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Impaired lymphocyte development and antibody class switching and increased malignancy in a murine model of DNA ligase IV syndrome

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Nonstandard abbreviations used: CCG, chicken γ globulin; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSB, DNA double-strand break; ENU, ethylnitrosourea; HEL, hen egg lysozyme; IgH, Ig heavy-chain locus; LigIV, DNA ligase IV; MEF, mouse embryonic fibroblast; NHEJ, nonhomologous end-joining; NP, 4-hydroxy-3-nitrophenylacetyl aminoethylcarboxymethyl; TC, Tricolor; VHL, variable heavy light; XLF, XRCC4-like factor.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Hypomorphic mutations in DNA ligase IV (LIG4) cause a human syndrome of immunodeficiency, radiosensitivity, and growth retardation due to defective DNA repair by the nonhomologous end-joining (NHEJ) pathway. Lig4-null mice are embryonic lethal, and better mouse models are needed to study human LigIV syndrome. We recently identified a viable mouse strain with a Y288C hypomorphic mutation in the Lig4 gene. Lig4Y288C mice exhibit a greater than 10-fold reduction of LigIV activity in vivo and recapitulate the immunodeficiency and growth retardation seen in human patients. Here, we have demonstrated that the Lig4Y288C mutation leads to multiple defects in lymphocyte development and function, including impaired V(D)J recombination, peripheral lymphocyte survival and proliferation, and B cell class switch recombination. We also highlight a high incidence of thymic tumors in the Lig4Y288C mice, suggesting that wild-type LigIV protects against malignant transformation. These findings provide explanations for the complex lymphoid phenotype of human LigIV syndrome.

Introduction

Nonhomologous end joining (NHEJ) is one of the main pathways for repair of DNA double-strand breaks (DSBs) (1). The pathway comprises 6 core components: Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRC4, DNA ligase IV (LigIV) (1), and the recently discovered XRCC4-like factor (XLF) (2, 3). The end-joining process starts with the binding of Ku70/ Ku80 heterodimers to the DNA ends, followed by DNA-PKcs and other enzymes involved in end processing. LigIV is subsequently recruited to the DNA ends in complex with XRCC4 and, together with XLF, catalyzes the final end-joining reaction.

At least 10 hypomorphic mutations in the Lig4 gene have been identified in the human population, and in homozygous or compound heterozygous states, they cause a condition known as LigIV syndrome (4–11) (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI32743DS1). The clinical presentation of the syndrome is complex and heterogeneous and may include radiosensitivity, varying degrees of lymphopenia, growth retardation, and microcephaly (4–12). At the molecular level, all the features of the LigIV syndrome are thought to arise from impaired DNA DSB repair, and the complexity of the syndrome highlights the functions of the NHEJ pathway in many physiological processes.

The embryonic lethality of the Lig4-null mouse strains (13, 14) limits their utility as models of LigIV syndrome. Other NHEJ-deficient strains – Ku70, Ku80, and DNA-PKcs–null mice – are viable and share some of the phenotypic features of LigIV syndrome (15–19). However, the essential nature of Lig4 but not Ku70, Ku80, or DNA-PKcs attests to differing impact of these genes. Further, at the molecular level, the impact of these alleles is not equivalent to that of Lig4-null or –mutant alleles. For example, the functions of Ku70, Ku80, and DNA-PKcs are not limited to DNA DSB repair, and all 3 proteins are also required for normal telomere maintenance (20, 21). Additionally, residual DSB rejoining can occur in the absence of DNA-PKcs, and to a lesser extent Ku, in G0 phase cells despite being LigIV dependent (19, 22). Furthermore, and possibly underlying the differences in DSB rejoining, the “accessibility” of DNA ends to alternative repair pathways is believed to differ in cells deficient in different NHEJ components (23, 24). The differences between Ku and LigIV deficiencies are further highlighted by the surprising observation that a loss of Ku can partly rescue the lethality of the Lig4-null strain (23). Thus, at least at the molecular level, some features of the previously characterized NHEJ-null mouse strains are distinct from those of the Lig4-null mouse and the human LigIV syndrome, suggesting that a LigIV-mutant mouse strain is a more appropriate model for human LigIV syndrome than the existing NHEJ-deficient lines.

