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Interferon (IFN)-g and Herpes simplex virus/tumor necrosis factor-a synergistically induce nitric oxide synthase 2 in macrophages through cooperative action of nuclear factor-kB and IFN regulatory factor-1

Abstract

The radical nitric oxide (NO) constitutes an important part of the innate immune response to many viruses, and among these notably Herpes simplex virus (HSV).

We have previously shown that HSV/tumor necrosis factor-a (TNF-a) and IFN-g synergistically induce NO production in macrophages, and here we have investigated the molecular mechanism underlying this phenomenon. The enhancement of NO production was regulated at the level of NO synthase 2 (NOS2, iNOS) transcription. The ISRE element of the NOS2 promoter, which binds IFN regulatory factor (IRF)-1, was essential both for full responsiveness to IFN-g and the synergistic response. The GAS motif, binding signal transducer and activator of transcription 1 (STAT1), did not contribute to the cross-talk with virus/TNF-induced signals, but was necessary for full responsiveness to IFN-g. The distal binding site for nuclear factor (NF)-kB was important for the cooperative response, while the proximal kB site was not involved in the cooperative promoter activation but played a role in full promoter inducibility. By ectopic expression of IRF-1 and NF-kB (p65), we found that these factors synergistically induce NO accumulation. Together, our results show that binding of IRF-1 and NF-kB to their respective sites in the distal domain of the NOS2 promoter, creates a potent trans-activating complex with the ability to induce NOS2 transcription synergistically in response to simultaneous HSV-2/TNF-a and IFN-g treatment.

Full text

INTRODUCTION

The first line of defence against viral infections involves auto- and paracrine secretion of cytokines, notably interferons (IFN)s and tumour necrosis factor (TNF)-alpha. The early produced IFN-gamma is important for activation of macrophages [1]. Activated macrophages in turn play a pivotal role in clearance of a number of viral infections [2-5]. Macrophages are endowed with a whole battery of mechanisms to combat infections. These include receptor-mediated phagocytosis, cytokine and chemokine secretion and production of the oxygen and nitrogen radicals superoxide anion and nitric oxide (NO), respectively [6-9]. For a number of viral infections, and among these notably *Herpes simplex* virus (HSV), NO has been shown to exhibit significant antiviral activities [10-16], whereas its role in other infections seems minor [17-19].

Because of the non-selective mechanism of action of NO, it is potentially toxic to the host, as demonstrated by its involvement in the pathogenesis of *e.g.* insulin-dependent diabetes mellitus [20], lymphocytic choriomeningitis [21] and

HSV-1-induced pneumonia and encephalitis [22, 23]. It is thus important for the organism to keep the production of NO under tight control. NO is produced by NO synthase (NOS), of which three isoforms exist [9, 24]. Of these, NOS2/iNOS, is responsible for high out-put NO production, which can be induced in a number of cell types especially macrophages [9]. Lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria is a strong inducer of NOS2 expression, while IFN-gamma alone is able to induce NOS2 accumulation to a more moderate extent [25]. This induction of NOS2 can, however, be markedly enhanced by co-exposure of cells to a number of entities, including TNF-alpha, bacterial DNA, mycobacterial lipoarabinomannan, and dsRNA [26-30]. Studies from our laboratory have shown that HSV-2 infection of murine peritoneal cells and murine macrophage cell lines synergistically augments IFN-gamma-induced NOS2 expression [26, 31].

NOS2 production is mainly regulated at the level of transcription, and functional sites have been characterized in the murine NOS2 promoter [25, 32, 33]. Different laboratories have localized two regions responsible for the inducibility of NOS2 by LPS and IFN-gamma [25, 34, 35]. The proximal region contains binding sites for the transcription factors nuclear factor (NF)-kappaB, NF-IL-6 and Oct-1/2. In the distal region, binding sites for signal transducer and activator of transcription (STAT) and IFN-regulatory factor (IRF) family members are present as well as an NF-kappaB binding site. IRF-1 and its cognate DNA-binding site, termed the IFN-stimulated response element (ISRE), are essential for induction of NOS2 by IFN-gamma [34, 35] and the gamma activation site (GAS), to which STATs bind, is necessary for full inducibility of NOS2 by IFN-gamma [36]. The kappaB site in the proximal region of the NOS2 promoter is critical for LPS-mediated induction of NOS2 [37]. We have previously shown that the ability of HSV-2 infection to synergistically enhance IFN-gamma-induced NOS2 expression in macrophages can be abolished by inhibitors of NF-kappaB activation [26].

It is well known that IFN-gamma works in synergy with a range of other pro-inflammatory molecules, and knowledge of the mechanisms underlying this phenomenon is increasing [38]. IRF-1 and NF-kappaB are necessary for the ability of IFN-gamma and TNF-alpha to synergistically induce transcription of many genes [39, 40]. These two transcription factors interact *in vitro* [41, 42] and are responsible for bending of the NOS2 promoter, which may be an important step in the formation of a functional enhanceosome [43]. STAT1 and

NF-kappaB may also work in concert in the activation of various promoters [44-46], but the mechanisms through which it occurs have not been revealed. In addition to the above-described cooperation between NF-kappaB and IFN-gamma-activated transcription factors, other mechanisms of synergy have been demonstrated in a variety of experimental systems [47-49].

In this study, we have investigated the molecular mechanisms responsible for the ability of HSV-2 infection or TNF-alpha treatment of macrophages to synergistically enhance IFN-gamma-induced NOS2 expression. We show that while STAT1 is important for maximal induction of NOS2 by IFN-gamma, the synergistic response is primarily mediated by cooperation between IRF-1 and NF-kappaB.

METHODS

Cytokines, antibodies and enzymes

Murine recombinant IFN-gamma was obtained from Pharmingen. The antibodies used were: monoclonal mouse anti-STAT1 and monoclonal mouse anti-NOS2 (Transduction Laboratories); polyclonal rabbit anti-STAT6, polyclonal rabbit anti-IRF-1, polyclonal rabbit anti-IRF-2, polyclonal goat anti-IFN consensus sequence binding protein; polyclonal rabbit anti-NF-kappaB p50, polyclonal goat anti-NF-kappaB p65 (all Santa Cruz Biotechnology); HRP-conjugated anti-rabbit immunoglobulin (Transduction Laboratories). The enzymes used in the study were T4 polynucleotide kinase, Taq2000 DNA polymerase (Stratagene) and Expand Reverse Transcriptase (Roche).

Virus and cell cultures

The MS strain of HSV-2 used in these experiments was from a stock with an infectivity titre of 3.2×10^7 PFU/ml and was prepared as described earlier [50]. The murine macrophage cell line RAW 264.7 and the simian kidney fibroblast cell line COS7 were grown in DMEM with 1% Glutamax I (Life Technologies), supplemented with 5% LPS-free fetal calf serum (Hyclone), 200 IU/ml penicillin and 200 mug/ml streptomycin.

Measurement of NO accumulation

RAW 264.7 cell cultures were set up by seeding 1 x 10^5 cells in 150 mul medium in 96-well tissue culture plates. The cells were allowed to settle for 2 hours before stimulation with cytokines and infection with HSV-2, to a final volume of 200 mul per well. Supernatants were harvested 24-36 hours after stimulation/infection and assayed for NO production by measuring nitrite (NO_2^-) concentrations by the Griess assay as described earlier [31].

Nuclear run-on analysis

RAW 264.7 cells were seeded in 25 cm^2 plates at a density of 3.5 x 10^6 cells/plate in 5 ml medium. Six hours post-stimulation/infection, the cells were washed in ice-cold PBS and resuspended in a hypotonic buffer (10 mM HEPES pH 7.6, 25 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) and left for 10 min on ice. NP-40 was added to 0.5% followed by incubation on ice for 5 min and centrifugation at 2,000 x g for 10 min. The resulting nuclear pellet was washed twice in 20 mM Tris-HCl pH 7.6, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 1 mM DTT and resuspended in 200 mul of the same buffer supplemented with ATP, GTP, CTP and UTP (Roche), each at a concentration of 0.5 mM. The mixture was incubated at 30° C for 30 min. followed by RNA isolation by the TriZOL method (Life Technologies). The isolated RNA was reverse transcribed using random hexamer-priming and amplified by PCR with the following primers: NOS2: 5'-CAC ATT CAG ATC CCG AAA CGC TTC ACT TC-3' (sense), 5'-TGG TCC TGG GAG GAG CTG ATG GAG TAG T-3' (anti-sense); beta-actin: 5'-CCA ACC GTG AAA AGA TGA CC-3' (sense), 5'-GCA GTA ATC TCC TTC TGC ATC C-3' (anti-sense).

