

Leishmania major Infection Activates NF- κ B and Interferon Regulatory Factors 1 and 8 in Human Dendritic Cells[∇]

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The salient feature of dendritic cells (DC) is the initiation of appropriate adaptive immune responses by discriminating between pathogens. Using a prototypic model of intracellular infection, we previously showed that *Leishmania major* parasites prime human DC for efficient interleukin-12 (IL-12) secretion. *L. major* infection is associated with self-limiting cutaneous disease and powerful immunity. In stark contrast, the causative agent of visceral leishmaniasis, *Leishmania donovani*, does not prime human DC for IL-12 production. Here, we report that DC priming by *L. major* infection results in the early activation of NF- κ B transcription factors and the up-regulation and nuclear translocation of interferon regulatory factor 1 (IRF-1) and IRF-8. The inhibition of NF- κ B activation by the pretreatment of DC with caffeic acid phenethyl ester blocks *L. major*-induced IRF-1 and IRF-8 activation and IL-12 expression. We further demonstrate that IRF-1 and IRF-8 obtained from *L. major*-infected human DC specifically bind to their consensus binding sites on the *IL-12p35* promoter, indicating that *L. major* infection either directly stimulates a signaling cascade or induces an autocrine pathway that activates IRF-1 and IRF-8, ultimately resulting in *IL-12* transcription.

Leishmania major is the causative agent of cutaneous leishmaniasis, which is characterized by the development of lesions at sand fly bite sites. These cutaneous lesions ulcerate, resolve, and ultimately stimulate powerful immunity against the disease. Robust induction of this immunity is the basis of leishmanization, an effective vaccination procedure in which the inoculation of live *L. major* has been used with great success (12); safety concerns, however, have led to the abandonment of such vaccination (17). In contrast to *L. major*, *Leishmania donovani* causes visceral leishmaniasis, a severe systemic illness that is often fatal if untreated.

Healing of cutaneous leishmaniasis has been attributed to the development of a strong Th1 immune response in the vertebrate host. Interleukin-12 (IL-12) is up-regulated in *L. major*-infected human dendritic cells (DC), whereas visceral leishmaniasis agents do not induce IL-12 production (28). Those previous studies revealed that *L. donovani* does not actively inhibit IL-12 production; rather, *L. major* primes human DC for IL-12 production. This strong induction of IL-12 by *L. major*-infected DC sets the stage for a strong and robust Th1 immune response that leads to lesion healing and immunity against the disease. Elucidation of the mechanisms that mediate the strong immunity elicited by *L. major* will undoubtedly have consequences for vaccine development against all *Leishmania* species as well as other infections, where strong cell-mediated immune responses are essential for resistance.

IL-12 belongs to a family of cytokines including IL-23, IL-27, and ciliary neurotrophic factor receptor (CNTFR). The bioactive form of this proinflammatory cytokine is a unique heterodimeric protein composed of p35 and p40 subunits that are encoded by independent genes located on separate chromosomes (40, 45). The induction and secretion of bioactive IL-12 (IL-12p70) are regulated by the independent activation of p35 and p40 subunits. The IL-12p40 subunit can be secreted as homodimers or monomers and is tightly regulated by *IL-12p40* gene expression in activated DC and other hematopoietic phagocytes (macrophages, monocytes, and neutrophils) (49).

The secretion of free IL-12p40 and IL-12p70 by activated macrophages and DC is dependent on the regulation of the *IL-12p40* gene by an array of transcription factors including CAAT/enhancer binding protein (C/EBP) (39), Rel proteins (39), interferon regulatory factor 1 (IRF-1) (21), and gamma interferon (IFN- γ) consensus sequence binding protein, also known as IRF-8 (52). Although IL-12p70 secretion is specifically dependent on IL-12p40 secretion, recent studies indicate that IL-12p35 expression is also tightly regulated, and hence, it plays an important role in the regulation of IL-12p70 expression. The IL-12p35 subunit is regulated at both the transcriptional and translational levels (43, 48, 57). Putative common transcription factors between murine and human *IL-12p35* promoters include activator protein 2 (AP-2), Sp-1, C/EBP, CREB (CRE binding protein), AP-1, Rel proteins, IRF-1, and IRF-8 (18, 21). IRF-1 has been shown to actively regulate *IL-12p35* induction (22), while the activities of the other transcription factors remain to be elucidated.

Quantitative reverse transcription (RT)-PCR analysis of *IL-12p35* and *IL-12p40* expression revealed a 10-fold increase in the message level of *IL-12p35* and *IL-12p40* due to *L. major* as opposed to *L. donovani* infection (28). These data suggest that the *L. major*-specific up-regulation of IL-12 production is de-

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pendent on the regulation of both the *IL-12p35* and *IL-12p40* genes. The *IL-12p35* and *IL-12p40* promoters share DNA elements and likely attract a number of common transcription factors including IRF-1, IRF-8, c-Rel, and NF- κ Bp50, suggesting a specific role for these factors in the activation of both subunits in response to *L. major* infection. Here, we delineate the upstream molecular events of IL-12p70 induction in human DC infected with *L. major* and demonstrate that NF- κ B and IRF transcription factors play a specific role in the induction of IL-12 in *L. major*-infected DC.

MATERIALS AND METHODS

DC generation. CD14⁺ monocytes were isolated from peripheral blood mononuclear cells of healthy human donors (Central Indiana Regional Blood Center, Indianapolis, IN) by positive selection (AutoMACS separator; Miltenyi Biotec, Auburn, CA). The purity of separated monocytes was determined by flow cytometry analysis of CD14, CD19, CD45, and CD3 expression. Enriched monocytes were treated with granulocyte-macrophage colony-stimulating factor (1,000 U/ml) and IL-4 (400 U/ml) on days 0, 3, and 6 after plating in six-well plates at 2.5×10^6 cells/ml in complete RPMI medium (10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine). Immature DC were harvested on day 7 and plated in 48-well plates at 2×10^5 cells/300 μ l per well in the absence of exogenous cytokine. The phenotypes of DC were determined similarly by flow cytometry analysis of CD1a, CD14, HLA-DR, CD40, CD80, and CD86 expression. Antibody-stained cells were evaluated using an MCL500 flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

Parasites and infection. All infections were performed with *L. major* NIH Friedlin V1 (MHOM/IL/80/FN), isolated from a patient with localized cutaneous leishmaniasis in Israel, and *L. donovani* strain 9515 (MHOM/IN/95/9515), isolated from a splenic aspirate of a patient with visceral leishmaniasis in India. Infective-stage metacyclic promastigotes were isolated by use of a Ficoll gradient (47) and opsonized with 5% normal human serum prior to infection at a concentration of five parasites to one dendritic cell. Infection rates were determined at the end of each experiment by DiffQuick staining of cytopins and visualization by light microscopy. In all cases, there were no significant differences between infecting species. All parasite strains were cultured at 26°C without CO₂ in medium 199 containing 20% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml biotin. Parasites tested negative for mycoplasma using a Mycoplasma PCR detection set (Takara Bio, Inc., Madison, WI) and tested below the detection limits for endotoxin using a Limulus Amoebocyte Lysate kit (Charles River Endosafe, NC).

