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KEAP1 editing using CRISPR/Cas9 for therapeutic NRF2 activation in primary human T lymphocytes

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Abstract

Oxidant stress modifies T lymphocyte activation and function. Previous work demonstrated that murine T cell specific *Keap1* deletion enhances antioxidant capacity and protects from experimental acute kidney injury. Here, we used CRISPR technology to develop clinically translatable human T cell-specific *KEAP1* deletion. Delivery of *KEAP1* exon 2 specific Cas9:gRNA in Jurkat T cells led to significant (~70%) editing and upregulation of NRF2 regulated antioxidant genes *NQO1* (upto 11 fold), *HO1* (upto 11 fold) and *GCLM* (upto 2 fold). In primary human T cells, delivery of *KEAP1* exon 2 target site 2-specific ATTO 550-labeled Cas9:gRNA 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 24h after electroporation. Assessment of ATTO 550 positive cells showed *KEAP1* editing in ~55% cells. There was no detectable off-target cleavage in top 3 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 to 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 to 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 Treg cells. *KEAP1* edited T cells could potentially be used for treating multiple human diseases.

Introduction

T lymphocytes in concert with other immune mediators elicit adaptive immune responses following an antigen exposure. In addition to mounting antigen-specific immune response, T lymphocytes sense and respond to varying oxygen concentrations (1, 2). Significant experimental and clinical data indicates 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).

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Previous research demonstrated that T lymphocyte specific genetic deletion of *Keap1* (kelch like-ECH-associated protein 1), used to upregulate nuclear Nrf2 (nuclear factor erythroid-derived 2 like 2) activation, significantly enhanced antioxidant responses, while adoptive transfer of *Keap1*-deficient T lymphocytes protected wild type (WT) 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 pro-survival genes including *NQO1* (NADPH dehydrogenase quinone 1) and *HO1* (heme oxygenase 1) and thus an attractive therapeutic target for various oxidative stress-related diseases (7-9). Although, genetic deletion of *Keap1* using *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 CRISPR (clustered regularly interspaced short palindromic repeats) technology as a novel tool for *ex vivo KEAP1* editing in primary human T cells to develop T lymphocyte based antioxidant therapy with 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 knock-out and knock-in of virtually any gene (10-12). In spite of its immense success to edit genome in large number of cell types and initial approval to use in human clinical trial 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 term of identification and enrichment of the edited cells. Nonetheless, some research groups have reported successful use of the CRISPR technology to knock-out CXC chemokine receptor type 4 (CXCR4) and programmed cell death receptor 1 (PD1) as well as targeted nucleotide replacement (all expressed on cell surface) in human $CD4^+$ T cells (14-18).

Here, we present data to demonstrate successful targeting of *KEAP1* gene in primary and immortalized human T cells that 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 to 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 American Type Culture Collection (ATCC, 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 $CD4^+CD25^+CD127^{dim/-}$ Treg cell isolation kit (Miltenyi Biotech, Auburn, CA). T cells and Treg cells were cultured in CTS OpTmizer T cell expansion media (ThermoFisher, 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 (10μg/ml) and anti-CD28 (10μg/ml) in the presence of interleukin 2 (IL-2) (50–1000 U/ml) for 48h prior to electroporation as described elsewhere (15). A total of 5×10^6 cells were used for each electroporation. These studies were approved by Johns Hopkins institutional review board.

Cas9:guide RNA 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*, 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 1). A complex of Cas9 (1.5 or 3 μM) and gRNA (1.8 or 3.6μM) along with Alt-R Cas9 electroporation enhancer oligo (1.8 or 3.6μM) was electroporated with a Neon transfection kit and device (Invitrogen). Control cells were electroporated in the absence of Cas9:gRNA complex. Electroporation efficiency was assess by eGFP mRNA (TriLink Biotechnologies, San Diego, CA) or ATTO 550 labeled tracrRNA. Editing of *KEAP1* gene as well as top three off-target genes was estimated by Surveyor mutation detection assay at different time points after electroporation using target specific primers (Table 2 and 3).

