A Severe Form of Human Combined Immunodeficiency Due to Mutations in DNA Ligase IV

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A Severe Form of Human Combined Immunodeficiency Due to Mutations in DNA Ligase IV

Anselm Enders,* Paul Fisch,‡ Klaus Schwarz,§ Ulrich Duffner,* Ulrich Pannicke,‡ Elisabeth Nikolopoulos,† Anke Peters,* Marzenna Orlowska-Völk,† Detlev Schindler,§ Wilhelm Friedrich,‡, Barbara Selle,† Charlotte Niemeyer,* and Stephan Ehl2*

DNA ligase IV (LigIV) deficiency was identified as the molecular basis for a severe form of combined immunodeficiency in two microephatic siblings with cellular radiosensitivity. In one patient the diagnosis was made directly after birth, allowing analysis of the role of LigIV in the development of specific immune cells. Absolute numbers of B cells were reduced 100-fold and αβ T cells 10-fold, whereas γδ T cells were normal. Spectratyping of all three cell populations showed a diverse repertoire, but sequencing of IGH V(D)J junctions revealed shorter CDR3 regions due to more extensive nucleotide deletions among D and J elements and fewer N nucleotide insertions. Clonal restriction of IgG-expressing, but not IgM-expressing, B cells and the lack of primary and secondary lymph node follicles indicated impaired class switch recombination. Observations in the older sibling showed that this rudimentary immune system was able to mount specific responses to infection. However, partial Ab responses and extensive amplification of γδ T cells could not prevent a life-threatening course of viral and bacterial infections, the development of an EBV-induced lymphoma, and immune dysregulation reflected by severe autoimmune cytopenia. Impaired generation of immune diversity under conditions of limited LigIV activity can cause a human SCID variant with a characteristic immunological phenotype. The Journal of Immunology, 2006, 176: 5060–5068.

The V(D)J recombination process induces double-strand DNA breakage and rejoining. It forms the basis for the generation of diversity in the specific immune system (1). Early lymphoid progenitors express the RAG-1 and RAG-2 proteins, which bind at the recombination signal sequences flanking the genetic V, D, or J elements, induce double-strand DNA breaks to excise intervening DNA sequences, and generate molecular hairpin structures at the remaining (coding) ends. DNA rejoining is then conducted by nonhomologous DNA end joining (NHEJ), a double-strand break repair pathway active in all nucleated cells (2). As a first step in NHEJ, the Ku proteins bind to the DNA ends and associate with the DNA-dependent protein kinase (PK) catalytic subunit. The resulting DNA-PK complex associates with Artemis and activates its endonuclease activity. The Artemis/DNA-PK complex opens the hairpinned V, D, or J ends. End processing by the endonuclease activity of Artemis in combination with template-dependent as well as template-independent polymerase activity generates junctional diversity. The DNA-dependent PK catalytic subunit then also acts as a scaffold for the x-ray repair cross-complementing protein 4 (XRCC4)/ligase IV (LigIV) complex, which finally rejoins the DNA ends (reviewed in Ref. 3).

The key role for some of the enzymes involved in V(D)J recombination for human lymphocyte development is well illustrated in patients with primary immunodeficiencies (4). Patients with null mutations in RAG-1, RAG-2, or Artemis completely lack B and T cells (5, 6) and present with SCID, a uniformly lethal disorder unless treated by bone marrow transplantation or gene therapy (7). Sensitivity to ionizing irradiation differentiates Artemis-deficient patients from RAG-deficient patients (5), illustrating a more general role of the NHEJ pathway in the repair of double-strand DNA breaks. Patients with null mutations in the other genes involved in V(D)J recombination have not been reported. This may in part be explained by their essential role in development. Mice with a targeted deletion of DNA LigIV die at an early embryonic stage, suggesting other essential roles of that enzyme in development (8).

