# α-Galactosylceramide treatment before allergen sensitization promotes iNKT cell–mediated induction of Treg cells, preventing Th2 cell responses in murine asthma

Received for publication, August 17, 2018, and in revised form, January 18, 2019 Published, Papers in Press, February 11, 2019, DOI 10.1074/jbc.RA118.005418

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**Asthma is a common inflammatory pulmonary disorder involving a diverse array of immune cells such as proinflammatory T helper 2 (Th2) cells. We recently reported that intraperitoneal injection of α-galactosylceramide (α-GalCer) can stimulate the lung invariant natural killer T (iNKT) cells and does not lead to airway inflammation in WT mice. Other studies indicate that iNKT cells play an important role in inducing regulatory T cells (Treg cells) and peripheral tolerance. Using iNKT cell–knockout mice, functional inactivation of Treg cells, and co-culture experiments in murine asthma models, we investigated the immunoregulatory effects of α-GalCer treatment before allergen sensitization on Th2 cell responses. We also studied whether α-GalCer's effects require lung Treg cells induced by activated iNKT cells. Our results disclosed that intraperitoneal administration of α-GalCer before allergen sensitization could promote the expansion and suppressive activity of lung $CD4^+FoxP3^+$ Treg cells. These effects were accompanied by down-regulated Th2 cell responses and decreased immunogenic maturation of lung dendritic cells in WT mice. However, these changes were absent in $CD1d^{-/-}$ mice immunized and challenged with ovalbumin or house dust mites, indicating that the effects of α-GalCer on Treg cells mainly require iNKT cells. Moreover, functional inactivation of Treg cells could reverse the inhibitory ability of this α-GalCer therapy on Th2 cell responses in a murine asthma model. Our findings indicate that intraperitoneal administration of α-GalCer before the development of asthma symptoms induces the generation of lung Treg cells via iNKT cells and may provide a potential therapeutic strategy to prevent allergic asthma.**

Bronchial asthma is a common chronic inflammatory disorder of the respiratory tracts, and a diverse array of cell types are involved in the development of airway inflammation of asthma. The predominant paradigm is that T helper 2 (Th2)[2] cells drive inflammatory responses through the release of Th2 cell cytokines, including IL-4, IL-5, and IL-13, which result in airway inflammation, B cell isotype shifting, mucus overproduction, and airway hyper-responsiveness (AHR) (1). The development of Th2 cells is modulated by regulatory T (Treg) cells, which can maintain homeostasis and inhibit aberrant immunity (2). Previous data have shown that Treg cells have a reduced number and impaired function in patients with asthma, and thereby asthma is a condition of dysregulated immune responses (3, 4). Several reports indicated that $CD4^+CD25^+$ Treg cells adoptively transferred into immunized mice before allergen challenge can inhibit the initiation of asthma (5, 6), whereas adoptive transfer of $CD4^+CD25^+$ Treg cells after acute exacerbation of asthma can reduce established allergic airway inflammation (7). These studies raise some important evidence that Treg cells can prevent initiation and progression of asthma. As a result, the induction of lung Treg cells may be a potential therapeutic protocol for the treatment of asthma (8).

Invariant natural killer T (iNKT) cells constitute a distinct and relatively minor T lymphocyte subset, which expresses a semi-invariant T-cell receptor (TCR) that can recognize glycolipid antigens presented by the nonpolymorphic major histocompatibility complex-like molecule CD1d (9, 10). α-Galactosylceramide (α-GalCer), a derivative from a marine sponge, can specifically and strongly activate iNKT cells to result in the secretion of both Th1 and Th2 cell cytokines, such as IFN-γ and IL-4, which probably account for their immunomodulatory functions (11–13). Recent studies have shown that iNKT cells activated by α-GalCer can prevent experimental autoimmune disorders such as type 1 diabetes and myasthenia gravis through induction of $CD4^+CD25^+$ Treg cells (14, 15). Interestingly, Ronet *et al.* (16) reported that iNKT cells play a key role in the initiation of the lethal ileitis after *Toxoplasma gondii* infection, but activation of iNKT cells by intraperitoneal administration of α-GalCer before *T. gondii* infection can enhance the frequency of IL-10–secreting Treg cells to control the inflamma-

This work was supported by the National Natural Science Foundation of China Grant 81770036. The authors declare that they have no conflicts of interest with the contents of this article.

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[2] The abbreviations used are: Th2, T helper 2; iNKT cell, invariant natural killer cell; OVA, ovalbumin; Treg cell, regulatory T cell; LDC, lung dendritic cell; HDM, house dust mite; AHR, air hyper-responsiveness; TCR, T-cell receptor; α-GalCer, α-galactosylceramide; BMDC, bone marrow-derived dendritic cell; PBS, phosphate-buffered saline; Cdyn, dynamic compliance; RL, airway resistance; Mch, methacholine; BALF, bronchoalveolar lavage fluid; H&E, hematoxylin-eosin; PAS, periodic acid–Schiff; MHC, major histocompatibility complex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MNC, mononuclear cell; Ab, antibody.



