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The Single IgG IL-1–Related Receptor Controls TLR Responses in Differentiated Human Intestinal Epithelial Cells

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Intestinal epithelial cells (IECs) are constantly exposed to enteric microbes. Although IECs express TLRs that recognize bacterial products, the activation of these TLRs is strictly controlled through poorly understood mechanisms, producing a state of hyporesponsiveness and preventing unwanted inflammation. The single IgG IL-1–related receptor (Sigirr) is a negative regulator of TLRs that is expressed by IECs and was recently shown to inhibit experimental colitis. However, the importance of Sigirr in IEC hyporesponsiveness and its distribution within the human colon is unknown. In this study, we investigated the role of Sigirr in regulating epithelial-specific TLR responses and characterized its expression in colonic biopsy specimens. Transformed and nontransformed human IECs were cultured as monolayers. Transient gene silencing and stable overexpression of Sigirr was performed to assess innate IEC responses. Sigirr expression in human colonic biopsy specimens was examined by immunohistochemistry. Bacterial infection of IECs and exposure to flagellin transiently decreased Sigirr protein expression, concurrent with secretion of the neutrophil chemokine IL-8. Sigirr gene silencing augmented chemokine responses to bacterial flagellin, Pam3Cys, and the cytokine IL-1β. Conversely, stable overexpression of Sigirr diminished NF-κB–mediated IL-8 responses to TLR ligands. We also found that Sigirr expression increased as IECs differentiated in culture. This observation was confirmed in biopsy sections, in which Sigirr expression within colonic crypts was prominent in IECs at the apex and diminished at the base. Our findings show that Sigirr broadly regulates innate responses in differentiated human IECs; therefore, it may modulate epithelial involvement in infectious and inflammatory bowel diseases. ***The Journal of Immunology*****, 2010, 184: 2305–2313.**

The intestinal epithelial cells (IECs) lining the human colon undergo continuous transition from an undifferentiated and proliferative state at the base of colonic crypts to a mature and differentiated phenotype at the crypt apex (1). Throughout this transition, IECs are exposed to a multitude of commensal and pathogenic microbes, including their products, such as LPS and flagellin. Under steady-state conditions, IECs remain generally quiescent and tolerant of these products, because they have evolved critically important, yet poorly defined, mechanisms to remain hyporesponsive to these stimuli (2). In part, this tolerance is maintained by limiting the activation of innate receptors in IECs, which express a specific subset of TLRs, including TLR5 and TLR9 (required for detecting bacterial flagellin and CpG DNA, respectively) (3, 4), as well as low levels of TLR2 and TLR4 (5). Numerous studies showed that these innate receptors are involved in the host's recognition of enteric pathogens, such as *Shigella*, *Salmonella typhimurium*, and enteropathogenic *Escherichia coli* [EPEC (2, 6, 7)], triggering inflammation and playing a central role in host defense.

Although innate receptors normally provide protection against bacterial and viral pathogens, dysregulated activation of TLRs in the gastrointestinal (GI) tract could impair host defenses as well as lead to chronic inflammation and cancer (8). Therefore, it is not surprising that IECs have evolved strategies to control innate sensing, including the expression of negative regulators of TLRs. Negative regulators inhibit TLR signaling by binding key adaptor proteins, such as IL-1R–associated kinase and TNFR-associated factor 6, thereby impeding downstream signaling to NF-κB (9). Only negative regulators of TLR4, such as Tollip, have been identified within IECs (9), but considering that colonic IECs express relatively little TLR4 or its coreceptor myeloid differentiation-2 (10), the impact of such regulation on gut homeostasis is unclear. In contrast, negative regulators of the innate receptors expressed at higher levels by IECs, such as TLR5 (11), have not been reported. However, it seems likely that such regulators do exist, and they may play a significant role in controlling the innate and inflammatory responsiveness of the mucosal epithelium within the GI tract. Identifying these negative regulators and clarifying their roles in TLR responses and expression patterns in human IECs could significantly increase our understanding of GI health and disease.

The single IgG IL-1–related receptor (Sigirr) was first described as a negative regulator of IL-1β and TLR4 signaling (12). Expressed throughout the human GI tract, with the highest expression in the colon (13), Sigirr was recently shown to regulate inflammation as well as mucosal homeostasis in a mouse model of chemical colitis (14); however, the mechanisms involved and the cellular distribution of Sigirr within the intestine remain obscure. Therefore, the objectives of this study were to characterize the expression and elucidate the role of Sigirr in regulating the immune responsiveness of human IECs. We

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Abbreviations used in this paper: DPP, dipeptidyl dipeptidase; EPEC, enteropathogenic *Escherichia coli*; GI, gastrointestinal; HK, heat-killed; IEC, intestinal epithelial cell; Ihh, Indian hedgehog; NCM, nontransformed colonic mucosal; NSC, nonsilencing control; Sigirr, single IgG IL-1–related receptor; siRNA, small interfering RNA; UBC, University of British Columbia.

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show that Sigirr inhibits responses to several bacterial-derived TLR ligands as well as signaling due to the proinflammatory cytokine IL-1β. Moreover, the innate response to infection by the attaching and effacing bacterial pathogen EPEC, which causes diarrheal disease, was also modulated by Sigirr. In contrast, IEC responses to IFN-γ and PMA were unaffected by Sigirr. Interestingly, we found that Sigirr expression directly correlated with the maturation state of cultured IECs, a finding that was corroborated in the epithelium of human colonic biopsy sections. These findings identify Sigirr as a critical modulator of IEC responses in the human colon and highlight the need to address Sigirr function in the context of host responses to enteric pathogens and idiopathic intestinal diseases, including inflammatory bowel disease.

Materials and Methods

Cell culture

Caco-2, HT-29 (ATCC), and TLR5-expressing HEK-293T cells (Invivogen, San Diego, CA) were grown in DMEM with 10% serum and antibiotics. Nontransformed colonic mucosal (NCM)460 is a human nontransformed colonic mucosal IEC line (15) grown in M3 medium. CHO cells stably cotransfected with human TLR5 and NF-κB luciferase reporter were gifted by Dr. Stuart Turvey (University of British Columbia [UBC]) and were maintained as described (16). IECs were used for experiments 3–5 d after confluence.

Sigirr gene silencing and overexpression studies

Gene silencing was performed with Sigirr and control small interfering RNA (siRNA) duplexes for transient transfection using the Hiperfect transfection reagent (Qiagen, Valencia, CA), as per the manufacturer's recommendations. IEC uptake of siRNA was confirmed by fluorochrome-tagged control siRNA. Two sets of Sigirr 27-mers siRNA (Nucleic Acid Protein Service, UBC) duplexes were tested simultaneously with negative control siRNA. A single duplex that produced greater knockdown of the target gene was selected for transfection. Monolayers of IECs (10^4 cells)/well were transfected in 24-well plates and assessed after 48–72 h. The pUNO-Sigirr mammalian expression vector and empty pUNO control vector (Invivogen) were used to transfect IECs and generate stable clones, as per the manufacturer's specifications. Briefly, pUNO-transformed *E. coli* was grown in Luria broth and plated on blasticidin-containing agar plates for selection. Bacteria were then grown in recommended culture medium, and purified pUNO control and Sigirr-containing plasmids were extracted for stable transfection of epithelial cells with the Effectene transfection reagent (Qiagen). Stably transfected IECs were selected and maintained in DMEM with serum and blasticidin (20–40 μg/ml). Sigirr knockdown and overexpression were analyzed by RT-PCR and Western blot analysis.

