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CRISPRi-mediated knock-down of PRDM1/BLIMP1 programs central memory differentiation in *ex vivo*-expanded human T cells

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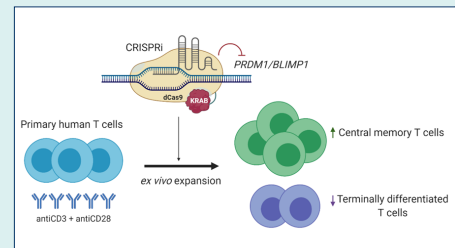
Article Info

Abstract

Introduction: B lymphocyte-induced maturation protein 1 (BLIMP1) encoded by the positive regulatory domain 1 gene (*PRDM1*), is a key regulator in T cell differentiation in mouse models. BLIMP1-deficiency results in a lower effector phenotype and a higher memory phenotype.

Methods: In this study, we aimed to determine the role of transcription factor BLIMP1 in human T cell differentiation. Specifically, we investigated the role of BLIMP1 in memory differentiation and exhaustion of human T cells. We used CRISPR interference (CRISPRi) to knock-down BLIMP1 and investigated the differential expressions of T cell memory and exhaustion markers in BLIMP1-deficient T cells in comparison with BLIMP1-sufficient ex vivo expanded human T cells.

Results: BLIMP1-deficiency caused an increase in central memory (CM) T cells and a decrease in effector memory (EM) T cells. There was a decrease in the amount of TIM3 exhaustion marker expression in BLIMP1-deficient T cells; however, there was an increase in PD1 exhaustion marker expression in BLIMP1-deficient T cells compared with BLIMP1-sufficient T cells.

Conclusion: Our study provides the first functional evidence of the impact of BLIMP1 on the regulation of human T cell memory and exhaustion phenotype. These findings suggest that BLIMP1 may be a promising target to improve the immune response in adoptive T cell therapy settings.

Introduction

The B lymphocyte-induced maturation protein 1 (BLIMP1) encoded by the positive regulatory domain 1 gene (*PRDM1*), is a critical regulator of plasma cell differentiation in B cells.[1] In order to have proper induction of effector functions in mouse T cells, BLIMP1 is essentially needed in CD4+ T lymphocytes[2-6] and in CD8+ T lymphocytes.[3,5,7-10] Mouse models that are deficient in BLIMP1 have reduced T lymphocyte cytotoxic activity,[3,11,12] increased memory phenotype population,[3,5,11] and enhanced survival of effector cells (persistence).[5]

However, there is no direct experimental evidence for the role of BLIMP1 in regulating human T cell fate and function.

Elucidation of the regulatory functions of BLIMP1 in human T cells is not only important for understanding the mechanism of the cellular immune response against infections and cancer, but also may provide insights for improving T cell-based immunotherapies. Ex vivo expanded human T cells are particularly important in human cancer immunotherapy because of their ability to mediate cancer regression in patients.[13] The duration of T

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cell persistence after the adoptive transfer is correlated with clinical response, and this has led to strategies to enhance in vivo persistence. These strategies consist of extrinsic modification of the host environment, conditioning lymphodepletion, intrinsic manipulation of the T cell itself, cytokine modulation to enhance cellular replicative potential,[14] phenotype-based selection, and genetic engineering.[15] The importance of the differentiation state of the infused cells has been shown by studies in mouse models.[16,17] Antigen-independent persistence and self-renewal are considered to be specific memory T cell characteristics.[7]

The effects of BLIMP1 knock-down on T cell differentiation have been extensively studied in mouse models.[1-12] To the best of our knowledge, this research has not been conducted on human T cells. Therefore, we intend to study the effects of BLIMP1 knock-down on human T cells. We focused on ex vivo expanded primary human T cells due to their application in human T cell therapies. We knocked down the *PRDM1* gene in primary human T lymphocytes to determine the effects of BLIMP1 deficiency on T cell phenotype and T cell exhaustion.

Materials and Methods
Materials
Esp3I enzyme (#FD0454), TurboFect Transfection Reagent, and Dynabead Human T-Activator CD3/CD28 (#11131D) were purchased from Thermo Fisher Scientific. RPMI-1640 medium, fetal bovine serum, and L-glutamine were purchased from Sigma-Aldrich. Lymphodex (#002041500) was obtained from Inno-train, the RNeasy Micro Kit (#74004) from Qiagen, PrimeScript RT Reagent Kit from Takara, SYBR Green Master Mix (#4367659) from Applied Biosystems Life Technologies, Inc., True-Nuclear™ Buffer Set from BioLegend, Rat IgG2a antibody from BD Biosciences, and human IL-2 from Royan Institute. The anti-CD3-FITC (clone HIT3a, #555339), anti-CD4-PerCP (clone SK3, #566316), anti-CD8-PE (clone HIT8a, #555635), anti-CD45RA-PE (clone 5H9, #556627), anti-CD45RA-APC (clone 5H9, #561210), and anti-BLIMP1-PE (clone 6D3, #564702) antibodies from BD Biosciences, the anti-CD8-Pacific Blue (clone SK1, #344718) and anti-CD62L-FITC (clone DREG-56, #304812) antibodies from BioLegend, the anti-CD3-APC-Alexa Flouor 750 (clone UCHT1, #A66329), anti-CD4-Krome Orange (clone 13B8.2, #A96417) antibodies from Beckman Coulter, and the anti-CD107a-PE (clone eBioH4A3, #12-1079-42) antibody from eBioscience were used for the flow cytometry experiments.

