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18β-Glycyrrhetinic Acid Triggers Curative Th1 Response and Nitric Oxide Up-Regulation in Experimental Visceral Leishmaniasis Associated with the Activation of NF-κB[1]

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The efficacy of 18β-glycyrrhetinic acid (GRA), a pentacyclic triterpene belonging to the β-amyrin series of plant origin, was evaluated in experimental visceral leishmaniasis. GRA is reported to have antitumor and immunoregulatory activities, which may be attributable in part to the induction of NO. Indeed, an 11-fold increase in NO production was observed with 20 μM GRA in mouse peritoneal macrophages infected with *Leishmania donovani* promastigotes. In addition to having appreciable inhibitory effects on amastigote multiplication within macrophages (IC_{50}, 4.6 μg/ml), complete elimination of liver and spleen parasite burden was achieved by GRA at a dose of 50 mg/kg/day, given three times, 5 days apart, in a 45-day mouse model of visceral leishmaniasis. GRA treatment resulted in reduced levels of IL-10 and IL-4, but increased levels of IL-12, IFN-γ, TNF-α, and inducible NO synthase, reflecting a switch of $CD4^+$ differentiation from Th2 to Th1. This treatment is likely to activate immunity, thereby imparting resistance to reinfection. GRA induced NF-κB migration into the nucleus of parasite-infected cells and caused a diminishing presence of IκB in the cytoplasm. The lower level of cytoplasmic IκBα in GRA-treated cells resulted from increased phosphorylation of IκBα and higher activity of IκB kinase (IKK). Additional experiments demonstrated that GRA does not directly affect IKK activity. These results suggest that GRA exerts its effects at some level upstream of IKK in the signaling pathway and induces the production of proinflammatory mediators through a mechanism that, at least in part, involves induction of NF-κB activation. *The Journal of Immunology,* **2005, 175: 1161–1169.**

Visceral leishmaniasis is a fatal and chronic protozoan infection caused by *Leishmania donovani*, an obligate intramacrophage parasite (1). Currently available antileishmanial drugs are few, and the regimen used is lengthy. Moreover, they produce serious side effects, and the refractory cases are a problem (2). The disease is associated with immunological dysfunctions of T cells, NK cells, and macrophages (3–5). Studies performed on mice infected with *Leishmania* demonstrated that host defense against this infection depends on IL-12-driven expansion of the Th1 subset, with production of cytokines such as IFN-γ that activate macrophages for parasite killing through the release of NO (6). NO, which mediates many of the nonspecific cytotoxic and inflammatory responses of macrophages after infection by pathogens, is generated after the up-regulation of expression of the inducible form of NO synthase (iNOS).[4] Similarly, the biological activity of IL-12 (a heterodimeric cytokine comprising p35 and p40 subunits), which is the key cytokine driving Th1 cell development (7, 8), is regulated by the induction of the p40 subunit (9). The expression of these immunomodulatory proteins appears to be regulated primarily at the level of transcription; indeed, NF-κB activation has been shown to be a key factor in the regulation of induction in both cases (10–12). The NF-κB family is comprised of five members: NF-κB1 (p50/p105 (p50 precursor)), NF-κB2 (p52/p100 (p52 precursor)), Rel A (p65), Rel B (p68), and c-Rel (p75) (13). Although most members are capable of maintaining homo- and heterodimeric complexes, the most prevalent activated form of NF-κB is the heterodimer p50-p65, which possesses the transactivity domains necessary for gene regulation (11, 14, 15). In most cells, NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm, but upon activation by extracellular stimuli or other factors, NF-κB rapidly translocates to the nucleus and activates gene expression (12). The exact molecular mechanism by which various extracellular stimuli lead to the activation of NF-κB is not well understood. However, most signals induce the activity of a large multisubunit protein kinase, called IκB kinase (IKK). Activation of the IKK complex leads to specific IκBα phosphorylation/degradation and subsequent release of NF-κB, which then translocates to the nucleus and activates transcription of multiple κB-dependent genes, including iNOS and Th1 cytokines. The ability to interfere with the degradation of IκB is one of the strategies used by some pathogens to inhibit the action of NF-κB. Although the exact mechanism is still unclear, it has been shown that *L. donovani* promastigotes are able to prevent phosphorylation and subsequent degradation of IκB in normal bone marrow-derived macrophages, which effectively shuts down the NF-κB-dependent expression of proinflammatory cytokines (16). Thus, the promastigotes successfully avoid activating the macrophages for the production of NO. Activation of the NF-κB pathway may, therefore, prove a promising approach for the therapy of visceral leishmaniasis.

Despite the tremendous progress in synthetic chemistry, medicinal plants continue to play an important role as a source of biologically active agents (17). Of late, a large number of dietary

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[4] Abbreviations used in this paper: iNOS, inducible NO synthase; GRA, 18β-glycyrrhetinic acid; IKK, IκB kinase; LDU, Leishman-Donovan unit; NMMA, N^G-monomethyl-L-arginine; pNPP, *p*-nitrophenylphosphate; SLA, soluble leishmanial Ag.

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components have been evaluated as potential chemopreventative agents (18). The root of the medicinal plant licorice (*Glycyrrhizza glabra* L) is widely used as a flavoring and sweetening agent in food products. Its major water-soluble component, 18β-glycyrrhetinic acid (GRA), a pentacyclic triterpene derivative of the β-amyrin type, has long been used in traditional or folk medicine of the east. It is known to exhibit a variety of pharmacological effects, including antiulcerative, antihepatotoxic, and immunoregulatory activities (19–21). GRA also produces a wide variety of antitumor activities, including inhibition of tumorigenesis (22) and tumor production (23) and the induction of tumor cell differentiation (24). Immunomodulatory and antitumor effects of GRA may be mediated by the released NO from macrophages; recent reports have demonstrated that GRA stimulates NO and is able to upregulate iNOS through NF-κB transactivation in resting macrophages (25). However, the mechanism of action for GRA is not well understood.

Because the L-arginine-NO pathway is implicated in the macrophage-induced killing of *Leishmania* spp., we tested the capability of GRA to establish and maintain a Th1 curative response for visceral leishmaniasis and to redirect the Th2 response associated with disease progression. Our investigation was also aimed toward understanding the molecular mechanism of GRA action on the IκB/NF-κB signaling pathway, which may be associated with suppressing the functional differentiation of Th2-type $CD4^+$ T cells and, in turn, augmenting Th1 response. In this study we present data demonstrating that GRA can induce the generation of NO and proinflammatory cytokines, resulting in the abrogation of parasite infection. Our results also demonstrate that GRA induces IκBα phosphorylation and degradation by activating IKK in macrophages, leading to enhancement of NF-κB DNA binding activity and translocation of NF-κB p65 into the nucleus.

