PKCθ Signals Activation versus Tolerance In Vivo

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Abstract

Understanding the pathways that signal T cell tolerance versus activation is key to regulating immunity. Previous studies have linked CD28 and protein kinase C-θ (PKCθ) as a potential signaling pathway that influences T cell activation. Therefore, we have compared the responses of T cells deficient for CD28 and PKCθ in vivo and in vitro. Here, we demonstrate that the absence of PKCθ leads to the induction of T cell anergy, with a phenotype that is comparable to the absence of CD28. Further experiments examined whether PKCθ triggered other CD28-dependent responses. Our data show that CD4 T cell–B cell cooperation is dependent on CD28 but not PKCθ, whereas CD28 costimulatory signals that augment proliferation can be uncoupled from signals that regulate anergy. Therefore, PKCθ relays a defined subset of CD28 signals during T cell activation and is critical for the induction of activation versus tolerance in vivo.

Key words: anergy • CD28 • CTL • protein kinase C • LCMV

Introduction

Protein kinase C-θ (PKCθ) is a member of the calcium-independent PKC subfamily of serine/threonine kinases that is primarily expressed in the muscle and T lymphocytes (for review see references 1, 2). Papers have demonstrated that TCR/CD28 stimulation of Jurkat cells leads to PKCθ-mediated activation of the transcription factors AP-1 (3, 4) and NF-κB (4–6). Papers have also linked PKCθ activation with the induction of stress-activated protein kinase/c-Jun NH$_2$-terminal kinase (JNK) and IL-2 production (7–9). There is evidence that PKCθ can synergize with calcineurin and influence apoptosis by altering FasL expression (10, 11). Impaired calcium mobilization and NFAT activation has been reported in T cells from PKCθ-deficient mice (12). Activation of peripheral T cells from PKCθ-deficient mice confirmed the involvement of PKCθ in AP-1 and NF-κB activation (12, 13); however, there was no apparent defect in JNK activation (13). Although papers have suggested that TCR stimulation alone can lead to PKCθ activation, it is clear that costimulatory CD28 signals augment PKCθ activation (14, 15). Collectively, evidence suggests that PKCθ may play an important role in the activation of several signaling pathways downstream of the TCR, and PKCθ activation is significantly enhanced by CD28-mediated costimulation.

T cell activation is associated with the formation of the supramolecular activation complex (SMAC) or the immunological synapse. The colocalization of the TCR, CD28, and other molecules is effective in coordinating optimal T cell activation (16). Importantly, PKCθ is the only PKC isoform that has been detected in the SMAC after antigen-specific T cell engagement with APCs (17, 18). The translocation of PKCθ from the cytosol to the membrane is an important step that regulates the activation of this kinase. Papers have shown that engagement of the TCR and CD28 leads to the movement and activation of PKCθ to the lipid rafts in the SMAC (15, 19). This process involves other signaling molecules including lck, Vav, and PI3K (14, 20, 21).

CD28 is the most extensively studied costimulatory molecule that contributes to T cell activation by multiple mechanisms. CD28 is well recognized for its role as "signal 2" that is required to prevent the induction of anergy (22, 23). CD28 has been shown to augment T cell activation by

Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte; gp, glycoprotein; JNK, c-Jun NH$_2$-terminal kinase; LCMV, lymphocytic choriomeningitis virus; PKCθ, protein kinase C-θ; SMAC, supramolecular activation complex; VSV, vesicular stomatitis virus.

reducing the number of TCRs that must be triggered for T cell activation (24, 25). This may reflect the ability of CD28 engagement to trigger cytoskeletal movement and recruit lipid rafts and associated proteins to the immunological synapse (26, 27). CD28 also promotes T cell survival by enhancing IL-2 production and stimulating the PI3K–Akt–BclX$_L$ pathway (28–31).

Given the evidence that links CD28 and PKCθ with optimal T cell activation, our goal was to investigate whether PKCθ played an important role in the induction of T cell activation or anergy in vivo. Using PKCθ-deficient mice and CD28-deficient mice, we compared viral immune responses in vivo and in vitro.

Materials and Methods

Mice. The P14 TCR transgenic mouse line (32), CD28-deficient mice (33), and PKCθ-deficient mice (13) were described previously. The P14 TCR transgenic mice were bred with the CD28-deficient or the PKCθ-deficient mice to yield P14/CD28$^{-/-}$ and P14/PKCθ$^{-/-}$ mice, respectively.

Peptides. Peptide glycoprotein (gp) 33 (KAVYNFATM) is the major cytotoxic T lymphocyte (CTL) epitope of the lymphocytic choriomeningitis virus (LCMV) gp and is H-2D^b restricted (34). The peptide AV (SGPSNTPPEI) is an H-2D^b–restricted peptide derived from adenovirus and does not stimulate proliferative responses from P14 transgenic T cells.

CTL Assays. Mice were immunized intravenously with 5,000 PFU of LCMV Armstrong, and after 8 d, CTL assays were done. Alternatively, mice were immunized intravenously with 10 μg gp33 peptide in HBSS, and CTL assays were performed 1 or 3 d later. Single cell suspensions were prepared from the spleen in IMDM supplemented with 10% FCS, glutamine, and β-mercaptoethanol and used as effector cells. Effector cells were plated in duplicate in 96-well round-bottom plates at 9×10^5 cells/well and three threefold serial dilutions were made. EL4 target cells were prepared by incubating with 400 μCi ml^{-1} ^{51}Cr (Perkin-Elmer) and 10^{-5} M peptide for 2 h at 37°C. Target cells were washed three times and added to the effector cells at 10^4 cells/well. Plates were spun briefly and incubated for 5 h at 37°C. 70 μl/well of supernatant was counted for 60 s using a Wallac Wizard γ-counter (PerkinElmer). Maximum release was induced by adding 1 M HCl to targets. Specific lysis was calculated as (cpm sample release − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release) × 100.

For experiments examining secondary CTL activity, mice were immunized with LCMV. After 40 d, splenocytes were stimulated in vitro. Cultures were set up in 24-well plates with 3×10^6 spleen cells/well and 10^6 C57Bl/6 spleen cells/well with 10^{-7} M gp33 peptide and incubated at 37°C for 5 d.