Microarray data collection and analysis
Two publicly available microarray gene expression datasets were obtained from the NCBI Gene Expression Omnibus. The CD4+ T cell[16] (accession GSE23321) and CD8+ T cell[18] (accession GSE61697) datasets were used for differential gene expression analysis. Both datasets contained the expression data of four human T cell phenotypes: naïve (N), stem cell memory (SCM), central memory (CM), and effector memory (EM). We used R statistical programming language and applied log2 transformation to raw expression levels, and the datasets were merged by similar gene symbols. Cleaning and normalization,[19] and adjustments for the batch effect amongst these two datasets were done using an empirical Bayesian method described by Johnsen et al,[20] which is implemented in the SVA package.[21] A pairwise comparison was performed between each two consecutive stages (i.e., N vs. SCM, SCM vs. CM, and CM vs. EM) by using linear models for microarray (LIMMA). Statistically significant differentially expressed genes (DEGs) were identified by applying a threshold of 0.05 to the adjusted P-value, which was based on the false discovery rate using the Benjamini-Hochberg procedure[22] and more than 1.5-fold change. Next, we computed the intersection of the DEG sets with the purpose of locating genes that upregulated during the course of differentiation from N T cells to EM T cells. In order to identify the regulators of EM T cell-upregulated genes, a list of 107 DEGs that had more than two-fold higher expressions in EM T cells compared with CM T cells were subjected to TF gene enrichment using Enrichr[23] and the ARCHS⁴ TF Co-expression database.[24]

Genetic vectors
The lentiviral CRISPR interference (CRISPRi) plasmid used in this study, pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro, was a gift from Charles Gersbach (Addgene plasmid #71236; http://n2t.net/addgene: 71236; RRID: Addgene_71236).[25] pLV-hU6-sgPRDM1-5-hUbC-dCas9-KRAB-T2a-Puro (sgPRDM1-5), pLV-hU6-sgPRDM1+35-hUbC-dCas9-KRAB-T2a-Puro (sgPRDM1+35), and pLV-hU6-sgPRDM1+61-hUbC-dCas9-KRAB-T2a-Puro (sgPRDM1+61) were generated using Esp3I and T4 DNA ligase enzymes (Thermo Fisher Scientific, #FD0454, UK). Table 1 lists the sequences of these three sgRNAs. In order to ensure the specificity of the sgRNA sequences, we confirmed that there is no potential off-targets in the human genome with less than 3 mismatches using CRISPOR (http://crispor.tefor.net/) and CRISPR RGENE Tool (http://www.rgenome.net/cas-offinder/).

In order to produce the lentiviral particles, the aforementioned pLV plasmids and packaging plasmids

Table 1. sgRNA oligonucleotides integrated into the CRISPR interference (CRISPRi) construct

sgRNA	Strand	Sequence
sgPRDM1-5	Sense	CACCGTGGCTAAGGAAATCTTAAGC
	Antisense	AAACGCTTAAGATTTCCTTAGCCAC
sgPRDM1+35	Sense	CACCGCACTGGAGGGCCGAGTGTCA
	Antisense	AAACTGACACTCGGCCCTCCAGTGC
sgPRDM1+61	Sense	CACCGCACTCGGCCCTCCAGTGTTG
	Antisense	AAACCAACACTGGAGGGCCGAGTGC

psPAX2 and pMD2.G were transfected into HEK293T cells using a TurboFect transfection reagent (Thermo Scientific). psPAX2 and pMD2.G were gifts from Didier Trono (Addgene plasmid #12259; http://n2t.net/addgene:12259; RRID: Addgene_12259 and Addgene plasmid #12260; http://n2t.net/addgene:12260; RRID: Addgene_12260). The Lentivirus-containing supernatants were collected at 48- and 72-hour post-transfection, filtered through a 0.45 μm filter, and concentrated by centrifugation at 20 000 g for 2 hours.

Primary T cell culture and transduction
Peripheral blood mononuclear cells (PBMCs) were obtained from 5 ml blood obtained from healthy blood donors. The donor signed a written informed consent to authorize use of their blood sample. The Institutional Review Board, Royan Institute (Tehran, Iran) approved this research study and study-related informed consent. PBMCs were isolated by Lymphodex (Inno-train, REF 002041500) by gradient centrifugation. The PBMCs were subsequently cultured with Dynabead Human T-Activator CD3/CD28 (ThermoFisher Scientific, REF 11131D) in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich). The culture was placed in a humidified incubator in 5% CO_2 at 37°C. After 24 hours, the activated T cells were transduced with lentiviral supernatants in the presence of 100 U/ml of human IL-2 (Royan Institute, Iran), and maintained in the presence of 50 U/mL of human IL-2 for the rest of the culture period. In order to enrich the transduced T cells, puromycin (2.5 μg/mL) was added to the media at 48 hours post-transduction and continued for six days.

For the co-culture experiment, K562 cells engineered by a membrane-bound single-chain variable fragment (scFV) from an anti-CD3 antibody (K562-OKT3) were used as universal target cells. Transduced T cells were cultured in a 1:1 ratio with the K562-OKT3 cells in the absence of IL-2. The percentages of the CD3- target cells and CD3+ T cells, and the expression of CD107a were ascertained by flow cytometry analysis.

RNA isolation, cDNA synthesis, and RT-qPCR analysis.
Total RNA was isolated from the PBMCs by using an RNeasy Micro Kit (Qiagen, 74004) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA by using a PrimeScript RT Reagent Kit (Takara, Japan). A total of 2 μL of diluted cDNA template (25 ng) was added per reaction to achieve a final volume of 20 μL of the reaction mixture. Duplicate RT-qPCR reactions were designed with SYBR Green Master Mix (Applied Biosystems Life Technologies, Inc., REF 4367659). Gene expression analysis was assessed by RT-qPCR for the candidate genes with an ABI StepOnePlus (Applied Biosystems Life Technologies System) according to the following program: 95°C for 10 minutes, 40× (95°C for 15 seconds, 60°C for 1 minute), followed by melting curve acquisition at a ramp from 60°C to 95°C.

All of the target genes were normalized to *GAPDH*, an endogenous control, in order to analyze the gene expression results, which were finally calibrated against a control group. Relative gene expression levels were determined by the $2^{-(\Delta\Delta CT)}$ method. Table 2 lists the primers used in the RT-qPCR analysis.

Flow cytometry
Transduced T cells were used in this experiment. The T cells were washed with phosphate-buffered saline (PBS), centrifuged, and then blocked with BSA. The cells were subsequently washed with PBS. Primary antibodies were added to the test samples according to the manufacturer's recommendations. The cells in both groups were rinsed with PBS. After surface staining and to perform intracellular staining of BLIMP1, initially the cells were fixed and permeabilized with a True-Nuclear™ Buffer Set (BioLegend) according to the manufacturer's instructions and then stained with rat anti-human BLIMP1 antibody or rat IgG2a antibody (BD Biosciences, San Jose, CA, USA). Finally, the cells were assessed by flow cytometry (BD FACSCalibur or BD FACSCanto, BD Biosciences) and analyzed with FlowJo v. 10 software.

