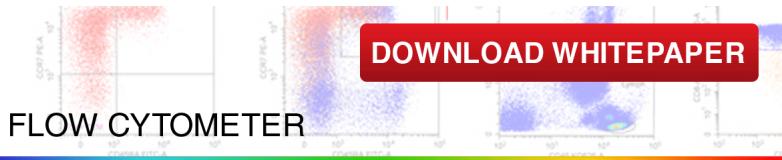


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Batf2/Irf1 Induces Inflammatory Responses in Classically Activated Macrophages, Lipopolysaccharides, and Mycobacterial Infection

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Basic leucine zipper transcription factor Batf2 is poorly described, whereas Batf and Batf3 have been shown to play essential roles in dendritic cell, T cell, and B cell development and regulation. Batf2 was drastically induced in IFN- γ -activated classical macrophages (M1) compared with unstimulated or IL-4-activated alternative macrophages (M2). Batf2 knockdown experiments from IFN- γ -activated macrophages and subsequent expression profiling demonstrated important roles for regulation of immune responses, inducing inflammatory and host-protective genes *Tnf*, *Ccl5*, and *Nos2*. *Mycobacterium tuberculosis* (Beijing strain HN878)-infected macrophages further induced Batf2 and augmented host-protective Batf2-dependent genes, particularly in M1, whose mechanism was suggested to be mediated through both TLR2 and TLR4 by LPS and heat-killed HN878 (HKTB) stimulation experiments. Irf1 binding motif was enriched in the promoters of Batf2-regulated genes. Coimmunoprecipitation study demonstrated Batf2 association with Irf1. Furthermore, Irf1 knockdown showed downregulation of IFN- γ - or LPS/HKTB-activated host-protective genes *Tnf*, *Ccl5*, *Il12b*, and *Nos2*. Conclusively, Batf2 is an activation marker gene for M1 involved in gene regulation of IFN- γ -activated classical macrophages, as well as LPS/HKTB-induced macrophage stimulation, possibly by Batf2/Irf1 gene induction. Taken together, these results underline the role of Batf2/Irf1 in inducing inflammatory responses in *M. tuberculosis* infection. *The Journal of Immunology*, 2015, 194: 000–000.

Macrophages are widely distributed cells of the immune system that play an indispensable role in a vast range of biological processes by acquiring diverse states of

activation in response to tissue signals and environmental stresses. The various immunological processes are carried out by phenotypically polarized macrophages called classically activated macrophages (M1) (1) and alternatively activated macrophages (M2) (2, 3). M2 is induced by IL-4 and IL-13 and involved in the regulation of inflammation in many biological processes, including type 2 infectious diseases (4), tissue repair, insulin resistance (5), and thermogenesis (6) among many others. M1 is induced by IFN- γ , mainly secreted from Th1 cells and NK cells, leading to the secretion of proinflammatory mediators, the release of reactive oxygen, and nitrogen intermediates (7–10), and is thus important for killing effector function, particularly for intracellular pathogens. M1 is usually associated with the control of acute infections.

Macrophages are the most important target cell population in which *Mycobacterium tuberculosis* resides and multiplies by challenging the host-protective immune response. Protective immunity against *M. tuberculosis* is dependent on an IL-12-driven dominant Th1 immune response and consequent microbicidal activities by M1 (11). This is supported by clinical data collected during the early phase of *M. tuberculosis* infection from patients, which demonstrated that macrophages were polarized toward an M1 profile (12). The major components of immune target genes for IFN- γ stimulation include MHC classes I and II, inflammatory and pyrogenic cytokines such as TNF- α , IL-6, IL-12, TGF- β , and IL-10, chemokines Ccl2, Ccl3, Ccl4, and RANTES (Ccl5), chemokine receptors Cxcl2, Cxcl3, Cxcl4, and Cxcl5 (13, 14), and antimicrobial proteins such as inducible NO synthase (Nos2) (7, 15). M1 also produces phagocyte oxidase and immune GTPases, which are major components of innate immunity (14). NO is usually associated with the control of acute infection (16), but it may be also responsible for necrosis and tissue damage, which leads to infectious pathogenesis

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59210>) under accession number GSE59210.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMDM, bone marrow-derived macrophage; CAGE, capped analysis of gene expression; GO, Gene Ontology; HKTB, heat-killed *Mycobacterium tuberculosis* strain HN878; IRF, IFN regulatory factor; M1, classical macrophage; M2, alternatively activated macrophage; MOI, multiplicity of infection; qRT-PCR, quantitative real-time RT-PCR; shRNA, short hairpin RNA.

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when uncontrolled (17, 18). M1 also leads to the induction of immune regulatory transcription factors such as IFN regulatory factors (Irf5), Stats, NfkB, and Ap1 (19, 20), which modulate inflammatory gene expression. Irf1, the first member of the IRF family to be identified, targets different sets of genes in various cell types in response to diverse cellular stimuli and evokes appropriate innate and adaptive immune responses (21). Irf8, also known as IFN consensus sequence-binding protein, seems to have broader effects on microbe-induced cytokine production than does Irf1. Irf8 can function both as a transcriptional repressor and as an activator, depending on the partners with which it interacts, and it plays crucial roles in macrophage activation (21). Previous studies indicated that Irf8 activates IL-12 p35 gene transcription in synergy with Irf1 (21, 22) and enhances Ccl5 gene transcription in cooperation with PU.1, NfkB, and Irf1 (23).

Despite those extensive studies, there seems to be many unknown molecular mechanisms that govern this complicated transcriptional regulation in M1. In the present study, we report that a transcription factor Batf2 is involved in classical activation of macrophages. We found that *Batf2* is drastically induced in IFN- γ -activated classical macrophages but not in IL-4-activated alternative macrophages. Batf2 knockdown experiments in IFN- γ - or LPS-stimulated macrophages revealed that Batf2 plays an important role in innate immune response by controlling expression of several important immune regulatory genes, such as *Nos2*, *Tnf*, *Ccl5*, *Cxcl9*, *Cxcl11*, *Ccr5*, *Cxcr3*, *Il6*, and *Niarcl*, possibly by the Batf2/Irf1 complex. *M. tuberculosis* infection amplified Batf2 expression in M1 via the TLR pathway for induction of inflammatory response in macrophages. Hence, the Batf2 pathway might be a potential target for drug and vaccine development against infectious disease.

Materials and Methods

Ethics statement

All experiments were performed in accordance with the Animal Research Ethics Committee of the Riken Yokohama Institute (25-007) and the South African National Standard (SANS 10386:2008) and the University of Cape Town practice for laboratory animal procedures. The protocol (permit number: 012/036) was approved by the Animal Ethics Committee, Faculty of Health Sciences, University of Cape Town (Cape Town, South Africa).

Bone marrow-derived macrophage generation and cell stimulation

Bone marrow-derived macrophages (BMDMs) were generated from 8- to 12-wk-old BALB/c male mice as described previously (24). Briefly, bone marrow was harvested from femurs and differentiated into macrophages in sterile tissue culture grade 140 \times 20-mm petri dishes with Vent (Nunc, Roskilde, Denmark) containing Plutznik media (DMEM containing 10% FCS, 5% horse serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM 2-ME, 30% L929 cell-conditioned medium, 100 U/ml penicillin G, and 100 μ g/ml streptomycin). After 10 d of differentiation, macrophages were harvested and plated into six-well plates (Nunc) at a concentration of 2.5 \times 10⁶ cells per well. Following overnight incubation, cells were either left untreated or stimulated with IFN- γ (100 U/ml, BD Biosciences, San Jose, CA), LPS from *Escherichia coli* 0111:B4 (100 ng/ml, Sigma Aldrich), heat-killed *M. tuberculosis* HN878 (HKTB; 5 μ g/ml), or with synthetic triacylated lipoprotein Pam3CSK4 (100 ng/ml, InvivoGen) and incubated at 37°C under 5% CO₂. At 2, 4, 6, and 24 h after stimulation for IFN- γ and 4 h for LPS, HKTB, or Pam3CSK4, supernatants and RNA were collected. Total RNA was extracted by an miRNAeasy kit (Qiagen, Valencia, CA).

