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Intranasal but not intravenous delivery of the adjuvant α-galactosylceramide permits repeated stimulation of natural killer T cells in the lung

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Abstract

Efficient induction of antigen-specific immunity is achieved by delivering multiple doses of vaccine formulated with appropriate adjuvants that can harness the benefits of innate immune mediators. The synthetic glycolipid α-galactosylceramide (α-GalCer) is a potent activator of NKT cells, a major innate immune mediator cell type effective in inducing maturation of DCs for efficient presentation of co-administered antigens. However, systemic administration of α-GalCer results in NKT cell anergy in which the cells are unresponsive to subsequent doses of α-GalCer. We show here that α-GalCer delivered as an adjuvant by the intranasal route, as opposed to the intravenous route, enables repeated activation of NKT cells and DCs, resulting in efficient induction of cellular immune responses to co-administered antigens. We show evidence that after intranasal delivery, α-GalCer is selectively presented by DCs for the activation of NKT cells, not B cells. Furthermore, higher levels of PD-1 expression, a potential marker for functional exhaustion of the NKT cells when α-GalCer is delivered by the intravenous route, are not observed after intranasal delivery. These results support a mucosal route of delivery for the utility of α-GalCer as an adjuvant for vaccines, which often requires repeated dosing to achieve durable protective immunity.

Keywords

Adjuvants; Anergy; DCs; α-GalCer; Mucosal immunity; NKT

Introduction

Vaccination is the ideal approach for sustained protection against infectious diseases and cancer. The administration of multiple doses of candidate vaccines is often necessary to induce the strongest and most long-lived antigen-specific immune responses. Potent vaccine

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formulations include appropriate adjuvants to increase the immunogenicity of co-administered antigens and also to help overcome immune tolerance, generally through harnessing the potential of a variety of innate immune modulators. Systemic administration of the synthetic glycolipid α-galactosylceramide (α-GalCer) by the intravenous route leads to CD1d-mediated presentation by APCs which activates NKT cells to induce the maturation of DCs for more efficient priming of T-cell responses to co-administered antigens [1]. This has led to the exploration of α-GalCer as an adjuvant for the induction of pathogen- and tumor-specific immune responses [2–4]. However, clinical development efforts of α-GalCer administration have been hampered by the realization that after the initial activation, the NKT cells become unresponsive to additional doses of α-GalCer delivered by the systemic route, a state referred to as anergy, when the NKT cells fail to produce cytokines and proliferate [5, 6]. We reported earlier that repeated immunization by the intranasal or oral route using α-GalCer as an adjuvant induced systemic and mucosal immune responses to co-administered antigens [7]. Here we investigated the mechanism for the effectiveness of α-GalCer as a mucosal adjuvant by characterizing the NKT cell responses after delivering primary and booster doses of α-GalCer admixed with the ovalbumin (OVA) antigen by the intranasal route. We observed activation of NKT cells in terms of IFN-γ production and proliferation after each dose of α-GalCer leading to DC activation in the lung and lung-draining LNs along with induction of OVA-specific T-cell responses.

Results

We have previously reported on the effectiveness of α-GalCer as a mucosal adjuvant for inducing systemic and mucosal immune responses specific to co-administered antigens delivered two or more times by the intranasal or oral routes [7]. Here, we investigated the mechanism for the potent mucosal adjuvant activity of α-GalCer by analyzing the kinetics and functional properties of NKT cells, relative to both priming and boosting by the intranasal route.

I.n. immunizations employing α-GalCer induce repeated activation and expansion of NKT cells in the lung

Groups of mice immunized by the intranasal or intravenous route with either OVA and α-GalCer (α-GalCer group) or OVA alone (control group) were sacrificed on days 1, 3, 5, 6, 8, and 10 post-immunization (Fig. 1A). A second (booster) immunization was delivered in each case to additional groups of mice on day 5 and sacrificed on days 6, 8, and 10 (i.e. days 1, 3, and 5 respectively, relative to the second dose). Single-cell suspensions prepared from spleen and lung tissues were analyzed for functional activation of NKT cells in terms of IFN-γ production (Fig. 1B). We observed a significant increase in the number of IFN-γ-producing NKT cells after intranasal immunization in mice from the α-GalCer group, relative to that in the control group animals, with peak activity at one day after the first as well as the second dose. In contrast to these results, mice immunized by the intravenous route showed a significant increase in the number of IFN-γ-producing NKT cells at day one after only the first dose, and not the second dose (Fig. 1C). These results from mice immunized by the intravenous route are consistent with the reports in the literature showing that a single dose of systemic α-GalCer administered either by the intravenous or intraperitoneal route induced NKT cell anergy, where NKT cells become unresponsive to a second or booster dose of α-GalCer administered by the same route, in terms of an inability to produce IFN-γ or proliferate [5, 6, 8, 9].

Along with increased IFN-γ production, expansion of NKT cells also occurred in the α-GalCer group with the peak levels observed at day 5 after the priming immunization by the intranasal route in the lung (Fig. 1D). Of importance is the observation of a second wave of

expansion of the NKT cells in the lung between days 6 and 10 (i.e. days 1 and 5 respectively, after the second intranasal immunization) that is significantly higher when compared with the percentages of NKT cells at the corresponding time point in the mice that did not receive the second immunization or the control group of mice that received two doses of OVA only (Fig. 1D). In the mice immunized by the intravenous route with two doses of α-GalCer, there was a slight increase in the NKT population at day 8, which corresponds to day 3 post-boost (Fig. 1D); however, this increase was smaller and less sustained than what was observed in the intranasal group and did not correspond to increased IFN-γ production (Fig. 1C).

The reactivation of NKT cells paralleled an increase in the CD86 expression on $CD11c^+$ DCs (Fig. 2A and B) in the spleen and lung after the second intranasal dose of α-GalCer + OVA when compared with the OVA control group on day 1 after the second immunization, a trend similar to that observed for activation of DCs on day 1 after the primary immunization (Fig. 2A and B). However, CD86 expression was not elevated on $CD11c^+$ DCs after the second intravenous dose of α-GalCer + OVA when compared with the OVA control group (Fig. 2A and B).

Thus, each dose of α-GalCer adjuvant delivered by the intranasal route resulted in the activation and expansion of NKT cells with IFN-γ producing potential along with an increase in activated DCs. On the other hand, a second dose of α-GalCer administered by the intravenous route resulted in only a slight increase in NKT cell proliferation, with no concurrent increase in IFN-γ production by NKT cells and no increase in activated DCs. Finally, the significant increase in the activation and reactivation of NKT cells and DCs from the booster immunization by the intranasal route with α-GalCer + OVA also translated into significant increases in antigen-specific cytotoxic T lymphocyte (CTL) activity and IFN-γ-producing cells after the booster dose, which was not observed after the intravenous booster immunization (Fig. 2C and D respectively).

