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Neutrophils Promote Amphiregulin Production in Intestinal Epithelial Cells through TGF-β and Contribute to Intestinal Homeostasis

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Neutrophils are the first responders to sites of inflammation when the intestinal epithelial barrier is breached and the gut microbiota invade. Despite current efforts in understanding the role of neutrophils in intestinal homeostasis, the complex interactions between neutrophils and intestinal epithelial cells (IECs) is still not well characterized. In this study, we demonstrated that neutrophils enhanced production of amphiregulin (AREG), a member of the EGFR ligand family, by IECs, which promoted IEC barrier function and tissue repair. Depletion of neutrophils resulted in more severe colitis in mice because of decreased AREG production by IECs upon dextran sodium sulfate (DSS) insult. Administration of AREG restored epithelial barrier function and ameliorated colitis. Furthermore, neutrophil-derived TGF-β promoted AREG production by IECs. Mechanistically, TGF-β activated MEK1/2 signaling, and inhibition of MEK1/2 abrogated TGF-β–induced AREG production by IECs. Collectively, these findings reveal that neutrophils play an important role in the maintenance of IEC barrier function and homeostasis. ***The Journal of Immunology*****, 2018, 201: 2492–2501.**

As the largest mucosal surface in the body, the intestinal epithelium serves as a critical relay station between microbiota and mucosal immune cells. Multiple pattern recognition receptors allow intestinal epithelial cells (IECs) to sense microbes and translate the signals to mucosal immunity (1). Meanwhile, the thick layer of mucus and deep invaginated intestinal crypt structure buffer microbial signals to prevent excessive Ag exposure and subsequent overactivation of the immune system (2). The capacity of IECs for rapid self-renewal protects the host from continuous exposure to microbial stimuli and environmental insults (3). In contrast, tissue-resident immune cells closely interact with IECs to support barrier function and regulate luminal microbiota. We have previously reported that Th17 cells upregulate polymeric Ig receptor (pIgR) on IECs as a way to promote intestinal IgA responses to microbiota, thus contributing to the maintenance of intestinal homeostasis (4). Accumulating evidence also shows that innate lymphoid cells (ILCs) are capable of producing IL-22 and amphiregulin (AREG) to exert beneficial effects on IECs (5, 6). Dysfunction of IECs has detrimental effects on the host, resulting in increased bacterial translocation and risk of developing inflammatory diseases, including inflammatory bowel disease (IBD). It has been reported that individuals with altered IEC gene expression are more susceptible to IBD (7). Moreover, it has been shown that patients with IBD have increased intestinal permeability, much of which was attributed to compromised IEC barrier function (8). However, the cells and factors that regulate IEC function are still not well understood.

Emerging evidence has demonstrated that AREG plays an important role in the regulation of intestinal homeostasis (9). As a member of the epidermal growth factor family, AREG is essential in regulating cell differentiation and proliferation. It has been reported that AREG-deficient ($AREG^{-/-}$) mice spontaneously develop gastric tumors (10). In the context of intestinal injury after dextran sodium sulfate (DSS) administration, $AREG^{-/-}$ mice develop more severe colitis compared with wild-type (WT) B6 mice, which suggests a crucial role for AREG in wound healing and tissue repair (5). Additionally, AREG is thought to play a role in type 2–mediated immune resistance and tolerance. Lung-resident Th2 cells and group 2 ILCs produce AREG to promote tissue homeostasis following an infection of influenza virus and nematodes, allowing for increased barrier protection (11, 12). Regulatory T cells (Treg) have also been identified as an important

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The microarray data presented in this article have been submitted to the ArrayExpress database at the European Molecular Biology Laboratory–European Bioinformatics Institute (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6179.

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Abbreviations used in this article: ADAM17, ADAM metallopeptidase domain 17; AREG, amphiregulin; DSS, dextran sodium sulfate; gRNA, guide RNA; HNF4α, hepatocyte NF 4 alpha; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; ILC, innate lymphoid cell; MA, microarray analysis; MSIE, mouse small intestinal epithelium; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; Treg, regulatory T cell; WT, wild-type.

source of AREG during early influenza infection (13), and AREG has been reported to promote the suppressive capacity of Treg during inflammation (14). Among various hematopoietic and nonhematopoietic AREG producers, IECs remain a critical source for both paracrine and autocrine AREG responses. Within the lung, epithelium-derived AREG can enhance barrier defense against pathogens (15). Despite these advances, relatively little is known about the factors that regulate IEC production of AREG and the functional significance of IEC-derived AREG in regulating intestinal immune responses.

The association between massive infiltration of neutrophils into the intestines and compromised IEC function found in IBD patients suggests a central role for dysregulated neutrophil–IEC interaction in the pathogenesis of IBD (16). Neutrophils are the most abundant leukocytes in circulation and are pertinent in responding to microbial invasion at epithelial surfaces. At sites of microbial invasion, neutrophils perform several functions to control inflammation, including direct phagocytosis of invading pathogens, formation of neutrophil extracellular traps, and production of matrix metalloproteases, elastase, and other proteolytic enzymes (17). Neutrophils are also potent cytokine producers. We have previously reported that neutrophils protect the intestines from inflammation, and neutrophil-derived IL-22 ameliorated colitis by promoting epithelial integrity (18, 19). In addition, IL-22–producing neutrophils have been shown to enhance IEC antimicrobial peptide production, which aids in barrier defense (20). Moreover, the infiltration of neutrophils can rapidly deplete O_2 levels in the microenvironment via reactive oxygen species production, which also contributes to barrier protection by stabilizing IEC hypoxia-inducible factor expression (21). It has been shown that dysregulated neutrophil transepithelial migration results in altered expression of tight junction proteins in IECs, and neutrophil-derived proinflammatory mediators further affect IEC viability (16, 22). Hence, proper cross-talk between IECs and neutrophils is crucial for maintaining a delicate balance of local immune response and intestinal homeostasis. However, it is still unknown if neutrophils regulate intestinal epithelial function and intestinal homeostasis through regulating IEC AREG expression.

In the current study, we demonstrated that neutrophils induce AREG production from IECs through production of TGF-β. Upon DSS insult, depletion of neutrophils results in more severe colitis because of decreased AREG production by IECs. Administration of AREG to mice after depletion of neutrophils alleviates intestinal injury. Thus, our study demonstrates that neutrophils augment epithelial protection by promoting AREG production by IECs and reveals a critical pathway of neutrophil-mediated tissue protection.

Materials and Methods

Mice

Specific pathogen–free C57BL/6 (B6) mice, which were norovirus and *Helicobacter* free, were obtained from The Jackson Laboratory and bred and maintained in the animal facilities at the University of Texas Medical Branch. Both male and female mice were used. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch.

