

Loss of PKC λ/ι impairs Th2 establishment and allergic airway inflammation in vivo

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The differentiation of T cells along different lineages is central to the control of immunity. Here we have used a conditional gene knockout system to delete PKC λ/ι selectively in activated T cells. With this system we have demonstrated that PKC λ/ι is necessary for T-helper cell (Th2) cytokine production and optimal T-cell proliferation and allergic airway inflammation in vivo. Our data demonstrate that the activation of the transcription factors nuclear factor of activated T cells and NF- κ B is impaired in PKC λ/ι -deficient activated T cells. In addition, we present genetic knockout evidence in ex vivo experiments with primary T cells that PKC λ/ι is critical for the control of cell polarity during T-cell activation. Therefore PKC λ/ι emerges as a critical regulator of Th 2 activation.

Asthma | NF-kappaB | T-cell activation | polarity | NF-AT

CD4⁺ T cells are central in the control of immunity thanks to their ability to differentiate into different subsets of T-helper (Th) cells (1, 2). It now is well established that naïve CD4⁺ T cells can differentiate in response to antigen stimulation into 3 distinct subsets of effector cells, Th1, Th2, or Th17 cells, which display distinct cytokine profiles and immune regulatory functions (3). Th17 cells are the latest addition to the group of effector T cells and are induced in vitro by the combined actions of TGF β and IL-6, are characterized by the secretion of the proinflammatory cytokine IL-17, and have been shown to play an essential role in autoimmunity (4, 5). In contrast, Th1 cells mainly produce IFN- γ and are essential for cell-mediated immune responses against intracellular pathogens. Th2 cells produce a different set of cytokines, including IL-4, IL-5, IL-10, and IL-13, and are important in the control of humoral immunity and allergy (6). In this regard, the pathology of asthma, a chronic lung inflammatory disease with increased prevalence in developed countries (7), is associated with aberrant activation and/or differentiation of CD4⁺ Th2 lymphocytes (8). The molecular mechanisms controlling Th2 differentiation and function have been the object of much research because of their relevance to inflammation disorders, such as asthma, for which better therapies are sorely needed, and because these mechanisms are a very interesting model system of crosstalk between different signaling cascades during a complex biological process.

IL-4 is important for induction and maintenance of differentiated Th2 cells (9). IL-4 and IL-13 share interactions with the IL-4 receptor chain and activate the transcription factor Stat6 through a Jak1/Jak3 signaling pathway (6, 10). We recently have presented in vitro, ex vivo, and in vivo genetic evidence that the atypical PKC (aPKC) isoform, PKC ζ , is necessary for optimal activation of the IL-4 signaling cascade upstream of Jak1 (11). Furthermore, in vivo adoptive transfer experiments using PKC $\zeta^{-/-}$ mice and cells demonstrated that PKC ζ in Th2 cells is required for allergy-induced airway inflammation (11). These findings established PKC ζ as an important mediator of Th2 differentiation in vivo in the IL-4 signaling pathway. However, the fact that IL-4 is required for Th2 differentiation but at the same time must be produced by Th2 cells seems paradoxical. It suggests that other signals must trigger the initial activation of the Th2 polarization event, and that the Th2-produced IL-4 serves to amplify and maintain that response.

In fact, recent studies propose that activated antigen-presenting cells (APCs) trigger a new set of signals that serve to instruct T cells toward the different differentiated lineages (2). In dendritic cells, for example, Notch ligands, as well as the receptor tyrosine kinase, c-Kit, and its ligand, SCF, signal T lymphocytes to polarize to the Th1 or Th2 lineages, depending on the type of APC-stimulating trigger (12–15).

The role of the other aPKC member, termed PKC λ/ι , in the control of the immune response has not yet been investigated using in vivo genetic systems. PKC λ/ι is highly homologous to PKC ζ (16), and because the latter has been shown to be important in Th2 differentiation, our goal in this study was to test whether PKC λ/ι plays a role in this process. Our data shown here clearly establish that PKC λ/ι is required for optimal Th2 cytokine production and allergic airway inflammation and that its loss correlates with impaired activation of key transcriptional factors and cell polarity.

Results

Generation of Conditional PKC λ/ι Knockout Mice on Activated T Cells.

