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Diacylglycerol kinase synthesized by commensal *Lactobacillus reuteri* diminishes Protein Kinase C phosphorylation and histamine-mediated signaling in the mammalian intestinal epithelium

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Abstract

Lactobacillus reuteri 6475 (Lr) of the human microbiome synthesizes histamine and can suppress inflammation via type 2 histamine receptor (H2R) activation in the mammalian intestine. Gut microbes such as Lr promote H2R signaling and may suppress H1R pro-inflammatory signaling pathways in parallel by unknown mechanisms. In this study, we identified a soluble bacterial enzyme known as diacylglycerol kinase (Dgk) from Lr that is secreted into the extracellular milieu and presumably into the intestinal lumen. DgK diminishes diacylglycerol (DAG) quantities in mammalian cells by promoting its metabolic conversion and causing reduced PKC phosphorylation (pPKC) as a net effect in mammalian cells. We demonstrated that histamine synthesized by gut microbes (Lr) activates both mammalian H1R and H2R, but Lr-derived Dgk suppresses the H1R signaling pathway. Phospho-PKC and IκBα were diminished within the intestinal epithelium of mice and humans treated by WT Lr, but pPKC and IκBα were not decreased in treatment with *ΔdgkA* Lr. Mucosal *IL-6* and systemic IL-1α, eotaxin and G-CSF

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Authors Contribution

BPG was involved in the study design, completion of all experiments, data analysis and interpretation and manuscript preparation. AH contributed to the dgkA mutant *L. reuteri*. SA helped with western blot analysis and scientific discussion. JWN took part in mRNA isolation and scientific discussion. RF took part in flow analysis. AH performed histamine quantification by MS. ME and ML helped with tissue harvesting AM performed all the fIHC staining. SV performed the MAGPIX assay. MW helped with Swiss Webster animal experimental set up at MIT, CA. MW, JGF and JV provided guidance, help design the experiments and write the manuscript.

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were suppressed in WT Lr, but not in *ΔdgkA* Lr colonized mice. Collectively, the commensal microbe Lr may act as a "microbial antihistamine" by suppressing intestinal H1R mediated pro-inflammatory responses via diminished pPKC-mediated mammalian cell signaling.

Keywords

antihistamine; cytokines; diacylglycerol kinase; gut; H1R; H2R; histamine receptors; interleukin; intestine; *Lactobacillus*; lipid; inflammation; microbe; protein kinase C; signaling

Introduction

Diacylglycerol kinases (DGK) are key enzymes in mammalian lipid metabolism that phosphorylate diacylglycerol (DAG) formed by the turnover of membrane phospholipids to phosphatidic acid (PA) [1,2]. Many mammalian hormones, growth factors, and other cell stimuli evoke a transient increase in the amounts of cellular diacylglycerol (DAG) through hydrolysis of phosphoinositides by phospholipase C (PLC) [3] upon G-protein coupled receptor activation [4]. As an example, when histamine binds to a G-protein coupled histamine receptor [5], DAG is an established second messenger and an allosteric activator of PKC [4,6,7] for important biological processes [3,8]. In mammals, DAG is a precursor in phospholipid metabolism, but it also serves as an intracellular lipid signal that activates protein kinase C (PKC) and is involved in cell cycle regulation, cell survival, tumorigenesis and apoptosis [1,9]. DGK consumes DAG to produce PA and is a potential terminator of DAG signaling. By attenuating DAG levels, DGK may downregulate membrane localization of PKC and may terminate transient receptor-induced PKC activation, thereby inhibiting signaling pathways downstream of PKC [3].

In bacteria, the head-group of phosphatidylglycerol is extensively used in the biosynthesis of membrane components, and removal of the head group generates DAG [8]. DAG is converted to PA by Dgk [2] and recycles DAG into the cytidine diphosphate-diacylglycerol pathway for phospholipid synthesis, thereby preventing the lethal accumulation of DAG in bacterial membranes [8]. *Lactobacillus reuteri* 6475 (Lr) genome has been sequenced [10], and this bacterial genome contains a gene encoding diacylglycerol kinase (Dgk). Gram positive bacteria have been documented to express soluble isoforms of Dgk [8,11]. Since bacterial Dgk shares 19% sequence identity with mammalian DGK [8] we speculated that Lr-derived Dgk enzyme might inhibit DAG downstream signaling in bacterial and mammalian cells [3,8].

A key biogenic amine, histamine, signals via H1R to affect PKC signaling in mammalian cells. Histamine serves a primary role as a mediator of allergic inflammation [12,13]. Histamine is also a neurotransmitter that plays a key role in areas of the central nervous system enriched with histaminergic neurons such as the hippocampus [5,14,15]. Apart from mammals, microbes can synthesize histamine to maintain intracellular pH [16,17]. One such bacterium, *Lactobacillus reuteri* 6475 belongs to the phylum Firmicutes and is indigenous to the gastrointestinal tracts of avian and mammalian species [18]. This organism is considered to be a generally recognized as safe (GRAS) and beneficial microbe, and has been used globally as a probiotic for more than two decades. A recent pangenomic study showed that Lr 6475 strain is distinctive among gut microbes by containing a complete chromosomal *hdc*

gene cluster (genes *hdcA*, *hdcB*, *hdcP*) and the genetic capacity to convert histidine to histamine [19]. Lr–derived histamine suppressed pro-inflammatory cytokines in intestinal epithelial cells, monocytes [1720] and intestinal inflammation in different rodent models [21,22]. However, the molecular mechanisms behind the interactions of Lr-derived histamine and the mammalian intestinal epithelium are not clearly understood.

Histamine interacts with the host using four different mammalian receptors (histamine receptor 1 (H1R), H2R, H3R and H4R) [23]. The majority of histamine receptors in the gut are known to be H1R and H2R [24,25]. H1R is known to initiate pro-inflammatory pathways by increasing phosphorylation of protein kinase C (pPKC) [5,26,2728]. H2R, by contrast, suppresses inflammation by inducing protein kinase A (PKA) phosphorylation [5,29] or by suppressing TLR-mediated inflammation [30]. H2R antagonists increased infection risk and necrotizing enterocolitis in low birth weight infants [31,32] and doubled the risk of hospitalization and surgery for patients with Crohn's disease [33]. Suppression of intestinal inflammation depended on the presence of dietary L-histidine and histamine-generating Lr 6475 in a murine colitis model via a H2R-mediated mechanism [34]. Histidine decarboxylase (HDC) is the unique enzyme responsible for generation of a biogenic amine, histamine, via conversion of L-histidine in mammals, including humans [14,35]. HDC deficiency in mice was shown to promote inflammation-associated colorectal cancer (CRC) by accumulation of $CD11b^{+}Gr\text{-}1^{+}$ immature myeloid cells (IMCs) in bone marrow and spleen compared to wild-type mice [14]. This deficiency was associated with increased concentrations of pro-inflammatory cytokines, especially IL-6 and IL-1, and excessive quantities of IL-1 were generated at least partly by immature myeloid cells in the circulation [14]. Histamine plays a key role in the maturation of immature myeloid cells (IMCs) via HDC) expression [36].

Histamine derived from *L. reuteri* differentially promotes H2R pathway signaling and may antagonize H1R signaling in parallel. Microbiome-generated Dgk synthesized by Lr may interact with host epithelial DAG and inhibit H1R downstream signaling by converting DAG to PA, thereby reducing quantities of DAG, preventing PKC phosphorylation and likely reducing intestinal inflammation. To better understand the molecular interactions of a single commensal bacterium Lr with the intestinal epithelium and its impact on shaping gut immune maturation, the mammalian intestine was colonized with WT or mutant (*ΔhdcA* or *ΔdgkA*) Lr strains deficient in diacylglycerol kinase or histamine production using inbred and outbred germ-free (GF) mouse models. The results described in this manuscript show that bacterial DgK is secreted into the extracellular milieu and modulates PKC signaling in mouse and human intestinal epithelial cells. Furthermore, microbial DAG kinase appears to modulate myeloid cell maturation and cytokine production *in vivo*.

