Identification of NOXA as a pivotal regulator of resistance to CAR T-cell therapy in B-cell malignancies

Xin Yan1,2, Deyun Chen2, Yao Wang2, Yelei Guo2, Chuan Tong2, Jianshu Wei2, Yajing Zhang2, Zhiqiang Wu2,3 and Weidong Han2,3

Despite the remarkable success of chimeric antigen receptor (CAR) T-cell therapy for treating hematologic malignancies, resistance and recurrence still occur, while the markers or mechanisms underlying this resistance remain poorly understood. Here, via an unbiased genome-wide CRISPR/Cas9 screening, we identified loss of NOXA, a B-cell lymphoma 2 (BCL2) family protein in B-cell malignancies, as a pivotal regulator of resistance to CAR T-cell therapy by impairing apoptosis of tumor cells both in vitro and in vivo. Notably, low NOXA expression in tumor samples was correlated with worse survival in a tandem CD19/20 CAR T clinical trial in relapsed/refractory B-cell lymphoma. In contrast, pharmacological augmentation of NOXA expression by histone deacetylase (HDAC) inhibitors dramatically sensitized cancer cells to CAR T cell-mediated clearance in vitro and in vivo. Our work revealed the essentiality of NOXA in resistance to CAR T-cell therapy and suggested NOXA as a predictive marker for response and survival in patients receiving CAR T-cell transfusions. Pharmacological targeting of NOXA might provide an innovative therapeutic strategy to enhance CAR T-cell therapy.

INTRODUCTION

Chimeric antigen receptor (CAR) T-cell therapy has dramatically shifted the landscape of treatment for hematologic malignancies.1-4 To date, four US Food and Drug Administration (FDA)-approved CD19-targeted CAR T-cell products have been approved for treating acute lymphoblastic leukemia (ALL), relapsed/refractory (R/R) certain types of large B-cell lymphoma, or chronic lymphocytic leukemia.5-8 However, despite these encouraging advancements, ~20% of CD19+ R/R B-cell ALL patients who received an infusion of CD19 CAR T cells had no complete remission within 3 months, and approximately 50% of patients relapsed within a year.9 Hence, the key challenge is uncovering the underlying resistance mechanisms to CAR T-cell therapy and surmounting these barriers. Mechanisms hampering CAR T-cell therapy are traditionally categorized into two forms: antigen loss or reduction in tumor cells and T-cell dysfunction.10-15 Numerous studies, including ours, have attempted to create novel therapeutic strategies to overcome resistance to CAR T-cell therapy by expanding CAR T-cell antigen targets or ameliorating CAR T-cell dysfunction; however, a subset of patients still have no response or eventually relapse,16-19 suggesting that the mechanisms responsible for resistance to CAR T-cell therapy are diverse. These two traditional mechanisms of tumor escape and T-cell dysfunction do not fully explain the causes of CAR T-cell failure, suggesting nonantigen-dependent factors in tumor cells might play an important role in resistance to CAR T-cell therapy. Recently, sporadic laboratories began to focus on the effect of nonantigen-dependent factors in tumor cells on the efficacy of CART therapy.20-22 However, the underlying intrinsic mechanism of tumor cell resistance to CAR T-cell therapy remains unclear and needs further exploration.

Genome-wide CRISPR/Cas9 screening is a powerful tool extensively used to discover novel targets or molecular resistance mechanisms for cancer therapy.23 Genome-wide CRISPR screening in various models has identified that intrinsic regulators of cancer cells mediated the evasion of T cell-mediated killing.24-26 Due to the different structures and non-MHC-dependent killing forms, the mechanisms of cancer cell resistance to CAR T cells are not completely identical to those of T cells.27 Thus, unbiased CRISPR/Cas9 screening provided us with a favorable way to discover novel mechanisms of tumor cells escaping from CAR T-cell therapy. In this study, we systematically explored a novel mechanism of resistance to CAR T cells using genome-wide CRISPR/Cas9 screening, and highlighted a central role for NOXA, a B-cell lymphoma 2 (BCL2) family protein, in predicting susceptibility to CAR T-cell therapy in B-cell malignancies. Our work provided evidence for developing mechanism-based combination therapies to improve the efficacy of CAR T-cell therapy in B-cell malignancies.

