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Innate PI3K p110δ Regulates Th1/Th17 Development and Microbiota-Dependent Colitis

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Genetic variants that confer susceptibility to the human inflammatory bowel diseases (IBD) Crohn’s disease (CD) and ulcerative colitis highlight the importance of innate immune interactions with the enteric microbiota in both initiating and controlling inflammation (1). Commensal and pathogenic microorganisms are recognized through conserved molecular microbial patterns by pattern-recognition receptors, of which TLRs form integral components (2). Although mechanisms by which the host distinguishes commensal from pathogenic bacteria are not well defined, under normal conditions TLR signaling initiated by the enteric microbiota is protective (3). PI3Ks have emerged as important regulators of TLR signaling (4, 5). Class IA PI3Ks have five different regulatory subunits and three p110 catalytic subunits: p110α and p110β are expressed ubiquitously in tissues, whereas p110δ is enriched in leukocytes (6). Agents that activate macrophages to produce IL-12p40, the common subunit of the proximal inflammatory cytokines IL-12 and IL-23, also activate class Iα PI3K (7). Activation of PI3K in turn blocks the expression of IL-12p40 mRNA (IL12b) (7). Although inflammatory responses are essential for eradicating pathogenic microbes, excessive/prolonged activation of innate immunity is harmful to the host. PI3K-mediated negative feedback of IL-12p40 is important to prevent excessive innate immune responses.

The clearest role for PI3K in chronic inflammation is described in a mouse harboring a kinase-dead p110δ catalytic subunit of PI3K (p110δKD) (8). These mice demonstrate B and T cell defects, including defective Ag receptor signaling and impaired humoral responses. Notably, the occurrence of spontaneous colitis was demonstrated in PI3K p110δKD mice (9). Expression of IL-12p40, Th1, and Th17 cytokines was described in the intestinal and systemic immune compartments. Consistent with a homeostatic role for p110δ in the intestine, wild-type (WT) mice raised in a germfree (GF) environment markedly upregulated colonic p110δ expression when Pik3cd knockout mice were raised in the same conditions did not (9).

Given the role of the PI3K p110δ subunit in innate immune processes fundamental to the pathogenesis of IBD, host-enteric microbiota and APC–T cell interactions in p110δKD mice were further characterized. We describe a requirement for the enteric...
microbiota to drive intestinal inflammation in p110<sup>KD</sup> mice. Microbial-innate immune interactions, through p110<sup>KD</sup>, maintain homeostasis through regulation of both protective (IL-10) and inflammatory (IL-12p40) cytokines. Furthermore, p110<sup>KD</sup> orchestrates innate regulation of adaptive immune responses. Importantly, in human CD, decreased intestinal PIK3CD gene expression is demonstrated that inversely correlates with intestinal IL12B:IL10 ratios. Thus, p110<sup>KD</sup> appears to be a central homeostatic switch in the intestine, governing the critical balance between microbiota-induced IL-12/23 and IL-10, shaping the subsequent T cell response. Counter to prevailing paradigms in which microbiota-induced IL-12/23 and IL-10, shaping the sub-

Materials and Methods

Mice

All mice were maintained on a C57BL/6 background in conventional or GF housing. P13K p110<sup>KD</sup><sup>−/−</sup> mice were previously obtained from R. Vanhaesebroeck (Queen Mary University of London, London, U.K.). GF p110<sup>KD</sup> mice were Caesarian derived, as previously described (12), and were maintained according to standard techniques in the University of North Carolina National Gnotobiotic Resource Center. OT-II (C57BL/6-Tg[TCraTcrb]425Cbn/J) male mice were provided by J. Ting (University of North Carolina, Chapel Hill, NC). All animal experiments were in compliance with protocols approved by the International Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Reagents

LPS from Escherichia coli and Pam3CSK<sub>4</sub> were purchased from Invivogen (San Diego, CA). Zymosan A from Saccharomyces cerevisiae was purchased from Sigma-Aldrich (St. Louis, MO). Inhibitors IC87114, rapamycin, and Pam3CSK<sub>4</sub> were purchased from Invivogen (San Diego, CA). IL-12p35 and IL-12p40 and IL-10 (BD Biosciences, San Jose, CA) and IFN-

Histology

Slides were prepared for H&E staining, and a pathologist (L.B.B.) blinded to the study groups performed histological analysis using established criteria for p110<sup>KD</sup> mice (9). In T cell adoptive transfer studies, the following scoring system was used: tissue changes were categorized into inflammatory and epithelial changes and graded for severity (0 = normal, 1 = mild, 2 = moderate, 3 = marked, and 4 = severe); the sum of the two grades comprised the histopathology score. For inflammation, a score of 0 (normal) signified rare small lymphoplasmytic aggregates confined to the lamina propria; scores of 1 (mild) and 2 (moderate) represented increasing numbers of multifocal inflammatory aggregates that were predominantly confined to the lamina propria, with occasional submucosal infiltration; a score of 3 (marked) was assigned if inflammatory infiltrates frequently extended into the submucosa and muscular layers; a score of 4 (severe) was designated if transmural inflammation was common. Epithelial changes, characterized by hypertrophy, were scored (0–4) with increasing severity and prevalence of the observed change.

Cell isolation

Bone marrow–derived macrophages (BMDMs) were cultured, as described previously (15). Splenocytes were isolated, as described (16), and further separated into CD11c<sup>+</sup> and CD11c<sup>−/−</sup> cells by MACS with anti-CD11c microbeads (Miltenyi Biotec, Auburn, CA). Lamina propria mononuclear cells (LPMCs) were isolated from mouse colons, as described previously (17). LPMCs were further separated into CD11b<sup>+</sup> and CD11b<sup>−</sup> cells by MACS with anti-CD11b microbeads (Miltenyi Biotec).

