FULL PAPER

cis-Element clustering correlates with dose-dependent pro- and antisignaling effects of IL18

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We examine the effects of IL18 on monocytes by performing microarray experiments using cell line KG1. Based on sensitivity to IL18, we identified three functionally distinct gene expression clusters (EC). We see little proinflammatory gene induction at low IL18 concentrations, but instead observe induction of diverse NFκB signaling inhibitors. Conversely, intermediate concentrations of IL18 induced proinflammatory genes including the activating subunits of NFκB. At the highest IL18 concentration, we observe a third gene cluster containing the proapoptotic Fas gene among others. Clustering of IL18-responsive genes based on cis-elements in their promoters agreed well with the ECs. We conclude that IL18 produces a dose-dependent transcriptional response that can in part be attributed to the composition of cis-elements in the promoters of IL18-responsive genes. These results also support a model for regulatory mechanisms that prevent spurious immune response due to weak cytokine fluctuations and a separate mechanism enabling induction of proinflammatory functions by higher levels of cytokine.

Introduction

IL18 is a proinflammatory cytokine structurally related to IL1. It is considered to play a critical role in the Th1 polarization of T cells during an immune response.1,2 It is also thought to activate NK cells3-4 and monocytes, including the myelomonocytic cell line KG-1.5-10 IL18 signal transduction is mediated via a heterodimeric receptor11 that engages IRAK via Myd8812-14 leading to activation of the NFκB pathway1,15 via NIK, as well as the activation of AP1 via JNK.13 Whether IL18 receptor engagement activates other pathways, is not known. Thus far, most reports have studied the function of IL18 in concert with other proinflammatory cytokines such as IL12, especially with respect to interferon gamma gene regulation.16-19 In fact, IL18 alone does not induce interferon gamma expression in most experimental model systems. IL12 cooperation with IL18 has been speculated to function via IL18 receptor upregulation and STAT4 activation.17,20 Apart from interferon gamma expression, little is known about other genes regulated by IL18 alone or in combination with other cytokines.

In this study, we use oligonucleotide microarrays to investigate the gene expression patterns induced by physiologically relevant concentrations of IL18 in the monocytic cell line KG1.21 Using various clustering and visualization techniques, we demonstrate that IL18 alone is sufficient to induce expression of specific genes in a dose-dependent manner. Low concentrations of IL18 appear to provoke an anti-NFκB signaling response; higher concentrations result in expression of many chemokines and other monocyte/macrophage activation markers, whereas the highest concentration of IL18 results in activation of a third set of genes including proapoptotic FAS, Fc receptor γ and IL2 receptor. These results suggest a model whereby a feedback mechanism limiting activation of NFκB-signaling at low or spurious cytokine concentration and a mechanism producing inflammatory responses at higher concentrations of IL18.

We also performed computational analysis on the promoter regions of the IL18-responsive genes determined by microarray experiments. We discovered that a very high percentage of the genes responding to low and intermediate concentrations of IL18 contain putative NFκB family binding sites. Using a computer program CLOVER (Frith MC, Hansen U, Weng Z, manuscript in preparation), we determined cis-elements in the proximal promoters of all IL18-responsive genes. We then clustered these genes based on their cis-element contents. Surprisingly, the resulting cis-element-based clusters agree well with gene clusters that are based on expression profile. We conclude that IL18 produces a dose-dependent transcriptional response that can be accounted for in part by the composition of cis-elements representing common signal transduction end points.