Stimulation and infection of IECs

Recombinant purified and endotoxin-free bacterial flagellin (*S. typhimurium*), LPS, Pam3Cys, and TLR5-neutralizing Abs (Invivogen) were prepared in DMSO, according to the manufacturer's specifications. rIL-1β, IFN-γ, and PMA (Sigma-Aldrich, St. Louis, MO) were prepared as recommended by the manufacturer. To assess innate responses, IECs were stimulated for 6 h with the TLR ligands in DMEM and serum without antibiotics, and IL-8 concentration was quantified by ELISA. The following TLR ligands were used in this study: bacterial flagellin (FliC; 10–20 ng/ml), LPS (20 ng/ml), and Pam3Cys (TLR2 ligand; 20 μg/ml). IECs were also stimulated with IL-1β (5 ng/ml) for 3 h, IFN-γ (100 ng/ml), and PMA (50 ng/ml) for 6 h. The wild-type EPEC strain 2348/69 was used to infect IECs in this study (17). Caco-2 cells cultured in 12-well plates were infected by EPEC in DMEM/Nutrient Mixture F-12 Ham for 3 h. At this time point, the multiplicity of infection ranged from 30–40. Postinfection, cells were washed twice with DMEM/Nutrient Mixture F-12 Ham, and gentamicin (1 μg/ml) was added to prevent the overgrowth of extracellular bacteria; cell culture supernatant was collected 24 h later for IL-8 ELISA, as described previously (7).

IL-8 ELISA and Western blot analysis

IECs were cultured as confluent monolayers in six-well plates, washed twice in 2 ml DMEM, and exposed to various TLR ligands in 1 ml DMEM. After 6 h, cell culture supernatant was collected for IL-8 quantification by an ELISA kit (BD Biosciences, San Jose, CA), as per the manufacturer's specifications. For Western blot analysis, IECs were washed twice with 2 ml ice-cold HBSS (Sigma-Aldrich). Cells were then lysed in 350–500 μl lysis buffer (50 mm Tris [pH 7.5], 150 mm NaCl, 1 mm EDTA, 1 mm EGTA, 1% Triton X-100, 2.5 mm sodium pyrophosphate, 1 mm β-glycero-phosphate, 1 mm Na3VO4, 1 μg/ml leupeptin, and 1 mm PMSF) on ice for 5–10 min and then scraped into microcentrifuge tubes. The tubes were centrifuged at 13,000 × *g* for 5 min to pellet debris, and the supernatant was transferred to another tube for Western blots. Caco-2 proteins (30–50 μg in cleared cell lysate) were resolved by 9–10% $NaDodSO_4$-PAGE (SDS-PAGE) and transferred to 0.2 μm polyvinylidene fluoride membranes. Blots were blocked for 1 h with 5% nonfat milk in TBST. Membranes were incubated with primary Ab in TBST overnight at 4°C and probed with the respective secondary Ab on the following day for 1 h at room temperature. Rabbit polyclonal Ab to Sigirr (ProSci, Poway, CA) was used to detect protein expression in IECs.

Conventional semiquantitative RT-PCR analysis

IECs were scraped in RLT lysis buffer (Qiagen) for 30 s in an Eppendorf tube and centrifuged for 5 min at 13,000 × *g* at room temperature. One to 2 ml cleared supernatant was used for extraction of RNA using the RNeasy Protect Minikits (Qiagen) spin columns. Total RNA from Caco-2 cells grown in 12–24-well plates was extracted by RNeasy Protect Minikits, as per the manufacturer's recommendations. One to 2 μg RNA were reverse transcribed into cDNA for 60 min at 37°C in 20 μl reaction buffer containing 10 mM Tris-HCl (pH 8.3), 50 mmol/l KCl, 5.0 mmol/l $MgCl_2$, 100 μmol/l pooled deoxynucleotide triphosphates, 100 U of Moloney murine leukemia virus reverse transcriptase, 4 μg random hexamer primers, and 20 U RNase inhibitor. Random hexamers were used to provide a one-step reverse transcription reaction that yielded cDNA for amplification by PCR. A semiquantitative conventional PCR method of amplification was used because the target mRNA was expressed at low copy numbers. Aliquots of reverse-transcribed cDNA were added to PCR reaction buffer to obtain a reaction mixture containing 20 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 100 μmol/l pooled deoxynucleotide triphosphates, 1–2 U Taq DNA polymerase, and 0.25 μmol/l each of GAPDH and gene-specific oligonucleotide primers (Nucleic Acid Protein Service , UBC). PCR conditions were optimized for different genes of interest, as reported previously (7). Primers used in this study are listed in Table I.

Human TLR5–NF-κB luciferase assay

CHO cells were stably transfected with human TLR5 cDNA (cloned into the pEF6 V5/His TOPO vector [Invitrogen, Carlsbad, CA]) and Elam-Luc plasmids (Promega, Madison, WI]), as reported previously (16). CHO cells expressing TLR5 and NF-κB luciferase reporter were stably transfected with control and Sigirr-expressing plasmids, as described earlier in *Materials and Methods*. CHO cells stably expressing Sigirr were stimulated with bacterial flagellin for 6 h before assaying for luciferase activity (Promega).

Intestinal biopsy sampling and immunofluorescent staining

Two colonic biopsy specimen were obtained from each patient at a single pass using standard crocodile biopsy forceps. Informed consent was obtained from all patients undergoing diagnostic colonoscopy for lower GI symptoms, such as abdominal pain and altered bowel movements. Study approval was obtained from the UBC Clinical Ethics Review Board. Colonic biopsy samples were collected in 4% formaldehyde solution and rapidly processed for immunohistochemical analysis, as described below.

Immunofluorescence staining of colon tissues

Colonic tissue samples were rapidly processed for immunofluorescence, and staining was performed as described previously (18). In brief, tissues were rinsed in ice-cold PBS, embedded in optimal cutting temperature compound (OCT, Sakura Finetech, Tokyo, Japan), frozen with isopentane (Sigma-Aldrich) and liquid N_2, and stored at −70°C. Serial sections were cut at a thickness of 6–8 μm and fixed in ice-cold acetone for 10 min. Tissue sections were directly blocked with 1% BSA, followed by the addition of mouse monoclonal and goat polyclonal Abs to Sigirr (R&D Systems, Minneapolis, MN), mouse mAbs to claudin-3 (Invitrogen), and rabbit polyclonal Abs to Indian hedgehog (Ihh; Santa Cruz Biotechnology, Santa Cruz, CA), all at 1:200 dilution. These were followed by secondary Alexa Fluor 568/488-conjugated goat anti-rabbit IgG Abs (Molecular Probes, Eugene, OR) and Prolong Gold antifade reagent containing DAPI (Invitrogen). Cells and tissues were visualized at 350 and 594 nm using a Leica DM 4000B microscope (Leica Microsystems, Deerfield, IL).

