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B7-H3-targeted 4-1BB activation potentiates CD8 T cell-dependent antitumor immunity without systemic toxicity

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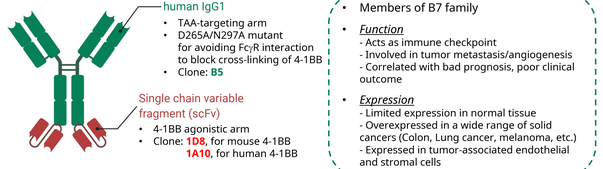
Abstract

Cancer immunotherapy with 4-1BB agonists has limited further clinical development due to dose-limiting toxicity. Here, we developed a bispecific antibody (bsAb, B7-H3×4-1BB), targeting human B7-H3 and mouse or human 4-1BB, to restrict the 4-1BB stimulatory activity in tumors. B7-H3×m4-1BB bsAb elicited a 4-1BB-dependent antitumor response in hB7-H3-overexpressing murine tumor models without systemic immune-related adverse events (irAEs). B7-H3×4-1BB bsAb primarily targets CD8 T cells in the tumor and increases their proliferation and cytokine production. Among the CD8 T cell population in the tumor, 4-1BB is solely expressed on PD-1$^+$ Tim-3$^+$ "terminally differentiated" subset, and bsAb potentiates these cells for eliminating the tumor. Furthermore, the combination of bsAb and PD-1 blockade synergistically inhibits tumor growth accompanied by further increasing terminally differentiated CD8 T cells. B7-H3×h4-1BB bsAb also shows antitumor activity in h4-1BB-expressing mice. Our data suggest that B7-H3×4-1BB bsAb is an effective and safe therapeutic agent against B7-H3-positive cancers as monotherapy and combination therapy with PD-1 blockade.

Introduction of B7-H3×4-1BB bsAb



B7-H3 (CD276)
- Members of B7 family
- *Function*
  - Acts as immune checkpoint
  - Involved in tumor metastasis/angiogenesis
  - Correlated with bad prognosis, poor clinical outcome
- *Expression*
  - Limited expression in normal tissue
  - Overexpressed in a wide range of solid cancers (Colon, Lung cancer, melanoma, etc.)
  - Expressed in tumor-associated endothelial and stromal cells

4-1BB (CD137, *tnfrsf9*)
- Members of TNFR superfamily expressed on immune cells (activated T cell, NK cell, T_{reg}, ···)
- *Function*; *Costimulation*
  - Enhances T, NK-cell effector function (cytokine production, cytolytic activity), proliferation and survival
  - Increases endothelial adhesion molecules resulting in CD8 homing to tumor site
- 4-1BB agonistic mAbs exhibit potent antitumor activity in preclinical models, but display dose-limiting toxicity (e.g. hepatotoxicity) or insufficient efficacy in clinic

