

Host CD40 Is Essential for DCG Treatment Against Metastatic Lung Cancer

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Abstract. *Background/Aim:* For the application of invariant natural killer T (iNKT) cells in cancer therapy, the CD40-CD40L interaction is indispensable in administering alpha-galactosylceramide (α GalCer). We hypothesized that CD40 plays an important role in dendritic cells (DC) pulsed with α GalCer (DCGs) in the treatment of lung metastases. *Materials and Methods:* Wild-type (WT) and CD40^{-/-} mice were treated with DCGs isolated from WT or CD40^{-/-} mice in a B16F10 lung metastases model and NK and NKT cell activity in lungs and the spleen were examined. *Results:* DCG treatment improved WT mice survival but CD40^{-/-} hosts received no survival benefit. Conversely, attenuation of a therapeutic effect in mice treated with CD40^{-/-} DCGs was not observed. The functional activities of NK and NKT cells in DCG-treated CD40^{-/-} mice were partially suppressed. *Conclusion:* Host CD40 is essential for DCG treatment to have a therapeutic effect on B16F10 lung metastases.

Invariant natural killer T cells (iNKT) cells are unique, innate-like populations of T lymphocytes bearing a distinctive invariant T cell receptor (TCR) α chain, known as V α 14J α 18 in mice and V α 24J α 18 in humans. The TCRs of iNKT cells are selected for the recognition of a variety of lipid antigens when bound to a major histocompatibility class I-like molecule, CD1d (1, 2). Ongoing progress in research

on a specific ligand of iNKT cells, alpha-galactosylceramide (α GalCer), and treatment with dendritic cells (DCs) pulsed with α GalCer (DCG) has led to diverse approaches for modulating immune responses, including those involved in vaccination against various types of cancers (3-5).

Following encounters with CD1d/ α GalCer complexes displayed by antigen-presenting cells (APCs), iNKT cells not only produce a large amount of interferon (IFN)- γ , but also up-regulate surface expression of CD40 ligand (CD40L). Ligation of CD40 expressed by DCs is especially important for mediating the maturation and functional activation of these cells, the subsequent up-regulation of co-stimulation molecules, such as CD80 or CD86, and the amplified production of IFN- γ (6, 7). Furthermore, matured DCs are potent producers of interleukin (IL)-12, which induces sustained IFN- γ production by iNKT cells (8, 9). In coexistence with a tumor antigen, mature DCs also support the priming of CD8⁺ T cells and, eventually, the formation of effector and memory cells (10). Finally, iNKT cell production of IFN- γ leads to activation and proliferation of natural killer (NK) cells and, in turn, their production of IFN- γ . Through these sequential events, α -GalCer-induced iNKT cells play a crucial role in these antitumor effects (11).

Based on these findings, we hypothesized that host CD40 also plays an important role in the therapeutic effect of DCG treatment on lung metastases. To further examine this, we established a lung metastasis animal model using B16F10 melanoma cells, as previously described (12). Wild-type (WT) control and CD40^{-/-} mice were treated with DCG derived from WT control (WT-DCG) and CD40^{-/-} mice (CD40^{-/-}-DCG). Here, we first showed the antitumor effect of DCG treatment in CD40^{-/-} mice in the lung metastatic model; however, a therapeutic effect on their survival was not observed. We then performed functional analyses of NKT and NK cells in CD40^{-/-} host mice. We found that IFN- γ was produced after DCG treatment and after

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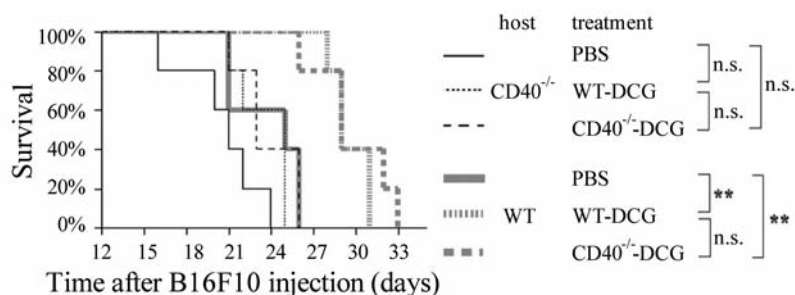


Figure 1. *CD40^{-/-}* hosts did not show a survival benefit after DCG treatment in a B16F10 lung metastases model. Wild-type(WT) C57BL/6 and *CD40^{-/-}* mice were injected intravenously (i.v.) with B16F10 melanoma cells (5×10^5 cells/mouse) on Day 0 and with PBS, WT-DCGs or *CD40^{-/-}*-DCGs (3×10^5 /mouse) on Day 3. The survival of treated mice was monitored. Experiments were repeated three times with similar results. $n=5$ per group. * $p<0.05$; ** $p<0.01$; n.s.: not significant.

re-stimulation and that the cytotoxicity of NK cells was partially suppressed compared to those of WT controls.

