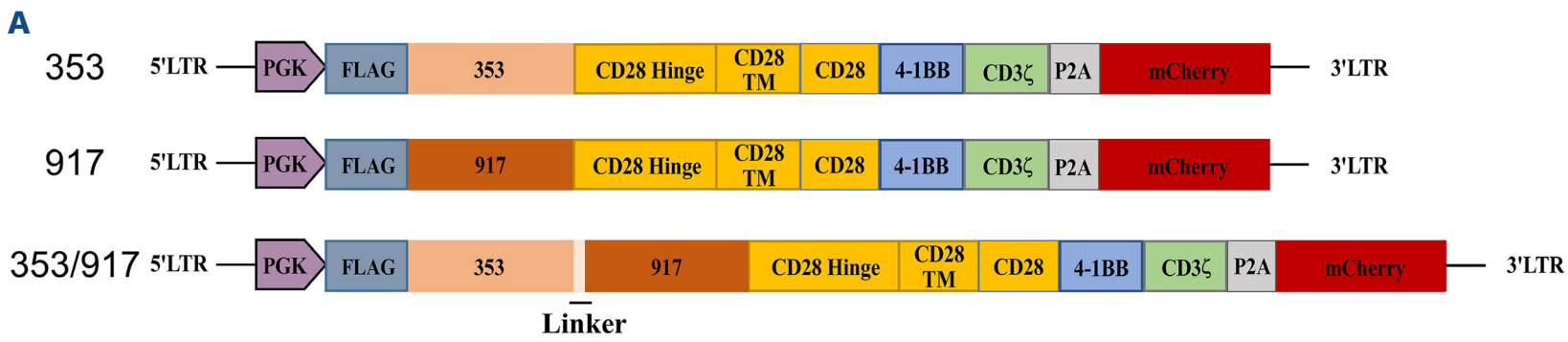
The nuclear factor-κ B inhibitor SN50 enhances the efficacy of B-cell maturation antigen-targeted chimeric antigen receptor T-cell therapy

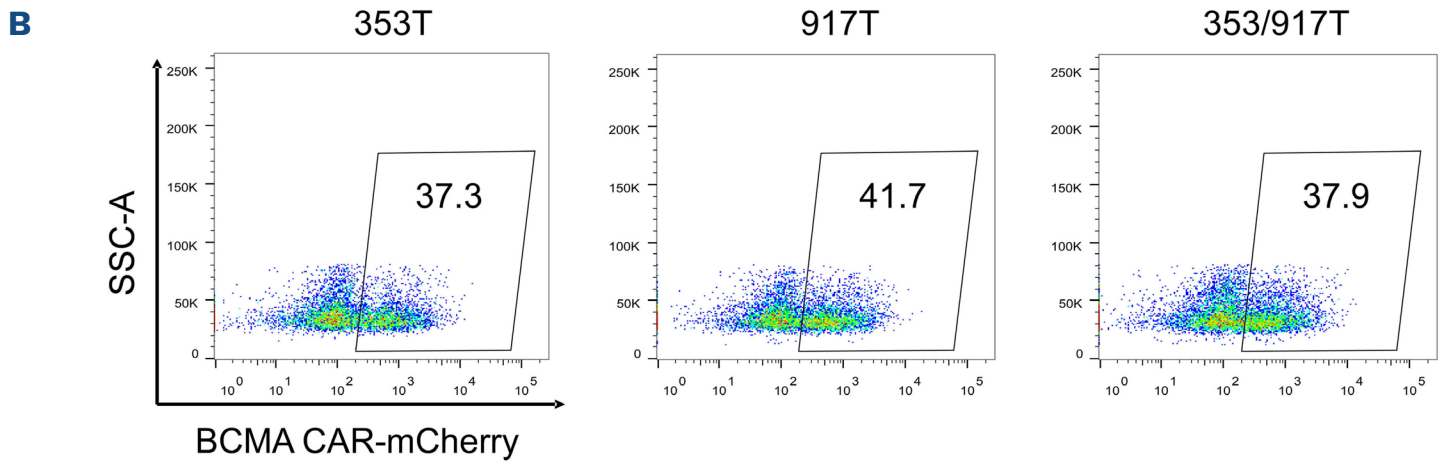
Multiple myeloma (MM) is the second most common hematologic malignancy in high-income countries. Although the prognosis of MM has been significantly improved with the use of proteasome inhibitors, immunomodulators, monoclonal antibodies, and stem cell transplantation, disease progression or relapse is still an inevitable reality.[1] The introduction of chimeric antigen receptor (CAR) T-cell therapy marks a significant breakthrough in the field of cancer treatment. B-cell maturation antigen (BCMA) is a member of the tumor necrosis factor receptor superfamily. It is specifically expressed at high levels in both normal and malignant plasma cells, making it an ideal target antigen for novel immunotherapies in MM.[2] BCMA CAR T cells have been proven to be highly effective in treating relapsed or refractory MM. However, some patients still experience relapse,[3] highlighting the urgent need for measures to enhance the efficacy of BCMA CAR T-cell therapy. This study investigates the cytotoxic efficacy of BCMA CAR T-cell therapies, comparing two monoepitopic BCMA CAR T cells (353T and 917T) and a bi-epitopic BCMA CAR T cells (353/917T). Our results demonstrate that the bi-epitopic BCMA CAR T cells exhibit superior cytotoxic effects against MM cell lines compared to its mono-epitopic counterparts. We observed that the differential activation levels of the nuclear factor-κ B (NF-κB) pathway contribute to the variations in BCMA CAR T-cell efficacy, with 353T showing the highest activation, followed by 917T, and 353/917T the lowest. The overactivation of the NF-κB pathway promotes the survival and resistance of MM cells. We further explored the combination effect of BCMA CAR T cells with the NF-κB pathway inhibitor SN50, finding that this combination enhances the cytotoxic effects of BCMA CAR T cells *in vitro* and reduces tumor growth in a non-obese diabetic/severe combined immunodeficiency disease mouse model. These findings suggest that SN50 can enhance the efficacy of BCMA CAR T-cell therapy.