Although the expected presentation of patients with null mutations in genes involved in V(D)J recombination is a T−B− SCID, patients with hypomorphic mutations may retain some recombination activity and develop a rudimentary specific immune system (9, 10). Secondary modification by an infectious or self Ag can then induce characteristic phenotypes such as Omenn syndrome (10–12), a disorder characterized by generalized erythoderma, hepatosplenomegaly, eosinophilia, elevated IgE, and oligoclonal T cell expansion. Both hypomorphic Artemis and RAG mutations can lead to Omenn syndrome (10, 13), indicating that the partially developed specific immunity and not the particular genetic defect may be responsible for the phenotype. Thus, patients with hypomorphic mutations can provide important insights on how the human immune system develops and acts under limiting conditions.

Reports on six patients with hypomorphic mutations in LigIV (14, 15) have been published to date. The first patient developed leukemia at the age of 14 and was diagnosed on the basis of his increased sensitivity to radiation therapy (16). Another patient in a

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3 Abbreviations used in this paper: NHEJ, nonhomologous end joining; LigIV, DNA ligase IV; PK, protein kinase; XRCC4, x-ray repair cross-complementing protein 4.

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recently studied a newly developed T cell leukemia at the age of 4.5 years and showed a phenotype resembling Nijmegen breakage syndrome with microcephaly and growth retardation (17). The other four patients were diagnosed between 9 and 48 years of age with various abnormalities, including developmental delay, microcephaly, and pancytopenia (14). Although in two patients the pancytopenia was severe enough to warrant bone marrow transplantation, none of the patients has been described as being severely immunodeficient (18). Although the crucial role of LigIV for human V(D)J recombination has been established in a gene-targeted human pre-B cell line (19) and in cell lines obtained from LigIV-deficient patients (20–23), the phenotypic consequences of LigIV deficiency for human lymphocyte development and immunity to new infections remain poorly defined.

Here we describe the immunological profile of two siblings who were found to be compound heterozygous for a null allele and a hypomorphic mutation in DNA LigIV and who presented with severe immunodeficiency. One patient was identified after multiple severe infections and the other was identified directly after birth, providing the opportunity to study the impact of LigIV deficiency both on early lymphocyte development and on immune defense.

Materials and Methods

Inforned consent

Informed consent for the performed studies was obtained from the patient’s family in accordance with the guidelines of the local ethics committee.

Irradiation experiments

Primary diploid fibroblast-like cells were grown from skin biopsy specimens using standard cell culture techniques. Confluent, quiescent (G0 phase) cultures were trypsinized, and aliquots were irradiated at the indicated dosage levels using a 6-MV linear accelerator (Siemens) at a dose rate of 2 Gy min⁻¹. For colony survival studies, aliquots of trypsinized fibroblasts were irradiated at 0, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 Gy and seeded in triplicate into petri dishes as described (24). Eagle’s MEM with 15% FBS was replaced every 3–4 days. After a growth period of 2 wk, colonies (>50 cells) were stained with 1% crystal violet in 20% ethanol. Means and SDs of the ratio of colony number relative to the number of seeded cells were calculated individually and plotted as colony survival vs radiation dosage for the two patient and the two normal control strains. Dose-response curves were fitted to the linear quadratic model SF = exp(−αX − βX²), where SF is the survival fraction, X the radiation dose, and α and β are fitting parameters. Curves and statistics were generated using the Origin software (MicroCal). For 72-h cell cycle analysis, fibroblasts irradiated with 0 and 1.5 Gy were seeded at a density of 4000 cells/cm² into parallel cultures. Growth medium was Eagle’s MEM substituted with 15% FBS, 1 × 10⁻⁴ M BrdU, and 1 × 10⁻⁴ M 2-deoxyctydine. After the growth period, the cells were trypsinized and stained with Hoechst 33258 and propidium iodide. Bivariate histograms were recorded on an LSR flow cytometer (BD Biosciences) and analyzed quantitatively using MULTID and MCYCLE software (25).

Genetic analysis

The LigIV gene was amplified with primer pairs yielding overlapping amplimers. These amplimers were sequenced in both directions. Primer sequences for amplification and sequencing are available on request (klaus.schwarz@medizin.uni-ulm.de). Nucleotide and amino acid counts are according to RefSeq database sequences NM_002312 and NP_002303, respectively.

