Validation of monoclonal anti-PKC isozyme antibodies for flow cytometry analyses in human T cell subsets and expression in cord blood T cells

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T cells from neonates (cord blood) with a tendency to develop allergic diseases express low PKCζ levels. More extensive investigations into PKC isozyme levels in T cell subsets and changes during neonatal T cell maturation are hampered by limitations of Western blot analyses. We have undertaken to validating the specificity of commercially available antibodies marketed for flow cytometry to measure PKCα, βI, βII, δ, ε, η, θ, ζ, ι/λ and μ. Western blot analyses of human peripheral blood mononuclear cell (PBMC) lysates demonstrated that some antibodies were unsuitable for flow cytometry assays. A panel of antibodies with the desirable specificity and reliability in the flow cytometry assay were identified using both PBMC and whole blood assays. The results showed that all PKC isozymes were expressed in CD4+ and CD8+ T cells, monocytes and neutrophils. Murine lymphocytes showed similar patterns of expression. A major finding was that 35.2% and 38.5% of cord blood samples have low PKCζ (≤ the 5th percentile of adult levels) in the CD4+ and CD8+ subsets, respectively, consistent with the incidence of allergy development in the population. Furthermore, these low PKCζ levels ‘normalised’ within 24 h after initiation of maturation of these cells in culture, providing a ‘window of opportunity’ for altering PKCζ levels.

Protein kinase Cs (PKCs) are a family of phospholipid-dependent serine/threonine protein kinases which consists of at least 11 isoforms, each with tissue-specific distribution and individual functional properties. On the basis of structure, requirement for Ca2+ for activation and unique binding ability with phorbol myristate acetate (PMA) or diacylglycerol (DAG), they have been divided into three groups: classical or conventional PKC (consisting of PKCα, βI, βII and γ), novel (PKCδ, ε, η and θ) and atypical (PKCζ, ι/λ and μ) isozymes. The role of PKC isozymes in cell proliferation, differentiation1, motility and survival2 has been well established. Metabolic disorders3, immune immaturity4, cancer5,6 and cardiovascular malfunction7 are some of the diseases that have been associated with mutation/altered expression of specific PKC genes. Differences in levels of PKCs or the abnormal activation have been reported in autoimmune diseases, heart failure, acute and chronic heart disease, kidney and lung diseases, diabetes, various dermatological diseases, psychiatric diseases, cancer as well as neurological conditions8. Involvement of PKC in these abnormalities highlights the critical role that these kinases perform in cell signalling and regulation of health and disease development.

Reduced T cell PKCζ levels are associated with Th2 dominance at birth and the subsequent failure to develop towards T helper (Th)1 phenotype whereas high T cell PKCζ expression at birth was associated with non-allergy development in infants9–12. The studies also showed that the levels of PKCζ in cord blood T cells (CBTC) could be used as a biomarker for assessing whether children were likely to develop allergic diseases/sensitisation10,11. This
At their expected molecular weights, around 80 kDa, except for PKCε in Fig. 1b show the Western blot analyses of PBMC lysates probed with these antibodies. All isozymes were detected in the expected 115 kDa. In all cases there was a high level of specificity for each of these isozymes.

The analysis was performed by SSC and FSC properties of murine cells, after exclusion of doublets by FSC-H. Flow cytometry assays to measure intracellular levels of PKC isozymes. Following validation of their specificity and adaptation to flow cytometry assays, we demonstrated the presence of PKCα, β, δ, ε, η, ζ, ι, λ, θ and μ in T cell subsets, monocytes and neutrophils. These measurements could be made in whole blood assays. The antibodies were also able to detect all of these PKC isotypes in mouse splenic and peripheral blood lymphocytes. Examination of PKC isozyme levels in CBTC demonstrated that 30–40% (of the cord bloods) were deficient in PKCζ, a deficiency rate expected for high risk of allergy development in the population. Furthermore, we were able to show that these low levels rapidly normalise following the initiation of CBTC maturation in a culture model system.

Results

Specificity of anti-PKC isozyme antibodies. To validate the specificity of commercially available anti-PKC isozyme antibodies for flow cytometry, we examined their specificities against their respective targets by Western blot using lysates from human PBMC. Four of the antibodies (anti-PKCα, -PKCβ, -PKCλ and -PKCδ antibodies; clones E-5, E-3, E-7 and G-9; see Supplementary Table S1) that were designated for use in Western blot and flow cytometry assays were found not to be suitable for the flow cytometry assay (Fig. 1a). The anti-PKCα antibody was not able to detect the PKC isozyme in these lysates; that against PKCδ detected an additional protein and the anti-PKCλ showed several protein bands. The antibody to PKCδ also detected other proteins apart from this PKC isozyme. While these antibodies have been recommended for use in both Western blot and flow cytometry, it is evident that for human leukocytes they are not appropriate for use in the latter.