Statistical analyses
All the experiments were carried out in triplicate. The data are expressed as mean ± SD. The independent student's *t* test with either SPSS 24.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism 8.0.2 (GraphPad Software, Inc., La Jolla, CA, USA) was applied to analyze group differences in the data. Significance was set at 5%. The statistics symbol for meaningful differences is an '*' for the student's *t* test.

Results
Gene expression analysis of human T cell memory subpopulations
First, we compared the transcriptomes of the N, SCM, CM, and EM subpopulations of the human T cells to identify

Table 2. Primers used for RT-qPCR

Target gene/element	Forward primer	Reverse primer
PRDM1	ACGTGTGCCCTTTGGTATGT	CTGGGATTCTGGTGCTGATG
WPRE	CGTTGTCAGGCAACGTG	CTGACAGGTGGTGGCAAT
GAPDH	CTCATTTCCTGGTATGACAACGA	CTTCCTCTTGTGCTCTTGCT

PRDM1: Transcription factor PR/SET domain 1

potential regulators of memory T cell differentiation. We identified eight genes: *CCL5*, *CST7*, *BHLHE14*, *PRDM1*, *MYO1F*, *ERN1*, *MAF*, and *CD58*, which upregulated during the course of differentiation from the N to the EM T cell phenotypes (Fig. 1A). We also performed a transcription factor enrichment analysis on the genes that upregulated in the EM T cells compared to the CM T cells (Table S1, Supplementary file 1) to identify the transcription factors associated with differentiation of the memory T cell subpopulations (Figs. 1B and 2A). According to our observation, *PRDM1* was associated with the highest number of EM T cell upregulated genes (19 genes), which included three transcription factors (FOSL2, PLEK, and ID2) and, in turn, link *PRDM1* to more target genes in the built network (Fig. 2B). We then confirmed a graded increase in PRDM1/BLIMP1 expression during T cell differentiation from the N to EM T cells by measuring BLIMP1 protein expression in the different memory T cell subpopulations in unstimulated human PBMCs. The highest BLIMP1 median fluorescence intensity (MFI) was detected in the EM T cell and terminally effector (TE) subpopulations; the lowest MFI was observed in naïve like T cells (NL) that comprised the N T cell and SCM T cell populations (Fig. 1C, D). These results, along with the previous knowledge on the function of mouse BLIMP1 in T cell differentiation, prompted us to investigate the role of PRDM1/BLIMP1 in human memory T cell differentiation by using a loss-of-function approach.

Efficient knock-down of the transcription factor PR/SET domain 1 (PRDM1) gene with sgPRDM1/Cas9-KRAB

In order to efficiently knock-down BLIMP1 expression using the CRISPRi method, we tested three different sgRNAs that targeted different positions, -5, +35 and +61, relative to the *PRDM1* transcription start site (TSS) (Fig. 3A). The three designed sgRNAs were integrated into a lentiviral CRISPRi construct that expressed a Cas9-KRAB fusion protein along with a puromycin resistant gene (Fig. 3B). The three constructs performed differently in reducing *PRDM1* mRNA expression. While sgPRDM1+61 and sgPRDM1-5 did not make a significant change in *PRDM1* expression, sgPRDM1+35 dramatically decreased PRDM1 gene expression at the mRNA level (Fig. 3C). Thus, sgPRDM1+35 (subsequently denoted as sgPRDM1 in this paper) was selected for *PRDM1* suppression for the following experiments.

The role of B lymphocyte-induced maturation protein 1 (BLIMP1) in determining T cell memory phenotype

We sought to determine whether BLIMP1 deficiency impacted the T cell phenotypes. To this extent, we measured CD4, CD8, CD45RA, and CD62L cell surface markers by extracellular staining and flow cytometry analysis on day 18 and day 22 after T cell activation (Fig.
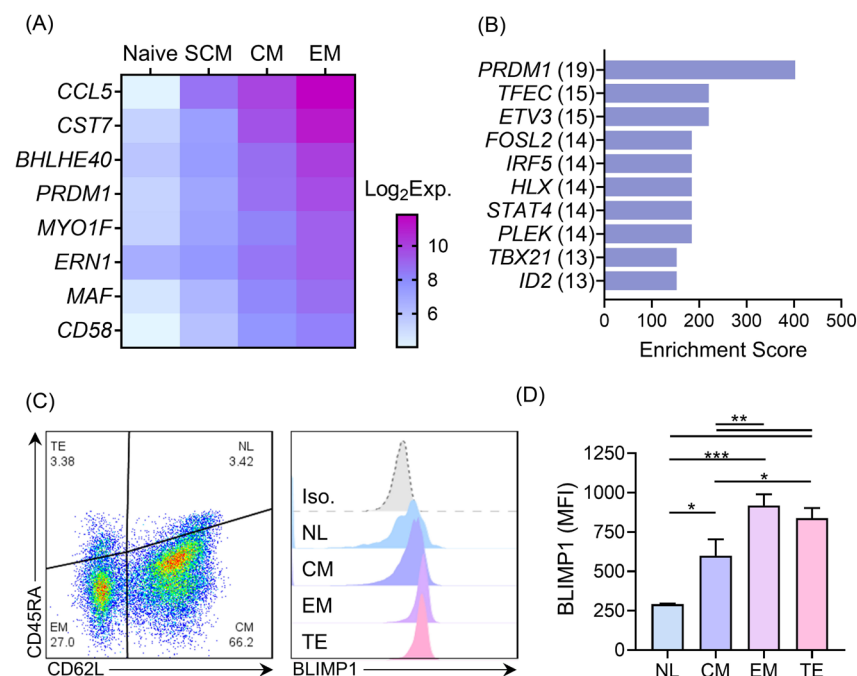