For the secondary CTL response examining anergy induction, cultures were set up 3 d after gp33 immunization with 2×10^6 spleen cells/well and 10^{-6} M gp33 peptide with or without 10% vol/vol ConA supernatant in 24-well plates. Alternatively, 10 U ml^{-1} IL-2 was added to the cultures. The CTL assay was performed after 3 d of culture at 37°C.

Proliferation Assays. Mice were immunized intravenously with 10 μg gp33 peptide in HBSS. 3 d later, single cell suspensions were prepared from the spleen and plated in triplicate at 10^5 cells/well in a 96-well flat-bottom plate with given concentrations of gp33 peptide with or without 10% vol/vol ConA super-

natant or IL-2 (10 U ml^{-1}). Cells were incubated for 24–48 h at 37°C and pulsed with 1 μCi [^{3}H]thymidine per well overnight. Proliferation assays using MACS-sorted CD8$^+$ T cells were performed by adding 3×10^4 P14 T cells/well and 10^5 spleen cells/well from C57Bl/6 mice. In the indicated experiments, CD8$^+$ T cells were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) and washed before coculture in vitro.

Flow Cytometric Analyses. Cells were stained with PE-conjugated antibody recognizing CD8α or biotin-conjugated antibodies recognizing CD44, CD62L, CD49d, CD69, or Vα2 (BD Biosciences). Biotin-conjugated antibodies were detected using streptavidin–Cy-Chrome. For tetramer staining, cells were incubated with H-2D^b/gp33-PE tetramers and CD8α-FITC for 1 h on ice. Tetramers were provided by the tetramer core facility, National Institute of Allergy and Infectious Diseases. Stained cells were fixed in 2% paraformaldehyde/PBS. Flow cytometry was performed on a FACScan™ (Becton Dickinson) and analyzed using CELLQuest™ (Becton Dickinson) or FlowJo (Tree Star) software. Live events were gated based on forward and side scatter profiles, and division number was determined from CFSE intensity. In the indicated experiments, mean fluorescence intensity of cells that have undergone division have been analyzed as described previously (35).

Vesicular Stomatitis Virus (VSV) Assay and Immunohistochemistry. Mice were immunized intravenously with 2×10^5 PFU of VSV serotype Indiana, and peripheral blood was collected after 4 and 8 d. Neutralizing titers of sera were determined as described previously (36). For immunohistochemistry, spleens were immersed in PBS and snap frozen in liquid nitrogen and processed as described previously (37).

Results

Normal Expansion and Effector Function of LCMV-specific T Cells Deficient for PKCθ. Previous works have shown that T cells from PKCθ-deficient mice had impaired proliferative responses in vitro (12, 13). To examine whether PKCθ-deficient mice were able to mount an immune response in vivo, PKCθ-deficient mice were immunized with LCMV. 8 d after virus infection, PKCθ$^{-/-}$ mice mounted an efficient CTL response against the major LCMV gp epitope gp33, which was comparable to control mice (Fig. 1 A). Expansion of the LCMV-specific T cells detected by a H-2D^b/gp33 tetramer was also similar in the presence or absence of PKCθ (Fig. 1, B and C). The secondary CTL response after LCMV infection was also examined in PKCθ$^{-/-}$ mice. 40 d after mice were immunized with LCMV, the secondary CTL activity from PKCθ-deficient mice was similar to control mice (Fig. 1 D). Therefore, the induction of an immune response to LCMV was not impaired in the absence of PKCθ.

In Vitro Antigen-specific Proliferation Is Influenced by PKCθ. It is possible that in vivo infection with pathogens stimulates the innate immune system, which in turn provides appropriate signals that circumvent the requirement for CD28 and/or PKCθ signals in vivo. Therefore, to further characterize the role of PKCθ, we wanted to evaluate the immune response in the absence of pathogen-induced signals.
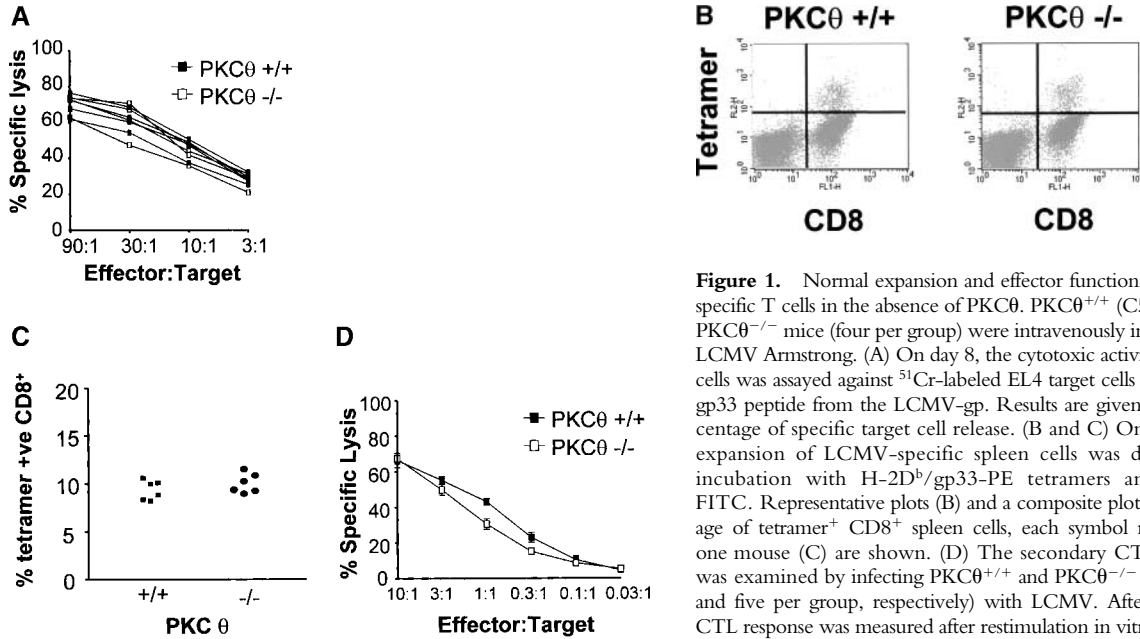
Figure 1. Normal expansion and effector function of LCMV-specific T cells in the absence of PKCθ. PKCθ^{+/+} (C57Bl/6) and PKCθ^{−/−} mice (four per group) were intravenously injected with LCMV Armstrong. (A) On day 8, the cytotoxic activity of spleen cells was assayed against ^{51}Cr-labeled EL4 target cells pulsed with gp33 peptide from the LCMV-gp. Results are given as the percentage of specific target cell release. (B and C) On day 8, the expansion of LCMV-specific spleen cells was detected by incubation with H-2D^b/gp33-PE tetramers and CD8α-FITC. Representative plots (B) and a composite plot of percentage of tetramer$^+$ CD8$^+$ spleen cells, each symbol representing one mouse (C) are shown. (D) The secondary CTL response was examined by infecting PKCθ^{+/+} and PKCθ^{−/−} mice (three and five per group, respectively) with LCMV. After 40 d, the CTL response was measured after restimulation in vitro.

