

ORIGINAL ARTICLE

TGF β -dependent gene expression profile during maturation of dendritic cells

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Primary immune response to pathogens involves the maturation of antigen-presenting dendritic cells (DC). Bacterial lipopolysaccharide (LPS) is a potent inducer of DC maturation, whereas the transforming growth factor β (TGF β) attenuates much of this process. Here, we analyzed the global gene expression pattern in LPS-treated bone marrow derived DC during inhibition of their maturation process by TGF β . Exposure of DC to LPS induces a pronounced cell response, manifested in altered expression of a large number of genes. Interestingly, TGF β did not affect most of the LPS responding genes. Nevertheless, analysis identified a subset of genes that did respond to TGF β , among them the two inflammatory cytokines interleukin (IL)-12 and IL-18. Expression of IL-12, the major proinflammatory cytokine secreted by mature DC, was downregulated by TGF β , whereas the expression level of the proinflammatory cytokine IL-18, known to potentiate the IL-12 effect, was upregulated. Expression of the peroxisome proliferator-activated receptor γ (PPAR γ) increased in response to TGF β , concomitantly with reduced expression of chemokine receptor 7 (CCR7). This finding supports the possibility that TGF β -dependent inhibition of CCR7 expression in DC is mediated by PPAR γ .

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Introduction

Dendritic cells (DC) are sparsely distributed, migratory bone marrow-derived cells that are specialized in uptake, processing and presentation of antigens to T cells.^{1,2} The murine DC compartment is defined by surface expression of major histocompatibility complex class II (MHCII) and the β 2-integrin CD11c, found on all DC. At the immature state, DC monitor the antigenic environment for the presence of foreign antigens. Detection of damage or pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) by tissue-resident DC, initiates DC maturation and migration to the regional lymph nodes (LN).¹ Maturation is associated with upregulation of MHCII molecules and costimulatory molecules such as CD80 and CD86.¹ Mature DC are unrivaled in their potential to stimulate naïve T cells. Hence, activation of DC is continuously attenuated by immunosuppressive cytokines³ such as transforming growth factor β (TGF β).^{4–6}

TGF β belongs to a well-defined multi-potent cytokine family involved in many pathophysiological events.⁷ Three isoforms (TGF β 1, TGF β 2 and TGF β 3) that bear overlapping activities are expressed in mammals. Of the three, TGF β 1 is the most investigated and best character-

ized prototype. Loss-of-function mutation in TGF β 1 in mice⁸ results in a severe, multifocal inflammatory response leading to early death, proving its essential role in maintenance of immune homeostasis. TGF β promotes DC development *in vitro*⁹ but suppresses their final maturation.¹⁰ When modulated by TGF β -expressing adenoviral vectors, DC become tolerogenic and suppress T-cell alloreactivity.¹¹ Immature DC can lead to peripheral tolerance by inducing differentiation of regulatory T cells.¹² Thus, DC exposed *in vitro* to TGF β are able to ameliorate experimental autoimmune myasthenia gravis.¹³ It was also demonstrated that tumors evade the host immune attack by secreting TGF β , which inhibits DC migration to the draining LN and thereby preventing the presentation of tumor antigens to T cells.^{14,15} To gain insights into the molecular mechanism underlying the inhibitory effect of TGF β on maturation and function of DC, we characterized the effect of TGF β on global gene expression of DC, when cells were driven to maturation by exposure to LPS.

Results

In order to assess the effect of TGF β on DC maturation, five independently derived bone marrow dendritic cells (BMDC) cultures, each from a separate mouse, were generated (see Materials and methods). Each such culture was then divided into two plates, one of which was treated on day zero with TGF β . To induce DC maturation, on day 7, cultures were treated with LPS and harvested at 0, 2, 4, 16 and 24 h. CD11c⁺ DC were

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positively selected by CD11c magnetic beads, total RNA was prepared and processed for microarray analysis.

