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Activation of the Epidermal Growth Factor Receptor in Macrophages Regulates Cytokine Production and Experimental Colitis

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Macrophages regulate innate immunity to maintain intestinal homeostasis and play pathological roles in intestinal inflammation. Activation of the epidermal growth factor receptor (EGFR) promotes cellular proliferation, differentiation, survival, and wound closure in several cell types. However, the impact of EGFR in macrophages remains unclear. This study was to investigate whether EGFR activation in macrophages regulates cytokine production and intestinal inflammation. We found that EGFR was activated in colonic macrophages in mice with dextran sulfate sodium (DSS)–induced colitis and in patients with ulcerative colitis. DSS-induced acute colitis was ameliorated, and recovery from colitis was promoted in Egfrfl/fl/LysM-Cre mice with myeloid cell–specific deletion of EGFR, compared with LysM-Cre mice. DSS treatment increased IL-10 and TNF levels during the acute phase of colitis, and increased IL-10 but reduced TNF levels during the recovery phase in Egfrfl/fl/LysM-Cre mice. An anti–IL-10 neutralizing Ab abolished these effects of macrophage-specific EGFR deletion on DSS-induced colitis in Egfrfl/fl/LysM-Cre mice. LPS stimulated EGFR activation and inhibition of EGFR kinase activity enhanced LPS-stimulated NF-κB activation in RAW 264.7 macrophages. Furthermore, induction of IL-10 production by EGFR kinase-blocked RAW 264.7 cells, in response to LPS plus IFN-γ, correlated with decreased TNF production. Thus, although selective deletion of EGFR in macrophages leads to increases in both pro- and anti-inflammatory cytokines in response to inflammatory stimuli, the increase in the IL-10 level plays a role in suppressing proinflammatory cytokine production, resulting in protection of mice from intestinal inflammation. These results reveal an integrated response of macrophages regulated by EGFR in intestinal inflammatory disorders. The Journal of Immunology, 2014, 192: 000–000.

The macrophage, as one of the effector cells of the innate immune system, recognizes pathogen-associated molecular patterns from both pathogens and commensal microorganisms through TLRs. Intestinal macrophages, which are continuously exposed to commensal bacteria in the intestinal tract, express low levels of TLRs and other activating receptors, but exhibit high phagocytic efficiency (1). Thus, under physiological conditions, macrophages in the intestinal tract, as compared with macrophages in most other organs, are hyporesponsive to TLR activation, resulting in rapid microbial uptake and killing in the absence of strong inflammatory responses.

Macrophages display protective and detrimental functions in patients with inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease, and in animal models of colitis (2). IBD may be caused by dysregulation of the mucosal immune system, which might be due to excessive immune responses to normal microflora. Such immune responses might be initiated in genetically susceptible hosts by alterations in the composition of intestinal microflora and/or deranged epithelial barrier function (3–5). Innate immunity plays an important role in the onset and severity of IBD (6). Macrophages in the inflamed intestinal mucosa are considered part of the destructive force behind IBD, as a result of their involvement in regulating inflammation by production of inflammatory cytokines and chemokines, such as TNF, IL-6, and IL-8 (7, 8).

Protective effects of macrophages in intestinal inflammation include release of cytoprotective PGs in the peri-cryptal stem cell niche.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; COX-2, cyclooxygenase-2; DSS, dextran sulfate sodium; EGF, epidermal growth factor; EGFR, EGF receptor; IBD, inflammatory bowel disease; MPO, myeloperoxidase; PMN, polymorphonuclear cell; TNBS, 2,6,4-trinitrobenzenesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate; UC, ulcerative colitis; WT, wild-type.
Egf receptor in macrophages regulates colitis

to promote epithelial cell wound healing (9). Another mechanism by which macrophages can play a protective role in inflammation is through production of IL-10. IL-10 is expressed by a variety of cells of the innate and adaptive immune systems, including neutrophils, dendritic cells, and macrophages. It is a suppressive cytokine that inhibits the production of proinflammatory cytokines (10). Macrophages serve not only as an important source of IL-10 but also as a target cell for IL-10. IL-10 can inhibit the production of proinflammatory mediators by macrophages, such as release of IL-6, IL-8, and TNF by these cells in response to endotoxin and IFN-γ stimulation (11, 12). IL-10 also enhances the production of anti-inflammatory mediators such as soluble TNF-Rs (13) and inhibits the capacity of monocytes and macrophages to present Ags to T cells (14). Therefore, production of IL-10 in macrophages may represent an autocrine feedback mechanism to constrain activation of macrophages, thereby influencing the development of adaptive immune responses.

Epidermal growth factor (EGF) is the prototypal ligand for EGF receptor (EGFR). EGF production is submandibular and Brunner’s glands under physiological conditions (15). However, EGF can be synthesized by other cell types under pathological conditions, such as by intestinal epithelial cells in response to injury (16). Unlike exocrine EGF, other EGFR ligands, including TGF-α, heparin-binding EGF, and amphiregulin, are expressed by intestinal epithelial cells and myofibroblasts, suggesting that these ligands act in an autocrine or paracrine manner. EGFR is a type I tyrosine kinase receptor with an extracellular ligand-binding domain and an intracellular portion that contains a tyrosine kinase domain (17, 18). EGFR can be activated by direct ligand binding and can be transactivated by a wide variety of pharmacological and physiological stimuli, including TNF (19) and bacterial products (20) such as ligands of TLR4 activation (21) in intestinal epithelial cells and myofibroblasts, suggesting that these cells (25). However, very little is known regarding the impact of EGFR activation on regulating immune responses in general and in macrophages, specifically.

