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Selective Expression of Type I IFN Genes in Human Dendritic Cells Infected with Mycobacterium tuberculosis

Maria Elena Remoli,* Elena Giacomini,* Georges Lutfalla,¶ Elisabetta Dondi,§ Grazia Valenza,¶ Angela Battistini,§ Gilles Uzé,¶ Sandra Pellegrini,§ and Eliana M. Coccia 2*

Type I IFN regulates different aspects of the immune response, inducing a cell-mediated immunity. We have recently shown that the infection of dendritic cells (DC) with Mycobacterium tuberculosis (Mtbc) induces IFN-α. In this work we have monitored a rapid induction of IFN-β followed by the delayed production of the IFN-α1 and/or -α13 subtypes. The Mtbc infection rapidly activates the NF-κB complex and stimulates the phosphorylation of IFN regulatory factor (IRF)-3, events known to induce IFN-β expression in viral infection. In turn, the autocrine production of IFN-β induces the IFN-stimulated genes that contain binding sites for activated STATs in their promoters. Among the IFN-stimulated genes induced in DC through STAT activation are IRF-1 and IRF-7. The expression of IRF-1 appears to be dependent on the sequential activation of NF-κB and STAT-1. Once expressed, IRF-1 may further stimulate the transcription of IFN-β. Induction of IRF-7 is also regulated at the transcriptional level through the binding of phosphorylated STAT-1 and STAT-2, forming the IFN-stimulated gene factor-3 complex. In turn, the IRF-1 and IRF-7 expression appears to be required for the delayed induction of the IFN-α1/13 genes. Although correlative, our results strongly support the existence of a cascade of molecular events in Mtb-infected DC. Upon infection, constitutively expressed NF-κB and IRF-3 are activated and likely contribute to the rapid IFN-β expression. In turn, IFN-β-activated IRF-1 and IRF-7 may cooperate toward induction of IFN-α1/13 if infection persists and these factors are activated. The Journal of Immunology, 2002, 169: 366–374.

The IFN are a heterogeneous family of cytokines, originally identified on the basis of their ability to induce cellular resistance to viral infections (1). The family can be divided in two classes (type I and II) on the basis of structure, function, and cell of origin. Indeed, while type I IFN (IFN-α/β) is secreted by virtually all cells in response to viral infection, type II (IFN-γ) is mainly produced as a result of stimulation of T lymphocytes and NK cells (1). In line with the different cell origin, type I and II IFN mediate diverse functions, including antiviral and antigrowth effects and immunomodulatory activities (1). Although IFN-γ possesses antiviral activity, it primarily promotes antimicrobial and antitumor activities in macrophages (2). Conversely, type I IFN, which are potent antiviral agents, have been found to regulate the immune system through the control of the proliferation, differentiation, activation, and maturation of different leukocytic populations, such as dendritic cells (DC), NK cells, Th1 cells, and memory CD87 lymphocytes (3–6). Several immunoregulatory effects are shared by all IFN. Indeed, type I and type II IFN induce overlapping but not identical sets of genes via different cell surface receptors (7). A major immunomodulatory role of IFN is exerted on the generation, transport, and presentation of antigenic peptides displayed on MHC class I (8–11). Conversely, other effects appear to be induced uniquely by IFN-γ, such as the MHC class II proteins, or by IFN-α/β, like the β2 subunit of the IL-12R on human T cells (9).

Recent studies have highlighted the production of type I IFN following bacterial infections (12, 13). It has been shown that the infection of THP-1 cells with Mycobacterium tuberculosis (Mtb) leads to secretion of type I IFN (13), and we have recently reported on the ability of Mtb-infected DC to produce type I IFN (14). Moreover, Cella et al. (15) found that plasmacytoid DC produce type I IFN in Mtb-infected lymph nodes. Despite these converging data, the mechanisms that regulate type I IFN expression in Mtb-infected cells are poorly defined.

In humans, all the known intronless type I IFN genes are clustered on the short arm of chromosome 9 spanning 400 kb (16). While IFN-β is encoded by a single gene, IFN-α are encoded by a large family of related genes composed of 12 functional IFN-α and a single IFN-ω. The expression of the IFN-α genes appears coordinately induced in virus-infected cells and may differ among cell types, reflecting the transcriptional activity of the corresponding promoter regions (17). A virus-responsive element (VRE) has been identified in the IFN-α1 gene promoter (VRE-A1) that contains a positive regulatory domain (PRD)/I-like sequences, thus suggesting the involvement of IFN regulatory factor (IRF) proteins (18–21). The regulation of the IFN-β gene has been studied extensively. The VRE of IFN-β is composed of positive and negative regulatory elements (22). The PRDIV binds a heterodimer of activating transcription factor-2 and c-Jun, whereas PRDII binds NF-κB. The PRDI and III elements are known to bind specifically members of the IRF family. Among the nine members of this

