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Function of C/EBPδ in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals

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The innate immune system is like a double-edged sword: it is absolutely required for host defense against infection, but when uncontrolled, it can trigger a plethora of inflammatory diseases. Here we use systems-biology approaches to predict and confirm the existence of a gene-regulatory network involving dynamic interaction among the transcription factors NF-κB, C/EBPδ and ATF3 that controls inflammatory responses. We mathematically modeled transcriptional regulation of the genes encoding interleukin 6 and C/EBPδ and experimentally confirmed the prediction that the combination of an initiator (NF-κB), an amplifier (C/EBPδ) and an attenuator (ATF3) forms a regulatory circuit that discriminates between transient and persistent Toll-like receptor 4–induced signals. Our results suggest a mechanism that enables the innate immune system to detect the duration of infection and to respond appropriately.

The innate immune system must provide stable, specific and protective responses in a diverse pathogenic environment while at the same time attenuating the collateral damage inflicted by the inflammation associated with such responses[1–8]. Much has been learned about the recognition mechanisms that facilitate the specificity of innate immune responses. In general, pattern-recognition receptors such as the Toll-like receptors (TLRs) recognize microbial components[9–11] and activate intracellular signaling pathways that lead to the transcriptional induction of genes critical for protective inflammatory responses[12,13].

Bacterial lipopolysaccharide (LPS) is a principal surface component of Gram-negative bacteria and is detected by TLR4 (A002296)[14]. LPS stimulation leads to macrophage activation characterized by changes in extracellular and intracellular microbe-killing systems, the production and secretion of proinflammatory cytokines and chemokines, enhanced expression of costimulatory receptors essential for efficient T cell activation and enhanced production of arachidonic acid metabolites[15,16]. These and other inflammatory responses in macrophages are driven mainly at the level of transcription[17,18]. However, the gene-regulatory program of TLR-induced activation of macrophages is not well understood. It is known that macrophages express more than 500 transcription factors[19], of which approximately 100 are induced by LPS; this suggests a high degree of complexity in the regulation of TLR4-induced responses.

In this report we use the tools of systems biology[20–24] to identify a transcriptional circuit leading to the TLR4-activated state in macrophages. We analyzed temporal activation of macrophages by LPS by microarray and then clustered these data to show regulated 'waves' of transcription. It is well established that genes that are regulated together often share *cis*-regulatory elements and that transcriptional programs are propagated by sequential cascades of transcription factors[25,26]. We therefore identified transcription factors in the first cluster of expressed genes (cluster 1) and used computational motif scanning to predict which genes in cluster 2 contained in their promoters binding sites for cluster 1 transcription factors. We then confirmed those predictions by chromatin immunoprecipitation (ChIP), a method that also allowed us to establish the kinetics of promoter occupancy. These kinetic data allowed mathematical modeling of the transcriptional circuitry, which in turn allowed the prediction of previously unknown functions not easily identified by conventional approaches. We then tested the functional predictions in cell culture systems and in mice.

We used this strategy to identify a previously unknown regulatory circuit involving the transcription factors NF-κB (A002052), ATF3 (A003217) and C/EBPδ. We predicted and confirmed that C/EBPδ acts as an amplifier of NF-κB responses and that it discriminates between transient and persistent TLR4 signals. By combining ChIP with microarray technology ('ChIP-on-chip' analysis), we identified 63 LPS-induced C/EBPδ targets, many of which are linked to host defenses against bacterial infection. Integration of the kinetic and functional data suggested a mechanism by which C/EBPδ participates in the control of persistent bacterial infection.

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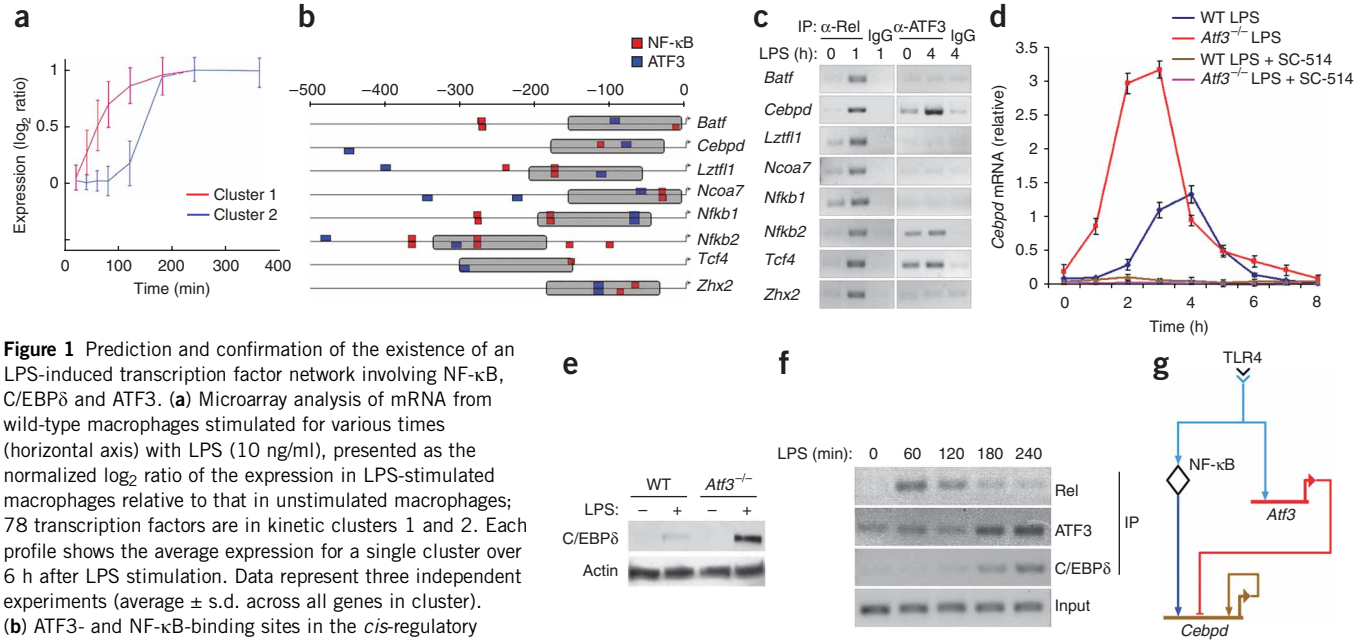

