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Influenza A Virus-Induced IFN-α/β and IL-18 Synergistically Enhance IFN-γ Gene Expression in Human T Cells[1]

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T cells contribute significantly the to host's early defense against viral and bacterial infections and are essential for clearance of the pathogen. IFN-γ, a product of activated T and NK cells, has, in addition to its direct antimicrobial activity, a major role in activating cell-mediated immunity. Here we report that cytokines secreted by influenza A virus-infected macrophages are able to induce IFN-γ synthesis in human T cells. Influenza A virus-infected human peripheral macrophages secreted IFN-α/β, TNF-α, IL-1β, and a recently identified cytokine, IL-18 (or IFN-γ-inducing factor), whereas the production of IL-12 was not detected. Supernatants collected from virus-infected macrophages induced rapid IFN-γ mRNA expression and protein production in T cells. This was down-regulated by the addition of neutralizing anti-IFN-α/β Abs, whereas neutralizing anti-IL-12 Abs had no effect on IFN-γ gene expression. Exogenously added IFN-α/β also rapidly stimulated the synthesis of IFN-γ mRNA in T cells independently of protein synthesis. IL-18 synergized with IFN-α to up-regulate IFN-γ gene expression and protein production. The data suggest that IFN-α/β and IL-18 produced by macrophages during virus infection may act together to induce IFN-γ synthesis and, consequently, may play an important role for both of these cytokines in the development of Th1-type immune responses. ***The Journal of Immunology,*** **1998, 160: 6032–6038.**

The activation of macrophages is one of the first responses in natural immunity against viral and other intracellular infections. Intracellular pathogens activate these cells to produce proinflammatory cytokines, which further activate phagocytic cells and other inflammatory responses. These early, non-Ag-specific responses of natural immunity are effective in eliminating pathogens or at least in significantly reducing their ability to multiply (1). Virus infections generally induce immune responses that lead to a long lasting immunologic memory (2). In many viral infections, the complete elimination of the pathogen is mediated by this Ag-specific adaptive immunity, whose major contributors are B and T cells. IFN-α/β is one of the most abundant cytokines released by macrophages during viral infections. The role of IFN-α/β as a direct antiviral substance has been well documented, but its role as a significant immunoregulatory molecule in T cell responses has also been suggested (3, 4).

IFN-γ is an important regulator of immune responses in vivo (5–7). It is a product of activated T and NK cells and plays a critical role in the host defense against microbial pathogens (8, 9). The expression of the IFN-γ gene is strictly controlled at the transcriptional level, the molecular mechanisms of which are presently not very well understood (10, 11). IL-12 is a heterodimeric cytokine (12) produced primarily by APCs, including monocytes, macrophages, and dendritic cells, especially in bacterial infections (13, 14). It has a crucial role in inducing IFN-γ production by T lymphocytes, and in the generation of IFN-γ-producing Th1 cells (15–17). Some other costimulatory factors, such as TNF-α and IL-1β, may also be needed for optimal IFN-γ production (18, 19). Data obtained from experiments with IL-12 p40 knockout mice have suggested that alternative pathways exist for IFN-γ induction (20). A novel cytokine, IL-18 (IFN-γ-inducing factor (IGIF)[3]), which shares some but not all the biologic activities of IL-12, has been identified and molecularly cloned from both mice and humans (21–23). Human IL-18 has been reported to induce IFN-γ and granulocyte/macrophage CSF (GM-CSF) production in PBMC, decrease IL-10 production, and enhance NK cell cytotoxicity (22).

In the present report we analyze the expression of IFN-γ in human T lymphocytes during influenza A virus infection. We show that IFN-α/β produced by virus-infected macrophages directly stimulates IFN-γ production in T cells. Macrophages also respond to influenza A virus infection by producing IL-18, which synergizes with IFN-α to stimulate IFN-γ gene expression and protein secretion. In our experimental setting, macrophages infected with influenza A virus did not produce IL-12. Thus, in influenza A virus infection, macrophage-derived IFN-α/β and IL-18 may promote Th1-type immune responses.

Materials and Methods

Generation of macrophages and T cell blasts from PBMCs

PBMCs were isolated from freshly collected buffy coats of normal blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) by density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were incubated in six-well plates (Falcon Multiwell, Becton Dickinson, Franklin Lakes, NJ) at 2×10^7 cells/well for 1 h at 37°C in serum-free RPMI 1640 medium supplemented with 20 mM HEPES, 2 mM glutamine, 0.6 μg/ml penicillin, and 60 μg/ml streptomycin. Adherent cells (monocytes) were thoroughly washed with PBS and grown in Macrophage-SFM medium (Life Technologies, Gaithersburg, MD) containing 10 ng/ml of GM-CSF (Leucomax, Schering-Plough, Innishannon, Ireland) and antibiotics. The medium was replaced every 2 days, and the cells were used in the experiments 1 to 2 wk after isolation. At this

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[3] Abbreviations used in this paper: IGIF, IFN-γ-inducing factor; GM-CSF, granulocyte/macrophage-CSF; CHX, cycloheximide; NP, nucleoprotein.

