IL-33 promotes recovery from acute colitis by inducing miR-320 to stimulate epithelial restitution and repair

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Defective and/or delayed wound healing has been implicated in the pathogenesis of several chronic inflammatory disorders, including inflammatory bowel disease (IBD). The resolution of inflammation is particularly important in mucosal organs, such as the gut, where restoration of epithelial barrier function is critical to reestablish homeostasis with the interfacing microenvironment. Although IL-33 and its receptor ST2/ILRL1 are known to be increased and associated with IBD, studies using animal models of colitis to address the mechanism have yielded ambiguous results, suggesting both pathogenic and protective functions. Unlike those previously published studies, we focused on the functional role of IL-33/ST2 during an extended (2-wk) recovery period after initial challenge in dextran sodium sulfate (DSS)-induced colitic mice. Our results show that during acute, resolving colitis the normal function of endogenous IL-33 is protection, and the lack of either IL-33 or ST2 impedes the overall recovery process, while exogenous IL-33 administration during recovery dramatically accelerates epithelial restitution and repair, with concomitant improvement of colonic inflammation. Mechanistically, we show that IL-33 stimulates the expression of a network of microRNAs (miRs) in the Caco2 colonic intestinal epithelial cell (IEC) line, especially miR-320, which is increased by >16-fold in IECs isolated from IL-33–treated vs. vehicle-treated DSS colitic mice. Finally, IL-33–dependent in vitro proliferation and wound closure of Caco-2 IECs is significantly abrogated after specific inhibition of miR-320A. Together, our data indicate that during acute, resolving colitis, IL-33/ST2 plays a crucial role in gut mucosal healing by inducing epithelial-derived miR-320 that promotes epithelial repair/restitution and the resolution of inflammation.

IL-33/ST2 | miR-320 | IBD | DSS colitis | mucosal healing

The resolution of inflammation at mucosal surfaces is a fundamental physiological process that promotes restitution of the epithelial barrier and appropriate tissue repair in an attempt to restore normal organ function and homeostatic conditions with the interfacing microenvironment. Dysfunction and/or delay in mucosal healing has been implicated in the pathogenesis of several chronic inflammatory disorders, including psoriasis, idiopathic pulmonary fibrosis, and inflammatory bowel disease (IBD) (1). Crohn's disease (CD) and ulcerative colitis (UC), two main forms of IBD, are chronic, relapsing inflammatory disorders of the gastrointestinal (GI) tract resulting from dysregulated immune responses toward environmental factors in genetically predisposed individuals. In this setting, achieving efficient resolution of inflammation and mucosal healing is one of the most important goals to achieve to maintain long-term remission in patients with IBD.

Although the precise etiology is currently unknown, it is widely accepted that an imbalance of pro- and antiinflammatory mediators is a key mechanism in the pathogenesis of IBD (2); among these, a wealth of data supports the role of the IL-1 family of cytokines (3). IL-33/IL-1F11 is the most recently identified member of this family and is widely expressed in various organs, particularly those positioned at mucosal surfaces, such as the GI tract (4). The main cellular sources of IL-33 within the GI tract are predominantly nonhematopoietic in nature and include intestinal epithelial cells (IECs), endothelial cells, subepithelial myofibroblasts (SEMFs), and smooth muscle cells (4–9); however, IL-33 is also expressed in cells of hematopoietic origin, particularly professional antigen-presenting cells, such as macrophages (4, 5). IL-33 serves as a protein with dual functions that can act both as a classic cytokine and as an intracellular nuclear factor (4, 10). Similar to IL-1α, IL-33 functions as an endogenous danger signal, or "alarmin," that is released from stressed, damaged, or necrotic cells and alerts the innate immune system to tissue injury during trauma or infection (11). As a classic signaling cytokine, IL-33 exerts its biological effects through binding to its cognate receptor, IL-1 receptor-like 1 (IL1RL1)/IL-1R4, also known as "ST2L," pairing with its

Significance

We clarify that the normal, inherent function of IL-33 following acute, resolving colitis is protection, inducing proliferation and restitution of ST2L-bearing intestinal epithelial cells (IECs). Importantly, this response occurs in otherwise healthy, immunocompetent C57BL/6J (B6) mice and may be different in other models possessing genetic and/or immunologic abnormalities that predispose to colitis, similar to patients with inflammatory bowel disease. Mechanistically, although the molecular processes responsible for control of microRNA (miR) biogenesis in response to challenge remain largely unknown, we report that IL-33 augments epithelial miR-320, which increases IEC proliferation and wound closure that is significantly diminished upon specific miR-320 inhibition. This study provides the rationale for the potential therapeutic use of either IL-33 or miR-320A to obtain optimal gut mucosal healing and the resolution of inflammation.

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coreceptor, IL-1 receptor accessory protein (IL1RAcP)/IL-1R3, and activating MAPK and NF-κB pathways (4, 12). A secreted, soluble isoform of ST2 (sST2) also exists, which results from differential splicing of *IL1RL1* and which down-regulates IL-33's bioactivity by functioning as an antagonist decoy receptor (13). Several cell types within the GI tract express ST2, most prevalently type 2 innate lymphoid cells, eosinophils, mast cells, alternatively activated M2 macrophages, Th2 lymphocytes, Tregs, IECs, SEMFs, and adipocytes (4, 5, 14–16). While one of the first reported and main functions of IL-33 is to promote the Th2 immune responses (4), IL-33 is now well recognized as playing a role in several biological functions aside from immune regulation (17).

The role of the IL-33/ST2 axis in the pathogenesis of IBD was first reported in 2010, revealing a strong association with UC (5–8). In subsequent attempts to determine the precise mechanistic role of IL-33 in IBD, investigations have mainly been performed using chemically induced mouse models of colitis (*SI Appendix*, Table S1). The most commonly used of these is acute administration of dextran sodium sulfate (DSS), which has long been established as an effective model of epithelial damage that results in a highly reproducible acute colitis with weight loss, bloody diarrhea, and mucosal ulceration (18). Interestingly, investigations into the role of IL-33 in the development of colitis using variations of this model have generated ambiguous results, revealing both protective and pathogenic functions (*SI Appendix*, Table S1). Nevertheless, although DSS-induced colitis does not recapitulate all features of IBD [e.g., colitis occurs in the absence of adaptive immune responses that are a hallmark feature of the human condition (19)], several variations are routinely used to study different aspects of colitis. In fact, studying the recovery phase following DSS challenge is a useful approach to specifically evaluate potential mechanism(s) of epithelial restitution and repair as well as mucosal healing. Therefore, unlike previously published studies investigating the role of the IL-33/ST2 axis in DSS-induced colitis, we focused on an extended 2-wk recovery period after DSS challenge to mechanistically evaluate how IL-33 and ST2 affect the resolution of inflammation and mucosal healing.