The purpose of this work was to determine potential roles and mechanisms of EGFR activation in macrophages for regulating cytokine production and experimental colitis. In this study, we report that EGFR is activated in colonic macrophages in mice with dextran sulfate sodium (DSS)–induced colitis and in patients with UC. Selective deletion of Egrf in macrophages leads to an increase in IL-10 levels, which plays a role in suppressing proinflammatory cytokine production and protects mice from colitis.

Materials and Methods

Generation of EgrfLoxP/LysM-Cre mice

All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at Vanderbilt University (Nashville, TN). Mice harboring a floxed allele of EGFR (EgrfLoxP) on a C57BL/6 background were crossed with homozygous LysM-Cre mice on a mixed C57BL/6J and C57BL/6N background (The Jackson Laboratory, Bar Harbor, ME) to generate EgrfLoxP/LysM-Cre mice. LysM-Cre mice express Cre recombinase under the control of the endogenous LysM locus in myeloid cells, including monocytes, mature macrophages, and granulocytes. In EgrfLoxP/LysM-Cre mice, Cre-mediated recombination results in deletion of the Egrf gene in the myeloid cell lineage. Correct excision of the floxed Egrf cassette was confirmed in offspring by PCR-based genotyping. Sequences of PCR primers used for genotyping are available upon request. The EGRF expression level in macrophages was tested using Western blot analysis.

Mice and treatment

Eight- to 10-wk-old wild-type (WT) C57BL/6 (The Jackson Laboratory), LysM-Cre, and EgrfLoxP/LysM-Cre mice were used for this study. All experimental groups contained balanced numbers of age-matched male and female mice.

Mice were treated with 3% DSS (m.w. 36–50 kDa; MP Biomedical, Solon, OH) in drinking water (w/v) for 2, 3, or 4 d. Four-day DSS treatment was followed by a 3- or 7-d recovery period. LysM-Cre and EgrfLoxP/LysM-Cre mice treated with 4-d DSS, followed by 3-d recovery, also received treatment with a neutralizing anti–IL-10 Ab or IgGl1 isotype control Ab (BioLegend, San Diego, CA) via i.p. cavity injection at 50 μg/day for 4 d (days 2 and 4 after DSS treatment and days 1 and 2 of recovery).

Mice were also treated with 100 μl 70 mM 2,6,4-trinitrobenzenesulfonic acid (TNBS) in 50% ethanol intrarectally. Control mice received 100 μl 50% ethanol intrarectally. Mice were sacrificed 4 d after TNBS treatment (20).

Analysis of colitis

Paraffin-embedded tissue sections of Swiss-rolled whole colon were stained with H&E for light-microscopic examination to assess colon injury and inflammation. Samples from the entire colon were examined by a pathologist blinded to Treatment conditions. For assessing DSS-induced colitis, a modified combined scoring system, as previously described (21), including degree of epithelial regeneration (scale of 0–3), distortion/branching (scale of 0–3), inflammation (scale of 0–3) and crypt damage (0–4), percentage of area involved by inflammation (0–4) and crypt damage (0–4), and depth of inflammation (0–3), was applied. The total score is 0 (normal) to 24 (severe colitis).

The scoring system used to assess TNBS-induced colitis was modified from a previous scoring system (26, 27); lamina propria mononuclear cell and polymorphonuclear cell infiltration, enterocyte loss, crypt inflammation, and epithelial hyperplasia were scored from 0 to 3, yielding an additive score between 0 (no colitis) and 15 (maximal colitis).

Colonic cell isolation for immunophenotyping

The colon was weighed, cut into small pieces, and diced in DMEM containing 1 mg/ml dispase, 0.25 mg/ml collagenase A, and 25 U/ml DNase at 37˚C for 20 min with shaking. The suspension was passed through a 70-μm cell strainer (BD Bioscience, San Jose, CA) via i.p. cavity injection at 50 μg/day for 30 min at room temperature. The cells were then fixed in 0.1% paraformaldehyde/PBS at room temperature and processed by multicolor flow cytometry to determine the percentage of positive cells using a BD LSRII system (BD Biosciences). The results were calculated as follows: % of cells × total immune cells/colon weight (gram) = number of cells/gram of colon weight.

Myeloperoxidase assay

Colon tissues were weighed and homogenized in tissue suspension buffer containing 50 mM potassium phosphate (pH 6.0) and 5 mg/ml hexadecyltrimethylammonium bromide (Sigma-Aldrich) at a ratio of 50 mg tissue to 1 ml buffer. Myeloperoxidase (MPO) level was detected using the reaction buffer containing 17% o-dianisidine (Sigma-Aldrich), 5 mM potassium phosphate, and 0.0005% H2O2, as described before (28). Pure MPO (Calbiochem/EMD Biosciences, Darmstadt, Germany) was used to prepare a concentration curve. Tissue suspension buffer was used as negative control. The results were calculated as U/g colon tissue.

Colonic macrophage isolation

The colon was cut and digested with 1 mg/ml dispase (Sigma-Aldrich), 0.25 mg/ml collagenase A (Sigma-Aldrich), and 25 U/ml DNase (Roche Diagnostics, Indianapolis, IN) for 20 min at 37˚C. Cells were harvested by passing the suspension through a 70-μm cell strainer (BD Biosciences, San Diego, CA), followed by centrifugation. Colonic macrophages were isolated from colonic cells using a biotin-conjugated anti-mouse F4/80 Ab (Invitrogen, Carlsbad, CA) and streptavidin-conjugated magnetic beads (BD Biosciences, San Jose, CA), as described before (29).