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point, practically 100% of the cells were macrophages (24, 25), as determined by their initial CD14 expression, typical morphology, and lack of other cell types. T cells were further purified from nonadherent cells using nylon wool columns. Purified T cells were primarily stimulated with plate-bound anti-CD3 mAbs (26) and cultured in RPMI supplemented with 10% FCS (Integro, Zaandam, The Netherlands) and 100 IU/ml IL-2 (Chiron Corp., Emeryville, CA) for 6 days. Growing T cell cultures were expanded every 3 to 5 days with RPMI supplemented with FCS and IL-2, and used 2 wk after isolation. These cells are referred to as T cell blasts. As determined by flow cytometry, >99% of the cells were CD3 positive, consisting of $CD4^+$ (30%) and $CD8^+$ (70%) cells. In all the experiments, the T cells were removed from the IL-2-containing medium 16 h before stimulation.

Virus and infection

The stock of human pathogenic influenza virus A/Beijing/353/89 (H3N2), originating from the National Institute of Medical Research (London, U.K.), was grown in the allantoic cavities of 11-day-old embryonated hen eggs and stored at −70°C. The virus stock had a hemagglutination titer of 128 when a standard method was used (27). Macrophages grown to confluence in six-well plates were infected with the virus (1/10 dilution) in a 2-ml volume of Macrophage-SFM medium per well. After 1 h, the virus was removed, and the infected macrophages were washed with PBS and incubated further in RPMI with FCS. The cells or cell culture supernatants were collected at different times after infection. Allantoic fluid from uninfected embryonated eggs was used as a mock control. Both the virus and control stocks were endotoxin free, as tested by the *Limulus* assay. Macrophages or T cells from two to four donors were used in each experiment. In those experiments in which macrophage supernatants and T cells were used in a mixture, they always originated from the cells of the same donor.

Cytokines

Highly purified human leukocyte IFN-α (13×10^6 IU/ml) was provided by Dr. Hannele Tölö (Finnish Red Cross Blood Transfusion Service) and used at 100 IU/ml, unless otherwise stated. Human IFN-β (0.2×10^6 IU/ml) was obtained from Bioferon (Laupheim, Germany). Human rIL-1β, rIL-12, and rTNF-α were obtained from R&D Systems (Abingdon, U.K.) and used at 10, 5, and 10 ng/ml, respectively. *Escherichia coli*-produced highly purified human rIL-18 (22) was used at 10 ng/ml.

Antibodies

A 1/200 dilution of sheep antiserum specific for natural human IFN-α/β (containing 450,000 neutralizing U/ml for IFN-α and 3,000 neutralizing U/ml for IFN-β) was used. This antiserum has previously been described (28). Neutralizing anti-human IL-12 polyclonal Ab was obtained from R&D Systems. The Ab concentration used (5 μg/ml) was determined to be able to fully neutralize exogenously added IL-12 (1 ng/ml) to induce IFN-γ mRNA synthesis in T cells.

RNA isolation and analysis

RNA was isolated from pooled cell samples from different donors by lysing cells in guanidinium thiocyanate and pelleting the RNA through a 5.7-M $CsCl_2$ cushion. Equal amounts (20 μg) of total cellular RNA were size fractionated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane (Hybond, Amersham, Aylesbury, U.K.), and hybridized with the cDNA probe encoding human IFN-γ (29). Glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe, β-actin cDNA probe, and ethidium bromide staining of ribosomal RNA bands were used to ensure equal RNA loading. The probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) using a random primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). The membranes were hybridized under conditions of high stringency (50% formamide, 5× SSPE, 5× Denhardt's solution, and 0.5% SDS) at 42°C and washed twice at room temperature and once at 65°C in 1× SSC/0.1% SDS for 30 min each time. The filters were exposed to Kodak AR X-OMAT film (Eastman Kodak, Rochester, NY) at −70°C using an intensifying screen.

Assay for IFN-α/β bioactivity and cytokine ELISAs

The medium from the cell cultures was collected, pooled, and stored in aliquots at −70°C until assayed. For the IFN-α/β assay, the samples were dialyzed against acidic glycine buffer (pH 2) to destroy possible IFN-γ and other cytokine activity, followed by two dialyses against PBS and one against MEM. The IFN-α/β titers were determined by means of a vesicular stomatitis virus plaque reduction assay in the HEp2 cells (30). The biologic assay for IFN-α/β was specified by the addition of neutralizing Abs against IFN-α/β. The results are presented as international units per milliliter using a standard IFN-α preparation as a control. The amounts of IFN-γ, TNF-α, IL-1β, and IL-12 in the culture supernatants were measured by specific ELISA (R&D Systems). The IL-18-specific ELISA assay was obtained from Hayashibara Biochemical (Okayama, Japan) (31).