Materials and Methods

Parasite, NO production, and in vitro killing assay

L. donovani (MHOM/IN/1983/AG83) parasites were cultured as promastigotes in medium 199 (Invitrogen Life Technologies) with Hanks' salt containing HEPES (12 mM), L-glutamine (20 mM), 10% heat-inactivated FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Adherent peritoneal macrophages (BALB/c) or the murine macrophage cell line RAW 264.7 were cultured at 37°C with 5% CO_2 in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Nitrite production was determined in macrophage culture medium by the Griess reaction, as previously described (26). Cell viability was assessed using an MTT-based colorimetric assay kit (Roche) according to the manufacturer's instructions. For in vitro killing assays, adherent macrophages on glass coverslips (18 mm^2; 5×10^5 macrophages/coverslip) in 0.5 ml of RPMI 1640/10% FCS were infected with promastigotes at a ratio of 10 parasites/macrophage. Infection was allowed to proceed for 4 h, and the cells were washed to remove excess parasites, as previously described (26). Cells were then resuspended in medium with GRA for various time periods at 37°C. Intracellular parasite numbers were determined by staining with Giemsa.

Infections and GRA treatment

For experimental infections, BALB/c mice (20–25 g) were injected with 10^7 promastigotes through the tail vain, and for reinfection, the same number of promastigotes was injected 45 days after the first infection. GRA (Sigma-Aldrich) was given i.p. in various doses on days 10, 15, and 20 after infection. Spleen and liver were removed from 45-day infected mice, and multiple impression smears were prepared and stained with Giemsa. Spleen or liver parasite burdens, expressed as Leishman-Donovan units (LDU), were calculated as the number of parasites per 1000 nucleated cells × organ weight (in grams) (27).

Analysis of cytokines

Every 15 days after infection up to 45 days, single-cell suspensions were prepared using splenocytes from infected mice as described previously (28). Cells were cultured in 96-well tissue culture plates at 4×10^6 cells/ml and stimulated with 20 μg/ml soluble leishmanial Ag (SLA) for 48 h. SLA was prepared as previously described (26). Supernatants were assayed directly using an ELISA kit (BD Pharmingen) for IL-12, IFN-γ, TNF-α, IL-10, and IL-4, as recommended by the manufacturer. mRNA profiles for these cytokines along with β-actin as the internal control were analyzed by RT-PCR. RT of 1 μg of RNA was performed according to the manufacturer's protocol for the Superscript One-Step RT-PCR system (Invitrogen Life Technologies). Oligonucleotide primers for these cytokines were selected from published cDNA sequences. After the appropriate number of PCR cycles, the amplified DNA was separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Inducible NOS expression by RT-PCR and immunoblot analysis

RT-PCR was performed to determine the mRNA profile for iNOS along with β-actin as an internal control. For immunoblot analysis, 20 μg of whole cell extracts were resolved by SDS-PAGE, electroblotted to nitrocellulose membrane, and probed with murine anti-iNOS Ab (BD Transduction Laboratories). After extensive washing, the Ag-Ab complexes were detected using the ECL kit (Amersham Biosciences) with HRP-conjugated anti-rabbit IgG (1/2000 dilution).

Preparation of nuclear and cytoplasmic extracts

The nuclear extracts were prepared from normal and infected macrophages in the presence or the absence of GRA as described by Yaron et al. (29). Briefly, sedimented cells were resuspended in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT) and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer (Kontes). The nuclei were separated by spinning at 3300 × *g* for 5 min at 4°C. The supernatant was used as the cytoplasmic extract. The nuclear pellet was extracted in nuclear extraction buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, and 0.5 mM DTT) for 30 min on ice and centrifuged at 12,000 × *g* for 30 min. The supernatant was used as nuclear extract.

EMSA

For nuclear mobility shift assay, each 10 μg of nuclear extracts were preincubated with 1 μg of poly(dI-dC) in a binding buffer (25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl) for 10 min at room temperature. As a control, a 100-fold molar excess of unlabeled NF-κB competitor oligonucleotide was added. After preincubation, 0.5 ng of ^{32}P end-labeled NF-κB oligonucleotide probe (5′-CGGGGACTTTCCGCTGGGGACTTTCCGCTTGAGCT-3′) was added to the reaction mixture and incubated for 30 min. The DNA-protein complex was then electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.5× TBE buffer (50 mM Tris, 50 mM borate, and 1 mM EDTA) and analyzed by autoradiography. For supershift assay, the nuclear extracts from GRA-treated cells were incubated with anti-p65 or anti-p50 polyclonal Ab (Santa Cruz Biotechnology) for 30 min at 25°C and analyzed by EMSA in the presence of all components of the binding reaction described above. As controls, the nuclear extracts were also treated with normal rabbit IgG.

Western blot analysis

For detection of p65 translocation, normal and infected macrophages were treated with GRA. The nuclear extracts were prepared as described above, and the protein concentrations were measured with a Bio-Rad protein assay. The nuclear extract (30 μg) was resolved by SDS-PAGE, then electrotransferred to nitrocellulose membrane. The membranes were incubated with p65 Ab, washed, incubated further with HRP-conjugated anti-rabbit IgG (1/2000 dilution), and detected using an ECL detection kit. For IκBα phosphorylation studies, GRA-treated normal and infected macrophages were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 2 mM PMSF), and the protein concentrations in cleared supernatants were measured using a Bio-Rad protein assay. The supernatant (lysates) containing equal amount of total protein (30 μg) was resolved by SDS-PAGE and electrotransferred from gel to nitrocellulose membranes. The membranes were incubated with rabbit anti-phospho-IκBα Ab (1/500 dilution), additionally incubated with HRP-conjugated anti-rabbit IgG, and detected by ECL detection system (Amersham Biosciences) according to the manufacturer's instructions. To demonstrate the effect of GRA on IκBα degradation, cytoplasmic extracts after resolution by SDS-PAGE were probed with anti-IκBα Ab (1/500 dilution) and detected using the ECL detection system as described above.

IKK assay

IKK activity was measured as described by Philip and Kundu (30). GRA-treated normal and infected macrophages were lysed in cold kinase assay lysis buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 10 mM *p*-nitrophenylphosphate (pNPP), 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.25% Nonidet P-40). The supernatant was obtained by centrifugation at 12,000 $\times$ *g* for 10 min at 4°C. Protein concentrations were measured using a Bio-Rad protein assay. The cell lysates (300 μg) were immunoprecipitated with anti-IKKα/IKKβ Ab in immunoprecipitation buffer (40 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 10 mM pNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.1% Nonidet P-40). Immunoprecipitated samples were incubated with recombinant IκBα (4 μg) in kinase buffer (20 mM HEPES (pH 7.7), 2 mM $MgCl_2$, 10 μM ATP, 3 μCi of [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 10 mM pNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) at 30°C for 1 h. The kinase reaction was stopped by addition of SDS-sample buffer. The sample was resolved by SDS-PAGE, dried, and autoradiographed. To determine the total amounts of IKKα and IKKβ in each sample, 30 μg of the whole cell extract protein was subjected to SDS-PAGE and analyzed by Western blot using anti-IKKα and IKKβ Ab.