RESULTS

CRISPR screening identifies essential regulators of resistance to CAR T-cell therapy

We performed a genome-scale CRISPR/Cas9 screening in the CD19+ human ALL cell line Nalm6 to systematically identify genetic perturbations that regulate resistance to CAR T-cell therapy. We transduced Nalm6 cells with the CRISPR/Cas9...
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Fig. 1 Genome-wide CRISPR/Cas9 screening in Nalm6 cells cocultured with CD19 CAR T cells. a Schematic of the CRISPR/Cas9 screening. b Volcano plots of essential enriched sgRNAs after screening. c KEGG, GO, and Reactome pathway enrichment analysis of genes identified in CRISPR screening.
against CAR T cell-mediated killing, transcriptome differences between NOXA\textsuperscript{−/−} Nalm6 cells and Nalm6 cells with control sgRNA in the presence of CAR T cells were analyzed. Transcriptomic analysis showed significant changes in gene expression in NOXA\textsuperscript{−/−} tumor cells compared to sgCONT tumor cells under CD19 CAR T-cell pressure (Supplementary Fig. 3a). Pathway enrichment analysis showed that the differentially expressed genes were mainly enriched in DNA replication, apoptotic signaling pathway, cell cycle, and MAPK cascade (Fig. 3d). NOXA\textsuperscript{−/−} tumor cells revealed downregulated expression of genes associated with apoptosis, including \textit{PRELIDE}, \textit{BAX}, \textit{BNIP3}, and \textit{HRK}. Cell growth-related genes such as \textit{CDK6}, \textit{WAPL}, \textit{CDK14}, and \textit{BCL2} were also significantly upregulated in NOXA\textsuperscript{−/−} tumor cells (Fig. 3e).

Consistently with transcriptomic analysis, we also observed marked differences in the mRNA levels of \textit{BAX}, \textit{HRK}, \textit{CDK6}, \textit{CDK14}, and \textit{BCL2} between the sgCONT and NOXA\textsuperscript{−/−} tumor cells after co-incubated with CD19 CAR T cells (Fig. 3f).

Taken together, our data indicate that NOXA ablation might impair apoptosis of tumor cells, rendering resistance to CAR T-cell therapy.

Loss of NOXA in tumor cells exhibits lower susceptibility to CAR T-cell therapy in vivo.

To assess the effect of NOXA expression in tumor cells on the antitumor efficacy of CAR T cells in vivo, we engrafted NOD-Prkdcscid Il2rg-null (NPG) mice with sgCONT or NOXA\textsuperscript{−/−} Nalm6 via tail vein, and then infused control T cells or CD19 CAR T cells intravenously (Fig. 4a). Consistent with in vitro findings, CD19 CAR T cells (1e\textsuperscript{5}/mouse) had poor ability to eliminate tumors established with NOXA\textsuperscript{−/−} Nalm6 cells (Fig. 4b, c). Of note, we
also observed a significant reduction in the survival of NOXAKO Nalm6 tumor-bearing mice (Fig. 4d), suggesting that mice xenografted derived from NOXAKO Nalm6 cells exhibited lower susceptibility to CD19 CAR T cells than xenografted mice from sgCONT Nalm6 cells.

