



INHERITED DISEASE

RESEARCH ARTICLE

Anti-inflammatory action of type I interferons deduced from mice expressing interferon β

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Type I interferons (IFN) are widely used for the therapeutic treatment of viral infections, tumor growth and various chronic diseases such as multiple sclerosis. Antagonism between type I IFNs and IFN- γ has been described in cells of the immune system, in particular in the activation of macrophages. To study the systemic effects of type I IFNs we used transgenic mice carrying a human IFN- β (hIFN- β) gene under the control of the rat insulin I promoter. These animals expressed high levels of hIFN- β in β -pancreatic cells, and the ability of the macrophages to respond to pro-inflammatory stimuli was analyzed. Transgenic mice exhibited an increased extravasation of cells to the peritoneal cav-

ity after eliciting with thioglycollate broth. The expression of the inducible form of nitric oxide synthase and cyclooxygenase-2, two enzymes involved in inflammation, was impaired in transgenic animals challenged with lipopolysaccharide and IFN- γ . Analysis of the mechanisms leading to this attenuated inflammatory response showed a decrease in the serum levels of TNF- α and an inhibition of the activation of the transcription factor NF- κ B in various tissues. These results indicate that systemic administration of IFN- β might influence the response to pro-inflammatory stimuli, in particular through the antagonism of IFN- γ signaling. Gene Therapy (2000) 7, 817–825.

Keywords: transgenic mice; macrophage; interferon β ; nitric oxide; nuclear factor κ B

Introduction

Type I IFNs are pleiotropic cytokines, involved in host defense against viral infection, that are synthesized by most cell types in response to virus.¹ Three families of type I IFNs have been characterized, α , β and γ . There is only one gene coding for IFN- β , but at least 30 genes coding for IFN- α have been identified, 12 in mice.² Type I IFNs exhibit closely related structures, interacting with identical cell membrane receptors. However, their binding with the receptor appears to be slightly different, which might explain the observed specificities in their biological actions.^{3,4} In addition to the antiviral effects of type I IFNs, cumulative evidence supports the view that these cytokines exert an inhibitory or regulatory action on the activation of cells of the immune system, in particular macrophages.^{5–8}

Expression of inducible nitric oxide synthase (iNOS) constitutes a key step for commitment of the physiological functions of activated macrophages.^{9–11} Multiple pro-inflammatory cytokines and bacterial cell wall products are involved in the expression of iNOS, acting in a concerted cooperative pathway.^{12–14} The synergism between lipopolysaccharide (LPS) and IFN- γ in the transactivation of the inducible iNOS promoter has been well documented using mice lacking molecules involved in the intracellular signaling of IFN- γ ,^{15–17} and this synergistic

pathway appears to be one of the mechanisms relevant for the expression of iNOS in the process of defense against pathogens and in the deletion of tumor cells.^{13,15} However, inappropriate expression of iNOS and overproduction of NO have adverse effects on the organism, these alterations participating in the onset of several pathologies such as various forms of neurodegeneration, chronic inflammation and autoimmune diseases.^{18,19} For these reasons, the characterization of pathways involved in the negative regulation of macrophage activation constitutes a field of interest.²⁰ In this vein, inhibition by type I IFNs of iNOS expression has been described in macrophages stimulated *in vitro* with IFN- γ , and this has been explained in terms of the antagonism between type I and type II IFNs.^{5,7,21–23} Indeed, the antagonism between type I and II IFNs has been observed in other cells, for example in the expression of MHC class II proteins, where type I IFNs down-regulate the process.²⁴

Regarding the expression of iNOS in the macrophage, multiple sites for the binding of factors modulated by IFN- γ and type I IFNs have been recognized in the promoter region of the gene, including IFN-stimulated response element (ISRE), γ -activated site (GAS) and IFN-regulatory factor element (IRF-E).^{13,14} However, controversy exists about the mechanism of action of type I IFNs in the transcriptional control of iNOS: IFN- α and - β are synthesized by macrophages stimulated with LPS and IFN- γ , and neutralizing antibodies against type I IFNs decreased the expression of iNOS.⁸ Alternatively, challenge of macrophages with IFN- α/β before IFN- γ stimulation impaired the transcription of iNOS through a mechanism that involved a decreased activity of NF- κ B

and inhibition of IFN- γ -dependent signaling. Interestingly, these effects were observed only during the initial 2-h period of stimulation, indicating that type I IFNs inhibited the recruitment or the function of the transcription factors responsible for the activation of the iNOS promoter, but they did not affect the stability of the iNOS mRNA and protein.^{5,22} Keeping in mind this perspective, it is reasonable to develop pharmacological approaches intended to regulate the function of inflammatory cells, based on the administration of type I IFN. In this regard, taking advantage of the development of transgenic mice expressing a human IFN- β gene (hIFN- β) in β -cells under the control of the rat insulin I promoter,²⁵ we evaluated the ability of elicited macrophages from these animals to respond after pro-inflammatory stimulation. Our results show that these animals exhibited an increased accumulation of macrophages in the peritoneal cavity and have an attenuated response to LPS and IFN- γ challenge. Interestingly, the inhibitory effects due to the expression of the hIFN- β transgene were lost when macrophages were isolated and stimulated in culture. These data reinforce the view of an impaired macrophage activation in those situations in which type I IFN synthesis occurs, for example, after viral infection, or in patients treated pharmacologically with IFN- β , such as those suffering multiple sclerosis.^{26,27}