Cell culture experiments

BMDMs or splenocytes were cultured at 1 × 10<sup>6</sup>/ml in the presence of LPS (1 ng/ml), zymosan A (10 μg/ml), Pam3CSK<sub>4</sub> (5 ng/ml), or PBS, and supernatants were harvested after 0 or 24 h (BMDMs or splenocytes, respectively). Inhibitors IC87114 (0.1 or 1 μM), rapamycin (1 or 10 μM), SB-216763 (1 or 10 μM), or DMSO were added 1 h prior to stimulation with LPS or zymosan A. CD11b<sup>+</sup> LPMCs were treated with IC87114 (10 μM) for 30 min prior to exposure to heat-killed E. coli (HKEC; multiplicity of infection [MOI] = 100) for 3 h. Total RNA was assessed for Il12β and Il10 expression by quantitative PCR.

Western blot analysis

BMDMs were cultured at 1 × 10<sup>6</sup>/ml with LPS (1 μg/ml), and cecal tissue from GF to conventionalized (CNV) mice was collected. Cell or tissue lysates were collected in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors. Equal amounts of protein were loaded and run on a 10% SDS-PAGE gel. Blots were probed for phosphorylated glycogen synthase kinase-3β (GSK-3β) (Ser<sup>9</sup>) and total GSK-3β with Abs from Cell Signaling Technology (Danvers, MA) and phosphorylated CREB (Ser<sup>133</sup>) (EMD Millipore, Billerica, MA) and total CREB (Santa Cruz Biotechnology, Dallas, TX). Equal protein loading was confirmed by probing blots with anti-β-actin (mAbcam 8226) from Abcam (Cambridge, MA).

Quantitative RT-PCR

Quantitative RT-PCR was performed on total RNA, as described (14). Murine primer sequences will be provided upon request. The following human primer sequences were used: PIK3CD, forward, 5′-GGCGCGG-GACGATAAAGGAGTC-3′; and reverse, 5′-GCTGCCACAGGCTGTC-ACCT-3′; IL10, forward, 5′-GCATACACTGCTCTGAGATC-3′; and reverse, 5′-TGTAGTGTGGCTTGTTCGTC-3′; IL22, forward, 5′-GCTCTG-TGCCCTGGACCTAGCGC-3′; and reverse, 5′-GCTGAAATTGAT- GGTATCCCCG-3′; GAPDH, forward, 5′-GCTGAGGCTCGGACTCAAC-GGA-3′; and reverse, 5′-GAGGGATCTCGCTCTGGAAGA-3′.

ELISAs

IL-12p40 and IL-10 (BD Biosciences, San Jose, CA) and IFN-γ concentrations (R&D Systems, Minneapolis, MN) were determined by sandwich ELISA, according to manufacturer’s instructions.

APC–CD4<sup>+</sup> T cell coculture

Splenic APCs from WT or p110<sup>KD</sup> mice were isolated by negative selection using CD90.2 microbeads (Miltenyi Biotec). Splenic APCs were incubated overnight with CBL (50 ng/ml), and, after washing several times to remove extracellular Ag, APCs were cocultured with negatively selected CD4<sup>+</sup> T cells (CD8<sup>+</sup>/B220/MHC II microbeads; Miltenyi Biotec) from WT or IL10<sup>−/−</sup> mice at a 3:2 ratio (APC:T cell) for 72 h. For Ag-specific studies, APCs were incubated overnight with LPS (10 ng/ml) and OVA peptide (332–339) at 10 μM (13). After washing to remove extracellular Ag, APCs were cocultured with negatively selected CD4<sup>+</sup> T cells (CD8<sup>+</sup>/B220/MHC II microbeads; Miltenyi Biotec) from mice expressing a transgenic TCR that recognizes OVA epitope residues 323–339 (OT-II mice) at a 3:2 ratio (APC:T cell) for 72 h. CD4<sup>+</sup> T cells were analyzed for intracellular cytokine expression (IFN-γ and IL-17A) by flow cytometry.

Flow cytometry

CD4<sup>+</sup> T cells were stimulated for 4 h with PMA (100 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiStop (BD Biosciences). Cells were then washed and stained with allophycocyanin-conjugated anti-CD3 (clone 17A2; eBioscience, San Diego, CA). After fixing and permeabilizing the cells with BD Cytofix/Cytoperm (BD Biosciences), staining for intracellular PE-conjugated anti-IFN-γ (clone XMG1.2; eBioscience) and FITC-conjugated anti-IL-17A (clone eB17B7; eBioscience) was performed. Flow cytometry samples were run on a CyAn ADP Analyzer (Beckman Coulter, Brea, CA) and analyzed using Summit v4.3 (Beckman Coulter).