Clinical relevance of NOXA protein levels to CAR T-cell therapy in R/R B-cell lymphoma

Based on our preclinical observations, we next investigated the clinical relevance of NOXA protein levels in patients with R/R B-cell lymphoma to CAR T-cell therapy outcomes. Immunohistochemistry
(IHC) was used to examine the NOXA protein levels in all available tumor tissue samples collected prior to tandem CD19/20 CAR T-cell therapy\textsuperscript{16,35} from responders who achieved a complete or partial response ($n = 27$) and from non-responders ($n = 7$). The results showed that the level of NOXA expression was significantly higher in the responders than in the non-responders ($P = 0.033$, Fig. 5a, b). Areas under the ROC curve and 95% CIs were 0.745 (0.55–0.94) for response status within the range of the NOXA IHC scores, indicating the strong distinguishing ability of the NOXA IHC scores (Fig. 5c). We quantified NOXA expression in IHC images and classified samples into low NOXA expression group (IHC scores <4) and high NOXA expression group (IHC scores ≥4).\textsuperscript{36} We identified that patients with low NOXA expression responded worse to CAR T-cell therapy than those with high NOXA expression ($P = 0.007$, Fig. 5d). Notably, a Kaplan–Meier survival analysis of the expression of NOXA and clinical outcomes further showed that a low protein level of NOXA was related to worse progression-free survival (PFS) in this patient cohort ($P = 0.004$, Fig. 5e). A similar trend was shown in the overall survival analysis ($P = 0.024$, Fig. 5f). In addition, it was confirmed that tumor burden, lymphoma type, CAR T-cell dose, CD4/CD8 ratio, and memory phenotype were not significantly distinct between the low and high NOXA expression groups, which ruled out the interference of these factors (Fig. 5g–k). Together, these data provide overwhelming clinical evidence that NOXA might serve as a great prognostic marker in B-cell malignancies.

Histone deacetylase inhibitors upregulate NOXA expression and enhance tumor cell vulnerability to CAR T cells

Several histone deacetylase (HDAC) inhibitors have been shown to induce cell death of hematologic malignancies by modulating NOXA expression.\textsuperscript{33,37,38} Based on our findings that overexpression of NOXA via gene transfer improved the susceptibility of tumor cells to CAR T cells, we attempted to investigate whether HDAC inhibitors might obtain similar biological responses by pharmacologic upregulation of NOXA. We identified that panobinostat, a pan-HDAC inhibitor, caused an increase in NOXA protein levels in Raji and Daudi cells (Fig. 6a). A class I HDAC inhibitor, entinostat, was also shown to upregulate NOXA protein levels in Raji and Daudi cells (Fig. 6b). Prior to the addition of CD19 CAR T cells, we pretreated tumor cells with 30 nM panobinostat or 0.5 \( \mu \)M entinostat for 24 h, both of which showed no effect on the viability of Raji and Daudi cells (Supplemental Fig. 4a–d). Notably, when tumor cells were pretreated with HDAC inhibitors, we observed a dramatically increased cytotoxic effect of CAR T cells.
More importantly, we found that HDAC inhibitors did not increase the susceptibility of NOXAKO Nalm6 cells to CAR T-cell therapy (Fig. 6e), indicating that HDAC inhibitors enhanced the therapeutic efficacy of CAR T cells dependently on NOXA expression. Finally, we explored the efficacy of the combination of HDAC inhibitors and CD19 CAR T-cell therapy in a mouse xenograft model using Raji cells (Fig. 6f). Consistent with our in vitro results, the growth of Raji tumors was significantly suppressed by the combination of panobinostat and CD19 CAR T cells as compared with either panobinostat or CD19 CAR T cells alone (Fig. 6g–i). Notably, we also observed in vivo that panobinostat in synergy with CD19 CAR T cells increased the expression of NOXA and cleaved caspase3 in the tumors of xenografted mice (Fig. 6j).

Altogether, our in vitro and in vivo results suggested that pharmacological targeting NOXA via HDAC inhibitors might be a promising strategy to overcome resistance to CAR T-cell therapy in B-cell malignancies.

**DISCUSSION**

Despite the inspiring advances in CAR T-cell therapy for hematologic tumors, some patients still do not respond to this treatment. The mechanism enabling evasion of antigen-positive tumor cells from CAR T-cell therapy is currently presumed to be CAR T-cell dysfunction. In this study, we performed an unbiased CRISPR/Cas9 screening to reveal novel tumor-intrinsic perturbations that lead to CAR T-cell therapy failure.