Immunophenotyping and IgH and T cell CDR3 spectratyping

Lymphocyte phenotyping was performed with four-color flow cytometry using Abs from BD Pharmingen and Immunotech as described previously (26). TCR CDR3 spectratyping was performed as described by Pannetier et al. (27, 28) for analysis of the TCR-β chain. The primers and details for spectratyping of the TCR-β chains have been published (26). IgH spectra were established according to previously published methods (29) and new work (E. Nikodopoulos, and P. Fisch, manuscript in preparation). In brief, the CDR3 regions were amplified using 35 cycles of PCR with a forward (sense) primer specific for the V region family and a backward (antisense) primer for the constant region. In a second round, three cycles of a primer extension reaction with a fluorescent (5′ FAM-labeled) nested antisense primer specific for the constant region was performed (runoff reaction). The products were then analyzed on an ABI 3100 capillary sequencer (Applied Biosystems) to determine the length distribution of the fluorescent fragments. Clonal populations generally appear as sharp peaks, whereas polyclonal populations typically show Gaussian distributions but may show one or several prominent peaks within the polyclonal peaks if the repertoire is dominated by a few clones. The PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen Life Technologies), amplified, and sequenced. Ig gene segments were identified using the international ImMunoGeneTics (IMGT) database (imgt.cines.fr) (30). Gene junctions were analyzed using the IMGT/ JunctionAnalysis tool (31). All N nucleotide sequences of seven or more nucleotides were individually screened for the presence of a second D element in both orientations. A homology of at least 7 bp and appropriate chromosomal localization relative to the first D element were required for identification of a second D element.

Results

Clinical course of two siblings with microcephaly, radiosensitivity, and severe immunodeficiency

A 2-year-old girl (P1) born to nonconsanguineous parents of German origin was admitted to our hospital with a history of chronic diarrhea, failure to thrive, autoimmune cytopenia, and recurrent infections. At birth the girl was microcephalic with no further dysplastic stigmata. She developed mild intermittent diarrhea and growth delay. At 1 year of age she suffered from pneumococcal sepsis two weeks after measles, mumps, and rubella vaccination. Three months later she presented with severe neutropenia (80/μl) and thrombocytopenia (18,000/μl). PCR was positive for human herpes virus 6 from bone marrow and peripheral blood. She had varying lymphopenia with reduced T cells, almost absent B cells, and normal NK cells. The thymus was small for her age but present. Motor development and cognitive development were normal, and all developmental milestones had been achieved in time. At 20 mo of age she was diagnosed with persistent Norwalk virus-positive diarrhea with pronounced failure to thrive and the need for parenteral nutrition. Four months later, she was referred to our hospital with high fever, cervical lymphadenopathy, hepatomegaly, and a pronounced necrotizing mucositis. PCR for EBV was positive in peripheral blood (2,900 genome equivalents/ml) and bone marrow (640,000 genome equivalents/ml). Imaging studies revealed multiple ovaloid mass lesions in the brain and the lung. A biopsy showed an EBV-encoded latent membrane protein 1-positive, monoclonal, diffuse, large cell non-Hodgkin B cell lymphoma. Intensive chemotherapy reduced the tumor mass but was followed by severe mucositis with oral and gastrointestinal bleeding. The patient developed rapidly progressive aspergillosis and died during preparative therapy for allogeneic stem cell transplantation.

