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# α-Galactosylceramide Can Act As a Nasal Vaccine Adjuvant Inducing Protective Immune Responses against Viral Infection and Tumor[1] FREE

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# α-Galactosylceramide Can Act As a Nasal Vaccine Adjuvant Inducing Protective Immune Responses against Viral Infection and Tumor[1]

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**α-Galactosylceramide (α-GalCer) is a ligand of invariant Vα14$^+$ NKT cells and is presented by CD1d molecule on APC. NKT cells produce a large amount of Th1 and Th2 cytokines in response to α-GalCer-presented APC. In this study, we assessed whether α-GalCer could act as an effective nasal vaccine adjuvant for mucosal vaccine that would be capable of inducing systemic as well as mucosal immune responses. When α-GalCer was administered with OVA via the intranasal route to C57BL/6 and BALB/c mice, significant OVA-specific mucosal secretory IgA, systemic IgG, and CTL responses were induced with mixed Th1 and Th2 cytokine profiles seen in both strains of mice. Interestingly, as BALB/c mice were intranasally immunized with PR8 hemagglutinin Ag isolated from influenza virus A/PR/8/34 together with α-GalCer, significant protection was afforded against influenza viral infection. When α-GalCer was coimmunized with a replication-deficient live adenovirus to BALB/c mice, it significantly induced both humoral and cellular immune responses. In addition, intranasal administration of OVA with α-GalCer showed complete protection against EG7 tumor challenge in C57BL/6. The adjuvant effects induced by intranasal coadministration with α-GalCer were blocked in CD1d$^{-/-}$ mice, indicating that the immune responses were exclusively mediated by CD1d molecule on APC. Most interestingly, intranasally coadministered α-GalCer activated naive T cells and triggered them to differentiate into functional effector T cells when CFSE-labeled OT-1 cells were adoptively transferred into syngeneic mice. Overall, our results are the first to show that α-GalCer can act as a nasal vaccine adjuvant inducing protective immune responses against viral infections and tumors.** *The Journal of Immunology,* **2005, 175: 3309–3317.**

As the main entry site of most environmental Ags, mucosal surfaces, such as those of the respiratory, gastrointestinal, and genital tract, act as the first line of defense against pathogenic Ags. Not surprisingly then, many studies have focused on developing mucosal vaccines capable of effectively inducing both mucosal and systemic immune responses (1–4). However, mucosal vaccines comprised solely of protein Ags induce only a weak immune response or tolerance. Therefore, a new mucosal vaccine adjuvant must be identified to develop an effective mucosal vaccine (5).

α-Galactosylceramide (α-GalCer),[3] a glycolipid originally extracted from marine sponges, is a ligand of the invariant Vα14$^+$ NKT cell and is presented by CD1d molecule on APC (6). Upon activation, NKT cells produce a large amount of both IFN-γ and IL-4, which can modulate the immune responses to certain diseases, especially to autoimmune diseases, or infections (7–13). Previous studies have reported that activated NKT cells direct immune responses toward Th2 immune responses (14–16), whereas other studies have shown that activated NKT cells skew immune responses toward Th1 immune responses (17, 18). Recently, it has been shown that the coadministration of α-GalCer with OVA can induce full maturation of dendritic cells (DCs), thereby generating functional Ag-specific Th1 CD4$^+$ T cells and CTLs that are resistant to OVA-expressing tumors (19, 20). In addition, our previous study showed that the administration of α-GalCer triggers the full maturation of mesenteric DCs in vivo, which in turn contributes to the division of T cells in vitro and blocks the tolerance induced by both high and low doses of orally administered OVA (21). These results strongly suggest that α-GalCer could act as an effective and versatile mucosal adjuvant, one that would be capable of biasing the immune response into Th1 and CTL or Th2 immune response.

Several previous studies assessed the potential of α-GalCer as a systemically delivered vaccine adjuvant. Indeed, α-GalCer has been shown to act as an effective adjuvant against infections (22) and tumors (17, 19). Of the available mucosal vaccination routes, the intranasal one is preferred, although adjuvants such as cholera toxin and mutant cholera toxins are known to redirect vaccine protein into olfactory tissues when delivered intranasally (23). Further, the intranasal route requires much lower doses of both adjuvant and coadministered vaccine protein than does oral immunization (24). In this study, α-GalCer was evaluated as a nasal vaccine adjuvant for the induction of humoral as well as cellular immune responses against coadministered Ag in both C57BL/6 and BALB/c mice. We have proven in this study for the first time that α-GalCer is a potent nasal vaccine adjuvant capable of inducing both humoral and cellular immune responses and of affording protection in antiviral and antitumor nasal vaccines.

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[3] Abbreviations in this paper: αGalCer, α-galactosylceramide; DC, dendritic cell; S-IgA, secretory IgA; CLN, cervical lymph node; MLN, mesenteric lymph node; HA, hemagglutinin.



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## Materials and Methods

### *Mice and immunization protocols*

Female C57BL/6 and BALB/c mice age 6–8 wk, purchased from the Charles River Breeding Laboratories, as well as $CD1d^{-/-}$ mice were used in this experiment. All mice were housed in the experimental animal facility of the College of Pharmacy, Seoul National University (Seoul, Korea) until use and kept in specific pathogen-free conditions during the entirety of the experiment. Mice were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and immunized intranasally on days 0, 7, and 14 with 20 μl (10 μl/nostril) of PBS-diluted vehicle solution containing 100 μg of OVA alone or together with α-GalCer, unless otherwise noted. In the preliminary experiment, we have examined optimal frequency of nasal immunization (a single, two, or three times) and the result revealed that three times immunization showed sufficient immune responses by α-GalCer nasal vaccine adjuvant (data not shown). In the influenza virus studies, BALB/c mice were used. PR8 hemagglutinin (HA) vaccines (split-product virus vaccines), kindly provided by Dr. S.-I. Tamura (Osaka University, Osaka, Japan), were prepared from influenza virus A/PR/8/34 (PR8, H1N1) by the method of Davenport et al. (25). In the adenovirus studies, BALB/c mice were intranasally immunized with $10^6$ PFU of replication-deficient adenovirus harboring β-galactosidase gene (Ad-LacZ) or together with 0.125 μg of α-GalCer two times at 2-wk interval. Ad-LacZ is the type 5 adenoviral vector, which is replication defective with the El deletion and was kindly donated by ViroMed (26). For the examination of the activation and differentiation of OVA-specific $CD8^+$ T cells into effector cells, $1 \times 10^7$ cells of CFSE-labeled OT-1 cells were i.v. transferred into syngeneic mice. One day later, OVA alone or together with 2.0 μg of α-GalCer was administered intranasally. All experiments were done in compliance with relevant laws and institutional guidelines.

### *Synthesis of α-GalCer*

α-GalCer was prepared by coupling of phytosphingosine with hexacosanoic acid followed by the protection/deprotection and galactosylation scheme as described (27) and kindly provided from Dr. S. Kim (Seoul National University, Seoul, Korea). α-GalCer was dissolved in 0.5% Tween 20 in PBS, which is used as a vehicle in all experiments.

### *Sample collection*

One week after the final immunization, serum, nasal wash, and lung wash were collected to assess OVA- and β-galactosidase-specific Ab responses. Nasal wash specimens were collected by gently flushing the nasal passages with 100 μl of sterile PBS (28). To obtain lung wash, bronchoalveolar lavage fluids were taken as previously described (29).

### *ELISA for Abs*

OVA-specific IgG was determined as previously described (29). To assess HA-specific and β-galactosidase-specific Ab responses, ELISA plates with 96-wells were coated with 100 ng of HA or β-galactosidase, respectively. For the assessment of β-galactosidase-specific Ab responses, nasal wash and lung wash were examined at dilutions of 1/5, 1/15, and 1/45, and sera were examined at dilutions of 1/300, 1/900, and 1/2700. For the measurement of IgA, IgG1, and IgG2a titers in other samples, 2-fold serially diluted samples were used and the titers were determined. For the determination of IgA titers, HRP-conjugated goat anti-mouse IgA and the peroxidase substrate tetramethylbenzidine were used. Next, 0.5 N HCl was added to stop the development, and the OD was detected at 450 nm. To determine IgG, IgG1, and IgG2a titers, we used alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, and IgG2a and the phosphatase substrate, *p*-nitrophenyl phosphate. Goat anti-mouse IgA, IgG1, and IgG2a were purchased from Southern Biotechnology Associates.

