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Familial NK Cell Deficiency Associated with Impaired IL-2- and IL-15-Dependent Survival of Lymphocytes

Céline Eиденшёнк,* Emmanuelle Jouanguy,* Alexandre Alcaïs,* Jean-Jacques Mention,† Benoît Pasquier,‡ Ingrid M. Fleckenstein,§ Anne Puel,* Laure Gineau,* Jean-Claude Carel,‖ Eric Vivier,¶ François Le Deist,** and Jean-Laurent Casanova***

We previously reported the clinical phenotype of two siblings with a novel inherited developmental and immunodeficiency syndrome consisting of severe intrauterine growth retardation and the impaired development of specific lymphoid lineages, including transient CD8 αβ T lymphopenia and a persistent lack of blood NK cells. We describe here the elucidation of a plausible underlying pathogenic mechanism, with a cellular phenotype of impaired survival of both fresh and herpesvirus saimiri-transformed T cells, in the surviving child. Clearly, NK cells could not be studied. However, peripheral blood T lymphocytes displayed excessive apoptosis ex vivo. Moreover, the survival rates of CD4 and CD8 αβ T cell blasts generated in vitro, and herpesvirus saimiri-transformed T cells cultured in vitro, were low, but not nil, following treatment with IL-2 and IL-15. In contrast, Fas-mediated activation-induced cell death was not enhanced, indicating a selective excess of cytokine deprivation-mediated apoptosis. In keeping with the known roles of IL-2 and IL-15 in the development of NK and CD8 T cells in the mouse model, these data suggest that an impaired, but not abolished, survival response to IL-2 and IL-15 accounts for the persistent lack of NK cells and the transient CD8 αβ T lymphopenia documented in vivo. Impaired cytokine-mediated lymphocyte survival is likely to be the pathogenic mechanism underlying this novel form of inherited and selective NK deficiency in humans. The Journal of Immunology, 2006, 177: 8835–8843.

The role of murine NK cells in protective immunity to viruses has been clearly established (1), but that of human NK cells remains unclear, in the absence of a well-defined primary immunodeficiency associated with selective NK deficiency. Four types of SCID, characterized by intrinsically impaired T cell development (2), are associated with a lack of NK cells. Adenosine deaminase deficiency and reticular dysgenesis also result in a lack of B lymphocytes. X-linked and autosomal recessive SCID due to mutations in the common γ-chain and JAK3, respectively (3)–both of which are components of the IL-2, 4, 7, 9, 15, and 21 pathways–are associated with a lack of T and NK lymphocytes but normal B cell development (4). A patient lacking NK cells but with a less severe T cell deficiency has also been described (5). He displayed impaired IL-2R β-chain expression but no genetic lesion was identified. Patients with T and NK cell defects have been reported to suffer from various infections, but the contribution of NK lymphopenia to the development of these infections remains unclear, given the known dominant contribution of T cells, as exemplified by IL-7R-deficient patients (6) who selectively lack T cells and present with a clinical phenotype comparable to that of NK-deficient SCID patients.

Only a couple of cases of apparently selective defects in human NK cell development have been reported (7). The first patient, reported in 1989, was an adolescent girl who initially presented disseminated, life-threatening varicella infection (8). She subsequently developed CMV pneumonitis and cutaneous HSV infections. However, NK cell counts were not determined before viral illnesses and varicella-zoster virus was recently shown to cause a decrease in NK cell numbers in otherwise healthy children (9). The patient remained healthy until the age of 13 and the patient was 17 when reported in 1989. NK deficiency persisted 6 years after the last infection (10), but a more recent follow-up indicated that she subsequently died of myelodysplasia (7), strongly suggesting that she probably suffered from an acquired, as opposed to inherited, hematopoietic deficiency (11). Moreover, this case was sporadic and the parents of the child were not consanguineous. A second patient with NK deficiency and recurrent varicella infections was recently reported; this patient died from one such infection at the age of 2 years, precluding any clinical and immunological follow-up (12). This case was sporadic too, but the parents were consanguineous, suggesting that their child may have had an inherited defect. These
two patients (8, 12) may have suffered from an inherited defect of NK cells, suggesting that human NK cells may be involved in antiviral immunity. However, the lack of data for NK cell counts in these two patients before the onset of viral disease, the absence of identified familial cases, and the lack of disease-causing cellular phenotype make it impossible to draw firm conclusions.

