

T-cell activation genes differentially expressed at birth in CD4⁺ T-cells from children who develop IgE food allergy

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Abstract

Background: Presymptomatic immaturity in neonatal T-cell function is a consistent antecedent of allergic disease, including reduced responsiveness to polyclonal activation.

Methods: To elucidate the underlying mechanisms, we examined for differences in T-cell gene expression in longitudinal samples collected at birth and at 1 year of age in children with ($n = 30$) and without IgE-mediated food allergy ($n = 30$). We employed a low-level soluble anti-CD3 stimulus to activate the T-cell receptor (TCR) and surveyed gene expression by DNA microarray in purified CD4⁺ T-cells. Allergen-specific responses were assessed in parallel functional studies.

Results: At birth, the allergic group showed a reduced number of genes up regulated in response to anti-CD3 treatment on the microarray and a reduced lympho proliferative capacity, suggesting clear differences in T-cell signalling pathways. Polymerase chain reaction (PCR) validation of candidate genes confirmed significantly lower expression of a number of genes in the allergic group including RELB, NF κ B2, LIF and FAS. By 12 months of age, there were marked changes in the anti-CD3 response in all infants, culminating in upregulation of cytokine genes (IL-5, IL-13, IL-17 and IL-22). Neonatal differences were no longer apparent. Instead, the allergic group, all symptomatic by this age, showed differential expression of T-cell lineage pathways including GATA-3, MAL and FcER1 in unstimulated T-cells. Allergen stimulation induced significantly higher cytokines production (IL-5, IL-13 and IFN γ) in the allergic group.

Conclusion: Although transient, suboptimal neonatal T-cell activation pathways that signal through the NF- κ B complex may affect the developmental transition of T-cell phenotypes in the periphery shortly after birth and may increase the risk of food allergy.

Significant differences in cord blood T-cell function have been consistently observed in neonates who subsequently develop allergic disease (1–4). One of the most striking and reproducible antecedents of the allergic phenotype is relative T-cell immaturity compared with neonates who do not go on to develop allergic disease, including attenuated capacity for T-cell expansion (5) and reduced production of IFN- γ (6–8) and other cytokines in response to polyclonal T-cell activation. These mitogen-induced disparities provide compelling evidence of putative differences in neonatal T-cell function, at an age when allergen-specific responses are unrelated to allergen exposure (5, 9) or classical memory (10), and are therefore of uncertain significance. Whilst this is not well understood, these variations in neonatal T-cells appear to be an important predisposing factor for the development of

allergic diseases. Furthermore, the increasing rate of allergic disease in infancy suggests that variations in T-cell immunity are likely to reflect specific T-cell pathways modified by gene–environment interactions during pregnancy. Characterizing the genes and molecular pathways involved may reveal novel targets for therapeutic intervention.

We have recently provided evidence that T-cell signalling proteins are expressed at lower levels in neonates who subsequently develop allergic disease (11), placing firm emphasis on signalling cascades induced downstream of the T-cell receptor (TCR). To further characterize the T-cell signalling pathways altered at birth, we have adopted a genome-wide approach to examine for differences in cord blood T-cell gene expression in allergic and nonallergic individuals based on the well-defined subsequent allergic outcomes. We used an

antibody specific for the T-cell receptor to examine for differences in signalling when T-cells are activated. We profiled gene expression following activation of the TCR with low-level soluble anti-CD3 in purified populations of neonatal CD4⁺ T-cells and compared the response between children with early allergic outcomes (IgE-mediated food allergy in the first year of life) with children who remained nonallergic. The specific focus on food allergy was driven by the dramatic recent rise in this condition (12) and the pressing need to explore underlying mechanisms.

Our findings demonstrate that specific aspects of cord blood T-cell function are attenuated at birth in children destined to develop food allergy in early life.

Methods

Cohort

The cohort for this study consisted of sixty children ($n = 30$ with IgE-mediated food allergy and $n = 30$ nonallergic) selected from existing bio-banked specimens with defined allergic outcomes determined by prospective follow-up assessments at 1, 2.5 or 5 years of age. The cohort was recruited through Princess Margaret Hospital for Children under approval from the institutional ethics committee.

The allergic group consisted of children who developed IgE food allergy to the three most common food allergies, egg, milk and peanut. Food allergy status was determined by a paediatric allergist based on outcomes at the 12-month physical assessment, case histories and allergy testing. These children were subsequently followed up at 2.5 and 5 years where possible. Food allergy was defined by clear immediate symptoms (within 1–2 h) on exposure to egg, milk or peanut (including anaphylaxis, angioedema or urticaria) and confirmed IgE-mediated sensitivity to the triggering food (egg, cow's milk or peanut) by virtue of a positive skin prick test at (> 3 mm wheal size) 12 months of age. Nonallergic children showed no evidence of either allergic disease or sensitization (by skin prick test) at any of the postnatal visits (at 1, 2.5 or 5 years of age).

Cell preparation and culture methodologies

We used a soluble anti-CD3 mitogen at low doses to study the T-cell activation gene programme. Cryopreserved stocks of peripheral blood mononuclear cells (PBMC) or cord blood mononuclear cells (CBMC) were thawed and seeded at 2.5×10^5 cells per well in 96-well round-bottom polystyrene plates with 20 replicate wells (5×10^6 cells) per condition (treated and untreated). Cells were cultured in AIM-V media plus β -mercaptoethanol (4×10^{-5} mol/l), alone or in the presence of optimal levels of soluble anti-CD3 monoclonal antibody (0.5 μ g/ml; Miltenyi, New South Wales, Australia) with recombinant human IL-2 (10 U; Sigma Aldrich, Sydney, Australia). Optimal concentrations of cell culture stimulants were determined in preliminary titration experiments using a range of functional readouts. Following 24 h in culture, replicate wells were combined and CD4⁺ T-cells were isolated from stimulated and unstimulated cultures by positive selec-

tion using magnetic Dynabeads (Invitrogen, Victoria, Australia) to 85–95% purity (as determined by flow cytometry). Cytokine production (IL-5, IL-10, IL-13, IL-17, TNF- α and IFN- γ) to anti-CD3 was monitored in cell culture. Supernatants were quantified with Luminex Xmap multiplexing technology (Luminex Corp, Austin, TX, USA). The limits of detection were 3–10 000 pg/ml for all cytokines, and all data were shown as increases above unstimulated controls. For the assessment of immune function, PBMC or CBMC were cultured in media alone or in the presence of ovalbumin (OVA) (100 μ g/ml; Sigma) for 48 h before aspiration of supernatants for luminex analysis. Proliferative responses to anti-CD3 (0.5 μ g/ml) + IL-2 (10 U) were also measured where cell numbers permitted in a small subset of individuals after 72 h in culture as previously described (13). Statistical analysis by Mann–Whitney *U*-test was performed in the SPSS software (IBM, New South Wales, Australia).

