Regulatory T cells hamper the efficacy of T-cell-engaging bispecific antibody therapy

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Abstract

T-cell-engaging bispecific antibodies (T-BsAb) have produced impressive clinical responses in patients with relapsed/refractory B-cell malignancies, although treatment failure remains a major clinical challenge. Growing evidence suggests that a complex interplay between immune cells and tumor cells is implicated in the mechanism of action and therefore, understanding immune regulatory mechanisms might provide a clue for how to improve the efficacy of T-BsAb therapy. Here, we investigated the functional impact of regulatory T (Treg) cells on anti-tumor immunity elicited by T-BsAb therapy. In a preclinical model of myeloma, the activation and expansion of Treg cells in the bone marrow were observed in response to anti-B-cell maturation antigen (BCMA) T-BsAb therapy. T-BsAb triggered the generation of induced Treg cells from human conventional CD4 cells after co-culture with tumor cells. Moreover, T-BsAb directly activated freshly isolated circulating Treg cells, leading to the production of interleukin-10 and inhibition of T-BsAb-mediated CD8 T-cell responses. The activation of Treg cells was also seen in bone marrow samples from myeloma patients after *ex vivo* treatment with T-BsAb, further supporting that T-BsAb have an impact on Treg homeostasis. Importantly, transient ablation of Treg cells in combination with T-BsAb therapy dramatically improved effector lymphocyte activities and disease control in the preclinical myeloma model, leading to prolonged survival. Together, this information suggests that therapy-induced activation of Treg cells critically regulates anti-tumor immunity elicited by T-BsAb therapy, with important implications for improving the efficacy of such treatment.

Introduction

T-cell-engaging bispecific antibodies (T-BsAb) have emerged as a powerful “off-the-shelf” immunotherapy against B-cell malignancies. T-BsAb can simultaneously engage the tumor-associated antigen on the malignant cell and CD3 on the T cell, leading to the activation of T cells and tumor elimination. In 2014, blinatumomab (a tandem single-chain variable fragment T-BsAb targeting CD19) was approved for the treatment of relapsed or refractory B-cell precursor acute lymphoblastic leukemia.[1] More recently, anti-CD20 T-BsAb have produced impressive clinical responses in patients with relapsed or refractory B-cell lymphoma.[2,3] In multiple myeloma, T-BsAb against B-cell maturation antigen (BCMA), G-protein coupled receptor family C group 5 member D, and Fc receptor-homolog 5 have demonstrated clinical benefits in heavily pretreated patients.[4-6]

T-BsAb-based immunotherapy is expected to play a key role in the treatment of multiple types of hematologic malignancies. However, treatment failure and relapse remain major clinical challenges. Multiple factors can be involved in the mechanisms of resistance, including loss of target tumor antigens, exhaustion of cytotoxic T cells, and an immunosuppressive microenvironment,[7-11] although it remains largely unknown how better clinical responses can be achieved.

We recently reported that innate immune activation critically contributes to antitumor immune responses elicited by an anti-BCMA T-BsAb in preclinical myeloma models, indicating that a complex cross-talk between immune subsets is implicated in effector mechanisms.[12] Given that T-BsAb therapy triggers dynamic changes in the immune microenvironment, understanding immune regulatory

mechanisms might provide a clue for how to overcome therapeutic resistance. In this context, we hypothesize that regulatory T (Treg) cells may act as key players in regulating the efficacy of T-BsAb therapy. Growing evidence suggests that myeloma favors the generation and accumulation of Treg cells in the bone marrow (BM).[13,14] While Treg cells are a small population in the myeloma BM, modulation of their numbers or activities has shown great potential for improving anti-myeloma immunity.[14-19] Nevertheless, their contribution to the immunological mechanism of T-BsAb therapy remains to be understood. In this study, we aimed to characterize the impact of Treg cells on anti-myeloma immune responses elicited by T-BsAb therapy.

Methods

A list of reagents and antibodies is shown in the *Online Supplementary Information*.

The preclinical model of myeloma

Transplantable Vk14451 myeloma cells expressing enhanced green fluorescent protein (GFP) were maintained as described previously.[12] C57BL/6 wild-type and Foxp3^{DTR} mice[15] were bred and maintained in-house. Mice were intravenously challenged with 2×10^6 Vk14451 cells. Tumor-bearing mice with paraproteinemia (producing clonal IgG2b) were treated with anti-mouse BCMA T-BsAb (25 μg, intraperitoneally). In some experiments, Foxp3^{DTR} mice were treated with diphtheria toxin (DT; 250 ng, intraperitoneally) to deplete Treg cells. For survival experiments, tumor-bearing mice were monitored over time for clinical endpoints, typically hind-limb weakness. All experiments were approved by the Animal Ethics Committee of QIMR Berghofer Medical Research Institute.

Multiple myeloma cell lines

Human myeloma cell lines, JJN-3 and RPMI8226 cells,[20] were cultured in RPMI1640 medium supplemented with sodium pyruvate, non-essential amino acids, penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum (complete RPMI), and maintained at 37°C in 5% CO_2. JJN-3 cells stably expressing GFP were generated by retrovirus transfection using a pMX-IRES-GFP vector, as described previously.[21,22] Negativity for *Mycoplasma* was routinely tested and confirmed by MycoAlert™.

Clinical samples

Clinical samples from patients with newly diagnosed multiple myeloma were collected at the Princess Alexandra Hospital, Brisbane. All samples used in this study were supplied with written informed consent. 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).

Preparation of primary immune cells

Human peripheral blood mononuclear cells were isolated from buffy coats from healthy donors using Lymphoprep™ density gradient medium. Isolation of CD8 T cells, CD4 T cells, and CD25$^+$ CD127^{low} Treg cells was performed by immune magnetic separation using EasySep™ isolation kits. Induced Treg (iTreg) cells were generated by co-culture of CD4 T cells and JJN-3 myeloma cells in the presence of T-BsAb at a 4:1 CD4 T-to-target cell ratio for 4 days. After the removal of CD138$^+$ myeloma cells by magnetic beads, T-BsAb-mediated iTreg cells were used for subsequent assays. Control CD4 T cells were generated by stimulation with anti-CD3/CD28 beads (ImmunoCult™ Human CD3/CD28 T-Cell Activator) according to the manufacturer's instructions.

