Science Signaling
AAAS
www.sciencesignaling.org/cgi/content/full/3/135/ra62/DC1

Supplementary Materials for

Arginine Usage in Mycobacteria-Infected Macrophages Depends on Autocrine-Paracrine Cytokine Signaling

Joseph E. Qualls, Geoffrey Neale, Amber M. Smith, Mi-Sun Koo, Ashley A. DeFreitas, Huiyuan Zhang, Gilla Kaplan, Stephanie S. Watowich, Peter J. Murray*

*To whom correspondence should be addressed. E-mail: peter.murray@stjude.org

Published 17 August 2010, *Sci. Signal.* **3**, ra62 (2010)
DOI: 10.1126/scisignal.2000955

The PDF file includes:

Materials and Methods
Fig. S1. The expression of Arg1 and C/EBPβ during infection with BCG is dependent on MyD88.
Fig. S2. Principal components analysis (PCA) of the Affymetrix transcript array.
Fig. S3. C/EBPβ is partially required for the expression of *Arg1* after stimulation with supernatant from BCG-infected cells.
Fig. S4. BCG-induced expression of *Arg1* is independent of the alternative activation of macrophages.
Fig. S5. IL-6, IL-10, and G-CSF are produced by wild-type, but not *Myd88*$^{-/-}$, macrophages after infection with BCG.
Fig. S6. Expression of *Arg1* is induced by recombinant IL-6, IL-10, and G-CSF.
Fig. S7. The expression of *Arg1* is intact in BMDMs from *Il6*$^{-/-}$ mice after infection with BCG.

Materials and methods

Mice

C57BL/6, BALB/c, *Il6*$^{-/-}$, *Il10*$^{-/-}$, *Il4ra*$^{-/-}$, *Stat6*$^{-/-}$, and *Cebpb*$^{-/-}$ mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and bred within the Animal Resource Center at St. Jude Children's Research Hospital. *Myd88*$^{-/-}$ mice were obtained from D. Green (St. Jude Children's Research Hospital, Memphis, TN) and were originally a gift from S. Akira (Osaka University, Osaka, Japan). *Socs3*$^{fl/fl}$ mice were a gift from D. Hilton and W. Alexander (Walter and Eliza Hall Institute, Melbourne, Australia) and were bred to Tie2cre mice to induce deletion of *Socs3* in hematopoietic cells. Bones from *Stat3*$^{+/fl}$ and *Stat3*$^{\Delta/fl}$;Tie2cre mice were provided by S. Watowich (M.D. Anderson, Houston, TX). All mice in this study were used according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at St. Jude Children's Research Hospital.

Isolation of macrophages

Unless otherwise noted, all experiments involving the isolation and treatment of macrophages used three animals of each genotype. To prepare FLDMs, fetal liver cells were isolated from embryos (embryonic days 12 to 14) derived from the intercross of *Cebpb*$^{+/-}$ female and *Cebpb*$^{+/-}$ male mice. Cells were grown for 7 to 8 days in L-cell conditioned media (LCCM). FLDMs were plated in 24-well plates at 5 x 10^5 cells/well in LCCM (500 µl) and allowed to adhere overnight. Media was changed to Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) 1 hour before infection. To prepare peritoneal-derived macrophages (PDMs), 4 to 8 week old mice were injected intraperitoneally with sterile thioglycolate (5 ml). Three days later, PDMs were collected by lavage. Cells were plated at 1 x 10^6 cells/well in 12-well plates in RPMI containing 10% FBS, penicillin, and streptomycin. For the preparation of BMDMs, femurs and tibias were removed from 4 to 8 week old mice, and bone marrow was flushed with sterile PBS. Bone marrow cells were resuspended and grown for 5 to 6 days in LCCM. BMDMs were plated in 6-well plates (at 2 x 10^6 cells/well in 2 ml of medium) or in 12-well plates (1 x 10^6 cells/well in 1 ml of medium) and allowed to adhere overnight. Media was changed to DMEM containing 10% FBS 1 hour before infection.

