Activation of Epidermal Growth Factor Receptor in Macrophages Mediates Feedback Inhibition of M2 Polarization and Gastrointestinal Tumor Cell Growth*1

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EGF receptor (EGFR) in tumor cells serves as a tumor promoter. However, information about EGFR activation in macrophages in regulating M2 polarization and tumor development is limited. This study aimed to investigate the effects of EGFR activation in macrophages on M2 polarization and development of gastrointestinal tumors. IL-4, a cytokine to elicit M2 polarization, stimulated release of an EGFR ligand, HB-EGF, and transactivation and down-regulation of EGFR in Raw 264.7 cells and peritoneal macrophages from WT mice. Knockdown of HB-EGF in macrophages inhibited EGFR transactivation by IL-4. IL-4-stimulated STAT6 activation, Arg1 and YM1 gene expression, and HB-EGF production were further enhanced by inhibition of EGFR activity in Raw 264.7 cells using an EGFR kinase inhibitor and in peritoneal macrophages from Egfrwa5 mice with kinase inactive EGFR and by knockdown of EGFR in peritoneal macrophages from Egfrwa5 LysM-Cre mice with myeloid cell-specific EGFR deletion. Chitin induced a higher level of M2 polarization in peritoneal macrophages in Egfrwa5/WT but suppressed that in Egfrwa5/LysM-Cre mice. Accordingly, IL-4-conditioned medium stimulated growth and epithelial-to-mesenchymal transition in gastric epithelial and colonic tumor cells, which were suppressed by that from Raw 264.7 cells with HB-EGF knockdown but promoted by that from Egfrwa5 mice and Egfrwa5/LysM-Cre peritoneal macrophages. Clinical assessment revealed that the number of macrophages with EGFR expression became less, indicating decreased inhibitory effects on M2 polarization, in late stage of human gastric cancers. Thus, IL-4-stimulated HB-EGF-dependent transactivation of EGFR in macrophages may mediate inhibitory feedback for M2 polarization and HB-EGF production, thereby inhibiting gastrointestinal tumor growth.

Macrophages contribute to immune responses through possessing phenotypic plasticity. Classically activated macrophages (M1 polarization) are driven by IFN-γ and pathogen-derived signals, such as LPS-activated canonical interferon-regulatory factor-3 (IRF-3) and STAT1 pathways. Macrophages with M1 polarization are characterized by production of proinflammatory cytokines and NOS2 (1). Alternatively activated macrophages (M2 polarization) are elicited by IL-4 and IL-13-activated STAT6 and IL-10-activated STAT3 signals. M2-polarized macrophages exhibit increased transcription of genes, including arginase 1 (Arg1), mannose receptor 1 (Mrc1), resistin-like α (Retnla, Fiz1), and chitinase 3-like 3 factor (Chi3l3, YM1) (1) and possess functions of anti-inflammation, angiogenesis, and tissue repair, thus having tumor-promoting properties (1, 2). In addition, other transcriptional factors, such as NF-κB (3) and IL-4-induced epigenetic changes (4) have been found to participate in directing polarization in macrophages.

Tumor-associated macrophages (TAMs) in the tumor microenvironment usually exhibit M2 polarization in human and mouse models of cancers (5, 6) and possess pro-tumorigenic activities in most cancers, including promoting tumor cell proliferation, migration, and invasion, increasing angiogenesis, and suppressing immunity through secretion of growth factors such as HB-EGF, EGF, CSF-1, VEGF, and PDGF, enzymes, including metalloproteinases (MMPs), and cytokines, such as IL-6, IL-10, and TNF (7). Clinical studies have revealed that

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there is a strong correlation between the abundance of TAMs and poor prognosis in most cancers. However, both positive and negative associations between TAMs and clinical outcomes have been reported in lung, gastric, prostate, and bone cancers, depending on tumor types and stages (8–10).

It is known that cytokines, such as IL-4, IL-10, IL-13, IFN-γ, TNF, macrophage colony-stimulting factor (MCSF), TGFβ, and PGE2 produced by lymphocytes and tumor cells promote M2 polarization and functions of TAMs (11). However, the mechanism underlying the diverse functions of TAMs in different tumor microenvironment remains unclear.

EGF receptor (EGFR) belongs to the ErB family of tyrosine kinases. EGFR can be activated by direct ligand binding and transactivated by a wide variety of pharmacological and physiologial stimuli, including TNF (12) and bacterial products, such as LPS (13) in intestinal epithelial cells, through stimulation of EGFR ligand release, such as EGF and HB-EGF. The cytoplasmic domain of EGFR contains the kinase domain as well as autophosphorylation sites at tyrosine residues. Ligation of EGFR leads to receptor dimerization, phosphorylation, and increased tyrosine kinase activity (14). These phosphorylated amino acids provide docking sites for a variety of signaling molecules that regulate intracellular signaling networks and ultimately define biological responses, such as proliferation, differentiation, migration, and survival (15, 16). Activated EGFR is subsequently sorted by incompletely understood mechanisms for either recycling to the plasma membrane or destruction (17, 18). Excessive functions of EGFR signaling are involved in initiation and progression of cancers in many human cancers of epithelial origin (19, 20) and are implicated in numerous animal models of gastrointestinal tumorigenesis (21–23).

