Conditional knockout of polarity complex (atypical) PKCζ reveals an anti-inflammatory function mediated by NF-κB

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ABSTRACT The conserved proteins of the polarity complex made up of atypical PKC (aPKC, isoforms ζ and η), Par6, and Par3 determine asymmetry in several cell types, from Caenorhabditis elegans oocytes to vertebrate epithelia and neurons. We previously showed that aPKC is down-regulated in intestinal epithelia under inflammatory stimulation. Further, expression of constitutively active PKCζ decreases NF-κB activity in an epithelial cell line, the opposite of the effect reported in other cells. Here we tested the hypothesis that aPKC has a dual function in epithelia, inhibiting the NF-κB pathway in addition to having a role in apicobasal polarity. We achieved full aPKC down-regulation in small intestine villi and colon surface epithelium using a conditional epithelium-specific knockout mouse. The results show that aPKC is dispensable for polarity after cell differentiation, except for known targets, including ROCK and ezrin, claudin-4 expression, and barrier permeability. The aPKC defect resulted in increased NF-κB activity, which could be rescued by IKK and ROCK inhibitors. It also increased expression of proinflammatory cytokines. In contrast, expression of anti-inflammatory IL-10 decreased. We conclude that epithelial aPKC acts upstream of multiple mechanisms that participate in the inflammatory response in the intestine, including, but not restricted to, NF-κB.

INTRODUCTION
Partition-deficient (PAR) mutant genes encoding PAR proteins and PCK-3 (the orthologue atypical PKC [aPKC]) were identified in Caenorhabditis elegans as essential components of cell polarity mechanisms (Guo and Kemphues, 1996). These proteins are highly conserved in metazoan evolution and participate in polarization of various cell types, including epithelial apicobasal polarity (Suzuki and Ohno, 2006; Pieczynski and Margolis, 2011; Chen and Zhang, 2013). Typically, the aPKC-Par6-Par3 polarity complex is highly conserved and essential for epithelial cell differentiation and polarized structure. The conserved proteins of the polarity complex made up of atypical PKC (aPKC, isoforms ζ and η), Par6, and Par3 determine asymmetry in several cell types, from C. elegans oocytes to vertebrate epithelia and neurons. The PKCζ (intestine) cells (Suzuki et al., 2007). However, most of the evidence supporting a role of aPKC in epithelial apicobasal polarity in vertebrates was obtained in polarized tissue culture epithelial cell lines, such as Madin–Darby canine kidney (MDCK) and Caco-2 (intestine) cells (Suzuki et al., 2004; Liu et al., 2007; Zihni et al., 2014). Global mouse knockout models for both aPKC isoforms (PKCζ and η) were not informative regarding the role of aPKC in polarity in vivo. The PKCζ knockout model displays a mild phenotype with defective immune response and compromised NF-κB activation in B-lymphocytes (Martin et al., 2002).

Two other groups independently reported the use of conditional PKCζ-deficient mice for studies of epithelia (Imai et al., 2006; Calcagno et al., 2011). In one case, defects of adherens junctions in neuroepithelial cells were found (Imai et al., 2006), whereas in the other, the status of apicobasal polarity was not reported. Calcagno et al. (2011), however, reported an intriguing hypersensitivity of PKCζ-knockout mice to chemical colitis, compatible with an anti-inflammatory role of this kinase, but evidence for a specific role in modulating the NF-κB pathway was lacking. In contrast, the role of aPKC in other cell types has been extensively studied, especially in relationship to its role in glucose metabolism (Farese and Sajan, 2010).
Our interest in the possible role of aPKC in inflammatory pathways was sparked when we observed that aPKC is deeply down-regulated in intestinal epithelia under proinflammatory stimuli in Caco-2 cells, a mouse model of colitis (Mashukova et al., 2011), and samples from human inflammatory bowel disease (IBD) patients (Wald et al., 2011). We demonstrated that aPKC down-regulation is posttranslational, due to destabilization, and under the control of NF-κB activation (Mashukova et al., 2011). It was natural to ask what consequences, if any, result from loss of aPKC in inflammation. Overexpressing wild-type and constitutively active PKCι in Caco-2 cells, we found inhibition of basal NF-κB activity. Furthermore, there was no detectable effect on apicobasal polarity by a constitutively active mutant defective in the PB1 domain and, thus, depolarized. These results were intriguing because they are not consistent with prevalent views of aPKC activating NF-κB. We hypothesized that different effects of aPKC on NF-κB might be tissue specific or perhaps due to the transformed status of Caco-2 cells. This work was undertaken to test the hypothesis that PKCι down-regulation affects both apical polarity and inflammation in vivo. To that end, we used a PKCιflox/flox mouse developed in our facility to achieve a conditional knockout in intestinal epithelia. The results showed a modest role of aPKC in the maintenance of apical polarity and a critical control of NF-κB activation.

