

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Neonatal protein kinase C zeta expression determines the neonatal T-Cell cytokine phenotype and predicts the development and severity of infant allergic disease

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Keywords

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Abstract

Background: Previous studies have demonstrated that reduced T-cell protein kinase C zeta (PKC ζ) expression is associated with allergy development in infants born to atopic mothers. This study examined whether this relationship extends to a general population and addressed the basis for the association.

Methods: A flow cytometry assay was developed for the measurement of T-cell PKC ζ levels in PBMC, cord blood mononuclear cell and whole blood. Cord blood T-cell PKC ζ levels were measured in 135 neonates, and allergic disease was evaluated by skin prick test and clinical examination at 12 months of age.

Results: Allergic children (particularly those with eczema) had significantly lower neonatal T-cell PKC ζ expression than nonallergic children ($P < 0.001$). PKC ζ levels predicted allergic disease with optimal specificity of 86% and sensitivity of 54%. The sensitivity was increased in the children of allergic mothers, who had significantly lower PKC levels than the children of nonallergic mothers. Cord blood PKC ζ levels did not affect T-cell maturation in culture as assessed by CD45RA/RO expression, but low PKC ζ expression was associated with reduced capacity for IFN γ production by matured T cells. Low cord blood PKC expression was further associated with increased IL-13 responses at 6 months.

Conclusions: The findings suggest a potential role for the use of PKC ζ levels in cord blood T cells as a presymptomatic test to predict allergy risk in children, particularly offspring of allergic mothers, and that the basis of this relationship is related to cytokine patterns in mature T cells.

With rising allergy prevalence, there is a growing need for more effective early prediction and targeted primary prevention strategies. Recent studies indicate that around 70% of infants are at

'high risk' based on their immediate family history (1). Elucidating the pathways driving early immune dysregulation will help define disease pathogenesis and potentially provide targets for novel intervention and screening strategies.

While immature T-cell function is a feature of all newborns, more exaggerated immaturity has been a consistent association with allergic predisposition (2–6). This has been largely detected by relative deficiency in the production of Th1 cytokine, IFN γ , implicated in the inhibition of the allergy response (2–5, 7, 8). Interestingly in separate studies, we have found that the immune immaturity in the newborn

Abbreviations

BLG, β -lactoglobulin; CBMC, cord blood mononuclear cell; CD, cluster of differentiation; ICS, intracellular staining; MC, mononuclear cells; MFI, mean fluorescence intensity; PKC ζ , Protein kinase C zeta; ROC, curve receiver operating characteristics curve; SEB, Staphylococcus aureus enterotoxin B (superantigen); SPT, skin prick test.

was associated with low levels of protein kinase C zeta (PKC ζ) in T cells (9) and that PKC ζ levels in cord blood T cells predicted the development of allergy disease by the age of 3 years in high-risk infants (10). We thus speculated that lower neonatal PKC ζ expression and reduced rates of maturation could be implicated in the immaturity of T-cell function, which precedes the development of allergic disease (2–6) and is still evident in later infancy (7, 11).

To increase our understanding of the clinical and diagnostic implications of these observations, studies were undertaken to (i) develop a simple flow cytometry assay for T-cell PKC ζ expression compared to western blot assays, (ii) apply this to a second high-risk cohort, (iii) extend the significance of this relationship to a more 'general' population and (iv) gain insights into the basis linking cord blood T-cell PKC ζ levels to functional T-cell profiles.

Materials and methods

Study design

Cord blood samples were used to assess the relationship between neonatal T-cell PKC ζ expression and subsequent allergic outcomes in 135 infants.

The population

Infants were selected from a birth cohort ($n = 670$) recruited between 2005 and 2008 from the Western Australian community. Mothers were skin-prick-tested in the third trimester of pregnancy and allergies in the family recorded. Infants were considered at 'high risk' of allergy if mothers had a history of allergic disease and were skin prick test (SPT) positive to at least one allergen. Some infants in the present study ($n = 29$) received a fish oil supplement (in the postnatal period only) as documented elsewhere (12). This had no effect on allergy outcomes, but was examined as a confounding factor here for completeness. The selection of subjects ($n = 135$) for the present study was based on the availability of cord blood samples, complete allergy follow-up data and a clear-cut diagnosis of allergy (or no allergy).

Clinical outcomes and allergy definitions

The development of eczema, food allergy and allergic sensitization was assessed in infants at 12 months of age through detailed history and clinical examination. Information on recurrent wheeze and physician-diagnosed asthma was collected, but the limitations are recognized at this age. A child was classified as having 'allergic disease' if they had a physician diagnosis of IgE-mediated food allergy, eczema or asthma at these assessments. The diagnostic criteria conformed to the recently published clinical guidelines (13). A diagnosis of eczema was made in infants with typical skin lesions (14). The extent and severity of the eczema was determined by the standardized SCORAD severity index (14). IgE-mediated food allergy was defined as a history of immediate symptoms (within 60 min) following contact with and/or ingestion of

food and a positive skin prick test (SPT). A diagnosis of asthma was based on a history of recurrent wheeze that was demonstrated to be responsive to bronchodilator medications.