Statistical analysis

The significance of the data was evaluated using a two-tailed *t* test.

Results

Effect of GRA on nitrite production

The generation of NO is an important host defense mechanism, including antileishmanial protection. Because GRA might act as an immunomodulator through NO production, we first investigated the ability of GRA to up-regulate NO in *L. donovani*-infected macrophages. As shown in Fig. 1*A*, GRA could up-regulate NO production in peritoneal macrophages in a concentration-dependent manner. The effect was maximal with a concentration of 20 μM after incubation for 24 h. To demonstrate that the effect observed was related to the L-arginine-NOS pathway, we used the specific NOS inhibitor, N^G-monomethyl-L-arginine (NMMA). The addition of NMMA almost completely abolished nitrite production. GRA exhibited no cytotoxicity at 20 μM, although there was some effect at higher concentrations (>30 μM). GRA-induced up-regulation was observed even in *L. donovani*-infected macrophages, which exhibited an 11-fold increase in NO production after GRA treatment (20 μM for 24 h) compared with untreated cells (Fig. 1*B*). Preincubation of GRA with polymyxin B, an LPS inhibitor, did not alter NO production, suggesting that the up-regulation of NO by GRA in peritoneal macrophages is not due to LPS contamination. This is confirmed by the observation that GRA could induce comparable NO-generating ability in a bone marrow-derived, LPS nonresponder, macrophage cell line (10ScNCr/23 from mouse strain C57BL; American Type Culture Collection; Fig. 1*B*). The level of nitrite produced by various regimens is also consistent with the expression of iNOS mRNA, which, after isolation of total RNA, was subjected to RT-PCR analysis (Fig. 1*C*).

In vitro and in vivo antileishmanial effects

Because GRA could up-regulate NO production, which is the most effective antileishmanial mechanism, we checked the efficacy of GRA on intracellular growth of *L. donovani*. Fig. 2*A* shows that the in vitro effect of GRA on amastigote multiplication within macrophages was dependent on both the concentration of the drug and the duration of treatment. For each concentration of drug, the inhibitory effect was found to be directly proportional to the duration of treatment up to 24 h. No additional increase in antileishmanial activity was achieved by increasing the treatment time to 48 h. The IC_{50} for 24-h treatment of GRA was 4.6 μg/ml. To ascertain the involvement of NO in the inhibition of intracellular parasite growth, the infection index was measured in the presence

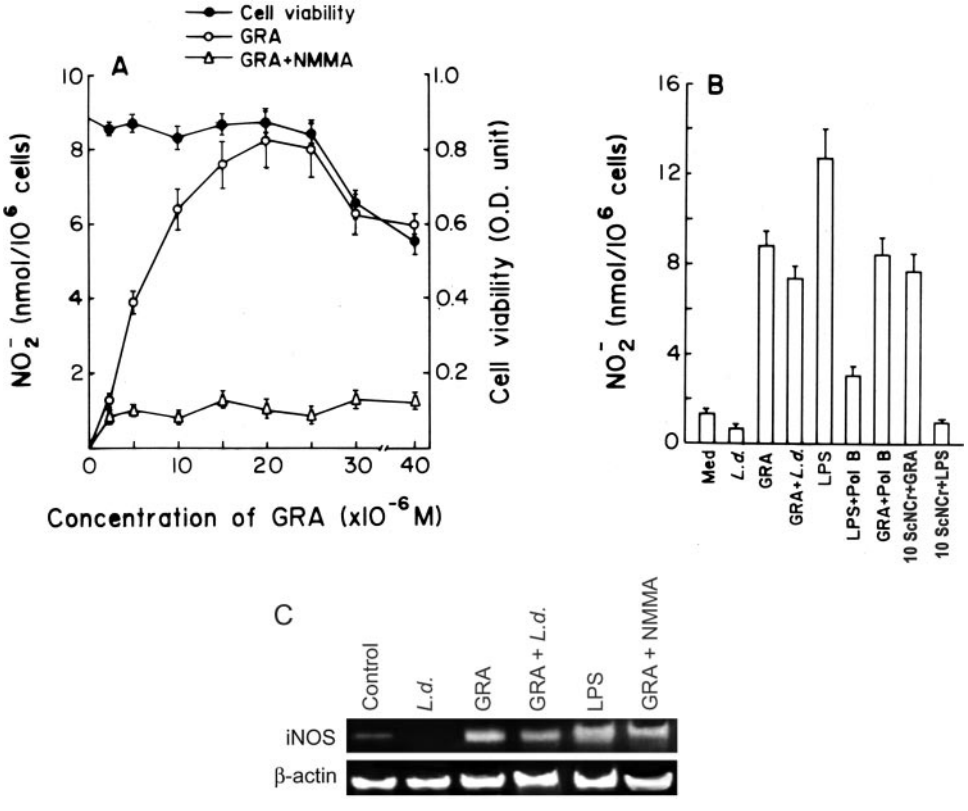

FIGURE 1. Effect of GRA on NO production. *A*, Peritoneal macrophages (10^6 cells/ml) were treated with various concentrations of GRA, either alone or in the presence of NMMA (2.5 μM), for 24 h at 37°C. Cell viability was assessed by an MTT assay. *B*, Macrophages (10^6 cells/ml) were incubated with *L. donovani* (macrophage:parasite ratio, 1:10), GRA (20 μM), GRA plus *L. donovani*, LPS (1 μg/ml), LPS plus polymyxin B preincubation (10 U/ml for 2 h), and GRA plus polymyxin B preincubation. LPS nonresponder macrophages (10ScNCr/23 from mouse strain C57BL; 10^6 cells/ml) were incubated with GRA (20 μM) and LPS (1 μg/ml). Each bar shows the mean $\pm$ SD of three independent experiments. *C*, The nature of iNOS expression was determined by RT-PCR in macrophages treated with *L. donovani* (*lane 2*), GRA (*lane 3*), GRA plus *L. donovani* (*lane 4*), LPS (*lane 5*), and GRA plus NMMA (*lane 6*). *Lane 1* represents untreated macrophages.

