Negative Regulation of Interleukin-12 Production by a Rapamycin-Sensitive Signaling Pathway: A Brief Communication

SUWANNEE UTHAISANGSOOK,[1] NOORBIBI K. DAY, REMI HITCHCOCK, AARON LERNER, MICHELLE JAMES-YARISH, ROBERT A. GOOD, AND SOICHI HARAGUCHI[2]

Division of Allergy and Immunology, Department of Pediatrics, University of South Florida, All Children's Hospital, St. Petersburg, Florida 33701

Interleukin-12 (IL-12), an important cytokine in host defense against microbial pathogens, regulates natural killer and T-cell function(s) including the induction of γ-interferon production. The major cellular sources of IL-12 are monocytes/macrophages. Bacteria, bacterial products, and intracellular parasites are the most efficient inducers of IL-12 production. In the present study we show that a signal transduction pathway sensitive to rapamycin may have an important role in the regulation/suppression of *Staphylococcus aureus*–induced IL-12 production *in vitro*. Human peripheral blood mononuclear cells, monocytes, or a human monocytic cell line THP-1 were stimulated with *S. aureus* Cowan strain 1 (SAC) in the presence or absence of rapamycin and investigated for production of IL-12 protein by enzyme-linked immunosorbent assay and IL-12 p40 mRNA accumulation by RNase protection assay or real-time quantitative polymerase chain reaction. The results show that rapamycin significantly enhances SAC-induced IL-12 p70 protein production and IL-12 p40 mRNA accumulation. Further the results demonstrate that wortmannin enhances SAC-induced IL-12 p40 mRNA accumulation, whereas Ly294002 does not. These data indicate that a rapamycin-sensitive signaling pathway may act as a negative feedback cascade in the regulatory mechanisms of IL-12 production. Exp Biol Med 228:1023–1027, 2003

Key words: interleukin 12; rapamycin; negative regulation; signal transduction

Interleukin-12 (IL-12), a proinflammatory cytokine, is a 70-kDa heterodimer (p70) composed of p40 and p35 subunits, the co-expression of which is necessary for biological activity. The expression of IL-12 p40 mRNA is highly regulated and induction dependent, whereas the p35 transcripts are ubiquitously expressed in most cell types (1). IL-12 is produced by phagocytes, dendritic cells, and Langerhans' cells of skin, B cells, or other major histocompatibility complex class II–positive cells (1). Induction of IL-12 occurs when these cells become stimulated with bacteria, intracellular pathogens, fungi, viruses, or their products *in vitro* either by a T-cell-dependent pathway through interaction between CD40 and CD40 ligand, or by a T-cell-independent pathway (2, 3). IL-12 is rapidly produced in response to infections with bacteria or intracellular pathogens and released by phagocytic cells through a T-cell-independent mechanism at an early stage of infection. This process may represent a possible bridge between innate resistance and adaptive immune response, and the early response may be important for the activation of the phagocytic system as a first line of defense against infection (4).

Under pathological conditions, patients who have IL-12 p40 deficiency or IL-12 receptor defect are susceptible to infections including *Mycobacterium*, *Salmonella*, or pneumococcal infections (5–7). *In vitro* studies and studies using animal models have shown that IL-12 induces tumoricidal natural killer, lymphokine-activated killer, and cytotoxic T-cell activities (4). Overproduction of IL-12, however, may result in destructive inflammation (4, 8). It is therefore crucial to study the molecular/intracellular signaling mechanisms of IL-12 p40 gene expression that are triggered by the interactions between pathogens and immune systems (4, 8–12).

