Interleukin-12-induced Interferon-gamma Production by Human Peripheral Blood T Cells is Regulated by Mammalian Target of Rapamycin (mTOR)

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Running title: Effect of rapamycin on IL-12-induced IFN-γ production

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ABSTRACT

Depending on the type of external signals, T cells can initiate multiple intracellular signaling pathways that can be broadly classified into two groups based on their sensitivity to the immunosuppressive drug cyclosporin A (CsA). IL-12-mediated IFN-γ production by activated T cells has been shown to be CsA insensitive. In this report, we demonstrate that the IL-12-induced CsA-resistant pathway of IFN-γ production is sensitive to rapamycin. Rapamycin treatment resulted in the aberrant recruitment of Stat3, Stat4 and phospho-c-Jun to the genomic promoter region resulting in decreased IFN-γ transcription. IL-12-induced phosphorylation of Stat3 on Ser727 was affected by rapamycin, which may be due to the effect of rapamycin on the IL-12-induced interaction between mTOR and Stat3. In accordance with this, reduction in mTOR protein level by siRNA resulted in suppression of Stat3 phosphorylation and decreased production of IFN-γ after IL-12 stimulation. These results suggest that mTOR may play a major role in IL-12-induced IFN-γ production by activated T cells.
INTRODUCTION

During an immune reaction in which antigen presenting cells (APC) interact with T cells, both cell types get activated expressing both cell surface molecules and soluble factors that in turn are involved in further activating each cell type. The cell surface molecules, CD40 ligand, IL-12- and IL-18-receptors, among others, are expressed on T cells, whereas B7 molecules, IL-12, and IL-18 are expressed by APCs. One of the cytokines produced by activated T cells is IFN-γ, which is a potent activator of APCs. Activated APCs produce IL-12 and IL-18, which are potent activators of T cells, which in turn produce IFN-γ. This circular pathway of mutual stimulation is important in eliciting a complete cell-mediated immune response.

IL-12 is an important cytokine involved in the production of IFN-γ by T cells and NK cells, and plays an important role in the differentiation of T helper 1 (Th1) cell population (1-4). A unique aspect of IL-12 signaling is that it works together with IL-18 and the T cell co-receptor CD28 in inducing IFN-γ production (1,5,6). Although IL-12-induced IFN-γ expression has been shown to occur mostly at the level of transcription, the synergy between IL-12 and αCD28 or IL-12 and IL-2 has been shown to occur mostly at the post-transcriptional level, e.g., message stability and mobilization of nuclear mRNA (7-9).
Upon binding to its receptor, IL-12 activates Janus family kinases, Tyk2 and Jak2, which then phosphorylate the tyrosine residues of Stat3 and Stat4 (10). These tyrosine phosphorylations are responsible for the formation of Stat4/Stat4 homodimer and Stat3/Stat4 heterodimers (10-12). These dimers then translocate to the nucleus and bind to the promoters of IL-12-responsive genes (11,13). IL-12 stimulation also induces serine phosphorylation of Stat3 and Stat4, which are important for their transcriptional activity (14-16). Recently, it has been shown that Stat4 binds to the IFN-\(\gamma\) promoter along with AP1 in response to IL-12 stimulation of activated T cells. This is the same promoter region where synergy between IL-12 and IL-18 occurs (17,18).

Depending on the type of external signals, T cells can initiate multiple intracellular signaling pathways that can be broadly classified into two groups: one, which includes T cell receptors (TCRs), CD3, and phorbol esters plus ionomycin, and is sensitive to cyclosporin A (CsA); the other one includes IL-12 alone, or in combination with either IL-18 or \(\alpha\)CD28, and is resistant to CsA (1,19,20). The CsA-resistant pathway has been thought to be involved in graft-versus-host disease during allogeneic bone marrow transplantation (21). We have shown that the CD28-mediated CsA-resistant co-stimulatory pathway of T cell activation is sensitive to rapamycin (22). Rapamycin is a macrolide antibiotic with potent immunosuppressive properties. It is structurally related to the immunosuppressant FK506 and binds to the same intracellular receptor FKBP12 (FK506-binding protein) (23). However, the principal target proteins are different for these two compounds. Whereas FK506-FKBP12 inhibits the serine-threonine phosphatase calcineurin, rapamycin-FKBP12 inhibits the serine-threonine
kinase mTOR (24-27). Interestingly, the target of CsA and its intracellular receptor cyclophilin is also calcineurin (24,25). Rapamycin treatment has been shown to cause G1 cell cycle arrest in a variety of cell types, including T cells (28,29).