We recently reported the first kindred with an inherited selective NK deficiency (13). Four related children from large, inbred Irish kindred were found to have very low counts of NK cells in the blood (below 4% and generally below 100 cells/mm³). One patient presented with EBV-driven lymphoproliferative disorder and two patients with severe pneumonitis of probable viral origin. The fourth patient has remained clinically healthy so far. The NK cell deficiency is a specific and inherited defect in this family, as it is a familial trait that was documented before any clinical infectious disease in at least one of the four patients. Moreover, it segregates as an autosomal recessive trait that is linked to the centromeric region of the chromosome 8 (logarithm of the odds score 4.51). This NK cell defect was therefore registered as a distinct nosological entity in the Online Mendelian Inheritance in Man (OMIM) database (OMIM:609981), unlike the previously reported patients (8, 12). The clinical phenotype of these patients strongly suggests that NK cells in human are also involved in antiviral immunity and also perhaps in antitumoral immunity (13). Although it is clear that the patients from this kindred suffer from a selective deficit of NK cells, including NKT cells, the pathogenic mechanism remains obscure. The disease-causing gene has not been identified and no disease-causing cellular phenotype was identified either.

We previously reported the clinical phenotype of a new developmental and immunological syndrome observed in two sisters, born to nonconsanguineous parents (14). The sisters showed developmental and immunological syndrome observed in two sisters, disease-causing cellular phenotype was identified either. The patients from this kindred were found to have very low counts of NK cells in the blood (below 4% and generally below 100 cells/mm³). One patient presented with EBV-driven lymphoproliferative disorder and two patients with severe pneumonitis of probable viral origin. The fourth patient has remained clinically healthy so far. The NK cell deficiency is a specific and inherited defect in this family, as it is a familial trait that was documented before any clinical infectious disease in at least one of the four patients. Moreover, it segregates as an autosomal recessive trait that is linked to the centromeric region of the chromosome 8 (logarithm of the odds score 4.51). This NK cell defect was therefore registered as a distinct nosological entity in the Online Mendelian Inheritance in Man (OMIM) database (OMIM:609981), unlike the previously reported patients (8, 12). The clinical phenotype of these patients strongly suggests that NK cells in human are also involved in antiviral immunity and also perhaps in antitumoral immunity (13). Although it is clear that the patients from this kindred suffer from a selective deficit of NK cells, including NKT cells, the pathogenic mechanism remains obscure. The disease-causing gene has not been identified and no disease-causing cellular phenotype was identified either.

We previously reported the clinical phenotype of a new developmental and immunological syndrome observed in two sisters, born to nonconsanguineous parents (14). The sisters showed severe prenatal and postnatal growth retardation, with strictly normal psychomotor development. They lacked detectable blood NK cells and displayed transient CD8αβ T lymphopenia, affecting the CD45RO memory cells in particular. They had neutropenia, but presented no neutropenia-associated infectious diseases. The older sister died of CMV infection at the age of 18 mo (14). The severity of this infection may have resulted from NK cell deficiency, consistent with findings for the mouse model of mouse CMV infection (1, 15–19). However, NK cell deficiency was diagnosed after the onset of CMV disease; moreover, other phenotypic features, such as low CD8 cell counts, may also have been involved (20, 21). NK cell deficiency was diagnosed in the younger sister in the neonatal period. She is now doing well at 8 years of age and she has not yet been infected by any of the known human-tropic herpesviruses (this report). This familial congenital syndrome thus consisted of a specific NK cell deficiency associated with intrauterine growth retardation revealing the first possible link between NK cell differentiation and in utero development. We describe here a plausible pathogenic mechanism of NK cell deficiency and the probable disease-causing cellular phenotype, characterized by enhanced apoptosis of lymphoid cells due to impaired cell survival in response to IL-2 and IL-15.

Materials and Methods

Patients

We previously reported a novel complex syndrome, observed in two French sisters (patients P1 and P2) born to healthy, nonconsanguineous parents (14). This syndrome is characterized by severe pre- and postnatal growth retardation (~3 SDs for height and weight), facial dysmorphism, and immunodeficiency. No retardation of psychomotor development is observed (occipitofrontal circumference at birth: 32 cm for P1 and 33 cm for P2). The immunological phenotype consists of leukopenia with a lack of detectable NK cells (and no detectable NK cytotoxic activity), a transiently small CD8αβ CD45RO T cell fraction and neutropenia (typically 400–700/mm³). The eldest sister (P1) was born in 1993, at 37 wk of gestation. She died due to CMV infection at the age of 18 mo. The second child (P2) was born in 1998, at 38.5 wk of gestation. Bone marrow aspirate, collected 10 mo after birth, was normal, indicating that the leukopenia was a peripheral defect. The small proportion of CD8αβ CD45RO T cells in this patient was found to be a transient defect. Indeed, since the age of 3 years, P2 has presented a normal number of CD8αβ CD45RO T cells. T cell proliferation was normal after mitogen (PHA Ag, CD3) and Ag (tetanus toxoid and poliovirus stimulation, as was the TCR Vβ2 profile. No major abnormality was found in the number of αβ T cells or their activity (14). Since our previous report, P2 has remained healthy and she is now 7 years old and has no prophylactic treatment. However, she remains seronegative for Abs against the herpesviruses commonly associated with illnesses of childhood (HSV, CMV, EBV, varicella-zoster virus). The findings of the most recent immunological investigation conducted for this child, at the age of 7 years, are reported in Results. Growth hormone (GH) treatment was initiated at the age of 4 years, to correct growth retardation. P2 had a height and weight four SDs below the mean at the beginning of treatment. By the age of 6 years, she had attained a height of 108 cm (~2SD) and a weight of 14.8 kg (~2SD). The parents of this patient gave informed consent for all investigations reported here. This work has been approved by appropriate institutional committee (Necker Comités Consultatifs de Protection des Personnes dans la Recherche Biomédicale (CCPBPR)).