Fig. 1. Global gene expression and BLIMP1 protein expression in different human T cell memory phenotypes. A) Gene expression of eight genes on a significantly meaningful (adj. *P*>0.05) graded scale of increase were expressed in different phenotypes, from N to EM T cells. B) The top ten transcription factors with enriched targets among the EM T cell-upregulated genes, based on the ARCHS[4] TF co-expression database. The number of detected targets for each TF is shown in parentheses. C) The scatter plot (left) shows a representative data of memory phenotypes of CD3+ T cells in unstimulated human PBMCs detected based on CD62L and CD45RA expressions. Stacked histograms (right) show BLIMP1 MFI, which is gated on different memory phenotypes. D) The summary quantitative data of BLIMP1 MFI across different memory phenotypes of human T cells. The data are presented as mean ± SD, *P<0.05, **P<0.01, and ***P<0.001, n = 9 in transcriptome data, n = 4 in BLIMP1 staining. BLIMP1: B lymphocyte-induced maturation protein 1; MFI: Median fluorescent intensity; N: Naïve; EM T cell: Effector memory T cell; PBMC: Peripheral blood mononuclear cells.
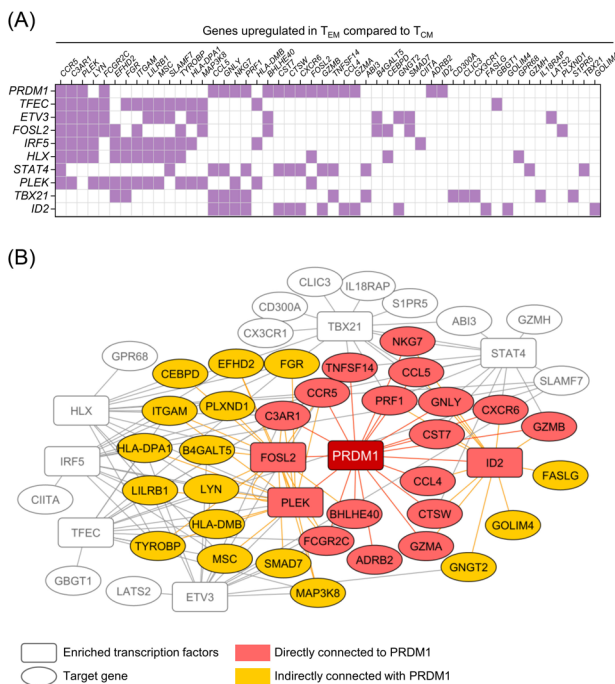
(A)

(B)

Fig. 2. Transcription factor enrichment analysis on a list of genes upregulated in EM T cells compared to CM T cells. A) Heatmap representation of the overlap of the list of top ten enriched transcription factors (vertical axis) and corresponding EM T cell upregulated genes (horizontal axis). Some of the enriched transcription factors, FOSL2, PLEK and ID2, are also present in the EM T cell upregulated gene list. B) A network model built based on top ten enriched transcription factors illustrates the genes directly (red) and indirectly (orange) connected to PRDM1 through other enriched transcription factors. The network was visualized with Cytoscape software. EM T cell: Effector memory T cell; CM T cell: Central memory T cell; PRDM1: transcription factor PR/SET domain 1.

4A). We did not observe any association between PRDM1 knock-down and the CD4+/CD8+ ratio (Fig. 4B, C). No statistical changes were observed in the expressions of the

CD45RA and CD62L cell surface markers in BLIMP1-sufficient and BLIMP1-deficient T cells at day 18 (Fig. 5). However, flow cytometry analysis of T cell memory phenotypes in the whole T cell population at day 22 revealed a meaningful increase in the BLIMP1-deficient CM T cells (P=0.003) and a significant decrease in the EM T cell population (P=.019) in comparison with the BLIMP1-sufficient counterpart T cells (Fig. 4D, E). Phenotypic analysis of CD4+ T cells at day 22 revealed a significant increase (P=0.010) in the CM T cell population (Fig. 4F), while a more significant increase was observed in CM (P=0.002) versus a significant decrease in NL (P=0.032), EM (P=0.00027), and TE (P=0.043) T cells in the CD8+ population at day 22 (Fig. 4G).

The effect of B lymphocyte-induced maturation protein 1 (BLIMP1) suppression on the exhaustion marker expressions

We next explored the expressions of exhaustion markers on sgPRDM1-transduced BLIMP1-deficient human T cells. We first assessed PD1 expression by flow cytometry on day 18 of culture. Although statistically not significant, there was an elevated proportion of PD1-expressing cells in the BLIMP1-sufficient T cells at this time point (Fig. 6 A-C). In order to further assess the influence of BLIMP1 on T cell exhaustion, PD1 and TIM3 exhaustion markers were measured by flow cytometry on day 25. In the whole T cell population on day 25, there was a statistically significant lower frequency of PD1−TIM3+ cells observed in the BLIMP1-deficient T cells, but no statistical difference was seen in the percentage of PD1+TIM3− and PD1+TIM3+ cells (Fig. 7A, B). No statistically significant difference was seen for PD1+TIM3− cells in the BLIMP1-deficient CD4+ T cell populations on day 25 (Fig. 7C); however, there were statistically fewer PD1+TIM3− cells
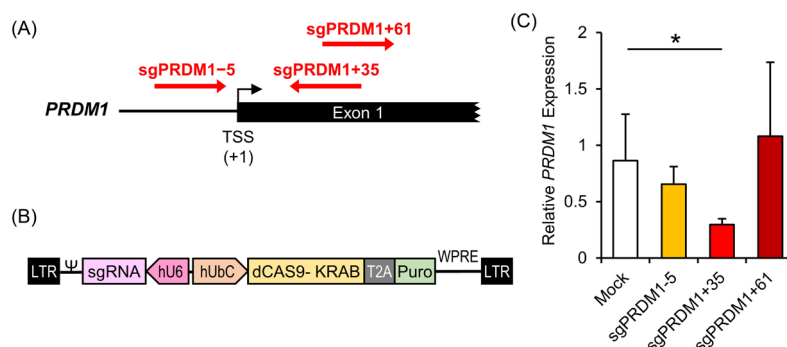