Western blotting analysis

The proteins were separated on 12% SDS-PAGE in accordance with the Laemmli buffer system (32), and then transferred electrophoretically onto Immobilon membranes (Millipore, Bedford, MA). Polyclonal rabbit anti-influenza A nucleoprotein antiserum (1/1000 dilution) (33) was allowed to bind in PBS containing 5% nonfat milk for 2 h at room temperature followed by secondary Ab binding with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) for 30 min at room temperature. The protein bands were visualized by the ECL chemiluminescense system (Amersham). The protein sample concentrations were measured using a Bio-Rad protein assay kit.

Cycloheximide (CHX) treatment

For protein synthesis inhibition, T cells were incubated for 30 min in the presence of 10 μg/ml CHX (Sigma, St. Louis, MO). The concentration of CHX used was found previously to block protein synthesis in human blood mononuclear cells (33).

Results

Supernatant from influenza A virus-infected macrophages induces IFN-γ mRNA and protein production in T cells

Our aim was to study, using human peripheral blood cells, whether the cytokines produced by influenza A virus-infected macrophages are responsible for inducing IFN-γ gene expression in T cells. Macrophages were obtained from peripheral blood monocytes by culturing them with GM-CSF as described previously (34, 35). Macrophages originating from different blood donors were infected separately with pathogenic influenza virus A/Beijing/353/89 (H3N2). After 1-h infection, the unabsorbed virus was removed, and fresh medium was added. The macrophages were cultured further, and samples of the supernatant were collected at different times after virus infection. The supernatants were then used to stimulate potential IFN-γ production in T cell cultures originating from the same donor. After 3-h incubation, the majority of the cells were collected, and total cellular RNA was prepared for Northern blot analysis. Elevated IFN-γ mRNA levels were detected in T cells incubated with macrophage supernatant collected at 6 h postinfection (Fig. 1*A*, *lane 4*), and the maximum IFN-γ mRNA expression was seen after stimulation with supernatants collected 16 and 24 h postinfection (Fig. 1*A*, *lanes 6* and *7*). The remainder of the cells were incubated further for 24 h, and IFN-γ secreted into the culture medium was analyzed by ELISA. Supernatants collected at 16 or 24 h after influenza A virus infection were equally effective in inducing IFN-γ production in T cells (Fig. 1*B*). The data suggest that cytokines or other soluble factors produced by virus-infected macrophages may induce IFN-γ gene expression in T cells.

To determine the kinetics of influenza A virus infection and analyze its relation to cytokine gene expression, macrophages were collected at different times after infection and pooled, and the total cellular RNA was isolated. Viral nucleoprotein (NP) mRNA accumulation was observed from 6 h post infection (Fig. 2*A*, *lane 4*), continued to accumulate up to 16 h, and declined thereafter. Western blotting analysis showed that NP was detectable at 2 h (Fig. 2*B*, *lane 2*), apparently originating from the input virus, but started to accumulate at 6 h postinfection and peaked at 16 h after infection. Influenza A virus-infected T cells had kinetics very similar to those of the macrophages. However, the amount of synthesized NP was much smaller, as determined from mRNA and protein analysis (data not shown). Human T cells infected with influenza A virus exhibited very low IFN-γ mRNA expression at 2 h after infection, but this disappeared totally thereafter. Very low levels of secreted

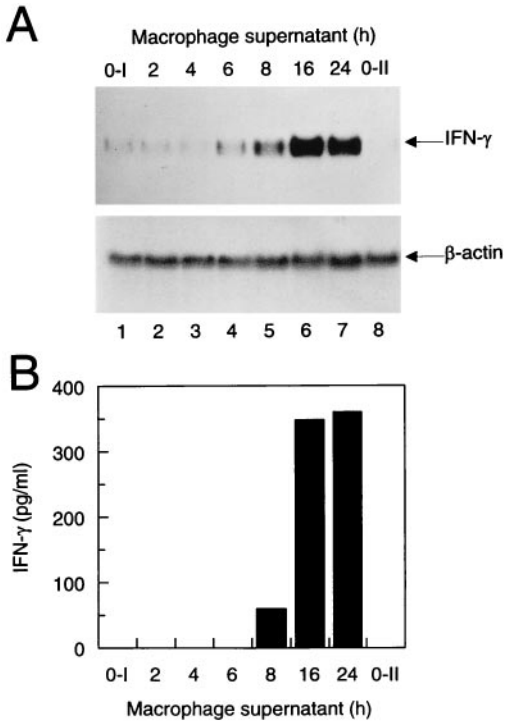

FIGURE 1. Induction of IFN-γ production in T cells in response to the cell culture supernatants harvested from macrophages at different times after influenza A virus infection. Human macrophages were infected with influenza virus strain A/Beijing/353/89 (H3N2), and the supernatants were collected at the times indicated in the figure. *A*, Human T cells were incubated with the supernatants for 3 h, the cells were collected, total cellular RNA was isolated, and Northern blot analysis was conducted using IFN-γ and β-actin cDNA probes. *B*, T cells were incubated with macrophage supernatants for 24 h, and then the amount of IFN-γ in the T cell supernatants was measured by IFN-γ ELISA. The results shown are from one experiment but are representative of five individual experiments. 0-I and 0-II are the supernatants of the uninfected macrophages after 3- and 16-h incubations, respectively.