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family, IFN-1, -3, -7, and -9 have been identified as positive regulators of IFN-α/β genes (23, 24). Although IFN-1 was first identified by its ability to bind the hexamer (AAATGTA) repeats in the IFN-β promoter and to induce transcription, the analysis of mice null for this gene showed that IFN-1 is dispensable for IFN gene expression (25). Evidence has been provided for the involvement of the IFN-stimulated gene factor (ISGF)-3, a heterotrimeric complex consisting of IRF-9, STAT-1, and STAT-2, in the IFN-α/β expression (26–28). More recently, it was found that IRF-3 and IRF-7 are involved in IFN-α/β induction (29). IRF-3 undergoes phosphorylation and nuclear translocation following virus infection (30). This phosphorylation leads to the association of IRF-3 with the general coactivator p300/CREB-binding protein and the binding to the IFN-β PRD1 element (31–35). While IRF-3 is constitutively expressed in growing cells, IRF-7 expression is induced by IFN-α/β through the activation of ISGF-3 (36). Similar to IRF-3, IRF-7 undergoes virus-induced serine phosphorylation and nuclear translocation (33, 36–38).

Once secreted from infected cells, IFN induce in an autocrine and paracrine fashion the activation of Janus kinases and STAT transcription factors, leading to expression of target genes (39). Tyrosine phosphorylation of STAT-1 and STAT-2 occurs in most cell types (40), although activation of STAT-3, -4, -5, and -6 has also been reported (41–44). Upon phosphorylation, dimerization, and translocation into the nucleus, STATs bind to STAT-binding element (SBE) or to the IFN-stimulated response element (ISRE) in the promoter of target genes. Interestingly, several primary response genes are themselves transcription factors that are required for induction of secondary effectors of the cellular response to IFNs and to other cytokines. Among these, IRF-1 and IRF-7 are transcriptionally regulated by SBE and ISRE sites present within their respective promoters. Thus, the response to IFN as well as its production can be amplified and potentiated through the enhanced expression of IRF factors.

Mtb is an intracellular pathogen that infects human macrophages and DC (14, 45, 46). The outcome of the infection is dependent upon both innate and acquired immune responses (47, 48). We have previously shown that the infection of DC with Mtb induces the expression of markers involved in Ag presentation and T cell interaction. Moreover, a typical pattern of Th1/IFN-γ-inducing cytokine production is elicited in infected DC that secrete IL-12 and IFN-γ (49, 50). The outcome of the infection is determined by positive sorting of IFN-α/β-producing cells from freshly collected buffy coats obtained from healthy voluntary blood donors (Blood Bank of University La Sapienza, Rome, Italy) by density gradient centrifugation using Lymphocyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada). Monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The recovered cells were >99% CD14+ as determined by flow cytometry with anti-CD14 Ab. DC were generated by culturing adherent monocytes in six-well tissue culture plates (Costar, Cambridge, MA) with 25 ng/ml GM-CSF and 1000 U/ml IL-4 (R&D Systems, Abingdon, U.K.) for 5 days at 0.5 × 10^6 cells/ml in RPMI with supplements as above. No antibiotics were added to the cultures. At day 5, the cells were analyzed for the expression of surface markers associated with DC differentiation. Resulting cells were 70–80% CD1a- and 95% CD14+. DC were infected with Mtb at a Mtb/cell multiplicity of infection of 5, as previously described (14). Where indicated, IFN-α/β (recombinant human IFN-α2b; provided by D. Gewirtz, QT Genetics, Cambridge, U.K.) was used at 0.1 nM to monitor STAT activation. Rabbit polyclonal antiserum raised against IFN-α and -β was used at 20 μg/ml (PBL Biomedical Laboratories, New Brunswick, NJ).

**IFN determination**

Supernatants from control and Mtb-infected DC cultures were harvested at different times after infection, filtered (0.2-μm filters), and stored at −80°C. Ab pairs used in ELISA for IFN-α and IFN-β were from PBL Biomedical Laboratories. Supernatants from 6–10 separate experiments were considered. The ELISA were conducted according to the manufacturer’s instructions.