Since the primary immunization with α-GalCer + OVA resulted in the expansion of NKT cells that peaked at day 5 in the lung and did not decrease to base-line levels even at day 10 post-immunization (Fig. 1D), we evaluated whether the second increase in NKT cells is a consequence of the continued effect of the priming dose of α-GalCer or the effectiveness of the second dose delivered on day 5. For this, we delayed the booster immunization until day 23 post-priming and characterized NKT cells and DCs in different tissues on days 24, 26, and 28 (i.e. days 1, 3, and 5 respectively, relative to the booster dose, Fig. 3A). Significant increases in the percentages of IFN-γ-producing NKT cells were observed in the spleen and lung of mice immunized with the booster dose of α-GalCer + OVA at day 24 (i.e. day 1 after the booster immunization, Fig. 3B) and furthermore, significant expansion of NKT cells was observed in the lung between days 1 and 5 after the booster immunization (Fig. 3D) compared with that in either the OVA only control group of mice or those that received only the priming dose of α-GalCer + OVA. We also found $CD11c^+$ DCs expressing slightly increased levels of the CD86 activation marker on day 24 (i.e. day 1 after the booster dose), when compared with the DCs from mice in the OVA control group (Fig. 3F). These results from mice that received the priming and boosting doses of α-GalCer + OVA by the intranasal route 23 days apart (the longer immunization scheme) were similar to those observed when the two doses were delivered 5 days apart (the shorter immunization scheme). Thus, regardless of the timing of the second dose, α-GalCer administration by the intranasal route leads to repeated activation of NKT cells, primarily in the lung. These results employing α-GalCer as an adjuvant delivered by the intranasal route are in contrast to those where primary and booster immunizations of α-GalCer + OVA delivered by the intravenous route 23 days apart. These intravenous immunizations did not result in either repeated activation or expansion of NKT cells or repeated activation of DCs in any of the

tissues studied (Fig. 3C, E and G). Thus, our data together with literature reports suggest a potential influence of the systemic versus mucosal administration of α-GalCer for inducing anergy in NKT cells.

CD11c$^+$ and not B220$^+$ cells present α-GalCer in the lung and draining lymph node after i.n. immunization

We investigated whether the tissue of origin and/or the phenotype of the α-GalCer-presenting cells influenced the anergy observed for NKT cells after intravenous versus intranasal route of administration. At one day after intranasal immunization, cells isolated from the spleen, lung, and several mucosal-draining lymph nodes of mice from either the α-GalCer group or OVA control group were co-cultured with an NKT cell clone (DN32.D3), and IL-2 production was assessed as a measure of α-GalCer presentation by cells from the various tissues [10]. We observed strong activation of the NKT cell clone by cells isolated from the lung and a lower but sustained level of activation by cells from the mediastinal lymph nodes (MdLNs) through day 5 suggesting that lung and MdLNs (lung-draining LNs) are the primary sites for α-GalCer presentation after intranasal immunization (Fig. 4A). These results, together with the data showing significantly higher NKT cell activation/expansion in the lung, described above (Figs. 1–3), support the lung as the major responding tissue for the α-GalCer adjuvant delivered by the intranasal route.

We further investigated the cellular phenotype presenting α-GalCer in the lung on day 1 after intranasal immunization with α-GalCer + OVA by isolating the CD11c$^+$ or B220$^+$ populations (potentially DCs and B cells respectively) for co-culturing with the DN32.D3 NKT cell clone, and analyzing the supernatants for IL-2 production. We observed that only the CD11c$^+$ cells but not B220$^+$ cells, from the lungs of mice in the α-GalCer group induced IL-2 production while neither cell type from lungs of mice immunized with OVA alone activated the NKT cell clone (Fig. 4B). These data suggest that most likely DCs and not B cells are involved in selectively presenting α-GalCer to NKT cells in the lung after intranasal administration of α-GalCer.

Expression of PD-1 on NKT cells after i.v. but not i.n. immunization employing α-GalCer as an adjuvant

Recent reports in the literature implicate increased PD-1 protein expression on NKT cells for the observed anergy resulting from administration of α-GalCer by the systemic routes [11–13]. To test this, NKT cells from different tissues of mice immunized either by the intravenous or intranasal route with α-GalCer + OVA were examined for surface PD-1 expression by flow cytometry. Consistent with the literature reports, we observed significantly higher PD-1 levels on NKT cells from spleen (3.7-fold, $p = 0.019$) and liver (11.5-fold, $p = 0.0016$) of mice at day 1 after immunization with α-GalCer + OVA by the intravenous route when compared with that on NKT cells from mice immunized with OVA alone (Fig. 5A). However, after intranasal immunization PD-1 levels on the NKT cells from spleen and lung tissues of mice from the α-GalCer group were not similarly increased when compared with PD-1 expression on NKT cells from mice in the OVA control group (Fig. 5B). Thus, NKT cells in the lungs of mice immunized by the intranasal route using α-GalCer as adjuvant exhibit no changes in the PD-1 expression on day one post-immunization and no signs of functional anergy, in terms of cytokine production and expansion. These results support the hypothesis that mucosal, as opposed to systemic administration of α-GalCer, (i.e. intranasal versus intravenous route) may lead to different consequences for NKT cells in terms of induction of anergy or functional competence in response to repeated α-GalCer delivery.

Discussion

The results from this investigation strongly support mucosal delivery as an efficient approach to harness the adjuvant potential of α-GalCer for priming as well as boosting cellular immune responses to co-administered immunogens. This is due to the repeated activation of NKT cells and DCs achieved after intranasal immunization with α-GalCer as an adjuvant. Meanwhile, systemic immunization by the intravenous route resulted in the unresponsiveness of the NKT cells to booster doses of α-GalCer, a phenomenon known as NKT cell anergy. These results are consistent with our earlier published studies which demonstrated the effectiveness and necessity of α-GalCer for repeated immunization by mucosal routes for the induction of strong cellular immune responses to the co-administered antigen [7].

