

PKC ζ activation promotes maturation of cord blood T cells towards a Th1 IFN- γ propensity

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Abstract

A significant number of babies present transiently with low protein kinase C zeta (PKC ζ) levels in cord blood T cells (CBTC), associated with reduced ability to transition from a neonatal Th2 to a mature Th1 cytokine bias, leading to a higher risk of developing allergic sensitisation, compared to neonates whose T cells have 'normal' PKC ζ levels. However, the importance of PKC ζ signalling in regulating their differentiation from a Th2 to a Th1 cytokine phenotype propensity remains undefined. To define the role of PKC ζ signalling in the regulation of CBTC differentiation from a Th2 to a Th1 cytokine phenotype we have developed a neonatal T cell maturation model which enables the cells to develop to CD45RA⁻/CD45RO⁺ T cells while maintaining the Th2 immature cytokine bias, despite having normal levels of PKC ζ . The immature cells were treated with phytohaemagglutinin, but in addition with phorbol 12-myristate 13-acetate (PMA), an agonist which does not activate PKC ζ . This was compared to development in CBTC in which the cells were transfected to express constitutively active PKC ζ . The lack of PKC ζ activation by PMA was monitored by western blot for phospho-PKC ζ and translocation from cell cytosol to the membrane by confocal microscopy. The findings demonstrate that PMA fails to activate PKC ζ in CBTC. The data show that CBTC matured under the influence of the PKC stimulator, PMA, maintain a Th2 cytokine bias, characterised by robust IL-4 and minimal interferon gamma production (IFN- γ), and lack of expression of transcriptional factor, T-bet. This was also reflected in the production of a range of other Th2/Th1 cytokines. Interestingly, introduction of a constitutively active PKC ζ mutant into CBTC promoted development towards a Th1 profile with high IFN- γ production. The findings demonstrate that PKC ζ signalling is essential for the immature neonatal T cells to transition from a Th2 to a Th1 cytokine production bias.

KEYWORDS

allergy, CD4⁺ and CD8⁺ T cells, cord blood T cells, cytokines, neonate, PKC isozymes, PKC ζ , T cell maturation, Th1 and Th2 subsets

INTRODUCTION

Humans are born with a T helper 2 (Th2) bias, reflecting the hormonal and cytokine milieu of pregnancy, which transitions to more mature Th1 responses over the first years of life [1]. This appears to be less efficient or delayed in children who develop allergic diseases, who have deficient Th1 responses at birth and ongoing predisposition to Th2 responses with age [1]. Environmental exposures, including microbes, pollutants and dietary factors during this critical window of development period have been shown to alter this maturation trajectory to modify disease risk [1–3]. Therefore, a deeper understanding of the fundamental mechanisms underpinning alterations in Th1 development is likely to provide a target for preventative intervention strategies.

In this context, protein kinase C zeta (PKC ζ) expression has been identified as a potential cord blood biomarker for children at risk of Th2 persistence and allergy development [3, 4]. We have previously shown that lower PKC ζ levels in cord blood T cells (CBTC) predict a high risk of developing allergy in the first years of life [3–5]. Further, when CBTC with low PKC ζ are matured in vitro, those with lower PKC ζ expression have a propensity to produce higher Th2 cytokines such as IL-4/IL-9 and lower interferon-gamma (IFN- γ) [4, 6, 7]. This relationship between low PKC ζ , Th2 propensity and allergic sensitisation is not evident with other PKC isozymes [3, 7]. Interestingly, we have shown that early intervention with omega 3 polyunsaturated fatty acids (fish oil) during pregnancy resulted in higher levels of PKC ζ and reduced development of allergy in the infants [3], potentially involving epigenetic regulation of the PKC ζ gene through the acetylation of histone H3 [8]. However, a critical unfulfilled task, the outcome of which can provide compelling evidence for a paradigm shift in mechanisms of healthy development, is to decipher the role of PKC ζ activation and signalling in regulating neonatal T cell development. To address this, we have established an in vitro neonatal T cell maturation model to examine the role of PKC ζ activation in promoting T cell maturation towards a Th1 cytokine phenotype.

MATERIALS AND METHODS

Reagents

The mouse monoclonal IgG PKC β II AF647 (clone F-7), PKC θ PE (clone E-7) and PKC ζ PE (clone H-1) isozymes antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, US) and rabbit monoclonal IgG PKC ϵ AF488 (clone EPR1482 [2]) was purchased from Abcam

(Cambridge, UK) and these antibodies were previously validated by our laboratory [5]. The corresponding isotype controls were purchased from BD (Franklin Lakes, NJ, US). RPMI 1640 tissue culture medium (Cat# R0883), PMA (Cat# P8139), phytohaemagglutinin (PHA), human AB serum and β -mercaptoethanol (M6250) were from Sigma Aldrich (St. Louis, Missouri, US), while rhIL-2 was purchased from PeproTech (Rocky Hill, New Jersey, US). X-VIVO 15 medium (cat# 04418Q, Lonza, BSL, Switzerland), foetal calf serum (FCS) and L-glutamine were purchased from SAFC Biosciences (Lenexa, KS).

Preparation of mononuclear cells

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 from healthy full-term neonates with informed consent from pregnant women undergoing elective caesarean section.

Mononuclear cells from cord blood (CBMC) or adult peripheral blood (PBMC) were isolated by density gradient centrifugation on Ficoll[®] Paque Plus (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. The harvested cells were washed in cX-VIVO medium (X-VIVO-15 supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% FCS). Cells were immediately cultured in cX-VIVO or cryopreserved for later functional analysis [5].

Isolation of T cells

T cell isolation was performed as previously described [6] using EasySep[™] Human CD3⁺ T cell Isolation Kit (Stem Cell Technologies, Vancouver, Canada) by negative isolation, according to manufacturer protocols. Cryopreserved CBMC or PBMC were rapidly thawed in a 37°C water bath and washed in PBS supplemented with 2% FCS and 1 mM EDTA (separation buffer). Viability was assessed by Trypan blue dye exclusion assay and found to be approximately 90%. Cells were resuspended in 0.25–2 mL of separation buffer, in 12 \times 75 mm polystyrene round-bottom tubes (Corning, Cat #352058), maintaining a cell concentration of 5 \times 10⁷ cells/mL. EasySep[™] Human CD3⁺ T Cell Isolation Cocktail was added at 50 μ L/mL to the suspension, mixed and incubated at room temperature (RT) for 5 min.

EasySep™ Dextran Rapid Spheres™ (50 μ L/mL) were added and samples were reconstituted to 2.5 mL separation buffer. The T cells were retrieved using an EasySep™ Magnet (Stemcell Technologies, Cat #18000), with purity consistently greater than 97%.

CBTC maturation

Cord blood T cells were matured using different stimulation methods. In the classical [7] CD3/CD28 co-stimulation cultures, anti-CD3 antibody (clone OKT3, Abcam, Cambridge, UK) was added to 24-well tissue culture plates at a final concentration of 2.5 μ g/mL in HBSS and incubated at 4°C overnight or at 37°C for 3 h, and then washed with HBSS. At the initiation of culture, anti-CD28 antibody (clone CD28.2, eBiosciences, San Diego, CA, USA) was added to a final concentration of 1 μ g/mL with 1×10^6 CBTC in each well in a final volume of 1 mL. To develop a new neonatal T cell maturation model independent of PKC ζ activation, 1×10^6 CBTC/mL were cultured with either PHA (2 μ g/mL) and PMA (40 nM); or PMA (40 nM) and ionomycin (0.5 μ g/mL).

