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Residual Type 1 Immunity in Patients Genetically Deficient for Interleukin 12 Receptor β1 (IL-12Rβ1): Evidence for an IL-12Rβ1-independent Pathway of IL-12 Responsiveness in Human T Cells

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Abstract

Genetic lack of interleukin 12 receptor β1 (IL-12Rβ1) surface expression predisposes to severe infections by poorly pathogenic mycobacteria or Salmonella and causes strongly decreased, but not completely abrogated, interferon (IFN)-γ production. To study IL-12Rβ1-independent residual IFN-γ production, we have generated mycobacterium-specific T cell clones (TCCs) from IL-12Rβ1-deficient individuals. All TCCs displayed a T helper type 1 phenotype and the majority responded to IL-12 by increased IFN-γ production and proliferative responses upon activation. This response to IL-12 could be further augmented by exogenous IL-18. IL-12Rβ2 was found to be normally expressed in the absence of IL-12Rβ1, and could be upregulated by IFN-α. Expression of IL-12Rβ2 alone, however, was insufficient to induce signal transducer and activator of transcription (Stat)4 activation in response to IL-12, whereas IFN-α/IFN-αR ligation resulted in Stat4 activation in both control and IL-12Rβ1-deficient cells. IL-12 failed to upregulate cell surface expression of IL-18Rα, integrin α6, and IL-12Rβ2 on IL-12Rβ1-deficient cells, whereas this was normal on control cells. IL-12-induced IFN-γ production in IL-12Rβ1-deficient T cells could be inhibited by the p38 mitogen-activated protein kinase (MAP) kinase inhibitor SB203580 and the MAP kinase kinase (MEK) 1/2 inhibitor U0126, suggesting involvement of MAP kinases in this alternative, Stat4-independent, IL-12 signaling pathway.

Collectively, these results indicate that IL-12 acts as a partial agonist in the absence of IL-12Rβ1. Moreover, the results reveal the presence of a novel IL-12Rβ1/Stat4-independent pathway of IL-12 responsiveness in activated human T cells involving MAP kinases. This pathway is likely to play a role in the residual type 1 immunity in IL-12Rβ1 deficiency.

Key words: mycobacteria • Th1 • interleukin 12 (receptor) • interleukin 18 (receptor) • interferon-γ
Introduction

The subdivision of CD4+ T lymphocytes into functionally distinct subsets, based on their cytokine production profile, has provided a basis for understanding the regulatory role of T cells in intracellular infections, allergic manifestations, and autoimmune diseases in both animals and humans (1-5). Th1 cells producing IFN-γ and IL-2 are known to sustain cell-mediated immunity against intracellular pathogens by activation of microbicidal mechanisms in infected macrophages. Th2 cells secrete IL-4 and IL-5, favoring allergic responses and immunity to extra-cellular pathogens. A key factor in human T cell differentiation is the presence of IL-12 and IL-4; whereas IL-12 promotes the generation of Th1 cells and additionally stimulates the growth and IFN-γ production of T cells and NK cells (6, 7). IL-4 is the most potent stimulus for Th2 differentiation (8).

Recently, several studies in humans have shown that genetic deficiencies in type 1 cytokines (IL-12) or cytokine receptors (IL-12Rβ1, IFN-γRI, IFN-γRII) strongly affect cell-mediated immunity-dependent resistance to mycobacteria and Salmonella (9-11). Other groups and ours have reported that IL-12Rβ1 deficiency, caused by deleterious genetic mutations in the IL-12Rβ1 gene, causes a predisposition to severe infections due to otherwise poorly pathogenic mycobacteria or Salmonella, and is associated with strongly reduced, yet not fully abrogated IFN-γ production by such patients' PBMCs (9, 10). Interestingly, these patients did not develop unusual infections with other viral, bacterial, or fungal pathogens, suggesting that IL-12Rβ1 deficiency may selectively cause a predisposition to severe infections with certain intracellular bacteria. IL-12Rβ1-deficient patients develop severe, yet mostly curable infections; in contrast, patients with complete IFN-γR deficiencies often develop fatal and treatment-resistant infections (12, 13). This suggests that IFN-γR deficiency is an obligatory step towards immunity to intracellular pathogens in humans, and that in IL-12Rβ1 deficiency low grade (type 1) immunity to intracellular pathogens persists in the absence of IL-12Rβ1. We have hypothesized that the observed low production of IFN-γ in IL-12Rβ1 deficiency is a key factor in controlling the residual type 1 immunity in these patients (11), but the IL-12Rβ1-independent pathway involved in this low IFN-γ remains unknown.

