Influenza A and Sendai Viruses Induce Differential Chemokine Gene Expression and Transcription Factor Activation in Human Macrophages

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Chemokines regulate leukocyte traffic and extravasation into the site of inflammation. Here we show that influenza A or Sendai virus-infected human macrophages produce MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, MIP-3α, IP-10, and IL-8, whereas no upregulation of MIP-3β, eotaxin, or MDC production was detected. Influenza A virus was a better inducer of MCP-1 and MCP-3 production than Sendai virus, whereas MIP-1α, MIP-1β, RANTES, MIP-3α, and IL-8 were induced preferentially by Sendai virus. Infection in the presence of protein synthesis inhibitor indicated that ongoing protein synthesis was required for influenza A virus-induced expression of MCP-1, MCP-3, and IP-10 genes, whereas Sendai virus-induced chemokine mRNA expression took place in the absence of de novo protein synthesis. Neutralization of virus-induced IFN-α/β resulted in downregulation of virus-induced IP-10, MCP-1, and MCP-3 mRNA expression. IFN-α or IFN-γ were found to directly enhance MCP-1, MCP-3, and IP-10 mRNA expression. Both influenza A and Sendai viruses similarly activated transcription factor NFκB. In contrast to NFκB, IRFs and STATs, the other transcription factors involved in the regulation of chemokine gene expression, were differentially activated by these viruses. Influenza A virus more efficiently activated ISGF3 complex formation and Stat1 DNA-binding compared to Sendai virus, which in turn was a more potent activator of IRF-1. Our results show that during viral infections macrophages predominantly produce monocyte and Th1 cell attracting chemokines. Furthermore, virus-induced IFN-α/β enhanced chemokine gene expression in macrophages emphasizing the role of IFN-α/β in the development of Th1 immune responses.

INTRODUCTION

Influenza A and Sendai (murine parainfluenza type 1) viruses infect primarily upper respiratory tract epithelial cells. In addition, they infect T cells and monocytes/macrophages (Cantell et al., 1981; Ronni et al., 1995, 1997; Bussfeld et al., 1997, 1998; Sareneva et al., 1998; Pirhonen et al., 1999). Macrophages are involved in initiating innate and adaptive immune responses against viruses and other intracellular pathogens (Unanue, 1993). During viral infections macrophages produce a number of different cytokines and chemokines. Influenza A virus-infected monocytes/macrophages produce, e.g., IL-1β, IL-6, TNF-α, IFN-α/β, and IL-18 (Gong et al., 1991; Peschke et al., 1993; Hofmann et al., 1997, 1998; Sareneva et al., 1998; Pirhonen et al., 1999), which are important activators of T cells, macrophages, and dendritic cells (Van der Meer et al., 1998; Dinarello, 1999). Recent evidence suggests that IFN-α/β, which is essential for antiviral immunity (Vilcek and Sen, 1996; Zinkernagel and Hengartner, 1997), also modulates cell-mediated immune responses. For instance, IFN-α/β enhances survival of activated T cells (Marrack et al., 1999; Matikainen et al., 1999), up-regulates IFN-γ gene expression in T cells, and favors the development of the Th1 immune response (Rogge et al., 1997; Sareneva et al., 1998).

Chemokines play an important role in immune and inflammatory reactions, as well as in viral infections. They regulate the recruitment and functional activation of leukocytes (Bagnoliini, 1998; Luster, 1998). Some chemokines, e.g., MDC and SDF-1, are produced constitutively by specialized cells and organs while the production of most chemokines requires specific activation signals. Monocytes/macrophages have been shown to respond to virus infection by producing chemokines (Sprenger et al., 1996; Hofmann et al., 1997; Bussfeld et al., 1998). Differential expression of chemokine receptors in different leukocyte populations regulates their responsiveness to chemokines (Bagnoliini, 1988; Luster, 1998; Salustro et al., 1998a). Monocytes and immature dendritic cells express CCR1, CCR2, and CCR5 and are thus responsive for MIP-1α/β, RANTES, and various MCPs. Eosinophils, basophils, and Th2 cells respond to these.

