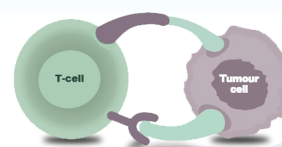


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Regulation of Asymmetric Division and CD8⁺ T Lymphocyte Fate Specification by Protein Kinase C ζ and Protein Kinase C μ

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During an immune response against a microbial pathogen, activated naive T lymphocytes give rise to effector cells that provide acute host defense and memory cells that provide long-lived immunity. It has been shown that T lymphocytes can undergo asymmetric division, enabling the daughter cells to inherit unequal amounts of fate-determining proteins and thereby acquire distinct fates from their inception. In this study, we show that the absence of the atypical protein kinase C (PKC) isoforms, PKC ζ and PKC μ , disrupts asymmetric CD8⁺ T lymphocyte division. These alterations were associated with aberrant acquisition of a pre-effector transcriptional program, detected by single-cell gene expression analyses, in lymphocytes that had undergone their first division *in vivo* and enhanced differentiation toward effector fates at the expense of memory fates. Together, these results demonstrate a role for atypical PKC in regulating asymmetric division and the specification of divergent CD8⁺ T lymphocyte fates early during an immune response. *The Journal of Immunology*, 2015, 194: 2249–2259.

The generation of effector and memory CD8⁺ T lymphocyte subsets is a key function of an adaptive immune response against microbial pathogen. Short-lived, terminally differentiated effector T (T_{SLE}) cells provide acute host defense, whereas memory lymphocytes provide long-term protection against reinfection (1). Among long-lived memory lymphocytes, effector memory T (T_{EM}) cells patrol the peripheral tissues and execute immediate effector functions after re-encountering microbe, whereas central memory T (T_{CM}) cells patrol the lymphoid tissues and retain the capacity to proliferate upon rechallenge (2).

Recent reports have suggested that another lymphocyte subset, so-called long-lived effector T cells, may persist into the memory phase and mediate a potent protective response upon reinfection, but these cells seem to lack the same capacity for long-term survival as T_{EM} or T_{CM} cells (3, 4).

Although it has been shown that a single activated naive CD8⁺ T cell can generate all of the diverse cellular fates necessary for a robust immune response (5, 6), it remains unclear when the differentiation pathways leading to these disparate cellular fates diverge. One possibility is that the progeny of an activated CD8⁺ T lymphocyte progress along a linear differentiation pathway, initially becoming effector cells, with a subset of these cells later acquiring the memory fate (7). Another possibility is that an activated CD8⁺ T cell might undergo asymmetric division, thereby enabling lymphocyte fates to diverge early during an immune response (8–10). During asymmetric division, cellular components and fate determinants are unequally partitioned into the two daughter cells, which may subsequently acquire distinct fates as a result of differences in size, morphology, gene expression, or protein abundance (11). In T lymphocytes, potential fate determinants that undergo asymmetric partitioning during the first division include the transcription factor T-bet and the IL-2 and IFN- γ receptors (8–10). Because signals downstream of these pathways have been implicated in effector CD8⁺ T cell differentiation (12–17), these observations suggest a key role for asymmetric division in regulating CD8⁺ T lymphocyte fate specification.

Asymmetric division has been shown to control fate specification of many different cell types and tissues in *Caenorhabditis elegans* embryos and *Drosophila* neuroblasts (11, 18, 19). In these model systems, evolutionarily conserved polarity proteins, most notably atypical protein kinase C (aPKC), have been shown to regulate asymmetric cell division and, in turn, control the balance between terminal differentiation and self-renewal (20–22). Chemical inhibition and small interfering RNA knockdown approaches have suggested a similar role for aPKC in the regulation of asymmetric division by CD8⁺ T cells (9, 23), but the extent and specific role of

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Abbreviations used in this article: aPKC, atypical PKC; BCL2, B cell lymphoma 2; CMAC, 7-amino-4-chloromethylcoumarin; IRF4, IFN regulatory factor 4; KLRG1, killer-cell lectin-like receptor G1; Lm-OVA, *Listeria monocytogenes*-OVA; MTOC, microtubule-organizing center; PCA, principal component analysis; PKC, protein kinase C; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{MP}, putative memory precursor T cell; T_{SLE}, short-lived effector T cell.

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each aPKC isoform, protein kinase C (PKC) ζ or PKC λ/μ , remain unanswered. Additionally, whereas both PKC ζ and PKC λ/μ have been implicated in CD4⁺ T cell differentiation (24, 25), it remains to be seen whether the aPKC isoforms regulate differentiation of CD8⁺ T cells. In this study, we show that PKC ζ and PKC λ/μ regulate asymmetric localization of effector fate-associated factors during the first CD8⁺ T cell division in vivo, thereby influencing the specification of T lymphocyte fates.

Materials and Methods

Mice

All animal work was done in accordance with the Institutional Animal Care and Use Guidelines of the University of California, San Diego. All mice were housed in specific pathogen-free conditions prior to use. *Prkcz*^{-/-} mice were obtained from the European Conditional Mouse Mutagenesis Program. *Prcki*^{fl/fl} mice (26) were bred to *Cd4*^{Cre} mice. *Prkcz*^{-/-} and *Cd4*^{Cre}*Prcki*^{fl/fl} mice were bred with OT-I TCR transgenic mice that recognize chicken OVA peptide SIINFEKL (residues 257–264)/K^b. Wild-type C57BL/6J recipient mice were purchased from The Jackson Laboratory.

CFSE labeling and in vitro cell culture

Splenocytes were isolated from OT-I mice and labeled with 5 μ M CFSE for 9 min at 37°C. Reactions were quenched with FBS, and CD8⁺ T cells were isolated with a negative selection magnetic microbeads kit (Miltenyi Biotec), according to the manufacturer's protocol. Splenocytes were harvested from wild-type mice and irradiated for 15 min at 3000 rad. T cells were depleted using magnetic microbeads (Miltenyi Biotec), and the remaining splenocytes were pulsed with 1 μ M OT-I peptide (SIINFEKL). Cells were cultured together for 3 d and analyzed on an Accuri C6 (BD Biosciences) flow cytometer with FlowJo software (Tree Star).

Immune synapse assay

Wild-type mice were injected i.p. with 10⁶ Flt3-ligand-secreting B16 tumor cells (27), which were provided by G. Dranoff (Dana Farber Cancer Institute, Boston, MA). Ten days postinjection, splenocytes were harvested and CD11c⁺ dendritic cells were isolated with a positive selection magnetic microbeads kit (Miltenyi Biotec), according to the manufacturer's protocol. Following isolation, cells were labeled with 1 μ M CellTracker Blue 7-amino-4-chloromethylcoumarin (CMAC) dye for 30 min at 37°C. Reactions were quenched with FBS, and cells were pulsed with 1 μ M OT-I peptide for 1 h at 37°C. Splenocytes were isolated from OT-I mice, and CD8⁺ T cells were isolated as above. CD11c⁺ cells and CD8⁺ T cells were incubated together for 30 min at 37°C and then prepared for immunofluorescence, as described below. Dendritic cell–T cell conjugates were identified by the presence of an unlabeled cell (T cell) contacting a CMAC-labeled cell (dendritic cell).

Adoptive cell transfers and infections

For primary infections, 5 \times 10³ OT-I CD45.1⁺CD8⁺ T cells were adoptively transferred into wild-type CD45.2⁺ recipients, followed by infection i.v. 1 d later with 5 \times 10³ CFU *Listeria monocytogenes* expressing full-length chicken OVA (Lm-OVA). Blood was collected from mice at 7, 14, 35, or 50 d postinfection in 5 mM EDTA solution. Blood samples were lysed with Red Cell Lysing Buffer (Sigma-Aldrich) for 15 min before staining for indicated markers. Splenocytes were isolated from recipient mice at 7, 14, 35, or 50 d postinfection. For rechallenge experiments, 10⁴ memory OT-I CD45.1⁺CD8⁺ T cells were adoptively transferred into wild-type CD45.2⁺ recipients, followed by infection i.v. 1 d later with 10⁵ CFU Lm-OVA. Blood was collected on day 8 postrechallenge and analyzed as above. To measure bacterial burden, spleens were removed on day 5 postrechallenge and homogenized. Duplicate serial dilutions were plated on brain–heart infusion (BD Biosciences) agar plates containing 1 μ g/ml erythromycin, and bacterial colonies were counted following incubation for ~36 h at 37°C. To isolate cells that had undergone their first division, 2 \times 10⁶ OT-I CD8⁺ T cells were first labeled with CFSE, as above, prior to adoptive transfer, and splenocytes from recipient mice were harvested at 48 h postinfection. Lymphocyte fate-tracking experiments were performed, as previously described (8). Briefly, 500 sorted IL-2R α ^{high} or IL-2R α ^{low} first-division cells were adoptively transferred into infection-matched recipients. At day 50 posttransfer, recipients were rechallenged with 10⁵ CFU Lm-OVA, and population expansion was tracked in the blood, as above. Cells were sorted on a MoFlo (Beckman Coulter) or FACS Aria II (BD Biosciences) flow cytometer.

