Antigen mRNA-transfected, allogeneic fibroblasts loaded with NKT-cell ligand confer antitumor immunity

Shin-ichiro Fujii,1 Akira Goto,1 and Kanako Shimizu1,2

1Research Unit for Cellular Immunotherapy and 2Research Unit for Therapeutic Model, The Institute of Physical and Chemical Research (RIKEN), Research Center for Allergy and Immunology (RCAI), Yokohama, Japan

Introduction

Dendritic cells (DCs) play a pivotal role in determining the character and magnitude of an immune response. Because of this, many studies have developed methods to manipulate DCs to either augment the subsequent cellular immune response (to treat infectious diseases or cancer) or mute the immune response (to treat autoimmune diseases).1,2 In the context of cancer therapy, 2 approaches are the most promising: ex vivo generation of antigen-loaded mature DCs (ex vivo DC strategy), and targeted delivery of antigen to in vivo DCs followed by subsequent DC maturation (in vivo DC strategy). Previous studies based on tumor antigen-loaded DCs have produced encouraging results for some types of cancer. Antigen loaded onto DCs can take several forms: peptides,3 whole proteins,4 dying tumor cells,5-7 leukemic DCs,8 tumor-cell–derived mRNA,9 and DNA.10,11 Short peptides corresponding to epitopes encoding mRNA, thus combining the advantageous effects of INKT-cell activation with delivery of antigen to DCs in vivo. We found that these cells produce antigen protein and activate NK and INKT cells. When injected into major histocompatibility complex (MHC)–mismatched mice, they elicited antigen-specific T-cell responses and provided tumor protection, suggesting that these immune responses depend on host DCs. In addition, antigenexpressing fibroblasts loaded with α-GalCer lead to a more potent T-cell response than those expressing NK cell ligands. Thus, glycolipid-loaded, mRNA-transfected allogeneic fibroblasts act as cellular vectors to provide INKT-cell activation, leading to DC maturation and T-cell immunity. By harnessing the innate immune system and generating an adaptive immune response to a variety of antigens, this unique tool could prove clinically beneficial in the development of immunotherapies against malignant and infectious diseases. (Blood. 2009; 113:4262-4272)
matured by activated iNKT cells to become potent stimulators of the cytolytic immune response.38,43 This concept has been explored with several strategies: tumor cells loaded with α-GalCer (tumor/Gal),44,45 combination therapy with α-GalCer, anti-DR5 Ab, and anti–4-1-BB Ab;46 combination therapy with α-GalCer and TLR-ligands;47 and α-GalCer–loaded, soluble CD1d-fused anti–HER2-scFv fusion protein.48 In our recent studies, we proved that syngeneic tumor/Gal activated iNKT cells and NK cells,10,44 which in turn attacked the tumor cells. Tumor cells killed by activated iNKT cells were then captured by endogenous DCs in a milieu of inflammatory cytokines, leading to the generation of tumor-specific cytotoxic T cells and subsequent antitumor immunity.45

Our current study modifies the strategy of tumor/Gal: instead of tumor cells as a source of antigen, we use allogeneic fibroblasts transfected with mRNA encoding tumor antigen. This approach could prove clinically useful in situations where access to autologous tumor is limited or response to a specific tumor antigen is desired.

### Methods

#### Mice and cell lines

Pathogen-free C57BL/6 (B6) mice were purchased from CLEA Japan (Tokyo) at 6 to 8 weeks of age, and B6 CD4<sup>−/−</sup> and CD8<sup>−/−</sup> female mice were purchased from Jackson Laboratory (Bar Harbor, ME). OT-I T-cell receptor (TCR) transgenic mice were generously provided by Dr Heath (Walter and Eliza Hall Institute, Victoria, Australia). CD11c-DTR/GFP mice were a kind gift of Dr Littman (New York University, New York, NY) and were backcrossed 12 generations to C57BL/6 mice. Jo18<sup>−/−</sup> mice were generously provided by Dr Taniguchi (RIKEN) and were backcrossed more than 9 generations to C57BL/6 mice. These mice were maintained under specific pathogen–free conditions and studied in compliance with our institutional guidelines. B16, EL4, and EG7 cell lines were obtained from ATCC (Rockville, MD), and NIH3T3 cells were obtained from RIKEN Cell Bank. As previously described,44 for introduction of CD1d, pMX-mCD1d-IRES-GFP carrying mCD1d was retrovirally transduced into B16 melanoma or NIH3T3 cells, and cells were subsequently sorted based on the expression of green fluorescent protein (GFP) by the FACS Vantage cell sorter.