In this report, we investigated the effect of rapamycin on IL-12-induced IFN-\(\gamma\) production by activated human peripheral blood T cells. Our results show that IL-12-induced IFN-\(\gamma\) production was partially sensitive to rapamycin pretreatment. Rapamycin treatment resulted in the aberrant recruitment of Stat3, Stat4 and phospho-c-Jun to the genomic promoter region resulting in decreased IFN-\(\gamma\) transcription. IL-12-induced phosphorylation of Stat3 on Ser727 was affected by rapamycin, which might be responsible for the aberrant recruitment of transcription factors to the promoter region. Our data also show that the IL-12-induced interaction between mTOR and Stat3 was affected by rapamycin pretreatment, and the reduction in mTOR protein level by siRNA resulted in decreased production of IFN-\(\gamma\) after IL-12 stimulation. Thus, the present study illustrates a mechanism by which the IL-12-induced CsA-resistant signaling pathway can be manipulated by another immunosuppressive drug, rapamycin, providing another rationale for combination therapy approaches to diseases requiring T cell suppression.
EXPERIMENTAL PROCEDURES

Reagents-Anti-human IL-4 mAb, anti-IFN-γ mAb, and IL-12 were purchased from R&D Systems (Minneapolis, MN). Anti-CD3 and anti-CD28 mAbs were purchased from Pharmingen (San Diego, CA). Anti-STAT3 (sc-482), anti-STAT4 (sc-486) antiserum and anti-phospho (Y705)-STAT3 mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mTOR antiserum was from Cell Signaling Technology (Beverly, MA), anti-Nucleoporin (p62) was from BD Transduction Laboratories (San Diego, CA), anti-phospho (S727)-STAT3 was from Upstate Biotechnology (Lake Placid, NY), and anti-phospho (Y693)-STAT4 antiserum was from Zymed laboratories (San Francisco, CA). Cyclosporin A and rapamycin were purchased from Calbiochem (San Diego, CA).

Cells and tissue cultures-Peripheral blood mononuclear cells (PBMC) collected from healthy donors (who provided informed consent) were isolated by Ficoll-Hypaque density gradient centrifugation. Total resting T cells were purified from PBMC with the Human T-cell Enrichment Column Kit (R&D System). Cells were cultured in RPMI 1640 with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. T cells were activated with plastic bound anti-CD3 (200 ng/mL) plus anti-CD28 Ab (1 µg/ml) for 48 hours. Preactivated T cells were washed with phosphate buffered saline (PBS), and were then stimulated with 1 ng/ml of recombinant IL-12 and/or 100 ng/ml of anti-CD28 mAb clone 9.3 (kindly provided by Dr. Carl June, University of Pennsylvania Medical Center, Philadelphia, PA) for 1 hour. Pretreatment with CsA and rapamycin were done 30 minutes before IL-12 stimulation. For
measurement of IFN-γ production, T cells were stimulated for 20 hours and the supernatants were analyzed by Cytometric Beads Array (CBA) Kit (BD Bioscience, San Diego, CA) according to the manufacturer’s instructions. For differentiation of T cells into Th1 and Th2 populations, resting T cells were cultured as described previously (30).

**RT-PCR**—Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using SuperScript II RT (Invitrogen, Carlsbad, CA) and oligo-dT 3’ primer in a total volume of 20 µl. 2 µl of cDNA was amplified by PCR. The primers used were as follows: IFN-γ, 5’-AAGAGTGTGGAGACCATCAA-3’ and 5’-CTGACTCCTTTTTTCGCTTCC-3’. GAPDH, 5’-TGTCATACCAGGAAATGAGCTTGAC-3’ and 5’-CCTGGCCAAGGTCATCCATGACAAC-3’. The reaction volume of 25 µl contained each primer at 0.5 µM, dNTP at 200 µM, 2 mM magnesium chloride and 0.5 U of Taq polymerase. PCR conditions were as follows: 94°C for 4 min; 38 cycles at 94°C for 30s, 52°C for 30s and 72°C for 30s; final elongation at 72°C for 10 min. PCR was done with an iCycler (Bio-Rad laboratories, Hercules, CA). The PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

**Immunoprecipitation and Immunoblotting**—Nuclear extracts were prepared as described previously (31). 100 µg of nuclear extracts were immunoprecipitated according to the procedure described earlier (22). In brief, protein A Sepharose (10 µl 50% slurry) was first incubated with the antibody for 1 hour at 4°C. The antibody-protein A-Sepharose was then washed and exposed to the lysates overnight at 4°C. After extensive washing, the immunocomplexes were eluted with NuPAGE LDS sample buffer (Invitrogen). The
samples were boiled for 5 minutes, resolved on 4-12 % NuPAGE Novex Bis-Tris Gels (Invitrogen), and transferred to PVDF membrane (Invitrogen). Detection method was carried out with ECL-Plus detection reagent (Amersham Pharmacia Biotech, U.K.). Whole cell lysates were prepared as described previously (32). 300 µg lysates were used to immunoprecipitate with 4 µg anti-mTOR antibody.