Isolation of spleen lymphocytes, PBMC, and polymorphonuclear cells

The spleen was mashed, suspended in DMEM, and passed through a 70-μm cell strainer (BD Biosciences, San Diego, CA). The suspended cells were

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centrifuged at 1500 rpm for 3 min. Lymphocytes were isolated using Lympholyte-M (Cedarlane Laboratory, Burlington, NC), according to the manufacturer’s instructions.

PBMC and polymorphonuclear cells (PMN) were isolated from whole mouse blood using Ficoll gradient centrifugation (Sigma-Aldrich). RBC were removed from PMN fraction by lysis of RBC in water.

Colonic epithelial cell isolation

Colonic epithelial cells were isolated using a modified protocol (28). The colon tissues were incubated with 0.5 mM DTT and 3 mM EDTA at room temperature for 1.5 h without shaking. Crypts were released from the colon by vigorous shaking. Epithelial cells were sorted using a biotin-labeled E-cadherin Ab (Invitrogen Life Technologies, Grand Island, NY) and streptavidin magnetic beads (Invitrogen).

Real-time PCR analysis

Total RNA was isolated from homogenized colon tissue and colonic macrophages using a RNA isolation kit (Qiagen, Valencia, CA) and was treated with RNase-free DNase. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit and the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The data were analyzed using Sequence Detection System V1.4.0 software. All primers were purchased from Applied Biosystems: TNF (Mm00443429), IL-10 (Mm00439614), IL-6 (Mm00446190), KC (Mm00433859), and cyclooxygenase-2 (COX-2) (Mm00478374). The relative abundance of GAPDH mRNA was used to normalize levels of the mRNAs of interest. All cDNA samples were analyzed in triplicate.

Macrophage culture and treatment

The mouse RAW 264.7 monocyte/macrophage cell line (ATCC) and primary mouse bone marrow–derived macrophages (BMDMs) isolated from NF-κB reporter mice (HIV long-term repeat/luciferase) and immortalized, as described before (30), were cultured in DMEM containing 10% FBS, 1% glutamine, 100,000 IU/L penicillin, and 100 mg/L streptomycin at 37°C with 5% CO2.

ELISA

RAW 264.7 macrophages were treated with LPS (1 μg/ml; Sigma-Aldrich; catalogue L2939) plus mouse IFN-γ (2.5 ng/ml; PeproTech, Rocky Hill, NJ) in the presence or absence of mouse IL-10 at 2, 120, or 200 μg/ml (PeproTech), neutralizing anti–IL-10 Ab (10 μg/ml) or IgG1 isotype control Ab (10 μg/ml), or AG1478 (300 nM; Calbiochem, San Diego, CA) for 8, 18, 24, or 48 h. Cell culture media were collected to determine the levels of TNF using mouse TNF ELISA Ready-Set-Go kits (eBioscience, San Diego, CA) and IL-10 using mouse IL-10 platinum ELISA kits (eBioscience), according to the manufacturer’s instructions.

Luciferase activity

To measure NF-κB transcriptional activity, BMDMs isolated from NF-κB reporter mice were treated with LPS (200 ng/ml) in the presence or absence of AG1478 (300 nM) for 6 h. Cellular lysates were collected for detecting luciferase activity using a standard assay (Promega, Madison, WI), according to the manufacturer’s instructions. Luciferase activity was expressed as fold change compared with the control group.

Cellular lysate preparation and Western blot analysis

Total cellular lysates were prepared by solubilizing cells using cell lysis buffer, containing 1% Triton X-100, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and protease and phosphatase inhibitor mixture (Sigma-Aldrich). The protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL). The lysates were mixed with Laemmli sample buffer for SDS-PAGE.

Nuclear fractions were prepared as described before (31). Cells were washed with ice-cold PBS and scraped into buffer A (10 mM HEPES [pH 7.5], 10 mM KCl, 2 mM MgCl2, 1% Nonidet P40, and protease inhibitors), passed through a 21-gauge needle, and centrifuged. The pellet (nuclear fraction) was resuspended in buffer B (20 mM HEPES [pH 7.5], 10 mM KCl, 2 mM MgCl2, 50 mM NaCl, and 25% glycerol), centrifuged, and kept on ice for 30 min. After centrifugation, the supernatant was mixed with Laemmli sample buffer for SDS-PAGE.

Western blot analysis was performed using anti-EGFR (Millipore, Billerica, MA), anti-phospho-EGFR (Tyrosine 1068), anti-IκBα, anti–phospho-p38, anti-total p38, anti-phospho stress-activated protein kinase/JNK, anti-total JNK, anti-total ERK1/2 (Cell Signaling Technology), anti–phospho-ERK1/2 (Promega, Madison, WI), anti–β-actin (Sigma-Aldrich), and anti-Ki67 Abs.

Immunohistochemistry

Colon sections were deparaffinized and rehydrated. To detect macrophages, Ag was unmasked using EDTA solution. Mouse sections were stained with a rat anti-mouse F4/80 Ab (Invitrogen), and human sections were stained with a mouse anti-human CD68 Ab (Dako, Carpinteria, CA). Both sections were counterstained with rabbit anti-phospho-EGFR-Tyr1068 Ab (Cell Signaling Technology). For F4/80 and EGFR-Tyr1068 containing, the sections were incubated sequentially with FITC-labeled anti-rabbit IgG (Invitrogen), followed by tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-rat IgG Abs (Sigma-Aldrich). For CD68 and EGFR-Tyr1068 containing, the sections were incubated sequentially with FITC-labeled anti-rabbit IgG, followed by Cy3-labeled anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were then mounted using mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) for nuclear staining. Slides were observed using fluorescence microscopy, FITC, TRITC, and DAPI and FITC, Cy3, and DAPI images were taken from the same field.