#### Cell preparation

Bone marrow–derived DCs were generated from bone marrow progenitors as previously described.49 On day 6, α-GalCer (100 ng/mL) was added to DCs for 40 hours, and 100 ng/mL of lipopolysaccharide (LPS) was added for the last 16 hours. For loading of α-GalCer to other cell lines, fibroblasts (NIH3T3 or CD1d<sup>−/−</sup>-NIH3T3) or tumor cells were cultured for 48 hours in the presence of 500 ng/mL of α-GalCer. These α-GalCer–loaded cells were washed 3 times before injection. CD1d<sup>−/−</sup>-NIH3T3 was prepared as previously described.

CD70-NIH3T3, Rae1<sup>e</sup>-NIH3T3, Rae1γ-NIH3T3, and Multz1-NIH3T3 were prepared as follows. Briefly, the mouse CD70 complementary (c) DNA, Rae1<sup>e</sup>cDNA, Rae1γ cDNA, and Multz1 cDNA were cloned into the retroviral vector carrying pMX-ligand CDNA-IRES-GFP and infected into NIH3T3. The cells were subsequently sorted by expression of GFP.

#### Preparation of EGFP, OVA, and TRP-2 mRNA

Each full length of cDNA (EGFP, OVA, TRP-2) was subcloned using the pSP64 poly(A) vector (Promega, Madison, WI; Document S1 and Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). The vector carrying each cDNA was amplified and then linearized by cutting with the enzymes EcoRI (in the case of EGFP or OVA) or PvuII (in the case of TRP-2). After making capped-mRNA, Ribo m<sup>G</sup> cap analog (Ambion, Austin, TX) was incorporated during the RiboMax transcription reaction for amplifying mRNA with RiboMax large-scale RNA production systems–SP6 (Promega).

#### Transfection of mRNAs

In vitro transcribed (IVT) RNAs were transfected into different cell lines with the TransMessenger transfection kit (QIAGEN, Valencia, CA) following the manufacturer’s protocols. Briefly, 2 × 10<sup>5</sup> cells were seeded one day before transfection on 60-mm tissue-culture Petri dishes. The next day, cells were washed with phosphate-buffered saline (PBS) 3 times and transfected with different amounts of IVT RNAs. The ratio of mRNA, enhancer solution, and transmessenger reagent was 1:2:4. The cells were transfected for different lengths of time and then either directly harvested or incubated for an additional 16 hours in RPMI 1640 containing 10% fetal bovine serum. In Figure 1D through F, (1°) refers to the different initial transfection times (2, 4, 8, or 16 hours), and (2°) refers to the additional period of incubation, either 0 or 16 hours. These cells were analyzed for EGFP by fluorescence-activated cell sorter (FACS) and a laser-scanning confocal microscope (TCS-SP2 Leica DMIRE; Leica, Heidelberg, Germany), or measured for OVA Protein by enzyme-linked immunosorbent assay (ELISA; Morinaga Institute of Biological Science, Yokohama, Japan).

#### Real-time polymerase chain reaction assay

Total RNAs from different cell lines were isolated with either RNaseasy kits (QIAGEN) or Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols. In the case of the total RNA isolation from a small number of cells (<2 × 10<sup>5</sup> cells) with Trizol reagent, 5 μg of glycogen (Roche, Indianapolis, IN) was used for the coprecipitation. After the synthesis of cDNA from 1 μg of total RNAs, the quantification of mRNA expression was performed by real-time polymerase chain reaction (PCR) using Taqman probe primers (Applied Biosystems, Foster City, CA).

#### In vivo tumor studies

Mice were immunized intravenously with antigen-encoding mRNA–transfected NIH3T3 fibroblasts loaded with α-GalCer (5 × 10<sup>5</sup> cells/mouse). In experiments evaluating the development of protective immunity to tumor challenge, immunized mice were challenged with tumor cells subcutaneously 2–3 weeks later and then the tumor size was measured. In some experiments, CD4<sup>+</sup> and CD8<sup>+</sup> mice were used as recipient mice.