Reagents

Neutralizing Ab against Ly-6G (1A8) was purchased from Bio X Cell. Recombinant AREG was purchased from Leinco Technologies. Thioglycollate broth and Percoll were purchased from Sigma-Aldrich. Culture medium RPMI 1640, HEPES, penicillin/streptomycin, 2-ME, sodium pyruvate, L-glutamine, and insulin–transferrin–selenium were purchased from Life Technologies. HBSS and DMEM were purchased from Corning. DMEM/F12 medium (12634-010), L-glutamine (25030), and B27 (12587-010) were purchased from Invitrogen. Mouse recombinant cytokines were purchased from BioLegend. Retinoic acid was purchased from Sigma-Aldrich. Matrigel was purchased from BD Biosciences. Recombinant EGF (2028-EG), noggin (1967-NG/CF), R-spondin (3474-RS), Wnt3a (35036-WN/CF), and N2 supplement (AR009) were purchased from R&D Systems. SMARTpool small interfering RNAs (siRNAs) specific for murine MEK1, MEK2, and nontargeting siRNA were purchased from Dharmacon. Inhibitor U0126 and PD98059 were purchased from Promega. Western blot Abs against phosphorylated ERK1/2, β-actin, and anti-rabbit secondary Ab conjugated with HRP were purchased from Cell Signaling Technology.

Neutrophil isolation

Neutrophils were collected from the peritoneal cavity as previously described (18). Briefly, peritoneal cells were collected by lavage with 10 ml PBS supplemented with 5% FBS 5 h after 1 ml of 3% thioglycollate broth i.p. injection. Neutrophils were separated from other cell types by using 50% Percoll. After spinning for 20 min at 260 g, the neutrophil pellet was collected. Neutrophil purity was >90% as tested by flow cytometry after CD11b and Ly-6G staining. Neutrophil supernatant was harvested after 24 h of culture in complete culture medium.

Enteroid culture

After cleaning, small intestines were removed from the euthanized mice, cut into 0.5-cm pieces, and rocked in a Falcon tube with ice-cold PBS for 15 min at 4˚C. The intestinal tissues were treated with 2 mM EDTA for 30 min at 4˚C. The tissues were then transferred into a new tube with 5 ml of cold PBS containing 43.3 mM sucrose and 54.9 mM sorbitol. After shaking for 2 min, the tissues were filtered through a 70-μm cell strainer and rinsed with 5 ml of shaking buffer. Supernatant was collected and centrifuged at 150 × *g* for 10 min at 4˚C. The resulting pellet containing detached crypts was resuspended gently in Matrigel with 0.5 μg/ml recombinant EGF, 1 μg/ml recombinant noggin, 5 μg/ml recombinant R-spondin, and 1 μg/ml recombinant Wnt3a. Next, 50 μl of Matrigel with 500 crypts was plated in each well of the prewarmed (37˚C) 24-well plate. After polymerization of Matrigel for 30 min, 500 μl of prewarmed, advanced DMEM/F12 medium with 2 mM L-glutamine, 1% penicillin/streptomycin, 10 μM HEPES, 1X N2 supplement, and 1X B27 was gently added to each well. Enteroids were used after 5 d of culture, and 250 μl of medium was replaced with neutrophil supernatant for microarray study. For enteroid extraction, the medium was removed and the Matrigel was gently washed with ice-cold PBS. Following washing, 1 ml of cold PBS was added to each well, and the Matrigel was mechanically dissociated using a pipette tip. The dissociated Matrigel was collected and centrifuged for 8 min at 350 × *g*. The resulting enteroid pellet was collected for subsequent studies.

Microarray analysis

RNA expression analysis was conducted using the Affymetrix Mouse Transcriptome Assay 1.0 kit (part number 902919; Affymetrix). Total RNA was isolated using the TRIzol method (Invitrogen); RNA quality and quantity were analyzed by the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), and only RNA with an RNA integrity number >7.0 were included in the microarray analysis (MA). Gene expression analysis was done using Affymetrix GeneChip Mouse Transcriptome Array 1.0, which evaluates the expression of more than 66,100 different genes. Total RNA sample processing, labeling, and hybridization were performed using the Affymetrix GeneChip WT PLUS with the WT Terminal Labeling Kit according to the manufacturer's guidelines (Affymetrix). Scanning and data extraction of the microarray were followed by the transformation of fluorescence data into CEL files employing the Affymetrix GeneChip Command Console software. Microarray expression data were further analyzed using Transcriptome Analysis Console 4.0 Software. Microarray data have been deposited in the ArrayExpress database at the European Molecular Biology Laboratory–European Bioinformatics Institute (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6179.

Epithelial cell culture

Mouse small intestinal epithelium (MSIE) cells, a conditionally immortalized epithelial cell line established from the intestines of WT mice (23), were cultured in RPMI 1640 medium with 5 U/ml murine IFN-γ, insulin–transferrin–selenium (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid), 100 U/ml penicillin/streptomycin, and 5% FBS at 33˚C and 5% CO_2. After reaching 80% confluence, cells were starved in RPMI 1640 medium with 100 U/ml penicillin/streptomycin and 0.5% FBS at 37˚C for 16 h before subsequent experiments. All subsequent experiments were performed at 37˚C. Caco-2 cells were cultured in DMEM supplemented with 10% FBS at 37˚C and 5% CO_2.

siRNA transfection

siRNA transfection of MSIE cells was performed by using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. A total of 2×10^5 MSIE cells were incubated with 30 pmol of siRNA and 3 μl of Lipofectamine RNAiMAX transfection reagent in Opti-MEM medium (1 ml/well) for 6 h, followed by 1 ml of normal medium per well for 24 h at 33˚C and 5% CO_2. MSIE cells were then cultured in medium containing only 0.5% FBS for 16 h at 37˚C and 5% CO_2 before treatment. Transfection efficiency was determined at 24 h post transfection (Supplemental Fig. 3A).

Knockout of MEK1 by using CRISPR

LentiCRISPR vector (plasmid no. 52961; Addgene, Cambridge, MA) established by the Zhang laboratory (24) was used to create knockouts of MEK1. The design and cloning of the target guide RNA (gRNA) sequences were performed as recommended by the Zhang laboratory GeCKO Web site (http://www.genome-engineering.org). Briefly, the suitable target sites for gRNA sequence design against MEK1 were identified using the CRISPR design tool software at http://crispr.mit.edu. Cas9 target sites for the indicated genes were designed in http://www.genome-engineering.org. Then, synthetic gRNA (Integrated DNA Technologies) containing target sites were subcloned into the lentiCRISPR vector. The newly constructed lentiCRISPR plasmids were then transfected into MSIE cells. After antibiotic positive selection, cells were established as a stable cell line. Transfection efficiency was determined at 24 h posttransfection via quantitative real-time PCR (qRT-PCR) (Supplemental Fig. 3C). gRNA oligo sequences for lentiCRISPR are listed in Supplemental Table I.

qRT-PCR

RNA was extracted from homogenized tissue or cultured cells with TRI Reagent (Molecular Research Center) and followed by cDNA synthesis with qScript cDNA Supermix (Quanta BioSciences). qRT-PCR was performed with SYBR Green gene expression assays. Predesigned primers were ordered from Life Technologies and normalized against *Gapdh* mRNA expression. The specific primer sequences are listed in Supplemental Table I.