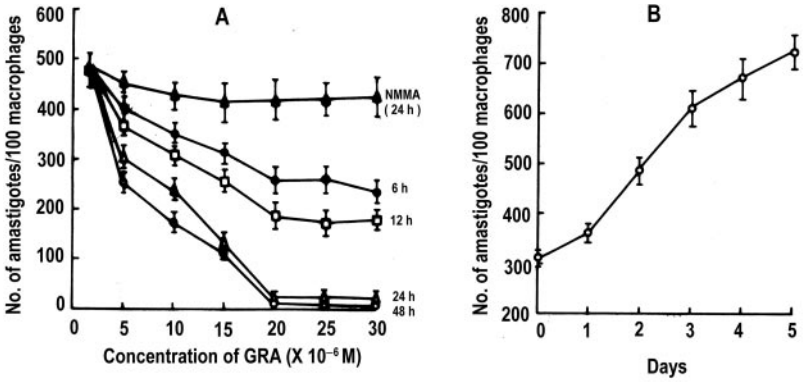

FIGURE 2. Effects of concentration and duration of treatment of GRA on amastigote proliferation within peritoneal macrophages. *A*, Macrophages were infected with *L. donovani* promastigotes (cell:parasite ratio, 1:10), and noningested promastigotes were removed. Infected macrophages were treated with various concentrations of GRA for different time periods and washed; after culture for a total of 48 h, the number of parasites inside macrophages was counted. In one set of experiments, NMMA (2.5 μM) was used along with GRA for 24 h. *B*, Growth rate of *L. donovani* amastigotes within macrophages in the absence of GRA. The data represent the mean ± SD of three independent determinations.

of a specific NOS inhibitor, NMMA. Increased leishmaniacidal activity induced by GRA in infected macrophages was almost totally abrogated by treatment with NMMA, suggesting that the antileishmanial activity of GRA may be correlated with increased production of NO. The potent in vitro antileishmanial activity of GRA in the absence of obvious cytotoxicity on macrophage host cells forms the basis for evaluating its efficacy in a murine model of visceral leishmaniasis. The dose titration experiment assessed the efficacy of GRA against established (10-day) infections with a dose range of 1–100 mg/kg/day, given three times, 5 days apart. The infection was allowed to proceed for 45 days, after which the animals were killed, and antileishmanial potency was assessed in terms of parasite burden in liver and spleen. During the experiment, no marked effect on body weight was noted in any of the experimental groups. In the placebo-treated infected controls, a high burden of amastigotes was present in liver (3981 ± 385 LDU) and spleen (316 ± 28 LDU). At a lower dose of GRA (1 mg/kg/day), there was almost no control of infection (2 and 5% reductions in liver and spleen, respectively), but a dose-related inhibition was noted at higher doses, and at 50 mg/kg/day the inhibition was almost complete, with greatly reduced levels of liver and spleen parasite burden (Fig. 3*A*). Complete clearance of parasitemia was also noticed when progression of visceral leishmaniasis was followed in the presence of 50 mg/kg/day GRA administered on days 10, 15, and 20 (Fig. 3*B*). The organs of the treated animals did not show the presence of any viable transformed promastigotes when their homogenates were cultured for 2 wk, as described previously (26). The results suggest that 50 mg/kg/day might be regarded as the lowest active dose of GRA for curative treatment 10 days after infection.

That the GRA therapy might also have effected a protective immunity was indicated by a reinfection experiment. Reinfection of animals after 45 days resulted in only a slight and transient increase in organ parasite burden (Fig. 3*C*). To ascertain whether GRA-treated mice also control the infection by an NO-dependent mechanism, NMMA (50 mM) was administered 1 wk after reinfection. Within 3–4 days of NMMA administration, the organ parasite burden started to increase. The infection was under control again after NMMA was withdrawn (Fig. 3*C*).

Effect of GRA on cytokine production

To evaluate the type of immunological response in *L. donovani*-infected mice after GRA treatment, mRNA transcription levels for IL-10, IL-4, IL-12 p40, IFN-γ, and TNF-α were determined on isolated spleen cells every 15 days after infection. The results clearly demonstrated a high level of IL-10 and some IL-4 expression in infected untreated controls and a low expression level of transcripts for IL-12, IFN-γ, and TNF-α (Fig. 4*A*). In contrast, a high level of IL-12, IFN-γ, and TNF-α expression and a low level of IL-10 and IL-4 expression were detected in infected GRA-treated cases (Fig. 4*B*). The differential cytokine expression was found to peak on day 30 after infection. Inducible NOS was also found to be up-regulated in infected GRA-treated cells (Fig. 4*C*). For additional confirmation, cytokine production was assessed in

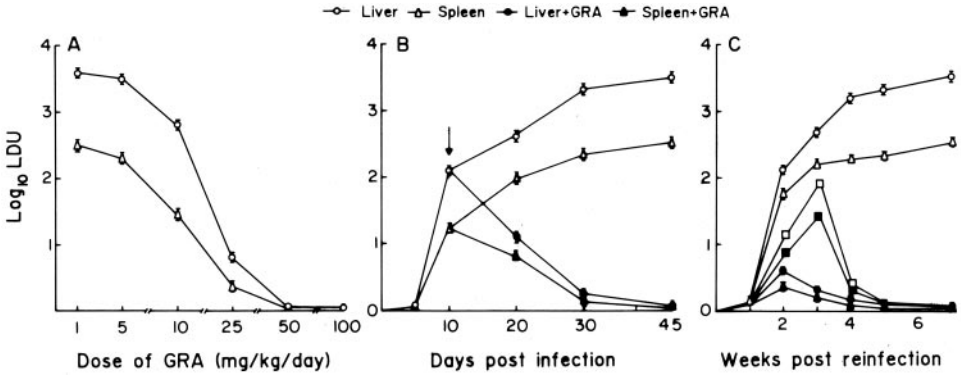

FIGURE 3. Effect of GRA treatment on visceral infection in BALB/c mice. *A*, Various doses of GRA ranging from 1 to 100 mg/kg/day were given i.p. on days 10, 15, and 20 after infection. The parasite burdens in liver and spleen were then determined at 45 days after infection. *B*, The course of visceral infection was followed in BALB/c mice that had received three i.p. injections of GRA (50 mg/kg/day) on days 10, 15, and 20 after infection vs untreated controls. *C*, The course of visceral reinfection was studied by i.v. administration of 1×10^7 *L. donovani* promastigotes into naive, age-matched BALB/c mice and cured (50 mg/kg/day GRA-treated) mice. In one group of cured mice, NMMA (50 mM) was given in the drinking water 1 wk after reinfection for 2 wk. Determining liver and spleen parasite burdens, expressed as $\log_{10}$LDU, monitored the progression of infection in all the cases. Results are from three experiments and indicate the mean ± SD for five to seven mice at each time point.