Western blot analysis. Nuclear extracts were obtained from *L. major*- and *L. donovani*-infected DC using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL). Twenty to thirty micrograms of nuclear extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Novex system (Invitrogen Life Technologies, Carlsbad, CA). After transfer onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA), blots were blocked in 1% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. Primary antibodies diluted in 1% bovine serum albumin in phosphate-buffered saline were used at concentrations suggested by the manufacturer. Blots were extensively washed before the addition of secondary antibody (BD Biosciences, Franklin Lakes, NJ). Proteins were detected using Supersignal West stable peroxide solution (Pierce Endogen, Rockford, IL), followed by exposure to X-ray film. Densitometry analysis was performed with a Bio-Rad GS-800 densitometer and QuantityOne software. The optical density of the transcription factors was normalized to lamin A/C from each condition.

EMSA. Electrophoretic mobility shift assays (EMSAs) were performed using IRF-1 (24) and IRF-8 (10) consensus probes. The minimal inducible promoter region carrying the consensus regions 5'-TGAAATCCC-3' was labeled with [³²P]ATP and mixed with nuclear extracts of *Leishmania*-infected human DC. The complexes were fractionated through 4% polyacrylamide gels, and the shifted complexes were detected by autoradiography.

ChIP analysis. Chromatin immunoprecipitation (ChIP) was performed using an assay kit according to the manufacturer's instructions (Upstate Biotechnology, Charlottesville, VA). Briefly, 10⁶ DC were infected with *L. major* or *L. donovani* and then cross-linked with 1% formaldehyde for 10 min at 37°C. Cross-linked chromatin was sheared by sonication to obtain DNA fragments

ranging from 200 to 1,000 bp. Sheared chromatin was precleared by protein A-agarose beads, followed by overnight immunoprecipitation with 2 μ g anti-IRF-1, anti-IRF-8, or isotype control antibody at 4°C. Cross-linking was reversed by heating at 65°C for 4 h, followed by proteinase K digestion and ethanol-chloroform purification of immunoprecipitated DNA. Input and purified immunoprecipitated DNAs were subjected to PCR using primers encompassing the IRF-1 site in the human *IL-12p35* promoter (5' primer GCGAACATTTCGCTTTCATT and 3' primer ACTTTCCCGGGACTCTGGT). Products were amplified for 34 cycles using GoTaq (Promega Corp., Madison, WI) and analyzed on a 2% agarose gel.

Cytokines and antibodies. The following antibodies were used for flow cytometry: anti-CD14-phycoerythrin (PE) (clone M5E2), immunoglobulin G2a(κ) [IgG2a(κ)]-PE (clone MOPC-173), anti-CD1a-PE (clone HI149), IgG1(κ)-PE (clone MOPC-2), anti-HLA-DR-PE (clone L243), IgG2b(κ)-PE (clone MPC-11), anti-CD40-PE (clone HB14), anti-CD80-PE (clone 2D10), and anti-CD86-PE (clone IT2.2), purchased from BioLegend, Inc. (San Diego, CA), and anti-CD19-PE (clone J4.119), IgG1-PE (clone 679.1Mc7), anti-CD3-fluorescein isothiocyanate (clone UCHT.1), and anti-CD45-PE-Cy5 (clone Immu19.2), purchased from Beckman Coulter, Inc. (Fullerton, CA). The following antibodies were used for Western blotting: anti-lamin A/C (clone N-18), anti-IRF-1 (clone C-20), anti-IRF-8 (clone H-70), anti-NF- κ Bp50 (clone E-10), anti-NF- κ Bp65 (clone F6), and anti-c-Rel (clone N), purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-pSTAT1 (polyclonal) and anti-tSTAT1 (polyclonal), purchased from Cell Signaling Technology, Inc. (Danvers, MA). Horseradish peroxidase-conjugated secondary antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Recombinant human granulocyte-macrophage colony-stimulating factor (specific activity of $\geq 1 \times 10^7$ units/mg), IL-4 (specific activity of $\geq 5 \times 10^6$ units/mg), and IFN- γ (specific activity of $\geq 2 \times 10^7$ units/mg) were purchased from Peprotech, Inc. (Rocky Hill, NJ).

Real-time quantitative RT-PCR (Taqman). Relative levels of *IL-12p35*, *IL-12p40*, and *IL-23p19* mRNA were determined by real-time PCR. Total mRNA was prepared with the RNeasy Mini kit (Qiagen, Inc., Valencia, CA). One microgram of total mRNA was reverse transcribed using random primers with the Superscript III synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For analysis of *IL-12p35*, *IL-12p40*, *IL-23p19*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene mRNA expression, Taqman predeveloped assay kits were purchased from Applied Biosystems (Foster City, CA). Taqman reactions were performed according to the manufacturer's recommendations by using an ABI Prism 7500 sequence detection system (Perkin-Elmer, Foster City, CA). The relative number of mRNA copies for *p35*, *p40*, and *p19* were determined using the comparative method by following formula: number of copies = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ equals $\Delta C_{T(\text{experimental})} - \Delta C_{T(\text{calibrator})}$. ΔC_T equals $C_{T(\text{experimental})} - C_{T(\text{GAPDH})}$. C_T is the cycle number where a statistically significant increase in the emission intensity over the background is detected, and $\Delta C_{T(\text{calibrator})}$ equals the mean ΔC_T for the uninfected and unstimulated control from each donor.

Statistical analyses. A paired Student's *t* test on raw (quantitative RT-PCR) or log₁₀-transformed (Western blot) data was utilized to determine statistical differences between experimental groups.

RESULTS

Transcriptional induction of *IL-12p35* and *IL-12p40* in *L. major*-infected DC. Although to a lesser extent than in the presence of CD40L, both *IL-12p35* and *IL-12p40* mRNAs are induced in DC after 8 h of *L. major* infection in the absence of CD40L (28). In order to determine the kinetics of this transcriptional induction, DC were infected with *L. major* and *L. donovani* for 1, 2, 4, 6, 8, 12, and 16 h, followed by the isolation of total RNA at these time points and quantification of IL-12 (*IL-12p35* and *IL-12p40*) by real-time quantitative RT-PCR. The levels of *IL-12p35* (Fig. 1A) and *IL-12p40* (Fig. 1B) were significantly up-regulated in DC infected with *L. major* by 4 h, as opposed to *L. donovani*-infected DC. Generally, the highest level of message was detected by 8 h postinfection, followed by a drop in *IL-12p35* levels 12 h postinfection and sustained expression of *IL-12p40*. *IL-23p19* was also induced preferentially by *L. major* infection (data not shown). The similar kinetic inductions of *IL-12p35* and *IL-12p40* gene expression