### *ELISA for cytokine*

For the examination of cytokine production, single cells from spleen and lymph nodes were collected by passing through 70-μm nylon mesh and RBC were lysed with ammonium chloride. Cells were cultured at $5 \times 10^6$ cells/ml with or without 500 μg/ml OVA for 4 days. Culture supernatants were harvested and the concentrations of IL-4 and IFN-γ were measured by using mouse IL-4 and IFN-γ OptEIA set ELISA kits (BD Pharmingen) according to the manufacturer's instructions.

### *Intracellular cytokine staining*

Intracellular staining was performed with BD Cytofix/Cytoperm Plus (with GolgiPlug; BD Pharmingen) according to the manufacturer's instructions. For the measurement of IFN-γ producing $CD8^+$ T cells in BALB/c mice, $2 \times 10^6$ cells/ml cells were incubated with 500 μg/ml OVA for 4 days and 1 μl/ml GolgiPlug was added during the last 6 h. For the measurement of epitope-specific cellular immune response in C57BL/6 mice and cytokine producing $CD8^+$ T cells in OT-1 transferred C57BL/6 mice, $2 \times 10^6$ cells/ml cells were incubated with 5 μM $OVA_{257–264}$ peptide and 1 μl/ml GolgiPlug for 6 h. For the measurement of β-galactosidase-specific IFN-γ-producing $CD8^+$ T cells in adenovirus study, $2 \times 10^6$ cells/ml spleen cells were incubated with 2.5 μg/ml β-galactosidase for 5 days and 1 μl/ml GolgiPlug was added during the last 6 h. Stainings were performed directly with FITC-conjugated CD3 mAb (clone 145-2C11) and PE-conjugated CD8 mAb (clone 53-6.7; Biolegend), and intracellularly with allophycocyanin-conjugated IL-2 mAb (clone JES6-5H4; Biolegend), and allophycocyanin-conjugated IFN-γ mAb (clone XMG1.2; Biolegend). Stained cells were analyzed with FACSCalibur (BD Biosciences) and CellQuest Pro software (BD Biosciences).

### *In vivo cytotoxicity assay*

Splenocytes from naive C57BL/6 mice were separated, RBC were removed with ammonium chloride and divided into two equal populations, one of which was pulsed with 1 μM $OVA_{257–264}$ at 37°C for 90 min. The cells were labeled with CFSE (Molecular Probes) at a final concentration of 20 μM for the peptide-pulsed population ($CFSE^{high}$) and 2 μM for the peptide-unpulsed population ($CFSE^{low}$) at 37°C for 15 min. The two populations were mixed equally and $2 \times 10^7$ cells injected i.v. into the immunized mice. After 24 h, single cells from spleen and lymph nodes were isolated and CFSE intensities were analyzed using FACSCalibur and CellQuest Pro software. The percentage of specific killing was calculated as follows: percentage of lysis = $[1 - (\text{ratio}_{\text{unprime}}/\text{ratio}_{\text{prime}})] \times 100$; ratio = percentage $CFSE^{low}$/percentage $CFSE^{high}$.

### *Protection assay against EG7*

Two weeks after the final immunization, 100 μl of PBS containing $3 \times 10^6$ OVA-expressing EG7 cells was injected s.c. into the left flank of the immunized C57BL/6 mice. Fourteen days after the tumor challenge, the mice were sacrificed and palpable tumors were weighed.

### *Protection assay against influenza virus A/PR/8/34*

Two weeks after the final immunization, the immunized BALB/c mice were infected intranasally by dropping 20 μl (10 μl/nostril) of PBS containing influenza virus A/PR/8/34 suspension with 20 $LD_{50}$ per mouse. Mice were divided into two populations in each group. After 3 days, one population was sacrificed and nasal wash, lung wash, and serum were taken and then PR8 HA-specific Ab responses were examined. The other population was monitored for weight loss and survival rates every other day for 14 days.

### *Transfer of OT-1 cells*

OVA-specific $CD8^+$ T cells (OT-1 cells) were isolated from OT-1 mice using CD8α (Ly-2) magnetic beads (Miltenyi Biotec). These cells (>94% were Vα2-positive) were labeled with 10 μM CFSE at 37°C for 15 min and transferred i.v. into the syngeneic mice.

### *Data and statistical analysis*

Results are expressed as mean ± SE. Statistical analyses were performed upon comparisons made between the control groups and treated groups using the Student *t* test. Each experiments was repeated at least twice.

## Results

### *Intranasal administration of α-GalCer induced OVA-specific mucosal secretory IgA (S-IgA) and systemic IgG Ab responses in C57BL/6 mice*

To clarify mucosal adjuvanticity of α-GalCer, C57BL/6 mice were immunized with α-GalCer plus OVA three times at 1-wk intervals. The mice were sacrificed 1 wk after the last immunization and OVA-specific Ab responses were measured by ELISA. OVA-specific IgA responses in nasal and lung wash were significantly induced in the group receiving 2.0 μg of α-GalCer plus OVA compared with those receiving vehicle or OVA alone (Fig. 1*A*). To eliminate the possibility that the IgA responses were due to contamination of the sera, the amount of total IgG in nasal wash was assessed; no total IgG Ab was detected (data not shown). In addition, intranasal administration of different doses of α-GalCer

