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Impairment of IL-12-Dependent STAT4 Nuclear Translocation in a Patient with Recurrent Mycobacterium avium Infection

Hidemi Toyoda,†‡ Masaru Ido,‡ Tatsuya Hayashi,† Esteban C. Gabazza,† Koji Suzuki,‡ Jun Bu,‡ Shigeki Tanaka,‡ Takashi Nakano,§ Hitoshi Kamiya,§ James Chipeta,¶ Rodrick R. Kisenge,*, Jian Kang,* Hiroki Hori,* and Yoshihiro Komada2*

We examined the immunological abnormality in a patient with recurrent Mycobacterium avium infection. T cells from the patient showed decreased ability both to produce IFN-γ and to proliferate in response to IL-12. Despite decreased expression of IL-12Rβ1 and β2 chains in the patient’s PHA-activated T cells, there was no difference in IL-12-induced tyrosine and serine phosphorylation of STAT4 in PHA-activated T cells between the patient and healthy subjects, suggesting that IL-12R signals are transmitted to STAT4 in the patient’s PHA-activated T cells. Using EMSA, confocal laser microscopy, and Western blotting, we demonstrated that the nuclear translocation of STAT4 in response to IL-12 is reduced in PHA-activated T cells from the patient when compared with those from healthy subjects. Leptomycin B was used to examine whether nuclear export of STAT4 is increased in the patient’s T cells. However, leptomycin B treatment did not reverse impaired IL-12-induced nuclear accumulation of STAT4. Although the exact mechanism responsible for the impaired STAT4 nuclear translocation in this patient remains unclear, the absence of mutation in the IL-12Rβ1, IL-12Rβ2, STAT4, and STAT4-binding sequence of the IFN-γ gene and preservation of STAT4 tyrosine and serine phosphorylation suggest the existence of a defective STAT4 nuclear translocation. This defect is likely responsible for the impaired STAT4 nuclear translocation in IL-12-stimulated T cells, leading to impairment of both IFN-γ production and cell proliferation. To the best of our knowledge, this is the first report of a patient with atypical mycobacterial infection associated with impairment of STAT4 nuclear translocation. The Journal of Immunology, 2004, 172: 3905–3912.

Interleukin L-12 is a heterodimeric cytokine composed of two disulfide-bound glycoprotein subunits, p35 and p40; it is secreted from macrophages and dendritic cells (1). IL-12 stimulates T cells and NK cells to produce IFN-γ, which activates macrophages. This cascade is critical for host defense against intracellular pathogens such as Mycobacteria, Salmonella, Leishmania, Toxoplasma, and Listeria.

So far, mutations in five genes have been reported in patients with the so-called idiopathic Bacillus Calmette-Guérin and environmental nontuberculous mycobacteria infections, and the presence of these mutant genes has been associated with Mendelian susceptibility to mycobacterial infection (MIM209950). The reported mutations include: IL12B (2), encoding the p40 subunit of IL-12; IL12RB1 (3, 4), encoding the β1 chain of IL-12R; IFNGR1 (5, 6) and IFNGR2 (7), encoding the two chains of IFN-γR; and STAT1 (8), encoding an essential molecule in the IFN-γR signaling pathway. The severity of the clinical manifestations differs among affected individuals. Patients with IFN-γR deficiency are highly susceptible to mycobacterial infection in early childhood and die prematurely (5, 6), but those with partial deficiency of IFN-γR1 caused by a missense mutation in its extracellular domain, have only a mild clinical course (9). Patients with IL-12Rβ1-chain deficiency tend to have a mild clinical course (3); mycobacterial infection in these patients can be effectively controlled with antibiotics and IFN-γ supplementation. These studies demonstrated that human IL-12-dependent IFN-γ-mediated immunity is essential for the control of mycobacterial infection. Molecular diagnosis and pathophysiology-based treatment are essential for these pathological conditions, but no clear genetic etiology has been identified in many of these cases.