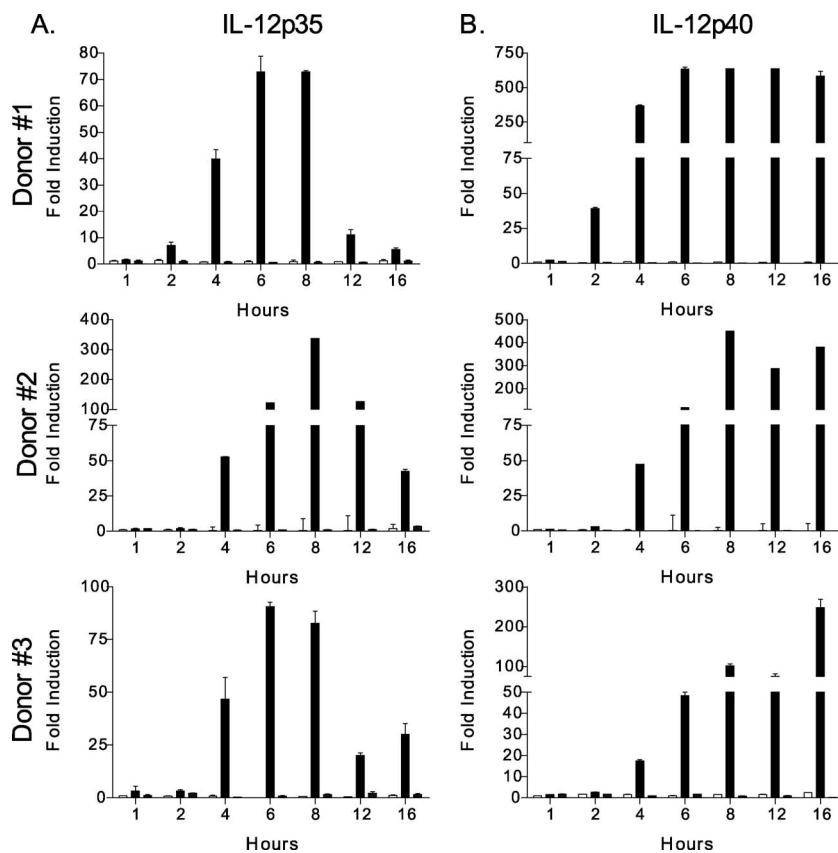


FIG. 1. Kinetics of *IL-12p35* and *IL-12p40* induction in *Leishmania*-infected DC. RNA isolated from uninfected DC (open bars) or DC infected with *L. major* (black bars) or *L. donovani* (gray bars) for 1, 2, 4, 6, 8, 12, and 16 h was analyzed for *IL-12p35* (A) and *IL-12p40* (B) transcript levels by quantitative real-time RT-PCR. The induction of *IL-12p35* and *IL-12p40* transcripts in *Leishmania*-infected DC was calculated using the comparative $\Delta\Delta C_T$ method and normalized to *GAPDH*. Values are means \pm standard errors of the means (SEM) of triplicate culture wells. *L. major*-infected DC from all donors exhibited significant up-regulation of *IL-12p35* and *IL-12p40* mRNAs by 4 h postinfection ($P \leq 0.05$).

after *L. major* infection of DC suggest the activation of both genes by common transcription factors.

Gene expression in *Leishmania*-infected DC. Assessment of the expression of genes known to be regulated by specific transcription factors can be a first clue to the signaling pathways that are activated in response to specific stimuli or infections. To identify transcription factors involved in signaling cascades initiated by *L. major* infection in human DC, we reanalyzed previously published microarray data. These microarray experiments were carried out using total RNA obtained from human DC infected with *L. major* and *L. donovani* for 16 h. Pooled cRNAs from seven donors were hybridized to the Affymetrix HU95A microarray (4). Here, we reanalyzed these data to assess changes in genes regulated by transcription factors known to bind IL-12 promoters. Genes regulated by IFN- γ -stimulated response elements (ISREs) (guanylate binding protein 1 [GBP-1], GBP-2, IP-10, and COX-2), GAS (IFN- γ -activated sequence) (IRF-1 and MIG), or NF- κ B ($I\kappa$ B ϵ , $K\kappa$ B β , COX-2, and IRF-1) binding elements were assessed.

The reanalysis of the microarray information indicated the up-regulation of certain genes that are regulated by IRF-1 and IRF-8 in DC infected with *L. major* (Fig. 2). Among the genes that are preferentially up-regulated by *L. major* infection, guanylate binding proteins (*GBP-1* and *GBP-2*) (8) and *COX-2* (51) possess ISREs that bind IRF-1. In addition, IRF-1 mRNA levels are up-regulated specifically by *L. major* infection, fur-

ther implicating IRF-1 as a mediator of IL-12 production in *L. major*-infected DC. *IP-10*, however, is not differentially regulated in *L. major*- and *L. donovani*-infected DC. Although the *IP-10* promoter contains an ISRE, previous studies indicated

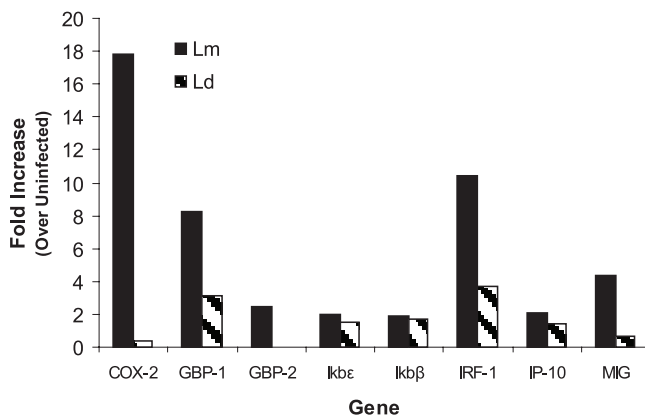


FIG. 2. Up-regulation of genes in *L. major*-infected DC. Data obtained from microarray analysis of total RNA obtained from DC infected with *L. major* (Lm) or *L. donovani* (Ld) for 16 h were reanalyzed (4) to examine genes regulated by NF- κ B, ISRE, and GAS regulatory elements. Indicated changes in transcript levels in *Leishmania*-infected DC are over basal levels present in uninfected DC.