RESULTS
Effects of the conditional PKCι knockout
Exon 4 of the gene encoding PKCι (Prkci) (Figure 1A) was floxed by breeding Prkci(flox/flox) animals with transgenic mice expressing CRE under a 12.4-kb villin promoter. In these animals, CRE is expressed in the intestinal epithelium (Madison et al., 2002), active in all lineages, and possibly expressed in stem cells (Koo et al., 2009). To analyze the phenotype, we performed studies with an antibody that recognizes the phosphorylated turn domain in both aPKC isoforms, ι and ζ (T555 and T560, respectively). Because the turn domain is auto-phosphorylated, it reports that the kinase is in the active conformation. Therefore this strategy enabled us to identify cells lacking the ι isoform. The characteristic signal of aPKC in the tight junction region in villus enterocytes (Figure 1B, arrows) and colonocytes (Figure 1E, arrows) was missing in the Prkci(flox/flox) Vil-CRE+ (hereafter referred to as KO). However, we found aPKC signal in the intestinal glands (“crypts”;

FIGURE 1: Effect of conditional Prkci(flox/flox) Vil-CRE+ (KO) on aPKC protein expression. (A) Structure of the targeted flox/flox insertion in the Prkci locus after homologous recombination. Numbers in gray represent Prkci exons. Boxes represent the selection genes. Triangles represent the targets for Flp (FRP) and CRE (loxP) recombinases. Arrows represent orientation of genotyping primers. (B) Villus enterocytes or (C) crypts in small intestine frozen sections were stained with anti–phosphorylated aPKC turn domain antibody, which recognizes the active conformation of PKCι/λ and ζ. (D) Crypts in the small intestine mucosa stained with an isoform-specific antibody against PKCζ that does not recognize the ι isoform. (E) Large intestine mucosa sections were processed with anti–p–PKCι/λ antibody as in B and C. Arrows point at aPKC signal in the apical domain. Bars, 10 μm (B), 25 μm (C, E), 20 μm (D).
no changes in the number or localization of Paneth cells (Figure 2B, arrows), suggesting that the loss of PKCζ does not affect lineage determination, at least in the presence of compensatory PKCζ. The intestinal barrier permeability, measured by diffusion of 3-kDa fluorescent dextran into the blood, showed a significant threefold increase in the KO animals (Figure 2C). Such an increase was abolished by prior treatment with oral sulfasalazine (SS), an inhibitor of NF-κB, which interferes with IKKα and β (Wahl et al., 1998; Weber et al., 2000). Sulfasalazine is approved by the U.S. Food and Drug Administration for use in IBD patients (Nielsen and Munck, 2007) and is also safe for use in rodents. These results led us to ask about the extent to which, if any, the total loss of aPKC in villus enterocytes would affect apicobasal polarity or junction structure.

**aPKC is dispensable for maintenance of epithelial polarity in differentiated enterocytes but necessary for correct localization of direct targets**

The question of how loss of aPKC affects apicobasal polarity is relevant because these kinases are deeply down-regulated in intestinal inflammation (Wald et al., 2011). We tested polarized proteins and tight junction (TJ) markers. Several markers remained well polarized in the villus enterocytes despite the lack of aPKC. Brush border actin, NHE3, and occludin (Figure 3), as well as alkaline phosphatase, Par3, and ZO-1 (Figure 4A), were normally localized in the KO. An increase in the basolateral F-actin signal was noted in the KO villus enterocytes (Figure 3, arrow). The ultrastructure of brush borders (Figure 4, B and C) and apical junctional complexes (Figure 4, D and E) was also indistinguishable (Figure 4, D and E). Claudin-4, which is not confined to TJ.s in intestinal epithelia and is expressed almost exclusively in villi (Rahner et al., 2001), was deeply down-regulated in the KO (Figure 3). Finally, as expected, the homologue of *Drosophila* lethal giant larvae, LLGL2, a known aPKC target (Kjaer et al., 2013), changed its distribution from diffuse
cytoplasmic signal in control enterocytes to cortical, including the apical pole in KO (Figure 3).