IL-12 binds with high affinity to heterodimeric IL-12Rβ1/β2 complexes on T cells and NK cells (14-16). In contrast to the IL-12Rβ1 chain, the human IL-12Rβ2 chain contains functional tyrosine-based signaling motifs in its cytoplasmic domain (15, 16). The expression of the IL-12Rβ2 chain is therefore thought to be a critical factor in controlling T cell development and functioning. At present, several lines of evidence suggest that IL-12/IL-12R ligation signals through the Janus kinase (JAK)−signaling transducer and activator of transcription (Stat) signal transduction pathway in both mice and humans (17-19). In particular, the essential role of Stat4 in IL-12R signaling has been demonstrated in Stat4 knockout mice, in which IL-12 induced IFN-γ production and proliferation of T cells and NK cells were almost completely abrogated (20, 21). Th1 cells can express both IL-12Rβ1 subunits, but IL-12Rβ2 expression is lost in Th2 cells (22, 23), and consequently, IL-12 induces tyrosine phosphorylation of JAK2 and Stat4 in human Th1, but not Th2 cells (22, 24). One factor that is thought to contribute to the activity of IL-12 is IL-18, which strongly amplifies IFN-γ production of T cells in response to IL-12 (25).

Previous transfection studies have shown that IL-12 can bind with low affinity to the independently expressed human IL-12Rβ2 chain when ectopically expressed in Ba/F3 or COS-7 cells, but a role for endogenous IL-12Rβ1 could not be excluded (15, 16). Genetically IL-12Rβ1-deficient patients provide a unique opportunity to investigate IL-12Rβ2 expression and IL-12 functioning in the absence of IL-12Rβ1. We have isolated mycobacterium (myc)-responsive T cell clones (TCCs) from IL-12Rβ1-deficient patients and found that these cells display a Th1 phenotype and respond to IL-12 by increased proliferative responses and IFN-γ production upon activation.

Materials and Methods

Patients

Patients (P1, P3, P4, and P17) presented with (recurrent) severe mycobacterial and Salmonella infections in the absence of a recognized immunodeficiency. All patients failed to express cell surface IL-12Rβ1 due to individually different homozygous recessive mutations or deletions in the IL-12Rβ1 gene, leading to premature stop codons in the extracellular domain of the IL-12Rβ1 molecule. Genetic mutations or deletions have been described elsewhere for P1, P3 (9), and P4 (10). The genetic deficiency in the IL-12Rβ1 gene of P17 was identified more recently and will be described elsewhere. In brief, P1, a 27-year-old female, developed a severe Salmonella paratyphi septis at the age of 3 yr and at the age of 22 yr presented with disseminated Mycobacterium avium infection. P3 is a 4-year-old female who developed progressive Mycobacterium avium BCG infection after vaccination at the age of 1 yr, followed by severe Salmonella typhimurium septis at the age of 2 yr. P4, a 31-year-old man, suffered from a Salmonella enteritis infection at the ages of 11 and 20 yr and developed M. avium infection at the age of 24 yr. P17, a 5-year-old boy, who has now died, presented with disseminated BCG infection at the Hacettepe University Children's Hospital.

Generation and Cytokine Secretion Profile of Myc-responsive TCCs

To generate myc-responsive T cells, standard procedures were used (26). PBMCs from P1 and P3 were stimulated with an M. avium sonicate (2 μg/ml; Royal Tropical Institute) for 6 d, and further expanded in the presence of 10 U/ml IL-2 (Proleukin; Chiron Corp.) for another 7 d. Culture medium IMDM (Life Technologies) supplemented with 10% pooled heat-inactivated normal human serum (pooled screened serum from donors from The Neth-

Abbreviations used in this paper: DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK kinase; myc, mycobacterium; Stat, signal transducer and activator of transcription; TCC, T cell clone.
erlands), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Expanding T cells were cloned in 96-well flat-bottomed plates by limiting dilution in the presence of 10^5 irradiated allogenic feeder cells from five random blood donors and M. avium sonicate (2 μg/ml). After 10–17 d, individual TCCs were further expanded in 24-well plates by standard mitogenic stimulation in the presence of irradiated allogenic feeder cells and 0.5% PHA (Murex Biotech Ltd.). After 3 d, 10 U/ml IL-2 was added to the cultures. 10 d later, TCCs were screened for their responsiveness to mycobacteria by testing 10^5 cells in 200 μl culture medium in a flat-bottomed 96-well plate for 3 d in the presence of whole sonicated M. avium, M. tuberculosis, or purified protein derivative of M. tuberculosis (PPD, 2 μg/ml; Statens Serum Institute) in the presence of irradiated PBMCS (5 × 10^4 cell/well) from an HLA-matched donor. The cultures were incubated for an additional 16 h in the presence of [3H]thymidine (0.5 μCi/well). Cultures were harvested and incorporated radioactivity was measured by liquid scintillation counting. Control myc-responsive TCCs (R1E4 and R2F10) generated from leprosy patients were used (1). Cytokine production of TCC was tested at least 10 d after the last restimulation. 10^5 cells were incubated in immobilized anti-CD3 mAb (OKT3) and soluble PMA in a total volume of 200 μl/well in a flat-bottomed 96-well plate. From a panel of TCCs, 5 × 10^4 cells were incubated with M. avium sonicate (2 μg/ml) in the presence of irradiated PBMCS (5 × 10^4 cell/well) from an HLA-matched donor. Control TCCs were incubated with M. tuberculosis lepra sonicate (5 μg/ml). After 24 h, 100 μl cell-free supernatants were collected, pooled, and stored at −20°C until testing. The remaining cultures were incubated for an additional 24 h in the presence of IFN-γ (0.5 μCi/well) to confirm stimulation. The results are expressed as mean cpm of triplicate cultures. Measurement of IFN-γ and IL-4 concentrations in the supernatants of stimulated or unstimulated TCCs was performed with a specific solid-phase sandwich ELISA for IFN-γ (Ucyc-Tech; University of Utrecht), and for IL-4 (The Central Laboratory of The Netherlands Red Cross Blood Transfusion Service).