CD4<sup>+</sup> CD45RB<sup>high</sup> T cell–adaptive transfer colitis

T cell–mediated colitis was induced in Rag1<sup>−/−</sup> and Rag1<sup>−/−</sup>/p110<sup>KD</sup> (RKO8<sup>KD</sup>) mice at 8 wk of age, as described previously (18). CD4<sup>+</sup> T cells were isolated by negative selection (CD8<sup>+</sup>/B220/MHC II microbeads; Miltenyi Biotec) and stained with FITC-conjugated anti-CD4 (clone GK1.5; eBioscience) and PE-conjugated anti-CD45RB (clone 16A; BD Pharmingen, San Jose, CA). CD4<sup>+</sup> T cells were sorted into CD45RB<sup>high</sup> and CD45RB<sup>low</sup> populations using a MoFlo XDP Cell Sorter (Beckman Coulter). Mice were i.p. injected with 4 × 10<sup>5</sup> CD4<sup>+</sup>CD45RB<sup>low</sup> cells, as described (18). Clinical scores were assigned, as described (19).
**Human intestinal samples**

Intestinal samples were obtained from surgical resections from CD patients and subjects requiring surgical intervention for noninflammatory conditions (e.g., colon cancer). In CD patients, when available, paired inflamed and noninflamed intestinal segments, as determined by gross appearance by the processing pathologist, were obtained for analysis. The University of North Carolina Institutional Review Board approved collection of de-identified samples, and written informed consent was obtained from all patients.

**Statistical analysis**

Statistical significance for data subsets was assessed by the two-tailed Student *t* test. The *p* values <0.05 were considered to be significant. All data are expressed as mean ± SEM.

**Results**

**Presence of the enteric microbiota is necessary for the development of colitis in p110KD mice**

To determine whether the microbiota is necessary for the development of colitis, p110KD mice were derived GF. GF p110KD mice up to 30 wk of age did not develop histological colitis (Fig. 1A, Supplemental Fig. 1A). Interestingly, GF p110KD mice produced significantly less colonic IL-10 compared with GF WT mice (Fig. 1B, left). GF p110KD and WT mice were then transitioned to CNV housing, and colonic inflammation was assessed at days 7 and 14 after transfer. Compared with GF to CNV WT mice, colons from GF to CNV p110KD mice demonstrated increased colitis scores (Fig. 1A, Supplemental Fig. 1A). However, GF to CNV p110KD mice gained weight similarly to GF to CNV WT mice (Supplemental Fig. 1B). Furthermore, colonic explants from day 7 and 14 GF to CNV p110KD mice produced significantly less IL-10 (Fig. 1B) compared with GF to CNV WT mice. At day 14, GF to CNV p110KD mice produced significantly elevated IL-12p40 (Fig. 1C) compared with WT GF to CNV mice. IL-10 is important for the maintenance of intestinal homeostasis in part through inhibition of IL-12p40 (20). The ratio of colonic IL-12p40 to IL-10 protein production therefore reflects the overall balance of intestinal pro- and anti-inflammatory cytokines. Indeed, colons from days 7 and 14 GF to CNV p110KD mice demonstrated significantly higher ratios of IL-12p40:IL-10 production (Fig. 1D) compared with GF to CNV WT mice.

Given the recent report of coregulation of IL-10 and IFN-β by p110δ in dendritic cells (21), we measured IFN-β levels in colonic...
explant tissue cultures from GF to CNV WT and p110<sup>KD</sup> mice. Interestingly, IFN-β production was enhanced in colonic explants from day 7 GF to CNV p110<sup>KD</sup> mice compared with WT mice (Fig. 1E), in contrast to decreased levels of IL-10 production in p110<sup>KD</sup> mice. IFN-β production was increased in LPS-stimulated p110<sup>KD</sup> compared with WT BMDMs (Fig. 1F). Additionally, LPS-stimulated II10<sup>−/−</sup> BMDMs produced more IFN-β compared with WT BMDMs (Fig. 1F), consistent with previously reported regulation of IFN-β by IL-10 (21). To assess the direct contribution of IFN-β production from colonic macrophages, CD11b<sup>+</sup> LPMCs, comprising mostly macrophages, were isolated from p110<sup>KD</sup> mice and stimulated with HKEC. Similar to BMDMs, HKEC-stimulated p110<sup>KD</sup> CD11b<sup>+</sup> LPMCs produced significantly more IFN-β compared with WT CD11b<sup>+</sup> LPMCs (Fig. 1G), suggesting that p110<sup>KD</sup> differentially regulates IL-10 and IFN-β in macrophages.

PI3K p110 regulates macrophage production of IL-10 in response to pathogen-associated molecular patterns

WT and p110<sup>KD</sup> BMDMs were exposed to TLR agonists (LPS [TLR4], 5 ng/ml; Pam<sub>3</sub>CSK<sub>4</sub> [TLR2/1], 5 ng/ml; zymosan A [TLR2/6], 5 µg/ml), and cytokine production was measured. BMDMs from p110<sup>KD</sup> mice produced less IL-10 in response to all TLR agonists tested compared with WT BMDMs (Fig. 2A).

**FIGURE 2.** Defective p110<sup>KD</sup> activity alters macrophage production of IL-10 and IL-12p40 in response to bacterial products. BMDMs were stimulated with LPS (5 ng/ml), Pam<sub>3</sub>CSK<sub>4</sub> (5 ng/ml), or zymosan A (5 µg/ml) for 8 h. Supernatants were collected and assayed for IL-10 (A) and IL-12p40 (B) production by ELISA. Error bars represent mean ± SEM from three independent experiments (*p < 0.05). (C) The ratio of IL-12p40 to IL-10 from individual experiments was calculated. Error bars represent mean ± SEM from three independent experiments (*p < 0.05, **p < 0.005).