Data presentation and statistical analysis

All of the results are expressed as mean ± SEM and were analyzed with GraphPad Prism software (GraphPad, San Diego, CA). Results are from one of at least three representative experiments. Statistical analysis was performed using the one-way ANOVA test and the Student *t* test, with $p < 0.05$ considered significant. Quantity-one software (Chemidoc XRS, Bio-Rad, Hercules, CA) was used for densitometry.

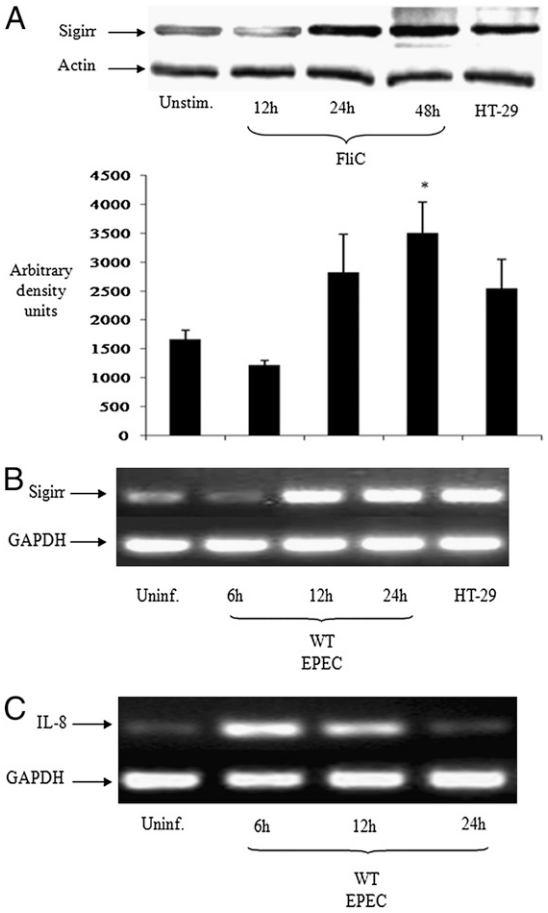

FIGURE 1. *A*, Exposure to bacterial flagellin induces Sigirr protein expression in NCM IECs. NCM IECs were grown in M3 growth media and exposed to 10 ng/ml purified flagellin (FliC). Cell lysates were prepared as described in *Materials and Methods*. Thirty to 50 μg of cleared cell lysates were subjected to SDS-PAGE and probed with rabbit polyclonal Ab to Sigirr. Blots were developed in ECL, and bands were analyzed by densitometry. Result is representative of three separate experiments. *B*, EPEC infection increases Sigirr message levels in Caco-2 IECs. Cells were grown to confluence in 12-well plates and infected with wild-type (WT) EPEC for 3 h. Postinfection, cells were washed twice, and fresh media were added with gentamicin to prevent bacterial growth. Total RNA extracted at different time points was subjected to semiquantitative RT-PCR to measure gene expression, as described in *Materials and Methods*. GAPDH was included as internal control. Results are representative of three independent experiments. *C*, EPEC infection increases IL-8 expression in Caco-2 IECs. Cells were grown to confluence in 12-well plates and infected with wild-type (WT) EPEC for 3 h, as above. Cells were washed twice, and fresh media were added with gentamicin. Total RNA was extracted and subjected to semiquantitative IL-8 RT-PCR. GAPDH was included as internal control. Results are representative of two separate experiments.

Results

Bacterial flagellin induces Sigirr expression in human IECs

Although Sigirr was shown to regulate the inflammatory response to LPS, it is unclear whether it regulates the responses to other TLR ligands, such as flagellin. We exposed human NCM IECs to flagellin (FliC), which transiently decreased Sigirr protein levels by 50% after 12 h. However, prolonged exposure to flagellin (24–48 h) significantly increased Sigirr protein levels by 50–100% over baseline levels in unstimulated cells (Fig. 1*A*). To assess whether enteric bacterial infection also alters Sigirr expression, we infected Caco-2 colonic IECs with the diarrheal pathogen EPEC. We recently showed that the inflammatory response to EPEC is triggered by flagellin activation of TLR5 in IECs (7). Through RT-PCR analysis, we observed transient downregulation of Sigirr message by ~50% after 6 h, which subsequently increased by 50–60% over baseline 24–48 h after EPEC infection (Fig. 1*B*; primer sequences shown in Table I). These alterations in Sigirr expression inversely correlated with the induction of IL-8 mRNA in Caco-2 IECs infected with EPEC (Fig. 1*C*). These results indicate that Sigirr expression is temporally regulated in response to flagellin and bacterial stimulation of IECs, and this regulation occurs within the same time point as the IL-8 response.

Sigirr gene silencing augments responses to bacterial flagellin and heat-killed bacteria

The transient downregulation of Sigirr gene expression and its correlation with IL-8 release suggest a possible intrinsic mechanism in IECs, in which a temporary reduction of Sigirr protein facilitates TLR activation, leading to chemokine responses. Therefore, we assessed whether Sigirr gene silencing would lead to augmentation of TLR responses in IECs. Transient gene silencing reduced Sigirr protein levels by 50% in Caco-2 IECs (Fig. 2*A*). Following exposure to FliC at 48–72 h posttransfection, these cells produced a 2-fold increase in IL-8 secretion compared with cells transfected with nonsilencing control (NSC) siRNA (1280.25 ± 68.6 pg/ml versus 615 ± 152.9 pg/ml; $p < 0.05$) (Fig. 2*B*). Time-course analysis indicated that this effect was transient, but it confirmed that the effect of Sigirr knockdown was maximal at 48–72 h posttransfection (data not shown), so we conducted the remainder of the gene-silencing studies within these time points. Thus, Sigirr deficiency also exaggerated the IL-8 response to proinflammatory IL-1β used as control (1492.6 ± 223.3 pg/ml versus 721.6 ± 107.4 pg/ml; $p < 0.05$). In contrast, the IL-8 response to IFN-γ stimulation, which involves the JAK-STAT pathway, instead of IL-1R–associated kinase and TNFR-associated factor 6, was unaffected by Sigirr knockdown (Fig. 2*B*). We also exposed these cells to heat-killed (HK) EPEC to examine what impact Sigirr knockdown would have on the IL-8 response to the multiple TLR ligands expressed by enteric bacteria. Similar to the results obtained from purified flagellin, HK EPEC-treated Sigirr-deficient Caco-2 cells released 3-fold more IL-8 compared with cells transfected with NSC siRNA, suggesting that Sigirr modulates the responses to whole bacteria (Fig. 2*C*).