#### Statistical analysis

Differences in the in vitro data were analyzed using the Mann-Whitney U test. To correct for multiple testing, the Bonferroni correction analysis was used. The conservative Bonferroni corrections were 0.05/2 = 0.025 in Figures 2 and 3, and 0.05/3 = 0.017 in Figure 5. Consequently, P values less than .025 for data in Figures 2 and 3 and P values less than .017 for data in Figure 5 were considered statistically significant.

#### Results

**Determination of optimal conditions to introduce antigen-encoding mRNA into allogeneic cells**

We determined optimal transfection conditions for introducing mRNA into cells by a chemical method using lipofection (Figure S1).50,51 To determine the dose-dependent transduction rate of antigen-encoding mRNA into cells, the expression of EGFP mRNA transcribed in vitro from the linearized EGFP carrying SP64 vector was evaluated. The expression of EGFP in transfected B16 melanoma cells (H2-K<sup>b</sup>) or NIH3T3 fibroblasts (H2-K<sup>b</sup>) was analyzed by fluorescent microscopy at graded doses of mRNA (Figure 1A). By comparing different doses of mRNA transfections, we determined that 5 μg of EGFP mRNA was sufficient to express
The percent of EGFP evaluated by flow cytometry for expression of EGFP. The number shown represents means plus or minus SEM of 4 mice per group. Data are representative of 3 independent experiments.

Figure 1. Determination of optimal conditions to introduce antigen-encoding mRNA into cell lines. (A,B) Graded doses of EGFP mRNA were transduced into B16 melanoma cells (H2-Kb) and 5 μg EGFP mRNA to NIH3T3 fibroblasts (H2-Kq). Levels of EGFP expression were evaluated by confocal microscopy (20×/0.7 NA oil objective). (C) A total of 5 μg EGFP mRNA–transduced B16 and NIH3T3 cells were evaluated by flow cytometry for expression of EGFP. The number shown represents the percent of EGFP+ cells. Data are representative of 3 independent experiments.

(D-F) Whole OVA gene-carrying vector SP64 was linearized, and 5 μg of OVA mRNA was transfected into B16, NIH3T3, or EL4 cells for different incubation periods to determine optimal transduction time (incubation time, 1°). Levels of OVA protein from cell lysates were measured by an ELISA kit (Morinaga Institute of Biological Science) at 2 time points after transduction: 0 hours and 16 hours (incubation time, 2°). Data shown are means plus or minus SEM of 4 mice per group.

Transduction of the CD1d gene into cell lines lacking costimulatory molecules

We previously showed that even CD1d-expressing tumor cells lacking costimulatory molecules were able to present αGalCer to primary iNKT cells. Here, we verified that NIH3T3 fibroblasts and B16 melanoma cells did not express CD40, CD70, CD86, and MHC class II (data not shown). We analyzed parental cell lines (NIH3T3 and B16) for the levels of CD1d expression and established stable variants that had been transduced with a retrovirus expressing high levels of murine CD1d as previously reported (Figure 2A,B). The stable CD1dαβ cell lines were selected by sorting with a FACSVantage cell sorter to a purity greater than 98% (Figure 2B left). Parental B16 melanoma cells and NIH3T3 cells expressed lower levels of CD1d than bone marrow–derived DCs (Figure 2A). We found the highest expression of CD1d on CD1dαβ-NIH3T3 cells compared with other cell lines and DCs by real-time PCR. This finding was verified by FACS (Figure 2B right).

Direct antigen-presenting activity of mRNA-transduced cell lines to T cells in vitro

Established cell lines expressing either high or low levels of CD1d were transfected with OVA mRNA, and cell lysates were analyzed for levels of OVA expression. Parental B16 and CD1dαβ-B16 transfectants demonstrated an almost equivalent amount of OVA production as NIH3T3 or CD1dαβ-NIH3T3 transfectants (Figure 2C). We verified that not only cell lines, but also transfectants, including EGFP-NIH3T3 or CD1dαβ-NIH3T3, were able to be stably transduced with OVA mRNA.