Fig. 3. Knock-down of the transcription factor PR/SET domain 1 (*PRDM1*) gene by designed CRISPR interference (CRISPRi) constructs in human T cells. A) The sgRNA target sites near the *PRDM1* transcription start site (TSS). The *PRDM1* gene is located on chromosome 6. sgPRDM1-5 targets five nucleotides downstream of the TSS; sgPRDM1+35 targets 35 nucleotides upstream of the TSS; and sgPRDM1+61 targets 61 nucleotides upstream of the TSS. B) The CRISPRi construct to knock-down *PRDM1*. The vector is created to knock-down the *PRDM1* gene by the designed sgRNA and the CAS9-KRAB enzyme. By pairing dCas9 with a sequence-specific sgRNA, the dCas9–sgRNA complex can interfere with transcription elongation by blocking RNA polymerase (Pol). It can also impede transcription initiation by disrupting transcription factor binding. In order to achieve enhanced repression, the Krüppel-associated box (KRAB) is fused to the carboxyl terminus of dCas9. Together with a target-specific sgRNA, the dCas9–KRAB fusion proteins can efficiently repress endogenous genes. The puromycin resistant gene expression product is segregated by T2A, a self-cleaving peptide, from the other products of the vector. C) RT-qPCR data depicts expression of the *PRDM1* gene. The bar graph shows mRNA expression in different T cell groups transduced with CRISPRi constructs that comprise the designed sgRNAs. The three constructs perform differently in reducing the expression of *PRDM1* mRNA. sgPRDM1+35 dramatically decreased PRDM1 gene expression at the mRNA level. The data are presented as mean ± SD, *P<0.05, n = 3.
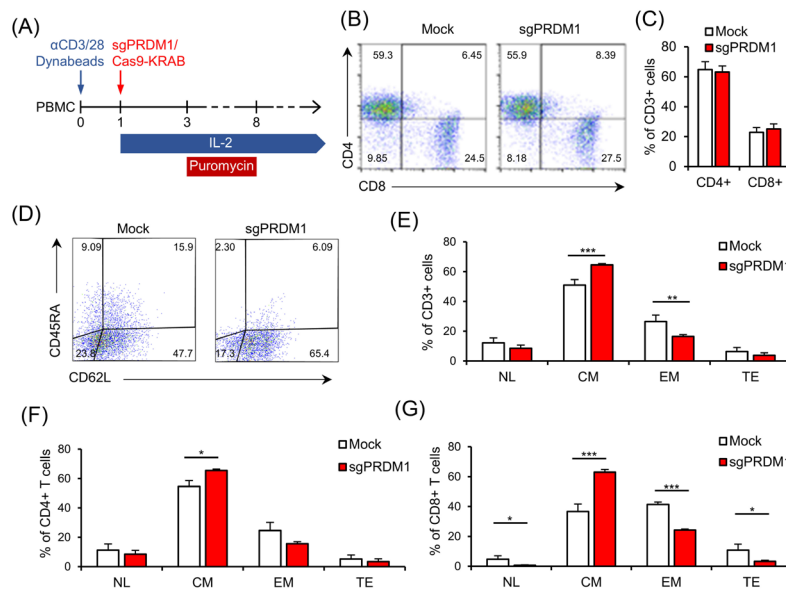
Fig. 4. BLIMP1 knock-down prevents EM and TE T cell phenotypes and increases CM T cell differentiation. A) The schematic timeline of the experimental setting. PBMC was activated by anti-CD3/CD28 Dynabead on day zero. The sgPRDM1/Cas9-KRAB genetic constructs that contain the designed sgRNAs were transduced into T cells of different groups on the first day. T cells received IL-2 from the first day until the end of the experiment, while puromycin treatment was carried out for six days. Flow cytometry assessments were done at different days after the puromycin treatment. B) The scatter plot represents the percentages of the CD4+ and CD8+ T cell population on day 10 in a representative donor. This assessment was fulfilled after puromycin selection on the transduced groups. C) The bar graph represents the percentages of CD4+ and CD8+ T cell populations on day 10. There was no observed association between transcription factor PR/SET domain 1 (PRDM1) knock-down and the CD4+/CD8+ ratio. D) Scatter plot shows the percentages of CD62L and CD45RA expression in the whole T cell population on day 22 in the Mock group and in the whole T cell population on day 22 in the Mock and sgPRDM1 groups. A meaningful increase in BLIMP1-deficient CM T cells and a significant reduction in EM T cells is shown. F) Bar graph depicts the different T cell phenotypes in the CD4+ T cell population on day 22 in the Mock and sgPRDM1 groups. G) Bar graph depicts the different T cell phenotypes in the CD8+ T cell population on day 22 in the Mock and sgPRDM1 groups. The data are presented as mean ± SD, *$P<0.05$, **$P<0.01$ and ***$P<0.001$, n = 3. EM T cell: Effector memory T cell; TE T cell: Terminally effector T cell; CM T cell: Central memory T cell; BLIMP1: B lymphocyte-induced maturation protein 1; PBMC: Peripheral blood mononuclear cells.

in the BLIMP1-deficient CD8+ T cell populations on day 25 (Figs. 6D, E and 7D).

Next, we measured the MFI in the whole T cell population as well as the CD4+ and CD8+ subpopulations to determine if there were any additional differences between BLIMP1-sufficient and BLIMP1-deficient T cells for the expressions of PD1 and TIM3 on day 25. A decreased TIM3 MFI level was observed in BLIMP1-deficient T cells
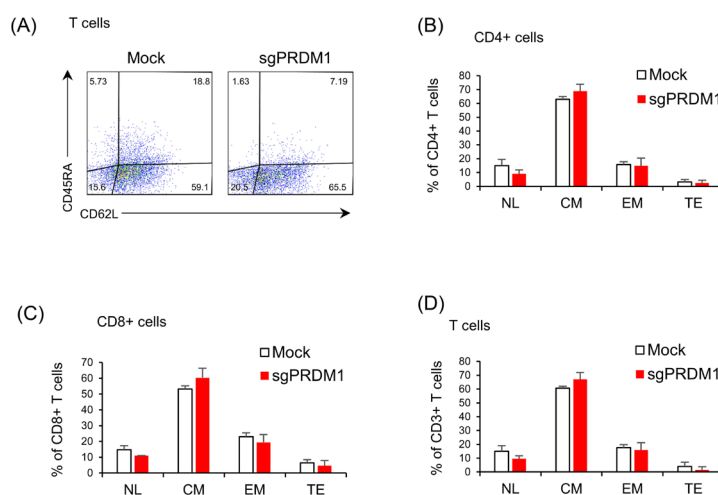