Figure 1 Prediction and confirmation of the existence of an LPS-induced transcription factor network involving NF-κB, C/EBPδ and ATF3. (**a**) Microarray analysis of mRNA from wild-type macrophages stimulated for various times (horizontal axis) with LPS (10 ng/ml), presented as the normalized $\log_2$ ratio of the expression in LPS-stimulated macrophages relative to that in unstimulated macrophages; 78 transcription factors are in kinetic clusters 1 and 2. Each profile shows the average expression for a single cluster over 6 h after LPS stimulation. Data represent three independent experiments (average ± s.d. across all genes in cluster). (**b**) ATF3- and NF-κB-binding sites in the *cis*-regulatory regions of genes encoding transcription factors (in cluster 2). Predicted targets were filtered with the additional constraint of a 150–base pair proximity limit (gray shading) between putative ATF3- and NF-κB-binding sites. (**c**) ChIP analysis of nuclear Rel and ATF3 immunoprecipitated from wild-type macrophages stimulated for various times (above lanes) with LPS (10 ng/ml); genes (left margin) were amplified by PCR from transcription factor–bound DNA. IP, immunoprecipitation; α-, anti-; IgG, negative control. Results are representative of two independent experiments. (**d**) Expression of *Cebpd* mRNA by wild-type (WT) and *Atf3*$^{-/-}$ macrophages stimulated for various times (horizontal axis) with LPS (10 ng/ml) in the presence or absence of the NF-κB inhibitor sc-514 (25 μM). Data are representative of three experiments (average of three values ± standard error). (**e**) Immunoblot analysis of lysates of wild-type and *Atf3*$^{-/-}$ macrophages left unstimulated (–) or stimulated for 4 h with LPS (10 ng/ml; +). Actin, loading control. Results are representative of three independent experiments. (**f**) ChIP and PCR analysis (as described in **c**) of the recruitment of Rel, C/EBPδ and ATF3 to the *Cebpd* promoter in wild-type macrophages stimulated for various times (above lanes) with LPS (10 ng/ml). Input, DNA without immunoprecipitation. Data are representative of three independent experiments. (**g**) Transcription factor network model, presented as a BioTapestry diagram[37]. Arrow colors indicate the controlling molecule: light blue, TLR4; dark blue, NF-κB; red, *Atf3*; brown, *Cebpd*.

RESULTS

NF-κB and ATF3 control C/EBPδ expression

We designed a strategy to identify regulatory circuits in LPS-stimulated macrophages (**Supplementary Fig. 1** online). Transcriptome analysis showed that LPS induced the expression of two temporal clusters of transcription factors within 3 h: the early cluster (cluster 1) was composed of 23 transcription factors, and the intermediate cluster (cluster 2) contained 55 transcription factors (**Fig. 1a** and **Supplementary Table 1** online). We focused on the transcriptional circuitry involving two transcription factors in cluster 1, NF-κB (Rel) and ATF3, as it has been shown that Rel activates and ATF3 attenuates a subset of LPS-induced genes[27]. We therefore scanned the promoters of the genes encoding the 55 transcription factors in cluster 2 for ATF3- and NF-κB-binding sites and identified 8 genes whose promoters contained candidate binding sites for both ATF3 and NF-κB within 1,500 base pairs of the transcriptional start site and within 150 base pairs of each other (**Supplementary Table 2** online). This subset included *Batf*, *Cebpd*, *Lztfl1*, *Ncoa7*, *Nfkb1*, *Nfkb2*, *Tcf4* and *Zhx2* (**Fig. 1b**). ChIP analysis demonstrated that LPS induced the binding of NF-κB (at 1 h) and ATF3 (at 4 h) to the promoters of *Cebpd*, *Nfkb2* and *Tcf4* (**Fig. 1c**). We focused on *Cebpd* (which encodes C/EBPδ) in our subsequent experiments, as LPS stimulated the binding of NF-κB and ATF3 to the *Cebpd* promoter (**Fig. 1c**). Transcription of *Cebpd* was induced by LPS (**Fig. 1d**). Pharmacological inhibition of NF-κB blocked LPS-induced *Cebpd* transcription (**Fig. 1d**), and there was much more LPS-induced *Cebpd* mRNA (**Fig. 1d**) and C/EBPδ protein (**Fig. 1e**) in *Atf3*-null macrophages. These data collectively demonstrate that LPS-induced transcription of *Cebpd* is absolutely dependent on NF-κB and that NF-κB-dependent production of *Cebpd* mRNA is attenuated by ATF3. ChIP analysis demonstrated rapid and transient recruitment of Rel to the *Cebpd* promoter; maximum binding occurred 1 h after stimulation with LPS (**Fig. 1f**). In contrast, LPS-stimulated binding of ATF3 to the *Cebpd* promoter (over basal amounts) occurred after 3 h and was sustained (**Fig. 1f**). Notably, motif scanning of the 5′ *cis*-regulatory region of *Cebpd* predicted that this transcription factor can bind to its own promoter (**Supplementary Table 3** online); we confirmed this prediction by ChIP (**Fig. 1f**). The kinetics of the binding of C/EBPδ to its own promoter paralleled those of the binding of ATF3.

