Targeted gene editing restores regulated CD40L function in X-linked hyper-IgM syndrome

Nicholas Hubbard,1 David Hagin,1 Karen Sommer,1 Yumei Song,1 Iram Khan,1 Courtnee Clough,1 Hans D. Ochs,1,2 David J. Rawlings,1-3 Andrew M. Scharenberg,1,3 and Troy R. Torgerson1,2

1Center for Immunity and Immunotherapies and Program for Cell and Gene Therapy, Seattle Children’s Research Institute, Seattle, WA; and 2Department of Pediatrics and 3Department of Immunology, University of Washington, Seattle, WA

Key Points

- The CD40LG locus can be specifically targeted and repaired in primary human T cells by insertion of a spliced CD40LG complementary DNA.
- Gene editing restores regulated CD40L expression in X-HIGM T cells, reconstituting B-cell immunoglobulin class switching.

Loss of CD40 ligand (CD40L) expression or function results in X-linked hyper-immunoglobulin M (IgM) syndrome (X-HIGM), characterized by recurrent infections due to impaired immunoglobulin class-switching and somatic hypermutation. Previous attempts using retroviral gene transfer to correct murine CD40L expression restored immune function; however, treated mice developed lymphoproliferative disease, likely due to viral-promoter–dependent constitutive CD40L expression. These observations highlight the importance of preserving endogenous gene regulation in order to safely correct this disorder. Here, we report efficient, on-target, homology-directed repair (HDR) editing of the CD40LG locus in primary human T cells using a combination of a transcription activator-like effector nuclease–induced double-strand break and a donor template delivered by recombinant adeno-associated virus. HDR-mediated insertion of a coding sequence (green fluorescent protein or CD40L) upstream of the translation start site within exon 1 allowed transgene expression to be regulated by endogenous CD40LG promoter/enhancer elements. Additionally, inclusion of the CD40LG 3’-untranslated region in the transgene preserved posttranscriptional regulation. Expression kinetics of the transgene paralleled that of endogenous CD40L in unedited T cells, both at rest and in response to T-cell stimulation. The use of this method to edit X-HIGM patient T cells restored normal expression of CD40L and CD40–murine IgG Fc fusion protein (CD40-mulg) binding, and rescued IgG class switching of naive B cells in vitro. These results demonstrate the feasibility of engineered nuclease-directed gene repair to restore endogenously regulated CD40L, and the potential for its use in T-cell therapy for X-HIGM syndrome. (Blood. 2016;127(21):2513-2522)

Introduction

CD40–CD40 ligand (CD40L) interactions play a critical role facilitating T- and B-cell cross-talk following antigen recognition.1,2 CD40 is expressed constitutively on the surface of B cells and dendritic cells, whereas CD40L is inducibly expressed on the T-cell surface following T-cell receptor (TCR) activation.3,4 Engagement of CD40 by CD40L represents a critical checkpoint in the adaptive immune response: this T-cell:B-cell interaction, commonly known as “T-cell help” leads to the initiation of B-cell proliferation, long-term memory responses, and antibody production through class-switch recombination (CSR) and somatic hypermutation.5,9 Additionally, CD40:CD40L interactions play an essential role in dendritic cell/antigen-presenting cell licensing.10

CD40L, or CD154, is a small (32-39 kDa, depending on posttranslational modifications) type II transmembrane protein from the tumor necrosis factor superfamily.2,11,12 Similar to other tumor necrosis factor family ligands, CD40L exists as a functional trimer with secreted soluble forms also capable of acting on CD40.11,14 In resting T cells, preformed CD40L exists within intracellular secretory vesicles. Upon TCR activation, preformed CD40L is rapidly translocated to the cell surface, accompanied by increased transcriptional activity of CD40LG.5

X-linked hyper-immunoglobulin M (IgM) syndrome (X-HIGM) type I is a striking example of the importance of CD40L in T-cell help and adaptive immunity. X-HIGM arises in humans carrying mutations in CD40LG, and is characterized by recurrent infections, low serum immunoglobulins G, A, and E (IgG, IgA, and IgE) with normal or elevated IgM levels, reduced numbers of memory B cells, and the absence of class-switched memory B cells.15-17 Autosomal-recessive forms of HIGM syndrome also exist, resulting from mutations within other genes required for CD40 signaling, CSR, and somatic hypermutation, notably CD40, AID, UNG, ATM, and NEMO.18-37 In most cases, HIGM syndrome is treated by regular administration of IV immunoglobulin, or by early matched bone marrow transplantation.38,39 Both represent effective therapy options, although possibilities for complications exist and may be improved by the development of an autologous therapy.