In the present study we show that, although IL-33 can initially sustain colonic inflammation in mice immediately after acute DSS challenge, its primary role is to promote mucosal wound healing during recovery. In fact, both *Il33* and *St2* deficiency considerably dampen epithelial restitution and repair and exacerbate ulcer formation up to 2 wk into recovery. Conversely, exogenous IL-33 administration during recovery is potently effective in accelerating mucosal healing and decreasing colitis severity. In vitro studies demonstrate that IL-33 has a direct effect on the Caco-2human colonic IEC line by inducing cell proliferation and promoting wound closure. Microarray analysis of IL-33–stimulated IECs confirmed the activation of intracellular proliferative pathways and showed increased expression of a network of microRNAs (miRs), of which *MIR320A* was one of the most highly expressed. Mechanistically, miR-320A inhibition in Caco-2 cells significantly decreases IL-33–dependent cell proliferation and wound closure, while increased *Mir320* is observed in IECs isolated from IL-33–treated vs. vehicle-treated DSS colitic mice. Taken together, our data indicate that during acute, resolving colitis, the IL-33/ST2 axis plays a critical role in gut mucosal wound healing by inducing epithelial-derived miR-320, promoting epithelial repair and restitution, overall restoration of barrier integrity, and the resolution of inflammation.

Results

IL-33 and Its Receptor ST2 Are Up-Regulated and Localized to the Epithelium During Recovery from DSS-Induced Colitis. Using a highly reproducible model of epithelial damage and repair with extended recovery, we found that colonic *Il33* increased dramatically (by 4.1 ± 0.2-fold, $P < 0.001$) in C57BL/6J (B6) mice (*SI Appendix*, Fig. S1) immediately after DSS challenge (hereafter, "DSS B6 mice") compared with control B6 mice administered regular drinking water and was further augmented (by 5.1 ± 0.5-fold vs. control mice, $P < 0.001$) after 1-wk recovery during peak inflammation (Fig. 1*A*). After 2-wk recovery, when mucosal healing was evident, *Il33* in DSS-induced colitic mice decreased markedly (by 80.8%, $P < 0.001$) compared with levels at 1-wk recovery and was similar to levels in uninflamed control mice (Fig. 1*A*). Similar trends were observed for colonic ST2 mRNA levels in DSS-treated mice (Fig. 1*B*). *Il1rl1* expression of cell-surface ST2L was elevated after 5 d of DSS administration (2.4 ± 0.2-fold vs. control mice, $P < 0.001$) and increased, reaching peak levels at 1-wk recovery (3.5 ± 0.2-fold vs. control mice, $P < 0.001$ and 1.5 ± 0.3-fold vs. 5-d DSS challenge, $P < 0.01$). After 2-wk recovery, *Il1rl1* (ST2L) was reduced compared with 1-wk recovery and compared with 5-d DSS challenge (by 62.8 and 45.9%, respectively, both $P < 0.001$) and reached baseline levels close to those of control mice (Fig. 1*B*, *Left*). Similarly, *Il1rl1* (sST2) increased at 5-d DSS challenge (3.3 ± 0.6-fold vs. control mice, $P < 0.01$), rose further after 1-wk recovery (5.2 ± 0.9-fold vs. control mice, $P < 0.01$), and subsequently decreased (by 58.4% vs. 1-wk recovery, $P < 0.05$) but, interestingly, remained elevated compared with control mice at 2-wk recovery (2.2 ± 0.6-fold, $P < 0.05$) (Fig. 1*B*, *Right*).

At the protein level (Fig. 1 *C* and *D*), while the abundance of colonic IL-33 and total ST2 remained relatively constant throughout the experimental period in control mice, both were considerably increased in colitic mice, reaching peak levels at 1-wk recovery and remaining elevated after 2-wk recovery ($P < 0.001$ and $P < 0.01$, respectively, vs. control mice), when epithelial restitution and repair is achieved, eventually decreasing from 1-wk to 2-wk recovery ($P < 0.05$) (Fig. 1 *C* and *D*, *Left*). Western blots for IL-33 showed the presence of 30 and 20–22 kDa bands, corresponding to full-length (f) IL-33, the most bioactive form, and cleaved (c)-IL33, a less bioactive isoform (20), respectively. f-IL-33 was up-regulated in the colons of DSS-challenged mice compared with control mice, with little post-translational modification into c-IL-33, which was equally low in all groups (Fig. 1*C*, *Right*). The difference in total ST2 protein was mainly contributed by sST2 (~60 kDa), which was the prevalent form up-regulated in colons of DSS colitic mice, while cell-surface ST2L (~120 kDa), the predominant form in control mice, diminished considerably after 5-d DSS challenge but began to reappear during recovery (Fig. 1*D*, *Right*).

IL-33 and ST2 immunolocalized to colon tissues from both DSS-treated and control mice, although the intensity and distribution varied among experimental groups (Fig. 2). In uninflamed control mice, IL-33 localized primarily to IECs but also to lamina propria mononuclear cells (LPMCs), while ST2 was found mainly in surface IECs (Fig. 2, *Left*). Following 5-d DSS challenge and at 1-wk recovery, IL-33 increased dramatically in inflamed colonic tissues (Fig. 2, *Upper Middle*), with intense staining primarily localized to IECs, particularly restituting epithelium (black arrows). ST2 was similarly augmented in inflamed tissues after 5-d DSS challenge (Fig. 2, *Lower Middle*) but was most notably localized to ulcerated lesions and areas of reepithelialization upon recovery and initial reformation of crypts (Fig. 2, *Lower Right*).

Overall, these data suggest that IL-33 is potently up-regulated following acute DSS challenge and also during active recovery; as such, the overall functional effects of IL-33 and whether its expression is a consequence of inflammation or healing are still unclear. However, the potential role of ST2 is better defined. Total ST2 is also elevated, which functionally likely represents the observed increase in sST2 in an attempt to decrease the pathogenic inflammatory activity of IL-33 or, alternatively, up-regulation of epithelial ST2L to promote IL-33–dependent repair and restitution of barrier integrity.

