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Myeloid Cell Expression of LACC1 is Required for Bacterial Clearance and Control of Intestinal Inflammation

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Abstract

Background & Aims: Loss of function variants in the laccase domain containing 1 (*LACC1*) gene are associated with immune-mediated diseases, including inflammatory bowel disease. It is not clear how LACC1 balances defenses against intestinal bacteria vs intestinal inflammation or what cells are responsible for this balance in humans or mice.

Methods: *Lacc1*$^{-/-}$ mice and mice with myeloid specific disruption of *Lacc1* (*Lacc1* ^{mye}) were given oral *Salmonella* Typhimurium or dextran sodium sulfate. CD45RB^{hi}CD4^{+}T cells were transferred to *Lacc1*$^{-/-}$*Rag2*$^{-/-}$ mice to induce colitis. Organs were collected and analyzed by histology and protein expression. Bone marrow-derived macrophages and dendritic cells, lamina propria macrophages and mesenteric lymph node dendritic cells were examined. We performed assays to measure intestinal permeability, cell subsets, bacterial uptake and clearance, reactive oxygen species, nitrite production, autophagy, signaling, mRNA, and cytokine levels.

Results: *Lacc1*$^{-/-}$ mice developed more severe T cell-transfer colitis than wild-type mice and had an increased burden of bacteria in intestinal lymphoid organs, which expressed lower levels of T-helper 1 (Th1) and Th17 cytokines and higher levels of Th2 cytokines. Intestinal lymphoid organs from mice with deletion of LACC1 had an increased burden of bacteria after oral administration of *S.* Typhimurium and following administration of dextran sodium sulfate compared with wild-type mice. In macrophages, expression of LACC1 was required for toll like receptor-induced uptake of bacteria, which required PDK1, and for MAPK- and nuclear factor κB-dependent induction of reactive oxygen species, reactive nitrogen species, and autophagy.

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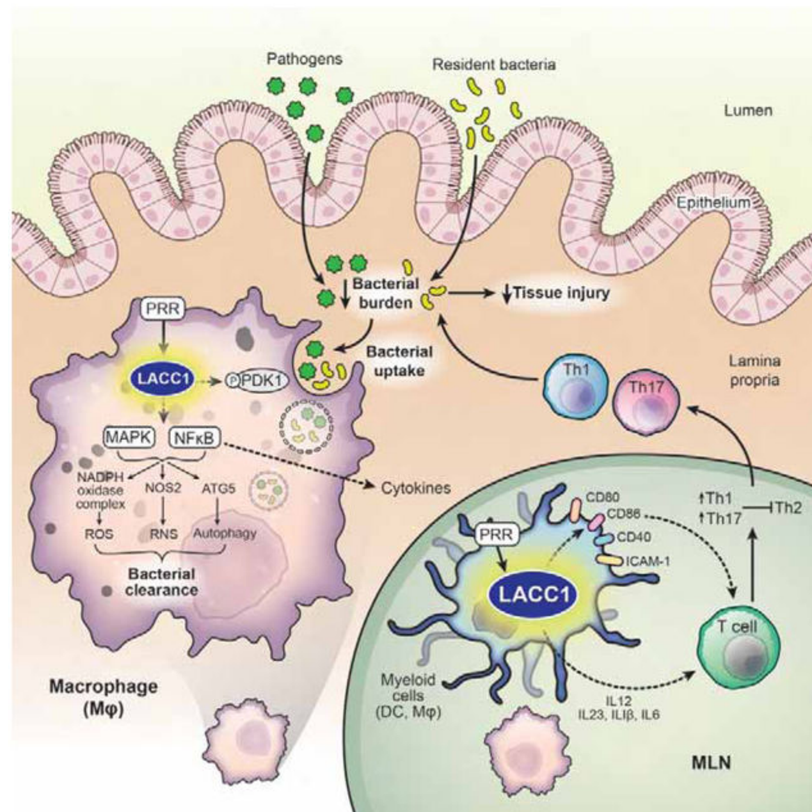
Expression of LACC1 by dendritic cells was required for increasing expression of Th1 and Th17 cytokines and reducing expression of Th2 cytokines upon co-culture with $CD4^{+}$ T cells. Mice with LACC1-deficient myeloid cells had an increased burden of bacteria and altered T-cell cytokines in intestinal lymphoid organs, similar to *Lacc1*$^{-/-}$ mice. Complementation of cytokines produced by myeloid cells to co-cultures of LACC1-deficient myeloid cells and wild-type $CD4^{+}$ T cells restored T cell cytokine regulation. When *S*. Typhimurium-infected *Lacc1* ^{mye} mice were injected with these myeloid cell-derived cytokines, intestinal tissues increased production of Th1 and Th17 cytokines and bacteria were reduced.

Conclusions: Disruption of *Lacc1* in mice increases the burden of bacteria in intestinal lymphoid organs and intestinal inflammation following induction of chronic colitis. LACC1 expression by myeloid cells in mice is required to clear bacteria and to regulate adaptive T-cell responses against microbes.

Graphical Abstract



Lay Summary:

The LACC1 gene, which is altered in some patients with inflammatory bowel diseases, regulates the immune response to microbes and colon inflammation in mice.

Keywords

innate immunity; genetics; Crohn's disease; signaling

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Introduction

Inflammatory bowel disease is characterized by dysregulated host:microbial interactions and cytokine production[1]. Microbial recognition and responses are mediated by host pattern recognition receptors (PRRs). Either loss-of-function or gain-of-function in PRR-mediated signaling and downstream outcomes can be associated with intestinal inflammation[1], thereby highlighting the balance in regulation of PRR-mediated outcomes as critical in intestinal tissues. An important role for host:microbial interactions is highlighted by Crohn's disease (CD)-associated loss-of-function polymorphisms in the PRR *NOD2*[1], as well as in additional pathways regulating microbial clearance mechanisms, such as autophagy and NADPH oxidase complex-mediated generation of reactive oxygen species (ROS)[2]. The success in inflammatory bowel disease (IBD)-associated loci discoveries[3] has provided an important opportunity to understand pathways contributing to intestinal immune homeostasis. One such region is on chromosome 13 (*C13orf31*) encompassing the *LACC1* gene[3].

Polymorphisms in *LACC1* are associated with Crohn's disease, ankylosing spondylitis, leprosy and juvenile idiopathic arthritis[3–6]; these polymorphisms lead to a loss-of-function in LACC1[7–9]. Only a few recent reports have described roles for mammalian LACC1[7–11]. LACC1 is expressed in myeloid cells and is required for optimal fatty acid oxidation and mtROS production, PRR-induced cytokine secretion, and bacterial clearance in human and mouse macrophages in vitro[7, 9,10]. LACC1 roles in vivo have, to our knowledge, been limited to two reports. One study shows that $Lacc1^{-/-}$ mice have reduced early systemic LPS responses[7]. The second study surveyed $Lacc1^{-/-}$ mice outcomes in a range of models, including arthritis, psoriasis, DSS-induced injury and *C. rodentium* infection[11]. Specific LACC1-expressing cell subsets mediating intestinal outcomes in vivo and in-depth examination of mechanisms in intestinal immune pathogenesis were not examined.

Here, we define an essential role for LACC1 in clearing intestinal pathogens and resident luminal microbiota and in limiting chronic intestinal inflammation in vivo. We further identify that myeloid cell-intrinsic LACC1 is required for these roles in vivo and establish pathways and mechanisms through which myeloid cell-intrinsic LACC1 regulates innate and adaptive immune outcomes. These studies provide insight into mechanisms through which LACC1 mediates intestinal bacterial clearance and regulates mucosal immunity in vivo.

Materials and Methods

Mice

$Lacc1^{tm1a(KOMP)/Wtsi}$ mice ($Lacc1^{-/-}$ mice; C57BL/6N, from KOMP repository [Davis, CA]) were crossed with C57BL/6N or $Rag2^{-/-}$ mice. $Lacc1^{tm1a(KOMP)/Wtsi}$ mice were also crossed with FLPe mice (stock #019100, Jackson Laboratory) to generate $Lacc1^{fl/fl}$ mice. $Lacc1^{fl/fl}$ mice were then crossed with Lyz-Cre (stock #018956, Jackson Laboratory) to delete *Lacc1* from myeloid cells ($Lacc1^{\ mye}$). OTII mice were used for T cell isolation. Mice were maintained in a specific pathogen-free facility, and cohoused, littermate mice were used between 2–5 months of age, with studies confirmed in female and male

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mice. Experiments were performed per Yale University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Abs and staining reagents

Fluorophore-conjugated Abs to cell surface proteins or intracellular proteins were used on a FACS Calibur or LSR II (BD Biosciences): CD4, CD45, CD3, CD11b, Ly6G, I-A/I-E, Ly6C (Biolegend), CD4, CD80, CD54 (BD Biosciences); CD4, F4/80, CD40, CD86 (eBioscience); p-PDK1, p-p44/p42 (ERK1/2), p-p38, ATG16L1 (Cell Signaling Technology); LACC1, p47phox, NOS2, ATG5, LC3B, p-IκBα (Santa Cruz Biotechnology). 7-AAD (Biolegend) was used as a viability stain. Intracellular cytokines were assessed after stimulation with 50 ng/ml Phorbol-12-myristate-13-acetate and 750 ng/ml ionomycin (Alfa Aesar) for 4h and addition of 10μg/ml Brefeldin A (Cayman) using fluorophore-labelled antibodies: IFNγ, IL17A, IL4, IL5 (Biolegend), IL13 (eBioscience). ELISA antibodies were to: IL17, IL5, IL13, IL23 (eBioscience); IFNγ, IL4, IL5, IL6, TNF, IL10, IL12p40 (Biolegend); and IL1β (Peprotech).

CD45RB^{hi} CD4^{+} T cell transfer colitis

Colitis was induced with sorted CD45RB^{hi}CD4^{+} T cells (5×10^5) transferred into *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ or *Lacc1*$^{-/-}$*Rag2*$^{-/-}$ mice. H&E-stained colon sections were scored by a pathologist (JRT) blinded to treatment using a five-tier scale (0–3 score for mucosal thickness, architectural distortion and crypt regeneration, surface damage, neutrophil infiltration, mononuclear cell infiltration) for a total possible score of 15.

Salmonella Typhimurium in vivo infection

Mice were orally inoculated with Streptomycin (20mg/mouse) (VWR International) and 24h later orally inoculated with 1×10^6 colony forming units (CFU) of *Salmonella enterica* serovar Typhimurium (strain SL1344). Mice were euthanized (prior to time frame for mortality) and organs collected in PBS. In some cases mice were injected i.p. with 100ng IL12(mouse):Fc, 500ng IL23(mouse):Fc (AdipoGen Life Sciences) or Fc isotype controls (Bio X Cell) prior to *S*. Typhimurium infection.