Results

IL18 alone directly regulates gene expression in KG1 cells

To determine the set of transcripts regulated directly by IL18, we titrated recombinant IL18 into duplicate KG1
cell cultures in the presence and absence of the protein synthesis inhibitor cycloheximide. mRNA samples were prepared and analyzed using Affymetrix Hu6800 Genechips® (see Online Supplemental Information and Methods for MIAME data). Figure 1a is a heatmap representation of 60 genes whose expression levels were most significantly regulated by IL18. We did not consider downregulated transcripts in this analysis due to the well-documented effects of cycloheximide on mRNA stability. Compared to vehicle treatment, these genes increased expression under at least one IL18 concentration (see Figure 1a). The genes comprised a variety of functional categories including transcription factors, kinases and surface molecules and secreted proteins of which many have been previously identified as markers of activated macrophages and monocytes. IL18 treatment causes transcriptional activation of at least five chemokines and three cytokines. The upregulated chemokines (MIP1-α and -β, MIP2-α and -β and IL8) are all reported to attract predominantly monocytes and neutrophils. We also detected upregulation of the cytokines IL1-β, TNF-10 and the macrophage growth factor CSF-1. Among the surface molecules upregulated under these conditions were ICAM-1, CD83 and CD36, which have established roles in macrophage function. We also detected upregulation of at least 15 transcription factors including six members of the NFκB pathway (see below). The heatmap also indicates that some but not all transcripts had a dose-dependent response to IL18, which we examined more closely.

Bioinformatics analysis alone predicts IL18-induced NFκB signaling
Since some genes appeared to respond differently depending on IL18 concentration, we attempted to determine computationally whether the genes in the

![Figure 1](image-url)

Figure 1 Genes induced by IL18 in KG1 cells. (a) Response of the 60 genes most significantly induced by IL18. KG1 cells were treated with the indicated amounts of IL18 in the presence or absence of cycloheximide. The heatmap was generated by selecting transcripts that were upregulated ≥2-fold with $P<0.05$ by t-test in IL18 and CHX + IL18 samples, as compared to vehicle-treated samples, for at least one concentration of IL18, or were up ≥1.5-fold and had a $P<0.01$ by t-test in the IL18 and CHX + IL18 samples, as compared to vehicle-treated samples, for at least one concentration of IL18. Each column represents the arithmetic average of biological duplicates. Indicated at the right by purple bars are the genes containing NFκB sites recognized by TRANSFAC. (b) NFκB binding site prediction using TRANSFAC. Each of the genes depicted herein was analyzed for NFκB regulation by literature searching using PubMed and by using the binding site identification tool TRANSFAC. In all, 25 of 27 literature-annotated genes were also identified by TRANSFAC as containing NFκB sites. TRANSFAC also predicted another 13 genes from the gene set in panel (a) to contain sites. These sites were not further validated. The high preponderance of NFκB site containing genes indicates a role for this factor in the IL18 pathway. (c) NFκB binding ELISA assay. Nuclear extracts were prepared from cells treated identically to those used for panel (a). These extracts were used in an ELISA-based assay using plate bound p50 and p65 binding sites as indicated. Soluble wild-type or mutant competitor oligonucleotides were included in parallel reactions as indicated. Reaction conditions and oligonucleotide sequences are described in Materials and Methods. Differences between 10 and 50 ng/ml IL18 for both p65 and p50 are significant to $P<0.01$ using Student’s t-test. (d) NFκB mobility shift. Nuclear extracts from cells were mixed with a 32P-labeled oligonucleotide probes encoding an NFκB binding site and electrophoresed through a polyacrylamide gel. → indicates the position of NFκB p65/p50 heterodimers and → indicates the position of NFκB p50/50 homodimers. Lanes 1–7 contain nuclear extracts from KG1 cells treated with the indicated amounts of recombinant IL18. Lane 8 contains a 32P-labeled AP2 binding site and nuclear extract from KG1 cells incubated with 10 ng/ml IL18. Lanes 9 and 10 contain nuclear extracts from control and LPS-treated mouse peritoneal exudate cells. (e) NFκB antibody super shifts. Each panel contains EMSA analysis of KG1 nuclear extracts from control cells or cells incubated with 10 ng/ml IL18 as indicated incubated with a 32P-labeled oligonucleotide. → indicates the position of NFκB p65/p50 heterodimers and → indicates the position of NFκB p50/50 homodimers. Indicated above each lane is the type of anti-NFκB monoclonal antibody or cold oligonucleotide competitor coincubated in each reaction.
groups were related to each other in terms of transcription factor binding sites in their proximal promoters. First, we used the MATCH program from the TRANSFAC database to scan the 5' upstream regions of these genes to identify NFkB binding sites. The program identified 38 (out of 60) genes. We also performed literature searches to determine which of the IL18-response genes had been previously annotated as NFkB regulated. The comparison is summarized as a Venn diagram in Figure 1b. MATCH identified most of the genes annotated as NFkB-regulated (25 out of 27, or 97%). It also predicted 13 genes not previously annotated. The rightmost column of Figure 1a denotes which genes contained predicted NFkB binding sites. Because these predicted sites are biased toward the top of the heatmap we inferred a potential transcriptional basis for the differential response to IL18 meriting further biochemical and computational analysis.

