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IFN- β Impairs Superoxide-Dependent Parasite Killing in Human Macrophages: Evidence for a Deleterious Role of SOD1 in Cutaneous Leishmaniasis¹

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Type I IFNs (IFN- α/β) have only recently gained considerable attention as immunomodulators in nonviral infectious diseases. IFN- β has been shown to protect, in a NO-dependent manner, against murine Old World leishmaniasis caused by *Leishmania major*, but data in New World leishmaniasis are lacking. We found that IFN- β dose-dependently increases parasite burden in *Leishmania amazonensis*- as well as *Leishmania braziliensis*-infected human macrophages, independent of endogenous or exogenous NO. However, IFN- β significantly reduced superoxide release in *Leishmania*-infected as well as uninfected human macrophages. This decrease in superoxide production was paralleled by a significant IFN- β -mediated increase in superoxide dismutase 1 (SOD1) protein levels. Additionally, IFN- β inhibition of leishmanicidal activity was mimicked by SOD1 and antagonized by either pharmacological or small interfering RNA-mediated inhibition of SOD1. Finally, pronounced SOD1 expression in situ was demonstrated in biopsies from New World cutaneous leishmaniasis patients. These findings reveal a hitherto unknown IFN- β /SOD1 axis in *Leishmania* infection and suggest that inhibition of SOD-associated pathways could serve as strategy in the treatment of *L. amazonensis* as well as *L. braziliensis* infection, major human pathogens. *The Journal of Immunology*, 2009, 182: 2525–2531.

Leishmaniasis is endemic in several parts of the world, with a global prevalence of more than 12 million cases and 1,500,000 new cases emerging every year (1). The infection is caused by protozoan parasites of the genus *Leishmania*, transmitted through the bite of the sand fly vector. Several *Leishmania* species are able to cause a wide spectrum of clinical manifestations, ranging from the mild cutaneous form, the disfiguring mucosal form, and the life-threatening visceral form, also known as kala-azar. Remarkable differences have been described regarding host immunological responses, leishmaniasis clinical manifestation, and parasite resistance between Old World cutaneous leishmaniasis and New World cutaneous leishmaniasis in vivo and in

vivo models (2). In Brazil, *Leishmania braziliensis* causes cutaneous and mucosal disease, and *Leishmania amazonensis* causes cutaneous and, sporadically, diffuse cutaneous or visceral disease. The murine model of Old World leishmaniasis (*Leishmania major*) has been instrumental for the elaboration of the Th1/Th2 paradigm, inasmuch as the preferential action of Th1 (IFN- γ , IL-12) or Th2 cytokines (IL-4, IL-13) results in cure or progression of the disease, respectively (3, 4). In New World human and murine leishmaniasis, this Th1/Th2 dichotomy is much less explicit for in vitro or ex vivo cytokine production (2, 5). Patients with localized cutaneous leishmaniasis display a diminished Th1 response during the early phase of disease, which is reverted after treatment (6). In mucosal leishmaniasis, on the other hand, an exacerbated Th1 response with increased IFN- γ and TNF- α levels is thought to provoke tissue destruction (7). In patients with visceral leishmaniasis, characterized by immunosuppression and absence of in vitro IFN- γ production (8), IFN- γ was the first cytokine to show in vivo benefit (9). In contrast to type II IFN (IFN- γ), fewer data are available on the role of type I IFN (IFN- α/β) in New World leishmaniasis. A protective, strictly NO-dependent role has been demonstrated in murine *L. major* infection for both endogenous IFN- α/β (10) and exogenous recombinant IFN- β (11). Likewise, IFN- β enhanced parasite killing in human macrophages infected with *L. major* (12). However, IFN- β has also been shown to antagonize IFN- γ induction of the respiratory burst (13) and Fc γ RI expression (14). Therefore, we examined the possible effect of IFN- β in New World *Leishmania*-infected human macrophages. We found that IFN- β increases parasite burden in *L. amazonensis*- and *L. braziliensis*-infected human macrophages through a superoxide-dependent, NO-independent mechanism, reinforcing the striking differences between the Old World and New World models of leishmaniasis, which may have important therapeutic implications.

