Enteric commensal bacteria potentiate epithelial restitution via reactive oxygen species-mediated inactivation of focal adhesion kinase phosphatases

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The mechanisms by which enteric commensal microbiota influence maturation and repair of the epithelial barrier are relatively unknown. Epithelial restitution requires active cell migration, a process dependent on dynamic turnover of focal cell-matrix adhesions (FAs). Here, we demonstrate that natural, commensal bacteria stimulate generation of reactive oxygen species (ROS) in intestinal epithelia. Bacteria-mediated ROS generation induces oxidation of target cysteines in the redox-sensitive tyrosine phosphatases, LMW-PTP and SHP-2, which in turn results in increased phosphorylation of focal adhesion kinase (FAK), a key protein regulating the turnover of FAs. Accordingly, phosphorylation of FAK substrate proteins, focal adhesion formation, and cell migration are all significantly enhanced by bacterial contact in both in vitro and in vivo models of wound closure. These results suggest that commensal bacteria regulate cell migration via induced generation of ROS in epithelial cells.

intestine | gastroenterology | phosphoprotein phosphatases | probiotics | lactobacillus

The mammalian gastrointestinal tract is home to an extraordinarily large group of commensal bacteria that mediate homeostatic effects on their host and influence a wide range of systemic metabolic, nutritional, and immune functions (1, 2). Additionally, the intestinal microbiota can directly affect the function of the epithelial cells that form a physical interface between the host and the luminal contents. For example, gut commensal bacteria have been implicated in regulation of epithelial proliferation, survival, barrier function, and resolution of epithelial wounds (3–6). In this report, we investigated the mechanisms by which the intestinal microbiota influence epithelial cell restitution.

Epithelial cell restitution is a process during which wounds or breaks in the epithelial lining are repaired by migration of the surrounding epithelial cells. Cells at the leading edge flatten and move into the wounded area by rapidly extending lamellipodia, which are stabilized to the underlying matrix at specialized points called focal adhesions (FAs). The rapid disassembly of FAs at the rear end and assembly of FAs at the leading edge of the cells provides the traction force necessary for the cells to move forward (7). Additionally, FAs serve as signaling nidus points where multiple intracellular and extracellular signals integrate to coordinate cell migration. FAs are composed of protein complexes including transmembrane integrins, cytoplasmic signaling adaptors, and components of the actin cytoskeleton (8). A key regulatory protein of FA dynamics is focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase that is phosphorylated in response to many extracellular signals. Models of cell migration demonstrate that phosphorylation of FAK and Src accompanies the formation of the FA complex, which subsequently mediates the turnover of adhesions and affects cell migration (7).

Recently, we reported that commensal bacteria induce the generation of reactive oxygen species (ROS) in intestinal epithelial cells (IECs) (9, 10). Whereas high levels of ROS are associated with molecular damage to cellular components and consequent tissue injury, nonradical ROS such as H_2O_2 are enzymatically generated in response to external stimuli and have an increasingly recognized role in modulating signal transduction pathways due to their ability to oxidize low pK_a cysteines within catalytic sites of a subset of enzymes (11–14). In accordance, we demonstrated that bacteria-elicited ROS modulate the NF-κB signal transduction pathway (9). ROS induced in response to endogenous receptor-mediated signals (such as integrin ligation and growth factors) have been shown to mediate a regulatory role in FA assembly, directed cell migration, and wound healing (15, 16). A role for ROS in cell migration is further supported by the demonstration that ROS-generating enzymes of the NADPH oxidase (Nox) family colocalize and physically interact with FA proteins (16). Chiarugi et al. (17) demonstrated that physiological integrin ligation results in generation of ROS, which causes oxidative inactivation of low molecular weight protein tyrosine phosphatase (LMW-PTP), a phosphatase that acts on FAK. Consequently, this results in activation of FAK and subsequent events necessary for cell adhesion and spreading. In the present study, we propose that commensal bacteria-elicited ROS stimulate phosphorylation of FAK and thus augment restitution of injured intestinal epithelial monolayers. In doing so, we have identified a mechanism by which commensal bacteria can contribute to epithelial homeostasis.