For Ki67 staining, Ag retrieval was carried out by using Ag unmasking solution (Vector Laboratories). The sections were then incubated with a rabbit anti-Ki67 mAb (Biocare Medical, Concord, CA) at 4°C overnight, followed by 1-h incubation with a goat anti-rabbit polymer-HRP secondary Ab (Biocare Medical) for 1 h at room temperature. The sections were developed using the ImmPACT dianamobenzidine substrate (Vector Laboratories). Sections were counterstained with hematoxylin and observed using light microscopy.

Human samples

 Archived paraffin-embedded colonic tissue sections of biopsies from patients with UC and healthy controls were accessed from the Human Tissue Acquisition and Pathology Shared Resources at Vanderbilt University. Colon biopsy specimens were obtained from the endoscopically affected colon segment in UC patients and from the distal colon in healthy controls. Samples from patients with moderate to severe UC were obtained at diagnosis, prior to therapy. Only demographic information pertinent to the study design (age at the diagnosis and sex) was collected from patient records (Table I). The study was approved by the Vanderbilt University Institutional Review Board.

Statistical analysis

Statistical significance was determined by one-way ANOVA, followed by Newman–Keuls analysis using Prism 5.0 (GraphPad Software, San Diego, CA) for multiple comparisons and t test for paired samples. A p value <0.05 was defined as statistically significant. Data are presented as mean ± SEM.

Results

Induction of EGFR activation in colonic macrophages in mice with experimental colitis and in patients with UC

EGFR regulates multiple aspects of cell homeostasis, including proliferation, differentiation, migration, and survival in many cell types. However, the impact of EGFR activation on regulating immune responses in general remains unclear. As reported before that EGFR is expressed in macrophages (24, 25), our data showed that mouse peritoneal and colonic macrophages expressed EGFR (Fig. 1A, 1B). However, EGFR expression was not detected in blood PMNs, PBMCs, or splenic lymphocytes (Fig. 1A). Thus, we determined the EGFR activation status in colonic and peritoneal macrophages during intestinal inflammation.

DSS induces colitis in mice by disrupting intestinal epithelial barrier function and activating nonlymphoid cells such as macrophages and PMNs. Increased production of proinflammatory cytokines, including TNF and IL-6, by macrophages and PMN phagocytes directly or indirectly suppresses intestinal mucosal barrier repair (32, 33). We therefore selected the DSS colitis model to investigate the role of EGFR in macrophages in controlling intestinal inflammation. EGFR activation, as evidenced by increased tyrosine phosphorylation, was demonstrated by Western blot analysis of colonic and peritoneal macrophages (Fig. 1B) and by immunostaining of colon tissues (Fig. 1C) prepared from mice treated with DSS for 4 d to induce acute colitis. Analysis of the fold change of relative density showed that EGFR expression levels in peritoneal and colonic macrophages from control mice

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EGF RECEPTOR IN MACROPHAGES REGULATES COLITIS

DSS-treated mice (Fig. 1B), suggesting that EGFR is more activated in colonic macrophages than peritoneal macrophages during intestinal inflammation.

Macrophages have been shown to contribute to the pathology of IBD. Therefore, we assessed the EGFR activation status in macrophages in colonic tissues from patients with UC (Fig. 2A, Table I). Immunostaining was performed to detect EGFR phosphorylation in macrophages expressing CD68 (Fig. 2B). The number of macrophages with activated EGFR in UC patients was significantly higher than those observed in healthy controls (Fig. 2C). These data suggested that EGFR is activated in colonic macrophages from patients with intestinal inflammatory disorders.

Deletion of EGFR in macrophages ameliorates colitis and enhances recovery in mice treated with DSS

To determine the role of EGFR expression by macrophages in immune responses in vivo, we employed Egfrfl/flLysM-Cre mice. Although the LysM locus is expressed in myeloid cells (34), including monocytes, mature macrophages, and granulocytes, we did not detect EGFR expression in blood PMN and PBMC (Fig. 1A). Thus, the contributing factor to the immune phenotype of Egfrfl/flLysM-Cre mice might be from mature macrophages. In Egfrfl/flLysM-Cre mice, the EGFR expression level in colonic and peritoneal macrophages was significantly decreased, compared with that in LysM-Cre mice (Fig. 3A). EGFR expression in colonic epithelial cells was similar between Egfrfl/flLysM-Cre and LysM-Cre mice (Fig. 3A). Thus, Egfrfl/flLysM-Cre mice exhibit a selective deletion of EGFR in macrophages.

To study the effect of EGFR activation in macrophages from mice with DSS-induced colitis, we evaluated colon inflammation and injury in mice treated with DSS for 4 d to induce acute colitis and in mice treated for 4 d with DSS, followed by 3 or 7 d of recovery. DSS-treated LysM-Cre control mice exhibited injury and acute colitis with massive colon ulceration, crypt damage, and severe inflammation during both the acute and recovery phases. These abnormalities were reduced in Egfrfl/flLysM-Cre mice (Fig. 3B). In LysM-Cre mice, the inflammation and injury score in the 4-d DSS treatment group was 9.41 ± 3.79, and scores in the 3- and 7-d recovery groups were 15.19 ± 2.11 and 11.05 ± 3.24,
Colon length was longer in DSS-treated Egrf<sup>fl/fl</sup>LysM-Cre mice, as compared with LysM-Cre mice (Fig. 3D). In addition, the corresponding treatment group in LysM-Cre mice.