Because PKC $\lambda/\iota^{-/-}$ mice die at very early embryonic stages, probably because of defects in cell polarity (Diaz-Meco, Leitges, and Moscat, unpublished observations), we generated a conditional PKC λ/ι knockout (PKC $\lambda/\iota^{fl/fl}$) mouse line in which PKC λ/ι is specifically deleted in activated T cells. To do so, we crossed PKC $\lambda/\iota^{fl/fl}$ mice (17) with Cre^{OX40} mice in which the expression of Cre is under the control of the *Tnfrsf4* locus (18). OX-40 is expressed almost exclusively in activated T cells, especially CD4⁺ cells, upon stimulation (18). In this mutant mouse line, PKC λ/ι is expressed at normal levels in immature thymocytes and naïve T cells and, as predicted, is deleted only upon T-cell activation. This strategy is advantageous in that it avoids embryonic lethality and prevents potential confounding effects resulting from the deletion of PKC λ/ι during development or in resting cells. This approach has been used previously to delete the *GATA3* gene specifically in activated T cells during Th2 differentiation experiments (18). PCR genotyping was used to screen for homozygous conditional PKC λ/ι -deficient (PKC $\lambda/\iota^{fl/fl}$ Cre^{OX40}), heterozygous (PKC $\lambda/\iota^{fl/wt}$ Cre^{OX40}), and wild-type (PKC $\lambda/\iota^{wt/wt}$ Cre^{OX40}) mice. No Cre-mediated effects were detected when PKC $\lambda/\iota^{wt/wt}$ Cre^{OX40} and PKC $\lambda/\iota^{fl/fl}$ mice were compared (data not shown). The deletion of PKC λ/ι in activated CD4⁺ T cells was confirmed by Western blot. That is, upon anti-CD3 plus anti-CD28 stimulation under non-skewed conditions or after differentiation of the cells under Th0, Th1, or Th2 conditions, we observed a significant up-regulation of PKC λ/ι

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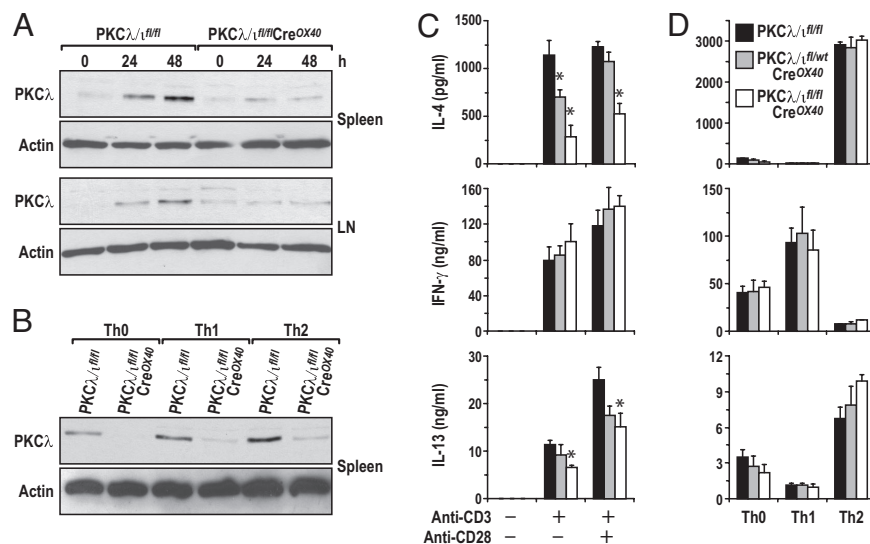


Fig. 1. PKC ζ is up-regulated upon T-cell activation and differentiation and is required for Th2-cytokine secretion. (A) Purified naïve splenic CD4⁺ T cells or cells from cervical, axillary, inguinal, and popliteal lymph nodes (LN) were stimulated with anti-CD3/CD28 for 24 or 48 hours. (B) Splenic CD4⁺ T were differentiated under Th0, Th1, and Th2 conditions for 4 days. Cells in A and B were restimulated with anti-CD3/CD28 for 2 days. Cells were collected for Western blot analysis. Results shown are from a single experiment representative of 2 independent experiments. (C and D) Purified naïve splenic CD4⁺ T cells were stimulated with (C) anti-CD3/CD28 for 3 days or (D) differentiated under Th0/Th1/Th2 conditions for 4 days and restimulated with plate-bound anti-CD3 for 1 day. Supernatants from cell cultures were collected for ELISA assays to detect cytokines. Data (mean $\pm$ SE) are from 4 experiments with triplicated determinations in each sample. * $P < 0.05$.

