

Immune Suppression by Recombinant Interleukin (rIL)-12 Involves Interferon γ Induction of Nitric Oxide Synthase 2 (iNOS) Activity: Inhibitors of NO Generation Reveal the Extent of rIL-12 Vaccine Adjuvant Effect

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Summary

Recombinant interleukin 12 (IL-12) can profoundly suppress cellular immune responses in mice. To define the underlying mechanism, recombinant murine (rm)IL-12 was given to C57BL/6 mice undergoing alloimmunization and found to transiently but profoundly suppress *in vivo* and *in vitro* allogeneic responses and *in vitro* splenocyte mitogenic responses. Use of neutralizing antibodies and genetically deficient mice showed that IFN- γ (but not TNF- α) mediated rmIL-12-induced immune suppression. Splenocyte fractionation studies revealed that adherent cells from rmIL-12-treated mice suppressed the mitogenic response of normal nonadherent cells to concanavalin A and IL-2. Addition of an inhibitor of nitric oxide synthase (NOS) restored mitogenic responses, and inducible (i)NOS^{-/-} mice were not immunosuppressed by rmIL-12. These results support the view that suppression of T cell responses is due to NO produced by macrophages responding to the high levels of IFN- γ induced by rmIL-12. When a NOS inhibitor was given with rmIL-12 during vaccination of A/J mice with irradiated SCK tumor cells, immunosuppression was averted and the extent of rmIL-12's ability to enhance induction of protective antitumor immunity was revealed. This demonstrates that rmIL-12 is an effective vaccine adjuvant whose efficacy may be masked by its transient immunosuppressive effect.

Key words: interleukin 12 • immunosuppression • interferon γ • nitric oxide • nitric oxide synthase 2

Interleukin (IL)-12 is an immunoregulatory cytokine with potent antitumor, antiviral, and antimicrobial effects (1, 2). Many of its activities are attributable to its ability to induce Th1 CD4⁺ T cell differentiation, CD8⁺ T cell cytotoxicity, and NK cell activation. IL-12 is proinflammatory through its ability to induce production of IFN- γ , TNF- α , GM-CSF, and other cytokines by T and NK cells. A particularly important mediator of IL-12 effect is IFN- γ , which, among other actions, activates macrophages and induces the production of nitric oxide (NO).¹ IFN- γ also acts on many

other types of cells, including tumor cells, and its ability to upregulate MHC expression, slow cell proliferation, and inhibit angiogenesis (3–5) may contribute to IL-12's antitumor effects.

Therapeutic use of rIL-12 can be accompanied by severe toxicities. Dose- and schedule-dependent toxicities have been seen during clinical trials (6, 7) and in mice (8). Administration of recombinant murine (rm)IL-12 during lymphocytic choriomeningitis virus (LCMV) infection in mice has been associated with adverse immunological effects manifest by higher viral loads, decreased antiviral CTL activity, and poorer outcome (9). While studying the effects of rmIL-12 during vaccination with genetically modified tumor cells, we identified a dose-dependent transient suppression of the immune response that was accompanied by suppressed *in vitro* splenocyte responses to T cell mitogens (10). These effects of high-dose rmIL-12 were generalized,

¹Abbreviations used in this paper: D-NAME, *N*-nitro-*D*-arginine methyl ester; D-NMMA, *N*-methyl-*D*-arginine; DTH, delayed-type hypersensitivity; iNOS, inducible nitric oxide synthase; LCMV, lymphocytic choriomeningitis virus; L-NAME, *N*-nitro-*L*-arginine methyl ester; L-NMMA, *N*-methyl-*L*-arginine; NO, nitric oxide; SCK.GM cells, SCK tumor cells engineered to secrete GM-CSF.

affecting responses to allogeneic vaccination and splenocyte mitogenic responses in naive mice of many strains, and appeared to result from impairment of immune effector function rather than failure to induce immunity. Studies described in this manuscript examine the mechanism of rmIL-12 immune suppression that appears to result from inhibition of T cell proliferation by NO generated by macrophages activated by the IFN- γ produced in response to rmIL-12. When inhibitors of NO generation were given with rmIL-12 during vaccination, it prevented immune suppression and allowed the potent vaccine adjuvant effect of rmIL-12 to be fully revealed.

Materials and Methods

Mice and Cell Lines. 5–8-wk-old female C57BL/6 (H-2^b) mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). IFN- γ ^{-/-} and iNOS^{-/-} C57BL/6 mice and wild-type controls were purchased from The Jackson Laboratory (Bar Harbor, ME). IFN- γ R1^{-/-} C57BL/6 \times SV129 mice and controls stemmed from breeding pairs that were gifts from Dr. Michel Aguet (University of Zurich, Zurich, Switzerland; reference 11). TNF- α p55 and p75 receptor^{-/-} C57BL/6 \times SV129 mice and controls were provided by Dr. Philip Scott and Michelle Nashleas (University of Pennsylvania, Philadelphia, PA) with permission from Genentech (South San Francisco, CA) and Dr. Horst Bluethmann of Roche Pharmaceuticals (Basel, Switzerland; references 12, 13). 5–8-wk-old female A/J (H-2^a) mice were purchased from The Jackson Laboratory. HKB cells were established from a spontaneous tumor that arose in an aged, unmanipulated female A/J mouse and are maintained in RPMI with 10% FCS and penicillin/streptomycin. They are MHC class I⁺ and are non-tumorigenic in A/J mice when 10⁶ cells are injected subcutaneously. SCK murine mammary carcinoma cells and SCK tumor cells engineered to secrete GM-CSF (SCK.GM cells) were maintained as previously described (10).

