Macrophages, tissue-resident phagocytic cells of the innate immune system, are critical sentinels in the detection and containment of infectious microbes and the initiation of inflammatory type I immune responses. In addition to these functions, collectively referred to as classical activation, macrophages may also undergo alternative activation, resulting in distinct noninflammatory programs that are important in type II immune responses, wound healing, and tissue homeostasis. Alternatively activated macrophages, tissue-resident phagocytic cells of the innate immune system, are critical sentinels in the detection and containment of infectious microbes and the initiation of inflammatory type I immune responses. In addition to these functions, collectively referred to as classical activation, macrophages may also undergo alternative activation, resulting in distinct noninflammatory programs that are important in type II immune responses, wound healing, and tissue homeostasis. Given the central role of macrophages in diverse immune functions, it is important to develop a more systematic understanding of the transcriptional networks that govern their activation and polarization.

One recently developed tool that may yield great insight into mechanisms of macrophage activation is regulatory network analysis, a statistical method for identifying components of a dataset that covary across a broad range of samples or conditions. Furthermore, this study provides experimental validation for our network modeling approach, which will facilitate the future use of gene expression data from open databases to reveal novel, physiologically relevant regulatory relationships. The Journal of Immunology, 2015, 194: 000–000.

Macrophages are crucial for restriction of microbial infection but may also promote inflammatory pathology in a wide range of both infectious and sterile conditions. The pathways that regulate macrophage activation are therefore of great interest. Recent studies in silico have putatively identified key transcription factors that may control macrophage activation, but experimental validation is lacking. In this study, we generated a macrophage regulatory network from publicly available microarray data, employing steps to enrich for physiologically relevant interactions. Our analysis predicted a novel relationship between the AP-1 family transcription factor Junb and the gene Il1b, encoding the pyrogen IL-1β, which macrophages express upon activation by inflammatory stimuli. Previously, Junb has been characterized primarily as a negative regulator of the cell cycle, whereas AP-1 activity in myeloid inflammatory responses has largely been attributed to c-Jun. We confirmed experimentally that Junb is required for full expression of Il1b, and of additional genes involved in classical inflammation, in macrophages treated with LPS and other immunostimulatory molecules. Furthermore, Junb modulates expression of canonical markers of alternative activation in macrophages treated with IL-4. Our results demonstrate that Junb is a significant modulator of both classical and alternative macrophage activation. Further, this finding provides experimental validation for our network modeling approach, which will facilitate the future use of gene expression data from open databases to reveal novel, physiologically relevant regulatory relationships. The Journal of Immunology, 2015, 194: 000–000.

A wealth of macrophage transcriptional data is available in public databases, but such data are generally considered unsuitable for network analysis due to the confounding effects of technical variation resulting from the use of diverse nucleic acid amplification procedures and expression profiling platforms. In this study, we present the results of a regulatory network analysis approach that is based on mutual information and data processing inequality procedures applied to strictly standardized and normalized public datasets. We further improved the power of this approach to identify physiological relationships by using existing literature to strengthen predictions in a series of steps that we term “knowledge-based enrichment.”

Our network model led us to examine the AP-1 transcription factor Junb for its role in myeloid immune activation. Although Junb has historically been studied primarily in the contexts of cell cycle regulation and differentiation, several recent bioinformatic studies, like the one presented in this study, have predicted a role for Junb in the regulation of myeloid immune responses. However, there is currently little experimental evidence to support this prediction. To directly test the importance of Junb in macrophage activation, we characterized the transcriptional responses of Junb-deficient macrophages to diverse stimuli. Confirming our network prediction, we found that Junb modulates subsets of immune-related genes in macrophages treated with microbial ligands referred to as classically activated, or M(LPS), macrophages as well as with the cytokine IL-4, which stimulates polarization of alternatively activated M(IL-4) macrophages (10). To our knowledge, this is one of the first reports of a transcription factor that promotes polarization of both M(LPS) and M(IL-4) macrophages. Furthermore, this study provides experimental validation for several recent predictions made in silico, demonstrating the power of network analysis to lead to new insights into immune regulation.

**Materials and Methods**

*Gene Expression Omnibus data preprocessing*

All mouse macrophage microarray datasets warehoused in the Gene Expression Omnibus (GEO) or ArrayExpress database as of 2010 were
downloaded. Data were log₂ transformed, and each experimental sample was normalized to a baseline sample (e.g., untreated or time zero) to reduce interdataset technical variation. Each microarray dataset was then z-scored to minimize distribution variation. This dataset consisted of 40 studies and 243 samples, which were subsequently collapsed by averaging technical and biological replicates to reduce bias from studies that used larger sample groups. Author-provided gene identifiers were used to map datasets to one another, and studies that failed to map to at least 40% of the total gene set were removed, leaving 87 samples from 18 studies (Supplemental Table I). Data were further filtered by removal of genes not present in at least 60% of the remaining arrays, resulting in probes for 11,816 unique genes.