Stimulation of TCCs with IL-12 and IL-18

10 d after the last restimulation, TCCs were tested for IL-12 and IL-18 responsiveness upon activation. 5 × 10^4 cells were cultured in 200 μl culture medium in a flat-bottomed 96-well plate for 72 h in the presence or absence of 5 U/ml IL-12 (a gift from G. Trinchieri, Wistar Institute, Philadelphia, PA) or 2.5 ng/ml IL-12 (R&D Systems), and/or different concentrations of IL-18 (0–100 ng/ml; Medica and Biological Laboratories Co.), or different concentrations of IL-18 (0–100 ng/ml; Medica and Biological Laboratories Co.). T cells were activated with M. avium sonicate (2 μg/ml) in the presence of irradiated PBMCS (5 × 10^4 cell/well) from an HLA-matched donor. Control TCCs were incubated with M. leprae sonicate (5 μg/ml). Mitogenic stimuli were soluble murine mAb to human CD28 (CLB-CD28/1), three murine mAbs to human CD2 (CLB-T11/1, CLB-T11.2/1, Hic 27), and immobilized anti-CD3 mAb. mAbs to CD28 and CD2 were a gift from R.A.W. van Lier (The Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). After 72 h, supernatants were collected, pooled, and stored at −20°C before determining IFN-γ levels. During the last 16 h, the cultures were incubated in the presence of 0.5 μCi/well [3H]thymidine to determine proliferation. The results are expressed as mean cpm of triplicate cultures.

Generation and Stimulation of PHA Blasts

PBMCS were isolated from heparinized blood by Ficoll-Amidotrizoate density gradient centrifugation. Cells (10^4/ml) were incubated in a 24-well plate in standard culture medium and 0.5% PHA. After 3 d, the cells were expanded in the presence of 10 U/ml IL-2. PHA blasts (10^5 cells/ml) on day 10 after activation were incubated in 24-well plates (Costar) with 1,000 U/ml IFN-γ (Pharma Biotechnologie Hannover) for 16 h or with 25 U/ml IL-12 (R ochte) for 20–24 h in the presence of 20 U/ml IL-2.

Cell Surface Staining

Cells (5–20 × 10^4) were labeled with either FITC-conjugated mouse mAb to CD3 (Becton Dickinson), unlabeled purified rat anti-hu-IL-12 p1 mAb (2B10, 1 μg/ml) (29), rat anti-hu-IL-12 p2 mAb (2B6, 1 μg/ml), mouse anti-hu-IL-18 (117-10C, 5 μg/ml), FITC-conjugated mAb to αβ-integrin (CD49a; BD Pharmingen), or isotype-matched control antibodies (BD Pharmingen) at 4°C for 1 h. mAbs 2B10 and 2B6 were provided by D. Presky (Department of Inflammation/Autoimmune Diseases, Hoffmann-La Roche, Nutley, NJ). The cells labeled with mAb 2B10, 2B6, or isotype control mAb were subsequently incubated with biotinylated sheep anti–rat Ig (Amersham Pharmacia Biotech) for 30 min at 4°C, followed by incubation with Avi- dIn-FITC (1:500; Vector Laboratories) for 30 min. The cells labeled with mAb 117-10C were subsequently incubated with FITC-labeled anti-mouse IgM (Southern Biotechnology Associates Inc.) for 30 min. Cells were analyzed with a FACScan™ flow cytometer (Becton Dickinson).