The findings reported above suggested a model in which TLR4 activates NF-κB, which then binds to the promoter of *Cebpd* and activates it (**Fig. 1g**). TLR4 also activated *Atf3* transcription[27] (data not shown) and later stimulated the recruitment of ATF3 to the *Cebpd* promoter. The binding of ATF3 to the *Cebpd* promoter inhibited its NF-κB-dependent transcription. C/EBPδ was also recruited to its own promoter in a TLR4-dependent way, which suggested autoregulation.

Regulatory circuit involving NF-κB, C/EBPδ and ATF3

Motif-scanning analysis predicted the presence of binding sites for NF-κB, C/EBPδ and ATF3 in the *cis*-regulatory regions of 146 LPS-induced genes (**Supplementary Table 4** online). Many of these genes have well-established functions in regulating the immune response; we selected *Il6* (which encodes interleukin 6 (IL-6)) for further study because its regulation by NF-κB and ATF3 has been explored[27]. Motif scanning predicted the existence of NF-κB-, ATF3- and

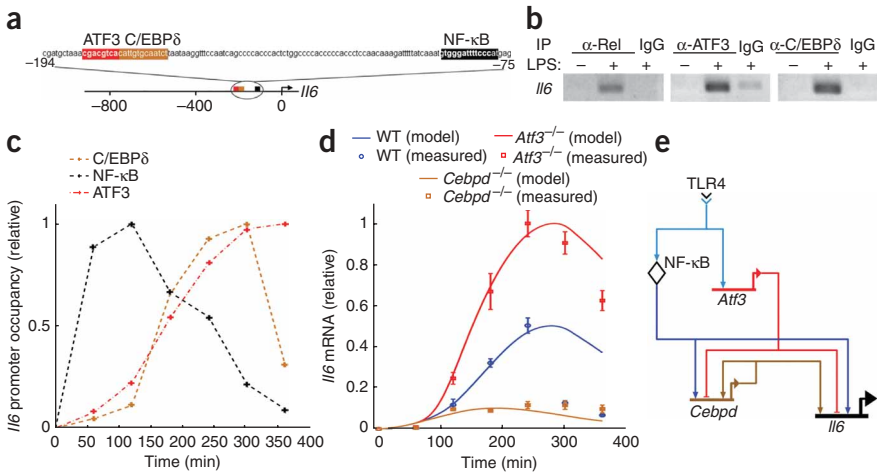

Figure 2 Mathematical model characterizing the transcriptional regulation of *Il6* in TLR4-stimulated macrophages. (**a**) NF-κB-, ATF3- and C/EBPδ-binding sites in the *Il6* promoter relative to the transcription start site, as predicted by motif scanning. (**b**) ChIP and PCR analysis (as described in **Fig. 1c**) of the binding of NF-κB (Rel), ATF3 and C/EBPδ to the *Il6* promoter in wild-type macrophages stimulated for 6 h with LPS (10 ng/ml). Results are representative of three independent experiments. (**c**) ChIP analysis (as described in **Fig. 1c**) of wild-type macrophages stimulated for various times (horizontal axis) with LPS (10 ng/ml), followed by quantitative real-time RT-PCR analysis of the binding of immunoprecipitated NF-κB (Rel), ATF3 and C/EBPδ to the *Il6* promoter, normalized to the amount of PCR product loaded. Data represent three independent experiments (average). (**d**) Predicted *Il6* expression in wild-type, *Cebpd*$^{-/-}$ and *Atf3*$^{-/-}$ examples of the kinetic model (lines) and measured *Il6* mRNA in wild-type, *Cebpd*$^{-/-}$ and *Atf3*$^{-/-}$ macrophages (symbols). Data represent three experiments (average of three values ± standard error). (**e**) Extended transcriptional network model, presented as a BioTapestry diagram[37]. Arrow colors indicate the controlling molecule: light blue, TLR4; dark blue, NF- κB; red, *Atf3*; brown, *Cebpd*.

C/EBPδ-binding sites in the *cis*-regulatory region of *Il6* (**Fig. 2a**), and we confirmed this prediction by ChIP (**Fig. 2b**). Notably, the binding of NF-κB, ATF3 and C/EBPδ to the *Il6* promoter occurred in an LPS-dependent way (**Fig. 2b**). The Rel subunit of NF-κB was transiently recruited to the *Il6* promoter with maximum binding at 2 h after LPS stimulation (**Fig. 2c**), as was true for RelA (data not shown). ATF3 and C/EBPδ demonstrated slower kinetics of recruitment with maximum binding at 4–5 h after LPS stimulation (**Fig. 2c**).