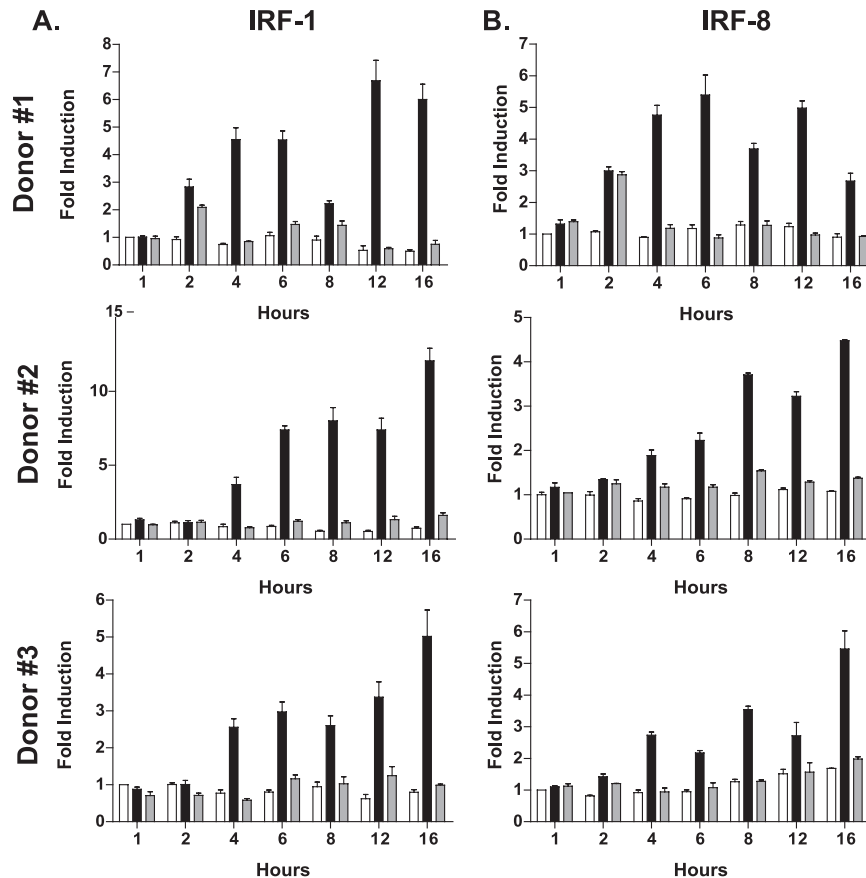


FIG. 3. Kinetics of *IRF-1* and *IRF-8* induction in *Leishmania*-infected DC. RNA isolated from uninfected DC (open) or DC infected with *L. major* (black bars) or *L. donovani* (gray bars) for 1, 2, 4, 6, 8, 12, and 16 h was analyzed for *IRF-1* (A) and *IRF-8* (B) transcript levels by quantitative real-time RT-PCR. The induction of *IRF-1* and *IRF-8* transcripts in *Leishmania*-infected DC was calculated using the comparative $\Delta\Delta C_T$ method and normalized to *GAPDH*. Values are means \pm SEM of triplicate culture wells. *L. major*-infected DC from all donors exhibited significant up-regulation of *IRF-1* and *IRF-8* mRNAs by 4 h postinfection ($P \leq 0.05$).

that IRF-1 binding is not essential for ISRE-mediated transcription of the human *IP-10* promoter (26). Interestingly, *IRF-1* (38, 46) and *MIG* (56), genes which contain GAS promoter elements, are preferentially up-regulated in *L. major*-infected DC (Fig. 2), suggesting that there is an increase in IRF-8 binding.

Both *COX-2* (51) and *IRF-1* (46) promoters have NF- κ B elements, implicating a role for NF- κ B activation. Of interest is that I κ B gene promoters also contain NF- κ B binding sites (13); these genes are not induced preferentially by *L. major* 16 h postinfection in DC (Fig. 2). Although NF- κ B has been implicated in several nuances of IL-12 regulation (32, 35), it appears that it does not play a role in *L. major*-specific priming for IL-12 secretion by DC according to the microarray analysis (Fig. 2). However, it is possible that I κ B proteins might be regulated by other members of the NF- κ B family in this system or that the induction of the I κ B genes occurs earlier than 16 h after infection of DC with *L. major*.

RT-PCR analysis of IRF-1 and IRF-8 in *L. major*-infected DC. Other studies have shown that IRF-1 and IRF-8 are critical for the activation of both *IL-12p35* and *IL-12p40* promoters in murine macrophage systems (21, 22, 49), and our previous results strongly implicate these factors in response to *L.*

major infection. In order to gauge the transcriptional induction of *IRF-1* and *IRF-8* in *L. major*-infected DC, we analyzed mRNA levels of *IRF-1* and *IRF-8* by real-time quantitative RT-PCR. Our results indicate that *IRF-1* mRNA levels steadily increase in *L. major*-infected DC compared to *L. donovani*-infected DC starting at 4 h (Fig. 3A) postinfection. A similar kinetic pattern of induction was revealed for *IRF-8* expression (Fig. 3B).

Nuclear translocation of IRF-1 and IRF-8. To directly assess the activation of the implicated transcription factors, nuclear extracts of *L. major*-infected DC were analyzed using Western blotting, probing for IRF-1 and IRF-8 (Fig. 4). As the kinetics of *p35* and *p40* induction revealed a time-dependent up-regulation of transcripts in response to *L. major* infection, nuclear extracts were obtained from *L. major*-infected DC at 1, 8, and 16 h postinfection to observe changes in nuclear translocation of the above-mentioned transcription factors. The significant increase in *IRF-1* transcript levels in *L. major*-infected DC starting at 4 h (Fig. 3A) corresponds to the increased nuclear translocation of IRF-1 in *L. major*-infected DC, as opposed to *L. donovani*-infected DC by 8 h postinfection (Fig. 4A). This increase in IRF-1 nuclear translocation observed in *L. major*-infected DC is sustained at 16 h postinfection. Similar activa-

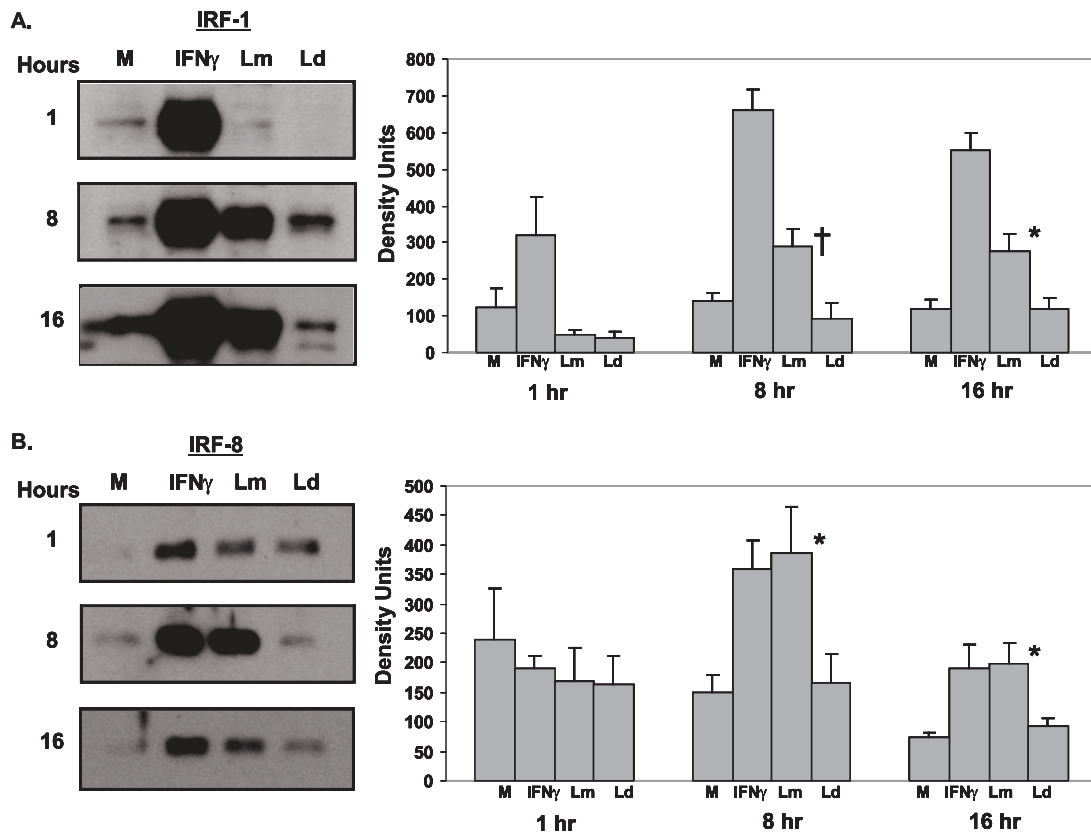


FIG. 4. Nuclear translocation of IRF-1 and IRF-8 in *L. major*-infected DC. Nuclear extracts obtained from DC infected with *L. major* (Lm) or *L. donovani* (Ld) for 1, 8, or 16 h were analyzed by Western blotting for IRF-1 (A) and IRF-8 (B). IFN- γ (1,000 U/ml)-stimulated DC were used as a positive control. Densitometry readings for each blot were normalized to lamin A/C. Western blots for one representative donor are shown. Values are means \pm SEM of 3 to 12 donors. †, $P \leq 0.1$; *, $P \leq 0.05$. M, medium control.

