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# Activation of Natural Killer T Cells by α-Galactosylceramide Rapidly Induces the Full Maturation of Dendritic Cells In Vivo and Thereby Acts as an Adjuvant for Combined CD4 and CD8 T Cell Immunity to a Coadministered Protein

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## Abstract

The maturation of dendritic cells (DCs) allows these antigen-presenting cells to initiate immunity. We pursued this concept in situ by studying the adjuvant action of α-galactosylceramide (αGalCer) in mice. A single i.v. injection of glycolipid induced the full maturation of splenic DCs, beginning within 4 h. Maturation was manifest by marked increases in costimulator and major histocompatibility complex class II expression, interferon (IFN)-γ production, and stimulation of the mixed leukocyte reaction. These changes were not induced directly by αGalCer but required natural killer T (NKT) cells acting independently of the MyD88 adaptor protein. To establish that DC maturation was responsible for the adjuvant role of αGalCer, mice were given αGalCer together with soluble or cell-associated ovalbumin antigen. Th1 type $CD4^+$ and $CD8^+$ T cell responses developed, and the mice became resistant to challenge with ovalbumin-expressing tumor. DCs from mice given ovalbumin plus adjuvant, but not the non-DCs, stimulated ovalbumin-specific proliferative responses and importantly, induced antigen-specific, IFN-γ producing, $CD4^+$ and $CD8^+$ T cells upon transfer into naive animals. In the latter instance, immune priming did not require further exposure to ovalbumin, αGalCer, NKT, or NK cells. Therefore a single dose of αGalCer i.v. rapidly stimulates the full maturation of DCs in situ, and this accounts for the induction of combined Th1 $CD4^+$ and $CD8^+$ T cell immunity to a coadministered protein.

Key words: α-galactosylceramide • dendritic cell maturation • dendritic cells • exogenous pathway • T cell–mediated immunity

## Introduction

It is important to identify new immune adjuvants to improve the efficacy of vaccines against human tumors and many infectious diseases such as tuberculosis, malaria, and AIDS (1, 2). Available adjuvants, like alum and CFA, are suboptimal in that alum can polarize the immune response toward a Th2 type (3), while CFA can induce suppressive macrophages (4) and cytokines (5).

Adjuvants potentially could act at many sites in the immune response, e.g., directly on T cells (6) or on DCs (7). DCs charged with antigens ex vivo are able to induce adaptive immunity upon injection into rodents and humans, both $CD4^+$ and $CD8^+$ T cells. To do so, the injected DCs need to be stimulated ex vivo to undergo an intricate differentiation process termed maturation (8–12). In this way, the injected antigen-bearing mature DCs serve as "nature's adjuvants."

It would be desirable to identify adjuvants that would harness the DC system directly in situ for the purpose of controlling immunity. For example DCs are specialized to present nonreplicating antigens on MHC class I products to $CD8^+$ T cells (for reviews, see references 13 and 14). DC stimulation through toll-like receptors (15–17) and by $CD4^+$ T cells (18, 19) induces some features of DC maturation in vivo. In recent experiments, antigen also has been targeted selectively to DCs in vivo together with an agonistic anti-CD40 antibody to stimulate differentiation of DCs and many other cell types. The combination of antigen targeting to DCs combined with anti-CD40 leads to strong Th1 $CD4^+$ (20) and $CD8^+$ (21, 22) T cell responses. The

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presentation of antigen leading to the induction of effector T cells and memory is consistent with the full maturation of DCs. While these approaches draw a correlation between DC maturation and the development of immunity, it remains to be shown directly that antigen-capturing maturing DCs in vivo are the mediators of immunization.

The synthetic glycolipid α-galactosylceramide (αGalCer)* enhances resistance to tumors (23–26) and several intracellular infections (27–29). The glycolipid is presented to NKT cells by CD1d molecules particularly on DCs (30). Presentation by DCs leads to a rapid innate response and then a more prolonged production of IFN-γ by the NKT cells (31). It is also known that αGalCer acts as an adjuvant for $CD8^+$ T cell–dependent protection against malaria infection, when the glycolipid is administered together with an irradiated sporozoite vaccine (32). These results are surprising because αGalCer has been used to reduce T cell–mediated autoimmune diseases (33–38). The mechanism of adjuvant action of αGalCer is therefore of interest to decipher, especially in the contexts of adjuvant action and DC maturation. Also this glycolipid appears to lack major toxicity in humans and may be useful in the design of vaccines and therapies (39). In this paper, we describe the capacity of αGalCer to act as a stimulus for the full maturation DCs in mice, and we show with an adoptive transfer approach that the mature DCs exclusively mediate the glycolipid's role as an adjuvant to help prime antigen-specific, Th1 $CD4^+$ and $CD8^+$ T cell–mediated immunity.

## Materials and Methods

*Mice.* Pathogen-free C57BL/6 (B6), and $TAP^{-/-}$ female mice at 6–7 wk were purchased from The Jackson Laboratory. We were generously provided with OT-I and OT-II TCR transgenic mice by Dr. F. Carbone (University of Melbourne, Parkville, Victoria, Australia), $MyD88^{-/-}$ mice by Dr. S. Akira (Osaka University, Osaka, Japan), and $J\alpha281^{-/-}$ mice by Dr. M. Taniguchi (Chiba University, Chiba, Japan). Mice were maintained under specific pathogen-free conditions in the animal facility of the Rockefeller University. All experiments were done in compliance with relevant laws and institutional guidelines.

*Reagents.* αGalCer (2S, 3S, 4R-1-O(a-galactopyranosyl)-2(N-hexacosanoylamino)-1,3,4-octadecanetriol) was provided by the Pharmaceutical Research Laboratory, Kirin Brewery (Gunma, Japan) and diluted in PBS. OVA protein was purchased from Seikagaku Corp. LPS was purchased from Sigma-Aldrich, and CpG-DNA from TriLink BioTechnologies. The following mAbs were purchased from BD Biosciences: FITC-conjugated anti-CD8α or PE-conjugated anti-CD11c, CD44, CD62L, biotinylated-isotype control, anti-CD40, CD80, CD86, $I\text{-}A^b$, and Vα2. Biotinylated mAbs were detected with streptavidin-APC. The following mAbs were purified from hybridoma culture supernatants: anti-CD4, anti-CD8, anti-DEC205, and agonistic FGK45.5 anti-CD40 Ab. Rabbit polyclonal anti-asialo GM1, rabbit-IgG, and rat-IgG were purchased from Wako Pure Chemical USA Industries and Jackson ImmunoResearch Laboratories, respectively.

*Flow Cytometry.* Cells were preincubated with 2.4G2 culture supernatant to block Fcγ receptors, then washed and incubated with the indicated mAb conjugates for 30 min. Cells were washed and analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson). For intracellular cytokine staining by FACS®, $5 \times 10^6$ spleen cells were cultured in 24-well plates for 6 or 8 h in the absence or presence of 1 μM $OVA_{257–264}$ peptide (for $CD8^+$ T cells), or 2 μM $OVA_{323–339}$ peptide (for $CD4^+$ T cells) with GolgiPlug (BD Biosciences) to accumulate cytokines intracellularly. The cells were incubated for 15 min at 4°C with the 2.4G2 anti-FcγR mAb to block nonspecific staining. Then we stained cells with anti-CD4 or anti-CD8-FITC, or Vα2-biotin and streptavidin-APC, for 20 min at room temperature. The cells were permeabilized using Cytofix/Cytoperm Plus™ (BD Biosciences) and stained with PE-conjugated anti-IFN-γ (XMG1.2) or anti-IL-4 (11B11) mAb for 15 min at room temperature. Flow cytometry used a FACSCalibur™ instrument and CELLQuest™ software (BD Biosciences) or FlowJo (Tree Star).

*DC Preparation from Spleen.* DCs were isolated from spleens using prior methods (40). In brief, splenocytes were released by homogenization followed by treatment with collagenase (collagenase D; Roche Diagnostics Corporation). A DC-enriched cell population was obtained as a low-density cell fraction using gradients. Collagenase-treated spleens were suspended in a dense BSA solution, overlaid with 1 ml of PBS, and centrifuged for 30 min. We separated low density $CD11c^+$ and $CD11c^-$ fractions using anti-CD11c coated magnetic beads (Miltenyi Biotech). To isolate DC subsets on the basis of CD8α expression (41), we first depleted the low density spleen cells of $CD5^+$ and $CD19^+$ lymphocytes (T and B cells) with magnetic beads. From the lymphocyte-negative fraction, we selected $CD8^+$ and then $CD11c^+$ cells ($CD8^-$ DCs). No difference in viability was observed between $CD8\alpha^+$ and $CD8\alpha^-$ DCs.

