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Mechanisms of IL-12 Synthesis by Human Dendritic Cells Treated with the Chemical Sensitizer NiSO4

Diane Antonios, Philippe Rousseau, Alexandre Larangé, Saadia Kerdine-Römer, and Marc Pallardy

Allergic contact dermatitis, caused by metallic ions, is a T cell-mediated inflammatory skin disease. IL-12 is a 70-kDa heterodimeric protein composed of IL-12p40 and IL-12p35, playing a major role in the generation of allergen-specific T cell responses. Dendritic cells (DCs) are APCs involved in the induction of primary immune responses, as they possess the ability to stimulate naive T cells. In this study, we address the question whether the sensitizer nickel sulfate (NiSO4) itself or in synergy with other signals can induce the secretion of IL-12p70 in human monocyte-derived DCs (Mo-DCs). We found that IL-12p40 was produced by Mo-DC in response to NiSO4 stimulation. Addition of IFN-γ concomitantly to NiSO4 leads to IL-12p70 synthesis. NiSO4 treatment leads to the activation of MAPK, NF-κB pathways, and IFN regulatory factor 1 (IRF-1). We investigated the role of these signaling pathways in IL-12 production using known pharmacological inhibitors of MAPK and NF-κB pathways and RNA interference-mediated silencing of IRF-1. Our results showed that p38 MAPK, NF-κB, and IRF-1 were involved in IL-12p40 production induced by NiSO4. Moreover, IRF-1 silencing nearly totally abrogated IL-12p40 and IL-12p70 production provoked by NiSO4 and IFN-γ. In response to NiSO4, we observed that STAT-1 was phosphorylated on both serine and tyrosine residues and participated to NiSO4-induced alteration of the redox status of the cell. These results indicate that p38 MAPK, NF-κB, and IRF-1 are activated by NiSO4 in Mo-DC and cooperate for IL-12 production.

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ickel is a metallic allergen causing contact dermatitis following dermal exposure (1). Reports by the North American Contact Dermatitis Group have consistently ranked nickel as the number one allergen in frequency of positive patch test reactions. Nickel’s allergy is mainly due to jewelry, piercing, and coins (2, 3).

Allergic contact dermatitis (ACD) caused by metallic ions and other reactive hapten is a T cell-mediated inflammatory skin disease (4–6). ACD is characterized by two phases: a sensitization phase and an elicitation phase. During the sensitization phase, skin dendritic cells (DCs) capture the metal bound to self-protein, migrate through the lymphatic vessels, and present the haptendprotein complex to naive T cells in the draining lymph node (7, 8). Moreover, using human in vitro DC models, several groups have shown that chemical sensitizer treatment can induce phenotypic modifications with HLA-DR, CD86, CD80, CD83, and CCR7 upregulation and IL-12 and IL-8 production (9–12).

IL-12 consists of two chains (p40 and p35) covalently linked to give rise to a heterodimeric (p70) molecule. The induction and secretion of bioactive IL-12 are regulated by the independent production of p35 and p40 subunits. IL-12 is primarily produced by macrophages and DCs in response to danger signals such as TLR agonists and plays a major role in IFN-γ production by immune cells, driving a Th1/Tc1-type response (13–16). Many authors have demonstrated that CD8+ cytotoxic T lymphocytes are the main effector cells of ACD and observed their early recruitment in the skin postchallenge with chemical sensitizers (6, 17). Injection of IL-12 during the sensitization phase favors CD8+ T cell differentiation and increases the ACD reaction (18). In vivo studies also demonstrated that 2,4-dinitrofluorobenzene–mediated ACD was significantly blocked by anti-IL-12–neutralizing Abs, supporting the conclusion that IL-12 is an important effector in the pathogenesis of ACD (19).

The secretion of free IL-12p40 and IL-12p70 by activated macrophages and DCs is dependent on the regulation of the il-12p40 promoter by an array of transcription factors including C/EBP, NF-κB, AP-1, IFN regulatory factor (IRF)-1, and IRF-8 (20–25). IL-12p35 expression is also tightly regulated both at the transcriptional and translational level (26, 27). Specificity protein 1, AP-1, IRF-1, IRF-3, and IRF-8 have been described to play a role in the expression of human IL12A (23, 28, 29). RNA interference experiments showed a critical requirement for the NF-κB p50 subunit in the production of IL-12 by human monocyte-derived DCs (Mo-DCs) after CD40L and IL-1 stimulation (24). Moreover, IRF-1−/− splenic DCs were markedly impaired in their ability to produce IL-12 after LPS stimulation (25). In vivo, a defective IL-12 production was also observed in LPS-treated macrophages obtained from MAP kinase kinase 3-deficient mice (30). All of these reports suggest that MAPKs, NF-κB, and IRF pathways are involved in the production of IL-12 by DCs.