While the first patient was being treated for the lymphoma, a sister was born (P2) who was also microcephalic (Fig. 1). At 2 wk of age she had an absolute lymphocyte count of 936/μl with a similar subset distribution as her older sister. At 4 mo of age she received a bone marrow transplant from a matched unrelated donor following nonmyeloablative conditioning with fludarabine and fludarabine and...
of cells in G2 phase among all cycling cells. The ratios from fibroblasts of
The percentage of cells in G1/G2 phase is shown as a function of the ratio
propidium iodide, and cell cycle status was determined by flow cytometry.
irradiation with 1.5 Gy, fibroblasts were stained with Hoechst 33258 and
8 mo after transplantation with full chimerism and ongoing thymic
compensated renal insufficiency. She is presently clinically stable
vere hemolytic-uremic syndrome, presumably triggered by cyclo-

FIGURE 1. Sensitivity to ionizing radiation in two patients with micro-
cerephaly and immunodeficiency. A, Colony survival. Dose vs radiation re-
response curves of fibroblasts from the two patients, P1 (filled triangle) and
and P2 (inverted filled triangle), are compared with those of two random nor-
mal controls, C1 (open circle) and C2 (closed circles), and studied in par-
allel. Error bars denote one SD. B, G2 phase accumulation. 72 h after
irradiation with 1.5 Gy, fibroblasts were stained with Hoechst 33258 and
propidium iodide, and cell cycle status was determined by flow cytometry.
The percentage of cells in G1/G2 phase is shown as a function of the ratio
of cells in G2 phase among all cycling cells. The ratios from fibroblasts of
patient P1 (filled triangle) and patient P2 (inverted filled triangle) are
within the range of radiosensitive ataxia telangiectasia fibroblast strains
(gray diamonds; n = 18), whereas normal control strains (gray circles; n =
15) show a much lower ratio. The high noncycling cell fractions of
the patient cultures relative to positive (ataxia telangiectasia) and negative
(normal) controls indicate their poor growth. C, Growth charts showing
development along the 25th to 50th percentile (Fig. 1C, upper panels). By contrast, the head circumference was disproportionally
small and was below the 3rd percentile in both patients (Fig. 1C, lower panel). Although no severe immunodeficiency had been re-
ported in the six previously published patients (14, 15, 17, 18), we
decided to sequence the LIG4 gene. We identified a point mutation
(1118A→T, resulting in H282L) on the maternal allele and a de-
letion of five bases (1544AAAGA1548) on the paternal allele, leading
to a frame shift and an altered amino acid sequence starting
after D423 and premature termination of translation after amino
acid 442 (Fig. 2A) in both patients. The parents were heterozygous
for these mutations.

Impaired B cell development, but remaining specific Ab
production in LigIV deficient patients
The absolute numbers of peripheral B cells were reduced ∼100-
fold in both siblings (Table I). Nevertheless, serum Igs and specific
Abs could be detected. When analyzed at 4 wk of age in the ab-
ence of obvious infections, P2 had low amounts of IgM, whereas
IgA was absent. IgG levels could not be interpreted due to remain-
ing maternal Abs. Nevertheless, IgM hemagglutinins were detect-
able. Moreover, 7% of the B cells expressed the memory marker
CD27, similar to age-matched controls. P1 was analyzed after se-
vere infections and showed surprisingly high levels of serum Igs.
Total IgG and IgM were elevated, but IgA was in the normal
range. Despite three vaccinations, the patient did not have specific
Abs to tetanus or diphtheria and showed only low titers to pneu-
monococcus despite having undergone culture-proven sepsis. By
contrast, significant IgG responses could be detected against the
measles and mumps vaccines and to the human herpes virus 6
infection documented by PCR. Response to the rubella vaccine
was limited to IgM, and no Ab response could be detected against
EBV. In addition to these partial antimicrobial Ab responses, the
patient developed high titers of autoantibodies against platelets,
resulting in severe thrombopenia.

Reduced BCR diversity in LigIV deficiency
To analyze the B cell repertoire generated under conditions of
limited LigIV activity, we performed CDR3 spectratyping of the
VH genes using IgM- and IgG-specific primers in P2 and a healthy
age-matched control. The spectratyping analysis was performed
three times from the same RNA samples and found to be reproducible, especially for the more commonly used \( V_{H1} \), \( V_{H3} \), and \( V_{H4} \) families (33). Several differences were noted in comparison to the control (Fig. 3A). First, diversity was more restricted among some \( V_{H} \) families such as \( V_{H5} \) and \( V_{H6} \). Second, there were more seemingly clonal expansions, e.g., in the \( V_{H2} \), \( V_{H3} \), \( V_{H5} \), and \( V_{H6} \) families. Third, the sizes of the CDR3 fragments were smaller in the patient than in the healthy control, most obvious in the \( V_{H3} \) and \( V_{H4} \) families. CDR3 length diversity was less pronounced in both infants when IgG-specific primers were used (Fig. 3B). Because the analysis was performed 8 wk after birth, this finding may reflect the largely naive state of the B cell system.