For *M. tuberculosis* infection, the *M. tuberculosis* HN878 strain was cultivated in 7H9 broth and grown to exponential phase. BMDMs were cultured overnight into six-well plates (Nunc) at a concentration of 4.5 \times 10⁶ cells per well and then left untreated or stimulated with IFN- γ or IL-4 (100 U/ml each, BD Biosciences, San Jose, CA) overnight. After 4 h of infection with *M. tuberculosis* (multiplicity of infection [MOI] of 5), BMDMs were washed once with prewarmed culture media to remove extracellular bacteria and incubated with media containing fresh IFN- γ or IL-4 activators and 10 μ g/ml gentamicin. At 4, 12, 24, 48, and 96 h after infection, supernatants were collected and RNA was extracted.

Thioglycollate-elicited peritoneal macrophage generation and stimulation

Three percent Brewer-modified thioglycollate (Becton Dickinson) was prepared according to the manufacturer's instructions. Sterile thioglycollate medium (1 ml) was injected into BALB/c mice i.p. Peritoneal exudate cells were harvested 7 d after the injection using 15 ml ice-cold sterile PBS with 5 mM EDTA. Cells were washed and RBCs were lysed. After washing twice with medium, peritoneal macrophages were seeded in six-well plates (2 \times 10⁶ cells/well). After overnight incubation, cells were washed and used in stimulation experiments.

Microarray

Total RNA (500 ng) was amplified using the Ambion total RNA amplification kit (Ambion, Carlsbad, CA). cRNA was hybridized to an Illumina mouse Sentrix BeadChip WG-6V2 array (Illumina, San Diego, CA) according to the manufacturer's protocol. Scanning of the chip was performed using Illumina BeadScan and BeadStudio software packages, and data were generated using BeadStudio (version 3.1). Quantile normalization of the microarray data was executed using limma packages of Bioconductor in the R statistical language. Three biological replicates were analyzed for the microarray experiments. Microarray data were deposited in the Gene Expression Omnibus database, accession number GSE59210 (<http://www.ncbi.nlm.nih.gov/geo/>).

Quantitative real-time RT-PCR

Total RNA (500 ng) was reverse transcribed using the ThermoScript RT-PCR system (Life Technologies, Carlsbad, CA), and quantitative PCR was performed using SYBR Premix Ex Taq (TaKaRa Bio) and the 7900HT real-time PCR system (Applied Biosystems). Relative expression of mRNA was determined after normalization with *Gapdh* using the $\Delta\Delta CT$ method. A Student *t* test was used to evaluate statistical significance.

Western blotting

Protein was isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, MA). Protein concentration was measured using a BCA kit (Thermo Fisher Scientific). Five micrograms nuclear proteins in each lane was subjected to 4–12% bis Tris Novex gel electrophoresis (Life Technologies) and transferred to nitrocellulose membrane. The membrane was probed with Batf2 Ab (L-24; Santa Cruz Biotechnology, Santa Cruz, CA), incubated with HRP-conjugated anti-rabbit IgG (Abcam, Cambridge, MA), and developed using an ECL plus kit (GE Healthcare, Buckinghamshire, United Kingdom). Chemoluminescence was recorded with a Fuji LAS-3000 luminescent analyzer. The membrane was stripped and reprobed with control Abs against β -actin (Sigma-Aldrich, St. Louis, MO).

Immunostaining

BMDM cells were seeded in Nunc Lab-Tek Chamber Slide (Sigma-Aldrich) and stimulated with IFN- γ for 6 h. Cells were then fixed with methanol, washed, quenched with 0.3 M glycine, blocked with 0.3 M glycine in 0.1% Tween 20, followed by incubation overnight with Batf2 Ab (Santa Cruz Biotechnology). Cells were washed with PBS, incubated for 1 h with secondary Ab, Alexa Fluor 488 goat anti-rabbit IgG (Abcam) diluted in block solution. Cells were washed with PBS and incubated for 1 h with Alexa Fluor 594 WGA (Abcam) to stain the plasma membrane. Cells were washed and mounted in the ProLong Gold antifade reagent (Life Technologies) and viewed by confocal microscope (Leica TCS SPE system; Leica, Wetzlar, Germany) at \times 63 magnification (HCX Plan Apochromat with a 63 \times /1.40-0.06 oil objective lens; Leica) using acquisition software (LAS AF/TCS SPE; Leica).

Knockdown experiment using lentivirus short hairpin RNA

Control short hairpin RNA (shRNA), Batf2 shRNA, and Irf1 shRNA clones were purchased from Open Biosystems (Thermo Fisher Scientific). Plasmids were purified by endotoxin-free plasmid midiprep kit (Promega, Fitchburg, WI) according to the manufacturer's instruction. Seventeen micrograms plasmid, 10 μ g HIV-gp, and 10 μ g vesicular stomatitis virus envelope genes were cotransfected into 4 \times 10⁶ 293T cells using FuGENE HD (Roche Applied Science, Mannheim, Germany) in Opti-MEM (Life Technologies) medium containing 5% FBS at 37°C, 5% CO₂. After 2 d of incubation, the supernatant was collected and centrifuged at 19,400 rpm for 2 h at 20°C. Pellets were dissolved in 100 μ l HBSS and stored at -80°C for future use.

For lentivirus infection, BMDM cells (1.5 \times 10⁶ cell/well in a six-well plate) were incubated overnight followed by media change and replenishment with 8 μ g/ml Polybrene (EMD Millipore, Billerica, MA) con-

taining DMEM (with 10% FBS, antibiotics). BMDM cells were infected with lentivirus for negative control shRNA and *Batf2* shRNA at an MOI of 1:10. After 24 h, the media were removed and 4 ml fresh DMEM with 10% FBS was added. The media were changed every third day. On day 6 of the transfection, medium was replenished with 3 μ g/ml puromycin (Sigma-Aldrich) containing DMEM with 10% FCS. On day 10 of the transfection, cells were washed and used for the stimulation experiments.

For identification of *Batf2*-regulated genes, gene expression profiles were compared between IFN- γ -stimulated BMDMs at 4 h and unstimulated control, both negative shRNA treated, and between IFN- γ -stimulated *Batf2* knockdown BMDMs and negative shRNA-treated IFN- γ -stimulated BMDMs. Genes with >1.6 -fold change significantly ($p < 0.001$) were selected as up- and downregulated gene. Genes upregulated by IFN- γ stimulation and downregulated in IFN- γ -stimulated *Batf2* knockdown were selected as positively regulated genes. Additionally, genes downregulated by IFN- γ stimulation and upregulated in IFN- γ -stimulated *Batf2* knockdown were selected as negatively regulated genes.

Measurement of NO in culture supernatants

Cell culture supernatants were analyzed for the production of NO₂⁻ using the Griess reagent assay as previously described (4, 25).

Measurement of TNF α and IL-12p40 in culture supernatants

TNF- α and IL-12p40 levels in the culture supernatants were measured by sandwich ELISA using tetramethylbenzidine substrate solution (Roche Diagnostics, Mannheim, Germany) for development (4).

