The Journal of Immunology

KEAP1 Editing Using CRISPR/Cas9 for Therapeutic NRF2 Activation in Primary Human T Lymphocytes

Sanjeev Noel, Sul A. Lee, Mohanraj Sadasivam, Abdel R. A. Hamad and Hamid Rabb

J Immunol 2018; 200:1929-1936; Prepublished online 19 January 2018;
doi: 10.4049/jimmunol.1700812
http://www.jimmunol.org/content/200/5/1929

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

KEAP1 Editing Using CRISPR/Cas9 for Therapeutic NRF2 Activation in Primary Human T Lymphocytes

Sanjeev Noel,* Sul A. Lee,* Mohanraj Sadasivam,† Abdel R. A. Hamad,† and Hamid Rabb*

Oxidant stress modifies T lymphocyte activation and function. Previous work demonstrated that murine T cell–specific kelch like-ECH-associated protein 1 (*Keap1*) deletion enhances antioxidant capacity and protects from experimental acute kidney injury. In this study, we used CRISPR technology to develop clinically translatable human T cell–specific *KEAP1* deletion. Delivery of *KEAP1* exon 2 specific Cas9:guide RNA in Jurkat T cells led to significant (~70%) editing and upregulation of NRF2-regulated antioxidant genes NADPH dehydrogenase quinone 1 (*NQO1*) (up to 11-fold), heme oxygenase 1 (*HO1*) (up to 11-fold), and *GCLM* (up to 2-fold). In primary human T cells, delivery of *KEAP1* exon 2 target site 2-specific ATTO 550–labeled Cas9:guide RNA edited *KEAP1* in ~40% cells and significantly ($p \leq 0.04$) increased *NQO1* (16-fold), *HO1* (9-fold), and *GCLM* (2-fold) expression. To further enrich *KEAP1*-edited cells, ATTO 550–positive cells were sorted 24 h after electroporation. Assessment of ATTO 550–positive cells showed *KEAP1* editing in ~55% cells. There was no detectable off-target cleavage in the top three predicted genes in the ATTO 550–positive cells. Gene expression analysis found significantly ($p \leq 0.01$) higher expression of *NQO1* mRNA in ATTO 550–positive cells compared with control cells. Flow cytometric assessment showed increased ($p \leq 0.01$) frequency of CD4-, CD25-, and CD69-expressing *KEAP1* edited cells whereas frequency of CD8- ($p \leq 0.01$) and IL-17– ($p \leq 0.05$) expressing cells was reduced compared with control cells. Similar experimental conditions resulted in significant *KEAP1* editing, increased antioxidant gene expression, and frequency of CD69 and IL-10 positive cells in highly enriched *KEAP1*-edited regulatory T cells. *KEAP1*-edited T cells could potentially be used for treating multiple human diseases. *The Journal of Immunology*, 2018, 200: 1929–1936.

In concert with other immune mediators, T lymphocytes elicit adaptive immune responses following an Ag exposure. In addition to mounting an Ag-specific immune response, T lymphocytes sense and respond to varying oxygen concentrations (1, 2). Significant experimental and clinical data indicate T lymphocyte involvement during ischemia reperfusion (IR)-induced tissue injury and repair, where oxidative stress–dependent mechanisms appear to modulate T cell responses (3, 4).

Previous research demonstrated that T lymphocyte–specific genetic deletion of kelch like-ECH-associated protein 1 (*Keap1*), used to upregulate nuclear NF erythroid-derived 2 like 2 (NRF2) activation, significantly enhanced antioxidant responses, whereas adoptive transfer of *Keap1*-deficient T lymphocytes protected wild-type mice from experimental IR-induced acute kidney injury (AKI) (5). KEAP1 is an adapter protein for the E3 ubiquitin ligase complex that tags NRF2 for ubiquitination and proteasomal degradation (6). NRF2 is a b-ZIP transcription factor that regulates multiple prosurvival genes including NADPH dehydrogenase quinone 1 (*NQO1*) and heme oxygenase 1 (*HO1*), and thus an attractive therapeutic target for various oxidative stress–related diseases (7–9). Although genetic deletion of *Keap1* using the *Cre/lox* system effectively increases T lymphocyte–specific NRF2 activity, which renders protection from IR injury in mice, this method is not clinically viable. Therefore, we harnessed clustered regularly interspaced short palindromic repeats (CRISPR) technology as a novel tool for ex vivo *KEAP1* editing in primary human T cells to develop T lymphocyte–based antioxidant therapy with the potential for clinical translation.

Genome editing using CRISPR technology, comprising of a Cas9 (*Streptococcus pyogenes*–derived RNA guided endonuclease) protein and a gene-specific guide RNA (gRNA), allows effective knockout and knockin of virtually any gene (10–12). Despite its immense success to edit genomes in a large number of cell types and initial approval for use in human clinical trials to treat certain cancers, the delivery of Cas9:gRNA or the ribonucleoprotein (RNP) complex in some cell types such as primary human T lymphocytes has been challenging (13). Moreover, targeting genes that encode for intracellular proteins poses additional difficulty in terms of identification and enrichment of the edited cells. Nonetheless, some research groups have reported successful use of the CRISPR technology to knockout CXCR type 4 and programmed cell death receptor 1 as well as targeted nucleotide replacement (all expressed on the cell surface) in human $CD4^+$ T cells (14–18).

*Division of Nephrology, Department of Medicine, Johns Hopkins University, Baltimore, MD 21205; and †Department of Pathology, Johns Hopkins University, Baltimore, MD 21205

ORCIDs: 0000-0003-2121-721X (S.A.L.); 0000-0002-3132-2948 (M.S.).

Received for publication June 5, 2017. Accepted for publication December 18, 2017.

This work was supported by grants from the National Institutes of Health (R01 DK111209) and the Living Legacy Foundation to H.R.

Address correspondence and reprint requests to Dr. Hamid Rabb, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Ross Research Building, Room 965, Baltimore, MD 21205. E-mail address: hrabb1@jhmi.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AKI, acute kidney injury; CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, CRISPR RNA; gRNA, guide RNA; HO1, heme oxygenase 1; IR, ischemia reperfusion; *Keap1*, kelch like-ECH-associated protein 1; *NQO1*, NADPH dehydrogenase quinone 1; Nrf2, nuclear NF erythroid-derived 2 like 2; RNP, ribonucleoprotein; T2, target site 2; tracrRNA, *trans*-activating crRNA; Treg, regulatory T.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1700812

Table I. List of crRNA sequences used for targeting *KEAP1*, exon 2

T1	5′-AGCCGCCCGCGGTGTAGATC-3′
T2	5′-CTACCTGGTCAAGATCTTCG-3′
T3	5′-GGAAGTTCGGCGTCAACGAG-3′

Table II. List of PCR primers used for detecting *KEAP1*-specific editing

Sense	5′-AGCCGCCCGCGGTGTAGATC-3′
Antisense	5′-CTACCTGGTCAAGATCTTCG-3′

In this study, we present data to demonstrate successful targeting of *KEAP1* gene in primary and immortalized human T cells, which significantly enhances their antioxidant potential. Our data show that CRISPR-based *KEAP1* editing results in significant upregulation of NRF2-dependent antioxidant genes. *KEAP1* editing was also found to induce immunological changes in T lymphocytes in addition to an increased antioxidant gene expression. Additionally, this study presents a strategy to enrich edited cells while targeting genes that encode intracellular proteins. This *KEAP1* editing and enrichment strategy in purified regulatory T (Treg) cells resulted in significant *KEAP1* gene editing, upregulated NRF2 regulated antioxidant genes, and induced immunological changes compared with control Treg cells. Successful expansion of *KEAP1*-edited cells can lead to the development of novel, ready to use, immune cell–based antioxidant therapy for a broad range of human diseases.