Immunization and Challenge. Immunization of C57BL/6 mice with HKB cells or A/J mice with SCK or SCK.GM cells was performed with cells suspended in PBS at 10⁷ trypan blue-excluding cells/ml. Cells were irradiated with 6,000 rads from a ¹³⁷Cs source, and mice were vaccinated with 10⁶ cells subcutaneously (day 0). Mice given rmIL-12 (Genetics Institute, Andover, MA) were injected intraperitoneally with 500 ng/day (all strains except A/J) or 250 ng/day (A/J strain) on days 0–4 and 7–11 (10 injections) unless otherwise noted, whereas control mice received PBS injections. Where indicated, vaccinated mice received 1 mg of anti-IFN- γ (XMG6) or anti-TNF- α (XT22) mAbs on days –1, 3, and 7. Where indicated, mice received 0.2 mg *N*-nitro-L-arginine methyl ester (L-NAME) or *N*-nitro-D-arginine methyl ester (D-NAME) on days 0–4 and 7–11. Vaccinated and naive A/J mice were challenged with 2.5 \times 10⁴ trypan blue-excluding SCK cells to assay for the presence of tumor immunity.

Mitogen and Alloantigen Stimulation of Splenocytes. In vitro mitogenic stimulation of splenocytes with 2.5 μ g/ml Con A or 100 U/ml rmIL-2 was performed as previously described (10). Proliferative responses to allogeneic antigens (MLR) were measured when splenocytes were stimulated with 10⁵ mitomycin C-treated A/J splenocytes. Splenocyte fractionation was performed by allowing 10⁵ splenocytes to adhere for 90 min in 96-well plates after which the nonadherent cells were removed and cocultured with adherent cells from different wells for assay. When added,

antibodies (XMG6 for IFN- γ , XT22 for TNF- α , AE5 for IL-10, and C17.8 for IL-12) were used at 10 μ g/ml final concentration and *N*-methyl-L-arginine (L-NMMA) and *N*-methyl-D-arginine (D-NMMA) were used at 500 μ M final concentration (Sigma Chemical Co., St. Louis, MO). After 72 h of exposure to mitogen, cultures were pulsed with 1 μ Ci [³H]thymidine for 16 h, cells were harvested and ³H incorporation was measured by scintillation counting. Supernatants from cultures assayed for IFN- γ by RIA (using antibodies AN18 and XMG6; reference 14) were harvested 24 or 72 h after stimulation.

Delayed Type Hypersensitivity Assessment. For assessment of allogeneic delayed type hypersensitivity (DTH) responses, mice were injected with 50 μ l PBS containing 10⁶ irradiated SCK cells in the right footpad and with 50 μ l PBS in the left footpad. Footpad thickness measurements were taken just before injection and 24 h later using a Starrett pocket gauge (Athol, MA). Data are presented as the difference in footpad swelling induced by SCK cells and by PBS.

Measurement of Nitrite Production. NO production measured as nitrite concentration in stimulated cell culture supernatants was measured by the Greiss assay (15). Supernatant (100 μ l) was added to 96-well plates; 100 μ l of a 1:1 mixture of 1% sulfanilamide dihydrochloride in 2.5% H₃PO₄ and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄ was then added to samples. Plates were incubated at room temperature for 10 min and A540 was determined using a microplate reader with reference to sodium nitrite standard curves. SNAP (S-nitroso-N-acetyl-penicillamine; Sigma Chemical Co.) was used as an acellular source of NO in cultures.

Results

IFN- γ Mediates rmIL-12-induced Immunosuppression. In studies of rmIL-12 effects in A/J mice on vaccination with irradiated SCK tumor cells, we previously had found that high doses of the cytokine transiently suppressed tumor protection in vivo and proliferative responses of splenocytes to T cell mitogens in vitro (10). We also had found that rmIL-12 exerted similar effects (suppressed in vivo DTH and in vitro mitogenic and alloproliferative responses) in C57BL/6 (H-2^b) mice vaccinated with irradiated allogeneic HKB (H-2^a) cells. Since most effects of IL-12, both beneficial and inhibitory, are mediated by IFN- γ , and because TNF- α is implicated in rmIL-12-suppressive effects during LCMV infection (16), we examined the roles of these two cytokines in rmIL-12-induced suppression of responses to alloimmunization. Studies using neutralizing mAbs in C57BL/6 mice given irradiated HKB cells and a course of rmIL-12 on days 0–4 and 7–11 showed that XMG6 (anti-IFN- γ) completely restored DTH responses, XT22 (anti-TNF- α) only partially restored responses, and XMG6 + XT22 restored responses no better than XMG6 alone (data not shown). Although these results suggested that IFN- γ is crucial for rmIL-12 suppression of immune responses and that the role of TNF- α is less certain, we examined the role of these cytokines more definitively by testing the effect of rmIL-12 on alloimmunization in mice genetically deficient for these cytokines or their receptors. After vaccination with HKB cells, a course of rmIL-12-suppressed DTH responses to background levels in wild-