T-cell-engaging bispecific antibody assays

For T-cell proliferation assays, CD8 T cells (1×10^5) were labeled with CellTrace™ Violet (CTV), and co-cultured with JJN-3 myeloma cells (5×10^4) in complete RPMI supplemented with interleukin (IL)-2 (100 U/mL), using 96-well, U-bottomed plates. These cells were stimulated with anti-human BCMA T-BsAb in the presence of different ratios of Treg cells. CTV dilution was measured by flow cytometry. In order to phenotype activated CD4 T cells, peripheral blood mononuclear cells were stained with the indicated markers 4 and 10 days after T-BsAb stimulation. To assess immunosuppressive activities, isolated T-BsAb-mediated iTreg cells or CD4 T cells activated by anti-CD3/CD28 beads were added to co-cultures of CD8 T cells (1×10^5) and JJN-3 cells (1×10^5) in the presence of T-BsAb (0.25 μg/mL). For conjugation assays, CTV-labeled CD8 T cells, PKH26-labeled iTreg cells, and GFP-expressing RPMI8226 myeloma cells were co-cultured at a 1:1:1 ratio. Cells were fixed with Image-iT™ Fixative Solutions, and then evaluated by flow cytometry and confocal microscopy. To assess T-BsAb-induced IL-10 production, isolated CD4$^+$CD25$^+$CD127^{low} Treg cells (2×10^5) were co-cultured with JJN-3 myeloma cells (1×10^5) with the indicated concentrations of anti-BCMA T-BsAb for 3 days, followed by measurement of IL-10 levels in culture supernatants by enzyme-linked immunosorbent assay. For Treg suppression assays, isolated CD4$^+$CD25$^+$CD127^{low} Treg cells were co-cultured with CTV-labeled CD8 T cells (1×10^5) in the presence of T-BsAb and JJN-3 myeloma cells (1×10^5) at different Treg/CD8 T-cell ratios. The suppressive activity (% suppression) was calculated as follows: 100 – (% proliferating CD8 T cells with Treg cells) / (% proliferating CD8 T cells without Treg cells) × 100. The supernatant level of interferon-γ was determined by enzyme-linked immunosorbent assay. For *ex vivo* stimulation in primary samples, bulk BM mononuclear cells (1×10^6) were stimulated with anti-human BCMA T-BsAb (0.25 μg/mL) in complete RPMI

medium supplemented with IL-2 (100 U/mL) for 4 days.

Flow cytometry

Single-cell suspensions were immunostained according to standard protocols. Cell surface staining was performed in the presence of the indicated monoclonal antibodies. For intracellular staining, cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer, and then stained with specific monoclonal antibodies. For intracellular cytokine staining, BM cells were stimulated for 3 h by Cell Stimulation Cocktail plus a protein transport inhibitor according to the manufacturer's instructions, followed by intracellular staining. Tumor burden was determined by GFP-positive tumor cells in the femoral BM. The numbers of cells of each subset were calculated by counting beads. The flow cytometry-based conjugation assays were performed as described previously.[23] Samples were acquired by a BD LSR Fortessa Flow Cytometer and analyzed with FlowJo version 10. The t-distributed stochastic neighbor embedding (t-SNE) analysis of concatenated samples was visualized using the built-in FlowJo plugin and Adobe Illustrator.

Confocal imaging

For conjugate formation, CTV-labeled CD8 T cells, PKH26-labeled iTreg cells, and GFP-expressing RPMI8226 myeloma cells were co-cultured for 30 min, and then fixed with image-iT™ Fixative Solutions for 30 min. In order to image the immunological synapse, freshly isolated Treg cells were co-cultured with RPMI8226-GFP cells at a 1:1 ratio in the presence or absence of anti-human BCMA T-BsAb (0.25 μg/mL) for 2 h. For the last 30 min, cells were incubated with biotin anti-CD11a/LFA-1 monoclonal antibody, and stained with Alexa Fluor 647 streptavidin. Cells were fixed with image-iT™ Fixative Solutions for 30 min, followed by permeabilization with 0.05% Triton X-100 for 10 min. The cells were then stained with ActinRed 555 Ready Probes to detect F-actin. Confocal fluorescence images were obtained using a Zeiss 780-NLO confocal microscope; cells were examined with a 63x objective for conjugate formation and a 100× objective for immunological synapses. Images were captured using dual-channel excitation and analyzed using Zeiss software.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8 software. A Mann-Whitney U test or a paired *t* test was used for comparisons between two groups. For multiple comparisons, two-way analysis of variance (ANOVA) with a *post-hoc* Tukey multiple comparisons test was used. For multiple comparisons of paired samples, a repeated measures ANOVA with *post-hoc* Tukey multiple comparisons test or Dunnett multiple comparisons test was used. Differences in survival were evaluated with a Mantel-Cox test. *P* values <0.05 were considered statistically significant (*P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001).

Results

Activation and expansion of regulatory T cells in the myeloma bone marrow after T-cell-engaging bispecific antibody therapy.

To examine the impact of T-BsAb therapy on the immune microenvironment, C57BL6 wild-type mice were intravenously challenged with Vk14451 multiple myeloma cells. In this preclinical myeloma model, mice typically develop IgG2b paraproteinemia 4 weeks after tumor challenge, followed by progressive tumor growth in the BM.[12] In response to T-BsAb therapy, both CD8 T cells and Foxp3$^+$ Treg cells upregulated CD69 expression within 24 h (*Online Supplementary Figure S1*), indicating rapid T-cell activation in the myeloma BM.

To understand late immune responses, BM cells were analyzed 2 weeks after treatment (Figure 1A). Treatment with the anti-BCMA T-BsAb dramatically reduced tumor burden (Figure 1B). Intriguingly, t-SNE plots of BM TCR-β$^+$ cells revealed enrichment of Foxp3$^+$ Treg cells in the myeloma BM from the treatment group (Figure 1C). Indeed, while an increased number of BM CD8 T cells was observed after T-BsAb therapy, the number of Foxp3$^+$ Treg cells was also significantly increased (Figure 1D). As a result, the CD8/Treg ratio was decreased in the myeloma BM (Figure 1E). Phenotypically, BM CD8 T cells from the treatment group showed lower expression levels of PD-1 and CD38, which might be due to the expansion of non-exhausted CD8 T cells (Figure 1F). By contrast, BM Treg cells from the treatment group showed high expression of PD-1, Tigit, and CD38 (Figure 1F), which are known to be upregulated in activated Treg cells in the tumor microenvironment.[24,25] These results suggest that T-BsAb therapy has a strong impact on the numbers and phenotypes of Treg cells in myeloma BM.