Our studies comparing the intravenous and intranasal routes for delivering α-GalCer revealed similar kinetics of activation of NKT cells and DCs in terms of peak levels of IFN-γ production by NKT cells and DC activation at one day after a single immunization and are consistent with literature reports [5, 8, 14]. The key finding from our investigation is that a booster immunization employing α-GalCer as an adjuvant by the intravenous and intranasal routes revealed vastly different effects on NKT cells and DCs. While a single intravenous administration of α-GalCer, as demonstrated in this manuscript and reported in the literature, leads NKT cells to become unresponsive in terms of inability to produce cytokines in response to a booster dose of α-GalCer and also an inability to proliferate [5, 6, 8], our data demonstrates that after booster intranasal administration of α-GalCer, a potent activation of the NKT cells is observed for a second time in the lung, including IFN-γ production and expansion as well as DC activation. This repeated activation of NKT cells and DCs occurs regardless of the timing for the administration of the booster dose (i.e. day 5 or 23), suggesting that immunization by the intranasal route is a potential means to allow repeated dosing of the α-GalCer adjuvant without the induction of NKT cell anergy. A recent report published during the preparation of this manuscript showed delivery of α-GalCer by the intradermal route to be effective in avoiding NKT cell anergy, but mechanistic details are not described [15].

Of note, NKT cell activation and proliferation occurs in multiple tissues after primary intranasal administration of α-GalCer, but NKT cells are fully re-activated in the lung after the second intranasal administration of α-GalCer, suggesting that the lung is the major site of α-GalCer presentation after intranasal administration. This was confirmed by the observation that α-GalCer presentation to the DN32.D3 NKT cell clone occurs mainly in the lung and to a lesser extent in the lung-draining lymph node up to 5 days after intranasal administration. However, it is unclear as to how NKT cells and DCs are activated in more distal tissues, such as the spleen and liver, after a primary intranasal immunization with α-GalCer. It is possible that either activated DCs and/or activated NKT cells migrate from the lung after stimulation with α-GalCer, or alternatively the cytokine milieu resulting from NKT cell stimulation with α-GalCer may induce activation of these cell types in other tissues. In this regard it has been reported that a decrease in NKT cell populations in the liver coincided with an increase in the blood NKT cell levels after intraperitoneal immunization with α-GalCer, suggesting potential trafficking of NKT cells [16].

It has been observed that multiple administrations of DCs pulsed ex vivo with α-GalCer, as opposed to free α-GalCer, do not induce NKT cell anergy [5, 8]. On the other hand, it has also been shown that injection of B cells pulsed ex vivo with α-GalCer does induce NKT cell anergy [5, 17]. Here we have shown that after intranasal administration, $CD11c^+$ cells, not $B220^+$ cells, more efficiently present α-GalCer in the lung, suggesting that the intranasal route of immunization preferentially targets α-GalCer presentation to DCs. Interestingly,

Hermans et al. [18] showed that presentation of both α-GalCer and peptide antigen by the same DC was required for the strong activation of antigen-specific T-cell responses. Futhermore, Ko et al. [14] showed that the responding DC-presenting antigen in the lung-draining LNs also expresses a $CD8\alpha^-$ phenotype. This suggests that the DCs presenting α-GalCer in the lung should show a similar phenotype, which would be intriguing to pursue in the future.

In addition to the potential influence of the phenotype of cells presenting α-GalCer to induce NKT cell anergy, recently it has been reported that expression levels of the cell surface marker PD-1 on NKT cells may also be an important factor for anergy induction. In T cells, higher levels of PD-1 expression were observed to be associated with functional exhaustion resulting from interactions with either of its ligands, PD-L1 or PD-L2, which are both commonly expressed on APCs including B cells, DCs, and macrophages [19–21]. It has also been observed that PD-1 expression is up-regulated on the 'exhausted' $CD8^+$ T cells in HIV-infected patients and blocking of the PD-1/PD-L1 interaction could rescue the exhausted T cells in terms of restoring functional properties [22, 23]. Multiple groups have also shown that PD-1 is up-regulated on NKT cells very early after systemic administration of α-GalCer, and that blockade of the PD-1/PD-L1 interaction can reverse the unresponsiveness of the NKT cells [11–13]. We observed that while NKT cells from mice administered with α-GalCer by the intravenous route exhibited high levels of PD-1 expression at day 1 post-immunization, those in mice where α-GalCer was delivered by the intranasal route did not (Fig. 5). Furthermore, PD-1 expression on NKT cells coincided with functional exhaustion and unresponsiveness at 24 h after a second dose of α-GalCer by the intravenous route but not when α-GalCer was delivered by the intranasal route where NKT cells were fully functional in terms of IFN-γ production and expansion (Figs 1 and 3). Thus, in addition to the cell type mediating α-GalCer presentation (i.e. DCs versus B cells), the phenotype of NKT cells in terms of PD-1 expression could be another important factor for the avoidance of NKT cell anergy resulting from mucosal α-GalCer delivery (e.g. intranasal route), as opposed to systemic delivery (e.g. intravenous route). These observed differences between intravenous versus intranasal route of α-GalCer delivery may enable the repeated activation of NKT cells to aid in promoting DC activation which allows α-GalCer to serve as an efficient mucosal adjuvant for inducing immune responses to co-administered antigens. In fact, as shown in Fig. 2 a booster dose of α-GalCer administered by the intranasal route resulted in a subsequent increase in antigen-specific immune responses, while a booster dose of α-GalCer administered by the intravenous route did not correspond to an increase in antigen-specific immune responses.

In addition to the differences in terms of NKT cell anergy induction or the lack thereof, our investigation revealed several other differences for NKT cell activation after intravenous versus intranasal administration of α-GalCer. First, the timing of NKT cell activation and expansion appeared to be prolonged after intranasal administration of α-GalCer because the peak levels of NKT cell expansion were observed at day 5 post-immunization in the lung, the main responding tissue for this route of immunization. These results differ from that seen after the intravenous immunization where the NKT cell population peaked at day 3 in all tissues tested. In this regard, Fujii et al. [8] reported that intravenous administration of DCs pulsed ex vivo with α-GalCer, as opposed to free α-GalCer, which is shown to be a potential approach to avoid anergy to NKT cells, resulted in a prolonged NKT cell response, as measured by IFN-γ production. Second, we observed a decrease in the NKT cell population in the spleen and liver at day 1 after the priming immunization by the intravenous route, which is consistent with the literature reports that attribute the decrease in population to the down-regulation of the TCR as the underlying mechanism, but no such decrease in TCR was observed for NKT cells in mice that received the priming immunization via the intranasal route. Incidentally, Fujii et al. [8] reported a phenomenon describing NKT cell

turnover, a decrease in the NKT cell population on day 1 after α-GalCer administration later found to be due to TCR down-regulation, after administration of free α-GalCer that was "less rapid and severe" when DCs pulsed with α-GalCer were administered.

