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*J Immunol* 2011; 186:5885-5895; Prepublished online 11 April 2011;
doi: 10.4049/jimmunol.1003447
http://www.jimmunol.org/content/186/10/5885

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/04/11/jimmunol.1003447.DC1

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Transcriptional Suppression of IL-27 Production by Mycobacterium tuberculosis-Activated p38 MAPK via Inhibition of AP-1 Binding

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*Mycobacterium tuberculosis* remains a major global challenge to human health care, and the mechanisms of how *M. tuberculosis* evades host immune surveillance to favor its survival are still largely unknown. In this study, we found that bacillus Calmette–Guérin (BCG) and viable *M. tuberculosis* as well as *M. tuberculosis* lysates could activate IL-27 expression in human and mouse macrophages by induction of p28 subunit transcription. However, in parallel with these effects, BCG and *M. tuberculosis* lysate stimulation of macrophages induced activation of p38 MAPK signaling molecules MLK3/MKK3/MK2 to prevent maximal IL-27 production. *M. tuberculosis* lysate-induced p28 transcription was dependent on MyD88 signaling pathway. AP-1/c-Fos was shown to bind directly to the p28 promoter and induce p28 expression after *M. tuberculosis* lysate stimulation. Overexpression of p38 inhibited the binding of c-Fos to the p28 promoter but had no effect on c-Fos protein expression or phosphorylation in response to *M. tuberculosis* lysate stimulation. Furthermore, blockade of p38 by SB203580 enhanced *M. tuberculosis*-induced AP-1 binding to the p28 promoter. Importantly, we show that adding exogenous IL-27 to increase the levels produced by PBMCs stimulated with live mycobacteria enhanced the ability of BCG-expanded T cells to inhibit intracellular mycobacterial growth in human macrophages. Taken together, our data demonstrate that mycobacterial stimulation induces both IL-27 production and p38 MAPK activation. Strategies designed to tip the balance toward positive regulation of p28 induction by mycobacteria could lead to enhanced protective tuberculosis immunity. *The Journal of Immunology*, 2011, 186: 5885–5895.
17). However, though the CFU were reduced, the infected WSX-1 knockout mice died much earlier than infected WT mice, presumably due to excessive inflammation and pathology in the lungs and liver (17). It is worth noting that these two studies were performed using WSX-1 knockout mice, and it is possible that the newest member of the IL-12 cytokine family, IL-35 (18), might also induce signals in immune cells through WSX-1. If so, the previous results generated in WSX-1 knockout mice may be difficult to interpret because of a potential deficiency of both IL-27 and IL-35 signaling. Therefore, further evaluation of the effects of IL-27 on tuberculosis (TB) immunity in humans is needed. Recently, it has been reported that vaccine-induced protection against virulent M. tuberculosis is associated with robust production of IL-27 and IFN-γ (19). Other human studies also have shown that vaccination of infants with bacillus Calmette–Guérin (BCG) could induce potent IL-27 responses (20), and that IL-27 was highly expressed in Th1-associated granulomas in TB patients (21). Taken together, these data indicate that IL-27 may play an important role in mycobacterial immunity both by enhancing immune responses critical for inhibition of bacterial growth and by preventing immune pathology associated with TB disease progression.

In this study, we found that IL-27 production in response to M. tuberculosis stimulation is tightly controlled by regulation of the p28 subunit at the transcriptional level. The transcription factor AP-1/c-Fos mediates M. tuberculosis lysate-induced p28 gene transcription. However, in parallel the maximal expression of p28 is suppressed by M. tuberculosis lysate-activated p38 MAPK through inhibition of c-Fos binding to the p28 promoter. Our data also clearly show that IL-27 enhances the ability of M. tuberculosis-specific T cells to inhibit intracellular mycobacterial growth in human macrophages. Our work demonstrates important cross-regulation between p38 MAPK and the regulation of IL-27 expression during M. tuberculosis stimulation of macrophages with implications for development of immunotherapeutic approaches for the control of TB infection.

Materials and Methods

Cells
The murine macrophage cell lines RAW264.7 (RAW) and J774A.1 (J774) were obtained from American Type Culture Collection and maintained in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (Endotoxin NMT 10.0 EU/ml; Sigma, St. Louis, MO). Peritoneal macrophages were obtained by lavage of C57Bl/6 mice, kindly provided by Dr. Aihao Ding (Weill Cornell Medical College, New York, NY) with permissions from Dr. John M. Kyriakis (Tufts University School of Medicine, Boston, MA), Dr. Jim Woodgett (Samuel Lunenfeld Research Institute, Toronto, ON, Canada), Dr. Jiahui Han (The Scripps Research Institute, La Jolla, CA), and Dr. Matthias Gaetel (Medical School Hannover, Hannover, Germany). Expression vectors A-Fos and c-Fos were originally provided by Dr. Thomas Curren (St. Jude Children’s Hospital, Memphis, TN) and Dr. Charles Vinson (National Cancer Institute, Bethesda, MD). All plasmid DNA was prepared with Endo-free Maxi-Prep kits (Qiagen).

Reagents
Abs against c-Fos and c-Jun were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Abs against phosphorylated p38, total p38, phosphorylated c-Fos, and total c-Fos were purchased from Cell Signal Technology (Danvers, MA). Recombinant mouse IFN-γ was purchased from Genzyme (Boston, MA). LPS produced by Escherichia coli 0217:B8 was purchased from Sigma-Aldrich (St. Louis, MO). Mouse and human M-CSF were purchased from R&D Systems (Minneapolis, MN) and PeproTech (Rocky Hill, NJ), respectively.

