GLP-1R Agonists Modulate Enteric Immune Responses Through the Intestinal Intraepithelial Lymphocyte GLP-1R

Obesity and diabetes are characterized by increased inflammation reflecting disordered control of innate immunity. We reveal a local intestinal intraepithelial lymphocyte (IEL)-GLP-1 receptor (GLP-1R) signaling network that controls mucosal immune responses. Glp1r expression was enriched in intestinal IEL preparations and copurified with markers of T αβ and T γδ IELs, the two main subsets of intestinal IELs. Exendin-4 increased cAMP accumulation in purified IELs and reduced the production of cytokines from activated IELs but not from splenocytes ex vivo. These actions were mimicked by forskolin, absent in IELs from Glp1r−/− mice, and attenuated by the GLP-1R agonist exendin (9-39) consistent with a GLP-1R-dependent mechanism of action. Furthermore, Glp1r−/− mice exhibited dysregulated intestinal gene expression, an abnormal representation of microbial species in feces, and enhanced sensitivity to intestinal injury following administration of dextran sodium sulfate. Bone marrow transplantation using wild-type C57BL/6 donors normalized expression of multiple genes regulating immune function and epithelial integrity in Glp1r−/− recipient mice, whereas acute exendin-4 administration robustly induced the expression of genes encoding cytokines and chemokines in normal and injured intestine. Taken together, these findings define a local enteroendocrine-IEL axis linking energy availability, host microbial responses, and mucosal integrity to the control of innate immunity.

The enteroendocrine system comprises a complex network of specialized endocrine cells situated within the epithelial lining of the stomach and small and large intestine. Gut endocrine cells synthesize and secrete dozens of peptide hormones that function as local neurotransmitters, propagating signals through engagement of the enteric nervous system. Enteroendocrine-derived hormones also act as classical hormones controlling metabolism through regulation of energy intake, digestion, absorption, and the disposal and storage of digested nutrients (1). The majority of gut hormones are secreted at low basal levels in the fasting state, and feeding produces a rapid rise in circulating levels, enabling activation of gut hormone action in response to energy intake. Plasma levels of some gut hormones, including the glucagon-like peptides, also increase rapidly in the presence of intestinal injury or mucosal inflammation (2).

Proglucagon-producing L cells are among the most intensively studied enteroendocrine cells predominantly localized to the distal small bowel and colon (3). Proglucagon liberates multiple proglucagon-derived peptides with unique biological activities, including glicentin, oxyntomodulin, and GLP-1 and GLP-2 (4). Although the actions of glicentin and oxyntomodulin remain incompletely understood, both GLP-1 and GLP-2 exert well-defined actions through distinct G-protein-coupled receptors. GLP-1 controls gut motility and food intake and stimulates insulin while simultaneously inhibiting glucagon secretion (4,5). In contrast, GLP-2 acts more locally within the gut to increase nutrient absorption and maintain the integrity of the gut epithelium (6).

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Considerable evidence supports the importance of the GLP-1 system as an integral component of the incretin axis. Indeed, transient blockade of GLP-1 receptor (GLP-1R) signaling reduces insulin, increases glucagon, and impairs glucose tolerance in multiple species (4). Similarly, genetic interruption of the murine Glp1r impairs glucose control, establishing basal GLP-1R signaling as essential for regulation of β-cell function and glucose homeostasis. Nevertheless, the low levels of circulating active GLP-1 together with the distal location of L cells have led some to question whether the major role of GLP-1 is to function as a circulating incretin hormone (7).

More recent studies have expanded concepts of L-cell biology to encompass control of GLP-1 secretion by cytokines and bacterial products. Interleukin (IL)-6 enhances secretion of GLP-1, actions mediated by direct interaction of IL-6 with gut L cells (8). GLP-1 secretion is also induced by bacterial endotoxin, enabling lipopolysaccharide (LPS)-mediated glucoregulation through the GLP-1R (9,10). Furthermore, plasma GLP-1 levels are significantly increased in hospitalized critically ill patients (10), supporting a correlation between systemic inflammation and L-cell secretion. Nevertheless, the importance of GLP-1 in the setting of localized or systemic inflammation remains uncertain. We describe a new axis linking GLP-1R signaling to the control of immune responses in intestinal intraepithelial lymphocytes (IELs), a specialized gut immune cell population that modulates innate immunity and controls gut barrier function, epithelial turnover, and the host response to enteric pathogens (11). These findings extend our understanding of enteroendocrine-immune interactions beyond traditional glucoregulatory concepts of GLP-1 action, with implications for use of GLP-1R agonists in the treatment of metabolic disorders characterized by enhanced activity of the innate immune system.

**RESEARCH DESIGN AND METHODS**

**Mice**

Male or female C57BL/6 wild-type (WT) mice were from The Jackson Laboratory (Bar Harbor, ME) and acclimated to the animal facility for a minimum of 1 week before analysis. Whole-body Glp1r−/− mice on the C57BL/6 genetic background (12) and Glp1r+/+ control mice were generated by crossing Glp1r+/− mice; 9–18-week-old animals from the same litter or family were studied and housed under specific pathogen-free conditions in microisolation cages and maintained on a 12-h light/dark cycle with free access to standard rodent diet and water unless otherwise noted. All experiments were carried out in accordance with protocols and guidelines approved by the Animal Care Committee at the Toronto Centre for Phenogenomics.

