

FRONTLINE:

 α -Galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: Role of IFN- γ

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Allergic asthma is a multifaceted syndrome consisting of eosinophil-rich airway inflammation, bronchospasm, and airway hyper-responsiveness (AHR). Using a mouse model of allergic asthma, we previously reported that invariant NKT (iNKT) cells increase the severity of this disease. Herein, we demonstrate that a single i.v. injection of α -galactosylceramide (α -GalCer), 1 h before the first airway allergen challenge of OVA-sensitized mice, abrogates elicitation of AHR, airway eosinophilia, IL-4 and IL-5 production in bronchoalveolar lavage fluid, and specific anti-OVA IgE antibodies. Further, α -GalCer administered intranasally also strongly inhibited the major symptoms of asthma in sensitized and challenged mice. α -GalCer treatment induces iNKT cell accumulation in the lungs, and shifts their cytokine profile from pro-asthmatic IL-4 to a protective IFN- γ production. The role of IFN- γ from iNKT cells in protection was shown by adoptive transfer of sorted iNKT cells from OVA-sensitized and α -GalCer-treated mice which protected immunized recipients from manifesting asthma by an IFN- γ -dependent pathway. Our findings demonstrate for the first time that α -GalCer administered locally inhibits asthma symptoms, even in predisposed asthmatic mice, through an iNKT cell- and IFN- γ -dependent pathway.

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Abbreviations: α -GalCer: α -galactosylceramide · AHR: airway hyper-responsiveness · BALF: bronchoalveolar lavage fluid · CD1d/ α -GalCer tetramer: α -GalCer-loaded CD1d/ β 2 m tetramer · HE: hematoxylin/eosin · i.n.: intranasal · iNKT: invariant natural killer T

Introduction

Recent advances in understanding the pathogenesis of allergic asthma have identified a complex interplay between Th2 effector cells, recruited inflammatory eosinophils, local IgE-activated mast cells, and released inflammatory cytokines and chemokines, as well as mediators of airway spasm and hyper-reactivity [1–5]. Locally, released Th2-type cytokines like IL-4 and IL-5 have been shown to generate mucosal eosinophil-rich

inflammation with goblet cell proliferation and increased mucus production [1–5]. Importantly, Th2-dependent mechanisms are also responsible for airway hyper-responsiveness (AHR), and for the production of IgE antibodies that lead to local mast cell activation [1–5]. Additionally, current concepts postulate that the Th2-type effector responses can possibly be regulated by suppressor CD4⁺CD25⁺ T cells, counter-regulatory Th1 cells, or immunoregulatory NKT cells [3, 6–8].

Invariant V α 14 natural killer T (iNKT) cells constitute a distinctive subpopulation of mature T lymphocytes, which co-express a highly restricted T cell receptor (TCR) repertoire composed of a single invariant V α 14J α 18 chain in mice and a V α 24J α 18 chain in humans, preferentially paired with limited TCR V β chains [9–12]. This semi-invariant TCR reflects positive selection by the non-polymorphic MHC class I-like molecule, CD1d [9–12]. iNKT cells specifically recognize the exogenous CD1d-bound glycolipid, α -galactosylceramide (α -GalCer), obtained from a marine sponge, but absent in mammalian cells [13]. A large range of cytokines are promptly generated by iNKT cells in response to α -GalCer, including IL-3, IL-4, IL-10, IL-13, IFN- γ , TNF- α and GM-CSF [10–14]. Recently, Bendelac and collaborators have identified the glycolipid iGb3 as one of the endogenous molecules that positively select and stimulate iNKT cells [15].

We and others have previously demonstrated that iNKT cells are required for the severity of allergic asthma since iNKT cell-deficient mice develop mild disease [6–8]. Herein, we hypothesized that allergic asthma could be modulated by treatments targeting iNKT cells. Knowing that iNKT cells specifically respond to α -GalCer and that injection of this glycolipid is protective in some experimental models of human disease [16–21], we reasoned that activation of iNKT cells by α -GalCer might prevent allergic asthma.

Our results show that a single administration of α -GalCer to sensitized mice, both prior or after antigen challenge, completely abolished their airway eosinophilia and AHR. This protective effect is dependent on the IFN- γ produced by iNKT cells and can be adoptively transferred to sensitized mice.

Results

Systemic α -GalCer treatment of previously sensitized mice inhibits the major asthma hallmarks

We previously demonstrated that the adoptive transfer of iNKT cells to pre-sensitized J α 18^{-/-} (iNKT cell-deficient) mice 1 h before the first OVA airway challenge reconstituted allergic asthma [6]. This finding prompted

us to examine whether α -GalCer could protect against asthma when injected 1 h before the first OVA challenge.

Similar to previous studies [6–8], we found that mice sensitized with OVA (i.p.) and challenged with allergen *via* the airways had AHR significantly increased compared to OVA-immunized and NaCl-challenged control mice (Fig. 1A, B). In contrast, mice treated with a single i.v. injection of α -GalCer 1 h before the first OVA challenge did not develop AHR (Fig. 1A, B). The protective effect of α -GalCer treatment was observed in both C57BL/6 and Balb/c mice, while it did not occur following injection of controls (vehicle used to dilute α -GalCer) (Fig. 1A, B). Further, α -GalCer treatment significantly decreased eosinophils, neutrophils and lymphocytes in bronchoalveolar lavage fluid (BALF) as compared to controls (Fig. 1C). Histochemical staining also revealed that treatment with α -GalCer markedly reduced the characteristic eosinophil infiltrates in the lung of OVA-immunized and -challenged mice (Fig. 1D). In addition, typical Th2 cytokines induced by OVA challenge, namely IL-4 and IL-5, were significantly reduced in the BALF of α -GalCer-treated mice, while IFN- γ production was enhanced (Fig. 2A). Finally, α -GalCer treatment inhibited the peripheral response to immunization and airway challenge, as measured by serum OVA-specific IgE levels (Fig. 2B). Thus, several hallmarks of the Th2 asthmatic response were markedly inhibited by i.v. treatment of sensitized mice with α -GalCer 1 h before challenge.

