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A Potent CD1d-binding Glycolipid for iNKT-Cell-based Therapy Against Human Breast Cancer

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Abstract. *Background/Aim: Invariant natural killer T-cells (iNKT) stimulated by CD1d-binding glycolipids have been shown to exert antitumor effects by a number of studies in a mouse model. Breast cancer is a devastating disease, with different types of breast cancer recurring locally or distant as metastatic/advanced disease following initial treatment. The aim of this study was to examine the tumoricidal effect of a CD1d-binding glycolipid, called 7DW8-5, against a highly invasive human breast cancer cell line both in vitro and in vivo. Materials and Methods: Parental MDA-MB-231 cells and MDA-MB-231 cells transduced with human CD1d were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), followed by loading with glycolipids. After co-culturing with human iNKT cells, the cells were permeabilized and stained with Alexa Flour 647-conjugated antibody to active caspase-3, and analyzed using a BD LSR II. For the in vivo tumoricidal effect, MDA-MB-231 cells transduced with human CD1d and luciferase genes were injected into the mammary fat pad of female NOD/SCID/IL2rγnull (NSG) mice, followed by the injection of human iNKT cells with or without 7DW8-5, and the levels of luminescence were analyzed with whole-body imaging. Results: Human iNKT cells could kill CD1d-expressing human breast cancer cells in vitro in the presence of 7DW8-5, but not α-GalCer. As for in vivo, the adoptive transfer of human iNKT cells into tumor-challenged NSG mice significantly inhibited the growth of CD1d+ MDA-MB-231 human breast cancer cells in the presence of 7DW8-5. Conclusion: CD1d-binding, glycolipid-based iNKT-cell therapy is suggested as a potent and effective treatment against breast cancer in humans.*

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Breast cancer is a devastating disease, with different types of breast cancer recurring locally (1, 2) or distant as metastatic/advanced disease (3) following initial treatment. Metastatic breast cancer usually occurs from months to years after first treatment, and is typically found in the lungs, liver, bones or brain. Nonetheless, breast cancer recurrence is different in each patient; those differences are based on tumor biology (*e.g.* hormone receptor status), stage of disease when first diagnosed, or even on the chemotherapy received by the patient following diagnosis. A recent and promising treatment approach against various forms of cancer is immunotherapy, which takes advantage of the patient's own immune system to attack the tumor cells.

Natural killer T-cells (NKT) are a unique subset of T-cells that share properties of both T-cells and natural killer cells. NKT cells recognize lipid antigens presented by the non-polymorphic major histocompatibility complex (MHC) class I-like molecule, CD1d (4-6). There are two major subpopulations of NKT cells: Type I NKT cells, also called invariant NKT (*i*NKT) cells, and type II NKT cells (7-10). The prototypic antigen for type I NKT cells is α-galactosylceramide (α-GalCer), which stimulates *i*NKT cells to release large amounts of interferon-γ (IFNγ), which helps activate both $CD8^+$ T-cells and antigen-presenting cells such as dendritic cells (DCs) and macrophages (11, 12). Functionally, type I NKT cells exert a protective immune response against tumors (13-17), whereas type II NKT cells are typically associated with immunosuppression (18, 19). More recently, we identified a synthetic analog of α-GalCer, named 7DW8-5, which elicits the most potent *i*NKT-cell response among 100 analogs tested (20). As shown in Figure 1A, this analog differs from α-GalCer in that it possesses a fluorinated benzene ring at the end of a C8 length fatty acyl chain (20). Thus, 7DW8-5 has been shown to have stronger bioactivity towards *i*NKT cells and CD1d-bearing DCs (20-26). In addition, 7DW8-5 also has tumoricidal activity against human medulloblastoma *in vivo,* using humanized mice (17).

In the current study, using a highly invasive and poorly differentiated triple-negative breast cancer cell line, MDA-MB-231, we investigated the ability of α-GalCer and its

analog, 7DW8-5, to display NKT cell-dependent cytotoxicity against these human breast cancer cells *in vitro*; we then analyzed the *in vivo* antitumor-activating activity of 7DW8-5 using immunodeficient mice.