Clustering analysis

The super paramagnetic clustering (SPC) algorithm¹⁶ was used for performing cluster analysis. In the resulting dendrogram (Figure 1), stable (i.e., statistically significant) gene clusters are identified; these are denoted G2–G12 (see Supplementary Information for the complete list of genes in each cluster). A cluster C is registered as stable only if it exceeds a certain size and when $Stab(C)$, its stability index defined in terms of the range of resolution parameters, T , through which cluster C 'lives',¹⁷ exceeds a certain threshold.

Transcriptional profile of LPS-treated DC

In agreement with the human data (Perrier *et al.*¹⁸ and references therein; Huang *et al.*¹⁹), treatment with LPS had a profound effect on the transcriptional profile of the mouse DC. Additionally, the set of mouse genes modulated by LPS corresponds to those reported for human. Figure 1 displays the expression profiles of 500 genes that were most influenced in the experiment (i.e., largest standard deviation over all samples, see Methods). The effect of LPS on gene expression was more prominent than that of TGF β , as reflected by the difference between time point 0, before the addition of LPS, and the following time points after LPS addition. Out of 11 stable gene clusters (Figure 2), six displayed no difference in gene expression with or without TGF β (G3, G4, G5, G9, G11 and G12) (Figure 2). Three displayed very small differences (G6, G7 and G10) and only two clusters (G2 and G8) showed expression affected by TGF β , albeit in opposite directions. Expression levels of the genes clustered in G2 increased by TGF β treatment (Table 1), whereas expression levels of genes in G8 decreased (Table 2). Not only was the effect of LPS on gene expression more pronounced than that of TGF β , it also exhibited unique kinetics. A sharp transient increase in expression within the first four hours is seen in G3,

and a sharp nontransient increase is seen in G9, G10 and G11. The expression profiles of G9, G10 and G11 are very similar, and they comprise cluster G12, which is highly enriched for immune response genes. G7 genes are transiently downregulated, and G6 genes are nontransiently downregulated within the first four hours after LPS

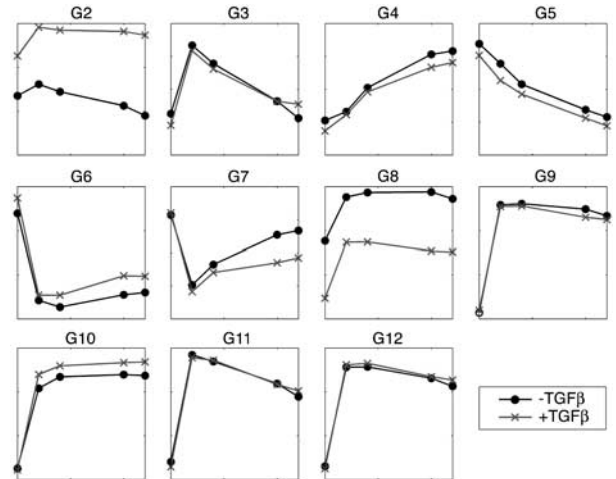


Figure 2 Expression levels versus time, with and without TGF β ; the average expression level of the genes of each stable cluster is presented. The expression profile of each gene was normalized as described in Materials and methods. Samples were collected at 0, 2, 4, 16 and 24 h after adding LPS (see text).

Table 1 G2 (TGF β upregulated genes)

Gene symbol	Gene name
<i>Chemokines and receptors</i>	
Cxcl14	Chemokine ligand 14
<i>Cytokines and receptors</i>	
IL-18	Interleukin-18
<i>Growth factors</i>	
Areg	Amphiregulin
Ereg	Epiregulin
Emr4	EGF-like module containing, mucin-like, hormone receptor like sequence 4
<i>Inflammatory response</i>	
Chi3l3	Chitinase 3-like 3
Chi3l4	Chitinase 3-like 4
<i>Transcription factors</i>	
PPAR γ	Peroxisome proliferator activated receptor γ
<i>Proteases</i>	
Mcpt1	Mast cell protease 1
Mcpt2	Mast cell protease 2
Cpa3	Carboxypeptidase A3, mast cell
<i>Cell adhesion</i>	
Vcam1	Vascular cell adhesion molecule 1
Thbs4	Thrombospondin4
<i>Others</i>	
S100a9	S100 calcium binding protein a9 (calgranulin B)