Materials and Methods

Jurkat T cell culture

Jurkat E6-1 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 containing 10% FBS, 10 mM HEPES, and 100 U/ml penicillin and streptomycin. A total of 2×10^5 cells were used per electroporation for each experimental condition.

Human T cell isolation and culture

Primary T cells were isolated from blood collected from healthy individuals by Ficoll gradient centrifugation and negative selection using EasySep human T cell isolation kit (Stemcell Technologies, Cambridge, MA). Treg cells were isolated using a $CD4^+CD25^+CD127^{dim/-}$ Treg cell isolation kit (Miltenyi Biotec, Auburn, CA). T cells and Treg cells were cultured in CTS OpTmizer T cell expansion media (Thermo Fisher Scientific, Waltham, MA) containing 2% OpTmizer T cell expansion supplement, 10 mM HEPES, and 100 U/ml penicillin and streptomycin, and stimulated with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) in the presence of IL-2 (50–1000 U/ml) for 48 h prior to electroporation as described elsewhere (15). A total of 5×10^6 cells were used for each electroporation. These studies were approved by the Johns Hopkins institutional review board.

Cas9:gRNA delivery and editing analysis

All CRIPSR-related reagents were purchased from IDT (San Jose, CA). Cas9:gRNA complex was prepared immediately before each experiment. Briefly, *KEAP1* and exon 2 specific CRISPR RNAs (crRNAs) were mixed in equimolar concentrations (200 μM) with *trans*-activating crRNA (tracrRNA) and allowed to form a gRNA (Table I). A complex of Cas9 (1.5 or 3 μM) and gRNA (1.8 or 3.6 μM) along with Alt-R Cas9 electroporation enhancer oligo (1.8 or 3.6 μM) was electroporated with a Neon Transfection kit and device (Invitrogen). Control cells were electroporated in the absence of Cas9:gRNA complex. Electroporation efficiency was assessed by eGFP mRNA (TriLink BioTechnologies, San Diego, CA) or ATTO 550–labeled tracrRNA. Editing of *KEAP1* gene as well as the top three off-target genes was estimated by a Surveyor mutation detection assay at different time points after electroporation using target-specific primers (Tables II, III).

Enrichment of KEAP1-edited primary T cells

ATTO 550–positive cells were flow-sorted using a MoFlo XDP (Beckman Coulter, Indianapolis, IN) cell sorter 24 h after Cas9:gRNA electroporation. Propidium iodide was added before sorting to exclude dead cells. Sorted cells were assessed visually with a Leica fluorescent microscope for purity and a Surveyor mutation detection assay for *KEAP1* editing.

Phenotypic and intracellular cytokine analysis of edited cells

Fluorochrome-conjugated Abs to the following human Ags were used for flow cytometric analysis of *KEAP1*-edited cells: TCR-BV421 (BioLegend, San Diego, CA), CD4-PerCP-Cy5.5 (BD Biosciences, Franklin Lakes, NJ), CD8-APC (BioLegend), CD25-BV605 (eBioscience, San Diego, CA), FOXP3-APC (eBioscience), or Alexa 488 (BioLegend), CD69-APC-Cy7 (BD Biosciences, Franklin Lakes, NJ), IFN-γ–PE (BD Biosciences), TNF-α–FITC (BD Biosciences), IL-4–Alexa Fluor 488 (BioLegend) IL-10–PE or APC (eBioscience), and IL-17–BV421 or PE (BioLegend). T lymphocytes ($\sim 5 \times 10^5$) were stimulated with leukocyte activation mixture (BD Pharmigen, San Jose, CA) containing PMA, ionomycin, and brefeldin A before staining for surface markers and intracellular cytokines. Labeled samples were analyzed with an LSRII flow cytometer (BD Biosciences). Unstained and unstimulated samples were used to correctly identify and gate cell populations during analysis using FlowJo software (Tree Star, Ashland, OR).

Antioxidant gene expression analysis

Total RNA from purified T cells was isolated with an RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed using RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific). Gene-specific TaqMan primer and probe sets (Applied Biosystems) were used to assess the transcriptional status of *NQO1*, *HO1*, *GCLM*, and *GCLC* in CFX96 real-time PCR (Bio-Rad, Hercules, CA). The expression value for each gene was normalized to β-actin and the relative fold expression values calculated using a ΔΔ cycle threshold method.

Statistical analysis

Data are presented as mean ± SEM or SD, and are compared by a paired, two-tailed Student *t* test for a single comparison between two groups. Statistical significance of difference was defined as a p value ≤ 0.05.

Results

Delivery of KEAP1-specific CRISPR/Cas9 results in NRF2 activity in Jurkat T cells

We used Jurkat cells (clone E6-1), a lymphoblastic human T cell line, to optimize *KEAP1* editing using CRISPR technology. We targeted three different target sites against *KEAP1* exon 2 (Table I) using two different electroporation conditions (1600 V per 10 ms per three pulses and 1700 V per 10 ms per three pulses) based on the previous published literature to determine the most effective *KEAP1* editing [Tables II and III, (16, 17)]. There was no effect on cell viability 72 h after electroporation under these electroporation conditions, although we observed fewer cells after 1700 V, indicating an initial deleterious effect of high-voltage electroporation on cell viability (Supplemental Fig. 1). Flow cytometric analysis showed comparable electroporation efficiencies (≥95% GFP-positive

Table III. List of PCR primers used for detecting off-target editing in top three predicted genes

PIGS (NM_033198)	
Sense	5′-GGTAGATGGAAGGCACAGTAAG-3′
Antisense	5′-CCTGACAGACAAAGCCAACTA-3′
HELZ2 (NM_001037335)	
Sense	5′-GAGACGCAGTGAAGGAAGAC-3′
Antisense	5′-GTCCACAGTGAAGGTCAAGAA-3′
TNIP1 (NM_001258456)	
Sense	5′-TCAGCGGAGTGAAAGGATTG-3′
Antisense	5′-AGAGAAGAAGGGAGGGAGAAA-3′