Preparation of viable M. tuberculosis and M. tuberculosis whole-cell lysates
Erdman strain M. tuberculosis and GFP-expressing BCG (23) were grown to mid-log phase in Middlebrook 7H9 media supplemented with ADC and 200 ng/ml amphotericin B. Bacteria were pelleted, washed, resuspended in 1/10 volume fresh media, and frozen at −70°C until use. Dilutions of this stock were plated on Middlebrook 7H10 agar supplemented with OADC for determination of bacterial concentration (in CFU). Whole-cell lysates of the Erdman strain of M. tuberculosis were prepared from mid-logarithmic phase cultures grown in glycerol–alanine salts broth (24). After washing with PBS, mycobacteria were heat killed (80°C for 1 h) then disrupted by sonication or bead vortex before passage through 0.2-μm filters. M. tuberculosis lysates were quantified by Bradford protein assay. Viable M. tuberculosis and BCG were diluted with complete RPMI media and added to cultured macrophages at multiplicities of infection (MOIs) as indicated. After different times, culture supernatants and total RNA were collected for measurement of protein and mRNA expression, respectively.

Mycobacterial infection assay
T cell-mediated inhibition of intracellular mycobacteria was measured as described previously (25, 26). Total PBMCs isolated from purified protein derivative (PPD) healthy donors were rested in medium alone or stimulated with live BCG in the presence of different amounts of human recombinant IL-27 for 7 d. Simultaneously, adherent monocytes were matured to macrophages with human serum for 6 d, followed by infection with live BCG overnight at an MOI of 3:1. On day 7, the infected macrophages were carefully washed to remove extracellular mycobacteria followed by addition of BCG-expanded PBMCs at a ratio of 10:1 (T cells versus macrophages). After 3-d coculture of BCG-expanded cells with the infected macrophages, saponin was added to release BCG from infected macrophages, and viable BCG were quantitated by tritiated uridine incorporation.

Flow cytometric analysis
T lymphocytes were stimulated with PMA (50 ng/ml)/ionomycin (1 μg/ml) in the presence of GolgiStop (4 μl of BD GolgiStop for every 6 ml of cell culture) for 5 h. Then, cells were harvested and incubated with fixation/permeabilization buffer, followed by suspending the cells with 50 μl of Perm/Wash buffer and 20 μl tube of human Th1/Th17 phenotype mixing (BD Biosciences) and incubating at room temperature for 30 min in the dark, followed by FACs analysis.

Quantitative real-time PCR
Reverse-transcription reactions were carried out as described previously (27). Briefly, 1-μl aliquots of total RNA were used for cDNA syntheses. cDNA samples were diluted at different concentrations and mixed with PCR primers (10 μM) targeting different mouse and human genes with SYBR Green PCR master mix (Applied Biosystem, Carlsbad, CA) in 15 μl volumes. The sequences of primers used for quantitative real-time PCR (qRT-PCR) are listed in Supplemental Fig. 1.

ELISAs
Supernatants from human T cell, murine peritoneal macrophage, or BDMM cultures were harvested after stimulation with IFN-γ, LPS, BCG, and/or M. tuberculosis whole-cell lysates and stored at −70°C. Mouse IL-27 p28 and IL-12 p70 were detected using Quantikine ELISA kits (R&D Systems) and BD OptEIA kits (BD Biosciences, San Jose, CA), respectively, according to the manufacturers’ instructions. Human IFN-γ and IL-17 were measured.
by ELISA kits purchased from PeproTech and from BioLegend (San Diego, CA), respectively. Concentrations were calculated by regression analysis of a standard curve.

**Transfection assay**
Transient transfections in RAW or J774 cells were performed by electroporation as described previously (27).

**Nuclear extract preparation and LightShift Chemiluminescent EMSA**
Isolation of nuclear extracts and performance of EMSA were carried out as described previously (27).

**Primary transcript measurement**
To determine the rate of p28 gene primary transcript, cDNA was synthesized with random primers using 1 µg DNase-treated RNA generated from mouse macrophages stimulated with different amounts of *M. tuberculosis* lysates as indicated. The two sets of primers used for measurement of p28 primary transcripts by qRT-PCR are listed in Supplemental Fig. 1.

**Western blotting**
SDS-PAGE was performed with 40–100 µg protein extracts. Gels were transferred to polyvinylidene fluoride membranes and blocked in 5% nonfat milk in Tris buffer, pH 8. Primary Ab was added at a concentration of 1 µg/ml in 5% milk/Tris buffer and left overnight at 4˚C. After washing extensively, secondary Ab conjugated to HRP was added at a 1:3000 dilution in 5% milk/Tris buffer. After extensive washing, blots were subjected to ECL detection (PerkinElmer Life Sciences, Boston, MA).

**Chromatin immunoprecipitation assay**
The chromatin immunoprecipitation (ChIP) procedure was performed using an assay kit following the manufacturer’s instructions (Upstate Biotechnology, Lake Placid, NY). Briefly, 1 × 10⁷ J774 cells were stimulated for 2 h and then cross-linked by addition of 1% formaldehyde. Nuclei were fragmented by sonication. Chromatin fractions were precleared with protein A-agarose beads followed by immunoprecipitation overnight with 2 µg anti–c-Fos or control Abs. Cross-linking was reversed followed by proteinase K digestion. DNA was purified and subjected to PCR. The input and immunoprecipitated DNA were amplified by PCR using primers encompassing the AP-1–RE in the mouse p28 promoter (see Supplemental Fig. 1), and analyzed by electrophoresis on 1.2% agarose gel.

**Statistical analysis**
Student *t* tests were performed wherever applicable. SD of the mean is shown in figures unless otherwise indicated.