IL-12R is a heterodimer, composed of β1 and β2 chains. Both chains are essential for the high affinity binding of IL-12 to its receptor (10). STAT4 resides in the cytoplasm as a monomer in resting states, but after activation with IL-12 (11, 12) it is recruited to a tyrosine-based motif in the IL-12Rβ2 chain via the STAT4 Src homology 2 domain (13). After tyrosine phosphorylation, STAT4 forms a homodimer via reciprocal interaction through conserved Src homology 2 domains, and then the STAT4 dimer translocates to the nucleus where it regulates the transcription of IFN-γ (11, 12, 14–16). STAT4 knockout mice are susceptible to intracellular pathogens including Leishmania major and Toxoplasma gondii and are unable to control their replication (17, 18). Lymphocytes from STAT4 knockout mice are unable to produce sufficient IFN-γ, proliferate, and lyse target cells in response to IL-12 (19, 20). Cells reconstituted with the high-affinity IL-12Rβ1/IL-12Rβ2 plus various STATs are able to activate only STAT4 when stimulated with IL-12 (13). In addition, CD4 T cells from STAT4 knockout mice bearing an IL-12Rβ2 chain transgene are unable to exhibit IL-12-mediated IFN-γ production (16). These results indicate that STAT4 activation is the main mechanism involved in lymphocyte response to IL-12.

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In the present report, we describe a patient with recurrent Mycobacterium avium infection whose T cells could not proliferate or produce IFN-γ in response to IL-12, despite expression of wild-type IL-12Rβ1 and IL-12Rβ2. Whereas IL-12 was able to stimulate tyrosine and serine phosphorylation of STAT4 in response to IL-12, the IL-12-induced nuclear translocation of STAT4 was reduced in the patient’s T cells. To our knowledge, this is the first report of a patient with defective nuclear translocation of STAT4 upon IL-12 stimulation as a main mechanism of susceptibility to mycobacterial infection.

Materials and Methods

Case report

A Japanese boy presented with lung abscess, hepatosplenomegaly, and generalized lymphadenopathy at the age of 1 year and 3 mo. Treatment of the patient with cefazolin sodium resulted in exacerbation of his lung abscess, hilar lymphadenopathy, and hepatosplenomegaly. Mantoux test (10 × 9 mm erythema, 2 × 2 mm induration) was positive and the culture of gastric juice specimens demonstrated the presence of M. avium. Hilar lymph node and lung biopsy showed histiocytic granuloma, and culture of the samples showed the presence of M. avium, confirming the diagnosis of disseminated mycobacterial infection. The immunological workup, such as the complement and surface immunophenotype of T, NK, and B cells, NK cell activity, neutrophil chemotaxis and superoxide production test, Ig levels, and proliferative response to mitogens such as lectin PHA or Con A were normal, and he was HIV seronegative. After treating the patient with kanamycin, isoniazid, and rifampicin for 12 mo, reduction in lymph node swelling and hepatosplenomegaly was observed, and the drugs were discontinued. But then the lymph nodes enlarged again, and thus antibiotics were resumed. The patient showed recurrent episodes of generalized lymphadenopathy, and from the age of 11 years he was treated with s.c. injection of 3 × 10^6 U recombinant IFN-γ three times per week (21). He has tolerated his childhood immunizations without adverse reactions and had normal Ab production for diphtheria toxin, pertussis, measles, and rubella. There was no family history of recurrent infections among his parents, 10-year-old brother, and other relatives. The patient’s parents are not consanguineous.

Isolation and activation of PBMCs

Heparinized blood samples were obtained from the patient, his parents, and healthy adult volunteers. PBMCs were isolated by density centrifugation using Ficoll Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). For T cell activation studies, PBMCs were cultured for 96 h in RPMI 1640 (Sigma-Aldrich) containing 15% FCS, 5 μg/ml PHA (Sigma-Aldrich), and 100 U/ml IL-2 (Shionogi, Osaka, Japan) as described (22). After incubation, cells were >95% CD3 positive and CD56 negative.

Proliferation assays and measurement of IFN-γ production

After activation with PHA plus IL-2 for 96 h, T cells were incubated in 96-well, U-bottom plates at a density of 2 × 10^5 cells/well with or without the indicated concentrations of IL-2 or IL-12 (R&D Systems, Minneapolis, MN) at 37°C for 72 h. Cell proliferation was assayed by pulse labeling of cells with 1 μCi of [3H]thymidine (Amersham, Piscataway, NJ) for 8 h before harvesting. For assessing IFN-γ production, supernatants were harvested and the IFN-γ concentration was assayed using an ELISA kit (eBioScience, San Diego, CA).

