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A LGG-derived protein promotes IgA production through up-regulation of APRIL expression in intestinal epithelial cells

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Abstract

p40, a *Lactobacillus rhamnosus* GG (LGG)-derived protein, transactivates epidermal growth factor receptor (EGFR) in intestinal epithelial cells, leading to amelioration of intestinal injury and inflammation. To elucidate mechanisms by which p40 regulates mucosal immunity to prevent inflammation, this study aimed to determine the effects and mechanisms of p40 on regulation of a proliferation-inducing ligand (APRIL) expression in intestinal epithelial cells for promoting IgA production. p40 up-regulated *April* gene expression and protein production in mouse small intestine epithelial (MSIE) cells, which were inhibited by blocking EGFR expression and kinase activity. Enteroids from $Egfr^{fl/fl}$, but not $Egfr^{fl/fl}$-Vil-Cre mice with EGFR specifically deleted in intestinal epithelial cells, exhibited increased *April* gene expression by p40 treatment. p40-conditioned media from MSIE cells increased B cell class switching to IgA^+ cells and IgA production, which was suppressed by APRIL receptor neutralizing antibodies. Treatment of B cells with p40 did not show any effects on IgA production. p40 treatment increased *April* gene expression and protein production in small intestinal epithelial cells, fecal IgA levels, IgA^+B220^+, IgA^+CD19^+, and IgA^+ plasma cells in lamina propria of $Egfr^{fl/fl}$, but not $Egfr^{fl/fl}$-Vil-Cre mice.

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Thus, p40 up-regulates EGFR-dependent APRIL production in intestinal epithelial cells, which may contribute to promoting IgA production.

Keywords

APRIL; B cell; epidermal growth factor receptor; IgA; intestinal epithelial cell; Lactobacillus GG; p40

INTRODUCTION

Immunoglobulin A (IgA), a dominant immunoglobulin isotype secreted into the intestinal lumen, serves as the first line of defense for maintaining mucosal homoeostasis by shaping homeostatic communities of commensal bacteria and protecting the host against pathogenic infections.[1] It is well known that IgA is able to neutralize microbial toxins and pathogens using a high-affinity binding system and prevents commensal bacteria from breaching the mucosal surface using a low-affinity binding system, which is called immune exclusion.[2, 3] Remarkably, intestinal IgA achieves both immune protection and immune exclusion in a non-inflammatory manner, thereby exerting beneficial effects on the establishment of a sustainable host–microbial mutualism.[4] Thus, strategies targeting on regulation of IgA production has the potential for enhancing innate defense against commensals and pathogens.

Microbial colonization of the intestine plays an important role in IgA production. Indeed, the number of IgA-secreting B cells is dramatically reduced in the intestine of germ-free animals,[5] which can be counteracted by introduction of the bacterial flora.[6] Commensal bacteria contribute to IgA class switching recombination in B cells through regulation of cytokine production in $CD4^+$ T cell-dependent and -independent manners[2]. For T cell-independent cytokine production, both intestinal dendritic cells[7, 8] and epithelial cells[9, 10] participate in this process. Studies regarding the roles of intestinal epithelial cells in IgA production reveal that antigens from commensal bacteria stimulate intestinal epithelial cells to secret two tumor necrosis factor family members, a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF), which direct B cell class switching towards IgA secreting plasma cells.[9, 11] These plasma cells migrate to the lamina propria under the influence of intestinal epithelial cell-derived chemokines such as CCL28.[12] Eventually, IgA binds to a polymeric Ig receptor (pIgR) on the basolateral surface of intestinal epithelial cells and translocates to the mucosal surface to exert its functions[13].

Lactobacillus rhamnosus GG (LGG), as a model commensal probiotic organism, has shown possible benefits on prevention and/or treatment of several diseases, including ulcerative colitis,[14] infectious diarrhea[15] and antibiotic-associated diarrhea.[16] To elucidate the mechanisms underlying the beneficial effects of LGG, our group has purified and cloned a LGG-derived protein, p40[17] and demonstrated that p40 transactivates epidermal growth factor receptor (EGFR) in intestinal epithelial cells through activation of a disintegrin and metalloproteinase domain-containing protein-17 (ADAM-17) for HB-EGF release.[18] Activation of EGFR in intestinal epithelial cells by p40 is required for amelioration of intestinal injury and inflammation.[19]

To further elucidate the mechanisms underlying prevention of inflammation by p40, this study was focused on investigating the effects of p40 on IgA production in the intestine. We found that p40 up-regulated APRIL expression in intestinal epithelial cells in an EGFR-dependent manner, thereby increasing IgA class switching in B cells and IgA production in the intestine. Thus, these results provide new information for understanding the roles of p40 in maintaining intestinal immunological homoeostasis through promoting IgA production, which may contribute to p40-mediated prevention of intestinal inflammation.

RESULTS

p40 stimulates *April* gene expression in mouse small intestine epithelial (MSIE) cells, which promotes IgA production in B cells

It has been shown that intestinal bacteria trigger T-cell-independent B cell class switching in lamina propria for IgA production through expression of cytokines, such as APRIL.[9] LGG has been reported to strengthen the immune response to viral vaccines by increasing production of IgA.[20, 21] Thus we investigated the effects of p40-regulated intestinal epithelial cell responses on promoting IgA production.

First, we examined whether p40 stimulated MSIE cells to produce factors for promoting activation-induced cytidine deaminase (AID) expression, IgA class switching, and IgA production in B cells. Naïve B cells isolated from the mouse spleen were cultured for 4 days with the treatment of p40-conditioned medium from MSIE cells. B cells were also treated with p40 to examine whether p40 had direct effects on B cells. B cell IgA class switching was examined using flow cytometry analysis. The proportion of IgA^+B220^+ cells was higher in B cells treated with p40-conditioned medium than that treated with the control-conditioned medium (Figure 1A and 1B). Supernatants from B cell culture were prepared for ELISA to detect the IgA level. The level of IgA produced by B cells treated with p40-conditioned medium was significantly higher than that by B cells treated with the control-conditioned medium (Figure 1C). Furthermore, the p40-conditioned medium increased AID expression level in B cells (Figure 1D). However, B cells treated with p40 directly did not show effects on AID expression, IgA class switching and IgA production (Figure 1A–1D). In addition, we found that neither p40-conditioned medium nor p40 direct treatment affected B cell proliferation (Supplemental Figure 1).

Next, we determined which factors in p40-conditioned medium mediate increased IgA production. APRIL is a known cytokine secreted by intestinal epithelial cells to trigger IgA production.[9] We found that p40 up-regulated *April* gene expression in MSIE cells in a time and concentration-dependent manner (Figure 2A). The production of APRIL was examined by Western blot analysis of cellular lysates and ELISA analysis of APRIL release in cell culture medium. Increased cellular level of APRIL was identified in MSIE cells with p40 treatment (Figure 2B). The p40 treatment stimulated release of APRIL in MSIE cell culture medium after 6-hour treatment, as compared to control medium with the same treatment time (Figure 2C).

