Nicotinamide mononucleotide enhances the efficacy and persistence of CD19 CAR-T cells via NAD + –Sirt1 axis

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Article

Keywords: NMN, NAD+, lifespan, Sirt1, CAR-T

Posted Date: April 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1483519/v1

Abstract

Chimeric antigen receptor (CAR)-modified T cells targeting antigens expressed on tumor cells have exhibited significant antitumor effects on several types of hematological malignancies, including acute myeloid leukemia, lymphoma, B cell maturation antigen, and CD19-positive acute lymphoblastic leukemia. However, several drawbacks, particularly the poor persistence, limit the use of CAR-T cell-based therapy. Nicotinamide mononucleotide (NMN), the precursor of nicotinamide adenine dinucleotide (NAD^+), has been shown to reverse age-related complications and slow the aging rate by enhancing NAD^+ levels in the body. However, whether NMN treatment could enhance the longevity and persistence of CAR-T cells and improve their antitumor efficacy is still unclear. In this study, we observed that NMN treatment limited differentiation and significantly increased stem cell–like memory (Tscm) and stem cell–like memory (Tcm) in CD19 CAR-T cells. In addition, both telomerase length and proliferation ability were increased, whereas apoptosis was reduced after NMN treatment of CD19 CAR-T cells. High-throughput sequencing data indicated that NMN treatment upregulated Sirt1 expression in CD19 CAR-T cells and downregulated genes downstream of Sirt1, such as *NF-κB*, *TP53*, and *Bax*. Animal experiments showed that NMN treated CAR-T cells exerted great antitumor efficacy in human xenografted mouse models. In conclusion, NMN enhances the efficacy and longevity of CD19 CAR-T cells via the NAD^+–Sirt1 axis.

Introduction

Chimeric antigen receptor T (CAR-T) cell therapy is regarded as an effective solution for relapsed or refractory tumors, especially the hematological malignancies[1]. However, there are multiple challenges in treating tumors with CAR-T cell therapy, such as poor T cell persistence, T cell senescence, and T cell exhaustion[2–3]. Cell senescence is characterized by decreased telomerase length, β-galactosidase aggregation, increased apoptosis and cell proliferative capacity. Improving the persistence of CAR-T cells is critical for the in vivo efficacy of CAR-T cell-based therapy.

Nicotinamide mononucleotide (NMN), the precursor of nicotinamide adenine dinucleotide (NAD^+), is a biologically active nucleotide which is formed by the reaction of a phosphate group with a riboside containing ribose and nicotinamide[4]. Administration of NMN surprisingly demonstrated amelioration of the pathological conditions in some age-related diseases, including neurodegenerative diseases, cardiovascular diseases, metabolic diseases and so on[5–8]. Emerging evidence demonstrates that systemic NMN administration in mice effectively enhances NAD^+ biosynthesis in various peripheral tissues [9]. NAD^+, as a co-factor in redox metabolism, plays a decisive role in various biological processes, including aging, metabolism, cell death, DNA repair, and gene expression. Aging is related to cellular NAD^+ depletion, and therefore NAD^+ replenishment extends lifespan, and improves health span[10–11]. NAD^+ also plays a decisive role in the regulation of the activity of NAD^+-consuming enzymes, such as sirtuins [12–14], which are implicated in the cellular aging regulation [15– 16].

Sirtuins are an evolutionarily conserved family of NAD^+-dependent deacetylases and ADP-ribosyltransferases, among which, Sirt1 plays a vital role in a broad range of normal and abnormal physiological processes, including caloric restriction-related longevity, metabolism, DNA damage response, aging, and tumorigenesis [17–22].

T lymphocytes are a part of the adaptive immune system, which is characterized by the ability to participate in immune responses that are highly specific to certain antigens and result in the formation of immunological memory[23] .The stem memory T cells (TSCM) is a self-renewing population of T cells and is responsible for maintaining memory [24]. Memory T cells (Tcm) can instantly trigger effector functions, secrete inflammatory cytokines and kill infected cells. Although these cells lack immediate function, TSCM and Tcm cells recall responses to antigens and differentiate into effector T cells following antigen stimulation. Therefore, increasing the percentage of TSCM and Tcm in CAR-T cells is important for optimizing the antigen immune response. In this study, we investigated the effect of NMN on the longevity of CD19 CAR-T lymphocyte cells *in vitro* and *in vivo*, where NMN increased the NAD/NADH ratio, Sirt1 expression level, and the Sirt1 downstream related genes.

Materials And Methods

Cell Culture and NMN treatment

Human peripheral blood from healthy donors were diluted with PBS at the ratio of 1:1, lymphoprep (MP Biomedicals) was used to centrifugate and isolated the blood by low-density centrifugation. Human peripheral blood mononuclear cells (PBMC) were activated with anti-CD3 and anti-CD28 beads and transduced with retrovirus on two consecutive days by centrifugation on retronectin-coated culture dish (Thermo). Six days after transduction, T cells were cultured in X–VIVO15 serum-free medium (Lonza) which containing 5% Human Serum AB (Gemini Bio) and IL-2 (SL PHARM). For weekly stimulations, cells were treated by100uM NMN (St. Louis, MO, USA N3501). This research was approved by the Beijing Shijitan Hospital Institutional Review Board and informed consent was obtained from all the participants. All methods were performed in accordance with the relevant guidelines and regulations

Cell lines

NALM-6 was obtained from ATCC and cultured in RPMI-1640 medium (Lonza) supplemented with 10% fetal bovine serum (Biosera), 10,000 IU/mL penicillin/10,000 μg/mL streptomycin (EallBio Life Sciences) at 37°C in a humidified 5% CO2 incubator. NALM-6 cells were transduced to express firefly luciferase-GFP.

Cytotoxicity assays

The firefly luciferase-GFP expressed NALM-6 cells were served as the target cells. The effector (E) and tumor target (T) cells were co-cultured in triplicates. The E/T ratio was 0.5:1 using 96-well plates with 1x 10^4 target cells in a total volume of 200 μl T cells medium each well. Target cells alone were plated at the same cell density to determine the maximal luciferase expression.24 hours later, 20μl luciferase substrate

(Bright-Glo, Promega) was added to each well directly. Emitted light was detected on IVIS Series III imaging system (Lunma), and quantified using Living Image software (Lunma).

Antigen provide and proliferation assays

NALM-6 expressing firefly luciferase-GFP cells were used as antigen-presenting cells. For adequate antigen to sustain the cell alive, 1x 10^5NALM-6 cells and 1x 10^6 CAR-T cells were co-culture in 24-well plates within XVIVO 15, 5% human serum AB and 200 U IL-2 /ml. Every 7 days, cells were counted with trypan blue.