Dextran Sodium Sulfate Colitis

Dextran Sodium Sulfate, MW ca 40,000 (Alfa Aesar) 2.5% (w/v) was added to the drinking water of mice with *ad libitum* access. Tissues were plated on brain heart infusion agar for bacterial colony counts. H&E-stained colon sections were examined by pathologists blinded to treatment (JRT, XZ).

Generation and culture of primary cells

Bone marrow cells were cultured in DMEM containing 10% L929-conditioned medium (for BMMs), fed fresh medium every 3 days and used at 6–8 days. In some cases, BMMs were first treated with lipid A (Peptides International). BMDCs were generated by conditioning with 20ng/ml GM-CSF (Peprotech). Colonic lamina propria cells were isolated as previously described[12].

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Bacterial entry/uptake

FITC-labeled *E. coli* bioparticles (1.5×10^{6}) (Invitrogen) or 5:1 MOI *S.* Typhimurium-GFP (generously provided by Jorge E. Galan) were co-cultured with BMMs for 20min, cell surface fluorescence was quenched with 0.25mg/ml trypan blue, and then analyzed by flow cytometry. In some cases 1μM GSK 2334470 (Tocris) (PDK1 inhibitor) or 3μM PS48 (Santa Cruz Biotechnology) (PDK1 activator) was used.

Transfection of DNA vectors

Vectors expressing 4μg Atg5 (Addgene plasmid #24922; kindly deposited by Toren Finkel)[13], 2μg Ncf1 (p47phox; BC055836; Transomic Technologies; subcloned into pcDNA.3), 2μg Nos2 (BC062378; Transomic Technologies; subcloned into pcDNA.3), 6μg pMCL-MKK1 (R4F) (constitutively active ERK kinase)[14] (generous gift from Dr. Ben Turk), 4μg pcDNA3-FLAG MKK6(glu) (constitutively active p38 kinase) (Addgene plasmid 13518; kindly deposited by Roger Davis[15]), 4μg IKK-2 S177E S181E (constitutively active NFκB) (Addgene plasmid 11105; kindly deposited by Anjana Rao[16]), or empty vector were transfected for 48h into BMMs using Amaxa nucleofector technology (Lonza).

In vitro T cell activation

Splenic OTII $CD4^{+}$ T cells were isolated using $CD4^{+}$ microbeads (Miltenyi Biotec) (purity 95%). 5×10^{4} OTII $CD4^{+}$ T cells were cultured with BMDCs (10:1) and 20mg/ml chicken OVA antigen (MP Biomedicals) in a 96-well round-bottom plate for 72 hours. In some cases, recombinant cytokines were added to the cultures, including 10ng/ml IL12, 5ng/ml IL23, 50ng/ml IL1β, or 20ng/ml IL6.

mRNA expression

RNA (Trizol, Life Technologies) from cells was isolated, reverse transcribed, and quantitative RT-PCR performed as previously described[17]. Primers sequences in Supplementary Table 1.

Protein analysis

Colon tissue was homogenized and cytokines (ELISA) were normalized to tissue weight. Western blot was as per[17] with antibodies to: LACC1, p38 (Santa Cruz Biotechnology), LC3B, p-ERK, ERK, p-p38, p-IκBα, IκBα (Cell Signaling Technology) and GAPDH (EMD Millipore and Proteintech).

Intracellular bacterial clearance

BMMs were infected in triplicate with *S.* Typhimurium, AIEC, *S. aureus* at 5:1 MOI or *C. rodentium* at 10:1 MOI for 20min, washed three times with PBS, incubated in HBSS containing 30μg/ml gentamicin and then cultured for an additional 4h. Cells were washed, lysed with 1% Triton X-100 and plated on MacConkey or LB agar. In some cases BMMs were treated with inhibitors: 20μM BAY 11–7082, 10μM PD98059, 10μM SB202190 (Calbiochem), 20mM *N*-acetylcysteine (NAC; MilliporeSigma), 10mM N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), or 10mM 3-methyl adenine (3-MA) (Acros Organics).

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Myeloperoxidase Assay

Colonic tissue was homogenized in hexadecyltrimethylammonium bromide (MilliporeSigma) buffer. MPO was assayed using o-dianisidine dihydrochloride (MilliporeSigma) and H_2O_2 (change in optical density at 450nm).

Lipocalin

Frozen fecal samples were reconstituted in PBS/0.1% Tween-20. Supernatants were assessed for lipocalin (R&D Systems).

FITC-dextran permeability assay

Mice were orally gavaged with FITC-dextran (MilliporeSigma) (40 mg/100g body weight) 4h before sacrifice[18,19]; earlier time measures reflect small intestinal permeability while measures between 3–8h can also reflect colonic permeability[18]. Serum concentration of FITC-dextran at 488nm.

Intracellular ROS measurement

ROS was measured by flow cytometry using 10 μM of 2’,7’-dichlorodihydrofluorescein diacetate (H_2DCFDA). Mitochondrial ROS was measured by flow cytometry using 5μM MitoSOX (Life Technologies).

Nitric oxide measurement

Griess reagent was incubated with samples and absorbance measured at 548nm.

Statistical analyses

Statistical comparisons were assessed using a one-way ANOVA with a Tukey’s posttest or Dunnett’s posttest, or a two-tailed Student’s t test along with a Bonferroni-Holm correction for multiple comparisons where appropriate. Values of $p<0.05$ were considered significant.

Results

LACC1 is required for downregulating inflammation in chronic experimental colitis

To address the role of LACC1 in chronic colitis, we crossed *Lacc1*$^{-/-}$ mice to *Rag2*$^{-/-}$ mice and examined *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$x*Rag2*$^{-/-}$ mice in the adoptive T cell-transfer model of chronic colitis. Relative to *Lacc1*$^{+/+}$*Rag2*$^{-/-}$ mice, *Lacc1*$^{-/-}$*Rag2*$^{-/-}$ mice demonstrated greater weight loss (Figure 1a) and histological inflammation (Figure 1b–c) after transfer with CD45RB^{hi} CD4^{+} T cells. Paradoxically, colonic myeloperoxidase activity (MPO, measure of neutrophil infiltration) and stool lipocalin (measure of intestinal inflammation[20]) (Figure 1d–e) were reduced, as were the Th1-associated cytokines IL12 and IFNγ, the pro-inflammatory cytokine TNF, the Th17 cytokine IL17 (Figure 1f), and the anti-inflammatory cytokine IL10 (Figure 1f). In contrast, the Th2 cytokines IL4, IL5 and IL13 were increased (Figure 1f). Given the increased weight loss and intestinal inflammation despite the reduced Th1 and Th17 immune responses, we asked if the mice less effectively cleared resident luminal bacteria, which could then lead to the increased injury observed. This was the case, with an increased bacterial burden in both MLN and

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spleen of *Lacc1*$^{-/-}$*Rag2*$^{-/-}$ mice adoptively transferred with CD45RB^{hi}CD4^{+} T cells (Figure 1g). Therefore, consistent with the increased susceptibility to IBD[3] in loss-of-function *LACC1* carriers, *Lacc1*$^{-/-}$ mice demonstrate an increased severity of experimental chronic colitis.

Lacc1$^{-/-}$ mice infected with oral intestinal pathogens demonstrate impaired immune responses and an increased bacterial burden

As *Lacc1*$^{-/-}$ mice demonstrate an increased burden of resident luminal bacteria during chronic colitis (Figure 1g), we asked if LACC1 also regulates responses to intestinal pathogens in vivo. We ensured that *Lacc1* mRNA in *Lacc1*$^{-/-}$ colon was reduced (Supplementary Fig 1a). *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice were orally infected with the enteric pathogen *Salmonella enterica* serovar Typhimurium. Tissue injury was equivalent with infection in *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice (Supplementary Fig 1b–c). However, *Lacc1*$^{-/-}$ demonstrated increased weight loss (Supplementary Figure 2a) and a higher bacterial burden in the colon and feces, as well as in systemic organs relative to *Lacc1*$^{+/+}$ mice five days after infection (Supplementary Figure 2b). Myeloid cells such as neutrophils play an important role in the clearance of enteric pathogens, including *S.* Typhimurium[21]. Colonic MPO levels were reduced in *S.* Typhimurium-infected *Lacc1*$^{-/-}$ relative to *Lacc1*$^{+/+}$ mice (Supplementary Figure 2c). Immune cell-derived cytokines are also critical in regulating enteric pathogens, including *S.* Typhimurium[21]. Colonic tissue from *S.* Typhimurium infected *Lacc1*$^{-/-}$ mice demonstrated decreased levels of Th1 (IL12, IFNγ), Th17 (IL17), and pro-inflammatory (TNF) cytokines relative to infected *Lacc1*$^{+/+}$ mice (Supplementary Figure 2d). The anti-inflammatory cytokine IL10 was similarly reduced (Supplementary Figure 2d). In contrast, and similar to what we observed with chronic colitis (Figure 1f), the Th2 cytokines IL4, IL5 and IL13 were elevated (Supplementary Figure 2d). Supernatants from colonic punch biopsies showed a similar cytokine pattern (Supplementary Fig 3). Similar patterns were also observed in serum (Supplementary Figure 2e). *Lacc1*$^{+/-}$ mice infected with *S.* Typhimurium generally demonstrated an intermediate phenotype (Supplementary Figure 2–3). Similar patterns were observed with another intestinal pathogen, *C. rodentium* (Supplementary Figure 4–5). In summary, LACC1 regulates colonic myeloid cells, Th1 and Th17 cytokine production, and clearance of enteric pathogens.