**IL18-induced NFkB binding**

To confirm the results of our preliminary bioinformatics analysis, we set out to verify experimentally that NFkB activation occurred under our IL18 treatment conditions. The results of NFkB DNA-binding ELISA assays using two different NFkB-specific oligonucleotides are shown in Figure 1c. These experiments demonstrated induction of NFkB binding activity that was apparently bimodal. At the lowest IL18 concentration (0.5 ng/ml), we observe a slight increase in NFkB binding activity above background level. The activity is further increased at 2 ng/ml, but it remains unaltered when the concentration is increased to 10 ng/ml. A slight increase in the NFkB binding activity is observed at 50 ng/ml. Based on the high incidence of NFkB binding sites and concordance with binding data, these results validate the concept of computational identification of transcription factor binding sites in conjunction with transcript profiling data. Hence, we took a hypothesis-independent computational approach to identify other cis-elements associated with the dose-dependent IL18 response (see below). The results of such an analysis would also be useful in determining whether other pathways, in addition to NFkB activation, could be triggered by IL18 stimulation. IL18 treatment preferentially activates NFkB-p65/p50 heterodimers over p50/p50 homodimers. We used electrophoretic mobility shift assays (EMSA) to identify the forms of NFkB activated by IL18 treatment. Heterodimers have been shown to be transcriptional activators whereas p50 homodimers are believed to act most often as transcriptional repressors. As shown in Figure 1d, nuclear extracts prepared from KG1 cells treated with increasing amounts of IL18 were incubated with a 32P-labeled oligonucleotide probe capable of binding both p65/p50 heterodimers and p50 homodimers. Consistent with the results shown in Figure 1c, we detected a dose-dependent increase in a single band likely to correspond to the p65/p50 heterodimer. A very faint band corresponding to the p50 homodimer could also be detected upon longer exposure (Figure 1e). For comparison, nuclear extracts from LPS-treated mouse peritoneal exudate cells gave rise to two bands of similar intensity corresponding to homo and heterodimers. To confirm the identity NFkB species induced by IL18, we used antibodies specific for p50 and p65 to interrogate the complexes. As shown in Figure 1e, formation of both NFkB containing complexes dependent is perturbed by an antibody specific for NFkB p50 (lane 6) whereas a p65-specific antibody perturbs only the upper complex (lane 7). Both complexes fail to appear in the presence of a molar excess of an unlabeled oligonucleotide containing an NFkB p50 binding site (lane 8).

**Bioinformatics analysis reveals feedback mechanisms that limit inflammatory response to IL18**

To examine the apparent IL18 dose-dependent response more carefully we created another heatmap based on more stringent filtering criteria. We selected transcripts upregulated at least two-fold (P<0.05) at 50 ng/ml IL18 both in the absence and presence of cycloheximide. In Figure 2a, these 34 transcripts are ordered by hierarchical clustering using the Euclidean distance as a similarity measure. We observe a clear dose response since the number of transcripts upregulated ≥ two-fold increases across the 0.5–50 ng/ml IL18 concentration range (bottom of Figure 2a). Binning the transcripts induced at least two-fold by the incremental IL18 concentrations defines three distinct ECs designated by white boxes in Figure 2a. Interestingly, the apparent IL18 dose-dependent increases in gene activation parallels the dose dependent increases in NFkB binding. To confirm the presence of the ECs, we used two other clustering methods: K-means clustering and Principle Component Analysis (PCA). K-means clustering (Figure 2a, rightmost column) indicated that the transcripts fell into three clusters similar to those defined by hierarchical clustering. For PCA, the expression values represented in Figure 2a were pivoted and reduced to three principle components. In Figure 2b, plotting principle component 3 against principle component 1 for all genes clearly stratifies the genes according to EC. Thus, all three clustering methods (hierarchical, K-means and PCA) support the observation of three classes of IL18-responsive genes.

