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Non-canonical NFKB signaling endows suppressive function through FOXP3-dependent regulatory T cell program

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ABSTRACT

Regulatory T cells (Tregs) play a central role in modulating adaptive immune responses in humans and mice. The precise biological role of non-canonical nuclear factor 'κ-light-chain-enhancer' of activated B cells (NFKB) signaling in human Tregs has yet to be fully elucidated. To gain insight into this process, a Treg-like cell line (MT-2) was genetically modified using CRISPR/Cas9. Interestingly, NFKB2 knockout MT-2 cells exhibited downregulation of FOXP3, while NFKB1 knockout did not. Additionally, mRNA expression of FOXP3-dependent molecules was significantly reduced in NFKB2 knockout MT-2 cells. To better understand the functional role of the NFKB signaling, the NFKB1/NFKB2 loci of human primary Tregs were genetically edited using CRISPR/Cas9. Similar to MT-2 cells, NFKB2 knockout human Tregs displayed significantly reduced FOXP3 expression. Furthermore, NFKB2 knockout human Tregs showed downregulation of FOXP3-dependent molecules and a diminished suppressive function compared to wild-type and NFKB1 knockout Tregs. These findings indicate that non-canonical NFKB signaling maintains a Treg-like phenotype and suppressive function in human Tregs through the FOXP3-dependent regulatory T cell program.

1. Introduction

Regulatory T cells (Tregs) are a unique cell population that regulates the immune system and maintains self-tolerance [1]. FOXP3 is a master transcription factor for Treg development and suppressive function [2,3]. The importance of FOXP3 in immune regulation is exemplified by the genetic loss of FOXP3, which results in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans [4,5]. Not limited to FOXP3, the deficiency of other Treg-related molecules, such as cytotoxic T-lymphocyte associated protein 4 (CTLA4), interleukin 2 receptor subunit alpha (IL2RA, also known as CD25), and lipopolysaccharide responsive beige-like anchor protein, also result in the systemic autoimmunity, referred to as "Tregopathies" [6]. The presence of Tregopathies suggests that Treg function and development are precisely controlled by FOXP3 and other Treg-related molecules across species. The growing recognition of genetic autoimmune diseases, facilitated by the development of genetic and clinical diagnoses, is reflected in the increasing number of patients identified with genetic autoimmune diseases [7]. Therefore, understanding Treg biology in healthy donors and disease patients has become more important than in the past as the field of Treg biology has progressed.

The canonical nuclear factor 'κ-light-chain-enhancer' of activated B cells (NFKB) signal is an essential signaling pathway related to cell proliferation, differentiation, and apoptosis among different cell lineages across species [8]. In the immune system, B cells and

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antigen-presenting cells, such as monocytes and macrophages, are predominantly dependent on canonical NFKB signaling. T cells also express NFKB subunit 1 (NFKB1) upon activation. The functional role of NFKB1 in T cells has not yet been fully elucidated. Non-canonical NFKB signaling in Tregs has been studied mainly in transgenic mice. Nfkb2 knockout Treg mice display reduced Foxp3 expression [9]. However, the functional role of non-canonical NFKB signaling in human Tregs has not yet been fully elucidated.

The biological role of NFKB signaling in the human immune system is exemplified by the primary immune deficiency caused by mutations in NFKB family members including NFKB1 [10], NFKB2 [11], RELA [12], RELB [13] and RELC [14]. Multiple immune cell lineages have been shown to be affected by mutations in NFKB signaling. Among the mutations in NFKB family members, NFKB2 mutation has been associated with Treg impairment [15,16]. However, the functional role of NFKB2 in human Tregs is not fully understood due to the rarity and clinical heterogeneity partially modulated by the presence of immunosuppressant usage and infections.

CRIPSR/Cas9 is an emerging gene editing technology that makes it possible to knockout target genes and knock-in homologous donor templates. $CD4^+$ T cells (Teffs) and Tregs have been successfully edited by CRISPR/Cas9 for the understanding the biology [17] and correcting disease causing mutations in Tregopathies including FOXP3 [18], CTLA-4 [19] and IL2RA [20]. The role of Treg-related molecules in Treg function can be investigated using CRISPR/Cas9 gene editing [21]. Therefore, it is hypothesized that NFKB1/NFKB2 gene editing will provide information on Treg biology associated with NFKB signaling. The association between NFKB signaling and Tregs was suggested by the presence of human disease and knockout mouse model. However, the association remains unclear due to the lack of human samples and suitable molecular and immunological approaches.

To better understand the functional role of NFKB signaling, the NFKB1/NFKB2 loci of human primary Tregs were genetically edited using CRISPR/Cas9. Here, we demonstrate that NFKB2 maintains FOXP3 expression and FOXP3-dependent regulation of Tregs.

2. Materials and methods

2.1. Cell line and isolation

Jurkat and MT-2 cells were provided by the Japan National Institute of Biomedical Innovation, Health and Nutrition, Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Peripheral blood mononuclear cells (PBMCs) isolated from healthy subjects were purchased from Precision for Medicine (n = 4) and Lonza (n = 2). The biological profiles of PBMCs donors are summarized in Supplementary Table 1. Teffs were enriched from PBMCs using the EasySep Human $CD4^+$ Isolation Kit (STEMCELL Technologies). Tregs were isolated from frozen PBMCs using the EasySep Human $CD4^+$ CD127low $CD25^+$ Regulatory T cell isolation kit (STEMCELL Technologies). The purity of enriched Teffs (%CD4) and Tregs (%CD25 + CD127-) was >95 % for Teffs and >90 % for Tregs immediately after isolation. Representative dot-plots of isolated Tregs was shown in Supplementary Fig. 1.

2.2. Cell culture

The cell lines were cultured in Roswell Park Memorial Institute medium (Life Technologies) supplemented with 10 % fetal bovine serum (Gibco). Mycoplasma testing (MycoAlert Quick Mycoplasma Detection Kit, Lonza) was routinely conducted. Mycoplasma contamination was not detected during the study period. Teffs and Tregs were cultured in ImmunoCult XF T cell expansion medium (STEMCELL Technologies) in the presence of 100 U/ml of recombinant human (rh) IL-2 (Peprotech). ImmunoCult Human CD3/CD28/CD2 T cell activator (STEMCELL Technologies) was added on day 0 (activation) and days 10–13 (re-stimulation), according to the manufacturer's protocol.

2.3. CRISPR/Cas9

Freshly isolated Teffs and Tregs were activated for 3–5 days using ImmunoCult Human CD3/CD28/CD2 T cell activator (STEMCELL Technologies). A ribonucleprotein (RNP) complex was made by incubating 15 μ g of Cas9 protein (Alt-R® S. p. Cas9 Nuclease V3; Integrated DNA Technologies) with 8 μ g of synthetic single guide RNA (sgRNA; Synthego) targeting NFKB1 with the sequence 5′-2′O-methyl [OMe](U (phosphorothioate [ps])A (ps)U (ps))AUAGAUCUGCAACUAUG or NFKB2 with the sequence 5′-2′OMe(U (ps)C (ps)C (ps))CUCGUAGUUACAGAUCU, followed by 80-mer SpCas9 scaffold at room temperature for 15 min. The RNP complex, together with a 5 μ g of donor plasmid containing homologous arms and enhanced green fluorescent protein (EGFP) or tdTomato expression cassette under the control of the PGK promoter (Genscript), was transduced into Teffs or Tregs using the NEPA21 Electroporator (Nepagene). The electroporation conditions are provided in the Supplementary Table 2. The EGFP/tdTomato-positive population was sorted by fluorescence-activated cell sorting (FACS) 4–5 days after transduction. After sorting, edited cells were expanded by CD3/CD28/CD2 T cell activator and harvested 10–13 days after re-stimulation.

2.4. PCR

Genomic DNA was isolated from the edited cells using a DNeasy kit (QIAGEN). Primer sequences for the in-out PCR were EGFP (forward primer, 5′-CACCGGTCGCCACCATGGTGAGCAAGGGCGAGGA-3′), tdTomato (forward primer, 5′-ATGGTGAGCAAGGGCGAGGA-3′), NFKB1 (reverse primer, 5′-GCAGCAGAGAGCTGAAATGA-3′), and NFKB2 (reverse primer, 5′-GGGCAGTAGGTACATGTGTGA-3′). PCR reactions were performed using the KOD One PCR Master Mix (TOYOBO) in a T100 Thermal Cycler (Bio-Rad).

Full un-adjusted images are provided in Supplementary Fig. 9. PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific).