Intranasal administration of α -GalCer to previously sensitized mice inhibits AHR, eosinophilia and BALF Th2 cytokine production

We addressed the question whether α -GalCer could exert the same protective effect when administered intranasally (i.n.). This proved to be the case (Fig. 3) since α -GalCer administered i.n. 1 h before the first OVA challenge significantly inhibited both AHR and eosinophil recruitment in the airways (Fig. 3A, B). Moreover, the decrease of IL-4 was concomitant with an increase of IFN- γ (Fig. 3C), indicating that a shift from a pro-asthmatic Th2 to a protective Th1 response had occurred in the airways.

Local α -GalCer administration inhibits asthma elicitation in OVA-sensitized and -challenged mice

We further analyzed whether local α -GalCer treatment could abrogate asthma symptoms in mice that were previously sensitized and challenged with OVA. Indeed, α -GalCer administered i.n. 24 h after the first OVA challenge inhibited AHR (Fig. 3A), airway eosinophilia (Fig. 3B) and BALF IL-4 production while BALF IFN- γ

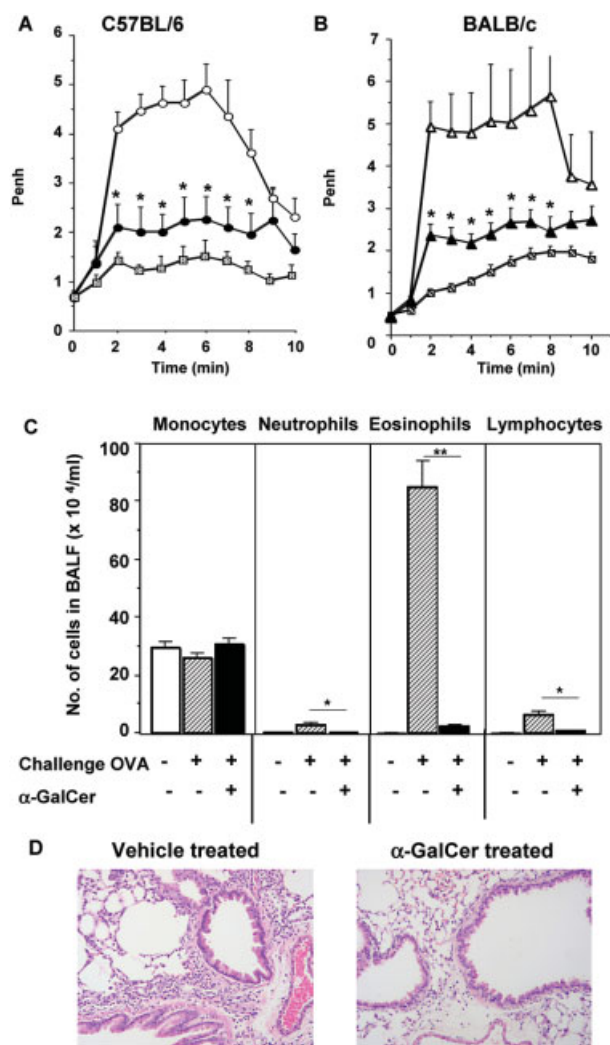


Figure 1. Analysis of antigen-induced airway responses in α -GalCer-treated mice. (A,B) α -GalCer treatment inhibits the development of AHR. Airway reactivity to methacholine was measured 1 day after the last challenge with OVA (circles and triangles) or NaCl (squares) in both C57BL/6 (A) and Balb/c (B) immunized mice treated with a single i.v. injection of α -GalCer (filled circles and triangles) or vehicle (empty circle and triangle) 1 h before the first challenge. Similar results were obtained when immunized mice were treated with α -GalCer i.v. or vehicle (data not shown) and then challenged with NaCl (squares represent data obtained with α -GalCer). Data represent the mean $\pm$ SEM enhanced pause (Penh) values from groups of 6–20 sensitized mice. OVA-immunized and -challenged mice treated with α -GalCer exhibit decreased AHR when compared to vehicle-treated animals (* p < 0.05). (C) Airway eosinophilia is reduced after α -GalCer treatment. BALF from mice in (A) was analyzed immediately after AHR measurement. Results are shown as the number of cells per mL in BALF. Data represent the mean $\pm$ SEM of six mice. * p < 0.05, ** p < 0.01. (D) Representative lung sections stained with HE (magnification $\times$ 10) from OVA-sensitized mice, treated with vehicle (left panel) or α -GalCer (right panel) i.v. 1 h before the first OVA challenge.

