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The p110δ subunit of class IA PI3K modulates signaling in innate immune cells. We previously demonstrated that mice harboring a kinase-dead p110δ subunit (p110δKD) develop spontaneous colitis. Macrophages contributed to the Th1/Th17 cytokine bias in p110δKD mice through increased IL-12 and IL-23 expression. In this study, we show that the enteric microbiota is required for colitis development in germfree p110δKD mice. Colonic tissue and macrophages from p110δKD mice produce significantly less IL-10 compared with wild-type mice. p110δKD APCs cocultured with naïve CD4+ T cells also produce significantly less IL-10 and induce more IFN-γ– and IL-17A–producing CD4+ T cells compared with wild-type APCs. Illustrating the importance of APC–T cell interactions in colitis pathogenesis in vivo, Rag1−/−/p110δKD mice develop mild colonic inflammation and produced more colonic IL-12p40 compared with Rag1−/− mice. However, CD4+CD45RBhigh/low T cell Rag1−/−/p110δKD recipient mice develop severe colitis with increased percentages of IFN-γ– and IL-17A–producing lamina propria CD3+CD4+ T cells compared with Rag1−/− recipient mice. Intestinal tissue samples from patients with Crohn’s disease reveal significantly lower expression of PIK3CD compared with intestinal samples from non–inflammatory bowel disease control subjects (p < 0.05). PIK3CD expression inversely correlates with the ratio of IL12B:IL10 expression. In conclusion, the PI3K subunit p110δ controls homeostatic APC–T cell interactions by altering the balance between IL-10 and IL-12/23. Defects in p110δ expression and/or function may underlie the pathogenesis of human inflammatory bowel disease and lead to new therapeutic strategies. The Journal of Immunology, 2014, 192: 3958–3968.

Genetic variants that confer susceptibility to the human inflammatory bowel disease (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 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 intestines, wild-type (WT) mice raised in a germfree (GF) environment markedly upregulated colonic p110δ (Pik3cd) expression when the enteric microbiota were introduced, but colitis-prone Il10−/− mice 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

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; CBL, cecal bacterial lyase; CD, Crohn’s disease; CNN, conventionalized; GF, germfree; GS–3β, glycogen synthase kinase 3β; HKEC, heat-killed Escherichia coli; IBD, inflammatory bowel disease; LPMC, lamina propria mononuclear cell; MLN, mesenteric lymph node; MOI, multiplicity of infection; mTOR, mammalian target of rapamycin; TRIF, Toll–IL-1R domain–containing adaptor inducing IFN-β; WT, wild-type.

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microbiota to drive intestinal inflammation in p110KD mice. Microbial-innate immune interactions, through p110, maintain homeostasis through regulation of both protective (IL-10) and inflammatory (IL-12p40) cytokines. Furthermore, p110 orchestrates innate regulation of adaptive immune responses. Importantly, in human CD, decreased intestinal PI3KCD gene expression is demonstrated that inversely correlates with intestinal IL12B/IL10 ratios. Thus, p110 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 p110 inhibition is a strategy in inflammatory diseases (10, 11), induction of p110 could be a potential therapeutic approach in human IBD.

Materials and Methods

Mice

All mice were maintained on a C57BL/6 background in conventional or GF housing. PI3K p110KD (p110KD) mice were previously obtained from B. Vainheebroock (Queen Mary University of London, London, U.K.). GF p110KD 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-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 Pam3CSK4 were purchased from Invivogen (San Diego, CA). Zymosan A from Saccharomyces cerevisiae was purchased from Sigma-Aldrich (St. Louis, MO). Inhibitors IC87114, rapamycin, and SB-216763 were purchased from Selleck Chemicals (Houston, TX). Cecal bacterial lysates (CBL) from C57BL/6 mice were prepared, as described previously (13). The peptide corresponding to residues 323–339 of OVA was purchased from AnaSpec (Fremont, CA).

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 p110KD 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 lymphoplasmacytoid aggregates confined to the lamina propria; scores of 1 (mild) and 2 (moderate) represented increasing severity and prevalence of the observed change.

Cell culture experiments

BMDMs or splenocytes were cultured at 1 × 10^6/ml in the presence of LPS (1 μg/ml), zymosan A (10 μg/ml), Pam3CSK4 (5 μg/ml), or PBS, and supernatants were harvested after 4 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+ 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 IL12b and IL10 expression by quantitative PCR.

