Harnessing the cytotoxic granule exocytosis to augment the efficacy of T-cell-engaging bispecific antibody therapy

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Abstract

T-cell-engaging bispecific antibody (T-BsAb, also known as BiTE) therapy has emerged as a powerful therapeutic modality against multiple myeloma. Given that T-BsAb therapy redirects endogenous T cells to eliminate tumor cells, reinvigorating dysfunctional T cells may be a potential approach to improve the efficacy of T-BsAb. While various immunostimulatory cytokines can potentiate effector T-cell functions, the optimal cytokine treatment for T-BsAb therapy is yet to be established, partly due to a concern of cytokine release syndrome driven by aberrant interferon (IFN)-γ production. Here, we functionally screen immunostimulatory cytokines to determine an ideal combination partner for T-BsAb therapy. This approach reveals interleukin (IL)-21 as a potential immunostimulatory cytokine with the ability to augment T-BsAb-mediated release of granzyme B and perforin, without increasing IFN-γ release. Transcriptome profiling and functional characterization strongly support that IL-21 selectively targets the cytotoxic granule exocytosis pathway, but not pro-inflammatory responses. Notably, IL-21 modulates multiple steps of cytotoxic effector functions including upregulation of co-activating CD226 receptor, increasing cytotoxic granules, and promoting cytotoxic granule delivery at the immunological synapse. Indeed, T-BsAb-mediated myeloma killing is cytotoxic granule-dependent, and IL-21 priming significantly augments cytotoxic activities. Furthermore, *in vivo* IL-21 treatment induces cytotoxic effector reprogramming in bone marrow T cells, showing synergistic anti-myeloma effects in combination with T-BsAb therapy. Together, harnessing the cytotoxic granule exocytosis pathway by IL-21 may be a potential approach to achieve better responses by T-BsAb therapy.

Introduction

Multiple myeloma, a plasma cell neoplasm, has historically been recognized as an incurable disease. Over the past decade, the treatment landscape of multiple myeloma has dramatically evolved with T-cell redirection immunotherapies such as T-cell-engaging bispecific antibody (T-BsAb, also known as BiTE) therapy and chimeric antigen receptor (CAR) T-cell therapy.[1-3] T-BsAb therapy can redirect endogenous polyclonal T cells to recognize and eliminate tumor cells by simultaneously engaging target antigens on tumor cells and CD3 on T cells. As the cancer-immunity cycle is frequently dysregulated in multiple myeloma,[4] T-BsAb therapy is recognized as a potential approach to stimulate adaptive immunity to control multiple myeloma. Recently, T-BsAb therapies targeting B-cell maturation antigen (BCMA), G-protein coupled receptor family C group 5 member D, and Fc Receptor-Like 5 have shown impressive clinical responses in heavily pretreated patients with relapsed/refractory multiple myeloma.[1,2,5]

Despite impressive clinical responses, treatment failure and relapse remain major obstacles. Still, it remains largely unknown how we can overcome therapeutic resistance mediated by loss of target antigen, immunosuppression, and T-cell hyporesponsiveness.[4,6,7] Given that T-BsAb stimulate endogenous T cells and that T cells are functionally impaired in the myeloma bone marrow (BM),[4,8-10] reprogramming dysfunctional T cells might be necessary to achieve better disease control by T-BsAb therapy. Indeed, in a recent preclinical study, we showed that priming of cytotoxic lymphocytes by innate immune activation can improve T-BsAb efficacy, supporting the importance of harnessing effector T-cell functions prior to T-BsAb therapy.[11] Immunostimulatory cytokines including common γ-chain

cytokines (IL-2, IL-7, IL-15, and IL-21) and interferon (IFN)-γ-inducing cytokines (IL-12 and IL-18) are recognized as potent adjuvants for cancer immunotherapy by their ability to support either T-cell proliferation or modulating effector functions.[12-14] Currently, therapeutic administration of these recombinant cytokines is widely being tested in combination with immune checkpoint inhibitors and other immunotherapy.[12] While cytokine-based therapy can be a possible combination partner for T-cell redirection therapy, overstimulation of T cells can drive activation-induced cell death and increase the risk of severe immune-related adverse events, such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome. Activated T cells release pro-inflammatory cytokines including IFN-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), all of which are known to fuel the vicious cycle of CRS by stimulating monocytes/macrophages to produce IL-1β and IL-6.[15-17] Above all, IFN-γ plays a crucial role in the immunopathology of CRS, as blockade or deletion of IFN-γ can ameliorate cytokine-related toxicities in CAR T-cell therapy[18,19] By contrast, the perforin/granzyme-mediated cytotoxic granule pathway negatively regulates macrophage activation syndrome (MAS) and hemophagocytic lymphohistiocytosis (HLH) by killing activated monocytes/macrophages. Indeed, familial HLH is seen in patients with mutations in the perforin gene (*PRF1*) or genes related to cytotoxic granule exocytosis.[20,21] More recently, Ishii *et al.* showed that adoptive transfer of perforin-deficient CAR T cells, but not wild-type CAR T cells, triggers the MAS/HLH-like preclinical phenotype with the excessive release of IL-1 family cytokines.[22] These results provide evidence that the cytotoxic granule pathway negatively regulates aberrant macrophages inflammatory responses during T-cell redirection immunotherapy. Importantly, accumulated evidence supports that IFN-γ is dispensable for the efficacy of CAR T therapy against hematological malignancies, but not solid malignancies.[18,19,23] Thus, we hypothesize that a cytokine-based therapy selectively harnessing the cytotoxic granule pathway might be an ideal strategy to achieve better responses by T-BsAb therapy. By performing a functional screening of immunostimulatory cytokines, this study aims to determine the optimal approach for cytokine-mediated priming of effector T cells and to establish proof-of-concept for improving the efficacy of T-BsAb therapy against multiple myeloma.

Methods

Details on experimental procedures and materials are included in the *Online Supplementary Appendix*.

Multiple myeloma cell lines

Human multiple myeloma cell lines (JJN-3 cells, KMS-11 cells, and RPMI8226)[24] were cultured in RPMI1640 media supplemented with sodium pyruvate, non-essential amino acids, penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum (complete RPMI [cRPMI]) Luciferase was transduced by retrovirus transfection of the MSCV-mCherry-IRES-luc2 vector, as described previously.[25] Mycoplasma negativity was confirmed by the Mycoplasma Detection Kit. The transplantable Vk14451 myeloma cells expressing enhanced green fluorescent protein (EGFP) were maintained as previously described.[11,26]

Primary cells and clinical samples

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors using Lymphoprep™ density gradient medium. CD8 T cells were magnetically isolated by EasySep™ Human CD8+ T Cell Isolation Kit. Clinical samples from patients with newly diagnosed multiple myeloma were collected at the Princess Alexandra Hospital, Brisbane. The study was approved by the QIMR Berghofer Human Research Ethics Committee (P2125) and by the Metro South Hospital and Health Service Human Research Ethics Committee (9448).

Screening assays

PBMC (4×10^5) from healthy donors and patients with newly diagnosed multiple myeloma were co-cultured with JJN-3 myeloma cells (1×10^5) in the presence of anti-BCMA T-BsAb (0.1 μg/mL) and one of the following recombinant human immunostimulatory cytokines: rIL-2 (500 U/mL), rIL-7, rIL-12, rIL-15, rIL-18 and rIL-21 (50 ng/mL). The levels of granzyme B, perforin, IFN-γ, TNF-α, and GM-CSF in culture supernatants were determined by enzyme-linked immunosorbant assay (ELISA) 3 days after co-culture. Results were calculated as fold changes relative to control (T-BsAb stimulation in the absence of immunostimulatory cytokines).