To explore relative function of each transcription factor in LPS-induced production of IL-6, we examined *Cepbd*$^{-/-}$ and *Atf3*$^{-/-}$ macrophages (**Fig. 2d**), as well as macrophages treated with NF-κB inhibitors (**Supplementary Fig. 2** online). LPS-induced production of *Il6* mRNA was much higher in *Atf3*$^{-/-}$ macrophages and much lower in *Cebpd*$^{-/-}$ cells than in wild-type macrophages (**Fig. 2d**). Notably, LPS-induced activation of NF-κB and expression of ATF3 were unaltered in *Cebpd*$^{-/-}$ macrophages (data not shown). These data collectively suggest a model in which *Il6* expression is controlled by superposition of three network motifs: first, positive autoregulation, mediated by the binding of C/EBPδ to its own promoter; second, feed-forward transcriptional activation of *Il6*, mediated by NF-κB and C/EBPδ; third, feed-forward transcriptional inhibition mediated by the binding of ATF3 to the *Cebpd* and *Il6* promoters (**Fig. 2e**).

We developed a mathematical model of this regulatory network (kinetic modeling, **Supplementary Methods** online). We assumed that the rate of transcriptional initiation of *Il6* depends on the fractional occupancy of its promoter by NF-κB, ATF3 and CEBPδ, that NF-κB acts as an activator of the transcription of *Cebpd* (**Fig. 1d**) and *Il6* (**Supplementary Fig. 2**), that ATF3 attenuates the transcription of *Cebpd* (**Fig. 1d**) and *Il6* (**Fig. 2d**) and that C/EBPδ acts together only with NF-κB (**Supplementary Fig. 3** online). We fit our model to the measurements of LPS-induced *Il6* expression in wild-type, *Atf3*$^{-/-}$ and *Cebpd*$^{-/-}$ macrophages reported above (**Fig. 2d**). We determined seven parameters of the kinetic model by minimizing the prediction error for recapitulating the time-course data for gene and

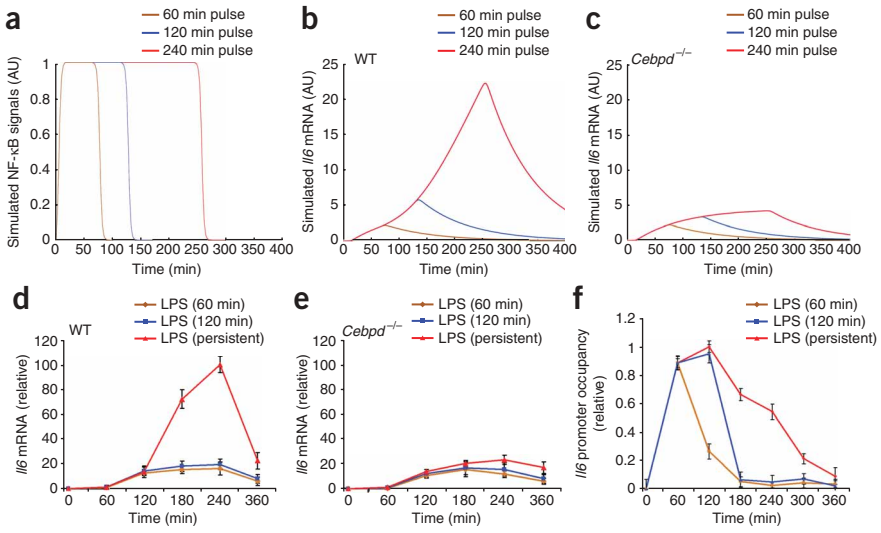

Figure 3 Computational simulations of the transcriptional response of *Il6* to TLR4 signals of varying duration identify a threshold effect. (**a**) Computational simulation of transient and persistent NF-κB signals; for simulation of LPS pulsing (key), the NF-κB signal was computationally varied over time. NF-κB signals of the same amplitude but different duration are presented in arbitrary units (AU). (**b**,**c**) Outputs of computationally simulated *Il6* transcriptional response to transient LPS signals (Pulse; key) in wild-type macrophages (**b**) and *Cebpd*$^{-/-}$ macrophages (**c**). (**d**,**e**) Quantitative real-time RT-PCR analysis of *Il6* mRNA in wild-type macrophages (**d**) and *Cebpd*$^{-/-}$ macrophages (**e**) stimulated for 1 or 2 h or persistently with LPS (10 ng/ml) and analyzed at various times (horizontal axis), presented relative to *Eef1a1* expression. Data are representative of three experiments (average of three values ± standard error). (**f**) ChIP analysis (as described in **Fig. 1c**) of wild-type macrophages stimulated for 1 h or 2 h or persistently with LPS (10 ng/ml), followed by quantitative real-time RT-PCR analysis of immunoprecipitated Rel on the *Il6* promoter, normalized to the amount of PCR product loaded. Data are representative of two experiments (average of three values ± standard error).