**FIGURE 3.** A p110<sup>KD</sup>-specific inhibitor decreases IL-10 and augments Il-12p40 production in WT macrophages stimulated with bacterial products. WT, p110<sup>KD</sup>, and II10<sup>−/−</sup> BMDMs were cultured with a p110<sup>KD</sup>-specific inhibitor (IC87114, 0.1, 1, or 10 µM) for 1 h prior to stimulation with LPS (1 ng/ml, BMDMs). Supernatants from WT and p110<sup>KD</sup> BMDMs were collected after 8 h of culture and assayed for IL-10 (A) and IL-12p40 (B) production by ELISA. Error bars represent mean ± SEM from three independent experiments (*p < 0.05, **p < 0.005, ***p < 0.0005). (C) Supernatants from WT and II10<sup>−/−</sup> BMDMs were collected after 8 h of culture and assessed for IL-12p40. Error bars represent mean ± SEM from three independent experiments (*p < 0.05, **p < 0.005, ***p < 0.0005). (D and E) WT and p110<sup>KD</sup> CD11b<sup>+</sup> LPMCs were incubated with a p110<sup>KD</sup>-specific inhibitor (IC87114, 10 µM) 1 h prior to stimulation with HKEC (MOI = 100) for 3 h. Quantitative RT-PCR was performed in duplicate for II10 (D) and II12b (E), expression levels normalized to β-actin expression and calculated as fold induction over unstimulated cells. Error bars represent mean ± SEM for three independent experiments (*p < 0.05).
Additionally, p110\textsuperscript{KD} BMDMs exposed to TLR agonists produced significantly more IL-12p40 compared with WT BMDMs (Fig. 2B), in agreement with our previously published data (9). Consequently, the ratio of IL-12p40:IL-10 production in TLR ligand–treated p110\textsuperscript{KD} BMDMs was consistently increased compared with WT BMDMs (Fig. 2C). LPS- or zymosan A–stimulated CD11b\textsuperscript{+} and CD11c\textsuperscript{+} splenocytes from p110\textsuperscript{KD} mice also produced less IL-10 and more IL-12p40 than WT splenic cells (Supplemental Fig. 2A–D).

To further validate these findings, LPS-stimulated WT BMDMs were treated with p110 isoform–specific chemical inhibitors. LPS-activated WT BMDMs demonstrated a dose-dependent decrease in IL-10 production (Fig. 3A) and increase in IL-12p40 (Fig. 3B, left) with specific chemical inhibition of p110 (IC87114). Interestingly, p110\textsuperscript{KD} inhibition in WT BMDMs markedly enhanced IL-12p40 production above levels produced by p110\textsuperscript{KD} BMDMs. It is possible that there is compensation by other PI3K isoforms in p110\textsuperscript{KD} BMDMs but not in chemically inhibited WT BMDMs. Indeed, we have previously shown that phosphorylation of Akt in LPS-stimulated p110\textsuperscript{KD} BMDMs is not completely abrogated (9). PI3K p110\textsuperscript{a} and p110\textsuperscript{b} inhibition (PIK-90 and TGX-221, respectively) did not alter LPS-stimulated IL-10 production (Supplemental Fig. 2E, 2F) or IL-12p40 (Supplemental Fig. 2G, 2H) in WT BMDMs, in agreement with reported results in dendritic cells (21). As a control, p110\textsuperscript{KD} BMDMs did not alter IL-10 or IL-12p40 expression (Fig. 3A, 3B). However, in p110\textsuperscript{KD} BMDMs, p110\textsuperscript{BD} inhibition decreased IL-10 (Supplemental Fig. 2F) production, and p110\textsuperscript{a}- or p110\textsuperscript{BD}-specific inhibition modestly enhanced LPS-induced IL-12p40 expression (Supplemental Fig. 2G, 2H), suggesting that, in the absence of p110\textsuperscript{a} function, other isoforms may have modest effects on IL-10/IL-12p40 regulation.

Chemical inhibition of p110 in \textit{Il10}\textsuperscript{–/–} BMDMs led to a dose-dependent increase in IL-12p40 production (Fig. 3C), suggesting that p110-mediated decreases in IL-12p40 are in part independent of the inhibitory actions of IL-10. Relevant to mucosal innate inflammatory responses, WT CD11b\textsuperscript{+} colonic LPMC treated with HKEC demonstrated diminished \textit{Il10} (Fig. 3D) and enhanced \textit{Il12b} (Fig. 3E) expression in the presence of the p110\textsuperscript{BD}-specific inhibitor. As a control, expression of neither cytokine was altered in CD11b\textsuperscript{+} colonic LPMC from p110\textsuperscript{KD} mice treated with the p110\textsuperscript{BD}-specific inhibitor.

![FIGURE 4. Phosphorylation of p110 targets is altered in macrophages and colons from p110\textsuperscript{KD} mice. (A and B) BMDMs from WT and p110\textsuperscript{KD} mice were treated with LPS (1 \mu g/ml), and cell lysates were collected for Western blot analysis after the indicated times. (A) Blots were probed for phosphorylated GSK-3\textbeta (Ser9) and total GSK-3\textbeta. Figure shows representative results from three independent experiments. Quantitative analysis of p-GSK-3\textbeta relative light units (RLU) is normalized to total GSK-3\textbeta and actin. Error bars represent mean ± SEM (*p < 0.05, **p < 0.005 compared with WT). (B) Blots were probed for phosphorylated CREB (Ser133) and total CREB. Representative results from two independent experiments are displayed. Quantitative analysis of p-CREB RLU is normalized to total CREB. Error bars represent mean ± SEM from three independent experiments (*p < 0.05 compared with WT). (C) GF WT and p110\textsuperscript{KD} mice were transferred to CNV housing and colonized with a commensal enteric microbiota (GF:WT, \textit{n} = 2; p110\textsuperscript{KD}, \textit{n} = 2; GF to CNV: 7-d WT, \textit{n} = 1; 7-d p110\textsuperscript{KD}, \textit{n} = 3; 14-d WT, \textit{n} = 2; 14-d p110\textsuperscript{KD}, \textit{n} = 4). Mice were sacrificed at the indicated times after colonization, and cecal tissue was collected and protein extracted. Blots were probed for the indicated proteins/phosphoproteins. Quantitative analysis of p-GSK-3\textbeta RLU is normalized to total GSK-3\textbeta and actin. Error bars represent mean ± SEM (*p < 0.05, **p < 0.005 compared with WT).]
Mammalian target of rapamycin and GSK-3β act downstream of p110δ in macrophages to regulate cytokine production