This study suggests that host CD40 is essential for DCG treatment to have a therapeutic effect on lung metastases, thus highlighting a new strategy for cancer immunotherapy with NKT cells.

Materials and Methods

Mice and cell lines. Procedures used in mice studies have been described previously (13). Mice were obtained from commercial vendors. Briefly, pathogen-free C57BL/6 female mice (6–8-weeks-old) were purchased from CLEA Japan (Tokyo, Japan). *CD40^{-/-}* mice and IL-12 (p35)^{-/-} mice with a C57BL/6 background were purchased from The Jackson Laboratory (Sacramento, CA, USA). All mice were maintained under specific, pathogen-free conditions and used in compliance with the institutional guidelines of Kobe University (approval numbers: P100305 and P110102).

Cell preparation. Procedures used in immune cell isolation have been described previously (14). Primary cells were isolated from the spleen, lungs and bone marrow of mice and filtered using cell strainers (BD Bioscience, San Diego, CA, USA). Red blood cells were lysed with lysis buffer to obtain single cell suspensions. Immune cells were isolated after lungs were harvested and minced and single cells obtained using 2% collagenase A and 0.75% DNase I (Roche, Mannheim, Germany) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 20°C for 60 min. Purified single cells were stained with a 7-aminoactinomycin D (7-AAD) viability staining solution (BioLegend, San Diego, CA, USA) to label dead cells. Bone marrow-derived DCs were generated in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) as described previously (12). On day 6, cells were incubated with 100 ng/ml LPS and 100 ng/ml α -galactosylceramide (α -GalCer; KR7000; Funakoshi, Tokyo, Japan) for 16 h.

Antibodies and flow cytometry. Flow cytometry has been described previously (12). The following monoclonal antibodies (mAbs) were purchased from BD Biosciences: anti-mouse Cluster of Differentiation (CD)3e, anti-Natural Killer (NK) 1.1 and anti-type

I IFN- γ . Anti-mouse CD1d- α GalCer tetramer was purchased from Proimmune Limited (Oxford, UK). Cells were stained in a 96-well round-bottom plate for 20–30 min at 4°C and washed with PBS containing 5% FBS. Flow cytometry data were obtained using a FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software version 7.6.5 (TreeStar, Ashland, OR, USA).

Cytokine secretion assay. Samples of 4×10^5 spleen and lung cells from naive WT and DCG-immunized mice were cultured in 200 μ l RPMI-1640 medium containing 10% FBS in 96-well round-bottom plates, with or without 100 ng α GalCer/ml for 16 h. Supernatants were collected and IFN- γ production was measured using a CBA assay kit (BD Biosciences). The serum concentrations of IFN- γ and IL-12p70 were also measured using CBA assay kits. For intracellular cytokine staining of NK or NKT cells, splenocytes were incubated in GolgiPlug (BD Biosciences) for 2 h and, then, incubated with anti-NK1.1-allophycocyanin (APC) and CD3e-FITC mAb. After the cell surface was labeled with mAb, cells were permeabilized in Cytotfix/Cytoperm Plus (BD Biosciences) and stained with anti-IFN- γ -phycoerythrin (PE).

⁵¹Cr release assays. B16F10 target cells were labeled with ⁵¹Cr for 1 h at 37°C, washed three times and incubated in triplicate with effector cells at the indicated effector to target cell (E:T) ratio. After a 5-h incubation, ⁵¹Cr release into supernatants was assayed with a scintillation counter (15).

Statistical analysis. Differences were analyzed using Student's *t*-test and a log-rank test. A value of $p<0.05$ was considered statistically significant.