Both LysM-Cre and F4/80 and CD11b by flow cytometric analysis, was increased in macrophages into the colon, as detected by double staining of Egrf<sup>fl/fl</sup>LysM-Cre mice treated with DSS for 4 d showed a significant difference in macrophage numbers between these two groups (Supplemental

F0llowing3dof recovery, but there was no difference in macrophage numbers between these two groups (Supplemental

FIGURE 3. Deletion of EGFR in macrophages ameliorates acute colitis and promotes recovery from colitis in DSS-treated mice. To confirm the deletion of EGFR in macrophages (Mac), colonic and peritoneal macrophages and colon epithelial (Epi) cells were isolated from LysM-Cre and Egrf<sup>fl/fl</sup>LysM-Cre mice. Total cellular lysates were prepared from cells for Western blot analysis to detect EGFR expression. Anti-β-actin Ab was used as a loading control (A). The colonic macrophage lane represents the combination of the same number of cells pooled from five mice. Peritoneal macrophage and epithelial cell lanes represent images from five mice. LysM-Cre and Egrf<sup>fl/fl</sup>LysM-Cre mice were treated with 3% DSS in drinking water for 4 d to induce colitis, and then mice were provided with water alone for 3 or 7 d for recovery before sacrifice (DSS4D3DR or DSS4D7DR). Control mice received water alone. Paraffin-embedded colon sections were stained with H&E for light-microscopic assessment of epithelial damage (B). Colon injury/ inflammation scores are shown (C). MPO activity in the colonic tissue was detected (D). The length of the colon was measured when mice were euthanized (E). *p < 0.05 compared with the water group of LysM-Cre mice, †p < 0.05 compared with the corresponding treatment group in LysM-Cre mice.

Egfr<sup>fl/fl</sup>LysM-Cre mice were treated with 3% DSS in drinking water for 3 or 7 d for recovery before sacrifice (DSS4D3DR or DSS4D7DR). There were no significant body weight changes found in mice treated with DSS for 4 d and following 3 and 7 d of recovery.

We also sought to determine the role of EGFR in the TNBS-induced colitis model. Mice were administered TNBS intrarectally and euthanized after 4 d of TNBS treatment. TNBS-induced colitis in Egrf<sup>fl/fl</sup>LysM-Cre mice was attenuated as compared with LysM-Cre mice (Supplemental Fig. 2).

These results suggested that deletion of EGFR in macrophages ameliorates colitis induced by DSS and TNBS and enhances recovery in mice treated with DSS.

Deletion of EGFR in macrophages differentially regulates IL-10 and TNF production during DSS-induced colitis

Increased proinflammatory cytokine production by macrophages is a hallmark pathological factor in IBD (7, 8) and experimental colitis (32, 35). Activated macrophages in colitis also produce the anti-inflammatory cytokine IL-10 to limit colitis development (10). Therefore, we tested the effects of EGFR deletion in macrophages on pro- and anti-inflammatory cytokine production during DSS-induced colitis. We employed real-time PCR analysis to detect mRNA levels in the colon tissue and colonic macrophages. Our results showed that IL-10 levels in the colonic tissue of DSS-treated Egrf<sup>fl/fl</sup>LysM-Cre mice were significantly increased as compared with untreated Egrf<sup>fl/fl</sup>LysM-Cre and LysM-Cre mice during both the acute (at 2, 3, and 4 d after DSS treatment) and recovery phases (at 3 and 7 d of recovery) of colitis. However, such changes in IL-10 production were not observed in LysM-Cre mice (Fig. 4A). TNF levels in colon tissue of DSS-treated Egrf<sup>fl/fl</sup>LysM-Cre mice compared with LysM-Cre mice were increased during acute colitis (at 2 and 3 d after DSS treatment), but decreased at 4 d after DSS treatment and during the recovery phase.
Levels of IL-6 and KC, which are macrophage-derived proinflammatory mediators, were persistently higher in the colonic tissues of LysM-Cre mice, compared with Egfr<sup>fl/fl</sup>LysM-Cre mice (Fig. 4A). There was no difference in the production of these cytokines in the water treatment group of Egfr<sup>fl/fl</sup>LysM-Cre mice, compared with LysM-Cre mice (Fig. 4A). We also tested the cytokine levels produced by colonic macrophages isolated from mice following 4 d of DSS treatment and following a 3-d recovery phase. Consistent with the cytokine profile in colonic tissues, macrophages from Egfr<sup>fl/fl</sup>LysM-Cre mice had increased IL-10 levels, but reduced TNF, IL-6, and KC levels, as compared with macrophages from LysM-Cre mice (Fig. 4B). Thus, decreased colitis in Egfr<sup>fl/fl</sup>LysM-Cre mice was associated with increased IL-10 and decreased proinflammatory cytokine and chemokine levels in these animals.

**IL-10 plays a critical role in suppressing proinflammatory cytokines and colitis in Egfr<sup>fl/fl</sup>LysM-Cre mice**

