Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

T-cell engager bispecific antibodies enable T cells to eliminate Multiple Myeloma by targeting tumor CD38 in combination with PD-L1

Shanshan Luo

2018XH0258@hust.edu.cn

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Chaofan Wang

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Wanting Wang

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Moran Wang

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

https://orcid.org/0000-0001-7019-0768

Mengling Yang

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Jun Deng

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Bahgat Fayed

Pharmaceutical and Drug Industries research institute, National Research Centre

Huafang Wang

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

https://orcid.org/0000-0001-6226-072X

Mei Heng

Hua Zhong University of Science and Technology

De-sheng Hu

Huazhong University of Science and Technology

Frank Momburg

German Cancer Research Center (DKFZ)

Yu Hu

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Chunyan Sun

Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Article

Keywords:

Posted Date: December 8th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-8174877/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: (Not answered)

Version of Record: A version of this preprint was published at Cell Death & Disease on August 21st, 2026. See the published version at https://doi.org/10.1038/s41419-026-09190-2.

Abstract

Bispecific antibodies are increasingly used in multiple myeloma (MM) treatment. Those targeting CD3 and CD28 mimic T cell activation's dual-signal system to boost anti-tumor response. CD38 is highly expressed on MM cells and PD-L1 is an immunosuppressive molecule related to poor prognosis in MM patients. This study aimed to explore bispecific antibody combinations' cytotoxic effects on MM for potential clinical use. First, we genetically engineered bispecific antibodies (BiMabCD3-CD38, BiMabCD28-CD38, BiMabCD28-PD-L1, BiMabCD28-SLAMF7) and divided them into three groups: (i) BiMabCD3-CD38 and BiMabCD28-PD-L1, (ii) BiMabCD3-CD38 and BiMabCD28-CD38, (iii) BiMabCD3-CD38 and BiMabCD28-SLAMF7. In in vitro tests, bispecific antibody combinations could bind to T cells and MM simultaneously. Co-culture experiments showed they enhanced T cell activation, proliferation, differentiation and cytokine release. When CD38 expression on myeloma cells was low, groups i and iii, especially BiMabCD3-CD38 and BiMabCD28-PD-L1, had an edge in tumor cell elimination. In a mouse subcutaneous tumor xenograft model, we confirmed the anti-tumor effects and T cell recruitment/activation ability of bispecific antibody combinations. Through in vitro and in vivo experiments, our study explored how bispecific antibodies clear MM by activating T cells. Notably, targeting PD-L1 and CD38, bispecific antibodies had enhanced anti-tumor effects, especially when MM cells downregulated surface CD38.

Introduction

Multiple myeloma (MM) is the second most prevalent hematological malignancy, comprising approximately 10% of all malignant hematological disorders.[1] Over the past decade, the introduction of proteasome inhibitors (PIs), immunomodulatory drugs (IMiDs), and autologous stem cell transplantation (ASCT) has markedly enhanced the prognosis of MM patients, with a median survival extending to 7–10 years.[2, 3] Nevertheless, despite these therapeutic breakthroughs, patients inevitably experience relapse as a result of clonal evolution during disease progression, which enables tumor cells to escape the cytotoxic effects of treatment regimens.[4, 5]

To address challenges such as tumor drug resistance and recurrence, novel therapies targeting tumor antigens have emerged as a focal point in translational research for MM.[6] Consequently, antibody engineering-based immunotherapy has been developed. [7-9]This approach achieves its cytotoxic effect by targeting tumor-associated antigens (TAAs) while simultaneously binding to and recruiting effector cells to sites of tumor. [10, 11] As a result, it demonstrates enhanced specificity and targeting capability during treatment, thereby minimizing adverse reactions.[12]

With the advancement of antibody engineering, several tumor markers have been successively identified in multiple myeloma, including B-cell maturation antigen(BCMA),[12] CD38,[13]G protein-coupled receptor class C group 5 member D（GPRC5D）,[14]Fc Receptor-Like Protein 5（FcRH5）[15] and Signaling Lymphocyte Activation Molecule Family Member 7(SLAMF7).[16] In terms of effector T cells, continuous

stimulation of the signal transduction pathways via T cell receptor CD3 in the absence or presence of co-stimulatory molecules such as CD28 has been recognized as a critical factor contributing to the unresponsiveness or apoptosis of effector T cells induced by bispecific antibodies (bsAbs). [17-19]Further research has demonstrated that bispecific antibodies incorporating CD28 can mimic the second signal required for T cell activation, forming a synergistic combination therapy with CD3-based bispecific antibodies, which significantly enhances T cell activity, thereby effectively amplifying the tumor-killing efficacy mediated by bispecific antibodies.[20, 21] However, MM cells often downregulate the surface antigens to escape the immune attack and drug effects under the selection pressure. Therefore, combinations of different bispecific antibodies targeting multiple surface antigens can be applied to increase their therapeutic efficacy.

Therefore, this study aims to investigate and optimize the immunotherapy of bispecific antibodies. Bispecific antibodies targeting distinct antigens on the surface of multiple myeloma (MM) cells and T-cell surface antigens (CD3, CD28) were engineered via gene editing. Combination therapies were implemented (i) BiMabCD3-CD38 and BiMabCD28-PD-L1; (ii) BiMabCD3-CD38 and BiMabCD28-CD38; (iii) BiMabCD3-CD38 and BiMabCD28-SLAMF7, and their anti-tumor efficacy and associated molecular mechanisms were systematically evaluated using MM cell lines and mouse subcutaneous tumor models. The results demonstrated that various combinations of bispecific antibodies could effectively suppress MM cell proliferation and further eradicate MM cells by promoting T-cell proliferation, differentiation, activation, and secretion of pro-inflammatory cytokines, thereby achieving substantial anti-tumor effects. Especially the combinations of BiMab-CD38xCD3 with BiMab-PD-L1xCD28 displayed a high priority to eliminate MM cells ,which downregulates CD38. These findings provide a robust theoretical foundation and valuable data for the clinical application of bispecific antibodies.

Materials and Methods

Gene cloning and construction of bispecific antibodies against MM

The surface target antigens of multiple myeloma were recognized by single-chain variable fragments (scFvs). The bispecific antibody is a ~170 kD homodimer consisting of two copies of myeloma antigen-specific scFvs connected via a glycine-serine (G4S)3 linker to the N-termini of a modified IgG1 Fc domain containing mutations C224S and N297Q, followed by CD3-specific scFvs derived from the variable domains of OKT3, or CD28-specific scFvs derived from antibody 9.3. C-terminally. Bispecific antibodies (BiMAb), with Strep-tag II between the Fc domain and the C-terminal single-chain scFvs, were expressed in HEK293 cells. The BiMAbs were purified using biotin-labeled affinity chromatography followed by size exclusion chromatography. The purity of all the biMabs was verified by Coomassie staining and Western blotting.

Binding of BiMabs to MM

The binding capacity of bispecific antibodies to multiple myeloma (MM) cells was verified by flow cytometry. Initially, two MM cell lines, RMIP-8226 and U266B1, were counted and adjusted to 4×10^5 cells per sample. Subsequently, 1 nM concentrations of the bispecific antibodies BiMabCD3-CD38, BiMabCD28-CD38, BiMabCD28-PD-L1, and BiMabCD28-SLAMF7 were co-cultured with the respective myeloma cell lines RMIP-8226 and U266B1 at 37 °C for 30 minutes. Following this step, fluorescently labeled goat anti-human IgG Fc APC (Biolegend) was added, and the samples were incubated in the dark for 30 minutes. Following extensive washing, the binding affinity was subsequently analyzed using flow cytometry.

Binding of BiMabs to T cells in PBMC

Human Peripheral Blood Mononuclear Cells (PBMCs) isolated from peripheral blood were evenly distributed into four groups, with each group containing 10^6 cells. Each group of PBMCs was co-incubated with 1 nM of BiMabCD3-CD38, BiMabCD28-CD38, BiMabCD28-PD-L1, or BiMabCD28-SLAMF7 at 37 °C for 30 minutes, respectively. Subsequently, fluorescence-labeled specific antibodies, including anti-CD4 (BB515) (Biolegend), anti-CD8 (PE) (Biolegend), and anti-IgG Fc (APC) (Biolegend), were added to the samples. After a 30-minute incubation on ice in the dark, the samples were washed and further analyzed by flow cytometry.

Assessment of T cell activation

PBMCs and BiMAbs were added to 96-well cell culture plates with U266B1 cells pre-cultivated in individual wells. After 48 hours of incubation, the supernatants and cell pellets were collected. ELISA was performed to quantify the cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-2, and IL-4 secreted by T cells in the supernatants. Flow cytometry was applied to analyze the T cell activation, differentiation, and proliferation capacity for each sample.

The BiMabs mediated eliminationof myeloma by human PBMCs

The cultured RPMI-8226 cells and U266B1 cells were first stained with CFSE, subsequently co-cultured with PBMCs isolated from healthy donors in the presence or absence of different bispecific antibody combinations. These bispecific antibody combinations consisted of four individual bispecific antibodies forming three distinct combinations: (i) BiMabCD3-CD38 and BiMabCD28-PD-L1; (ii) BiMabCD3-CD38 and BiMabCD28-CD38; (iii) BiMabCD3-CD38 and BiMabCD28-SLAMF7. Such co-culture experiments were conducted under varying conditions, including time gradients (24h, 48h, 72h), antibody concentration gradients (0.01nM, 0.1nM, 1nM), and effector-to-target ratios (2:1, 5:1, 10:1). After the co-cultivation, cells were further stained with 7-AAD. Finally, the cytotoxic activity of T cells triggered by the bispecific antibody combinations against two multiple myeloma (MM) cell lines (RPMI-8226 cells and U266B1 cells) was assessed using flow cytometry.