Antigen-specific cellular immune responses were measured after each dose of the α-GalCer adjuvant and OVA antigen mixture, similar to our previously reported studies with a different antigen [7]. Both these studies demonstrate that multiple doses of α-GalCer, administered by the intranasal route, are necessary to induce efficient antigen-specific cellular immune responses, regardless of the mouse strain used. In addition to the antigen-specific cellular immune responses, effectiveness of α-GalCer as an adjuvant after intranasal immunization to induce humoral immune responses, in terms of antigen-specific IgA and IgG responses has been described in the literature [24] and also observed in other unrelated studies in our laboratory (data not shown). Thus, our studies provide mechanistic support for mucosal delivery of α-GalCer adjuvant as an attractive strategy for vaccination regimens.

It is also important to note potential inflammatory effects from the intranasal administration of α-GalCer. Different mouse model studies revealed that intranasal administration of α-GalCer can induce airway infiltration of a combination of eosiniphils, neutrophils, and/or monocytes [25, 26]. Preliminary studies in our lab showed increase in the percentages of eosinophils but not neutrophils or monocytes (data not shown). However, clinical trials performed by Kunii et al. [4] showed that administration of α-GalCer by a nasal sub-mucosal route was safe.

Overall, this investigation has shown that α-GalCer can be administered by the intranasal route for primary and booster immunizations to induce cellular immune responses to co-administered antigens, without inducing NKT cell anergy. This is in striking contrast to α-GalCer administration by the intravenous route, in which a single dose leads to NKT cell anergy and a reduction in the ability of the adjuvant to boost adaptive immune responses to co-administered antigen. Thus, our data support the intranasal route of immunization as an attractive route for immunization especially because the ability to deliver multiple doses of the vaccine is essential for most therapeutic applications against infectious diseases and cancer.

Materials and methods

Animals

Female C57Bl/6 mice aged 6–10 wk were purchased from the National Cancer Institute. All procedures on the animals were carried out in accordance with institutionally approved protocols. The animals were housed in microisolator cages and provided with sterile food and water. The animal facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animals Care International. The studies were conducted according to the National Institute of Health Guidelines on the care and use of Laboratory Animals.

Cell lines and cell cultures

The cell line EL-4 (C57BL/6, H-2b, Thymoma) was maintained in RPMI complete media (CM) supplemented with 10% heat-inactivated FBS, 50 U/mL of penicillin–streptomycin and 50 μg/mL gentamycin.

Peptides and reagents

The synthetic peptide corresponding to the CTL epitopes of chicken ovalbuman (SIINFEKL) was purchased from American Peptide (Sunnyvale, CA, USA), dissolved in

dimethyl sulfoxide, DMSO (Sigma, St. Louis, MO, USA) and diluted in 1 × PBS at a final concentration of 1 mg/mL for cell culture studies. The OVA protein was purchased from Sigma.

α-GalCer

α-GalCer was purchased from Diagnocine LLC (Hackensack, NJ, USA) and dissolved in DMSO (Sigma) at a concentration of 1mg/mL.

Immunizations

Mice were immunized by the intranasal or intravenous routes 1–2 times at 0 and 5 or 23 days with a mixture of the OVA protein at 100 μg/mouse/dose and the synthetic glycolipid α-GalCer at 2 μg/mouse/dose. For intranasal immunizations, mice were anaesthetized by intraperitoneal (i.p.) injection of ketamine–xylaxine mixture, and 10 μl of the adjuvant–antigen mixture in 1 × PBS was introduced into each nostril as reported earlier [7, 27]. For intravenous immunizations, 200 μl of the adjuvant–antigen mixture in 1 × PBS was injected into the tail vein of the mouse. At various time point post-immunization, mice were sacrificed and perfused and cell suspensions were prepared from the spleen, lung, liver, and lymph nodes by homogenization or enzymatic dissociation using collagenase type IV (Sigma). Lymphocytes from liver were further isolated through a percoll (Sigma) gradient of 44 and 67%.

Analyses of antigen-specific cytolytic activity by the ^{51}Cr release assay

The CTL responses in single-cell suspension from spleens of immunized mice were assayed as described previously [28]. Briefly, spleen cells were re-stimulated for 5 days with the OVA peptide (SIINFEKL). These effector cells were tested for cytolytic activity against ^{51}Cr-labeled syngeneic EL-4 target cells that were pre-incubated with either medium alone or OVA peptide. The percentage (%) of specific lysis was calculated using the following formula: % specific lysis = (experimental release−spontaneous release)/(maximum release−spontaneous release) × 100, where the spontaneous release represents the radioactivity obtained when the target cells were incubated in culture medium without effectors and maximum release represents the radioactivity obtained when the target cells were lysed with 5% Triton X-100.

IFN-γ ELISpot assay

Cells isolated from the lung and MdLN of immunized mice were subjected to ELISpot assay for enumerating the numbers of antigen-specific IFN-γ-producing cells as described earlier [29] using the reagent kit from BD Biosciences (San Jose, CA, USA). The spots, representing individual IFN-γ-producing cells as spot forming cells (SFC), on the membrane were enumerated by Zellnet Consulting, New York, NY using the KS-ELISPOT automatic system (Carl Zeisis, Thornwood, NY, USA). Responses were considered positive when they were above 10 SFC/well and at least double the number obtained in cells cultured with medium alone.

Fluorescence-labeled antibodies and flow cytometry

Single-cell suspensions isolated from the various tissues of immunized mice were analyzed for NKT cells by staining with Pacific Blue-conjugated CD3 (clone 500A2, BD Biosciences), FITC-conjugated PD-1 (clone J43, eBioscience, San Diego, CA, USA) and the allophycocyanin-conjugated mouse CD1d tetramer loaded with PBS57 (provided by NIAID tetramer facility at Emory University, Atlanta, GA, USA). The NKT cells were stained first with Aqua Live/Dead reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then cells were washed and incubated with the CD1d

tetramer for 30 min in dark at 37°C. Cells were then incubated with a combination of surface markers (CD3 and PD-1) for an additional 30 min at 4°C, and then washed and fixed with Cytofix/Cytoperm Buffer (BD Biosciences for 10 min at 4°C. The percentages of DCs and their activation status were analyzed by staining for FITC-conjugated CD11b (clone M1/70, BD Biosciences), allophycocyanin-conjugated CD11c (clone HL3, BD Biosciences), PE-conjugated CD86 (clone GL1, BD Biosciences), and incubated with a combination of surface markers for 30 min at 4°C. After staining all cells were analyzed on an LSRII flow cytometer (BD Biosciences) and the data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA). For NKT cell analysis, lymphocytes were first gated using the forward scatter and side scatter plots. Next live cells were gated using side scatter and Aqua plots. Finally, the NKT cell population was determined by plotting PB-CD3 against the CD1d tetramer and these cells were analyzed further for surface marker expression and cytokine production. For DC analysis, lymphocytes were first gated using the forward scatter and side scatter plots. Next $CD11c^+$ cells were gated and then CD86 expression was determined by histogram plots.