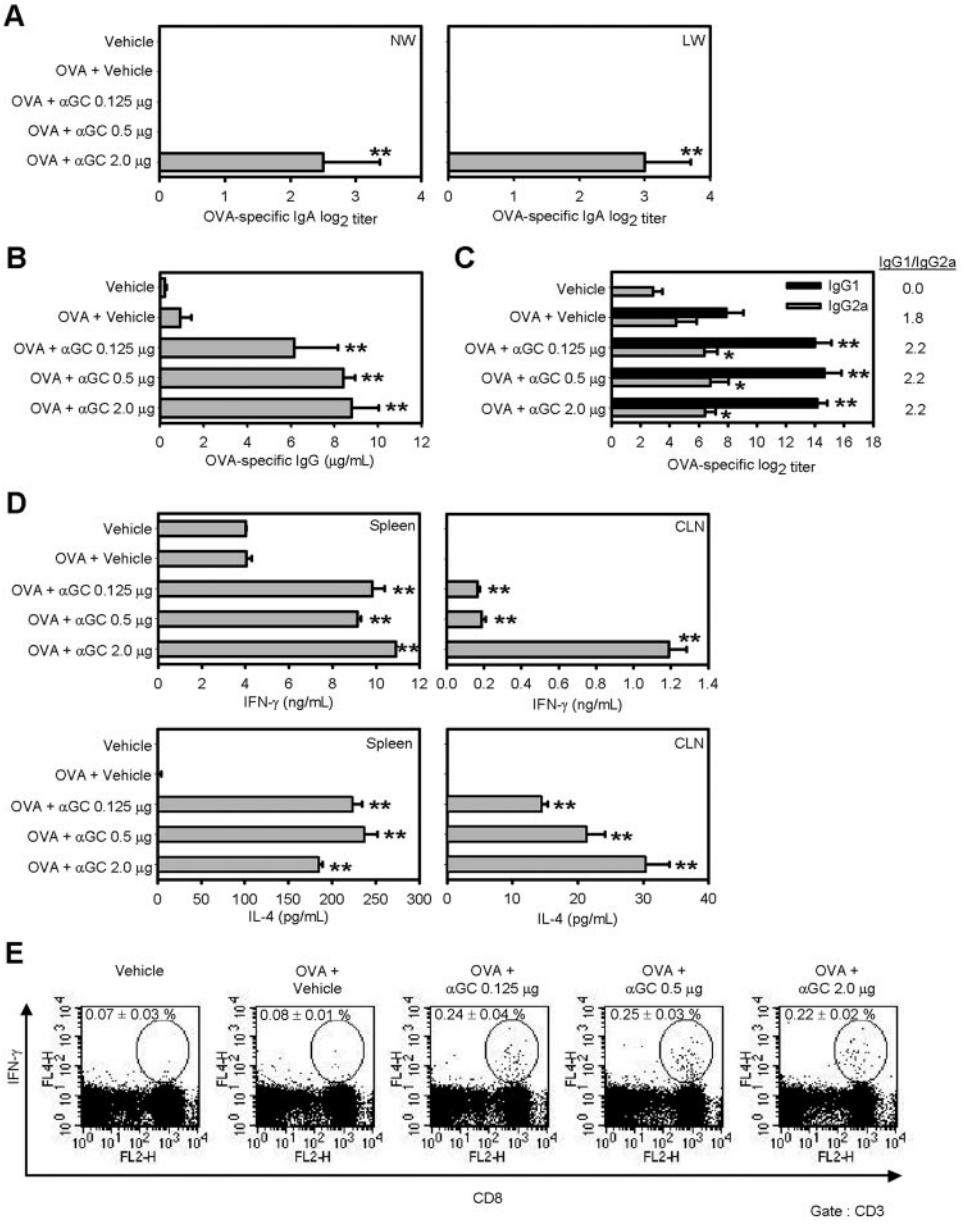


**FIGURE 1.** Coadministration of OVA with α-GalCer induced OVA-specific mucosal S-IgA and systemic IgG responses and showed both Th1 and Th2 cytokine profiles in C57BL/6 mice. C57BL/6 mice were immunized with OVA alone or together with α-GalCer three times at 1-wk intervals. One week after the final immunization, the immunized mice were sacrificed. OVA-specific S-IgA titers were determined in nasal wash and lung wash (*A*). The levels of OVA-specific systemic IgG (*B*) and the titers of IgG isotypes (*C*) were examined in serum. Splenocytes and cells from lymph nodes were collected and cultured with 500 μg/ml whole OVA for 4 days, and the levels of IFN-γ and IL-4 in the supernatant were examined by sandwich ELISA (*D*). A total of $2 \times 10^6$ cells/ml spleen cells were cultured with 1 μg/ml $OVA_{257–264}$ peptide and 1 μl/ml GolgiPlug for 6 h. IFN-γ-producing $CD8^+$ T cells were analyzed using BD Cytofix/Cytoperm kit and flow cytometry according to the manufacturer's instructions. Flow cytometric analysis was performed by gating on T cells (*E*). Bars represent mean Ab titer or amount ± SE in each group. Each group consists of six mice. Data are representative of three separate experiments except in *E*, which is that of two independent experiments. *, $p < 0.05$; **, $p < 0.01$ vs Vehicle and OVA+Vehicle.

plus OVA elicited significantly higher levels of OVA-specific IgG in sera than did intranasal administration of the vehicle or OVA alone (Fig. 1*B*). To determine indirectly whether Th1 or Th2 immune responses predominate in the humoral immune response to OVA, the IgG isotypes in sera were determined and the ratios of IgG1 to IgG2a were calculated. Coadministration of OVA with α-GalCer significantly enhanced both OVA-specific IgG1 and IgG2a levels, indicating that α-GalCer induces both Th1 and Th2 immune responses (Fig. 1*C*). These results indicate that α-GalCer is a potent mucosal adjuvant for the induction of Ag-specific mucosal S-IgA and systemic IgG Ab responses and indirectly suggest that α-GalCer induced both Th1 and Th2 immune responses in C57BL/6 mice.

### *Intranasal administration of α-GalCer enhanced both Th1 and Th2 cytokines in C57BL/6 mice*

To assess directly whether α-GalCer skews immune responses toward Th1 or Th2 immune response, mononuclear cells from spleen and cervical lymph node (CLN) were obtained 1 wk after the final immunization and cultured with whole OVA for 4 days. The secretion patterns of IFN-γ and IL-4 in the culture supernatants were examined by sandwich ELISA. Both IFN-γ and IL-4 production were significantly increased in spleen and CLN (Fig. 1*D*). A very high level of IFN-γ was induced by a high dose of α-GalCer and levels of IL-4 increased proportionally to the increasing dose of α-GalCer in CLN. To assess the epitope-specific cellular immune response induced by coimmunization of α-GalCer via intranasal route, IFN-γ-producing $CD8^+$ T cells were analyzed after stimulating splenocytes with $OVA_{257–264}$ peptide. The number of IFN-γ-producing $CD8^+$ T cells was significantly increased in all groups coimmunized with OVA and α-GalCer compared with those in vehicle- or OVA alone-treated groups ($p < 0.01$, vehicle and OVA vs 0.125, 0.5, and 2.0 μg) (Fig. 1*E*). These results demonstrate that intranasal coadministration of α-GalCer induces mixed Th1 and Th2 immune responses in both systemic and mucosal compartments.

### *α-GalCer induced potent CTL responses in C57BL/6*

It has been well established that α-GalCer induces CTL responses when it is administered by the i.v. or oral routes (19, 30). To test

whether α-GalCer induces CTL responses when it is coadministered intranasally, a mixture containing equal amounts of $OVA_{257–264}$-pulsed $CFSE^{high}$ and $OVA_{257–264}$-unpulsed $CFSE^{low}$ splenocytes of syngeneic mice was injected i.v. into the mice 1 wk after the final intranasal immunization. Twenty-four hours later, the specific lysis of peptide-pulsed CFSE-labeled cells in the spleen, mesenteric lymph node (MLN) and CLN was analyzed using flow cytometry. All groups of α-GalCer-treated mice showed potent lytic activities in a dose-dependent manner (Fig. 2, *A* and *B*). Interestingly, the cytotoxic activities were induced both mucosally (CLN) and systemically (spleen and MLN). These results show that α-GalCer is a potent nasal vaccine adjuvant for the induction of CTLs in both local and systemic lymphoid organs.

### *Intranasal administration of α-GalCer adjuvant induced both humoral and cellular immune responses in BALB/c mice*

We then examined whether α-GalCer could also be used as a potent nasal vaccine adjuvant in BALB/c mice. In BALB/c mice, intranasal administration of different doses of α-GalCer plus OVA elicited significantly higher levels of OVA-specific IgA responses in nasal wash and lung wash as well as of OVA-specific IgG responses in sera than did intranasal administration of vehicle or OVA alone (Fig. 3, *A* and *B*). Additionally, intranasal administration of OVA with α-GalCer significantly enhanced both OVA-specific IgG1 and IgG2a levels (Fig. 3*C*). Further, significant levels of both IFN-γ and IL-4 were induced in all α-GalCer-administered groups (Fig. 3*D*). Interestingly, the levels of IgG Ab in serum and IL-4 production in spleen were the highest in the mice intranasally receiving 0.5 μg of α-GalCer and then declined as the dose of α-GalCer was increased. Furthermore, mucosal IgA in lung wash and IL-4 production in CLN was inversely proportional to the coadministered dose of α-GalCer. Collectively, it seems likely that a high dose of α-GalCer induces tolerance to coadministered Ag in BALB/c mice.

As there was no known MHC class I-binding peptide of OVA in BALB/c mice, the numbers of IFN-γ-producing $CD8^+$ T cells were examined to test the cytotoxic activity induced by α-GalCer adjuvant in BALB/c mice. As doses of α-GalCer increased, the number of IFN-γ-producing $CD8^+$ T cells decreased ($p < 0.05$, 0.125 μg vs 0.5 μg; $p < 0.01$, 0.125 μg and 0.5 μg vs 2.0 μg) (Fig. 3*E*). Although the amount of IFN-γ detected by sandwich ELISA did not show dose dependency, the number of IFN-γ-producing CTLs was inversely proportional to the dose of α-GalCer. As a possible explanation for this discrepancy, it should be noted that the amount of IFN-γ detected by sandwich ELISA represents that from all kinds of cells producing IFN-γ, such as $CD4^+$, $CD8^+$ T cells or APCs, whereas the number of effector CTLs detected by FACS represents only $CD8^+$ T cells. Overall, these results indicate that α-GalCer also represents a potent nasal vaccine adjuvanticity in BALB/c mice.

### *α-GalCer showed a nasal vaccine adjuvanticity for the induction of antiviral immune responses against influenza virus A/PR/8/34 infection*

To determine the level of mucosal protection afforded by α-GalCer against viral infection, BALB/c mice were intranasally immunized with PR8 HA Ag alone or together with α-GalCer three times at 1-wk intervals and then intranasally infected with 20 $LD_{50}$ of influenza virus 2 wk after the final immunization. Three days after the viral infection, PR8 HA-specific Ab responses were measured

