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Interleukin-12 Gene-Expression of Macrophages Is Regulated by Nitric Oxide

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Interleukin-12 is a heterodimeric cytokine, mainly produced by macrophages. In our present study we demonstrate that interleukin-12 expression is regulated by nitric oxide. Incubation of the macrophage cell line IC 21 with interferon-γ gave rise to both interleukin-12 p40 mRNA and nitric oxide production. The concurrent addition of the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine inhibited nitrite production and in parallel completely suppressed interleukin-12 p40 mRNA formation. This indicated that endogenous nitric oxide synthase activity was required for IL-12 p40 gene expression. Exposure of the cells towards the nitric oxide generating compounds nitroprusside or S-nitroso-N-acetyl-penicillamine induced interleukin-12 p40 mRNA. Maximal mRNA levels were induced with nitric oxide donors at 1 μM concentration. We conclude that nitric oxide may exert an autoregulatory and paracrine control of interleukin-12 gene expression.

Interleukin-12 (IL-12) is a 75 kDa heterodimeric glycoprotein consisting of two disulfide linked subunits p35 and p40. The production of biologically active recombinant IL-12 requires the coexpression of the IL-12 p40 and p35 subunits (1-3). Although initially described as a cytokine activating spontaneously cytotoxic lymphocytes the significance of IL-12 lies in its effect on T-helper cells (3). The T-helper subsets type 1 (Th1) and type 2 (Th2) are distinguished by their secretion of different cytokine patterns. Th1 cells preferentially produce the cytokines interferon-γ (IFN-γ), IL-2 and/or tumor necrosis factor-β (TNF-β), and mediate delayed hypersensitivity responses (4, 5). In contrast Th2 cells are characterized by the cytokines IL-4, IL-5, IL-6 and/or IL-10, and are particularly good helper cells for B lymphocytes, promoting the synthesis of several antibody isotypes (6, 7). IL-12 conditions naive T-helper cells to differentiate along the Th1 pathway, and stimulates Th0 or Th2 cells to transiently produce IFN-γ (8). Furthermore, IL-12 is known to be involved in infectious and organ specific autoimmune diseases (9-11). In addition to promoting Th1 responses via the secretion of IL-12, macrophages upon activation also released mediators counteracting Th1 reactivities. One such compound is IL-10, and it has been shown that IL-10 exerts its inhibitory activity on Th1 cells by downregulation of IL-12 gene expression (12). Another major product of activated macrophages is nitric oxide (NO) which is generated in large amounts by the inducible NO synthase. Since NO has recently been reported to interfere with Th1 but not Th2 reactivities (13, 14) we analysed for a regulatory role of NO in IL-12 gene expression. We report here that NO induces IL-12 p40 gene expression.

MATERIALS AND METHODS

Cells. The murine macrophage cell line IC 21, was provided by ATCC (Rockville, Maryland, USA). The cells were cultured in RPMI 1640 medium (GIBCO, Heidelberg, Germany) supplemented with 10 % (v/v) fetal calf serum (FCS, GIBCO), and 2 mM L-glutamine.

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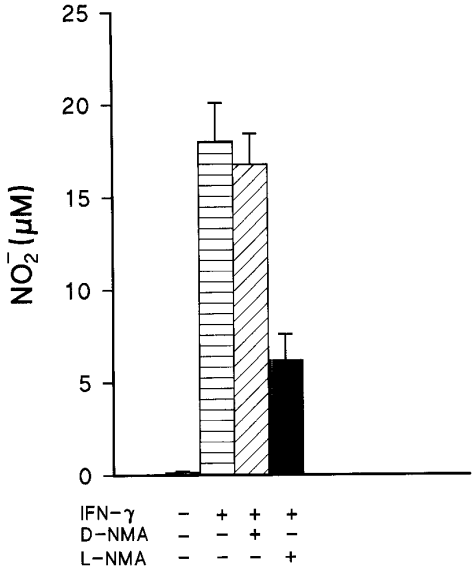

FIG. 1. Nitric oxide production of IFN-γ activated IC 21 cells. IC 21 cells (10^6/ml) were incubated with 100 U IFN-γ for 24 hrs. Inhibition of the NO production was done by adding 1 mM L-NMA, D-NMA for 3 hrs as control. Shown are the mean values of nitrite levels in supernatants of five seperate experiments, done in triplicate. Bars indicated SD.