Allergen skin prick testing (SPT)

Skin prick test was assessed at 12 months, using allergen extracts (milk, peanut, house dust mite, cat, grass, mould; Hollister-Stier Laboratories, Spokane, WA, Australia) and whole egg. Histamine and glycerine were used as positive and negative controls, respectively. A wheal diameter of ≥ 2 mm was considered positive.

Measurement of PKC ζ levels

Monoclonal mouse anti-human PKC ζ (H-1) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and labelled using Lightning-Link R-Phycoerythrin Conjugation kit (Innova Biosciences, Cambridge, UK).

Peripheral blood mononuclear cells (PBMC) prepared from healthy adult volunteers, infants and cord blood (CBMC) were isolated by Ficoll-Hypaque gradient centrifugation and either used immediately or cryopreserved in DMSO (15%) and FCS (85%). CBMC or PBMC (1×10^5) were fixed in 2% paraformaldehyde. The leucocytes were permeabilized in buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.05% NP40, 0.25% λ -carrageenan and 0.02% NaN₃ (15). After 10 min at room temperature, the cells were washed and the cell pellets were resuspended in 10 μ l of 100 μ g/ml mouse immunoglobulin (IgG) to block nonspecific binding. PE-conjugated anti-PKC ζ monoclonal antibody (15 μ g/ml) or PE-conjugated anti-IgG 2a (isotype control) (15 μ g/ml) and FITC-conjugated anti-CD3 monoclonal antibody (1 μ l) and permeabilizing buffer to a total of 90 μ l were added. After 30 min, the cells were washed and fluorescence was analysed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Using Cell-Quest software (BD Biosciences), gates were set around the lymphocyte population, within which the PKCzeta staining intensity was assessed in the CD3-positive population.

When comparing samples, we standardized conditions to correct for any interexperimental variation. Standards (1 neonate CBMC preparation from an individual with many samples and 1 adult PBMC preparation from a healthy volunteer) were run concurrently with samples. Data are displayed as the mean fluorescence intensity (MFI) values expressed as % of the neonate standard MFI value.

T-cell responses

Naïve CBMC (1×10^6 MC/ml) from 55 participants from the main study cohort were stimulated with either phytohaemagglutinin (PHA) (2 μ g/ml, Ramel, Kansas) or superantigen *Staphylococcus aureus* enterotoxin B (1 μ g/ml, SEB; Sigma, St. Louis, MO, USA) in serum free in AIM V medium (Gibco, Life Technology, Grand Island, NY, USA) with 2-mercaptoethanol (ME) for 48 h. Supernatants were analysed for cytokine content of IL-1b, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17a, TNF α and IFN γ .

For the measurement of cytokine production in matured T cells, cord blood from an additional 10 individuals (not related to the main cohort) was collected, matured (as described below) and stimulated with PHA as for naïve CBMC, and supernatants were assessed for IL-2, IL-4, IL-10, IL-13, TNF α , IFN γ and TGF β contents.

PBMC from 6-month-old infants were stimulated with allergens house dust mite (HDM) *D. pteronyssinus* (20 μ g/ml, 2×10^6 cells/ml, Department of Cell Biology, Institute for Child Health Research, Perth, WA, USA), ovalbumin (OVA 100 μ g/ml, 2×10^6 cells/ml) or betalactoglobulin (BLG, 100 μ g/ml, 2×10^6 cells/ml) (both Sigma-Aldrich) and PHA and SEB as above. Supernatants were analysed for cytokine contents (IL-5, IL-10, IL-13, IL-17a, TNF α , IFN γ) using Luminex Xmap multiplexing technology.

T-cell maturation

Cord blood T cells and monocytes were isolated as previously described (16) and matured as previously described using PHA and IL-2 (17). Maturation was gauged by measuring the expression of CD45RA and CD45RO using anti-CD45RA-APC, anti-CD45RO-PE, anti-CD3-FITC or isotype mix (IgG2b-APC, IgG2a-PE and IgG1-FITC) (BD Biosciences) by flow cytometry.

Statistical analysis

Neither PKC data nor cytokine data were normally distributed. Where possible, data were log-transformed to obtain a log-normal distribution. Pearson's correlations were calculated for normal data, and Spearman's or Kendall's Tau b correlations for nonparametric data. Means of continuous variables were compared using the Student's *t*-test or Mann-Whitney *U*-tests depending on data normality. Logistic regression was used to determine the weighted effects of neonatal variables on clinical outcomes and linear regression to determine the weighted effects on neonatal PKC expression. The diagnostic relationship between allergic disease and PKC ζ expression was examined using a receiver operator characteristics (ROC) curve analysis. All statistical analyses were performed using Macintosh SPSS (version 16.0 for Mac OS X) (IBM, Armonk, NY, USA) and STATA software (version 10) (StataCorp, TX, USA). A *P*-value of <0.05 was considered statistically significant for all analyses.