DSS model of colitis and neutrophil depletion

A total of 1.5–2% DSS w/v (Gojira Fine Chemicals) was dissolved into drinking water and administered ad libitum for 7 d, followed by 3 d of water without DSS. Body weights were monitored daily. For neutrophil depletion, Ly-6G–depleting Ab (1A8) (4 mg/kg) or control Ab were administered via i.p. injection to DSS-treated mice every 3 d. Mice were sacrificed by using carbon dioxide asphyxiation on day 10.

Histopathological assessment

At necropsy, colons were harvested and Swiss rolls prepared. Colons were fixed in 10% buffered formalin and were paraffin embedded. Five-micrometer sections were sliced, stained with H&E, and blindly scored by an experienced pathologist. Histological scoring was performed using a modification of a previously published scoring system (25). Briefly, longitudinal sections were examined for epithelial damage based on hyperplasia, crypt degeneration, and loss; goblet cell loss; crypt exudate; lamina propria and submucosal inflammatory cell accumulation; submucosal edema; mucosal ulceration; and transmural inflammation. Each lesion component was scored for intensity and extent. Intensity was scored a 0, 1, 2, or 3 for absent, mild, moderate, or severe inflammation, whereas extent was scored a 1, 2, 3, or 4, corresponding to 25, 50, 75, or 100% of the total tissue affected. The total lesion severity score was calculated by summing the extent and intensity scores for each individual lesion component.

Isolation of primary IECs

After cleaning, small intestines and large intestines were removed from euthanized mice, cut into small pieces, and rinsed thoroughly with cold PBS. After a 40-min incubation with 5 mM EDTA in HBSS buffer containing 5% FBS at 37˚C, IECs were collected by passing the supernatant through a 100-μm cell strainer (BD Falcon). After washing with PBS, IECs were then underlaid with a solution of 20% Percoll and 40% Percoll. After spinning for 20 min at 830 × *g* and 25˚C, IECs were collected at the 20/40 interface.

Ex vivo colonic tissue cultures

After cleaning, two colonic punctures (2 mm) were obtained from the proximal and distal colon in each mouse. Tissue segments were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin/streptomycin, 10% FBS, 100 mM sodium pyruvate, 10 mM HEPES, and 50 μM 2-ME in a 24-well culture plate for 24 h at 37˚C and 5% CO_2.

Statistics

Statistical significance was calculated with GraphPad (Prism 6.0) using paired or unpaired Student *t* tests and one-way ANOVA for multiple comparisons. The Mann–Whitney *U* test was used for assessing pathology scores. Where appropriate, mean ± SEM is represented on each graph. A p value <0.05 was considered statistically significant and shown as an asterisk (*).

Results

Neutrophils induce AREG expression in IECs

Our previous studies have demonstrated that neutrophils protected the intestines from inflammation, and depletion of functional neutrophils in mice led to increased intestinal permeability upon DSS insult (18). As IECs serve as the first line of defense against gut bacterial invasion, we hypothesized that tissue-infiltrating neutrophils could directly regulate IEC functions as a way to restore intestinal homeostasis. To investigate whether neutrophils regulate IEC functions, we used intestinal epithelial enteroids, a three-dimensional primary culture system for mouse IECs. It is practically hard to isolate neutrophils from intestines because of the short $t_{1/2}$ of neutrophils, as it requires a long processing time, and most neutrophils isolated from intestines would have died by the end of preparation. Thus, we used peritoneal neutrophils, as they are readily isolated in large quantities (18). We first cultured peritoneal neutrophils for 24 h and then collected the supernatants to serve as neutrophil-conditioned medium. Next, we cultured the enteroids in the presence or absence of conditioned medium for 6 h. RNA was extracted for MA using Affymetrix Mouse Gene 1.0 ST microarrays. Using the criteria of >2-fold increase or decrease, we observed 579 genes with significantly altered expression between the two groups of enteroids (Fig. 1A). To better understand the functional interaction of these genes, we analyzed the 579 probes using Cytoscape software. Data analysis revealed that neutrophil-conditioned medium differentially regulated genes that are pertinent for IEC turnover and barrier function. The heat map in Fig. 1A demonstrates the differentially expressed transcripts associated with IEC proliferation (AREG, Anxa1, and Anxa2), differentiation (AREG, Skil, Id1, Cd44, and Nox1), and junction formation (Cldn4, Lamc2, Ace2, and Ace). Compared with the control, enteroids treated with neutrophil-conditioned medium had increased AREG transcripts (Fig. 1A, 1B). We further verified these findings with qRT-PCR (Fig. 1C). Additionally, neutrophil-conditioned medium upregulated AREG transcription in epithelial cells from both the small and large intestines (Fig. 1D) as well as MSIE cells (Fig. 1E), a conditionally immortalized epithelial cell line established from the intestines of normal mice (23). Taken together, these results demonstrated that neutrophils induce IEC production of AREG through production of soluble factors.

AREG protects against DSS-induced colitis

AREG is a growth factor capable of inducing proliferation, differentiation, and maturation of a variety of mesenchymal and epithelial cells (9). Previous studies have also detailed the importance of AREG in the development of intestinal epithelial structure and integrity (10, 26). Therefore, we sought to determine if administration of exogenous recombinant AREG conferred epithelial protection in the DSS colitis model. WT mice were fed DSS in drinking water, and recombinant AREG was administered to one group of the mice every 2 d. Consistent with previous reports (10, 26), after 7 d of DSS exposure followed by 3 d of water without DSS, mice that received exogenous AREG exhibited less