The direct presentation activity of each transfectant as antigen-presenting cells was monitored. To analyze OVA-specific T-cell responses in vitro, a class I highly expressing B16 cell line was established by exposure to recombinant IFN-γ for 12 hours (data not shown). Parental cells or transfectants were cocultured with OVA-specific TCR transgenic CD8+ T cells (OT-I cells) for 48 hours, and IFN-γ levels were measured from the supernatants. IFN-γ secretion was elevated in response to the supernatant from OVA mRNA–transfected B16 cells (B16-ova), but not OVA mRNA–transfected NIH3T3 (NIH3T3-ova; Figure 2D), even though these 2 cell lines secreted similar levels of OVA protein (Figure 2C). This indicates that OVA peptide was expressed in the context of MHC class I molecules, which are the same for OT-I and B16 (Kb), but mismatched on NIH3T3 cells (Kq).
We examined CD1d RNA expression levels in B16 cells, NIH3T3 cells, and murine bone marrow–derived DCs (mBM-DCs) as well as B16 and NIH3T3 cells after retrovirus-mediated transfer of a murine CD1d gene (CD1dhi-B16, CD1dhi-NIH3T3) by real-time PCR. (B) Because the retroviral vector contained both murine CD1d and GFP genes, the stable CD1dhi cell lines were evaluated by FACS (right panel). (C) After a 4-hour transduction, the expression of OVA protein was measured from cell lysates by ELISA. In this experiment, the B16 cell line was pretreated with IFN-γ 16 hours after immunization. The supernatants were collected and IFN-γ in NIH3T3/Gal-activated mice were similar to B16/Gal or CD1dhi-B16/Gal, respectively (Figure 3B). These data further indicate that CD1dhi-NIH3T3/Gal as well as CD1dhi-B16/Gal act as antigen-presenting cells for innate iNKT-cell and NK cell responses in vivo.

Figure 2. Antigen-presenting activity in mice immunized with mRNA-transfected cells loaded with α-GalCer. (A) We examined CD1d RNA expression levels in B16 cells, NIH3T3 cells, and murine bone marrow–derived DCs (mBM-DCs) as well as B16 and NIH3T3 cells after retrovirus-mediated transfer of a murine CD1d gene (CD1dhi-B16, CD1dhi-NIH3T3) by real-time PCR. (B) Because the retroviral vector contained both murine CD1d and GFP genes, the stable CD1dhi cell lines were selected to a purity of more than 98% on a FACSVantage cell sorter (left panel). The CD1d expression of parental cell lines or CD1d transfectants was analyzed by FACS (right panel). (C) After a 4-hour transduction, the expression of OVA protein was measured from cell lysates by ELISA. (D) The antigen-presenting activity was evaluated by coculturing OVA-transgenic CD8+ T cells (OT-I cells) with each type of transfectant for 48 hours. The supernatants were collected and IFN-γ secretion was measured by ELISA. In this experiment, the B16 cell line was pretreated with IFN-γ for 12 hours to enhance MHC class I expression before coculture. (E) C57BL/6 mice were given 2 × 10^5 OT-I cells and then immunized 24 hours later with OVA mRNA-transfectants loaded with or without α-GalCer. Absolute numbers of OT-I cells in the spleen were measured 3 days later. α-GalCer–loaded CD1dhi-B16 transfectant with OVA mRNA (CD1dhi-B16/Gal-ova) was administered to mice as a positive control. Data are representative of 2 independent experiments with 2 mice per group. Data are means plus or minus SEM of 4 mice per group. *P < .025 (CD1dhi-NIH3T3-ova vs CD1dhi-NIH3T3/Gal-ova, CD1dhi-B16-ova vs CD1dhi-B16/Gal-ova).

α-GalCer–loaded fibroblasts activate allogeneic NK and iNKT cells in vivo

We wanted to measure the capacity of allogeneic cells with or without α-GalCer to stimulate the innate immune system in vivo. NK cell responses were analyzed by flow cytometry for the expression of CD69 and IFN-γ 16 hours after immunization. NK cells up-regulated CD69 and secreted IFN-γ in mice given CD1dhi-NIH3T3/Gal. Only a weak allogeneic response was seen in NK cells from mice injected with NIH3T3- or CD1dhi-NIH3T3–injected mice (Figure 3A).

