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Computational modelling

Identification of helper T cell master regulator candidates using the polar score method

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ABSTRACT

The T helper paradigm is currently being revised from the Th1–Th2 dichotomy to a multi-state paradigm involving a number of different cell phenotypes. Transcriptional profiling using microarrays has been used to study the development of these phenotypes. There is however no clear consensus on how to approach the analysis of this data, especially in the context of cells that are triggered to expand rapidly, and massively change their gene expression pattern.

We develop a method we call 'polar score' to identify genes that are related to T helper cell polarization. This method is designed to identify polarizing genes in a set where many genes change expression. To illustrate the use of this technique, we apply it to published T cell microarray data and compare it to conventional analysis methods. With the new method, we find evidence for the existence of IL9 producing T cells ('Th9 cells') that are induced by a combination of TGF β and IL4. We identify several candidate master regulator genes for this phenotype. Furthermore, treatment with TGF β and IL12 results in a Treg and Th17 hybrid cell phenotype.

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1. Introduction

Helper T cells are CD4+ T lymphocytes that play an important role in determining the nature and function of the adaptive immune response. Classically, helper T cells were thought to consist of two major subtypes (Mosmann et al., 1986), but the recent discovery of Th17 cells indicates that there are more phenotypes (Weaver et al., 2006; Awasthi and Kuchroo, 2009). The discovery of Th17 cells also links the differentiation of FOXP3+ regulatory T (Treg) cells to that of helper T phenotypes, because the set of cytokines inducing either Treg or Th17 cells overlaps. New Th phenotypes are currently being suggested, e.g. Th9 (Veldhoen et al., 2008), reviewed in (Soroosh and Doherty, 2009), and T_{FH} cells (Nurieva et al., 2008).

With the discovery of these new phenotypes, the Th1/Th2 dichotomy is currently being replaced by extended helper T cell

differentiation models including three or more phenotypes, which prompts one to reexamine previous work in the light of the new paradigm. For instance, several mouse models for human disease that were formerly thought to be Th1 mediated, are now attributed to the Th17 phenotype, e.g. experimental autoimmune encephalomyelitis (EAE) (Cua et al., 2003), collagen-induced arthritis (CIA) (Langrish et al., 2005), and inflammatory bowel disease (IBD) (Murphy et al., 2003). Furthermore, hyper IgE syndrome in humans (also known as Job's syndrome) seems to originate from a lack of Th17 cells (Milner et al., 2008; Ma et al., 2008).

Given the importance of the T helper response in, for instance, vaccine effectiveness and safety (Openshaw and Tregoning, 2005), several studies have tried to elucidate the precise transcriptional profile of different phenotypes. If the details of T helper response gene regulation were understood, vaccine formulations could potentially be adjusted to raise particular immune response phenotypes. Additionally, comprehensive mRNA profiling of the T helper response may identify novel biomarkers that could be used for diagnosis and prognosis (Van den Ham et al., 2009).

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There have been several attempts to comprehensively describe the genetic profile of different T helper cells using microarray technology (Lund et al., 2003, 2005, 2007; Stockis et al., 2009; Wei et al., 2009), which aimed to identify novel genes that were associated with certain Th phenotypes. Indeed, microarray profiling of T helper cells led to the discovery of the putative Th9 phenotype (Veldhoen et al., 2008), and is being used by several groups to fish for genes associated with these T helper phenotypes (Haines et al., 2009). The analysis of microarray data is however far from trivial, and the outcome often depends on the analysis methods used (Millenaar et al., 2006). One of the analysis environments that is frequently used is R/BioConductor, which provides linear modelling methods (e.g. package Limma) for determining differential gene expression. There is no clear consensus in the field on the best method to analyse microarray data, and the best method of analysis depends on the biological system studied, and on the questions being asked.

We develop and apply a novel analysis method that we call polar score. Rather than being a generic method, this method is based on the specific behaviour of key helper T cell genes that involves both upregulation and polarization of expression level. By designing a method that specifically identifies polarizing genes in an environment where many genes are changing expression level, we expect to have here a better method than the generic approaches. To show this, the polar score method is used to reanalyse and reinterpret existing microarray data (Lund et al., 2003, 2005, 2007). We show that for identifying polarizing genes, this method is superior to the analysis methods that are currently used. The new method provides evidence for the existence of the Th9 phenotype, and proposes candidate master regulator genes that may control this phenotype.

2. Methods

2.1. Experimental design and microarray studies

We analyse data that were published by Lund et al. (2003, 2005, 2007), and are available from NCBI GEO Series accession number GSE2770. For details concerning the experimental setup, please refer to the original publication. Briefly, CD4+ T cells were isolated from cord blood (Turku University Central Hospital, Turku, Finland), and were activated with platebound α CD3 (500–1000 ng/ml for coating) and soluble α CD28 (500 ng/ml). To polarize cells into different phenotypes, the cells were stimulated with IL12 (2.5 ng/ml), IL4 (10 ng/ml), and/or TGF β (3 ng/ml). A subset of the cells was cultured under neutral conditions, i.e., without polarizing cytokines. At timepoints 0, 2, 6, and 48 h, cells were harvested and gene profiles were generated using microarrays. Using two complementary microarrays (Affymetrix HG-U133A and HG-U133B), 44527 transcripts were profiled. In total, 34 microarrays (4 biological replicates of naive T cells, 2 biological replicates for each experimental condition) were run with varying treatments (Table 1).

2.2. Microarray preprocessing and analysis

Microarray preprocessing was performed within R version 2.8.1 (R Development Core Team, 2008) and the corresponding BioConductor (Gentleman et al., 2004) packages. Using the affy

Table 1

Experimental design of the microarray experiments. The number of microarrays performed for each condition is shown for each treatment and timepoint.

| Treatment conditions | | Time (h) | | | |
|----------------------|-----------------------|----------|---|---|----|
| | | 0 | 2 | 6 | 48 |
| Naive | | 4 | – | – | – |
| α CD3 | neutral | – | 2 | 2 | 2 |
| | + IL-12 | – | 2 | 2 | 2 |
| + | + IL-12 + TGF β | – | 2 | 2 | 2 |
| | α CD28 | – | 2 | 2 | 2 |
| | + IL-4 | – | 2 | 2 | 2 |
| | + IL-4 + TGF β | – | 2 | 2 | 2 |

package (Gautier et al., 2004), microarrays were processed using RMA background correction, quantile normalization, and median polish summarization (i.e., standard RMA processing). Alternatively, the arrays were normalized using VSN, followed by median polish summerization (i.e., standard VSN-RMA processing). The two different array types (HG-U133A and U133B) were combined using the matchprobes package. Using the Limma package (Smyth, 2004), Limma analysis was performed for three different gene sets: those present on only U133A, only on U133B, or present on both using unpaired *t*-tests. Limma results were subsequently merged into a single table, after hierarchical adjustment for multiple testing (FDR of 0.05). Genes were annotated using the annotate package (NCBI annotation Sept. 2008).