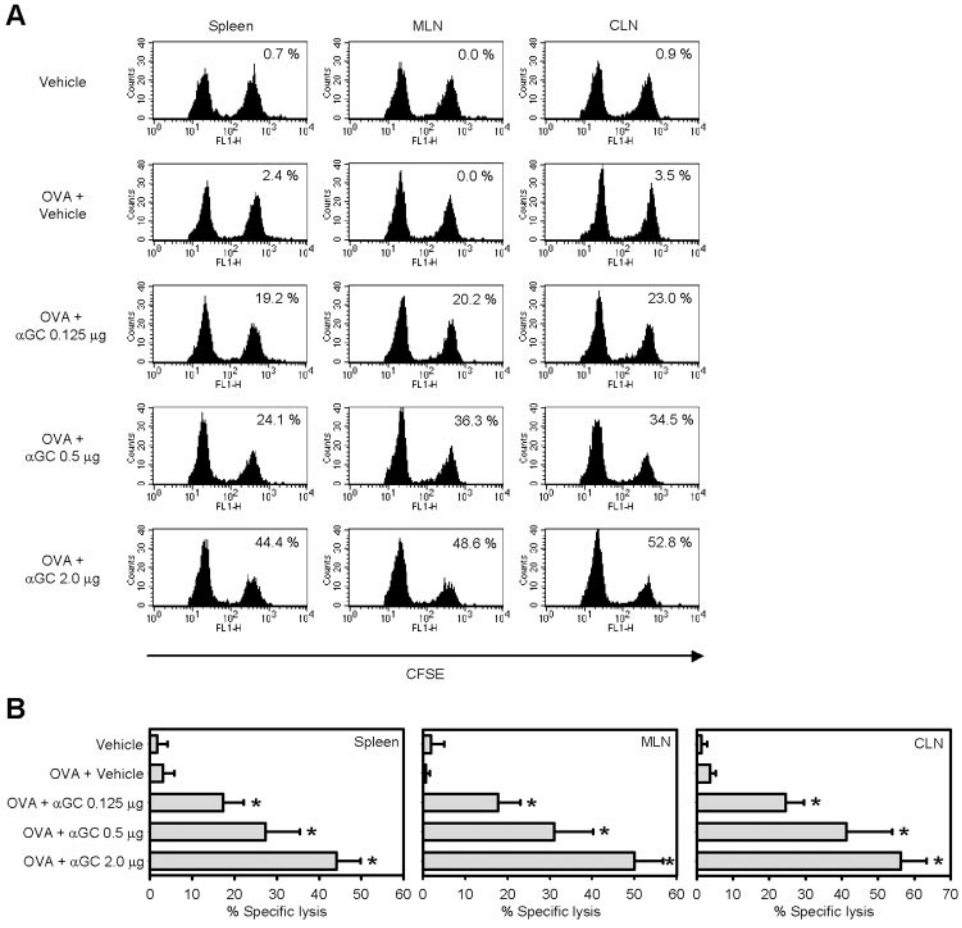


**FIGURE 2.** α-GalCer induced potent CTL responses in vivo in C57BL/6 mice. One week after the final immunization, $2 \times 10^7$ cells of an equal mixture of $OVA_{257–264}$-pulsed $CFSE^{high}$ splenocytes and unpulsed $CFSE^{low}$ splenocytes were injected i.v. into the immunized mice. Twenty-four hours later, the mice were sacrificed, and the killing of peptide-loaded splenocytes in spleen and lymph nodes was analyzed using flow cytometry. *A*, Representative profiles of three individual mice from each group. *B*, In vivo CTL killing was calculated and expressed as the percentage of specific lysis for each group. Bars represent the mean percentage of specific lysis ± SE in each group. Each group consists of three mice, and data are representative of two separate experiments. *, $p < 0.01$ vs Vehicle and OVA+Vehicle.

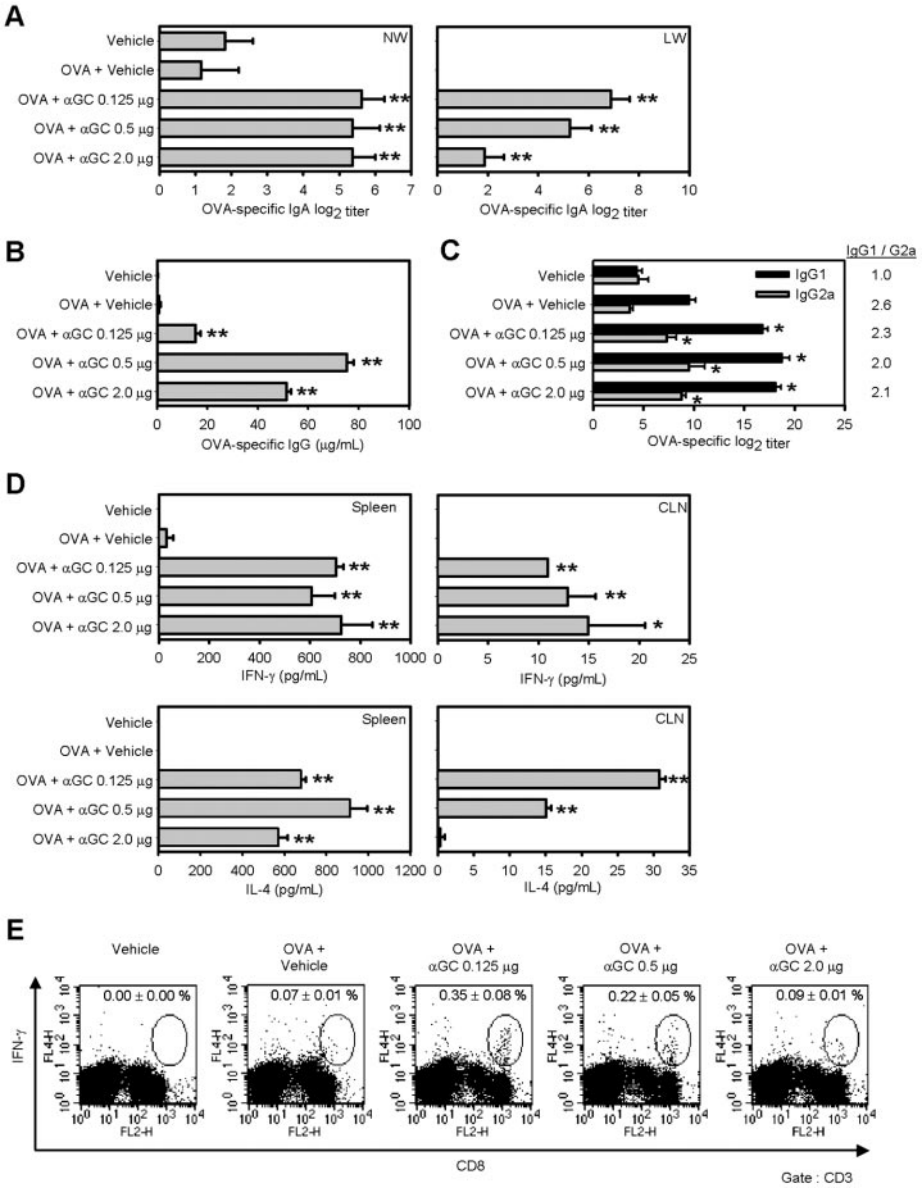


**FIGURE 3.** Coadministration of OVA with α-GalCer represented OVA-specific Ab responses, both Th1 and Th2 cytokine profiles and CTL activities in BALB/c mice. One week after the final immunization, the immunized BALB/c mice were sacrificed, and nasal wash, lung wash, and serum were collected. The titers of OVA-specific S-IgA were determined in nasal wash and lung wash (*A*). The levels of OVA-specific systemic IgG (*B*) and the titers of IgG isotypes (*C*) were examined in serum. One week after the final immunization, splenocytes and cells from lymph nodes were collected. A total of $5 \times 10^6$ cells/ml were stimulated with 500 μg/ml whole OVA for 4 days, and the levels of IFN-γ and IL-4 in the supernatant were examined by sandwich ELISA (*D*). A total of $2 \times 10^6$ cells were cultured with 500 μg/ml whole OVA for 4 days and 1 μl/ml GolgiPlug was added for the last 6 h. IFN-γ-producing CTLs were analyzed using BD Cytofix/Cytoperm kit and flow cytometry according to the manufacturer's instructions. Flow cytometric analysis was performed by gating on T cells (*E*). Bars represent mean value ± SE in each group. Each group consists of six mice and data are representative of two separate experiments. *, $p < 0.02$; **, $p < 0.01$ vs Vehicle and OVA+Vehicle.

in the nasal wash, lung wash, and sera. In addition, the weight loss and survival rates of the infected mice were observed every other day. Interestingly, high levels of PR8 HA-specific IgA Abs were detected in the nasal wash, lung wash, and sera of all the α-GalCer-treated groups (Fig. 4*A*). Further, high levels of PR8 HA-specific IgG Abs were also detected in sera of all groups of α-GalCer-treated mice (Fig. 4*B*). These results indicate that α-GalCer is a potent nasal adjuvant inducing mucosal S-IgA as well as systemic IgG Ab responses against virus-derived Ag. Most interestingly, much greater pathogenesis was observed in the mice immunized without α-GalCer than in the mice coimmunized with α-GalCer, as indicated by survival rate, weight loss, and time to weight loss recovery (Fig. 4*C*). Although all mice died within 10 days after the viral infection in the group receiving vehicle alone and 57% of the mice treated with OVA alone died within 14 days, the mice intranasally immunized with α-GalCer plus OVA did not show any significant decrease in survival rate (Fig. 4*C*, *left*). These results indicate that α-GalCer is a potent nasal vaccine adjuvant inducing mucosal S-IgA Abs, systemic IgG Abs, and protection against viral infection.