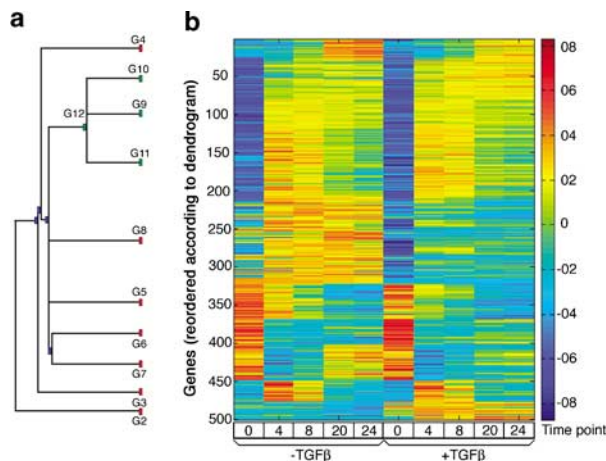


Figure 1 Clustering analysis of genes during DC maturation in the absence or presence of TGF β . (a) Dendrogram of genes including stable clusters of at least five genes. The boxes and letters G4, etc indicate the identified clusters. (b) Expression matrix of the genes reordered according to the dendrogram. The colors represent induction (red) or repression (blue).

Table 2 G8 (TGF β downregulated genes)

Gene symbol	Gene name
<i>Chemokines and receptors</i>	
Ccl7	Chemokine ligand 7
Ccl8	Chemokine ligand 8
Ccl12	Chemokine ligand 12
Ccr7	Chemokine receptor 7
<i>Cytokines and receptors</i>	
Il12b	Interleukin 12b
Il1r2	Interleukin 1 receptor, type 2
Il2ra	Interleukin 2 receptor, α chain
Tnfrsf9	Tumor necrosis factor receptor superfamily, member 9
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b
<i>Growth factors</i>	
Pgf	Placental growth factor
<i>Inflammatory response</i>	
Nos2	Nitric oxide synthase 2, inducible
Cd80	CD80 antigen
Cd86	CD86 antigen
<i>Transcription factors</i>	
Stat2	Signal transducer and activator of transcription 2
Stat5a	Signal transducer and activator of transcription 5A
<i>Proteases and inhibitors</i>	
Mmp14	Matrix metalloproteinase 14
Timp2	Tissue inhibitor of metalloproteinase 2
<i>Cell adhesion</i>	
Itga5	Integrin α 5
Thbs1	Thrombospondin 1
<i>Prostaglandin metabolism</i>	
Ptgs2	Prostaglandin endoperoxide synthase 2
<i>Cell cycle and apoptosis</i>	
Cdkn1a	Cylin dependent kinase inhibitor 1 A (p21)
Mdm2	Transformed mouse 3T3 cell double minute 2
Casp4	Caspase 4
Gadd45b	Growth arrest and DNA damage inducible 45 β
<i>Others</i>	
Cp	Ceruloplasmin
Pla1a	Phospholipase-A1 member A
Lta	Lymphotoxin A
Msr2	Macrophage scavenger receptor 2
Ldlr	Low density lipoprotein receptor

addition. Additional effect of LPS is evident in clusters G4 and G5, which display a steady increase and decrease of expression, respectively, in response to LPS.

Validation of the transcriptional profile

To validate the data of the microarray transcriptional analysis, semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), Western blotting and fluorescence-activated cell sorting (FACS) analysis were performed on a selected number of genes. We particularly searched for genes whose induction by LPS was