Studies regarding EGFR in macrophages have revealed that EGFR is phosphorylated at tyrosine and serine and threonine residues through stimulation of HB-EGF release and ligand-independent manner, respectively (24–26). EGFR signaling has been shown to be involved in TLR3 (27)- and IFN-γ (28)-dependent signaling pathways in macrophages. Our previous studies have demonstrated that EGFR is transactivated by LPS in macrophages (29). EGFR activation in macrophages suppresses both pro- and anti-inflammatory cytokines in response to inflammatory stimuli, and the decrease in the IL-10 plays a role in further up-regulating proinflammatory cytokine production, resulting in enhancing intestinal inflammation in dextran sulfate sodium-induced colitis (29). However, EGFR signaling in macrophages in directing M2 polarization for regulating gastrointestinal tumor development remains to be elucidated.

The purpose of this work is to determine the roles and mechanisms of EGFR activation in macrophages in regulating M2 polarization and gastric and colonic tumor growth. We demonstrated that IL-4 transactivated EGFR through stimulation of HB-EGF release in macrophages. Transactivation of EGFR by IL-4 in macrophages could serve as a negative feedback mechanism for M2 polarization and HB-EGF production, thereby inhibiting tumor growth. Our clinical assessment of human cancer samples revealed that EGFR was down-regulated in macrophages in the late stage of gastric cancers, which suggests that the less inhibitory effect of EGFR in macrophages may be associated with promoting cancer development by TAMs. These findings provide pivotal information for understanding the cell type-specific function of EGFR in tumorigenesis.

Results

IL-4 Stimulates Transactivation of EGFR in Macrophages, Which Requires HB-EGF Release—To determine whether EGFR signaling contributes to regulation of M2 polarization, we first studied the effects of IL-4 on EGFR activation in macrophages. Our data showed that IL-4 treatment stimulated transactivation of EGFR in Raw 264.7 cells (Fig. 1A) and in peritoneal macrophages isolated from WT mice (Fig. 1B). Degradation of EGFR occurred following its activation stimulated by IL-4 in macrophages (Fig. 1, A and B). As expected, an EGFR kinase inhibitor, AG1478, blocked IL-4-stimulated EGFR transactivation and degradation (Fig. 1A). Furthermore, IL-4 failed to stimulate EGFR activation and degradation in Egfr<sup>−/−</sup> peritoneal macrophages, which have kinase-defective EGFR. These data indicate that EGFR degradation in IL-4-stimulated macrophages is a subsequent event after EGFR activation.

TAMs produced EGFR ligands such as EGF and HB-EGF to promote tumor cell growth (30–32). We next tested whether EGFR ligand release mediated IL-4-induced EGFR transactivation in macrophages. Release of EGFR ligand by IL-4 treatment in macrophages was detected using ELISA assay. We found that IL-4 stimulated HB-EGF release in Raw 264.7 cells (Fig. 2A).

IL-4 treatment for 0.25–0.5 h induced EGFR transactivation (Fig. 1A), during which time points HB-EGF release was increased (Fig. 2A). Thus, the time point for HB-EGF release was correlated with EGFR transactivation. No effect of IL-4 on EGF production in Raw 264.7 cells was identified (data not shown).

Next we examined the requirement of HB-EGF release for IL-4-stimulated EGFR transactivation in macrophages. MMPs
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We further examined the effects of knockdown of EGFR in macrophages on regulation of gastrointestinal epithelial cell growth and EMT—It has been reported that HB-EGF produced by TAMs promotes tumor cell growth (30–32). We tested if inhibition of EGFR kinase activity and knockdown of EGFR expression in macrophages could affect the effects of IL-4-conditioned media from macrophages on regulation of gastrointestinal epithelial cell growth and EMT.

We treated peritoneal macrophages isolated from WT and Egfrwa5 mice with IL-4, then conditioned media were collected for treating ImSt and IMCEras cells (Fig. 6A). Conditioned medium from IL-4-stimulated Egfrwa5 peritoneal macrophages induced higher levels of cell growth in ImSt cells than those by conditioned medium from IL-4-stimulated WT peritoneal macrophages (Fig. 6B). In agreement with this finding, we found that conditioned media from IL-4-stimulated WT peritoneal macrophages promoted formation of colonies in IMCEras cells, as compared with control, which was further increased by conditioned media from IL-4-stimulated Egfrwa5 peritoneal macrophages (Fig. 6C).
Inhibition of EGFR kinase activity in macrophages increases IL-4-stimulated STAT6 activation and M2 polarization. Raw 264.7 mouse macrophages (A, B, and D) and peritoneal macrophages isolated from WT and Egfrwa5 mice (C and E) were treated with IL-4 (10 ng/ml) for the indicated time periods (A–D) and 24 h in the presence or absence of a 1-h pretreatment of AG1478 (450 nM) and TAPI-1 (10 μM) (E). Cellular lysates were collected for Western blot analysis of phosphorylation of STAT6 (P-STAT6) and total STAT6 expression (A, B, and C). The β-actin blot was used as a protein loading control. RNA was isolated from cells. Gene expression levels of STAT6 and IL-4-treated conditioned medium from WT peritoneal macrophages up-regulated Snail gene expression in IMSt and IMCEras cells, which was further increased by conditioned medium from IL-4-stimulated Egfrwa5 macrophages (Fig. 6, D and E).