T-cell-engaging bispecific antibody assays

For T-cell proliferation assays, PBMC were labeled with CellTrace™ Violet (CTV) and co-cultured with JJN-3 myeloma cells (1×10^5) in cRPMI supplemented with recombinant IL-2 (rIL-2, 100 U/mL) at the effector-to-target ratio (E: T ratio) of 4:1. These cells were stimulated with the indicated concentrations of rIL-21 and anti-BCMA T-BsAb for 3 days, and CTV-dilution in live CD8 T cells was measured by flow cytometry. In some experiments, unlabeled PBMC were used to examine expression levels of CD226 and CD28 in CD8 T cells. The levels of cytotoxic granules (granzyme B and perforin) and cytokines (IFN-γ, TNF-α, and GM-CSF) in culture supernatants were measured by ELISA. For *ex vivo* stimulation in primary samples, bulk bone marrow (BM) mononuclear cells were reconstituted in cRPMI supplemented with rIL-2 (50 U/mL) (8×10^5/mL). These cells were stimulated with anti-BCMA T-BsAb (0.2 μg/mL) in the presence or absence of rIL-21 (100 ng/mL) for 3 days.

In vivo treatment

C57BL/6 wild-type mice were bred and maintained in house. Mice were intravenously challenged with 2×10^6 Vk14451 cells expressing EGFP. Tumor-bearing mice with paraproteinemia were intraperitoneally (i.p.) treated with recombinant murine IL-21 (100 μg, i.p.), according to a dosing schedule reported previously.[27,28] In some experiments, tumor-bearing mice were treated with anti-mouse BCMA T-BsAb (25 μg, i.p.) either alone or in combination with rIL-21. Mice were monitored over time for clinical endpoints. All experiments were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (P3533).

Statistical analysis

Statistical differences were tested with GraphPad Prism 9 software. For comparisons between two groups, a Mann-Whitney U test or paired *t* test was used. For multiple comparisons, a Friedman test, a repeated measures ANOVA, or Kruskal-Wallis test was used, followed by indicated *post hoc* tests. Differences in survival were evaluated with a Mantel-Cox test. $P<0.05$ was considered to be statistically significant (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$).

Results

Functional screening of immunostimulatory cytokines to determine an ideal combination partner for T-cell-engaging bispecific antibody therapy

We sought to identify an immunostimulatory cytokine regulating the cytotoxic granule pathway with negligible impacts on the production of IFN-γ. To this end, PBMC from five healthy donors and five patients with newly diagnosed multiple myeloma were co-cultured with JJN-3 myeloma cells in the presence of anti-BCMA T-BsAb and one of the following immunostimulatory cytokines: rIL-2, rIL-7, rIL-12, rIL-15, rIL-18, or rIL-21 (Figure 1A). After 3 days of co-culture, levels of effector molecules in culture supernatants were determined. The release of granzyme B was enhanced by the addition of rIL-12, rIL-18, or rIL-21, whereas a significant increase in perforin levels was only seen by rIL-21 (Figure 1B, C). Consistent with the ability to drive type 1 immune responses,[29] both rIL-12 and rIL-18 dramatically augmented the release of IFN-γ (Figure 1D). However, notably, treatment with rIL-21 showed a negligible impact on IFN-γ production (Figure 1D). Moreover, treatment with rIL-21 did not increase levels of TNF-α and GM-CSF (Figure 1E, F). These results suggest that rIL-21 might be a potential adjuvant for enhancing the cytotoxic granule pathway, but not pro-inflammatory responses.

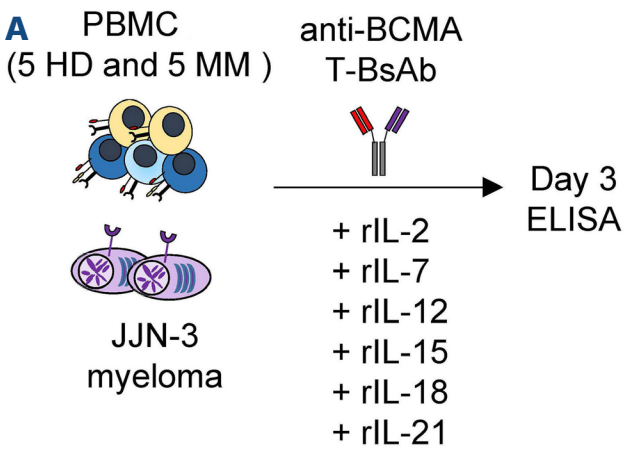
Dose-dependent effects of IL-21 on CD8 T cells stimulated with T-cell-engaging bispecific antibody

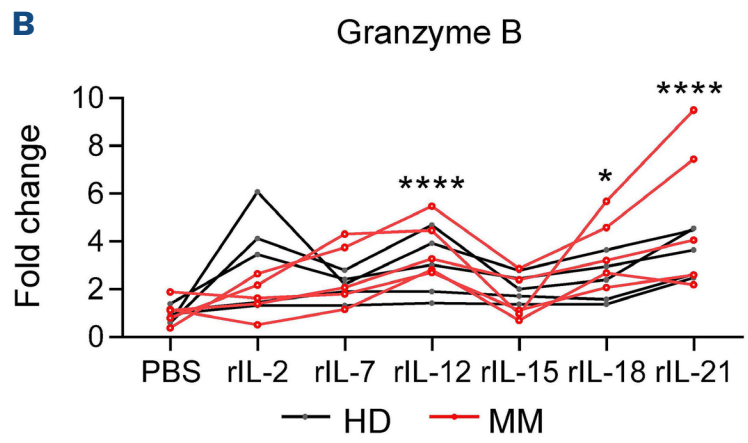
In order to validate the results from the immunostimulatory cytokine screening, we examined the dose-dependent effects of rIL-21 on T-BsAb-induced cytokine production from eight different healthy donor-derived PBMC. Indeed, treatment with rIL-21, at 20 ng/mL or higher, significantly increased the release of granzyme B and perforin without increasing levels of pro-inflammatory cytokines IFN-γ, TNF, and GM-CSF (Figure 2A). Intriguingly, levels of IFN-γ and GM-CSF were modestly but significantly reduced in the presence of rIL-21 (Figure 2A), further supporting the differential impact of IL-21 on T-BsAb-induced release of cytotoxic granules and pro-inflammatory cytokines.

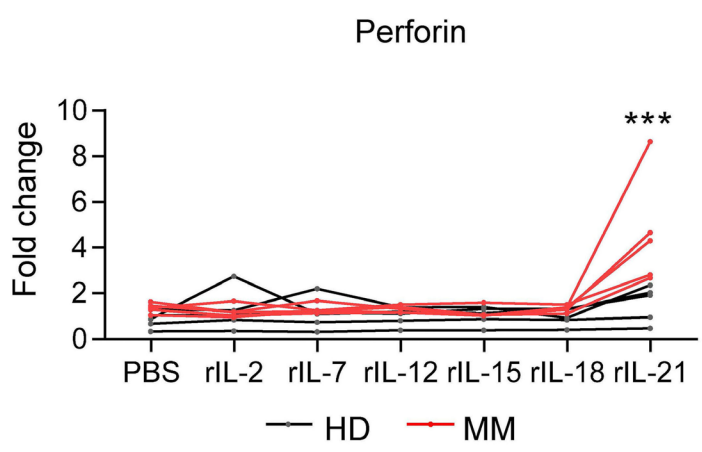
Next, we addressed whether rIL-21 could modulate the activation of CD8 T cells. The upregulation of an early activation marker CD69 was detectable on CD8 T cells 5 hours after T-BsAb stimulation, especially at high concentrations of T-BsAb (Figure 2B). Frequencies of CD69-expressing CD8 T cells significantly increased in the presence of rIL-21, even at suboptimal concentrations of T-BsAb, suggesting that treatment with rIL-21 lowered the activation threshold in CD8 T cells (Figure 2B). By contrast, treatment with rIL-21 showed negligible impacts on T-BsAb-mediated proliferation (Figure 2C). Intriguingly, treatment with rIL-21 upregulates the expression levels of CD226 and CD28, key receptors for tumor recognition and co-stimulation, respectively (Figure 2D).[30-32] Together, these results suggest that IL-21 modulates selective effector functions, rather than broadly stimulates CD8 T cells.