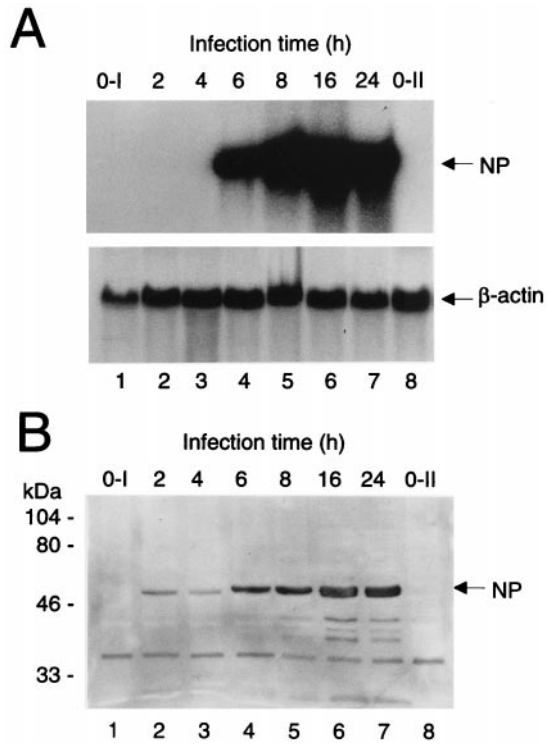

FIGURE 2. Kinetics of influenza A virus infection in human macrophages. Macrophages from different donors were separately infected with influenza A virus. Samples of the cells were collected at different times after infection. *A*, Total cellular RNA was isolated from the pooled cell samples from different donors. Equal amounts of RNA (20 μg/ml) were size fractionated by formaldehyde-agarose gel electrophoresis and subjected to Northern blot analysis with a ^{32}P-labeled influenza A virus NP probe. *B*, Protein samples (10 μg) prepared from virus-infected macrophages were separated on 12% SDS-PAGE, and Western blots were stained with specific Abs for influenza NP. 0-I and 0-II are the uninfected cells after 3- and 16-h incubations, respectively. NP, influenza A virus nucleoprotein.

IFN-γ were observed in the T cell culture supernatant using a biologic assay sensitive for this cytokine (data not shown).

Cytokines secreted by macrophages in response to influenza A virus infection

Several cytokines have previously been reported to enhance IFN-γ production, including IFN-α, TNF-α, IL-1β, IL-12, and IL-18 (18, 19, 21, 36, 37). To analyze whether these cytokines were produced in our infection model, macrophages were infected with influenza virus as described above. Supernatant samples were collected at different times after infection and analyzed for the presence of various cytokines using a biologic assay (IFN-α/β) or cytokine-specific ELISAs. Influenza A virus-infected macrophages secreted IFN-α/β, TNF-α, and low amounts of IL-1β (Fig. 3). In addition, IL-18 was detected in the supernatant of the virus-infected macrophages (Fig. 3). Expression of IL-12 was not observed at either the mRNA (Northern blot) or the protein (ELISA) level (data not shown). These results indicate that the possible cytokines involved in the induction of IFN-γ synthesis in influenza A virus infection include at least IFN-α/β, TNF-α, IL-1β, and IL-18.

IFN-α/β produced by influenza A virus-infected macrophages activates IFN-γ synthesis in T cells

To identify the cytokine(s) responsible for IFN-γ gene expression, samples of the supernatants collected from the macrophages infected with influenza A virus for 6 or 16 h were treated with neutralizing anti-IFN-α/β or anti-IL-12 Abs for 30 min before addition to the T cell cultures. The neutralizing capacity of both these Abs was pretested to totally block the induction of IFN-γ mRNA synthesis by IFN-α/β (1000 IU/ml) or IL-12 (1 ng/ml) in T cells (data not shown). Treatment of the supernatants with anti-IFN-α/β Abs significantly down-regulated IFN-γ mRNA expression (Fig. 4, *lanes 3* and *6*), whereas the anti-IL-12 Abs had no effect on IFN-γ gene expression (Fig. 4, *lanes 4* and *7*). This effect was seen with both the 6 and 16 h supernatants, but not with the untreated supernatants or the supernatants pretreated with anti-IL-12 Abs (Fig. 4). T cells were also incubated with the supernatants pretreated with neutralizing anti-TNF-α or anti-IL-15 Abs or with normal sheep serum, but none of these treatments affected the cell culture supernatant-induced IFN-γ mRNA expression (data not shown).

