

Effect of IL-4 and IL-13 on IFN- γ -induced production of nitric oxide in mouse macrophages infected with herpes simplex virus type 2

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Abstract Interleukin (IL)-4 and IL-13 share a wide range of activities. Prominent among these is the ability to antagonize many interferon (IFN)- γ -induced activities. Here we demonstrate that IL-4 and IL-13 totally abrogate IFN- γ -induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) mRNA and protein synthesis in a murine macrophage cell line. IFN- γ -treated cells infected with herpes simplex virus type 2 (HSV-2) or costimulated with tumor necrosis factor (TNF)- α showed an enhanced reactivity, which was only partially reduced by IL-4/13. The results indicate that IL-4 and IL-13 function by intervening with a step prior to iNOS transcription by antagonizing IFN- γ -induced signal(s) without counteracting synergistic virus- or TNF- α -induced signals. The beneficial effect of a sustained NO production in foci of virus infection is suggested.

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Key words: Interleukin-4; Interleukin-13; Interferon- γ ; Herpes simplex virus; Macrophage; Nitric oxide

1. Introduction

Nitric oxide (NO) is a gaseous molecule with properties of a free radical due to an unpaired electron. Consequently it is very reactive and can cause severe damage on substances such as DNA, proteins, lipids and carbohydrates [1–4]. This probably works through generation of peroxynitrite via reaction with O_2^- , nicely illustrating how two pathways each generating relatively deleterious compounds together become even more powerful. It has been found that NO is produced in large amounts by activated macrophages [5] and is involved in the antitumor [6], antimicrobial [7,8] and antiviral [9,10] action of these cells.

NO is produced from arginine by the enzyme inducible nitric oxide synthase (iNOS). The main inducers of iNOS are interferon- γ (IFN- γ), lipopolysaccharide (LPS) and, in conjunction with these, tumor necrosis factor- α (TNF- α) [11,12]. They function by activating interferon regulatory factor 1 (IRF-1), signal transducer and activator of transcription 1 (STAT1) and nuclear factor (NF)- κ B which bind to sites in

the iNOS promoter [11,13,14]. Studies in IRF-1 knock-out mice and analyses of cycloheximide sensitivity have revealed that de novo IRF-1 synthesis is essential in iNOS transcription [13,15,16].

Because NO is extremely reactive, it is potentially toxic to the host. Therefore, the production must be tightly regulated. In recent years it has become clear that many effects induced by the T-helper cell type 1 (Th1) cytokine IFN- γ are counteracted by Th2 cytokines like interleukin (IL)-4 and IL-13 [17–20]. IL-4 is primarily produced by activated Th2 cells but also by some other T-cell subpopulations as well as by cells of the basophil/mast cell lineage [21,22]. IL-13 has thus far only been found to be expressed by activated Th2 cells [23,24]. The two cytokines share a wide range of functions [20,24,25].

In mouse macrophages we have previously shown that infection with herpes simplex virus type 2 (HSV-2) synergizes with IFN- γ in induction of NO production and that this synergy is largely due to autocrine secretion of TNF- α [26]. To get a broader understanding of iNOS regulation during a virus infection we have analyzed putative down-regulators of iNOS and initiated studies on the molecular mechanisms underlying their actions.

In the present study we have analyzed the ability of IL-4 and IL-13 to antagonize IFN- γ -induced NO production in the murine macrophage cell line J774A.1 in the presence or absence of infection with HSV-2. We have found that both IL-4 and IL-13 completely abolish IFN- γ -induced NO production and that HSV-2- and TNF- α -mediated signals can partly overcome this down-regulating effect, thus allowing some NO production despite the presence of high levels of IL-4 and IL-13. The two Th2 cytokines work by diminishing iNOS mRNA levels and we suggest they act primarily by antagonizing intracellular IFN- γ signals.

2. Materials and methods

2.1. Cells, media, virus and reagents

The experiments were done with the mouse macrophage cell line J774A.1 (ATCC TIB 67) grown in Dulbecco's modified Eagle's medium with 5% fetal calf serum (Hyclone, LPS free) and antibiotics.