Western blot analysis

BMDMs were cultured at 1 × 10^6/ml with LPS (1 μg/ml), or 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^9) and total GSK-3β with Abs from Cell Signaling Technology (Danvers, MA) and phosphorylated CREB (Ser^133) (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: PI3KCD, forward, 5′-GGGCGGAGCACGATAAGAGTC-3′, and reverse, 5′-GCTGCCCAGGGGCTCTACCT-3′; IL10, forward, 5′-GCTTCAACATGCTTCGGAGATC-3′, and reverse, 5′-TGATGTTGTTGTCGTGGTCCT-3′; IL12b, forward, 5′-GCTCTGTTGAGCGTCGGAGCATC-3′, and reverse, 5′-CTGTAAGTGTGGTGGATTGTTATCCGGG-3′; GAPDH, forward, 5′-GGTGAAGTTGGCTGATCAACGGA-3′, and reverse, 5′-GAGGGATCTCCTGTCCTGGAGA-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.

Flow cytometry

CD4+ 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 eBio17B7; eBioscience) was performed. Flow cytometry samples were run on a CyAn ADF Analyzer (Beckman Coulter, Brea, CA) and analyzed using Summit v4.3 (Beckman Coulter).

CD4+ CD45RBhigh low T cell–adoptive transfer colitis

T cell–mediated colitis was induced in Rag1−/− and Rag1−/−p110KD (p110KD) mice at 8 wk of age as described previously (18). CD4+ T cells were isolated by negative selection (CD8α/220/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+ T cells were sorted into CD45RBhi and CD45RBdim populations using a MoFlo XDP Cell Sorter (Beckman Coulter). Mice were i.p. injected with 4 × 10^6 CD4+CD45RBhi or CD45RBdim cells, as described (18). Clinical scores were assessed, 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 infamed and noninfamed 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 p110\(^{KD}\) mice

To determine whether the microbiota is necessary for the development of colitis, p110\(^{KD}\) mice were derived GF. GF p110\(^{KD}\) mice up to 30 wk of age did not develop histological colitis (Fig. 1A, Supplemental Fig. 1A). Interestingly, GF p110\(^{KD}\) mice produced significantly less colonic IL-10 compared with GF WT mice (Fig. 1B, left). GF p110\(^{KD}\) 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 p110\(^{KD}\) mice demonstrated increased colitis scores (Fig. 1A, Supplemental Fig. 1A). However, GF to CNV p110\(^{KD}\) mice gained weight similarly to GF to CNV WT mice (Supplemental Fig. 1B). Furthermore, colonic explants from day 7 and 14 GF to CNV p110\(^{KD}\) mice produced significantly less IL-10 (Fig. 1B) compared with GF to CNV WT mice. At day 14, GF to CNV p110\(^{KD}\) 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 p110\(^{KD}\) 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-\(\beta\) by p110\(^{8}\) in dendritic cells (21), we measured IFN-\(\beta\) levels in colonic...
explant tissue cultures from GF to CNV WT and p110\textsuperscript{KD} mice. Interestingly, IFN-\(\beta\) production was enhanced in colonic explants from day 7 GF to CNV p110\textsuperscript{KD} mice compared with WT mice (Fig. 1E), in contrast to decreased levels of IL-10 production in p110\textsuperscript{KD} mice. IFN-\(\beta\) production was increased in LPS-stimulated p110\textsuperscript{KD} compared with WT BMDMs (Fig. 1F). Additionally, LPS-stimulated \(\text{II}10^{-/}\) BMDMs produced more IFN-\(\beta\) compared with WT BMDMs (Fig. 1F), consistent with previously reported regulation of IFN-\(\beta\) by IL-10 (21). To assess the direct contribution of IFN-\(\beta\) production from colonic macrophages, CD11b\(^+\) LPMCs, comprising mostly macrophages, were isolated from p110\textsuperscript{KD} mice and stimulated with HKEC. Similar to BMDMs, HKEC-stimulated p110\textsuperscript{KD} CD11b\(^+\) LPMCs produced significantly more IFN-\(\beta\) compared with WT CD11b\(^+\) LPMCs (Fig. 1G), suggesting that p110\textsuperscript{d} differentially regulates IL-10 and IFN-\(\beta\) in macrophages.

PI3K p110\textsuperscript{d} regulates macrophage production of IL-10 in response to pathogen-associated molecular patterns

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

![FIGURE 2.](attachment:image.png)

A. WT and p110\textsuperscript{KD} BMDMs were cultured with LPS (5 ng/ml), Pam\textsubscript{3}CSK\textsubscript{4} (5 ng/ml), or zymosan A (5 \(\mu\)g/ml) for 8 h. Supernatants were collected and assayed for IL-10 and IL-12p40 by ELISA. Error bars represent mean \(\pm\) SEM from three independent experiments (*\(p < 0.05\)).