Ethical approval

The research and ethics committees of relevant hospitals approved the study. All women gave informed consent.

Results

Development and validation of the flow cytometry method for T-cell PKC ζ expression

Greater than 99% of the CD3+ T-cell population in the PBMC and CBMC stained positive for PKC ζ (Fig. 1A,B). PKC ζ levels were significantly lower in cord blood T cells

(Fig. 1B). Titration of the anti-PKC ζ antibody against the isotype control suggested a working concentration of 10–15 μ g/ml (Fig. 1C). The staining of PKC ζ could be inhibited by a peptide that blocks the binding of antibody to this PKC isotype (Fig. 1D), confirmed also by western blotting (data not presented). Assays were also optimized for whole blood with identical results (data not shown).

Association between neonatal PKC ζ expression and allergic disease by 1 year of age

Table I displays the characteristics of this study population based on infant allergies at 12 months of age. Table II shows the characteristics divided by maternal allergy. The data showed that PKC ζ expression (expressed as MFI relative to control) was significantly lower in the cord blood of children who developed allergic disease by 1 year of age (mean $78.12 \pm$ standard error 5.6% MFI) compared with nonallergic children ($103.29 \pm 4.3\%$ MFI) ($P < 0.001$) Fig. 2A. For specific allergic outcomes, lower neonatal PKC ζ levels were associated with subsequent development of eczema ($P < 0.001$) independent of infant sensitization. Those who developed IgE-mediated food allergy also had significantly lower neonatal PKC ζ levels ($P = 0.006$). There was a trend for lower PKC ζ levels in sensitized children, but this was not statistically significant ($P = 0.131$). Recurrent wheezing during the first year of life was also associated with lower PKC ζ levels ($P = 0.016$) (data not shown), although it is recognized that this symptom is nonspecific at this age, and only one child was diagnosed with asthma.

These predictive relationships of PKC levels with eczema, allergic disease and IgE-food allergy (as shown in Fig. 2A) remained significant after controlling for potential confounding factors by multivariate logistic regression (maternal age, paternal allergy, delivery mode, gestation, dietary supplements, infant feeding practices (duration of breastfeeding and solids introduction), or exposure to other children (siblings or at day care) or pets. The relationship with sensitization was not statistically significant after adjusting for these interactions.

Sensitivity and specificity of PKC ζ expression as a predictive tool for allergic disease

Receiver operating characteristic (ROC) curves were generated to determine the optimal 'cut-off' levels for predicting allergic disease in all 135 infants. This demonstrated that PKC ζ expression below 0.725 (72.5% of control) predicted allergic disease with a sensitivity of 55.4% and a specificity of 86% (Fig. 3A), resulting in an accuracy of 74.3% (% children correctly classified). In the high-risk infants, PKC ζ expression predicted allergic disease with a sensitivity of 69.7% and specificity of 81.6%, resulting in an accuracy of 76.8% (Fig. 3B).

Comparison of PKC ζ and family history as predictors of allergic disease

Protein kinase C zeta levels were significantly lower in infants born to allergic mothers compared to infants born to nonallergic mothers ($P < 0.001$).