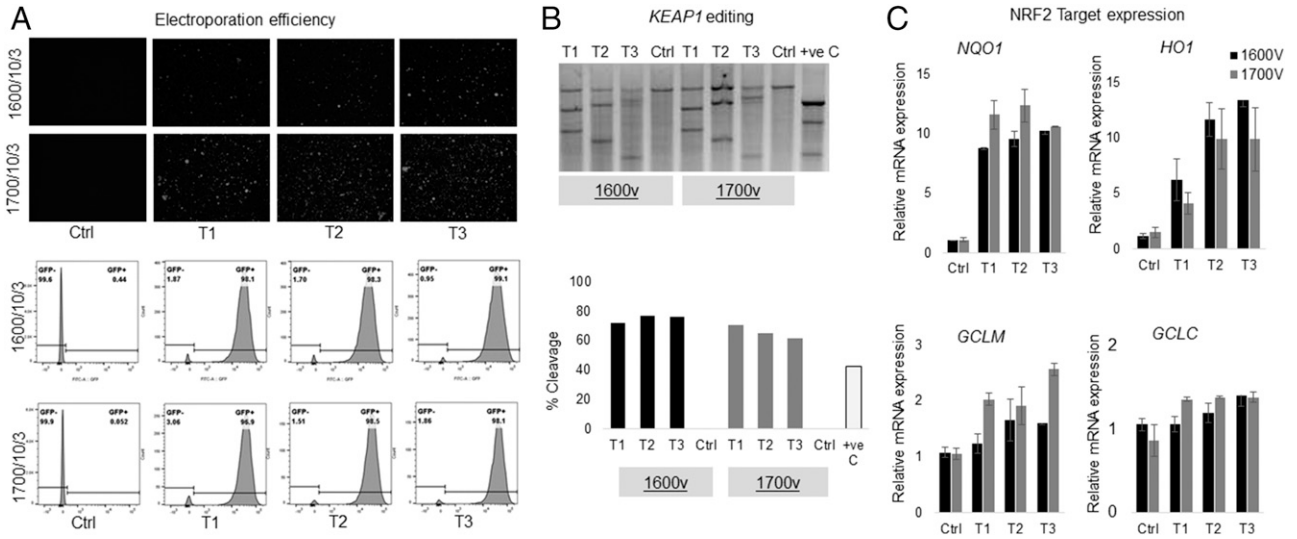

FIGURE 1. Delivery of *KEAP1*-specific RNP complex resulted in gene editing and NRF2 activation in Jurkat T cells. We tested three gRNAs to target three different target sites in *KEAP1*, exon 2. The RNP complexes were delivered using a Neon Transfection system at two different electroporation conditions (1600 V per 10 ms per three pulses and 1700 V per 10 ms per three pulses). (**A**) Flow cytometric analysis of GFP-positive cells 72 h after electroporation showed ≥95% electroporation efficiencies under both electroporation conditions. (**B**) Genomic cleavage analysis indicated *KEAP1* editing in ∼70% cells under 1600 V and ∼65% editing in 1700 V. (**C**) Real-time PCR–based assessment of NRF2 target genes 72 h after RNP complex delivery showed significant ($p \leq 0.05$) increases in *NQO1* (up to 11-fold), *HO1* (up to 11-fold), and *GCLM* (up to 2-fold) under these electroporation conditions.

cells) under these electroporation conditions (Fig. 1A). Genomic cleavage analysis indicated *KEAP1* editing in ∼70% cells under 1600 V and ∼65% editing in 1700 V (Fig. 1B). Analysis of NRF2 target genes 72 h after electroporation of the Cas9:gRNA complex resulted in significant ($p \leq 0.05$) increases in *NQO1* (up to 11-fold) and *HO1* (up to 11-fold) for all three target sites and under both electroporation conditions, whereas *GCLM* (up to 2-fold) levels increased mildly for all three target sites under 1700 V electroporation conditions (Fig. 1C). Similarly, delivery of all three *KEAP1*-specific Cas9:gRNA complexes under these conditions resulted in a significant ($p \leq 0.05$) increase in *NQO1* (up to 21-fold), *HO1* (up to 40-fold), *GCLM* (up to 6-fold), and *GCLC* (up to 3-fold) expression in primary human T cells, although electroporation efficiency was less (<70% compared with >95% GFP-positive cells) than that of Jurkat cells (Supplemental Fig. 2). In general, primary T cells were more vulnerable at high voltage electroporation as we observed low cell numbers and reduced NRF2 activation at 1700 V compared with 1600 V.

KEAP1 editing using CRISPR/Cas9 upregulates NRF2 activity in primary human T cells

Because we observed significant editing with all three *KEAP1*-specific gRNA tested in Jurkat T cells and primary T cells in our optimization studies, we decided to use target site 2 (T2) specific gRNA in primary T cells. Furthermore, we used ATTO 550–labeled tracrRNA to directly measure electroporation of the RNP complex in primary T cells instead of enhanced GFP mRNA, which does not directly represent Cas9:gRNA complex electroporation properties. ATTO 550–labeled tracrRNA is an effective tool for monitoring transfection efficiency using microscopy and flow cytometry as it forms an integral part of the RNP complex, thus providing a more reliable electroporation measurement of the RNP complex. Labeling of tracrRNA with ATTO 550 does not affect specificity of gRNA and activity of Cas9 protein. Flow cytometric analysis of primary T cells 24 h after electroporation showed up to ∼80% ATTO 550–positive cells, confirming RNP complex delivery (Fig. 2A). *KEAP1* editing analysis showed cleavage in ∼40% of

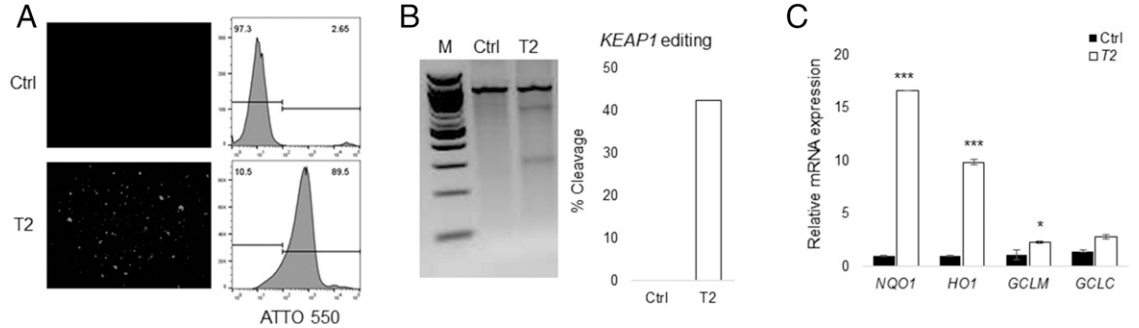

FIGURE 2. *KEAP1* editing resulted in significant NRF2 activation in primary T cells. We used a T2-specific RNP complex containing ATTO 550–labeled tracrRNA in primary T cells ($n = 3$). ATTO 550–labeled tracrRNA forms an integral part of the RNP complex and provided better assessment of transfection efficiency. (**A**) Microscopic and flow cytometric analysis of primary T cells 24 h after electroporation showed up to ∼80% cells received RNP complex. (**B**) *KEAP1* editing analysis using a Surveyor mutation assay showed cleavage in ∼40% cells that received RNP complex in comparison with control cells. (**C**) There was a significant increase in *NQO1* (16-fold), *HO1* (9-fold), and *GCLM* (2-fold) mRNA expression in primary T cell, 72 h after RNP complex electroporation, compared with control cells. *$p \leq 0.05$, ***$p \leq 0.001$.

