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Functional analysis of naturally occurring DCLRE1C mutations and correlation with the clinical phenotype of ARTEMIS deficiency

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Abstract

Background—The endonuclease ARTEMIS, encoded by the *DCLRE1C* gene, is a component of the non-homologous end-joining (NHEJ) pathway, and participates in hairpin opening during the V(D)J recombination process and repair of a subset of DNA double strand breaks. Patients with ARTEMIS deficiency usually present with severe combined immunodeficiency (SCID) and cellular radiosensitivity, but hypomorphic mutations may cause milder phenotypes (leaky SCID).

Objective—We sought to correlate the functional impact of human *DCLRE1C* mutations on phenotypic presentation in patients with ARTEMIS deficiency.

Methods—We studied recombination and DNA repair activity of 41 human *DCLRE1C* mutations in *Dclre1c*$^{-/-}$ v-abl kinase transformed pro-B cells retrovirally engineered with a

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construct that allows quantification of recombination activity by flow-cytometry. For assessment of DNA repair efficacy, resolution of γH2AX accumulation was studied after ionizing radiation.

Results—Low or absent activity was detected for mutations causing a typical SCID phenotype. Most of leaky SCID patients were compound heterozygous for one loss of function (LOF) and one hypomorphic allele with significant residual levels of recombination and DNA repair activity. Deletions disrupting the C-terminus result in truncated, but partially functional proteins, and are often associated with leaky SCID. Overexpression of hypomorphic mutants may improve the functional defect.

Conclusions—Correlation between the nature and location of *DCLRE1C* mutations, functional activity, and the clinical phenotype, has been observed. Hypomorphic variants that have been reported in the general population may be disease-causing if combined in *trans* with a LOF allele. Therapeutic strategies aimed at inducing overexpression of hypomorphic alleles may be beneficial.

Keywords

V(D)J recombination; non-homologous end-joining; DNA repair; ARTEMIS deficiency; *DCLRE1C* mutations; severe combined immunodeficiency

Introduction

The endonuclease ARTEMIS, encoded by the gene *DCLRE1C* (NG_007276.1; ENSG00000152457), is an essential component of the V(D)J recombination machinery during T and B cell development, and plays an important role in the non-homologous end joining (NHEJ)-mediated DNA double-strand break (DSB) repair pathway [1].

The V(D)J recombination process is initiated by the recombination-activating gene products [2] RAG1 and RAG2, which bind to recombination signal sequences (RSS) adjacent to the coding V-, D-, and J-segments and induce a DNA double strand break, leaving hairpin structure at coding ends. Upon phosphorylation by the DNA protein kinase catalytic subunit (DNA-PKcs) complex, Artemis is recruited, and mediates hairpin opening via its endonuclease activity [3]. The open ends at the overhangs can be modified by ARTEMIS' endo- and exonuclease activities before the XRCC4/XLF/Ligase 4 complex seals the DNA strands. Defects in ARTEMIS result in aberrant hairpin opening and give rise to increased numbers of P-nucleotides in the coding joints [4]. In contrast to what observed in defects of other members of the NHEJ pathways, the blunt ends formed at the RSSs cleavage sites (yielding excision circles or inversions) rejoin normally in the absence of ARTEMIS.

The ARTEMIS protein is a member of the metallo-β-lactamase superfamily and is organized into an N-terminal region containing the β-lactamase homology domain, a central β-CASP domain, and a C-terminal region. The first two domains have critical catalytic function, whereas the C-terminus plays a role in protein stabilization and regulation of function [5, 6].

ARTEMIS is also involved in an ATM-dependent slow-kinetic NHEJ DNA repair pathway, where it is required to modify non-ligatable ends of DNA breaks before they can be rejoined [7, 8]. This type of breaks occur in a small fraction (around 10%) of DNA DSB after

exposure to ionizing irradiation and require more time for repair, resulting in delayed repair kinetics.

By affecting V(D)J recombination, mutations of the *DCLRE1C* gene impair T and B cell development, leading to T^-B^- severe combined immunodeficiency (SCID), associated with a mild form of cellular radiosensitivity [9].

The most common causes for *DCLRE1C* loss-of-function (LOF) alleles are large deletions affecting the first 4 exons, and a nonsense founder mutation which has been described in Athabascan-speaking Native Americans [10]. However, missense mutations affecting highly conserved residues can also abrogate protein function [11, 12]. On the other hand, hypomorphic mutations in *DCLRE1C* retaining residual function have been reported in patients with $B^{-/low}$ T^{low} "leaky" SCID or Omenn syndrome (OS)[13].

We have recently reported correlation between recombination activity of naturally occurring RAG1 mutant proteins and clinical and immunological phenotype associated with *RAG1* mutations [14]. In that study, RAG1 recombination activity was studied in murine *Rag1*$^{-/-}$ v-abl kinase transformed pro-B cell lines retrovirally engineered with a recombination construct and transduced with RAG1-expressing vectors, thus allowing GFP expression as a read-out of recombination proficiency. Using a similar approach, we have attempted to measure V(D)J recombination and DNA repair activity levels of 41 naturally occurring mutated *DCLRE1C* products caused by point mutations or small deletions.

Methods

Patient selection based on genotype

Clinical and molecular data from patients with ARTEMIS deficiency caused by missense mutations or small deletions in *DCLRE1C* were collected from previous reports, or were provided by physicians from Europe and the United States, according to protocols approved by local institutional review boards. Based on the clinical and immunological presentation, each patient was assigned to subgroups of T^-B^-SCID or a leaky/atypical presentation referred to as "leaky SCID", the latter including patients with OS, according to criteria defined by the Primary Immune Deficiency Treatment Consortium [15].