in T cells from wild-type mice but not in those from conditional PKC ζ -knockout mice (Fig. 1A and B). The heterozygous activated T cells display PKC ζ levels that are intermediate to those of wild-type and PKC ζ -deficient activated T cells (supporting information (SI) Fig. S1). Therefore, PKC ζ normally is induced during sustained T-cell stimulation and differentiation and is deleted effectively in the mutant cells. This observation in itself is interesting because it suggests that in unstimulated conditions PKC ζ levels are very low and that this kinase is induced only when T cells are activated for a relatively long period (Fig. 1A and B) and that PKC ζ might be required for the sustained signaling leading to T-cell differentiation but not for early cell activation.

Of note, conditional PKC ζ -deficient mice appeared to develop normally (data not shown), and the percentages of T and B cells (CD4⁺, CD8⁺, and B220⁺ cells) in the spleens and lymph nodes of these mice were similar to those in wild-type mice (Table S1). The number of double-positive cells (CD4⁺CD8⁺) and single-positive cells (CD4⁺ or CD8⁺) in the thymus also did not differ in conditional PKC ζ -deficient and wild-type mice (Table S2).

Reduced Th2 Cytokine Secretion by CD4⁺ T Cells from Conditional PKC ζ -Deficient Mice. Because PKC ζ has been shown to play a critical role during Th2 differentiation (11), and because of the high homology between the 2 aPKCs, we sought to determine whether the conditional deletion of PKC ζ in activated T cells would produce an effect similar to that of PKC ζ deficiency. To do so, we stimulated purified naïve CD4⁺ T cells from mice of different genotypes with anti-CD3 in the absence or in the presence of anti-CD28; the levels of different secreted cytokines were detected by ELISA. Interestingly, reduced secretion of IL-4 was observed in PKC ζ -deficient cells as compared with wild-type littermate controls when both types of cells were activated with anti-CD3 plus anti-CD28 (Fig. 1C). In the case of heterozygous PKC ζ mice (PKC ζ ^{fl/fl}Cre^{OX40}), this reduction was apparent only under anti-CD3 activating conditions (Fig. 1C). These results indicate that PKC ζ is required for Th2 cytokine production. Of note, when IFN γ secretion was determined under the same conditions, it was clear that its production was unaf-

fected in the PKC ζ -deficient T cells (Fig. 1C). Consistently, secretion of IL-13, another Th2 cytokine, also was reduced in the PKC ζ -deficient T cells (Fig. 1C) stimulated under the same conditions. Because these data suggest that PKC ζ is required for the control of the balance between Th2 and Th1 cytokines, we next wanted to test whether PKC ζ likewise is necessary during Th2/Th1 differentiation in vitro. To address this possibility, isolated CD4⁺ T cells were cultured for 4 days under non-skewing (Th0) or under Th1 or Th2 conditions. Afterwards, cells were washed and restimulated with anti-CD3 for 24 hours, and cytokine secretion was determined by ELISA. Surprisingly, the loss of PKC ζ in activated T cells did not impair IL-4 or IL-13 secretion in Th2-polarized T cells or the secretion of IFN- γ in Th1-polarized T cells (Fig. 1D). Collectively these results could be interpreted to mean that PKC ζ is required for tilting the balance of Th2 vs. Th1 cytokine secretion but that, under the conditions of in vitro T-cell differentiation, the PKC ζ pathway