Both total ezrin and p-T567 ezrin signals were significantly decreased in the KO villus enterocytes, with the exception of positive patches (Figure 4A). However, ezrin was normally localized to the apical domain in the crypts (Figure 4A, inset). This is consistent with our finding that PKCι phosphorylates ezrin in cultured intestinal Caco-2 cells (Wald et al., 2008). Because this result seems to contradict the normal ultrastructure of brush borders in KO enterocytes (Figure 4C), we further quantified apical and cytoplasmic ezrin signals in confocal images. There was a 15-fold decrease in average pixel intensity for the brush border (Figure 5, A and B, black bars; note the difference in the pixel-value scales) in the KO enterocytes. However, despite the fact that it is difficult to see when the images are acquired at the same gain, there was a significant remnant of apical ezrin, well above the background level in the KO (Figure 5B). We conclude that a substantial fraction of T567 ezrin phosphorylation depends on aPKC activity.

With regard to basolateral markers, except for LLGL2 (Figure 3), we did not find any mistargeting to the apical domain. Na+-K’ATPase images showed variability (Figure 6A) but no apical localization. We also analyzed another basolateral marker, E-cadherin (Figure 6B), and reached a similar conclusion. Finally, we determined pixel values in the basolateral domain for both markers (after background subtraction; Figure 6C). The results indicate that there were no significant differences in the E-cadherin signal and a small but significant increase in Na+-K’ATPase signal in the KO mice. In summary, the phenotype of basolateral markers in Prkci<sup>flox/flox</sup> Vil-CRE+ villus enterocytes is complex. There are differences in the steady-state levels of expression of different markers: claudin-4 decreases considerably, E-cadherin does not change, and Na+-K’ATPase slightly increases in the KO. Therefore it is difficult to conceive that a single mechanism can account for all these observations. In addition, considering the normal images of the junctions (Figure 4, B–E), we conclude that there was no global disruption of the lateral domain in the KO enterocytes, although there were changes in the expression of some specific basolateral proteins.

**aPKC is an inhibitor of NF-κB activity in vivo**

Because PKCι overexpression inhibits NF-κB activity in the Caco-2 (intestinal epithelia) cell line (Forteza et al., 2013), we hypothesized that the conditional PKCι KO would display intestinal NF-κB activation and, possibly, inflammation. Up to ~30% of the animals developed anal prolapse after 5 mo of age, which suggested the existence of a mild colitis. A role of PKCι in colitis has been shown (Calcagno et al., 2011). Therefore we wanted to determine whether PKCι-dependent pathways inhibit NF-κB in the absence of or with minimal external proinflammatory stimuli. Accordingly, we focused our attention on the small intestine, where there are small microbial loads and fewer infiltrating myeloid cells than in the colon and local inflammation levels are generally low. Studies in the large intestine and ileum are more common and clinically relevant due to the localization of lesions in IBD. However, the complexity of the interactions among microbiota, epithelia, and immune cells in the colon obscures mechanistic conclusions. Specifically, we analyzed villus enterocytes in which the aPKC defect was complete (Figure 1).

To test the hypothesis, we used various independent criteria of NF-κB activation in conditional PKCι-knockout mouse enterocytes. First, we examined NF-κB nuclear translocation. In control (Im)
duodenum villus enterocytes, relA(p65) signal was found excluded from the nuclei. Conversely, Prkci<sup>fl</sup>/<sup>fl</sup> Vil-CRE<sup>+</sup> (KO) enterocytes showed nuclear relA (Figure 7A). The average ratio of nuclear/cytoplasmic relA signal doubled in KO enterocytes, and the difference was significant (Figure 7B). Second, we semiquantitatively measured pSer536 relA signal in epithelial and lamina propria connective tissue (examples of regions of interest [ROIs] in Figure 7C, yellow squares). The overall epithelial signal was lower than the lamina propria signal in control cells (Im) but showed similar intensities in KO (Figure 7, C and D). In other words, the levels of phospho-relA signal in the lamina propria were indistinguishable between control and KO, but the levels in epithelial cells significantly increased threefold in the KO enterocytes. As a negative control, we treated the animals with SS and observed minimal levels of phospho-relA signal (Figure 7, C and D).