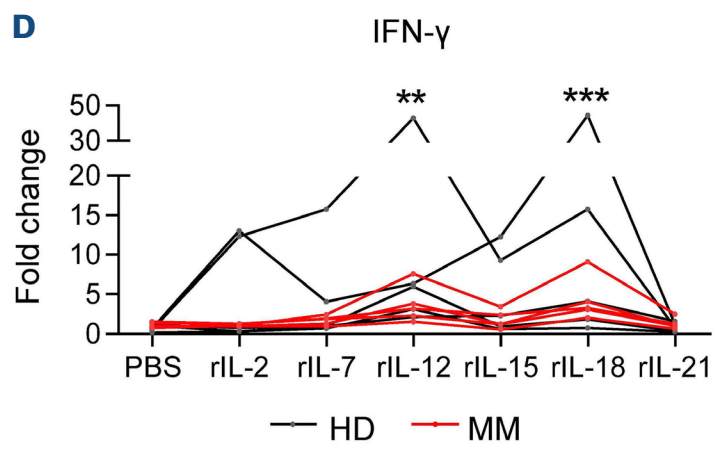
Treatment with IL-21 transcriptionally regulates the cytotoxic granule pathway in CD8 T cells stimulated with T-cell-engaging bispecific antibody

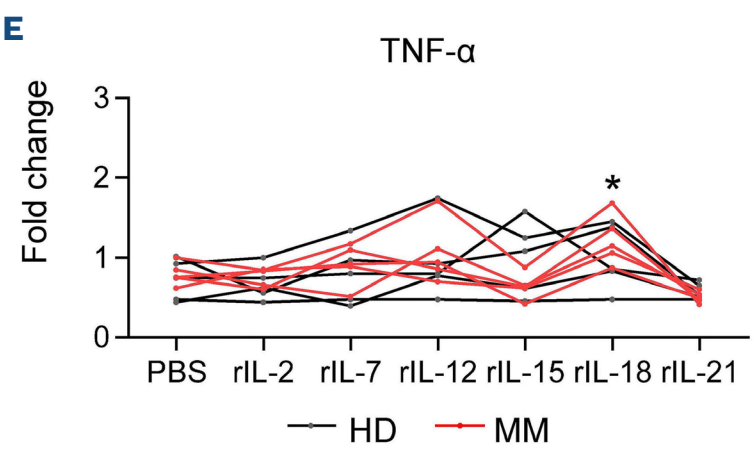
It is appreciated that inflammatory cytokines act as a third signal for functional maturation of CD8 T cells.[29] In order to obtain a comprehensive profiling of effector programs regulated by IL-21, we performed bulk RNA sequencing (RNA-seq) of T-BsAb-stimulated CD8 T cells from six healthy donors in the absence (control) or presence of rIL-21 using a paired design (Figure 3A; *Online Supplementary Figure 1A*; *Online Supplementary Table S1*). Principal components analysis (PCA) revealed samples clustered by donor along principal component (PC) 1 and PC2, whilst PC2 *versus* PC3 separated control CD8 T cells and IL-21-primed CD8 T cells (Figure 3B). Differential expression analysis between IL-21-primed and control CD8 T cells identified 2,609 differentially expressed genes, of which 1,280 were upregulated, and 1,329 were downregulated (false discovery rate <0.05; *Online Supplementary Table S2*). Reactome pathway analysis of the genes upregulated in IL-21-primed CD8 T cells revealed enrichment of neutrophil degranulation (granule exocytosis) as well as Rho and other GTPase pathways (Figure 3C; *Online Supplementary Table S3*), key signaling pathways for cytoskeletal reorganization in cytotoxic T cells.[33] By contrast, pro-inflammatory pathways such as IFN, TNF, and IL-1 signaling pathways were enriched based on Reactome pathway analysis of the genes downregulated in IL-21-primed CD8 T cells (*Online Supplementary Figure*











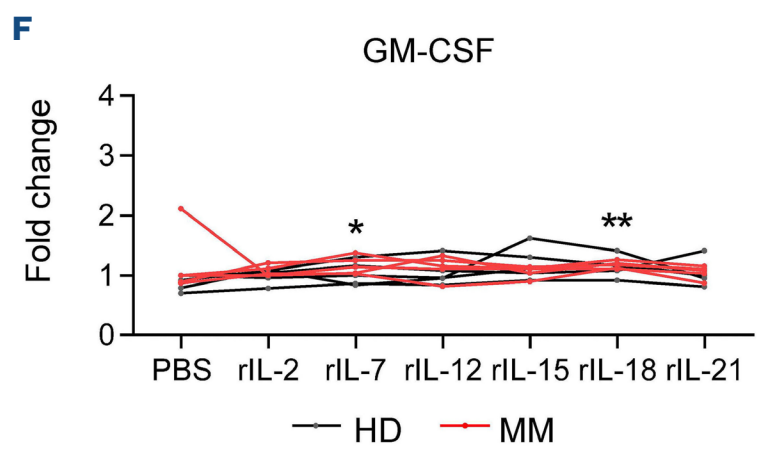


Figure 1. Functional screening of immunostimulatory cytokines to determine an ideal combination partner for T-cell-engaging bispecific antibody therapy. (A) Schematic illustrates the screening of immunostimulatory cytokines. Peripheral blood mononuclear cells (PBMC, 4×10^5) from healthy donors (HD, N=5) and patients with multiple myeloma (MM, N=5) were co-cultured with JJN-3 myeloma cells (1×10^5) in the presence of T-cell-engaging bispecific antibody targeting B-cell maturation antigen (anti-BCMA T-BsAb, 0.1 μg/mL) and each of following recombinant immunostimulatory cytokines: recombinant interleukin-2 (rIL-2) (500 U/mL), rIL-7, rIL-12, rIL-15, rIL-18 and rIL-21 (50 ng/mL). (B-F) Individual graphs showing the levels of granzyme B (B), perforin (C), interferon-γ (IFN-γ) (D), tumor necrosis factor-α (TNF-α) (E), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (F) in culture supernatants 3 days after stimulation, determined by enzyme-linked immunosorbant assay (ELISA). Data are shown as fold changes, relative to T-BsAb stimulation without cytokine treatment. Results from 1 experiment are shown, and differences were tested for statistical significance using a Friedman test with a Dunn's multiple comparisons test. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

S1B; Online Supplementary Table S4), supporting the cytotoxic granule pathway as a key target of IL-21. Indeed, genes encoding cytotoxic granules (*GZMB, GZMH, NKG7*, and *GNLY*) were significantly increased in IL-21-primed CD8 T cells (Figure 3D; *Online Supplementary Table S2*). Together, IL-21 transcriptionally induces the cytotoxic effector program in CD8 T cells.

IL-21 priming augments cytotoxic granule exocytosis and myeloma-killing activity by T-cell-engaging bispecific antibody

The results from transcriptome profiling strongly suggest that IL-21 priming could enhance cytotoxic granule-dependent tumor-killing activities. Thus, we assessed T-BsAb-mediated cytotoxic activities of CD8 T cells primed with or without rIL-21 (Figure 4A). Indeed, IL-21 priming markedly augmented T-BsAb-mediated cytotoxicity against three