B. The ratio of IL-12p40 to IL-10 from individual experiments was calculated. Error bars represent mean \(\pm\) SEM from three independent experiments (*\(p < 0.05\), **\(p < 0.005\)).

C. Supernatants from WT and Il10\textsuperscript{2/2} BMDMs were collected after 8 h of culture and assayed for IL-12p40. Error bars represent mean \(\pm\) SEM from three independent experiments (*\(p < 0.05\), **\(p < 0.005\)).

D. WT and p110\textsuperscript{KD} CD11b\(^+\) LPMCs were incubated with a p110\textsuperscript{d}-specific inhibitor (IC87114, 10 \(\mu\)M) 1 h prior to stimulation with HKEC (MOI = 100) for 3 h. Quantitative RT-PCR was performed in duplicate for Il10 (D) and Il12b (E), expression levels normalized to \(\beta\)-actin expression and calculated as fold induction over unstimulated cells. Error bars represent mean \(\pm\) SEM for three independent experiments (*\(p < 0.05\)).
Additionally, p110^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^KD^ BMDMs was consistently increased compared with WT BMDMs (Fig. 2C). LPS- or zymosan A–stimulated CD11b^+ and CD11c^+ splenocytes from p110^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^KD^ inhibition in WT BMDMs markedly enhanced IL-12p40 production above levels produced by p110^KD^ BMDMs. It is possible that there is compensation by other PI3K isoforms in p110^KD^ BMDMs but not in chemically inhibited WT BMDMs. Indeed, we have previously shown that phosphorylation of Akt in LPS-stimulated p110^KD^ BMDMs is not completely abrogated (9). PI3K p110^Ka^ and p110^Kb^ 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^K-specific inhibition of LPS-activated p110^KD^ BMDMs did not alter IL-10 or IL-12p40 expression (Fig. 3A, 3B). However, in p110^Kb^ BMDMs, p110^Kb^ inhibition decreased IL-10 (Supplemental Fig. 2F) production, and p110^Ka^-or p110^K-specific inhibition modestly enhanced LPS-induced IL-12p40 expression (Supplemental Fig. 2G, 2H), suggesting that, in the absence of p110 function, other isoforms may have modest effects on IL-10/IL-12p40 regulation.

Chemical inhibition of p110 in Il10^−/−^ 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^+ colonic LPMC treated with HKEC demonstrated diminished Il10 (Fig. 3D) and enhanced Il12b (Fig. 3E) expression in the presence of the p110-specific inhibitor. As a control, expression of neither cytokine was altered in CD11b^+ colonic LPMC from p110^KD^ mice treated with the p110-specific inhibitor.

**FIGURE 4.** Phosphorylation of p110 targets is altered in macrophages and colons from p110^KD^ mice. (A and B) BMDMs from WT and p110^KD^ mice were treated with LPS (1 μg/ml), and cell lysates were collected for Western blot analysis after the indicated times. (A) Blots were probed for phosphorylated GSK-3β (Ser9) and total GSK-3β. Figure shows representative results from three independent experiments. Quantitative analysis of p-GSK-3β RLU is normalized to total GSK-3β. Error bars represent mean ± SEM from three independent experiments (*p < 0.05 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^KD^ mice were transferred to CNV housing and colonized with a commensal enteric microbiota (GF:WT, n = 2; p110^KD^, n = 2; GF to CNV:7-d WT, n = 1; 7-d p110^KD^, n = 3; 14-d WT, n = 2; 14-d p110^KD^, 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-3β RLU is normalized to total GSK-3β 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 Thx21 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).
compared with WT APCs. WT and p110ΔKD APCs cultured with hIL10−/− CD4+ T cells demonstrate that IL-10 expression is largely derived from APCs (Fig. 6E). As expected, CBL-pulsed p110ΔKD 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 p110ΔKD APCs. OVA-loaded p110ΔKD 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 p110ΔKD APCs (Supplemental Fig. 4A–C). These data suggest that cytokine production by p110ΔKD APCs directs differentiation of Ag-specific Th1 and Th17 CD4+ T cells.