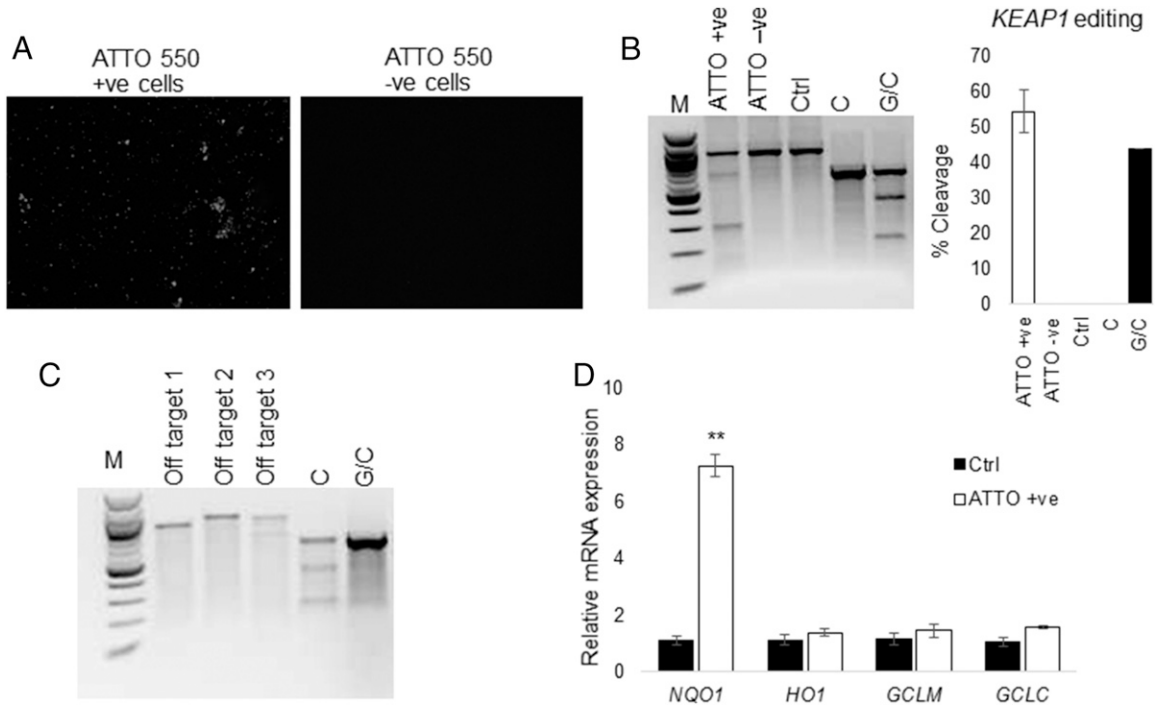

FIGURE 3. Enrichment of *KEAP1*-edited primary human T cells. Because ATTO 550 tracrRNA forms an integral part of the RNP complex we sorted ATTO 550–positive cells ($n = 4$) 24 h after electroporation and assessed *KEAP1*-editing and Nrf2 activity. (**A**) Fluorescent microscopic images of ATTO 550–positive and negative cells 90 h after sorting. (**B**) Assessment of *KEAP1* editing, 90 h after sorting (120 h after electroporation), showed cleavage in ∼55% ATTO 550–positive T cells with no detectable editing in the ATTO 550–negative cells or control cells. (**C**) Surveyor enzyme–based genomic mutation detection analysis showed no detectable off-target effect in the ATTO 550–positive cells. (**D**) Real-time PCR–based gene expression analysis showed significantly ($p \leq 0.01$) higher expression of *NQO1* mRNA in ATTO 550–positive cells compared with control cells. There was no difference in the mRNA level of other NRF2 target genes between ATTO 550–positive and control cells at this time point.

RNP-treated cells in comparison with control cells (Fig. 2B). Moreover, we observed a significant increase in the expression of NRF2 target genes *NQO1* ($p \leq 0.01$; 16-fold), *HO1* ($p \leq 0.01$, 9-fold), and *GCLM* ($p \leq 0.04$; 2-fold) in primary T cells, 72 h after RNP complex electroporation, compared with control cells (Fig. 2C).

Enrichment of KEAP1-edited primary T cells

Because there is no established method to enrich edited cells in situations where the target gene encodes for an intracellular protein, we decided to use ATTO 550–labeled tracrRNA for enrichment of *KEAP1*-edited primary T cells. We sorted ATTO 550–positive cells 24 h after electroporation of the RNP complex (Fig. 3A). Analysis of *KEAP1* editing, 90 h after sorting, using the Surveyor mutation detection assay showed cleavage in ∼55% ATTO 550–positive T cells ($n = 4$) with no detectable editing in the ATTO 550–negative cells, indicating that labeled tracrRNA could be useful to enrich edited cells under these conditions (Fig. 3B). In addition to the on-target editing effects of RNP complex on *KEAP1* gene, we also examined for potential off-target effects of CRISPR-mediated *KEAP1* editing. We selected the top three off-target genes, identified using CRISPR design tool (crispr.mit.edu). Mutation detection analysis showed no detectable off-target effect for the selected genes in this study (Fig. 3C). Real-time PCR–based NRF2 target gene expression analysis of ATTO 550–positive cells showed significantly ($p \leq 0.01$) higher expression of *NQO1* (6.5-fold) in comparison with control cells, 120 h after RNP delivery (Fig. 3D). There was no significant difference in the expression of other NRF2 target genes between ATTO 550–positive and control cells 120 h after RNP delivery.