**Isolation of Mouse Intestinal IELs, Splenocytes, and Thymocytes**

Murine intestinal IELs were obtained following established protocols (13). After flushing luminal contents and removing Peyer’s patches, intestines were opened longitudinally and cut in 5–10-mm pieces. Tissue fragments were incubated twice in 10 mmol/L calcium- and magnesium-free Hanks’ balanced salt solution containing 1 mmol/L dithiothreitol and 5% FBS at 37°C followed by vortexing. Supernatants were combined, filtered through a 40-μm strainer, and pelleted (crude IEL preparations). IELs were further purified by centrifugation in a discontinuous 44/67% Percoll gradient. IELs were collected at the interface (Percoll-purified IEL preparations). At this stage, cells were routinely 50–75% lymphocytes, with epithelial elements accounting for the remainder. Mouse splenocytes and thymocytes were isolated by mechanical dissociation of the spleen and thymus, respectively, followed by red blood cell lysis.

**cAMP Analysis**

Sorted IELs, splenocytes, or thymocytes were allowed to recover for 1 h and then challenged for 15 min at 37°C with exendin-4 (Ex-4) (CHI Scientific, Maynard, MA), prostaglandin E2 (5 μmol/L; Sigma-Aldrich Canada), or the adenylyl cyclase activator forskolin (Fk) in Hanks’ balanced salt solution buffer containing 0.1% BSA and 300 μmol/L 3-isobutyl-1-methylxanthine. cAMP concentration was measured using a LANCE cAMP Detection Kit (PerkinElmer, Waltham, MA) and an EnVision Multilabel Plate Reader (PerkinElmer).

**Activation Assays in IELs and Splenocytes**

Freshly isolated splenocytes and sorted untouched IELs were stimulated in vitro with plate-bound anti-CD3 (clone 145-2C11) and soluble anti-CD28 (clone 37.51) monoclonal antibodies (mAbs) in culture medium (RPMI + 10% FBS + 50 μmol/L β-mercaptoethanol) in the presence or absence of Ex-4, exendin (9–39) (Bachem, Torrance, CA), or Fk. After incubation for 5 h at 37°C, cells were harvested for quantitative RNA and cAMP analyses.

**Dextran Sodium Sulfate–Induced Colitis**

Glp1r+/+ or Glp1r−/− female mice were maintained on drinking water containing 3% (weight for volume) dextran sodium sulfate (DSS) (molecular weight 40,000–50,000; USB, Cleveland, OH) or regular drinking water ad libitum for 7 days. Water intake and body weights were determined daily. Animals were euthanized on day 8, and for each mouse, a disease activity index score was calculated (14). Colon damage scores were determined as described (15). In acute studies, C57BL/6 male mice were maintained on drinking water containing 3% (weight for volume) DSS ad libitum for 4 days. On the fourth day, mice were switched to regular drinking water and given two subcutaneous injections of 10 nmol/kg Ex-4 or PBS separated by 12 h. Mice were killed the following morning for analysis of the small bowel and colon.

**Histology and Immunohistochemistry**

Intestinal tissue segments were fixed in 10% neutral buffered formalin and paraffin embedded. Digital image
acquisition and morphometry were performed on 5-µm histological sections stained with hematoxylin-eosin as described (16). To quantify IELs, sections were stained with a rabbit polyclonal anti-CD3 antibody (Dako Canada, Burlington, ON, Canada) and counterstained with hematoxylin. A minimum of 20 villi/section from three to four sections per tissue sample were scored.

**Generation of Bone Marrow Chimeras**

Bone marrow chimeras were generated by lethally irradiating Glp1r+/+ or Glp1r−/− female mice (1,100 cGy split into two equal doses 4 h apart) followed by reconstitution with 5 × 10⁶ bone marrow cells from donor C57BL/6 male mice as described (17). Mice were euthanized 12–14 weeks after bone marrow transplantation. Chimerism was assessed by quantitative PCR (qPCR) analysis for the sex-determinant Y chromosome (Sry) using spleen DNA from recipient mice. The efficiency of reconstitution of WT Glp1r expression within the intestinal compartment of recipient mice was assessed by qPCR.

**RNA Isolation and Analysis of mRNA Expression**

Total RNA was extracted using guanidinium isothiocyanate (18). cDNA synthesis and assessment of full-length Glp1r mRNA expression were performed as described (16,19). Real-time qPCR was performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems). Quantification of transcript levels was performed by the 2−ΔΔct method using 18S rRNA, cyclophilin, Tbp, or β-actin for normalization.

**Gut Microbiota Analysis**

Fecal DNA extraction and 16S rRNA gene amplification were carried out as previously described (20). Microbial population similarities within and between groups of Glp1r+/+ and Glp1r−/− mice were determined using metagenomic analysis as described (21). A cluster analysis tree was generated using UPGMA (unweighted pair group method with arithmetic mean) cluster analysis of Bray-Curtis dissimilarity coefficients.