**ARACNe network prediction**

A regulatory network model was generated using a mutual information metric with p value thresholding and application of the data processing inequality as implemented in ARACNe (4, 5). Briefly, missing data were estimated in R using K-nearest neighbors as implemented in the impute package and pairwise mutual information was calculated using ARACNe. Thresholds for p values were evaluated from 10⁻⁵ to 10⁻¹⁵, and the data processing inequality was applied at a tolerance of 0.10. For each p value threshold, a false discovery rate was estimated by generating a background model based on the average number of edge predictions in >1000 random iterations. Random iterations were based on datasets in which gene expression values were unchanged (to preserve the distribution) but randomly ordered for each array.

A p value was chosen for thresholding based on the F-score calculated from precision and recall. Calculation of these parameters is limited by the lack of availability of a suitably large database of mouse macrophage transcriptional regulation interactions; the most extensive such database, InnateDB (11), contains <200 such relationships. To obtain a more robust estimation of precision and recall, we based our calculations on all human and mouse interactions cataloged in BioGRID (12), including physical associations, under the assumption that coexpressed genes would be enriched in these data because a physical association implies some degree of coexpression. The maximal F-score was found to occur at p value of 10⁻⁴, which was chosen for thresholding and subsequent data processing inequality application.

**Knowledge enrichment**

Knowledge enrichment was performed in two steps. First, triangular regulatory subnets were identified by retrieving triangular subnets (i.e., those with three nodes and three edges) from the ARACNe-predicted full network, followed by removal of triangular subnets without exactly one transcription factor as defined by comparison with the RIKEN transcription factor database (13). Triangular subnets were subjected to knowledge enrichment by searching PubMed for each transcription factor–gene combination (a strong association), the rationale being that these subnets were further enriched for true biological interactions. Triangular subnets were recombined into a complete network delineating transcription factor–gene relationships, but not the originally predicted gene–gene relationships.

Testing the degree of enrichment is limited by the absence of a robust regulatory interaction reference database. In our case, testing is further limited by the fact that existing databases are based on published literature, which is similarly used to enrich our network predictions. For testing, we therefore eliminated all strong associations and based our enrichment analysis only on weak associations. Using all mouse and human interactions (including physical) in InnateDB, as well as all human regulatory interactions in the Human Transcriptional Regulation Database (14), allowed testing of weak associations against a reference database. Similar comparisons were performed with BioGRID and other subnets of InnateDB (e.g., mouse-specific interactions or transcriptional regulation interactions), but insufficient associations were discovered to perform the analysis. Transcription factor–specific subnets were parsed using in-house scripts, and network predictions were visualized in Cytoscape (15). All analysis described above was performed using customized Perl scripts.

**Mice**

Junb<sup>fl/fl</sup> mice (from E. Passegué, University of California, San Francisco [UCSF]) and Lyz2<sup>Cre/Cre</sup> mice were crossed in-house to generate Junb<sup>fl/fl</sup> × Lyz2<sup>Cre/Cre</sup> mice. All mouse work was conducted with the approval of the UCSF Institutional Animal Care and Use Committee in strict accordance with the guidelines of the Office of Laboratory Animal Welfare.

**Derivation and stimulation of macrophages**

Macrophages were derived from bone marrow by culturing for 8 d in RPMI 1640 supplemented with 10% serum, 10% supernatant from 3T3–M-CSF cells, and 1 mM sodium pyruvate, with feeding on day 5. Resident peritoneal macrophages were isolated by peritoneal lavage with 10 ml ice-cold PBS containing 1 mM EDTA and 3% FBS. Unless otherwise indicated, macrophages were plated in 12-well dishes in a density of 8 × 10⁴ cells/well and treated with LPS (100 ng/ml; Sigma–Aldrich), CpG (1.5 μg/ml; ODN1826; Invivogen), imiquimod (5 μg/ml; Invivogen), polyinosinic-polyribidylic acid (2.5 μg/ml; Sigma–Aldrich), IL-4 (20 ng/ml; Peprotech), or transfected cyclic di-GMP (10 μg/ml; kind gift of D. Burdette and R. Vance, University of California, Berkeley, Berkeley, California). Lipofectamine 2000 (Life Technologies) was used for transfection.