Previous studies evaluating genetic therapies for X-HIGM used viral delivery to randomly integrate a CD40L expression cassette into the T-cell genome.40-42
human21,22 or mouse hematopoietic cells.23 Retroviral delivery of CD40L to bone marrow or thymocytes in a mouse model of X-HIGM was sufficient to restore adaptive immunity23; however, the majority of mice developed thymic lymphoproliferative disorders likely due to unregulated overexpression of CD40L, demonstrating the need for future gene therapy to recapitulate tightly regulated endogenous CD40L expression. Lentiviral transfer of an expression cassette using endogenous CD40LG promoter elements to drive expression of CD40L, complementary DNA (cDNA) in human T cells facilitated activation-dependent expression,22 and transferring a trans-splicing pre-messenger RNA (mRNA) fragment of CD40LG to mouse bone marrow also corrected X-HIGM deficiencies,24 although the latter approach is unlikely to be therapeutically effective due to the low efficiency of trans-splicing. Both gene-transfer approaches share 2 challenges: the possibility for multimerization of the transgene/spliced product with defective endogenous CD40L25 thus not fully correcting the defect, and the risk of insertional mutagenesis and/or gene silencing. Some X-HIGM patients have CD40LG mutations causing leaky exon skipping, and which result in the coexpression of both wild-type (WT) and mutant CD40LG, and the presence of WT:mutant CD40L complexes at the cell surface. This finding suggests that mutant CD40L acts as a dominant-negative inhibitor of the WT protein.26 Additionally, random X-chromosome inactivation of all hematopoietic cells in carrier females results in stable mosaic expression of 2 populations of circulating T lymphocytes, one expressing the WT CD40LG allele and the other expressing the mutated one.26

In contrast, targeted gene editing using engineered nucleases to induce a double-strand break (DSB) allows targeted endogenous gene modification. DSBs may be repaired either through nonhomologous end joining (NHEJ), an error-prone pathway resulting in a high frequency of nucleotide insertions or deletions (in-dels), or homology-directed repair (HDR) where DSBs are seamlessly repaired using homologous DNA.27–29 Natural HDR mechanisms may be leveraged for therapeutic purposes to insert a transgene into the DSB using an engineered donor DNA template.

Transcription activator-like (TAL) effector nucleases (TALENs) have been used to target full-length β-globin cDNA to the endogenous β-globin locus, preserving the regulatory environment of transgene expression.30 Recently, we reported high-efficiency gene editing in human primary T cells and CD34 cells, achieved by nuclease-mediated gene disruption and codelivery of recombinant adeno-associated virus (rAAV).31 We hypothesized and report here that a similar approach using a combination of TALEN nucleases32,33 and rAAV donor template.

T-cell cultures were maintained with interleukin-2 (IL-2; 50 ng/mL), interleukin-7 (IL-7; 5 ng/mL), and interleukin-15 (IL-15; 5 ng/mL).

### In vitro mRNA synthesis

pUC57 CD40LG-TALEN constructs were linearized using BstXI and gel purified with standard molecular biology techniques. In vitro transcription, 5′-capping, and polyA synthesis were performed using the T7 mScript Standard mRNA Production System (Cellscript) according to the manufacturer’s protocols. Briefly, linearized template was in vitro transcribed into unmodified mRNA transcripts, capped (5′-7-methylguanylate cap) with cap-1 mRNA structure (2′-O-methyltransferase), and subsequently polyA tails were synthesized using provided enzymes. Final purification was performed using the Qiagen RNeasy kit and manufacturer protocols.

### Molecular assays

Polymerase chain reaction (PCR) amplifications were performed using either Platinum Taq (Invitrogen) or Q5 Polymerase (New England Biolabs). Sequencing analyses were performed using BigDye v3.1 reagents (Thermo Scientific). PCR cloning for in-del analysis was performed using the CloneJET PCR Cloning kit and manufacturer protocols, followed by colony PCR and sequencing analysis. Primers for PCR and sequencing analyses are listed in supplemental Table 1 (available on the Blood Web site).