After 3 days of culture by all methods, cells were counted and reseeded at 1×10^6 /mL with/without the addition of rhIL-2 (10 ng/mL), and this process was repeated on day 5. On day 7, the CD45RA/RO surface expression was measured by flow cytometry.

T cell proliferation

T cell proliferation was determined using the radioactive [3 H]-thymidine method as described previously [8]. Briefly, in 96-well round-bottom plates, T cells were added at 2×10^5 /well in RPMI/2.5% AB serum with or without PHA (2 μ g/mL) or PMA (10 ng/mL) in triplicate, and incubated at 37°C, 95% humidity and 5% CO $_2$ for 72 h. Cells were pulsed with 1 μ Ci [3 H]-thymidine 6 h prior to harvesting onto glass filter paper using a Titertek Multiple Cell Harvester. The glass fibre filter cutouts were applied to vials with liquid scintillation cocktail (PerkinElmer) and the disintegrations per minute (DPM) were determined in a Wallac 1409 scintillation counter. Lymphoproliferation was expressed as DPMs.

Flow cytometry

Apoptosis and cell viability

Early and late apoptosis was ascertained using Annexin V and 7-AAD, respectively. The staining panel is

described in Table S2. At the indicated time points, 2×10^5 cells were harvested, washed in 1 mL of Annexin V Binding buffer (10 mM HEPES, pH 7.4, 0.14 M NaCl, 0.25 mM CaCl $_2$ and 0.5% BSA) and incubated with the staining panel for 20 min at RT in the dark. After two washes with Annexin V Binding buffer, samples were stained with 7-Aminoactinomycin D (7-AAD, BD, cat# 51-68981E) for 5 min at RT and analysed by flow cytometry.

Naïve and memory T cell immunophenotyping

To ascertain T cell maturation, CD45RA and CD45RO expression was examined at various time points of T cell culture. For each time-point, 2×10^5 cells were harvested, washed in PBS/1% FCS (wash solution) followed by incubating with a cell surface antibody panel (Table S3) for 20 min at RT in the dark. Following two washes in the wash solution, the samples were acquired on a BD FACSCanto and analysed using FlowJo v10.8.1 (Ashland, OR, USA). Lymphocytes were gated based on their high CD45 $^+$ expression and were further gated on CD3 $^+$ T cells to examine the surface expression of CD45RA and CD45RO.

Detection of intracellular PKC isozymes at single-cell levels

PKC isozymes were assessed in unstimulated cells by a previously described method [5]. Briefly, either whole blood or 2×10^5 CBMCs were incubated with anti-CD3 APC-H7 and anti-CD8 PE-Cy7, both from BD Biosciences (Franklin Lakes, NJ, USA) for surface staining for 15–20 min. The cells were then fixed with BD Cytofix/Cytoperm (BD, 555028) and permeabilised with NET-Gel. Furthermore, 1 μ g of mouse/rabbit IgG Fc blocking reagent was added for 10 min. The optimal amount of fluorochrome-conjugated anti-PKC isozymes antibodies or isotype controls (Table S4) were added as appropriate. After 30 min of incubation at RT in the dark, the cells were washed twice. They were then analysed on a FACS-Canto II (BD Biosciences, NJ, USA).

Detection of intracellular cytokines

Intracellular cytokines were measured in stimulated mature T cells, using the BD Cytofix/Cytoperm™ Plus Permeabilisation Kit with GolgiPlug as described previously [6]. Briefly, 1×10^6 cells/mL in RPMI/2.5% AB serum were stimulated with 50 nM PMA and 2 μ g/mL

PHA or anti-CD3/-CD28 antibodies in the presence of Brefeldin A, and incubated at 37°C/5% CO₂ for 16–20 h. Cells were then washed in the wash solution and resuspended in 200 µL of wash solution for surface staining with anti-CD3 PE-Cy5 (HIT3a), and anti-CD45 APC-H7 (2D1), for 15–20 min at RT in the dark. The cells were stained with the BD Horizon™ Fixable Viability Stain 510 (FVS510) Stock Solution (1:1000) in sodium azide- and protein-free PBS. At the end of 15 min incubation, cells were washed twice with 2 mL of wash solution.

The cells were then fixed with 250 µL BD Cytofix/CytoPerm™ Fixation and Permeabilisation Solution for 20 min at RT in the dark, and then permeabilised with 1 mL of BD Perm/Wash for 10 min at RT in the dark. A selected panel of antibodies for intracellular cytokine detection was then added to the cells (Tables S5–S7) and then incubated for 30 min at RT in the dark. In transfection experiments where enhanced green fluorescent protein (EGFP) expressing Plasmids were used, IFN-γ PE (Clone: 4S.B3, eBiosciences, San Diego, CA, USA) was used instead of IFN-γ FITC to avoid false-positive signals for IFN-γ in FITC channel. After two washes with BD Perm/Wash, the samples were acquired on either a FACSCanto or FACS-Canto II, as appropriate, with a minimum of 10 000 lymphocyte events acquired. Data analysis was performed using FlowJo v10.8.1. The lymphocyte population was gated by forward and side scatters, followed by gating of the CD3⁺CD8[−] population (negative gating for CD4⁺) and CD3⁺CD8⁺ cells, with cytokine expression and percentage positive cells analysed after exclusion of doublets and dead cells by gating on the FVS510[−] cell population.

Cytometric bead array

The quantification of secretory cytokines from culture supernatants was performed as previously described [9] by using BD™ Cytometric Bead Array kits for IFN-γ, TNF-α, IL-2, IL-6, IL-8 and IL-10 (BD Biosciences) following adaptation of the manufacturer's protocols for assay in 96-well v-bottom plates, with the acquisition on a BD FACSCanto with an attached BD™ High Throughput Sampler (HTS), and analysis with FCAP Array v3 software (BD Biosciences).

ELISA

Interleukin (IL)-2 was quantitated in cell culture supernatants by sandwich ELISA as previously described for our laboratory [10]. Cell culture supernatants were applied to anti-human IL-2 polyclonal antibody (Thermo Fisher Scientific) coated Nunc™ MaxiSorp™ flat-bottom 96-well plates.

The plates were washed, followed by the addition of biotinylated labelled anti-human IL-2 monoclonal antibody, then HRP-Conjugated Streptavidin. Colourimetric development was achieved by the addition of 3,3',5,5'-tetramethylbenzidine, and terminated by the 0.5 M H₂SO₄. The absorbance was measured at 450 nm using a Victor™ X4 Multilabel plate reader (PerkinElmer, Waltham, MA, US).

Production of T cell PKCZ transfectants

Plasmid preparation and purity check

Prevalidated constitutively active PKCZ (EGFP-PKCZdel239, cat# 110513), control plasmids expressing pEGFP-N1-FLAG (cat#60360) or wild-type EGFP-PKCZ.WT (cat#110512) or kinase-dead mutant FLAG.PKCZeta.K/W (cat# 10800) in transformed competent *Escherichia coli* were purchased from Addgene (Cambridge, MC, US). These were propagated on Luria broth agar supplemented with 100 µg/mL kanamycin. Plasmids were then extracted using NucleoBond® Xtra Midi Plus Endotoxin Free kit (Düren, Germany) according to manufacturer's protocol. Plasmid integrity was checked by 1% agarose gel electrophoresis. Plasmid yield and quality were quantified by UV spectrophotometry.