We next tested the effects of conditioned medium from macrophages with inhibition of EGFR expression on colonic tumor growth. IMCEras cells were treated with conditioned media from IL-4-stimulated peritoneal macrophages from Egfrβ/β and Egfrβ/β LysM-Cre mice for examining EMT. IL-4-stimulated conditioned medium from Egfrβ/β peritoneal macrophages up-regulated Snail gene expression in IMCEras cells, which was further increased by conditioned medium from IL-4-stimulated Egfrβ/β LysM-Cre macrophages (Fig. 7A).

To further assess the effects of EGFR activation in macrophages on colonic tumor growth, we used a xenograft tumor model. We inoculated Rag2−/− mice with IMCEras cells with co-treatment of conditioned media from peritoneal macrophages with and without IL-4 treatment. In the control media, IL-4 was added into the non-treated conditioned medium before inoculation to exclude the effects of IL-4 presenting in IL-4-conditioned media. Conditioned media from IL-4-stimulated Egfrβ/β peritoneal macrophages significantly increased tumor volumes after 6 days of implantation, as compared with those with control media from Egfrβ/β peritoneal macrophages and RPMI media (Fig. 7B). Conditioned media from IL-4-stimulated Egfrβ/β LysM-Cre peritoneal macrophages significantly increased tumor volumes after 6 days of implantation, as compared with those by control media from Egfrβ/β LysM-Cre macrophages and IL-4-stimulated media from Egfrβ/β macrophages (Fig. 7B). In addition, significant increases in cell proliferation, detected by immunohistochemistry of a proliferation marker, Ki-67 (Fig. 7, C and D) and Snail expression by RT-PCR analysis in tumor cells (Fig. 7E), were observed in xenograft treated with IL-4-stimulated Egfrβ/β LysM-Cre macrophage-conditioned medium, as compared with those by IL-4-stimulated Egfrβ/β macrophage-conditioned medium.

These results indicate that transactivation of EGFR in macrophages is capable of inhibiting colon cancer cell growth and EMT. It should be noted that IL-4 did not show any direct effects on growth and expression of Snail in IMSt cells (Fig. 8, B and C). Together, these data suggest that transactivation of EGFR in IL-4-stimulated macrophages suppresses the effects of conditioned medium from macrophages on growth and EMT in IMSt and IMCEras cells.

HB-EGF Mediates IL-4-conditioned Medium-stimulated Tumor Growth and EMT—To determine whether increased production of HB-EGF by IL-4 treatment in macrophages serves as a functional factor for promoting tumor growth, we...
first evaluated the effects of IL-4-conditioned medium on EGFR activation, cell growth, and EMT in WT and Egfr/H11002/H11002 ImSt cells. Conditioned medium from IL-4-stimulated Raw 264.7 cells activated EGFR in WT ImSt cells (Fig. 8A). Compared with control-conditioned medium from untreated Raw 264.7 cells, conditioned medium from IL-4-stimulated Raw 264.7 cells stimulated higher levels of cell growth and Snail and Vimentin gene expression in WT but not Egfr/H11002/H11002 ImSt cells (Fig. 8, B and C). These data indicate that EGFR in ImSt mediates IL-4-conditioned medium regulation of growth and EMT. Furthermore, IL-4-conditioned media from Raw 264.7 cells transduced with siRNA HB-EGF failed to stimulate EGFR activation and Snail and Vimentin gene expression in WT ImSt cells (Fig. 8, A and D).

These results suggest that HB-EGF may contribute to promoting tumor cell growth and EMT by IL-4-conditioned media from macrophages. Because EGFR activation inhibits HB-EGF production, the effects of EGFR in macrophages on inhibiting tumor development may be through decreasing HB-EGF production.

Expression of EGFR in Macrophages Is Decreased in the Late Stage of Human Gastric Cancers—TAMs are associated with poor prognosis in gastric cancer (36). To provide some information regarding EGFR in macrophages in human cancers, we sought to study whether there is a correlation between the level of EGFR expression in macrophages in cancer tissues and tumor development.

The gastric tissue array, including normal and cancer tissues (Fig. 9A), was analyzed using immunohistochemistry to detect EGFR expression in CD68-positive macrophages (Fig. 9B). The immunohistochemistry data showed that the percentage of macrophages with EGFR expression was similar to that in normal gastric tissues (p > 0.05); however, the percentages of macrophages with EGFR expression were significantly decreased in stage II-IV gastric cancer tissues, as compared with that in normal and stage 1 gastric cancer (p < 0.05) (Fig. 9C). These results indicate that the level of EGFR expression in macrophages may be associated with roles of TAMs in gastrointestinal tumor development.