Preparation of whole cell and nuclear extracts

To prepare whole cell extracts, cells were washed with ice-cold PBS and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton-X 100, 10% glycerol, 0.5 mM EDTA, 0.5 mM PMSF, 0.2 mM leupeptin, 0.2 mM pepstatin A). After 30 min on ice, the lysates were centrifuged at 10,000 x g for 10 min at 4° C and supernatants were isolated. Nuclear extracts were isolated as previously described [26].

Western blotting

Proteins were extracted as described above and subjected to SDS-PAGE followed by blotting onto a PVDF membrane (Novex), and blocking with TBS (10

mM Tris, 140 mM NaCl) supplemented with 0.05% Tween-20 and 5% skimmed milk powder before the primary antibody was added for overnight incubation at 4° C. The membrane was washed 4 times for 10 min in washing buffer (10 mM) Tris, 140 mM NaCl, 0.05% Tween-20) and incubated for 1 hour at room temperature with a polyclonal HRP-conjugated secondary antibody. The membrane was washed as above, plus once in washing buffer without Tween-20. Finally, the HRP-conjugated antibody was visualized using LumiGLO (New England BioLabs).

Immunoprecipitation and Western blotting

The cells were washed in ice-cold PBS and lysed in immunoprecipitation lysis buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 0.2 mM Na_3VO_4, 0.5 mM PMSF). Specific proteins were targeted by mixing 800 mug protein extract with 5 mug of antibody in a total volume of 1 ml immunoprecipitation lysis buffer and incubating for 1 hour at 4° C with rotation. The antigen-antibody complexes were precipitated by adding Protein A/G PLUS-agarose (Santa Cruz Biotechnology) followed by incubation overnight at 4° C with rotation. The precipitate was washed 4 times in lysis buffer and the final pellet was resuspended in SDS-PAGE sample buffer and subjected to Western blotting. Western blotting was performed following a previously described protocol [26].

Electrophoretic mobility shift assay (EMSA)

To assay for DNA-binding activity, 18 mug protein in 4 mul whole cell extract buffer was mixed with 3 mug poly[d(I-C)] and 20,000 cpm [^{32}P] phosphate-labeled probe in 25 mul. The final concentrations for the kappaB assay were: 8.4 mM Tris-HCl, pH 8.0, 20 mM HEPES pH 8.0, 63 mM NaCl, 5.3 mM $MgCl_2$, 0.22% Triton-X 100, 0.7 mM EDTA, 1 mM DTT, 12.2% glycerol. The final concentrations for the GAS assay were: 2.4 mM Tris-HCl, pH 8.0, 20 mM HEPES pH 8.0, 50 mM NaCl, 5.3 mM $MgCl_2$, 0.22% Triton-X 100, 0.5 mM EDTA, 1 mM DTT, 10% glycerol. After 25 min of incubation at room temperature, the reaction mixture was subjected to electrophoresis on a non-denaturing 5% polyacrylamide gel in 0.5 x TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The gel was dried and analyzed by autoradiography. The distal kappaB site of the NOS2 promoter was used as the kappaB probe: 5'-TAG-GGG-GAT-TTT-CCC-CTC-3' while the GAS probe

corresponded to the GAS site of the NOS2 promoter: 5'-CCT-TTT-CCC-CTA-ACA-CT-3'.

Plasmids

To generate the constructs for yeast two-hybrid analysis, fragments corresponding to residue 1-155 of murine IRF-1 and full-length murine p65 were PCR amplified with the high-fidelity polymerase Pfu using the following primers (DNA Technology): IRF-1: 5'-GAA TTC ATG CCA ATC ACT CGA ATG-3' (sense), 5'-GTC GAC TAG AGC TGC TGA GTC CAT-3' (anti-sense); p65: 5'-GAA TTC GAA CCA TGG ACG ATC TGT TT-3' (sense) and 5'-CTC GAG TTA GGA GCT GAT CTG ACT-3' (anti-sense). Restriction sites used for subcloning are underlined. The PCR fragments were cloned into the EcoRV site of pBluescript KS and subcloned into the appropriate sites in pAS2-1 and pACT2 (Clonetech), respectively. The pcDNA-derived vectors for STAT1, IRF-1 and p65 were kind gifts from Drs. Ulrike Schindler, Thomas Maniatis and Tucker Collins, respectively. The NOS2 CAT reporter constructs were provided by Dr. Tom Evans, while the NOS2 luciferase reporter plasmids were donated by Dr. William Murphy.

Transient transfection

RAW 264.7 cells were seeded in 75 cm^2 culture flasks at a density of 15 x 10^6 cells and left for 16 hours. The cells were transfected with reporter plasmid using TransFast (Promega). Briefly, 10 mug of DNA was added to 5 ml of serum-free DMEM and mixed gently. TransFast reagent was added and the mixture was vortexed gently and incubated for 15 min at room temperature. The medium above the cells was exchanged with the DNA-TransFast mixture and transfection was allowed to occur for 1 hour at 37° C. Ten ml of complete medium was added and the cells were left for 5 hours, at which point they were replated onto 10 cm^2 plates at a density of 1.5 x 10^6 cells per plate. In cases where the cells were stimulated/infected subsequent to transfection, this was done 24 hours after re-seeding, followed by a 24 hours incubation period at 37° C before lysing the cells.

Reporter gene assays

To prepare cell lysates for luciferase assay, the cells were lysed in 1 x reporter lysis buffer (Promega) by 2 cycles of freeze-thawing. The lysates were assayed

for luciferase content in a luminometer. For chloramphenicol acetyl transferase (CAT) assay, cells were lysed in 250 mM Tris-HCl pH 7.8 by 2 cycles of freeze-thawing. Equal amounts of protein were incubated for 2 h at 37° C with 0.05 muCi of [^{14}C]-chloramphenicol (Amersham) and 20 muM of acetyl-CoA (Roche) in a total volume of 150 mul. The chloramphenicol and acetylated derivatives were extracted with ethyl acetate and separated by thin layer chromatography followed by exposure to X-ray films.

Yeast two-hybrid assay

Yeast cells (strain Y190, Clonetech) were made competent for transformation by the lithium acetate method. Briefly, cells growing in the exponential phase were harvested by centrifugation (10 min at 2,000 x g), washed in 1 x TE and resuspended in freshly made TE/LiAc buffer (1 x TE, 100 mM lithium acetate). For transformation, plasmid and denatured herring testes carrier DNA (Clonetech) were mixed with competent cells. Six time excess of freshly made PEG/LiAc (1 x TE, 100 mM lithium acetate, 40% PEG 4,000) was added and the suspension was incubated for 30 min at 30° C. The cells were heat-shocked for 15 min at 42° C followed by 2 min on ice. The cells were resuspended in 1 x TE and spread over agar plates of SD/÷TRP/÷LEU-medium. Co-transformed cells were re-streaked on SD/÷TRP/÷LEU-medium agar plates and incubated 3 days at 30° C. A dry Whatman #5 filter was placed on top of the colonies and allowed to wet completely. The cells were permeabilized by one freeze-thaw cycle and the filter paper was placed on a Whatman #5 filter pre-soaked in freshly made X-GAL/Z-buffer (50 mM Na_2HPO_4, 30 mM NaH_2PO_4 pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 0.27% beta-mercaptoethanol, 0.44 mg/ml X-GAL) and incubated at 30° C until the blue color was of appropriate intensity.