Enrichment of KEAP1 edited primary T cells

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

Phenotypic and intracellular cytokine analysis of edited cells

Fluorochrome conjugated antibodies to following human antigens 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, San Diego, CA), CD25-BV605 (eBioscience, San Diego, CA), FoxP3-APC (eBioscience, San Diego, CA), or Alexa488 (BioLegend, San Diego, CA) CD69-APC-Cy7 (BD Biosciences, Franklin Lakes, NJ), IFNγ-PE (BD Biosciences, Franklin Lakes, NJ), TNFα-FITC (BD Biosciences, Franklin Lakes, NJ), IL4-AlexaFluor 488 (BioLegend, San Diego, CA) IL-10-PE or APC (eBioscience, San Diego, CA), and IL17-BV421 or PE (BioLegend, San Diego, CA). T

lymphocytes (~5×10^5) were stimulated with leukocyte activation cocktail (BD Pharmigen, San Jose, CA) containing PMA (Phorbol 12-Myristate 13-Acetate), ionomycin and brefeldin A before staining for surface markers and intracellular cytokines. Labelled samples were analyzed with LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ). Unstained and unstimulated samples were used to correctly identify and gate cell populations during analysis using FlowJo software (Tree Star Inc., Ashland, OR).

Antioxidant gene expression analysis

Total RNA from purified T cells was isolated with RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed using RevertAid first strand cDNA synthesis kit (ThermoFisher, Waltham, MA). Gene specific Taqman primer and probe sets (Applied Biosystems) were used to assess transcriptional status of *NQO1*, *HO1*, *GCLM* and *GCLC* in CFX96 real time PCR (BioRad, Hercules, California). The expression value for each gene was normalized to β-actin and the relative fold expression values calculated using δδCT method.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM) or standard deviation (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 cell

We used Jurkat cells (clone E6-1), a lymphoblastic human T cell line, to optimize *KEAP1* editing using CRISPR technology. We targeted 3 different target sites against *KEAP1* exon 2 using two different electroporation conditions (1600V/10ms/3pulse and 1700V/10ms/3pulse) based on the previous published literature to determine most effective *KEAP1* editing (Table 1 and (16, 17)). There was no effect on cell viability 72h after electroporation under these electroporation conditions, though we observed fewer cells after 1700V indicating an initial deleterious effect of high voltage electroporation on cell viability (Supplemental figure 1). Flow cytometric analysis showed comparable electroporation efficiencies (≥95% GFP positive cells) under these electroporation conditions (Figure 1A). Genomic cleavage analysis indicated *KEAP1* editing in ~70% cells under 1600V and ~65% editing in 1700V (Figure 1B). Analysis of NRF2 target genes 72h after electroporation of Cas9:gRNA complex resulted in significant ($p\leq0.05$) increases in *NQO1* (upto 11 fold) and *HO1* (upto 11 fold) for all three target sites and under both electroporation conditions where as *GCLM* (upto 2 fold) levels increased mildly for all three target sites under 1700V electroporation condition (Figure 1C). Similarly, delivery of all 3 *KEAP1* specific Cas9:gRNA complexes under these conditions resulted in a significant ($p\leq0.05$) increase in *NQO1* (upto 21 fold), *HO1* (upto 40 fold), *GCLM* (upto 6 fold) and *GCLC* (upto 3 fold) expression in primary human T cells, though electroporation efficiency was less (<70% compared to > 95% GFP positive cells) than that of Jurkat cells (Supplemental figure 2). In general, primary T cells were more vulnerable at high voltage electroporation as we observed low cell number and reduced NRF2 activation at 1700V compared to 1600V.

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

Since we observed significant editing with all three *KEAP1* sfpecific gRNA tested in Jurkat T cells and primary T cell 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 RNP complex in primary T cells instead of eGFP mRNA that 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 more reliable electroporation measurement of 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 24h after electroporation showed upto ~80% ATTO 550 positive cell confirming RNP complex delivery. *KEAP1* editing analysis showed cleavage in about 40% of RNP treated cells in comparison to control cells. 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 cell, 72h after RNP complex electroporation, compared to control cells.