NiSO4 has been shown to activate both MAPK and NF-κB pathways in human Mo-DCs or in human DCs differentiated from CD34+ cells (CD34-DC) (9–11, 31, 32). Inhibition of p38 MAPK, JNK, and ERK abrogated the upregulation of CD86, CD83, and CCR7 expression induced by NiSO4, whereas expression of HLA-DR and CD40 and the production of IL-6, IL-12p40, and IL-8 were...
mainly dependent on the NF-κB pathway (10, 11, 31–33). Current hypothesis in the field of contact allergy suggests that nickel and other chemical sensitizers could be perceived as a danger signal by DCs, leading to MAPK and NF-κB activation and DC phenotypic modifications.

In this study, we address the question whether NiSO₄ itself or in synergy with other signals can induce the secretion of IL-12p70 in human Mo-DCs. We showed that: 1) nickel induced the production of IL-12p40; 2) IL-12p70 synthesis needed the presence of NiSO₄ and IFN-γ, and both signals were also required for the expression of the p35 subunit of IL-12; and 3) the production of IL-12p70 in response to NiSO₄ and IFN-γ was dependent on p38 MAPK, NF-κB, and IRF-1 activation. We also found that IRF-1 was activated in response to NiSO₄ in a STAT-1–dependent manner and played a crucial role in NiSO₄-induced IL-12 production.

**Materials and Methods**

**Generation of Mo-DCs**

PBMCs were purified from buffy coats obtained from Etablissement Français du Sang (Ivry-Sur-Seine, France) by density centrifugation with Ficoll gradient (Eurobio, Les Ulis, France). Monocytes were isolated from the mononuclear fraction through magnetic positive selection using MiniMacs separation columns (Miltenyi Biotec, Bergish Gladbach, Germany) and anti-CD14 Abs coated on magnetic beads following the provider’s instructions (Direct CD14 isolation kit, Miltenyi Biotec). Monocytes were cultured at 1 × 10⁶ cells/ml in the presence of GM-CSF (550 U/ml) and IL-4 (550 U/ml) (both from Abcys, Paris, France) in RPMI 1640 containing Glutamax I.
supplemented with 10% heat-inactivated FCS, 1 mM sodium pyruvate, 0.1 mg/ml streptomycine, and 100 U/ml penicillin (RPMIC) (all from Life Technologies/ Invitrogen, Paisley, U.K.). Within 5 d, monocytes differentiated into DCs with immature phenotype (iDCs).

Chemical treatment of iDCs

Mo-DCs (at day 5) were washed three times in RPMIC, and their concentration was adjusted to 1 × 10⁶ cells/ml. iDCs were stimulated or not with different concentrations of NiSO₄ (Sigma-Aldrich, St. Louis, MO) with or without IFN-γ (1000 U/ml; Abcys) for the indicated time. To study the involvement of signaling pathways in IL-12 production, Mo-DCs were pretreated for 30 min with SP600125 (20 μM) or SB203580 (20 μM) for 1 h with Bay 11-7085 (3 μM) or for 2 h with Jak inhibitor 1 (0.5 μM) (all from Merck Chemicals, Darmstadt, Germany). N-acetylcysteine (NAC) was purchased from Sigma-Aldrich.

Western blot analysis

iDCs (10⁶ cells/ml) were exposed to NiSO₄ (500 μM) or IFN-γ (1000 U/ml). After an adequate time of stimulation, cells were washed with ice-cold PBS. Cell lysates were prepared by resuspending cell pellet in lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA (pH 7.4), 1% Triton, 25 mM β-glycerophosphate, 1 mM NaF, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin]. The homogenates were centrifuged at 15,000 rpm for 20 min at 4°C. A total of 50 μg denatured protein were loaded onto 12.5% SDS-PAGE gel and transferred on polyvinylidene difluoride membrane (Amersham Biosciences, Les Ulis, France). Membranes were then incubated with Abs directed against the phosphorylated forms of p38 MAPK (Thr180/Tyr182), JNK 1/2 (Thr183/Tyr185), STAT-1 (Tyr 701), or STAT-1 (Ser 727) (all from Cell Signaling Technology, Ozyme, St-Quentin en Yvelines, France) or against IRF-1 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were detected by chemiluminescence (ECL solution, Amersham Biosciences). p38 MAPK or STAT-1 were used as a loading control and revealed with Abs raised against p38 MAPK (p38 N20, Santa Cruz Biotechnology) or STAT-1 (Cell Signaling Technology, Ozyme). Bands were quantified by densitometry using the ImageQuant software. The intensity of the specific band was normalized to the intensity of loading control protein band. Folds represent the ratio of the normalized intensity of treated cells divided by the normalized intensity of nontreated cells.

