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Research Article

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OX40 ligand expressed by DCs costimulates NKT and CD4+ Th cell antitumor immunity in mice

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The exceptional immunostimulatory capacity of DCs makes them potential targets for investigation of cancer immunotherapeutics. We show here in mice that TNF-α–stimulated DC maturation was accompanied by increased expression of OX40 ligand (OX40L), the lack of which resulted in an inability of mature DCs to generate cellular antitumor immunity. Furthermore, intratumoral administration of DCs modified to express OX40L suppressed tumor growth through the generation of tumor-specific cytolytic T cell responses, which were mediated by CD4+ T cells and NKT cells. In the tumors treated with OX40L-expressing DCs, the NKT cell population significantly increased and exhibited a substantial level of IFN-γ production essential for antitumor immunity. Additional studies evaluating NKT cell activation status, in terms of IFN-γ production and CD69 expression, indicated that NKT cell activation by DCs presenting α-galactosylceramide in the context of CD1d was potentiated by OX40 expression on NKT cells. These results show a critical role for OX40L on DCs, via binding to OX40 on NKT cells and CD4+ T cells, in the induction of antitumor immunity in tumor-bearing mice.

Introduction

DCs are professional antigen-presenting cells that undergo phenotypic changes from immature DCs to mature DCs with a reduced capacity for antigen uptake but with an exquisite capacity for antigen-specific immune activation (1, 2). The maturation process is accompanied by the upregulation of surface costimulatory molecules such as B7 family members (CD80/B7-1 and CD86/B7-2) and TNF family members (CD134/OX40 ligand [CD134/OX40L] and CD137/4-1BB ligand), and thus mature DCs become capable of eliciting adaptive immunity by providing costimulatory signaling as well as antigenic stimulation principally to CD4+ Th cells (1–5).

Besides the well-established role of DCs in the adaptive immune system, it is now evident that DCs are also implicated in the innate immune system (1, 2, 6–8). Mature DCs not only prime CD4+ Th cells but also activate innate lymphocytes, including invariant NKT cells, which then release cytokines like IFN-γ for concerted stimulation of innate and adaptive immunity (7, 9–13). With regard to the molecular mechanisms for activating NKT cells, α-galactosylceramide (α-GalCer) and several other glycolipids are currently thought to be NKT cell–stimulatory antigens presented by the nonpolymorphic MHC class I–like molecule CD1d of antigen-presenting cells such as DCs (11, 14, 15). Adding to the antigenic stimulation of NKT cells via CD1d-mediated presentation, the functional consequences of DC maturation appear to promote NKT cell activation, perhaps by increased costimulatory interactions between them (2, 7). However, the costimulatory molecules with which mature DCs stimulate NKT cell activation still need to be determined (2, 7, 12, 13).

Owing to the immunostimulatory activity of DCs in antigen-nonspecific innate and antigen-specific adaptive immunity, DCs are increasingly used as adjuvants for vaccination, and the clinical application has now been investigated in patients with cancer (16–19). In the present study, while evaluating the immunogenic properties of mature DCs with the goal of exploiting their potential in therapeutic vaccines against cancer, we found that induced expression of OX40L on DCs was critical and sufficient for the cells' capacity to promote tumor-specific T cell responses via the engagement of OX40 on NKT cells as well as on CD4+ T cells. We also noted that triggering through OX40 on NKT cells potentiated NKT cell activation mediated by CD1d-restricted recognition of α-GalCer. These findings raise the possibility that OX40-OX40L interactions could couple innate to adaptive immunity and potentially serve as a molecular target for the clinical development of DC-based therapies for cancers.

Results

Essential role of OX40L expressed on DCs in their immunostimulatory capacity. It has previously been noted that the ability of DCs to induce T cell–mediated immunity is dependent on their maturation/activation stage, which can be triggered by a variety of factors, including inflammatory cytokines such as TNF-α (16, 17). To confirm these earlier findings, the immunogenicity of TNF-α–stimulated DCs was examined in an immunization challenge model using OVA as a model antigen. As expected, immunization of C57BL/6 mice with TNF-α–stimulated DCs pulsed with OVA markedly suppressed tumor growth of OVA-expressing EG7-OVA cells relative to control immunizations ($P < 0.0001$ versus all other groups; Figure 1A). The tumor-suppressive effect was well associated with the OVA-specific cytotoxic activity of splenocytes from the immunized mice; animals immunized with TNF-α–stimulated DCs pulsed with OVA caused 37% lysis of EG7-OVA

Nonstandard abbreviations used: Ad, adenovirus; α-GalCer, α-galactosylceramide; LLC, Lewis lung carcinoma; OX40L, OX40 ligand.

Conflict of interest: The authors have declared that no conflict of interest exists.

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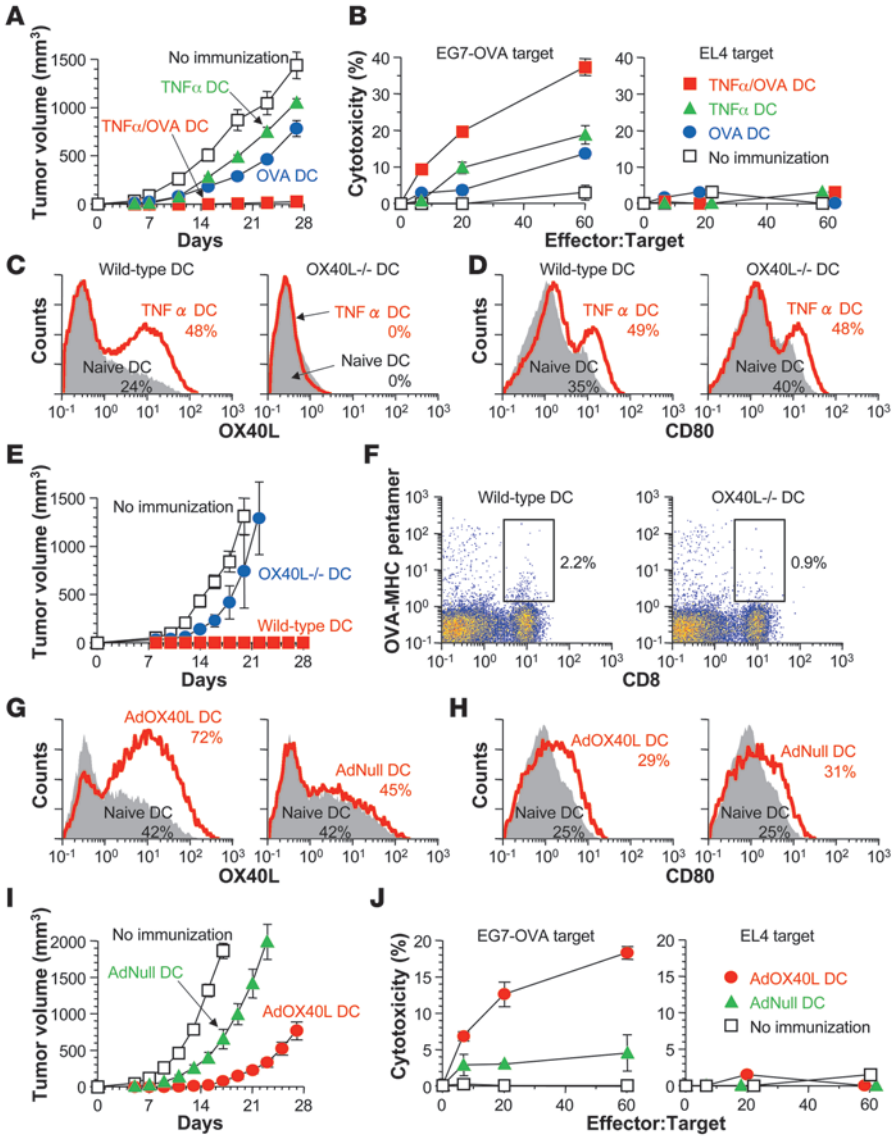