Discussion

Diverse functions of TAMs have been reported in different types and stages of tumors (8–10). Thus understanding of the mechanisms underlying these different functional programs in macrophages in response to signals in tumor microenvironment is important for cancer therapy. A finding from our stud-
ies demonstrates that EGFR activation in macrophages exerts a negative feedback for M2 polarization. Therefore, EGFR signaling may be involved in the functional plasticity of macrophage development in the tumor microenvironment. However, the mechanisms underlying inhibition of M2 polarization by EGFR in macrophages is under investigation. It is known that in addition to activation of STAT6 pathway for M2 polarization, IL-4 induces negative feedback to inhibit STAT6 phosphorylation by up-regulating suppressor of cytokine signaling 1 (SOCS1) (1). In fact, inhibition of EGFR kinase activity in EgfΔwa5 macrophages inhibits IL-4-stimulated SOCS1 production (supplemental Fig. 1). This evidence suggests that SOCS1 may serve as a target of EGFR activation for inhibition of M2 polarization.

It is important to elucidate the mechanisms underlying regulation of EGFR activation in macrophages in the tumor microenvironment. Our data support IL-4-stimulated HB-EGF release for induction of EGFR transactivation in macrophages. HB-EGF activates EGFR in Raw 264.7 cells in the same time-dependent manner as IL-4-conditioned media do (supplemental Fig. 2). We did not find EGF production in Raw 264.7 cells and in peritoneal macrophages from WT and EgfΔwa5 mice with and without IL-4 treatment by using a Proteome Profiler Mouse Angiogenesis Array kit and ELISA (data not shown). Because regulation of proteolytic activity of ADAMs (a disintegrin and metalloproteinases), which are membrane-anchored proteases, to cleave the extracellular domains of membrane-bound proteins including EGFR ligands is still poorly defined (37, 38), more studies are needed for elucidating the mechanisms involved in specific release HB-EGF by IL-4 in macrophages.

We cannot exclude the possibility that other cytokines produced by lymphocytes and tumor cells, such as IL-10 and TNF, which play a central role in directing polarization and functions of ATMs (11), stimulate release of EGF, leading to activation of EGFR in macrophages.

It should be noted that compared with DMEM and the control medium from EgfΔ/Δ LysM-Cre macrophages, the control medium from EgfΔ/Δ LysM-Cre peritoneal macrophages induced higher levels of tumor growth, cell proliferation, and Snail expression in the IMCEΔras xenograft model (Fig. 7). However, HB-EGF production in untreated EgfΔ/Δ LysM-Cre peritoneal macrophages.
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IL-4 (10 ng/ml) was added to the media from untreated macrophages. IL-4, but not LPS, up-regulates HB-EGF gene expression in macrophages. More importantly, IL-4-stimulated EGFR transactivation mediates inhibitory feedback for HB-EGF production. However, LPS-stimulated EGFR transactivation does not affect HB-EGF production (supplemental Fig. 3). We have shown that LPS-stimulated EGFR transactivation mediates inhibition of LPS-stimulated NF-κB activation and proinflammatory and anti-inflammatory cytokine production in Raw 264.7 macrophages (29). Thus, EGFR activation may exert different functions in macrophages upon M1 and M2 stimulation.

We have reported that deletion of EGFR in macrophages leads to an increase in the IL-10 level in response to inflammatory stimuli, such as LPS, which plays a role in suppressing proinflammatory cytokine production, resulting in protection of mice from intestinal inflammation colitis (29). Current studies show that blocking EGFR in macrophages up-regulates M2 polarization. Therefore, it should be noted that more studies are needed to elucidate the roles of EGFR activation in macrophages in inflammation-associated tumorigenesis. In fact, EGFR in macrophages in liver has shown tumor-promoting effects on hepatocellular carcinoma formation induced by diethylnitrosamine/phenobarbital (39).

Because excessive EGFR signaling in tumor cells is known to promote tumorigenesis (19, 20), EGFR is one of the key targets of the therapeutic strategy designed to treat cancers. Currently, two anti-EGFR monoclonal antibodies have been approved for the treatment of metastatic colorectal cancer (cetuximab and panitumumab) (40). However, therapies to inhibit the EGFR activity are not universally efficacious, suggesting possible resistance of EGFR inhibition in tumor cells or divergent roles of EGFR in other cell types involved in tumor development. Accordingly, we demonstrated that transactivation of EGFR in macrophages inhibits M2 polarization and exerts an anti-tumor effect. The decreased level of EGFR expression in macrophages might be associated with pro-tumor effects of TAMs in late stage of cancers. Our results are supported by the reported study that cetuximab increases activities of tumor-promoting M2 macrophages in the colorectal tumor microenvironment (41). Thus, our studies broaden the understanding of the mechanisms of EGFR signaling in tumor development by supporting the concept of cell type-specific (epithelial cell versus macrophage) EGFR activation in regulation of tumor establishment and progression. This concept represents a new direction to elucidate the mechanisms of EGFR signaling in tumorigenesis and assess therapeutic application of EGFR inhibitors.

In summary, these findings revealed previously unrecognized roles of EGFR signaling in regulation of M2 polarization in macrophages and demonstrate the association between the EGFR expression level in macrophages and the roles of TAMs in cancer development. These results should be taken into account for the application of anti-EGFR antibodies for cancer treatment.
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Experimental Procedures

Human Tissue Microarrays—A human gastric tissue microarray was accessed from the Human Tissue Acquisition and Pathology Shared Resources at Vanderbilt University Medical Center. There were 78 paraffin-embedded stomach tissue samples (8 normal mucosa and 70 tumors) in the gastric tissue microarray cores. All tissue samples were coded and de-identified in accordance with Institutional Review Board approved protocols. Clinical staging was evaluated according to the American Joint Committee on Cancer criteria. The adenocarcinomas were analyzed ranging from well to poorly differentiated (stages I to IV). Gastric tumors included a mix of intestinal- and diffuse-type tumors. The histology of all tissue samples was verified using H&E staining. The tissue microarray was used for immunohistochemistry to analyze EGFR expression in CD68-positive macrophages.