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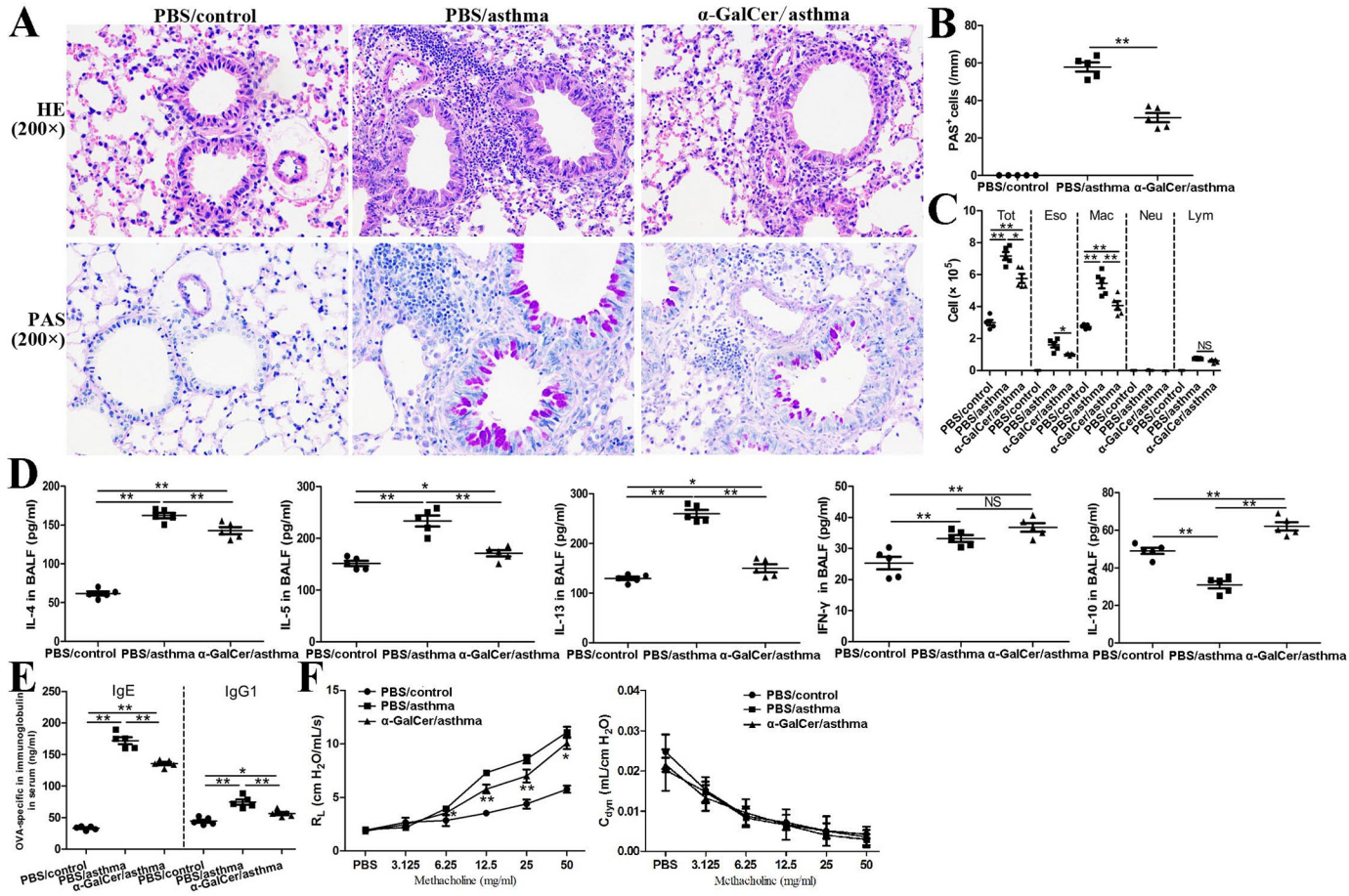



**Figure 1. α-GalCer treatment before OVA sensitization alleviates Th2 cell responses in an OVA-induced murine asthma model.** WT mice, intraperitoneally treated with α-GalCer or PBS before OVA sensitization, were sacrificed 24 h after the final OVA challenge. *A,* histopathologic analysis of lung tissue H&E and PAS staining. *B,* lung goblet cell hyperplasia is indicated as the number of PAS-positive cells per unit of length (mm) of the basement membrane. *C,* total and differential cell counting in BALF. *Tot,* total cell counts; *Eos*, eosinophils; *Mac*, macrophages; *Neu*, neutrophils; and *Lym*, lymphocytes. *D,* concentrations of IL-4, IL-5, IL-10, IL-13, and IFN-γ in BALFs were analyzed by ELISA. *E,* OVA-specific IgE and IgG1 concentrations in the serum. Data are expressed as the means ± S.D. of three independent experiments ($n = 5$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$; *NS*, not significant. *F,* airway response to increasing concentrations of methacholine was examined. Significant differences between asthmatic mice administered α-GalCer or PBS are shown as *, $p < 0.05$, and **, $p < 0.01$.

tory intestinal disorder. These data suggested that iNKT cells activated by α-GalCer play an important role in inducing the development of Treg cells and peripheral tolerance (17). In our previous study, we showed that intraperitoneal injection of α-GalCer has the capability to stimulate iNKT cells, but activation of iNKT cells alone does not lead to airway inflammation in wildtype (WT) mice in the absence of ovalbumin (OVA) sensitization and challenge (18). Moreover, our previous data found that immature bone marrow-derived dendritic cells (BMDCs) adopt immunogenic maturation status upon interplay with iNKT cells in the presence of OVA, but immature BMDCs undergo a tolerogenic maturation condition upon interplay with iNKT cells alone without OVA (19). Thus, it was hypothesized that α-GalCer treatment before antigen sensitization may prevent Th2 cell responses through the induction of lung Treg cells by iNKT cells in asthmatics.

To elucidate this hypothesis, we have investigated the immunoregulatory effects of α-GalCer treatment before allergen sensitization on Th2 cell responses and maturation status of lung dendritic cells (LDCs), and we further analyzed whether the influence of this α-GalCer therapy requires lung Treg cells induced by iNKT cells using iNKT cell–knockout mice, specific functional inactivation of Treg cells, and co-culture experiments in murine asthma models. Our findings demonstrated that α-GalCer treatment before allergen sensitization promotes iNKT cell-mediated induction of Treg cells, preventing Th2 cell responses in murine asthma models.

## Results

### α-GalCer treatment before allergen sensitization alleviates Th2 cell responses in an OVA- or HDM-induced murine asthma model

A recent study has shown that activation of iNKT cells by intraperitoneal administration of α-GalCer before *T. gondii* infection can enhance the frequency of IL-10–secreting Treg cells to control the inflammatory intestinal disorder, although iNKT cells play a key role in the initiation of the lethal ileitis after *T. gondii* infection (16). Thus, we sought to determine the influence of α-GalCer treatment before allergen sensitization on Th2 cell responses of asthma. As shown in Fig. 1, α-GalCer treatment before OVA sensitization markedly reduced inflammatory cell infiltration in the respiratory tract (Fig. 1*A*), PAS-positive goblet cells in the airway epithelium (Fig. 1, *A* and *B*),