Results

Increased macrophage extravasation in animals carrying a hIFN- β transgene

To study the potential role of IFN- β in counteracting macrophage activation, we used a transgenic animal model that expressed human IFN- β specifically in pancreatic β -cells.²⁵ These animals had a CD-1 genetic background, since they were obtained by backcrossing C57/Bl6/SJL transgenic mice (RIP-I/hIFN- β transgene) to CD-1 mice. Two transgenic lines, Tg-1 and Tg-2 (which corresponded to N7 and N5 backcrosses to CD-1, respectively) were used. Both Tg-1 and Tg-2 mice exhibited similar serum levels of hIFN- β , representing a 65-fold increase over the control value of immunoreactive protein measured in transgene-negative littermates (Figure 1). When elicited peritoneal macrophages were

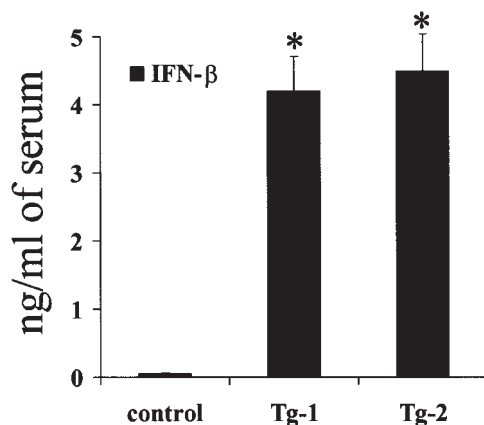


Figure 1 Determination of IFN- β levels in animals carrying a hIFN- β transgene. The serum levels (samples obtained between 9.00 and 11.00 h) of hIFN- β of the Tg-1 and Tg-2 lines were determined by ELISA using recombinant human IFN- β as standard. Results show the mean \pm s.e.m. of eight animals of each group. *, $P < 0.01$ versus control animals.

prepared from these animals, an increased extravasation to the peritoneal cavity was observed systematically in Tg-1 and Tg-2 animals, in agreement with the measured serum levels of hIFN- β in both lines (Figure 2). On analysis, this increased cell content in the peritoneal fluid corresponded to CD11b positive cells, suggesting an enhancement in the recruitment of monocyte/macrophages from the circulation in these animals. In addition to this cell extravasation, the total amount of protein detected in the peritoneal fluid also increased (Figure 2). Since both transgenic lines exhibited a similar behavior, experiments were performed using only the Tg-1 colony.

Macrophage activation was impaired in animals carrying a hIFN- β transgene

Macrophage activation was evaluated in control and transgenic animals both *ex vivo* and *in vivo* after challenge with low doses of LPS and IFN- γ , and following the synthesis of NO as a marker of cell activation. As Figure 3 shows, when elicited peritoneal macrophages were stimulated *ex vivo* with LPS or LPS plus IFN- γ , identical results were obtained in control and Tg-1 animals in terms of the synthesis of NO. Incubation of the cells with IFN- α/β inhibited (58%) the activation of macrophages challenged with LPS and IFN- γ , data in agreement with previous reports.^{7,28} However, when animals were i.p. injected with a solution containing LPS plus IFN- γ and peritoneal macrophages were isolated and kept in culture an important inhibition (61%) in the synthesis of NO was observed in cells from Tg-1 animals when compared with the corresponding control animals. When both groups of mice were treated *in vivo* with IFN- α/β , followed by LPS and IFN- γ challenge, a decrease of NO synthesis (54%) was measured in control animals, although this cytokine did not modify the response of the Tg-1 group. Moreover, the inhibitory effect of hIFN- β on the activation of macrophages *in vivo* was absent when Tg-1 animals were treated only with LPS, in the absence of IFN- γ . These results indicate that expression of hIFN- β in transgenic animals mediates the attenuation of the inflammatory response, a mechanism that was lost when macrophages were isolated and activated *in vitro*.

To evaluate better the effect of IFN- β in macrophage

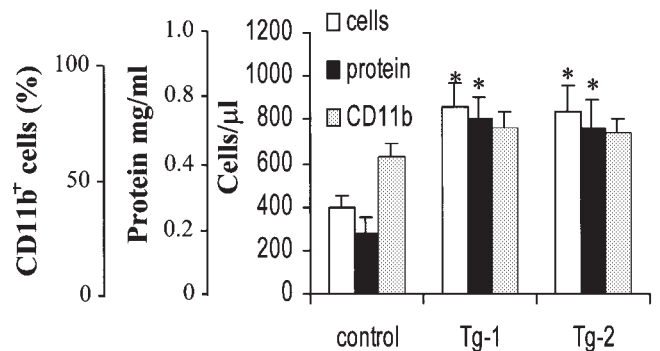


Figure 2 Peritoneal extravasation of elicited macrophages from animals carrying a hIFN- β transgene. Control, Tg-1 and Tg-2 mice were i.p. injected 1 ml of thioglycollate broth and 4 days later the peritoneal cavity was washed with 10 ml of PBS and the cell concentration, the percentage of CD11b⁺ cells (measured by flow cytometry) and protein content were determined. Results show the mean \pm s.e.m. of four experiments ($n = 4-6$). *, $P < 0.01$ versus control animals.