2.3. Polar contrast and summary score computation

Conceptually, the polar score method quantifies the change in gene expression level by calculating the surface area between the expression at a given timepoint, the expression at time is 0 h (naive T cell expression), and the diagonal (Fig. 1a). All scores in a time series are summed to produce a final score (Fig. 1b). All functions were implemented in R, and code is available upon request.

- Preprocessing: Microarray data is processed with RMA (rather than VSN) in order to obtain a robust estimate of expression without performing extra normalization. The expression data is represented as the mean \log_2 probeset intensity of all replicates.
- Polar coordinates: Every contrast is transformed to polar coordinates using the naive expression level (time point 0 h) as the origin. The angle θ is calculated relative to the $y=x$ diagonal.
- Polar contrast score: For every gene a polar contrast score is calculated. This score is the surface area of the sector between the origin (naive expression), the diagonal (no polarizing expression) and the mean expression value, i.e., is computed by $\frac{1}{2}\theta r^2$ where θ is the angle, and r is the distance to the origin (Fig. 1a). Conceptually, the radius r represents the fold change in expression for both conditions relative to the expression at $t=0$, and the angle θ is similar to the log ratio. We use polar score to summarize the effect of an experimental treatment by summing over the scores of all timepoints of that treatment.
- Polar profile: Using all possible polar contrasts over all conditions, one can construct a polar profile that describes the polar score of all possible pairs of treatment conditions.

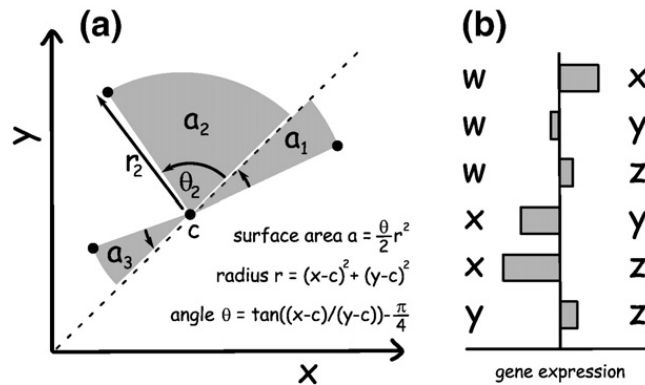


Fig. 1. Explaining the polar score method. (a) Gene expression at $t = 0$ is taken to be the origin (c). Expression of the same gene in different conditions is plotted as x and y . The sector (grey area) between the origin, the diagonal and the x - y coordinate is most easily calculated when the expression values are converted to polar coordinates. The polar score of a timeseries is the sum of these surface areas of a particular gene. (b) The polar scores are computed for all pairwise combinations of experimental conditions (i.e., $w:x$, $w:y$, $w:z$, $x:y$, $x:z$, and $y:z$). The set of polar scores is represented as a barplot, which we call a gene profile. This particular profile schematically shows a gene that is upregulated in condition x .

When a certain treatment affects a gene, it is expected to be regulated accordingly in all pairs of treatment. Genes that are specific for a certain treatment, e.g. IL12, are expected to be differentially regulated in all contrasts containing this treatment ('discriminating' polar contrasts) (Fig. 1b), and have no differential regulation of this gene in all other contrasts ('non-discriminating' polar contrasts). In this way, a 'search image' for a certain treatment condition can be constructed.

- Ranking polar profiles: All genes that fit to a particular search image (i.e., a treatment) can be ranked to identify the most strongly polarizing genes. A gene fits to a search image when its discriminating polar scores have matching directions. For every treatment, genes are then ranked by the smallest value of their discriminating polar contrast scores (Fig. 1b). Genes that are inconsistently regulated in the discriminating contrasts (e.g., up in IL12 vs IL4, but down in IL12 vs IL4 + TGF β) are not included in the ranking for a particular search image. We determined the cutoff for differential gene expression by using the total distribution of minimum correct polar scores, setting a cutoff such that the top 0.25% of differentially regulated genes are selected.
- Variation control: To visualize the variation in the array measurement, we calculate the polar contrast scores *between duplicate arrays*, thereby capturing the biological and technical noise. A standard deviation is calculated for every gene from all duplicate array pairs.
- Visualization of gene behaviour: Gene expressions can be visualized as polar profiles using bargraphs of the polar contrast scores and the trajectory plots of gene behaviour in a particular contrast. The standard deviation of biological-technical variation is plotted as a dotted line in the bargraph.

3. Results

3.1. Most differentiation gene expression is due to T cell activation rather than cytokine treatment

As a first step in the analysis, we establish the principle cause of changes in gene expression over the whole experiment. Using Variance Stabilization Normalization (VSN) and median polish to preprocess the microarray data, the variability

of individual probesets over all arrays can be visualized. To visualize the effect of time, we compute the variance of probeset expressions over all time points. Using naive and neutral arrays only, i.e., arrays with naive T cells and T cells stimulated with α CD3 + α CD28 in the absence of cytokines (in total 10 arrays; Fig. 2a), the variance is plotted against rank mean intensity of the probeset. To visualize the effect of cytokine treatment, we plot the variation of probesets at a single timepoint over all treatments (10 arrays per timepoint, only within-timepoint comparisons). Comparing the variance between treatments with the variance over time should reveal which of the two leads to the greatest differences in gene expression. To quantify the number of highly variable probesets in each plot, we use a variance cutoff of 0.04. In time, over 800 probesets are highly variable, whereas the treatment conditions lead to only 70 to 100 highly variable probesets (Fig. 2b–d). This suggests that the activation of cells over time generates much more variation in gene expression than does treatment with polarizing cytokines.

To further quantify the difference between time and treatment, we performed a Limma (Linear Modelling of Microarrays) analysis on the data, normalized with either RMA or VSN. Both RMA and VSN perform well, but VSN is more conservative than RMA. Analysing the neutral arrays, Limma finds that many probesets are differentially expressed over time, both with RMA and VSN processing (> 1000 probesets, Fig. 2e). However, using all possible within-timepoint contrasts, a Limma analysis shows that the effect of the cytokine polarization is small when quantified by the number of differentially expressed probesets (16 genes, Fig. 2f). In both cases, there is some overlap with the highly variable probesets identified earlier (Fig. 2e–f). These findings demonstrate that activation of T cells leads to much more differential gene expression than does the additional cytokine treatment, which is after all not an unexpected result.