PI3Ks modulate multiple downstream signaling pathways, of which mammalian target of rapamycin (mTOR) and GSK-3β have been previously shown to regulate cytokine secretion in macrophages (22). PI3K p110δ KD BMDMs treated with LPS demonstrated impaired phosphorylation of GSK-3β and CREB (Fig. 4A, 4B, respectively), but not p70 S6 kinase (downstream of mTOR; data not shown). Furthermore, colonic expression of p-GSK-3β was attenuated in GF to CNV p110δ KD mice compared with GF to CNV WT mice (Fig. 4C). BMDMs from WT and p110δ KD mice were exposed to mTOR or GSK-3β inhibitors (rapamycin or SB-216763, respectively) prior to LPS stimulation. Rapamycin decreased IL-10 (Fig. 5A, 5C) and increased IL-12p40 (Fig. 5B, 5D) protein and mRNA expression in WT and p110δ KD TLR-stimulated BMDMs. These same trends were observed in WT and p110δ KD CD11b+ and CD11c+ splenocytes (Supplemental Fig. 3A–D). Inhibition of GSK-3β in p110δ KD BMDMs and splenocytes increased IL-10 protein (Fig. 5E, Supplemental Fig. 3E, 3F) and mRNA expression (Fig. 5G), whereas p110δ KD BMDMs and splenocytes decreased IL-12p40 protein (Fig. 5F, Supplemental Fig. 3G, 3H) and mRNA (Fig. 5H) expression. Hence, mTOR and GSK-3β are downstream of p110δ and are relevant for regulation of IL-10 and IL-12p40.

APC p110δ regulates T cell cytokine production

To begin to determine whether resident APCs regulate intestinal T cell phenotype and function, T cell cytokines and lineage markers were measured in p110δ KD colons. Colonic Tbx21 and Rorc transcripts (Fig. 6A, 6B), the hallmark transcription factors of Th1 (23) and Th17 cells (24), respectively, were increased in GF to CNV p110δ KD mice at days 7 and 14 posttransition compared with matched GF to CNV WT mice. Likewise, increased Ifng and Il17a transcripts (Fig. 6C, 6D) were detected in cecal tissue from days 7 and 14 GF to CNV p110δ KD compared with matched GF to CNV WT mice.

Consequently, we next investigated whether T cell–dependent IL-12p40 and IL-10 expression was altered in p110δ KD APCs. Splenic CD4+ T cells from WT mice were cultured with either WT or p110δ KD splenic APCs pulsed with CBL. CBL-pulsed p110δ KD APCs cultured with naive WT CD4+ T cells produced decreased levels of IL-10 (Fig. 6E) and increased levels of IL-12p40 (Fig. 6F).

**FIGURE 5.** IL-10 and IL-12 p40 production in macrophages is mTOR and GSK-3β dependent. WT and p110δ KD BMDMs were cultured with the mTOR inhibitor rapamycin (A–D) or the GSK-3β inhibitor SB-216763 (E–H) for 1 h prior to stimulation with LPS (1 ng/ml) for 4 h (quantitative PCR) or 8 h (ELISA). Supernatants were collected and assayed for IL-10 (A, E) and IL-12p40 (B, F) production by ELISA. Error bars represent mean ± SEM from three independent experiments (*p < 0.05, **p < 0.005). Total RNA was assayed for Il10 (C, G) and Il12b (D, H) expression normalized to β-actin expression by quantitative RT-PCR. Error bars represent mean ± SEM from three independent experiments (*p < 0.05).
compared with WT APCs. WT and p110^KD^ APCs cultured with I110^KD^ CD4^+^ T cells demonstrate that IL-10 expression is largely derived from APCs (Fig. 6E). As expected, CBL-pulsed p110^KD^ APCs also produced significantly less IL-10 (Fig. 6E) and more IL-12p40 (Fig. 6F) compared with WT APCs in the absence of CD4^+^ T cells.

Next, to study Ag-specific APC–T cell interactions, splenic CD4^+^ T cells from OVA-specific transgenic TCR mice (OT-II mice) were cocultured with OVA-pulsed and LPS-activated WT and p110^KD^ APCs. OVA-loaded p110^KD^ APCs induced significantly more IFN-γ–producing (Fig. 6H, 6I) and IL-17A–producing (Fig. 6H, 6J) CD4^+^ T cells compared with OVA-loaded WT APCs (Fig. 6G, 6I, 6J). However, T cell proliferation was induced to a similar extent by both WT and p110^KD^ APCs (Supplemental Fig. 4A–C). These data suggest that cytokine production by p110^KD^ APCs directs differentiation of Ag-specific Th1 and Th17 CD4^+^ T cells.