**Results**
**BCG and *M. tuberculosis* activate IL-27 expression through induction of the p28 subunit**
To determine the effects of BCG and *M. tuberculosis* on IL-27 expression, we treated macrophages with different amounts of live

![](https://example.com/Figure1.png)

**FIGURE 1.** IL-27 p28 and EBI3 mRNA expression in BCG- and *M. tuberculosis*-treated macrophages. Fully differentiated BMDMs (2 × 10⁶) derived from WT B6 mice were infected with live *M. tuberculosis* at different MOIs as indicated for 6 h, and then total RNA was isolated from the infected BMDMs for measurement of p28 (A) and EBI3 (B) mRNA expression by qRT-PCR. Fully differentiated human macrophages (2 × 10⁶) were treated with different amounts of live BCG and *M. tuberculosis* whole-cell lysate as indicated for either 48 h for measurement of IL-27 protein production by ELISA (C) or for 6 h before RNA extraction for detection of IL-27 p28 (D) and EBI3 (E) mRNA expression by qRT-PCR. Fully differentiated BMDMs (3 × 10⁶) were treated with live BCG and *M. tuberculosis* whole-cell lysates as indicated for different times as described above. Murine IL-27 p28 protein production (F) and p28 (G) as well as EBI3 (H) mRNA expression were measured by ELISA and qRT-PCR, respectively. qRT-PCR data were normalized relative to GAPDH mRNA expression levels in each respective sample and further normalized to the results from the untreated group (Med). Data shown are means plus SD from three experiments. Mtb, *M. tuberculosis*; Mtb-L, *M. tuberculosis* whole-cell lysates.
M. tuberculosis for 6 h, followed by measurement of IL-27 p28 and EBI3 mRNA expression. Viable M. tuberculosis significantly induced p28 mRNA expression in a dose-dependent manner (Fig. 1A), whereas EBI3 was only slightly induced (Fig. 1B), suggesting that live mycobacteria could induce IL-27 expression in macrophages. We next wanted to determine whether BCG and M. tuberculosis lysates could also induce IL-27 expression in human and mouse macrophages. We stimulated human macrophages with different amounts of live BCG and M. tuberculosis lysates for different times, followed by measurement of protein and mRNA expression. Similar to viable M. tuberculosis infection, live BCG and M. tuberculosis lysates dose-dependently induced IL-27 protein production (Fig. 1C) and p28 mRNA expression (Fig. 1D). The EBI3 mRNA expression was only slightly induced by BCG and M. tuberculosis lysates compared with that of untreated cells (Fig. 1E), which is also in line with our previous studies showing that p28 is the limiting and inducible subunit for making biologically activated IL-27, whereas EBI3 is constitutively expressed (22). Similar effects of BCG and M. tuberculosis lysates on IL-27 production as well as on p28 and EBI3 mRNA expression were observed in mouse BMDMs (Fig. 1F–H) and in mouse macrophage cell lines (Supplemental Fig. 2A, 2B). These data indicate that the p28 subunit is responsible for BCG/M. tuberculosis-induced IL-27 production and is regulated similarly in human and mouse macrophages.

FIGURE 2. The role of p38 MAPK in BCG- and M. tuberculosis-induced IL-27 p28 production. BMDMs (1 × 10⁶) were treated with different amounts of a p38 MAPK antagonist SB203580 for 1 h prior to addition of various stimuli as indicated. An identical amount of dissolvent DMSO was used for negative control in each experiment. Forty-eight hours later, the cell-free supernatants were collected and used for measurement of IL-27 p28 (A, B) and IL-12 p70 (C) production by ELISA. Data shown are means plus SD of three experiments. Five micrograms of different expression plasmids encoding different molecules involved in the p38 MAPK signaling pathway including p38α (D), DN-MK2 (E), MLK3 (F), MKK3 (G), and MKK6 (H) or empty vector were transiently transfected with Lipofectamine. Forty-eight hours after transfection, the cells were treated with LPS (1 μg/ml) or M. tuberculosis lysates (1 μg/ml) for 6 h, followed by measurement of p28 mRNA expression by qRT-PCR. qRT-PCR data were normalized relative to GAPDH mRNA expression levels in each respective sample and further normalized to the results from the untreated group (Med). Data shown are means plus SD from three experiments. Mtb-L, M. tuberculosis whole-cell lysates.

p38 MAPK inhibits BCG- and M. tuberculosis-induced IL-27 p28 production through MLK3/MKK3/MK2 molecules

It has been reported that many cytokines, including IL-12, IL-1β, IL-6, TNF-α, and IL-10, play important roles in immune responses directed against infection and are regulated by p38 MAPK (28, 29). To investigate whether M. tuberculosis-induced IL-27 production was regulated by p38 MAPK, we blocked p38 MAPK signaling using a specific inhibitor SB203580 prior to BCG and M. tuberculosis lysate stimulation. The results showed that blockade of p38 MAPK signaling resulted in dose-dependent inhibition of IL-27 p28 production.
enhancement of BCG- and M. tuberculosis lysates-induced (Fig. 2A) as well as LPS-induced (Fig. 2B) p28 protein production. Consistent with previous reports (30, 31), blockade of p38 with SB203580 significantly augmented LPS-induced IL-12 p70 production (Fig. 2C). To confirm the effect of p38 on M. tuberculosis-induced p28 expression, we overexpressed p38 in J774 cells, followed by M. tuberculosis lyse stimulation. Overexpression of p38 suppressed M. tuberculosis lyse-induced p28 mRNA expression compared with that of empty vector transfected cells (Fig. 2D), further demonstrating that p38 activation inhibits IL-27 p28 induction. To identify which molecules in the p38 signaling cascade were involved in inhibition of M. tuberculosis-induced p28 expression, we overexpressed upstream molecules (MLK3/MKK3/MKK6) or a dominant negative mutant of the downstream molecule (MK2) in J774 cells, followed by measurement of p28 mRNA expression after M. tuberculosis lyse stimulation. As shown in Fig. 2E, blocking MK2 with an MK2 dominant negative mutant (DN-MK2) significantly enhanced M. tuberculosis lysate-induced p28 mRNA expression (p = 0.0038). In addition, overexpression of MLK3 (Fig. 2F) and MKK3 (Fig. 2G) suppressed M. tuberculosis lysate-induced p28 mRNA expression, whereas MKK6 had little effect (Fig. 2H), indicating the involvement of MLK3 and MKK3 in inhibition of p28 expression. Taken together, these data demonstrate that p38 activated by M. tuberculosis lyse stimulation suppresses IL-27 p28 expression through upstream MLK3 and MKK3 and downstream MK2 molecules, which could limit maximal production of IL-27 and prevent optimal protective immune responses during M. tuberculosis infection.