APRIL binds two receptors on B cells, BCMA and TACI. These interactions are necessary for IgA class switching and IgA production.[22] To examine whether MSIE-derived APRIL

stimulated by p40 mediated IgA induction, B cells were treated with p40-conditioned medium with APRIL receptor neutralizing antibodies, BCMA-Ig and TACI-Ig, which block APRIL binding to its receptor. MSIE cells treated with IgG were used as control. BCMA-Ig and TACI-Ig, but not control IgG, significantly reduced p40-conditioned medium-up-regulated proportion of IgA^+B220^+ B cells and production of IgA (Figure 2C and 2D), suggesting that APRIL mediates IgA production by conditioned-media from p40-treated MSIE cells.

Interestingly, control-conditioned medium from MSIE cells up-regulated the proportion IgA^+B220^+ B cells (Figure 1B), and this effect was not affected by BCMA-Ig and TACI-Ig (Figure 2C). These data indicate that MSIE cells secrete some factors, in addition to APRIL, in a manner independent of p40 treatment, for regulating B cell class switching.

Taken together, these data indicate that p40 enhanced-IgA production by B cells is through stimulation of APRIL secretion by intestinal epithelial cells. However, p40 does not have direct effects on B cell activity.

Activation of EGFR by p40 mediates *April* gene expression in MSIE cells

EGFR is a transmembrane receptor composed of an extracellular-ligand binding domain and an intracellular tyrosine kinase domain. Activation of EGFR leads to intracellular signaling networks and ultimately defines biological responses, such as proliferation, differentiation, migration, and survival.[23] We have shown that p40 transactivates the EGFR through its downstream target, PI3K/Akt, in colonic epithelial cells.[17, 19] We further found that p40 transactivated EGFR and Akt in MSIE cells (Figure 3A). To investigate the requirement of EGFR and Akt for p40-stimulated APRIL expression, we treated MSIE cells with p40 in the presence or absence of AG1478, a EGFR kinase inhibitor, and LY294002, an inhibitor of phosphoinositide 3-kinase to block Akt activation). p40- and EGF-stimulated expression of APRIL in MSIE cells (Figure 3B) and release of APRIL (Supplemental Figure 2) were suppressed by AG1478 and LY294002.

To confirm these results, we transfected mouse EGFR siRNA into MSIE cells to decrease EGFR expression. The gene expression and protein level of APRIL were significantly suppressed in MSIE cells with decreased EGFR expression, as compared to those in non-transfected and non-targeting transfected cells (Figure 3C and 3D).

It has been reported that NF-κB serves as a transcriptional factor for *April* gene expression,[24] and EGFR signaling mediates NF-κB activation.[25–27] We examined whether p40 activated NF-κB and whether EGFR-dependent APRIL expression by p40 was through activating NF-κB. We found that p40 activated NF-κB detected by increased NF-κB p65 subunit nuclear translocation in MSIE cells, which was inhibited by EGFR and Akt inhibitors (Figure 3E). These results suggest that p40 activates NF-κB in MSIE cells, which is mediated by EGFR activation. To examine whether NF-κB mediates p40-stimulated *April* expression, MSIE cells were co-treated with p40 and a NF-κB inhibitor, PDTC. PDTC blocked p40-stimulated NF-κB p65 translocation (Figure 3F) and APRIL expression (Figure 3G) in MSIE cells.

Furthermore, *Egfr*$^{fl/fl}$-Vil-Cre mice with constitutive deletion of EGFR in the intestinal epithelial cells generated by our laboratory,[19] and their littermate, *Egfr*$^{fl/fl}$ mice, were used for generating small intestinal enteroids. Consistent with our previous findings that p40 stimulates intestinal epithelial cell proliferation,[17] p40 treatment stimulated growth of enteroids from *Egfr*$^{fl/fl}$, but not from *Egfr*$^{fl/fl}$ -Vil-Cre mice (Figure 4A and 4B). In enteroids from *Egfr*$^{fl/fl}$, *April* expression was significantly increased by p40 treatment, as compared to un-treated group. However, p40 failed to stimulate *April* expression in enteroids from *Egfr*$^{fl/fl}$-Vil-Cre mice (Figure 4C).

These results suggest that activation of EGFR and Akt by p40 mediates APRIL expression in MSIE cells. Furthermore, EGFR-dependent NF-κB activation by p40 may be involved in transcriptional regulation of APRIL expression.

Expression of EGFR in the intestinal epithelium mediates p40-stimulated APRIL expression, B cell class switching, and IgA production in mice

We have demonstrated that p40 transactivation of EGFR in the intestinal epithelium plays a protective role in intestinal inflammatory disorders.[19] It is important to determine the role of EGFR in intestinal epithelial cells in p40-stimulated IgA production, which may contribute to protective effects of p40 on intestinal inflammation.

To investigate whether p40 promoted IgA$^+$ B cell development and IgA production *in vivo*, WT mice were gavaged with p40 containing pectin/zein beads for 3 weeks. Pectin/zein beads without p40 were used to treat mice as controls. The fecal IgA level was significantly elevated in the mice at 2 and 3 weeks after p40 treatment as detected in our experiment. Control bead treatment did not affect the fecal IgA levels (Figure 5A). Consistent with the results, the mRNA level and protein production of APRIL in small intestinal epithelial cells were significantly increased in mice with p40 treatment (Figure 5B–5D). Furthermore, p40 treatment increased the number of AID (Figure 5E and 5F) and IgA (Figure 5G and 5H) expressing cells in the lamina propria of the ileum in wt mice. These findings suggest that p40 plays a role in up-regulation of APRIL expression in intestinal epithelial cells for IgA production *in vivo*.

Our *in vitro* data indicate that p40 stimulates APRIL expression in an EGFR-dependent manner. Results from *in vivo* studies further showed that p40 failed to up-regulate fecal IgA levels (Figure 6A), *April* gene expression (Figure 6B) and protein production (Figure 6C and 6D), and the number of AID expressing cells in the lamina propria of the small intestine (Figure 6E and 6F) in *Egfr*$^{fl/fl}$ -Vil-Cre mice.

Flow cytometry analysis was performed to examine cell populations of B220 and CD19 with IgA expression in the lamina propria of the small intestine and Peyer's patches. p40 treatment did not affect the total number of B220$^+$ and CD19$^+$ cell populations in the lamina propria of the small intestine and Peyer's patches in *Egfr*$^{fl/fl}$ and *Egfr*$^{fl/fl}$-Vil-cre mice (Supplemental Figure 3). p40 treatment increased populations of IgA$^+$B220$^+$ B and IgA$^+$CD19$^+$ B cells and IgA$^+$B220$^-$ and IgA$^+$CD19$^-$ plasma cells in the lamina propria in *Egfr*$^{fl/fl}$, but not *Egfr*$^{fl/fl}$-Vil-cre mice (Figure 7A and 7B). However, no significant effect of p40 on regulating B cell class switching was found in the Peyper's patches in *Egfr*$^{fl/fl}$ and

Egfr$^{fl/fl}$-Vil-cre mice (Supplemental Figure 4). Thus, these data suggest that the effect of p40 on stimulation of B cell class switch and IgA production occurs in the lamina propria of the small intestine, and this effect is through up-regulation of EGFR-dependent APRIL expression in the intestinal epithelial cells.