Materials and Methods

Animals, glycolipids and a human breast cancer cell line, MDA-MB-231. A total of 13 female NOD/SCID/IL2rγnull (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained under specific pathogen-free conditions in the animal facilities at the Comparative Bioscience Center of The Rockefeller University. α-GalCer was purchased from Enzo Life Sciences (Farmingdale, NY, USA). 7DW8-5 was synthesized as previously described (20). The structural difference between the two glycolipids is shown in Figure 1A. A human breast cancer cell line, MDA-MB-231, which is a highly aggressive, invasive and poorly differentiated triple-negative breast cancer cell line that lacks estrogen receptor (ER) and progesterone receptor (PR) expression, as well as human epidermal growth factor receptor 2 (27, 28), was purchased from the American Type Culture Collection (Manassas, VA, USA).

A human breast cancer cell line transduced with human CD1d and luciferase genes. A human CD1d-human β2-microglobulin (β2m) (hCD1d–hβ2m) gene was amplified with polymerase chain reaction from a plasmid consisting of a human CD1d gene covalently linked to a human β2m gene that was previously constructed in our laboratory (26). A firefly luciferase (Luc) gene was amplified from a plasmid that was previously established in our laboratory (29). The amplified hCD1d–hβ2m gene and Luc gene were then linked with a T2A linker sequence (ggaagcggagagggcagaggaagtctgctaacatgcggtg acgtcgaggagaatcctggacct). The hCD1d–hβ2m-T2A linker-Luc gene complex was then inserted into a lentivirus vector, pLV-EF1a-IRES-Blast, purchased from Addgene (Cambridge, MA, USA) (30), using a Gibson Assembly® method (New England Biolabs, Ipswich, MA, USA) (31, 32). The final constructs were verified with Sanger sequencing. Viral supernatants were produced by the transfection of 5×10^6/10 mI 293 T-cells in 10-cm petri dishes with 8 μg of the CD1d-Luc plasmid, and the packaging plasmids, psPAX2 (6.5 μg; Addgene) and pMD2.G (2 μg; Addgene) for 24 h. MDA-MB-231 cells (4×10^5) in 12-well plates were infected with 2 mI of the viral supernatants followed by antibiotic selection with 8 μg/ml of blasticidin. (ThermoFisher Scientific, Waltham, MA, USA). After 16-17 days, the successful infection and transduction of human CD1d were confirmed by staining the infected cancer cell line with an allophycocyanin-labeled anti-human CD1d antibody (BioLegend, San Diego, CA, USA), followed by a flow cytometric assay. The successful transduction and expression of Luc were confirmed by measuring the *in vitro* firefly luciferase activity, using luminometry (33).

In vitro cytotoxic effects of human iNKT cells against human breast cancer cells. Parental MDA-MB-231 cells and MDA-MB-231 cells transduced with human CD1d were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) (34, 35), followed by loading with the respective glycolipid at 0.1 μg/mI for 30 min. Human *i*NKT cell lines were established from peripheral blood mononuclear cells, as described elsewhere (36). CFSE-labeled MDA-MB-231 cells pulsed with glycolipid (1×10^5) in 100 μl were then mixed with the same number and volume of human *i*NKT cells as effector cells, in conical polypropylene Costar cluster tubes (Costar, Corning, NY, USA). The cell mixture was centrifuged at low speed ($10 \times g$) for 1 min and incubated for 5 h at 37˚C (34, 37). The cells were then washed with culture media at room temperature, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA, USA) for intracellular staining. Next, the cells were washed twice with Perm/Wash buffer (BD Biosciences) and resuspended in staining buffer. After incubation for 1 h on ice with Alexa Fluor 647-conjugated monoclonal antibody to active caspase-3 (BD Biosciences) (38, 39), the cells were washed and analyzed using a BD LSR II instrument (BD Biosciences).

A less invasive orthotopic injection of human breast cancer cells into the mammary fat pad of female NSG mice. Human breast cancer cells were inoculated into the mammary fat pad of female NSG mice as described elsewhere (40). Briefly, the mice were first anesthetized using 3% inhalant isoflurane. Hair around the abdominal and inguinal fat pads was trimmed and the skin was sterilized with alcohol. With the aid of magnifying surgical loupes, a small incision of less than 3 mm was made externally and caudally to the fourth nipple. The nipple was then lifted, and with the tip of a micro-dissecting scissor blade, the skin was detached from the fascia below and the fat pad exposed. Two million MDA-MB-231/Luc/hCD1d cells were then injected into the fat pad in a small volume (<100 μl) using an insulin syringe. With the aid of a Q-Tip dipped in an antibiotic mixture solution, the exposed fat pad was gently pushed inward, allowing it to easily glide back into place. Finally, the small incision was sealed using tissue adhesive (Vetbond; 3M, St Paul, MN, USA), followed by applying post-operative analgesia (bupivacaine) to the area.