Atypical PKC phosphorylates Rho-associated protein kinase (ROCK) in S1333, T1334, T1337, and S1341. These residues comprise the ROCK Rho-PH binding domain. Phosphorylation renders ROCK inactive and detached from the membrane (Ishiuchi and Takeichi, 2011). In turn, it was shown that ROCK activates IKK (Guo et al., 2012; Kim et al., 2014). To test the hypothesis that aPKC acts on NF-κB activation pathway through ROCK, we determined the subcellular distribution of ROCK1/2. As expected from evidence in MDCK cells (Ishiuchi and Takeichi, 2011), ROCK cortical and supranuclear distribution increased dramatically in KO enterocytes, but no changes were noted in lamina propria cells (Figure 7E). It is of note that the increase in ROCK cortical localization was more prominent in the apical pole but was also observed in the basolateral domain (Figure 7E). Consistent with this result, the ROCK inhibitor Y27632 significantly rescued >50% of the increase in phospho-Ser536 relA in the KO enterocytes, with little effect on the same signal in lamina propria cells (Figure 7, C and D). These results suggest that ROCK1/2 may be involved in NF-κB activation in aPKC-deficient enterocytes. We speculate that the widespread subcellular distribution of potentially active ROCK may multiply the effect of highly localized aPKC.

The third criterion to assess NF-κB activity was nonmuscle myosin II light chain phosphorylation (pMLC). NF-κB increases myosin light chain kinase (MLCK) transcription (He et al., 2012), which phosphorylates myosin II regulatory light chain (MLC), enabling assembly of functional nonmuscle

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**FIGURE 4:** Apical and junction markers and intestinal epithelium ultrastructure in Prkci<sup>fl</sup>/<sup>fl</sup> Vil-CRE<sup>+</sup> (KO) mice. (A) Frozen sections of villus enterocytes from KO and control littermates (Im) were processes with the following antibodies: alkaline phosphatase (iAP), active (pT567) ezrin, ezrin, ZO-1, and human homologue of Par3. Ezrin inset, cross sections of the crypts displaying normal apical ezrin levels. Bars, 10 μm (iAP, pT567ezrin), 45 μm (ezrin), 25 μm (ZO-1, Par3). (B–E) Electron microscopy images of villus enterocytes from control (B, D) or KO (C, E) animals. Bars, 10 μm (B, C), 0.5 μm (D, E).
myosin II (Graham et al., 2006). Phosphorylation of MLC by MLCK in enterocytes is a hallmark of intestinal inflammation and inflammation-associated carcinogenesis (Suzuki et al., 2014). pMLC signal was consistently higher in all KO animals studied (Figure 8). Treatment of KO animals with SS abrogated pMLC signal increase. These results independently suggest NF-κB activation in Prkct<sup>flx/flx</sup> Vil-CRE<sup>+</sup> enterocytes. It is important to note that activation of myosin II is considered a major cause of TJ opening and barrier permeabilization (Al-Sadi et al., 2010), which is consistent with the results in Figure 2C.

Finally, to assess directly NF-κB transcriptional activity, we measured CXCL-1 (GRO-1) and CXCL-2 (GRO-2) mRNAs, broadly accepted as NF-κB target genes (El-Guendy and Sinai, 2008; Patterson et al., 2013). Reverse transcription-PCR (RT-PCR) was performed on RNA extracts from purified enterocytes. Both cytokines were found to be significantly increased (ninefold and fivefold, respectively) in KO enterocytes. However, KO animals treated with SS or Y27628 did not show increased CXCL-1 mRNA levels (Figure 9). These data provide additional evidence for NF-κB activation in the absence of PKCζ, likely through the canonical IKK pathway via ROCK.

Two other cytokines have been found to be essential in the development of intestinal inflammation: interleukin 1α (IL-1α) and IL-10. The former is proinflammatory, and the latter is anti-inflammatory (Kole and Maloy, 2014; Scarpa et al., 2015). These cytokines have been commonly studied in myeloid cells but are known to be secreted by the intestinal epithelium as well (Bersudsky et al., 2014; Olszak et al., 2014; Scarpa et al., 2015). IL-1α mRNA was significantly increased in the KO. Conversely, IL-10 in the KO was found to be ~25% the levels of the control animals, a significant reduction (Figure 9). It is worth highlighting that these two cytokines are not known to be directly under NF-κB transcriptional control. The possibility that these results may be due to a contamination with cells of mesenchymal lineage, including myeloid cells, was controlled by measuring mRNAs that are not expressed in epithelial cells. Spleen parenchymal cells (S) were used as a positive control of mesenchymal/myeloid lineage cells and were compared with purified enterocytes (E) from the same animal (Figure 9). Two mRNAs not expressed in epithelial cells were used. Vimentin mRNA showed a 200-fold enrichment of epithelial over mesenchymal RNAs in our preparations. IL-1β mRNA is transcribed at lower levels than cytoskeletal proteins mRNAs. By in situ hybridization, it has been shown that IL-1β mRNA is expressed in lamina propria but not in epithelial cells in the intestine (Woywodt et al., 1999). Because IL-1β expression was 400-fold higher in a spleen cells compared with enterocytes, we further concluded that the contamination of our purified enterocyte mRNA with RNAs from other cells was minimal. These data support the conclusion that basal (unstimulated) cytokine expression in Prkct<sup>flx/flx</sup> Vil-CRE<sup>+</sup> enterocytes is proinflammatory via NF-κB and possibly other, unidentified pathways as well.