We also analyzed iNKT-cell activation in mice by injecting either parental, CD1d-transfected NIH3T3, or B16 cells with or without α-GalCer. We restimulated spleen cells in an IFN-γ–producing ELISPOT assay with or without 100 ng/mL α-GalCer (filled bar and open bar in Figure 3B). The number of IFN-γ–producing spots in NIH3T3/Gal– or CD1dhi-NIH3T3/Gal–injected mice were similar to B16/Gal or CD1dhi-B16/Gal, respectively (Figure 3B). These data further indicate that CD1dhi-NIH3T3/Gal as well as CD1dhi-B16/Gal act as antigen-presenting cells for innate iNKT-cell and NK cell responses in vivo.

Innate lymphocyte-mediated antitumor effects generated by α-GalCer–loaded allogeneic fibroblasts

To assess antitumor effects by innate immune cells in response to injected NIH3T3 in an MHC-mismatched manner, we used a lung metastasis model in which mice were given B16 melanoma cells intravenously and injected 3 hours later with NIH3T3, NIH3T3/Gal, CD1dhi-NIH3T3, or CD1dhi-NIH3T3/Gal. As we have previously shown, resistance to the establishment of lung metastases does not require T cells, but mainly depends on NK and iNKT cells. Mice given allogeneic fibroblasts without α-GalCer readily developed lung metastases (Figure 3C second and fourth columns). However, this did not occur in mice given NIH3T3/Gal or CD1dhi-NIH3T3/Gal (Figure 3C third and last columns). Je18−/− mice, which do not have iNKT cells, did not demonstrate this resistance to tumor metastases (data not shown). These results indicate that the activation of innate lymphocytes by NIH3T3/Gal or CD1dhi-NIH3T3/Gal in vivo is sufficient to block the establishment of lung metastases.

A crucial role of in vivo DC maturation in response to α-GalCer–loaded allogeneic fibroblasts

In our previous studies, we injected mice with α-GalCer–loaded tumor cells and demonstrated that host DCs needed to undergo maturation after capturing antigen to generate T-cell responses. In the current study, as shown in Figure 3A,B, NIH3T3/Gal activated expanded OT-I cells in immunized mice was analyzed 3 days later. As shown in Figure 2E, mice given α-GalCer–loaded, OVA mRNA–transfected CD1dhi-NIH3T3 (CD1dhi-NIH3T3/Gal-ova) showed the OT-I cell proliferation more than mice given OVA mRNA–transfected CD1dhi-NIH3T3 (CD1dhi-NIH3T3-ova). The number of OT-I cells in mice given CD1dhi-NIH3T3/Gal-ova was equivalent to those found in mice given tumor/Gal (ie, CD1dhi-B16/Gal-ova; Figure 2E). Thus, CD1dhi-NIH3T3-ova was unable to stimulate OT-I cells in vitro due to MHC class I mismatch (Figure 2D); however, CD1dhi-NIH3T3/Gal-ova were able to generate OT-I cellular proliferation in vivo. This proliferation despite MHC class I mismatch suggests cross-presentation by endogenous DCs in the allogeneic hosts.
Innate lymphocytes. We then tested whether host DCs were induced by the presentation of OVA antigen to OT-I cells, using CD11c-diphtheria toxin receptor (DTR) transgenic (CD11c-DTR/GFP) mice treated with diphtheria toxin (DT) to ablate host CD11c+ DCs in vivo. In these mice, there was little proliferation of OT-I cells, demonstrating the role of DCs in the cross-presentation of antigens in mice given CD1dhi-NIH3T3-ova–Gal-ova (Figure 4D).

Immunization of C57BL/6 mice with CD1dhi-NIH3T3-ova–Gal-ova leads to a strong adaptive immune response