Diagnostic restriction endonuclease digest

The PKCZ inserts were verified in plasmids by restriction digest. Briefly, a reaction mix was prepared for each plasmid by firstly combining 1 µg of plasmid with 5 µL of 10X CutSmart™ buffer (Cat#B7204S) and water to final volume 50 µL following addition of the appropriate restriction enzymes: for constitutively active or wild-type PKCZ plasmid, 1 µL EcoR1-HF (Cat#R3101S) with or without 3' cloning site with 1 µL of Sall-HF (Cat#R3138S). The empty vector backbone was digested with EcoR1-HF. All buffer and enzymes were from New England Biolabs (Ipswich, MC, US). The reaction mixtures were then incubated at 37°C for 1 h. After the incubation, 100 ng of each sample or undigested plasmid was used as control and subjected to electrophoresis in 1% agarose gel (Figure S1).

Nucleofection of human CBTC with constitutively active PKCZ plasmid

Nucleofection of human CBTC with constitutively active EGFP-PKCZ or control EGFP plasmid was carried out essentially as described previously [8]. The human T cell Nucleofection kit was from Amaxa (Lonza, Walkersville, MD, USA). Freshly purified CBTCs were rested in cX-

VIVO media containing β -mercaptoethanol (50 μ M) for at least 2 h at 37°C. Approximately 10^7 CBTCs were added to 5 μ g of control or PKC ζ specific plasmids in cuvettes and the cells were transfected using programme V-024 for high transfection, according to the manufacturer's instructions. After transfection, CBTCs were cultured for 4 h before setting for maturation with PHA/PMA. An aliquot of the cultures was used to confirm the transfection efficiency (Figure S5) and viability analysis by flow cytometry.

PKC ζ activation assay

PKC ζ activation in purified T cells was assessed by Western blot following culture without or with either PHA (2 μ g) and PMA (100 nM), or anti-CD3/-CD28 (as described under CBTC maturation) at 2×10^6 /mL in HBSS for various periods. Cultures were ended using cold HBSS. The cells were recovered by using rubber scraper and centrifuged at 600 \times g, 5 min at 4°C.

Cell lysates were prepared from the harvested cells and applied to SDS-PAGE and Western blot as described previously [5]. The transferred nitrocellulose membranes were incubated with the rabbit anti-phospho-PKC ζ (Thr410) Monoclonal Antibody (clone S.447.6), or mouse monoclonal anti-PKC ζ isozyme antibody (clone H-1) followed by washing and incubation with HRP-conjugated goat anti-rabbit or rabbit anti-mouse Ig secondary antibodies (Dako, Glostrup, Denmark) as appropriate. Immunoreactive material was detected using the Western LightningTM Chemiluminescence Reagent Plus (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. The protein bands on the membranes were visualised by a ChemiDoc XRS+ Imaging System and quantitated using Image LabTM 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 anti-GAPDH antibody (clone 71.1, Sigma-Aldrich, used at 1/20000) to confirm equal loading and protein integrity.

Immunofluorescence and confocal microscopy

Immunofluorescent imaging of cultured T cells utilised the flow cytometric techniques to fix and permeabilise cells as described earlier. Briefly, T cells purified from adult healthy volunteers were first surface stained with anti-CD3 FITC antibody for 15 min then left untreated or treated with PMA, or PHA/PMA or anti-CD3 antibody for 15 min at 37°C/5%

CO₂. The reaction was stopped immediately by the addition of ice-cold washing buffer, followed by centrifugation, fixation, permeabilisation and intracellular staining for either PE-conjugated anti-PKC ζ or PKC θ antibodies for 25 min. DAPI nuclear stain was added for a further 5 min incubation. At the end of the assay, cells were transferred to Superfrost[®] microscope slides (Thermo Scientific, Waltham, MA) using a Cytospin 3 centrifuge (Shandon Scientific, Cheshire, UK). Cytospin T cells were air-dried and mounted with a fluorescent mounting medium (Dako, Santa Clara, CA, US). The samples were visualised either under an Olympus BX51 Fluorescence Microscope (Olympus, Tokyo, Japan) at 100 \times magnification with oil or Olympus FV3000 Confocal Microscope at 60 \times magnification with oil and the images were acquired by using FV31S-SW Image software tools. The images were processed by using ImageJ FIJI image software (version 1.8.0; WS Rasband, National Institute of Health, Bethesda, MD, USA) [11].

PCR assays

The standard or quantitative reverse transcribed PCR (qRT-PCR) assays were performed as previously described [12]. In brief, CBTC were cultured with maturation inducers either anti-CD3/CD28 or PHA/PMA and RNA was extracted on day-4 or 5 by using TRIzol reagent (Invitrogen). cDNA was prepared using iScript cDNA synthesis kit (Bio-Rad).

The standard PCR was performed by using AmpliTaq GoldTM 360 Master Mix. The polymerase reaction was performed by Activation of AmpliTaq Gold[®] 360 Master Mix at 95°C 5 min, then 40 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extending at 72°C for 60 s/kb and final extension at 72°C for 7 min. The PCR product was loaded on 2% agarose gel to visualise the size of the different PCR products by a ChemiDoc XRS+ Imaging System.

qRT-PCR analysis was performed using PowerUpTM SYBRTM Green Master Mix (Thermo Fisher Scientific) with the following conditions: initial activation of UDG for 2 min at 50°C followed by activation of Dual-LockTM DNA polymerase. Then 40 cycles of denaturation for 15 s at 95°C and anneal/extend at 60°C for 1 min, 60°C for 30 s and 72°C for 30 s using an iQ5 Real-Time Detection System with iQ5 Optical System v2.1 software (Bio-Rad). Data were normalised to the expression of a control gene GAPDH for each experiment. The primer pairs used were for human T-BX21 (Forward: 5'-GTCCAACAATGTG ACCCAGAT-3'; Reverse: 5'-ACCTCAACGATATGCAG CCG-3'), GATA3 (Forward: 5'-GCCCCCTCATTAAGCCC AAG-3'; Reverse: 5'-TTGTGGTGGTCTGACAGTTCCG-3') and GAPDH (Forward: 5'-GAGTCAACGGATTTGGTTCG T-3'; Reverse: 5'-GACAAGCTTCCCGTTCTCAGCCT-3').

