs from infiltrating neutrophils that promote cleavage of cadherins, thereby influencing epithelial barrier properties. Analysis of functional effects of cadherin ectodomain fragments on epithelial barrier function revealed decreased transepithelial electrical resistance with corresponding increase in 4-kDa dextran flux in response to sDsg2 and sE-cadherin but not sDsc2 (Supplemental Figure S2, A and B). These results are supported by a previous report that treatment of a Dsg2 antibody directed against extracellular domain disrupts intestinal epithelial barrier function (Schlegel et al., 2010). In this scenario, compromised intercellular adhesion was associated with decreased Dsg2 and E-cadherin in the lateral membrane (Figure 3D). We propose that the shed Dsg2 and E-cadherin ectodomains interfere with trans interactions among desmosomal cadherins, which are important for mediating intercellular adhesion (Chitaev and Troyanovsky, 1997; Waschke et al., 2007; Nie et al., 2011). These findings are consistent with the results of studies showing that the soluble N-terminal region of E-cadherin decreases intercellular adhesion (Wheelock et al., 1987; Noe et al., 1999; Symowicz et al., 2007). In this study, we also observed that Dsc2 distribution in the lateral membrane was decreased in epithelial cells exposed to Dsg2 EC1–4 or E-cadherin EC1–5. However, the extent of Dsc2 relocation from the lateral plasma membrane was not as prominent as observed for Dsg2 and E-cadherin (Figure 3D).

Cadherins mediate bidirectional signaling with receptor tyrosine kinases to control cell behavior (Wheelock and Johnson, 2003). For example, EGFR/HER1 regulates desmosome assembly and function in squamous carcinoma cells by decreasing the cell surface distribution of desmosomal cadherins (Lorch et al., 2004). In
addition, EGFR/HER1, in concert with ADAM17, regulates the shedding and endocytic trafficking of Dsg2 (Klessner et al., 2009). We observed that Dsg2 ectodomains not only inhibit intercellular adhesion, but also increase epithelial cell proliferation. However, we did not observe a change in EGFR/HER1 phosphorylation/activation in response to sDsg2 exposure. The mammalian HER receptor family consists of four closely related growth factor receptors (HER1–4), which mediate cell adhesion, proliferation, and migration (Citri and Yarden, 2006). Our results showing that sDsg2 activates HER2 and HER3 but not EGFR/HER1 are consistent with the results of a study by Najy et al. (2008) in breast cancer cells. This unique mechanism is also supported by other reports that proinflammatory cytokines induce the phosphorylation of HER2 in intestinal epithelial cells and that HER2 activation requires ADAM17-dependent shedding of the ligand neuregulin-1 (Finigan et al., 2011; Jijon et al., 2012). On ligand binding, HER receptors dimerize, activating the cytoplasmic kinase domain and initiating downstream signaling (Hynes and lane, 2005). HER2 is the preferred dimerization partner, and HER2-containing heterodimers generate intracellular signals that are stronger than signals emanating from other HER combinations (Pinkas-Kramarski et al., 1996). In this study, we found that sDsg2-induced HER2 and HER3 phosphorylation and downstream activation of Akt/mTOR and MAPK promote IEC proliferation (Figure 4) and HER2–HER3 heterodimerization (Supplemental Figure S4J). Similarly, a previous study demonstrated that ADAM15-mediated E-cadherin cleavage in cancer cells stabilizes HER2 and HER3 heterodimerization and downstream Erk signaling (Najy et al., 2008). We also observed that pharmacological inhibition of Akt and Erk signaling is sufficient to inhibit the hyperproliferative effects of sDsg2. It is known that Akt and/or MAPK activation promote mTORC1 signaling through parallel mechanisms (Winter et al., 2011). Therefore, mTORC1 could be a downstream link between Akt and MAPKs signaling and IEC proliferation during inflammation. In addition, IEC proliferation caused by the release of soluble cadherins in the inflamed intestinal mucosa may be mediated by the generation or binding of HER ligands.

A continuous turnover of intercellular junction proteins is observed in healthy individuals and is required for the maintenance of epithelial barriers. Physiologically low levels of cadherin cleavage products have been identified in the serum of healthy individuals. Increased E-cadherin has been detected in the serum of cancer patients (Wilman et al., 2004; Gogali et al., 2010), and their generation likely contributes to tumor biology and metastasis. Our studies suggest that sDsg2 in addition to E-cadherin are generated in the intestinal mucosa during inflammation. Thus not only does the presence of such cadherin fragments influence epithelial homeostasis, but their detection in the serum could also serve as a biomarker of active inflammation in the mucosa.