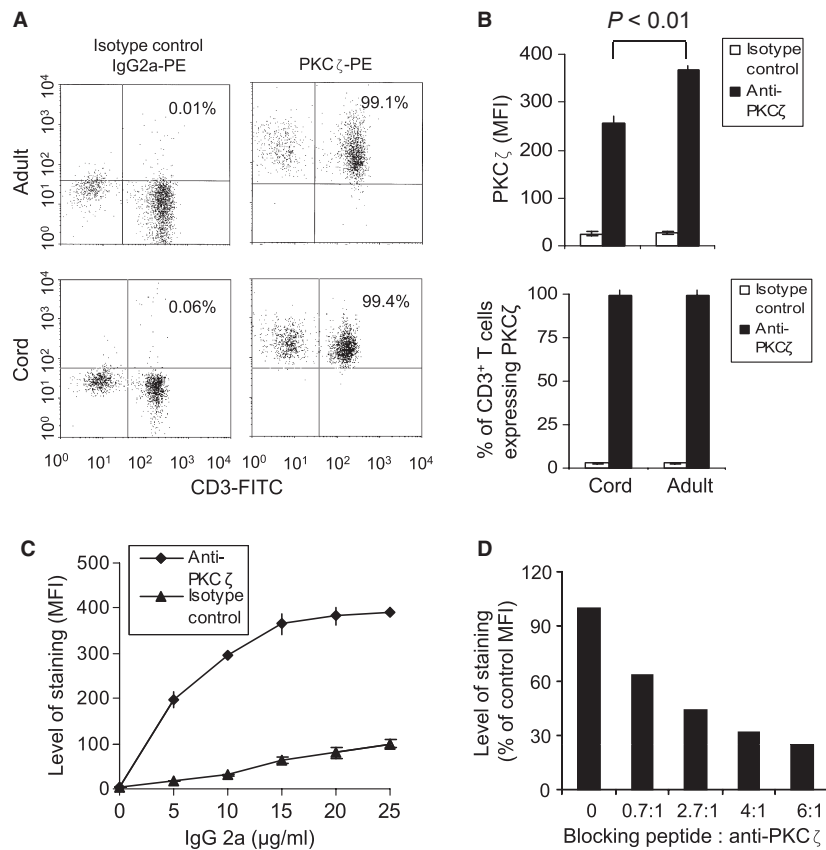


Figure 1 Measurement of protein kinase C zeta (PKC ζ) in CD3⁺ T cells using flow cytometry. (A, B) Comparison of isotype and anti-PKC ζ antibody (15 μg/ml) staining of T cells in cord blood mononuclear cell and adult blood PBMC. Results are presented as mean $\pm$ SEM of five individuals/experiments (C) Antibody titration

As shown in Fig. 2B, allergic disease was further predicted in children at 'high risk' (based on positive maternal history) by low PKC ζ expression. Relationship between PKC ζ expression and disease remained significant after adjustment for potential confounding factors (as mentioned earlier). The predictive value of PKC ζ expression (for allergic outcomes) was greater in high-risk infants partly because there were fewer allergic children in the low-risk group. In this population, paternal allergy status was not associated with PKC ζ levels.

We compared the predictive value of PKC ζ to that of family history by converting PKC ζ values to a binary variable (above or below cut-off 0.725) and comparing areas of ROC curves generated using family history and PKC ζ , respectively. PKC ζ was a significantly better predictor (P values ranging from 0.015 to 0.000) than any measures of family history. Furthermore, no measure of family history improved the allergy prediction by PKC ζ (data not shown).

The relationship between disease severity and PKC ζ expression at birth

In 45 infants with SCORAD assessments available at 12 months, there was a significant correlation between neo-

natal PKC ζ expression and degree of intracellular staining. Results are presented as mean $\pm$ SEM of three individuals/experiments (D) Examination of the specificity of the PKC ζ staining using a peptide that inhibits the interaction between the anti-PKC ζ antibody and PKC ζ .

natal PKC ζ expression and eczema severity ($\tau = -0.299$; $P = 0.046$) (Fig. S1A).

T-cell PKC ζ expression after *in vitro* maturation

A subgroup of 20 infants (10 nonallergic and 10 allergic infants) were chosen for T-cell maturation cultures to assess any relationship between baseline PKC levels and T-cell function. Individuals ($n = 20$) who differed in baseline cord blood T-cell PKC ζ levels (10 with low PKC ζ levels and 10 with high PKC ζ levels) all showed a significant increase in PKC ζ expression after 8 days ($P < 0.001$) of *in vitro* maturation (data not shown), but PKC ζ expression on day 8 was not associated with baseline PKC ζ levels or allergy development.

The relationship between PKC ζ expression at birth and T-cell CD45RA/RO expression

The majority of cells were CD45RA⁺/RO⁻ (naïve) on day 0 and a large proportion of T cells acquired CD45RO expression by day 8 (data not shown). There were no significant correlations between PKC ζ expression on day 0 or day 8 and

Table 1 Characteristics of the population ($n = 135$)

Characteristics	Allergic at 12m	Non allergic at 12m	<i>P</i> value
Number of infants (% total)	56 (41.4)	79 (58.6)	
Parental characteristics			
Mean maternal age (years)	33.9	33.2	0.303
Maternal atopy (SPT+) (%)	39 (69.6)	50 (63.3)	0.719
Paternal allergy (%)	36 (64.3)	49 (62)	0.855
Maternal smoking (%)	0 (0)	0 (0)	
Paternal smoking (%)	5 (8.9)	3 (3.8)	0.276
Birth characteristics			
Mean gestation (weeks)	39.1	39.2	0.790
Vaginal birth (%)	29 (51.8)	44 (55.7)	
Elective caesarean (%)	16 (28.6)	28 (35.4)	
Emergency caesarean (%)	11 (19.6)	7 (8.9)	
Mean birth weight (g)	3532	3397	0.080
Mean birth length (cm)	50.69	50.14	0.204
Birth head circumference (cm)	34.88	34.77	0.693
Male (%)	31 (55.4)	37 (46.8)	0.384
Infant characteristics			
Breastfed ever (%)	55 (98.2)	76 (96.2)	0.632
Mean age solid introduction (months)	5.7	5.7	0.994
Number of siblings at home	0.68	0.72	0.749
Pets at home (%)	22 (39.3)	44 (55.7)	0.079
Infant allergies by 12 months			
Sensitized (%)	23 (41.1)	5 (6.3)	0.000
Sensitized foods (%)	13 (23.2)	0 (0)	0.000
IgE mediated food allergy symptoms (%)	13 (23.2)	0 (0)	0.000
Wheeze ever (%)	31 (55.4)	16 (20.3)	0.000
Diagnosed asthma (%)	1 (1.8)	0 (0)	0.415
Eczema (%)	54 (96.4)	0 (0)	0.000

CD45RA/CD45RO expression on day 8. There were also no differences in neonatal T-cell CD45RA/CD45RO expression between allergic and nonallergic groups.