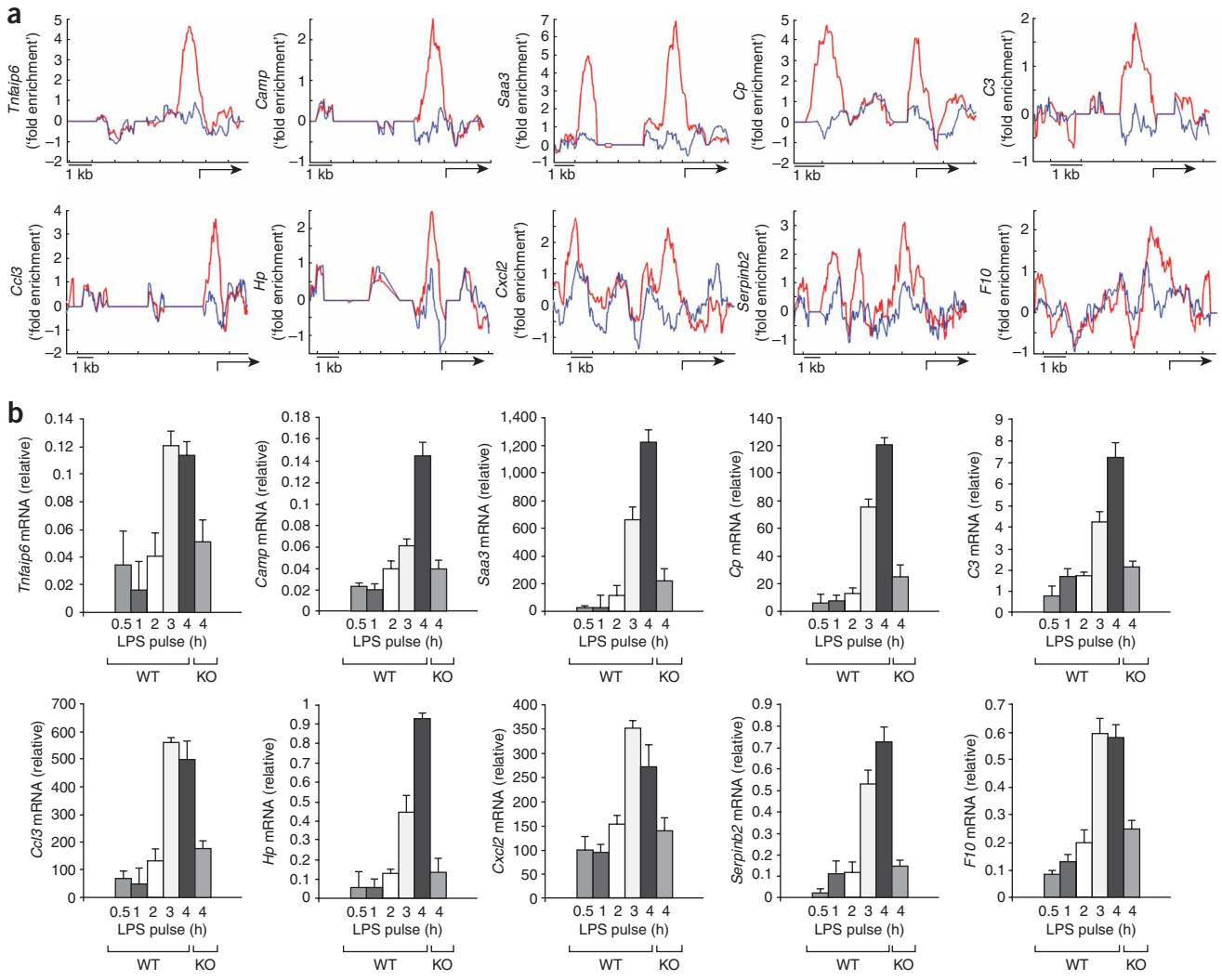

Figure 4 Identification of direct targets of C/EBPδ. (**a**) Whole-genome (ChIP-on-chip) analysis of the binding of immunoprecipitated C/EBPδ to the promoters of target genes (vertical axes) in wild-type macrophages left unstimulated (blue lines) or stimulated for 6 h with LPS (10 ng/ml; red lines), presented as the normalized value relative to that of the negative control (normal rabbit serum). Bent arrows indicate transcription start sites. Data are representative of two experiments (average of two values). (**b**) Quantitative real-time RT-PCR analysis of mRNA transcripts (vertical axes) in wild-type (WT) and *Cebpd*$^{-/-}$ (KO) macrophages stimulated for various times (horizontal axes) with LPS (1 ng/ml) and analyzed 4 h later, presented relative to *Eef1a1* expression. Data are representative of three experiments (average of three values and standard error).

protein expression in LPS-stimulated macrophages of the three genotypes (wild-type, *Atf3*$^{-/-}$ and *Cebpd*$^{-/-}$). The parametric complexity of the model, with a ratio of 1.17 fit parameters per dynamic variable and a ratio of approximately six measured data points per fit parameter, was comparable to that of published kinetic models[28,29]. Consistent with a model that is not over-fitted, the predicted *Il6* transcriptional response was robust in terms of simultaneous variation of the seven parameters (**Supplementary Fig. 4** online).