Cell purification and culture

PBMCs. PBMCs were isolated from whole blood by Ficoll-Hypaque (Amersham Biosciences) density centrifugation. They were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% AB serum (SAB) at 37°C, in an atmosphere containing 5% CO2.

PHA-activated T cell blasts. PBMCs were activated by incubation with PHA (1/700; BD Biosciences) for 3 days and then reisolated on a Ficoll density gradient and resuspended in Panserin 401 (Biotech), 5% SAB, 2 mM l-glutamine (Invitrogen Life Technologies), and 40 IU/ml recombinant human pro-IL-2 (Chiron).

Herpesvirus saimiri-infected T cell lines. Transformed T cell lines were generated as previously described (22) and cultured in Panserin 401/RPMI 1640 (v/v), 2 mM l-glutamine, 10% SAB, and 40 IU/ml IL-2.

Cell stimulation

PBMCs were plated in 96-well plates at a density of 10⁶ cells/ml and were activated by incubation with PHA (1/700), anti-CD3 Ab (5 ng/ml; OKT3, Ortho Pharmaceutical), IL-2 (Chiron), or IL-15 (147-IR-100, R&D Systems). After 6 days of culture, PHA-activated T cell blasts were also plated in 96-well plates at a density of 10⁶ cells/ml and activated by incubation with IL-2 or IL-15. Herpesvirus saimiri-infected T cell lines were plated at a density of 5 × 10⁵/ml and activated by incubation with IL-2.

Apoptosis

Whole blood and PBMCs were stained with Annexin VFITC, 7-aminomycin D (7AAD) (559925) and Abs against human CD4-PE (555347), CD8-PE (555367), CD14-PE (555398), CD19-PE (555413), or CD15-PE (555402). All these Abs were supplied by BD Biosciences/BD Pharmingen. From whole blood, RBC were lysed by BD Pharm Lyse (555899) (BD Biosciences/BD Pharmingen). DNA fragmentation was assessed in PBMCs, PHA-activated T cell blasts, and herpesvirus saimiri-infected T cells, by washing cells in 0.9% NaCl and incubating the cells in a hypotonic solution containing propidium iodide (50 μg/ml), 0.1% sodium citrate, and 1/100 Triton X-100. We also conducted annexin V and propidium iodide staining (Annexin VFITC Detection kit II (BD Biosciences/BD Pharmingen) on PHA-activated T cell blasts, according to the manufacturer’s instructions. The staining patterns obtained were analyzed by flow cytometry (FACScan; FACS; BD Biosciences). For Fas-induced apoptosis, PHA-activated T cell blasts were stimulated on day 4 with a human anti-Fas mAb (APO-1-3; Coger). For this stimulation, 2 × 10⁵/ml cells in a 96-well plate were activated by incubation for 1 h in medium supplemented with 20 IU/ml IL-2 and the anti-Fas Ab (at a concentration of 100 or 250 ng/ml). We then added 10 μg/ml rabbit anti-mouse IgG Fcγ (Jackson ImmunoResearch Laboratories). Cells were incubated for 24 h and stained.

2 Abbreviations used in this paper: 7AAD, 7-aminoactinomycin D; GH, growth hormone; SAB, AB serum; PMN, polymorphonuclear neutrophil; mTOR, mammalian target of rapamycin. 

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**In vitro proliferation assays**

Cell proliferation was assayed by adding 1.0 μCi [3H]thymidine to the medium for the last 18 h of culture. Radioactivity was counted with a Skatron apparatus (Osl). Cell proliferation was also measured by CFSE labeling: 3 × 10^6 PBMC were labeled at room temperature by incubation for 10 min with 0.1 mg/ml CFSE. They were then washed with FCS (Invitrogen Life Technologies), resuspended in RPMI 1640 supplemented with 10% SAB, and stimulated. On day 3, cells were stained with PE-conjugated Abs against human CD4, CD8, or CD56. CFSE incorporation was evaluated by flow cytometry.