Microarray methodologies

Total RNA was harvested from purified CD4⁺ T-cells using RNeasy mini kits (Qiagen, Victoria, Australia). RNA integrity was assessed on the Agilent 2100 Bioanalyzer using the RIN method and quantified by Nanodrop spectrophotometry. All RINs were between 7.2 and 10 with a minimum requirement for RIN 7.0 to pass QC. We utilized an RNA-pooling strategy to maximize the number of individuals in the microarray study to increase statistical power and representation of intra-individual variability in gene expression. This strategy has been shown to provide benefits in smaller scale microarray studies (14). Equimolar amounts of RNA (1 : 1 ratio of CD4⁺ T-cells from each donor) from six independent donors were pooled prior to labelling and hybridization to Affymetrix microarrays. Five independent pools (biological replicates) were created for each class (allergic/nonallergic), and there were two treatment conditions (stimulated/unstimulated), consistent with consensus microarray practices (15, 16). Atopics were randomized across pools to provide an even mix of food allergy phenotypes across arrays. Pool membership was preserved between cord blood experiments and 12-month experiments in a repeated measures design. Pooled RNA samples were concentrated using RNeasy minelute kits (Qiagen), and 100ng pooled RNA was prepared for microarrays using the Affymetrix WT sense labelling kit according to manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Hybridization to Affymetrix Human Gene 1.0 ST arrays, washing, staining and scanning were performed by the Molecular Genetics Research Services at Sir Charles Gardiner Hospital, Perth, Australia.

Quantitative RT-PCR

Quantitative PCR was carried out as previously described (17). Absolute quantitation was performed using standard curves derived from serially diluted RT-PCR products, and data were normalized to the housekeeping gene UBE2D2 (18). QC was performed for each assay by means of melt curve analysis to ensure specificity, and baselines were set at

a maximum of two cycles below the beginning of the amplification curve. Correlation coefficients were measured for each standard curve and were routinely between 0.992 and 0.999. Data were expressed as gene expression above unstimulated controls and multiplied by a scaling factor to obtain whole numbers. Statistical analysis by Mann–Whitney *U*-test was performed in the SPSS software.

Statistical analysis of microarray data and quality control

The quality of the microarray data was assessed using QC metrics in the Expression Console software (Affymetrix), with the average positive versus negative AUC 0.8695 ($n = 80$, ± 0.017) for all microarray experiments. The microarray data were preprocessed with the PLIER algorithm (gcbg background subtraction, quantile normalization and iterPLIER summarization) (17). Data were variance stabilized by adding 16 to all data points, followed by log₂ transformation in the R environment (19). Clustering and mds plot analysis were performed using the gplots package (20) to confirm the absence of batch effects, sex effects or outlying individuals. An empirical Bayes statistical procedure was employed to identify genes significantly modulated above unstimulated controls in allergics and nonallergics using the method of McCarthy (21). The *P* values derived from the moderated *t*-statistics were adjusted to control the false discovery rate (FDR) (22). Differentially expressed genes were identified by specifying a contrast matrix to the fitted linear model, using the cut-offs log₂ fold change > 1.5 above background and FDR-adjusted *P*-value < 0.05 . To identify differentially expressed pathways, the GSA gene sets test (23) was performed on the data sets. A minimum of 1000 permutations were performed to estimate *P*-values, and an FDR cut-off of 0.01 was specified. The array data were interrogated with the c2-gene sets, and canonical pathways hosted by the BROAD institute (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>). Ontology enrichment was performed using the GOrilla bioinformatics tool under the default settings (24).

Results

Characteristics of the sample population

All children in the allergic group had a history of IgE-mediated clinical reactions to foods (egg, milk or peanut) by 12 months of age and confirmed sensitization to that food on Skin Prick Test (SPT). The characteristics of the groups and their subsequent phenotypes are shown on Table 1. The non-allergic children had no symptoms or sensitization to foods at 12 months or at the follow-up visits. There were no differences in birth characteristics, sex and infant feeding practices (including the timing of introduction of complimentary foods) between the groups.

Immune responses to allergens

We first examined allergen-specific responses to OVA (as the majority of allergic children were known to be egg-sensitized)

using whole mononuclear cell populations, as a marker of the allergic phenotype. Consistent with previous studies (3, 6), allergen-induced cytokine responses were significantly lower at birth in subsequently atopic children, including lower production of IFN- γ , IL-13, IL-5 and IL-10 compared with the nonallergic group. As noted previously, the nature of allergen-specific responses in the neonatal period is not clear (10), but appear to reflect the general immaturity of T-cell responsiveness that is also seen with polyclonal stimulation compared with nonallergic individuals at this age (5–8). At 12 months, when the allergen-stimulated culture responses more clearly reflect the activity of memory (CD45RO) T-cells specific for allergen (OVA), we observed significantly higher IL-5, IL-13 and IFN- γ responses in the allergic group (Fig. 1). These responses are consistent with similar previous studies (25, 26) and reflect the greater expansion of this memory (CD45RO) T-cell population in allergic than nonallergic children.

Polyclonal TCR activation for microarray analysis

To explore the more generalized differences seen in early T-cell signalling (5–8), we profiled the polyclonal response to anti-CD3 using a gene expression microarray in purified CD4⁺ T-cells from the same individuals. We used a moderated *t*-test (27) to identify genes significantly modulated to the anti-CD3 stimulus specifying a cut-off of fold change > 1.5 and adjusted *P*-value < 0.05 . The anti-CD3-induced gene response was markedly reduced in the allergic group at birth consisting of 2143 probes compared with 3173 probes in nonallergic group. We performed a parallel assessment of the magnitude of expansion to this stimulus and found a significantly reduced proliferative response in the allergic group (Fig. 2). Ontology enrichment of the anti-CD3 response in allergics and nonallergics (combined response) indicates dedicated sets of genes with specific functions related to the activity of the TCR receptor, accompanied by genes involved in cell proliferation, transcription and growth (detailed description provided in Data S1).

We used a gene sets test (23) to identify differentially expressed genes between allergics and nonallergics. This test has the added benefit of detecting coordinate changes in gene pathways. A two-class comparison was performed on the background-adjusted gene expression profiles from allergics and nonallergics. In the birth data set, we detected 8 differentially expressed sets of genes including TNF-dependent NF- κ B genes, G-protein signalling genes, type 1 interferon genes, IL-6 dependent and Notch-dependent genes (Table 2). We ranked the genes in each set by test score and found that the majority of these sets were enriched for genes in the NFKB family. We selected the top-ranked genes in each gene set identified for validation. Validation was performed by qRT-PCR on individual RNA samples from the same cohort, and we identified RELB ($P = 0.018$), NFKB2 ($P = 0.045$), LIF ($P = 0.045$) and FAS ($P = 0.04$) to be significantly reduced in the allergic group, with a notable trend for downregulation of NFKB1 ($P = 0.075$) and NFKB1A ($P = 0.085$) (Fig. 3).