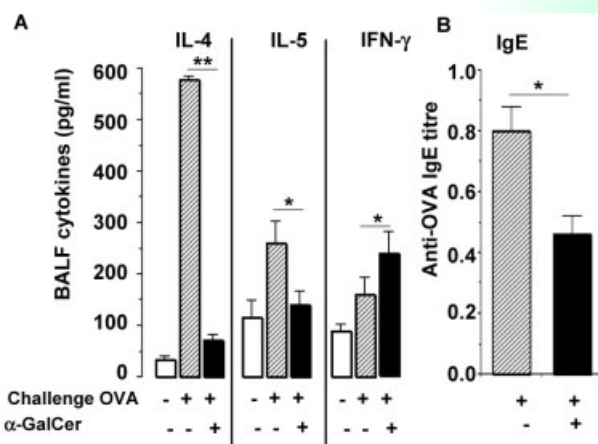


Figure 2. Analysis of antigen-induced cytokine responses in α -GalCer-treated mice. (A) A shift in the Th2/Th1 cytokine response occurs in BALF of α -GalCer-treated mice. IL-4, IL-5 and IFN- γ were measured in BALF of OVA-immunized mice treated or not with α -GalCer i.v. and then challenged with OVA or NaCl. Data are the means $\pm$ SEM of ten mice. (B) Evaluation of OVA-specific IgE in α -GalCer-treated mice. OVA-specific IgE was measured by ELISA in sera from mice sensitized, and α -GalCer-treated or not, and challenged with OVA. Data represent the mean $\pm$ SEM of six mice. * p < 0.05, ** p < 0.01.

levels were enhanced (Fig. 3C), compared to untreated OVA-immunized and -challenged mice. These findings demonstrate that α -GalCer administered locally not only inhibits the elicitation of asthma in sensitized mice but also abrogates the major symptoms of asthma in mice that already were airway challenged with OVA, thus supporting a possible therapeutic use of this glycolipid.

IFN- γ is required for the protective effect of α -GalCer treatment

To further investigate the mechanism underlying the protective effect mediated through α -GalCer, we examined involvement of cytokines like IL-4, IFN- γ and IL-10. We blocked their activity *in vivo* by injecting neutralizing antibodies against the corresponding cytokine or its specific receptor, as in the case of IL-10. Only anti-IFN- γ antibody injection abrogated the protective effect of α -GalCer and completely restored airway eosinophilia and BALF IL-4 production (Fig. 4A, B). Importantly, AHR was also recovered in these conditions (Fig. 4C), supporting the notion that protection from allergic asthma could be afforded by a single administration of α -GalCer acting through IFN- γ , as shown in Fig. 3C.

α -GalCer treatment enhances both the frequency of lung iNKT cells and their capacity to produce IFN- γ

We thus examined the cytokine production profile of lung iNKT cells. iNKT cells are already present in the lung

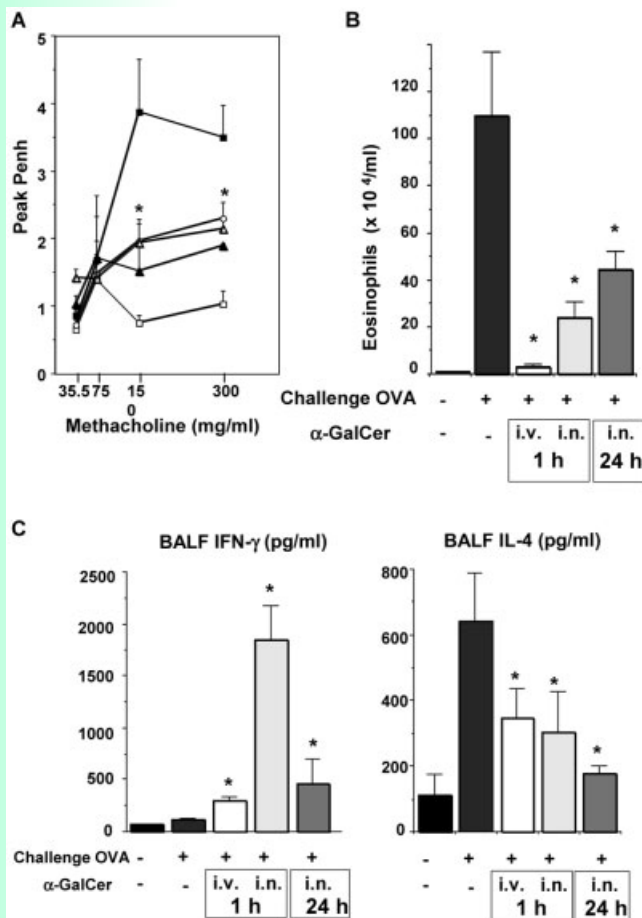


Figure 3. Effect of i.n. α -GalCer treatment on antigen-induced airway responses. (A) I.n. α -GalCer treatment inhibits the development of AHR. Airway reactivity to methacholine was measured 1 day after the last challenge with OVA (filled triangles and squares and both empty circles and triangles) or NaCl (empty squares) in C57BL/6 immunized mice treated with a single i.v. (circles) or i.n. (triangles) injection of α -GalCer 1 h before the first (empty circles and triangles) or 24 h after the first (filled triangles) OVA challenge. Data represent the mean $\pm$ SEM peak enhanced pause (Penh) values from groups of three to six sensitized mice. OVA-immunized and -challenged mice treated with α -GalCer i.n. or i.v. before the first OVA challenge exhibit decreased AHR when compared to untreated OVA-challenged animals ($p < 0.05$). (B, C) Airway eosinophilia and IFN- γ and IL-4 BALF levels after α -GalCer treatment. BALF from mice in (A) was analyzed immediately after AHR measurement. 1 h, α -GalCer injected 1 h before the first OVA challenge; 24 h, α -GalCer injected 24 h after the first OVA challenge. Data are the means $\pm$ SEM; $p < 0.05$.

of OVA-primed mice, and their frequency is enhanced following challenge with OVA compared to NaCl (Fig. 5A). Notably, α -GalCer treatment significantly augments the percentage of the iNKT cells among lung T cells in OVA-primed and -challenged mice (Fig. 5A). Further, lung iNKT cells in OVA-primed, α -GalCer-treated and OVA-challenged mice were significantly activated as assessed by their forward scatter (Fig. 5A).