Reagents. Recombinant rat IFN-γ was purchased from Genzyme (Cambridge, MA, USA). N^G-monomethyl-D-arginine (D-NMA) and L-NMA were purchased from Sigma (Deisenhofen, Germany). S-nitroso-N-acetyl-penicillamine (SNAP) was a kind gift of Dr. K-D Kröncke (University of Düsseldorf, Düsseldorf, Germany). Nitroprusside was purchased from Merck (Darmstadt, Germany).

Cell culture. IC21 cells were cultured in petri-dishes at 1×10^6/ml at 37°C. Pretreatment with 1 mM D-NMA or L-NMA was done for 3 hrs before stimulating the cells with 100 U IFN-γ/ml for 24 hours. For mRNA studies cells were cultured with SNAP or nitroprusside for 24 hrs. Nitroprussid and SNAP were dissolved in medium immediately before use. Cyanide ions generated during nitroprusside decomposition were inactivated by 8 U /ml rhodanese (Sigma) and 5 mM sodium thiosulfate (Merck).

mRNA-analysis. Total RNA was isolated after removal of the supernatant and lysis of the cells in petri-dishes with 1 ml Trizol (GIBCO). Determination and quantification of specific mRNA was performed by reverse transcriptase polymerase chain reaction (RT-PCR) as described elsewhere (11, 15). Specific primers for β-actin were purchased from Clontech (Clontech Labortories Inc., Palo Alto, CA, USA). The specific primers for IL-12 p40 and p35 subunits were described elsewhere (9). After a total of 35 cycles the products were subjected to electrophoresis on a 2% agarose gel followed by hybridization with specific ^{32}P labelled probes binding at sites between the primer sequences. Signals were quantified by measuring the ^{32}P stimulated luminescence (PSL) by a phosphorimager. Relative PSL was calculated by normalization of the measured PSL to the strength of the β-actin signal (15).

Nirite production by macrophages. Supernatants of the macrophage culture were collected before mRNA isolation nad nitrite levels were determined by the Griess method as described previously (16).

Statistical analysis. All data derived from three to five separate experiments. Mean values were calculated and are presented with their standard deviation. Mean values were compared by Student's *t*-test.

RESULTS AND DISCUSSION

Macrophages are known to respond to activation with the production of NO and IL-12. Stimulation of the macrophage line IC 21 with 100 U /ml IFN-γ for 24 hrs resulted in the accumulation of 18 μM of nitrite in the supernatant (Fig. 1). In the presence of the NO synthase inhibitor L-NMA nitrite levels were decreased by 75 % ($p<0.005$). The nonreactive stereoisomer D-NMA did not affect nitrite formation (Fig. 1).

In parallel experiments, the cells were analysed for IL-12 p40 gene expression. Incubation

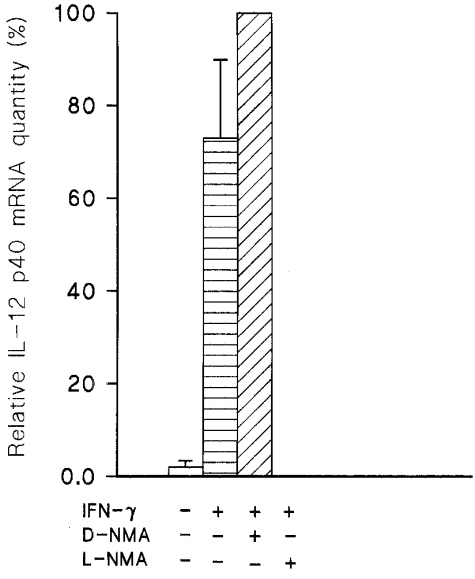

FIG. 2. IL-12p40 gene expression in IFN-γ activated IC 21 cells. IC21 cells (10^6/ml) were incubated with 100 U IFN-γ in the presence or absence of 1 mM L-NMA or D-NMA. RNA was isolated after 24 hrs. The amount of IL-12 p40 mRNA induced by IFN-γ was set as 100%, after calibration to the amount of β actin mRNA.

with IFN-γ led to an about fiftyfold increase of IL-12 p40 mRNA levels (Fig. 2). Addition of L-NMA completely suppressed IL-12 p40 gene transcription. This effect of L-NMA appeared to be due to the interaction with macrophage NO synthase since addition of the non inhibitory isomer D-NMA did not decrease IL-12 p40 levels (Fig. 2). These findings indicated that the stimulatory effect of IFN-γ on IL-12 p40 gene expression required the prior induction of NO synthase in macrophages.