**Statistical Analysis**

Except where indicated, data are presented as mean ± SD or SE. Statistical significance was determined by unpaired two-tailed Student t test or one- or two-way ANOVA with Bonferroni post hoc analysis using GraphPad Prism version 5.02 software (San Diego, CA). P < 0.05 was considered statistically significant.

**RESULTS**

**The Glp1r Is Expressed in Murine Intestinal IELs**

Recent studies highlighted limitations of commercially available antisera for detection of immunoreactive GLP-1R expression (16,22). To localize cellular sites of intestinal Glp1r expression beyond the enteric nervous system (23), we fractionated the murine small intestine by sequential collagenase digestion followed by analysis of Glp1r expression and simultaneous assessment of cell lineage–specific RNA transcripts. Quantitatively, Glp1r expression segregated with markers of the villus epithelial compartment (Supplementary Fig. 1A, fraction 1) rather than the crypt compartment, mesenchyme, or smooth muscle layer (Supplementary Fig. 1A, fraction 2). Unexpectedly, a marked increase in relative Glp1r expression was observed in RNA obtained from intestinal cell preparations enriched in IELs (Fig. 1A and B). Consistent with IEL Glp1r expression, we detected abundant expression of IEL markers such as integrin α E (Itgae) and the T-cell surface glycoprotein CD3 γ (CD3g) in RNA from purified small bowel IELs (Fig. 1A). Moreover, the level of Glp1r expression in the intestinal IEL compartment was considerably higher than in other mouse primary and secondary lymphoid organs (Fig. 1B). A 1.4-kb product corresponding to the entire coding region of the canonical mouse Glp1r was amplified from RNA obtained from Percoll-purified IELs at levels approximating those detected in RNA from mouse lung (24) (Fig. 1C). Cloning and sequencing of the 1.4-kb IEL Glp1r mRNA transcript demonstrated that it corresponded to the sequence of the canonical murine Glp1r (data not shown).

Because Tcβ and Thy IELs represent the two major subsets of intestinal IELs, we assessed Glp1r expression in RNA from Percoll-purified and FACS-sorted IEL subsets isolated from the small intestine, revealing abundant Glp1r expression in both IEL subsets (Fig. 1D). Furthermore, we detected a strong correlation between levels of Glp1r mRNA and the relative abundance of transcripts corresponding to the IEL markers Itgae and CD3g along the entire murine small intestine and colon (Supplementary Fig. 1B). Taken together, these findings identify the IEL as a previously unrecognized site of Glp1r expression in the small and large bowel.

**Neither Loss of GLP-1R Signaling nor Chronic GLP-1R Agonist Treatment of WT Mice Alters Intestinal IEL Number or Subset Composition**

To determine whether the presence or absence of GLP-1R signaling alters IEL homeostasis, we compared the frequency of IELs in the small intestine and colon of Glp1r−/− versus Glp1r+/+ mice and in C57BL/6 mice treated with liraglutide or saline for 1 week (Fig. 2). No significant differences in the phenotypic composition of the IEL population were detected in the small intestine or colon of Glp1r−/− versus Glp1r+/+ littermate control mice (Fig. 2A). Furthermore, loss of GLP-1R signaling did not affect IEL density in the jejunum (Fig. 2B). Likewise, liraglutide had no impact on IEL density in the small intestine (Fig. 2C). Thus, GLP-1R signaling is not critical for IEL development or recruitment to the intestinal niche.

**Activation of the GLP-1R on Murine Intestinal IELs Suppresses Expression of Inflammatory Cytokines**

To assess whether the IEL Glp1r mRNA transcript encodes a functional GLP-1R protein, we measured levels of
cAMP in sorted nonactivated IELs in response to Ex-4 (Fig. 3A and B). Ex-4 directly increased cAMP levels in a dose-dependent manner (half-maximal effective concentration 0.35 nmol/L). Furthermore, the relative extent of Ex-4-induced cAMP production was preserved in activated IELs and was comparable in magnitude to the cAMP response triggered by 10 μmol/L Fk (Fig. 3B). Surprisingly, IELs exhibited a modest cAMP response to the adenyl cyclase activator Fk compared with T cells from the thymus (Supplementary Fig. 1C). Because IELs produce a broad spectrum of cytokines that play key roles in intestinal inflammation, pathogen clearance, and epithelial barrier function (25), we examined whether GLP-1R signaling modified cytokine expression in sorted intestinal IELs in vitro. As a control, we examined splenocytes that express low levels of Glp1r mRNA. Following activation by immobilized anti-CD3 and soluble anti-CD28 antibodies, transcript levels of the proinflammatory cytokines IL-2, IL-17a, interferon γ, and tumor necrosis factor-α robustly increased in isolated IELs and splenocytes (Fig. 3C). Ex-4 significantly attenuated the induction of both mRNA expression and protein production of proinflammatory cytokines in IELs but not in splenocytes (Fig. 3C and Supplementary Fig. 1E). Notably, the magnitude of reduction in cytokine expression in IELs after Ex-4 treatment paralleled reductions observed with Fk (Fig. 3C). The actions of Ex-4 to suppress cytokine induction required the canonical GLP-1R because they were not detected in IELs from Glp1r−/− mice (Fig. 3D). Moreover, the GLP-1R antagonist exendin (9-39) attenuated the Ex-4-dependent suppression of cytokine mRNA expression (Supplementary Fig. 1D). In contrast, Fk robustly inhibited cytokine upregulation in IELs from Glp1r−/− mice (Fig. 3D). These findings link direct activation of GLP-1R signaling to attenuation of proinflammatory cytokine expression in IELs.