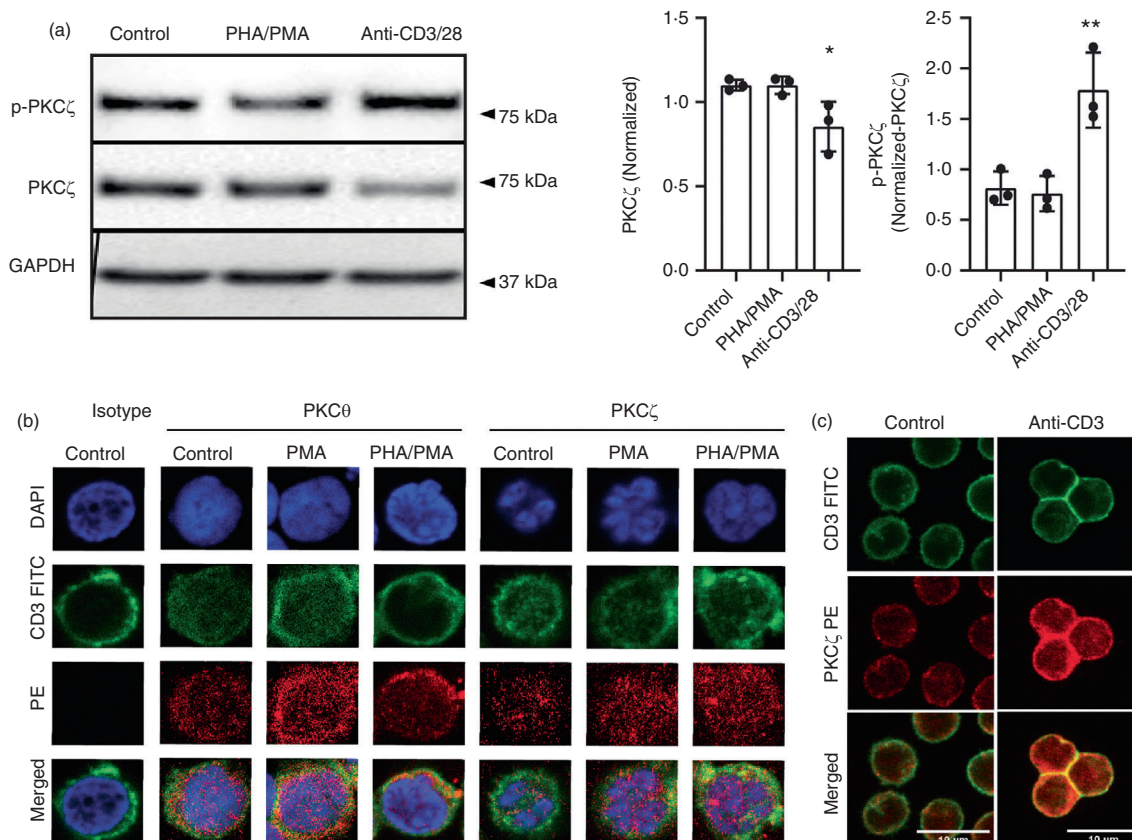


FIGURE 1 Activation of PKC ζ by anti-CD3/CD28 antibodies or PHA/PMA. (a) Western blot (left panel) showing levels of PKC ζ and phospho-PKC ζ in CBTC treated with PHA-PMA or anti-CD3/-CD28 antibodies for 60 min. Right panel shows quantitated data as mean \pm SD ($n = 3$ experiments). (b, c) Photomicrograph of changes in PKC ζ redistribution after treatment (representative of three independent experiments) with (b) PMA only, PHA-PMA or (c) anti-CD3 antibody for 15 min. PKC θ was a positive control. Cells were stained with PE-labelled anti-PKC ζ or PE-labelled anti-PKC θ antibodies. Significance of difference from unstimulated cells; * $p < 0.05$; ** $p < 0.01$, using One-way ANOVA with post-hoc Tukey's multiple comparisons test. 'n' represents the number of samples each from a different individual. CBTC, cord blood T cells; PHA, phytohaemagglutinin; PKC ζ , protein kinase C zeta; PMA, phorbol 12-myristate 13-acetate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, California, USA). Comparisons were performed using one-way ANOVA with post-hoc Tukey's multiple comparisons test or Student's *t*-test. A *p* value of <0.05 was considered statistically significant for all analyses.

RESULTS

Establishing an in vitro CBTC maturation model independent of PKC ζ activation

Phorbol 12-myristate 13-acetate (PMA) activates conventional and novel PKC isozymes but not atypical PKCs, such as PKC ζ [13–15]. We, therefore, developed a CBTC maturation model that uses PMA to promote this maturation, in the absence of PKC ζ activation. The model

involves using purified cord blood CD3⁺ T cells cultured in the presence of PHA and PMA over a 7 day period. This was compared with the classical model which uses anti-CD3/anti-CD28 antibodies plus rhIL-2 to induce maturation of CBTC [16–22]. Given the lack of data on PKC ζ activation in human T cells, we examined changes in PKC ζ phosphorylation by western blot as a readout of activation when CBTC were treated with the maturation stimuli. While treatment with anti-CD3/-CD28 antibodies increased phosphorylation of PKC ζ , treatment with PHA/PMA had no effect (Figure 1a). In addition, total PKC ζ protein was decreased (Figure 1a). This was accompanied by the appearance of immunoreactive material that migrated with an Mr of around 50 kDa (Figure S2), implicating the proteolysis/degradation of PKC ζ as has been reported for other PKC isozymes following cell activation [23, 24], hence providing further evidence of PKC ζ activation by anti-CD3/-CD28 antibodies. This degradation was not seen with PHA/PMA treatment. The lack of PKC ζ activation by PHA/PMA was also examined in