DISCUSSION

This work focused on innate immunity activity in epithelial cells under minimal exogenous proinflammatory signaling: The goal was to identify epithelium-specific functions of polarity complex aPKC. The evidence leads to two conclusions. First, aPKC is dispensable for the maintenance of apicobasal polarity in the short term, that is, the 2-d lifespan of postmitotic villus enterocytes. Second, aPKC is indeed a negative regulator of NF-κB activity in enterocytes. The latter is a tissue-specific effect of aPKC and indicates that results from other cell types, in which aPKC activates NF-κB, cannot be generalized to epithelia. Furthermore, although there are commonalities between epithelia in the small and large intestine, the conclusions from this work should not be generalized to colon epithelium.

Apical polarity persists in PKCζ KO enterocytes after PKCζ<sup>−/−</sup>KO is spontaneously down-regulated at the crypt/villus boundary for the short lifespan of the villus enterocytes. The Prkct<sup>flx/flx</sup> Vil-CRE<sup>+</sup> animals showed normal weight gains, in stark contrast with the phenotype of mutations disturbing apical membrane traffic, such as Rab8 (Sato et al., 2007) and Myo5b (Carton-Garcia et al., 2015) KO models. Even defects in single apical transporters, such as the GLUT8 transporter, have been shown to affect body weight (DeBosch et al., 2012). Overall, therefore, nutrient absorption in PKCζ KO animals must be normal. On the other hand, changes in the distribution of direct aPKC targets such as LLGL2 and ROCK were observed, confirming the loss of aPKC activity.

The loss of ezrin phosphorylation in the PKCζ KO enterocytes supports our previous observation in Caco-2 cells that PKCζ phosphorylates ezrin (Wald et al., 2008). The rapid turnover of the ezrin activation phosphosite (T567; Viswanatha et al., 2012) is consistent with dephosphorylation immediately after PKCζ<sup>−/−</sup>KO is down-regulated in the PKCζ KO enterocytes. This issue has been controversial: Mst4 was also shown to phosphorylate ezrin in intestinal epithelia (ten Klooster et al., 2012). Both splice variants of LBK1 are activated by aPKC (Zhu et al., 2013). Therefore the results shown here demonstrate that PKCζ is key for ezrin phosphorylation, either directly or indirectly via the LKB1-Mst4 pathway, and are consistent with observations in other systems (Liu et al., 2013).

It may be counterintuitive that KO enterocytes still show a normal brush border (Figure 4C). In ezrin-knockout mice, enterocytes lacking ezrin throughout their differentiation still show microvilli, albeit ones that are shorter and more disorganized (Saotome et al., 2004). PKCζ KO enterocytes, on the other hand, show a 15-fold decrease in apical ezrin but still display a sizable apical accumulation of ezrin. Considering that the ezrin decrease is short-lived due to the limited lifespan of the cells, it is not surprising that the brush border structure is not affected. In summary, the data suggest that persistence of a polarized phenotype may be more related to the temporal stability.
modulate claudin-2 as well (Lu et al., 2015). Finally, there is extensive evidence that ROCK activation mediates opening of TJs (Ivanov et al., 2009; Clark et al., 2013) by inhibiting MLC dephosphorylation (Kaneko-Kawano et al., 2012). The results from the PKC ι KO mouse presented here strongly suggest that, in addition to “local” regulation of nonmuscle myosin II via ROCK at the junctional level, aPKC exerts control over the canonical NF-κB activation pathway and, accordingly, is involved in transcriptional control. All of these non–mutually exclusive mechanisms can explain the increase in TJ permeability.