RESULTS

HSV-2 infection and IFN-gamma *synergize to induce NOS2 expression*

We have previously shown that HSV-2 infection of IFN-gamma-stimulated mouse peritoneal cells or macrophage cell lines leads to a synergistic enhancement of IFN-gamma-induced NO production [26, 31]. In Figure 1a and b, we confirm these findings in RAW 264.7 cells and show that the synergy is observed over a wide range of concentrations of IFN-gamma and HSV-2. At high virus concentrations (3 x 10^6 PFU/ml), the synergistic effect of virus infection on IFN-gamma-induced

NO production was impaired. This finding correlated with a strong viral cytopathic effect.

To examine the time course of NOS2 protein accumulation after viral infection and IFN-gamma stimulation, RAW 264.7 macrophages were harvested at various time points after treatment and whole cell extracts were prepared. The levels of NOS2 were determined by Western blotting. As seen in Fig. 1c, a low constitutive level of NOS2 protein was observed, which was not appreciably affected by HSV-2 infection. IFN-gamma treatment induced accumulation of NOS2 protein with a noticeable enhancement after 8 hours of treatment. HSV-2 infection synergistically augmented the IFN-gamma-induced NOS2 levels with an effect demonstrated at 4 hours, and with further accumulation throughout the 18 hours of the experiment. In separate experiments we have observed that NOS2 protein levels remain high at 24 and 36 hours post treatment (Paludan, unpublished).

A number of reports have shown that NOS2 induction by various stimuli is regulated at the level of transcription [25, 43, 51]. In order to examine whether the synergistic effect of HSV-2 infection on IFN-gamma-dependent NOS2 expression is regulated in a similar fashion we performed nuclear run-on analysis on nuclei from cells stimulated and infected for 6 hours. As seen in Fig. 1d, we did not detect NOS2 mRNA production in nuclei from untreated cells, despite a high level of beta-actin expression. IFN-gamma treatment *per se* activated NOS2 transcription to some extent, while concomitant HSV-2 infection strongly enhanced the IFN-gamma-induced response. Thus, HSV-2 infection of IFN-gamma-activated macrophages potentiates NO synthesis over a wide range of virus and cytokine concentrations through enhanced NOS2 transcription.

*HSV-2 and IFN-*gamma *synergistically induce NF-*kappa*B activation*

We have previously shown that various NF-kappaB inhibitors can abolish the cooperative effect of HSV-2 infection on IFN-gamma-dependent NOS2 expression [26], and others have shown that both STAT1 and IRF-1 contribute to the IFN-gamma-induced response [34, 36]. To explore how the DNA-binding of these transcription factors to their respective sites in the murine NOS2 promoter was affected by viral infection and IFN-gamma treatment, we stimulated and infected RAW 264.7 cells for different time intervals, prepared whole cell extracts and performed EMSA. As seen in Figure 2a (upper panel), IFN-

gamma induced a low and transient, yet reproducible, binding to the distal kappaB site of the NOS2 promoter. Following HSV-2 infection however, the binding was not induced until between 2 and 5 hours post-infection, after which it remained elevated past 9 hours and returned to basal levels 24 hours after infection. Interestingly, co-treatment with IFN-gamma and HSV-2 enhanced the level of kappaB-binding activity with peak levels at 9 hours of treatment. Moreover, the kinetics of the binding was also changed, with bands detectable already after 2 hours of treatment and the kappaB-binding activity remaining elevated for 24 hours. We have previously shown that the upper complex contains the NF-kappaB subunits p50 and p65 while the lower complex contains p50 [26].

GAS-binding activity was strongly and rapidly induced by IFN-gamma (Figure 2a, lower panel). The highest level was observed after 30 min of IFN-gamma stimulation, after which the intensity of the band slowly decreased. HSV-2 infection did not affect the GAS-binding activity of untreated or IFN-gamma-stimulated RAW 264.7 cells. Super-shift analysis revealed that the GAS-binding complex contained STAT1 but not STAT6 (data not shown).

When testing for ISRE-binding activity, we were unable either to detect induction of IRF-1 binding following IFN-gamma treatment, or to super-shift any of the constitutive bands with antibodies against IRF-1, IRF-2 and ICSBP. This was the case using either whole cell extracts or nuclear extracts despite the implementation of various previously published protocols [35, 52, 53]. However, given the convincing literature about the role of IRF-1 in NOS2 induction [34, 35], we chose to determine the levels of IRF-1 protein in nuclear extracts by Western blotting. Untreated RAW 264.7 cells contained a very low level of IRF-1 that was marginally increased following HSV-2 infection (Figure 2b). IFN-gamma induced IRF-1 expression, with a delayed kinetics relative to the activation of STAT1, which is consistent with the STAT1-dependent nature of the IRF-1 induction by IFN-gamma and parallels results from earlier studies [52, 54, 55]. The IRF-1 accumulation peaked after 2 hours and slowly decreased to basal levels. Co-infection with HSV-2 did not change this profile.

Altogether, these results show that IFN-gamma in a manner not affected by HSV-2, induces binding of STAT1 to the GAS site in the NOS2 promoter, and accumulation of IRF-1 in the nucleus of macrophages. Moreover, binding of NF-kappaB to the distal kappaB site in the murine NOS2 promoter is increased in

intensity and time in macrophages treated with both IFN-gamma and HSV-2, when compared with cells receiving only one of the treatments.

The ISRE element but not the GAS motif is essential for synergistic activation of the NOS2 promoter by HSV-2/TNF-alpha *and IFN*-gamma

Mice deficient in IRF-1 are unable to express NOS2 in response to IFN-gamma [34], and mutation of the ISRE element impairs the inducibility of the NOS2 promoter by IFN-gamma [36, 51]. In a similar fashion, the activity of a NOS2 promoter with a non-functional GAS motif is not fully inducible by IFN-gamma [36, 51]. We thus wanted to investigate how these two sites were involved in cooperation with HSV-2/TNF-alpha-induced signals. For this purpose, NOS2 promoter luciferase constructs were transiently transfected into RAW 264.7 cells, and promoter activation by IFN-gamma and HSV-2 was examined. While IFN-gamma was able to augment luciferase activity 3-fold in cells transfected with the wild type promoter (Figure 3), HSV-2 infection of IFN-gamma-stimulated RAW 264.7 cells led to a strong cooperative activation of the reporter gene. When testing the NOS2 promoter mutated in the GAS motif, we observed, in agreement with previous reports [36, 51], that the activation by IFN-gamma was compromised. This was also associated with a lower level of luciferase activity after simultaneous HSV-2 infection, as compared with the wild type promoter. Interestingly, however, HSV-2 was still able to synergize strongly with IFN-gamma. This was in marked contrast to the behavior of the NOS2 promoter mutated in the ISRE element. While the promoter activation by IFN-gamma was reduced to the same extent as with the promoter mutated in the GAS motif, the effect of HSV-2 infection was almost abolished. In another set of experiments, the cells were stimulated with TNF-alpha instead of virus infection and activation of the three promoter constructs were analyzed. The results obtained (here expressed as degree of synergism) were highly similar to those obtained with HSV-2: wt, 6.9; mGAS, 6.0; mISRE, 2.4.

These results thus indicate that while the GAS motif is essential for maximal expression of NOS2, the ability of IFN-gamma-induced factors to cooperate with TNF-alpha/HSV-2-activated signals is dependent on a functional ISRE element.

Infection of RAW 264.7 cells 5 hours after IFN-gamma *treatment does not affect the synergistic effect on NO production*

Given the results above, IRF-1 rather than STAT1 seems to be responsible for mediating the cooperation with virus-activated signals. To test this further, we treated RAW 264.7 cells with IFN-gamma and infected them with HSV-2, either simultaneously or 5 hours after IFN-gamma stimulation. At this time point the IFN-gamma-induced STAT1 activity has waned (Figure 2), while the IRF-1 levels remain elevated for several hours. As seen in Figure 4, postponing the HSV-2 infection by 5 hours relative to IFN-gamma challenge did not significantly affect the synergism between IFN-gamma and HSV-2. This indicates that generation of a synergistic response does not require overlapping STAT1 activation and HSV-infection.