Enrichment of KEAP1 edited primary T cells

Since 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 cell. We sorted ATTO 550 positive cells 24h after electroporation of RNP complex. Analysis of *KEAP1* editing, 90h after sorting, using Surveyor mutation enzyme 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. 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 top 3 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 (Figure 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 to control cells, 120h after RNP delivery. There was no significant difference in the expression of other NRF2 target genes between ATTO 550 positive and control cells 120h 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 cell (ATTO 550 positive) and control cells. *KEAP1* editing significantly increased the frequency of $CD4^+$ (47.5%±3.1% vs 24.1%±3%; $p \leq 0.01$) T cells whereas frequency of $CD8^+$ (47.8%±3.4% vs 68.5%±2.5%; $p \leq 0.01$) T cells was reduced compared to control cells, 120h post RNP delivery. Furthermore, *KEAP1* edited T cells expressed significantly higher level of CD25 (14.9%±2% vs 6.3%±0.4%; $p \leq 0.01$) and CD69 (9.1%±1.2% vs 1%±0.3%; $p \leq 0.01$) in comparison to control cells (Figure 4A). We found no significant difference in frequencies of cells producing TNF-α (13.5%±3.4% vs 9%±2.4%;

p=0.36), IFN-γ (9.3%±1.7% vs 10.8%±1.3%; p=0.54), IL-4 (13.7%±4.4% vs 6.5%±1.6%; p=0.20) or IL-10 (14.4%±4.5% vs 4.7%±1.3%; p= 0.09) between *KEAP1* edited and control cells. However, IL-17 production was significantly reduced (6.1%±1.1% vs 21.3%±5.5%; p=0.04) in *KEAP1* edited cells (Figure 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 (Figure 5A). Delivery of *KEAP1* specific (T2) Cas9:gRNA complex resulted in *KEAP1* editing in ~35% cells (Figure 5B) and significant increase in NRF2 target genes *NQO1* ($p \leq 0.001$), *HO1* ($p \leq 0.05$) and *GCLM* ($p \leq 0.01$) mRNA levels (Figure 5C). Furthermore, flow sorting based enrichment of ATTO 550 positive cells improved cleavage to ~63% cells (Figure 5D & E, n=3). Flow cytometric analysis of enriched *KEAP1* edited Treg cells showed increased frequency of CD69 (52.0%±2.7% vs 41.3%±2.8%, $p \leq 0.05$) and IL-10 (5.4%±1.3% vs 1.8%±0.4%, $p \leq 0.05$) expressing cells compared to control Treg cells (Figure 5F). Frequency of TNF-α (32.5%±2.7% vs 31.1% ±3.1%; p=0.75), IFN-γ (8.9%±1.1% vs 8.9%±0.9%; p=0.97), or IL-17 (2.2%±0.4% vs 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 that demonstrated significant upregulation of Nrf2 regulated antioxidant gene expression and protection from IR induced AKI (5) and myocardial injury (unpublished observation). Adoptive transfer of T cells with augmented antioxidant activity protected kidneys from AKI and improved survival in WT 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 enrichment strategy using fluorescently labeled tracrRNA resulted in *KEAP1* editing in about 55% primary T cells and 63% Treg cells. Despite modest editing efficiency, we observed significant augmentation of NRF2 target gene expression in total T cell as well as purified Treg cell population. This increase in basal antioxidant gene expression was expected and comparable to that of *Keap1* deficient T cells from our conditional knock out mice and other Nrf2 activation studies using pharmacologic activators (6, 20-22). We found multiple antioxidant genes (*NQO1, HO1* and *GCLM*) upregulated 72h after Cas9:gRNA delivery but only *NQO1* remained elevated at 120h time point 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 attenuated pro-inflammatory response upon NRF2 activation, Morzadec *et al.* found no effect of NRF2 activation on cytokine secretion by human T cells (20, 23, 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 (ROS) production by activated T cells triggers glutathione (GSH) response to scavenge increasing ROS and prevent cellular damage (25) Therefore, *KEAP1* editing could modulate metabolic integration and reprogramming during inflammatory T cell responses. Another possible affect could be on PD-1 that inhibits T cell activation (26). Furthermore, *KEAP1* editing may affect epigenetic elements such as histone deacetylases (HDACs) 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 that was found to improve hematopoietic progenitor stem cell (HSPC) 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 shift from oxidative to glycolytic energy production during iPSC reprogramming (28). Therefore, editing of *KEAP1* using CRISPR technology may be a useful tool for controlling iPSC 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 sensitivity of lung cancer for kinase targeted therapy (30). In a recent study Zagorski *et al.* 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 (24).