Animals and Treatment—All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center (Nashville, TN). WT C57BL/6j and Rag2−/− mice were subcutaneously inoculated into the flank of 6–8-week-old Rag2−/− mice. Conditioned medium was prepared from peritoneal macrophages isolated from the WT and Egfr−/− mice with and without IL-4 (10 ng/ml) treatment for 1 h. To exclude the potential effect of IL-4 in IL-4-conditioned medium on xenograft growth, IL-4 (10 ng/ml) was added to the media from untreated macrophages when inoculating with homozogous LysM-Cre mice on a mixed C57BL/6J and C57BL/6N background in our laboratory (29). Gene mutation was confirmed by genotyping. EGFR expression in macrophages was tested using Western blot analysis.

Chitin (Sigma) was solubilized in PBS, as described before (4). Egfrfl/fl LysM-Cre and Egfr−/− mice were treated with chitin (800 ng in 100 μl of PBS) intraperitoneally. Peritoneal macrophages were isolated 48 h after chitin administration.

Tumor Xenograft—1 × 10⁶ IMCEas cells in conditioned medium and in DMEM medium containing IL-4 (10 ng/ml) were subcutaneously inoculated into the flank of 6–8-week-old Rag2−/− mice. Conditioned media were prepared from peritoneal macrophages isolated from the Egfrfl/fl LysM-Cre and Egfr−/− mice with and without IL-4 (10 ng/ml) treatment for 1 h. To exclude the potential effect of IL-4 in IL-4-conditioned medium on xenograft growth, IL-4 (10 ng/ml) was added to the conditioned medium from untreated macrophages when inoculation as a control. Conditioned medium was injected into the xenograft again at 7 days after inoculation. Mice were euthanized 14 days after inoculation. Tumor volume was calculated using the formula of tumor volume = larger diameter × (smaller diameter)² × 0.5.

Cell Culture and Treatment—The mouse Raw 264.7 (ATCC® TIB-71™) monocyte/macrophage cell line and peritoneal macrophages from mice were cultured in DMEM medium containing 10% FBS, 1% glutamine, 100,000 IU/liter penicillin, and 100 mg/liter streptomycin at 37 °C with 5% CO₂. Peritoneal macrophages were isolated from mice and plated on cell culture dishes for 3 h. Unattached cells were removed by
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A

Gastric, normal

Gastric cancer, Stage IV

B

CD68 (Macrophage) EGFR/ DAPI (Nucleus)

Yellow = CD68 + EGFR

Normal

Cancer, Stage IV

C

% EGFR+CD68+ in CD68+ cells

Stage

Normal I II III IV

changing medium. For each experiment, peritoneal macrophages isolated from 4–5 mice were mixed for treatments.

Macrophages were treated with IL-4 at 10 ng/ml (R&D Systems, Inc., Minneapolis, MN) in the presence or absence of a 1-h pretreatment of an EGFR-tyrosine kinase inhibitor, AG1478, at 450 nM (Calbiochem-EMD Millipore Corp., Billerica, MA), and the broad-spectrum MMP inhibitors GM6001 at 2.5 μM (EMD Millipore) and TAPI-1 at 10 μM (Enzo Life Sciences, Farmingdale, NY). Cells were collected for isolation of total cellular lysate and RNA. Culture supernatants were collected for ELISA and as conditioned media for treatment of gastric and colonic epithelial cells and xenograft. We adjusted the ratio of cell-to-medium at 5 × 10^5 cells per 1 ml of medium.

Mouse conditionally immortalized stomach epithelial cells (ImSt) were isolated from the gastric epithelium of transgenic mice with a temperature-sensitive mutation of the simian virus 40 (SV40) large tumor antigen gene (tsA58) fused to the promoter of the mouse H-2Kb class I gene (H-2Kb-tsA58 mice) (42). The Egfr⁻/⁻/ImSt cell line was generated from the stomach epithelium of EGFR-null mice crossed to the Immortomouse (43).

The Immorto-Min colonic epithelial (IMCE) cell line was generated from the colonic epithelium of F1 Immorto-Apcmin/+ mouse hybrid (44). Thus, IMCE cells carry both the mutant Apcmin gene and a temperature-sensitive mutant of the SV40 large T gene. The IMCEax cell line was generated by overexpression of v-Ha-ras gene in IMCE cells (45).

ImSt, Egfr⁻/⁻/ImSt, and IMCEax were maintained in RPMI 1640 media supplemented with 5% FBS, 5 units/ml murine IFN-γ, 100 units/ml penicillin and streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid on collagen-coated plates at 33 °C (permissive condition) with 5% CO2. Cells were serum-starved for 16–18 h in RPMI 1640 containing 0.5% FBS and 100 units/ml penicillin and streptomycin (no IFN-γ) at 37 °C (nonpermissive conditions) before treatment.