These results suggest a model in which defective p110^d^, through regulation of IL-10 and IL-12p40, leads to inflammatory effector T cell development. To test this model in vivo, we generated

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**FIGURE 6.** APC p110^d^ regulates T cell differentiation. (**A–D**) GF WT and p110^KD^ mice were transferred to CNV housing. Colonic tissue was collected from WT and p110^KD^ mice sacrificed at days 0 (n = 12 and 10, respectively), 7 (n = 5 and 7, respectively), and 14 (n = 6 and 12, respectively) after transfer. Quantitative RT-PCR was performed in duplicate for Tbx21 (A), Rorc (B), Ifng (C), and Il17a (D) expression normalized to β-actin expression. Error bars represent mean ± SEM (*p < 0.05). (**E and F**) WT and p110^KD^ APCs were cultured overnight with CBL (50 μg/ml) and then cocultured with WT or I110^KD^ CD4^+^ T cells at a ratio of 3:2 for 72 h. Supernatants were collected and assayed for IL-10 (E) and IL-12p40 (F) production by ELISA. Error bars represent mean ± SEM for three independent experiments (*p < 0.05). (**G–J**) WT and p110^KD^ APCs were stimulated with LPS (10 ng/ml) and OVA peptide (5 μM) overnight and then cocultured with WT CD4^+^CD62L^+^ OT-II T cells at a ratio of 3:2 for 72 h. T cells were assayed for IL-17A and IFN-γ production by flow cytometry. CD4^+^ lymphocytes were gated using forward and side scatter. Representative flow cytometry plots show IFN-γ– and IL-17A–producing WT CD4^+^ OT-II T cells cocultured with LPS- and OVA-stimulated WT (G) and p110^KD^ (H) APCs. Plots are representative of results from three independent experiments with similar results. Quantification of the percentage of total CD4^+^ T cells producing IFN-γ (I) and IL-17A (J) was determined from the flow cytometry analysis. Error bars represent mean ± SEM from three independent experiments (*p < 0.05).
Rag1⁻/⁻ p110KD mice (RKO/6KD). Interestingly, colitis is present but attenuated in the absence of an adaptive immune system (Fig. 7A, 7B). Colonic explant cultures from RKO/6KD mice produced significantly decreased IL-10 (Fig. 7C) and increased IL-12p40 (Fig. 7D) compared with colonic tissue explant cultures from Rag1⁻/⁻ mice.

It was previously reported that p110KD CD4⁺ T cells adaptively transferred into Rag1⁻/⁻ recipients induce colitis owing to impaired T regulatory cell function (25). To study how p110 inactivation in nonlymphocyte populations affects T cell differentiation, admixed WT CD4⁺CD45RBhigh and CD4⁺CD45RBlow T cells were adaptively transferred into Rag1⁻/⁻ and RKO/6KD mice (CD45RB recipient mice) and monitored for colitis development. Adoptive transfer of T cells demonstrated reconstitution, with localization of CD3⁺ cells to the colons of recipient mice (Supplemental Fig. 4D). Total body weight of recipient mice was recorded until the experiment was terminated at day 24 due to severe clinical manifestations in the CD45RB recipient RKO/6KD mice. There was no difference in weight loss between CD45RB recipient Rag1⁻/⁻ and RKO/6KD mice (data not shown). Clinical colitis activity scores (Fig. 8A) and quantitative colonic histology analysis (Fig. 8B, 8C) from CD45RB recipient RKO/6KD mice were increased compared with the respective recipient Rag1⁻/⁻ mice. Colonic IL-10 production was significantly lower (Fig. 8D) and IL-12p40 production higher (Fig. 8E) in CD45RB recipient RKO/6KD mice compared with CD45RB recipient Rag1⁻/⁻ mice. Consequently, ratios of colonic IL-12p40/IL-10 production from CD45RB recipient RKO/6KD mice were significantly higher (Fig. 8F) than ratios from recipient Rag1⁻/⁻ mice. Furthermore, a greater percentage of IFN-γ-producing (Fig. 8G), but not IL-17A–producing (Fig. 8H), CD4⁺ T cells were isolated from mesenteric lymph nodes (MLNs) of CD45RB recipient RKO/6KD mice compared with recipient Rag1⁻/⁻ mice. Finally, a greater percentage of lamina propria CD3⁺CD4⁺ T cells from recipient RKO/6KD mice produced IFN-γ (Fig. 8I) and IL-17A (Fig. 8J; each data point represents three pooled samples), compared with recipient Rag1⁻/⁻ mice.

**Intestinal PIK3CD expression correlates with IL12B:IL10 ratios from patients with CD**

Expression of p110 (PIK3CD), IL-12p40 (IL12B), and IL-10 (IL10) mRNA was determined in human intestinal tissue from control subjects without intestinal inflammation and patients with CD. Significantly higher levels of PIK3CD mRNA were detected in noninflamed intestinal samples from control subjects compared with tissue from patients with CD (Fig. 9A). Paired macroscopically inflamed and noninflamed intestinal resections from the same subject were obtained from 14 CD patients. There was lower expression of PIK3CD in inflamed intestinal tissues compared with noninflamed tissues obtained from the same patient (Fig. 9B). Furthermore, ratios of IL12B:IL10 expression from individual CD patients demonstrated a strong and statistically significant inverse correlation with PIK3CD expression (Fig. 9C).