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 non-specific effects in *KEAP1* edited T cells. 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. In spite of its limitations CRISPR based *KEAP1* editing appears to be promising for permanent and specific NRF2 activation compared to reversible and non-specific pharmacologic or small interfering RNA (siRNA) 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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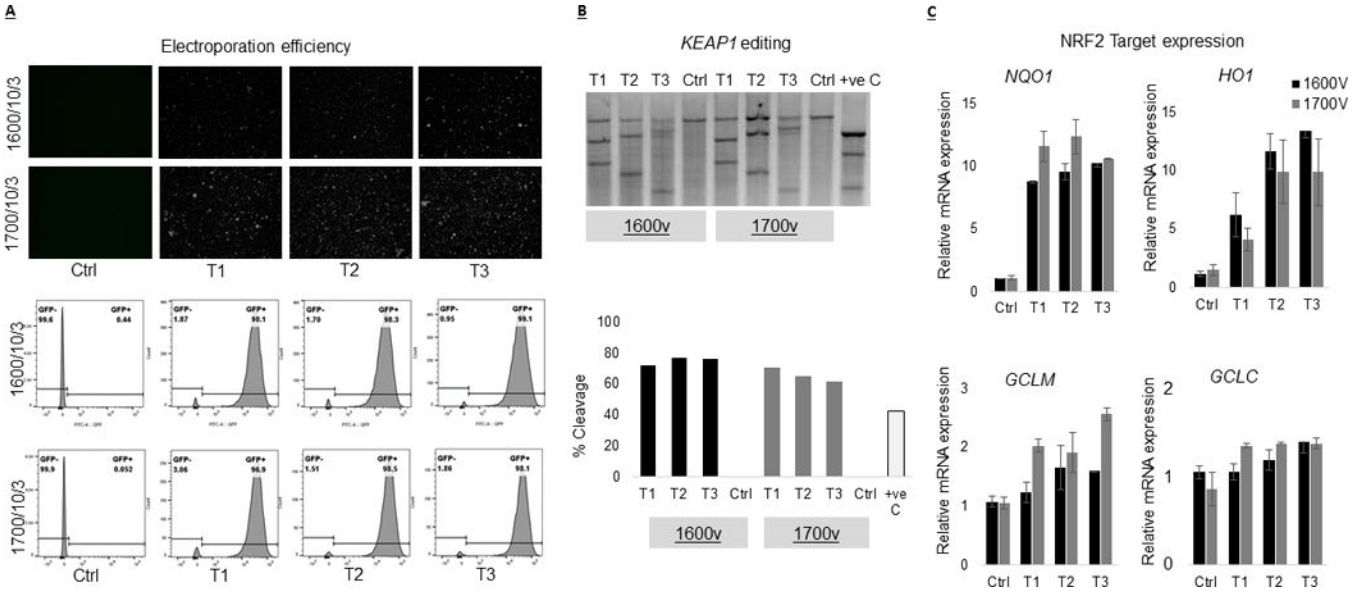

Figure 1.

Delivery of *KEAP1* specific RNP complex resulted in gene editing and NRF2 activation in Jurkat T cells. We tested guide RNA against three different target sites against *KEAP1*, exon 2. The RNP complexes were delivered using Neon Transfection system at two different electroporation conditions (1600V/10ms/3pulse and 1700V/10ms/3pulse). (A) Flow cytometric analysis of GFP positive cells 72h after electroporation showed ≥95% electroporation efficiencies under both electroporation conditions. (B) Genomic cleavage analysis indicated *KEAP1* editing in ~70% cells under 1600V and ~65% editing in 1700V. (C) Real time PCR based assessment of NRF2 target genes 72h after RNP complex delivery showed significant ($p \leq 0.05$) increases in *NQO1* (upto 11 fold), *HO1* (upto 11 fold) and *GCLM* (upto 2 fold) under these electroporation condition.