### Primary human T-cell gene editing

T-cell editing was performed as described previously using combination mRNA electroporation and adeno-associated virus (AAV) transduction.31 Briefly, thawed human T cells (CD4+ or CD3+ ) were activated using human T-activator CD3/CD28 Dynabeads (Gibco). The Neon Transfection System (100 μL; Buffer T; Invitrogen) was used for all mRNA transfections, at a cell concentration of 2 × 10⁶ cells per mL. TALEN mRNA (7.5 μg of each; 15 μg total) was used for all transfections. Previews experiments, experiments using blue fluorescent protein (BFP) mRNA transfection showed efficiencies of >98% in T cells.31 Following electroporation (3 pulses of 1400 V, 10-millisecond pulse width), cells were subdivided and cultured in 24-well plates (final volume, 1 mL; 1 × 10⁶ cells per mL). Cells were incubated for 2 hours at 30°C. Twenty-percent culture volume of AAV (200 μL) was added to each well and 30°C incubation was continued to 24 hours. Two hundred microliters of AAV typically corresponds to a multiplicity of infection (MOI) ranging 50 000 to 100 000 infectious units. After 24 hours, cells were transferred to 37°C and gene editing analyzed by flow cytometry 5 days posttransfection.

### Flow cytometry

Analysis of fluorescent markers and cell surface protein expression was performed on an LSR II flow cytometer (BD Biosciences), and data were analyzed using Flow Jo software versions 9.2 or 10.7 (TreeStar). Traditional forward scatter/side scatter gates encompassing the lymphocyte population were used for all experiments. For kinetic assays, T cells were stained at rest or following activation with phorbol myristate acetate (20 ng/mL) and ionomycin (1 μg/mL) (P/IL Sigma-Aldrich) at indicated time points. The following antibodies were used: phycoerythrin (PE)-CD40–murine IgG Fc fusion protein (CD40-muIg) (Anceil), PE-anti-CD40L (clone 24-31; ebioscience), Alexa 700-anti-CD4 (clone RPA-T4; BD Biosciences), Pacific Blue anti-IgM (clone MMH-88; BioLegend), PE-Cy7 anti-CD19 (clone HB19; BioLegend), and peridinin chlorophyll Cy5.5-anti-CD4 (clone RPA-T4; BioLegend), Alexa 700-anti-IgG (clone G15-145; BD Biosciences), and allophycocyanin anti-CD69 (clone FN50; BD Biosciences).

### Materials and methods

#### Cell isolation and culture

The research was approved by the Seattle Children’s Research Institute’s Institutional Review Board, and written informed consent was obtained for all healthy male donors and X-HIGM patients. Peripheral blood mononuclear cells were isolated from whole blood of healthy or X-HIGM male donors by Ficoll-Paque (GE Healthcare). CD3+, CD4+ T cells and naive B cells were isolated from peripheral blood mononuclear cells by negative selection using human T-cell or CD4+ T-cell and human naive B-cell enrichment kits (EasySep, Stemcell Technologies) according to the manufacturer’s protocols. Cells were frozen in 10% dimethylsulfoxide or used directly. All cells were cultured in RPMI 1640 supplemented with 20% fetal calf serum, 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 1× Corning GlutaGRO, and 55 μM 2-mercaptoethanol.

#### Primary mouse T-cell gene editing

TOD-scid IL2Rγnull mice (NSG; The Jackson Laboratory) were maintained in a specific pathogen-free facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Seattle Children’s Research Institute’s Institutional Animal Care and Use Committee.

Eight- to 15-week-old animals were transplanted via intraperitoneal injection of 2 × 10⁶ engineered T cells in phosphate-buffered saline, then killed 4 weeks posttransplant, and spleens were analyzed for engrafted cells.
B-cell class switch assay

Naïve B cells were isolated from healthy donors using EasySep Human Naïve B-Cell Enrichment Kits, customized on-request by the manufacturer’s addition of anti-human IgA and IgG antibodies (Stemcell Technologies). Alternatively, naïve B cells were selected using noncustomized naïve B-cell isolation kits (Stemcell Technologies) followed by fluorescence-activated cell sorter sorting IgG/IgA+B cells (BD FACSaria II).