Western blotting and histological analyses

For Western blotting analysis, cells were lysed in radioimmunoprecipitation (RIPA) buffer containing protease inhibitor cocktail tablets (Roche Diagnostics). Protein lysates were separated by Tris-HCl–buffered 4 to 15% gradient SDS PAGE (Bio-Rad), followed by transfer to Protran membranes (Sigma Aldrich). Membranes were blocked in 3% milk in Tris-buffered saline containing 0.05% Tween and then analyzed with the appropriate antibody. Antibodies for Western blotting included chicken polyclonal antibody against Arg1 (1:2000, a gift from S. Morris, University of Pittsburgh); rabbit polyclonal antibody against total STAT1 (1:1000, clone E-23, sc-346, Santa Cruz Biotechnology); rabbit polyclonal antibody against pSTAT1 (1:1000, pTyr^{701}, #9171, Cell Signaling Technology); rabbit polyclonal antibody against total STAT3 (1:1000, cat. #9132, Cell Signaling Technology); rabbit polyclonal antibody against pSTAT3 (1:1000, pTyr^{705}, cat. #9131, Cell Signaling Technology); total STAT4 (1:5000, a gift of James Ihle, St. Jude Children's Research Hospital); mouse monoclonal antibody against pSTAT4 (1:1000, pTyr^{693}, cat. #612738, BD Biosciences); mouse monoclonal antibody against total STAT5 (1:1000, cat. #S21520, BD Biosciences); rabbit polyclonal antibody against pSTAT5 (1:1000, pTyr^{694}, cat. #9351, Cell Signaling Technology); rabbit polyclonal antibody against

total STAT6 (1:1000, clone S-20, sc-621, Santa Cruz Biotechnology); rabbit polyclonal antibody against pSTAT6 (1:1000, $pTyr^{641}$, #9361, Cell Signaling Technology); and mouse monlclonal antibody against Grb2 (1:2000, clone 81, cat. #610112, BD Biosciences). Histology sections were stained by the St. Jude Veterinary Pathology Core with hematoxylin and eosin, standard staining for acid fast bacilli, and rabbit polyclonal antibody against Arg1 (clone H-52, sc-20150, Santa Cruz Biotechnology).

ELISAs

96-well plates were coated with capture antibodies against IL-6 (50 μl at 0.5 μg/μl, cat. #554400, BD Biosciences) or IL-10 (1 μg/μl, cat. #551215, BD Biosciences) diluted in PBS and incubated overnight at 4°C. After washing, plates were blocked for 2 hours at room temperature with PBS containing 10% FBS (50 μl). Plates were washed, and 50 μl of standards or supernatants from BCG-infected BMDMs was added and incubated overnight at 4°C. After washing, 50 μl of biotinylated antibodies against IL-6 (0.5 μg/ml, cat. #554402, BD Biosciences) or IL-10 (0.5 μg/ml, cat. #554423, BD Biosciences) were added and incubated for 1 hour at room temperature. Plates were washed and 50 μl of horseradish peroxidase (HRP)-conjugated streptavidin (diluted 1:1000 in PBS containing 10% FBS, Sigma Aldrich) was added and incubated for 30 min at room temperature. Plates were washed and 50 μl of TMB-peroxidase substrate solution (KPL) was added followed by 50 μl of 10% phosphoric acid to stop the reaction. Absorbance was measured at 405 nm on the SpectraMax 340 spectraphotometer with Softmax Pro 2.1 software (Molecular Devices).

Additional reagents

Recombinant IL-1β, IL-6, IL-10, and TNF-α, were obtained from BD Biosciences. G-CSF was obtained from R&D Systems.

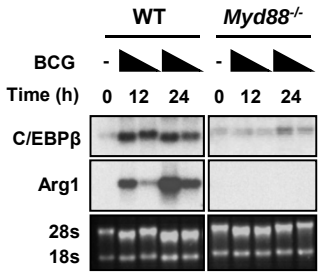

Fig. S1. The expression of Arg1 and C/EBPβ during infection with BCG is dependent on MyD88. BMDMs from wild-type and *Myd88*$^{-/-}$ mice were left untreated or were infected with BCG (at MOIs of 100 or 10). RNA was collected at 12 and 24 hours after infection and analyzed by gel electrophoresis and Northern blotting. Data are representative of two individual experiments with at least three mice per group.