Once our method of generating an immune response to mRNA-transfected fibroblasts was established using mice injected with transgenic OT-I cells, it is more important to pursue the immune response generated in wild-type mice. We then investigated the importance of iNKT-cell activation and CD1d-expressing fibroblasts in the induction of OVA-specific T-cell responses in wild-type mice using OVA mRNA–transfected fibroblasts loaded with α-GalCer. To study this, we immunized mice with variations of parental or CD1dhi-NIH3T3 cells transfected with OVA mRNA: NIH3T3-ova, NIH3T3/Gal-ova, CD1dhi-NIH3T3-ova, and CD1dhi-NIH3T3/Gal-ova. After 7 days, we collected spleen cells and analyzed the number of CD8+ T cells specific to the OVA peptide SIINFEKL by staining with the Kb/OV A257-264 tetramer. As shown in Figure 5A, the number of OVA-tetramer–positive cells increased in mice given NIH3T3/Gal-ova or CD1dhi-NIH3T3/Gal-ova, but not in mice given NIH3T3-ova or CD1dhi-NIH3T3-ova. These did not occur when Jα18-deficient mice were used as recipients (Figure 5B). In addition, C57BL/6 mice immunized with CD1dhi-NIH3T3-ova–Gal-ova generated a higher number of OVA257-264 peptide–specific T cells than mice given NIH3T3/Gal-ova.

We then compared the magnitude of T-cell responses after priming with the iNKT-cell ligand α-GalCer versus ligands of NK cells, such as retinoic acid early inducible-le (Rae1), Rae1y, CD70, and murine UL16-binding protein-like transcript 1 (Muli1). The NK cell ligands were cloned using an EGFP-carrying retrovirus vector. Coexpression of each molecule and EGFP was verified by FACS analysis (data not shown). T-cell proliferation was
evaluated with tetramer staining one week after immunization with CD70-NIH3T3-ova, Rae1-NIH3T3-ova, Mult1-NIH3T3-ova, or Rae1γ–NIH3T3-ova. As is shown in Figure 5C, the Rae1–NIH3T3–ova– and CD70–NIH3T3–ova–immunized groups demonstrated K/ova-specific tetramer–positive cell proliferation, but the other NK ligand–immunized groups did not. T-cell responses specific for OVA were also tested using IFN-γ ELISPOT. The number of IFN-γ–producing T-cell responses was much higher in mice given CD1^d–NIH3T3/Gal-ova than in mice given Rae1–NIH3T3–ova, Mult1–NIH3T3–ova, CD70–NIH3T3–ova, or CD1^d–NIH3T3–ova (Figure 5D). Therefore, α-GalCer–loaded, antigen-carrying fibroblasts lead to a stronger immune response by linking innate and adaptive immunity in naive mice.

**Vaccination with CD1^d–NIH3T3/Gal-ova induces antitumor T-cell immunity**

To evaluate whether the T-cell response in mice immunized with CD1^d–NIH3T3/Gal-ova can lead to antitumor immunity, mice were challenged subcutaneously with 2 × 10^5 EL4 thymoma or OVA-expressing EL4 (EG7) 2 weeks after immunization intravenously with 5 × 10^5 NIH3T3-ova, CD1^d–NIH3T3–ova, NIH3T3/Gal-ova, or CD1^d–NIH3T3–ova (Figure 6). Antitumor effects in mice given CD1^d–NIH3T3/Gal-ova were shown against EG7, but not EL4, indicating a tumor-specific immune response (Figure 6B). In 11 mice immunized with NIH3T3/Gal-ova, 3 mice demonstrated inhibition of tumor growth, but in 8 mice, the vaccination failed to provide the protective effect (Figure 6A top right). These results indicated that CD1d expression is for adaptive immunity rather than innate immunity, probably due to the α-GalCer–loading capacity. Mice given NIH3T3-ova (Figure 6A), CD1^d–NIH3T3–ova (Figure 6B), or CD1^d–NIH3T3–ova (data not shown) developed EL4 and EG7 tumors. Protection against tumor development after subcutaneous inoculation requires CD4^+ and CD8^+ T-cell responses (Figure 6C). We also tested if the CD1^d–NIH3T3/Gal-ova cells would provide the same protection from tumor development after irradiation with 30 Gy, and we found similar results in groups of mice receiving irradiated cells (data not shown).

**Antitumor effects driven by adaptive immunity in response to α-GalCer–loaded, trp2 mRNA–transfected NIH3T3 cells**

We demonstrated the link between innate and adaptive immunity by immunizing with α-GalCer–loaded, mRNA-transduced allogeneic cell lines in OVA models (Figure 6). We then applied this concept to real tumor models by immunizing mice with CD1^d–NIH3T3/Gal cells transduced with mRNA encoding the melanocyte differentiation antigen, tyrosinase-relating protein 2 (trp2). Trp2 expression in NIH3T3-trp2 was verified by reverse transcription (RT)–PCR (Figure 7A), quantified by real-time PCR (Figure 7B), and shown to be nearly 3 times that of trp2 endogenously expressed on B16 melanoma cells.