Animal experiments

Female BALB/C nude mice aged 6-8 weeks were raised in SPF-grade animal rooms to adapt to the environment for one week and receive humane care. The mice were provided by Beijing Weitonglihua Animal Technology Co., Ltd. and raised at the Animal Center of Tongji Medical College, Huazhong University of Science and Technology. All the mouse experimental protocols and operation procedures involved have been reviewed and approved by the Animal Ethics Committee of Tongji Hospital (IACUC number 4444). All the staff involved in the mouse experiments hold the qualification certificates for laboratory animal practitioners issued by Hubei Province.

Evaluation of the in vivo efficacy of bispecific antibody-mediated MM elimination

The MM cell line RPMI-8226 in the logarithmic growth phase was collected and subcutaneously inoculated into the right flank of nude mice at a dose of 1×10^7 cells in 0.1 ml per mouse. One week after tumor cell inoculation ,when the tumor volume reached approximately 100 mm^3, human PBMCs were resuscitated and injected via the tail vein at a dose of 5×10^6 cells in 0.1 ml per mouse. Mice were then randomly divided into three groups based on tumor volume: (1) physiological saline-treated group, (2) BiMabCD3-CD38 combined with BiMabC28-CD38 treated group (5 μg/mouse for each injection) , and (3) BiMabCD3-CD38 combined with BiMabCD28-PD-L1 treated group (5 μg/mouse for each injection). The bispecific characteristic antibodies were administered via tail vein injection twice weekly for a total of five doses. Throughout the experiment, body weight, tumor volume, and survival rate were monitored regularly.

The mice were euthanized 23 days post-treatment for tumor excision. The volume changes of subcutaneous tumors in the mice were quantitatively compared across different treatment groups. Additionally, tumor tissues were harvested and analyzed for the infiltration capacity of human CD4+ T cells and CD8+ T cells, as well as the cytokine release ,using immunohistochemistry and quantitative PCR.

Statistical analyses

In this experiment, FlowJo 10.8.1 and GraphPad Prism 9 were used to analyze the experimental data. As for the difference between the two groups, a Meta-analysis was performed using a One-way ANOVA test. $p<0.05$ indicates that the difference is statistically significant. The results of all data were expressed as mean ± standard deviation.

Results

Expression, purification, and qualitative research of bispecific antibodies

In order to develop immunosuppressants capable of simultaneously recruiting and activating T cells while targeting multiple myeloma, genetic engineering techniques were applied to construct various

bispecific antibody plasmids with distinct tumor-associated antigens on MM cells (CD38 or PD-L1) as well as T cell antigens (CD3 or CD28). (Fig. 1A) Then these plasmids were used to transiently transfect the 293T cell to prove whether the bispecific antibodies (BiMabs) can be successfully expressed and released in the culture supernatant. (Fig. 1B) Following a 48-hour culture period, the cell supernatants were harvested and subjected to electrophoresis analysis. The results demonstrated that most bispecific antibodies were successfully expressed. (Fig. 1C) Subsequently, 293T cells that highly expressed BiMabCD3-CD38, BiMabCD28-CD38, BiMabCD28-SLAMF7, and BiMabCD28-PD-L1 were selected for further expression and purification. After expansion culture, the supernatants were concentrated and purified using affinity chromatography and size exclusion chromatography. Lastly, the quality of the purified BiMabs was proved by Commaasie staining and Western blot. The results indicated that the purity of the four bispecific antibodies could reach more than 90% purity. (Fig. 1D)

Bispecific antibodies bind to T cells and myeloma cells simultaneously

To verify whether bispecific antibodies can simultaneously bind to T cells and myeloma cells, we treated two myeloma cell lines (RPMI-8226 and U266B1) and human peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood with four bispecific antibodies: BiMabCD3-CD38, BiMabCD28-CD38, BiMabCD28-PD-L1, and BiMabCD28-SLAMF7. The samples were co-cultured at 37°C for 30 minutes. Subsequently, fluorescence-labeled specific antibodies were added, and the binding of bispecific antibodies to both myeloma cells and T cells was assessed by flow cytometry. The results demonstrated that all tested bispecific antibodies could simultaneously bind to CD4 + and CD8 + T cells, as well as myeloma cells (Fig. 2A-D). As expected, BiMabCD3-CD38 binds to T cells with stronger intensity than CD28 ,as the CD3 is dominantly expressed on the surface of T cells. However, surprisingly, BiMabCD3-CD38 and BiMabCD28-CD38 bind to RPMI-8226 with higher intensity than to U266B1. In order to know the reason why different binding intensity was detected for these bispecific antibodies to two different tumor cell lines, the expression levels of CD38 on the surface of the two myeloma cell lines were further evaluated using flow cytometry. The data revealed that CD38 was highly expressed on RPMI-8226 cells, but weakly expressed on U266B1 cells (Fig. 2E). In summary, all four bispecific antibodies could simultaneously bind to T cells and myeloma cells. In addition, since RPMI-8226 cells exhibit higher CD38 expression than U266B1 cells, the binding affinity of BiMabCD3-CD38 and BiMabCD28-CD38 to RPMI-8226 is also stronger than to U266B1 cells, while BiMabCD28-PD-L1 and BiMabCD28-SLAMF7 bind equally to both RPMI-8226 and U266B1 cells.

Bispecific antibodies mediate T cells to eliminate myeloma cells

To investigate the bispecific antibodies-mediated cytotoxicity of T cells against myeloma cells, we first stained PMMI-8226 (high CD38 expression) and U266B1 (low CD38 expression) cells with carboxyfluorescein succinimidyl ester(CFSE). Subsequently, these stained cells were co-cultured with

PBMCs isolated from healthy donors in the presence of various bispecific antibody combinations: Combination one (BiMabCD3-CD38 X BiMabCD28-PD-L1), Combination two (BiMabCD3-CD38 X BiMabCD28-CD38), and Combination three (BiMabCD3-CD38 X BiMabCD28-SLAMF7). Different conditions, including time gradients (24h, 48h, 72h)(Fig. 3A), antibody concentration gradients (0.01nM, 0.1nM, 1nM)(Fig. 3B), and effector-to-target ratios (2:1, 5:1, 10:1) were screened in the co-culture experiments (Fig. 3C). After co-culture, cells were stained with 7-AAD to assess viability. Finally, the cytotoxic activity of T cells against both MM cell lines triggered by the bispecific antibody combinations was analyzed by flow cytometry. (Fig. 3D) The results demonstrated that the cytotoxicity of T cells positively correlated with co-culture times, antibody concentrations, and effector-to-target ratios. The longer the culture time, the higher the concentration, the higher the effector-to-target ratio, the better the killing efficacy is. Notably, BiMabCD3-CD38 together with BiMabCD28-PD-L1 exhibited a more pronounced cytotoxic effect on PMMI-8226 cells (high CD38 expression), whereas a weaker cytotoxic effect was observed for U266B1 cells (low CD38 expression).

Bispecific antibodies promote the proliferation and activation of T cells

To further investigate the activation of T cells mediated by bispecific antibody combinations, we isolated and extracted PBMCs from healthy individuals. These PBMCs were co-cultured with multiple myeloma cell lines in the presence or absence of three different bispecific antibody combinations. In this co-culture system, the antibody concentration was set at 1 nM, and the effector-to-target ratio was 1:1. After a 48-hour co-culture period, PBMCs were stained with fluorescent antibodies targeting CD3, CD25 ,and CD69.[22, 23] Following a 30-minute incubation in the dark, the expression levels of various T cell activation markers were further quantified by flow cytometry. The results demonstrated that for 8226 cells, all three combinations of bispecific antibodies induced a significant increase in expression of in both early (CD69+) and late (CD25+) activation markers compared to the control group. (Fig. 4A) For U266 cells, the bispecific antibody combinations also significantly activated T cells compared to the control group(Fig. 4B) .To further assess the proliferation of T cells induced by these bispecific antibody combinations, PBMCs were stained with CFSE with a 10-minute incubation at 37°C in the dark. Subsequently, PBMCs were co-cultured with two different myeloma cell lines (RPMI-8226 and U266B1) ,respectively ,at an effector-to-target ratio of 1:5 in the presence or absence of varying concentrations of the bispecific antibody combinations. After co-culture for 48 hours, PBMCs were stained with fluorecence-labeled anti-CD3 antibody in the dark and further analyzed by flow cytometry to quantify T cell proliferation. The findings revealed that all bispecific antibody combinations induced T cell proliferation. Notably, in the 8226 cells with high CD38 expression (Fig. 4C) ,the bispecific antibody combinations promote T cell proliferation more significantly than in the U266 cells with low CD38 expression. (Fig. 4D)

The combination of bispecific antibodies can promote the pro-inflammatory cytokine release by T cells

To investigate whether the bispecific antibody combination can promote the inflammatory cytokine release while activating T cells, we quantitatively analyzed the cytokine levels in the supernatants from the aforementioned co-culture system using specific cytokine detection kits. The results demonstrated that the combination of bispecific antibodies could effectively promote the release of cytokines IFN-γ(Fig. 5A), TNF-α(Fig. 5B), IL-2, and IL-4 by T cells(Fig. 5C-D), and as the antibody concentration increased, the concentration of pro-inflammatory factors also increased. Furthermore, we found that the concentrations of pro-inflammatory factors TNF-α and IFN-γ were the highest in the two types of myeloma cells. In U266 cells, the combination of BiMabCD3-CD38 X BiMabCD28-PD-L1 has a relative advantage in mediating the release of various cytokines by T cells. (Fig. 5E)