Rapamycin, a macrolide immunosuppressant, binds to and inhibits the FK506-binding protein proline rotamase. It prevents autophosphorylation of FK506-binding protein-rapamycin-associated protein (FRAP)/target of rapamycin (TOR), which is a highly conserved atypical protein kinase and an upstream signaling enzyme of 70-kDa S6 protein kinase (p70 S6 kinase), and selectively blocks signaling that leads to the activation of p70 S6 kinase (13–15). p70 S6

[1] Present address: Faculty of Medicine, Naresuan University, Tapoe, Muang, Phitsanulok 65000, Thailand.
[2] To whom requests for reprints should be addressed at the Department of Pediatrics, University of South Florida, All Children's Hospital, 801 Sixth Street South, St. Petersburg, FL 33701. E-mail: haragucs@allkids.org

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kinase, a mitogen-activated Ser/Thr protein kinase, is one of the downstream signaling enzymes of phosphatidylinositol 3-kinase (PI 3-K) and has an established role in protein translation and in the progression of the G_1 cell cycle (14, 15). Wortmannin, a fungal metabolite, selectively inhibits PI 3-K by covalently binding to p110 catalytic subunits of PI 3-K, a heterodimer composed of p85 and p110 subunits (16). Ly294002 is also a potent inhibitor of PI 3-K; it competitively binds to the ATP binding site of PI 3-K (17).

In the present study, using human cells and *Staphylococcus aureus* to induce IL-12 p70 production, we have focused on the effects of rapamycin on *S. aureus* Cowan strain 1 (SAC)-induced IL-12 production *in vitro*. We have also investigated the possible involvement of PI 3-K, one of the upstream signaling enzymes of TOR, in SAC-induced IL-12 production using wortmannin and Ly294002. We demonstrate herein the existence of a rapamycin-sensitive negative signaling pathway of IL-12 production by showing that rapamycin enhances SAC-induced IL-12 production.

Human peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density gradient centrifugation using Ficoll-paque PLUS (Amersham Pharmacia Biotech, Piscataway, NJ). Monocytes were obtained by incubating PBMCs (4×10^6) in 1 ml of serum-free Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) in a 12-well plate at 37°C for 1 hr. Nonadherent cells were removed, and adherent cells were rinsed twice with serum-free DMEM. The adherent cells were incubated overnight in complete DMEM supplemented with 10% fetal bovine serum (FBS) (HyClone, Iogan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamine (Invitrogen), and then nonadherent cells were removed (18).

PBMCs (2×10^6) were pre-treated with either 0, 0.01, 0.1, 1, 10, or 100 n*M* of rapamycin (Biomol, Plymouth Meeting, PA) for 2 hr in 1 ml complete Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamine using a 24-well plate. Adherent monocytes were pre-treated with either medium, 100 n*M* rapamycin, 100 n*M* wortmannin (Biomol), or 50 μ*M* Ly294002 (Biomol) for 2 hr in 1 ml complete DMEM. After pre-treatment with signaling inhibitor, PBMCs or monocytes were stimulated with SAC (1:10,000 wt/vol) (Pansorbin, Calbiochem, La Jolla, CA) for 24 hr. Cell-free supernatants were analyzed for IL-12 p70 by enzyme-linked immunosorbent assay (Quantikine, R&D, Minneapolis, MN).

For cell proliferation assay, PBMCs (1×10^5) were pre-treated with either 0, 10, or 100 n*M* of rapamycin for 1 hr, stimulated with SAC (1:10,000 wt/vol) for 48 hr, and then pulsed with [^{3}H]thymidine (0.5 μCi/well; Amersham) for another 24 hr in 200 μl RPMI-1640 supplemented with 50 m*M* HEPES buffer (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamine using a 96-well U-bottom plate.

For RNase protection assay (RPA), PBMCs (10×10^6) were pre-treated with either medium, 100 n*M* rapamycin, 100 n*M* wortmannin, or 50 μ*M* Ly294002 for 1 hr in 5 ml complete RPMI-1640 using a 6-well plate. PBMCs were then stimulated with SAC (1:10,000 wt/vol) for 4 or 16 hr. RNA was extracted from cells using RNAzol B (Tel-test, Friendswood, TX). RPA was performed using 15 μg RNA and BD RiboQuant RPA System (BD Biosciences Pharmingen, San Diego, CA). Autoradiographic films were analyzed by the Multiscan-R (Interactive Technologies International, St. Petersburg, FL).