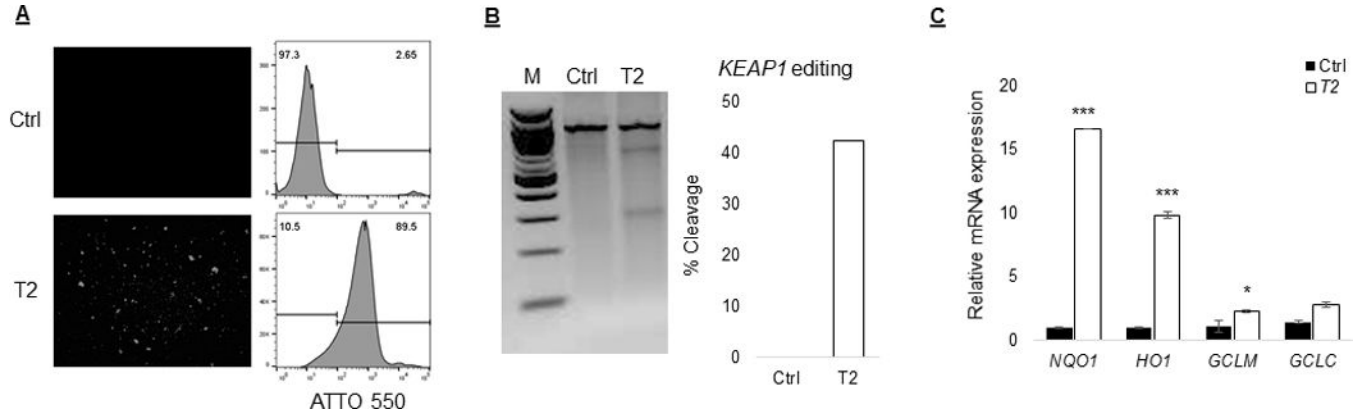

Figure 2.

KEAP1 editing resulted in significant NRF2 activation in primary T cells. We used target site 2 (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 cell 24h after electroporation showed upto ~80% cells received RNP complex. (B) *KEAP1* editing analysis using Surveyor mutation assay showed cleavage in about 40% cells that received RNP complex in comparison to control cells. (C) There was a significant increase in *NQO1* ($p \leq 0.01$; 16 fold), *HO1* ($p \leq 0.01$; 9 fold) and *GCLM* ($p \leq 0.04$; 2 fold) mRNA expression in primary T cell, 72h after RNP complex electroporation, compared to control cells.

