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Transcriptomic analysis of mouse EL4 T cells upon T cell activation and in response to protein synthesis inhibition via cycloheximide treatment

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ABSTRACT

T cell activation involves the recognition of a foreign antigen complexed to the major histocompatibility complex on the antigen presenting T cell to the T cell receptor. This leads to activation of signaling pathways, which ultimately leads to induction of key cytokine genes responsible for eradication of foreign antigens. We used the mouse EL4 T cell as a model system to study genes that are induced as a result of T cell activation using phorbol myristate acetate (PMA) and calcium ionomycin (I) as stimuli. We were also interested to examine the importance of new protein synthesis in regulating the expression of genes involved in T cell activation. Thus we have pre-treated mouse EL4 T cells with cycloheximide, a protein synthesis inhibitor, and left the cells unstimulated or stimulated with PMA/I for 4 h. We performed microarray expression profiling of these cells to correlate the gene expression with chromatin state of T cells upon T cell activation [1]. Here, we detail further information and analysis of the microarray data, which shows that T cell activation leads to differential expression of genes and inducible genes can be further classified as primary and secondary response genes based on their protein synthesis dependency. The data is available in the Gene Expression Omnibus under accession number GSE13278.

Specifications	
Organism/cell line/tissue	*Mus musculus*
Sex	N/A
Sequencer or array type	Affymetrix Mouse Gene 1.0 ST Array
Data format	Raw and analyzed
Experimental factors	EL4 T cells were pre-treated with either DMSO or cycloheximide in DMSO, followed by stimulation with phorbol myristate acetate (PMA) and Ionomycin (I) for 4 h or are left unstimulated.
Experimental features	Using mouse EL4 T cells as a model system to study T cell activation, we examined how inhibiting protein synthesis using cycloheximide prior to cell activation affects the inducibility of genes upon T cell activation. We used PMA/I stimulation for 4 h as a way to mimic T cell activation.
Consent	N/A
Sample source location	N/A

1. Direct link to deposited data

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13278 (Submission number GSE13278).

2. Experimental design, materials and methods

2.1. Cell culture

All reagents were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. EL4 T cells were cultured in RPMI 1640 medium with 10 mM HEPES, 10% fetal calf serum (CSL, Parkville, Victoria, Australia), 120 μg/ml penicillin, and 16 μg/ml gentamycin. Cells were pretreated with 10 μg/ml cycloheximide (CHX) for 30 min, and then stimulated with 10 ng/ml phorbol myristate acetate (PMA; Boehringer Mannheim, Mannheim, Germany) and 1 μM ionomycin (I; A23187).

2.2. Total RNA isolation and purification for microarray analysis

Total RNA was isolated from 5×10^6 cells/ml using TRI Reagent (Sigma-Aldrich) for DMSO-treated and CHX-treated EL4 T cells, unstimulated (0 h) or stimulated for 4 h with PMA/I as previously

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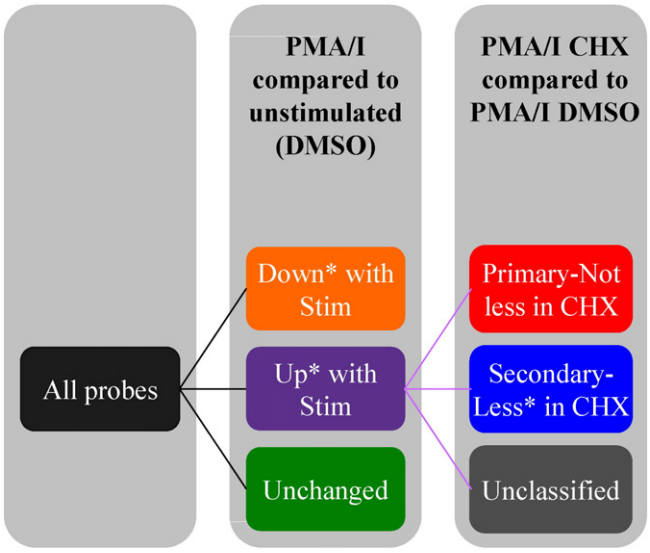