Chromatin Immunoprecipitation (ChIP) assay-The ChIP Assay Kit (Upstate Biotechnology) was used in this assay according to the procedure described earlier (30). Briefly, T cells (1x10⁶) were cross-linked using 1% formaldehyde for 10 min at 37°C. The reaction was terminated by addition of 125 mM glycine for 5 min. Cells were lysed in 200 µl of SDS lysis buffer. Chromatin was sheared by sonication and diluted 10 fold in ChIP dilution buffer. Immunoprecipitations were performed at 4°C overnight with 4 µl of anti-acetyl-histone H4, 10 µl of anti-phospho c-Jun (Upstate Biotechnology), 4 µg of anti-Stat3 (Santa Cruz), or 4 µg of anti-Stat4 (Zymed) rabbit polyclonal Ab. Immune complexes were collected with salmon sperm DNA/protein A-agarose for 1 hr. After washing, samples were eluted in elution buffer (1% SDS and 0.1 M NaHCO3) for 1st-ChIP, or in 10mM DTT for 2nd-ChIP (33). Samples for 2nd-ChIP were diluted in ChIP dilution buffer and reimmunoprecipitated with a second antibody. Immune complexes were collected with salmon sperm DNA/protein A-agarose, washed, and eluted in elution buffer. DNA cross-links were reversed by heating at 65°C for 4 hrs. After proteinase K digestion, DNA was extracted with phenol-chloroform and precipitated in ethanol. 1/20th of the total volume of DNA was used in each PCR using the following primer pairs: sense, 5'-TGCCTCAAAGAATCCCACC-3' (nt 105-123) and antisense, 5'-
CAGTAACAGCCAAGAGAACC-3' (nt 542-523). PCR conditions were as follows: 94°C for 4 min; 38 cycles at 94°C for 30s, 58°C for 30s and 72°C for 30s; final elongation at 72°C for 10 min. The PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

Treatment of peripheral blood T cells with mTOR-specific siRNA-Human peripheral blood T cells were transfected with mTOR-specific siRNA using Human T Cell Nucleofector kit (Amaxa Biosystems, Germany), according to the manufacturer’s instructions. Briefly, CD3/CD28-preactivated T cells (5x10⁶/point) were washed once with PBS, and were then transfected with 1.4 µg of either control siRNA or siRNA specific for mTOR (Dharmacon, Inc.). After transfection, cells were cultured for 48 hours, washed, and then stimulated with 1ng/ml of rIL-12 for either 1 hour to prepare lysates or 24 hours to collect supernatants for IFN-γ measurement.
RESULTS

Effect of rapamycin on IL-12-mediated IFN-γ production by T cells-It has been reported that IL-12-mediated IFN-γ production by T cells is insensitive to CsA, and that IL-12 synergizes with IL-18 or CD28-mediated costimulation in a CsA-resistant manner (1,19,20). Since the CsA-resistant pathway has been thought to be responsible for the limited effectiveness of CsA in the treatment of graft-versus-host disease following allogeneic bone marrow transplantation (21), we wanted to investigate the effect of rapamycin on IL-12-mediated IFN-γ production. To determine the effect of rapamycin on IL-12-induced IFN-γ production, we used freshly isolated human peripheral blood T cells that have been activated by αCD3 plus αCD28 for 48 hours. As shown in Figure 1a, IL-12 stimulation of preactivated T cells produced a significant amount of IFN-γ, which was insensitive to pretreatment of CsA, but was partially inhibited by rapamycin pretreatment. The levels of IFN-γ production were further increased significantly when cells were treated with IL-12 in the presence of αCD28 antibody, where αCD28 treatment alone had no effect (Fig. 1a). Interestingly, IL-12 plus αCD28-mediated IFN-γ production was also sensitive to rapamycin pretreatment, whereas CsA had no effect on IFN-γ production. The insensitivity of the IL-12 or IL-12 plus αCD28 treatment to CsA was not due to the ineffective dose of CsA (100 ng/ml), since the escalation of the dose up to 500 ng/ml had no effect on these treatments (Fig. 1b). The maximum inhibitory effect of rapamycin was found to be 20 ng/ml (Fig. 1b). Interestingly, even with the highest dose, the maximum inhibition by rapamycin was 60% indicating the involvement of a rapamycin-resistant pathway. These data suggested that IL-12-mediated IFN-γ production by activated T cells was insensitive to CsA, but partially sensitive to
rapamycin. Interestingly, the incremental increase in IL-12-induced IFN-γ production mediated by CD28 costimulation was almost completely inhibited by rapamycin.