Intracellular cytokine staining

For intracellular cytokine staining all cells were incubated with GolgiPlug (BD Biosciences) in CM for 4.5 h before any cellular staining. Cells were stained for surface markers and fixed as described in the flow cytometry section. Cells were then washed and incubated with PE-conjugated IFN-γ antibody (BD Biosciences) in 1 × Perm/Wash Buffer (clone XMG1.2, BD Biosciences) for 60 min at 4°C. Cells were then washed two more times in the Perm/Wash buffer and fixed in Cytofix/Cytoperm buffer (BD Biosciences), and samples were analyzed on the LSRII flow cytometer as described in the flow cytometry section.

In vivo presentation of α-GalCer

Cells isolated from immunized mice were co-cultured with the NKT cell hybridoma DN32.D3 for 24 h at a concentration of 1×10^6 lymphocytes to 1×10^5 hybridomas. Alternatively, single-cell suspensions from the lungs of immunized mice were purified using MACs beads (Miltenyi Biotec, Bergisch Gladbach, Germany) specific for PE-conjugated $CD11c^+$ or PE-conjugated $B220^+$ cells (BD Biosciences) as described in the literature [30]. The purified cells, as α-GalCer-presenting cells, were co-cultured at a concentration of 5×10^5 with 1×10^5 hybridoma cells. The NKT cell activation was assessed in terms of the release of IL-2 which was measured by the CTLL assay as described in the literature [31]. Briefly, supernatants were collected from the co-culture, serially diluted, and incubated with 5×10^3 CTLL cells for approximately 40 h at 37°C. Then, 1 μCi of ^{3}H-thymidine (Perkin Elmer, Waltham, MA, USA) was added for the final 16 h and cells were harvested and measured for ^{3}H incorporation.

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Abbreviations

α-GalCer	α-glactosylceramide
MdLN	mediastinal lymph node
PD-1	Programmed Death-1

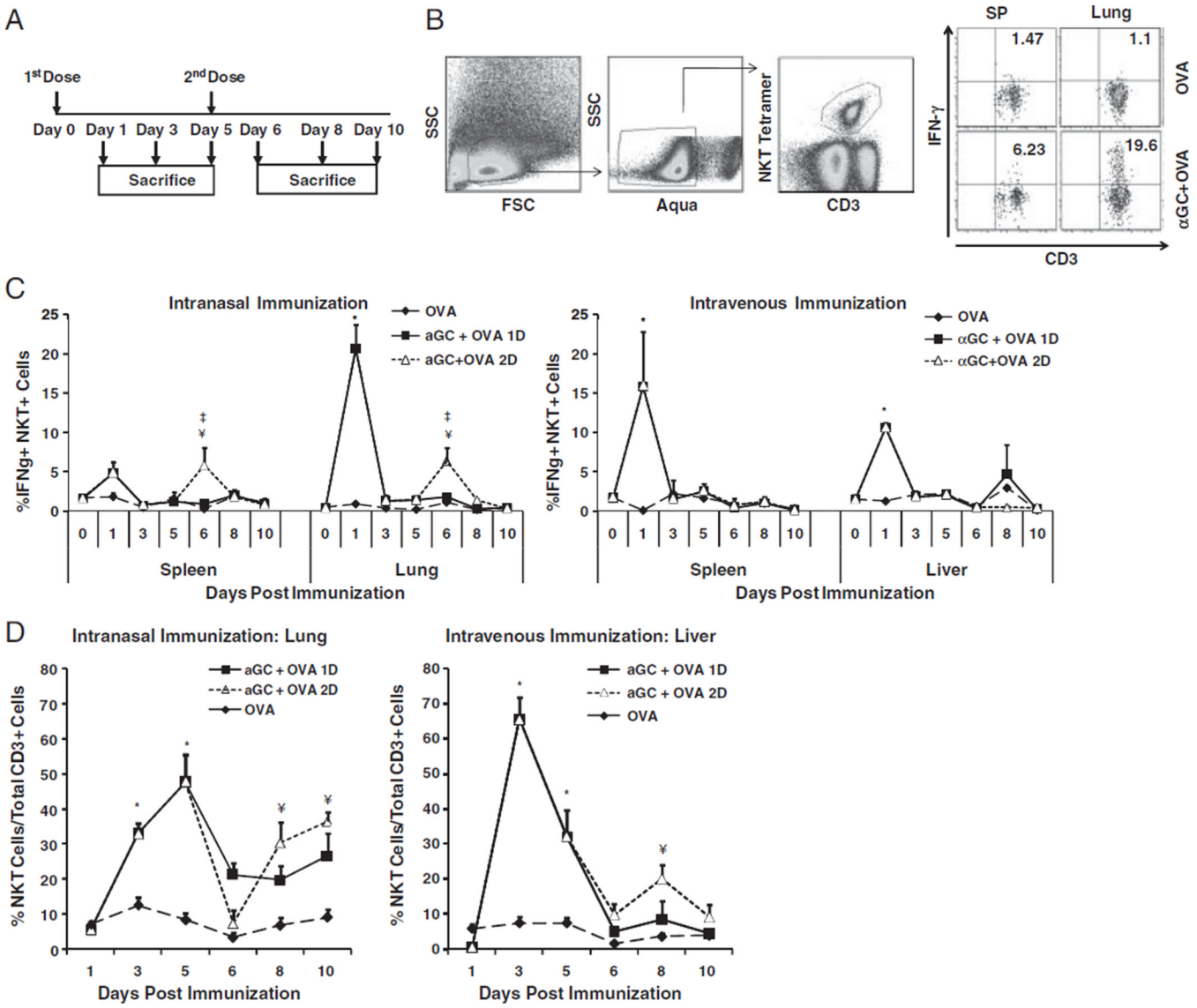

Figure 1.