**Cell extracts**

Nuclear cell extracts were prepared as described by Schreiber et al. (49). Briefly, cells (5 × 10^6) were resuspended in 1 ml of buffer A (0.5% Nonidet P-40, 10 mM EDTA, 10 mM EGTA, 10 mM KCl, 10 mM HEPES (pH 7.9) to which 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml trypsin inhibitor, and 1 μg/ml antipain were added). Nuclei were isolated (4°C) and incubated in EMSA buffer, centrifuging the lysates at 1,200 × g for 10 min. The nuclear pellets were resuspended in 30–40 μl with buffer C (1 mM EDTA, 1 mM EGTA, 0.4 M NaCl, 20 mM HEPES (pH 7.9), 5 mM MgCl2, 25% glycerol, with fresh addition as above) and incubated for 10 min on ice with occasional mixing. The suspensions were clarified by centrifuging at 15,000 × g for 10 min. The supernatants were recovered as nuclear extracts and were rapidly frozen in dry ice and stored at −80°C. Whole cell extracts were prepared as previously described (50). Briefly, cells (10^6) were lysed in 30–50 μl of ice-cold whole cell extraction buffer (20 mM HEPES (pH 7.9), 50 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 10 mM EDTA, 2 mM EGTA, 10 μg/ml leupeptin, 100 mM NaF, 0.5 mM PMSF, 10 mM sodium orthovanadate, and sodium molybdate). The lysate was incubated for 30 min on a shaker at 4°C and insoluble debris was removed by centrifugation (13,000 × g at 4°C for 10 min) and the lysate was stored at −80°C. Both whole and nuclear extracts from cells infected by Mtb were filter-sterilized before removal from BSL-3 containment.

**EMSA**

To measure the association of DNA-binding proteins with different DNA sequences, synthetic double-stranded oligonucleotides were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase. For the analysis of NF-κB complexes, nuclear cell lysates (5 μg) were used in EMSA experiments. Binding reaction mixture (20 μl final volume) contained labeled oligonucleotide probes (30,000 cpm) in binding buffer (4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 μg poly(dI)-poly(dC)). Nuclear lysates were added and the reaction mixture was incubated for 30 min at room temperature. For supershift analysis, 1 μg of anti-p50 (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction. For the analysis of IRF and STAT complexes, whole cell lysates (15 μg) were used in EMSA experiments. The labeled oligonucleotide probe (30,000 cpm) was mixed with 2 μg of poly(dI)-poly(dC) (Amersham Pharmacia Biotech, Little Chalfont, U.K.) and 1 μg of BSA (Sigma-Aldrich, St. Louis, MO), and incubated for 30 min at 20°C in a final volume of 20 μl of binding buffer (75 mM KCl, 20 mM Tris-HCl (pH 7.5), 1 mM DTT) containing 15 μg of cell extract. For Ab treatments, 1 μg of anti-STAT-1, STAT-2, and IRF-1 (Santa Cruz Biotechnology) were added to the reaction mixture. Glyceraldehyde was added to 13% (v/v) and samples were analyzed on 5% polyacrylamide gels with 0.5% TBE (1× TBE is 50 mM Tris-borate (pH 8.2) and 1 mM EDTA) for 1.5 h at 200 V at 18°C.

The oligonucleotides used were as follows: IFN-β B (5'-AGTGG GAAATTCCTCT-3') (22); IFN-β PRDIII-1 (5'-GAAATGCAGGAATGGTGAAG-3') (22); IFN-β b (5'-GGGCGGGGAATCCC CGCAGTAA-3') (51); IFN-1 B (5'-GATCGATTTCCGAAATGTA-3') (52); IFN-7 ISRE (5'-TTTTAGGTTGCCCTTTTCCGCG-3') (53); and IFN-α/β VRE (5'-GAGTGCATGAGGAAGGAAACAAAGCAAACGAAACGAAAATGGG AAAGTGGG-3') (54).

**DNA affinity purification assay**

Biotinylated oligonucleotides wild-type IFN-α VRE (5'-GAGTGCATGAGGAAGGAAACAAAGCAAACGAAACGAAAATGGG AAAGTGGG-3') were annealed with the corresponding antisense oligonucleotide in 1× STE buffer, containing 10 mM Tris-HCl (pH 8), 50 mM NaCl, and 2 mM EDTA. Biotinylated DNA oligonucleotides were...
mixed with 200 μg of total extract in 500 μl of binding buffer containing 20 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM DTT, and 5 μg/ml BSA in presence of 13% glycerol and 20 μg of poly(dI-dC) and incubated for 25 min at room temperature. Then streptavidin magnetic beads (Promega, Madison, WI), washed three times with 800 μl of binding buffer, were added to the reaction mixture and incubated for 30 min at 4°C and for 10 min at room temperature with mixing by rotation. The beads were collected with a magnet and washed three times with 1 ml of binding buffer. The bound proteins were eluted from the beads by boiling in sample buffer and were resolved on 10% SDS-PAGE followed by immunoblotting with Ab against IRF-1, IRF-3, and IRF-7.