Figure 1

Mice immunized with OVA-pulsed DCs in an OX40L-dependent manner. (**A**) Tumor growth in TNF-α–stimulated DCs. Mice were immunized with TNF-α–stimulated DCs pulsed with OVA (filled squares), DCs pulsed with OVA alone (circles), or TNF-α–stimulated DCs alone (triangles), and were challenged with EG7-OVA cells (day 0). Mice without any immunization (open squares) were used as controls. (**B**) OVA-specific cytotoxicity in TNF-α–stimulated DCs. Eight days after the immunization described in **A**, splenocytes were isolated and assayed for cytolytic function by using EG7-OVA or EL4 cells as target cells. (**C** and **D**) Flow cytometric analysis of TNF-α–stimulated DCs. DCs from wild-type or OX40L$^{-/-}$ mice were stimulated with TNF-α and analyzed 2 days later for OX40L (**C**) or CD80 expression (**D**). Overlay (filled) histograms depict naive DCs. The percentages of stained cells above isotype control staining are shown in each panel. (**E**) Role of OX40L on DCs and in tumor growth. TNF-α–stimulated OX40L$^{-/-}$ (circles) or wild-type DCs (filled squares) pulsed with OVA were used for the immunization challenge experiment. (**F**) Role of OX40L on DCs and OVA-specific cytotoxic T cells. Ten days after the immunization described in **E**, splenocytes were isolated and analyzed for OVA-reactive CD8$^+$ T cells (CD8$^+$H-2K^b/SIINFEKL pentamer$^+$, boxed) by flow cytometry. The percentage of positive cells is listed. (**G** and **H**) Flow cytometric analysis of AdOX40L-modified DCs. DCs were transduced with AdOX40L or AdNull and analyzed 2 days later for OX40L (**G**) or CD80 expression (**H**). The percentages of stained cells above isotype control staining are shown in each panel. (**I**) Tumor growth in AdOX40L-modified DCs. AdOX40L- (circles) or AdNull-modified DCs (triangles) pulsed with OVA were used for the immunization challenge experiment. (**J**) OVA-specific cytotoxicity of AdOX40L-modified DCs. Eight days after the immunization described in **I**, splenocytes were assayed for cytolytic function.

target cells at an effector/target ratio of 60:1, whereas control immunized animals caused approximately 19% lysis ($P < 0.01$; Figure 1B). The specificity of the detected EG7-OVA lysis was confirmed by the lack of apparent lysis against EL4 cells, OVA-negative parent cells (Figure 1B).

We next investigated the expression of surface markers characteristic of DC maturation and/or activation on TNF-α–stimulated DCs (Figure 1, C and D, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI32693DS1). Our results showed that, compared