To study the role of IFN-α/β in IFN-γ biosynthesis more closely, T cells were directly incubated with two different concentrations (100 and 1000 IU/ml) of highly purified human leukocyte IFN-α. The cells were harvested 1, 3, and 6 h after IFN-α stimulation and analyzed by Northern blotting with an IFN-γ-specific probe. The kinetics of the IFN-γ mRNA synthesis were rapid. At 1 h, IFN-γ mRNA levels were elevated with both doses of IFN-α (Fig. 5, *lanes 2* and *5*), and maximum steady state mRNA levels were detected 3 h after stimulation (Fig. 5, *lanes 3* and *6*). IFN-γ

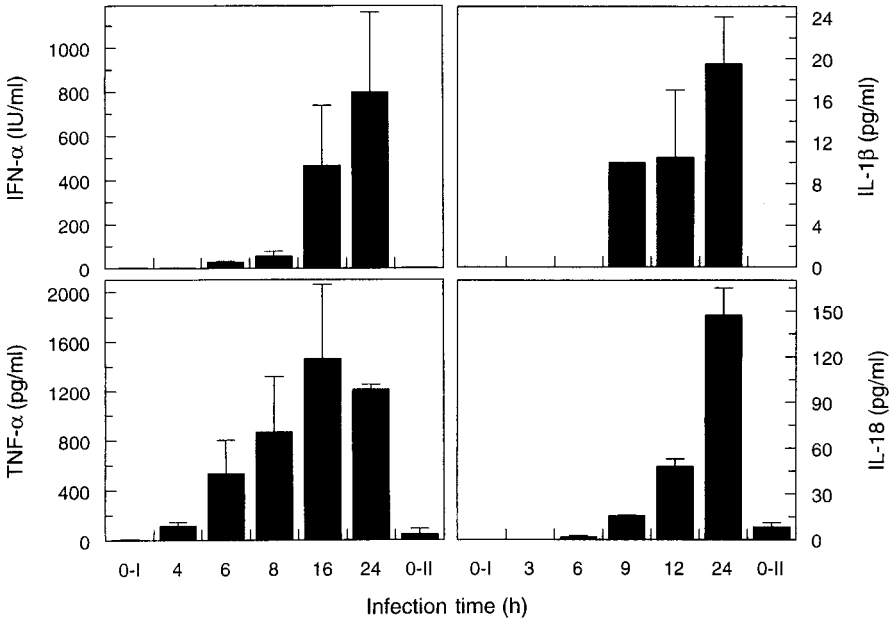

FIGURE 3. Production of cytokines from influenza A virus-infected macrophages. Macrophages from different blood donors were separately infected with influenza A virus. Supernatants from infected cells of different donors were collected at the times indicated and pooled. The IFN-α/β titers of the supernatants were determined by a biologic assay as described in *Materials and Methods*, and the amounts of TNF-α, IL-1β, and IL-18 were analyzed by the specific ELISAs. The mean of three separate experiments ($\pm$SD) is shown. 0-I and 0-II are the supernatants from uninfected cells after 3- and 24-h incubations, respectively.

mRNA levels decreased thereafter, but were still detectable at 6 h post stimulation. The induction of IFN-γ production by IFN-α was dose dependent. IFN-γ mRNA levels increased with 1 IU/ml IFN-α (data not shown), and maximum induction occurred with 100 IU/ml (Fig. 5).

To ascertain whether activation of the IFN-γ gene by IFN-α/β is independent of de novo protein synthesis, T cells were treated with CHX for 30 min before the addition of IFN-α or IFN-β. The steady state IFN-γ mRNA levels were analyzed 1 h after addition of these cytokines. Both IFN-α and IFN-β induced the expression of the IFN-γ gene in T cells (Fig. 6, *lanes 3* and *5*). Treatment of T cells with IFN-α or IFN-β in the presence of CHX resulted in the induction of IFN-γ transcripts (Fig. 6, *lanes 4* and *6*), which was clearly more efficient than with CHX alone (Fig. 6, *lane 2*). The data indicate that the rapid induction of IFN-γ gene expression in T cells by type I IFNs is direct and suggest that induction may not require ongoing protein synthesis.

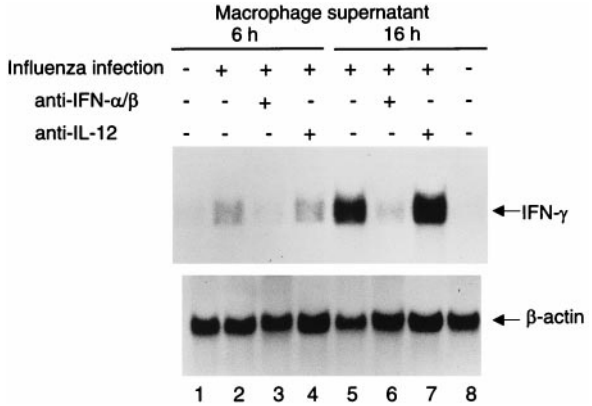

FIGURE 4. The effect of neutralizing anti-IFN-α/β and anti-IL-12 Abs on IFN-γ mRNA expression in T cells. The supernatants of the influenza A virus-infected macrophages, collected at 6 and 16 h postinfection, were untreated or were treated with neutralizing sheep anti-IFN-α/β antisera or anti-IL-12 Abs for 30 min before addition to T cells. After 3-h incubation, the cells were collected, total cellular RNA was isolated, and the expression of IFN-γ mRNA was analyzed by Northern blotting.