B cells were stimulated for 5 days using various combinations of the following cytokines: recombinant human IL-21 (PeproTech; 100 ng/mL), IL-15 (goat anti-human IgM; Southern Biotech; 1 µg/mL), human Toll-like receptor 9 (TLR9) agonist Cpg oligodeoxynucleotide (ODN) 1826 (Invivogen; 1 nM), and soluble CD40L (sCD40L; generated using techniques described previously, 3 µg/mL).

B-cell/T-cell coculture class-switch assays were performed with allogeneic CD4+ T cells. Prior to coculture, CD4+ T cells were washed and rested overnight in cytokine-free medium. T cells were then activated for 3 to 4 hours using CD3/CD28 Dynabeads at a 1:3 bead:cell ratio. After removing beads and washing cells with complete media, B and T cells were incubated in 100 µL of complete media at a 1:1 B-cell/T-cell ratio (1 x 10^6 cells per well total). T-cell activation was confirmed by CD69 and CD40L expression. After 5 days of coculture, cells were incubated for 5 hours with Brefeldin A (BD GolgiPlugg; 1 µL/mL) followed by surface staining for CD4, CD19, and IgM. Cells were then fixed and permeabilized, and stained for intracellular IgG using BD Cytofix/Cytoperm Plus.

Statistical analysis

Statistical analyses were performed with Prism 6 (GraphPad Software). Data are shown as mean ± standard deviation (SD) unless otherwise noted. Tests of statistical significance were performed using the unpaired 2-tailed Student t test; where noted, we corrected for multiple comparisons using the Holm-Sidak method with α = 0.05%. For multiple comparisons of normally distributed data, statistical analysis was determined with a 2-way analysis of variance using the Tukey multiple comparison test (α = 0.05%).

Results

The CD40LG locus is efficiently and accurately targeted by TALEN in human T cells

A TALEN pair was designed targeting the 5′-untranslated region (5′ UTR) in exon 1 of CD40LG (Figure 1A). An additional TALEN pair targeting downstream of the first ATG in exon 1 was also designed and tested (supplemental Figure 2). This TALEN was determined to be inferior in downstream editing analyses, and all further data presented utilizes the TALEN pair described in Figure 1 targeting the CD40LG 5′ UTR. T-cell electroporation with TALEN mRNA demonstrated transfection efficiencies >88% for either the sense or antisense TALEN half based upon efficiency of cell sorting. Following transfection, NHEJ rates (in-del frequency) reached 96% as determined by amplicon sequencing of the TAL target site within transfected BFP+ cells, demonstrating efficient delivery and cleavage in human T cells.

Potential off-target sites were identified using Prognos and TALE-NT. Each of the predicted off-target sites had at least 5 mismatches and were predicted to have low likelihoods of off-target cleavage. PCR cloning and sequencing of the 10 top-ranked off-target sites (combined top 5 Prognos and TALE-NT) detected no off-target activity (supplemental Figure 4).

Templates delivered by rAAV modify the CD40LG locus in human T cells via HDR

Gene-editing templates within rAAV packaging constructs were designed to introduce a green fluorescent protein (GFP) coding sequence directly downstream of the first ATG of CD40LG via HDR (Figure 1A). TALEN-binding sites were deleted from editing templates to avoid cleavage of donor DNA as well as later cleavage of potentially modified genomic DNA (gDNA). Using this approach, GFP expression is expected to replace endogenous CD40L expression, while conserving upstream promoter and intrinsic regulatory elements downstream of the inserted cDNA sequence. To assess the importance of 3′ UTR in expression and regulation of CD40L, 2 gene-editing templates were created: (1) CD40LG[GFP.3′ UTR], where the GFP transgene was followed by either the putative CD40LG 3′ UTR and polyadenylation (pA) signal or (2) CD40LG[GFP.WPRE], using a synthetic Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) 3′ UTR and synthetic pA signal. Both gene-editing templates contained identical upstream (5′) and downstream (3′) homology arms. All template sequences are available in supplemental Figure 5.