DISCUSSION

One of the significant functions of the intestinal epithelial cells is their contribution for regulating the mucosal immunity in response to the intestinal microbiota. Thus, we have expanded the scope of our work from understanding the mechanisms underlying p40-regulated intestinal epithelial cell survival and barrier function to elucidating the effects of p40 on IgA production in the intestinal tract. In the present study, we demonstrated that p40 stimulated APRIL expression in intestinal epithelial cells and B cell IgA class switching in the lamina propria, thereby increasing IgA production. Thus, our studies provide new information for understanding the roles of probiotics in regulation of mucosal immunity, and will help to guide clinical application of probiotics for host health and preventing diseases. However, although the requirement of APRIL for IgA production has been demonstrated by studies that *April*-deficient mice have selectively impaired IgA class switching and IgA production,[28] it would be important to determine the involvement of p40-stimuated APRIL production for increasing IgA production by using APRIL deficient mice. These experiments are undergoing in our laboratory.

This study expanded the scope of our previous finding of transactivation of EGFR by p40 in intestinal epithelial cells[19, 29] to identify the role of EGFR activation by p40 in regulation of APRIL expression in intestinal epithelial cells. The expression of APRIL in gene and protein levels was remarkably decreased in intestinal epithelial cells with EGFR knock down, resulting in suppressing of IgA production, in *in vitro* and *in vivo* experiments. Although EGFR signaling is well-known to regulate several cellular responses in epithelial cells, our study first reveal the involvement of EGFR signaling regulated by the microbiota-derived factor, p40, in regulation of APRIL expression, thereby supporting epithelial immune functions. Furthermore, activation of NF-κB has been shown to be involved in *April* expression.24 For example, the TLR pathway has been reported to mediate APRIL production through activation of NF-κ$^{B.10}$ We found that p40 activated NF-κB in MSIE cells, which was mediated by EGFR and Akt activation. Furthermore, blocking NF-κB suppressed the effect of p40 on the elevation the expression level of APRIL. All these results support the involvement of NF-κB pathway in the regulation of IgA production via EGFR activation by p40.

It should be noted that it has been reported that BAFF, secreted by intestinal epithelial cells, serves as another inducer of T cell-independent IgA production.[11] Interestingly, our studies found that p40 up-regulated BAFF gene expression and protein production in MSIE cells (Supplemental Figure 5A and 5B) and in intestinal epithelial cells in mice (Supplemental Figure 5E and 5F). These effects by p40 depended on EGFR expression in intestinal epithelial cells because p40 failed to stimulation of BAFF production in MSIE cells with EGFR knock down (Supplemental Figure 5C and 5D) and in *Egfr*$^{fl/fl}$ Vil-Cre mice (Supplemental Figure 5E and 5F). Thus, in addition to APRIL, BAFF may contribute to up-

regulation of IgA production by p40. Since the effect of p40 on up-regulation of APRIL production is more potent than that on BAFF production by intestinal epithelial cells, this study was focused on investigating the regulatory role of p40 in APRIL production.

Intestinal epithelial cells also produce chemokines in a toll-like receptor (TLR) 4 dependent manner, including CCL20 and CCL28, to recruit B cells into lymphoid tissues and lamina propria of the intestine.[10] We noticed that the proportion of $B220^+$ and $CD19^+$ B cell in lamina propria and Peyer's patches did not increase under p40 treatment in mice, which suggest that p40 does not influence the recruitment of B cells into the intestine and B cell proliferation and survival. This evidence was further supported by our *in vitro* studies that p40 and p40-conditioned medium did not stimulate B cell proliferation. However, the possibility that p40 treatment enhances the survival of the IgA^+ plasma cell population, in addition to B cell class switching, to increase IgA production cannot be fully excluded. Thus more experiments are needed to evaluate whether p40 treatment stimulates proliferation and survival of the IgA^+ plasma cell population, as a mechanisms for up-regulation of IgA production.

Regulation of IgA responses also occurs in a region-specific manner throughout the intestine. T cell-independent mucosal IgA production, which requires cytokines and growth factors for stimulation of B cell activation, has been reported to occur in isolated lymphoid follicles and in the lamina propria without the formation of germinal centers.[8, 30] For example, studies have demonstrated that RORγt+ innate lymphoid cells produce membrane-bound lymphotoxin γ to stimulate T cell-independent IgA induction in the lamina propria.[31] This concern is further supported by the study showing that microbial colonization influences early B-lineage development in the intestinal lamina propria.[32] To provide information about the biogeography of EGFR-induced APRIL and BAFF-mediated IgA induction by p40, our study demonstrated the effects of p40 on B cell class switching in the lamina propria of the small intestine. No inference on B cell class switching in the Peyper's patches was found in mice treated with p40. Thus, these data suggest that the effect of p40 on stimulation of B cell class switch and IgA production may occur in the lamina propria.

Recent studies have shown that IgA in breast milk promoted long-term intestinal homeostasis and ameliorates colonic damage caused by DSS in adult mice[33], and bacteria coated with IgA are potentially responsible for driving intestinal inflammation in patients with inflammatory bowel disease suggested the protective role of IgA in gut inflammation.[34] We have demonstrated that p40 treatment prevents and treats DSS-induced colon epithelial cell injury and inflammation and ameliorates oxazolone and TNBS-induced colitis in mice, which requires activation of EGFR in intestinal epithelial cells.[19] These effects are associated with p40-regulated EGFR-dependent reduction of intestinal epithelial apoptosis and disruption of barrier function in the colonic epithelial cells in these animal models of colitis.[19] We further found that p40 stimulated higher level of *April* expression in intestinal epithelial cells in $Egfr^{fl/fl}$ mice with DSS-induced colonic injury and colitis than that in $Egfr^{fl/fl}$-Vil-Cre mice (Supplemental Figure 6). In fact, *April* knock out mice have enhanced Th2 cytokine production under non-polarizing conditions.[35] Thus, results from this work further emphasize the importance of promotion of IgA production and enhancement of innate defenses by p40 in prevention and treatment of intestinal inflammatory diseases.

Since it has been reported that most commensal bacteria are targeted by T-independent IgA responses,[36] it is interesting to evaluate the effects of p40 treatment on IgA-coated intestinal bacteria, which might provide more mechanisms for understanding prevention of intestinal inflammation by p40.

p40 used for this study was purified from LGG culture broth. It is possible that this p40 preparation may be contaminated by other LGG components. Our previous works have provided evidence to confirm that the effects on regulation of intestinal epithelial cells are from p40, not from the contaminated LGG components.[19] We expressed the N-terminal 1-180 aa and the C-terminal portion (aa 181-412) of p40 as recombinant peptides and found that the p40 N-terminal peptide was sufficient to stimulate EGFR and Akt activation in intestinal epithelial cells and prevent DSS-induced colitis. However, the C-terminal portion of p40 had no effect on EGFR and Akt activation and DSS-induced colitis. These data not only suggest that the p40 functional domain localizes in the N-terminal 1-180 aa, but indicate that the regulatory effects on intestinal epithelial cells are not exerted by contaminated bacterial components in the p40 preparation.

In summary, results from this study demonstrate that p40-stimulated activation of EGFR in intestinal epithelial cells mediates the production of APRIL, B cell class switch in the lamina propria, and IgA production. These results reveal a mechanism of LGG in maintenance of intestinal mucosal homoeostasis through regulation of IgA production. Furthermore, these studies support the feasibility of applying microbiota-derived products as a strategy to promote intestinal health.