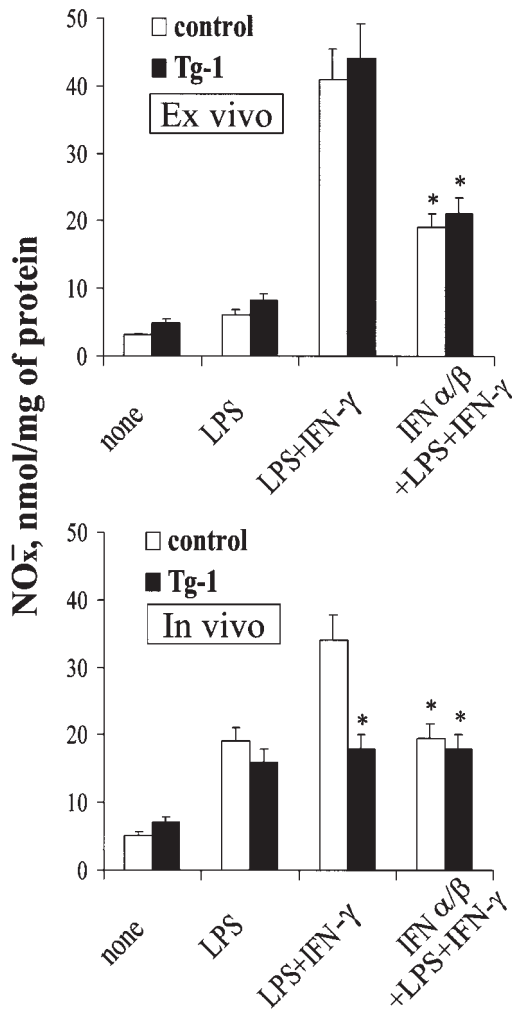


Figure 3 Ex vivo and in vivo activation of macrophages from mice overexpressing hIFN- β . Peritoneal macrophages from control and Tg-1 animals were isolated and cultured in RPMI 1640 medium. Cells were stimulated with 100 ng/ml of LPS, 20 U/ml of IFN- γ , 100 U/ml of IFN- α/β or combinations of these. After 18 h of incubation, the NO synthesis was determined (left panel). Alternatively, animals were i.p. injected 1 μ g of LPS, 150 ng of IFN- γ , 1000 U of murine IFN- α/β or combinations of these. After 2 h, macrophages were obtained from the peritoneal cavity, kept in culture for an additional 16 h period, and the release of NO was measured (right panel). Results show the mean \pm s.e.m. of three experiments ($n = 4$). *, $P < 0.01$ versus the macrophages from control animals treated with LPS plus IFN- γ ex vivo or in vivo, respectively.

function, control and transgenic animals were i.p. injected with LPS and IFN- γ and, at the indicated times, the cells present in the intraperitoneal cavity were aspirated and the levels of RNA and protein of COX-2 and iNOS, two genes involved in the onset of macrophage activation,^{9,29} were measured. As Figure 4 shows, the inhibitory action of endogenous hIFN- β was very important at early times (up to 4 h), with the effect decreasing at later periods of time.

hIFN- β attenuates the increase of TNF- α after LPS and IFN- γ challenge

To establish the effect of the expression of hIFN- β on the levels of pro-inflammatory cytokines, animals were i.p. injected with a solution containing LPS and IFN- γ , and at the indicated times samples of blood were collected