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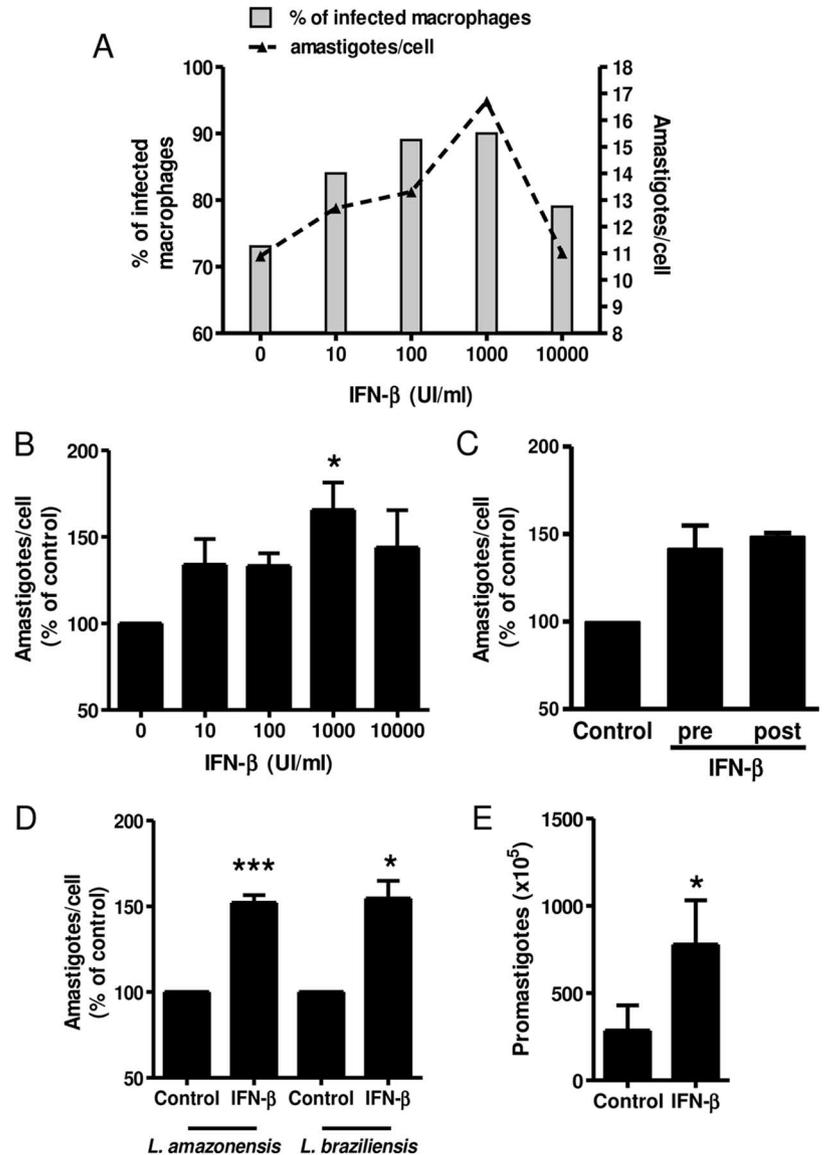
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FIGURE 1. IFN- β increases parasite burden in *Leishmania*-infected human macrophages. *A* and *B*, Monocyte-derived macrophages were infected with *L. amazonensis* promastigotes (5:1 ratio). After infection, cells were treated with increasing concentrations of IFN- β for 48 h and the number of intracellular amastigotes was quantified as described in *Materials and Methods*. *A*, Percentage of infected cells (bars) and number of intracellular amastigotes (dots with connected line) are indicated; representative results from three independent experiments are shown. *B*, Number of intracellular amastigotes; results are expressed as percentage of control. Each bar represents the mean \pm SEM of three donors (one-way ANOVA, Bonferroni's correction, *, $p < 0.05$). *C*, Monocyte-derived macrophages were treated with 1000 IU/ml IFN- β 24 h before (pre) or 24 h after (post) infection with *L. amazonensis* promastigotes. At 48 h of culture, the number of intracellular amastigotes was quantified as described in *Materials and Methods* (pre vs post, one-sample *t* test, $p = 0.69$). *D*, Monocyte-derived human macrophages infected with *L. amazonensis* or *L. braziliensis* were cultured with or without IFN- β (1000 IU/ml) for 48 h and the number of intracellular amastigotes was quantified as described in *Materials and Methods*. Results are expressed as percentage of control. Each bar represents the mean \pm SEM of seven donors for *L. amazonensis* experiments and three donors for *L. braziliensis* experiments (one-sample *t* test, ***, $p < 0.0001$ and *, $p < 0.05$, respectively). *E*, Intracellular survival of *L. amazonensis* amastigotes was quantified by transformation of proliferating extracellular motile promastigotes in Schneider's medium. Each bar represents the mean \pm SEM of eight experiments (Student's *t* test, *, $p < 0.05$).



Materials and Methods

Reagents

All chemicals were purchased from Sigma-Aldrich; cell culture media and sera were obtained from Invitrogen. Endotoxin-free sterile disposables were used in all experiments. Recombinant human IFN- β (specific activity 10^8 IU/mg) was a gift from Serono.

Cell culture

Monocytes were isolated from peripheral blood of healthy donors through Ficoll gradient centrifugation and plastic adherence, and they were differentiated *in vitro* into macrophages (7 days). Human monocytes and macrophages were cultivated in RPMI 1640 or DMEM media supplemented with 5% human AB serum. Macrophages were infected with *L. amazonensis* (MHOM/BR/87/BA125) or *L. braziliensis* (MHOM/BR/88/BA92) and treated after 4 h of infection with IFN- β , inducible NO synthase (iNOS) inhibitor (N^G -monomethyl-L-arginine, L-NMMA),³ NO donor (sodium nitroprusside, SNP), superoxide dismutase (CuZnSOD/SOD1), and/or superoxide dismutase inhibitor (diethyldithiocarbamate, DETC). *Leishmania* spp. cultures were maintained *in vitro* as proliferating promastigotes in Schneider's medium supplemented with 10% FCS.

³ Abbreviations used in this paper: L-NMMA, N^G -monomethyl-L-arginine; DETC, diethyldithiocarbamate; iNOS, inducible NO synthase; siRNA, small interfering RNA; SNP, sodium nitroprusside; SOD, superoxide dismutase.

Quantification of parasite burden

Infected and treated macrophage monolayers were extensively washed, stained with H&E, and amastigotes were counted by optical microscopy (in 100 cells, duplicates for each sample). Similar results were obtained with macrophages directly cultivated upon glass coverslips or macrophages removed by gentle scraping and deposited on glass slides in a cytocentrifuge (Cytospin).

Quantification of parasite survival

After 48 h, infected and treated macrophage monolayers were extensively washed, and medium was replaced by 0.5 ml of Schneider's medium supplemented with 10% FCS. Cultures were maintained at 23°C for 8 additional days. Intracellular survival of *L. amazonensis* amastigotes was quantified by counting proliferating extracellular motile promastigotes (15).

Quantification of NO and superoxide

NO production in supernatants was quantified using Griess reagent (16) and measuring its stable reaction product nitrite. Human iNOS enzymatic activity was quantified by [¹⁴C]arginine-citrulline conversion and iNOS mRNA expression by RT-PCR, as previously described (17). Superoxide production was quantified by three techniques: 1) by adding hydroxylamine (0.5 mM) (18) during cell culture, which converts superoxide into nitrite plus nitrate, and which was reduced by VCl₃ (19) and quantified using Griess reagent; 2) by measuring ferricytochrome C reduction spectrophotometrically in the absence or presence of SOD, as described (20);

and 3) by hydroethidine (Invitrogen) staining and quantification of bright (superoxide high) and dull (superoxide low) cells by flow cytometry (FACSsort; BD Biosciences) (21).

Quantification of intracellular SOD1

For measurement of cytoplasmic SOD1 levels, uninfected human monocytes cultured with or without IFN- β for 48 h were homogenized in cold 20 mM HEPES lysis buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, centrifuged at $1500 \times g$ for 5 min at 4°C, and supernatant was used for SOD1 ELISA (Calbiochem).

Small interfering RNA (siRNA)-mediated inhibition of SOD1

SOD1 and control siRNAs were purchased from Santa Cruz Biotechnology, with transfection being performed according to the manufacturer's instructions.

Immunohistochemistry

Histological sections from New World cutaneous leishmaniasis patients (endemic area of Jequié-Bahia, Brazil) were deparaffinized in xylene and rehydrated in alcohol and water. Inhibition of endogenous peroxidase was performed with a 30% hydrogen peroxide-methanol solution. Sections were incubated with normal goat serum (30 min at 37°C) and then with monoclonal anti-SOD1 Ab (Santa Cruz Biotechnology). Incubations with biotinylated goat anti-mouse Ab and with streptavidin-HRP complex (Sigma-Aldrich) were conducted for 30 min at 37°C. A positive reaction was detected with diaminobenzidine substrate (Sigma-Aldrich), followed by Harris hematoxylin counterstaining. Sections were examined microscopically, and images were recorded with a digital camera (Nikon). This study has been approved by the Centro de Pesquisas Gonçalo Moniz Ethical Committee.