**Western blot**

Cell lysates were harvested in RIPA buffer supplemented with 1 mM PMSF and 1X Complete Protease Inhibitor Cocktail (Roche). Proteins were separated on 10% NuPage bis-Tris gels (Life Technologies), transferred to polyvinylidene membrane, and immunoblotted with Abs to JUNB (Cell Signaling Technology) or β-actin (Abcam).

**Phagocytosis assay**

Bone marrow–derived macrophages (BMDM) were incubated with 3-μm FITC–labeled beads (ECFP-F1; Spherotech) at a cell/bead ratio of 5:1 or with 1-μm Fluosphere beads (Life Technologies) at 10:1 for 24 h. Cells were labeled with anti-F4/80 Ab as described below, and uptake of fluorescent beads was measured on an LSRII (BD Biosciences).

**Flow cytometry**

BMDM were blocked with anti-CD16/16 (2.4G2; UCSC mAb Core), stained with Abs to myeloid surface markers, and analyzed on an LSRII (BD Biosciences). Intracellular staining was performed after 4-h incubation (for BMDM) or 2-h incubation (for peritoneal macrophages) with GolgiPlug (BD Biosciences) and monensin (eBioscience), using Cytotox/Cytoperm (BD Biosciences) according to the manufacturer’s protocols. Results were analyzed using FACSDiva (BD Biosciences) and FlowJo (Tree Star) software. Abs to the following were used: Ly6C (clone HK1.4), CD11c (N418), IL-1β (JTN3), TNF (TN3-19), and rat IgG1 isotype (eB9R1), all from eBioscience; Ly6G (1A5) and S100 H (550) (BioLegend); Ly6B.2 (7/4) (Cedarlane Laboratories); CD11b (M1/70), F4/80 (BM8), and Gr-1 (RB6-8C5), all from eBioscience; Ly6G (1A8) and Siglec H (550) (BioLegend), Ly6C (500) (Cedarlane Laboratories); CD11b (M1/70), F4/80 (BM8), and Gr-1 (RB6-8C5), all from eBioscience; and CD68 (FA-11) (AbD Serotec). Myeloid populations were delineated as previously described (16): granulocytes (CD11b<sup>+</sup>Ly6G<sup>+</sup>), eosinophils (CD11b<sup>+</sup>Ly6C<sup>+</sup>CD205<sup>-</sup>), and monocytes (CD11b<sup>+</sup>F4/80<sup>+</sup>CD205<sup>-</sup>). Macrophages were segmented using FACS (CD11b<sup>+</sup>F4/80<sup>+</sup>CD11c<sup>+</sup>), conventional dendritic cells (DCs; CD11c<sup>+</sup>), and plasmacytoid DCs (CD11c<sup>+</sup>CD3<sup>-</sup>Siglec H<sup>+</sup>).

**Junb microarray analysis**

Following a 4-h LPS stimulation, RNA was isolated from BMDM using the RNeasy Micro kit (Ambion). Purified RNA was amplified (Amino Allyl MessageAmp II kit; Life Technologies) with minor modifications to the manufacturer’s instructions to generate amino allyl–incorporated amplified RNA. Amino allyl–incorporated amplified RNA was coupled to Cy3 dye (GE Healthcare Life Sciences) and hybridized overnight to a SurePrint G3 Mouse Gene Expression 8 × 60K microarray, which was washed and scanned per the manufacturer’s instructions (Agilent Technologies). Raw intensities were extracted using Feature Extraction software (Agilent Technologies) and quantile normalized using Limma (17). Differentially expressed genes were identified using Significance Analysis for Microarrays (SAM) (18), and data were visualized as heat maps using custom Perl scripts. Genes that were increased by at least 2-fold according to SAM (1% false discovery rate) in LPS-treated Junb<sup>fl/fl</sup> BMDM were considered to be LPS-induced. JUNB-dependent genes were identified using SAM (10% false discovery rate, 1.5-fold change threshold) to compare LPS-stimulated gene expression values, normalized to average baseline expression, between Junb<sup>fl/fl</sup> and Junb<sup>fl/fl</sup> macrophages. The set of genes regulated by JUNB in response to LPS was defined as the intersection of
Reverse transcription and quantitative PCR