Preparation of whole cell extract and DNA-affinity protein-binding assay

Following treatment with NiSO₄ (500 μM) or IFN-γ (1000 U/ml), cells were lysed in Nonident P-40 hypertonic lysis buffer. In brief, cell pellets from 8 × 10⁶ cells were resuspended in a buffer adjusted at pH 7.9 containing 0.2% Nonidet P-40, 20% glycerol, 20 mM HEPES-KOH, 1 mM DTT, 1 mM Na3VO4, 1 mM sodium pyrophosphate, 0.125 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin and incubated at 4°C for 30 min. Cellular debris were removed by centrifugation at 4°C, 15,000 rpm, for 20 min. The DNA affinity binding assay

FIGURE 3. NF-κB, MAPKs, and IRF-1 pathways are activated upon addition of NiSO₄ or NiSO₄ and IFN-γ treatment. At day 5, iDCs were treated or not for 30, 60, and 120 min with NiSO₄ (500 μM), IFN-γ (1000 U/ml), or their association. Poststimulation, cells were lysed, and the level of phosphorylated p38 MAPK and JNK was evaluated by Western blotting using anti–phospho-p38 MAPK or anti–phospho-JNK Abs. The membrane was then probed with anti-p38 MAPK Ab for loading control. Folds represent the ratio of the normalized intensity of phosphorylated form of MAPK band/p38 MAPK band) of treated cells divided by the normalized intensity of nontreated cells. Results are representative of three independent experiments. B, NF-κB activation by NiSO₄ or NiSO₄ and IFN-γ at 60 min. At day 5, iDCs were treated for 60 min with NiSO₄ (500 μM), IFN-γ (1000 U/ml), or their association. Whole-cell extracts were prepared and incubated with either biotinylated NF-κB probes (NF-κB) or mutated NF-κB probes (NF-κB-mut) and streptavidin-agarose beads. P65 was detected by Western blotting. Results are representative of three independent experiments. C, IRF-1 activation by NiSO₄ or NiSO₄ and IFN-γ at 90 min. At day 5, iDCs were treated for 90 min with NiSO₄ (500 μM), IFN-γ (1000 U/ml), or their association. Whole-cell extracts were prepared and incubated with either biotinylated ISRE probes (ISRE) or mutated ISRE probes (ISRE-mut) and streptavidin-agarose beads. IRF-1 was detected by Western blotting. p38 MAPK was used as a loading control in whole-cell extracts using Western blotting and anti-p38 MAPK Ab. Results are representative of three independent experiments. N.S., nonspecific.
FIGURE 4. Role of MAPKs and NF-κB in NiSO₄-induced IL-12p40 production. At day 5, iDCs were treated with either DMSO, SB203580 (20 μM), SP600125 (20 μM), or Bay 11-7085 (3 μM) and then stimulated with NiSO₄ at 500 μM. After 24 h, IL-12p40 production was measured by ELISA. Results are expressed in picograms per milliliter (values are mean ± SD of three independent experiments). *p ≤ 0.05 compared with NiSO₄-treated cells (control NiSO₄); †p ≤ 0.05 compared with NiSO₄- and DMSO-treated cells.

Electroporation of Mo-DCs with small interfering IRF-1

At day 4 of differentiation, Mo-DCs were washed once with serum-free medium and once with PBS. Cells were then resuspended in serum-free medium at 4 × 10⁶ cells/ml; 10 μg nonsilencing control small interfering RNA (siRNA) (siRDM) from Qiagen, Courtaboeuf, France) or IRF-1 siRNA (siIRF-1 ON-TARGETplus SMARTpool from Thermo Scientific Dharmacon, Lafayette, CO) were transferred into 4-mm cuvettes (Bio-Rad, Marne-La-Coquette, France) and filled up to a final volume of 100 μl with serum-free medium. The siIRF-1 ON-TARGETplus SMART-pool, which contained four individual duplexes, was chosen because it enhances siRNA specificity and reduces off-target effects. A total of 4 × 10⁶ cells was added and pulsed in a GenePulsar II (Bio-Rad) (300 V, 150 μF). Cells were then resuspended in complete medium with GM-CSF (550 U/ml) and IL-4 (550 U/ml) at a concentration of 10⁶ cells/ml for 24 h and then treated according to different protocols.

mRNA expression using semiquantitative and real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Cergy Pontoise, France) by the guanidium thiocyanate method as mentioned by the manufacturer. RNA was quantified by spectrophotometry. First-strand cDNA was synthesized from total RNA extracted in RNease-free conditions. The reaction was performed on 2 μg total RNA with oligo(dT) primers (MWG Biotech) and 2 U AMV RT (Promega, Charbonnières-les-Bains, France). For semiquantitative PCR, the reaction was performed using 1 U Taq polymerase (Qiagen, Montreal, Canada). The number of cycles and the hybridization temperature used for the PCR were optimized for all the genes studied: GAPDH (24 cycles, 60˚C), IRF1 (30 cycles, 54˚C), and GAPDH (24 cycles, 60˚C), IRF1 (30 cycles, 54˚C), and mRNA expression was evaluated using RT-PCR.