Flow Cytometry

Flow cytometry was performed and analyzed on a FACS Canto Plus instrument (BD Biosciences). The following antibodies were used to define antigen expression by flow-cytometry:CD3APC-CY7(BDBiosciences,560176);CD4-V450(BDBiosciences562424);CD8-FITC(BDBiosciences555634);CCR7-PECY7(BDBiosciences,557648);45RO-BV605(biolegend,304238);CD107a-FITC(Beckton-Dickinson Pharmingen, San Diego, CA, USA). For flow cytometry for detection of apoptosis, aliquots of 10^5 cells were washed and incubated in the dark for 15 min in PBS containing either 2 µl of annexin V-FITC (to detect early apoptosis) and 2ul 7-AAD/ml (to detect late apoptosis), Samples were analyzed 20 min later by FAC-Scan flow cytometry.

Elisa

The CAR-T cells treated with/without NMN were co-cultured with NALM-6 cells at an E: T ratio of 0.5:1 for 24 hours. The medium from the co-culture model was collected and centrifuged at 1500rpm to collect the supernatant. IFN-γ and IL-6 cytokines in the harvested supernatant were measured using a commercial ELISA kit in accordance with the manufacturer's instruction (R&D system).

NAD/NADHratio assay

NAD+ and NADH levels were measured using NAD+/NADH assay kits according to the manufacturer's instructions (Abbkine CheKine™). The principle of the method is based on glucose dehydrogenase cycling reaction. The product absorbance was measured at 565 nm, is proportionate to the NAD+ concentration of the sample[25] .

Telomerase activity measure

Telomerase activity of the samples were assayed using TRAPEZE Gel-Based Telomerase Detection Kit (Merck S7700). Briefly, 1µg protein was mixed with TRAP PCR mixture for PCR amplification and the products were electrophoresed on a 12 % non-denaturing polyacrylamide gel and chemiluminescence detection.

DNA Isolation and Measurement of Telomere Length

The genomic DNA was extracted from the CD19 CAR-T cells treated with/without NMN. FastPure Blood DNA Isolation Mini Kit V2 (Vazyme) was used following theinstruction book. The NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to detected the DNA concentration. Telomere length was assessed by Real-time quantitative PCR. Briefly, the primers F:CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT , R:GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT was used to amplify the telomeres (T) of the CD19 CAR-T cells.

F: CAGCAAGTGGGAAGGTGTAATCC R:CCCATTCTATCATCAACGGGTACAA were used to amplify the single-copy gene as S. The relative telomere length was measured by comparing the ratio of T repeat copy number and S copy number, expressed as the telomere length (T/S) ratio.

SA-β-gal staining

The SA-β-gal staining of CD19 CAR-T cells treated with/without NMN was investigated using SA-β-gal staining kit (Cell Signaling Technology). CD19 CAR-T cells were fixed and washed in 1.5ml tubes and incubated with the staining solution at 37°C overnight. Four random fields each well were imaged using the phase-contrast microscope (Olympus). Mean percentage of SA-β-gal-positive cells was calculated.

Quantitative real time polymerase chain reaction (Q-PCR)

Total RNA was extracted with TRIZOL reagent (Invitrogen) and reversed-transcribed using the Prime Script RT Master Mix Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The quantity and purity of RNA were measured using Nanodrop spectrophotometer (Thermo Fisher Scientific). The reverse transcription products were used as the templates and subjected to Q-PCR amplified using SYBR Green PCR Master Mix (Thermo Fisher Scientific) with gene-specific primers. The primer sequences for Q-PCR analysis are listed in the Supplementary Material. GAPDH was used as the internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Table 1.

Reverse Transcription-PCR

Total RNA was extracted from $1x10^6$ cells by using TRIZOL reagent (Invitrogen) and the cDNA was synthesized using the cDNA Reverse Transcription kit (Thermo Fisher Scientific).PCRs were performed using the following primers:SIRT1:F:- GCAGGTTGCAGGAATCCAAA.R:GGCAAGATGCTGTTGCAAAG.β-actin: F: GGGTCAGAAGGATTCCTATG, R:GGTCTCAAACATGATCTGGG.ThePCR cycle conditions were 94℃for 2 min, 55℃for 1 min, and 72℃for 1 min for a total of 35 cycles. Samples were resolved on a 1% agarose gel and visualized with ethidium bromide.

Animal experiment

We used 6 to12week-old NOD/SCID mice, under a protocol approved Beijing shijitan hospital in Capital Medical University Institutional Animal Care and Use Committee. All relevant animal use guidelines and

ethical regulations were followed. Mice were inoculated with 2x 10^6NALM-6 expressing firefly luciferase-GFP cells by tail vein injection using 200μl 1× PBS, followed by 2x 10^7 CD19 CAR-T cells injected for 3 days. The CD19 CAR-T cells treated with NMN (100uM) 24 hours in advance. NALM-6 expressing firefly luciferase-GFP produce even tumor burdens, NMN was administered intraperitoneal injection at 300 mg/kg/day. Bioluminescence imaging was performed by injecting mice weekly via tail vein with luciferin and quantifying luminescence using IVIS Lunma Series III imaging. The study is reported in accordance with ARRIVE guidelines.

RNA-seq analysis

2 μg total RNA samples were used as the input material for generating the sequencing libraries, The NEBNext®Ultra™ RNA Library Prep Kit was used (#E7530L, NEB). In brief, poly-T oligo-attached magnetic beads was used to purify mRNA from total RNA. Under the condition of elevated temperature and NEB Next First Strand Synthesis Reaction Buffer (5X), divalent cations was used to generate fragmentation. Random hexamer primers and RNase H was used to synthesize first-strand cDNA. Second strand cDNA synthesis was subsequently performed using buffer, DNA polymerase I, dNTPs and RNase H. The library fragments was purified by QiaQuick PCR kits and the product was eluted with EB buffer, and then terminal repair, A-tailing, and adapters were implemented. Index-coded samples was performed on a cBot Cluster Generation system with TruSeq SR Cluster kit, v3-cBot-HS (Illumina Inc). Afterwards, the library was subjected to an Illumina Nova Seq 6000 System platform (Illumina Inc). Raw data were first processed using custom Perl scripts. Clean data were read by removing reads containing poly-N with 5'-adapter contaminants, without 3'- adapter or the insert tag, or containing poly-A, -T, -G or -C from raw data, as well as low-quality reads. The genes expressed differentially were subjected to KEGG pathway, gene ontology (GO) and subcellular localization analysis using DAVID Bioinformatics Resources 6.8.

Statistical analysis

The statistical significance of differences between two groups was assessed with the two-tailed paired or unpaired t test. Comparisons of more than two groups were performed use the one-way ANOVA with multiple comparison tests. Data are shown as the mean ± standard deviation (SD). Difference were marked as NS, $P > 0.05$; *$P < 0.05$; **$P < 0.01$, and ***$P < 0.001$.*In vivo* experiment, P value of radiance based on Wilcoxon rank-sum test. The excel Kaplan-Meier and non-parametric Wilcoxon p value was used for survival curve comparison. All the data obtained from the study was analyzed using SPSS 22.0 (IBM, USA).