Western blot analysis

Whole cell extracts (30 μg) were separated on 10% SDS-PAGE gel and blotted onto nitrocellulose membranes. Blots were incubated with rabbit polyclonal Abs against IRF-1, IRF-3, IRF-7, STAT-1, and STAT-2 (Santa Cruz Biotechnology) and reacted with anti-rabbit HRP-coupled secondary Ab (Amersham Pharmacia Biotech) using an ECL system. Tyrosine-phosphorylated STAT-1 and STAT-2 were detected by immunoblotting with phosphospecific Abs and, after stripping, with the specific STAT Abs to evaluate loading. Anti-phospho-STAT-1 Abs were from New England Biolabs (Beverly, MA). Anti-phospho-STAT-2 was a generous gift of Dr. D. Levy (New York University School of Medicine, New York, NY).

RNA isolation and real-time PCR quantification

RNA was extracted from DC with RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions, which include a DNasel treatment. A phenol/chloroform extraction was performed to inactivate residual mycobacterial particles. Reverse transcription were primed with oligo(dT) and performed using the murine leukemia virus reverse transcriptase from mycobacterial particles. Reverse transcription were primed with oligo(dT) instructions, which include a DNaseI treatment and were collected at different time points after Mtb infection and analyzed with specific ELISA for IFN-α and IFN-β. The results represent the means ± SE of six separate experiments. B. Cells were infected with Mtb and were collected 24 h after infection. The expression of type I IFN subtypes was investigated by real-time RT-PCR using primers specific for sequences within the coding region of each isoform. The ratio of IFN:GAPDH transcript was determined by quantitative RT-PCR. The bars represent the 95% confidence limits of the ratio. C, DC were prepared from four different donors, which are represented by different symbols (squares, circles, triangles, and diamonds). Cells were collected at 0, 3, 8, 16, 24, and 43 h after the infection, and total cellular RNA was isolated and analyzed by real-time RT-PCR. The expression of IFN-β (filled symbols) and IFN-α/13 (open symbols) mRNA was investigated. The ratio of IFN/GAPDH transcript was determined by quantitative RT-PCR. The error bars represent the 95% confidence limits of the ratio. Because the same standard has been used for the quantification of IFN-β and IFN-α/13 mRNA, the values for both IFNs are directly comparable. The basal levels of both IFN-α/13 and -β have been measured in DC of all donors: the ratio of IFN-β/GAPDH is between 0 (undetectable IFN-β) in control DC derived from the donor □ and 1.9 for the donor ◇. The ratio of IFN-α/13/GAPDH is between 0 in control DC derived from the donors ○ and ◇ and 4.2 for the donor △.

FIGURE 1. IFN-β and IFN-α are expressed with different kinetics in Mtb-infected DC. A. Cells were infected with Mtb at a multiplicity of infection of 5 and supernatants were collected at different time points after infection and analyzed with specific ELISA for IFN-α and IFN-β. The results represent the means ± SE of six separate experiments. B. Cells were infected with Mtb and were collected 24 h after infection. The expression of type I IFN subtypes was investigated by real-time RT-PCR using primers specific for sequences within the coding region of each isoform. The ratio of IFN:GAPDH transcript was determined by quantitative RT-PCR. The bars represent the 95% confidence limits of the ratio. C, DC were prepared from four different donors, which are represented by different symbols (squares, circles, triangles, and diamonds). Cells were collected at 0, 3, 8, 16, 24, and 43 h after the infection, and total cellular RNA was isolated and analyzed by real-time RT-PCR. The expression of IFN-β (filled symbols) and IFN-α/13 (open symbols) mRNA was investigated. The ratio of IFN/GAPDH transcript was determined by quantitative RT-PCR. The error bars represent the 95% confidence limits of the ratio. Because the same standard has been used for the quantification of IFN-β and IFN-α/13 mRNA, the values for both IFNs are directly comparable. The basal levels of both IFN-α/13 and -β have been measured in DC of all donors: the ratio of IFN-β/GAPDH is between 0 (undetectable IFN-β) in control DC derived from the donor □ and 1.9 for the donor ◇. The ratio of IFN-α/13/GAPDH is between 0 in control DC derived from the donors ○ and ◇ and 4.2 for the donor △.

Results

IFN-α and -β are expressed with different kinetics in Mtb-infected DC

We have previously shown that DC infected with Mtb produce IFN-α (14). To identify the expression profile of type I IFN and to investigate the kinetics of production, cell culture supernatants were collected at different time points after Mtb infection and the levels of IFN-α and -β were determined by ELISA (Fig. 1A). DC
infected with Mtb produced both IFN-α and IFN-β subtypes, but with different kinetics: IFN-β was maximally detected at 16 h after infection, while IFN-α steadily increased from 24 h postinfection.