**Western blotting**

PHA-activated T cell blasts were left unstimulated or were stimulated on day 7 by incubation for 30 min with various doses of IL-2 and IL-15. Some cells were also treated with an inhibitor of PI3K, Ly294002, at a concentration of 50 μM (9901; Cell Signaling) 1 h before activation and during activation. Cells were lysed in 50 μl of lysis buffer (0.5% Nonidet P-40, 0.15 M NaCl, 10 mM NaF). Whole cell protein extracts were subjected to SDS-PAGE in 10% acrylamide gels and the separated protein bands were transferred to a nitrocellulose membrane. Blots were blocked by incubation for 1 h at room temperature in PBS (Invitrogen Life Technologies) supplemented with 0.05% Tween 20 (Sigma-Aldrich) and 5% BSA (Sigma-Aldrich), and then probed by incubation overnight at 4°C with Abs against phosphorylated-Ser473-Akt (9271; Cell Signaling), Akt1 (sc-5298; Santa Cruz Biotechnology), phosphorylated-Tyr705-Stat3 (sc-8059; Santa Cruz Biotechnology), Stat3 (sc-7179; Santa Cruz Biotechnology), phosphorylated-Tyr705-Stat5 (9351; Cell Signaling), and Stat5b (sc-1656; Santa Cruz Biotechnology). Blots were incubated with the secondary Ab (rabbit anti-mouse IgG Fcy and mouse anti-rabbit IgG Fcy (Jackson ImmunoResearch Laboratories)) for 1 h at room temperature. Immune complexes were detected by ECL, using an ECL kit (Amersham International) according to the manufacturer’s instructions.

**Bcl-2 and Bcl-x<sub>L</sub> induction**

PHA-activated T cell blasts were left unstimulated or were stimulated on day 6 with 10 or 100 U/ml IL-2 or with 1 or 10 ng/ml IL-15. On day 9, cells were stained with FITC-conjugated anti-human Bcl-2 oncogene Ab (clone 124; DakoCytomation), anti-human Bcl-x<sub>L</sub> Ab (clone BXL03; Chemicon International), and PE-goat anti-mouse (Caltag Laboratories) or with FITC-mouse IgG1 (555748; BD Biosciences/BD Pharmingen), purified IgG1 (BD Pharmingen) as isotype controls, using the Intrastain kit (K2311; DakoCytomation), according to the manufacturer’s instructions.

**Determination of cytokines by ELISA**

Whole blood was left unstimulated or stimulated with PHA (1:700). PBMCs were stimulated as described above. The supernatant was recovered after 72 h for PBMCs. ELISA was performed according to the manufacturer’s kit instructions for IL-2 (DY202; R&D Systems).

**Results**

**Immunological features**

At the age of 7 years, P2 had a lymphocyte count of 2200/mm<sup>3</sup> (data not shown). There were 10% CD19<sup>+</sup> B cells and B cells had a normal phenotype, with CD27-switched B cells present in normal numbers. The Ab response to recall protein and polysaccharide numbers. The Ab response to recall protein and polysaccharide

<table>
<thead>
<tr>
<th>Expression of NK receptors on CD3&lt;sup&gt;+&lt;/sup&gt; cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P2&lt;sup&gt;b&lt;/sup&gt; (2.5 years)</th>
<th>Controls&lt;sup&gt;c&lt;/sup&gt; (4 mo–16 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD94</td>
<td>8.2</td>
<td>3.08–8.3</td>
</tr>
<tr>
<td>CD159 (NK2G2A)</td>
<td>7.1</td>
<td>1.8–8.3</td>
</tr>
<tr>
<td>CD158a (KIR2DL1/2DS1)</td>
<td>0.3</td>
<td>0.5–2.2</td>
</tr>
<tr>
<td>CD158b (KIR2DL2/2DL3/2DS2)</td>
<td>1.3</td>
<td>1.3–6</td>
</tr>
<tr>
<td>CD158c (KIR3DL1/3DS1)</td>
<td>0.6</td>
<td>0.7–2.2</td>
</tr>
<tr>
<td>CD158i (KIR2DS4)</td>
<td>0.4</td>
<td>0.1–3</td>
</tr>
<tr>
<td>CD158k (KIR3DL2)</td>
<td>0</td>
<td>0.1–2.4</td>
</tr>
<tr>
<td>CD161 (NKRP1)</td>
<td>5.7</td>
<td>4–18.7</td>
</tr>
<tr>
<td>CD62L (L-selectin)</td>
<td>54.5</td>
<td>72.5–89.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of CD3<sup>+</sup> T cells expressing NK cell receptors, measured by FACScan analysis.