Cultures of J774A.1 cells were infected with 3×10^5 pfu/ml of the MS strain of HSV-2 prepared as previously described [27]. The murine recombinant cytokines were used at the following concentrations: IFN- γ (Pharmingen), 100 IU/ml (10 ng/ml); TNF- α (Genzyme), 250 U/ml (6.2 ng/ml); IL-4 (R&D Systems), 100 ng/ml; IL-13 (R&D systems), 100 ng/ml. Staurosporine (Sigma) was used at a concentration of 20 nM, which did not induce any overt toxicity in the cells.

2.2. iNOS induction and assay of NO

Cells (10^5 /well; 6×10^5 /ml) were seeded in 96-well tissue culture plates (Nunc). The cells were left to settle for 2 h before stimulation and/or infection. Supernatants were harvested 24 h later and nitrite concentrations, as a correlate of NO production, were determined by use of the Griess assay as described earlier [26].

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Abbreviations: IFN- γ , interferon- γ ; IL-4, interleukin-4; IL-13, interleukin 13; TNF- α , tumor necrosis factor- α ; NO, nitric oxide; iNOS, inducible nitric oxide synthase; HSV-2, herpes simplex virus type 2; IRF, interferon regulatory factor; STAT, signal transducer and activator of transcription; NF- κ B, nuclear factor- κ B; Jak, Janus kinase; Th, T-helper cell; RT-PCR, reverse transcribed-polymerase chain reaction

2.3. Western blotting of iNOS

For analysis of iNOS protein levels, 2×10^6 cells were seeded in 35 mm culture dishes (Nunc) at a concentration of 6×10^5 cells/ml and stimulated and/or infected 2 h later. After 24 h of treatment, cells were lysed (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.2% Triton-X100, 250 mM sucrose, protease inhibitors [28]) and cytoplasmic proteins harvested. Equal amounts of protein for each sample were subjected to SDS-PAGE and blotted onto a PVDF membrane (NOVEX). Western blotting was performed following the recommendations of the antibody manufacturer (Transduction Laboratories) using a mouse monoclonal antibody against mouse iNOS (cat. no. N39120), a horse-radish peroxidase-conjugated goat anti-mouse antibody (cat. no. M15345) and DAB chromogen tablets (DAKO).

2.4. iNOS mRNA detection by RT-PCR and Northern blot analysis

From cells cultured and stimulated as for the Western blot analysis, total cellular RNA was extracted by the guanidinium thiocyanate procedure as described by Chomczynski and Sacchi [29]. RT-PCR was performed as described earlier [26] using Oligo-(dT)₁₅ as primer for the RT and the following specific iNOS primers for the PCR: sense, 5'-CAC ATT CAG ATC CCG AAA CGC TTC TCT TC-3'; antisense, 5'-GAG CCT CGT GGC TTT GGG CTC CTC-3'. The amplified product spanned 1379 bp.

For Northern blot analysis total RNA was poly(A)-selected using Oligo-(dT)₂₅ beads (Dynal), following the manufacturer's instructions. The mRNA was subjected to denaturing electrophoresis and transferred to a Zeta probe blotting membrane (Bio-Rad) and UV-cross-linked. The hybridization and wash was done at 60°C using the buffers recommended by Bio-Rad. As probe we used the 1379 bp PCR product mentioned above purified from a 1% low melting temperature agarose gel (NuSieve GTG Agarose, FMC) and labeled using the Random Primed DNA Labeling Kit from Boehringer-Mannheim. RNA loading of the gel was assessed by hybridization with a full-length GAPDH-specific probe labeled as above.

3. Results

We have previously shown that IFN- γ induces NO production in mouse macrophages and that HSV-2 infection synergistically enhances this production [26]. To analyze the effect of possible down-regulators we tested the ability of the Th2 cytokines IL-4 and IL-13 to antagonize this IFN- γ and HSV-