KEAP1 editing induces immunological changes in primary T cells

To further understand the functional effects of *KEAP1* editing on primary T cells we investigated the expression of CD4, CD8, CD25, CD69, and T cell–specific intracellular cytokines in *KEAP1* edited cells (ATTO 550 positive) and control cells. *KEAP1* editing significantly increased the frequency of $CD4^+$ ($47.5 \pm 3.1\%$ versus $24.1 \pm 3\%$; $p \leq 0.01$) T cells, whereas frequency of $CD8^+$ ($47.8 \pm 3.4\%$ versus $68.5 \pm 2.5\%$; $p \leq 0.01$) T cells was reduced compared with control cells, 120 h post–RNP delivery. Furthermore, *KEAP1*-edited T cells expressed significantly higher levels of CD25 ($14.9 \pm 2\%$ versus $6.3 \pm 0.4\%$; $p \leq 0.01$) and CD69 ($9.1 \pm 1.2\%$ versus $1 \pm 0.3\%$; $p \leq 0.01$) in comparison with control cells (Fig. 4A). We found no significant difference in frequencies of cells producing TNF-α ($13.5 \pm 3.4\%$ versus $9 \pm 2.4\%$; $p = 0.36$), IFN-γ ($9.3 \pm 1.7\%$ versus $10.8 \pm 1.3\%$; $p = 0.54$), IL-4 ($13.7 \pm 4.4\%$ versus $6.5 \pm 1.6\%$; $p = 0.20$), or IL-10 ($14.4 \pm 4.5\%$ versus $4.7 \pm 1.3\%$; $p = 0.09$) between *KEAP1*-edited and control cells. However, IL-17 production was significantly reduced ($6.1 \pm 1.1\%$ versus $21.3 \pm 5.5\%$; $p = 0.04$) in *KEAP1*-edited cells (Fig. 4B).

Treg-specific KEAP1 editing upregulates antioxidant genes, CD69 and IL-10

We next tested this *KEAP1*-editing and enrichment strategy in purified human Treg cells. We used magnetic beads to enrich $CD4^+CD25^+CD127^{dim/-}$ Treg cells that contained over 90% cells positive for CD25 and Foxp3 (Fig. 5A). Delivery of *KEAP1*-specific (T2) Cas9:gRNA complex resulted in *KEAP1* editing in ∼35% cells (Fig. 5B) and significant increase in NRF2 target

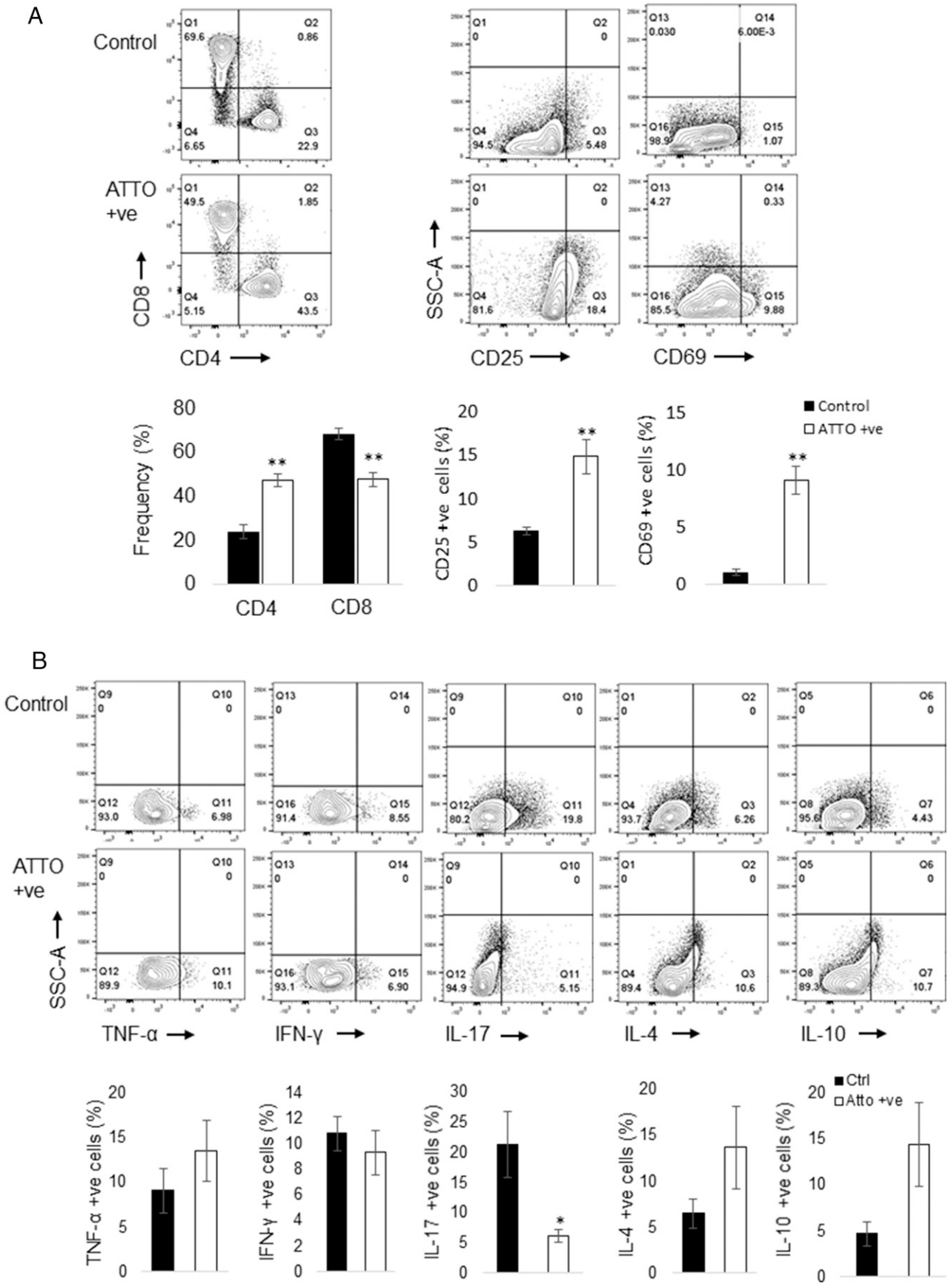

FIGURE 4. *KEAP1* editing–induced immunological changes in primary human T cells. (**A**) *KEAP1* editing significantly increased the frequency of CD4 cells whereas frequency of CD8 cells was reduced compared with control cells, 5 d post–RNP delivery. Furthermore, KEAP1-edited T cells expressed significantly higher levels of CD25 and CD69 in comparison with control cells. (**B**) The frequency of IL-17–producing cells was significantly reduced in ATTO 550–positive *KEAP1* edited cells in comparison with control cells. There was no difference in frequency of cells producing TNF-α, IFN-γ, IL-4, IL-10 between *KEAP1*-edited and control cells. $*p \leq 0.05$, $**p \leq 0.01$.