*Intranasal administration of α-GalCer with live virus enhanced immunogenicity against replication-deficient live virus*

To examine the adjuvanticity of α-GalCer against live virus, BALB/c mice were intranasally immunized with $10^6$ PFU of replication-deficient live Ad-LacZ alone or together with 0.125 μg of α-GalCer twice at 2-wk interval. One week after the last immunization, β-galactosidase-specific Ab responses were measured in nasal wash, lung wash, and serum. For determination of CTL activities, splenocytes were stimulated with 2.5 μg/ml β-galactosidase for 5 days and IFN-γ-producing $CD8^+$ T cells were assessed by intracellular cytokine staining. Interestingly, β-galactosidase-specific S-IgA and IgG responses were significantly higher in mucosal compartment and sera of α-GalCer-coimmunized group, respectively, compared with those in vehicle or Ad-LacZ alone-treated groups (Fig. 5, *A* and *B*). Moreover, the number of IFN-γ-producing $CD8^+$ T cells was significantly enhanced in the α-GalCer-treated group (Fig. 5*C*). Taken together, these results indicated that α-GalCer is an effective nasal vaccine adjuvant against the replication-deficient live virus.

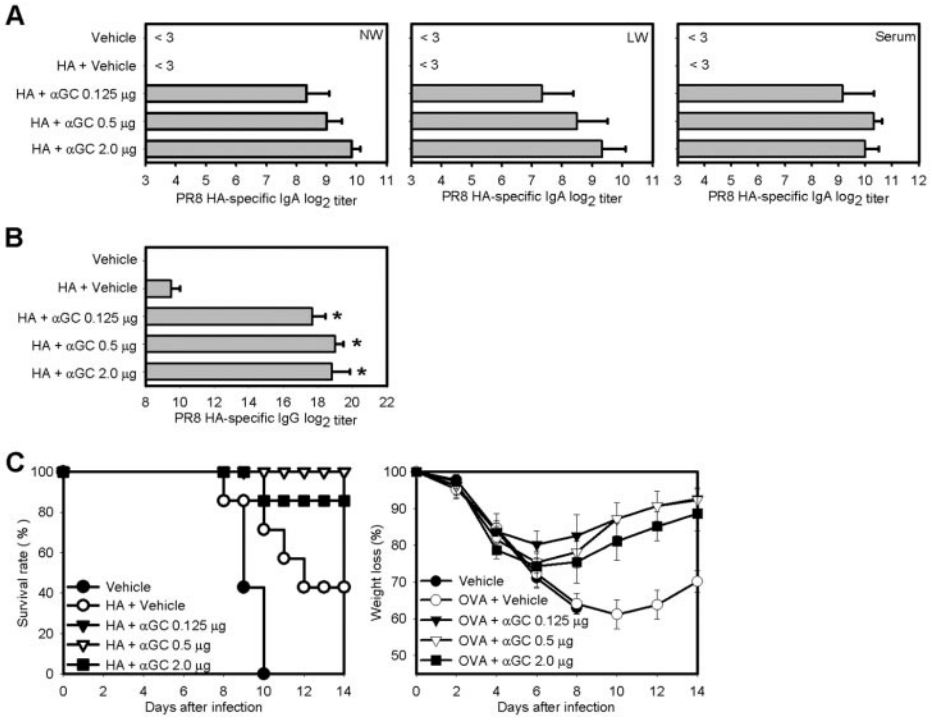


**FIGURE 4.** α-GalCer adjuvant induced mucosal S-IgA and systemic IgG responses and represented potent protection against influenza virus A/PR/8/34 infection in BALB/c mice. BALB/c mice were immunized intranasally with PR8 HA alone or together with α-GalCer three times at 1-wk intervals. Two weeks after the final immunization, 20 $LD_{50}$ of influenza virus A/PR/8/34 were infected via the intranasal route. Levels of PR8 HA-specific IgA titers in nasal wash, lung wash, and serum (*A*) and PR8 HA-specific IgG titers in serum (*B*) were assessed 3 days after the viral infection. Separately, the weight loss and survival rates of mice were observed every other day after the viral infection (*C*). Bars represent mean Ab titer ± SE in each group. Each group consists of seven mice and data are representative of two separate experiments. *, $p < 0.01$ vs Vehicle and OVA+Vehicle.

### *α-GalCer showed a nasal vaccine adjuvanticity for the induction of antitumor immune response against EG7 tumor*

To assess whether α-GalCer could be used as a nasal vaccine adjuvant for the induction of antitumor activity, C57BL/6 mice were immunized intranasally with OVA alone or together with α-GalCer three times at 1-wk intervals. Two weeks after the final immunization, $3 \times 10^6$ cells of EG7 tumor cells were inoculated s.c. into the left flank of the immunized mice. Fourteen days after the inoculation, the mice were sacrificed and palpable tumors were weighed. Palpable tumors were found in all mice receiving the vehicle or OVA alone and in one-third of those to which 0.125 μg of α-GalCer had been coadministered (Fig. 6). The weights of the

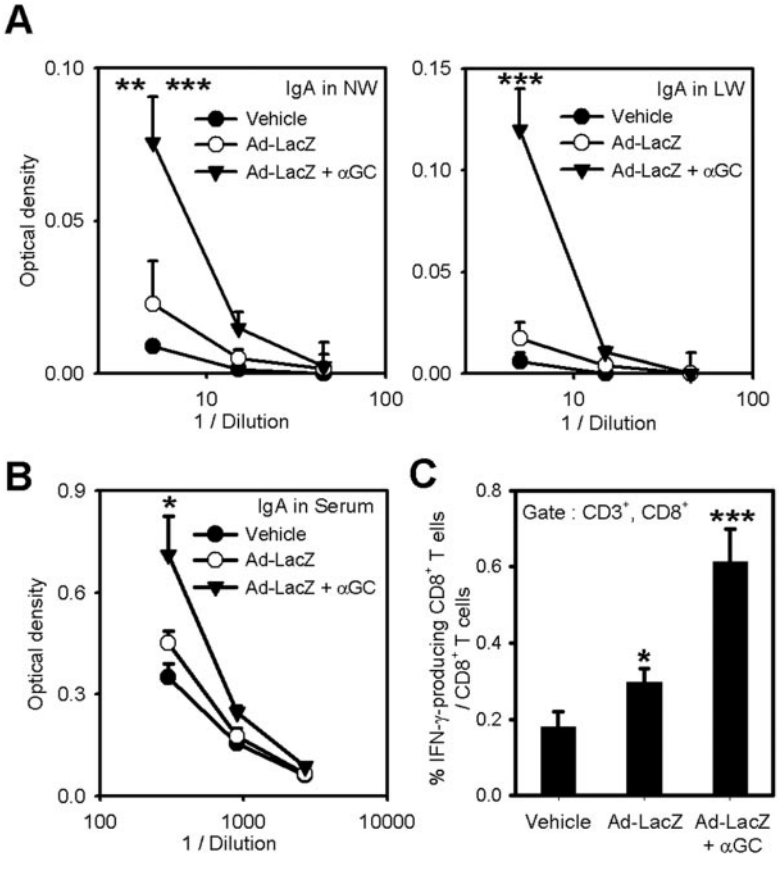


**FIGURE 5.** α-GalCer adjuvant induced higher levels of humoral as well as cellular immune responses against replication-deficient live adenovirus. BALB/c mice immunized with $10^6$ PFU of Ad-LacZ alone or together with 0.125 μg of α-GalCer twice at 2-wk interval. One week after the final immunization, the mice were sacrificed, and nasal wash, lung wash, and serum were obtained. The nasal and lung wash were examined for the presence of β-galactosidase-specific S-IgA at dilutions of 1/5, 1/15, and 1/45 (*A*). The serum was tested for the presence of β-galactosidase-specific IgG at dilutions of 1/300, 1/900, and 1/2700 (*B*). A total of $2 \times 10^6$ spleen cells were cultured with 2.5 μg/ml β-galactosidase for 5 days, and 1 μl/ml GolgiPlug was added during the last 6 h. IFN-γ-producing $CD8^+$ T cells were analyzed using BD Cytofix/Cytoperm kit and flow cytometry according to the manufacturer's instructions. Flow cytometric analysis was performed by gating on $CD8^+$ T cells (*D*). The SE values were shown. Each group consists of five mice, and data are representative of two separate experiments. **, $p < 0.02$ vs Ad-LacZ and ***, $p < 0.01$ vs Vehicle (*A*, *left*); and in the remaining figures, *, $p < 0.05$ and ***, $p < 0.01$ vs Vehicle and Ad-LacZ.