Flow cytometric analysis

IL-12R or IL-2R expression was detected using flow cytometric analysis. Briefly, cells (1 × 10^6) were incubated with PE-conjugated anti-IL-12Rβ1 (clone 2.4E6; BD Pharmingen, San Diego, CA), PE-conjugated anti-IL-12Rβ2 (clone 2B6/12β2; BD Pharmingen), PE-conjugated anti-IL-2R α-chain (clone M-A251; BD Pharmingen), PE-conjugated mouse IgG1 negative control Ab (clone MOPC-21; BD Pharmingen), or PE-conjugated rat IgG2a negative control Ab (clone R35-95; BD Pharmingen). Each sample was washed and the expression of each receptor was analyzed by FACS (20,000 cells/sample).

Preparation of cytosol and nuclear extract

Cytoplasmic and nuclear extract was prepared as described (23). After T cells were activated with PHA and IL-2 for 72 h, they were washed and recultured for an additional 18 h under starvation in RPMI 1640 medium before cytokine stimulation. Activated T cells were treated with or without 1 mM IL-12 for 30 min at 37°C. After stimulation, the cells were washed once with ice-cold PBS and then resuspended in extraction buffer (20 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM Na3VO4, 1 mM EDTA, 10% glycerol, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM DTT, and 0.2% Nonidet P-40) at 4°C for 5 min. After centrifugation at 4°C (8000 × g) for 10 s, the supernatant was collected and used as cytosol extract. The pellet was resuspended in high-salt buffer (hypotonic buffer with 20% glycerol and 420 mM NaCl) at 4°C for 30 min and centrifuged at 4°C (8000 × g) for 5 min, and the supernatant was used as nuclear extract.

Kinetics of STAT4 phosphorylation and dephosphorylation

To examine kinetics of STAT4 phosphorylation, T cells were stimulated with 1 nM IL-12, as described above, during several time intervals as indicated, and whole cell extract was prepared as described previously (22). To examine kinetics of STAT4 dephosphorylation, T cells were stimulated with 1 nM IL-12 for 30 min, washed three times with fresh medium, and resuspended in fresh medium during several time intervals, and whole cell extract was prepared (22). Ten micrograms of each protein was mixed with 5× Laemmli running buffer, heated at 95°C for 5 min, and subjected to Western blotting.

Western blotting

The cytosol (50 μg), nuclear (10 μg), or whole cell extract (10 μg), quantitated using a Bio-Rad protein assay (Bio-Rad, Hercules, CA), was separated by SDSPAGE and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blocking was performed by incubating the membrane in PBS containing 5% nonfat dry milk, and then the membrane was incubated in the presence of its respective Ab at room temperature for 2 h. After washing, the membrane was treated with appropriate HRP-conjugated secondary Ab for 2 h. The membrane was then washed, and the signals were detected using Western Lightening Chemiluminescence Reagent Plus (PerkinElmer Life Science, Boston, MA). The Ab raised against STAT4 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-STAT4 Ab anti-pY701STAT4, which recognizes the tyrosine 693-phosphorylated form of STAT4, was purchased from Zymed Laboratories (South San Francisco, CA).

EMSA

EMSA were performed essentially as described (24) using a 32P-labeled, double-stranded oligonucleotide (5′-GAGGCTCTATTCCCCGAATGATGAGC-3′) corresponding to the IFN-γ-responsive factor 1 gene promoter (24). Briefly, cytoplasmic or nuclear extract was incubated with a 32P-labeled probe in binding buffer for 15 min at room temperature. When used, 1 μl of anti-STAT4 Ab was incubated with nuclear extract for an additional 15 min at 4°C. Competitive inhibition was performed by incubating 50- and 100-fold excess of unlabeled probe or the IFN-γ-stimulated response element (ESE)-responsive factor 1 gene protein 15 (5′-GATCTATGCCTCGGCAAAGGGGACCCGAACTGAGCC-3′) (11) with cell extract for 10 min before addition of the probe.