Results

Wild type and mutant *L. reuteri* 6475 stably colonize the germ-free mouse intestine

Ten week-old BALB/c germ-free mice were either colonized with *L. reuteri* 6475 WT (WT) or *ΔhdcA L. reuteri* 6475 (*ΔhdcA*) or *ΔdgkA L. reuteri* 6475 (*ΔdgkA*) on day 1 and maintained for 22 days (Figure 1a). On day 23 the mice were euthanized and intestinal contents were collected and processed as described. Extracted DNA was quantified using qPCR. GF mice colonized for 22 days with WT or *ΔhdcA* or *ΔdgkA* Lr were successfully

colonized with a single Lr administration. We detected $\log_{10}8.56 \pm 0.72$ of WT Lr, $\log_{10}7.64 \pm 0.76$ of *ΔhdcA*, $\log_{10}7.06 \pm 0.66$ of *ΔdgkA* per gram wet weight of cecum contents of BALB/c gnotobiotic mice (Figure 1b). Similarly, 13 week-old Swiss Webster (SW) GF mice were stably colonized with *L. reuteri* 6475 WT (WT) for 22 days (Supplemental Figure 1a). However, SW GF mice yielded more abundant gut lactobacilli following colonization by Lr compared to BALB/c GF mice. On day 23 the SW mice were euthanized, and were colonized with $\log_{10}9.83 \pm 0.24$ of WT Lr per gram of cecal contents (Supplemental Figure 1b).

Systemic immune responses were suppressed by intestinal colonization with histamine-generating, DAG kinase-producing WT *L. reuteri*

To identify whether *hdcA*$^+$ *dgkA*$^+$ *L. reuteri* 6475 colonization had an impact on immunomodulation of the mouse intestine, pro-inflammatory cytokines including interleukin (IL)-1α, IL-6, TNF-α, IL-17, IL-22, IL-12, Eotaxin, G-CSF and IFN-γ were analyzed in the blood plasma of gnotobiotic and GF control mice. Interestingly mice colonized with WT *L. reuteri* yielded reduced circulating concentrations of IL-1α, compared with mice colonized by *L. reuteri ΔdgkA* or control GF mice (Figure 2a). Similar differences were observed for the chemokines, eotaxin and G-CSF, in peripheral blood quantified in BALB/c gnotobiotic mice colonized by WT *L. reuteri* and respective mutants (Figure 2b&2c). In addition, circulating quantities of IL-6 were reduced in SW (outbred) mice that received WT *L. reuteri* (Supplemental Figure 2e) by orogastric gavage. In addition, amounts of plasma IL-10, a cytokine known to suppress inflammation, were increased in mice colonized by WT *L. reuteri* (Supplemental Figure 2f), and whereas G-CSF and IL-6 were diminished (Supplemental figure 2a & 2e) in SW mice colonized with WT *L. reuteri* (Supplemental figure 2a–2f).

Intestinal mucosal pro-inflammatory cytokine suppression by *hdcA*$^+$ *dgkA*$^+$ WT *L. reuteri* in gnotobiotic mice

Mucosal immune responses due to the presence and absence of histamine and/or diacylglycerol kinase of wild type *L. reuteri* 6475 were measured in intestinal mucosal tissue samples. Cecal *IL-6* mRNA in the mucosa was significantly decreased in mice colonized by WT *L. reuteri*, compared to mice colonized by mutant *ΔdgkA L. reuteri* or control GF BALB/c mice (Figure 2d). However, other cytokine mRNAs such as TNF-α, IFN-γ, IL-17, IL-12, IL-1α and IL-22 were not altered or below the detection limit. Similarly, *IL-6* mRNA and protein quantities were significantly reduced in the cecal mucosa of SW mice colonized by WT *L. reuteri* compared to control GF mice (Supplemental Figure 2g, 2i–2j).

Colonization by wild type *dgkA*$^+$, but not mutant Δ*dgkA*, *L. reuteri* inhibits mucosal PKC phosphorylation and NFκB accumulation in intestinal epithelium

Since PKC phosphorylation depends on availability of DAG through G-protein coupled receptor activation [5], phosphorylation of p-PKC was compared among the cecal mucosae of mice colonized by WT and mutant *ΔhdcA* or *ΔdgkA L. reuteri*, in addition to control GF mice. To determine that the bacterial enzyme Dgk, presumably released into the extracellular milieu, inhibits DAG-mediated signaling and PKC phosphorylation in mammalian cells, immunoblot studies were performed targeting PKC phosphorylation using specific

antibodies. The phosphorylated protein kinase, p-PKC-α (Ser 657) was diminished in the cecal mucosa of gnotobiotic BALB/c mice colonized with WT Lr compared to control GF mice. In addition, BALB/c mice colonized by mutant *ΔhdcA L. reuteri* had reduced amounts of p-PKC when compared to control GF mice (Figure 3a). Interestingly, the greatest abundance of p-PKC was localized in the intestines of mice colonized by mutant *ΔdgkA L. reuteri*, compared to GF mice colonized by WT *L. reuteri* or mutant *ΔhdcA L. reuteri* (Figure 3a&3c). Consistent with the immunoblot data, pPKC was decreased in ceca of mice colonized by WT and mutant *ΔhdcA L. reuteri* when compared to mice colonized by mutant *ΔdgkA L. reuteri* (Figure 3b) evaluated by f-IHC. Swiss Webster GF mice colonized by WT *L. reuteri* also showed reduced levels of pPKC compared to corresponding non-phosphorylated PKC and GF PKC protein concentrations from the cecal mucosa (Supplemental Figure 3a).

Human jejunal enteroids treated with WT *L. reuteri* conditioned media (CM) showed decreased pPKC levels compared to human enteroids treated with mutant *ΔdgkA L. reuteri* CM (Supplemental Figure 4a). Additionally, *ΔhdcA* Lr CM treated enteroids showed a trend towards increased PKC phosphorylation (Supplemental Figure 4a). Consistent with immunoblot data, pPKC was decreased in human enteroids treated with WT *L. reuteri* CM compared to mutant *ΔdgkA* Lr CM, as examined by f-IHC (Supplemental Figure 4b). To explore mammalian intestinal epithelial DAG and pPKC signaling further, ileal enteroids from 10 week-old GF mice were treated with a DGK inhibitor *in vitro.* The enteroids treated with a DGK inhibitor (R59-022) yielded increased PKC phosphorylation in the presence of WT Lr CM, while enteroids lacking DGK inhibitor in the presence of WT Lr CM did not yield evidence of increase PKC phosphorylation (Supplemental Figure 5). Similarly, *in vitro* treatment of HT-29 human epithelial cells with *ΔdgkA* Lr CM yielded a nearly three-fold increase in PKC phosphorylation compared to treatment with Lr WT CM (Supplemental Figure 4c). Additionally, non-phosphorylated PKC protein concentrations increased over time in the presence of WT Lr CM and decreased with mutant *ΔdgkA* Lr CM treatment in HT-29 human epithelial cells (Supplemental Figure 4c).

Activation and accumulation of cytosolic NF-κB (IκBα-RelA:P50 complex) is dependent on phosphorylation of p-PKC [37]. The intestinal epithelial tissues obtained from the cecal mucosa of mice colonized by WT, mutant *ΔhdcA* or *ΔdgkA L. reuteri* were compared to control GF mice. To determine that the bacterial enzyme Dgk inhibits activation of NF-κB through reduced phosphorylation of PKC in mammalian cells, immunoblot studies were performed targeting NF-κB accumulation using specific antibodies. Total IκBα (L35A5) complex was diminished in the cecal mucosa of gnotobiotic BALB/c mice colonized with WT Lr, compared with control GF mice. The *ΔdgkA L. reuteri* colonized mice yielded increased synthesis of NF-κB (Figure 3d).