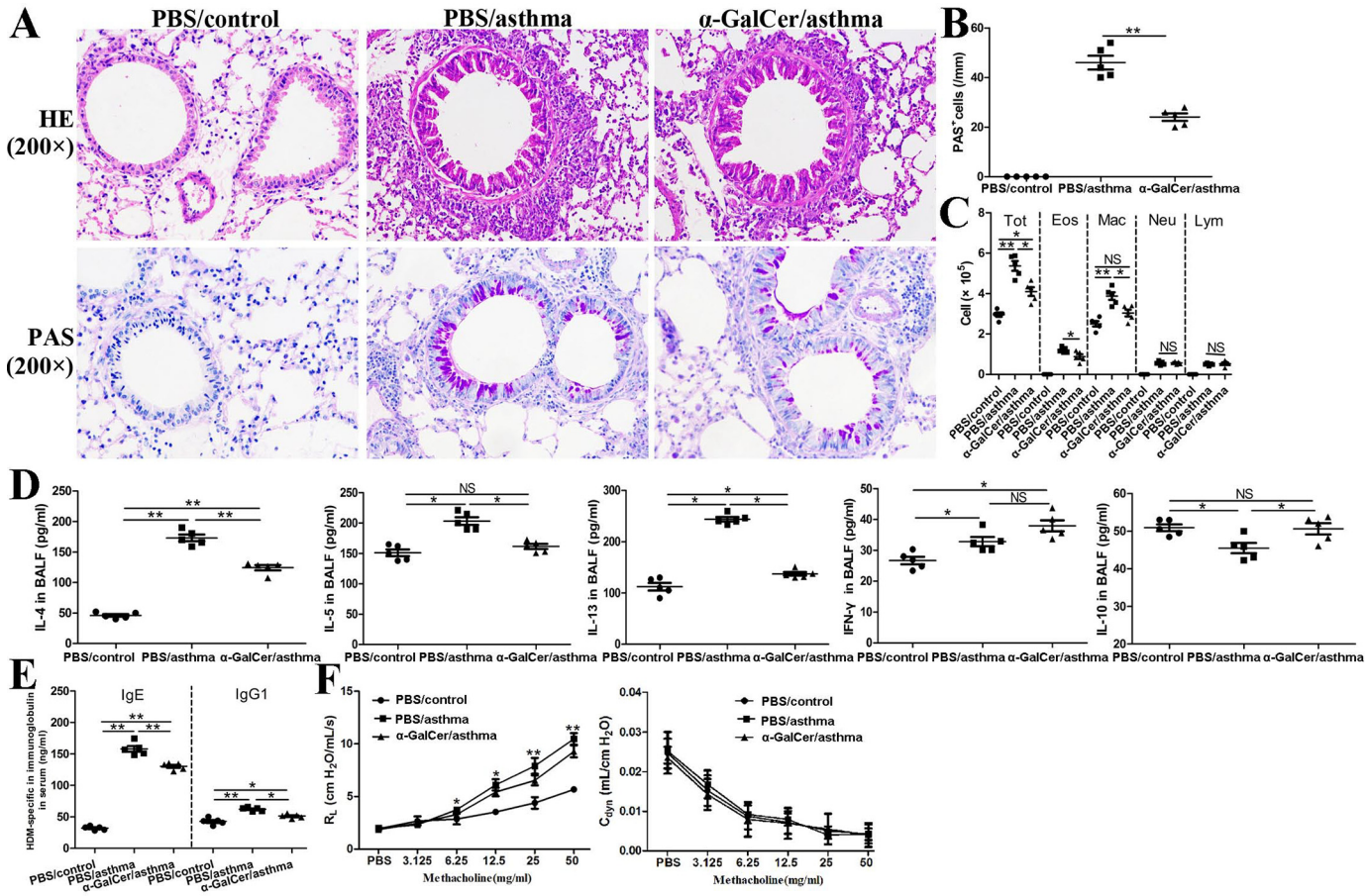



**Figure 2. α-GalCer treatment before HDM sensitization suppresses Th2 cell responses in HDM-induced murine asthma model.** WT mice, intraperitoneally treated with α-GalCer or PBS before HDM sensitization, were sacrificed 3 days after the final HDM challenge. *A,* histopathological analysis of lung tissue H&E and PAS staining. *B,* lung goblet cell hyperplasia is indicated as the number of PAS-positive cells per unit of length (mm) of the basement membrane. *C,* total and differential cell counting in BALF of mice. *Tot,* total cell counts; *Eos,* eosinophils; *Mac,* macrophages; *Neu,* neutrophils; and *Lym,* lymphocytes. *D,* BALF was collected 24 h after the final challenge, and the concentrations of IL-4, IL-5, IL-10, IL-13, and IFN-γ were analyzed by ELISA. *E,* HDM-specific IgE and IgG1 concentrations in the serum. Data are expressed as the means ± S.D. of three independent experiments ($n = 5$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$; *NS*, not significant. *F,* airway response to increasing concentrations of methacholine was examined. Significant differences between asthmatic mice treated with α-GalCer or PBS are shown as *, $p < 0.05$, and **, $p < 0.01$.

and the number of total cells, eosinophils, and macrophages in the BALF (Fig. 1*C*), as well as the levels of IL-4, IL-5, and IL-13 in the BALF (Fig. 1*D*) in the OVA-induced asthmatic mouse, compared with PBS administration ($p < 0.05$ or $p < 0.01$). However, the level of IL-10 in the BALF was significantly increased in the OVA-induced asthmatic mouse with α-GalCer treatment before OVA sensitization, compared with PBS administration (Fig. 1*D*) ($p < 0.05$). Furthermore, the concentrations of OVA-specific IgE and IgG1 in the serum were significantly reduced in OVA-induced asthmatic WT mice treated with α-GalCer before OVA sensitization, compared with PBS treatment (Fig. 1*E*) ($p < 0.01$). In addition, AHR was assessed by direct measurement of RL and Cdyn to increasing levels of Mch, ranging from 3.12 to 50 mg/ml in PBS. Our data showed that weakened RL to Mch was observed in OVA-induced asthmatic mice with α-GalCer treatment before OVA sensitization, compared with PBS treatment (Fig. 1*F*) ($p < 0.05$ or $p < 0.01$). By contrast, no difference was found in Cdyn between these two groups (Fig. 1*F*) ($p > 0.05$). Collectively, these data suggested that α-GalCer treatment before OVA sensitization can alleviate Th2 cell responses in an OVA-induced murine asthma model.

The HDM is one of the most common allergens in the living environment of asthmatic patients (20). Thus, we sought to determine the influence of α-GalCer treatment before HDM sensitization on Th2 cell responses of asthma. As shown in Fig. 2, administration of α-GalCer before HDM sensitization strongly attenuated inflammatory cell infiltration (Fig. 2*A*), mucus-secreting cells in the airways (Fig. 2, *A* and *B*), the number of total cells, eosinophils, and macrophages in the BALF (Fig. 2*C*), the release of cytokines, including IL-4, IL-5, and IL-13 in the BALF (Fig. 2*D*), and the levels of HDM-specific IgE and IgG1 in the serum (Fig. 2*E*) ($p < 0.05$ or $p < 0.01$). However, the level of IL-10 in the BALF was markedly elevated in the HDM-induced asthmatic mice with α-GalCer treatment before HDM sensitization, compared with PBS treatment (Fig. 2*D*) ($p < 0.05$). In addition, significantly alleviated RL to Mch was observed in HDM-induced asthmatic mice with α-GalCer treatment before HDM sensitization, compared with PBS treatment (Fig. 2*F*) ($p < 0.05$ or $p < 0.01$). However, no difference was observed in Cdyn between the two groups (Fig. 2*F*) ($p > 0.05$). Collectively, these data suggested that α-GalCer treatment before HDM sensitiza-