Sigirr deficiency enhances IL-8 responses to diverse TLR ligands

To ensure that the actions of Sigirr were not cell line specific, we silenced Sigirr in the enterocytic HT-29 IEC cell line. After 70% knockdown of Sigirr mRNA (Fig. 2*D*), we noted that IL-8 release doubled in response to FliC (971 ± 143.8 pg/ml versus 423.67 ± 85.5 pg/ml; $p < 0.05$) as shown in Fig. 2*E*. To confirm TLR5 involvement, we examined the FliC response in the presence of a neutralizing Ab to TLR5, which abolished IL-8 release in Sigirr-deficient HT-29 IECs (971 ± 143.8 pg/ml versus 153 ± 52 pg/ml; $p < 0.05$) (Fig. 2*E*) but did not affect IL-1β responses.

Table I. *Oligonucleotide primers used in this study*

Target Gene	Forward	Reverse
GAPDH	5′-ATGACCTTGCCCACAGCC-3′	5′-CCCATCACCATCTTCCAG-3′ (7)
Sigirr	5′-GCTGACTGCAAGGACAGAGA-3′	5′-ACTCGTGGAGGCTGTAGTGG-3′ (13)
IL-8	5′-TCTGCAGCTCTGTGTGAAGGTGCAGTT-3′	5′-TTCCTTTGACCCACGTCTCCCAA-3′ (18)

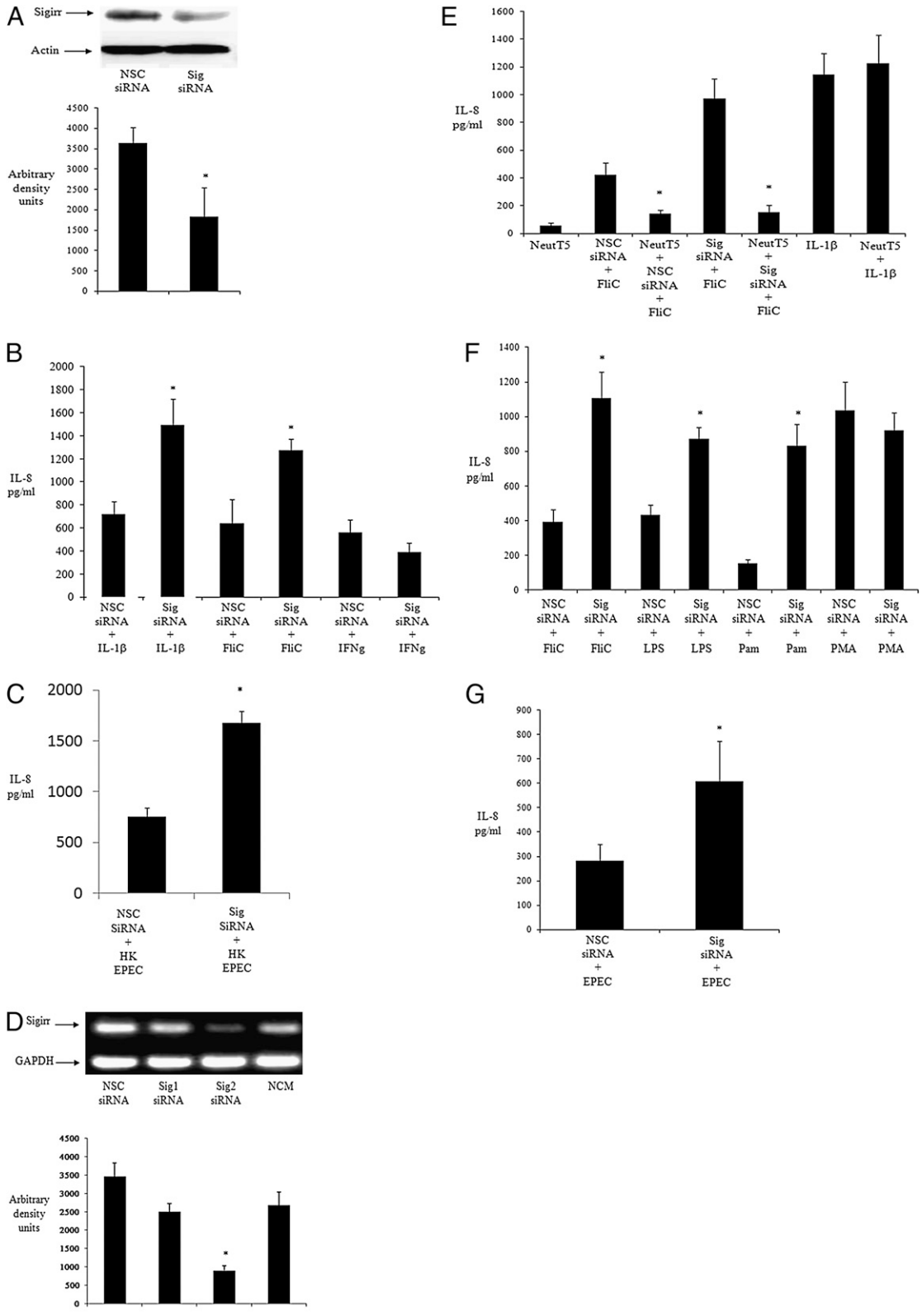

FIGURE 2. *A*, Transient gene silencing reduces Sigirr protein in Caco-2 cells. IECs (2×10^5/well) were seeded in 24-well plates. After 48 h, cells were transfected with Sigirr and NSC siRNA in Hiperfect transfection reagent. Sigirr protein was analyzed by SDS-PAGE, as described in *Materials and Methods*, and developed in ECL. Bands were quantified by densitometry with Bio-Rad quantity one software program. *B*, Sigirr gene silencing augments flagellin-induced IL-8 chemokine secretion from Caco-2 cells. Sigirr- and NSC siRNA-transfected Caco-2 cells were exposed to 10 ng/ml purified flagellin (FliC) and IFN-γ (100 ng/ml) in DMEM with serum and antibiotics. IL-1β (5 ng/ml) was included as a positive control. Cell culture supernatant was collected after 6 h, and IL-8 was quantified by ELISA. *C*, HK EPEC–induced IL-8 production is enhanced upon Sigirr gene silencing in Caco-2 cells. Sigirr- and NSC siRNA-transfected Caco-2 cells were exposed to HK-EPEC in DMEM with serum and antibiotics. Cell culture supernatant was collected after 24 h, and IL-8 was quantified by ELISA. *D*, Transient gene silencing decreases Sigirr gene expression in HT-29 cells. IECs (2×10^5/well) were seeded in 24-well plates and transfected after 48 h with Sigirr and NSC siRNA. Total RNA was extracted, and Sigirr gene expression was measured by semiquantitative RT-PCR. GAPDH was included as internal control. Results are