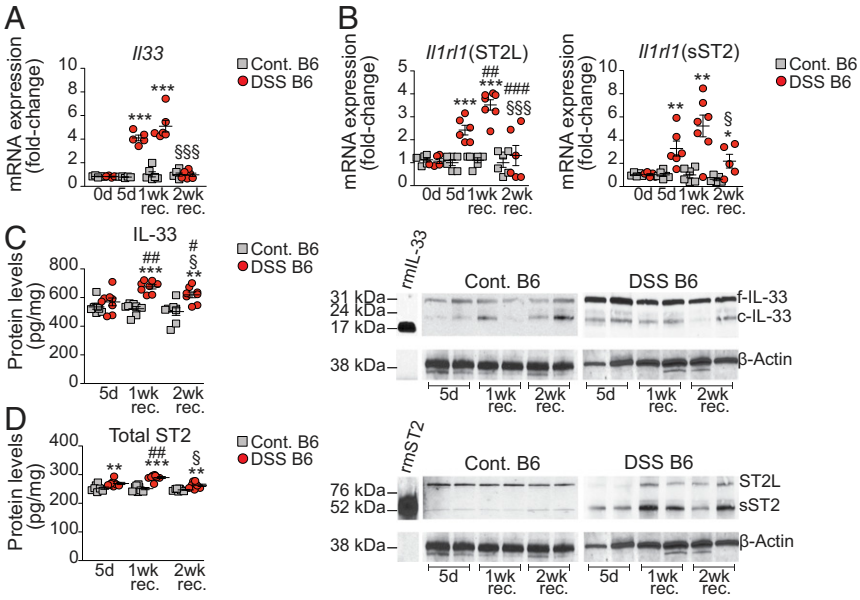

Fig. 1. IL-33 and ST2 are modulated, with differential expression of protein isoforms, during acute DSS challenge and recovery from colitis. (*A* and *B*) Il33 (*A*) and Il1rl1 (*B*) encoding ST2L (*B*, *Left*) and sST2 (*B*, *Right*) in colons from DSS B6 mice at baseline/steady state (day 0), after 5 d of DSS challenge, and at 1- and 2-wk recovery ($n = 5$–7). Vehicle-treated B6 mice served as controls (Cont. B6) and were killed at the same time points as DSS-treated mice. (*C* and *D*) IL-33 (*C*) and total ST2 (*D*), evaluated quantitatively ($n = 7$–8) (*Left*) and for expression of protein isoforms (Western blots representative of four separate experiments) (*Right*) to distinguish between full-length (f)- and cleaved (c)-IL-33, at 30 and 20–22 kDa, respectively (*C*, *Right*), and between ST2L (~120 kDa) and sST2 (~60 kDa) (*D*, *Right*). Either recombinant mouse IL-33 (rmIL) (~18 kDa) or ST2/IL-33R (~63 kDa) proteins were used as positive controls. Data are expressed as mean ± SEM with relative fold differences compared with control B6 mice at 5-d DSS challenge (arbitrarily set as 1); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. time point-matched control B6 mice; $^{\S}P < 0.05$, $^{\S\S\S}P < 0.01$ vs. 1-wk recovery in DSS B6 mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. DSS B6 mice at 5-d DSS challenge.

***Il33*$^{-/-}$ and *St2*$^{-/-}$ Mice Are Deficient in Their Ability to Recover from Acute DSS-Induced Colitis.** To address whether the role of IL-33 during recovery from DSS-induced colitis is protective or pathogenic, we performed the same experiment using *Il33*- and/or *St2*-deficient mice (Fig. 3). *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice exposed to DSS (DSS *Il33*$^{-/-}$ and DSS *St2*$^{-/-}$ mice, respectively) lost less of their initial body weight and displayed a decreased disease activity index (DAI) than DSS-challenged *Il33*$^{+/+}$*St2*$^{+/+}$ (DSS WT) controls early during DSS challenge and after 1-wk recovery (both $P < 0.01$), consistent with the observation that early IL-33 expression appears to possess pathogenic functional effects (Fig. 3*A*, *Left* and *Left Center*). At 2 wk, however, DSS *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice continued to lose weight and sustained an elevated DAI compared with DSS WT mice (both $P < 0.01$), which, like DSS B6 mice (*SI Appendix*, Fig. S1 *A* and *B*), showed efficient recovery as indicated by both weight gain almost back to baseline and a dramatic drop in DAI (Fig. 3*A*, *Left* and *Center Left*). Similarly, endoscopic examination of the colonic mucosa revealed progressive worsening of disease in DSS *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice, peaking at 2-wk recovery ($P < 0.05$ and $P < 0.01$,

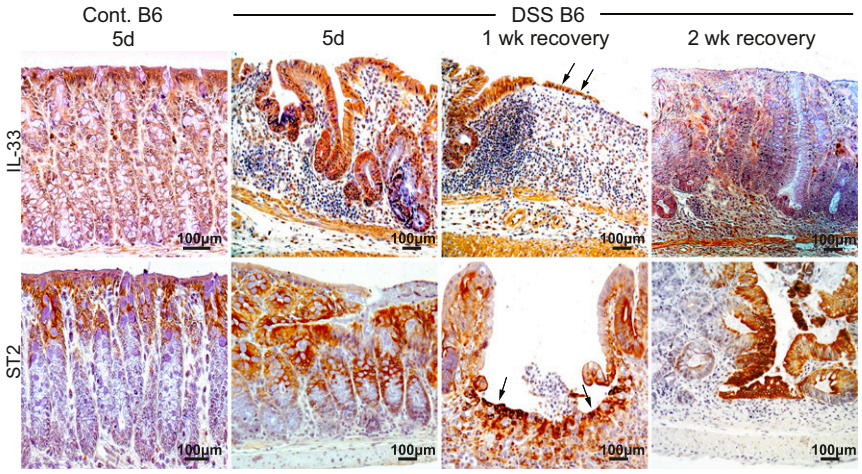

Fig. 2. IL-33 and ST2 are predominantly localized to the colonic epithelium during acute DSS challenge and recovery from colitis. Representative photomicrographs of colonic tissues stained for IL-33 (*Upper Row*) and ST2 (*Lower Row*) show primary localization to IECs in uninflamed (no DSS) 5-d control mice (*Far Left*), which is unchanged after 1 and 2 wk of recovery. During DSS challenge and 1 wk of recovery, both IL-33 and ST2 are potently increased, particularly in restituting epithelium (black arrows) overlying ulcer lesions (*Center*). After 2 wk of recovery (*Far Right*), crypt formation becomes more prominent, with IL-33–responsive, ST2-expressing cells limited to regenerating epithelium ($n = 8$) (original magnification: 20× + 1.25).