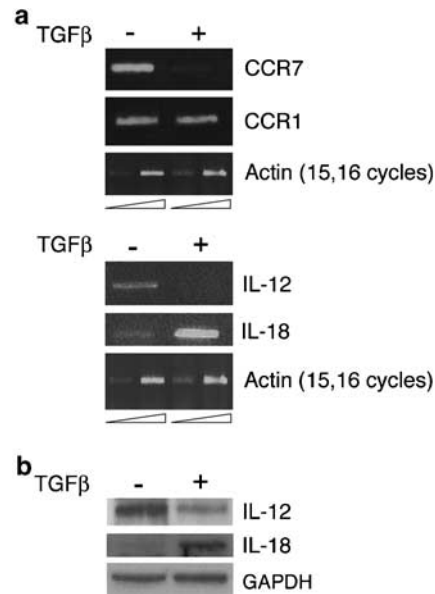


Figure 3 BMDC were incubated with GM-CSF (10 ng/ml) with or without TGF β (10 ng/ml). Day 7 cells (10⁶ cells/ml) were cultured overnight with 1 μ g/ml LPS, collected and RNA (a) or protein (b) was prepared. (a) RT-PCR was performed to detect the influence of TGF β on the expression of CCR7 and CCR1 (upper panel). RT-PCR was performed to detect the influence of TGF β on the expression of IL-12 and IL-18 (lower panel). (b) Western blot was performed to detect the influence of TGF β on the expression of IL-12 and IL-18.

affected by exposure of the DC to TGF β . In accordance with its known function, TGF β attenuated the maturation of LPS-treated DC, manifested in a low level of surface MHCII on a significant proportion of the DC population (FACS data not shown). In agreement with the microarray data, semiquantitative PCR confirmed that expression of chemokine receptor 7 (CCR7) was indeed downregulated by concomitant treatment with TGF β and LPS (Figure 3, upper panel). Accordingly, it was previously shown²⁰ that CCR7 expression is decreased at the protein level on treatment of BMDC with TGF β . Interleukin-12 (IL-12), the major proinflammatory cytokine secreted by mature DC, was also downregulated by TGF β (Figure 3a, lower panel, and 3b). Intriguingly, IL-18, another known proinflammatory cytokine that has been shown to potentiate the IL-12 effect,²¹ has been upregulated by the TGF β treatment. The effect of TGF β on DC maturation is not dependent on the mouse strain. We have tested the effects of TGF β on BMDC derived from ICR, MF1 or C57Bl/6 mouse strains and found no differences in either regulation of IL-12 and IL-18 expression (Figure 3) or inhibition of MHCII, CD80 and CCR7 expression (data not shown).

Discussion

LPS is a potent inducer of DC maturation, a process accompanied by an increase in surface expression of MHCII and several co-stimulatory markers.¹ TGF β attenuates much of this process, as shown by the gene expression data of co-stimulatory markers (Table 2: G8). In this study, we analyzed the global gene expression pattern in LPS-treated BMDC during inhibition of the

maturation process by TGF β . As TGF β was added to cultures on day zero, we cannot rule out the possibility that some of the observed transcriptional differences resulted from the effect of TGF β on DC differentiation. It should be noted, however, that in order to minimize the TGF β differentiation effect, cells were positively selected by CD11c beads before RNA extraction. Hence, only fully differentiated, CD11c-expressing BMDC were analyzed.

TGF β has long been known for its anti-inflammatory properties. Recently, however, a proinflammatory activity of TGF β through induction of T-helper 17 cells has been reported.²² Its pleiotropic anti-inflammatory activity is reflected in changes seen in a large number of genes, some of which are upregulated (G8) and the others downregulated (G2). Expression of the chemokines CCL7, CCL8 and CCL12 was downregulated in response to TGF β treatment, as was the expression of inflammatory cytokine receptors IL1 R2 and IL2 Ra. IL-12, the major proinflammatory cytokine secreted by mature DC was also downregulated by TGF β . On the other hand, the proinflammatory cytokine IL-18, known to potentiate the IL-12 effect, was upregulated by TGF β treatment. Both these results have been confirmed by RT-PCR and Western blotting (Figure 3). IL-18 plays an important role in regulating immune responses. It is a unique cytokine with a capacity to induce either T helper 1 (Th1) or Th2 polarization, depending on the immunologic context. Several recent reports have revealed a significant role for IL-18 in the biology of DC. IL-18 along with IL-12 plays a critical role in the reciprocal maintenance and expansion of CD8 α^+ DC and NK1.1 $^+$ NK cells, respectively.²¹ Monocyte-derived DC1 preferentially induce a Th1 response, and the plasmacytoid-derived DC (DC2) have been linked to a Th2 response. Consistent with its role in modulating Th1/Th2 responses, IL-18 also regulates DC1 and DC2 responses. IL-18 is constitutively released from DC1, but not DC2. Thus, TGF β -induced IL-18 secretion might have an important role during the DC-mediated initiation phase of an immune response, by determining the resulting Th1/Th2 balance.