2.5. Epigenetic qPCR

DNA was isolated from non-edited and edited cells using the DNeasy kit (QIAGEN). Bisulfite conversion was conducted using EZ DNA Methylation Kits (Zymo). Bisulfite-converted genomic DNA was amplified using methylated Treg-specific demethylated region (TSDR) qPCR primers (forward primer, 5′- GTATTTGGGTTTTGTTGTTATAGTTTTC -3'; reverse primer, 5′- TACAAAACAAAA-CAACCAATTCTCG -3'; probe, 5′- FAM CGACGCATC/ZEN/CGACCGCCA -3IABkFQ-3′) and unmethylated TSDR primers (forward primer, 5′-GTATTTGGGTTTTGTTGTTATAGTTTTT -3'; reverse primer, 5′-CTACAAAACAAAACAACCAATTCTCA -3'; and probe, 5′-FAM- ACCCAACAC/ZEN/ATCCAACCACCA –3IABkFQ-3′) as previously described [22].

2.6. qPCR

Total RNA was extracted from 1×10^6 Teffs and Tregs using an RNeasy Plus Mini Kit (QIAGEN). RNA (100 ng) was used to synthesize cDNA using Superscript IV VILO (Thermo Fisher Scientific). qPCR was performed using TaqMan Gene Expression Assay and TaqMan Universal Master mix II (Thermo Fisher Scientific) by QuantStudio 5 Real Time PCR (Thermo Fisher Scientific). Gene expression was calculated by ΔCT, where $\Delta CT = CT_{target} - CT_{housekeeping}$. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an internal control gene. The qPCR primers and TaqMan probe list are provided in Supplementary Table 3.

2.7. RNA-seq

RNA-seq was performed on isolated RNA from Jurkat and MT-2 cells. Briefly, purified RNA was assessed for quantity and quality using an Agilent 2100 Bioanalyzer and Qubit instrument. RNA-seq libraries were generated using Illumina TruSeq Stranded Total RNA kits. The resultant cDNA libraries were quantified via Qubit and assessed for quality via Bioanalyzer. Libraries were molecularly barcoded, pooled at equimolar concentrations and sequenced at 2×100 cycles on an Illumina Novaseq 6000. RNA-seq sequencing was performed according to the manual of Rhelixa Co., Ltd (Tokyo, Japan).

2.8. Western blot

Immunoblotting was performed to analyze NFKB1/NFKB2 expression. Briefly, total protein was extracted from the cell line and primary cells using the EzRIPA lysis kit (ATTO). Protein concentration was measured using a Pierce BC protein assay kit (Thermo Fisher Scientific). Five micrograms protein were loaded in each lane of a 4–20 % gradient sodium dodecyl sulfate polyacrylamide gel (Bio-Rad). Proteins resolved following electrophoresis were transferred to a polyvinylidene fluoride membrane (Bio-Rad) using a *Trans*-Blot Turbo Transfer System (Bio-Rad). Primary antibody against the target proteins was added overnight at a concentration of 1:1000. A 1:1000 dilution of anti-rabbit horseradish peroxidase conjugated IgG secondary antibody (Cell Signaling Technologies) was added for 1 h. Chemiluminescent substrate (Clarity Western ECL Substrate, Bio-Rad) was added to the membrane and the signal was captured using the ChemiDoc Touch Imaging System (Bio-Rad). Protein expression was quantified by ImageJ software ver 1.54. The antibodies list is provided in Supplementary Table 4. Full un-adjusted images are provided in Supplementary Fig. 8.

2.9. Flow Cytometry

Teffs were re-suspended in FACS buffer (PBS supplemented with 0.5 % bovine serum albumin and 2 mM EDTA) and stained with a cocktail of surface staining antibodies for 30 min. After surface staining, intracellular staining was performed using a Foxp3/Transcription Factor Staining buffer set (eBioscience) combined with intracellular staining antibody cocktail for 30 min. For the unconjugated antibodies (NFKB1, NFKB2, RELA, RELB, and RELC), anti-rabbit IgG conjugated with phycoerythrin was added as the secondary antibody and incubated for an additional 30 min. Data were acquired using a FACSAria III (BD Biosciences) and analyzed using FlowJo 10.7 software (FlowJo LLC). The antibodies are listed in Supplementary Table 5.

2.10. Suppression assay

Suppression assay was performed as previously described [23,24]. Briefly, responder $CD4^+$ T cells (50,000 cells) labeled with Cellstain CFSE (FUJIFILM/Wako Pure Chemical) and suppressor $CD4^+$ T cells (12,500–50,000 cells) labeled with Cellstain CytoRed (FUJIFILM/Wako Pure Chemical) were re-suspended in 100 μ l of ImmunoCult XF T cell expansion medium without rhIL-2. Responder and suppressor cells were co-cultured at different concentrations (1:0.25–1:1) after activation by Dynabeads Human T cell Activator CD3/CD28 (Gibco) at a 1:25 bead-to-cell ratio in 96 well plates. After 96 h of stimulation, proliferation of responder $CD4^+$ T cells was determined by Flow Cytometry. The suppression index was calculated as previously described [25].

2.11. Statistical analysis

GraphPad Prism Software ver 9.3.1 (GraphPad Software) was used for statistical analysis. One-way analysis of variance followed by

Tukey's multiple comparison tests or Kruskal Wallis tests were performed when comparing multiple conditions. $P < 0.05$ was considered significant. Data are shown as mean ± standard deviation (SD) or median ± interquartile range (IQR).

3. Results

3.1. NFKB2 signaling was activated in tregs and MT-2 treg-like cell line

To characterize NFKB signaling in Tregs, mRNA and protein were extracted from Tregs and the MT-2 Treg-like cell line after polyclonal TCR stimulation. NFKB1 was equally expressed in Tregs and MT-2 cells compared to Teffs and Jurkat cells, and NFKB2 was preferentially expressed in Tregs and MT-2 cells, as confirmed by qPCR (Fig. 1A) and Flow Cytometry (Fig. 1B). Moreover, Teffs and Tregs equally expressed NFKB1 as p50 (cleaved), not as p105 (precursor). In contrast to NFKB1, Tregs and MT-2 cells preferentially expressed NFKB2 as p52 (cleaved) as well as p100 (precursor) confirmed by western blotting (Fig. 1C). As it was technologically challenging to distinguish cleaved proteins (p50/p52) and their precursors (p105/p100) by qPCR or Flow Cytometry, Western blot analysis identified the involvement of NFKB2 signaling in Tregs and MT-2 cells upon TCR stimulation.

Analyses of NFKB family members RELA (p65), RELB, and RELC revealed no significant changes in Tregs compared to Teffs, using qPCR (Fig. 1D) and Flow Cytometry (Fig. 1E). Western blotting revealed comparable expression of RELA and RELC in Tregs and MT-2