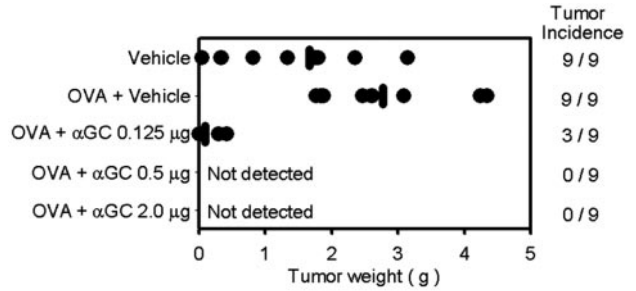


**FIGURE 6.** Coadministration of OVA with α-GalCer showed potent protection against the EG7 tumor in C57BL/6 mice. Two weeks after the final immunization, $3 \times 10^6$ cells of EG7 tumor cells were injected s.c. into the left flank of the immunized C57BL/6 mice. Palpable tumors were weighed and tumor incidences were observed 14 days after the tumor inoculation. Oval bar represents the mean value. Data are representative of two separate experiments.

tumors in the mice intranasally immunized with OVA alone were significantly greater than those in the mice immunized with vehicle alone ($p < 0.05$). Most interestingly, the formation of tumor masses was completely blocked in the mice intranasally administered with 0.5 and 2.0 μg of α-GalCer plus OVA (Fig. 6). These results indicate that α-GalCer can be used as a potent nasal vaccine adjuvant for the induction of antitumor immune responses.

### *Nasal adjuvant activity of α-GalCer was exclusively mediated via CD1d molecule*

To confirm that the α-GalCer-induced immune responses are mediated by CD1d molecule, we used $CD1d^{-/-}$ C57BL/6 mice, which lack the class I restricting element of NKT cells and therefore lack NKT cells (31). One week after the final intranasal administration, systemic IgG in serum and in vivo CTL activity were examined in wild-type and $CD1d^{-/-}$ C57BL/6 mice. As expected, systemic IgG Ab response was remarkably inhibited in $CD1d^{-/-}$ mice (Fig. 7*A*). Further, the lytic activity of CTL was blocked in the draining lymph node and systemic lymphoid organs of $CD1d^{-/-}$ mice (Fig. 7*B*). These results clearly demonstrate that the immune responses induced by α-GalCer in this study are exclusively mediated by the CD1d molecule.

### *Intranasal administration of α-GalCer activates naive T cells and triggers them to differentiate into effector cells*

To further clarify the role of α-GalCer on the activation of T cell responses, the expression of surface CD25 molecules was measured in adoptively transferred CFSE-labeled OT-1 cells into the syngeneic mice. One day after the adoptive transfer, the mice were intranasally administered with OVA alone or together with 2.0 μg of α-GalCer. Forty-eight hours later, the expression of CD25 molecules in CLN was analyzed using flow cytometry. A much more substantial population of OT-1 cells expressing CD25 could be detected in the mice intranasally administered with OVA plus α-GalCer compared with those receiving OVA alone, suggesting that α-GalCer nasal adjuvant induced the activation of naive T cells (Fig. 8*A*). To verify that the activated T cells were differentiated into functional CTLs, the cells were further stimulated with $OVA_{257–264}$ peptide for 6 h and then intracellular IL-2 and IFN-γ were measured. Much higher populations of IL-2- and IFN-γ-producing OT-1 cells were observed in the mice intranasally immunized with OVA plus α-GalCer than in those immunized with OVA alone (Fig. 8*B*). Collectively, these results demonstrate that intranasal coadministration of α-GalCer induces the activation of naive T cells and that the activated T cells exert a potent effector function.

## Discussion

In this study, we evaluated the adjuvanticity of α-GalCer as a nasal vaccine in both C57BL/6 and BALB/c mice. Whether the Ag used is OVA or PR8 HA, intranasal coadministration of the Ag with α-GalCer induced high levels of systemic IgG and mucosal S-IgA Abs in both C57BL/6 and BALB/c mice. The ratio of IgG1 and IgG2a in serum was not significantly affected by intranasal coadministration with α-GalCer. In addition, ex vivo measurement of cytokine profiles revealed high levels of IFN-γ and IL-4 in mucosal and systemic compartments. Also, Ag-specific CTLs were remarkably induced when Ag was administered together with α-GalCer by the intranasal route. Further, intranasal coadministration with α-GalCer afforded complete protection against influenza viral infection and tumor, and enhanced humoral as well as cellular immune responses against replication-deficient live adenovirus. Taken together, we have proven for the first time that α-GalCer is a potent nasal vaccine adjuvant inducing mixed Th1 and Th2 immune responses and that it can be use as an effective nasal vaccine adjuvant to enhance protection against viral infection and tumors.

A recent study demonstrated that repeated exposure to α-GalCer induced disappearance of NKT cells and increased expression of inhibitory Ly49, resulting in tolerance to Ag (32). We suspect that the amount of α-GalCer may affect the level of immune responses or the degree of tolerance induced to coadministered Ag. To clarify those issues, we evaluated the immune responses by α-GalCer in a dose-dependent manner. Interestingly, the optimal dose of

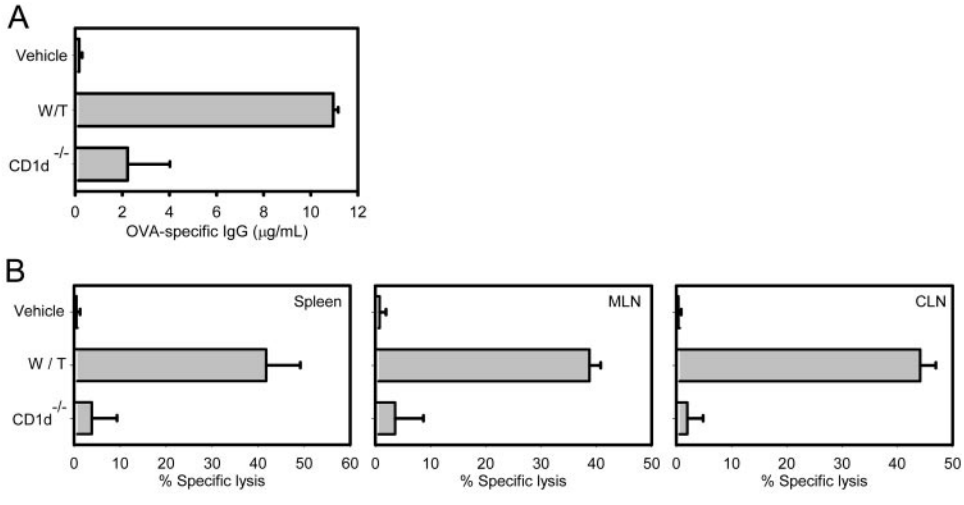


**FIGURE 7.** Adjuvant activity of α-GalCer is exclusively mediated by the CD1d molecule. Wild-type and $CD1d^{-/-}$ C57BL/6 mice were immunized intranasally with 100 μg of OVA and 2 μg of α-GalCer three times at 1-wk intervals. One week after the final immunization, mice were injected with $2 \times 10^7$ cells of an equal mixture of $OVA_{257–264}$-pulsed $CFSE^{high}$ splenocytes and unpulsed $CFSE^{low}$ cells. Twenty-four hours later, the mice were bled and the levels of OVA-specific IgG were measured (*A*). In vivo CTL activity was examined according to the protocol described in *Materials and Methods* (*B*). Bars represent mean percentage specific lysis ± SE in each group.

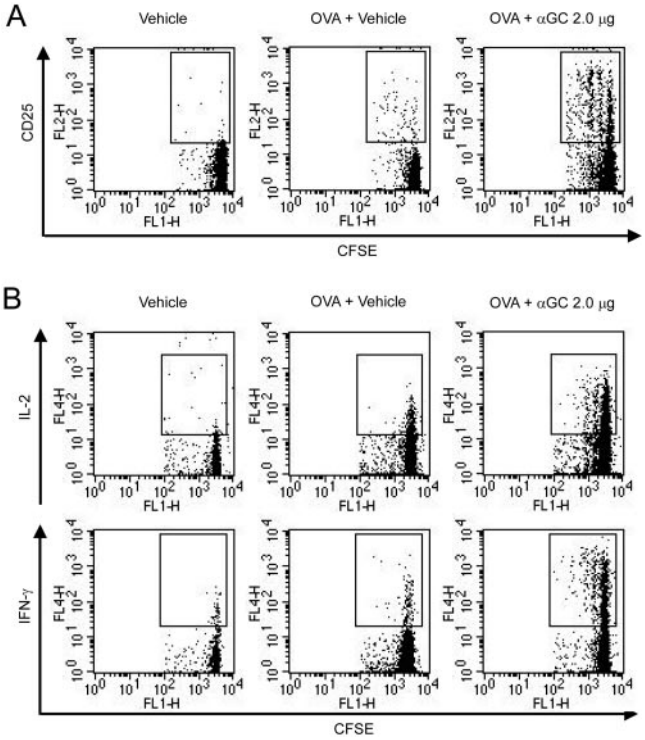


**FIGURE 8.** Coimmunization of OVA with α-GalCer activated naive $CD8^+$ T cells and triggered them to differentiate into effector cells. CFSE-labeled OT-1 cells were transferred i.v. into syngeneic mice, and 1 day later, OVA alone or together with 2.0 μg of α-GalCer was intranasally administered. Forty-eight hours later, lymphoid cells from CLN were analyzed for their surface expression of CD25 (*A*). The cells were further measured for intracellular IL-2 and IFN-γ after stimulation with 1 μM $OVA_{257–264}$ peptide for 6 h (*B*).