The combination of bispecific antibodies can induce T cell differentiation

To determine whether the bispecific antibody combinations could induce T cell differentiation, the aforementioned co-culture experiment was performed. After 48 hours of co-culture, T cells were specifically stained with fluorescent-labeled antibodies targeting CD45RA, CCR7, CD4, and CD8. Following a 30-minute incubation at 37°C in the dark, the bispecific antibody-mediated T cell differentiation was analyzed via flow cytometry. The results demonstrated that all three bispecific antibody combinations induced T cell differentiation in PBMCs. (Fig. 6A, C) In the cell co-culture system with RMIP-8226, combination one (BiMabCD3-CD38 X BiMabCD28-CD38) promoted significantly greater differentiation of T cells into effector memory T cells (TEM) and terminal effector memory T cells (TEMRA) compared to combination two(BiMabCD3-CD38 X BiMabCD28-SLAMF7) and combination three(BiMabCD3-CD38 X BiMabCD28-PD-L1). (Fig. 6B) It was reported that TEM and TEMRA exhibit the capacity for rapid response and potent cytotoxicity[24, 25] ,and these cells display direct or indirect killing in an antigen-independent manner and can reside within tissues, thereby contributing significantly to the anti-tumor immune responses.[26, 27] While in the cell co-culture system with U266, a weaker trend was observed. (Fig. 6D) Collectively, all three dual-antibody combinations effectively promote the differentiation of T cells. Combination one(BiMabCD3-CD38 X BiMabCD28-CD38) promotes T cell differentiation into more tumor-cytotoxic subtypes in 8226 cells, which are characterized by high CD38 expression, whereas combination two (BiMabCD3-CD38 X BiMabCD28-SLAMF7)and three(BiMabCD3-CD38 X BiMabCD28-PD-L1) exhibit greater efficacy in U266 cells with low CD38 expression.

The combination of bispecific antibodies inhibits tumor growth in multiple myeloma xenograft mice

To evaluate the *in vivo* therapeutic efficacy of different bispecific antibody combinations to control MM growth. We selected 6-week-old female nude mice and housed them in SPF-grade animal facilities. The MM cell line RPMI-8226 in the logarithmic growth phase was subcutaneously inoculated into the right flank of each nude mouse to establish a xenograft mouse model of multiple myeloma. (Fig. 7A) One week after tumor cell inoculation, pre-isolated human PBMCs were thawed and rested overnight, then

administered via the tail vein. At this time, the mice were randomly divided into three groups based on tumor sizes:(1) normal saline control group, (2) BiMabCD3-CD38 combined with BiMabC28-CD38 treatment group, and (3) BiMabCD3-CD38 combined with BiMabCD28-PD-L1 treatment group. Starting from the day of grouping, bispecific antibodies were injected intravenously twice weekly for a total of five administrations. Throughout the entire experiment, the body weight, tumor volume ,and survival rate of the mice were closely monitored. After 23 days of treatment, all were sacrificed, the tumors were collected ,and the peripheral blood was taken. Observe the anti-tumor situation of the bispecific antibody combination in mice. The results demonstrated that the bispecific antibody combination effectively inhibited tumor growth in multiple myeloma xenograft mice. (Fig. 7B-C, E) In peripheral blood, T cell activation induced by the bispecific antibody combination was also detected. (Fig. 7D) Both immunohistochemistry(Fig. 7J) and qPCR(Fig. 7F-I) confirmed the infiltration of CD4 + and CD8 + T cells into tumor tissues, as well as the expression of pro-inflammatory cytokines such as IFN-γ and TNF-α. Taken together, the synergistic combination of dual antibodies demonstrates potent efficacy in inhibiting the growth of subcutaneous tumors in xenografted mouse models. Significant activation of T cells is detected in peripheral blood, and the recruitment and cytotoxic activity of T cells are also observable at the sites of tumor tissues.

Discussion

Although significant progress has been achieved in the treatment of MM, the heterogeneity of clonal evolution remains a major challenge, contributing to drug resistance and high recurrence rates. For instance, CD38 is highly expressed on the surface of multiple myeloma cells and plays a critical role in tumor immune escape by consuming nicotinamide adenine dinucleotide (NAD+) and generating adenosine, which inhibits the functions of T cells and natural killer (NK) cells.[28, 29] Consequently, CD38 has emerged as an effective therapeutic target for multiple myeloma. Anti-CD38 monoclonal antibodies, such as Daratumumab and Isatuximab, have been approved for clinical use in treating multiple myeloma. [30] [31]Additionally, AMG424, a CD38xCD3 bispecific antibody, demonstrates potent anti-tumor activity and manageable toxicity through its dual-targeting mechanism and T cell activation capability.[7] However, the development of drug resistance in some patients was reported to be associated with the downregulation of CD38 expression. Thus, exploring optimized design strategies and combination therapies is essential for overcoming these challenges.[32]

PD-L1 is expressed on the surface of multiple myeloma cells and binds to PD-1 on T cells, thereby inhibiting T cell activity.[33] Clinical trials have evaluated monospecific anti-PD-L1 antibodies (Atezolizumab, Durvalumab), which, when combined with other therapies, have demonstrated certain synergistic effects.[34, 35] SLAMF7 is expressed on the surface of both multiple myeloma cells and NK cells.[36] Elotuzumab (an anti-SLAMF7 monospecific antibody) has shown significant efficacy and survival benefits in combination therapy regimens in several clinical trials and has been approved for relapsed/refractory multiple myeloma.[37] To our understanding, this approach can compensate for drug resistance in patients and the immune escape phenomenon of tumors caused by the downregulation of

CD38 expression. Additionally, preclinical studies have demonstrated that CD28 bispecific antibodies can significantly enhance the anti-tumor activity of CD3 bispecific antibodies, particularly in tumor models with low antigen expression.[20] Bispecific antibodies recruit and activate T cells to tumor tissues by targeting CD3, thereby the locally activated T cells further recognize and eliminate tumor cells due to the bridge effects of BiMabs. However, full activation of T cells typically requires co-stimulatory signals.[18]Therefore, bispecific antibodies targeting CD28 can provide co-stimulatory signals by binding to CD28 on T cells and surface antigens on tumor cells, promoting complete activation and proliferation of T cells, improving T cell-mediated anti-tumor effects by reactivating exhausted T cells, and maintaining long-lasting anti-tumor activity of T cells. Preliminary studies have indicated that CD28 bispecific antibodies enhance efficacy without significantly increasing toxicity.

In the current work, a combination of different bispecific pairs was employed, in which one BiMab can target both the T-cell surface molecule CD3 and the multiple myeloma biomarker CD38, while the other paired one can target CD28 and PD-L1 or SLAMF7. The use of these antibody combinations served to enhance the anti-tumor efficacy of T cells while compensating for the downregulation of tumor surface targets. Furthermore, in this study, gene cloning techniques were applied to artificially modify the Fc region (CH2-CH3 domain) of the original antibody, rendering it unrecognizable by the Fcγ receptor expressed on macrophages or neutrophils. This modification will prevent unnecessary antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), thereby reducing potential side effects and toxicity.

As for the mechanism for BiMab-mediated killing of MM cells, we found that in vitro, when MM cells were co-cultured with PBMCs in the presence of three different combinations of bispecific antibodies, robust expression of activation markers (CD25, CD69, CCR7, and CD45RA) on T cell surfaces was induced, accompanied by significant secretion of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-2, and IL-4) and T cell proliferation and differentiation. Further evaluation of MM cell viability revealed that the BiMab triggered T cells' activation, further mediated efficient killing and clearance of myeloma cells. These findings collectively demonstrate that the bispecific antibody combinations ,by tracking T cells to sites of tumor, effectively promote T cell activation for further tumor elimination, thereby validating the capacity of bispecific antibodies to induce T cell-mediated cytotoxicity against MM cells.

In a xenograft model, RPMI-8226 cells with a high expression level of CD38 were subcutaneously injected into nude mice to establish tumors. Following tumor formation, PBMCs and a bispecific antibody were administered via tail vein injection separately. Compared with the control group, the bispecific antibody-treated group significantly inhibited tumor growth. Flow cytometric analysis of peripheral blood further confirmed that T cells were activated in the treatment group. Immunohistochemistry and quantitative PCR analyses of subcutaneous tumors revealed extensive infiltration of activated T cells into the tumor microenvironment, and elevated levels of pro-inflammatory cytokines were also detected(TNF-α and IFN-γ), supporting the therapeutic efficacy of bispecific antibodies in vivo against MM by recruiting and activating T cells.

However, when constructing the subcutaneous tumor model, we attempted to inject U266B1 cells exhibiting low expression of the CD38 antigen for comparison. However, the tumorigenicity of this cell line was suboptimal, and we were only able to establish a subcutaneous tumor model using RPMI-8266 cells alone. Consequently, while the bispecific antibody demonstrated significant inhibition of tumor growth in the mouse model based on RPMI-8266 cells with high CD38 expression, further verification in models with low CD38 expression should be considered in subsequent studies.

In conclusion, our *in vitro* and *in vivo* studies have demonstrated that the combination of bispecific antibody pairs by recruiting T cells to sites of tumor specifically targets and kills multiple myeloma cells, which is both effective and promising. This approach may hold broad application prospects in the treatment of multiple myeloma. However, whether the potential toxicity can be effectively controlled and the extent of off-target effects remains to be further investigated in future studies.

Taken together, in this study, the CD3 bispecific antibody in synergy with the CD28 bispecific antibody concurrently engages T cells and myeloma surface markers, exerting direct or indirect (via the release of inflammatory cytokines) MHC-unrestricted cytotoxicity against tumor cells. This dual-targeting approach demonstrates enhanced efficacy compared to a single bispecific antibody, effectively addressing challenges such as drug resistance, immune evasion, and disease recurrence that arise from the downregulation of the target antigens during tumor evolution. These findings provide robust theoretical support and valuable data-driven insights for the clinical translation of bispecific antibodies in multiple myeloma therapy.