LACC1 is required for clearing resident luminal bacteria in acute DSS-induced intestinal injury

The role of a given immune pathway can be different in acute and chronic colitis or with distinct triggers and microbial exposures[22]. We therefore assessed if LACC1 also regulates inflammatory responses to resident intestinal bacteria during acute intestinal injury. We selected an intestinal injury model that disrupts the intestinal epithelial barrier and increases influx of intestinal resident bacteria, the dextran sodium sulfate (DSS) model. Acute DSS administration induced equivalent levels of injury between *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice (Figure 2a). Similar to chronic experimental colitis, *Lacc1*$^{-/-}$ mice administered DSS in drinking water demonstrated greater weight loss (Figure 2b) in the context of reduced colonic myeloperoxidase (Figure 2c) and fecal lipocalin (Figure 2d) compared to *Lacc1*$^{+/+}$ mice. In *Lacc1*$^{-/-}$ mice, colonic inflammatory cytokines (TNF), Th1-associated (IL12,

IFNγ) and Th17 (IL17) cytokines were decreased compared to *Lacc1*$^{+/+}$ mice administered DSS (Figure 2e). In contrast, Th2 cytokines (IL4, IL5, IL13) and IL10 were increased compared to *Lacc1*$^{+/+}$ mice (Figure 2e). Supernatants from colonic punch biopsies showed a similar cytokine pattern (Supplementary Figure 6). The cytokine pattern was also similar in serum (Figure 2f). Of note is that we observed a decrease in Th2 cytokines with colonic inflammation; an increase in Th2 cytokines is frequently observed under these inflammatory conditions[23]. The *Lacc1*$^{-/-}$ mice are on a C57BL/6N background which can demonstrate certain immunological differences relative to C57BL/6J mice[24,25]. In side-by-side studies, with DSS colon Th2 cytokines decreased in WT C57BL/6N mice, whereas they increased in C57BL/6J mice compared to mice receiving drinking water (data not shown). Given the evidence of reduced myeloid cell responses (e.g. reduced MPO activity and lipocalin) with DSS in *Lacc1*$^{-/-}$ mice, we directly assessed myeloid cell populations in the lamina propria. We focused on Gr1^{+} neutrophils and the pro-inflammatory P1 and P2, and anti-inflammatory P3/4 macrophage subsets previously described[26]. Frequencies of these cell subsets in the lamina propria of *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice were not different at baseline (Supplementary Fig 7a). However, following DSS, colonic lamina propria Gr1^{+} cell frequencies in *Lacc1*$^{-/-}$ mice were reduced compared to *Lacc1*$^{+/+}$ mice (Figure 2g, Supplementary Fig 7b), consistent with the myeloid cell measures assessed above. We confirmed reduced neutrophils by immunohistochemistry (Supplementary Fig 7c). Following DSS the frequency of pro-inflammatory macrophage subsets (P1, P2) was also reduced, whereas the frequency of anti-inflammatory P3/4 macrophages was increased in *Lacc1*$^{-/-}$ compared to *Lacc1*$^{+/+}$ mice (Figure 2g, Supplementary Fig 7b). In the context of the reduced pro-inflammatory myeloid cell response, the burden of resident luminal bacteria was increased in the MLN and spleen of *Lacc1*$^{-/-}$ compared to *Lacc1*$^{+/+}$ mice with DSS (Figure 2h). *Lacc1*$^{+/-}$ mice generally demonstrated an intermediate phenotype (Figure 2b–h). Increased intestinal permeability can contribute to an increased bacterial burden. Pro-inflammatory cytokines can promote intestinal epithelial permeability; however, these cytokines were actually reduced in *Lacc1*$^{-/-}$ mice with DSS (Figure 2e). *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice had equivalent low levels of intestinal permeability under homeostatic conditions (Supplementary Figure 7d). After DSS the elevated intestinal permeability remained equivalent across genotypes (Figure 2i). Therefore, LACC1 is required for mounting an immune response leading to clearance of resident intestinal bacteria during acute intestinal injury.

LACC1 is required for optimal TLR-induced PDK1-dependent bacterial uptake and bacterial clearance in peripheral and intestinal myeloid cells

We next assessed mechanisms through which LACC1 regulates bacterial clearance. We confirmed reduced LACC1 protein expression through both Western blot (Figure 3a) and intracellular flow cytometry (Supplementary Fig 8a) in *Lacc1*$^{-/-}$ compared to *Lacc1*$^{+/+}$ BMMs both at baseline and under lipid A (TLR4 ligand)-treated conditions where LACC1 expression is increased; *Lacc1*$^{+/-}$ BMMs were intermediate in expression. Given the consistency between LACC1 protein expression by Western blot and flow cytometry here and in our prior studies[9], and the advantages of flow cytometry[27], we will utilize flow cytometry in studies that follow.

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Myeloid cells in the intestinal lamina propria are exposed to microbial ligands on an ongoing basis, and we[28] and others[29,30] have found that chronic PRR stimulation of macrophages increases antimicrobial pathways. Bacterial uptake is the initial step in bacterial clearance and chronic lipid A treatment enhanced *S.* Typhimurium-GFP uptake in BMMs (Figure 3b). *Lacc1*$^{-/-}$ BMMs demonstrated less *S.* Typhimurium-GFP uptake at baseline and particularly after chronic lipid A treatment compared to *Lacc1*$^{+/+}$ BMMs (Figure 3b, Supplementary Figure 8b). A similar reduction was observed with fluorophore-labeled bacterial bioparticles (Supplementary Figure 8c). The PI3K pathway can promote bacterial uptake[31]. Lipid A treatment induced PDK1 activation, a contributor to PI3K activation, but this activation was impaired in *Lacc1*$^{-/-}$ BMMs (Figure 3c, Supplementary Figure 8d). We confirmed the requirement for lipid A-induced PDK1 activation in bacterial uptake using a PDK1 inhibitor (GSK2334470) (Figure 3d, Supplementary Figure 8e); cell viability was intact with the inhibitor (Supplementary Figure 8f). Complementing PDK1 activation with the PDK1 agonist PS48 in lipid A-treated *Lacc1*$^{-/-}$ BMMs (Supplementary Figure 8e) restored bacterial uptake (Figure 3d). Taken together, LACC1 is required for TLR4-induced PDK1-dependent bacterial uptake in macrophages.

We next examined intracellular clearance of the bacteria that had been taken up by myeloid cells. We confirmed that bacterial clearance was enhanced with chronic lipid A treatment (Figure 3e, Supplementary Fig 9a). *Lacc1*$^{-/-}$ BMMs demonstrated less effective clearance of intracellular *S.* Typhimurium and *C. rodentium* both under untreated and chronic lipid A-treated conditions (Figure 3e). Similar results were observed with adherent invasive *E. coli* (AIEC), a strain of *E. coli* enhanced in the ilea of Crohn's disease patients[32], and *S. aureus*, a resident bacteria (Supplementary Figure 9a). Intracellular bacterial clearance was also reduced in *Lacc1*$^{-/-}$ neutrophils (Supplementary Figure 9b). Consistent with the reduced colonic MPO activity with *S.* Typhimurium infection in vivo in *Lacc1*$^{-/-}$ mice, *Lacc1*$^{-/-}$ neutrophils demonstrated less MPO activity upon co-culture with *S.* Typhimurium (Supplementary Figure 9c). Therefore, the reduced colonic MPO activity in *S.* Typhimurium-infected *Lacc1*$^{-/-}$ mice is likely due to both reduced frequency (Figure 2g, Supplementary Fig 7c) and function (Supplementary Fig 9b–c) of neutrophils.

We next assessed levels of *S.* Typhimurium in *Lacc1*$^{-/-}$ macrophages over time so as to integrate bacterial uptake, bacterial growth and bacterial clearance. As expected, *S.* Typhimurium levels in *Lacc1*$^{-/-}$ BMMs were lower after 20 minutes of co-culture due to the reduced bacterial uptake (Supplementary Figure 10a). Over the next 2 hours the slope of the growth curve was similar in *Lacc1*$^{+/+}$ and *Lacc1*$^{-/-}$ BMMs (Supplementary Figure 10a). Consistently, *S.* Typhimurium genes governing growth rate[33] increased equivalently in *Lacc1*$^{+/+}$ and *Lacc1*$^{-/-}$ BMMs (Supplementary Figure 10b). After 2 hours *Lacc1*$^{+/+}$ BMMs began to reduce intracellular bacteria, whereas *Lacc1*$^{-/-}$ BMMs were unable to do so as effectively, such that the intracellular bacterial load in *Lacc1*$^{-/-}$ BMMs surpassed that in *Lacc1*$^{+/+}$ BMMs at these later times (Supplementary Figure 10a). Therefore, the increased bacterial burden with intestinal injury or infection with enteric pathogens in *Lacc1*$^{-/-}$ mice is likely a combination of reduced bacterial uptake leading to increased bacterial exposure and reduced intracellular bacterial clearance.

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Consistent with the reduced cytokines upon in vivo infection with *S.* Typhimurium in *Lacc1*$^{-/-}$ mice (Supplementary Figure 2), *Lacc1*$^{-/-}$ BMMs demonstrated reduced pro-inflammatory and anti-inflammatory cytokine secretion upon co-culture with *S.* Typhimurium (Supplementary Figure 11a) and upon treatment with lipid A (TLR4 ligand) and TLR2 and TLR5 ligands (Supplementary Figure 11b). In contrast, consistent with what we had observed in human macrophages[9], dectin-induced IL10 secretion was unaffected by *Lacc1* genotype (Supplementary Figure 11c).

Finally, we directly assessed intestinal macrophages. We confirmed reduced LACC1 protein expression in colonic macrophages from *Lacc1*$^{-/-}$ mice (Supplementary Figure 12a). Similar to BMMs, *S.* Typhimurium uptake at early times was reduced and intracellular *S.* Typhimurium clearance at later times was impaired in *Lacc1*$^{-/-}$ intestinal macrophages (Supplementary Figure 12b). TNF secretion from *Lacc1*$^{-/-}$ intestinal macrophages was also reduced upon *S.* Typhimurium co-culture (Supplementary Figure 12c). In contrast, IL10 secretion was increased (Supplementary Figure 12c), thereby demonstrating differences in regulation of cytokine secretion between peripheral and intestinal macrophages. Therefore, LACC1 in macrophages is required for optimal live bacterial-induced cytokine secretion and uptake and clearance of intracellular bacteria in peripheral and intestinal macrophages in vitro.

LACC1 promotes multiple antimicrobial pathways in macrophages

We next assessed LACC1-dependent mechanisms promoting intracellular bacterial clearance. Reactive oxygen species (ROS) can contribute to microbial clearance[1]. Furthermore, polymorphisms in NADPH oxidase complex genes required for ROS production are associated with increased risk for IBD[2,3]. We and others previously found that LACC1 is required for optimal mtROS production[7,9], which can be one contributor to cellular ROS. With chronic lipid A treatment ROS increased less effectively in *Lacc1*$^{-/-}$ compared to *Lacc1*$^{+/+}$ BMMs (Figure 3f). Similar results were observed with mtROS (Figure 3f). As above, an important mechanism for ROS induction is through regulation of the NADPH oxidase complex. Chronic lipid A treatment of BMMs induced expression of *p40phox*, *p47phox*, *p22phox* and *gp91phox* transcripts (Supplementary Figure 13a). However, *p47phox* and *p22phox* were less effectively induced in *Lacc1*$^{-/-}$ BMMs compared to *Lacc1*$^{+/+}$ BMMs (Supplementary Figure 13a). In contrast, *p67phox* was not induced, and *p40phox* and *gp91phox* were not regulated by *Lacc1* genotype (Supplementary Fig 13a). *Lacc1*$^{+/-}$ BMMs generally demonstrated an intermediate phenotype (Supplementary Fig 13a). We focused on p47phox in the experiments that follow and confirmed LACC1-dependent regulation of p47phox at the protein level (Figure 3g, Supplementary Figure 13b). We confirmed that ROS pathways were required for *S.* Typhimurium clearance using the ROS inhibitor, *N*-acetyl cysteine (Figure 3h). Importantly, complementing ROS production through the pharmacological inducer tBHP (Supplementary Figure 13c) or through expressing p47phox to the levels expressed in lipid A-treated *Lacc1*$^{+/+}$ BMMs (Supplementary Fig 13d) partially restored bacterial clearance (Supplementary Figure 13c, Figure 3i).