3.2. Differential gene expression between Th1 and Th2

Given the large number of genes that are differentially regulated over time, and the small number of genes that are differentially regulated between treatment conditions, we suspected that the variation over time obscures the variation between treatments. This idea was strengthened by the absence

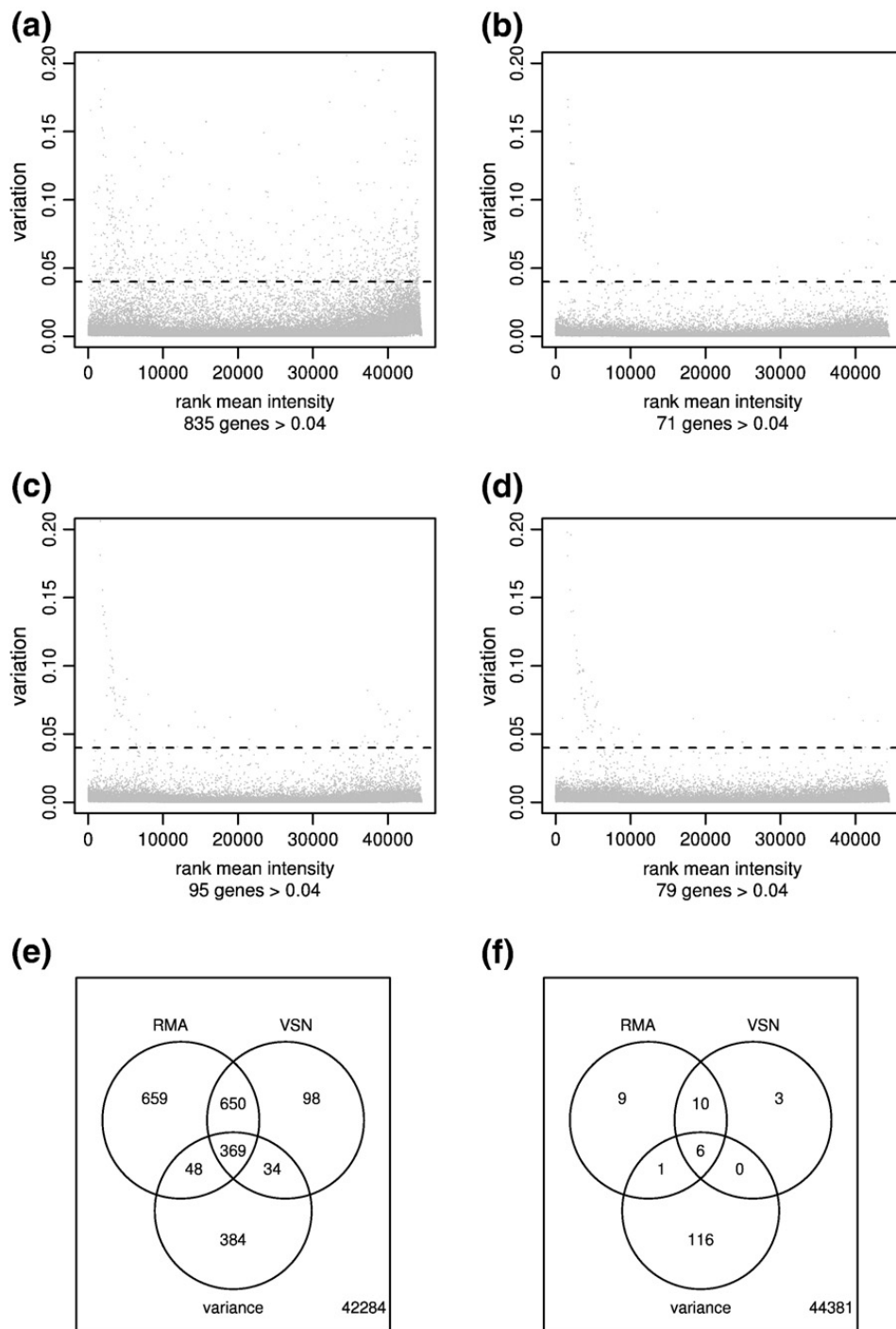


Fig. 2. Variation over time is much greater than that among cytokine treatments. Panels a–d show mean rank intensity of a probeset plotted against its variation over all arrays (a), and for each timepoint 2 h (b), 6 h (c), and 48 h (d), respectively. Panels e and f show a Limma analysis of the arrays over time or between treatments using RMA or VSNRMA processed data. The overlap in differentially regulated genes between these two methods, and with the most variable genes (panels a–d) is shown for time (panel e) and between treatments (panel f; 2 h, 6 h, and 48 h genes combined).

of dichotomous Th1/Th2 genes from the list of differentially regulated genes: when comparing Th1 to Th2, the well-established Th1 and Th2 master regulators TBX21 (Tbet) and GATA3, as well as Th2 cytokines IL4, IL5 and IL13, were not found to be differentially regulated (see the Limma analysis in Table 2). Using an alternative design matrix featuring time and TGF β as confounders, the Limma analysis identified 65 and 43 genes (RMA or VSN normalization, respectively) including GATA3 but not IFN γ in the Th1–Th2 contrast. Also notice that the genes that

we find using Limma in the data do not overlap with the gene lists published by (Lund et al., 2007). Apparently the somewhat different analysis methods yield quite different results, and our conjecture is that this is due to the fact that the variation induced by polarization is much smaller than that due to T cell activation.

To obtain more insight into the expression of genes under different treatments, we visualized their expressions over time using trajectory plots (Fig. 3a–f). For each available timepoint, the mean expression of a probeset under Th1 and Th2 conditions

Table 2

Th2 vs Th1 genes identified by Limma analysis. All genes with a false discovery rate of <0.05 in either the RMA or VSN analysis are shown. The log₂ expression values at timepoints 0 h, 2 h, 6 h, and 48 h shown are normalized by RMA. The right most columns show the genes marked present (+) or absent (–) in a certain analysis. Since this list does not contain the well-established Th1/Th2 genes, and fails to overlap with the analysis of Lund et al. (2007), we think it is biased too much by the genes upregulated by T cell activation.

| | Gene | Th2–Th1 difference | | | Mean expr. | RMA | VSN |
|-------------|---------|--------------------|-------|-------|------------|-----|-----|
| | | 2 h | 6 h | 48 h | | | |
| 221223_x_at | CISH | 3.00 | 1.50 | 0.60 | 8.50 | + | + |
| 223961_s_at | CISH | 2.90 | 1.90 | 0.60 | 7.70 | + | + |
| 244422_at | | 2.00 | 0.50 | 0.60 | 4.00 | + | + |
| 210715_s_at | SPINT2 | 1.00 | 2.40 | 1.80 | 7.70 | + | + |
| 225606_at | BCL2L11 | 1.60 | 0.70 | 2.00 | 8.50 | + | – |
| 203574_at | NFIL3 | 2.20 | 0.90 | 2.10 | 6.10 | + | + |
| 209999_x_at | SOCS1 | 0.90 | 2.20 | 1.90 | 5.90 | + | + |
| 213524_s_at | GOS2 | –0.10 | –0.20 | –3.40 | 5.50 | + | + |
| 210354_at | IFNG | 0.10 | –1.10 | –4.80 | 6.10 | + | + |
| 210145_at | PLA2G4A | 0.10 | –0.10 | 2.00 | 2.90 | + | + |
| 236787_at | | 0.10 | 0.70 | 1.80 | 5.10 | + | + |
| 222457_s_at | LIMA1 | 0.20 | –0.00 | 2.30 | 4.60 | + | – |
| 229764_at | TPRG1 | 0.30 | 2.60 | 3.30 | 5.50 | + | + |
| 217892_s_at | LIMA1 | –0.30 | –0.10 | 1.90 | 6.20 | + | – |
| 244267_at | | 2.00 | 0.90 | 1.20 | 6.00 | + | – |
| 205419_at | GPR183 | 1.00 | 1.80 | 2.30 | 7.80 | – | + |
| 206513_at | AIM2 | –0.20 | 0.00 | 2.00 | 5.40 | – | + |
| 230109_at | | 0.10 | –0.20 | 1.20 | 4.90 | – | + |