α -GalCer treatment did also increase the frequency of CD4⁺ cells among lung iNKT cells, as well as the expression of this marker by these cells (Fig. 5A). Previous reports suggested that IL-4 produced by iNKT cells potentiated the development of asthma [7]. Thus, we examined the effect of the α -GalCer treatment on the cytokine production profile of lung iNKT cells obtained 2 days post treatment. Intracytoplasmic cytokine staining in freshly isolated lung iNKT cells showed that *in vivo* they produce preferentially IL-4 after priming and challenge with OVA (Fig. 5B). This *in vivo* data confirms previous data showing that lung iNKT cells from sensitized and challenged mice could produce IL-4 after *in vitro* stimulation [7]. In contrast, α -GalCer treatment induced a shift in the cytokine production profile of lung iNKT cells away from IL-4 towards higher IFN- γ production (Fig. 5B). This indicates that lung iNKT cells of α -GalCer-treated mice preferentially secrete IFN- γ in the airways.

The adoptive transfer of iNKT cells recovered from OVA-sensitized mice treated with α -GalCer protects sensitized mice against asthma

It was important to determine whether the protective effect of α -GalCer could be transferred to wild-type sensitized mice. Thus, we examined whether splenocytes from OVA-immunized and α -GalCer-treated wild-type mice could inhibit airway eosinophilia and AHR in OVA-immunized recipients. Using intracellular cytokine staining, we observed, as expected [10], that the majority of donor iNKT splenocytes from OVA-primed α -GalCer-treated mice produce IFN- γ and IL-4 at the time of their transfer to recipients (Fig. 6). Splenocytes from OVA-immunized mice treated at day 8 with α -GalCer were transferred i.v. to OVA-immunized recipients, 1 h before the first OVA challenge. This resulted in significantly reduced OVA-induced airway eosinophilia (Fig. 7A; group D) and AHR (Fig. 7B). In contrast, transfer of splenocytes from naive (data not shown), or simply OVA-primed mice to OVA-immunized recipients (Fig. 7A, B; group C) had no such effect, confirming the central role of α -GalCer treatment. Importantly, pulmonary eosinophilia and AHR were not decreased in OVA-immunized recipients injected with splenocytes from OVA-primed α -GalCer-treated $J\alpha 18^{-/-}$ (iNKT cell-deficient) mice (Fig. 7A, B; group E) nor from IFN- γ -deficient mice (Fig. 7A, B; group F). These findings indicate that IFN- γ produced by iNKT cells is required for adoptive cell transfer of the inhibition of eosinophilia and airway hyper-reactivity.

To prove that iNKT cells actually were responsible for the inhibition of asthma following α -GalCer treatment, we sorted iNKT cells from the spleen of OVA-primed α -GalCer-treated mice after staining with α -GalCer-loaded