Responsiveness of PHA Blasts to IL-12

PHA blasts were restimulated with irradiated allogenic adherent cells T = 0.25–0.5 × 10^6 adherent cells, 1 × 10^6 PHA blasts were added in 1 ml culture medium per well in a 24-well plate, and incubated in the presence of 0.5% PHA and IL-2 (10 U/ml). On day 3 after stimulation PHA blasts were collected, washed, and tested for IL-12R p1 and IL-12R p2 expression (as described above) and for responsiveness to IL-12 based on Stat4 activation.

Stat4 Activation

Cell Extracts. Total proteins were extracted from PHA blasts (10^6 cells/ml) that were or were not exposed to 2.5 ng/ml IL-12 (R&D Systems) 1,000 U/ml IFN-γ (Pharma Biotechnologie Hannover), or control medium for 20 min. Cells were washed with ice-cold PBS and packed cells were resuspended in 50 μl of lysis buffer (20 mM Hepes KOH [pH 7.9], 350 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM dithiothreitol [DTT], 10 μg/ml aprotinin, 0.5% [vol/vol] of a saturated PMF solution in ethanol, 20% [vol/vol] glycerol, and 1% [vol/vol] N-P-40, Sigma-Aldrich). After 20 min incubation on ice, the lysate was cleared by 15 min centrifugation at 14,000 g. The supernatant was diluted with 1 vol of dialysis buffer (20 mM Hepes KOH [pH 7.9], 0.2 mM EDTA, 2 mM DTT, 0.5% [vol/vol] of a saturated PMF solution in ethanol, 20% [vol/vol] glycerol, and 0.25% NP-40) to reduce salt concentration. The final protein concentration was determined by Bradford microassay (Bio-Rad Laboratories), using a calibrated BSA (Sigma-Aldrich) solution as a reference.

Electrophoretic Mobility Shift Assay. DNA-binding reactions were conducted in a final volume of 20 μl. The reactions were start by adding 10 μg of total cell protein extract to a reaction mixture containing 2 μg of poly-deoxyxinonic-deoxyctydilic acid (Sigma-Aldrich), 2 μg of BSA, 2 μl of 10× binding buffer (100 mM Tris-HCl [pH 7.5], 500 mM NaCl, 75 mM EDTA, 50% glycerol, and 2 mM DTT), 0.5% [vol/vol] of a saturated PMF solution in ethanol, 2 μl of dilution buffer (supplemented with 100 mM KCl), and ~10,000 cpm (± 0.1 ng) of the [γ-32P]
ATP-labeled double-stranded DNA oligonucleotide. For super-shift experiments, binding reactions were incubated with affinity-purified rabbit anti-murine Stat4 (C-20; Santa Cruz Biotechnology, Inc.) during the last 20 min of incubation. All reactions were adjusted to the final volume of 20 μl with distilled water and incubated for 30 min at room temperature. The whole sample was then loaded on a 4% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. After electrophoresis for 2 h at room temperature at 10 V/cm, gels were dried, and separated protein–DNA complexes were visualized by autoradiography using XAR 5 films (Eastman Kodak Co.).

Oligonucleotides. The following double-stranded DNA oligonucleotides were used in electrophoretic mobility shift assay (EMSA) analyses: FcyRII- (5′-AGCATTTGTTTCAAGGATTGAG-ATGTGTTCGAGTTTTAC-3′), corresponding to the IFN-γ response region in the human FcyRII gene promoter (27), which is bound by various STAT family members, including Stat4, and a sequence (5′-CCCCTGGCCGTACATCCGGCCCCGCCCA-TC-3′) containing the herpes simplex virus SP-1 site. All oligonucleotides were purchased from Biosource International and labeled by T4 polynucleotide kinase (Roche).

Inhibition of Mitogen-Activated Protein Kinase Pathway

To assess the involvement of mitogen-activated protein kinases (MAPKs) and MAPK kinase (MEKs) in IL-12–induced IFN-γ production in IL-12Rβ1-deficient cells, myc-specific, mitogen-treated TCCs were incubated with IL-12 in the absence or presence of the p38 MAPK inhibitor SB203580 (Sigma-Aldrich), the mitogen-activated and extracellular signal-regulated kinase MEK1/MEK2 inhibitor PD098059 (Sigma-Aldrich) or the mitogen-activated and extracellular signal-regulated kinase MEK1/MEK2 inhibitor U0126 (Promega). In brief, TCCs were incubated with IL-12Rβ1–responsive IL-12Rβ1 wild-type (control) T1 TCCs (R1E4 or R2F10) were stimulated with mycobacterial stimuli (further details are given in the legend of Fig. 1). Two control TCCs (R1E4 and R2F10) were stimulated with mycobacterial sonicates (M. avium or M. leprae), or with CD3/PMA. 24-h supernatants were collected and analyzed for their IFN-γ and IL-4 production by ELISA. Data are given in pg/ml.