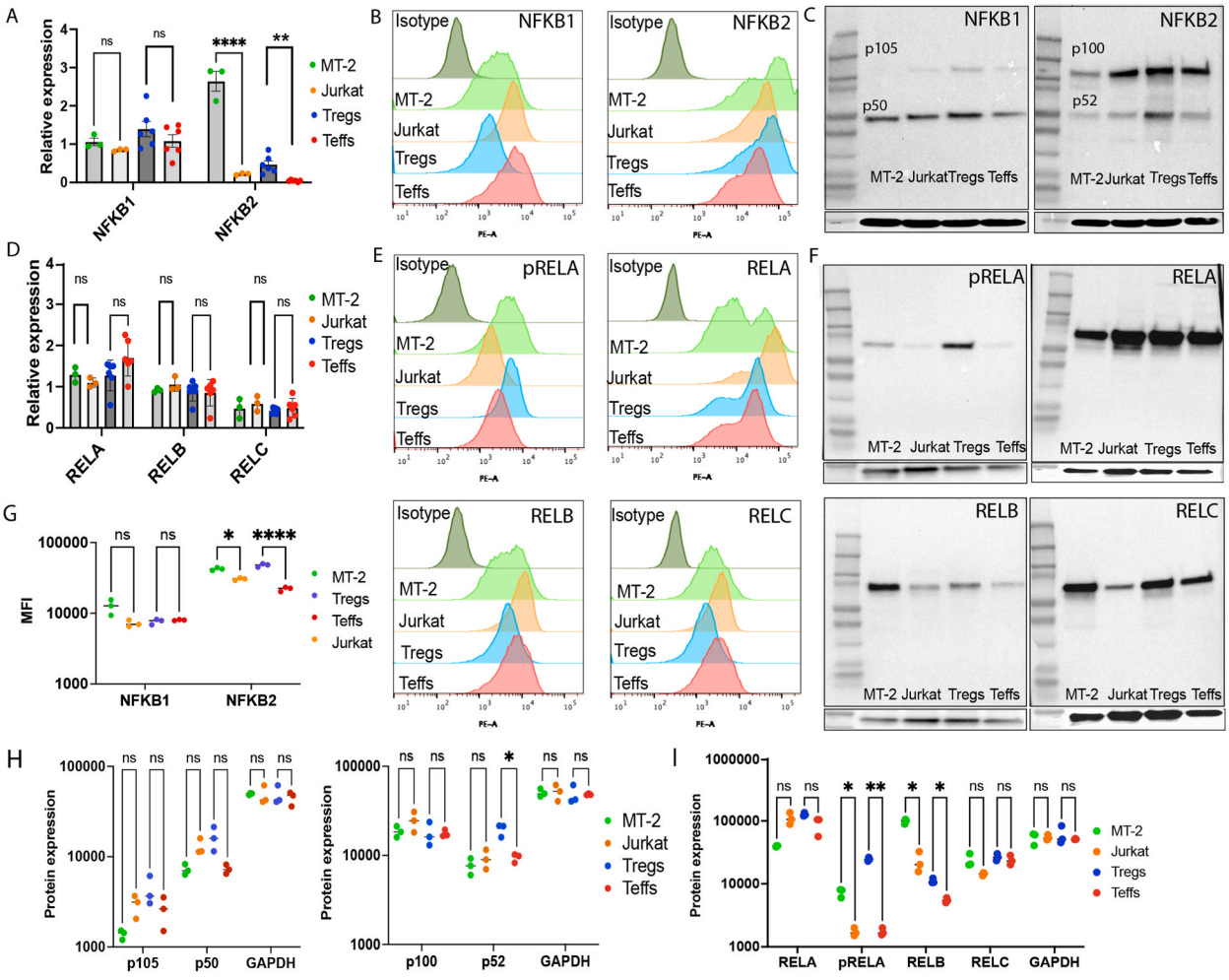

Fig. 1. NFKB2 is preferentially expressed by Tregs and Treg-like cell line. (A–B) NFKB1/NFKB2 expressions in Tregs and Treg-like cell lines were analyzed by (**A**) qPCR (n = 6) and (**B**) Flow Cytometry (n = 4). (**C**) Preferential NFKB2 expressions in Tregs and Treg-like cell lines were confirmed by Western blot (n = 4). Full un-adjusted images are provided in Supplementary Fig. 8. (**D-E**) Expressions of NFKB family members (RELA, RELB and RELC) were analyzed by (**D**) qPCR (n = 6) and (**E**) Flow Cytometry (n = 4). (**F**) Protein expression of NFKB family members (RELA, RELB and RELC) were confirmed by Western blot (n = 4). Full un-adjusted images are provided in Supplementary Fig. 8 (G) NFKB1/2 MFI of Tregs and Treg-like cell lines. (H) NFKB1/2 protein expressions of Tregs and Treg-like cell lines. (I) Protein expressions of NFKB family members. One-way analysis of variance followed by Tukey's multiple comparison tests were performed when comparing multiple conditions. Data were representative of three independently repeated experiments shown by mean ± SD (N = 2, n = 2,2).

cells compared to that in Teffs and Jurkat cells (Fig. 1F). In addition to the p52 expression in Tregs, RELB was upregulated in Tregs and MT-2 cells. Upregulation of p52 and RELB in Tregs supports the previous study demonstrating that NFKB2 p100 (precursor) could inhibit RelB in the murine immune system [9]. Notably, phospho-RELA was upregulated in Tregs and MT-2 cells compared with that in Teffs and Jurkat cells. Therefore, NFKB2 signaling was activated in Tregs and MT-2 cells, while other NFKB family members were similarly expressed in both Tregs and Teffs, except for phospho-RELA. It has been suggested that RELA is associated with Treg function [26]. However, the functional role of NFKB2 in human Tregs is not yet clearly understood. Based on the foregoing results, preferential NFKB2 expression in Tregs might be associated with a Treg-like phenotype and function.

3.2. *Knockout of NFKB1/NFKB2 by CRIPSR/Cas9 in jurkat cells*

To identify the biological role of NFKB signaling in human immune cells, the NFKB1/NFKB2 loci were knocked out by CRISPR/Cas9 mediated homologous recombination. Jurkat cells were targeted by CRISPR/Cas9 mediated gene disruption. Donor template plasmids and RNP complex were electroporated into target cells. Knock-in of the GFP cassette into either NFKB1 or NFKB2 loci was performed, followed by FACS purification according to GFP expression (Supplementary Fig. 2A). Both NFKB1 and NFKB2 expression were successfully disrupted by GFP cassette knock-in at the target loci, as confirmed by Flow Cytometry (Supplementary Fig. 2B). The average GFP knock-in efficiencies were 26.5 ± 6.1 % for NFKB1 and 24.5 ± 10.2% for NFKB2 loci (n = 4, Mean ± SD). Integrations of the GFP cassette in the NFKB1/2 loci were confirmed by in-out PCR (Supplementary Fig. 3A) and further validated by Sanger sequencing of the PCR products (Supplementary Fig. 3B). A significant reduction of NFKB1/NFKB2 by EGFP knock-in was confirmed by Flow Cytometry (Supplementary Fig. 2C) and western blotting (Supplementary Fig. 2D). It was possible to knockout NFKB1/NFKB2 by CRISPR/Cas9 mediated gene disruption in Jurkat cells.

