Dendritic Cell Internalization of α-Galactosylceramide from CD8 T Cells Induces Potent Antitumor CD8 T-cell Responses

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Abstract

Dendritic cells (DC) present α-galactosylceramide (αGalCer) to invariant T-cell receptor–expressing natural killer T cells (iNKT) activating these cells to secrete a variety of cytokines, which in turn results in DC maturation and activation of other cell types, including NK cells, B cells, and conventional T cells. In this study, we showed that αGalCer-pulsing of antigen-activated CD8 T cells before adoptive transfer to tumor-bearing mice caused a marked increase in donor T-cell proliferation, precursor frequency, and cytotoxic lymphocyte activity. This effect was interleukin (IL)-2 dependent and involved both natural killer T cells (NKT) and DCs, as mice lacking IL-2, NKTs, and DCs lacked any enhanced response to adoptively transferred αGalCer-loaded CD8 T cells. iNKT activation was mediated by transfer of αGalCer from the cell membrane of the donor CD8 T cells onto the αGalCer receptor CD1d which is present on host DCs. αGalCer transfer was increased by prior activation of the donor CD8 T cells and required AP-2–mediated endocytosis by host DCs. In addition, host iNKT cell activation led to strong IL-2 synthesis, thereby increasing expansion and differentiation of donor CD8 T cells. Transfer of these cells led to improved therapeutic efficacy against established solid tumors in mice. Thus, our findings illustrate how αGalCer loading of CD8 T cells after antigen activation *in vitro* may leverage the therapeutic potential of adoptive T-cell therapies. *Cancer Res; 71(24); 7442–51. ©2011 AACR.*

Introduction

Natural killer T cells (NKT) have been focused on a subset of NKT cells that express an invariant T-cell receptor (iTCR), termed iNKT cells. The murine iTCR is encoded by Vα14/Jα18 gene segments, whereas human iTCR is encoded by Vα24/Jα18 gene segments. iNKT cells are potent immunomodulators by virtue of their ability to secrete both Th1 and Th2 cytokines, which are associated with various autoimmune diseases, immune tolerance, tumor resistance, and protection against infections (1). Although iNKT cells display heterogenicity in terms of their cytokine profiles and tissue localization (2, 3), these cells are all restricted by CD1d molecules.

CD1d proteins, MHC class I–like molecules, can present lipids and glycolipids to iNKT cells and are constitutively expressed by many cells, including dendritic cells (DC), macrophages, and B and T lymphocytes (1). Like protein antigens, glycolipids on CD1d appear to require processing and internalization for successful presentation to iNKT cells. The internalization of CD1d from the plasma membrane and trafficking to endosomes, which involve adaptor proteins (4, 5), are essential for lipid processing and loading onto CD1d. Such processing is inhibited in the absence of saposins, microsomal triglyceride transfer protein, and adaptor protein complex 3 (AP-3; refs. 5–8). Saposins, known as endosomal lipid transfer proteins, might assist lipid extraction from the membrane for presentation on CD1d in endosomes (6, 7). Microsomal triglyceride transfer protein is predominantly found in the endoplasmic reticulum (ER) and might serve as a chaperone responsible for transferring lipids to the newly synthesized CD1d before delivery from the ER to the plasma membrane (8). AP-3 is known to be responsible for trafficking of CD1d to lysosomes (5).

α-Galactosylceramide (αGalCer), glycolipid identified from marine sponge, has high affinity for CD1d (9). Presentation of αGalCer on CD1d by DCs induces iNKT cells to rapidly synthesize large quantities of cytokines. These secreted cytokines, in turn, activate a variety of other cell types, including NK cells, DCs, B cells, and T cells (10). These properties have been shown to enable αGalCer to act as a novel vaccine adjuvant that induces potent CD4 and CD8 T-cell responses in animal models (11–13), although clinical responses have been less impressive (14–17).

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Here, we show that iNKT activation can involve a novel form of cell-to-cell interaction in which activated CD8 T cells absorb αGalCer via a CD1d-independent mechanism *in vitro* and then transfer αGalCer to host DCs after injection into normal mice. Interestingly, the transfer of αGalCer from CD8 T cells to DCs appeared to require DC internalization, which occurs more efficiently when CD8 T cells are activated. CD1d-restricted presentation of the transferred αGalCer by DCs activates host iNKT cells to synthesize interleukin (IL)-2 and thereby induces the donor CD8 T cells to further proliferate and mediate enhanced tumor rejection.

Materials and Methods

Mice

C57BL/6, IL-2$^{-/-}$, IFN-γ$^{-/-}$, MHC class II$^{-/-}$, IL-12RII$^{-/-}$, IL-17$^{-/-}$, and LYSTEG mice were purchased from Charles River Laboratories (Shizuoka) and kept in the POSTECH (Pohang, Korea) animal care facility. Jα18$^{-/-}$, CD1d$^{-/-}$, and CD11c-DT mice were provided by Dr. M. Taniguchi (RIKEN, Yokohama, Japan), Dr. Hua Gu (NIAID-Taconic facility of NIH) and Dr. Gunter J. Hammerling (DKFZ, Heidelberg, Germany), respectively (18). Thy1.1$^+$OT-I mice were generated as described previously (19). IL-2$^{-/-}$ and Thy1.1$^+$2C mice were kept in the animal care facility at the Garvan Institute of Medical Research (Darlinghurst, NSW, Australia). All mice were B6 background.

Reagents and cell lines

αGalCer and tyrphostin (A23 and A51) were purchased from Alexis-Biochemicals. EG7 (OVA-expressing EL4 cells) and NKT hybridoma (DN32.D3) were purchased from the American Type Culture Collection; both were maintained in RPMI-1640 (Welgene) supplemented with 10% FBS (Hyclone), and 100 U/mL penicillin–100 μg/mL streptomycin (Invitrogen).