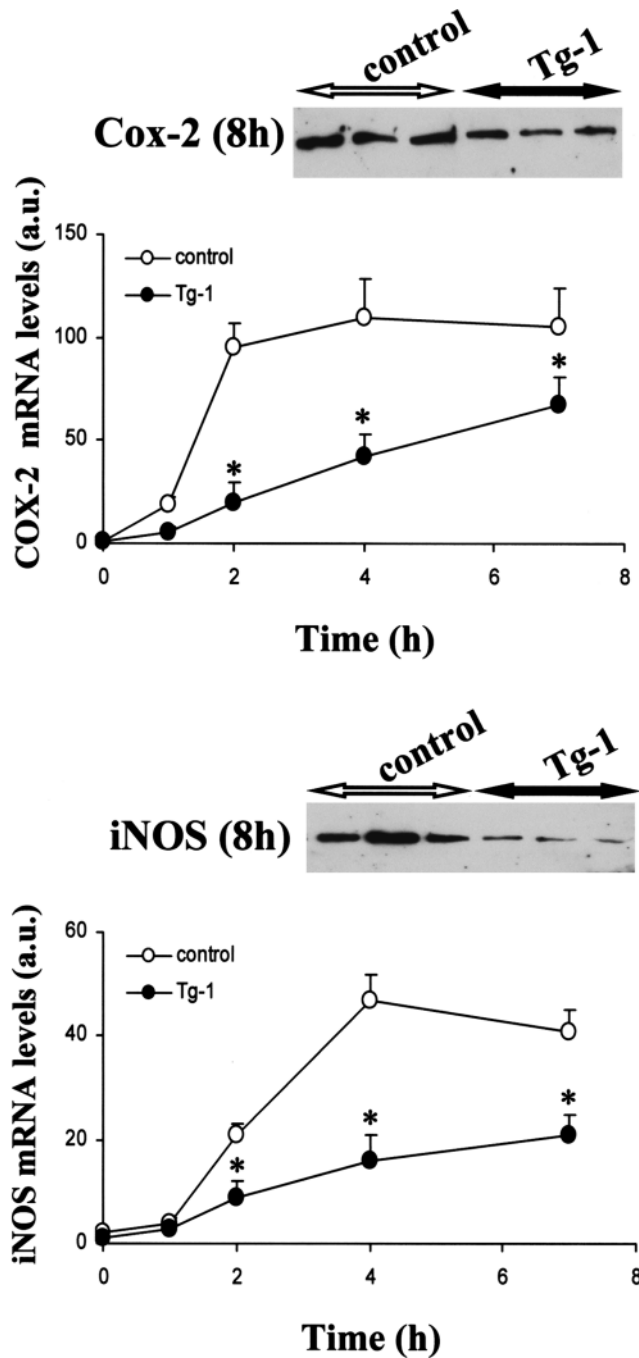


Figure 4 Time-course of COX-2 and iNOS expression in macrophages from animals carrying a hIFN- β transgene. Control or Tg-1 mice were i.p. injected 1 μ g of LPS and 150 ng of IFN- γ and at the indicated times macrophages were isolated and the mRNA levels of COX-2 and iNOS were determined by Northern blot. Cell extracts obtained after 8 h of stimulation were used to determine the corresponding COX-2 and iNOS protein levels. Results show the mean \pm s.e.m. of three experiments ($n = 3$), or a representative experiment when the protein was determined. *, **, denotes $P < 0.05$, $P < 0.01$ versus the corresponding control animals at the indicated times.

and the amount of TNF- α and IL-1 β was determined by a specific murine EIA (enzyme immunoassay). As Figure 5a shows, lower levels of TNF- α were measured in Tg-1 animals challenged with the pro-inflammatory stimulus when compared with the control group. This decreased

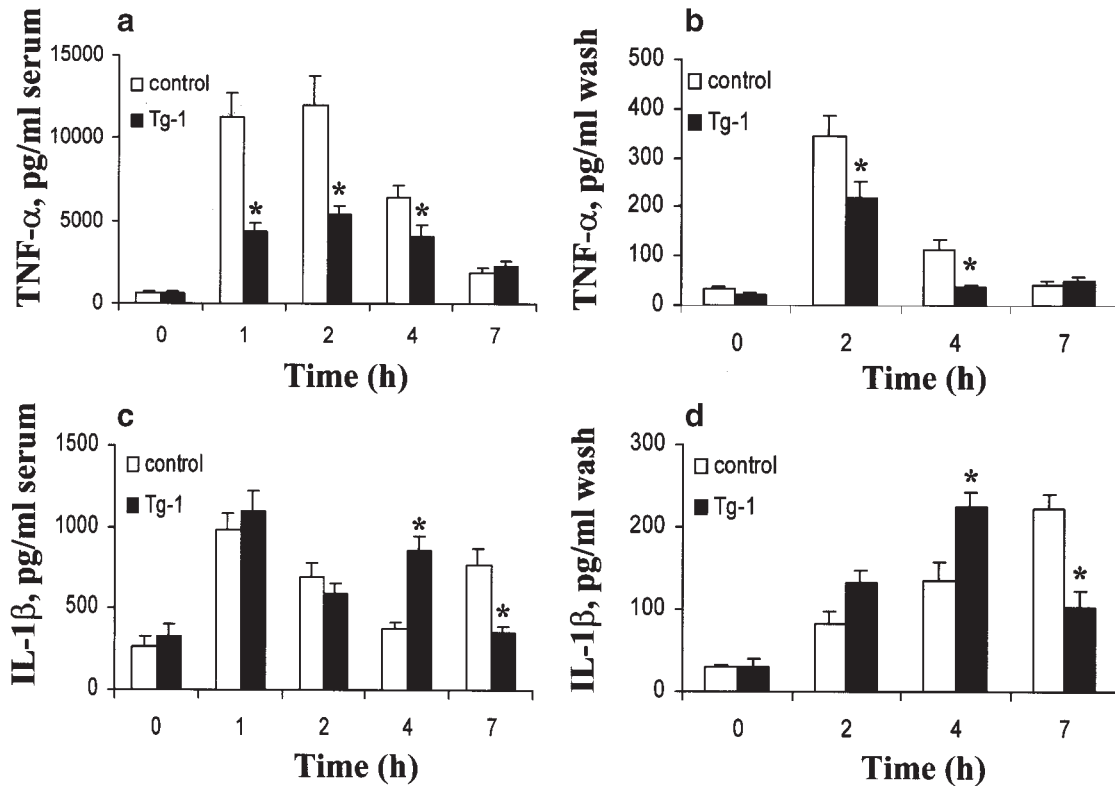


Figure 5 TNF- α and IL-1 β levels in serum and peritoneal wash from animals treated with LPS and IFN- γ . Control or Tg-1 animals were *i.p.* injected 1 μ g of LPS and 150 ng of IFN- γ as indicated in Figure 4. Samples of serum (panels a and c) or from the peritoneal wash (10 ml per animal; panels b and d) were collected and the amount of TNF- α and IL-1 β was determined by a specific murine ELISA. Results show the mean \pm s.e.m. of six animals. *, denotes $P < 0.05$ versus the corresponding control animals at the indicated times.

response was observed at short periods of time (less than 4 h), and was not followed by a delayed increase in the levels of this cytokine in Tg-1 animals. However, when the amount of IL-1 β was measured in this experimental model (Figure 5c), the response was similar between control and Tg-1 animals during the initial 2-h period, but changed significantly at later periods of time (4 and 7 h). Interestingly, the profile of TNF- α and IL-1 β determined in the peritoneal fluid obtained after removing the cells paralleled the changes observed in serum (Figure 5b and d).