are plotted on the x- and y-axis, respectively. We take advantage of the fact that we have two replicates for every treatment, and use these to draw a box around the mean expression of every timepoint to visualize the replicate variation. Connecting the timepoints by a line yields a trajectory that visualizes gene expression in both polarization conditions, and concurrently shows the variability of the two replicates. A gene that hardly changes expression over the course of the experiment will stay in the center of the plot. Importantly, the trajectory of a gene that changes with time, but not with treatment, will remain close to the (dashed) diagonal line. A major advantage of these trajectory plots is that they visualize the original expression values in a very direct manner, and on a per-gene basis separate the effect of activation and polarization.

The trajectory plots of the key Th1/Th2 transcription factors show that TBX21 and GATA3 are clearly differentially regulated with respect to the Th1 and Th2 treatment conditions (Fig. 3a,c), while they failed to show up in the conventional analysis shown above. Of the key cytokines, IFN γ is differentially regulated, i.e., is correctly moving into the right-bottom corner (Fig. 3b), while IL13 shows only minor changes with respect to treatment (Fig. 3f). IL4 and IL5 do not undergo any change at all, and fluctuate around the naive expression level (Fig. 3d–e). The upregulation of TBX21,

GATA3 and IFN γ all follow a similar pattern. Initially, their expression increases in both Th1 and Th2 treatment, followed by a downregulation away from the diagonal depending on the treatment. We call this effect ‘up and out’: an initial increase in expression during both treatments, i.e., by activation, after which the expression turns either left or right, i.e., the cell polarizes to either the Th1 or Th2 phenotype, respectively. In summary, depicting the original data as trajectories reveals that the T helper genes do polarize during the experiment, despite the fact that this was not picked up by a global Limma analysis.

3.3. The polar score method

To take advantage of the specific profiles of gene regulation like the ‘up and out’ behaviour, we designed a scoring system quantifying the deviation from the diagonal. Conceptually, we score for every gene in a particular contrast the surface area between the diagonal, the timepoint of each mean expression value, and the naive T cell expression (Fig. 3g). Summing over the three surface areas obtained from the three different timepoints in the experiment, we compute a final score of the deviation of a gene from the diagonal (Fig. 1b). The computation of this surface area is most convenient when we switch to polar coordinates, i.e., angles and vector lengths. Technically, we compute the area of the sector between the diagonal and the timepoint of each mean expression value, using the naive T cell expression as the origin. We refer to this as a ‘polar contrast score’.

Since every polar contrast score results from a pair of polarizing conditions, we compute it for every combination of cytokine treatments. Over the whole experiment, there are 10 such possible combinations. A gene that is truly controlled by a particular cytokine is expected to be differentially regulated in all contrasts containing this particular treatment. Coloring the discriminating bars white, and non-discriminating bars grey, we can summarize the skewing of a gene in all 10 contrasts by a single bargraph (Fig. 1b). For example, IFN γ expression is upregulated by IL12. This means that in contrasts containing IL12 treatment (the discriminating contrasts, i.e., neutral vs IL12, IL4 vs IL12, IL12 vs IL4 + TGF β , and IL12 vs IL12 + TGF β), this gene should be differentially regulated. In the other polar contrasts (non-discriminating contrasts) IFN γ should not change. To visualize the technical and biological noise in the experiment, we compute all possible polar contrast scores between replicate arrays, and depict the standard deviation of these scores as a dotted line in the bargraphs.

One can search for differentially regulated genes by using the magnitude of the expected discriminating contrast scores and sort the genes on their minimum discriminating contrast score, i.e., the smallest white bar in the barplot. We find that the well-established T helper genes have relatively high polar scores in

Fig. 3. Trajectories for selected genes in the Th1–Th2 contrast (top panels). The expression levels are plotted as a trajectory with gene expression in response to IL12 or IL4 on the horizontal and vertical axes, respectively. The corners of the boxes around the mean expression values indicate the values of individual arrays. The panels in the middle show a schematic representation of polar score computation. The contrast point is plotted, and the polar coordinates are indicated in grey. The polar score that is computed for a single contrast corresponds to the surface area of the grey ‘slice of pie’ (left panel). The score for each of contrast can be plotted and represented as a bargraph (right panel). To find the effect of IL12 treatment we shade scores white of the treatment that contains IL12 only; otherwise the score is shaded grey. The smallest white bar is used to rank the genes for every cytokine treatment. Using all data put together, a cutoff was defined such that we obtained the 0.25% most differentially regulated genes.