Confocal laser microscopy analysis

PHA-activated T cells from the patient and healthy subjects were studied under multiple conditions (see figure legends). IFN-α2b was provided by Schering-Plough (Madison, NJ). IFN-γ was purchased from R&D Systems, and leptomycin B (LMB) was purchased from Sigma-Aldrich. After cytokine stimulation, cells were fixed with Intracellular Fixation Buffer (eBioscience) and then treated with Permeabilization Buffer (eBioscience). The cells were incubated with rabbit polyclonal Ab to STAT1, STAT4, or STAT5α (Santa Cruz Biotechnology) for 30 min, followed by incubation with FITC-conjugated Ab to rabbit Ab for 30 min (DAKO, Glostrup, Denmark). Distribution of STATs was observed using confocal laser microscopy (BX51; Flowview Laser Scanning Microscope; Olympus, Melville, NY).

RNA and DNA isolation, RT-PCR, and sequencing

Total cellular RNA was extracted with RNAzol B (Tel-Test, Friendswood, TX) from T cells activated for 96 h with PHA and IL-2. Total RNA (5 μg) was used for the synthesis of the first-strand cDNA using the Superscript first-strand synthesis kit (Life Technologies, Rockville, MD). Genomic DNA was purified from the patient’s and healthy subjects’ cells using the genomic DNA purification kit (Gentra Systems, Minneapolis, MN). PCR

3 Abbreviations used in this paper: LMB, leptomycin B; CRM1, chromosomal region maintenance 1; NES, nuclear export signal.
was conducted using Takara Taq polymerase (Takara Biomedicals, Shiga, Japan). The primers used for amplification are described in Table I. PCR products were run on 2% agarose gel and stained with ethidium bromide. For determination of nucleotide sequence, PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and sequencing was performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and the ABI 373 automated DNA sequencer (Applied Biosystems).

Statistical analysis
Data were expressed as the mean ± SE. The difference between groups was analyzed by Student’s t test, and p < 0.05 was considered statistically significant.

Results
Impairment of IL-12-induced proliferation and IFN-γ production in lymphocytes from the patient
Disseminated M. avium infection usually occurs in association with HIV infection or genetic alteration that causes an immunodeficient state. It is well known that the IL-12-IFN-γ system plays a major role in host defense against mycobacterial infection. Therefore, we assessed the response of PHA-activated T cells to IL-12 stimulation. T cells isolated from the patient failed to proliferate (Fig. 1A) or to produce IFN-γ (Fig. 1B) in response to increasing concentrations of IL-12 ranging from 10 to 1000 pM. In contrast, both the patient’s and healthy subjects’ T cells responded to IL-2 proliferated and produced IFN-γ at the same level (Fig. 1, C and D). We also analyzed the response of PHA-activated T cells to IL-12 stimulation in the patient’s parents. Cell proliferation and IFN-γ production induced by IL-12 in T cells from his parents were similar to those of control (data not shown).

Expression of IL-12Rβ1 and β2 chains
Because the functional response of lymphocytes to IL-12 depends on the expression of high-affinity IL-12R, we evaluated whether IL-12Rβ1 and β2 chains are present on PHA-activated T cells by flow cytometry using IL-12Rβ1- and β2-specific mAbs. The expression of both IL-12Rβ1 and β2 chains was decreased in PHA-activated T cells derived from the patient: the mean fluorescence channel of IL-12Rβ1 chain in the patient was 62.9% of that of healthy subjects, whereas the mean fluorescence channel of IL-12Rβ2 chain in the patient was 46.6% of that of healthy subjects (Fig. 2). No difference was observed in the expression of CD25 between T cells from the patient and from healthy subjects.

We then evaluated whether the patient had mutation in the IL-12Rβ1- or IL-12Rβ2-coding regions. However, no mutation was observed in the cDNA nucleotide sequences of these genes (data not shown).