Generation of donor cells

Donor OT-I cells and CD8-depleted splenocytes were isolated from Thy1.1$^+$OT-I mice and C57BL/6 mice by magnetic-activated cell sorting (MACS; Miltenyi Biotec), respectively. They were cultured with an equal number in the presence or absence of 1 μg/mL OVA peptide (SIINFEKL) and/or αGalCer (200 ng/mL) for 1 day, and then OT-I cells were purified by MACS (Miltenyi Biotec). Donor OT-I cells are referred to as OT-I, OT-I/αGalCer, OT-I/OVAp, and OT-I/OVAp/αGalCer, respectively. Donor CD8 T cells were isolated from CD1d$^{-/-}$ or C57BL/6 mice (Miltenyi Biotec) and incubated on anti-CD3/CD28 (BD Bioscience/SouthernBiotech) antibody-coated plates for 2 days. After stimulation, CD8 T cells were pulsed with αGalCer (200 ng/mL) for 30 minutes or left untreated. After transfer of donor cells (1×10^5 per mouse), the frequency of transferred cells was measured by assessing the proportion of Thy1.1$^+$ or Thy1.2$^+$ cells.

Generation of bone marrow DCs

Bone marrow DCs were derived from bone marrow progenitors of following culture with granulocyte macrophage colony-stimulating factor (GM-CSF; PeproTech; ref. 20). Approximately,

90% of the bone marrow DCs were positive for CD11c and IA/IE. Bone marrow DCs were cultured with an equal number of donor CD8 T cells. After culturing for 6 hours, bone marrow DCs were isolated using CD11c-microbeads (Miltenyi Biotec). Isolated bone marrow DCs were cultured with an equal number of DN32.D3 for 24 hours, and IL-2 levels were assessed with supernatant by ELISA (OPTEIA, BD Bioscience). IL-2 level represented the values obtained after subtracting the value for supernatants of bone marrow DCs and DN32.D3 cultured alone.

Generation of tumor-bearing mice

Mice were inoculated s.c. with EG7 (1×10^6 per mouse). Donor OT-I cells (1×10^5 per mouse) were transferred i.v. on day 7 when tumor diameters reached an average of 6 to 8 mm. Mice that developed tumors larger than 2,000 mm^3 were sacrificed for ethical reasons.

Surface and intracellular staining

Cells were stained with the appropriate combination of the following monoclonal antibodies (mAb): anti-CD8, anti-Thy1.1, and anti-Thy1.2 (BD Bioscience); and anti-IFN-γ and anti-TNFα (eBioscience). For intracellular cytokine staining, cells were stimulated with 1 μg/mL OVAp for 6 hours in the presence of 5 μg/mL Brefeldin-A (Sigma-Aldrich), anti-CD107a (BD Bioscience), and anti-CD107b (eBioscience). The cells were permeabilized with the Cytofix/Cytoperm (BD Biosciences) and stained for 45 minutes with anti-cytokine mAbs. Flow cytometry was carried out using a Gallios flow cytometer (Beckman Coulter).

Measurement of immune response

The *in vivo* OVA-specific cytolytic activity of CD8 T cells was measured as previously described (21). ELISPOT and ELISA assays were carried out with anti-IFN-γ and anti-IL-2 mAbs (BD Bioscience) for capture and detection as described previously (22).

Statistical analysis

Statistical differences were determined using Student *t* tests. Differences between tumor survival rates were analyzed using log-rank tests.

Results

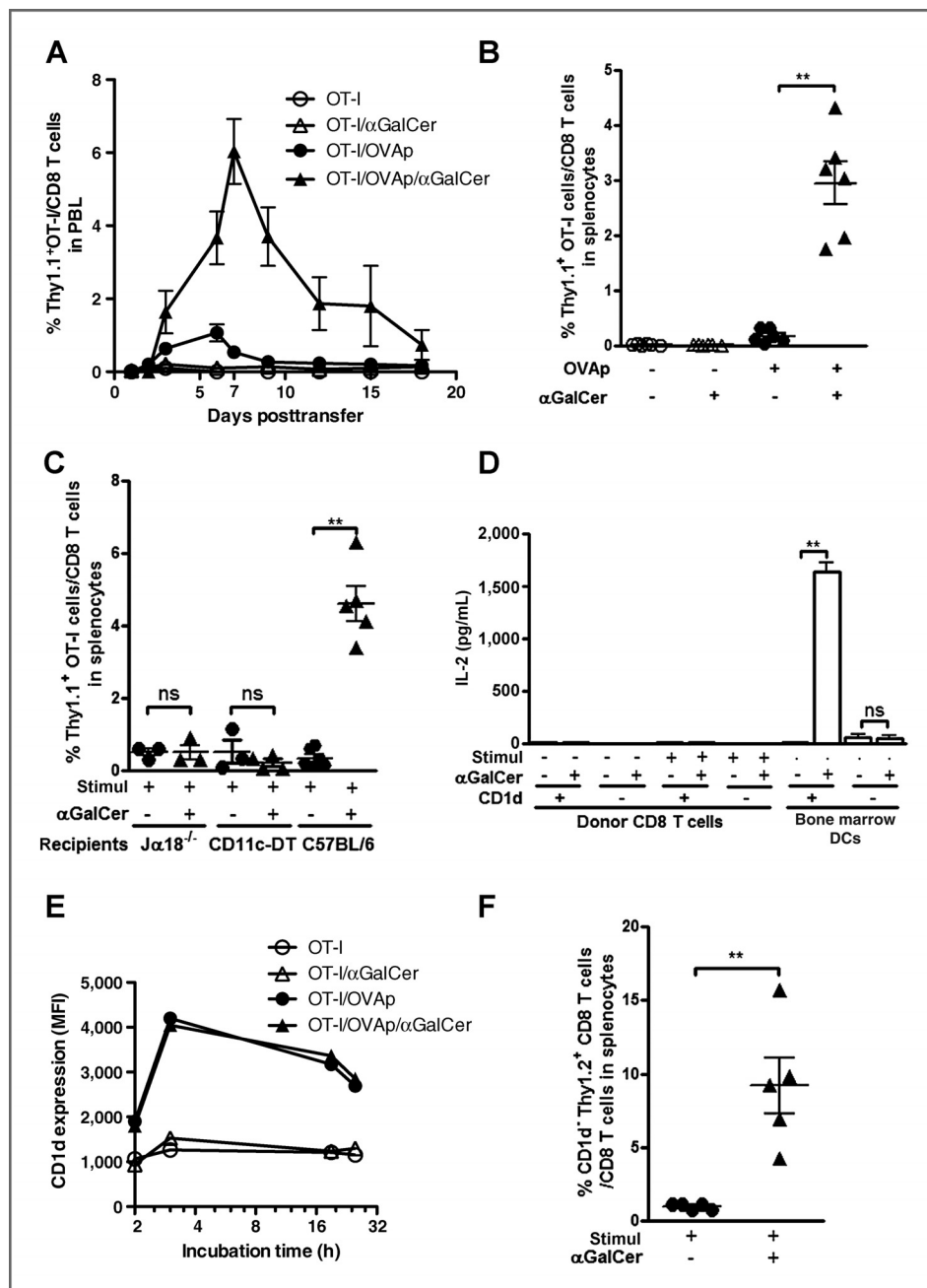### The frequency of activated CD8 T cells is enhanced *in vivo* by αGalCer loading