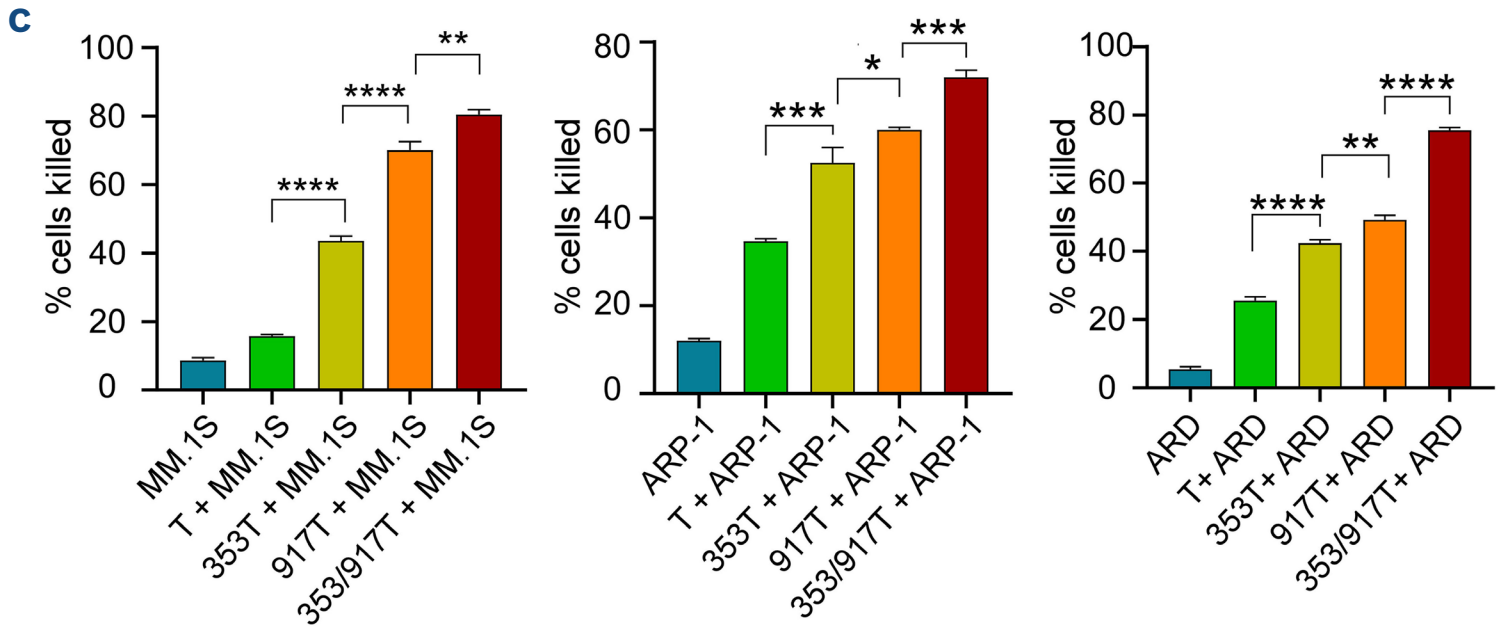
Cilta-cel is a CAR T-cell therapy product that includes two tandem nanobodies targeting BCMA. Its unique bi-epitopic BCMA CAR structure has demonstrated exceptional therapeutic efficacy in clinical practice.[4-6] In this study, we constructed two mono-epitopic BCMA CAR (named 353 and 917) and one bi-epitopic BCMA CAR (named 353/917) based on the structure of Cilta-cel (Figure 1A). All constructs were efficiently transduced into primary human activated T cells, resulting in detectable CAR expression, as confirmed by flow cytometry (Figure 1B). Our aim was to compare the killing efficacy of these two types of CAR T cells and investigate the reasons behind any observed differences. This would enable us to develop targeted combination therapy strategies to further enhance the clinical efficacy of BCMA CAR T cell in treating MM.