Results

Commensal Bacteria Induce ROS in Model Wounded Intestinal Epithelial Cells. We first investigated the effects of commensal microbiota on ROS generation in wounded model IECs. IECs contacted with *Lactobacillus rhamnosus* strain GG (LGG), a natural commensal and commonly used probiotic, stimulated ROS generation as observed by the rapid increase in fluorescence of Hydro-Cy3, a newly developed dye sensitive to superoxide (Fig. 1*A*). ROS generation could be effectively abolished by pretreatment of the cells with a nonspecific ROS scavenger, *N*-acetyl cysteine (NAC) (Fig. S1). Generation of ROS was also observed in IECs treated with insulin, a growth factor known to induce ROS (Fig. 1*A*) (18). To extend these findings in vivo, we orally gavaged mice with LGG for 1 h and used Hydro-Cy3 to look for ROS production by IECs. Whole mount preparations of murine proximal small intestinal

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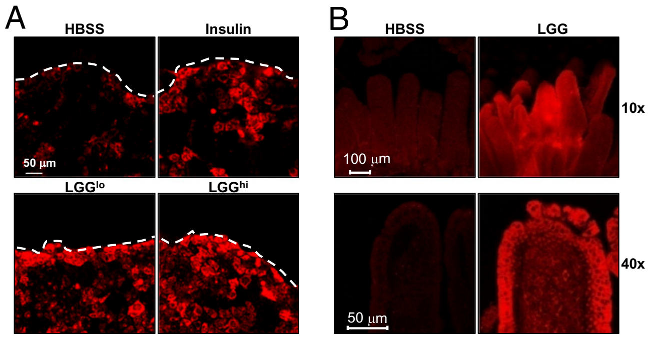

Fig. 1. Commensal bacteria induce generation of ROS in intestinal epithelia. (*A*) Fluorescent images of ROS generation in scratch-wounded Caco-2 monolayers treated with media (HBSS) for 15 min, LGG^{lo} (1.5×10^7 cfu/mL) for 5 min, LGG^{hi} (5.0×10^7 cfu/mL) for 5 min, or 50 μm insulin for 15 min, and then loaded with 100 μm Hydro-Cy3. (*B*) Fluorescently labeled whole mount preparations of proximal small intestine taken from mice injected with Hydro-Cy3 followed by oral gavage with HBSS or 10^8 cfu/mL LGG for 1 h. (*Upper*) 10× magnification; (*Lower*) 40× magnification. Data are representative of two independent experiments with *n* = 3 mice per group.

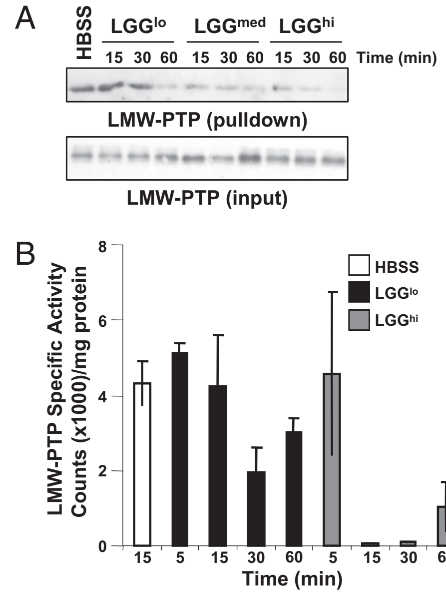

Fig. 2. Commensal bacteria oxidize and inactivate LMW-PTP. (*A*) Western blots of LWM-PTP levels in migrating T84 cells cultured in buffer containing biotin maleimide (BM) and treated with HBSS, LGG^{lo} (1.5×10^7 cfu/mL), LGG^{med} (5.0×10^7 cfu/mL), or LGG^{hi} (1.5×10^8 cfu/mL) for the indicated times. Representative Western blots show LWM-PTP levels before (input; *Lower*) and after precipitation (pulldown, *Upper*). (*B*) Specific phosphatase activity of LMW-PTP–transfected HeLa cells treated with HBSS, LGG^{lo} (5.0×10^7 cfu/mL), or LGG^{hi} (1.5×10^8 cfu/mL) for the indicated times. Data are expressed as counts/mg of protein and are means ± SE of triplicate samples. Two independent experiments were performed and representative data from one experiment are shown.

mucosa, a region of the gut with low absolute numbers of resident microflora, revealed a marked increase in ROS production in the intestinal villi (Fig. 1*B*). Together, these findings indicate that commensal bacteria stimulate rapid generation of ROS in migrating IECs.