We found that expression of the peroxisome proliferator-activated receptor γ (PPAR γ) is increased by TGF β treatment of DC (Table 1). Others²³ have shown that TGF β affects PPAR γ -mediated signaling in macrophages. The PPARs are nuclear eicosanoid receptor transcription factors that regulate adipocyte differentiation and metabolism. It has been shown that PPAR γ activation by selective agonists (i.e. rosiglitazone and ciglitazone) impairs the migration of intratracheally injected BMDC from the lung to the thoracic LN.²⁴ It was hypothesized that this might be due to reduced expression of the CCR7 receptor necessary for DC migration to the draining nodes, as rosiglitazone-treated DC failed to respond to the CCR7 ligand SLC and ELC. The end result of PPAR γ activation by rosiglitazone is diminished recruitment of eosinophils to the lung in an induced asthma mouse model. The process of DC maturation involves upregulation of specific chemokine receptors, which direct the migration of DC to the T-cell zones of the regional LNs. A switch in CCR expression²⁵ concomitantly with DC maturation enables activated mature DC to leave sites of inflammation and migrate to the T-cell zones of the draining LN via afferent lymphatics. Upregulation of CCR7 on DC maturation plays a pivotal role in guiding mature DC to sites of T-cell recruitment and activation,

initiating the adaptive immune response. It has been shown²⁶ that TGF β causes a complete shut off of CCR7 transcription even in the presence of LPS. We now show that TGF β causes increased expression of PPAR γ associated with downregulation of CCR7 (Table 2). This finding lends further support to the possibility that TGF β -mediated inhibition of CCR7 expression proceeds through induction of PPAR γ , which in turn transcriptionally represses CCR7 expression. It has also been reported that as a consequence of TGF β -mediated inhibition of DC maturation, expression of T-cell costimulatory molecules decreases, as clearly seen in the G8 cluster for CD80, CD86. Interestingly, however, expression of CD40 – another co-stimulatory molecule – was not changed in response to TGF β .

In conclusion, the data reveal the global changes in DC gene expression, when their maturation is attenuated in response to treatment with TGF β . It highlights the intricate downregulation of the chemokine receptor CCR7, which, as shown here, is accompanied by upregulation of the transcription factor PPAR γ and the opposite effect of TGF β on the expression of the inflammatory cytokines IL-12 and IL-18. The biological significance of these findings with respect to DC immune regulation remains to be elucidated.

Materials and methods

Bone marrow cultures

BMDC were prepared as described briefly.²⁷ Briefly, mice were killed and BM was extracted from femurs and tibiae by flushing the shaft with 5 ml RPMI-1640 (Invitrogen, Carlsbad, CA, USA). Animal experiments followed the guidelines of the Institutional Animal Care and Use Committee of the Weizmann Institute. Red blood cells were lysed in 1.66% NH $_4$ Cl, and cells seeded into non-tissue culture plates at a density of 1×10^6 cells/ml in medium: RPMI-1640, 5% FCS, 5×10^{-5} M 2-mercaptoethanol, penicillin/streptomycin (all from Invitrogen) containing 10 ng/ml murine recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ, USA). Medium was replenished every 3 days. The cells were divided into two cultures; to one culture, TGF β (10 ng/ml) (Peprotech) was added (we refer to this culture as +TGF β). To induce DC maturation, day 7 cultures were treated with LPS (1 μ g/ml); (Sigma, Rehovot, Israel) and harvested after 0, 2, 4, 16 and 24 h. CD11c $^+$ DC Cells were enriched on magnetic activated cell sorter (MACS) columns (Miltenyi Biotec, Bergisch Gledbad, Germany), yielding >90% CD11c $^+$ cells, before preparation of RNA.