L. reuteri DAG kinase is secreted into the extracellular milieu

Since we observed negligible PKC phosphorylation in intestinal mucosal tissues of mice colonized with WT or *ΔhdcA* compared to *ΔdgkA* Lr, Dgk (approximately 15-kDa) [11,38] is secreted by the bacterium Lr, and the extracellular microbial enzyme may effectively decrease DAG activity in mammalian cells. By suppressing DAG lipid-mediated signaling,

DAG kinase from lactobacilli may suppress H1R signaling via reduced PKC phosphorylation. To explore the possibility that bacterial Dgk may be secreted into the intestinal lumen and interact with substrate DAG in the host epithelium, Dgk was detected in bacterial culture supernatants following growth in liquid media. WT and mutant *hdcA* or *ΔdgkA L. reuteri* 6475 were cultured in LDM4 for 12 hours in the presence and absence of DAG, and liquid culture supernatants were evaluated.

Extracellular proteins were fractionated by size, processed for tryptic digestion, and analyzed by LC-MS/MS followed by amino acid sequence analysis. Amino acid sequences of Dgk peptide fragments (Supplemental Figure 6) matched the expected Dgk protein sequences (Figure 4a, 4b & 4c). As expected, Dgk was present in WT *L. reuteri* supernatants, but absent in mutant *ΔdgkA L. reuteri* cultures (Figure 4c). WT Lr *dgkA* mRNA levels were significantly increased in the presence of DAG compared to absence of DAG in the growth medium after 12 hours *in vitro* (Figure 4d) and bacterial *dgkA* gene expression was increased in cecal luminal contents of the gnotobiotic BALB/c mouse intestine colonized by WT *L. reuteri* (Figure 4f). In addition, clade II *L. reuteri* 6475 *dgkA* gene was present in greatest quantities during the elongation phase (Supplemental Figure 7). Bacterial mRNA was obtained from the cecal contents and quantified using RT-PCR. Bacterial *dgkA* expression was increased in the lumens of both WT and *ΔhdcA* Lr colonized gnotobiotic BALB/c mice compared to *ΔdgkA* Lr colonized mice, whereas GF control BALB/c mice showed no evidence of dgkA expression (Figure 4f). In addition, mammalian DGK quantified from the cecal intestinal epithelium by immunoblot did not yield significant differences between control mice and the GF mice colonized with WT or mutant *L. reuteri* (Supplemental Figure 3b).

Bacterial histidine decarboxylase mRNA and luminal histamine present in the intestinal lumen of gnotobiotic BALB/c mice colonized by *L reuteri*

Luminal contents were treated with 99% methanol and the supernatants obtained were used for histamine quantification using LC-MS. WT *L. reuteri* 6475 and mutant *ΔdgkA L. reuteri* colonized BALB/c mice yielded detectable luminal histamine (qualitative data not shown). Interestingly *ΔdgkA* Lr culture grown for 24 hours in lactobacillus defined media, LDM3 or LDM4, generated > 2-fold increased histamine concentrations compared to WT Lr culture. Mutant *ΔhdcA* Lr yielded absence of detectable histamine in liquid media (Supplemental Table 1). However, mutant *ΔhdcA* Lr lacked detectable histamine in culture supernatants (Supplemental Table 1). Bacterial histidine decarboxylase mRNA, presumably at least partly from *L. reuteri*, was also detectable in the intestinal lumens of gnotobiotic BALB/c mice colonized by wild type and mutant *ΔdgkA L. reuteri* (Figure 4e).

L. reuteri resides adjacent to the intestinal epithelium and modifies the intestinal mucus layer and GPCRs signal of gnotobiotic mice

Bacterial FISH demonstrated that Lr resides in the mucus layer, penetrates the inner mucus layer, and establishes intimate contact with intestinal enterocytes in the murine large intestine (Figure 5a–5f). The data suggest that Lr directly communicates with the host intestinal epithelium irrespective of WT or mutant (*hdcA*, *dgkA*) status. Similarly, Lr was

adjacent to the human epithelium when Lr was co-cultured with human jejunal enteroid monolayers (Figure 5g & 5h) and HT-29 cells (Figure 1c).

To determine H1R and H2R G-protein-coupled receptor (GPCR) protein quantities, fluorescence immunohistochemistry (f-IHC) analysis was performed as described in the Materials and Methods. Both H1R and H2R protein expression were similar between the WT, *ΔhdcA*, *ΔdgkA* Lr colonized compared to control GF mice (Figure 5i–5p). However, H1R was present in greater abundance than H2R in the intestinal epithelium.

Mucus serves as the primary contact between the intestinal epithelial layer and luminal antigens [39,40]. Since GF mice were colonized with Lr, it was of interest to evaluate mucus maturation after probiotic colonization using lectin staining. We observed significantly increased mucus fucosylation with UEA agglutinin staining of the terminal mucus fucose in WT Lr colonized compared to control BALB/c GF mice (Supplemental Figure 8a(i) & 8a(ii)). Similarly, increased fucosylation was observed in mice colonized with *ΔhdcA* and *ΔdgkA* Lr compared to control BALB/c GF mice. However, *ΔdgkA* colonized mice had decreased mucus fucosylation compared to WT Lr colonized mice (Supplemental Figure 8a(i)–8a(iv)). Similarly, SW mice colonized with WT Lr also showed increased mucus fucosylation with increased mucus secreting goblet cells (Supplemental Figure 8a(v)–8a(viii), 8b)).

Histamine-dependent enhancement of CD11b^{+}Gr-1^{+}Ly6G^{high} splenic myeloid cell maturation was offset by the absence of DAG kinase in *L. reuteri*

To determine if bacterial derived histamine has immunomodulatory effects by targeting myeloid cell maturation, GF mice were colonized with WT, *ΔhdcA, ΔdgkA* Lr and a control group without any bacteria. The absence of endogenous histamine results in increased proportions of immature myeloid cells (IMCs) and has been associated with cancer progression in mammals [14]. AOM/DSS treated *Hdc*$^{-/-}$ male mice showed significantly reduced CD11b^{+}Gr-1^{+} IMCs (80%) in spleens after oral administration of *hdc*$^{+}$ Lr 6475 (WT) treatment [41]. In the current study, BALB/c gnotobiotic mice colonized with WT *L. reuteri* strain yielded significantly decreased proportions of CD11b^{+}Gr-1^{+} IMCs in the spleen (Figure 6b), suggesting that histamine-generating Lr promotes maturation of splenic CD11b^{+}Gr-1^{+} IMCs (Figure 6a & 6b). The lack of bacterial histidine decarboxylase in *ΔhdcA* Lr corresponded with a trend towards increased populations of IMCs in the spleen (Figure 6b). In addition, GF mice colonized with *ΔdgkA* Lr did not result in suppression of IMCs in the spleen (Figure 6b); suggesting that loss of microbiome-derived DAG kinase and enhanced H1R signaling may offset effects via H2R on myeloid cell maturation. Additionally, we observed reduced Ly6G^{high} and Ly6G^{medium} populations in the spleens of mice that were colonized with WT *L. reuteri,* compared to mice colonized by mutant *ΔhdcA* or *ΔdgkA L. reuteri* (Figure 6a).

L. reuteri colonization leads to increased microRNA mmu-miR-669k-3p in the intestinal epithelium of GF BALB/c mice

Because basal cytokine responses were suppressed in the presence of WT *L. reuteri* in the intestines of gnotobiotic BALB/c mice (Figure 2), changes in inflammatory responses were

related to microRNA modification. Eighty-four individual microRNAs considered as candidates for regulating inflammatory pathways were selected. The microRNA expression patterns in the intestinal mucosa were evaluated for alterations by Lr-derived histamine and/or Dgk. The miRNA mmu-miR-669k-3p was found to be increased by 2-fold in mice colonized with WT Lr compared to *ΔhdcA*, *ΔdgkA* Lr and control GF mice (Figure 6d).

Discussion

The mammalian microbiome contains microbial cells with genomes encoding the capacity for microbial-mediated conversion of amino acids to bioactive signals such as biogenic amines [17]. As shown in this study, bacterial enzymes such as diacylglycerol kinase may abrogate mammalian cell signaling pathways by effectively "removing" mammalian lipid signals such as diacylglycerol. The gut commensal microbe, *L. reuteri*, secretes Dgk and due to its close proximity to the gut epithelium, this enzyme may convert mammalian DAG to PA. The presence of dgkA-producing *L. reuteri* suppresses phosphorylation of PKC downstream of the histamine type 1 receptor. Dgk secreted by Lr 6475 potentially interacts with epithelial DAG lipid signaling, thereby inhibiting pro-inflammatory H1R and allowing "net positive" activation of H2R by histamine-generating Lr. Ultimately this reduced phosphorylation of PKC may result in diminished signaling via NF-kB and suppression of mucosal inflammation.