Further examination, we identified ten antibodies that had the specificities needed for the detection and estimation of PKCα, β, δ, ε, η, ζ, ι, λ, θ or μ by flow cytometry (clones outlined in Fig. 1 legend). The data in Fig. 1b show the Western blot analyses of PBMC lysates probed with these antibodies. All isozymes were detected at their expected molecular weights, around 80 kDa, except for PKCμ that migrated in SDS-PAGE gels at the expected 115 kDa. In all cases there was a high level of specificity for each of these isozymes.

Flow cytometry validation of the PKC isozyme measurements in whole blood assays. The above Western blot-validated antibodies were then examined for suitability in flow cytometry assays for detecting intracellular PKC isozymes. Anti-PKCα, β, δ and μ (AF647 conjugated) and PKCγ ε and δ (AF488) and PKCζ, η and λ (PE) conjugated antibodies were titrated in parallel with the isotype control. Supplementary Fig. S1 shows the gating strategies. Data for the titration of the anti-PKC antibodies is presented as signal-to-noise ratios in Fig. 2. The results showed that lymphocytes stained for all the PKC isozymes in a concentration related manner.

The data in Fig. 3 show the expression of the various PKC isozymes in both CD4+ and CD8+ T cells from healthy donors. There was a wide variation in levels of specific isozymes expressed between these individuals. However, the levels expressed in the different isozymes were similar between the two T cell subsets (Fig. 3). Further analyses on the monocyte and neutrophil populations showed also the expression of all the isotypes in these cells (Figs 4 and 5).

A small proportion of lymphocytes lacked PKCη (Fig. 5a). Further, analysis for PKCη in CD4+ and CD8+ T cells, B cells and NK cells revealed that these all expressed the PKC isozymes except for B cells which lacked PKCη (Fig. 5b). Examination of neutrophils and monocytes for all PKC isozymes revealed that neutrophils and monocytes express all PKC isozymes (Fig. 4; Fig. S3). The data displayed in Supplementary Fig. S3 show the differential expression of these isozyme in different leukocyte subpopulations. Thus PKCα, β, δ and μ are the most highly expressed in neutrophils, PKCδ and ε in monocytes and PKCη in T cells.

Analysis of PKC isozymes in murine lymphocytes. The analyses were extended to expression of the PKC isozymes in murine lymphocytes. The above antibodies specific for the PKC isozymes were used to examine their expression in murine splenic lymphocytes, using outbred Swiss white mice. Examination of lysates by Western blotting revealed a similar level of specificity (Fig. 6a) as for the human PBMC (Fig. 1). The unidentified lower molecular weight bands of approximately 25–35 kDa in the PKCα, β, δ and ε blots were unlikely to be due to degraded PKC or to non-specific staining by the primary antibodies since it was also detected in the absence of a primary antibody (Supplementary Fig. S4), suggesting that it is due to the secondary antibody. However, this would not have any implications in the flow cytometry assay which is based on direct staining of the PKC isozymes.

Flow cytometry analysis was conducted per human PBMC for detection of the different isoforms in splenic lymphocytes as well as in whole blood assays. As we were only interested in whether these antibodies were able to detect the isoforms in murine lymphocytes by flow cytometry assay, no subset characterisation was undertaken. The analysis was performed by SSC and FSC properties of murine cells, after exclusion of doublets by FSC-H and FSC-A. All PKC isozymes were detected in murine lymphocytes from blood and splenic cells (Fig. 6b,c). Furthermore, like human leukocytes, mouse blood lymphocytes and splenic cells showed two distinct populations based on presence or absence of PKCη (Fig. 6b,c). A more prominent population of PKCη negative was seen in splenic than blood since the former contain ~50% B cells.
Levels of PKC isozymes in cord blood CD4⁺ and CD8⁺ T cells, monocytes and neutrophils.

Previously, we have examined, by Western blot analyses, the levels of a limited number of PKC isozymes in T cells from cord bloods of subjects with a family history of allergic diseases. Here we have used a non-selected antibody approach to examine the levels of all PKC isozymes in T cells, monocytes, and neutrophils from cord bloods of healthy newborns.