Activation and expansion of NKT cells after repeated intranasal administration of α-GalCer. (A) Mice were immunized by the intranasal or intravenous route with one or two doses of either OVA alone or admixed with α-GalCer (α-GC + OVA). The second dose was administered at day 5 after the primary immunization and the mice were sacrificed on days 1, 3, 5, 6, 8, and 10. (B) Cells isolated from the spleens and lungs or livers were stained with fluorescently labeled reagents: anti-CD3-PB, NKT tetramer-allophycocyanin, anti-IFN-γ-PE and Aqua live/dead stain. The gating strategy for enumerating live NKT^+ $CD3^+$ cells producing IFN-γ is shown. (C) The percentages of IFN-γ-producing NKT cells between days 1 and 10 after intranasal or intravenous immunization with OVA alone (OVA) and one or two doses of α-GalCer + OVA (α-GC + OVA 1D or 2D respectively) are shown. Data are presented as mean and standard deviations for all the mice tested. *p<0.05 between groups of mice that were immunized with OVA alone and one dose of α-GalCer + OVA, ¥p<0.05 between mice immunized with OVA alone and two doses of α-GalCer + OVA, and ‡ p<0.05 between one and two doses of α-GalCer + OVA. (D) The percentages of NKT $tetramer^+$ cells out of total $CD3^+$ cells in the lungs (left) or livers (right) of all three groups

of mice between days 1 and 10 after intranasal (left) or intravenous (right) immunization are shown. Significant differences, as determined by Student's *t*-test ($p<0.05$), were observed between days 1 and 3 or 1 and 5 after one dose of α-GalCer + OVA (*). Additionally, significant differences as determined by Student's *t*-test ($p<0.05$), were observed between days 6 and 8 or days 6 and 10 for the two-dose immunization of α-GalCer + OVA (¥). Data are shown as the average with standard deviation for three mice and representative of three experiments.

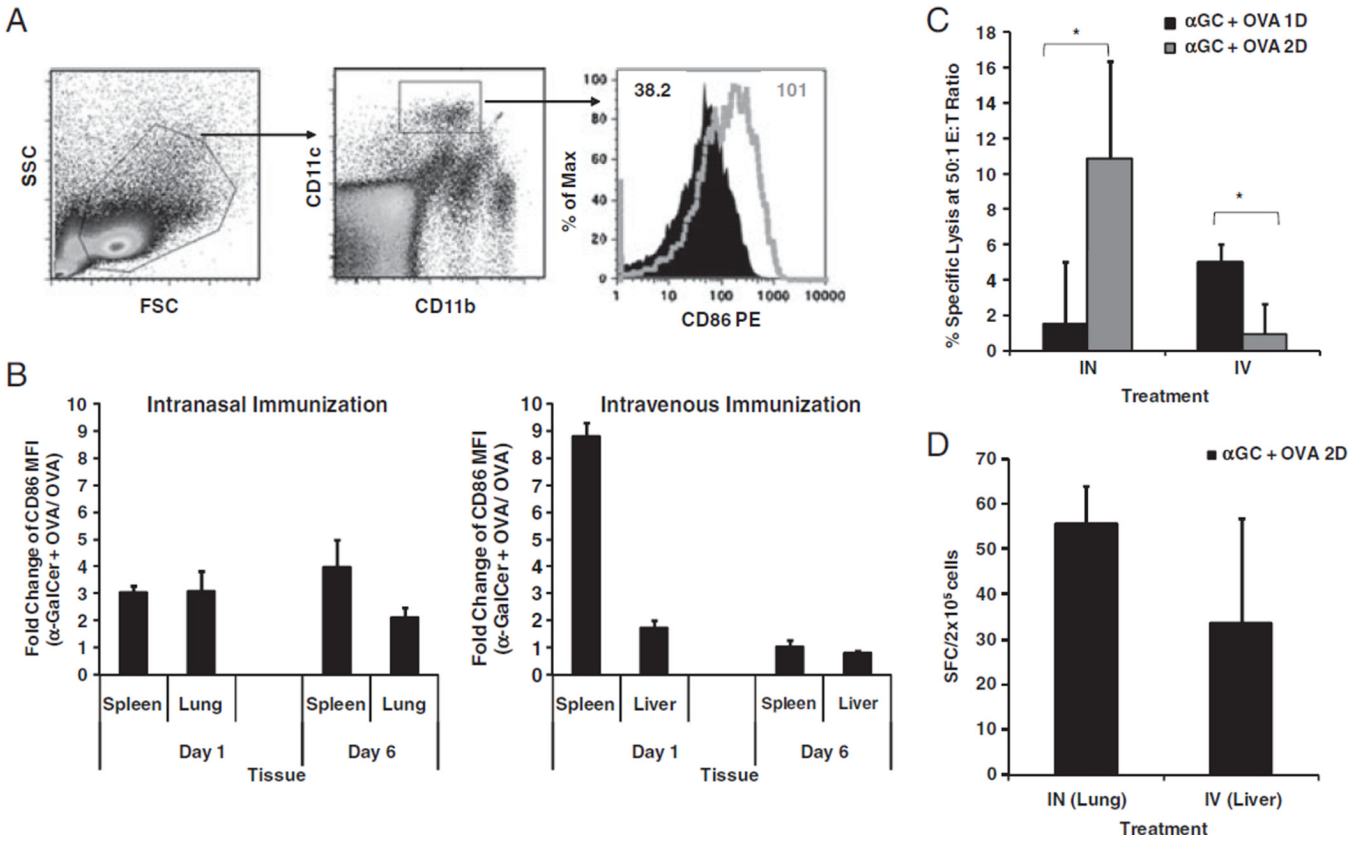

Figure 2.

Activation of DCs and antigen-specific immune responses after repeated intranasal immunization employing α-GalCer as an adjuvant. Mice were immunized by the intranasal or intravenous route with either OVA alone or admixed with α-GalCer (α-GC + OVA) and sacrificed 1 day post-immunization. Cells isolated from the spleens and lungs were stained with fluorescently labeled antibodies: anti-CD11b-FITC, anti-CD11c-allophycocyanin and anti-CD86-PE. (A) The gating strategy for $CD11c^+$ DCs expressing the activation marker CD86 and a histogram for the percentage of activated $CD11c^+$ cells in the spleen in one mouse each, immunized with OVA alone (filled histogram) or α-GC + OVA (open histogram) are shown. (B) Fold change of CD86 expression in $CD11c^+$ cells from the spleens and lungs or livers of mice at day 1 after either the 1st or 2nd immunization (day 1 and day 6 respectively) with α-GC + OVA administered by either the intranasal (lung) or intravenous (liver) route over the values from mice immunized with OVA alone. Data are presented as mean + SD. (C) Fold change of antigen-specific cytotoxic T lymphocyte activity in spleen cells from mice immunized by either intranasal (IN) or intravenous routes (IV), 5 days apart, assessed by the standard chromium release assay using syngeneic EL4 target cells pulsed with the OVA peptide. Data were adjusted for background by subtracting control values (target cells not pulsed with the OVA peptide) and fold change was calculated between 2 dose and 1 dose immunization groups at each effector to target ratio tested. The data are shown as the mean + SD. (D) Fold change in the number of antigen-specific IFN-γ-producing cells. Single-cell suspensions from the lungs of mice immunized by the intranasal (IN) or the liver of mice immunized by the intravenous route (IV) with one or two doses of α-GC + OVA administered 5 days apart (1 dose and 2 doses, respectively) were stimulated and IFN-γ measured by ELISpot. Fold changes were calculated between the 1 dose and 2 dose immunization groups after adjustment for medium stimulation and data are shown as

mean + SD. Data are shown as the average with standard deviation for three mice and representative of three experiments.