Determination of relative recombination activity levels associated with mutations in *DCLRE1C*

Analysis of recombination activity was performed in a cellular system of murine *Dclre1c*$^{-/-}$ Abelson murine leukemia virus (A-MuLV)-transformed pro-B cells (Fig. E1, A) [16], as previously described [1714]. Cells were retrovirally transduced to insert an inverted GFP cassette flanked by two RSS, and subjected to limiting dilution to isolate pro-B cells containing only one copy of such cassette (Fig. E1, B). Finally, in vitro mutagenized h*DCLRE1C* cDNAs were individually introduced into engineered *Dclre1c*$^{-/-}$ A-MuLV-pro-B cells using retroviral vectors. Efficient mutagenesis was confirmed by Sanger sequencing in constructs and transduced cells.

Targeting of the RSS contained in the recombination cassette allows GFP expression as a functional read-out of recombination activity that can be quantified by flow-cytometry upon gating on cells expressing both hCD4 and hCD2. For assessment of recombination and DNA repair activities, *Dclre1c*$^{-/-}$ abl pro-B cells were blocked in G0/G1 cell cycle phases for 72h by culture in 3μM of the v-abl kinase inhibitor imatinib (STI-571) (Novartis, Basel, Switzerland), which allows the Rag1/Rag2 complex to initiate the recombination process with higher efficiency (Fig. E2). Experiments were carried out in wild-type (WT), mock transduced cells and sets of up to 8 mutants at a time and were run in triplicates. Recombination activity levels were assessed as percentages of GFP expression detected in cells transduced with WT-*DCLRE1C*, after subtraction of background activity observed in mock-transduced cells.

Determination of DNA repair activity levels

NHEJ-mediated DNA repair was assessed in WT, mock-transduced and mutant-transduced cells at 1h, 8h, 24h, and 36h after ionizing radiation (IR) with 10Gy. Phosphorylation of the histone protein H2AX (γH2AX) was quantified by flow-cytometry (Phospho Histo H2A.X, (Ser139) (20E3), Cell Signaling, 5763S; Rabbit (DA1E) IgG XP isotype control, Cell Signaling, 5742). Since chromosomal replication is known to cause formation of γH2AX foci [18], also for this study cell cycles were blocked in G0/G1 for 72h. DNA repair efficiencies were calculated based on mean fluorescent intensities (MFI) at 36h after IR. For cells transduced with either WT or mutant constructs of *DCLRE1C*, the γH2AX MFI was subtracted from mock MFI, and activities were assessed as percentages of WT construct performance at 36h after exposure to 10Gy IR.

Southern Blotting

To assess the number of *DCLRE1C* vector integrations in transduced cells, 15μg genomic DNA extracted from *Dclre1c*$^{-/-}$ abl pro B cells were digested with EcoR1, HindIII, and NcoI (NEB, Ipswich, MA), run on TBE gels and blotted on a 0.45μm nylon membrane (Whatman Turbo Blotter Transfer System, Fisher Scientific, Waltham, MA). Membranes were hybridized with digoxigenin-labeled probes detecting h*CD4* and h*CD2* respectively, and signal detection was performed using an anti-dioxigenin antibody based system (Roche, Basel, Switzerland) following manufacturers instructions.

Quantitative real-time PCR

Quantitative PCR (qPCR) was performed using the ΔΔCT method, and normalized to WT gene expression. Additional information and primer sequences are listed in the online repository material.

Statistical analysis

Recombination and DNA repair activity levels of Artemis proteins were analyzed with the Mann-Whitney *U* test. Gene expression levels were compared using the unpaired two-tailed students *t* test, and $p \leq 0.05$ was considered significant. Correlation between recombination and DNA repair activity levels was assessed with the Pearson product-moment correlation coefficient (*r*), two-tailed, 95% confidence interval (CI).

Results

Recombination and DNA repair activity in ARTEMIS mutated proteins

We analyzed recombination and DNA repair activity levels for 22 missense and 6 nonsense mutations, 2 insertions (max. 8bp) and 11 small deletions (max. 17bp) detected in the *DCLRE1C* gene in a cohort of 40 ARTEMIS deficient patients (Table 1) [1, 3, 4, 9–11, 13, 19–28].

Chromosomal V(D)J recombination activity was tested in a cellular model previously described [14, 29]. V-abl kinase transformed murine *Dclre1c*$^{-/-}$ A-MuLV pro-B cells [16], from now on referred to as *Dclre1c*$^{-/-}$ abl pro-B cells, were engineered with a stable single integration of the pMX-INV GFP cassette flanked by two coding RSSs (Fig.1, A; Fig. E1, B), followed by transduction with a retroviral vector containing WT, mock or mutagenized h*DCLRE1C* cDNAs. Subsequently, GFP expression was analyzed as a read-out of recombination activity (Fig. 1, B) and γH2AX levels were assessed at 1h, 8h, 24h and 36h after IR with 10Gy to measure DNA repair activity (Fig. 1, C).

Due to alternative splicing, 16 h*DCLRE1C* transcript isoforms are known, where isoform a, b, and c encode for proteins with 692, 577 and 572 amino acids, respectively. In a preliminary set of experiments, we tested the functional activity of each of these products, by transducing *Dclre1c*$^{-/-}$ abl pro-B cells with *DCLRE1C* transcript isoforms a, b, and c (NM_001033855; NM_022487; NM_001033858). Only variant a (further referred to as WTa), which encodes for the 692aa canonical sequence, induced high levels of GFP expression, as did also the murine *Dclre1c* transcript isoform a (Fig. E3). Consequently, isoform WTa was used for mutagenesis experiments. Expression of *DCLRE1C* transcript was confirmed by qPCR (Fig. E4).

In contrast to what observed with *Rag1*$^{-/-}$ abl pro B-cells [14], minimal levels of GFP expression were observed in mock-transduced *Dclre1c*$^{-/-}$ abl pro-B cells (Fig. 1, B), indicating occurrence of construct rejoining in a small fraction of cells even in the absence of Artemis.