The preceding results suggest that the expression of hIFN- β affects events occurring mainly at early times after stimulation, in particular the synthesis of TNF- α . To characterize better this process, we investigated the activation of NF- κ B and Stat1 α as transcription factors integrating the early pro-inflammatory stimulation involved in the transcriptional activation of genes that participate in the onset of inflammation.^{8,30–33} As Figure 6 shows, NF- κ B activation in macrophages from animals challenged *in vivo* with LPS and IFN- γ was impaired in Tg-1 when compared with the corresponding controls, at least during the initial 2-h period after stimulation. Analysis of the bands by supershift assays suggested the presence of p65.p50 and p50.p50 complexes in the upper and lower bands, respectively. This decrease of NF- κ B activation determined by electrophoretic mobility shift assays (EMSA) was confirmed by the reduced levels of p65 detected in the nucleus of Tg-1 animals, reflecting an inhibition of the release of active NF- κ B complexes from

the cytosol towards the nucleus. The amount of C/EBP β in nuclear extracts was determined by Western blot to evaluate the homogeneity of lane charge in the EMSA and immunoblot of p65. The effect of endogenous hIFN- β on IFN- γ signaling was analyzed by measuring the sub-cellular distribution of Stat1 α and its phosphorylation state in macrophages as described for the analysis of NF- κ B (Figure 7). IFN- γ induced a similar phosphorylation and translocation to the nucleus of Stat1 α in control and Tg-1 animals (60 min after challenge). However, the levels of IRF1 measured at 90 min were notably lower in Tg-1 animals. Analysis of the binding of nuclear proteins to a consensus GAS site showed a lower binding in Tg-1 animals, whereas when the same extracts were analyzed using a consensus SIE/ISRE site the binding was enhanced in IFN- γ challenged Tg-1 animals. These results suggest interference between the hIFN- β and IFN- γ signaling pathways involved in the transcriptional control of specific genes.

Discussion

Type I IFNs have been used for the pharmacological treatment of several forms of cancer, neurodegenerative and infectious diseases.^{26,27,34,35} Among type I IFNs, IFN- β has potent antiproliferative activity against most human tumor cells *in vitro* in addition to its known immunomodulatory activities.^{36–40} For these reasons, the availability of an animal model expressing IFN- β might help to understand the biological properties of this cyto-

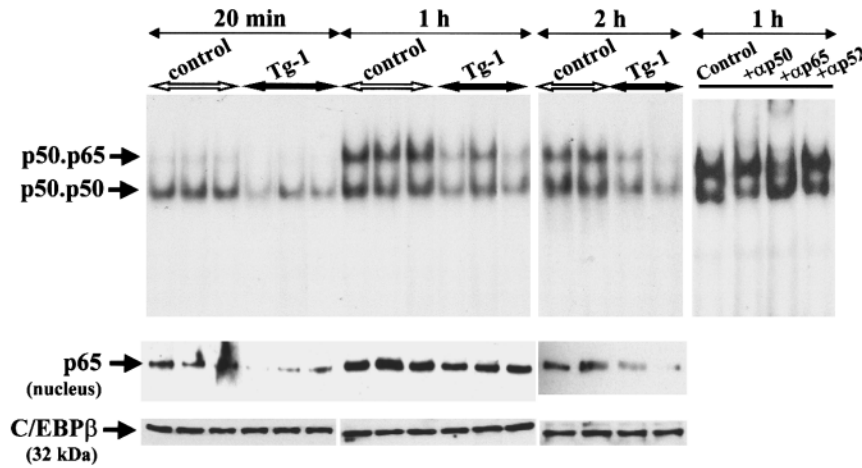


Figure 6 NF- κ B activation is impaired in mice expressing hIFN- β . Animals were i.p. injected a solution containing LPS and IFN- γ as described in Figure 4. At the indicated times nuclear extracts from peritoneal cells were prepared and the activation of NF- κ B was followed by EMSA using the κ B motif of the murine iNOS promoter. The bands corresponded mainly to p50-p65 dimers (upper band) or p50 homodimers (lower band) as deduced by supershift assays. The levels of p65 in the nuclear extracts were determined by Western blot using the samples analyzed by EMSA. The amount of C/EBP β in the nuclear extracts was measured to ensure equal protein loading per lane. The results show a representative experiment out of two ($n = 3$).