M. tuberculosis-induced p28 gene expression is mediated through MyD88 at the transcriptional level

Our previous studies show that IL-27 p28 expression is dependent on the MyD88 signaling pathway in macrophages treated with LPS (22). We wondered whether the M. tuberculosis/BCG-induced IL-27 is also dependent on MyD88. As shown in Fig. 3A,

![FIGURE 3. MyD88 is required for M. tuberculosis-induced p28 transcription. A and B, BMDMs (3 x 10^5) differentiated from BM cells of WT and MyD88^-/- mice were stimulated with different amounts of M. tuberculosis lysates (A) and live BCG (B) as indicated for 6 h, followed by extraction of total RNA to measure p28 mRNA expression by qRT-PCR. C, For measuring p28 gene primary transcript, 3 x 10^5 J774 cells were stimulated with M. tuberculosis lysates (1 μg/ml) for different times as indicated, and total RNA was extracted from the stimulated cells for measurement of p28 mRNA expression by qRT-PCR. D, RAW cells (10 x 10^5) were transiently transfected with 5 μg p28 luciferase reporter constructs (−3.2 kb and −835 p28 promoters). Forty hours later, the transfected cells were stimulated with different amounts of M. tuberculosis lysates as indicated for 7 h, followed by lysis and measurement of luciferase activity by luminometer. Luciferase activities in M. tuberculosis-treated cells were normalized to the results from the untreated group (Med). E and F, Five micrograms of various p28 promoter deletion constructs as indicated (E) and an AP-1 mutant construct (F) were transiently transfected into J774 cells. The transfected cells were treated with M. tuberculosis lysates (1 μg/ml) for 7 h, followed by measurement of luciferase activity. Results shown are means plus SD of three to five separate experiments. Mtb-L, M. tuberculosis whole-cell lysates.](http://www.jimmunol.org/)
M. tuberculosis lysate-induced p28 expression was markedly reduced in MyD88-deficient cells compared with that of WT macrophages. The same was true for BCG-induced p28 expression (Fig. 3B). However, the EBI3 mRNA expression was not affected by deletion of MyD88 in cells treated with either BCG or M. tuberculosis lysates (data not shown), indicating that the expression of p28 but not EBI3 is dependent on MyD88 signaling pathway during M. tuberculosis lysate stimulation.

To investigate the molecular mechanisms of M. tuberculosis-induced p28 expression, we measured primary transcript rates of the p28 gene in response to M. tuberculosis lysates in J774 cells, with two pairs of primers corresponding with two different regions of the p28 gene. The use of primers specific for an intron/exon boundary region allowed us to assess the primary transcript rate of p28 inside the nucleus. Supplemental Fig. 3A shows a schematic of the primers used in these experiments. As shown in Fig. 3C and in Supplemental Fig. 3B, p28 primary transcript was induced by M. tuberculosis lysates within 30 min and reached a peak 3 h after stimulation, indicating transcriptional induction of p28 expression by M. tuberculosis lysates. To confirm further whether M. tuberculosis lysates activate p28 gene transcription, we transiently transfected mouse p28 promoter–luciferase constructs into RAW cells by electroporation, followed by stimulating the transfected cells with different amounts of M. tuberculosis lysates and measuring luciferase activity in cell lysates. As shown in Fig. 3D, the p28 promoters (including 3.2 kb or 835 bp upstream of the transcription initiation site) were activated by M. tuberculosis lysates in a dose-dependent manner, further confirming that M. tuberculosis-induced p28 gene expression is regulated at the level of transcription.

To elucidate the molecular basis of M. tuberculosis-mediated transcriptional induction of p28, we decided to localize the functional M. tuberculosis response element in the p28 promoter. RAW cells were transiently transfected with several 5' deletion constructs of the p28 promoter, stimulated with M. tuberculosis lysates for 24 h, and then luciferase activity was measured in cell lysates. The responses to M. tuberculosis lysates were significantly reduced in cells transfected with the −392 plasmid compared with the cells transfected with the −421 plasmid (Fig. 3E), indicating that a major M. tuberculosis “response element” (RE) in the p28 promoter was likely located between −421 and −392. By searching this region, we noticed that a putative AP-1 binding site is presented between −421 and −392. Then, we mutated this putative AP-1 binding site and transiently transfected the WT and AP-1 mutant, as well as the downstream deletion construct (−392 with the AP-1 site deleted), into RAW cells. Responses of the mutant and downstream deletion promoter constructs to M. tuberculosis lysates were almost completely abolished (Fig. 3F), whereas the responses to IFN-γ plus LPS stimulation were only slightly affected by mutation of the AP-1 binding site (data not shown), indicating that this putative AP-1 binding site is indeed critical for responsiveness of the p28 promoter to M. tuberculosis lysate but not LPS stimulation.