While DNA breaks are repaired over time, progressive de-phosphorylation of the H2AX histone occurs. Since ARTEMIS is only required for processing of a small fraction of DNA breaks repaired in a slow-kinetic pathway [7], differences in phosphorylated H2AX (γH2AX) expression in *Dclre1c*$^{-/-}$ abl pro-B cells expressing WT or mutant ARTEMIS constructs can be best appreciated at later time points, such as 24h–36h (Fig. 2).

Correlation between functional activity and clinical phenotype

ARTEMIS mutants were divided into two major subgroups: a) missense mutations and in frame deletion; and, b) frameshift mutations. When recombination and DNA repair activity were plotted against location of the individual mutations along the protein, significant variability of protein function was observed for missense/inframe mutations affecting the β-lactamase or the β-CASP domain, including missense mutations within the same codon (eg, T134A and T134R, or D165N and D165V) (Fig. 3, A and B; Table 2). Frameshift mutations disrupting these domains almost completely abrogated ARTEMIS function. By contrast, frameshift mutations disrupting the C-terminus consistently retained high activity levels

when expressed in *Dclre1c*$^{-/-}$ abl pro-B cells (Table 2), consistent with the less severe phenotype in patients harboring these mutations.

Although there was a trend for slightly higher DNA repair activities, particularly in the β-CASP domain, recombination activities correlated well with DNA repair activities ($r_{b\text{-}lact}$= 0.964, p<0.001; $r_{b\text{-}CASP}$= 0.818, p= 0.001; $r_{C\text{-}term}$= 0.968, p<0.001) (Fig. 3, C–E). This may reflect the notion that ARTEMIS plays a more critical role in V(D)J recombination than in NHEJ-mediated DNA repair.

To correlate recombination and DNA repair activity levels of mutated ARTEMIS proteins with the clinical presentation, patients were divided into two cohorts: a) typical SCID: and, b) milder, atypical presentation (leaky SCID). Definition of SCID was done in accordance to PIDTC criteria [15], which included T cell count below 300/μl and/or presence of maternal T cell engraftment. In cases where this information was not available, we assumed the interpretation of the authors of the original report.

As expected, most of the patients in the SCID group presented with severe and opportunistic infections early in life; by contrast, patients with leaky SCID often developed autoimmune cytopenias (6/15), other autoimmune manifestations (2/15), lymphoproliferation (5/15), or malignancies in the form of lymphoma (4/15) or squamous cell carcinoma (1/15) (Table 1). Two patients in this group had mutations affecting the *DCLRE1C* translation initiation site, one presenting with OS, the other one with leaky SCID, but without skin manifestations.

To attempt correlation with the clinical phenotype, activity levels of the mutated proteins were analyzed in patients with SCID vs. leaky SCID. For patients with compound heterozygous mutations, we considered the mutant with the higher activity level. As shown in Fig. 4, A and B, both recombination and DNA repair activity levels were significantly higher for mutations observed in patients with leaky SCID (p≤ 0.001). However, while robust levels of protein function were uniformly recorded for mutations associated with leaky SCID, significant variability was observed for mutations associated with SCID. Importantly, patients with leaky SCID were either homozygous for frameshift mutations affecting the C-terminus and that retained residual activity, or were compound heterozygous for a hypomorphic mutation (or a putative polymorphism) and a LOF allele (Fig. 4C).

Correlation of predicted pathogenicity with functional activity

To predict the pathogenicity of the *DCLRE1C* mutations analyzed, we used the online bioinformatics tools PolyPhen-2[30], and SIFT[31] (Table E1).

All, but three genetic variants (G153R, T71P and P171R) were predicted as disease causing by all tools. The variant P171R is a known polymorphism and has been reported before [24]. All three variants showed high activity levels in our study.

To investigate the frequency of putatively damaging *DCLRE1C* genetic variants in the general population, we analyzed data of the Exome Aggregation Consortium (ExAC) of the Broad Institute Cambridge, MA (URL: http://exac.broadinstitute.org) [12/2014], that includes whole exome sequencing data from over 60,000 individuals. Most of the mutations

reported in patients with SCID or leaky SCID and analyzed in this manuscript were not reported in the ExAC database. However, two of them (G153R and P171R) were reported at rather high frequency (0.01 and 0.09). Furthermore, homozygosity for these variants was demonstrated in a considerable number of individuals (12 and 612, respectively) in the ExAC database (Table E1). The fully normal VDJ activity of these variants is consistent with their polymorphism status.

The mutations M1T and M1V, which had been identified in compound heterozygous patients presenting with OS and leaky SCID ([13] and unpublished), were predicted by Mutation T@ster [32] to use an alternative translation initiation site 7 amino acids downstream. We observed high activity levels for both mutants in our system suggesting residual function.

Protein overexpression restores activity of hypomorphic Artemis variants

A feature of the cellular platform described here is that retrovirus-mediated transduction typically results in generation of a population of cells harboring a different number of copies of *DCLRE1C* cDNA. The number of vector copies in the pool of unselected cells harboring these mutations consistently ranged between 4–8 per cell (data not shown). To investigate whether high levels of activity could reflect protein overexpression, we selected the hypomorphic mutants M1T and T432*16, and the polymorphism P171R, all of which showed high recombination and DNA repair activities when tested in bulk culture of *Dclre1c*$^{-/-}$ abl pro-B cells (80% – 100% of wild-type *DCLRE1C*, Table 2). Upon limiting dilution subcloning of transduced *Dclre1c*$^{-/-}$ abl pro-B cells harboring these mutations, we selected clones with more than six copies of vector, and compared their activity levels to that of clones with two vector copies (Fig. E5). Both the recombination and the DNA repair activities of the M1T and T432*16 mutants were significantly lower in clones harboring 2 vector copies than in those with 6 copies. By contrast, high activity was observed for the P171R, even in clones with two copies of this variant (Fig. 5). These data are consistent with P171R being a polymorphism. Furthermore, they indicate that overexpression can partially restore functional activity of hypomorphic ARTEMIS mutants.