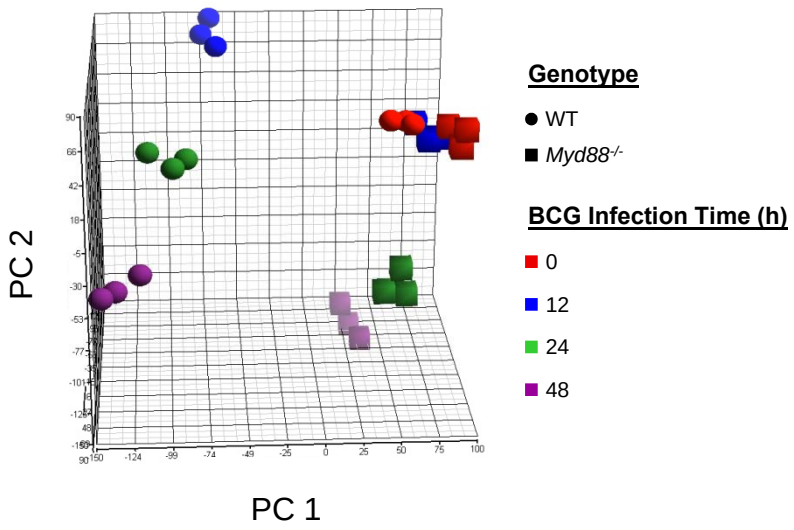

Fig. S2. Principal components analysis (PCA) of the Affymetrix transcript array. Wild-type and *Myd88*$^{-/-}$ BMDMs were left untreated or were infected with BCG (at an MOI of 10). RNA was collected and subjected to Affymetrix expression analysis. Data are grouped by wild-type (spheres) and *Myd88*$^{-/-}$ (cubes) samples from individual mice (n = 3) that were uninfected (red) or were infected with BCG for 12, 24, or 48 hours (blue, green, and purple, respectively).