T lymphocyte confocal microscopy

Immunofluorescence of T cells was performed, as previously described (10), with the following Abs: anti- β -tubulin (T8328) (Sigma-Aldrich); anti-IL-2R α (PC61.5), anti-T-bet (4B10), and anti-LFA-1 (M17/4) (eBioscience); anti-proteasome 20S C2 (ab3325) (Abcam); anti-IFN- γ R (2E2) (BioLegend); and anti-mouse Alexa Fluor 488, anti-rat Alexa Fluor 488, anti-mouse Alexa Fluor 647, anti-rabbit Alex Fluor 647, streptavidin Alexa Fluor 647, and anti-rat Alexa Fluor 647 (Life Technologies). DAPI (Life Technologies) was used to detect DNA. Premitotic cells were identified by a single microtubule-organizing center (MTOC) with β -tubulin staining; mitotic blasts were identified by the presence of two microtubule-organizing centers; and cells undergoing cytokinesis were identified by dual nuclei and pronounced cytoplasmic cleft by brightfield. Acquisition of image stacks was performed, as previously described (10), using a FV1000 laser-scanning confocal microscope (Olympus). The volume of three-dimensional pixels (voxels) containing the designated protein fluorescence was quantified within each hemisphere or within nascent daughter in cytokinetic cells, as previously described (10), using ImageJ software.

Abs and flow cytometry

The following Abs were used: T-bet (4B10), Eomes (Dan11mag), B cell lymphoma 2 (BCL2; BCL/10C4), IFN regulatory factor 4 (IRF4; IRF4.3E4), CD27 (LG.7F9), CD8a (53-6.7), CD45.1 (A20), CD62L (MEL-14), killer-cell lectin-like receptor G1 (KLRG1; 2F1), IL-7R (A7R34), CD44 (1M7), V α 2 (B20.1), CD4 (RM4-5), IFN- γ (XMG1.2), TNF- α (MP6-XT22), IL-2 (JES6-5H4), and F(ab')₂ anti-rabbit anti-IgG, and were obtained from BioLegend or eBioscience. Rabbit anti-T cell factor-1 (C63D9) Ab was obtained from Cell Signaling Technology. Anti-human PE-conjugated granzyme B (GB11) was obtained from Life Technologies. For intracellular staining of T-bet, Eomes, BCL2, TCF-1, IRF4, and granzyme B, Foxp3/Transcription Factor Staining Buffer Kit was used (eBioscience). For intracellular detection of IFN- γ , TNF- α , and IL-2, CD8⁺ T cells were stimulated for 6 h at 37°C ex vivo with 1 μ M OT-I peptide in the presence of brefeldin A (Sigma-Aldrich); cells were fixed in 4% paraformaldehyde (Electron Microscopy Services) and permeabilized before staining. All samples were analyzed on an Accuri C6 or FACSCanto (BD Biosciences) flow cytometer with FlowJo software (Tree Star).

Single-cell gene expression assays, data processing, and analysis

Single CD8⁺ T cells were sorted and analyzed in 96.96 Dynamic Arrays on a BioMark system (Fluidigm), as previously described (8). BioMark data processing and principal component analysis were performed, as previously described (8).

Results

Loss of PKC ζ or PKC λ/μ does not affect CD8⁺ T cell activation

To study the role of PKC ζ and PKC λ/μ in CD8⁺ T cell asymmetric division and differentiation, we used *Prkcz*^{+/+} (wild-type) and *Prkcz*^{-/-} (PKC ζ -deficient) mice or *Prcki*^{fl/fl} (wild-type) and *Cd4*^{Cre}*Prcki*^{fl/fl} (PKC λ/μ -deficient) mice. PKC ζ -deficient mice have been shown to develop a normal immune system (28), whereas conditional *Prcki*^{fl/fl} mice (26), which were used to overcome the previously reported embryonic lethality of germline *Prcki*^{-/-} mice (29), have yet to be characterized. To assess T cell development and homeostasis, thymus, spleen, and lymph nodes from pairs of wild-type and PKC ζ -deficient mice or wild-type and PKC λ/μ -deficient mice were analyzed. We observed similar percentages of double-negative, double-positive, and single-positive CD4 and CD8 T cells in the thymus (Fig. 1A) and similar percentages of CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes (Fig. 1B). Previous activation of CD8⁺ T cells to environmental Ags was assessed by staining for CD44 and CD62L to delineate between naive (CD44^{low}CD62L^{high}) and Ag-experienced (CD44^{high}) cells. Compared with wild-type controls, PKC ζ - and PKC λ/μ -deficient mice exhibited normal percentages of naive and Ag-experienced CD8⁺ T cells in the spleen and lymph nodes (Fig. 1C).

Previous reports have suggested that aPKC activity is required for effective scanning of dendritic cells (30) and polarization of