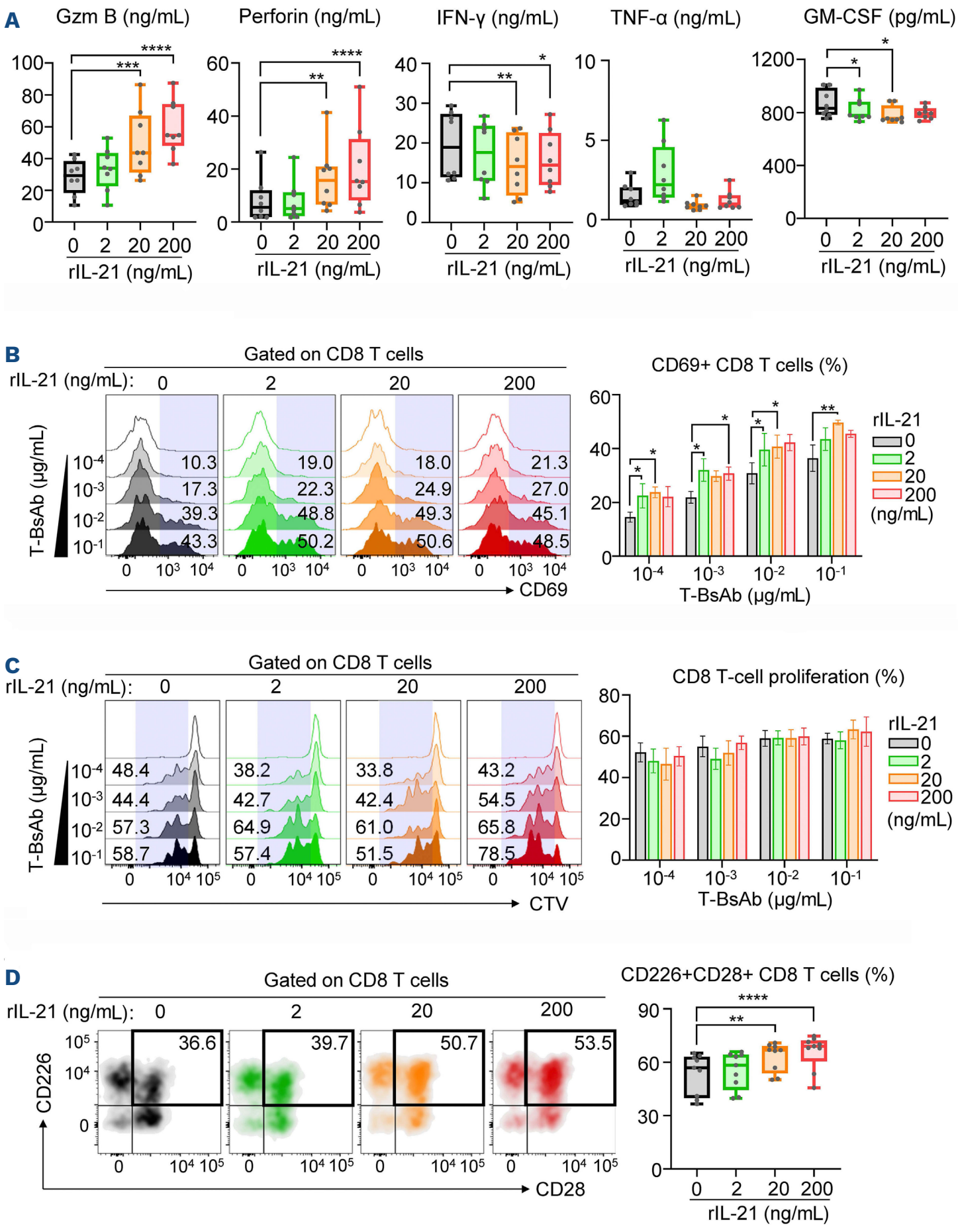


Figure 2. Treatment with recombinant IL-21 augments cytotoxic granule release without affecting the production of pro-inflammatory cytokines. (A) Peripheral blood mononuclear cells from healthy donors and JJN-3 myeloma were co-cultured for 3 days in the presence of T-cell-engaging bispecific antibody targeting B-cell maturation antigen (anti-BCMA T-BsAb) and indicated concentrations of recombinant interleukin-21 (rIL-21). Box-and-whisker plots showing levels of indicated effector proteins and cytokines in culture supernatants (N=8). (B) Representative histograms (left) and bar graphs (right), showing expression levels of CD69 in CD8 T cells 5 hours after stimulation with indicated concentrations of anti-BCMA T-BsAb and rIL-21. Data are shown as mean ± standard error of mean (N=5). (C) CTV-labeled peripheral blood mononuclear cells and JJN-3 myeloma cells were co-cultured for 3 days in the presence of indicated concentrations of anti-BCMA T-BsAb and rIL-21. Representative histograms (left) and bar graphs (right) showing CD8 T-cell proliferation (N=4). Data are shown as mean ± standard error of mean. (D) Representative histograms (left) and box-and-whisker plots (right) showing expression levels of CD28 and CD226 in CD8 T cells after 3 days of co-culture in the presence of T-BsAb (0.1 µg/mL) and indicated concentrations of IL-21 (N=9). Data were pooled from 2 experiments, and differences were tested for statistical significance using a repeated measures ANOVA with a *post hoc* Dunnett's multiple comparisons test. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. CTV: CellTrace™ Violet; Gzm B: granzyme B; INF-γ: interferon-γ; TNF-α: tumor necrosis factor-α.

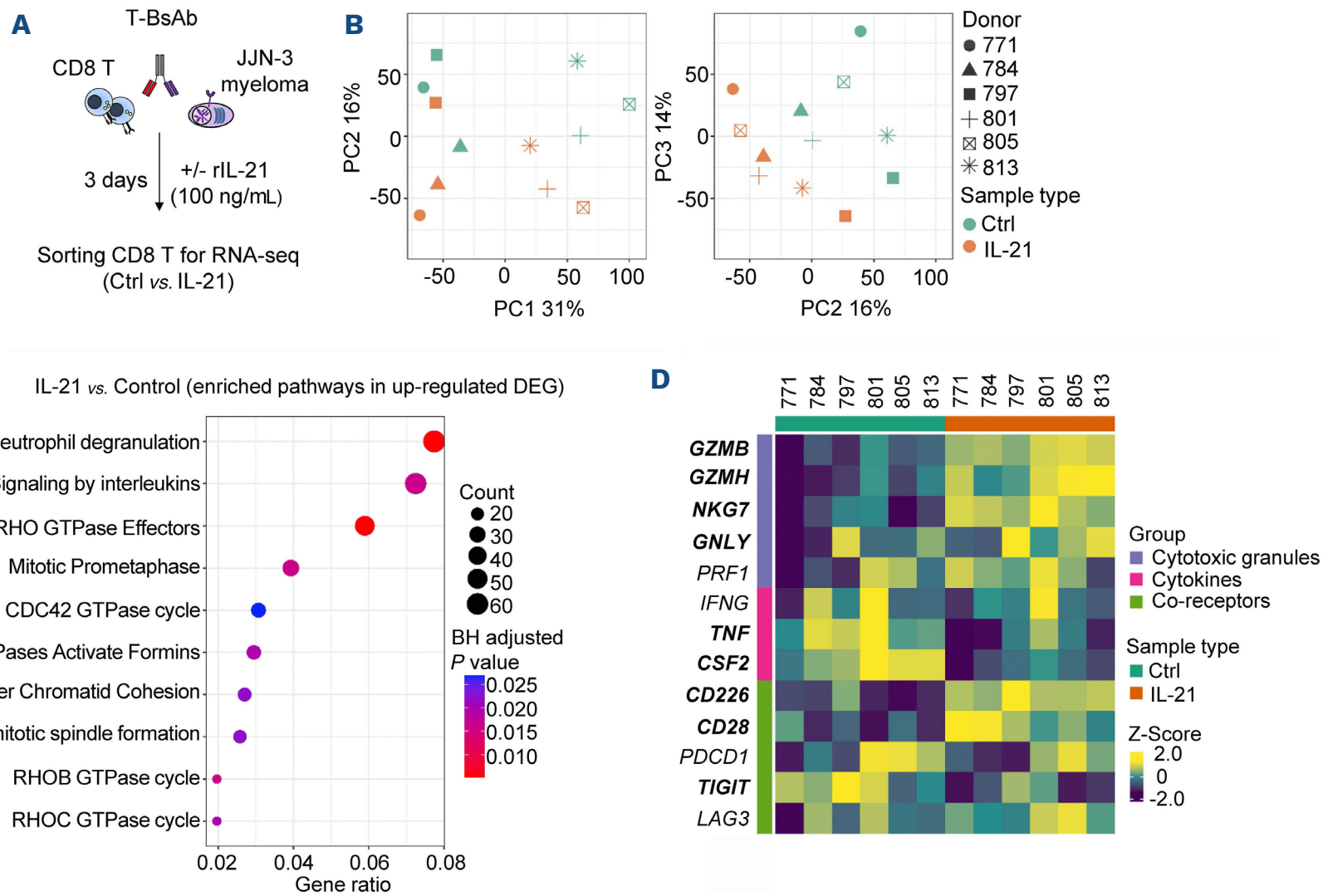


Figure 3. IL-21 transcriptionally modulates the cytotoxic granule exocytosis pathway. (A) Isolated CD8 T cells were co-cultured with JJN-3 in the presence of T-cell-engaging bispecific antibody targeting B-cell maturation antigen (anti-BCMA T-BsAb, 0.1 µg/mL) with or without recombinant interleukin-21 (rIL-21, 100 ng/mL), followed by sorting of CD8 T cells for RNA sequencing (RNA-seq). A schematic illustrates the sample preparation. (B) Principal component analysis (PCA) of log2-transformed normalized protein-coding genes across all samples. Principal component (PC) 1 *versus* PC2 (left) and PC2 *versus* PC3 (right) of the scaled and centered protein-coding genes from 6 different donors colored by sample type. (C) Enriched Reactome pathways in differentially expressed genes (DEG) significantly upregulated in IL-21-primed CD8 T cells compared with control CD8 T cells. (D) Heatmap showing z-scored mRNA abundances of genes related to cytotoxic granules (*GZMB, GZMH, NKG7, GNLY*, and *PRF1*), pro-inflammatory cytokines (*IFNG, TNF*, and *CSF2*), and co-receptor (*CD226, CD28, PCDC1, TIGIT, LAG3*). DEG are indicated in bold text. Ctrl: control.