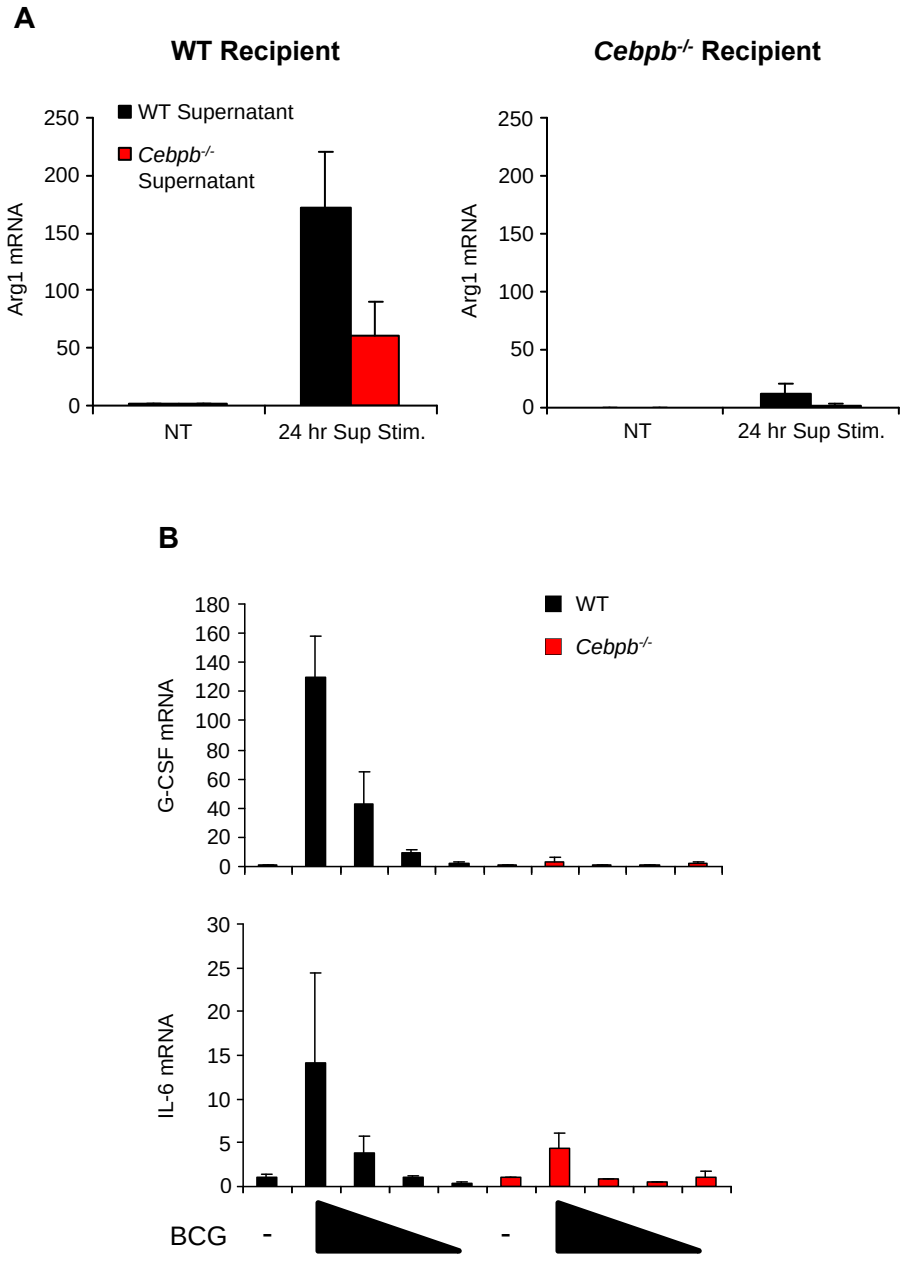

Fig. S3. C/EBPβ is partially required for the expression of *Arg1* after stimulation with supernatant from BCG-infected cells. (**A** and **B**) Wild-type and *Cebpb*$^{-/-}$ FLDMs (n = 3 mice) were infected with BCG at MOIs of 500, 100, 25, and 5 for 24 hours. (A) Pooled supernatants (at a 1:2 dilution) from the cells infected at MOIs of 500 and 100 were used to stimulate FLDMs from wild-type of *Cebpb*$^{-/-}$ mice (n = 3) for 24 hours. The expression of Arg1 was determined by qRT-PCR. (**B**) RNA from BCG-infected FLDMs was analyzed by qRT-PCR.