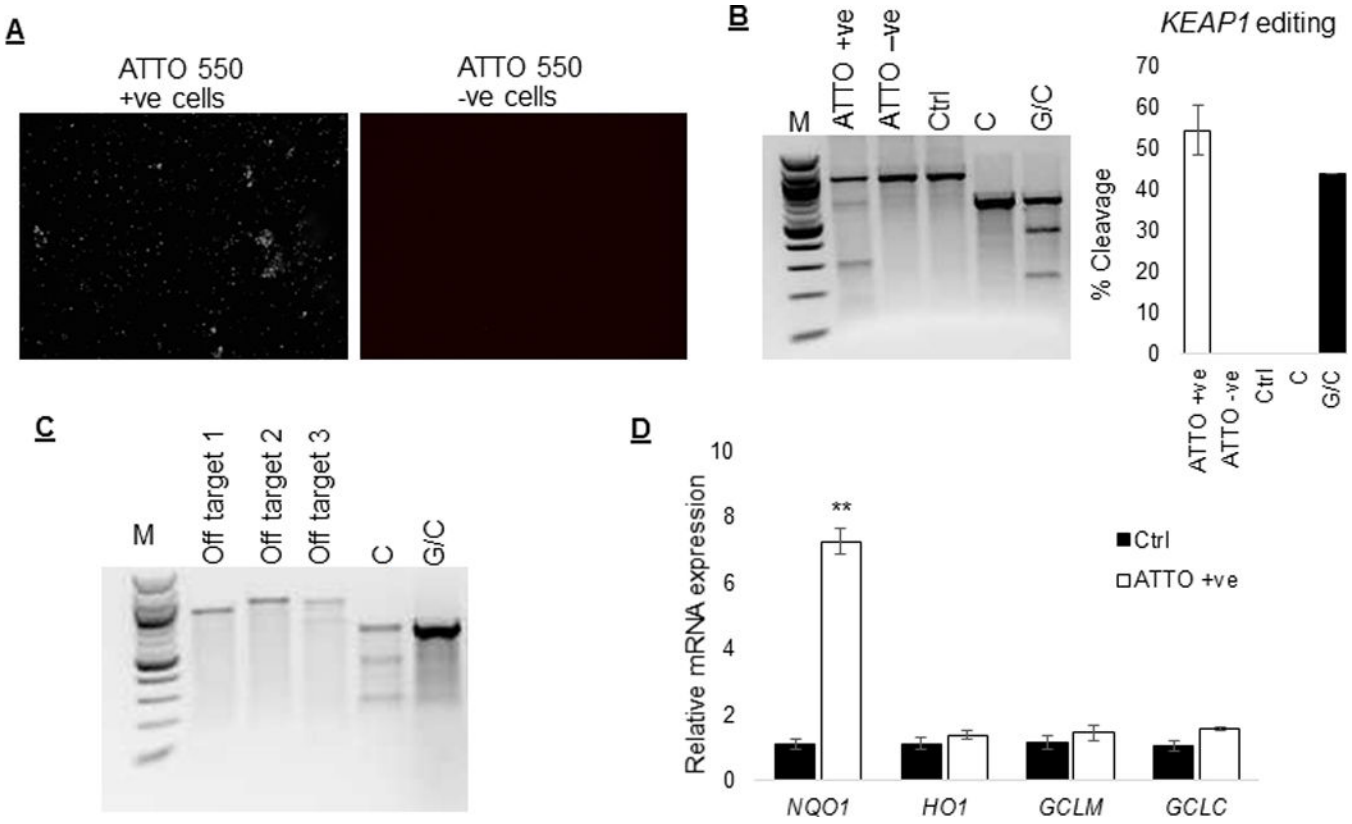

Figure 3.

Enrichment of *KEAP1* edited primary human T cells. Since ATTO 550 tracrRNA forms an integral part of the RNP complex we sorted ATTO550 positive cells (n=4) 24h after electroporation and assessed *KEAP1* editing and Nrf2 activity. (A) Fluorescent microscopic images of ATTO 550 positive and negative cells 90h after sorting. (B) Assessment of *KEAP1* editing, 90h after sorting (120h 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 to 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.