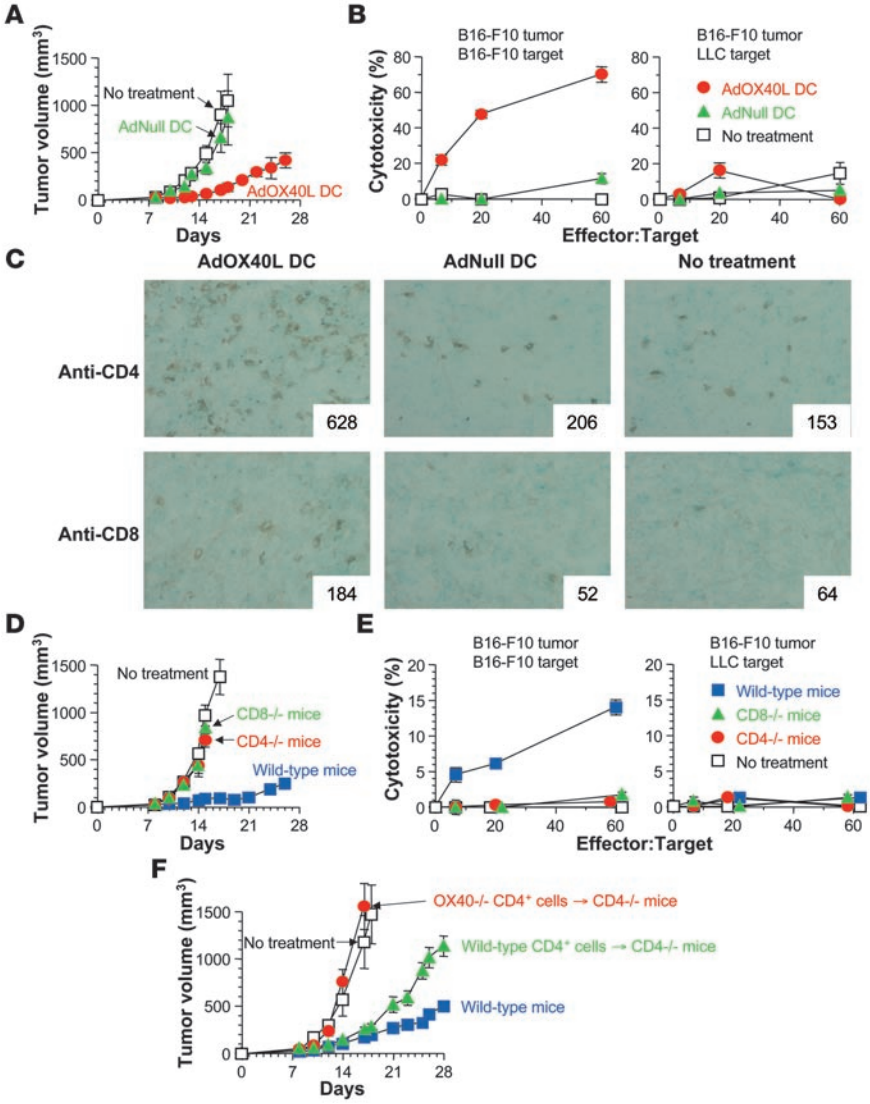

Figure 2
Tumor-bearing mice treated with intratumoral administration of AdOX40L-modified DCs. (**A**) Tumor growth. B16-F10 tumor–bearing mice were treated by intratumoral injection of DCs modified with AdOX40L (circles) or AdNull (triangles). Tumor-bearing mice without any treatment (squares) were used as controls. (**B**) Tumor-specific cytotoxic T cell response. Ten days after the treatment described in **A**, splenocytes were isolated and then assayed for cytolytic function by using B16-F10 or LLC cells as target cells. (**C**) Immunohistochemical evaluation of tumors' $CD4^+$ and $CD8^+$ cells. Three days after the treatment described in **A**, the tumors were dissected, and the frozen tumor sections were stained with anti-CD4 or anti-CD8 antibodies. Numbers at bottom right of each panel denote the number of positive cells per 10 random high-power fields (original magnification, ×400). (**D**) Role of $CD4^+$ and $CD8^+$ T cells in tumor growth. The study was similar to that in **A**, but $CD4^+$ T $cell^{-/-}$ (circles), $CD8^+$ T $cell^{-/-}$ (triangles) or wild-type mice (blue squares) bearing B16-F10 tumors were treated with AdOX40L-modified DCs. (**E**) Role of $CD4^+$ and $CD8^+$ T cells in tumor-specific cytotoxic T cell response. Ten days after the treatment as in **D**, splenocytes were isolated and assayed for cytolytic function. (**F**) Role of OX40 on $CD4^+$ T cells and in tumor growth. The study was similar to that in **D**, but the $CD4^+$ T $cell^{-/-}$ mice were reconstituted with $OX40^{-/-}$ (circles) or wild-type $CD4^+$ T cells (triangles) 1 day before the treatment.

with naive DCs, TNF-α–stimulated DCs had an increase in the number of positive cells for all surface markers examined, and especially exhibited a marked increase of OX40L expression (OX40L, 24% versus 48%; CD54/ICAM-1, 70% versus 81%; CD70, 8% versus 22%; CD80/B7-1, 35% versus 49%; CD86/B7-2, 26% versus 37%; CD137L/4-1BBL, 7% versus 10%).

Thus, the role of OX40L for the induced specific cellular immune response was examined using DCs prepared from $OX40L^{-/-}$ mice for the immunization (Figure 1E). Even when stimulated with TNF-α and pulsed with OVA, $OX40L^{-/-}$ DCs provided no protection against the subsequent EG7-OVA tumor challenge, whereas wild-type DCs showed complete protection ($P < 0.01$, $OX40L^{-/-}$ DCs versus wild-type DCs; Figure 1E). The antitumor effect of the DC/TNF-α/OVA immunization was related to the appearance of OVA-reactive $CD8^+$ T cells in splenocytes from the immunized mice (Figure 1F and Supplemental Figure 2). In this context, the DC/TNF-α/OVA immunization protocol using wild-type DCs induced significant expansion of $CD8^+H\text{-}2K^b$/OVA pentamer$^+$ T cells compared with that in $OX40L^{-/-}$ DCs ($P < 0.005$ versus all other immunization protocols; Figure 1F and Supplemental Figure 2). Although our results showed that $OX40L^{-/-}$ DCs lost the ability to induce antigen-specific cellular immune responses, we found no difference in the surface expression of phenotypic markers between wild-type and $OX40L^{-/-}$ DCs except for OX40L (OX40L, wild-type 48% versus $OX40L^{-/-}$ 0%; CD54, wild-type 81% versus $OX40L^{-/-}$ 84%; CD70, wild-type 22% versus $OX40L^{-/-}$ 23%; CD80, wild-type 49% versus $OX40L^{-/-}$ 48%; CD86, wild-type 37% versus $OX40L^{-/-}$ 34%; CD137L/4-1BBL, wild-type 10% versus $OX40L^{-/-}$ 11%; Figure 1, C and D, and Supplemental Figure 1).

We therefore hypothesized that only the increased expression of OX40L on DCs would be sufficient to improve their immunostimulatory capacity. To evaluate this concept, we used an $E1^-$ adenovirus (Ad) gene transfer vector expressing OX40L cDNA (AdOX40L) to genetically modify DCs, and confirmed that AdOX40L modification of DCs enhanced the expression of OX40L, but did not affect other DC-lymphocyte costimulatory molecules compared with control AdNull modification (OX40L, AdOX40L 72% versus AdNull 45%; CD54, AdOX40L 44% versus AdNull 43%; CD80, AdOX40L 29% versus AdNull 31%; CD86, AdOX40L 19% versus AdNull 18%; Figure 1G and data not shown).