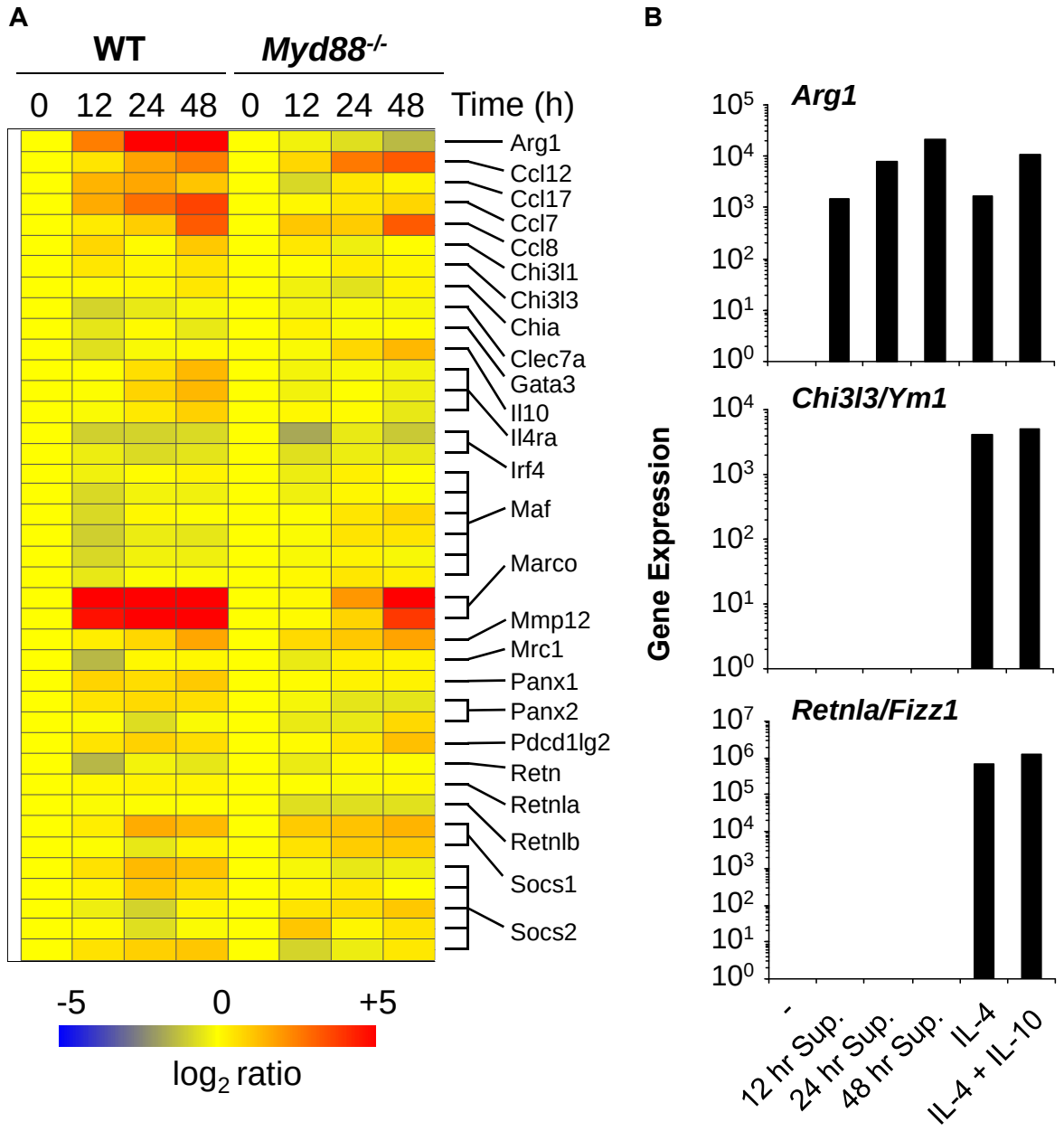

Fig. S4. BCG-induced expression of *Arg1* is independent of the alternative activation of macrophages. (**A**) The $\log_2$ ratio heat map representing the expression of genes associated with the alternative activation of macrophages after infection with *M. bovis* BCG. (**B**) qRT-PCR analysis of macrophages after stimulation with supernatants from BCG-infected cells. Stimulations with IL-4 alone or together with IL-10 were used as positive controls for the alternative activation of macrophages.

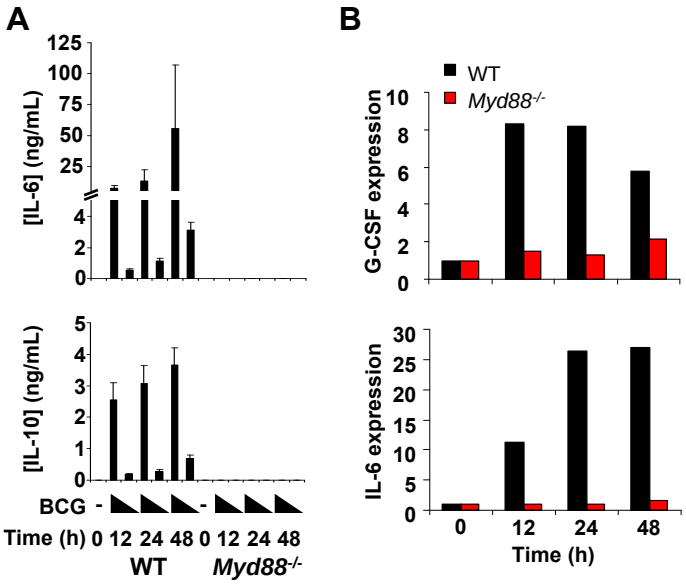

Fig. S5. IL-6, IL-10, and G-CSF are produced by wild-type, but not *Myd88*$^{-/-}$, macrophages after infection with BCG. (**A**) IL-6 and IL-10 in the supernatants of BCG-infected BMDMs from wild-type and *Myd88*$^{-/-}$ mice (n = 3) were detected by enzyme-linked immunosorbent assay (ELISA). (**B**) BMDMs from wild-type and *Myd88*$^{-/-}$ mice were subjected to Affymetrix expression analysis as described for Fig. 1D. Data are presented as the mean fold-increase in the abundance of G-CSF and IL-6 mRNAs.