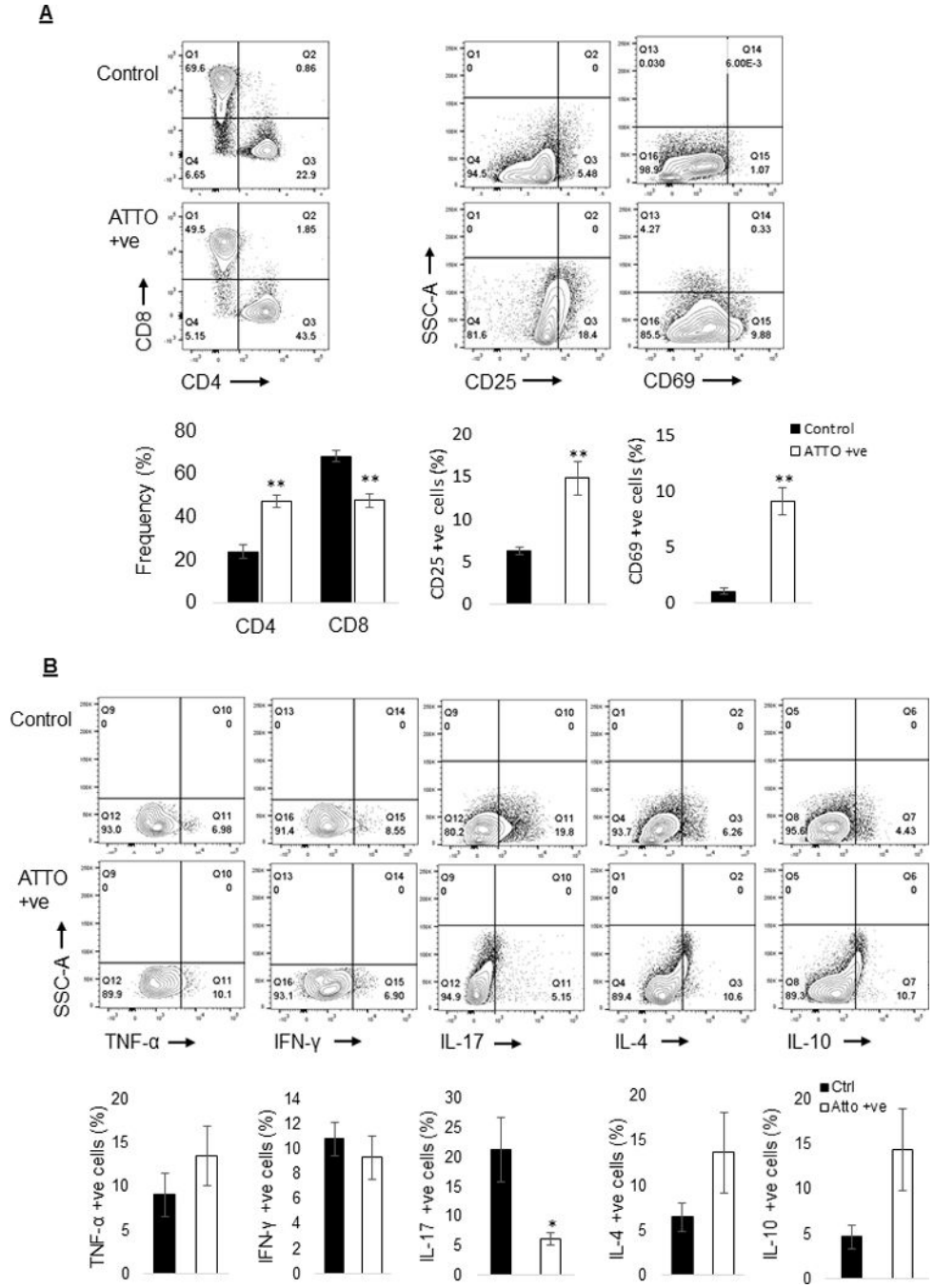

Figure 4.

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 to control cell, 5 days post RNP delivery. Furthermore, KEAP1 edited T cells expressed significantly higher level of CD25 and CD69 in comparison to control cells. (B) The frequency of IL-17 producing cells was significantly reduced in ATTO 550 positive *KEAP1* edited cells in comparison to control cells. There was no difference in

frequency of cell producing TNF-α, IFN-γ, IL-4, IL-10 between *KEAP1* edited and control cells.