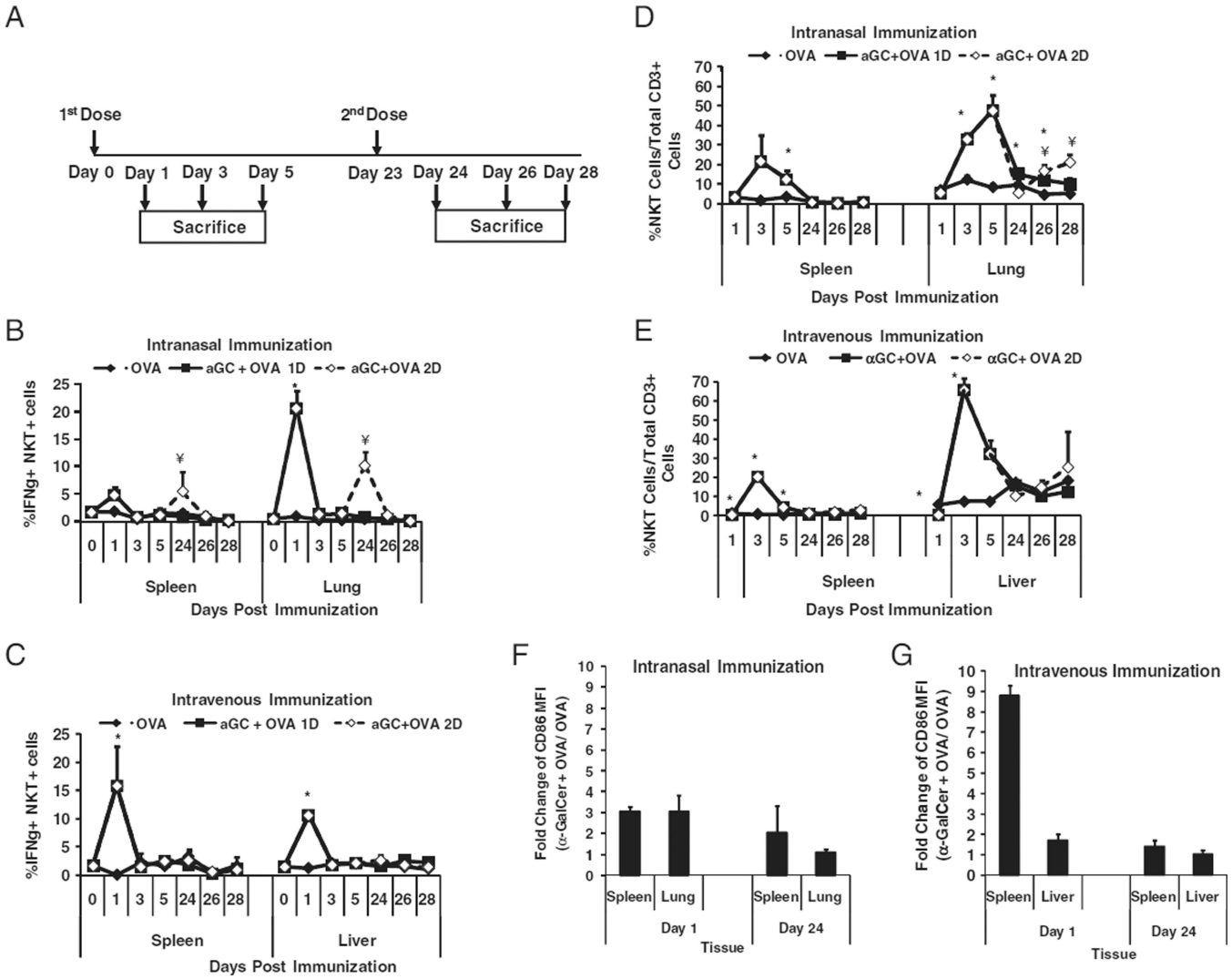

Figure 3.

Efficient activation and expansion of NKT cells along with activation of DCs after intranasal but not intravenous booster immunization employing α-GalCer adjuvant. (A) Mice were immunized by the intranasal or intravenous route with a primary dose of OVA alone (OVA) or admixed with α-GalCer (α-GC + OVA 1D) and sacrificed 1, 3 and 5 days post-immunization. A separate group received a booster dose of OVA alone or α-GC + OVA (α-GC + OVA 2D) at day 23 after the primary immunization followed by the sacrifice of the mice on days 24, 26, and 28, which are 1, 3 and 5 days after the booster immunization. (B, C) Cells isolated from the spleen, lung and liver of mice immunized (B) intranasally or (C) intravenously were stained with fluorescently labeled reagents: anti-CD3-PB, NKT tetramer-allophycocyanin, anti-IFN-γ-PE and Aqua live/dead stain. The live NKT^+ $CD3^+$ cells producing IFN-γ were enumerated for each tissue between days 1–5 and 24–28 post-immunization and significant differences, as determined by Student's t-test ($p < 0.05$), between the different groups of mice immunized with OVA alone (OVA) and one or two doses of α-GalCer + OVA (α-GC + OVA 1D or 2D) were marked with * or ¥ respectively. (D, E) The percentage of NKT $tetramer^+$ cells from total $CD3^+$ cells from mice immunized (D) intranasally and (E) intravenously was measured to determine the kinetics of NKT cell expansion between days 1–5 and 24–28 post-immunization for the OVA and α-GC + OVA

1D and 2D groups of mice and significant differences between the different groups were marked with * or ¥ as above. (F, G) The activation status of DCs from mice immunized (F) intranasally and (G) intravenously was assessed in terms of surface CD86 expression on $CD11c^+$ cells as measured by flow cytometry from spleen, liver on day 1 after the 1^{st} and 2^{nd} immunization (days 1 and 24 respectively) and the fold change was calculated between groups. Data are shown as the average with standard deviation for three mice and representative of two experiments.