Discussion

The endo- and exonuclease ARTEMIS has a crucial function in opening the hairpin-sealed coding ends of V, D and J elements during V(D)J recombination in developing T and B lymphocytes [3], and in processing overhangs that occur in a small fraction of DNA double strand breaks [8]. The severity of ARTEMIS deficiency depends on the amount of residual nuclease activity. While lack of protein expression or function completely abrogates T and B cell development, hypomorphic mutant proteins allow V(D)J recombination, albeit with reduced efficacy. This results in a milder immunodeficiency, but also in susceptibility to develop autoimmunity, lymphoproliferation, and malignancy. In this regard, the clinical phenotype of leaky ARTEMIS deficiency resembles that observed in patients with hypomorphic RAG1 or RAG2 defects [33]. To gain more insights into genotype-phenotype correlation in ARTEMIS deficiency, we analyzed recombination and DNA repair activity in a large series of naturally occurring ARTEMIS mutations, and we correlated these results

with the clinical phenotype. To this purpose, we used a similar cellular platform to the one we have recently reported to investigate genotype-proteotype-phenotype correlation in RAG1 deficiency [14]. Importantly, an advantage of the *Dclre1c*$^{-/-}$ abl pro B-cell system is that it allows at the same time to analyze recombination activity and DNA repair.

While ARTEMIS plays a critical role in V(D)J recombination, its major function as a component of the NHEJ pathway of DNA repair is to process specific DNA double strand breaks, which are repaired in a slow-kinetic process. By analyzing the kinetics of H2AX histone dephosphorylation in pro-B cell lines expressing mutant (or WT) ARTEMIS at various time points after exposure to IR, we observed different levels of DNA repair activity for the various mutants analyzed, with good correlation with the data observed in the recombination assay. However, slightly higher levels of DNA repair than of recombination were observed in mutants with absent or low recombination activity, which was particularly the case for mutations affecting the β-CASP domain. It has been proposed that the β-CASP domain participates with the β-lactamase region in forming the catalytic core of ARTEMIS [5]. Our findings suggest that ARTEMIS may play a less critical role in DNA repair than in V(D)J recombination.

Consistent with previous observations [12, 25], we found that mutations affecting highly conserved residues in the catalytically active β-lactamase or β-CASP domains completely abrogate function, and are associated with typical features of SCID. Mutations affecting other residues in the same domains may cause conformational changes that may variably affect protein function, as indicated by heterogeneous levels of activity even for missense mutations involving the same codon.

Mutations disrupting the C-terminus take a special position. In contrast to the β-lactamase and β-CASP domain, this region is dispensable for the catalytic function [5], but plays a critical role in regulation of V(D)J recombination and DNA repair through the interaction with DNA-PKcs and DNA Ligase 4 [6, 34]. It has recently been reported that ARTEMIS also interacts with PTIP, a BRCT-domain containing effector protein in the 53BP1 pathway, which antagonizes the homologous recombination DNA repair pathway [35]. The importance of this region for genomic stability has been studied in detail in a mouse model harboring a C-terminal 7-nt deletion resulting in a frameshift mutation at D449 (corresponding to D451*11 in humans) [36]. This mutation was shown to cause aberrant intra- and interchromosomal V(D)J joining events predisposing to lymphoma [37]. Five out of 8 patients reported with deletions in this region presented with leaky SCID, four had features of autoimmunity and two developed EBV-associated lymphomas [4, 20, 26–28]. Except for two mutations affecting the most proximal part of the C-terminal domain and reported in SCID patients, we observed very high activity levels for all other truncating C-terminal mutations. Interestingly, 50% of the leaky SCID patients with C-terminal mutations were homozygous for such defects. This contrasts with the observation that the remainder of the patients with leaky SCID carried a hypomorphic missense mutation on one allele, and a deletion on the second allele.

Our system allows to assess functional activity of ARTEMIS mutated proteins on an intrachromosomal substrate in a relevant cell type (pro-B cells), which represents a stage of

B cell development where V(D)J recombination occurs physiologically. Other approaches to test ARTEMIS activity are based on co-transfection of a reporter construct and RAG1 and RAG2 expression plasmids into the cytoplasm of ARTEMIS mutated fibroblast cell lines [5, 12]. However, it is important to mention that none of the cellular platforms available allow to fully model functional activities as they occur in patients. In particular, in the cellular platform described here, ARTEMIS expression is driven under a viral promoter and influenced by random genomic integration and by the number of vector copies. All of these factors may contribute to variable expression, including overexpression. To adjust for this limitation, we normalized mutant activities to WT activity and subtracted background activities obtained in mock-transduced cells. Furthermore, to assess the impact of protein overexpression on recombination and DNA repair activity we performed limiting dilution analysis of vector copy numbers. By titrating the expression levels of the hypomorphic mutations M1T and T432*16, we observed that overexpression was associated with improved functional activity. This was not the case for the P171R variant, for which high levels of activity were consistently observed, even in clones harboring only two vector copies. Our data differ from previous observations that had reported that the P171R variant had significantly lower than normal activity, which could be partially restored by increased product expression [24]. Importantly, the observation that overexpression of the M1T and T432*16 mutants was associated with improved functional activity in *Dclre1c*$^{-/-}$ abl pro-B cells raises the interesting hypothesis that novel therapeutic approaches, aimed at enhancing gene transcription, might be efficacious in patients with hypomorphic *DCLRE1C* mutations. However, overexpression of *DCLRE1C* has also been associated with cytotoxicity [38], suggesting that regulated control of increased expression may be necessary for therapeutic purposes.