The level of expression of all known IFN-α subtypes, IFN-α1, and IFN-β was then studied in DC infected for 24 h with Mtb. For this, mRNA levels were quantified by real-time RT-PCR using primers specific for sequences within the coding region of each subtype. Surprisingly, Mtb-infected cells did not induce a mixture of isotypes, but induced mainly IFN-α1 and/or IFN-α13 (Fig. 1B). These two nonallelic genes differ only in few nucleotides in the 5' and 3' noncoding regions and their mRNAs give rise to the same protein (55). To compare the kinetics of IFN-β and IFN-α1/13 gene expression, RNA was isolated from DC at different times after Mtb infection and the steady-state mRNA levels were measured by real-time RT-PCR (Fig. 1C). At 16 h postinfection, a clear correlation was observed between the secretion of IFN-β and the up-regulation of its transcript. The expression of the IFN-α1/13 mRNA was delayed, being induced 16 h after exposure of the DC to Mtb and increasing up to 24 h. At 43 h, the level of both IFN-β and IFN-α1/13 mRNAs decreased sharply.

**Infection of DC with Mtb induces the activation of NF-κB and IRF transcription factors**

NF-κB is a major player in the transcription of the IFN-β gene. In particular, the p50/p65 heterodimer is known to bind to the −66 to −57 region of the IFN-β promoter, termed PRDII (56–59). To study whether Mtb infection was able to induce binding of NF-κB to the PRDII regulatory sequence, DC were stimulated with live bacteria. Nuclear extracts were prepared at different times and analyzed by EMSA using an oligonucleotide corresponding to the κB site of the IFN-β promoter (Fig. 2A). NF-κB DNA binding was detected at 1 h after infection and reached a maximal level at 8 h. The identity of the complex was confirmed by supershift experiments using Abs raised against the p50 subunit.

The IFN-β promoter contains also the PRDI and III elements that are known to bind different IRF family members. We thus investigated whether infection of DC by Mtb would induce IRF DNA-binding activity as measured by EMSA using an oligonucleotide corresponding to the PRDIII-I (Fig. 2B). A clear induction of IRF-1 DNA binding activity was observed as early as 3 h postinfection and a sustained activity was still present at 24 h. The identity of the complex was confirmed by reaction with anti-IRF-1 Ab. This activation well correlated with the induction of the IFN-β protein (see Fig. 4B). Because we were unable to detect IRF-3 binding activity by EMSA profile, we investigated whether Mtb infection could induce IRF-3 phosphorylation, as demonstrated in the event of virus infection (60, 61). Immunoblots were performed on total cell extracts prepared at different times following infection (Fig. 2C). As expected, IRF-3 was constitutively expressed in uninfected cells in its nonphosphorylated form. Mtb infection resulted in a evident increase of IRF-3 phosphorylation, monitored by the appearance of slower migrating forms in 7% SDS gels (IRF-3P). The Mtb-induced phosphorylation was rapid and transient, starting at 1 h and decreasing at 16 h postinfection. The total IRF-3 content was better evaluated in a shorter gel run (Fig. 2C, lower panel), wherein it appeared to decrease upon longer infection times, suggesting that proteasome-dependent degradation could operate in Mtb-infected cells, as previously described in virus-infected cells (30).

**Type I IFN production activates STAT phosphorylation following Mtb infection of DC**

Type I IFN exerts multiple biological functions through the activation of STAT-1 and STAT-2 transcription factors. Therefore, the extent of STAT phosphorylation was analyzed in Mtb-infected DC. A short kinetics of infection, from 30 min to 8 h (Fig. 3, left panel), and a longer one up to 48 h (Fig. 3, right panel), were performed. As a control, immature DC were treated for 30 min with a saturating dose of IFN-α2 (0.1 nM). Weak tyrosine phosphorylation of STAT-1 and STAT-2 was detected as early as 3 h of infection; it increased at 8 h and started to decrease at 48 h. At any time point, the extent of phosphorylation was considerably less than in immature DC treated with exogenous IFN-α. Moreover, a significant increase in STAT-1 and STAT-2 protein levels (3- to 5-fold) was consistently observed at 16 h (Fig. 3, right panel). This increase was specific, because the level of the tyrosine kinase 2, involved in the IFN-α signaling pathway, was unchanged (data not shown). To characterize in more detail the role of Mtb-induced IFN-αβ production on the observed STAT-1 and STAT-2 activation, neutralization experiments were conducted. DC cultures were...
stimulated with live Mtb in the presence or the absence of neutralizing anti-IFN-β Abs (Fig. 3, left panel). A clear reduction in STAT-1 and STAT-2 phosphorylation was observed in the presence of anti-IFN-β Abs. Moreover, the kinetics of IFN-β production induced by Mtb infection correlated with the kinetics of STAT activation, strongly suggesting that IFN-β mediates the Mtb-induced STAT phosphorylation.