Fig. 5. The early influence of BLIMP1 inhibition on T cell phenotype. No statistical changes were observed in the expressions of the CD45RA and CD62L cell surface markers in BLIMP1-sufficient and BLIMP1-deficient T cells. A) The scatter plots represent the percentages of CD62L and CD45RA expression in the whole T cell population in the Mock and sgPRDM1 groups on day 18. Bar graphs depict: B) different T cell phenotypes in the CD4+ T cell population in the Mock and sgPRDM1 groups on day 18; C) different T cell phenotypes in the CD8+ T cell population on day 18 in the Mock and sgPRDM1 groups; and D) different T cell phenotypes in the whole T cell population on day 18 in the Mock and sgPRDM1 groups. BLIMP1: B lymphocyte-induced maturation protein 1.
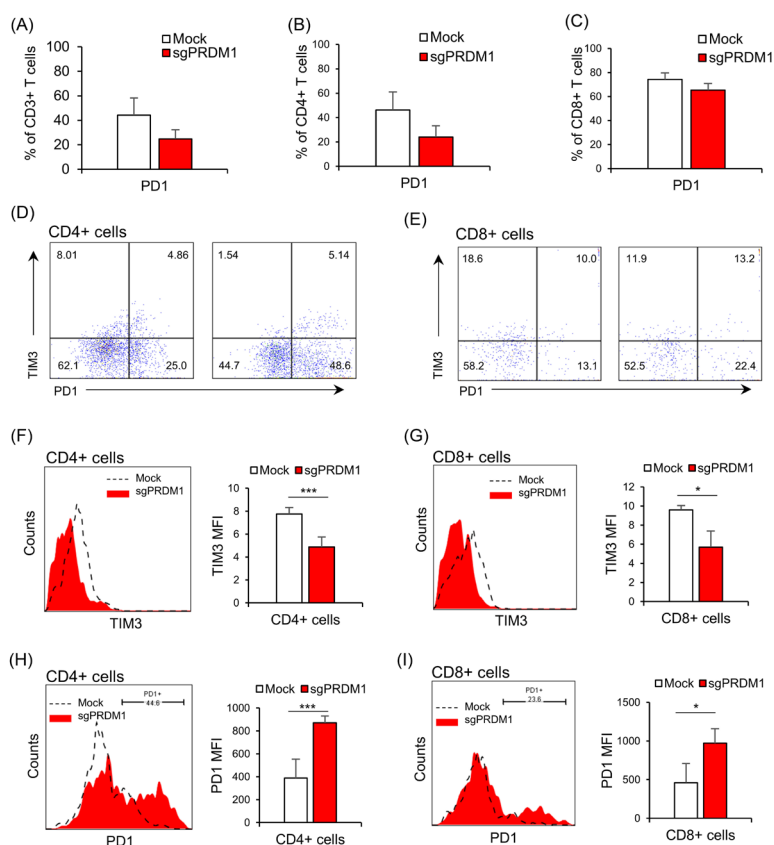
Fig. 6. The impact of BLIMP1 knock-down on exhaustion markers PD1 and TIM3 in the Mock and sgPRDM1 groups in CD4+ and CD8+ T cells. On day 18, there were no statistically meaningful differences observed in the expression levels of PD1 and TIM3 between BLIMP1-sufficient and BLIMP1-deficient T cells. However, on day 25, there was a decline in TIM3 expression and an increase in PD1 expression in BLIMP1-deficient T cells rather than BLIMP1-sufficient T cells. Bar graphs show: A) expression of the exhaustion marker PD1 in the whole T cell population for the Mock and sgPRDM1 groups on day 18 and B, C) expression of the exhaustion marker PD1 in the CD4+ and CD8+ T cell populations, respectively, for the Mock and sgPRDM1 groups on day 18. D, E) Scatter plots depict the expression of the exhaustion markers PD1 and TIM3 in the CD4+ and CD8+ T cell populations, respectively, for the Mock and sgPRDM1 groups on day 25. F, G) The representative histogram and bar graph delineate TIM3 expression in the CD4+ and CD8+ T cell populations, respectively, on day 25. H, I) The representative histogram plot and bar graph show PD1 expression in the CD4+ and CD8+ T cell populations, respectively, on day 25. BLIMP1: B lymphocyte-induced maturation protein 1.

(Figs. 6F, G and 7E, F). On the other hand, PD1+ T cells showed higher PD1 MFI in the BLIMP1-deficient group (Figs. 6H, I and 7G, H,). Overall, this data suggests that BLIMP1 knock-down results in elevated PD1 and reduced TIM3 expressions in ex vivo expanded human T cells.

The effect of B lymphocyte-induced maturation protein 1 (BLIMP1) knock-down on functional characteristics of human T cells

The preferential differentiation of BLIMP1-deficient T cells into cells with a CM phenotype suggested that these cells would have lower proliferation and death rate. Examination of T cell proliferation and death, with an adjusted number of 1×10^5 cells for each group after Puromycin selection (10 days after PBMC isolation), showed that the proliferation rate for BLIMP1-deficient T cells was half and these cells had longer longevity compared to BLIMP1-sufficient T cells (Fig. 8A, B).

We sought to investigate the effect of BLIMP1 knock-down on T cell activation and cytotoxicity by co-culturing

the transduced T cells with an engineered K562 cell line that expresses a membrane-bound anti-CD3 protein (K562-OKT3), and serves as a universal target cell for T cells. Next, we examined the activation marker and changes in T cell-target cell ratio. As shown in Fig. 8C, D, the percentage of target cells was lower in the control group compared to the BLIMP1-deficient group. On the other hand, the MFI of CD107a, a degranulation marker, showed no significant diference btween the groups (Fig. 8E, F). Collectively, these results suggest that downregulation of BLIMP1 is associated with increased overall killing by T cells that might result from their improved persistance instead of increasaed cytotoxic activity.