To address whether the inhibition of innate signaling by Sigirr was limited to TLR5, we tested other TLR ligands in the LPS-responsive HT-29 cells (19). In these IECs (Fig. 2*F*), we saw a >2-fold increase in their FliC response (1108 ± 149.9 pg/ml versus 392.3 ± 72.5 pg/ml; $p < 0.05$), a 2-fold increase in their LPS response (870.6 ± 66.9 pg/ml versus 435.3 ± 57.7 pg/ml; $p < 0.05$), and a 4-fold increase in response to Pam3Cys (833.3 ± 123.5 pg/ml versus 154.6 ± 20.4 pg/ml; $p < 0.05$). We also tested TLR9 responses and found similar elevations in IL-8 release (data not shown). Notably, although Sigirr gene silencing amplified inflammatory responses to TLR ligands, it had no effect on IL-8 responses to PMA (Fig. 2*F*). To assay the potential impact of Sigirr in regulating the IL-8 response to a live enteric bacterial pathogen, we infected HT-29 cells with EPEC. We noted that EPEC induced a significantly higher IL-8 response in Sigirr-deficient cells (609.79 ± 163 pg/ml versus 281.5 ± 67.3 pg/ml; $p < 0.05$) (Fig. 2*G*), but the overall response was much lower than that obtained with HK EPEC, likely reflecting the previously described suppressive abilities of virulence factors expressed by EPEC (7). Even so, these results clearly demonstrate that the loss of Sigirr enhances the chemokine response to this bacterial pathogen.

Immunofluorescence analysis of Sigirr expression in human IECs

Although IECs express Sigirr, its localization within these cells has not been reported. To visualize Sigirr, we first assessed Sigirr staining in transfected HEK293T cells. Although no signal was detected in cells transfected with control vector (Fig. 3*A*), immunostaining of cells stably overexpressing Sigirr revealed a homogenous green signal, corresponding to Sigirr protein, present in the cytoplasm and the cell membrane (Fig. 3*B*). Specificity was confirmed by incubation with the Sigirr peptide, which abolished Sigirr immunoreactivity (Fig. 3*C* versus 3*D*). Next, we analyzed the pattern of native Sigirr expression by immunocytochemistry in Caco-2 IECs as well as Caco-2 and HT-29 cell lines stably overexpressing Sigirr. Interestingly, unlike in HEK293T cells, Sigirr expression in native spontaneously differentiating Caco-2 cells was patchy and clustered in cells growing in smaller colonies (Fig. 3*E*, 3*F*), with staining localized to the cell membrane as well as in a punctate pattern within the cytoplasm. We observed increased Sigirr immunoreactivity in Caco-2 cells seeded at higher cell densities (Fig. 3*F* versus 3*E*) and in Caco-2 (Fig. 3*H* versus 3*G*) and HT-29 cells (Fig. 3*J* versus 3*I*) overexpressing Sigirr relative to their controls.

Overexpression of Sigirr dampens NF-κB–mediated TLR responses

We next assessed the impact of increased Sigirr levels on TLR- and cytokine-induced responses, using our stably Sigirr-overexpressing Caco-2 and HT-29 IECs (Fig. 4*A*), as well as Sigirr-overexpressing CHO cells (transfected with NF-κB luciferase reporter) and TLR5-expressing HEK293T cells. NF-κB luciferase activity in response to FliC decreased by ~25% in CHO cells overexpressing Sigirr (Fig. 4*B*), whereas in Caco-2 IECs, IL-8 release in response to FliC decreased significantly by 30% (Fig. 4*C*, *left panel*) compared with control (pUNO) transfected cells (870.33 ± 150 pg/ml versus 581.6 ± 103 pg/ml; $p < 0.05$). These data were reproduced in HEK293T cells stably coexpressing TLR5 and Sigirr (571.6 ± 109.4 pg/ml versus 295.8 ± 90.3 pg/ml; $p < 0.05$) (Fig. 4*C*, *right panel*). Overexpression of Sigirr in HT-29 IECs also significantly reduced IL-8 responses to FliC, LPS, and Pam3Cys (Fig. 4*D*) relative to control cells. Moreover, consistent with our results observed with Sigirr-deficient Caco-2 cells, overexpression of Sigirr in Caco-2 cells led to the production of less IL-8 when exposed to HK EPEC compared with pUNO-

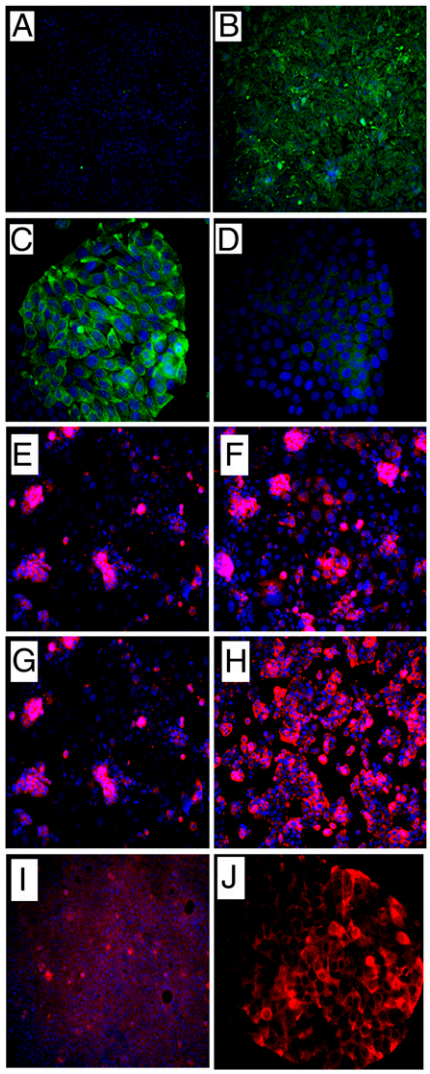

FIGURE 3. Immunofluorescent staining for Sigirr expression in HEK293T cells and human IECs. HEK293T cells grown on coverslips were stably transfected with pUNO Sigirr overexpression vector (*A*) and the control vector (*B*). After formalin fixation, Sigirr expression was analyzed using a rabbit polyclonal Ab and Alexa Fluor 468 (green)-conjugated secondary Ab. Sigirr Ab was preincubated with full-length Sigirr peptide and used for staining Sigirr-overexpressing cells (*D*) versus Sigirr Ab alone (*C*); nuclei (blue) were stained with ProlongGold DAPI. Native Caco-2 cells grown on coverslips were assessed for Sigirr expression with a goat polyclonal primary Sigirr Ab and Alexa Fluor 568-conjugated and DAPI nuclear stain (blue). Caco-2 cells seeded at a lower density (*E*) and a higher density (*F*). Immunostaining in Caco-2 cells overexpressing Sigirr (*H*) and control (*G*). HT-29 cells overexpressing Sigirr (*J*) and control vector (*I*). Original magnification ×20.

representative of three experiments, and bands were analyzed by densitometry. *E*, Sigirr gene silencing augments TLR5-mediated IL-8 secretion. siRNA-transfected HT-29 cells were exposed to FliC (10 ng/ml), in the presence or absence of a specific TLR5 neutralizing Ab. Cell culture supernatant was collected after 6 h, and IL-8 was quantified by ELISA. IL-1β (5 ng/ml) was included as positive control. *F*, Sigirr gene silencing augments diverse TLR responses in IECs. siRNA-transfected HT-29 cells were exposed to FliC (10 ng/ml), LPS (20 ng/ml), Pam3Cys (Pam-20 μg/ml), or PMA (50 ng/ml) for 6 h in DMEM with serum and antibiotics. Cell culture supernatant was collected, and IL-8 was quantified by ELISA. *G*, EPEC infection upregulates chemokine response in Sigirr-deficient HT-29 cells. IECs were grown to confluence and infected with wild-type EPEC for 3 h. Postinfection, cells were washed twice, and fresh media were added with gentamicin treatment. Cell culture supernatant was collected after 12 h for IL-8 ELISA. $*p < 0.05$.