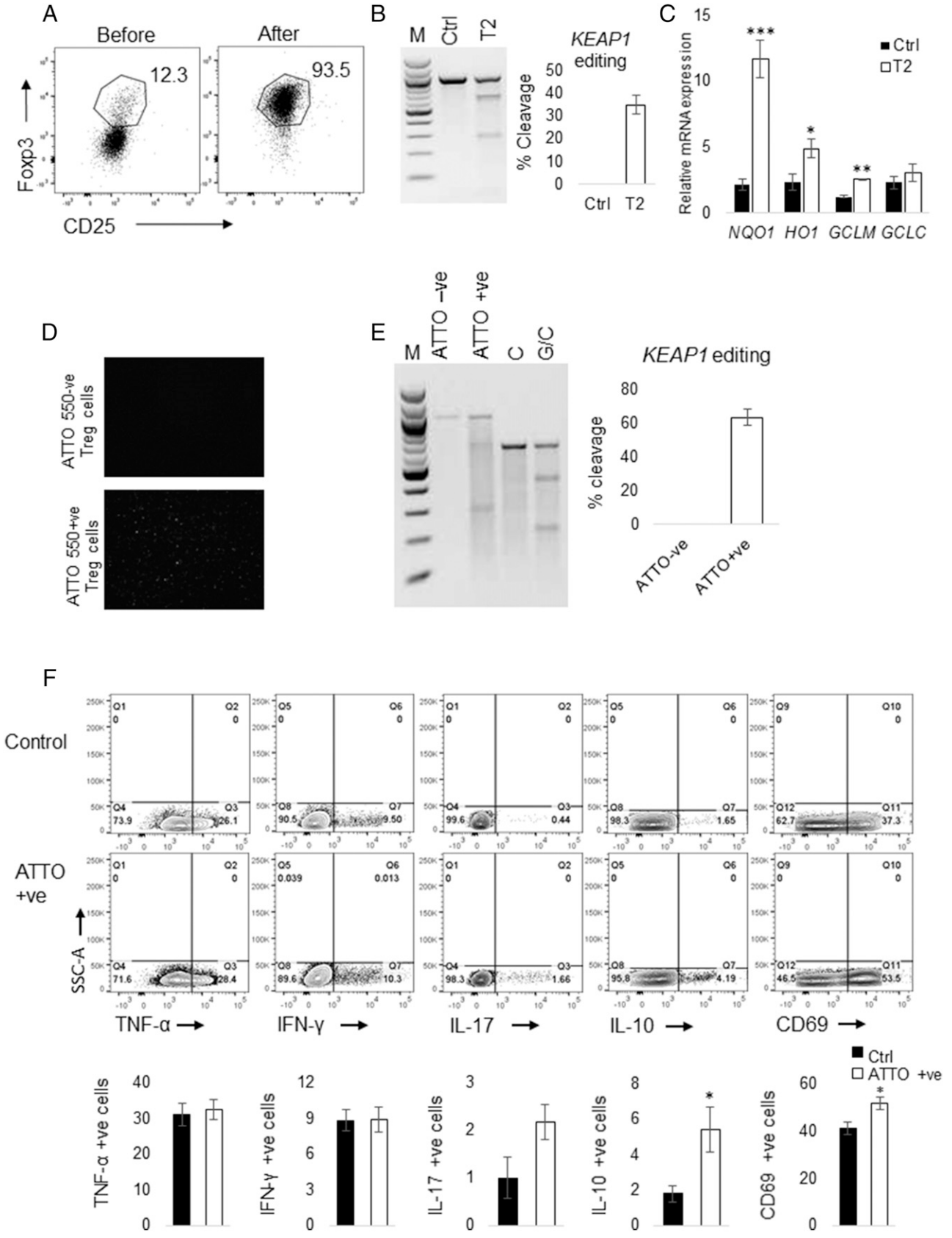

FIGURE 5. Treg-specific *KEAP1* editing increases NRF2 regulated antioxidant gene expression and frequency of CD69- and IL-10–positive cells. (**A**) Isolation of $CD4^{+}CD25^{+}CD127^{dim/-}$ cells using magnetic beads resulted in the enrichment of highly purified (>90%) Treg cells that were positive for CD25 and Foxp3. (**B**) Delivery of *KEAP1*-specific Cas9:gRNA complex resulted in *KEAP1* editing in ~35% cells. (**C**) *KEAP1*-edited cells had significantly increased *NQO1* (5-fold), *HO1* (2-fold), and *GCLM* (2-fold) mRNA levels compared with control cells. (**D**) ATTO 550–positive, *KEAP1*-edited cells were enriched using flow sorting and assessed using fluorescent microscope. (**E**) Enrichment of ATTO 550–positive *KEAP1*-edited cells improved percent cleavage to ~63% cells. (**F**) Flow cytometric analysis of ATTO 550–positive *KEAP1*-edited *(Figure legend continues)*

genes *NQO1* ($p \leq 0.001$), *HO1*($p \leq 0.05$), and *GCLM* ($p \leq 0.01$) mRNA levels (Fig. 5C). Furthermore, flow sorting–based enrichment of ATTO 550–positive cells improved cleavage to ~63% cells (Fig. 5D, 5E, $n = 3$). Flow cytometric analysis of enriched *KEAP1*-edited Treg cells showed increased frequency of CD69- (52.0 ± 2.7% versus 41.3 ± 2.8%, $p \leq 0.05$) and IL-10– (5.4 ± 1.3% versus 1.8 ± 0.4%, $p \leq 0.05$) expressing cells compared with control Treg cells (Fig. 5F). Frequency of TNF-α– (32.5 ± 2.7% versus 31.1 ± 3.1%; $p = 0.75$), IFN-γ– (8.9 ± 1.1% versus 8.9 ± 0.9%; $p = 0.97$), or IL-17– (2.2 ± 0.4% versus 1.0 ± 0.4%; $p = 0.10$) producing cells was not significantly different between *KEAP1* edited and control Treg cells.

Discussion

CRISPR-based genome editing allows specific gene targeting and is revolutionizing the field of experimental medicine (19). Our present study demonstrates that CRISPR technology can be used to successfully engineer both immortalized and primary human T cells for therapeutic enhancement of NRF2-regulated antioxidant capacity. The rationale to edit T lymphocyte *KEAP1* was based on our previous data in T cell–specific *Keap1*-deficient mice, which demonstrated significant upregulation of NRF2-regulated antioxidant gene expression and protection from IR-induced AKI (5) and myocardial injury (S. Noel and H. Rabb, unpublished observations). Adoptive transfer of T cells with augmented antioxidant activity protected kidneys from AKI and improved survival in wild-type mice, indicating that transfer of engineered T cells with enhanced antioxidant activity could potentially be used as immune cell–based therapy for various oxidative stress–driven diseases.