The relationship between PKC ζ expression at birth and cytokine responses

In CBMC ($n = 55$) cultured *ex vivo* for 48 h (with no maturation), there were no correlations between PKC ζ expression and cytokine production (IL-1b, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17a, TNF α or IFN γ) to PHA or SEB stimulation (data not shown).

In CBMC (from 10 individuals not related to the main study population) matured for 8 days in culture, low PKC ζ expression at baseline (day 0) was associated with significantly ($P = 0.007$) reduced capacity for IFN γ production following maturation (Fig. 4). There were no significant differences in the production of other cytokines (IL-2, IL-4, IL-10, IL-13, TNF α or TGF β) between the two groups (Fig. 4).

Twenty two of the high-risk infants from the main study population of 135 had cytokine responses assessed at

Table 2 Characteristics of the population based on maternal allergic history as a risk factor for infant allergic disease ($n = 132$)

Characteristics	Infants of nonallergic mothers*	Infants of allergic mothers†	<i>P</i> value
Number of infants (% total)	56 (42.4)	76 (57.6)	
Parental characteristics			
Mean maternal age (yrs)	33.5	33.6	0.913
Paternal allergy (%)	32 (62.7)	52 (62.7)	0.946
Maternal smoking (%)	0 (0)	0 (0)	
Paternal smoking (%)	3 (5.9)	5 (6.0)	0.973
Birth characteristics			
Mean gestation (weeks)	38.9	39.3	0.091
Vaginal birth (%)	26 (51)	47 (56.5)	
Elective caesarean (%)	17 (33.3)	26 (31.3)	
Emergency caesarean (%)	8 (15.7)	10 (12)	
Mean birth weight (g)	3457	3453	0.952
Mean birth length (cm)	50.5	50.3	0.699
Birth head circumference (cm)	34.7	34.9	0.433
Male (%)	25 (49)	42 (50.6)	0.859
Infant characteristics			
Breastfed ever (%)	53 (96.4)	75 (98.7)	0.572
Mean age solid introduction (months)	5.69	5.75	0.699
Number of siblings at home	0.82	0.64	0.180
Pets at home (%)	23 (45.1)	43 (51.8)	0.475
Infant allergies by 12 months			
Sensitized (%)	8 (15.7)	20 (24.1)	0.379
Sensitized foods (%)	1 (2)	12 (14.5)	0.017
IgE mediated food allergy symptoms (%)	1 (2)	12 (14.5)	0.017
Wheeze ever (%)	14 (27.5)	33 (39.8)	0.192
Diagnosed asthma (%)	0 (0)	1 (1.2)	
Eczema (%)	19 (37.3)	33 (39.8)	0.856

*Nonallergic mothers: with no or unconfirmed history of allergic disease, and no evidence of sensitisation to common allergens.

†Maternal allergy: defined as history of allergic disease with confirmed sensitisation to common allergens.

6 months of age. There was a significant negative correlation between PKC ζ expression at birth and PBMC IL-13 responses to OVA ($\tau = -0.49$; $P = 0.019$) (Fig. S1B), BLG ($\tau = -0.59$; $P = 0.006$) (Fig. S1C) and SEB ($\tau = -0.43$; $P = 0.049$) (Fig. S1D). PKC ζ expression at birth also negatively correlated with IL-10 responses to BLG ($\tau = -0.49$; $P = 0.029$) (Fig. S1E). There were no further significant relationships between PKC expression and cytokine responses at 6 months (to any stimulations).