For real-time polymerase chain reaction (PCR), THP-1 cells (2×10^6) were pre-treated with either medium or rapamycin (10 or 100 n*M*) for 1 hr in 1 ml complete RPMI-1640 using a 24-well plate. THP-1 cells were then stimulated with SAC (1:10,000 wt/vol) for 4 hr. RNA was extracted from cells using TRIzol (Invitrogen). cDNA was reverse transcribed from 2 μg total RNA by MultiScribe Reverse Transcriptase and random hexamers from the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Real-time PCR for IL-12 p40 mRNA was performed on an ABI Prism 7900HT Sequence Detection System using Pre-Developed TaqMan Assay Reagents (Taqman PDARs) with TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocols. Ribosomal RNA control reagent was used as an endogenous control for PCR quantitation studies. Relative expression profiles for these cytokine mRNAs were performed using a comparative threshold method with the data obtained.

Student's *t* test was used to evaluate the significance of the results.

As shown in Figure 1A, when PBMCs were stimulated with SAC in the presence of rapamycin, SAC-induced IL-12 p70 production was significantly enhanced. SAC-induced IL-12 p70 production plateaued at the dose of 10 and 100 n*M* of rapamycin, indicating that rapamycin enhanced IL-12 production in a dose-dependent manner. Rapamycin by itself (10 or 100 n*M*) did not induce IL-12 production (data not shown). Additional experiments were then performed using adherent monocytes. In these experiments we also investigated a possible involvement of PI 3-K using wortmannin and Ly294002. The results showed that rapamycin and wortmannin enhanced SAC-induced IL-12 p70 production in monocytes, whereas Ly294002 suppressed the production (Fig. 1B).

To examine the effects of rapamycin, wortmannin and Ly294002 on SAC-induced IL-12 p40 mRNA levels, PBMCs were pre-treated with either medium, 100 n*M* of rapamycin, 100 n*M* wortmannin, or 50 μ*M* of Ly294002 for 1–2 hr followed by SAC stimulation for 4 and 16 hr. At 4 hr stimulation with SAC, rapamycin and wortmannin enhanced IL-12 p40 mRNA levels, whereas Ly294002 significantly suppressed accumulation of the transcript (data not shown). At 16 hr, SAC-induced IL-12 p40 mRNA levels