First, we selected the MM.1S, ARP-1, and ARD cell lines as target cells for *in vitro* cytotoxicity validation. Co-cultures of 353T, 917T, and 353/917T with target cells were conducted at an effector-to-target (E:T) ratio of 1:5 for 16 hours. Compared to control T cells, all three BCMA CAR T cells exhibited specific cytotoxic effects against MM cells. Among them, 353/917T showed the strongest killing effect on MM cell lines, followed by 917T, and 353T demonstrated the weakest effect ($P<0.05$; N=3) (Figure 1C).

Next, we explored whether the differences in killing efficacy between 353/917T, 353T, and 917T are due to variations in the internal effects induced within MM cells following their interaction with these three types of CAR T cells. The activation of BCMA downstream pathways can promote the proliferation and survival of MM cells, and it can also contribute to the formation of an immunosuppressive microenvironment.[7-9] One of the main pathways downstream of BCMA is the NF-κB pathway. Overactivation of the NF-κB pathway can lead to abnormal survival and enhanced anti-apoptotic mechanisms in MM cells, thereby promoting the growth and survival of MM cells.[10] The activation of the NF-κB pathway in MM cells during interactions between BCMA-directed CAR T cells and tumor cells has been reported.[11] Therefore, we co-cultured 353T, 917T, and 353/917T with MM cell lines at an E:T ratio of 1:50 for 16 hours, sorted the MM cells by flow cytometry after co-culture (*Online Supplementary Figure S1A*), extracted the proteins from the MM cell lines, and performed western blot experiments. It was found that the interaction between the three types of BCMA CAR T cells and MM cells could all activate the NF-κB pathway in MM cells, with 353T showing the most significant activation, followed by 917T, and 353/917T being the least. NF-κB activation was associated with an increased expression of the anti-apoptotic protein BCL-2, which partially resisted the cytotoxic effects of BCMA CAR T cells (Figure 1D). Notably, cytokines such as TNF-α secreted by CAR T cells are also known to activate the NF-κB pathway in MM cells.[12] To further validate this finding, we treated MM cells with the supernatants collected from CAR T cells/MM cells co-culture systems, which contain cytokines such as TNF-α and IFN-γ. In this setting, the supernatants did not induce NF-κB activation in MM cells (*Online Supplementary Figure S1B*), suggesting that NF-κB activation observed in our study primarily due to direct interaction between BCMA CAR T cells and MM