Generation of induced regulatory T cells after stimulation with T-cell-engaging bispecific antibodies

We next examined the impact of T-BsAb therapy on human Treg cells. To this end, we co-cultured bulk peripheral blood mononuclear cells from healthy donors and BCMA$^+$ JJN-3 myeloma cells in the presence of anti-human BCMA T-BsAb (Figure 2A and *Online Supplementary Figure S2A*). Treatment with T-BsAb triggered the proliferation of CD8 T cells and CD4 T cells in a concentration-dependent manner (*Online Supplementary Figure S2B*). Intriguingly, an increased frequency of CD4 T cells expressing CD25 and FOXP3 was observed after T-BsAb treatment (Figure 2B). These cells co-expressed ICOS and CTLA-4 (Figure 2B), which are recognized as key markers for activated human Treg cells with highly immunosuppressive activities.[26] While murine CD4 T cells can differentiate into

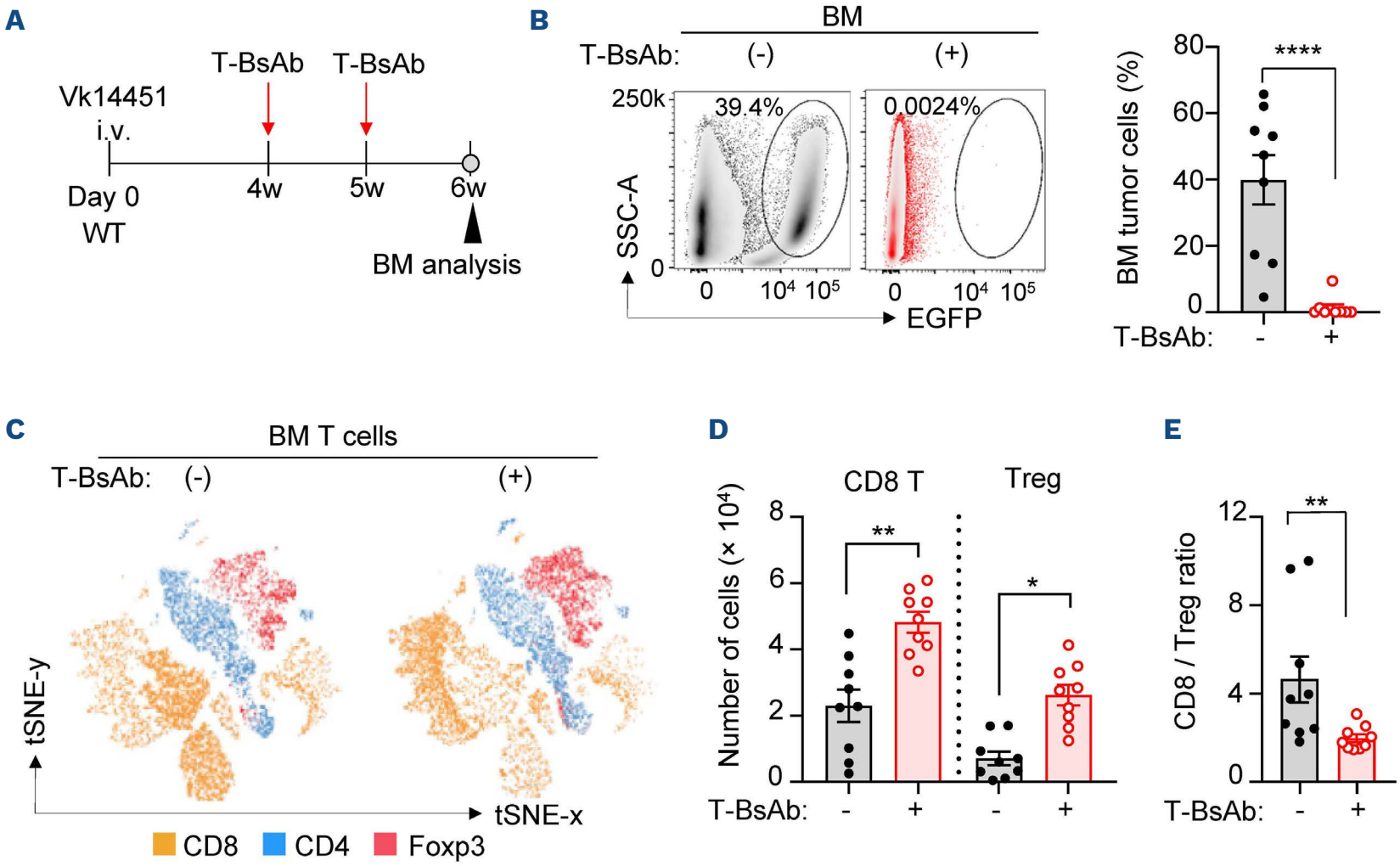

Figure 1. T-cell-engaging bispecific antibody therapy triggers the activation of regulatory T cells in myeloma bone marrow. (A) C57BL6 wild-type mice were challenged with Vk14451 myeloma cells, and treated with anti-mouse B-cell maturation antigen T-cell engaging bispecific antibody. The schematic illustrates the experimental design. (B) Representative plots and graphs showing frequencies of enhanced green fluorescent protein-positive tumor cells in the bone marrow (BM) (N=9 per group), pooled from two experiments. (C) t-distributed stochastic neighbor embedding plots showing the density of events of indicated subsets in BM TCRβ-positive cells from one experiment (N=4 per group). (D, E) Graphs showing numbers of indicated immune cells (D) and the CD8/Treg ratio (E) in the myeloma BM (N=9 per group), pooled from two experiments. (F) Histograms and graphs showing expression levels of PD-1, Tigit and CD38 on CD8 T cells and Treg cells in the myeloma BM. Representative results from two experiments are shown (N=4-5). Numbers indicate mean fluorescence intensity. Data are shown as mean ± standard error of mean. Differences were tested for statistical significance, using a Mann-Whitney U test (B, D, and E) and Student *t* test (F). **P*<0.05, ***P*<0.01, *****P*<0.0001. WT: wild-type; T-BsAb: T-cell-engaging bispecific antibody; BM: bone marrow; SSC; side scatter; EGFP: enhanced green fluorescent protein; t-SNE: t-distributed stochastic neighbor embedding; Treg: regulatory T cells; FMO: fluorescence minus one; MFI: mean fluorescence intensity.