<sup>b</sup> Patient PBMC, when P2 was 2.5 years old.

<sup>c</sup> Normal values of CD3<sup>+</sup> cells positive for NK cell receptors expression in healthy controls (n = 5) aged from 4 mo to 16 years.

The persistent lack of peripheral NK, NKT cells, and the transient lack of CD8 memory T cells suggested that our patient might present impaired lymphoid stimulation by IL-15. Indeed, mice lacking IL-15 or the α- or β-chains of its receptor present very low levels of NK cells, NKT cells, and memory CD8 αβ T cells (24–27). As IL-15 and the closely related cytokine IL-2 induce the proliferation of T and NK cells in vitro (28–33), we assessed the cellular responses of P2 to these two cytokines. T cell proliferation was normal following the stimulation of PBMC (71% lymphocytes and 11% monocytes) by incubation for 3 days with PHA (data not shown) or anti-CD3 (Fig. 1a), as demonstrated by assessing [3H]thymidine incorporation. T cell proliferation was almost normal following stimulation with a CD3-specific Ab plus rIL-2 (Fig. 1a). However, PBMC from our patient stimulated with IL-2 alone showed lower levels of proliferation than did PBMC from six controls, including one age-matched healthy child (Fig. 1b). The dose-dependent proliferation of control blood cells induced by IL-2 in this assay is primarily due to expansion of the NK cell and CD8 T cell populations (data not shown). No IL-2-driven proliferation of CD8 T cell lines was detected by measuring CFSE incorporation in the patient (data not shown), even though the patient had normal numbers of these cells at the time of the experiment, suggesting that the patient’s CD8 T cells did not respond well to the stimulation of PBMC by IL-2. Following CD3 plus IL-15 stimulation, the patient’s PBMC displayed levels of proliferation similar to those observed for the six controls (Fig. 1c). The patient’s PBMC also responded poorly to stimulation with IL-15 alone (Fig. 1d). Impaired proliferation was also observed if PBMC from a healthy control were depleted of NK cells and stimulated with IL-15, despite the proliferation of CD4 and CD8 T cells (data not shown). The defect in lymphocyte proliferation upon IL-15 stimulation observed in P2 therefore results largely from the absence of NK cells. Overall, no apparent defect in the proliferation of the patient’s T cells was observed if PBMC were stimulated with PHA and CD3, alone or with IL-2 or IL-15. In contrast, proliferation in response to IL-2 or IL-15 was found to be impaired, due to the
absence of NK cells (IL-2 and IL-15) or to a poor response of CD8 cells (IL-2).

Apoptosis of fresh T cells

IL-2 and IL-15 have also been reported to induce the survival of T cells and NK cells in vitro and in vivo in the mouse model and in vitro in humans (34–36). We assessed apoptosis in total PBMC by flow cytometry analysis of propidium iodide incorporation. The patient’s PBMC presented a much higher rate of apoptosis than control cells when cultured in serum-supplemented medium devoid of cytokines (Fig. 2a). This experiment was repeated five times on cells from P2 and we also tested six controls included one age-matched control. Mean apoptosis rates in control PBMC were close to 12% (7.4–17.1%) on day 2 and reached 22% (17–28.1%) by day 3. For P2, >30% (28–32%) of PBMC were apoptotic on day 2 and >38% (34.7–47.4%) were apoptotic on day 3, these proportions being much higher than the upper limit of the normal range. PBMC were also stained with an Ab against a lineage-specific surface marker and annexin V/7AAD, to enable us to determine which populations were apoptotic. More cells from CD8 and CD4 T cells subsets were apoptotic in the patient than control lymphocytes (Fig. 2b), despite the normal CD4 T cell counts of P2. The patient’s B cells also presented a higher rate of apoptosis than control B cells at days 2 and 3 (data not shown). This suggested that the apoptotic phenotype documented on T cells was broader, possibly involving IL-2 and/or IL-15 (37, 38). PBMC apoptosis was also assessed by flow cytometry analysis of propidium iodide incorporation upon stimulation. The stimulation of PBMC with IL-2, with or without anti-CD3 Ab, induced a dose-dependent decrease in the percentage of apoptotic control cells (Fig. 2, c and d). A dose-dependent decrease in apoptosis was also observed for the patient’s PBMC, but the decrease (in percentage) was lower than