Commensal Bacteria Mediate Inhibition of FAK Phosphatases. ROS elicited by endogenous stimuli have been shown to mediate oxidative inactivation of LMW-PTP, a FAK phosphatase (17). We confirmed that LMW-PTP is redox sensitive by labeling recombinant LMW-PTP with a thiol-reactive chemical, biotin-maleimide (BM), which efficiently reacts with reduced thiols (the constitutive physiological form present in low pK_a cysteines); therefore, its incorporation reflects the amount of reduced proteins. Under physiological conditions, recombinant LMW-PTP was efficiently labeled by BM, whereas in the presence of H_2O_2 there was a dose-dependent decrease in labeling, indicating oxidation of reactive cysteines in this protein (Fig. S2*A*). Importantly, IECs contacted with LGG also paralleled the effects observed with exogenous H_2O_2 (Fig. 2*A*). Incubation of SHP-2, which also regulates FAK phosphorylation (19), with LGG also resulted in dose- and time-dependent reduction in BM labeling (Fig. S2*B*). Bacterial-mediated oxidative changes were not observed in the redox-sensitive PTEN tyrosine phosphatase, indicating FAK phosphatase specificity (Fig. S2*D*).

Next, we measured the enzymatic activity of LMW-PTPase in cells contacted with LGG. Incubation of the recombinant protein with exogenous H_2O_2 reduced the PTP activity of the recombinant protein by more than 90% (Fig. S2*C*). To examine the intracellular effects of LGG on the enzymatic activity of LMW-PTP, we transfected full-length Myc-tagged LMW-PTP into HeLa cells and assayed the Myc-precipitated protein for phosphatase activity. Significantly, treatment of transfected cells for 15 min with LGG caused a dose- and time-dependent decrease in LMW-PTP enzymatic activity, which was partially reversible after 60 min (Fig. 2*B*). Collectively, these results indicate that LGG-induced ROS can directly oxidize and inactivate FAK phosphatases (LMW-PTP and SHP-2).

Commensal Bacteria Mediate Phosphorylation of FAK in Model IECs. We next assessed whether oxidative inactivation of LMW-PTP and SHP-2, correlated with increase phosphorylation of FAK. Phosphorylation of FAK at multiple tyrosines is known to regulate migration in multiple cell types (20, 21). Coculture of wounded IEC monolayers with LGG dramatically increased phosphorylation of FAK at tyrosine residue 861 (pFAK-Y861) over baseline conditions in a dose- and time-dependent manner (Fig. 3*A*), an effect reproduced by several other strains of commensal bacteria (Fig. S3*A*). Interestingly, high concentrations of LGG led to rapid but short-lived FAK phosphorylation. This kinetic pattern of FAK activation is very similar to the dose–response of commensal bacterial-elicited ROS observed in unwounded intestinal epithelial cells (9) and is consistent with the transient oxidative inactivation of LMW-PTP (Fig. 2*B*). Finally, the specificity of LGG-mediated FAK activation was confirmed by the fact that LGG had much weaker effects on a separate tyrosine kinase, Pyk2 (Fig. S3*B*).

Also of note, LGG mediated a marked increase in the numbers of pFAK-Y861 containing FAs that was more pronounced at the leading edge of migrating cells (Fig. 4). Furthermore, costaining the same monolayers with the actin-specific marker, phalloidin, revealed that contact with LGG induced the formation of dense parallel arrays of basal F-actin fibers terminating in FAs, consistent with new actin polymerization. These data implicate a role for commensal bacteria in accelerating FA formation and cytoskeletal rearrangement at the leading edge of migrating cells.