**Effect of rapamycin on IL-12-induced transcription of IFN-γ gene**-To determine whether the effect of rapamycin on IL-12-mediated IFN-γ production was due to the decreased IFN-γ transcription, total RNAs were isolated from activated T cells treated under different conditions as shown in Fig. 2. Total RNAs were then used to measure the expression levels of IFN-γ gene by semi-quantitative RT-PCR. Like protein level, the expression level of IFN-γ mRNA was increased upon IL-12 treatment, and the IFN-γ mRNA level was unaffected by CsA pretreatment, but was reduced by rapamycin pretreatment. IL-12 plus αCD28 treatment caused a slight increase in expression levels of IFN-γ mRNA over IL-12 treatment alone, whereas αCD28 treatment alone had no effect. However, the addition of αCD28 had a greater effect on cytokine production than on message levels. Again, IL-12 plus αCD28 treated expression of IFN-γ mRNA was partially sensitive to rapamycin, but insensitive to CsA. These data suggested two things: first, IL-12 induced IFN-γ production by activated T cells occurred mainly at the level of transcription, as has been reported earlier (7), and rapamycin affected the steady state levels of IFN-γ mRNA; and, second, the additive effect of αCD28 on IL-12-induced IFN-γ production might not be at the level of transcription. In agreement with this, the synergy between αCD28 and IL-12 has been shown to occur at the level of message stability (8).
Rapamycin affects IL-12-induced recruitment of Stat proteins at IFN-γ promoter-It has been shown that Stat4 binds to the IFN-γ promoter along with AP1 in response to IL-12 stimulation of activated T cells (17,18). Since we found that IL-12 induced IFN-γ transcription was partially sensitive to rapamycin, we wanted to determine whether rapamycin pretreatment had any effect on the IL-12-induced recruitment of the Stat4/AP1 complex to the IFN-γ promoter. For this purpose, we performed a ChIP assay. Besides Stat4 and phospho-c-Jun, we also examined the status of Stat3 and acetylated histone H4. It has been shown that IL-12 stimulation also activates Stat3 (10), and acetylated histone H4 was chosen to monitor the activation status of the chromatin, since acetylation of histone H4 has been correlated to the transcriptionally active chromatin (34). As shown in Fig. 3a, Stat4 was recruited to the promoter after IL-12 stimulation (lane 2 vs 1), and that level of recruitment was slightly increased after IL-12/αCD28 treatment (lane 6 vs 2). Pretreatment with CsA did not affect either IL-12- or IL-12/αCD28-induced recruitment of Stat4 (lane 3 vs 2, and lane 7 vs 6), but the recruitments were reduced by rapamycin in both IL-12 and IL-12/αCD28 stimulations (lane 4 vs 2, and lane 8 vs 6). The recruitments of Stat3 and phospho-c-Jun were similar to the Stat4 recruitment, except for the fact that the level of Stat3 after IL-12 stimulation alone was not significantly higher than the control level present due to the preactivation of T cells. Input controls demonstrated that equivalent amounts of materials were added to each immunoprecipitation. The degree of acetylation of histone H4 was comparable among different treatments indicating the transcriptionally active nature of the IFN-γ promoter. In lanes 9, 10, and 11, the recruitment status of the individual transcription factor was examined as a function of T-cell subset. The results showed that Stat3, Stat4 and
phospho-c-Jun were recruited to Th1 cells, but not precursor or Th2 cells. We and others have shown recently that during in vitro differentiation of human peripheral blood T cells to Th1 or Th2, the degree of acetylation of histone H4 was greater in Th1 compared to Th2, indicating that the IFN-γ promoter was more active transcriptionally in Th1 than in pTh and Th2 cells (30,35).