Table 1 Clinical characteristics of the 60 infants selected for this study

Characteristic	Food allergy (n = 30)		Healthy (n = 30)	P value
Gender* (n(%))				
Male	20 (67)		15 (50)	0.2949
Female	10 (33)		15 (50)	
Family history * (n(%))	30 (100)		24 (92)	
Gestation* (w(SE))	38.9 (0.28)		39.1 (0.329)	0.772
Birth weight* (g(SD))	3596 (70.8)		3476.4 (113.4)	0.359
Length* (g(SD))	50.5 (0.465)		49.8 (0.55)	0.303
Head circumference* (cm(SD))	35.1 (0.25)		34.55 (0.35)	0.197
Number of siblings* (n(SD))	0.48 (0.13)		0.91 (0.2)	0.06
Age of first weaning* (m(SD))	5.3 (0.22)		4.8 (0.22)	0.138
Age milk introduced* (m(SD))	8.9 (0.51)		9.3 (0.52)	0.645
Age egg introduced* (m(SD))	10.2 (0.32)		9.1 (0.54)	0.086
Age peanut introduced* (m(SD))	10 (0.84)		9.7 (1.1)	0.831
Clinical outcomes	Food allergy		Nonallergic	
Sensitization† (n(%))	12 months	2.5 years	12 months	2.5 years
Any food	30 (100)	18 (85.7)‡	0 (0)	0 (0)
Egg	26 (86.7)	12 (57)	0 (0)	0 (0)
Milk	6 (20)	3 (14.3)	0 (0)	0 (0)
Peanut	10 (33.3)	7 (33.3)	0 (0)	0 (0)
Any inhalant	8 (27)	12 (57)	0 (0)	0 (0)
HDM	3 (10)	12 (57)	0 (0)	0 (0)
Mould	2 (7)	1 (4.7)	0 (0)	0 (0)
Cat	5 (17)	6 (28.6)	0 (0)	0 (0)
Symptoms* ((n(%))				
Anaphylaxis	4 (13)	4 (19)	0 (0)	0 (0)
Respiratory	6 (20)	5 (24)	0 (0)	0 (0)
Angiodema	10 (33)	4 (19)	0 (0)	0 (0)
Urticaria	21 (68)	7 (33)	0 (0)	0 (0)
Gastrointestinal	3 (10)	0 (0)	0 (0)	0 (0)
Asymptomatic	0 (0)	5 (24)	30 (100)	30 (100)

†Determined by skin prick test.

*Determined by questionnaire data.

‡SPT Data on 21 of 30 children at 2.5 years.

Statistical comparisons by Chi square and *t*-test for continuous data.

We used the same polyclonal stimulation in the samples collected from the same individuals at 12 months, to allow comparison with gene expression patterns at birth. There were marked differences in the pattern of postnatal compared with neonatal responses in all individuals (Fig. 4) highlighting the significant and rapid developmental changes over the first year of life. The moderated *t*-test identified 640 genes that are differentially expressed between the neonatal and postnatal response (of all individuals regardless of atopic status). Ontology enrichment indicates these genes are heavily involved in reorganization of the nucleosomes and chromatin restructuring and were enriched for histone modifier genes (HIST1HK1 and HIST1HK2 family), centromere proteins (CENP family) and cell cycle control genes. This response was accompanied by large-scale upregulation of cytokines IL-5, IL-13, IL-17 and IL-22. The latter was confirmed by differences in analysis of protein levels from these cultures (Fig. 4),

although we did not observe phenotypic differences in these developmental pathways by cytokine analysis or PCR (data not shown).

In this population, comparison of the 'change' in gene expression (above unstimulated cells) patterns following polyclonal stimulation did not reveal significant differential response profiles between allergic and nonallergic infants at 12 months. This may indicate (1) that there may be baseline constitutive differences in gene expression that are not revealed in background-corrected TCR activation profiles and/or (2) that the differences in gene expression are restricted to the very small subpopulation of allergen-specific CD4⁺ T-cell clones. To test the first hypothesis that there were constitutive differences in the resting (unstimulated) gene expression profiles, we used the gene sets test to perform a two-class comparison of the unstimulated samples. The test returned nine gene sets differentially expressed with a false

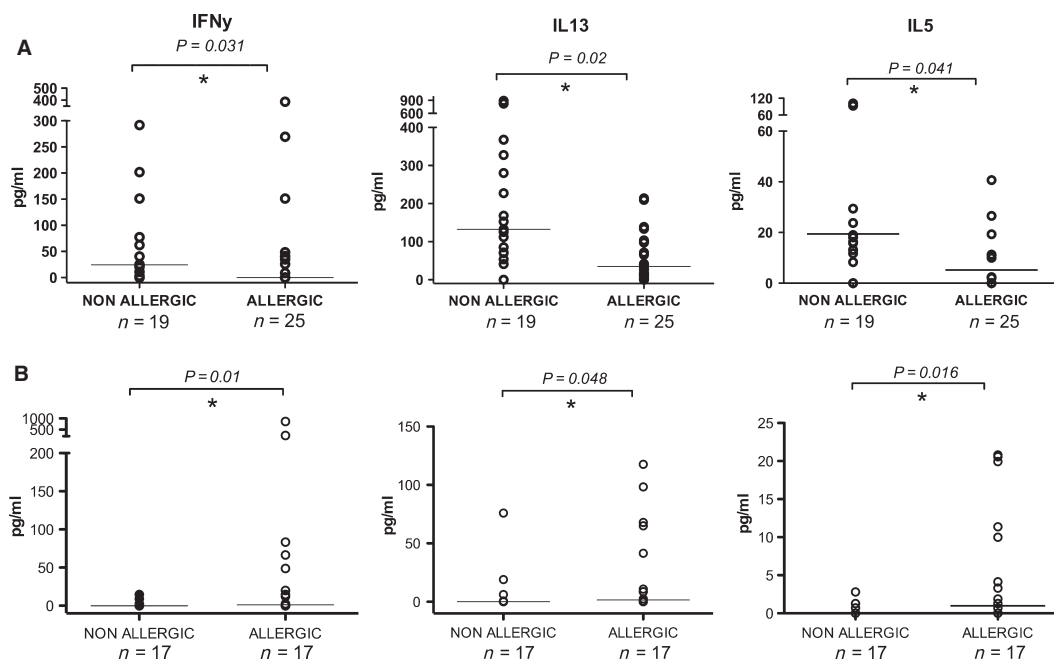


Figure 1 Allergen-specific (ovalbumin) cytokine responses at birth (A) and 12 months (B). The data are presented as background-

corrected cytokine levels, and the median is indicated by the black line. Statistical analysis by Mann-Whitney *U*-test. **P* < 0.05.