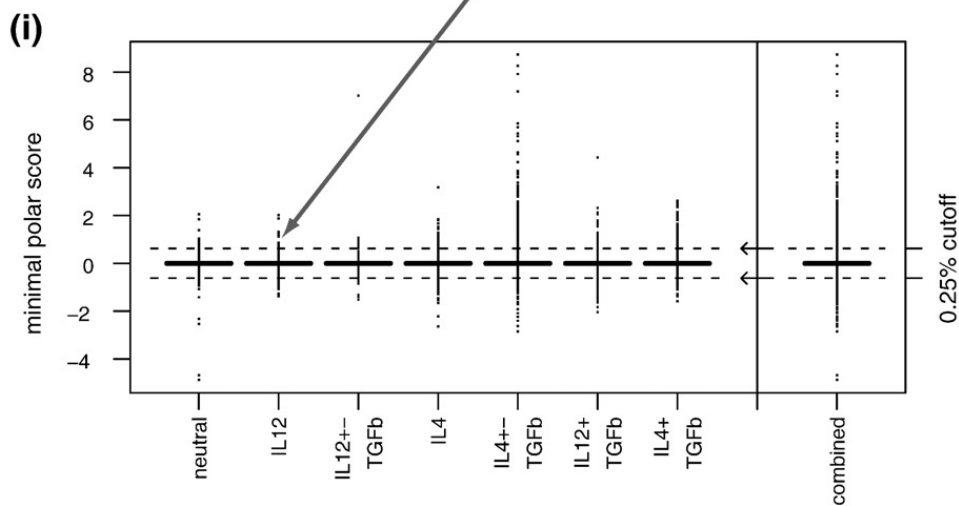
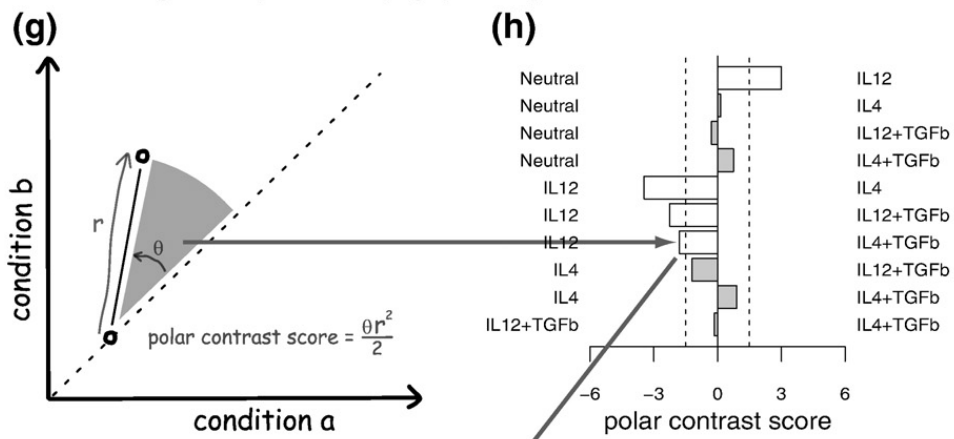
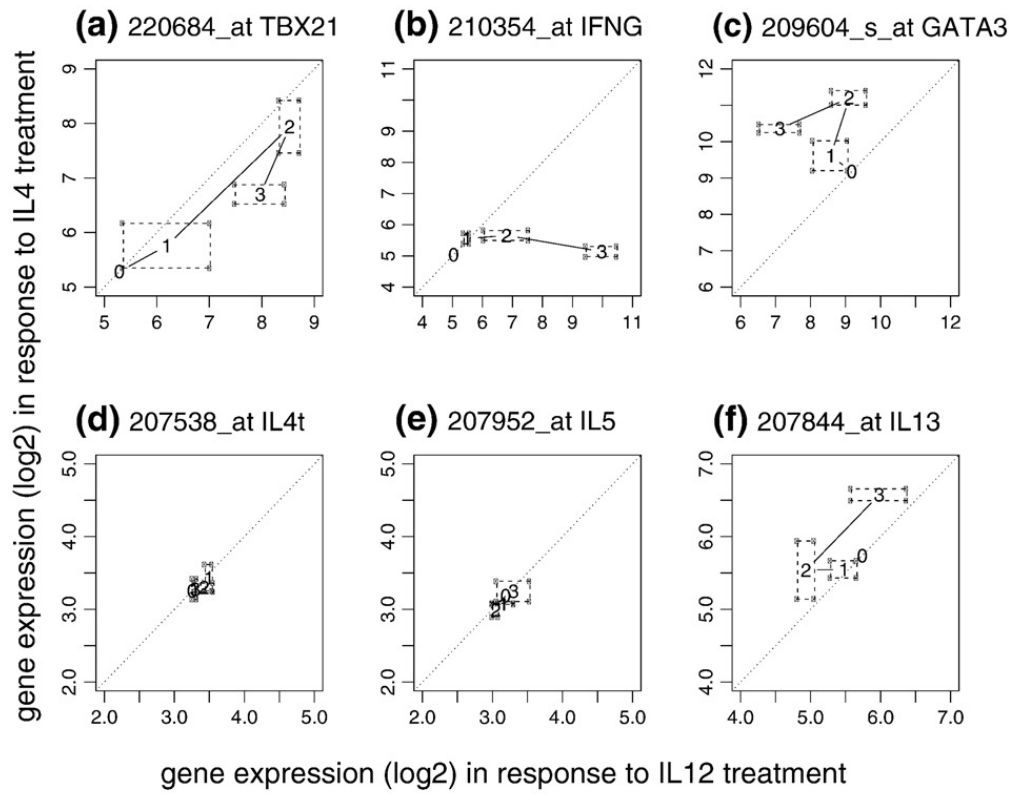


Table 3

Ranks of genes scored out of 44,524 probesets with the polar score method. High ranking (i.e., upregulated) genes are represented by positive rank numbers, and low ranking (i.e., downregulated) genes are represented by negative rank numbers. Ranks $> \pm 1000$ are represented by a dot (.). Please note that some genes have more than one probeset, and that all probesets available for a certain gene are in the table. '# genes that fit profile' indicates the number of genes that were assigned a rank for a certain search image. '# genes in 0.25%' indicates the number of genes that are differentially regulated (# up \uparrow ↓ down) with a 0.25% cutoff.

| | AffyID | Symbol | none | IL12 only | IL12 \pm TGF β | IL4 only | IL4 \pm TGF β | IL12 + TGF β | IL4 + TGF β |
|---|-------------|--------|--------------------|--------------------|------------------------|--------------------|-----------------------|--------------------|---------------------|
| Th1 | 220684_at | TBX21 | . | 11 | 4 | . | -75 | . | -32 |
| | 210354_at | IFNG | . | 3 | 1 | . | . | . | . |
| Th2 | 209603_at | GATA3 | . | . | . | 141 | 50 | . | . |
| | 209602_s_at | GATA3 | . | . | -250 | 5 | 31 | -113 | . |
| | 209604_s_at | GATA3 | . | . | -17 | 184 | 20 | -246 | . |
| | 207538_at | IL4 | . | . | . | . | . | . | . |
| | 207539_s_at | IL4 | . | . | . | . | . | . | . |
| | 207952_at | IL5 | . | . | . | . | . | . | . |
| | 207844_at | IL13 | . | . | . | 527 | . | . | . |
| Treg | 221333_at | FOXP3 | . | . | . | . | . | 351 | . |
| | 221334_s_at | FOXP3 | . | . | . | . | . | 188 | . |
| | 224211_at | FOXP3 | . | . | . | -760 | . | 76 | . |
| | 203084_at | TGFB1 | . | . | . | . | . | . | -249 |
| | 203085_s_at | TGFB1 | -33 | . | . | . | . | 184 | . |
| 207433_at | IL10 | -487 | . | . | . | . | . | . | |
| Th17 | 206419_at | RORC | . | . | . | . | . | 548 | . |
| | 228806_at | RORC | . | . | . | . | . | 110 | . |
| | 210426_x_at | RORA | . | . | . | . | . | . | . |
| | 210479_s_at | RORA | . | . | 30 | -341 | . | . | . |
| | 239550_at | RORA | . | . | . | . | . | . | . |
| | 208402_at | IL17A | . | . | . | . | . | . | . |
| | 216876_s_at | IL17A | . | . | . | . | . | . | . |
| | 234408_at | IL17F | . | . | . | . | . | . | . |
| Th9 | 208193_at | IL9 | . | . | . | . | . | . | 36 |
| # genes that fit profile | | | 17946 | 17827 | 10055 | 15763 | 10283 | 19159 | 17997 |
| # genes in 0.25% (up \uparrow ↓ down) | | | 25 \uparrow ↓ 14 | 21 \uparrow ↓ 35 | 12 \uparrow ↓ 17 | 71 \uparrow ↓ 45 | 229 \uparrow ↓ 57 | 56 \uparrow ↓ 61 | 109 \uparrow ↓ 26 |