type C57BL/6 mice but had no suppressive effect in IFN- γ ^{-/-} C57BL/6 mice (Fig. 1 A). Similarly, rmIL-12 suppressed DTH responses in wild-type but not in IFN- γ R1^{-/-} C57BL/6 \times SV129 mice vaccinated with HKB cells (data not shown). From these results, we conclude that IFN- γ is crucial for rmIL-12-induced immunosuppression. To examine the role of TNF- α , we studied C57BL/6 \times SV129 mice deficient for both the p55 and p75 TNF- α receptors. HKB-vaccinated TNFR^{-/-} mice treated with rmIL-12 had depressed DTH responses like wild-type C57BL/6 \times SV129 mice (Fig. 1 B), indicating that TNF- α responses were dispensable for rmIL-12 immune suppression. However, DTH responses without rmIL-12 were lower in the TNFR^{-/-} mice, suggesting that TNF- α responses might be necessary for maximal responses.

In our earlier studies, suppression of *in vitro* splenocyte mitogenic responses correlated well with suppression of *in vivo* immune responses. This correlation held up in studies of IFN- γ ^{-/-} and TNFR^{-/-} mice: Con A, IL-2, and allogeneic stimulation of splenocytes from rmIL-12-treated IFN- γ ^{-/-} mice resulted in normal proliferative responses, whereas responses of splenocytes from rmIL-12-treated TNFR^{-/-} mice were suppressed (data not shown).

Adherent Cells Mediate IL-12-induced Suppression of Splenocyte Mitogenesis. To identify the cell population responsible for the suppressed splenocyte mitogenic responses, splenocytes from C57BL/6 mice given rmIL-12 or PBS were fractionated by adherence to plastic and reconstituted in various combinations of adherent and nonadherent cells before stimulation with Con A or IL-2. As expected, cultures of adherent and nonadherent splenocytes from rmIL-12-treated mice had suppressed mitogenic responses compared with cultures of adherent and nonadherent cells from

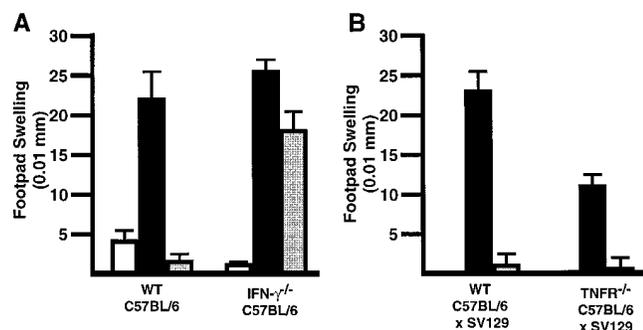


Figure 1. IFN- γ but not TNF- α is required for rmIL-12-induced immunosuppression. (A) C57BL/6 mice genetically deficient for IFN- γ and wild-type controls were vaccinated with 10^6 irradiated HKB cells and given 500 ng rmIL-12 or PBS on days 0–4 and 7–11 or left unvaccinated. They were challenged with 10^6 irradiated SCK cells in the right foot, with PBS control injection in the left foot on day 12. Mean footpad swelling (+ SE) is presented for unvaccinated mice (white bars; two mice) and vaccinated mice given PBS (black bars; four mice) or rmIL-12 (gray bars; four mice). (B) C57BL/6 \times SV129 mice deficient for both the p55 and p75 TNF- α receptors and control mice were vaccinated with 10^6 irradiated HKB cells and given 500 ng rmIL-12 (gray bars) or PBS (black bars) on days 0–4 and 7–11. DTH assessment was performed on day 12 as described and mean footpad swelling (+ SE, three mice per group) is presented.

PBS-treated mice (Fig. 2). Nonadherent cells from rmIL-12-treated mice cocultured with adherent cells from PBS-treated mice had normal mitogenic responses, indicating that mitogenesis of T cells from rmIL-12-treated mice is not intrinsically or irreversibly defective. When nonadherent cells from control mice were mixed with adherent cells from rmIL-12-treated mice, proliferative responses were severely impaired whether the stimulus was Con A, IL-2, or alloantigen (Fig. 2). These results indicate that T cells from rmIL-12-treated mice can respond to mitogens and generate an antigen-specific mitogenic response in the presence of adherent cells from normal mice, and that adherent cells are largely responsible for the defect after rmIL-12 therapy.