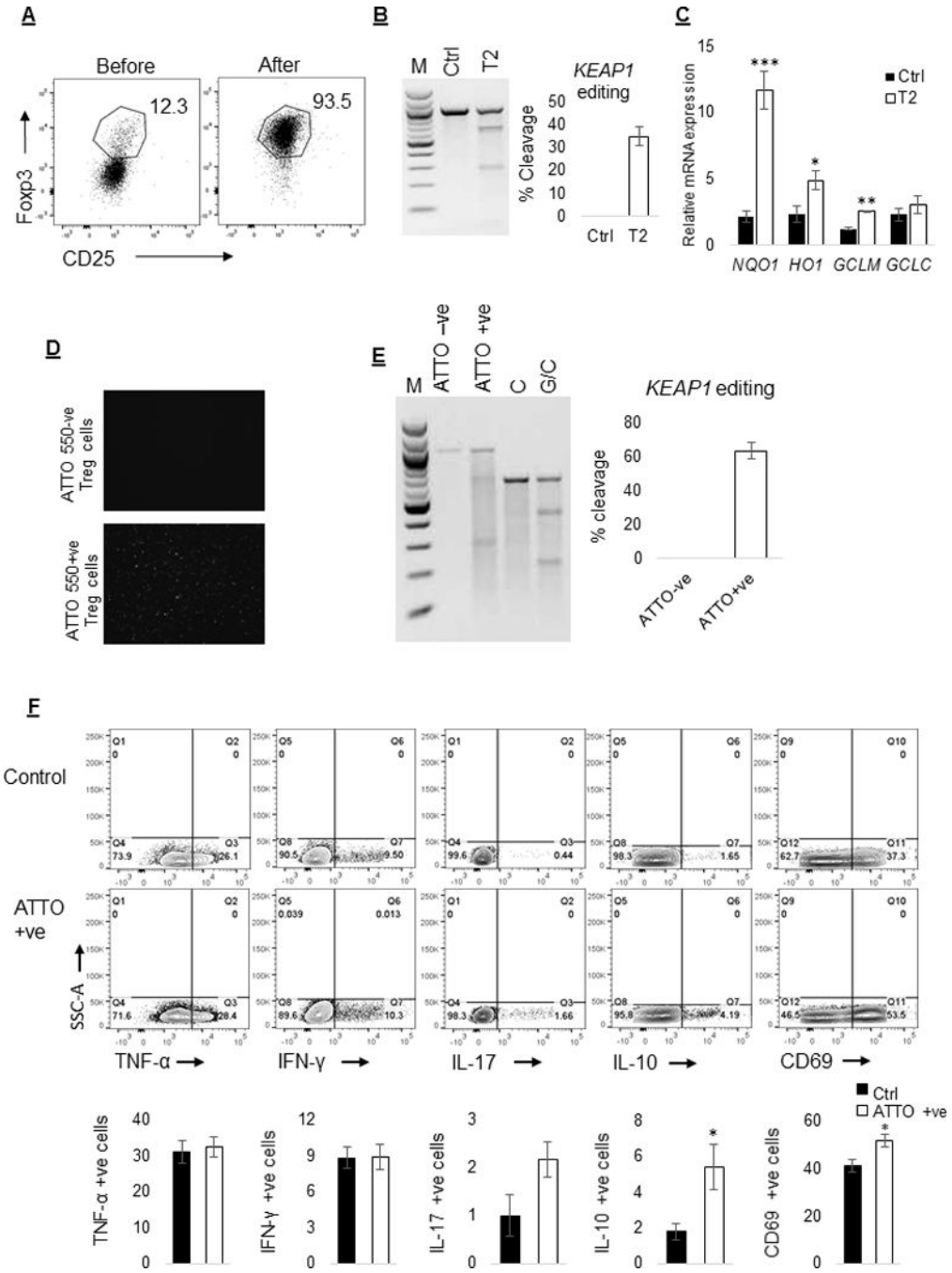

Figure 5.

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* ($p \leq 0.001$; 5 fold), *HO1* ($p \leq 0.05$; 2 fold) and *GCLM* ($p \leq 0.01$; 2 fold) mRNA levels compared to 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 Treg cells showed increased frequency of CD69 (52.0±2.7 vs 41.3%±2.8, $p \leq 0.05$) and IL-10 (5.4±1.3 vs 1.8±0.4, $p \leq 0.05$) positive cells compared to control Treg cells. The frequency of TNF-α (32.5% ±2.7% vs 31.1%±3.1%; p=0.75), IFN-γ (8.9%±1.1% vs 8.9%±0.9%; p=0.97), and IL-17 (2.17%±0.4% vs 1.0%±0.4%; p= 0.10) positive cells was not different between *KEAP1* edited and control Treg cells.

Table 1

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

Target site 1 (T1)	AGCCGCCCGCGGTGTAGATC
Target site 2 (T2)	CTACCTGGTCAAGATCTTCG
Target site 3 (T3)	GGAAGTTCGGCGTCAACGAG

Table 2

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

Sense	AGCCGCCCGCGGTGTAGATC
Anti-sense	CTACCTGGTCAAGATCTTCG

Table 3

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

PIGS (NM_033198)	
Sense	GGTAGATGGAAGGCACAGTAAG
Anti-sense	CCTGACAGACAAAGCCAACTA
HELZ2 (NM_001037335)	
Sense	GAGACGCAGTGAAGGAAGAC
Anti-sense	GTCCACAGTGAAGGTCAAGAA
TNIP1 (NM_001258456)	
Sense	TCAGCGGAGTGAAAGGATTG
Anti-sense	AGAGAAGAAGGGAGGGAGAAA