their respective treatment group: IFN γ (3rd), TBX21 (11th), and GATA3 (5th, 141th, 184th) are all ranked in the top 200 out of 45,000 probesets. Despite the upregulation of GATA3, Th2 cytokine genes show little or no response to the cytokine treatment (IL4, IL5 and IL13 ranks above 1000) (Table 3). IL4 is known to be a 'slow' cytokine, i.e., it takes 3–4 cell divisions before it is expressed (Gett and Hodgkin, 1998), hence it is possibly not picked up by these experiments that range from 2 to 48 h. Additionally, the absolute mRNA expression is low, and changes in these genes may therefore not be measurable. When we compare our findings to the original analysis by Lund et al. (2007), a limited number of genes are found to be differentially regulated in both studies (Fig. 4). Furthermore, Lund et al. (2007) identified TBX21 as differentially expressed, but none of the other canonical Th1/Th2 genes are differentially regulated in their results. This shows that our method is better at detecting helper T cell specific genes in this particular dataset.

In addition to IL12 and IL4 treatment without TGF β (i.e., exclusively IL12 and IL4 regulated genes), one can also score the conditions in which IL4 or IL12 was added, regardless of TGF β (i.e., constitutively IL12 and IL4 regulated genes). Using the constitutive instead of the exclusive treatment as a search image (i.e., for IL4 the contrasts: neutral vs IL4, neutral vs IL4 + TGF β , IL12 vs IL4, IL12 vs IL4 + TGF β , IL4 vs IL12 + TGF β , and IL4 + TGF β vs IL12 + TGF β), the results for IL4- and IL12- specific genes improve: IFN γ (1st, i.e., highest scoring gene), TBX21 (4th), GATA3 (20th, 31st, 50th) (Table 3). This indicates that these Th genes are activated regardless of additional TGF β .

Using the polar score method, we searched for other potential IL4 and IL12 specific genes changing by Th1/Th2 skewing.

Focussing on immune related genes and transcription factors, we manually searched for interesting genes. For Th1 treatment, the IL18 receptor and accessory proteins (IL18R1 and IL18RAP), which are known to be upregulated by IL12 stimulation, are upregulated. Furthermore, BCL6, SLAMF7 (involved in NK cell activation), and IRF8 (involved in interferon signalling) were upregulated (BCL6 and SLAMF7 are shown in Supplemental Fig. 1). Conversely, SOX4 (TCR signalling) and BCL2L11 (apoptotic regulator) were downregulated (Supplemental Fig. 1). These proteins were differentially regulated in response to IL12 treatment without TGF β . If we extend the search, and look at all arrays treated with IL12, regardless of TGF β treatment, we find that IL2 is upregulated as well as TBX21 and IFN γ as shown before (Fig. 3a,b), whereas that chemokine lymphotaxin (XCL1 and XCL2) is downregulated (Supplemental Fig. 1).

For the Th2 treatment, upregulated genes include GATA3, but not IL4, IL5 and IL13 as mentioned before, but also IL3 and IL3-inducer NFIL3, AIM2, and KLF6, which are involved in transcriptional activation (Supplemental Fig. 2). Downregulated are LT α , CCL20 and TNF α , consistent with the induced Th2 phenotype (Supplemental Fig. 2). If we disregard TGF β as before, and look at any treatment condition involving IL4, we additionally find CD47, CD109 (negatively affects TGF β signalling), chemokine ligand XCL2, CISH (SOCS), SOCS3, MAPK1, RNF125 (positive regulator of TCR signalling) and CXCR4 that are upregulated by IL4; conversely, STAT1, IL2, IL8, IL21R and granzyme A are downregulated (Supplemental Figs. 3 and 4). Differential regulation of these genes is consistent with a Th2 phenotype.

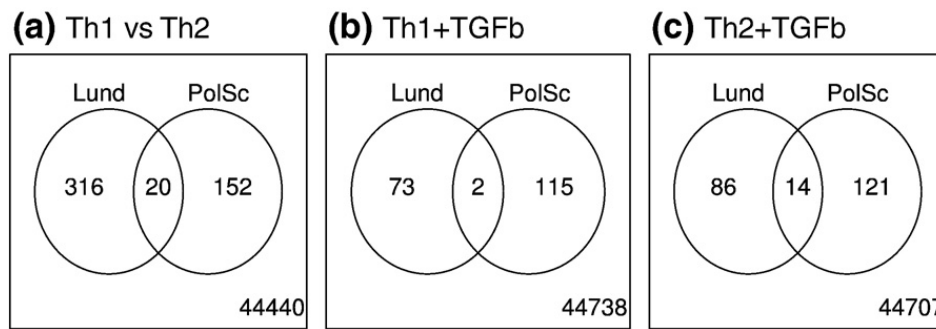


Fig. 4. Overlap between Lund et al. (2007) and our analysis. Overlap including all timepoints of (a) the Th1/Th2 contrast, (b) the Th1 + TGFβ contrast, and (c) the Th2 + TGFβ contrast.

3.4. Identification of Th9-specific genes

To identify genes that are associated with the putative IL9 producing T helper phenotype ('Th9', (Veldhoen et al., 2008)), we use the polar score as a search image to obtain differentially expressed genes. We select the genes and plot the polar contrast scores and a relevant trajectory for every gene. The trajectory plots, and polar contrast scores of the top 0.25% of differentially expressed genes are shown in the Supplementary data. These plots were then manually curated for genes of interest based on polar contrast profile and trajectory in a particular contrast. To find 'Th9' genes, we use the polar contrast Th2 (IL4) vs 'Th9' (IL4 + TGFβ) to plot gene trajectories as these will show genes of interest most clearly.