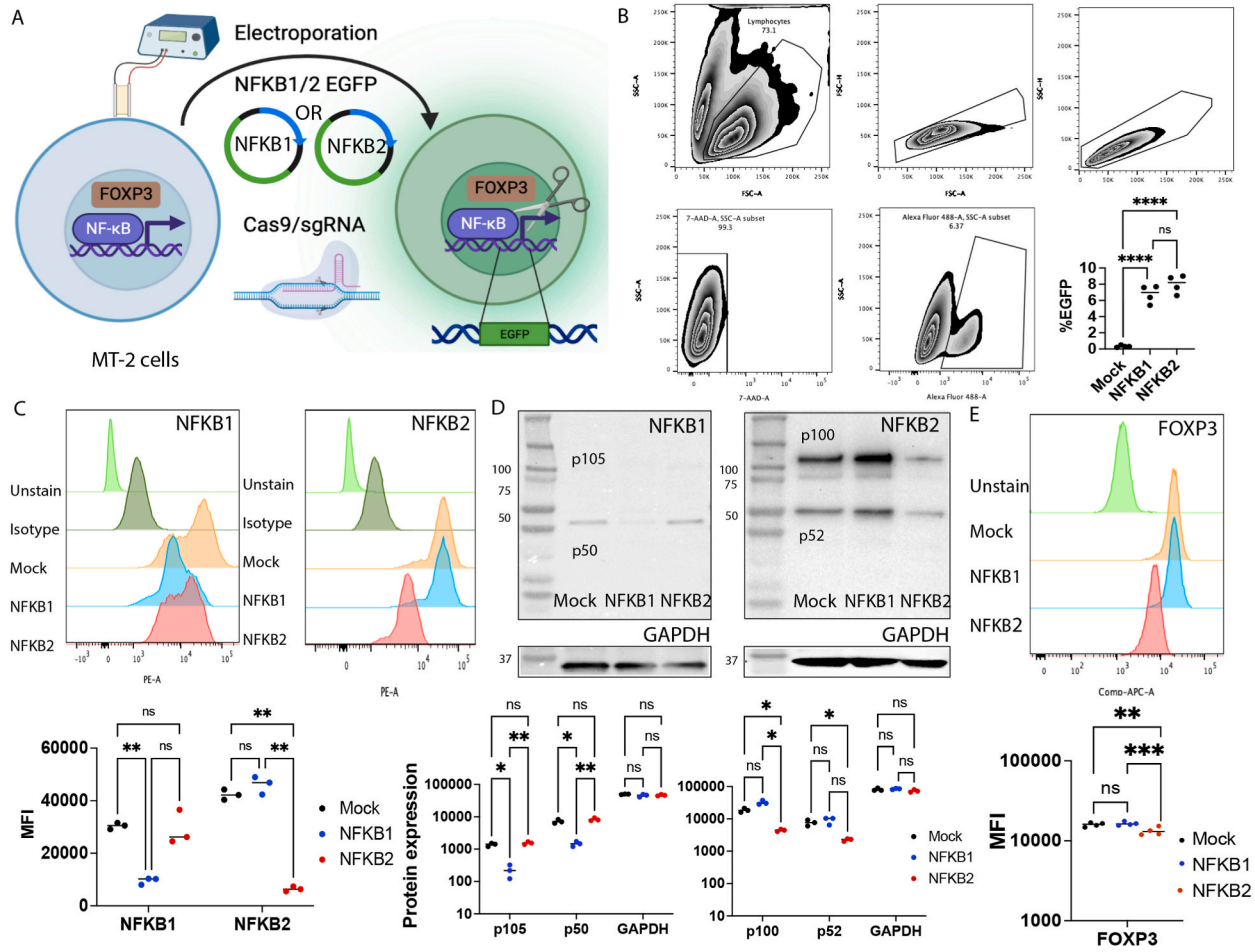

Fig. 2. NFKB1/NFKB2 can be knocked out by CRIPSR/Cas9 in MT-2 cells. (**A**) NFKB1/NFKB2 Editing strategy of MT-2 cells. (**B**) EGFP knock-in cassette was successfully inserted in NFKB1/NFKB2 loci. NFKB1/NFKB2 knock-out was confirmed by (**C**) Flow Cytometry (n = 4) and (**D**) Western blot (n = 4). Full un-adjusted images are provided in Supplementary Fig. 8. (**E**) FOXP3 expression of NFKB1/NFKB2 knock-out in MT-2 cells was analyzed by Flow Cytometry (n = 4). One-way analysis of variance followed by Tukey's multiple comparison tests were performed when comparing multiple conditions. Data were representative of two independently repeated experiments shown by mean ± SD (N = 2, n = 2,2).

FOXP3 expression is minimal in Jurkat cells [27]. Thus, the effect of NFKB1/NFKB2 knockout on FOXP3 expression was assessed in Jurkat cells. Residual FOXP3 expression in Jurkat cells was not significantly influenced by NFKB1/NFKB2 knockout, as confirmed by Flow Cytometry (Supplementary Fig. 2E). Treg-related molecules (cytotoxic T-lymphocyte associated protein 4 [CTLA4], inducible

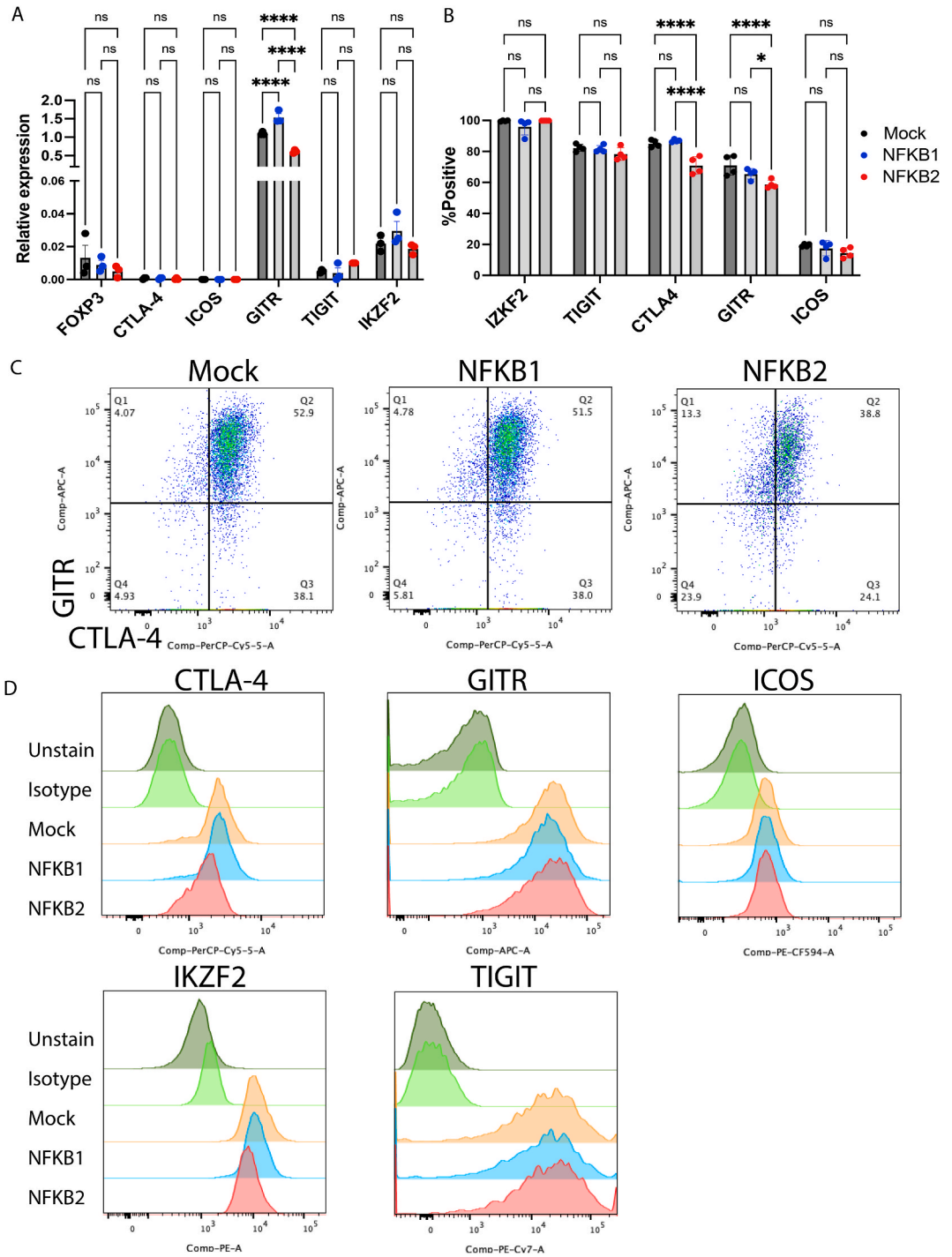

Fig. 3. FOXP3 expression in MT-2 cells were reduced by NFKB-2 knockout. (**A**) FOXP3 and Treg-related molecules mRNA expression analyzed by qPCR (n = 3). (**B**) Expressions of Treg-related molecules were analyzed by Flow Cytometry (n = 4). (**C**) Representative dot plots of CTLA-4 and GITR. (**D**) Representative histograms of CTLA-4, GITR and ICOS. One-way analysis of variance followed by Tukey's multiple comparison tests were performed when comparing multiple conditions. Data were representative of two independently repeated experiments shown by mean ± SD (N = 2, n = 2,2).

T-cell stimulator [ICOS], glucocorticoid-induced tumor necrosis factor receptor-related protein [GITR], and T cell immunoreceptor with Ig and ITIM domains [TIGIT]), which were not preferentially expressed in Jurkat cells, remained low, except for IKAROS family zinc finger 2 (IKZF2), as shown by qPCR (Supplementary Fig. 3C) and Flow Cytometry (Supplementary Fig. 3D), as expected.

Hence, NFKB1/NFKB2 was efficiently knocked out by CRISPR/Cas9 mediated gene disruption in Jurkat cells. However, it was not possible to assess the relationship between FOXP3 and NFKB1/NFKB2 signals due to the limited FOXP3 expression in Jurkat cells. Therefore, other Treg-like cell lines, such as MT-2 cells, were tested, as described next.

3.3. *Reduced FOXP3 expression by NFKB-2 knockout leads to downregulation of FOXP3 dependent molecules*

Since it was not possible to fully investigate the relationship between FOXP3 and NFKB1/NFKB2 in Jurkat cells due to limited FOXP3 expression, a similar editing strategy was attempted in MT-2 cells, a Treg-like cell line characterized by constitutive FOXP3 expression [28]. To clarify the relationship between NFKB signaling and the Treg-like phenotype, including FOXP3 expression, NFKB1/NFKB2 loci of MT-2 cells were knocked out by CRISPR/Cas9 mediated homologous recombination. It was possible to knock-in the GFP cassette into either NFKB1 or NFKB2 loci and then purify it by FACS according to GFP expression (Fig. 2A). Both NFKB1 and NFKB2 expression was disrupted by GFP cassette knock-in at the target loci (Fig. 2B). The average GFP knock-in efficiencies were 6.8 ± %1.1 for NFKB1 and 8.0 ± 1.1% for NFKB2 loci (n = 4, mean ± SD). Integrations of the GFP cassette in the NFKB1/2 loci were confirmed by in-out PCR (Supplementary Fig. 4A) and further validated by Sanger sequencing of the PCR products (Supplementary Fig. 4B). Reduced expression of NFKB1/NFKB2 was confirmed by Flow Cytometry (Fig. 2C) and western blotting (Fig. 2D). Therefore,