Because the ability of FAK to transduce downstream signals depends on its phosphorylation at multiple additional tyrosine residues, we examined the effects of LGG on phosphorylation of FAK at tyrosine position 397 and 566/567. Again, increased phosphorylation of FAK at these positions was observed after IEC contact with LGG (Fig. 3*B*). FAK *cis*-phosphorylation at multiple sites enables transphosphorylation of other component proteins of FAs, such as p130Cas and paxillin (PAX) (22). Consistently, LGG stimulation of IECs induced phosphorylation of p130Cas at tyrosine position 410 (pp130Cas-Y410) and PAX

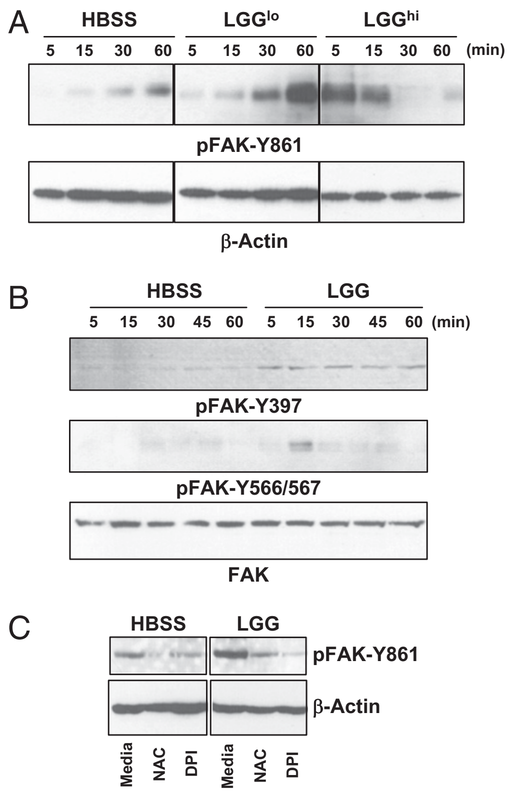

Fig. 3. Commensal bacteria stimulate phosphorylation of FA proteins via elicitation of ROS. (*A*) Western blots of FAK phosphorylation at tyrosine residue 861 (pFAK-Y861) in migrating Caco-2 cells after treatment with HBSS, LGG^{lo} (1.5×10^7 cfu/mL), LGG^{med} (5.0×10^7 cfu/mL), or LGG^{hi} (1.5×10^8 cfu/mL) for the indicated times. (*B*) Western blots of FAK phosphorylation at tyrosine residues 397 (pFAK-Y397) and 566/567 (pFAK-Y566/567) in migrating Caco-2 cells after treatment with HBSS or 5.0×10^7 cfu/mL LGG for the indicated times. Western blots for unphosphorylated FAK were used as controls. (*C*) Western blots of pFAK-Y861 in migrating Caco-2 cells treated first with media, NAC (20 mM) or DPI (40 μM) followed by HBSS or 5.0×10^7 cfu/mL LGG. β-Actin was used as a loading control for all Western blots, except for *B*. All experiments were repeated three times.

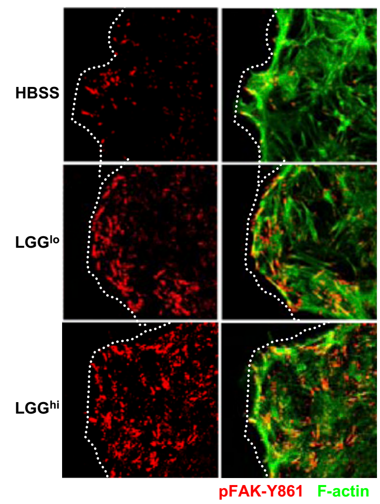

Fig. 4. Commensal bacteria mediate changes in subcellular localization of FA. Immunohistochemical images of pFAK-Y861 (red) (*Left*) or pFAK-Y861 (red) and F-actin (green) (*Right*) expression in migrating T84 cells treated with HBSS, LGG^{lo} (1.5×10^7 cfu/mL), or LGG^{hi} (5.0×10^7 cfu/mL) for 5 min. Photomicrographs are representative of three independent wounds, obtained in three independent experiments.

at tyrosine position 118 (pPAX-Y118) (Fig. S3*C*). An increase in distribution of FAs was also observed in wounded monolayers contacted with LGG and fluorescently labeled with pPAX-Y118 (Fig. S4). No significant changes in the absolute levels of unphosphorylated FAK, p130Cas, and PAX were observed over a period of 60 min of contact with LGG (Fig. 3*B* and Fig. S3*C*).