Results

Host CD40 is essential for DCG treatment to increase survival in a B16F10 lung metastases model. Previous studies have demonstrated that the CD40-CD40L interaction is required for NKT cell activation after α GalCer administration (16). We previously showed that allogeneic DCG treatment induced survival benefits in a B16F10 melanoma lung metastases model (12), a well-accepted,

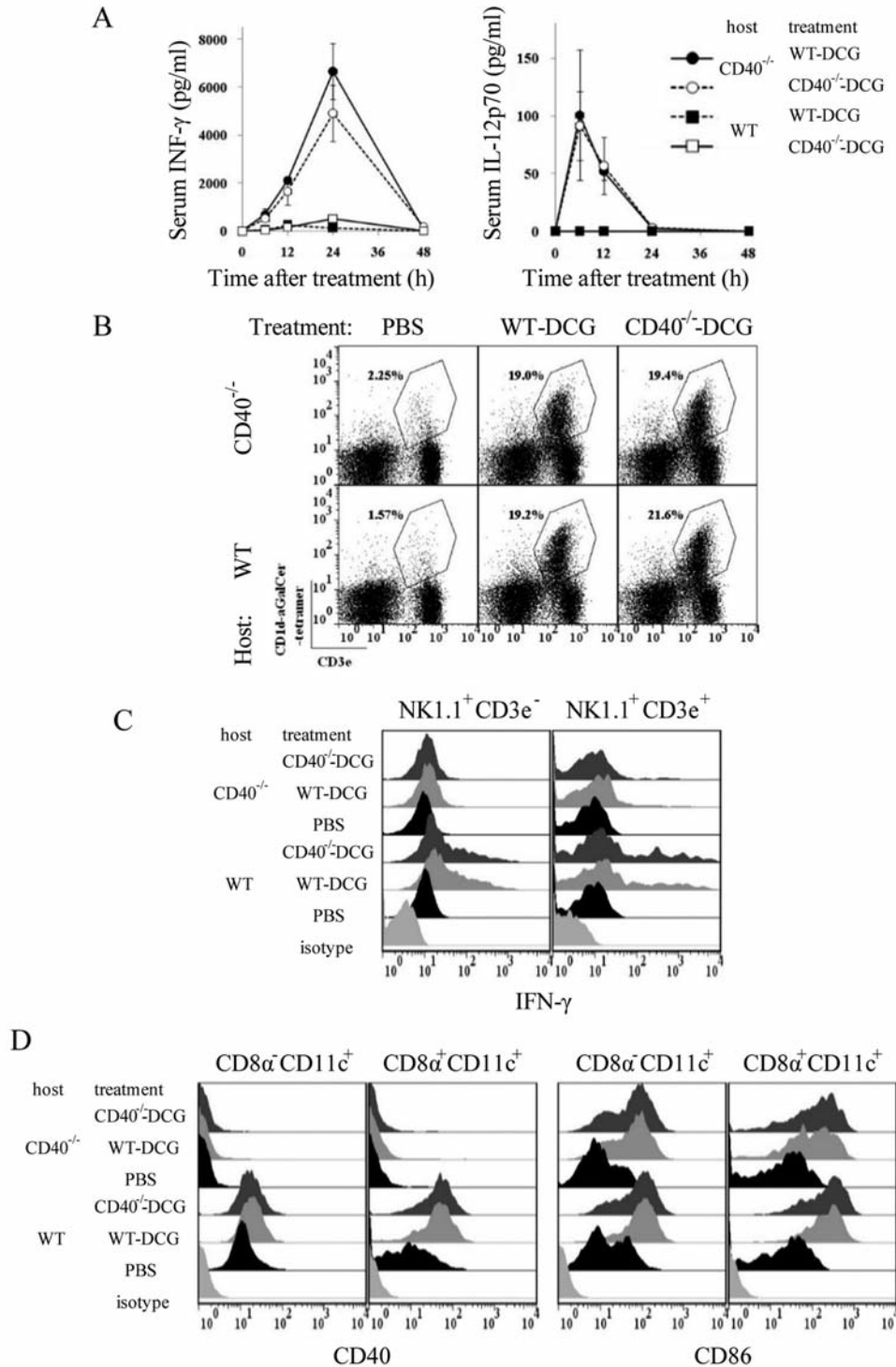


Figure 2. Functional analysis of NK and NKT cells. A: The serum concentrations of IFN- γ and IL-12p70 were significantly and severely suppressed in CD40^{-/-} mice compared to WT controls (** $p < 0.01$). WT and CD40^{-/-} mice were injected i.v. with WT-DCG or CD40^{-/-}-DCGs. After 6, 12, 24 and 48 h, the serum concentrations of IFN- γ and IL-12p70 were measured using a CBA assay kit. B: NKT cell proliferation in the spleens of CD40^{-/-} hosts after DCG treatment on Day 7 in lungs was analyzed for the percentage of CD11a-GalCer-tetramer⁺CD3e⁺ iNKT cells, total cell counts and iNKT cell counts. C: IFN- γ expression in NK and NKT cells from CD40^{-/-} mice was significantly and partially suppressed after DCG treatment compared to that in WT controls (* $p < 0.05$). Similar to A, spleens were analyzed for IFN- γ production by NK1.1⁺CD3e⁻ NK and NK1.1⁺CD3e⁺ NKT cells. D: As with WT controls, CD8 α ⁺ or CD8 α ⁻ DCs from CD40^{-/-} mice showed increased expression of CD86 after DCG treatment. Similar to A, spleens were analyzed for CD40 and CD86 expression on CD8 α -CD11c⁺ and CD8 α ⁺CD11c⁺ dendritic cells. Experiments were repeated three times with similar results. $n = 5$ per group.