![FIGURE 1. PBMC from healthy controls and from the patient (P2) were not stimulated or were stimulated for 72 h with (a) CD3 plus various doses of IL-2, (b) various doses of IL-2, (c) CD3 plus various doses of IL-15, (d) various doses of IL-15; proliferation was assessed by measuring [3H]thymidine incorporation. a–d, The mean of six controls and of five independent experiments on PBMC from the patient. Error bars indicate the minimum and maximum values.](http://www.jimmunol.org/)

![FIGURE 2. Apoptosis of PBMC from healthy controls and from the patient (P2) was assessed by measuring propidium iodide incorporation. a, We assessed the apoptosis of unstimulated PBMC on 3 consecutive days. b, Apoptosis of PBMC from a healthy control (Control) and from the patient (P2) was measured over 3 days by staining with 7AAD. Cells were also stained with CD4 and CD8. Apoptosis of PBMC measured on day 3 (c) following stimulation with CD3 and various doses of IL-2, (d) following stimulation with various doses of IL-2, (e) following stimulation with CD3 and various doses of IL-15, and (f) upon stimulation with various doses of IL-15. a, c, d, e, and f, The mean of six controls and four independent experiments on PBMC from the patient. Error bars indicate the minimum and maximum values.](http://www.jimmunol.org/)
that for control cells (Table II). The highest concentrations of IL-2 were almost associated with a normalization of the phenotype, suggesting that the patient’s cells may respond better to high doses of IL-2. Similar results were obtained with IL-15, with a less profound phenotype for the patient’s cells (Fig. 2, e and f, and Table II). We did not succeed in generating NK cells in vitro by stimulating PBMC with high doses of IL-2, possibly reflecting the poor response of the patient’s NK cells to this cytokine. In conclusion, we observed excess spontaneous and IL-2/IL-15-antagonized apoptosis of the patient’s T cells in assays conducted with fresh PBMC.

**Proliferation and apoptosis of PHA-activated T cell blasts**

We generated PHA-activated T cell blasts and assessed their proliferation following stimulation with IL-2 and IL-15. PBMC were stimulated with PHA, isolated by Ficoll density centrifugation on day 3, and cultured in medium supplemented with IL-2 (40 IU/ml). PHA-activated T cell blasts were composed of a mixture of CD4 and CD8 T cells (data not shown). On day 6, cells were washed and IL-2 (10, 20, 40, or 100 IU/ml) or IL-15 (0.5, 1, 5, or 10 ng/ml) was added. T cell proliferation was assessed on day 9 by evaluating $[^3]$H]thymidine incorporation. PHA-activated T cell blasts from the patient proliferated normally in response to IL-2 or IL-15 stimulation (Fig. 3, a and b), confirming the results obtained with fresh PBMC stimulated with anti-CD3 Ab and a cytokine. The percentage of apoptotic T cell blasts was also determined in these experiments. On day 9, in the absence of cytokine stimulation, the number of apoptotic T cells was normal for the patient (70%). Following the addition of low concentrations of IL-2 (10 IU/ml) on day 6, control T cells displayed a marked decrease in apoptosis on day 9, whereas the patient’s T cells did not. Addition of 10 IU/ml IL-2 resulted in a lower decrease of apoptosis in the patient’s than in the control’s cells (Fig. 3c, Table III). The percentage of apoptotic cells decreased to levels similar to those for control PHA-activated T cell blasts following stimulation with high concentrations of IL-2 (100 IU/ml) (Fig. 3c). Control cells responded much better than the patient’s cells to low concentrations of IL-15 (0.5

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**Table II. Percentage of decrease of apoptotic PBMC upon cytokine stimulation**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>P2</th>
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<tbody>
<tr>
<td></td>
<td>−CD3</td>
<td>+CD3</td>
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<tr>
<td>IL-2$^c$ (IU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10$^d$</td>
<td>29.62</td>
<td>31.66</td>
</tr>
<tr>
<td>40</td>
<td>52.05</td>
<td>48.96</td>
</tr>
<tr>
<td>100</td>
<td>59.84</td>
<td>50.19</td>
</tr>
<tr>
<td>IL-15$^c$ (mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>5</td>
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<td>10</td>
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</table>

|            | Percentage of decrease of apoptotic PBMC from healthy controls and patient (P2) measured on day 3 following stimulation with various doses of IL-2, following stimulation with CD3 and various doses of IL-2, following stimulation various doses of IL-15 and following stimulation with CD3 and various doses of IL-15. The corresponding rates of apoptosis were illustrated on Fig. 2, e-f. |       |
|            | The stimulating CD3 Ab used was 5 ng/ml. |       |
|            | Recombinant cytokine used for stimulation. |       |
|            | Concentration of cytokine used. |       |