Coimmunoprecipitation

Coimmunoprecipitation experiments were done using the Dynabeads protein G kit (Life Technologies). Briefly, 10 μ g Irf1 (Cell Signaling Technology) and Irf8 (Santa Cruz Biotechnology) Abs was diluted in 200 μ l PBS containing Tween 20. Ab was incubated with the Dynabeads protein G at 4°C for 2 h. Nuclear protein was prepared from unstimulated and IFN- γ - or LPS-stimulated BMDM cells after 6 h. The Ab-conjugated Dynabeads were washed and incubated overnight with 5 μ g nuclear protein extract. The beads

were washed and protein was eluted using 20 μ l elution buffer. Ten microliters premixed NuPAGE LDS (Life Technologies) sample buffer and NuPAGE sample reducing agent was added to the samples. The sample was heat denatured and subjected to Western blot experiment.

Results

Induction of *Batf2* in IFN- γ -stimulated macrophages

To identify novel regulation mechanisms in M1, mouse BMDMs were stimulated with IFN- γ (100 U/ml) in a time-dependent manner and total RNA was subjected to an Illumina beads microarray (Fig. 1A). *Batf2* mRNA was highly induced in IFN- γ -stimulated BMDMs in a transient manner, together with known induced transcription factors *Irf1*, *Irf8*, *Stat1*, *Stat3*, *NfkB1*, *Jun*, and *Crem* (Fig. 1B). The expression level of *Batf2* was close to the detection limit at time 0, but drastically induced after 2 h of IFN- γ stimulation (28.0 \pm 12.5-fold in comparison with the unstimulated control), keeping a high level until 6 h and downregulated at 24 h after stimulation. Because *Batf2* is a member of the AP-1 basic leucine zipper transcription factor (activating transcription factor) family (26–29), we also explored expression profiles of other family members *Batf* and *Batf3* (Fig. 1C). *Batf* and *Batf3* showed low expression and were not induced by IFN- γ stimulation. Those intensities were quantitatively confirmed by the capped analysis of gene expression (CAGE) measurement (30), which shows that *Batf2* was not induced by IL-4, IL-13, or IL-4/IL-13 (Supplemental Fig. 1A, 1B) in activated BMDMs, suggesting that *Batf2* induction was specific for M1. In contrast, we found weak *Batf* and *Batf3* induction by IL-4-stimulated M2 in the CAGE measurement (Supplemental Fig. 1C). The *Batf2* induction was further confirmed using independent experiments by quantitative real-time RT-PCR (qRT-PCR) (Fig. 1D),

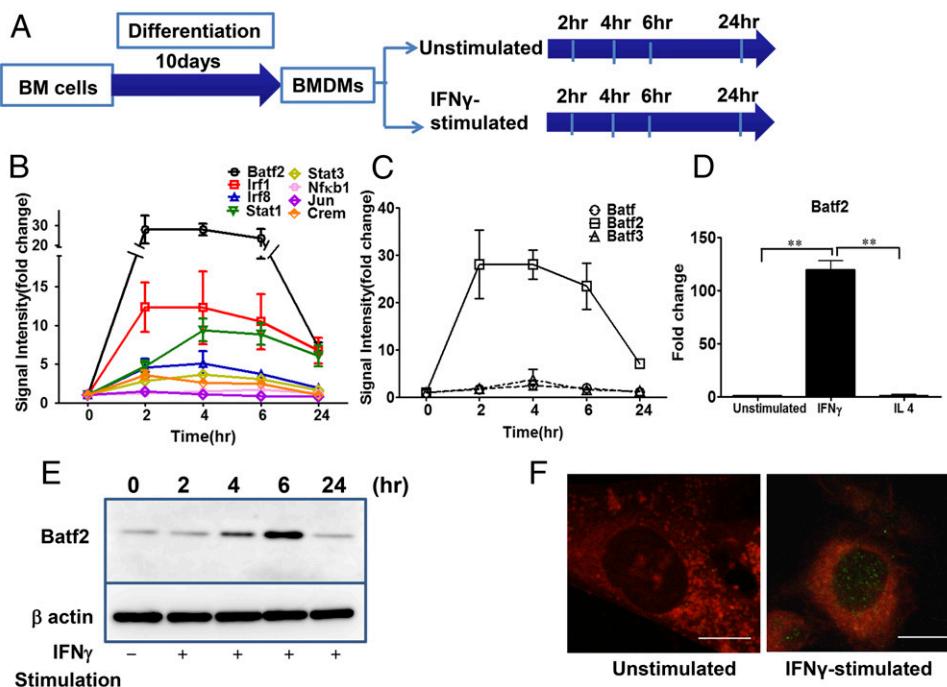


FIGURE 1. Induction of *Batf2* in IFN- γ -stimulated classically activated macrophages. **(A)** Schematic representation of preparation and stimulation of BMDMs from BALB/c mice. After 10 d of differentiation, BMDMs were stimulated with IFN- γ followed by microarray analysis at 2, 4, 6, and 24 h. Three independent biological replicates were used. **(B)** Relative signal intensity of *Irf1*, *Batf2*, *Irf8*, *Stat1*, *NfkB1*, *Jun*, and *Fos* in IFN- γ -stimulated cells was plotted in comparison with unstimulated control. The data were plotted as means \pm SEM. **(C)** Relative signal intensity was plotted for *Batf* family genes. The data were plotted as means \pm SEM. **(D)** *Batf2* mRNA is not induced by IL-4 stimulation. The relative expression level of *Batf2* in IFN- γ - and IL-4-stimulated cells was plotted in comparison with unstimulated control by using qRT-PCR data at 4 h. Three independent experiments were used. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. ** p < 0.01 versus control. **(E)** Western blot analysis of IFN- γ -mediated *Batf2* induction. Three independent experiments were performed. **(F)** Immunofluorescence staining of *Batf2* protein at 6 h after stimulation with IFN- γ . *Batf2* and cell membranes were stained by secondary Ab Alexa Fluor 488 (green) and by Alexa Fluor 594 WGA (red), respectively. Scales bars, 10 μ m. Three independent experiments were performed.

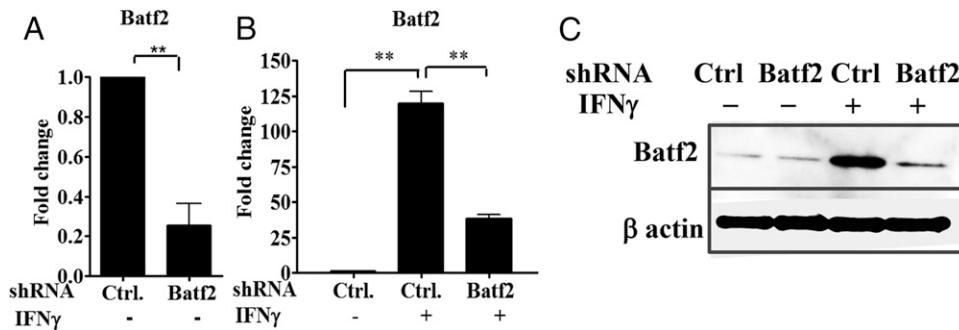


FIGURE 2. Knockdown of Batf2 from IFN- γ -stimulated macrophages. **(A)** *Batf2* knockdown in unstimulated BMDMs. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. ** p < 0.01 versus control. **(B)** Suppression of IFN- γ -mediated *Batf2* mRNA induction by *Batf2* shRNA at 4 h. Three independent experiments were performed. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. ** p < 0.01 versus control. **(C)** Western blots analysis for downregulation of IFN- γ -induced Batf2 by *Batf2* shRNA. Three independent experiments were performed.