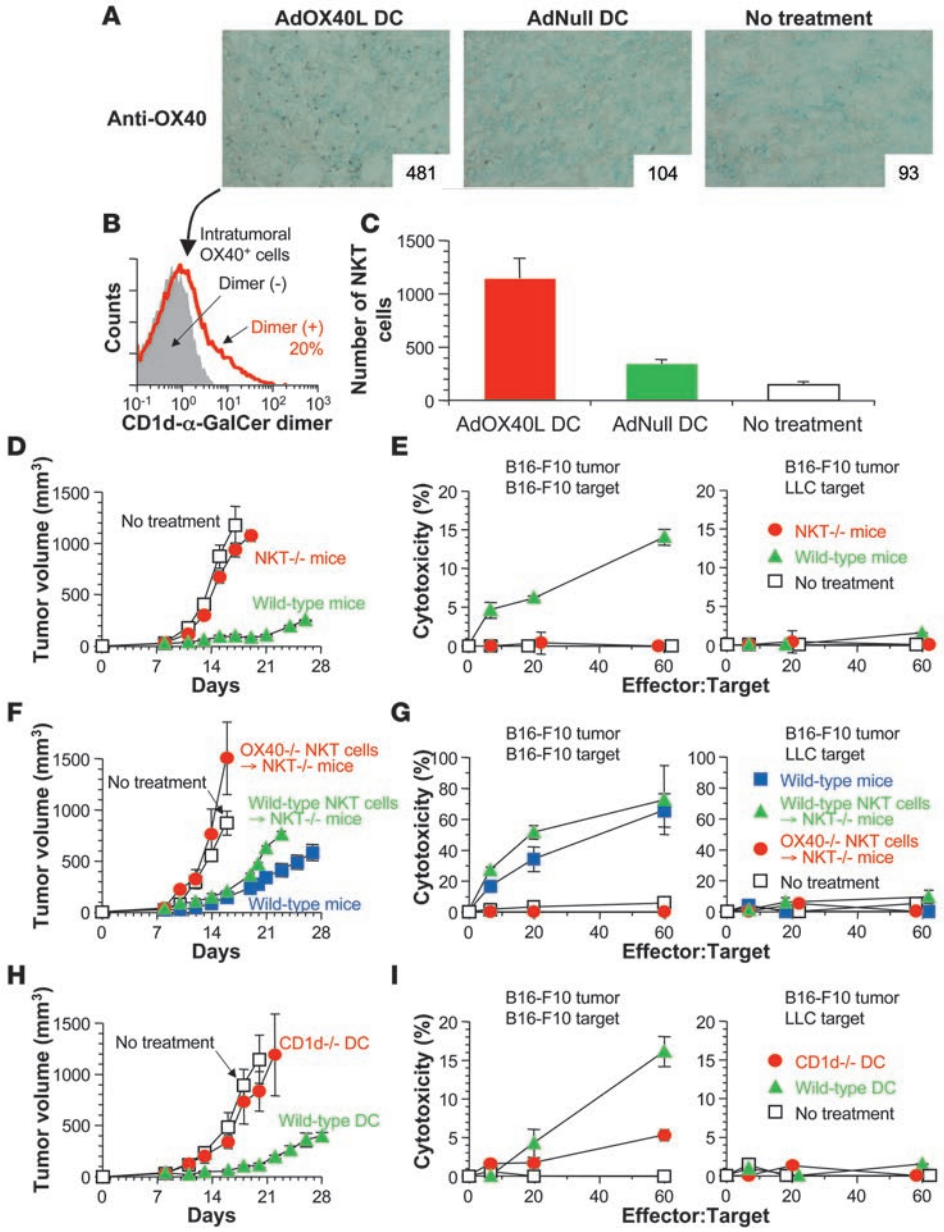

Figure 3
Involvement of NKT cells in the therapeutic effect elicited by intratumoral administration of AdOX40L-modified DCs. (**A**) Immunohistochemical evaluation of tumors for OX40$^+$ cells. Three days after injection of AdOX40L- or AdNull-modified DCs to B16-F10 tumors, frozen sections of the tumors were stained with anti-mouse OX40 antibody. Numbers at bottom right denote the number of positive cells per 10 random high-power fields (original magnification, ×400). Untreated tumors were used as controls. (**B**) OX40$^+$CD1d/α-GalCer dimer$^+$ cells. OX40$^+$ cells from tumors treated with AdOX40L-modified DCs were analyzed for the CD1d/α-GalCer dimer binding by flow cytometry. Overlay (filled) histogram depicts OX40$^+$ cells stained without dimer. The percentage of CD1d/α-GalCer dimer$^+$ cells above control staining is shown. (**C**) Quantification of intratumoral NKT cells. The number of CD1d/α-GalCer dimer$^+$ NKT cells in tumors treated as in **A** was determined by flow cytometry. (**D** and **E**) Role of NKT cells. NKT cell$^{-/-}$ (circles) or wild-type mice (triangles) bearing B16-F10 tumors were treated with AdOX40L-modified DCs. Tumor-bearing wild-type mice without any treatment (squares) were used as controls. (**F** and **G**) Role of OX40 on NKT cells. The NKT cell$^{-/-}$ mice were reconstituted with OX40$^{-/-}$ (circles) or wild-type NKT cells (triangles) 1 day before the treatment. (**H** and **I**) Role of CD1d on DCs. CD1d$^{-/-}$ (circles) or wild-type DCs (triangles) were used to prepare AdOX40L-modified DCs for the treatment. (**D**, **F**, and **H**) The size of each tumor was assessed to evaluate tumor growth. (**E**, **G**, and **I**) At 10 days after treatment, splenocytes were isolated and assayed for cytolytic function using B16-F10 or LLC cells as target cells.

Using the AdOX40L-modified DCs, we examined whether DCs genetically modified to express OX40L alone could efficiently induce antigen-specific cellular immunity. Immunization of mice with AdOX40L-modified DCs pulsed with OVA before EG7-OVA tumor challenge resulted in a significant slowing of the tumor growth relative to control immunizations ($P < 0.001$ versus all other groups; Figure 1I). Consistent with this finding, effector cells from mice immunized with AdOX40L-modified DCs pulsed with OVA exhibited an apparent lysis of EG7-OVA target cells, but not EL4 target cells (Figure 1J). These data indicate that genetic modification of DCs to express OX40L enabled DCs to develop the effective antigen-specific cellular immunity underlying the antitumor effects in the immunization challenge experiments.

DCs modified to express OX40L elicit therapeutic immunity against preexisting tumors. To further examine the immunostimulatory potential of the AdOX40L-modified DCs, we tested the idea of suppressing the growth of preexisting tumors by direct administration of these cells. In these experiments, we injected AdOX40L- or AdNull-modified DCs into B16-F10 tumors established for 8 days in C57BL/6 mice and found a significant growth suppression of tumors treated with AdOX40L-modified DCs ($P < 0.05$ versus all other groups; Figure 2A). Moreover, marked cytolytic activity against B16-F10 tumor cells was also observed in mice treated with AdOX40L-modified DCs, whereas only minimal cytolytic activity was observed in control mice (Figure 2B). As a control for the specificity of the cytolytic activity, no apparent lysis was detected against irrelevant Lewis lung carcinoma (LLC) cells (Figure 2B). Similar results were obtained in the Colon-26 tumor model in BALB/c mice (data not shown).

Next, we performed immunohistochemical analysis to delineate the immunological mechanisms that mediate the antitumor effect of AdOX40L-modified DCs. Our data demonstrated that AdOX40L-modified DCs injected into established subcutaneous

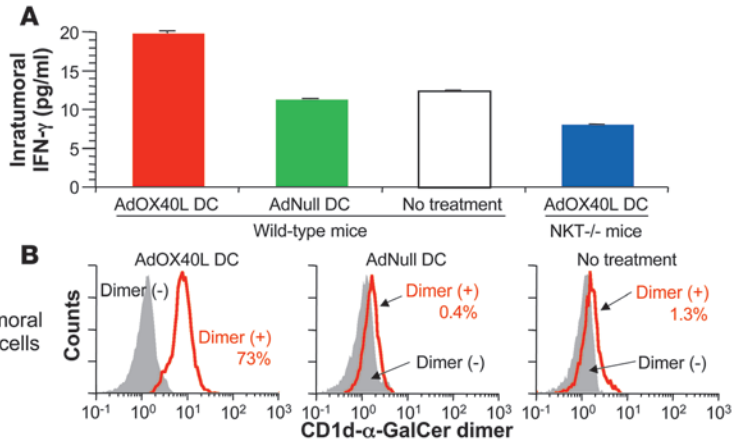

Figure 4
IFN-γ production of NKT cells in tumors treated with AdOX40L-modified DCs. (**A**) IFN-γ levels in tumors. Five days after intratumoral injection of AdOX40L-modified DCs to 8-day established B16-F10 tumors in wild-type or NKT cell$^{-/-}$ mice, the tumors were dissected, and the IFN-γ levels in the tumor homogenates were measured by ELISA. Tumor-bearing mice treated with AdNull-modified DCs as well as those without any treatment were used as controls. (**B**) IFN-γ^{+}CD1d/α-GalCer dimer^{+} cells in tumors. A single-cell suspension was prepared from the B16-F10 tumors treated as in **A**, and the IFN-γ^{+} cells were analyzed for the CD1d/α-GalCer dimer binding by flow cytometry. Overlay (filled) histogram depicts IFN-γ^{+} cells stained without dimer. The percentages of CD1d/α-GalCer dimer^{+} cells above control staining are shown in each panel.

tumors enhanced the intratumoral infiltration of both CD4^{+} T cells and CD8^{+} T cells compared with control tumors (CD4^{+} T cells per 10 high-power fields, AdOX40L DCs 628, AdNull DCs 206, no treatment 153; CD8^{+} T cells per 10 high-power fields, AdOX40L DCs 184, AdNull DCs 52, no treatment 64; Figure 2C). Accordingly, we examined the role of CD4^{+} T cells and CD8^{+} T cells in the antitumor responses; B16-F10 tumors established in wild-type, CD4$^{-/-}$, or CD8$^{-/-}$ C57BL/6 mice were treated by intratumoral injection with AdOX40L-modified DCs. In marked contrast to the tumor suppression of treated wild-type mice, tumors in CD4$^{-/-}$ and CD8$^{-/-}$ mice were not diminished by the treatment of AdOX40L-modified DCs ($P < 0.005$, CD4$^{-/-}$ and CD8$^{-/-}$ versus treated wild-type; Figure 2D). The relevance of the in vivo tumor suppression to the in vitro tumor lysis was demonstrated by showing that the intratumoral treatment of B16-F10 tumor–bearing mice with AdOX40L-modified DCs enhanced the cytolytic activity specific for B16-F10 cells in wild-type mice, but not in CD4$^{-/-}$ or CD8$^{-/-}$ mice (Figure 2E).