METHODS

MSIE cell culture and treatment

MSIE cells were isolated from the small intestinal epithelium of H-2Kb-tsA58 mice (Immortomouse).[37] MSIE were maintained in RPMI 1640 media supplemented with 5% fetal bovine serum (FBS), 5 U/ml of murine IFN-γ, 100 U/ml penicillin and streptomycin, and ITS (5 Rg/ml insulin, 5 Rg/ml transferrin, 5 ng/ml selenous acid) at 33°C (permissive condition) with 5% CO2. Prior to experiments, cells were serum-starved for 16–18 hours in RPMI 1640 containing 0.5% FBS and 100 U/ml penicillin and streptomycin at 37°C (non permissive conditions).

p40 was isolated from LGG culture supernatant, as previously described.[17] Cells were treated with p40 and human EGF (PeproTech, Rock Hill, NJ) in the presence or absence of an EGFR tyrosine kinase inhibitor, AG1478 (Calbiochem - EMD Millipore Corporation, Billerica, MA), a phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 (Calbiochem), and a NF-κB inhibitor, PDTC (Sigma-Aldrich, St Louis, MO).

Transient transfection of siRNA

MSIE cells were transiently transfected with either mouse EGFR siRNA (Dharmacon, Lafayette, CO) or nontargeting siRNA using Lipofectamine RNAiMAX reagent (Invitrogen, Waltham, MA) for 6 hours, according to the manufacturer’s instructions. Cells were cultured

for 24 hours after transfection at 33 °C and then were serum-starved under the nonpermissive condition for 18 hours before treatment.[29]

Isolation, culture, and treatment of B lymphocytes

B cells were isolated from the spleen of C57BL/6 mice. The spleen was mashed, suspended in Magnetic cell sorting (MACS) buffer, and passed through a 70 μm cell strainer (BD Biosciences, San Diego, CA). B cells were purified using Mouse B lymphocyte enrichment set-DM (BD IMag™, BD Biosciences) according to the manufacturer's instructions. The biotin mouse B lymphocyte enrichment cocktail containing monoclonal anti-mouse CD4, anti-mouse CD43, and anti-mouse TER-119/erythroid cells antibodies was used for the negative selection of resting conventional ($CD5^-$) B cells.

Naive B cells (1×106/well) were cultured in 96-well dish in 100 μL of RPMI 1640 medium containing 10% FBS, 5 μM 2-mercaptoethanol (2-ME), and 100 U/ml penicillin and streptomycin for 6 hours, Then B cells were treated with p40 and conditioned media from MSIE cells with or without p40 treatment in the presence and absence of two goat anti-mouse neutralizing antibodies against APRIL binding receptors on B cells, TACI/TNFRSF13B, which recognizes Phe5-Thr129 in TACI (1 μg/ml) and BCMA/TNFRSF17, which recognizes Met1-Thr49 in BCMA (1μg/ml) (R&D Systems, Minneapolis, MN). Goat IgG (1 μg/ml) was used a control.

Mice and treatment

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Vanderbilt University. *Egfr*$^{fl/fl}$ mice on a C57BL/6 background were crossed with villin-Cre mice on a C57BL/6 background to generate constitutive intestinal epithelium–specific EGFR-knockout (*Egfr*$^{fl/fl}$-Vil-Cre) mice.[19] Their littermates, *Egfr*$^{fl/fl}$ mice were used as controls.

To deliver p40 to the small intestine and the colon, p40-containing pectin/zein beads were prepared using the method, as described in our previous publication,[19] with a modification. p40 was dissolved in pectin solution (4% w/v) in water, and then encapsulated by zein solution (0.5% w/v in 85% ethanol) containing $CaCl_2$ (0.5% w/v). Each bead contains 5 μg of p40 with the volume of 50 μl. As negative controls, pectin/zein beads without p40 were prepared.

Four- to six-week old mice were gavaged with pectin/zein beads containing p40 (10 μg/day) and pectin/zein only beads, as control, for three weeks. Fecal samples were collected before and after p40 treatment. Small intestinal epithelial cells were isolated for RNA isolation and cellular lysates preparation. Small intestinal issues were fixed in 10% formalin for preparation of paraffin-embedded sections for immunohistochemistry.

Culture of small intestinal enteroids

The ileum tissues were cut into small fragments and washed in cold PBS buffer. Tissues were incubated in chelation buffer containing 5.6 mM Na_2HPO_4, 8 mM KH_2PO_4, 96.2 mM NaCl, 1.6 mM KCl, 43.4 mM sucrose, 54.9 mM D-sorbitol and 2 mM EDTA for 20 minutes

at 4 °C. Then villi were released by shaking tissues in cold chelation buffer containing 0.5 mM DTT. Supernatent were filterd through 70 μm cell stainer (BIOLOGIX, Lenexa, KS) and centrifuged. Villi were washed in cold chelation buffer, mixed with Matrigel containing 25% of IntestiCult™ Organoid Growth Medium (STEMCELL™, Vancouver, Canada) and overlayed with this medium. Culture was performed in the absence or presence of p40 (100 ng/ml) in matrigel and medium.

Isolation of lymphocytes from mouse lamina propria and Peyer's patches

As described previously,[38] after removing Peyer's patch, the small intestinal tissues were flushed with ice-cold calcium- and magnesium-free HBSS containing 2% FBS, cut into small pieces, and incubated in HBSS supplemented with 1 mM DTT for 40 minutes with shaking at 37 °C, followed by HBSS containing 1.3 mM EDTA for 1 hour at 37°C. Tissues were further incubated in 1 U/mL collagenase D in RPMI medium for 1.5 h at 37 °C. Lamina propria lymphocytes were isolated on a 44/67% Percoll density gradient.

Lymphocytes from Peyer's patches were isolated by gently crushing the patches and incubating in Dispase and DNase solution with constant stirring at 37 °C. Lymphocytes from the supernatant were washed with RPMI.

Flow cytometry analysis

Cultured B cells and lymphocytes isolated from lamina propria and Peyper’s patches of the mouse small intestine were labeled using the following antibodies: PE-anti-CD45R (B220), eBioscience, San Diego, CA), FITC-anti-IgA, PE-Cy7-CD19, (BD Pharmingen, San Diego, CA), PE-rat IgG2a isotype control (eBioscience), and FITC-rat IgG1 isotype control (BD Pharmingen) for 1 h at room temperature. Cells were analyzed using multi-color flow cytometry to determine the percentage of positive cells using a BD LSRII system (BD Biosciences).

Isolation of small intestine epithelial cells from mice

Small intestinal epithelial cells were isolated using a modified protocol.[39] The small intestine was cut in to small pieces and incubated with 0.5 mM dithiothreitol and 3 mM EDTA at room temperature for 1.5 hours without shaking. After gently removing the solution, PBS was added to the small intestine. Crypts released from the small intestine by shaking the tubes. Cells were washed with PBS by centrifuging and then were solubilized in 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 cocktail (Sigma-Aldrich). The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc.).