Statistical analysis

Student's *t* test, one-way ANOVA, with Bonferroni's multiple test correction, or two-way ANOVA was used, all two-tailed. Data are presented as means \pm SEM, and differences were considered significant at *p* values of ≤ 0.05 .

Results

IFN- β increases parasite burden in *Leishmania*-infected human macrophages

To explore the effects of type I IFNs on New World strains of *Leishmania*, we first investigated the effect of IFN- β on parasite burden of *L. amazonensis*-infected human macrophages. As shown in Fig. 1A, IFN- β increased both the percentage of infected cells and the number of intracellular amastigotes, being effective at 10 and 100 IU/ml and optimal at 1000 IU/ml (Fig. 1B), a dose response curve strikingly similar to our previous observations regarding CD64 expression (14). A fixed concentration of 1000 IU/ml IFN- β was used in all additional experiments. The IFN- β -induced increase in parasite burden was already significant at 24 h and slightly increased at 48 h (data not shown), which was chosen for all additional experiments. Despite the strong donor-to-donor variation usually observed with human macrophages, IFN- β was able to increase parasite load in 22 of 27 donors tested, demonstrating the reproducibility of our observations. In contrast to type I IFN in murine macrophages (22), IFN- β exerted its effect in human macrophages independent of the sequence of infection and treatment, since 24 h before treatment with IFN- β or treatment 24 h after infection were equally effective in increasing parasite load (Fig. 1C). To investigate whether the IFN- β effect was *L. amazonensis*-specific, we subsequently infected human macrophages with *L. braziliensis*. IFN- β treatment increased parasite burden to the same extent in *L. braziliensis* ($55 \pm 10\%$ increase, $n = 3$) as in *L. amazonensis*-infected ($52 \pm 4\%$ increase, $n = 7$) human macrophages (Fig. 1D). To evaluate if increased parasite burden also resulted in enhanced survival of *Leishmania* following IFN- β treatment, intracellular parasite survival was quantified by transformation of amastigotes into proliferating extracellular motile promastigotes in Schneider's medium (15). In fact, the effect

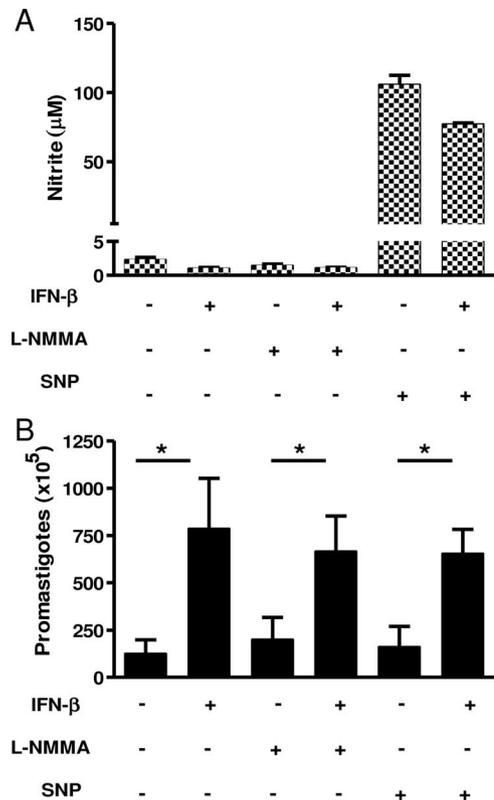


FIGURE 2. IFN- β impairs NO-independent parasite killing in human macrophages. Human macrophages were treated 4 h after infection with IFN- β (1000 U/ml) in the absence or presence of an iNOS inhibitor (2 mM L-NMMA) or a NO donor (1 mM SNP). *A*, Supernatants were collected after 48 h; nitrite was measured using the Griess reaction. Each bar represents the mean \pm SEM of four donors. *B*, Intracellular survival of *L. amazonensis* amastigotes was quantified by transformation of proliferating extracellular motile promastigotes in Schneider's medium. Each bar represents the mean \pm SEM of four experiments (two-way ANOVA, IFN- β , *, $p < 0.05$).

on parasite survival was even more pronounced, since *L. amazonensis* promastigote viability increased 3-fold following IFN- β treatment (Fig. 1E).

IFN- β inhibition of leishmanicidal activity of human macrophages is NO-independent

Since Mattner et al. (11) had demonstrated that the protective effect of IFN- β in murine leishmaniasis was strictly iNOS-dependent, we investigated possible NO production and/or iNOS expression in untreated and IFN- β -treated *Leishmania*-infected macrophages. First, nitrite levels were quantified in cell supernatants in the absence or in the presence of an iNOS inhibitor (2 mM L-NMMA) or a NO donor (1 mM SNP), as a positive control. Nitrite levels above background (2–3 μ M) were not detected in macrophage culture supernatants, independent of infection or treatment (Fig. 2A). As expected, SNP treatment induced significant accumulation (up to 90 μ M) of nitrite in cell supernatants (Fig. 2A). Second, we did not detect iNOS enzymatic activity by [¹⁴C]arginine-citrulline conversion, nor did we detect iNOS mRNA expression by RT-PCR in four donors tested, independent of treatment or infection (data not shown). In contrast, [¹⁴C]arginine-citrulline conversion was easily detected in IFN- γ plus LPS-stimulated murine macrophages used as a positive control (data not shown). Third, we investigated whether endogenous or exogenous NO might interfere with *in vitro* killing of *Leishmania*-infected human macrophages or with its inhibition by IFN- β . Both basal and IFN- β -induced parasite burden remained unaltered in the presence of