According to our model (**Fig. 2e**), TLR4 stimulates translocation of NF-κB to the nucleus, where it activates a small amount of *Il6* transcription. Concomitant with that, NF-κB induces expression of C/EBPδ, which then binds to the *Il6* promoter and acts together with NF-κB to stimulate maximum transcription of the cytokine-encoding gene; this is known as 'coherent feed-forward type I regulation'[30]. Additional features of the model include autoregulation of *Cebpd* (positive feedback) and ATF3-mediated attenuation of the transcription of *Cebpd* and *Il6*.

C/EBPδ discriminates transient from persistent TLR4 signals

Coherent feed-forward type I regulation protects biological systems from unwanted responses to fluctuating inputs[30]. Given the 'double-edged sword' nature of inflammation, it is critical that the macrophage be able to recognize a persistent versus a transient insult; this would enable the cell to discriminate between real and spurious threats. For this reason, we hypothesized that the 'architecture' of the regulatory circuit involving NF-κB, ATF3 and C/EBPδ might have evolved to serve the function of discriminating between transient and persistent innate immune stimuli. To test our hypothesis, we simulated *Il6* transcriptional activation after LPS pulsing by computationally varying the NF-κB activation signal (**Fig. 3a** and **Supplementary Methods**). Short-duration pulses were predicted by the model to induce only weak production of *Il6* mRNA, whereas persistent stimulation was predicted to 'super-induce' *Il6* transcription (**Fig. 3b**). Furthermore, the 'super-induction' of *Il6* induced by persistent stimulation was predicted to be absent from *Cebpd*$^{-/-}$

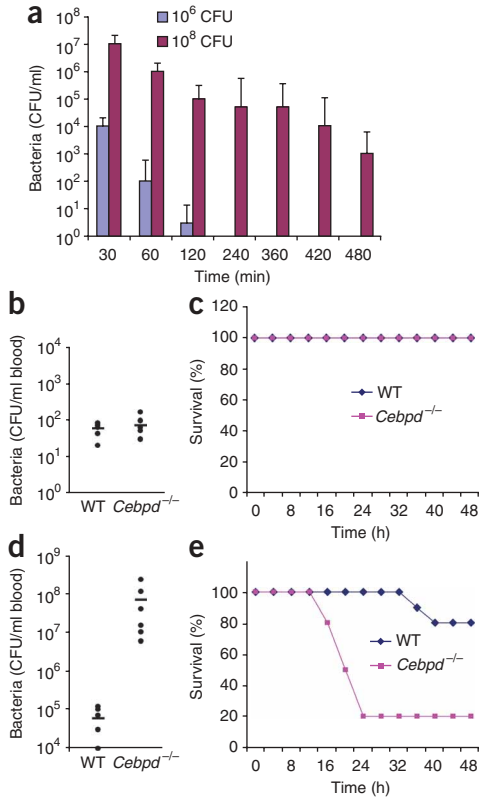

Figure 5 The function of C/EBPδ in the restriction of transient and persistent bacterial infections. (**a**) Peritoneal bacteria in wild-type mice challenged intraperitoneally with a low dose (1×10^6 CFU) or high dose (1×10^8 CFU) of *E. coli* H9049. Data are representative of three experiments (average and standard error; $n = 6$ mice per data point). (**b**) Bacterial burden in the blood 18 h after intraperitoneal infection of wild-type and *Cebpd*$^{-/-}$ mice with 1×10^6 CFU *E. coli* H9049. Dots represent individual CFU values; small horizontal lines indicate the average. Data are from one experiment of three ($n = 6$ mice per group). (**c**) Survival of wild-type and *Cebpd*$^{-/-}$ mice infected intraperitoneally with 1×10^6 CFU *E. coli* H9049 ($n = 10$ mice per group). Data are representative of three independent experiments. (**d**) Bacterial burden in the blood 18 h after intraperitoneal infection of wild-type and *Cebpd*$^{-/-}$ mice with 1×10^8 CFU *E. coli* H9049, presented as described in **b**. Data are from one experiment of three ($n = 6$ mice per group). (**e**) Survival of wild-type and *Cebpd*$^{-/-}$ mice after intraperitoneal challenge with 5×10^8 CFU *E. coli* H9049 ($n = 10$ mice per group). Data are representative of three independent experiments.

macrophages (**Fig. 3c**). Measurements of *Il6* expression in wild-type and *Cebpd*$^{-/-}$ macrophages were in qualitative agreement with those model predictions (**Fig. 3d,e**). Notably, the measured dynamics of the binding of Rel to the *Il6* promoter (**Fig. 3f**) were in qualitative agreement with computationally simulated NF-κB inputs (**Fig. 3a**). We reconfirmed our model predictions using measured Rel binding (**Fig. 3f**) as an input for computational simulation of the production of *Il6* mRNA by wild-type and *Cebpd*$^{-/-}$ macrophages (**Supplementary Fig. 5** online). These results collectively suggest that the overall function of the feed-forward motifs involving NF-κB, C/EBPδ and ATF3 is to detect and to respond to persistent signals while filtering out brief inputs. It is also possible that C/EBPδ mediates a mechanism to sense the dose rather than the duration of the response. This possibility is less likely, as the LPS concentration required for half-maximum induction of *Il6* transcription (with continuous stimulation) was similar in wild-type and *Cebpd*$^{-/-}$ macrophages (about 0.4 ng/ml).