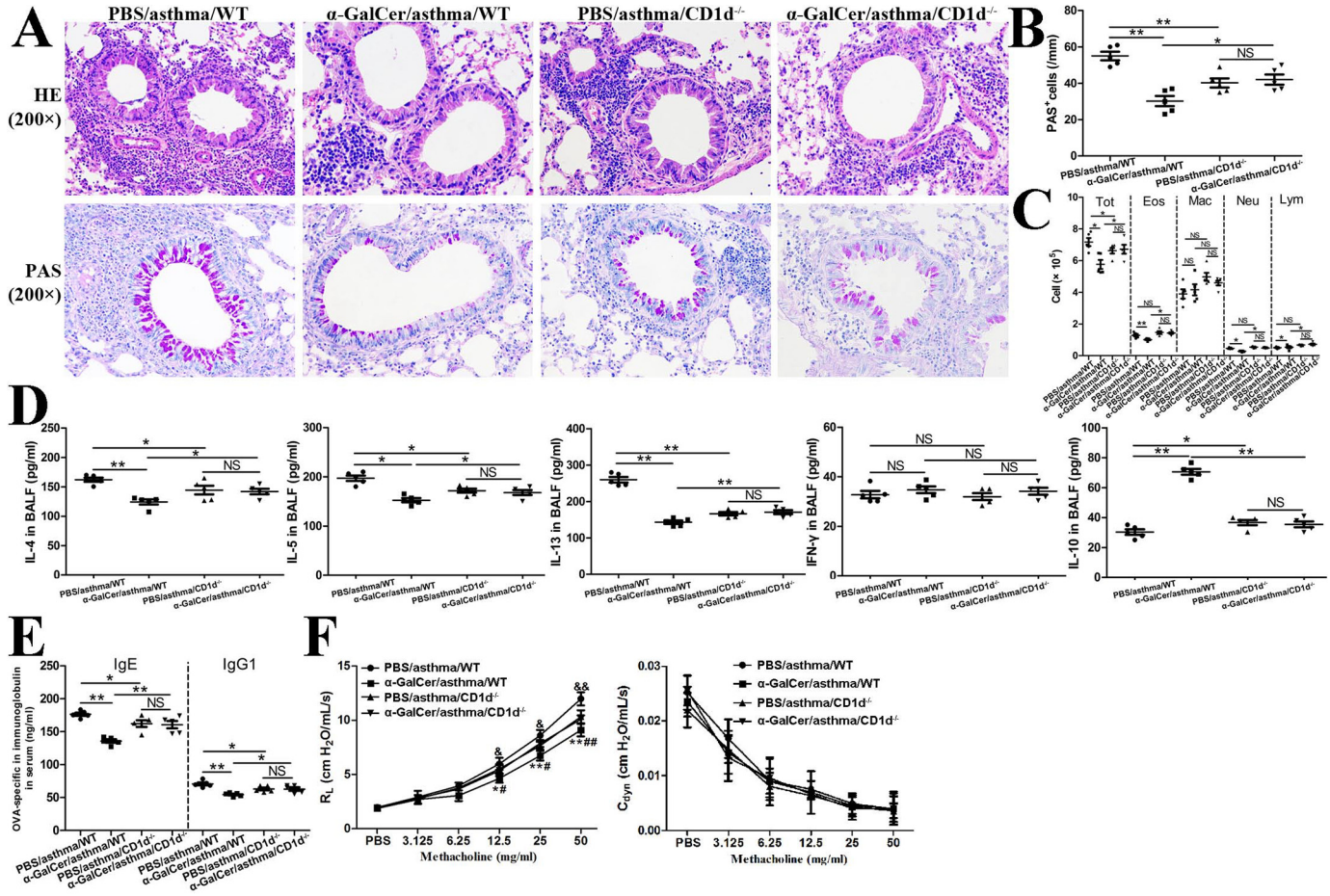



**Figure 3. α-GalCer–mediated inhibition of Th2 cell responses requires iNKT cells in asthmatic mice.** WT mice and CD1d$^{-/-}$ mice, immunized and challenged with OVA, were intraperitoneally injected with α-GalCer or PBS 24 h before the first sensitization. *A,* histopathological analysis of lung tissue H&E and PAS staining. *B,* lung goblet cell hyperplasia is indicated as the number of PAS-positive cells per unit of length (mm) of the basement membrane. *C,* total and differential cell counting in BALF of mice. *Tot*, total cell counts; *Eos*, eosinophils; *Mac*, macrophages; *Neu*, neutrophils; and *Lym*, lymphocytes. *D,* concentrations of IL-4, IL-5, IL-10, IL-13, and IFN-γ in BALF from mice analyzed by ELISA. *E,* OVA-specific IgE and IgG1 concentrations in the serum. Data are expressed as the means ± S.D. of three independent experiments ($n = 5$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$; *NS*, not significant. *F,* airway response to increasing concentrations of methacholine was examined. Significant differences between WT mice and CD1d$^{-/-}$ mice treated with α-GalCer are shown as *, $p < 0.05$, and **, $p < 0.01$.

tion can suppress Th2 cell responses in HDM-induced asthmatic mice.

### α-GalCer–mediated inhibition of Th2 cell responses requires iNKT cells in asthmatic mice

α-GalCer, a glycolipid ligand originally derived from marine sponges, presented by the CD1d molecule, can stimulate iNKT cells and is widely used to determine the role of iNKT cells in multiple diseases, such as asthma, tumors, and autoimmunity (21–23). We sought to determine whether the inhibition of Th2 cell responses mediated by α-GalCer required iNKT cells in asthmatic mice. As outlined in Fig. 3, α-GalCer treatment before OVA sensitization markedly reduced inflammatory cell infiltration (Fig. 3*A*), mucus-secreting cells in the airways (Fig. 3, *A* and *B*), the number of total cells, eosinophils, and macrophages in the BALF (Fig. 3*C*), the release of such cytokines as IL-4, IL-5, and IL-13 in the BALF (Fig. 3*D*), as well as the levels of OVA-specific IgE and IgG1 in the serum (Fig. 3*E*) in WT mice sensitized and challenged with OVA as compared with CD1d$^{-/-}$ mice sensitized and challenged with OVA ($p < 0.05$ or $p < 0.01$). However, α-GalCer treatment before OVA sensitization strongly enhanced the level of IL-10 in the BALF from WT mice sensitized and challenged with OVA, compared with CD1d$^{-/-}$ mice sensitized and challenged with OVA (Fig. 3*D*) ($p < 0.01$). In addition, α-GalCer treatment before OVA sensitization markedly attenuated RL to Mch in WT mice sensitized and challenged with OVA, compared with CD1d$^{-/-}$ mice sensitized and challenged with OVA (Fig. 3*F*) ($p < 0.05$ or $p < 0.01$). However, no difference was observed in Cdyn between the two groups (Fig. 3*F*) ($p > 0.05$). Interestingly, no difference was found in airway inflammation and AHR between CD1d$^{-/-}$ asthmatic mice treated with α-GalCer and PBS (Fig. 3, *A–F*) ($p > 0.05$). Collectively, these data showed that α-GalCer–mediated inhibition of Th2 cell responses requires iNKT cells in asthmatic mice.