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Reactive nitrogen species (RNS) can also contribute to bacterial clearance, and a combination of ROS and RNS pathways is central in maintaining homeostasis in the intestinal mucosa[34]. With chronic lipid A treatment, nitrite, a product of nitric oxide, was induced in WT BMMs, whereas *Lacc1*$^{-/-}$ BMMs demonstrated a less effective increase in nitrite (Figure 3j), *Nos2* mRNA (Supplementary Figure 13e) (the molecule regulating nitric oxide production) and NOS2 protein (Figure 3k, Supplementary Figure 13f). RNS pathways were required for optimal bacterial clearance as per the RNS inhibitor L-NAME (Figure 3l). Complementing RNS production in *Lacc1*$^{-/-}$ BMMs through the pharmacological inducer SNAP (Supplementary Figure 13g) or through expressing NOS2 to the levels expressed in lipid A-treated *Lacc1*$^{+/+}$ BMMs (and thereby increasing nitrite) (Supplementary Figure 13h) partially restored bacterial clearance (Supplementary Figure 13g, Figure 3m).

Autophagy is another key bacterial clearance mechanism induced with PRR stimulation, and polymorphisms in the autophagy-associated genes *ATG16L1* and *IRGM* confer an increased risk for Crohn's disease[3]. With 48h lipid A treatment LC3II expression increased as assessed by Western blot in WT BMMs (Figure 3n); LC3II to LC3I ratios increased under these conditions (Supplementary Figure 14a). *Lacc1*$^{-/-}$ and *Lacc1*$^{+/-}$ BMMs did not as effectively induce LC3II (Figure 3n, Supplementary Figure 14a). We confirmed increased LC3II by flow cytometry (Supplementary Figure 14b). Similar results were observed per LC3-expressing punctae (Supplementary Figure 14c). To address the pathways leading to autophagy induction, we examined select autophagy-associated molecules. *Atg5*, *Atg12* and *Beclin* transcripts were induced, and this induction was impaired in *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ BMMs (Supplementary Figure 14d). In contrast, *Atg16l1* induction did not depend on *Lacc1* genotype (Supplementary Figure 14d). We confirmed similar regulation of ATG5 and ATG16L1 protein (Figure 3o, Supplementary Figure 14e). We also confirmed that autophagy pathways were required for optimal bacterial clearance using the autophagy inhibitor 3-MA (Figure 3p). Complementing autophagy induction in *Lacc1*$^{-/-}$ BMMs through the pharmacological inducer rapamycin (Supplementary Figure 14f) or through expressing ATG5 to the levels expressed in lipid A-treated *Lacc1*$^{+/+}$ BMMs (and thereby increasing autophagy; Supplementary Figure 14g) partially restored bacterial clearance (Supplementary Figure 14f, Figure 3q).

As complementing each of the LACC1-dependent antimicrobial pathways individually only partially restored bacterial clearance, we complemented each of the pathways in combination through either the pharmacological (Supplementary Figure 14h) or transfection (Figure 3r) approaches and found that they cooperated to mediate bacterial clearance downstream of LACC1. Therefore, LACC1 is required for optimal induction of multiple key antibacterial pathways and intracellular bacterial clearance in mouse macrophages with chronic PRR stimulation.

LACC1-dependent MAPK and NFκB pathways regulate LACC1-dependent antimicrobial pathways

We next assessed LACC1-dependent signaling pathways that might be regulating the antimicrobial pathways we had defined. In human macrophages, with PRR stimulation LACC1 assembles in a complex with TRAF6 and IRAK1 common to multiple to PRRs, and

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in turn, regulates the MAPK and NFκB pathways downstream of this complex[9]. LACC1 also contributed to optimal levels of TLR4-induced NFκB and MAPK signaling in mouse BMMs as assessed by Western blot (Figure 4a) and flow cytometry (Supplementary Figure 15a). Inhibiting each of the NFκB, ERK or p38 pathways reduced lipid A-induced ROS and nitrite production (Figure 4b–c). Inhibiting these signaling pathways also reduced efficacy of bacterial clearance in both untreated and 48h lipid A-treated BMMs (Figure 4d). Cell survival was intact with these inhibitors (Supplementary Figure 15b). Restoring NFκB, ERK, or p38 activation in lipid A-treated *Lacc1*$^{-/-}$ BMMs to levels observed in lipid A-treated *Lacc1*$^{+/+}$ BMMs through transfection of vectors leading to constitutive activation of these respective pathways (Supplementary Figure 15c) partially restored each of the LACC1-dependent ROS, RNS, and autophagy antimicrobial pathways (Supplementary Figure 15d–f) and intracellular bacterial clearance (Supplementary Figure 15g). Restoring these signaling pathways in combination more effectively restored each of the antimicrobial pathways (Figure 4e) and intracellular bacterial clearance (Figure 4f). Therefore, LACC1 contributes to TLR4-induced NFκB and MAPK signaling and these signaling pathways, in turn, cooperate for optimal induction of LACC1-dependent antimicrobial pathways.

Myeloid cell-intrinsic LACC1 regulates T cell differentiation in vitro

We next sought to dissect mechanisms through which LACC1 regulates T cell-associated cytokines during intestinal infection and inflammation. We first assessed if these cytokines were, in fact, being produced by T cells in the lamina propria. Frequencies of IFNγ– and IL17-producing CD4^{+} T cells were reduced and frequencies of IL4-, IL5- and IL13-producing CD4^{+} T cells were increased in *Lacc1*$^{-/-}$ compared to *Lacc1*$^{+/+}$ colonic lamina propria during DSS-induced injury (Figure 5a). Of note is that at baseline the frequency of CD4^{+} T cells and of various transcription factor-expressing CD4^{+} T cells in the lamina propria was not altered with *Lacc1* genotype (Supplementary Fig 16a–b). Transcription factor mRNA expression in the lamina propria was similarly not regulated by *Lacc1* genotype (Supplementary Fig 16c). However, *Lacc1*$^{-/-}$ colonic lamina propria CD4^{+} T cells demonstrated slightly reduced activation markers (Supplementary Figure 16d).

While there is a spectrum of macrophage phenotypes, IFNγ/LPS-differentiated ('M1') and IL4-differentiated ('M2') macrophages are two commonly described phenotypes, and during antigen presentation to T cells, these cells can result in Th1/Th17 and Th2 cells, respectively[35]. However, both M1 and M2 differentiation of *Lacc1*$^{-/-}$ BMMs was impaired as assessed by transcriptional regulation (Supplementary Figure 17a) and protein expression (Supplementary Figure 17b–e) of pertinent markers and cytokines, indicating that there was not a selective increase in M2 differentiation in *Lacc1*$^{-/-}$ macrophages that might be accounting for the increased Th2 cytokines from T cells.

As dendritic cells are key antigen presenting cells, we next assessed if *Lacc1*$^{-/-}$ BMDCs could directly lead to altered T cell differentiation through in vitro co-culture. WT OTII CD4^{+} T cells (expressing a T cell receptor recognizing chicken ovalbumin [OVA] antigen) were co-cultured with *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ BMDCs along with OVA antigen. Proliferation was equivalent in the co-culture system at the time assessed (Supplementary Figure 18a). In contrast, differentiation outcomes paralleled those observed in vivo, with

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reduced IFNγ and IL17, and increased Th2 (IL4, IL5 and IL13) cytokine secretion (Figure 5b). IL10 secretion was also increased (Figure 5b), and this was accompanied by an increased frequency in Foxp3^{+}-expressing CD4^{+} T cells (Supplementary Figure 18b). As a control, T-bet-expressing CD4^{+} T cells frequencies were reduced (Supplementary Figure 18b). *Lacc1*$^{-/-}$ BMMs in co-culture demonstrated similar results (data not shown). Similar cytokine outcomes were observed upon co-culture with *Lacc1*$^{-/-}$ DCs from mesenteric lymph nodes (MLNs) (Supplementary Figure 18c). These studies implicate a myeloid cell-intrinsic role for LACC1 in regulating T cell differentiation.

We next assessed LACC1-dependent mechanisms in myeloid cells regulating T cell differentiation. We assessed both costimulatory molecules and myeloid cell-secreted cytokines that can regulate T cell differentiation outcomes. Lipid A-induced co-stimulatory molecules (CD40, CD80, CD86 and ICAM-1) were reduced in *Lacc1*$^{-/-}$ BMDCs (Figure 5c, Supplementary Figure 19a). Similar results were observed in lipid A-treated BMMs (Supplementary Figure 19b). Complementing the reduced costimulatory molecules through recombinant costimulatory proteins upon co-culture of *Lacc1*$^{-/-}$ BMDCs with WT OTII CD4^{+} T cells restored T cell cytokine secretion patterns (Supplementary Figure 19c).

We next examined the inflammatory cytokines secreted by BMDCs that condition differentiation of Th1 (through IL12) or Th17 (through IL23, IL1β, IL6) cells; these were reduced in lipid A-treated *Lacc1*$^{-/-}$ BMDCs (Supplementary Figure 20a). We also confirmed IL23 reduction in *S*. Typhimurium-infected and DSS-administered *Lacc1*$^{-/-}$ colons (Supplementary Figure 20b). Th2 cytokines were also decreased in lipid A-treated *Lacc1*$^{-/-}$ BMDCs (Supplementary Figure 20c) and BMMs (Supplementary Figure 20d), such that the mechanism for the increased Th2 cytokines from CD4^{+} T cells (Figure 5b) was not due to an increase in these cytokines from antigen presenting cells. Importantly, upon complementing IL12 during co-culture of *Lacc1*$^{-/-}$ BMDCs with WT OTII CD4^{+} T cells and OVA, the Th1 cytokine IFNγ was restored to the levels of that observed with *Lacc1*$^{+/+}$ BMDC co-culture (Figure 5d). With IL23 or IL1β and IL6, the Th17 cytokine IL17 was partially restored (Figure 5d); IL17 was more completely restored with a combination of these cytokines (Figure 5d). With complementation of either of these Th1- or Th17-inducing cytokine conditions, the high levels of Th2 cytokines and IL10 were reduced (Figure 5d). Upon complementation of these cytokines in combination, Th1 and Th17 cytokines were increased and Th2 cytokines and IL10 were reduced to an even greater degree (Figure 5d). Therefore, LACC1 in myeloid cells is required for both induction of costimulatory molecules and secretion of cytokines regulating T cell differentiation.