Supplemental Fig. 1D) and thioglycollate-elicited peritoneal macrophages following IFN- γ stimulation (Supplemental Fig. 1E). Next we confirmed Batf2 induction at a protein level using Western blot analysis (Fig. 1E). As expected from the gene expression analysis, Batf2 was transiently upregulated at 4 h with a peak at 6 h and was downregulated to the unstimulated level at 24 h, which showed slightly delayed responses by protein induction in comparison with mRNA expression profiles. We further explored the localization of IFN- γ -induced Batf2 by immunohistochemical staining, which demonstrated a predominant localization in the nucleus (green fluorescence) at 6 h IFN- γ -stimulated BMDMs (Fig. 1F). Taken together, these results show that Batf2, but not Batf and Batf3, is transiently induced in BMDMs by IFN- γ stimulation.

Batf2 is essential for IFN- γ -induced Nos2 and Tnf expression

To understand the functional importance of Batf2 in IFN- γ stimulation, we assessed the effect of *Batf2* knockdown in IFN- γ -stimulated BMDMs. Because macrophages are one of the most difficult

cells for lipofection-based gene knockdown by siRNA, we prepared lentivirus expressing *Batf2* shRNA. BMDMs were infected by the lentivirus at an MOI of 1:10, and total RNA was extracted after 10 d of infection followed by qRT-PCR analysis. The mRNA expression indicated that knockdown efficiency of 75% on average was achieved in *Batf2* shRNA-transfected cells as compared with the negative control shRNA-transfected cells (Fig. 2A). Additionally, significant knockdown (p < 0.01) of *Batf2* mRNA level was also obtained after IFN- γ stimulation at 4 h (Fig. 2B), which was further confirmed at the protein level by Western blot at 6 h (Fig. 2C).

The consequences of *Batf2* knockdown in IFN- γ -stimulated BMDMs was explored by qRT-PCR measurement of representative classical effector genes. IFN- γ -mediated induction of the major classical effector genes, NO synthase 2 (*Nos2*) and TNF- α (*Tnf*), were strikingly downregulated by *Batf2* knockdown (Fig. 3A, 3C). The induction of the *Nos2* enzyme leads to the production of NO, measured as nitrite in the culture medium by the Griess reagent assay. Nitrite was drastically reduced in *Batf2* knockdown cells

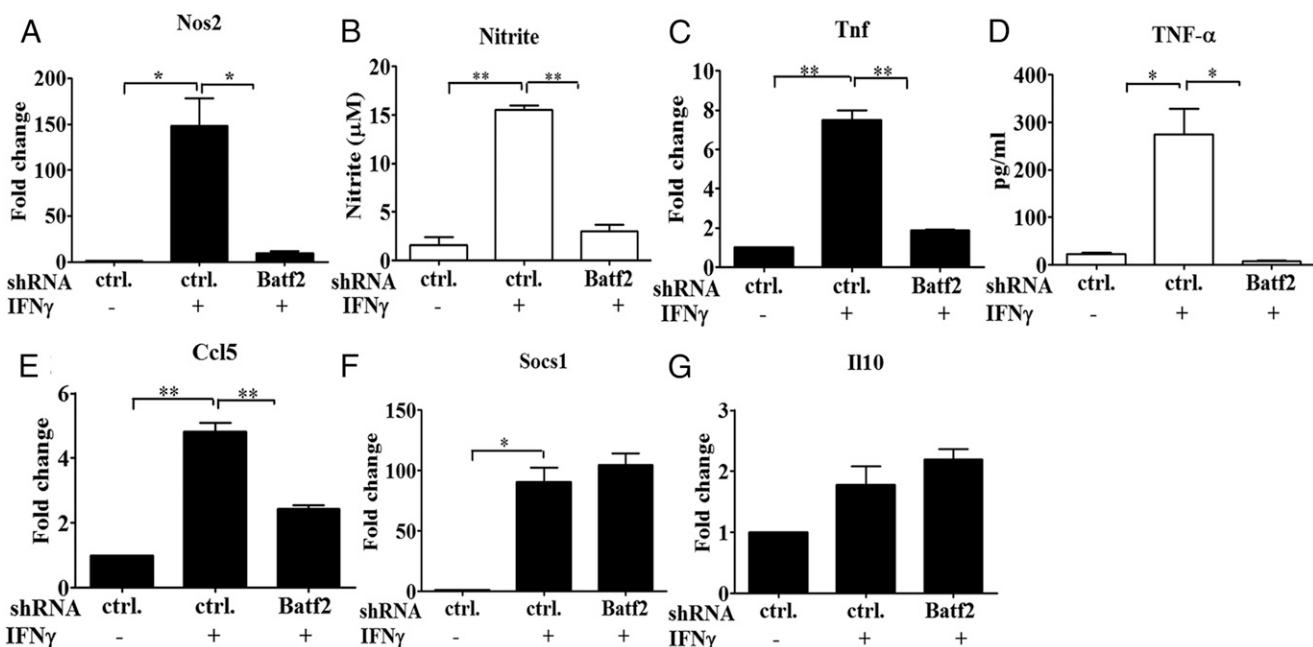


FIGURE 3. Effect of *Batf2* knockdown to representative classical effector genes in IFN- γ -stimulated BMDMs. Relative expression levels of *Nos2*, *Tnf*, *Rantes* (*Ccl5*), *Socs1*, and *Il10* at 4 h, analyzed by qRT-PCR, are shown in **(A)**, **(C)**, **(E)**, **(F)**, and **(G)**, respectively. Three independent experiments were done. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. **(B)** Measurement of nitrite (NO_2^-) in the culture medium, the converted form of NO free radical, by Griess reagent assay at 48 h. **(D)** Measurement of secreted TNF- α in the culture medium by ELISA. Three independent experiments were performed at 48 h. * p < 0.05, ** p < 0.01 versus control.

compared with the negative control cells (Fig. 3B), validating downregulation of IFN- γ -induced Nos2. We also confirmed by ELISA assay that IFN- γ -mediated induction of secreted TNF- α in the culture medium was significantly reduced by *Batf2* knockdown (Fig. 3D). Of note, IFN- γ -mediated weak induction of the chemokine *Rantes* (*Ccl5*) also revealed downregulation by *Batf2* knockdown (Fig. 3E). In contrast, IFN- γ -mediated induction of suppressor of cytokine signaling 1 gene (*Socs1*) was not perturbed by *Batf2* knockdown (Fig. 3F). IL-10 (*Il10*) was induced by IFN- γ but not perturbed by *Batf2* knockdown (Fig. 3G). Taken together, this demonstrates that important early immune response genes, including macrophage killing effector genes, are regulated by *Batf2*.