DNA DSBs can form as the result of random DNA damage and also during programmed DNA rearrangement events, namely V(D)J recombination and Ig class switching (25, 26). The phenotypic effects of impaired V(D)J rearrangement are widely known and account for a complete arrest in lymphocyte development in Lig4-null (13) and other NHEJ-null mice (15–19) and most likely
also for the lymphopenia in LigIV syndrome (4, 6–8). The phenotypic effects of the impaired repair of nonprogrammed DNA damage are more diverse and difficult to study. They include the increased apoptosis in the developing brain causing the lethality of the Lig4-null mice (13, 14), the progressive decline in hematopoietic stem cells in the Lig4Y288C strain (27), and the impaired ex vivo lymphocyte proliferation (28, 29) and increased accumulation of chromosome breaks in the lymphocytes in LigIV syndrome (4). Although such defects in cell survival and proliferation are likely to have an impact on the immune system, their contribution to the immunodeficiency of the LigIV syndrome remains unknown.

Another physiological source of DNA DSBs is immunoglobulin class switching (30, 31), which is a somatic rearrangement of the Ig heavy-chain (IgH) locus, occurring in B cells during the immune response and resulting in a switch in the antibody isotype. The DNA rearrangement is initiated by activation-induced deaminase, and the uracil lesions introduced by activation-induced deaminase into the switch-region DNA are processed to DSBs. Overwhelming evidence indicates that NHEJ has a role in the rejoining of the breaks, including evidence from the earlier studies of Ku- and DNA-PKcs-null mice (28, 29, 32–34) and the recent work on the conditional XRCC4 knockout, Lig4Y288C, and XLF-null mouse

Figure 1
Partial arrest in lymphocyte development in Lig4Y288C strain. (A) Flow cytometry profiles of the thymus of WT, Rag1−/−, and Lig4Y288C mice stained for CD4, CD8, CD44, and CD25 and gated on total thymocytes (upper panels) or CD4−CD8− double-negative thymocytes (lower panels). Numbers represent the percentages of cells in the plot that fall within the different regions, corresponding to different stages of thymocyte differentiation. (B) Absolute number of CD4−CD8− double-negative (DN), CD4+CD8+ double-positive (DP), and CD4 and CD8 single-positive cells in the thymus of WT, Rag1−/−, and Lig4Y288C mice. Bars represent mean ± 95% CI; n ≥ 6. (C) Flow cytometry of the bone marrow of WT, Rag1−/−, and Lig4Y288C mice stained for B220, IgM, IgD (upper panels), or for B220 and CD43 (lower panels) and gated on B220+ cells or total lymphocytes, respectively. Numbers represent the percentages of cells in the plot that fall within the different regions, corresponding to different stages of B cell differentiation. (D) Absolute numbers of B220+CD43− pro–B, B220+CD25− pre–B, and B220+IgM−IgD− immature B cells in the bone marrow of WT, Rag1−/−, and Lig4Y288C (1 tibia and femur). Bars represent mean ± 95% CI; n ≥ 5.
The recent studies indicate that, although NHEJ is involved, an alternative microhomology-mediated repair pathway can to some extent compensate, with residual levels of switching in the NHEJ-null B cells being as high as 25%–50% of the WT level. The alternative pathway was also implicated in class switching in the LigIV syndrome patients, based on the analysis of the structures of switch junctions (39). However, the quantitative impact of hypomorphic LigIV mutations on switching remains unknown.

Aberrant repair of DNA DSBs arising through any of the mechanisms described above could lead to chromosomal translocations, transformations, and cancer. Previous studies in mice indicate that the loss of NHEJ on its own results in only a mild predisposition to cancer in some strains (40–42). However, a combined loss of NHEJ and DNA damage cell-cycle checkpoints leads to a dramatic increase in lymphoid malignancies, as seen in the NHEJ-deficient p53−/− mouse strains (43–45). In these mice, the tumors arise due to aberrant resolution of DNA DSBs during V(D)J recombination and carry translocations involving IgH and c-myc loci. Lymphomas were also reported in some LigIV syndrome patients (5, 8, 9, 11), although their frequency and the mechanism of carcinogenesis are difficult to ascertain. They may have arisen as a direct result of impaired DNA repair and genomic instability or as a secondary effect of immunodeficiency (8, 9). Furthermore, there may be significant bias in our estimates of lymphoma predisposition in this syndrome as, on the one hand, patients with severe forms of the disease commonly undergo bone marrow transplantation (7, 8, 10), while on the other hand, some patients with milder forms of the condition are diagnosed with LigIV syndrome only following adverse responses to radio- or chemotherapy (5, 11).