tion kinetics were observed for IRF-8 by *L. major* infection (Fig. 4B). Thus, increased nuclear translocation of IRF-1 and IRF-8 in *L. major*-infected DC at 8 h and 16 h of infection suggests that both these transcription factors are likely related to the induction of the *IL-12p35* and *IL-12p40* genes. IFN- γ stimulation of DC was used as a positive control for the nuclear translocation of both transcription factors; IFN- γ activation was further substantiated by increases in transcript levels of *IRF-1* and *IRF-8* in DC treated with IFN- γ (data not shown).

***L. major*-induced IRF-1 and IRF-8 interact with the *IL-12p35* promoter.** The up-regulation of both *IL-12p35* and *IL-12p40* transcription and the increased nuclear translocation of IRF-1 and IRF-8 in *L. major*-infected DC compared to that of *L. donovani*-infected DC suggest a role for IRF-1 and IRF-8 in the activation of *L. major*-induced IL-12 expression. To determine if these factors are active in *L. major*-infected DC, we assessed the abilities of IRF-1 and IRF-8 present in nuclear extracts obtained from *L. major*-infected DC to bind their consensus binding sites by EMSA. This experiment revealed that crude nuclear extracts from *L. major*-infected DC do bind to IRF-1 and IRF-8 consensus sequences (data not shown). In order to determine if this binding occurs endogenously in *L. major*-infected DC, ChIP assays were performed on *Leishmania*-infected DC. Following 6 h of infection, the protein-DNA complexes were cross-linked with paraformaldehyde and immunoprecipitated with antibodies reacting to IRF-1 or IRF-8

(Fig. 5). Binding to the *IL-12p35* promoter was assessed by amplifying the IRF-E promoter element by PCR. Binding of both IRF-1 and IRF-8 to the *IL-12p35* promoter was observed specifically in *L. major*-infected DC compared to *L. donovani*-infected DC (Fig. 5).

Up-regulation of phosphorylated STAT1 in *L. major*-infected DC. The *IRF-8* promoter contains a GAS element, rais-

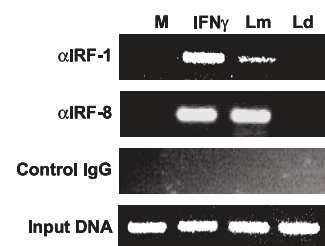


FIG. 5. Endogenous IRF-1 and IRF-8 interact with the *IL-12p35* promoter in *L. major*-infected DC. DC were infected with *L. major* (Lm) and *L. donovani* (Ld) for 6 h, followed by cross-linking and immunoprecipitation of protein-DNA complexes with antibodies against IRF-1 and IRF-8 or an isotype control. The presence of the *IL-12p35* IRF-E element was assessed by PCR. IFN- γ (1,000 units/ml)-treated DC served as a positive control at all time points. Input DNA indicates that all samples contain equal amounts of DNA. One representative of two independent experiments is shown. M, medium control.

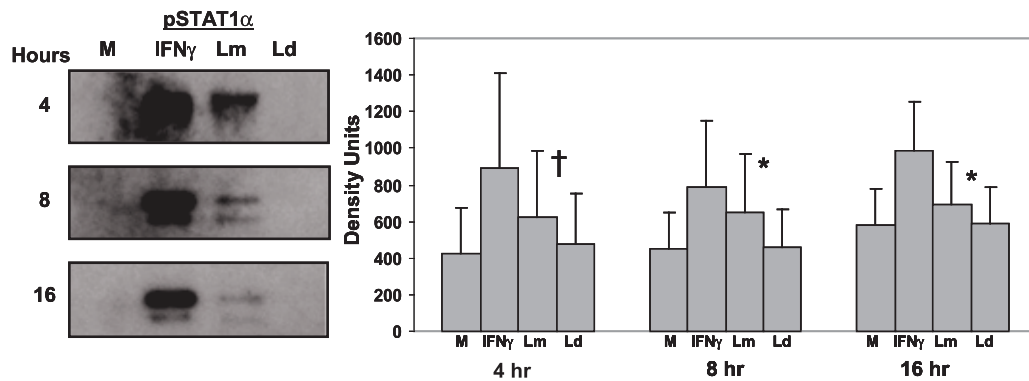


FIG. 6. Activation and nuclear translocation of STAT1 in *L. major*-infected DC. Nuclear extracts obtained from DC infected with *L. major* (Lm) or *L. donovani* (Ld) for 4, 8, and 16 h were analyzed for phosphorylated STAT1 α . IFN- γ (1,000 U/ml)-treated DC served as a positive control. Lamin A/C was used as a loading control. Western blots of one representative donor are shown. Values are means \pm SEM for four to seven donors. †, $P \leq 0.1$; *, $P \leq 0.05$. M, medium control.

ing the possibility that STAT1 α mediates the activation of this promoter in *L. major*-infected DC. Therefore, we investigated the nuclear translocation of STAT1 α in *Leishmania*-infected DC. As STAT1 α only is active when phosphorylated, we utilized an anti-STAT1 α -phosphospecific antibody. Our results indicate elevated levels of phosphorylated STAT1 α in nuclear extracts of *L. major*-infected DC as early as 4 h postinfection compared to *L. donovani*-infected DC (Fig. 6). Phosphorylation of STAT1 α in the cytosol of *L. major*-infected DC was not evident until 2 to 4 h postinfection (data not shown).

Nuclear translocation of c-Rel, NF- κ Bp65, and NF- κ Bp50. The microarray analysis of *L. major*- and *L. donovani*-infected DC indicated that some NF- κ B-regulated genes may not be differentially induced by *Leishmania* species. However, both *IRF-1* and *IRF-8* promoters contain NF- κ B elements and are specifically induced only by *L. major* infection as early as 4 h postinfection, suggesting that the timing of the microarray experiment may have hindered our ability to detect differentially expressed NF- κ B-regulated genes. Therefore, we assessed the nuclear translocation of NF- κ B transcription factors in *Leishmania*-infected DC as early as 1 h postinfection (Fig. 7). Our results demonstrate increased nuclear translocation of NF- κ Bp65 in DC infected with *L. major* for 1, 8, and 16 h (Fig. 7B) and NF- κ Bp50 at 8 and 16 h postinfection (Fig. 7C), suggesting a role for NF- κ Bp65 and NF- κ Bp50 in the activation of *IRF-1* and *IRF-8* by *L. major* infection. c-Rel has been associated strongly with the maturation of DC (3) in addition to the activation of *IL-12p35* and *IL-12p40*. Although somewhat more variable than NF- κ Bp65, c-Rel also translocates into the nucleus after *L. major* infection compared to *L. donovani* infection (Fig. 7A).