Discussion

Our findings suggest that low PKC ζ expression at birth is associated with reduced Th1 cytokine production at birth, increased IL-13 responses to allergens at 6 months and subsequent allergic disease. Apart from supporting previous findings (10) in an independent cohort, our present data reveal that this association is maintained in a cohort more

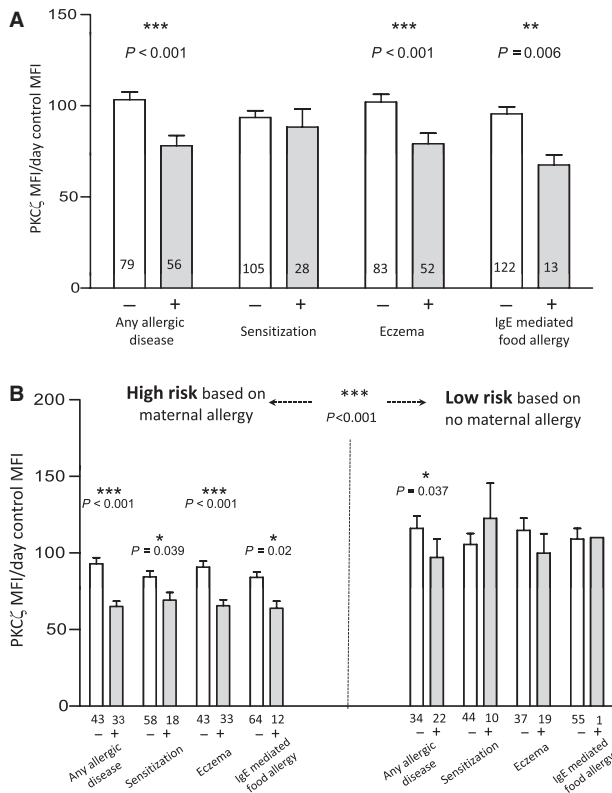


Figure 2 Neonatal CD3+ T-cell protein kinase C zeta (PKC ζ) expression and infant allergies. PKC ζ levels shown relative to (%) control infant PKC ζ expression. Levels are shown as means and SEMs, and significance levels denote significant differences in expression between children positive (+) or negative (-) for specific allergic symptoms by 1 year of age (* P < 0.05, ** P < 0.01, *** P < 0.001). (A) includes the whole population (n = 135). (B) shows population divided by maternal sensitization skin prick test. Numbers on and under bars represent numbers of participants.

representative of a 'general' population and provide new insight into factors associated with neonatal PKC expression.

In the light of a growing need for improved predictive assays, which may in turn allow targeted interventions, we suggest that measuring neonatal T-cell PKC ζ expression may increase the accuracy of allergy prediction which currently rely on the insensitive measure of cord blood IgE (15, 18) and the crude measure of 'positive family history'.

The observation that reduced PKC ζ expression was associated with maternal allergic disease (but not paternal allergy) strongly suggests a direct effect of maternal immune status on the developing foetal immune system and T-cell programming during pregnancy. The relationships were strongest in infants born to allergic mothers, which may suggest that a potential future screening test would be most applicable to this subgroup.

The stronger association with eczema, a T cell-mediated condition (compared with sensitization), suggests that PKC ζ may be implicated more in the cutaneous manifestations of disease, rather than atopy *per se*. However, these conditions

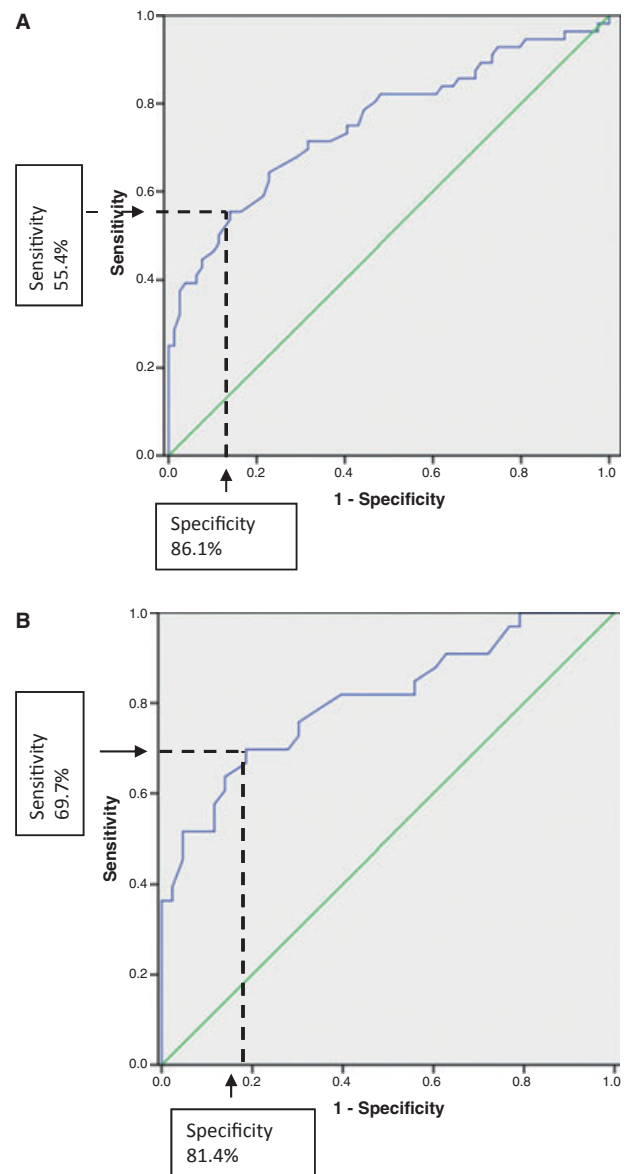


Figure 3 Sensitivity and specificity of neonatal CD3+ protein kinase C zeta (PKC ζ) in predicting allergic disease at 1 year of age. (A) In all infants (n = 135), PKC ζ expression of <72.5% of control infant predicted allergic disease with optimal specificity (86.1%) and sensitivity (55.4%). (B) In infants born to allergic mothers (n = 76), PKC ζ expression of <72.5% of control infant predicted allergic disease with optimal specificity (81.4%) and sensitivity (69.7%).