In DSS-treated Egfr<sup>fl/fl</sup>LysM-Cre mice, we observed a persistent increase in IL-10 production throughout the disease process, and increased TNF production in the early stage of acute colitis, but decreased TNF production during the late stage of acute colitis and the recovery phase (Fig. 4). Because IL-10 has been shown to inhibit proinflammatory cytokine production in macrophages (11, 12), we examined whether the persistent increase in IL-10 production played a role in the development of and recovery from colitis in Egfr<sup>fl/fl</sup>LysM-Cre mice. An IL-10 neutralization was used to test the effects of inhibition of IL-10 on recovery of DSS-induced colitis in LysM-Cre and Egfr<sup>fl/fl</sup>LysM-Cre mice. In Egfr<sup>fl/fl</sup>LysM-Cre mice, as compared with control IgG, treatment with anti–IL-10 neutralizing Ab abolished the effects of macrophage-specific EGFR deficiency on enhanced recovery from colitis, with an inflammation and injury score of 10.60 ± 2.30 in IgG-treated mice and 13.6 ± 1.14 in anti–IL-10–treated mice, respectively (p < 0.05; Fig. 5A, 5B). As shown in our previous study (20) and in Fig. 4 that there was no increase in the IL-10 level in DSS-treated WT mice, the treatment with anti–IL-10 neutralizing Ab did not affect colitis in LysM-Cre mice (13.60 ± 1.85 in IgG-treated mice and 14.4 ± 1.36 in anti–IL-10–treated mice; Fig. 5A, 5B). Importantly, the inflammation/injury scores in DSS- and anti–IL-10 Ab-treated Egfr<sup>fl/fl</sup>LysM-Cre mice were similar (p > 0.05) to those observed in LysM-Cre mice treated with DSS and anti–IL-10 Ab or control IgG (Fig. 5A, 5B). These results indicate that increased production of the anti-inflammatory cytokine IL-10 by EGFR-deficient macrophages plays an important role in amelioration of DSS-induced colitis.

Furthermore, in Egfr<sup>fl/fl</sup>LysM-Cre mice, IL-10 mRNA levels in mice treated with anti–IL-10 Ab were similar to those of mice treated with isotype control, whereas TNF, IL-6, and KC mRNA levels were significantly increased in mice treated with the anti–IL-10 Ab (Fig. 5C). These data suggest that increased production of the anti-inflammatory cytokine IL-10 by EGFR-deficient macrophages plays an important role in downregulating proinflammatory cytokine production, and thus amelioration of DSS-induced colitis.

**Increased regenerative responses in Egfr<sup>fl/fl</sup>LysM-Cre mice**

It has been reported that macrophages, but not neutrophils or lymphocytes, are necessary for the regenerative response of colonic epithelial progenitors during DSS-induced colitis. Furthermore, macrophages in the peri-cryptal stem cell niche express genes such as COX-2 that are associated with promotion of epithelial cell proliferation (9). Thus, we tested whether EGFR activation in macrophages affects COX-2 expression, and we determined the role of COX-2 in colon epithelial cell proliferation in mice during recovery from DSS-induced colitis. We detected colon epithelial cell proliferation by Ki67 staining in colon tissues isolated from DSS-treated mice following a recovery period of 3 or 7 d. In both groups of animals, colon epithelial cell proliferation rates were higher in Egfr<sup>fl/fl</sup>LysM-Cre mice compared with LysM-Cre mice (Fig. 6A, 6B).

We examined COX-2 mRNA levels in colonic macrophages isolated from mice after 4 d of DSS treatment, followed by a 3-d recovery period. There was no difference between levels of COX-2 expression in colonic macrophages from untreated Egfr<sup>fl/fl</sup>LysM-Cre mice compared with LysM-Cre mice. We were unable to detect significant changes in COX-2 mRNA levels in colonic macrophages isolated from LysM-Cre mice upon DSS treatment. However, the COX-2 mRNA level was significantly increased in colonic macrophages isolated from DSS-treated Egfr<sup>fl/fl</sup>LysM-Cre mice, as compared with the water-treated group (Fig. 6C).

In colonic tissues isolated from LysM-Cre mice, COX-2 mRNA expression levels were increased in the 3-d recovery group, but no significant changes were observed in the 7-d recovery group.
FIGURE 5. IL-10 mediates amelioration of DSS-induced colitis in Egfr<sup>fl/fl</sup>LysM-Cre mice. LysM-Cre and Egfr<sup>fl/fl</sup>LysM-Cre mice were treated with 3% DSS in drinking water for 4 d to induce colitis, and then mice were provided with water for 3 d for recovery before sacrifice. Control mice received water alone. Mice were also treated with anti–IL-10 Ab or IgG1 isotype control Ab via i.p. injection at 50 mg/day for 4 d (days 2 and 4 after DSS treatment and days 1 and 2 of recovery). Paraffin-embedded colon sections were stained with H&E for light-microscopic assessment of epithelial damage (A). Colon injury/inflammation scores are shown (B). mRNA was isolated from colon tissues for real-time PCR analysis of the indicated cytokine mRNAs (C). The cytokine mRNA expression level in the water group of LysM-Cre mice was set as 100%, and mRNA expression levels in treated mice were compared with the water group. In (C), *<i>p</i> < 0.05 compared with the water group of LysM-Cre mice, *<i>p</i> < 0.05 compared with the control IgG treatment group of the corresponding mouse genotypes, and $<i>p</i> < 0.05 compared with the corresponding treatment group in LysM-Cre mice. n = 5 mice for each treatment group.

Inhibition of EGFR kinase activity in macrophages regulates LPS- and IFN-γ-stimulated cytokine production in vitro

It is well known that macrophages are classically activated by exposure to two signals (36). IFN-γ primes macrophages for activation, but does not in itself substantially activate macrophages. The second signal is TNF or an inducer of TNF. Under physiological conditions, endogenous TNF is produced by TLR ligation on macrophages. Thus, classically activated macrophages develop in response to IFN-γ and exposure to a microbe or microbial product such as LPS. Inflammatory mediators induced by LPS plus IFN-γ in monocytes/macrophages play important roles in sepsis (37) and inflammatory conditions such as colitis (7, 8). Thus, we determined the role of EGFR in regulating cytokine production in response to LPS plus IFN-γ stimulation in the mouse macrophage cell line RAW 264.7. We used AG1478, an EGFR kinase inhibitor, to inactivate EGFR in macrophages. IL-10 production was up-regulated in macrophages under both conditions, with or without EGFR kinase inhibition, upon LPS/IFN-γ treatment for 8–48 h, with a peak at 24 h of treatment. However, in macrophages with EGFR inhibition, the levels of IL-10 production induced by LPS/IFN-γ treatment for 8–24 h were higher than those in macrophages with EGFR activity. However, there was no difference in IL-10 production at the 48-h time point of LPS/IFN-γ treatment in macrophages with intact EGFR kinase activity, compared with macrophages undergoing EGFR inhibition (Fig. 7).