### α-GalCer treatment before allergen sensitization down-regulates surface maturation markers and proinflammatory cytokine production of LDCs in an OVA-induced murine asthma model

The phenotypic maturation of DCs is mainly based on surface markers, including co-stimulatory molecules CD40, CD80,

and CD86 as well as MHC II molecules (24, 25). To assess the modulatory effect of α-GalCer treatment before OVA sensitization on the phenotypic maturation of LDCs, the expression of surface maturation markers of LDCs was analyzed in asthmatic mice intraperitoneally treated with α-GalCer treatment before OVA sensitization. Our results indicated that LDCs from asthmatic mice treated with α-GalCer displayed significantly decreased expression of CD86, CD80, CD40, and MHC II, compared with PBS treatment (Fig. 4, *A* and *B*) ($p < 0.05$ or $p < 0.01$), but exhibited significantly elevated expression of CD86, CD80, CD40, and MHC II, compared with LDCs from WT mice unimmunized and unchallenged with OVA (Fig. 4, *A* and *B*) ($p < 0.05$ or $p < 0.01$).

Functionally mature DCs can secrete large quantities of proinflammatory cytokines like IL-12, IL-6, and TNF-α (25), but they do not secrete or they secrete negligible amounts of tolerogenic cytokine IL-10 (26). To determine the modulatory effect of α-GalCer treatment before OVA sensitization on the functional maturation of LDCs, LDCs sorted from WT asthmatic mice intraperitoneally treated with α-GalCer treatment before OVA sensitization were cultured for 72 h, and culture supernatants were harvested to measure the spontaneous secretion of IL-12p70, IL-6, TNF-α, and IL-10. As shown in Fig. 4*C*, the purity of LDCs, *i.e.* $CD11c^+$ $F4/80^-$ cells, was over 99%. Our results showed that the concentrations of IL-12p70, IL-6, and TNF-α were markedly lower in the culture supernatants of LDCs from asthmatic mice treated with α-GalCer before OVA sensitization, compared with PBS treatment (Fig. 4*D*) ($p < 0.05$ or $p < 0.01$). In contrast, the concentration of IL-10 was significantly higher in the culture supernatant of LDCs from asthmatic mice treated with α-GalCer before OVA sensitization, compared with PBS treatment (Fig. 4*D*) ($p < 0.05$). However, the levels of IL-12p70, IL-6, TNF-α, and IL-10 in the culture supernatant of LDCs from WT mice unimmunized and unchallenged with OVA were negligible (Fig. 4*D*). Furthermore, our findings showed that $CD11c^+$ cells (*red*) in the lung tissues could be detected by immunofluorescence in OVA-induced asthmatic mice treated with α-GalCer or PBS before OVA sensitization and WT mice treated with α-GalCer or PBS (Fig. 4, *E–G*). However, $CD11c^+TNF\alpha^+$ cells, $CD11c^+IL\text{-}12^+$ cells, and $CD11c^+IL\text{-}10^+$ cells (*yellow*) in the lung tissues could be found in OVA-induced asthmatic mice treated with α-GalCer or PBS before OVA sensitization, but not in WT mice treated with α-GalCer or PBS (Fig. 4, *E–G*). In addition, $CD11c^+TNF\alpha^+$ cell and $CD11c^+IL\text{-}12^+$ cell infiltration was reduced, but $CD11c^+IL\text{-}10^+$ cell infiltration was increased in the lung tissues from asthmatic mice treated with α-GalCer before OVA sensitization (Fig. 4, *E–G*). Taken together, these results indicated that α-GalCer injection before OVA sensitization down-regulates immunogenic maturation of LDCs in an OVA-induced murine asthma model.

### α-GalCer treatment before allergen sensitization can enhance the expansion and suppressive activity of lung $CD4^+FoxP3^+$ Treg cells in an OVA-induced murine asthma model