The distal kappa*B element in the NOS2 promoter is essential for maximal synergism between HSV-2/TNF*-alpha *and IFN*-gamma

Inhibitors of NF-kappaB activation abolish the synergy between IFN-gamma and HSV-2 infection in NOS2 induction [26]. To test more thoroughly the involvement of NF-kappaB in the synergy and to investigate how the two kappaB sites in the NOS2 promoter were involved in this process, we performed reporter gene analysis with NOS2 promoters mutated in the kappaB sites. In agreement with the results shown in Figure 3, IFN-gamma and HSV-2 cooperatively activated the wild type NOS2 promoter (Figure 5). Mutation of the proximal kappaB site reduced the inducibility of the promoter moderately in response to IFN-gamma in infected and uninfected cells. The cooperation between IFN-gamma and HSV-2, however, was not altered by the disruption of the proximal kappaB site. A more pronounced effect was found when the NOS2 promoter, mutated in the distal kappaB motif, was used. In this case, the ability of IFN-gamma to induce reporter gene expression was strongly impaired and the co-operative effect of concomitant HSV-2 infection was also affected. When both kappaB sites were mutated, responsiveness to IFN-gamma and HSV-2 was lost.

These results suggest that IFN-gamma-activated factors cooperate with constitutive and HSV-2/TNF-gamma-inducible NF-kappaB family members to induce NOS2 expression and that notably the distal kappaB site in the NOS2 promoter is responsible for this effect.

Physical interaction between NF-kappa*B (p65) and IRF-1*

It has previously been demonstrated that bacterially expressed IRF-1 and NF-kappaB subunits can interact physically [41, 42]. In order to look for interactions between the transcription factors when expressed by mammalian cells COS7 cells were transfected with STAT1, IRF-1 and the NF-kappaB subunit p65, respectively. The cell lysates were subjected to immunoprecipitation followed by Western blotting. When using a specific antibody against p65 for detection, we observed that the NF-kappaB subunit was detected in samples immunoprecipitated with antibodies against p65 and IRF-1, but not by antibodies against STAT1 (Figure 6a, upper panel). Likewise, we observed that IRF-1 was found in immunoprecipitates performed with antibodies against IRF-1 and p65, but not in anti-STAT1 immunoprecipitates (Figure 6a, middle panel), although they did contain the specific STAT1 protein (Figure 6a, lower panel).

To characterize the interaction between IRF-1 and p65 further, we performed yeast two-hybrid assay using constructs where the DNA-binding domain of GAL4 was fused to the amino-terminal 155 residues of IRF-1 and the *trans*-activating domain of GAL4 was fused to full-length p65. Yeast cells were transformed with the constructs and the GAL4-driven reporter gene beta-galactosidase was used as a measure of interaction between the co-expressed proteins. As seen in Figure 6b co-expression of the fusion proteins containing p65 and IRF-1 led to a strong beta-galactosidase activity in contrast to the control transformations.

Overexpression of p65 and IRF-1 synergistically induces NO production

To investigate if NF-kappaB and IRF-1 are sufficient to mediate cooperative NOS2 induction, we transiently transfected RAW 264.7 cells with expression constructs encoding these transcription factors and measured the accumulation of nitrite in the supernatant. As seen from Figure 7, transfection with empty vector induced significant amounts of nitrite. Expression of p65 or IRF-1 alone enhanced the NO production above the levels induced by empty vector. When co-expressing p65 and IRF-1, nitrite accumulation was further enhanced to a level that was beyond the additive effect of IRF-1 and p65 alone (degree of synergism, 2.3). Thus, ectopically expressed p65 and IRF-1 synergistically induce NO production in RAW 264.7 cells.

DISCUSSION

An effective host defence against HSV infections is dependent on multiple cell types, with macrophages playing a pivotal role [2, 5]. One of the main effector mechanisms of macrophages is the production of the free radicals superoxide anion and NO [8, 9]. Over the past few years, NO has been demonstrated to inhibit replication of a large number of viruses, among these HSV [10-16]. We have previously demonstrated that HSV-2 infection synergistically enhances IFN-gamma-induced NOS2 expression and NO synthesis in macrophages [26, 31].

In the present study, we show that the ability of HSV-2 infection to synergistically enhance IFN-gamma-induced NOS2 expression in RAW 264.7 cells involves cooperative action of the transcription factors NF-kappaB and IRF-1, while STAT1 is essential for maximal NOS2 expression but not for the synergy. This conclusion is supported by several pieces of evidence. (i) Mutation of the ISRE element in the NOS2 promoter abolished the synergistic induction, whereas mutation of the GAS motif still allowed a substantial degree of synergy. (ii) A reporter construct where the proximal kappaB site of the NOS2 promoter was rendered inactive, was inducible to a lesser extent, but displayed an unaltered degree of synergy in response to HSV-2 and IFN-gamma. Inactivation of the distal kappaB site hampered the inducibility of the promoter as well as the synergy. (iii) The kinetics of NF-kappaB DNA-binding activity following HSV-2 infection showed a high degree of overlap with the presence of IRF-1, in the nucleus of IFN-gamma-treated macrophages. (iv) Infection of RAW 264.7 cells, after STAT1 activation had waned, did not affect the synergistic effect of HSV infection on NO production. (v) Overexpression of IRF-1 and p65 resulted in synergistic induction of NO production.

Synergistic induction of proteins involved in innate and acquired immunity has been described for many pro-inflammatory agents [38]. In the majority of cases the synergy is at least partly regulated at the transcriptional level. In recent years, the molecular biology of this synergy has received increasing attention, and molecular models have been proposed. Most genes induced synergistically by TNF-alpha/LPS and IFN-gamma, are driven by promoters with kappaB sites alongside ISRE or GAS motifs, and it has been demonstrated that these sites, as well as the associated transcription factors, are essential for the cooperative gene induction [34, 40, 45, 46, 56]. In addition, other mechanisms influence a synergistic response between IFN-gamma and proinflammatory agents. For instance, enhanced phosphorylation of STAT1 on serine 727 in response to LPS

[47], increased Jak2 activation by IFN-gamma following co-stimulation with TNF-alpha [48] and IFN-gamma-mediated potentiation of NF-kappaB activation by TNF-alpha [49].

The data presented in this study show that cooperation between IRF-1 and NF-kappaB is a major contributor to the synergistic expression of NOS2 by IFN-gamma and HSV-2. By contrast, STAT1 did not contribute significantly to generation of the synergistic response, but was involved in establishing maximal NOS2 induction in response to IFN-gamma, in agreement with previous reports [36]. This could be due to the observation that there is only a narrow time window where both STAT1 and NF-kappaB are active in IFN-gamma-treated macrophages infected with HSV-2, whereas the kinetics of IRF-1 and NF-kappaB are much more overlapping. Moreover, using yeast two-hybrid assays and immunoprecipitation we have found that IRF-1, but not STAT1, is able to interact with the NF-kappaB subunit p65. This may imply that IRF-1 can exert a stronger cooperation with NF-kappaB than STAT1. A recent study from Saura *et al.* [43] has provided evidence supporting the hypothesis that physical interaction between IRF-1 and NF-kappaB is indeed involved in the formation of a functional enhanceosome on the NOS2 promoter. In that study, it was shown that interaction between IRF-1 and NF-kappaB on the NOS2 promoter led to bending of the promoter as determined by a cyclization assay. Promoter bending and the resulting dense packing of multiple transcription factors, is believed to be an important step in the formation of enhanceosomes able to initiate high out-put transcription [57].