Results

NMN treatment enhances CD19$^+$ CAR-T cells proliferation and maintains effector function *in vitro*

To investigate the function of NMN on CAR-T cells, we investigated the effect of NMN on CAR-T cell proliferation and the killing ability toward target cells. CD19 CAR-T cells were treated with 100 μM NMN and co-cultured with NALM-6 cells at a ratio of 10:1 to provide sufficient antigen to maintain sustainable

survival for two days (Figure1. a). NMN-treated CD19 CAR-T cells proliferated significantly more than CD19 CAR-T cells without NMN treatment. After co-culturing with NALM6 cells three times, the number of NMN-treated CD19 CAR-T cells was 180 times higher than the starting number, whereas almost all non-NMN-treated CD19 CAR-T cells died (Figure1. b). Meanwhile, we verified this observation by evaluating the cell proliferation rate via CCK8 assay at different concentrations. Within the 100 μM NMN concentration, the OD450 value of CD19 CAR-T cells after NMN treatment was double that of that of the untreated group after 96 hours (FigureS1. a). We observed a decreased IL-6 and an increased IFN-γ release in NMN-treated CD19 CAR-T cells (Figure1. c. d). As shown in Figure1. e. and f., NMN treatment in CD19 CAR-T cells displayed increased antitumor activity and exhibited similar CD107a levels as compared with the control. Notably, the NMN-treated CD19 CAR-T cells remained capable of killing and expressed high IFN-γ levels, whereas the untreated cells died (FigureS1. b. and c.). The above results indicate that NMN can instantly enhance antitumor activity and effectively promote cell survival in CD19 CAR-T cells.

NMN treatment increased TSCM/TCM percentage and delay senescence in CAR-T cells

As the precursor of NAD^+, NMN can lead to an increase in intracellular NAD^+ levels. Accordingly, we found higher NAD/NADH ratios in the CD19 CAR-T cells after NMN treatment (Figure2. a.) TSCM and Tcm are classified into subsets according to their phenotypes, functions, and homing potential [26] . Maintenance of long-lasting immunity is thought to depend on TSCM, which have superior self-renewal capacity, longevity, and proliferative potential. Antigen-specific Tcm have been considered the main mediator of maintenance and expansion of T cell immunity, following secondary antigen exposure, due to their ability to differentiate into effector T cells[24,27,28].Our data showed that the percentages of TSCM and Tcm in the NMN-treated CD19 CAR-T cells were higher than those in the non-NMN-treated CD19 CAR-T cells (Figure2. b) thus suggesting that NMN can affect CAR-T cell differentiation. Telomerase, the enzyme responsible for maintenance of the length of telomeres, is critically important in T cell division and function and its expression is decreased in T cell differentiation, aging, and diseases[29]. Moreover, in addition to β-galactosidase aggregation, and increased apoptosis, decreased telomeres length is a feature of cell senescence. To determine the effect of NMN on senescence of CD19 CAR T cells, we assessed both telomerase activity and the level of senescence-associated beta-galactosidase (SA-β-gal). After NMN treatment, telomerase activity increased and SA-β-gal level decreased in CD19 CAR-T cells (Figure2. c. and d and FigureS2). NMN-treated CD19 CAR-T cells showed lower apoptosis (Figure2. e), which might explain, in part, the higher rate of cell proliferation after NMN treatment. These results provide us with great confidence that NMN can enhance CD19 CAR-T cell longevity.

NMN treatment altered gene expression profile of CD19 CAR-T cells.

To explore why NMN-treated CD19 CAR-T cells have higher anti-aging activity thanthecontrol group, high-throughput RNA sequencing was used to examine the transcriptome profiles of the two experimental conditions. Upon differential expression analysis, we foundthe upregulated and downregulated genes in the NMN-treated CD19 CAR-T cells compared with control cells (Figure3. a). The differentiallyexpressed

geneswere subjected to gene ontology analysis. We found that genes regulating NAD-dependent protein deacetylase activity, positive regulation of cell proliferation, negative regulation of cellular senescence, positive regulation of adaptive immune response,and intrinsic apoptotic signaling pathway in response to DNA damage were upregulated in NMN-treated cells (Figure3. b). After protein–protein interaction (PPI) analysis, we found two gene clusters, one of which is mainly linked with NAD^+ conversion, and the other is associated with the Sirtuins family. In the PPI network Sirt1 serves as a focus between NAD^+ and functional regulatory genes, such as TP53, Bax, and NF-κB (Figure3. c). Furthermore, *Sirt1*, as a focus gene, was part of a cell longevity pathway in the KEGG database (FigureS3. b).

NMN regulates senescence related genes through upregulating Sirt1 expression

To further elucidate the mechanism of NMN anti-aging function in CD19 CAR-T cells, we examined the expression of sirtuins and found an upregulation of sirtuins in the NMN-treated CD19 CAR-Tcells. Thus,sirtuins may contribute to the protection of the CD19 CAR T cells after NMN administration.Sirtuinsare an evolutionarily conserved family of NAD^+-dependent deacetylases and ADP-ribosyltransferases that play important roles in biological activities[17] . Among mammalian sirtuins, Sirt1 plays significate roles in many physiological and disease-related processes such as caloric restriction-related longevity, metabolism and so on [30].We verified that NMN treated CD19 CAR-T cells showed higher expression of Sirtuin genes (Figure4. a. b). Interestingly, Sirt1-related gene, such as, TP53,NF-κB and Bax, also underwent significant changes and their expression was significantly reduced by the Sirt1 inhibitor EX527 (Figure4. c-f). These genes were linked with cell longevity in the KEGG database. We also found that NMN treatments reduced the secretion of inflammatory factors, such as IL-6 (Figure1d), which may be linked with the decrease in NF-κB, thus suggesting that NMN may reduce inflammatory responses in immunotherapy. Overall, these results suggest that in CD19-CAR-T cells, NMN retarded the senescence of CD19 CAR-T cells by increasing the level of Sirt1 expression.

NMN treatment enhances CD19 CAR-T cell antitumor activity in B-ALL xenograft mouse model

Finally, we tested whether the NMN treatment affected the properties of CD19 CAR-T cells *in vivo* (Figure5. a-d). NALM6-Luc cells were injected subcutaneously into NOD-SCID mice to generate a xenograft mouse model. The next three days, CD19 CAR-T and NMN-treated CD19 CAR-T cells were injected by tail vein injection. NMN (300 mg NMN/kg body weight) was injected intraperitoneally daily. Bioluminescent imaging (IVIS system) was used to monitor tumor development. As shown in Figure 5, NMN-treated CD19 CAR-T cells exhibited longer-lasting tumor suppression. Mice treated with NMN had a lower tumor burden than that of the untreated mice. Fluorescence analysis and survival time were shown in Table 2. Based on the results established herein, we propose a model for the NMN-Sirt1 axis that enhances CD19 CAR-T cell longevity, and, therefore, may benefit the therapeutic approach against B lymphoid leukemia (Figure 6).