**Reduced V(D)J junctional diversity in LigIV deficiency**

To test whether the limited LigIV activity resulted in a particular pattern of V(D)J recombination, we cloned and sequenced 63 PCR products from IgM-specific CDR3 amplifications and 58 products from IgG-specific CDR3 amplifications. V, D, and J elements as well as N and P nucleotides were identified using the IMGT database. Additional analysis of N/P nucleotide sequences longer than six nucleotides revealed the presence of two D elements in 1/70 sequences from the patient and 1/51 sequences from the control. Fig. 4 shows an alignment of the V(D)J regions obtained with primers for \( V_{H3} \) and the constant region of IgM (upper panel) or the constant region of IgG (lower panel) for P2 and the age-matched control. The differences were subtle when single \( V_{H} \) families were compared but became clearer after 70 sequences from the patient and 51 sequences from the control had been analyzed.
First, there was significant clonal identity among IgG sequences from the patient. Although among IgM sequences 23/24 control and 37/39 patient sequences were unique, only 15/31 IgG sequences from the patient compared with 26/27 from the control showed a unique sequence. Thus, in contrast to IgM, the clonal diversity of IgG-expressing B cells appeared to be significantly more restricted in P2 than in the control. Second, N nucleotide sequences were used less often and, if used, were shorter in the patient (Fig. 5A). This could be seen among both IgM and IgG sequences (Fig. 5 and data not shown). By contrast, the number of sequences that harbored P nucleotides was similar in both infants (15/52 in P2 vs 16/49 in the control). Third, more deletions of the end nucleotides could be observed among the J and D elements of the patient. Fig. 5 shows the number of nucleotides deleted from the J elements (Fig. 5B) and the D elements (Fig. 5C) among the unique IgM and IgG sequences from P2 and the control.

**FIGURE 4.** VH3 junctional sequences among IgM and IgG B cells. PCR products from the IgH CDR3 spectratyping (see Fig. 3) were isolated, cloned into the pCR2.1-TOPO vector, amplified, and sequenced. Sequences amplified using VH3- and IgM-specific (upper panel) or VH3- and IgG-specific primers (lower panel) are shown for P2 and a healthy age-matched control. The sequences are shown as displayed by the IMGT software. The dots in the aligned V and J regions as well as in the D regions reflect deletions of the germline end nucleotides. The far right column shows the number of amino acids in the CDR3 region.

**FIGURE 5.** Fewer N nucleotides and more gene end deletions among J and D elements in LigIV deficiency. The unique VH3 junctional sequences amplified using IgM- and IgG-specific primers from the patient (52 unique/70 total sequences) and the control (49 unique/51 total sequences) were aligned using the IMGT software. A, The number of N nucleotides was determined in the individual sequences. B and C, J and D elements were compared with the germline sequences, and the number of nucleotides deleted from 5’ and 3’ ends during V(D)J recombination was determined for the individual sequences. B, Number of nucleotides deleted from the J elements. C, Number of nucleotides deleted from the D elements. Data are displayed as box plots with the median, 25/75th, and 10/90th percentiles indicated as horizontal bars.
Absence of primary and secondary lymph node follicles

A cervical lymph node from P2 was removed. H&E staining revealed a poorly structured fibrotic node with pronounced lymphopenia, lack of follicular areas, and interfollicular zones (Fig. 6A). Immunohistochemical analysis using B cell Abs (CD20; Fig. 6, B and C) showed a proper number of follicles (normal anlage) but of small size and with different severity of B cell depletion. The follicles lacked CD3⁺ T cells but contained abundant CD68⁺ macrophages (Fig. 6D). These cells can frequently be observed in the granuloma-like structures forming in lymph nodes of SCID patients. There were no secondary follicles, no centroblasts, and no signs of germinal center activity as shown by negative staining for the proliferation marker Ki-67 (data not shown).