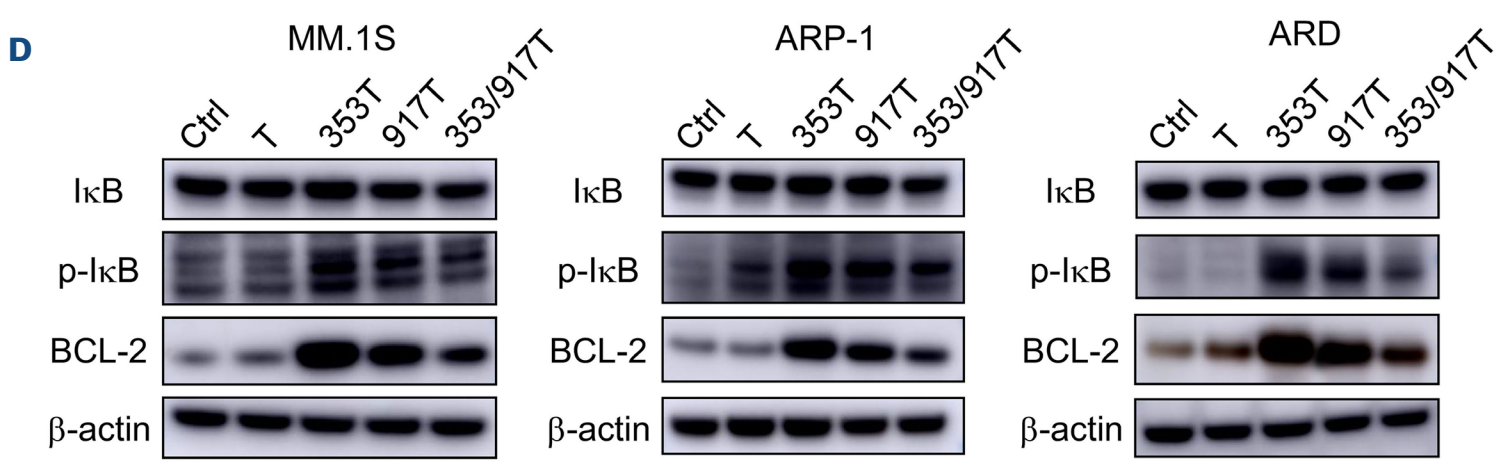


Figure 1. Comparison between mono-epitopic and bi-epitopic B-cell maturation antigen-targeted chimeric antigen receptor T cells. (A) Schematic diagram of chimeric antigen receptor (CAR) structure: 353, 917, 353/917. (B) Transduction efficiency of B-cell maturation antigen-targeted CAR in human T cells. (C) 353T, 917T, and 353/917T cells were evaluated for their cytotoxic activity against multiple myeloma (MM) cell lines MM.1S, ARP-1, and ARD. The killing efficiency was assessed using flow cytometry. Target cells were co-cultured with T cells or CAR T cells at an effector-to-target ratio of 1:5 for 16 hours. Cell viability was determined by staining with annexin V and 7-AAD, and the percentage of dead target cells was quantified. Results are presented as mean ± standard deviation from 3 independent experiments. Statistical significance was determined by *t* test, with *P* values indicated as follows: **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; NS: not significant. (D) MM cell lines were co-cultured with T, 353T, 917T, and 353/917T cells at an effector-to-target ratio of 1:50 for 16 hours. Following co-culture, MM cells were harvested and lysed for protein extraction. The activation of the nuclear factor-κ B pathway was assessed by western blot analysis.