**Figure 1.** Specificity of the anti-PKC isozyme monoclonal antibodies. Lysates from PBMC from human blood of adult volunteers were subjected to Western blot analysis using isoenzyme-specific monoclonal anti-PKC antibodies as indicated in figures. These were developed with an HRP-conjugated anti-mouse/anti-rabbit IgG antibody. (a) Shows the reactivity of antibodies recommended for flow cytometry. The blots show either one or two samples (lanes 1 and 2) with blots stripped and reprobed to detect GAPDH. These antibodies either did not show any immunoreactivity towards the intended PKC isozyme or they showed non-specific binding to multiple proteins. Antibodies: anti-PKCε, -PKCβI, -PKCλ/ι and -PKCδ antibodies were from clones E-5, E-3, E-7 and G-9; (Supplementary Table S1). (b) Specificity of antibodies selected for flow cytometry assay. These anti-PKC antibodies were selected based on their specificities, as they each showed specific binding to a single protein band. The clones for the anti-PKCα (H-7), βI (EPR18512), βII (F-7), δ (EPR17075), ε (EPR1482(2)), η (EPR18513), θ (E-7), ζ (H-1), ι/λ (H-12) and μ (EP1493Y) are shown in parentheses (see also Supplementary Table S1). The blots are presented individually for each staining, intact without splicing, with lanes in their entirety.

Previously, we have examined, by Western blot analyses, the levels of a limited number of PKC isozymes in T cells from cord bloods of subjects with a family history of allergic diseases. Here we have used a non-selected approach to examine the levels of all PKC isozymes in T cells, monocytes, and neutrophils from cord bloods of healthy newborns.
Figure 2. Signal-to-noise ratios of titrated anti-PKC isozyme antibodies in whole blood assay of lymphocytes. The lymphocyte fraction of whole blood was gated and assessed for staining by the anti-PKCα, βI, βII, δ, ε, ζ, λ/ι, μ and η antibodies along with the appropriate fluorochrome-conjugated isotype controls. Signal-to-noise ratios were calculated as described in the methods. The final concentration of each antibody with the respective isotype control is indicated in the graphs. The MFI graphs are shown in Supplementary Fig. S2.

Figure 3. Expression of PKC isozymes in CD4+ and CD8+ T cells by flow cytometry analysis. Flow cytometry analysis of PKC isozyme expression in CD4+ and CD8+ T cells. (a) Representative histogram for PKC isozymes in whole blood assay gated on CD3+CD8− T-cells (left) and individual and mean values showing the variation in the population (n = 11) (right). (b) Representative histogram for PKC isozymes in whole blood assay gated on CD3+CD8+ T cells and values representing the variation in the population (n = 11). Dashed lines represent the isotype controls and solid lines represent anti-PKC isozyme antibody staining. Bar graphs show values for each individual and the mean ± SD expressed as change in median fluorescence intensity (ΔMFI) obtained after subtracting the isotype control MFI values from respective PKC isozyme MFI values.
population of cord bloods to examine the levels by flow cytometry. Adult donor blood and cryopreserved PBMC from healthy adult donors were run in parallel as quality assurance for the assay. CB samples were analysed for PKC isozymes expression in CD4$^{+}$ and CD8$^{+}$ cell populations. The results showed that for both of these a proportion of the cord bloods displayed low PKC$\zeta$ and $\delta$ (Fig. 7a). We also show that these were deficient in monocytes but this was less evident in neutrophils (Fig. 7b).

**Changes in PKC isozyme levels in cord blood T cells during maturation in culture.** In this aspect of the study we selected those CBTC which showed low or deficient levels of PKC$\zeta$. These low levels were based on our normal range (5th–95 percentiles) of 35.2–90.5% for CD4$^{+}$ T cells and 38.5–92.3% for CD8$^{+}$ T cells. Since PKC is important in T cell responses once the cells have matured, it was expected that those with low levels would normalise during the maturation process. Using a previously described in vitro T cell maturation system$^{9–13}$, we examined whether the PKC isozyme levels normalized in those cord cells which expressed low levels. The data presented in Fig. 8a for CD4$^{+}$ T cells and Fig. 8b for CD8$^{+}$ T cells, show that within 24 h of the commencement of the maturation process, the PKC isozymes had already increased to mature T cell levels, with no further increases after this time (Fig. 8).