IFN-α/β and IL-18 synergistically enhance IFN-γ mRNA expression and protein production

When we compared the levels of IFN-γ mRNA induced in T cells by the macrophage supernatant obtained from influenza A virus infection or by purified IFN-α preparation, we found that the former was a much better inducer. This raises the possibility that different cytokines in the supernatants of virus-infected macrophages function synergistically in enhancing IFN-γ production in T cells. Therefore, we incubated T cells in the presence of purified IL-1β, IL-18, or TNF-α, with combinations of these with IFN-α, as well as with IFN-α alone and analyzed the induction of IFN-γ gene expression and protein production. IL-1β or TNF-α alone or combined with IFN-α did not markedly up-regulate IFN-γ mRNA synthesis (Fig. 7*A*, *lanes 3*, *5*, *6*, and *8*) or protein production (Fig. 7*B*). IL-18 alone had no effect on IFN-γ transcription (Fig. 7*A*, *lane 4*) or protein production (Fig. 7*B*). Remarkably, the combination of IFN-α and IL-18 had a very strong synergistic effect on both IFN-γ mRNA synthesis (Fig. 7*A*, *lane 7*) and protein production (Fig. 7*B*).

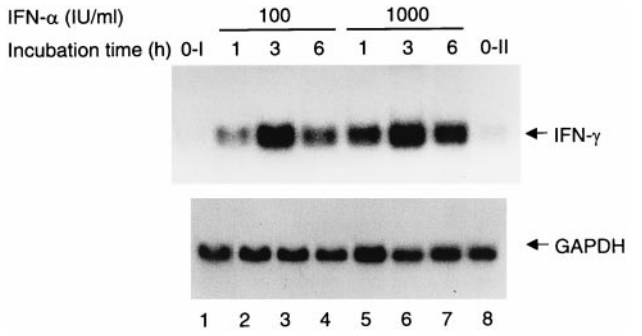

FIGURE 5. Kinetics of IFN-γ mRNA expression in T cells after treatment with IFN-α. Human T cells were treated with two doses (100 and 1000 IU/ml) of highly purified IFN-α originating from human leukocytes. The cells were collected at different times after addition of IFN-α and were prepared for mRNA analysis with ^{32}P-labeled IFN-γ and glyceraldehyde phosphate dehydrogenase (GAPDH) probes. The results are representative of three separate experiments. 0-I and 0-II are the untreated cells after 1- and 6-h incubations, respectively.

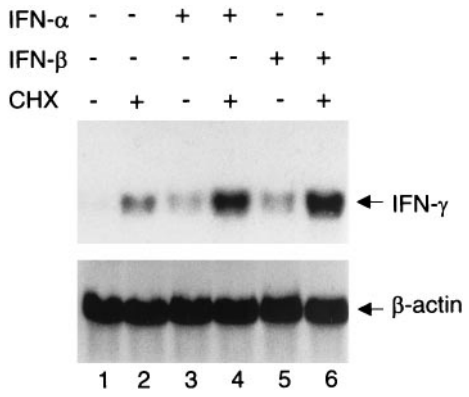

FIGURE 6. Induction of IFN-γ mRNA synthesis in T cells by IFN-α/β in the presence of protein synthesis inhibitor. Human T cells (3×10^6 cells/ml) were untreated or were treated with the protein synthesis inhibitor CHX (10 μg/ml) for 30 min before addition of IFN-α or IFN-β (100 IU/ml of each). After 1-h incubation, the cells were collected and prepared for RNA analysis by Northern blotting with IFN-γ- and β-actin-specific probes. The experiment was repeated three times with similar results.

Next we examined whether IL-12 was able to stimulate IFN-γ synthesis in our cell system. T cells responded to IL-12 by inducing IFN-γ mRNA synthesis (Fig. 8, *lane 3*) and protein production