Functional Characterization of B7-H3×4-1BB bsAb

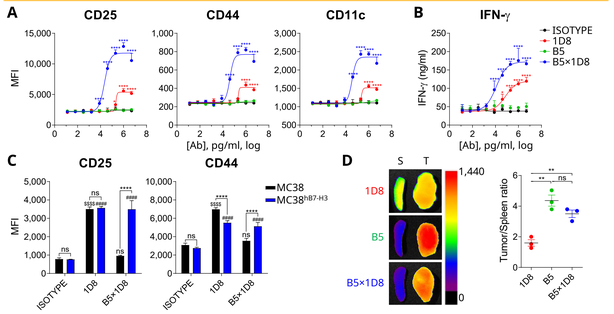


Figure 1. (**A** and **B**) Dose-dependent costimulatory activity of human IgG1 isotype, 1D8, B5, and B5×1D8 on CD8 T cells stimulated with anti-CD3ε (1 μg/ml) and irradiated MC38$^{hB7\text{-}H3}$ cells. Flow cytometric analysis of surface expressions on CD8 T cells (A) and IFN-γ secretion by ELISA (B) 72 hours after stimulation. (**C**) Flow cytometric analysis of surface expressions on CD8 T cells stimulated with anti-CD3ε (1 μg/ml) and irradiated MC38 or MC38$^{hB7\text{-}H3}$ cells with indicated antibodies (1 μg/ml) 72 hours after stimulation. (**D**) Representative *ex vivo* fluorescence images of spleen (S) and tumor (T) (left), and tumor-to-spleen ratio (right) from MC38$^{hB7\text{-}H3}$ tumor-bearing mice 24 hours after intravenous injection of 37.5 μg of 680XL-labeled mAb (1D8 and B5) or 50.0 μg of 680XL-labeled B5×1D8 (*n* = 3/group). $^{*/\$/\#}P$ < 0.05; $^{**/\$\$/\#\#}P$ < 0.01; $^{***/\$\$\$/\#\#\#}P$ < 0.001; and $^{****/\$\$\$\$/\#\#\#\#}P$ < 0.0001, two-way ANOVA with Bonferroni posttests compared with hIgG1 isotype group (A and B); two-way ANOVA with Bonferroni posttests (C); and one-way ANOVA with Bonferroni's multiple comparison test (D). ns, not significant. For (D), * compares two cell lines, $ (for MC38) and # (for MC38$^{hB7\text{-}H3}$) compare each treatment in one cell line. Data presented as mean ± SD.

Absence of irAEs following B7-H3×4-1BB bsAb Treatment

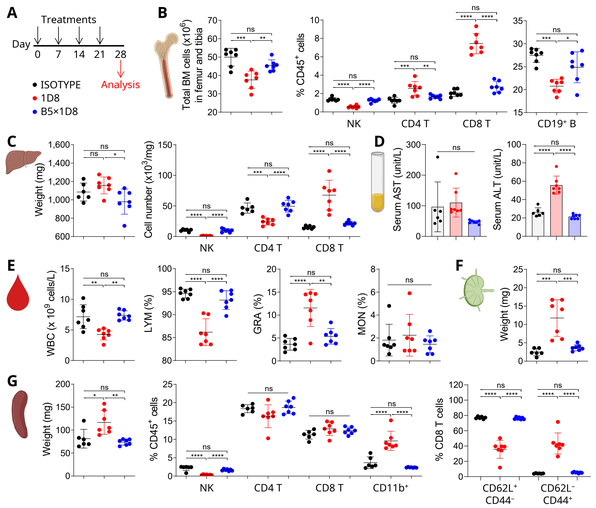


Figure 2. (**A**) Experimental scheme. C57BL/6 naïve mice (*n* = 6-7 per group) were treated with indicated antibodies once a week. Systemic alterations in each organ of antibody-treated mice were addressed 7 days after the last treatment. (**B**) Number of bone marrow (BM) cells (left), NK-, CD4 T-, and CD8 T cell frequency (middle), B cell frequency (right) from femur and tibia. (**C**) Serum AST (left) and ALT (right). (**D**) Liver weight (left), liver-infiltrated NK-, CD4 T-, and CD8 T cell number (right). (**E**) Peripheral blood cell population analyzed by the CBC counter. WBC; white blood cells, LYM; lymphocytes, GRA; granulocytes, MON; monocytes. (**F**) Weight of inguinal lymph node. (**G**) Spleen weight (left), NK-, CD4 T-, CD8 T-, and CD11b$^+$ myeloid cell population (middle), and subtypes of CD8 T cell (right). The immune population in BM, liver, and spleen, was analyzed by flow cytometry. $^*P$ < 0.05; $^{**}P$ < 0.01; $^{***}P$ < 0.001; and $^{****}P$ < 0.0001, one-way ANOVA with Bonferroni's multiple comparison test for (B to G). ns, not significant. Data presented as mean ± SD. All icons were "Created with BioRender.com."