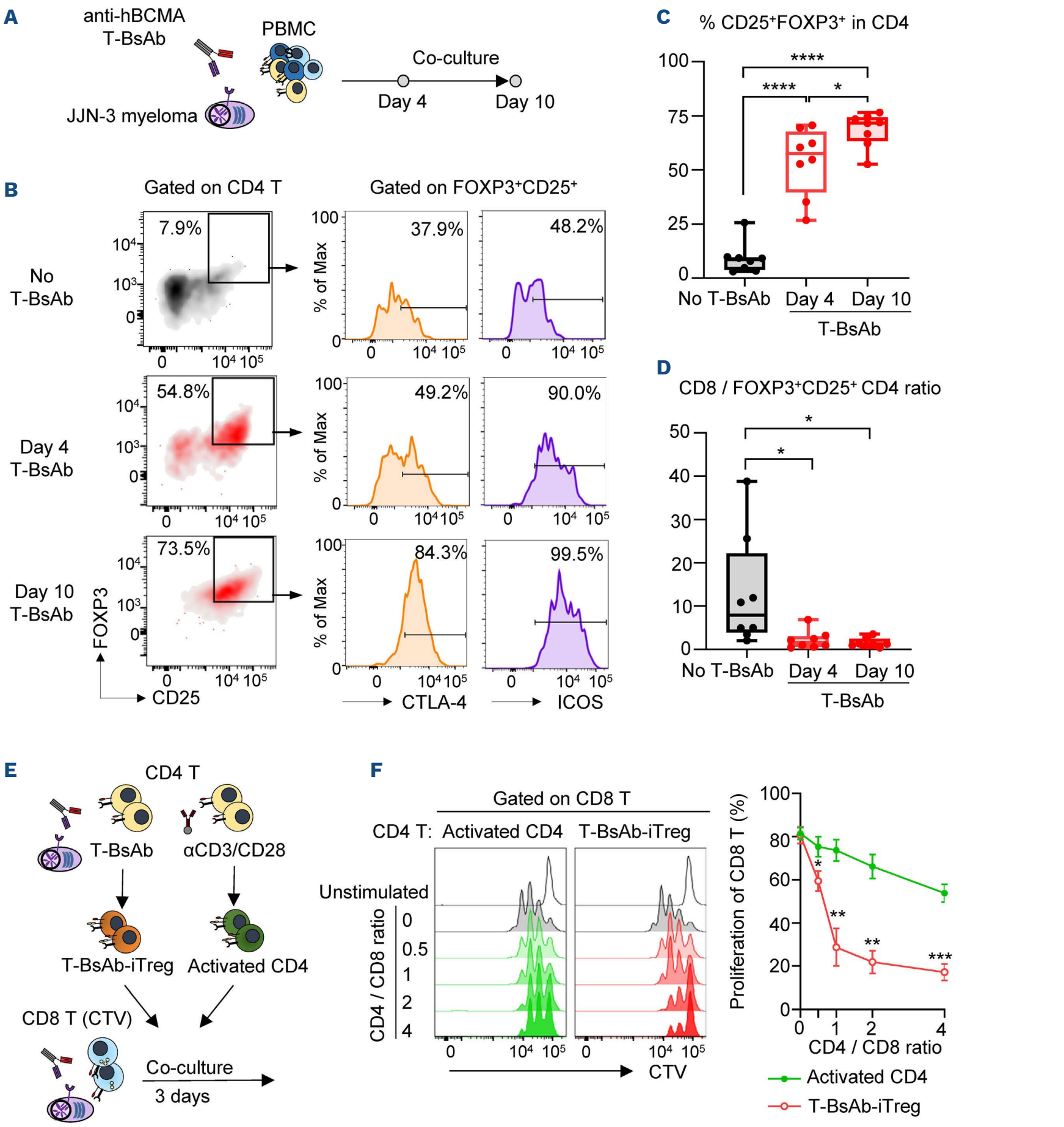

Figure 2. *In vitro* differentiation of induced regulatory T cells by T-cell-engaging bispecific antibody therapy. (A) Peripheral blood mononuclear cells were co-cultured with JJN-3 myeloma cells in the presence of anti-human B-cell maturation antigen T-cell-engaging bispecific antibody (T-BsAb). A schematic illustrating the experimental design. (B) Representative flow cytometry plots showing the frequency of CD4 T cells expressing FOXP3 and CD25 at the indicated time points (left). Representative histograms showing expression levels of CTLA-4 and ICOS in CD25$^+$FOXP3$^+$CD4 T cells (right). (C, D) Box and whisker plots showing the frequency of FOXP3$^+$CD25$^+$ cells in CD4 T cells (C), and the CD8/FOXP3$^+$CD25$^+$CD4 ratio (D) at indicated time points after treatment with T-BsAb. Pooled results from two experiments are shown (N=8). (E, F) Isolated CD4 T cells were stimulated with T-BsAb or anti-CD3/CD28 beads for 4 days. Subsequently, T-BsAb-mediated induced regulatory T cells and activated CD4 T cells were co-cultured with CellTrace™ Violet-labeled CD8 T cells to test immunosuppressive activities. A schematic illustrating the experimental design (E). Representative histograms and graphs showing CD8 T-cell proliferation at the indicated CD4/CD8 ratios 3 days after stimulation (F). Data are shown as mean ± standard error of mean, pooled from two experiments (N=6). Differences were tested for statistical significance using repeated measures analysis of variance with a *post-hoc* Tukey multiple comparisons test (C, D) and a paired *t* test (F). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. hBCMA; human B-cell maturation antigen; PBMC: peripheral blood mononuclear cells; iTreg; induced regulatory T cells; CTV: CellTrace™ Violet.

iTreg cells by T-cell receptor (TCR) stimulation in the presence of TGF-β, activated human conventional CD4 T cells are known to transiently upregulate FOXP3 without acquiring immunosuppressive activity.[27,28] However, we observed that T-BsAb-stimulated CD4 T cells maintained FOXP3 expression even 10 days after stimulation (Figure 2B-D), raising the possibility that these cells might have acquired iTreg phenotypes. Of note, iTreg-like cells were not observed after treatment with control T-BsAb (*Online Supplementary Figure S2C, D*), supporting the idea that T-BsAb can induce iTreg-like phenotypes in a target antigen-specific manner.

To examine their immunosuppressive activity, T-BsAb-mediated iTreg-like cells were isolated 4 days after co-culture with JJN-3 myeloma cells. Subsequently, these cells were co-cultured with CTV-labeled CD8 T cells from the same donor (Figure 2E). Indeed, T-BsAb-induced CD8 T-cell proliferation was potently inhibited in the presence of iTreg-like cells, compared to control CD4 T cells (Figure 2F). These results support the generation of iTreg cells from conventional CD4 T cells after stimulation with T-BsAb.

To further understand the interaction between iTreg cells and tumor cells, we next co-cultured CTV-labeled CD8 T cells, PKH26-labeled iTreg cells, and GFP-expressing RPMI8226 myeloma cells in a 1:1:1 ratio in the presence of T-BsAb (Figure 3A). Notably, iTreg cells preferentially formed aggregates around myeloma cells, compared to CD8 T cells (Figure 3B-D). These results suggest that iTreg cells can actively interact with myeloma cells, leading to negative regulation of CD8 T-cell responses elicited by T-BsAb.

Regulatory T cells and tumor engagement negatively regulate CD8 T-cell responses

In addition to the differentiation of iTreg cells from conventional CD4 T cells, we hypothesized that T-BsAb therapy could directly trigger the activation of circulating Treg cells. To address this possibility, we next used freshly isolated human $CD4^+CD25^+CD127^{low}$ Treg cells from peripheral blood mononuclear cells (Figure 4A). In general, engagement of the TCR/CD3 complex leads to immunological synapse formation at the contact site, which acts as a molecular platform for T-cell activation.[29] To investigate immunological synapse formation, freshly isolated human $CD4^+CD25^+CD127^{low}$ Treg cells were cultured with RPMI8226 cells expressing GFP. In the presence of T-BsAb, clustering of LFA-1, a leukocyte adhesion molecule, and reorganization of F-actin were observed at the contact site (Figure 4B), supporting the immunological synapse formation between Treg cells and tumor cells. Production of an anti-inflammatory cytokine, IL-10, is a key feature of activated Treg cells in the tumor microenvironment.[30] Indeed, T-BsAb triggered IL-10 production from isolated Treg cells in a concentration-dependent manner (Figure 4C). These results provide direct evidence for the activation of Treg cells by T-BsAb.