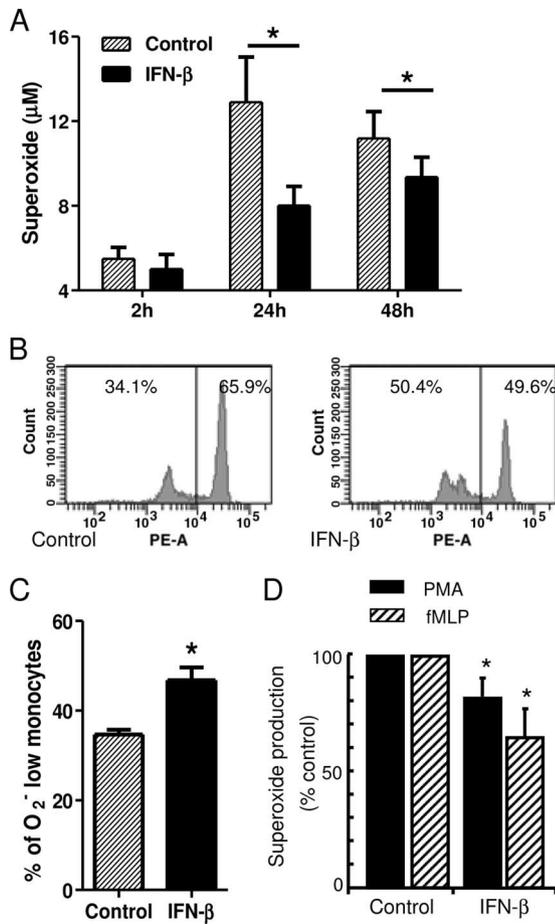


FIGURE 3. IFN- β reduces superoxide release by *Leishmania*-infected human macrophages. *A*, *L. amazonensis*-infected human macrophages were cultured with or without IFN- β (1000 U/ml) in the presence of hydroxylamine (0.5 mM), and supernatant was collected at 2, 24, and 48 h. Superoxide production was quantified as described in *Materials and Methods*. Each bar represents the mean \pm SEM of seven donors (two-way ANOVA: IFN- β , *, $p < 0.05$; time, ***, $p < 0.0001$; interaction, $p = 0.18$). *B* and *C*, Uninfected monocytes were cultured for 48 h in the absence or presence of IFN- β and stained with hydroethidine for 45 min, and during the last 15 min cells were triggered with PMA. Superoxide production was measured through flow cytometry technique. Each bar represents the mean \pm SEM of four donors. *D*, Uninfected monocyte-derived macrophages were cultured for 48 h in the absence or presence of IFN- β and triggered with PMA or fMLP for 30 min. Superoxide was measured spectrophotometrically by ferricytochrome C reduction in the absence or presence of SOD, as described in *Materials and Methods*. Each bar represents the mean \pm SEM of three donors.

L-NMMA (Fig. 2*B*). Surprisingly, parasite survival did not significantly decrease by NO-generating SNP treatment, even at supraphysiological nitrite levels ($\pm 90 \mu\text{M}$). Moreover, IFN- β was still able to strongly increase parasite survival, independent of an excess of exogenous NO (Fig. 2*B*). Two-way ANOVA confirmed IFN- β as an independent determinant of *Leishmania* survival ($p = 0.037$), whereas SNP/L-NMMA were not ($p = 0.84$), and, more importantly, no significant interaction was observed between the effect of IFN- β and SNP/L-NMMA upon parasite survival ($p = 0.43$).

IFN- β impairs superoxide release of uninfected and *Leishmania*-infected human macrophages

Since IFN- β impaired human macrophage leishmanicidal activity by an apparently NO-independent mechanism, we next investigated if this phenomenon might be superoxide-dependent. Super-

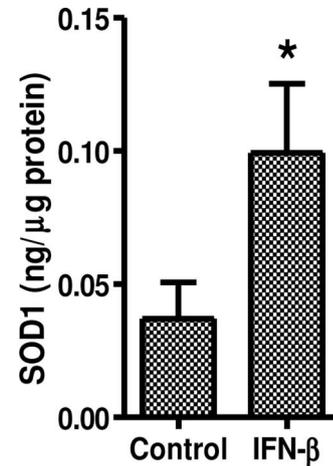


FIGURE 4. IFN- β induces SOD1 expression in human monocytes. Uninfected human monocytes were cultured with or without IFN- β (1000 U/ml). SOD1 expression of protein extraction of these cells was quantified by ELISA. Each bar represents the mean \pm SEM of seven donors (Student's *t* test, *, $p = 0.023$).

oxide production by human macrophages was constitutive, but it gradually increased during the first 4 h of infection with *L. amazonensis* (data not shown), as previously demonstrated for *Leishmania chagasi* infection (23). Conversely, IFN- β treatment reduced superoxide release at 24 and 48 h of treatment, compared with untreated infected human macrophages (two-way ANOVA:

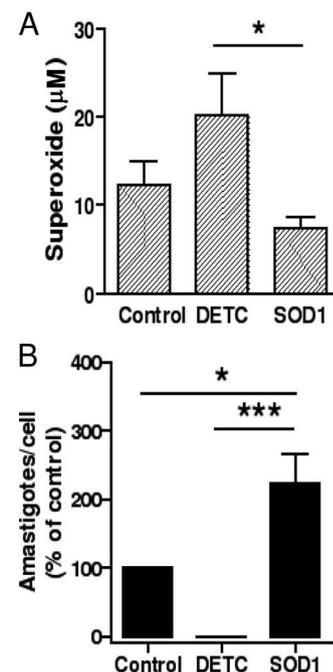


FIGURE 5. SOD1 and SOD1 inhibitor DETC reciprocally regulate parasite killing in *Leishmania*-infected human macrophages. Infected human macrophages were cultured in the presence of SOD1 (175 U/ml) protein or DETC (2 mM). *A*, Supernatant in the presence or absence of hydroxylamine (0.5 mM) was collected at 48 h. Superoxide production was quantified as described in *Materials and Methods*. Each bar represents the mean \pm SEM of three donors (one-way ANOVA, $p = 0.025$; Bonferroni's correction, $p < 0.05$). *B*, Number of intracellular amastigotes was quantified as described in *Material and Methods*. Results are expressed as percentage of control. Each bar represents the mean \pm SEM of five donors (one-way ANOVA, $p = 0.0005$; Bonferroni's correction, ***, $p < 0.0001$ and *, $p < 0.05$).