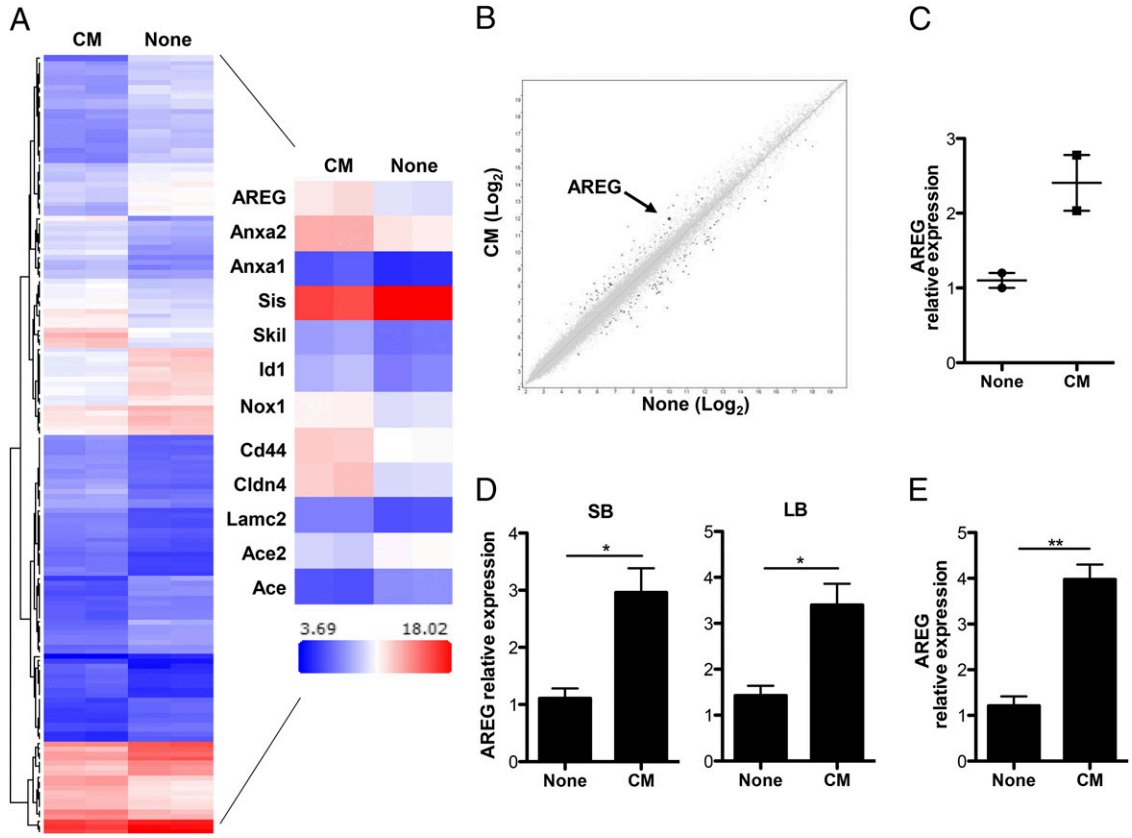

FIGURE 1. Neutrophil-conditioned medium induces AREG expression by IECs. Intestinal enteroids were cultured with or without neutrophil-conditioned medium for 6 h followed by MA. (**A**) Hierarchical clustering of genes that were differentially (|Fold change| >2) expressed between the two groups ($n = 2$). Heat map of some differentially expressed probes between the two groups ($n = 2$). (**B**) Scatterplot displaying the $\log_2$ fold change in expression between the two groups ($n = 2$). (**C**) mRNA expression levels of AREG in the enteroids were measured by qRT-PCR and normalized to GAPDH. (**D**) Primary IECs from either small intestines (SB) or large intestines (LB) treated with or without neutrophil-conditioned medium for 6 h and mRNA expression level of AREG measured by qRT-PCR and normalized to GAPDH. (**E**) mRNA expression level of AREG in the MSIE cells treated with neutrophil-conditioned medium (CM) for 6 h. Data are presented as mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$, Student *t* test. None, medium alone.

severe systemic disease as evidenced by less weight loss compared with mice fed DSS without AREG treatment (Fig. 2A). Consistent with this finding, histopathological assessment showed that AREG treatment protected against the development of severe colitis (Fig. 2B, 2C). Taken together, these data indicated that AREG contributes to the maintenance of intestinal homeostasis.

Neutrophils confer protection against colitis through induction of AREG by IECs

It has been shown that AREG has a potent effect on maintaining epithelial barrier integrity and repair. To determine whether neutrophils protect mice from intestinal inflammation through inducing IEC production of AREG, we treated WT mice with either IgG control or Ly-6G–specific neutralizing Ab to deplete neutrophils (Supplemental Fig. 1). Mice were then fed DSS and treated with or without exogenous AREG. As shown in Fig. 3, neutrophil-depleted mice experienced more weight loss compared with control IgG-treated mice. In contrast, neutrophil-depleted mice that received AREG treatment experienced less weight loss compared with anti–Ly-6G-treated mice without treatment of AREG, experiencing similar weight loss to that of IgG-treated mice (Fig. 3A). Histologically, neutrophil-depleted mice developed more severe colitis compared with IgG-treated mice, characterized by increased infiltrates and higher pathology scores (Fig. 3B, 3C). Interestingly, AREG supplementation resulted in less severe colitis in anti–Ly-6G-treated mice, as evidenced by decreased infiltrates at sites of inflammation and lower pathology scores (Fig. 3B, 3C). Moreover, ex vivo culture of colonic tissues from anti–Ly-6G-treated mice that received AREG showed decreased production of proinflammatory cytokines, including IL-6 and IFN-γ, as compared with anti–Ly-6G-treated mice without treatment of AREG (Fig. 3D), suggesting that AREG can, at least partially, compensate for the loss of neutrophils. Importantly, neutrophil depletion decreased IEC expression of two genes that play a critical role in epithelial integrity, hepatocyte NF 4 alpha (HNF4α) and TJP1, which were partially restored with AREG treatment (Fig. 3E). To investigate whether AREG regulates epithelial barrier permeability, we fed mice FITC–dextran and examined the serum for fluorescence. Higher serum FITC–dextran concentrations were observed in neutrophil-depleted mice as compared with IgG-treated mice, and AREG treatment decreased the serum FITC–dextran concentrations in anti–Ly-6G-treated mice (Fig. 3F). Taken together, these data indicate that neutrophils play a protective role in intestinal inflammation at least partially through AREG.

TGF-β induces AREG production in IECs

To identify the factors that potentially induce IEC AREG production, we treated MSIE cells with different cytokines, including TGF-β1 (hereafter referred to as TGF-β), IL-17, IL-22, IL-10, IL-1β, IL-4, TNF-α, IFN-γ, and IL-6 for 6 h (19, 27–31). We also treated MSIE cells with retinoic acid, LPS, and IL-33, which

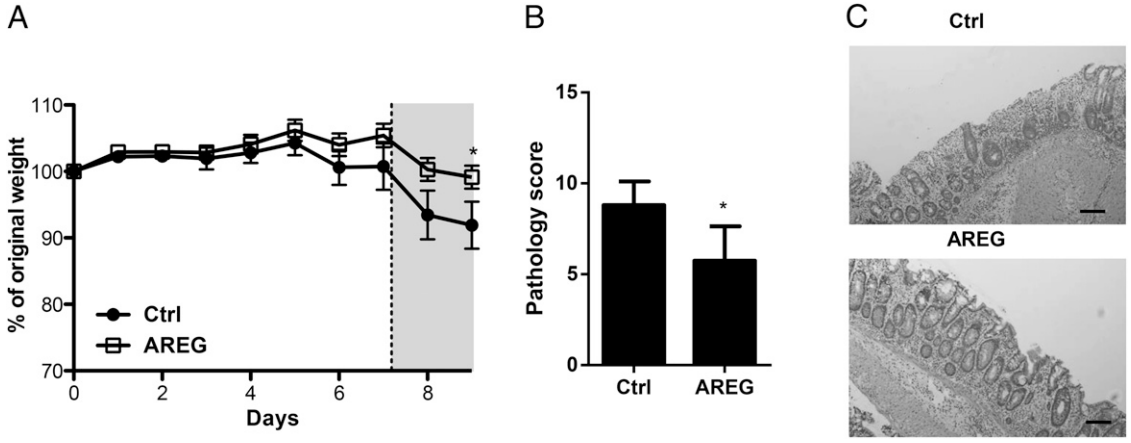

FIGURE 2. AREG protects the intestinal epithelium from DSS-induced injury. WT mice were administered 2% DSS in drinking water for 7 d, followed by 3 d of water. One group of mice received 15 μg of AREG every 2 d. (**A**) Relative body weight change. (**B**) Blinded histopathological scoring. (**C**) Representative images of H&E-stained colon sections. Scale bars, 100 μm. Data are representative of three independent experiments ($n = 4$ per group per experiment). $^*p < 0.05$, Student t test.