Luciferase expression by noninvasive bioluminescent imaging. On 3, 10, 20 and 30 days after intra-mammary fat pad injection of MDA-MB-231/Luc/hCD1d cells, whole-body images of luciferase expression in NSG mice were monitored using IVIS®Lumina with Living Image software (Caliper LifeScience, Hopkinton, MA, USA), as we and others have described (29, 34). Briefly, after anesthetizing the mice, 200 μl of 15 mg/ml D-luciferin (Gold Biotechnology, St Louis, MO, USA) was injected intra-peritoneally, and whole-body *in vivo* imaging analysis was performed for 30 s to 2 min, using an *in vivo* imaging system (IVIS®Lumina). Luciferase expression data were then quantified using Living Image software (Caliper LifeScience) in a fixed region of interest in terms of photons/s/cm^2/sr.

Data analysis. Statistical analysis of experimental and control data was evaluated by Student's *t*-test. A value of $p<0.05$ was considered statistically significant. Statistical analysis of survival rates was performed by using Gehan-Breslow Wilcoxon test, as previously described (25).

Results

In vitro cytotoxic effects of human iNKT cells triggered by α-GalCer versus 7DW8-5 against human breast cancer cells. We first established a human breast cancer line that expresses human CD1d, namely MDA-MB231/hCD1d, by