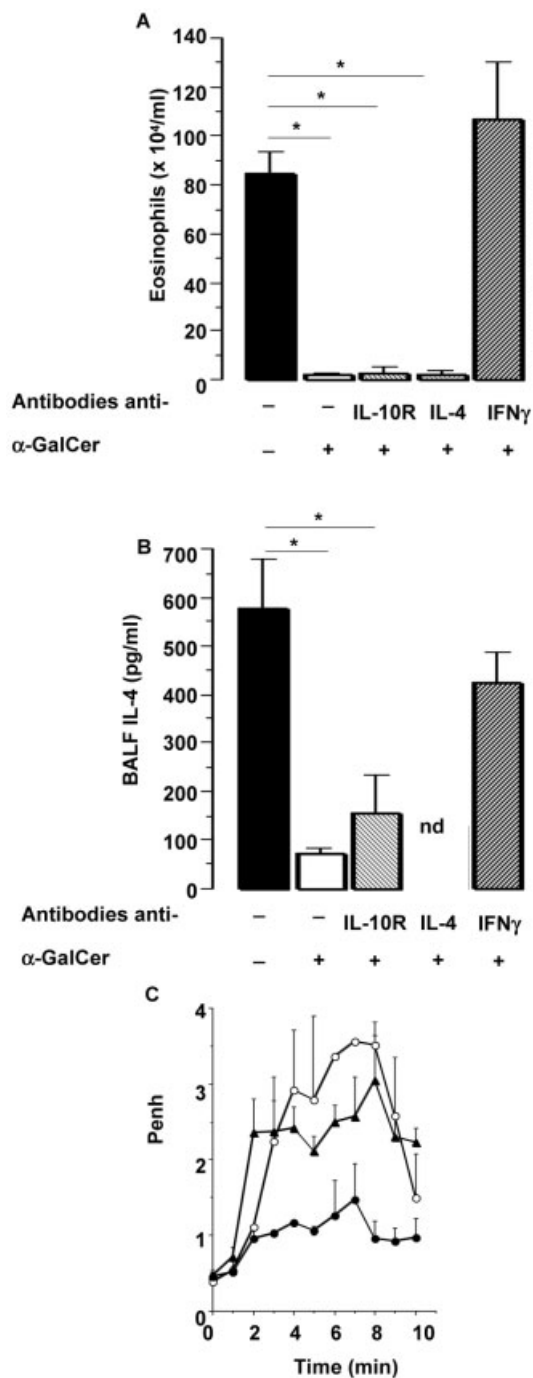


Figure 4. Anti-IFN- γ mAb treatment blocks the protective effect of α -GalCer injection. (A,B) Anti-IFN- γ treatment restores airway eosinophilia and IL-4 production in the BALF. Groups of OVA-immunized C57BL/6 mice were treated with anti-IL-4, anti-IL-10R, anti-IFN- γ mAb or control rat IgG (see Materials and methods), treated or not with α -GalCer i.v. and challenged with OVA 1 h later. At 24 h after the last challenge, BALF were collected. Eosinophil numbers (A) and IL-4 (B) were measured in BALF. (C) Anti-IFN- γ treatment restores AHR. Airway reactivity to methacholine was measured 1 day after the last challenge with OVA in mice treated (filled circles, triangles) or not (empty circle) with a single injection i.v. of α -GalCer 1 h after the second anti-IFN- γ treatment (triangles). No significant effect was observed when isotype controls were used (data not shown). Results represent means $\pm$ SEM from five to ten mice. * $p < 0.05$.

Discussion

Our findings clearly demonstrate that a single systemic (i.v.) or local (i.n.) administration of α -GalCer 1 h before the first airway challenge to OVA-sensitized mice abrogates the major asthma hallmarks such as AHR, airway eosinophilia, IL-4 and IL-5 production in BALF, and specific anti-OVA IgE antibodies. Importantly, local i.n. α -GalCer administration also effectively blocked AHR and airway eosinophilia following challenge in allergen-sensitized mice. These findings support the possible therapeutic use of α -GalCer treatment since it is effective when administered locally and protection is observed also in mice already challenged with the antigen.

Our results differ from previous reports showing that α -GalCer, co-administrated with OVA immunization, exacerbated allergic asthma probably by amplifying Th2 responses in the lung [22]. The protocol used in the paper of Kim *et al.* [22] is quite different from ours. They administered OVA i.n. on three consecutive days to lead to systemic unresponsiveness on rechallenge with the same antigen. α -GalCer was co-administered i.n. with OVA to act as an adjuvant, which broke the tolerance, thus inducing allergic asthma [22]. Other reports have already demonstrated that α -GalCer treatment could block the induction of tolerance by an IL-4-dependent mechanism or by triggering dendritic cell maturation when co-administered with the antigen [23, 24]. In contrast, here we demonstrated that a single i.n. administration of α -GalCer abrogates asthma symptoms in predisposed asthmatic mice, since these animals were immunized and challenged with OVA before the treatment. Another recent report also showed that α -GalCer treatment could inhibit allergic airway inflammation [25]. However, our paper brings new important data because we demonstrated that α -GalCer could act locally and even in mice already challenged with OVA,

CD1d/ β 2 m tetramers (CD1d/ α -GalCer tetramers) and assessed their protective effect after injection into OVA-immunized recipients. We found that antigen-induced airway eosinophilia and AHR were significantly reduced by the transfer of only 10 000 sorted iNKT cells from OVA-immunized and α -GalCer-treated mice (Fig. 7A, B; group G), proving the high efficiency of iNKT cells in suppressing both eosinophilic inflammation and airway hyper-reactivity.