Adaptive antitumor responses to injected trp2-encoding mRNA-transfected CD1^d–NIH3T3/Gal were assessed. Mice were immunized intravenously with CD1^d–NIH3T3/Gal-trp2, CD1^d–NIH3T3/Gal, or CD1^d–NIH3T3–ova. When the mice were given subcutaneous challenge of B16 melanoma cells 2 weeks later to assess antitumor protection, growth of B16 tumor cells was inhibited in mice that received CD1^d–NIH3T3/Gal-trp2 (Figure 7C bottom left), but not in mice receiving CD1^d–NIH3T3-trp2 (Figure 7C middle left) or CD1^d–NIH3T3/Gal (Figure 7C top right). None of the groups of immunized mice demonstrated any antitumor immunity against EL4 thymoma cells.

We then assessed the effects of immunization with trp2-encoding mRNA-transfected CD1^d–NIH3T3/Gal on established tumors. As shown in top of Figure 7D, 9 mice were injected with B16 cells subcutaneously. The mice were then injected (intravenously) with 5 × 10^5 CD1^d–NIH3T3/Gal-trp2 cells on days 5 and 12, and tumor size was evaluated. Inhibition of tumor growth was
apparently seen in immunized mice until day 20 (Figure 7D), although no mouse demonstrated complete rejection of the tumor.

Discussion

In this study, we have established an antitumor vaccination strategy in which allogeneic fibroblasts are loaded with α-GalCer and transduced with mRNA encoding tumor antigen as a cellular vector. The use of antigen-derived mRNA is beneficial in some instances because mRNA can be derived from tumor cell lines or tumors from third-party patients without the need for human leukocyte antigen (HLA) matching. The advantage of our strategy over previous approaches using tumor-derived mRNA-transduced DCs is the addition of iNKT-cell help, which acts to mature DCs in situ and drives an effective immunogenic immune response.

Instead of transferring DCs that have been transduced with antigen-encoding mRNA, our strategy uses in vivo DCs to capture mRNA-derived antigen proteins in the presence of activated innate lymphocytes, which resulted in DC maturation. As shown in Figure 2D, mRNA-transfected, MHC-mismatched NIH3T3 cells did not present antigen directly to T cells due to the MHC disparity, but MHC-matched B16 cells did. However, mice given OVA-specific OT-I cells followed by immunization with OVA mRNA-transfected NIH3T3/Gal cells demonstrated enhanced proliferation of OT-I cells, indicating that host DCs process antigen from mRNA-transfected cells and cross-prime onto MHC class I molecules (Figures 2E, 4D).

α-GalCer works as an immunologic agent by activating iNKT cells, which in turn mature DCs. The matured DCs then prime T cells in the context of inflammation, thus generating cytotoxic T cells as opposed to tolerized T cells. The use of allogeneic cells, which are easily killed when transferred in vivo, may lead to an enhanced NK as well as iNKT-cell response. However, as is shown in Figure 3, administration of allogeneic NIH3T3 fibroblasts or CD1d−/−NIH3T3 cells alone did not lead to the strong activation of NK or iNKT cells; that is, simple alloantigens did not strongly activate NK or iNKT cells, but α-GalCer loading onto fibroblasts caused both NK and iNKT cells to respond with the secretion of IFN-γ.

Several studies have shown that tumor cells transfected with NK-cell ligands lead to NK cell– and/or CD8+ T cell–mediated tumour rejection due to constitutive cell-surface expression of NKGD2 on almost all NK cells and activated CD8+ T cells. Therefore, we evaluated the ability of known NKGD2 ligands, such as Rae-1e, Rae-1γ, and Mult1 as well as CD70, to act as adjuvants for the generation of T-cell immunity. To compare
iNKT cell–mediated T-cell immunity with NK cell–mediated immunity, we established 3 groups of NKG2D transfectants and one CD70 transfectant (Figure 5). When NK cell ligand transfectants expressing OVA were given, small numbers of tetramer-positive cells were shown in mice given Rae1<sup>-</sup>/H9280-NIH3T3-ova and CD70-<sup>-</sup>NIH3T3-ova, but none were seen in mice given Rae1<sup>+</sup>-NIH3T3-ova or Mult1-NIH3T3-ova. In papers observing the efficacy of NKG2D ligands as adjuvants, CD8<sup>+</sup>/H11001 T-cell responses in mice given Rae-1<sup>-</sup>/H9253–transfected cells were generated (0.25%) only after boosting with additional Rae-1<sup>-</sup>/H9253–transfected cells. These results suggest that targeted activation of iNKT cells leads to a more powerful immunogenic response than activation of NK cells under the same conditions.