**The Severity of Intestinal Injury Is Increased in Glp1r−/− Mice**

IELs play an important role in maintaining the integrity of the epithelial barrier (15). Baseline small intestinal permeability assessed following 14C-mannitol oral gavage was not different in Glp1r+/+ versus Glp1r−/− mice (Supplementary Figure E1). The thymus (Supplementary Fig. 1C). Because IELs produce a broad spectrum of cytokines that play key roles in intestinal inflammation, pathogen clearance, and epithelial barrier function (25), we examined whether GLP-1R signaling modified cytokine expression in sorted intestinal IELs in vitro. As a control, we examined splenocytes that express low levels of Glp1r mRNA. Following activation by immobilized anti-CD3 and soluble anti-CD28 antibodies, transcript levels of the proinflammatory cytokines IL-2, IL-17a, interferon γ, and tumor necrosis factor-α robustly increased in isolated IELs and splenocytes (Fig. 3C). Ex-4 significantly attenuated the induction of both mRNA expression and protein production of proinflammatory cytokines in IELs but not in splenocytes (Fig. 3C and Supplementary Fig. 1E). Notably, the magnitude of reduction in cytokine expression in IELs after Ex-4 treatment paralleled reductions observed with Fk (Fig. 3C). The actions of Ex-4 to suppress cytokine induction required the canonical GLP-1R because they were not detected in IELs from Glp1r−/− mice (Fig. 3D). Moreover, the GLP-1R antagonist exendin (9-39) attenuated the Ex-4-dependent suppression of cytokine mRNA expression (Supplementary Fig. 1D). In contrast, Fk robustly inhibited cytokine upregulation in IELs from Glp1r−/− mice (Fig. 3D). These findings link direct activation of GLP-1R signaling to attenuation of proinflammatory cytokine expression in IELs.
To evaluate the importance of \textit{Glp1r} in the setting of mucosal injury, we compared the severity of DSS-induced colitis in \textit{Glp1r}^{2/2} versus \textit{Glp1r}^{+/+} mice. \textit{Glp1r}^{2/2} mice lost significantly more weight despite similar intake of DSS (Fig. 4A and B); colon lengths were shorter at baseline yet reduced to a comparable extent after DSS (Fig. 4C). Furthermore, \textit{Glp1r}^{2/2} mice exhibited significantly increased disease activity scores (Fig. 4D) and greater epithelial damage (Fig. 4E) reflected by a greater colon damage score (Fig. 4F).
Murine intestinal IELs express a functional GLP-1R. A: Ex-4 stimulates cAMP accumulation in nonactivated IELs from Glp1r+/+ mouse small intestine. Levels of cAMP were quantified in sorted untouched IELs following incubation with increasing concentrations of Ex-4. Prostaglandin E2 (PGE2) (5 μmol/L) was used as a positive control. Shown are combined data (mean ± SD) of two independent batches of sorted cells assayed in triplicate.

B: Ex-4 and Fk 10 μmol/L induce comparable increases in cAMP in both activated and nonactivated IELs. Sorted untouched IELs were incubated for 5 h without (nonactivated IELs) or with (activated IELs) anti-CD3/CD28 antibodies. The cells were then challenged with Ex-4, Fk, or vehicle alone (Veh) for 15 min, and cAMP content was quantified. Data are relative to the cAMP levels in Veh-treated nonactivated IELs and are mean ± SD of four independent experiments with two or three replicates each.

C and D: Ex-4 suppresses the induction of cytokine expression in activated IELs from Glp1r+/+ (C) but not Glp1r−/− (D) mouse small intestine. Splenocytes and sorted untouched IELs were activated with anti-CD3/CD28 for 5 h in the presence of Ex-4, Fk, or Veh. mRNA levels of the indicated cytokines were assessed by qPCR. Data in C are mean ± SD of one out of five independent experiments with similar results assayed in duplicate or triplicate. Data in D are from a single experiment assayed in triplicate.