We identified and confirmed the key role of NOXA in CAR T-cell cytotoxicity in cancer cells through knockout and overexpression of NOXA in B-lymphoid cell lines using a variety of CAR T cells, including CD19 CAR T, CD20 CAR T, and tandem CD19/20 CAR.
Histone deacetylase inhibitors could enhance tumor cell vulnerability to CAR T cells. a, b Western blot analysis of Raji cells and Daudi cells treated with panobinostat (a) or entinostat (b) for 24 h. c, d Cytotoxic analysis of Raji cells and Daudi cells pretreated with 30 nM panobinostat (c) or 0.5 μM entinostat (d) for 24 h and then cocultured with CD19 CAR T cells over time. e Cytotoxic analysis of NOXA knockout Nalm6 cells treated with 30 nM panobinostat or 0.5 μM entinostat for 24 h and then cocultured with CD19 CAR T cells over time. Values in (a–e) were shown as the mean ± SD of triplicates. f Experimental timeline comparing the antitumor ability of vehicle alone, panobinostat alone, the combination of vehicle and CD19 CAR T cells, and combination of panobinostat and CD19 CAR T cells in mice bearing Raji-luc xenograft tumors (n = 5 mice per group). g Raji tumor progression as evaluated by bioluminescence imaging. h Mouse tumor burden (average radiance). The indicated P value was determined by two-way ANOVA test. i Survival analyses of mice treated with vehicle alone, panobinostat alone, the combination of vehicle and CD19 CAR T cells, and combination of panobinostat and CD19 CAR T cells. Log-rank tests were used to analyze the significance between four groups. j Representative images of IHC staining for NOXA and cleaved caspase3 from Raji tumor xenografts treated with the indicated treatments. Magnification: ×40, Scale bars: 20 μm. Quantification of IHC staining using IHC scores was represented as mean ± SD of five mice per group. P values were calculated with the two-tailed Student’s t test. k Schematic drawing summarizing our findings. Values are shown as the mean ± SD. ns: not significant (P > 0.05); *P < 0.05, **P < 0.01, and ***P < 0.001
T cells. Furthermore, we revealed that knockout of NOXA attenuated CAR T cell-mediated apoptosis of tumor cells, thereby conferring tumor cell resistance to CAR T cells. In addition to in vitro experiments, analysis involving R/R B-cell lymphoma patients treated with tandem CD19/20 CAR T-cell treatment revealed poorer response and survival when patients had low NOXA protein levels in tumor samples (Fig. 6k).

T cells kill target cells through induction of apoptosis, either via death receptors to initiate the extrinsic apoptotic pathway, or by secreting granzymes and perforin to trigger the mitochondrial-mediated intrinsic apoptotic pathway. Recently, using CRISPR/Cas9 screening, two laboratories demonstrated that impaired extrinsic apoptotic signaling in tumor cells dampened CD19 CAR T cytototoxicity and drove CAR T-cell dysfunction, supporting the significance of apoptosis pathway in the CAR T-cells effectiveness. Consistent with their results, we identified that extrinsic apoptotic signaling molecules, including CASP8 and FADD, were enriched in our CRISPR screening. Moreover, mitochondrial-mediated intrinsic apoptosis molecule represented by NOXA was also enriched, possibly attributed to the low effect-target ratio, long duration, and repeated CAR T-cell challenges from the coculture model used in our screening system. Our CRISPR screening system might mimic the in vivo tumor conditions and enable discovery of more candidate proteins driving tumor cells to escape CAR T-cell therapy.

NOXA protein, known as a sensitizer for inducing apoptosis, is a pro-apoptotic BCL2 family protein. Several studies have reported the significance of NOXA in regulating the cytotoxic effect of a variety of chemotherapies, BCL2 inhibitors and targeted therapies. A recent study also identified disruption of NOXA conferred resistance to natural killer cell cytotoxicity by a CRISPR screening in SUDHL4 cells, a B-cell lymphoma cell line, implying that NOXA might be a critical regulator of the antitumor response of cancer cells to distinct modalities of therapies. Future work needs to further explore whether patients with high expression of NOXA would respond better to a range of immune-based therapies given its important role in regulating apoptosis.