Conclusion

Our research comprehensively investigated the bispecific antibody- mediated elimination of MM by the activation of T cells through both in vitro and in vivo experiments. Notably, we highlighted that when targeting the immunosuppressive factor PD-L1 in combination with CD38, bispecific antibodies demonstrate a significantly enhanced anti-tumor effect, especially when MM cells downregulate surface CD38.

Declarations

Author contributions: C.F.W. and S.S.L. performed the experiments and wrote the main manuscript; W.T.W. and M.R.W. performed partial experiments, analyzed the data, and wrote the manuscript; M.L.Y., J.D., and B.F. provided technical support and analyzed the data; H.F.W., H.M., and D.S.H. provided conceptual advice and revised the manuscript; Y. H., C.Y.S., and S.S.L. designed the whole work, discussed the data, and revised the manuscript. All authors reviewed the manuscript.

Funding: This work was supported by the National Natural Science Foundations of China (No. 82070136 to SL, No.82274317, 81974249 to DH), and the National Key R&D Program of China (No. 2022YFC2304600 to YHu)

Declaration of interests: There is no conflict of interest.

References

1. Kocoglu M, Badros A. The Role of Immunotherapy in Multiple Myeloma [J]. Pharmaceuticals (Basel), 2016, 9(1).
2. Cowan A J, Green D J, Kwok M, et al. Diagnosis and Management of Multiple Myeloma: A Review [J]. JAMA, 2022, 327(5): 464–477.
3. Zangari M, van Rhee F, Anaissie E, et al. Eight-year median survival in multiple myeloma after total therapy 2: roles of thalidomide and consolidation chemotherapy in the context of total therapy 1 [J]. Br J Haematol, 2008, 141(4): 433–444.
4. Chen Q, Zhang M, Zheng S, et al. Therapeutic progress in relapsed/refractory multiple myeloma [J]. Ann Hematol, 2024, 103(6): 1833–1841.
5. Kumar S K, Lee J H, Lahuerta J J, et al. Risk of progression and survival in multiple myeloma relapsing after therapy with IMiDs and bortezomib: a multicenter international myeloma working group study [J]. Leukemia, 2012, 26(1): 149–157.
6. Yang W C, Lin S F. Mechanisms of Drug Resistance in Relapse and Refractory Multiple Myeloma [J]. Biomed Res Int, 2015, 2015: 341430.
7. Perrot A, Hulin C, Boumendil A, et al. Teclistamab in relapsed refractory multiple myeloma: a multi-institutional real-world study from the French early access program [J]. Haematologica, 2025, 110(4): 990–994.
8. Lesokhin A M, Tomasson M H, Arnulf B, et al. Elranatamab in relapsed or refractory multiple myeloma: phase 2 MagnetisMM-3 trial results [J]. Nat Med, 2023, 29(9): 2259–2267.
9. Chari A, Minnema M C, Berdeja J G, et al. Talquetamab, a T-Cell-Redirecting GPRC5D Bispecific Antibody for Multiple Myeloma [J]. N Engl J Med, 2022, 387(24): 2232–2244.
10. Brischwein K, Parr L, Pflanz S, et al. Strictly target cell-dependent activation of T cells by bispecific single-chain antibody constructs of the BiTE class [J]. J Immunother, 2007, 30(8): 798–807.
11. Offner S, Hofmeister R, Romaniuk A, et al. Induction of regular cytolytic T cell synapses by bispecific single-chain antibody constructs on MHC class I-negative tumor cells [J]. Mol Immunol, 2006, 43(6): 763–771.
12. Shah N, Chari A, Scott E, et al. B-cell maturation antigen (BCMA) in multiple myeloma: rationale for targeting and current therapeutic approaches [J]. Leukemia, 2020, 34(4): 985–1005.
13. van de Donk N, Richardson P G, Malavasi F. CD38 antibodies in multiple myeloma: back to the future [J]. Blood, 2018, 131(1): 13–29.
14. Zhou D, Wang Y, Chen C, et al. Targeting GPRC5D for multiple myeloma therapy [J]. J Hematol Oncol, 2024, 17(1): 88.
15. Stewart A K, Krishnan A Y, Singhal S, et al. Phase I study of the anti-FcRH5 antibody-drug conjugate DFRF4539A in relapsed or refractory multiple myeloma [J]. Blood Cancer J, 2019, 9(2): 17.

16. Roders N, Nakid-Cordero C, Raineri F, et al. Dual Chimeric Antigen Receptor T Cells Targeting CD38 and SLAMF7 with Independent Signaling Demonstrate Preclinical Efficacy and Safety in Multiple Myeloma [J]. Cancer Immunol Res, 2024, 12(4): 478–490.
17. Judokusumo E, Tabdanov E, Kumari S, et al. Mechanosensing in T lymphocyte activation [J]. Biophys J, 2012, 102(2): L5-7.
18. O'Connor R S, Hao X, Shen K, et al. Substrate rigidity regulates human T cell activation and proliferation [J]. J Immunol, 2012, 189(3): 1330–1339.
19. Sedwick C E, Morgan M M, Jusino L, et al. TCR, LFA-1, and CD28 play unique and complementary roles in signaling T cell cytoskeletal reorganization [J]. J Immunol, 1999, 162(3): 1367–1375.
20. Riha P, Rudd C E. CD28 co-signaling in the adaptive immune response [J]. Self Nonself, 2010, 1(3): 231–240.
21. Jakobs J, Rink L. Zinc Ionophore Pyrithione Mimics CD28 Costimulatory Signal in CD3 Activated T Cells [J]. Int J Mol Sci, 2024, 25(8).
22. Adamczyk M, Bartosinska J, Raczkiewicz D, et al. The Expression of Activation Markers CD25 and CD69 Increases during Biologic Treatment of Psoriasis [J]. J Clin Med, 2023, 12(20).
23. Liu A, Liu D, Liu X, et al. The prognosis prediction value of CD69 + CD8 + tissue-resident memory T cell as a novel indicator of pathologic complete response heterogeneity following different neoadjuvant therapy regimen in esophageal squamous cell carcinoma [J]. Cancer Immunol Immunother, 2025, 74(5): 147.
24. Tian Y, Babor M, Lane J, et al. Dengue-specific CD8 + T cell subsets display specialized transcriptomic and TCR profiles [J]. J Clin Invest, 2019, 129(4): 1727–1741.
25. Tavukcuoglu E, Yanik H, Parveen M, et al. Human memory T cell dynamics after aluminum-adjuvanted inactivated whole-virion SARS-CoV-2 vaccination [J]. Sci Rep, 2023, 13(1): 4610.
26. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance [J]. Annu Rev Immunol, 2004, 22: 745–763.
27. Rodriguez R M, Suarez-Alvarez B, Lavin J L, et al. Epigenetic Networks Regulate the Transcriptional Program in Memory and Terminally Differentiated CD8 + T Cells [J]. J Immunol, 2017, 198(2): 937–949.
28. Guerreiro S, Privat A L, Bressac L, et al. CD38 in Neurodegeneration and Neuroinflammation [J]. Cells, 2020, 9(2).
29. Li W, Liang L, Liao Q, et al. CD38: An important regulator of T cell function [J]. Biomed Pharmacother, 2022, 153: 113395.
30. Sonneveld P, Dimopoulos M A, Boccadoro M, et al. Daratumumab, Bortezomib, Lenalidomide, and Dexamethasone for Multiple Myeloma [J]. N Engl J Med, 2024, 390(4): 301–313.
31. Moreau P, Dimopoulos M A, Mikhael J, et al. Isatuximab, carfilzomib, and dexamethasone in relapsed multiple myeloma (IKEMA): a multicentre, open-label, randomised phase 3 trial [J]. Lancet, 2021, 397(10292): 2361–2371.

32. Wu H T, Zhao X Y. Regulation of CD38 on Multiple Myeloma and NK Cells by Monoclonal Antibodies [J]. Int J Biol Sci, 2022, 18(5): 1974–1988.
33. Rosenblatt J, Avigan D. Targeting the PD-1/PD-L1 axis in multiple myeloma: a dream or a reality? [J]. Blood, 2017, 129(3): 275–279.
34. Ebinama U, Sheshadri A, Anand K, et al. Pulmonary Immune-Related Adverse Events of PD-1 Versus PD-L1 Checkpoint Inhibitors: A Retrospective Review of Pharmacovigilance [J]. J Immunother Precis Oncol, 2023, 6(4): 177–184.
35. Schjesvold F, Paiva B, Ribrag V, et al. Cobimetinib Alone and Plus Venetoclax With/Without Atezolizumab in Patients With Relapsed/Refractory Multiple Myeloma [J]. Clin Lymphoma Myeloma Leuk, 2023, 23(1): e59-e70.
36. King E M, McClendon J, Trinh T, et al. Slamf7 is dispensable in mouse models of acute lung injury [J]. ERJ Open Res, 2024, 10(5).
37. Martino E A, Palmieri S, Galli M, et al. Elotuzumab plus pomalidomide and dexamethasone in relapsed/refractory multiple myeloma: Extended follow-up of a multicenter, retrospective real-world experience with 321 cases outside of controlled clinical trials [J]. Hematol Oncol, 2024, 42(4): e3290.