To examine antigen-specific responses in vitro, P14 transgenic mice expressing a TCR specific for LCMV gp peptide gp33 and H-2D^b were bred with PKCθ-deficient mice. Spleen cells from P14/PKCθ^{+/+} and P14/PKCθ^{−/−} mice were cultured with different concentrations of the gp33 peptide, and proliferation was measured after several time points. Fig. 2 shows that a TCR-specific signal could promote proliferation in the absence of PKCθ, but 10–100-fold more antigen was necessary to induce a similar degree of proliferation. This, together with the findings from LCMV infection, suggests that PKCθ is not absolutely essential for T cell function, but rather alters the threshold for T cell activation.
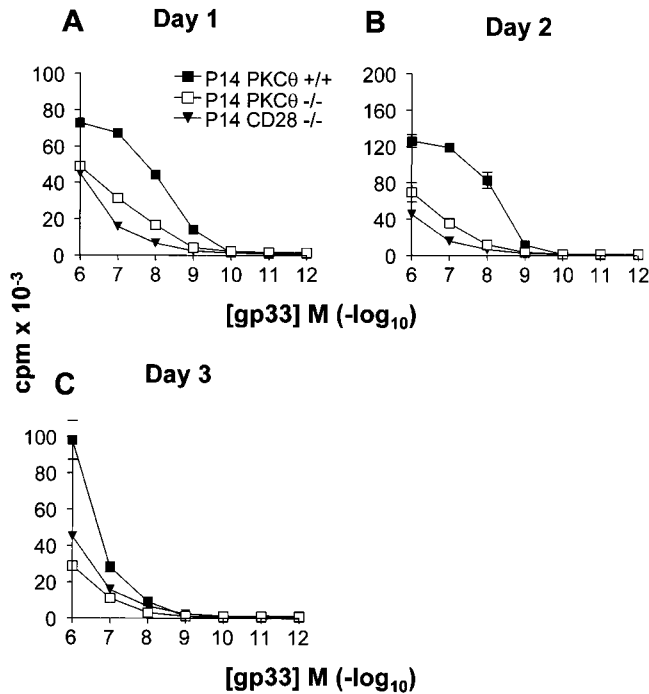
One possible interpretation for the reduced proliferation is that PKCθ-deficient T cells receive a suboptimal activation signal, and lack important costimulatory signals that are required for efficient T cell activation in vitro. Because previous analyses clearly show that CD28 augments PKCθ activation downstream of TCR signals (4, 5, 14, 15), we compared the proliferative response of P14/PKCθ^{−/−} T cells with P14/CD28^{−/−} T cells (Fig. 2). A similar functional phenotype was observed in P14/PKCθ^{−/−} and P14/CD28^{−/−} mice, supporting the idea that PKCθ-deficient T cells lack important costimulatory signals.

gp33 Peptide Can Induce Transient CTL Activity in PKCθ-deficient T Cells In Vivo. Further analyses were performed to examine the ability of P14 T cells to respond to the gp33 peptide in vivo. P14/PKCθ-deficient and P14 control animals were given 10 μg gp33 intravenously. Both P14 and P14/PKCθ^{−/−} T cells up-regulated various activation markers such as CD69, CD44, and down-regulated CD62L and the TCR to a similar but not identical extent (unpublished data). Because the T cells appeared to be activated by peptide in vivo, we investigated whether gp33 could induce CTL activity. Fig. 3 A shows that the CTL activity 1 d after peptide administration was comparable to WT animals, whereas the CTL response was clearly diminished after 3 d in the absence of PKCθ (Fig. 3 B).