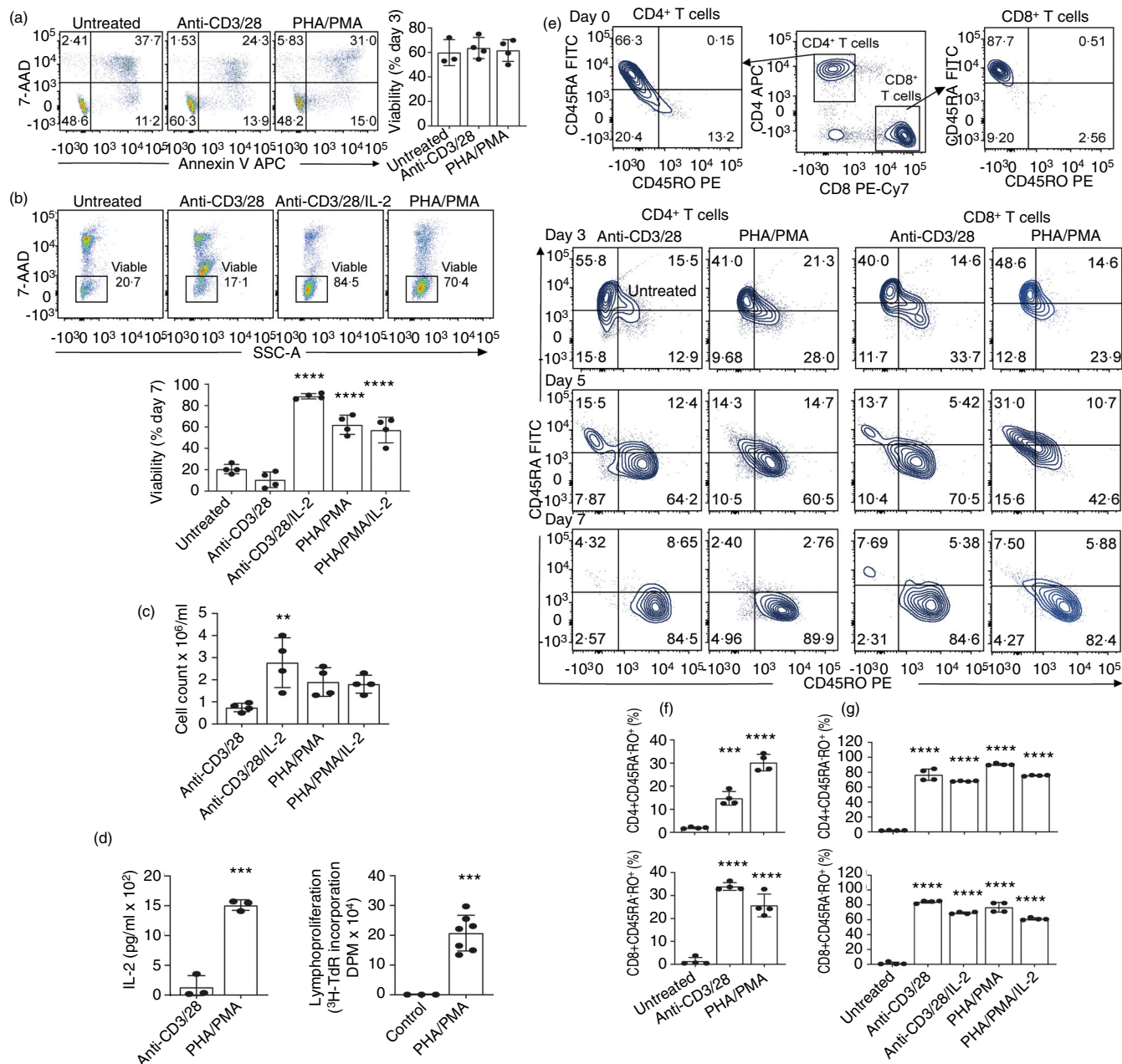


FIGURE 2 Maturation and viability of CBTC cultured in the absence or presence of PKC ζ activation. CD3⁺ T cells were matured in the presence of the indicated (x-axis) maturation agents. Data show representative flow dot plots and experimental data for viable cells (Annexin V⁻ and 7-AAD⁻ cells) on (a) day 3 or (b) day 7. (c) The cell number was also recorded on day 7. (d) shows IL-2 production in culture cell fluids from CB CD3⁺ T cells ($n = 3$ experiments, each with cells from a different individual) and lymphoproliferation as DPM ($n = 7$ experiments) on day 3. (e) Representative flow contour plots for the CD45RO and CD45RA expression on day 3, 5 and 7 in CD4⁺ and CD8⁺ T cells treated with the maturation agents. Level of CD45RA⁻RO⁺ cells on (f) day 3 and (g) day 7 in CD4⁺ and CD8⁺ T subsets. (a–c, f, g; $n = 4$ experiments). Bars show mean \pm SD. Significance of difference from unstimulated cells or anti-CD3/CD28; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ using either One-way ANOVA with post-hoc Tukey's multiple comparisons test (a–c, f, g) or student's t -test (d). 'n' represents the number of samples each from a different individual. CBTC, cord blood T cells; PHA, phytohaemagglutinin; PKC ζ , protein kinase C zeta; PMA, phorbol 12-myristate 13-acetate.

adult blood T cells by determining the intracellular relocalisation of the PKC isozyme. There was no change in the localisation of PKC ζ when purified T cells were incubated with either PMA or PHA/PMA (Figure 1b). In

contrast, the positive control isoform, PKC θ , translocated to the cell periphery and colocalised with CD3, indicating translocation to the plasma membrane (Figure 1b). When using anti-CD3 antibody to activate the cells,

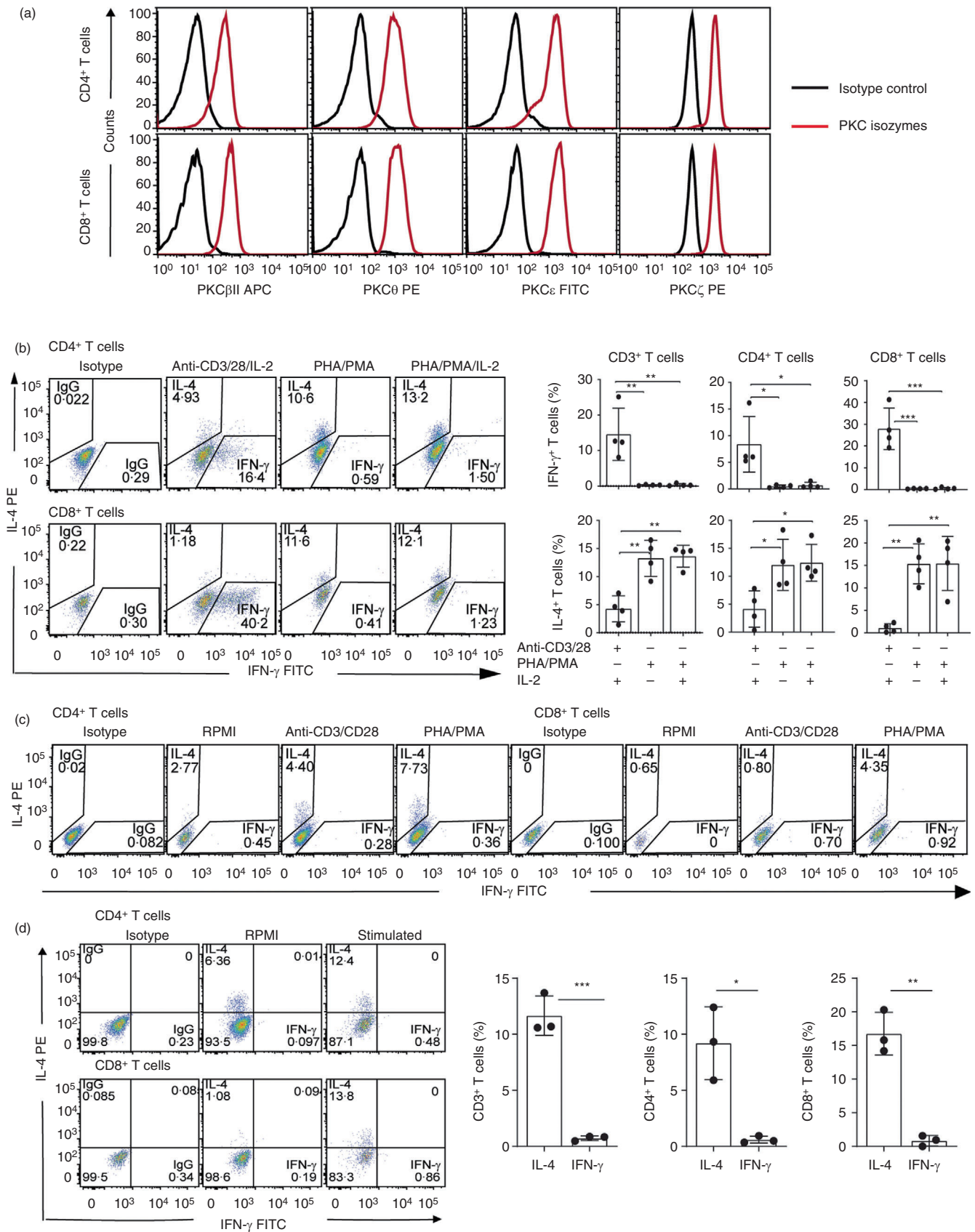


FIGURE 3 Legend on next page.

translocation of PKC ζ to the cell periphery was evident (Figure 1c). In these adult T cells, the activation of PKC ζ was confirmed by examining the phospho-PKC ζ changes by western blot (Figure S3).

Lack of PKC ζ activation does not affect the viability or maturation of CBTC

Analysis of CBTC viability shows a similar rate of early apoptosis with both types of maturation inducers. During T cell maturation with PHA-PMA the cell viability is similar to that observed with the conventional inducer anti-CD3/-CD28 antibodies and rhIL-2, observed at 3 (Figure 2a) and 7 days of culture (Figure 2b). At day 7 of culture, the lymphocyte counts were similar for both maturation inducers (Figure 2c).