Human-derived clade II *L. reuteri* strains contain a complete chromosomal *hdc* gene cluster with genes encoding histidine decarboxylase (HdcA) and the histidine: histamine transporter (HdcP) [19], culminating in suppression of human TNF production [17]. Gut microbes like *L. reuteri* clade II strains provide a useful model system to study the effects of luminal histamine on the mammalian gut mucosa. By introducing individual gut microbes into germ-free mice, our data show that WT and mutant *L. reuteri* each colonized the mammalian intestine successfully with a single oral dosing. Although immunologists have accumulated insights regarding how histamine works in allergic inflammation [42] and in the context of mucosal mast cells [12], the role of histamine in the lumen of the gastrointestinal tract is not well understood. The effects of luminal histamine may differ from that of mucosal histamine simply based on the relative distributions of histamine receptors in different cell types. Our studies suggest that luminal histamine does impact mucosal and systemic immunity, as well as mammalian epithelial cell signaling pathways. Suppression of interleukin-6 production within the cecal mucosa is one example of histamine-dependent suppression of gut inflammation in an acute colitis model [34]. By investigating the role of luminal histamine produced by gut microbes, we may gain a deeper mechanistic understanding of the interrelationships between the gut microbiome, the intestinal mucosa, and immunity.

Luminal histamine may act through the histamine type 2 receptors (H2Rs) to suppress the production of pro-inflammatory cytokines [30] and cell signaling via MAP kinases (Thomas et al, 2012; Gao et al, 2015). However, both histamine type 1 (H1R) and type 2 (H2R) receptors are present in the mouse and human intestinal epithelium, so the role of H1R in promoting gastrointestinal mucosal inflammation must be considered [5]. Our studies provide insights about a potential "brake" directed at H1R-mediated signaling. A bacterial enzyme, diacylglycerol kinase (Dgk), may modify the lipid signal diacylglycerol to abrogate H1

receptor-mediated signaling via protein kinase C (PKC). By contrast, histamine binding to H2R activates protein kinase A (PKA) and binds cAMP via adenylate cyclase [43] to suppress MAP kinase activation. Instead, H1R signaling involves the conversion of phosphatidylinositol 2 (PIP2) by phospholipase C (PLC) into diacylglycerol (DAG) and inositol triphosphate (IP3) [25,43,44]. DAG stimulates phosphorylation of protein kinase C (PKC) [5,23,44] by binding to the catalytic domain 1 and recruiting PKC to the membrane. The interaction of PKC with the membrane causes release of pseudosubstrate from the catalytic domain leading to availability of serine, threonine phosphoacceptor residues causing PKC activation [45]. Phosphorylated PKCα (p-PKC) is known to induce pro-inflammatory responses such as NF-κB activation [43,46]. Thus a net reduction of p-PKC in the intestinal mucosa by the gut microbiome may help to keep the immune system “in check” and promote immunologic homeostasis.

Beneficial microbes in the intestine may produce enzymes that inhibit mammalian cell signaling pathways by effective signal depletion. DGK is an enzyme known to inhibit DAG-mediated signaling by converting DAG to phosphatidic acid (PA) utilizing adenosine triphosphate (ATP) as the phosphate source [3,47–49]. Conversion of DAG to PA depletes DAG, and this reduced amount of DAG may suppress protein kinase C (PKC) activity [47,49], thereby suppressing pro-inflammatory immune responses in the intestine (Figure 7). On that note, *E. coli* has increased amounts of DAG kinase. Resting human platelets (10^9 cells have 0.111 nmol DAG) treated with *E. coli* DAG kinase results in conversion of lipid DAG to PA [50]. We propose that the mammalian microbiome may synthesize Dgk adjacent to the intestinal epithelium, effectively depleting mammalian DAG and inhibiting H1R downstream activation. In support of our proposed hypothesis, *L. reuteri* is positioned adjacent to the intestinal epithelium and could deliver DAG kinase in close proximity to the epithelial cell membranes. PKC phosphorylation was drastically reduced in the intestinal epithelium of WT compared to *ΔdgkA* Lr colonized mice. Dramatically increased phosphorylation of PKC was evident in the intestinal mucosa of mice colonized by *ΔdgkA* Lr and control germ-free BALB/c mice, but not in mice colonized by WT or *ΔhdcA L. reuteri*. Increased PKC phosphorylation was observed in human cells such as the human intestinal epithelial cell line HT29 treated with *ΔdgkA* Lr-CM. These findings raise intriguing possibilities of bacterial enzymes from the mammalian microbiome converting compounds within the mammalian cell milieu and altering signaling pathways in mammalian cells. Mammalian epithelial cells may, for brief periods of time, behave like “chimeric” cells with bacterial and mammalian enzymes affecting intra-mammalian cell signaling pathways.

Previous findings showed that bacterial Dgk shares 18 to 19% amino acid sequence identity with human DGK [8] which can have soluble isoforms [8,11,38]. Similarly, *Staphylococcus aureus* DAG kinase B structure has been identified to be directly relevant to the human enzymes that function in cell signaling because the key structural and active site residues are conserved in the sequences of both enzymes [8]. The bacterial Dgk superfamily provides candidate enzymes and substrates for evaluation of small molecule therapeutics to combat gram-positive organisms and to modulate lipid signaling pathways in humans [8]. Mammalian DGK is found both in soluble and membrane-bound forms actively converting lipid DAG to PA [51], thereby suppressing PKC phosphorylation via DAG phosphorylation to PA [52]. We

hypothesized that Dgk released by Dgk-positive intestinal lactobacilli can suppress H1R downstream signaling by modifying mammalian cell membrane-associated DAG. It is not understood if a bacterial kinase from the microbiome may directly convert membrane-associated DAG in adjacent mammalian epithelial cells. We present evidence that downstream mammalian PKC is activated in the absence of gut microbes, but suppressed in the presence of histamine-generating microbes with an intact DAG kinase gene. In support of the hypothesis, Lr-derived Dgk could not suppress intestinal epithelial PKC phosphorylation in the presence of a DGK inhibitor.

Prior evidence indicates that bacterial DAG kinase may be secreted or released by microbes into the extracellular milieu [8,50]. Prior studies have also indicated that mammalian DAG kinases may be released [51] however; mammalian DAG kinase was not significantly altered by the presence of *L. reuteri* DAG kinase in gnotobiotic mice. DAG kinases bind to lipid DAG and generate PA in the lipid bilayer and these compounds may be available for effects on intracellular signaling pathways. *Bacillus subtilis* has been shown to produce 34-kDa soluble Dgk enzyme [11]. We looked for soluble Dgk proteins/peptides in the supernatant obtained from WT Lr culture for 12 hours in LDM4. By LC-MS/MS analysis we generated the presence of 6 peptide sequences from bacterial culture supernatants, and these peptide sequences were identical to segments of the Dgk protein (http://www.ncbi.nlm.nih.gov/protein/EGC15283.1) from Lr 6475. In addition, Lr 6475 was found to colonize the germ-free mouse intestine, adjacent to the intestinal epithelium. *L. reuteri* is known to both possess secretory and extracellular proteins (secretome) and these secreted proteins include enzymes involved in lipid metabolism [53,54]. DAG is a well-known mammalian membrane lipid compound that flip-flops rapidly in the lipid bilayer [55]. Dgk secreted by *L reuteri* may interact with host DAG in the gut epithelium, reducing amounts of DAG and thereby restricting PKC phosphorylation and NFκB activation[37] following downregulation of mucosal cytokines like IL-6. Such a reduction in mucosal inflammation or dampening of mucosal immunity may result in altered maturation programs of splenic myeloid cells [14].