To understand the properties of LGG that mediated the activation of FAK, we compared live and physically disrupted bacterial preparations from equivalent numbers of organisms. Nonviable, sonicated bacterial preparations of LGG induced FAK phosphorylation (Fig. S3*D*), suggesting that these effects are receptor mediated rather than due to bacterial invasion. Next, we treated IECs with various ROS inhibitors before LGG stimulation to confirm that LGG mediates its effects via ROS signaling. IECs treated with LGG in the presence of NAC suffered a marked attenuation in phosphorylation of FAK (Fig. 3*C*), whereas the respiratory chain inhibitor rotenone (5 μM) had little suppressive effect, excluding the involvement of mitochondria in production of ROS (Fig. S3*E*). Interestingly, diphenylene iodonium (DPI), a flavoprotein inhibitor, did impair the phosphorylation of FAK, suggesting the involvement of NADPH oxidases (Fig. 3*C*).

Commensal Bacteria Stimulate Phosphorylation of FAK in Vivo. Having demonstrated commensal bacteria-mediated FAK activation using cultured IECs, we wanted to confirm these results using live tissue and in vivo-based systems. First, excised segments of murine colon were incubated ex vivo with PBS, commensal bacteria, or H_2O_2. Treatment of mouse colon with LGG or H_2O_2 caused a significant increase in pFAK-Y861 (Fig. 5*A* and Fig. S5). In a second in vivo assay, LGG injected into surgically closed ileal loops within live mice led to an increase in pFAK-Y861 that was primarily confined to the epithelial cells at the luminal surface (Fig. 5*B*). Finally, we tested whether bulk commensal bacteria populations from mice stimulate FAK phosphorylation in IECs. Bacterial suspensions prepared from the luminal contents of mice ceca and diluted to a density comparable to previously used LGG concentrations were equally effective in inducing pFAK-Y861 activation in vitro (Fig. 5*C*). These results suggest that dynamic changes in FAK phosphorylation is also a feature of native small bowel IECs and murine commensal bacteria.

Commensal Bacteria Enhance Wound Closure. To correlate LGG-mediated changes in phosphorylation and localization of FA proteins with functional alterations, we used an established in vitro model of epithelial wound restitution (23). Using time lapse video microscopy of scratch-wounded epithelia, we observed that LGG accelerated wound closure in a dose-dependent fashion (Fig. 6*A* and Fig. S6*A*). To confirm the role of FAK in these processes, we used a newly characterized inhibitor of FAK, 1,2,4,5-benzenetetraamine tetrahydrochloride (FAK-14) (24). We first showed that this inhibitor effectively attenuated activation of FAK by Western blot (Fig. S6*B*). Significantly, pretreatment of wounded monolayers with FAK-14 slowed cell migration even after cells were treated with LGG (Fig. 6*B*). Because phosphorylation of FAK mediates adhesion of cells to the extracellular matrix, we also monitored the effects of LGG on cell adhesion. Treatment of IECs with LGG significantly increased cell adhesion to fibronectin (Fig. S6 *C* and *E*) and collagen 1 (Fig. S6 *D* and *F*). Additionally, the LGG-mediated effects on both wound closure and cell migration were mitigated by inhibiting ROS signaling (Fig. 6*B* and Fig. S6 *E* and *F*). On the basis of these analyses, we conclude that colonization of intestinal epithelial cells with LGG significantly enhances motility of IECs via ROS- and FAK-mediated signaling pathways.

Finally, we monitored the effects of LGG on facilitating resolution of epithelial wounds in an in vivo model of restitution. To