To further examine the functional impact of T-BsAb-activated Treg cells, freshly isolated Treg cells and CD8

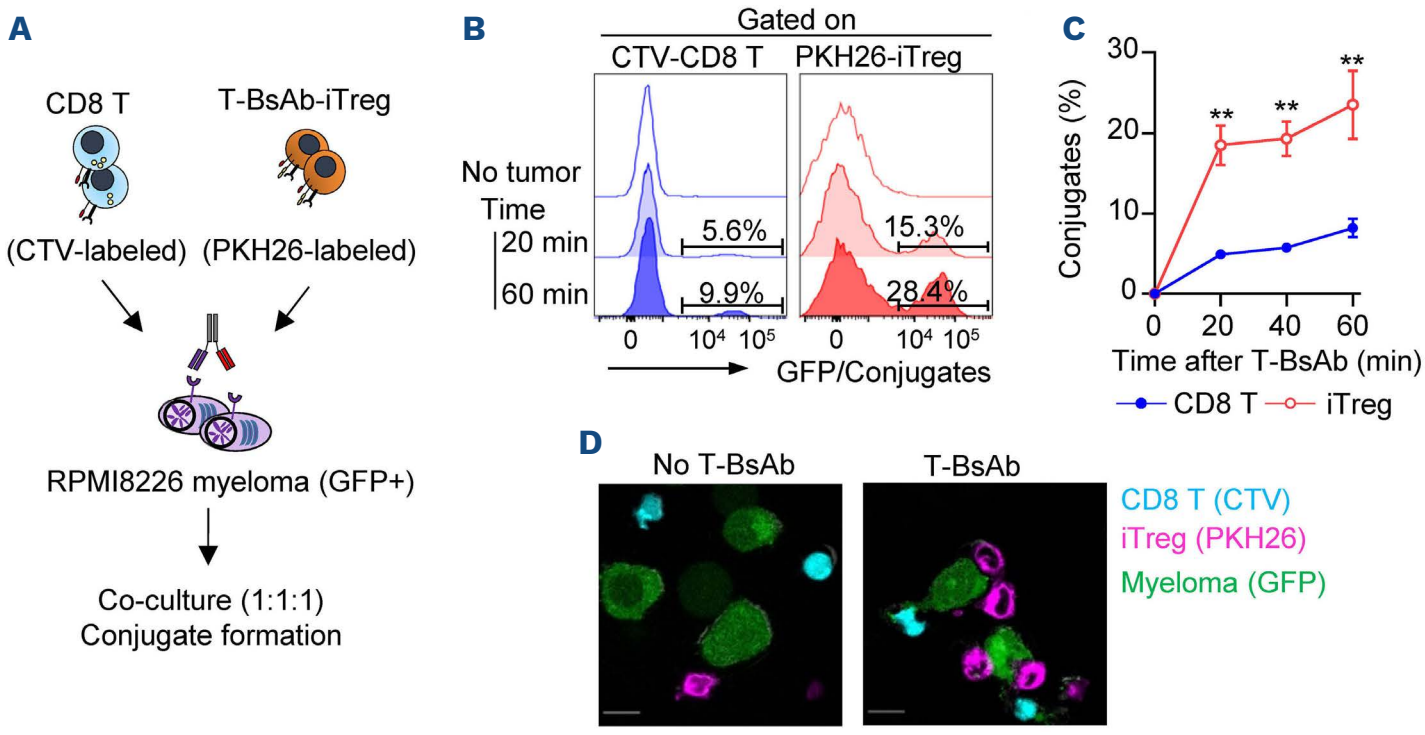

Figure 3. Induced T regulatory cells preferentially form aggregates around tumor cells. (A) CellTrace™ Violet-labeled CD8 T cells, PKH26-labeled T-cell-engaging bispecific antibody (T-BsAb)-mediated induced regulatory T cells (iTreg) and green fluorescent protein (GFP)-expressing RPMI8226 myeloma cells were co-cultured in a 1:1:1 ratio in the presence of anti-B-cell maturation antigen T-BsAb (0.1 μg/mL). The schematic illustrates the experimental design. (B, C) Representative histograms (B) and graphs (C) showing conjugate formation with GFP^+ myeloma cells by CD8 T cells or iTreg cells. Data are shown as mean ± standard error of mean, pooled from two experiments (N=6). Differences were tested for statistical significance using a paired *t*-test. $^{**}P<0.01$. (D) Confocal images showing conjugate formation 30 min after co-culture (scale bar: 10 μm). Representative images from six donors are shown. CTV: CellTrace™ Violet.