IFN- γ was readily detected by RIA in cocultures of adherent cells from rmIL-12-treated mice and nonadherent cells from PBS-treated mice at both 24 and 72 h after stimulation with Con A, IL-2, or alloantigen (data not shown). Addition of anti-IFN- γ antibody to these cocultures restored mitogenic responses, whereas addition of antibodies to IL-12, IL-10, or TNF- α had little effect (Fig. 3 A). These antibodies did not suppress mitogenic responses in cocultures containing adherent and nonadherent splenocytes from PBS-treated mice (data not shown), indicating that they had no intrinsic suppressive effects that could have shrouded beneficial effects of cytokine neutralization. These data support the results of *in vivo* experiments showing a critical role for IFN- γ in rmIL-12 immune suppression.

Adherent Cell-derived NO Inhibits Proliferative and Immune Responses. Knowing that adherent cells are important for

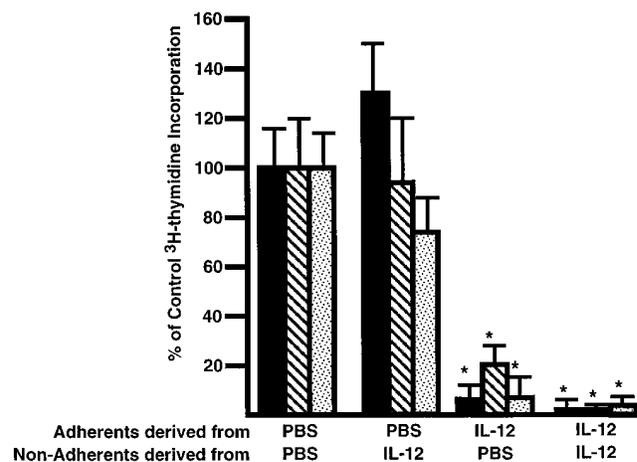


Figure 2. Adherent cells from rmIL-12-treated mice confer immunosuppression. Splenocytes from rmIL-12- and PBS-treated C57BL/6 mice were allowed to adhere to 96-well plates for 90 min. Nonadherent cells were overlaid on adherent cells and cocultures were stimulated. Data are from one of five experiments that produced similar results and are shown as the percentage (+ SE) of stimulation elicited in cultures of adherent and nonadherent cells from spleens of PBS-treated mice. Cocultures were stimulated with either 2.5 μ g/ml Con A (black bars), 100 U/ml IL-2-treated (hatched bars) or 10^5 mitomycin C-treated A/J (H-2^a) splenocytes (stippled bars). Data are from triplicate determinations and are significantly different from control mixtures (adherent and nonadherent cells from PBS treated mice, $P < 0.05$) where indicated (*).

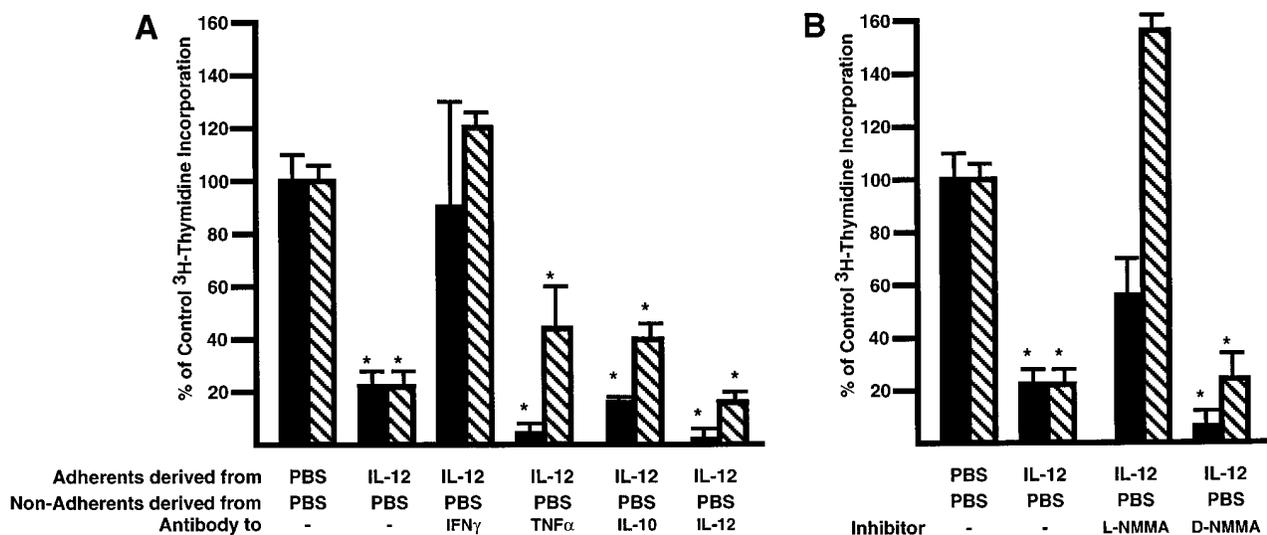


Figure 3. IFN- γ -induced NO mediates rmIL-12-induced immunosuppression. Cocultures were established from splenic adherent cells and nonadherent cells of PBS-treated mice or from the adherent cells of rmIL-12-treated mice and nonadherent cells from PBS-treated mice. (A) Antibodies XMG6 (to IFN- γ), XT22 (to TNF- α), AE5 (to IL-10), and C17.8 (to IL-12) were added to a final concentration of 10 mg/ml in cocultures containing adherent cells from spleens of rmIL-12-treated mice. Data from Con A- (black bars) and IL-2-stimulated (hatched bars) cultures are from triplicate determinations and are significantly different from control cocultures ($P < 0.05$) where indicated (*). (B) L-NMMA and D-NMMA were added to the coculture containing adherent cells from rmIL-12-treated mice at a final concentration of 500 mM. Data from Con A- (black bars) and IL-2-stimulated (hatched bars) cultures are from triplicate determinations and are significantly different from control cocultures ($P < 0.05$) where indicated (*).