In summary, we report on a cellular platform that permits to analyze both recombination activity and DNA double strand break repair function of naturally occurring ARTEMIS mutations. Good correlation was observed between the functional activity of the mutated protein and the clinical phenotype of affected patients. Use of this cellular system may allow to assess the possible disease causing role of novel *DCLRE1C* genetic variants identified in patients. Finally, the observation that overexpression of hypomorphic mutants may significantly rescue function, opens the way to possible future therapeutic interventions based on modulation of gene expression for patients with ARTEMIS deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

A-MulV	Abelson murine leukemia virus
ATM	Ataxia telangiectasia mutated protein
CID	Combined immunodeficiency
DSB	Double strand break
DNA-PKcs	DNA protein kinase catalytic subunit
GFP	Green fluorescent protein
IR	Ionizing radiation
LOF	Loss of function
MFI	Mean fluorescent intensity
NHEJ	Non-homologous end-joining
OS	Omenn syndrome
RAG	Recombination activation gene
RSS	Recombination signal sequence
SCID	Severe combined immunodeficiency

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Key messages

- We describe a cellular model that allows rapid analysis of functional activity of recombination and DNA repair associated with human *DCLRE1C* mutations.
- Phenotypic diversities in ARTEMIS deficiency depend on residual activity levels on both alleles, which, for hypomorphic variants, can be increased with higher levels of expression.
- These findings can potentially help guide gene therapy strategies

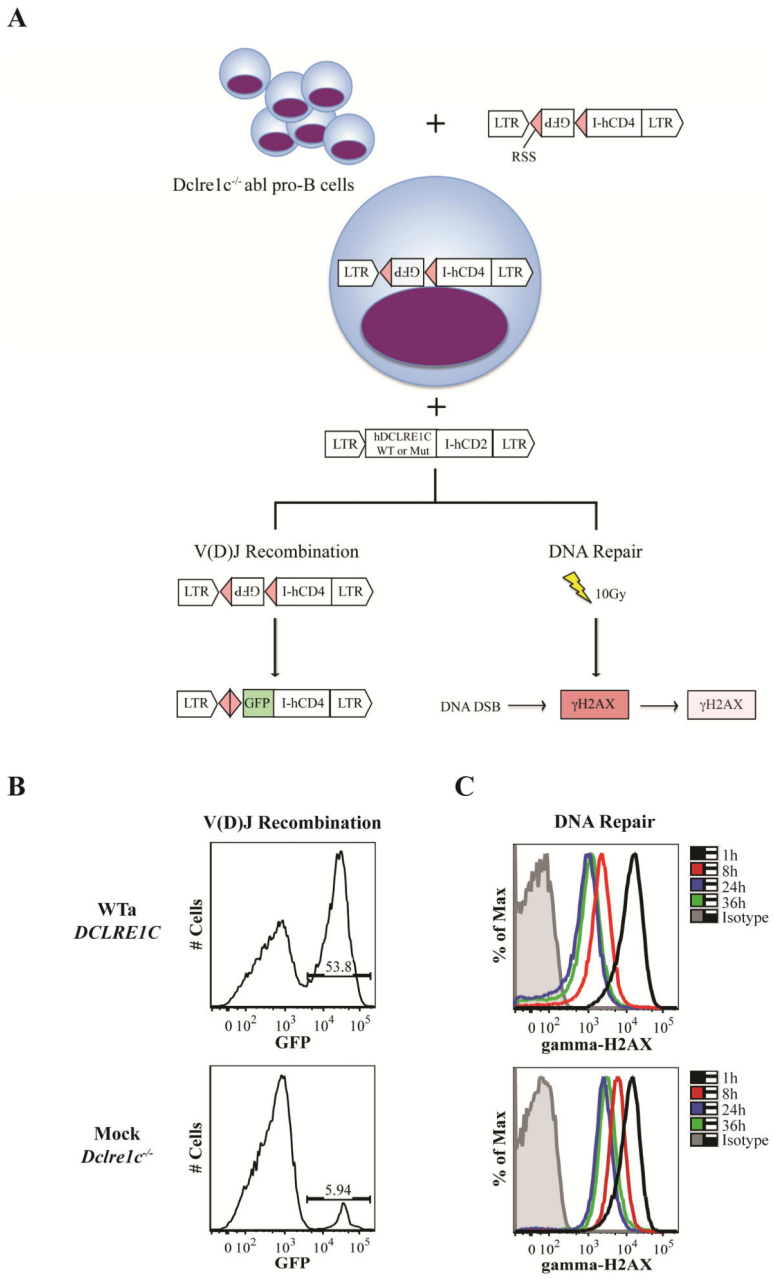

FIG. 1. Schematic representation of the experimental outline and readout

A, *Dclre1c*$^{-/-}$ abl pro-B cells were retrovirally engineered with a single construct containing an inverted GFP cassette flanked by two RSS and the h*CD4* sequence for positive selection. Subsequently, cells were transduced with a second construct containing either WT or mutated h*DCLRE1C* (isoform a) and the h*CD2* sequence. Sufficient targeting of RSS by the Rag1/Rag2 complex and completion of the recombination process, which involves Artemis, allows the GFP cassette to flip into sense orientation. **B**, GFP expression can be used for quantification of V(D)J recombination activity in cells expressing both vectors (CD4^{+}CD2^{+}). **C**, To assess DNA repair activity, cells were irradiated with 10Gy and γH2AX de-phosphorylation was quantified by flow-cytometry at 1h, 8h, 24h, and 36h in

cells expressing the DCLRE1C-hCD2 vector. The difference in GFP expression and down-regulation of γH2AX is shown for *Dclre1c*$^{-/-}$ abl pro B-cells transduced with WTa *DCLRE1C* or a mock plasmid, respectively.