Of importance, although a threefold increase in solute permeability seems modest, it mimics the levels of increased permeability in IBD patients in remission or in asymptomatic relatives (Buhner et al., 2006), including the barrier permeability in the small intestine (Buning et al., 2012). Accordingly, we speculate that the conditional PKC ι-null mouse model might be clinically relevant to mimic increased barrier permeability close to the threshold that triggers colitis.

**aPKC defect increases NF-κB activation**

The evidence presented here strongly supports activation of NF-κB in epithelial cells within an environment with low levels of inflammatory stimuli as compared with the colon. Bearing in mind that in other cell types, aPKC activates NF-κB (Diaz-Meco and Moscat, 2012), the identity of the pathway(s) connecting a highly localized aPKC to inhibition of NF-κB activation is important. Two pieces of evidence suggest that the effect must be exerted on the canonical NF-κB activation pathway. First, expression of constitutively active PKCι results in IkB accumulation (Forteza et al., 2013). Second, PKCι deficiency increased pSer 536 relA signal (Figure 7, C and D), an effect rescued by two different small molecules (SS and Y27632), which both inhibit IKKs directly or indirectly via ROCK. On the other hand, the transcriptional effects of an aPKC defect on IL-1α and IL-10 suggest that additional pathways might be involved. Evidence supports the possibility that aPKC might signal through STAT3 (Guyer and Macara, 2015), which in turn activates IL-10. Moreover, aPKC also signals through CREB-binding protein (CBP) by direct phosphorylation in S436 (He et al., 2009). In turn, CBP is involved in the control of NF-κB and IL-10 transcription (Alvarez et al., 2009; Mukherjee et al., 2013). Dissecting all of these possible mechanisms is beyond the scope of this work.

Evidence from other laboratories has been contradictory regarding the effects of the Rho pathway in NF-κB activation. However, there is support for the notion that RhoA potentiates NF-κB activation (Perez-Moreno et al., 2006; Bist et al., 2015). On the basis of the poor effect of Y27632 on phospho-relA in lamina propria cells, we speculate that this pathway of aPKC inhibition of ROCK, which in turn activates IKK (Anwar et al., 2004; Kim et al., 2014), might...
FIGURE 7: Activation of NF-κB in of Prkci^flx/flx Vil-CRE± (KO) villus enterocytes. (A) Frozen sections of villus enterocytes from untreated KO or littermate control (Prkci^flx/flx Vil-CRE- or Prkci^flx/− Vil-CRE+; lm) animals processed with anti-relA(p65) (red channel) and DAPI (blue channel). (B) Quantification of the data in A. Average pixel values were measured in ROIs in the cytoplasm or the nucleus. Several cells were measured in four to seven random sections. Each dot represents the average of all of the measured cells from one animal. Horizontal bars show averages of each group. *Kruskal–Wallis test, H₀ = control and KO populations are equal, p < 0.003. (C) Frozen sections of intestinal villi were processed with anti-pSer536 relA (red channel) antibody. Animals were untreated controls (lm) or KO, as well as KO mice treated with ROCK inhibitor Y27628 or SS. Yellow squares show examples of randomly located ROIs in epithelia or lamina propria cells used for measurements in D. (D) Quantification of pixel values in images like those in C using ROIs in epithelial cells (black bars) or in lamina propria cells (gray bars) for control littermate (lm) or KO animals or KO animals treated with Y27628 or SS. Data shown as average and SD. *t test for difference between average in untreated KO epithelial cells (n = 22) and corresponding controls (n = 28) or between untreated KO epithelial cells and KO treated with Y27628 (n = 29) or between untreated lamina propria cells in KO compared with SS-treated cells, p < 0.001. (E) The sections were processed with anti-ROCK1/2 antibody. Bars, 10 μm.
provide clues for the tissue specificity of the effect of PKCι on NF-κB. Additional studies are necessary to further test this hypothesis.

Recently Guyer and Macara (2015) confirmed our original observation that Par3 is also an inhibitor of NF-κB (Forteza et al., 2013) in carcinoma cells. The data presented here, however, challenge the notion that loss of Par3 activates NF-κB through the activation of aPKC. The results in this work, conversely, support our interpretation that the anti-NF-κB activity of Par3 is exerted independently of aPKC (Forteza et al., 2013). It is possible that the differences arise from the use of different cell types in culture in the cited work and the analysis in vivo in this work.