**STAT activation regulates the expression of IRF transcription factors in Mtb-infected DC**

Next, we asked whether the Mtb-induced phosphorylation of STAT-1 and STAT-2 correlated with induction of their DNA binding activity. Among the IFN-stimulated genes induced through STAT activation are members of IRF family, IRF-1 and IRF-7. In particular, the expression of IRF-1 is dependent on the activation of STAT-1 recognizing the SBE motif within the IRF-1 promoter. Thus, binding of STAT-1 homodimers was analyzed by EMSA on the SBE motif present within the IRF-1 promoter (52), using whole cell extracts from Mtb-infected DC. A robust induction of binding activity was evident 8 h after Mtb infection and remained high at later time points (Fig. 4A). To verify whether this activity correlated with the induction of the IRF-1 protein, whole cell extracts were prepared at various times after Mtb infection and were analyzed for IRF-1 expression by immunoblot. A 1-h infection with Mtb resulted in induction of the IRF-1 protein, which increased further at 8 h and then leveled off (Fig. 4B). This early rise in IRF-1 suggests a STAT-independent regulatory mechanism. Because it is known that IRF-1 induction is also regulated by NF-κB (51, 62), we investigated whether the rapid IRF-1 expression observed in Mtb-infected DC could be ascribed to the activation of the NF-κB pathway. Nuclear extracts were prepared from DC infected with Mtb at different time points and analyzed by EMSA using the κB sequence of the IRF-1 promoter. As shown in Fig. 4C, Mtb infection readily activated NF-κB DNA binding, which remained high thereafter. As shown by supershift, the NF-κB DNA binding complex contained the p50 subunit.

**FIGURE 4.** Mtb infection induces the expression of IRF-1 via activation of NF-κB and STAT-1. Cell extracts were prepared from Mtb-infected cells at different time points. A, Fifteen micrograms of total extracts were subjected to EMSA analysis with IRF-1 SBE sequences as described in Materials and Methods. Supershift assays were performed after incubation of cell extracts with anti-STAT-1 Abs, as indicated. A representative EMSA experiment, of three performed with different extracts, is shown. B, Whole cell extracts were prepared and analyzed by immunoblot with anti-IRF-1 Abs. C, Five micrograms of nuclear proteins were subjected to EMSA analysis using oligonucleotide the κB sequence of the IRF-1 promoter. Supershift assays were performed after incubation of cell extracts with anti-p50 Abs (α-p50), as indicated.

In addition, we analyzed the expression of IRF-7 along the infection of DC because recent studies demonstrated the critical role of this transcription factor in virus-mediated induction of IFN-α (36–38). We measured the Mtb-induced STAT DNA binding activity on the ISRE motif that was recently identified within the promoter of the IRF-7 gene (53). A low mobility complex was transiently induced at 8 h postinfection and was shown, by supershift assays, to contain STAT-2 (Fig. 5A, ISGF-3). A higher mobility complex was also detected at 8 h after stimulation and persisted to 48 h. This complex was shown to contain IRF-1 by supershift assays (Fig. 5A, IRF-1). Consistent with the EMSA experiment, the IRF-7-specific transcript was detected at 8 h after Mtb infection and increased at 24 h, as evaluated by quantitative RT-PCR (Fig. 5B). The IRF-7 protein accumulated at 16 h after stimulation (Fig. 5C). Altogether, these strong correlative results suggest that IRF-1 factor and ISGF-3 may cooperate toward a sustained expression of IRF-7 in Mtb-infected DC.

**IRF-1 regulates the expression of IFN-α1/13 in Mtb-infected DC**

It has been recently described that the relative level of IRF-1, IRF-3, and IRF-7 modulates the expression profile of IFN-α subtypes (54). To evaluate the relative contribution of IRF factors to
the delayed expression of the IFN-α/13 gene. EMSA experiments were performed on the VRE sequence present within IFN-α/13 promoter using whole cell extracts prepared at various times after Mtb infection (Fig. 6A). Surprisingly, only IRF-1 binding was detected, starting from 8 h after infection. The identity of the complex was confirmed by supershift experiments using anti-IRF-1 Abs. As an alternative approach to EMSA, we performed a DNA affinity purification assay, which represents a more sensitive analysis of tran-
crption factor binding to target consensus sequences. Biotinyl-
at ed oligonucleotides, corresponding to the wild-type VRE of the IFN-α/13 promoter and a mutant form, were immobilized onto streptavidin-coated magnetic beads and then incubated with whole cell extracts from uninfected or 24-h Mtb-infected DC. The isolated complexes were examined by immunoblotting with IRF-1-, IRF-3-, and IRF-7-specific Abs. Despite the presence of both IRF-3 and IRF-7 in the cell extracts used (data not shown), Mtb infection resulted in retention of only IRF-1 (Fig. 6B). The binding was specific because the mutated VRE oligonucleotide did not retain IRF-1 from the same cell extract.