**Discussion**

We previously described the development of spontaneously occurring Th1- and Th17-mediated colitis in p110KD (9). In the present series of experiments, we further elucidate intestinal host-microbial and APC–T cell interactions mediated by p110. Colitis in p110KD mice is dependent on host responses to the enteric microbiota, as has been described in other murine colitis models (26). In the absence of the enteric microbiota, p110KD mice did not develop intestinal inflammation, whereas, after reconstitution with commensal enteric microbiota, colons from p110KD mice demonstrated histological inflammation, impaired IL-10, and increased IL-12p40 production (Fig. 1). Consequently, altered IL-10 and IL-12p40 production by p110KD APCs in response to microbial products and cognate interactions with T cells orchestrates pathogenic adaptive immune responses contributing to intestinal inflammation.

Class I, PI3Ks regulate macrophage and dendritic cell responses to bacteria (5). Taken together, our results and those of others (21) elucidate a model in which p110 is an intracellular integrator of environmental signals that is involved in the restoration of inflammatory responses to homeostasis, mediated in part by IL-10. Regulation of IL-10 expression involves both PI3K-dependent and independent pathways. Moreover, IL-10 signaling in macrophages has been shown to activate the PI3K pathway (27). Indeed, we have shown that colonic p110 expression is attenuated in colitis-prone Il10⁻/⁻ mice, suggesting that IL-10 regulation of IL-12p40 occurs in part via induction of p110 (9). Our current and previous findings (9) demonstrate that bacterial products and IL-10 induce p110 gene expression in macrophages and suggest that transcription regulation of p110 can determine functional immunologic consequences. Most studies have focused on posttranslational...
regulation of p110β (21, 28, 29) as the sole biologic determinant of function. Thus, transcriptional regulation of p110β expression in macrophages may have unexpected biological significance and needs to be further explored.

Aksoy et al. (21) recently demonstrated that p110β signaling in dendritic cells dampens responses to LPS by sequestering TLR4 signaling components and facilitating the switch from Toll–IL-1R domain–containing adaptor protein/MyD88–dependent inflammatory cytokine production (IL-12, IL-6, TNF-α) to Toll–IL-1R domain–containing adaptor inducing IFN-β (TRIF)–related adaptor molecule/TRIF–dependent anti-inflammatory cytokine production (IFN-β, IL-10). It is possible that LPS-induced p110β signaling in macrophages also facilitates the switch to TRIF–related adaptor molecule–TRIF signaling, leading to the enhanced production of IL-10 and IFN-β. Indeed, this agrees with our finding that p110βKD macrophages produce less LPS-induced IL-10. However, we have previously shown that CD11b+ LPMCs from Trif⁻/⁻ mice produce higher levels of basal and bacterially stimulated IL-10 compared with WT mice (30, 31). Conversely, BMDCs from Trif⁻/⁻ mice produce less LPS-induced IL-10 compared with WT mice (32). These findings suggest that the TRIF pathway negatively regulates IL-10 production uniquely in intestinal macrophages. Furthermore, CD11b+ LPMCs produce high levels of IL-10 in GF conditions (30), suggesting that microbial signals are not necessary for driving constitutive expression of IL-10. However, TRIF signaling, perhaps through the recognition of endogenous ligands, remains vital for IL-10 production in intestinal macrophages, as HKEC-stimulated MyD88⁻/- CD11b+ LPMCs do not produce detectable levels of IL-10 (30). Additionally, our data suggest that IFN-β and IL-10 demonstrate differential regulation in CD11b+ LPMCs, in contrast to the recent study demonstrating convergent regulation in LPS–stimulated BMDCs (21). Indeed, Kaiser et al. (33) demonstrated cell type–specific differences in IL-10 and IFN-β production in response to LPS: BMDCs and splenic macrophages did not make detectable amounts of IFN-β but made significant amounts of IL-10 in response to LPS, whereas BMDCs produced both IFN-β and IL-10, suggesting that macrophages use distinct pathways to regulate IFN-β and IL-10. Thus, further studies are necessary to elucidate specific intestinal macrophage signaling pathways required for IL-10 production.

In macrophages, mTOR and GSK-3β are central regulators of IL-12p40 and IL-10 downstream of PI3K. Bacterial products induce MyD88–dependent PI3K activation, leading to phosphorylation of its downstream effector molecule Akt. Akt inactivates tuberous sclerosis complex, a negative regulator of mTOR (34). Both Akt- and PI3K-dependent mTOR activation modulate IL-12p40 and IL-10 production by suppressing GSK-3β activity (22, 35). GSK-3β constitutively represses IL-10 by blocking CREB binding to and activation of the Il10 promoter (36). PI3K- and mTOR–mediated inhibition of GSK-3β thus releases IL-10 from suppression by GSK-3β (36). Interestingly, mTOR activation targets were not altered in LPS–stimulated p110βKD BMDCs compared with WT BMDCs, suggesting that compensatory activation pathways sustain mTOR signaling in p110βKD BMDCs.
Indeed, we have previously shown dysregulation of IL-ways. In this study, we showed that inhibition of GSK-3 thus p110litis in mice (40). GSK-3a therapeutic target in IBDs to induce IL-10 production. Indeed, IL12B (* paired and compared with noninflamed sites (Fig. 6). CD4+ T cells induced greater production of IL-12p40 by APCs, whereas APCs from p110 colitogenic WT CD4+CD45RBhigh cells did not protect proliferative signaling through the B cell and TCRs (8). PI3K development, APC–CD4+ T cell coculture experiments were performed (Fig. 8). mTOR is activated through many pathways, including cellular energy sensing and Wnt signaling (37). Addi-
tionally, the balance of IL-10 and IL-12p40 production, as well as immune-driven colonic inflammation in the absence of adaptive immune cells has been well described (42, 43). Recently, p110 inhibiton has been targeted for the treatment of chronic rejection of tissue transplants, systemic lupus erythematosus, and certain lymphoid cell malignancies (10, 11, 44). Although preliminary clinical results are promising, this finding does not rule out functional defects in p110 T regulatory cells. It is entirely possible, and in fact likely, akin to human IBD pathogen-
esis, that innate and adaptive immune defects interact to drive colitis.