A deficiency of histidine decarboxylase (HDC) and lack of endogenous histamine was shown to promote inflammation-associated colorectal cancer (CRC) by accumulation of $CD11b^{+}Gr\text{-}1^{+}$ immature myeloid cells (IMCs) [14,56]. Modulation of histamine receptors through its antagonist modifies CRC progression [57–59]. We observed suppression of splenic (not bone marrow) IMCs in germ-free mice colonized with WT *L. reuteri*. This suppression could be due to reduced IL-6 expression in intestinal mucosa because increased IL-6 was previously shown to increase splenic IMC infiltration [14,60]. Suppression of *IL-6* facilitates maturation of IMCs [14,36]. However, *ΔdgkA* Lr colonized mice did not suppress splenic IMCs or reduce *IL-6* mRNA expression, suggesting that *dgkA* is important in alteration of H1R-mediated signaling and maturation of myeloid cells in peripheral compartments. Bacterial genes, *hdcA* and *dgkA* are important for *L. reuteri* to generate sustained immunosuppressive effects in the intestine. In addition to reduced *IL-6* gene levels we also observed reduced IL-1α in blood plasma of mice received WT *L. reuteri* compared to the GF control mice. Interestingly, *ΔdgkA* Lr colonized mice had increased levels of plasma IL-1α, a pro-inflammatory cytokine predominantly secreted by macrophages [61]. IL-1α may promote IL-6 production [62]. Since we observed decreased IL-1α protein levels in blood plasma of WT Lr colonized mice but not in *ΔhdcA* and *ΔdgkA* Lr colonized mice, we

speculate that luminal histamine and luminal DAG kinase may both be important for IL-1α production by mucosal macrophages. Furthermore, we observed diminished mucosal *IL-6* mRNA levels in the presence of WT Lr 6475 with increase in microRNA (mmu-miR)-669k-3p of BALB/c mice. Therefore, we speculate that mmu-miR-669k-3p expression modulates *IL-6* gene expression and may have an impact on myeloid cell maturation. However, detailed studies are required to further characterize the microRNA mmu-miR-669k-3p on intestinal immunomodulation.

The mammalian microbiome includes dozens of bacterial taxa that possess the genetic capacity to perform intestinal luminal conversion of diverse biological compounds. These compounds may be derived from the diet or mammalian cells and provide substrates for bacterial enzymes. The model commensal microbe, *L. reuteri,* has yielded insights regarding luminal conversion by gut microbes and effects on epithelial and immune cell signaling pathways. Gut lactobacilli may generate small amines like histamine from amino acids and suppress inflammation via specific receptors. Gut microbes may also provide enzymes that convert lipid signals, thereby resulting in diminished signaling via receptors that promote allergic inflammation. The net effect is suppression of intestinal inflammation. Future advances in microbiome science may lead to new therapeutics that target bacterial enzymes acting in bacterial cells or mammalian cells (or both cell types) to alter cell signal or immune responses. Probiotics generating “microbial antihistamine factors” like DAG kinase may abrogate intestinal inflammation and promote the development of new microbiome-harvested treatment strategies.

Materials and Methods

Bacterial Strains

The bacterial strains used in this study were: *Lactobacillus reuteri* 6475 (or also known as MM4-1A), *ΔhdcA* Lr 6475 and *ΔdgkA* Lr 6475. All strains were routinely cultured at 37°C in Man, Rogosa and Sharpe (MRS) or LDM4 media and incubated overnight under strictly anoxic conditions using N_2/CO_2 (80/20; v/v) as the gas phase.

Gnotobiotic mouse model – bacterial colonization studies

GF BALB/c mice (5 males and 5 females per group) were bred in Trexler-type isolators. After weaning, all mice (ten weeks old) were colonized by gavaging with *L. reuteri* 6475 (WT strain), *ΔhdcA* Lr 6475 (*hdcA* mutant strain) or *ΔdgkA* Lr 6475 (*dgkA* mutant strain) ($1*10^9$ bacterial cells) in the presence of a 4% L-histidine diet on Day 1 and were euthanized on Day 23. All mouse experiments were performed in a germ-free animal facility, according to an Institutional Animal Care and Use Committee (IACUC)-approved mouse protocol at Baylor College of Medicine (BCM), Houston, TX and the Massachusetts Institute of Technology (MIT) Committee on Animal Care. (Elaborative protocols see supplemental text)

Immunoblot studies of phospho-PKC (p-PKC) signaling

Proteins were extracted from tissue samples in RIPA buffer. A total of 30 or 20 μg of protein from each sample were separated by SDS-PAGE, and transferred onto nitrocellulose

membranes. Membranes were incubated with specific primary antibodies, non-phosphorylated – PKC ^{63} and phospho – p-PKCα (Ser 657) (diluted 1:1000) (Santa Cruz, USA).

Statistics

Data were tested for normal distribution using the Kolmogorov–Smirnov test. Normally distributed data are presented as means with standard error while the medians with their range are given for non-normally distributed data. Significance of differences between Control, WT Lr, ΔhdcA and ΔdgkA mutant Lr colonized BALB/c mice were analyzed using the One-way analysis of variance test for normally distributed data (or) the Kruskal-Wallis test for non-normally distributed data, followed by either Bonferroni/Tukey or Dunn's comparison post-hoc tests. Differences between with and without DAG treated bacterial culture gene expression and Swiss Webster mice with and without WT Lr were analyzed using students t-test followed by the Mann-Whitney test for non-normally distributed data. Differences between the groups were considered significant at $*P < 0{\cdot}05$, $**P<0.01$, $***p<0.001$. SPSS 16.0 (IBM, USA) for Windows 7 was used for data analysis. Prism 5.0 software (Graph Pad Software, Inc., La Jolla, CA, USA) for Windows, was used for data presentation and also for data analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DAGK/DGK	mammalian diacylglycerol kinase
Dgk	microbial diacylglycerol kinase
dgkA	diacylglycerol kinase bacterial gene
hdcA	histidine decarboxylase gene
Δ	mutant

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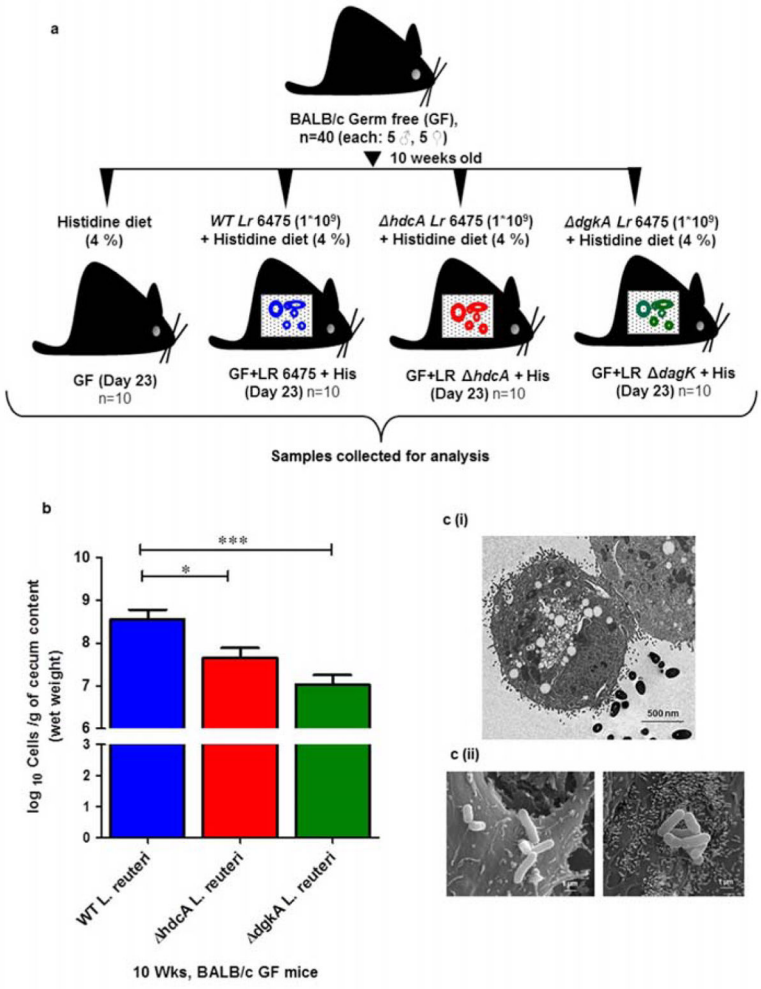