rmIL-12 suppression of in vitro mitogenic and immunological responses and that IFN- γ is necessary for this effect, we considered that NO from activated macrophages might mediate suppression. To examine this possibility, we added an inhibitor of iNOS, L-NMMA, to cocultures of adherent cells from rmIL-12-treated mice and nonadherent cells from control mice. We found that it reduced NO levels in the culture supernatant by 58 and 94% in two independent measurements and restored mitogenesis (Fig. 3 B) when compared with addition of D-NMMA, a noninhibitory isoform. If secreted NO is responsible for suppression of mitogenesis, an acellular source of NO should have a similar effect. We added SNAP (S-nitroso-*N*-acetyl-penicillamine), an NO donor, to splenocytes from HKB-vaccinated C57BL/6 mice and found that cultures with NO levels as low as 2.6–3.5 mM inhibited mitogenic responses 83–98%. Together, these data suggest that adherent splenocytes (probably macrophages) activated by rmIL-12 treatment to secrete NO are responsible for impaired T cell mitogenic responses.

The ability of an iNOS inhibitor to reverse rmIL-12-induced suppression of mitogenesis in vitro suggested that mice lacking iNOS might be resistant to the immunosuppressive effects of rmIL-12. iNOS^{-/-} and wild-type C57BL/6 mice were vaccinated with irradiated HKB cells and given a course of rmIL-12 or PBS. iNOS^{-/-} mice receiving rmIL-12 had DTH responses that were at least as great as those of PBS-treated iNOS^{-/-} and wild-type mice and that were substantially higher than those of wild-type mice given rmIL-12 (Fig. 4 A). Although rmIL-12 induced splenomegaly in iNOS^{-/-} mice as in wild-type mice (17),

their splenocytes had proliferative responses like those of splenocytes from control mice after in vitro stimulation with mitogens or alloantigens (Fig. 4 B). Together, these data show that macrophage-derived NO is essential for rmIL-12-

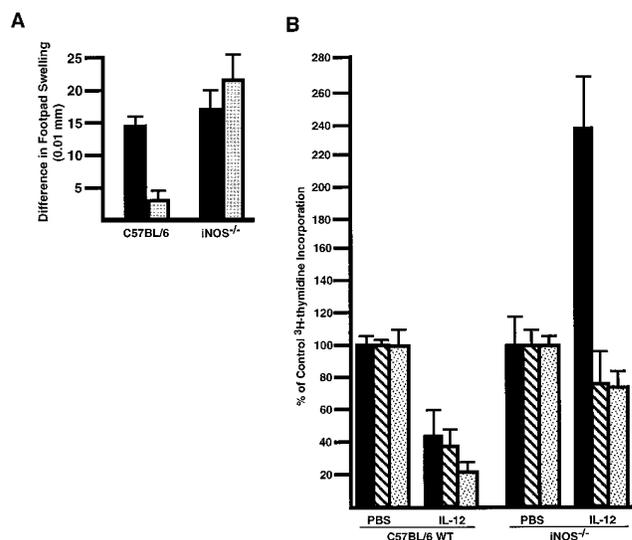


Figure 4. iNOS^{-/-} mice do not experience rmIL-12-induced immunosuppression. iNOS^{-/-} and wild-type C57BL/6 mice were vaccinated with irradiated HKB cells and received rmIL-12 (gray bars) or PBS (black bars) injections. (A) Footpad injections for DTH assessment were performed on day 12, and swelling 24 h later is presented as the mean (+ SE) from three mice in each treatment group. (B) Mitogenic (Con A, black bars; IL-2, hatched bars) and allogeneic stimulation (stippled bars) of splenocytes was performed as described in the legends to Figs. 2 and 3.

induced immunosuppression, whereas rmIL-12-induced splenomegaly and associated pathological changes are not.