Impaired T cell development but normal T cell repertoire diversity

The number of circulating CD4⁺ and CD8⁺ T cells was reduced in both patients with a reduced proportion of CD45RA⁺ naive T cells among total CD4⁺ T cells. T cell proliferation assessed by [³H]thymidine incorporation was significantly reduced in response to PHA and anti-CD3 stimulation, and there was no response to tetanus in P1. Using a CFSE dilution, we could demonstrate significant proliferation of CD4⁺ T cells obtained from P2 in response to PHA and anti-CD3/anti-CD28, whereas there was little response from CD8⁺ T cells (data not shown). All of the seven Vβ chains analyzed were used by T cells from P2, and there were only limited shifts compared with published controls (Fig. 7, A and B). CDR3 spectratyping of the Vβ chain revealed a polyclonal repertoire that resembled the diversity of an age-matched control (Fig. 7C).

Predominance of γδ T cells

Both patients showed a predominance of γδ T cells (Table II). In the absence of any obvious infection in P2, 29% of CD3⁺ lymphocytes expressed the γδ TCR. Up to 89% of CD3⁺ lymphocytes expressed the γδ TCR in P1 after a history of severe bacterial and viral infections. The diversity of δ-chains was analyzed in T cells from P2 by TCR CDR3 spectratyping analysis (Fig. 8). Significant genetic diversity could be documented among the more common Vδ1, Vδ2, and Vδ3 chains, whereas the more rare Vδ4, Vδ5, and Vδ6 chains showed an oligoclonal spectrum. The overall pattern of CDR3 sizes was as diverse as that of an age-matched control (Fig. 8), arguing against Ag-driven oligoclonal expansion as the basis for the γδ T cell predominance in P2.