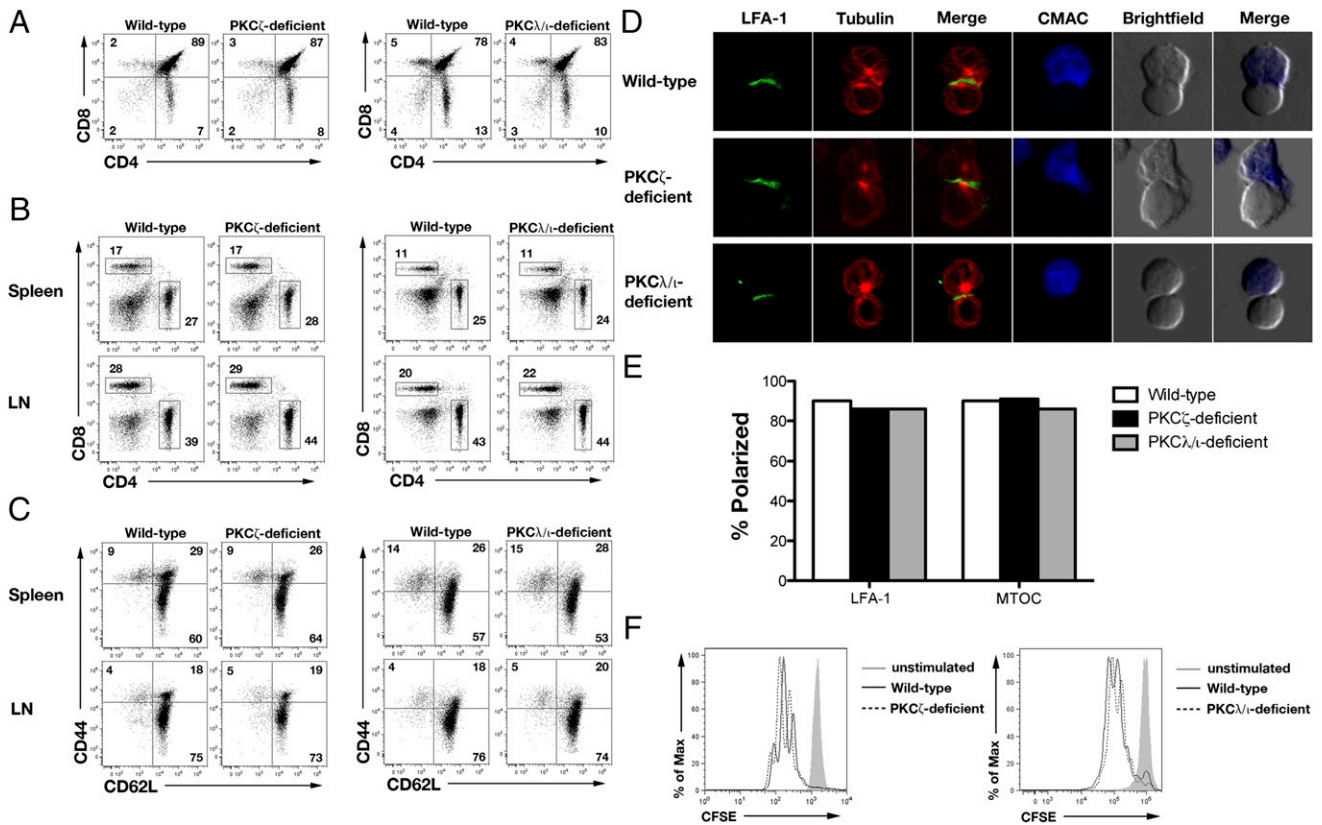


FIGURE 1. PKC ζ - and PKC λ/ι -deficient mice develop normal immune systems. **(A)** Frequencies of double-negative, double-positive, and single-positive CD4 and CD8 T cells from thymi of wild-type, PKC ζ -deficient, and PKC λ/ι -deficient mice. **(B)** Frequencies of CD4⁺ and CD8⁺ T cells from spleens and lymph nodes of wild-type, PKC ζ -deficient, and PKC λ/ι -deficient mice. **(C)** Frequencies of naive CD8⁺ T cells (CD62L^{high}CD44^{low}) and effector memory or central memory CD8⁺ T cells (CD62L^{low}CD44^{high} and CD62L^{high}CD44^{high}, respectively) from spleens and lymph nodes of wild-type, PKC ζ -deficient, and PKC λ/ι -deficient mice. **(D)** Confocal microscopy of LFA-1 (green) and β -tubulin (red) in wild-type, PKC ζ -deficient, or PKC λ/ι -deficient OT-I CD8⁺ T cells interacting with OVA-pulsed CD11c⁺ dendritic cells labeled with CellTracker Blue CMAC dye (blue) after 30 min of incubation. Original magnification $\times 40$. **(E)** Incidence of polarized localization of LFA-1 or the MTOC in wild-type ($n = 21$), PKC ζ -deficient ($n = 22$), or PKC λ/ι -deficient ($n = 21$) T cells toward a dendritic cell, as shown in (D). **(F)** CFSE dilution of unstimulated (gray filled), wild-type (solid black), and PKC ζ -deficient (dashed black) CD8⁺ OT-I TCR transgenic T cells (*left*) or unstimulated (gray filled), wild-type (solid black), and PKC λ/ι -deficient (dashed black) CD8⁺ OT-I TCR transgenic T cells (*right*) after 3 d in vitro culture with IL-2 and irradiated, T cell-depleted splenocytes pulsed with 1 μ M OVA peptide. Data are representative of three experiments.

surface receptors and T cell secretory machinery toward the immunological synapse in activated T cells (31–33), raising the possibility that loss of PKC ζ or PKC λ/ι might affect immunological synapse formation, activation, or proliferation of naive CD8⁺ T cells. We bred wild-type, PKC ζ -deficient, and PKC λ/ι -deficient mice with OT-I TCR transgenic mice to generate mice with CD8⁺ T cells that recognize aa 257–264 (SIINFEKL) of chicken OVA (34). Expression of the OT-I TCR did not affect T cell development in PKC ζ - or PKC λ/ι -deficient mice (Supplemental Fig. 1). To investigate the formation of immunological synapses, purified wild-type, PKC ζ -deficient, or PKC λ/ι -deficient naive CD8⁺ T cells were incubated in vitro for 30 min with purified OVA peptide-pulsed CD11c⁺ dendritic cells labeled with CellTracker Blue CMAC dye. Examination via confocal microscopy of LFA-1 and the microtubule-organizing center, polarization of which indicate a fully formed immunological synapse (35), revealed no deficiency in the ability of PKC ζ - or PKC λ/ι -deficient CD8⁺ T cells to polarize either component toward the interface shared with a dendritic cell, compared with wild-type controls (Fig. 1D, 1E). This finding suggests that PKC ζ and PKC λ/ι are not required for the initial engagement of dendritic cells or the establishment of a polarized immunological synapse by naive CD8⁺ T cells. To assess the ability of naive PKC ζ - or PKC λ/ι -deficient CD8⁺ T cells to respond to Ag following dendritic cell engagement, purified CD8⁺ T cells of each genotype

were labeled with CFSE and cultured in vitro with OVA peptide-pulsed splenocytes. We observed that PKC ζ - and PKC λ/ι -deficient CD8⁺ T cells proliferated comparably to wild-type controls (Fig. 1F), suggesting that Ag-induced activation of naive CD8⁺ T cells is not affected by the loss of either isoform. Together, these data show that loss of PKC ζ or PKC λ/ι does not alter development, immunological synapse formation, or activation of naive CD8⁺ T cells.

Loss of PKC ζ or PKC λ/ι impairs asymmetric CD8⁺ T cell division

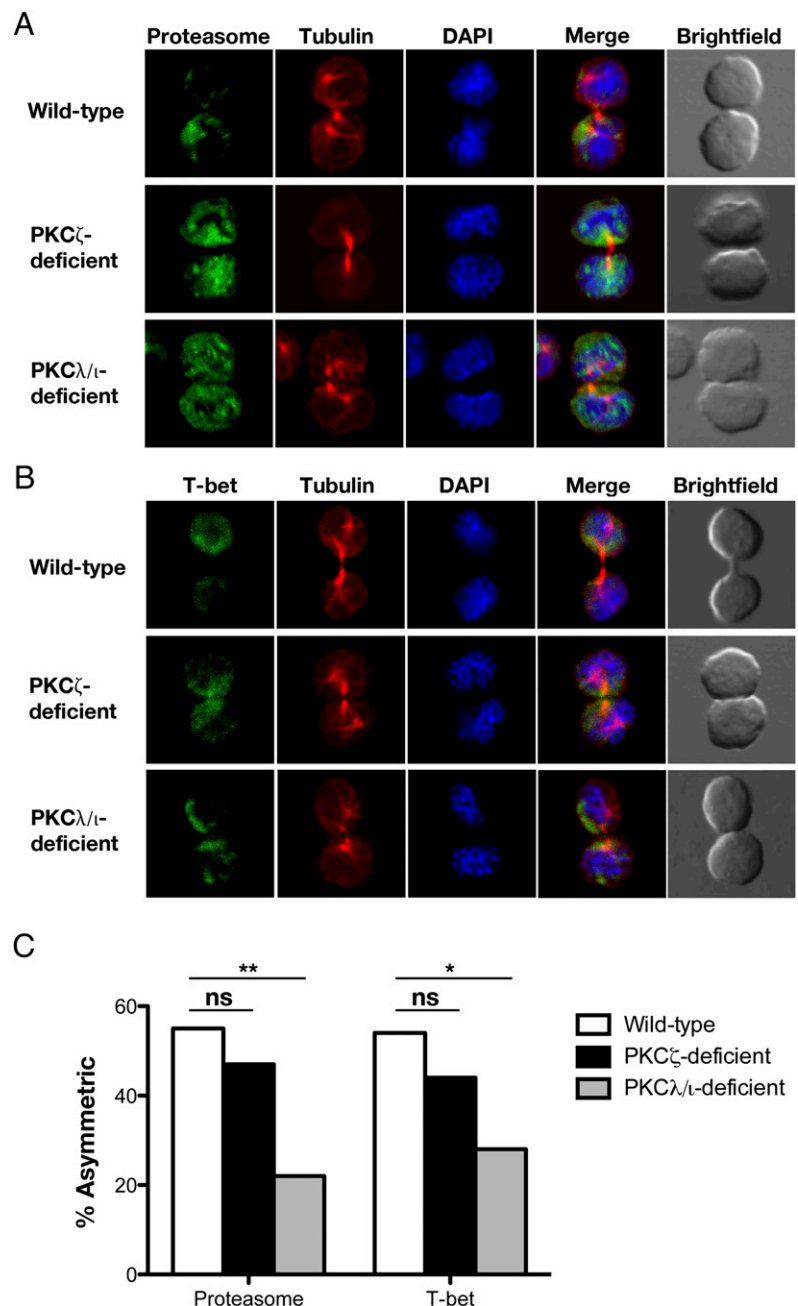
Previous reports have implicated aPKC in the regulation of asymmetric CD8⁺ T cell division (9, 23). It has been previously shown that asymmetric segregation of the proteasome degradation machinery during mitosis enables localized destruction of T-bet within a dividing CD8⁺ T lymphocyte, yielding daughter cells proximal or distal to the immunological synapse that inherit different amounts of T-bet and the proteasome. Knockdown or pseudosubstrate inhibition of PKC ζ resulted in a loss of proteasome asymmetry during mitosis, leading to the loss of preferential T-bet localization to the proximal daughter cell (9). To investigate the individual roles of PKC ζ and PKC λ/ι in the localization of these two factors during asymmetric CD8⁺ T cell division, we used a previously established in vivo method to isolate activated naive CD8⁺ T cells undergoing their first division in response to

a microbial pathogen (10). OT-I CD8⁺ T cells of each genotype were labeled with CFSE and adoptively transferred into mice that had been infected 24 h prior with recombinant Lm-OVA. Undivided donor CD8⁺ T cells were isolated by flow cytometry 36 h after adoptive transfer and examined by confocal microscopy. We observed a modest, but statistically insignificant decrease in proteasome and T-bet asymmetry in cytokinetic PKC ζ -deficient CD8⁺ T cells and a more prominent defect in asymmetry of these two components in cytokinetic PKC λ/ι -deficient CD8⁺ T cells (Fig. 2). This result raises the possibility that the prior experimental approach (9) targeting aPKC may have affected both isoforms, which is unsurprising given the high degree of homology between PKC ζ and PKC λ/ι (36).