myeloma cell lines, JJN-3, RPMI8226, and KMS-11 (Figure 4B). Target myeloma cells expressed IL-21 receptor; however, treatment with rIL-21 in these cells showed negligible impacts on expression levels of BCMA and ligands for co-receptors in tumor cells (*Online Supplementary Figure S2*). While we observed that the expression level of CD226 was upregulated by rIL-21 (Figure 2D), anti-CD226 blocking monoclonal antibody (mAb) did not inhibit T-BsAb-mediated cytotoxicity (Figure 4C), suggesting that the enhanced *in vitro* cytotoxic activity was explained by other mechanisms. Importantly, T-BsAb-mediated tumor-killing activities were not ameliorated by anti-IFN-γ neutralizing mAb, regardless of IL-21 treatment (Figure 4C). By contrast, pretreatment with a perforin inhibitor, concanamycin A, largely abrogated cytotoxic activities in both control CD8 T cells and IL-21-primed CD8 T cells, indicating that the T-BsAb-mediated myeloma-killing activity was cytotoxic granule-dependent (Figure 4C). Additionally, IL-21-primed CD4 T cells showed upregulation of cytotoxic granules and enhanced myeloma-killing activities (*Online Supplementary Figure S3*). Thus, IL-21 induces the cytotoxic program in both CD8 T cells and CD4 T cells.

The cytotoxic immunological synapse acts as a molecular platform, triggering dynamic cytoskeletal reorganization and delivery of cytotoxic granules via exocytosis.[34] Given that granule exocytosis and signaling pathways related to cytoskeletal reorganization were transcriptionally upregulated by IL-21 (Figure 3C), we next evaluated whether treatment with rIL-21 could promote immunological synapse formation and degranulation. Indeed, IL-21-primed CD8 T cells more rapidly expressed the degranulation marker CD107a, compared to control CD8 T cells (Figure 4D). The polarization of microtubule-organizing center (MTOC) toward the contact site is recognized as a crucial step for delivering cytotoxic granules.[34] In IL-21-primed CD8 T cells, rapid MTOC polarization was observed after T-BsAb stimulation (Figure 4E, F). Together, IL-21 priming promotes the cytotoxic granule exocytosis pathway.

IL-21 treatment enhances the *in vivo* efficacy of T-cell-engaging bispecific antibody therapy by modulating cytotoxic T-cell phenotypes in the myeloma bone marrow

In order to further understand the impact of rIL-21 treatment on T-cell phenotypes *in vivo*, we used the Vk14451 myeloma preclinical model.[11] Tumor-bearing mice with paraproteinemia were treated with rIL-21, according to a published dosing schedule[27] (Figure 5A). Treatment with rIL-21 showed negligible impacts on tumor burden (Figure 5B) and the number of immune subsets in the myeloma BM (Figure 5C; *Online Supplementary Figure S4*). However, circulating levels of granzyme B, but not IFN-γ, were significantly increased by rIL-21 treatment (Figure 5D). The downregulation of co-receptors CD226 has been recognized as a key feature of dysfunctional T cells in patients with multiple myeloma.[30,31] Importantly, rIL-21 treatment significantly increased BM CD8 T cells and