Figure 3—Murine intestinal IELs express a functional GLP-1R. A: Ex-4 stimulates cAMP accumulation in nonactivated IELs from Glp1r+/+ mouse small intestine. Levels of cAMP were quantified in sorted untouched IELs following incubation with increasing concentrations of Ex-4. Prostaglandin E2 (PGE2) (5 μmol/L) was used as a positive control. Shown are combined data (mean ± SD) of two independent batches of sorted cells assayed in triplicate. B: Ex-4 and Fk 10 μmol/L induce comparable increases in cAMP in both activated and nonactivated IELs. Sorted untouched IELs were incubated for 5 h without (nonactivated IELs) or with (activated IELs) anti-CD3/CD28 antibodies. The cells were then challenged with Ex-4, Fk, or vehicle alone (Veh) for 15 min, and cAMP content was quantified. Data are relative to the cAMP levels in Veh-treated nonactivated IELs and are mean ± SD of four independent experiments with two or three replicates each. C and D: Ex-4 suppresses the induction of cytokine expression in activated IELs from Glp1r+/+ (C) but not Glp1r−/− (D) mouse small intestine. Splenocytes and sorted untouched IELs were activated with anti-CD3/CD28 for 5 h in the presence of Ex-4, Fk, or Veh. mRNA levels of the indicated cytokines were assessed by qPCR. Data in C are mean ± SD of one out of five independent experiments with similar results assayed in duplicate or triplicate. Data in D are from a single experiment assayed in triplicate.
Intestinal GLP-1R Signaling Modulates the Expression of Genes Involved in Immune Regulation and Epithelial Protection and Repair

To understand how GLP-1R signaling controls mucosal integrity, we examined expression of genes important for epithelial repair, barrier function, and immune regulation in the colon of Glp1r−/− and Glp1r+/+ mice in the presence or absence of DSS-induced colitis (Fig. 5 and Supplementary Fig. 2). Remarkably, even in the absence of experimental gut injury, Glp1r−/− mice exhibited significant reductions in expression of genes that contribute to epithelial protection and repair (trefoil factor [Tff]-1 and -2, transforming growth factor [Tgf]-b1 and -3, epidermal growth factor receptor [Egfr], keratinocyte growth factor [Fgf7], hepatocyte growth factor [Hgf]), the innate immune response (Il6, Il1b), and inflammation (Il12b). Furthermore, induction of gut injury with DSS produced differential colonic gene expression (Tff3, Tgfβ2, Ifng, Hgf) in Glp1r+/+ versus Glp1r−/− mice. These findings illustrate that GLP-1R signaling regulates genes important for intestinal homeostasis in the presence and absence of gut inflammation.

Bone Marrow Radiation Chimeras Establish the Essential Role of the IEL GLP-1R for Control of Intestinal Gene Expression

Because dysregulated intestinal gene expression in Glp1r−/− mice potentially reflects loss of GLP-1R signaling in intestinal and extraintestinal cell types, we reassessed colonic gene expression after transplantation of bone marrow from C57BL/6 male donor mice into Glp1r−/− or Glp1r+/+ recipient females. In such a setting, Glp1r−/− mice are reconstituted with Glp1r+/+ hematopoietic stem cells, including IELs. Chimerism, assessed in spleen DNA from the recipient mice by qPCR, was 84.3 ± 6.4%. Analysis of Glp1r expression in jejunum and sorted IELs from Glp1r−/− and Glp1r+/+ recipient females demonstrated that IELs derived from transplanted marrow repopulated the intestinal compartment (Supplementary Fig. 3B). The reconstitution efficiency of Glp1r expression in the jejunum and IELs of Glp1r−/− recipients 12 weeks after bone marrow transplantation was 64% and 73%, respectively. We focused our analysis on genes shown in Fig. 5 to be dysregulated in the colon of Glp1r−/− mice. Selective re-establishment of WT IELs in the intestinal mucosa of Glp1r−/− mice normalized gene expression profiles for the majority of genes examined (Fig. 6).

Acute Ex-4 Administration Attenuates Edema and Increases Expression of Immunomodulatory and Antimicrobial Genes in the Small Intestine

IELs control intestinal barrier function and repair and protect the intestinal mucosal epithelium by promoting pathogen clearance and lysing pathogen-infected cells (26). Accordingly, we examined expression of immunomodulatory genes in the jejunum of C57BL/6 mice in response to Ex-4 administration (Fig. 7A). Ex-4 markedly upregulated levels of Il1b, Il6, Il22, Il12b, Tnfa, Cd2, Cxcl1,
and Cxcl2 mRNA transcripts. The augmentation of gene expression by Ex-4 was rapid, detectable by 4 h, and transient, with levels of most mRNA transcripts returning to normal by 24 h. Increases in IL-6 and tumor necrosis factor-α protein levels were also observed 4 h after Ex-4 administration (Supplementary Fig. 3C). Ex-4 also significantly increased expression of genes that encode antimicrobial proteins (regenerating islet-derived protein 3 [RegIII]γ and RegIIIβ) or play a role in pathogen clearance (IL-5, IL-13). For some genes, the Ex-4–dependent induction of this group of genes, particularly RegIIIγ and RegIIIβ, was sustained with continued Ex-4 treatment (Fig. 7A). These data suggest that activation of GLP-1R signaling establishes a generalized cytoprotective response in the murine intestine.