Because aPKC has been established as a key regulator of asymmetric cell division in other model systems (37), we hypothesized that PKC ζ and PKC λ/ι might regulate the segregation of other T cell components that are known to localize asymmetrically

during the first division of an activated naive T cell (8, 10). IL-2R α and IFN- γ R are two such components and were selected for investigation because they have been shown to mediate signals that influence T cell fate decisions (14–17). Examination of these two cytokine receptors in premitotic cells showed that both receptors were polarized similarly in wild-type, PKC ζ -deficient, and PKC λ/ι -deficient CD8⁺ T cells early during activation, prior to division (Supplemental Fig. 2). During the first CD8⁺ T cell division, however, a decrease in the asymmetric localization of both proteins in PKC ζ - and PKC λ/ι -deficient CD8⁺ T cells was observed (Fig. 3), indicating that PKC ζ and PKC λ/ι are important for maintaining polarity through the late stages of CD8⁺ T cell activation. Taken together, these results suggest that the function of aPKC as a regulator of asymmetric division is conserved in CD8⁺ T cells, but PKC ζ and PKC λ/ι may have some nonredundant roles in segregating specific proteins unequally into the proximal or distal daughter cells.

FIGURE 2. Loss of PKC ζ or PKC λ/ι impairs asymmetric segregation of proteasome and T-bet during the first division of an activated naive CD8⁺ T cell. Confocal microscopy of (A) proteasome or (B) T-bet (green), β -tubulin (red), and DNA (blue; stained with the DNA-intercalating dye DAPI) in sorted wild-type, PKC ζ -deficient, or PKC λ/ι -deficient OT-I CD8⁺ T cells undergoing their first division after adoptive transfer into Lm-OVA-infected recipient mice. Original magnification $\times 40$. (C) Incidence of asymmetric protein localization from T cells shown in (A) and (B). The number of dividing cells from two experiments is indicated in parentheses, as follows (wild-type, PKC ζ deficient, PKC λ/ι deficient): proteasome (58, 30, 27) and T-bet (28, 34, 43). * $p < 0.05$, ** $p < 0.01$ (one-tailed unconditional Fisher's exact test). ns, not significant.



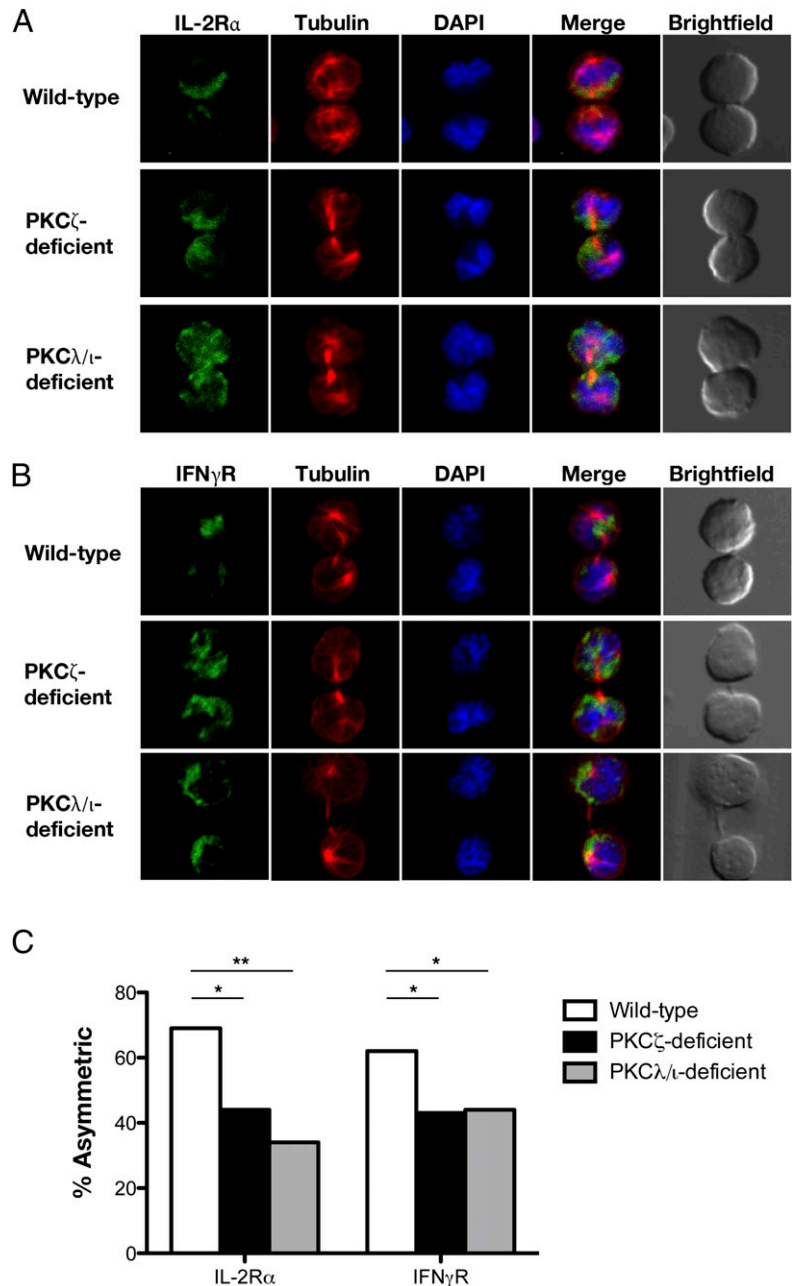


FIGURE 3. PKC ζ and PKC λ/ι regulate asymmetric localization of IL-2R α and IFN- γ R during the first division of an activated naive CD8⁺ T cell. Confocal microscopy of **(A)** IL-2R α or **(B)** IFN- γ R (green), β -tubulin (red), and DNA (blue) in sorted wild-type, PKC ζ -deficient, or PKC λ/ι -deficient OT-I CD8⁺ T cells prepared as in Fig. 2. Original magnification $\times 40$. **(C)** Incidence of asymmetric protein localization from dividing T cells shown in (A) and (B). The number of dividing cells from two experiments is indicated in parentheses, as follows (wild-type, PKC ζ deficient, PKC λ/ι deficient): IL-2R α (36, 36, 32) and IFN- γ R (78, 30, 41). * $p < 0.05$, ** $p < 0.01$ (one-tailed unconditional Fisher's exact test).

PKC ζ - and PKC λ/ι -deficient CD8⁺ T lymphocytes exhibit reduced differentiation into putative memory precursor T cells

Because signals downstream of T-bet, IL-2R α , and IFN- γ R facilitate the differentiation of effector T cells (12–17), we hypothesized that the symmetric distribution of these determinants during the first division of an activated naive CD8⁺ T cell would, as a result of PKC ζ or PKC λ/ι deficiency, alter subsequent differentiation into effector and memory T cells. To investigate this possibility, we adoptively transferred wild-type, PKC ζ -deficient, or PKC λ/ι -deficient OT-I CD45.1⁺CD8⁺ T cells into separate CD45.2⁺ wild-type recipients, which were infected 24 h later with Lm-OVA. Compared with wild-type CD8⁺ T lymphocytes, we observed no difference in the kinetics of the PKC ζ - or the PKC λ/ι -deficient CD8⁺ T cell response following infection (Fig. 4A, Supplemental Fig. 3A).