Discussion

In this study, we used the CRISPRi genome editing tool. It is noteworthy to mention the merits of CRISPRi over siRNA and CRISPR/Cas9. The main advantage of CRISPRi over CRISPR/Cas9 is its transient knock-down effects compared to the full knock-out effects of CRISPR/
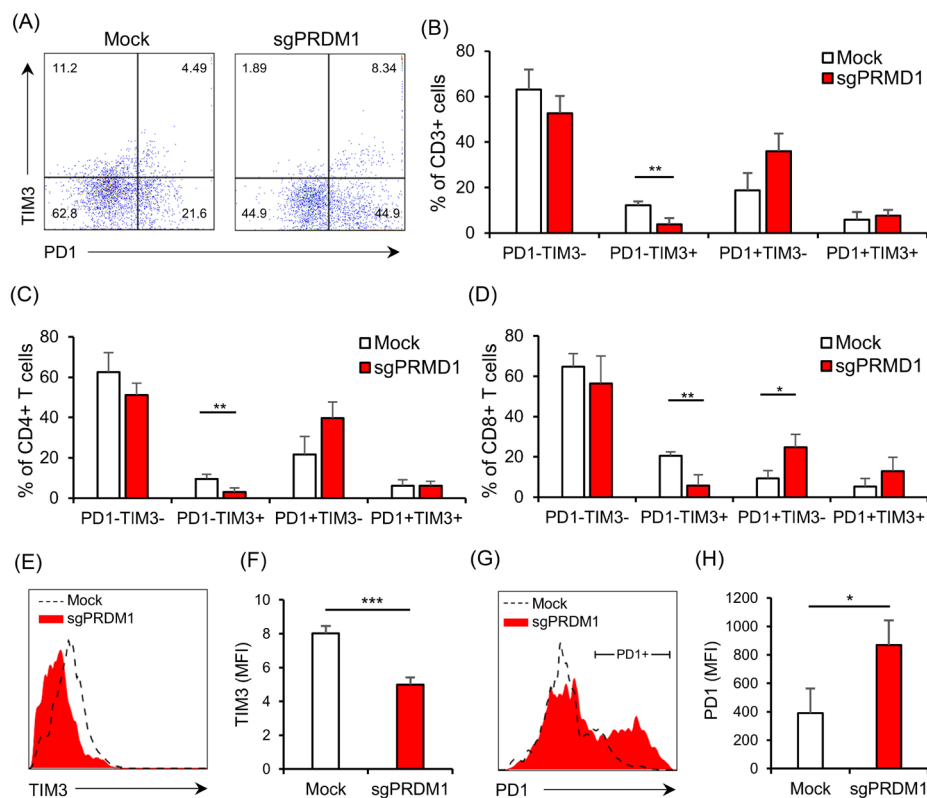
Fig. 7. BLIMP1 knock-down affects the expression of the exhaustion markers PD1 and TIM3 in human T cells. A) The representative scatter plot illustrates PD1 and TIM3 expression for the whole T cell population on day 25. B) The bar graph depicts the percentage of the T cell subpopulations based on the expression of the exhaustion markers PD1 and TIM3 in the whole T cell population for the Mock and sgPRDM1 groups on day 25. C) Percentages of PD1 and/or TIM3 expressing cells in the CD4+ T cell population on day 25. D) Percentages of PD1 and/or TIM3 expressing cells among the CD8+ T cells on day 25. E) The representative histograms compare TIM3 expression between the Mock and sgPRDM1 groups for the whole T cell population on day 25. F) The bar graph represents the MFI for the expression of the exhaustion marker TIM3 in the whole T cell population for the Mock and sgPRDM1 groups on day 25. G) The representative histograms of PD1 expression in the Mock and sgPRDM1 groups for the whole T cell population on day 25. H) The bar graph represents the MFI for the expression of PD1 in PD1+ cells for the Mock and sgPRDM1 groups on day 25. The data are presented as mean ± SD, *$P<0.05$, **$P<0.01$ and ***$P<0.001$, n = 3. BLIMP1: B lymphocyte-induced maturation protein 1; MFI: Median fluorescent intensity.

Cas9.[26] Gene knock-down via CRISPRi is not dependant on ploidy or frame shift mutations, which is a major benefit.[27] CRISPRi appears to produce a more consistent and robust knock-down given the same number of effector RNAs rather than RNAi. For instance, six out of eight sgRNAs were found to repress GFP levels by at least 75%[28] and CRISPRi shows significantly stronger loss-of-function phenotypes for gene inhibition compared with RNAi.[29] In addition, reversible *PRDM1* knock-down, which is possible by inducible CRISPRi through the Tet on/off system rather than RNAi, was used in this study because the BLIMP1 protein is necessary for the proper function of effector T cells and for the recall response of memory T cells.[30] Therefore, we considered CRISPRi to be the best genome editing tool for this study.

We observed that *PRDM1* knock-down enhanced CM T cell ($P=0.003$) and decreased EM T cell ($P=0.019$) phenotypes in human T cells. Less of the TIM3 exhaustion marker was observed in BLIMP1-deficient CD8+ T cells; however, there was higher, but not statistically meaningful, PD1 expression in the BLIMP1-deficient T cell population.

To the best of our knowledge, previous studies on the

impacts of BLIMP1-deficiency were mostly conducted on mouse T cells. It has been reported that BLIMP1 influences effector versus memory subsets of CD4+ T cells in mouse models,[3,5,31] which we confirmed by our findings in the human CD4+ T cell population. Our data showed that BLIMP1-deficiency caused a meaningful increase in the CM T cell phenotype ($P=0.010$) in CD4+ human T cells. In terms of CD8+ T cells, previous studies on mouse T cells depicted a critical regulatory role for BLIMP1 in driving the terminal differentiation of effector T cells,[3,5,9,10] which was completely compatible with our results on human CD8+ T cells. The current study results had enough compelling data for BLIMP1-deficient CD8+ T cells to have a higher CM T cell phenotype ($P=0.002$) versus lower EM ($P=0.00027$), TE ($P=0.043$), and NL ($P=0.032$) T cell phenotypes. Overall, for the whole T cell population in human T cells, we showed that a BLIMP1 deficiency increased the CM T cell phenotype and lowered the EM T cell phenotype, which confirmed results of previous studies on mouse models.[3,5]