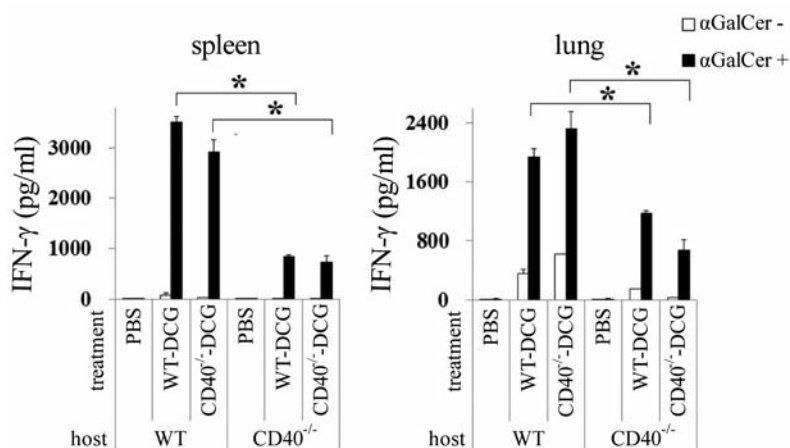


Figure 3. *IFN-γ* expression by spleen and lung cells from *CD40*^{-/-} mice treated with DCGs after re-stimulation was less than that of WT control mice. WT and *CD40*^{-/-} mice were injected i.v. with WT or *CD40*^{-/-}-DCGs. Two days later, 4×10^5 spleen and lung cells/200 μ l of RPMI 1640 medium with 10% FBS were incubated, with or without α GalCer (100 ng/ml), for 16 h. The concentration of *IFN-γ* in the medium was measured using a CBA assay kit. Experiments were repeated three times with similar results. *IFN-γ* production after re-stimulation with α GalCer after DCG treatment is significantly and partially suppressed in *CD40*^{-/-} mice (* $p < 0.05$).

experimental therapeutic model for this disease. Based on these reports, we investigated the effects of CD40 during DCG treatment in *CD40*^{-/-} hosts using this metastatic model. To this end, we first challenged each WT and *CD40*^{-/-} mouse with 5×10^5 B16F10 melanoma cells and, then, immunized mice on Day3 with PBS or 3×10^5 DCGs/mouse isolated from WT (WT-DCGs) or *CD40*^{-/-} mice (*CD40*^{-/-}-DCGs). The treatment of WT mice with WT-DCGs or *CD40*^{-/-}-DCGs significantly improved their survival compared to PBS control WT mice (Figure 1; $p < 0.01$). As expected, the survival benefit of DCG treatment in the lung metastases model was significantly impaired in *CD40*^{-/-} hosts (Figure 1). In summary, a significant difference in survival time was noted when WT controls were immunized with WT-DCGs or *CD40*^{-/-}-DCGs, but not *CD40*^{-/-} mice (Figure 1).