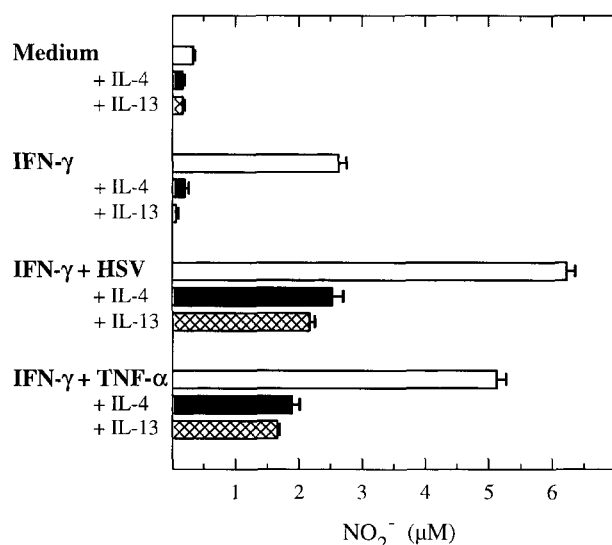


Fig. 1. Effect of IL-4 and IL-13 on IFN- γ -induced NO production in J774A.1 cells. The cells were treated with 100 IU/ml IFN- γ , 100 ng/ml IL-4, 100 ng/ml IL-13 and 100 U/ml TNF- α and infected with 3×10^5 pfu/ml of HSV-2 as indicated. After 24 h the amounts of nitrite in the supernatants were measured by the Griess assay. The results are expressed as mean \pm S.E.M. of triplicate cultures. Essentially similar results were obtained in two other experiments.

IFN- γ	-	+	+	+	-	+	+	+
HSV-2	nd	nd	nd	nd	-	+	+	+
IL-4	-	-	+	-	-	-	+	-
IL-13	-	-	-	+	-	-	-	+

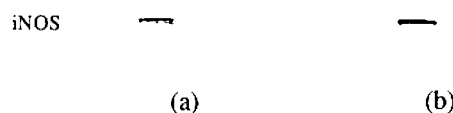


Fig. 2. Effect of IL-4 and IL-13 on iNOS protein levels in uninfected and virus-infected J774A.1 cells treated with IFN- γ . The cells were treated with 100 IU/ml IFN- γ , 100 ng/ml IL-4 and 100 ng/ml IL-13 and infected with 3×10^5 pfu/ml of HSV-2 as indicated. After 24 h the iNOS protein levels were assessed by Western blotting. nd = not done.

2-induced activity in the murine macrophage cell line J774A.1. As shown in Fig. 1 the IFN- γ -induced NO production was totally abrogated by IL-4 and IL-13. Infection of the macrophages with HSV-2 did not in itself result in NO production (data not shown), but synergistically enhanced the IFN- γ -induced NO production. The synergistic enhancement was also reduced by IL-4 and IL-13, but the two cytokines were not able to eliminate NO production in virus-infected cells, even when a 10-fold higher dose of IL-4/IL-13 was used (data not shown). To investigate the mechanism employed by IL-4 and IL-13 to down-regulate NO production in murine macrophages, we replaced virus infection with TNF- α , which in an autocrine manner is responsible for the effect of the virus infection [26]. HSV-2 infection and TNF- α (250 U/ml) had the same effect on NO production, regardless what other stimuli were given (Fig. 1). This supports that HSV-2 works through TNF- α -induction and indicates that IL-4 and IL-13 can abolish IFN- γ -induced signal(s), but not counteract the TNF- α -triggered signals, which are able to synergize with remaining IFN- γ -induced signals.

Having found that IL-4 and IL-13 down-regulate the ability of IFN- γ to induce NO production, we decided to analyze at which stage of the induction pathway they work. Using Western blotting we found that the iNOS protein levels correlated with the determined nitrite concentrations in both uninfected and virus-infected macrophages (Fig. 2a,b). Thus, IL-4 and IL-13 do not seem to modulate the activity of translated iNOS protein, but rather interfere with an earlier stage in NO production.

By RT-PCR we showed that iNOS mRNA synthesis was completely shut down in IFN- γ -stimulated cells treated with either IL-4 or IL-13 (Fig. 3a). In contrast, Northern blot analysis of virus-infected cells revealed that although the two Th2 cytokines were able to substantially reduce the amount of iNOS mRNA transcribed, they were not able to completely eliminate iNOS transcription when IFN- γ -treated cells were infected with HSV-2 (Fig. 3b). From these results we conclude that IL-4 and IL-13 work by modulating the level of iNOS mRNA, whereas the activity of translated iNOS seems not to be affected. Furthermore, virus infection of the cells mediates signals, which can still cause iNOS transcription in spite of apparent extinction of the IFN- γ signal by IL-4 or IL-13.