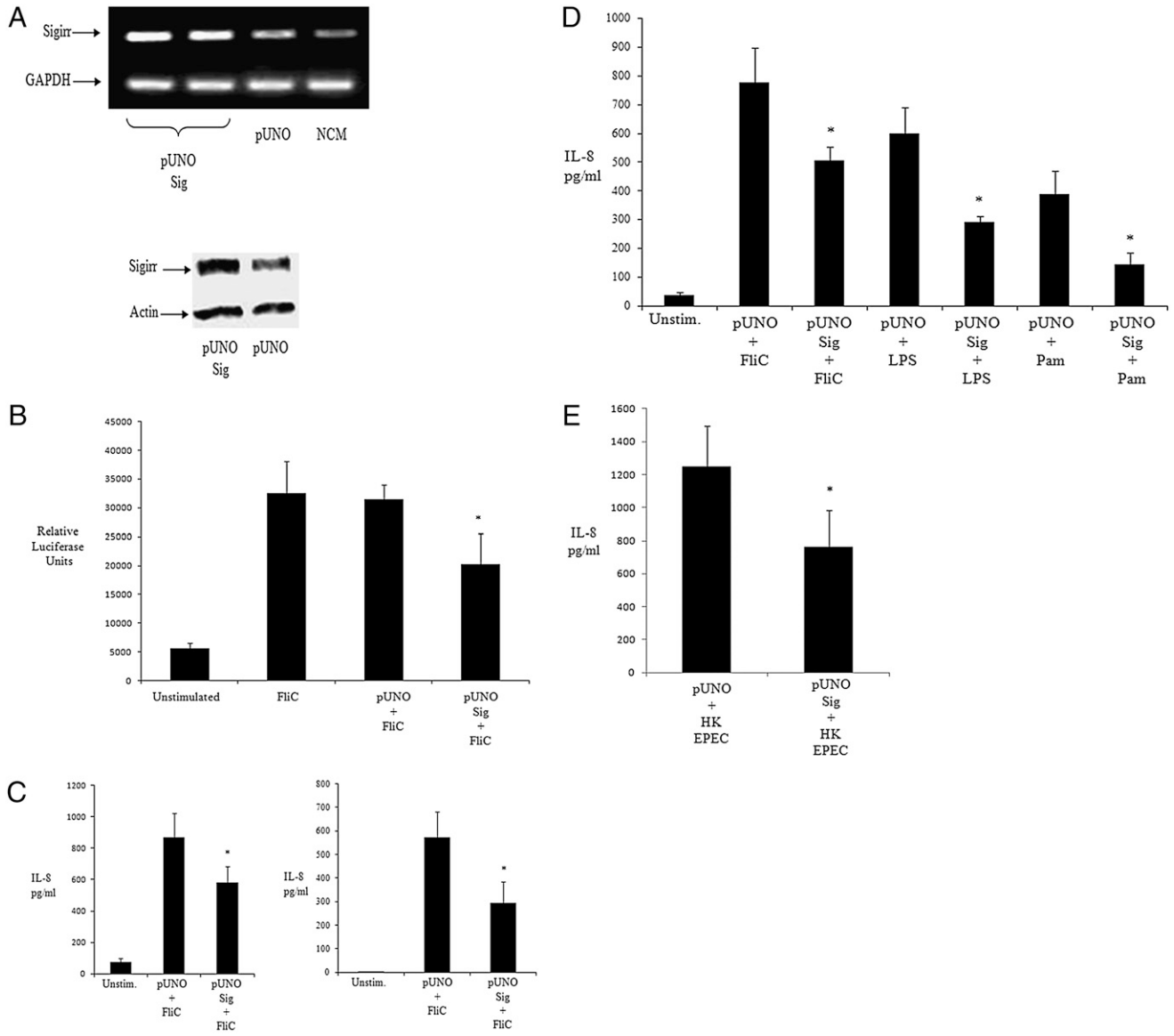

FIGURE 4. Sigirr overexpression depresses NF-κB–mediated diverse TLR responses. *A*, HT-29 and Caco-2 IECs were stably transfected with pUNO Sigirr mammalian expression vector or empty pUNO vector with Effectene transfection reagent. Clones overexpressing Sigirr were maintained in blastocidin and were subsequently assessed for Sigirr by RT-PCR and immunoblotting. Results are representative of two experiments. *B*, CHO cells containing an NF-κB luciferase reporter were stably transfected with Sigirr-overexpressing pUNO vector and control vector. Clones were maintained in blastocidin and subsequently tested for flagellin (FliC) responses. Whole-cell lysate was collected after 6 h, and relative luciferase activity was measured by luminometry. $*p < 0.05$ versus pUNO + FliC and unstimulated cells. *C*, Overexpression of Sigirr blunts FliC-induced chemokine response. HT-29 IECs (*left panel*) and HEK293T cells (*right panel*) overexpressing Sigirr were exposed to 10 ng/ml of flagellin (FliC) for 6 h, and IL-8 was quantified by ELISA in cell culture supernatant $*p < 0.05$. *D*, Overexpression of Sigirr blunts IL-8 responses to diverse TLRs and inflammatory cytokines. Stably transfected HT-29 cells overexpressing Sigirr were exposed to 10 ng/ml of flagellin (FliC), 20 ng/ml of LPS, and 20 μg/ml of Pam3Cys (Pam) for 6 h in DMEM, and IL-8 secretion was quantified by ELISA. $*p < 0.05$ versus pUNO + FliC, pUNO + LPS, and pUNO + Pam. *E*, HK EPEC-induced IL-8 production is abrogated upon Sigirr overexpression in Caco-2 cells. Stably transfected Caco-2 cells overexpressing Sigirr were treated with HK EPEC for 24 h in DMEM, and IL-8 secretion was quantified by ELISA, as above. $*p < 0.05$ versus pUNO + HK EPEC.

transfected cells (Fig. 4*E*). These results confirm that increasing Sigirr expression attenuates TLR signaling and depresses IEC responses to bacterial-derived products.