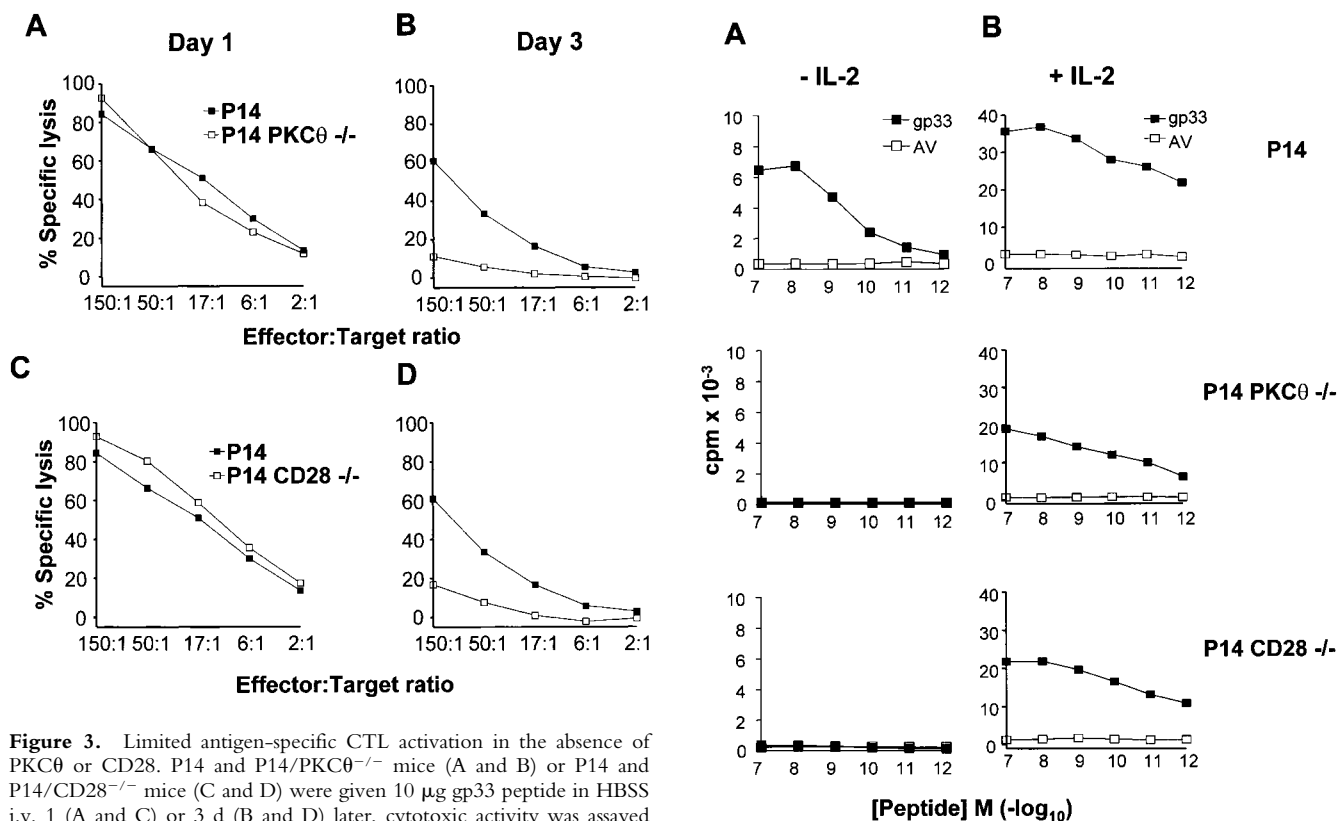Because of the link between CD28 stimulation and PKCθ activation (Fig. 2; references 4, 5, 14, 15) and the

Figure 2. PKCθ signals contribute to antigen-specific CD8 proliferation in vitro. Spleen cells from P14/PKCθ^{+/+}, P14/PKCθ^{−/−}, or P14/CD28^{−/−} mice were cultured in the presence of different concentrations of gp33. After 24 (A), 48 (B), and 72 (C) h, proliferation was measured by thymidine incorporation. The data shown are representative of three experiments.

Figure 3. Limited antigen-specific CTL activation in the absence of PKCθ or CD28. P14 and P14/PKCθ$^{-/-}$ mice (A and B) or P14 and P14/CD28$^{-/-}$ mice (C and D) were given 10 μg gp33 peptide in HBSS i.v. 1 (A and C) or 3 d (B and D) later, cytotoxic activity was assayed against ^{51}Cr-labeled EL4 target cells pulsed with gp33 peptide. Results are given as the percentage of specific target cell release and depict one representative mouse of five.

Figure 4. Peptide-induced unresponsiveness in the absence of CD28 or PKCθ. P14, P14/PKCθ$^{-/-}$, and P14/CD28$^{-/-}$ mice were given 10 μg gp33 peptide in HBSS i.v. After 3 d, mice were killed, and proliferation assays from total splenocytes (A and B) or sorted CD8$^+$ T cells (C and D) were set up with various concentrations of stimulatory gp33 peptide or nonstimulatory AV peptide in the absence (A and C) and presence (B and D) of IL-2.

evidence for CD28 in the protection from anergy, it is possible that the lack of PKCθ leads to T cell anergy. The CTL response from P14/CD28$^{-/-}$ mice after gp33 peptide administration was compared with the CTL response from P14/PKCθ$^{-/-}$ mice. As seen in the P14/PKCθ-deficient mice, the absence of CD28 did not affect the CTL response 1 d after peptide administration (Fig. 3 C), whereas impaired CTL responses were observed after 3 d (Fig. 3 D; reference 38). Therefore, P14/PKCθ-deficient T cells were able to mount a transient gp33-specific CTL response, similar to P14/CD28$^{-/-}$ T cells. It is possible that the rapid decline in CTL activity in PKCθ$^{-/-}$ and CD28$^{-/-}$ T cells may be due to the induction of anergy.