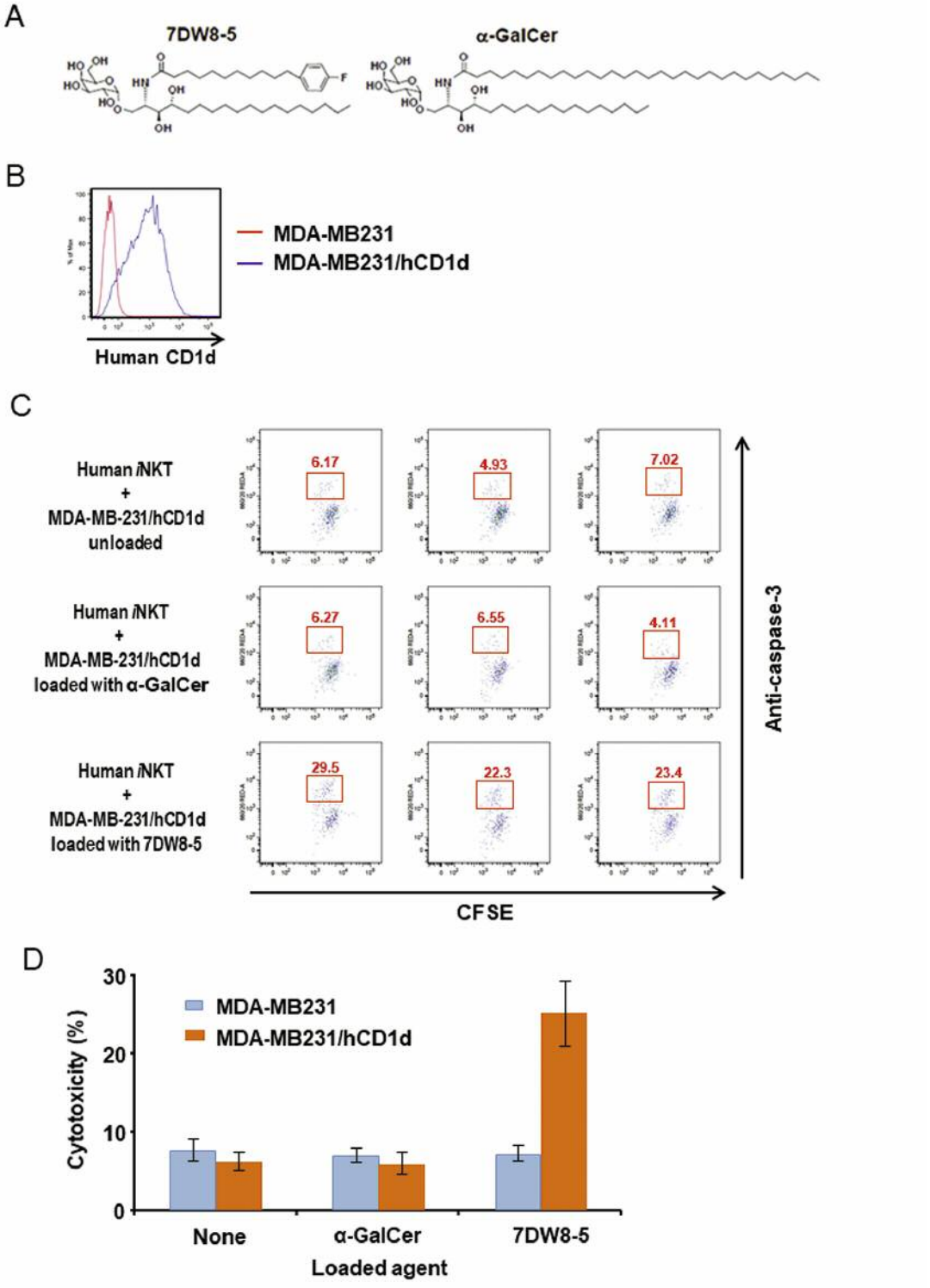

Figure 1. *Structure of glycolipids 7DW8-5 and α-galactosylceramide (α-GalCer) and their ability to trigger cytotoxic activity of human invariant natural killer T- (iNKT) cells against human breast cancer cells in vitro. A: Structural comparison of 7DW8-5 and α-GalCer. B: The expression of human CD1d by MDA-MB-231/hCD1d cells, but not by MDA-MB231 cells by flow cytometric analysis. C: Human iNKT cells were used as effector cells. MDA-MB-231 and MDA-MB-231/hCD1d cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and used as target cells. These two cell populations were co-cultured in the presence or absence of the respective glycolipid, and in-vitro CTL assays were performed by measuring the amount of caspase-3 within the target cells. D: Quantification of the data shown in C. Data are shown from one of three independent experiments with similar results.*

transducing MDA-MB231 line with a plasmid consisting of a human CD1d gene covalently linked to a human β2m gene, as described in the Materials and Methods. Figure 1B shows successful expression of human CD1d by MDA-MB231/hCD1d cells as shown by flow cytometric analysis. In order to determine the *in vitro* cytotoxic activity of α-GalCer and its analog 7DW8-5, the human breast cancer lines MDA-MB231 and MDA-MB231/hCD1d were labeled with CFSE, and used as target cells. After establishing human *i*NKT cell lines, we co-cultured the human breast cancer line with the human *i*NKT cell line as effector cells in the presence or absence of α-GalCer or 7DW8-5. As shown in Figure 1C and D, we found that human *i*NKT cells exerted significant *in vitro* cytotoxic activity against 7DW8-5-pulsed, but not against α-GalCer-pulsed or non-pulsed MDA-MB-231 cells that co-expressed human CD1d, as determined by the level of active caspase-3 activity. Neither of the glycolipids was able to induce killing activity against CD1d-deficient MDA-MB-231 cells (Figure 1C and D).

In vivo tumoricidal effect of 7DW8-5 against human breast cancer cells. Next, we assessed the antitumor effects of 7DW8-5 against the human MDA-MB-231 breast cancer line *in vivo* using immunodeficient NSG mice. These highly immunodeficient mice lack almost the entire mouse-derived immune system, including murine B-cells, T-cells, and NK cells (41). We first inoculated 2×10^6 MDA-MB-231/Luc/hCD1d cells into female NSG mice using a less invasive orthotopic injection into the mammary fat pad (40). Four and 11 days later, 200 μl of medium containing 4×10^6 human *i*NKT cells and 0.1 μg of 7DW8-5, or human *i*NKT cells only or PBS as a control, was administered intravenously to a group of tumor-challenged mice. Finally, we assessed the levels of luminescence by whole-body *in vivo* imaging on days 3, 10, 20 and 30 post-tumor inoculation (Figure 2A). We first ensured that the tumor masses were established in all NSG mice challenged with MDA-MB-231/Luc/hCD1d cells before treating with human *i*NKT cells/7DW8-5, as determined by IVIS (Figure 2C). We then observed that both groups of NSG mice that received human *i*NKT cells and 7DW8-5, as well as human *i*NKT cells only, showed a lower luciferase signal on day 27 post treatment (Figure 2B and C). Nevertheless, a statistical difference was only observed between a group of NSG mice that received human *i*NKT cells and 7DW8-5, as compared with PBS-treated mice (Figure 2C). Furthermore, compared to the PBS-treated mice, all four NSG mice that received human *i*NKT cells and 7DW8-5 survived significantly longer upon tumor challenge (Figure 2D). When we challenged mice with MDA-MB-231/Luc cells that lacked human CD1d gene expression, the glycolipids failed to exert a significant antitumor effect (data not shown).