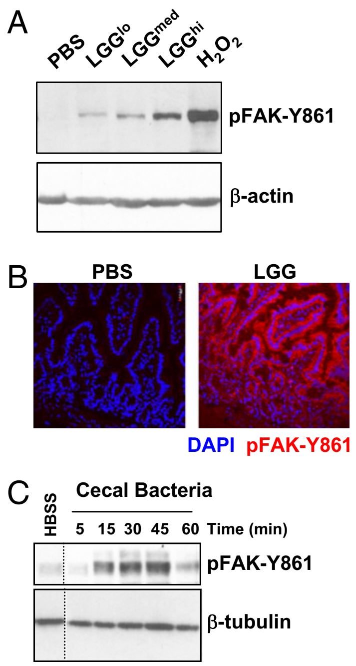

Fig. 5. LGG or murine bacterial preparations mediate phosphorylation of FAK in IECs. (*A*) Western blots of pFAK-Y861 expression in mouse colon explants treated with HBSS, LGG^{lo} (1.5×10^7 cfu/mL), LGG^{med} (5.0×10^7 cfu/mL), LGG^{hi} (1.5×10^8 cfu/mL) or H_2O_2 (1 mM). β-Actin was used as a loading control. Two independent experiments were performed with $n = 4$. (*B*) Immunohistochemical images of DAPI (blue) and pFAK-Y861 (red) expression within sections of distal small intestine loops instilled with HBSS or LGG for 30 min. $n = 3$ mice per group and representative images are shown. (*C*) Western blots of pFAK-Y861 in migrating T84 cells treated with HBSS or murine (C57BL/6) cecal microbiota preparations (5.0×10^7 cfu/mL) as described in *Materials and Methods*. β-Tubulin was used as a loading control. $^*P < 0.05$.

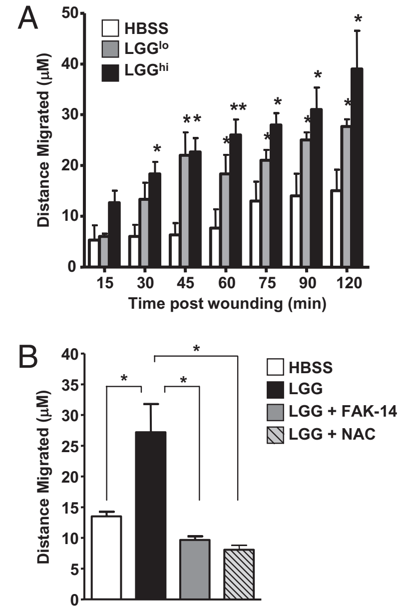

Fig. 6. Commensal bacteria enhance migration and adhesion of model IECs. (*A*) Migration distance of wounded Caco-2 monolayers treated with DMEM, LGG^{lo} (1.5×10^7 cfu/mL), or LGG^{hi} (5.0×10^7 cfu/mL) as calculated from morphometric analysis of static images shown in Fig. S6*A*. Data represent means ± SE of two independent experiments with $n = 2$. (*B*) Migration distance of Caco-2 cells pretreated with DMEM for 30 min and then incubated with DMEM or LGG (5.0×10^7 cfu/mL) for 60 min, or Caco-2 cells pretreated with 100 μM FAK-14, or 20 mM NAC for 30 min and then incubated with LGG (5.0×10^7 cfu/mL) for 60 min. Data represent means ± SE with $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$.

induce epithelial injury, mice were given low dose (3.5%) dextran sodium sulfate (DSS) in drinking water. DSS was withdrawn after 9 d to permit healing. We focused our investigation on the small bowel because DSS induces less severe injury in the small intestine relative to the colon, and we sought to evaluate the effects of commensal bacteria-mediated recovery from epithelial barrier injury rather than mucosal ulceration, which would require epithelial cell proliferation (rather than migration) to heal. Post DSS treatment, mice were orally gavaged with PBS or LGG for 1 d, after which, barrier function was assessed by measuring systemic translocation of the orally administered permeability tracers FITC-dextran and HRP. Intestinal injury was also monitored by histology and serum markers of inflammation. As expected, all DSS-treated mice had a significant increase in the translocation of HRP and FITC-dextran, as well as characteristically elevated local (myeloperoxidase, MPO, and histological ileitis) and systemic (blood KC and lipocalin-2) inflammatory markers (Fig. 7 and Fig. S7). Importantly, LGG treatment enhanced recovery from DSS-induced injury as measured by all indices (Fig. 7). To assess the role of FAK in these observations, DSS-treated mice received FAK-14 24 h before receiving LGG by oral gavage. As shown in Fig. 7 *D–F* and Fig. S7*D*, the beneficial effects of LGG post-DSS treatment were nullified by FAK-14. These data demonstrate that commensal bacteria augment mucosal repair following DSS-induced injury via the FAK signaling pathway.