We therefore tested the hypothesis that NO might induce transcription of the IL-12 p40

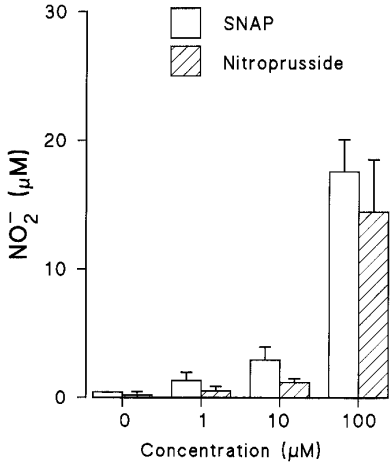

FIG. 3. NO donor dependent nitrite production. SNAP or nitroprusside were added to nonactivated macrophages and the supernatant analysed after 24 hrs. Shown are mean values of three seperate experiments, done in triplicate. Bars indicate SD.

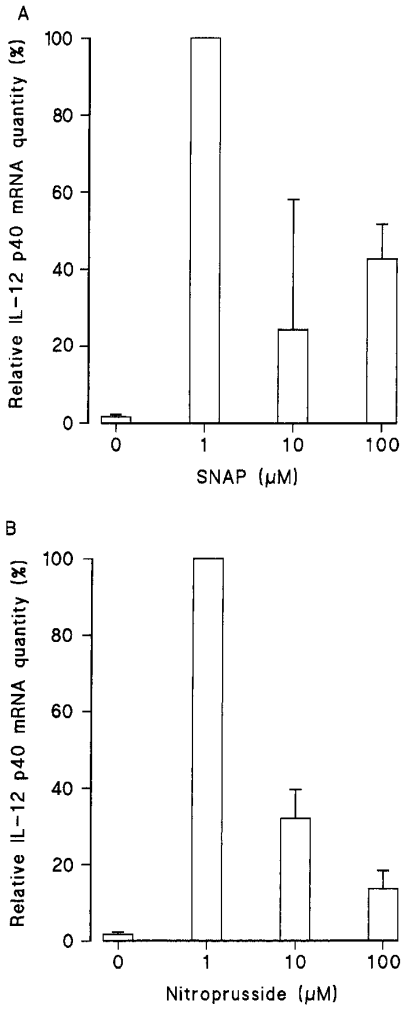

FIG. 4. Induction of IL-12p40 mRNA by NO-donors IC21 cells (10^6/ml) were incubated with dose dependent amounts of the NO donors SNAP (A) and nitroprusside (B). RNA was isolated after 24 hrs. The amount of IL-12 p40 mRNA induced by 1 mM SNAP or nitroprusside was set as 100%, after calibration to the amount of β actin mRNA. Shown are mean values of 3 different experiments, bars indicate SD.

gene. Macrophages were exposed to the NO generating compounds SNAP and nitroprusside. As expected, addition of the NO donors caused a dose dependent increase of nitrite levels in the supernatant (Fig. 3). In parallel, the cells were analysed for IL-12 p40 mRNA levels. As shown in Fig. 4, the addition of SNAP or nitroprusside induced substantial levels of IL-12 p40 mRNA. Specific mRNA was seen for both NO donors, and at all concentrations tested. Levels of mRNA were highest with the lowest dose of SNAP or nitroprusside (Fig. 4). At the higher concentrations, the well known depressive effects of NO on cell respiration and proliferation (17, 18) may limit RNA production.

It is of interest to note that IL-12 p40 gene expression was affected by exogenous NO as well as by inhibiting endogenous NO synthase activity. These observations indicate that NO not only has autoregulatory function in macrophage IL-12 gene expression, but also may act on neighbouring cells in a paracrine fashion. Hence, the expression of inducible NO synthase

at sites of infection or in organ specific autoimmune disease (3, 15) may have a severe impact on IL-12 homeostasis. As a consequence, potentiation or inhibition of Th1 type immune reactions may result, depending on the local level of IL-12 p35 gene expression. With sufficient p35 peptides and an increase in IL-12 p40 gene expression high levels of the bioactive heterodimer IL-12 will be produced. However, in the absence of p35, enhanced expression of IL-12 p40 will lead to formation of the p40 homodimer. The latter has been shown to antagonize IL-12 activities and hence would interfere with Th1 reactions (19, 20). IC 21 cells were therefore tested for IL-12 p35 gene expression in response to exogenous NO under the same conditions as described in Fig. 4. IL-12 p35 mRNA was absent in nontreated cells and was not inducible by exposure of cells to the two NO donors. We conclude that NO selectively caused IL-12 p40 gene expression. This implies a possible regulatory role of NO in the downregulation of IL-12 by induction of the IL-12 antagonist IL-12 p40.

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