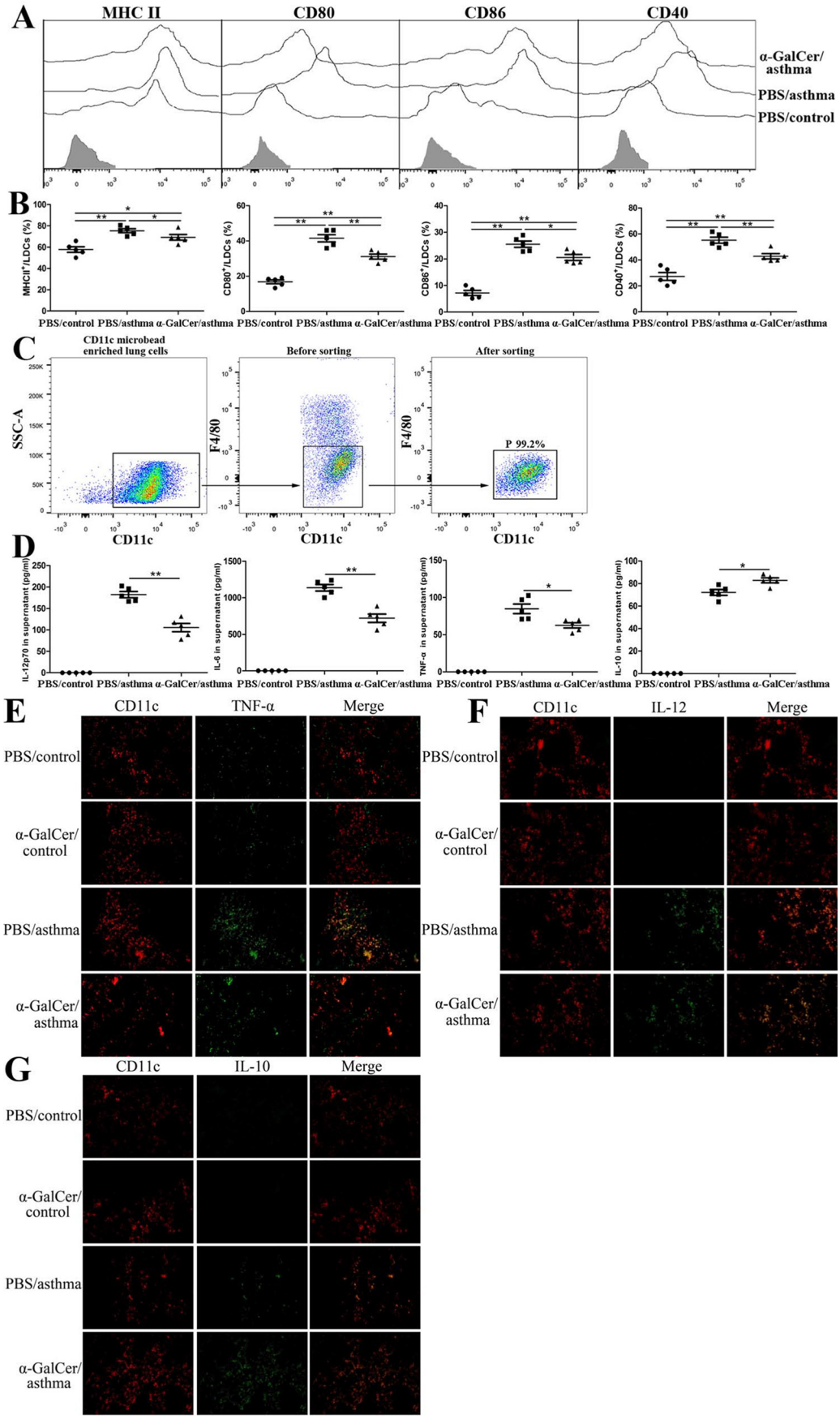
Previous data have shown that Treg cells have a reduced number and impaired function in patients with asthma (3, 4), and the induction of lung Treg cells may be a potential therapeutic protocol for the treatment of asthma (8). Thus, we assessed whether α-GalCer treatment before allergen sensitization could affect the expansion and function of $CD4^+$ $FoxP3^+$ Treg cells in asthma. As shown in Fig. 5, the number of lung $CD4^+FoxP3^+$ Treg cells in OVA-induced asthmatic WT mice with α-GalCer treatment before OVA sensitization was significantly elevated, compared with PBS treatment (Fig. 5, *A* and *B*) ($p < 0.05$). In addition, the IL-10 production in culture supernatants of Treg cells, whose sorted purity was ~98% (Fig. 5*C*), from OVA-induced asthmatic WT mice with α-GalCer treatment before OVA sensitization was markedly elevated, compared with PBS treatment (Fig. 5*D*) ($p < 0.01$). To further determine the suppressive activity of Treg cells *in vitro*, $CD4^+CD25^-$ T cells were co-cultured with the indicated ratio of $CD4^+CD25^+$ Treg cells for 3 days, and then culture supernatants were collected to detect the levels of IL-4 and IFN-γ by ELISA. As outlined in Fig. 5, the suppressive activity of Treg cells on cytokine production was gradually increased with increasing numbers of Treg cells. However, the lung Treg cells from asthmatic WT mice treated with α-GalCer before OVA sensitization were more effective in suppressing cytokine secretion of $CD4^+CD25^-$ T cells, compared with PBS treatment (Fig. 5*E*) ($p < 0.05$ or $p < 0.01$). Collectively, our findings showed that α-GalCer treatment before OVA sensitization can promote the expansion and suppressive activity of Treg cells in an OVA-induced murine asthma model.

### iNKT cells contribute to the expansion and suppressive activity of Treg cells induced by α-GalCer treatment in asthmatic mice

Recent reports have shown that iNKT cells activated by α-GalCer play an important role in inducing the development of Treg cells and peripheral tolerance (14, 15). Therefore, we investigated whether iNKT cells are responsible for the enhanced number and inhibitory activity of Treg cells in asthmatic mice treated with α-GalCer before allergen sensitization. As outlined in Fig. 6, the number of lung $CD4^+FoxP3^+$ Treg cells (Fig. 6, *A* and *B*), the level of IL-10 in culture supernatant of Treg cells (Fig. 6*C*), as well as the suppressive capacity of Treg cells on cytokine production of $CD4^+$ $CD25^-$ T cells (Fig. 6*D*) were significantly increased in OVA-immunized and OVA-challenged WT mice with α-GalCer treatment before OVA sensitization, compared with OVA-immunized and OVA-challenged $CD1d^{-/-}$ mice with same treatment ($p < 0.05$ or $p < 0.01$). Interestingly, no difference was observed in the number and suppressive capacity of Treg cells among OVA-immunized and OVA-challenged $CD1d^{-/-}$ mice with α-GalCer or PBS treatment, and OVA-immunized and OVA-challenged WT mice with PBS treatment (Fig. 6, *A–D*) ($p > 0.05$). Taken together, these data suggested that iNKT cells contribute to the expansion and suppressive activity of Treg cells induced by α-GalCer treatment in asthmatic mice.

### Treg cells contribute to the inhibitory effects of α-GalCer treatment on Th2 cell responses in an OVA-induced murine asthma model

Controlling the function of Treg cells can modulate the induction of Th2 cells, which plays a vital role in suppressing aberrant immune responses and maintaining homeostasis (2).


A
MHC II
CD80
CD86
CD40
α-GalCer/ asthma
PBS/asthma
PBS/control
B
MHCII+/LDCs (%)
CD80+/LDCs (%)
CD86+/LDCs (%)
CD40+/LDCs (%)
PBS/control PBS/asthma α-GalCer/asthma
C
CD11c microbead enriched lung cells
Before sorting
After sorting
SSC-A
F4/80
CD11c
P 99.2%
D
IL-12p70 in supernatant (pg/ml)
IL-6 in supernatant (pg/ml)
TNF-α in supernatant (pg/ml)
IL-10 in supernatant (pg/ml)
E
CD11c
TNF-α
Merge
PBS/control
α-GalCer/ control
PBS/asthma
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Previous studies have shown that anti-CD25 mAb delivery can lead to functional inactivation of Treg cells *in vivo* (14, 27). To determine whether Treg cells contributed to the inhibitory effects of α-GalCer on Th2 cell responses in asthma, mice were intravenously administered anti-CD25 mAb or IgG isotype mAb, rested for 1 day, followed by intraperitoneal administration of α-GalCer, then rested for 1 day, and subsequently sensitized and challenged with OVA. Airway inflammation and AHR were measured 24 h after the final OVA challenge. Our findings showed that anti-CD25 mAb delivery could reverse the protective effects of α-GalCer treatment before OVA sensitization on the level of inflammatory cell infiltration in the airways and mucus-secreting cells in the respiratory tracts (Fig. 7, *A* and *B*), the number of total cells, eosinophils, and macrophages in the BALF (Fig. 7*C*), the concentrations of such cytokines as IL-4, IL-5, and IL-13 in the BALF (Fig. 7*D*), the OVA-specific IgE and IgG1 in the serum (Fig. 7*E*), as well as AHR (Fig. 7*F*) in the OVA-induced asthmatic WT mice, compared with those treated with IgG isotype mAb ($p < 0.05$ or $p < 0.01$). Furthermore, anti-CD25 mAb administration significantly reduced the level of IL-10 in the BALF from the OVA-induced asthmatic WT mice, compared with those treated with IgG isotype mAb (Fig. 7*D*) ($p < 0.01$). Collectively, these data showed that Treg cells contribute to the inhibitory effects of α-GalCer on Th2 cell responses in an OVA-induced murine model of asthma.