Our study provides novel insights into the mechanism by which proinflammatory cytokines released into the epithelial milieu during mucosal inflammation promote cadherin ectodomain shedding, thereby altering homeostasis and barrier function of the intestinal epithelium. Although the compromised epithelial barrier may exacerbate mucosal inflammation, soluble cadherin fragments may also have beneficial effects by promoting the loosening of intercellular contacts and inducing cellular proliferation to stimulate repair of the epithelial barrier (Figure 6).

**MATERIALS AND METHODS**

**Antibodies and reagents**

The following primary monoclonal and polyclonal antibodies were used to detect proteins by immunofluorescence microscopy and/or immunoblot analysis: mouse anti-Dsg2 (clone AH12.2, generated in-house; Nava et al., 2007; Kolegara et al., 2011b), anti-Dsg2 (clone 4B2, a kind gift from K. J. Green, Northwestern University, Evanston, IL), rabbit anti-Dsg2 (clone EPR6767), anti-MMP2 (Novus Biologicals, Littleton, CO), anti-Dsg2 (clone EPR6768), mouse anti-His (clone HIS.H8; Abcam, Cambridge, MA), guinea pig anti-Dsc2 (American Research Products, Waltham, MA), mouse anti-Dsc2/3 (clone 7G6; Life Technologies, Carlsbad, CA), anti-E-cadherin (clone HECDC-1, a generous gift from A. S. Yap, University of Queensland, Brisbane St Lucia, Australia; Shimmoyama et al., 1989), anti–E-cadherin (clone 36; BD Biosciences, San Jose, CA), rat anti–uvomorulin/E-cadherin (clone DECA-1), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), anti-actin (Sigma-Aldrich, St. Louis, MO), mouse anti-JAM-A (clone J10.4, generated in-house; Liu et al., 2000), anti-SIRPα (clone SAF17.2, generated in-house; Lee et al., 2010), anti–Myc-tag (clone 9B11), rabbit anti–phospho-EGFR (Tyr-1068), anti-EGFR, anti–phospho-HER2 (Tyr-1196), anti-HER2, anti–phospho-HER3 (Tyr-1289), anti-HER3, anti–phospho-Akt (Thr-308), anti–phospho-Akt (Ser-473), anti–pan Akt, anti–phospho-S6 ribosomal protein (Ser-235/Ser-236), anti–S6 ribosomal protein, anti–phospho-Erk1/2 (Thr-202/Tyr-204), anti–Aurora1/2, and anti–MMP9 (Cell Signaling Technology, Danvers, MA). Fluorophore-conjugated secondary antibodies (Alexa dye series) were obtained from Life Technologies. Horseradish peroxidase (HRP)–conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant human IFN-γ, TNF-α, mouse IL-1β, IFN-γ, and TNF-α were purchased from PeproTech (Rocky Hill, NJ). Recombinant human IL-1β was obtained from EMD Millipore (Billerica, MA).

**Cell and organ culture**

The human T84 intestinal epithelial cell line and CHO cells were cultured as previously described (Ivanov et al., 2007; Kolegara et al., 2011b). For the noncontact coculture system, T84 cells were seeded on a 3-μm–pore size filter in the inner chamber, and CHO cells were seeded in the outer chamber (Transwell; Corning, Corning, NY). Tissue cultures were prepared from the murine distal colon, which was opened longitudinally and cleaned of fecal contents with cold Hank’s balanced salt solution containing 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). The gut was cut into 2-mm² pieces and then transferred onto an uncoated 12-well plate (Corning). The tissue pieces were incubated in 1.5 ml RPMI-1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO₂ incubator for 6 h. Culture supernatants were collected for immunoblot analysis or zymography.

**Inhibitor treatment**

Confluent T84 cells were treated with the following inhibitors 1 h before treatment with sDsg2 (10 μg/ml): 20 μM TAPI-2, 5 μM MMP2 inhibitor IV, 100 μM MMP9 inhibitor II, 2.5 μM MMP2/9 inhibitor II, 2.12 μM Akt inhibitor VIII, 20 μM PD98059 (EMD Millipore), 1–10 μM inhibitor Gi254023X (a kind gift from A. Ludwig, RWTH Aachen University, Aachen, Germany; Hundhausen et al., 2003), 100 nM Aryl-380 (Selleckchem, Houston, TX), or 2 nM rapamycin (Tocris Bioscience, Bristol, United Kingdom).