Type 1 Cytokine Secretion Profiles (IFN-γ/IL-4) of TCCs Generated from IL-12Rβ1-deficient Patients. Since IL-12 is known to be a key factor in Th1 cell development (6, 7, T cell differentiation in IL-12Rβ1-deficient mice might be anticipated to be Th2 skewed. To study this, myc-specific TCCs were generated from two IL-12Rβ1-deficient individuals with distinct recessive null mutations (P1 and P3), and cytokine profiles of 25 myc-responsive TCCs were determined after standard stimulation with anti-CD3 mAb plus PMA. All myc-responsive IL-12Rβ1-deficient TCCs were CD4+CD8− and showed a clear Th1 phenotype, producing significant levels of IFN-γ but low levels of IL-4 (shown in Table I for 10 representative TCCs, compared with two control myc-responsive TCCs). Antigen-specific stimulation of these TCCs induced similar cytokine profiles, although lower levels of IFN-γ tended to be produced.

To exclude the influence of skewing by microenvironmental cytokines possibly present during the generation of TCCs, separate cloning experiments were performed by direct limiting dilution of PHA-stimulated PBMCs from

<table>
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<tr>
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</tr>
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</tr>
<tr>
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P1 and P3. Cytokine secretion profiles of these TCCs (with unknown specificity) were determined after mitogenic stimulation. Also in these experiments TCCs with a clear Th1 phenotype were observed (data not shown).

Myeloma-reactive Th1 TCCs from IL-12Rβ1-deficient Patients Can Still Respond to IL-12. Using the myc-responsive Th1 TCCs from genetically IL-12Rβ1-deficient patients, we next wanted to elucidate the pathway(s) involved in the remaining low IFN-γ production that was previously observed in PBMCs from these patients (9), and now also in TCCs (this study). Since IL-12 is a key factor in Th1 activation, we first determined whether IL-12Rβ1-deficient Th1 TCCs were still capable of responding to IL-12. To this end, IL-12Rβ1-deficient Th1 TCCs (n = 10) or IL-12Rβ1 wild-type (control) Th1 TCCs (n = 2) were stimulated independently with four different stimuli, in the presence or absence of exogenous IL-12, as indicated in Fig. 1. TCCs were stimulated with antigen presented by HLA-matched APCs (Fig. 1 A), or by myeloma antibodies in the absence of any added APCs, using either a mix of two or three mAbs to CD2 (CD2d [Fig. 1, B and C] or CD2t [Fig. 1 D], respectively) or a mAb to CD3 (Fig. 1 E) to which in each case costimulatory anti-CD28 mAb was added (Fig. 1, B–E) (28). All experiments were repeated between two and seven times, and in each experiment TCCs were stimulated by one to four of these different stimuli (further details are given in the legend of Fig. 1).
As shown in Fig. 1, IL-12 was found to induce a significant response (defined as >50% increase in proliferation or IFN-γ production) in 8 out of the 10 tested IL-12R β1-deficient TCCs. The observed IL-12-induced responses were consistent and reproducible, and representative experiments are shown. The IL-12-mediated enhancement of proliferation and IFN-γ production in the case of stimulation with CD2d/CD28 (Fig. 1, B and C) or CD2t/CD28 (Fig. 1 D) indicated a direct effect of IL-12 on IL-12R β1-deficient T cells in the absence of accessory cells. Although in some cases these stimuli already induced relatively high levels of IFN-γ secretion, this could be further augmented by IL-12. IL-12 also clearly enhanced the response of several IL-12R β1-deficient clones upon antigen-specific stimulation (Fig. 1 A) or activation by CD3/CD28 mAbs (Fig. 1 E). 2 of the 10 IL-12R β1-deficient TCCs (K4D8 and K3F12) repeatedly did not respond to IL-12 upon any of the stimulation modes used (Fig. 1, A–E), whereas the two IL-12R β1 wild-type control TCCs (R1E4 and R2F10) responded strongly to IL-12 as expected (Fig. 1, A–E).

Taken together, the results show that IL-12R β1-deficient patients are capable of developing Th1 T cells, and that the majority of the Th1 TCCs are capable of responding to IL-12 in a specific and consistent fashion by en-
enhanced IFN-γ production and/or proliferation. Thus, these results reveal an IL-12Rβ1-independent pathway of IL-12 responsiveness in human T cells.

The IL-12–dependent response of Myc-reactive, IL-12Rβ1-deficient Th1 TCCs is further augmented by IL-18. Since IL-18 is known to enhance IL-12-induced IFN-γ production in healthy individuals (25), we investigated the effect of IL-18 on IL-12-induced IFN-γ production also in IL-12Rβ1-deficient Th1 TCCs (n = 7). Representative results of one out of three independent experiments are shown in Fig. 2. In two IL-12Rβ1-deficient TCCs (SBA5 and K2E9), IL-18 synergized with IL-12 in a dose-dependent fashion by further increasing proliferation as well as IFN-γ production. A similar synergy was seen in control TCCs (R1E4) although the levels of increased proliferation and IFN-γ production were higher. In a third IL-12Rβ1-deficient TCC (K8D10), the response to IL-12 and IL-18 was additive, and this TCC already responded to IL-18 in the absence of IL-12. Thus, the IL-12-induced proliferation and IFN-γ production of IL-12Rβ1-deficient T cells can be further augmented by IL-18.