*Cytokine Production by DCs.* After mice were given αGalCer, LPS, or PBS i.v., $CD11c^+$ and $CD11c^-$ cells were isolated with anti-CD11c magnetic beads and cultured at $2 \times 10^5$ or $3 \times 10^5$ cells/well for 48–72 h in the absence or presence of FGK45.5 anti-CD40 mAb (10 μg/ml). The titers of IFN-γ, IL-4, IL-10, IL-12p40, and IL-18 in the culture supernatants were determined by OptEIA™ Kit (BD Biosciences), and IL-12p70 was determined by Quantikine ELISA kit (R&D Systems).

*Stimulation of the Mixed Leukocyte Reaction by DCs.* Spleen DCs were isolated using anti-CD11c magnetic beads 8 h after administration of αGalCer, LPS, or PBS. Graded numbers of spleen DCs from C57BL/6 mice were irradiated and cultured with $2 \times 10^5$ allogeneic BALB/C or syngeneic T cells, isolated using T cell enrichment columns (R&D Systems), in a 96-well flat-bottom plate for 88 h. During the final 16 h, $^3$H-thymidine (1 μCi/well) was added. In some experiments, DCs were fixed with 0.75% paraformaldehyde (Electron Microscopy Science) for 30 min on ice.

*DC Presentation of OVA.* To assess the role of αGalCer as an adjuvant for a T cell–mediated immune response, we used OVA as antigen in part because antigen presentation can be readily monitored using $CD4^+$ and $CD8^+$ OVA-specific TCR transgenic T cells. OVA was administered to mice i.v. either as a soluble protein (where 5 mg was required) or in association with dying osmotically shocked, syngeneic, $TAP^{-/-}$ splenocytes (where only 1 μg was associated with the injected cells; the dying cells efficiently target to DCs in spleen, accounting for the high efficiency of presentation as described [42]). Briefly, spleen cells were incubated with hypertonic medium at 37°C in the presence or absence of 10 mg/ml OVA for 10 min, and further incubated

*Abbreviations used in this paper:* TLR, Toll-like receptor; MLR, mixed leukocyte reaction; αGalCer, α-galactosylceramide.

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with hypotonic medium for 2 min to induce apoptosis, followed by washing with cold PBS (42). After injection of OVA in either soluble or cell-associated forms, the following tests for antigen presentation were done. (a) $CD11c^+$ DC-enriched and $CD11c^-$ DC-depleted spleen cells were isolated 4 h after OVA injection and used to stimulate proliferation of OT-I or OT-II T cells in culture as described (42). (b) 7 d after OVA injection, mice were tested for T cell priming by quantifying OVA-specific, IFN-γ and IL-4 producing T cells in the spleen as described above. (c) Mice were given $10^6$ OT-I OVA-specific T cells i.v. and 1 d later the animals were primed with OVA and αGalCer; 3 d later, the OVA-specific T cells were monitored for expansion in cell numbers and intracellular IFN-γ production (22). (d) $CD11c^+$ DC-enriched and $CD11c^-$ DC-depleted spleen cells were isolated 4 h after OVA injection and used at a dose of 1 and $10 \times 10^6$ respectively to prime naive recipients, assessed as in approach (b) above.

*Tumor Protection Experiments.* $2 \times 10^7$ OVA pulsed apoptotic spleen cells were used to immunize mice, either mice given OT-I OVA-specific T cells i.v. 1 d earlier or naive animals, (42). 3 d later, $2 \times 10^6$ EG7, OVA-transduced tumor cells (American Type Culture Collection; CRL-2113) were inoculated s.c. The parental non-OVA transduced EL-4 thymoma was used as control tumor. To identify protective cells, we used blocking antibodies given i.p. 2 d before tumor inoculation and every 2 d after. The antibodies were control rat IgG, rat anti-CD4 (GK1.5) and CD8 (53–6.72), and rabbit anti-asialoGM1 Ab or control rabbit IgG in PBS. Mice were killed when tumor growth exceeded 400 $mm^2$.

*Statistical Analysis.* The statistical significance of differences between the experimental groups was determined by the Mann-Whitney exact rank sum test.

## Results

*Maturation of the DC Surface After a Single I.V. Injection of αGalCer.* αGalCer enhances resistance to tumors and infections and is also presented by DCs to NKT cells (Introduction). We used several criteria to test if αGalCer leads to in vivo maturation of DCs, including their capacity to simultaneously capture and present antigen to diverse TCRαβ T cells and induce immunity. After i.v. administration of αGalCer, both $CD8^+$ and $CD8^-$ DC subsets up-regulated several markers consistent with maturation. This included molecules involved in T cell costimulation (CD40, 80, 86), as well as antigen capture and presentation (MHC class II and the DEC-205 endocytic receptor; Fig. 1 A, compare the tracings for αGalCer with the black tracing for the vehicle control). These responses to glycolipid paralleled those seen with other known stimuli for DC maturation in vivo (Fig. 1 A), i.e., LPS (15), CpG deoxyoligonucleotides (16, 17), and agonistic anti-CD40 mAb (20–22).

When the kinetics of the splenic DC response to αGalCer was examined, as shown for CD86, maturation was evident within 4 h, and then began to subside at 48 h, returning to baseline at 72 h (Fig. 1, B and C). Axillary lymph node DCs from the same mice only showed minimal increases in CD86 and CD40 (Fig. 1 D). The kinetics of the splenic DC response to αGalCer was similar to that seen with LPS treatment (unpublished data). However, αGalCer was unable to directly stimulate DC maturation from bone marrow progenitors in culture, in contrast to LPS (Fig. 1 E). Therefore, αGalCer acts as a rapid and efficient inducer of splenic DC maturation in vivo, comparable in efficacy to other stimuli, but it does not have a direct effect on DCs, unlike other maturation stimuli.

*NKT Cells Mediate DC Maturation in a MyD88-independent Manner.* NKT cells respond quickly to the presentation of αGalCer on CD1d molecules (43). To address the role of NKT cells in the rapid maturation of DCs, we tested mice lacking these T cells because of the deletion of essential TCR Jα281 sequences (44). The DCs from $J\alpha281^{-/-}$ mice did not mature in response to αGalCer in vivo and expressed comparable levels of CD86 and other maturation markers (CD40, CD80, MHC II) to wild-type mice given the PBS vehicle control (Fig. 2 A, left). As a positive control, DCs from $J\alpha281^{-/-}$ mice were shown to mature in response to LPS and express high levels of CD86 (Fig. 2 A, right). We then tested DCs from $MyD88^{-/-}$ mice, where MyD88 is an essential adaptor protein for signaling cytokine production via Toll-like receptors (TLRs; references 45 and 46). The DCs from $MyD88^{-/-}$ mice did respond to αGalCer, maturing as effectively as DCs from wild type mice (Fig. 2 B). DCs from NK cell-depleted animals (using anti-asialoGM1 antibody treatment [31]) also matured in response to αGalCer (Fig. 2 C). Therefore, αGalCer in concert with NKT cells rapidly matures DCs in situ, as assessed by the surface markers of DCs in spleen, but the response does not require MyD88-based signaling or NK cells.

*Functional Maturation of αGalCer-stimulated DCs.* To demonstrate that DCs from αGalCer treated mice could stimulate resting T cells, we isolated DCs with anti-CD11c magnetic beads and tested them in some standard assays for DC maturation, e.g., as stimulators for allogeneic T cells in the primary mixed leukocyte reaction (MLR). DCs from all groups of mice (controls and mice treated with αGalCer or LPS) stimulated allogeneic T cell proliferation (Fig. 3 A) and to a much lesser extent, syngeneic T cell proliferation (Fig. 3 B). As splenic DCs are able to mature "spontaneously" in culture (47), we also tested DCs that were fixed in paraformaldehyde immediately upon isolation, to assess their MLR stimulating activity at the time of isolation. The fixed DCs from control mice no longer had stimulating activity for the MLR (Fig. 3 C), confirming prior work that most DCs in a spleen are functionally immature (20–22). In contrast, fixed DCs from αGalCer treated mice were potent stimulators of the allogeneic MLR (Fig. 3 C), suggesting the DCs had matured in the mice before isolation and fixation. With both live and fixed cells, $CD11c^-$ spleen cells lacked MLR stimulating activity at the doses tested (Fig. 3, A–D, open symbols). To prove that enhanced MLR stimulation by $CD11c^+$ DCs was dependent upon NKT cells, we repeated the experiments on fixed DCs with $J\alpha281^{-/-}$ mice. The increase in MLR stimulation was now ablated (Fig. 3 E).