AP-1/c-Fos activates p28 gene transcription in response to M. tuberculosis lysate stimulation

A-Fos is a dominant negative mutant of c-Fos that can effectively compete for dimerization between c-Fos and other AP-1 members and therefore block AP-1 function. To confirm that AP-1 controls p28 gene transcription, we first blocked AP-1 function by trans-

**FIGURE 4.** AP-1/c-Fos activates p28 gene transcription. A, Five micrograms of A-Fos or empty vector (PIRES) was transiently transfected into J774 cells with Lipofectamine. Forty-eight hours after transfection, the cells were treated with LPS (1 μg/ml) or M. tuberculosis lysate (1 μg/ml) for 6 h, followed by measurement of p28 mRNA expression by qRT-PCR. qRT-PCR data were normalized relative to GAPDH mRNA expression levels in each respective sample and further normalized to the results from the untreated group (Med). B and C, J774 cells (10 × 10⁶) were transiently cotransfected with 5 μg p28 luciferase reporter construct (−835 p28 promoter) and A-Fos or Pires at different ratio (effector versus reporter). Forty hours later, the transfected cells were stimulated with either M. tuberculosis (B) or IFN-γ plus LPS (C) for 7 h, followed by lysis and measurement of luciferase activity by luminometer. D, Five micrograms of p28 promoter construct (−835) was cotransfected with c-Jun or c-Fos expression vectors or empty vector at different ratios (effector versus reporter) into J774 cells. Forty hours later, the cells were either treated with M. tuberculosis (1 μg/ml) or not for 7 h, followed by measurement of luciferase activity in cell lysates. Transfection data are representative of four to six independent experiments. Mtb-L, M. tuberculosis whole-cell lysates.
fection of A-Fos into J774 cells and studied the effects on p28 induction by *M. tuberculosis* lysate stimulation. As shown in Fig. 4A, p28 mRNA expression induced by *M. tuberculosis* lysates was significantly reduced in A-Fos–transfected cells compared with that of empty vector-transfected cells. In addition, p28 promoter activation induced by *M. tuberculosis* lysates was also completely abolished in cells transfected with A-Fos in comparison with that of cells transfected with empty vector (Fig. 4B). Notably, p28 mRNA expression induced by LPS (Fig. 4A) and promoter activity activated by IFN-γ plus LPS (Fig. 4C) were not affected by overexpression of A-Fos, indicating that AP-1 mainly mediates *M. tuberculosis* but not LPS-induced p28 transcription and mRNA expression. c-Fos and c-Jun are the most common AP-1 proteins in mammalian cells, and c-Fos and c-Jun dimers provide the majority of AP-1 functional activity. To identify further which AP-1 components mediate *M. tuberculosis*–induced p28 gene transcription, we cotransfected J774 cells with the p28 promoter and either c-Fos or c-Jun expression plasmids, or empty vector, and studied the effects on *M. tuberculosis* lysate and IFN-γ plus LPS stimulations. Overexpression of c-Fos dose-dependently activated p28 promoter activity, whereas c-Jun suppressed p28 promoter activation in response to *M. tuberculosis* lysate induction (Fig. 4D). Taken together, these data demonstrate that AP-1/c-Fos plays a critical role in *M. tuberculosis* lysate-induced p28 transcription and mRNA expression.

c-Fos induced by *M. tuberculosis* lysates specifically binds to the p28 promoter both in vitro and in vivo

To explore further the molecular mechanisms responsible for *M. tuberculosis*–induced p28 gene transcription, we performed EMSA using nuclear extracts isolated from BMDMs stimulated with *M. tuberculosis* lysates and probes harboring either WT or AP-1 mutant p28 promoter sequence. As shown in Fig. 5A, there was one major nuclear DNA binding complex formed with the WT probe, and the binding intensity of this complex was increased upon *M. tuberculosis* lysate stimulation. This binding activity was completely abolished with the AP-1 mutant probe (Fig. 5A). Competition EMSA further confirmed the sequence specificity of this binding to the p28 promoter: binding with the labeled WT probe was completely competed by unlabeled WT probe (cold probe) but not by the mutant and unrelated probes (Fig. 5B). To confirm that AP-1 was present in the DNA–nuclear protein binding complex, we performed a “supershift” experiment with anti-c-Fos and anti-c-Jun Abs along with anti–PU-1 Ab and rabbit IgG as controls. As shown in Fig. 5C, the DNA–nuclear protein complex indeed contained c-Fos as anti–c-Fos but not anti-c-Jun specific Ab was able to abolish the slowly mobilized binding, whereas anti–PU-1 Ab and IgG had no effect on the binding. Notably, anti-JunB Ab was also able to supershift the faster mobilized binding (the last lane in Fig. 5C), indicating that JunB is also a member of this binding complex. To determine whether *M. tuberculosis*-stimulated interactions between AP-1/c-Fos and the p28 promoter occur in vivo, ChIP assays were performed in J774 cells. Fig. 5D illustrates the region of the p28 promoter containing the AP-1–RE that was examined in the ChIP assay. Fig. 5E demonstrates that *M. tuberculosis* lysate stimulation induced specific binding between the p28 promoter and c-Fos in J774 cells, which could be immunoprecipitated with anti-c-Fos but not control Ab.