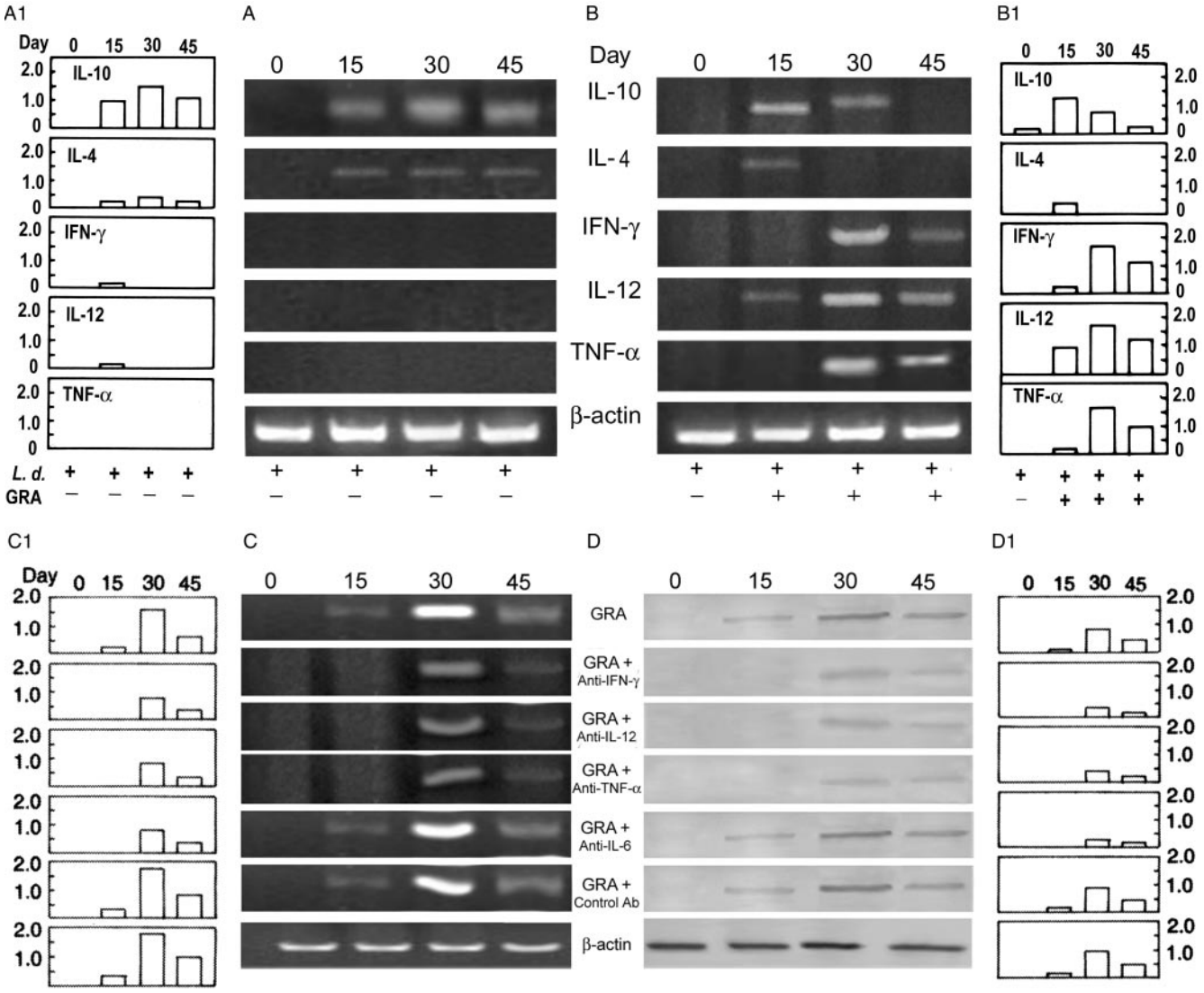

FIGURE 4. A time-course pattern of cytokine expression in *L. donovani*-infected mice. RT-PCR analysis was performed for the expression of IL-10, IL-4, IFN-γ, IL-12p40, and TNF-α mRNA by splenocytes of infected (*A*) and GRA-treated (50 mg/kg; on days 10, 15, and 20 after infection; *B*) mice. RT-PCR products were visualized by ethidium bromide staining. RNA samples were obtained from five mice in each group. Results are representative of three separate samples. β-Actin expression levels were used as controls for RNA content and integrity. Semiquantitative analysis of mRNA expression by densitometry is shown in the *extreme left* and *right panels* (A_1 and B_1). Inducible NOS expression by RT-PCR (*C*) and Western blot (*D*) in splenocytes of *L. donovani*-infected mice treated with GRA (50 mg/kg; on days 10, 15, and 20 after infection) and GRA along with various anti-cytokine mAbs (200 μg/mouse; on days 10, 15, and 20 after infection) is shown. Control infected mice received normal mouse IgG (200 μg) along with GRA. Band intensities were analyzed by densitometry (C_1 and D_1).

spleen cells of GRA-treated infected animals at the protein level by ELISA, which showed significantly more IL-12 p40, IFN-γ, and TNF-α than those from untreated infected controls (Fig. 5). These results not only support the balance of Th1/Th2 cytokines in the predominant profile of disease progression and resolution in leishmaniasis, but also show the potential of GRA as a proper effector molecule, which might induce protective responses. It should be mentioned that the antileishmanial efficacy of conventional antimony chemotherapy also required host T cells and an intact Th1 cell-type response with IL-12 and IFN-γ secretion (31). To determine whether up-regulation of Th1 cytokines by GRA is the mechanism by which GRA up-regulates iNOS in vivo, we applied 200 μg of various anti-cytokine mAbs (Genzyme) along with GRA (i.e., on days 10, 15, and 20) to *L. donovani*-infected mice. The anti-cytokine mAbs reactive against IFN-γ, IL-12, or TNF-α greatly reduced GRA-mediated splenocyte iNOS induction at both mRNA and protein levels, whereas splenocyte iNOS in mice treated with GRA plus control Abs was similar to that in mice treated with GRA alone (Fig. 4, *C* and *D*). Neutralizing mAbs to other cytokines, such as IL-6, also had little effect on GRA-mediated iNOS induction. The therapeutic effect of GRA, therefore, may be attributed to the up-regulation of NO resulting from the direct effect of GRA as well as through activation by Th1 cytokines.