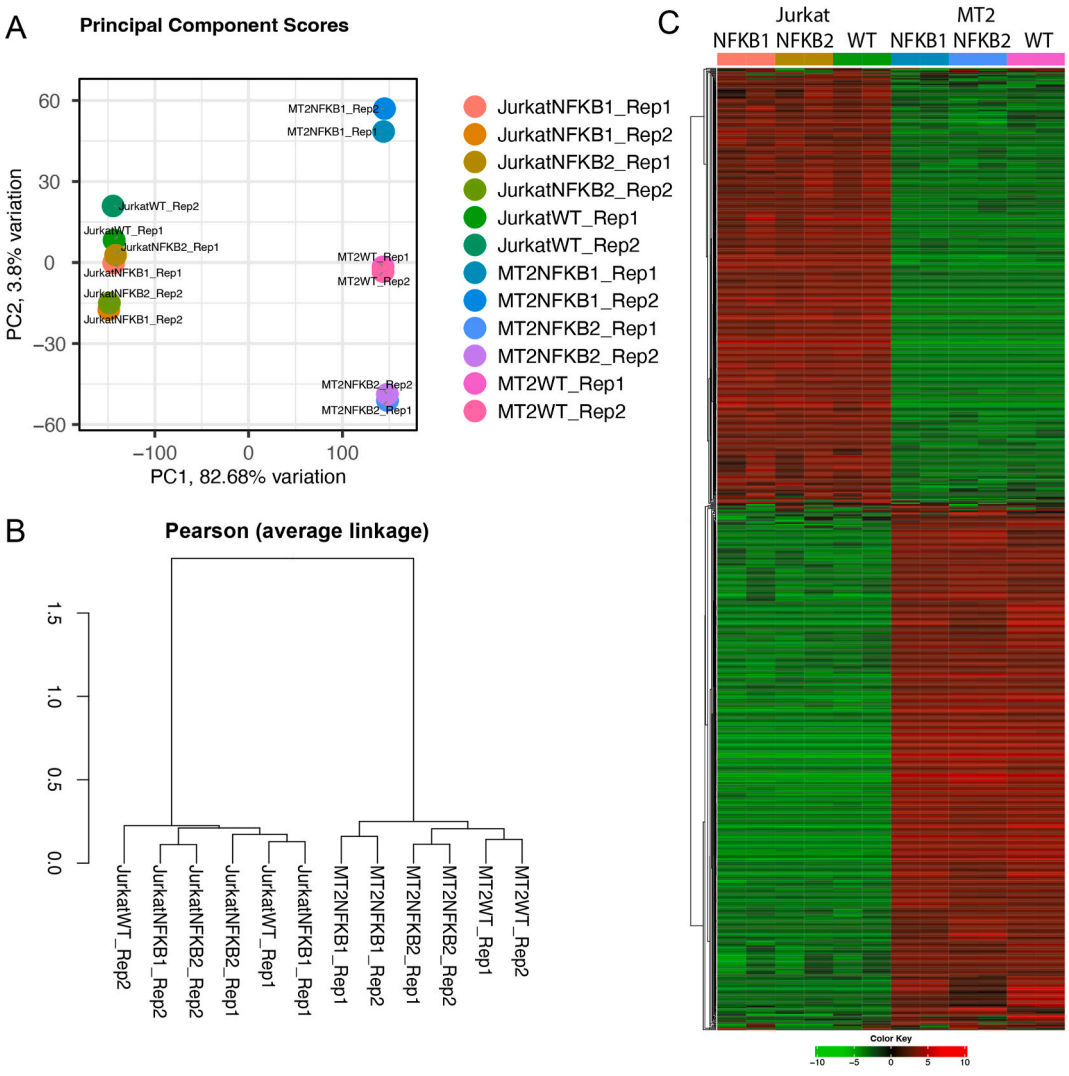

Fig. 4. RNA-seq analysis on Jurkat and MT-2 cells. (**A**) Principle component analysis (PCA) (**B**) Hierarchical cluster analysis (HCA) (**C**) Heat map of differentially expressed genes (DEGs) between Jurkat and MT-2 cells.

NFKB1/NFKB2 was successfully knocked out by CRISPR/Cas9 mediated gene disruption in MT-2 cells.

Next, the Treg-like phenotype was analyzed in NFKB1/NFKB2 knockout MT-2 cells. FOXP3 expression in MT-2 cells was significantly reduced by NFKB2 knockout but not by NFKB1 knockout, as confirmed by Flow Cytometry (Fig. 2E). Interestingly, FOXP3 mRNA expression was not reduced in NFKB1/NFKB2 knockout MT-2 cells (Fig. 3A). To confirm that FOXP3 downregulation was not caused by epigenetic changes, TSDR analysis was performed. It was not possible to identify any TSDR methylation changes in Jurkat/MT-2 cells after NFKB1/NFKB2 knockout (Supplementary Fig. 4C). Regardless of the Treg-like phenotype, TSDR was almost fully methylated in the MT-2 cells. Based on these results, it was suggested that the reduction in FOXP3 expression is most likely caused by instability of FOXP3 protein, and not by the reduced gene expression due to the methylation change during CRISPR/Cas9 gene editing.

To address the downstream effect of the reduction of FOXP3, the mRNA expression of Treg-related molecules (CTLA-4, ICOS, IKZF2, GITR, and TIGIT) was analyzed by qPCR. It was possible to detect downregulation of FOXP3-dependent molecules in NFKB2 knockout MT-2 cells, including GITR, while FOXP3 independent molecules, such as TIGIT, remained unchanged (Fig. 3A). The protein expression of Treg-related molecules, including CTLA-4, and GITR, previously shown to be preferentially expressed by MT-2 cells [28] was reduced by NFKB2 knockout, while TIGIT, ICOS, and IKZF2 were not (Fig. 3B–D).

Overall, these results suggest that NFKB2 signaling may contribute to the stabilization of FOXP3 expression in MT-2 cells at the protein level. The Treg-like phenotype of MT-2 cells, such as CTLA-4 and GITR expression, was partially lost by NFKB2 knockout-mediated FOXP3 downregulation.

3.4. *NFKB1 and NFKB2 differently modulate transcriptional profile of MT-2 cells*

In addition, to further investigate the effects of NFKB signaling, RNA-seq analysis was performed on both Jurkat and MT-2 cells. It was observed that either NFKB1 or NFKB2 knockout could alter the gene expression profile in MT-2 cells compared to Jurkat cells, as confirmed by principle component analysis (Fig. 4A) and hierarchical clustering analysis (Fig. 4B). Gene expression profiles of NFKB1/NFKB2 knockout Jurkat cells were compared to the mock edited Jurkat cells. On the other hand, it was hypothesized that the gene expression profile of MT-2 cells can be more drastically altered by NFKB1 or NFKB2 knockout (Fig. 4C). NFKB2 knockout MT-2 cells