FIGURE 5. IRF-1 is needed for IL-12p40 and IL-12p70 production in response to NiSO₄ and IFN-γ. At day 4 of differentiation, cells were electroporated with 10 μg siRNA random (RDM) or siRNA IRF-1. For the next 24 h, cells were incubated with GM-CSF (550 U/ml) and IL-4 (550 U/ml). Cell treatments were proceeded 24 h postelectroporation. A, Effect of IRF-1 siRNA treatment on IL-12p40 production. Cells were treated with NiSO₄ (500 μM) and IFN-γ (1000 U/ml). After 24 h, IL-12p40 production was measured by ELISA. Results are expressed in pg/ml (values are mean ± SD of three independent experiments). B, Effect of IRF-1 siRNA treatment on IL-12p70 production. Cells were treated with NiSO₄ (500 μM) and IFN-γ (1000 U/ml). IL-12p70 production was measured after 24 h by ELISA. Results are expressed in picograms per milliliter (values are mean ± SD of three independent experiments). C, IL12A mRNA (IL-12p35) expression. Cells were treated with NiSO₄ (500 μM) and IFN-γ (1000 U/ml) for 8 h. IL12A mRNA expression was evaluated using RT-PCR. GAPDH was used as the housekeeping gene. Results are representative of three independent experiments.

D, siRNA IRF-1 effect on IRF-1 protein levels. Cells were stimulated with IFN-γ for two hours and lysed. IRF-1 expression was evaluated by Western blotting. Membrane was then probed with anti-p38 MAPK Ab for loading control. Numbers represent the ratio of the intensity of IRF-1 band/intensity of p38 MAPK band. Results are representative of three independent experiments.
IL12A (IL-12p35) (35 cycles, 59.2°C). The specific primers used were (forward and reverse primers, respectively): GAPDH: 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3'; IRF1: 5'-ACC CTG GCTAGAGAT GCAGA-3' and 5'-TTT TCC CCT GCT TGT ATC G-3'; and IL12A: 5'-ACC ACT CCC AAA ACC TGC-3' and 5'-CCA GGC AAC TCC CAT TAG-3'. PCR products were visualized by addition of ethidium bromide on 1% agarose gel.

Real-time PCR was performed using the SYBR Green technology on a LightCycler rapid thermal cycler (Roche Diagnostics, Meylan, France). Forward and reverse primers were each designed on a different exon of the target gene sequence, eliminating the possibility of amplifying genomic DNA. To confirm the specificity of the amplification, the PCR product was subjected to a melting curve analysis and agarose gel electrophoresis. PCR amplification was performed in duplicate in a total reaction volume of 10 µl. The reaction mixture consisted of 5 µl diluted template, 2 µl FastStart DNA MasterPLUS SYBR Green kit, and 0.5 µM forward and reverse primers. After an 8 s activation of Taq polymerase at 95°C, amplification was proceeded for 30–45 cycles, each consisting of denaturation at 95°C for 5 s, annealing at 60°C for 5 s, and extension at 72°C for 9 s. Specific primers were used in the PCR reaction mixture (forward and reverse primers, respectively): IL12A: 5'-ACC ACT CCC AAA ACC TGC-3' and 5'-CCA GGC AAC TCC CAT TAG-3'; and β-actin: 5'-GGC ATC CTC ACC CTG AAG TA-3' and 5'-GCA GCA GCT CTG GCT AG-3'. Results were expressed as fold induction calculated using the standard curve method. β-actin was used to control and calibrate cDNA synthesis. Fold induction represents the normalized ratio of treated samples divided by untreated samples.

FIGURE 6. IRF-1 is activated by NiSO₄ and regulates IL-12p40 production. A, IRF-1 activation in response to NiSO₄ at 4 and 6 h. Whole-cell extracts were prepared and incubated with biotinylated ISRE probes (ISRE) or mutated ISRE probes (ISRE-mut) and streptavidin-agarose beads. IRF-1 was detected by Western blotting. Results are representative of three independent experiments. p38 MAPK was used as a loading control in whole-cell extracts using Western blotting and anti-p38 MAPK. B, IRF1 mRNA expression in NiSO₄ or NiSO₄- and IFN-γ–treated cells. At day 5, iDCs were treated or not (control) with either NiSO₄ (500 µM), IFN-γ (1000 U/ml), or their association. IRF1 mRNA expression was evaluated using RT-PCR at 2, 4, and 6 h. GAPDH was used as the housekeeping gene. Folds represent the ratio of the normalized intensity of treated cells divided by the normalized intensity of nontreated cells. Results are representative of three independent experiments. C, IRF-1 regulates NiSO₄-induced IL-12p40 production. At day 4 of differentiation, cells were electroporated with 10 µg siRNA random (RDM) or siRNA IRF-1. Twenty-four hours later, cells were treated with NiSO₄ (500 µM), and IL-12p40 production was measured by ELISA. Results are expressed in picograms per milliliter (values are mean ± SD of three independent experiments). p ≤ 0.05.
FIGURE 7. IRF-1 expression in response to NiSO₄ is dependent on Jak-induced STAT-1 phosphorylation but not on p38 MAPK. A, STAT-1 phosphorylation in response to NiSO₄ or NiSO₄ and IFN-γ. At day 5, iDCs were treated or not (control) with either NiSO₄ (500 μM), IFN-γ (1000 U/ml), or their association for 2, 3, or 4 h. Cells were lysed, and the level of STAT-1 phosphorylation (p-tyr 701 and p-Ser 727) was evaluated by Western blotting. Membrane was then probed with an anti–STAT-1 Ab for loading control. Folds represent the ratio of the normalized intensity of treated cells divided by the
ELISA assay for measuring IL-12p40 and IL-12p70 production