**Immunoblotting**

For in vitro cell cultures, confluent monolayers were collected in lysis buffer (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM ethylene glycol tetraacetic acid, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.4) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). After sonication, the cell lysates
were cleared by centrifugation (5000 x g at 4°C for 10 min). For studies with culture supernatants, cells were grown to confluency and switched to serum-free medium 24 h before sample collection. The supernatants were cleared by centrifugation to remove cell debris and concentrated 10-fold using Amicon Ultra Centrifugal Filters (3K MW; EMD Millipore). For analysis with human and mouse colonic mucosa specimens, the samples were resuspended in lysis buffer, homogenized, and sonicated as described. Protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Marietta, OH). The samples were then resuspended in NuPAGE LDS Sample Buffer (Life Technologies) and loaded onto polyacrylamide gels (15–20 μg protein/lane). After electrophoresis, the separated protein bands were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA) and probed with primary antibodies. Secondary antibodies conjugated to HRP were used to visualize the protein bands. Staining with Ponceau S solution (Sigma-Aldrich) was used to show equal loading of proteins. Immunoblots were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

Zymography
Culture supernatants were concentrated using an Amicon Ultra Centrifugal Filter (3K MW) according to the manufacturer's instructions and loaded on a Novex 10% Zymogram Gel containing 0.1% (wt/vol) gelatin (Life Technologies). After electrophoresis, the gels were washed twice in renaturing buffer (Life Technologies) and then incubated at 37°C for 24 h in developing buffer (Life Technologies). To visualize proteinase activity, the gels were stained with Simply Blue SafeStain (Life Technologies).

Generation of stable cell lines
CHO cells were transfected with the empty neomycin-resistant pcDNA3.1 expression vector (Life Technologies) as a control or pcDNA3.1 vectors expressing soluble Dsg2 EC1–4, Dsg2 EC3+4, Dsg2 EC1, Dsc2 EC1–4, or E-cadherin EC1–5 (generated by the Emory Cloning Core Facility, Atlanta, GA) and grown in the presence of G418 Sulfate (Mediatech, Manassas, VA) to select transfected cells. Monoclonal cell populations were isolated by the limiting dilution method.

Dispase assay
Cell monolayers were treated with 2.4 U/ml Dispase II (Roche Diagnostics, Mannheim, Germany) in phosphate-buffered saline (PBS) with calcium and magnesium at 37°C for 30 min. The cells were then transferred to 15-ml conical tubes containing 5 ml of PBS, which were gently mixed by rotating in a tube rotator 10–30 times. Monolayer fragments were transferred to 12-well tissue culture plates (Corning), imaged using a dissecting microscope, and counted.

Immunofluorescence microscopy
Cells grown on filters were fixed and permeabilized with ice-cold absolute methanol at –20°C for 20 min or 4% paraformaldehyde
at room temperature for 10 min, followed by treatment with 0.5% Triton X-100 at room temperature for 5 min. Nonspecific protein binding was blocked by 3% bovine serum albumin (Sigma-Alrich) in PBS at z for 1 h, and the cells were incubated with primary antibodies at 4°C overnight, washed in PBS, and subsequently labeled with secondary antibodies at room temperature for 1 h. Nuclei were stained using TO-PRO-3 iodide (Life Technologies) in PBS at room temperature for 15 min. Samples were mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA) and visualized on a Zeiss LSM 510 Meta Confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

Immunoprecipitation
Confluent T84 cell monolayers were homogenized and lysed in lysis buffer containing 100 mM KCl, 10 mM NaCl, 3.5 mM MgCl2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 1% Nonidet P-40, and protease and phosphatase inhibitor cocktails. The lysates were cleared by centrifugation (4000 rpm at 4°C for 4 min) and then incubated overnight with mouse anti-Dsg2 (clone 4B2), anti-E-cadherin (clone 36), or isotype-matched control immunoglobulin G (IgG; Sigma-Alrich) or incubated overnight with rabbit anti-HER2, anti-HER3, or isotype-matched control IgG. After addition of Protein A/G Sepharose Fast Flow (GE Healthcare, Little Chalfont, United Kingdom), the supernatants were incubated at 4°C for 3 h. The immunocomplexes were washed four times, boiled in NuPAGE LDS Sample Buffer, and then separated by SDS–PAGE for protein analysis.

Recombinant proteins
The Dsg2 extracellular domain, E-cadherin, JAM-A, and SIRPα were cloned into pcDNA3.0 with a His tag and transfected into CHO or HEK293T cells. The supernatants were collected, and the His-tagged proteins were purified using nickel beads (Thermo Fisher Scientific). Purified proteins were analyzed by Coomassie staining (Imperial Protein Stain; Thermo Fisher Scientific) of gels and immunoblotting (Supplemental Figure S3).