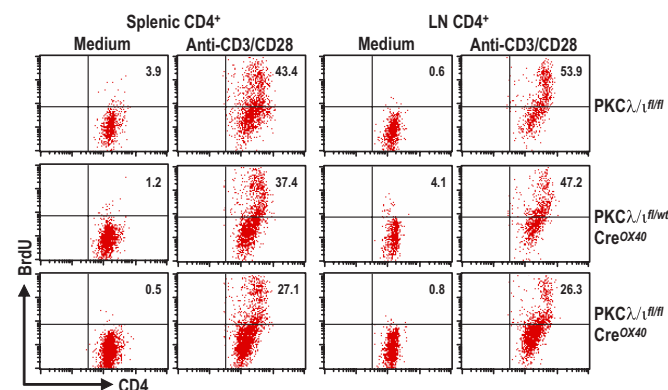
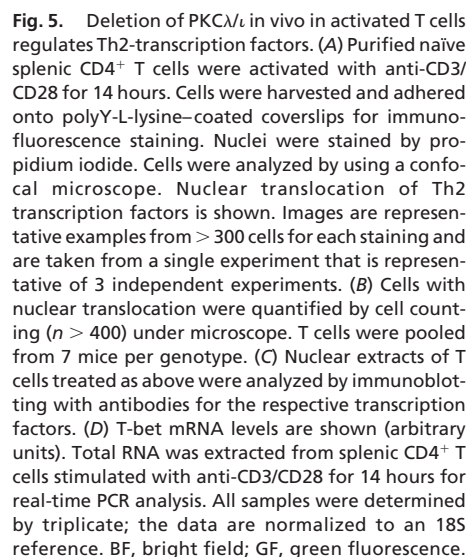


Fig. 2. Deletion of PKC ζ impairs CD4⁺ T-cell proliferation. Purified naïve CD4⁺ T cells from spleens or whole lymph node (LN) cells were stimulated with plate-bound anti-CD3 plus anti-CD28 for 3 days. Then cell proliferation was assessed by BrdU intracellular staining by FACS. Percentages of BrdU-positive cells are shown.



determine the signaling pathways affected by the loss of PKC λ/ι in activated T cells. Because Th2 cytokine secretion is a long-term process that requires sustained activation of T-cell receptor (TCR) signals, we determined which of the transcription factors reported to be essential for this process were affected by the loss of PKC λ/ι . Specifically, activation of GATA3 (indicated by localization to the nucleus), which is necessary and sufficient to drive T cells to the Th2 lineage, was determined by confocal microscopy in cell cultures of wild-type or PKC λ/ι -deficient CD4⁺ T cells that had been incubated under non-skewing conditions with anti-CD3 plus anti-CD28 for 14 hours. Fig. 5A shows a representative image demonstrating that GATA3 was localized to the nucleus in activated wild-type CD4⁺ T cells but was excluded almost entirely from the nucleus in activated PKC λ/ι -deficient cells. Fig. 5B shows the percentage of cells displaying nuclear GATA3 staining based on counts of 400 cells and demonstrates that, although more than 70% of activated wild-type T lymphocytes had nuclear GATA3, nuclear GATA3 was detected in only about 25% of the PKC λ/ι -deficient T cells. These results are very important because they show the relevance of PKC λ/ι for the activation of a Th2-specific transcription factor and are consistent with this kinase being relevant to Th2 cytokine production. Interestingly, when the activation states of other transcription factors important for this process, such as nuclear factor of activated T cells (NFAT), p65, or Stat6, were determined, it was clear that the nuclear levels of all of these transcription factors were reduced severely by the loss of PKC λ/ι (Fig. 5A and B). NFAT and p65 stimulation are linked to the activation of the TCR (20),