iDCs were treated with NiSO4 with or without IFN-γ for 24 h. In some experiments, cells were pretreated for 30 min with MAPK inhibitors or with Bay 11-7085 or DMSO for 1 h and then stimulated. Culture supernatants were collected after 24 h and stored at −80°C. ELISA assays were performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The OD proportional to the intensity of the color was measured at 450 nM and 540 nM with a microplate reader (Thermo Labsystems-Multiskan, Philadelphia, PA). Results were expressed in picograms per milliliter. The sensitivity of the method was 31 pg/ml for IL-12p40 and 7.8 pg/ml for IL-12p70 according to the manufacturer.

Data analysis

All data are represented as mean ± SD of the mean (based on the entire population). Differences between the production of IL-12p40 or IL-12p70 in chemical-treated cells compared with respective controls were evaluated using an unpaired Student t test. p ≤ 0.05 was considered to be statistically significant. All tests were performed using the software GraphPad Instat 3 (GraphPad, San Diego, CA).

Results

NiSO4 triggers the production of IL-12p40 in human Mo-DC cells

In the first part of the study, we measured the production of IL-12p40 and IL-12p70 after NiSO4 addition in Mo-DCs. iDCs were treated or not (control) with NiSO4 (300, 400, and 500 μM) for 24 h. NiSO4 induced the production of IL-12p40 in a concentration-dependent manner (Fig. 1A). IL-12p40 production was significantly augmented at 300 μM when compared with control cells. A significant amount of IL-12p40 was present in untreated Mo-DCs as previously described (35). In the same conditions, an average of 100 ng/ml IL-12p40 was present in untreated Mo-DCs as previously described (3). In the same conditions, an average of 100 ng/ml IL-12p40 was present in untreated Mo-DCs as previously described (3). In the same conditions, an average of 100 ng/ml IL-12p40 was present in untreated Mo-DCs as previously described (3).

The presence of IFN-γ in association with NiSO4 is necessary for the expression of IL12A mRNA

IL-12p70 depends on the synthesis of both p40 and p35 chains. iDCs were treated or not (control) for 4 or 8 h with NiSO4 (500 μM), IFN-γ (1000 U/ml), or their association. IL12A mRNA expression was measured using real-time PCR (Fig. 2A) and semiquantitative PCR (Fig. 2B). These times of stimulation were chosen according to preliminary kinetics experiments measuring IL12A mRNA expression (data not shown). IL12A mRNA expression was chosen as a readout as there are no robust methods to measure IL-12p35 at the protein level. Results showed that very low levels of IL12A mRNA were detected when NiSO4 or IFN-γ were added. The presence of both NiSO4 and IFN-γ was necessary to induce the expression of detectable levels of IL12A mRNA. Moreover, results obtained with inhibitors of the p38 MAPK, JNK, and NF-κB pathways showed that in addition to IRF-1, other pathways are involved in IL-12 p35 mRNA expression (Supplemental Fig. 1).

NiSO4 and IFN-γ activate complementary signaling pathways: MAPKs, NF-κB, and IRF-1

The promoters of p35 and p40 are composed of several DNA binding sites for AP-1, NF-κB, and IRFs. These pathways have also been previously described to participate in IL-12 synthesis in DCs and macrophages (24, 25, 30). We first evaluated the kinetics of JNK and p38 MAPK activation in iDCs stimulated by NiSO4, IFN-γ, or their combination. Our results showed that NiSO4 and IFN-γ induced the phosphorylation of p38 MAPK and JNK at 30 min. The association of both NiSO4 and IFN-γ slightly upregulated p38 MAPK (at 30 min) and JNK phosphorylation (at 30 min and 60 min) compared with NiSO4 alone (Fig. 3A). Binding of the p65 subunit of NF-κB to specific NF-κB probes using a DNA-binding assay after 1 h of treatment was detected with both NiSO4 and IFN-γ with IFN-γ (Fig. 3B). We then assessed the binding of the transcription factor IRF-1 to specific IL-12p35 ISRE using a DNA-binding assay after 90 min of treatment with NiSO4 and IFN-γ (Fig. 3B). Results showed that IFN-γ and NiSO4 associated to IFN-γ showed comparable binding of IRF-1 on specific ISRE DNA probes (Fig. 3C). However, no binding of IRF-1 was detectable after 90 min of NiSO4 treatment alone.