Maturation stimuli also can prime DCs in vivo to produce large amounts of immune enhancing cytokines such

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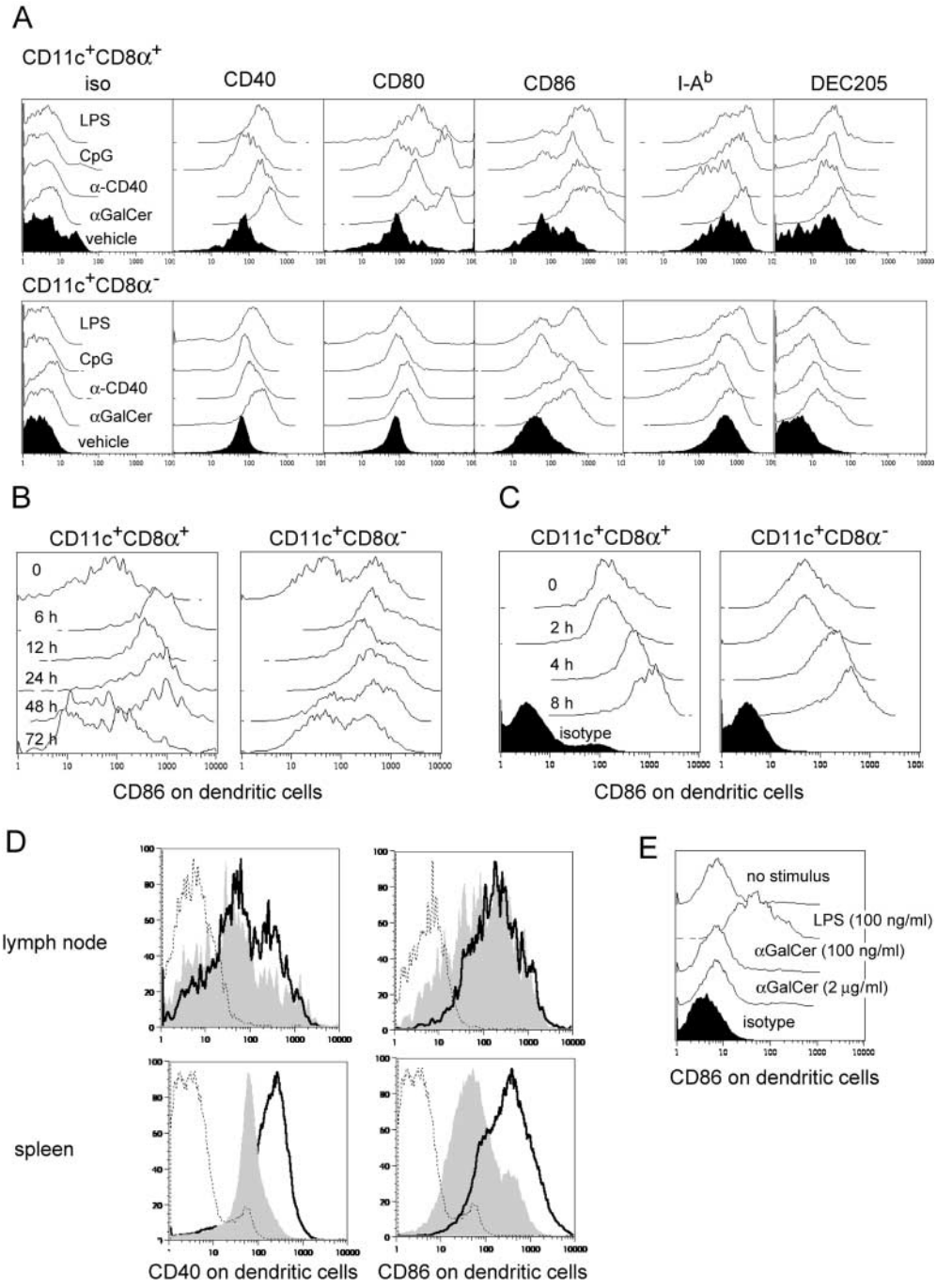


**Figure 1.** Maturation of DC surface markers with αGalCer and other stimuli in vivo. (A) 24 h after i.v. administration of LPS (25 μg), CpG ODN (10 μg), agonistic anti-CD40 mAb (50 μg), αGalCer (2 μg), or PBS (black tracing) to C57BL/6 mice, low density spleen cells were stained with PE-CD11c and FITC-CD8α (to identify DCs and their $CD8^+$ or $CD8^-$ subsets) and with biotinylated isotype control, anti-CD40, CD80, CD86, $I\text{-}A^b$, and DEC205/CD205 followed by streptavidin APC. (B and C) As in (A), but the surface markers of $CD8^-$ or $CD8^+$ DCs were followed at the indicated time points after i.v. administration of the maturation stimulus; data shown are for CD86 expression. (D) Minimal up-regulation of CD40 and CD86 on lymph node DCs (relative to splenic DCs) 24 h after α-GalCer i.v. Dotted line is the isotype control, gray shadow is antigen expression at time 0, and bold black line is CD40 and CD86 stain at 24 h. (E) Inability of αGalCer to induce the maturation of DCs from bone marrow progenitors, in contrast to LPS, as shown for up-regulation of CD86 on $CD11c^+$ DCs from day 6–7 in culture.

as IL-12 (48), IFN-γ (49), and IFN-α (50). We studied cytokine production by cells from mice stimulated 8 h in vivo with αGalCer i.v., with LPS in comparison. We prepared $CD11c^+$ DC-enriched and $CD11c^-$ DC-depleted cells, and further fractionated the DCs into $CD8^+$ and $CD8^-$ subsets. The $CD11c^+$ DCs contained few contaminating T and NK cells (0.5% $CD3^+$ and $<0.1\%$ $NK1.1^+$; unpublished data), while the DC subsets ($CD8^+$ and $CD8^-$) were obtained after depletion of T cells with anti-CD5 (Materials and Methods). $CD11c^+$ DCs from αGalCer treated mice produced very high levels of IFN-γ (Fig. 3 F). As prior work (48) showed that IL-12 production by DCs requires sequential stimuli from a microbe and then a T cell, e.g., through CD40 stimulation, we restimulated the DCs primed to αGalCer in vivo with anti-CD40 in culture. Now the DCs from αGalCer primed mice also produced IL-12 p70, in contrast to DCs from either PBS or LPS treated mice (Fig. 3, F and G). DCs from PBS, LPS, and αGalCer treated mice all produced IL-12 p40, possibly because of stimuli arising during their isolation and culture, but as in the case of IFN-γ, production of IL-12 p40 and p70 were most active in the $CD8^-$ DC subset