We observed that BLIMP1-deficiency in human T cells resulted in a non-meaningful PD1 increase despite a
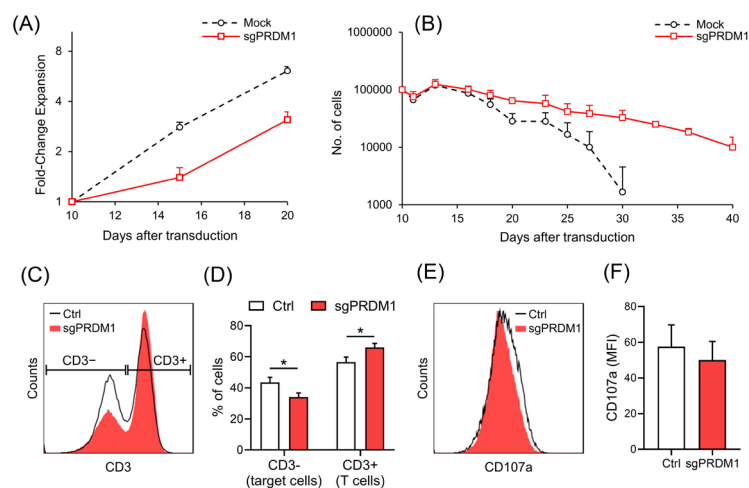
Fig. 8. The effect of BLIMP1 knock-down on the functional characteristics of T cells. A) The line graph represents fold change expansion in the Mock and sgPRDM1 groups during a 10 day-expansion period. B) The line graph shows the trend for T cell death in the Mock and sgPRDM1 groups during a 30 day-expansion period after a re-stimulation on day 3. C) Representative flow cytometry data of a co-culture of T cells with target cells (K562-OKT3) analyzed after three days. T cells were distinguished from target cells based on CD3 expression. D) A summary of data observed from the co-culture experiment (n = 3) shows significantly fewer target cells in the BLIMP1-deficient T cell group (sgPRDM1) compared to untransduced T cells (Ctrl). E) Representative data for the expression of a degranulation marker, CD107a, on T cells in the co-culture experiment. F) CD107a median fluorescent intensity (MFI) measured in the co-culture experiment (n = 3). The data are presented as mean ± SD, *P<0.05. BLIMP1: B lymphocyte-induced maturation protein 1.

meaningful TIM3 decrease in the whole T cell population. In line with our findings, BLIMP1 expression has previously been reported to repress PD1, such that there is an increase in PD1 expression in BLIMP1–deficient CD8+ T cells in mouse models.[32] Our findings that PD1 increased this level in human T cells supported the results of those studies. It has previously been reported that exhausted T cells have higher expression of BLIMP1 and, in contrast, BLIMP1-deficient CD8+ T cells resist exhaustion and exhibit improved cell numbers.[6] There is higher PD1 expression in BLIMP1 deficient CD8+ T cells.[12] BLIMP1 deficiency has been shown to lead to higher/increased levels of PD1 expression during T follicular helper (Tfh) and regulatory T (Treg) cell differentiation.[20,33] A compensatory effect between a number of checkpoint inhibitors including PD-1, LAG-3, and CTLA-4 has been proposed.[34] However, there is no data on such compensatory effect between TIM3 and any of the known BLIMP1 targets. On the other hand, there are several reports about upregulation of PD1 and TIM3 in exhausted T cells,[31,35-41] which is indirectly in line with our findings on TIM3 downregulation in BLIMP1 deficient human T cells because BLIMP1 is a key up-regulator of exhaustion markers.[42]

Of note, BLIMP1 knock-down reduces T cell proliferation. Our results showed a three-fold expansion for BLIMP1 knock-down T cell groups and a six-fold expansion for BLIMP1 sufficient T cells. However, BLIMP1 knock-down improved T cell persistence and cytotoxicity. Since our knock-down system only partially deplete BLIMP1, our cytotoxicity result is in line with previous data showing that a genetic mice model with haploinsufficiency of BLIMP1 in CD8+ T cells (conditional heterozygous) controls infection more rapidly than wild-type or BLIMP1-null mice.[6]

It can be inferred from the results of our study and previous studies on knocking down *Prdm1* in mouse models that BLIMP1 is a master inducer of effector phenotype in human T cell differentiation. A BLIMP1 deficiency leads to a reduction in effector phenotype through an increase in memory phenotype, a reduction in TIM3, but an increase in PD1 markers. Although we provided the first functional evidence on the significant role of BLIMP1 in human T cell differentiation, additional research should be conducted to determine the impact of BLIMP1 function on memory T cell recall response in human T cells in addition to a thorough investigation of this effect in subsequent in vivo experiments. Our findings can also be assessed in an adoptive immunotherapy model, most importantly the CAR T cell models.

In conclusion, the results of this study showed the specific impacts of BLIMP1 deficiency on the human T cell phenotype and exhaustion. We uncovered the critical role of BLIMP1 in the human T cell fate; therefore, it can be determined how BLIMP1 could be a potential key factor in regulating T cell persistence in human immunotherapies.

We suggest that further studies be conducted to ascertain the mechanism of action for BLIMP1. Whether BLIMP1 increases CD62L+ memory T cells through an outgrowth in their population or through its direct impact on increasing the expression level of CD62L remains to be determined. The result of our study proved the effects of BLIMP1 knock-down on human T cell phenotypes and showed that BLIMP1 knock-down changed the cell surface markers. Further molecular research on BLIMP1 targets that change their expression levels due to BLIMP1 knock-down could be conducted to determine the effects

Research Highlights

What is the current knowledge?
√ CBLIMP1 is a T cell differentiation regulator in mice.
√ Activated human T cells express PRDM1/BLIMP1 at different levels.

What is new here?
√ Knock-down of PRDM1/BLIMP1 increases the central memory (CM) phenotype in ex vivo expanded human T cells.
√ PRDM1/BLIMP1-deficient human T cells express higher levels of PD1 and lower levels of TIM3.

of BLIMP1 on the functional properties of T cells at the molecular level.

Ethical statement
This study was approved by the Research Ethics Committee at Royan Institute (ref. no. IR.ACECR.ROYAN.REC.1379.112).

Competing interests
The authors declare that they have no competing interests.

Authors' contribution
MA, MB and AA contributed to the study design. MA, AS and MB performed the experiments and data analysis. NN, SH, HM and BK performed the in silico analyses. MA drafted the manuscript. All authors contributed to data interpretation, discussed the results, and revised the manuscript.

Supplementary Materials
Supplementary file 1 contains Table S1.

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