### *Treg cells contribute to the inhibitory effects of α-GalCer treatment on immunogenic maturation of LDCs in asthmatic mice*

Furthermore, we investigated the effects of functional inactivation of Treg cells with anti-CD25 mAb delivery on surface maturation markers and proinflammatory cytokine production of LDCs in an OVA-induced murine asthma model intraperitoneally administered α-GalCer. Our data showed that anti-CD25 mAb administration could reverse the inhibitory influences of α-GalCer treatment on the expression levels of MHC II, CD80, CD86, and CD40 of LDCs (Fig. 8, *A* and *B*), as well as the capacity to secrete IL-12p70, IL-6, and TNF-α of LDCs (Fig. 8*C*) from OVA-induced asthmatic WT mice, compared with those treated with IgG isotype mAb ($p < 0.05$ or $p < 0.01$). However, anti-CD25 mAb treatment could markedly down-regulate the level of IL-10 in the culture supernatant of LDCs from OVA-induced asthmatic mice treated with α-GalCer, compared with IgG isotype mAb treatment (Fig. 8*C*) ($p < 0.05$). Thus, these data indicated that the suppressive capacity of α-GalCer on surface maturation markers and proinflammatory cytokine production of LDCs may require Treg cells in an OVA-induced murine asthma model.

### *α-GalCer treatment before allergen sensitization can enhance the level of IL-2 in vivo*

The generation, survival, and suppressive capacity of Treg cells are closely related to the IL-2 signal (28, 29). Thus, we further determined whether α-GalCer treatment before allergen sensitization could promote IL-2 production in the lung by iNKT cells in OVA-induced murine asthma. The level of IL-2 in the BALF and the expression of *il-2* mRNA in lung tissue were measured by ELISA and quantitative RT-PCR, respectively. Our findings showed that α-GalCer treatment before OVA sensitization could enhance the level of IL-2 in the BALF (Fig. 9*A*), as well as the expression of *il-2* mRNA in the lung tissue (Fig. 9*B*) in OVA-induced murine asthma, compared with PBS treatment ($p < 0.01$). However, no difference was detected in the level of IL-2 in the BALF and the expression of *il-2* mRNA in the lung tissue between OVA-sensitized and OVA-challenged CD1d$^{-/-}$ mice with α-GalCer and PBS treatment (Fig. 9, *A* and *B*) ($p > 0.05$). Furthermore, the level of IL-2 in the BALF and the expression of lung *il-2* mRNA from asthmatic WT mice treated with α-GalCer were markedly higher than asthmatic CD1d$^{-/-}$ mice treated with α-GalCer (Fig. 9, *A* and *B*) ($p < 0.01$). Furthermore, our data showed that the lung IL-2$^+$iNKT cells in asthmatic mice treated with α-GalCer before OVA sensitization were markedly elevated, compared with asthmatic mice treated with PBS before OVA sensitization and WT mice treated with α-GalCer or PBS (Fig. 9, *C* and *D*) ($p < 0.01$). Also, our findings indicated that lung CD4$^+$IL-2$^+$T cells in asthmatic mice treated with α-GalCer before sensitization were significantly reduced compared with asthmatic mice treated with PBS before OVA sensitization, but were strongly increased compared with those in WT mice treated with α-GalCer or PBS (Fig. 9, *E* and *F*) ($p < 0.01$). Surprisingly, the number of IL-2$^+$ LDCs was very limited in asthmatic mice treated with α-GalCer before OVA sensitization (Fig. 4, *G* and *H*). Therefore, these results suggested that α-GalCer treatment before OVA sensitization can enhance the production of IL-2 in the lung through iNKT cells in OVA-induced murine asthma.

**Figure 4. α-GalCer treatment before allergen sensitization down-regulates surface maturation markers and proinflammatory cytokine production of LDCs in an OVA-induced murine asthma model.** WT mice, intraperitoneally treated with α-GalCer or PBS before OVA sensitization, were sacrificed 24 h after the final OVA challenge. Then the LDCs (CD11c$^+$F4/80$^-$) were isolated using a combination of magnetic microbead selection and flow cytometry. LDCs were quantified by CD11c and F4/80 staining using flow cytometry. *A,* cells were stained for maturation-associated surface markers and detected by flow cytometry. Expression of MHC II, CD80, CD86, and CD40 was assessed in CD11c$^+$F4/80$^-$ cells and isotype controls. *B,* expression levels of MHC II, CD80, CD86, and CD40 in CD11c$^+$F4/80$^-$ cells. *C,* flow cytometry determined the purity of LDCs, which were stained with both CD11c and a mAb against F4/80 (*i.e.* the proportion of LDCs cells was over 99%). *D,* LDCs from OVA-induced asthmatic mice with PBS or α-GalCer treatment before OVA sensitization were cultured *in vitro* for 72 h. The concentrations of IL-12p70, IL-6, TNF-α, and IL-10 in the culture supernatants were determined by ELISA. Data are represented as means ± S.D. from three independent experiments ($n = 5$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$. *E,* immunofluorescence of the lung tissues was performed. Paraffin-embedded tissue sections were stained with anti-rabbit CD11c and anti-goat TNFα Abs followed by Alexa Fluor 594–conjugated goat anti-rabbit IgG (*red*) or Alexa Fluor 488–conjugated donkey anti-goat IgG (*green*). *Red* indicates CD11c$^+$ cells; *green* indicates *tnf-α*$^+$ cells, and *yellow* indicates CD11c$^+$*tnf-α*$^+$ cells. *F,* paraffin-embedded lung tissue sections were stained with anti-rabbit CD11c and anti-goat *il-12/il-35 p*35 Abs followed by Alexa Fluor 594–conjugated goat anti-rabbit IgG (*red*) or Alexa Fluor 488–conjugated donkey anti-goat IgG (*green*). *Red* indicates CD11c$^+$ cells; *green* indicates *il-12*$^+$ cells, and *yellow* indicates CD11c$^+$*il-12*$^+$ cells. *G,* paraffin-embedded lung tissue sections were stained with anti-rabbit CD11c and anti-goat *il-10* Abs followed by Alexa Fluor 594–conjugated goat anti-rabbit IgG (*red*) or Alexa Fluor 488–conjugated donkey anti-goat IgG (*green*). *Red* indicates CD11c$^+$ cells; *green* indicates IL-10$^+$ cells, and *yellow* indicates CD11c$^+$*il-10*$^+$ cells.