NF- κ B elements are required for induction of *IL-12*, *IRF-8*, and *IRF-1*. The increased nuclear translocation of NF- κ Bp50 and NF- κ Bp65 in *L. major*-infected DC at early time points after infection suggests a role for these transcription factors in the *L. major*-specific induction of *IL-12p35* and *IL-12p40*. To determine the necessity of NF- κ B for *L. major*-induced *IL-12* expression, the NF- κ B pathway in *Leishmania*-infected DC was blocked with caffeic acid phenyl ester (CAPE), a specific NF- κ B inhibitor, and *IL-12* expression was assessed by real-time quantitative RT-PCR. Although infection levels were not

altered, *IL-12p35* (Fig. 8A) and *IL-12p40* (Fig. 8B) mRNA levels dropped drastically in *L. major*-infected DC pretreated with CAPE compared to untreated *L. major*-infected DC. CAPE treatment also affects *IRF-8* and *IRF-1* transcription, as evidenced by reduced *IRF-1* (Fig. 8C) and *IRF-8* (Fig. 8D) mRNA levels in treated *L. major*-infected DC compared to those in untreated *L. major*-infected DC. The reduction in *IRF-8* transcription corresponds to reduced *IRF-8* nuclear translocation in CAPE-treated *L. major*-infected DC, a reduction that was not observed in untreated *L. major*-infected DC (Fig. 9). Thus, it is evident that NF- κ B family members coordinate gene activation during *L. major* infection, resulting in downstream events required for the activation of *IL-12p35* and *IL-12p40* promoters.

To test if de novo protein synthesis is required for *L. major*-induced *IL-12* transcription, we blocked protein translation with cycloheximide prior to infection. Cycloheximide pretreatment resulted in elevated amounts of *IL-12p35* (169-fold \pm 104-fold increase for *L. major* and 108-fold \pm 168-fold increase for *L. donovani* over infection plus vehicle control) and *IL-12p40* (28-fold \pm 34-fold increase for *L. major* and 29-fold \pm 43-fold increase for *L. donovani* over infection plus vehicle control), suggesting that protein synthesis is required for the production of a natural hemostatic mechanism that influences *IL-12* production.

DISCUSSION

The goal of this study was to elucidate the mechanism of *IL-12* regulation in *L. major*-infected human DC. *L. major* initiates a proinflammatory Th1 immune response mediated by *IL-12* to establish and sustain immunity. Here, we investigated the specific induction of *IL-12p35* and *IL-12p40* in human DC in response to *L. major* infection and demonstrate a critical role for NF- κ B, IRF-1, and IRF-8 in mediating this *IL-12* production.

Elevated levels of *IL-12p35* and *IL-12p40* transcripts are induced in *L. major*-infected DC in the presence and absence of CD40L (28). The induction of both subunits of *IL-12* by *L. major* in the absence of CD40L was observed at 8 h after infection of DC. However, information regarding the initial

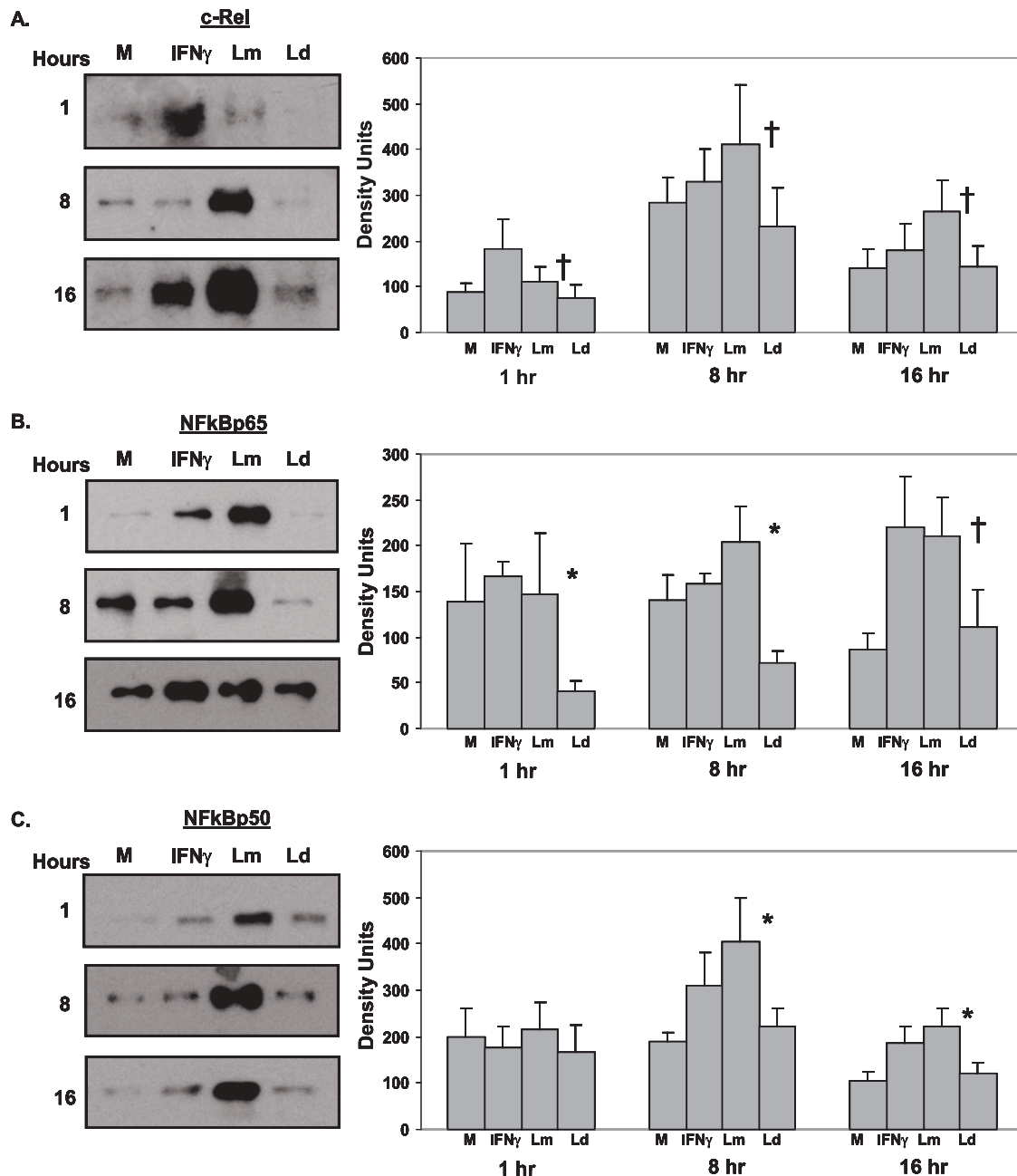


FIG. 7. Nuclear translocation of NF- κ B transcription factors in *L. major*-infected DC. Nuclear extracts obtained from DC infected with *L. major* (Lm) or *L. donovani* (Ld) for 1, 8, or 16 h were analyzed by Western blotting for c-Rel (A), NF- κ Bp65 (B), and NF- κ Bp50 (C). IFN- γ (1,000 U/ml)-stimulated DC were used as a positive control. Densitometry readings for each blot were normalized to lamin A/C. Western blots of one representative donor are shown. Values are means \pm SEM for three to nine donors. †, $P \leq 0.1$; *, $P \leq 0.05$. M, medium control.