Table I. Primers used for amplification

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12Rβ1 chain</td>
<td>TGGGGCTTGGGGCTCTACGTG</td>
</tr>
<tr>
<td>542 to 521</td>
<td>CATGGGCTTGGGGCTCTACGTG</td>
</tr>
<tr>
<td>305 to 326</td>
<td>AGGTGGGGCTTGCTTGCGTGA</td>
</tr>
<tr>
<td>1056 to 1036</td>
<td>GTGGTTCCAGCCCTAGATT</td>
</tr>
<tr>
<td>1004 to 1027</td>
<td>CTGCCGACACCCACAGAACCAG</td>
</tr>
<tr>
<td>1521 to 1498</td>
<td>GAGGTGACTTTGCTCTGCTG</td>
</tr>
<tr>
<td>1451 to 1477</td>
<td>GCCGAGAGAGAGACAGAAACAGG</td>
</tr>
<tr>
<td>2017 to 1997</td>
<td>GTCACCTACCCTCTCTGAGCC</td>
</tr>
<tr>
<td>IL-12Rβ2 chain</td>
<td>CGTGGAGAGATCCGAGATGCTCTACGT</td>
</tr>
<tr>
<td>542 to 505</td>
<td>CATGGGCTTGGGGCTCTACGTG</td>
</tr>
<tr>
<td>351 to 370</td>
<td>GAGGTGACTTTGCTCTGCTG</td>
</tr>
<tr>
<td>927 to 908</td>
<td>GTCACCTACCCTCTCTGAGCC</td>
</tr>
<tr>
<td>725 to 744</td>
<td>CTGCCGACACCCACAGAACCAG</td>
</tr>
<tr>
<td>2415 to 2396</td>
<td>GAGGTGACTTTGCTCTGCTG</td>
</tr>
<tr>
<td>2143 to 2162</td>
<td>GCCGAGAGAGAGACAGAAACAGG</td>
</tr>
<tr>
<td>3272 to 3253</td>
<td>GTCACCTACCCTCTCTGAGCC</td>
</tr>
<tr>
<td>STAT4</td>
<td>ACCAAGCTGTAAGAGGTCCAG</td>
</tr>
<tr>
<td>176 to 157</td>
<td>TGGGGCTTGGGGCTCTACGTG</td>
</tr>
<tr>
<td>1119 to 1000</td>
<td>CATGGGCTTGGGGCTCTACGTG</td>
</tr>
<tr>
<td>1050 to 1069</td>
<td>GAGGTGACTTTGCTCTGCTG</td>
</tr>
<tr>
<td>2292 to 2273</td>
<td>GTCACCTACCCTCTCTGAGCC</td>
</tr>
<tr>
<td>IFN-γ promoter fragment</td>
<td>CCCCTTTGAAAGGGTTGAGAGG</td>
</tr>
<tr>
<td>−530 to −507</td>
<td>ATGGTGGCCTTGGCTCTGTCG</td>
</tr>
<tr>
<td>STAT binding region in IFN-γ gene (first intron of IFN-γ gene)</td>
<td>CATTGGAGAGTAGTGGTT</td>
</tr>
<tr>
<td>213 to 232</td>
<td>GTCACCTACCCTCTCTGAGCC</td>
</tr>
<tr>
<td>1100 to 1081</td>
<td>GTCACCTACCCTCTCTGAGCC</td>
</tr>
</tbody>
</table>
IL-12 activates STAT4 in T cells from the patient

Because studies in STAT4 knockout mice have shown that STAT4 is necessary for lymphocyte IL-12 responsiveness, we examined whether the inability of the patient’s PHA-activated T cells to proliferate or to produce IFN-γ/H9253 in response to IL-12 was due to a loss of STAT4 activation by IL-12. As shown in Fig. 3A, IL-12 was able to induce both tyrosine and serine phosphorylation (14) of STAT4 in PHA-activated T cells from the patient and from healthy subjects. To clarify whether phosphorylated STAT4 is able to bind DNA, cytoplasmic extract prepared from unstimulated or IL-12-stimulated PHA-activated T cells was incubated with a radiolabeled probe containing the STAT4 binding site of the IFN-γ/H9253 gene. The STAT4/DNA complex increased in the cytoplasmic extract of IL-12-stimulated T cells from both healthy subjects and the patient (Fig. 3B, lanes 2 and 4).

Decreased IL-12-induced nuclear translocation of STAT4 in T cells from the patient