Discussion

There is growing appreciation for the diverse influences of the mammalian gut microbiota on the biology of the mammalian host. Interestingly, recent data have indicated intriguing roles for the interactions between the microbiota and epithelial cells. Bacterial-epithelial contact has been shown to enhance epithelial proliferation during development and after injury (4). Studies have also linked probiotics and endogenous commensals to alterations of intercellular junctional protein expression and turnover that resulted in improved barrier properties both in vitro and in vivo (25). However, commensal bacteria have not been implicated in stimulation of epithelial cell motility or enhancement of wound restitution. Interestingly, stimulation of phagocyte actin dynamics and motility by bacterial products is a well-established process in innate immunity (26). Additionally, induced alterations in the epithelial cytoskeleton is a classic mechanism by which enteric pathogens such as *Salmonella*, *Shigella*, and other bacteria mediate intracellular invasion (27)

Here we describe biochemical events elucidating how commensal bacteria mediate epithelial movement and recovery from injury (summarized in Fig. S8). Our data demonstrate that damaged epithelial monolayers respond to commensal bacteria with ROS generation. This effect is most pronounced at the leading edge of the migrating epithelial sheet, where there is constant remodeling of the actin and turnover of FA. Furthermore, our data demonstrate that bacteria-induced ROS lead to oxidative inactivation of the PTPs, LMW-PTP and SHP-2, both of which are known regulators of FAK phosphorylation. Concomitantly, we show an increase in auto-phosphorylation of FAK, as well as other proteins of the FA and increased formation of FAs and F-actin bundling. Finally, commensal bacteria significantly increased the velocity of cell migration in vitro and enhanced recovery of barrier function postinjury in vivo.

Our laboratory demonstrated that intestinal epithelial cells generate ROS in response to contact with commensal bacteria (9, 10). This phenomenon is seen in all metazoans, as well as plants, and is a cardinal cellular response to bacteria by phagocytes (respiratory burst) (28–31). In mammalian cells, ROS have been

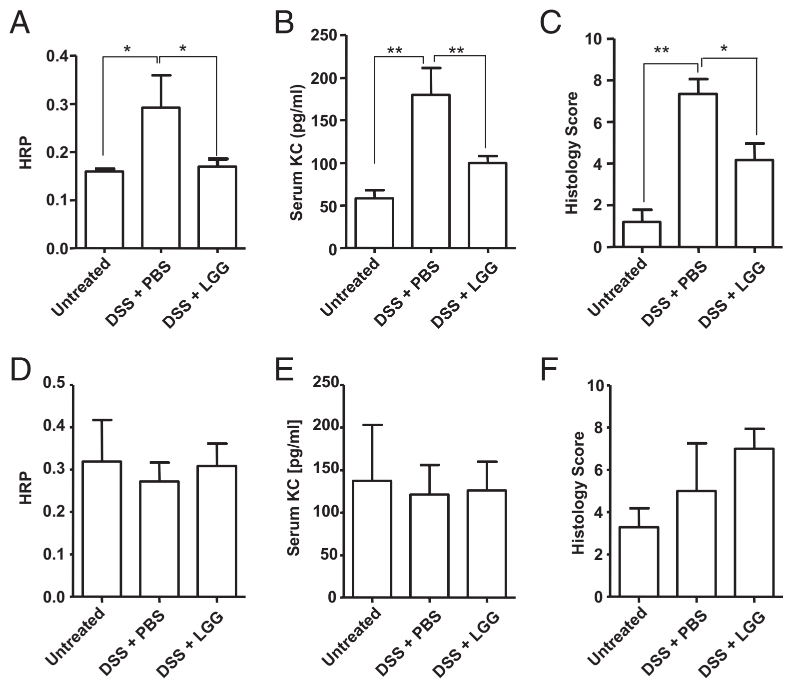

Fig. 7. Commensal bacteria attenuate ileitis in mice via FAK activation. Eight-week-old mice were weighed and given water supplemented with DSS for 9 d. On the ninth day, DSS was withdrawn and recovering mice were treated with PBS or LGG (10^8 cfu/mL) for 24 h (*A*–*C*) or 30 mg/kg FAK-14 (day 8) + PBS or LGG (day 9) for 24 h (*D*–*F*). (*A* and *D*) Serum concentration of HRP. (*B* and *E*) Serum KC levels. (*C* and *E*) Small bowel histology scores. $*P < 0.05$, $**P < 0.01$. Data represent means ± SE with $n = 6$.

shown to serve as both microbiocidal agents and critical secondary messengers in multiple signal transduction pathways (31, 32). Neutrophil and macrophage ROS can be generated by the enzymatic activation of NADPH oxidases (Nox). The Nox enzymes are widely conserved across the animal and plant kingdoms and are often responsible for ROS generation in response to bacterial stimuli (32). In humans, paralogs of Nox2 are found in many tissues, two of which, Nox1 and Duox2, are predominately expressed in colonic tissue (28). In flies, the ortholog of Duox2, dDuox, plays a vital role in epithelial homeostasis in response to commensal bacteria (33). Our data showing suppression of bacterially induced FAK activation by the flavoprotein inhibitor DPI suggest that the observed ROS generation is, at least in part, mediated by Nox enzymes. These data are consistent with observations that ROS generating NADPH oxidases (Nox2) localize at sites of lamellipodial focal complexes in migrating endothelial cells (34–36).