Global analysis of *Batf2* knockdown in IFN- γ -induced gene expression

To globally understand *Batf2*-regulated genes in IFN- γ -stimulated BMDMs, the *Batf2* knockdown samples were subjected to the Illumina beads microarray. Out of 670 IFN- γ -induced genes, 78 genes (11.6%) were found as positively regulated genes by *Batf2* (Supplemental Fig. 2A), where 66 of 78 genes are early response genes with peak expression up to 6 h of IFN- γ stimulation. In addition to *Tnf*, *Rantes* (*Ccl5*) and *Nos2* that were identified by qRT-PCR, well-characterized classical effector genes, such as *Ccr5*, *Ccl11*, *Cxcl9*, *Cxcl11*, *Ifitm1*, *Niacr1*, *Igf2bp2*, *Il6ra*, and *Irg1*, were regulated by *Batf2*. Because transcription factor genes *Klf6*, *Crem*, *Bcl3*, and *Batf3* were also under the regulation of *Batf2*, it is possible that some of the 78 affected genes may be directly regulated by those transcription factors. Gene Ontology (GO) analysis for the *Batf2* positively regulated genes revealed significant enrichment of the ontology term for immune response such as “immune response” (GO 0006955), “cytokine-mediated signaling pathway” (GO 0019221), “innate immune response” (GO 0045087), “response to wounding” (GO 0009611) “regulation of T cell activation” (GO 0050863), and “regulation of interleukin-6 production” (GO 0032675) (Fig. 4). Alternatively, of 458 IFN- γ -downregulated genes, 43 genes were selected as negatively regulated genes by *Batf2* ($p < 0.001$) (Supplemental Fig. 2B). Although we found classical effector genes such as *Cxcr3* and *Slc17a9* among those genes, GO analysis of *Batf2* negatively regulated genes did not show significant enrichment of ontology terms for the positively regulated genes, except for the term “fatty acid elongation” (GO 0019367). These results indicate that *Batf2* plays an important role for the positive regulation of immune responses by altering expression of cytokines and chemokines and therefore possibly maintaining the balance in inflammatory processes.

M. tuberculosis induces *Batf2* in macrophages

To test the biological relevance of *Batf2* regulation in an infectious disease model, BMDMs were either left unstimulated or stimulated with IFN- γ or IL-4 for 24 h (Fig. 5A). The treatment of BMDMs with IFN- γ resulted in rapid induction of *Batf2* at 2 h after treatment, keeping the high level until 6 h and downregulation at 24 h (blue line in Fig. 5A). Subsequent infection with a clinical *M. tuberculosis*, Beijing strain HN878 (Fig. 5A time point 0, at 24 h), in a time course CAGE expression analysis demonstrated induction of *Batf2* within 4 h after infection in unstimulated BMDMs (yellow line in Fig. 5A), indicating that *M. tuberculosis* itself is able to induce *Batf2* independently of IFN- γ stimulation. However, *Batf2* induction was strongest and prolonged in *M. tuberculosis*-infected IFN- γ -stimulated macrophages with up to 6-fold increased *Batf2* gene expression at 4 and 12 h postinfection (green line in Fig. 5A), compared with unstimulated or IL-4-activated macrophages. These results indicated that IFN- γ and *M. tuberculosis* infection may act in concert to upregulate *Batf2*. Of note, infection also induced *Batf* in

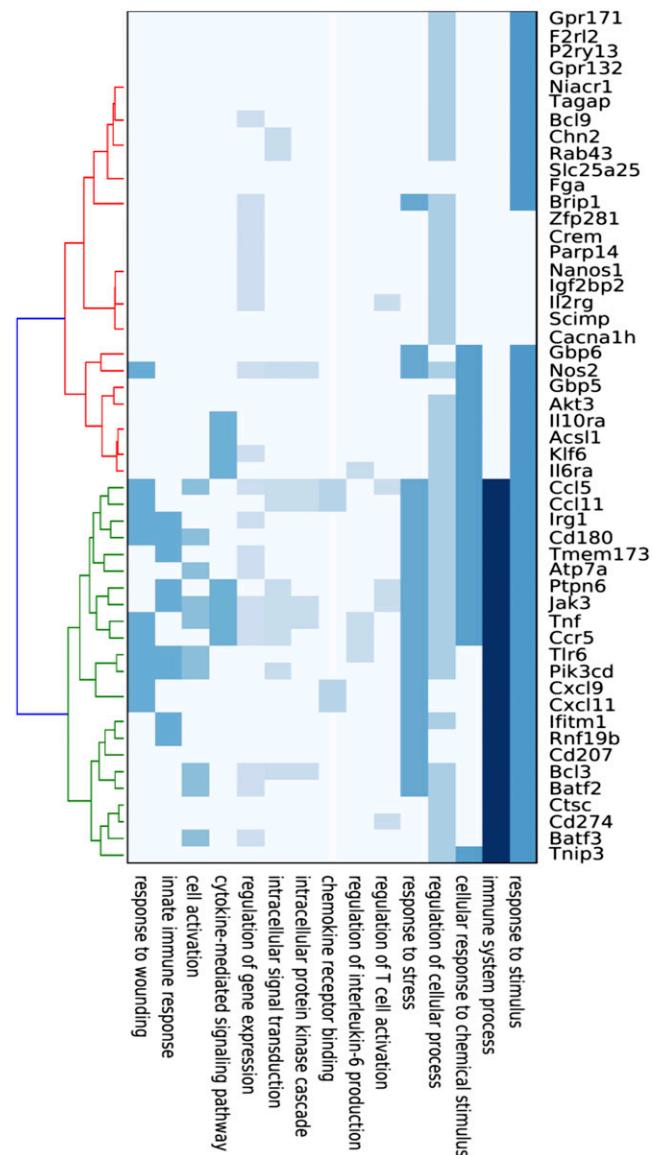


FIGURE 4. GO analysis of *Batf2*-perturbed genes. The heat map shows the association of selected highly enriched significant GO terms of *Batf2*-perturbed gene. The negative logarithm of the *q* value (false discovery rate-adjusted *p* values) is shown. The higher the value (the lower the *q* value), the stronger the color in the heat map, the more significant the association. A *q* value of 1 ($-\log(1) = 0$; white color in the heat map) was used for non-association between the GO term and the gene.

unstimulated macrophages and in IFN- γ -stimulated macrophages (Fig. 5B, 5C), whereas *M. tuberculosis* infection did not induce *Batf3*. A comparison from the *Batf2* knockdown experiment in IFN- γ -stimulated macrophages (Supplemental Fig. 2A) demonstrated that many of the perturbed genes, including *Tnf*, *Ccl5*, and *Irg1*, but not all, were also induced by *M. tuberculosis* infection (Supplemental Fig. 3A). These results indicate that *M. tuberculosis* infection has an overlapping effect on the perturbation of *Batf2* target genes with IFN- γ , but has also distinct differences. Biologically important for host defense, *M. tuberculosis* infection in IFN- γ -stimulated macrophages drastically induced known host-protective genes such as *Tnf*, *Ccl5*, and *Cxcl11* (Supplemental Fig. 3A), including *Nos2* (Fig. 5D, 5E, Supplemental Fig. 3B, 3C). Interestingly, *M. tuberculosis*-infected unstimulated and IL-4-stimulated macrophages also induced *Nos2* to a certain level, which resulted in residual NO production in IL-4-stimulated