Discussion

CAR-T cell therapy has been shown to be an effective cancer immunotherapy for different types of human cancers[31]. However, as a result of cell senescence, exhaustion, among the several reasons, long-term remission is still uncommon and most patients relapse[32]. Therefore, there is an imperative need to improve the therapeutic efficacy of CAR-T cells, particularly for hematological malignancies. Improving the persistence of CAR-T cells is critical for their *in vivo* efficacy, which is indeed dependent on the viability and proliferation ability of CAR-T cells. Multiple strategies have been developed to facilitate T cell proliferation and persistence, such as integration of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal into the CAR design[4], addition of a third cytokine signal[5], and providing additional co-stimulation signals[6]. In this study, we found that NMN can increase cell rejuvenation and cell proliferation by upregulating Sirt1 and provide us with great confidence that NMN can enhance CD19 CAR-T cell longevity and anti-tumor efficiency.

As a biosynthetic precursor of NAD^+, NMN is known to promote cellular NAD^+ production and counteract age-related pathologies associated with a decline in NAD^+ levels. There is assertive evidence that in aged mice, NAD^+ biosynthesis increasing by NMN treatment reverses age-related mal-function in multiple organs, including the eye[33], skeletal muscle[34], and peripheral arteries[22]. participate in metabolism, NAD^+ also serves as a cofactor in fuel breakdown, which can regulate the bioactive proteins and improve the cell function. In this study, we evaluated the reproductive capacity of CD19 CAR-T cells after NMN addition. Our findings demonstrate that NMN can enhance the proliferative potential and enhance the functional properties of CAR-T cells. Next, we aimed to understand how NMN enhances the proliferation of CAR-T cells.

Telomeres are non-coding DNA repetitive sequences (TTAGGG) which associated with specialized proteins at the ends of linear chromosomes. They protect the chromosome from damage, especially during recombination[35]. Telomere length is an important feature of aging[36]. Ghimire *et al.* studied the pertinence between telomere length and aging from the data of 7,826 adults based on the National Health and Nutrition Examination Survey (1999–2002, age > 20 to > 80 years) and found that aging is related to a shorter telomere length [37]. In the present study, we observed longer telomere length in CD19 CAR-T cells with NMN addition, which suggests the potential molecular mechanisms of NMN-mediated improved lifespan.

β-galactosidase is a typical senescence biomarker, and its expression is significantly increased in senescent cells. After co-culture with the ALL cell line NALM-6, a 3-fold reduction in β-galactosidase was observed in NMN-treated CD19 CAR-T cells. Apoptosis is an important index of cell senescence, and in our study, after co-culture with NALM-6, NMN treated CD19 CAR-T cells were less apoptotic by flow cytometry). In summary, in the present study, we observed longer longevity of CD19 CAR-T cells upon NMN treatment and investigated the potential molecular mechanisms of NMN-mediated improved lifespan. Similar effects have been observed in animal models.

T cell subsets are important indicators of T cell differentiation. In our study, flow cytometry analysis showed a higher frequency of Tcm subset and TSCM in NMN-treated CD19 CAR-T cells. TSCM represents a small group of Tcm cells with enhanced proliferation and differentiation ability, and they can produce more terminally differentiated daughter cells which are vitally important for cancer immunotherapy. Tcm may contribute to their prolonged persistence, enhanced survival and self-renewal ability, and increase antitumor ability in vivo after adoptive transfer. After co-culturing with NALM-6, it is plausible that NMN treated CD19 CAR-T cells have a higher percentage of Tcm subset and TSCM subset. These results suggest that NMN has anti-CAR-T cell senescence ability and inhibits cell differentiation.

Researchers have reported that NMN enhances NAD^+ biosynthesis and ameliorates various pathologies in mice and humans over age [38]. In this study, the expression of NAD^+ increased in CD19 CAR-T cells after NMN addition, thus enhancing NAD^+ biosynthesis from NAD^+ intermediates. Indeed, the NMN anti-aging effect has been predicted in the concept of the “NAD World,” a hypothetical systemic regulatory network for the control of aging and longevity in mammals [39]. This effect of long-term NMN administration carve a path for the NAD^+ intermediated development of effective anti-aging ability.

The results of RNA-sequencing showed that NMN can upregulate the Sirtuins family, which is known to mediate beneficial anti-aging effects. Previous studies suggested that NMN treatment rectorates NAD + levels by activating the NAD^+ -dependent histone deacetylase enzyme Sirt1 in aged cells [34]. Sirt1 has been previously documented as an anti-aging gene. This study shows that NMN boosted NAD^+ levels, which are associated with increased Sirt1 expression in CD19 CAR-T cells. Supporting this theory, enhancing Sirt1 biosynthesis can extend the healthy lifespan of CAR-T cells.

Many major transcription factors and cofactors deactivated by Sirt1 dependent deacetylation for example: NF-κB and tumor suppressor TP53. The transcription factor TP53 translates various stress signals into diverse cellular outcomes, such as DNA repair, cell cycle arrest, or cell death. In this study, upregulation of Sirt1 expression by NMN caused downregulation of TP53expression level. Furthermore, to further verify the relationship between Sirt1 and its downstream genes, the Sirt1 inhibitor EX527(10UM) was used. The addition of EX527 inhibited the downregulation of TP53 induced by NMN, which indicates that NMN affects the proliferation of CD19 CAR-T cells by upregulating Sirt1 levels and causing a decrease in TP53 levels. Meanwhile, the additional NMN downregulated the Sirt1 relative downstream molecules, such as TP53, NF-κB and Bax.

NF-κB is a family of derivable transcription factors which play a central role in inflammation and senescence-associated secretory phenotype (SASP). The downregulation of NF-κB induced the decline of IL-6, which is the main manifestation of the inflammatory response. Bax is an important apoptosis-related factor, and the addition of NMN induces apoptosis of CD19 CAR-T cells. The above results show that NMN upregulates Sirt1 and leads to downregulation of TP53, Bax, and NF-κB, thereby inhibiting apoptosis and inflammatory response. Meanwhile, cell function assay show that NMN increase the ability the anti-tumor activity of CAR-T cells in a short time, suggesting that NMN supplementation can

improve the metabolic level of CAR-T cells by increasing the level of NAD^+, and thus enhance cell function.

In summary, the NMN administration study provides compelling support to an effective CAR-T cell anti-aging intervention using NMN, a key NAD^+ intermediate. Our findings highlight the clinical potential of NMN for CAR-T cell immunotherapy for human cancers, attributed to their central memory phenotype. The cells are more viable, proliferative, and long-lived in vivo. Furthermore, these CAR-T cells exerted great antitumor efficacy against tumors in human xenograft mouse models. Detailed molecular mechanisms responsible for the pleiotropic effects of NMN need to be investigated further.

Declarations

Funding Statement

Beijing Municipal Science and Technology Commission, Brain Science Research Fund (Z161100000216136);

Authorship

Contribution: Zhen Yu and Shuai Tong designed the protocol, conducted experiments, analyzed data, and wrote the paper; Can Zhang and Qinghui Zhuang provided the CAR-T cells preparation; Yue Bai and Zhijing An provided flow cytometry technical suppor; Chang Xu operated animal experiments; Yi Hu edited the paper; and Xiaosong Zhong designed the protocol, interpreted data, and edited the paper.