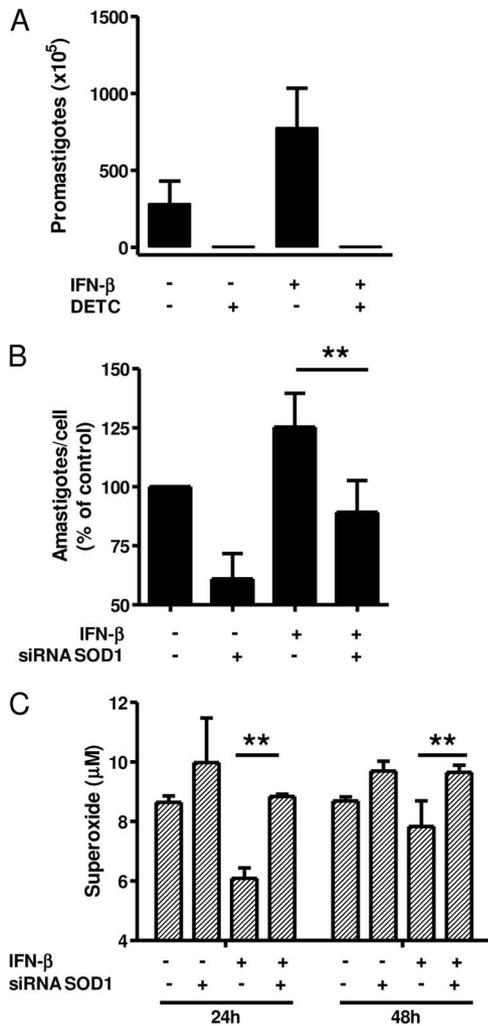


FIGURE 6. SOD1 inhibitor antagonizes IFN- β inhibition of leishmanicidal activity. *A*, Infected human macrophages were treated with IFN- β (1000 U/ml) and/or SOD inhibitor (2 mM DETC). Intracellular survival of *L. amazonensis* amastigotes was quantified by transformation of proliferating extracellular motile promastigotes in Schneider's medium. Each bar represents the mean \pm SEM of four experiments. *B*, Infected human macrophages were previously transfected or not with SOD1 siRNA and were cultured with or without IFN- β . Number of intracellular amastigotes was quantified as described in *Materials and Methods*. Each bar represents the mean \pm SEM of eight donors (one-way ANOVA, ***, $p = 0.0005$; Bonferroni's correction, $p < 0.001$). *C*, Supernatant in the presence or absence of hydroxylamine (0.5 mM) was collected at 24 and 48 h. Superoxide production was quantified as described in *Materials and Methods*. Each bar represents the mean \pm SEM of three donors (two-way ANOVA: SOD1 siRNA, **, $p = 0.0015$; time, *, $p = 0.030$; interaction, $p = 0.37$).

IFN- β , $p = 0.023$; time, $p < 0.0001$), although no significant interaction was observed between IFN- β and time (two-way ANOVA: interaction, $p = 0.37$) (Fig. 3*A*). Additionally, IFN- β treatment reduced superoxide release triggered by PMA and fMLP in uninfected human monocytes and macrophages (Fig. 3*B–D*), suggesting that IFN- β down-regulates human macrophage superoxide release triggered by several stimuli, instead of simply antagonizing the *L. amazonensis* effect.

IFN- β up-regulates the expression of SOD1 in human monocytes

Due to its primarily cytoplasmic location, the SOD1 isozyme rather than mitochondrial SOD2 or extracellular SOD3 might be

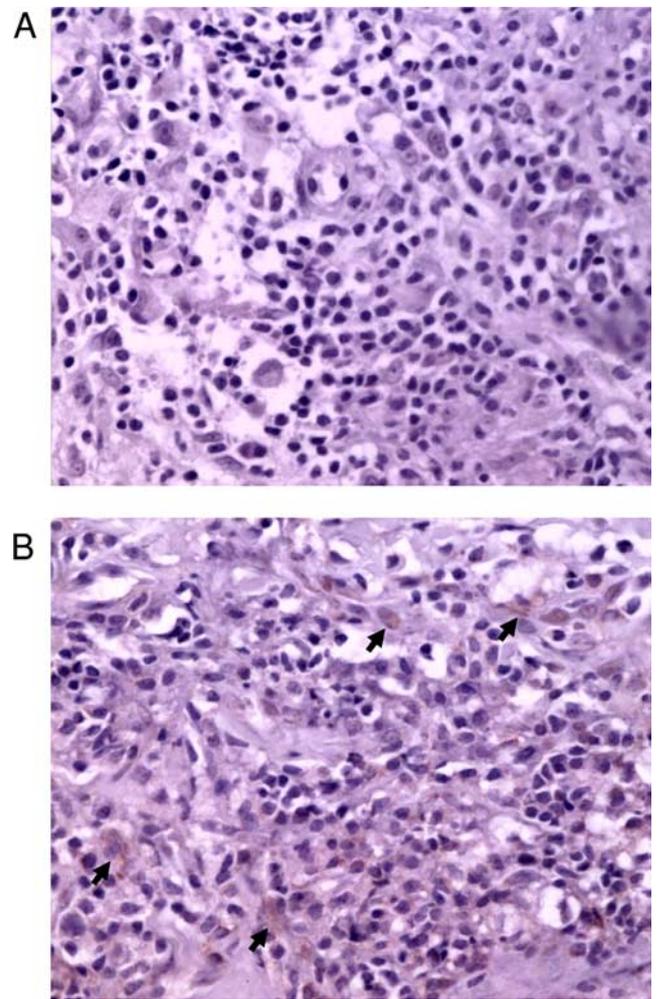


FIGURE 7. In situ expression of SOD1 in biopsies of New World cutaneous leishmaniasis patients. A representative biopsy of four patients with typical mononuclear inflammatory infiltrate (magnification $\times 400$) is shown. *A*, Negative IgG1 isotype control; *B*, anti-SOD1 mAb. Arrows show SOD1-positive macrophages.

responsible for the decrease in superoxide release measured upon IFN- β treatment. Therefore, we decided to evaluate the role of IFN- β in the regulation of SOD1 expression. We detected an almost 3-fold increase of SOD1 in soluble protein extracts of IFN- β -human uninfected monocytes when compared with control cells (Fig. 4) (Student's *t* test, $p = 0.023$), as quantified by ELISA.

SOD1 and SOD1 inhibitor DETC reciprocally regulate parasite killing in Leishmania-infected human macrophages

To test the hypothesis that SOD1 might be involved in *Leishmania* killing due to a decrease of superoxide release, DETC (a SOD1 inhibitor) and purified SOD1 enzyme were used. As expected, DETC enhanced superoxide release by 65%, whereas addition of SOD1 enzyme reduced superoxide release by 40% (Fig. 5*A*) (one-way ANOVA, $p = 0.025$; Bonferroni's correction, $p < 0.05$). As shown in Fig. 5*B*, parasite burden was strongly correlated to superoxide release and reciprocally regulated by both stimuli: SOD1 treatment led to a 124% increase in parasite load, whereas SOD1 inhibition by DETC resulted in total elimination of intracellular infection (Fig. 5*B*) (one-way ANOVA, $p = 0.0005$; Bonferroni's correction, $p < 0.0001$, $p <$

0.05), further stressing the importance of superoxide in human macrophage leishmanicidal activity.