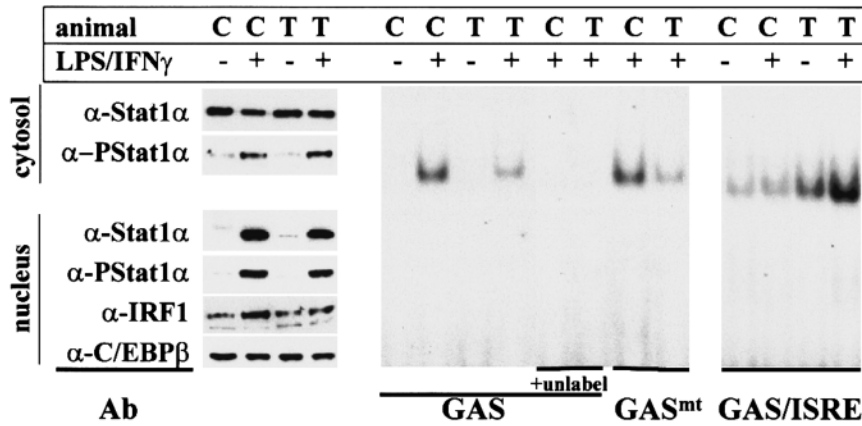


Figure 7 IFN- γ signaling is altered in mice expressing hIFN- β . Animals were treated as described in Figure 6 and at 60 and 90 min cytosolic and nuclear extracts were prepared and pooled ($n = 5$). The levels of Stat1 α and phosphoStat1 α were determined at 60 min in the cytosol and nucleus using specific Ab. IRF1 was determined at 90 min in the nuclear extracts. The binding of nuclear proteins to a GAS and GAS/ISRE sequences was determined by EMSA. A GAS mutated sequence (GAS^{mt}) and an excess of unlabelled oligonucleotide (30-fold) were used to establish the specificity of the binding. C, control; T, Tg-1 animals.

kinase in the different pathological and pharmacological circumstances in which IFN- β is present. For example, using these animals, it has been reported that hIFN- β favors the development of a pre-diabetic state that was evidenced after administration of low doses of streptozotocin.²⁵ Tg-1 mice released relatively large amounts of hIFN- β , but because of the much lower affinity of the human cytokine for the murine type I IFN receptor, it exerts systemic effects outside the pancreas where the cytokine is produced,⁸ a condition which constitutes an advantage of this model over a murine IFN- β -expressing alternative. We detected levels of hIFN- β that are in the range of 4–5 ng/ml of serum as deduced by ELISA assays. However, the amount of type I IFNs might be even higher if the binding to circulating proteins is considered. Moreover, the amount of other type I IFNs such as IFN- α , could be elevated in these animals due to the priming effect over type I IFN expression exerted by IFN- β in peripheral tissues, as deduced using mice with inactivated IFN- β gene ($-/-$ mice).^{2,7}

In this work we used these animals to evaluate the ability of systemic hIFN- β to modulate macrophage activation. Signaling through type I and II IFN receptors is known in detail and involves the activation of in part overlapping proteins of the JAK/Stat pathway. IFN- α and - β mediate their action through the phosphorylation and dimerization of Stat1/Stat2, members of the signal transducers and activation of the transcription (Stat) protein family, and p48, a member of the IRF1 transcription factors.^{32,41} These complexes bind preferentially to ISRE motifs. The effects of IFN- γ are mediated through the formation of Stat1 α dimers that bind to GAS motifs.^{41–43} The role of type I IFNs in the activation of macrophages has been a subject of controversy. Some investigators reported a potentiation of the activation process,^{37,44} and others described an anti-inflammatory contribution.^{5,7,22,45} However, these data can be concealed if the nature of the pro-inflammatory stimuli used to activate macrophages and the time at which macrophages produce endogenous type I IFNs synthesis are considered. Challenge of macro-

phages with LPS, in the absence of IFN- γ synergism, is independent of the action of IFN- α/β ^{37,44} (and this work); however, macrophage activation in the presence of IFN- γ is significantly abrogated by type I IFNs.^{5,22} Moreover, following viral infection (for example with flavivirus), an abrogation has been shown of the expression of iNOS in macrophages challenged with IFN- γ and TNF- α , due to the overproduction of IFN- α/β by infected cells.^{46,47} Synthesis of IFN- α/β by cells stimulated with pro-inflammatory cytokines is required for the effective expression of iNOS, as deduced by the observation that inhibition of IFN- α/β activity with neutralizing antibodies abolished the induction of iNOS in animals infected with intracellular microbes, giving a phenotype similar to that of animals with a null mutation of iNOS.^{8,48,49} In addition to these effects on macrophages, it has been shown that IFN- β antagonizes with IFN- γ on the expression of high affinity Fc γ receptors in peripheral blood monocytes, a process which may partially explain the beneficial effects of IFN- β treatment of patients with multiple sclerosis.²³ The mechanism by which hIFN- β inhibits macrophage activation in intact animals challenged with LPS and IFN- γ involves an important decrease of the synthesis of pro-inflammatory cytokines, in particular, TNF- α . TNF- α is mainly produced by cells of the macrophage lineage, and this cytokine is involved in multiple inflammatory reactions. In mice suffering experimental allergic encephalomyelitis, an autoimmune disease of the central nervous system with similarities to multiple sclerosis, it has been shown that local synthesis of TNF- α is one of the key factors involved in the etiology of the disease since treatment of the animals with soluble TNF- α receptors notably ameliorates the symptoms. Indeed, IFN- α treatment of these animals also favors the decrease of pro-inflammatory cytokines, including TNF- α .⁵⁰ Also, IFN- β reduces the TNF- α production by microglia, and possibly contributes through this mechanism to the alleviation of the symptoms in multiple sclerosis patients.⁵¹