PKCθ Signals Prevent the Induction of Anergy In Vivo. Analysis has shown that activated T cells remain in the PKCθ-deficient mice 3 d after gp33 peptide treatment (unpublished data). Therefore, the absence of a CTL response in P14/PKCθ$^{-/-}$ mice may correspond to an anergic population of cells. Anergy has been defined as a state of T cell unresponsiveness that can be reversed by the addition of IL-2 in some models (39). To test whether these T cells were anergic, splenocytes from gp33 peptide–treated P14, P14/PKCθ$^{-/-}$, and P14/CD28$^{-/-}$ mice were tested for their proliferative and cytotoxic response in vitro, with or without the addition of IL-2 (Figs. 4 and 5). 3 d after peptide administration in vivo, splenocytes were cultured with gp33 peptide in the presence or absence of IL-2 for 48 h
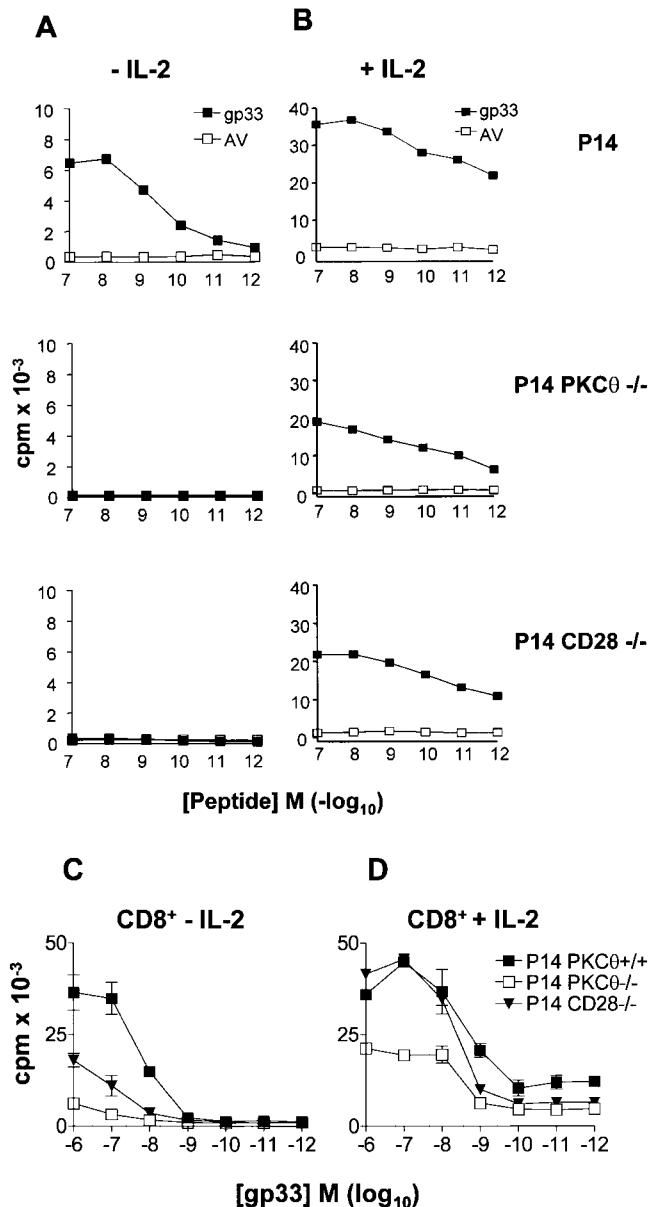
and pulsed with [^{3}H]thymidine. In the absence of additional IL-2, only splenocytes from the control P14 mice proliferated in response to antigen (Fig. 4 A). However, the proliferative response from P14 T cells was also reduced. This may reflect the possibility that P14 T cells were also on their way to becoming anergic, and the absence of CD28 or PKCθ may have altered the kinetics of the induction of anergy. When IL-2 was added to the culture, splenocytes from all three mice showed a strong proliferative response (Fig. 4 B). To confirm that this response was di-
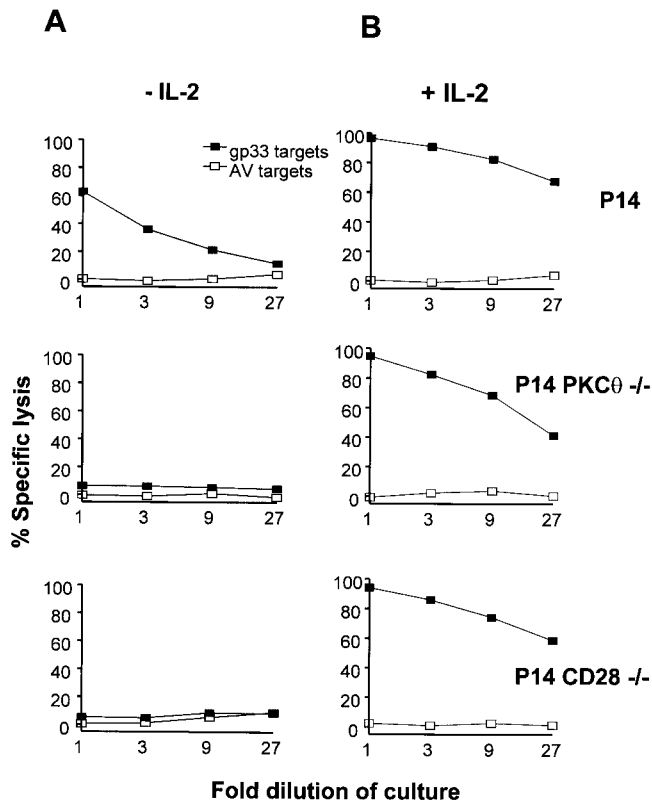
A **B**

- IL-2   + IL-2

gp33 targets
AV targets

P14

P14 PKCθ -/-

P14 CD28 -/-

% Specific lysis

Fold dilution of culture

Figure 5. CTL anergy in the absence of CD28 or PKCθ. P14, P14/PKCθ$^{-/-}$, and P14/CD28$^{-/-}$ mice were given 10 μg gp33 peptide in HBSS i.v. After 3 d, mice were killed, and total splenocytes were restimulated with gp33 in vitro peptide in the absence and presence of IL-2. After 3 d, cytotoxic activity was assayed with target cells pulsed with the gp33 peptide or the control nonstimulatory AV peptide.

rectly due to CD8$^+$ T cells, CD8$^+$ T cells were sorted from P14, P14/PKCθ$^{-/-}$, and P14/CD28$^{-/-}$ mice treated as aforementioned. Similar findings were observed (Fig. 4, C and D). This suggests that the P14/PKCθ$^{-/-}$ and P14/CD28$^{-/-}$ transgenic T cells taken from gp33 peptide–treated mice were anergic and could proliferate after the addition of exogenous IL-2.

Evaluation of CTL responses also supported the induction of anergy in T cells that did not express PKCθ or CD28. In the absence of IL-2, only splenocytes from the control P14 transgenic mice demonstrated an effective CTL response against gp33-pulsed target cells (Fig. 5 A). The addition of IL-2 allowed effective CTL activity from the P14/PKCθ$^{-/-}$ and P14/CD28$^{-/-}$ T cells (Fig. 5 B). Together, these data suggest that PKCθ signals prevent the induction of anergy in vivo.

It remained possible that the IL-2–dependent proliferative responses detected from the CD28$^{-/-}$ and PKCθ$^{-/-}$ T cells were a result of cells that never encountered antigen in vivo, and reflected an IL-2–dependent proliferative response of a small population of naive T cells from the spleen. To exclude this possibility, the following analysis was done in vitro. Sorted CD8$^+$ T cells from P14, P14/PKCθ$^{-/-}$, and P14/CD28$^{-/-}$ mice were labeled with

CFSE and stimulated for 3 d with macrophages pulsed with the gp33 peptide. All cells divided as seen by CFSE dilution (Fig. 6 A) and, therefore, had received an antigen-specific stimulus. These cells were counted, replated at 3×10^4 cells/well, and stimulated with 10^5 C57Bl/6 splenocytes/well, with or without IL-2 (Fig. 6, B and C). Both P14/PKCθ$^{-/-}$ and P14/CD28$^{-/-}$ T cells only proliferated upon the addition of IL-2, consistent with our previous results, which suggest that both CD28 and PKCθ signals are important to prevent the induction of anergy.