Pharmacological or siRNA-mediated SOD1 inhibition antagonizes IFN- β -mediated blunting of leishmanicidal activity

To test the hypothesis that IFN- β -impaired *Leishmania* killing was mediated by SOD1, SOD1 inhibitor DETC and SOD1 siRNA were used in the absence or presence of IFN- β . As shown in Fig. 6A, DETC treatment completely antagonized the IFN- β -induced increase in parasite burden. Additionally, SOD1 siRNA significantly reverted (one-way ANOVA with Bonferroni's correction, $p < 0.001$) antileishmanicidal-IFN- β activity (Fig. 6B). Again, restoration of human macrophage leishmanicidal activity by SOD1 siRNA (Fig. 6C) was paralleled by significantly increased superoxide levels (two-way ANOVA, $p = 0.0015$). However, similar to Fig. 3A, no significant interaction was observed between siRNA and time (two-way ANOVA, interaction, $p = 0.37$).

SOD1 is expressed in situ in biopsies of New World cutaneous leishmaniasis patients

To explore a possible in vivo role for SOD1 in human leishmaniasis, we investigated whether SOD1 might be expressed in situ in cutaneous lesions of New World cutaneous leishmaniasis patients. As shown in Fig. 7, cutaneous biopsies of leishmaniasis patients presented a remarkable density of mononuclear inflammatory infiltrate, mainly composed of macrophages. These cells displayed pronounced SOD1 expression with intracytoplasmic patterns of staining (Fig. 7B), whereas no staining was observed with a control mAb (Fig. 7A).

Discussion

After a few decades of confinement as merely antiviral cytokines, type I IFNs (IFN- α/β) have gained considerable attention as immunomodulators in nonviral infectious diseases in recent years (24). However, no apparent picture of a protective vs deleterious role for type I IFNs emerges from the literature, with often contrasting effects for different doses and/or treatment schemes (25). We found that IFN- β dose-dependently increases parasite burden in *Leishmania*-infected human macrophages through a superoxide-dependent, NO-independent mechanism. Our results emphasize the striking differences between the human and murine models of Old World vs New World leishmaniasis, considering the conflicting role of type I IFN, as well as its dependence on NO. This implies that in vivo IFN- β production in human leishmaniasis might be deleterious, especially in clinical forms with high parasite burden, such as diffuse cutaneous and visceral leishmaniasis, despite the encouraging results in the murine (11) and human (12) *L. major* model. However, a small-scale clinical trial of topical IFN- β in human leishmaniasis caused by *L. major* was unsuccessful (26), showing neither a deleterious nor a protective effect of IFN- β treatment. In light of our present results, SOD-directed therapy, rather than type I IFN therapy, might be a valuable therapeutic option in human leishmaniasis. Most likely candidates are diffuse cutaneous leishmaniasis patients with typically nonhealing lesions and abundant parasitism, commonly refractory to standard antimonial treatment. In addition to a more pronounced effect upon parasite survival (Fig. 6A), pharmacological SOD inhibition seems more plausible than does siRNA-mediated SOD inhibition from an economical and clinical point of view. Moreover, the superior effect of DETC, as compared with SOD1 siRNA, might be partially mediated through inhibition of other host (mitochondrial SOD2 and extracellular SOD3) or even *Leishmania* SOD isoforms. A role for parasite SOD in intramacrophage survival has been previously described in *L. tropica* (27) and *L. chagasi* (28). Interestingly, treat-

ment of infected human macrophages with purified SOD1 in vitro led to strongly increased parasite burden and the appearance of large parasitophorous vacuoles (R. Khouri and J. Van Weyenbergh, unpublished results), reminiscent of typical nodular lesions in diffuse leishmaniasis (29). Moreover, the same histopathological pattern was observed in CBA mice infected with *L. amazonensis*, but not with *L. major* (30), confirming the striking differences between New World and Old World *Leishmania* species. Additionally, when compared with *L. major*, *L. amazonensis* was highly resistant to NO-mediated leishmanicidal activity in murine macrophages in vitro (31, 32), but Mukbel et al. (32) found parasite killing to be dependent on superoxide production, in agreement with our findings. In parallel with New World leishmanial infection, killing of *M. tuberculosis* was shown to be NO-independent in human macrophages (33), and *M. bovis* bacterial load was significantly increased upon IFN- α/β treatment (34). Hence, type I IFN inhibition of human macrophage leishmanicidal and bactericidal activity in vitro uncovers a striking similarity to TGF- β (35, 36), the major macrophage-deactivating cytokine. However, macrophage deactivation induced by both cytokines seems to operate through distinctive mechanisms. Although in this study, the IFN- β -mediated increase in parasite burden was superoxide-dependent, Chang et al. (37) have demonstrated that TGF- β has no effect upon superoxide levels during *L. chagasi* infection in human macrophages. Besides their antagonistic effect upon H₂O₂ (13), HLA-DR (38), transferrin receptor (39), and CD64 (14), IFN- β and IFN- γ seem to exert opposite effects on macrophage microbicidal activity (this study and Refs. 23, 34, 40). Taken together, an opposite role for type I and type II IFN might be a generalized finding in human infections with intracellular pathogens. Likewise, increased susceptibility to opportunistic infections has been occasionally described (41, 42) as an undesirable side effect during treatment of viral infections or cancer with type I IFN, without further evidence of the molecular and cellular mechanism(s) involved.

In this study, we have unexpectedly identified human SOD1 as an IFN-regulated protein, which had not been revealed in several microarray studies (43–46), including in human PBMCs (44–46). Since a significant increase of SOD1 protein was only observed following 48 h of IFN- β treatment, and array experiments have been limited to 2–24 h of IFN treatment (43–46), a delayed and, most probably, intermediate protein synthesis-dependent mechanism of SOD1 mRNA induction cannot be excluded. However, the absence of IFN regulation of SOD1 in previous studies might be due to posttranscriptional and/or cell-specific mechanisms. Therefore, an increase in SOD1 mRNA in monocytes might have been obscured in total PBMC RNA studies (44, 45). In fact, a major part of the in vivo effect of IFN- β might be monocyte- or myeloid cell-specific, as previously suggested by us in multiple sclerosis (14, 47) and recently demonstrated in an experimental autoimmune encephalomyelitis model (48).