Sigirr expression depends on the maturation state of IECs

As Caco-2 cells grow in culture they form colonies in which cell–cell contact induces their differentiation from an immature state toward a mature enterocytic phenotype (20). As these cells form monolayers, they acquire a cylindrical polarized morphology with microvilli on their apical surface, tight junctions between adjacent cells, and express intestinal hydrolase enzymes, such as alkaline phosphatase, sucrose isomaltase, and dipeptidyl dipeptidase (DPP) (21, 22). In this context, the patchy staining for Sigirr observed in the midst of cultured Caco-2 cell colonies in Fig. 3*E–G* suggested that Sigirr expression was possibly related to the maturation state of IECs. We examined this possibility using sodium butyrate, an agent known to induce IEC differentiation (23). Exposure to sodium butyrate progressively increased Sigirr protein levels in Caco-2 cells after 24, 48, and 72 h compared with preconfluent untreated IECs (Fig. 5*A*). Sigirr expression also increased significantly in untreated spontaneously differentiating Caco-2 cells. Therefore, we tested whether the seeding density of Caco-2 cells affected Sigirr expression, because cells seeded at higher densities reach maturation sooner than cells seeded at lower densities. As seen in Fig. 5*B*, cells seeded at higher densities, i.e., 3×10^6 cells/well, expressed progressively higher levels of Sigirr

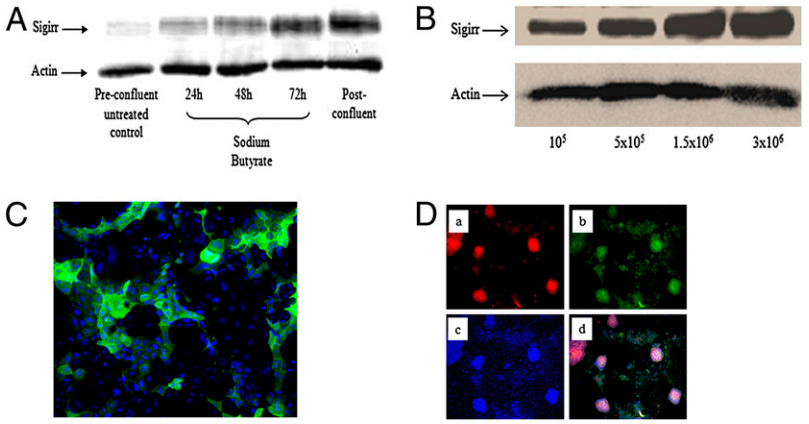

FIGURE 5. Sigirr expression in Caco-2 cells is dependent on the differentiation state. *A*, Caco-2 monolayers were exposed to sodium butyrate (2 mM) for 24–72 h in DMEM supplemented with serum and antibiotics. Thirty to 50 μg of total protein from preconfluent sodium butyrate-treated and spontaneously differentiating postconfluent cells were probed with Sigirr rabbit polyclonal Ab in Western blots. Results are representative of two experiments. *B*, Caco-2 cells were seeded at increasing densities as indicated, and 30 μg of total protein from postconfluent cells were probed with Sigirr Ab in Western blots, as above. *C*, Caco-2 cells express the differentiation marker DPP in IEC monolayers. Formalin-fixed Caco-2 monolayers grown on coverslips were incubated with mouse monoclonal DPP Ab conjugated with Alexa Fluor 488 (green). Cell nuclei were stained with DAPI. Original magnification ×40. Result is representative of three independent experiments. *D*, Sigirr colocalizes with the differentiation marker DPP in Caco-2 cells. Cells grown at a higher density (5×10^5/well) were fixed in 4% formalin and incubated simultaneously with goat polyclonal Sigirr Ab (*a*: red) with mouse monoclonal DPP Ab conjugated to Alexa Fluor 488 (*b*: green) and nuclear DAPI stain (*c*: blue). Images taken with a Zeiss fluorescent microscope were merged to show colocalization (*d*). Results are representative of two independent experiments (original magnification ×20).

compared with cells seeded at lower densities (i.e., 1×10^5 cells/well). These results indicate that the level of Sigirr expression in IECs is linked to their differentiation state. To verify this, we investigated the expression of DPP, a brush border-associated marker of differentiation in colonic IECs (24). Immunocytochemical analysis revealed that DPP was patchy and confined to small clusters of cells (Fig. 5*C*), remarkably similar to the Sigirr staining pattern seen in Fig. 3*E* and 3*F*. Further, Sigirr staining colocalized with DPP expression (Fig. 5*D*), suggesting that Sigirr expression by Caco-2 cells coincides with the expression of this brush border enzyme.

Sigirr is expressed by differentiated epithelial cells in the human colon

To examine the in vivo relevance of our findings, we stained for Sigirr expression in human colonic biopsy sections. By immunofluorescence, we detected maximal Sigirr expression on the apical surface of colonic crypts (Fig. 6*A*), with the signal diminishing in cells at the base. Additional staining was observed in cells within the colonic lamina propria, which likely reflect immune cells, such as dendritic cells and T lymphocytes, resident in the lamina propria (25, 26). To address whether Sigirr expression was mostly localized to IECs, we stained for the tight junction protein claudin-3 (27). As expected, claudin-3 staining was maximal on the lateral intercellular junctions of the IECs, whereas Sigirr staining was most intense on the apical surface of these IECs (Fig. 6*B*). To confirm that Sigirr was predominantly expressed by differentiated epithelial cells, we further stained for Ihh, a marker of differentiated apical IECs in small intestine and colonic epithelial cells (28–30). We found abundant Ihh expression in epithelial cells that were also expressing Sigirr (Fig. 6*C*). These findings were consistent with our data in cultured IECs, and confirmed that, within the human colon, Sigirr is predominantly expressed by mature and differentiated IECs present on the luminal surface of the crypts.

Discussion

This study aimed at elucidating the inhibitory functions of Sigirr in the human colonic epithelium. Our results indicated that Sigirr plays a major role in maintaining the hyporesponsiveness of IECs to a variety of bacterial and proinflammatory stimuli. We show for the first time that bacterial flagellin, as well as direct infection of IECs with the enteric bacterial pathogen EPEC, causes a transient decrease, followed by a substantial and prolonged increase, in Sigirr expression that inversely correlates with the expression and release of the neutrophil chemokine IL-8. Sigirr gene silencing in IECs caused a significant and selective enhancement of IL-8 secretion in response to TLR ligands and augmented responses to the proinflammatory cytokine IL-1β. Conversely, stable overexpression of Sigirr diminished the same TLR and cytokine responses. While exploring different IEC densities, we determined that Sigirr expression increased with the differentiation/maturation state of these cells in culture. Correspondingly, Sigirr expression was minimal in cells at the base of human colonic crypts and maximal in the differentiated epithelial cells at the crypt apex, providing a basis for the previously identified hyporesponsiveness of mature IECs to bacterial products (1, 19).