As a preliminary experiment, OT-I cells expanded considerably *in vivo* after prior stimulation with OVAp and αGalCer *in vitro* for up to 48 hours. The extent of expansion, measured in blood, depended on the duration of *in vitro* stimulation (Supplementary Fig. S1A), and reached a maximum with a 24-hour incubation with OVAp plus αGalCer prior to transfer. As expected, there were significant differences in activation markers (i.e., CD44 and CD25) by incubation with OVAp, but not with αGalCer for up to 48 hours (Supplementary Fig. S1B).

To assess the relative importance of OVAp versus αGalCer for inducing cell expansion after transfer, we cultured naïve OT-I cells *in vitro* for 24 hours in the presence or absence of OVAp and/or αGalCer; thereafter, OT-I cells were purified and

transferred into naïve mice. Upon transfer of OT-I/αGalCer, the frequency of OT-I cells in peripheral blood was very low and remained unchanged over time (Fig. 1A). With injection of OT-I/OVAp, the frequency of OT-I cells in blood increased to a maximum of 1% of CD8 T cells at 6 days after transfer (Fig. 1A). However, injection of OT-I/OVAp/αGalCer increased the frequency of OT-I cells in blood to about 6% 7 days after injection (Fig. 1A). Similar results were also observed in splenocytes 6 days after transfer (Fig. 1B).
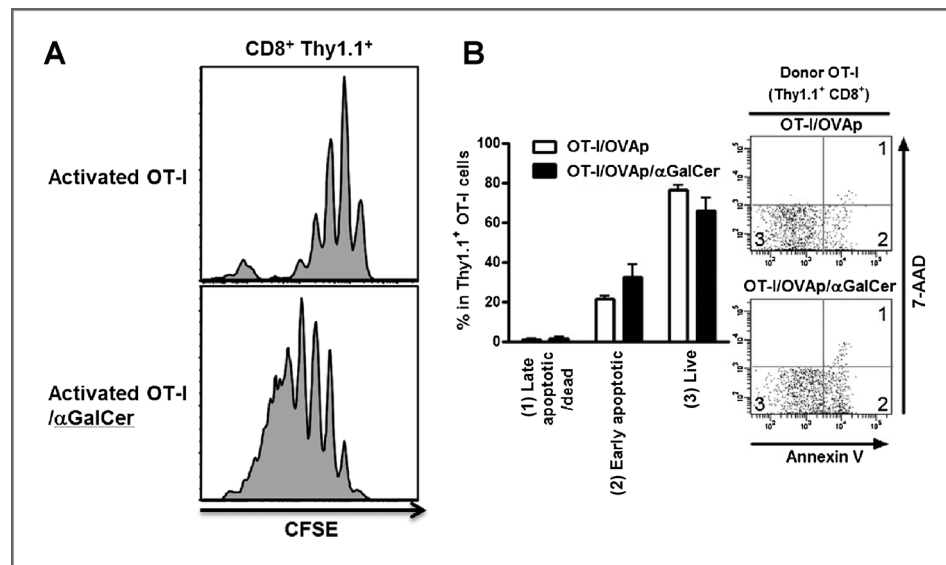
To rule out the possible contamination of antigen-presenting cells (APC) from the coculture with donor OT-I cells, fluorescence-activated cell sorting (FACS)-sorted OT-I cells

were stimulated with anti-CD3/CD28 mAbs in the presence or absence of αGalCer and then transferred into naïve B6 mice. As shown in Fig. 2A, proliferation defined by carboxyfluorescein succinimidyl ester dilution was much greater for αGalCer-loaded OT-I cells than for unloaded OT-I counterparts. These results clearly exclude an *in vivo* role of contaminated APCs that may capture αGalCer and antigenic peptide from *in vitro* coculture. Thus, it is likely that the effect of αGalCer loading may apply to a broad range of polyclonal or TCR transgenic CD8 T cells either activated in an antigen-dependent or antigen-independent manner. It should be noted that the marked expansion of activated CD8 T-cell population by

Figure 1. iNKT cells enhance the frequency of αGalCer-loaded, activated OT-I cells *in vivo* via a mechanism that requires CD1d of DCs, but not of CD8 T cells. After transfer of donor OT-I cells into C57BL/6 mice, Jα18$^{-/-}$, or CD11c-DT mice (n = 3–6), the frequency of donor cells was examined in peripheral blood lymphocytes (PBL; A) and splenocytes (B and C) at the indicated time points and 6 days after transfer, respectively. D, after culture of an equal number of DN32. D3 and donor CD8 T cells for 24 hours (2×10^5 per well), IL-2 levels were assessed with supernatants. E, mean fluorescence intensity of CD1d on OT-I cells was examined at the indicated time points during *in vitro* culture. F, the frequency of donor CD1d$^{-/-}$ CD8 T cells in splenocytes was examined 6 days after transfer into Thy1.1$^+$ naïve mice (1.5×10^5 per mouse; n = 5); stimul, stimulation of donor cells. Data are expressed as mean ± SEM. Similar results were obtained in 2 or 3 independent experiments. **, $P < 0.01$; ns, not statistically significant.

Figure 2. The proliferation and apoptosis of activated OT-I cells by αGalCer loading. A, FACS-sorted OT-I cells were stimulated with anti-CD3/CD28 mAbs for 6 hours in the presence or absence of αGalCer (500 ng/mL). After stimulation, OT-I cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into naïve mice (1×10^5 per mouse; $n = 6$). CFSE dilution of OT-I cells was examined at 6 days in lymph nodes. B, apoptosis of donor OT-I cells was examined at 3 days after transfer using 7-aminoactinomycin D (7-AAD) and Annexin V. Left, the percentage of OT-I cells in right. Data are expressed as mean ± SEM. Similar results were obtained in 2 independent experiments.