Effect of GRA on NF-κB DNA binding and activation

Because iNOS and many proinflammatory gene expressions are regulated by NF-κB, the effects of GRA on NF-κB binding activity were examined by EMSA in the nuclear protein extracts of RAW 264.7 cells (Fig. 6). GRA (20 μM) treatment caused greatly increased NF-κB binding activity (*lane 4*) compared with that in untreated cells (*lane 1*). Significantly, GRA treatment also resulted

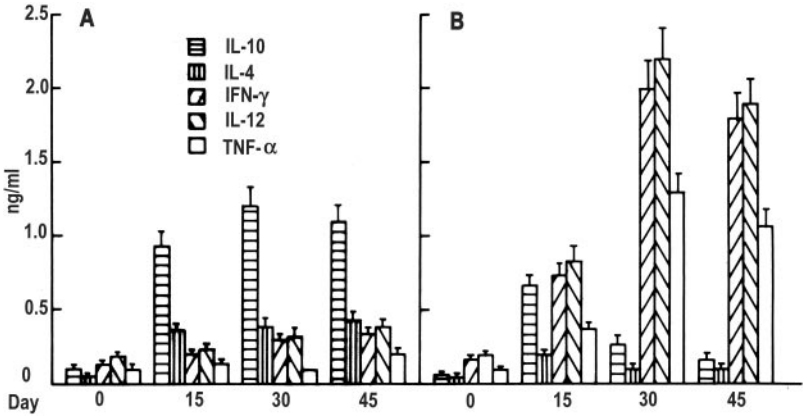

FIGURE 5. A time-course pattern of cytokine levels in *L. donovani*-infected (*A*) and GRA-treated (50 mg/kg; on days 10, 15, and 20 after infection; *B*) mice. Spleen cells were isolated on the indicated days after infection, plated aseptically (4×10^6 cells/ml), and stimulated with SLA (20 μg/ml) for 72 h. Ongoing cytokine production was determined by ELISA. Results represent the mean ± SD of three mice per group studied independently.

in marked induction of NF-κB binding in *L. donovani*-infected macrophages (*lane 5*), although *L. donovani* infection caused a down-regulation of NF-κB activation in naive macrophages (*lane 3*). Densitometric quantification of the NF-κB-specific bands indicated that there was a 3.3-fold increase in DNA binding in GRA-treated infected cells compared with untreated infected cells. The specificity of binding was confirmed by incubating the nuclear extract with a 100-fold excess of unlabeled oligonucleotide, which resulted in complete displacement of the NF-κB-specific band (*lane 6*). To further ascertain the authenticity of the NF-κB band in GRA-treated cells (Fig. 5*B*), nuclear extracts were incubated with anti-p65, anti-p50 Ab, or both before analysis by EMSA. A shift of the NF-κB specific band to higher m.w. when the nuclear extracts were treated with anti-p65 (*lane 5*), anti-p50 (*lane 4*), or both Abs (*lane 6*) suggested that the GRA-activated complex consisted of p65 and p50 subunits. Normal rabbit IgG, as a control, had no effect on NF-κB mobility (*lane 3*).

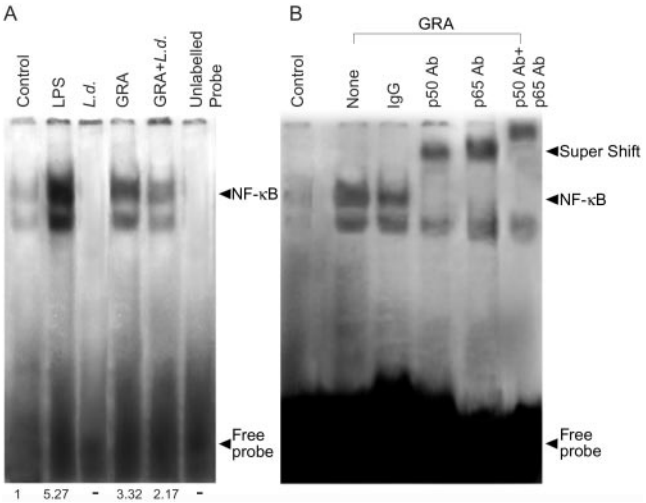

FIGURE 6. Effect of GRA treatment on activation of NF-κB in RAW 264.7 cells. *A*, Labeled NF-κB probe was incubated with nuclear extracts from normal and infected cells treated in the presence or the absence of GRA (20 μM) at 37°C for 3 h, and EMSA was performed. Normal macrophages stimulated with LPS (1 μg/ml) served as a positive control. The specificity of binding was determined by adding to nuclear extracts from GRA-treated normal cells a 100-fold molar excess of unlabeled NF-κB oligonucleotide. The bands were analyzed densitometrically, and fold changes are indicated. *B*, For supershift assay, nuclear extracts from GRA-treated cells were incubated with anti-p65, anti-p50, or both Abs for 30 min before EMSA. Normal rabbit IgG was used as a control. The results are representative of one of three separate experiments.

Effect of GRA on the translocation of p65

To determine the effects of GRA on the migration of p65 into the nucleus, Western blot analysis of nuclear protein fractions was conducted. As shown in Fig. 7*A*, GRA induced p65 translocation into the nucleus in a time-dependent manner. Gradual accumulation of p65 in the nuclear protein fraction of GRA-treated cells started after 30 min and continued up to 120 min. Exposure of *L. donovani*-infected macrophages to GRA also caused nuclear translocation of p65 within 30 min of addition of GRA. In contrast, in untreated infected cells, most of the p65 was localized in cytosol up to 120 min.

Role of GRA in IκBα phosphorylation

As a necessary prerequisite, for NF-κB translocation to the nucleus, cytoplasmic IκBα must be phosphorylated, ubiquitinated, and degraded. To ascertain the effects of GRA on this event, normal and infected cells were treated with GRA (20 μM) for 0–120 min and lysed. The lysates containing an equal amount of total proteins were resolved by SDS-PAGE, and levels of phosphorylated IκBα were detected by Western blot analysis using anti-phospho IκBα Ab. Preincubation of both normal and infected cells with GRA markedly induced the phosphorylated IκBα signal as early as 10 min, and it had almost disappeared after 60 min (Fig. 7*B*). In untreated infected cells, no IκBα phosphorylation was observed. To gain insight into the phosphorylation-mediated degradation of IκBα, a Western blot analysis was performed with GRA-treated normal and infected cells using anti-IκBα Ab. With GRA treatment, normal cells exhibited a rapid disappearance of cytosolic IκBα during the first 60 min; after that, IκBα reappeared, possibly caused by NF-κB, within 120 min (Fig. 7*C*). GRA-treated infected cells followed the same pattern. Untreated infected cells did not exhibit any transient change in cytoplasmic IκBα. The bands were quantified by densitometry, and the fold changes compared with the control were calculated.