Regulation of T cells IL-12Rβ2 expression in the absence of IL-12Rβ1. To further delineate this unexpected IL-12 responsiveness to IL-12Rβ1 deficiency, we determined surface expression of the IL-12Rβ2 signaling chain on IL-12Rβ1-deficient and control TCCs. At the same time, we studied the expression of the IL-18R that, in mice, has been suggested to be selectively expressed on Th1 cells (29). As can be seen in Fig. 3, PHA-stimulated IL-12Rβ1-deficient TCCs express IL-12Rβ2 and IL-18R. The level of expression differed among different TCCs, which may correlate with the activation state (23) and the capacity of the TCCs to respond to IL-12 and/or IL-18 as described above. Control TCCs expressed both receptors.

To investigate the dynamics of IL-12Rβ2 expression, PHA-activated PBMCs (PHA blasts) from four IL-12Rβ1-deficient patients and four healthy controls were assayed at different time points after stimulation (Fig. 4). No IL-
expression could be detected on patient-derived PHA blasts whereas control PHA blasts showed maximal levels of IL-12Rβ1 expression on day 3 after activation, declining thereafter to baseline expression. In line with the results in TCCs, PHA blasts from both patients and controls showed equally strong IL-12Rβ2 expression, which was highest on day 3 after activation and declined to low or undetectable levels on day 11 after activation. On day 3 after activation equal levels of IL-18R expression among patients and controls were seen (data not shown).

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It has previously been reported that culturing T cells with IL-12 or IFN-α upregulates surface expression of IL-12Rβ2 receptor in the absence of TCR stimulation (22). Other molecules that have recently been described to be upregulated by IL-12 are IL-18R and integrin α6 (30, 31).

To study the effect of IL-12 or IFN-α on IL-12Rβ2 expression in IL-12Rβ1-deficiency, PHA blasts of patients and controls (10 d after activation) were incubated with IL-12 or IFN-α for 20 and 16 h, respectively (23). Whereas IFN-α upregulated the surface expression of IL-12Rβ2 both in patients and in controls, exposure to IL-12 only induced the expression of IL-12Rβ2 on cells from healthy controls, but not from patients (Fig. 5). Similarly, IL-12 induced upregulation of IL-18R and integrin α6 expression in controls, but not in patients (Fig. 6).

Taken together these results demonstrate that (a) human IL-12Rβ2 expression can occur normally in the absence of IL-12Rβ1 and can be upregulated by IFN-α independent from IL-12Rβ1; and (b) IL-12Rβ2, IL-18R, and integrin α6 expression can only be upregulated by IL-12 in the presence of IL-12Rβ1, suggesting that IL-12Rβ2 expression alone is not sufficient to confer full IL-12 responsiveness to human T cells.

IL-12 Fails to Induce Stat4-containing Complexes in Human IL-12Rβ1 Deficiency. Since the IL-12Rβ2 chain is essential in phosphorylating Stat4, a key signal transduction and activator of transcription in IL-12-induced T cell responses (17, 19–22), we investigated whether IL-12 signaling in IL-12Rβ2-expressing, IL-12Rβ1-deficient T cells was involved in Stat4 activation. Total cell extracts of 3-d PHA-stimulated blasts of IL-12Rβ1-deficient patients (n = 4) and healthy controls (n = 2) that expressed optimum levels of IL-12Rβ2 (see above) were examined by EMSA for formation of Stat4-containing complexes and binding to the oligonucleotide probe FcγR1, which contains high affinity binding sites for Stat4. As shown in Fig. 7 A, IL-12 induced binding of the FcγR1 probe in the IL-12-responsive PHA blasts from controls (lanes 11 and 14), but no activity binding was found in cells from patients (lanes 2, 5, and 8). As a positive control, IFN-α induced similar levels of Stat4 activation in samples from patients and controls (lanes 3, 6, 9, 12, and 15). Unstimulated samples were always negative (lanes 1, 4, 7, 10, and 13). Competition analysis with unla-
beled FcγR1 oligonucleotide (Fig. 7 B, lanes 1–3) indicated the specificity of this DNA–protein interaction, and this was further confirmed in supershift experiments showing that Stat4-specific antiserum interfered with the formation of IL-12–induced (Fig. 7 C, lane 8) as well as IFN-α–induced (Fig. 7 C, lanes 6 and 10) protein–DNA complexes. As an additional control for the quality of total cell extracts, no differences were found between patient versus controls and cytokine-stimulated versus unstimulated samples when DNA binding activity of the constitutively expressed transcription factor SP-1 to a specific SP-1 binding oligonucleotide was examined (data not shown).