were previously reported as capable of inducing AREG expression in different cells (5, 32, 33). Among all the candidates, only TGF-β induced AREG expression in MSIE cells (Fig. 4A). Consistently, we observed increased AREG expression in primary IECs treated with TGF-β but no other cytokines tested (Supplemental Fig. 2A), indicating that TGF-β is an inducer of AREG in IECs. Next, we performed ELISA analysis and confirmed the presence of TGF-β in neutrophil-conditioned medium (Fig. 4B). To determine the kinetics of TGF-β induction of AREG in IEC, we treated the MSIE cells with TGF-β and measured AREG expression with respect to time over the course of 48 h. The increase in AREG expression started as early as 3 h posttreatment with TGF-β and peaked at 24 h before declining (Fig. 4C). To determine whether TGF-β mediated neutrophil-conditioned medium induction of AREG by IECs, we pretreated neutrophil-conditioned medium with anti–TGF-β Ab or control Ab to neutralize TGF-β before applying the medium to cultures of MSIE cells. Addition of anti–TGF-β Ab abrogated the effect of neutrophil-conditioned medium in the induction of AREG by MSIE cells at both RNA and protein levels (Fig. 4D, 4E). These results were replicated using primary IECs from both the small and large intestines, suggesting that neutrophil-derived TGF-β contributes to the AREG production by IECs (Fig. 5F).

Neutrophils produce TGF-β, which regulates IEC production of AREG in the setting of inflammation

To determine if neutrophils produce TGF-β, which possibly regulates IEC production of AREG in the setting of inflammation, neutrophils were isolated to measure the expression of TGF-β. Because neutrophils are extremely fragile and it is almost practically impossible to isolate purified neutrophils from intestinal lamina propria, as almost all neutrophils are dead after long process time (normally over 12 h), we isolated neutrophils from the bone marrow, peritoneal cavity, and spleen of mice and measured TGF-β expression by qRT-PCR. As it has been well established that Treg produce high levels of TGF-β (34), we also generated Treg from $CD4^+$ T cells to serve as positive controls. As shown in Fig. 5A, splenic neutrophils from DSS-fed mice expressed TGF-β at a similar level to peritoneal neutrophils and Treg, which is much greater than splenic neutrophils from healthy WT mice. Moreover, ex vivo colonic organ cultures showed higher TGF-β levels in IgG-treated mice after DSS administration, which was decreased in neutrophil-depleted mice (Fig. 5B). Interestingly, lower levels of soluble AREG were detected in the supernatants of ex vivo colon cultures in neutrophil-depleted mice as compared with control mice (Fig. 5C). Consistently, primary IECs isolated from neutrophil-depleted mice showed decreased expression of AREG at both RNA and protein levels (Fig. 5D, 5E). It has been shown that AREG is first expressed as a transmembrane precursor in the cells (35). Shedding of AREG requires ADAM metallopeptidase domain 17 (ADAM17), a TNF-α–converting enzyme found on IECs (36). We found that depletion of neutrophils resulted in decreased ADAM17 in the IECs of mice upon DSS insult (Fig. 5F). Furthermore, we cultured enteroids in the presence or absence of neutrophil-conditioned medium for 6 h, and ADAM17 expression was increased with neutrophil-conditioned medium as compared with controls. Together, these data indicated that neutrophils induce IEC expression of AREG and ADAM17, which sheds AREG, thus promoting AREG production by IECs. It also suggests a role for TGF-β–producing neutrophils in an AREG-dependent protective pathway in the intestines.

TGF-β induction of AREG in IECs is MEK1/2 dependent

Last, we investigated the mechanisms underlying TGF-β–induced AREG production by IECs. Previous studies have suggested a positive feedback loop in TGF-β signaling through upregulating the expression of TGF-β receptors in different cell types (37, 38). We first set out to assess whether the phenomenon was consistent in IECs. However, we observed no significant change in the expression of either TGF-β type I receptor (TGF-β–RI) or TGF-β type II receptor (TGF-β–RII) in MSIE cells with TGF-β treatment (data not shown). In addition to activating the cascade of SMADs, it is known that the activation of the TGF-βR complex can also lead to the subsequent activation of MAPK pathways (39). We then sought to determine if MEK–ERK signaling regulates AREG production in IECs. Western blot analysis revealed that TGF-β activated ERK1/2 in MSIE cells, as evidenced by increased phosphorylation of ERK1/2 after treatment with TGF-β (Fig. 6A). To determine if activation of ERK mediates TGF-β induction of AREG in IECs, we used two selective inhibitors of the ERK-activating enzyme MEK, PD98059 and U0126, which are small molecules capable of readily crossing the cell membrane to block ERK activation. We showed that addition of PD98059 and U0126 greatly decreased AREG expression induced by TGF-β (Fig. 6B). To further confirm these results, we used MEK siRNA, which specifically inhibits the expression of MEK. Transfection of MSIE