Importantly, we find IL9 to be differentially regulated, suggesting that IL4 and TGFβ do indeed induce the production of IL9 at the mRNA level in this experiment (Table 3, Fig. 5b). Furthermore, we found a number of other molecules to be associated with 'Th9' differentiation including cytokines IL1RN and TNFSF11 (RANKL), and chemokine CCL17 (Fig. 5c–d). Additionally, ALOX5AP is required for leukotriene synthesis; leukotrienes are implicated in (potential Th9-related) inflammation in diseases such as asthma, psoriasis and arthritis (Soroosh and Doherty, 2009). Several transcription factors were also upregulated including RBPJ (Fig. 5f), which is known to bind to the TBX21 promotor Ansel et al. (2006); Rothenberg et al., 2008), and perhaps CBFβ (Fig. 5e), which is a transcription factor belonging to the PEBP2/CBF transcription factor family known for master-regulating a host of genes specific to hematopoiesis. This identifies CBFβ and RBPJ as potential master regulators, providing interesting suggestions for experimental verification.

Downregulated immune genes include TNFSF8 (Fig. 5g), BTLA (which is a marker for Th1 cells), the PGE receptor (PTGER4, activator of T cell factor signaling; skin immune responses), LY9 (T cell adhesion, Fig. 5h), and granzyme A. Downregulated transcription factors include TOB1, which inhibits T cell proliferation and transcription of cyclins and cytokines (including IL2), and ZBTB32, which putatively prevents GATA3 protein from binding its promotor. This is consistent with GATA3 having a role in this phenotype (Veldhoen et al., 2008).

3.5. No evidence for a 'Th12' phenotype

In addition to the analysis of the effects of TGFβ and IL4, we investigated the combined effect of IL12 and TGFβ on activated helper T cells. We find a number of immune-related

molecules that are differentially regulated, most notably LTα (Fig. 6b), which has previously been found specific for Th0, Th1, and Th17 cells (Chiang et al., 2009), LIF (Fig. 6c; which is thought to have a skewing effect toward Treg as opposite to IL6 for Th17 (Gao et al., 2009)), TNFSF13b (BAFF) growth factor for Th17 (Lai Kwan Lam et al., 2008) (Fig. 6d), and innate defense molecules APOBEC3F and -G (Fig. 6e–f). Using the ranks of Th phenotype specific genes (Table 3), we observe that both Treg and Th17 specific genes are induced by this treatment, i.e., the Treg master regulator FOXP3, and the Th17 master regulators RORA and RORγt as well as Th17 chemokine CCL20. No upregulation of transcription factors could be measured by microarray, suggesting that there are no master genes involved in inducing this phenotype. Conversely, several transcription factors are downregulated, such as FYB (T cell signalling and modulates IL2 expression), SATB1 (which has a role in removing nonfunctional T cells, Fig. 6g), and CBLB (dampens TCR signalling, Fig. 6h). In summary, TGFβ + IL12 treatment seems to induce a hybrid phenotype between the Th1, Th17 and Treg phenotype.

4. Discussion

Numerous methods have been proposed for the analysis of microarray data. Rather than producing a 'yes' or 'no' answer, the polar score method that we propose allows for the gene expression values to be visualized directly, and pull out polarizing genes from an environment where many more genes are changing expression level. Our method has been specifically designed to find polarizing genes in T cells, but we think that it is applicable to any situation where generic differential expression of many genes obscures the identification of a particular subset. A comparison of the polar score method, Limma and the original analysis (Lund et al., 2007) shows that there is some overlap between results. However, the polar score method is superior at identifying T helper cell specific polarizing genes.

Note that the polar score will also pick up genes that have different dynamics under different conditions. Genes with different dynamics will deviate from the diagonal at intermediate time points, but ultimately they may approach the diagonal and no longer be polarized. Because such dynamical differences in protein levels can have downstream consequences, we consider it an advantage that the polar score method should pick up these genes. In conditions where the majority of the genes has different dynamics, one could consider rescaling time for one of the conditions to compensate for this.

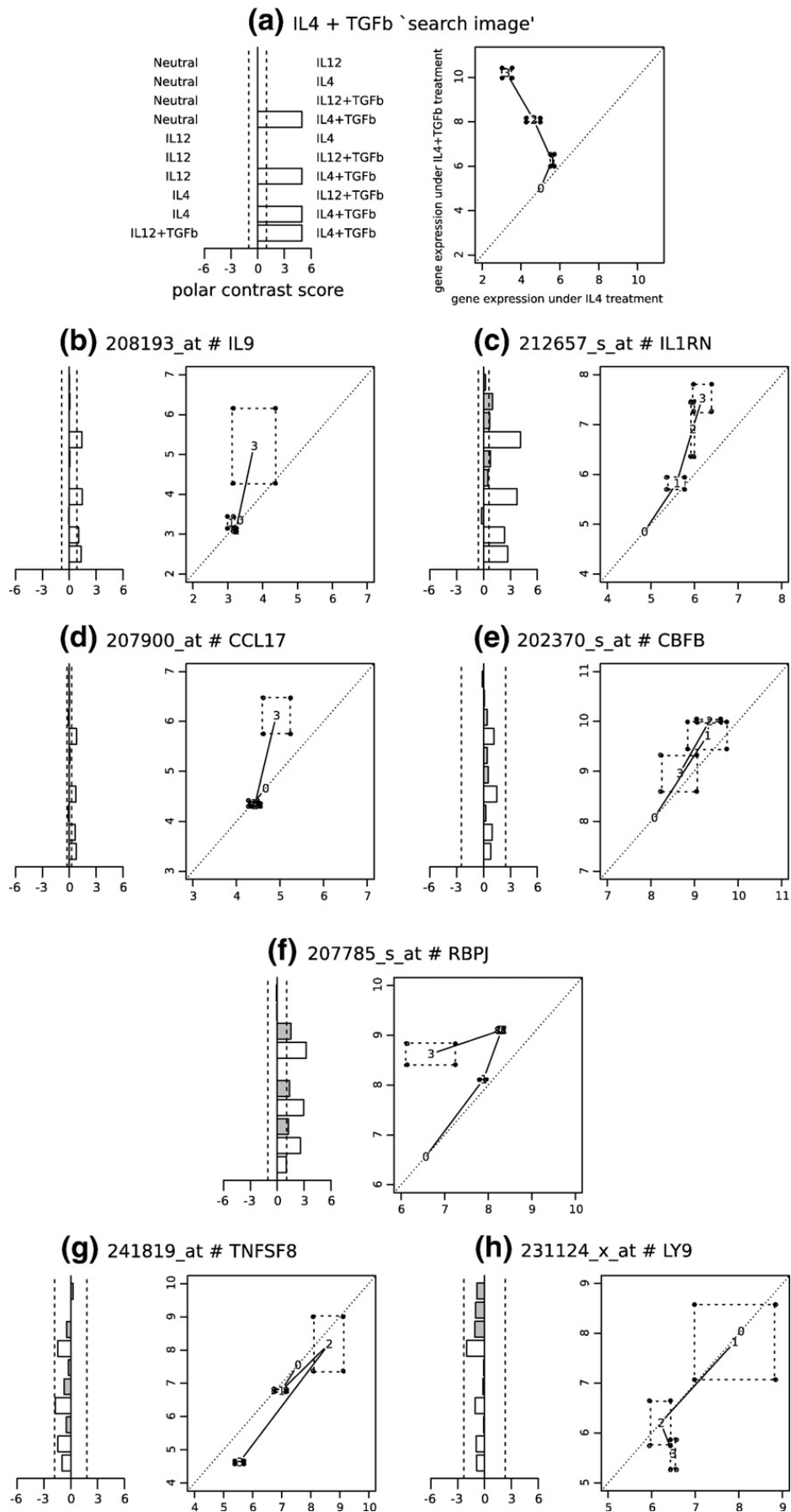


Fig. 5. 'Th9' gene candidates identified by the polar score method. The trajectories were plotted as gene expressions in response to IL4 treatment and IL4 + TGF β treatment on the x- and y- axis, respectively. Affymetrix probeset ID and gene symbols are shown above the plots.