Figures

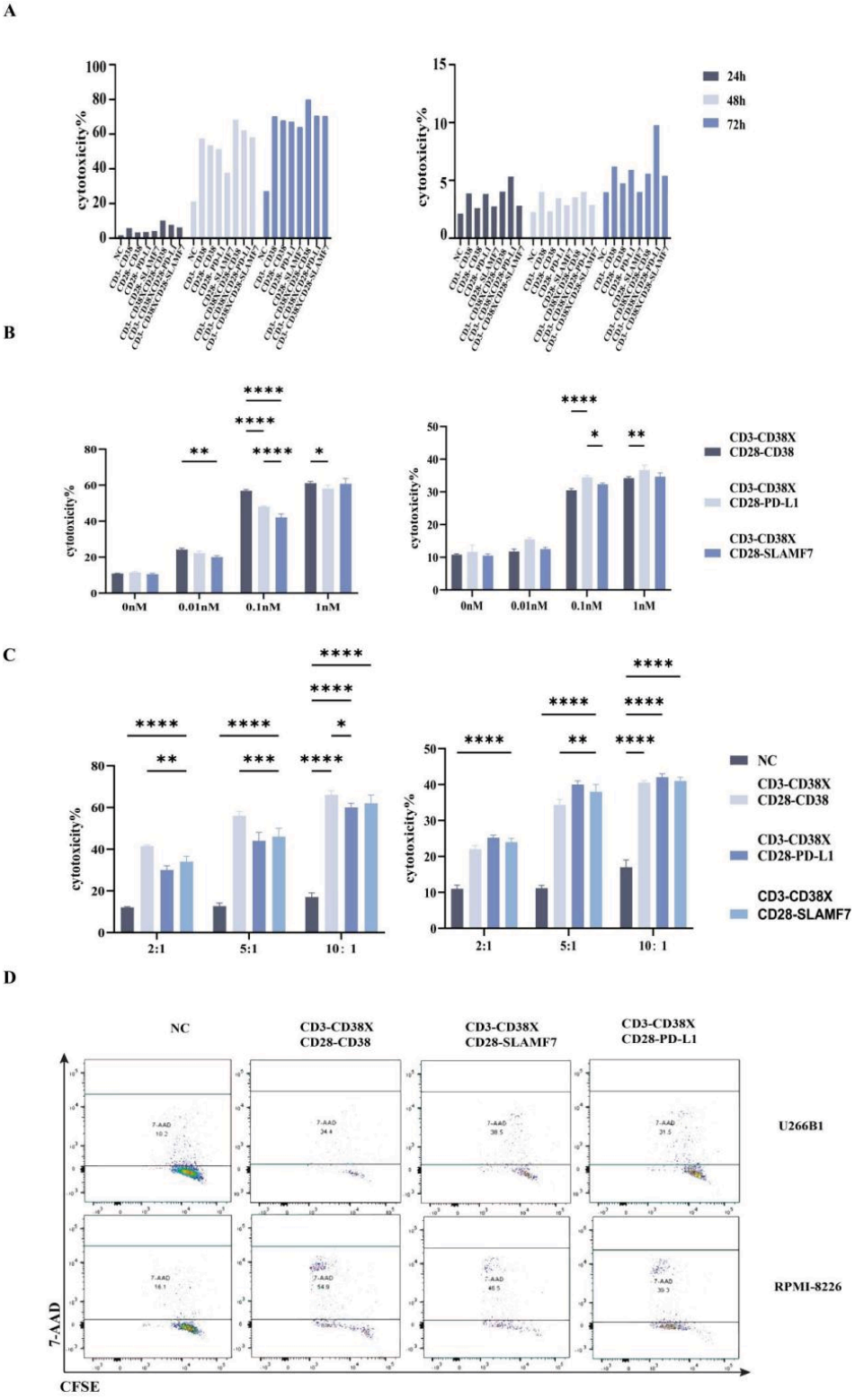


Figure 1

Expression, purification ,and qualitative research of bispecific antibodies. (A) Schematic diagram of the structure of the bispecific antibody constructed. **(B)**The bispecific antibody model was constructed after plasmid extraction, transfection, expression ,and purification by affinity chromatography column. **(C)** The expression of four antibodies in the cell culture supernatant 48 hours after transient transfection (WB detection, reduced form) showed that the upper band was the long chain and the lower band was the

short chain. **(D)** The WB (left image) and Coomax brilliant blue staining (right image) of the four double antibodies purified by affinity column chromatography (reduced form).

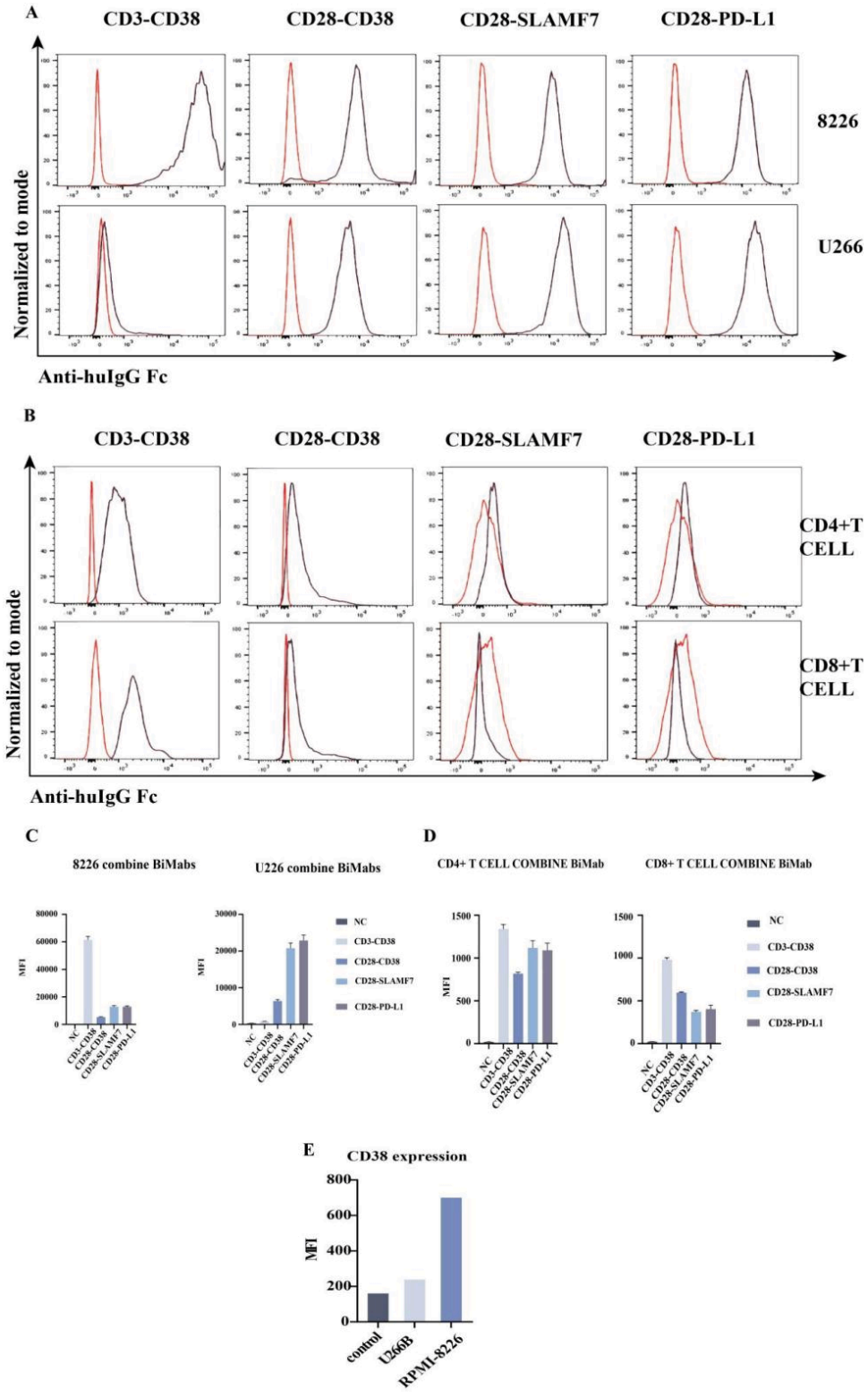


Figure 2

Bispecific antibodies bind to T cells and myeloma cells simultaneously. (A) Detection of bispecific antibodies to MM cell lines by flow cytometry. (B) Detection of bispecific antibodies to CD4+T and

CD8+T cells by flow cytometry. (C-D) MFI indicating the binding ability of four BiMabs to MM cells (C) and T cells (D). By flow cytometry detection, the same dose of bispecific antibodies (1nM) could bind to two types of MM cells, RMIP-8226 and U266B1, respectively, as well as CD4+T cells and CD8+T cells simultaneously. It was also found that for the same dose of double antibodies, the binding ability to the CD28 molecule on the surface of T cells was lower than that of CD3. (E) The expression of the target antigen CD38 molecules on two MM cell lines was detected by flow cytometry. Data are shown as means ± SD, or from one of three independent experiments. The p-values were calculated by the Student's t-test. *p<0.05, **p<0.01,***p<0.001.