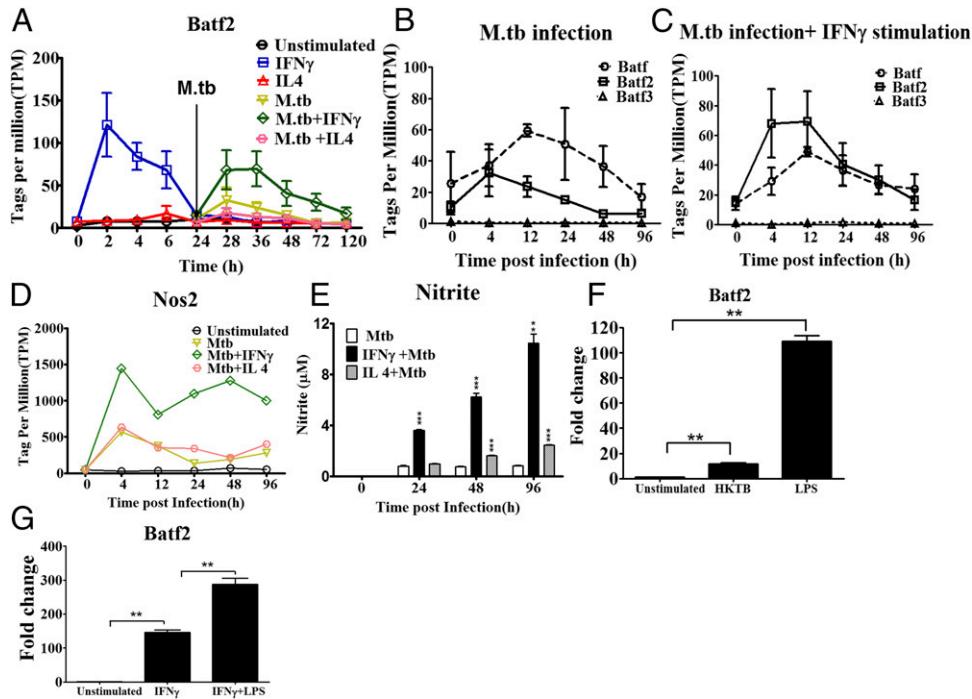


FIGURE 5. *M. tuberculosis* infection induces Batf2 in BMDMs. BMDMs were stimulated with IFN- γ (100 U/well), IL-4 (100 U/well), or left untreated for 24 h. Cells were then infected with HN878 strain of *M. tuberculosis* (MOI of 5), and at the indicated time points, RNA and supernatant were collected for CAGE analysis and ELISA, respectively (30), for the nucleotide sequence-based expression analysis. Expression intensity of each gene is represented by tags per million. The data were obtained from three independent biological experiments and plotted after calculating means and SEM. **(A)** Cytokine-induced Batf2 expression and subsequent *M. tuberculosis* infection at 24 h followed by *M. tuberculosis*-induced Batf2 expression with or without cytokine preactivation. Data were plotted as means \pm SEM. **(B)** Expression of Batf family genes after *M. tuberculosis* infection only, and **(C)** expression of Batf family gene in *M. tuberculosis* infection with IFN- γ stimulation. Data were plotted as means \pm SEM. **(D)** *M. tuberculosis* induced Nos2 expression with or without cytokine stimulation in a time course CAGE experiment. One of the replicate was shown as representative figure. **(E)** Measurement of nitrite (NO_2^-) in the culture supernatant of *M. tuberculosis*-infected cells with or without cytokine stimulated BMDMs. Data were plotted as means \pm SEM. One of the replicates was shown as representative figure. **(F)** Relative expression levels of Batf2 in HKTB and LPS-stimulated BMDMs were analyzed by qRT-PCR at 4 h. **(G)** Relative expression level of Batf2 was analyzed in IFN- γ - or IFN- γ /LPS-stimulated BMDMs by qRT-PCR at 4 h. Three independent experiments were performed. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. * p < 0.05, ** p < 0.01 versus control.

macrophages but not in unstimulated macrophages (Fig. 5D, 5E, Supplemental Fig. 3B, 3C). This might indicate that additional factors are needed to produce sufficient NO, depending on the activation status of host macrophages. Taken together, these results demonstrate that *M. tuberculosis* infection induces Batf2, particular in IFN- γ classical macrophages, which upregulates important inflammatory and host-protective effector genes.

LPS and heat-killed *M. tuberculosis* induce Batf2 effectively in macrophages

M. tuberculosis-induced gene expression change is known to be mediated by TLR-2 and TLR-4 (31), a different signal transduction pathway from IFN- γ stimulation by its receptors *Ifngr1* and *Ifngr2*. To explore how *M. tuberculosis* activates Batf2 expression, BMDMs were stimulated with bacteria-derived LPS and HKTB, which induce macrophage activation through TLR-4 and TLR-2, respectively (31–33). Batf2 expression was induced in both LPS and HKTB (Fig. 5F) similarly to IFN- γ (Fig. 1D), although the induction was relatively weak in HKTB. TLR-2-mediated Batf2 induction in BMDM cells was also confirmed by stimulation with Pam3CSK4, a specific activator for TLR-2 (Supplemental Fig. 3D). Furthermore, we confirmed that costimulation with IFN- γ and LPS synergistically induced Batf2 mRNA in both BMDM cells (Fig. 5G) and thioglycollate-elicited peritoneal macrophages (Supplemental Fig. 3E).

Subsequently, we performed Batf2 knockdown experiments in LPS-stimulated BMDMs (Fig. 6A) and explored the effect to the above found set of representative classical effector genes (Fig. 6B–J).

LPS stimulation induced Nos2, *Tnf*, and *Ccl5* mRNA expression, which were significantly downregulated by Batf2 knockdown experiments (Fig. 6B–F). Interestingly, LPS-mediated *Socs1* mRNA induction was significantly downregulated by Batf2 knockdown (Fig. 6G), which was not observed in IFN- γ stimulation. Furthermore, we observed LPS-mediated induction of *Il10*, although it was not downregulated by Batf2 knockdown (Fig. 6H). Finally, *Il12b* was induced by LPS stimulation, which was significantly downregulated by Batf2 knockdown (Fig. 6I, 6J). The same experiment was also performed in HKTB-stimulated BMDMs (Fig. 6K–O). HKTB stimulation also induced Nos2, *Tnf*, *Ccl5*, and *Il12b* mRNA, which were significantly downregulated by Batf2 knockdown experiments (Fig. 6L–O). Taken together, these results demonstrate that Batf2 is induced by LPS and HKTB stimulation in macrophages via TLR-4/TLR-2, leading to Nos2, *Tnf*, and *Ccl5* mRNA expression, also seen in M1 and *M. tuberculosis* infection.

Batf2 associates with Irf1

Because basic leucine zipper transcription factors, including Batf2, lack the DNA binding domain, they regulate downstream genes by physical association with other transcription factors. Therefore, we performed the oPOSSUM program, an enrichment analysis of transcription factor binding motif (34). We identified RELA, SPIB, FOXA2, MZF1_1–4, IRF1, and NF- κ B as significantly enriched motifs at the promoter sites of Batf2-regulated genes (Fig. 7A). Motif analysis exhibit that 27 of 79 Batf2-perturbed genes have Irf1 binding sites (Supplemental Table I).