An Inhibitor of NO Generation Prevents rmIL-12 Suppression of Vaccine Efficacy and Reveals the Extent of rmIL-12 Adjuvant Activity. Finding that rmIL-12 does not suppress allogeneic responses in iNOS^{-/-} mice, we investigated whether iNOS inhibitors would prevent immunosuppression in mice given rmIL-12 during tumor cell vaccination. Previously, we showed that vaccinating A/J mice with irradiated SCK.GM cells was highly protective, but that administration of rmIL-12 abrogated protection 2 wk after vaccination (but had no deleterious effect 4 wk after vaccination) (18). We gave A/J mice undergoing SCK.GM vaccination and rmIL-12 treatment either L-NAME, an inhibitor of iNOS that acts similarly to L-NMMA, or D-NAME, the inactive isoform. As expected, SCK.GM vaccination protected the great majority of mice from tumor cell challenge 2 wk after vaccination (19% developed tumors), and rmIL-12 severely impaired this protection (94% developed tumors). L-NAME, but not D-NAME, prevented this impairment (Fig. 5; the difference in tumorigenesis between rmIL-12-treated mice given L-NAME versus either D-NAME or nothing is significant at $P < 0.05$). In mice not treated with rmIL-12, L-NAME, and D-NAME had no effect on SCK.GM-induced protection (data not shown), showing that L-NAME acts by preventing rmIL-12 suppression of SCK.GM vaccine efficacy. rmIL-12 also impairs tumor protection in A/J mice with established SCK immunity if it is given just before tumor cell rechallenge (18). We found that L-NAME but not D-NAME given with the rmIL-12 prevented this impair-

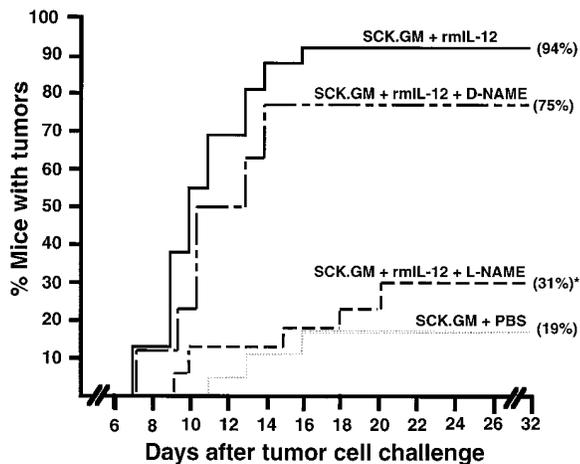


Figure 5. Inhibition of iNOS function reverses rmIL-12 suppression of immunologic protection. Female A/J mice were vaccinated with 10^6 irradiated SCK.GM cells and received either PBS (gray lines), rmIL-12 (solid black lines), rmIL-12 + L-NAME (hatched black lines), or rmIL-12 and D-NAME (double dashed black lines) on days 0–4 and 7–11. Mice were challenged 14 d after vaccination with 2.5×10^4 SCK cells in the opposite flank. Tumorigenesis was scored daily. The * designates statistical differences at $P < 0.05$ for rmIL-12- and L-NAME-treated mice versus rmIL-12- and D-NAME-treated mice and rmIL-12- and L-NAME-treated mice versus rmIL-12-treated mice. Data are compiled from two separate experiments that produced consistent results (15–17 mice per group total).

ment of immune rejection: only 25% of rmIL-12-treated mice given L-NAME developed tumors, whereas 75% of rmIL-12-treated mice given D-NAME developed tumors (data not shown). Thus, L-NAME prevents rmIL-12 suppression of established antitumor immune responses. In these studies, levels of NO were not consistently measurable in mice given rmIL-12 (at or below the sensitivity limits of the assay), so lower levels in mice also given L-NAME could not be demonstrated.

Previously, we had shown that vaccination of A/J mice with irradiated wild-type SCK cells protected only ~10% of mice from a tumor cell challenge, i.e., SCK cells are intrinsically poorly immunogenic (18). Giving rmIL-12 with vaccination did not improve protection when mice were challenged 14 d after vaccination but did improve protection when they were challenged at 28 d. Since an iNOS inhibitor prevented transient immunosuppression by rmIL-12, we asked whether its use might reveal rmIL-12's effectiveness as a vaccine adjuvant at the earlier time point. As shown in Fig. 6, only 38% of mice given L-NAME with irradiated SCK cells and rmIL-12 developed tumors when they were challenged on day 14, whereas 75% of mice given D-NAME developed tumors. This indicated that rmIL-12 improves SCK cell vaccine efficacy markedly and rapidly but that the improvement at day 14 was obscured by rmIL-12's immunosuppressive effect. The level of protection with L-NAME at 14 d (62%) was similar to the level of protection seen at 28 d in SCK-vaccinated mice given rmIL-12 alone (75%) or rmIL-12 with L-NAME (50%) or D-NAME (50%), indicating that use of L-NAME did not impair long-term protection afforded by rmIL-12 and SCK vaccination.