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**FIGURE 3.** PHA-activated T cell blasts from controls (Controls) and from the patient (P2) were left unstimulated or were stimulated with (a) various doses of IL-2 or (b) various doses of IL-15 over 3 days and proliferation was assessed by measuring $[^3]$H]thymidine incorporation. This figure shows the mean of seven controls and three independent experiments on cells from the patient. Apoptosis of PHA-activated T cell blasts from healthy controls and from the patient was assessed by measuring propidium iodide incorporation after 3 days in the absence of cytokine stimulation or (c) following stimulation with various doses of IL-2. This figure shows the mean of seven controls and five independent experiments on cells from the patient, (d) following stimulation with various doses of IL-15. This figure represents the mean of four controls and three independent experiments on cells from the patients. e, PHA-activated T cell blasts from controls and from the patient (P2) were left unstimulated or were stimulated with the rabbit anti-mouse Ig alone (RaM-Ig), with anti-Fas Ab at 100 and 250 ng/ml. This figure represents the mean of three controls. Error bars indicate the minimum and maximum values.
and 1 ng/ml), with a marked decrease in apoptosis (Fig. 3d, Table III). At high concentrations of IL-15 (5 and 10 ng/ml), the patient’s T cell blasts responded almost normally. We compared the apoptosis levels of CD4 and CD8 T cell blasts. These two subpopulations presented similar defects in their responses to IL-2 and IL-15 (data not shown). In conclusion, the patient’s T cell blasts required concentrations of IL-2 or IL-15 at least 10 times higher than normal to prevent cytokine-starvation apoptosis and to ensure survival. In contrast, the level of apoptosis was not greater in the patient’s cells than in control cells following Fas stimulation, and may even have been lower (Fig. 3e). Thus, the pathways of starvation-mediated apoptosis and cytokine-mediated cell survival are affected in the patient, whereas death-domain-dependent-Fas-mediated apoptosis is not affected (39–41).

**Apoptosis and proliferation of herpesvirus saimiri-transformed T cells**

A herpesvirus saimiri-transformed T cell line was generated as previously described (22). Herpesvirus saimiri-transformed T cells were cultured in medium supplemented with IL-2 (40 IU/ml) every 3 days. To measure proliferation and apoptosis, the cells were washed and incubated with (20 or 200 IU/ml) or without IL-2. Proliferation was assessed on day 4 poststimulation by IL-2 by evaluating $[^{3}H]$thymidine incorporation. Proliferation of the patient’s saimiri-transformed T cells was normal, like that of the patient’s PBMC (Fig. 4a). Apoptosis was measured every day until day 6 poststimulation with IL-2. At day 6, nearly 40% of the patient’s saimiri-transformed T cells were apoptotic, compared with <20% of control saimiri-transformed T cells. Like the patient’s fresh T cells, the patient’s saimiri-transformed T cells thus presented with an excess of apoptosis. A partial complementation of the phenotype was observed with the addition of 20 IU/ml IL-2 and a complete normalization was observed with 200 IU/ml IL-2 (Fig. 3b). Like with P2 PBMC, herpesvirus saimiri-transformed T cells from P2 are therefore excessively apoptotic in the absence of the survival factor IL-2 and respond only to supplementation with high doses of rIL-2.