p38 MAPK, NF-κB, and IRF-1 contribute to the production of IL-12

To investigate the implication of MAPKs and NF-κB in IL-12p40 synthesis induced by NiSO4, we used three well-described pharmacological inhibitors: SB203580, a p38 MAPK inhibitor, SP60-0125, a JNK inhibitor, and Bay 11-7085, an NF-κB inhibitor. At day 5, cells were washed and pretreated with SB203580 (20 μM), SP600125 (20 μM) for 30 min, or Bay 11-7085 (3 μM) for 1 h and then stimulated for 24 h with NiSO4. These concentrations of inhibitors were not cytotoxic, and the optimal concentration for each inhibitor was determined based on preliminary experiments (data not shown). As JNK and NF-κB inhibitors were diluted in DMSO, cells were also pretreated for 1 h with DMSO (0.1%) as an internal control. Results showed that IL-12p40 production induced by NiSO4 was mainly dependent on p38 MAPK and NF-κB pathways; this production was independent of the JNK pathway (Fig. 4).

To investigate whether IRF-1 was critically required for IL-12 production induced by both signals, we used RNA interference to inhibit IRF-1 expression in iDCs. The levels of IL-12p40 and IL-12p70 production were measured by ELISA. RNA interference experiments revealed that IRF-1 was critical for IL-12p40 and IL-12p70 production induced by NiSO4 associated to IFN-γ (Fig. 5A, B, STAT-1 phosphorylation (p-Ser 727) in response to NiSO4 is controlled by p38 MAPK. At day 5, iDCs were pretreated or not (control) with SB203580 at 20 μM for 30 min and then treated with NiSO4 (500 μM) for 3 h. Cells were lysed, and the level of phosphorylation of STAT-1 (p-Ser 727) and the level of IRF-1 was evaluated by Western blotting. Membrane was then probed with STAT-1 Ab for loading control. Results are representative of three independent experiments. C, Specificity of Jak inhibitor 1 and SB203580 for Ser and Tyr STAT-1 phosphorylation, respectively. At day 5, iDCs were pretreated or not (control) with SB203580 at 20 μM for 30 min or Jak inhibitor at 0.5 μM for 2 h and then treated with NiSO4 (500 μM) for 3 h. Cells were lysed, and the level of phosphorylated STAT-1 was evaluated by Western blotting. Membrane was then probed with STAT-1 Ab for loading control. Results are representative of two independent experiments. D, Jak control IRF-1 expression and STAT-1 tyrosine phosphorylation induced by NiSO4. At day 5, iDCs were pretreated or not (control) with either DMSO or the Jak inhibitor at 0.5 μM for 2 h and then treated with NiSO4 (500 μM) for 3 h. Cells were lysed, and the level of phosphorylation of STAT-1 (p-Tyr 701) and IRF-1 were evaluated by Western blotting. Membrane was then probed with STAT-1 Ab for loading control. Results are representative of three independent experiments. E, p38 MAPK inhibition does not control IRF-1 expression. At day 5, iDCs were pretreated or not (control) with SB203580 at 20 μM for 30 min and then treated with NiSO4 (500 μM) for 3 h. Cells were lysed, and the level of IRF-1 was evaluated by Western blotting. Membrane was then probed with STAT-1 Ab for loading control. Results are representative of the mean of two independent experiments.
and IRF-1 expression were not dependent on p38 MAPK activity (Fig. 7E). The next question was to elucidate if STAT-1 tyrosine phosphorylation resulted from a direct effect of NiSO4 on Jak activity or through the synthesis of an intermediate factor. Indeed, type I IFNs are well-known inducers of STAT-1 tyrosine phosphorylation in Mo-DCs (38). We found that NiSO4 upregulated the ifn-β mRNA, but IFN-β was undetectable in the supernatants of Mo-DC treated for 24 h by NiSO4 (data not shown). We then used puromycin, a well-known protein synthesis inhibitor. Mo-DCs were pretreated for 1 h with puromycin at 10 μM and then treated for 3 h with NiSO4. The optimal concentration of puromycin for protein synthesis inhibition was determined based on preliminary experiments (data not shown). We showed that STAT-1 tyrosine phosphorylation induced by NiSO4 was not modified by puromycin, suggesting that STAT-1 phosphorylation did not depend on an intermediate product synthesized by Mo-DC in response to NiSO4 (Fig. 8A). IRF-1 protein expression was strongly inhibited as a consequence of puromycin treatment, showing that puromycin was an effective inhibitor of protein synthesis in the conditions used (Fig. 8A). Preincubation of cells with NAC (a precursor of glutathione) is often used to reinforce the redox potential of cells. The optimal concentration of NAC was determined based on preliminary experiments (data not shown). We showed that pretreatment with NAC inhibited STAT-1 phosphorylation and completely abolished NiSO4-induced IRF-1 expression (Fig. 8B).