Immunohistochemistry

To unmask antigens, small intestinal sections were boiled in Antigen Unmasking Solution (C, Inc. Burlingame, CA). Then sections were blocked using 10% goat serum and stained with a rat anti-mouse IgA antibody (BD Pharmingen) and a rat anti-mouse AID antibody (eBioscience) overnight at 4°C, followed by a goat FITC-labeled anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. Sections were

then mounted using Mounting Medium containing DAPI (Vector laboratories) for nuclear counter-staining and observed using fluorescence microscopy. FITC and DAPI images were taken from the same field. Positive cells in 500 vili in ileum were counted.

Real-time PCR analysis

Total RNA was isolated from MSIE cells, enteroids, intestinal epithelial cells isolated from mice using the Qiagen RNA isolation kit and was treated with RNase-free DNase. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit for Real Time-PCR reactions, using the April primer mix (Mm03809849-s1), cDNA template, and Taqman Gene Expression Master Mix. Real-Time PCR was performed using the Bio-Rad PCR System (Bio-Rad, Hercules, CA). The data were analyzed using the Bio-Rad CFX manager software. The relative abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize levels of the mRNAs of interest. All cDNA samples were analyzed in triplicate.

Preparation of cellular lysates and Western blot analysis

MSIE cells and intestinal epithelial cells isolated from mice were solubilized in cell lysis buffer to prepare total cellular lysates. Nuclear fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc.).

Cellular and nuclear lysates were mixed with Laemmli sample buffer and proteins were separated by SDS-PAGE for Western blot analysis using anti-phospho-Ser473(P)-Akt (Cell Signalling Danvers, MA), anti-phospho-Tyr1068-EGFR (Cell Signalling), anti-total EGFR (Millipore, Billerica, MA), anti-APRIL (Thermo scientific), anti-BAFF (Enzo Biochem, Inc, Farmingdale, NY), anti-AID (eBioscience), anti-p65 (Santa Cruze, Dallas, TX), anti-fibrillrin (Santa Cruze), and anti-β actin (Sigma-Aldrich) antibodies.

ELISA analysis

For detecting IgA levels in mouse feces and B cell culture supernatants, feces were weighted and homogenized in PBS (10% wt:vol) using TissueLyser. Cell culture supernatants were prepared from B cell culture. Ninety-six-well flat bottom plates (Corning Inc, Corning, NY) were coated with anti-mouse IgA (Sigma-Aldrich) in PBS at 4 °C overnight. The plates were washed with wash buffer (0.05% Tween 20 in PBS) and blocked by 1% BSA in PBS for 1 hour at room temperature. Then the plates were incubated with supernatants of fecal samples and culture supernatants from B cells for 2 hours at room temperature, followed by a FITC-conjugated anti-mouse IgA (Sigma-Aldrich) in PBS for 1 hour at room temperature. Fluorescent intensity at 475-nm wavelength was measured using a fluorescent plate reader. Purified mouse IgA (Sigma-Aldrich) was used for generating a standard concentration curve. The IgA concentration in feces was calculated as of IgA μg/g feces and in B cell culture supernatants as IgA ng/ml culture supernatant.

For testing APRIL release in cell culture supernatants, cell culture supernatants were prepared from cultured MSIE cells and the level of APRIL was measured using a mouse

APRIL ELISA kit (Neobiolab, Woburn, MA), according to the manufacturer's instruction. The IgA concentration was calculated as of IgA ng/mg of cellular proteins.

Statistical analysis

Statistical significance was determined by one-way ANOVA analysis for multiple comparisons and T-test for two sets of comparisons using Prism 6 (GraphPad Software, Inc. San Diego, CA). A p value < 0.05 was defined as statistically significant. Data are presented as mean±S.E.D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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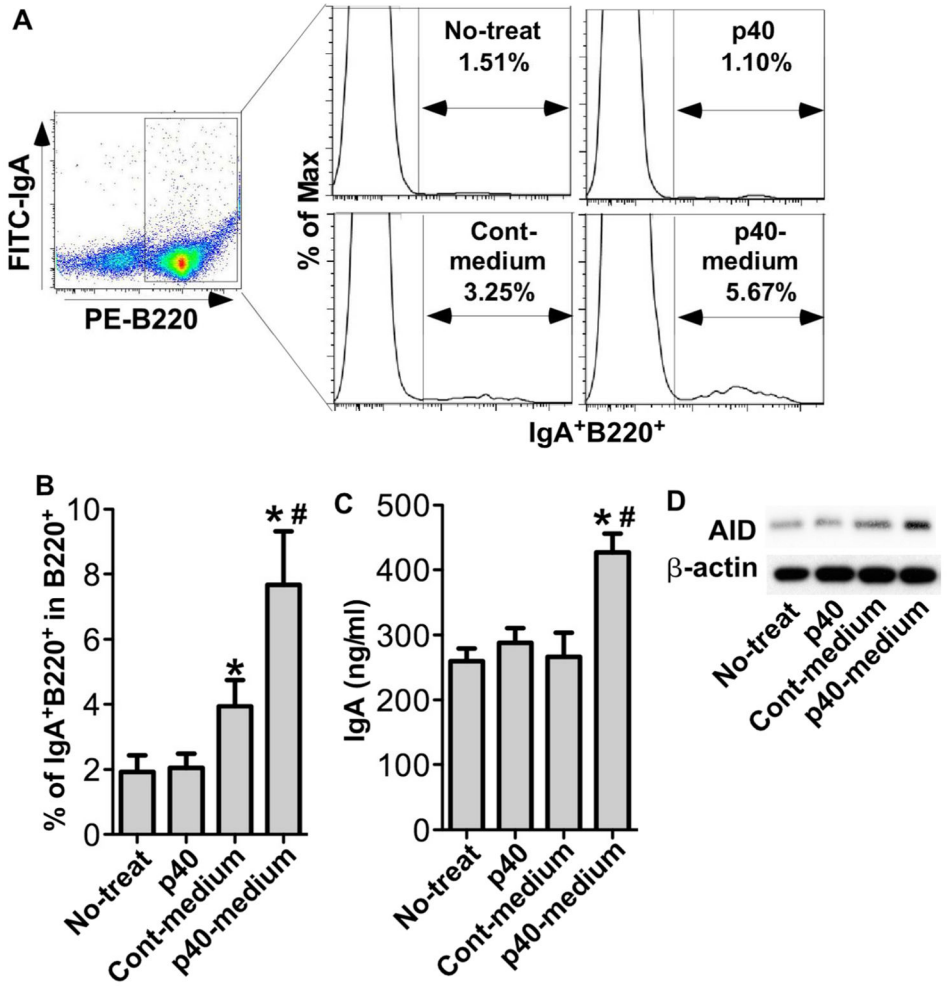

Figure 1. p40-conditioned medium from MSIE cells, but not p40, promotes IgA production in B cells

MSIE cells (5×10^5cells/well) were treated with p40 at 10 ng/ml in RPMI 1640 medium containing 0.5% FBS for 6 h to prepare conditioned media. B cells isolated from wt mouse spleen (10^6/well) were cultured in 100 μl of RPMI containing 10 % FBS and 5 μM 2-ME for 6 h, then treated without (No-treat) and with p40 at 10 ng/ml (p40) in 100 μl of B cell culture medium, conditioned media from untreated (Cont-medium) and p40-treated (p40-medium) MSIE cells (adjust FBS to 10 % and 2-ME to 5 μM) for 4 days. (A and B) Characterization of IgA class switching in B cells was performed by staining B cells using FITC-labeled anti-IgA and PE-labeled anti-B220 antibodies and analyzed using flow cytometry. $B220^+$ cells were selected for analyzing the percentage of IgA^+ cells in $B220^+$ cells. (C) Supernatants from B cell culture were collected for analysis of the IgA level using ELISA . (D) Total cellular proteins from B cells were prepared for Western blot analysis of the AID protein level. β-actin blot was used as the protein loading control. In B abd C, * $p<0.05$ compared to the No-treat group. # $p<0.05$ compared to the Cont-medium group. Data in B and C are quantified from three independent experiments. Data in D are representative of three independent experiments.