Myeloid cell-intrinsic LACC1 regulates resident intestinal bacteria during acute colonic injury

We next sought to establish if myeloid cell-intrinsic LACC1 contributes to clearance of intestinal bacteria and altered T cell-associated cytokines in vivo. To address this, we crossed *Lacc1*$^{fl/fl}$ mice with LysM-Cre mice (*Lacc1*^{mye}). LACC1 expression was reduced in BMMs from *Lacc1*^{mye} mice with and without lipid A treatment (Supplementary Figure 21a). In contrast, LACC1 expression in skin fibroblasts was not decreased in *Lacc1*^{mye} mice (Supplementary Figure 21a), thereby demonstrating expected cell subset selectivity in

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LACC1 deletion. We further confirmed that BMMs from *Lacc1*^{mye} mice demonstrated decreased cytokine secretion to a range of PRR ligands (Supplementary Figure 21b). BMMs from *Lacc1*^{mye} mice also demonstrated decreased efficacy in bacterial clearance (Supplementary Figure 22a), along with reduced induction of antimicrobial pathways (Supplementary Figure 22b–d). These mice also demonstrated reduced serum cytokines in vivo upon LPS injection compared to *Lacc1*$^{fl/fl}$ mice (Supplementary Figure 23). Therefore, *Lacc1*^{mye} mice demonstrate effective LACC1 deletion in myeloid cells and functional consequences of this deletion in vitro and in vivo.

We next assessed if myeloid cell-intrinsic LACC1 is required for reducing the burden of resident luminal bacteria observed during acute DSS-induced injury. Upon inducing equivalent levels of injury in *Lacc1*$^{fl/fl}$ and *Lacc1*^{mye} mice with oral administration of DSS (Figure 6a), outcomes in *Lacc1*^{mye} mice were similar to that observed in *Lacc1*$^{-/-}$ mice, with increased weight loss (Figure 6b), reduced colonic MPO and fecal lipocalin (Figures 6c–d), reduced colonic and serum inflammatory cytokines (e.g. TNF, Th1 and Th17 cytokines), and increased colonic and serum Th2 and anti-inflammatory cytokines (Figures 6e–f), along with colonic cytokine-producing $CD4^{+}$ T cells corresponding to these differentiation patterns (Figure 6g) compared to *Lacc1*$^{fl/fl}$ mice. Moreover, with DSS-induced injury *Lacc1*^{mye} mice demonstrated reduced pro-inflammatory and increased anti-inflammatory colonic macrophages (Figure 6h & Supplementary Figure 24), and in turn, increased levels of bacteria in MLN and spleen (Figure 6i). Epithelial permeability was equivalent in *Lacc1*$^{fl/fl}$ and *Lacc1*^{mye} mice (Figure 6j). Therefore, upon intestinal injury leading to high levels of intestinal bacterial exposure, myeloid cell-intrinsic LACC1 is required for induction of the inflammatory responses and mechanisms required to regulate the intestinal resident bacterial burden observed.

Myeloid cell-intrinsic LACC1 is required for optimal clearance of S. Typhimurium in vivo

Finally, we assessed if myeloid cell-intrinsic LACC1 is required for regulating oral *S.* Typhimurium infection and the T cell cytokine responses required for regulating infection. Similar to *Lacc1*$^{-/-}$ mice, *Lacc1*^{mye} mice orally infected with *S.* Typhimurium demonstrated increased weight loss, increased burden of *S.* Typhimurium in colonic tissues, feces and systemically, decreased colonic tissue MPO activity, and decreased Th1-associated (IL12, IFNγ), Th17 (IL17) and additional pro-inflammatory (TNF) cytokines, as well as IL10 in both colonic tissues and serum, whereas Th2 (IL4, IL5, IL13) cytokines were increased relative to *Lacc1*$^{fl/fl}$ controls (Supplementary Figure 25). Importantly, upon complementing IL12 or IL23 alone, and particularly in combination, in *Lacc1*^{mye} mice during oral *S.* Typhimurium infection, body weight increased (Figure 7a), bacterial burden decreased (Figure 7b) and colonic cytokine patterns more closely resembled those observed in *Lacc1*$^{fl/fl}$ mice (Figure 7c). Therefore, myeloid-cell intrinsic LACC1 is required for the induction of inflammatory responses, T cell differentiation, and regulation of oral *S.* Typhimurium infection in mice in vivo.

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Discussion

This study defines mechanisms through which the IBD-associated gene *LACC1* contributes to intestinal inflammation in mice in vivo. It finds that LACC1 is required for properly clearing both intestinal pathogens and intestinal resident bacteria. As such, during chronic experimental colitis, the absence of LACC1 leads to a higher resident intestinal bacterial burden and more severe intestinal injury. Moreover, myeloid cell-intrinsic LACC1 is critical in regulating these outcomes in vivo. Mechanistically, LACC1 is required for PDK1-dependent bacterial uptake and for NFκB- and MAPK-dependent induction of ROS, RNS and autophagy pathways in macrophages, which in turn regulate intracellular bacterial clearance in these cells. Further, myeloid cell-intrinsic LACC1 is required for costimulatory molecule induction and the cytokines conditioning Th1 and Th17 differentiation, and in turn, Th2 reduction. *Lacc1*$^{+/-}$ mice demonstrate intermediate LACC1 expression levels and generally demonstrate an intermediate phenotype. Therefore, myeloid cell-intrinsic LACC1 regulates the balance of intestinal infections and intestinal inflammation through regulating both innate and adaptive immune outcomes (Supplementary Figure 26). These results are consistent with the increased IBD risk conferred by loss-of-function *LACC1* genetic variants.

A recent study examining collagen-induced arthritis and psoriasis found that *Lacc1*$^{-/-}$ mice developed more severe disease, accompanied by increased TNF expression and IL17-producing CD4^{+} T cells[11]; this cytokine regulation was in distinction to the cytokine regulation observed in our study. This prior study also examined limited measures in DSS-induced colitis and *C. rodentium* infection in *Lacc1*$^{-/-}$ mice[11]; similar to our findings the histology was not regulated by *Lacc1* genotype during acute DSS. With *C. rodentium* infection, the prior study found that intestinal histology was slightly worse and accompanied by increased *Tnf* mRNA while the bacterial burden was not different in *Lacc1*$^{-/-}$ mice[11]. In contrast, our study found histology to be similar between *Lacc1*$^{+/+}$ and *Lacc1*$^{-/-}$ mice with *C. rodentium* infection, but bacterial burden to be higher in the context of reduced myeloid cell and Th1 and Th17 responses. Our study demonstrating an increased bacterial burden of resident microbes upon epithelial barrier disruption and upon enteric infection with either *S.* Typhimurium or *C. rodentium* is consistent with the various LACC1-dependent antimicrobial functions identified by both us (current study and[9]) and others[7]. Whether the distinct cytokine regulation and overall outcomes are due to differences in facility microbial colonization or other potential contributors is unclear.

A significant challenge with the success in genetic discoveries in immune-mediated diseases has been to clearly define the mechanisms by which the implicated genes are contributing to disease pathogenesis. In the case of IBD, it has become increasingly apparent that either an inadequate or an excessive response to intestinal microbiota can lead to intestinal inflammation[1]. Moreover, despite the contribution of pro-inflammatory cytokines to inflammation, they can also result in protective effects, as with IL18[36]. As such, with deficient LACC1 in myeloid cells, the reduced myeloid cell responses, and in turn, T cell responses, to microbiota ultimately lead to an increased bacterial burden with intestinal injury, thereby leading to more severe tissue damage. That loss-of-function in *LACC1* confers risk for IBD, and that LACC1 deficiency in myeloid cells is sufficient to

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confer an increased risk for intestinal inflammation, highlights the potential for enhancing antimicrobial pathways as a therapeutic strategy for those individuals who are *LACC1* risk carriers or directly enhancing LACC1 function in myeloid cells for IBD patients more broadly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

BMDCs	bone marrow derived dendritic cells
BMMs	bone marrow derived macrophages
DSS	dextran sodium sulfate
PRRs	pattern recognition receptors
ROS	reactive oxygen species
RNS	reactive nitrogen species

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What you need to know:

BACKGROUND AND CONTEXT:

Variants in the laccase domain containing 1 (LACC1) gene are associated with immune-mediated diseases, including inflammatory bowel diseases.

NEW FINDINGS:

Disruption of Lacc1 in mice and in myeloid cells of mice increases the burden of bacteria in intestinal lymphoid organs, alters T-cell responses against microbes, and increases intestinal injury.

LIMITATIONS:

This study was performed in mice; additional studies are needed in humans.

IMPACT:

These findings provide insight into the mechanisms by which loss of function variants in LACC1 increase risk of inflammatory bowel disease in humans.

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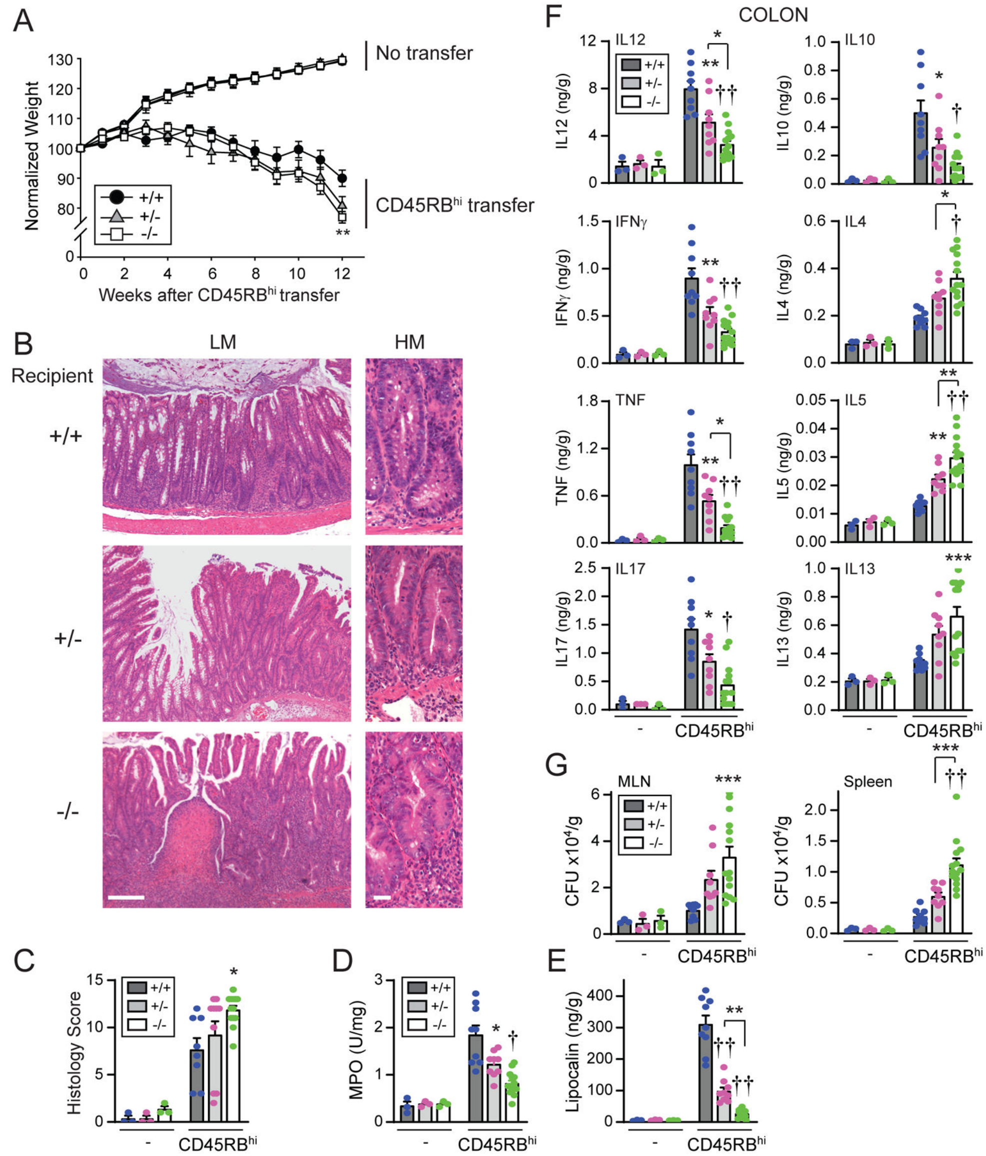