Effect of GRA on IKK

Because IκBα is phosphorylated by the IKK multiprotein complex, the effect of GRA on the status of intrinsic cellular IKK activation was determined. Accordingly, normal or *L. donovani*-infected macrophages were treated with GRA for 1 h. Cells were lysed and immunoprecipitated with anti-IKKα and IKKβ Ab. The immunoprecipitated samples were used for kinase assay with recombinant IκBα as substrate. The radiolabeled, phosphorylated IκBα-specific band was detected in GRA-treated cells, demonstrating that GRA induced IKK activity in both normal and infected cells (Fig. 8*A*, *lanes 2* and *4*). In contrast, IKK activity was negligible in the normal untreated as well as infected untreated

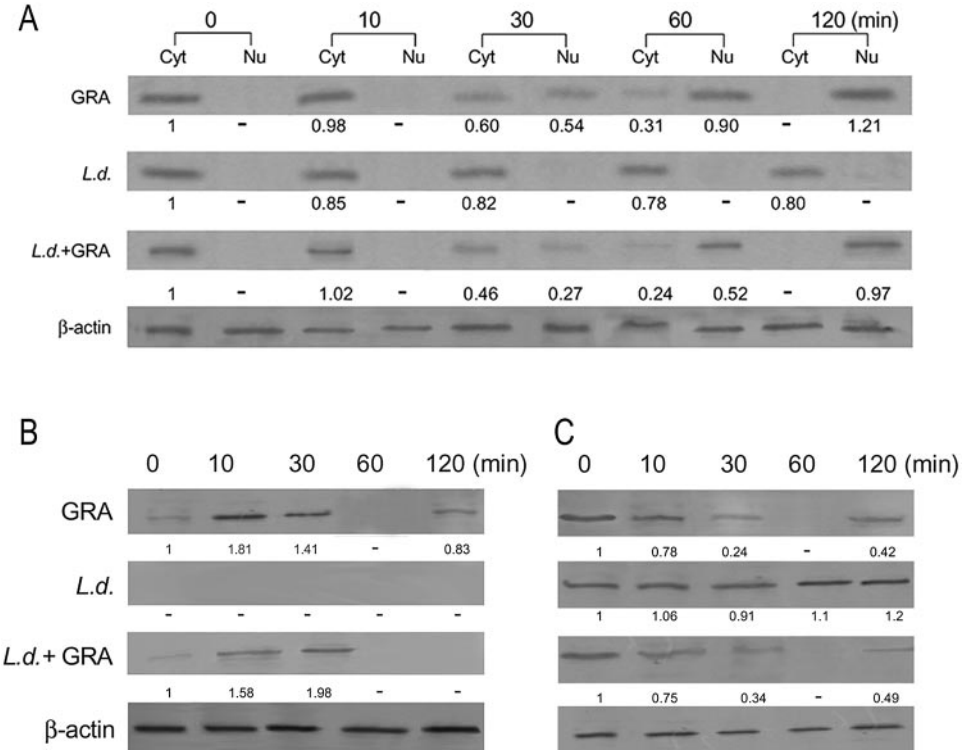

FIGURE 7. Nuclear translocation of p65 and phosphorylation and subsequent degradation of IκBα in GRA-treated RAW 264.7 cells. Normal and infected cells were incubated in the presence or the absence of GRA (20 μM) for the indicated time periods at 37°C before preparing cytosolic and nuclear protein extracts for Western blot analysis. Bands reacting with primary Abs against p65 (*A*) phosphorylated IκBα (*B*), and IκBα (*C*) were visualized by ECL. The blots were analyzed densitometrically, and the values were normalized to β-actin. The relative values in terms of fold changes are indicated. Similar results were obtained in two additional independent experiments.

macrophages (*lanes 1* and *3*). Western blot analysis with whole cell lysates using anti-IKKα and IKKβ Ab (Fig. 8*A*) showed identical levels of expression of IKK, suggesting that IKK was equally expressed in cells with different treatments. To ascertain whether GRA had any direct effect on IKK, it was immunoprecipitated from normal macrophages, and GRA was added at the initiation of the assay for IKK activity. The activity of IKK was unaltered by the presence of GRA (Fig. 8*B*), suggesting that GRA exerts its effect by stimulating upstream signaling pathways that activate IKK, rather than by directly activating IKK activity.

Discussion

The use of biologically active natural products is becoming an increasingly attractive approach for the treatment of various diseases as an alternative to traditional medicine. However, limited scientific evidence regarding the effectiveness of these natural derivatives in conjunction with a lack of mechanistic understanding have prevented their incorporation into the mainstream of medical care. The present study has demonstrated that BALB/c mice with visceral leishmaniasis may be completely cured of their parasite burden by GRA, a relatively nontoxic, naturally occurring triterpenoid, found in the licorice plant (*Glycyrrhiza glabra*), with a number of medicinal properties. Even though BALB/c mice differ from symptomatic human subjects with *L. donovani* infection in that the infection is chronic, but not fatal, there is no evidence of wasting, and the parasite burden decreases during the course of infection, it serves as a good experimental model for the disease. GRA is an immunomodulatory and antiproliferative triterpene that has been shown to induce the production of NO and proinflammatory cytokines such as IL-12 (25, 32). The killing of intracellular *Leishmania* parasites by GRA correlated with the induction of the NOS pathway, because it correlated with the generation of nitrogen derivative production and was abrogated in the presence of NMMA, a competitive inhibitor of the NOS pathway. That the mice treated with GRA were indeed cured was indicated by the complete suppression of liver and spleen parasite burdens. Moreover, this therapy was effective in mice with ongoing infections in which a disease-progressive Th2 response had been established. After treatment and the resulting resolution of parasitism, the cytokine profile in these mice indicated a switch to a protective Th1 pattern associated with up-regulation of NO. Because NF-κB, the *Rel* family of inducible transcription factors, plays a central role in the evolution of inflammation through the regulation of genes essentially involved in encoding proinflammatory cytokines and inflammatory mediators such as NO, the molecular mechanism of GRA action on the IκB/NF-κB signaling pattern was determined in parasite-infected macrophages. Our results demonstrate that GRA can induce NF-κB activation, which was suppressed by *L. donovani* infection. GRA induced NF-κB activation through the induction of IκBα phosphorylation by stimulating the signal leading to IKK activation without directly interfering with IKK. This ultimately caused the degradation of IκBα and the translocation of NF-κB in the nucleus with the possible transcriptional activation of iNOS and proinflammatory cytokines. It is also possible that GRA may act directly on T cells to induce NF-κB activation. Recently, NF-κB activation within T cells has been implicated in the Th1 response, independently of the role for this transcription factor in APC production of IL-12 (33). Furthermore, NF-κB induction is required not only for clonal expansion, but also for full differentiation and substantial IFN-γ production by each committed T cell. Although GRA exhibited potent in vitro activity against intracellular *L. donovani* amastigotes (IC_{50}, 4.3 μg/ml), it is devoid of any obvious cytotoxicity on macrophage host cells, because the cytotoxic concentration causing 50% cell death was ~100 μg/ml (data not shown).