It has been found that the broad-specificity kinase inhibitor staurosporine inhibits STAT6 phosphorylation [30]. Since STAT6 is known to be involved in many IL-4/IL-13-mediated

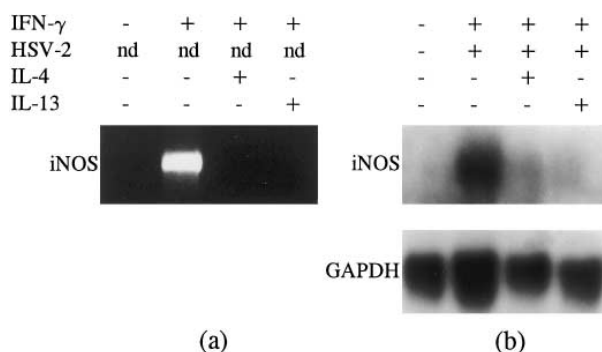


Fig. 3. Effect of IL-4 and IL-13 on iNOS mRNA levels in uninfected and virus-infected J774A.1 cells treated with IFN- γ . The cells were treated with 100 IU/ml IFN- γ , 100 ng/ml IL-4 and 100 ng/ml IL-13 and infected with 3×10^5 pfu/ml of HSV-2 as indicated. After 10 h the cells were lysed and total cellular RNA isolated. The presence of iNOS mRNA was assessed by (a) RT-PCR with iNOS-specific primers and (b) by Northern blot analysis of poly(A)-selected mRNA with an iNOS-specific probe. RNA loading of the gel for Northern blot analysis was assessed by hybridization with a GAPDH-specific probe.

functions, we tested if staurosporine was able to influence the ability of IL-4 and IL-13 to down-regulate IFN- γ -induced NO production. As shown in Fig. 4, staurosporine abolished the ability of IL-4 and IL-13 to counteract IFN- γ both in the presence and absence of HSV-2 infection. Furthermore, it is seen that the synergistic effect of HSV-2 on IFN- γ -induced NO production is also abolished upon staurosporine treatment.

4. Discussion

In the present study we have analyzed the effect and mechanism of action of the Th2-cytokines IL-4 and IL-13 on the production of NO in a murine macrophage cell line stimulated with IFN- γ and the influence of infection with HSV-2 on this process. A prominent activity described for both IL-4 and IL-13 is to counteract IFN- γ -induced activities [17,18,20]. Among these, down-regulation of iNOS, which catalyzes the production of NO from arginine, has been reported [28].

We have previously shown that infection of murine peritoneal macrophages with HSV-2 synergistically enhances IFN- γ -induced production of NO, mainly or exclusively through autocrine secretion of TNF- α [26]. Our present data show that IL-4 and IL-13 totally abrogate IFN- γ -induced NO production during HSV-2 infection or after treatment with TNF- α . These results indicate that IL-4 and IL-13 work primarily by antagonizing a step in the IFN- γ signal downstream of the IFN- γ receptor, and that signals induced by virus infection (or exogenously added TNF- α) are not or to a lesser extent affected by IL-4 and IL-13. These signals, which by themselves are not able to induce production of NO, exert a synergistic effect on the otherwise down-regulated IFN- γ signal and induce some iNOS transcription and NO production in spite of high concentrations of IL-4 or IL-13.

It is known that the transcription factors IRF-1 and STAT1 are essential for the transcription of the iNOS gene and many other IFN- γ -induced genes [31,32]. IRF-1 is itself induced by STAT1 [33]. This, together with our finding that IL-4 and IL-13 down-regulate iNOS at the mRNA level, suggest two pos-

sible mechanisms by which the two Th2 cytokines might counteract IFN- γ : (1) inhibition of the Janus kinase (Jak)/STAT pathway, possibly through activation of a phosphatase that dephosphorylates the IFN- γ receptor, Jak1, Jak2 or STAT1; (2) inhibition of IRF-1 activity, mediated by IL-4-induced factor(s) transcribed via a staurosporine sensitive mechanism. In this context it is interesting that IL-4 and IL-13 are unable to down-regulate IFN- γ -induction of iNOS in STAT6-deficient mice [34] and that STAT6 phosphorylation is inhibited by staurosporine [30]. This could point to STAT6 as being involved in the above process. Another possible mechanism also exists, namely that IL-4 and IL-13 destabilize iNOS mRNA. However, this has been tested by Bogdan et al. for IL-4 [28] and found not to be the case. For IL-13, this has not been explored yet but is a subject of future investigations.