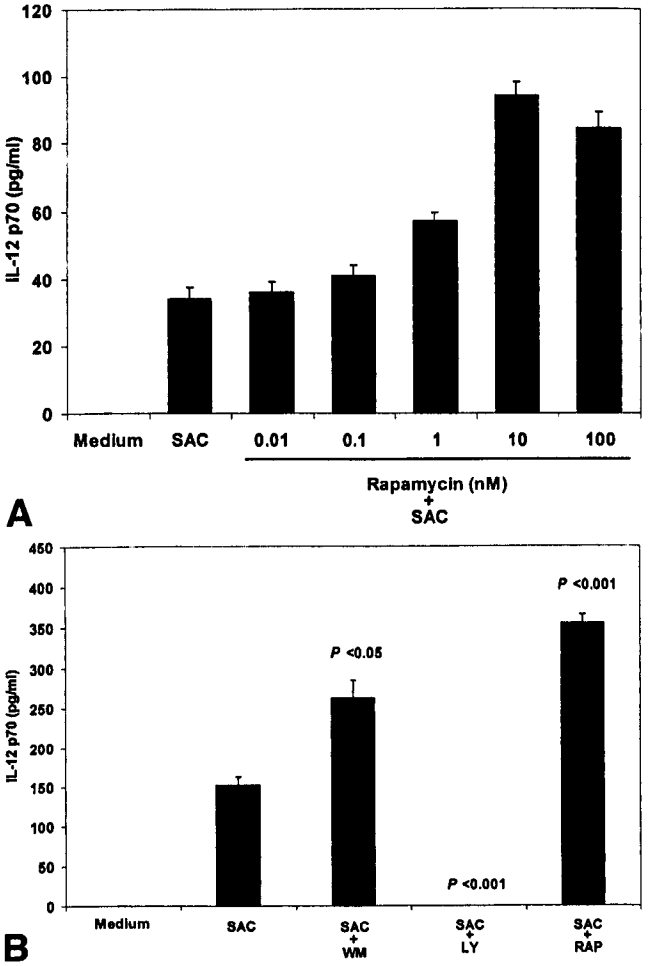

Figure 1. The effect of rapamycin, wortmannin, or Ly294002 on SAC-induced IL-12 p70 production. (A) PBMCs were pre-treated with 0, 0.01, 0.1, 1, 10, or 100 n*M* rapamycin for 2 hr in complete RPMI, and then stimulated with SAC (1:10,000 wt/vol) for 24 hr at 37°C. Results are expressed as the mean ± SD of triplicate cultures. Similar results were obtained in two additional experiments. (B) Adherent monocytes were pre-treated with medium, 100 n*M* wortmannin (WM), 50 μ*M* Ly294002 (LY), or 100 n*M* rapamycin (RAP) for 2 hr in complete DMEM, and then stimulated with SAC (1:10,000 wt/vol) for 24 hr at 37°C. Results are expressed as the mean ± SD of triplicate cultures. Similar results were observed in three separate experiments. *P* values show a significant difference versus SAC alone when calculated using the results obtained in three separate experiments. In all experiments cell-free supernatants were analyzed for IL-12 p70 by enzyme-linked immunosorbent assay. Viability of cells, as assessed by the trypan blue dye exclusion, after the treatment with inhibitors was >90% and was the same as the control cultured in medium alone.

were still enhanced by rapamycin and wortmannin (Fig. 2A), although the mRNA levels were less than those found at 4 hr. In contrast, Ly294002 consistently inhibited the mRNA levels at 16 hr (Fig. 2A). Densitometric analyses confirmed that the IL-12 p40 mRNA levels (in arbitrary optical density units) were significantly enhanced by rapamycin and wortmannin as compared with IL-12 p40 mRNA levels in SAC stimulation alone (Fig. 2B). The enhancing effect of rapamycin was further confirmed using real-time quantitative PCR and a human monocytic cell line THP-1 (Fig. 2C).

Of interest, using DO11.10 T-cell receptor transgenic mice, rapamycin has been shown to be a unique immunosuppressive agent that does not inhibit cytokine synthesis, while inhibiting bone marrow-derived dendritic cell–induced, ovalbumin-specific T-cell proliferation (19). In accordance with the previous report (19) our data show that rapamycin suppresses SAC-induced proliferative responses of human PBMCs in a dose-dependent manner. Percent inhibition by rapamycin (expressed as the mean of triplicate cultures) is as follows: 24.9% at 10 n*M* rapamycin and 79.4% at 100 n*M* rapamycin. This experiment was performed twice with similar outcomes. Coefficient of variation (SD/mean × 100) is always less than 15%. Thus, rapamycin inhibits SAC-induced cell proliferation, whereas it enhances SAC-induced IL-12 production.

The data in the present study show that rapamycin consistently upregulates IL-12 p40 mRNA expression as well as IL-12 p70 secretion, indicating that a rapamycin-sensitive signaling pathway may regulate or suppress IL-12 p40 mRNA accumulation. In this context, it should be noted that IL-12 p40 mRNA carries adenosine + uridine (AU)-rich or AUUUA-repeat elements (ARE) in the 3′ untranslated regions, which are thought to be involved in mRNA degradation (10). Various factors that interact with ARE have been demonstrated, including a *de novo* synthesized protein(s), which interacts with this specific ARE of IL-12 p40 mRNA sequence and may be involved in the regulation of IL-12 p40 mRNA degradation (10). As mentioned before, FRAP/TOR and/or p70 S6 kinase, which are direct and indirect targets of rapamycin, have been shown to play an important role in protein translation. Taking these previous reports into consideration, it is tempting to speculate that rapamycin may interfere with the *de novo* synthesis of a protein(s), which interacts with ARE on IL-12 p40 mRNA and promotes the IL-12 p40 mRNA decay. This issue deserves further study.