Figure 1. Colonization status of histamine-generating probiotic strain (*L. reuteri* 6475) in adult BALB/c mice

(a) BALB/c mice (10 weeks old) were colonized with *L. reuteri* (Lr) 6475 on Day 1 and euthanized on Day 23. The mice were fed with a histidine-rich (4%) diet during the bacterial colonization period. Four groups differed by probiotic colonization status: group 1 GF control mice which lacked bacteria, group 2 mice received WT *L. reuteri* 6475, group 3 received *ΔhdcA L. reuteri* 6475 and group 4 received *ΔdgkA L. reuteri* 6475 (n = 10 mice per groups, 5 males and 5 females). **(b)** Cecal intestinal contents showed positive bacterial colonization of WT, *ΔhdcA* and *ΔdgkA* Lr after day 22 days of colonization. GF mice showed no amplification indicating the germ-free status. Data are expressed as mean ± SEM. **P<0.05, **P<0.01, ***P<0.001*. n = 10 mice per group. One-Way analysis of variance with Bonferroni correction. **(c-i)** Transmission electron microscopy or **(c-ii)** Scanning electron microscopy of HT29 cells co-cultured with WT Lr 6475 for 2 (i) or 6 (ii) hours under anoxic condition.

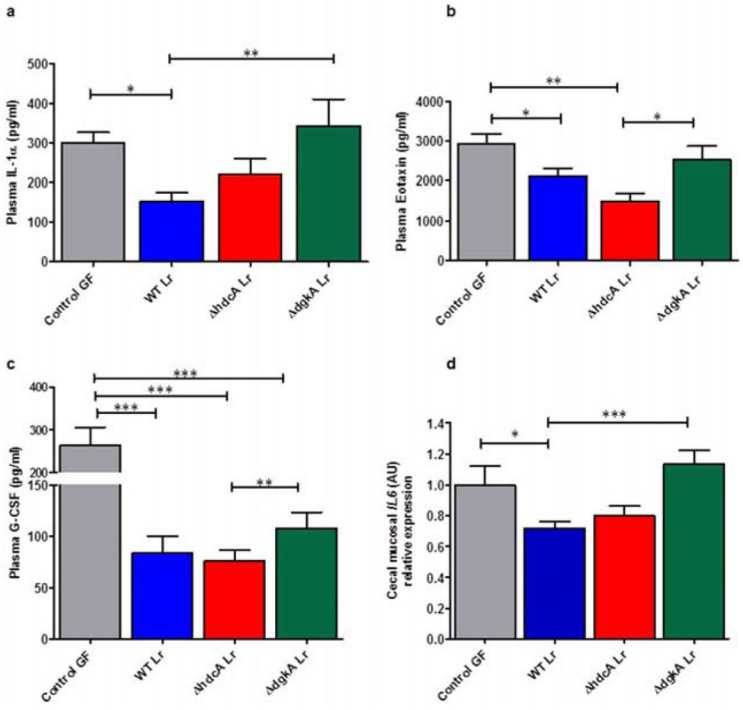

Figure 2. *L. reuteri* colonization reduced pro-inflammatory responses in mono-associated BALB/c mice

BALB/c mice (10 weeks old) were colonized with *L. reuteri* (Lr) 6475 on Day 1 and euthanized on Day 23 showed reduced inflammatory biomarkers. MAGPIX analysis on plasma of mice colonized with WT, *ΔhdcA* and *ΔdgkA* Lr showing differences in protein levels of **(a)** interleukin (IL)-1α, **(b)** Eosinophilic chemoattractant chemokine (Eotaxin), **(c)** Granulocyte colony stimulating factor (G-CSF) and **(d)** cecum mucosal mRNA relative expression of *IL6* by qRT-PCR analyzed in BALB/c mice colonized with WT, *ΔhdcA* and *ΔdgkA* Lr *and GF*. Data are expressed as mean ± SEM. **P<0.05, **P<0.01, ***P<0.001*. n = 10 mice per group. One-Way analysis of variance with Bonferroni correction.

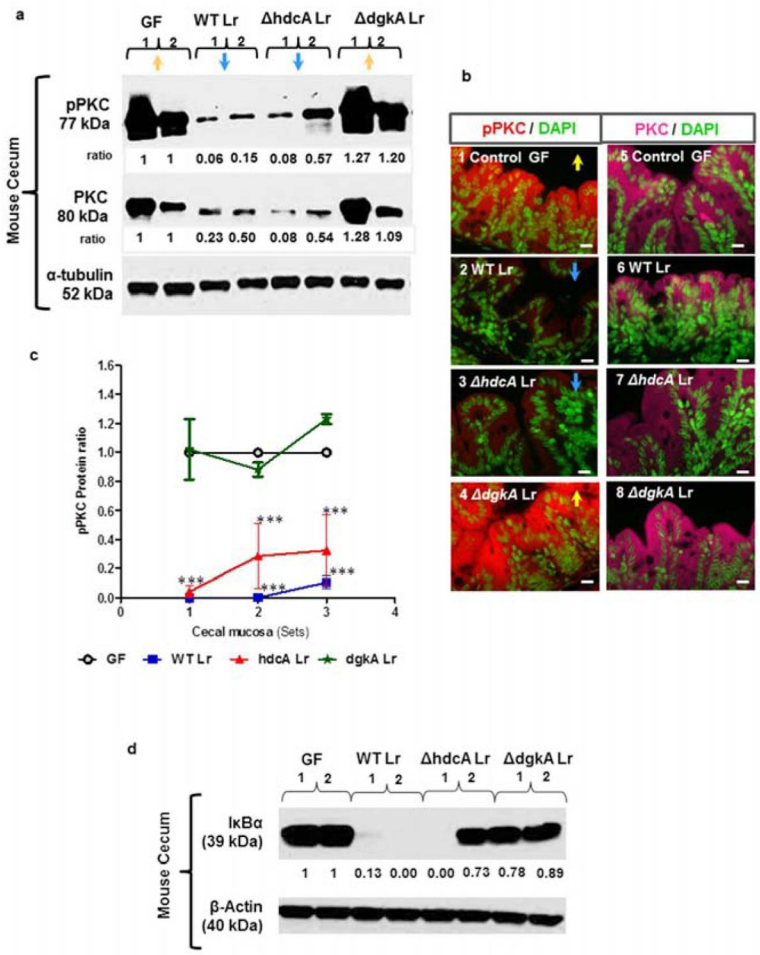

Figure 3. *L. reuteri* derived-Dgk synthesis prevents H1R mediated PKC phosphorylation and NFκB accumulation in mammalian intestinal epithelium

(a) BALB/c mice (10 weeks old) were colonized with *L. reuteri* (Lr) 6475 on Day 1 and sacrificed on Day 23 showed suppressed cecal p-PKC. Western blot analysis of cecal mucosa proteins from WT, *ΔhdcA*, *ΔdgkA* Lr colonized and GF mice incubated with antibody targeting phospho-PKC (p-PKC) or non-phospho-PKC (PKC). One (1) represents proteins pooled from all the 5 female mice and two (2) represents protein pooled from all the 5 male mice per group. The proteins were normalized for housekeeping protein α-tubulin. The p-PKC and PKC ratio was obtained by image-J analysis where p-PKC and PKC from GF control mice was set to 1 and used as baseline. 25 μg of proteins were loaded into each wells of the SDS gel. Proteins were isolated from the pooled samples (1 to 5=1 and 6 to 10 =2) 3 individual times and proteins were estimated by Bio-Rad analysis and western blot was performed for 3 individual times. **(b)** p-PKC and PKC analyzed using fluorescence immunohistochemistry from cecum tissue of BALB/c mice colonized with WT, *ΔhdcA, ΔdgkA L. reuteri* 6475 and control GF mice. Red color indicates p-PKC whereas pink color indicates PKC distribution of lamina propria and green color indicates nuclei staining by DAPI. Bar indicates 10 μm. Images shown are representative of 5 individual mouse sections per group. The proteins were normalized for housekeeping protein α-tubulin. **(a&b)** Blue arrow indicates downregulation of p-PKC and yellow arrow indicates upregulation of p-

PKC. **(c)** Mean values of 3 independent western blot gel showing pPKC protein concentration (loaded 25 ug) and quantified by densitometry using imageJ. GF control groups were set to 1. **P<0.05, **P<0.01, ***P<0.001*. n = 10 mice per group. One-Way analysis of variance with Bonferroni correction. **(d)** Western blot analysis of cecal mucosa proteins from WT, *ΔhdcA*, *ΔdgkA* Lr colonized and GF mice incubated with antibody targeting IκBα (L35A5) NFκB complex.