It has been previously shown that CD8⁺ T lymphocytes responding to microbial infection can be divided into T_{SLE} cells and putative memory precursor (T_{MP}) cells at 7 d postinfection (38). T_{SLE} cells can be identified by high expression of the lectin-

like receptor, KLRG1, and low expression of IL-7R (KLRG1^{high}IL-7R^{low}), whereas T_{MP} cells can be identified by reciprocally high expression of IL-7R and low expression of KLRG1 (KLRG1^{low}IL-7R^{high}) (38). We observed that mice that received PKC ζ - or PKC λ/ι -deficient CD8⁺ T cells exhibited an increase in the percentage of T_{SLE} cells and a decrease in the percentage of T_{MP} cells in both the blood and spleen at day 7 postinfection (Fig. 4B, Supplemental Fig. 3B). Additionally, increased numbers of T_{SLE} cells and decreased numbers of T_{MP} cells continued to be observed through day 35 postinfection in mice that received PKC ζ - or PKC λ/ι -deficient CD8⁺ T cells (Supplemental Fig. 3C, 3D). Despite these alterations in the differentiation patterns of PKC ζ - and PKC λ/ι -deficient CD8⁺ T cells, examination of key transcription factors, T-bet and Eomes, and the cytotoxic molecule, granzyme B, revealed no differences between PKC ζ - or PKC λ/ι -deficient CD8⁺ T cells and wild-type controls (Fig. 4C). Together these results show that aPKC regulates CD8⁺ T lymphocyte differentiation without affecting proliferation or expression of key effector-associated molecules.

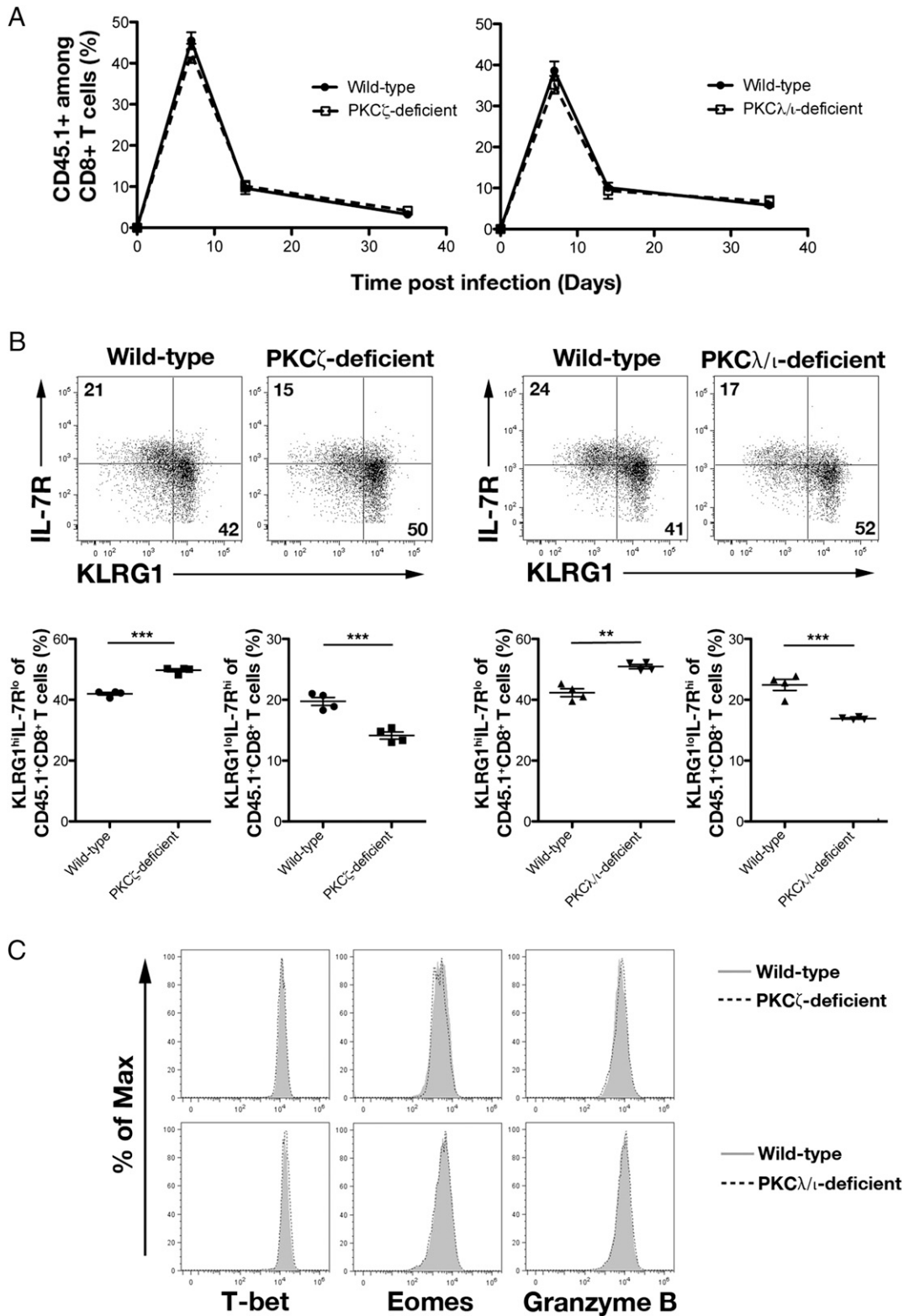


FIGURE 4. PKC ζ - and PKC $\lambda/1$ -deficient CD8⁺ T cells give rise to reduced KLRG1^{low}IL-7R^{high} T cells at day 7 postinfection. **(A)** Percentages of CD45.1⁺ cells of CD8⁺ T cells on days 7, 14, and 35 in the blood of mice that received 5×10^3 wild-type (closed circles) or PKC ζ -deficient (open squares) OT-I CD45.1⁺CD8⁺ T cells (*left*) or wild-type (closed circles) or PKC $\lambda/1$ -deficient (open squares) OT-I CD45.1⁺CD8⁺ T cells (*right*) and were infected with Lm-OVA; points represent mean \pm SEM; $n \geq 4$ /group. **(B)** Expression of KLRG1 and IL-7R by CD45.1⁺CD8⁺ T cells in the spleen on day 7 postinfection (*top*). Frequencies of KLRG1^{high}IL-7R^{low} and KLRG1^{low}IL-7R^{high} cells (*bottom*). Each point represents an individual mouse, and lines indicate the mean \pm SEM ($n = 4$ /group). ** $p < 0.01$, *** $p < 0.001$ (two-tailed unpaired *t* test). **(C)** Expression of T-bet, Eomes, and granzyme B by wild-type (gray filled) and PKC ζ -deficient (dashed black) CD45.1⁺CD8⁺ T cells (*top*) or wild-type (gray filled) and PKC $\lambda/1$ -deficient (dashed black) CD45.1⁺CD8⁺ T cells (*bottom*) in the spleen on day 7 postinfection. Data are representative of three experiments.

PKCζ and PKCλ/ι regulate differentiation into long-lived CD8⁺ T lymphocyte fates

Next, we investigated the differentiation of CD8⁺ T cells into memory lymphocytes. The decreased numbers of T_{MP} cells through day 35 postinfection in mice that received PKCζ- and PKCλ/ι-deficient CD8⁺ T cells (Supplemental Fig. 3D) suggested that there might be a corresponding decrease in the formation of memory lymphocytes at later time points. Indeed, at day 50 postinfection, mice that received CD8⁺ T cells lacking PKCζ or PKCλ/ι displayed a 1.5- to 2-fold reduction of CD62L^{high} T_{CM} cells in the blood and spleen with no changes in the percentages of total CD8⁺ T lymphocytes surviving into the memory phase (Fig. 5A, Supplemental Fig. 4A). Recent reports describing an additional subset of effector-like CD8⁺ T lymphocytes, termed long-lived effector T cells, that survives into the memory phase (3, 4) raised the possibility that the defective ability of PKCζ- and PKCλ/ι-deficient CD8⁺ T lymphocytes to become T_{CM} cells might be a consequence of enhanced differentiation toward this long-lived effector fate. Long-lived effector T cells are phenotypically KLRG1^{high}CD127^{low}

CD27^{low}CD62L^{low} (3, 4). We confirmed that KLRG1^{high} lymphocytes at day 50 postinfection in our system were also CD62L^{low} and CD27^{low} (Supplemental Fig. 4B, 4C) and therefore used CD27 expression to distinguish between the long-lived effector (CD27^{low}CD62L^{low}) and T_{EM} (CD27^{high}CD62L^{low}) cell subsets. Assessment of these CD8⁺ T lymphocyte populations revealed that mice receiving PKCζ- or PKCλ/ι-deficient CD8⁺ T lymphocytes had increased percentages of long-lived effector T cells and decreased percentages of T_{EM} cells (Fig. 5B).