Given the importance of the NF-κB–C/EBPδ–ATF3 circuit, it is likely that in addition to *Il6*, other genes of the innate immune system are regulated in this way. To begin to define this set of genes, we did whole-genome location analysis and found that activation by TLR4 stimulated recruitment of C/EBPδ to the promoters of 63 LPS-induced genes at 6 h after LPS stimulation, including *Serpinb2*, *Cp*, *Saa3*, *Hp*, *Camp*, *C3*, *Tnfaip6*, *Ccl3*, *Cxcl2* and *F10* (**Fig. 4a** and **Supplementary Table 5** online). Transcription of genes in response to persistent LPS stimulation was considerably blunted in *Cebpd*$^{-/-}$ macrophages (**Fig. 4b**), which confirmed the idea that C/EBPδ regulates these genes. However, transcription induced in response to TLR4 stimulation of a short duration was similar in wild-type and *Cebpd*$^{-/-}$ macrophages (data not shown). Overall, these results suggest that, as with *Il6*, C/EBPδ discriminated between transient and persistent signals leading to the activation of these genes. Many of the C/EBPδ-regulated genes are known to be associated with host defense against infection (**Supplementary Table 5**).

We therefore tested whether C/EBPδ could discriminate between transient and persistent infection *in vivo*. We established a model of Gram-negative bacterial peritoneal infection in mice in which a low dose of *Escherichia coli* strain H9049 (ref. 31; 1×10^6 colony-forming units (CFU)) was cleared rapidly, whereas a high dose of bacteria (1×10^8 CFU) resulted in a persistent infection (**Fig. 5a**). Next we compared the ability of wild-type and *Cebpd*$^{-/-}$ mice to clear low and high doses of *E. coli* by assessing the bacterial burden in the blood at 18 h after infection. Wild-type and *Cebpd*$^{-/-}$ mice cleared the low-dose bacterial infection with similar efficiency (**Fig. 5b,c**). However, infection of *Cebpd*$^{-/-}$ mice with a high dose of bacteria resulted in severe bacteremia; *Cebpd*$^{-/-}$ mice had a bacterial load in the blood 1,000-fold higher than that of wild-type mice (**Fig. 5d**). In addition, whereas neither wild-type nor *Cebpd*$^{-/-}$ mice succumbed after infection with a low dose (**Fig. 5c**), 80% of *Cebpd*$^{-/-}$ mice but no wild-type mice succumbed within 24 h of infection with a high dose of bacteria (**Fig. 5e**).

DISCUSSION

It is becoming increasingly apparent that the tools of systems biology are invaluable in deciphering the complex immune system and in predicting new drug targets[20–24]. Stimulation by TLR4 results in the induction of a complex gene-regulatory network that 'programs' macrophage activation, resulting in an effective host response to pathogens[12,13,15,16]. We have shown here that the TLR4 agonist LPS regulates the transcription of approximately 2,000 genes within 24 h in macrophages[32]. It is well established that transcriptional programs are propagated by sequential cascades of transcription factors[25,26]. We have shown here that stimulation of macrophages with LPS induced the transcription of two clusters of transcription factors within 3 h; the first cluster included 23 transcription factors and the second cluster included 55 transcription factors. Next we used a combination of mathematical and biological experiments to predict and confirm the existence of a transcriptional network involved in TLR4 activation. The power of the approach lies in its ability to rapidly identify complex interactions between transcription factors and to define the functional emergent properties of the system, which in turn suggest the molecular underpinnings of the biological response. Analysis of the transcription factors in clusters 1 and 2 predicted the existence of many networks involved in the TLR4 response.

We focused on an NF-κB (Rel)–ATF3–C/EBPδ subnetwork; each of these transcription factors has been shown before to participate in host defense[27,33,34], but their interaction and the consequences of this interaction in the innate immune response have not been described

before. High-density temporal measurements of LPS-induced binding of these transcription factors to the *Il6* promoter, combined with gene-deletion studies, enabled us to construct a model of a regulatory circuit that participates in the transcription of this cytokine-encoding gene. In this model, TLR4 stimulates translocation of NF-κB to the nucleus, where it activates weak transcription of *Il6*. Concomitant with that, NF-κB induces C/EBPδ, which then binds to the *Il6* promoter and acts together with NF-κB to stimulate maximum transcription of *Il6*. At a later time point, ATF3 attenuates transcription of *Cebpd* and *Il6*. ATF3 recruits histone deacetylase 1 to the *Il6* promoter in an LPS-dependent way. The ATF3-associated histone deacetylase 1 then deacetylates histones, resulting in the closure of chromatin and inhibition of *Il6* transcription[27]. It is known that C/EBPδ binds to and recruits the histone acetylase CBP to its target promoters, leading to more histone acetylation and chromatin opening[35]. It is therefore possible that the NF-κB (Rel)–ATF3–C/EBPδ regulatory network is regulated by epigenetic chromatin remodeling.