In this study, we first optimized *KEAP1*-editing conditions in Jurkat T cells and subsequently targeted *KEAP1* in primary human T cells as well as purified Treg cells. Present experimental conditions and the enrichment strategy using fluorescently labeled tracrRNA resulted in *KEAP1* editing in ~55% primary T cells and ~63% Treg cells. Despite modest editing efficiency, we observed significant augmentation of NRF2 target gene expression in total T cells as well as purified Treg cell populations. This increase in basal antioxidant gene expression was expected and comparable to that of *Keap1*-deficient T cells from our conditional knockout mice and other NRF2 activation studies using pharmacologic activators (6, 20–22). We found multiple antioxidant genes (*NQO1, HO1*, and *GCLM*) upregulated 72 h after Cas9:gRNA delivery, but only *NQO1* remained elevated at 120 h, indicating that the edited cells attain a more stable transcriptional status following an initial surge under basal conditions. In addition to an increased antioxidant gene expression, we observed distinct phenotypic (increased CD4 and reduced CD8 cell frequency in total T cell population) and functional (high CD25- and CD69- and low IL-17–positive cell frequency in total T cells and high CD69- and IL-10–positive cell frequency in purified Treg cells) differences between the *KEAP1* edited primary T cell and control cells. The exact reason for increased CD4 and reduced CD8 frequency is not clear but could be due to better electroporation of ATTO 550–labeled RNP complex in CD4 cells than CD8 cells. Although some studies suggest an attenuated proinflammatory response upon NRF2 activation, Morzadec et al. (23) found no effect of NRF2 activation on cytokine secretion by human T cells (20, 24). Thus, it is not entirely clear how KEAP1/NRF2 modulates cytokine expression in T cells, however, *KEAP1* editing in primary T cells may induce regulatory features that increase anti-inflammatory and suppressive functions.

CRISPR-based *KEAP1* editing may induce additional changes that were not investigated in this study. For example, reactive oxygen species production by activated T cells triggers glutathione response to scavenge increasing reactive oxygen species and prevent cellular damage (25) Therefore, *KEAP1* editing could modulate metabolic integration and reprogramming during inflammatory T cell responses. Another possible effect could be on programmed cell death receptor 1, which inhibits T cell activation (26). Furthermore, *KEAP1* editing may affect epigenetic elements such as histone deacetylases and histone acetyltransferases that modulate suppressive function, mainly of Treg cells. In addition to these, *KEAP1* editing may modulate T cell differentiation via Notch signaling. Notch signaling has been shown to be a critical regulator of T cell differentiation, which was found to improve hematopoietic progenitor stem cell function and myelosuppression following radiation exposure in an NRF2-dependent manner (27).

Apart from T cell–specific *KEAP1* editing, CRISPR/Cas9-based *KEAP1* editing can be carried out in additional cell types. KEAP1 has been shown to modulate metabolic shifts from oxidative to glycolytic energy production during induced pluripotent stem cell reprogramming (28). Therefore, editing of *KEAP1* using CRISPR technology may be a useful tool for controlling induced pluripotent stem cell reprogramming. Furthermore, CRISPR-based gene editing appears to produce more robust functional effects (29). *KEAP1* ablation using CRISPR technology has also been found to modulate the sensitivity of lung cancer for kinase-targeted therapy (30). In a recent study Zagorski et al. (24) employed CRISPR/Cas9 to delete *NRF2* in Jurkat T cell line and found significant reduction in *NQO1* mRNA but no immunological effect at steady state.

Even though we did not observe any detectable off-target effects of CRISPR-based *KEAP1* editing, this study lacks a comprehensive sequence-based analysis of nonspecific effects in *KEAP1*-edited T cells. The lack of suitable animal models further limits functional characterization of these *KEAP1*-edited human T cells in in vivo disease models. Moreover, this study lacks investigation into NRF2-independent effects of *KEAP1* editing. Despite its limitations CRISPR-based *KEAP1* editing appears to be promising for permanent and specific NRF2 activation compared with reversible and nonspecific pharmacologic or small interfering RNA–based approaches (31). Successful expansion of *KEAP1*-edited T cells is expected to result in the development of a novel T cell–based antioxidant therapy.

Acknowledgments

We thank all the volunteers for donating blood samples for this study and a research gift from Rogelio Miro of Panama.

Disclosures

The authors have no financial conflicts of interest.

Treg cells showed increased frequency of CD69- (52.0 ± 2.7 versus 41.3 ± 2.8%) and IL-10– (5.4 ± 1.3 versus 1.8 ± 0.4) positive cells compared with control Treg cells. The frequency of TNF-α– (32.5 ± 2.7 versus 31.1 ± 3.1%; $p = 0.75$), IFN-γ– (8.9 ± 1.1% versus 8.9 ± 0.9%), and IL-17– (2.17 ± 0.4% versus 1.0 ± 0.4%) positive cells was not different between *KEAP1* edited and control Treg cells. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