Finally, further research will be necessary to explore the role of superoxide and/or SOD1 in the in vivo effects of IFN- β , of particular interest in immune-mediated and neurodegenerative diseases, such as multiple sclerosis, where IFN- β is currently a major therapeutic option (49), or HTLV-1-associated myelopathy/tropical spastical paraparesis, where IFN- β has been recently introduced (50).

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Disclosures

The authors have no financial conflicts of interest.

References

- Desjeux, P. 2004. Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis.* 27: 305–318.
- McMahon-Pratt, D., and J. Alexander. 2004. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniasis or the visceral disease? *Immunol. Rev.* 201: 206–224.
- Coffman, R. L., R. Chatelain, L. M. Leal, and K. Varkila. 1991. *Leishmania major* infection in mice: a model system for the study of CD4⁺ T-cell subset differentiation. *Res. Immunol.* 142: 36–40.
- Reiner, S. L., and R. M. Locksley. 1995. The regulation of immunity to *Leishmania major*. *Annu. Rev. Immunol.* 13: 151–177.
- Bogdan, C. 2008. Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. *Cell Microbiol.* 10: 1221–1234.
- Rocha, P. N., R. P. Almeida, O. Bacellar, A. R. de Jesus, D. C. Filho, A. C. Filho, A. Barral, R. L. Coffman, and E. M. Carvalho. 1999. Down-regulation of Th1 type of response in early human American cutaneous leishmaniasis. *J. Infect. Dis.* 180: 1731–1734.
- Ribeiro-de-Jesus, A., R. P. Almeida, H. Lessa, O. Bacellar, and E. M. Carvalho. 1998. Cytokine profile and pathology in human leishmaniasis. *Braz. J. Med. Biol. Res.* 31: 143–148.
- Carvalho, E. M., R. Badaró, S. G. Reed, T. C. Jones, and W. D. Johnson, Jr. 1985. Absence of gamma interferon and interleukin 2 production during active visceral leishmaniasis. *J. Clin. Invest.* 76: 2066–2069.
- Badaró, R., E. Falcoff, F. S. Badaró, E. M. Carvalho, D. Pedral-Sampaio, A. Barral, J. S. Carvalho, M. Barral-Netto, M. Brandely, L. Silva, et al. 1990. Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. *N. Engl. J. Med.* 322: 16–21.
- Diefenbach, A., H. Schindler, N. Donhauser, E. Lorenz, T. Laskay, J. MacMicking, M. Rollinghoff, I. Gresser, and C. Bogdan. 1998. Type 1 interferon (IFN α/β) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* 8: 77–87.
- Mattner, J., A. Wandersee-Steinhauser, A. Pahl, M. Rollinghoff, G. R. Majeau, P. S. Hochman, and C. Bogdan. 2004. Protection against progressive leishmaniasis by IFN- β . *J. Immunol.* 172: 7574–7582.
- Passwell, J. H., R. Shor, and J. Shoham. 1986. The enhancing effect of interferon- β and - γ on the killing of *Leishmania tropica major* in human mononuclear phagocytes in vitro. *J. Immunol.* 136: 3062–3066.
- Garotta, G., K. W. Talmadge, J. R. Pink, B. Dewald, and M. Baggiolini. 1986. Functional antagonism between type I and type II interferons on human macrophages. *Biochem. Biophys. Res. Commun.* 140: 948–954.
- Van Weyenbergh, J., P. Lipinski, A. Abadie, D. Chabas, U. Blank, R. Liblau, and J. Wietzerbin. 1998. Antagonistic action of IFN- β and IFN- γ on high affinity Fc γ receptor expression in healthy controls and multiple sclerosis patients. *J. Immunol.* 161: 1568–1574.
- Ribeiro-Gomes, F. L., A. C. Otero, N. A. Gomes, M. C. A. Moniz-de-Souza, L. Cysne-Finkelstein, A. C. Arnholdt, V. L. Calich, S. G. Coutinho, M. F. Lopes, and G. A. DosReis. 2004. Macrophage interactions with neutrophils regulate *Leishmania major* infection. *J. Immunol.* 172: 4454–4462.
- Saltzman, B. E. 1954. Colorimetric microdetermination of nitrogen dioxide in the atmosphere. *Anal. Chem.* 26: 1949–1955.
- Zhao, H., N. Dugas, C. Mathiot, A. Delmer, B. Dugas, F. Sigaux, and J. P. Kolb. 1998. B-cell chronic lymphocytic leukemia cells express a functional inducible nitric oxide synthase displaying anti-apoptotic activity. *Blood* 92: 1031–1043.
- Elstner, E. F., and A. Heupel. 1976. Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. *Anal. Biochem.* 70: 616–620.
- Miranda, K. M., M. G. Espey, and D. A. Wink. 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* 5: 62–71.
- Lipinski, P., and H. Retmanska. 1996. Horse spleen ferritin inhibits superoxide production by equine blood monocytes in vitro. *Free Radic. Biol. Med.* 20: 729–734.
- Rothe, G., and G. Valet. 1990. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J. Leukocyte Biol.* 47: 440–448.
- Mattner, J., H. Schindler, A. Diefenbach, M. Rollinghoff, I. Gresser, and C. Bogdan. 2000. Regulation of type 2 nitric oxide synthase by type 1 interferons in macrophages infected with *Leishmania major*. *Eur. J. Immunol.* 30: 2257–2267.
- Gantt, K. R., T. L. Goldman, M. L. McCormick, M. A. Miller, S. M. Jeronimo, E. T. Nascimento, B. E. Britigan, and M. E. Wilson. 2001. Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. *J. Immunol.* 167: 893–901.
- Stetson, D. B., and R. Medzhitov. 2006. Type I IFN in host defense. *Immunity* 25: 373–381.
- Bogdan, C., J. Mattner, and U. Schleicher. 2004. The role of type I interferons in non-viral infections. *Immunol. Rev.* 202: 33–48.
- Trau, H., M. Schewach-Millet, J. Shoham, T. Doerner, R. Shor, and J. H. Passwell. 1987. Topical application of human fibroblast interferon (IFN) in cutaneous leishmaniasis. *Isr. J. Med. Sci.* 23: 1125–1127.
- Ghosh, S., S. Goswami, and S. Adhya. 2003. Role of superoxide dismutase in survival of *Leishmania* within the macrophage. *Biochem. J.* 369: 447–452.