Use of an iNOS inhibitor to alleviate rmIL-12 immune suppression would be problematic if it reduces the antitu-

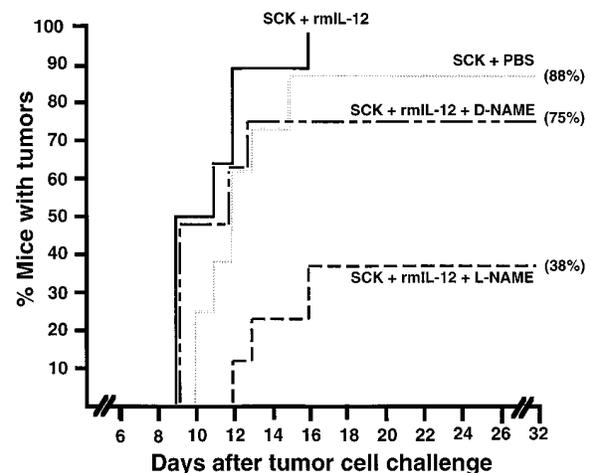


Figure 6. Inhibition of iNOS function reveals rmIL-12 adjuvant effects. Female A/J mice (8 mice per group) were vaccinated with 10^6 irradiated SCK cells and received either PBS (gray lines), rmIL-12 (solid black lines), rmIL-12 + L-NAME (hatched black lines), or rmIL-12 and D-NAME (double dashed black lines) on days 0–4 and 7–11. Mice were challenged 14 d after vaccination with 2.5×10^4 SCK cells in the opposite flank. Tumorigenesis was scored daily.

mor efficacy of the cytokine, an important consideration since the antitumor effects of rmIL-12 are diverse and some are not immunological (3, 4). Thus, we tested the effect of L-NAME on the antitumor activity of rmIL-12 against SCK tumors. Similar to what was seen in the past (19), rmIL-12 injections started on the day of SCK cell injection delayed tumor appearance by about 5 d (Fig. 7; medians of 9 d to tumor appearance without rmIL-12 and 14 d to tumor appearance with rmIL-12). Mice given L-NAME with their rmIL-12 developed tumors after a median of 17 d which was 8 d later than in untreated mice and 3 d later than in mice given rmIL-12 alone or with D-NAME. L-NAME given without rmIL-12 had no effect on SCK tumorigenesis (data not shown). Together, these data indicate that iNOS inhibitors can prevent rmIL-12 immune suppression and reveal its effectiveness as a vaccine adjuvant without adversely affecting rmIL-12 antitumor activity.

Discussion

rmIL-12 is a proinflammatory cytokine with potentially useful effects against tumors and infectious agents. However, recently it has been shown to suppress cellular immune responses in mice (10). To understand the mechanisms underlying this suppression without the confounding influence of tumor burden or host infection with pathogens, we studied the effects of rmIL-12 on allogeneic immune responses. In vivo and in vitro evidence indicates that alloimmunization is transiently but profoundly suppressed by high-dose rmIL-12. It seems to impair immune effector mechanisms because responses in mice with established immunity are also suppressed, and may not impair

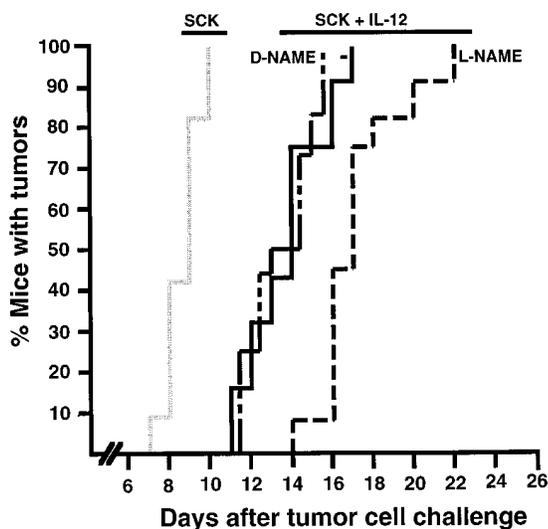


Figure 7. Inhibition of iNOS function enhances rmIL-12–induced delay of SCK tumorigenesis. Female A/J mice were injected with 2.5×10^4 SCK cells and either PBS (gray lines), rmIL-12 (solid black lines), rmIL-12 + L-NAME (hatched black lines), or rmIL-12 and D-NAME (double dashed black lines) on days 0–4 and 7–11. Tumorigenesis was scored daily. Data are compiled from two separate experiments that produced consistent results (11–12 mice per group total).

induction of immunity because rmIL-12 given during tumor cell vaccination provides enhanced protective antitumor immunity after the period of immunosuppression. rmIL-12 impairment of cellular immune responses is consistently associated with and likely due to impaired T cell mitogenic responses. In this study, we provide in vivo and in vitro evidence that anti-IFN- γ , but not antibodies to other cytokines, prevents rmIL-12–induced suppression. The importance of IFN- γ was confirmed by studies in IFN- $\gamma^{-/-}$ and IFN- γ R1 $^{-/-}$ mice that are not immunosuppressed by rmIL-12. Studies using splenocyte fractionation, an inhibitor of NO generation, and iNOS $^{-/-}$ mice revealed that adherent cells of the spleen, through stimulated production of NO by iNOS, are responsible for suppressing T cell mitogenesis in vitro and cellular immune responses in vivo. Having identified the probable mechanism, we used an iNOS inhibitor with rmIL-12 during tumor cell vaccination that prevented immune suppression and allowed the full extent of rmIL-12 adjuvant activity to be revealed.