Ethics approval and consent to participate

This research was approved by the Beijing Shijitan Hospital Institutional Review Board.

Competing Interests

The authors declare no competing interests.

Data availability

The GEO accession is GES200367

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Figures

Figure 1

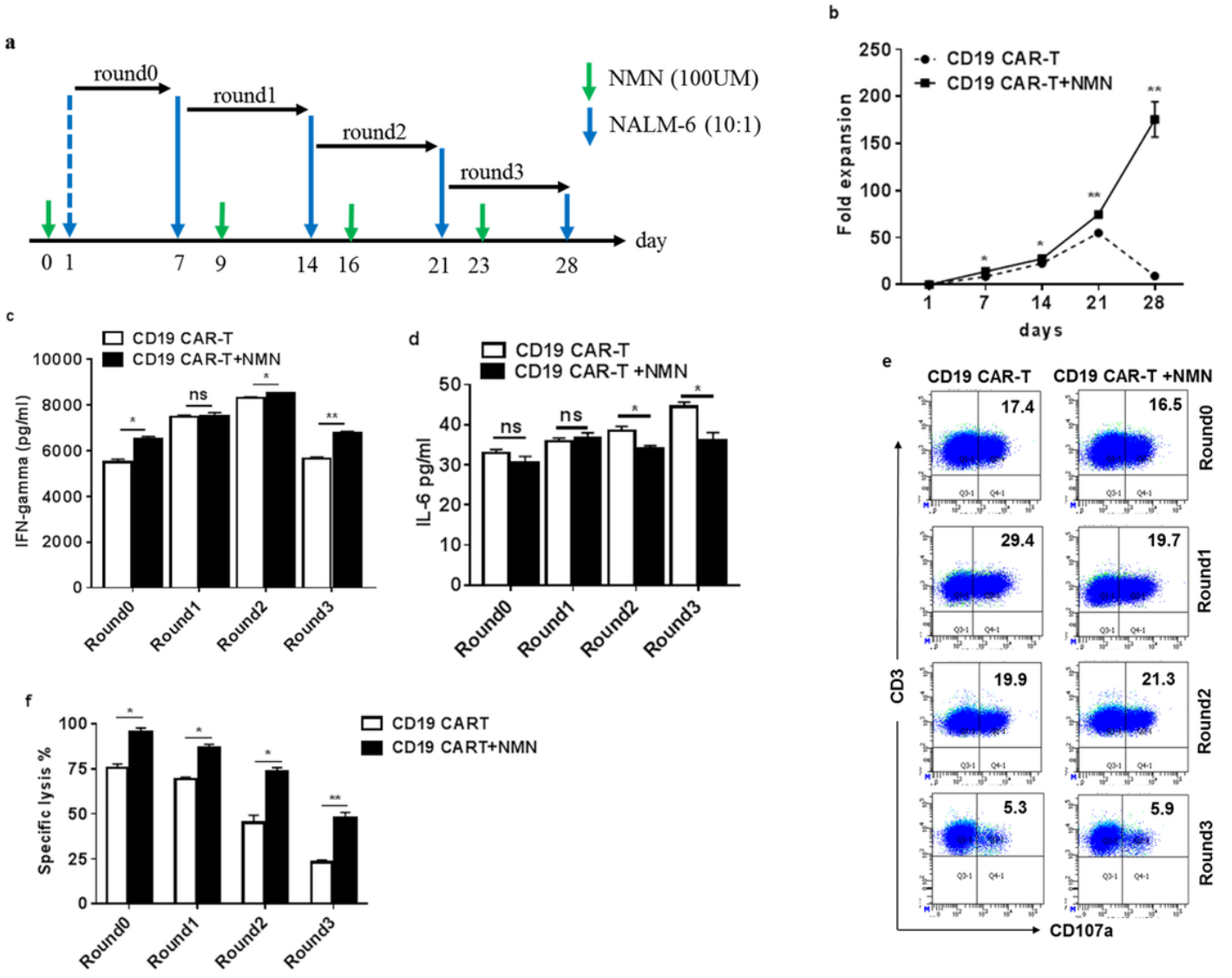

Figure 1

Impact of NMN on CAR-T cell counting and T cells effector function

(a) The skeleton of the experiment. The green arrow means the NMN treatment. The blue arrow means the co-culture with NALM-6. The dashed line means non co-culture. (b) CD19-CAR-T cells were co-cultured with NALM-6 cells for 28 days. The cells were treated with100UM NMN upon weekly co-cultured with NALM6 target cells at an E: T ratio of 10:1. Number of viable CAR-T cells determined by trypan blue exclusion. Fold expansion of CAR-T cells was calculated. (c. d) The supernatants were collected 24hours later to evaluate IFN-γ levels and IL-6 by ELISA (E: T = 0.5:1). (e)The CD107a degranulation was calculated by flow cytometry. (f) The cells were co-cultured with firefly-luciferase-transduced NALM-6 cell line (E: T ratio=0.5:1). The luciferase signal produced by surviving NALM-6 cells was measured after 24 hours later. Results were analyzed by one-way ANOVA, and statistical significance was set at *p < .05, **P < .01, ***p < .001