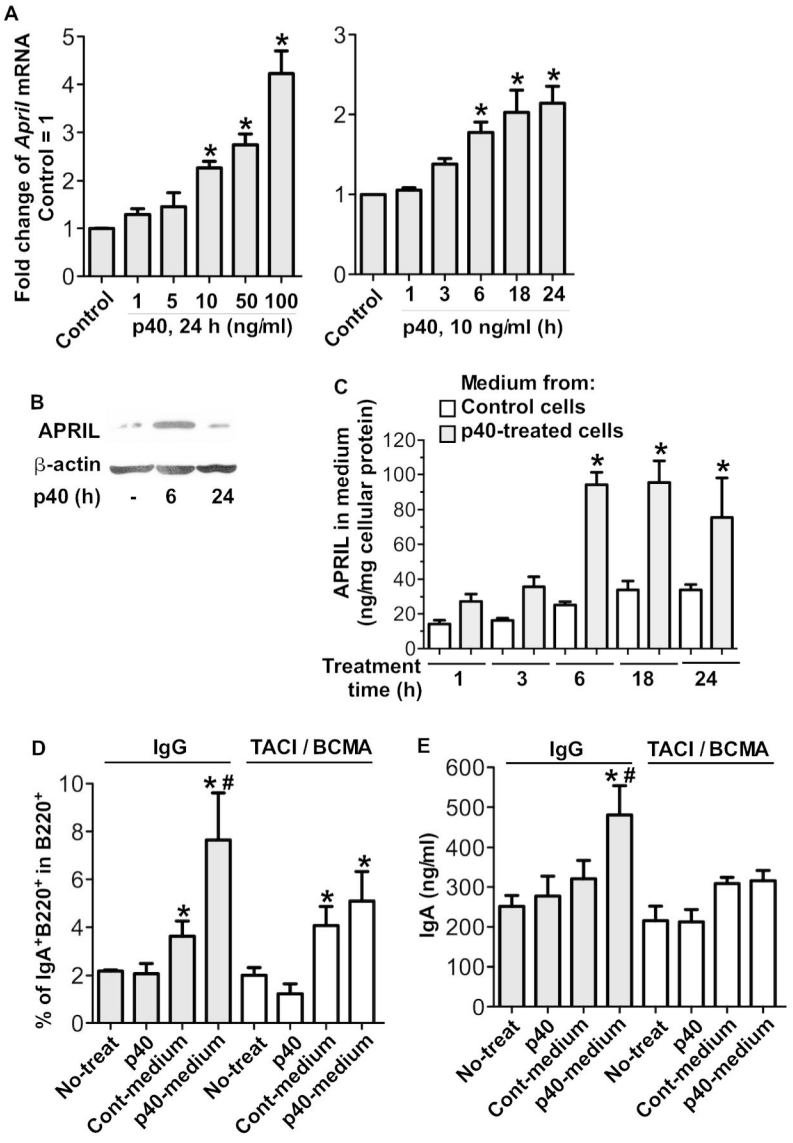

Figure 2. p40 stimulates *April* gene expression and protein production in MSIE cells, which mediates up-regulation of IgA production in B cells by p40-conditioned media

(A) MSIE cells were treated with p40 at the indicated concentrations for indicated times. RNA was isolated for real-time PCR analysis of the *April* mRNA level. The *April* mRNA expression level in the control group was set as 1, and mRNA expression levels in treated groups were compared to the control group. (B) MSIE cells were treated with p40 at 10 ng/ml for indicated times. Total cellular proteins were prepared for Western blot analysis of the APRIL protein level. β-actin blot was used as the protein loading control. (C) Supernatants from MSIE cell culture with and without p40 (10 ng/ml) treatment for the indicated times were collected for analysis of the APRIL release using ELISA . (D and E) B cells were treated with p40 and p40-conditioned media for 4 days, as described in Figure 1, in the presence of neutralizing antibodies, TACI-Ig (1 μg/ml) and BCMA-Ig (1 μg/ml), to block APRIL function, and IgG (1 μg/ml) as control. IgA class switching and IgA production in B cell culture supernatants were examined as described in Figure 1. The

percentage of IgA^+B220^+ cells in $B220^+$ cells (D) and IgA levels in B cell culture supernatants (E) are shown. * $p<0.05$ compared to the control group (A), control group with the same treatment time (C), and No-treat group (D and E), # $p< 0.05$ compared to the Cont-medium group (D and E). Data in A, C, D, and E are quantified from at least three separate experiments. Data in B are representative of three independent experiments.

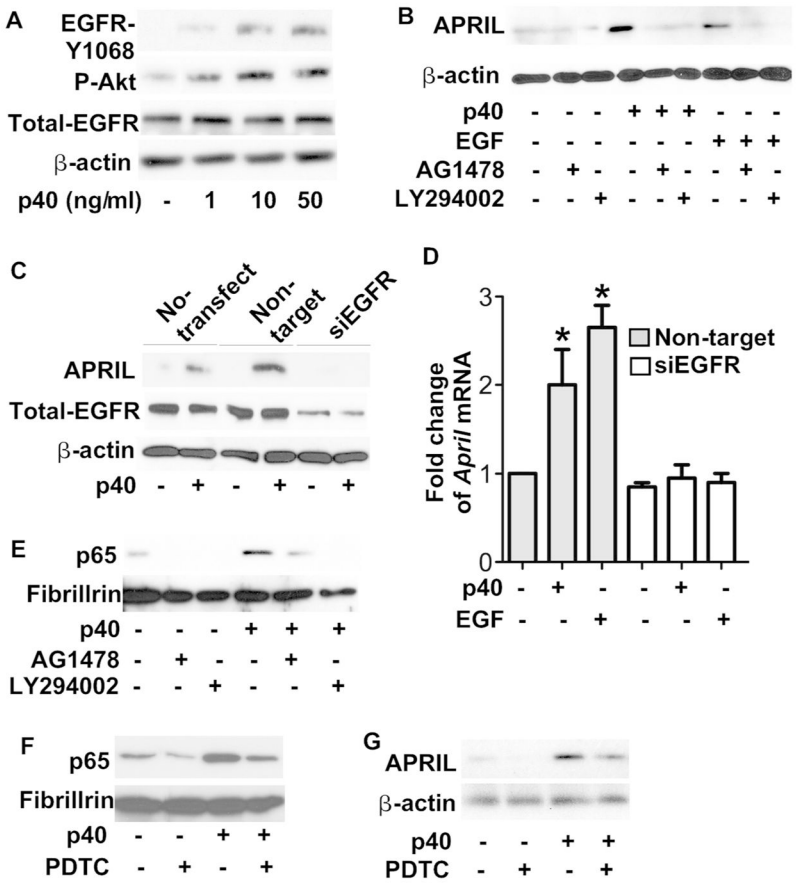

Figure 3. Activation of EGFR mediated p40-promoted APRIL expression in MSIE cells