Discussion

In this report LigIV deficiency has been identified as a new genetic cause for a variant of severe combined immunodeficiency in humans. It adds to the wide phenotypic spectrum of LigIV syndrome (Online Mendelian Inheritance in Man no.601837), a previously described heterogenous disorder with radiosensitivity, growth failure, microcephaly, developmental delay, predisposition to lymphoma, and milder forms of immunodeficiency. In all patients described it is caused by hypomorphic LigIV mutations (14, 15, 17, 34). This event is not unexpected, because mice with a targeted deletion of LigIV die during embryonal development (8). Neither of the LigIV mutations identified in this study have been reported previously (Fig. 2A). The first mutation leads to a frameshift after D423 and a premature stop after amino acid 442, presumably representing a null allele. The most similar mutation that has been characterized in detail is the R580X mutation, likely representing a null allele, because the protein is not stably expressed, does not interact with XRCC4, and does not enter the nucleus (14). The second most likely hypomorphic mutation (H282L) is close to the conserved ligase motif that includes the active site K273. It is in the vicinity of the R278H mutation (15) that leads to 5–10% residual adenylation and double-strand ligation activity (20, 22, 23). It has been reported that this activity is further reduced below 1% by the additional polymorphisms A3V and T9I (34). These polymorphisms were not present in our patients. Because the functional consequences of the H282L mutation have not yet been studied by in vitro assays, we cannot exclude the possibility that additional uncharacterized defects may also contribute to the impaired class switch recombination.
deficiency in vivo (14, 15). In plasmid-based assays, cell lines none of the mutations described to date caused a severe immuno-
quency and fidelity of V(D)J recombination in vitro (14, 20 – 23), patients (14). However, despite various abnormalities in the fre-
tibility to infection have been reported in some LigIV-deficient
topenia of several hemopoietic lineages and an increased suscep-
possibility of a SCID phenotype in human LigIV syndrome. Cy-
NHEJ identified in mice (8) and human cell lines (19) predicted the
which a particular mutation impairs immunological development.
neurodevelopmental outcome cannot be predicted by the extent to
and motor development. Our observations also imply that the neu-
ral defect is, however, highly variable. Although LigIV-
deficient mice die during embryonal development (8), the devel-
neurological defect is, however, highly variable. Although LigIV-
deficient mice die during embryonal development (8), the develop-
mental delay in patients with hypomorphic mutations ranges from
no defects (patient 118BR) (15) to significant developmental
mental delay (patient 99P0149) (14). This can only in part be
explained by the localization of the individual mutations, because
our two siblings differed markedly in their cognitive development
and motor development. Our observations also imply that the neu-
rodevelopmental outcome cannot be predicted by the extent to
which a particular mutation impairs immunological development.
The crucial role of LigIV in DNA double-strand repair and
HEJ identified in mice (8) and human cell lines (19) predicted the
possibility of a SCID phenotype in human LigIV syndrome. Cy-
topenia of several hemopoietic lineages and an increased suscep-
tibility to infection have been reported in some LigIV-deficient
patients (14). However, despite various abnormalities in the fre-
quency and fidelity of V(D)J recombination in vitro (14, 20 – 23),
one of the mutations described to date caused a severe immuno-
deficiency in vivo (14, 15). In plasmid-based assays, cell lines
obtained from patients 180BR and 411BR only showed a 2–3-fold
reduction in the frequency of V(D)J recombination (14, 20, 23),
whereas it was reduced 1000-fold in the gene-targeted LigIV-de-
cient human pre-B cell line N114P2 (19). In our patients, the
LigIV mutations led to a significant impairment of lymphocyte
development. Obviously, the repertoire of peripheral B and T cells
detected in the periphery represents the complex result of develop-
mental impairment, homeostatic proliferation, and peripheral
modification by infectious and other types of Ags. However, the
younger sibling was studied only a few weeks after birth without
obvious infections, and the spectratyping analysis revealed a
largely undisturbed repertoire. The severely reduced number of
Circulating B and T lymphocytes may, therefore, allow a rough
estimation of the efficiency of V(D)J recombination. Interestingly,
the impact was different on various cell populations. Although B
cell numbers were reduced ~ 100-fold, there was only a 10-fold
reduction in αβ T cells and no reduction in γδ T cells. This could
be due to cell type-specific differences in LigIV requirements, an
interpretation that is supported by the finding that the repertoire
diversity was more restricted among B cells than among T cells.
An additional factor may be thymic selection. Under conditions
of limiting production of precursor cells, a relatively higher propor-
tion of T cells may be able to pass selection in the thymus such that
the lack of mature T cells may be relatively less pronounced than
the lack of T cell precursors. The predominance of γδ T cells
observed in both patients may have been influenced by the less
stringent thymic selection of γδ T cells that is probably not influ-
ced by MHC restriction. Following the infections in P1, the
γδ T cells may have been triggered and further expanded by microbial
Ags recognized by the γδ TCR (40–43) or other triggering mecha-
isms of the innate immune system.
LigIV deficiency significantly affected the fidelity of the joining
reaction in V(D)J recombination. Previous studies (20) with trans-
fected recombination substrates revealed signal joint, but not cod-
ing joint, deletions during recombination in the 180BR cell line.
The defects in cell lines from patients 2304 and 411BR were more
pronounced and included deletions, lack of end protection, and
reduced insertion of N nucleotides by TdT (14, 20, 21). In the
sequences obtained from peripheral B cells of P2 we found sig-
ificant deletions of D and J elements and reduced N nucleotide
insertion. This may suggest a disturbed interaction between LigIV
and TdT, but a direct interaction between these two enzymes has
not been proven biochemically. The increased number of nucleo-
tide deletions could be due to lack of end protection by LigIV (21).
Another possible explanation is that, due to limited LigIV activity,
the DNA ends are accessible to nucleases such as Artemis for a
longer period of time, leading to a relative overactivity of these en-
zymes.