NKT cells proliferate in spleen even in CD40^{-/-} hosts after DCG treatment. The serum concentration of *IFN-γ* was severely suppressed in *CD40*^{-/-} mice compared to WT mice after DCG treatment; also, the concentration of IL-12p70 was significantly and severely suppressed in *CD40*^{-/-} mice (Figure 2A; $p < 0.01$). Additionally, similar effects were noted in the production of these cytokines in mice treated with WT-DCGs or *CD40*^{-/-}-DCGs (Figure 2A). In order to determine the cause of suppression of *IFN-γ* in *CD40*^{-/-} mice, we examined NKT cell activation, which can lead to induction of NKT cells in the spleen, *IFN-γ* production in NK and NKT cells, as well as maturation of DCs. Firstly, we examined NKT cell expansion in the spleen. WT control or *CD40*^{-/-} mice were immunized with PBS, WT-DCGs or

CD40^{-/-}-DCGs to verify the ability to induce NKT cells in the spleen. In spite of a lack of CD40, *CD40*^{-/-} mice showed the same rate of NKT cells expansion in the spleen compared to WT mice, indicating that the absence of CD40 did not have a significant impact on the expansion of NKT cells after DCG (Figure 2B). *IFN-γ* expression in NK and NKT cells was only partially suppressed in the spleen after DCG immunization (Figure 2C). Previous studies have demonstrated that activated iNKT cells have the ability to induce maturation of DCs *in vivo* (6). Matured DCs express increased co-stimulatory molecules, such as CD40, CD80 and CD86. To check such characteristics of DCs, we examined the expression of CD40 and CD86 in splenic *CD8α*⁺ or *CD8α*⁻ DCs after DCG treatment. As with the WT control, the two types of DCs from *CD40*^{-/-} mice showed increased expression of CD86 after DCG administration (Figure 2C).

IFN-γ production after re-stimulation with α GalCer and cytotoxicity of tumor cells after DCG treatment are partially suppressed in *CD40*^{-/-} mice. Previous reports have demonstrated that an immunotherapeutic strategy of cancer immunotherapy, based on DCG administration, is to activate iNKT and NK cells to mediate antitumor effects (12, 17). In these settings, the number of *IFN-γ*-producing innate lymphocytes positively correlated with the extent of antitumor responses (17, 18). We found that *IFN-γ* expression by spleen cells from *CD40*^{-/-} mice treated with DCGs was significantly smaller than that from the spleen and lungs of WT control mice immunized with DCGs (Figure 3; $p < 0.05$ for all). In our previous study, we