Our next step was to identify whether Stat3 and Stat4 were recruited simultaneously to IFN-γ promoter upon IL-12 stimulation. For that purpose we performed sequential ChIP analysis as described in Methods and Materials. First immunoprecipitation was done with either anti-Stat3 or anti-Stat4 (designated as 1st ChIP), and the immunoprecipitated sample was subjected to a second round of immunoprecipitation with either normal IgG, anti-Stat4 or anti-Stat3 (designated as 2nd ChIP). As shown in Fig. 3b, the immunoprecipitated complexes after the 1st ChIP with either anti-Stat3 or anti-Stat4 showed the presence of Stat4 or Stat3, respectively, in the DNA/protein complex as demonstrated by the PCR amplification of the promoter region. The products obtained from the 2nd ChIP were specific since no PCR product was obtained from the 2nd ChIP performed with normal IgG. These results demonstrated that the recruitment of Stat3 and Stat4 to the IFN-γ promoter were simultaneous upon IL-12 stimulation, and these inducible recruitments were insensitive to CsA, but were sensitive to rapamycin pretreatment (Fig. 3b). Without additional experiments, we cannot conclude from these ChIP assays if Stat3 and Stat4 were recruited to the IFN-γ promoter as a complex.
Rapamycin affects IL-12-induced phosphorylation of Stat3 on ser727-To determine the mechanism underlying the effect of rapamycin on IL-12 signaling, we performed co-immunoprecipitation analysis of the nuclear lysates prepared from activated T cells treated with different stimuli as shown in Fig. 4a. The results demonstrated that IL-12 stimulation induced interaction between Stat3 and Stat4, which was unaffected by treatment with either CsA or rapamycin. The Stat4-associated Stat3 was both tyrosine (position 705) and serine (position 727) phosphorylated, whereas Stat4 was tyrosine phosphorylated at position 609. Interestingly, rapamycin, but not CsA pretreatment, only decreased IL-12-induced serine phosphorylation of Stat3 at position 727 without affecting tyrosine phosphorylation of Stat3 and Stat4. This was confirmed by western blot analysis using nuclear extracts of activated T cells treated with various stimuli. As shown in Fig. 4b, only rapamycin, but not CsA pretreatment, inhibited IL-12-induced serine phosphorylation of Stat3 at position 727, but this pretreatment did not affect the tyrosine phosphorylation of either Stat3 or Stat4. The decrease in serine phosphorylation was not due to a decreased level of nuclear Stat3 as revealed by the presence of comparable levels of Stat3 present in different treatments. To rule out the possibility that the anti-phospho-Stat3 (S727) antiserum did not cross-react with the serine phosphorylated Stat4, we performed immunoprecipitation analysis with either anti-Stat3 or anti-Stat4 antiserum using IL-12-stimulated nuclear lysate, which was boiled in 1% SDS to disrupt the protein-protein interaction. As shown in Fig. 4c, the phospho-Stat3 was detected only in the sample that had been immunoprecipitated with anti-Stat3, but not with anti-Stat4 (upper panel). The cross-reactive band, designated by * in the upper panel, was a non-specific band since it was not competed by the cognate peptide (data not
shown). In the lower panel, more Stat4 was detected in Stat4-immunoprecipitated, but not in Stat3-immunoprecipitated sample. These results indicated the lack of cross-reactivity between anti-phospho-Stat3 (S727) antiserum and phosphorylated Stat4. Collectively, these data suggest that the effect of rapamycin on IL-12-induced IFN-γ production might be due to the decreased serine phosphorylation of Stat3 at position 727, although we cannot rule out the role of serine phosphorylation of Stat4 and c-Jun.

Role of mTOR in IL-12-induced IFN-γ production by activated T cells-Since IL-12-induced serine phosphorylation of Stat3 was inhibited by rapamycin, we tested whether mTOR was interacting directly with Stat3. Whole cell lysates of activated T cells treated with either medium alone or IL-12 in the presence or absence of rapamycin pretreatment were used for co-immunoprecipitation analysis. As shown in Fig. 5, IL-12 treatment caused inducible interaction between mTOR and Stat3, which was decreased by pretreatment with rapamycin. We were unable to detect any association between mTOR and Stat4 (data not shown). To further confirm the role of mTOR in IL-12-induced IFN-γ production, T cells were transfected with either control siRNA or siRNA specific for mTOR. Transfected cells were then treated with either medium alone or rIL-12. As shown in Fig. 6a, the treatment with mTOR-specific siRNA resulted in decreased production of IFN-γ upon IL-12 stimulation as compared to control siRNA-treated cells, and this effect was due to the decrease in mTOR protein levels by mTOR-specific siRNA (Fig. 6b). To determine the effect of mTOR level on IL-12-induced serine phosphorylation of Stat3, cell lysates from mTOR-specific siRNA-treated cells were analyzed for phospho-Stat3. As shown in Fig. 6c, a decrease in mTOR level also resulted
in the decrease in serine 727 phosphorylation of Stat3. This decrease in serine phosphorylation was not due to the decrease in total Stat3 level, since total Stat3 was comparable between the IL-12-treated samples. These data demonstrated that mTOR might be directly involved in IL-12-induced IFN-\(\gamma\) production by activated T cells.