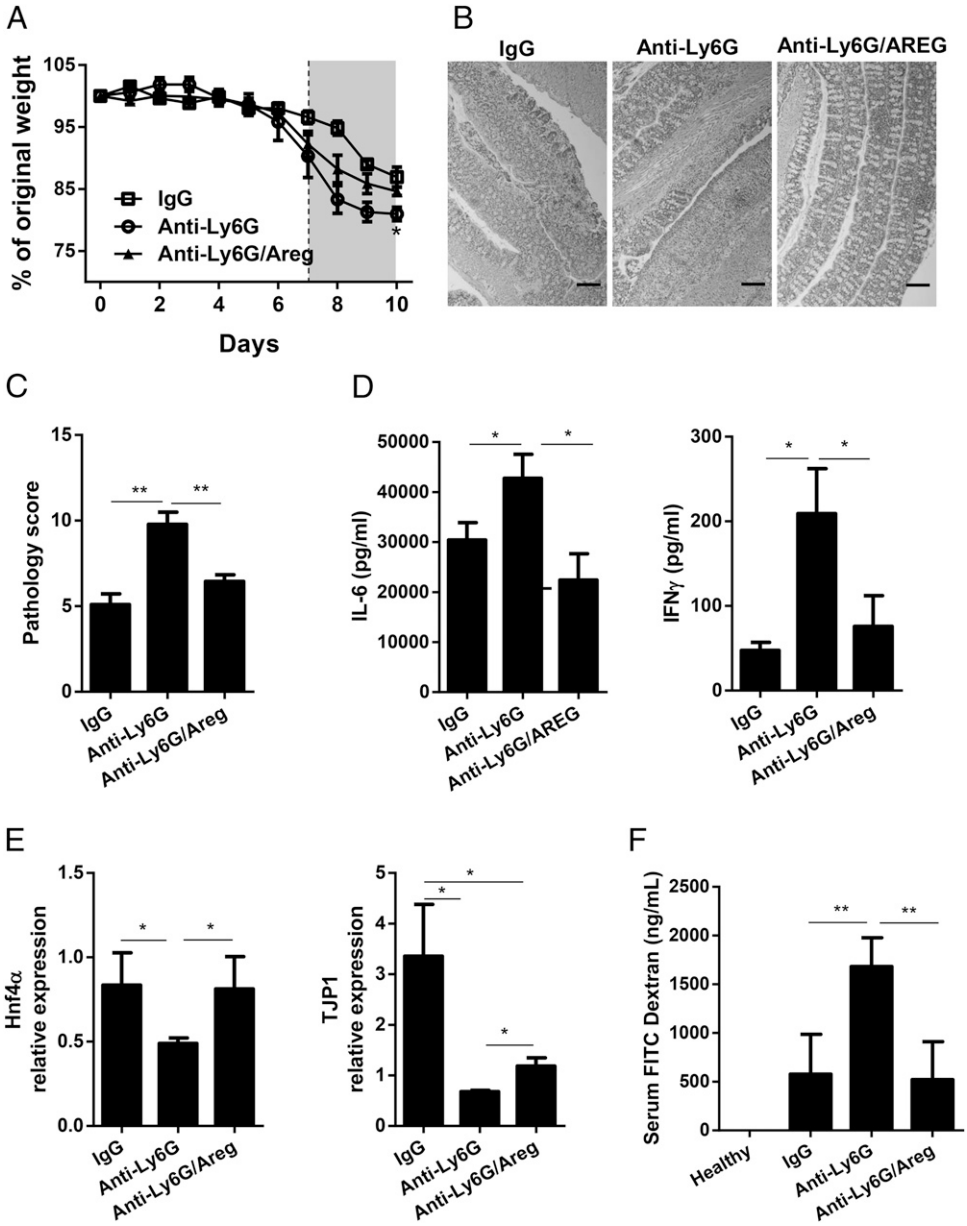

FIGURE 3. Neutrophils confer protection against colitis through induction of AREG by IECs. WT mice were administered 2% DSS in drinking water for 7 d, followed by 3 d of water. The mice were given Ly-6G–depleting Ab i.p. every 3 d. One group of mice received AREG every 2 d and the other group received PBS. One group of mice treated with DSS were administrated with IgG to serve as control. (**A**) Relative change in body weight. (**B**) Representative images of H&E-stained colon sections. Scale bar, 100 μm. (**C**) Blinded histopathological scoring. (**D**) Protein level of inflammatory cytokines in colonic culture supernatant. (**E**) mRNA expression level of HNF4α and TJP1 in isolated IECs. (**F**) FITC–dextran level in plasma was determined. Data are representative of three independent experiments ($n = 4$ per group). $^*p < 0.05$, $^{**}p < 0.01$, unpaired Student t test, one-way ANOVA, nonparametric Mann–Whitney U test.

cells with MEK1 or MEK2 siRNA decreased expression of ERK-activating enzymes (Supplemental Fig. 3A). Additionally, transfection with either MEK1 or MEK2 siRNA decreased AREG expression in TGF-β–treated MSIE cells compared with nontargeting controls (Fig. 6C). Furthermore, CRISPR knockout of MEK1 caused a marked decrease in AREG production induced by TGF-β in MSIE cells as compared with WT controls (Supplemental Fig. 3B). Taken together, these data indicated that MEK1/2 is required for TGF-β induction of AREG production by IECs.

Discussion

Tightly regulated intestinal barrier function is crucial in the maintenance of intestinal homeostasis. In this report, we demonstrate that neutrophils protect the intestines from inflammation, at least partially, through the induction of AREG by IECs. Administration of exogenous AREG mitigated tissue damage following depletion of neutrophils upon DSS challenge by restoring epithelial barrier function. Our findings thus reveal a previously unappreciated immune interaction between neutrophils and IECs in maintenance of intestinal homeostasis.

Despite many advances made in studying the interaction between epithelial cells and immune cells, the interplay between IECs and neutrophils remains poorly defined. We previously reported that IL-22–producing neutrophils enhance intestinal barrier integrity in the context of colitis, suggesting that neutrophil–IEC cross-talk plays a critical role during the inflammatory state (18). To further delineate the mechanisms underlying neutrophil regulation of IECs, we used intestinal epithelial enteroids,