αGalCer loading before transfer correlated with extensive proliferation of the cells (Fig. 2A). In contrast, there was no detectable change in the rate of apoptosis between OT-I/OVAp and OT-I/OVAp/αGalCer (Fig. 2B). Thus, these results indicate that the frequency of CD8 T cells could be significantly increased *in vivo* by prior αGalCer-pulsing *in vitro*, especially when CD8 T cells were activated; loading-activated CD8 T cells with αGalCer before transfer caused the cells to proliferate extensively rather than to inhibit cell death on transfer.

Enhanced proliferation of CD8 T cells on transfer is mediated by iNKT cells and involves host, but not donor, CD1d

Because *in vivo* responses to αGalCer are mediated by iNKT cells (12, 13), we tested whether the αGalCer-loaded CD8 T cells could proliferate in mice that lacked iNKT cells, namely Jα18$^{-/-}$ mice. Proliferation was totally ablated in these mice, showing that proliferation was iNKT dependent (Fig. 1C). To investigate the potential of CD8 T cells to absorb αGalCer for direct presentation to iNKT cells, we tested whether donor CD8 T cells elicited IL-2 production *in vitro* by DN32.D3, which are known to recognize αGalCer–CD1d complexes and subsequently produce IL-2. IL-2 production was undetectable in this context, which contrasted with high-level IL-2 synthesis elicited by αGalCer-loaded bone marrow DCs (Fig. 1D). It is known that DCs play the major role for iNKT activation (23), which is consistent with our finding that the proliferative response of CD8 T cells by αGalCer-pulsing was ablated in CD11c.DT-Tg mice depleted of CD11c$^+$-DCs by treatment with diphtheria-toxin receptor (Fig. 1C). Thus, the implication of these observations is that proliferation was mediated via stimulation of host iNKT cells by αGalCer presented by CD1d$^+$ host DCs. However, this model left open the question of how αGalCer could be transferred from CD8 T cells to host DCs and, in particular, whether surface CD1d on CD8 T cells plays a role in αGalCer transfer. On this point, we found that activating OT-I cells *in vitro* with OVAp, but not with αGalCer, caused strong upregulation of CD1d (Fig. 1E).

To address this question, we activated non-TCR transgenic CD1d$^{-/-}$ CD8 T cells by CD3/CD28 ligation *in vitro* with or without αGalCer loading. Interestingly, prior αGalCer loading of activated CD1d$^{-/-}$ CD8 T cells enhanced expansion of these cells (Fig. 1F). Hence, uptake of αGalCer by CD8 T cells *in vitro* might be CD1d-independent, and there is no direct presentation of αGalCer-loaded CD8 T cells to iNKT cells.

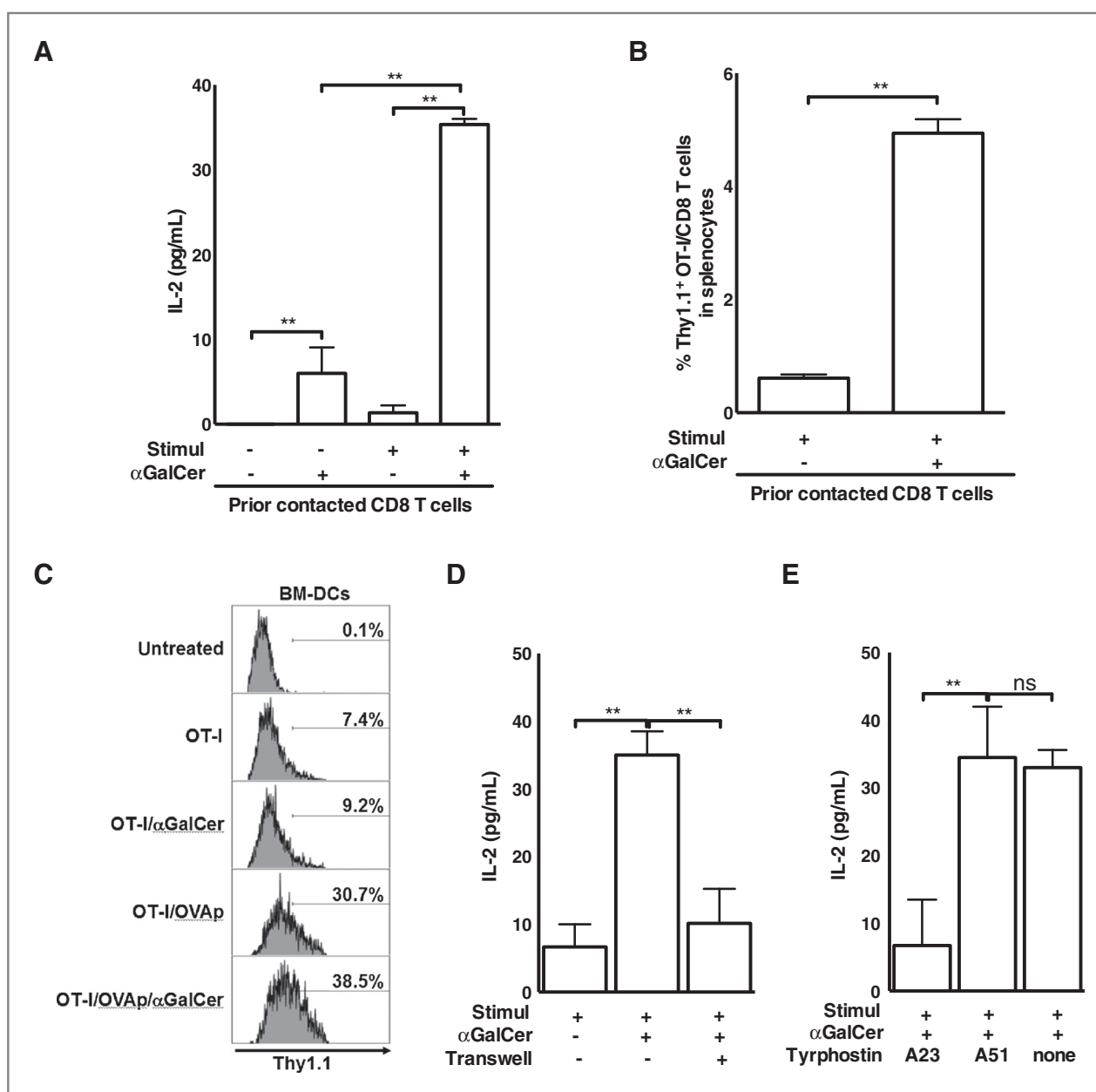Collectively, these data provide strong support for a model in which activated CD8 T cells absorb αGalCer by a CD1d-independent mechanism *in vitro*; after transfer, αGalCer is then somehow conveyed to host DCs for CD1d-restricted presentation to iNKT cells, leading to expansion of donor CD8 T cells.