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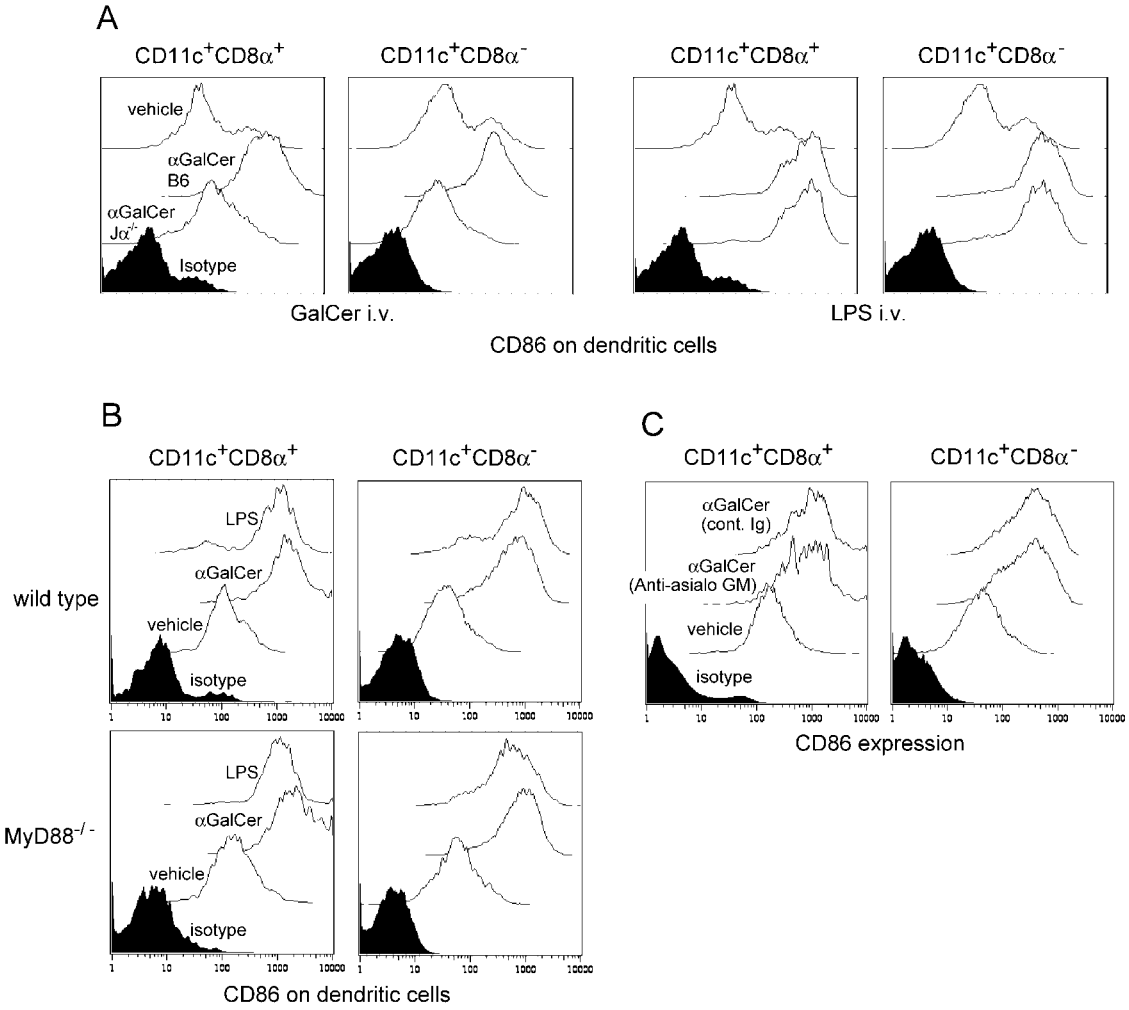


**Figure 2.** DC maturation by αGalCer in vivo requires NKT cells. (A) Maturation, assessed by increased CD86 expression, did not occur in Jα281$^{-/-}$ mice (lacking NKT cells) exposed to αGalCer (left), but did occur with LPS (right). (B) Maturation of DCs from MyD88 $^{-/-}$ mice in response to αGalCer and LPS, and (C) from mice depleted of NK cells by anti-asialoGM1.

(Fig. 3, F and H). In contrast to these findings with DC stimulated in vivo with αGalCer, Ohteki et al. showed that the CD8$^+$ DC subset from *Listeria*-infected mice was more active in IFN-γ production after stimulation with IL-12 in vitro (49). We could not detect IL-4 and IL-10 (<10 pg/ml) in DCs from αGalCer primed mice (unpublished data). Interestingly, DCs stimulated by LPS in vivo responded to CD40 restimulation in culture by producing IL-18 rather than IL-12 (unpublished data). In sum, αGalCer matures DCs to produce enhancing cytokines and to more actively stimulate quiescent T cells in the mixed leukocyte reaction.

*DCs Responding to OVA plus αGalCer Prime CD4$^+$ and CD8$^+$ T Cells in Culture.* To prove that DC maturation was associated with enhanced immunogenicity, we next injected mice with the protein antigen, OVA, either as a soluble protein or in association with dying TAP$^{-/-}$ splenocytes (the TAP$^{-/-}$ deletion ensured OVA presentation by MHC class I of recipient DCs rather than the injected splenocytes [21]), without or with αGalCer. Then we isolated the DCs and non-DC fractions from spleen and measured their capacity in tissue culture to stimulate CD8$^+$ and CD4$^+$, OVA-specific, naive OT-I and OT-II, TCR transgenic T cells respectively (Fig. 4 A). With both soluble (Fig. 4 B) and cell-associated OVA (Fig. 4 C) as antigen, the DCs selectively and actively stimulated proliferative responses by the naive CD4$^+$ and CD8$^+$ T cells in culture; CD11c$^-$ cells were inactive (Fig. 4, B and C). The T cells produced substantial IFN-γ, >10 ng/ml culture, but no detectable IL-4 (unpublished data), indicating that antigen-capturing DCs were able to polarize the Th1 type of T cell differentiation.

The apparent lack of T cell stimulating activity in the CD11c$^-$ fractions was puzzling, because these cells should include marginal zone B cells that express high levels of CD1d (51, 52) and are activated by αGalCer in vivo (53). Therefore, in the OVA experiments of Fig. 4, we also enriched CD19$^+$ CD21$^+$ CD23$^-$ marginal zone B cells and

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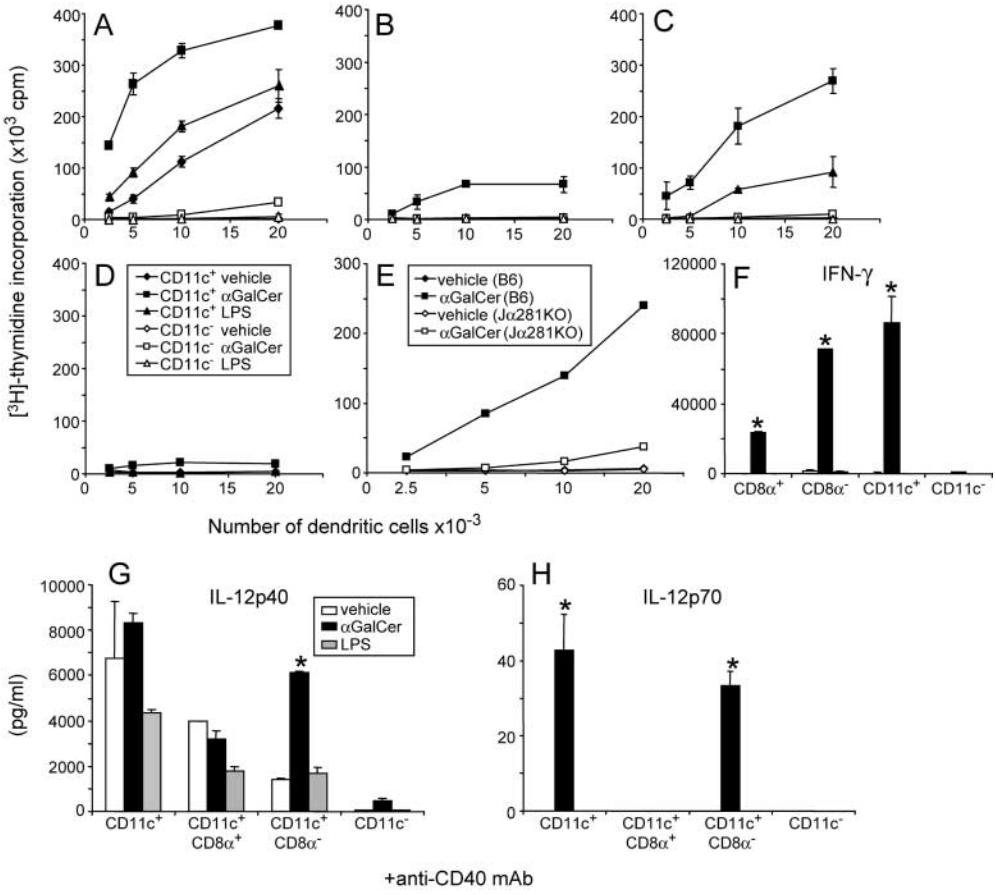