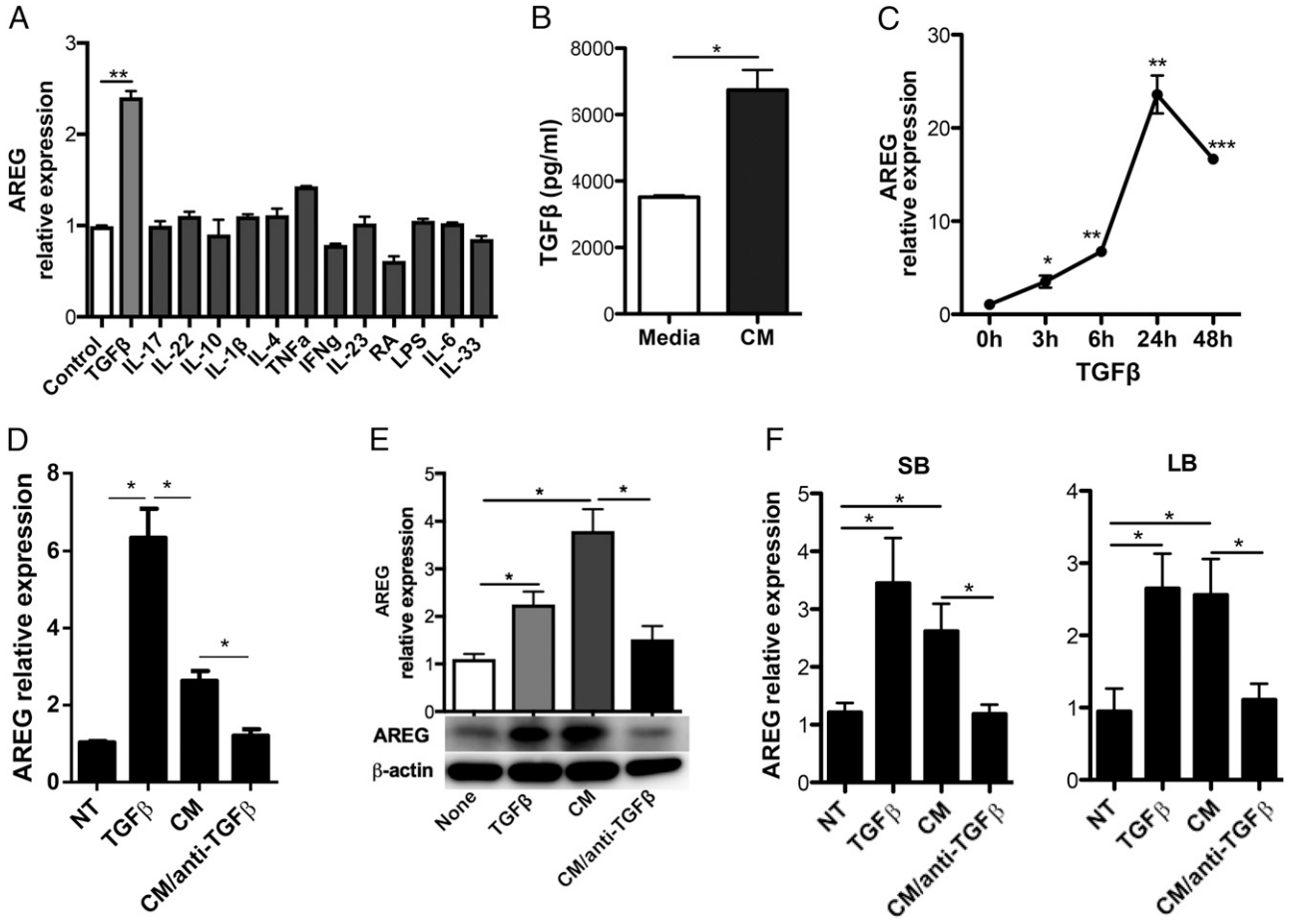

FIGURE 4. TGF-β induces AREG production in IECs. (**A**) MSIE cells were treated with different cytokines/factors for 6 h, and mRNA expression level of AREG was measured by qRT-PCR and normalized against GAPDH. (**B**) Level of TGF-β was determined in neutrophil-conditioned medium versus medium control. (**C**) mRNA expression level of AREG in the MSIE cells treated with 20 μg/ml TGF-β over time, determined by qRT-PCR and normalized against GAPDH. (**D** and **E**) Neutrophil-conditioned medium was pretreated with 10 μg/ml anti-TGF-β Ab and then added into MSIE cell cultures. (D) mRNA expression level (24 h) and (E) protein level of AREG (24 h) were measured by qRT-PCR and Western blot, respectively. (**F**) mRNA expression level of AREG in primary IECs isolated from either SB or LB. Data are presented as mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student *t* test, one-way ANOVA. CM, neutrophil-conditioned medium; None, medium alone.

which recapitulate the diverse composition and complexity of IECs. Analysis of microarray data showed that neutrophil-conditioned medium–treated enteroids were enriched in mRNA responsible for wound healing and junction formation, indicating a role for neutrophils in tissue repair. We also observed an increase in mRNA encoding proteins for IEC proliferation and differentiation; AREG is essential in both processes (40). We confirmed that neutrophil-conditioned medium induced AREG expression by MSIE cells, a mouse small IEC line.

AREG acts as an EGFR ligand and has been shown to be crucial in protection against intestinal inflammation (5, 40). We showed that the increased levels of AREG corresponded with improved disease outcomes in the DSS-induced colitis model. Moreover, additional exogenous AREG administration ameliorated disease and significantly improved the integrity of the epithelial barrier. Given that IBD is rooted in chronic dysregulated immune responses against gut microbiota, any initial contact with the microbiota by the immune system would lead to overwhelming inflammation that perpetuates itself into a chronic diseased state. As such, the key for preventing aberrant immune activation is to possess a strong, intact epithelial barrier to prevent penetration by the microbiota and leave the immune system quiescent. We speculate that deficiency in AREG production leads to a less robust epithelial barrier, thereby conferring susceptibility to IBD. In supporting this notion, a recent report using transcriptomic MA demonstrated an increased intestinal ERRFI1 in patients with either Crohn disease or ulcerative colitis. ERRFI1 mediates inhibition of EGFR, thereby blocking signaling of AREG and other EGF family members (41). Interestingly, IECs themselves also express EGFR (42). As accumulating evidence has shown that AREG enhances epithelial function (5, 40), we thus treated MSIE cells with exogenous AREG to investigate if AREG can further amplify its own production. However, there was no evidence for a change in expression of AREG in IECs (Supplemental Fig. 2B).

Consistent with our previous finding using the T cell–induced colitis model (18), depletion of neutrophils led to more severe inflammation in a DSS colitis model. These data reinforce the idea that innate immune components, especially neutrophils, are indispensable regulators for resolving inflammation. We found that expression of TGF-β was impaired in the intestines of mice receiving neutrophil-depleting treatment. Similar to lung epithelium, we demonstrated that TGF-β induced robust expression of AREG in IECs. However, in comparison with other cell types, including ILCs (5), Treg (13), cholangiocytes (32), epidermal cells (33), or fibroblasts (43, 44), TGF-β remains the only inducer among the cytokines/factors tested capable of eliciting AREG production in IECs. Although the first report of neutrophil production of TGF-β