**Discussion**

While it has been reported that low levels of PKC$\zeta$ in CBTC may be a risk factor for development of allergy, the acceptance of this phenomenon awaits more profound studies. This includes the relationship in expression of this isozyme to other PKC isozymes, expression in CD4$^{+}$ and CD8$^{+}$ T cells and kinetics of normalisation of the levels during CBTC maturation. Advancements can be made if levels of the PKC isozymes can be measured by flow cytometry, together with the characterization in T cell subsets and with assessing of cytokine production. To facilitate such studies, we have shown that translating assays from Western blot to flow cytometry requires validation of these antibodies. Our data show that despite claims that monoclonal antibodies are suitable for flow cytometry, we have shown that when tested on Western blot some of them may not be specific for the indicated PKC isozymes. By subjecting the antibodies to Western blot analysis we were able to identify a panel which could...
specifically detect the PKC isozymes. This enabled the identification of levels of these isozymes in human lymphocytes, T cell subsets, B cells, NK cells, monocytes and neutrophils in whole blood assays.

The advantages of using flow cytometry are evident from the finding that we detected a PKC η positive and negative populations in peripheral blood lymphocytes population. It was evident that CD4⁺, CD8⁺ and NK cells are positive for PKC η, while B cells lack this PKC isozyme in both human and mice. This has been reported in developing B cells, which expressed high PKC η at mRNA level, while mature or resting B cells have low levels of PKC η. This finding is consistent with our data from flow cytometry studies.

Overall, all PKC isozymes were detectable in CD3⁺ T cells in whole blood assays. The data show that levels of each PKC isozyme in CD4⁺ and CD8⁺ T cell populations were comparable. The low expression of PKC β in human monocytes is consistent with the literature. PKC has been reported to play various roles in T cell function. The atypical PKCζ and PKCλ/ι play a role in regulation of asymmetric CD8⁺ T cell division involving the differentiation of CD8⁺ T cells to a long-lived effector phenotype with reduced memory T cell development. PKCθ plays an important role in Th2 cell type development but has no effect on Th1 cell responses. PKCε is important for CD4 T cell proliferation.

While PKC isozyme expression in monocytes is well documented, by Western blot analysis, their expression in neutrophils remains either incompletely characterized or discordant. In rat neutrophils, Tsao and Wang reported the expression of PKCα, β, δ, ε, θ, μ, θ, λ, ζ and γ (with γ being normally detected in neuronal cells) albeit at various levels. In contrast, Dang, et al. examined the expression of six PKC isozymes in human neutrophils and was unable to detect PKCζ, ε and θ. Balasubramanian, et al. examined the expression of all PKC isozymes in human neutrophils and were able to detect all except PKCζ, ζ and η. The basis for these differences is unclear even though an argument based on species differences could be made for the findings of Tsao and Wang as well as those of Balasubramanian, et al. as both groups used antibodies from the same commercial source.

We optimised our flow cytometry-based assay on whole blood to examine their expression in these cells. All PKC
isozymes are expressed in monocytes and neutrophils. All PKC isozymes were detectable in mouse lymphocytes from blood and splenic cells.

Using the flow cytometry assay, a number of cord blood samples were examined for PKC expression in a random population. It was evident from these results that only about 40% of these were considered to be low or deficient in PKCζ compared to adult control values. This was evident in both CD4+ and CD8+ T cells. Although we previously only examined the CD3+ T cell population, it was evident that a high proportion of that cohort, which comprise babies born to women who had a family history of allergic diseases, displayed low expression of PKCζ. Our present findings provide further support to the proposal that PKCζ expression levels in T cells are prognostic.

Figure 6. Detection of PKC isozyme expression in mouse lymphocytes by Western blot and flow cytometry. Mouse spleens were removed and cells were separated. (a) Cell lysates were prepared for Western blot analysis for detection of PKC isozymes by using PKC isozymes specific antibodies as shown in figures, n = 1–2. The blots are presented individually for each staining, intact without splicing, with lanes in their entirety. (b) Murine whole blood or (c) splenic lymphocytes were stained for intracellular PKC isozymes expression as outlined in methods. Histogram data from murine lymphocyte population are shown. Shaded histogram represents isotype control, dashed line represents unstained population and solid line represents PKC isozymes. Quantitated data (mean ± SD) from mouse blood lymphocytes (n = 5) and mouse splenic cells (n = 2–5) are presented in the right hand panels.
for risk of allergy development since it has been claimed that 40% of the population are at risk of developing allergic diseases. Interestingly, the only other isozyme found to be deficient in cord blood CD4\(^+\) and CD8\(^+\) T cells was PKC\(\delta\). Previously we have not found the levels of this isozyme to be correlated with development of allergic diseases. Amongst the other leukocyte subpopulations neutrophils did not show the PKC isozyme deficiency but monocytes from some cord bloods presented with deficient isozymes, PKC\(\delta\) and PKC\(\zeta\). It will be interesting to follow up the consequence of these low levels in some cord blood monocytes. Thus the isozyme levels can be determined in whole blood without the need to purify the subpopulations of leukocytes.