Resistance to apoptosis is a hallmark of tumor biology, often conveyed by halted apoptosis machinery. Hence, targeting apoptotic machinery of tumor cells provides novel opportunities to sensitise the tumor cells to multiple therapeutic options. Numerous previous studies have reported that NOXA expression was up-regulated at the epigenetic and transcript level. HDAC inhibitors have been repeatedly shown to induce NOXA expression in lymphoma cells in preclinical studies. Our in vitro and in vivo study confirmed that HDAC inhibitors (panobinostat and entinostat) dramatically increased the cytotoxic efficacy of CAR T-cell therapy. To date, four HDAC inhibitors including panobinostat, a pan-deacetylase inhibitor, have been approved by the FDA for treating hematologic tumors in clinic. Entinostat is a highly selective class 1 HDAC inhibitor, and has exhibited synergy in preclinical models when combined with rituximab for treating B-cell lymphoma. In addition to panobinostat and entinostat, other HDAC inhibitors, such as kendine 92 and SAHA, have also shown NOXA protein increases in chronic lymphocytic leukemia cells, which may be used as reference drugs in combination with CAR T-cell therapy in hematologic cancers. Our exciting in vitro and in vivo results, although requiring further exploration, may provide a strong mechanistic rationale for the incorporation of HDAC inhibitors into CAR T-cell-based therapy in B-cell malignancies.

In summary, our genome-scale CRISPR/Cas9 screening identified a novel mechanism of resistance to CAR T cells, potentially revealing that NOXA might be a good marker for predicting the susceptibility of CAR T-cell therapy. In addition, pharmacologic induction of NOXA would be a promising strategy to enhance sensitivity to CAR T-cell therapy in B-cell malignancies.

**MATERIALS AND METHODS**

**Cell lines and cell culture**

Nalm6, Raji, and Daudi cells were purchased from ATCC (Manassas, VA). Their identities were verified by short tandem repeat DNA analysis. These cells were infected with the lentivirus pRLSIN-EF-1a-GFP-luc to express GFP virus and firefly luciferase. Nalm6 cells were infected with a pRLSIN-EF-1a-mCherry-luc virus to express mCherry and firefly luciferase. Infected cells were sorted based on GFP or mCherry expression by flow cytometry. All the above cells were tested for mycoplasma contamination before the experiment. All cell lines were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 μM penicillin/streptomycin (Gibco).

**CAR T-cell manufacturing**

All CAR T designs used in this study have been described in our previous reports. Different CAR constructs in this study are displayed in supplemental Fig. 5a, b. The CAR constructs were synthesized and cloned into the lentivirus pRLSIN-EF-1a-GFP vector. pRLSIN and PSPAX2 and PMD2G packaging plasmids were transfected into 293T cells. Lentiviral supernatants were harvested 48 h later and stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were derived from healthy donors. One microgram/mL anti-CD3 monoclonal antibody (OKT3, Takara, Japan) and RetroNectin (Takara) were precoated overnight at 4°C to activate T cells. Primary T cells were cultured in X-VIVO 15 medium (Lonza, Switzerland) containing 10% fetal bovine serum (Gibco) and 300 μM recombinant human IL-2 (PeproTech, USA). After 2 days, the virus supernatant was added to RetroNectin-coated plates and centrifuged for 2 h at 2000xg. Activated T cells and 4 μg/mL polybrene (Sigma–Aldrich, USA) were added to the plates and centrifuged at 1000xg for 10 min. After 24 h, CAR T cells were replaced with fresh medium to reduce the toxicity of polybrene. CAR T cells were maintained in X-VIVO 15 medium containing 300 μM recombinant human IL-2.