Mechanism of αGalCer transfer from CD8 T cells to DCs

To further investigate how αGalCer-loaded CD8 T cells were able to induce stimulation of host iNKT cells, we incubated bone marrow DCs for 6 hours with donor CD8 T cells. The purified bone marrow DCs were then cocultured for 24 hours with an equal number of DN32.D3. Notably, bone marrow DCs that had been incubated with αGalCer-loaded, activated CD8 T cells induced strong IL-2 secretion by DN32.D3; IL-2 synthesis was substantially lower when naïve T cells were used (Fig. 3A). To investigate whether similar findings applied to iNKT cell stimulation *in vivo*, purified bone marrow DCs, derived from the culture of bone marrow DCs and donor CD8 T cells, were injected *in vivo* together with additional OT-I/OVAp. Significantly, the transferred bone marrow DCs induced strong expansion of the OT-I/OVAp, but only if the bone marrow DCs had been precultured *in vitro* with αGalCer-loaded, activated CD8 T cells (Fig. 3B). Similar results were obtained using donor OT-I cells (Supplementary Fig. S2).

The above results indicate that αGalCer is transferred from CD8 T cells to DCs, which then induced iNKT cells to become activated and synthesize IL-2. Interestingly, incubating bone marrow DCs with activated versus naïve Thy1.1$^+$OT-1 cells led to significant increase in the appearance of Thy1.1 on the bone marrow DCs (7.4% vs. 30.7% and 9.2% vs. 38.5%; Fig. 3C). These

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Figure 3. iNKT cells are activated by DCs via CD8 T-cell–associated αGalCer uptake in a manner that requires cell-to-cell contact and endocytosis. Donor CD8 T cells were cultured with an equal number of bone marrow DCs for 6 hours. A, isolated bone marrow DCs were cultured with DN32.D3. After culturing cells for 24 hours, IL-2 levels in supernatant were assessed. B, an equal number of isolated bone marrow DCs were cotransferred with OT-I/OVAp into naïve mice ($n = 3$). The frequency of OT-I/OVAp in splenocytes was examined at 6 days after transfer. C, Thy1.1 expression in isolated bone marrow DCs is displayed as a histogram. Number, percentage of gated population (CD11c$^+$Thy1.1$^{dim/negative}$). D and E, isolated bone marrow DCs from culture with donor CD8 T cells (D) in Transwells (0.4 μm pore) or (E) in the presence of tyrphostin A23 or A51 (30 μmol/L) were cultured with DN32.D3. After culturing cells for 24 hours, IL-2 levels in supernatants were assessed; stimul, stimulation of donor cells. Data are expressed as mean ± SEM. Similar results were obtained in 2 or 3 independent experiments. **, $P < 0.01$; ns, not statistically significant.

results are in line with a previous report that DCs acquire lipids and proteins from other live cells, including leukocytes, via cell-to-cell contact (24), and further indicate that such transfer is more prominent with activated than resting CD8 T cells. It should be noted that transfer of αGalCer from CD8 T cells to DCs appeared to require direct cell-to-cell contact, as stimulation of iNKT cells by bone marrow DCs failed to occur *in vitro* when

bone marrow DCs were cultured with αGalCer-loaded CD8 T cells separated by a cell-impermeable membrane (Fig. 3D).

Transfer of αGalCer from CD8 T cells to DCs might require internalization by DCs. On this point, 4 adaptor proteins (AP-1, -2, -3, and -4) are known to bind integral membrane proteins and direct their selective localization to intracellular compartments (25, 26). Because AP-2 is an essential component of the

endocytic clathrin-coat involved in internalization, we used tyrphostin A23, a known inhibitor of AP-2–mediated internalization; tyrphostin A51 was used as a negative control (27). Significantly, addition of tyrphostin A23 during culture of αGalCer-pulsed, activated CD8 T cells and bone marrow DCs substantially reduced the subsequent capacity of the DCs to elicit IL-2 secretion by DN32.D3 (Fig. 3E). These results thus support the notion that transfer of αGalCer from CD8 T cells to DCs requires internalization by DCs prior to association of αGalCer with CD1d on the cell surface.

Influence of αGalCer loading on the effector function of CD8 T cells after adoptive transfer

To investigate if αGalCer loading influenced the effector function of the transferred CD8 T cells, we stained donor cells for intracellular cytokines 3 days after transfer. As shown in Fig. 4A, OT-I/OVAp/αGalCer markedly increased the absolute number of IFN-γ–positive cells expressing CD107 (a functional marker of CD8 T-cell cytotoxicity). In addition, the fold-change in mean fluorescence intensity (MFI) of IFN-γ or TNF-α on OT-I cells 3 days after transfer was dramatically increased by in vitro αGalCer-pulsing; the MFI of CD107 on activated OT-I cells was not changed by αGalCer loading (Fig. 4B). Notably, this pattern was maintained for up to 76 days after transfer (Supplementary Fig. S3). Moreover, mice injected with OT-I/OVAp/αGalCer also generated more in vivo cytotoxicity than did OT-I/OVAp (Fig. 4C). Taken together, these results indicate that αGalCer loading of activated CD8 T cells before transfer considerably increases the long-term survival and effector function of the donor cells.

To further investigate the effector function induced by αGalCer loading, we transferred OT-I/OVAp or OT-I/OVAp/αGalCer into EG7 tumor-bearing mice. Significantly, marked inhibition of tumor growth occurred following injection of OT-I/OVAp/αGalCer and thus 70% of these mice survived to the end of the experiment, whereas 100% mortality was observed among mice receiving OT-I/OVAp (Fig. 4D). The enhanced therapeutic potential of OT-I/OVAp/αGalCer was correlated with a marked increase in the total number of donor cells in blood after transfer (6-fold) and the number of OVAp-specific IFN-γ–secreting CD8 T cells (10-fold; Fig. 4E).