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2 Abbreviations used: CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; IRF, IFN regulatory factor; ISGF3, IFN-stimulated gene factor 3; IP-10, inflammatory protein-10; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and secreted; RSV, respiratory syncytial virus; SDF, stroma-derived factor.
chemokines as well, but in addition, they contain eotaxin receptor, CCR3 (Sallusto et al., 1997). Th1 cells are responsive to IP-10, since they selectively express CXCR3 (Bonecchi et al., 1998; Sallusto et al., 1998b).

The transcriptional systems activated during Influenza A and Sendai virus infections include IRFs, NF-κB, and STATs. IRF-3 is activated by Sendai virus leading to the induction of RANTES gene expression (Lin et al., 1999). Both Influenza A and Sendai viruses are known to activate transcription factor NF-κB (Garoufalis et al., 1994; Pahl and Bauerle, 1995; Ronni et al., 1997). NF-κB enhances the expression of many cytokine and chemokine genes including IL-8, IP-10, MCP-1, and RANTES (Choi and Jacoby, 1992; Garofalo et al., 1996; Ueda et al., 1997; Cheng et al., 1998; Thomas et al., 1998). Viral infections apparently do not activate STATs directly, rather virus-induced cytokines, especially IFN-α, can activate multiple STAT proteins. STATs are involved at least in the activation of IP-10 and MCP-1 gene expression (Majumder et al., 1998; Valente et al., 1998; Ito et al., 1999).

In the present study we have analyzed chemokine production and activation of transcription factors that are involved in the induction of chemokine gene expression in macrophages infected with Influenza A or Sendai viruses. The chemokines analyzed are representative members of the chemokine families corresponding to the ligands for CCR1–CCR7 and CXCR1–CXCR3 chemokine receptors. We show that Influenza A and Sendai viruses induce differential chemokine production and activation of transcription factors involved in the regulation of chemokine genes.

RESULTS

Kinetics of chemokine mRNA synthesis in macrophages infected with Influenza A or Sendai viruses

To study virus-induced chemokine gene expression we infected macrophages with Influenza A or Sendai viruses. At different time periods after infection cells and cell culture supernatants were collected, total cellular RNA was isolated, and chemokine mRNA expression was analyzed by Northern blotting. MIP-1α, MIP-1β, MCP-1, MCP-3, and IP-10 mRNAs were induced by both viruses and peak mRNA levels were detected at 6 and 12 h after infection (Fig. 1). In contrast, virus-induced MIP-3α mRNA levels peaked at 3 and 6 h after infection (Fig. 1). MIP-1α, MIP-1β, RANTES, and MIP-3α mRNA levels in macrophages infected with Sendai virus were higher compared to that of Influenza A virus-infected cells. MIP-3β (ELC), the ligand for CCR7 (Baggiolini, 1998; Luster, 1998; Sallusto et al., 1998a), was not expressed in uninfected or virus-infected macrophages (data not shown). Both viruses clearly induced IP-10, MCP-1, and MCP-3 mRNA expression (Fig. 1), whereas IL-8 gene expression was upregulated only by Sendai virus. MDC mRNA was constitutively expressed in macrophages, although some reduction in MDC mRNA levels was seen in Influenza A virus-infected cells. This may result from the ability of Influenza A virus polymerase protein complex to destroy host cell pre-mRNAs (Lamb and Krug, 1996).

Chemokine secretion from virus-infected macrophages

As shown in Fig. 1, the expression of several chemokine genes was turned on in virus-infected macrophages. To study whether virus infection leads to enhanced chemokine protein production, we measured chemokine levels in cell culture supernatants by ELISA. MIP-1α, RANTES, IP-10, and IL-8 were detected in supernatants collected at 3 h after infection and the peak levels were found at 12 and 24 h after infection (Fig. 2). Consistent with the Northern blot data, both viruses in-
duced the production of MIP-1α, RANTES, and IP-10, although differences in expression levels were found. Influenza A virus infection enhanced the production of MCP-1 and MCP-3, whereas Sendai virus infection poorly induced these chemokines (Fig. 2). However, in Sendai virus-infected cells enhanced levels of MCP-1 and MCP-3 mRNAs were found (Fig. 1). This may suggest that additional stimuli provided by influenza A, but not by Sendai virus infection, are required for efficient translation of MCP mRNAs and production of the respective proteins (Fig. 2). Eotaxin production was not detected in virus-infected macrophages (data not shown).