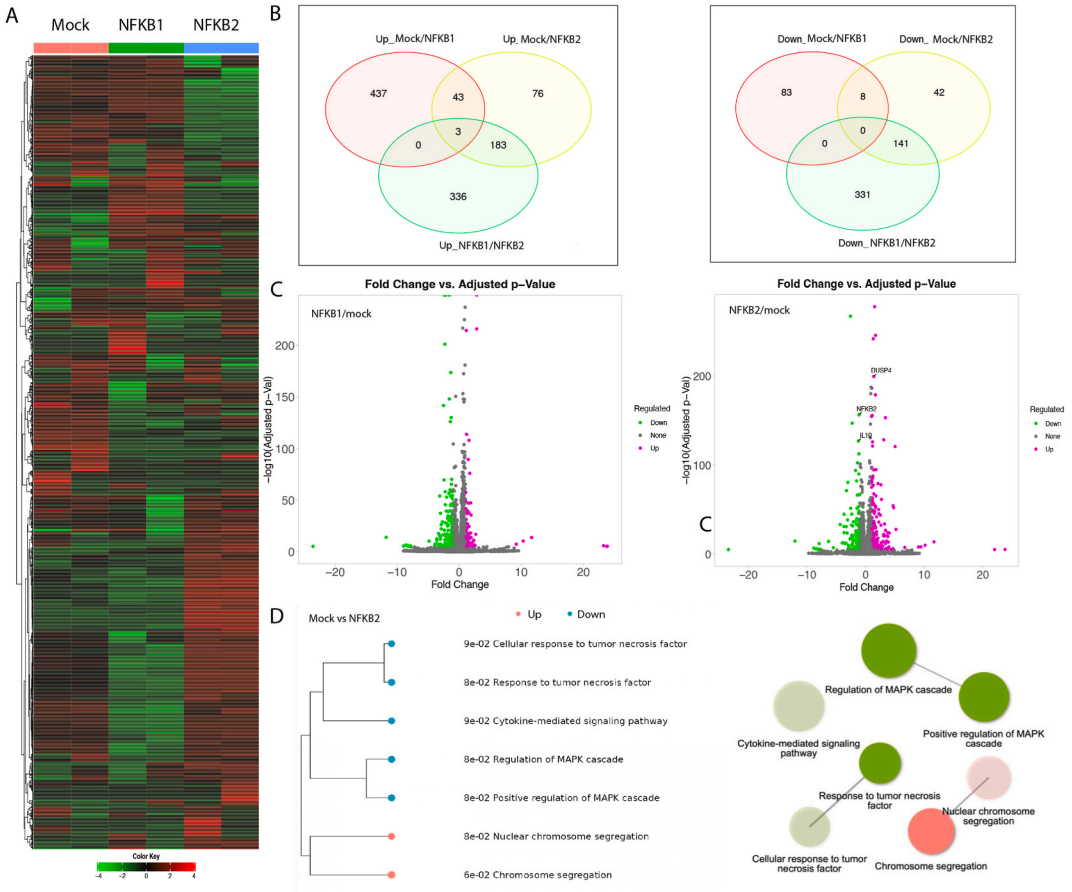

Fig. 5. RNA-seq analysis of NFKB1/2 knockout MT-2 cells. (**A**) Heat map of differentially expressed genes (DEGs) between NFKB1/2 knockout MT-2 cells. (**B**) Venn diagram of DEGs between NFKB1/2 knockout MT-2 cells. (**C**) Volcano plots of NFKB1/2 knockout MT-2 cells. (**D**) Pathway analysis of NFKB2 knockout MT-2 cells.

exhibited a unique gene expression profile compared to those of NFKB1 knockout MT-2 cells and mock edited MT-2 cells (Fig. 5A). The differentially expressed genes (DEGs) against NFKB2 knockout MT-2 cells were shared by NFKB1 knockout MT-2 cells and mock edited MT-2 cells (Fig. 5B, Supplementary Tables 6–7). As expected, NFKB1/NFKB2 were downregulated via NFKB1/NFKB2 knockout (NFKB1, $p < 2.7E\text{-}6$; NFKB2, $p < 3.5E\text{-}157$). Similar to the qPCR analysis, the expression of Treg-related genes, including FOXP3, was not highly upregulated in the MT-2 cells; however, it was possible to detect the downregulation of GITR (TNFRSF18) only in NFKB2 knockout MT-2 cells (Supplementary Table 8). Furthermore, pathway analysis identified alterations in the TNF and MAPK signaling pathways, contributing to Treg stability and function.

3.5. *Bi-allelic NFKB1/NFKB2 knockout can be achieved in teffs*

Primary $CD4^+$ T cells have limited *in vitro* survival compared to immortalized cell lines, such as Jurkat and MT-2 cells. To understand the role of NFKB2 signaling in primary human T cells, bi-allelic NFKB1/NFKB2 knockout was performed to obtain fully knocked out populations within a few rounds of expansion (Supplementary Fig. 5A). Teffs simultaneously expressing both EGFP and tdTomato were identified and sorted by FACS (Supplementary Fig. 5B). The average GFP knock-in efficiencies were 23.9 ± 5.3% for NFKB1 and 25.1 ± 4.6 % for NFKB2 loci (n = 4, mean ± SD). The average tdTomato knock-in efficiencies were 16.9 ± 3.0 % for NFKB1 and 15.4 ± 3.5% for NFKB2 loci (n = 4, mean ± SD). Bi-allelic integration of marker genes (EGFP and tdTomato) was confirmed by in-out PCR (Supplementary Fig. 6A) and further validated by Sanger sequencing of PCR products from the FACS-sorted double-positive population (Supplementary Fig. 6B). A significant reduction of NFKB1/NFKB2 was confirmed by Flow Cytometry (Supplementary Fig. 5C) and western blotting (Supplementary Fig. 5D). Similar to Jurkat cells, FOXP3 expression was not significantly reduced by NFKB1 or NFKB2 knockout, as confirmed by Flow Cytometry (Supplementary Fig. 5E) and western blotting (Supplementary Fig. 5F). As expected, Treg-related molecules were not highly expressed in Teffs, except for TIGIT, which is normally expressed by activated memory T cells, and not altered by NFKB1/NFKB2 knockout, as shown by qPCR (Supplementary Fig. 6C) and Flow Cytometry (Supplementary Fig. 6D). Therefore, NFKB1/NFKB2 was completely knocked out by CRISPR/Cas9 mediated bi-allelic gene disruption

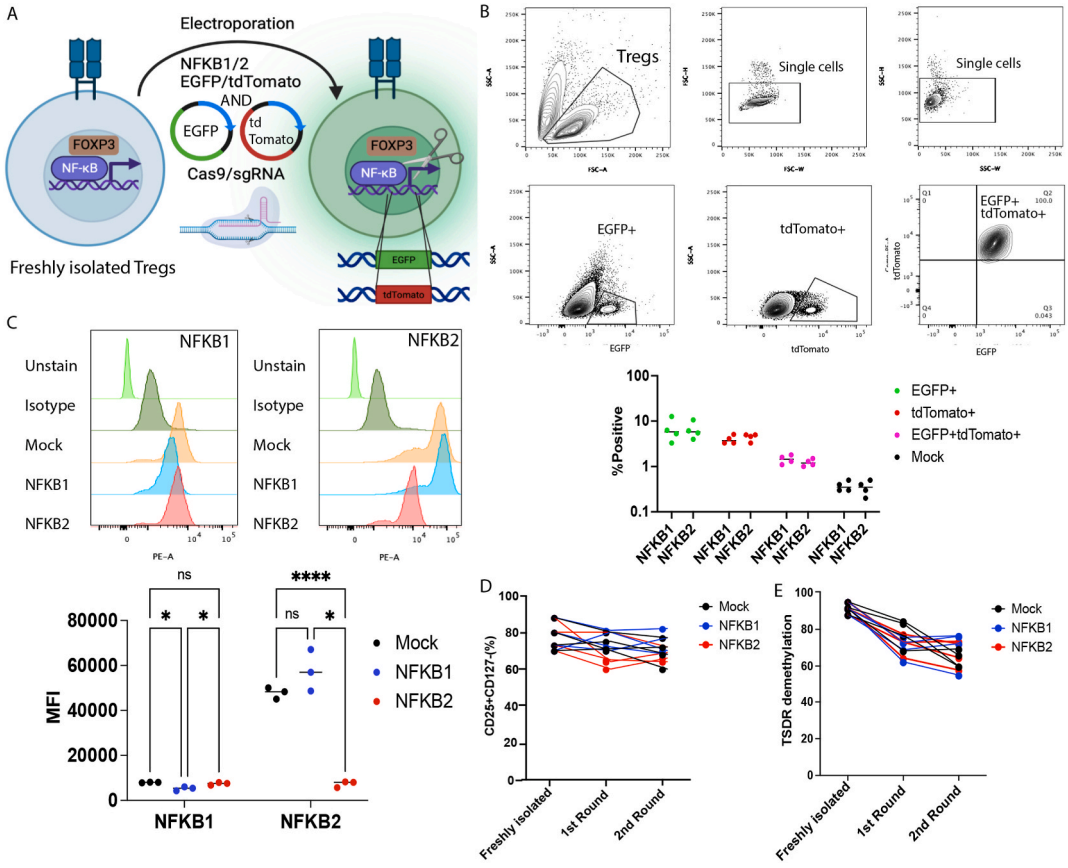

Fig. 6. Bi-allelic NFKB1/NFKB2 knockout can be achieved in primary human Tregs. (**A**) NFKB1/NFKB2 Editing strategy of Tregs. (**B**) EGFP/tdTomato knock-out cassette was inserted in NFKB1/NFKB2 loci. (**C**) NFKB1/NFKB2 knock-out was confirmed by Flow Cytometry (n = 4). (**D**) Treg-like phenotype of NFKB1/NFKB2 knock-out Tregs was analyzed by Flow Cytometry (n = 4). (**E**) TSDR demethylation of NFKB1/NFKB2 knock-out Tregs was analyzed by epigenetic qPCR (n = 4). One-way analysis of variance followed by Kruskal Wallis tests were performed when comparing multiple conditions. Data were representative of three independently repeated experiments shown by mean ± SD (N = 3, n = 1,1,2).