Examination of PKC\(\zeta\) and \(\delta\) during CD4\(^+\) and CD8\(^+\) T cell maturation in the culture model showed that when fully matured, both isotypes were expressed at mature T cell levels. In fact, their expression normalised within 24 h of initiating maturation, and before the expression of maturation markers CD45RA\(^-\)/RO\(^+\). Our previously published data demonstrated that PKC\(\zeta\) is required to prevent cells from maintaining an immature Th2 allergic cytokine profile, a characteristic of neonatal T cells. Taken together these results lead to an understanding that

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**Figure 7.** Cord blood PKC isozyme expression in CD4\(^+\) and CD8\(^+\) T cells, monocytes and neutrophils. Whole blood from cord (n = 9) and adult donor blood (n = 4) were stained with anti-CD3 and anti-CD8 antibodies and co-stained for anti-PKC isozymes. The data are expressed as a percentage of cryopreserved adult donor PBMC that were analysed in parallel. PKC levels were quantified as change in median fluorescent intensity (ΔMFI) by flow cytometry and then expressed as percentage of cryopreserved adult control cells. (a) Expression of PKC isozymes in cord (open bars) and adult (shaded bars) blood CD4\(^+\) and CD8\(^+\) T cells. (b) Expression of PKC isozymes in cord blood monocytes and neutrophils as compared to adult control blood. These levels were quantified as change in ΔMFI after subtracting the MFI of respective isotype control by flow cytometry and shown as median. Data are presented as mean ± SD.

**Figure 8.** Kinetics of PKC\(\zeta\) and \(\delta\) expression in cord blood CD3\(^+\)CD8\(^-\) and CD3\(^+\)CD8\(^+\) T cells during maturation in culture. Cord blood T cell subsets expressing low levels of PKC\(\zeta\) at day-0 were matured in the presence of PHA (2 µg/ml), and IL-2 (10 ng/ml). Levels of the PKC isozymes were analysed by intracellular staining method as per method section at the indicated time point in (a) CD4\(^+\) and (b) CD8\(^+\) T cells during culture. ΔMFI of PKCs expressed as percentage of ΔMFI in cryopreserved PBMCs (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
not only PKCζ, may be a biomarker for propensity to develop allergic diseases but may also have a functional role in development of T cells towards a non-allergic cytokine profile. The significance of our finding also extends to identification of an early period during which this development can be ‘re-programmed’.

**Materials and Methods**

**Reagents, chemicals and antibodies.** Information about the antibodies for intracellular staining for PKC isozymes, leukocyte surface markers and the labelling kit used for antibody-fluorescent dye conjugation isotype controls and mouse and rabbit IgG blocking antibodies, are summarized in Supplementary Table S1. RPMI 1640 tissue culture medium, foetal calf serum (FCS) and L-glutamine were purchased from SAFC Biosciences (Lenexa, Kansas, USA).

**Ethics statement.** The procurement of human blood and all experimental procedures were approved by the Human Research Ethics Committee of the Women's and Children's Health Network (WCHN), Adelaide, South Australia, in accordance to The National Statement on Ethical Conduct in Human Research (2007, updated 2018) (National Health and Medical Research Council Act 1992). Venous blood was collected from healthy adult volunteers with their informed consent, and umbilical cord blood with informed consent from mothers undergoing elective caesarean section.

All mouse cell experimental procedures, including the collection and use of murine blood and spleen, were approved by the WCHN Animal Ethics Committee and conducted in accordance to the Australian code for the care and use of animals for scientific purposes. Blood and spleens were collected as scavenger tissue.

**Preparation of human PBMC.** PBMC were prepared by layering the samples on Ficoll® Paque Plus (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. The interphase layer containing PBMC was harvested and cells were washed in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS.