Recently, it has been shown that iNKT-cell activation leads to up-regulation of CD70 on DCs and is a key component for the induction of T-cell immunity. Taraban et al showed that CD70–CD27 interaction between DCs and T cells follows CD40–CD40L coupling between DCs and iNKT cells. The importance of this interaction was demonstrated in studies where CD70–CD27 interactions were blocked, abolishing the ability of iNKT cells to generate a cytotoxic T-cell response. As is shown in Figure 4A and B, we immunized mice and measured expression levels of costimulatory molecules after 12 hours (to allow for up-regulation of CD40, CD86, and down regulation of CD119) and 40 hours (to allow for up-regulation of CD70; Figure 4B). In the current study, we found that T-cell priming in situ can be broken down into 2 phases: an initial phase of NKT-cell activation and functional maturation of DCs through CD40–CD40L interaction, and a second phase in which naive T cells are cross-primed by endogenous mature DCs through CD70–CD70L interaction. In particular, CD8<sup>a</sup>– DCs expressed more CD70 and secreted more IL-12 than CD8<sup>b</sup>– DCs, thus suggesting an important role for these cells in this type of immunotherapy (Figure 4).

In terms of adjuvant effect, some Toll-like receptor (TLR) agonists for TLR3, TLR7, and TLR9 have begun for patients with several types of cancer in clinical studies. Imiquimod, a Food and Drug Administration (FDA)–approved topically applied TLR7 agonist, especially has shown clinical efficacy against basal cell carcinoma and has been used in combination with NY-ESO-1 protein in patients with malignant melanoma. Allogeneic cells carrying antigen and transduced with the gene encoding GM-CSFs have been shown to be safe in trials using GM-CSF gene–transduced irradiated cancer vaccine (GVAX) therapy. In the current study, we demonstrated that low concentrations of antigen together with the glycolipid-loaded,
allogeneic fibroblast effectively matured in situ DCs and led to a protective cytotoxic T-cell response.

Immunologically, CD1d expression level may be one of the key factors linking innate and adaptive immunity.\(^4^1\) \(\alpha\)-GalCer–loaded, nontransfected NIH3T3 cells activate NK and iNKT cells, blocking tumor establishment in the lung in mice injected with B16 melanoma (Figure 3C). Nevertheless, mice given NIH3T3/Gal-ova exhibited weaker T-cell responses (Figure 5A) and only partial antitumor effects (Figure 6) compared with mice given CD1dhi-NIH3T3/Gal-ova. To ease clinical application of this method, further investigations into enhancing the use of parental NIH3T3 cells could eliminate the need to transfect cells to overexpress CD1d. Possible alterations in the current method are an increase in the number of vaccinations or the amount of protein, \(\alpha\)-GalCer, or cell dose used.

The combination strategy of mRNA-encoded antigen and iNKT-cell ligand packaged in allogeneic cells offers the advantage of supplying antigen to host DCs in the context of activated iNKT cells. Our strategy replicates the cascade of events occurring in vivo that lead to an effective adaptive immune response. This approach could be translated into clinical trials with the intended outcome of a better antitumor response and fewer side effects compared with conventional cancer therapies.

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Authorship

Contribution: S.F. and K.S. designed and performed research, analyzed data, and wrote the paper; and A.G. performed research and analyzed data.

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Correspondence: Shin-ichiro Fujii, Research Unit for Cellular Immunotherapy, RCRI, RIKEN, Yokohama, Kanagawa, 230-0045, Japan; e-mail: fujii@rcai.riken.jp.
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Antigen mRNA-transfected, allogeneic fibroblasts loaded with NKT-cell ligand confer antitumor immunity

Shin-ichiro Fujii, Akira Goto and Kanako Shimizu