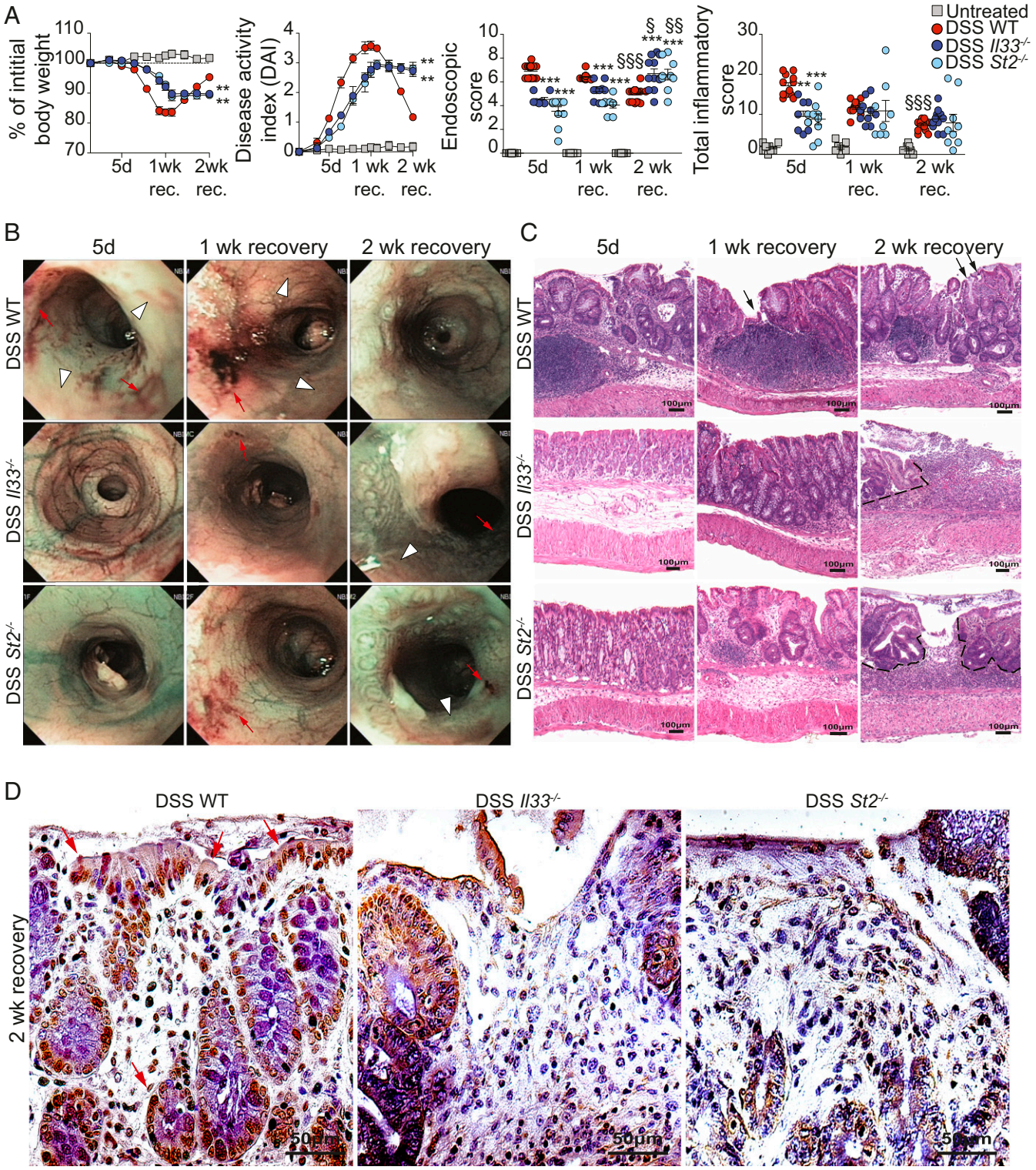

Fig. 3. Absence of the IL-33/ST2 receptor–ligand pair disrupts the ability to recover effectively from DSS-induced colitis. (*A*) Body weight, DAI, and endoscopic and histologic analyses of colons from Il33$^{-/-}$, St2$^{-/-}$, and Il33$^{+/+}$St2$^{+/+}$ (WT) mice (color-coded circles) after 5-d DSS challenge and at 1 and 2 wk of recovery; untreated mice not exposed to DSS (gray squares), regardless of genotype, are grouped since no significant differences were observed among groups or with untreated B6 mice (*SI Appendix*, Fig. S1). (*B*) Representative endoscopic images; red arrows indicate frank bleeding, and white arrowheads indicate edema. (*C*) Representative photomicrographs of H&E-stained colonic tissues (original magnification: 10× + 1.25); black dashed lines demarcate ulcerated mucosal lesions (also evident on corresponding endoscopic images), and black arrows indicate reepithelialization and healing of mucosa. (*D*) Representative photomicrographs of colonic tissues stained for BrdU at 2-wk recovery (original magnification: 40× + 1.25); red arrows indicate actively proliferating BrdU^{+} cells. For each time point, n = 7–10 untreated mice, n = 18 = 21 DSS WT mice, n = 8–12 DSS Il-33$^{-/-}$ mice, and n = 8–10 DSS St2$^{-/-}$ mice; data are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. time point-matched DSS WT mice; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ vs. strain-matched mice challenged with DSS for 5 d.

respectively, vs. 5-d DSS challenge) and compared with DSS WT mice at 2-wk recovery (both $P < 0.001$) (Fig. 3*A*, *Center Right*). These mice showed persistent bleeding (red arrows) and edema (white arrowheads), with abundant and large mucosal ulcerations, particularly at 2-wk recovery (Fig. 3*B*, *Bottom Right*), while DSS WT mice displayed improved overall endoscopic scores and mucosal healing (Fig. 3*B*, *Top Right*).

Histologic analyses confirmed these results: Like DSS B6 mice (*SI Appendix*, Fig. S1 *A–C*), WT mice during DSS challenge showed severe colitis, which improved progressively over 2-wk recovery ($P < 0.001$ vs. 5-d DSS challenge) (Fig. 3*A*, *Right*). Conversely, both *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice displayed moderate colitis during DSS challenge, which did not change significantly even after 2 wk of recovery (Fig. 3*A*, *Right*). In fact, at 1-wk recovery, areas of regenerating epithelium (black arrows) overlying ulcerated inflammatory lesions were present in DSS WT mice (Fig. 3*C*, *Top Center*) but were much less evident in DSS *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice even at 2-wk recovery (Fig. 3*C*, *Bottom Right*). Moreover, BrdU staining showed reduced expression and decreased epithelial distribution of proliferating cells in both DSS *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice as compared with WT mice, with no apparent differences between *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice (Fig. 3*D*). In addition, mice not exposed to DSS (untreated), independent of genotype (here, grouped together for *Il33*$^{-/-}$, *St2*$^{-/-}$, and WT controls), did not show any significant changes (Fig. 3*A*) and were similar to results obtained in (untreated) control B6 mice (*SI Appendix*, Fig. S1). Overall, these data indicate that the lack of either IL-33 or ST2 impedes the overall recovery process after acute DSS colitis and appears to affect epithelial-specific proliferation and repair.

Exogenous Administration of IL-33 During Recovery After DSS Challenge Enhances Mucosal Healing and the Resolution of Colitis. To confirm our results thus far and to test the potential therapeutic role of IL-33 in promoting epithelial repair/restitution and ultimate wound healing, we exogenously administered a pharmacological dose (33 μg/kg) of recombinant IL-33 (rIL-33) during the recovery period following acute DSS colitis (Fig. 4*A*). IL-33–treated mice showed accelerated recovery from body weight loss compared with vehicle-treated controls ($P < 0.01$), reaching initial baseline levels after 2-wk recovery, and lowered DAI ($P < 0.01$), decreasing disease activity almost to zero (Fig. 4*B*). The general endoscopic appearance of the colonic mucosa was also dramatically improved upon IL-33 treatment ($P < 0.001$ at both 1- and 2-wk recovery vs. vehicle-treated controls), with greater magnitude and efficacy between 1 and 2 wk of recovery (Fig. 4*C*). Less edema (white arrowheads) and bleeding (red arrows) were observed at 1-wk recovery (Fig. 4*C*), and increased translucency of the mucosal surface, prominent vascularization, and centralized, tubular-shaped lumens became evident at 2-wk recovery (Fig. 4*D*).