As described in “Materials and methods,” TALEN mRNA and donor-template AAV was sequentially delivered to activated T cells within a 3-day time frame (Figure 1B). In cells receiving AAV alone, GFP expression was low to undetectable, whereas cells receiving both TALEN mRNA and AAV produced stable GFP expression by day 5 following gene editing (Figure 1C), consistent with HDR. PCR amplification of gDNA at the CD40LG locus using primers outside of the gene-editing template homology arms, confirmed targeted CD40LG editing in GFP+ edited T cells. Thus, DNA bands corresponding to the predicted size of an HDR product are visible within GFP-enriched populations from both CD40LG[GFP.3′ UTR] and CD40LG[GFP.WPRE] editing templates (Figure 1D). No editing band was visible within populations receiving AAV or TALEN alone. Sequencing analysis further confirmed predicted faithful HDR events (supplemental Figure 6).

The observation of resting GFP expression is consistent with the known expression pattern of CD40L in resting T cells where it is constitutively expressed within cytoplasmic vesicles. Although initial experiments using male CD3+ T cells demonstrated editing of both the CD4 and CD8 compartments (supplemental Figure 7), all subsequent experiments were performed using male CD4+ T cells to avoid an in vitro expansion bias of the CD8 compartment and to focus on T-cell helper function in repaired cells, as X-HIGM syndrome is recognized to be primarily a CD4+ T-cell help defect.

CD40LG is replaced following HDR of gene-editing templates

Consistent with the prediction that gene editing will replace endogenous CD40L expression, following stimulation with PI, no surface CD40L expression was observed in edited GFP+ T cells (Figure 1E). Predictably, binding of soluble CD40-muIg was also lost in GFP+ T cells (data not shown). Although CD40L expression was undetectable on the surface of edited GFP+ cells, stimulation with PI induced an approximately half-log shift of GFP mean fluorescent intensity (MFI), suggesting preserved CD40LG promoter integrity driving GFP expression. These “knockout” templates therefore demonstrate gene replacement with an HDR-editing approach, a significant advantage in cases where background expression of the endogenous mutated gene product is undesirable, as with X-HIGM.

Previously, we showed that HDR-mediated gene editing is significantly more efficient than non-homology-directed insertion of AAV-delivered genetic material at a DSB. We recapitulated that result here using AAV delivery of “homology-less” BFP under an MND (myelo-proliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted) promoter (MND.BFP) codelivered with TALEN (Figure 1F). When MND.BFP and CD40LG[GFP.WPRE] templates were codelivered at equivalent MOIs, GFP+ editing outcomes were ~10-fold greater than BFP+ ones, demonstrating a preference toward HDR editing over AAV insertion in this setting.
Gene editing with CD40LG replacement templates results in regulated CD40L expression

CD40LG[GFP.3'UTR] and CD40LG[GFP.WPRE] gene-editing templates were modified with a 2A-linked CD40L cDNA directly downstream of GFP (Figure 2A). As described in supplemental Methods, the donor CD40L cDNA sequence was codon diverged with synonymous mutations to avoid the previously described possibility of unpredictable HDR between the gene-editing template and homologous premature (nonspliced exons) CD40LG genomic DNA.30 Pairwise alignment of WT to the diverged CD40L coding region was 68.4%. As before, either the CD40LG 3'UTR or WPRE 3'UTR were used to generate, respectively, CD40LG[GFP,CD40L.3'UTR] and CD40LG[GFP,CD40L.WPRE]. Unlike the knockout approach, these templates were expected to reconstitute CD40L expression in addition to replacing endogenous gene expression. Editing of healthy male donor CD4 T cells using CD40LG[GFP,CD40L.3'UTR] or CD40LG[GFP,CD40L.WPRE] resulted in average editing rates...
binding its cognate target. Replacement, but also expression of functional protein capable of response to P/I (Figure 2D), demonstrating not only regulated gene binding in edited cell populations mirrored that of nonedited cells in soluble CD40-muIg fusion protein (PE-CD40-muIg). CD40-muIg CD40L-CD40 binding was tested innonedited T cells using recombinant increased mRNA transport and stability.

expression, suggesting the WPRE 3'UTR provides, as predicted, CD40L.3'UTR (top) and CD40LG[CD40L.WPRE] (bottom). Total AAV packaging sizes are 4616 and 4234 bp, respectively. (B) Average HDR rate (percentage GFP+; n = 4 separate healthy male donors) of each gene-editing template in CD4+ T cells. (C) GFP (x-axis) and CD40L (y-axis) coexpression or (D) CD40-muIg binding in T cells edited with cDNA gene replacement templates. (Top row) Resting; (bottom) P/I activated. *Plots in panels C and D were generated at separate times, using different donor T cells.