Antitumor Efficacy of B7-H3×4-1BB bsAb

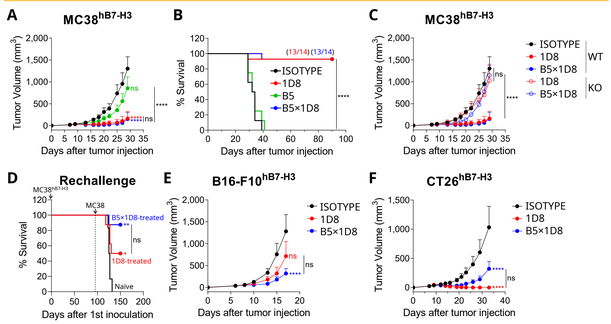


Figure 3. (**A** to **C**) MC38$^{hB7\text{-}H3}$ tumor-bearing C57BL/6 or 4-1BB KO mice (*n* = 7-14 per group) were treated with indicated antibodies on day 7 and 10 after tumor injection and analyzed for tumor growth (A), survival (B) for C57BL/6 mice, and tumor growth for C57BL/6 or 4-1BB KO mice (C). Numbers in survival curves indicate tumor-free mice/total mice at the end of the experiment. (**D**) Long-term survivors (*n* = 6-8 per group) from 4-1BB agonist treatments (A) were rechallenged with MC38 and analyzed for survival. (**E**) B16-F10$^{hB7\text{-}H3}$ tumor-bearing C57BL/6 mice (*n* = 10 per group) were treated with indicated antibodies on day 6, 9, 12, and 15 after tumor injection and analyzed for tumor growth. (**F**) CT26$^{hB7\text{-}H3}$ tumor-bearing BALB/*c* mice (*n* = 11 per group) were treated with indicated antibodies on day 7 and 10 after tumor injection and analyzed for tumor growth. 10.0 μg for mAb and 13.3 μg for bsAb were intraperitoneally administered in all experiments. $^*P$ < 0.05; $^{**}P$ < 0.01; $^{***}P$ < 0.001; and $^{****}P$ < 0.0001, two-way ANOVA with Bonferroni posttests for (A, E, and F); and Log-rank (Mantel-Cox) test for (B and D). ns, not significant. Data presented as mean ± SEM.

Changes in CD8 TILs following B7-H3×4-1BB bsAb Treatment

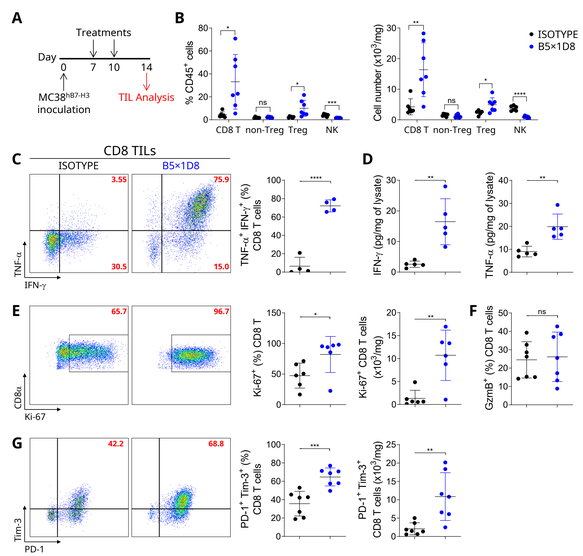


Figure 4. (**A**) MC38$^{hB7\text{-}H3}$ tumor-bearing C57BL/6 mice (*n* = 4-7 per group) were intraperitoneally treated with 10.0 μg of hIgG1 isotype or 13.3 μg of B5×1D8. and tumor tissues were analyzed 4 days after last treatment. (**B** and **C**) Flow cytometric analysis of TIL composition (B, left), cell count per mg of tumor (B, right), and TNF-α and IFN-γ in restimulated CD8 TILs (B). (**D**) The protein level of TNF-α and IFN-γ in the tumor lysate by ELISA. (**E** to **G**) Flow cytometric analysis of Ki-67 (E), GzmB (F), and PD-1/Tim-3 (G) expression in CD8 TILs. $^*P$ < 0.05; $^{**}P$ < 0.01; $^{***}P$ < 0.001; and $^{****}P$ < 0.0001, unpaired Student's *t*-test for (B to G). ns, not significant. Data presented as mean ± SD.