The data presented herein also show that wortmannin as well as rapamycin enhances SAC-induced IL-12 p70 production, but Ly294002 does not. The precise reasons for the differential influence of wortmannin or Ly294002, both of which are PI 3-K inhibitors, are unknown at present, but similar differential effects of wortmannin and Ly294002 have been previously reported (20–22). This phenomenon might be attributable to either PI 3-K or enzymes other than PI 3-K. Assuming that PI 3-K is not involved in the signaling pathway of SAC-induced IL-12 p70 production, perhaps an enzyme other than PI 3-K that is sensitive to wortmannin may act as a negative regulator of IL-12 production. Indeed, wortmannin has been shown to inhibit other protein kinases (23). At least eight PI 3-Ks have been found in mammals and it is unknown which of these are sensitive to wortmannin or Ly294002 (23). In light of these findings, another possibility is that a member of the PI 3-K family that is sensitive to wortmannin, but not affected by Ly294002, may act as a negative regulator of IL-12 production. In addition, a recent study (24) examining specificity and the mecha-

nisms of action of serine/threonine-specific protein kinases should be taken into consideration. The study showed that rapamycin and wortmannin, but not Ly294002, exhibited impressive selectivity profiles (24).

Several observations by others (25, 26) are consistent with the idea that the PI 3-K pathway negatively regulates IL-12 production. It is remarkable that Fukao *et al.* (25) have recently demonstrated PI 3-K-mediated negative feedback regulation of IL-12 production using dendritic cells from PI 3-K$^{-/-}$ mice. It is also interesting to note another report that described a novel and unexplored pathway to selectively downregulate IL-12 production (26). This previous report showed that wortmannin (50–100 n*M*) and low doses of Ly294002 (0.1–2 μ*M*) restored CD47 ligation-mediated downregulation of IL-12 production that was induced by SAC plus γ-interferon (26). In addition, it has been demonstrated that extracellular signal-regulated kinase suppresses production of IL-12, whereas p38 mitogen-activated protein (MAP) kinase promotes IL-12 (27). Of interest, the report by Fukao *et al.* (25) showed that lipopolysaccharide-induced activity of p38 MAP kinase is higher in PI 3-K$^{-/-}$ dendritic cells than in PI 3-K$^{+/+}$ dendritic cells and enhanced by wortmannin in PI 3-K$^{+/+}$ dendritic cells. Thus, further investigation on the cross-talk between a rapamycin-sensitive signaling pathway(s), PI 3-K, and MAP kinases seems to be very much in order.

It is also important to discuss our data from a viewpoint of clinical application. Because IL-12, a proinflammatory cytokine, generates Type 1 T-helper (T_H1) cell-mediated immune responses (4, 8), the IL-12 augmenting activity along with the inhibitory activity of cell proliferation may provide a unique advantage to clinical use of rapamycin. It should be noted, however, that IL-12 has also been found to cause considerable toxicity due to its ability to foster T_H1 responses, inducing an inflammatory response (4, 8). These observations should be considered in the therapeutic strategies for rapamycin (sirolimus), particularly in immunocompromised patients.

In conclusion, we propose that a rapamycin-sensitive signal transduction pathway(s) is a possible negative regulator of IL-12 production. Although further identification and characterization of the rapamycin-sensitive negative regulator (pathway/molecule) are required, our data provide a novel insight into the molecular mechanisms that control IL-12 p40 gene expression as well as a novel modality of rapamycin, an immunosuppressant and a promising anticancer drug.