The observation that IFN-gamma *per se* induced low levels of NF-kappaB in macrophages has not been reported previously, but IFN-gamma has been shown to augment activation of NF-kappaB in response to LPS and TNF-alpha [49, 58]. The growth medium and serum used in our studies did not contain detetable levels of LPS (data not shown), and the IFN-gamma preparation contained less that 200 pg/ml LPS, giving a maximum possible level of 2 pg/ml at the dilution used in our studies. This argues against LPS being responsible for the phenomenon, but we cannot formally rule out that trace amounts of LPS or other compounds present in the growth medium or the IFN-gamma preparation cooperate with IFN-gamma to induce low levels of NF-kappaB. Recent data have shown that activation of NF-kappaB by LPS plus IFN-gamma, in murine macrophages is sensitive to inhibition of the IkappaB kinase (IKK) by cyclopentone prostaglandins [59]. This suggests that IFN-gamma-dependent

signaling to NF-kappaB involves IKK. When IFN-gamma was given to HSV-2-infected macrophages, NF-kappaB activation was increased in time and intensity relative to what was seen in cells receiving IFN-gamma or HSV-2 alone. Although this is as yet not explained mechanistically, a couple of possibilities can be suggested. Firstly, a previous study from our laboratory has shown that IFN-gamma synergistically enhances virus-induced secretion of TNF-alpha [26], which in turn could be responsible for the activation of NF-kappaB. Secondly, it has been reported that while TNF-alpha stimulation of pre-neuronal cells was associated only with degradation of the NF-kappaB inhibitor IkappaBalpha, co-stimulation with IFN-gamma also led to degradation of IkappaBbeta, resulting in a sustained NF-kappaB activation [49]. Which, if any, of these mechanisms are responsible for the observed effect is currently being investigated in our laboratory.

The ability of many viruses and viral products to induce NOS2 expression in macrophages has been reported [30, 60-63]. Moreover, many virus infections activate NF-kappaB and it has been demonstrated that the effect of some viruses and viral products on NOS2 expression is NF-kappaB-dependent [26, 30, 60]. Thus, the findings of the present study may represent the molecular mechanism employed by most viruses to enhance NOS2 expression.

In previous studies we showed that the synergy between HSV-2 and IFN-gamma is dependent on autocrine TNF-alpha secretion [26, 31], and that the effect of HSV-2 infection on NO secretion can be mimicked by addition of exogenous TNF-alpha [55]. Interestingly, very similar observations have been done in the *Leishmania major* infection model. Nacy and associates showed that IFN-gamma-treated macrophages could kill *L. major* amastigotes through an NO-dependent pathway, and that this was dependent on autocrine TNF-alpha secretion [64]. These two sets of observations illustrate an interesting principle that may have physiological implications: macrophages recruited to the site of infection secrete TNF-alpha locally which alone does not affect NO production but together with IFN-alpha allows high out-put of the anti-microbial and cytotoxic radical. The requirement for dual stimulation to obtain high expression levels of NOS2 may be a means to keep NO production under tight control.

CONCLUSION

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Figures

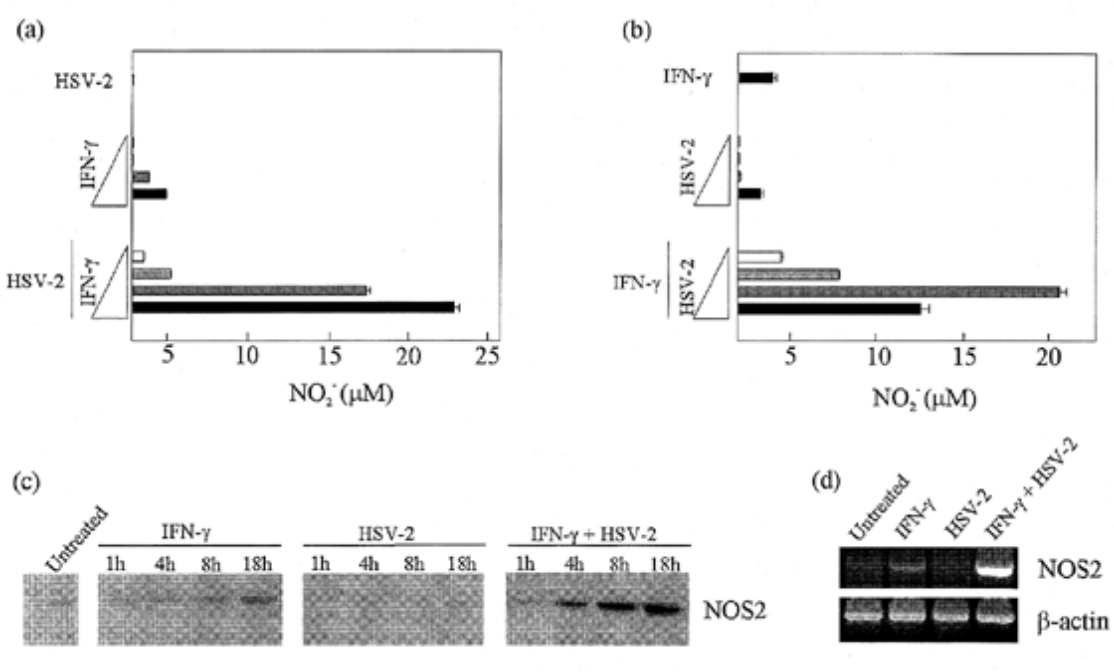


Figure 1

Enhancement of NO synthesis and NOS2 expression in HSV-2-infected RAW 264.7 cells stimulated with IFN-gamma.

For measurement of NO, the cells were stimulated with **(a)** 0.1, 1.0, 10 and 100 IU/ml of IFN-gamma and infected with 3×10^5 PFU/ml of HSV-2 or **(b)** stimulated with 100 IU/ml of IFN-gamma and infected with 3×10^3, 3×10^4, 3×10^5 and 3×10^6 PFU/ml of HSV-2. Supernatants were harvested after 36 hours and nitrite content was

measured. © Whole cell extracts from RAW 264.7 cells treated with 100 IU/ml of IFN-gamma and 3 x 10^5 PFU/ml of HSV-2 for different time intervals were tested for the presence of NOS2 protein by Western blotting. Thirty micrograms of protein were loaded to each lane. **(d)** Nuclei from cells treated for 6 hours with 100 IU/ml of IFN-gamma and 3 x 10^5 PFU/ml of HSV-2 were isolated and subjected to nuclear run-on analysis. The RNA levels were assessed by RT-PCR.

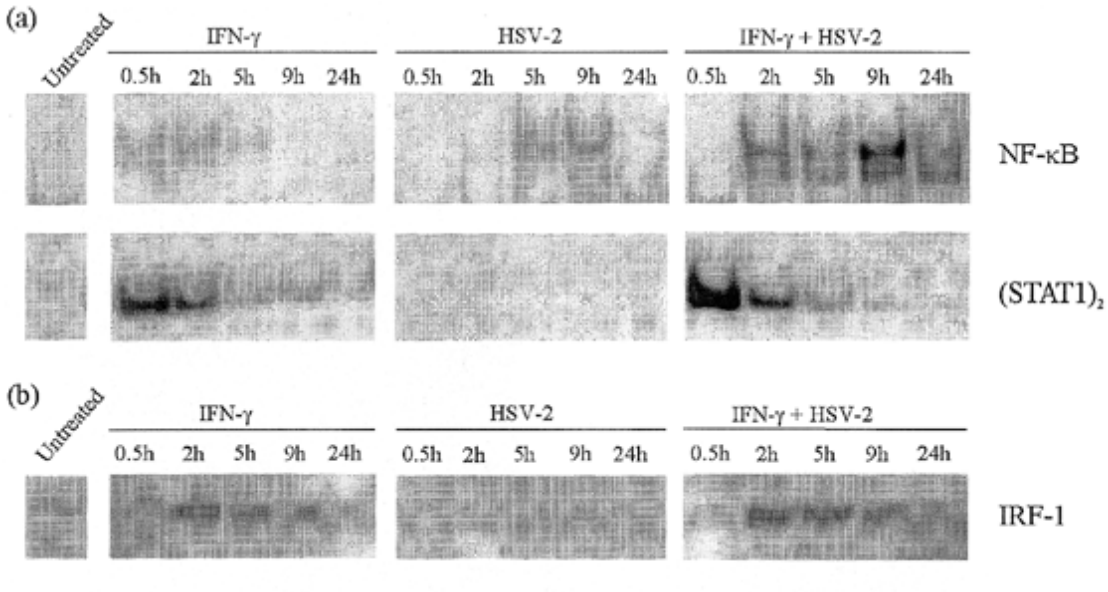


Figure 2

Activation and induction of transcription factors involved in NOS2 transcription.