These observations led us to propose that events leading to immune suppression by high-dose rmIL-12 administration are initiated by its induction of IFN- γ production by host lymphocytes. Levels of IFN- γ production high enough to activate macrophages and induce iNOS activity generate levels of NO that impair the proliferation of T cells in response to mitogens. That adherent cells rather than T cells are primarily responsible for the pathogenesis of rmIL-12 immune suppression is supported by the fact that T cells from the spleens of rmIL-12–treated mice are normally mitogenic when cocultured with adherent cells from normal mice or from rmIL-12–treated iNOS $^{-/-}$ mice. Impairment is transient presumably because T cell proliferative responses recover as IFN- γ production and consequent macrophage activation wanes after completion of rmIL-12 therapy. How NO impairs T cell proliferative responses is uncertain, but a recent study indicates that NO allows initial T cell activation up to and including IL-2 receptor expression and IL-2 production but impedes effective signal transduction by reversibly inhibiting JAK3 and STAT5 phosphorylation (20). Other studies have suggested that NO may induce apoptosis in conjunction with drug therapy or infection (21, 22).

Our identification of NO as a mediator of rmIL-12–induced immunosuppression is consistent with its known activities. NO, a key component of host defense mechanisms against invading pathogens, is produced by iNOS in macrophages activated by IFN- γ and other cytokines. Although other NOS (nNOS or NOS1 and eNOS or NOS3) also generate NO, iNOS (or NOS2) is the high-output source of NO, and phagocytic cells are the major source of iNOS during inflammation (23). Impaired splenocyte mitogenesis during *Salmonella typhimurium*, *Trypanosoma cruzi*, *Toxoplasma gondii*, and *Listeria monocytogenes* infections is due to NO production associated with high levels of IFN- γ , endogenous IL-12, and/or other proinflammatory cytokines (24–27). When splenocytes from these mice are fractionated, the adherent population con-

taining macrophages and producing NO appears to suppress splenic T cell mitogenic responses, and this suppression is reversed by inhibitors of iNOS (28). Thus, diverse processes that induce NO production by macrophages (24, 25, 29) can impair T cell proliferative responses. To this list can be added high-dose rmIL-12 that, through induction of high levels of IFN- γ and in the absence of additional inflammatory stimuli, sufficiently activates macrophages to engender immunosuppression.

Although NO is a mediator of impaired T cell mitogenesis, the cytokines that impair mitogenesis or induce NO production may vary with the circumstance. In studies of parasite infection, IFN- γ appears to play a significant role, and IL-10 plays a minor role in impaired mitogenesis (30). In studies of rmIL-12-induced immunosuppression during LCMV infection, TNF- α has been implicated as a mediator (16). Although endogenously produced IL-12 is important for controlling *Toxoplasma gondii* infection, it also suppresses splenocyte mitogenic responses seen during infection (24). IL-12-directed pathways are also implicated in immune suppression after vaccination with attenuated *Salmonella typhimurium* (26). These studies raise the point that agents that naturally induce strong inflammatory responses may engender immunosuppression through endogenous production of proinflammatory cytokines. The specific or spectrum of cytokines involved may vary with the initiating agent, but suppression of antigen-specific immune responses is likely to develop if the result is vigorous activation of phagocytic cells and their production of NO. In our studies of rmIL-12-induced immune suppression, we were only able to identify IFN- γ as an essential mediator, which may reflect the simplicity or paucity of pathways activated by rmIL-12 compared with infectious agents. The importance of other proinflammatory cytokines for suppressing T cell responses associated with infection and giving rmIL-12

during LCMV infection probably reflects a contribution of these cytokines to the inflammatory response and macrophage activation under these circumstances.

Therapeutic applications of rmIL-12 may benefit from reduction or elimination of its transient immunosuppressive side effects, and the mechanism elucidated here suggests several potential approaches. Reducing or eliminating rmIL-12 immunosuppression by using lower or fewer doses of the cytokine is one option. Although this approach may be beneficial (31), finding the "proper" regimen of rmIL-12 may be involved, and the results may be idiosyncratic, e.g., we have not found a lower dose of rmIL-12 that improves its adjuvant effect during tumor cell vaccination (data not shown). Inhibiting IFN- γ action is another alternative for avoiding rmIL-12 immunosuppression but is an approach that would be difficult to implement (other than in mice) and that may be self-defeating because IFN- γ may be an essential mediator of rmIL-12 therapeutic effects. The promising approach tested here, inhibition of NO generation, alleviates rmIL-12 immune suppression and allows its adjuvant effects to be fully revealed. This finding suggests that some studies testing rmIL-12 as an immunological adjuvant should be reevaluated since studies performed during the period of immune suppression are unlikely to reflect its true adjuvant potential. Of course, use of inhibitors of NO generation in conjunction with rmIL-12 must be examined for its independent effect on desired therapeutic endpoints. As use of the iNOS inhibitor, L-NAME, enhanced and did not impair rmIL-12's limited efficacy against SCK tumors, our studies do not reveal any problems with its use. Clearly, it will be important to determine whether recombinant human IL-12 produces similar immune suppressive effects in patients and, if so, whether inhibitors of iNOS function might be beneficial in this setting.

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