whereas activation of Stat6 probably is caused by the secretion of IL-4 in activated T cells. IL-4 production is reduced in PKC λ/ι -deficient activated T cells (see Fig. 1C), and this reduction is likely to account for the reduced nuclear Stat6 levels detected in PKC λ/ι mutant T cells and is consistent with our data demonstrating the lack of a role for PKC λ/ι in IL-4 signaling in Stat6 activation (Diaz-Meco, *et al.*, unpublished data). Immunoblot analysis of a similar experiment demonstrates a dramatic inhibition of GATA3, NFAT, and p65 nuclear levels in the PKC λ/ι -deficient T cells activated as described earlier in this article (Fig. 5C), consistent with the data in Fig. 5A and B. Interestingly, the nuclear levels of the Th1 transcription factors Stat1 and Stat4 were not affected by the loss of PKC λ/ι in these experiments (Fig. 5A–C). Of note, the expression of the Th1 master regulatory gene, *T-bet*, was induced in PKC λ/ι -deficient activated T cells to levels comparable to those of the wild-type cells (Fig. 5D). Results shown in Fig. S2 demonstrate that the *OX40-Cre* transgene is expressed at 14 hours of stimulation and, consistently, PKC λ/ι is up-regulated at this time point in wild-type T cells and is effectively depleted in the knockout cells. (Fig. S3). The activation of Akt and ERK was not affected by the loss of PKC λ/ι (Fig. S3). Deletion of PKC λ/ι in Jurkat T cells by RNA interference also inhibited NFAT and NF- κ B activities (Fig. S4). Collectively, these results could be interpreted to mean that PKC λ/ι plays a decisive role in the TCR activation of p65 and NFAT, which leads to GATA3 activation and the synthesis of IL-4, thus influencing the stimulation of Stat6. Therefore, we conclude that PKC λ/ι is induced during and is required for the sustained activation of key transcriptional events of Th2 differentiation.

Role of PKC λ/ι in T-cell Polarity. The aPKCs have been implicated in the control of cell polarity in several mammalian *in vitro* cell-culture experiments (21). More recently, the potential role of polarity in T-cell activation has been shown also (22, 23). However, no genetic evidence from *ex vivo* experiments has been produced to test directly the involvement of the aPKCs in T-cell polarity. A recent study identified Crtam as a scaffold protein that binds the cell polarity protein Scribble and through this interaction regulates cell polarity, proliferation, and the secretion of cytokines (24). Therefore, a potential explanation for the findings reported here is that PKC λ/ι could be necessary for T-cell proliferation and Th2 cytokine secretion because of its potential role in T-cell polarization. To address this question, we incubated CD4⁺ T cells from either wild-type or PKC λ/ι -mutant mice with anti-CD3 plus anti-CD28 for 14 hours, following exactly a previously described procedure to induce and assess T-cell polarization (24). We then determined T-cell polarity by monitoring the ability of the polarity marker Scribble to localize to one of the poles of the activated T cell (24). Fig. 6 *A* and *B* strongly suggests that the lack of PKC λ/ι during T-cell activation leads to a significant loss of T-cell polarity. To establish this conclusion more firmly, we analyzed the localization of CD44 and CD3 in double immunofluorescence analysis. From the data of Fig. 6C it is clear that CD44 is asymmetrically polarized relative to CD3, consistent with the induction of cell polarity in the activated T cells. More importantly, this polarization is severely impaired in the PKC λ/ι -deficient T cells (Fig. 6 *C* and *D*). Interestingly, PKC λ/ι colocalizes with Crtam and is required for its proper polarization in activated T cells (Fig. 6 *E* and *F*). Similar conclusions were obtained when the role of PKC λ/ι was investigated in late T-cell polarity using polystyrene beads coated with anti-CD3 plus anti-CD28 (Fig. S5). Of note, the lack of PKC λ/ι does not affect T-cell migration under these experimental conditions (Fig. S6). Collectively these results indicate that PKC λ/ι deficiency in activated T cells leads to impaired polarity during late T-cell activation.