To reduce fluctuations because of variability in mice and culture, for each time point cDNA was generated from pooled RNA derived from five parallel cultures, each of a different mouse.²⁸ LPS treatment of BMDC induced a high expression level of MHCII with respect to untreated controls. TGF β treatment attenuated much of this LPS effect although not completely.²⁹ As indicated in Table 2, TGF β treatment of DC caused downregulation of the maturation markers CD80 and CD86.

RNA preparation and RT-PCR analysis

Total BMDC RNA (derived from separate experiments) was extracted using EZ-RNA (Biological Industries,

Kibbutz Beit Haemek, Israel) according to the manufacturer's instructions. The RNA content was determined spectrophotometrically. Total RNA was reverse transcribed using the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was subjected to PCR reactions using SuperTherm polymerase (Hoffman-La-Roche, Basel, Switzerland).

Western blotting

BMDC were washed in PBS and then lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA), containing protease inhibitors. After determination of protein content, samples were boiled for 5 min with loading buffer containing 2-mercaptoethanol. Samples were then run on SDS-polyacrylamide gels and transferred to a Hybond-P membrane. Membranes were incubated with blocking buffer (5% dry milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20), washed and reacted with the appropriate first antibody: rabbit anti-mouse IL-18 (gift from Dr Charles Dinarello); rat anti-mouse IL-12/IL-23 (p40 subunit) (eBioscience, San Diego, CA, USA); mouse anti-GAPDH monoclonal antibody (CHEMICON 28820, Temecula, CA, USA). After washing, the membrane was incubated with the respective secondary antibody: goat anti-rabbit HRP and goat anti-mouse HRP (Jackson ImmunoResearch, West Grove, PA, USA) and signal detected by an ECL kit (Amersham, Bucks, UK).

Sample preparation and hybridization to gene chips

Double-stranded cDNA was generated from 12 μ g of total RNA using the Super Script Choice System (Gibco BRL, Rockville, MD, USA), using an oligo(dT) 24 primer containing a T7 promoter site at the 3' end (Genset, La Jolla, CA, USA). The cDNAs were purified via a phenol-chloroform extraction followed by an ethanol precipitation. Purified cDNA was subjected to *in vitro* transcription (IVT), with T7 RNA polymerase and biotin-labeled ribonucleotides, using the ENZO BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, New York, NY, USA). Labeled transcripts were purified over RNeasy mini-columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Labeled cRNA was fragmented at 94°C for 35 min in fragmentation buffer (40 mM Tris-acetate, pH 8.1/100 mM potassium acetate, 30 mM magnesium acetate), and a hybridization mix was generated by the addition of herring sperm DNA (0.1 mg/ml), acetylated BSA (0.5 mg/ml; Invitrogen), sodium chloride (1 M), Tris-acetate (10 mM) and Tween 20 (0.0001%). A mixture of four control bacterial and phage cRNA (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre) was included to serve as an internal control for hybridization efficiency.

Aliquots of each sample (12 μ g cRNA in 200 μ l hybridization mix) were hybridized to Genechip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). After hybridization, each array was washed according to the procedures developed by the manufacturer (Affymetrix), and stained with streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, OR, USA). The hybridization signal was amplified by using biotinylated anti-streptavidin antibodies (Vector Laboratories, Burlingame, CA, USA), followed by restaining

with streptavidin-phycoerythrin. Arrays were scanned by the Gene Array scanner G3000 (Affymetrix, Santa Clara, CA, USA), and scanned images were visually inspected for hybridization imperfections. Low-level analysis of the arrays was performed using the GCOS software (Affymetrix), scaling to an average intensity of 250.

Preprocessing and filtering

Gene-expression values lower than 50 were adjusted to 50 to eliminate noise from the data, and then all values were log₂-transformed. Standard deviation of the log₂-transformed expression values was calculated over all five time points and two treatments (5 \times 2 chips). A variation filter was applied; the 500 genes with the highest standard deviation over all samples were selected. The log₂-transformed expression values of each gene at all time points and treatments were centered and normalized such that the mean expression is set to zero, and the standard deviation is set to one.

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Supplementary Information accompanies the paper on Genes and Immunity website (<http://www.nature.com/gene>)