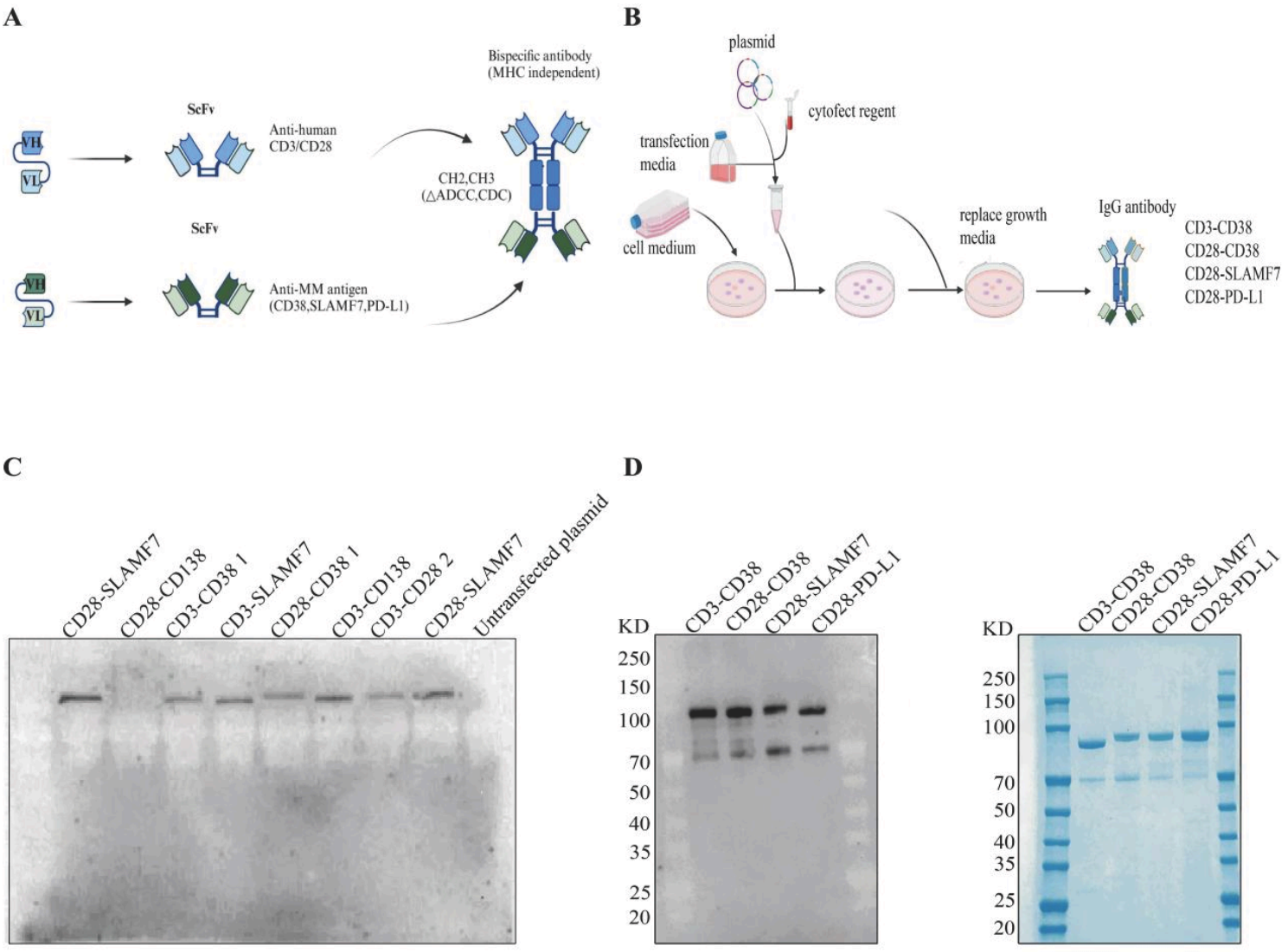


Figure 3

Bispecific antibodies can mediate T cells to eliminate myeloma cells. (A) Flow cytometry plots of bispecific antibody-mediated cytotoxicity of myeloma over time(24h、48h、72h). **(B)** Flow cytometry plot of the combination of bispecific antibodies mediating the killing of myeloma with the variation of antibody concentration (0.01、0.1、1nM). **(C)** Flow cytometry plots of multiple myeloma killing mediated by bispecific antibody combinations varying with the effector-to-target ratio(2:1,5:1,10:1). **(D)** Dot plots of

the killing of myeloma cells by the three combinations after co-incubation at a concentration of 1nM with a fixed effector-to-target ratio of 5:1 for 48 hours. Four bispecific antibodies constituted three antibody combinations: Combination One is CD3-CD38XCD28-PD-L1, Combination Two is CD3-CD38XCD28-CD38, and Combination Three is CD3-CD38XCD28-SLAMF7. The mortality rate of tumor cells was detected by 7AAD and CFSE staining on a flow cytometer. The CFSE-positive cell population was the target cells, and the proportion of the 7-AAD-positive cell population within this population was the mortality rate of the target cells. Experiment A was repeated only once, with three replicate Wells. The remaining experiments were repeated twice, with three replicate Wells. Data are shown as means ± SD;the p values were calculated by the Two-way ANOVA. *p<0.05, **p<0.01,***p<0.001.

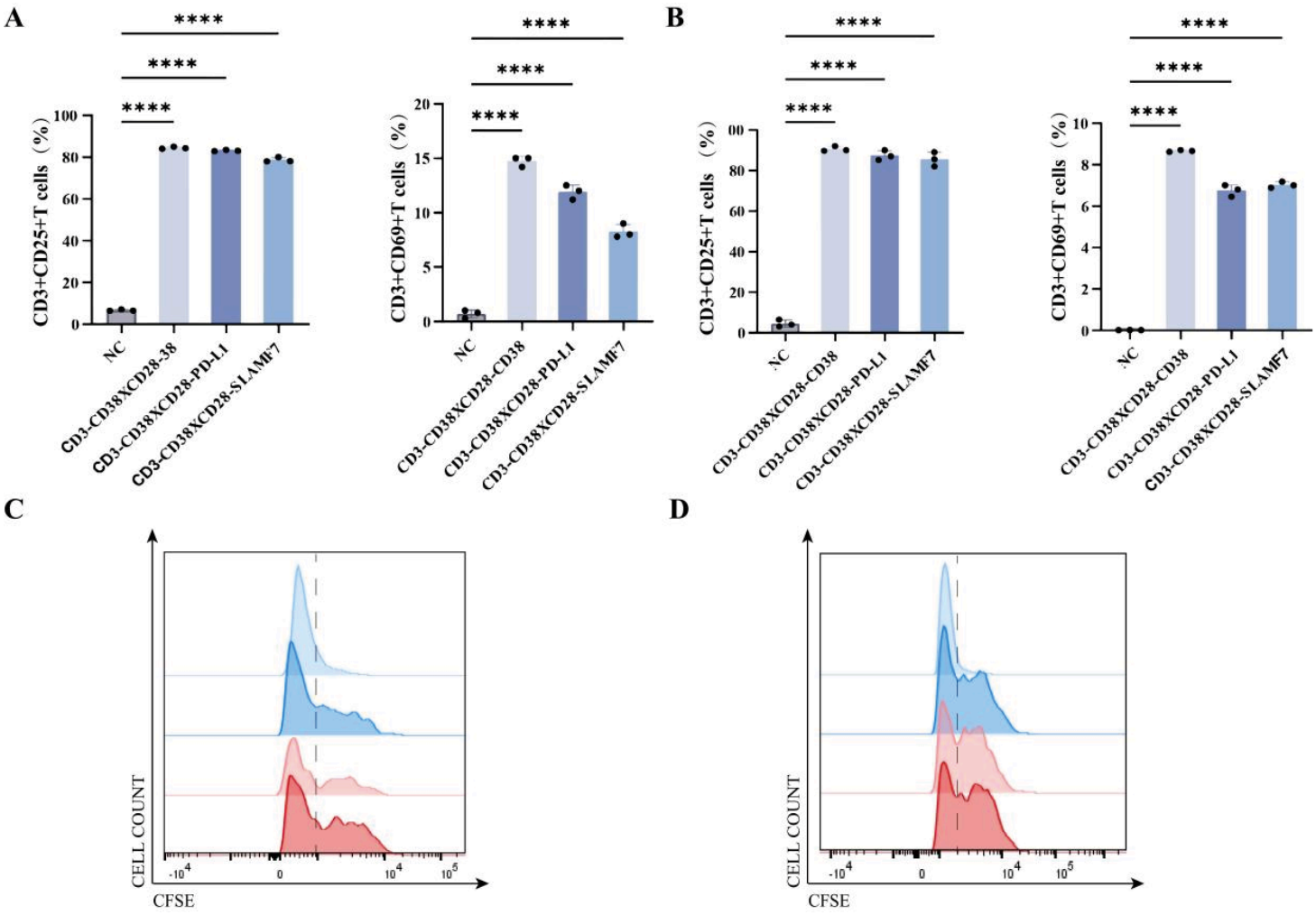


Figure 4

The combination of three bispecific antibodies promotes the proliferation and activation effect of T cells. The percentages of CD25+ (late activation marker) and CD69+ (early activation marker) in CD3+T cells after co-incubation for 48 hours were detected by flow cytometry. **(A)** shows that the combination of bispecific antibodies can mediate the activation of T cells that kill PMMI-8226. **(B)** represents the activation of T cells that mediate the killing of U266 cells.A one-way ANOVA test was used for differences between groups. *P<0.05,**P<0.01,***P<0.001,****P<0.0001 indicated significant differences

compared with the control group. **(C)** The proliferation of T cells in the RMIP-8266 co-culture system. When the effector-to-target ratio was fixed, with the increase of antibody concentration, after co-incubation with 8266 cells for 48 hours, T cell proliferation could be induced, and the higher the concentration, the more obvious the proliferation peak. **(D)** The proliferation of T cells in the U266B1 co-culture system: Due to the low expression of CD38 molecules in U266 cells, after co-incubation with antibodies, the induced proliferation of T cells was not as obvious as that of 8266 cells.