Figure 1. *Lacc1*$^{-/-}$*Rag2*$^{-/-}$ mice demonstrate more severe chronic colitis when transferred with WT CD45RB^{hi} CD4$^+$ T cells.

CD45RB^{hi} CD4$^+$ T cells were adoptively transferred into *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ *Rag2*$^{-/-}$ mice (n=3–12/genotype). **(A)** Body weights. **(B)** Representative H&E of colon sections. Low magnification (LM) scale bar=200 µm. High magnification (HM) scale bar=20 µm. **(C)** Histology scores. **(D)** Colon myeloperoxidase. **(E)** Fecal lipocalin. **(F)** Colon cytokines. **(G)** Bacteria in MLN and spleen. Mean + SEM. Representative of 2 independent experiments. Significance is to *Lacc1*$^{+/+}$ mice within the transferred group or as indicated. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; ††, $p<1\times10^{-5}$.

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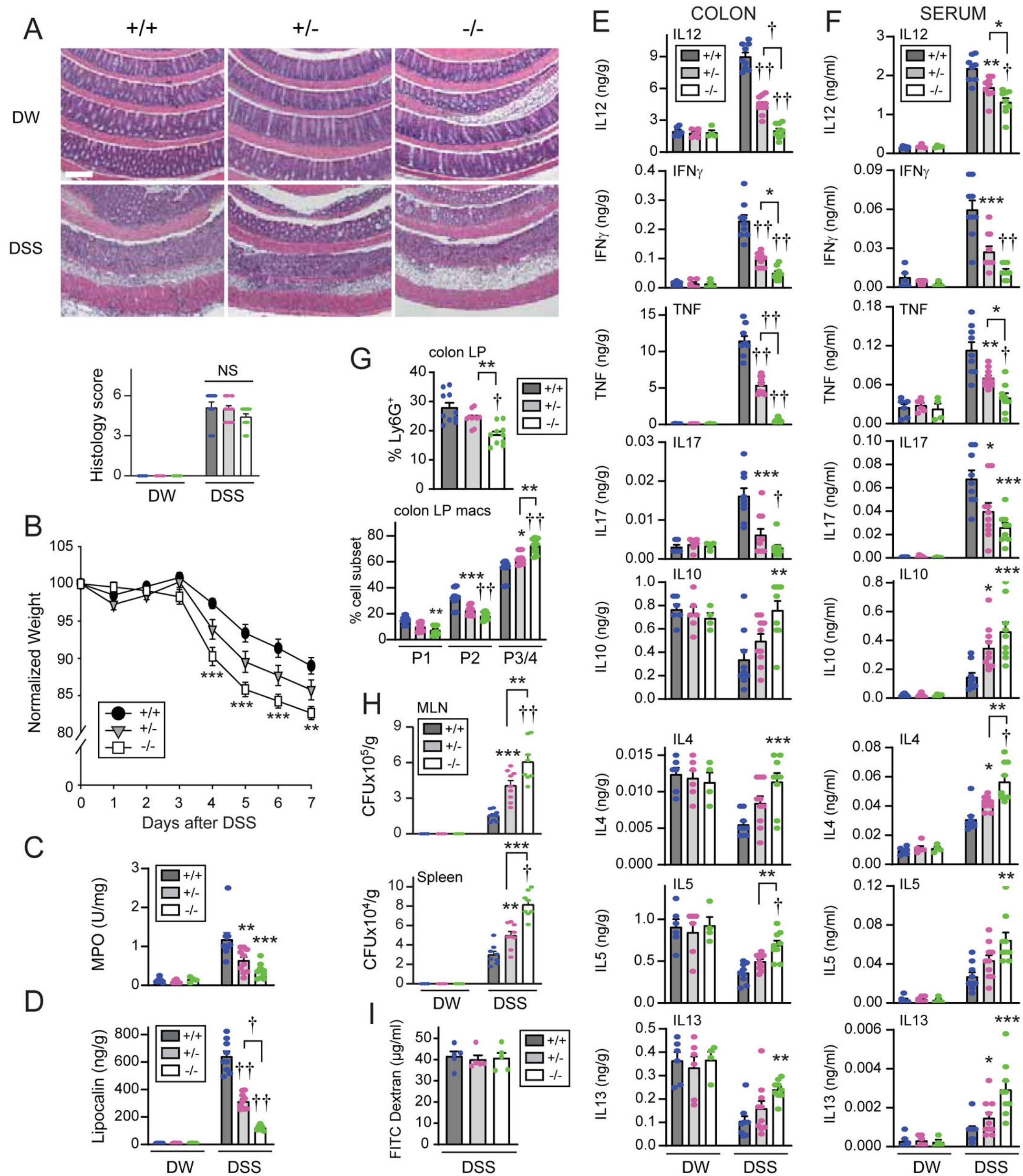

Figure 2. *Lacc1*$^{-/-}$ mice demonstrate greater weight loss and an increased bacterial burden with DSS-induced injury.

Lacc1$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice were given 2.5% DSS in the drinking water (DW). **(A)** Representative H&E of colon sections and histology scores. Scale bar=200 μm. **(B)** Body weights. **(C)** Colon myeloperoxidase. **(D)** Fecal lipocalin. **(E)** Colon cytokines. **(F)** Serum cytokines. **(G)** Percentage colonic lamina propria myeloid cell-derived populations (n=9–10 mice). **(H)** Bacteria in MLN and spleen. **(I)** Intestinal permeability (n=5; representative of 2 independent experiments). Mean + SEM. **A–B, I.** DSS for 7d (n=3–10; representative of 2 independent experiments). **C–H**: DSS for 7d and then water for an additional 2d. **C–F, H**: (n=3–10; representative of 2 independent experiments). Significance is to *Lacc1*$^{+/+}$ mice within the DSS group or as indicated. Macs, macrophages; NS, not significant. *, p<0.05; **, p<0.01; ***, p<0.001; †, $p<1\times10^{-4}$; ††, $p<1\times10^{-5}$.

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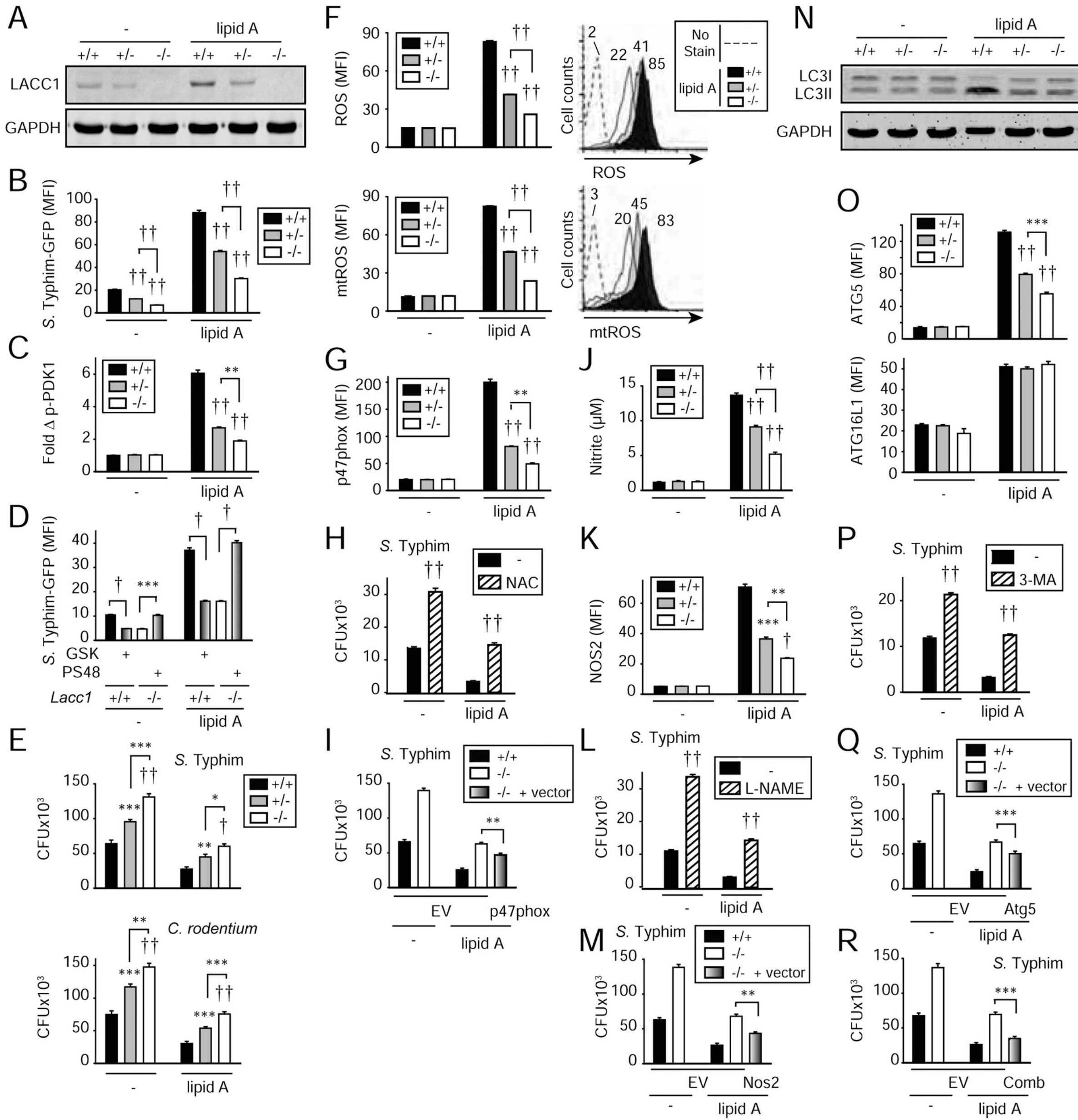

Figure 3. BMMs from *Lacc1*$^{-/-}$ mice demonstrate reduced bacterial uptake and intracellular bacterial clearance and reduced PRR-induced ROS, RNS and autophagy pathways.