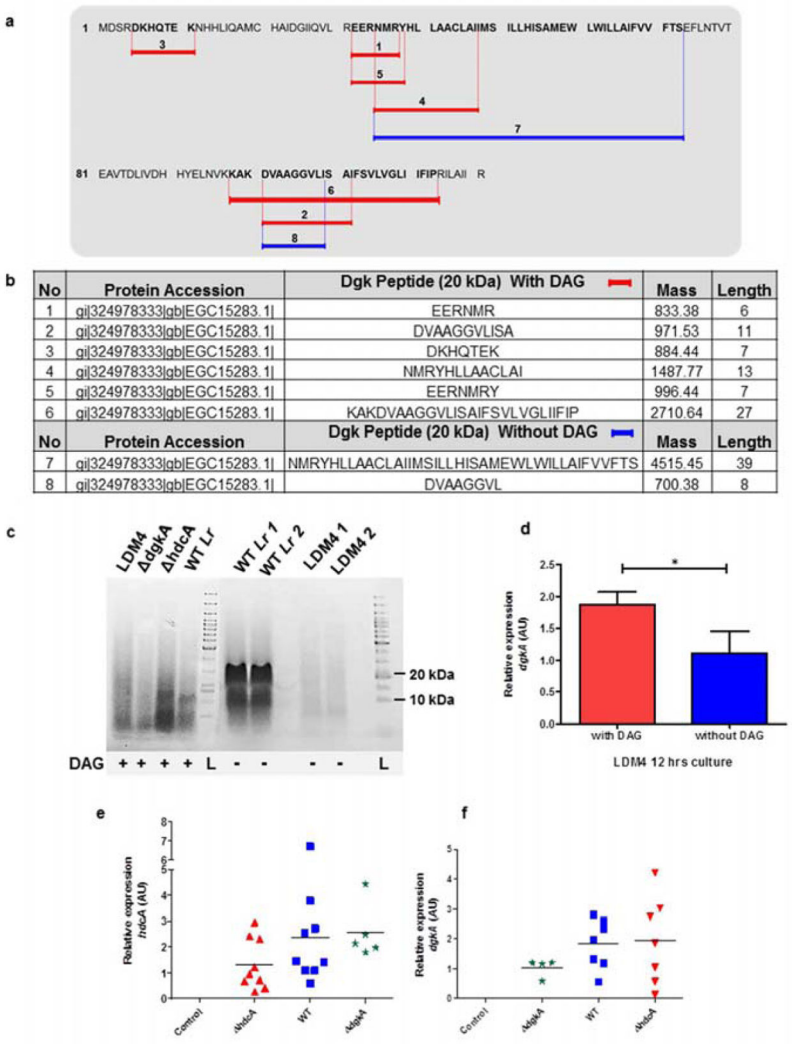

Figure 4. *L. reuteri* 6475 derived-Dgk secreted into the medium

(a) Conditioned media (CM) from WT *L. reuteri* (Lr) 6475 grown in LDM4 with lipid diacylglycerol (DAG) supplements for 12 hours were analyzed using Ekisgent nanoLC and the ABCIEX TripleTOF 5600 mass spectrometer. The obtained sequences were matched with the NCBI database with the Analyst software version 1.6 (ABCIEX Inc.). Red line indicates the protein sequence matching *L. reuteri* diacylglycerol kinase (Dgk) protein in the database from medium grown in the presence of lipid DAG whereas blue line indicates the protein sequence matching *L. reuteri* Dgk in the database from medium grown in absence of lipid DAG. The protein sequences were obtained from 20 kDa band (also see section C). **(b)** CM from WT, *ΔhdcA* and *ΔdgkA* Lr 6475 grown in LDM4 without lipid DAG supplement for 12 hours were analyzed as mentioned in section A. **(c)** One-dimensional SDS gel showing positive bands at 20 kDa and 10 kDa, from *L. reuteri* derived CM with and without DAG supplementation. **(a – c)** 20 kDa protein band was cut and followed by tryptic digestion and were used to analyze as mentioned in **a&b** section. **(d)** mRNA expression of *dgkA* in *WT L. reuteri* cultured for 12 hours with and without lipid DAG supplementation in LDM4 minimal media in N2/CO2 at 37°C and analyzed by RT-PCR. All experiments were

repeated 3 times individually. n=3 per group. Data are expressed as mean ± SEM. **P<0.05,* One-Way analysis of variance with Bonferroni correction.

Graph showing cecal bacterial **(e)** *hdcA* and **(f)** *dgkA* gene expression levels of BALB/c mice colonized with WT, *ΔhdcA, ΔdgkA Lr* and GF control. **(e & f)** Data are expressed as median with range. **P<0.05, **P<0.01, ***P<0.001*. n = 10 mice per group. Kruskal-Wallis test with Dunn's correction.

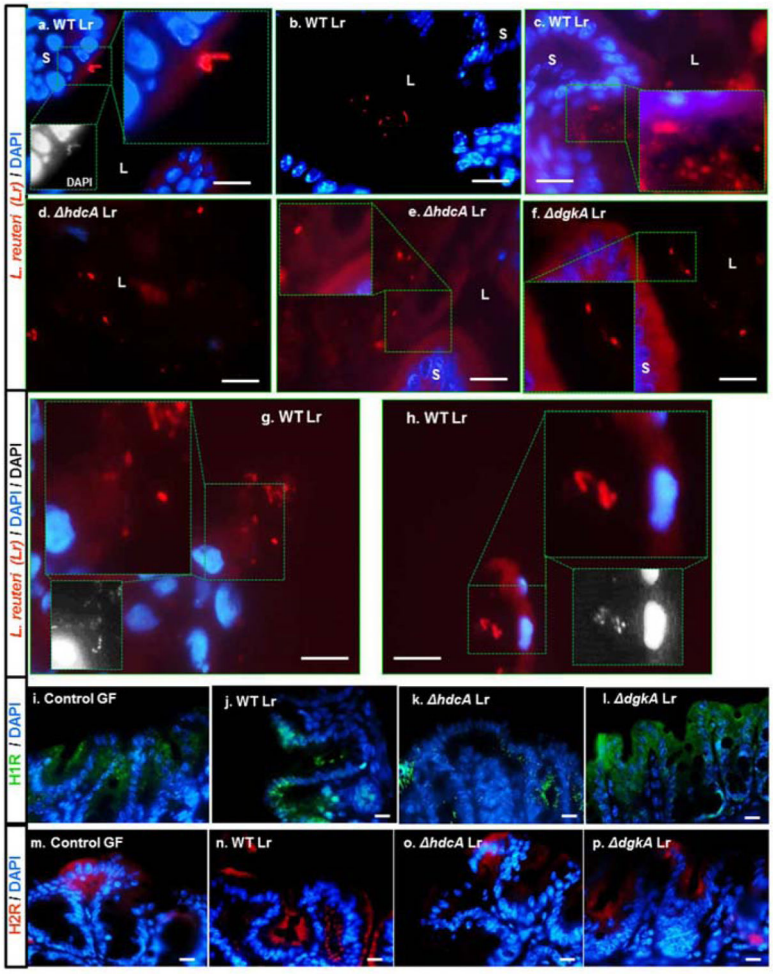

Figure 5. *L. reuteri* resides close to intestinal epithelium and epithelial histamine receptors