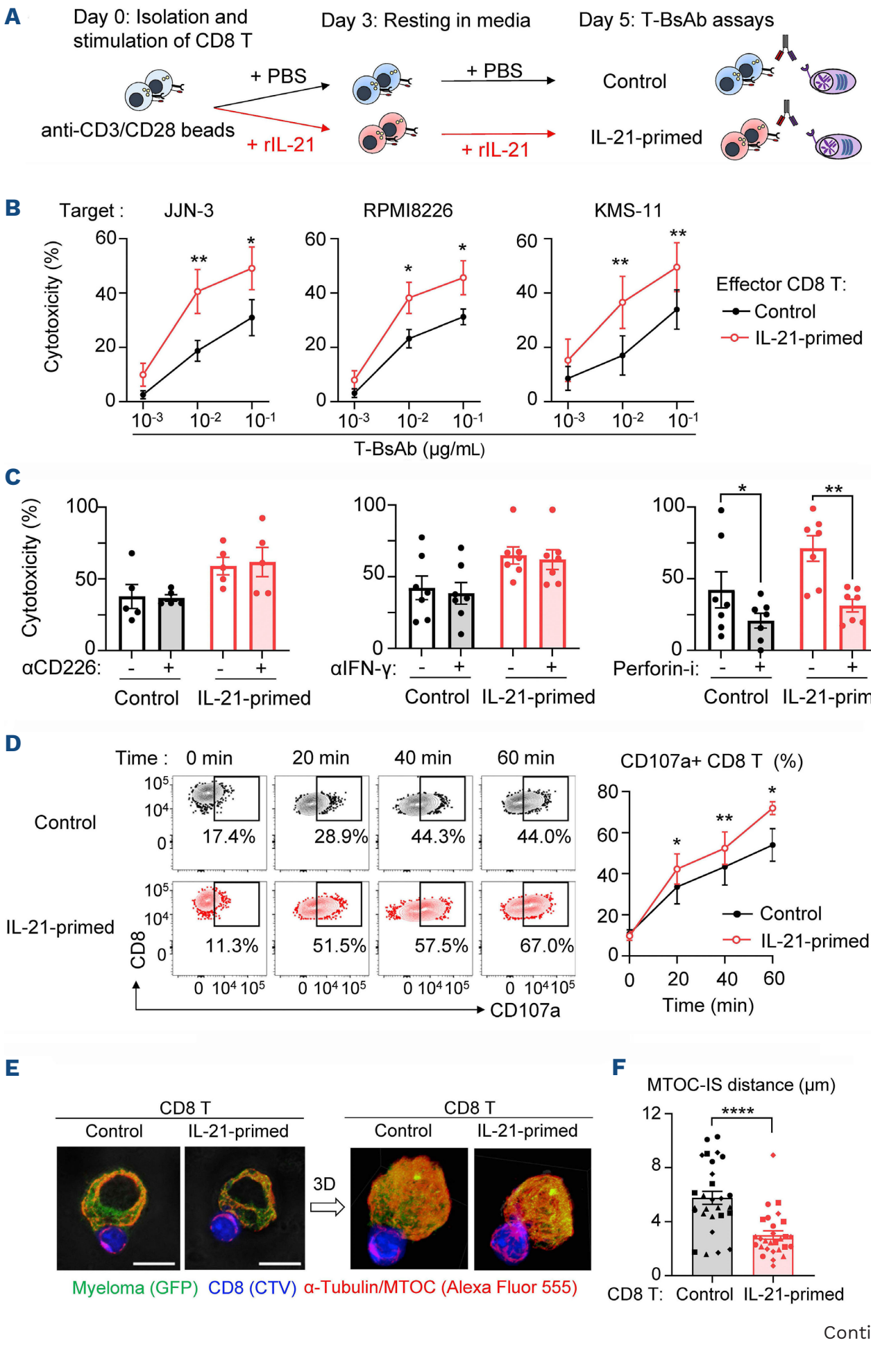


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Figure 4. Priming by IL-21 augments granule-dependent myeloma-killing activity. (A) Isolated CD8 T cells were stimulated with anti-CD3/CD28 beads in the presence or absence of recombinant interleukin-21 (rIL-21), followed by maintenance for 2 days without anti-CD3/CD28 beads. After the washout of rIL-21, these effector CD8 T cells were used for assays. Schematic illustrates the preparation of control and IL-21-primed CD8 T cells. (B) CD8 T cells were co-cultured with target myeloma cells expressing luciferase at a 2:1 effector to target cell ratio (E: T ratio) for 4 hours. Graphs showing target cytotoxicity with different concentrations of T-cell-engaging bispecific antibody targeting B-cell maturation antigen (anti-BCMA T-BsAb) (N=7). (C) CD8 T cells were co-cultured with RPMI8226 cells at a 3:1 E: T ratio in the presence of anti-BCMA T-BsAb (0.1 μg/mL). Graphs showing the impact of anti-CD226 blocking monoclonal antibody (mAb, 5 μg/mL) (left), anti-interferon-γ (IFN-γ) neutralizing mAb (5 μg/mL) (middle), or pretreatment with a perforin inhibitor (Perforin-I, 50 nM) (right) on target cytotoxicity (N=5-7). (D) Representative plots (left) and graphs (right) showing frequencies of $CD107a^+$ cells in CD8 T cells after co-culture with RPMI8226 myeloma cells for indicated periods in the presence of anti-BCMA T-BsAb (N=7). (E, F) CTV-labeled CD8 T cells were co-cultured with RPMI8226-GFP myeloma cells for 15 minutes at a 1:1 E: T ratio in the presence of anti-BCMA T-BsAb. Representative confocal images showing polarization of the microtubule-organizing center (MTOC) to the immunological synapse (IS) with 3D-reconstituted images (E). Scale bars indicate 10 μm. Graphs showing the distance between the MTOC and IS (right), with each symbol representing 1 donor (N=4) (F). Data are shown as mean ± standard error of mean. Results were pooled from 2 experiments. Differences were tested for statistical significance using a paired *t* test (B, D), a repeated measures ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test (C), and a Mann-Whitney U test (F). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. Perforin-I: perforin inhibitor; GFP: green fluorescent protein; CTV: CellTrace™ Violet.

CD4 T cells expressing CD226 and granzyme B (Figure 5E, F). These results support that rIL-21 treatment can enhance the cytotoxic lymphocyte program in the myeloma BM.

We next asked whether rIL-21 can enhance the efficacy of anti-BCMA T-BsAb therapy in the Vk14451 myeloma preclinical model (Figure 6A). Consistent with *in vitro* results, pretreatment with rIL-21 did not enhance T-BsAb-mediated IFN-γ release, while the combination approach significantly increased plasma levels of granzyme B (Figure 6B). Strikingly, the combination therapy reduced serum paraprotein levels (Figure 6C), and significantly prolonged survival compared to T-BsAb monotherapy (Figure 6D). Together, IL-21 priming is a potential approach to enhance the efficacy of T-BsAb against multiple myeloma.

Treatment with IL-21 modulates effector functions in primary bone marrow CD8 T cells from patients with multiple myeloma

Exhausted CD8 T cells are frequently observed in BM, compared to peripheral blood in patients with multiple myeloma.[35] Thus, we addressed whether rIL-21 treatment could augment cytotoxic effector phenotypes in myeloma BM T cells. To this end, patient-derived BM mononuclear cells were cultured in the presence of T-BsAb with or without rIL-21 for 3 days (Figure 7A). In line with our results from PBMC, treatment with rIL-21 significantly upregulated CD226 in T-BsAb-stimulated myeloma BM CD8 T cells, while the upregulation of CD69 and CD28 was mainly driven by T-BsAb (Figure 7B-D). Importantly, rIL-21 treatment markedly enhanced the release of granzyme B and perforin, but not pro-inflammatory cytokines in T-BsAb-stimulated myeloma BM mononuclear cells (Figure 7E). Taken together, IL-21 can augment cytotoxic effector functions in patient-derived BM CD8 T cells.

Discussion

In this study, we demonstrated that harnessing the cytotoxic granule exocytosis pathway through IL-21 can augment T-BsAb-mediated anti-myeloma effects *in vitro* and *in vivo*. Unlike other immunostimulatory cytokines, IL-21 treatment did not enhance the release of IFN-γ and other pro-inflammatory cytokines, indicating that IL-21 priming preferentially harnesses cytotoxic effector functions, and can augment granule-dependent myeloma-killing activities by T-BsAb therapy.

Recent results from single-cell immune landscape profiling revealed an accumulation of terminally differentiated, functionally impaired T cells in the myeloma BM, especially in patients with relapsed/refractory multiple myeloma.[9,36] Since the effector mechanism of T-BsAb therapy largely depends on endogenous T cells, functional impairment of T cells may limit T-BsAb efficacy against multiple myeloma. Indeed, Friedrich *et al.* recently demonstrated that the presence of exhausted-like CD8 T cell clones was associated with treatment failure in patients treated with anti-BCMA T-BsAb therapy,[7] providing a rationale for reprogramming dysfunctional CD8 T cells to achieve better clinical responses. Over the past few years, new approaches have been developed to improve the functionality of CAR T cells during *ex vivo* manufacturing.[37,38] By contrast, to improve the efficacy of T-BsAb therapy, systemic therapy will be inevitable to reinvigorate endogenous T cells in the myeloma BM. However, the optimal therapeutic approach remains to be established due to the risk of CRS exacerbation. In this context, results from this study reveal IL-21 as an ideal partner for T-BsAb therapy, providing important translational implications. We showed that treatment with rIL-21 did not enhance IFN-γ release both *in vitro* and *in vivo*. Given that IFN-γ is a key driver for CRS and MAS/HLH,[18,19,39] but dispensable for eliminating malignant B cells by immunotherapy,[18,23] avoiding the excess release of IFN-γ will be an important consideration for T-BsAb therapy against hematological malignancies. Of note, conflicting results have been reported on whether or not IL-21 can induce Th1 responses and IFN-γ production, possibly due to context-dependent effects of IL-21 on T-cell