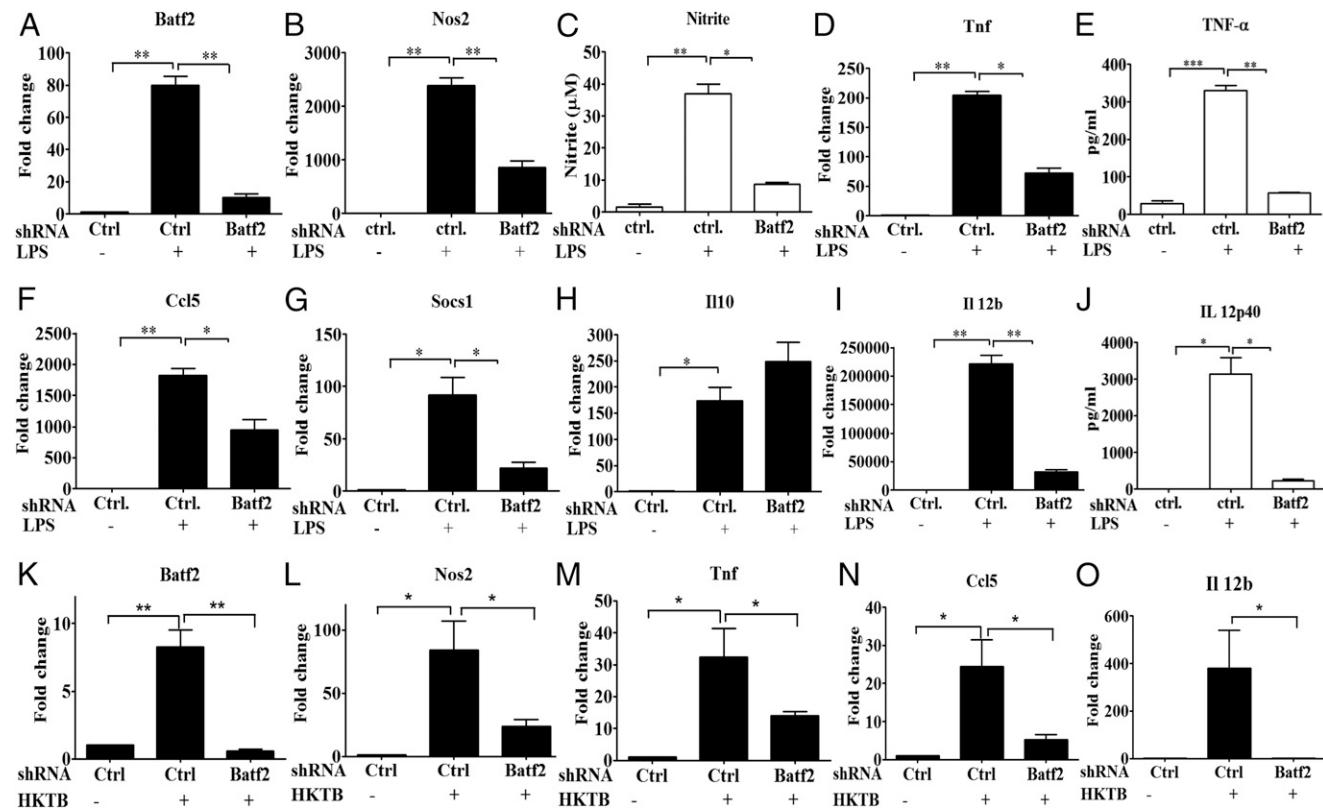


FIGURE 6. Effect of Batf2 knockdown in LPS/HKTB-stimulated BMDMs at 4 h. **(A and K)** Suppression of Batf2 mRNA in Batf2 knockdown BMDMs stimulated with LPS and HKTB, respectively. Batf2 knockdown was analyzed by qRT-PCR at 4 h. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. **p* < 0.05, ***p* < 0.01 versus control. **(B), (D), (F), (G), (H), and (I)** show relative expression levels of Nos2, Tnf, Rantes (Ccl5), Socs1, IL10, and IL12b, respectively, that were analyzed by qRT-PCR in Batf2 knockdown BMDMs stimulated with LPS at 4 h. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. Three independent experiments were performed. **p* < 0.05, ***p* < 0.01 versus control. **(C), (E), and (J)** show effects of Batf2 knockdown in LPS-stimulated BMDMs that were confirmed at the protein level. After 48 h of LPS stimulation, the culture supernatants were collected for detection of (E) nitrite (NO_2^-) by Griess reagent assay, (G) TNF, and (J) IL-12p40 by ELISA. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. **p* < 0.05, ***p* < 0.01 ****p* < 0.001 versus control. **(L–O)** Effects of Batf2 knockdown in HKTB-stimulated BMDMs. Relative expression levels of Nos2, Tnf, Rantes (Ccl5), and IL12b were analyzed by qRT-PCR after Batf2 knockdown in HKTB-stimulated BMDMs at 4 h. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. Three independent experiments were performed. **p* < 0.05, ***p* < 0.01 versus control.

So far it has been recently shown that Batf/Irf4 and Batf/Irf8 associations regulate compensatory dendritic cell development through the AICE motif (35–38), suggesting that Batf2 may also associate with Irf proteins. In fact, 17 of 78 Batf2 positively regulated genes possess the AICE motif significantly ($p = 2.581 \times 10^{-5}$). Thus, we explored physical association of Batf2 with IFN- γ -induced Irf5, Irf1, and Irf8 using immunoprecipitation. We found that Batf2 is coimmunoprecipitated by Irf1 but not by Irf8 (Fig. 7B). To further explore the role of Irf1 we performed Irf1 knockdown experiments in LPS-stimulated BMDMs (Fig. 7C) and explored the effect of the above found (Supplemental Table I) set of classical effector genes (Fig. 7D–G). Irf1 knockdown significantly downregulated IFN- γ - and LPS-activated classical effector genes Nos2, Tnf, Ccl5, and IL12b in LPS-stimulated BMDMs (Fig. 7D–G). These results indicate that Batf2 and Irf1 regulated the same classical genes and suggest that some of the Batf2-regulated genes may be regulated through Batf2/Irf1 interaction.

Discussion

Batf2 belongs to the AP-1 basic leucine zipper transcription factor (activating transcription factor) family proteins, composed of Batf, Batf2, and Batf3 (26–29, 35), with Batf2 having very limited functional information available. Batf family proteins do not have a DNA binding domain and bind to DNA indirectly with support

of other transcription factors. In this study, we demonstrate that Batf2 is involved in gene regulation of IFN- γ -activated classical macrophages as well as LPS/TLR-induced macrophage stimulation. Batf2, most probably in complex with the transcription factor Irf1, induces important classical effector genes in macrophages, among them Tnf, Nos2, Ccl5, and IL12b for inflammatory responses against type 1 infectious diseases, such as *M. tuberculosis*.

Gene expression studies in IFN- γ -activated M1 and in TLR-stimulated macrophages revealed a strong induction of the transcription factor Batf2, which was not observed in unstimulated or IL-4-activated M2, and hence Batf2 can be classified as a new marker for M1. Batf2 can compensate for Batf3 in CD8 $^+$ and CD103 $^+$ dendritic cell development during *Toxoplasma gondii* infection (36). In contrast, expression of Batf and Batf3 were not altered during macrophage stimulation; however, *M. tuberculosis* infection induced Batf in macrophage infection. A recent publication showed that Batf is involved in IL-12 production in CD103 $^+$ dendritic cells in response to mycobacteria and *Listeria* infection (36), associated with class switch recombination in B cells (39, 40), and is essential for the development of Th17 cells, T follicular helper cells, Th1 cells, and Foxp3 $^+$ T cells (41, 42), effector function of CD8 $^+$ T cells (43), as well as maintenance of hematopoietic stem cells (44). Moreover, Batf3 has been shown to be essential for the development of CD8 $^+$ classical dendritic cells and related