BMMs were harvested from *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ or *Lacc1*$^{-/-}$ mice. **(A–B)** Cells were treated with 0.1 μg/ml lipid A for 48h. **(A)** Western blot LACC1. **(B)** *S*. Typhimurium-GFP uptake (4 replicates; representative of 3 independent experiments). **(C)** Cells were treated with 0.1 μg/ml lipid A for 15min. Fold p-PDK1 induction (3 replicates; representative of 2 independent experiments). **(D)** Cells were treated with 0.1 μg/ml lipid A for 48h. Cells were additionally treated with a PDK1 inhibitor (GSK2334470) or a PDK1 activator (PS48) and uptake of *S*. Typhimurium-GFP was assessed (3 replicates; representative of 2 independent experiments). **(E–G, I–K, M–O, Q–R)** Cells were treated with 0.1 μg/ml lipid A for 48h. **(E)** Intracellular clearance of *S*. Typhimurium or *C. rodentium*

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(CFU) (6 replicates; representative of 4 independent experiments). **(F)** ROS (4 replicates; representative of 4 independent experiments) and mtROS (4 replicates; representative of 3 independent experiments). **(G, K, O)** Expression of the indicated proteins by flow cytometry (3 replicates). **(I, M, Q)** Cells were transfected with an empty vector (EV) or **(I)** p47phox-expressing, **(M)** Nos2-expressing, or **(Q)** Atg5-expressing vectors. Intracellular *S.* Typhimurium clearance (6 replicates, representative of 3 independent experiments). **(J)** Nitrite (5 replicates; representative of 4 independent experiments). **(N)** LC3II by Western blot. **(H, L, P)** *Lacc1*$^{+/+}$ BMMs (8 replicates) were treated with 0.1 μg/ml lipid A for 48h in the absence (control vehicle) or presence of inhibitors for: **(H)** ROS (NAC), **(L)** RNS (L-NAME), or **(P)** autophagy (3-MA) and then intracellular *S.* Typhimurium clearance was assessed. **(R)** Cells were transfected with p47phox-, Nos2-, Atg5-expressing vectors in combination (comb). Intracellular *S.* Typhimurium clearance (6 replicates, representative of 2 independent experiments). Significance is to *Lacc1*$^{+/+}$ BMMs in each respective treatment group or as indicated for **(B-C, E-G, J-K, O)**. Mean + SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; ††, $p<1\times10^{-5}$.

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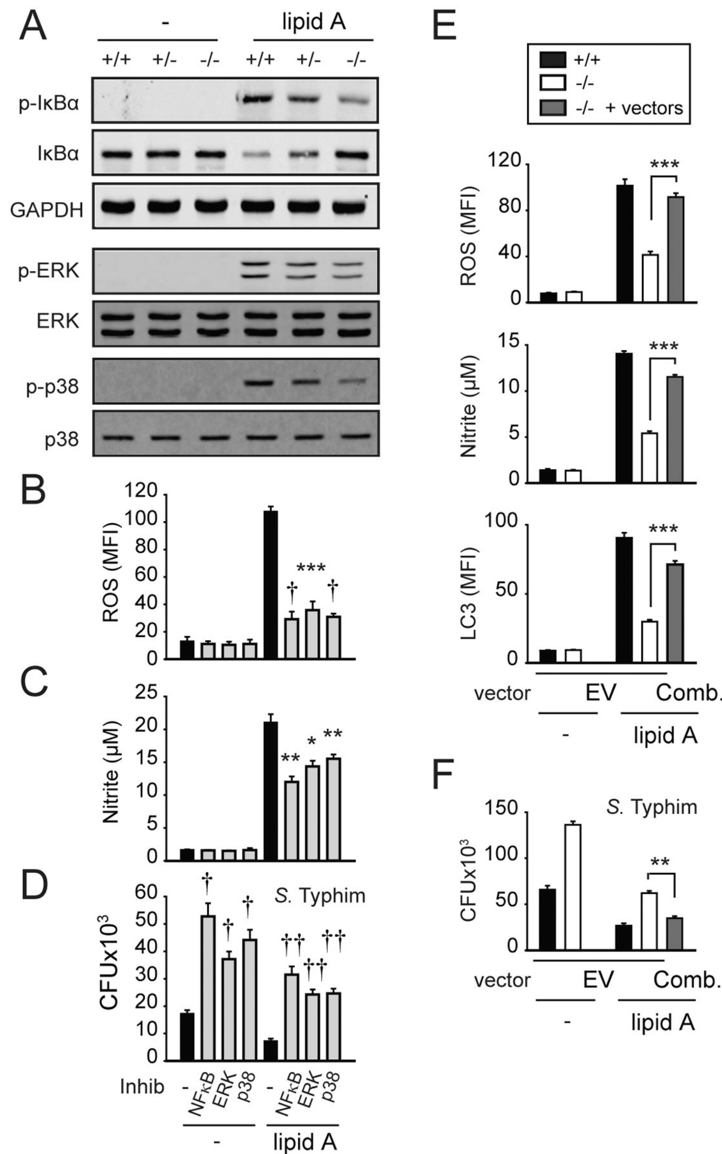

Figure 4. LACC1-dependent MAPK and NFκB signaling pathways contribute to the LACC1-dependent antimicrobial pathways defined in BMMs.

(A) BMMs from *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice were treated with 0.1 μg/ml lipid A for 15min and assessed for phospho-IκBα, phospho-ERK, and phospho-p38 by Western blot. **(B–D)** WT BMMs were treated 0.1 μg/ml lipid A for 48h along with BAY 11–7082 (inhibits NFκB), PD98059 (inhibits ERK) or SB202190 (inhibits p38). **(B)** ROS (4 replicates). **(C)** Nitrite (4 replicates). **(D)** Intracellular bacterial clearance (8 replicates). **(E–F)** Cells were transfected with empty vector (EV) or a combination of vectors leading to constitutive activation of NFκB-, ERK- and p38 (comb), and then treated with 0.1 μg/ml lipid A for 48h. **(E)** ROS (5 replicates), nitrite (5 replicates) and LC3 (5 replicates) (each representative of 2 independent experiments). **(F)** Intracellular bacterial clearance (6 replicates, representative of 2 independent experiments). Mean + SEM. Significance is compared to vehicle control from each respective treatment condition for **(B–D)** or as

indicated for **(E–F).** Inhib, inhibitors. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; †† $p<1\times10^{-5}$.

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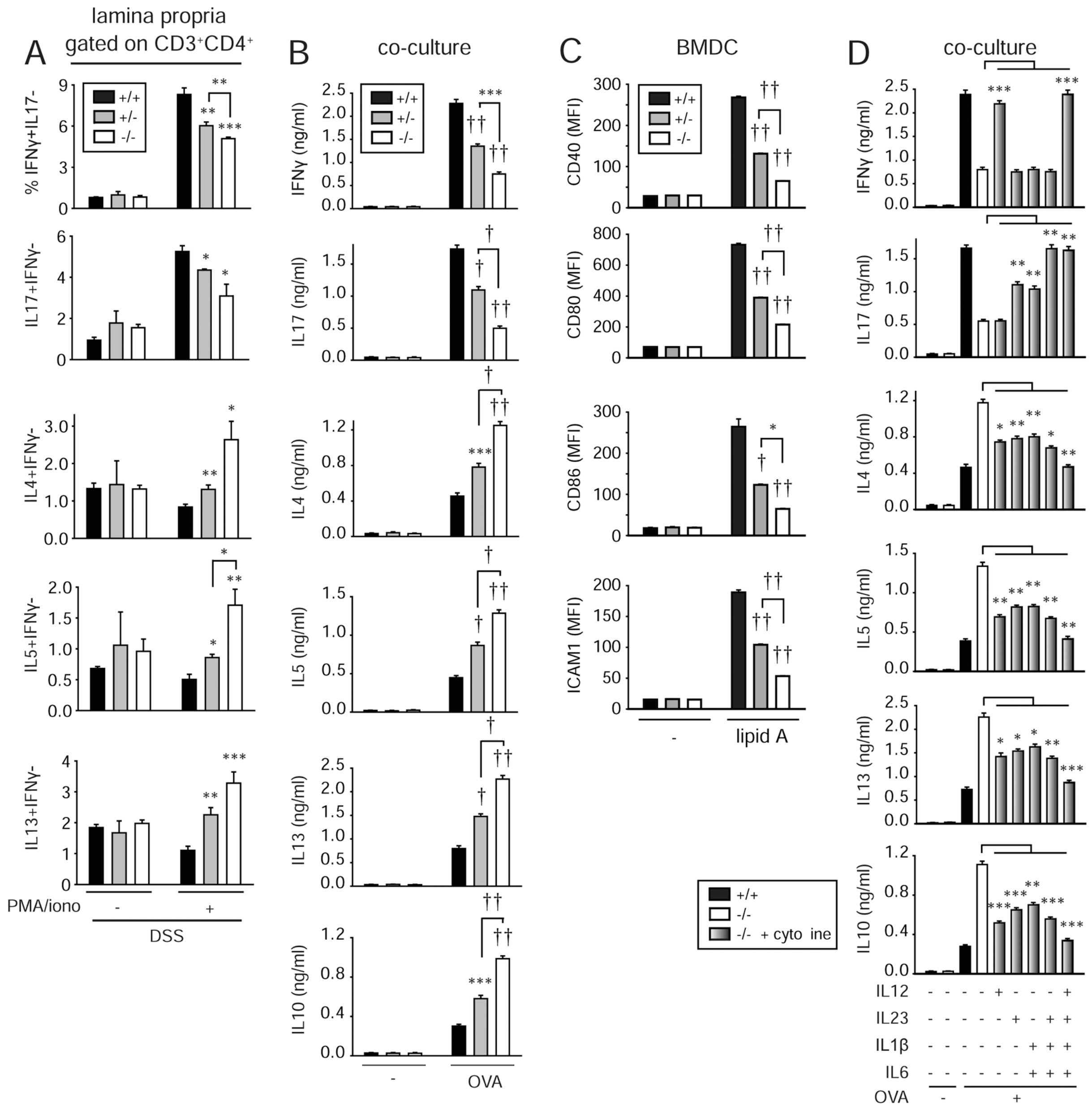

Figure 5. Myeloid cell-intrinsic LACC1 increases Th1 and Th17 and reduces Th2 cytokines.
(A) *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice were given 2.5% DSS in the drinking water for 7d, and then plain drinking water x 2d. Percentage of cytokine-producing cells within colonic lamina propria CD3^{+}CD4^{+} T cells (n=6). **(B)** BMDCs from *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ mice matured with 0.1 μg/ml lipid A for 24h were co-cultured with OTII CD4^{+} T cells and 20mg/ml OVA for 72h. Cytokine secretion (4 replicates; representative of 3 independent experiments). **(C)** *Lacc1*$^{+/+}$, *Lacc1*$^{+/-}$ and *Lacc1*$^{-/-}$ BMDCs were treated with 0.1 μg/ml lipid A for 24h. Co-stimulatory molecules by flow cytometry (MFI) (4 replicates; representative of 2 independent experiments). **(D)** *Lacc1*$^{-/-}$ BMDCs matured with 0.1 μg/ml lipid A for 24h were co-cultured with OTII CD4^{+} T cells + 20mg/ml OVA ± IL12 or IL23 or IL1β + IL6, or combinations of these cytokines for 72h. Cytokine secretion (4 replicates;

representative of 2 independent experiments). *Lacc1*$^{+/+}$ BMDCs as a control. Mean + SEM. Significance is to *Lacc1*$^{+/+}$ in the same group or as indicated for **(A–C)**. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; †† $p<1\times10^{-5}$.