We recently identified an ethylnitrosourea-induced (ENU-induced) hypomorphic mutation in the Lig4 gene in a mouse strain that displays the common features of the LigIV syndrome, including lymphopenia and growth retardation (27). The in vivo LigIV activity in the Lig4Y288C mice is reduced over 10-fold, similar to many of the LigIV syndrome patients (4, 46). One of the effects of the Lig4Y288C mutation is a progressive attrition of hematopoietic stem cells in aged mice, although this is not limiting in young animals (27). Here, we provide a detailed characterization of the effects of the Lig4Y288C mutation on the immune system: describing a severe defect in lymphocyte development, a partial arrest in B cell class switching, and a defect in the survival of long-lived lymphocyte populations. Antibody responses to T cell–dependent and –independent antigens are dramatically impaired. We also describe the unexpectedly high incidence of thymic malignancies in the Lig4Y288C mice and show that these can arise from aberrant resolution of V(D)J breaks leading to translocation of the T cell receptor locus. These findings provide important insights into the underlying basis of immune pathology of LigIV syndrome.

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total nucleated cells (%)</th>
<th>Blood</th>
<th>Spleen</th>
<th>Mesenteric lymph nodes</th>
<th>Peritoneum</th>
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<td></td>
<td>B cell</td>
<td>CD4+</td>
<td>CD8+</td>
<td>B cell</td>
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<tr>
<td>WT</td>
<td>31 ± 5</td>
<td>12 ± 2</td>
<td>5.0 ± 0.8</td>
<td>754 ± 148</td>
<td>141 ± 42</td>
</tr>
<tr>
<td>Lig4Y288C</td>
<td>2.9 ± 0.7</td>
<td>1.7 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>16 ± 11</td>
<td>10 ± 3</td>
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Numbers indicate means ± 95% CI; n ≥ 9. FACS plots representing B cell and CD4+ T cell populations in the blood previously published in ref. 27.

Results

Partial arrest in lymphocyte development in the Lig4Y288C strain. The Lig4Y288C mouse strain was identified in an ENU mutagenesis program as a recessive C57BL/6 line with growth retardation and peripheral blood lymphopenia (27). The mice carry a Y288C substitution in the catalytic domain of LigIV, which reduces in vivo LigIV activity at least 10-fold and severely impairs the repair of DNA breaks.

Figure 2

Impaired humoral immunity in Lig4Y288C mice. (A) Serum antibody isotype levels in naive WT and Lig4Y288C mice. Data points and bars represent the individual mice and the means of each dataset, respectively. (B) Antigen-specific antibody responses to T cell–independent (NP-Ficoll) and T cell–dependent (CGG) antigens in WT and Lig4Y288C mice (analyzed by ELISA, optical density plotted against the serum dilution factor). Error bars represent mean ± SEM. **P < 0.01.
The effects of the mutation at the molecular and cellular levels closely resemble those in human LigIV syndrome patients (4, 27).

We assessed the impact of the mutation on lymphocyte development by comparing primary lymphoid organs of Lig4−/− mice with those of age-matched WT and Rag1−/− mice, all on a C57BL/6 background. This revealed a severe but incomplete arrest in lymphocyte development in the Lig4−/− strain (Figure 1). The total thymic cell count was 100-fold lower than in the WT and comparable to that in the Rag1−/− mice. The loss of thymocytes increased progressively at each developmental transition, and the proportion of thymocytes at the CD25−CD4 CD8− stage preceding the onset of TCRβ rearrangement was increased, consistent with impaired V(D)J recombination (Lig4−/− = 48% ± 1% and WT = 26% ± 6% of double-negative thymocytes, mean and 95% CI; Figure 1, A and B).

The absolute numbers of pro–B (B220−CD43−), pre–B (B220−CD25−), and immature B cells (B220−IgM−IgD+) in the bone marrow of the Lig4−/− mice also showed progressive cell loss, and a larger proportion of B220− cells was arrested at the pro–B stage (Lig4−/− = 33% ± 11% and WT = 13% ± 4% of B220− bone marrow cells; Figure 1, C and D), consistent with impaired rearrangement of the IgH locus. Mature (B220+IgM+IgD−) B cells in the bone marrow could not be reliably detected but were found in low numbers in the periphery.