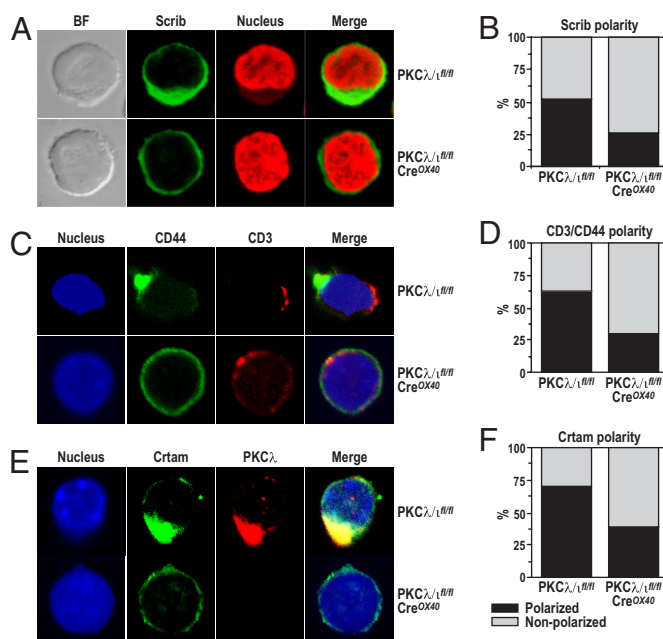


Fig. 6. PKC λ / ϵ control cell polarity. (A, C, and E) Purified naïve splenic CD4 $^{+}$ T cells were activated with anti-CD3/CD28 for 14 hours. Cells were treated for immunofluorescence staining as in Fig. 5. Polarity of Scrib (B), CD3/CD44 (D), and Crtam/PKC λ (F) are shown. Images are representative of > 300 cells for each staining. Cells with polarity were quantified by cell counting ($n > 400$) under a microscope. CD4 $^{+}$ T cells were pooled from 7 conditional PKC λ / ϵ -deficient mice and their wild-type littermates. The results are from a single experiment that is representative of 2 independent experiments with identical results.

Discussion

Unraveling the signaling pathways that promote T-cell differentiation along the different lineages is of paramount importance on 2 levels: for the general insight it provides on the interplay between different cell-signaling pathways in the regulation of complex cellular processes and, more specifically, for an increased understanding of T-cell biology under normal and pathological conditions. Here we show that PKC α/ι , 1 of the 2 members of the aPKC family, plays a critical role in allergic airway inflammation *in vivo*, most likely because it is required for the production of Th2 cytokines. *Ex vivo* experiments with PKC α/ι -deficient T cells demonstrate that this kinase is required for the activation of transcription factors critical for adequate Th2 cell function and differentiation. We also provide genetic evidence using conditional knockout cells that PKC α/ι is essential for T-cell polarity, an event that has been suggested to be relevant to T-cell function (22). Considered collectively, these results suggest that defects in cell polarity caused by the lack of PKC α/ι in activated T cells, along with alterations in gene expression programs, are responsible for the defects in Th2 cytokine production detected in the *ex vivo* experiments and account for the impaired lung inflammatory response observed in the PKC α/ι mutant mice when challenged with an allergic stimulus.

Our findings bring up a number of questions that deserve discussion before a consensus model emerges on the links between cell polarity, signaling, and function, at least in the immune system. How alterations in polarization correlate with defects in gene expression signaling need more in-depth investigation, both in T lymphocytes and in other cell systems in which polarity is essential for relevant cellular functions. In this regard, the identification of a Scribble-interacting partner, Crtam, constitutes an important step in linking polarity and T-cell function in a cause-effect type of experiment (24). Interestingly, the loss of Crtam leads to impaired T-cell polarity, which is accompanied by increased proliferation and

decreased production of the Th1 and Th17 cytokines, IFN γ and IL-17, respectively, but not of the Th2 cytokine IL-4 (24). At first glance, these published data could be interpreted to mean that T-cell polarization is essential for T-cell differentiation toward Th1 but not Th2 lineages. However, our results shown here establish that the loss of polarity as a consequence of PKC λ /i ablation in activated T cells does not correlate with increased proliferation, but rather with reduced proliferation, and leads to the impairment of the production of IL-4 but not of IFN γ .

A potential explanation for these different observations is that PKC λ /i, in addition to binding the Phox and Bem1p-1 domain (PB1)-containing polarity adapter Par-6 (16), also binds a non-polarity PB1-containing signaling adaptor, termed p62, whose genetic knockout gives a phenotype in vivo and ex vivo very similar to that of PKC λ /i in terms of T-cell activation (3). This possibility could be interpreted as meaning that PKC λ /i, because of its ability to bind p62 through their respective PB1 domains (16), will influence late T-cell signaling, ultimately resulting in modulation of Th2 cytokine production and of the response to allergic airway inflammation. On the other hand, probably through its interaction with polarity adapters such as the PB1 scaffold Par-6 and other polarity proteins (16), PKC λ /i affects the localization of these molecules in polarized regions of the T cell. Therefore, PKC λ /i generates 2 independent kinds of signals, depending on its different binding partner, and these signals direct PKC λ /i participation to distinctive signaling cascades. These data are consistent with a model in which the inactivation of different proteins with different polarity would have different cellular consequences because of their association with different signaling complexes.