Histologic analyses provided further support for the efficacy of IL-33 treatment in promoting epithelial repair/restitution and mucosal healing, showing decreased inflammation in treated DSS-induced colitic mice at 1- or 2-wk recovery ($P < 0.001$) (Fig. 4*E*). In particular, the emergence of restituting epithelium (black arrows), goblet cells, and organized colonic crypts (Fig. 4*F*, *Lower Row*), as well as increased expression of BrdU^{+} cells (Fig. 4*G*, red arrows), mainly localized to epithelial crypt cells, was evident in IL-33–treated colitic mice as compared with untreated controls after 1- and 2-wk recovery. Taken together, these findings indicate that pharmacologic treatment of DSS-induced colitic mice with rIL-33 during recovery dramatically accelerates epithelial restitution and repair, with concomitant improvement of intestinal inflammation.

IL-33 Directly Induces IEC Proliferation and Wound Healing by Specific Up-Regulation of miR-320. To mechanistically determine the direct effects of IL-33 on IECs, we performed a series of in vitro experiments using the colonic Caco-2 IEC line stimulated with human rIL-33. IL-33–treated Caco-2 cells showed increased proliferation compared with (untreated) controls in a dose-dependent manner after both 6 and 12 h, with maximum effects (21.7% vs. control, $P < 0.01$) at 6 h using 10 ng/mL IL-33 (Fig. 5*A*). More dramatic results were observed when measuring in vitro wound healing, demonstrating that IL-33 accelerated IEC wound closure by 33.1% ($P < 0.01$ vs. control), with maximum effects at 24 h (Fig. 5*B*). Microarray analysis of IL-33–regulated genes in IECs revealed gene targets primarily involved in cell-cycle function, cell morphology, cell-to-cell signaling and interaction, and cellular development and maintenance (*SI Appendix*, Table S2). Among these, a specific network of highly expressed miRs was identified that are associated with the activation of intracellular proliferative pathways (*SI Appendix*, Table S3). To confirm these results, we performed in vitro experiments that were identical to the previous experiments except for miR enrichment. Our results showed that after miR enrichment, *MIR320A* was the most highly expressed miR in colonic IECs stimulated with IL-33 (by 12.58 ± 2.63-fold vs. controls, $P < 0.02$) (Fig. 5*C*). These findings were verified in vivo; IECs isolated from DSS-induced colitic mice treated with IL-33 during recovery (Fig. 4*A*) expressed 16.63 ± 1.42-fold elevated *Mir320* after 2 wk than vehicle-treated DSS-induced colitic mice ($P < 0.001$) (Fig. 5*D*).

To determine the functional role of miR-320 in IECs, we transfected Caco-2 cells with small ssRNA molecules designed specifically either to inhibit miR-320A (mirVana miR-320A inhibitor) or to serve as a negative control (scrambled mirVana). After stimulation with IL-33, reduced *MIR320A* was confirmed after specific inhibition of miR-320A (by 84.8% vs. control, $P < 0.01$) (Fig. 5*E*), and IL-33–dependent proliferation was partially, albeit significantly, blocked, by 35.7% vs. control after 6 h ($P < 0.05$) and by 32.9% vs. control at 24 h ($P < 0.01$) (Fig. 5*F*). More impressively, specific miR-320A inhibition decreased in vitro wound closure by 66.4% vs. control after 6 h ($P < 0.05$) and by 74.5% vs. control at 24 h ($P < 0.01$) (Fig. 5*G*). Taken together, these data indicate that IL-33 has a direct effect on epithelial proliferation and wound healing that is due, in part, to miR-320 regulation.

Discussion

While the increased expression of IL-33 and its association with IBD has been firmly established, dissecting the precise mechanistic role of IL-33 during intestinal inflammation has generated ambiguous results, suggesting both protective and pathogenic functions. In the great majority of studies using experimental models of colitis, healthy immunocompetent mice have been challenged with 1.5–5% DSS continuously for 5–13 d (in some cases, DSS exposure was repeated with a 5- to 7-d recovery period between cycles), and colitis was evaluated either immediately without recovery or after a recovery period of up to 7 d (*SI Appendix*, Table S1). Under these conditions, IL-33 was generally shown to have pathogenic effects when colitis was evaluated without recovery or after up to 7 d of recovery and when acute DSS was administered (without cycling) at a moderate to high dose (2–5%). These results are consistent with our observation that deletion of either *Il33* or *St2* in mice in which colitis was induced by 3% DSS (which allowed almost 100% survival) was effective in decreasing DAI, improving endoscopic appearance of the colonic mucosa, and dampening overall disease severity when evaluated either during DSS challenge or after 7 d of recovery. Importantly, however, when allowed to recover for an extended period of 2 wk, DSS *Il33*$^{-/-}$ and *St2*$^{-/-}$ mice were deficient in their ability to appropriately restitute epithelium, abrogate mucosal ulcerations, and effectively heal the colonic mucosa as compared with WT controls. These data suggest that