As a result of this altered differentiation pattern, we observed a 2-fold reduction of PKCζ- and PKCλ/ι-deficient CD8⁺ T lymphocytes in lymph nodes compared with wild-type controls (Fig. 5C), consistent with the prior observation that CD62L expression is required for entry into secondary lymphoid organs (39). Moreover, because long-lived effector T cells mediate a more potent protective response while exhibiting minimal proliferation upon re-exposure to Ag (3, 4), we hypothesized that PKCζ- and PKCλ/ι-deficient CD8⁺ T lymphocytes surviving into the memory phase would maintain an ability to clear pathogen, but generate an impaired secondary pro-

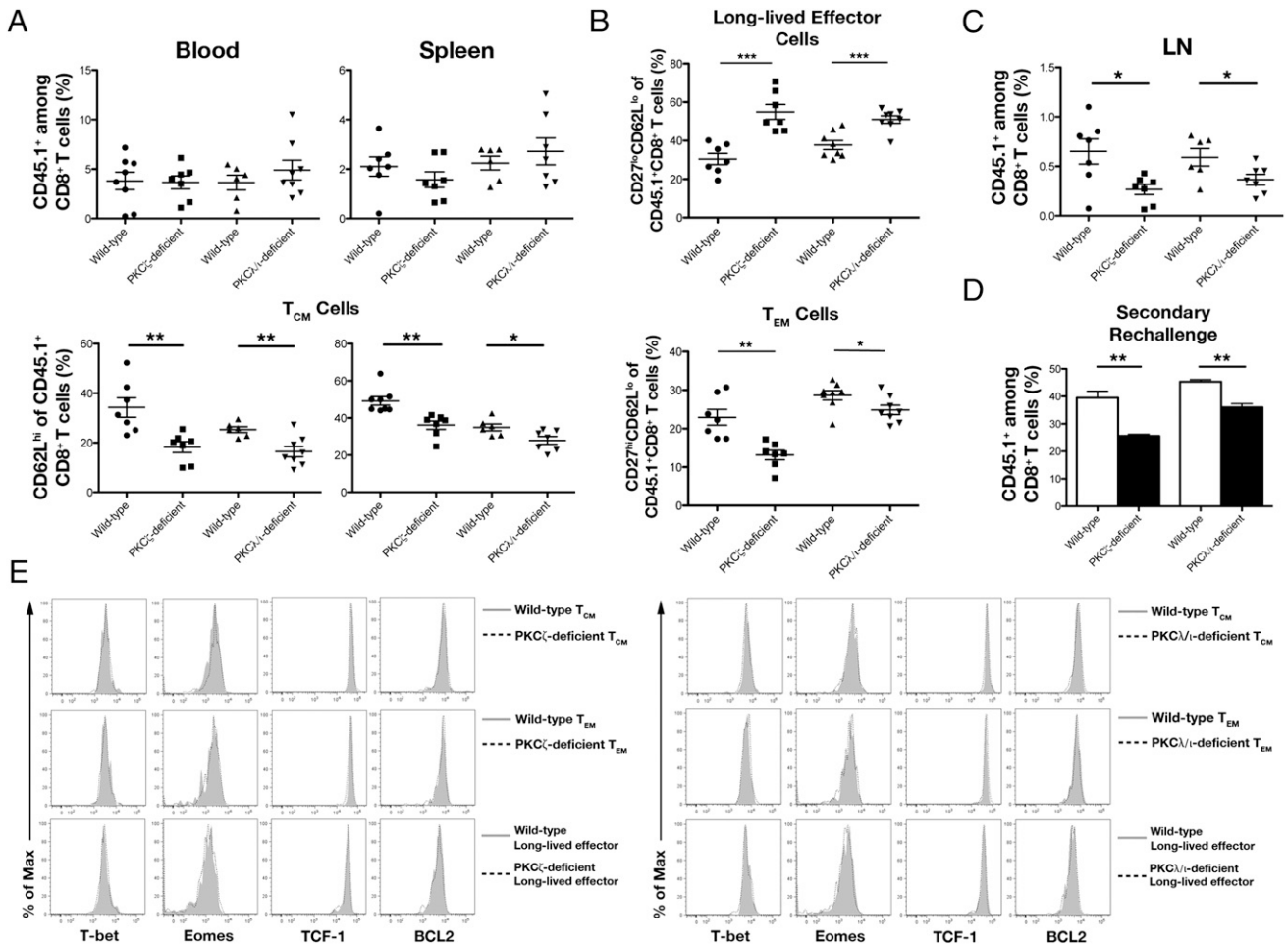


FIGURE 5. PKCζ and PKCλ/ι regulate central and effector memory CD8⁺ T cell differentiation. **(A)** Frequencies of CD45.1⁺CD8⁺ T cells (*top*) and CD62L^{high}CD45.1⁺CD8⁺ T_{CM} cells (*bottom*) in the blood and spleen on day 50 postinfection. Each point represents an individual mouse, and lines indicate the mean ± SEM (*n* = 6–7/group). **(B)** Frequencies of CD27^{low}CD62L^{low}CD45.1⁺CD8⁺ long-lived effector T cells (*top*) and CD27^{high}CD62L^{low}CD45.1⁺CD8⁺ T_{EM} cells (*bottom*) in the spleen on day 50 postinfection. Each point represents an individual mouse, and lines indicate the mean ± SEM (*n* = 7–8/group). **(C)** Frequencies of CD45.1⁺CD8⁺ T cells in the lymph nodes on day 50 postinfection. Each point represents an individual mouse, and lines indicate the mean ± SEM (*n* = 6–7/group). **(D)** Frequencies of CD45.1⁺CD8⁺ T cells on day 8 in the blood of mice that received 10⁴ wild-type, PKCζ-deficient, or PKCλ/ι-deficient CD45.1⁺CD8⁺ T cells on day 50 postinfection and were subsequently challenged with 10⁵ CFU Lm-OVA. Bars indicate the mean ± SEM (*n* = 4/group). **(E)** Expression of T-bet, Eomes, TCF-1, and BCL2. *Left*, Wild-type (gray filled) and PKCζ-deficient (dashed black) CD45.1⁺CD8⁺ T_{CM} cells (*top*), T_{EM} cells (*middle*), and long-lived effector T cells (*bottom*). *Right*, Wild-type (gray filled) and PKCλ/ι-deficient (dashed black) CD45.1⁺CD8⁺ T_{CM} cells (*top*), T_{EM} cells (*middle*), and long-lived effector T cells (*bottom*) in the spleen on day 50 postinfection. For (A)–(D), **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (two-tailed unpaired *t* test). Data are representative of two (D) or three (A–C, and E) experiments.

liferative response to reinfection. Indeed, PKC ζ - and PKC λ/ι -deficient CD8⁺ T lymphocytes did not display any changes in their ability to produce cytokine compared with wild-type controls (Supplemental Fig. 4D), and, upon adoptive transfer of equal numbers of CD45.1⁺ CD8⁺ T cells followed by secondary rechallenge, PKC ζ - and PKC λ/ι -deficient CD8⁺ T lymphocytes displayed similar abilities to clear bacteria in the spleen at day 5 postrechallenge compared with wild-type controls (Supplemental Fig. 4E). However, PKC ζ - and PKC λ/ι -deficient CD8⁺ T lymphocytes failed to proliferate in response to rechallenge as well as wild-type control cells (Fig. 5D). Taken together, these results demonstrate that, in the absence of PKC ζ or PKC λ/ι , CD8⁺ T lymphocytes differentiate toward a long-lived effector fate at the expense of the T_{CM} and T_{EM} cell fates.