Transient Transfection of HB-EGF siRNA—Raw 264.7 cells were transiently transfected with either 20 nm non-targeting siRNA or 20 nm mouse HB-EGF siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) at 80% confluence using Lipofectamine 2000 (Invitrogen) for 6 h according to the manufacturer’s instructions. Cells were then cultured for 48 h before treatment.

Colonies were treated with mouse IL-4 (10 ng/ml) in 500 μl of macrophage-conditioned medium with 5 units/ml of murine IFN-γ and cultured at 33 °C with 5% CO2 for 14 days. Conditioned medium was changed every 4 days. Cells were stained at the end of the experiment using the CellTiterH120 AQueousOne Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instructions. The area covered by colonies of >0.50 mm was measured (46).

ELISA—Raw 264.7 macrophages and peritoneal macrophages were treated with mouse IL-4 (10 ng/ml) in the presence or absence EGFR inhibitor AG1478 (450 nm) in 1 ml of medium for 1, 2, 8, and 24 h. The ratio of cell:medium was 5 × 10^5 cells/1 ml of medium. Cell culture media were collected for determining the levels of HB-EGF using mouse HB-EGF ELISA kits (DuoSet® ELISA Development System, R&D Systems, Inc.), according to the manufacturer’s instructions. The HB-EGF concentration in the cell culture medium was calculated as pg/ml.

Cell Viability Assay—WT and Egfr⁻/⁻/ImSt cells were plated in 96-well plates (5000 cells/well) and cultured overnight under permissive condition followed by culture in the starved medium under non-permissive condition for 8–12 h. Then cells were treated with macrophage-conditioned media at 37 °C for 24 h. Cell viability was assessed using the CellTiter®890 AQueousOne Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions. The cell number standard curve was generated to determine the cell number in each experimental 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
the phosphatase inhibitor mixture (Sigma). Protein concentrations of lysates were determined using a protein assay kit (Thermo Fisher Scientific, Rockford, IL). The lysates were mixed with Laemmli sample buffer, and the equal amounts of protein from samples were loaded for SDS-PAGE. Western blot analysis was performed using anti-total EGFR (Millipore), anti-phospho-EGFR (Tyr-1068) (Cell Signaling Technology), anti-phospho-STAT6 (Abcam Cambridge, MA), anti-total STAT6 (R&D Systems, Inc.), anti-HB-EGF (R&D Systems, Inc.), and anti-β-actin (Sigma) antibodies.

Real-time PCR Analysis—Total RNA was isolated from cultured cells and xenograft tissues using a RNA isolation kit (Qiagen, Valencia, CA) and 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, including Arg1 (Mm00475988_ml), YM1 (Mm00657889_ml), FIZZ (Mm00445109_ml), Snail (Mm00441533_gl), vimentin (Mm01333430_ml), and HB-EGF (Mm00439306_ml). The relative abundance of GAPDH mRNA was used to normalize the levels of the mRNAs of interest. All cDNA samples were analyzed in triplicate.

Immunohistochemistry—Tissues sections were deparaffinized and rehydrated. Sections on human gastric tissue microarray were unmasked using EDTA solution and stained using mouse anti-human CD68 (Dako, Carpinteria, CA) and rabbit anti-human EGFR antibodies (RayBiotech, Inc.). The sections were incubated sequentially with FITC-labeled anti-rabbit IgG followed by Cy3-labeled anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) antibodies. Sections were then mounted using mounting medium containing DAPI (Vector laboratories, Inc Burlingame, CA) for nuclear staining. Slides were observed under fluorescence microscopy. FITC, Cy3, and DAPI images were taken from the same field.

For Ki-67 staining, antigen retrieval was carried out by using antigen unmasking solution (Vector Laboratories). The sections were then incubated with a rabbit anti-Ki-67 monoclonal antibody (Biocare Medical, Concord, CA) at 4 °C overnight followed incubation with a goat anti-rabbit polymer-HRP secondary antibody (Biocare Medical) for 1 h at room temperature. The sections were developed using the ImmPACT™ DAB substrate (Vector Laboratories). Sections were counterstained using hematoxylin and the observed light microscopy.

Statistical Analysis—Statistical significance was determined by one-way analysis of variance followed by Newman–Keuls analysis using Prism 5.0 (GraphPad Software, Inc. 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 the mean ± S.E.