In summary, PKCι expression in differentiated enterocytes is critical to control transcription factors that mediate effects on MLC and barrier permeability (NF-κB), proinflammatory cytokine transcription inhibition (CXCL-1 and -2, IL-1α), and anti-inflammatory cytokine expression (IL-10, possibly through STAT3 or CBP). All of these mechanisms seem to be additional to apicobasal polarity functions and sufficient to predispose the intestinal mucosa to inflammation. It seems reasonable to speculate that this function might be general to polarized epithelia under conditions of low inflammatory stimulation. Whether this mechanism interacts with, for example, activated TLR4 or TNFR pathways remains to be determined. However, the downregulation of aPKC during intestinal inflammation is likely to contribute to the intensity or persistence of the inflammatory response. The data presented here add to a growing body of evidence (Bersudsky et al., 2014; Olszak et al., 2014; Scarpa et al., 2015) that intestinal epithelial cells are important regulators of inflammation, not only as a passive barrier, but also through the secretion of cytokines.

MATERIALS AND METHODS

Animal model

All studies in animals were conducted according to guidelines in the Public Health Service Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the local Institutional Animal Care and Use Committee. Embryonic stem cells carrying the recombinant insertions in the Prkci gene shown in Figure 1A were obtained from the International Mouse Knockout Consortium (www.mousephenotype.org/; Project 71671). The cells were selected, cloned, and injected into mouse blastocysts in the C57BL/6 genomic background. For genotyping, we used the following primers (Figure 1A, GenBank accession JN952602.1):

1. 14729: CAGGCCCTCTGCAAGATATCTTC
2. 21131: GGCTGCATACGCTTGATCCG
3. 22182: CCAACAAAAAGTCCCTCCCCT

Genotyping PCR was performed as follows: primers 2 and 3 were used for germline transmission, amplimer 1051 base pairs; primers 1–3 yielded a 350–base pair amplimer in WT and a 550–base pair amplimer in the flox insertion. After germline transmission was verified, the animals were bred with B6(C3)-Tg(Pgk1-FLPo)10Sykr/J mice (Jackson Laboratory) to remove the FRT insert. After removal of the Neo cassette, animals carrying the flox insertions flanking exon 4 were backcrossed with WT C57BL/6 mice for five generations. Finally, these animals were further bred with B6. Cg-Tg(Vil-cre)997Gum/J (Jackson Laboratory). Prkcιfloxflox/Vil-CRE+ animals. Expression of PKCι was verified independently by immunofluorescence in each animal.

Reagents

Reagents used were as follows: Texas red–dextran (3000 Da; Invitrogen, Carlsbad, CA), Alexa Fluor 546–phalloidin (Invitrogen), SS (Fluka, Sigma-Aldrich, St. Louis, MO), and Y27632 (Tocris, R&D Systems, Minneapolis, MN). The antibodies used in this work were PKCζ (directed against a synthetic peptide comprising amino acids 174–203 of the human orthologue; human–mouse similarity in this region, 100%; identity, 90%; ThermoFisher, Waltham, MA), PKCι (5282; Abcam, Cambridge, MA), pT555 PKCζ′ (GeneTex, Irvine, CA), CD68 (Bio-Rad, Hercules, CA), ezrin (Abcam), pT567 ezrin (Cell Signaling, Danvers, MA), intestinal alkaline phosphatase (iAP; Accurate), ZO-1 (Invitrogen), Par3 (Millipore, Billerica, MA), NF-κB p65 relA (Cell Signaling), occludin (ThermoFisher), NHE3 (Abcam), claudin-4 (Invitrogen), LLGL2 (Bioss, Woburn, MA), Na+/K+ ATPase (Accurate, Westbury, NY), pSer21-MLC (Abcam), pSer 536 relA (Invitrogen), and ROCK1/2 (Assay Biotech, Sunnyvale, CA).
FIGURE 9: Cytokine mRNA expression from isolated small intestine enterocytes from Prkci\textsuperscript{flox/flox} Vil-CRE\textsuperscript{±} (KO). Each data set comprises mRNAs from two different groups of animals for control littermates (Im) and KO, except for CXCL-1, which also comprises animals treated with oral SS or intraperitoneal Y27628. For each group, values were normalized to one control animal (Im). Horizontal lines represent average. Vimentin and IL-1\textbeta mRNA were measured in preparations of isolated enterocytes (E) or spleen (S) from the same animals to assess connective tissue contamination of the epithelium preparations. Kruskal–Wallis test, $H_0$ = control Im and KO populations are equal, $p < 0.005$ (CXCL-1), $p < 0.05$ (CXCL-2), $p < 0.05$ (IL-1\textalpha), SS or Y27628 groups, not significant. For vimentin and IL-1\textbeta, $H_0 = $ spleen data are equal to enterocytes, $p < 0.001$.