The observation that IFN- γ after HSV-2 infection or after addition of TNF- α is able to induce some iNOS activity despite the presence of IL-4 or IL-13 might be due to activation of NF- κ B. TNF- α induces translocation of NF- κ B to the nucleus [35] and the iNOS promoter contains two NF- κ B sites [36]. It therefore seems plausible that HSV-2 infection via TNF- α secretion activates NF- κ B, which in turn induces some degree of iNOS transcription in the presence of IFN- γ -activated factors and that IL-4 and IL-13 do not work at this level. That the synergistic enhancement of IFN- γ -induced NO production by HSV-2 is mainly due to NF- κ B activation is indicated by our data, showing that staurosporine abolishes the synergistic effect of HSV-2 infection on NO production,

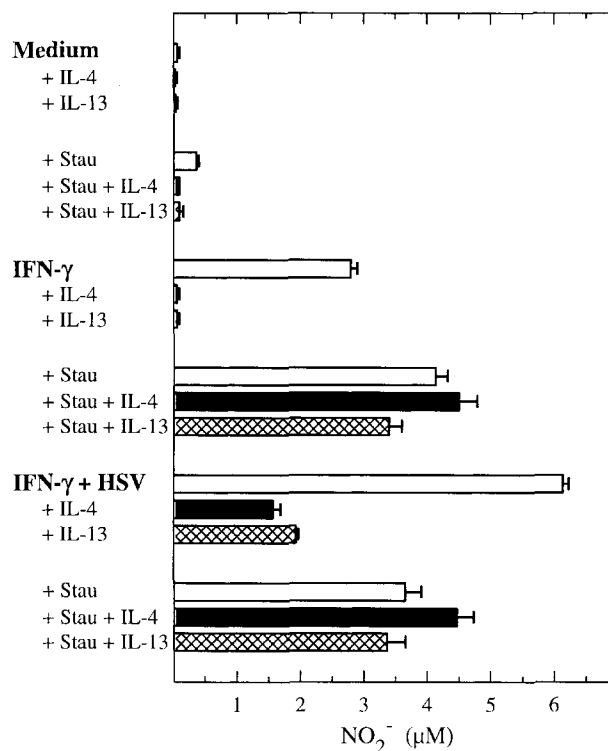


Fig. 4. Effect of staurosporine on the ability of IL-4 and IL-13 to down-regulate IFN- γ -induced NO production in J774A.1 cells. The cells were treated with 100 IU/ml IFN- γ , 100 ng/ml IL-4, 100 ng/ml IL-13 and 20 nM staurosporine (Stau) and infected with 5×10^5 pfu/ml of HSV-2 as indicated. After 24 h the amounts of nitrite in the supernatants were measured. The results are expressed as mean \pm S.E.M. of triplicate cultures. Essentially similar results were obtained in another two experiments.

and by the fact that staurosporine is known to inhibit I- κ B phosphorylation, thus hindering NF- κ B activation [37].

Taken together, our results show that the Th2 cytokines IL-4 and IL-13 completely abrogate the induction of iNOS by the Th1 cytokine IFN- γ and that virus infection of the cells, possibly through autocrine TNF- α secretion, exerts a signal which partially compensates for the action of the Th2 cytokines. The level of action of IL-4/IL-13 is prior to iNOS transcription. We are currently studying in more details the molecular mechanisms underlying the down-regulation, which might give a clue to the immunologically central dichotomy of the relationship between IL-4/IL-13 and IFN- γ . In the context of a virus infection our findings suggest a mechanism whereby the body might down-regulate a harmful systemic NO production while allowing a sustained production of this potential antiviral principle in infectious foci.

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