Gene ontology revealed functional trends within each EC (Table 1). EC1 contains genes that are upregulated by the lowest dose of IL18. Only one chemokine gene, CXCL2, is in this group. Instead, EC1 contains several genes whose products attenuate activation of NFkB. These include IkB-α and -ε, which both directly block NFkB entry to the nucleus; TNFAIP3, BIRC3 and IER3, which encode distinct proteins annotated as attenuating various steps in the TNF signaling pathway downstream of TRAF2-5. We confirmed IL18 induction of many transcript changes by quantitative PCR using KG1 RNA samples and RNA samples prepared from whole human blood treated with recombinant IL18 (Table 1). The results of the KG1 cell analysis suggest that the overall response to weak IL18 stimulation is to activate expression of genes whose function is to limit activation of NFkB. By contrast, the response to intermediate IL18 concentrations appears to be largely proinflammatory. Nine proinflammatory molecules are induced including two Rel family members that are activating members of NFkB signaling pathway (RELB and NFkB2). These results suggest a model whereby the difference in responses to low and intermediate IL18 concentrations indicate a feedback mechanism that could establish an IL18 signal threshold for activation of proinflammatory genes. Our results also suggest a third transcript group induced by higher concentrations of IL18 containing IL2 receptor alpha, Rel and Fas among others (Table 1).
cis-Element clusters showed similarity to EC clusters

We then investigated whether the similar IL18-response behavior of genes in the ECs could result from similar sets of cis-elements in their proximal promoters (defined as 1000 bp upstream of the transcription start site). We used an in-house program CLOVER, which can examine all cis-element matrices in TRANSFAC and determine if any are over- or under-represented in a given sequence (or in a set of regulatory regions). We performed the analysis in two ways: (1) unsupervised (regardless of our prior knowledge of expression cluster composition) clustering of all the genes based on the CLOVER-identified cis-elements in individual promoters: (2) detection of over-represented cis-elements in the promoters of all genes in each EC. We expected the first analysis to reveal whether the dose-dependent IL18 responsiveness correlated with similar promoter compositions among IL18-responsive genes, and the second analysis to indicate specifically which cis-elements distinguished the ECs.

Promoters were clustered based on log2 (raw CLOVER score) for each cis-element in TRANSFAC using Euclidean distance as the similarity measure. The results are summarized as a dendrogram in Figure 3a. The genes in Figure 2a were separated into distinct branches of the dendrogram. In order to facilitate comparison with the ECs, we cut the dendrogram into three cis-element clusters (CCs), with two neighboring branches lumped into CC1 (Figure 3a). The number of genes shared by CCs and ECs are listed in the figure, with 11 out of 12 EC1 genes in CC1 and all CC2 genes in EC2. The rightmost column on the heatmap in Figure 3b shows alignment of
the CCs and ECs. The Exact Analysis of contingency tables with sparsely populated cells showed a statistically significant agreement between the ECs and CCs ($P$-value $= 0.004$).

* cis-Elements that distinguish the ECs were identified by running CLOVER on the promoters of each EC, corresponding to genes in individual ECs. Genes in EC1 and EC2 (responding to low and intermediate concentrations of IL18) share a large number of cis-elements, including four NFκB-response elements. By contrast, NFκB elements are not apparent in the promoters of EC3 genes. Thus, we predict that the genes in EC3 respond to IL18 in an NFκB-independent manner, suggesting IL18 activation of another pathway. This is consistent with the only slight increase in NFκB detected in response to the highest concentration of IL18. Several cis-elements unique to EC3 were identified and could play a role in mediating an NFκB independent process. Unusual NFκB sites or those requiring cooperative binding interactions for functions would have escaped our detection. Several cis-elements distinguish EC1 from EC2, including E2F that may play a role in modifying the sensitivity of these genes to NFκB. Only ubiquitous TATA box and TANTIGEN are common to all three ECs (Figure 3c).