Characteristic phenotypic features of most patients described
with LigIV syndrome include microcephaly and radiosensitivity (Fig. 2B).
Growth defects and radiosensitivity can also be observed
in mice deficient in the NHEJ enzymes Ku (35–37), as well as in
mice deficient in XRCC4 or LigIV, when bred on a p53-deficient
background to overcome their embryonic lethality (38, 39). Although
microcephaly has not been reported in these mice, exces-

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<th>P1</th>
<th>P2</th>
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<tr>
<td>Age</td>
<td>1.5–2.5 years</td>
<td>2 wk–4 mo</td>
</tr>
<tr>
<td>Lymphocytes/μl</td>
<td>360–1300 (2600–10,400)</td>
<td>583–936 (3700–9600)</td>
</tr>
<tr>
<td>CD3+/μl</td>
<td>280–1074 (1600–6700)</td>
<td>176–228 (2300–6500)</td>
</tr>
<tr>
<td>CD45RA (percentage of CD4)</td>
<td>1–4</td>
<td>14–22 (64–95)</td>
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<tr>
<td>PHA</td>
<td>Reduced</td>
<td>Reduced</td>
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<tr>
<td>Tetanus</td>
<td>Reduced</td>
<td>ND</td>
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<tr>
<td>TCR γδ (percentage of CD3)</td>
<td>65–81 (2–10)</td>
<td>23–29 (1–10)</td>
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<tr>
<td>TCR γδ/μl</td>
<td>72–262 (85–215)</td>
<td>51–68 (85–215)</td>
</tr>
</tbody>
</table>

*Age-matched normal values are given in parentheses.

FIGURE 8. Normal γδ T cell repertoire. CDR3 length profile of the
indicated Vδ populations. cDNA was prepared from PBMC RNA, ampli-
fied using Vδ specific primers, and analyzed by spectratyping (see legend
to Fig. 3). The frequency of PCR fragments of a given size is plotted as a
function of the size of the PCR fragment. Dark peaks represent size mark-
ers, and light peaks represent PCR products.
The severe immunodeficiency observed in our patients included susceptibility to life-threatening bacterial and viral infections, development of an EBV-positive B cell lymphoma, and severe autoimmune cytopenia. These features developed in the presence of normal to elevated total levels of serum IgS, indicating that a significant number of Ab-producing plasma cells could be generated from the few developing B cells. However, specific IgM and IgG responses were only detected against some vaccines and infections, presumably reflecting an additional defect in the periphery. In line with this interpretation, the frequent finding of identical sequences showed that the repertoire diversity of IgG Ig receptors was more limited than that of IgM. This could either reflect limited helper T cell activity or, more likely, indicate a role for LigIV in class switch recombination. An altered pattern of recombination at the Sμ-Sα switch junctions has recently been observed in DNA obtained from patients 2303/4 and 411BR (44). The absence of primary and secondary lymph node follicles in P2 was also consistent with a role for LigIV in class switch recombination. The cytopenia in our patients was not due to ineffective hematopoiesis (data not shown) but was caused by autoantibodies with peripheral destruction. It remains unclear to what extent autoimmunity contributed to the cytopenia observed in the previously reported LigIV-deficient patients.

A predominance of γδ T cells and Ab-mediated autoimmunity despite minimal B cell and severely reduced αβ T cell numbers has recently been reported as a characteristic phenotype of patients with a SCID variant due to hypomorphic RAG mutations and CMV infection (26, 45). This report describes another molecular cause for this phenotype. Neither of our patients showed evidence of CMV infection. It is conceivable that EBV infection could have a similar effect on the T cell repertoire. However, the predominance of γδ T cells was also observed in the “naïve” younger sibling. As argued previously (26), it is therefore plausible to assume that limited V(D)J recombination activity may lead to a developmental advantage for γδ T cells. Under conditions of αβ T lymphopenia, herpes viruses infections may then additionally amplify these cells in the periphery.


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Disclosures
The authors have no financial conflict of interest.

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