Once activated by tyrosine phosphorylation, STAT4 forms a homodimer, translocates to the nucleus, binds to the specific DNA sequence, and activates transcription of the IFN-γ gene (11, 12, 14–16). To evaluate whether phosphorylated STAT4 of the patient’s T cells is able to translocate to the nucleus, we performed Western blotting using nuclear extract prepared from unstimulated or IL-12-stimulated PHA-activated T cells. Phosphorylated STAT4 increased in the nuclear extract of the IL-12-stimulated healthy subject’s T cells (Fig. 4A). However, only a small amount of phosphorylated STAT4 was detected in the IL-12-stimulated patient’s T cells (Fig. 4A). To evaluate DNA binding activity of STAT4, nuclear extract prepared from unstimulated or IL-12-stimulated PHA-activated T cells was incubated with a radiolabeled probe containing the STAT4 binding site of the IFN-γ gene. The STAT4/DNA complex was detected in the nuclear extract of healthy subject’s T cells (Fig. 4B, lanes 3 and 4). Excess unlabeled oligonucleotide corresponding to the STAT4-related sequence competed with the radiolabeled probe for STAT4 binding, but not for IFN-α-stimulated response element (data not shown). However, nuclear proteins prepared from the patient’s IL-12-stimulated T cells weakly bound to 32P-labeled STAT4 oligonucleotide (Fig. 4B, lanes 7 and 8). When anti-STAT4 Ab was added to the binding mixture, a supershifted complex appeared in control samples (Fig. 4B, lane 4). To exclude the possibility of a mutation in either STAT4 gene or STAT4 binding site of the IFN-γ gene as a causative factor for the decreased nuclear translocation of STAT4, we determined the cDNA sequence of the STAT4 gene and the promoter region (−530 to −122) and intron.

**FIGURE 2.** IL-12R expression in the patient and in a normal healthy subject. PBMCs from the patient or a healthy subject were activated with PHA and IL-2 and then were stained using Abs specific for CD25 (IL-2 receptor-α chain) or IL-12Rβ1 or β2 chain. Dotted lines indicate negative control; solid lines indicate treatment of cells with Abs specific for each protein. Results are representative of five separate experiments using cells from the patient and from two different healthy subjects.

**FIGURE 3.** Analysis of STAT4 activation by IL-12. PBMCs isolated from the patient or healthy subjects were activated with PHA and IL-2 for 72 h. After keeping at a resting state for 18 h, cells were stimulated with or without 1 nM IL-12 for 30 min. A, The extent of Tyr<sup>693</sup> phosphorylation of STAT4 in the cytoplasm was assessed by Western blotting using a specific Ab (anti-pYSTAT4) (upper panel). Serine phosphorylation of STAT4, as shown by a electrophoretic shift in the STAT4 band (14), was determined by Western blotting using a STAT4 Ab (lower panel). B, Cytoplasmic extract was incubated with 32P-labeled oligonucleotide corresponding to the IFN-γ-responsive factor 1 gene promoter. Results are representative of three separate experiments using cells from the patient and from two different healthy subjects. S, DNA-binding complex induced by IL-12.
nance 1 (CRM1), an LMB-sensitive export protein (25–28). LMB was added to the culture of patient’s and control cells before and after IL-12 stimulation to assess the possible role of LMB in STAT4 export from the nucleus and to determine whether STAT4 nuclear export is up-regulated in the patient. Treatment of the patient’s and healthy subject’s T cells with LMB resulted in increased stage 2 and 3 cells compared with untreated cells (Fig. 5, B and C). Although stimulation of the patient’s T cells with IL-12 plus LMB induced a slight increase of STAT4 nuclear accumulation compared with IL-12 stimulation alone (Fig. 5, B and C), these treatments did not dramatically increase STAT4 nuclear accumulation. Furthermore, stimulation of the patient’s T cells with IL-12 plus LMB restored neither IFN-γ production nor cell proliferation (data not shown). We also stimulated PHA-activated T cells with IL-12 for 60 min and obtained the same results as observed when the T cells were stimulated for 30 min (data not shown). To clarify whether the impairment of nuclear translocation is specific for STAT4, we evaluated the nuclear translocation of STAT1 in response to IFNs and that of STAT5 in response to IL-2. IFN-α (Fig. 6) or IFN-γ (data not shown) stimulation induced nuclear translocation of STAT1, and IL-2 stimulation induced nuclear translocation of STAT5 in both control and patient’s T cells (Fig. 6).