Discussion
The induction of type I IFN in infected cells represents an event central to innate immunity, because these cytokines mediate a variety of immunoregulatory effects, whose major outcome is the stimulation of CD4+ Th1 cells with production of high levels of IFN-γ and consequent induction of cell-mediated immune response. The importance of CD4+ T cells, IFN-γ production, and microbicidal activities in the control of acute Mycobacterium infections has been confirmed in a variety of experimental models (47, 48). Moreover, lung human CD4+ T cells were reported to exert some cytolytic functions on infected macrophages via Fas ligand-induced apoptosis (47).

We have previously shown that DC infected with Mtb are pri-
marily involved in inducing an antimycobacterial T cell immune response (14). After interacting with the pathogen, DC mature and acquire the ability to stimulate T cells through surface expression of MHC and costimulatory molecules, as well as secretion of immunoregulatory cytokines, such as IL-12 and type I IFN (14). Given the role played by type I IFN in stimulating NK cells and CD4+ T lymphocytes, we sought to characterize the pro-
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marily involved in inducing an antimycobacterial T cell immune response (14). After interacting with the pathogen, DC mature and acquire the ability to stimulate T cells through surface expression of MHC and costimulatory molecules, as well as secretion of immunoregulatory cytokines, such as IL-12 and type I IFN (14). Given the role played by type I IFN in stimulating NK cells and CD4+ T lymphocytes, we sought to characterize the profile of IFN subtypes produced in human DC infected by Mtb. We show in this work that the contact between Mtb and DC results in an elevated and rapid expression of IFN-β, followed by the delayed production of IFN-α. Whereas in virally infected DC most IFN-α subtypes are
induced (E. M. Coccia and G. Uzé, unpublished observation), in Mtbinfected DC the production is restricted to the IFN-α/13 subtype, which possesses low antiviral and antiproliferative activities compared with the others (63). Whether this IFN subtype exerts some unique biological effects toward the regulation of immune functions in the course of Mtbinfection is presently unknown.

The kinetics of type I IFN measured in our experimental model is reminiscent of the two-wave expression profile described in virally infected fibroblasts, where IFN-β production is followed by the delayed induction of specific IFN-α genes (36). The stimulation of IFN synthesis in virally infected cells is mainly regulated at the transcriptional level by factors belonging to the NF-κB and the IRF families. While both are important for the induction of IFN-β, NF-κB does not participate in IFN-α induction, as no κB sequences have been identified in the promoter region of IFN-α genes. It has been reported that Mtbin can induce NF-κB activation in human monocytes (64). Accordingly, we observed the rapid formation of the NF-κB complex on the κB sequences of the IFN-β VRE, confirming that the interaction between Mtbinfection and DC readily activates a signaling pathway leading to NF-κB activation. We also investigated the involvement of IRF family members in the induction of IFN in Mtbinfected DC. Interestingly, the kinetics of IRF-3 activation paralleled IFN-β induction. Indeed, the rapid IRF-3 phosphorylation, observed within the first 8 h after infection, is likely involved in the induction of the IFN-β mRNA, whose expression peaks at 16 h following Mtbinfection. Although the involvement of IRF-3 as regulator of IFN gene expression is extensively documented in studies using recombinant or ectopically expressed protein (29, 30), EMSA binding of the endogenous IRF-3 protein to the IFN-β promoter has never been reported, probably reflecting its low affinity to the PRDIII and I regulatory sequences (33). Accordingly, our attempts in Mtbinfected DC have been unsuccessful. However, using a chromatin immunoprecipitation approach, Wathelet et al. (33) were able to demonstrate virally induced IRF-3 association with the IFN-β promoter. Based on these findings, we propose that IRF-3 and NF-κB, activated by phosphorylation following Mtbinfection, might cooperate to up-regulate the IFN-β expression in human DC. Conversely, the involvement of IRF-3 in the delayed induction of IFN-α/13 is unlikely, due to its decay at 24 h postinfection.

IFN-β secreted from the infected DC is likely to act in an autocrine and paracrine loop, through the binding to cognate receptors and the activation of the Janus kinase/STAT pathway. The formation of heterotrimeric ISGF-3 and homodimeric STAT-1 complexes contributes to the expression of target genes. Among the genes controlled by the prompt IFN-β production from Mtbinfected DC are IRF-1 and IRF-7, which could be involved in the second wave of transcription leading to IFN-α/13 expression. Indeed, the neutralization of IFN-β in Mtbinfected DC abolished IFN-α expression (data not shown), and the expression of IRF-1 and IRF-7 paralleled the IFN-α/13 mRNA accumulation. Our data suggest that the long-lasting IRF-1 expression is dependent on STAT-1 homodimers induced by IFN-β, whereas the delayed expression of IRF-7 requires both the IRF-1 and the ISGF-3 complex. IRF-7 was shown to be indispensable for IFN-α gene expression in virally infected human and mouse cells (36–38, 65). Despite a consistent increase in IRF-7 content observed upon Mtbinfection, our multiple attempts to detect association of this factor to the IFN-α1 VRE sequences by EMSA or DNA affinity purification assay have failed. One possible explanation is that the modification(s) induced by Mtbinfection differs, both quantitatively and/or qualitatively, from those induced by a virus, and this difference could account for the distinct subsets of IFN-α genes induced in the two infectious scenarios. It is conceivable that IRF-7 undergoes serine phosphorylation following Mtbinfection, because its carboxyl