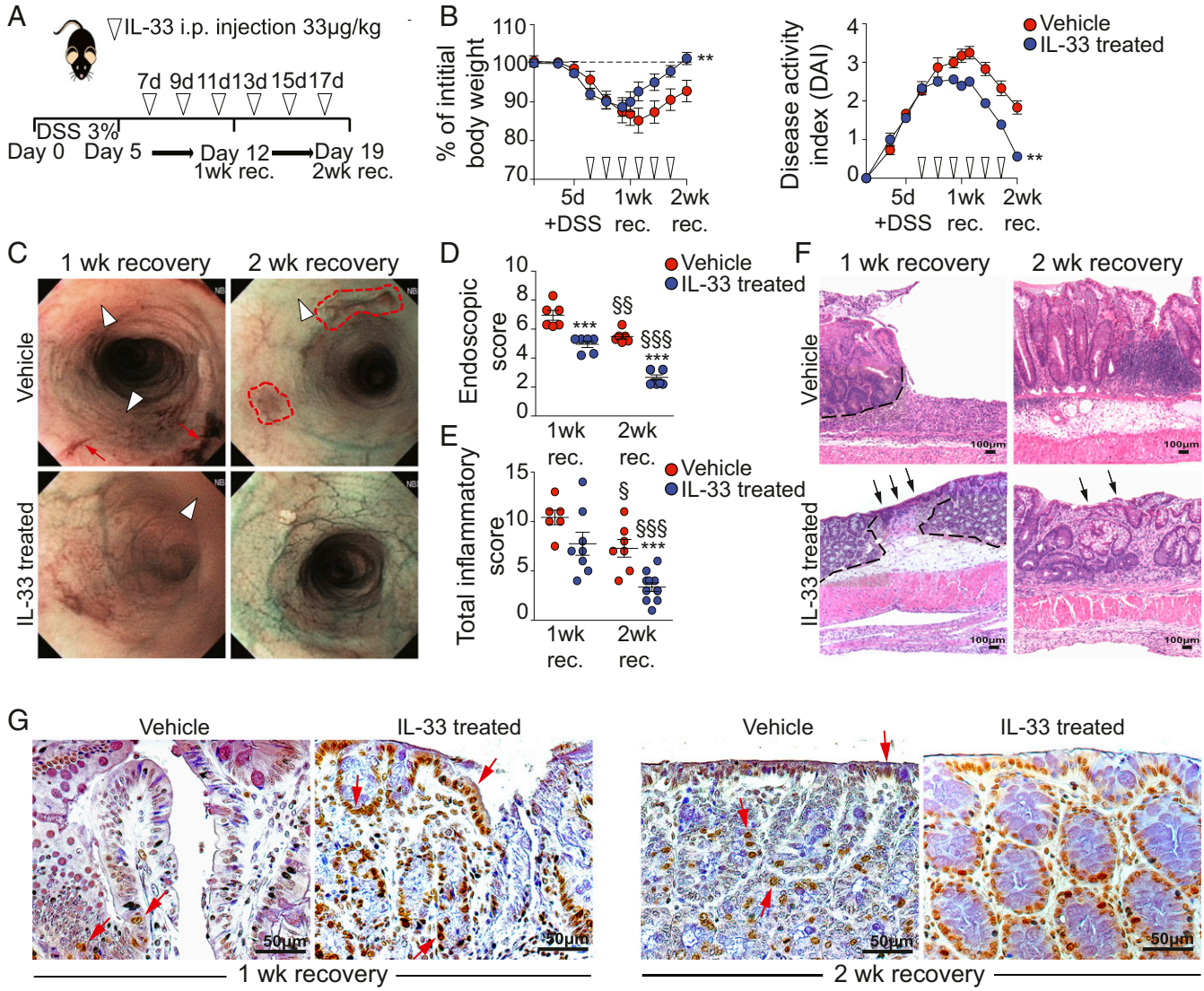

Fig. 4. IL-33 treatment during recovery from DSS accelerates mucosal healing and decreases colitis severity. (*A*) Strategy for IL-33 treatment (open arrowheads) in the DSS colitis model with extended recovery. (*B*) Resulting body weight (*Left*) and DAI (*Right*). (*C*) Representative images showing areas of frank bleeding (red arrows) and edema (white arrowheads). Red dashed lines outline healing ulcers. (*D*) Semiquantitative scores. (*E* and *F*) Histologic analysis showing total inflammatory scores (*E*) and representative photomicrographs (original magnification: 10× + 1.25) (*F*) of H&E-stained colonic tissues. Black dashed lines in *F* outline ulcerated mucosal lesions, and black arrows indicate single-layer reepithelialization overlying healing ulcers. (*G*) Representative photomicrographs of colonic tissues stained for BrdU at 1- and 2-wk recovery, comparing vehicle- and IL-33–treated DSS-induced colitic mice (original magnification: 40× + 1.25). Red arrows indicate actively proliferating $BrdU^+$ cells, with greatest abundance at 2-wk recovery following IL-33 treatment. For each time point, $n = 6$–7 vehicle-treated mice and $n = 8$–13 IL-33–treated mice; data are expressed as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. vehicle; $^{§}P < 0.05$, $^{§§}P < 0.01$, $^{§§§}P < 0.001$ vs. the same treatment at 1-wk recovery.

in healthy mice, when assaulting factors (e.g., DSS) are removed, the normal function of endogenously produced IL-33 within the gut mucosa is protection.

This concept is further supported by our results showing that exogenous supplementation of rIL-33 at pharmacological doses during recovery from DSS is able to significantly decrease DAI, promote ulcer healing, restore normal epithelial architecture, and globally improve gut mucosal wound healing. This finding may also account for results from previously published studies in which IL-33 administered during recovery periods between DSS cycling, commonly used to produce chronic colitis, demonstrated protective effects (21, 22). In fact, other studies treating colitis with exogenous, supraphysiological doses of IL-33 generally resulted in disease improvement in acute and chronic models (14, 23). Our results, however, conflict directly with those of Sedhom et al., (24), who reported that inhibiting the IL-33/ST2 pathway either by genetic ablation or by treatment with a specific blocking antibody against ST2 ameliorated colitis in two models of colitis [i.e., DSS- and 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced] by enhancing mucosal healing. Unlike our study that allowed recovery for 2 wk after ceasing DSS challenge, experimental mice in the study by Sedhom et al. were not permitted to recover, and colitis was assessed on the last day of chemical insult, which may, as in our results, represent a time point when IL-33–dependent disease activity is still increasing and colonic inflammation is most severe. As such, treatment with an anti-ST2 antibody during this time period or performing the same protocol in ST2-deficient mice would predictably decrease colitis severity. In the same study, exogenous IL-33 treatment was shown to exacerbate colitis and decrease effective wound