Fig. 1. Categorization of expression array probes according to their kinetics of induction. The probes on the Affymetrix Mouse Gene 1.0ST expression arrays were categorized according to their kinetics of induction. * indicates a statistical test was used (False Discovery Rate < 0.1). Three biological replicates for each treatment were carried out for the expression profiling experiments. See text for more details.

described [2]. Briefly, cells were pelleted at 1500 rpm (Beckman Allegra 6R Centrifuge) for 5 min at room temperature, resuspended in 1 ml of TRI Reagent and incubated at room temperature for at least 10 min to allow complete dissociation of nucleoprotein complexes. 200 μL of chloroform was added and samples were vortexed vigorously and incubated on ice for 15 min. The samples were then centrifuged at 13 000 rpm (Eppendorf Centrifuge 5415 R) for 15 min at 4 °C, after which the aqueous phase was transferred to a new 1.5 mL tube and mixed with 400 μl of isopropanol. Samples were incubated at −70 °C overnight to precipitate the RNA. Then the samples were centrifuged at 13 200 rpm (Eppendorf Centrifuge 5415 R) for 15 min at 4 °C, following which RNA pellets were washed with 500 μl of 70% ethanol at 13 200 rpm (Eppendorf Centrifuge 5415 R) for 15 min at 4 °C. RNA pellets were briefly air-dried and resuspended in 20 μl diethyl pyrocarbonate (DEPC)-treated Millipore-purified water. The RNA was purified another round to generate high quality total RNA using the QIAGEN® RNeasy Mini Kit (QIAGEN). The QIAGEN® RNeasy Mini Protocol for RNA Clean-up was followed according to the manufacturers' instructions, with the exception of the final elution of total RNA was performed twice in 10–12 μl volumes of RNase-free water (QIAGEN) with 1 min incubations on the RNeasy® mini column (QIAGEN). RNA concentrations were determined using Nanodrop® ND1000 Spectrophotometer (Nanodrop Technologies). RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) by checking the RNA Integrity Number and examining the electropherogram profile generated.

2.3. Expression microarrays

Total RNA prepared were submitted to the ACRF/Biomolecular Resource Facility (JCSMR, ANU), which processed the samples by performing the target preparation, hybridization, staining and scanning of Affymetrix™ Mouse Gene 1.0ST arrays as per manufacturers' instructions. Three biological replicates for each treatment were used for the expression arrays. The data was analyzed using Quantile normalisation and Robust Multichip Average (RMA) background correction adjusting for probe sequence using the Partek Software (Partek, USA). These programs were used to generate gene expression levels from the Mouse Gene 1.0ST arrays and an ANOVA test was used to identify genes induced with PMA/I stimulation or not induced ('unchanged'). Genes with higher expression in DMSO treated stimulated cells (than unstimulated, p-value <0.016 equivalent to a false discovery rate (FDR) of <0.1) and whose expression in CHX treated stimulated cells was not less than that in DMSO treated stimulated cells, were classified as primary response genes. Genes with higher expression in DMSO treated stimulated cells (than unstimulated, p-value <0.016) and with