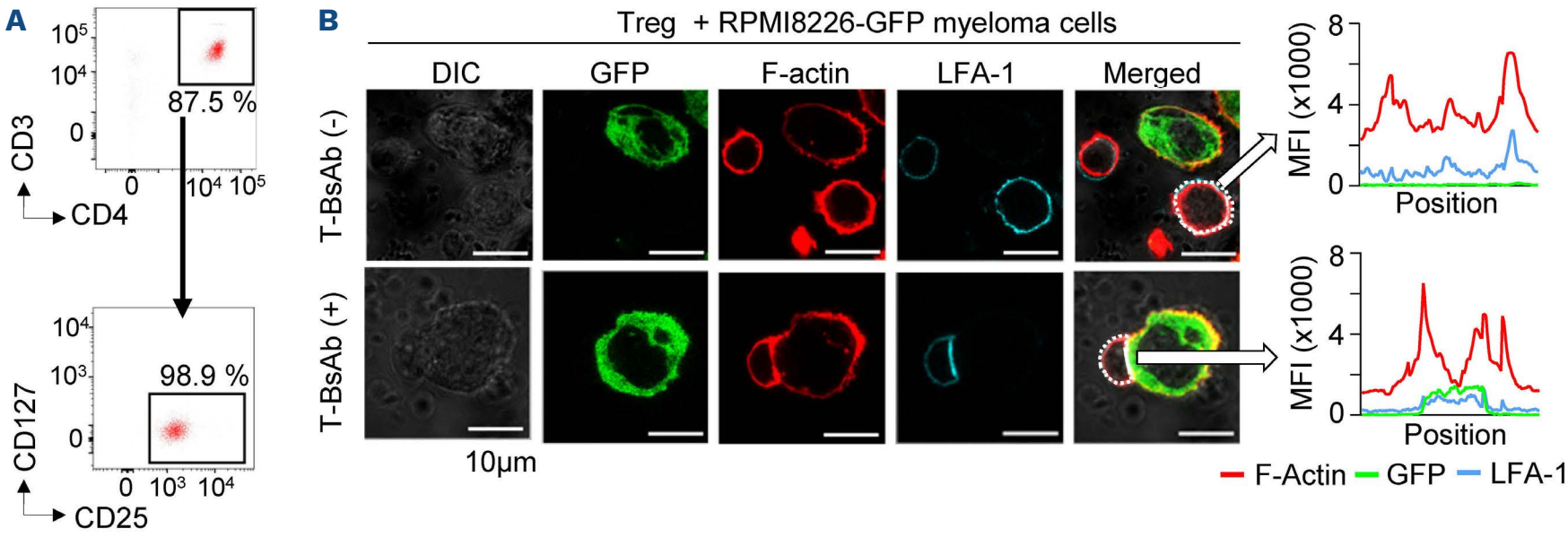

Figure 4. T-cell-engaging bispecific antibody-activated regulatory T cells negatively regulate CD8 T-cell responses. (A) Representative flow cytometry plots showing purity of $CD25^{+}CD127^{low}$ regulatory cells (Treg cells) isolated from peripheral blood mononuclear cells. (B) Freshly isolated human Treg cells were co-cultured with RPMI8226 myeloma cells expressing green fluorescent protein in the presence or absence of anti-B-cell maturation antigen (BCMA) T-cell-engaging bispecific antibody (T-BsAb). Representative confocal images and graphs showing accumulation of LFA-1 and F-actin between Treg cells and multiple myeloma cells after stimulation by anti-BCMA T-BsAb (scale bar: 10 µm). Images were processed using MetaMorph software. Representative results from two experiments are shown. (C) $CD25^{+}CD127^{low}$ Treg cells (2×10^{5}) were co-cultured with JJN-3 myeloma cells (1×10^{5}) with indicated concentrations of anti-BCMA T-BsAb for 3 days. The graphs show levels of interleukin-10 in culture supernatants. Data are shown as mean ± standard error of mean (N=4). (D) $CD4^{+}CD25^{+}CD127^{low}$ Treg cells and CD8 T cells were co-cultured with JJN-3 myeloma cells at different Treg/CD8 ratios in the presence of anti-BCMA T-BsAb for 3 days. The schematic illustrates the experimental design. (E, F) Representative histograms showing CD8 T-cell proliferation at indicated Treg/CD8 ratios (E, left). Individual graphs showing the suppressive effect of Treg cells on CD8 T-cell proliferation (E, right). Individual graphs showing interferon-γ production at different Treg/CD8 ratios (F). Pooled results from three experiments are shown (N=8). Differences were tested for statistical significance using a repeated measures analysis of variance with a *post-hoc* Dunnet multiple comparisons test (C) and Tukey multiple comparisons test (E, F). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. DIC: differential interference contrast; GFP: green fluorescent protein; LFA-1: lymphocyte function-associated antigen 1; MFI: mean fluorescent intensity; CTV: CellTrace™ Violet; IL: interleukin; IFN: interferon.

T cells were co-cultured with JJN-3 cells (Figure 4D). T-BsAb-induced proliferation of CD8 T cells (Figure 4E) and production of interferon-γ (Figure 4F) were markedly attenuated in the presence of Treg cells. These results indicate that the T-BsAb-mediated interaction between Treg cells and myeloma cells negatively regulates CD8 T-cell responses.

Transient ablation of regulatory T cells augments *in vivo* anti-tumor effects by T-cell-engaging bispecific antibody therapy

To further investigate the impact of Treg cells on anti-tumor effects, we challenged Vk14451 myeloma cells in mice expressing diphtheria toxin receptor (DTR) under the control of the Foxp3 promoter, and treated with T-BsAb in combination with DT (Figure 5A). Ablation of Treg cells augmented systemic release of effector molecules including interferon-γ and granzyme B by T-BsAb therapy (Figure 5B). In the absence of Treg cells, BM CD8 T cells showed higher levels of expression of interferon-γ in response to anti-BCMA T-BsAb therapy (Figure 5C), highlighting the strong impact of Treg cells on effector lymphocyte activities. Strikingly, transient ablation of Treg cells in combination with anti-BCMA T-BsAb therapy significantly improved disease control, leading to prolonged survival (Figure 5D). Together, these results demonstrate that Treg cells potently inhibit the efficacy of T-BsAb therapy.

T-cell-engaging bispecific antibody-induced activation of bone marrow regulatory T cells in multiple myeloma patients

To further confirm that treatment with T-BsAb could contribute to the activation of Treg cells in the myeloma BM, BM mononuclear cells from newly diagnosed patients were cultured in the presence of T-BsAb for 4 days (Figure 6A). FOXP3^{+}CD25^{+}CD4 T cells were indeed markedly