**Figure 3.** Functional maturation of splenic DCs from αGalCer treated mice. 8 h after i.v. administration of αGalCer, LPS or PBS, as in Fig. 1, spleen DCs were isolated using anti-CD11c–coated magnetic beads (purity >95 ± 2%). Graded numbers of spleen DCs from C57BL/6 mice were irradiated (30 Gy) and added to $2 \times 10^5$ allogeneic BALB/c (A) or syngeneic (B) T cells for 3 d in flat bottomed 96-well plates. In parallel, DCs were fixed with paraformaldehyde for 30 min, to block their maturation during the mixed leukocyte reaction, and also irradiated, followed by addition to allogeneic (C) and syngeneic (D) T cells. Proliferative responses were measured by [$^3$H]-thymidine incorporation (Materials and Methods). The results are representative of four independent experiments. In E, the experiment in B was repeated comparing fixed DCs from wild-type and Jα281$^{-/-}$ NKT deficient mice given αGalCer or vehicle. The results are averages of three independent experiments. (F and G) Mice were given PBS, αGalCer, or LPS i.v. and 8 h later, CD11c$^+$ DC enriched and CD11c$^-$ DC-depleted, low density spleen cells were isolated with α-CD11c coated magnetic beads. In parallel, to test cytokine production by DC subsets, the low density spleen cells were first lymphocyte depleted with CD5$^+$ and CD19$^+$ magnetic beads, and then CD8$^+$ and CD11c$^+$ cells were selected successively with magnetic beads, providing enriched CD8$^+$ and CD8$^-$ DC subsets respectively. IFN-γ secretion (pg/ml) by each cell subset was measured by ELISA assays after culturing the cells for 48 h without further stimulation in 96 well plates at $2 \times 10^5$/well (F), while IL-12 production was measured in cells cultured 72 h in the presence of 10 μg/ml agonistic FGK45.5 anti-CD40 antibody at $3 \times 10^5$/well (G and H). All results required pools of three mice and represent averages of three independent experiments (*$P < 0.05$).

CD19$^+$ CD21$^-$ CD23$^+$ follicular B cells. We confirmed that the former had higher levels of CD1d and that both types of B cell up-regulated CD86 in response to αGalCer (Fig. 4 D, left part of figure). Both B cell types failed to present OVA (Fig. 4 D, right part of figure), especially when compared with the CD11c$^+$ DCs isolated from these same animals (Fig. 4 B). Thus, DCs are the major cell type that acquires stimulating activity for naive T cells in response to the combined administration of a foreign protein and αGalCer.

*OVA Together with αGalCer as Adjuvant Primes CD8$^+$ T Cells In Vivo and Increases Resistance to OVA-expressing Tumors.* To monitor the activation of OVA-specific CD8$^+$ T cells more directly in vivo, mice were injected i.v. with $10^6$ CFSE-labeled CD8$^+$, OVA-specific, TCR transgenic T cells. 1 d later, OVA-loaded splenocytes were given without or with αGalCer. 3 d after injection of the OVA-loaded splenocytes, there was extensive expansion of the injected OT-I T cells and dilution of the CFSE label (proliferation). The injection of αGalCer together with antigen increased the frequency of cells in the spleen by threefold (Fig. 5 B) and induced them to express high levels of the activation antigen CD44 (unpublished data). The spleen cells were also cultured with or without OVA peptide to determine if the T cells, gated for expression of the Vα2 OT-I TCR, had been primed to produce IFN-γ. The use of the αGalCer adjuvant induced high level IFN-γ production in most OT-I T cells (Fig. 5 B), but no detectable IL-4 (unpublished data). To verify that αGalCer-dependent priming was associated with enhanced resistance to OVA-expressing tumors, we gave mice $10^6$ OT-I T cells i.v. and 1 d later, we administered αGalCer and OVA-loaded cells also i.v. 3 d later, we injected $2 \times 10^6$ EG7 cells into the skin, where EG7 is a murine thymoma transduced to express OVA in a secretory form. The mice primed with TAP$^{-/-}$ / OVA and αGalCer showed strong resistance to the tumor, whereas mice primed with TAP$^{-/-}$/ OVA splenocytes alone showed some resistance, but the EG7 tumors recurred (Fig. 5 C). We repeated the experiments in mice depleted of CD8$^+$ or CD4$^+$ T cells with monoclonal antibodies, or NK cells with anti-asialo GM1 (these depletions were begun 1 d after administering αGal-

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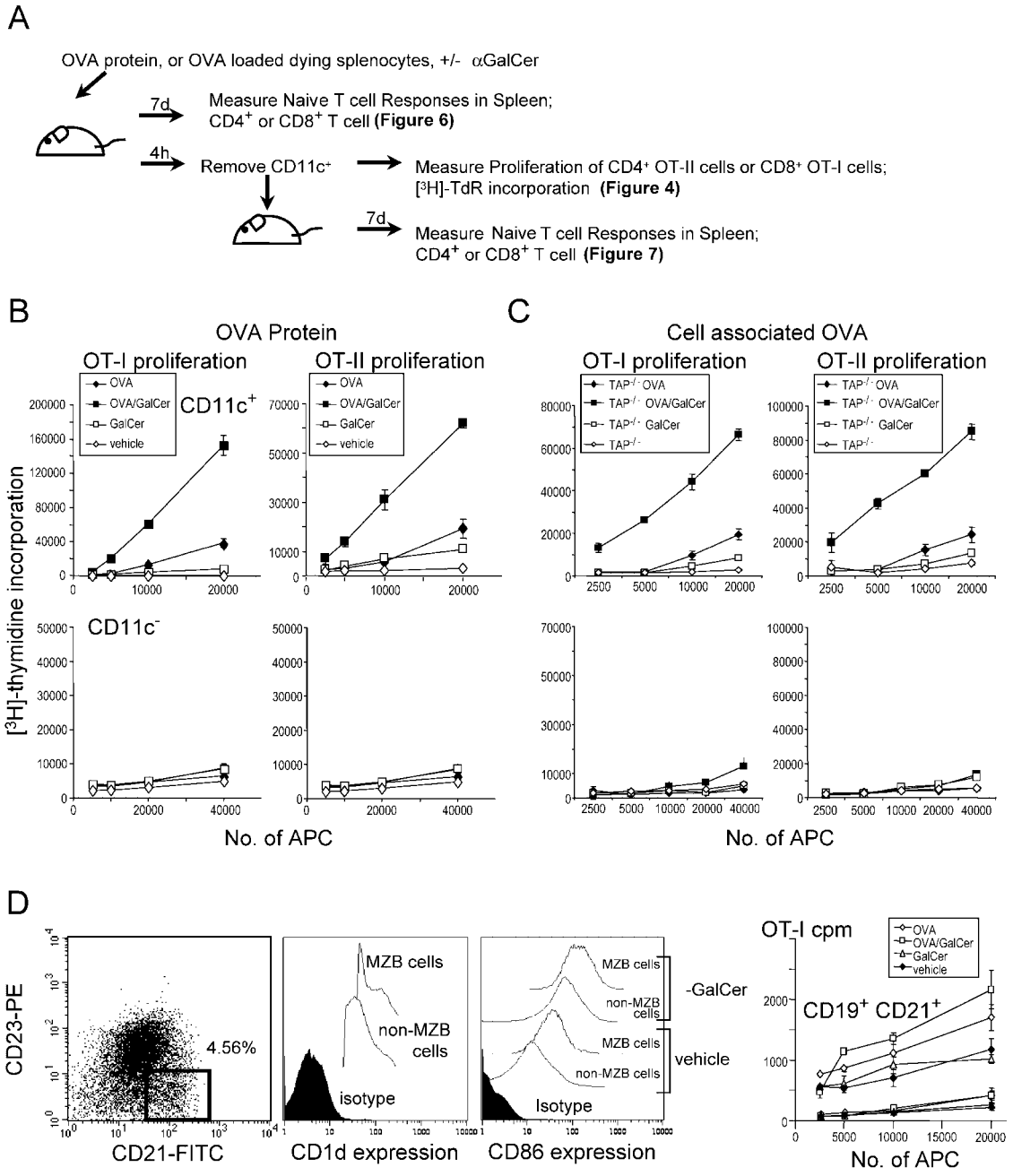


**Figure 4.** DCs capturing OVA protein and matured by αGalCer in vivo efficiently stimulate naive $CD4^+$ and $CD8^+$ T cells in culture. (A) Schematics for the experiments in Figs. 4, 6, and 7. Mice were given αGalCer or vehicle (PBS) together with OVA protein (5 mg/mouse) i.v. or osmotically shocked syngeneic splenocytes loaded with OVA protein as described (reference 21). 4 h later, $CD11c^+$ cells or $CD11c^-$ cells were isolated and tested for their capacity to stimulate $CD8^+$ and $CD4^+$ TCR transgenic T cells from OT-I and OT-II mice, respectively. T cell proliferation was measured after $[^3H]$-thymidine at 36–48 h. In some experiments (Fig. 5), OVA-specific T cell responses in spleen were evaluated 1 wk after immunization, while in others (Fig. 7), $CD11c^+$ cells were transferred into naive mice to prime T cells in vivo. (B and C) The responses of $CD4^+$ (OT-II) and $CD8^+$ (OT-I) T cells to $CD11c^+$ (top) and $CD11c^-$ (bottom) spleen cells from mice given soluble OVA (left panels) or cell-associated OVA (right panels) in the absence or presence of αGalCer; representative of three independent experiments. (D) As in B, but enriched populations of $CD21^{high}$ marginal zone B cells and $CD21^{low}$ follicular B cells were isolated by cell sorting and studied for CD1d and CD86 expression, as well as presentation of the injected soluble OVA to OT-I T cells. $CD21^{high}$ marginal zone B cells are the upper group of data on the right, while $CD21^{low}$ follicular B cells are the lower group (note much lower values on y-axis for D vs. B and C).