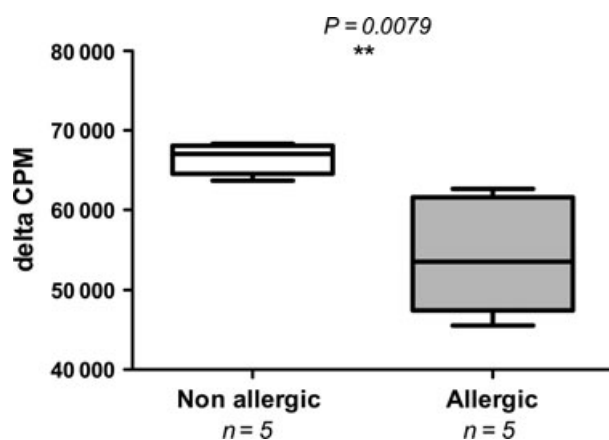


Figure 2 T-cell proliferative responses to anti-CD3/IL2. CBMC were cultured alone or with anti-CD3/IL-2 for 72hrs, and incorporated thymidine was measured as a surrogate for cell proliferation. Data are presented as median background-corrected counts per minute with range. Statistical analysis by Mann-Whitney *U*-test.

discovery rate <0.01. These included the TCR pathway, FCER1 pathway, GATA-3 pathway, MAL pathway, CTLA4 pathway insulin pathway, colorectal cancer pathway, PYK2 pathway and a pathway called 'downstream signal transduction' (Table 3). Genes in these sets that were expressed at higher levels in allergic infants at 1 year include PRKACG, MAP2K3, JUNB, IL-4, NFKBIE, CSF2 and others (full list and gene scores provided in the Data S1). Many of these

genes are logical candidates based on their recognized functional relevance in CD4⁺ T-cells. The second hypothesis that differences in gene expression at this age are restricted only to a small subset of CD4⁺ T-cells is more difficult to test. Other studies have suggested that T-cells dysfunction in infants with allergic predisposition is not restricted to isolated allergen-specific clones (28, 29) and it was not feasible to isolate allergen-specific CD4⁺ T-cells for the microarray experiments performed here.

We also specifically examined whether genes that previously showed attenuated expression (at birth) in the allergic group (in the TNF-NF- κ B pathway) were also differentially expressed at 12 months of age compared with the nonallergic population (Fig. 5). In PCR validation studies, we observed residual trends for reduced expression for these specific genes in allergic infants (NFKB1A, NFKB12 LIF and LIF), but these differences were no longer significant (Fig. 5C) reflecting the significant developmental changes over this period.

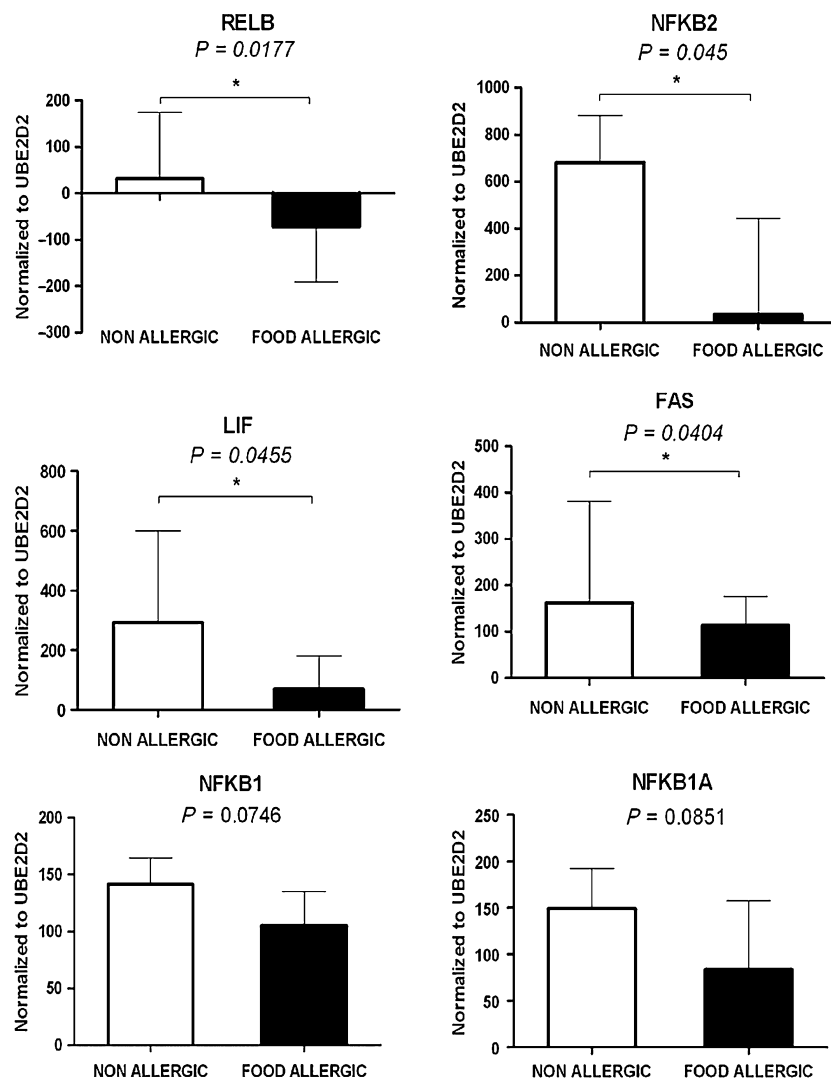
Together these observations demonstrate clear age-related changes in patterns of gene expression and suggest that pathways that were differentially expressed in allergic individuals in the presymptomatic period (at birth) may no longer be relevant in the symptomatic period.

Discussion

To our knowledge, this is the first study to explore the underlying gene pathways implicated in the well-recognized deficiencies in polyclonal T-cell responses of neonates who go on to develop allergic disease. Specifically, we found that

Table 2 Differentially expressed gene pathways in the neonatal data set

Gene set	Gene set description	Score	P-value	FDR
1538	Genes involved in TNF-NF κ B signalling	1.055	<0.01	<0.01
420	G-alpha 13 signalling	0.427	<0.01	<0.01
1357	Type I interferon genes	0.515	<0.01	<0.01
861	TNF-dependent, NF κ B-dependent genes	0.71	<0.01	<0.01
532	IL-6 pathway	0.475	<0.01	<0.01
882	C/EBP-induced early response genes	0.59	<0.01	<0.01
936	Notch signalling in cell differentiation	0.306	<0.01	<0.01
524	TNF-responsive genes	0.849	<0.01	<0.01

**Figure 3** qRT-PCR validation of microarray candidate genes at birth. The figure shows normalized background-corrected (stimulated-unstimulated) gene expression from individual samples in the

validation cohort. *P* values are derived from the Man-Whitney *U*-test. Bars represent medians with interquartile range.

impaired lymphoproliferation in subsequently allergic children was associated with a reduced number of upregulated genes in response to stimulation at birth and that genes

showing reduced expression were enriched for genes sets associated with cell signalling including TNF-dependent NF- κ B genes, G-protein signalling genes and type 1 interferon genes.