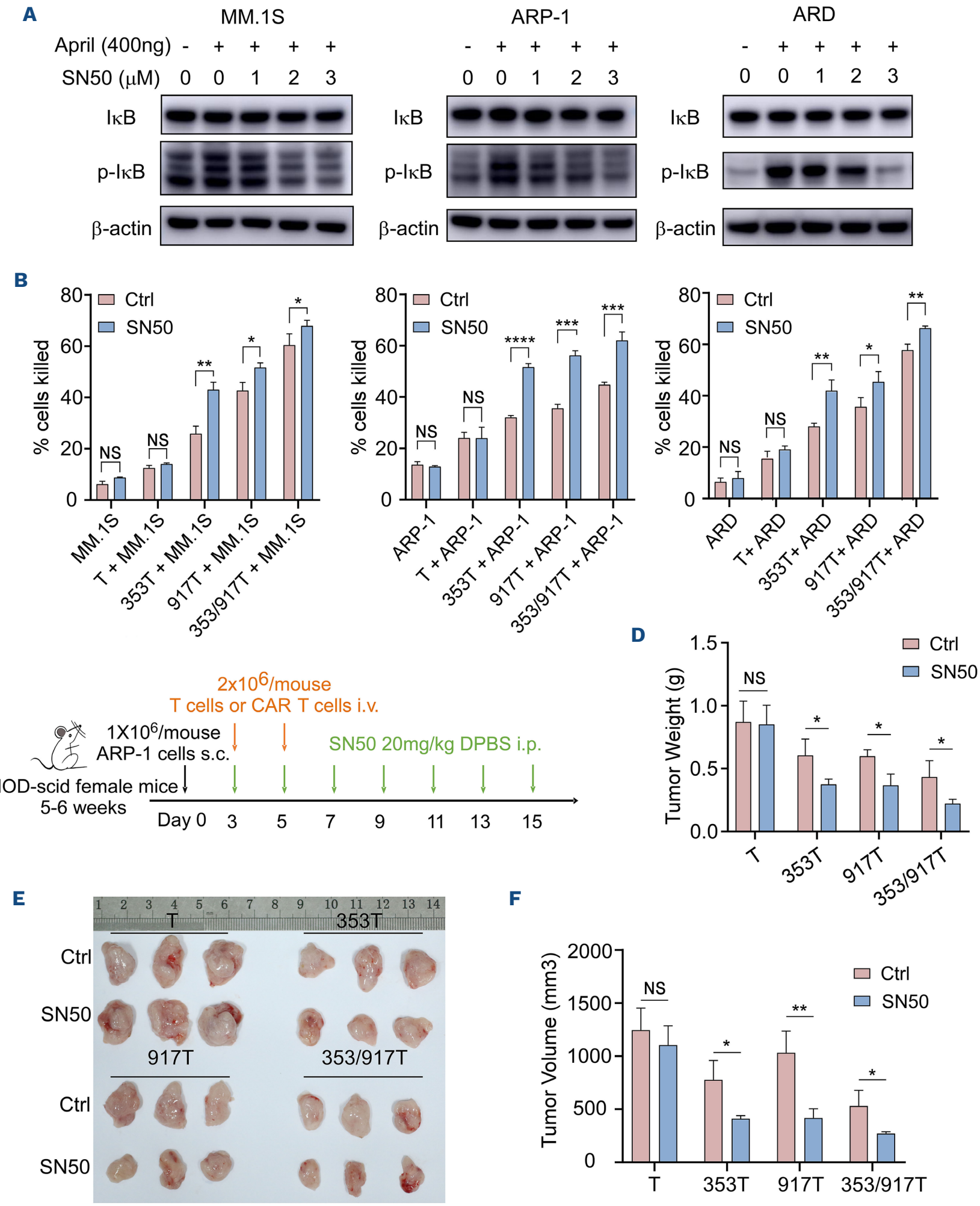


Figure 2. Enhancement of B-cell maturation antigen-targeted chimeric antigen receptor T cells cytotoxicity by the nuclear factor-κ B pathway inhibitor SN50. (A) Multiple myeloma (MM) cell lines (MM.1S, ARP-1 and ARD) were treated with a proliferation inducing ligand recombinant protein (400 ng/mL) to activate the nuclear factor-κ B (NF-κB) pathway, serving as a positive control (Ctrl). Simultaneously, MM cells were incubated with the NF-κB pathway inhibitor SN50 at various concentrations (0, 1.0, 2.0, and 3.0 μM) for 16 hours. After treatment, protein extracts were prepared from the cells and subjected to western blot analysis. (B) MM cell lines were co-cultured with T cells or B-cell maturation antigen-targeted chimeric antigen receptor T cells (353T, 917T, and 353/917T), in the presence of the NF-κB pathway inhibitor SN50. MM.1S and ARP-1 cells were treated with SN50 at a concentration of 2.0 μM, while ARD cells were treated with SN50 at a concentration of 3.0 μM. The effector-to-target ratio was 1:10, and co-cultures were maintained for 16 hours. The cytotoxic activity was assessed using flow cytometry by staining the cells with