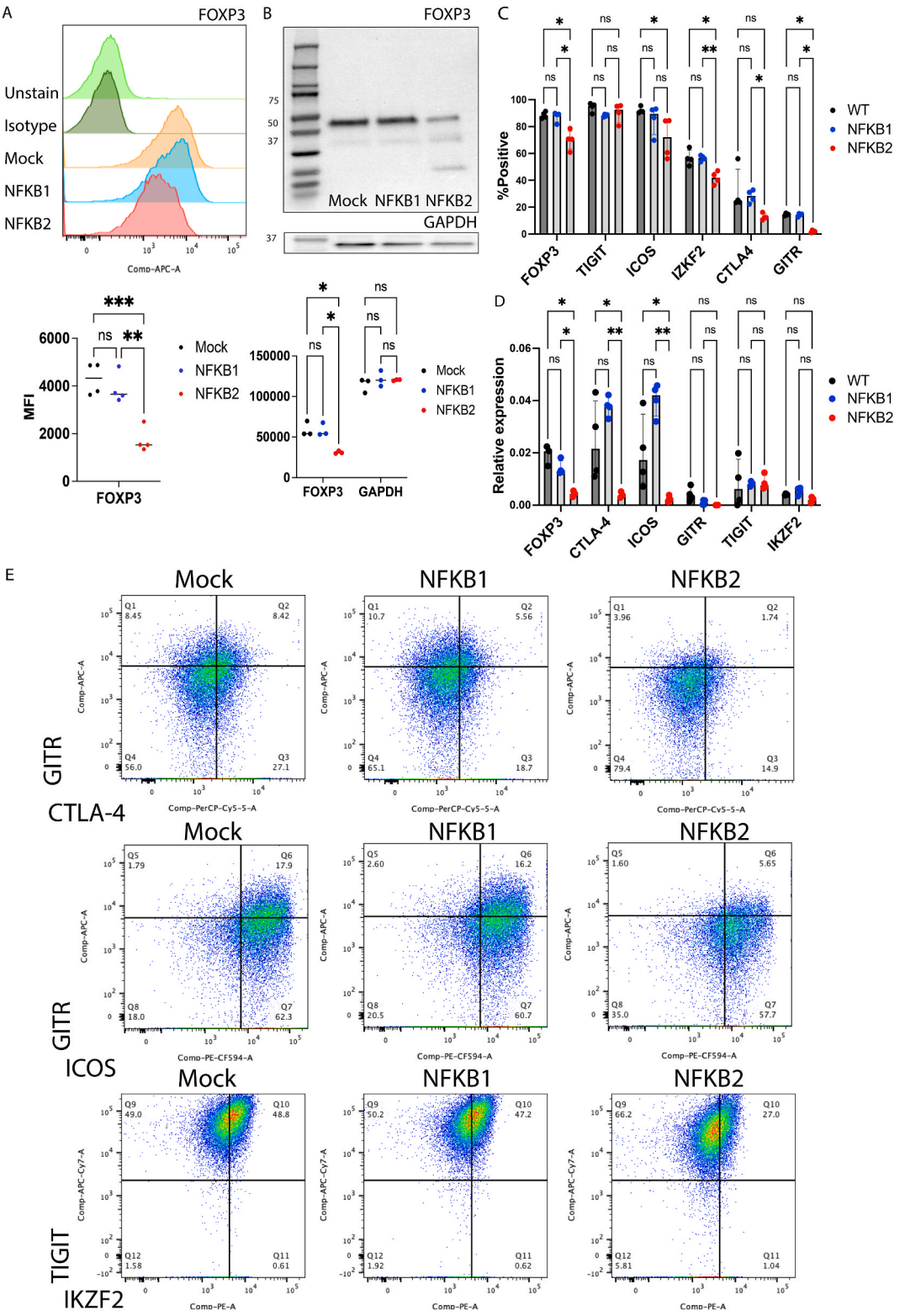

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Fig. 7. FOXP3 expression in primary Tregs, not in CD4$^+$ T cells was reduced by bi-allelic NFKB2 knockout. (A–B) FOXP3 expression of NFKB1/NFKB2 knockout Tregs was analyzed by (**A**) Flow Cytometry (n = 4) and (**B**) Western blot (n = 3). Full un-adjusted images are provided in Supplementary Fig. 8. (***C*-D**) Expressions of Treg-related molecules were analyzed by (**C**) Flow Cytometry (n = 4) and (**D**) qPCR (n = 3). (**E**) Representative dot plots of CTLA-4, GITR and ICOS. One-way analysis of variance followed by Kruskal Wallis tests were performed when comparing multiple conditions. Data were representative of three independently repeated experiments shown by median ± IQR (N = 3, n = 1,1,2).

in human primary CD4$^+$ T cells. However, it was not possible to assess the relationship between NFKB1/NFKB2 due to limited FOXP3 expression in Teffs.

3.6. FOXP3 expression in primary tregs, but not CD4$^+$ T cells, is reduced by bi-allelic NFKB2 knockout

To better understand the biological role of NFKB2 signaling in human Tregs, primary Tregs were isolated from human PBMCs and edited using CRISPR/Cas9 (Fig. 6A). Similar to Teffs, Tregs were successfully edited by CRISPR/Cas9. The edited Tregs expressed both EGFP and tdTomato, as confirmed by Flow Cytometry (Fig. 6B). The average GFP knock-in efficiencies were 6.9 ± 4.0 % for NFKB1 and 6.6 ± 2.8 % for NFKB2 loci (n = 4, mean ± SD). The average tdTomato knock-in efficiencies were 8.0 ± 0.9% for NFKB1 and 4.5 ± 0.8% for NFKB2 loci (n = 4, mean ± SD). Survival and proliferation of sorted cells were comparable between NFKB1/NFKB2 knockout Tregs. Reduced expression of NFKB1/NFKB2 in human Tregs was confirmed by Flow Cytometry (Fig. 6C). Moreover, Treg-like phenotypes, such as CD25$^+$CD127^{low} (Fig. 6D) and TSDR demethylation (Fig. 6E), were maintained after CRISPR/Cas9 mediated gene disruption and *in vitro* expansion. Thus, it was possible to knockout NFKB1/NFKB2 in Tregs using CRISPR/Cas9 and maintain a Treg-like phenotype without the outgrowth of contaminated Teffs. Therefore, NFKB1/NFKB2 was completely knocked out in Tregs mediated by CRISPR/Cas9 mediated bi-allelic gene disruption without losing Treg-like phenotypic hallmarks.

Treg-like phenotypes and functions were tested in NFKB1/NFKB2 knockout Tregs. Similar to MT-2 cells, FOXP3 expression was significantly reduced in NFKB2 knockout Tregs, but not in NFKB1 knockout Tregs, as confirmed by Flow Cytometry (Fig. 7A) and western blotting (Fig. 7B). FOXP3 mRNA expression (Fig. 7D) was significantly altered by the NFKB2 knockout, but not by the NFKB1 knockout. On the other hand, TSDR remained almost fully unmethylated and not influenced by NFKB1/NFKB2 knockout (Fig. 6E). These results indicate that reduced FOXP3 expression in Tregs is mainly explained by the instability of the FOXP3 mRNA/protein due to the lack of NFKB2 signaling, and not by changes in TSDR methylation.

Similar to MT-2 cells, FOXP3-dependent Treg-related molecules, including CTLA-4, ICOS, IKFZ2, and GITR, were downregulated in NFKB2 knockout Tregs, but not in NFKB1 knockout (Fig. 7C). In contrast, TIGIT was not inhibited by NFKB1/NFKB2 knockout. The mRNA expression of Treg-related molecules was also inhibited by NFKB2 knockout, but not by NFKB1 knockout (Fig. 7D). The suppression assay revealed reduced suppressive function in NFKB2 knockout Tregs, but not in NFKB1 knockout Tregs (Fig. 8A–B). The collective findings indicate that NFKB2 knockout Tregs partially lost their Treg-like function owing to reduced FOXP3 expression.