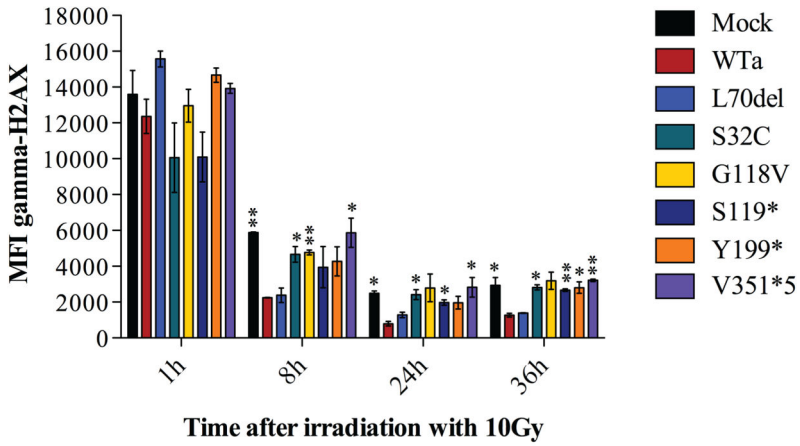

FIG. 2.

DNA repair activities were analyzed in *Dclre1c*$^{-/-}$ abl pro-B cells blocked in G0/G1 cell cycle phases. Mean fluorescence intensities (MFIs) for γH2AX at 1h, 8h, 24h and 36h after irradiation with 10Gy are shown for one representative experiment testing 6 ARTEMIS mutants compared to WTa and mock **(A–G)**. Error bars indicate standard deviations, * p≤0.05; **p≤0.01; ***p≤0.001.

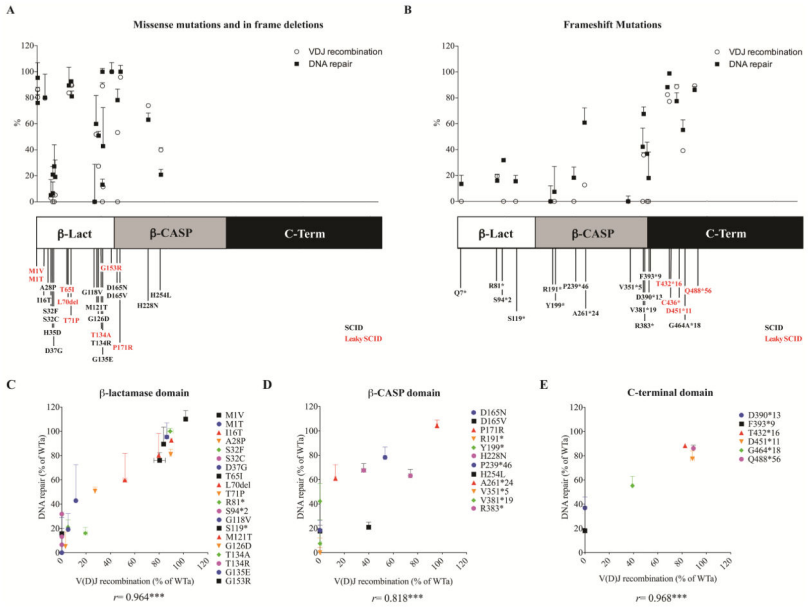

FIG. 3. Correlation of V(D)J recombination with DNA repair activities

Recombination and DNA repair activity levels of 41 genetic *DCLRE1C* variants were studied in 23 missense mutations and in frame deletions **(A)** and 18 frameshift mutations **(B)** affecting various domains: Metallo-β-lactamase domain (amino acids 1–155), β-CASP domain (amino acids 156–385) and the C-terminal region (amino acids 386–692) [5]. V(D)J recombination activity levels of mutations affecting the β-lactamase (**C**), β-CASP (**D**) and C-terminal domain (**E**) of the protein are plotted against DNA repair activities. Activity levels are expressed as percentages of WTa performance. Error bars indicate standard deviations, ***p≤0.001. *r*, Pearson correlation coefficient.

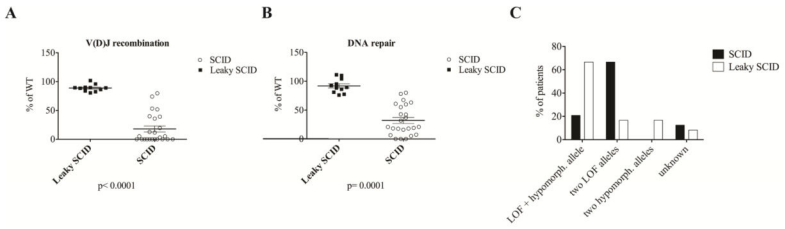

FIG. 4.

The activity levels for recombination **(A)** and DNA repair **(B)** of the mutations with the highest activity (in case of compound heterozygosity) found in patients presenting with SCID and leaky SCID are shown ($p < 0.0001$, and $p = 0.0001$). The percentages of patients being heterozygous or homozygous for a LOF or a hypomorphic *DCLRE1C* allele are shown for SCID and leaky SCID patients **(C)**. *LOF*, loss of function.