(A and B) Cells were treated with p40 at indicated concentrations for 1 h (A) or p40 at 10 ng/ml and EGF at 10 ng/ml for 6 h in the presence and absence of AG1478 (a EGFR kinase inhibitor, 150 nM) and LY294002 (a PI3K inhibitor to inhibit Akt activation, 10 μM) (B). Total cellular lysates were prepared for Western blot analysis of phosphorylated (P) and total EGFR and APRIL levels. (C and D) Cells transduced with siRNA against EGFR or non-targeting siRNA were treated with p40 (10 ng/ml) for 1 h (C) and p40 and EGF for 6 h (D). Cellular lysates were prepared for Western blot analysis of and total levels of EGFR and APRIL (C). RNA was isolated for real-time PCR analysis of the *April* mRNA level. The *April* mRNA expression level in the control group with non-target transfection was set as 1 (D). (E–G) Cells were treated with p40 (10 ng/ml for 1 h) in the presence and absence of AG1478 (150 nM), LY294002 (10 μM), and PDTC (50 μM). Nuclear proteins (E and F) and total cellular proteins (G) were prepared for Western blot analysis of NF-κB p65 subunit nuclear translocation (E and F), and APRIL expression (G). β-actin and Fibrillirin blots were used as the protein leading control for total cellular and nuclear proteins, respectively. *$p < 0.05$ compared to the control group with non-target transfection (D). Images shown in A–C and E–G are representative of three independent experiments. Data in (D) are quantified from three independent experiments.

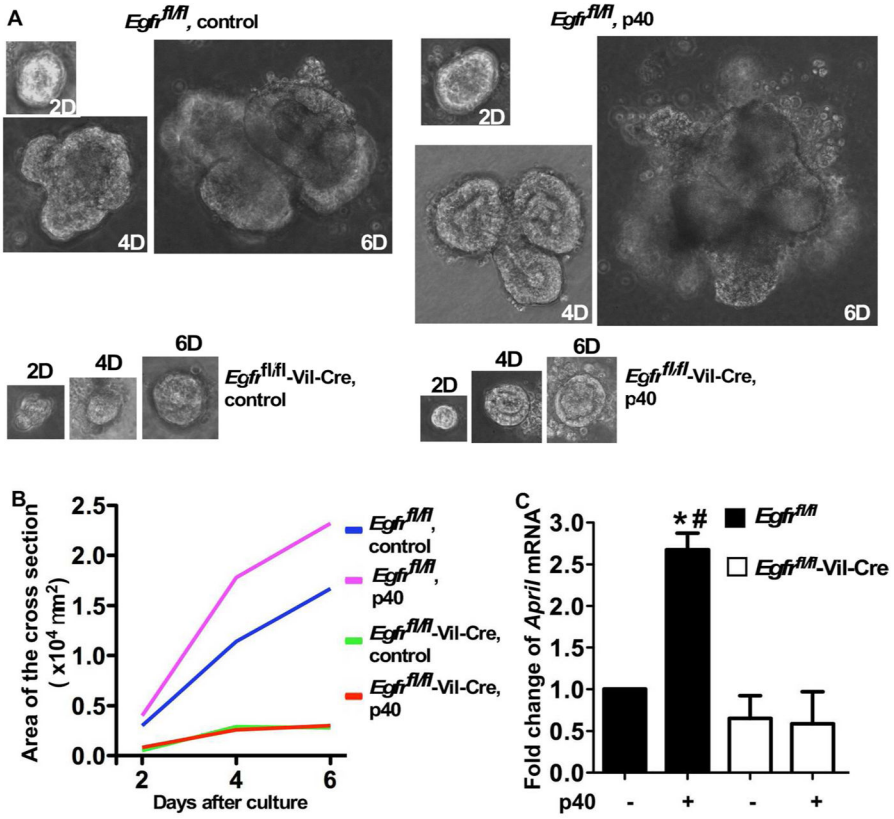

Figure 4. p40 up-regulates *April* expression in small intestinal enteroids, which requires EGFR

Enteroids generated from villi of the ileum of *Egfr*$^{fl/fl}$ and *Egfr*$^{fl/fl}$ -Vil-Cre mice were cultured in IntestiCult™ organoid growth medium in the presence and absence of p40 (100 ng/ml). (A and B) Representative images and the areas of enteroids at indicated days after culture are shown. (C) RNA was isolated from enteroids cultured for 6 days for real-time PCR analysis of the *April* mRNA level. The *April* mRNA expression level in the control group from *Egfr*$^{fl/fl}$ mice was set as 1. *p < 0.05 compared to the control group in *Egfr*$^{fl/fl}$ mice. #p < 0.05 compared to the p40-treated group in *Egfr*$^{fl/fl}$ −Vil-Cre mice. Data in B represent the average of at least 5 enteroids in each condition. Data in C are quantified from three independent enteroid cultures.

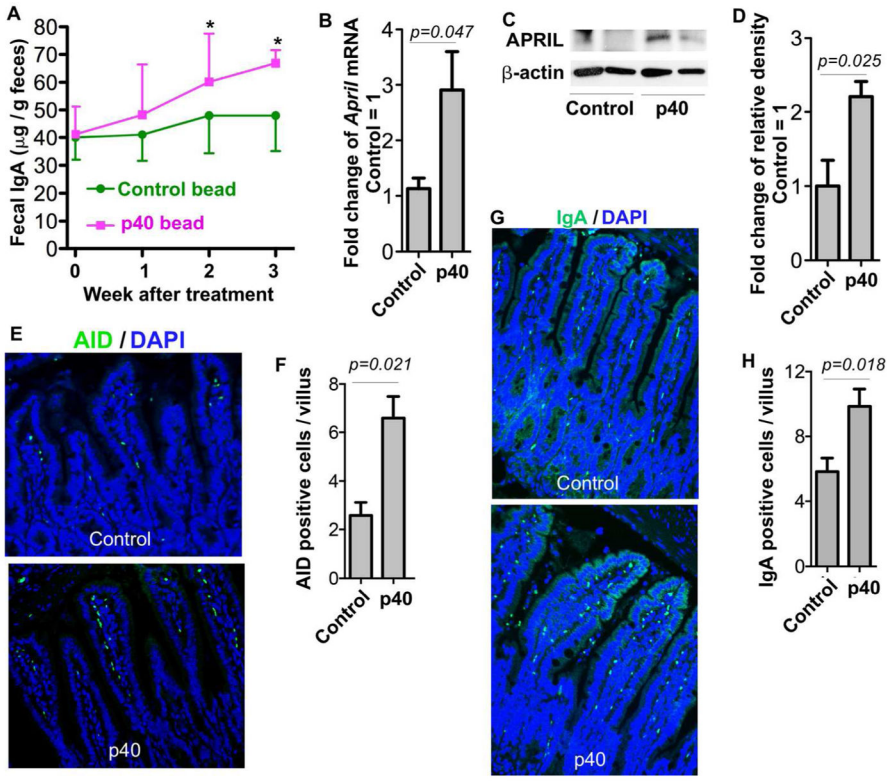

Figure 5. p40 stimulates IgA production in the intestine in wt mice

C57BL/6 wt mice were gavaged with p40 (10 μg/day) in pectin/zein beads and beads without p40 as control. (A) Feces were collected at 1, 2, and 3 weeks after p40 treatment for detecting the IgA level using ELISA. (B–D) Small intestinal epithelial cells were isolated from mice with 3-week p40 treatment. RNA was prepared for real-time PCR analysis of the *April* mRNA level (B). Total cellular lysates were prepared for Western blot analysis of the APRIL protein level (C). The relative density of protein bands was determined by comparing the density of APRIL to that of β-actin bands in the same mouse sample (D). The average of the *April* mRNA level (B) and the relative density (D) in the control group was set as 1. (E–H) Ilea tissues were prepared for AID (E) and IgA (G) immunostaining. Nuclei were stained using DAPI. The numbers of AID (F) and IgA (H) positive cells per villus are shown. * $p<0.05$ compared to the control group with the same p40 treatment time in A. N=5 in each group.