Macrophage RNA was harvested 4 h poststimulation using the RNeasy kit (Qiagen) with on-column Turbo DNase treatment (Ambion). Reverse transcription was performed with Superscript III (Life Technologies) primed with dT(20)V. Quantitative PCR (qPCR) was performed in a Step One Plus RT PCR System (Applied Biosystems) using PerfeCTa 2× qPCR mix (Quanta). Transcript levels were normalized to levels of actin mRNA. The following primer sequences were used: Il1b forward (F) 5'-CAACAGACCACACCACTAGTGC-3' and reverse (R) 5'-GGCAGTGTAACTCTTCTGCAT-3'; Il4ra F 5'-CAGGGCGTTCAAGAAGC-3' and R 5'-TTGGGGATGAC-3'; Tnf F 5'-GATCCACACTCTCCAGCTGCA-3' and R 5'-GGCAGTGTAACTCTTCTGCAT-3'; Rel F 5'-TGGGAGGCCTATCTTACAGA-3' and R 5'-AGATGCTGCTGTAGATGAAGGC-3'; Arg1 F 5'-TGAGGAGCTCTATCTACAGA-3' and R 5'-CA- TGTGGGCGGATCAC-3'; Retna F 5'-GGTAGCAACTTGTTAAATGG-3' and R 5'-GCACACCCATGTTAGCT-3'; Il1b F 5'-CAAGCTTGTCTGACGAGG-3' and R 5'-CGAAATTTGTTGACTC-3'; Junb F 5'-AATGCTGCTGAGCG GGAGGATGGAAG-3' and R 5'-AGATGTCTGCTGTAGATGAAGGC-3'; Ifnb F 5'-GCTCTTGATGTTGAACTTCAAGTCC-3' and R 5'-TGGGAGGCCTATCTTACAGA-3'; Tnf F 5'-GATCCACACTCTCCAGCTGCA-3' and R 5'-GGCAGTGTAACTCTTCTGCAT-3'; Rel F 5'-TGGGAGGCCTATCTTACAGA-3' and R 5'-CA- TGTGGGCGGATCAC-3'; Retna F 5'-GGTAGCAACTTGTTAAATGG-3' and R 5'-GCACACCCATGTTAGCT-3'; Il1b F 5'-CAAGCTTGTCTGACGAGG-3' and R 5'-CGAAATTTGTTGACTC-3'; Mox1 F 5'-TCCTGTCTACGATTGAGGAC-3' and R 5'-CGAATTTGTTGACTC-3'; TGGGATCAGCTTC-3'.

A role for the JUNB transcription factor in macrophage transcriptional responses to LPS

Because we were interested in pathways affecting macrophage activation, we focused on network predictions surrounding the gene Il1b, which encodes the proinflammatory cytokine IL-1β. IL-1β is a potent pyrogen produced by macrophages in response to a variety of proinflammatory stimuli. As an internal control, our network analysis correctly predicted a correlationary relationship between Il1b and the gene Rel, which encodes a subunit of NF-κB, a transcription factor known to be required for Il1b induction in macrophages (22) (Fig. 1B).

The network analysis also predicted a relationship between Il1b and Junb, a member of the AP-1 family of transcription factors, which are activated downstream of MAPK signaling and regulate a wide variety of cell processes, including differentiation, proliferation, activation, and apoptosis (23). The dimeric AP-1 is assembled from subunits of the JUN (c-JUN, JUNB, or JUND), FOS (c-FOS, FOSB, FRA-1, and FRA-2), or ATF protein families. Interestingly, JUNB was recently reported to be an important factor in the chromatin of LPS-treated DCs (9) and was also identified as one of the most highly expressed and extensively connected transcription factors in a regulatory network model from human monocyte-derived macrophages (3). However, despite these observations, no role has been demonstrated for JUNB in the regulation of Il1b, or indeed of any gene expressed in activated macrophages. In fact, in myeloid cells, AP-1 activity is response to inflammatory stimuli has until recently been attributed almost exclusively to c-JUN/c-FOS heterodimers (24–26), which represent AP-1’s canonical and most well-characterized composition.

Systemic deletion of Junb results in embryonic lethality (27). Therefore, to test whether JUNB does indeed regulate Il1b in macrophages, we crossed mice bearing floxed alleles of Junb to mice expressing Cre recombinase under control of the Lyz2 promoter, resulting in excision of Junb in a subset of myeloid lineages (monocytes, macrophages, granulocytes, and a small fraction of DCs) (28). The resulting mice are healthy, breed as expected, and exhibit no gross pathology. Previously, conditional deletion of