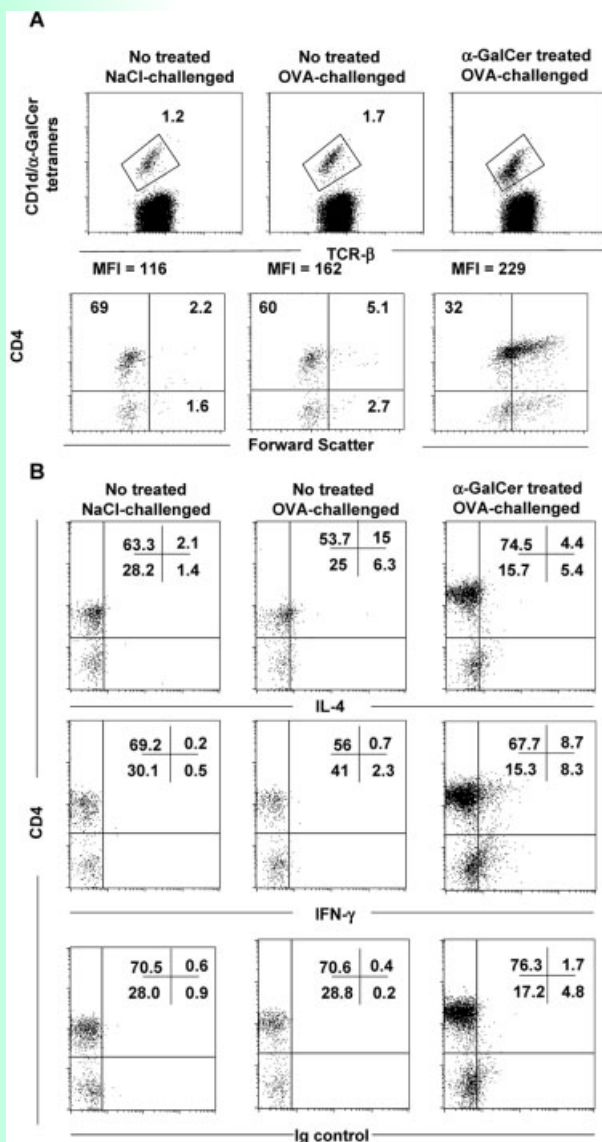


Figure 5. Effect of α -GalCer treatment on the cytokine production of lung iNKT cells. (A) Activation of lung iNKT cells increases in airways from mice treated with α -GalCer before being challenged with OVA. Mice were sensitized, treated or not with α -GalCer i.v. and challenged with OVA or NaCl 1 h later. Lung cells were isolated and stained with α -GalCer-loaded CD1d tetramers and mAb against TCR β and CD4. Upper panels show the percentage of iNKT cells among lung T cells. Lower panels show the percentage of CD4⁺ cells among gated lung iNKT cells as well as their forward scatter. The mean fluorescence intensity (MFI) for the CD4 expression among CD4⁺ iNKT cells is also indicated. Data represent one experiment out of three. (B) α -GalCer treatment shifts the cytokine production profile of pulmonary iNKT cells. Lung cells isolated from immunized and α -GalCer-treated (right) or untreated control (middle) before OVA-challenged mice were obtained 1 h after the last challenge and immediately stained with α -GalCer-loaded CD1d tetramers, anti-TCR β , anti-CD4 and anti-IL-4 or anti-IFN- γ mAb. The profile represents IL-4 (upper) and IFN- γ (low) production in gated lung iNKT cells. Data represent one experiment out of three.

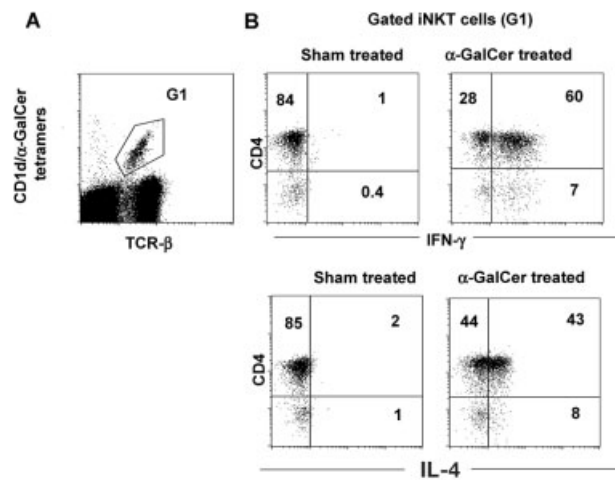


Figure 6. Cytokine production by iNKT donor splenocytes. (A,B) Representative FACS profiles showing IFN- γ and IL-4 production by iNKT splenocytes following α -GalCer injection. Mice were sensitized and treated or not with α -GalCer i.v. After 1 h, splenocytes were recovered, stained with α -GalCer-loaded CD1d tetramers, anti-TCR β , anti-CD4 mAb and anti-IFN- γ , anti-IL-4 mAb or isotype control antibody. These cytokines were then measured in gated (G1) iNKT cells. The percentage of positive cells in each quadrant is represented. Data represent one experiment out of three.

and that α -GalCer induced suppressive iNKT cells that transferred inhibition of asthma.

We have previously demonstrated that iNKT cells are implicated in the severity of asthma [6]. The mechanisms are still unclear, despite recent reports that point to the major role of endogenous antigens capable of stimulating iNKT cells [15, 26]. It is suggested that an increased expression of some self glycolipids associated with the pro-Th2 environment in the asthma model could amplify the IL-4- and IL-13-producing capacity of iNKT cells to enhance asthma. Our present findings demonstrate that we can counteract the deleterious effect of iNKT cells in allergic asthma by a single α -GalCer administration. These cells promptly produce large amounts of both IL-4 and IFN- γ in response to α -GalCer [10, 27]. However, it is noteworthy that while serum IL-4 production is very rapid (2–4 h) but transient, IFN- γ is still detected in the serum 24 h later [27]. Thus, it is possible that sustained IFN- γ production by iNKT cells induced by α -GalCer treatment, even in the airways as we demonstrated here, could be responsible for the protective effect of α -GalCer treatment. Indeed, the protective effect of α -GalCer is IFN- γ dependent since it was completely blocked by the injection of anti-IFN- γ mAb (Fig. 4). Previous results demonstrated that IFN- γ injection could inhibit asthma severity [28, 29]. The mechanism through which IFN- γ could influence elicitation of asthma in α -GalCer-treated mice is still unclear, but the protection probably results from