Contrary to the important decrease of TNF- α in animals treated with LPS and IFN- γ , the observation of minimal effects, if not opposite, in the case of IL-1 β , suggests that these cytokines are regulated by IFN- β at a different level in intact Tg-1 animals, and that IL-1 β plays a minor role in the expression of iNOS under these conditions. According to this suggestion, we observed that activation of NF- κ B, not only in macrophages but in other tissues such as liver and kidney (not shown) was significantly impaired in Tg-1 mice. NF- κ B can be considered as an integrator of the response to cell stress since this factor is activated in response to different stimuli, such as heat-shock, pro-inflammatory cytokines, oxidant molecules, virus and bacteria.^{30,52} In this regard, it has been described that IFN- β inhibits the production of reactive oxygen species in monocytes⁵³ and these molecules have been suggested as potential mediators of NF- κ B activation.^{54,55}

The increased extravasation of monocytes/macrophages to the peritoneal cavity detected in Tg-1 animals parallels results obtained via adenoviral vector-mediated IFN- β delivery.³⁴ To establish the mechanism responsible for this effect more work is required, but activation of PI3-kinase has been shown to be important for the extravasation of various cell types, and this kinase is indeed activated by engagement of type I IFN receptor.⁵⁶

Finally, the half-life of IFN- β is rather short and con-

tinuous synthesis or disposal is required to maintain substantial levels of this cytokine. Therefore, this model of mice releasing a 'low-affinity-like' IFN- β allowed us to define anti-inflammatory effects of type I IFNs, a situation reminiscent of that occurring in the course of viral infections or after IFN- β therapy of several diseases.^{57,58}

Materials and methods

Chemicals

Reagents were from Sigma (St Louis, MO, USA), Boehringer (Mannheim, Germany) or Merck (Darmstadt, Germany). Cytokine assay 'Biotrak' kits were from Amersham (Bucks, UK). Serum and media were from Biowhittaker (Walkersville, MD, USA).

Animals

Transgenic mice (C57Bl6/SJL genetic background) expressing a rat insulin I promoter/human IFN- β (RIP/IFN- β) chimeric gene were backcrossed to CD-1 mice.²⁵ In this study, we used N5 and N7 generations of transgenic mice, which had about 96.9% and 99.2%, respectively, CD-1 genetic background. At 3 weeks of age, animals were tested for the presence of the transgene by Southern blot analysis of tail DNA (10 μ g) digested with *Bgl*III.²⁵ Blots were hybridized with the 1.3-kb *Sac*I-*Xho*I fragment containing the entire RIP-I/hIFN- β chimeric gene, radiolabeled with α -³²P dCTP (3000 Ci/mmol) by random oligoprimering (Amersham). In the experiments described below, heterozygous male mice aged 2 to 3 months were used. Transgene-negative littermates were used as control. Mice were fed *ad libitum* with a standard diet (Panlab, Barcelona, Spain) and kept under a light-dark cycle of 12 h (lights on at 8 am).

Treatment of animals and isolation of peritoneal macrophages

Animals were used as follows. Four days before the assay, mice were i.p. injected 1 ml of 10% (w/v) of thioglycollate broth.⁵⁹ Elicited peritoneal macrophages were prepared from light-ether anesthetized mice (four to six animals per condition), killed by cervical dislocation and injected i.p. 10 ml of sterile RPMI 1640 medium. The peritoneal fluid was carefully aspirated avoiding hemorrhage and kept at 4°C to prevent the adhesion of the macrophages to the plastic. An aliquot of the cell suspension was used to determine the cell density in the peritoneal fluid. The cells were centrifuged at 200 g for 10 min at 4°C and the pellet was washed twice with 25 ml of ice-cold PBS. Cells were seeded at $1 \times 10^6/\text{cm}^2$ in RPMI 1640 medium supplemented with 10% of heat inactivated FCS and antibiotics. After incubation for 1 h at 37°C in a 5% CO₂ atmosphere, nonadherent cells were removed by extensive washing with PBS. Experiments were carried out in phenol red-free RPMI 1640 medium supplemented with 0.5 mM arginine and 10% of heat inactivated FCS plus antibiotics.⁵⁹ For *in vivo* experiments, animals were i.p. injected 0.5 ml of a solution containing 1 μ g of LPS from *S. tiphimurium*, 150 ng of murine recombinant IFN- γ , 1000 units of IFN- α/β , or combinations of these.⁶⁰

FACS analysis

Cells obtained from the peritoneal cavity were incubated with anti-CD11b (clone M1/70, PharMingen, San Diego,

CA, USA) and assayed by immunofluorescence staining with flow cytometry, following the instructions of the supplier of the antibody.

Preparation of blood samples

Blood was collected from the tail or by cardiac puncture at the indicated times and serial dilutions of the serum were assayed for the cytokines indicated following the recommendations of the suppliers of the tests.

Determination of NO synthesis

NO release was determined spectrophotometrically by the accumulation of nitrite and nitrate in the medium (phenol red-free). Nitrate was reduced to nitrite as described.⁵⁹ Nitrite was determined with Griess reagent by adding 1 mM sulfanilic acid and 100 mM HCl (final concentration). After a first reading of the absorbance at 548 nm, naphthylethylenediamine (1 mM in the assay) was added and the absorbance was compared with a standard of NaNO₂. Results were expressed as the amount of nitrite and nitrate released per milligram of cell protein.