Results

Knowledge-enriched analysis of macrophage regulatory networks

To identify novel transcriptional relationships that regulate macrophage activation, we generated a de novo regulatory network model based on microarray expression data obtained from the public GEO database (http://www.ncbi.nlm.nih.gov/geo). Our initial dataset consisted of 243 microarrays from 40 individual studies, each of which examined the transcriptional responses of mouse macrophages to various stimuli (Supplemental Table I). The data from each individual study were internally normalized to a baseline condition, reducing variance inherent in technical differences between studies and allowing physiological changes in gene expression. These data were used to generate a multiinformation-based network linking genes for which expression covaried over a range of conditions (see Materials and Methods and Supplemental Fig. 1).
Junb in hematopoietic stem cells was shown to result in a marked expansion of myelomonocytic progenitors (29); importantly, the myeloid compartment of Junb<sup>jfl/fl</sup> × Lyz2<sup>Cre/Cre</sup> mice (referred to henceforth as Junb<sup>mLy2<sup>−/−</sup></sup>) is indistinguishable from Junb<sup>jfl/fl</sup> controls (Fig. 1C). We confirmed loss of Junb expression in BMDM produced from Junb<sup>mLy2<sup>−/−</sup></sup> mice by both quantitative RT-PCR (Fig. 1D) and Western blot (Fig. 1E). Junb<sup>mLy2<sup>−/−</sup></sup> BMDM had typical morphology (M.F. Fontana and C.C. Kim, unpublished observations) and normal levels of macrophage-specific surface markers (Fig. 1F), and they phagocytosed fluorescent beads as efficiently as JUNB-sufficient macrophages (Fig. 1G). Together, these data indicate that myeloid-restricted deletion of Junb does not disrupt macrophage proliferation, differentiation, or phagocytic function.

In validation of our network model prediction, we observed inducted peritoneal macrophages from Junb<sup>mLy2<sup>−/−</sup></sup> mice stimulated with LPS ex vivo, confirming that JUNB deficiency affects cytokine production in a distinct type of macrophage (Fig. 2E).

Next, we used whole-transcriptome microarrays to assess the global impact of Junb on macrophage responses to LPS. We found that ~34% of LPS-induced genes exhibited significantly diminished upregulation in JUNB-deficient macrophages relative to their expression in JUNB-sufficient macrophages (Fig. 3A; 469 JUNB-dependent genes, Supplemental Table IIA versus 918 JUNB-dependent genes, Supplemental Table IIB). JUNB-deficient macrophages were not merely poorly responsive to LPS in general, as the majority of genes was induced equally in both JUNB-deficient and JUNB-sufficient macrophages (Fig. 3B; 469 JUNB-dependent genes, Supplemental Table IIA versus 918 JUNB-dependent genes, Supplemental Table IIB). In both JUNB-deficient and JUNB-sufficient macrophages, several of which were previously shown to be decreased following knockdown of Junb in DCs treated with LPS (30). Expression of a small number of genes, such as Il12b, which encodes a subunit of the cytokine IL-12, was drastically reduced in the absence of JUNB, consistent with the previous observation that JUNB binds to an enhancer in the Il12b promoter (31). However, ~70% of affected genes, like Il1b, were

**FIGURE 1.** Network analysis predicts a regulatory interaction between Junb and Il1b in macrophages. (A) Gene–gene relationships (edges) identified in the initial expression analysis (ARACNe) were enriched for biological relevance based on known interactions; the graph depicts the percent of edges remaining after each of two sequential refinements (triangular subnets and knowledge-enriched) that also appear in two databases of known biological interactions (InnateDB and HTRI). Numbers above bars indicate fold enrichment from the initial to the final datasets. (B) Regulatory network analysis identified significant covariance between Il1b and 7 transcription factors, including Rel and Junb. (C) Splenocytes from 4-wk-old mice (n = 4 of each genotype) were analyzed by flow cytometry to quantify frequencies of the indicated myeloid cells. (D-F) Macrophages were differentiated from the bone marrow of Junb<sup>jfl/fl</sup> or Junb<sup>jfl/fl</sup> × Lyz2<sup>Cre/Cre</sup> (Junb<sup>mLy2<sup>−/−</sup></sup>) mice. (D) Deletion of Junb was assessed by qPCR on BMDM genomic DNA. Means ± SD for three technical replicates are shown. (E) BMDM from the indicated genotypes were stimulated with LPS for 4 h. Expression of JUNB (top panel) and β-actin (bottom panel) was measured in lysates by Western blot. (F) BMDM were labeled with Abs to the indicated myeloid surface markers and analyzed by flow cytometry. Data shown are means ± SD averaged from three independent experiments. (G) BMDM were incubated for 24 h with fluorescent beads of the indicated diameter, and phagocytosis was measured by flow cytometry. **p < 0.01 by t test. cDC, conventional DC; pDC, plasmacytoid DC; RPM, red pulp macrophage.
reduced by only 2- to 3-fold (Fig. 3B). Together, these data suggest that JUNB is not absolutely required for activation of gene expression, but that it fine-tunes gene expression and, by inference, modulates the output of other transcription factors. Quantitative RT-PCR was used to validate additional predicted targets (Il12b, Tnf), confirming their JUNB dependence (Fig. 3C). Additionally, intracellular cytokine staining revealed a defect in production of TNF protein in JUNB-deficient macrophages following LPS stimulation (Fig. 3D, 3E). Thus, JUNB regulates production of multiple cytokines and other immune-related genes in macrophages treated with LPS, affecting levels of both transcript and protein.