Discussion

NKT cells, an innate cytotoxic lymphocyte subset, are possessed by all humans (7, 8). In addition, the CD1d molecule, which is highly conserved (4-6) and expressed by some breast cancer cells (29, 43), is able to present a glycolipid, such as 7DW8-5 and its parental glycolipid, α-GalCer, both of which activate NKT cells. 7DW8-5, which possesses a much stronger stimulatory activity against NKT cells than α-GalCer, has already been shown to display a more potent antitumor effect than α-GalCer in mice (17). Therefore, it is conceivable that 7DW8-5 elicits a potent NKT cell-directed attack on CD1d-expressing breast cancer cells in humans. In the current study, we demonstrated that human *i*NKT cells are able to kill CD1d-expressing human breast cancer cells *in vitro* in the presence of 7DW8-5, but not α-GalCer. The distinct difference in the ability of the two glycolipids to mediate *in vitro* killing by human *i*NKT cells may be due to the difference in their binding affinity to CD1d (20) but further investigation is needed to clarify the issue.

For *in vivo* tumoricidal effects, we opted to test only 7DW8-5, as α-GalCer failed to stimulate antitumor effects of human *i*NKT cells *in vitro*. Since NSG mice lack almost all mouse-derived adaptive immunity, as well as NK cells (42), it was possible for human *i*NKT cells to be adoptively transferred to NSG mice without facing an immediate GVH reaction, allowing us to observe the activity of human *i*NKT cells *in vivo* for several weeks. Although not statistically significantly, the adoptive transfer of human *i*NKT cells appeared to suppress tumor growth compared to PBS-treated NSG mice. It is possible that human *i*NKT cells may recognize endogenous glycolipids in the context of CD1d molecules expressed by the tumor. Nevertheless, the inclusion of 7DW8-5 concomitant with the adoptive transfer of human *i*NKT cells into tumor-challenged NSG mice significantly inhibited the growth of $CD1d^+$ MDA-MB-231 human breast cancer cells. In addition, it is noteworthy that the co-administration of human *i*NKT cells and 7DW8-5 to a group of tumor-bearing NSG mice resulted in a significant ($p<0.05$) increase in the survival rate of mice compared with that of PBS-treated NSG mice. 7DW8-5 failed to display its antitumor effect against human breast cancer cells that did not express human CD1d. In this regard, it is certainly imperative to investigate whether 7DW8-5 is able to trigger antitumor effects of human *i*NKT cells *in vivo* in the presence of other cell populations, such as DCs and macrophages that highly express human CD1d molecules. It is also plausible that 7DW8-5 would act as an adjuvant to enhance tumor antigen-specific $CD8^+$ T-cells (25, 43), which in turn can attack human breast cancer cells regardless of CD1d expression. The availability of a humanized mouse model, which possesses an almost entire human immune system that includes not only human *i*NKT cells but also