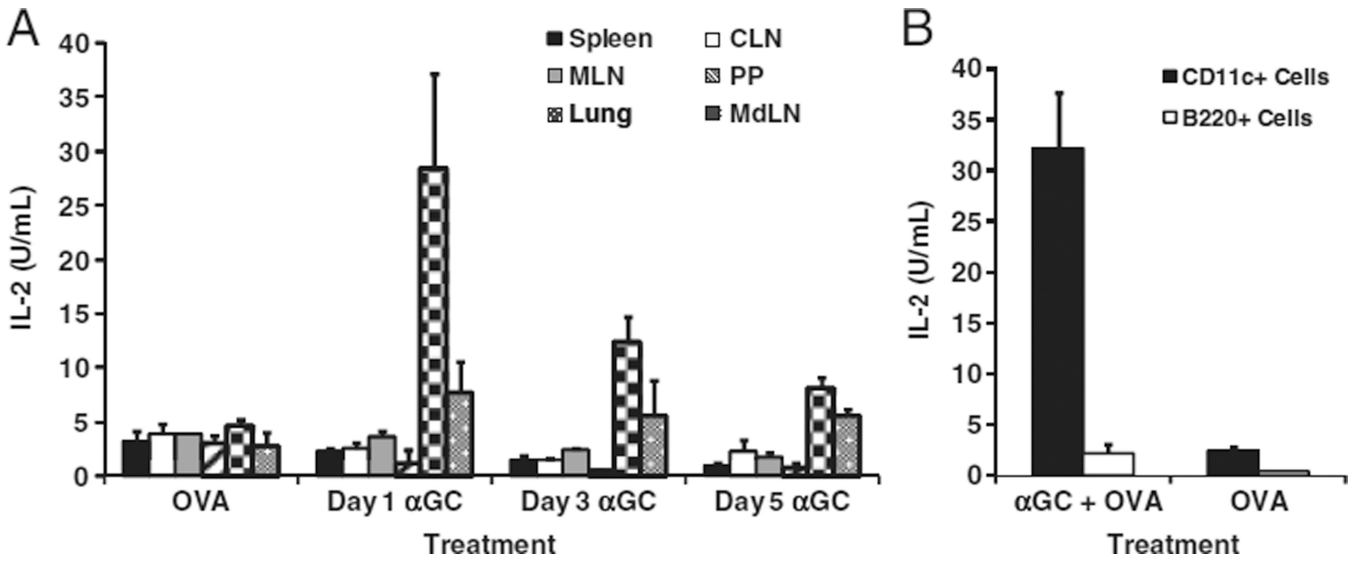

Figure 4.

Tissue of origin and cell type for α-GalCer presentation after intranasal immunization. Mice were immunized by the intranasal route with either OVA alone or admixed with α-GalCer (α-GC + OVA) and sacrificed on 1, 3 and 5 days post-immunization. (A) Cells isolated from the spleen, lung, MdLNs, cervical lymph nodes (CLNs), mesenteric lymph nodes (MLNs), and Peyer's patches (PP) were tested for the presentation of α-GalCer by co-culturing with the NKT cell clone DN32.D3. Culture supernatants were analyzed for IL-2 production by culturing IL-2-dependent CTLL cells with the supernatants and assessing CTLL cell proliferation by ^{3}H incorporation. (B) Separate groups of mice were immunized by the intranasal route with OVA alone or admixed with α-GalCer (α-GC + OVA) and sacrificed on day 1 post immunization. The lung cells were then stained for either CD11c-PE or B220-PE and purified by MACs sorting with PE-conjugated MACs beads. The isolated cells representing $CD11c^{+}$ and $B220^{+}$ populations were separately co-cultured with the NKT cell clone DN32.D3 and the culture supernatants in each case were analyzed for IL-2 production. Data are shown as the average with standard deviation for three mice and representative of three experiments.

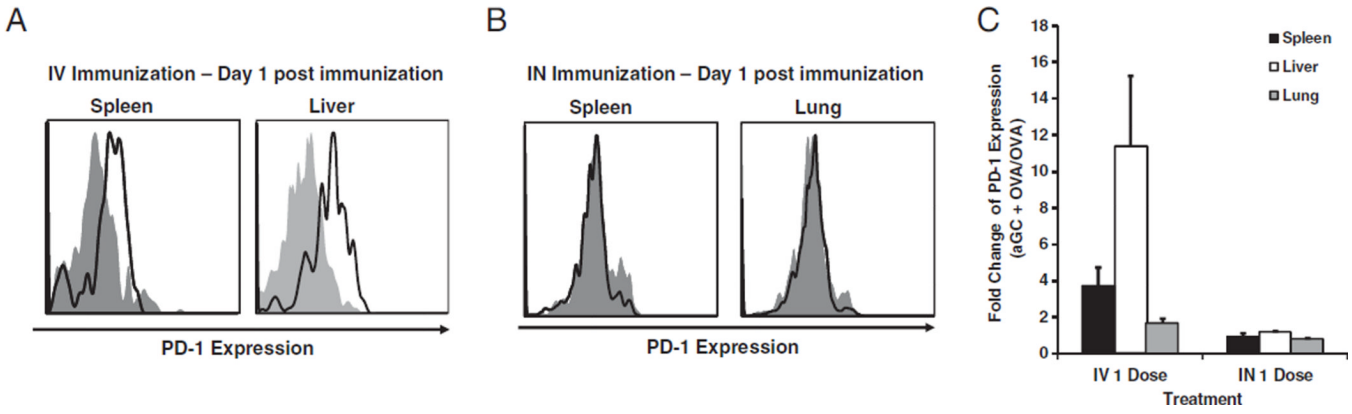

Figure 5.

Elevated PD-1 expression on NKT cells after intravenous but not intranasal administration of α-GalCer. Mice were immunized by the intravenous or intranasal route with either OVA alone (OVA) or admixed with α-GalCer (α-GC + OVA) and sacrificed on day 1 post-immunization. Cells isolated from the spleen and lung or liver tissues were stained with fluorescently labeled reagents: anti-CD3-PB, NKT tetramer-allophycocyanin, anti-PD-1-FITC and Aqua live/dead stain and analyzed by flow cytometry. The live NKT^+ $CD3^+$ cells expressing PD-1 were enumerated and representative histograms show data for (A) the spleen and liver tissues from one mouse immunized by the intravenous route or (B) spleen and lung tissues of one mouse immunized by the intranasal route with either OVA alone (filled histogram) or α-GC + OVA (open histogram). (C) Fold difference between the OVA and α-GC + OVA groups of mice for the expression of PD-1 on NKT cells at day 1 after one immunization by the intravenous (IV) and intranasal (IN) routes. Data are shown as the average with standard deviation for three mice and representative of three experiments.