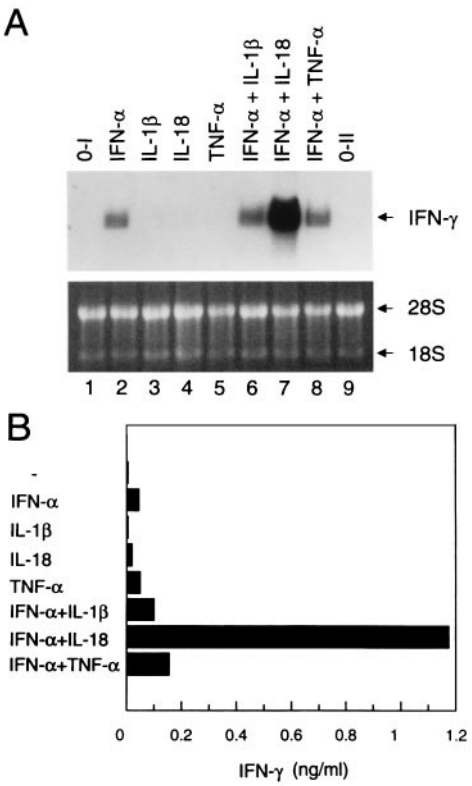

FIGURE 7. Roles of IFN-α, IL-1β, IL-18, and TNF-α on T cell IFN-γ production. *A*, Human T cells (3×10^6 cells/ml) were incubated with IFN-α (100 IU/ml), IL-1β (10 ng/ml), IL-18 (10 ng/ml), TNF-α (10 ng/ml), or the combination of each cytokine with IFN-α for 3 h. Most of the cells were collected, and RNA was isolated and used in Northern analysis with an IFN-γ probe. For controlling RNA loading, 1 μg of the total RNA was size fractionated by gel electrophoresis, and the ribosomal RNA bands were visualized by staining with ethidium bromide. *B*, The rest of the cells were incubated further for 24 h, and IFN-γ levels in the cell culture supernatants were determined by ELISA. The results shown are representative of three independent experiments.

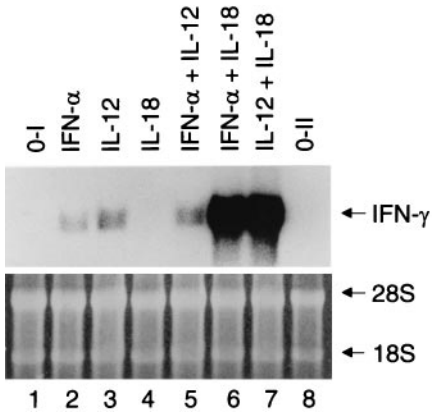

FIGURE 8. Induction of IFN-γ production in T cells in response to IL-12. Human T cells (3×10^6 cells/ml) were incubated with IFN-α (100 IU/ml), IL-12 (5 ng/ml), IL-18 (10 ng/ml), or combinations of these cytokines for 3 h. The cells were collected, and total cellular RNA was isolated and prepared for Northern blot analysis with an IFN-γ probe. As a control for RNA loading, ethidium bromide staining of ribosomal RNA bands was used. Comparable data were obtained in three independent experiments, each consisting of pooled T cells from two different donors.

(data not shown). Actually, in our cell system IL-12 was a slightly more potent inducer of IFN-γ mRNA synthesis than was IFN-α (Fig. 8, *lanes 2* and *3*). However, IL-12 had no synergy with IFN-α on IFN-γ gene expression (Fig. 8, *lane 5*). Our data, in accordance with the findings of previous experiments conducted in a murine Th1 cell clone (21), show a very effective synergy with IL-12 and IL-18 in IFN-γ production (Fig. 8, *lane 7*). These results demonstrate that the combination of IFN-α/β and IL-18 and the combination of IL-12 and IL-18 efficiently induce IFN-γ production in human T cells.

Discussion

Viral infections often induce strong immune responses that are essential for the clearance of the infection. Direct cellular interactions between virus-infected cells and T and B cells and the cytokines produced by virus-infected cells determine the quality and quantity of the specific immune responses. IFN-γ is one of the key cytokines directing T cell immune responses toward Th1-type and cell-mediated immunity (15). IFN-γ production in T lymphocytes and NK cells can be triggered by contact with an Ag-presenting cell or target cell, respectively. In addition, several monocyte/macrophage-derived cytokines, including IL-1β, IL-12, IL-18, and TNF-α, can enhance IFN-γ production (18, 19, 21, 37). Although experimental viral infections in vivo (38) and in vitro (33) have been demonstrated to induce IFN-γ production at early stages of infection, the molecular mechanisms of IFN-γ gene regulation have remained unresolved. Studies conducted in IL-12 knockout mice have shown that IL-12 is essential in enhancing IFN-γ gene expression following endotoxin administration (20).

To understand in more detail how IFN-γ gene expression is induced during viral infections, we used an in vitro macrophage/T cell infection model. Human leukocytes have previously been shown to be susceptible to influenza A virus infection and to be able to produce different cytokines during the infection (33, 39, 40). Human macrophages were readily infected with influenza A virus. Virus-infected T cells showed practically no enhancement of cytokine gene expression (data not shown), whereas macrophages responded actively by producing several different cytokines. The mechanisms by which macrophages or T cells respond to intracellular pathogens by producing different cytokines are not very well

understood. The data presented here suggest that macrophage-produced cytokines have a significant biologic role in enhancing IFN-γ gene expression in T cells. The cell culture supernatant of the influenza A virus-infected macrophages efficiently enhanced IFN-γ gene expression. Determination of cytokine levels in the cell culture supernatants revealed that influenza A virus infection enhances IFN-α/β, TNF-α, IL-1β, and IL-18 production in macrophages, whereas IL-12 was not induced at either the mRNA or the protein level. This led us to assume that in the absence of IL-12, other IFN-γ-inducing molecules are involved. A number of potential inducers could contribute to the induction of IFN-γ, including IFN-α, TNF-α, IL-1β (18, 19, 36), and IL-18 (21, 22). The addition of neutralizing anti-IFN-α/β Abs, but not anti-IL-12 Abs, effectively inhibited IFN-γ gene expression by the macrophage supernatant. This result clearly suggests that IFN-α/β is able to induce IFN-γ gene expression in T cells. This observation was further confirmed by the fact that purified IFN-α was able to enhance IFN-γ gene expression. Induction experiments performed in the presence of CHX indicated that both IFN-α and IFN-β can directly activate IFN-γ gene expression and suggest that the induction of IFN-γ mRNA is at least partly independent of de novo protein synthesis.