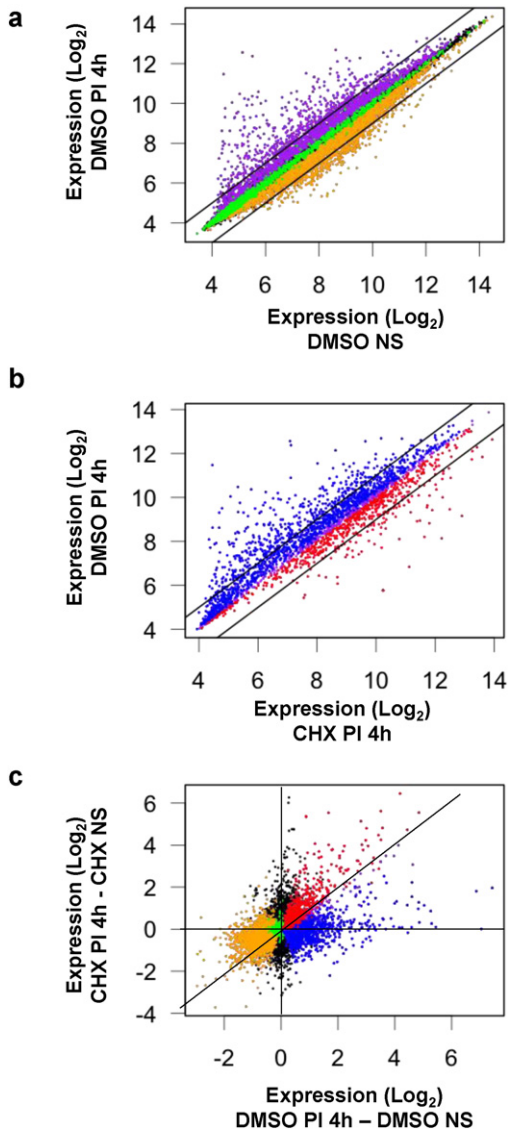

Fig. 2. Correlation of genes based on their expression kinetics. Scatter plot of genes from expression arrays categorized based on their response to CHX and PMA/I (4 h): (a) comparing genes that were unchanged with stimulation (●), genes that were induced with PMA/I stimulation (●), and genes that were inhibited by PMA/I stimulation (●); (b) comparing genes induced by PMA/I stimulation and not inhibited by CHX (●; primary response genes) and genes whose induction by PMA/I stimulation was inhibited by CHX (●; secondary response genes); (c) the distributions of the average Log_2 RMA values from the unstimulated cells were shown for all genes on the array (●), genes that were unchanged with stimulation (●), genes that were induced with stimulation (●), genes that were inhibited by stimulation (●), primary response genes (●) and secondary response genes (●). Genes with higher expression in DMSO treated stimulated cells (than unstimulated, p-value <0.016 equivalent to a false discovery rate (FDR) of <0.1) and whose expression in CHX treated stimulated cells was not less than that in DMSO treated stimulated cells, were classified as primary response genes. Genes with higher expression in DMSO treated stimulated cells (than unstimulated, p-value <0.016) and with lower expression in CHX-treated, stimulated cells (than DMSO-treated, stimulated, p-value <0.024, FDR < 0.1) were classified as secondary response genes. Genes with p-values >0.1 for all factors (stimulation, treatment, replicates and stimulation*treatment) were classified as unchanged genes.

Table 1
Number of genes in each expression bin groups.

Basal expression levels (Log 2 values)									
Gene group	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–12	12–13
All	13,154	3136	1738	2050	2193	1977	1107	500	193
Up	456	275	310	395	422	380	186	80	39
Down	216	241	279	458	562	542	318	130	30
Primary	36	48	77	126	125	103	41	22	10
Secondary	240	173	176	203	204	198	102	45	18
Unchanged	5583	744	150	126	100	84	58	29	6

lower expression in CHX-treated, stimulated cells (than DMSO-treated, stimulated, p-value <0.024, FDR < 0.1) were classified as secondary response genes. Genes with p-values >0.1 for all factors (stimulation, treatment, replicates and stimulation * treatment) were classified as unchanged genes. Groups were then subdivided further depending on their average basal expression level. Raw and normalized data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE13278.

3. Discussion

3.1. Categorisation of gene probes on expression arrays

Expression profiling was performed on non-stimulated or PMA/I-treated (4 h) EL4 T cells with or without cycloheximide (CHX) treatment, and inducible genes were identified. 2727 probes were significantly (False Discovery Rate (FDR) < 0.1) induced with PMA/I stimulation for 4 h (in DMSO treated cells) and were thus classified as induced or 'Up with Stimulation', while 2971 were significantly down-regulated or 'Down with Stimulation' (FDR < 0.1) and 7357 had p values >0.2 for both the drug (CHX/DMSO) and stimulation factors and thus were classified as 'unchanged' (Fig. 1). Of the induced probes, 645 had an average level of expression in stimulated CHX treated cells that was equal to or higher than the level of expression in stimulated DMSO treated cells and these were classified as 'primary response genes'. 1454 of the induced probes were significantly (FDR < 0.1) less in CHX stimulated cells than DMSO stimulated cells and these were classified as secondary response genes. It should be noted that some induced probes were on average less in CHX stimulated cells than DMSO stimulated cells but not significantly so and thus were not classified.