Various activation markers were also followed by flow cytometry by gating on the CFSE$^+$ population. After coculture with gp33, PKCθ-deficient T cells down-regulated Vα2 to similar levels as the P14 control cells (Fig. 6 A). This suggests that the absence of PKCθ did not affect the TCR triggering process, despite its central location in the SMAC. In addition, the up-regulation of CD69 was similar at early time points (Fig. 6 A, day 2). However, when the mean fluorescence intensity of CD69 expression was plotted against the different cell divisions, it was clear that maximal CD69 up-regulation does not occur in the absence of CD28 or PKCθ (Fig. 6 D). In addition, the level of CD69 expression on CD8$^+$ cells is reduced 3 d after stimulation of T cells from P14/PKCθ$^{-/-}$ and P14/CD28$^{-/-}$ mice, whereas expression is maintained on the P14 transgenic T cells from the wild-type background (Fig. 6 A). Therefore, the intensity and kinetics of CD69 expression are altered in the absence of PKCθ. These findings may explain the previously reported discrepancy in which one group has shown that PKCθ plays a role in CD69 up-regulation (13), whereas another group does not find impaired CD69 up-regulation in activated T cells that do not express PKCθ (12).

Our analysis also showed a reduction in the intensity of CD44 expression on activated PKCθ$^{-/-}$ and CD28$^{-/-}$ T cells (Fig. 6 A). By plotting the mean fluorescence intensity versus cell division, the data show that CD44 was not maximally up-regulated on PKCθ$^{-/-}$ or CD28$^{-/-}$ T cells (Fig. 6 E). Therefore, differences in various activation markers are strikingly similar in TCR transgenic T cells deficient for either CD28 or PKCθ. This suggests that the kinetics of T cell stimulatory signals are altered in the absence of the costimulatory CD28–PKCθ pathway and/or that the threshold of activation is increased in the absence of these molecules.

PKCθ-deficient T Cells Proliferate in Response to CD28 Costimulation In Vitro. The aforementioned analyses suggest that CD28–PKCθ relays signals that prevent the induction of anergy. To further understand the connections between downstream signaling pathways, we examined whether CD28 signals that augment proliferation were also defective in PKCθ$^{-/-}$ T cells. The cytoplasmic domain of CD28 has several sites that link to different signaling molecules, including PI3K, Grb2, and stress-activated protein kinase/JNK (for review see reference 40). To determine whether CD28 signals that promote proliferation are dependent on PKCθ, T cells from PKCθ$^{+/+}$, PKCθ$^{+/-}$, and PKCθ$^{-/-}$ mice were stimulated with different concentrations of anti-CD3 and/or anti-CD28 (Fig. 7). Surprisingly,

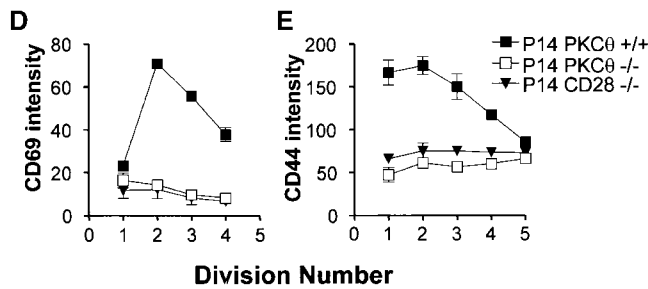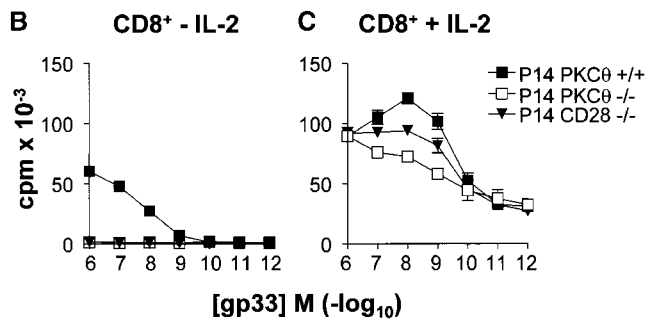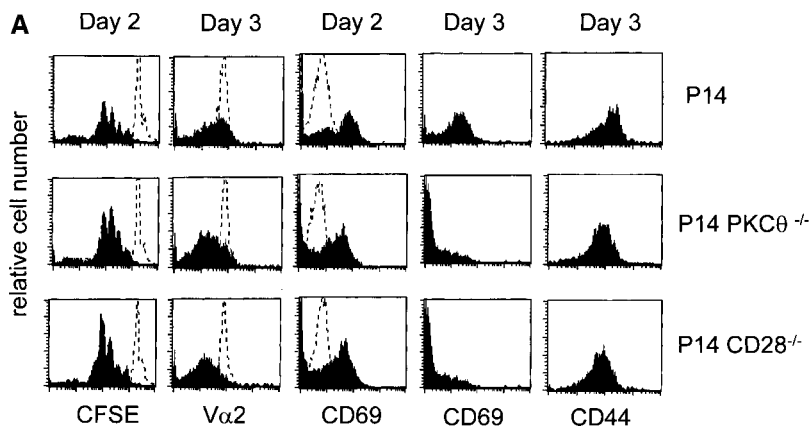**Figure 6.** Induction of anergy of PKCθ$^{-/-}$ T cells in vitro. Sorted CD8$^+$ T cells from P14, P14/PKCθ$^{-/-}$, and P14/CD28$^{-/-}$ mice were labeled with CFSE and stimulated with macrophages pulsed with the gp33 peptide for 3 d. Each day, cell division was followed by CFSE dilution, or CFSE$^+$ gated cells were analyzed with anti-Vα2, anti-CD69, and anti-CD44 as shown in A. Dashed lines indicate background staining on day 0. Solid lines indicate antibody staining for the corresponding days. These cells were counted and replated at 3 × 10^4 cells/well and stimulated with 10^5 C57Bl/6 splenocytes/well at the indicated peptide concentrations with (B) or without IL-2 (C). The mean fluorescence intensity of CD69 (D) and CD44 (E) expression per cell division is also shown from cultures depicted in A.