LPS/IFN-γ stimulated TNF production at 8, 18, and 24 h of treatment, but had no effect on TNF production at 48 h of treatment in RAW 264.7 macrophages in the presence or absence of EGFR kinase inhibition. Furthermore, compared with the TNF level in RAW 264.7 macrophages with intact EGFR kinase activity, inhibition of EGFR kinase activity decreased TNF production in macrophages at 8 and 18 h, but increased TNF production at 24 h of LPS/IFN-γ treatment (Fig. 7).

These kinetic data suggest that inactivation of EGFR in macrophages enhances IL-10 production at the early stage (8–24 h) but not at the later stage (48 h) of LPS/IFN-γ treatment. IL-10 plays a role in inhibiting the production of proinflammatory cytokines in response to endotoxin or IFN-γ stimulation (11, 12), suggesting that this transient increase in IL-10 production may lead to suppression of TNF production by inactivation of EGFR at the early stage of LPS/IFN-γ treatment (8–18 h). These data are consistent with our in vivo result that the TNF levels decrease as IL-10 production increases in DSS-treated Egfr<sup>fl/fl</sup>LysM-Cre mice (Fig. 4).

Inhibition of EGFR kinase activity in macrophages enhances LPS-stimulated NF-κB activation

Cytokine gene expression is regulated by a balance between positive and negative signal transduction pathways. LPS engages TLR4 in macrophages and triggers downstream signaling through MyD88-dependent and -independent pathways, involving the activation of NF-κB and the p38, JNK, and ERK1/2 members of the MAPK family. However, mechanisms and signaling pathways that limit the magnitude of induction of these genes are poorly understood. We found that LPS stimulation induced EGFR activation in RAW 264.7 cells (Fig. 8A).
Next, we tested the role of EGFR activation in regulating LPS-induced signaling pathways in macrophages. We detected the effects of EGFR inhibition on IκBα degradation, which leads to NF-κB activation, in RAW 264.7 macrophages treated with LPS for up to 6 h. IκBα degradation occurred at 15 min of LPS treatment, and IκBα levels returned to the untreated levels at 60 min in RAW 264.7 macrophages with EGFR activity. Under conditions of EGFR inhibition, IκBα degradation also occurred at 15 min of LPS treatment, but IκBα levels returned to untreated levels at 180 min. Thus, these data suggest that inhibition of EGFR stimulates extended but not persistent NF-κB activation (Fig. 8A, 8B).

To further confirm the effect of EGFR inhibition on increasing LPS-stimulated NF-κB activation, nuclear proteins from RAW264.7 macrophages were collected for Western blot analysis of NF-κB p65 subunit nuclear translocation. EGFR inhibition promoted LPS-stimulated NF-κB p65 nuclear translocation (Fig. 8C). In addition, Luciferase activity assays were performed to measure NF-κB transcriptional activity using BMDMs isolated from NF-κB reporter mice. LPS-stimulated NF-κB transcriptional activity was upregulated by EGFR inhibition (Fig. 8D).

Statistical analysis of the fold change of the relative density suggested that EGFR inhibition does not affect LPS activation of p38, ERK1/2, and JNK in RAW 264.7 macrophages (Supplemental Fig. 3). This transient increase of LPS-stimulated NF-κB activation by inhibition of EGFR correlates with the finding that inhibition of EGFR increases IL-10 production at early stage of LPS/IFN-γ treatment, leading to decreased TNF production in RAW 264.7 cells (Fig. 7). Thus, these data suggest that transactivation of EGFR decreases LPS activation of NF-κB, influencing cytokine production upon LPS treatment of macrophages.

**Discussion**

Studies have revealed diverse functions of macrophages in intestinal inflammation, with tissue damage caused by production of proinflammatory cytokines (7, 8), and protective effects mediated by...
factors that are immunosuppressive and/or aid in tissue repair (9, 38). In the current study, we demonstrate that EGFR in macrophages is activated by LPS treatment, in UC patients and during DSS-induced colitis in mice. Therefore, EGFR signaling may be involved in regulating macrophage functions. It has been reported that LPS transactivation of EGFR may involve release of EGFR ligands by intestinal epithelial cells (39) and bronchial epithelial cells (40). It has also been shown that macrophages express EGFR ligands such as EGF, induced in response to CSF-1 (41). Thus, LPS transactivation of EGFR in macrophages may involve release of EGFR ligands by macrophages. Other cell types may contribute to EGFR ligand production during LPS stimulation in vivo as well.

Transactivation of EGFR in intestinal epithelial cells has shown cytoprotective effects (19). Indeed, EGFR in epithelial cells is protective in murine colitis models (23). However, our data suggest that deletion of EGFR in macrophages ameliorates colitis development and promotes recovery, which is associated with decreased levels of TNF and other proinflammatory cytokines during the late stage of colitis and during the recovery phase, due to upregulation of IL-10 production. Thus, the biological function of EGFR in colitis appears to be cell specific.