Fluorescent in-situ hybridization analysis used to see *L. reuteri* (Lr) localization in the mouse intestine **(a, b&c)** WT, **(d&e)** *ΔhdcA* and **(f)** *ΔdgkA* Lr 6475. Red color indicates the bacterium and blue color (or) white color indicates the nuclei stained with DAPI. Magnification 100x. Representative images of 6 mice per group. Bar indicates 200 pixels. **(g & h)** WT *L. reuteri* 6475 interaction on the human jejunal enteroids monolayer visualized by FISH. Red color indicates the bacterium and blue color (or) white color indicates the nuclei stained with DAPI. Magnification 100x. Bar indicates 200 pixels. L-luminal, S- serosal side. Histamine receptor 1 analyzed using fluorescence immunohistochemistry from cecum tissue of 10 weeks old **(i)** GF BALB/c mice colonized with **(j)** WT, **(k)** *ΔhdcA,* **(l)** *ΔdgkA* Lr 6475. Green color indicates H1R distribution of cecal epithelium and blue color indicates nuclei by DAPI staining. Histamine receptor 2 analyzed using fluorescence immunohistochemistry from cecum tissue of 13 weeks old **(m)** GF BALB/c mice colonized with **(n)** WT, **(o)** *ΔhdcA* and **(p)** *ΔdgkA L. reuteri* 6475. Red color indicates H2R distribution and blue color shows nuclei by DAPI staining. **(i – p)** Magnification 60x. Bar indicates 10 μm. Representative images of 6 mice per group.

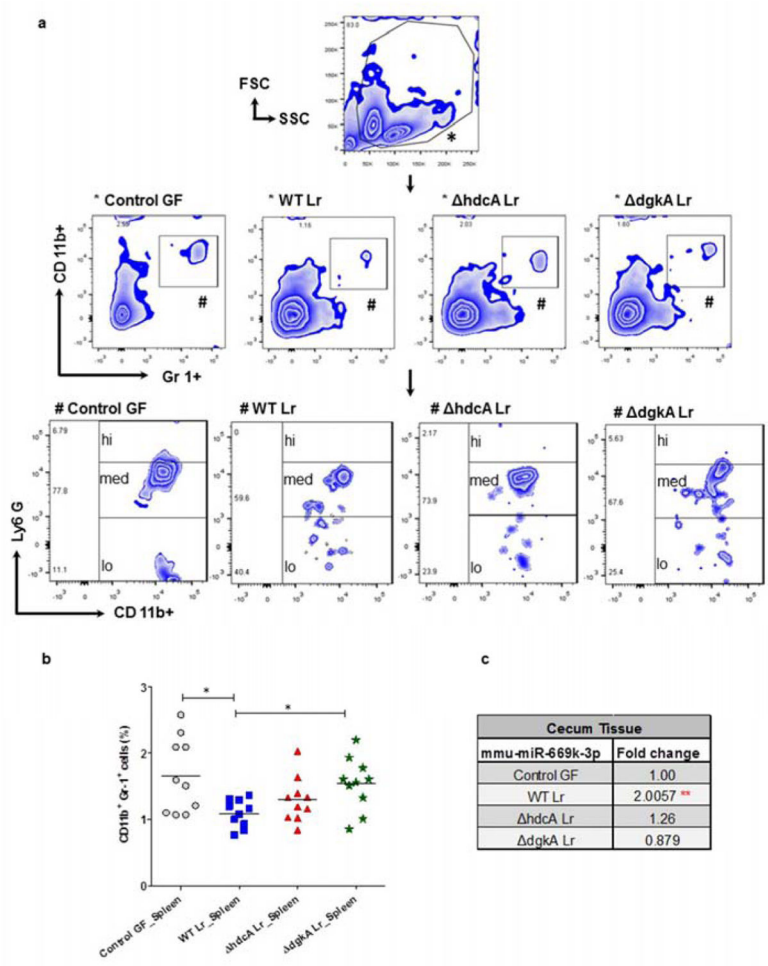

Figure 6. *L. reuteri* colonization reduces splenic IMCs with increased cecal mmu-miR-669k-3p

BALB/c mice (10 weeks old) were colonized with WT, *ΔhdcA*, *ΔdgkA L. reuteri* (Lr) 6475 and GF control. **(a)** Zebra plot showing $CD11b^{+}Gr1^{+}$ **(a*)** immature myeloid cells (IMC) with **(a#)** $Ly6G^{hi}$, $Ly6G^{med}$ and $Ly6G^{lo}$ in spleen of mice. Representative images of n=10 mice per group. **(b)** Splenic IMCs of WT, *ΔhdcA, ΔdgkA* Lr colonized and control GF mice. Data are expressed as mean ± SEM. **P<0.05, **P<0.01, ***P<0.001.* n = 10 mice per group. One-Way analysis of variance with Bonferroni correction. **(c)** Table shows microRNA data obtained from cecum mucosa of WT, *ΔhdcA, ΔdgkA* Lr colonized and control GF mice quantified using ViiA7 rtRT-PCR. From 84 miRNAs quantified one miRNA, mmu-miR-669k-3p (**), showed significant increase. Each group represents cecum mucosal miRNA pooled from 10 mice. Significant differences and ΔCt were obtained by web-based BmiRNA PCR Array Data Analysis software (QIAGEN). (hi-high, med-medium, lo-low).

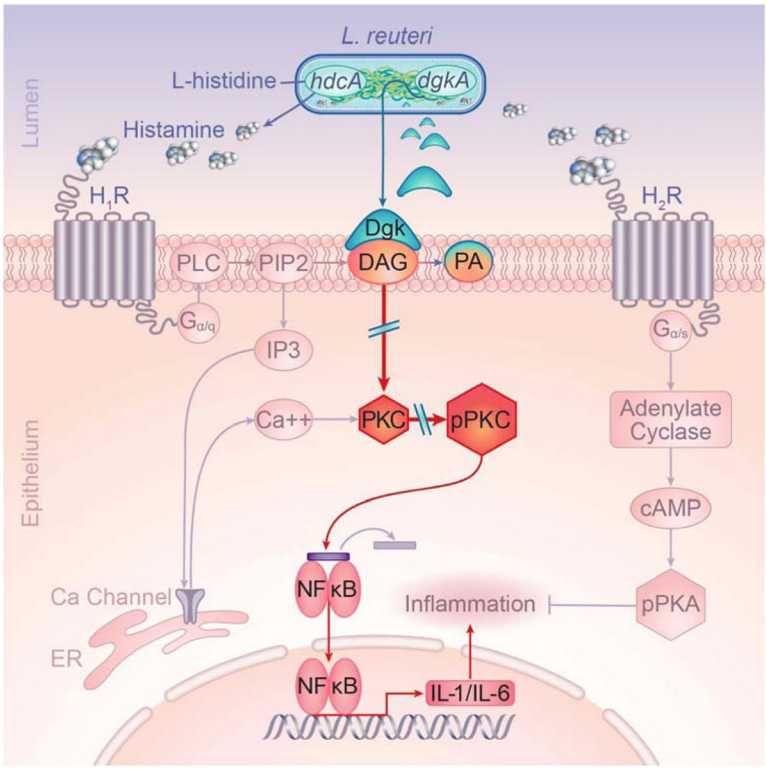

Figure 7. Schematic description of *L. reuteri* derived metabolites signaling through intestinal epithelium in mammals

Histamine secreted by *Lactobacillus reuteri* 6475 activates G-protein coupled histamine receptors H1 and H2. Diacylglycerol kinase (Dgk) synthesized by *L. reuteri* inhibits epithelial H1R downstream signaling and reduces PKC phosphorylation with decreased proinflammatory biomarkers. HR– histamine receptor, PK- phosphorylated protein kinase (C or A), ER- endoplasmic reticulum, PLC- phospholipase C, PIP2 – phosphatidylinositol 2, Hdc – histidine decarboxylase, DAG – diacylglycerol, Dgk- DAG kinase, PA- phosphatidic acid, IP_3- inositol triphosphate.