References

1. Clever, D., R. Roychoudhuri, M. G. Constantinides, M. H. Askenase, M. Sukumar, C. A. Klebanoff, R. L. Eil, H. D. Hickman, Z. Yu, J. H. Pan, et al. 2016. Oxygen sensing by T cells establishes an immunologically tolerant metastatic niche. *Cell* 166: 1117–1131.e14.
2. De Ciuceis, C., C. Agabiti-Rosei, C. Rossini, P. Airò, M. Scarsi, A. Tincani, G. A. M. Tiberio, S. Piantoni, E. Porteri, L. Solaini, et al. 2017. Relationship between different subpopulations of circulating CD4+ T lymphocytes and microvascular or systemic oxidative stress in humans. *Blood Press.* 26: 237–245.
3. Burne, M. J., F. Daniels, A. El Ghandour, S. Mauiyyedi, R. B. Colvin, M. P. O'Donnell, and H. Rabb. 2001. Identification of the CD4(+) T cell as a major pathogenic factor in ischemic acute renal failure. *J. Clin. Invest.* 108: 1283–1290.
4. Gandolfo, M. T., H. R. Jang, S. M. Bagnasco, G. J. Ko, P. Agreda, S. R. Satpute, M. T. Crow, L. S. King, and H. Rabb. 2009. Foxp3+ regulatory T cells participate in repair of ischemic acute kidney injury. *Kidney Int.* 76: 717–729.
5. Noel, S., M. N. Martina, S. Bandapalle, L. C. Racusen, H. R. Potteti, A. R. Hamad, S. P. Reddy, and H. Rabb. 2015. T lymphocyte-specific activation of Nrf2 protects from AKI. *J. Am. Soc. Nephrol.* 26: 2989–3000.
6. Noel, S., A. R. Hamad, and H. Rabb. 2015. Reviving the promise of transcription factor Nrf2-based therapeutics for kidney diseases. *Kidney Int.* 88: 1217–1218.
7. Wu, Q. Q., Y. Wang, M. Senitko, C. Meyer, W. C. Wigley, D. A. Ferguson, E. Grossman, J. Chen, X. J. Zhou, J. Hartono, et al. 2011. Bardoxolone methyl (BARD) ameliorates ischemic AKI and increases expression of protective genes Nrf2, PPARγ, and HO-1. *Am. J. Physiol. Renal Physiol.* 300: F1180–F1192.
8. Wright, M. M., J. Kim, T. D. Hock, N. Leitinger, B. A. Freeman, and A. Agarwal. 2009. Human haem oxygenase-1 induction by nitro-linoleic acid is mediated by cAMP, AP-1 and E-box response element interactions. *Biochem. J.* 422: 353–361.
9. Ano, S., A. Panariti, B. Allard, M. O'Sullivan, T. K. McGovern, Y. Hamamoto, Y. Ishii, M. Yamamoto, W. S. Powell, and J. G. Martin. 2017. Inflammation and airway hyperresponsiveness after chlorine exposure are prolonged by Nrf2 deficiency in mice. *Free Radic. Biol. Med.* 102: 1–15.
10. Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816–821.
11. Mali, P., K. M. Esvelt, and G. M. Church. 2013. Cas9 as a versatile tool for engineering biology. *Nat. Methods* 10: 957–963.
12. Mali, P., L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville, and G. M. Church. 2013. RNA-guided human genome engineering via Cas9. *Science* 339: 823–826.
13. Han, X., Z. Liu, M. C. Jo, K. Zhang, Y. Li, Z. Zeng, N. Li, Y. Zu, and L. Qin. 2015. CRISPR-Cas9 delivery to hard-to-transfect cells via membrane deformation. *Sci. Adv.* 1: e1500454.
14. Hultquist, J. F., K. Schumann, J. M. Woo, L. Manganaro, M. J. McGregor, J. Doudna, V. Simon, N. J. Krogan, and A. Marson. 2016. A Cas9 ribonucleoprotein platform for functional genetic studies of HIV-host interactions in primary human T cells. *Cell Reports* 17: 1438–1452.
15. Schumann, K., S. Lin, E. Boyer, D. R. Simeonov, M. Subramaniam, R. E. Gate, G. E. Haliburton, C. J. Ye, J. A. Bluestone, J. A. Doudna, and A. Marson. 2015. Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *Proc. Natl. Acad. Sci. USA* 112: 10437–10442.
16. Liang, X., J. Potter, S. Kumar, Y. Zou, R. Quintanilla, M. Sridharan, J. Carte, W. Chen, N. Roark, S. Ranganathan, et al. 2015. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J. Biotechnol.* 208: 44–53.
17. Liang, X., J. Potter, S. Kumar, N. Ravinder, and J. D. Chesnut. 2017. Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. *J. Biotechnol.* 241: 136–146.
18. Su, S., Z. Zou, F. Chen, N. Ding, J. Du, J. Shao, L. Li, Y. Fu, B. Hu, Y. Yang, et al. 2016. CRISPR-Cas9-mediated disruption of PD-1 on human T cells for adoptive cellular therapies of EBV positive gastric cancer. [Published erratum appears in 2017 *Oncoimmunology* 6: e1311485.] *OncoImmunology* 6: e1249558.
19. Miyagi, A., A. Lu, and B. D. Humphreys. 2016. Gene editing: powerful new tools for nephrology research and therapy. *J. Am. Soc. Nephrol.* 27: 2940–2947.
20. Rockwell, C. E., M. Zhang, P. E. Fields, and C. D. Klaassen. 2012. Th2 skewing by activation of Nrf2 in CD4(+) T cells. *J. Immunol.* 188: 1630–1637.
21. Turley, A. E., J. W. Zagorski, and C. E. Rockwell. 2015. The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells. *Cytokine* 71: 289–295.
22. Liu, M., N. M. Reddy, E. M. Higbee, H. R. Potteti, S. Noel, L. Racusen, T. W. Kensler, M. B. Sporn, S. P. Reddy, and H. Rabb. 2014. The Nrf2 triterpenoid activator, CDDO-imidazolide, protects kidneys from ischemia-reperfusion injury in mice. *Kidney Int.* 85: 134–141.
23. Morzadec, C., M. Macoch, L. Sparfel, S. Kerdine-Römer, O. Fardel, and L. Vernhet. 2014. Nrf2 expression and activity in human T lymphocytes: stimulation by T cell receptor activation and priming by inorganic arsenic and tert-butylhydroquinone. *Free Radic. Biol. Med.* 71: 133–145.
24. Zagorski, J. W., T. P. Maser, K. T. LIby, and C. E. Rockwell. 2017. Nrf2-dependent and -independent effects of tBHQ, CDDO-Im, and H2O2 in human Jurkat T cells as determined by CRISPR/Cas9 gene editing. *J. Pharmacol. Exp. Ther.* 361: 259–267.
25. Mak, T. W., M. Grusdat, G. S. Duncan, C. Dostert, Y. Nonnenmacher, M. Cox, C. Binsfeld, Z. Hao, A. Brüstle, M. Itsumi, et al. 2017. Glutathione primes T cell metabolism for inflammation. *Immunity* 46: 675–689.
26. Pan, F., H. Fan, Z. Liu, and S. Jiang. 2012. T cell signaling targets for enhancing regulatory or effector function. *Sci. Signal.* 5: pe32.
27. Kim, J. H., R. K. Thimmulappa, V. Kumar, W. Cui, S. Kumar, P. Kombairaju, H. Zhang, J. Margolick, W. Matsui, T. Macvittie, et al. 2014. NRF2-mediated Notch pathway activation enhances hematopoietic reconstitution following myelosuppressive radiation. *J. Clin. Invest.* 124: 730–741.
28. Hawkins, K. E., S. Joy, J. M. Delhove, V. N. Kotiadis, E. Fernandez, L. M. Fitzpatrick, J. R. Whiteford, P. J. King, J. P. Bolanos, M. R. Duchen, et al. 2016. NRF2 orchestrates the metabolic shift during induced pluripotent stem cell reprogramming. *Cell Reports* 14: 1883–1891.
29. Eyquem, J., J. Mansilla-Soto, T. Giavridis, S. J. van der Stegen, M. Hamieh, K. M. Cunanan, A. Odak, M. Gönen, and M. Sadelain. 2017. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* 543: 113–117.
30. Krall, E. B., B. Wang, D. M. Munoz, N. Ilic, S. Raghavan, M. J. Niederst, K. Yu, D. A. Ruddy, A. J. Aguirre, J. W. Kim, et al. 2017. KEAP1 loss modulates sensitivity to kinase targeted therapy in lung cancer. *eLife* 6: e18970.
31. Schmidt, H. H., R. Stocker, C. Vollbracht, G. Paulsen, D. Riley, A. Daiber, and A. Cuadrado. 2015. Antioxidants in translational medicine. *Antioxid. Redox Signal.* 23: 1130–1143.