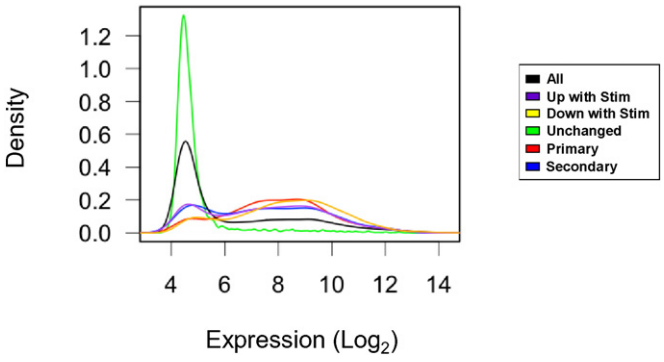

Fig. 3. Density plot of the basal expression values for the different gene groups identified with expression microarrays. EL4 T cells were stimulated with PMA/I for 4 h with and without pre-treatment with cycloheximide (CHX). Expression microarrays were performed with RNA from these cells (3 biological replicates per treatment) and genes were grouped according to the kinetics of their response. The distributions of the average Log_2 RMA values from the unstimulated cells are shown for all genes on the array (black line), genes that were unchanged with stimulation (green line), genes that were induced with stimulation (purple line), genes that were inhibited by stimulation (yellow line), genes induced by stimulation and not inhibited by CHX (red line) and genes whose induction by stimulation was inhibited by CHX (blue line).

3.2. Distribution of genes based on basal expression values and protein synthesis requirement

In order to confirm that the genes are grouped correctly according to their response to PMA/I and CHX treatment, the genes were scattered based on their basal mRNA expression levels (in log_2) to examine the distribution of genes based on their response to PMA/I and/or CHX in a graphical manner. In general, there was a spread of genes from low expression level to high expression level for the different groups of genes with most genes showing less than 2 fold change (Fig. 2a and b, between the two black lines, Table 1). Genes in which expression is induced upon PMA/I stimulation and genes that are inhibited by PMA/I stimulation were distributed accordingly with the unchanged genes that were distributed in between the genes that change with stimulation as expected (Fig. 2a). The primary response genes are distributed separately from secondary response genes thus these genes are classified correctly (Fig. 2b). Finally all the different groupings of genes were also plotted in a scatter plot to compare the distribution of all the different groupings based on the differences in expression levels when genes are stimulated with PMA/I and treated with CHX. The different groups of genes are distributed as expected and separately from each other (Fig. 2c). Thus, the different groups of genes selected and classified from the expression profiling show the expected distribution profiles and are classified accurately.

From the scatterplot profiles, there seems to be a variation in the distribution of genes across the different basal expression levels (Fig. 2a and b). To examine how the different gene groups are distributed based on the basal expression levels, a density plot of the number of genes within a certain basal expression level was generated (Fig. 3). Both the primary and secondary response gene groups displayed a wide spread of basal mRNA expression levels but on average the basal expression levels are higher compared with the unchanged group or all genes, suggesting that many inducible genes are already producing detectable transcripts (Fig. 3). Genes with different basal expression levels may display distinct basal chromatin characteristics. Therefore, to ensure comparison of genes with similar basal expression levels, the gene groups were binned according to their basal mRNA expression levels (Table 1). The number of primary response genes in the lowest expression bin (log2 3–4) was small and thus could not be treated in a sound statistical manner (data not shown).

Thus, inducible genes were categorized into primary and secondary response genes and further grouped according to their basal mRNA expression level, creating a grid of gene groups that can be used for comparison of epigenomic marks. This grouping of genes is important for comparing chromatin states as increasing basal expression level is associated with an increase in the levels of the histone modifications and RNA polymerase II suggesting that the levels of these factors correlate with transcriptional activity [1,3–5]. In summary, T cell activation leads to differential expression of genes and inducible genes can be classified as primary response and secondary response based on their dependency on protein synthesis.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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