(a) RAW 264.7 cells were stimulated with 100 IU/ml of IFN-gamma and infected with 3 x 10^5 PFU/ml of HSV-2. At the indicated time points after stimulation/infection whole cell extracts were prepared and the DNA-binding activity of NF-kappaB and STAT1 was assayed. The distal kappaB site and the GAS element of the murine NOS2 promoter were used as probes. (**b**) IRF-1 levels were determined in

nuclear extracts from cells treated as above. The nuclear proteins were subjected to SDS-PAGE and Western blotting using a specific antibody against IRF-1.

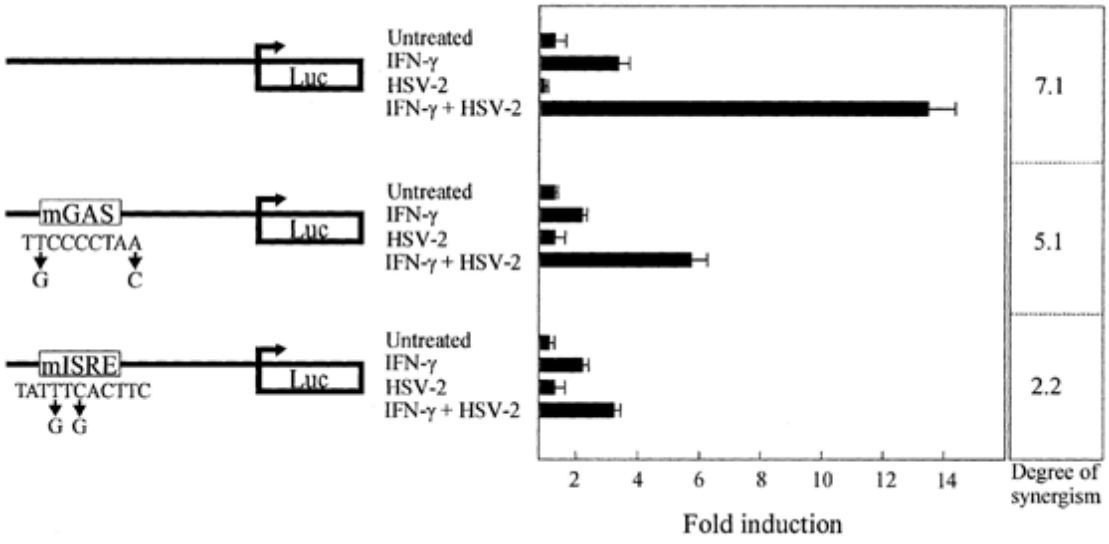


Figure 3

Involvement of the GAS motif and the ISRE element in NOS2 transcription in response to IFN-gamma and HSV-2.

RAW 264.7 cells were transfected with the wild type, GAS-mutated (mGAS) or ISRE-mutated (mISRE) NOS2 promoter constructs. Five hours later, the cells were re-seeded and left for 24 hours, followed by stimulation with 100 IU/ml of IFN-gamma and infection with 3×10^5 PFU/ml of HSV-2. Twenty-four hours later, the cells were lysed and assayed for luciferase activity. The degree of synergism was calculated by dividing the luciferase activity in cells receiving IFN-gamma and HSV-2 by the sum of the activities in cells given IFN-gamma or HSV-2. Prior to calculation, the luciferase activity in untreated transfected cells was subtracted. The mutations introduced are indicated by arrows.

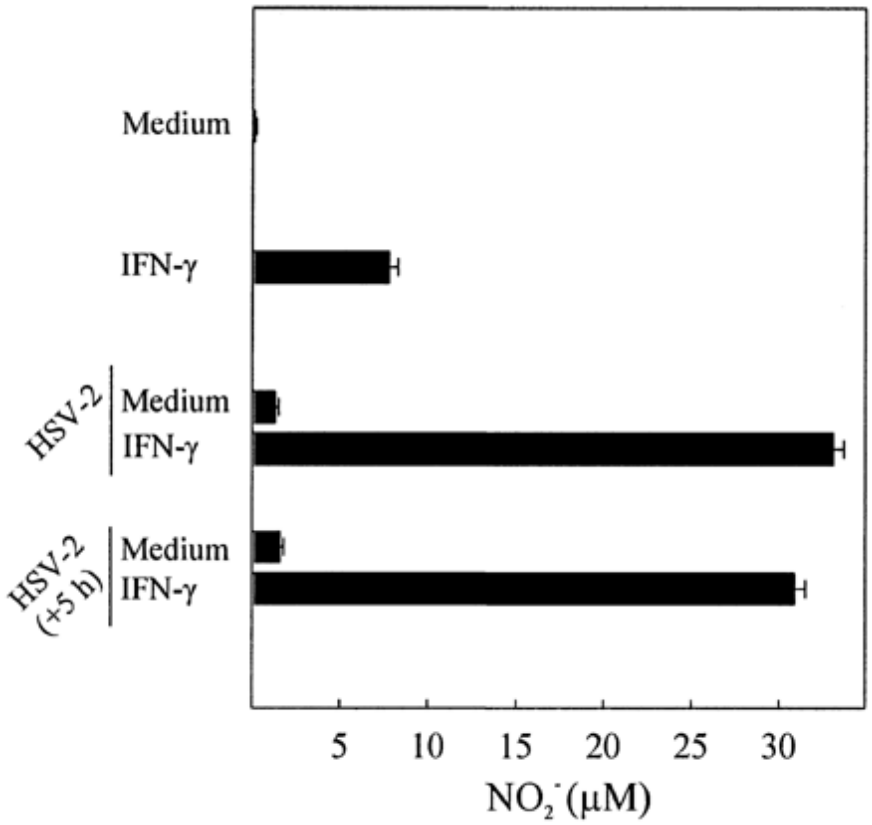


Figure 4

Effect of delayed HSV-2 infection on IFN-gamma-induced NO synthesis.

RAW 264.7 cells were stimulated with 100 IU/ml of IFN-gamma and infected with HSV-2 (3×10^5 PFU/ml), either at the time of IFN-gamma treatment or 5 hours later. Thirty-six hours after IFN-gamma stimulation, the supernatants were harvested and nitrite contents were measured.

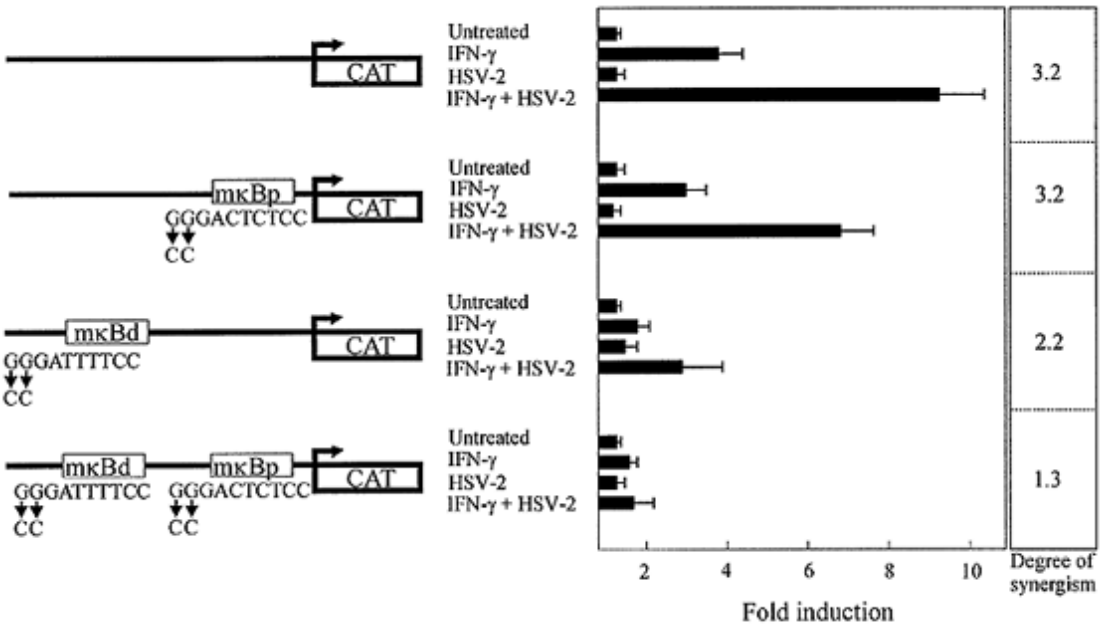


Figure 5

Involvement of the kappaB motifs in NOS2 trancription in response to IFN-gamma and HSV-2.