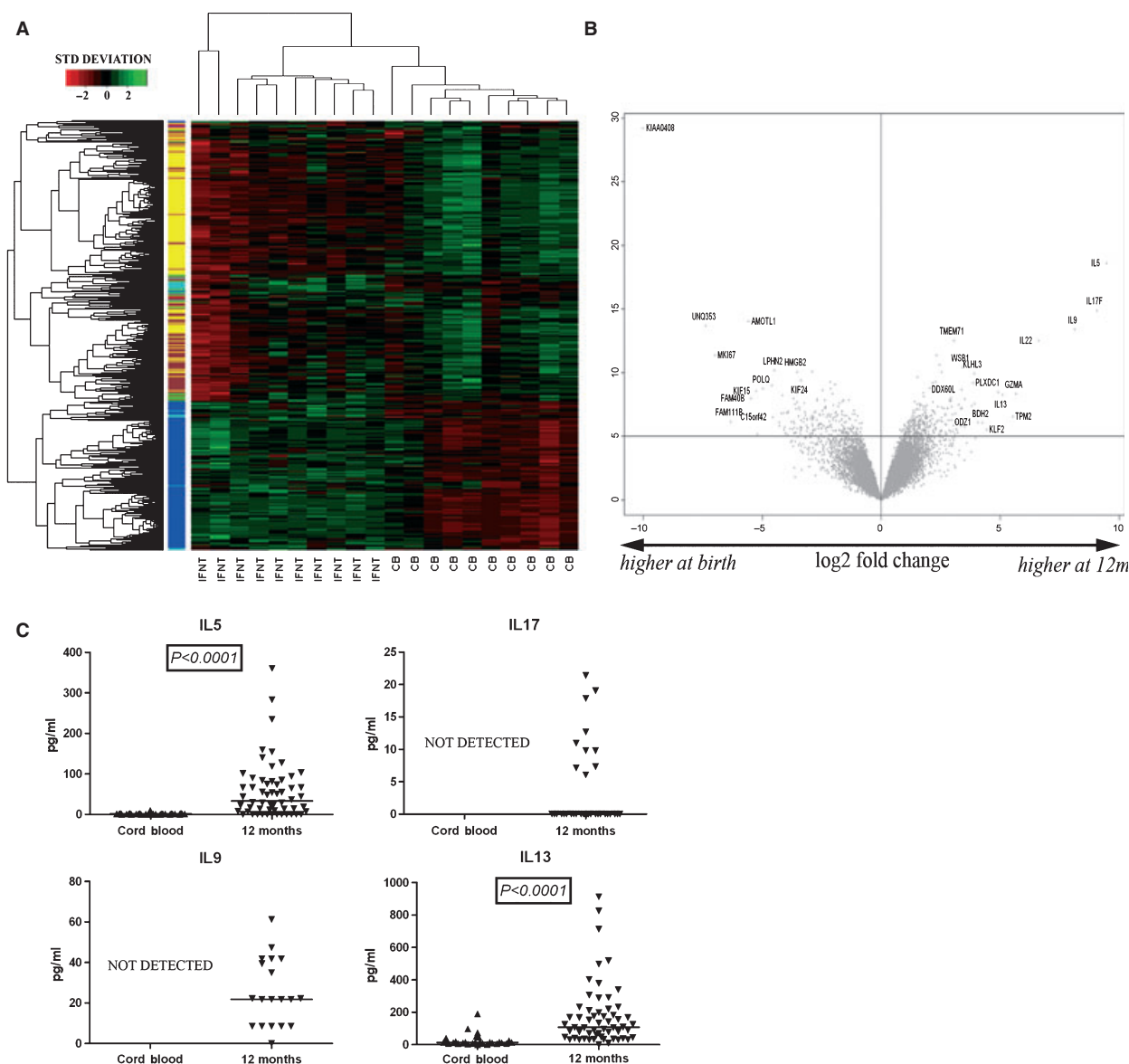


Figure 4 Differences between cord blood and peripheral blood CD4⁺ T-cell activation response to anti-CD3 in all individuals. (A) Shows heat map of combined cord blood and peripheral blood microarray data set filtered for 3,331 T-cell activation genes identified in neonatal experiments. Rows represent genes, columns represent samples. Cells are colored according to level of expression. (B) Volcano plot visualization of changes in gene expression from birth to 12 months in all individuals. Genes are

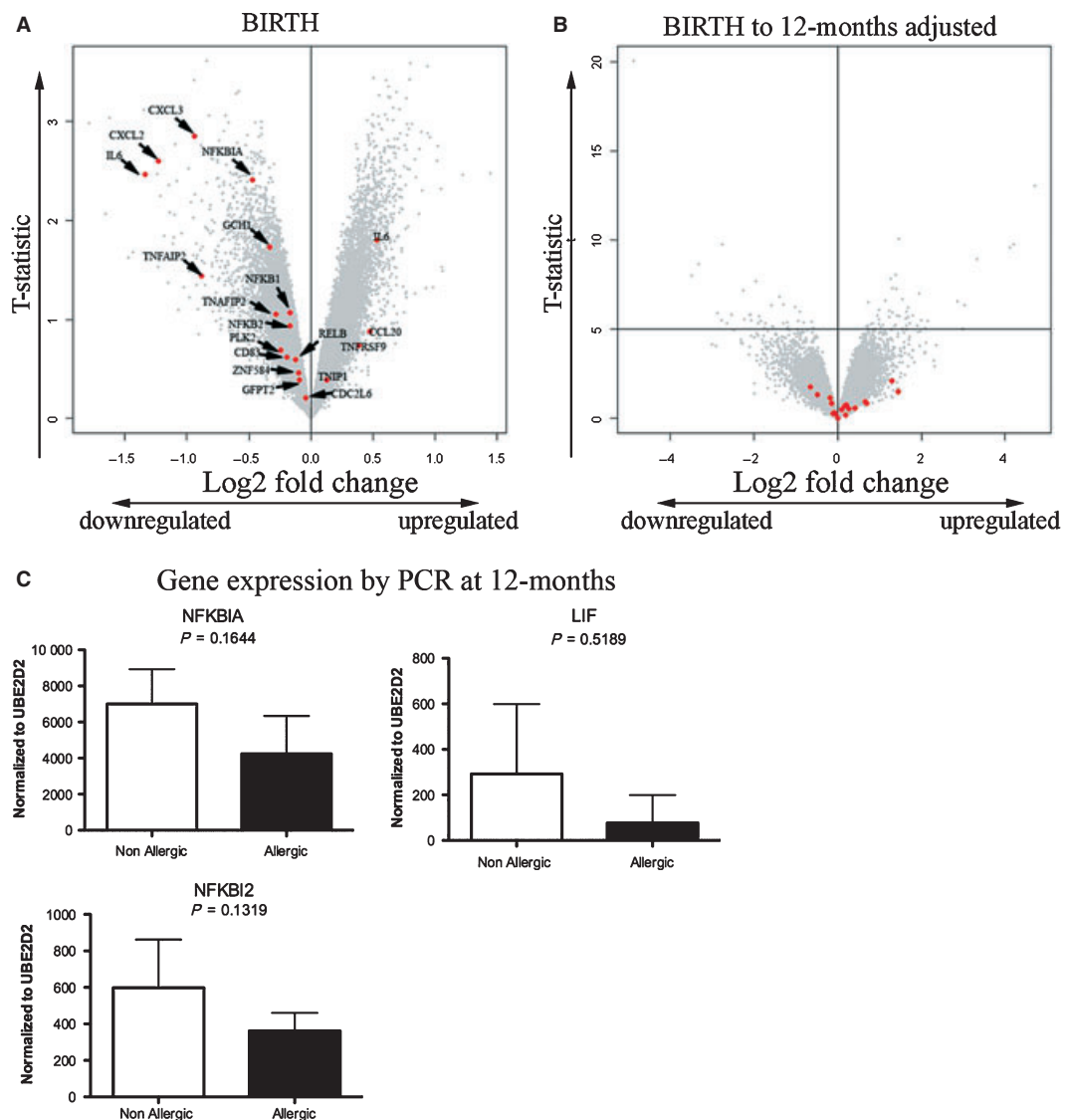
ranked on the plot by log2 fold change and moderated *t*-test score for differential expression (between birth and 12 months). The horizontal line is a visual guide only. (C) Cytokine production to anti-CD3 in all individuals measured at birth and 12 months. Data are presented as background-adjusted median cytokine levels and statistical analysis by Mann-Whitney *U*-test. *CB* = cord blood samples, *INFT* = peripheral blood samples.