Kinetics of STAT4 phosphorylation and dephosphorylation

In the present study described above, we identified that tyrosine phosphorylation of STAT4 after treatment of cells with 1 nM IL-12 for 30 min was similar between control and the patient’s T cells. However, the amount of STAT4 in the nucleus was markedly reduced in the patient’s T cells. These observations led us to examine whether decreased nuclear translocation of STAT4 in the patient’s T cells is due to decreased tyrosine kinase activity or increased phosphatase activity. To examine kinetics of phosphorylation, control and patient’s T cells were stimulated with 1 nM IL-12 during several time intervals as indicated, and then whole cell extract was prepared and analyzed with Western blotting using anti-pYSTAT4 or anti-STAT4 Abs. The kinetics of STAT4 phosphorylation induced by IL-12 in T cells from the patient were similar to those of control (Fig. 7A). To examine kinetics of dephosphorylation, control and patient’s T cells were treated with 1 nM IL-12 for 30 min and incubated in fresh medium for various periods of time, and then whole cell extract was prepared and analyzed with Western blotting as described above. We identified that the kinetics of STAT4 dephosphorylation after treatment of cells with 1 nM IL-12 were similar between control and patient’s T cells (Fig. 7B). These findings suggest that IL-12-induced STAT4 tyrosine phosphorylation and the subsequent dephosphorylation process were similar between the control and patient’s T cells.

Discussion

In this report, we have characterized an immunodeficiency that is caused by a defective nuclear translocation of STAT4 upon IL-12 stimulation. The STAT4-mediated IL-12 signaling pathway is critical for activation of host defense mechanisms against intracellular pathogens (17, 18). IL-12 stimulation results in activation of Janus kinase 2 and tyrosine kinase 2, which in turn phosphorylate IL-12R, creating docking sites for STAT4 (12, 29). Receptor-bound STAT4 is phosphorylated on Tyr<sup>593</sup> by Janus kinase 2 and tyrosine kinase 2, promoting STAT4 dimerization. STAT4 dimer accumulates in the nucleus and stimulates Th1 differentiation, proliferation, and IFN-γ production (11, 14). Ser<sup>725</sup> of STAT4 is also phosphorylated upon IL-12 stimulation in a p38 mitogen-activated protein kinase-dependent manner and is important for optimal IFN-γ production and Th1 differentiation (30).
Initially, we suspected that decreased expression of IL-12Rβ1 and β2 chains in PHA-activated T cells from the patient was responsible for the inability of IL-12 to stimulate lymphocyte IFN-γ production and proliferation. However, the patient’s T cells were found to have wild-type IL-12Rβ1 and β2 chains. In addition, stimulation of T cells with IL-12 resulted in both tyrosine and serine phosphorylation of STAT4, and the phosphorylated STAT4 is able to bind to the IFN-γ-responsive factor 1 gene promoter. These findings suggest that decreased expression of IL-12Rβ1 and β2 chains in PHA-activated T cells exerted no influence on IL-12-mediated tyrosine and serine phosphorylation of STAT4. Furthermore, no difference was observed in the kinetics of STAT4 phosphorylation and dephosphorylation between the control and patient’s T cells, suggesting that tyrosine kinase activity is not decreased and that phosphatase activity is not increased in the patient’s cells. Despite the presence of wild-type STAT4 and the absence of mutation in the STAT4 binding site on the IFN-γ gene in the patient’s T cells, IL-12 could not induce nuclear translocation of STAT4 in the patient’s T cells.

The decreased level of expression of both IL-12Rβ1 and β2 chains observed in this patient’s T cells is not surprising, because activation of STAT4 is needed for the maintenance of IL-12Rβ2 chain expression (31). This was supported by recent studies that have demonstrated that initial expression of IL-12Rβ2 chain (as measured by β2 chain mRNA expression) is greatly reduced in TCR-stimulated T cells from STAT4 knockout mice (31) and that STAT4 knockout T cells exhibit decreased IL-12Rβ1 and β2 chain expression (as measured by surface β1 and β2 chain protein expression) (16). These findings agree with our observation that impaired nuclear translocation of STAT4 resulted not only in decreased IL-12-mediated IFN-γ production but also in decreased IL-12Rβ1 and β2 chain expression.

In the present study, we identified that IL-12 or IFN-α stimulation of the patient’s T cells is unable to induce STAT4 nuclear translocation. However, IFN-α or IFN-γ stimulation of the patient’s T cells induced nuclear translocation of STAT1. Furthermore, treatment of the patient’s T cells with IL-2 induced nuclear translocation of STAT5. These data suggest that molecules responsible for nuclear translocation of STAT1 and STAT5 are different from those responsible for STAT4 translocation.