Discussion

DCs are activated by danger signals such as proinflammatory cytokines, bacterial products, viruses, and chemicals. Sensitizers have also been demonstrated to induce the expression of markers related to function and maturation of DCs, the production of proinflammatory cytokines, and the phosphorylation of members of the MAPK family and NF-κB pathway, thus mimicking the effects of danger signals (9–11, 31–33).

IL-12 is a key factor in the generation of Th1 and cytotoxic T cells (14–16), thus playing a major role in the generation of allergenspecific T cell response in ACD. In this study, we address the question if nickel, the most prevalent of all metal sensitizers (39), can trigger specific signaling pathways in DCs, leading to the production of IL-12 as previously described for danger signals. We showed that NiSO4 induced the production of IL-12p40 in Mo-DCs in a concentration-dependent manner. We and others have previously shown that NiSO4 induced the production of IL-12p40 in

**FIGURE 8.** STAT-1 phosphorylation (Tyr 701) in response to NiSO4 is not dependent on de novo protein synthesis and is redox sensitive. A. STAT-1 tyrosine phosphorylation induced by NiSO4 was not modified by puromycin. At day 5, Mo-DCs were pretreated for 1 h with 10 μM puromycin and then treated for 3 h with NiSO4. Cells were lysed, and the level of STAT-1 phosphorylation (p-Tyr 701) was evaluated by Western blot. Membrane was probed with an anti-IRF-1 to evaluate IRF-1 protein synthesis inhibition or with an anti–STAT-1 Ab for loading control. Results are representative of three independent experiments. B. STAT-1 phosphorylation in response to NiSO4 is redox sensitive. At day 5, iDCs were pretreated or not (control) with NAC at 25 mM for 30 min. Postremoval of NAC containing medium, Mo-DCs were treated with NiSO4 (500 μM) for 4 h. Cells were lysed, and the level of phosphorylation of STAT-1 (p-Tyr 701, p-Ser 727) and IRF-1 expression were evaluated by Western blotting. Membrane was then probed with STAT-1 Ab for loading control. Results are representative of three independent experiments.
NiSO₄, detectable amounts of IL-12p70 were then produced by mRNA, suggesting a crucial role for IRF-1 in IL-12p40 and IL-12p70 significantly inhibited when IRF-1 was downregulated by RNA silencing, showed that both the levels of IL-12p40 and IL-12p70 were significantly inhibited. Our results have in accordance with other works that have previously shown that the production of IL-12p70 in Mo-DCs required the presence of two complementary signals such as LPS, IFN-γ, or CD40L to induce a high amount of IL-12p70 synthesis (35, 40, 41).

These results showed the necessity of IFN-γ for high IL-12 production after NiSO₄ treatment in Mo-DCs. O’Leary et al. (42) showed that NK cells can mediate contact hypersensitivity reaction in mice with SCID or deficient in RAG2 (Rag2−/−), which lack T cells (but have NKs). NK cells are present in low frequency in normal human skin, and an influx of NK cells is found in dermal and epidermal inflammatory infiltrates in human contact hypersensitivity (43). NiSO₄ has also been described to induce the production of IFN-γ by splenic NK cells (44). We suggest that IFN-γ produced by NK cells may participate in the production of IL-12 by NiSO₄-stimulated DCs.

The production of IL-12p40 in activated professional APCs is generally in great excess over that of the p35 chain, making the latter molecule a limiting step in the formation of bioactive IL-12 (45). As NiSO₄ alone induced a high level of IL-12p40 but not of IL-12p70, we evaluated the expression of IL12A mRNA (IL-12p35) in response to NiSO₄ and IFN-γ. Our results showed that the combination of NiSO₄ and IFN-γ induced a high expression of IL12A mRNA.

The regulation of both IL-12p40 and IL12A genes is generally controlled by AP-1, NF-κB, and IRF-1 factors (20, 23, 28, 46). We then addressed the question of the signaling pathways involved in the regulation of IL-12p70 after NiSO₄ and IFN-γ treatment. NiSO₄ has been described to activate MAPKs and NF-κB in CD34-DCs and Mo-DCs (9, 11, 32). Our results showed that NiSO₄ provoked p38 MAPK and JNK phosphorylation and the binding of p65 on specific DNA probes in Mo-DCs. The association of NiSO₄ and IFN-γ slightly augmented p38 MAPK and JNK activation compared with NiSO₄-treated DCs. Moreover, IFN-γ or NiSO₄ associated to IFN-γ induced a detectable DNA-binding activity of IRF-1 at 90 min. The early activation of p38 MAPK, JNK, NF-κB, and IRF-1 by NiSO₄ and IFN-γ is probably responsible for the synergistic production of IL-12p40 and IL-12p70 induced by both signals compared with NiSO₄ alone.