**Preparation of mouse blood and splenic mononuclear cells.** Scavenged spleens of adult Swiss mice were collected during training sessions from the Adelaide University Medical School animal house. The mice were given injectable anaesthesia and blood was collected by cardiac puncture. The mice were killed by cervical dislocation. Spleens were removed by dissecting the mice aseptically and placed in RPMI-1640 containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mmol/l). The spleens were mashed in between the slides to prepare spleen cells. The blood or spleen cells were layered on Ficoll® Paque Plus according to the manufacturer’s protocol and after three washes, cells were used for Western blot or flow cytometry assays. Cell viability was determined by the trypan blue-exclusion method.

**Collection of cord blood samples.** Cord blood samples (n = 9) were collected following elective caesarean sections with no complications at birth. These samples were analysed for the PKC isozymes levels within 2 hours of collection. Additionally, cord blood mononuclear cells (CBMC) were isolated by centrifugation over Ficoll® Paque Plus according to the manufacturer's protocol and cryopreserved for later functional analysis.

**Cryopreservation of cells.** freshly isolated MC from human adult or cord blood, mouse blood or spleen were cryopreserved in freezing media containing 90% heat-inactivated FCS and 10% DMSO. Cells were incubated in a ‘Mr. Frosty’ Freezing Container (Thermo-Fisher Scientific, Scoresby Vic, Australia) overnight in a −80°C freezer, then transferred into liquid nitrogen storage.

**Western blot.** Western blots were conducted essentially as described previously. Cell lysates were prepared from either human PBMC or mouse splenic cells in a buffer containing 20 mmol/L HEPES, pH 7.4, 0.5% Nonidet P-40 (v/v), 100 mmol/L NaCl, 1 mmol/L EDTA, 2 mmol/L Na3VO4, 10 μg/ml of each protease inhibitor (benzamidine, leupeptin, pepstatin A, purchased from Sigma-Aldrich, St. Louis, Missouri, USA), and aprotinin (Calbiochem, Merck, Darmstadt, Germany). Lysate proteins were quantitated by using the Qubit® Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, US), prior to the addition of Laemmli buffer. The samples were boiled at 100°C in the presence of Laemmli buffer for 5 min before loading 25 μg of sample per well in 10% SDS Stain-Free™ FastCast™ Acrylamide gels (Bio-Rad Laboratories, Hercules, California, USA) for electrophoresis using the Bio-RAD Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories) at ~75 V for 1 h. The proteins were electrophoretically transferred to nitrocellulose membrane by using a Trans-Blot® Turbo™ transfer system (Bio-Rad Laboratories). After blocking, the membranes were incubated with the appropriate mouse or rabbit monoclonal anti-PKC isozyme antibodies, individually, followed by washing and incubation with HRP-conjugated secondary rabbit anti-mouse Ig (Dako, Glostrup, Denmark), or HRP-conjugated goat anti-rabbit Ig secondary antibodies (Dako, Glostrup, Denmark) as appropriate. Immunoreactive material was detected using the Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. The protein bands on the membranes were visualized by a ChemiDoc XRS+ Imaging System and quantitated using Image Lab™ Software, Version 3.0 (Bio-Rad Laboratories). Some blots were stripped using ReBlot Plus Mild Antibody Stripping Solution (Merck-Millipore) and re-probed with mouse monoclonal GAPDH antibody (clone 71.1, Sigma-Aldrich, used at 1/15000) to confirm equal loading and protein integrity.

**Conjugation of fluorophores to the anti-PKCζ, μ and ξ antibodies.** Conjugation of fluorophores to anti-PKCζ, anti-PKCξ and anti-PKCμ monoclonal antibodies was performed by using an Alexa Fluor 488 (AF488) or AF647 labelling kit (Thermo Fisher Kit, Waltham, MA, US), and unreacted dye was removed according to the manufacturer's instructions. Prior to conjugation, glycerol and sodium azide were removed using an
Flow cytometric detection of PKC isozymes in whole blood. Each PKC isozyme was assessed in whole blood from healthy donors or umbilical cords, using cell surface and intracellular flow cytometry staining methods. All staining steps were performed at room temperature with incubations in the dark where possible. To 50 μl of blood per tube, varying combinations of fluorochrome-conjugated monoclonal antibodies to CD3, CD4, CD8, CD19, CD16, and CD56, were added and incubated for 15 min. This was followed by erythrocyte lysis with the addition of 2 ml of BD FACS™ Lysing Solution for a further 10 min incubation, then a centrifugation to pellet leukocytes (500 g/3 min), with the supernatant discarded. The cells were washed with 2 ml of PBS with 1% FCS, and then fixed in 250 μl of BD Cytofix/Cytoper™ Fixation and Permeabilization Solution for 10 min. After another centrifugation and supernatant removal, the cells were permeabilised with 2 ml NET-Ge14 for 10 min. After supernatant removal, the fixed/permeabilised cells were Fc blocked with 1 μg of mouse/rabbit IgG for 10 min, followed by the addition fluorochrome-conjugated monoclonal antibodies to PKC isoforms or corresponding isotype control, in a final staining volume of approximately 50 μl, and incubated for 30 min. The stained cells were then washed twice with PBS + 1% FCS, and then acquired within 1 h on a BD FACSCanto, with at least 10,000 leukocyte events recorded.