Interestingly in the PHA-PMA promoted maturation, rhIL-2 was not required. This is not surprising as unlike cells treated with anti-CD3/-CD28 antibodies, T cells treated with PHA-PMA produced IL-2 on day 3 (Figure 2d), while also showing substantial cell proliferation (Figure 2d). Assessing cell maturity by examining changes in the cell surface makers from CD45RA⁺/RO⁻ to CD45RA⁻/RO⁺, the PHA-PMA model demonstrates a similar degree of T cell maturation to the cultures treated with anti-CD3/-CD28 antibodies and rhIL-2 (Figure 2e–g). This suggests that PKC ζ activation is not essential for either T cell viability or their maturation, with PHA-PMA promoting this maturation in both CD4⁺ and CD8⁺ T cell subsets.

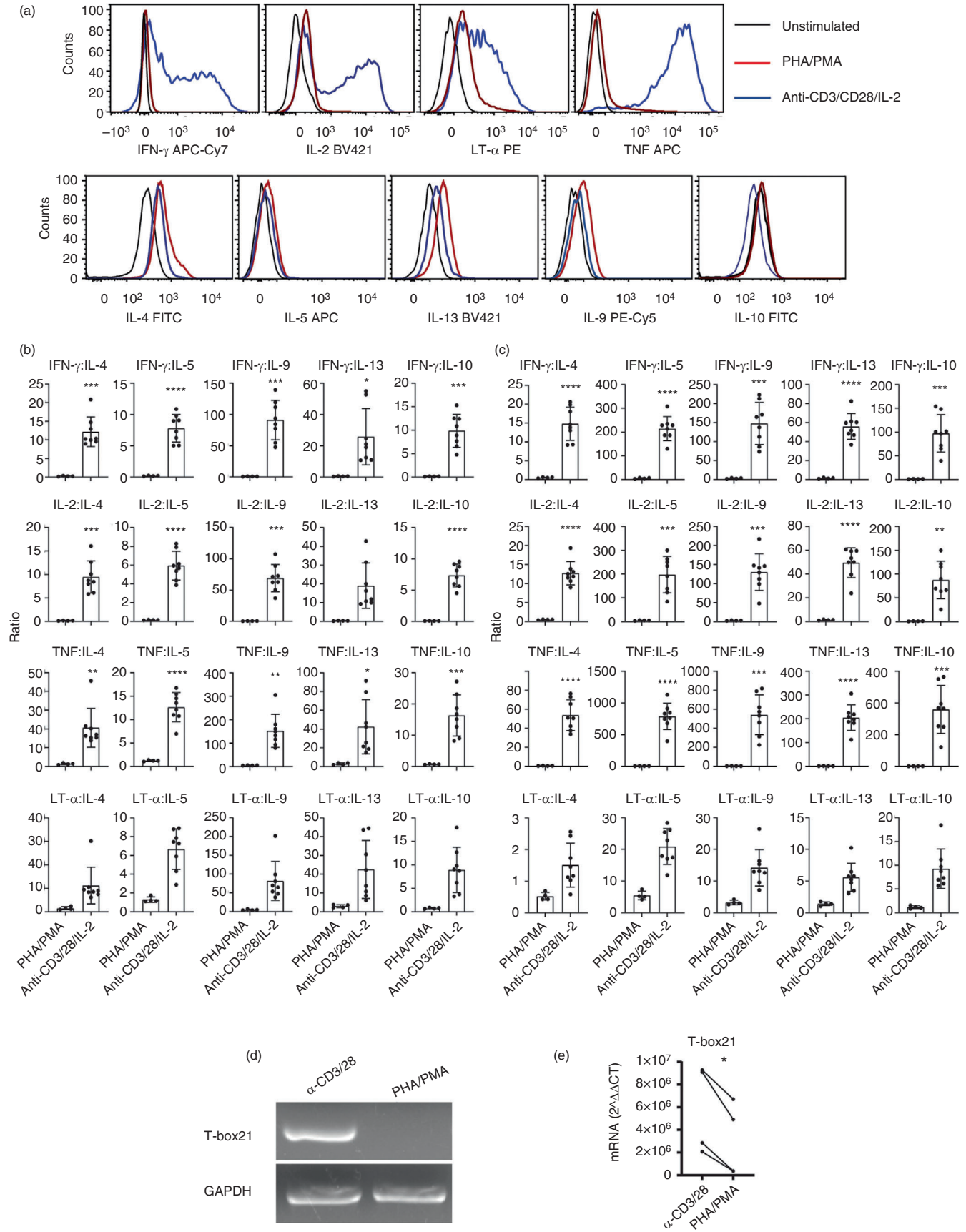
PKC ζ activation-independent maturation of CBTC retain Th2/IL-4 cytokines profile in both CD4 and CD8 T cells

With PMA treatment known to deplete PKC isozymes [25], the levels of PKC isozymes in the CBTC were

measured on day 7 following their maturation with PHA-PMA. PKC isozyme levels were abundant in the mature T cells (Figure 3a) implying that mature cell PKC levels were not compromised for the challenge phase of the analysis on day 7. We then examined the ability of the matured T cells to produce Th1 or Th2 cytokines. Purified CBTC were cultured with the maturation-inducing agents, either PHA-PMA or anti-CD3/-CD28 antibodies and rhIL-2, for 7 days. IL-4 and IFN- γ cytokine production was then examined in response to PHA-PMA in T cells and T cell subsets by intracellular staining and flow cytometry. The data demonstrate that PHA-PMA induced maturation leads to high IL-4 but little IFN- γ production in CD4⁺ T cells (Figure 3b). In comparison when anti-CD3/-CD28 was used the cytokines are skewed towards IFN- γ production (Figure 3b). Thus PMA causes the development of cells with a propensity to give rise to Th2 responses. This difference in response promoted by the two types of maturation agents is also evident when these cytokines are measured in the CD8⁺ T cell subset (Figure 3b). When the PHA-PMA matured T cells were stimulated with anti-CD3/-CD28 antibodies, similar results are obtained as for PHA-PMA stimulation, showing the response of the matured T cells is not stimulus-dependent (Figure 3c). PMA/ionomycin induced maturation also resulted in the inability of the mature cells to produce Th1 cytokines (Figure 3d). These results suggest that the model presented could be deployed universally to examine the biology of neonatal T cell maturation.

The analysis of purified CBTC matured in the presence of PHA-PMA was expanded to other cytokines. When these matured T cells were stimulated with anti-CD3/-CD28 antibodies they showed poor production of Th1 cytokines relative to Th2 cytokines, shown by the low Th1:Th2 cytokine ratio (Figure 4; Figure S4; Table S1) demonstrated by a low ratio of IFN- γ :IL-4 (Figure 4b) and extending to the ratio of IFN- γ : IL-5/IL-9/IL-13/IL-10 (Figure 4b). In contrast, when T cells were matured with anti-CD3/-CD28 antibodies and