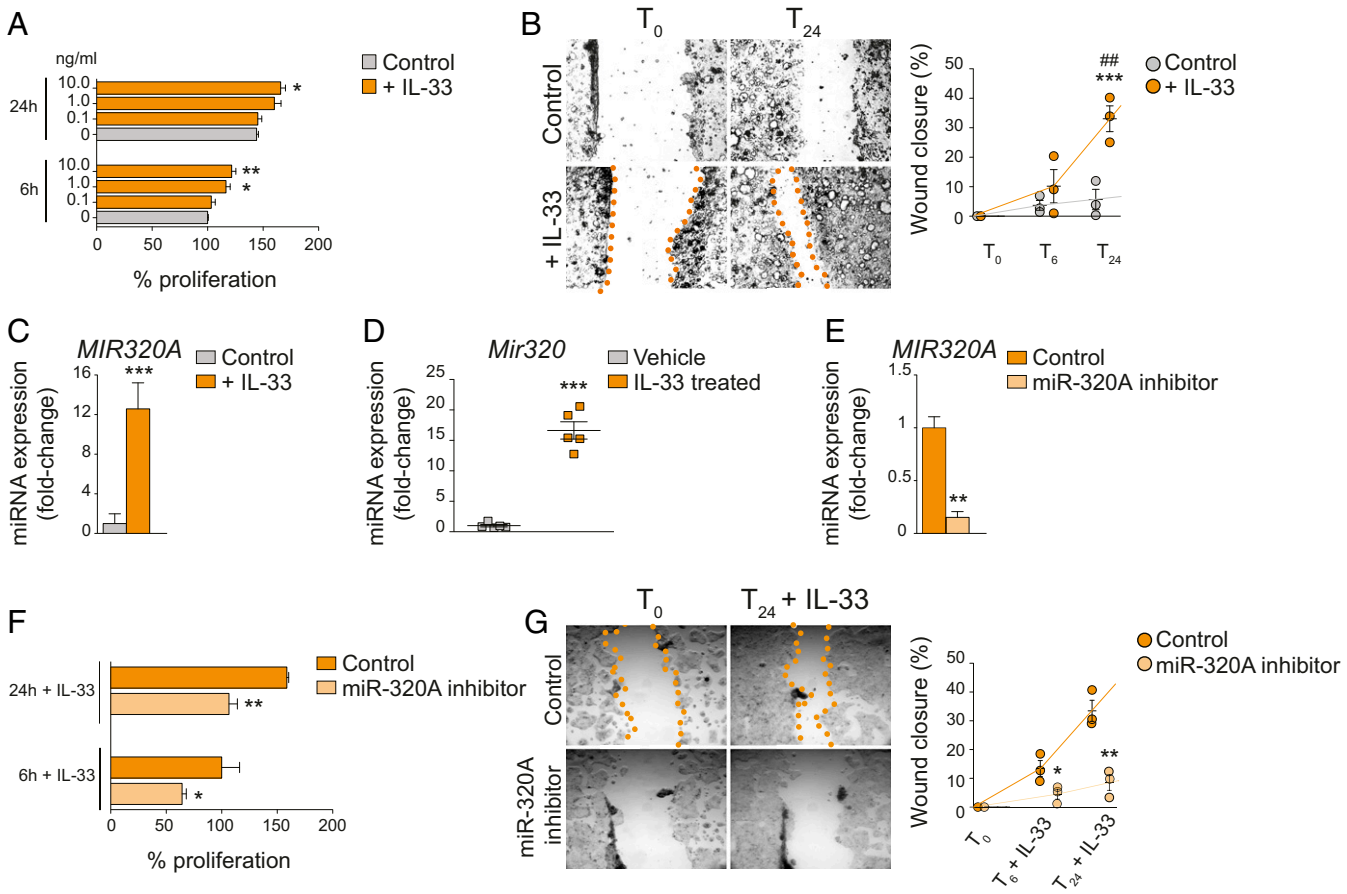

Fig. 5. IL-33 promotes epithelial-specific proliferation and wound healing through up-regulation of miR-320. (*A*) IL-33 dose–response on IEC percentage proliferation at 6 and 24 h after IL-33 stimulation; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. time-matched (vehicle) control. (*B*) Representative images (*Left*) and quantitation (*Right*) of IEC wound closure at baseline (T_0) and after 6 h (T_6) and 24 h (T_{24}) of IL-33 stimulation; $^{***}P < 0.001$ vs. time-matched control, $^{\#\#}P < 0.01$ vs. baseline. (*C*) *MIR320A* in IECs ± IL-33 IL-33 after miR enrichment. Data are shown as relative fold difference compared with vehicle (control) (arbitrarily set as 1). (*D*) *Mir320* in IECs isolated from in vivo IL-33–treated DSS-induced colitic mice. Data are shown as relative fold difference compared with vehicle-treated DSS-induced colitic mice (arbitrarily set as 1); $n = 5$. (*E–G*) *MIR320A* expression (*E*), percentage IEC proliferation (*F*), representative images (*G*, *Left*), and quantitation (*G*, *Right*) of wound closure in IL-33–stimulated IECs transfected with either a miR-320A–specific inhibitor or scrambled (negative) control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control for all in vitro studies; *n* (number of repeated measures for each condition for single experiment) = 3–4 and are representative of three or four separate experiments (*A–G*).

healing (24). However, an additional observation to note is that during and immediately after DSS challenge, lamina propria enriched with activated innate immune cells is maximally exposed to microbial antigens, since epithelial injury and denudation peak, with loss of ST2L-bearing IECs that then are unable to respond to IL-33 and promote proliferation and repair. This concept is further supported by our Western blot data demonstrating the specific loss of ST2L during DSS challenge. Interestingly, ST2 was also found to be absent or decreased in the epithelium of UC and Crohn's colitis patients but was present and increased within LPMCs during active disease (5), which may explain the skewing toward immune activation (vs. epithelial-induced repair and protection) that promotes chronic inflammation such as found in IBD.

In fact, the apparent discrepancies in dissecting the precise role of IL-33 using variations of DSS colitis, as well as other acute and chronic models of colitis, may simply be explained by the fact that DSS colitis in healthy, immunocompetent mice represents the response of a normal animal to acute intestinal injury, whereas other models possess genetic and immunologic abnormalities that predispose these mice to chronic intestinal inflammation, similar to patients with IBD. Such is the case with the SAMP1/YitFc (hereafter, "SAMP") mouse strain, which possesses multifactorial genetic, immunologic, and regional GI epithelial barrier defects predisposing these mice to spontaneously occurring chronic CD-like ileitis that escalates in severity over time (25). In SAMP mice, IL-33 has deleterious effects overall, inducing eosinophil infiltration and activation of pathogenic Th2 immune responses, resulting in chronic intestinal inflammation that is dependent on the gut microbiome (26). In fact, neutralization of IL-33 with an anti-ST2 antibody in this model was effective in preventing the massive influx of eosinophils into the gut mucosa and decreasing the overall severity of ileal inflammation when used as either a preventive treatment before the onset of inflammation or a therapeutic treatment of established disease. However, although the effect was consistent and significant, disease severity was reduced by only 30% (26), which, while reflecting efficient blockade of inflammation, may also compromise effective IL-33–dependent epithelial restitution/repair and mucosal healing. This concept of the dichotomous roles of IL-33 during chronic intestinal inflammation is consistent with other innate-type cytokines, including several members of the IL-1 family such as IL-1, IL-18, and IL-36, which can possess both protective and proinflammatory functions, depending upon the immunological status of the host and/or the type and phase of the ongoing inflammatory process (3, 27, 28).

In the present study, transcriptional profiling of the Caco-2 human colorectal epithelial cell line revealed that IL-33 stimulation resulted in the up-regulation of several miRs, in particular miR-320A, that have the ability to modify molecular patterns involved in various functions associated with cell proliferation and cell maintenance (*SI Appendix*, Table S2). In general, miRs are small, noncoding RNAs of ∼20–24 nt that posttranscriptionally repress the expression of target genes (29). Although the exact function of miRs in human development and physiology remains largely unknown, differential expression of given miRs during disease progression suggests that miRs have an especially crucial role in human pathologic conditions, but they also have been shown to modulate GI mucosal growth and repair after injury (30, 31). In fact, several GI-specific miRs are highly expressed in the intestinal epithelium and are critical for the maintenance of normal barrier integrity and regulation of tight junction proteins during disease states (30, 31).

Relevant to our results, *MIR320A* was shown to be abnormally expressed in mucosal biopsies from IBD patients, and *MIR320A* was decreased in UC patients as compared with uninflamed, healthy controls (32). These findings were later confirmed in a pediatric population with early-onset IBD, in which *MIR320A* was decreased in inflamed vs. uninflamed colonic biopsies from both UC and CD patients (33). The latter study further showed in vitro evidence that NOD2 served as a target for *MIR320A*, which negatively regulated its expression, specifically in IECs and under inflammatory conditions (33). *NOD2* encodes a cytosolic protein receptor that recognizes the bacterial cell wall product muramyl dipeptide and normally promotes its clearance by activating a proinflammatory cascade and/or by autophagy to maintain normal gut homeostasis. Mutations in *NOD2* were the first and strongest reported genetic associations shown to confer susceptibility to CD (34, 35), suggesting that carriers of such mutations have a dysfunction in handling normal bacterial loads. The aforementioned findings, along with the results from our current study, pose conceptually interesting possibilities regarding the potential role of epithelial-derived miR-320 in the setting of IBD. First, decreased miR-320 can lead to NOD2 overexpression and amplification of aberrant NOD2-induced proinflammatory events, influenced by carriage of *NOD2* mutations. Alternatively, inherent decreased miR-320 may result in the inability to achieve appropriate epithelial repair and restitution, leading to impaired gut mucosal healing. Both processes have the ability to promote uncontrolled immune responses and impair effective resolution of inflammation.