![EMSA](image)

**FIGURE 5.** c-Fos specifically binds to the p28 promoter both in vitro and in vivo. A, Nuclear extracts were isolated from BMDMs stimulated with LPS or *M. tuberculosis* lysate for 4 h. LightShift Chemiluminescent EMSA was performed with 10 μg nuclear extract for each sample and a double-stranded biotin-labeled oligonucleotide probe containing the AP-1–RE from the p28 promoter (sequence given with the critical AP-1–RE underlined) or the AP-1–RE mutated sequence. As shown in Fig. 5A, p28 promoter activity activated by IFN-γ plus LPS (Fig. 4C) were not affected by overexpression of A-Fos, indicating that AP-1 mainly mediates *M. tuberculosis* but not LPS-induced p28 transcription and mRNA expression. B, Competitive EMSA was performed with biotin-labeled WT “hot” probe and various unlabeled “cold” competitors as indicated (molar ratio of 50 to 1 between cold and hot probes) with lysates from *M. tuberculosis*-stimulated BMDMs. C, “Supershift” EMSA was performed with the WT probe and nuclear extracts from *M. tuberculosis*-stimulated BMDMs. Two micrograms of a series of AP-1 Abs and isotype-matched control rabbit IgG were used. The c-Fos– and JunB-related complex is indicated by an arrow. D, Sequence of mouse p28 promoter harboring the AP-1 binding sites. The Journal of Immunology 5891 D
**p38 MAPK induced by M. tuberculosis lysates inhibits the binding of c-Fos to the p28 promoter**

Our data demonstrated that p38 gene expression was suppressed by p38 signaling (Fig. 2) but enhanced by AP-1/c-Fos (Fig. 4). To explore a potential cross-regulation between p38 MAPK and AP-1 in p38 gene transcription, we evaluated the phosphorylation of these two proteins in BMDMs treated with *M. tuberculosis* lysates or LPS. *M. tuberculosis* lyse-induced p38 phosphorylation occurred more rapidly than LPS-induced p38 phosphorylation; p38 phosphorylation peaked 5 min after *M. tuberculosis* lyse stimulation and 15 min after LPS stimulation, respectively (upper panel in Fig. 6A). Phosphorylated c-Fos showed similar expression patterns with delayed kinetics comparing LPS to *M. tuberculosis* lyse stimulation (third panel in Fig. 6A). Expression of total p38 and c-Fos was similar among all groups after either LPS or *M. tuberculosis* lyse treatment (Fig. 6A). To determine whether p38 MAPK affected c-Fos phosphorylation, we either overexpressed p38α (Fig. 6B) or blocked p38 by SB203580 (Fig. 6C) in J774 cells prior to *M. tuberculosis* lyse and LPS treatment. From cells stimulated for 15 min with *M. tuberculosis* lyse or LPS, we collected whole-cell lysates to detect phosphorylated p38 and c-Fos. Overexpression of p38α increased p38 phosphorylation under all conditions compared with that of cells transfected with empty vector (Fig. 6B), indicating a successful transfection of p38α. However, the levels of phosphorylated c-Fos were not different between p38α expressing and nonexpressing cells. Furthermore, although SB203580 successfully abolished p38 phosphorylation induced by LPS or *M. tuberculosis* lyse stimulation (upper panel in Fig. 6C), the phosphorylated c-Fos expression was not affected (third panel in Fig. 6C), suggesting that p38 MAPK has no effect on c-Fos phosphorylation and protein expression.

To investigate further the interactions between p38 MAPK and c-Fos involved in the regulation of p38 expression, we wanted to know whether p38 MAPK could affect the binding of c-Fos to the p28 promoter. We first overexpressed p38α in J774 cells and then stimulated the cells with *M. tuberculosis* lysates, followed by extraction of nuclear proteins for EMSA. As shown in Fig. 6D, specific binding occurred in cells treated with *M. tuberculosis* lysates, and this binding was abolished in cells overexpressing p38α. Accordingly, blockade of p38 by SB203580 enhanced *M. tuberculosis* lysate-induced binding (Fig. 6E). The supershift assay in the same experiment confirmed c-Fos binding, as the major binding was abolished by anti-c-Fos but not IgG or anti-c-Jun Abs (Fig. 6E). Taken together, these data demonstrate that p38 MAPK activation does not affect c-Fos phosphorylation or protein production but instead inhibits the binding of c-Fos to the p28 promoter preventing maximal induction of p28 and therefore IL-27 production during *M. tuberculosis* stimulation.

**FIGURE 6.** p38 MAPK induced by *M. tuberculosis* inhibits the binding of c-Fos to the p28 promoter. **A**, BMDMs (5 × 10⁵) were stimulated with LPS (1 μg/ml) or *M. tuberculosis* lysates (1 μg/ml) for various times as indicated, followed by collection of whole-cell lysates for Western blot. Sixty micrograms of whole-cell lysates was used for detection of phosphorylated p38. The same blot was stripped and reanalyzed with Abs directed against total p38, phosphorylated c-Fos, and total c-Fos. **B**, Five micrograms of p38α and empty vector (pcDNA3.1) were transfected into J774 cells by Lipofectamine. Forty-eight hours later, the transfected cells were stimulated with LPS (1 μg/ml) or *M. tuberculosis* lysates (1 μg/ml) for 15 min, followed by collection of whole-cell lysates to detect phosphorylated p38 levels by Western blot. The same membrane was stripped and reblotted with Abs directed against total p38, phosphorylated c-Fos, and total c-Fos. **C**, BMDMs (5 × 10⁵) were pretreated with different amounts of SB203580 as indicated for 1 h, then treated by *M. tuberculosis* (1 μg/ml) or LPS (1 μg/ml) for an additional 15 min, followed by collection of whole-cell extracts for detection of phosphorylated p38 expression. The same membrane was stripped and reused as described above. **D**, Five micrograms of p38α and empty vector (pcDNA3.1) were transfected into J774 cells by Lipofectamine. Forty-eight hours later, the transfected cells were stimulated with LPS (1 μg/ml) or *M. tuberculosis* lysates (1 μg/ml) for 4 h, followed by collection of nuclear extract for EMSA as described previously. **E**, J774 cells (5 × 10⁵) were treated with different amounts of SB203580 as indicated for 1 h prior to *M. tuberculosis* treatment (1 μg/ml). Four hours later, nuclear extracts were collected, and 10 μg nuclear extracts was used for EMSA and supershift assays as described previously. Two micrograms of anti-c-Fos and c-Jun Abs were used for supershift assays. Data shown are representative of three experiments showing similar results. FP, free probe; M, medium only; Mtb-L, *M. tuberculosis* whole-cell lysates.
IL-27 enhances BCG-specific T cell-mediated inhibition of intracellular mycobacterial growth in human macrophages