Lymphopenia and failure in antibody responses to challenge in the Lig4−/− mice. The Lig4−/− strain was originally identified based on the decreased frequency of recirculating lymphocytes (27), and the numbers of B and T cells in the blood of the Lig4−/− mice were severely reduced (Table 1). Surprisingly, this was associated with elevated serum IgM (Figure 2A), which resembles some features of hyper-IgM immunodeficiencies seen in other DNA repair syndromes, particularly those affecting B cell class switching (12). Elevated IgM levels were also reported in some patients with mutations in LigIV and XLF (2, 8). Serum IgG levels in the Lig4−/− mice were significantly reduced, whereas serum IgG1 and IgA were comparable to those in WT (Figure 2A).

The numbers of B and T cells in the secondary lymphoid organs, such as the spleen and mesenteric lymph nodes, were severely reduced (Table 1). CD4+ and CD8+ T cells were 10- to 25-fold down in numbers and stained positive for TCRβ (Figure 2B). Interestingly, most CD4+ T cells had an activated phenotype with increased expression of CD44 and reduced CD45RB (Supplemental Figure 1). Such T cell activation is frequently seen in other partial T cell immunodeficiency syndromes in humans and mouse models, for example, in the Omenn syndrome, in which a reduction in T cell numbers caused by hypomorphic mutations in the RAG1/2 genes affects T cell tolerance and activation, probably by reducing competition for limiting amounts of prosurvival cytokines or costimulatory molecules (reviewed in refs. 47, 48).

The absolute numbers of B cells in the secondary lymphoid organs were reduced nearly 50-fold (Table 1), with a large relative increase in the proportion of transitional B cells (B220+CD23−CD21+). The transitional B cell population constituted 86% ± 7% of B220+ splenocytes in the Lig4−/− mice compared with only 32%...
Partial defect in immunoglobulin class switching in Lig4<sup>y288C</sup> mice. (A) Anti-HEL antibody levels in the serum of WT-VHL (WT) and Lig4<sup>y288C</sup>-VHL (Lig4<sup>y288C</sup>) mice. Bars represent geometric means and 95% CI, n ≥ 3; comparisons by Mann-Whitney test, *P < 0.05. (B–D) Class switching of Lig4<sup>y288C</sup>-VHL and control WT-VHL B cells at day 4 of in vitro stimulation with LPS, LPS plus IL-4, and anti-CD40 plus IL-4. (B) CFSE fluorescence as a measure of the proliferation of Lig4<sup>y288C</sup>-VHL (black lines) and WT-VHL (gray lines) CD19<sup>+</sup> B cells, representative of 5 mice per group. (C) CD19<sup>+</sup> versus IgG flow cytometry profiles of WT-VHL and Lig4<sup>y288C</sup>-VHL splenocytes gated on the highly proliferated CFSE-low cells. Numbers indicate the percentages of class-switched B cells within each plot. (D) IgG<sup>+</sup> cells as a percentage of the highly proliferated CFSE-low CD19<sup>+</sup> B cells in Lig4<sup>y288C</sup>-VHL, WT-VHL, Lig4<sup>y288C</sup>-VHL (Lig4<sup>y288C</sup>), and Rag<sup>1<sup>−/−</sup></sup>-VHL cultures at day 4 of in vitro stimulation. Error bars represent means ± 95% CI; n = 6 for WT and Lig4<sup>y288C</sup>; n = 3 for Lig4<sup>het</sup> and Rag<sup>1<sup>−/−</sup></sup>. **P < 0.01; ***P < 0.001 using ANOVA.
Impaired peripheral lymphocyte survival contributes to immunodeficiency in Lig4<sup>Y288C</sup> mice. (A) Flow cytometry profiles of the spleen of Lig4<sup>Y288C-Rag1<sup>−/−</sup></sup> and Lig4<sup>Y288C-Rag1<sup>−/−</sup></sup> mice stained for B220, CD21, and CD23 and gated on B220<sup>+</sup> cells. Numbers indicate the percentages of splenic B cells within the transitional CD21<sup>−</sup>-CD23<sup>−</sup>, follicular CD21<sup>−</sup>-CD23<sup>+</sup>, and marginal zone CD21<sup>−</sup>-CD23<sup>−</sup> gates. (B) Absolute number of B cells in the bone marrow, mesenteric lymph nodes (MLN), and spleen of Lig4<sup>Y288C-Rag1<sup>−/−</sup></sup> (WT) and Lig4<sup>Y288C-Rag1<sup>−/−</sup></sup> mice. Data points and bars represent individual mice and the mean of each dataset, respectively. Bone marrow immature B cells were gated as B220<sup>+</sup>IgM<sup>+</sup>HLA<sup>+</sup> and mature B cells as B220<sup>+</sup>IgM<sup>+</sup>HLA<sup>−</sup> splenic transitional B cells were gated as B220<sup>−</sup>CD21<sup>−</sup>-CD23<sup>−</sup>, follicular as B220<sup>−</sup>CD21<sup>−</sup>-CD23<sup>+</sup>, and marginal zone as B220<sup>−</sup>CD21<sup>−</sup>CD23<sup>+</sup>; B cells in the MLN were gated as B220<sup>+</sup>IgM<sup>+</sup>IgD<sup>+</sup>. *P < 0.05, 2-tailed Student’s t test.