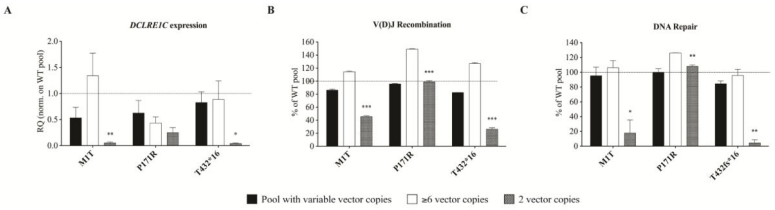

FIG. 5. Recombination and DNA repair activity levels of hypomorphic *DCLRE1C* variants with various vector copy numbers

Expression levels of two hypomorphic *DCLRE1C* variants and one polymorphism were titrated by varying vector copy numbers to ≥6 or 2. Expression of human *DCLRE1C* was assessed by quantitative PCR in cDNA samples and was normalized to WTa (indicated by dashed line) **(A)**. Activity levels for V(D)J recombination **(B)** and DNA repair **(C)** were compared between ≥6 or 2 vector copies, or the pool containing variable copy numbers per cell. Error bars indicate standard deviations. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Table 1

Genotype and clinical presentation of ARTEMIS-deficient patients

Allele 1	Allele 2	Protein	Age at diagnosis	Infections	Autoimmunity/ Lymphoproliferation	Malignancy	Immunology	Radiosensitivity	Reference
SCID									
c.47T>C	c.356C>G	I16T; S119*	1mo	LRI, oral thrush			$T^-B^-NK^+$	sensitive	20
c.82G>C	c.82G>C	A28P					$T^-B^-NK^+$	sensitive	11
c.95C>T	del Ex 1-3	S32F							19
c.95C>G	[2]N/A	S32C							1
c.110A>G	c.110A>G	D37G	2mo	moniliasis, gastroenteritis			$T^-B^-NK^+$	sensitive	4
c.241C>T	c.241C>T	R81*							9
c.281delC	c.281delC	S94fs*2					$T^{mat}B^-NK^+$		11
c.353G>T	c.353G>T	G118V	5mo	LRI, PJP, CMV			$T^-B^-NK^+$	sensitive	22
c.353G>T	c.353G>T	G118V					$T^-B^-NK^+$		11
c.362T>C	del Ex 1-3	M121T	8mo	LRI			$T^-B^-NK^+$ agamma		unpublished
c.404G>A	c.404G>A	G135E	4mo	VZV				sensitive	22
c.493G>A	c.493G>A	D165N							3
c.494A>T	c.494A>T	D165V					$T^-B^-NK^+$		11
c.571C>T	N/A	R191*							unpublished
c.597C>A	c.597C>A	Y199*		oral and genital ulcers					10
c.682C>A	c.682C>A	H228N					$T^-B^-NK^+$	sensitive	11
c.716delC	c.716delC	P239Lfs*46	8mo	recurrent URI, oral thrush, chronic diarrhea			$T^-B^-NK^+$		unpublished
c.761A>T	c.309delG (splice site)	H254L; K103fs							25
c.781delG	c.781delG	A261Qfs*24							9
c.1050delA	c.1050delA	V381Lfs*5					$T^-B^-NK^+$		11
c.1140_1146delAGTTCAC	c.1140_1146delAGTTCAC	V383Efs*19					$T^{mat}B^-NK^+$	sensitive	11
c.1147C>T	c.1147C>T	R383*					$T^{mat}B^-NK^+$		11
c.1167_1168insAG	c.delEx3	D390Rfs*13	2mo	URI			$T^-B^-NK^+$ hypogam	sensitive	26
c.1179delT	c.1179delT	F393Lfs*9	3mo	LRI, PJP, oral thrush, CMV			$T^-B^-NK^+$ hypogam	sensitive	20

Allele 1	Allele 2	Protein	Age at diagnosis	Infections	Autoimmunity/ Lymphoproliferation	Malignancy	Immunology	Radiosensitivity	Reference
c.1391_1395delGAATC	c.1391_1395delGAATC	G464Afs*18	1mo	LRI, GI			$T^-B^-NK^+$	sensitive	4
Omenn Syndrome									
c.2T>C	c.103C>G	M1T; H35D	5mo	sepsis, BCG	alopecia, erythrodermia, lymphadenopathy, hepato/splenomegaly, granuloma	lymphoma	$T^{hi}B^-NK^+$ hypogam TCR repertoire 25% of WT	sensitive	13
Leaky SCID									
c.1A>C	c.401C>G	M1V; T134R	6mo	pharyngitis, stomatitis, gastroenteritis			$T^-B^-NK^+$		unpublished
c.19C>T	c.457G>A	Q7*; G153R					73% CD3 3% CD19 of MNC in BM		19
c.194C>T	N/A	T65I	18mo	pulmonary TB			$T^-B^-NK^+$		unpublished
c.211A>C	del Ex 1-3	T71P	10mo (onset)	URI, *Pseudomonas* soft tissue infection	AIHA, neutropenia, thrombocytopenia, lymphadenopathy hepato/splenomegaly		$T^{lo}B^{lo}NK^+$ low IgA and IgG2; absent response to vaccine (Hib and tetanus); proliferation to PHA initially normal, later absent		21
c.211A>C	del Ex 1-3	T71P	birth	URI, oral candidiasis	AIHA		$T^{lo}B^-NK^+$ low IgG, absent IgA; decreased TRECs; poliferation to PHA initially normal, later absent		21
c.207_209delGTT	c.377G>A	L70del; G126D	4.5y	URI, Candida	AIHA		$T^{lo}B^+NK^+$ hypogam no specific Ab response	sensitive	23
c.400A>G	del Ex 1+ (not specified)	T134A			lymphadenopathy		T^{lo}, polyclonal T cells, proliferation to PHA >30% of normal		unpublished
c.512C>G	c.insInt2b	P171R	27y	Candida, HPV, fungal brain abscess	neutropenia, thrombocytopenia	bilateral carcinoma in situ of nipples	$T^{lo}B^{lo}NK^+$ abnormal T cell proliferation	mildly sensitive	24
c.512C>G	c.1299_1306dupAGGATGCT	P171R; C436*	7y	URI, LRI	lymphadenopathy, granulomatous inflammation	Hodgkin lymphoma	$T^{lo}B^-NK^+$ absent IgA		24
c.1290_1306delACAAACCCCAGGATGCT	c.1290_1306delACAAACCCCAGGATGCT	T432fs*16	4y	LRI, GI	cholangitis and liver cirrhosis (died at 16y)		$T^{lo}B^{lo}$ hypogam		27
c.1353_1359delTTGTGAA	c.delEx1-3	D451Kfs*11	birth	LRI, Candida	AIHA, ITP, cervical lymphadenopathy	lymphoma (9mo)	$T^-B^-NK^+$ monoclonal IgG, absent IgA		27
c.1353_1359delTTGTGAA	c.delEx1-3	D451Kfs*11	birth	LRI, Candida		lymphoma	$T^{lo}B^{lo}$ hypogam		27
c.1353_1359delTTGTGAA	c.delEx1-3	D451Kfs*11	1y	URI, LRI, CNS Toxo			$T^{lo}B^-NK^+$ hypogam	sensitive	27
c.1464delG	c.1464delG	Q488fs	1mo	URI, LRI, CMV	oral ulcers, sclerosing cholangitis, thrombocytopenia, hepatosplenomegaly		$T^{lo}B^-NK^+$ LGL cells, hypogam		28