At present, the role of IL-33–dependent regulation of miR-320A during IBD is unknown and is the major focus of ongoing studies in our laboratory at Department of Pathology, Case Western Reserve University (CWRU). Although our current results definitively indicate that in normal, immunocompetent BL6 mice IL-33 induces increased epithelial miR-320 expression that promotes epithelial repair/restitution and confers normal mucosal healing, it has yet to be revealed how this process is defective in inherently colitis-prone mice and/or in patients with IBD. In fact, the molecular processes responsible for the control of miR biogenesis in response to challenge or stress remain largely unknown and are an area of growing investigation. Based on our results and prior studies, it is tempting to speculate that dysfunction of IL-33 as a prototypic alarmin and/or increased epithelial-specific sequestration of IL-33 may negatively regulate or repress miR-320 synthesis, since primary processing of all animal miRs is achieved first in the nucleus, with subsequent, secondary processing occurring within the cytoplasm. As such, the elevated levels of IL-33 in both the nuclear and cytoplasmic compartments of IECs commonly observed in IBD patients (5, 6, 8) would potentially allow increased, and easy, accessibility of IL-33 to a number of different proteins involved in miR processing and specifically in miR-320 processing. An alternative hypothesis to consider is that IL-33 can also function as an intracellular nuclear factor with potent transcriptional-repressor properties (10), and in this setting IL-33 may regulate various proteins that impact miR-320 processing or may affect miR-320 transcription directly. In both scenarios, IL-33–dependent down-regulation of miR-320 could be achieved.

Additionally, we cannot rule out the possibility that intestinal tissue protection may require cooperative action from both hematopoietic and stromal cell lineages that could be initiated by rapid epithelial damage-induced alarmins, such as IL-33. One potential mechanism involves type 2 innate lymphoid cells (ILC2s), which have the ability to produce amphiregulin (AREG), an EGF receptor ligand, in response to IL-33 induced by intestinal injury that consequently mediates epithelial repair and eventual healing (23). This process can be further enhanced by IL-33–dependent differentiation of Tregs and tolerogenic $CD103^+$ dendritic cells, initiated by IEC activation and release of IL-33 and the subsequent production of Th2 cytokines, ultimately leading to intestinal mucosal protection (14–16). As such, IL-33/ST2 may orchestrate an integrated framework involving IECs, intestinal ILC2s, and Tregs through modulation of miR-320A, AREG, and Th2 cytokines, respectively, with the end goal of promoting gut health.

In summary, the results of the present study indicate that, upon challenge, the inherent role of endogenous IL-33 within the gut mucosa is protection, potentially through a mechanism that augments miR-320 expression, inducing epithelial restitution and repair and overall epithelial barrier integrity. In the setting of IBD, particularly during early disease stages, this process may be defective, leading to impaired healing and exacerbation of colitis into a more chronic and sustained inflammatory phenotype. Results from this study also provide the rationale for the potential therapeutic use of either IL-33 or miR-320A to obtain optimal gut mucosal wound healing and to promote the resolution of inflammation.

Materials and Methods

Mice. All experimental mice were bred and maintained under specific pathogen-free conditions in the Animal Resource Center at Case Western Reserve University. For a full description, see *SI Appendix, Materials and Methods*. All procedures performed were approved by the Institutional Animal Care and Use Committee at CWRU and followed the American Association for Laboratory Animal Care guidelines.

Model of DSS-Induced Colitis with Extended Recovery. Induction of colitis was performed on adult C57BL/6J (B6), *Il33*$^{-/-}$, and *St2*$^{-/-}$ mice and their WT (*Il33*$^{+/+}$*St2*$^{+/+}$) littermate controls by 5-d administration of 3% DSS, as previously described (36), but with an extended 2-wk recovery period. *Il33*$^{-/-}$, *St2*$^{-/-}$, and WT mice not exposed to DSS served as untreated controls. In separate experiments, acute colitis was induced in B6 mice, and these mice were injected i.p. with either murine rIL-33 (33 μg/kg) or vehicle (PBS) control during the 2-wk recovery period. For a full description, see *SI Appendix, Materials and Methods*.

Colonoscopy. Macroscopic progression of colitis was assessed by endoscopy of left colon and rectum following a standardized protocol (37). For a full description, see *SI Appendix, Materials and Methods*.

Tissue Harvest and Histologic Assessment of Colitis. When mice were killed, whole colons were taken for analysis and histology. Disease severity was evaluated as previously described (18, 36). For a full description, see *SI Appendix, Materials and Methods*.

Immunohistochemistry. Immunohistochemistry (IHC) was performed as previously described (5) using specific antibodies against murine IL-33 and ST2, and in select experiments IHC was performed for BrdU to detect cell proliferation. For a full description, see *SI Appendix, Materials and Methods*.

ELISA and Western Blot Analysis. IL-33 and ST2 protein levels were quantified by ELISA and evaluated by Western blots to detect specific IL-33 and ST2 isoforms as previously described (5). For a full description, see *SI Appendix, Materials and Methods*.

IEC Isolation. Freshly isolated colons from vehicle- and IL-33–treated DSS colitic mice were harvested and processed as previously described (5, 38). For a full description, see *SI Appendix, Materials and Methods*.

Cell Culture and in Vitro Assays. Caco-2 cells (HTB-37; ATCC) were grown to 80% confluency and were cultured with or without human rIL-33. Cells were collected after 6 and 24 h and were evaluated for cell proliferation using the XTT Cell Proliferation Assay Kit (ATCC) and measuring wound closure. For full description, see *SI Appendix, Materials and Methods*.

Microarray Analysis. Caco-2 cells were cultured with or without human rIL-33, and total RNA was prepared as described below. RNA (100 ng/μL) was submitted to the Case Comprehensive Cancer Center's Gene Expression and Genotyping Core at Case Western Reserve University for microarray analysis. For a full description, see *SI Appendix, Materials and Methods*.

Total RNA Extraction, miR Enrichment, and qPCR. Total RNA extraction and/or miR enrichment were performed on tissue homogenates and IEC preparations, and qPCR was done using specific primers for *Il33*, *Il1rl*, *MIR320A*, and *Mir320*. For a full description, see *SI Appendix, Materials and Methods*.

Inhibition of miR-320A. Single-stranded inhibitor RNA molecules (mirVana inhibitors), designed to specifically target miR-320A, miR-Let7c (positive control), or scrambled sequences (negative control), were transfected into Caco-2 cells. Transfection efficiency was confirmed by qPCR analysis of the miR-Let7c target gene, *HMGA2*, in miR-Let7c–targeted Caco-2 cells. For a full description, see *SI Appendix, Materials and Methods*.

Statistical Analysis. Statistical analyses were performed using one-way ANOVA with Bonferroni correction for multiple comparisons and individual *t* test comparisons adjusted for unequal variance (Welch's correction), as appropriate.

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