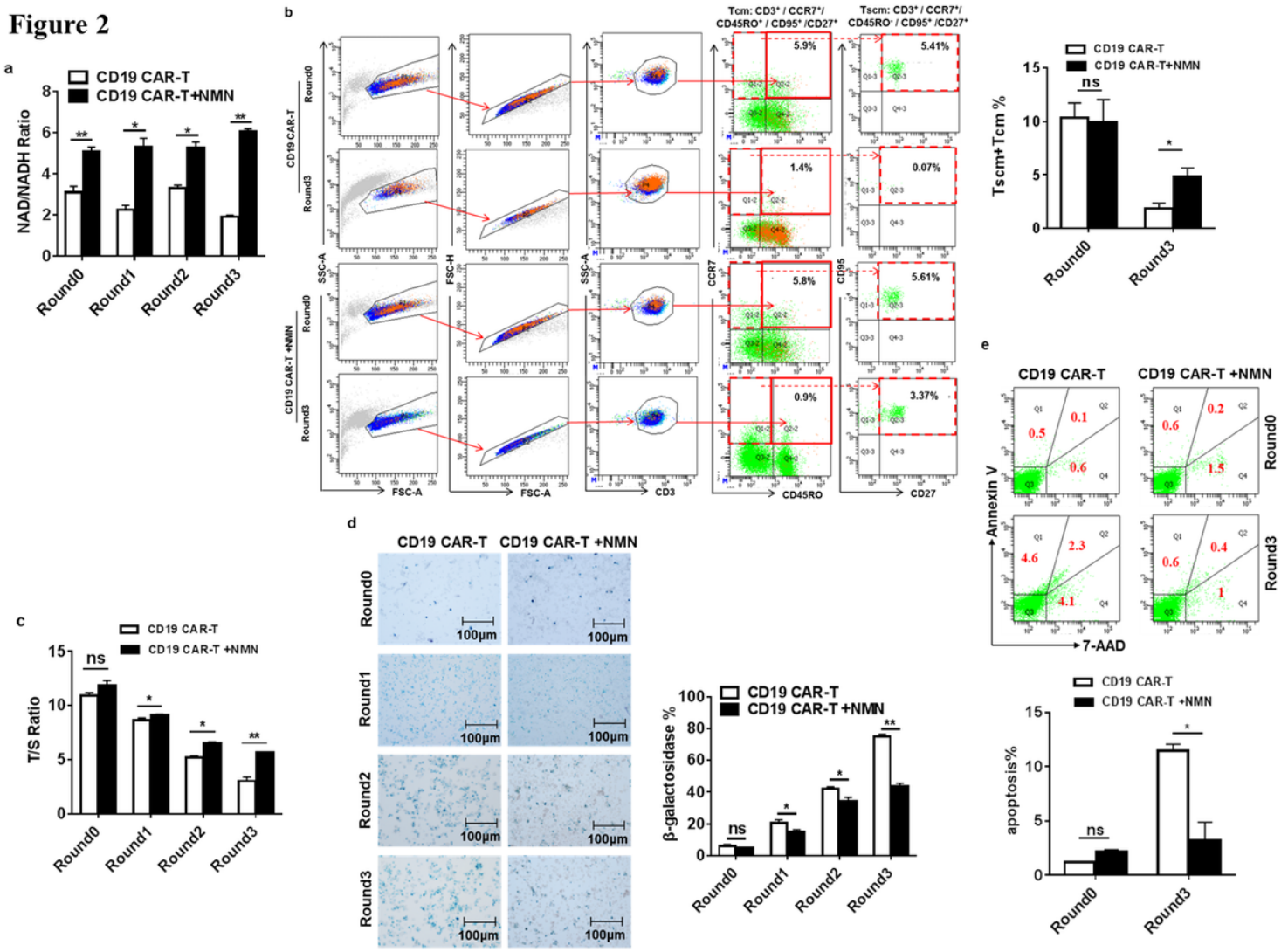

Figure 2

Effects of NMN on CD19 CAR-T cell phenotype and anti-aging

(a) NMN enhanced the NAD+/NADH ratio in CD19 CAR-T cells. NAD+/NADH levels were measured complete medium. The Round0 and Round3 represent the times of co-culture with NALM-6 cells. (b) The percentage of Tcm and Tscm treated with NMN is higher than that without NMN treated cell. (c) The effect of NMN on the telomere activity of the CD19 CAR-T cells treated with/without NMN treatment. (d) The SA-β-Gal activity in CD19 CAR-T cells and CD19 CAR-T cells treated with NMN. SA-β-Gal staining was observed in dark blue (Scale bar: 100μm). (e) Annexin-V and 7-ADD were introduced to evaluate cells that were programmed to die. Results were analyzed by one-way ANOVA, and statistical significance was set at *p < .05, **P < .01, ***p < .001

Figure 3

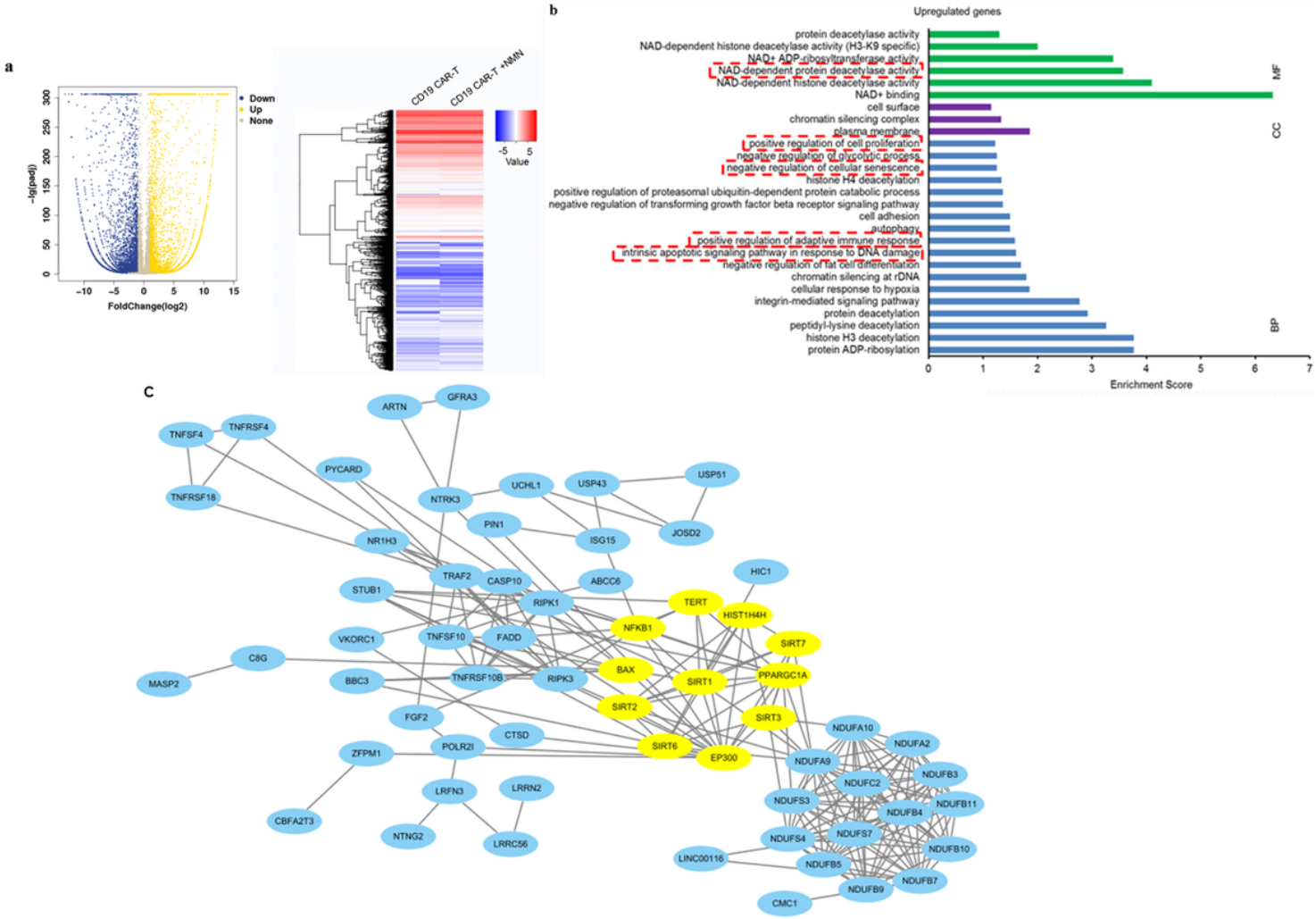

Figure 3

Gene ontology enrichment analysis of differentially expressed gene.