The effect of protein synthesis inhibition on chemokine gene expression

To further analyze the molecular mechanisms of virus-induced chemokine gene expression, we infected macrophages in the presence of cycloheximide. In cycloheximide-treated control cells elevated MIP-1α, MIP-1β, MIP-3α, and IL-8 mRNA levels were detected (Fig. 3). In Sendai virus-infected macrophages mRNA expression of MIP-1α, MIP-1β, RANTES, MIP-3α, and IL-8 genes was upregulated in the presence of cycloheximide. In contrast, cycloheximide treatment resulted in a significant reduction in Sendai virus-induced MCP-1 mRNA levels and in some reduction in Sendai virus-induced MCP-3 mRNA expression. In influenza A virus-infected cells cycloheximide reduced or almost completely blocked MCP-1, MCP-3, and IP-10 mRNA expression, suggesting that ongoing protein synthesis was required for virus-induced activation of these genes (Fig. 3).

The role of IFN-α/β in virus-induced chemokine gene expression

IFN-α/β is essential for host antiviral responses (Vilcek and Sen, 1996; Zinkernagel and Hengartner, 1997) and its production is induced in leukocytes and macrophages by influenza A and Sendai viruses (Cantell et al., 1981; Ronni et al., 1995; Cantell and Pirhonen, 1996; Hua et al., 1996; Sareneva et al., 1998; Pirhonen et al., 1999). Therefore, we analyzed whether IFN-α/β had a role in virus-induced chemokine gene expression. Influenza A- or Sendai virus-infected macrophages were treated with neutralizing anti-IFN-α/β Abs and chemokine gene ex-

FIG. 2. Kinetics of chemokine production by virus-infected macrophages. Macrophages were infected with influenza A or Sendai viruses and supernatants were collected at different time points after infection and analyzed with ELISAs. The results represent the means (±SD) of three individual experiments.
pression was analyzed by Northern blotting at 4 and 6 h after infection. Treatment of virus-infected macrophages with anti-IFN-α/β Abs had no effect on MIP-1α, MIP-1β, or RANTES mRNA synthesis (Fig. 4), suggesting that IFN-α/β does not have a major role in the regulation of these genes. MCP-1 and MCP-3 mRNA levels were readily induced by both viruses and anti-IFN-α/β Abs inhibited their expression to some extent. Influenza A virus-induced expression of IP-10 mRNA was almost completely inhibited by anti-IFN-α/β Abs, suggesting that IFN-α/β may be a primary regulator of IP-10 gene expression. Anti-IFN-α/β Abs had only marginal effect on Sendai virus-induced IP-10 gene expression suggesting that both IFN-dependent and IFN-independent activation pathways exist.

IFNs upregulate MCP-1, MCP-3, and IP-10 gene expression

To further study the role of IFNs in the regulation MCP-1, MCP-3, and IP-10 gene expression we stimulated macrophages with IFN-α or IFN-γ. As shown in Fig. 5, both IFN-α and IFN-γ upregulated MCP-1, MCP-3, and IP-10 mRNA synthesis. Already at 1 h after IFN treatment, enhancement of MCP-1, MCP-3, and IP-10 mRNA expression was detected. Results from Northern blot analyses support the idea that IFNs may regulate the expression of MCPs and IP-10. In macrophages IFN-α and IFN-γ enhanced IP-10 gene expression equally well. MIP-1α and MIP-3α genes were not induced by IFN-α or IFN-γ, whereas RANTES mRNA expression was weakly induced by IFN-α (data not shown).
Virus- and IFN-induced Stat1 DNA-binding to the MCP-1 GAS