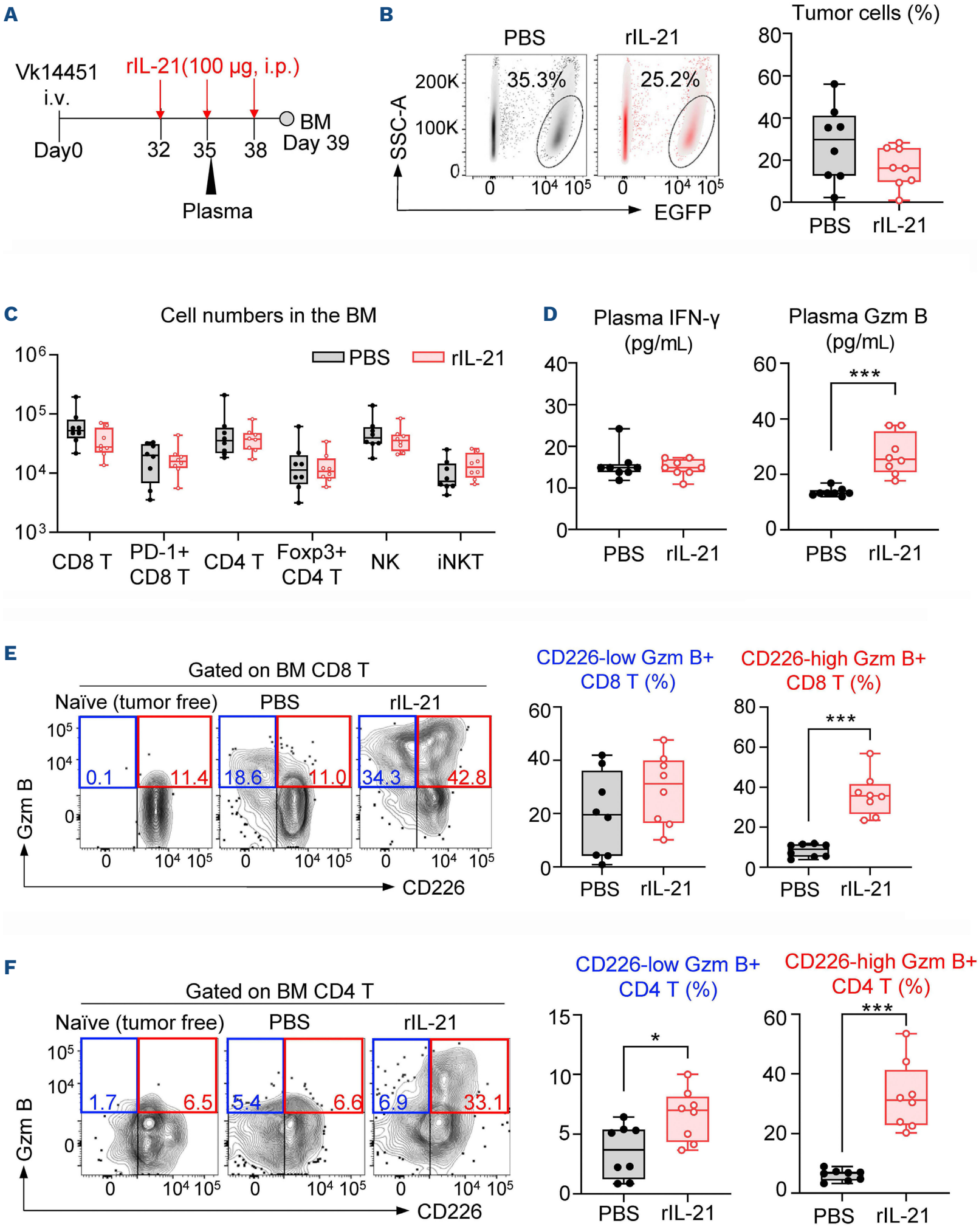

Figure 5. Treatment with recombinant IL-21 drives cytotoxic T-cell programming in the myeloma bone marrow. (A) Schematic illustrates the experimental design for murine recombinant interleukin-21 (rIL-21) treatment in the preclinical Vk14451 myeloma model. (B) Representative flow cytometry plots (left) and graphs showing frequencies (right) of EGFP$^+$ tumor cells in the bone marrow (BM). (C) Graphs showing numbers of indicated immune subsets in the myeloma BM. (D) Graphs showing plasma levels of interferon-γ (IFN-γ) (left) and granzyme B (Gzm B) (right) 6 hours after the second injection of rIL-21. (E, F) Representative flow cytometry plots (left) and graphs (right) showing frequencies of CD8 T cells (E) and CD4 T cells (F) expressing CD226 and Gzm B in the myeloma BM. Data are shown as box-and-whisker plots pooled from 2 experiments (N=8 per group). Differences were tested for statistical significance using a Mann-Whitney U test. *P<0.05; ***P<0.001. SSC: side scatter; EGFP: enhanced green fluorescent protein; PBS: phosphate-buffered saline.

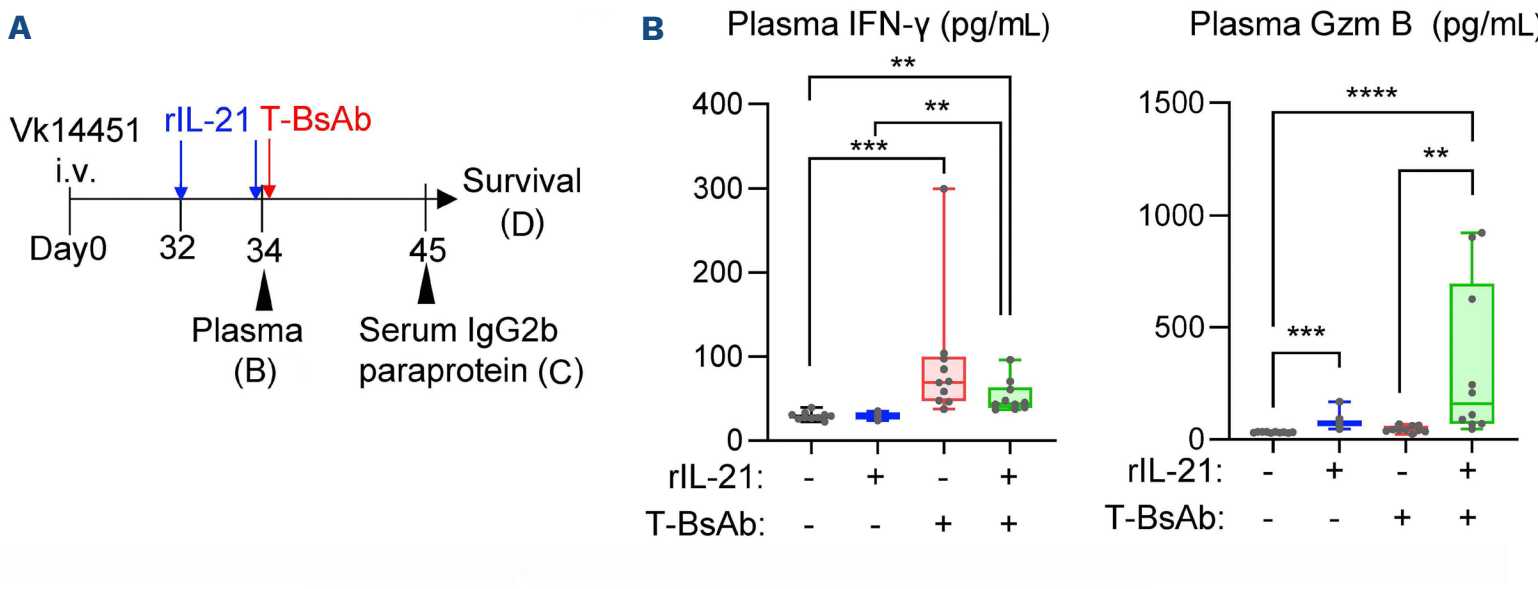

Figure 6. Pretreatment with recombinant IL-21 dramatically improves the efficacy of T-cell-engaging bispecific antibody therapy in the preclinical myeloma model. (A) C57BL6 wild-type mice were challenged with Vk14451 myeloma cells, and intraperitoneally (i.p.) treated with 2 shots of murine recombinant interleukin-21 (rIL-21, 100 μg), followed by T-cell-engaging bispecific antibody targeting mouse B-cell maturation antigen (anti-mouse BCMA T-BsAb, 25 μg). A schematic illustrates the experimental design. (B) Box-and-whisker plots showing plasma levels of interferon-γ (IFN-γ) and granzyme B (Gzm B) 6 hours after treatment. (C) Box-and-whisker plots showing serum levels of immunoglobulin (Ig)G2b paraprotein 11 days after treatment. (D) Kaplan-Meier survival curves of mice after indicated treatment. Data are pooled from 2 experiments (N=7-11 per group). Differences were tested for statistical significance using a Kruskal-Wallis test with *post hoc* Dunn's multiple comparisons test (B, C) and a Mantel-Cox test (D). **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

differentiation and effector functions.[40-42] However, our comprehensive profiling strongly supports the cytotoxic granule pathway as a key target of IL-21. Various immunostimulatory cytokines are known to improve anti-tumor functions by natural killer cells and CD8 T cells.[14,43] Although the upregulation of cytotoxic granules by IL-21 has been reported,[42,44] our results indicate that the multiple steps of the cytotoxic granule pathway are modulated by IL-21. It is noteworthy that priming CD8 T cells by IL-21 transcriptionally and functionally promoted cytoskeletal reorganization and granule degradation at the immunological synapse. Overall, IL-21 has unique roles in modulating effector functions.