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References
1. Gordon, S. (2003) Alternative activation of macrophages. Nat. Rev. Immunol. 3, 23–35
2. Lang, R., Patel, D., Morris, J. J., Rutschman, R. L., and Murray, P. J. (2002) Shaping gene expression in activated and resting primary macrophages by IL-10. J. Immunol. 169, 2253–2263
3. Hagemann, T., Lawrence, T., McNeish, I., Charles, K. A., Kulbe, H., Thompson, R. G., Robinson, S. C., and Balkwill, F. R. (2008) “Re-educating” tumour-associated macrophages by targeting NF-κB. J. Exp. Med. 205, 1261–1268
4. Satoh, T., Takeuchi, O., Vandenbon, A., Yasuda, K., Tanaka, Y., Kumagai, Y., Miyake, T., Matsushita, K., Okazaki, T., Saitoh, T., Honma, K., Matsuyama, T., Yui, K., Tsujimura, T., Standley, D. M., Nakashima, K., Nakai, K., and Akira, S. (2010) The Imj3-Irfl axis regulates M2 macrophage polarization and host responses against helminth infection. Nat. Immunol. 11, 936–944
5. Biswas, S. K., Gangi, L., Paul, S., Schioppa, T., Saccani, A., Sironi, M., Bottazzi, B., Doni, A., Vincenzo, B., Pasqualini, F., Vago, L., Nebuloni, M., Mantovani, A., and Sica, A. (2006) A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). Blood 107, 2112–2122
6. Guiducci, C., Vicari, A. P., Sangaletti, S., Trinchieri, G., and Colombo, M. P. (2005) Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res. 65, 3437–3446
7. Condeelis, J., and Pollard, J. W. (2006) Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 124, 263–266
8. Komohara, Y., Jinushi, M., and Takeya, M. (2014) Clinical significance of macrophage heterogeneity in human malignant tumors. Cancer Sci. 105, 1–8
9. Ruffell, B., and Coussens, L. M. (2015) Macrophages and therapeutic resistance in cancer. Cancer Cell 27, 462–472
10. Zhang, Q. W., Liu, L., Gong, C. Y., Shi, H. S., Zeng, Y. H., Wang, X. Z., Zhao, Y. W., and Wei, Y. Q. (2012) Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. PLoS ONE 7, e50946
11. Sica, A., Schioppa, T., Mantovani, A., and Allavena, P. (2006) Tumor-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur. J. Cancer 42, 717–727
12. Yamaoka, T., Yan, F., Cao, H., Hobbis, S. S., Dix, R. S., Tong, W., and Polk, D. B. (2008) Transactivation of EGF receptor and ErbB2 protects intestinal associated macrophages in solid tumor: a meta-analysis of the literature. Proc. Natl. Acad. Sci. U.S.A. 105, 11772–11777
13. Fukata, M., Chen, A., Yamadevan, A. S., Cohen, J., Breglio, K., Krishnareddy, S., Hsu, D., Xu, R., Harpaz, N., Dannenberg, A. J., Subbaramaiah, K., Cooper, H. S., Itzkowitz, S. H., and Abreu, M. T. (2007) Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterology 133, 1869–1881
14. Olajuyon, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J. 19, 3159–3167
15. Yarden, Y. (2001) The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. Eur. J. Cancer 37, S3–S8
16. Yarden, Y., and Slwidekowski, M. X. (2001) Untangling the ErbB signaling network. Nat. Rev. Mol. Cell Biol. 2, 127–137
17. Buccire, M., Roviozo, F., Cicala, C., Sessa, W. C., and Cirino, G. (2000) Geldanamycin, an inhibitor of heat shock protein 90 (Hsp90) mediated signal transduction has anti-inflammatory effects and interacts with glucocorticoid receptor in vivo. Br. J. Pharmacol. 131, 13–16
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18. de Renzi, S., Sönnichsen, B., and Zerial, M. (2002) Divalent Rab effectors regulate the sub-compartamental organization and sorting of early endosomes. Nat. Cell Biol. 4, 124–133

19. Walther, A., Johnstone, E., Swanton, C., Midgley, R., Tomlinson, I., and Kerr, D. (2009) Genetic prognostic and predictive markers in colorectal cancer. Nat. Rev. Cancer 9, 489–499

20. Goel, G. A., Kandel, A., Achkar, J. P., and Lashner, B. (2011) Molecular pathways underlying IBD-associated colorectal neoplasia: therapeutic implications. Am. J. Gastroenterol 106, 719–730

21. Goldstein, N. S., and Armin, M. (2001) Epidermal growth factor receptor immunohistochemical reactivity in patients with American Joint Committee on Cancer Stage IV colon adenocarcinoma: implications for a standardized scoring system. Cancer 92, 1331–1346

22. Roberts, R. B., Min, L., Washington, M. K., Olsen, S. J., Settle, S. H., Coffey, R. J., and Threadgill, D. W. (2002) Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. Proc. Natl. Acad. Sci. U.S.A. 99, 1521–1526

23. Allerez, D., Wilkinson, R. W., Watkins, J., Poulsom, R., Mandir, N., Wedge, S. R., Pyrah, I. T., Smith, N. R., Jackson, L., Ryan, A. J., and Goodlad, R. A. (2008) Dual inhibition of VEGFR and EGFR signaling reduces the incidence and size of intestinal adenomas in Apc(Min/+ ) mice. Mol. Cancer Ther. 7, 590–598

24. Gao, P., Wang, X. M., Qian, D. H., Jin, J., Xu, Q., Yuan, Q. Y., Li, X. J., and Si, L. Y. (2013) Induction of oxidative stress by oxidized LDL via membrane-α receptor-mediated epidermal growth factor receptor in macrophages. Cardiovasc. Res. 97, 533–543

25. Nishimura, M., Shin, M. S., Singhirunnusorn, P., Suzuki, S., Kawanishi, M., Koizumi, K., Saiki, I., and Sakurai, H. (2009) TAK1-mediated serine/threonine phosphorylation of epidermal growth factor receptor via p38/extracellular signal-regulated kinase: NF-κB-independent survival pathways in tumor necrosis factor α signaling. Mol. Cell. Biol. 29, 5529–5539