Previous analyses have shown that STATs are involved in the activation of MCP-1 gene expression (Valente et al., 1998). To study the mechanisms of virus- and IFN-induced MCP-1 gene expression in human macrophages, we performed electrophoretic mobility shift assay (EMSA) with MCP-1 GAS probe. Both influenza A and Sendai viruses induced detectable DNA-binding complexes to MCP-1 GAS at 3 h after infection (Fig. 6A). However, Sendai virus-induced protein/DNA complex was very weak compared to that induced by influenza A virus. The virus-induced complex consisted of Stat1 proteins since only anti-Stat1 Abs supershifted the complex (Fig. 6A). Stimulation of macrophages with IFN-α or IFN-γ resulted in a clearly detectable MCP-1 GAS binding complex (Fig. 6B). IFN-α-induced DNA-binding complex disappeared at 3 h after stimulation, whereas IFN-γ-induced protein/DNA complex persisted at least for 9 h after stimulation. As in the case of virus infection, supershift analyses suggested that IFN-induced DNA-binding complexes consisted mainly of Stat1 protein (Fig. 6B).

Influenza A and Sendai virus infection differentially induce ISGF3 complex formation and IRF-1 DNA-binding in macrophages

In addition to STATs, IRFs and NF-κB-transcription factors regulate chemokine gene expression. To study virus-induced activation of IRF and NF-κB we performed EMSA with ISRE15 and RANTES NF-κB oligonucleotides. ISGF3, a heterotrimer of Stat1, Stat2, and p48 proteins, binds to ISRE15 in response to IFN-α/β stimulation. The IRF-1 DNA-binding site overlaps with that of ISGF3 and IRF-1 DNA-binding to ISRE15 is induced by IFNs (Mamane et al., 1999). Both influenza A and Sendai viruses induced IRF-1 and ISGF3 DNA-binding at 3 h after infection (Fig. 7A). However, influenza A virus more efficiently induced ISGF3 complex formation compared to Sendai virus, whereas Sendai virus was a much more potent inducer of IRF-1 DNA-binding (Fig. 7A). The identity of the ISGF3 complex was verified with supershift experiments with anti-Stat1, anti-Stat2, and anti-p48 Abs (data not shown). The IRF-1 DNA-binding complex was most likely IRF-1 homodimer, since it was supershifted with anti-IRF-1 Abs but not with anti-ICSBP, anti-IRF-2, or anti-p48 Abs (data not shown). In Sendai virus-infected macrophages, we detected a slower migration DNA-binding complex (Fig. 7A), which did not react with anti-IRF-1, anti-IRF-2, anti-p48, anti-Stat1, anti-Stat5 Abs (data not shown).

Both Influenza A and Sendai viruses induced DNA-binding activity to the RANTES NF-κB site already at 1 h after infection (Fig. 7B). Thereafter, NF-κB DNA-binding declined and was strongly reinduced at 6–9 h after the infection. Similar results were obtained with consensus NF-κB oligonucleotide (data not shown).

DISCUSSION

In this study we analyzed chemokine gene expression in influenza A- and Sendai virus-infected human macrophages. Macrophages are found in most tissues where they produce cytokines, phagocytose cell debris and microbes, and present antigens (Unanue, 1993). Virus-infected macrophages produce proinflammatory cytokines, IL-1, IL-6, and TNF-α (Gong et al., 1991; Peschke et al., 1993; Hofmann et al., 1997; Sareneva et al., 1998; Pirhonen et al., 1999), as well as IFN-α/β and IL-18, which favor the development Th1 immune responses (Rogge et al., 1997; Sareneva et al., 1998; Dinarello, 1999).