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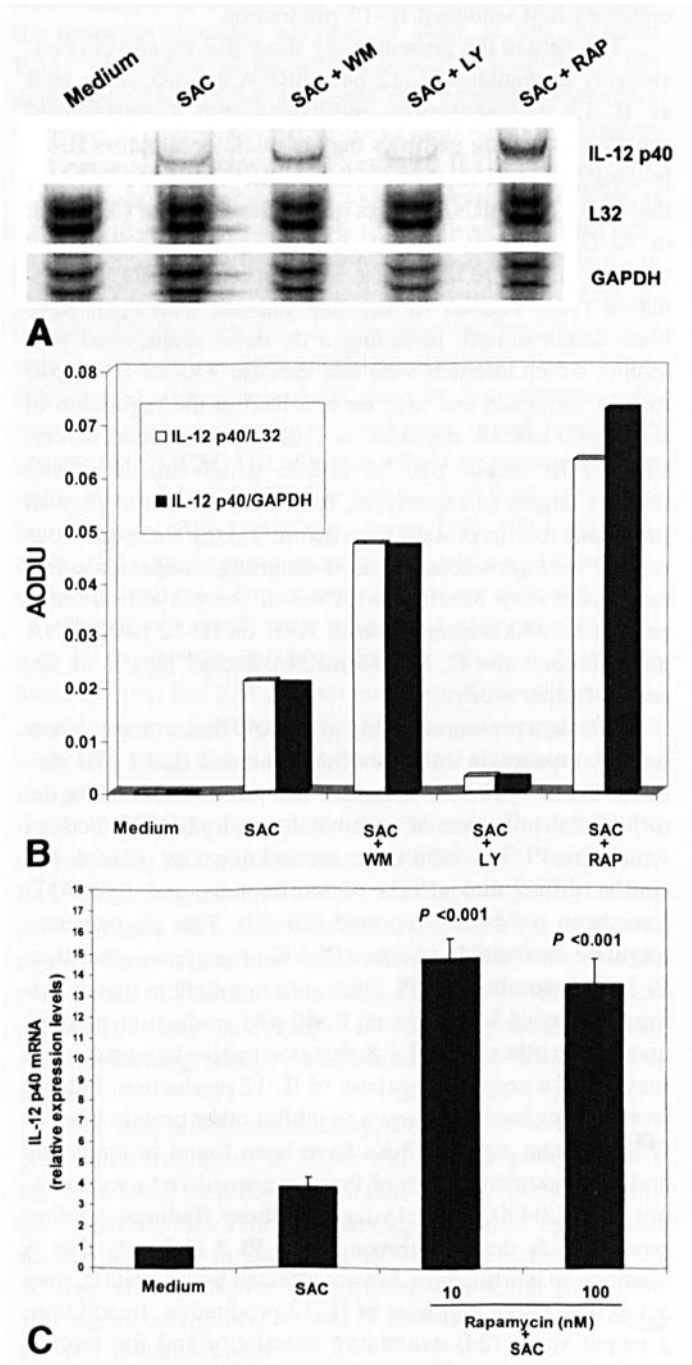

Figure 2. The effect of rapamycin, wortmannin, or Ly294002 on SAC-induced IL-12 p40 mRNA accumulation. (A) PBMCs were pre-treated with medium, 100 n*M* wortmannin (WM), 50 μ*M* Ly294002 (LY), or 100 n*M* rapamycin (RAP) for 1 hr in complete RPMI, and then stimulated with SAC for 16 hr at 37°C. RNA was extracted and analyzed by RPA. (B) Densitometric analyses of the autoradiographs shown in panel A. The values of IL-12 p40 mRNA accumulation were normalized to the mRNA levels of L32 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and expressed as arbitrary optical density units (AODU). The data show normalized IL-12 p40 mRNA levels stimulated by SAC in the presence or absence of inhibitors in AODU. Similar results were observed in three separate experiments. *P* values from the results obtained in three separate experiments are $P < 0.05$ in wortmannin, $P < 0.001$ in Ly294002, and $P < 0.001$ in rapamycin when compared with SAC alone. (C) THP-1 cells were pre-treated with medium or rapamycin (10 or 100 n*M*) for 1 hr in complete RPMI, and then stimulated with SAC for 4 hr at 37°C. RNA was extracted and analyzed by real-time quantitative PCR for IL-12 p40. Results are expressed as the mean ± SD of three separate experiments. *P* values show a significant difference versus SAC alone. Viability of cells, as assessed by the trypan blue dye exclusion, after the treatment with inhibitors was >90% and was the same as the control cultured in medium alone.

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