These results suggest a model in which defective p110Δ, through regulation of IL-10 and IL-12p40, leads to inflammatory effector T cell development. To test this model in vivo, we generated
Rag1<sup>-/-</sup>/p110<sub>KD</sub> mice (RKO/δ<sub>KD</sub>). Interestingly, colitis is present but attenuated in the absence of an adaptive immune system (Fig. 7A, 7B). Colonic explant cultures from RKO/δ<sub>KD</sub> mice produced significantly decreased IL-10 (Fig. 7C) and increased IL-12p40 (Fig. 7D) compared with colonic tissue explant cultures from Rag1<sup>-/-</sup> mice.

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

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

Expression of p110<sup>δ</sup> (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 p110<sup>KD</sup> (9). In the present series of experiments, we further elucidate intestinal host-microbial and APC–T cell interactions mediated by p110<sup>δ</sup>. Colitis in p110<sup>KD</sup> 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, p110<sup>KD</sup> mice did not develop intestinal inflammation, whereas, after reconstitution with commensal enteric microbiota, colons from p110<sup>KD</sup> mice demonstrated histological inflammation, impaired IL-10, and increased IL-12p40 production (Fig. 1). Consequently, altered IL-10 and IL-12p40 production by p110<sup>KD</sup> APCs in response to microbial products and cognate interactions with T cells orchestrates pathogenic adaptive immune responses contributing to intestinal inflammation.

Class I<sub>1</sub> 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<sup>δ</sup> 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<sup>δ</sup> expression is attenuated in colitis-prone Il10<sup>-/-</sup> mice, suggesting that IL-10 regulation of IL-12p40 occurs in part via induction of p110<sup>δ</sup> (9). Our current and previous findings (9) demonstrate that bacterial products and IL-10 induce p110<sup>δ</sup> gene expression in macrophages and suggest that transcription regulation of p110<sup>δ</sup> can determine functional immunologic consequences. Most studies have focused on posttranslational...
CD11b+ LPMCs produce high levels of IL-10 in GF conditions and produce less LPS-induced IL-10 compared with WT mice (32). Bars represent mean ± SEM (*p < 0.05). (A) Clinical disease activity scores were determined, as described in Materials and Methods. Error bars represent mean ± SEM (*p < 0.05). (B) (original magnification ×20, H&E) Colons from Rag1<sup>–/–</sup> and RKO/ΔKD recipient mice were taken for histological evaluation. Representative sections are shown. (C) H&E slides of colonic tissue were scored for colitis severity by a pathologist (L.B.B.) blinded to the experimental groups, as described in Materials and Methods. Error bars represent mean ± SEM (***p < 0.005). (D–F) Supernatants from 24-h colonic tissue explants were collected and assayed for IL-10 (D) and IL-12p40 (E) production by ELISA and are expressed as the amount of cytokine (pg/ml) per 50 mg colonic tissue weight. Error bars represent mean ± SEM (***p < 0.005). (F) IL-12p40 and IL-10 protein levels from colonic tissue explant culture in individual mice were used to determine the ratio of IL-12p40 to IL-10. Error bars represent mean ± SEM (***p < 0.005). (G and H) MLNs from Rag1<sup>–/–</sup> and RKO/ΔKD recipient mice were analyzed by flow cytometry for intracellular IFN-γ (G) and IL-17A (H) expression in CD3<sup>+</sup>CD4<sup>+</sup> T cells. Each point on the graphs represents MLN cells from one mouse. Error bars represent mean ± SEM (**p < 0.01, ***p < 0.005). (I and J) LPMCs were analyzed by flow cytometry for IFN-γ (I) and IL-17A (J) expression in CD3<sup>+</sup>CD4<sup>+</sup> T cells. Each point on the graphs represents pooled LPMCs from three mice. Error bars represent mean ± SEM (p < 0.05).

### Regulation of PI3K p110β

MyD88<sup>+/–</sup> CD11b<sup>+</sup> 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<sup>+</sup> 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 p110<sup>βKD</sup> BMDCs compared with WT BMDCs, suggesting that compensatory activation pathways sustain mTOR signaling in p110<sup>βKD</sup> BMDCs.
In this study, we showed that inhibition of GSK-3 (9). Thus, p110
in colons from GF to CNV p110
expression from patients with CD was assessed at inflamed sites (Fig. 6). CD4+ T cells induced greater production of IL-12p40 by

dysregulation of IL-10 and IL-12p40 production, as well as

FIGURE 9. Human intestinal PIK3CD expression is decreased in patients with CD and inversely correlates with IL12B:IL10 ratios. Macroscopically inflamed and noninflamed colonic or ileal tissue was obtained from patients with CD (n = 14) or non-IBD control patients (n = 20) undergoing surgical resection. (A) Total RNA from samples was assessed for PIK3CD expression by quantitative RT-PCR in duplicate normalized to GAPDH expression. Error bars represent mean ± SEM (**p < 0.05). (B) Intestinal PIK3CD expression normalized to GAPDH expression from patients with CD was assessed at inflamed sites (n = 14) and compared with noninflamed sites (n = 14) from the same patient. Lines connect samples from individual patients. Data were analyzed using a paired t test (***p < 0.005). (C) Total RNA from samples was assessed for IL12B and IL10 expression in duplicate normalized to GAPDH expression. IL12B:IL10 ratios in patients were correlated with PIK3CD expression (r² = 0.2363; p = 0.014).