- Plewes, K. A., S. D. Barr, and L. Gedamu. 2003. Iron superoxide dismutases targeted to the glycosomes of *Leishmania chagasi* are important for survival. *Infect. Immun.* 71: 5910–5920.
- Barral, A., J. M. Costa, A. L. Bittencourt, M. Barral-Netto, and E. M. Carvalho. 1995. Polar and subpolar diffuse cutaneous leishmaniasis in Brazil: clinical and immunopathologic aspects. *Int. J. Dermatol.* 34: 474–479.
- Lemos de Souza, V., J. Ascencao Souza, T. M. Correia Silva, P. Sampaio Tavares Veras, and L. A. Rodrigues de-Freitas. 2000. Different *Leishmania* species determine distinct profiles of immune and histopathological responses in CBA mice. *Microbes Infect.* 2: 1807–1815.
- Gomes, I. N., A. F. Calabrich, S. Tavares Rda, J. Wietzerbin, L. A. de Freitas, and P. S. Veras. 2003. Differential properties of CBA/J mononuclear phagocytes recovered from an inflammatory site probed with two different species of *Leishmania*. *Microbes Infect.* 5: 251–260.
- Mukbel, R. M., C. Patten, Jr., K. Gibson, M. Ghosh, C. Petersen, and D. E. Jones. 2007. Macrophage killing of *Leishmania amazonensis* amastigotes requires both nitric oxide and superoxide. *Am. J. Trop. Med. Hyg.* 76: 669–675.
- Thoma-Uzynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolsckei, M. Wagner, et al. 2001. Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science* 291: 1544–1547.
- Bouchonnet, F., N. Boechat, M. Bonay, and A. J. Hance. 2002. Alpha/beta interferon impairs the ability of human macrophages to control growth of *Mycobacterium bovis* BCG. *Infect. Immun.* 70: 3020–3025.
- Barral, A., M. Teixeira, P. Reis, V. Vinhas, J. Costa, H. Lessa, A. L. Bittencourt, S. Reed, E. M. Carvalho, and M. Barral-Netto. 1995. Transforming growth factor β in human cutaneous leishmaniasis. *Am. J. Pathol.* 147: 947–954.
- Gantt, K. R., S. Schultz-Cherry, N. Rodriguez, S. M. Jeronimo, E. T. Nascimento, T. L. Goldman, T. J. Recker, M. A. Miller, and M. E. Wilson. 2003. Activation of TGF- β by *Leishmania chagasi*: importance for parasite survival in macrophages. *J. Immunol.* 170: 2613–2620.
- Chang, H. K., C. Thalhofer, B. A. Duerkop, J. S. Mehling, S. Verma, K. J. Gollob, R. Almeida, and M. E. Wilson. 2007. Oxidant generation by single infected monocytes after short-term fluorescence labeling of a protozoan parasite. *Infect. Immun.* 75: 1017–1024.
- Lu, H. T., J. L. Riley, G. T. Babcock, M. Huston, G. R. Stark, J. M. Boss, and R. M. Ransohoff. 1995. Interferon (IFN) beta acts downstream of IFN-gamma-induced class II transactivator messenger RNA accumulation to block major histocompatibility complex class II gene expression and requires the 48-kD DNA-binding protein, ISGF3-gamma. *J. Exp. Med.* 182: 1517–1525.
- Testa, U., L. Conti, N. M. Sposi, B. Varano, E. Tritarelli, W. Malorni, P. Samoggia, G. Rainaldi, C. Peschle, F. Belardelli, et al. 1995. IFN-beta selectively down-regulates transferrin receptor expression in human peripheral blood macrophages by a post-translational mechanism. *J. Immunol.* 155: 427–435.
- Murray, H. W., A. Szuro-Sudol, D. Wellner, A. J. Oca, A. M. Granger, D. M. Libby, C. D. Rothermel, and B. Y. Rubin. 1989. Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. *Infect. Immun.* 57: 845–849.
- Pesce, A., E. Rosenthal, H. Vinti, B. Taillan, G. Garnier, P. Dujardin, and J. P. Cassuto. 1993. Opportunistic infections and CD4 lymphocytopenia with interferon treatment in HIV-1 infected patients. *Lancet* 341: 1597.
- Toren, A., A. Ackerstein, D. Gazit, R. Or, D. Raveh, U. Kupolovic, D. Engelhard, and A. Nagler. 1996. Oral tuberculosis following autologous bone marrow transplantation for Hodgkin's disease with interleukin-2 and alpha-interferon immunotherapy. *Bone Marrow Transplant.* 18: 209–210.
- Der, S. D., A. Zhou, B. R. Williams, and R. H. Silverman. 1998. Identification of genes differentially regulated by interferon α , β , or γ using oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* 95: 15623–15628.
- Rani, M. R., J. Shrock, S. Appachi, R. A. Rudick, B. R. Williams, and R. M. Ransohoff. 2007. Novel interferon- β -induced gene expression in peripheral blood cells. *J. Leukocyte Biol.* 82: 1353–1360.
- Satoh, J., Y. Nanri, H. Tabunoki, and T. Yamamura. 2006. Microarray analysis identifies a set of CXCR3 and CCR2 ligand chemokines as early IFN β -responsive genes in peripheral blood lymphocytes in vitro: an implication for IFN β -related adverse effects in multiple sclerosis. *BMC Neurol.* 6: 18.
- Singh, M. K., T. F. Scott, W. A. LaFramboise, F. Z. Hu, J. C. Post, and G. D. Ehrlich. 2007. Gene expression changes in peripheral blood mononuclear cells from multiple sclerosis patients undergoing β -interferon therapy. *J. Neurol.* Sci. 258: 52–59.
- Van Weyenbergh, J., J. Wietzerbin, D. Rouillard, M. Barral-Netto, and R. Liblau. 2001. Treatment of multiple sclerosis patients with interferon- β primes monocyte-derived macrophages for apoptotic cell death. *J. Leukocyte Biol.* 70: 745–748.
- Prinz, M., H. Schmidt, A. Mildner, K. P. Knobeloch, U. K. Hanisch, J. Raasch, D. Merkler, C. Detje, I. Gutcher, J. Mages, et al. 2008. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. *Immunity* 28: 675–686.
- Tourbah, A., and O. Lyon-Caen. 2007. Interferons in multiple sclerosis: ten years' experience. *Biochimie* 89: 899–902.
- Oh, U., Y. Yamano, C. A. Mora, J. Ohayon, F. Bagnato, J. A. Butman, J. Dambrosia, T. P. Leist, H. McFarland, and S. Jacobson. 2005. Interferon- β 1a therapy in human T-lymphotropic virus type I-associated neurologic disease. *Ann. Neurol.* 57: 526–534.