Interestingly, in the absence of T cells, RKO/KD mice developed mild histopathologic colonic inflammation. The development of mild colonic inflammation in RKO/KD mice could be explained by the presence of non–hepaticus Helicobacter species in our mouse colony (data not shown). The ability of H. hepaticus to induce innate immune-driven colonic inflammation in the absence of adaptive immune cells has been well described (42, 43).

Recently, p110 inhibition has been targeted for the treatment of chronic rejection of tissue transplants, systemic lupus erythematosus, and certain lymphoid cell malignancies (10, 11, 44). Although preliminary clinical results are promising, this study highlights a potentially untoward consequence of p110 inhibition—enhanced intestinal and innate inflammatory processes initiated by APCs.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. GF to CNV p110δKD develop histologic colitis.
GF WT (n = 6) and p110δKD (n = 12) mice were transitioned to conventional housing and monitored for colitis by weight and histology. (A) Representative H&E stained colonic tissue from GF to CNV WT and p110δKD mice at 14 days after transfer. (B) Percentage of initial weight of GF to CNV WT and p110δKD mice after transfer. Error bars represent mean ± SEM (NS, not significant).
Supplemental Figure 2. Splenic macrophages and dendritic cells demonstrate altered cytokine production, but inhibition of the Class IA PI3K isoforms p110α and p110β in WT macrophages has no effect on IL-10 and IL-12p40 production. (A-D) Splenic macrophages (CD11b<sup>+</sup>CD11c<sup>-</sup>) and dendritic cells (CD11b<sup>-</sup>CD11c<sup>+</sup>) were stimulated with TLR agonists (LPS, 1 ng/ml; Zymosan A, 10 μg/ml) for 8 hours. Supernatants were collected and assessed for IL-10 (A and B, macrophages and DCs, respectively) and IL-12p40 (C and D, macrophages and DCs, respectively) production by ELISA. Error bars represent mean ± SEM from three independent experiments (NS, not significant; *, p<0.05). (E-H) WT and p110δ<sup>KD</sup> BMDMs were stimulated with LPS (1 ng/ml) 1 hour after exposure to p110α- and p110β-specific inhibitors (PIK-90 and TGX-221, respectively) for 8 hours. Supernatants were collected and assessed for IL-10 (E, F) and IL-12p40 (G, H) by ELISA. Error bars represent mean ± SEM for three independent experiments (NS, not significant; *, p<0.05).
Supplemental Figure 3. LPS stimulated splenic macrophages and DCs modulate IL-10 and IL-12p40 expression in the presence of mTOR and GSK-3β inhibitors. (A-D) One hour after exposure to rapamycin (1 or 10 μM), splenic macrophages (CD11c+/CD11b+) and DCs (CD11c+) were stimulated with LPS (10 ng/ml) for 24 hours. Supernatants were assessed for IL-10 (A and B, macrophage and DCs, respectively) and IL-12p40 (C and D, macrophage and DCs, respectively) production by ELISA. Error bars represent mean ± SEM from three independent experiments (NS, not significant; *, p<0.05). (E-H) One hour after exposure to SB216763 (1 or 10 μM), splenic macrophages (CD11c+/CD11b+) and DCs (CD11c+) were stimulated with LPS (10 ng/ml) for 24 hours. Supernatants were collected and assessed for IL-10 (E and F, macrophage and DCs, respectively) and IL-12p40 (G and H, macrophage and DCs, respectively) production by ELISA. Error bars represent mean ± SEM from three independent experiments (NS, not significant; **, p<0.005).
A

WT APC

40%, MFI=298

p110δKD APC

42%, MFI=243

CD4

CFSE

B

Percent proliferation

NS

WT

p110δKD

C

Percent proliferation

NS

WT

p110δKD

D

Rag1−/−

- T cells

RKO/δKD

+ T cells
Supplemental Figure 4. WT and p110δKD APCs induce similar antigen-specific CD4+ T cell proliferation and repopulate the colonic lamina propria in CD4+CD45RB T cell recipient Rag1−/− and RKO/δKD mice. (A-C) APCs (T cell-depleted splenocytes) from WT and p110δKD mice were activated with LPS (100 ng/ml) and pulsed overnight with OVA peptide (5 μM). After washing extracellular antigen, APCs were co-cultured with CFSE-labeled OT-II (OVA-tg TCR) CD4+CD62L+ T cells for 72 hours. CD4+ T cells were analyzed by flow cytometry. (A) Lymphocytes were gated on forward- and side-scatter and CD4. Figures are representative results from 3 independent experiments. (B) Percentage of proliferating CD4+ T cells was quantified from 3 independent experiments (NS, not significant). (C) Mean fluorescence intensity (MFI) of the CFSE signal was quantified from 3 independent experiments (NS, not significant). (D) (10X, IHC staining for CD3) Histological sections were taken from colons from Rag1−/− and RKO/δKD mice with and without reconstitution with CD4+CD45RB T cells were immunostained for CD3.