α-GalCer following intranasal administration differed for C57BL/6 and BALB/c mice, demonstrating that dose must be determined based upon strain. Overall, our present results suggest that the precise determination of the optimal dose of α-GalCer as a nasal vaccine adjuvant should be performed in nonhuman primates before clinical trial.

Renegar et al. (33) demonstrated that S-IgA and systemic IgG are important for the protection against viral infection in the murine respiratory tract. Belyakov and Berzofsky (34) indicated that mucosal S-IgA and CTL are responsible for the potential protective local immune responses against HIV infection. In the current study, our results have shown that intranasally delivered α-GalCer adjuvant induced significant levels of mucosal S-IgA, systemic IgG and both systemic and mucosal CTLs, as well as providing complete protection against influenza viral infection. Several other groups have also demonstrated that α-GalCer adjuvant exhibits antitumor activity (19, 20), and that the antitumor effects of α-GalCer are mainly mediated by NK cells in a perforin-independent manner (36), by anti-angiogenesis of IFN-γ secreted by activated NKT cells (37) and by CTLs (35). During the preparation of our manuscript, Silk et al. (30) showed that oral administration of OVA plus α-GalCer provoked antitumor responses. In our study, intranasal administration of OVA plus α-GalCer induced significant IFN-γ production and afforded complete protection against the EG7 tumor. Interestingly, the tumors of the group treated with OVA alone were heavier than those treated with vehicle alone. Previous studies demonstrated that intranasal exposure to protein Ag alone leads to immunological tolerance and CLN is responsible for the induction of the tolerance (38, 39). Importantly, our previous study indicated that the administration of α-GalCer triggered full maturation of mesenteric DC and thereby blocked the induction of oral tolerance (21). In this point of view, the heavier tumor mass in the group treated with OVA alone is due to the induction of tolerance to intranasally administered-OVA Ag, speculating that the tolerance may be blocked by intranasal coadministration with α-GalCer. Taken together, these results demonstrate that α-GalCer is a potent nasal adjuvant inducing complete protection against viral infection and tumors by blocking mucosally induced tolerance to coadministered Ag.

We have demonstrated here that intranasal α-GalCer exerts its adjuvant activity via the CD1d molecule, and activates naive T cells and triggers them to differentiate into potent effector cells. α-GalCer-presented DCs activate NKT cells and, in turn, NKT cells make DCs mature, which is mediated by IFN-γ, TNF-α, or other unknown mediators or mechanisms (30). Oral administration of α-GalCer induces full maturation of mesenteric DCs (21). In addition, when cholera toxin is administered intranasally, plamacytoid DCs induce tolerance and myeloid DCs induce inflammation against coadministered Ag in mice (40). NKT cells are divided into $CD4^+$ and $CD4^-$ subpopulations in humans (41). Although $CD4^-$ NKT cells selectively produce Th1 cytokines, $CD4^+$ NKT cells potently produce both Th1 and Th2 cytokines. However, to our knowledge, no such studies have been conducted using intranasally administered α-GalCer. Therefore, further study is needed to determine which subpopulations of DC and/or NKT cells are involved in the adjuvanticity of intranasally delivered α-GalCer.

α-GalCer has been used in clinical studies in antitumor immunotherapy, and shown to induce NKT cell memory and then more vigorous immune responses than those observed in control groups (42, 43). In addition, those studies showed that α-GalCer provoked no dose-limiting toxicity (50–4800 $\mu g/m^2$) and is well tolerated in a dose escalation trial, which indicated that α-GalCer could be used safely. In contrast, a previous study demonstrated that cholera toxin, a GM1-binding molecule, redirects vaccine protein into olfactory tissues when given intranasally (23). In other words, intranasal administration of adjuvant may induce the redirection of coadministered Ag or adjuvant itself into the brain, provoking unwanted effects. With its high m.w. and its hydrophilic group, however, α-GalCer would be hard to redirect to the CNS. In addition, the population of immune cells including APC and T cells in the brain is thought to be so small that it would not be easy for activated NKT cells by α-GalCer to exert their toxic action. In fact, even though mild side effects were induced when α-GalCer was given i.v. in phase I clinical study, these effects were easily prevented by paracetamol pretreatment, and the liver toxicity induced in mice by parenteral administration of α-GalCer was not seen in this clinical study (44). Moreover, no side effects were reported in mice receiving doses up to 2200 μg/kg α-GalCer (43). Taken together, these findings demonstrate that α-GalCer is a safe and effective adjuvant for use in intranasally delivered antiviral and antitumor vaccines.

It is generally accepted that far lower doses are required in nasal drug delivery than in oral delivery because in the former the drug is more rapidly absorbed, has a higher bioavailability and avoids liver first pass effect and metabolism by gastrointestinal tract (24). Actually, much less α-GalCer was required by the intranasal route in this study than was required by the oral route to protect against the EG7 tumor, demonstrating once again that α-GalCer can be used as a safer mucosal adjuvant (30).

Recently, spray formulation of cold-adapted live attenuated vaccine has been used commercially because it is safer and more convenient than live vaccines (45). It seems likely that α-GalCer adjuvant would be suited to such vaccine formulations.

In conclusion, we have proven in this study for the first time that α-GalCer is a potent nasal vaccine adjuvant inducing both humoral

and cellular immune responses and is suitable for use in antiviral and antitumor nasal vaccines.

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## Disclosures

The authors have no financial conflict of interest.