To explore the effects of IL-27 on human T cell-mediated inhibition of intracellular mycobacterial growth, we performed an in vitro protection assay with both T cells and monocytes isolated from PPD+ healthy blood donors. T cells were expanded with live BCG in the presence of different amounts of IL-27 for 6 d and then added to BCG-infected autologous macrophages for 3 additional days before the macrophages were lysed to release intracellular BCG. As shown in Fig. 7A, medium-rested T cells had no effects on intracellular BCG growth compared with cultures of BCG-infected macrophages alone, whereas BCG-expanded T cells significantly inhibited intracellular BCG growth (p < 0.0001 compared with macrophages cocultured with medium-rested T cells). More importantly, addition of IL-27 during the expansions of M. tuberculosis-specific T cells resulted in significant enhancement of T cell-mediated inhibition of intracellular BCG growth. Because the importance of IFN-γ in protective responses to M. tuberculosis infection is well established (32), we next measured the levels of IFN-γ in IL-27 and BCG-expanded PBMCs. As shown in Fig. 7B, 7C, IL-27 dose-dependently increased IFN-γ but inhibited IL-17 protein production in BCG-expanded T cells. In addition, IL-27 dose-dependently increased BCG-specific Th1 cells from 13.1 to 16.95% and suppressed BCG-specific Th17 cells from 0.52 to 0.26% (Fig. 7D). Taken together, these data indicate that exogenous IL-27 can enhance the ability of mycobacterial-specific Th1 cells to inhibit intracellular mycobacterial growth in human macrophages.

Discussion

A number of studies have implicated the MAPKs as important cellular targets for infectious organisms, including Yersinia enterocolitica, L. donovani, Candida albicans, and Mycobacterium (33). During infection, these organisms evolved to protect themselves from host immune responses by modulating MAPK activation in host cells including macrophages (33). It has been reported that macrophages infected with pathogenic compared with nonpathogenic mycobacteria are restricted in their activation of MAPK pathways in that inhibition of p38 MAPK decreased virulent (SmT) but not avirulent (34) Mycobacterium avium growth in macrophages (35). Our data demonstrate that inhibition of p38 MAPK by SB203580 (Fig. 2A) or blocking the downstream molecule MK2 with DN-MK2 (Fig. 2E) enhanced M. tuberculosis lysate-induced p28 expression. Consistently, overexpression of p38α and the upstream molecules MLK3/MKK3 suppressed M. tuberculosis lysate-induced p28 mRNA expression (Fig. 2D, 2F–H), indicating that the p38 cascade including MLK3/MKK3/p38/MK2 can inhibit M. tuberculosis-induced IL-27 production, which

FIGURE 7. IL-27 enhances the ability of M. tuberculosis-specific T cells to inhibit intracellular BCG growth in human macrophages. A, Monocytes (3 × 10⁵) were cultured in 96-well plates for 6 d in RPMI 1640 with 10% AB pooled human serum prior to overnight infection with BCG at an MOI of 3:1. Purified autologous PBMCs (3 × 10⁶) were expanded with live BCG at an MOI of 0.3:1 for 7 d in the presence of different amounts of recombinant human IL-27 as indicated. The BCG-expanded PBMCs were washed and added to macrophages infected with live BCG at a ratio of 10:1 for 3 d. After thoroughly washing to remove extracellular BCG, the macrophages were lysed, and viable mycobacteria were determined by radiolabeling with tritiated uridine. Data shown are percentages of BCG growth in cultures incubated with BCG-stimulated T cells compared with cultures incubated with medium-rested T cells. B and C, Cell culture supernatants collected from BCG-expanded T cells without or with different amounts of IL-27 were used to measure IFN-γ (B) and IL-17 (C) production by ELISA. D, BCG-expanded T cells with or without IL-27 treatment were cultured for 6 d followed by stimulation with PMA/ionomycin for 5 h to measure Th1 and Th17 cells by FACS. Data shown are representative of three experiments showing similar results.
may prevent optimal immunity against TB infection. It is worth noting that MKK3 and MKK6 appeared to play differential roles in inhibition of p28 expression, with the expression of MKK3 but not MKK6 inhibiting *M. tuberculosis* lysate-induced p28 mRNA expression (Fig. 2G, 2H). These differential effects indicate that targeting a selective component like MKK3 in the p38 signaling cascade might be an alternative approach for TB treatments.