RAW 264.7 cells were transfected with wild type or mutated proximal (mkappaBp) and/or distal (m(Bd) (kappaB NOS2 promoter constructs. Five hours later, the cells were re-seeded and left for 24 hours, followed by stimulation with 100 IU/ml of IFN-gamma and infection with 3×10^5 PFU/ml of HSV-2. Twenty four hours later the cells were lysed and assayed for CAT activity. Degree of synergism was calculated by dividing the CAT activity in cells receiving IFN-gamma and HSV-2 with the sum of the activities in cells given IFN-gamma or HSV-2. Prior to calculation, the CAT activity in untreated transfected cells was subtracted. The mutations introduced are indicated by arrows.

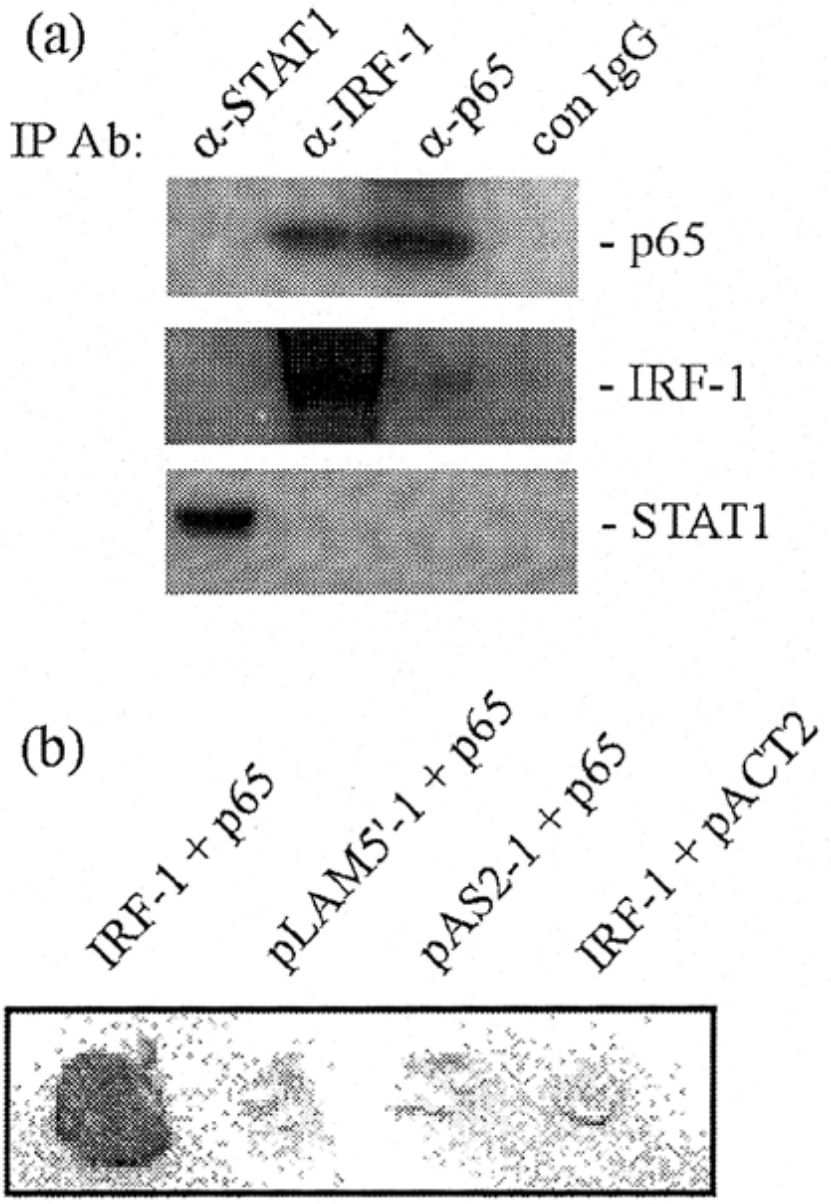


Figure 6

Interactions between STAT1, IRF-1 and p65.

a: COS7 cells were transfected with expression constructs encoding STAT1, IRF-1 and p65, respectively. The COS7 cells were lysed 32 hours after transfection and the lysates were combined in the following manner (left to right), Upper panel: p65 lysate was mixed with STAT1 lysate, IRF-1 lysate or immunoprecipitation buffer; Middle panel: IRF-1 lysate was mixed with STAT1 lysate, immunoprecipitation buffer or p65 lysate; Lower panel:

STAT1 lysate was mixed with immunoprecipitation buffer, IRF-1 lysate or p65 lysate. Specific proteins were immunoprecipitated with 5 mug of antibody and a protein-A/G-agarose mixture. The precipitates were separated by SDS-PAGE and proteins were detected by Western blotting. **b:** Yeast strain Y190 cells were transformed with constructs encoding either the DNA-binding domain of GAL4 (pAS2-1) alone or fused to laminin (pLAM5'-1) or the amino-terminal 155 residues of IRF-1 and co-transformed with constructs encoding the *trans*-activating domain of GAL4 (pACT2) alone or fused to p65. The transformed cells were assayed for beta-galactosidase activity, used as a measure of interaction between the proteins encoded by the yeast expression constructs.

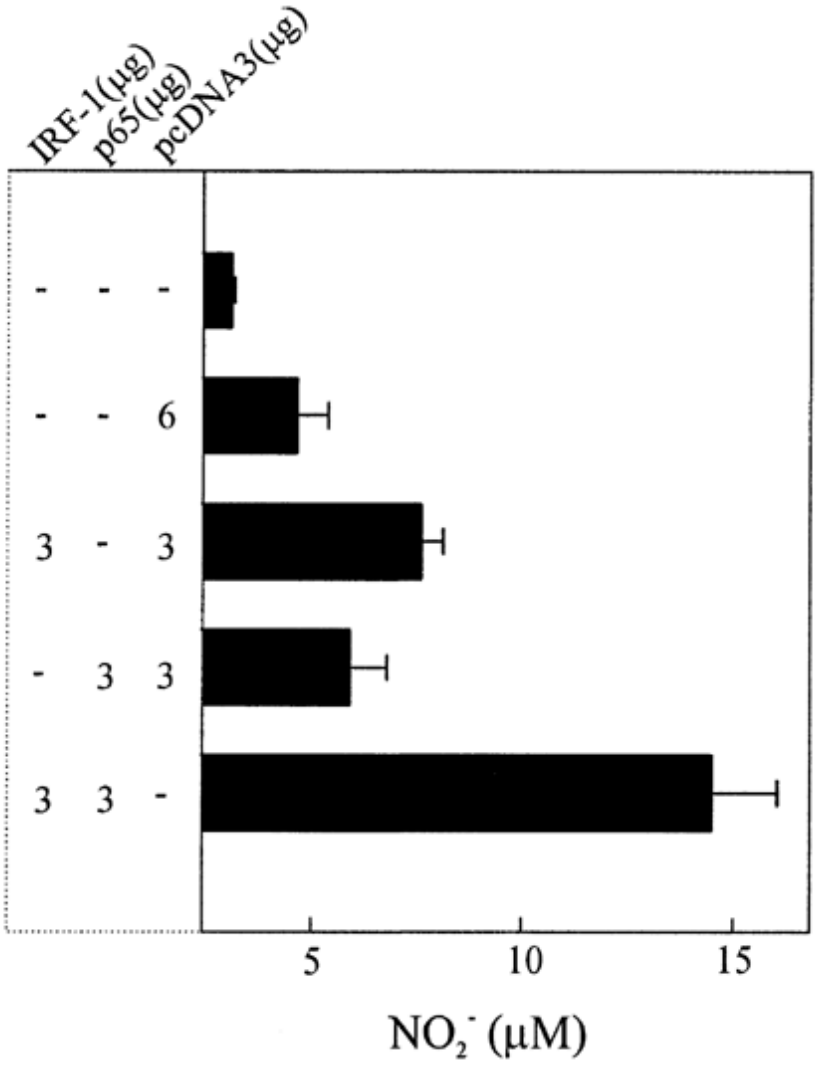


Figure 7

Synthesis of NO in RAW 264.7 cells expressing IRF-1 and p65 ectopically.