The relationship between NF-κB and C/EBPδ suggests coherent feed-forward type I regulation[30]. This type of regulation has been suggested to protect biological systems from unwanted responses to fluctuating inputs[30]. The inflammatory response is like a double-edged sword, and it is therefore critical that inflammatory cells be able to discriminate between real and perceived threats. The coherent feed-forward type I regulatory circuit described above could in principle enable immune cells to 'filter' transient insults from more dangerous persistent attacks. Exploration of this idea required computational simulation of the system; therefore, we used time-delay differential equations to simulate pulses of NF-κB activation and to examine transcriptional responses *in silico*. These simulations demonstrated a threshold effect in the transcriptional regulation of *Il6* and a critical function for C/EBPδ in a regulatory circuit that discriminates between transient and persistent TLR4 stimulation. We confirmed our predictions in LPS-stimulated macrophages and an *in vivo* model of bacterial infection.

We used a combination of motif-scanning, microarray and ChIP-on-chip analysis to identify many LPS-induced targets of C/EBPδ. These genes showed differences in transcriptional responsiveness to persistent and transient LPS-dependent stimulation of macrophages *in vitro*, and many have ascribed functions in host defenses against bacterial infection. Consistent with our *in vitro* studies, *Cebpd*-null mice were able to resist transient infection with a low dose of *E. coli* H9049 but were highly susceptible to persistent infection with a higher dose. In summary, we have used the tools of systems biology to show that TLR4-induced inflammatory responses are regulated by the integration of transcriptional 'on' and 'off' switches with 'amplifiers' and 'attenuators'. In addition, we have demonstrated a mechanism by which the macrophages are able to discriminate between real and perceived threats. Collectively, these regulatory elements may facilitate the maintenance of effective host defense and the prevention of inflammatory disease.

METHODS

Mouse bone marrow–derived macrophages. Bone marrow–derived macrophages were isolated from C57BL/6, *Atf3*$^{-/-}$ and *Cebpd*$^{-/-}$ mice essentially as described[27]. Bone marrow cells collected from femurs were plated on non–tissue culture–treated plastic in complete RPMI medium containing 10% (vol/vol) FBS (Hyclone Laboratories), 2 mM L-glutamine, 100 IU/ml of penicillin and 100 μg/ml of streptomycin (all from Cellgro, Mediatech) and supplemented with recombinant human macrophage colony-stimulating factor (50 ng/ml; Chiron). Bone marrow–derived macrophages were stimulated for various times with high-purity LPS (10 ng/ml; *Salmonella minnesota*; List Biologicals). LPS-induced activation of NF-κB was inhibited with 25 μM sc-514 (Calbiochem).

Microarray analysis. Total RNA was isolated with TRIzol reagent (Invitrogen) and overall quality was analyzed with an Agilent 2100 Bioanalyzer. Sample mRNA was amplified and labeled with the Affymetrix One-Cycle Eukaryotic Target Labeling Assay protocol and reagents. Biotinylated cRNA was hybridized to an Affymetrix GeneChip Mouse Genome 430 2.0 array with standard protocols and reagents from Affymetrix. Probe intensities were measured with the Affymetrix GeneChip Scanner 3000 and were processed into image analysis (.CEL) files with Affymetrix GeneChip operating software. Probe intensities were adjusted to background, normalized and summarized by probeset by the robust multi-chip average method with the software Bioconductor, then were exported to the MATLAB software framework for numerical computation (MathWorks) for further analysis.

Quantitative real-time PCR. For measurement of the expression of mRNA transcripts in macrophages, total RNA was isolated with Trizol reagent (Invitrogen), was reverse-transcribed and was analyzed by real-time PCR with TaqMan Gene Expression assays (Applied Biosystems; comprehensive list, **Supplementary Methods**). Data were acquired on a 7900HT Fast Real-Time PCR system (Applied Biosystems) and were normalized to the expression of *Eef1a1* mRNA transcripts (encoding eukaryotic translation elongation factor 1 α1) in individual samples.

ChIP assay and immunoblot analysis. For ChIP binding analysis, formalin-fixed cells were sonicated and processed for immunoprecipitation with antibody to Rel (anti-Rel; C), anti-ATF3 (C-19) and anti-C/EBPδ (M-17; all from Santa Cruz) essentially as described[27]. Immunoprecipitated DNA samples were amplified with target promoter–specific primers (**Supplementary Methods**). For immunoblot analysis, macrophages were lysed and processed as described[27].

ChIP-on-chip analysis. For these analyses, formalin-fixed cells were sonicated and processed for immunoprecipitation with polyclonal antibodies specific for C/EBPδ essentially as described[27]. Immunoprecipitated DNA samples were amplified and labeled according to the Affymetrix Chromatin Immunoprecipitation Assay protocol and were hybridized to a GeneChip Mouse Promoter 1.0R array. The ChIP-on-chip data were analyzed by model-based analysis of tiling arrays[36].

Additional methods. Information on motif scanning, gene-expression profiling, mice, analysis of the robustness of model prediction and kinetic modeling is available in the **Supplementary Methods**.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A002296, A002052 and A003217; GEO: microarray data, GSE14769 and GSE14812.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

V.L. designed experiments, did all experimental studies and drafted the manuscript; S.A.R. did data analysis and mathematical modeling; A.G.R., D.E.Z. and M.N. did computational analysis; K.A.K. did microarray experiments; A.E.L. provided technical assistance for experiments, including quantitative real-time PCR, ChIP and *in vivo* studies; I.S. supervised the computational analysis; and A.A. supervised the study and wrote the manuscript.

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