**DISCUSSION**

In this report we described the sensitivity of the CsA resistant IL-12 signaling pathway to rapamycin. Our data demonstrated that the production of IFN-\(\gamma\) after stimulation with either IL-12 alone or in combination with \(\alpha\)CD28 was partially sensitive to rapamycin. The degree of suppression of IFN-\(\gamma\) production remained the same with higher doses of rapamycin, indicating the possible presence of a rapamycin-resistant pathway. Our data shows that the IL-12 induced increase in the steady state level of IFN-\(\gamma\) mRNA was insensitive to CsA, while being partially sensitive to pretreatment with rapamycin. These data suggest that IL-12-induced IFN-\(\gamma\) gene transcription might be affected by rapamycin.

It has been shown that Stat4 binds to the IFN-\(\gamma\) promoter along with AP1 in response to IL-12 stimulation of activated T cells. This is the same promoter region where synergy between IL-12 and IL-18 occurs (17,18). To investigate the role of this promoter region in IL-12-induced IFN-\(\gamma\) gene expression, we performed ChIP assay to monitor the promoter recruitments of Stat4, Stat3, and phospho-c-Jun (Fig. 3a). The results demonstrated that IL-12 stimulation induced the recruitment of Stat4, Stat3 and
phospho-c-Jun to the promoter, and that these recruitments were sensitive to rapamycin, but not to CsA. Next, we wanted to know whether Stat4 and Stat3 were recruited to the same promoter simultaneously, since it was possible that the cells, which showed Stat4 recruitments, were different from the cells that had Stat3 recruitment. Sequential ChIP analysis revealed that the IL-12 treatment induced simultaneous recruitments of Stat4 and Stat3 to the same promoter (Fig. 3b). Although it has been shown that Stat4 forms a heterodimer with Stat3, and this heterodimer binds to DNA upon IL-12 stimulation ((11), and Fig. 4), without additional experiments, we cannot conclude from these ChIP assays if Stat4 and Stat3 were recruited to the IFN-γ promoter as a complex.

To identify the mechanism behind the IL-12-induced aberrant recruitments of Stat4 and Stat3 to the IFN-γ promoter upon rapamycin pretreatment, we tested the effect of rapamycin on the IL-12-induced heterodimer formation between Stat3 and Stat4. Interestingly, the degree of interaction between Stat3 and Stat4 upon IL-12 stimulation was unaffected by rapamycin pretreatment, although that same pretreatment caused a decrease in the serine phosphorylation of Stat3 associated with Stat4. We also confirmed this effect of rapamycin by western blot analysis. While IL-12-induced tyrosine phosphorylation of Stat3 and Stat4 was unaffected by rapamycin, IL-12-induced serine phosphorylation of Stat3 was decreased by rapamycin. This is in agreement with the published reports where Stat3 has been shown to be a substrate of mTOR (36). We were unable to determine the serine phosphorylation status of Stat4 due to the unavailability of phospho-Stat4-specific antiserum. These data suggested two things: first, serine phosphorylation of Stat3 had no influence on the IL-12-induced dimer formation with
Stat4; and second, Stat3 could be a direct substrate of mTOR, as has been reported earlier (36,37). To investigate the latter possibility, we performed co-immunoprecipitation analysis with mTOR (Fig.5). The results demonstrated that mTOR interacted with Stat3 in an IL-12 stimulation-dependent manner, and that the inducible interaction was inhibited by rapamycin. Whether mTOR directly phosphorylates Stat3 upon IL-12 stimulation needs to be determined. To further investigate the role of mTOR in IL-12 signaling, we used mTOR specific siRNA. The resulting lowered mTOR protein levels produced decreases in both Stat3 serine phosphorylation and IFN-γ production after IL-12 stimulation.