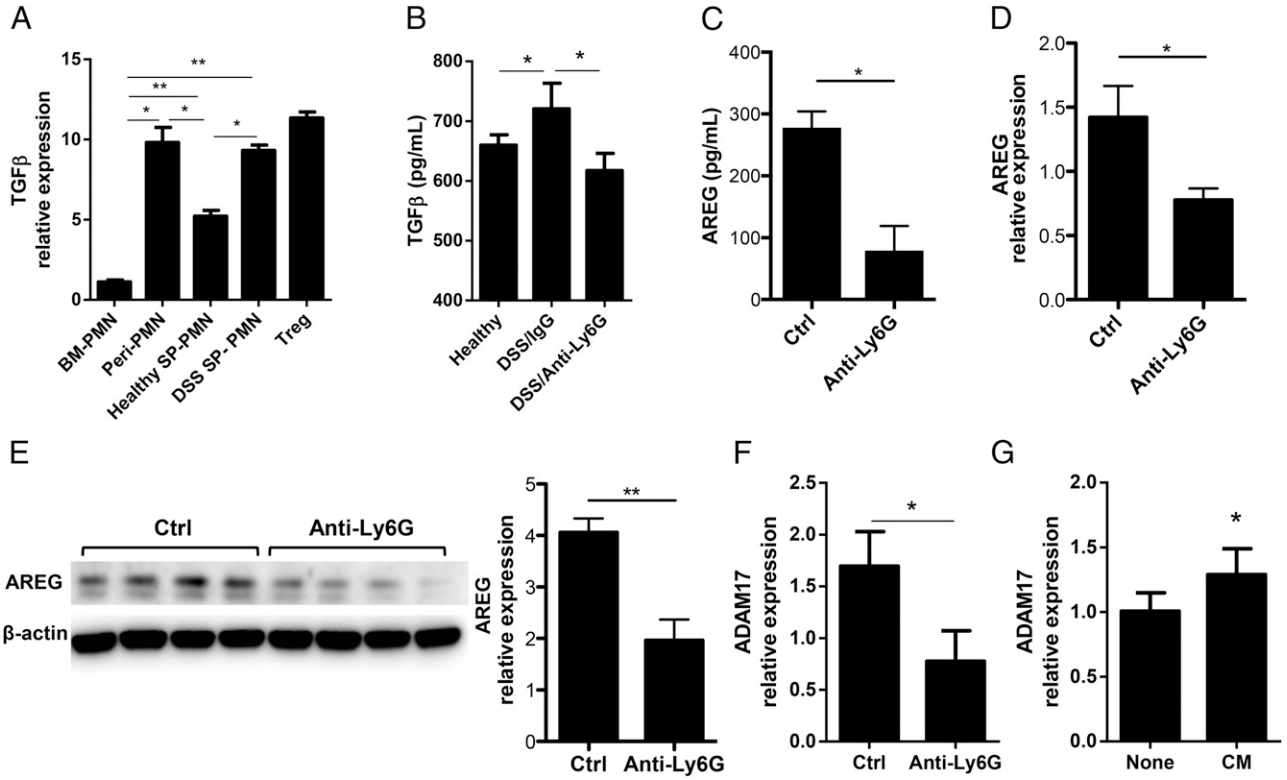

FIGURE 5. Neutrophils produce TGF-β, which regulates IEC production of AREG in the setting of inflammation. (**A**) mRNA expression level of TGF-β in bone marrow, peritoneal cavity, and spleen neutrophils, with Treg as positive control. (**B**) Protein levels of TGF-β in the colonic tissue culture were analyzed by ELISA. (**C**) Soluble AREG was measured in colonic culture supernatant via ELISA. (**D** and **E**) IECs were isolated from the colon of mice, and mRNA expression level (**G**) and protein level (E) of AREG were measured in IECs. (**F**) mRNA expression level of ADAM17 in the colonic IECs of mice. (G) Intestinal enteroids were cultured with or without neutrophil-conditioned medium for 6 h, and mRNA expression levels of ADAM17 in the enteroids were measured by qRT-PCR and normalized to GAPDH. Data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, unpaired Student *t* test, one-way ANOVA, nonparametric Mann–Whitney *U* test. PMN, polymorphonuclear neutrophil.

dates back to 1989 (45), attention has been mainly focused on neutrophil-derived products that facilitate TGF-β activation (46). Our data provide new evidence that neutrophils increase production of TGF-β under an inflammatory state. Correlation between impaired tissue TGF-β levels and decreased IEC production of AREG revealed a previously unappreciated role for neutrophil regulation of barrier function.

As an essential regulator of epithelial integrity, HNF4α has been identified as a susceptibility gene that confers risk of IBD (47). We demonstrated that depletion of neutrophils impaired HNF4α in colonic tissue (18), whereas exogenous AREG treatment rescued HNF4α expression in the colon of neutrophil-depleted mice, further restoring epithelial permeability. Additionally, neutrophil-conditioned medium induced Cldn4 encoding tight junction

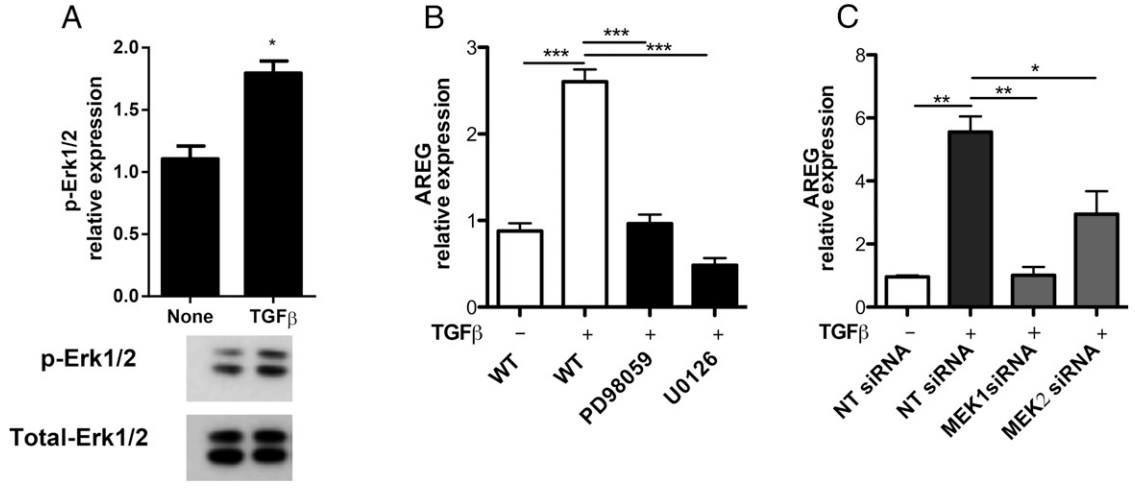

FIGURE 6. TGF-β induction of AREG in IECs is MEK1/2 dependent. MSIE cells were treated with or without TGF-β. (**A**) The phosphorylated ERK1/2 (p-ERK1/2) was determined by Western blot (1 h). (**B** and **C**) MEK1/2 was inhibited or knocked down in MSIE cells in the presence of TGF-β. mRNA expression level of AREG was measured by qRT-PCR in the MSIE cells treated with (B) specific inhibitors or (C) siRNA. Data are presented as mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, one-way ANOVA. None, no treatment; NT siRNA, nontargeting siRNA.

proteins and Lamc2 encoding extracellular matrix glycoproteins in enteroids. As reported, direct contact with IECs prevents neutrophil apoptosis (48). The question of whether direct physical interaction between neutrophils and IECs could also promote IEC barrier function warrants future investigation.

ADAM17, a transmembrane protease found on the surface of neutrophils, regulates shedding of various substrates, including AREG (49). Our finding that neutrophil depletion impaired IECs' expression of ADAM17 indicates a potential protective role for infiltrating neutrophils in the regulation of inflammation through localized AREG induction. Furthermore, neutrophil-facilitated AREG production is not only limited to epithelial origin but other cellular sources as well. Given the chemotactic effect of IECs on neutrophils under mucosal injury (50, 51), it is intriguing to speculate that IECs may recruit and retain neutrophils at the inflammatory site as a way to increase local TGF-β levels and promote AREG secretion during rampant inflammation. Such an occurrence would be consistent with the role of neutrophils in maintaining and/or restoring intestinal homeostasis.

In summary, our studies demonstrated a novel pathway of neutrophil regulation of intestinal inflammation through induction of AREG by IECs, which is dependent on TGF-β. As TGF-β has been considered to be one of the most important anti-inflammatory cytokines, our data provide a novel anti-inflammatory function of TGF-β in the intestines through induction of IEC production of AREG.

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Disclosures

The authors have no financial conflicts of interest.

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