Synergistic Effect of B7-H3×4-1BB bsAb with PD-1 Blockade

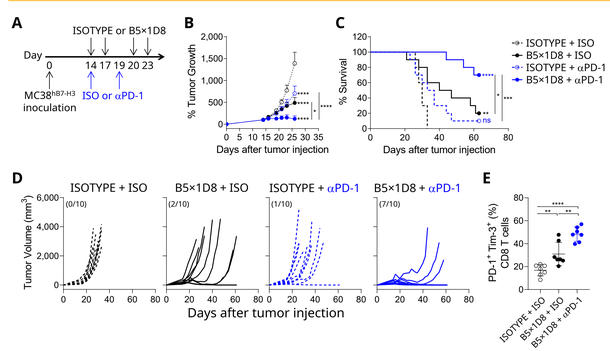


Figure 5. (**A**) Experimental scheme of combination therapy of B7-H3×4-1BB bsAb and anti PD-1 in MC38$^{hB7\text{-}H3}$ tumor-bearing C57BL/6 mice (*n* = 10/group). 37.5 μg of hIgG1 ISOTYPE or 50.0 μg or bsAb were administered intraperitoneally with 200 μg of rat IgG2a isotype (ISO) or anti-PD-1 (αPD-1) from day 14 after tumor injection (when the tumor reached an average volume of 100-200 mm^3). (**B** to **D**) Tumor growth (basal tumor volume at day 14) curves (B), survival curves (C), and tumor growth curves for individual mice (D). Numbers in in each plots in (D) indicate tumor-free/total mice ratios. (**E**) Flow cytometric analysis of PD-1$^+$ Tim-3$^+$ CD8 TILs at day 20 in (A). $^*P$ < 0.05; $^{**}P$ < 0.01; $^{***}P$ < 0.001; and $^{****}P$ < 0.0001, two-way ANOVA with Bonferroni posttests for (B); Log-rank (Mantel-Cox) test for (C); and one-way ANOVA with Bonferroni's multiple comparison test for (E). ns, not significant. Data presented as mean ± SEM for (B) and mean ± SD for (E).

B7-H3×h4-1BB bsAb in Human 4-1BB System

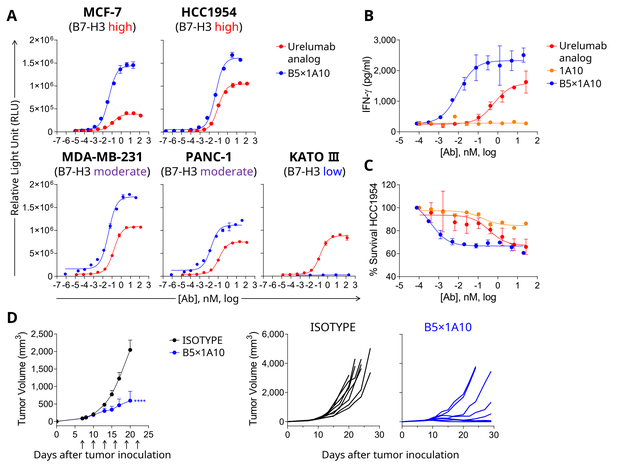
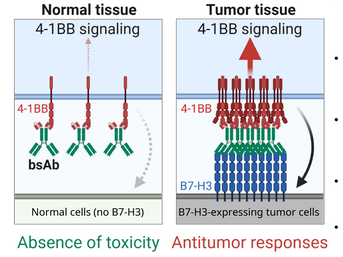


Figure 6. (**A**) Dose-dependent costimulatory activity of Urelumab analog and B5×1A10 on Jurkat-NFκB-luc2/h4-1BB reporter cells. Luminescence was measured 6 hours after stimulation with indicated cancer cells. (**B** and **C**) Dose-dependent costimulatory activity of Urelumab analog, 1A10, and B5×1A10 on PBMCs stimulated with anti-human CD3 (5 μg/ml) and HCC1954 cells. IFN-γ secretion by ELISA (B) and optical cellular density by cell counting kit (C) were analyzed 72 hours after stimulation. (**D**) MC38$^{hB7\text{-}H3}$ tumor-bearing h4-1BB KI mice (*n* = 8/group) were treated with 2.25 mg/kg of hIgG1 isotype of 3 mg/kg of B5×1A10. Black arrows (↑) indicate treatment points. Tumor growth curves of individual mice are shown on the right. $^{****}P$ < 0.0001, two-way ANOVA with Bonferroni posttests for (D). ns, not significant. Data presented as mean ± SD for (A to C) and mean ± SEM for (D).

Conclusion



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- B7-H3×4-1BB bsAb elicits potent T cell costimulatory activity and antitumor efficacy without apparent systemic irAEs.
- B7-H3×4-1BB bsAb increases proliferation and cytokine production of CD8 T cells within TME.
- 4-1BB stimulation on CD8 T cells induce PD-1$^+$ Tim-3$^+$ terminally differentiated population.
- B7-H3×4-1BB bsAb shows synergistic effect with PD-1 blockade.
- B7-H3×h4-1BB bsAb can activate human PBMC and induce antitumor activity in h4-1BB KI mice.

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