IL-2 production by iNKT cells is closely correlated with the enhanced proliferation of CD8 T cells induced by αGalCer loading

Injection of OT-I/OVAp/αGalCer led to a rapid, transient decrease in CD3$^+$NK1.1$^+$ cell numbers in the spleen (Supplementary Fig. S4A), presumably reflecting stimulation of NKT cells followed by downregulation of the CD3/NK1.1 surface marker on these cells (28). Moreover, OT-I/OVAp/αGalCer induced significant IFN-γ production by NK cells (Supplementary Fig. S4B), which is a known consequence of iNKT cell activation by αGalCer (29). Serum IFN-γ was detected rapidly in mice transferred with OT-I/OVAp/αGalCer (Fig. 5A, top). In addition, IL-2 levels were markedly elevated at the earlier time point than IFN-γ (peaking 6 hours after transfer) in animals receiving OT-I/OVAp/αGalCer only (Fig. 5A, bottom). Measurement of numbers of IFN-γ- or IL-2–producing iNKT cells
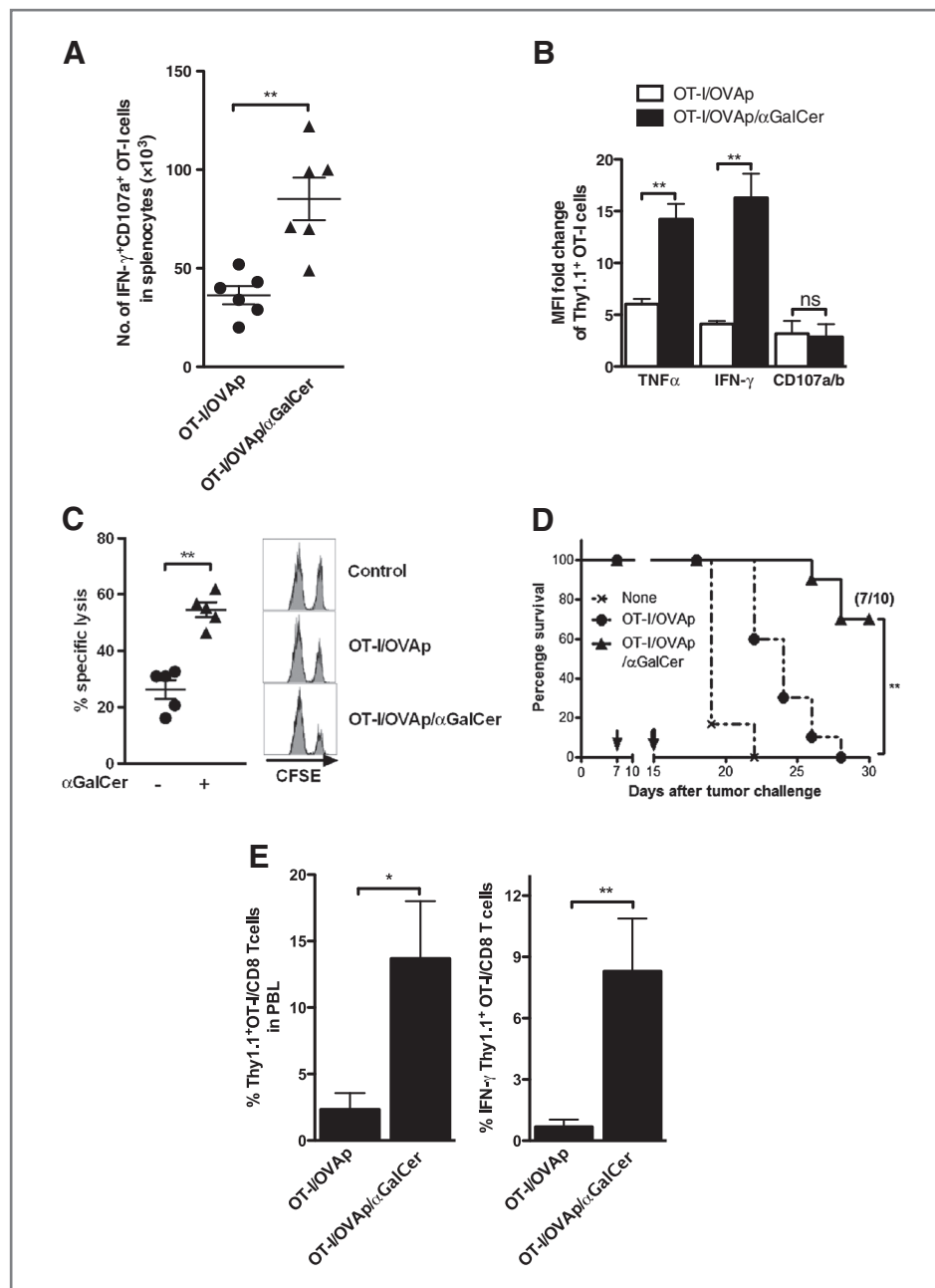
that were responsive to αGalCer 6 hours after transfer showed that OT-I/OVAp/αGalCer induced more IL-2 production significantly than IFN-γ production by iNKT cells (Fig. 5B); other cells, notably NK cells, showed prominent IFN-γ production (Supplementary Fig. S4B). These findings suggested that in vivo expansion of donor OT-I cells after prior αGalCer loading could be mediated largely by IL-2 produced by activated host iNKT cells.

To seek direct evidence on the role of host-derived IL-2 in expansion of the donor T cells, we transferred cells to various gene-knockout hosts, including IFN-γ$^{-/-}$, IL-12RII$^{-/-}$, MHC class II$^{-/-}$, LYSTEG, IL-17$^{-/-}$, and IL-2$^{-/-}$ mice. Interestingly, only IL-2$^{-/-}$ mice failed to enhance the proliferation of OT-I/OVAp/αGalCer (Fig. 5C). Although NKT cells in IL-2$^{-/-}$ mice showed different cytokine secretion capacity depending on the type of stimulation they received, they existed in the spleen and were somewhat functional (30; Supplementary Fig. S5). A similar result was observed with 2C transgenic CD8 cells, indicating that the crucial role for host IL-2 also applied to expansion of activated αGalCer-loaded 2C CD8 cells (Fig. 5D). It should be noted that strong expansion of αGalCer-loaded activated CD8 cells occurred after transfer to MHC class II$^{-/-}$ hosts but not to Jα18$^{-/-}$ hosts (Figs. 1C and 5C). Hence, IL-2 synthesis by host cells seemed to arise largely from iNKT cells and not CD4 T cells. Curiously, the expansion of activated OT-I cells was slightly enhanced in mice lacking NK cells, namely LYSTEG mice, by αGalCer loading, which is in agreement with the recent report that NK cells negatively regulated CD8 T-cell immune response by killing recent activated CD8 T cells (31).