To analyze the mechanisms that lead to carcinogenesis, we studied 1 of the thymic tumors for chromosome translocations involving TCR loci by looking for evidence of separation of red and green FISH probe pairs derived from BACs flanking the TCRα/β, -γ, and -δ loci. In this way, we found chromosomal separation of 54% (216/398) of TCRα/δ probes in the tumor cells, indicative of a translocation (Figure 3A). This observation suggests that thymic tumors in the LigIV syndrome can arise from aberrant resolution of DNA breaks at the TCR locus, probably during V(DJ) recombination.

The role of LigIV in B cell receptor class switching. To assess the impact of the Lig4<sup>Y288C</sup> mutation on B cell class switching, we crossed the following B cell receptor transgenes onto the Lig4<sup>Y288C</sup> line to generate Lig4<sup>Y288C-VHL</sup> (where VHL indicates variable heavy light) mice: an anti-<i>γ</i>-<i>δ</i> chain transgene and an "<i>γ</i>-<i>δ</i> knockin" that is capable of switching. To increase litter sizes, the cross was on a mixed C57BL/6 background and age-matched littersmates were used as controls in all the comparisons to minimize any differences in genetic background between the groups. In VHL mice, the vast majority of B cells showed specificity for HEL, and the VH transgene resulted in a partial rescue of B cell proliferation (33, 36, 50, 51). Specifically, cell proliferation in the Lig4<sup>Y288C</sup> strain was assessed and controlled for using CFSE labeling.
Discussion
The hypomorphic mouse strain Lig4<sup>Y288C</sup> generated in an ENU mutagenesis program is, we believe, the first viable animal model of the LigIV syndrome. Like LigIV syndrome patients, the Lig4<sup>Y288C</sup> mice show an over 10-fold reduction in LigIV activity and similar general phenotypic features of growth retardation and lymphopenia. In this study, we provide a detailed analysis of the effects of the hypomorphic LigIV deficiency on the mammalian immune system. This demonstrates how the generation of point mutations by ENU mutagenesis mimics naturally occurring hypomorphic mutations and allows an investigation of gene functions that are masked by embryonic lethality in gene-knockout lines.

We report profound immunodeficiency in Lig4<sup>Y288C</sup> mice, with primary and secondary lymphoid organ lymphopenia. Surprisingly, IgG, and IgA (but not IgG<sub>1</sub>) antibody isotypes in Lig4<sup>Y288C</sup> serum are maintained at normal levels, despite the very low numbers of peripheral B cells and impaired Ig class switching, which suggest that compensatory mechanisms must be present. These might operate at the level of B cell selection, T cell activation, competition for other B cell and plasma cell survival factors, or antibody clearance. When immunized, the Lig<sup>Y288C</sup> mice show an almost complete loss of antigen-specific antibody responses to T cell–dependent and T cell–independent antigens. This resembles features of the human LigIV syndrome (6, 8) and highlights the dramatic loss of immune system function caused by the LigIV mutations. We further demonstrate that, in addition to the well-characterized arrest in V(D)J rearrangement, impaired survival of long-lived lymphocyte populations also contributes to immunodeficiency. By creating a system in which Lig4<sup>Y288C</sup> B cells develop without undergoing V(D)J rearrangement or class switching and controlling for T cell numbers, we demonstrate the impact of background DNA damage on these long-lived lymphocyte populations and the role of NHEJ in their maintenance. We also report overall activation of peripheral T cells, retention of B1 cells, and some increase in IgM autoantibodies in the Lig4<sup>Y288C</sup> mice. These phenotypes have not been previously assessed in any of the LigIV syndrome patients; however, autoimmune thrombocytopenia was seen in at least 1 patient and also in patients with mutations in XLF (2, 8), suggesting that IgM autoantibodies may be relevant in NHEJ deficiency syndromes.