JUNB likely regulates transcription both directly and indirectly

To gauge whether JUNB regulates transcription through direct binding of regulatory elements in target genes (9, 30–32), we cross-referenced our microarray data with a previous study that performed ChIP-Seq on LPS-treated DCs, which are ontologically and functionally closely related to macrophages (9). We identified 178 genes, including Il1b and Il12b, that exhibited JUNB-dependent expression in macrophages and were also directly bound by JUNB in DCs (Supplemental Table III). These bound and regulated genes accounted for 38% of all JUNB-dependent genes examined. JUNB binding was not observed proximal to the remaining 291 JUNB-dependent genes (Supplemental Table III), consistent with indirect regulation of these targets by JUNB. Interestingly, 41% of JUNB-independent genes were also bound by JUNB (376 of 919 examined; Supplemental Table III), suggesting that binding of this transcription factor is not sufficient to alter gene expression. This observation is consistent with the previous report’s suggestion that JUNB likely cooperates with several other factors to activate transcription (9). Alternatively, these binding sites could represent sites at which JUNB binds without impacting gene expression, as has been observed for other transcription factors (33). Although we have not assessed binding of JUNB to target genes in macrophages in this study, our analysis of ChIP-Seq data from a closely related cell type, combined with a previous report of JUNB binding to the Il12b enhancer in macrophages (31), provides strong evidence that JUNB modulates transcription at least in part through direct activation.

In addition to binding directly to regulatory elements, JUNB may affect transcription indirectly by altering the levels of other transcription factors, such as other AP-1 family members. However, we observed no defect in mRNA expression of the AP-1 transcription factors c-Jun, Jund, and Fos in JUNB-deficient macrophages either at baseline or upon stimulation with LPS (Fig. 4A); in fact, expression of some of these factors was slightly increased in the absence of JUNB. Nevertheless, these data do not rule out the possibility that JUNB affects transcription indirectly; for example, although Jun and Fos transcript levels are intact in JUNB-deficient

![Graph](https://example.com/graph.png)

**FIGURE 2.** JUNB regulates IL-1β production in macrophages treated with LPS. (A) Following 4-h treatment with LPS, Il1b mRNA levels were assessed by RT-qPCR. (B) BMDM were pretreated for 3 h with LPS followed by addition of 5 mM ATP. IL-1β was measured in supernatant by ELISA. (C and D) BMDM were treated for 4 h with LPS, and intracellular pro–IL-1β was detected by flow cytometry. (C) Isotype staining and mock-treated controls were used to establish gates for F4/80+ BMDM. (D) The graph depicts the percent of BMDMs positive for intracellular pro–IL-1β. (E) Peritoneal macrophages were stimulated for 2 h with LPS and stained for intracellular pro–IL-1β as in (C) and (D). Data shown are means ± SE (D and E) or SD (A and B) and representative of three (A–D) or two (E) independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by t test. SSC, side scatter.
macrophages, it is possible that protein levels or activities are altered in the absence of JUNB or that other AP-1 family members are affected. Furthermore, we identified several other transcription factors, including Aff1, Mxd1, Crem, and Stat3, for which expression is defective in the absence of JUNB in macrophages (Supplemental Table IIA) and for which transcriptional initiation regions are directly bound by JUNB in DCs (9). Altered transcription downstream of these factors may therefore account for the JUNB-dependent regulation of genes we observed in the absence of direct JUNB binding. Interestingly, Junb itself is bound by JUNB (9), suggesting that its own expression may be subject to feedback. Altogether, our analyses indicate that JUNB is likely to affect transcription both directly, through binding of target gene regulatory regions, and indirectly, by altering levels of other transcription factors.
To understand whether JUNB preferentially regulates a specific category of genes, we attempted to classify the JUNB-dependent genes identified by microarray. Gene ontology analysis did not reveal enrichment of specific gene categories among JUNB-dependent genes; instead, JUNB modulated expression of a representative subset of LPS-induced gene ontology categories (M.F. Fontana and C.C. Kim, unpublished observations). Next, we examined 48 LPS-induced genes that were previously classified as primary or secondary response genes on the basis of their activation kinetics, as well as whether they require new protein synthesis for induction (discussed further below) (34, 35). Nine of these targets, including eight primary response genes and a single secondary response gene (Il12b), were determined to be JUNB-dependent by microarray (Fig. 4B). Although the pool of primary gene targets examined was larger (41 primary genes versus 7 secondary genes), the percentage of JUNB-dependent genes was similar in both categories (16% of primary and 10.5% of secondary genes examined) (Fig. 4B). Finally, we examined whether these 48 genes were tolerizable or nontolerizable according to the classification scheme proposed by Foster et al. (36), in which macrophages previously exposed to LPS will selectively upregulate nontolerizable genes, but not tolerizable genes, upon secondary LPS stimulation. Among the 48 primary/secondary response genes examined in (35), 19 genes were further classified as tolerizable or nontolerizable, including three JUNB-dependent genes (Fig. 4B). Thus, JUNB-dependent genes include both primary and secondary response genes as well as tolerizable genes. Although our analysis did not reveal any nontolerizable genes that are JUNB dependent, this is likely due to the small size of the gene sets that were searched for overlap (i.e., secondary genes versus nontolerizable genes versus JUNB-dependent genes). On the whole, we conclude that JUNB modulates the transcription of a broad range of genes from several ontological and regulatory categories without obvious bias toward any given category.