Figure 2. CD40LG gene replacement with a functional CD40LG transgene. (A) CD40LG cDNA gene replacement templates targeting the 5'UTR region of exon 1 of CD40LG. CD40LG[GGFP,CD40L.3'UTR] (top) and CD40LG[GGFP,CD40L.WPRE] (bottom). Total AAV packaging sizes are 4616 and 4234 bp, respectively. (B) Average HDR rate (percentage GFP+; n = 4 separate healthy male donors) of each gene-editing template in CD4+ T cells. (C) GFP (x-axis) and CD40L (y-axis) coexpression or (D) CD40-muIg binding in T cells edited with cDNA gene replacement templates. (Top row) Resting; (bottom) P/I activated. *Plots in panels C and D were generated at separate times, using different donor T cells.

<table>
<thead>
<tr>
<th></th>
<th>Non-edited</th>
<th>TALEN only</th>
<th>CD40LG[GGFP,CD40L.3'UTR]</th>
<th>CD40LG[GGFP,CD40L.WPRE]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40L surface expression</td>
<td>0.4%</td>
<td>0.8%</td>
<td>14.4%</td>
<td>3.0%</td>
</tr>
<tr>
<td>CD40L binding activity</td>
<td>0.3%</td>
<td>1.3%</td>
<td>14.9%</td>
<td>2.3%</td>
</tr>
</tbody>
</table>

In order to test the basic function of our codon-optimized transgene, CD40L-CD40 binding was tested in edited T cells using recombinant soluble CD40-muIg fusion protein (PE-CD40-muIg). CD40-muIg binding in edited cell populations mirrored that of nonedited cells in response to P/I (Figure 2D), demonstrating not only regulated gene replacement, but also expression of functional protein capable of binding its cognate target.

Comparison of the rates of NHEJ (percentage with in-del) vs HDR (percentage GFP+) showed that donor template with higher HDR rates (CD40LG[GGFP,CD40L.WPRE]) had lower NHEJ rates compared with CD40LG[GGFP,CD40L.3'UTR] edited cells (supplemental Figure 8). This inverse correlation between total HDR and NHEJ highlights the importance of donor template design for optimal HDR gene editing. Finally, it is conceivable that the 3'UTR element of donor template CD40LG[GGFP,CD40L.3'UTR] could be selected as a homology arm for HDR, despite its distance from the TALEN cleavage site (12 kb). Surprisingly, by PCR analysis we found that some HDR events did occur by this mechanism in a subpopulation of edited cells (supplemental Figure 9).

CD40LG editing in X-HIGM CD4+ rescues CD40L expression and CD40 binding

CD4+ T cells were obtained from X-HIGM patients who have CD40L mutations resulting in decreased/absent CD40L expression (supplemental Figure 10). Following editing with either cDNA template, surface expression of CD40L was rescued within GFP+ cells (Figure 3A). Expression levels are comparable to those seen in Figure 2 of either nonedited or edited healthy donor T cells. Additionally, CD40-muIg binding was restored to healthy donor levels (Figure 3B). This latter observation suggests that our approach succeeded in replacing any mutant CD40L protein that might have disrupted normal CD40L binding. Average editing rates using the CD40LG[GGFP,CD40L.3'UTR] or CD40LG[GGFP,CD40L.WPRE] templates were 14.3% and 29.3%, respectively (Figure 3C).