Cytokine assay

The serum or ascitic levels of TNF- α and IL-1 β were measured per duplicate using commercial kits (Biotrak, Amersham). The serum levels of hIFN- β were measured by ELISA using a commercial source of recombinant hIFN- β as standard.

Preparation of cell extracts

The macrophage layers were washed twice with ice-cold PBS and the plates filled with 0.4 ml of buffer A (20 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 10 mM KCl, 0.5 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 10 μ M leupeptin). The cells were scraped off the dishes using a rubber policeman and mixed with one volume of a 1% Nonidet P-40 solution. The tubes were gently vortexed for 15 s and nuclei were sedimented by centrifugation at 8000 g for 15 s. Aliquots of the supernatant were stored at -80°C (cytosolic extracts), and the nuclear pellets were resuspended in 100 μ l of buffer A supplemented with 0.4 M KCl. Nuclear proteins were extracted by centrifugation at 13000 g for 15 min and aliquots of the supernatant were stored at -80°C.⁵⁹ Proteins were measured using the Bio-Rad detergent-compatible protein reagent (Richmond, CA, USA). All steps of cell fractionation were carried out at 4°C.

Electrophoretic mobility shift assays

Oligonucleotides corresponding to the proximal κ B motif of the murine iNOS promoter^{13,61} 5'-CCAACTGGG GACTCT-CCCTTTGGGAACA-3', the Ly-6E GAS site 5'-GTCATATTC-CTGTAAGTG-3' or the GAS mutated sequence 5'-GTCATACCACCTGTAAGTG-3', and the GAS/ISRE sequence 5'-AAGTACTTTCAGTTTCATAT-TACTCTA-3' (50 ng) were annealed with the complementary sequence and end-labeled with Klenow enzyme in the presence of 50 μ Ci of α -³²P dCTP and the other unlabeled dNTP in a final volume of 50 μ l. The oligonucleotide was precipitated with ethanol and extracted with phenol/chloroform. Binding assays of nuclear extracts were carried out with 5 \times 10⁴ d.p.m. of the DNA probe as follows: 5 μ g of nuclear protein extract were incubated for 30 min at 4°C with the DNA and 1 μ g/ml of poly(dI-dC), 5% glycerol, 1 mM EDTA, 100 mM

KCl, 5 mM MgCl₂, 1 mM dithiothreitol and 10 mM Tris-HCl, pH 7.8, in a final volume of 20 μ l. The incubation mixture was applied to a 6% polyacrylamide gel which had been previously electrophoresed for 30 min at 100 V. Gels were run at 0.8 V/cm² in 45 mM Tris-borate, followed by transference to 3MM Whatman paper (Whatman, Maidstone, UK), drying under vacuum at 80°C and quantification of the band intensities in an autoradiograph (Fuji Bas 1000, Stamford, CT, USA). Analysis of competition with unlabeled oligonucleotides was performed using a 20-fold excess of double stranded DNA in the binding reaction, and adding the nuclear extracts as the last step in the binding assay. Supershift assays were carried out after addition of the antibody (0.5 μ g) to the binding reaction and incubation for 1 h at 4°C.⁶¹

Western blot analysis

Cytosolic and nuclear extracts were obtained as described previously. Samples containing equal amounts of protein (30 μ g and 10 μ g per lane of cytosolic and nuclear extracts, respectively) were boiled in 250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% β -mercaptoethanol and size-separated in 10% SDS-PAGE. The gels were processed as recommended by the supplier of the antibodies against the murine antigens (COX-2, iNOS, p65, p52, Stat1 α p90, phospho-Stat1, IRF1 and C/EBP β ; Santa Cruz Laboratories, Santa Cruz, CA, USA); and after blotting on to a PVDF membrane, proteins were revealed following the ECL technique (Amersham). Different exposure times of the films were used to ensure that bands were not saturated. Quantification of the films was performed by laser densitometry (Molecular Dynamics, Kemsing, UK).

RNA extraction and analysis

Total RNA (2-4 \times 10⁶ cells) was extracted following the guanidinium thiocyanate method.⁶² Equal amounts of RNA were denatured and size-separated by electrophoresis in a 0.9% agarose gel containing 2% formaldehyde and MOPS buffering system.⁶¹ The RNA was transferred to Nytran membranes (NY 13-N; Schleicher & Schüell, Dassel, Germany) with 10 \times SSC (10 \times SSC is 1.5 M NaCl, 0.3 M sodium citrate, pH 7.4) under low vacuum conditions, and the membranes were prehybridized for 6 h at 42°C in 50% formamide, 0.25 M NaCl, 0.1 M sodium phosphate, 7% SDS and 0.01% of salmon sperm DNA. An 817 bp fragment (nucleotides 1 to 817) from the cDNA of macrophage iNOS,¹² or the full-length COX-2 cDNA⁶³ were labeled with the Rediprime kit (Amersham) and used to detect the mRNA levels by Northern blot.⁶¹ The intensity of the bands was quantified in a Fuji BAS1000 autoradiograph, using the hybridization with a ribosomal 18S probe as internal control.

Statistical analysis

The data shown are the means \pm s.e.m. of three or four experiments. Statistical significance was estimated with Student's *t* test for unpaired observations. A *P* value of less than 0.05 was considered significant. In studies of Northern blot analysis, linear correlations between increasing amounts of input RNA and signal intensity were observed (correlation coefficients higher than 0.9).

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