To further characterize JUNB activity, we examined the kinetics of transcription of Il1b, a primary response gene, and Il12b, a secondary response gene, in macrophages treated with LPS. Primary response genes do not require new protein synthesis for induction, whereas secondary response genes do; furthermore, primary genes tend to be upregulated more swiftly than secondary genes, though exceptions exist (35). Consistent with previously reported activation kinetics, Il1b was markedly induced in wild-type macrophages as early as 1 h poststimulation, whereas robust upregulation of Il12b was not observed until 2 h poststimulation (Fig. 4C). Interestingly, Il1b induction was intact in JUNB-deficient macrophages at early time points, but by 4 h posttreatment, it lagged behind JUNB-sufficient controls (Fig. 4C). In contrast, upregulation of Il12b was almost completely abrogated in JUNB-deficient macrophages at all time points assayed (Fig. 4C). Thus, JUNB modulates expression of both primary and secondary genes with delayed kinetics and appears to be most important from 2 to 4 h poststimulation, perhaps reflecting the time needed to upregulate JUNB itself. In the case of the rapidly induced Il1b, this delayed involvement results in an initially intact response followed by a leveling off in JUNB-deficient macrophages, whereas with the more gradually induced Il12b, dramatic JUNB dependency is observed from the time that transcript levels first begin to increase.
their response to transfected cyclic di-GMP (Fig. 5B), which elicits robust expression of IFN-β (Ifnb) in wild-type macrophages through the cytosolic sensor STING (37). JUNB-deficient macrophages treated with a variety of TLR ligands produced less IL-12, IL-1β, and TNF protein than JUNB-sufficient macrophages, as measured by bead array (Fig. 5C) and intracellular cytokine staining (Fig. 5D), indicating defects in macrophage responses at the level of both transcript and protein. Taken together, these results demonstrate the broad involvement of JUNB in generating macrophage responses to multiple immunostimulatory ligands.

**JUNB regulates alternative macrophage activation**

Finally, we sought to determine whether JUNB modulated M(IL-4) macrophage responses in addition to M(LPS) responses. Our microarray analysis had revealed that LPS-treated macrophages upregulated Il33, a type II cytokine, in a JUNB-dependent manner (Figs. 3A, 6A). Because IL-4-driven alternative activation is a central feature of type II immunity, we evaluated the induction of M(IL-4) responses in JUNB-sufficient and JUNB-deficient BMDMs treated with IL-4. JUNB-deficient BMDMs exhibited marked defects both in the transcriptional induction of the M(IL-4) target genes Arg1 and Retnla (Fig. 6B) and in the upregulation of arginase activity (Fig. 6C). Importantly, expression of the IL-4R was intact in JUNB-deficient macrophages both at baseline and following treatment with IL-4, as was expression of another M(IL-4) target, Mrc1 (Fig. 6D, 6E). These data indicate that JUNB-deficient macrophages are not generally unresponsive to IL-4 signaling; rather, JUNB appears to act downstream of the IL-4 receptor to positively regulate some aspects of M(IL-4) polarization.