Involvement of MAPKs in IL-12 Responsiveness in IL-12Rβ1–deficient T Cells. To assess the possible involvement of MAPK signaling in the residual, Stat4-independent IL-12 responsiveness in IL-12Rβ1–deficient T cells, we measured IL-12–induced IFN-γ production in three TCCs that were stimulated with anti-CD2/anti-CD28, either in the presence or absence of the following inhibitors: the p38 stress MAPK inhibitor SB203580, the MEK1/2 inhibitor PD098059, or the MEK1/2 inhibitor U0126. Whereas SB203580 blocks p38 MAPK–mediated stress responses, PD098059 and U0126 prohibit signaling via the extracellular signal-regulated kinase (ERK)/MAPK–mediated pathway by interfering with the upstream MAPK kinases MEK1/2. Whereas U0126 inhibits the activated form of MEK directly, PD098059 merely prohibits activation of MEK by binding to its inactive form (32, 33). As shown in Fig. 8, the IL-12–mediated enhancement of IFN-γ production was significantly reduced (~50%) in the presence of SB203580 and U0126, but not PD098059, in the case of TCCs S8D3 and S8A5. In clone S3E8 there was only modest inhibition by SB203580 (20%), whereas U0126 again diminished IL-12–dependent IFN-γ production over 50%. No effects of these inhibitors were observed on cell viability, ruling out possibly toxic effects of these components. Thus, these results imply a significant role for the p38 MAPK, and the MEK1/2–p42 MAPK/ERK pathways in residual IL-12 responsiveness and IFN-γ production in IL-12Rβ1–deficient human T cells.

Discussion
The essential role of type 1 cytokines in protection against intracellular pathogens is illustrated by the extreme susceptibility of humans that are genetically deficient for type 1 cytokines (IL-12p40) or type 1 cytokine receptors (IL-12Rβ1, IFN-γR1, IFN-γR2) to severe infections with otherwise poorly pathogenic mycobacteria and Salmo-
We have hypothesized that this may be due to the residual competent IL-12R complexes remains obscure. The pathway that controls this in the absence of functionally expressed IL-12R is still largely unknown, a recent study showed a role for IL-18 in type 1 immunity against M. leprae, suggesting the involvement of IL-18 in mycobacterial immunity (34). Our data thus implicate that IL-12, either alone or together with IL-18, can regulate IL-12R β1-independent, low grade IFN-γ production by Th1 T cells. This low level of IFN-γ production presumably is necessary and sufficient to prohibit a fatal outcome of mycobacterial infectious disease in IL-12R β1-deficient patients.

This residual IL-12 response in IL-12R β1 deficiency compelled us to examine the expression and function of the IL-12R β2 signaling chain in the absence of the IL-12R β1. Previously, IL-12R β2 was suggested to bind IL-12 in the absence of IL-12R β1 (14–16). In support of this suggestion, Ba/F3 cells expressing the hUL-12R β2 subunit alone bound IL-12 with low affinity and proliferated in response to IL-12 (14, 35). In these studies, however, a role for endogenous IL-12R β1 could not be excluded. To our knowledge, our results are the first to show that in humans IL-12R β2 can be expressed at the cell surface in the absence of IL-12R β1 and that this expression can be upregulated by IFN-α. The expression of IL-12R β2 as well as IL-18R on IL-12R β1−/− TCCs is in line with their observed Th1 phenotype, since both receptors have been described to be selectively expressed by Th1, but not Th2 T cells (23, 29).

Although IL-12R β2-expressing IL-12R β1−/− T cells responded to IL-12 in the presence of antigens or mitogenic stimuli, IL-12 alone failed to induce upregulation of cell surface expression of IL-12R β2, IL-18R, and integrin α6, and failed to induce Stat4 activation, whereas in con-
trol cells all these markers of IL-12R signaling were present. Since several Stat4 binding sites have been identified on the IL-12R β2 promotor (36), the absence of IL-12-induced Stat4 activation in our patients may directly account for the lack of IL-12-induced upregulation of IL-12R β2. A similar explanation may account for the lack of IL-12-induced upregulation of IL-18R or integrin α6 expression. Although IL-12 activates multiple Stats (Stat1, Stat3, Stat4, and Stat5 [17, 19, 37]), the essential role of Stat4 in IL-12/IL-12Rβ2–induced signal transduction was demonstrated in Stat4 knockout mice that showed strongly reduced IL-12–dependent IFN-γ production and cellular proliferative responses in T cells and NK cells (20, 21). Furthermore, the selective expression of the IL-12R β2 chain on human Th1, but not Th2 cells has been shown to correlate with the capacity of IL-12 to induce activation of Stat4 in Th1, but not Th2 cells (22, 24). Here, however, we clearly show that in humans IL-12R β1 expression is also necessary for IL-12–dependent Stat4 activation. Taken together, our results show that IL-12 acts as a partial agonist in IL-12R β1 deficiency, inducing IFN-γ production and proliferation by activated T cells, whereas IL-12 alone failed to activate Stat4 and to upregulate expression of IL-12R β2, IL-18R, or integrin α6 to detectable levels.