*M. tuberculosis* lysate/BCG-activated p28 expression is dependent on the MyD88 signaling pathway because p28 mRNA expression was significantly reduced in MyD88-deficient cells (Fig. 3A, 3B). However, there were still relative amounts of p28 mRNA expression remaining in MyD88-deficient cells (Fig. 3A, 3B), indicating that although MyD88 signaling plays a major role in *M. tuberculosis* lysate/BCG-induced p28 expression, MyD88-independent signaling is also involved in p28 expression. To dissect the molecular mechanisms of *M. tuberculosis*-mediated p28 production, we measured p28 gene primary transcript and promoter activation in response to *M. tuberculosis* lysate stimulation. Our data showed that *M. tuberculosis* lysate-activated p28 induction was regulated at the transcriptional level (Fig. 3C–F), and further experiments clearly indicated that the transcription factor AP-1 was responsible for *M. tuberculosis* lysate-induced p28 gene transcription (Fig. 4). Notably, AP-1 is not involved in LPS-stimulated p28 induction because blocking AP-1 binding by a-Fos had little effect on both LPS-induced p28 mRNA expression (Fig. 4A) and promoter activation (Fig. 4C), suggesting that AP-1 might be a relatively specific transcription factor important for TB immunity. By overexpression of c-Fos and c-Jun individually in cells, we demonstrated that c-Fos, but not the c-Jun AP-1 family member, mediates p28 transcriptional induction (Fig. 4D), which was also consistent with the EMSA data that c-Fos but not c-Jun binds to the p28 promoter (Fig. 5C). We noticed that overexpression of c-Jun actually suppressed *M. tuberculosis*-activated p28 promoter activity, the opposite effect of c-Fos overexpression (Fig. 4D). These differential effects of c-Fos and c-Jun on p28 gene transcription indicate that c-Fos might dimerize with other members of the AP-1 family proteins. Indeed, we found that JunB bound to the p28 promoter at the same region as c-Fos (Fig. 5C). It has been reported that the biological properties of JunB differ from c-Jun in that these two proteins have opposing functions (36). Taken together, these data suggest that c-Fos may dimerize with JunB to form a heterodimer to activate p28 gene transcription.

It has been reported that MAPKs upregulate AP-1 activity through enhancing AP-1 protein phosphorylation (37). Because our data showed that p38 MAPK suppressed (Fig. 2) whereas the transcriptional factor AP-1/c-Fos activated (Figs. 4, 5) p28 gene transcription in response to *M. tuberculosis* lysate stimulation, we reasoned that the p38 MAPK might affect c-Fos phosphorylation. To our surprise, overexpression of p38 (Fig. 6B) or blockade of p38 (Fig. 6C) had no effects on c-Fos protein production and phosphorylation. Notably, overexpression of p38 abolished c-Fos binding to the p28 promoter (Fig. 6D), whereas blocking p38 enhanced p28 promoter binding (Fig. 6E). To our knowledge, this is the first report showing that p38 MAPK activated by *M. tuberculosis* lysates abrogates AP-1 binding to a proinflammatory cytokine promoter. The p38-mediated inhibition of AP-1 binding could be due to a conformational change of the AP-1 protein induced by p38 MAPK binding or to other transcriptional factors activated by the p38 MAPK pathway competing with AP-1 for binding to the p28 promoter. Nevertheless, these data demonstrate that during *M. tuberculosis* stimulation, two pathways with opposite effects on IL-27 production are triggered by *M. tuberculosis*. The transcription factor AP-1 activates p28 gene transcription and therefore IL-27 production. In contrast, p38 MAPK signaling pathway induction can suppress binding of AP-1 to the p28 promoter leading to reduced levels of IL-27 production.

IL-27 promotes Th1 (38) and inhibits Th2 as well as Th17 cells (10, 39, 40), suggesting important immunomodulatory roles for induction of protective immunity against invasive pathogens and prevention of host tissue damage secondary to excessive immune action. In this study, our data indicate that IL-27 enhanced the ability of BCG-specific T cells to inhibit intracellular mycobacterial growth in human macrophages (Fig. 7A), increased BCG-specific Th1 cells, and suppressed Th17 cells (Fig. 7B–D). These combined results suggest that the enhanced inhibitory effects of BCG-specific T cells expanded in the presence of increasing levels of IL-27 are due to the preferential skewing toward type I immune responses. In addition, it has recently been reported that IL-17 is involved in the pathological effects of *M. tuberculosis* infection in mice subjected to repeated BCG vaccination (41). Therefore, the inhibition of *M. tuberculosis*-specific Th17 cells shown by us to occur with increases in IL-27 might be beneficial for the prevention of tissue damage without altering protective immunity. In fact, IL-27 has been shown to enhance protective immunity but prevent immunopathology in other models of intracellular pathogens such as *T. cruzi* and *Toxoplasma* (10, 11), suggesting a broader role for IL-27 in the optimal balance of infection-induced immunity.

In summary, we report in this study the important finding that IL-27 can enhance the ability of *M. tuberculosis*-specific Th1 cells to inhibit intracellular mycobacterial growth in human macrophages. The outcome of IL-27 production during *M. tuberculosis* infection is determined by cross-regulation between p38 MAPK and AP-1 binding to the p28 promoter. Though *M. tuberculosis* could induce p28 expression, IL-27 production is suppressed by *M. tuberculosis*-induced p38 MAPK through inhibition of c-Fos binding to the p28 promoter (Supplemental Fig. 4). These opposing effects of *M. tuberculosis* stimulation provide a precarious balance between protective immunity and pathogen persistence. This study serves to lay an important foundation for further exploration of the cross-regulation between different signaling pathways during *M. tuberculosis* infection and may lead to the discovery of novel therapeutic targets for boosting protective TB immunity.

**Acknowledgments**

We thank Dr. Isaac Sakala for technical assistance with the mycobacterial inhibition assay.

**Disclosures**

The authors have no financial conflicts of interest.

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