OX40, a sole receptor for OX40L, is generally considered to be implicated in CD4^{+} T cell–mediated adaptive immunity, based on evidence that OX40 is expressed primarily on CD4^{+} T cells and that its engagement is required for effective priming of CD4^{+} T cells (3–5, 20–31). Therefore, to evaluate the contribution of OX40 on host CD4^{+} T cells to tumor-suppressive immunity, we reconstituted B16-F10 tumor–bearing CD4$^{-/-}$ mice by adoptively transferring CD4^{+} T cells from wild-type or OX40$^{-/-}$ mice before the intratumoral administration of AdOX40L-modified DCs. Strikingly, the reconstitution of wild-type CD4^{+} T cells into CD4$^{-/-}$ recipient mice restored the ability of the AdOX40L/DC treatment to enhance the antitumor immunity compared with treated wild-type mice, but that of OX40$^{-/-}$ CD4^{+} T cells did not ($P < 0.001$, wild-type versus OX40$^{-/-}$; Figure 2F). These findings indicate that, consistent with the current concept of OX40-OX40L interactions in effective CD4^{+} T cell responses, OX40L-expressing DCs induce the antigen-specific immunity through the OX40 engagement on CD4^{+} T cells.

Requirement of NKT cells for antitumor immunity generated by OX40L-expressing DCs. Further immunohistochemical analysis of the tumor treated with AdOX40L-modified DCs showed an increased number of tumor-infiltrating OX40^{+} cells compared with control tumors (OX40^{+} cells per 10 high-power fields, AdOX40L DCs 481, AdNull DCs 104, no treatment 93; Figure 3A). To characterize the OX40^{+} cells infiltrating the AdOX40L/DC-treated tumor, we stained these OX40^{+} cells with α-GalCer–loaded CD1d dimer, because CD1d molecules loaded with α-GalCer have been used to specifically detect NKT cells in vivo (32, 33). Notably, 20% of the infiltrating OX40^{+} cells bound CD1d/α-GalCer dimer (Figure 3B), indicating that the intratumoral treatment of AdOX40L-modified DCs promotes the infiltration of OX40-expressing NKT cells at this site. This was confirmed by detecting the intratumoral infiltration of CD3^{+}OX40^{+}CD1d/α-GalCer dimer^{+} cells in wild-type mice, which was not observed in NKT cell$^{-/-}$ mice (Supplemental Figure 3). We therefore examined the number of intratumoral NKT cells and observed a marked increased in the tumors treated with AdOX40L-modified DCs compared with control tumors (3- to 7-fold increase; $P < 0.05$; Figure 3C).

These findings prompted us to test whether host NKT cells are required for the tumor-specific immunity developed by the AdOX40L/DC treatment. We found that the B16-F10 tumor size treated with AdOX40L-modified DCs was remarkably suppressed in wild-type mice but not in NKT cell$^{-/-}$ mice ($P < 0.001$, NKT cell$^{-/-}$ versus wild-type; Figure 3D). In line with the in vivo data on tumor growth, splenocytes from B16-F10 tumor–bearing NKT cell$^{-/-}$ mice treated with AdOX40L-modified DCs exhibited no cytolytic activity regardless of the type of target cells, parent B16-F10 cells or irrelevant LLC cells, whereas splenocytes from B16-F10 tumor–bearing wild-type mice treated with the same regimen exhibited a specific lysis of B16-F10 (Figure 3E). The failure of NKT cell$^{-/-}$ mice to develop the AdOX40L/DC-mediated cellular immunity indicates that NKT cells represent an essential cell compartment for the developed immunity.

It was recently reported that blockage of OX40L on DCs with anti-OX40L blocking antibody inhibited the cells' ability to support NKT cell activation (34). Therefore, to extend our finding of surface OX40 expression on NKT cells (Figure 3B), we examined more closely the contribution of OX40 on NKT cells to the cellular immunity afforded by the AdOX40L/DC treatment. In these experiments, B16-F10 tumor–bearing NKT cell$^{-/-}$ mice were reconstituted with NKT cells isolated from wild-type mice or OX40$^{-/-}$ mice before the intratumoral administration of AdOX40L-modified DCs. Although NKT cell$^{-/-}$ mice reconstituted with wild-type NKT cells responded to the AdOX40L/DC treatment with a noticeable suppression of tumor growth, reconstitution with OX40$^{-/-}$ NKT cells failed to restore the responsiveness ($P < 0.01$, wild-type versus OX40$^{-/-}$; Figure 3F). Similar results were also achieved in the treatment using DCs pulsed with α-GalCer, although the tumor-suppressive effect of α-GalCer–pulsed DCs was partially comparable to