Influenza A and Sendai viruses have been used in studies concerning virus-host cell interactions and cytokine gene expression (Gong et al., 1991; Peschke et al., 1993; Ronni et al., 1995, 1997; Hofmann et al., 1997; Lin et al., 1998; Sareneva et al., 1998; Pirhonen et al., 1999). One of the advantages of these viruses is that a synchronous infection can be established in many cell types. In macrophages viral protein synthesis starts within 4–6 h after influenza A and Sendai virus infection (Sareneva et al., 1998; Pirhonen et al., 1999). Enhanced expression of
MIP-1α, RANTES, and IL-8 was seen during the first hours of infection (Figs. 1 and 2). This suggests that influenza A and Sendai viruses can rapidly activate the transcriptional systems involved in the upregulation of chemokine gene expression. These systems include NF-κB (Baldwin, 1996) and IRFs (Mamane et al., 1999), which are activated by influenza A virus (Pahl and Bauerle, 1995; Ronni et al., 1997) and by certain members of the paramyxoviridae (Lin et al., 1998, 1999; Marie et al., 1998; Yoneyama et al., 1998). NF-κB enhances IL-8 gene transcription (Kunsch et al., 1994; Garofalo et al., 1996) while both IRFs and NF-κB regulate RANTES gene expression (Matsukura et al., 1998; Thomas et al., 1998; Lin et al., 1999). IP-10 and MCP-1 gene expression is induced by IFNs (Luster et al., 1985; Satriano et al., 1993) and STATs are involved in the activation of these genes (Tebo et al., 1998; Valente et al., 1998; Ito et al., 1999). In our experiments influenza A and Sendai viruses similarly activated NF-κB DNA-binding. However, ISGF3 complex formation and IRF-1 and Stat1 DNA-binding were differentially activated by influenza A and Sendai viruses. This may, at least in part, explain the differential induction of chemokine gene expression. Compared to Sendai virus, influenza A virus was a much better inducer of ISGF3 and Stat1 DNA-binding although macrophages infected with Sendai virus produce 10- to 100-fold higher levels of IFN-α/β (Pirhonen et al., 1999). In contrast, Sendai virus was a more potent inducer of IRF-1 DNA-binding compared to influenza A virus. Recently, it was shown that Sendai virus C protein interferes with IFN-induced Stat1 expression (Garcin et al., 1999) which may explain, at least in part, poor STAT activation during Sendai virus infection.

Influenza A and Sendai viruses differentially activated chemokine gene expression in macrophages. Sendai virus was a potent inducer of MIP-1α, MIP-1β, and RANTES genes, which was seen both at mRNA and at protein expression levels. Up to 100-300 ng/ml of MIP-1α and RANTES was found in cell culture supernatants of Sendai virus-infected macrophages. These values were approximately 10-fold higher compared to those induced by influenza A virus. IL-8 production was induced preferentially by Sendai virus. The lack of IL-8 production by influenza A virus is consistent with previous observations in monocytes (Sprenger et al., 1996; Bussfeld et al., 1998). This suggests that in macrophages Sendai virus induces chemokines that attract cells expressing CCR1, CCR3, and CCR5 (receptors for MIP-1α, MIP-1β, and RANTES) and CXCR1 and CXCR2 (receptors for IL-8). Sendai virus-induced chemokines could favor the recruit-
 but not MIP-3α.

Previously, influenza A virus and RSV have been shown to induce IL-8 and IL-12p70 expression in airway epithelium cells (Choi and Jacoby, 1992; Garofalo et al., 1996; Mastronarde et al., 1996; Saito et al., 1996; Olszewska-Pazdruk et al., 1998).

Another difference between influenza A and Sendai viruses was found in MCP-1 and MCP-3 production. Although both viruses rapidly (at 3 h) induced the mRNA expression of these genes (Fig. 1), only macrophages infected with influenza A virus efficiently secreted MCP-1 and MCP-3. Induction of MCP-1 and MCP-3 mRNA was stronger in influenza A virus-infected cells, but it is unlikely that the difference in the mRNA expression levels alone could explain the differences in MCP protein production. It is possible that MCP-1 and MCP-3 synthesis is translationally or posttranslationally regulated during viral infections and only influenza A virus can efficiently enhance protein production-secretion. Enhanced secretion of MCP-1 and MCP-3 in influenza A virus infection and complete lack of eotaxin production could favor the selective recruitment of the blood mononuclear cell population to the site of influenza A virus infection.