Several lines of evidence have pointed to IL-12–triggered intracellular signaling pathways that are independent of Stat4. First, it has been shown that the cytoplasmic region of huli-12R β2 could associate with JAK2, and that IL-12–induced JAK2 activation correlated with Stat5 phosphorylation and cellular proliferation, whereas huli-12R β1 interacted directly with TYK2, and IL-12–induced activation of TYK2 correlated with Stat4 phosphorylation and IFN-γ production (35, 38). IL-12 responsiveness may, however, also involve the activation of MAPKs that have also been implicated in T cell receptor CD3/CD28–mediated signaling. A 42–44-kD MAPK was found to be activated by IL-12 in mitogen-stimulated human T cells (39). Interestingly, a more recent study showed that a p38 MAPK was activated by the synergistic action of IL-12 and IL-2, and subsequently induced Stat1 and Stat3 phosphorylation on serine residues, which contributed to the IL-12–induced functional effects (40). Moreover, inhibition of p38 MAPK led to reduced IFN-γ production in mouse Th1 cells (41). In agreement with these recent reports, we here show that inhibiting p38 MAPK by SB203580, as well as inhibiting MEK1/2 by U0126, both significantly reduced IL-12–dependent IFN-γ production in IL-12R β1–deficient T cells. In contrast, a second MEK1/2 inhibitor, PD098059, had no inhibitory effect up to a concentration of 25 μM. Since PD098059 blocks the activation of MEK, but does not prohibit signaling by already activated MEK, the failure of PD098059 to inhibit IL-12–induced IFN-γ production in IL-12R β1–deficient cells may suggest that pre-activation of the TCC with anti-CD2/anti-CD28 leads to activation of the MEK/ERK signaling pathway, which subsequently integrates with signaling via p38 MAPK in response to IL-12 to trigger IFN-γ production. However, future studies are required to unravel these signal transduction pathways and the putative cross-talk between various pathways involved in IL-12 responsiveness in IL-12R β1–deficient cells.

Finally, it cannot be ruled out that other cell surface molecules may associate with IL-12R β2 and engage in binding IL-12 and regulating IL-12 responsiveness. In one study an 85-kD protein was found to be associated with the IL-12R β1 subunit and suggested to represent a hitherto unidentified third component of the IL-12R complex (42).

The finding that all myc-responsive IL-12R β1-deficient TCCs appeared to be Th1 suggests significant Th1 cell development in IL-12R β1 deficiency. It is tempting to speculate that the residual IL-12 responsiveness shown in this paper may play a role in Th1 differentiation in these patients, although we cannot exclude that this is regulated by IL-12–independent mechanisms. Studies in IL-12p40β1−/− mice revealed polarized Th1 cell development in alloantigen responses or during viral infections (43–45), suggesting IL-12–independent pathways of Th1 induction. In humans, IL-12p40 protein deficiency was also found to be selectively associated with curable, but not fatal, disseminating M. bovis BCG and S. enteritidis infection (46). One alternative IL-12–independent Th1 sensitizing pathway has been shown to involve IFN-α. IFN-α, in humans can directly induce Th1 development and induces IFN-γ production independently of IL-12 (47). IFN-α was also found to synergize with IL-18 to induce IFN-γ production in human T cells (48). Here, we report normal responsiveness to IFN-α in IL-12R β1-deficient patients based on upregulated IL-12R β2 expression and Stat4 activation.

In conclusion, in this study we show that IL-12R β1−/− T cells can still develop into Th1 cells and can partially respond to IL-12 by cellular proliferation and IFN-γ production. Although IL-12R β2 can be expressed normally in the absence of IL-12R β1, this was most likely insufficient to confer IL-12 responsiveness to these cells, as judged by the lack of upregulation of Th1 cell surface markers and Stat4 activation. These results reveal a novel IL-12R β1–independent pathway of (residual) type 1 immunity in IL-12R β1 deficiency that may involve MAPK pathways. Thus, human IL-12R β1 deficiency provides a unique model to uncover novel intracellular signaling pathways and mechanisms involved in residual IFN-γ production and type 1 immunity.

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References