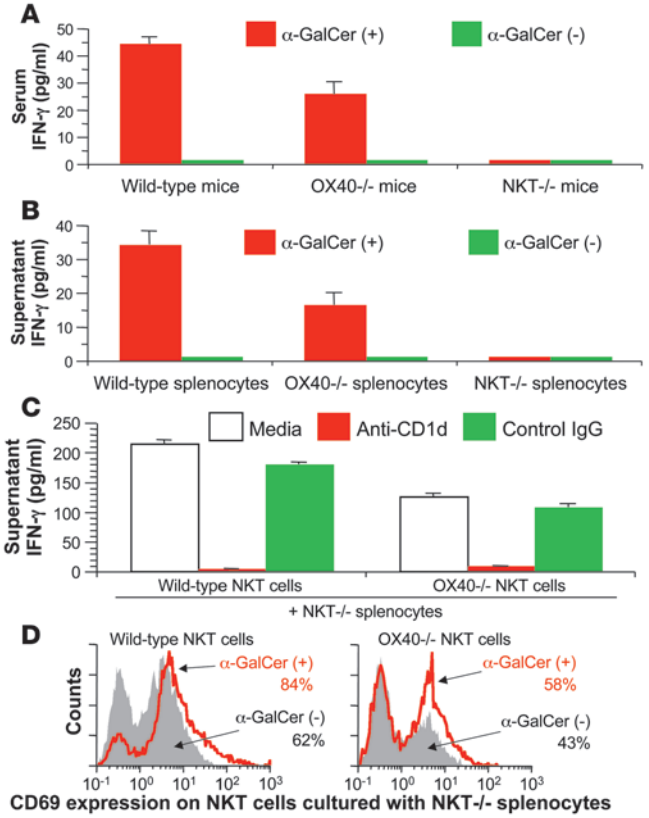

Figure 5
NKT cell activation in an OX40-dependent manner. (**A**) IFN-γ in serum. Wild-type, OX40$^{-/-}$, or NKT cell$^{-/-}$ mice were injected intravenously with α-GalCer or vehicle. The levels of IFN-γ were determined in serum by ELISA. (**B**) IFN-γ in splenocyte culture. Splenocytes were isolated from wild-type, OX40$^{-/-}$, or NKT cell$^{-/-}$ mice and cultured with α-GalCer or vehicle. The levels of IFN-γ in the culture medium were assayed by ELISA. (**C**) IFN-γ in splenocyte coculture. Splenocytes from NKT cell$^{-/-}$ mice were cocultured with NKT cells isolated from wild-type or OX40$^{-/-}$ mice in the presence of α-GalCer. Where indicated, anti-CD1d antibody or control IgG was added at the initiation of the coculture. The levels of IFN-γ were assayed by ELISA. (**D**) CD69 on NKT cells in splenocyte coculture. The study was similar to that in **C**, but at the end of coculture, NKT cells were analyzed for the surface expression of CD69 by flow cytometry. Overlay (filled) histograms depict NKT cells cocultured in the absence of α-GalCer as a control. The percentages of stained cells above isotype control staining are shown in each panel.

that of AdOX40L-modified DCs (days 8–19; $P > 0.1$, AdOX40L-modified DCs versus α-GalCer-pulsed DCs, Supplemental Figure 4A; $P < 0.05$, wild-type NKT versus OX40$^{-/-}$ NKT, Supplemental Figure 4B). This failure of reconstitution with OX40$^{-/-}$ NKT cells in the tumor-suppressive effect of the AdOX40L/DC treatment was paralleled by the tumor-specific cytolytic activity present in the spleens of treated mice (Figure 3G). With the intratumoral administration of AdOX40L-modified DCs to established B16-F10 tumors, the B16-F10–specific cytolytic response was observed in splenocytes from NKT cell$^{-/-}$ mice that had been reconstituted with wild-type NKT cells, but not OX40$^{-/-}$ NKT cells (Figure 3G). Our studies indicate that the development of tumor-specific cellular immunity after the AdOX40L/DC treatment requires NKT cells, especially OX40 on NKT cells of the tumor-bearing host.

Because the majority of NKT cells are thought to be restricted to CD1d-mediated antigen recognition (11, 12), we next evaluated the role of CD1d antigen presentation by DCs in the AdOX40L/DC treatment strategy. When modified with AdOX40L and administered intratumorally to preexisting B16-F10 tumors in wild-type mice, DCs obtained from CD1d$^{-/-}$ mice failed to exert an antitumor effect on the growth, which was significantly different from the result with DCs from wild-type mice ($P < 0.05$, CD1d$^{-/-}$ versus wild-type; Figure 3H). This abrogation of the AdOX40L/DC-mediated tumor suppression correlated with the tumor-specific cytolytic activity primed in vivo (Figure 3I). That is, splenocytes from B16-F10 tumor–bearing mice treated with CD1d$^{-/-}$ DCs displayed roughly 3-fold less cytotoxicity against parental B16-F10 target cells at an effector/target ratio of 60:1 than did splenocytes from mice treated with wild-type DCs (Figure 3I). These results show the requirement of CD1d-dependent antigen presentation by OX40L-engineered DCs for their immunostimulatory capacity, suggesting that OX40L-expressing DCs directly interact with CD1d-restricted NKT cells for the OX40-mediated costimulation.

Infiltrating NKT cells serve as a local source of IFN-γ. Given the observed link of NKT cells to the tumor-suppressive cytotoxic T cell activation of the AdOX40L/DC treatment, and given the notion that the secretion of cytokines by NKT cells influences several aspects of the immune responses (11, 13), we examined the intratumoral levels of IFN-γ, a potent stimulator of cell-mediated immunity, and their association with NKT cells. We injected B16-F10 established tumors with AdOX40L- or AdNull-modified DCs and, 5 days following the injection, measured the concentration of intratumoral IFN-γ. In wild-type mice, the IFN-γ levels of the AdOX40L/DC-treated tumors were increased approximately 2-fold compared with those of AdNull/DC-treated and untreated tumors ($P < 0.0001$; Figure 4A). Increased levels of intratumoral IFN-γ were not detected in NKT cell$^{-/-}$ mice despite the AdOX40L/DC treatment ($P < 0.0001$ versus AdOX40L/DC-treated wild-type, Figure 4A), indicating that the in vivo administration of AdOX40L-modified DCs locally stimulates IFN-γ production in an NKT cell–dependent manner. Consistent with this, CD1d/α-GalCer dimer$^+$ cells (i.e., NKT cells) constituted 73% of IFN-γ$^+$ cells in the AdOX40L/DC-treated B16-F10 tumors as assessed by flow cytometry, but less than 2% in control tumors (Figure 4B). Collectively, these in vivo findings suggest that when delivered intratumorally, DCs modified to express OX40L stimulate NKT cells to secrete IFN-γ in the local milieu and thereby potentiate Th1 and CD8$^+$ cytotoxic T cell responses that are necessary for effective antitumor immunity.

OX40 engagement on NKT cells contributes to their activation status. To investigate more clearly the immunological event of OX40-dependent NKT cell activation without confounding effects that might be caused by the intratumoral administration of genetically modified DCs, we examined the ability of α-GalCer to stimulate NKT cells in OX40$^{-/-}$ mice to produce IFN-γ in vivo and in vitro. Consistent with our knowledge that α-GalCer is a selective ligand that stimulates NKT cell functions such as IFN-γ production (14, 15), we found that intravenous administration of α-GalCer rapidly induced the production of IFN-γ in wild-type mice but not in NKT cell$^{-/-}$ mice (Figure 5A). OX40$^{-/-}$ mice also produced IFN-γ in response to the in vivo administration of α-GalCer, albeit at significantly lower levels than did wild-type mice ($P < 0.05$; Figure 5A). Similar results were obtained in the culture of spleen cells with α-GalCer (Figure 5B). When cultured in vitro with α-GalCer, spleen

cells from OX40$^{-/-}$ mice as well as wild-type mice produced strikingly elevated levels of IFN-γ in the culture supernatants, although the increased amount of IFN-γ observed in OX40$^{-/-}$ spleen cells was significantly less than that observed in wild-type control spleen cells ($P < 0.05$; Figure 5B). Spleen cells from NKT cell$^{-/-}$ mice did not produce detectable level of IFN-γ despite the addition of α-GalCer. These data suggest that the α-GalCer–mediated activation of NKT cells depends in part on the triggering of their OX40.