Assessing the effects of Ex-4 or liraglutide in mice with DSS-induced colitis was complicated by marked GLP-1R agonist–mediated reductions in water intake, which attenuated intestinal injury. Consequently, we first induced intestinal injury with DSS for 4 days and then treated mice with two injections of Ex-4 12 h apart. Acute Ex-4 had no effect on body weight, colon length, or colon damage score (Fig. 7B and Supplementary Fig. 3D) but significantly reduced colon weight (Fig. 7B) consistent with a reduction of DSS-induced colitis-associated edema. Ex-4 significantly upregulated expression of inflammatory, antimicrobial, and growth factor genes in the ileum (Fig. 7C). Because IELs play important roles in host mucosal defense and response to microbial pathogens, we examined the population of major microbial species by analysis of 16S bacterial rRNA genes in the feces of Glp1r<sup>−/−</sup> versus Glp1r<sup>+/+</sup> mice (Fig. 8A). Remarkably, highly significant differences in the relative abundance of Gammaproteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria were detected. Bacteroidetes and Firmicutes represented the majority of the bacterial population in both Glp1r<sup>−/−</sup> and Glp1r<sup>+/+</sup> mice.

Figure 5—GLP-1R signaling modulates the colonic expression of genes important for immune regulation and epithelial protection and repair. Gene expression was examined in colonic RNA samples from Glp1r<sup>+/+</sup> (WT) or Glp1r<sup>−/−</sup> (knockout [KO]) mice (n = 8–12 mice/group) maintained on regular drinking water or drinking water supplemented with 3% DSS for 7 days. *P < 0.05, **P < 0.01, ***P < 0.001 for Glp1r<sup>−/−</sup> vs. Glp1r<sup>+/+</sup> on regular drinking water; #P < 0.05, ##P < 0.01, ###P < 0.001 for Glp1r<sup>−/−</sup> vs. Glp1r<sup>−/−</sup> on DSS drinking water.
(Supplementary Fig. 4A). Less similarity of microbial profiles was seen between groups than within groups. Taken together, these findings suggest that GLP-1R signaling influences the establishment of the gut microbiome and the normal host immune response to intestinal injury.

**DISCUSSION**

GLP-1R is expressed in the small intestine and colon (23, 27); however, previous efforts to identify cellular sites of GLP-1R expression within the gut yielded conflicting results. Using a GLP-1R antibody and immunohistochemistry, Kedees et al. (28) reported GLP-1R expression in the intestinal mucosa, enteric nervous system, and Paneth cells, whereas studies using the GLP-1R promoter to direct expression of a fluorescent reporter protein localized intestinal GLP-1R expression to enteric neurons (23). GLP-1Rs have also been localized to gastric parietal cells in rats (29) and in the Brunner's gland of the duodenum, parietal cells and smooth muscle cells in the gastric muscularis externa, and myenteric plexus neurons throughout the primate small and large bowel (30). Given the lack of specificity of the majority of commercially available GLP-1R antibodies and existing discrepancies in studies reporting sites of intestinal GLP-1R expression, we initiated studies to localize the sites of endogenous GLP-1R mRNA expression in the murine gut.

Although mRNA transcripts encoding a functional GLP-1R were detected in rodent immune cells isolated from spleen, lymph nodes, and bone marrow (19), the current experiments reveal that IEL Glp1r expression is considerably more abundant relative to Glp1r mRNA levels in other mouse lymphoid organs. Several lines of evidence support the functional importance of the newly described IEL GLP-1R. First, Ex-4 directly increased cAMP accumulation in isolated IELs. Second, Ex-4 robustly suppressed expression of cytokines following activation of IELs from WT mice but had no effect on IELs from Glp1r2/2 mice. Furthermore, reconstitution of Glp1r2/2 mice with bone marrow from C57BL/6 donors normalized the expression of immune-related genes in the intestine. Taken together, these findings establish the functional importance of the intestinal IEL GLP-1R.

The delineation of an enteroendocrine-immune axis is not without precedent. Infection of severe combined immunodeficient mice with Trichuris muris reduced the number of enteroendocrine cells compared with WT controls, whereas the number of gut endocrine cells increased significantly following reconstitution of immunodeficient mice with CD4+ T cells from infected WT mice (31). Furthermore, bacterial-derived products such as LPS and flagellin independently modulated expression of genes encoding peptide hormones and multiple ligands and receptors important for the immune response in LCC-18 enteroendocrine cells (32). Conversely, receptors for vasoactive intestinal peptide and pituitary adenylate cyclase–activating peptide have been localized to intestinal IELs (33), whereas Toll-like receptors are expressed on enteroendocrine cells, and Toll-like receptor activation by bacterial products promotes secretion of cholecystokinin from gut endocrine cells (34).