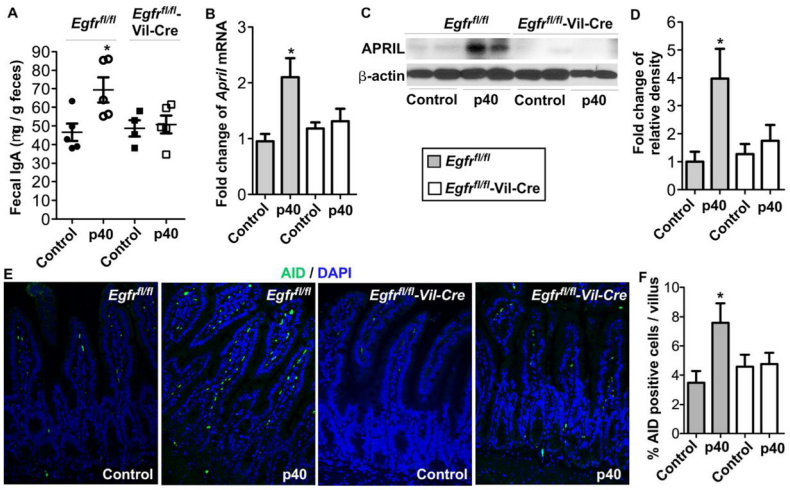

Figure 6. EGFR in intestinal epithelial cells mediates p40-stimulated APRIL and AID expression and IgA production in mice

Egfr$^{fl/fl}$ and *Egfr*$^{fl/fl}$-Vil-Cre mice were gavaged with p40 (10 μg/day) in pectin/zein beads or beads without p40 (control) for 3 weeks. (A) Feces were collected for detecting the level of IgA using ELISA. (B) RNA was prepared from small intestinal epithelial cells for real-time PCR analysis of the *April* mRNA level. (C–D)Total cellular proteins were prepared from small intestinal epithelial cells for Western blot analysis of APRIL protein level. The relative density of protein bands was determined as described in Figure 5. The average of the *April* mRNA level (B) and the relative density (D) in the control group was set as 1. (E) Ilea tissues were prepared for AID immunostaining. Nuclei were stained using DAPI. The numbers of AID positive cells per villus are shown (F). * $p<0.05$ compared to the control group in *Egfr*$^{fl/fl}$ mice. N=8–10 in each group.

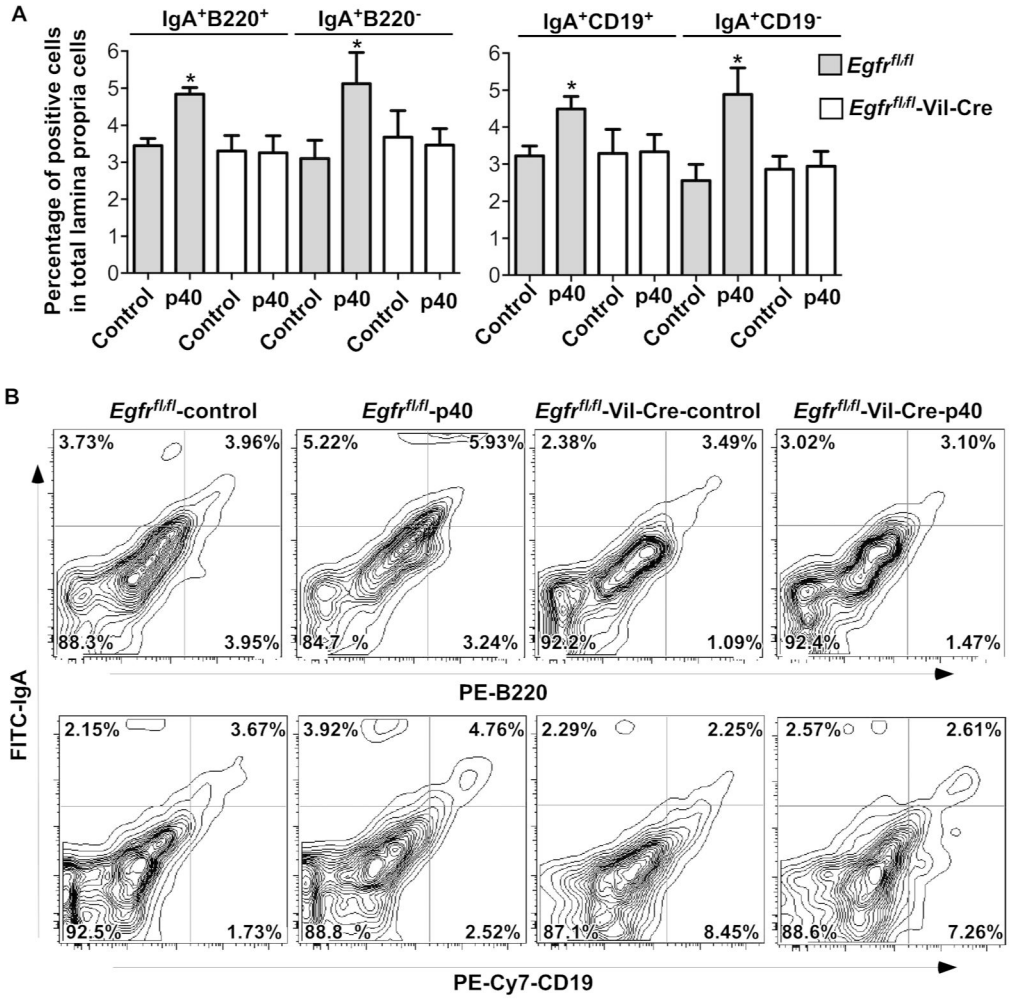

Figure 7. p40-stimulated B cell class switching in the lamina propria requires EGFR in mice

Egfr$^{fl/fl}$ and *Egfr*$^{fl/fl}$-Vil-Cre mice were treated with p40 (10 μg/day) for 3 weeks as indicated in Figure 6. Lamina propria cells were isolated from the small intestine for flow cytometry analysis of IgA, B220, and CD19 expressing cells. The percentages of indicated positive cells in total lamina propria cells (A) and couture plots (B) are shown. * $p<0.05$ compared to the control group in *Egfr*$^{fl/fl}$ mice. N=8–10 in each group.