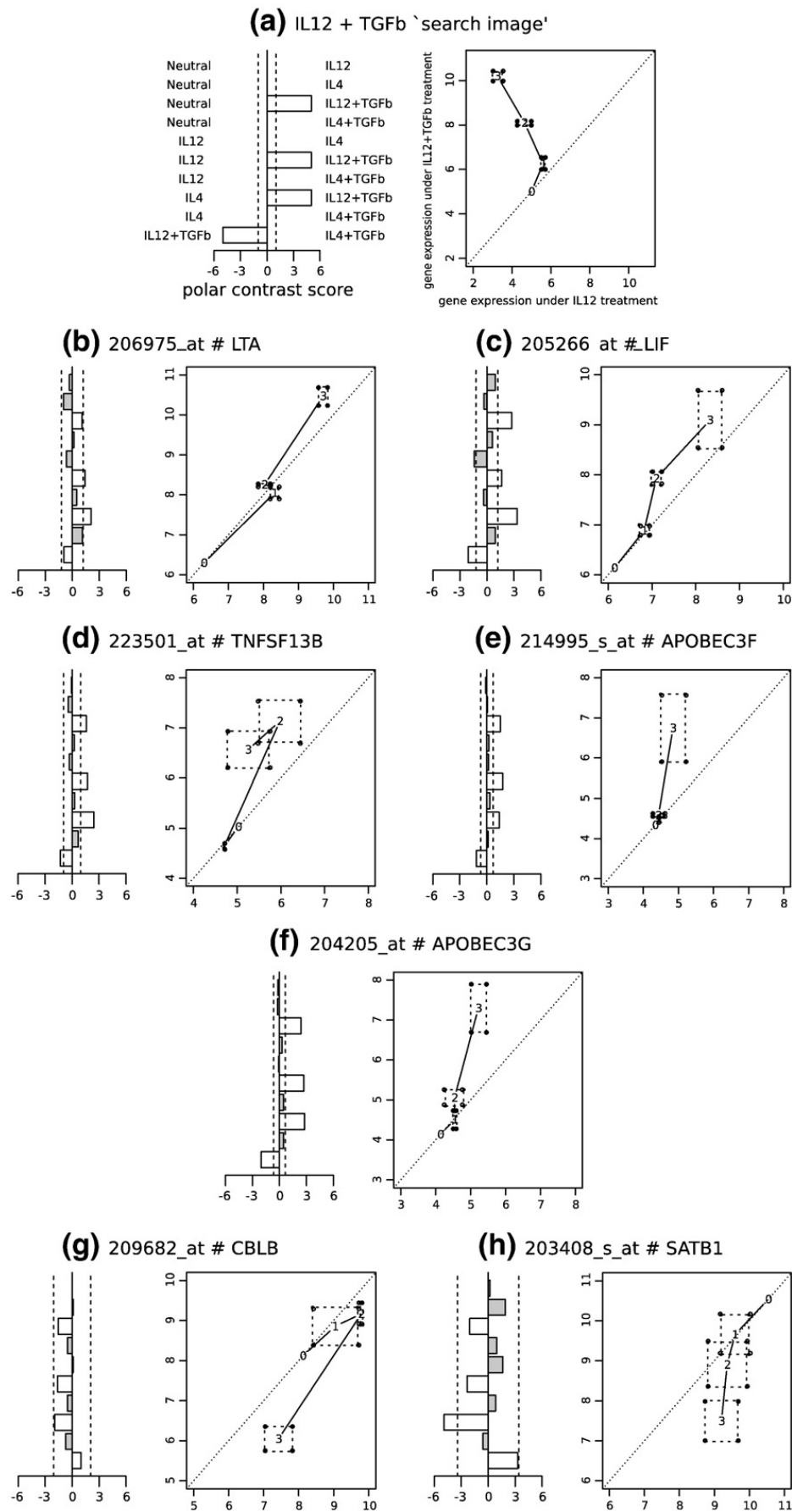


Fig. 6. Genes induced by IL12 + TGF β treatment that were identified by the polar score method. Trajectories were plotted for the treatments IL12 and IL12 + TGF β on the x- and y- axis, respectively. Gene identifiers and names are shown above the plots.

The recent discovery of Th17 cells, and the possibility of more phenotypes such as IL9-producing Th cells show that there are more phenotypes than previously acknowledged. By reanalysing published data containing the condition for generating the proposed Th9 cells, we indeed find evidence for this new phenotype. In response to cytokine treatment with IL4 + TGF β , T cells upregulate the cytokine IL9, and the cytokines CCL17 and IL1RN. Interestingly, the transcriptional regulator RBPJ is found to be upregulated using the polar score, but not in the original results (Lund et al., 2007) or using Limma. The fact that this molecule is known to participate in Notch signalling, and to interact with the TBX21 promoter (Ansel et al., 2006; Rothenberg et al., 2008), makes it a prime master regulator candidate for the Th9 phenotype. This calls for further experimental studies to verify the existence and stability of this phenotype.

Treatment with IL12 + TGF β treatment induces cells to express IL12-specific genes, such as TBX21 and IFN γ , but also FOXP3 and CCL20. These molecules are linked to the Th1, Treg and Th17 phenotypes, and suggest that this treatment induces an intermediate phenotype. Due to the fact that the mRNA is measured in thousands of cells simultaneously, individual cells could be differentiating toward the different phenotypes in response to this 'conflicting signal', or could be in an undecided state (Van den Ham and De Boer, 2008). The undecided state is characterised by the upregulation of multiple phenotype specific factors, whereby these phenotypes remain an option and each cell is in the process of choosing.

In conclusion, the polar score method that we propose allows identification of genes in a changing environment, and visualizes the data for direct inspection. Using this novel method, we find many expected genes of the Th1 and Th2 phenotype, as well as interesting genes for the proposed T helper 9 phenotype that awaits experimental verification.

Supplementary data to this article can be found online at doi:10.1016/j.jim.2010.07.009.

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