Our data suggests that the serine phosphorylation of Stat3 is an important event in the IL-12 induced IFN-γ production by activated peripheral blood T cells. The phosphorylation at serine 727 of Stat3 seems critical in the recruitments of Stat3, Stat4, and phospho-c-Jun to the IFN-γ promoter, although it has been reported that the serine phosphorylation of Stat3 at 727 has no influence on its DNA binding (38). However, there are several factors which can contribute to this discrepancy. The gel shift analysis which was used to support the previous conclusion has less physiological relevance than we show in our ChIP assay. Our studies use the IL-12-responsive region of the IFN-γ promoter which is a composite element consisting of Stats and AP1 binding sites, thus being more specific than the Stat consensus binding sequence used in the previous study. Lastly, the effect of rapamycin on IL-12 induced recruitments of transcription factors to the IFN-γ promoter could be due to the decreased serine phosphorylations of Stat4 and/or
c-Jun in addition to the Stat3 serine phosphorylation. This phosphorylation status of Stat4 and c-Jun is currently under investigation.

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Figure Legends

Figure 1. **Effect of CsA and rapamycin on IL-12- and IL-12/CD28-induced IFN-γ production.** Anti-CD3/CD28-pretreated T cells were stimulated with rIL-12 and/or anti-CD28 mAb in the presence or absence of CsA (100ng/ml) or rapamycin (20ng/ml). After 20 hr of stimulation, supernatants were analyzed for the production of IFN-γ. Data were collected from 10 different individuals (*P<0.05). (b) Effect of different doses of CsA and rapamycin on IL-12- and IL-12/CD28-induced IFN-γ production. Data were collected from three different donors (*P<0.05).

Figure 2. **Effect of CsA and rapamycin on IL-12- and IL-12/CD28-induced expression of IFN-γ gene.** Preactivated T cells were stimulated as described in Figure 1. Total RNAs were extracted to synthesize cDNAs. Different amounts of cDNAs were used for PCR amplification using primers for IFN-γ and GAPDH as described in Methods and Materials. PCR products were resolved on 1% agarose gel and stained with ethidium bromide. Every three lanes represent serial five-fold dilution of each sample DNA (a). (b) Quantitative representation of IFN-γ gene expression after normalization with the expression levels of GAPDH gene. Results were representative of three different experiments.

Figure 3. **Effect of CsA and rapamycin on IL-12-induced recruitment of transcription factors to the IFN-γ promoter.** (a) Preactivated T cells were stimulated with rIL-12 and/or anti-CD28 mAb in the presence or absence of CsA (100ng/ml) or
rapamycin (20ng/ml) for 1 hr and were used in the ChIP assay using control IgG, anti-Stat4, anti-Stat3, anti-phospho-c-Jun (p-c-Jun) and anti-acetylated histone H4 (Ac.HH4). Total resting peripheral blood T cells (pTh) were subjected to in vitro differentiation for 3 days. Differentiated cells were stimulated with PMA plus ionomycin for 30 min and were used for ChIP assay. Results were representative of three experiments. (b) Sequential ChIP assay. Preactivated T cells were stimulated with rIL-12 in the presence or absence of CsA or rapamycin for 1 hr and were used for sequential ChIP analysis. After immunoprecipitation with first antibody (1st ChIP), the immunocomplexes were eluted either with elution buffer (- in 2nd ChIP), or with 10 mM DTT and subjected to re-immunoprecipitation with control IgG, anti-Stat3 or anti-Stat4 (2nd ChIP). Results were representative of three experiments.

Figure 4. **Effect of rapamycin on IL-12-induced phosphorylation of Stat3 and Stat4.** Preactivated T cells were stimulated with rIL-12 and/or anti-CD28 mAb in the presence or absence of CsA (100ng/ml) or rapamycin (20ng/ml) for 1 hr. Nuclear lysates prepared from activated cells were used for immunoprecipitation with anti-Stat4 antibody, and the immunocomplexes (a) or the straight nuclear lysates (b) were analyzed by western blot analysis using antisera raised against tyrosine phosphorylated Stat3 (p-Stat3 Y705), serine phosphorylated Stat3 (p-Stat3 S727), tyrosine phosphorylated Stat4 (p-Stat4 Y693), and unphosphorylated Stat3 and Stat4. Bar graphs represent the different protein levels quantitated and normalized by either total Stat4 (a) or nucleoporin (b). (c) Specificity of the phospho-Stat3 (S727) antiserum. IL-12-treated nuclear lysates (400 µg/point) were boiled in TNT buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5%
Triton X-100) containing 1 % SDS for 5 minutes, diluted five fold with TNT buffer, and were immunoprecipitated with either anti-Stat3 or anti-Stat4 antiserum. The immunocomplexes were analyzed by western blot analysis using anti-phospho-Stat3 (S727) (upper panel) and anti-Stat4 antisera (lower panel). Results were representative of three experiments. * represents the non-specific band.