We show impaired class switching in Lig4<sup>Y288C</sup> B cells, demonstrating the involvement of LigIV and the NHEJ pathway in the class switching reaction. This confirms the recent conclusions of Yan et al. from studies of Lig4<sup>Y288C</sup> B cells (35), in this case using LigIV-deficient B cells without additional defects in DNA damage response pathways. Our experimental system directly assesses switching at the cellular level and shows that Lig4<sup>Y288C</sup> B cells are defective in class switching to 2 different antibody isotypes with 3 different stimuli, and this is independent of differences in B cell proliferation, B cell receptor specificity, or T cell help. Some switching in Lig4<sup>Y288C</sup> is probably due to the residual activity of LigIV, with possible contributions of alternative repair pathways (35, 39). However, despite the contribution of alternative repair pathways to class switching (35, 39), our findings show that hypomorphic
mutations in LigIV can significantly impair B cell class switching and contribute to the immunodeficiency of LigIV syndrome. We further report an unexpectedly high incidence of CD4+ CD8+ thymic tumors in Lig4<sup>Y288C</sup> mice, suggesting that LigIV deficiency predisposes to malignancies. The Lig4<sup>Y288C</sup> tumors are associated with chromosomal translocations at the TCRα/β locus, probably caused by aberrant repair of RAG-induced DNA breaks. The strong predisposition to cancer in Lig4<sup>Y288C</sup> mice suggests that the mutations that similarly impair LigIV activity in humans will also predispose to cancer and that T cell leukemias reported in some LigIV syndrome patients may arise through similar mechanisms (5, 11). This conclusion is of clinical interest, as the DNA repair deficiency is associated with adverse response to radio- and chemotherapy in this group of cancer patients (5, 11). It also further highlights the possibility that small alterations in LigIV activity in heterozygous carriers of Lig4 mutations may predispose to cancer, as suggested by the fact that Lig4 haploinsufficiency increases the frequency of soft-tissue sarcomas in INK4a/ARF-null mice (52) and by the association of a human Lig4 polymorphism with increased risk of lung carcinomas (53).

The high cancer susceptibility of the Lig4<sup>Y288C</sup> line compared with other NHEJ-deficient mouse strains is surprising, as it is widely believed that defects in NHEJ repair lead to carcinogenesis only when combined with loss of cell-cycle checkpoints, as in the NHEJ-deficient p53<sup>−/−</sup> strains (42–45). Although Ku70<sup>−/−</sup> mice were reported to develop CD4+ CD8<sup>−</sup> lymphomas similar to those of Lig4<sup>Y288C</sup> mice (54), this was disputed in recent reports arguing that both Ku70<sup>−/−</sup> and Ku80<sup>−/−</sup> strains are not tumor prone (40, 55). Furthermore, Lig4<sup>−/−</sup>, p53<sup>−/−</sup>, and RAG<sup>−/−</sup> mice with a defect in DNA damage apoptosis induction but normal cell-cycle arrest are tumor free (56). In contrast, we observed high rates of leukemia in Lig4<sup>Y288C</sup> mice despite normal G2/M checkpoint arrest and normal p53 activation, and the reason for increased cancer predisposition in Lig4<sup>Y288C</sup> compared with other NHEJ-null mouse strains is unknown. We can speculate that because the tumors arise from the CD4+ CD8<sup>−</sup> population, progression of some thymocytes to the CD4<sup>−</sup>CD8<sup>−</sup> stage may be a prerequisite for carcinogenesis. Thus, the increased incidence of malignancies in the Lig4<sup>Y288C</sup> mice may be the result of the hypomorphic nature of the phenotype that allows some “leaky” T cell development to take place, as is also seen in the tumor-prone and “leaky” Ku70<sup>−/−</sup> line (54). It is also possible to speculate that the accessibility of DNA breaks to alternative error-prone repair pathways may differ between the different NHEJ-deficient mouse lines, leading to increased predisposition to aberrant repair and translocations in some of the lines, such as Lig4<sup>Y288C</sup>. Although many questions remain unanswered, our findings with Lig4<sup>Y288C</sup> mice provide definitive evidence that hypomorphic LigIV deficiencies can predispose to leukemia.