**FIGURE 5.** Subcellular localization of STAT4. A, Lymphocytes are classified into three stages according to the distribution of STAT4 by confocal laser microscopy: Stage 1, STAT4 is in cytoplasm; Stage 2, STAT4 is found in both cytoplasm and nucleus; and Stage 3, STAT4 accumulates in the nucleus. B, PHA-activated T cells from a healthy subject and the patient were studied under five different conditions: unstimulated (Un); stimulated with 1 nM IL-12 for 30 min (IL-12); stimulated with 1000 U/ml IFN-α for 30 min (IFN-α); treated with 5 ng/ml LMB for 7 h (LMB); and pretreated with LMB (2 h, 5 ng/ml) and then switched to fresh medium containing LMB for 4.5 h (LMB/IL-12). C, Histogram analysis of the patient’s and control T cells according to the stages of STAT4 distribution. PHA-activated T cells from healthy subject (open bars) and the patient (filled bars) were studied under five different conditions as described in B. Cells (200 cells per each group) were scored by three independent reviewers blinded to stimulation group, according to the classification described in A. This figure shows the representative result of two independent experiments using cells from the patient and from different healthy subjects. Data are expressed as mean ± SE. *, p < 0.05.
translocation of STATs are different among STAT1, STAT4, and STAT5 and that the defect is downstream of STAT4.

The mechanisms that regulate STAT4 cytoplasmic-nuclear import and nuclear-cytoplasmic export are critical for IFN-γ production and proliferation of T cells. As was shown for STAT1 nuclear translocation, facilitated transport of STAT4 into the nucleus requires the presence of signal motifs (nuclear localization signals) that are recognized by specific soluble shuttling receptors of the importin/karyopherin family. Cytokine-induced transcription is a transient process, lasting only minutes to hours. Therefore, removal of activated STATs from the nucleus is required. The regulated export from the nucleus is dependent on the presence of a nuclear export signal (NES) consisting of a leucine-rich stretch of amino acids. CRM1/exportin 1 is thought to be a shuttling receptor that appears to bind NES sequences and to export STATs from nucleus to cytoplasm. In this regard, STAT1 mutants, which do not bind importin-α5, disrupt nuclear accumulation after IFN-γ stimulation. STAT1 protein defective for DNA binding does not accumulate into the nucleus only because it is effectively exported to the cytoplasm via an NES within the DNA binding domain.

If factors similar to importin-α5 and CRM/exportin for STAT1 are responsible for IL-12- or IFN-α-induced STAT4 nuclear translocation and export from the nucleus, respectively, the present data suggest that nuclear localization signals and NES of STAT4 are intact and that the mechanism of STAT4 nuclear translocation is impaired or that the STAT4 export mechanism is increased. We examined whether up-regulation of STAT4 export from the nucleus is the main cause of impaired nuclear accumulation of STAT4 in the patient’s T cells. To address this, LMB was added to control and the patient’s PHA-activated T cells before and after IL-12 stimulation. LMB treatment both without and with IL-12 stimulation slightly promoted nuclear accumulation of STAT4 in both control and the patient’s T cells, suggesting the existence of a functioning regulatory mechanism of STAT4 at the basal and stimulated state. However, treatment of the patient’s T cells with IL-12 plus LMB affected neither IFN-γ production nor cell proliferation.

Although the precise molecular mechanism of STAT4 nuclear accumulation is not fully understood, these findings in our patient suggest that increased nuclear export of STAT4 is not responsible for the impaired nuclear translocation of STAT4 and that specific shuttling receptors that facilitate translocation of STAT4 into the nucleus may exist. Some defect in this system may be responsible for the impaired STAT4 nuclear translocation in IL-12-stimulated T cells, leading to impairment of both IFN-γ production and proliferation and recurrent mycobacterial infection in the patient.

The patient examined in this study showed mild clinical course and had marked clinical improvement after treatment with IFN-γ. Although the lymphocytes from the patient were unable to proliferate and to produce IFN-γ in response to IL-12, they could produce IFN-γ in response to IL-2, which may have prevented mycobacterial infection from becoming a fatal disease in our patient. Future determination of the etiology of the defect in the IL-12 STAT4 signaling in this patient will provide new insight into the mechanism of STAT4 nuclear translocation and the genetic basis of the immunodeficiency in this patient.

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References