Whilst it was not possible to validate all potential genes expressed at lower levels in the allergic group, the top-ranking genes showed reduced expression in validation studies included NFKB2, RELB, LIF and FAS. Some of these genes are transcriptional co-activators that potentially have wide-ranging downstream effects on other genes. In particular, the proliferative response of lymphoid cells depends upon the transduction of activation signals through the NF- κ B/Rel

complex, and here we showed a reduction in NFKB2, RELB and a trend for reduced expression of other NFKB genes. Many will note that regulation of the NF- κ B complex occurs by post-transcriptional means whereby inhibitory proteins of the I κ B family sequester NF- κ B in the cytoplasm preventing nuclear entry. We report a reduction in the transcriptional levels of these genes from total mRNA, the effects of which may be amplified by inhibitory proteins in the cytoplasm.

Table 3 Differentially expressed gene pathways in resting CD4⁺ T-cells at 12 months

Gene set	Gene set name	Score	P-value	FDR	num genes
129	KEGG_T_CELL_RECEPTOR	-0.5758	<0.01	<0.01	108
163	KEGG_COLORECTAL_CANCER	-0.55342	<0.01	<0.01	62
258	BIOCARTA_FCER1_PATHWAY	-0.4756	<0.01	<0.01	39
264	BIOCARTA_GATA3_PATHWAY	-0.8469	<0.01	<0.01	16
292	BIOCARTA_INSULIN_PATHWAY	-0.6615	<0.01	<0.01	22
298	BIOCARTA_PYK2_PATHWAY	-0.7371	<0.01	<0.01	29
354	BIOCARTA_MAL_PATHWAY	-0.7544	<0.01	<0.01	19
462	REACTOME_CTLA4_INHIBITORY_SIGNALING	-0.8118	<0.01	<0.01	21
476	REACTOME_DOWN_STREAM_SIGNAL_TRANSDUCTION	-0.85058	<0.01	<0.01	35

**Figure 5** Volcano plot visualization of genes in the TNF-NF- κ B pathway in allergic subjects at birth (A) and at birth to 12 months adjusted (B). (C) qRT-PCR gene expression at 12 months: examining genes that were differentially expressed at birth. Data are pre-

sented as normalized background-corrected gene expression from individual RNA samples. P values are derived from the Man-Whitney U -test. Bars represent medians with interquartile range.

The immune system undergoes dramatic and dynamic changes in the early postnatal period (30). As anticipated in this context, comparison of gene expression patterns in all individuals between birth and 12 months indicated that a subset of T-cell activation genes undergo developmental regulation, including IL-5, IL-13, IL-17 and IL-22.

Within this same relatively short time frame, the allergic children have already established the classical Th2 responses to allergens, as overtly evidence in their IgE-mediated clinical responses to food. The significant change from impaired neonatal responses to significantly increased Th2 (IL-5, IL-13) and Th1 (IFN- γ) responses to allergens by 12 months highlights the dynamic transition of immune responses in this period. As allergen-responsive cells exist at low precursor frequency in the periphery, these increased responses are likely to reflect the greater expansion of these specific memory T-cells in the allergic infants. In the context of these dramatic changes, it is unsurprising that the patterns of gene expression seen at birth are no longer evident by 12 months of age. In keeping with other functional studies (5, 31), this suggests that deficient neonatal responses, whilst transient, are likely to play a key role in allergic predisposition. Our findings suggest that specific T-cell signalling genes are implicated, consistent with our previous observations of reduced protein expression of protein kinase C T-cell signalling molecules in neonates (11). Together this data underscore the importance of antenatal events in the modification of gene expression during pregnancy.

As we did not have samples from intervening time points between birth and 12 months, it is more difficult to identify specific gene pathways that may be implicated in the rapid transition of T-cell phenotypes. Although we have identified candidate pathways that appeared to be differentially expressed in unstimulated CD4⁺ T-cells of allergic infants at 12 months of age, these need further exploration. It is notable that these include the TCR pathway, the MAL T-cell differentiation factor pathways and the GATA-3 pathway, which is a master regulator of Th2 differentiation (32). Ideally, future studies with more frequent sampling over the course of early infancy are needed to determine the significance of this. Previous studies of early postnatal development have shown that infants at high genetic risk for atopy have delayed maturation of T-cell competence, with significantly lower immunocompetent T-cell precursors relative to low risk infants (29). This, together with studies showing that this altered postnatal immune responsiveness generalizes not only to polyclonal (33) but to other antigen responses (such as vaccines) (28), provided a sound basis for examining gene expression following polyclonal activation in this study. The lack of clear differences in gene upregulation could mean that the mitogenic stimulus did not permit detection of more subtle immaturities or differential patterns within subpopulations of CD4⁺ effector cells. This is an important avenue for further investigation.

In this case-control study, we selected children on the basis of IgE-mediated food allergy as an early, dramatic and

unequivocal expression of the allergic phenotype, which is reliably distinct from the nonallergic control group. However, it is not clear whether the differences in gene expression observed in the neonatal period are antecedents of an early allergic phenotype of food allergy *per se*. Our findings provide important direction for further studies to examine this in more detail.

It should be noted that whilst the microarray pooling strategy adopted here has been shown to improve power to detect differentially expressed genes (14), there is the potential to distort specific gene signatures pertaining to the different food allergy phenotypes surveyed here.

In summary, this study provides further evidence that differences in early T-cell signalling may predispose to allergic disease. Further studies are needed to identify the gene-environment interactions that are contribution to these neonatal differences as these may provide a major target for immunomodulation aimed at allergy prevention.

Statement of contribution

David Martino performed the laboratory work, data analysis and drafting of the primary manuscript.

Dr Anthony Bosco and Dr Kathy McKenna provided expertise in experimental design and statistical analysis and contributed to the drafting of the manuscript.

Dr Elysia Hollams and Dr Danny Mok provided advice and expertise on PCR validations.

Prof Patrick Holt and Prof Susan Prescott provided intellectual input, advice and expertise on characterization of the study cohort, design of the study and contributions to the drafting of the manuscript.

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Conflict of interest

Disclosure of potential conflict of interest: No potential conflicts of interest to disclose.

Supporting information

Additional Supporting Information may be found in the online version of this article found at: www.wileyonlinelibrary.com

Data S1. Genes expressed in CD4⁺ T-cells in response to anti-CD3.

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T-cell activation genes differentially expressed at birth in CD4⁺ T-cells from children who develop IgE food allergy.

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ONLINE SUPPLEMENT

Determination of the genes expressed in CD4+ T-cells in response to anti-CD3.

Anti-CD3 responsive genes were identified by average fold change > 1.5 above background, and FDR (313) adjusted p value <0.05 in all samples. Using this approach, a total of 3,332 genes were modulated after T-cell activation indicating substantial ‘reprogramming’ of the cell during the acute-phase response.