FIGURE 3 Analysis of IL-4 versus IFN- γ production by CBTCs matured under different maturation conditions. (a) The levels of the indicated PKC isozymes in PHA-PMA matured T cells were determined by flow cytometry. Isotype controls are in black. (b) Purified CD3⁺ T cells were matured with either PHA-PMA or anti-CD3/-CD28 plus IL-2 for 7 days and then examined for cytokine production in response to PHA-PMA. Cytokine production was assessed by permeabilising the cells, staining with the relevant antibodies and analysed by flow cytometry. Data are shown as representative flow dot plots for IL-4 and IFN- γ production by matured CD3⁺, CD4⁺ and CD8⁺ T cells, as well as pooled data from four experiments each conducted with cells from a different individual. (c) Cells were matured with PHA-PMA, and then stimulated with either PHA-PMA or anti-CD3/-CD28 antibodies as indicated and then analysed for the IL-4 and IFN- γ production in both CD4⁺ and CD8⁺ T cells (representative of two experimental runs). (d) Cells were matured with PMA/Ionomycin for 7 days, then stimulated with PHA-PMA and analysed for IL-4 and IFN- γ production by the CD3⁺, CD4⁺ and CD8⁺ T cells ($n = 3$ experiments, each conducted with cells from different individuals). Significance of difference between treatments: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, using either One-way ANOVA with post-hoc Tukey's multiple comparisons test (b) or student's t -test (d). CBTC, cord blood T cells; IFN- γ , interferon-gamma; PHA, phytohaemagglutinin; PKC ζ , protein kinase C zeta; PMA, phorbol 12-myristate 13-acetate.



rhIL-2 (Figure 4a) and then challenged/stimulated with PHA-PMA, the ratio of the Th1:Th2 cytokines was high. When the intracellular cytokine production was examined as mean fluorescence intensity (MFI), the findings were very similar to evaluating the % of cytokine producing T cells (Figure 4c). Also, examination of T-bet (T-box21) a transcription factor involved in Th1 cell development showed the anti-CD3/CD28 matured CBTC express higher levels of T-bet compared to PHA/PMA (Figure 4d,e). The analyses were extended to examine the ratio of other Th1 cytokines, IL-2, TNF and LT- α to the Th2 cytokines, IL-5/IL-9/IL-13/IL-10 and similar results were found (Figure 4b,c). This indicates that the major role of PKC ζ is in promoting the development of T cells skewed towards the production of IFN- γ and other Th1 cytokines.

PKC ζ activation is required for conversion of Th2 to Th1 cytokines profile in CBTC during maturation

Since PMA does not activate PKC ζ (Figure 1) [13–15], to determine a direct role for PKC ζ signalling in Th1 polarisation, we introduced a constitutively active or a kinase dead PKC ζ mutant into the CBTC by nucleofection. The naive CBTC were nucleofected with a plasmid carrying a wild type, a kinase-dead or an active PKC ζ mutant (Figure S1) and then matured the cells with PHA-PMA. There was an increase in the percentage of matured cells with a propensity to produce IFN- γ in both the CD4⁺ and CD8⁺ T subpopulations with little change in those producing IL-4, in samples nucleofected with active PKC ζ mutant but not with wild type or kinase-dead mutant (Figure 5a,b; Figure S6).

DISCUSSION

The mechanism through which neonatal T cells develop from an immature Th2 cytokine predominance to a Th1

cytokine bias remains essentially unknown, despite the availability of surrogate in vitro culture models representing this T cell maturation. Here we have developed a culture model of purified cord blood/neonatal T cell maturation/development in an attempt to gain an understanding of the mechanisms involved in regulating this development. In this study, we have used PHA-PMA which in contrast to anti-CD3/-CD28 antibodies as maturation inducers, does not activate PKC ζ . This creates a unique scenario whereby the T cells mature in the presence of PHA-PMA, determined by the expression of maturation markers CD45RA/RO, but do not develop towards a Th1 functional phenotype and maintain an immature Th2 cytokine bias. Hence, these matured cells are of the CD45RA⁺/RO⁺ phenotype but maintained a Th2/IL-4 cytokine bias. Previously it has been shown that PMA does not activate PKC ζ [13–15]. Here we have demonstrated that PKC ζ is not activated in human T cells including CBTC by PHA-PMA, by examining for phospho-PKC ζ as well as the degradation of the protein as a consequence of the activation of the T cells. The lack of PKC ζ activation is also demonstrated by confocal microscopy, by the absence of translocation of PKC isozyme to the membrane. While anti-CD3/-CD28 antibodies could induce maturation in isolated T cells, unlike with PHA-PMA, IL-2 has to be added to the cultures. Our data show that this may be due to the fact that PHA-PMA stimulation of immature T cells leads to the release of substantial amounts of IL-2 in cultures, compared to cultures stimulated with anti-CD3/-CD28 antibodies. It is also unlikely that external factors (like the addition of Th1 cytokines) at the time of differentiation can help the PHA/PMA matured cells to transit towards Th1 propensity as various other cytokines were also detected in PHA/PMA maturation model (Figure S7). This also explains why T cell viability was maintained in the PHA-PMA stimulated cultures without the addition of IL-2. However it is acknowledged that it is evident from the literature that the addition of exogenous T helper polarising cytokines can drive cells towards different paths when

FIGURE 4 Th1 or Th2 cytokine bias is regulated by the different maturation conditions in CBTC. Each batch of CBTC was matured in the presence of either PHA-PMA or anti-CD3/-CD28 antibodies plus IL-2 for 7 days, then washed and stimulated with anti-CD3/-CD28 antibodies or PHA-PMA, respectively, for 24 h. Intracellular cytokine levels were determined by flow cytometry. Dead cells were excluded by gating on the FVS510 negative population. (a) Representative histogram for unstimulated control samples (black), samples matured with either PHA-PMA (red) or anti-CD3/-CD28/rhIL-2 (blue). (b,c) The results are expressed as the ratio of Th1 to Th2 cytokines from (b) the percentage of cytokine-producing cells or (c) MFI levels. (d,e) For T-box21 expression, CBTC were treated with maturation-inducing agents for 4 or 5 days then the levels of T-box21 mRNA were determined by standard PCR (d) qRT-PCR (e) ($n = 4$ experiments for each treatment). X-axis shows the maturation agents. Data are presented as values for each individual as well as the mean \pm SD. (b,c) $n = 4$ experiments for PHA-PMA induced maturation or $n = 8$ experiments for anti-CD3/-CD28/rhIL-2 induced maturation. Significance of difference between the treatments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$ using student's t -test. 'n' represents the number of samples each from a different individual. CBTC, cord blood T cells; MFI, mean fluorescence intensity; PHA, phytohaemagglutinin; PKC ζ , protein kinase C zeta; PMA, phorbol 12-myristate 13-acetate.