Ab, antibody; *agamma*, agammaglobulinemia; *AIHA*, autoimmune hemolytic anemia; *BCG*, Bacillus Calmette-Guérin; *BM*, bone marrow; *CMV*, cytomegalovirus; *CNS*, central nervous system; *GI*, gastrointestinal infection; *HPV*, human papillomavirus; *hypogam*, hypogammaglobulinemia; *ITP*, idiopathic thrombocytopenic purpura; *LGL*, large granular lymphocytes; *LRI*, lower respiratory infections; *MNC*, mononuclear cells; *N/A*, not available; *PJP*, *Pneumocystis jirovecii* pneumonia; *PHA*, phytohemagglutinin; *TB*, Tuberculosis; *TCR*, T cell receptor; T^{mat}, maternal T cells; *Toxo*, Toxoplasmosis; *TRECs*, T cell receptor excision circles; *URI*, upper respiratory infection; *VZV*, varicella zoster virus

Table 2

Recombination and DNA repair activity levels of *DCLRE1C* mutated products

Mutations in *DCLRE1C*		Activity levels in % of WT activity			
Genomic	Protein	Recombination		DNA repair (36h after IR)	
		Mean	SD	Mean	SD
Metallo-β-Lactamase Domain					
c.1A>G	M1V	86.32	1.67	95.35	11.60
c.2T>C	M1T	80.41	4.77	76.04	6.43
c.19C>T	Q7*	0.00	0.35	13.52	6.66
c.47T>C	I16T	79.67	0.27	80.11	18.11
c.82G>C	A28P	3.00	0.15	5.13	11.95
c.95C>T	S32F	5.14	0.34	20.95	6.17
c.95C>G	S32C	0.00	0.40	6.56	8.73
c.103C>G	H35D	0.00	0.30	27.29	16.57
c.110A>G	D37G	5.24	0.65	19.15	13.04
c.194C>T	T65I	83.75	1.32	89.45	14.05
c.207_209delGTT	L70del	90.01	0.77	92.63	0.96
c.211A>C	T71P	89.52	0.80	81.03	4.18
c.241C>T	R81*	19.48	1.56	16.05	4.77
c.281delC	S94*2	0.00	1.01	31.86	0.04
c.353G>T	G118V	0.00	0.62	0.00	28.89
c.356C>G	S119*	0.00	0.27	15.59	4.51
c.362T>C	M121T	51.87	1.45	59.88	21.87
c.377G>A	G126D	27.40	0.21	50.80	3.31
c.400A>G	T134A	89.04	2.43	111.18	2.53
c.401C>G	T134R	0.00	0.44	13.21	4.25
c.404G>A	G135E	11.57	0.54	42.84	29.68
c.457G>A	G153R	101.82	0.45	110.04	7.03
β-CASP Domain					
c.493G>A	D165N	53.26	1.28	78.20	8.48

Mutations in *DCLRE1C*		Activity levels in % of WT activity			
Genomic	**Protein**	**Recombination**		**DNA repair (36h after IR)**	
		Mean	**SD**	**Mean**	**SD**
c.494A>T	D165V	0.00	0.62	17.71	4.91
c.512C>G	P171R	95.79	0.62	104.12	4.91
c.571C>T	R191*	0.00	0.87	0.00	12.11
c.597C>A	Y199*	0.00	1.21	7.46	19.56
c.682C>A	H228N	74.00	1.31	63.14	5.13
c.716delC	P239*46	0.00	0.73	18.30	8.29
c.761A>T	H254L	39.89	1.73	20.85	4.11
c.781delG	A261*24	12.69	0.61	60.92	11.34
c.1050delA	V351*5	0.00	0.29	0.00	4.21
c.1140_1146delAGTTCAC	V381*19	0.00	0.71	42.18	14.54
c.1147C>T	R383*	35.84	1.78	67.60	5.46
C-Terminal Domain					
c.1167_1168insAG	D390*13	0.00	0.30	36.84	9.00
c.1179delT	F393*9	0.00	0.42	18.07	19.99
c.1290_1306del17	T432*16	82.49	0.12	88.29	1.09
c.1299_1306dupAGGATGCT	C436*	77.26	0.42	98.89	1.26
c.1353_1359delTTGTGAA	D451*11	88.57	1.64	77.43	6.53
c.1391_1395delGAATC	G464*18	39.21	0.71	55.22	7.76
c.1464delG	Q488*56	89.38	0.46	86.14	2.59