transcriptional events of IL-12 expression in *L. major*-infected DC was lacking. This information was provided by a detailed kinetic analysis of *IL-12p35* and *IL-12p40* transcription at various time points after infection of DC with either *L. major* or *L. donovani* (Fig. 1). Both *IL-12* genes follow a similar pattern of up-regulation in DC in response to various durations of infection with *L. major*. These data add to a growing body of literature that reveals that along with *IL-12p40* transcriptional control, *IL-12p35* is also tightly regulated. In *L. major*-infected

DC, both subunits seem to contribute equally to the induction of IL-12 in DC.

The parallel inductions of *IL-12p35* and *IL-12p40* in *L. major*-infected DC suggest that these genes are activated by a common set of transcription factors. This speculation is supported by data from a previously published microarray analysis of *L. major*- and *L. donovani*-infected DC (4). We utilized these data by segregating genes into three categories based on promoter regulator elements. The *L. major*-specific up-regula-

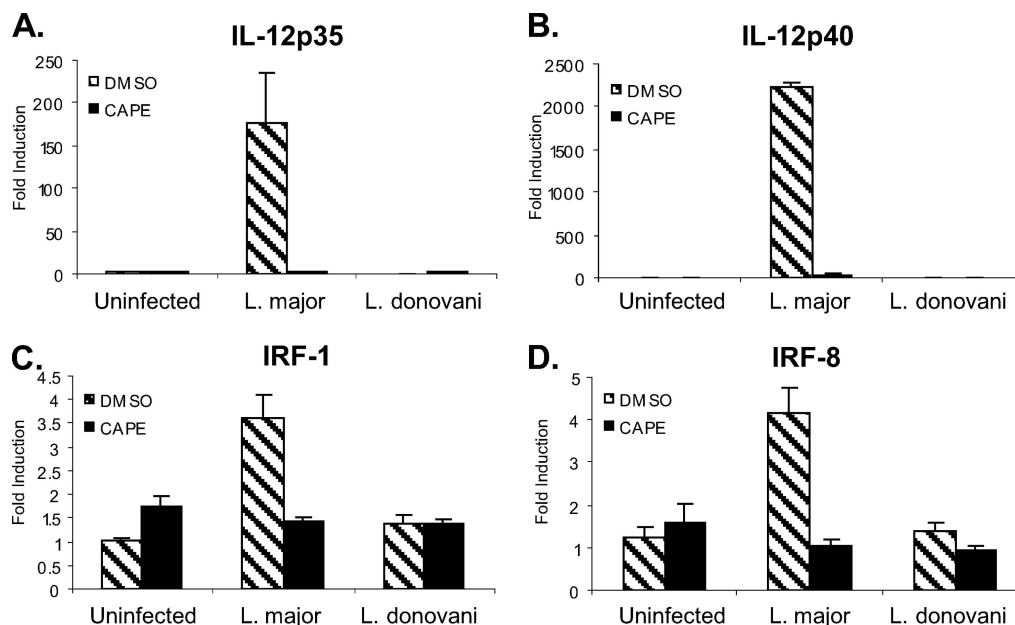


FIG. 8. Inhibition of NF-κB blocks IL-12 and IRF up-regulation in *L. major*-infected human DC. DC were pretreated with 52 μM CAPE for 1 h prior to infection with either *L. major* or *L. donovani* or treated with IFN-γ (1,000 U/ml). RNA isolated at 8 h postinfection was analyzed for *IL-12p35* (A), *IL-12p40* (B), *IRF-1* (C), and *IRF-8* (D) by quantitative real time RT-PCR. The induction of IL-12 or IRF transcripts in *Leishmania*-infected DC was calculated using the comparative ΔΔC_T method and normalized to GAPDH. Means ± SEM for three donors are shown. DMSO, dimethyl sulfoxide (vehicle control).

tion of *IRF-1* mRNA, in addition to genes regulated by IRF-1 (*GBP* genes and *COX-2*) (Fig. 2), strongly implicates IRF-1-mediated activation of these latter genes as well as *IL-12p35* and *IL-12p40* by *L. major* infection. The importance of IRF-1 is not surprising, as previous studies highlighted a key role for IRF-1 in IL-12 activation (21, 22), and murine studies demonstrated the necessity of IRF-1 in mediating resistance to *L. major* infection, a mechanism that requires IL-12 production (23, 27).

Our data also reveal that *IRF-8* and *MIG* are preferentially induced by *L. major* infection in human DC (Fig. 2 and 3). Interestingly, *IRF-1* (38, 46), *MIG* (56), and *IRF-8* (15) genes are triggered through GAS elements. GAS sequences bind IRF-8 as well as STAT1; it has been suggested that IRF-8 is

late-acting, potentiating or sustaining STAT1 activated immune activities (6). Here, we demonstrate that both IRF-8 (Fig. 4 and 5) and STAT1 (Fig. 6) are specifically activated by *L. major* infection, raising the possibility that either or both IRF-8 and STAT1 contribute to the *L. major*-induced IL-12 response in human DC. Interestingly, the lack of STAT1 in murine DC leads to lower IL-12 production in the draining lymph nodes and compromised Th1 cell responses during experimental *L. major* infection (14). One possible mechanism other than a direct activation of a STAT1-mediated signal transduction cascade by *L. major* to explain our results is the induction of autocrine-acting IFNs by *L. major* infection. We previously showed that neutralizing IFN-γ does not block *L. major*-induced CD40L-dependent IL-12p70 production (28); however, a role for type I IFNs or other cytokines remains to be elucidated.