We hypothesized that the altered differentiation patterns of PKC ζ - and PKC λ/ι -deficient CD8⁺ T lymphocytes might be due to a defect in the expression of key transcription factors and cell components within one or more of the long-lived T cell subsets. To investigate this possibility, we examined the expression of T-bet and Eomes in T_{CM}, T_{EM}, and long-lived effector T cells. We also assessed the expression of T cell factor 1 (TCF-1), which is important for differentiation and persistence of memory CD8⁺ T cells (40), and BCL2, an anti-apoptotic molecule thought to be important for T cell survival (41). Expression of these factors was similar between PKC ζ - or PKC λ/ι -deficient cells of each subset and their wild-type counterparts (Fig. 5E), suggesting that the increased differentiation of aPKC-deficient T lymphocytes into the long-lived effector fate was not caused by changes in transcriptional profiles at later time points.

PKC ζ and PKC λ/ι influence the transcriptional program of cells that have undergone their first division

The alterations in the types of effector and memory cells that differentiated in the absence of aPKC did not appear to be the result of defective expression of key effector- and memory-associated transcription factors at days 7 or 50 postinfection (Figs. 4, 5). These findings raised the possibility that disruptions to the transcriptional programs of aPKC-deficient T cells might be occurring early during the immune response. We hypothesized that the impaired asymmetric segregation of effector-associated molecules we observed in mitotic PKC ζ - and PKC λ/ι -deficient T lymphocytes (Figs. 2, 3) might result in a subsequent alteration in the transcriptional programs following the first division. To address this possibility, we performed single-cell gene expression analyses with Fluidigm 96.96 Dynamic Arrays in individual naive PKC ζ - or PKC λ/ι -deficient CD8⁺ T cells and cells that had completed their first division in vivo.

We used principal component analysis (PCA) to analyze the gene expression patterns of single naive and first-division PKC ζ - and PKC λ/ι -deficient CD8⁺ T cells compared with their wild-type counterparts. PCA is an unsupervised dimensionality reduction method that projects data into two dimensions by its coordinates in the first two principal components (PC1 and PC2). These principal components are linear combinations of the genes that account for the largest variations in the data. PCA revealed that naive T cells from each genotype clustered together (Fig. 6A), suggesting that any differences observed in the eventual fates of PKC ζ - and PKC λ/ι -deficient CD8⁺ T cells were not due to differences present prior to activation. Wild-type first-division cells exhibited substantial heterogeneity (Fig. 6A), reflective of distinct predispositions toward the effector and memory fates (8). In contrast, the majority of PKC ζ - and PKC λ/ι -deficient cells that had undergone their first division formed clusters that shared minimal overlap (Fig. 6A). This observation suggested that most of these cells lacking PKC ζ or PKC λ/ι were molecularly homo-

geneous and that the aPKC isoforms might have nonredundant roles in regulating asymmetric division.

This loss of molecular heterogeneity was driven, in part, by increased mRNA expression of *Irf4*, *Irf8*, and *Ii2ra* (Fig. 6A), which encode molecules related to effector T cell differentiation (14, 16, 17, 42–45). These changes in mRNA expression were accompanied by increased protein expression of IL-2R α and IRF4 by PKC ζ - and PKC λ/ι -deficient first-division CD8⁺ T cells (Fig. 6B). As we have previously shown that IL-2R α , in particular, may represent an early molecular switch in the differentiation pathway of an activated naive CD8⁺ T cell toward an effector fate (8), the finding of an increased proportion of IL-2R α ^{high} cells at the expense of IL-2R α ^{low} cells provides a potential mechanism underlying the impaired T_{EM} and T_{CM} differentiation observed in the setting of aPKC deficiency.

To test whether PKC ζ or PKC λ/ι deficiency might also alter cell fate once T lymphocytes have already diverged with respect to IL-2R α expression, sorted first-division IL-2R α ^{low} or IL-2R α ^{high} cells from wild-type or PKC λ/ι -deficient donor mice were adoptively transferred into infection-matched recipients, followed by a secondary rechallenge at day 50 posttransfer. Regardless of genotype, IL-2R α ^{low} cells exhibited robust proliferation in response to rechallenge, whereas IL-2R α ^{high} cells failed to do so (Fig. 6C). These results suggest that deletion of one aPKC isoform does not alter the fate of T lymphocytes once they have acquired differential amounts of IL-2R α . Instead, PKC ζ and PKC λ/ι appear to specifically regulate asymmetric division, which may allow each isoform to influence CD8⁺ T lymphocyte fate specification by controlling the relative proportion of IL-2R α ^{high} and IL-2R α ^{low} cells that are ultimately fated toward the effector and memory lineages.

Discussion

Asymmetric cell division has been previously observed in activated naive and memory lymphocytes (8–10, 23, 46–48), yet evidence for a functional role of asymmetric division in lymphocyte differentiation has been limited (49, 50). Our data suggest that asymmetric division by activated naive CD8⁺ T cells responding to a microbial infection may serve to exclude effector fate-promoting factors from distal daughter cells, thereby enabling them to initiate a prememory transcriptional program. In the setting of impaired asymmetric division, owing to the absence of either aPKC isoform, symmetric inheritance of key effector fate-promoting molecules seems to result in the acquisition of a pre-effector transcriptional program, detectable by single-cell gene expression analyses, by both daughter cells. This reduction in cells that would otherwise have acquired a prememory transcriptional program appears to yield an increased proportion of long-lived effector T cells at the expense of T_{CM} and T_{EM} cells.

Our results show that PKC ζ and PKC λ/ι are regulators of asymmetric CD8⁺ T cell division and indicate that each isoform may have some nonredundant roles. Regulation of proteasome and T-bet asymmetry during the first division appears to be PKC λ/ι specific. Nonetheless, loss of either isoform yields similar functional consequences with respect to CD8⁺ T lymphocyte differentiation during an immune response. Both PKC ζ and PKC λ/ι influence asymmetric partitioning of IL-2R α and IFN- γ R in activated naive CD8⁺ T cells undergoing their first division in vivo. Moreover, the absence of PKC ζ or PKC λ/ι in first-division CD8⁺ T cells seems to result in similar alterations in their transcriptional programs with a gain of genes that promote effector differentiation. Taken together, these results suggest that the shared functions of PKC ζ and PKC λ/ι may be more significant in determining CD8⁺ T lymphocyte fate than any unique role of either isoform.