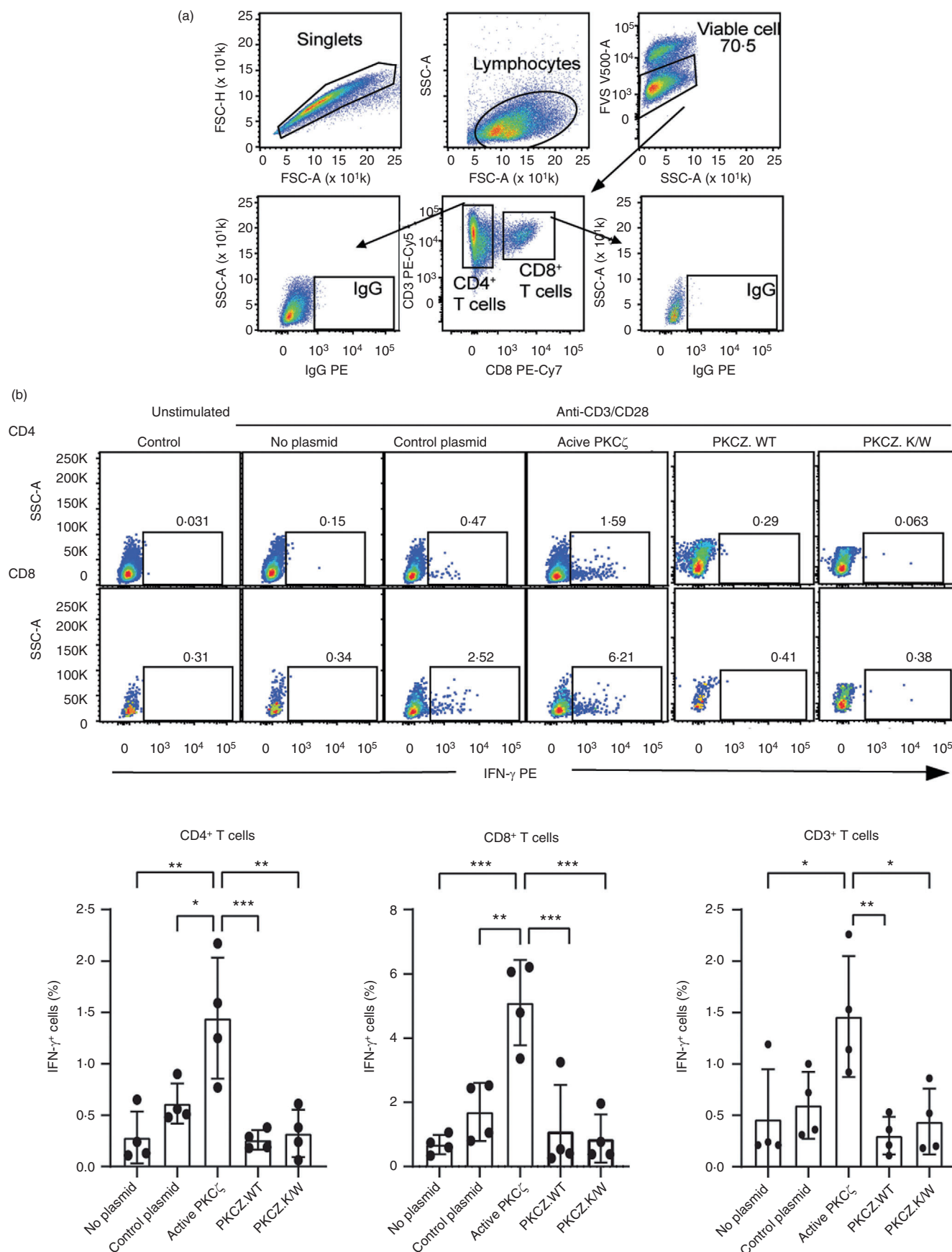


FIGURE 5 Legend on next page.

using anti-CD3/anti-CD28 stimulation [17, 26], but how this is affected when using anti-CD3/CD28 versus PHA/PMA remains to be studied, and would provide important/useful contextualisation for their effects. Thus, it is envisaged that the outcome seen here could be influenced/overcome by the addition of cytokines.

Our previous findings demonstrated that neonatal T cells/CBTCs could be characterised into those expressing low or normal/high PKC ζ levels [4]. Those expressing low levels maintained an immature Th2/Th9 cytokine bias when matured with either PHA-monocytes-IL2 or anti-CD3/-CD28/IL-2, in contrast to those with high PKC ζ levels. This however only provides indirect evidence for a role of PKC ζ . Knocking down the expression of PKC ζ in cord blood T cells that express normal levels of the kinase altered the T cell development towards a Th2 bias [8]. Our present findings advance our understanding of the role of PKC ζ as a key regulator of the balance between Th1 versus Th2 development from the immature Th2 propensity. First, in the absence of PKC ζ activation by treatment with PHA-PMA, the immature T cells expressing normal PKC ζ levels, developed into cells that maintained a Th2 cytokine bias. PKC activation is critical in promoting Th1 development as cells, expressing normal levels of PKC ζ , maintained a Th2 cytokine bias when matured with PHA-PMA. Clear evidence supporting a critical role of PKC ζ comes from the demonstration that when a constitutively active PKC ζ was introduced into the CBTCs, PHA-PMA was able to promote maturation of the cells towards a Th1 bias. Second, the extent to which PKC ζ is able to activate its downstream effectors also seems important.

Despite these findings which we have interpreted as being inherent differences between the different forms of stimulation, it does not exclude the possibility that they reflect different levels of stimulation strength for the T cells, which is known to influence Th1/Th2 balance. It could well be that different doses of the same agonist differentially activate PKC ζ which would still not negate our hypothesis but would suggest it is the specific dose of the agonist used rather than the type of agonist used that is critical.

Although our emphasis has been on CD4⁺ T cells, skewed cytokine propensity of CD8⁺ T cells is also

relevant to development or prevention of allergic diseases, such that these cells are being referred to as T cytotoxic 1/2 (Tc1) and Tc2 based on the cytokines they produce [27, 28]. Here we have demonstrated that development towards Tc1 cytokine bias was also dependent on PKC ζ activation.

Establishing and deploying a singular neonatal T cell development culture model independent of PKC ζ activation has enabled us to demonstrate a role for this PKC isozyme and its activation in the maturation of neonatal T cells towards a Th1 anti-allergy cytokine phenotype, strongly supporting a role for PKC ζ as a potential biomarker for allergy risk and target for allergy prevention in the newborn. There has been a significant increase in the incidence and burden of allergic diseases over recent decades, with high personal, societal and economic impact. Thus, providing compelling evidence for the importance of PKC ζ activation in regulating the development of neonatal T cells, towards either a Th1 or Th2 cytokine bias, provides a new focus in attempts to prevent and treat allergic diseases.

AUTHOR CONTRIBUTIONS

Conceptualization: Antonio Ferrante, Khalida Perveen, Charles S. Hii and Susan Prescott. *Methodology:* Khalida Perveen, Antonio Ferrante, Charles S. Hii, Michael J. Stark and Alex Quach. *Investigations:* Antonio Ferrante and Khalida Perveen. *Funding acquisition:* Antonio Ferrante, Charles S. Hii and Susan Prescott. *Supervision:* Antonio Ferrante, Charles S. Hii, Alex Quach and Simon C. Barry. *Writing—original draft:* Antonio Ferrante and Khalida Perveen. *Writing—review & editing:* Khalida Perveen, Antonio Ferrante, Charles S. Hii, Alex Quach, Susan Prescott, Simon C. Barry and Michael J. Stark. All authors contributed to the article and approved the submitted version.

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FIGURE 5 PHA/PMA treated CBTCs expressing constitutive active PKC ζ mature to T cells with a propensity to produce IFN- γ . Naive CBTC were nucleofected with indicated plasmid (Figure S1) and then matured with PHA-PMA. After 7 days, the cells were stimulated with anti-CD3/-CD28 antibodies and analysed for IFN- γ or IL-4 production in T cells and subsets. (a) Shows a representative gating strategy on viable lymphocytes. IgG-PE represents the isotype control. (b) Shows flow dot plots or graphs for the percentage positive for IFN- γ production in the matured CBTC for CD4⁺, CD8⁺, and CD3⁺ T cells ($n = 4$ experiments each conducted with cells from a different individual). The data are presented as values of each individual as well as the mean \pm SD. Significance of difference using One-way ANOVA with post-hoc Tukey's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. CBTC, cord blood T cells; IFN- γ , interferon-gamma; PHA, phytohaemagglutinin; PKC ζ , protein kinase C zeta; PMA, phorbol 12-myristate 13-acetate.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.


DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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