are clearly inter-related and the associations between reduced neonatal PKC ζ expression and both food allergy and food-specific responses are consistent with previously observed associations with food allergy (10), further suggesting an effect of PKC ζ on T-cell function.

While PKC ζ levels did not correlate with the ability of T cells to progress from CD45RA+ naïve T cells to CD45RO+ memory cells, the capacity for Th1 IFN γ production by

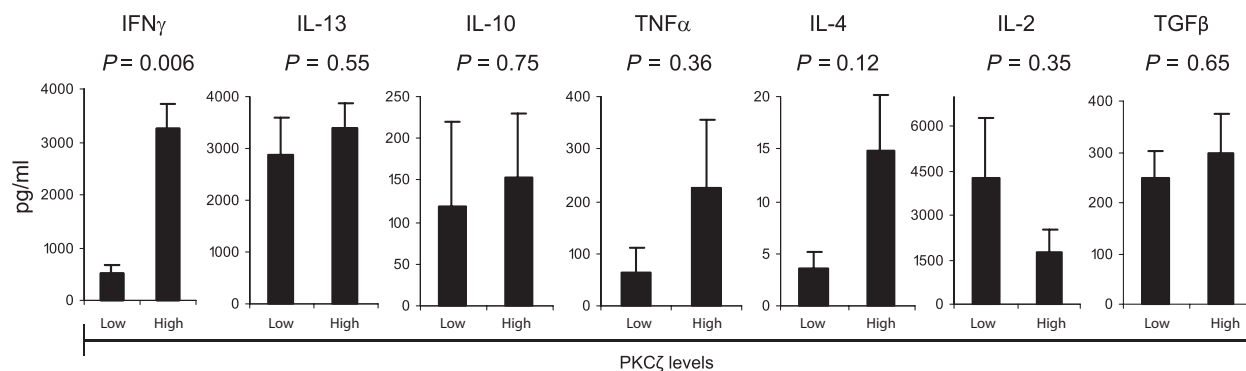


Figure 4 Association between protein kinase C zeta (PKC ζ) levels and the production of IFN γ in *in vitro* matured T cells (8 days). The cells were stimulated with phytohaemagglutinin and PMA (phorbol 12-myristate 13-acetate). In infants with low PKC ζ , mean level of

IFN γ produced was 464.5 ± 214.9 (mean + SEM) pg/ml, and in infants with high PKC ζ , mean level of IFN γ 3241.8 ± 879.3 pg/ml. Significance of difference: * $P < 0.05$. The results are presented as mean $\pm$ SEM of five CBMC.

matured cells correlated significantly with the level of PKC ζ expression in the naive state (prior to maturation). Thus, relatively low neonatal PKC ζ expression may be a risk factor for reduced Th1 maturation and this may predispose to eczema, particularly as impaired IFN γ production is seen in both established disease (19) and presymptomatically at birth (2). Approaches using intracellular cytokine staining and flow cytometric analysis may give better insights into the relationship between this PKC ζ and cytokine production. Interestingly, our preliminary unpublished observations suggest that PKC ζ may be important in regulating the survival of a proportion of maturing neonatal T cells and we speculate that in this manner there is a preference towards T cells that produce IFN γ upon stimulation.

In conclusion, our results suggest that relatively low neonatal PKC ζ expression is a marker of early T-cell dysfunction, a prelude to the expression of a cytokine profile conducive with the development of allergic disease in early infancy, and may be useful alone or as an adjunct to family history in predicting an early allergic phenotype. Our findings also suggest that developing neonatal screening strategies may be feasible using our standardized flow cytometry method.