![Figure 6](image-url)
Considerable evidence attests to the importance of inflammation and innate immunity in the control of metabolism (35,36); however, few studies have examined whether IELs play a role in the development and/or treatment of experimental models of type 2 diabetes or obesity. Emerging data link cytokine and chemokine activity with control of enteroendocrine cell responses. Direct administration of RANTES (regulated on activation, normal T cell expressed and secreted) to NCIH716 cells rapidly reduces GLP-1 secretion and cAMP accumulation, and RANTES reduces plasma levels of GLP-1 and blunts the rise in insulin levels after glucose administration in vivo.
GLP-1 and enhanced proglucagon expression in the murine brainstem (47) and distal gut (48), whereas microbial metabolites such as indole directly activate GLP-1 secretion from L cells (49). Collectively, these emerging findings link the gut microbiome and its metabolic byproducts with the control of central and peripheral GLP-1 production. Reduction of energy intake and malnutrition has been associated with defective immune responses, and accumulating evidence supports an intimate link among energy availability, nutrient sensing, and the regulation of the innate immune response (50). Of note, Glp1r−/− mice exhibit resistance to diet-induced obesity (51), increased relative abundance of Bacteroidetes, and reduced abundance of Firmicutes in their microbiota, whereas obese mice show a decrease in Bacteroidetes and an increase in Firmicutes (52). Collectively, the data establish a previously unrecognized local GLP-1-IEL-GLP-1R axis that may influence microbiome composition and the response to intestinal inflammation (Fig. 8B). Because considerable evidence supports a role for gut-derived inflammation in the pathophysiology of insulin resistance arising in subjects with obesity and diabetes (35), we hypothesize that GLP-1R agonists exert favorable nonglycemic actions in part through modulation of IEL-GLP-1R signaling. This hypothesis requires further validation through experimental models of metabolic dysfunction and inflammation.

**Figure 8**—A: Glp1r+/+ and Glp1r−/− mice exhibit significant differences in the relative abundance of several bacterial phyla. Comparisons were made using fecal samples from 11–14-week-old Glp1r+/+ and Glp1r−/− female mice maintained on standard rodent chow and housed in groups of the same genotype per cage (n = 3–5 mice/group). *P < 0.05 for Glp1r+/+ vs. Glp1r−/− mice. B: IELs and L cells define a local cellular network within the boundaries of the intestinal mucosal epithelium that translates information from the gut lumen (nutrients, bacterial products) through GLP-1 to control host mucosal immune responses through modulation of innate immunity.
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Cellular Fractionation of Mouse Small Intestine
Cellular fractionation of the mouse small intestine was performed as described (13). Briefly, the small intestine was removed, flushed with saline, opened and cut into 5-10 mm segments. The tissue was rinsed in calcium and magnesium-free Hank’s balanced salt solution (HBSS-/-) containing 10 mM EDTA and then subjected to sequential digestion with collagenase XI (1 mg/ml HBSS-/-, Sigma-Aldrich Canada, Oakville, ON). Cells released after each digestion (referred to as ‘fraction 1’ and ‘fraction 2’) were recovered by centrifugation and used for quantitative RNA analysis.

Flow Cytometry
Freshly isolated Percoll-purified IELs were treated with Fc block (anti-mouse CD16/CD32 mAb, BD Biosciences Canada, Mississauga, ON) for 15 min at 4°C. Cell surface staining was then performed by incubation for 30 min at 4°C with fluorochrome-coupled anti-mouse CD45, CD3ε, CD19, CD4, CD8α, CD8β, TCRβ and TCRγδ mAbs obtained from BD Biosciences or eBioscience (San Diego, CA). A viability marker (DAPI or 7-AAD) was included to discriminate live cells. Stained cells were analyzed on a Gallios flow cytometer (Beckman Coulter Canada, Mississauga, ON). Data analysis was performed using Kaluza software (Beckman Coulter Canada). For FACS enrichment, pooled Percoll-purified IELs from 2-6 mice were labeled with the appropriate antibodies and sorted on a MoFlo Astrios instrument (Beckman Coulter Canada). Live untouched intestinal IELs were also obtained from pooled Percoll-purified IELs by sorting using forward and side scatter features in combination with viability staining. Post-sorting purity was consistently >96% and viability >97%.

Determination of Cytokine/Chemokine Levels
Protein levels of IFNγ, IL-1β, IL-2, IL-6, IL-13, TNFα and Cxcl1 were quantified in jejuna extracts, and media from cultured IELs using a Cytometric Bead Array assay kit (BD Biosciences). Jejunal extracts were homogenized in PBS containing 0.5% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich Canada). Homogenates were centrifuged at 16,000g for 20 min at 4°C, supernatants collected, and total protein content determined using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL).
Supplementary Figure 1. (A) Glp1r expression segregates with markers of the villus epithelial compartment following cellular fractionation of the mouse small intestine. Fractions 1 and 2 correspond to cell fractions obtained following sequential collagenase XI digestion of the small intestine. Transcript levels for the different cell lineage markers are expressed relative to the corresponding jejunal mRNA levels and are mean±SD of 5 independent fractionation experiments. iFabp, intestinal fatty acid binding protein; Clca3, chloride channel calcium activated 3; ChrA, chromogranin A; Crypt5, cryptdin 5; Uchl1, ubiquitin carboxy-terminal hydrolase L1. (B) Glp1r expression along the mouse intestine correlates with the abundance of IEL markers. mRNA levels in duodenum (Duo), proximal (P), mid (M) and distal (D) jejenum (Jej), ileum (Ile) and colon are expressed relative to the corresponding transcript levels in the duodenum and are mean±SD (n=4 mice). (C) Contrasting sensitivity to forskolin among different murine lymphoid cell populations. Levels of cAMP were quantified in sorted untouched IELs, splenocytes, and thymocytes following incubation with either veh, Ex-4 or Fk. Data are expressed relative to the corresponding cAMP levels in veh-treated cells and are mean±SD from two independent experiments with 2-3 replicates each (IELs) or from a single experiment performed in triplicate (splenocytes and thymocytes). (D) Exendin(9-39) antagonizes exendin-4 suppression of cytokine induction in activated IELs. Sorted untouched IELs were activated with anti-CD3/CD28 for 5 h in the presence of Ex-4 (3 nM) or vehicle (Veh) with or without exendin(9-39) (1 μM). mRNA levels of the indicated cytokines were assessed by qPCR. Data are mean±SD of a single experiment assayed in triplicate. (E) Exendin-4 suppresses cytokine protein levels in activated IELs from Glp1r+/+ mice. Sorted untouched IELs were activated with anti-CD3/CD28 for 5 h (IFNγ, top panel) or 16 h (TNFα, bottom panel) in the presence of Ex-4 or vehicle (Veh). Levels of the indicated cytokines were assessed by cytometric bead assay. Data are mean±SD and representative of one out of three independent experiments. Values for the non-activated Veh-treated IELs were below the detection limit of the assay. ***= P, .001.
SUPPLEMENTARY DATA