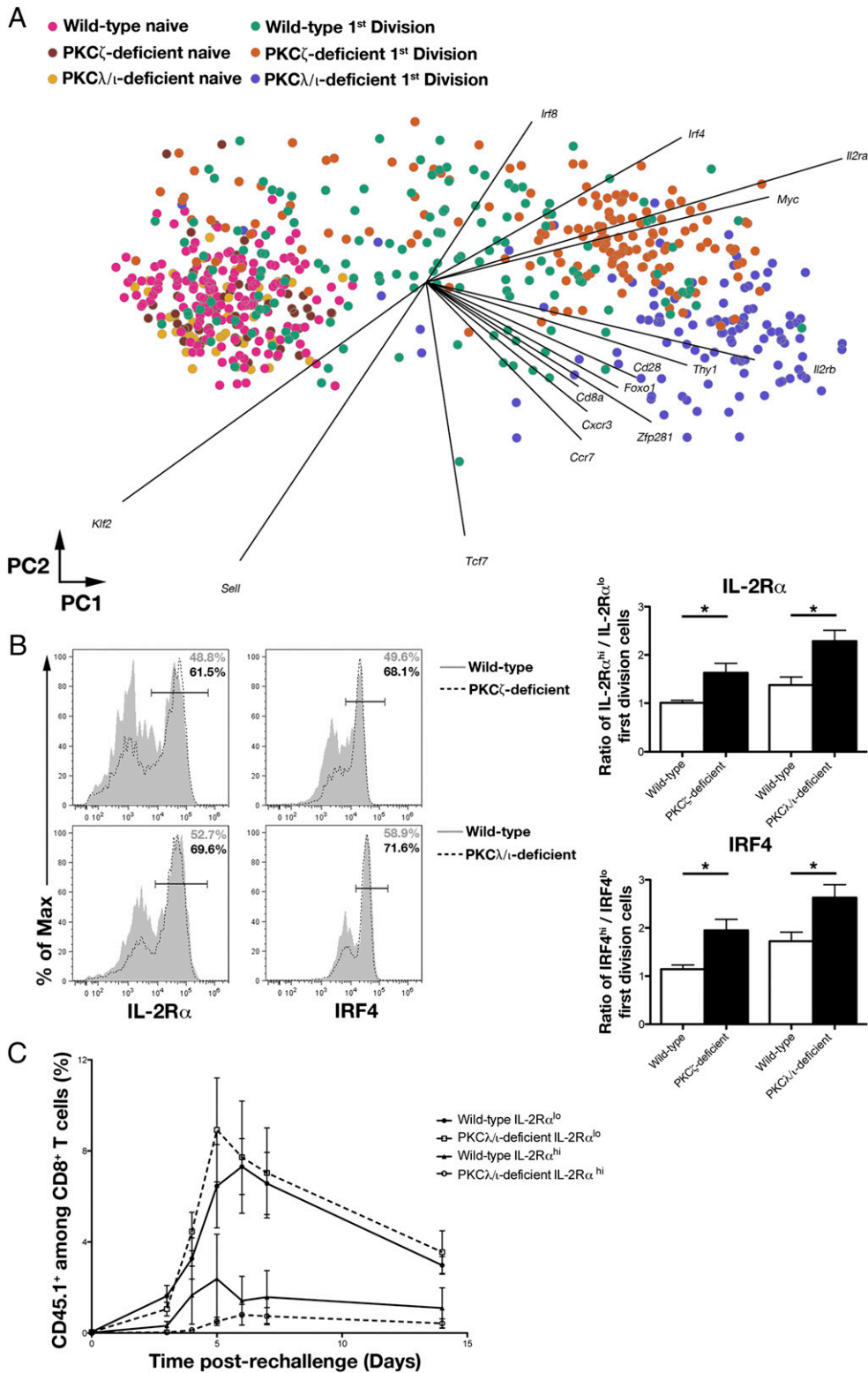


FIGURE 6. PKC ζ and PKC λ/ι regulate early molecular heterogeneity in cells that have undergone their first division in vivo. **(A)** PC projections (PC1, horizontal axis; PC2, vertical axis) for single-cell gene expression data derived from individual lymphocytes from populations of naive wild-type (pink), naive PKC ζ -deficient (brown), naive PKC λ/ι -deficient (yellow), first-division wild-type (teal), first-division PKC ζ -deficient (orange), and first-division PKC λ/ι -deficient (purple) OT-I CD8⁺ T cells sorted before or after adoptive transfer into Lm-OVA-infected recipient mice. Each circle represents an individual cell. Each vector emanating from the origin represents an individual gene. PC1 and PC2 account for 20 and 5% of the variance, respectively. **(B) Left**, Expression of IL-2R α and IRF4 by wild-type (gray filled) and PKC ζ -deficient (dashed black) first-division OT-I CD8⁺ T cells (*top*) or wild-type (gray filled) and PKC λ/ι -deficient (dashed black) first-division OT-I CD8⁺ T cells (*bottom*) after adoptive transfer into recipient mice and subsequent infection with Lm-OVA. **Right**, Ratios of IL-2R α ^{hi} and IL-2R α ^{lo} (*top*) or IRF4^{hi} and IRF4^{low} (*bottom*) first-division OT-I CD8⁺ T cells. Bars represent mean \pm SEM. **p* < 0.05 (two-tailed unpaired *t* test). **(C)** Population expansion of CD45.1⁺CD8⁺ T cells on days 3, 4, 5, 6, 7, and 14 postchallenge in blood obtained from Lm-OVA-infected CD45.2⁺ mice that received 500 sort-purified wild-type IL-2R α ^{low} (closed circles), wild-type IL-2R α ^{high} (closed triangles), PKC λ/ι -deficient IL-2R α ^{low} (open squares), or PKC λ/ι -deficient IL-2R α ^{high} (open circles) first-division CD45.1⁺CD8⁺ OT-I T cells on day 2 post-infection and were rechallenged with 10⁵ CFU Lm-OVA on day 50 posttransfer. Points represent mean \pm SEM (*n* = 3–4/group).

Through these shared functions in regulating asymmetric division, PKC ζ and PKC λ may enable the generation of first-division CD8⁺ T lymphocytes that are molecularly heterogeneous at the single-cell level. Our recent work suggested that this molecular heterogeneity may be indicative of pre-effector and prememory transcriptional programs that are predictive of the eventual fates of the cells (8). Lymphocytes that transition through a pre-effector state can undergo further differentiation to acquire the T_{SLE} fate, whereas cells transitioning through a prememory state can further diverge to give rise to T_{CM} or T_{EM} cells (8). The present findings suggest that disruption of asymmetric division by the absence of aPKC results in an increased proportion of first-division cells expressing high levels of IL-2R α and exhibiting a pre-effector transcriptional program.

This increased proportion of pre-effector cells was associated with an increased percentage of KLRG1^{high} cells, both at the peak of the immune response and in the memory phase. Because both T_{SLE} and long-lived effector T cells express high levels of KLRG1, one potential interpretation of these results is that a subset of T_{SLE} cells present at day 7 postinfection subsequently gives rise to long-lived effector T cells. Commitment to the T_{SLE} fate, however, is thought to correlate with increased proliferation of CD8⁺ T cells (51) and increased apoptosis following clearance of an infection (13). The lack of an alteration in the kinetics of the CD8⁺ T cell response in the absence of PKC ζ or PKC λ suggests that differentiation into T_{SLE} cells is unaffected by the loss of either aPKC isoform. An alternative possibility, therefore, is that cells transitioning through the pre-effector state (8) diverge in fate early during an immune response, giving rise to long-lived effector T cells as well as T_{SLE} cells that together comprise the KLRG1^{high} population at day 7 postinfection. The modest increase in the KLRG1^{high} population at day 7 postinfection may, therefore, represent a 2-fold change in long-lived T lymphocyte fates that has already occurred during an aPKC-deficient CD8⁺ T cell response.

Although aPKC-deficient CD8⁺ T lymphocytes exhibited increased differentiation toward the effector (T_{SLE} and long-lived effector) fates, the absence of aPKC did not result in a complete loss of memory (T_{CM} and T_{EM}) cells. The continued generation of aPKC-deficient T_{CM} and T_{EM} cells is most likely due to the continued presence of aPKC-deficient IL-2R α ^{low} prememory cells, which, unlike IL-2R α ^{high} pre-effector cells, maintain the capacity to differentiate into the memory fates following the first division in vivo. These prememory cells may be the product of asymmetric divisions still exhibited by some PKC ζ - and PKC λ -deficient cells, as the absence of either aPKC isoform did not completely disrupt the unequal segregation of IL-2R α . As such, PKC ζ - and PKC λ -deficient naive CD8⁺ T cells appear to give rise to reduced frequencies of IL-2R α ^{low} prememory cells, which subsequently reduce, rather than completely eliminate, memory differentiation. These findings indicate that the high and low expression of IL-2R α resulting from an asymmetric division may be important for the initial predisposition of CD8⁺ T lymphocytes to adopt either an effector fate or a memory fate. However, despite acquiring a tendency toward either the effector or the memory fates at the first division, specific commitment into a particular CD8⁺ T cell subset most likely requires additional input, signals that may include further exposure to cytokines (52), additional Ag encounter (53, 54), or migration into specific tissue sites during the immune response (55). Nonetheless, our results suggest that disruption of asymmetric division, via deletion of PKC ζ or PKC λ , alters the ratio of pre-effector and prememory cells, thereby influencing the transcriptional programming of first-division CD8⁺ T lymphocytes and the specification of effector and memory CD8⁺ T cell fates.

The finding that CD8⁺ T cells undergo asymmetric division (9, 10) suggested the possibility that lymphocyte fates may diverge early during an immune response to a microbial infection. Single-cell gene expression analyses revealed distinct molecular patterns, predictive of eventual fates, within lymphocytes that had undergone their first division in vivo (8). In this study, to our knowledge, we provide some of the first experimental evidence that an evolutionarily conserved regulator of asymmetric cell division influences CD8⁺ T lymphocyte fate specification by controlling unequal partitioning of fate-influencing molecules during mitosis. Disruption of asymmetric CD8⁺ T cell division, as a result of aPKC deficiency, was associated with striking changes in the transcriptional patterns exhibited by first-division lymphocytes and appeared to alter the balance of effector- and memory-fated progeny. These findings provide new evidence in support of the proposal that asymmetric division mediates a divergence in lymphocyte fates at the initiation of an adaptive immune response to microbial infection. Although the role of each CD8⁺ T cell subset in maintaining a long-term protective response is still being explored and debated (4, 56), the finding that asymmetric division plays a functional role in lymphocyte fate specification is likely to be important in understanding how to generate robust immunological protection against a variety of infectious diseases.

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Disclosures

The authors have no financial conflicts of interest.

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