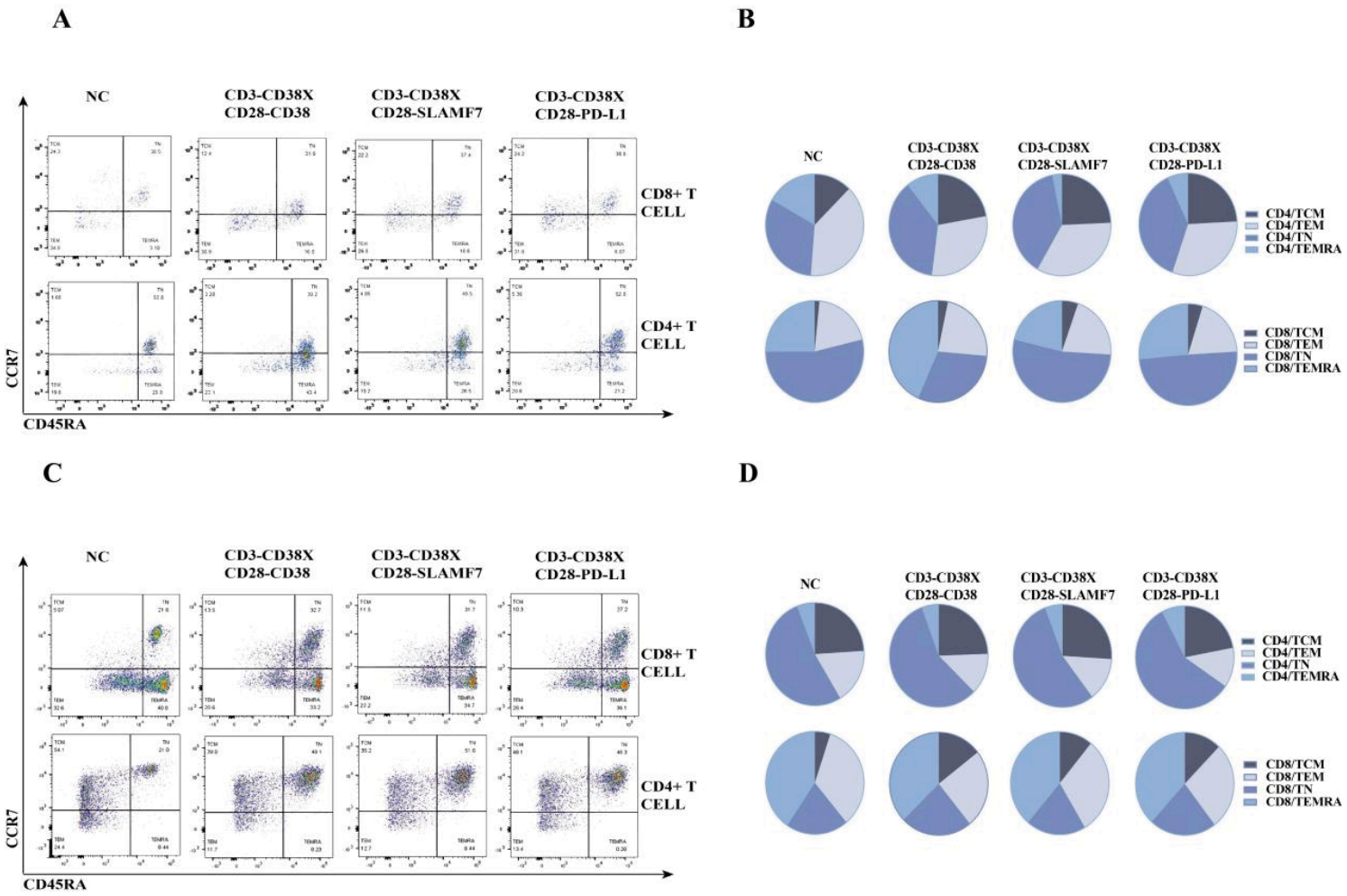


Figure 5

The combination of bispecific antibodies can promote the release of pro-inflammatory factors by T cells. After co-incubation for 48 hours, the concentrations of IFN-γ **(A)**, TNF-α **(B)**, IL-2 **(C)**, and IL-4 **(D)** in the supernatant were detected by ELISA kits. **(E)** After co-incubation for 48 hours, correlation analysis of the abundances of TNF-α, IFN-γ, IL-2, and IL-4 in the supernatant showed $R^2 > 0.99$.

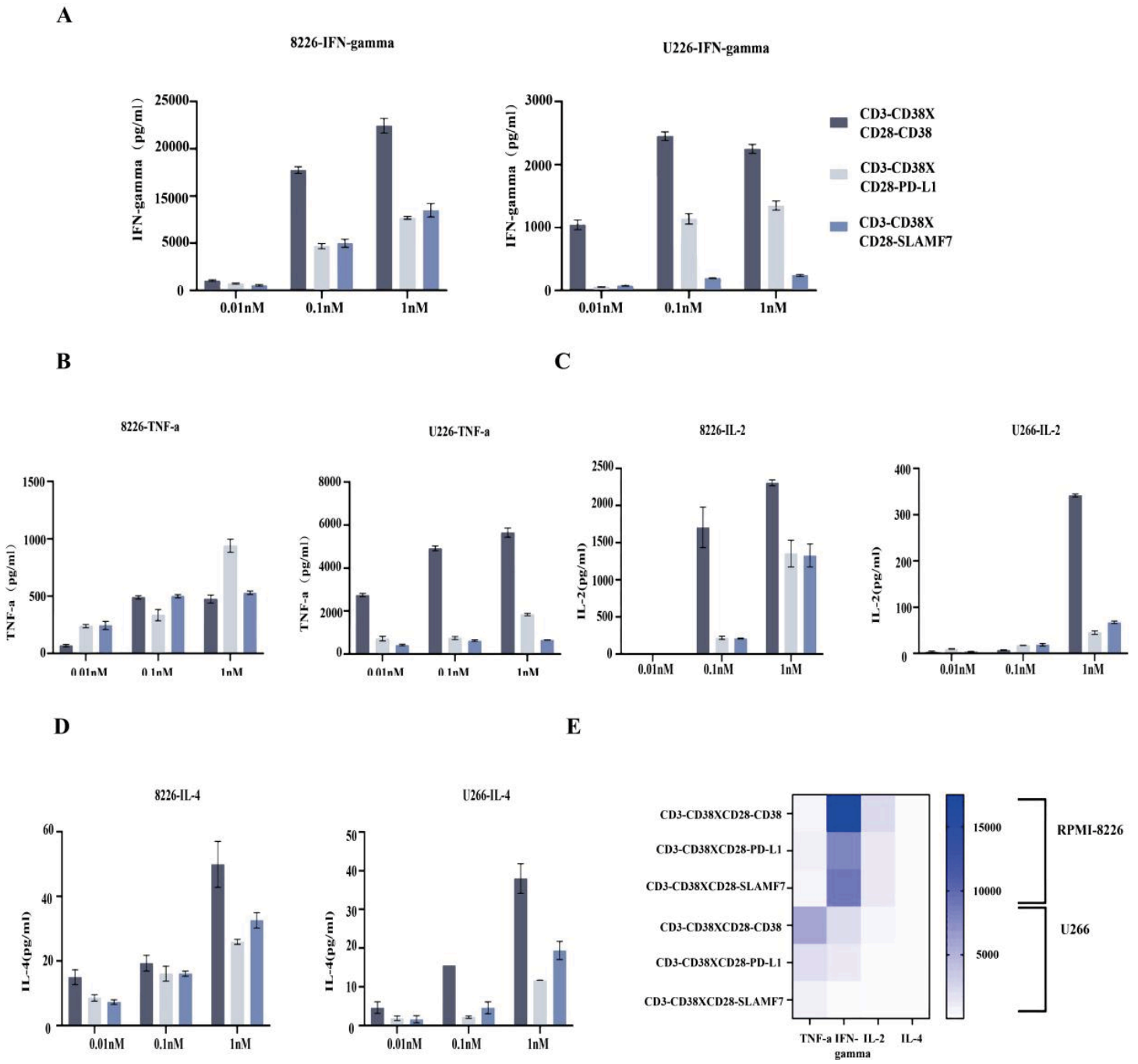


Figure 6

The combination of bispecific antibodies can induce T cell differentiation. (A) Flow cytometry diagrams of T cell differentiation induced by bispecific antibodies in the study of the killing function of PMMI-8226 cells. **(B)**The proportion of T cells differentiated into effector memory T cells and terminal effector memory T cells induced by the bispecific antibody combination CD3-CD38XCD28-CD38 was significantly higher than that of the other two combinations. **(C)**In the study of the killing function against U266 cells,a flow cytometry diagram of T cell differentiation induced by bispecific antibodies. **(D)** The proportion of T cells differentiated into effector memory T cells and terminal effector memory T cells induced by the bispecific antibody combination CD3-CD38XCD28-CD38 was lower than that of the other two combinations.