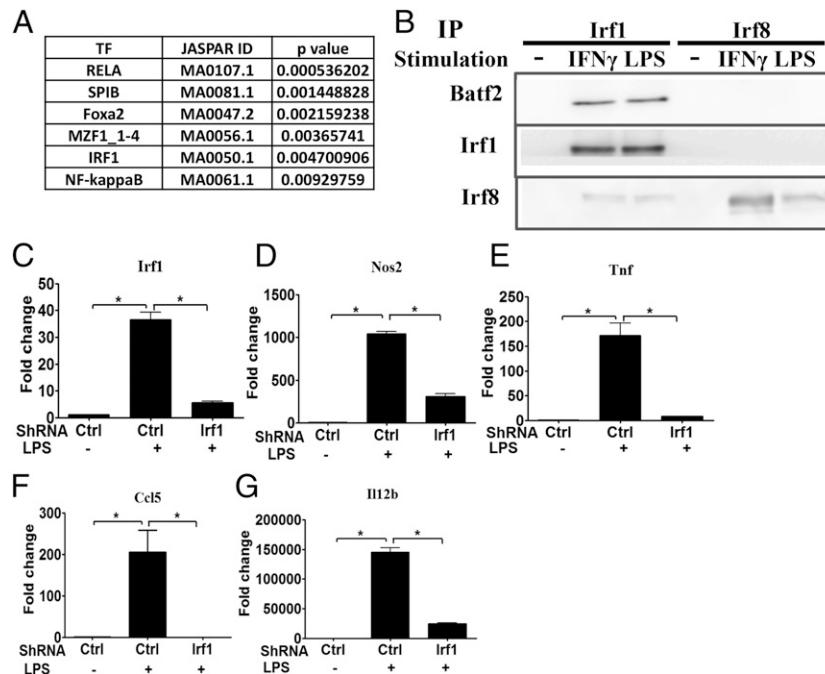


FIGURE 7. Batf2 associates with Irf1. **(A)** An enrichment analysis of transcription factor binding motifs (oPOSSUM program) was performed. We identified RELA, SPIB, FOXA2, MZF1_1-4, IRF1, and NF- κ B as significantly enriched motifs at the promoter sites of Batf2-regulated genes. **(B)** Coimmunoprecipitation of Batf2 with IFN- γ /LPS-induced Irf5. Nuclear extract was obtained from unstimulated (-) and IFN- γ - and LPS-stimulated BMDMs at 6 h. Ten micrograms Irf1 and Irf8 Ab was incubated with Dynabeads for 2 h. After incubation, the Dynabeads were washed and incubated with nuclear extract overnight at 4°C. The Irf complex was immunoprecipitated by Dynabeads with Abs against Irf1 or Irf8. The precipitants were analyzed by Western blot. Batf2 was detected as coprecipitant for Irf1 Ab, but not for Irf8 Ab. **(C–G)** Relative expression level of *Irf1* was analyzed after *Irf1* knockdown in LPS-stimulated BMDMs by qRT-PCR at 4 h. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. * p < 0.05, ** p < 0.01 *** p < 0.001 versus control. **(D–G)** Effect of *Irf1* knockdown from LPS-stimulated BMDMs. Relative expression levels of *Nos2*, *Tnf*, *Rantes* (*Ccl5*), and *Il12b* were analyzed by qRT-PCR after *Irf1* knockdown in LPS-stimulated BMDMs at 4 h. The data were plotted as means \pm SEM and statistical significance was calculated by a Student *t* test. Three independent experiments were performed. * p < 0.05, ** p < 0.01 versus control.

CD103⁺ dendritic cells that cross-present Ags to CD8⁺ T cells and produce IL-12 in response to pathogens (36). This is in agreement with our observation that induction of *Batf3* was not found in IFN- γ , IL-4, or *M. tuberculosis* infection in macrophages.

Batf2 protein was predominantly found in the nucleus after 6 h of IFN- γ stimulation, indicating its involvement in signal transduction-mediated gene regulation. This was confirmed by Batf2 knockdown experiments, which perturbed a limited number of inflammatory effector genes, demonstrating that Batf2 is an essential transcription factor for gene regulation and effector functions in classical macrophage activation. Owing to the incapability of currently available chromatin immunoprecipitation-grade Ab against Batf2, we were limited by coimmunoprecipitation studies where we could demonstrated that Batf2 associates with Irf1 in IFN- γ - and LPS-stimulated BMDMs. Transcription factor binding motif analysis showed that 27 of 78 Batf2-perturbed gene such as *Tnf*, *Ccl5*, *Ccr5*, *Irg1*, *Il6ra*, and *Cxcl9* has Irf1 binding sites (Supplemental Table I). Irf1 knockdown in LPS-stimulated BMDMs resulted in downregulation of classical effector genes. Thus, our results suggest that Batf2-mediated regulation of Irf1-regulated classical effector genes is carried out in a combinatorial way by association with Irf1. A recent publication revealed the importance of Batf/Irf complexes, where IRF4/BATF/JunB complexes bind to presumptive regulatory sequences in genes required by Th17 cells (37, 38, 45) and the Irf4, Irf8, and Batf complex is essential for the development of CD8⁺ classical dendritic cells (36, 37), supporting our findings. We observed that Socs1 expression is not affected by Batf2 knockdown in IFN- γ induction, but is affected in the LPS induction (46, 47). It is known that signal transduction of IFN- γ stimulation consists of Stat-dependent and -independent pathways where *Nos2* and *Tnf*

expression are regulated by the former pathway and Socs1 by the latter one (48), suggesting that Batf2 is not involved in the transcriptional regulation downstream of the Stat-independent pathway. However, our results show that Socs1 expression is alternatively transcriptionally regulated in a Batf2-dependent manner downstream of TLR-4-mediated signal transduction, where *Il12b* gene expression may be also under the same transcriptional regulation. Such complicated transcriptional regulation needs to be further explored to understand precise regulation mechanisms for classical effector genes.

M. tuberculosis infection induced Batf2 in macrophages, suggesting that IFN- γ stimulation and *M. tuberculosis* infection act in concert. This is supported by IFN- γ /LPS costimulation experiments in macrophages, which increased induction of Batf2 expression compared with single stimulation (Fig. 5G, Supplemental Fig. 3E) (36). Moreover, some of the downregulated genes found during Batf2 knockdown experiments in IFN- γ -stimulated macrophages were strongly upregulated during *M. tuberculosis* infection in macrophages, and particularly in infected IFN- γ -stimulated macrophages. One of the main functions of M1 is their inflammatory responses and antimicrobial functions, including the production of reactive nitrogen intermediates, which effectively kills *M. tuberculosis* and other intracellular pathogens in mouse macrophages. IFN- γ - and LPS-induced NO synthase is catalyzed by L-arginine to citrulline and NO, the later an effective antimicrobial radical and one of the main killers of *Mycobacterium* in mice, also demonstrated by *Nos2*-deficient mice, which are hypersusceptible to *M. tuberculosis* (16, 25). Global analysis of Batf2 knockdown genes show well-characterized classical effectors such as *Ccl11*, *Cxcl9*, *Ccr5*, *Niacr1*, *Il6ra*, and *Irg1* were downregulated after Batf2 knockdown.

Interestingly, *M. tuberculosis* infection experiments using knockout mice from our list of Batf2-regulated genes, such as *Nos2*, *Ccr5*, *Niacr1*, *Cxcr3*, *Irf1*, and *Stat1*, showed either susceptible or resistant phenotypes to tuberculosis infection (49–55). Therefore, Batf2 may play an important role in immune response by possibly maintaining the balance in the inflammatory process. Recently generated Batf2-deficient mice showed increased mortality to *T. gondii* infection compared with wild-type and Batf3-deficient mice. Parasite burden and cytokine levels were, however, similar compared with control and normal macrophage populations and were present in the peritoneum and liver but reduced in the lungs (36). However, a possible role of macrophages in toxoplasmosis was not explored. Host protection against *T. gondii* is also dependent on M1 effector functions, including NO. Together with our *M. tuberculosis* study, these results underline the role of Batf2/Irf1 in certain type 1 protective experimental human infectious diseases. Manipulating the Batf2 pathway may be therefore a valuable strategy to increase immunity to type 1 infectious diseases, particularly for tuberculosis.

Disclosures

The authors have no financial conflicts of interest.

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