The IFN-γ mRNA signal and the production of IFN-γ protein induced by the macrophage supernatant were greater than those observed with the maximal dose of IFN-α. Therefore, it was evident that some other cytokine(s) was functioning together with IFN-α to enhance IFN-γ gene expression. Since several other cytokines in addition to IFN-α/β were also produced by influenza A virus-infected macrophages, we systematically tested their ability, alone or combined with IFN-α, to induce IFN-γ gene expression. The ability of IL-1β and TNF-α to enhance IFN-α-induced IFN-γ gene expression was minimal (Fig. 7), even though it has previously been suggested that IL-1β and TNF-α contribute to enhanced IFN-γ gene expression in other cell systems (18, 19). IL-18, although alone a relatively weak inducer of the IFN-γ gene in our T cell model, was very effective when combined with IFN-α. There was a >10-fold increase in IFN-γ production when IFN-α was combined with IL-18 (Fig. 7*B*). It is also likely that IFN-α/β and IL-18 synergistically enhance IFN-γ gene expression in virus-infected macrophage/T cell cultures. The fact that both IFN-α/β and IL-18 are produced during virus infection in macrophages may indicate that the combination of these cytokines is essential in directing the immunity toward efficient Th1-type responses, which is often the case in viral infections. However, before any general statements can be made, systematic analyses of infections with other viruses will have to be conducted.

It has been shown recently that a cellular serine protease, caspase-1 (or IL-1β-converting enzyme) is involved in the proteolytic cleavage of pro-IL-18 and subsequent secretion of the mature biologically active form of IL-18 (41, 42). The activation of caspase-1 has been directly linked to programmed cell death, apoptosis (43). Since influenza A virus is capable of causing cell death by apoptotic mechanisms (44), it is possible that the virus infection itself is responsible for the presumed caspase-1 activation and IL-18 as well as for the IL-1β secretion from macrophages (Fig. 3). The IL-18 mRNA levels remained relatively stable during influenza A infection (data not shown), suggesting that there is an increase in the translation and/or processing of IL-18 in response to viral infection. Further analysis of the molecular mechanisms of IL-18 production during viral infection is clearly necessary.

Interactions between a cytokine and its receptor lead to the activation of several signaling molecules, including the family of STAT proteins (45, 46). IFN-α activates STAT1 and STAT2, which together with p48 protein (ISGF3 complex), bind to the promoter regions of genes that are under the transcriptional control of IFN-α. IL-12, in turn, induces the tyrosine phosphorylation and DNA binding of STAT4 (47), which is essential for IL-12 signaling, as shown by STAT4 gene knockout studies (48, 49). However, it has recently been demonstrated by Cho et al. (50) that STAT4 activation by IL-12 is not unique. IFN-α is also able to activate the tyrosine and serine phosphorylation and DNA binding of STAT4 in human T lymphocytes and NK cells (50). Similarly, in our experimental setting IFN-α induced the tyrosine phosphorylation and DNA binding activity of STAT4 in T cells (S. Matikainen, T. Sareneva, and I. Julkunen, unpublished observations), suggesting that there is a common molecular determinant of the overlapping functions of these two cytokines, e.g., in the induction of IFN-γ gene transcription. In addition, recently published results by Xu et al. (51) have demonstrated that STAT4 has a role in the regulation of IFN-γ gene expression.

IL-12, although not produced during influenza A infection by macrophages, is one of the key cytokines regulating IFN-γ gene expression. We analyzed the potential synergy among IFN-α, IL-12, and IL-18. It has previously been shown that IL-12 and IL-18 synergize very strongly in enhancing IFN-γ gene expression (52). In our cell model both IFN-α and IL-12 synergistically enhanced IFN-γ mRNA expression in combination with IL-18 (Fig. 8). The fact that IFN-α and IL-12 did not exhibit any synergy suggests that these cytokines can perhaps substitute for each other in the regulation of IFN-γ gene expression. These observations raise interesting biologic consequences. In viral infections IFNs and IL-18 are produced, whereas the production of IL-12 may be limited. However, in bacterial infections IL-12 and IL-18 (53–55) are produced, but IFN-α is not. As a consequence, IFN-α production in viral infections may be a significant factor leading to efficient Th1-type immune responses. Recently, the role of IFN-α as a Th1-type cytokine was supported by Rogge et al. (56), who demonstrated the up-regulation of IL-12R β2-chain synthesis by IL-12 and IFN-α in human T cells and that both of these cytokines have important roles in the functional activity and development of T cells.

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