PKC isozymes were assessed using FlowJo v10.1 (Ashland, Oregon, USA) on cell populations with the following gating strategy: doublets excluded using a forward scatter-height (FSC-H) versus -area (FSC-A) plot, then FSC-A versus side scatter-area (SSC-A) to ascertain lymphocyte, monocyte, and neutrophil populations. Specific lymphocyte subsets were then gated by their positivity to CD markers, or where only CD3 and CD8 were utilized in cell surface staining, CD3+/CD8− populations were considered as CD4 helper T-cells. PKC isoforms were gated positive based on fluorescence minus one and isotype controls. The data are presented as median fluorescent intensities (MFI). Signal-to-noise was calculated as ratio of positive (PKC isozyme)/negative (isotype control) MFI, as previously described25.

Maturation of cord blood T cells in culture. CBTC maturation was conducted as previously described19. CBMC were isolated from blood by the same process as described for PBMC isolation from adult blood and cryopreserved until assayed. Cells were thawed in a water bath and washed once with warm complete media. Viability was assessed by the trypan blue exclusion assay which revealed viability of 87–93%. CBMC cells at 1 × 10⁶/ml in complete media were seeded in a 12 well plate (total volume 2 ml). For each time point, separate wells were used. The cells were rested in a CO₂ incubator at 37°C for 2 h before adding phytohemagglutinin (PHA) at a final concentration of 2 μg/ml. IL-2 was added to the cells at a final concentration of 10 ng/ml on day three and media changed once a week. CBMC maturation was conducted as previously described10. The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

References
1. Lim, P. S., Sutton, C. R. & Rao, S. Protein kinase C in the immune system: from signalling to chromatin regulation. ImmunoLgy 146, 598–522, https://doi.org/10.1111/immm.12510 (2015).
2. Moscat, J., Rennert, P. & Diaz-Meco, M. PKCζ at the crossroad of NF-κB and Jak1/Stat6 signaling pathways. Cell Death & Differentiation 13, 702–711 (2006).
3. Nishizuka, Y. Studies and perspectives of protein kinase C. Science 233, 305–312 (1986).
4. Ferrante, A. & Prescott, S. L. Immunological Immaturity of the Neonate, Protein Kinase C Zeta and Allergy. J Neonatal Biol 3, e106, https://doi.org/10.14172/1267-0897.20100106 (2013).
5. Garg, R. et al. Protein kinase C and cancer: what we know and what we do not. Oncogene 33, 5225–5237, https://doi.org/10.1038/onc.2013.524 (2014).
6. Isakov, N. Protein kinase C (PKC) isoforms in cancer, tumor promotion and tumor suppression. Semin Cancer Biol 48, 36–52, https://doi.org/10.1016/j.semcancer.2017.04.012 (2018).
7. Singh, R. M., Cummings, E., Pantos, C. & Singh, J. Protein kinase C and cardiac dysfunction: a review. Heart Fail Rev 22, 843–859, https://doi.org/10.1007/s10741-017-9634-3 (2017).
8. Mochly-Rosen, D., Das, K. & Grimes, K. V. Protein kinase C, an elusive therapeutic target? Nat Rev Drug Discov 11, 937–957, https://doi.org/10.1038/nrd3871 (2012).
9. Hii, C. S. et al. Selective deficiency in protein kinase C isozenzyme expression and inadequacy in mitogen-activated protein kinase activation in cord blood T cells. Biochem J 370, 497–503, https://doi.org/10.1042/BJ20021122 (2003).
10. D’Vaz, N. et al. Neonatal protein kinase C zeta expression determines the neonatal T-Cell cytokine phenotype and predicts the development and severity of infant allergic disease. Allergy 67, 1511–1518, https://doi.org/10.1111/all.12027 (2012).
11. Prescott, S. L., Irvine, J., Dunstan, J. A., Hii, C. & Ferrante, A. Protein kinase C: a novel protective neonatal T-cell marker that can be upregulated by allergy prevention strategies. J Allergy Clin Immunol 120, 200–206, https://doi.org/10.1016/j.jaci.2007.03.045 (2007).
25. McLaughlin, B. E.