**Genome-wide CRISPR/Cas9 knockout library screening**

The Brunello sgRNA library consisting of 77441 sgRNAs targeting 19110 protein-coding genes (4 sgRNAs per gene) was used to transduce the target-cell line Nalm6. Cells transduced with the lentivirus were selected under puromycin pressure. Day 0 cells containing the library were collected as the control group. CD19 CAR T cells were cocultured with transduced Nalm6 cells at an effector-to-target (E:T) of 1:50, and CD19 CAR T cells were added at an E:T of 1:50 every 3 days. After 15 days, the remaining living Nalm6 cells were harvested for genomic DNA extraction. The sgRNA sequences were amplified by NEBNext® High-Fidelity 2X PCR Master Mix and subjected to massive parallel amplicon sequencing. The MACeGk v0.5.7 algorithm was used to analyze the sgRNA read counts. Candidate genes were identified based on log2 fold change and P value for all sgRNAs. To identify significant pathways enriched in the CRISPR screening, hypergeometric distribution statistics were used to compute overlapping gene sets with the top enriched genes (log2 fold change>2 and P < 0.05).

**Establishment of NOXA knockout and overexpression cell lines**

**NOXA knockout.** Two sgRNA sequences targeting NOXA were cloned into a lentivirus pCRISPRv2-GFP vector and confirmed by sequencing. The sgRNA sequences targeting NOXA were extracted from the CRISPR Brunello library. Nalm6 cells were infected with sgNOXA or control sgRNA lentivirus supernatants. Infected cells were sorted based on GFP by flow cytometry. All the sgNOXA sequences were as follows: sgNOXA-1: TTTGCTTTCCTTCTCAGAGC; sgNOXA-2: TTTGCTTTCCTTCTCAGACG.

**NOXA overexpression.** The NOXA construct was synthesized and cloned into the lentivirus pRLSIN-EF-1a-GFP vector. Raji cells

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were infected with NOXA or empty vector lentivirus supernatants. Infected cells were sorted based on GFP by flow cytometry.

**Drugs.** Panobinostat and entinostat were obtained from Selleck (USA) and APEX-BIO (USA), respectively.

**Growth competition assay.** NOXAKO (GFP−) or NOXADE cells (GFP+) and control tumor cells (mCherry+) were mixed at a 1:1 ratio with control or CAR T cells. The change in the GFP+/GFP+ + mCherry+) ratio in the mixed cells was detected by flow cytometry over time.

**Clinical tissue specimens and Ethics statement.** Tissue samples and patient information were obtained from a clinical trial of tandem CD19+20 CART-cell therapy in R/R B-cell lymphoma (NCT03097770), which was approved by the Ethics Committee of Chinese PLA General Hospital and conducted in accordance with principles of the Declaration of Helsinki. All patients provided informed consent and authorization.

**Immunohistochemistry and immunohistochemical scoring.** The samples were immunostained as described previously. NOXA and cleaved caspase3 staining were localized to the cytoplasm of tumor cells and scored according to intensity (0/1/2/3) and positive rate (grouped into quartiles (0–4)). The intensity and positive scores were multiplied to form an IHC score. Low expression scores ranged from 0 to 4, and the scores that exceeded 4 were identified as high expression.

**Additional methods information can be found in Supplementary Materials.**

**Statistical analysis**

Data were analyzed and visualized using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Two-tailed Student’s t or two-way ANOVA test was used to compare the significant differences between the relevant groups. The area under the receiver operating characteristic (ROC) curve was calculated to evaluate the sensitivity of NOXA IHC scores in predicting response to CAR T-cell therapy. Survival analysis was analyzed using the log-rank test. Significant association among groups was defined as *P < 0.05; **P < 0.01; ***P < 0.001.

**DATA AVAILABILITY**

All data that support the findings of this study are available to the researchers on reasonable request.

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**AUTHOR CONTRIBUTIONS**

X.Y., Z.W., and W.H. designed the research. X.Y. and D.C. performed the experiments. Y.W., Y.G., J.W., Y.Z., and C.T. assisted with the experiments. X.Y., D.C., Z.W., and W.H. analyzed the data and wrote the manuscript.

**ADDITIONAL INFORMATION**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41392-022-00915-1.

**Competing interests:** The authors declare no competing interests.

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