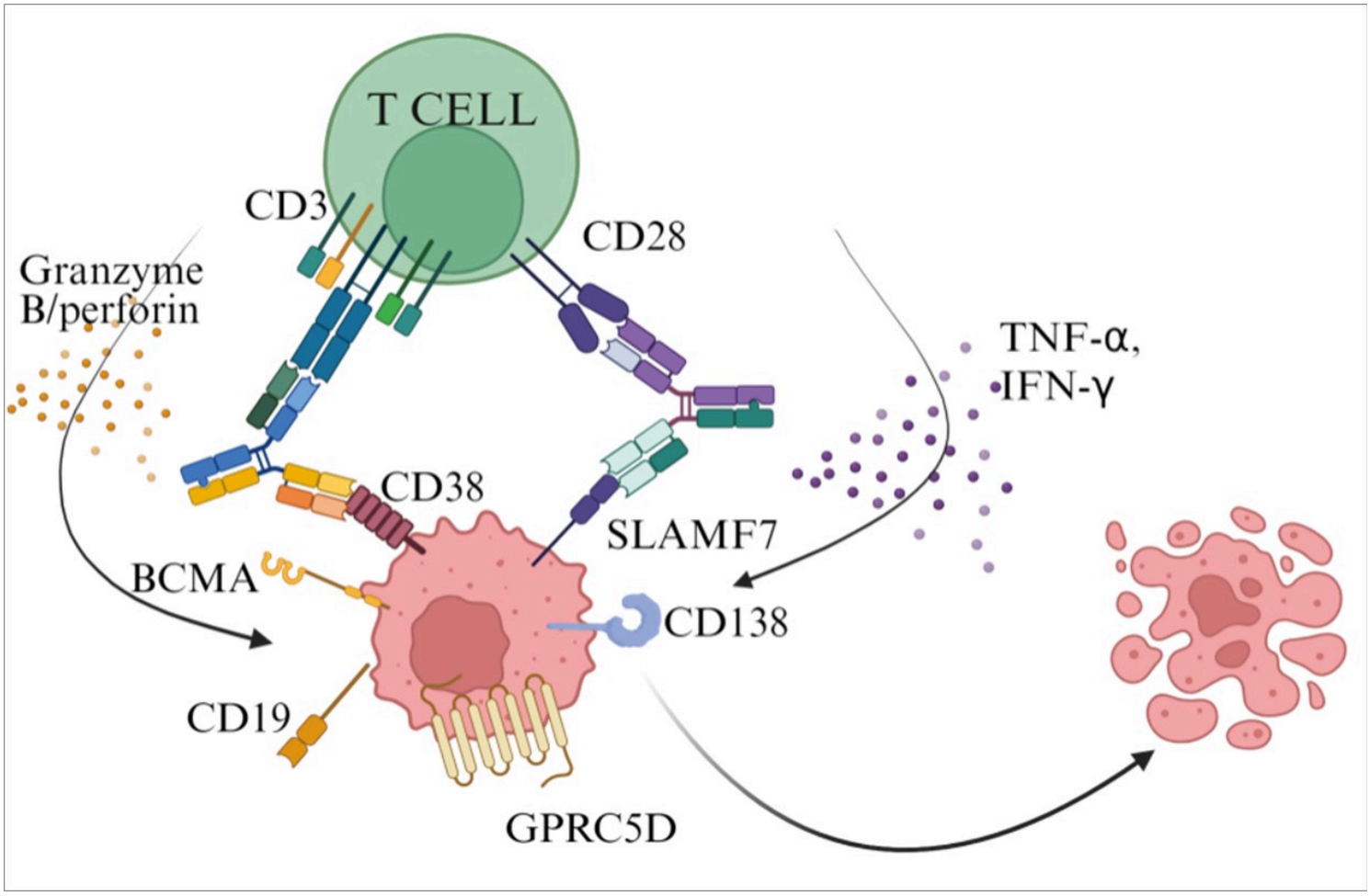


Figure 7

The combination of bispecific antibodies inhibits tumor growth in multiple myeloma xenograft mice. **(A)** The timeline of mouse treatment: The RPMI-8226 cell line in the exponential growth phase was inoculated subcutaneously into the right back of nude mice at a dose of 1×10^7/0.1 ml per mouse to construct a mouse model of multiple myeloma xenograft. After modeling, PBMCs were intravenously injected and administered continuously 5 times. **(B)** Tumor volume before and after administration. The inhibition of subcutaneous tumor volume in mice after administration shows that ,compared with the control group, the combination of the two bispecific antibodies can effectively inhibit tumor volume. **(C)** Comparison of tumor mass in different groups of mice after treatment. After the mice were sacrificed, compared with the control group, the tumor weight in the treatment group was significantly reduced, indicating a significant difference compared with the NC group. **(D)** Activation of T cells in peripheral blood. After 23 days, the peripheral blood of mice was taken to detect activated T cells. Compared with the normal saline group, T cells in both treatment groups were significantly activated. **(E)** After the treatment of mice in different groups, the appearance of the tumors was examined. 23 days later, when the subcutaneous tumors of different groups were removed, it could be clearly seen that ,compared with the control group, the tumor growth was inhibited in the treatment group. **(F-I)** The content of T cells and the secretion level of cytokines in tumor tissues. Some tumor tissues were taken, and the relative expression levels of CD4+, CD8+ T cells, IFN-γ, and TNF-α in the control group and the two treatment

groups were measured. Compared with the control group, the corresponding levels in both treatment groups increased significantly. Differences between groups using One-way ANOVA test * P < 0.05, P < 0.01, * * * * * P < 0.001, * * * * P < 0.0001; (J) Immunohistochemical map of T cell infiltration in tumor tissues. Some tumor tissues were taken ,and the infiltration of CD4+ and CD8+ ,two types of T cells in multiple myeloma tissues in the control group and the treatment group, was detected by immunohistochemistry.

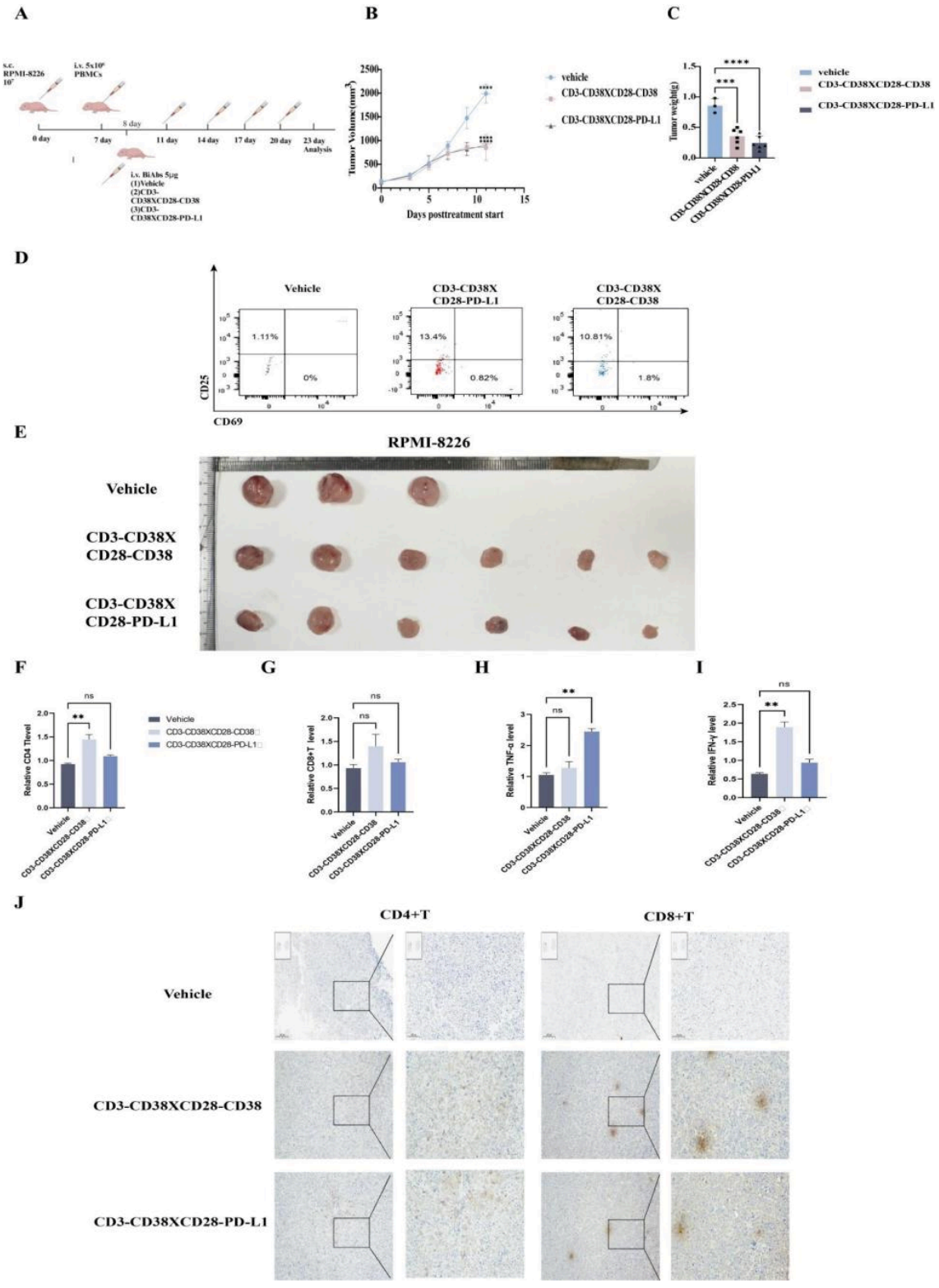


Figure 8

The mechanism of bispecific antibodies in inducing T cell-mediated cytotoxicity against myeloma. BCMA, CD38, GPRC5D, SLAMF7, CD19, and CD138 are among the most commonly expressed surface antigens on myeloma cells. Following intravenous administration, bispecific antibodies distribute systemically and employ their dual binding domains to simultaneously engage CD3 or CD28 on T cells and specific target antigens on myeloma cells. This interaction forms a physical bridge between T cells and tumor cells, thereby facilitating the recruitment of T cells to the vicinity of malignant plasma cells. The co-engagement of CD3 and CD28 mimics physiological T-cell receptor signaling, resulting in robust and sustained T-cell activation. Activated T cells subsequently release key cytotoxic effector molecules, including granzyme and perforin, as well as immunomodulatory cytokines such as interferon-γ, which collectively enhance the antitumor immune response. This cascade of events ultimately triggers apoptosis in myeloma cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TNFXXXXXqPCRdata.xlsx
- CD4TcellXXXqpcrdata.xlsx
- CD8TcellXXXqpcrdata.xlsx
- XXXXXX1.jpg
- westernblotdata.png
- IFNgammaXXXqpcrdata.pdf