We therefore extended this observation and confirmed the role of OX40 on NKT cells in their activation. To this end, NKT cells from wild-type mice or OX40$^{-/-}$ mice were cocultured in vitro with spleen cells from NKT cell$^{-/-}$ mice in the presence of α-GalCer and assessed for their IFN-γ responses to α-GalCer. When cocultured with NKT cell$^{-/-}$ splenocytes (containing CD1d-expressing antigen-presenting cells), NKT cells from OX40$^{-/-}$ mice, compared with NKT cells from wild-type mice, produced significantly reduced amounts of IFN-γ in response to α-GalCer ($P < 0.001$; Figure 5C). Similar results were achieved in the blockade of OX40/OX40L interactions with anti-OX40L antibody ($P < 0.005$ versus control IgG; Supplemental Figure 5). We observed no detectable amounts of IFN-γ in either wild-type or OX40$^{-/-}$ NKT cell culture alone (data not shown), eliminating the possibility that other immune effectors contaminating the NKT cell preparations could be responsible for the IFN-γ production observed in the coculture experiment. Moreover, these IFN-γ responses were dependent on CD1d-restricted NKT cell reactivity, because they were blocked by the addition of anti-CD1d antibody to prevent the interaction between NKT cells and CD1d-bearing splenocytes ($P < 0.0001$, anti-CD1d versus control IgG for both wild-type and OX40$^{-/-}$ NKT; Figure 5C). In parallel, we examined the degree of NKT cell activation as assessed by the expression of the CD69 activation marker on NKT cells cocultured with NKT cell$^{-/-}$ splenocytes. CD69 expression was upregulated on NKT cells from OX40$^{-/-}$ mice as well as those from wild-type mice in response to in vitro stimulation with α-GalCer, although the percentage of CD69^{+} cells in OX40$^{-/-}$ NKT cells cultured even in the presence of α-GalCer was lower than that in wild-type NKT cells cultured in the absence of α-GalCer (wild-type NKT, 62% to 84%; OX40$^{-/-}$ NKT, 43% to 58%; Figure 5D). Thus, the results show that OX40 on NKT cells contributes to their activation status regardless of the α-GalCer stimulation, and the involvement of OX40 becomes more apparent under conditions in which NKT cells are stimulated by CD1d-mediated recognition of α-GalCer.

Discussion

DCs represent the most potent antigen-presenting cells of the immune system because they have the capacity for coordinated stimulation of innate and adaptive immunity. Exploitation of this capacity therefore holds great promise for the treatment of cancer patients (16–19). However, the mechanisms of DC action leading to activation of innate lymphocytes such as NKT cells remain to be studied. We showed that OX40L — which is currently known as a major costimulatory molecule for CD4^{+} Th cells — expressed on DCs had costimulatory properties for OX40 not only on CD4^{+} Th cells but also on NKT cells. We confirmed further that the costimulation through OX40 on NKT cells contributed to their CD1d-dependent full activation associated with enhanced IFN-γ production that potentiates Th1-mediated adaptive immune responses. In fact, OX40L expression was upregulated on DCs after TNF-α–stimulated maturation and was responsible for their ability to generate tumor-specific cellular immunity in mouse tumor models, as shown here by using 2 experimental approaches: genetic deficiency and excess of OX40L in DCs. These results indicate that the OX40L expression on DCs is crucial in the development of antitumor immunity, possibly by coupling innate to adaptive immunity.

Whereas the role for DCs in adaptive immune responses is well established in the context of DC-mediated activation of CD4^{+} Th cells, it is generally believed that DCs are also involved in innate immune responses through the interaction with innate lymphocytes, including NK cells, NKT cells, and γδ T cells (1, 2, 6–8). In this regard, DC maturation that leads to priming adaptive T cell responses has been documented secondarily to the activation of all 3 innate lymphocytes, and TNF-α from innate lymphocytes has been identified as an inducer of DC maturation in these studies (35–39). Conversely, mature DCs have been also reported to stimulate NK cells, NKT cells, and γδ T cells, and several cytokines secreted from mature DCs contribute to the activation of these innate lymphocytes (37, 39–42).

With respect to the activation of NKT cells, intensive research efforts have been focused on glycolipid antigens presented by CD1d, and several types of these antigens have been defined, including α-GalCer (a marine-sponge–derived glycosphingolipid), bacterially derived glycosylceramides, and isoglobotrihexosylceramide (iGb3; a lysosomal glycosphingolipid) (43–46). Although CD1d-mediated recognition of these glycolipids may be critical for activating NKT cells to release potent immunomodulatory cytokines such as IFN-γ, their communication with CD1d^{+} DCs has been also shown to participate in the NKT cell activation (7, 11, 13). Several reports demonstrated that CD40 ligand on α-GalCer–stimulated NKT cells triggers DC maturation through the CD40 engagement, offering DCs the potential to secrete IL-12 that in turn affects IFN-γ production of NKT cells as a stimulator (35, 40, 47–50). In addition, Brigl et al. have shown that, in the case of infection with *Salmonella typhimurium*, NKT cells become activated depending on the maturation state of bystander DCs, which can sense microbial products and induce a variety of costimulatory molecules and cytokines, including IL-12, for NKT cell activation (51). However, except for IL-12, the identification and characterization of molecules that lead to NKT cell activation remain to be worked out (7, 11, 13). The results obtained in the present study establish that IFN-γ–producing NKT cell responses to the CD1d-presented antigen are amplified by OX40 engagement on NKT cells. Moreover, our study found an essential role for OX40L expressed on DCs in developing antigen-specific cellular immune responses, in which the triggering of OX40 on NKT cells as well as on CD4^{+} T cells provided a requisite signal for the development. These observations imply that OX40/OX40L interactions may be very important in linking innate and adaptive immunity, and therefore warrant consideration as a molecular target for therapeutic manipulation of the immune system.

Methods

Mice. C57BL/6 (H-2^{b}) mice were purchased from Japan Charles River. CD4^{+} T cell–deficient (B6.129S2-*Cd4*^{tm1Mak}) (52) and CD8^{+} T cell–deficient (B6.129S2-*Cd8a*^{tm1Mak}) (53) mice were obtained from The Jackson Laboratory. The derivation of OX40$^{-/-}$, OX40L$^{-/-}$, NKT cell$^{-/-}$, and CD1d$^{-/-}$ mice have been previously described (20, 54–56). All mice used in this study were age- (6–8 weeks old), sex- (female), and strain-matched (C57BL/6 background). Animals were housed under specific pathogen–free conditions in accordance with the protocols reviewed and approved by the Animal Care and Use Committee, Institute of Development, Aging and Cancer.

Cell preparation. The B16-F10 melanoma cell line, the LLC cell line, and the EL4 mouse lymphoma cell line were obtained from the Cell Resource Center for Biomedical Research of Tohoku University. The EG7-OVA cell line EL4, modified to express chicken OVA, was obtained from the American Type Culture Collection. B16-F10, LLC, and EL4 cells were maintained in complete RPMI-1640 media (10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin). EG7-OVA cells were grown in complete RPMI-1640 media containing 0.4 mg/ml G418 (Invitrogen). DCs were generated from mouse bone marrow precursors as previously described (57–59). DCs prepared from OX40L$^{-/-}$ or CD1d$^{-/-}$ mice were used in some experiments. Where indicated, CD4$^+$ T cells and NKT cells were purified from single-cell spleen suspensions by magnetic separation with MACS (Miltenyi Biotec) according to the manufacturer's instructions.