It is now known that the major antileishmanial oxidant produced by activated macrophages is NO, which is most relevant to killing established intracellular amastigotes (34). We demonstrated that macrophages pretreated with 20 μM GRA produced maximal NO production and iNOS transcription, which paralleled with maximal stimulation of leishmaniacidal activity. The exact molecular mechanism through which this radical promotes intracellular killing of

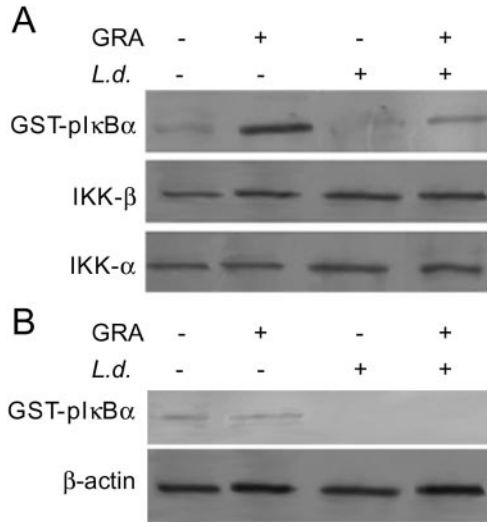

FIGURE 8. GRA-induced activation of IKK in RAW 264.7 cells. *A*, Normal and infected cells were incubated in the presence or the absence of GRA for 1 h at 37°C. Whole cell extracts were prepared and immunoprecipitated with Abs against IKKβ and IKKα. The activity of immunoprecipitated IKK was measured using GST-IκBα as substrate, and GST-phosphorylated IκBα was visualized by autoradiography. Relative amounts of IKKα and IKKβ in the whole cell extracts were determined by Western blots. *B*, Whole cell extracts were prepared from normal and infected macrophages, and IKK was immunoprecipitated with Abs against IKKβ and IKKα. The immune complex was treated with GRA (20 μM) for 30 min at 30°C. Unactivated cells served as a control. IKK activity was assessed using GST-IκBα as described in *A*. Equal protein loading was evaluated by β-actin. Similar results were obtained in three additional independent experiments.

Leishmania is not yet fully defined. However, it may be mentioned that NO also inhibits *Leishmania* cysteine proteinase activity, a known virulence factor, leading to lethal metabolic inhibition through irreversible chemical modification of reactive cysteine residues (35) as well as blocking the differentiation process from amastigotes to promastigotes (36). These observations are indicative of the essential role of reactive nitrogen intermediates in the control of intracellular parasites by murine macrophages, exerting lytic effects on both parasite developmental stages.

Polarization of Th responses to either Th1 or Th2 can lead to life or death outcomes to *Leishmania* infections in mice (37). A growing body of evidence supports the host protective role of IL-12-induced differentiation of Th1 cells that produce IFN-γ, which activates macrophages to produce NO (38). The acquired resistance to *L. donovani* infection by GRA therapy may be attributed to the switch of $CD4^+$ T cell-mediated immune responses from disease-promoting Th2 type to disease-resolving Th1 type, as evaluated by the mRNA levels of various cytokines in spleen cells at different time points after infection. Transcript levels of IL-10 and IL-4 were reduced in mice given GRA therapy, whereas those for IL-12 p40 and IFN-γ were significantly elevated. GRA also increased the levels of TNF-α, another inflammatory cytokine with well-defined antileishmanial effects that is known to act either alone or with IFN-γ to induce the production of reactive nitrogen and oxygen intermediates (39, 40). This type of an intervening, T cell-dependent mechanism is also reported to be required for optimal in vivo responsiveness to pentavalent antimony, the conventional chemotherapeutic agent for leishmaniasis (41). The secretion of the tested cytokine proteins was determined by ELISA. Although the results obtained by ELISA are in line with the findings using RT-PCR, it seems that the semiquantitative RT-PCR technique is more sensitive than ELISA.

There is increasing evidence that NF-κB family members play complex regulatory roles in controlling innate and adaptive responses after infection with *Leishmania*. Chronic infection in NF-κB2 $(p52)^{-/-}$ mice is associated with defective CD40-induced IL-12 responses, whereas susceptibility to infection in c-$Rel^{-/-}$ mice has been attributed to defective NO production and parasite killing (42, 43). In contrast, susceptibility to infection in NF-κB1 $(p50)^{-/-}$ mice exhibited a marked defect in the expansion of Ag-specific $CD4^+$ T cells secreting IFN-γ, underlying its role in the adaptive immune response (44). Very recently, p50/c-Rel heterodimer in monocytes has been found to be selectively induced by *L. major*, resulting in production of IL-10 (45). All these findings suggest the importance of need-based use of various NF-κB components for fine-tuning gene activation in response to external stimuli. Our present study has demonstrated that the transcriptional effects of GRA on genes regulating the expression of iNOS and Th1 cytokines might result in part from increased translocation of NF-κB into the nucleus of activated cells. GRA has been found to induce the complex composed of p50/p65 heterodimer in normal as well as *L. donovani*-infected macrophages. This observation is consistent with the recent report that infection by *L. major* amastigotes blocked nuclear translocation of a PMA-induced p50/p65 NF-κB complex in U937 cells (45). Induction of NF-κB was accounted for by the transient change as well as phosphorylation of IκBα by the IKK multiprotein complex, followed by ubiquitination and degradation by the 26S proteasome complex (46). The results of the present study suggest that the kinase activity of IKK was stimulated in cells that were activated in the presence of GRA through a mechanism that most likely involves upstream signaling pathways. This is substantiated by the lack of effect of GRA on IKK activity when GRA was added directly to the assay mixture containing IKK immunoprecipitated from normal macrophages. Although it is possible that GRA interferes with the regulatory interaction between NF-κB essential modifiers (IKKβ and IKKγ) (47), it seems more likely that GRA interferes with one of the key upstream kinases in the signaling pathway, e.g., NF-κB-inducing kinase, PI3K, or MAPK (48).

In macrophages, the MAPK cascade and the NF-κB pathway play important roles in the regulation of functions involved in inflammation and host defense. Previous reports demonstrated that in naive macrophages, *L. donovani* promastigotes evade the action of these pathways and thereby successfully avoid activating macrophages for the production of NO and proinflammatory cytokines (16). Thus, the agents that lead to activation of NF-κB pathway might prove attractive candidates to control infection with *Leishmania*. GRA, a triterpenoid isolated from the root of the licorice plant, is one such pharmacologically safe compound with known immunomodulatory properties. However, the development of new drugs that induce NF-κB activation at various points of the signal transduction pathway will require pharmacokinetic and toxicity studies in addition to clinical verification of in vivo activity. GRA has the advantage of being a relatively nontoxic compound. The pharmacological safety of GRA has been demonstrated by nontoxic consumption of up to 1.5 g/day in humans (49). Thus, the marked sensitivity of the macrophage effector responses of NO and proinflammatory cytokines to GRA suggest that this natural product could be used as a potential immunomodulator to generate the required immunity not only for the treatment of nonhealing leishmaniasis, but also for the treatment of other chronic infectious diseases.

Disclosures

The authors have no financial conflict of interest.

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