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annexin V and 7-AAD, and the percentage of dead target cells was quantified. (C) Schematic representation of the *in vivo* study design in NOD-SCID mice. NOD-SCID female mice were injected subcutaneously (s.c.) with 1×10^6 ARP-1 cells into the right upper limb axilla on day 0. On days 3 and 5, the mice received intravenous (i.v.) injections of 2×10^6 T cells or BCMA CAR T cells via the tail vein. Additionally, mice were administered intraperitoneal injections of SN50 (20 mg/kg) or DPBS on days 3, 5, 7, 9, 11, 13, and 15. (D) Subcutaneous tumors were excised from the right upper limb axilla on day 16. (E) Tumor weight from each treatment group. (F) Tumor volume from each treatment group. The tumor volume was calculated using the following formula: length × width^2 × 0.5. Results are presented as mean ± standard deviation from 3 independent experiments. Statistical significance was determined by *t* test, with *P* values indicated as follows: **P*<0.05; ***P*<0.01; *** *P*<0.001; *****P*<0.0001; NS: not significant.

cells. However, we cannot exclude the possibility that specific cytokine conditions or higher cytokine concentrations in other contexts might influence NF-κB activation. These results highlight that NF-κB pathway activation supports the resistance of MM cells to BCMA CAR T-cell therapy and partially explains the differential killing effects observed among 353T, 917T, and 353/917T. This also indicates that the combination of BCMA CAR T cells with NF-κB pathway inhibitors may be a potential therapeutic strategy to improve clinical outcomes for MM patients.

The NF-κB pathway inhibitor SN50 is a small molecule compound that serves as a cell-permeable NF-κB translocation inhibitor.[13] First, we need to determine the concentration at which SN50 can inhibit the activation of the NF-κB pathway in MM cells. A proliferation-inducing ligand (APRIL) binding to BCMA can promote B-cell proliferation and survival.[14] We treated MM cells with APRIL recombinant protein to activate the NF-κB pathway as a positive control while simultaneously treating with different concentrations of SN50. Western blot results indicated that SN50 effectively inhibited NF-κB pathway activation at 2 μM in MM.1S and ARP-1 cells, and at 3 μM in ARD cells (Figure 2A). Given the critical role of the NF-κB pathway in CAR T-cell function, we next evaluated whether SN50 affects CAR T-cell function. we treated CAR T cells with SN50 and demonstrated that SN50 at 2.5 μM, 5 μM, and 10 μM had no significant impact on the apoptosis (*P*>0.05; N=3) (*Online Supplementary Figure S2A*). At 2 μM, SN50 did not affect the differentiation phenotype or exhaustion of CAR T cells (*P*>0.05; N=3) (*Online Supplementary Figure S2B, C*), but it significantly increased CD69 expression and cytokine secretion (including IL-2, TNF-α, IFN-γ, granzyme B) in 353T cells (*P*<0.05; N=3). However, SN50 at 2 μM had no significant effect on CD69 expression or cytokine secretion in 917T or 353/917T cells (*P*>0.05; N=3) (*Online Supplementary Figure S2D-H*). These results demonstrate that SN50 at 2 μM does not impair CAR T-cell function. Next, we combined SN50 with these three types of BCMA CAR T cells. The results showed that blocking the activation of the NF-κB pathway enhanced the cytotoxic effects of BCMA CAR T cells on MM cells (*P*<0.05; N=3) (Figure 2B). To further validate the specificity of NF-κB inhibition in enhancing the efficacy of CAR T cells, we replicated the experiments using another NF-κB pathway inhibitor, IKKγ NBD inhibitory peptide TFA (IKK-NBD). Similar to SN50, IKK-NBD treatment enhanced the cytotoxic effects of BCMA CAR T cells on MM cells (*P*<0.05; N=3) (*Online Supplementary Figure S3A-C*).