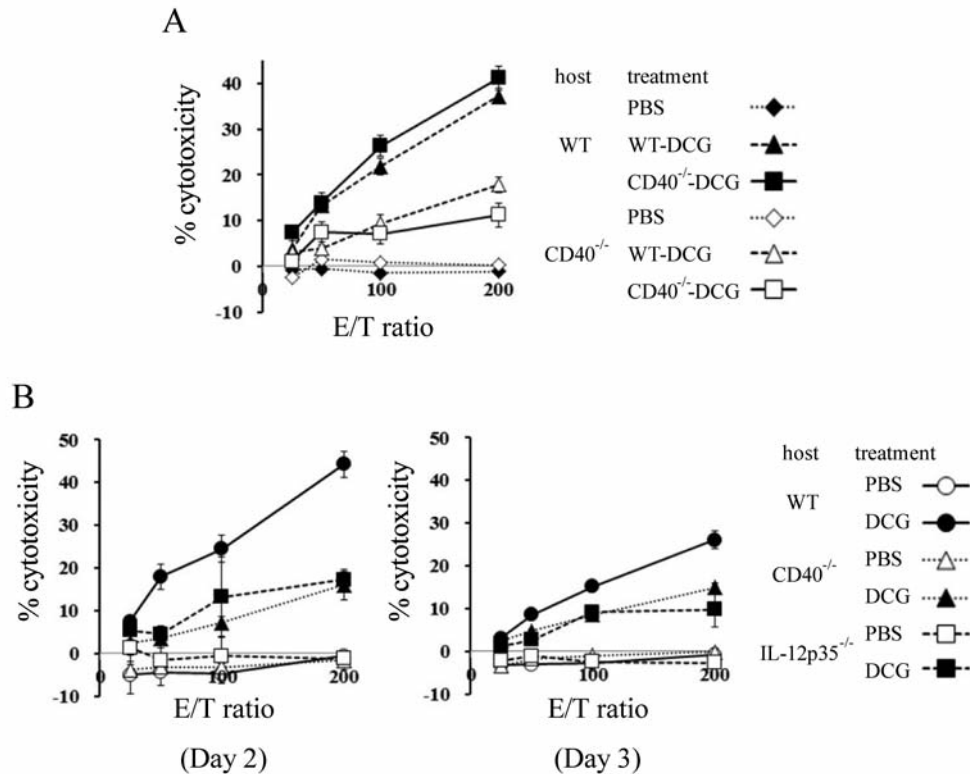


Figure 4. The tumor cytotoxicity of lung cells from CD40^{-/-} mice treated with DCG was impaired compared to that of lung cells from WT mice. A: WT and CD40^{-/-} mice were injected i.v. with PBS, WT-DCGs or CD40^{-/-}-DCGs and, two days later, tested for cytotoxicity by incubating lung cells with ⁵¹Cr-labelled target B16F10 melanoma cells for 5 h at the indicated E:T ratio. The tumor cytotoxicity of lung cells from CD40^{-/-} mice treated with DCG was significantly impaired compared to that of lung cells from WT mice (**p*<0.05). B: Similar to A, WT, CD40^{-/-} and IL-12p35^{-/-} mice were injected i.v. with PBS or WT-DCGs. Experiments were repeated three times with similar results.

demonstrated that lung cells from DCG-treated mice showed higher cytolytic activity that was mainly attributable to NK cells (12). Based on these data, we measured cytotoxicity in the lungs of WT control or CD40^{-/-} mice to estimate NK cell function after immunization with PBS, WT-DCGs or CD40^{-/-}-DCGs. The tumor cytolytic ability of lung cells from CD40^{-/-} mice after WT-DCGs or CD40^{-/-}-DCGs was significantly impaired to that of WT mice (Figure 4A). The tumor cytolytic ability of lung cells from IL-12^{-/-} mice was comparable to that of CD40^{-/-} mice (Figure 4B).

Discussion

The innate and adaptive immune systems do not work independently in antitumor immunity. Activated iNKT cells play a crucial role as mediators between innate and adaptive immune systems to give rise to an optimal immune response with antitumor effects (19). Several studies have demonstrated that after an initial antitumor response following DCG stimulation, NKT cells immediately release a large amount of IFN- γ . In turn, IFN- γ acts on DCs and NK

cells in the innate immune system and successively on CD8⁺ cytotoxic T cells and CD4⁺ Th1 cells to kill tumor cells and, eventually, eradicate tumors (10, 20).

Previous studies of iNKT activation after α GalCer administration have shown that the CD40-CD40L interaction plays a central role in T cell helper function (6, 16). In this study, we showed that host CD40 is essential for DCG treatment to have a therapeutic effect on lung metastases. Our results indicate that in a B16F10 lung metastases model, DCG treatment did not confer a survival benefit on CD40^{-/-} hosts. On the other hand, attenuation of a therapeutic effect when mice were treated with CD40^{-/-} DCGs was not observed. This result suggests that CD40 expression on DCs used for treatment would not be expected to affect therapy. A noteworthy report described how DCGs do not fail to induce anergy after re-stimulation by α GalCer, although the administration of free α GalCer leads to anergy after re-stimulation (17). Subsequent studies revealed that not only myeloid-derived suppressor cells (MDSC) and B cells, but also allogeneic fibroblasts (but only if these led to CD1d expression) can activate NKT cells to have an antitumor

effect (21-23). In mice treated with α GalCer loaded on a recombinant soluble CD1d molecule (α GalCer/sCD1d), repeated injections lead to sustained iNKT and NK cell activation associated with IFN- γ secretion (24). This suggested that binding of CD1d to α GalCer is required for the evasion of anergy after α GalCer re-administration. Consistent with these results, we directly demonstrated that the expression of CD40 on a α GalCer-loaded vehicle is not required for anergy evasion.

Functional analysis revealed that IFN- γ production after DCG administration and after re-stimulation (Figures 2 and 3), as well as the cytotoxicity of mainly NK cells (Figure 4A, 4B) had been only partially suppressed. This impairment did not exert a therapeutic effect on survival. We showed that production of IL-12 in CD40^{-/-} mice was more severely impaired than in WT controls. With regard to DCG treatment, sustained cytokine production by NK and NKT cells after IL-12 production would have been suppressed. Further study is required to elucidate the characteristics of antigen-specific T cells in CD40^{-/-} host mice.

In conclusion, we demonstrated that host CD40 is essential for DCG treatment to have a therapeutic effect on B16F10 metastases. These data suggest a new strategy of controlling malignant tumors by modulating immune responses of iNKT cells.

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Disclosure Statement

The Authors declare no financial or commercial conflicts of interest.

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