**Discussion**

Many reports have characterized the effects of IL18 and IL12 on IFNγ production from NK, T and monocytic cells. Although there is agreement that IL18 and IL12 synergize in this effect, detailed characterization of the effects of IL18 alone on other genes is lacking. Here, we present a transcript profile analysis of the effects of IL18 on the myelomonocytic cell line KG1. Our results suggest that IL18 may regulate expression of functionally distinct cohorts of genes relevant to macrophage/monocyte function. Bioinformatics analysis of the upregulated genes indicated that many of these genes harbor NFκB binding sites. This result was in part anticipated based on previous experiments demonstrating IL18-dependent activation of the NFκB pathway.1,15 We further illustrated dose-dependent activation of NFκB binding using biochemical assays.

Since we observed IL18-dependent activation in functional cohorts, rather than a single group, we classified all IL18-responsive genes according to the cis-element compositions of their promoters. The CCs we obtained agree well with clusters defined using transcript profiles at a statistically significant level. This analysis demonstrated a correlation between specific cis-elements and sensitivity to IL18 signaling. There are many difficulties in such analysis, namely, computationally identified cis-elements may not function in vivo; transcription regulation may require cis-elements outside the operationally defined 1000bp proximal promoters; incomplete lists of cis-elements in TRANSFAC; EC definition may not be perfect (despite agreement between the three clustering techniques) as some transcripts may be on the borderline between ECs. Nevertheless, our study has shown that bioinformatics techniques can be powerful in guiding functional genomics studies of novel cytokines, hormones and their receptors.
Bioinformatic analysis suggested other insights into the regulation of these genes. The three proinflammatory factors (REL, RELB, NFKB2) share some cis-elements in their promoter regions; however, the two IxB inhibitors do not have any cis-elements in common. This suggests independent regulatory pathways for the two inhibitors, providing another mechanism of avoiding spurious inflammatory reaction. Furthermore, there are some cis-elements that are over-represented in the activators and, at the same time, under-represented in inhibitors. These cis-elements might be of particular importance for distinguishing between pro- and antisignaling factors. Proinflammatory factors RELB and NFKB2 are both in EC2 while REL is in EC3. However, REL and RELB share 12 cis-elements, but RELB and NFKB2 share only five. This is intriguing because it suggests that only cis-elements shared between RELB and NFKB2, but not with REL, may confer greater sensitivity to IL18.

CLOVER analysis identified two such cis-elements, PAX5 and CREB. PAX5 is the deciding factor in B-cell commitment, while CREB together with REL are involved in T-cell activation. Involvement of these factors in addition to NFkB suggests a strengthened inflammatory response at intermediate concentrations of IL18.

Bioinformatic analysis of transcript profiles suggested a hierarchical response to IL18 and a threshold model that could prevent inappropriate activation by IL18. Low concentrations of IL18 induced little cytokine and chemokine expression yet induced expression of the genes encoding IxB and other inhibitory molecules. One interpretation of these results is that there is a mechanism, by which low-level signals are dampened or edited, preventing inappropriate activation. Consistent with this, at intermediate levels of IL18, we observed induction of cytokine gene expression and induction of Rel family members encoding activating subunits of NFkB. A model for dampening cytokine responses was recently proposed by Hoffmann et al., who described distinct NFkB responses comparing transient and prolonged stimuli. These authors observed that transient TNF stimulation resulted in degradation of IxB-α whereas IxB-β and IxB-ε were resistant. Degradation of the β and ε isoforms of IxB instead required prolonged TNF signaling. The authors also showed that duration of signal could differentially regulate the NFkB target genes IP10 and RANTES. Here, we test dose rather than duration of exposure yet our results are consistent with those of Hoffmann et al. in as much as a signal strength threshold may also be part of a mechanism preventing inappropriate activation of inflammatory responses. Failures in such a damping system could have profound consequences in the development of autoimmune diseases. Our study suggests another safeguard for regulating inflammatory response at the highest IL18 concentration, namely that the Fas gene is upregulated. This indicates the possibility of inhibiting excessive inflammatory activities via apoptosis. Indeed, Ohtsuki et al. reported IL18 upregulation of Fas ligand (FasL) and induction of apoptosis in KG-1 cells. Two other studies demonstrated that IL18 can induce apoptosis via the Fas–FasL pathway in a mouse NK cell line and in a canine cancer cell line, respectively.