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Conflict of interest

This study was funded by the National Health and Medical Research Council (NHMRC) of Australia and was conducted independently of any commercial entity. No conflict of interest exists for any author.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

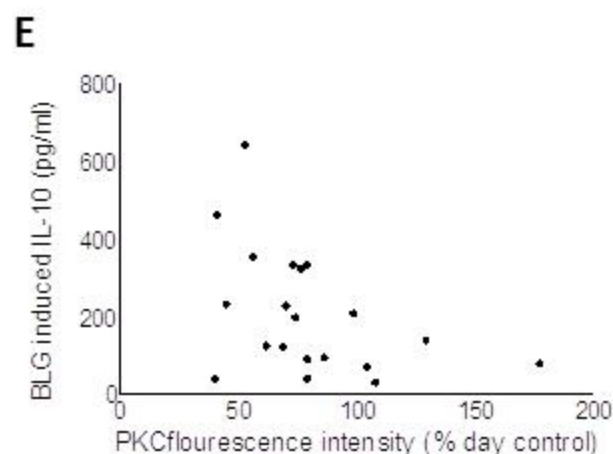
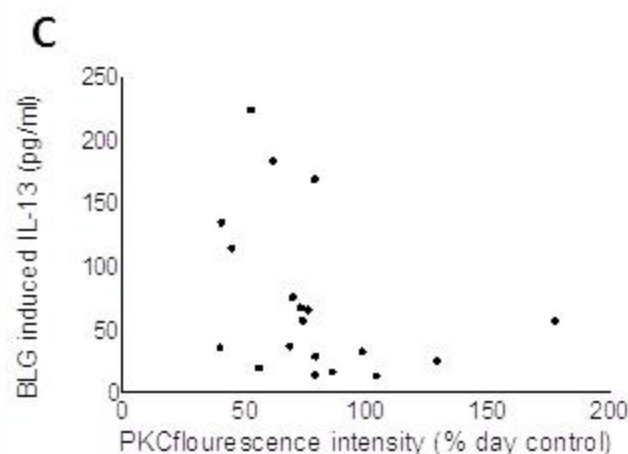
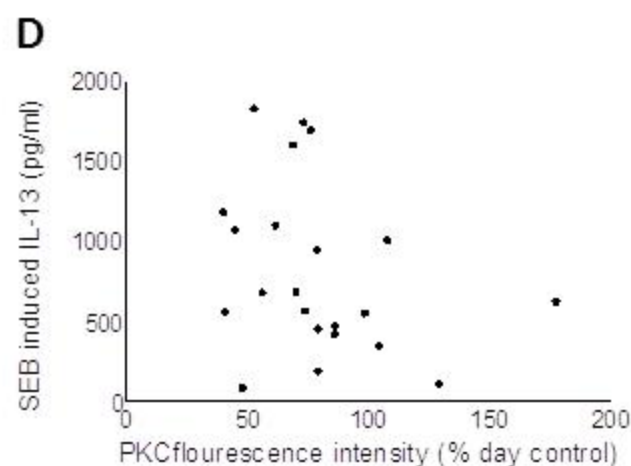
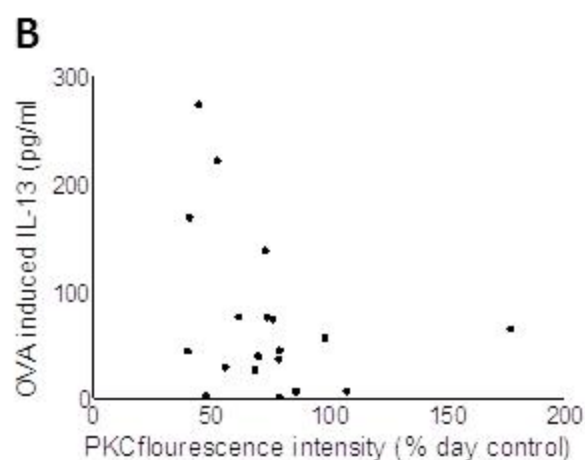
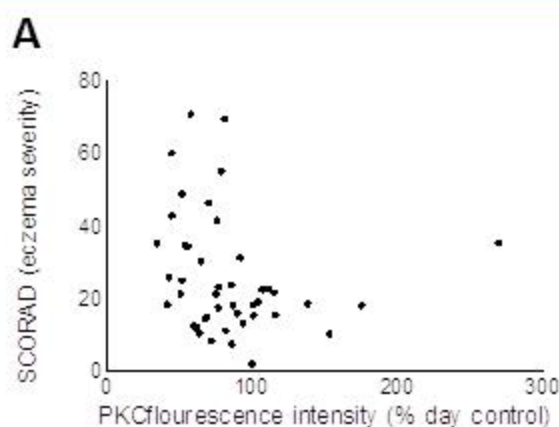
Figure S1. Association between PKC expression at birth (expressed relative to day control) and (A) Eczema severity at 12 months, (B) OVA induced IL-13 production at 6 months, (C) BLG induced IL-13 production at 6 months, (D) SEB induced IL-13 production at 6 months, and (E) BLG induced IL-10 production at 6 months.

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Supplementary figure 1



Supplementary Figure 1. Association between PKC expression at birth (expressed relative to day control) and **A**) Eczema severity at 12 months, **B**) OVA induced IL-13 production at 6 months, **C**) BLG induced IL-13 production at 6 months, **D**) SEB induced IL-13 production at 6 months and **E**) BLG induced IL-10 production at 6 months.