Cer to allow time for the NKT cells, which express CD4, to mature the DCs). Only CD8 depletion ablated the resistance induced by OVA-loaded splenocytes together with αGalCer (Fig. 5 D).

To determine if αGalCer could serve as an adjuvant for generating specific $CD4^+$ or $CD8^+$ T cell responses to OVA in naive animals, we injected $2 \times 10^7$ dying cells loaded with OVA i.v., and half the animals also received

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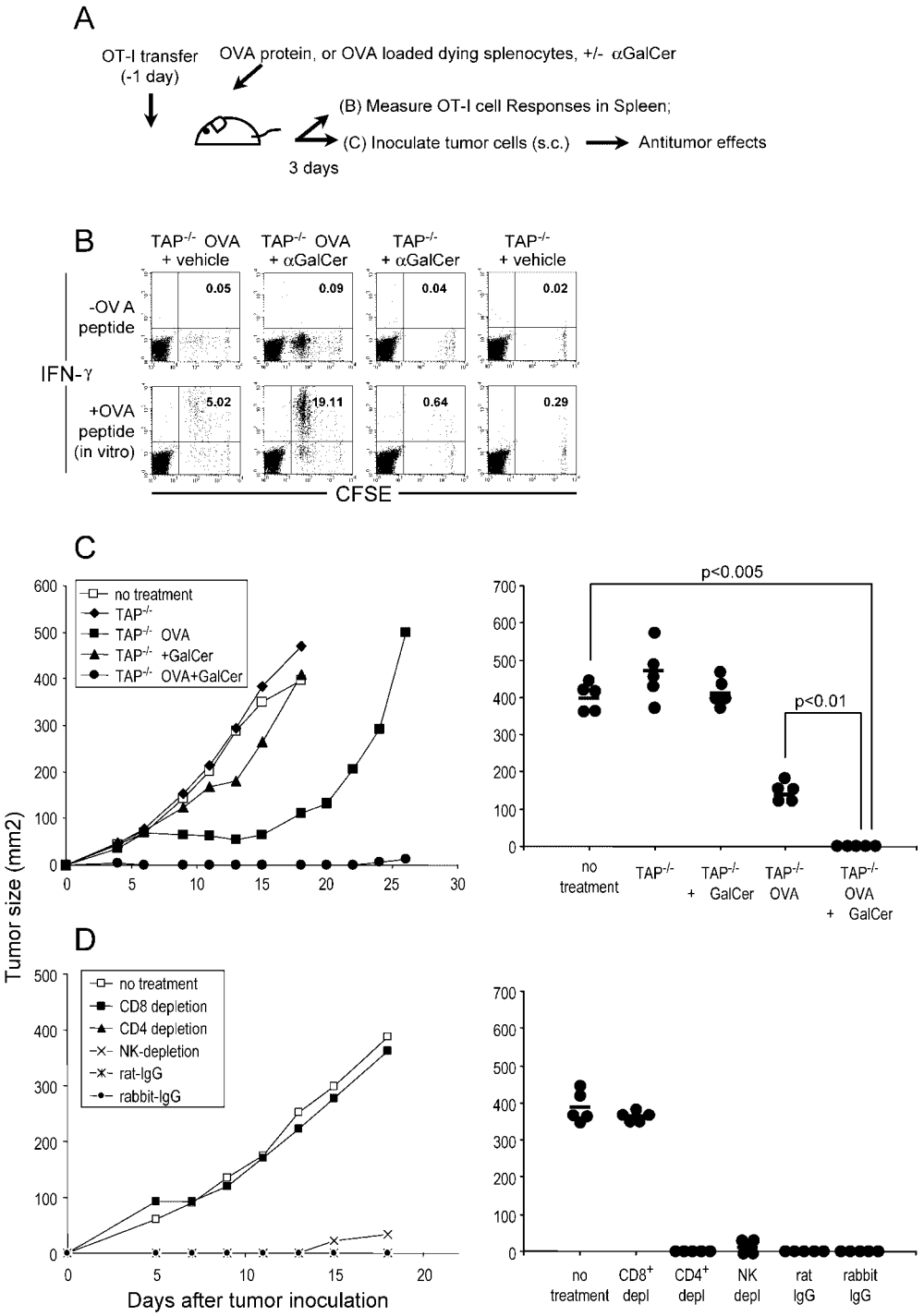


**Figure 5.** αGalCer enhances immunity to OVA-bearing DCs in vivo. (A and B) 1 d after transferring $10^6$ CFSE-labeled OT-I cells i.v. into C57BL/6 mice, four groups of mice were given either no treatment, osmotically shocked and OVA loaded $TAP^{-/-}$ spleen cells, αGalCer (2 μg/mouse), or both OVA loaded spleen cells and αGalCer. 3 d later, spleen cells were monitored for dilution of fluorescence in the CFSE labeled $V\alpha2^+$ OT-I cells in the spleen. IFN-γ production was monitored in tandem after culturing the spleen cells without or with $OVA_{257–264}$ (1 μM) for 6 h in the presence of GolgiPlug. Each panel shows the percentage of cytokine producing, CFSE-labeled, $V\alpha2^+$ cells in the spleen. (C and D) At day 3 after immunization as in A and B, the mice were injected s.c. with EG7 tumor cells, which stably express OVA as a secreted protein. Mean tumor sizes at 2 d intervals were plotted on the left, and the distribution of tumor sizes at day 18 in the individual animals shown on the right. The experiments in the bottom panels were similar to those in the top except that mice were immunized with OVA loaded spleen cells and αGalCer but depleted of individual lymphocyte subsets (from 2 d before tumor inoculation to the end of the experiments) with monoclonal antibodies as indicated.

αGalCer. 7 d later, we tested if splenic T cells would secrete IFN-γ in response to rechallenge with OVA peptides presented on MHC class I or II products (Fig. 4 A for diagram of protocol). αGalCer did serve as an adjuvant to prime $CD4^+$ and $CD8^+$ T cells in vivo, as illustrated by intracellular cytokine staining for IFN-γ in Fig. 6 A and summarized in Fig. 6 B. IL-4 production was not detectable (unpublished data). When we tried to prime $J\alpha281^{-/-}$ mice lacking NKT cells; however, αGalCer no longer served as an adjuvant (Fig. 6 B, bottom). To establish that the observed T cell immunity in wild-type mice was associated with protective resistance, we verified that naive mice immunized with the combination of $TAP^{-/-}$/OVA splenocytes and αGalCer showed resistance to OVA-transduced EG7 tumor cells, but no resistance to the parent EL-4 thymoma that was not transduced to express OVA (Fig. 6 C, compare left and right panels). In summary, a single intravenous dose of αGalCer acts as an adju-