(a) The volcano plot for differentially expressed genes (DEGs) (FC > 2 and adjusted p-value <0.05). The horizontal axis represents the fold change, and the vertical axis represents the adjusted p-value. The yellow and blue circles indicate up- and down- regulated genes, respectively. The gene cluster diagram as shown left. (b) The differentially expressed genes were subjected to GO analysis using the online bioinformatics tool: DAVID Bioinformatics Resources 6.8. Fisher's exact test was used for the gene enrichment analysis. BP, biological process; CC, cellular component; MF, molecular function. (c) The protein-protein interaction diagram.

Figure 4

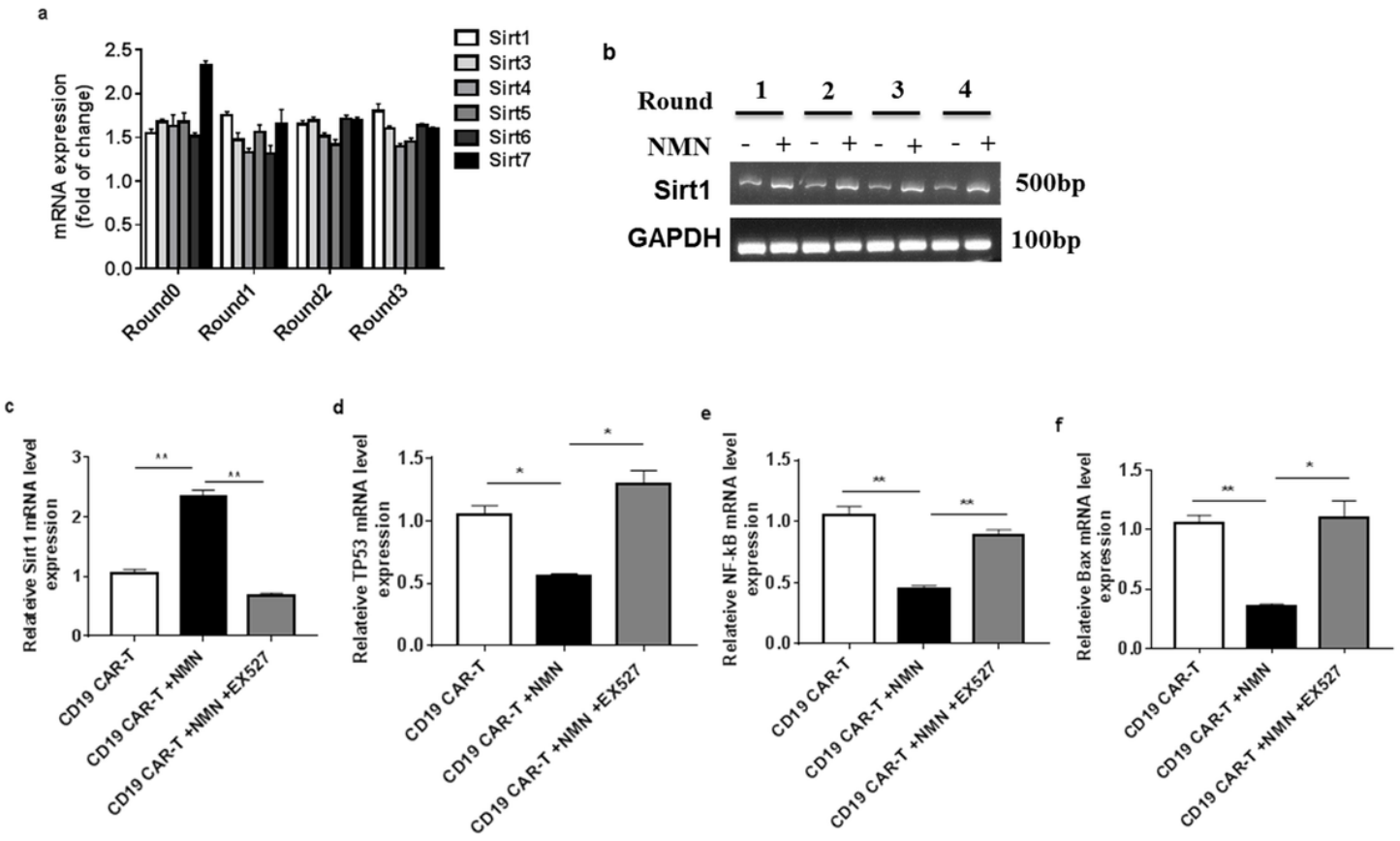

Figure 4

NMN regulated the expression of Sirt1 and senescence related genes

(a) The relative expression of Sirt1, Sirt3, Sirt4, Sirt5, Sirt6 and Sirt7 was measured by Q-PCR in CD19 CAR-T cells treated with/without 100UMNMN. (b) Result of agarose gel electrophoresis show NMN increases the level of Sirt1 expression in CD19-CAR-T cells. (c)The Sirt1 expression of NMN treated CD19 CAR-T cells are higher than non-NMN treated CD19 CAR-T cells by Q-PCR. After addition of Sirt1 inhibitor (EX527 10UM), The Sirt1 was significantly reduced. (b. c. d) The TP53, NF-κB and Bax expression of NMN treated CD19 CAR-T cells were detected by Q-PCR. Results were analyzed by one-way ANOVA, and statistical significance was set at *p < .05, **P < .01, ***p < .001