FIGURE 7. Model for the multistage induction of type I IFN gene expression following Mtbinfection. As early as 1 h after Mtbinfection, both NF-κB and IRF-3 are activated through mechanisms that are dependent on protein phosphorylation. They translocate into the nucleus, where they may drive the transcription of IFN-β promoter, resulting in the initial production of IFN-β. NF-κB induces also the transcription of IRF-1, which can cooperate in the IFN-β gene regulation. Starting from 8 h postinfection, secreted IFN-β stimulates the type I IFNR in an autocrine and paracrine fashion, leading to formation of both ISGF-3, which binds to the IRF-7 promoter, and STAT-1 homodimers, which induce IRF-1 expression. These transcription factors may cooperate in reinforcing IFN-β expression as well as in inducing IFN-α/13 expression. Dashed lines represent binding of transcription factors to regulatory elements, which has been documented in other studies but could not be experimentally demonstrated in Mtbinfected DC. Striped arrows indicate a possible activation event involving a novel cellular kinase and leading to IRF phosphorylation following Mtbinfection.
terminus is highly homologous to that of IRF-3, whose phosphorylation was detected (29, 34). As recently suggested by Levy et al. (66) in their study of virus-infected cells, an unknown kinase activated by Mtb infection could be responsible for IRF-3 and IRF-7 phosphorylation.

Binding of IRF-1 to the PRDIII-1 sequences of the IFN-β promoter and to the VRE motif of IFN-α1 promoter was clearly observed starting 8 h after infection. Therefore, IRF-1 could cooperate with IRF-3 and NF-κB in the regulation of IFN-β and thereafter with IRF-7 in the regulation of IFN-α/13, arguing for its repeated involvement in the coordinated expression of type I IFN genes. Although the original role attributed to IRF-1 in IFN gene induction was reconsidered following the analysis of IRF-1-null mice (25, 67), IRF-1 was recently shown to direct, together with NF-κB and activating transcription factor-2/c-Jun, the ordered recruitment of chromatin-modifying and general transcription factors to the IFN-β promoter (68). In addition, it has been suggested that IRF-1 may directly contribute to the transcriptional regulation of the IFN-α/1 and -α2 genes (54). Altogether, these and our results obtained in Mtb-infected primary cells revalidate the role of IRF-1 in the mechanism of type I IFN gene induction.

Our results also highlight the importance of STAT-1 in the induction of IRF factors involved in IFN-α regulation. Interestingly, the importance of STAT-1 in the establishment of an antmycobacterial activity has been demonstrated in patients with unexplained mycobacterial disease (69). In these subjects, an impairment of the IFN-γ response was associated to a defect in STAT-1 activation, due to a heterozygous substitution at nucleotide 2116 that severely impairs phosphorylation of tyrosine 701 and the subsequent homodimerization. The formation of a functional ISGF-3 complex in response to IFN-α was observed in these patients. Because both the IFN-α- and the IFN-γ-induced binding of STAT-1 homodimers to SBE were affected, it would be interesting to measure, in DC obtained from these patients, IRF-1 expression and IFN-α production following Mtb infection. These studies could help to clarify the role of type I IFN in the initiation of both innate and adaptive immunity against Mtb.

The working model that can be drawn from the analysis of the events occurring in human DC infected in vitro with live Mtb is illustrated in Fig. 7. The positive feedback regulation of type I IFN production appears to be mediated by a complex interplay of NF-κB, IRF, and STAT transcription factors, whose coordinated activation leads to a temporal and stimulus-specific IFN expression. In our experimental system, we propose IRF-1 as a key factor in the induction of type I IFN gene expression during Mtb infection. Interestingly, IRF-1 could be involved as well in a more general process linked to DC maturation, because it was recently found, by oligonucleotide microarrays, to be up-regulated in human DC induced to mature in response to different pathogens (70). The identification of the gene expression profile associated with DC maturation in response to Mtb infection will be crucial to the understanding of the events leading to the development of cell-mediated immunity and in turn to developing improved therapies to potentiate protective response against tuberculosis.

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