13. Early, E. & Reen, D. J. Rapid conversion of naive to effector T cell function counteracts diminished primary human newborn T cell responses. *Clin Exp Immunol* **116**, 527–533 (1999).

14. Morrow, T. A., Muljo, S. A., Zhang, J., Hardwick, J. M. & Schlissel, M. S. Pro-B-Cell-Specific Transcription and Proapoptotic Function of Protein Kinase Cγ. *Molecular and Cellular Biology* **19**, 5608–5618 (1999).

15. Pham, T. N. Q., Brown, B. L., Dobson, P. R. M. & Richardson, V. J. Protein kinase C-eta (PKC-η) is required for the development of inducible nitric oxide synthase (iNOS) positive phenotype in human monocyctic cells. *Nitrice Oxide* **9**, 123–134, https://doi.org/10.1016/j.niox.2003.09.006 (2003).

16. Metz, P. J. et al. Regulation of asymmetric division and CD8+ T lymphocyte fate specification by protein kinase Czeta and protein kinase Clambda/iota. *J Immunol* **194**, 2249–2259, https://doi.org/10.4049/jimmunol.1401652 (2015).

17. Metz, P. J., Soos, P. J., Spath, G., Littman, D. R. & Kopf, M. Protein kinase C-theta is critical for the development of in vivo T helper (Th)2 cell but not Th1 cell responses. *J Exp Med* **200**, 181–189, https://doi.org/10.1084/jem.20032229 (2004).

18. Tsao, L. T. & Wang, J. F. Translocation of protein kinase C isoforms in rat neutrophils. *Biochem Biophys Res Commun* **234**, 412–418, https://doi.org/10.1006/bbrc.1997.6656 (1997).

19. Tsao, L. T. & Wang, J. F. Translocation of protein kinase C Isoforms in Rat Neutrophils. *Biochem Biophys Res Commun* **234**, 412–418, https://doi.org/10.1006/bbrc.1997.6656 (1997).

20. Dang, P. M., Hakim, J. & Perianin, A. Immunochemical identification and translocation of protein kinase C-zeta in human newborn T cells. *FEBS Lett* **349**, 338–342, https://doi.org/10.1016/S0014-5793(94)00700-4 (1994).

21. Balasubramanian, N., Advani, S. H. & Zingde, S. M. Protein kinase C isozymes in normal and leukemic neutrophils: altered levels in leukemic neutrophils and changes during myeloid maturation in chronic myeloid leukemia. *Leuk Res* **26**, 67–81, https://doi.org/10.1016/S0145-2126(01)90098-4 (2002).

22. Prescott, S. L. & Tang, M. L. Australasian Society of Clinical, I. & Allergy. The Australasian Society of Clinical Immunology and Allergy position statement: Summary of allergy prevention in children. *Med J Aust* **182**, 464–467 (2005).

23. Mukaro, V. R. et al. Small tumor necrosis factor receptor biologics inhibit the tumor necrosis factor-p38 signalling axis and inflammation. *Nat Commun* **9**, 1365, https://doi.org/10.1038/s41467-018-03640-y (2018).

24. Lanuti, P. et al. A flow cytometry procedure for simultaneous characterization of cell DNA content and expression of intracellular protein kinase C-zeta. *J Immunol Methods* **315**, 37–48, https://doi.org/10.1016/j.jim.2006.06.015 (2006).

25. McLaughlin, B. E. et al. Nine-color flow cytometry for accurate measurement of T cell subsets and cytokine responses. Part I: Panel design by an empiric approach. *Cytometry A* **73**, 400–410, https://doi.org/10.1002/cyto.a.20555 (2008).

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Author Contributions

Conceived and designed the experiments: K.P. and A.F. Execution of experiments: K.P. Data analyses: K.P. and A.Q. Data interpretation K.P., A.F., A.Q., A.McP., C.S.H., S.B. and S.L.P. Wrote and/or critically read the manuscript: K.P., A.F., A.Q., A.McP., C.S.H., S.B. and S.L.P. Financial support from the National Health and Medical Research Council of Australia and the South Australian Channel 7 Children’s Research Foundation.

Additional Information

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