Ad vectors. AdOX40L and AdNull are structurally similar replication-deficient recombinant Ads with E1 and E3 deletions in which the mouse *OX40L* gene and no transgene, respectively, are under control of the cytomegalovirus immediate-early promoter and enhancer (60, 61). The propagation, purification, and titration of the Ad vectors were as previously described (62, 63). All vectors were free of replication-competent Ad.

Tumor therapy model. For the immunization challenge experiments, 2.5×10^5 DCs that had been incubated with 20 μg/ml TNF-α (BD Biosciences — Pharmingen), or AdOX40L or AdNull, at MOI 100 in the presence of 50 μg/ml OVA were injected subcutaneously in the left flank of mice, and 8 days later, 1×10^6 EG7-OVA cells were injected subcutaneously in the right flank. For intratumoral treatments, 3×10^5 B16-F10 cells were injected subcutaneously in the right flank of mice, and on day 8 the mice were injected with 50 μl of 7×10^5 DCs that had been transduced with AdOX40L or AdNull at MOI 100. The average tumor size on day 8 was approximately 30 mm^3. The size of each tumor was assessed by using calipers and was recorded as the tumor volume (length × width^2 × 0.52). Data are reported as the mean ± SEM tumor volume (in mm^3; $n = 5$ per group). For CD4$^+$ T cell$^{-/-}$ or NKT cell$^{-/-}$ mice reconstitution, 1×10^6 CD4$^+$ T cells or NKT cells, respectively, were adoptively transferred through intravenous injection 1 day before the intratumoral treatment.

Cytotoxicity assay. Tumor-specific cytotoxic activity was determined as previously described (61, 64). Briefly, effector cells were obtained by coculturing the splenocytes isolated from mice (3×10^6 cells/ml) with mitomycin C–treated EG7-OVA or B16-F10 cells (1×10^6 cells/ml) for 5 days. Their ability to lyse target cells was assessed using the lactate dehydrogenase (LDH) assay kit (Promega). The percentage of cytotoxicity was calculated as (experimental release – spontaneous release) / (maximal release – spontaneous release). Results are shown as mean ± SEM ($n = 3$ per data point).

Flow cytometric analysis. To characterize the cell surface phenotype, cells were stained with biotin-conjugated anti-OX40L antibody (clone RM134L; BD Biosciences — Pharmingen) plus streptavidin-PE conjugate (BD Biosciences — Pharmingen), biotin-conjugated anti-OX40 antibody (clone OX-86; BD Biosciences — Pharmingen) plus streptavidin-FITC conjugate (BD Biosciences — Pharmingen), FITC-conjugated anti-CD80 antibody (clone 16-10A1; BD Biosciences — Pharmingen), or FITC-conjugated anti-CD69 antibody (clone H1.2F3; BD Biosciences — Pharmingen). To detect OVA-specific CD8$^+$ T cells, splenocytes were stained with FITC-conjugated anti-CD8a antibody (clone 53-6.7; BD Biosciences — Pharmingen) and PE-conjugated H-2K^b/OVA pentamer (ProImmune). Intracellular IFN-γ staining was done using FITC-conjugated anti–IFN-γ antibody (clone XMG1.2; BD Biosciences — Pharmingen) as instructed by a fixation/permeabilization kit (BD Biosciences — Pharmingen). For NKT cell recognition, cells were stained with Cd1d/α-GalCer dimer plus PE-conjugated anti-mouse IgG_1 antibody (clone A85-1; BD Biosciences — Pharmingen); Cd1d/α-GalCer dimer was prepared by loading dimeric mouse CD1d:IgG_1 fusion protein (BD Biosciences — Pharmingen) with α-GalCer (Kirin Brewery) according to the manufacturer's passive loading protocol. For quantification of intratumoral NKT cells, the number of CD1d/α-GalCer dimer$^+$ NKT cells per 500,000 total cells from the B16-F10 tumors was determined, and the results are shown as mean ± SEM ($n = 3$ per group). The specificity of dimer binding to NKT cells has been confirmed by the absence of binding to splenocytes from NKT cell$^{-/-}$ mice. Cells were analyzed on an EPICS XL cytometer with EXPO32 ADC software (Beckman Coulter). To determine the percentage of stained cells, 1% of false-positive events was accepted in the control staining. Unless otherwise indicated, the percentages of stained cells above isotype control staining are shown.

Immunohistochemistry. After blocking nonspecific staining and endogenous peroxidase, acetone-fixed sections (5 μm thick) were incubated with 0.31 μg/ml anti-CD4 (clone RM4-5; BD Biosciences — Pharmingen), 10 μg/ml anti-CD8a (clone KT15; Serotec), or 10 μg/ml anti-OX40 (clone OX-86; Serotec) antibodies overnight at 4°C. After washing, the specimens were then incubated with 2.5 μg/ml biotinylated rabbit anti-rat Igs (DakoCytomation) for 15 min at room temperature. Signals were visualized with horseradish peroxidase–conjugated streptavidin and 3-3′-diaminobenzidine tetrahydrochloride (DAB) chromogen/substrate mixture (Nichirei). The sections were then incubated with 2.5% methyl green for nuclear counterstaining.

ELISA. To evaluate the IFN-γ levels in tumors, isolated tumors were homogenized with an equal volume of phosphate-buffered saline, pH 7.4. The homogenates were centrifuged to remove debris and then passed through a 0.22-μm filter. The IFN-γ levels in the homogenates were measured by ELISA. Results are presented as mean ± SEM ($n = 3$ per group). To evaluate in vivo α-GalCer–induced IFN-γ production, mice were injected intravenously with 2 μg α-GalCer and were bled 2 hours later. IFN-γ levels in the serum were measured by ELISA. Data are presented as mean ± SEM ($n = 4$ per data point). To evaluate in vitro α-GalCer–induced IFN-γ production, 5×10^5 splenocytes were incubated with 100 ng/ml α-GalCer in 96-well plates for 72 hours. The culture supernatants were collected and centrifuged to remove debris. The IFN-γ levels in the supernatants were measured by ELISA. Data are presented as mean ± SEM ($n = 3$ per data point). In another set of in vitro experiments, 5×10^5 splenocytes from NKT cell$^{-/-}$ mice were cocultured with 3×10^4 NKT cells from wild-type or OX40$^{-/-}$ mice in the presence of 100 ng/ml α-GalCer in a 96-well plate for 72 hours. Where indicated, blocking anti-mouse CD1d antibody (clone 1B1; BD Biosciences — Pharmingen) or isotype-matched control IgG (clone A95-1; BD Biosciences — Pharmingen) was added to the coculture medium at 10 μg/ml. IFN-γ concentrations were determined using ELISA kits for mouse IFN-γ (BioSource International). Data are presented as mean ± SEM ($n = 3$ per data point).

Statistics. Statistical comparison was made using 2-tailed Student's *t* test. A *P* value less than 0.05 was considered significant.

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