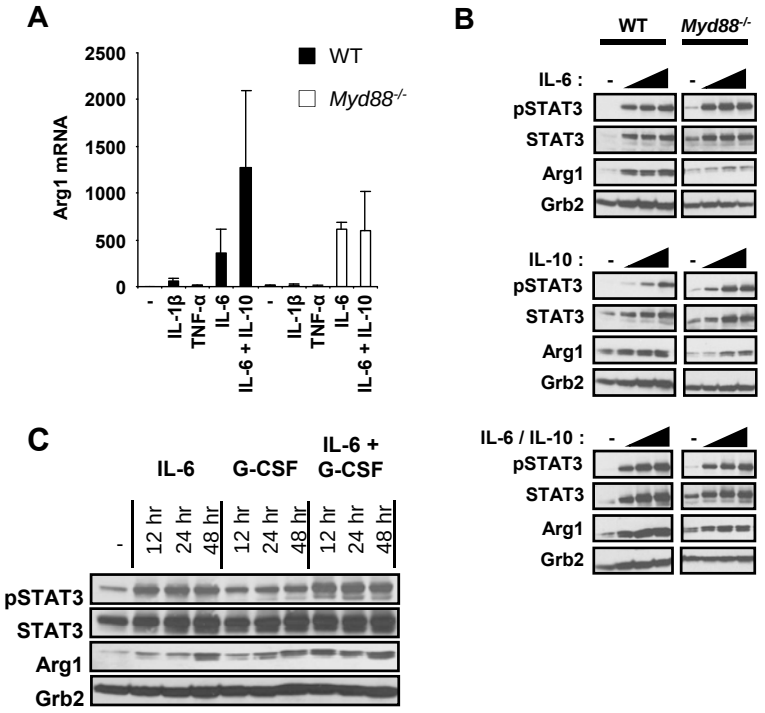

Fig. S6. Expression of *Arg1* is induced by recombinant IL-6, IL-10, and G-CSF. (**A**) BMDMs from wild-type mice (black bars) and *Myd88*$^{-/-}$ mice (white bars) were stimulated with IL-1β, TNF-α, IL-6, or IL-6 and IL-10 (all at 10 ng/ml) for 24 hours, and RNA was collected and analyzed by qRT-PCR. Data are presented as the mean fold-increase ± SD in the abundance of Arg1 mRNA normalized to that of GAPDH mRNA. (**B**) BMDMs from wild-type mice and *Myd88*$^{-/-}$ mice were untreated or were treated with IL-6, IL-10, or IL-6 and IL-10 for 24 hours at varying concentrations (2, 10, and 50 ng/ml). Whole-cell lysates were analyzed by Western blotting for the indicated proteins, with Grb2 acting as a loading control. (C) Pooled PDMs from three wild-type mice were stimulated with IL-6, G-CSF, or IL-6 and G-CSF (all at 10 ng/ml) for the indicated times. Whole-cell lysates were analyzed by Western blotting for the indicated proteins. Data are representative of two experiments with at least three mice per group (A and C), or of one experiment with one mouse per group (B).

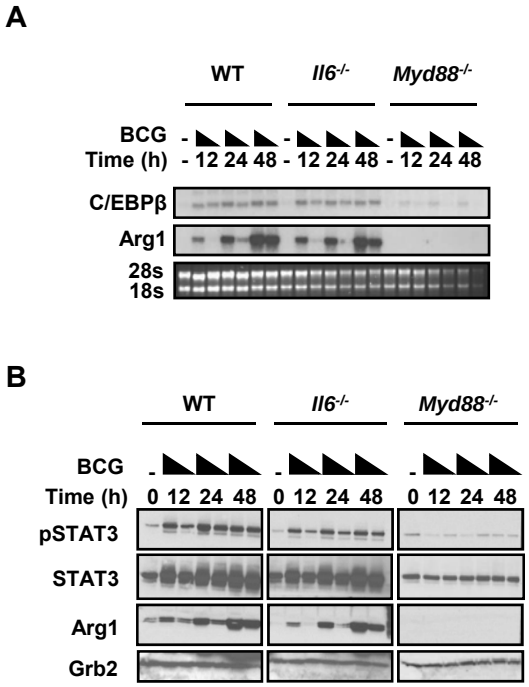

Fig. S7. The expression of *Arg1* is intact in BMDMs from *Il6*$^{-/-}$ mice after infection with BCG. (**A** and **B**) BMDMs from wild-type, *Il6*$^{-/-}$, and *Myd88*$^{-/-}$ mice were untreated or were infected with BCG (at MOIs of 100 and 10). RNA and whole-cell lysates were collected at 12, 24, and 48 hours after infection. (A) RNA was analyzed by Northern blotting. (B) Whole-cell lysates were analyzed by Western blotting for the indicated proteins, with Grb2 used as a loading control. Data are representative of two experiments with at least three mice per group.