A

B

C

D

E

Supplementary Figure 2. Gene expression in colon samples of Glp1r+/+ vs Glp1r−/− mice following 7 days of exposure to drinking water containing 3% DSS or regular drinking water (veh). n=9-12 mice/group. *, **, *** p<0.05, p<0.01 and p<0.001, respectively, for Glp1r+/+ vs Glp1r−/− on DSS drinking water.
Supplementary Figure 3. (A) Small bowel permeability of 3-month-old Glp1r+/+ and Glp1r−/− male mice. Mice were fasted overnight (16 h) and then given an oral gavage of 200 μl of PBS containing 0.4 μCi D-[1-14C]-mannitol (Perkin Elmer, Waltham, MA). Blood samples (50 μl) were collected from the tail vein after 0, 30, 60, 120 and 180 min and the amount of radioactivity in plasma determined by scintillation counting. (B) Quantitative assessment of Glp1r expression in jejunum (left panel) and sorted small intestinal IELs (right panel) from recipient Glp1r−/− female mice reconstituted with bone marrow from C57BL/6 donor male mice. The TaqMan expression assay targeting exons 3-4 of the murine Glp1r mRNA detects both wild-type and knockout Glp1r transcripts, whereas the assay targeting exons 5-6 only detects the wild-type Glp1r mRNA. Glp1r transcript levels are expressed relative to the Glp1r mRNA levels in Glp1r+/+ recipient mice (grey horizontal stripe). Shown are combined data from 2 independent bone marrow transfer experiments. Each data point corresponds to one mouse. (C) Cytokine protein levels in jejunal extract samples from C57BL/6 mice that were given a single i.p. injection of Ex-4 (10 nmol/kg) or PBS and then sacrificed 4 h later. n=5 samples per treatment. **, ***p<0.01 and p<0.001, respectively for Ex-4 vs PBS-treated mice. IL-6 and TNFα protein levels for PBS-treated mice were below the detection limit of the assay and assigned a value of 0. (D) Colon damage score in C57BL/6 mice maintained on drinking water supplemented with 3% DSS for 4 days followed by 2 injections (12 h apart) of Ex-4 (10 nmol/kg) or PBS. n=6 mice/group.
**SUPPLEMENTARY DATA**

**A**

**Intestinal permeability**

- **Glp1r**
- **Glp1r**

![Graph showing intestinal permeability over time](image)

**Time (min)**

**B**

**JEJUNUM**

**RELATIVE Glp1r mRNA LEVELS**

- **exons 3-4**
- **exons 5-6**

**Glp1r TaqMan assay**

- **Sorted CD45+ IELs**

**Glp1r expression in Glp1r** recipients

**C**

**IL-1β**

![Graph showing IL-1β levels](image)

**IL-6**

![Graph showing IL-6 levels](image)

**IFNγ**

![Graph showing IFNγ levels](image)

**TNFα**

![Graph showing TNFα levels](image)

**Cxcl1**

![Graph showing Cxcl1 levels](image)

**D**

**Colon Damage Score**

- **PBS**
- **Ex-4**

![Graph showing colon damage score](image)
Supplementary Figure 4. (A-B) Relative abundance of microbial communities in fecal samples from Glp1r<sup>+/+</sup> and Glp1r<sup>-/-</sup> mice. Bacteroidetes (light brown) and Firmicutes (yellow) represent the major bacterial populations in Glp1r<sup>+/+</sup> and Glp1r<sup>-/-</sup> mice. Each bar indicates the microbial composition of one mouse. MEtaGenome Analysis (MEGAN) was performed to determine microbial population similarities within and between groups of Glp1r<sup>+/+</sup> and Glp1r<sup>-/-</sup> mice. The cluster analysis tree was generated using UPGMA cluster analysis of Bray-Curtis dissimilarity coefficients.