**Discussion**

In this study, regulatory network analysis on community-generated gene expression data led us to predict a role for the AP-1 transcription factor Junb in macrophage activation. Whereas previous studies in silico have suggested such a role without providing experimental validation, we have demonstrated in this study that JUNB regulates macrophage responses to a variety of immunostimulatory ligands.
timulatory ligands. The defects we observed in JUNB-deficient macrophages included altered transcription of a subset of immune-related genes, decreased secretion of M(LPS)-associated cytokines in response to TLR ligands, and weakened M(IL-4) polarization following treatment with IL-4. It is notable that JUNB positively regulates both M(LPS) and M(IL-4) activation, because to our knowledge, virtually all previously described transcription factors with demonstrated roles in macrophage polarization specifically promote either M(LPS) or M(IL-4) activation, with the exception of C/EBPα (38). Importantly, at baseline, Junb<sup>M<sup>L/2</sup></sup> mice exhibit no gross defects in survival or development, suggesting that deletion of JUNB does not disrupt the homeostatic functions of monocytes and tissue macrophages but specifically affects activation. This selectivity raises the possibility that therapeutic targeting of JUNB in macrophages, for example through use of liposomes to deliver drugs selectively to phagocytic cells, might be an effective way to dampen harmful inflammatory responses while minimizing the toxicity associated with systemic inhibition of the canonical c-JUN–c-FOS AP-1 pathway (39).

Our results deepen our understanding of the contributions of individual AP-1 subunits to innate immune responses. Previous studies have provided conflicting data as to whether JUNB is a negative regulator of gene expression, a competitive inhibitor of JNK-mediated phosphorylation of c-JUN, or a transcriptional activator in its own right. Although c-JUN and JUNB appear to play antagonistic roles in processes such as cell cycle progression, JUNB can also compensate to transcribe c-JUN target genes in the absence of c-JUN (23, 40). Furthermore, during CD4<sup>T</sup> T cell and macrophage activation, JUNB appears to act as a transcriptional activator even in the presence of c-JUN (32 and this work). Although previous studies of AP-1 signaling in macrophages have largely focused on c-JUN and c-FOS, our data demonstrate that JUNB also shapes macrophage transcriptional responses to LPS and other stimuli. Together with previous studies, our results suggest that c-JUN and JUNB both serve as positive and nonredundant transcription factors in the induction of immune-related genes in macrophages.

The kinetics of JUNB-dependent transcription may offer a clue to how this transcription factor is regulated. Whereas expression of Il12b and Il1b was unaffected by the absence of JUNB in the first 2 h after LPS treatment, defects in transcription emerged in JUNB-deficient macrophages between 2 and 4 h after stimulation. The time frame of the transcriptional defect tracks with the expression pattern of JUNB itself: we were not able to detect JUNB in resting macrophages by Western blot, but observed robust accumulation of Junb mRNA and JUNB protein by 4 h after LPS stimulation. It is possible, therefore, that JUNB activity is regulated primarily at the level of expression, although phosphorylation by the upstream MAPK JNK has been reported in T cells (32).

Unlike an amplifier that promotes bistability, our data are consistent with a model in which JUNB serves to fine-tune the output of other transcription factors, including c-JUN. Several lines of evidence support this model: first, most (~70%) of the LPS-induced genes for which expression was defective in the absence of JUNB experienced only a modest (2- to 3-fold) decrease in expression. Second, in contrast to c-JUN, JUNB appears to activate only a subset of promoters containing AP-1 sites (32). Finally, a previous study in LPS-stimulated DCs proposed that JUNB be considered one of several primers that act in concert with a variety of other lineage- and environment-specific transcription factors to regulate the transcriptional output of the cell (9).

Importantly, the bioinformatic analysis that initially led us to examine JUNB in macrophage activation was performed on publicly available microarray data from a wide range of studies, each with its own set of protocols and sample treatments. Our work thus serves to demonstrate that despite the technical challenges inherent in starting with heterogeneous data, meaningful biological interactions can be identified with proper data processing. As increasingly extensive and diverse datasets become available, many of which introduce the additional variable of genetic heterogeneity across humans, future studies should not be deterred by these limitations, which can be overcome with sufficiently large datasets and appropriate analysis approaches.

The network method described in this study is also notable in that it permits enrichment for physiologically relevant predictions, as we have demonstrated through extensive experimental validation of one of these predictions. Furthermore, in revealing a novel cell type-specific function for JUNB in innate immune responses, this study lays the groundwork for future therapeutic targeting of the inflammatory response modulated by this key regulator.

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Disclosures

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References