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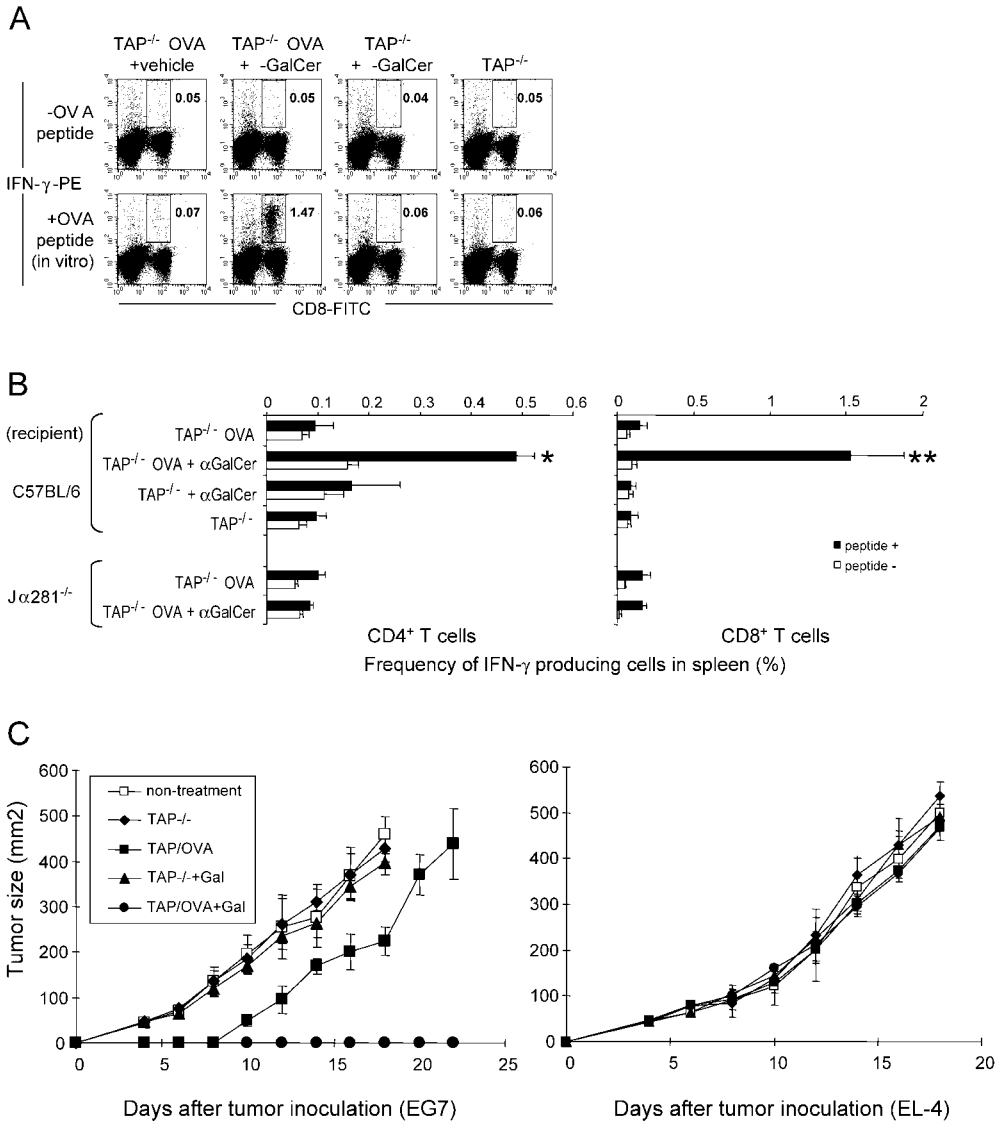


**Figure 6.** Induction of strong immunity in naive mice with cell associated OVA together with αGalCer. (A) Priming of naive mice with the combination of $2 \times 10^7$ OVA-loaded, $TAP^{-/-}$ spleen cells and αGalCer (2 μg/mouse) i.v. 7 d later, spleen cells were cultured without or with $OVA_{257–264}$ or $OVA_{323–339}$ peptides to stimulate OVA specific $CD8^+$ and $CD4^+$ T cells respectively and T cell production of IFN-γ was measured by intracellular cytokine staining (the frequency of T cells producing IL-4 was <0.04% for all groups). (B) Same as (A), but means are shown for four individual mice (*$P < 0.05$, **$P < 0.01$ for the wild-type mice primed with OVA-loaded, $TAP^{-/-}$ spleen cells and αGalCer vs. the other groups). $CD4^+$ and $CD8^+$ T cell responses were elicited by $OVA_{323–339}$ and $OVA_{257–264}$ peptides respectively. (C) 1 wk after immunization in each group, mice were challenged with either $2 \times 10^6$ EG7 or EL-4 tumor cells. The data are mean tumor sizes ± SEM of 6 (EG7) or 3–4 (EL4) individual mice in independent experiments. ($TAP^{-/-}$/OVA + αGalCer vs. the other groups at day 18; $P < 0.005$).

vant for combined Th1 type $CD4^+$ and $CD8^+$ T cell immunity in mice.

*The Adjuvant Action of αGalCer Is Due to Mature DCs.* Given the evidence for T cell responses to the coadministration of protein antigen and αGalCer, we wanted to prove that the mature DCs were responsible for the glycolipid's adjuvant action. To do so, we again gave mice OVA antigen as a soluble protein or in association with dying cells. Then we isolated $CD11c^+$ DCs from spleen 4 h later and tested their capacity to generate $CD4^+$ and $CD8^+$ effector T cells in naive animals (Fig. 4 A for diagram of the protocol). We monitored T cell priming by looking at day 7 for IFN-γ production by both T cell subsets using an intracellular cytokine secretion assay performed on lymph node and spleen cells cultured for 6 h with or without OVA peptides in the presence of brefeldin A (Materials and Methods).

We first obtained DCs from 4 groups of mice primed with vehicle, OVA only, αGalCer only, or both OVA and αGalCer. The latter DCs primed IFN-γ producing $CD4^+$ and $CD8^+$ T cells in naive mice (Fig. 7, A and B; top panels). The results were virtually identical in spleen (Fig. 7) and lymph node (unpublished data). Again, no IL-4 producing T cells could be detected (unpublished data). We were concerned that some of the primed IFN-γ producing cells might be NKT cells, but very few of the $CD4^+$ and $CD8^+$ cytokine producers labeled for the NK1.1 marker (unpublished data), presumably because at day 6, the NKT response to αGalCer on DCs has largely subsided (31).

It remained possible that the transferred mature DCs in Fig. 7 were not priming naive T cells directly, but still required either NK or NKT cells in the recipient animals. We therefore repeated the experiments but injected the DCs (from mice primed with OVA splenocytes and αGalCer) into three groups of recipients: control mice treated with rabbit Ig, mice treated with rabbit anti-asialoGM1 to deplete NK cells (31), and mice depleted of NKT cells through deletion of the Jα 281 genetic sequences (44). In