**Normal IL-2 and IL-15 signaling pathways**

We investigated the molecular basis of impaired responses to IL-2 and IL-15 in our patient by assessing levels of these cytokines and their signaling pathways. The production of IL-2 and IL-15 and the expression of their receptors (CD132, CD122, CD25, IL-15Rα) were found to be normal on T cell blasts (CD132, CD122, CD25) or monocytes (IL-15Rα) by flow cytometry (data not shown). The primary structure of the β-chain of these receptors (CD122) was also normal, as demonstrated by sequencing of the coding region of the cDNA (data not shown). We then investigated the IL-2- and IL-15-signaling pathways downstream from the receptors. We stimulated PHA-activated T cell blasts from a healthy control and from the patient with 10–1000 IU/ml IL-2. Western blots showed that Stat5, Stat3 (involved in T cell proliferation (42)) and Akt-1 (involved in T cell survival (43, 44)) were normally phosphorylated in the patient, even after stimulation with a low dose of IL-2 (10 IU/ml), which is known to decrease survival rates (Fig. 5a). Ly294002, an inhibitor of Akt-1 phosphorylation, served as a control. A similar result was obtained following stimulation with 1–10 ng/ml IL-15 (data not shown). We detected no defects in signaling between the IL-2 and IL-15 receptors and the Stat and Akt-1 proteins. We then studied the induction by IL-2 and IL-15 of Bcl-2 and Bcl-xL, crucial antiapoptotic factors in T lymphocytes (45–47). We used flow cytometry to determine intracellular Bcl-2 and Bcl-xL levels in PHA-activated T cell blasts following stimulation with IL-2 (10 or 100 IU/ml) or IL-15 (1 or 10 ng/ml) or in the absence of cytokine. In the absence of cytokine stimulation, both Bcl-2 and Bcl-xL levels were similar in the patient’s cells and in control cells. Dose-dependent Bcl-2 induction in response to IL-2 and IL-15 was observed with PHA-activated T cell blasts from both the patient and controls, even if concentrations of IL-2 known to impair survival were used (10 IU/ml) (Fig. 5b). The primary structure of Bcl-2 was also normal, as shown by sequencing of the coding region of the cDNA. The level of Bcl-xL is poorly induced in response to cytokine stimulations but comparable levels were observed in control and patient PHA-activated T cell blasts (data not shown). The mammalian target of rapamycin (mTOR), a central regulator of cell growth and proliferation, is activated by growth factors such as IL-2, via Akt. Rapamycin is an immunosuppressant that induces cell cycle arrest in G1 phase (48). Therefore, we treated PHA-activated T cell blasts for 72 h with rapamycin. Similar decreases in proliferation were observed for the patient’s and control cells (data not shown), with no additional apoptosis induced by rapamycin in either (data not shown). Thus,
known subsets of conventional \(T\) cells, notably of memory CD45RO \(CD8\) cells, and even B cells, some of which also express the receptors for IL-2 and IL-15 (37, 38). There is thus both global and partial impairment of lymphoid lineage survival, most pronounced in NK and NKT cells, suggesting that these cells are more dependent on IL-2 and IL-15 for survival than conventional \(CD8\) cells and B cells in humans. Nevertheless, until a disease-causing genotype is identified in this kindred, the cellular phenotype herein reported cannot be strictly proven to be disease causing. Although unlikely, it may be a mere consequence, rather than the cause, of the primary defect.

The impaired survival of lymphocytes probably accounts for the key immunological feature of our patients, the lack of NK cells. The small number of PMNs may reflect an impaired response to survival signals, with an enhanced apoptosis, or it may be a consequence of the lymphoid anomalies. The severe pre- and postnatal growth retardation clearly did not result from lymphoid or myeloid cytopenia. Instead, it may reflect a global impairment of cell survival. GH levels and responses to this hormone in vivo were found to be normal in our patient, and GH is not a major factor affecting development in utero. In contrast, insulin is known to be a major growth factor in utero. Its signaling pathway involves Akt and target genes also involved in the IL-2 signaling pathway (44, 49). Several mutant mice were shown to display in utero growth retardation, including IGF-1-, IRS-1-, AKT-1-deficient mice (50, 51), whose deleted genes are involved in the insulin-signaling pathway. Thus, impaired cell survival with increased apoptosis may account for the developmental and lymphoid phenotypes of our two patients. Identification of the disease-causing gene may shed new light on the survival pathways involving both cytokines and developmental growth factors. In any event, the cellular survival defect leading to excessive apoptosis probably accounts at least for NK and NKT cell deficiency. This in turn is the most threatening clinical feature associated with this syndrome, as P1 died of CMV infection. The survivor (P2) has not yet been infected by herpesviruses.

What genotype corresponds to this cellular phenotype of impaired cell survival? We provide compelling evidence that our patients’ \(T\) cells present a defect in survival in response to IL-2 and IL-15. Our results indicate that the genetic defect is downstream of the T-box transcription factor Eomesodermin (\(Eomes\)) in mice is responsible of a developmental defect resulting in early embryonic lethality (52). Mice that lack the T-bet transcription factor, heterozygotes for a loss-of-function mutation in \(Eomes\) present an immunological phenotype that most strikingly resembles that of our patients. In this specific genetic context, the deletion of one \(Eomes\) allele causes a deficiency of IL-15-dependent lymphoid lineages, due to an impaired regulation of CD122. The mice present with NK cell deficiency and an impaired development of cytotoxic memory CD8\(^+\) cells (53).
human EOMES gene and the fine regulation of CD122 should therefore be examined in detail in our patient. We also recently used panengenic microarrays to assess the expression pattern of IL-2 and IL-15 target genes following the stimulation of PHA-activated T cell blasts from the patient and a control. The microarray approach may provide a selection of candidate genes. An alternative approach is to recruit other kindreds with a similar phenotype. This should pave the way for the identification of the causal gene by means of a genome-wide scan. In conclusion, we report a plausible pathogenic mechanism underlying the first description of a familial form of inherited selective NK cell deficiency in humans, differing from the previously reported inborn nature. Our identification of a cellular phenotype, therefore be examined in detail in our patient.

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