Materials and Methods

Mice. PKC λ /i^{fl/fl} mice were reported previously (17). Cre^{OX40} mice were generated in the laboratory of N. Killeen at the University of California, San Francisco (18). Conditional PKC λ -deficient mice on activated T cells were generated by crossing PKC λ /i^{fl/fl} mice with Cre^{OX40} mice. Mouse genotyping was performed by PCR using primers for PKC λ /i^{fl/fl}, OX-40-Cre, and PKC λ /i deletion to screen homozygous conditional PKC λ -deficient (PKC λ /i^{fl/fl} Cre^{OX40}), heterozygous (PKC λ /i^{fl/wt} Cre^{OX40}), and wild-type (PKC λ /i^{fl/fl} or PKC λ /i^{wt/wt} Cre^{OX40}) mice. Age- (9–12 weeks) and sex-matched mice were used in each experiment.

CD4⁺ T-Cell Isolation and Differentiation. Splenocytes were prepared by disrupting spleens of 9- to 12-week-old mice. Naive CD4⁺ T cells were enriched with a Mouse CD4⁺ Isolation Kit using an AutoMACS Pro (Miltenyi Biotec). The purity (> 95%) and naive status of isolated CD4⁺ T cells were confirmed by FACS staining with conjugated mAbs to CD4, CD8, B220, CD44, and CD62L. Naive CD4⁺ T cells (10⁶/ml) were activated with plate-bound anti-CD3 (10

μg/ml) plus soluble anti-CD28 (2 μg/ml) or differentiated into Th0, Th1, or Th2 cells (11). The culture supernatants were collected at different times after activation for cytokine assays by ELISA.

Cytokine Assay. Cytokines in the supernatants and BAL fluid were assayed by ELISA. IL-4, IL-5, IL-6, IL-10, and IFN- γ were assayed with OptEIA kits (BD Pharmingen); and IL-13 and eotaxin were assayed with DuoSet ELISA kits (R&D Systems). ELISA plates were developed with TMB substrate (BD Pharmingen), and read by a microplate reader (model 550, Bio-Rad). Cytokine mRNA levels were measured by real-time quantitative PCR.

FACS. Spleen and lymph node cells or purified CD4⁺ cells were incubated with anti-CD16/32 (2.4G2) to block Fc γ R II/III and then were stained with various conjugated mAbs. Stained cells were analyzed by FACS Calibur with CellQuest software (Becton Dickinson).

Proliferation. CD4⁺ T-cell proliferation was assayed in vitro by measuring BrdU incorporation with a BrdU Flow kit (BD Pharmingen) following the manufacturers' protocols. Briefly, purified naive CD4⁺ T cells or whole lymph node cells were cultured with plate-bound anti-CD3 plus anti-CD28 for 2 to 3 days. BrdU (10 mM) was added to the cultures in the last 18 hours. After fixation, permeabilization, and DNase digestion, cells were stained with APC-conjugated anti-BrdU mAb and analyzed by FACS.

Immunofluorescence Staining. Purified naive CD4⁺ T cells were cultured with plate-bound anti-CD3 plus anti-CD28 for 14 hours. Cells were harvested and adhered onto poly-L-lysine (Sigma)-coated coverslips for 30 minutes at room temperature, and nonadherent cells were washed off with PBS. Adherent cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with different mAbs. The signals were amplified by the Tyramide Signal Amplification kit (Molecular Probes). For nuclear staining, cells were incubated with propidium iodide (Sigma) or TOPRO-3 (Invitrogen). Coverslips were mounted on Mowiol and examined with a Zeiss LSM-510 Meta Confocal Laser Scanning Microscope (Carl Zeiss Microimaging). Images from single plane were taken.

Real-Time PCR. Total RNA was extracted from lung tissues or cultured cells with the RNeasy Mini kit (Qiagen), and cDNA was prepared by the Omniscript Reverse Transcription kit (Qiagen). Quantitative PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems) on a Mastercycler ep realplex4 apparatus (Eppendorf). The data were normalized to the 18S reference. Primers for IL-4, IL-5, IL-13, eotaxin, MUC-5AC, Gob-5, and T-bet were designed with OLIG 4.0 software.

For more information, please see *SI Materials and Methods*.

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