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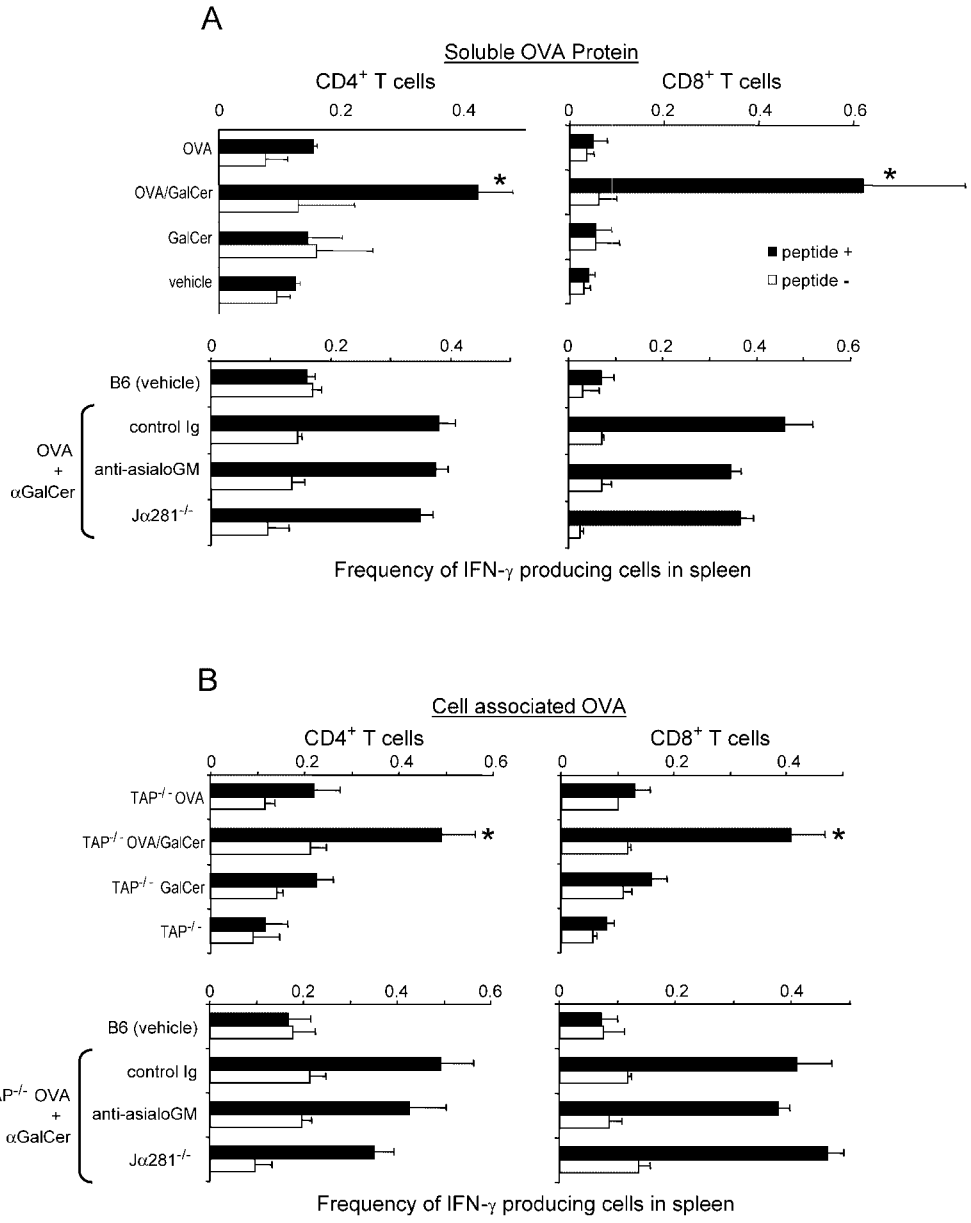


**Figure 7.** DCs isolated from mice given OVA together with αGalCer are able to prime naive animals. Mice were given αGalCer or vehicle together with either OVA protein (5 mg/mouse) i.v. (A) or with osmotically shocked $TAP^{-/-}$ splenocytes without or with OVA loading (B). 4 h after injection, $10^6$ $CD11c^+$ DCs from each group were transferred to naive syngeneic mice. In the top panels, 7 d after immunization with DCs but no further antigen or αGalCer, spleen cells were cultured without (open bars) or with (closed bars) OVA peptides with GolgiPlug to allow accumulation of intracellular IFN-γ as described in experimental procedures. Intracellular IFN-γ staining was performed with double labeling for CD4 and CD8. In the bottom panels, the DCs were taken from mice given αGalCer together with either OVA protein (A) or OVA loaded splenocytes (B), but the DCs were injected into either rabbit Ig (control)-treated mice or mice lacking NKT and NK cells (see Results) to test priming of $CD4^+$ and $CD8^+$, IFN-γ producing effector T cells. The data are mean ± SEM of three individual mice in independent experiments. *$P < 0.05$.

all cases, the DCs from mice given OVA together with αGalCer were able to prime the recipients (Fig. 7, A and B; lower rows). Therefore the NKT cell dependent maturation of antigen capturing DCs by αGalCer in donor mice leads to the priming of Th1 $CD4^+$ and $CD8^+$ effector T cells in naive recipient animals.

## Discussion

*Stimuli for the Maturation of DCs In Situ.* Most DCs in a mouse spleen are functionally immature in the steady-state, but they undergo rapid maturation in response to αGalCer. Several criteria have been used here to document the full maturation of DCs, which in prior studies has been monitored primarily at the level of increased expression of costimulatory molecules like CD86. Here we have emphasized the capacity of DCs in vivo to become potent stimulators of immunity, the classical criterion for maturation.

A useful functional marker for maturation is enhanced T cell stimulatory activity in the mixed leukocyte reaction. It seems that this is best assayed with chemically fixed DCs, as fixation should prevent DCs from undergoing "spontaneous" maturation in culture (47). When this is done, DCs from control mice are weak MLR stimulators, whereas formaldehyde-fixed DCs from αGalCer treated mice are potent. This maturation criterion is fully dependent upon NKT cells. It has also been reported that some features of DC maturation are induced by CD1d-restricted human NKT cell clones in vitro (54).

Maturation of DCs in mice also has been achieved by stimuli for toll like receptors, e.g., LPS (15) and CpG oligodeoxynucleotides (16, 17). Likewise TCRαβ T cells in-

duce DC maturation in vivo, as assessed by the up-regulation of surface costimulatory molecules (18, 19). In this paper, αGalCer harnesses NKT cells to fully mature DCs as evidenced by the induction of immunity to antigens coadministered with the glycolipid. In contrast to T cell–mediated maturation, which is a part of an adaptive immune response, NKT cells are innate cells that mature most of the DCs in spleen very quickly, within 4 h of administering αGalCer by the intravenous route.

*Mechanisms of Maturation via αGalCer.* The maturation of DCs after presentation of αGalCer to NKT cells seems to operate independently of TLR signaling. MyD88 is one of the adaptor proteins for signaling cytokine production through TLRs, but MyD88 is not required for the maturation of DCs by αGalCer. DCs have a MyD88-independent but TLR-dependent maturation pathway, especially through TLR4 (55, 56). However, this should lead to Th2 type responses (57), whereas DCs matured from αGalCer treated, MyD88 knockout mice induce Th1 type responses (unpublished data).

Transactivation of NK and B cells following administration of αGalCer is well known (58). As a high proportion of many cell types including DCs are activated in response to the glycolipid, a "cytokine storm" induced by αGalCer likely is playing a role. However, it is requiring a good deal of experimentation to identify the mechanisms of NKT-dependent DC maturation in vivo, and this will be the subject of a future report.

*αGalCer as an Adjuvant for Th1 Type Cell-mediated Immunity.* It may be surprising that αGalCer matures splenic DCs and also acts as an adjuvant for strong Th1 type $CD4^+$ and $CD8^+$ T cell responses to antigens given i.v., as numerous reports have used this same compound to suppress experimental, Th1-dependent autoimmune diseases (33–38, 59). Naumov et al. have reported that αGalCer treatment of autoimmune prone animals induces a form of tolerogenic DCs in the lymph nodes that drain the target organ for the autoimmune disease (37). The basis for these ostensibly major differences in immunologic outcome is unclear, i.e., polarization in our study to Th1 responses versus polarization toward Th2 in other studies. However, the experimental designs are different. We have used a single intravenous dose of glycolipid to activate DCs to produce Th1 polarizing cytokines such as IFN-γ and IL-12 at the same time that antigens are given i.v. for presentation by the maturing DCs. In contrast, repeated doses of αGalCer have been given to treat autoimmune prone mice. In other instances, a multiple dose regimen (60) or the s.c. administration of OVA together with CFA (61), have been associated with Th2 polarization to external antigens in response to αGalCer. A Th2 environment may originate from the finding that soluble αGalCer is able to anergize NKT cells after the initial cytokine response, such that the NKT cells can no longer respond to DCs and in particular to produce IFN-γ (31).

To document the role of DC maturation for adjuvant function in vivo, we have isolated the $CD11^+$ DC-enriched and $CD11c^-$ DC-depleted populations from the spleens after injection of either soluble or cell-associated OVA antigen in the absence or presence of αGalCer. Strong $CD4^+$ and $CD8^+$ T cell responses are induced by DCs from αGalCer-treated mice, whereas we do not detect immunogenicity with non-DCs from these animals. This adoptive transfer approach therefore establishes that mature DCs are responsible for the in vivo action of adjuvants, since the recipients of the DCs do not require additional exposure to OVA antigen, αGalCer adjuvant, or innate NKT and NK cells. In the case of cell-associated OVA, this antigen is known to induce peripheral tolerance in the absence of αGalCer (21), so that the protective tumor immunity we have observed overrides an otherwise tolerogenic situation. These data indicate that DCs are necessary and sufficient for the adjuvant role of αGalCer, including its capacity to enhance $CD8^+$ T cell responses to nonreplicating forms of a protein antigen. To harness the adjuvant properties of this glycolipid, however, DCs need to capture the antigen or vaccine in question and also present the αGalCer to NKT cells.

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