To visualize the genes invoked in the TCR activation program, a volcano plot was constructed, which ranks genes according to the moderated t test statistic for significance above background, and by fold change (Fig S1).

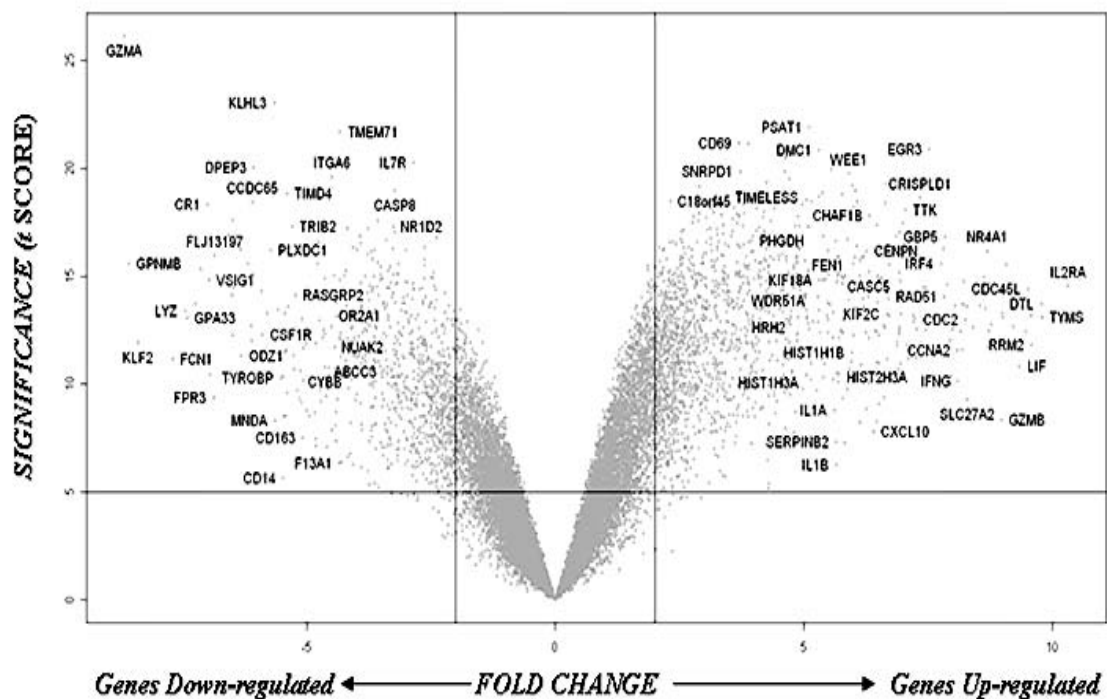


Figure S1 – Microarray analysis of the CD4+ T-cell activation response. Background corrected gene expression levels in all samples were compared employing a moderated t test (312). The data were visualized on a volcano plot that shows the value of the test statistic (y-axis) by fold change on the $\log_2$ scale (x-axis). The gates shown here are visual guides and do not reflect a cut-off for significance.

Clustering and description of the anti-CD3 response.

Next principle component analysis (PCA) and clustering was used to group genes together according to similarity of expression. Agglomerative hierarchical clustering was performed on the Pearson’s correlation coefficient calculated for each gene in the filtered data set, using the complete linkage method implemented in the cluster package (www.bioconductor.org). Clusters were resolved by the Dynamic Tree Cut method of Horvath (www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/BranchCutting). Genes were partitioned into colour-coded clusters and the data was represented on a heatmap below (Fig S2)

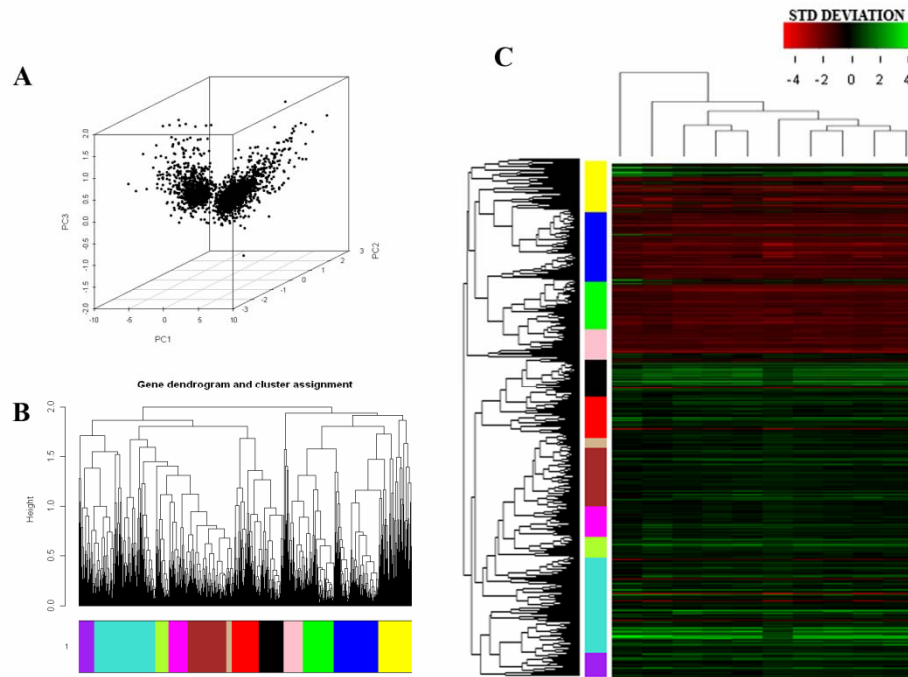


Figure S.2- Clustering of the T-cell activation response. (A) Principal component analysis on the centred and scaled data set reveals two main gradients indicated as distinct clouds. (B) Hierarchical clustering of genes and cluster assignment. (C) Heatmap of clustered gene expression profiles. Genes are represented as rows, samples as columns. Cells are colorized according to expression (red, down; green, up).

Twelve clusters in total were identified by the agglomerative hierarchical method. These clusters were significantly enriched for genes with similar functions. GO enrichment was performed on each cluster using the GOrilla online tool using the default parameters ($P < 10^{-3}$) to identify gene processes and functions and the results are summarized in the text below and listed in **Table S1**.