In summary, access to the ENU-induced hypomorphic Lig4<sup>Y288C</sup> mouse strain has made it possible to characterize the effects of LigIV deficiency on the mammalian immune system. Arrest in V(D)J rearrangement is the primary cause of lymphopenia, with impaired cell survival and proliferation due to random DNA damage making additional contributions to the loss of peripheral lymphocytes. We confirm the role of LigIV in normal B cell class switching and suggest that class switching deficiencies contribute to the impaired immune function in LigIV syndrome. We also show that a hypomorphic LigIV mutation can confer strong predisposition to lymphoid malignancies. The study reveals multiple NHEJ-dependent processes in immune system function and helps to explain the complex and multilayered immunodeficiency of the human LigIV syndrome.

Methods

Mouse strains. The Lig4<sup>Y288C</sup> mouse strain carries a hypomorphic Y288C mutation in the Lig4 gene on the C57BL/6 strain background (27). The C57BL/6 MD4 strain carries an anti-HEL immunoglobulin transgene (Ig<sup>HEL</sup>) encoding the heavy and light chains of IgM<sup>+</sup> and IgD<sup>+</sup> antibody isotypes, originally derived from the HyHEL10 hybridoma (57). The VHL strain has the same HyHEL10 antibody specificity, comprising an anti-HEL κ light chain transgene and a VDJ segment targeted to the heavy-chain locus and expressed with endogenous constant region exons (both gifts from Jason Cyster, UCSF, San Francisco, California, USA). C57BL/6 Rag1<sup>−/−</sup> mice were previously described (58). All mice were fewer than 14 weeks old and were maintained under specific pathogen-free conditions. All the experiments were approved by the Australian National University Animal Ethics and Experimentation Committee and the Oxford University Ethical Review Committee and were conducted under a United Kingdom Home Office license.

Flow cytometry. FITC-conjugated antibodies were against the following: CD21/CD35 (7G6), CD24 (HS, M1/69), IgD<sup>+</sup> (AMS9.1), IgM<sup>+</sup> (AF6-72) (BD Biosciences — Pharmingen); and B220, CD8α, CD11b (MAC1, M1/70.15), and CD44 (CALTAG; Invitrogen). PE-conjugated antibodies were against the following: CD3 (FcrRIII, B3B4), CD43 (Ly-1, 53-7.3), IgM<sup>+</sup> (DS-1), IgD<sup>+</sup> (217-170) (BD Biosciences — Pharmingen); and CD25 (PC6-1.53) and CD4 (CALTAG; Invitrogen). APC-conjugated antibodies were against the following: IgM (II/41) (BD Biosciences — Pharmingen) and B220 (RA3-6B2) and CD4 (CALTAG; Invitrogen). Tricolor-conjugated (TC-conjugated) antibody CD8<sup>+</sup> (16A) (CALTAG; Invitrogen) and biotin-conjugated anti-CD9 (KM58) (BD Biosciences — Pharmingen) followed by streptavidin-TC (CALTAG; Invitrogen) were also used. The data were collected on FACSCalibur or FACSCount (BD).

Mouse immunizations. Mice were immunized intraperitoneally with 50 µg of alum-precipitated CGG (Jackson ImmunoResearch Laboratories Inc.) or 25 µg NP-Ficoll (4-hydroxy-3-nitrophenylacetyl aminooethylcarboxymethyl—Ficoll; Biosearch Technologies) and bled from the tail vein on days 14 or 6 after immunization, respectively. Four weeks after the first immunization, mice were boosted with a secondary immunization of CCG and serum collected after 7 days.

Measurement of serum antibody titers. Serum antibody titers were measured by ELISA in 96-well plates (Greiner Bio-One) coated with 10 µg/ml HEL (Sigma-Aldrich), 5 µg/ml CGG, 20 µg/ml NP-BSA (Biosearch Technologies), or 2 µg/ml of antibodies for mouse IgM (II/41), IgA (C10-3), IgG1 (AS5-3), IgG2a (RII-89), or IgG1 (R2-38) (all from BD Biosciences — Pharmingen) in carbonate buffer, pH 9.8. The plates were blocked with 1% BSA (Sigma-Aldrich) in PBS. Detection antibodies were used the biotinylated anti-mouse IgM<sup>+</sup> (DS-1), IgA (C10-1), IgG1 (AS5-1), IgG2a (R2-40), and IgG1 (R40-82) (all from BD Biosciences — Pharmingen) followed by an avidin-alkaline phosphatase (AP) conjugate (Sigma-Aldrich). Alternatively, AP-conjugated goat anti-mouse polyclonal antibodies for IgM (Sigma-Aldrich), IgA, IgG1, IgG2a, and IgG1 (SouthernBiotech) were used. Plates were washed in PBS with 0.05% Tween-20, developed with 1 mg/ml Sigma-Alpha-2-naphthylphosphate (AP) conjugate (Sigma-Aldrich). The plates were run on a Bio-Rad 550 plate reader. Serum antibody concentration was determined by comparison with either a standard of known concentration or appropriate purified mouse isotype controls (BD Biosciences — Pharmingen).