ELISA
Immun 2 HB plates (Thermo Fisher Scientific) were coated with 5 μg of recombinant human Dsg2 Fc (R&D Systems, Minneapolis, MN) and incubated with casein (Roche Diagnostics) to block nonspecific protein binding and with 0.5 μg/ml sDsg2-His, sE-cadherin-His, or sJAM-A-His in PBS for 1 h. The binding of Dsg2, E-cadherin, and JAM-A was detected using mouse anti-His antibody and goat antimalouse HRP-conjugated antibody.

Enteroid culture
Intestinal crypts were isolated and cultured as described previously (Mahe et al., 2013) with modifications. Briefly, sections of the small intestine (∼10 cm) from 8-wk-old C57BL/6 mice were dissected and opened longitudinally. The sections were cut into 1-cm pieces, washed three times with cold PBS, and incubated with 5 ml of cell suspension buffer (2 mM EDTA in PBS) at 4°C for 30 min on a rotating wheel. The cell suspension was changed to 5 ml of cell dissociation buffer (PBS containing 43.3 mM sucrose and 54.9 mM sorbitol), and the epithelium was detached by vigorous shaking. The suspension was passed through a 70-mm cell strainer (BD Bioscien) to remove villi. The resulting crypt suspension was diluted with dissociation buffer, bringing the volume to 10 ml, and the detached crypts were counted in 20-μl droplets. The desired number of crypts was centrifuged at 150 × g for 10 min at 4°C in 10 ml of dissociation buffer. The pelleted crypts were resuspended in Matrigel (BD Biosciences) containing 50 ng/ml epidermal growth factor (EGF), 100 ng/ml Noggin, and 500 ng/ml R-spondin (R&D Systems) at 500 crypts/50 μl Matrigel, seeded in the middle of 24-well cell culture plate wells (Corning), and incubated for 30 min at 37°C. The gels were overlaid with 450 μl of minigut culture medium consisting of Advanced DMEM/F12, L-glutamine (2 mM), penicillin (100 U/ml)/streptomycin (100 μg/ml), HEPES (10 mM), B27 supplement (1:50; Life Technologies), and N2 supplement (1:100; R&D Systems). The minigut medium supplemented with EGF, Noggin, and R-spondin was replaced every 4 d, and the enteroids were passaged into fresh Matrigel every 8 d. Enteroids were cultured at 37°C in a 5% CO2 atmosphere for at least 3 d before stimulation with sDsg2 or sE-cadherin.

EdU incorporation assay
Cells and intestinal crypt organoids were pulse-labeled with EdU (10 nM; Click-IT EdU; Life Technologies) for 1 h and processed according to the manufacturer’s instructions.

Human colon samples
Human ulcerative colitis and normal colonic tissue specimens were obtained from the Human Tissue Procurement Service at Emory University. All procedures were approved by the Institutional Review Board at Emory University.

In vivo experiments
Female C57Bl/6J mice (6–8 wk old) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were intraperitoneally injected with recombinant mouse IL-1β (2.5, 5, or 7.5 μg/kg) or recombinant mouse TNF-α (2.5, 5, or 10 μg/kg) as previously described (Nava et al., 2014). To generate a mouse model of colitis, the mice were treated with DSS (2.5% [wt/vol]; molecular mass, 36–50 kDa; MP Biomedicals, Santa Ana, CA) for 7 d, as previously described (Cooper et al., 1993). All animal protocols were approved by the Institutional Animal Care and Use Committee of Emory University.

Measurement of transepithelial electrical resistance
Cells were cultured on Transwell inserts with 3-μm–pore size filters (Corning). Transepithelial electrical resistance was measured using an EVOM voltmeter with an ENDOHM-12 (World Precision Instruments, Sarasota, FL). Electrical resistance was expressed as Ω × cm2. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.

Measurement of paracellular permeability
To determine the paracellular flux, cells were cultured on Transwell filters with 3-μm pore size (Corning). A 4-kDa fluorescein isothiocyanate–dextran (FD4; Sigma-Alrich)–containing medium was added to the inner chamber. Samples were collected from the outer chamber at 30, 60, 90, and 120 min. Permeability across cell monolayers was assessed for fluorescence in a microplate fluorescence reader (FL-500; BioTek, Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm and was expressed as μg/h/cm2.

Statistics
For control-normalized experiments, group means were compared by the z test. Unpaired Student’s t test was used to analyze data from all other experiments. Results are displayed as means ± SEM; p < 0.05 was considered significant.
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