Table S1 – ontology enrichment of the T-cell anti-CD3 response

Cluster	Enriched Molecular Functions	Enriched Biological Process	Key Genes
Turquoise	IL2R binding MHC protein binding nuclease activity ribonucleotide binding	mitosis regulation of activated T-cell proliferation regulation of T cell homeostasis cell cycle DNA metabolism DNA replication/repair regulation of cell cycle negative regulation of IL10	TIMM50 - translocase of inner mitochondrial membrane 50 homolog (s. cerevisiae) IL21 - interleukin 21 IL2 - interleukin 2 LAG3 - lymphocyte-activation gene 3 TUBB - tubulin, beta CD8B - cd8b molecule CD8A - cd8a molecule IL2RA - interleukin 2 receptor, alpha FOXP3 - forkhead box p3 CASP3 - caspase 3, apoptosis-related cysteine peptidase
Greenyellow	ATPase activity nucleoside-triphosphatase activity hydrolase and phosphorylase activity	cell cycle DNA metabolism DNA replication/ DNA repair cell division	TAD3C - atpase family, aaa domain containing 3c RFC4 - replication factor c (activator 1) 4, 37kd KIF23 - kinesin family member 23 TNF - tumor necrosis factor (tnf superfamily, member 2) TNFSF8 - tumor necrosis factor (ligand) superfamily, member 8 TNFSF18 - tumor necrosis factor (ligand) superfamily, member 18
Magenta	RNA processing ncRNA processing and metabolism RNA/DNA metabolism mRNA processing DNA replication tRNA processing	methyltransferase activity folic acid transport activity folate carrier activity	NOP2 - nop2 nucleolar protein homolog (yeast) MRPS17 - mitochondrial ribosomal protein s17 NOP14 - nop14 nucleolar protein homolog (yeast) POLD1 - polymerase (dna directed), delta 1, catalytic subunit 125kda SLC19A1 - solute carrier family 19 (folate transporter), member 1 PRMT1 - protein arginine methyltransferase 1
Purple	nitrogen metabolism fatty acid metabolism amino acid metabolism small molecule metabolism enzyme activity RNA polymerase activity	cellular biosynthesis metabolic processes	ASNS - asparagine synthetase GOT2 - glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2) IL4 - interleukin 4 TNFSF4 - tumor necrosis factor (ligand) superfamily, member 4
Red	protein binding catalytic activity	Regulation of macromolecular structures protein metabolism catalytic activity ligase activity Protein folding	IL1B - interleukin 1, beta SOCS1 - suppressor of cytokine signaling 1 HDAC2 - histone deacetylase 2 NFKB1 - nuclear factor of kappa light polypeptide gene enhancer in b-cells 1 NFKBIA - nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha PSME2 - proteasome (prosome, macropain) activator subunit 2 (pa28 beta) PSMD1 - proteasome (prosome, macropain) 26s subunit, non-atpase, 1 HSPD1 - heat shock 60kda protein 1 (chaperonin) HSP90AB1 - heat shock protein 90kda alpha (cytosolic), class b member 1 FGF2 - fibroblast growth factor 2 (basic) ILF2 - interleukin enhancer binding factor 2, 45kda IRF4 - interferon regulatory factor 4 CAMK1 - calcium/calmodulin-dependent protein kinase i
Tan	binding to small nucleolar RNA	RNA processing rRNA metabolism Steroid biosynthesis Positive regulation of IL12	IL1A - interleukin 1, alpha IFNG - interferon, gamma IRF1
Black	DNA binding, nucleic acid binding	cell cycle, DNA metabolism, chromosome reorganization nuclear division DNA replication, DNA repair nucleic acid metabolism	HIST1H2BH - histone cluster 1, h2bh HIST1H2BK - histone cluster 1, h2bk HIST1H2BC - histone cluster 1, h2bc HIST2H2AB - histone cluster 2, h2ab TRAF4 - tnfr receptor-associated factor 4 NR4A2 - nuclear receptor subfamily 4, group a, member 2 BRCA1 - breast cancer 1, early onset POLE2 - polymerase (dna directed), epsilon 2 (p59 subunit) DNMT1 - dna (cytosine-5)-methyltransferase 1
Brown	Protein binding nucleoside binding ribonucleoside binding	mRNA transport RNA processing Negative regulation of DNA recombination metabolic process cell cycle cell division	STK3 - serine/threonine kinase 3 (ste20 homolog, yeast) NUP88 - nucleoporin 88kda NUP107 - nucleoporin 107kda NUP155 - nucleoporin 155kda HSP90AASP - heat shock protein 90kda alpha, class a member 5 TDP1 - tyrosyl-dna phosphodiesterase 1 PRPS1 - phosphoribosyl pyrophosphate synthetase 1 PRPS2 - phosphoribosyl pyrophosphate synthetase 2 SNRPF - small nuclear ribonucleoprotein polypeptide f TRMT5 - trm5 tRNA methyltransferase 5 homolog (s. cerevisiae) MRPL22 - mitochondrial ribosomal protein l22
Yellow	chemokine activity G-protein signaling signal transduction activity	immune response defense response regulation of immune response regulation of cytokine production regulation of T-cell differentiation in Thymus innate immune response regulation of T-cell differentiation oxidation/reduction steroid metabolism	IL4R - interleukin 4 receptor CD80 - cd80 molecule IL7R - interleukin 7 receptor CTLA4 - cytotoxic t-lymphocyte-associated protein 4 CD36 - cd36 molecule (thrombospondin receptor) TLR1 - toll-like receptor 1 VSI4 - v-set and immunoglobulin domain containing 4 CD180 - cd180 molecule TLR8 - toll-like receptor 8 CYP2C19 - cytochrome p450, family 2, subfamily c, polypeptide 19 CXCL16 - chemokine (c-x-c motif) ligand 16 CCR8 - chemokine (c-c motif) receptor 8 CCR5 - chemokine (c-c motif) receptor 5 CR2 - complement component (3d/epstein barr virus) receptor 2 CCR9 - chemokine (c-c motif) receptor 9 CCR1 - chemokine (c-c motif) receptor 1 IL4R - interleukin 4 receptor CCR1 - chemokine (c-c motif) receptor 1
Blue	Signal transduction activity molecular transduction activity cation binding ion binding metal ion binding transition metal ion binding zinc ion binding	Immune system process Regulation of the immune response cell signaling Cell death	CSF3R - colony stimulating factor 3 receptor (granulocyte) CSF1R - colony stimulating factor 1 receptor ITGB2 - integrin, beta 2 (complement component 3 receptor 3 and 4 subunit) CD40 - cd40 molecule, tnfr receptor superfamily member 5 CD46 - cd46 molecule, complement regulatory protein IFNGR1 - interferon gamma receptor 1 TNFRSF1A - tumor necrosis factor receptor superfamily, member 1a IL13RA1 - interleukin 13 receptor, alpha 1 GPR183 - g protein-coupled receptor 183 MAP3K1 - mitogen-activated protein kinase kinase 1 TLR4 - toll-like receptor 4 ZNF211 - zinc finger protein 211 BCL11B - b-cell cll/lymphoma 11b (zinc finger protein) IGF1R - insulin-like growth factor 1 receptor ADAM28 - adam metalloproteinase domain 28 ZNF484 - zinc finger protein 484 ZNF350 - zinc finger protein 350 FOXP1 - forkhead box p1 RUNX1 - runt-related transcription factor 1
Green	amylase activity	regulation of triglyceride metabolism proteoglycan synthesis	ZMAT1 - zinc finger, matrin type 1 ZBTB4 - zinc finger and btb domain containing 4 ADAMTS6 - adam metalloproteinase with thrombospondin type 1 motif, 6 CREBBP - creb binding protein
Pink	Nucleoside triphosphatase regulator activity GTPase regulator	No enrichment found	RGS18 - regulator of g-protein signaling 18 ARRB1 - arrestin, beta 1 DOCK9 - dedicator of cytokinesis 9 ARHGEF18 - rho/rac guanine nucleotide exchange factor (gef) 18