LPS-activated TLR4 stimulates cytokine production via recruitment of MyD88 and other adapter proteins, leading to activation of the NF-κB pathway and members of the MAPK signaling pathway, including p38, JNK, and ERB1/2 (42). We found that inhibition of EGFR increases the activation level and duration of LPS-stimulated NF-κB activation, which indicates that EGFR activation may suppress LPS-stimulated NF-κB signaling. Furthermore, the PI3K pathway has been reported to limit LPS activation of signaling pathways, including the NF-κB and MAPK pathways, and expression of inflammatory cytokines in human monocytic cells (43). Because PI3K is one of the EGFR targets, further studies are underway in our laboratory to elucidate the role of EGFR-regulated PI3K in the inhibition of LPS-stimulated NF-κB signaling pathways in macrophages. However, it is possible that there are other mechanisms underlying the effects of EGFR activation on TLR signaling. For example, regulation of dsRNA-activated TLR3 by EGFR involves binding of EGFR and Src sequentially to TLR3, and subsequent phosphorylation of the two tyrosine residues in the cytoplasmic domain of TLR3 induces recruitment of adaptor proteins and initiation of antiviral responses (44).
Our results further suggest that transactivation of EGFR decreases LPS activation of NF-κB; thus, EGFR activation has an inhibitory role in the induction of pro (TNF)- and anti-inflammatory (IL-10) cytokines by LPS plus IFN-γ in macrophages. Based on data from Western blot analyses of IkBα degradation and Luciferase activity assays to evaluate NF-κB transcriptional activity, IFN-γ does not affect NF-κB activity in the presence or absence of EGFR kinase activity in RAW 264.7 macrophages (data not shown). However, EGFR inactivation increases LPS-stimulated NF-κB activity (Fig. 8). Thus, enhancing LPS and IFN-γ-stimulated cytokine production by inhibition of EGFR kinase activity may occur by potentiating the effects of LPS on NF-κB activation. However, further investigations are needed to determine whether EGFR inactivation mediates priming of macrophages by IFN-γ via other signaling pathways.

It should be noted that, although EGFR inhibition in macrophages increases early TNF production in DSS-treated mice, sustained increases in IL-10 production as a result of EGFR inhibition in Egfrfl/fl LysM-Cre mice exert a negative feedback to decrease production of TNF and other proinflammatory cytokines. Because IL-10 inhibits production of proinflammatory cytokines and promotes production of anti-inflammatory mediators in other immune cells, inhibition of IL-10 production by macrophages through EGFR activation may affect immune responses mediated by other cell types, which further regulate colitis.

TLR signaling in intestinal macrophages has been shown to promote epithelial cell regeneration. COX-2 is one of the genes involved in epithelial cell regeneration that are upregulated in LPS-stimulated macrophages and in intestinal macrophages from DSS-treated mice (9). Colonic macrophages isolated from Egfrfl/fl LysM-Cre mice treated with DSS for 4 d followed by a 3-d recovery period showed increased levels of COX-2 mRNA expression, which correlated with increased intestinal epithelial cell proliferation rates in these animals. Whereas apoptosis in intestinal epithelial cells is a pathological factor for colitis, we were unable to observe alterations in intestinal epithelial cell apoptosis in DSS-treated Egfrfl/fl LysM-Cre mice, compared with LysM-Cre mice (data not shown). Therefore, EGFR activation in macrophages may negatively regulate production of factors that regulate the regenerative response in intestinal epithelial cells during colitis.

In this study, we used an anti-mouse F4/80 Ab to purify colonic macrophages. To address the important issue regarding the purity of cells enriched using this anti-F4/80 Ab, we performed phenotypic characterization of these enriched cells using FITC-conjugated anti-CD11b and PE-conjugated anti–Siglec-F (an eosinophil cell marker) Abs. Our data showed that, under normal conditions, the percentage of this enriched cell population expressing Siglec-F is very low (Siglec-F<sup>-</sup>, 1.31 ± 0.37%; CD11b<sup>+</sup>Siglec-F<sup>-</sup>, 0.68 ± 0.57%). These data are consistent with published results that resting mouse small intestinal eosinophils express CD11b, but not F4/80 (45). In DSS-induced colitis, the percentage of the enriched cell population expressing Siglec-F is significantly higher than that in the control group (Siglec-F<sup>-</sup>, 11.091 ± 4.43%; CD11b<sup>+</sup>Siglec-F<sup>-</sup>, 5.79 ± 2.31%). These data suggest that proinflammatory eosinophils express F4/80, as reported before that eosinophils express F4/80 in mice infected with Schistosoma mansoni (46). In addition, this evidence of increased colonic eosinophils in the DSS-treated mice has been observed in published paper (47). Although we found Siglec-F<sup>-</sup> cells in the cells enriched from DSS-treated mice, macrophages are the major cell type in this cell population. Thus, the results from enriched cells (cytokine production profiles in Figs. 4B and 6C) should predominantly represent functional changes in macrophages. Importantly, we have shown that PMN do not express EGFR (Fig. 1A).

Thus, the main contributing factor to the changes of cytokine expression levels in cells enriched from the colon of Egfrfl/fl LysM-Cre mice should be from knockdown of EGFR in macrophages.

In summary, although EGFR has been well studied for its role in promoting cell growth, few studies have investigated its immune regulatory functions. This study provides evidence that microbial products such as LPS activate EGFR in macrophages, which negatively regulates both pro- and anti-inflammatory cytokine production. During colitis, inhibition of IL-10 production by EGFR activation in macrophages may be involved in accelerating colitis and impairing disease recovery. Thus, these results provide important new information for understanding the mechanisms that regulate macrophage functions under physiological and pathological conditions.

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The authors have no financial conflicts of interest.

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