We observed that the expression of MIP-3α (LARC), but not MIP-3β (ELC), was induced by influenza A and Sendai viruses. CCR6, the receptor for MIP-3α, is found in immature dendritic cells (DC) (Sallusto et al., 1998b) and therefore virus-induced MIP-3α production could be involved in the recruitment of immature DCs to the site of infection. The lack of MIP-3β production in virus-infected macrophages is noteworthy. MIP-3β production may be restricted to activated DCs (Sallusto et al., 1999), which become CCR7 positive and by autocrine stimulatory mechanisms (via MIP-3β production) then migrate to local lymph nodes. This suggests that during viral infections macrophages and DCs have clearly different roles. Macrophages are involved, via MIP-3α production (Fig. 1), in recruitment of monocytes and immature DCs into the site of inflammation. Activated DCs in tissues are, after antigen sampling and via MIP-3β production (Sallusto et al., 1999), involved in initiating their own migration from the site of inflammation to local lymph nodes (Cyster, 1999; Sallusto et al., 1998b; Sozzani et al., 1998).

IP-10, initially described as an IFN-γ-inducible inflammatory protein (Luster et al., 1985), was readily secreted by influenza A- and Sendai virus-infected macrophages. Since IP-10 is regulated by IFN-γ and its promoter has IFN-responsive elements (Tebo et al., 1998; Ito et al., 1999) we reasoned that this gene could also be regulated by IFN-α/β during viral infections. Neutralization experiments with anti-IFN-α/β Abs clearly showed that influenza A virus-induced IP-10 gene expression is dependent on virus-induced IFN-α/β production. In the case of Sendai virus IFN-independent transcriptional activation of IP-10 gene was seen. As most viral infections lead to the production of IFN-α/β and subsequently IP-10, this phenomenon has relevance in the biology of viral infection. IP-10 specifically attracts Th1 cells, due to their expression of CXCR3 (Bonecchi et al., 1998; Sallusto et al., 1998b; Siveke and Hamann, 1998), leading to the recruitment of Th1 cells into the site of inflammation. Recent evidence suggests that IFN-α/β is an important cytokine in Th1 immune response, since it favors, together with macrophage-produced IL-12 and IL-18 (Marrack et al., 1996; Takeda et al., 1998), Th1 cell development (Rogge et al., 1997). IFN-α/β also enhances T cell survival (Marrack et al., 1999; Matikainen et al., 1999) and directly upregulates IFN-γ production in T cells (Sarneva et al., 1998). In addition, IFN-α has been shown to upregulate CCR1 and downregulate CCR3 expression, further favoring the Th1-type immune response (Sallusto et al., 1997). These observations emphasize the important role of IFN-α/β both in the development of cell-mediated immunity and as substances involved in mononuclear cell chemoattraction during viral infections.

**MATERIALS AND METHODS**

Isolation and culture of macrophages

PBMCs were isolated by Ficoll–Paque (Pharmacia Biotechnology, Uppsala, Sweden) density gradient centrifu-
igation from leukocyte-rich buffy coats of healthy human blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Cells were allowed to bind onto six-well plastic plates (Falcon Multwell, Becton–Dickinson, Franklin Lakes, NJ) for 1 h at 37°C in 5% CO₂. After incubation, nonadherent cells were removed and adherent cells (monocytes) were washed twice with PBS, pH 7.4. Monocytes were differentiated into macrophages in the presence of GM-CSF (10 ng/ml) (Leucomax; Schering-Plough, Innishannon, Ireland) as previously described (Pirhonen et al., 1999).

RNA isolation and analysis

Total cellular RNA was prepared using the guanidinium isothiocyanate/cesium chloride method (Glasin et al., 1974; Chirgwin et al., 1979). The amount of total cellular RNA was quantified photometrically. Equal amounts (20 μg) of total cellular RNA were size-fractionated on a 1% formaldehyde–agarose gel, transferred to a nylon membrane (Hybond, Amersham, Buckinghamshire, UK), and analyzed by Northern blotting. cDNA probes were labeled with [α-32P]dCTP (3000 Ci/m mole; Amersham, Buckinghamshire, UK) using a random primed DNA labeling kit (Boehringer Mannheim Corp., Indianapolis, IN). Hybridizations were done in a solution containing 50% formamide, 5× Denhardt's solution, 5× standard saline–phosphate–EDTA, and 0.5% SDS at 42°C. Membranes were washed twice with 1× SSC/0.1% SDS for 30 min at 42°C and once for 30 min at 60°C. cDNAs coding for human MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, and MDC genes were provided by Dr. Mantovani (Istituto di Ricerche Farmacologiche, Milan, Italy). IL-8, MIP-3α, and MIP-3β (Rossi et al., 1997) cDNAs were from DNAX Research Institute (Palo Alto, CA). IP-10 cDNA was cloned from total RNA obtained from IFN-γ-induced macrophages by RT-PCR using oligonucleotides TCCAGTGGATCCACATGAATCAACTGGCAGTTGAGATT (sense) and TGCTCCGGATCCGTTTTAAGGAGATCTTTAGACATTTT (antisense). Equal RNA loading in Northern blots was controlled by ethidium bromide staining of ribosomal RNA bands or by hybridization with glyceraldehyde phosphate dehydrogenase or β-actin cDNA probes.