Figure 5

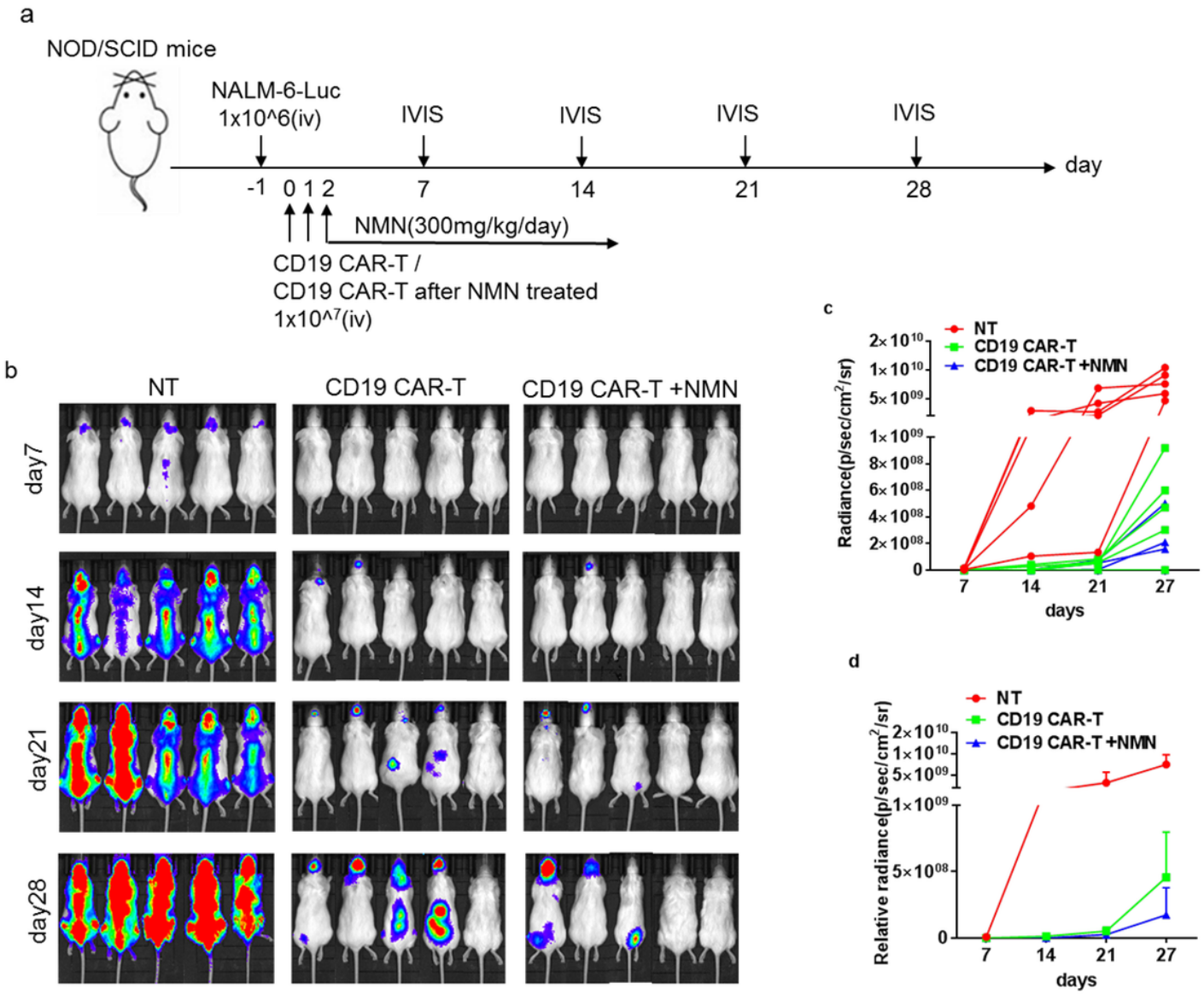

Figure 5

NMN treatment enhances CD19 CAR-T cell antitumor activity in B-ALL xenograft mouse model

(a)NOD/SCID mice were injected with NALM-6/luc cells via tail vein. The mice were divided into three groups. NT: PBMC; CD19 CAR-T; CD19 CAR-T +NMN: CD19 CAR-T cells treated with NMN (100UM) 24 hours in advance. The next 3 days, the above three groups cells were injected into the mice by tail vein injection. NMN or the equivalent volume of PBS (300 mg NMN/kg body weight) was intraperitoneal injected daily. All the mice were analyzed on the IVIS once a week. (b) Tumor burden over time showed bioluminescent signal quantified per mouse. (c. d) The statistics of tumor fluorescence values.

Figure 6

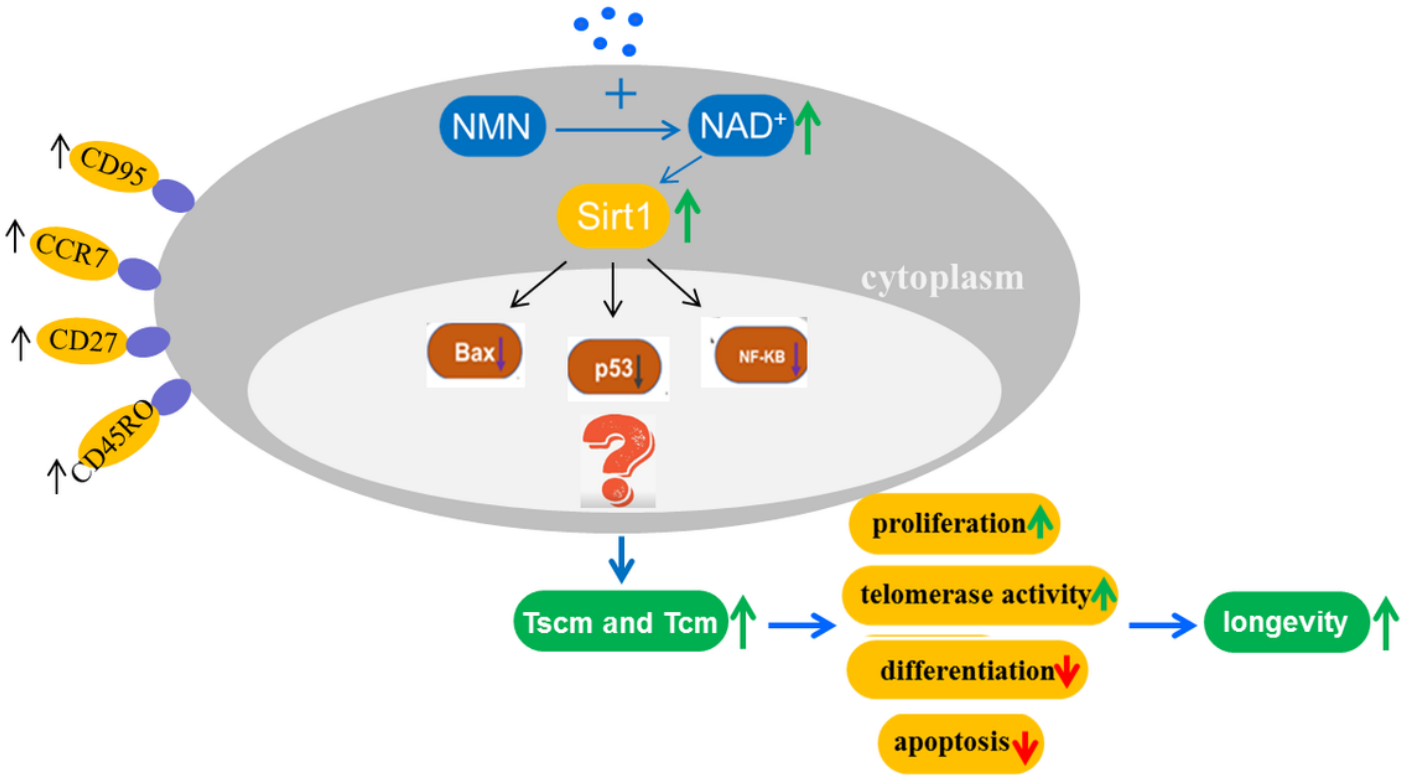

Figure 6

Experiment workflow and proposed model of better longevity efficacy within NMN treatment in CD19 CAR-T cells

Supplementary Files

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- supplementalMaterialstable.docx
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