The recent demonstration of exaggerated colitis and tumorigenesis in Sigirr-deficient mice designated this receptor as potentially important in GI inflammation; however, the underlying mechanism remained unclear. Although Sigirr is known to suppress TLR4 and IL-1β signaling, it was unknown whether exaggerated signaling through these receptors led to the resulting pathology. In part, this is because the potential role of Sigirr in regulating other innate receptors expressed at higher levels within the gut epithelium, such as TLR5, has not been examined. It is now evident that the inflammatory responses mediated through several innate and cytokine receptors are regulated by Sigirr within IECs. Based on the broad actions of Sigirr, we speculate that it may affect responses to other members of the IL-1 superfamily, such as IL-26 (31) and IL-22 (32), which were recently described to act on IECs to mediate inflammation and host defense, respectively.

Although a tumor-derived IEC line (HT-29) was shown to express Sigirr mRNA (12), its protein expression and function had not been examined in nontransformed human IECs or in human colonic tissues. Moreover, although IECs are known to differentiate while migrating apically in colonic crypts, becoming less responsive to bacterial products as they mature (19), the impact of differentiation on the expression of Sigirr by IECs was unknown. Interestingly, we found that the differentiation agent sodium butyrate (33) induced the expression of Sigirr in IECs. We noted a similar increase in Sigirr

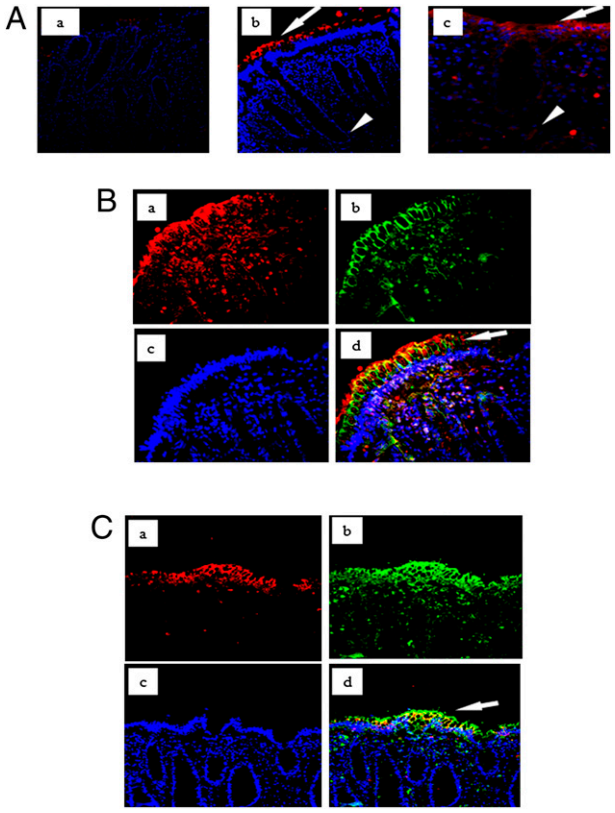

FIGURE 6. Immunohistochemical analysis of Sigirr expression in human colonic tissues. *A*, Colonic biopsy tissues were fixed in formalin and prepared as described in *Materials and Methods*. Eight- to 10-μM sections were mounted on slides and incubated with control Ab (*a*) or goat polyclonal Ab to Sigirr (*b* and *c*). Immunoreactivity to Sigirr is prominent on surface epithelial cells at the apex of the crypts (arrow) but is absent from cells at the base of the crypts (arrowhead). *B*, Epithelial Sigirr expression colocalizes with claudin-3 tight junction protein in human colon. Colonic biopsy tissues were prepared as described. Eight- to 10-μm sections were mounted on slides, and double immunostaining was performed with goat polyclonal Sigirr and mouse monoclonal claudin-3 Ab. Sigirr immunoreactivity (*a*: red) colocalized with claudin-3 (*b*: green) in epithelial cells on the crypt surface (*d*: arrow). Nuclei stained with DAPI (*c*: blue) stain. *C*, Ihh expression by IECs coincides with Sigirr expression in human colon. Colonic tissues from biopsy specimens were prepared as described. Double immunostaining was performed with a goat polyclonal Sigirr Ab and a rabbit polyclonal Ab to Ihh. Sigirr immunoreactivity (*a*: red) colocalized with Ihh (*b*: green) in epithelial cells on the crypt surface (*d*: arrow). Nuclei stained with DAPI (*c*: blue) stain. Original magnification ×20.

expression in spontaneously differentiating Caco-2 monolayers, indicating that the maturation of IECs promoted Sigirr expression. Immunohistochemical analysis of human colonic tissues confirmed that Sigirr protein levels were maximal in differentiated epithelial cells on the surface of the crypts.

These data are consistent with earlier studies in which IEC differentiation downregulated IL-1β–induced chemokine secretion (1). Moreover, previous immunohistochemical analyses of TLR4 and TLR5 in human colonic sections also demonstrated that these TLRs were preferentially expressed by mature IECs, therefore requiring the increased expression of negative regulators, such as Sigirr, in these type of cells (34, 35). Taken together, these studies support our findings that Sigirr expression within IECs is related to their differentiation state. We propose that in differentiated IECs, innate responses are controlled to prevent unwanted inflammatory responses against commensal microbes, whereas in less mature cells, limited Sigirr expression leads to heightened inflammatory responses, potentially contributing to the more sterile environment found at the base of crypts.

Despite the regulatory role of Sigirr, limited activation of specific epithelial TLRs may be required for the maintenance of colonic mucosal integrity (18, 36). In fact, TLR2, TLR4, and TLR9 signaling were shown to contribute to normal IEC function (4, 8, 36), at least in mice, and this TLR stimulation may be regulated by Sigirr, because epithelial homeostasis seems to be dysregulated in the absence of Sigirr (14). Although TLR5 is not known to play a role in intestinal homeostasis, IEC expression of TLR5 was shown to play a critical role in recognizing and responding to flagellated enteric pathogens for protection of the host (11, 37). Thus, Sigirr's regulation of TLR5 and other innate receptors may play a role in enteric host defense. The observed transient downregulation of Sigirr in response to flagellin and EPEC, followed by its prolonged upregulation, likely reflects a means to transiently recruit neutrophils to clear enteric bacterial pathogens, while attenuating collateral tissue damage and suppressing colonic inflammation postinfection.

Based on our findings, Sigirr dysregulation could dramatically impact IEC responsiveness, potentially contributing to the development of chronic inflammation, as seen in inflammatory bowel disease, not only in response to microbes, but possibly to other endogenous cytokines, such as IL-22, required for host defense against enteric pathogens (32). It is unclear whether single nucleotide polymorphisms within the Sigirr gene would alter its function and/or distribution; however, such assessment in human subjects should be a priority. These findings also indicate that modulation of Sigirr expression may prove to be of therapeutic benefit. In steady-state conditions, IECs are exposed to minimal amounts of flagellin and butyrate derived as a fermentation product from commensal bacteria in the gut. Both factors may facilitate the expression of Sigirr and perhaps other negative regulators and, thereby, offer attractive targets in the treatment of inflammatory disorders of the GI tract.

Disclosures

The authors have no financial conflicts of interest.

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