RAW 264.7 cells were transfected with the indicated amounts of pcDNA or derived constructs encoding IRF-1 or p65, and left for 6 hours after which time the overlaying medium was changed. Eighteen hours later the medium was changed again and the cells were incubated for another 24 hours. Nitrite levels in the supernatants were then quantified.

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In this issue

Regulation of helper T cell responses to staphylococcal superantigens

S.B. Cameron, M.C. Nawijn, W.W.S. Kum, H.F.J. Savelkoul, A.W. Chow
cytokines, Th1, Th2, Tr1, IL-10, staphylococcal superantigens.

Fibronectin increases the migration induced by stromal cell-derived factor-1a (SDF-1a) in pre-B acute lymphoblastic leukemia cells

E. Sbaa-Ketata, M. Vasse, B. Lenormand, P. Schneider, C. Soria, J.P. Vannier
acute lymphoblastic leukemia, SDF-1a, CXCR-4, VLA-4, VLA-5, fibronectin.

Hematopoietic growth factors in patients receiving intensive chemotherapy for malignant disorders: studies of granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and Flt-3 ligand (Flt3L)

O. Bruserud, B. Foss, H. Petersen
chemotherapy, acute leukemia, G-CSF, GM-CSF, IL-3, Flt3L.

Can interleukin-2 reverse anthracyclin chemoresistance in metastatic soft tissue sarcoma patients. Results of a prospective phase II clinical trial

G. Gravis, M. Mousseau, J.Y. Douillard, T. Dorval, M. Fabbro, B. Escudier, L. Mignot, P. Viens
immunotherapy, soft tissue sarcoma, interleukin-2, chemoresistance, anthracyclin.

Activation of two distinct anti-proliferative pathways, apoptosis and p38 MAP kinase-dependent cell cycle arrest, by tumor necrosis factor in human melanoma cell line A375

T. Hattori, H. Hayashi, T. Chiba, K. Onozaki
melanoma, tumor necrosis factor, apoptosis, cell cycle arrest, p38 MAPK.

Impaired interleukin-5 (IL-5) production by T cells as a prognostic marker of disease progression in human immunodeficiency virus type 1 (HIV-1)-infected children

S. Resino, S. Sanchez-Ramon, J.M. Bellon, J.L. Jimenez, M.A. Munoz-Fernandez
HIV-infected children, cytokine production, interleukin-5, PBMC, prognostic markers.

Cytokine-mediated inflammatory hyperalgesia limited by interleukin-13

B.B. Lorenzetti, S. Poole, F.H. Veiga, F.Q. Cunha, S.H. Ferreira
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thrombopoietin, umbilical vein endothelial cells.

Production of cytokines by immature erythroid cells derived from human embryonic liver

S.V. Sennikov, S.V. Krysov, T.V. Injelevskaya, A.N. Silkov, V.A. Kozlov
erythroid progenitor, IL-1b, IL-2, IL-4, IL-6, fetal liver.

Inflammatory cytokine production in interferon-g-primed mice, challenged with lipopolysaccharide. Inhibition by SK&F 86002 and interleukin-1b-converting enzyme inhibitor

S. Spinelle-Jaegle, P. Devillier, S. Doucet, S. Millet, C. Banissi, A. Diu-Hercend, E. Ruuth
interferon-g, lipopolysaccharide, interleukin-1b, cytokine, interleukin-1b-converting enzyme inhibitors.

The effect of Sambucol, a black elderberry-based, natural product, on the production of human cytokines: I. Inflammatory cytokines

V. Barak, T. Halperin, I. Kalickman
Sambucol, inflammatory cytokines, IL-1b, TNF-a, IL-6, IL-8.

Interferon (IFN)-g and Herpes simplex virus/tumor necrosis factor-a synergistically induce nitric oxide synthase 2 in macrophages through cooperative action of nuclear factor-kB and IFN regulatory factor-1

S.R. Paludan, L. Malmgaard, S. Ellermann-Eriksen, L. Bosca, S.C. Mogensen
macrophages, NOS2, Herpes simplex virus, NF-kB, IRF-1.

Negative regulation of onconstatin M signaling by suppressor of cytokine signaling (SOCS-3)

F. Magrangeas, O. Boisteau, S. Denis, Y. Jacques, S. Minvielle
cytokine signaling, inhibition, Jak-STAT pathway.

Increased expression of interleukin-8 and aminopeptidase N by cell-cell contact: interleukin-8 is resistant to degradation by aminopeptidase N/CD13

A. Kehlen, I. Egbert, K. Thiele, K. Fischer, D. Riemann, J. Langner
cell co-culturing, leucine aminopeptidase, plasma membrane preparations, CD13, proteolysis of IL-8, interleukin-8.

Functional characterization of the mouse lymphotoxin-beta receptor promoter

P. Muller, D.N. Männel, T. Hehlgans
LTbR-promoter, transcriptional activation, dexamethasone.

Glucose potentiates interleukin-1b (IL-1b)-induced p38 mitogen-activated protein kinase activity in rat pancreatic islets of Langerhans

A.M.E. Sprinkel, N.A. Andersen, T. Mandrup-Poulsen
interleukin-1b, p38, ERK1/2, JNK, nitric oxide, diabetes mellitus.

Sustained parasite burden in the spleen of Leishmania infantum-infected BALB/c mice is accompanied by

expression of MCP-1 transcripts and lack of protection against challenge

D. Rousseau, S. Demartino, F. Anjuère, B. Ferrua, K. Fragaki, Y. Le Fichoux, J. Kubar
murine leishmaniasis, acquired immunity, inflammation, chemokines, spleen cell subpopulations.

Effects of cytokines and prolactin on the replication of Toxoplasma gondii in murine microglia

N. Benedetto, A. Folgore, C. Romano-Carratelli, F. Galdiero
T. gondii, cytokine, PRL, MGC.

Strip test for bedside detection of interleukin-6 in cervical secretions is predictive for impending preterm delivery

H. Trébéden, F. Goffinet, G. Kayem, F. Maillard, E. Lemoine, D. Cabrol, B. Weill, F. Batteux
cytokine, IL-6, prematurity, cervicovaginal secretions, strip test.

Serum levels of interleukin-18 in patients with uncomplicated Plasmodium falciparum malaria

D. Torre, M. Giola, F. Speranza, A. Matteelli, C. Basilico, G. Biondi
interleukin-18, malaria, Plasmodium falciparum, gamma interferon.

The ecdysone inducible gene expression system: unexpected effects of muristerone A and ponasterone A on cytokine signaling in mammalian cells

S. Constantino, R. Santos, S. Gisselbrecht, F. Gouilleux
ecdysone, PI 3-kinase, AKT, survival.

Presence of estrogen receptors in human myeloid monocytic cells (THP-1 cell line)

M. Cutolo, B. Villaggio, A. Bisso, A. Sulli, D. Coviello, J.M. Dayer
THP-1 cells, macrophages, estrogen receptors, estrogens, monocytes.

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