Thus in our model for macrophage activation, IL18 activates three functionally distinct gene cohorts in a dose-dependent manner: EC1, inhibiting spurious inflammatory response; EC2, inflammation-committed; EC3, containing proinflammatory genes, but also potentially restraining inflammatory response through a proapoptotic mechanism. Our finding of similar trends among the promoters of the genes in each EC reinforces the integrity of the clusters. Other studies are necessary to test this model and determine whether this is a general feature of cytokine signaling or limited to IL18 and TNF family members.

Methods

Microarray experiments (MIAME check list)

Experimental design

Dose response to IL18 (see below for specifics).

Each condition performed in duplicate.

Comparisons were made between conditions using duplicate average values.

Quality control:


Microarrays: Present calls/chip ≥ 45%, Scaling factors all within 2×, RawQ < 10.

Samples, extract preparation and labeling. Approximately 3.0 × 10⁷ KG1 cells were used for each experimental condition. Cells were treated with 0, 0.5, 2.0, 10 or 50 ng/ml recombinant IL18 with or without a 30 min. pre-incubation with 10 mg/ml cycloheximide. After 2 h, cells were harvested for RNA. Total RNA was prepared using TRIZOL Reagent (Life Technologies). An initial phase separation was performed according to the manufacturer’s protocol and was followed by an additional extraction using a half volume of phenol:chloroform:i-soamyl alcohol (25:24:1, Life Technologies). RNA precipitation and wash were then performed according to the TRIZOL protocol. Approximately 3 μg of RNA were electrophoresed on a 1.0% agarose/formaldehyde denaturing gel to assess quality. For experiments requiring TNF preincubation, KG-1 cells were incubated for 12 h with 2 ng/ml TNF prior to stimulation with 2, 10 or 40 ng/ml of IL18.

Preparation of probe and target hybridization—10 μg of total RNA and the SuperScript Choice System for cDNA Synthesis (Gibco BRL) were used to synthesize double-stranded cDNA. The synthesis was carried out according to Affymetrix protocol, which requires T7(-dT)₅₀ oligomer primers (GENSET) in place of the oligo (dT) or random primers provided with the kit and which calls for incubations at 42°C during the temperature adjustment and first-strand synthesis steps. The resulting cDNA was cleaned with Phase Lock Gel Light 2 ml tubes (Eppendorf), and the pellet was resuspended in 12 μl of DEPC-H₂O. A volume of 5 μl of the cDNA was used in conjunction with the BioArray High Yield RNA Transcript Labeling Kit (Enzo) to produce biotin-labeled cRNA targets by in vitro transcription (IVT) from T7 RNA polymerase promoters. Free nucleotides were removed from the IVT reaction with RNase Mini Columns (Qiagen).

Hybridization. A measure of 15 μg of biotin-labeled cRNA was then fragmented according to Affymetrix protocol. The entire fragmented sample was combined with 5 μl control oligonucleotide B2 (Affymetrix), 15 μl
**Table 1  Genes contained within clusters 1–3**

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<th>Affymetrix</th>
<th>Blood</th>
<th>Locus link</th>
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<td>2.0</td>
<td>2.2</td>
<td>2.0</td>
<td>355</td>
</tr>
<tr>
<td>ACAD5</td>
<td>Short-chain acyl-CoA dehydrogenase</td>
<td>3.6</td>
<td></td>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>

Each table contains an annotated list of transcripts from the indicated Expression Cluster. Columns labeled *Taqman* indicate fold inductions measured using the Taqman method of quantitative PCR using KG1 mRNA samples. *Affymetrix* indicates the fold increases measured comparing the average difference values for IL18 treated and untreated KG1 RNA samples. *Blood* indicates the fold induction values for control and human whole blood treated with 50 ng/ml recombinant IL18 using Taqman. Transcripts colored red are literature annotated as proinflammatory whereas blue indicates antisignaling molecules. The proapoptotic gene Fas is indicated in green. N.D.: not done.