4. Discussion

Tregs control immune response and tolerance in humans and mice [29,30]. FOXP3+ Tregs share many biological features between humans and mice. However, they are not fully identical. For example, exon 2 and alternative splice variants are present only in humans [31]. Another functional difference is that activation-induced FOXP3 expression in T cells has been observed in humans, but not in mice [32]. Therefore, it is important to consolidate human Treg biology, especially for clinical translation, because Tregs are promising cell modalities, and the number of clinical trials using Tregs is increasing [33]. Many questions still need to be addressed in human Treg

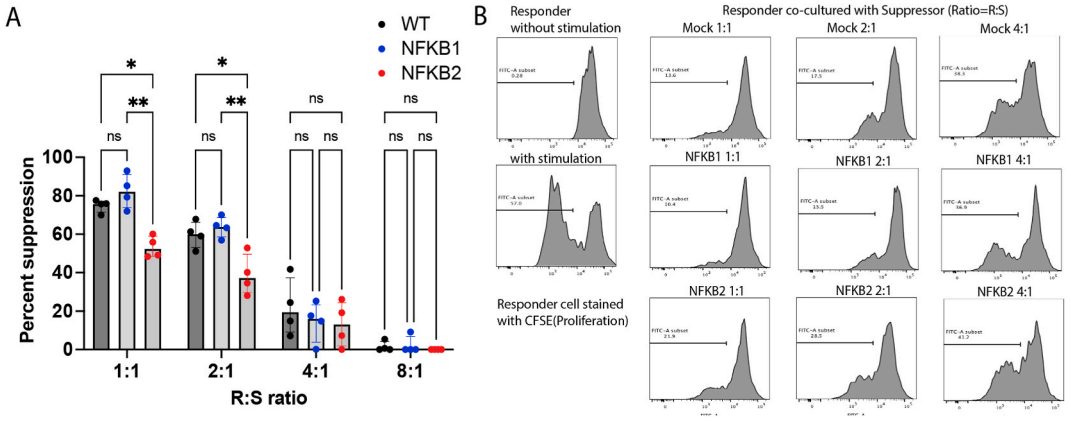

Fig. 8. Suppressive function in primary Tregs was reduced by bi-allelic NFKB2 knockout. (**A**) Suppression assays were conducted with NFKB1/NFKB2 knockout Tregs (n = 4). (**B**) Representative dot-plots of responder cells used in suppression assay. One-way analysis of variance followed by Kruskal Wallis tests were performed when comparing multiple conditions. Data were representative of three independently repeated experiments shown by median ± IQR (N = 3, n = 1,1,2).

biology. This reflects the difficulty in obtaining human primary Tregs compared to other model organisms, such as mice.

NFKB signaling is an essential transcription factor for cell proliferation, differentiation, and apoptosis [34]. Mutations in either NFKB1 or NFKB2 are known to result in the autoimmunity in humans, however, the precise disease mechanism is not yet fully understood [10,16]. Interestingly, NFKB2 deficiency, not NFKB1 deficiency, is shown to be associated with Treg deficiency or functional impairment. The role of NFKB2 in Tregs has been studied in NFKB2 knockout mice [9]. However, the functional role of NFKB2 in human Tregs is not yet fully understood, partially because of the lack of suitable biological resources. Moreover, the rarity and clinical heterogeneity, such as the presence of immune suppressants and active infections or inflammations, of patients with NFKB deficiency are other limitation in the study of Treg function in the absence of NFKB-related molecules.

The emergence of the CRISPR/Cas9 technology has enabled the generation of knock-in/knockout immune cells against specific loci. CRISPR/Cas9 gene editing in T cells and Tregs has become a standard tool for gene engineering in basic science and clinical applications against genetic and non-genetic autoimmunity [35]. Especially for IPEX syndrome, FOXP3 mutations have been successfully edited by CRISPR/Cas9 gene editing [18]. In addition to therapeutic purposes, CRISPR/Cas9 mediated FOXP3 knockout strategy is also contributing the understanding of human Treg biology [24,36]. The successful NFKB1/NFKB2 knockout in Teffs and Tregs using bi-allelic homologous recombination in the present study should have important implications.

In MT-2 cells, FOXP3 protein expression was reduced after NFKB2 knockout, as confirmed by Flow Cytometry and western blotting. Moreover, epigenetic and mRNA qPCR showed that TSDR methylation and mRNA expression were not significantly reduced, in contrast to FOXP3 downregulation at the protein level. These data imply that FOXP3 expression may be stabilized by NFKB2 signaling at the protein level. This is consistent with the autoinflammatory phenotype of patients with NFKB2 deficiency, which is explained by Treg dysfunction.

Analysis of NFKB2 knockout Tregs revealed reduced FOXP3 expression, similar to that in MT-2 cells. This finding indicates that NFKB2 maintains FOXP3 expression in human primary Tregs and Treg-like cell lines. In contrast, NFKB2 knockout did not change residual FOXP3 expression in $CD4^+$ T cells and the Jurkat T cell leukemic cell line. These findings are compatible with the clinical phenotype of NFKB2 deficiency, which is characterized by Treg dysfunction. Recently, TSDR analysis demonstrated that pharmacological NFKB1 inhibition improves Treg function and persistence of the Treg phenotype [37]. It was not possible to observe significant improvement in the Treg-like phenotype and functions in our system. However, it was possible to further confirm that canonical and non-canonical NFKB signaling independently and differently contribute to the Treg phenotype and function.

Treg-related molecules are divided into FOXP3-dependent and -independent molecules. CTLA-4, IKZF-2, and GITR are FOXP3-dependent molecules. In contrast, FOXP3-independent Treg-related molecules, such as TIGIT and ICOS, remained stable in MT-2 cells and primary human Tregs. Co-inhibitory molecules, such as CTLA-4, ICOS, and TIGIT, directly contribute to the suppressive function of Tregs. Antibody blockade of these Treg-related molecules results in reduced suppressive function [38]. Therefore, these molecules may be used as potential therapeutic targets in cancer immunotherapy to enhance the immune response against cancer antigens by blocking these co-inhibitory molecules. NFKB2 does not directly contribute to the suppressive function, but might maintain the suppressive function through FOXP3-dependent, Treg-related molecules. Therefore, tumor immunity might be enhanced by inhibiting non-canonical NFKB signaling.

A limitation of our study is the efficacy of CRISPR/Cas9 mediated NFKB1/NFKB2 knockout. Compared to the traditional knockdown, CRISPR/Cas9 can disrupt gene expressions through marker gene expressions. Therefore, it was possible to identify and enrich knockout cells by FACS sorting. However, it was possible to detect residual protein expressions by Flow Cytometry and western blotting. Interestingly, cleaved NFKB1(p50) and NFKB2 (p52) were significantly reduced through CRISPR/Cas9 gene disruption. Therefore, it was possible to downregulate NFKB1/NFKB2 signaling pathway and observe reduced suppressive function in NFKB2 knockout Tregs as observed in the Nfkb2 knockout murine Tregs.

Another limitation of our study is that we are not yet able to assess Treg development *in vivo* due to the technical limitations of editing hematopoietic stem cells. Previously, canonical NFKB signaling controls effector T cell differentiation, however it was not possible to fully discover the role of NFKB signaling in humans due to the lack of suitable materials [39]. In addition, Treg differentiation in humans is still not fully understood, but NFKB2 might be associated with Treg differentiation based on the Nfkb2/Relb knockout mice showing autoimmunity. Moreover, it is possible that NFKB2 signaling interacts with FOXP3 and influences Treg development. Tregs were developed in Nfkb2 knockout mice, in which Foxp3 expression was reduced and Nfkb2 deficient Tregs were less suppressive [9,40]. Furthermore, genome editing of hematopoietic stem cells has become feasible due to recent technical advances in molecular biology [41,42]. However, it is still challenging to edit long-term repopulating hematopoietic stem cells and achieve stable engraftment of edited cells in immunodeficient mice. Future studies are warranted to validate the functional role of NFKB1/NFKB2 in hematopoietic stem cell development and differentiation into effector T cells and Tregs. Overall, our results suggest that NFKB2 signaling stabilizes the Treg phenotype and function through a FOXP3-dependent regulatory T cell program.

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki. PBMC from normal healthy donors were purchased from a commercial source and it was declared that the cells were isolated after obtaining permission for their use in research applications by informed consent or legal authorization. Institutional Review Board (IRB) of the Jikei University School of Medicine granted the exemption of research use of deidentified PBMCs provided from commercial source isolated after obtaining permission for their use in research applications by informed consent or legal authorization. The use of deidentified cell lines was also considered exempt and do not require IRB review of the Jikei University School of Medicine.

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Data availability

Data will be made available on request. The RNA-seq datasets generated during the current study have been deposited at ArrayExpress under accession number E-MTAB-13408.

CRediT authorship contribution statement

Yohei Sato: Writing - review & editing, Writing - original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Erika Osada:** Writing - original draft, Visualization, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Yoshinobu Manome:** Writing - review & editing, Writing - original draft, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22911.

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