Figure 5. **IL-12-induced interaction between mTOR and Stat3 was sensitive to rapamycin.** Preactivated T cells were stimulated with rIL-12 in the presence or absence of rapamycin (20ng/ml) for 1 hr. Whole cell lysates were used for immunoprecipitation with anti-mTOR antiserum, and the immunocomplexes were analyzed by western blot analysis with anti-Stat3 and anti-mTOR antisera. Results were representative of three experiments. Bar graph represents the quantitation of the different levels of Stat3 and mTOR.

Figure 6. **Decrease in mTOR protein level affected IL-12-induced IFN-γ production.** CD3/CD28-preactivated T cells were transfected with either control siRNA (control) or mTOR-specific siRNA (siRNA). Transfected cells were restimulated with either medium alone or 1ng/ml of rIL-12 for either 1 hour (for lysate preparation; panel b) or 24 hours (for IFN-γ measurement; panel a). (c) Effect of mTOR siRNA on phosphorylation of Stat3 (S727). Preactivated T cells were transfected with either control siRNA or mTOR specific siRNA as above, and the lysates were analyzed by western blot analysis using anti-mTOR, anti-phospho-Stat3 (S727) and anti-Stat3. This is a representative experiment of three independent donors.
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Figure 1

(a) Bar graph showing IFN-γ (ng/mL) levels with different treatments. Treatments include Control, IL-12, IL-12/CsA, IL-12/RAP, CD28, IL-12/CD28, IL-12/28/CsA, IL-12/28/RAP.

(b) Line graph showing IFN-γ (ng/mL) levels with varying drug concentrations (1 ng/mL to 1000 ng/mL). Treatments include CD28/CsA, CD28/RAP, IL12/CsA, IL12/RAP, IL12/CD28/CsA, IL12/CD28/RAP.
Figure 2

(a) Bar chart showing relative expression of IFN-γ gene for different treatments: Control, CD28, IL-12, IL-12/CsA, IL-12/RAP, IL-12/CD28, IL-12/28/CsA, IL-12/28/RAP.

(b) Gel electrophoresis showing template cDNA for IFN-γ and GAPDH.
Figure 3a

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<th>IL-12/CD28</th>
<th>IL-12/CD28/CsA</th>
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1 2 3 4 5 6 7 8 9 10 11
Figure 3b

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Figure 4a

Control    IL-12    CD28    IL-12/CD28    IL-12/CsA    IL-12/RAP

IP: Stat4
IB:

p-Stat3 (Y705)

p-Stat3 (S727)

Stat3

p-Stat4 (Y693)

Stat4

Arbitrary Units

0    0.2    0.4    0.6    0.8    1    1.2

Control    IL-12    CD28    IL-12/CD28    IL-12/CsA    IL-12/RAP

p-Stat3 (Y705)
p-Stat3 (S727)
Stat3
p-Stat4 (Y693)
Figure 4b

**IB:**

- **p-Stat3 (Y705)**
- **p-Stat3 (S727)**
- **Stat3**
- **p-Stat4 (Y693)**
- **Stat4**
- **Nucleoporin**

**Graph:**

- **Arbitrary Units**

**Legend:**

- p-Stat3 (Y705)
- p-Stat3 (S727)
- Stat3
- p-Stat4 (Y693)
- Stat4
Figure 4c
Figure 5

[Image of a Western blot showing protein expression of Stat3 and mTOR under different conditions: Control, IL-12, and IL-12/RAP. The graph below the image displays the arbitrary units of Stat3 and mTOR expression in each condition.]
Figure 6

(a) Bar graph showing INF-γ levels in different conditions: Control siRNA, Control siRNA/IL-12, mTOR siRNA, mTOR siRNA/IL12.

(b) Western blot analysis comparing Control and siRNA conditions with or without IL-12 treatment. Bands for mTOR and Nucleoporin are shown.
Figure 6
Interleukin-12-induced IFN-gamma production by human peripheral blood T cells is regulated by mammalian target of rapamycin (mTOR)
Hitoshi Kusaba, Paritosh Ghosh, Rachel Derin, Meredith Buchholz, Carl Sasaki, Karen Madara and Dan L. Longo

J. Biol. Chem. published online November 1, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405204200

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