Discussion

Although αGalCer can elicit both humoral and cellular immunity in mice when co-injected with antigen (11, 13, 32), injection of αGalCer alone leads to selective activation of iNKT cells, followed by induction of energy in these cells (28). Moreover, co-injection of free αGalCer with CD8 T cells elicited splenomegaly compared with αGalCer loading of CD8 T cells, whereas their expansion of donor CD8 T cells is similar (Supplementary Fig. S6A and S6B). αGalCer-loaded B cells are also reported to reduce the functionality of iNKT cells to secrete cytokines (33), although they have been shown to generate antigen-specific T-cell immunity through interactions with iNKT cells (34). It was reported that αGalCer-loaded DCs enhanced induction of antigen-specific immunity in vivo (35). However, αGalCer-loaded DCs did not increase epitope-specific T-cell immunity (23), which is consistent with our data showing that in vitro pulsing of αGalCer on DCs with OVAp did not enhance OVA-specific T-cell response (Supplementary Fig. S6C). The discrepancy might be caused by difference in the amount of CTL-epitope, DC maturation status, and experimental condition. In addition, co-injection of αGalCer-loaded bone marrow DCs with CD8 T cells enhanced the proliferation of CD8 T cells, but appeared to be lower than transfer of αGalCer-loaded CD8 T cells (Supplementary Fig. S6D).

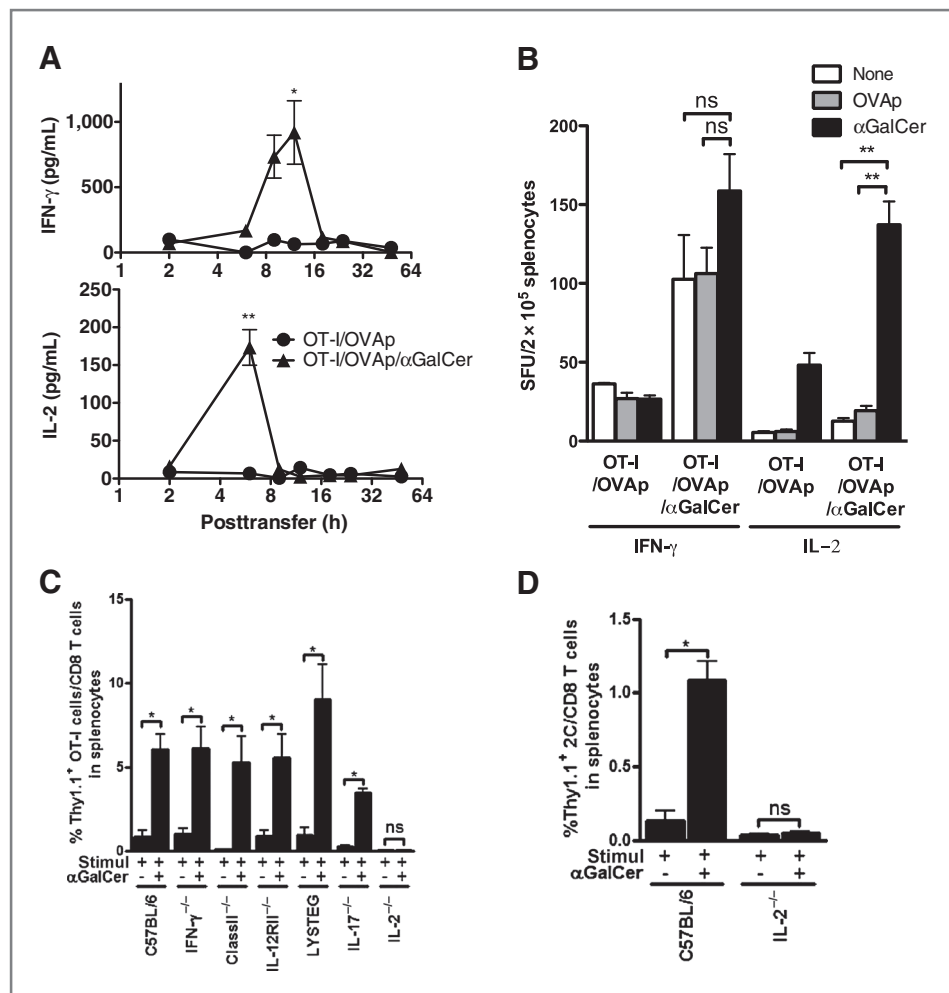It was previously reported that αGalCer-loaded DCs caused iNKT to produce IL-2 which then induced proliferation of

Figure 4. The efficacy of transferred CD8 T cells is enhanced by αGalCer loading. A–C, donor OT-I cells in splenocytes were examined at 3 days after transfer for (A) the absolute number of IFN-γ^+CD107a/b$^+$, (B) fold change of MFI (IFN-γ, TNFα, and CD107a/b) relative to unstimulated cells, and (C) *in vivo* CTL activity. D and E, donor OT-I cells were transferred into tumor-bearing mice (thin arrow). D, survival rates and (E) IFN-γ production of peripheral blood lymphocytes (PBL; thick arrow) were determined. Data were expressed as mean ± SEM. Similar results were obtained in 2 or 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.

CD4$^+$CD25$^+$ regulatory T cells (36). In contrast, we observed that the proportion of CD4$^+$CD25$^+$ regulatory T cells in the spleen was not significantly affected by injection of αGalCer-loaded activated CD8 T cells (Supplementary Fig. S7A). This discrepancy may reflect differences in cell types pulsed with αGalCer (CD8 T cells vs. DCs) and different experimental conditions. It may be noted that *in vitro* αGalCer-pulsing decreased *in vivo* frequency of transferred CD4 T cells (Supplementary Fig. S7B).