Viruses and infection

The stock of human pathogenic influenza virus (A/Beijing/353/89 H3N2) is from the National Institute of Medical Research (London, UK) and the murine Sendai virus stock (strain Cantell) is from our Institute. Both virus stocks were cultured in 8-day-old embryonated hen's eggs and stored at −70°C (Cantell et al., 1981; Ronni et al., 1995). The hemagglutination titers of influenza A and Sendai viruses were 128 and 6000, respectively. Macrophages were infected with influenza A (5 pfu/cell) or Sendai viruses (10 pfu/cell) in 2 ml of Macrophage-SFM medium. Flow cytometric analysis has confirmed that 95–99% of the cells were infected with the virus doses used in the experiments (Pirhonen et al., 1999). After 1 h, the virus was removed, plates were washed with PBS, and the cells were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. The cells and supernatants were collected at different time points after infection as indicated in each experiment. Macrophages from individual donors were grown and infected separately, and the samples of four individual donors were pooled in each experiment.

Chemokine ELISAs and assay for IFN-α/β

MIP-1α, RANTES, MCP-1, MCP-3, IP-10, eotaxin, and IL-8 concentrations in supernatants were measured using ELISA with Ab pairs and standards obtained from R&D Systems (Abingdon, UK). IFN-α/β levels were determined by a biological assay using a vesicular stomatitis virus plaque reduction assay in Hep2 cells (Cantell et al., 1991).

IFNs and anti-IFN-α/β antibodies

Human leukocyte IFN-α was prepared and purified as previously described (Cantell et al., 1981). Human IFN-γ was obtained from the Finnish Red Cross Blood Transfusion Service (Cantell et al., 1986). For neutralizing IFN-α/β activity 2300 neutralizing U/ml for IFN-α and 170 neutralizing U/ml for IFN-β were used (Mogensen et al., 1975).

Cycloheximide treatment

Cycloheximide (10 μg/ml; Sigma, St. Louis, MO) was used to block protein synthesis during virus infection and it was present throughout the experiment. The concentration of cycloheximide used is sufficient to totally block protein synthesis in human blood mononuclear cells (Roni et al., 1995).

Electrophoretic mobility shift assay

Nuclear extracts and nuclear protein/DNA-binding reactions were performed as described previously (Andrews and Faller, 1991; Matikainen et al., 1996), MCP-1 GAS: (5’-GATCGGCTCCCTCTACTCTCTTCCGAAATCCA-3’), RANTES NF-κB: (5’-GATCTTGGAACCTCCCTGAGGGATGTCCCC-3’), and ISRE15 (5’-GATCAGCTT-GATCGGAAAGGGAAACCAGAAGCCGA-3’) oligonucleotides were synthesized with an Applied Biosystems (Foster City, CA) oligonucleotide synthesizer and purified on PAGE in the presence of 8 M urea. The probes were labeled by Klenow-fill in reaction. The binding reaction was performed at room temperature for 30 min. The samples were analyzed by electrophoresis on 6% nondenaturing low-ionic-strength polyacrylamide gels in 0.25× TBE. The gels were dried and visualized by autoradiography. Anti-Stat1 (sc-345X, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Stat3 (sc-482X, Santa
Cruz), and anti-Stat5 (sc-835X, Santa Cruz) Abs (1:20 dilution) were used in supershift experiments.

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