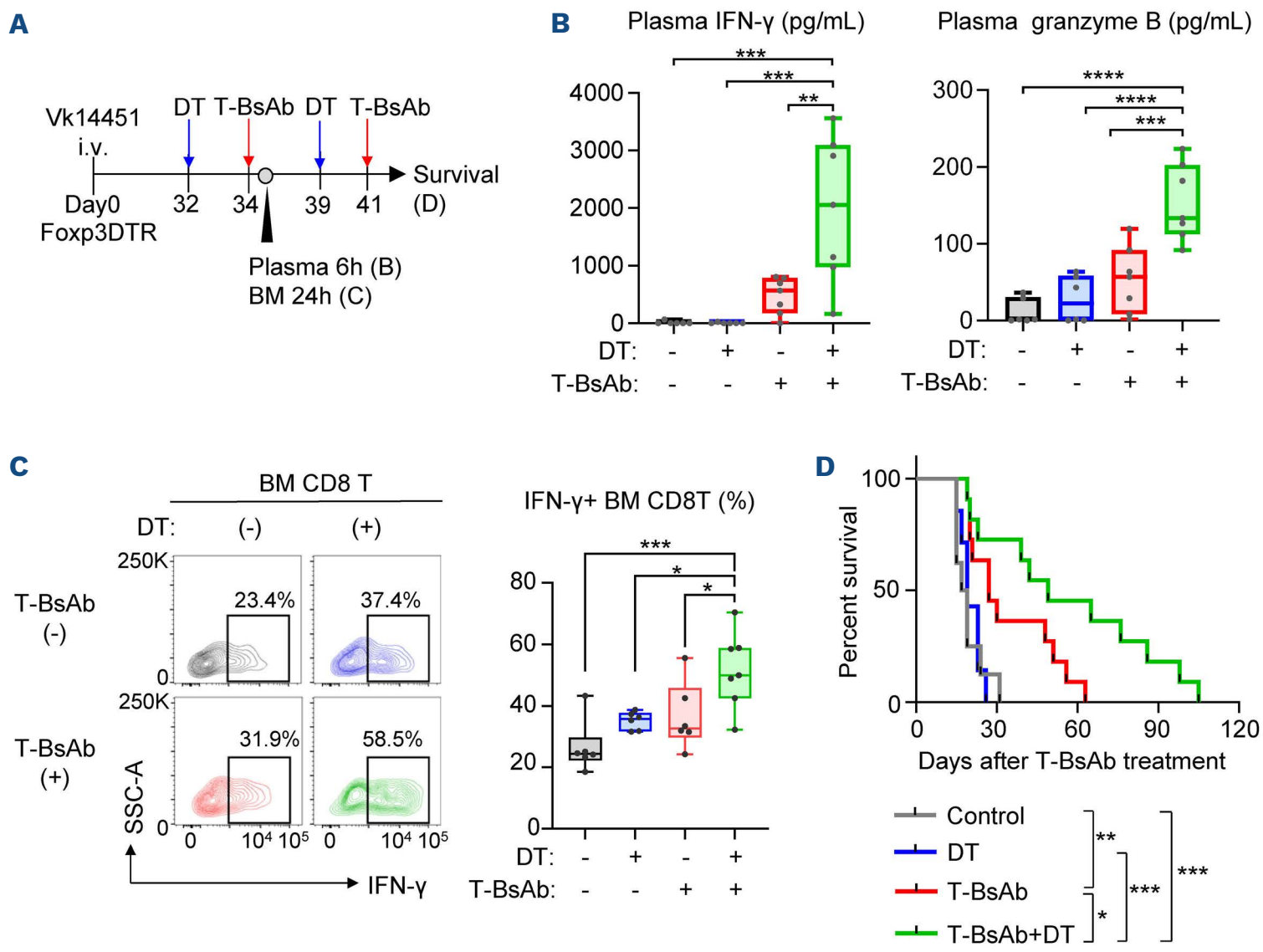

Figure 5. Transient ablation of regulatory T cells improves the efficacy of anti-B-cell maturation T-cell-engaging bispecific antibody therapy. (A) C57BL6 Foxp3^{DTR} mice were challenged with Vk14451 multiple myeloma cells, and treated with diphtheria toxin in combination with anti-mouse B-cell maturation antigen T-cell-engaging bispecific antibodies (T-BsAb). The schematic illustrates the the experimental design. (B) Tumor-bearing mice were given the indicated treatment. Box and whisker plots showing levels of interferon-γ and granzyme B in plasma 6 h after treatment (N=6-7 per group). (C) Representative flow cytometry plots and box and whisker plots showing frequencies of interferon-γ^{+} CD8 T cells in the myeloma bone marrow 24 h after treatment (N=6-7 per group). (D) Kaplan-Meier survival curves of mice after the indicated treatment (N=7-11 per group). Data are pooled from two independent experiments. Differences were tested for statistical significance using two-way analysis of variance with a *post-hoc* Tukey multiple comparisons test (B, C) and a Mantel-Cox test (D). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. DTR: diphtheria toxin receptor; DT: diphtheria toxin; T-BsAb: T-cell-engaging bispecific antibody; BM: bone marrow; IFN: interferon.