To investigate the implication of MAPK and NF-κB in the regulation of IL-12p40 by NiSO₄, we used well-described pharmacological inhibitors of these pathways. Our results showed that IL-12p40 production induced by NiSO₄ was dependent on p38 MAPK and NF-κB. Ade et al. (10) have recently showed that IL-12p40 production induced by NiSO₄ was inhibited by pharmacological inhibitors of p38 MAPK, JNK, and NF-κB pathways in CD34-DCs. Aiba et al. (11) have also shown an inhibition of IL-12p40 production by SB203580, a p38 MAPK inhibitor, in Mo-DCs. Moreover, in murine bone marrow-derived DCs, the production of IL-12p40 induced by CpG-DNA or PAM3CSK₄ depends on p38 MAPK and JNK pathways (47).

IRF-1, which has been shown to play a crucial role in IL-12p70 production, is known to regulate IL12A gene expression after LPS and IFN-γ treatment (20, 23, 28, 48). Gabriele et al. (25) have also observed that IRF-1−/− splenic DCs were markedly impaired for their ability to produce IL-12 after LPS treatment. Our results showed that both the levels of IL-12p40 and IL-12p70 were significantly inhibited when IRF-1 was downregulated by RNA silencing, suggesting a crucial role for IRF-1 in IL-12p40 and IL-12p70 production induced by NiSO₄ and IFN-γ. The downregulation of IL12A mRNA expression with IRF-1 siRNA correlated with the strong inhibition of IL-12p70 production. Interestingly, we showed that NiSO₄ alone was able to induce IRF1 mRNA expression at 2 h and a DNA-binding activity of IRF-1 to ISRE after 4 h of treatment compared with 90 min for NiSO₄ and IFN-γ. Downregulation of IRF-1 using siRNA significantly decreased IL-12p40 production induced by NiSO₄. These results suggested that IRF-1 was also a key factor for IL-12p40 production in DCs in response to NiSO₄ alone. This report is the first one describing IRF-1 activation by NiSO₄ in human DCs.

IRF-1 synthesis in Mo-DCs has been described to be mainly regulated by STAT1 in response to type I IFN (49). When Mo-DCs were treated with NiSO₄, we found that STAT1 was phosphorylated on both serine and tyrosine residues. Using Jak inhibitor I, we found that NiSO₄-induced IRF-1 expression was dependent on STAT1 tyrosine phosphorylation. In contrast, NiSO₄-induced STAT1 phosphorylation on serine 727 was regulated by p38 MAPK, but p38 MAPK did not play any role in NiSO₄-induced IRF-1 expression. As it is well described that TLR agonists induced type I IFN synthesis leading to STAT1 phosphorylation, IRF-1 activation, and IL-12 production, we addressed the question of whether NiSO₄-induced STAT1 phosphorylation was dependent on type I IFN production. STAT1 tyrosine phosphorylation induced by NiSO₄ was not affected by a pretreatment with puromycin, suggesting a direct activation of STAT1 by NiSO₄. Simon et al. (50) have shown in fibroblasts that the activation of the Jak-STAT pathway was dependent on the redox status of cells. NiSO₄ treatment can modify the redox status of human DCs in vitro, suggesting a possible link between activation of the Jak-STAT pathway by NiSO₄ and oxidative stress (51–54). We showed that NiSO₄-induced oxidative stress could regulate STAT1 phosphorylation and IRF-1 expression, suggesting that NiSO₄-induced IL-12p40 production following NiSO₄ treatment could be redox sensitive.

Finally, our results describe the specific signaling pathways induced by NiSO₄ for IL-12 production and found similarity with the one already described for danger signals. According to all published results on DC maturation induced by NiSO₄ (9–11, 31–33), we can speculate that NiSO₄ can be perceived by DCs as a danger signal, thus playing a double role of a hapten and a danger signal.

Disclosures
The authors have no financial conflicts of interest.

References
P38 MAPK, JNK and NFκB pathways participate to IL-12p35 mRNA expression. At day 5, iDC were treated with either DMSO, SB203580 (20 μM), SP600125 (20 μM) or Bay 11-7085 (3 μM) and then stimulated with NiSO₄ at 500 μM for 4 hours. il-12p35 mRNA expression was evaluated using RT-PCR. gapdh was used as the house-keeping gene. Results are expressed as the ratio of the normalized intensity of the different treatment (intensity of il-12p35 band/ intensity of gapdh band) divided by the normalized intensity of cells treated with NiSO₄ + IFN-γ. Results represent the mean of three independent experiments.