The signaling properties of physiologically generated ROS are mediated by rapid and transient oxidative inactivation of a number of regulatory enzymes. The sensitivity of these enzymes to ROS is conferred by catalytic cysteine residues that exist at low pK_a due to the effects of vicinal charged amino acids in the active site. These cysteines are thus maintained in a thiol anionic state at physiological pHs, a condition susceptible to oxidative inactivation (12, 13). The inactivation of these phosphatases establishes a feed-forward mechanism that activates downstream signaling events. Chiarugi et al. (17) initially described the physiologically mediated oxidative inactivation of LMW-PTP from integrin-induced ROS generation and showed consequent assembly of focal adhesions leading to cell spreading. Consistent with these studies, we show that LMW-PTP can be rapidly and transiently inactivated by ROS in cells cocultured with commensal bacteria resulting in activation of FAK. The transient nature of the inactivation shown in Fig. 2*B* (and the transient activation of FAK in Fig. 3*A*) is likely due to compensatory redox changes from up-regulation of endogenous redox sinks such as glutathione and thioredoxin, which are induced immediately after physiological ROS generation to reestablish a local reducing environment (11). Additionally, the dose dependency of ROS signaling results from the ratio of oxidized (inactive) to reduced (active) substrate enzyme (37). These properties ensure ROS signaling acts as a rheostat rather than a binary switch and could be a mechanism for the fine tuning of number of pathways controlled by redox signals. For example, signaling molecules involved in cell survival pathways such as Ubc12 (9), which controls the NF-κB and β-catenin pathways, as well as Dual specificity phosphatases (DUSPs), which modulate various MAPK activation pathways (38), could be regulated in this manner. Thus, bacteria-mediated ROS signaling may variably influence regulatory pathways in the gut. Plausibly, changes in the redox balance brought about by dynamic changes in bacterial numbers, such as during initial acquisition of the microbiota in the neonatal period, or during iatrogenic suppression of the flora that occurs with broad-spectrum antibiotic use, could have effects on cytoskeletal dynamics, epithelial restitution, and other processes. Bacterially stimulated ROS may represent a common mechanism by which the microbiota can influence the host. Potentially, some of the known beneficial effects of the normal microbiota and candidate probiotics on intestinal physiology may be mediated by the downstream effects of ROS generation.

Materials and Methods

Detection of Intracellular ROS. Synchronized migration of IEC monolayers were induced with scratch wounds inflicted immediately before coculture with HBSS, LGG, or insulin. ROS generation in IECs was detected by loading cells with the nontoxic ROS fluorescent dye Hydro-Cy3 (39).

Wound Healing Assays and Time Lapse Video Microscopy. Wounded cells were allowed to migrate in CO_2-independent medium (Invitrogen) alone or supplemented with LGG. Wound healing was recorded for up to 2 h using a CCD camera mounted on a Carl Zeiss inverted Meta 510 microscope equipped with a stage warmer maintained at 37 °C. Images were analyzed as described in *SI Materials and Methods*.

Induction and Evaluation of Experimental Ileitis. C57BL/6 mice (Jackson Laboratories) were given DSS (3.5%) for 9 d in drinking water. After the weights of the mice dropped ∼10%, mice were orally gavaged with 100 μL 10^6 cfu/mL of LGG. Control mice were kept on water and gavaged with PBS. After 24 h of LGG administration, in vivo assays of barrier function, inflammation, and tissue injury were performed as described in refs. 40, 41. Experiments with FAK-14 were set up as above, except, on the eighth day mice were IP injected with 30 mg/kg of FAK-14.

Statistics. Statistical significance was assessed by Student's *t* test, with *P* values of less than 0.05 considered significant.

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