anti-CD28 was able to enhance T cell proliferation in the absence of PKCθ. Therefore, CD28 signals that enhance proliferation are not absolutely dependent on PKCθ.
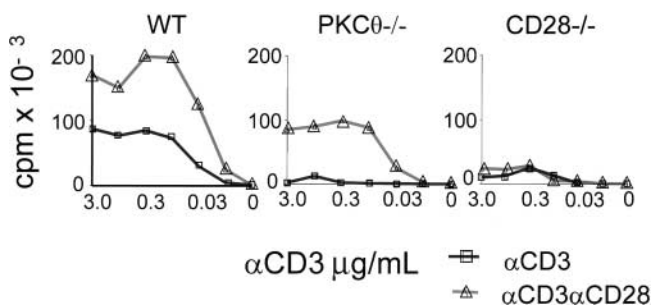

Figure 7. PKCθ-deficient T cells still respond to CD28 costimulatory signals. T cells from wild-type mice, PKCθ$^{-/-}$, or CD28$^{-/-}$ mice were stimulated with titrating amounts of anti-CD3 and anti-CD3/anti-CD28 (0.2 μg/ml). Proliferation was monitored by ^{3}[H]thymidine incorporation after 3 d in culture.

PKCθ Is Not Required for Th-dependent Antibody Isotype Switch or Germinal Center Formation. Previous papers have demonstrated that CD28 plays an important role in the production of neutralizing antibody responses as well as germinal center formation (33, 41, 42). To determine whether PKCθ had a role in these effector functions during antiviral responses, PKCθ$^{-/-}$, CD28$^{-/-}$, and control mice were immunized with VSV. Neutralizing anti–VSV titers were determined after 4 and 8 d. All mice had relatively high CD4-independent IgM titers 4 d after infection (Fig. 8 A). The CD4-mediated switch from IgM to IgG was impaired in the CD28-deficient mice at day 8. However, PKCθ-deficient mice showed a normal neutralizing antibody response to VSV, including a normal switch to IgG.

We also compared the formation of germinal centers in the absence of CD28 or PKCθ. As expected, CD28$^{-/-}$ spleens stained with PNA showed no evidence of germinal center formation (Fig. 8 B). However, the spleens from both control mice and PKCθ-deficient mice demonstrate normal formation of germinal centers (Fig. 8 B). Together,
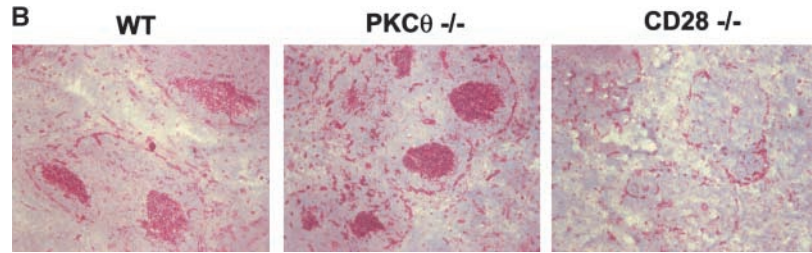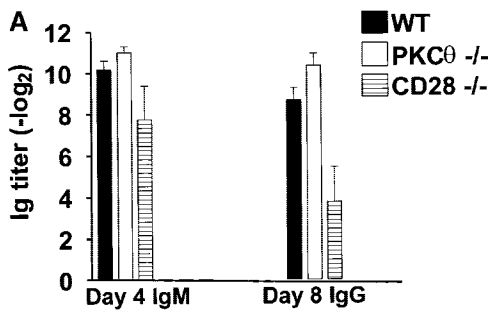
Figure 8. CD28 but not PKCθ is required for germinal center formation. WT, PKCθ^{−/−}, and CD28^{−/−} mice were immunized with VSV Indiana. (A) Serum was collected 4 and 8 d after immunization. Neutralizing IgM and IgG titers were determined. Error bars represent the SD. (B) Sections from the spleen of mice infected with VSV for 12 d were stained with PNA to detect germinal centers.

these data demonstrate that PKCθ does not play a role downstream of CD28 in the generation of neutralizing antibodies or germinal center formation.

Discussion

The Requirement for PKCθ Can Be Overcome by TCR Signals Combined with Costimulation by Pathogens. Previous papers suggested that PKCθ is crucial for T cell activation because PKCθ was the only reported PKC isoform found in the SMAC, and PKCθ-deficient T cells have impaired responses upon TCR triggering (12, 13, 17). Therefore, it was surprising that the LCMV response in PKCθ-deficient mice was comparable to wild-type mice (Fig. 1). The finding that in vitro responses are reduced, whereas in vivo responses to LCMV are normal has been characteristic for T cells from several gene-deficient mice, including CD28^{−/−} and calcineurin α[−/−] mice (33, 43). Studies with CD28^{−/−} mice suggested that the extended presence of antigen generated by viral replication was at least in part sufficient to overcome the induction of anergy in vivo (38). Because LCMV is a natural mouse pathogen, it is also possible that this virus can activate the innate immune system. Appropriate pathogen-derived "costimulatory" signals together with antigen-specific signals may lead to efficient T cell activation. In the absence of PKCθ, T cells may find alternate pathways of costimulation that augment effective T cell activation.

PKCθ: Linking T Cell Activation Thresholds, Costimulation, and Anergy. Optimal T cell activation in terms of the efficiency and kinetics of effector function can be influenced by antigen dose, the affinity of antigen (dissociation constant), and costimulation (38, 44–48). Previous work has shown that CD28 costimulatory signals influence T cell activation thresholds by altering the number of TCRs that need to be triggered for activation (24, 25). Studies have also shown that suboptimal TCR signals, such as altered peptide ligands or low avidity stimulation, may also lead to the induction of anergy (49, 50). Collectively, these experiments suggest that suboptimal stimulation by decreasing costimulatory signals or decreasing TCR stimulation (or the sensitivity of the TCR) may also lead to the induction of anergy. In this regard, PKCθ is a key candidate molecule that links these concepts because it alters the threshold for T cell activation (Figs. 2 and 7) and regulates the induction

of anergy (Figs. 4–6). However, one concern is whether the T cell proliferation and CTL function restored by the addition of IL-2 reflects a true rescue from anergy. Instead, there is a possibility that it represents the restoration of IL-2–dependent T cell function that was lacking under the suboptimal signaling conditions occurring in the absence of PKCθ. Therefore, it will be useful to examine molecular markers that characterize anergic CD8^{+} T cells because this may help to discriminate whether suboptimal signals alter T cell activation thresholds, anergy, or both.