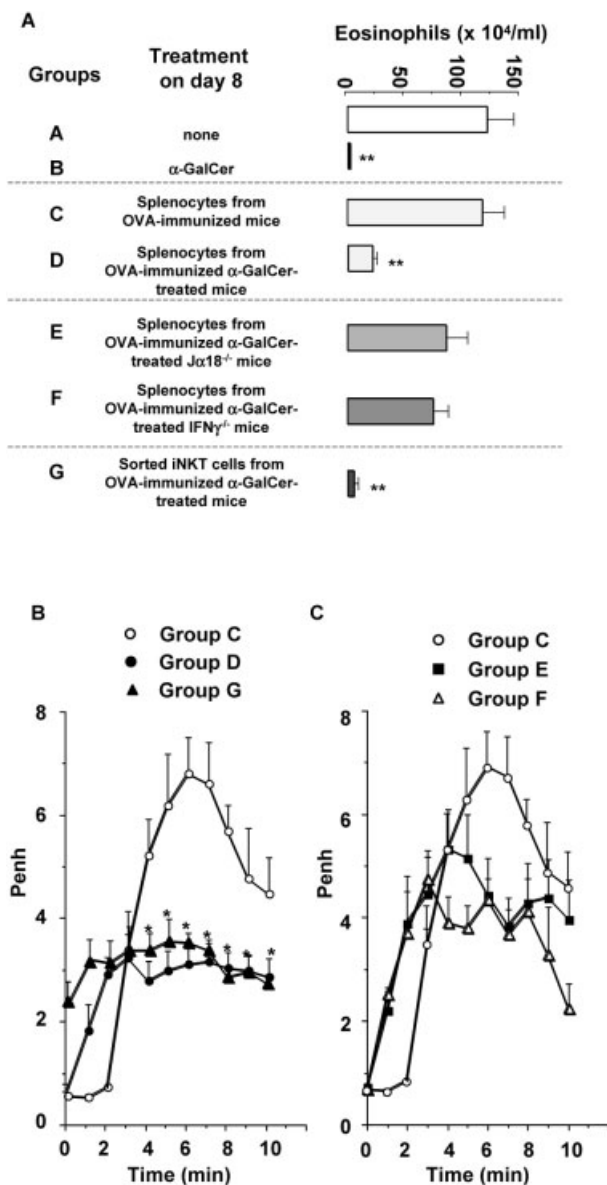


Figure 7. Transfer of iNKT splenocytes from OVA-immunized and α -GalCer-treated mice confers protection of recipient mice. (A) Donor splenocytes were obtained from wild-type (groups C and D), J α 18^{-/-} (group E) or IFN- γ ^{-/-} (group F) OVA-immunized mice that were treated (groups D–F) or not (group C) with α -GalCer i.v. These cells were transferred to wild-type OVA-immunized recipients 1 h before the first OVA challenge. Sorted CD1d/ α -GalCer tetramer-positive cells from OVA-immunized and α -GalCer i.v. treated mice (group G) were also transferred to recipient OVA-immunized mice 1 h before the first OVA challenge. Control mice were likewise sensitized and treated (group B) or not (group A) with α -GalCer and challenged with OVA. Eosinophil numbers in the BALF were measured 24 h after the last challenge. Results represent means $\pm$ SEM from five to ten mice. (B, C) Airway responsiveness is reduced after the transfer of cells from α -GalCer-treated mice. Airway reactivity to methacholine was measured 1 day after the last challenge with OVA from mice in (A). Donor splenocytes were obtained from wild-type (empty and filled circles and filled triangles), J α 18^{-/-} (filled squares) or IFN- γ ^{-/-} (empty triangles) OVA-immunized mice that had been treated (filled circles, filled and empty triangles and filled squares) or not (empty circles) with α -GalCer i.v. 1 h previously. These cells were transferred to wild-type OVA-immunized recipients 1 h before the first OVA challenge. Sorted CD1d/ α -GalCer tetramer-positive cells from OVA-immunized and α -GalCer i.v. treated mice (filled triangle) were also transferred to recipient OVA-immunized mice 1 h before the first OVA challenge. All these mice were challenged with OVA. * p < 0.05, ** p < 0.01.

immune deviation away from the harmful pulmonary Th2 response. We showed that α -GalCer treatment induces a shift in the cytokine production profile of lung iNKT cells by favoring their production of IFN- γ and decreasing their IL-4 secretion following OVA challenge.

Our experiments with adoptive transfer of purified iNKT cells from OVA-immunized and α -GalCer-treated mice reveal that, in addition to shifting the cytokine production profile of lung iNKT cells, α -GalCer treatment can also induce the emergence of protective IFN- γ -producing iNKT cells that inhibit pro-asthmatic Th2 responses. The induction of immunoregulatory IFN- γ -producing iNKT cells is supported by the fact that recipient immunized mice already contain harmful pro-Th2 iNKT cells that are counteracted by the adoptive transfer of small numbers of “protective” iNKT cells obtained from mice immunized and treated with α -

GalCer. Further studies are required to determine whether iNKT can directly or indirectly decrease Th2 or increase Th1 anti-OVA-specific immune responses, or even whether they influence other immunoregulatory cells such as CD4⁺CD25⁺ or dendritic cells that are implicated in controlling asthma severity [30–32].

In summary, we demonstrated that a single i.n. α -GalCer administration abrogates AHR and airway eosinophilia in mice predisposed to be asthmatic. The treatment counteracts the positive influence of iNKT cells on asthma pathology [6, 7] by shifting their cytokine production profile towards a preferential IFN- γ production, and by inducing the emergence of an immunoregulatory IFN- γ -producing iNKT cell population. The effectiveness of this population to inhibit asthma elicitation was confirmed using transfer of small numbers of sorted iNKT cells. Our findings strengthen the potential applicability of α -GalCer treatment, which can inhibit asthma severity in sensitized animals that otherwise mount several hallmarks of the disease. Our results thus raise the possibility that local α -GalCer treatment, acting specifically on iNKT cells, might provide novel therapeutic strategies to reduce allergic asthma.

Materials and methods

Animals

Male C57BL/6 wild-type, $J\alpha 18^{-/-}$ and $IFN-\gamma^{-/-}$ (backcrossed at least ten times with C57BL/6 mice) [33, 34] mice at 6–8 wk of age were bred in our own facilities. BALB/c mice were purchased from Janvier (France). All mice were kept in well-controlled animal housing facilities and had free access to tap water and pellet food. Animal experiments were performed according to the French institutional committee.

Sensitization and airway challenge

Animals were sensitized by an i.p. injection of 100 μ g OVA (Sigma) emulsified in 1.6 mg alum (Merck). After 8 days, mice were exposed to i.n. administered antigen (50 μ g/mouse) or sterile saline on three consecutive days (D8, D9 and D10). At 24 h after the last challenge (day 11), airway function was measured as described below, followed by collection of samples for further analyses.