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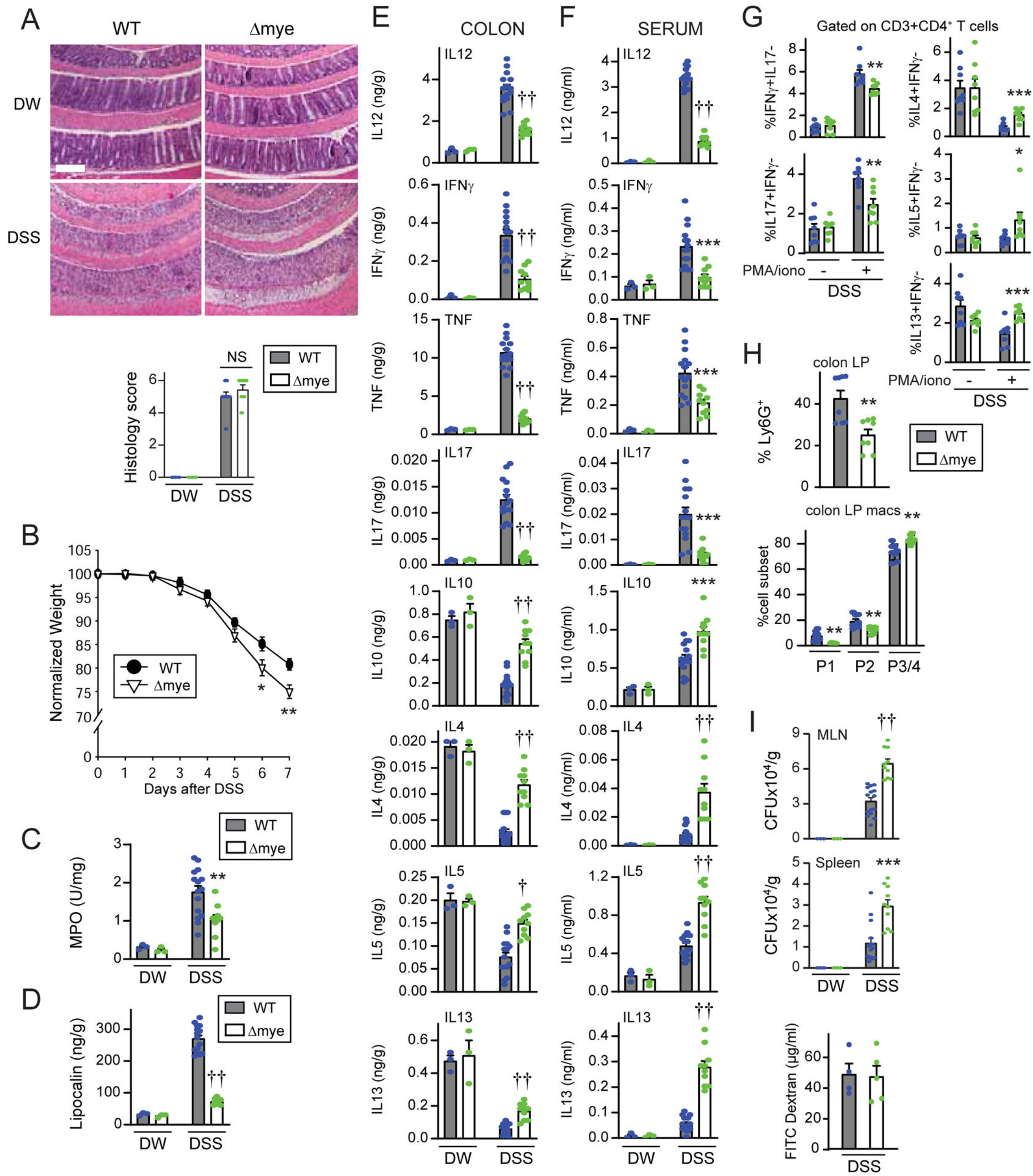

Figure 6.

Myeloid cell-intrinsic LACC1 contributes to clearance of resident microbiota during DSS-induced injury. $Lacc1^{WT}$ or $Lacc1$ ^{mye} mice were given 2.5% DSS in the drinking water (DW). **(A)** Representative H&E of colon sections and histology score. Scale bar=200 μm. **(B)** Body weights. **(C)** Colon myeloperoxidase. **(D)** Fecal lipocalin. **(E)** Colon cytokines. **(F)** Serum cytokines. (**A–F:** n=3–15). **(G)** Percentage of colonic lamina propria cytokine-producing cells within $CD3^{+}CD4^{+}$ T cells upon ex vivo PMA/ionomycin treatment (n=8). **(H)** Percentage colonic lamina propria myeloid cell-derived populations (n=8). **(I)** Bacteria in MLN and spleen (n=3–15; representative 2 independent experiments). **(J)** Intestinal permeability (n=4–5). Mean + SEM. **A–B, J:** DSS for 7d; **C–I**: DSS for 7d and water for

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an additional 2d. NS, not significant. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; †† $p<1\times10^{-5}$.

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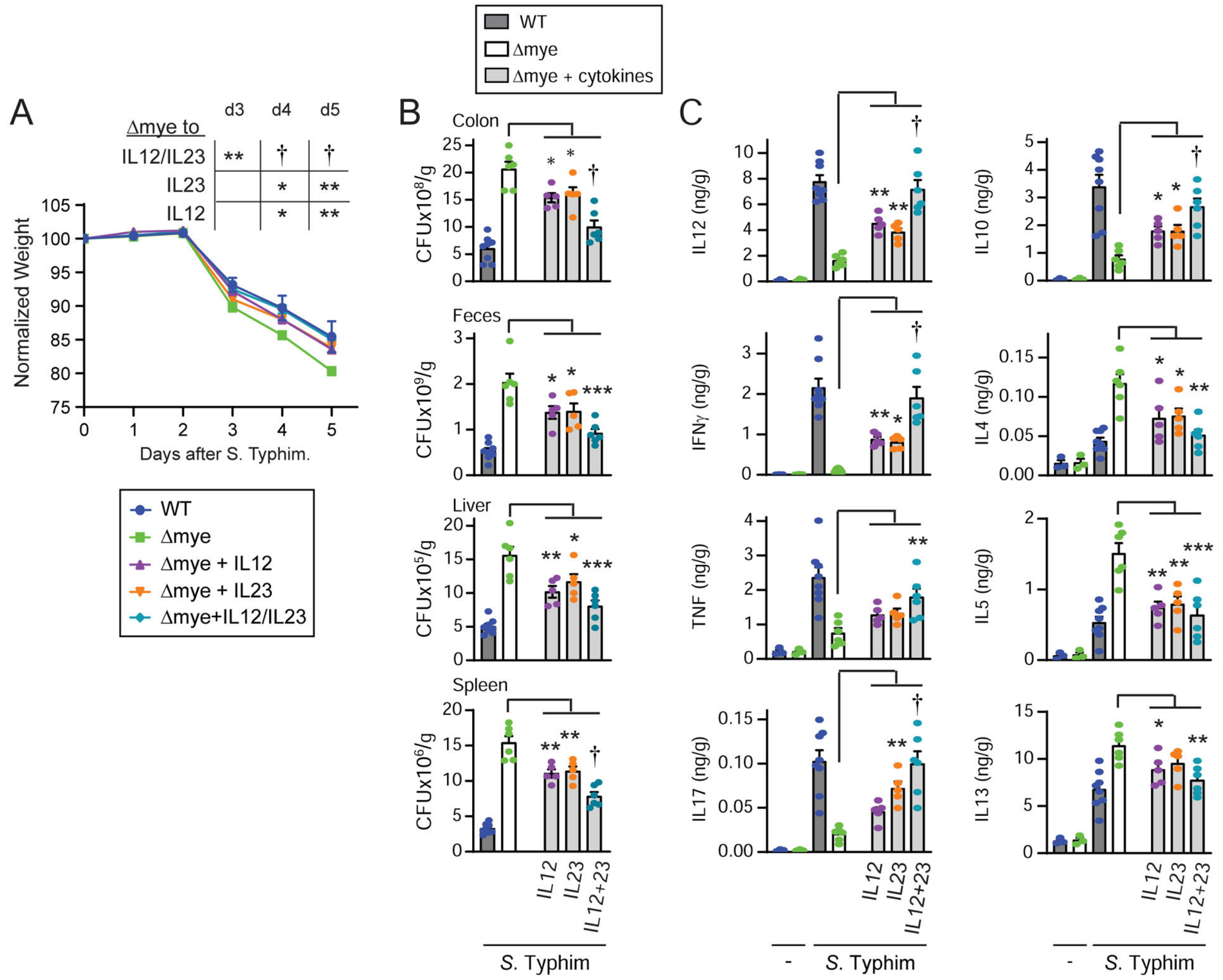

Figure 7.

IL12 and IL23 administration can rescue outcomes in *Lacc1* ^{mye} mice during oral *S*. Typhimurium infection. *Lacc1* ^{mye} mice (n=5–6; representative of 2 independent experiments) were orally inoculated with *S*. Typhimurium ± i.p. IL12:Fc or IL23:Fc (or Fc isotype control), alone or in combination. *Lacc1*^{WT} mice are shown as a control. **(A)** Normalized body weight. **(B)** *S*. Typhimurium in the indicated tissues. **(C)** Colon cytokines. Mean + SEM. *, p<0.05; **, p<0.01; ***, p<0.001; †, p<1×10^{-4}.