CD40LG editing restores physiological kinetics of CD40L surface expression on T cells

The surface expression of CD40L on edited T cells was monitored over a 54-hour time course following a 2-hour P/I activation, wash, then culture in cytokine-free media. Expression was analyzed at 0, 0.5, 2 (wash), 3, 8, 24, and 54 hours after P/I activation. CD40L expression in both nonedited healthy and edited CD40LG[GGFP,CD40L.3'UTR]...
X-HIGM cells peaked 3 to 8 hours postactivation and returned to baseline by 24 to 54 hours (Figure 4). Both the averaged MFI and the percentage of cells expressing surface CD40L (percentage CD40L<sup>+</sup>) were comparable throughout the time course for all 3 X-HIGM donors and editing templates (Figure 4B-C). Noticeably, cells edited with CD40LG[CD40L.3'UTR] demonstrated a consistently higher baseline percentage CD40L<sup>+</sup> and CD40L MFI compared with CD40LG[CD40L.WPRE] and nonedited healthy donors. Given that both repair constructs are inserted at identical locations within CD40LG, this is likely due to enhanced mRNA transport and/or stability associated with the WPRE 3'UTR. Kinetics in edited healthy donor cells recapitulated these results (supplemental Figure 11).

**Edited X-HIGM CD4 T cells induce naive B-cell class switching**

An in vitro class-switching assay to test the ability of edited T cells to induce naive B-cell CSR was developed. Cytokine conditions were tested in combination with sCD40L, assaying their ability to induce IgG expression in naive IgM<sup>+</sup>IgG<sup>+</sup>B cells (supplemental Figure 12). Optimal CSR was observed using a combination of the TLR9 agonist CpG ODN and IL-21 (CSR mix). Addition of anti-IgM antibody successfully increased IgG<sup>+</sup> CSR, but was dispensable for T:B cocultures.

Healthy donor resting or activated CD4<sup>+</sup>T cells cocultured with allogeneic naive B cells (1:1 ratio) induced IgG class switch at rates comparable to those previously observed using sCD40L (Figure 5A).
As expected, coculture with activated (as opposed to resting) T cells resulted in a greater percentage of class-switched B cells, likely due to increased CD40L surface expression (Figure 5B). Edited, healthy donor (sorted GFP<sup>1</sup>) T cells retained their ability to facilitate in vitro B-cell CSR, in contrast to those edited using a knockout gene-editing template (CD40LG<sup>[GFP.3'UTR]</sup>), which failed to induce B-cell CSR either when resting or activated (supplemental Figure 13). These findings demonstrate both the critical role of CD40L in this assay, and the effectiveness of this gene-replacement strategy.

As expected, activated X-HIGM CD4<sup>+</sup> T cells failed to induce B cells to undergo IgG class switch in vitro (Figure 5C). Strikingly, this important T-cell function was restored following gene editing, at rates similar to those seen with activated healthy donor T cells. In a comparison of B cells cocultured with activated, edited vs nonedited X-HIGM T cells, there is a significant increase in the percentage of those that are IgG<sup>+</sup> (fold-increase over resting) specifically within edited groups (Figure 5D), demonstrating rescued CD40L function in X-HIGM donors. Together, these results demonstrate restoration of functional CD40L in X-HIGM T cells by CD40LG editing. CD40LG-edited CD4<sup>+</sup> T cells are stable and retain regulatable CD40L expression in T cells after in vivo passage

The survival and stability of edited compared with nonedited T cells was evaluated in vivo by adoptive transfer of healthy or X-HIGM donor CD4<sup>+</sup> T cells into NOD-scid IL2R<sup>gnull</sup> (NSG) recipient mice. Within engrafted animals, editing percentage (percentage GFP<sup>+</sup>) of transferred cells remained stable pre- to posttransfer (Figure 6A). Additionally, edited CD4<sup>+</sup> T cells recovered from spleens retained their ability to express surface CD40L following P/I stimulation (Figure 6B), thereby demonstrating functional as well as genetic stability.

**CD40LG-edited CD4 T cells represent a broad TCR repertoire**

To determine whether a broad repertoire of T cells was edited using this approach, TCRV<sub>B</sub> spectratyping was performed on nonedited and CD40LG<sup>[GFP.CD40L.WPRE]</sup> edited (GFP<sup>+</sup> sorted) cells from healthy and X-HIGM donors. No differences in overall V<sub>B</sub> spectratype complexity were observed between edited vs nonedited T cells from any donor (Figure 6C). Additionally, all individual TCR fragments maintained a high level of complexity (supplemental Figure 14). These combined findings demonstrate the capacity for T-cell editing across a diverse TCR repertoire, a critical requirement for the application of T-cell therapy to X-HIGM syndrome.