In vivo treatment

In some experiments, OVA-sensitized mice received a single administration of 2 μ g α -GalCer (Kirin, Japan) i.v. or i.n. 1 h before the first OVA challenge or 24 h after the first challenge. Control mice received the vehicle (polysorbate-20, 0.5%). In other experiments, OVA-sensitized mice were treated i.p. with 500 μ g anti- $IFN-\gamma$ (clone R4-6A2), anti-IL-4 (clone 11B11), anti-IL-10R (clone 1B1) mAb or control rat IgG (Sigma-Aldrich) at 24 h and 1 h before the α -GalCer or vehicle injection. After 1 h, mice were challenged as described above.

Adoptive cell transfer

Spleen cells were harvested from wild-type or $IFN-\gamma^{-/-}$ mice previously OVA-sensitized and treated or not with 2 μ g α -GalCer i.v. 1 h before. These cells were injected (5×10^6 /mouse) i.v. into recipient wild-type OVA-immunized mice on day 8. After 1 h, recipient mice received their first challenge. In some experiments, donor cells were electronically sorted as $TCR\beta^+ CD1d/\alpha$ -GalCer tetramer $^+$ iNKT cells [35] also from spleens of mice previously OVA-sensitized and treated with 2 μ g α -GalCer i.v.

Sorting of iNKT cells

Splenocytes were depleted of $CD8^+$ T cells, $CD62L^+$, and $CD19^+$ B cells after labeling with the corresponding mAb (clone 53.67, clone Mel14, clone 1D3, respectively) using anti-rat Ig-coated magnetic beads (Dyna) [36]. The enriched negatively selected NKT spleen cells were stained with anti- $TCR\beta$ antibody and $CD1d/\alpha$ -GalCer tetramers. $TCR\beta^+ CD1d/\alpha$ -GalCer tetramer $^+$ iNKT cells were sorted using a FACSVantage (BD PharmMingen), and 10 000 purified cells were injected i.v. into immunized recipients. After re-analysis, 99% of the cells were found to be $CD1d/\alpha$ -GalCer tetramer positive.

Determination of AHR

Airway responsiveness was assessed as described [6]. Briefly, 24 h after the last challenge, conscious mice were placed into a whole-body plethysmograph (EMKA technologies, Paris), and 35.5, 75, 150 or 300 mM methacholine (Sigma-Aldrich) was delivered for 60 s. Respiratory parameters were then measured for 10 min. Airflow obstruction was expressed as enhanced pause (Penh) and calculated as: $Penh = [Te \text{ (expiratory time)}/Tr \text{ (relaxation time)}] - 1 \times [Pef \text{ (peak expiratory flow)}/Pif \text{ (peak inspiratory flow)}]$. The values of Penh expressed per minute were averaged from three determinations recorded every 20 s. The value of the peak Penh is calculated as the mean of three Penh values obtained at 3, 4 and 5 min after methacholine.

Collection and analysis of BALF

Immediately after assessment of AHR, mice were deeply anesthetized by i.p. injection of urethane (15 mg/10 g body weight) (Sigma-Aldrich), blood was collected, and the resulting serum stored. Airways were washed twice with saline, and differential cell counts were determined in BALF after May-Grünwald/Giemsa (Merck) staining of cells on cytopsin slides. Per slide, 200 cells were counted. Aliquots of BALF supernatants were stored for cytokine measurement. Cells were stained for flow cytometric analysis.

Histopathologic analysis

Immediately after BALF collection, the lungs were infused with 10% formalin and embedded in paraffin. Lung sections were cut (5 μ m thick) and stained with hematoxylin/eosin (HE) for optic microscopy examination.

Lung mononuclear cell preparation

The aorta and the inferior vena cava were sectioned and the lungs were perfused with PBS. The lobes of the lungs were sliced into small cubes and then incubated for 45 min in 2 mL RPMI 1640 solution containing DNase (1 mg/mL) (Roche Diagnostics), collagenase (2 mg/mL) and brefeldine (10 μ g/mL) (Sigma-Aldrich). Lung mononuclear cells (MNC) were separated by centrifugation on discontinuous Percoll (Amersham Biosciences) gradients (35%/70%).

Measurement of cytokines and anti-OVA IgE

The levels of IL-4, IL-5 and $IFN-\gamma$ in BALF and OVA-specific IgE serum levels were assessed by enzyme-linked immunosorbent assay (ELISA) [6]. OVA-specific IgE levels of samples were related to an internal standard from pooled sera of hyper-immunized BALB/c mice. Data were expressed as IgE index calculated as follows: $IgE \text{ index} = [OD \text{ sample} - OD \text{ buffer only}]/[OD \text{ positive control} - OD \text{ buffer only}]$.

Flow cytometry

Splenocytes and lung MNC were pre-incubated with monoclonal antibodies against Fc γ receptor (2.4G2 culture super-

nantant), washed and incubated with CD1d/ α -GalCer tetramer-allophycocyanin or control tetramers, as described [6]. Tetramers were prepared in our laboratory from the mCD1d/m β 2 m expression vector [35]. After washing, cells were further incubated with appropriate dilutions of anti-CD4-PerCP-Cy-5.5 (clone RM4-5) and anti-TCR β -FITC (clone H57-597) antibodies, all purchased from BD PharMingen. For intracellular staining, cells were then fixed with 4% paraformaldehyde, washed and permeabilized with 0.5% saponin (Sigma) and further incubated with anti-IL-4-PE, anti-IFN- γ -PE or isotype control antibody (PharMingen). The cells were washed and analyzed on a FACSCalibur (Becton Dickinson) using CellQuest software (PharMingen).

Statistical analysis

Nonparametric Mann-Whitney test was used to calculate significance levels for all measurements. Values of $p < 0.05$ were considered statistically significant.

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