Role of PKCs in the Induction of Lymphocyte Function or Anergy. The role of PKCs in lymphocyte function has been recently addressed in multiple studies. PKCδ, another member of the novel PKC subfamily, is predominantly expressed in the thymus, lymph nodes, spleen, intestine, and cerebellum (51). The absence of PKCδ leads to a B cell–mediated autoimmune disease characterized by spontaneous germinal center formation, multiorgan inflammation, and autoantibody production. Underlying defects in the induction of B cell anergy were seen, together with an increase in NFκB activation and IL-6 production (51, 52). These papers suggest that in B cells PKCδ negatively regulates NFκB activation and maintains self-tolerance by controlling the function of anergic B cells. This is in contrast to the role of PKCθ in T cells in which PKCθ-mediated signals are important in preventing anergy and act as a positive regulator of NFκB.

Other analyses have shown that B cell proliferation is impaired in the absence of another PKC isoform, PKCβ (53). In addition, PKCβ regulates the recruitment of IκB kinase α to lipid rafts and, therefore, acts as positive regulator of NFκB activation (54, 55). The absence of PKCβ also results in the inability to up-regulate Bcl-X$_L$ and Bcl-2 expression after BCR stimulation (54, 55). The ability of PKCβ to regulate NFκB and Bcl-X$_L$ is reminiscent of the activity described for PKBα in T cells (56). Notably, PKCθ and PKBα have been shown to interact in T cells and potentially synergize in vitro (57). In addition, studies have shown that PKCθ and IKKs coimmunoprecipitate after anti-CD3 and anti-CD28 stimulation in CD4 human T cells (19). Therefore, it is possible that PKCβ and PKCθ have similar roles in B cells and T cells, respectively.

This paper has demonstrated that PKCθ is important for preventing the induction of anergy in vivo. Previous analy-

ses have investigated CD4^{+} T cell subsets found in aged mice that express high levels of P-gp (58). Interestingly, this subset was found to be unresponsive to T cell stimulation in vitro, and no longer recruited PKCθ to the SMAC. These investigators associated T cell anergy with the absence of PKCθ.

PKCθ has also been shown to regulate genes that have been associated with T cell anergy in different models. PKCθ has been shown to be important in the activation of the transcription factor AP-1 (3, 13). Early work investigating the molecular mechanism of anergy using a mouse T cell clone rendered anergic by the addition of Concanavalin A has shown that AP-1 is a relevant molecular target in vitro (59). Further analyses have confirmed these findings and shown that these anergic T cells have a block in extracellular signal–regulated kinase and JNK signaling, therefore, preventing AP-1 activation (60–62). Both CD28 and PKCθ also regulate the activation of NFκB, and evidence also supports a role for impaired NFκB signaling in anergic B and T cells (60, 63). Because PKCθ can regulate both AP-1 and NFκB, we propose that it is the apical molecule that regulates the intracellular signal for preventing anergy in vivo.

Link between CD28 and PKCθ. Biochemical evidence from cell lines and immunofluorescence microscopy has demonstrated that CD28 together with TCR specific signals clearly increase the recruitment and activation of PKCθ and downstream transcription factors (4, 14, 15). However, there have not been any studies comparing the consequence of the absence of CD28 and PKCθ signaling in immune responses in vivo. In this paper, we have demonstrated that PKCθ plays an important role in preventing the induction of anergy in vivo and is strikingly similar to the role of CD28 costimulation in preventing anergy (38). Because lck plays a role in PKCθ activation (14, 64), and because CD28 can prolong lck autophosphorylation (65), it is possible that CD28 contributes to PKCθ activation via lck.

It is also notable that proliferative responses in T cells deficient for PKCθ still remain sensitive to CD28-mediated costimulation (Fig. 7). Therefore, it is possible to uncouple TCR/CD28-induced proliferative signals from those signals that lead to the induction of anergy.

Infection of PKCθ-deficient mice with VSV has further dissected the signaling pathways downstream of CD28. CD28-deficient mice have an impaired CD4-dependent neutralizing antibody response to VSV (33), whereas neutralizing antibody titres in PKCθ-deficient mice are comparable to wild-type animals (Fig. 8 A). In addition, CD28^{−/−} mice have impaired germinal center formation after VSV infection (42), whereas the PKCθ^{−/−} mice have normal germinal centers (Fig. 8 B). Previous studies using transgenic mice expressing CD28 with a mutation in the tyrosine residue at position 170 (tyrosine to phenylalanine) demonstrated that disrupting the signaling pathway downstream of Tyr170, did not alter the response to VSV. Normal neutralizing antibody production and germinal center formation was seen in mice expressing the mutation at the Tyr170 (29). Therefore, although the absence of CD28 leads to impaired T/B collaboration in vivo, CD28 signaling via Tyr170 or PKCθ does not play an essential role in these processes. Clearly, PKCθ is responsible for a defined subset of signals downstream of CD28, leading to defined alterations in immune function. In contrast, CD28 has a very complex signaling network that is integrated in many aspects of immune function in vivo.

Concluding Remarks. This paper begins to identify how signaling pathways trigger different aspects of T cell activation in vivo. These studies identified a potential role for PKCθ in preventing the induction of anergy and indicated that PKCθ is a key molecule in modulating T cell activation versus anergy. PKCθ becomes a novel target to regulate the induction of anergy versus activation in vivo. It will be important to determine the molecules and pathways that regulate the activity of PKCθ during T cell activation. Our studies also demonstrate that not all CD28-mediated signals are channeled through PKCθ. Costimulatory signals that augment T cell proliferation as well as T/B collaborative signals that enhance IgM/IgG switch and germinal center formation are independent of CD28–PKCθ pathways. Further in vivo studies will be required to identify how key signaling molecules and pathways interact to regulate immunity.

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