Although IRF-8 was originally discovered as a gene repressor (34, 53, 54), it assumes the role of an activator with regard to *IL-12p35* and *IL-12p40* promoters (22, 52). Moreover, studies demonstrated the requirement of IRF-8-mediated IL-12 production for resistance to murine cutaneous leishmaniasis (11). In addition to a GAS element, binding either STAT1 (15) or IRF-8 (19), the *IRF-8* promoter also binds IRF-1 (15). The activation of these codependent transcription factors by *L. major* likely induces a positive feedback loop, ensuring the activation of IL-12. The transactivation potential of IRF-8 is realized only when associated with other transcription factors including IRF-1, Rel proteins, and Pu.1 (19). Among the pool of transcription factors that might associate with IRF-8 to enable *L. major*-induced activation of IL-12, IRF-1 is the most likely candidate due to the following reasons: (i) the activation of the *IL-12p35* promoter in human monocytes by a complex

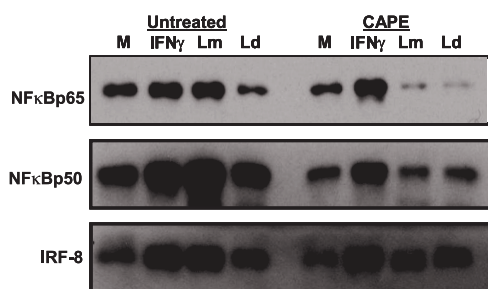


FIG. 9. Inhibition of NF-κB blocks IRF-8 nuclear translocation in *L. major*-infected human DC. DC were pretreated with 52 μM CAPE for 1 h prior to infection with either *L. major* (Lm) or *L. donovani* (Ld) or treated with IFN-γ (1,000 U/ml). Nuclear extracts isolated 8 h posttreatment or postinfection were analyzed by Western blotting for NF-κBp65, NF-κBp50, and IRF-8. One representative of two independent experiments is shown. M, medium control.

composed of IRF-1 and IRF-8 was reported previously (22), (ii) a large complex composed of IRF-1, IRF-8, Ets-2, and c-Rel has been implicated in the activation of the human *IL-12p40* promoter (49), (iii) our ChIP analysis indicates the binding of both IRF-8 and IRF-1 to the *IL-12p35* promoter in DC infected with *L. major*, and (iv) the early up-regulation of *IRF-8* mRNA in DC infected with *L. major* correlates with an increased nuclear translocation of IRF-8 in *L. major*-infected DC, corresponding to a similar pattern for IRF-1.

Our ChIP analysis indicates that both IRF-1 and IRF-8 bind the *IL-12p35* promoter in response to *L. major* infection, suggesting an interaction between IRF-8 and IRF-1. Of interest is that IRF-1 and IRF-8 do not bind the *IL-12p35* promoter in DC infected with *L. major* at later times of infection (data not shown). This absence of IRF-1 and IRF-8 binding to the *IL-12p35* promoter in DC infected with *L. major* for 12 h implies a subsequent halt of *IL-12p35* promoter activation. Accordingly, our results indicate a clear drop in *IL-12p35* levels in DC infected with *L. major* for 12 and 16 h (Fig. 1).

NF- κ B actively regulates *COX-2* (51), *IRF-1* (46), and *IRF-8* (15), in addition to *IL-12p35* (18, 21) and *IL-12p40* (39), all genes that are induced preferentially in human DC by *L. major* infection. By utilizing the NF- κ B-specific inhibitor CAPE, we demonstrate here that the inhibition of NF- κ B blocks *L. major*-induced *IL-12p35* and *IL-12p40* production (Fig. 8), supporting numerous previous studies implicating a specific role of NF- κ B factors in IL-12 transcription (18, 21, 39). Both *IL-12p35* and *IL-12p40* promoters have DNA elements that have been shown to bind NF- κ Bp50 and c-Rel as part of larger complexes (49). Our densitometry analysis revealed significant c-Rel nuclear translocation in response to *L. major* infection at all time points (Fig. 7A). NF- κ Bp50 was also reproducibly activated in response to *L. major* at 8 and 16 h postinfection (Fig. 7C), indicating that these factors are likely involved in the *L. major*-specific induction of IL-12 production. Interestingly, NF- κ Bp65 (Fig. 7B) was preferentially activated by *L. major* infection compared to *L. donovani* infection as early as 1 h postinfection. However, it appears that NF- κ Bp65 activation may actually be inhibited by *L. donovani* infection, an observation that we are currently investigating. Pretreatment with CAPE blocked NF- κ Bp50, NF- κ Bp65, and IRF-8 nuclear translocation (Fig. 9). NF- κ Bp50 was previously shown to bind the IRF-8 promoter (15); our data indicating the early activation of NF- κ Bp65 in response to *L. major* infection imply that this factor may also be involved in IRF-8 and possibly IRF-1 transcription.

Our data clearly demonstrate that CAPE treatment inhibits NF- κ B nuclear translocation, suggesting that NF- κ B factors are required for the induction of *IL-12p35*, *IL-12p40*, *IRF-1*, and *IRF-8* in response to *L. major* infection in human DC (Fig. 8). Interestingly, CAPE pretreatment did not inhibit NF- κ B nuclear translocation in response to IFN- γ (Fig. 9). NF- κ B activation is negatively regulated by I κ B (25). In response to activation stimuli, I κ B factors bound to NF- κ B proteins in the cytoplasm undergo phosphorylation, ubiquitination, and eventual proteasome-mediated degradation, resulting in NF- κ B nuclear translocation and promoter binding. While the JAK-STAT cascade is the major IFN- γ signaling pathway, IFN- γ induction of some genes requires NF- κ B activation through a pathway that likely involves double-stranded RNA-dependent

kinase (PKR) (16). The mechanism of CAPE inhibition of NF- κ B activation is unclear; preventing I κ B degradation in some studies (1), blocking nuclear translocation without significantly inhibiting I κ B degradation in other studies (33), and also suppressing the interaction of NF- κ B proteins with DNA (33). As the exact mechanism is unknown, it is tempting to surmise that the molecular basis for CAPE inhibition does not affect PKR activation of NF- κ B.

The complex process of *IL-12* transcription is coordinated by a group of transcription factors that respond to signaling cascades initiated by external stimuli. The induction of IL-12 has been best described in macrophage systems in response to IFN- γ and lipopolysaccharide stimulation (32, 39). Signaling cascades initiated by these stimuli activate a variety of transcription factors belonging to IRF, NF- κ B, and the Pu.1 family (21, 22, 32) that ultimately activate *IL-12p35* and *IL-12p40* promoters. Given this scenario, the idea of *L. major* initiating similar signaling events in DC is attractive. Our results invoke a model whereby *L. major* initiates a signaling cascade leading to the activation of NF- κ B elements followed by de novo synthesis and activation of IRF-1 and IRF-8 that further enhance *IL-12p35* and *IL-12p40* expression. This model predicts that blocking protein translation following *L. major* infection will inhibit the amplification of IL-12 in this system. Our attempts to test this model using cycloheximide revealed that the inhibition of protein synthesis resulted in large amounts of *IL-12p35* and *IL-12p40* mRNA under all conditions, suggesting that some fundamental protein necessary for regulating proinflammatory responses is absent in the presence of the inhibitor.

Signal transduction initiated by *Leishmania* in host cells results in the modulation of host immune responses. Specifically, *L. major* induces IL-12 production in DC, as opposed to *L. donovani*, resulting in strong Th1 immune responses followed by immunity. IL-12 production in response to *L. major* infection likely results from the initial interaction between the parasite and host receptors either by activating *IL-12* transcription directly or by inducing an autocrine pathway. Many receptors have been implicated in binding *Leishmania* parasites, including galectins (36, 37), Toll-like receptors (9, 31, 41), complement receptors 1 and 3 (7, 29), Fc receptors (30), mannose receptor (2, 55), scavenger receptor (44), and DC-specific ICAM1-grabbing nonintegrin (5). The interaction of one receptor or multiple receptors with *L. major* parasites or products may result in intracellular signals affecting downstream events, leading to the activation of NF- κ B, IRF-1, and IRF-8, ultimately activating *IL-12* promoters.

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