## References

1. Czerkinsky, C., F. Anjuere, J. R. McGhee, A. George-Chandy, J. Holmgren, M. P. Kieny, K. Fujiyashi, J. F. Mestecky, V. Pierrefite-Carle, C. Rask, and J. B. Sun. 1999. Mucosal immunity and tolerance: relevance to vaccine development. *Immunol. Rev.* 170: 197–222.
2. Belyakov, I. M., M. A. Derby, J. D. Ahlers, B. L. Kelsall, P. Earl, B. Moss, W. Strober, and J. A. Berzofsky. 1998. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. *Proc. Natl. Acad. Sci. USA* 95: 1709–1714.
3. Berzofsky, J. A., J. D. Ahlers, and I. M. Belyakov. 2001. Strategies for designing and optimizing new generation vaccines. *Nat. Rev. Immunol.* 1: 209–219.
4. Kozlowski, P. A., and M. R. Neutra. 2003. The role of mucosal immunity in prevention of HIV transmission. *Curr. Mol. Med.* 3: 217–228.
5. Yuki, Y., and H. Kiyono. 2003. New generation of mucosal adjuvants for the induction of protective immunity. *Rev. Med. Virol.* 13: 293–310.
6. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* 278: 1626–1629.
7. Chen, H., and W. E. Paul. 1997. Cultured NK1.1$^+$ CD4$^+$ T cells produce large amounts of IL-4 and IFN-γ upon activation by anti-CD3 or CD1. *J. Immunol.* 159: 2240–2249.
8. Wilson, M. T., C. Johansson, D. Olivares-Villagomez, A. K. Singh, A. K. Stanic, C. R. Wang, S. Joyce, M. J. Wick, and L. Van Kaer. 2003. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. *Proc. Natl. Acad. Sci. USA* 100: 10913–10918.
9. Gonzalez-Aseguinolaza, G., L. Van Kaer, C. C. Bergmann, J. M. Wilson, J. Schmieg, M. Kronenberg, T. Nakayama, M. Taniguchi, Y. Koezuka, and M. Tsuji. 2002. Natural killer T cell ligand α-galactosylceramide enhances protective immunity induced by malaria vaccines. *J. Exp. Med.* 195: 617–624.
10. Hong, S., M. T. Wilson, I. Serizawa, L. Wu, N. Singh, O. V. Naidenko, T. Miura, T. Haba, D. C. Scherer, J. Wei, et al. 2001. The natural killer T-cell ligand α-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat. Med.* 7: 1052–1056.
11. Sharif, S., G. A. Arreaza, P. Zucker, Q. S. Mi, J. Sondhi, O. V. Naidenko, M. Kronenberg, Y. Koezuka, T. L. Delovitch, J. M. Gombert, et al. 2001. Activation of natural killer T cells by α-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nat. Med.* 7: 1057–1062.
12. Singh, A. K., M. T. Wilson, S. Hong, D. Olivares-Villagomez, C. Du, A. K. Stanic, S. Joyce, S. Sriram, Y. Koezuka, and L. Van Kaer. 2001. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J. Exp. Med.* 194: 1801–1811.
13. Teige, A., I. Teige, S. Lavasani, R. Bockermann, E. Mondoc, R. Holmdahl, and S. Issazadeh-Navikas. 2004. CD1-dependent regulation of chronic central nervous system inflammation in experimental autoimmune encephalomyelitis. *J. Immunol.* 172: 186–194.
14. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W. E. Paul. 1995. Role of NK1.1$^+$ T cells in a TH2 response and in immunoglobulin E production. *Science* 270: 1845–1847.
15. Singh, N., S. Hong, D. C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and α-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 163: 2373–2377.
16. Laloux, V., L. Beaudoin, D. Jeske, C. Carnaud, and A. Lehuen. 2001. NK T cell-induced protection against diabetes in Vα14-Jα281 transgenic nonobese diabetic mice is associated with a Th2 shift circumscribed regionally to the islets and functionally to islet autoantigen. *J. Immunol.* 166: 3749–3756.
17. Hermans, I. F., J. D. Silk, U. Gileadi, M. Salio, B. Mathew, G. Ritter, R. Schmidt, A. L. Harris, L. Old, and V. Cerundolo. 2003. NKT cells enhance CD4$^+$ and CD8$^+$ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J. Immunol.* 171: 5140–5147.
18. Stober, D., I. Jomantaite, R. Schirmbeck, and J. Reimann. 2003. NKT cells provide help for dendritic cell-dependent priming of MHC class I-restricted CD8$^+$ T cells in vivo. *J. Immunol.* 170: 2540–2548.
19. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R. M. Steinman. 2003. 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. *J. Exp. Med.* 198: 267–279.
20. Fujii, S., K. Liu, C. Smith, A. J. Bonito, and R. M. Steinman. 2004. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J. Exp. Med.* 199: 1607–1618.
21. Chung, Y., W. S. Chang, S. Kim, and C. Y. Kang. 2004. NKT cell ligand α-galactosylceramide blocks the induction of oral tolerance by triggering dendritic cell maturation. *Eur. J. Immunol.* 34: 2471–2479.
22. Gonzalez-Aseguinolaza, G., C. de Oliveira, M. Tomaska, S. Hong, O. Bruna-Romero, T. Nakayama, M. Taniguchi, A. Bendelac, L. Van Kaer, Y. Koezuka, and M. Tsuji. 2000. α-Galactosylceramide-activated Vα14 natural killer T cells mediate protection against murine malaria. *Proc. Natl. Acad. Sci. USA* 97: 8461–8466.
23. van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee. 2000. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J. Immunol.* 165: 4778–4782.
24. Romeo, V. D., J. deMeireles, A. P. Sileno, H. K. Pimplaskar, and C. R. Behl. 1998. Effects of physicochemical properties and other factors on systemic nasal drug delivery. *Adv. Drug Deliv. Rev.* 29: 89–116.
25. Davenport, F. M., A. V. Hennessy, F. M. Brandon, R. G. Webster, C. D. Barrett, Jr., and G. O. Lease. 1964. Comparisons of serologic and febrile responses in humans to vaccination with influenza a viruses or their hemagglutinins. *J. Lab. Clin. Med.* 63: 5–13.
26. Choi, Y. K., Y. J. Kim, H. S. Park, K. Choi, S. G. Paik, Y. I. Lee, and J. G. Park. 2003. Suppression of glomerulosclerosis by adenovirus-mediated IL-10 expression in the kidney. *Gene Ther.* 10: 559–568.
27. Takikawa, H., S.-E. Muto, and K. Mori. 1998. Diastereoselective epoxidation of the double bond at C-4 of sphingosines to provide phytosphingosine relatives such as α-galactosylceramide KRN7000. *Tetrahedron* 54: 3141.
28. Yamamoto, M., D. E. Briles, S. Yamamoto, M. Ohmura, H. Kiyono, and J. R. McGhee. 1998. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. *J. Immunol.* 161: 4115–4121.
29. Chung, Y., J. Cho, Y. S. Chang, S. H. Cho, and C. Y. Kang. 2002. Preventive and therapeutic effects of oral tolerance in a murine model of asthma. *Immunobiology* 206: 408–423.
30. Silk, J. D., I. F. Hermans, U. Gileadi, T. W. Chong, D. Shepherd, M. Salio, B. Mathew, R. R. Schmidt, S. J. Lunt, K. J. Williams, I. J. Stratford, et al. 2004. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J. Clin. Invest.* 114: 1800–1811.
31. Park, S. H., A. Weiss, K. Benlagha, T. Kyin, L. Teyton, and A. Bendelac. 2001. The mouse CD1d-restricted repertoire is dominated by a few autoreactive T cell receptor families. *J. Exp. Med.* 193: 893–904.
32. Hayakawa, Y., S. P. Berzins, N. Y. Crowe, D. I. Godfrey, and M. J. Smyth. 2004. Antigen-induced tolerance by intrathymic modulation of self-recognizing inhibitory receptors. *Nat. Immunol.* 5: 590–596.
33. Renegar, K. B., P. A. Small, Jr., L. G. Boykins, and P. F. Wright. 2004. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. *J. Immunol.* 173: 1978–1986.
34. Belyakov, I. M., and J. A. Berzofsky. 2004. Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. *Immunity* 20: 247–253.
35. Nakagawa, R., I. Nagafune, Y. Tazunoki, H. Ehara, H. Tomura, R. Iijima, K. Motoki, M. Kamishohara, and S. Seki. 2001. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α-galactosylceramide in mice. *J. Immunol.* 166: 6578–6584.
36. Hayakawa, Y., K. Takeda, H. Yagita, S. Kakuta, Y. Iwakura, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Critical contribution of IFN-γ and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of α-galactosylceramide. *Eur. J. Immunol.* 31: 1720–1727.
37. Hayakawa, Y., K. Takeda, H. Yagita, M. J. Smyth, L. Van Kaer, K. Okumura, and I. Saiki. 2002. IFN-γ-mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, α-galactosylceramide. *Blood* 100: 1728–1733.
38. Wolvers, D. A., C. J. Coenen-de Roo, R. E. Mebius, M. J. van der Cammen, F. Tirion, A. M. Miltenburg, and G. Kraal. 1999. Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39. *J. Immunol.* 162: 1994–1998.
39. Tsitoura, D. C., R. H. DeKruyff, J. R. Lamb, and D. T. Umetsu. 1999. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4$^+$ T cells. *J. Immunol.* 163: 2592–2600.
40. Oriss, T. B., M. Ostroukhova, C. Seguin-Devaux, B. Dixon-McCarthy, D. B. Stolz, S. C. Watkins, B. Pillemer, P. Ray, and A. Ray. 2005. Dynamics of dendritic cell phenotype and interactions with CD4$^+$ T cells in airway inflammation and tolerance. *J. Immunol.* 174: 854–863.
41. Gumperz, J. E., S. Miyake, T. Yamamura, and M. B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J. Exp. Med.* 195: 625–636.
42. Nieda, M., M. Okai, A. Tazbirkova, H. Lin, A. Yamaura, K. Ide, R. Abraham, T. Juji, D. J. Macfarlane, and A. J. Nicol. 2004. Therapeutic activation of Vα24$^+$Vβ11$^+$ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* 103: 383–389.
43. Giaccone, G., C. J. Punt, Y. Ando, R. Ruijter, N. Nishi, M. Peters, B. M. von Blomberg, R. J. Scheper, H. J. van der Vliet, A. J. van den Eertwegh, et al. 2002. A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. *Clin. Cancer Res.* 8: 3702–3709.
44. Osman, Y., T. Kawamura, T. Naito, K. Takeda, L. Van Kaer, K. Okumura, and T. Abo. 2000. Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide. *Eur. J. Immunol.* 30: 1919–1928.
45. Belshe, R. B., P. M. Mendelman, J. Treanor, J. King, W. C. Gruber, P. Piedra, D. I. Bernstein, F. G. Hayden, K. Kotloff, K. Zangwill, et al. 1998. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *N. Engl. J. Med.* 338: 1405–1412.