**Discussion**

Gene-therapy strategies have traditionally relied on gene transfer using integrating (lentiviral or retroviral) or nonintegrating (adenoviral or
strategy replaces and restores normal regulated CD40L protein expression in primary human T cells, restoring helper T-cell function at efficiency rates amenable to clinical application. Of note, among our candidate gene-editing constructs, utilization of a synthetic 3'UTR (consisting of a WPRE and a synthetic poly A signal) reproducibly altered the expression patterns of our gene-edited product. Due to the relative overexpression of CD40L on the cell surface when using the WPRE construct, compared with endogenous CD40L expression in a healthy control, we feel that clinical application of these constructs will likely have utility in alternative targeted gene-manipulation approaches.

Here, we demonstrate the direct application of these advances in correction of X-HIGM syndrome through CD40LG gene editing, providing a promising pathway for near-term clinical application. Our strategy replaces and restores normal regulated CD40L protein expression in primary human T cells, restoring helper T-cell function at efficiency rates amenable to clinical application. Of note, among our candidate gene-editing constructs, utilization of a synthetic 3'UTR (consisting of a WPRE and a synthetic poly A signal) reproducibly altered the expression patterns of our gene-edited product. Due to the relative overexpression of CD40L on the cell surface when using the WPRE construct, compared with endogenous CD40L expression in a healthy control, we feel that clinical application should focus on the 3'UTR construct. However, although the WPRE regulatory element might be undesirable in this setting, this construct will likely have utility in other targeted gene-manipulation approaches.
In addition to maintaining associated endogenous locus-specific regulatory mechanisms, our optimal gene replacement approach also overcomes concerns regarding oligomerization of the WT CD40L derived from the transgene with residual nonfunctional protein expressed in X-HIGM patient T cells. Unlike gene replacement strategies that lack specific locus targeting, in this X-linked disorder, our gene repair approach always results in loss of the mutant protein. Here, we have rigorously demonstrated that introduction of a transgene upstream of the CD40L coding sequence is not only sufficient to remove endogenous protein expression, but also to rescue downstream T-cell help functions, demonstrating that residual mutant protein will not be a concern under this strategy.

Further analysis for off-target TALEN effects using alternative methodologies might be required prior to any clinical trial, but overall, our findings provide important preclinical data that will facilitate translation of this, or related therapeutic approaches, to the treatment of X-HIGM syndrome. Combined with highly efficient editing rates, we demonstrate expression patterns recapitulating endogenous CD40L, as well as effective rescue of downstream T-cell help. By correction of the primary defect associated with disease (absent T-cell help), an adoptive T-cell therapy using edited autologous T cells may provide a therapeutic benefit to recipient X-HIGM subjects. One approach could include hypervaccination in the period immediately following delivery of CD40LG gene-edited T cells in order to generate a repertoire of switched memory B cells. However, optimal benefit will likely require sustained engraftment of an edited CD4 T-cell product with a broad TCR repertoire in numbers sufficient to mediate T-cell help in response to varied infectious challenges. Previous transplant studies in X-linked severe combined immune deficiency have shown that an unconditioned, matched sibling transplant of whole marrow containing T cells effectively accelerates T-cell reconstitution and provides protective immunity not seen in T-cell–depleted marrow transplants. These data support the hypothesis that transfer of an edited T-cell product is also likely to provide protective immunity in the context of X-HIGM syndrome.

Although CD40L expression is traditionally associated with T cells, it is also expressed on a number of other lymphoid cells, including activated B cells, platelets, monocytes, natural killer (NK) cells, mast cells, and basophils. Therefore, it is important to extend this work to autologous hematopoietic stem cells, where targeting efficiencies using codeelivery of nuclease and AAV have now begun to reach clinically relevant levels. Thus, our combined observations support the potential of this methodology for treatment of X-HIGM syndrome and, likely, other primary immunodeficiency diseases including those where regulated expression of a transgene is required or highly advantageous.
References

Targeted gene editing restores regulated CD40L function in X-linked hyper-IgM syndrome

Nicholas Hubbard, David Hagin, Karen Sommer, Yumei Song, Iram Khan, Courtnee Clough, Hans D. Ochs, David J. Rawlings, Andrew M. Scharenberg and Troy R. Torgerson