Finally, we evaluated the antitumor activity of combining SN50 with BCMA CAR T cells in a non-obese diabetic/severe combined immunodeficiency disease mouse model. This study was conducted in accordance with the principles of the Declaration of Helsinki and relevant ethical guidelines for research involving human participants and animal studies. The research protocol was reviewed and approved by the Ethics Committee of Fujian Medical University Union Hospital on March 31, 2021 (approval file number: 2021KJT079). ARP-1 cells were subcutaneously implanted on day 0. BCMA CAR T cells were injected on days 3 and 5, and SN50 was administered on days 3, 5, 7, 9, 11, 13, and 15 at a dose of 20 mg/kg (Figure 2C). Compared to the control group, the SN50 group showed lower tumor weight (*P*<0.05; N=3) (Figure 2D, E) and smaller tumor volume (*P*<0.05; N=3) (Figure 2F), demonstrating that SN50 can enhance the efficacy of BCMA CAR T cells *in vivo*.

In summary, our results indicate that the combination of the NF-κB pathway inhibitor SN50 with BCMA CAR T cells can enhance the efficacy of MM treatment. This provides a scientific basis for further optimization of treatment strategies for MM patients and offers important references and insights for clinical practice and research.

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Disclosures

No conflicts of interest to disclose.

Contributions

ZX and HL designed the study. JL, SX, and WZ drafted the manuscript and prepared the figures. JL and YW performed the experiments. WD and RW participated in statistical analyses and data interpretation. All authors participated in the process of drafting and revising the manuscript. All the authors have read and approved the final manuscript.

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Data-sharing statement

For original data, please contact the corresponding authors.

References

1. Martino M, Canale FA, Alati C, et al. CART-cell therapy: recent advances and new evidence in multiple myeloma. Cancers (Basel). 2021;13(11):2639.
2. Yu B, Jiang T, Liu D. BCMA-targeted immunotherapy for multiple myeloma. J Hematol Oncol. 2020;13(1):125.
3. Manier S, Ingegnere T, Escure G, et al. Current state and next-generation CAR-T cells in multiple myeloma. Blood Rev. 2022;54:100929.
4. San-Miguel J, Dhakal B, Yong K, et al. Cilta-cel or standard care in lenalidomide-refractory multiple myeloma. N Engl J Med. 2023;389(4):335-347.
5. Zhao WH, Wang BY, Chen LJ, et al. Four-year follow-up of LCAR-B38M in relapsed or refractory multiple myeloma: a phase 1, single-arm, open-label, multicenter study in China (LEGEND-2). J Hematol Oncol. 2022;15(1):86.
6. Xu J, Chen LJ, Yang SS, et al. Exploratory trial of a biepitopic CAR T-targeting B cell maturation antigen in relapsed/refractory multiple myeloma. Proc Natl Acad Sci U S A. 2019;116(19):9543-9551.
7. Tai YT, Acharya C, An G, et al. APRIL and BCMA promote human multiple myeloma growth and immunosuppression in the bone marrow microenvironment. Blood. 2016;127(25):3225-3236.
8. An G, Acharya C, Feng X, et al. Osteoclasts promote immune suppressive microenvironment in multiple myeloma: therapeutic implication. Blood. 2016;128(12):1590-1603.
9. Shen X, Guo Y, Qi J, Shi W, Wu X, Ju S. Binding of B-cell maturation antigen to B-cell activating factor induces survival of multiple myeloma cells by activating Akt and JNK signaling pathways. Cell Biochem Funct. 2016;34(2):104-110.
10. Roy P, Sarkar UA, Basak S. The NF-κB activating pathways in multiple myeloma. Biomedicines. 2018;6(2):59.
11. Camviel N, Wolf B, Croce G, Gfeller D, Zoete V, Arber C. Both APRIL and antibody-fragment-based CAR T cells for myeloma induce BCMA downmodulation by trogocytosis and internalization. J Immunother Cancer. 2022;10(11):e005091.
12. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia. 2009;23(1):10-24.
13. Wu Y, Wang Y, Liu B, et al. SN50 attenuates alveolar hypercoagulation and fibrinolysis inhibition in acute respiratory distress syndrome mice through inhibiting NF-κB p65 translocation. Respir Res. 2020;21(1):130.
14. Ullah MA, Mackay F. The BAFF-APRIL system in cancer. Cancers (Basel). 2023;15(6):1791.