We provided preclinical proof-of-concept for IL-21 priming as a potential strategy to improve T-BsAb efficacy against multiple myeloma. Intriguingly, lenalidomide can induce IL-21 production from T cells, raising the possibility that IL-21 signaling might be implicated in its immunostimulatory mechanisms.[45] However, results from a recent preclinical study have shown that the addition of pomalidomide to anti-BCMA T-BsAb therapy only modestly improved the efficacy due to exhaustion of T cells in the myeloma BM,[46] suggesting that reprogramming of dysfunctional T cells by exogenous IL-21 might be necessary to fully augment T-cell functions. We observed that IL-21-primed human and mouse CD4 T cells markedly upregulated granzyme B expression levels. Given that T-BsAb-mediated generation and activation of regulatory T cells counteract anti-myeloma immunity elicited by T-BsAb,[24] the IL-21-driven cytotoxic program in CD4 T cells might contribute to overcome this negative feedback regulation. Alternatively, the activation of natural killer cells, natural killer T cells, and γδ T cells by IL-21 might also contribute to anti-myeloma immunity, as these cells are frequently exhausted in patients with relapsed/refractory myeloma.[9] Recently, Li *et al*. showed that IL-21 can enhance T-cell infiltration in solid malignancies.[47] Thus, IL-21 might be beneficial for controlling immunologically "cold" tumor

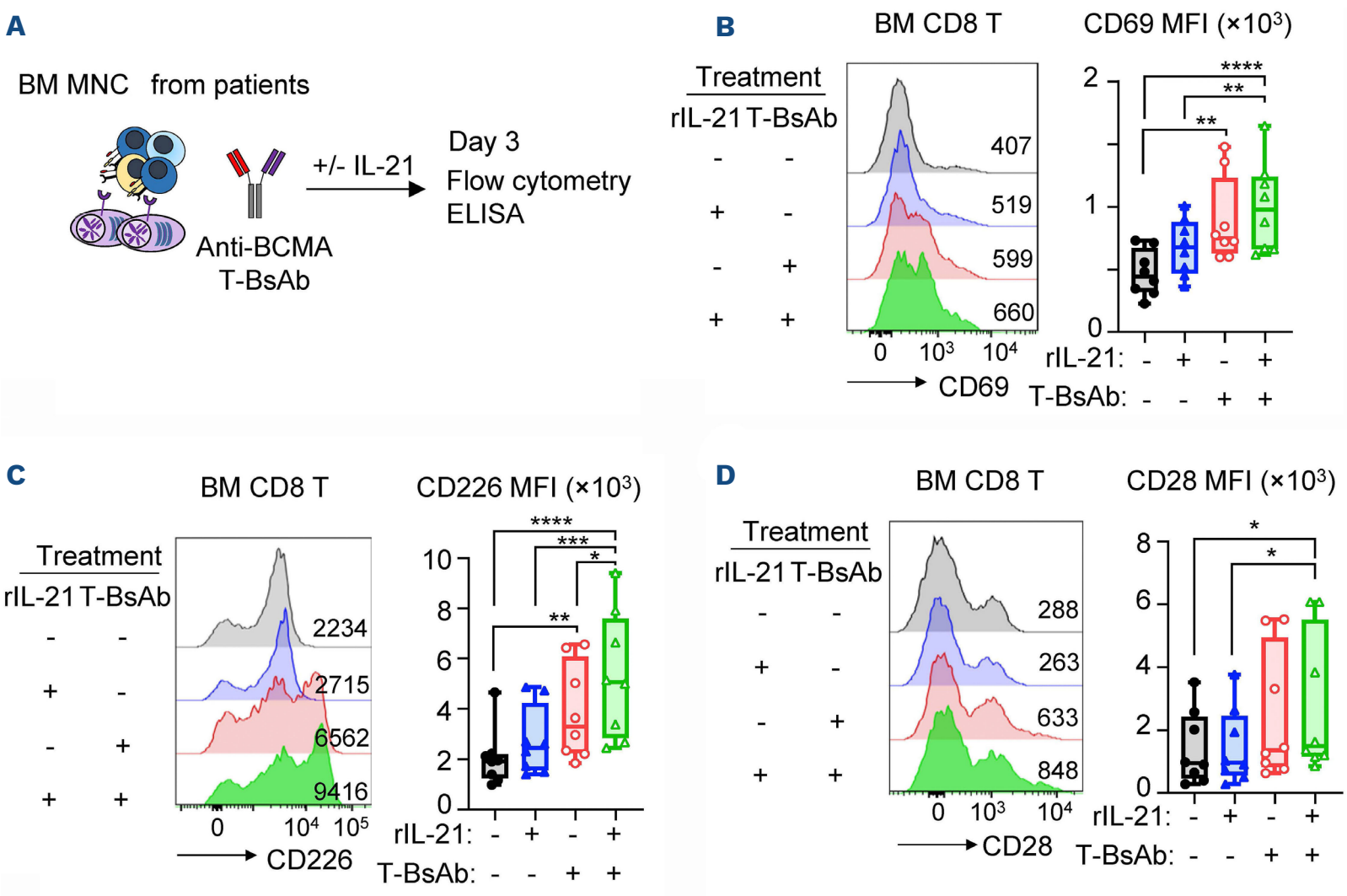

Figure 7. IL-21 improves effector functions in myeloma bone marrow CD8 T cells stimulated with T-cell-engaging bispecific antibody. (A) Primary bone marrow mononuclear cells (BM MNC, 8×10^5) from 8 patients with newly diagnosed multiple myeloma were stimulated with T-cell-engaging bispecific antibody targeting B-cell maturation antigen (anti-BCMA T-BsAb, 0.2 μg/mL) in the presence or absence of recombinant interleukin-21 (rIL-21, 100 ng/mL) for 3 days. A schematic illustrates the experimental design. (B-D) Representative histograms and box-and-whisker plots showing the expression levels of CD69 (B), CD226 (C), and CD28 (D) on bone marrow (BM) CD8 T cells. Numbers indicate mean fluorescence intensity (MFI). (E) Box and whisker plots showing levels of indicated effector proteins and cytokines in culture supernatants. Data are pooled from 2 experiments (N=8). Differences were tested for statistical significance using a repeated measures ANOVA with a *post hoc* Holm-Sidak's multiple comparisons test. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001. TNF-α: tumor necrosis factor-α; GM-CSF: granulocyte-macrophage colony-stimulating factor.

lesions such as extramedullary disease, though further studies are necessary to understand the impact of IL-21 on the myeloma immune microenvironment.

For clinical translation, rIL-21 treatment has been already tested in combination with rituximab in patients with relapsed chronic lymphocytic leukemia and low-grade B-cell lymphomas, and that weekly injection of rIL-21 (100 μg/kg) was tolerable in patients.[48] Besides, a recent preclinical study has also shown no major immune-mediated toxicities either alone or in combination with immune checkpoint inhibitors.[27] One of the limitations of our study is that we could not address whether or not the addition of rIL-21 has an

impact on CRS, as the CRS-like phenotype is not observed in preclinical T-BsAb models. Another limitation of the current study is that this study exclusively evaluated the impact of rIL-21 on anti-BCMA T-BsAb against multiple myeloma. It is reported that IL-21 can directly induce apoptosis in B-cell lymphomas, but not in multiple myeloma, possibly by up-regulating BIM (a member of the pro-apoptotic BH3-only members protein) in B-lymphoma cells.[49-51] In this context, beyond the IL-21-driven cytotoxic program, rIL-21 treatment may be able to lower the apoptotic threshold in B-lymphoma cells, leading to enhancement of anti-CD20 T-BsAb efficacy. Further studies are warranted to understand the tumor type-specific effect of rIL-21. Lastly, it is important to address whether IL-21 can improve T-cell immunity in patients with relapsed/refractory myeloma, although T-BsAb therapy is being tested as an earlier line of therapy (*clinicaltrials gov. Identifier: NCT05469893*). Despite these limitations, results from this study provide important translational implications for improving the efficacy of T-BsAb therapy against multiple myeloma and other hematological malignancies.

Disclosures

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Contributions

KN designed the research and wrote the manuscript. MC, CL, WYK and KN performed experimental work and analyzed the data. SMH and RLJ performed data analysis. SCL and MKG provided key materials. SCL, MKG and SJH contributed to critical discussion and data interpretation. KN conceived and supervised the study. All authors read and approved the final version of the manuscript.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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