(21). Indeed, mTOR is activated through many pathways, including cellular energy sensing and Wnt signaling (37). Additionally, the balance of IL-10 and IL-12p40 production, as well as other cytokines, is regulated by MAPK signaling downstream of TLR signaling (38). We previously reported that TLR-activated p110KD macrophages demonstrate impaired Akt phosphorylation and enhanced phosphorylation of p38 and JNK MAPK, but not ERK1/2, compared with WT macrophages (9). In this study, we show impaired phosphorylation of the downstream mediators GSK-3β and CREB in TLR-stimulated p110KD macrophages and in colons from GF to CNV p110KD mice. Furthermore, other cytokines such as TNF-α are affected by p110β, which regulates tubule fusion in TNF-α-containing vesicles bound for secretion (39). Indeed, we have previously shown dysregulation of IL-12p70, IL-23, and NO in TLR-activated p110KD macrophages (9). Thus, p110β may regulate cytokine secretion in multiple ways. In this study, we showed that inhibition of GSK-3β rescues LPS-induced IL-10 production in p110KD BMMDS (Fig. 5E, 5G). Our results therefore suggest that GSK-3β activity may be a therapeutic target in IBDs to induce IL-10 production. Indeed, GSK-3β inhibition has previously been shown to ameliorate colitis in mice (40).

PI3K p110KD B and T lymphocytes demonstrate impaired proliferative signaling through the B cell and TCRs (8). PI3K p110KD mice demonstrate impaired intrinsic T regulatory cell function, and p110KD CD4+CD45RBlow cells cotransferred with colitogenic WT CD4+CD45RBhigh cells did not protect Rag1−/− mice from T cell–mediated colitis (25). To determine how APC p110KD influences T cell subset differentiation and colitis development, APC–CD4+ T cell coculture experiments were performed (Fig. 6). CD4+ T cells induced greater production of IL-12p40 by APCs, whereas APCs from p110KD mice induced more Ag-specific IFN-γ– and IL-17A–producing T cells. Because only WT CD4+ T cells were used in coculture with APCs from both WT and p110KD mice, T cell phenotype can be attributed to the defect in p110KD APCs. As an in vivo correlate, Rag1−/− and RKO/RKO mice were reconstituted with admixed WT CD4+CD45RBhigh and CD45RBlow T cells (Fig. 8). Compared with respective recipient Rag1−/− mice, RKO/RKO recipient mice demonstrated significantly increased clinical and histology scores. More IFN-γ–producing T cells were isolated from MLNs and colonic lamina propria of RKO/RKO recipient mice compared with recipient Rag1−/− mice. IL-17–producing CD4+ T cells are rarely found in MLNs and other secondary lymphoid tissues but are found in abundant quantities at mucosal surfaces (41). Indeed, RKO/RKO recipient mice contained significantly higher percentages of IL-17A–producing T cells in the colonic lamina propria compared with the respective Rag1−/− recipient mice. We previously showed that bacterially stimulated p110KD macrophages produce significantly more IL-23, a cytokine necessary for the differentiation and maintenance of Th17 cells (9).

Although the development of pathogenic CD4+ T cells in CD45RB recipient mice was explored, we did not study T regulatory cell differentiation and function. During GF to CNV transition, colons from p110KD mice demonstrated significantly increased transcription of Foxp3, correlating with increased inflammation, compared with colons from WT mice (data not shown). However, this finding does not rule out functional defects in p110KD T regulatory cells. It is entirely possible, and in fact likely, akin to human IBD pathogenesis, that innate and adaptive immune defects interact to drive colitis.

Interestingly, in the absence of T cells, RKO/Δβ2 mice developed mild histopathologic colonic inflammation. The development of mild colonic inflammation in RKO/Δβ2 mice could be explained by the presence of non–hepatitis 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, p110δ 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 p110δ inhibition—enhanced intestinal and innate inflammatory processes initiated by APCs.

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Disclosures
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References