We showed that αGalCer loading enhanced proliferation and function of activated, but not naïve, CD8 T cells. There are a number of explanations for the predominant effect on activated CD8 T cells. First, activated CD8 T cells make stronger contact with DCs presumably via increased levels of costimulatory molecules, which may explain our finding that αGalCer transfer from T cells to DCs was more efficient when CD8 T cells were stimulated *in vitro* (Fig. 3A). Second, activated CD8 T cells express high levels of IL-2Rα (CD25; forming high-affinity trimeric IL-2R complexes; Supplementary Fig. S1B), therefore allowing these cells to have a greater sensitivity in responding to IL-2 (37). Finally, activated CD8 T cells have an enhanced ability to induce the maturation and survival of DCs through secreting crucial soluble factors or via cell-to-cell contact as previously described (38–41).

Figure 5. The number of IL-2–producing iNKT cells is correlated with the enhanced proliferation of OT-I cells induced by αGalCer loading. A, IL-2 and IFN-γ levels in serum were evaluated at the indicated time points after transfer ($n = 6$). B, 6 hours after transfer, IFN-γ and IL-2 ELISPOT assays were carried out by stimulating splenocytes with either OVAp (1 μg/mL) or αGalCer (200 ng/mL) for 18 hours ($n = 6$). C, the frequency of donor OT-I cells was examined 6 days after transfer into IFN-γ$^{-/-}$, MHC class II$^{-/-}$, IL-12RII$^{-/-}$, LYSTEG, IL-17$^{-/-}$, or IL-2$^{-/-}$ mice ($n = 3–6$). D, FACS-sorted CD44^{low}Thy1.1^{+}2C cells were stimulated with T-cell-depleted, irradiated syngeneic splenocytes pulsed with SIYR peptide (10 μmol/L) in the presence or absence of αGalCer (200 ng/mL). After culturing for 1 day, cells were isolated by MACS and transferred into C57BL/6 or IL-2$^{-/-}$ mice ($n = 3$). The frequency of transferred cells in splenocytes was examined 6 days after transfer. stimul, stimulation of donor cells. Data were expressed as mean ± SEM. Similar results were obtained in 2 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ns, not statistically significant. SFU, spot-forming units.
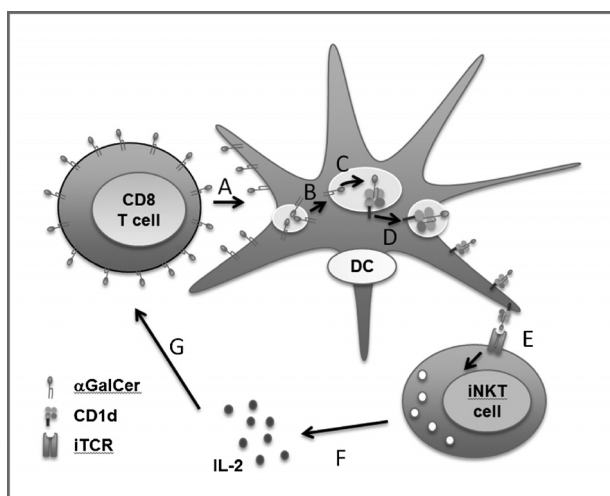
Shimizu and colleagues (42) and Fujii and colleagues (28) have suggested that transfer of cell-associated αGalCer to DCs is a reflection of cell death mediated by iNKT cells, which is enhanced by overexpression of CD1d on the αGalCer-loaded cells. However, it is also known that cell-associated proteins and lipids can be directly transferred to DCs from live cells via cell-to-cell contact (24). This finding is consistent with our observation that surface protein (Thy1.1) and lipid (αGalCer) on activated CD8 T cells were transferred to DCs, presumably through cell-to-cell contact (Fig. 3A–D). In addition, we showed that the percentage of apoptotic body was rather lower in activated CD8 T cells than in naïve CD8 T cells before and after in vitro culture for 6 hours (data not shown), excluding the possibility of DC capturing apoptotic CD8 T cells and its role in stimulating NKT cells. These results are consistent with the previous reports that OT-I cells stimulated by high-affinity agonist showed little evidence of death for up to 6 division rounds (43). Moreover, transfer of FACS-purified ex vivo memory (CD44^{hi}) phenotype CD8 T cells, a natural population responding to IL-2 (44), did show proliferation even when loaded with αGalCer in vitro for 45 minutes before transfer (Supplementary Fig. S8), which avoid long-term culture and presumably minimize in vivo apoptotic cell death right after transfer. Taken together, these results strongly support the idea that αGalCer transfer from T cells to DCs may be mainly caused by cell-to-cell contact rather than by capture of apoptotic bodies.

Significantly, CD1d was not required for both αGalCer loading of CD8 T cells and αGalCer transfer from CD8 T cells to DCs as enhanced proliferation of CD8 T cells after αGalCer loading was observed in CD1d$^{-/-}$ CD8 T cells (Fig. 1F). Instead, DC presentation of the absorbed αGalCer seemed to require initial endocytosis by DCs. Thus, presentation of cell-associated αGalCer to iNKT cells via DCs was inhibited by tyrphostin A23, which blocks AP-2–mediated endocytosis (ref. 27; Fig. 3E). Collectively, these findings suggest the following model (Fig. 6). First, αGalCer on CD8 T cells is transferred to DCs in the CD1d-independent manner, following cell–cell contact. The transferred αGalCer is then internalized via endocytosis into endosomes of DCs. Thereafter, αGalCer associates with CD1d of DCs, perhaps assisted by endosomal lipid transfer proteins (6, 7). Finally, αGalCer–CD1d complexes are transported from the endosome to the cell surface for presentation to iNKT cells (1).

Figure 6. Proposed mechanism for the enhanced efficacy of activated CD8 T cells by αGalCer loading. A, αGalCer transfer from activated CD8 T cells to DCs via cell-to-cell contact. B, internalization of αGalCer via endocytosis into endosome. C, αGalCer loading on CD1d by LTP. D, transport of αGalCer–CD1d complexes from endosome to DC surface. E, presentation of αGalCer to iNKT cells. F, secretion of IL-2 by iNKT cells. G, enhanced proliferation and functionality of CD8 T cells by IL-2.

With regard to clinical relevance, our findings may be applicable for CD 8 T-cell–based immunotherapy due to benefits of cost, simplicity, and safety in addition to efficacy. In this respect, there is an increasing need for a novel approach for expanding antigen-specific CD8 T cells in patients with cancer or chronic infections (45, 46). On the basis of the present study, joint incubation of CD8 T cells with antigen plus αGalCer before injection would be expected to promote expansion, survival, and effector function of the transferred CD8 T cells, resulting in enhanced therapeutic effect.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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