Fluorescence in situ hybridization. BAC probes (~100 kb in length) flanking the TCRα/β (RP24-336119 and RP23-9-18), TCRβ (RP24-164811 and RP23-97020), and TCRγ loci (RP23-97G21 and RP24-34719) were obtained.
In vitro assays to assess class switching while controlling for differences in cell proliferation were performed as previously described (33, 36, 50). Splenocytes from Vhl transgenic WT and Liggf2/c2 mice were analyzed on a mixed C57BL/6×NOD background were labeled with CFSE using Vybrant Kit (Molecular Probes; Invitrogen). Briefly, the cells were incubated at 2 × 10^5 cells/ml in 5 μM CFSE in PBS for 10 minutes at 37°C and washed 3 times in RPMI 1640 (Sigma-Aldrich). The cells were then cultured at 6.5 × 10^5 cells/ml in RPMI 1640 supplemented with 10% FCS (First Link [UK] Ltd.), 2 mM L-glutamine (Sigma-Aldrich), 10 mM HEPES, pH 7.4 (Gibco; Invitrogen), 100 μg/ml streptomycin, 100 U/ml penicillin (Sigma-Aldrich), and 50 μg/ml β-mercaptoethanol (Gibco; Invitrogen) at 37°C and 5% CO2. Some of the cultures were stimulated with 5 μg/ml of LPS (Sigma-Aldrich), or 5 μg/ml anti-mouse CD40 antibody (HM40-3; BD Biosciences — Pharmingen), with or without 5 ng/ml IL-4 (Sigma-Aldrich). At day 4, the cells were collected and stained with a biotin-conjugated anti-mouse CD19 antibody (1D3; BD Biosciences — Pharmingen) followed by polyclonal PE-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, IgG3 (SouthernBiotech), and streptavidin-TC (CALTAG; Invitrogen).

Checkpoint assays. p53 activation was used to assess G1/S checkpoint arrest, and changes in mitotic index after irradiation were used to monitor G2/M progression. For p53 analysis, primary MEFs were grown on glass cover slips to approximately 60% confluency, irradiated with 10 Gy, rested for 4 hours, fixed in 3% paraformaldehyde with 2% sucrose in PBS for 10 minutes, permeabilized with 0.2% Triton X-100 in PBS for 2.5 minutes, and washed in PBS. Cells were stained with anti–phospho-p53 antibody (Cell Signaling Technology) at 1:100 in PBS with 2% BSA for 30 minutes at 37°C. Following PBS washing, secondary antibody incubation was with Cy3 anti-rabbit IgG1 at 1:200 (Sigma-Aldrich). For G2/M analysis, transformed MEFs were grown on glass cover slips to approximately 60% confluency, irradiated with 1–5 Gy; rested for 2 hours, and fixed, permeabilized, and washed as above. All samples were stained with 4′,6-diamidino-2-phenyindole (Sigma-Aldrich) for 10 minutes at room temperature to reveal the nuclei. Cover slips were mounted in VECTASHIELD (Vector Laboratories) and cells scored under fluorescence microscope using x100 objective, counting approximately 200–500 cells per sample.

Brdu incorporation. BrdU (Sigma-Aldrich) was supplied at 0.25 mg/ml in drinking water supplemented with 1% glucose (Sigma-Aldrich) for 1 week. Cells were fixed in 0.5% paraformaldehyde in PBS and permeabilized in fresh 3 M HCl with 0.5% Tween-20, both incubations for 20 minutes at room temperature. The acid was neutralized with 0.1 M borax (Sigma-Aldrich) and the cells washed and stained with FITC-conjugated anti-BrdU antibody (BDI, BD Biosciences — Pharmingen).

Screen of mouse serum for autoantibodies. Screen for autoantibodies was performed using HEP-2000 ANA-Ro Test System slides (ImmunoConcepts; Alpha Laboratories) according to the manufacturer’s protocol.

Statistics. Statistical comparisons were performed with Prism 4.0 software (GraphPad Software) using 2-tailed Student’s t test for comparisons of 2 data sets and ANOVA for multiple comparisons. P ≤ 0.05 was considered significant.

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