*RT-qPCR reaction set-up.* A measure of 250 ng of total RNA and the Taqman Gold RT-PCR Kit (Applied Biosystems) were used to complete the RT-qPCR reactions following the manufacturer’s instructions. Reactions were carried out using an ABI 7900HT sequence detection system (Applied Biosystems). The reaction parameters were optimized to include a RT step at 48 °C for 30 min, followed by an AmpliTaq Gold activation step at 95 °C for 10 min. After the enzyme activation, 50 PCR cycles were run with a denaturing step at 95 °C for 15 s and an annealing/extension step at 60 °C for 1 min.
QPCR Data Analysis—qPCR measurements were analyzed using SDS 2.0 software (Applied Biosystems) for all dye layers and adjusting cycle thresholds to include the logarithmic growth phase for all the genes run on each plate. The data were finally exported to Microsoft Excel for statistical analysis and graphing. qPCR probes were purchased from Applied Biosystems using the following catalog numbers: ATF3, Hs00231069_m1; BIRC3, Hs00154104_m1; GAPDH, 433764F; GRO3, Hs00 171061_m1; ICAM1, Hs00164932_m1; IER3, Hs00174 674_m1; IL2RA, Hs00166229_m1; LTb, Hs00242737_m1; NFKB2, Hs00174517_m1; NFKBIA, Hs00153283_m1; NFKBIB, Hs00182115_m1; NFKBIE, Hs00234431_m1; PPIA, Hs99999904_m1; REL, Hs00231279_m1; RELB, Hs00232399_m1; SCYA3, Hs00234142_m1; SCYA4, Hs00 163653_m1; TRAF1, Hs00194638_m1; CD83, Hs0018 4866_m1.

Bioinformatic analysis of cis-elements. Statistical significance threshold is used in CLOVER to determine both the regulatory elements, presence of which in the sequence is highly likely (over-represented) and under-represented cis-elements, presence of which is highly unlikely. The 1000 bp upstream regions were obtained using the UCSC Genome Browser. All variants of GenBank and refseq mRNAs plus overlapping ESTs-nonrepeats were taken into account to identify the 5’-end of the gene. An upper limit of 0.5 was used for under-represented cis-elements while lower limit of 2.0 was used for over-represented cis-elements to ensure that ‘present’ and ‘absent’ cis-elements are distinguished by sign. A dendrogram was drawn by PhyloDraw software from Graphics Application Lab using neighbor-joining algorithm.

NFkB ELISA. ELISA results were obtained using a TransAM NFkB kit (Active Motif) following the manufacturer’s instructions. Briefly, 1 μg of each nuclear extract was incubated in the presence or absence of 40 ng (100-fold molar excess) competitor oligonucleotide. Results were read using a Molecular devices Spectra Max 250 plate reader.

NFkB EMSA. A measure of 5 μg of nuclear extract was incubated with a 100 molar excess of NFkB-specific oligonucleotide (Geneka) with the sequence 5’-GCCATGGGGGGATCCCC-GAAGTCC-3’. For supershift analysis, 2 μg of either NFkB p50 (Santa Cruz) or p65 (Santa Cruz) antibody was added to the mix. Controls contained only buffer. All tubes were incubated for 30 min at RT. 32P-ATP-labeled NFkB oligo probe (Geneka) and the reaction proceeded for an additional 30 min. Samples were separated on a 6% DNA retardation gel (Novex), fixed and dried and visualized by autoradiography.

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Supplementary Information accompanies the paper on Genes and Immunity’s website (http://www.nature.com/