26. Singhirunnusorn, P., Ueno, Y., Matsuo, M., Suzuki, S., Saiki, I., and Sakurai, H. (2007) Transient suppression of lipid-mediated activation of epidermal growth factor receptor by tumor necrosis factor-α through the TAK1-p38 signaling pathway. J. Biol. Chem. 282, 12698–12706

27. Yamashita, M., Chattopadhyay, S., Sen, G. C. (2012) EGFR is essential for Toll-like receptor 3 signaling. Sci. Signal. 5, ra50

28. Burova, E., Vassilenko, K., Dorosh, V., Gonchar, I., and Nikolovsky, N. (2007) Interferon γ-dependent transactivation of epidermal growth factor receptor. FEBS Lett. 581, 1475–1480

29. Lu, N., Wang, L., Cao, H., Liu, L., Van Kaer, L., Washington, M. K., Rosen, M. J., Dubé, P. E., Wilson, K. T., Ren, X., Hao, X., Polk, D. B., and Yan, F. (2014) Activation of the epidermal growth factor receptor in macrophages regulates cytokine production and experimental colitis. J. Immunol. 192, 1013–1023

30. Patialou, A., Wyckoff, J., Wang, Y., Goswami, S., Stanley, E. R., and Condeelis, J. S. (2009) Invasion of human breast cancer cells in vivo requires both paracrine and autocrine loops involving the colony-stimulating factor-1 receptor. Cancer Res. 69, 9498–9506

31. Rigo, A., Gottardi, M., Zambò, A., Mauri, P., Bonifacio, M., Krampera, M., Damiani, E., Pizzolo, G., and Vinante, F. (2010) Macrophages may promote cancer growth via a GM-CSF/HB-EGF paracrine loop that is enhanced by CXCL12. Mol. Cancer 9, 273

32. Goswami, S., Sahai, E., Wyckoff, J. B., Cammer, M., Cox, D., Pidley, F. J., Stanley, E. R., Segall, J. E., and Condeelis, J. S. (2005) Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res. 65, 5278–5283

33. Blobel, C. P. (2005) ADAMs: key components in EGFR signalling and development. Nat. Rev. Mol. Cell Biol. 6, 32–43

34. Blobel, C. P., Carpenter, G., and Freeman, M. (2009) The role of protease activity in ErbB biology. Exp. Cell Res. 315, 671–682

35. McLain, D. R., Lang, P. A., Maretsky, T., Hamada, K., Oshiki, K., Maney, S. K., Berger, T., Murthy, A., Duncan, G., Xu, H. C., Lang, K. S., Häussinger, D., Wakeham, A., Irie-Youten, A., Khokha, R., Ohashi, P. S., Blobel, C. P., and Mak, T. W. (2012) iRhom2 regulation of TACE controls TNF-mediated protection against Listeria and responses to LPS. Science 335, 229–232

36. Adrain, C., Zettl, M., Christova, Y., Taylor, N., and Freeman, M. (2012) Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. Science 335, 225–228

37. Lanaya, H., Natarajan, A., Komposch, K., Li, L., Amberg, N., Chen, L., Wculek, S. K., Hammer, M., Zenz, R., Peck-Radosavljevic, M., Sieghart, W., Trauner, M., Wang, H., and Sibilia, M. (2014) EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation. Nat. Cell Biol. 16, 972–981

38. Wheeler, D. L., Dunn, E. F., and Harari, P. M. (2010) Understanding resistance to EGFR inhibitors-impact on future treatment strategies. Nat. Rev. Clin. Oncol. 7, 493–507

39. Pander, J., Heusinkveld, M., van der Straaten, T., Jordanova, E. S., Baak-Pablo, R., Gelderblom, H., Morreau, H., van der Burg, S. H., Guchelaar, H. J., and van Hall, T. (2011) Activation of tumor-promoting type2 macrophages by EGFR-targeting antibody cetuximab. Clin. Cancer Res. 17, 5668–5673

40. Whitehead, R. H., VanEeden, P. E., Noble, M. D., Ataliotis, P., and Jat, P. S. (1993) Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2k-tsA58 transgenic mice. Proc. Natl. Acad. Sci. U.S.A. 90, 587–591

41. Díez, R. S., Frey, M. R., Whitehead, R. H., and Polk, D. B. (2008) Epidermal growth factor stimulates Rac activation through Src and phosphatidylinositol 3-kinase to promote colonic epithelial cell migration. Am. J. Physiol. Gastrointest. Liver Physiol. 294, G276–G285

42. Whitehead, R. H., and Joseph, J. L. (1994) Derivation of conditionally immortalized cell lines containing the Min mutation from the normal colonic mucosa and other tissues of an "Immortomouse/Min hybrid. Epithelial Cell Biol. 3, 119–125

43. D’Abaco, G. M., Whitehead, R. H., and Burgess, A. W. (1996) Synergy between Apc min and an activated ras mutation is sufficient to induce colon carcinomas. Mol. Cell. Biol. 16, 884–891

44. Guzmán, C., Bagga, M., Kaur, A., Westermark, J., and Abankwa, D. (2014) ColonyArea: an ImageJ plug-in to automatically quantify colony formation in clonogenic assays. Plas ONE 9, e92444