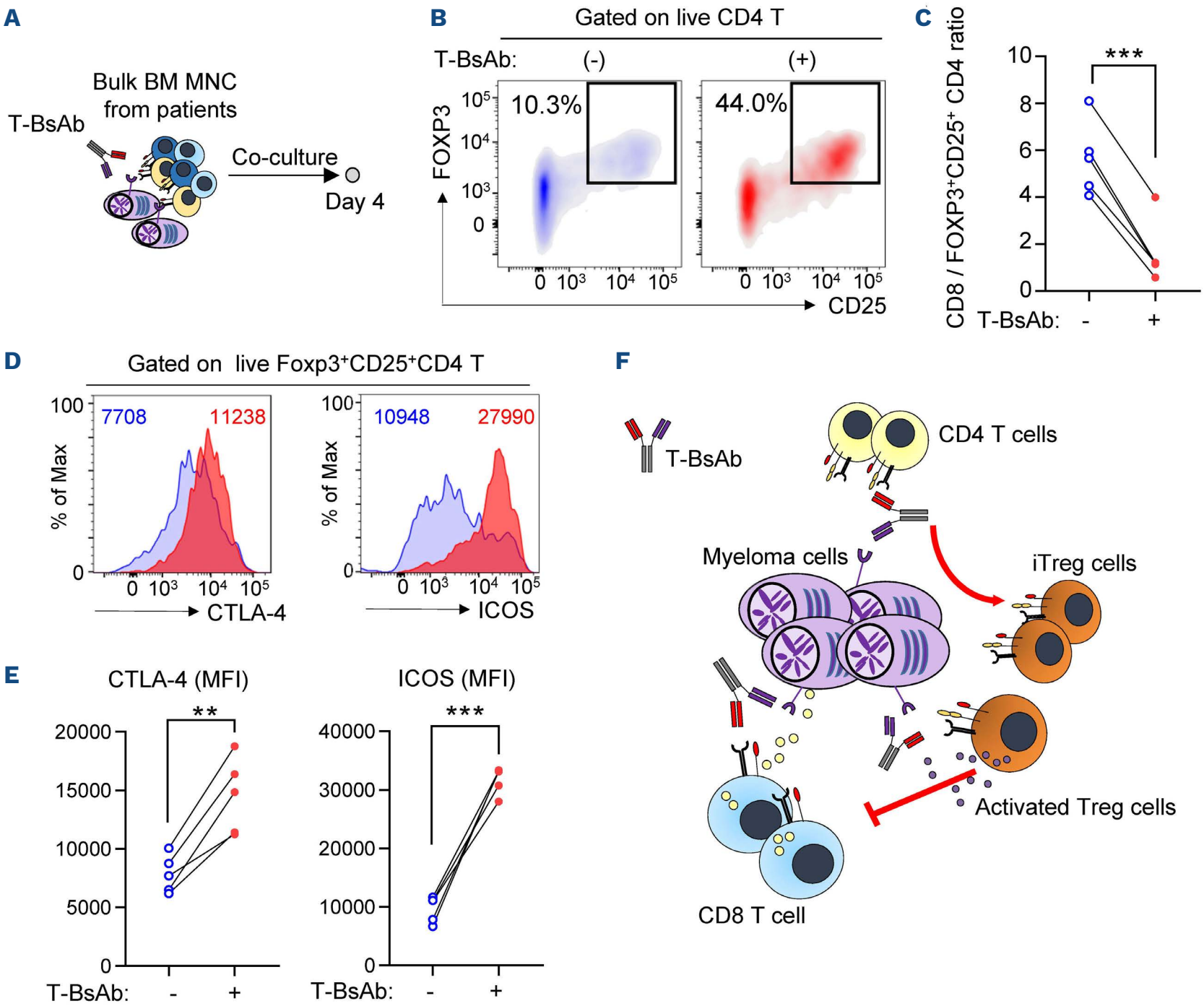

Figure 6. T-cell-engaging bispecific antibody-mediated activation of regulatory T cells in primary bone marrow samples from patients with multiple myeloma. (A) Bone marrow mononuclear cells (1×10^6 cells) from newly diagnosed patients with multiple myeloma were stimulated with anti-human B-cell maturation antigen T-cell-engaging bispecific antibody (T-BsAb). The schematic illustrates the the experimental design. (B, C) Representative flow cytometry plots showing frequencies of CD4 T cells expressing FOXP3 and CD25 4 days after treatment (B). Graphs showing the CD8/FOXP3$^+$CD25$^+$CD4 ratio 4 days after treatment (C). (D, E) Representative histograms (D) and graphs (E) showing expression levels of CTLA-4 and ICOS in FOXP3$^+$CD25$^+$CD4 T cells (D). Numbers indicate mean fluorescence intensity. Results from one experiment (N=5) are shown. Differences were tested for statistical significance using a paired *t*-test. $^{**}P<0.01$, $^{***}P<0.001$. (F) A graphical summary of the results. T-BsAb therapy triggers (i) differentiation of induced regulatory T cells from CD4 T cells, and (ii) activation of regulatory T cells, leading to suppression of CD8 T-cell responses. BM MNC: bone marrow mononuclear cells; MFI: mean fluorescence intensity; Treg: regulatory T cells; iTreg: induced regulatory T cells.

increased after T-BsAb stimulation (Figure 6B, C), with upregulation of activation markers including CTLA-4 and ICOS (Figure 6D, E). Together, these results indicate that the activation of Treg cells is an important concept for improving the efficacy of T-BsAb.

Discussion

In this study, we provide evidence that Treg cells negatively regulate anti-myeloma immune responses elicited by T-BsAb therapy. Our results suggest that T-BsAb therapy triggers at least two events for Treg homeostasis: (i) differentiation of iTreg cells from conventional CD4 T cells, and (ii) direct activation of Treg cells in the tumor microenvironment (Figure 6F).

Accumulation of Treg cells in tumor tissues can be explained either by recruitment and proliferation of circulating Treg cells, or by differentiation from conventional CD4 T cells, namely iTreg cells.[31] A recent study in solid malignancies showed that the TCR repertoires of tumor-infiltrating Treg cells display some overlap with those of

circulating Treg cells, but not with conventional CD4 T cells, implying limited contributions of iTreg cells.[32] However, it is plausible that myeloma-associated Treg cells are maintained differently, as myeloma predominantly grows in the BM, a lymphoid organ containing Treg cells with unique phenotypes and functions.[7,33,34] Intriguingly, Feng *et al*. reported that the co-culture of peripheral blood mononuclear cells with human myeloma cells can modestly, but significantly, increase the frequency of CD38$^+$ iTreg cells even without any additional stimulation.[14] Indeed, our results showed that the majority of CD4 T cells acquired an iTreg phenotype after T-BsAb stimulation, indicating that CD4 T-cell activation in the presence of myeloma cells contributes to the differentiation of iTreg cells. Notably, a recent study using single-cell TCR tracing of T cells from T-BsAb-treated patients showed that a fraction of naïve CD4 T cells exhibits a Treg cell-like phenotype after treatment, supporting the differentiation of iTreg cells under T-BsAb therapy.[8] Taken together, the myeloma microenvironment might favor the generation of iTreg cells, especially in the context of T-BsAb therapy. Various environmental factors might be implicated in the generation and functional maturation of myeloma-associated Treg cells, such as TGF-β, hypoxia-driven adenosine, and metabolic reprogramming,[7,35,36] although further studies are warranted to understand the mechanism.

Growing evidence suggests that Treg cells act as a major barrier to the optimal efficacy of immune checkpoint inhibitors. For example, Kamada *et al.* have shown that in gastric cancer patients who experienced hyper-progressive disease after PD-1 blockade, therapy-induced activation and proliferation of PD-1$^+$ Treg cells are implicated in the immunological mechanism.[37] Accordingly, the balance of PD-1 expression between CD8 T cells and Treg cells in the tumor microenvironment can predict clinical responses to PD-1 blockade in gastric cancer patients.[38] Similarly, CTLA-4 blockade can trigger the CD28-mediated activation and proliferation of tumor-associated Treg cells, supporting that T-cell reinvigoration by immune checkpoint inhibitors is not restricted to effector T cells.[39] Our results provide evidence that T-BsAb-activated Treg cells hamper anti-tumor immune responses. Importantly, TCR signaling in Treg cells is known to augment suppressive functions by altering cellular metabolism and enhancing Treg cell aggregation on antigen-presenting cells.[40,41] Indeed, we observed iTreg cells preferentially formed aggregates around tumor cells, compared to CD8 T cells. Thus, activated Treg cells play non-negligible roles in limiting anti-tumor immunity elicited by T-BsAb therapy. Together, therapy-induced activation of Treg cells will be an important consideration to achieve better clinical responses for T-cell–redirecting immunotherapy.

While our results strongly support the negative impact of Treg cells on the efficacy of T-BsAb therapy, a previous study found that Treg cells showed *in vitro* killing activities against solid tumor cells in the presence of anti-EGFRvIII T-BsAb therapy.[42] However, their cytotoxic potential remains controversial, as another group observed no direct cytotoxicity by human CD4$^+$CD25$^+$CD127^{low} cells.[43,44] Even if T-BsAb-activated Treg cells have cytotoxic potential, either by TCR-mediated killing or by death receptor-ligand interaction, their immunosuppressive activity would outweigh their tumoricidal activity, as supported by our results showing the benefit of transient ablation of Treg cells in the preclinical model.

For therapeutically targeting Treg cells, anti-CD38 monoclonal antibodies (daratumumab and isatuximab) have the ability to deplete CD38$^+$ Treg cells,[14,17] although the expression of CD38 can also be upregulated on activated effector lymphocytes. Alternatively, given that depletion of CD25$^+$ cells from grafts can safely delay the recovery of Treg cells after autologous stem cell transplant in patients with multiple myeloma,[45] Treg-depleted transplantation, followed by T-BsAb-based maintenance therapy may be a rational approach. While long-term ablation of Treg cells may theoretically increase the risk of immune-related adverse events, including cytokine release syndrome, the combination therapy was tolerable in our preclinical study. In addition, as T-BsAb therapy induces rapid clearance of myeloma cells from the BM,[4,5] limited-duration therapy may be preferable to minimize long-term side effects. One of the limitations of this study is that we have not designed the optimal therapeutic approach for targeting Treg cells in combination with T-BsAb therapy in a safe manner. Additionally, the impact of T-BsAb therapy on Treg homeostasis will need to be validated using on-treatment clinical samples. Despite these limitations, our results have important implications for improving the efficacy of T-BsAb therapy.

Disclosures

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Contributions

MC and KN designed the research and wrote the manuscript. MC, CL, WYK, and KN performed experimental work and analyzed the data. DC analyzed data. SCL and MKG provided key materials. 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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