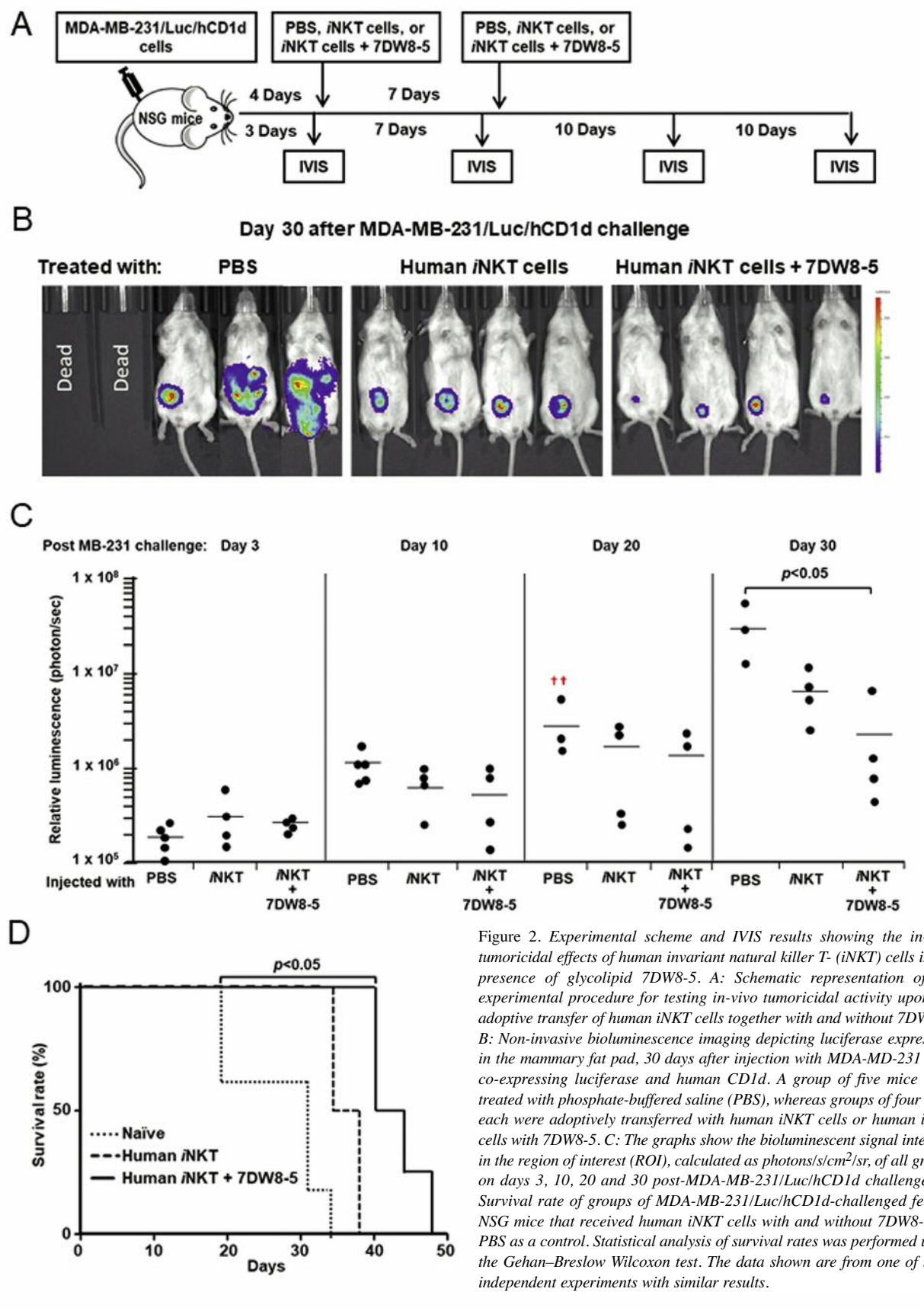

Figure 2. *Experimental scheme and IVIS results showing the in-vivo tumoricidal effects of human invariant natural killer T- (iNKT) cells in the presence of glycolipid 7DW8-5. A: Schematic representation of the experimental procedure for testing in-vivo tumoricidal activity upon the adoptive transfer of human iNKT cells together with and without 7DW8-5. B: Non-invasive bioluminescence imaging depicting luciferase expression in the mammary fat pad, 30 days after injection with MDA-MD-231 cells co-expressing luciferase and human CD1d. A group of five mice were treated with phosphate-buffered saline (PBS), whereas groups of four mice each were adoptively transferred with human iNKT cells or human iNKT cells with 7DW8-5. C: The graphs show the bioluminescent signal intensity in the region of interest (ROI), calculated as photons/s/cm^2/sr, of all groups on days 3, 10, 20 and 30 post-MDA-MB-231/Luc/hCD1d challenge. D: Survival rate of groups of MDA-MB-231/Luc/hCD1d-challenged female NSG mice that received human iNKT cells with and without 7DW8-5, or PBS as a control. Statistical analysis of survival rates was performed using the Gehan–Breslow Wilcoxon test. The data shown are from one of three independent experiments with similar results.*

$CD8^+$ T-cells and DCs (26, 34, 44), will enable us to address this aforementioned issue.

In conclusion, we believe that our current study has provided a proof of principle for using 7DW8-5 as a novel and potent anticancer immunotherapeutic agent *in vivo*, particularly against human $CD1d^+$ tumors. This initial finding dictates the necessity of a more in-depth mechanistic study, to investigate the mode of action and efficacy of 7DW8-5 against a myriad of human cancer cells.

Conflicts of Interest

The Authors have no conflict of interest in regard to this study.

Authors' Contributions

Designed studies: All Authors. Performed assays: TS, MT. Analyzed data: All Authors. Wrote manuscript: TS, MT. Reviewed manuscript: All Authors.

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