Intestinal permeability
The animals were food starved overnight but allowed free access to water. Then they received 100 μl of 4 mg/ml 3-kDa Texas red–dextran via stomach gavage. After 3 h, the animals were killed by deep anesthesia and exsanguination. In all cases, a Texas red–dextran bolus was macroscopically localized to the small intestine after death. Samples of the blood were anticoagulated with 2 mM EDTA, and plasma was separated by centrifugation. Texas red fluorescence was measured with a SpectraMax Gemini EM fluorometer (Molecular Devices) after dilution. Background in plasma from animals not receiving fluorescent dextran was subtracted. The results from different groups of animals are expressed as fluorescence relative to one control animal in the same group.

Frozen sections, immunofluorescence, and electron microscopy
Methods for these techniques have been published (Ameen et al., 2001; Mashukova et al., 2012). Immunofluorescence using phosphate antibodies was performed after fixation in trichloroacetic acid (Hayashi et al., 1999). All immunofluorescence images were collected with a Leica SP5 confocal microscope, and semiquantitative image analysis was performed with Leica software as described later. Electron microscope samples were prepared by standard techniques, and images were obtained with a Jeol JEM-1400 instrument, using Gatan software.

For fluorescence image analysis, pixel quantification was performed as described previously (Wald et al., 2011). Briefly, images were collected, avoiding pixel saturation in the channel to be measured. ROIs matching the size of positive signal images were defined and used throughout the sampling. Examples of ROIs are shown in Figures 5A, 6A, and 7C (yellow squares). The analyses were conducted blindly, and ROIs were positioned on the desired region of cells (e.g., apical edge, cytoplasm, or nucleus) in the 4′,6-diamidino-2-phenylindole (DAPI) channel to randomize the sampling. One average of pixel values in the ROI per cell was collected and used for statistics, which comprised several cells from different animals.

RT-quantitative PCR
Immediately after euthanasia, small intestine epithelial cells were purified by extraction in EDTA-dithiothreitol as described (McNicholas et al., 1994), with five to eight cycles of centrifugation and resuspension to wash mucus and separate nonepithelial cells. Spleen parenchymal cells were obtained by cutting the capsule with a razor blade in phosphate-buffered saline and shaking the organ with tweezers to separate the capsule. The resulting cell suspension was pipetted several times to separate debris. RNA was purified using mechanical homogenization by passing samples through needles 18G1/2 and 20G1/2 several times and TRIzol Reagent (Ambion Thermo Fisher Scientific). cDNA was synthesized from 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR amplification was performed using TaqMan gene expression assays (Life Technologies), including gene-specific primer and probe sets specifically designed against our molecules of interest (Mm04207460_m1 for CXCL-1, Mm00436450_m1 for CXCL-2, Mm00434151_m1 for IL-10, Mm00439620_m1 for IL-1\textalpha, Mm01333430-m1 for vimentin, and Mm00439620-m1 for IL-1\textbeta). The relative quantification method ($ΔΔC_{t}$) was used for analysis of mRNA fold change using endogenous control glyceraldehyde-3-phosphate dehydrogenase for normalization.

Sulfasalazine and Y27632 treatments
Sulfasalazine was dissolved at 37°C in tap water supplemented with 50 mM sucrose (for taste). The pH was tittered to 7.0 with NaOH. Accordingly, the SS vehicle for controls was tap water.
supplemented with 5 mM NaCl and 50 mM sucrose. The animals were allowed to drink ad libitum.

The Y27632 treatment was slightly modified from published protocols. The dose was at the low range of standard doses (2 mg/kg; Liao et al., 2013) and repeated twice a day. The rationale for this dosing was based on published bioavailability studies showing Y27632 half-life of ~90 min after intraperitoneal administration (Li et al., 2009). We aimed at peak plasma concentrations of ~1 μM. In addition, Y27632 doses were calculated to be above the EC50 of 0.14 μM for ROCK (Routhier et al., 2010) for much of the day, thus justifying two injections per day. At concentrations >20 μM, Y27632 blocks other kinases (Routhier et al., 2010), so higher doses were avoided. In summary, Y27628 was injected intraperitoneally, at 2 mg/kg twice a day.

**Statistics**

For normal distribution values, statistical significance was determined by Student’s t test. For data not conforming to normal distribution, the nonparametric Kruskal–Wallis test was used.

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