ASBMB

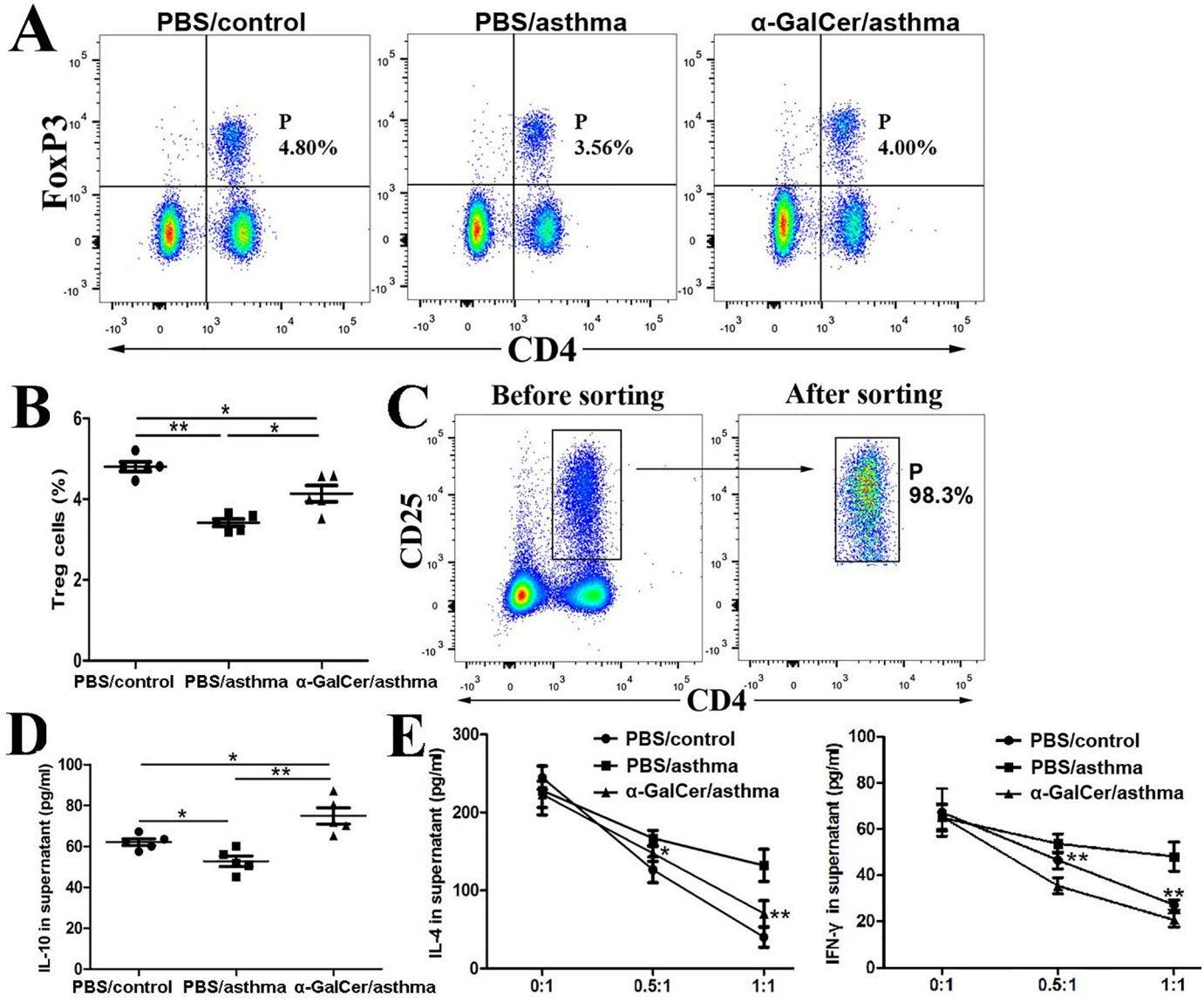


**Figure 5. α-GalCer treatment before allergen sensitization can enhance the expansion and suppressive activity of lung CD4$^+$FoxP3$^+$ Treg cells in an OVA-induced murine asthma model.** WT mice, intraperitoneally treated with α-GalCer or PBS before the first OVA sensitization, were sacrificed 24 h after the final OVA challenge. Subsequently, spleen Treg cells (CD4$^+$CD25$^+$ T cells) were isolated by magnetic microbead selection. CD4$^+$CD25$^-$ T cells were isolated from WT mice by magnetic microbead selection. *A,* lung Treg cells were confirmed by CD4 and FoxP3 staining in WT asthmatic mice intraperitoneally treated with α-GalCer or PBS before the first OVA sensitization using flow cytometry. The gating used for Treg cells (CD4$^+$FoxP3$^+$ Treg cells) and the corresponding percentages are shown in each dot plot. *B,* percentages of lung Treg cells in WT asthmatic mice intraperitoneally treated with α-GalCer or PBS before first OVA sensitization. *C,* flow cytometry determined the purity of spleen Treg cells, which were stained with both CD4 and a mAb against CD25 (*i.e.* the proportion of Treg cells was over 98%). *D,* Treg cells were cultured *in vitro* for 3 days, and then the concentrations of IL-10 in supernatants were determined by ELISA. *E,* CD4$^+$CD25$^-$ T cells co-cultured with the indicated ratio of Treg cells for 3 days. The potency of the Treg-mediated suppression was expressed as the relative inhibition of cytokine (IFN-γ and IL-4) production for each Treg/CD4$^+$CD25$^-$ T ratio. Data are shown as means ± S.D. of three independent experiments ($n$ = 5), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$.

### *Treg cells induced by α-GalCer–activated iNKT cells from OVA-induced murine asthma may involve in IL-2 in vitro*

To directly assess the role of IL-2 in the generation of Treg cells induced by α-GalCer–activated iNKT cells, we co-cultured iNKT cells from OVA-induced asthmatic WT mice intraperitoneally administered α-GalCer before OVA sensitization and CD4$^+$CD25$^-$ T cells from naive WT mice in the presence of anti-IL-2 mAb or IgG isotype mAb. As shown in Fig. 10*A*, the purity of iNKT cells, gated as PBS-57/mCD1d tetramer $^+$TCR-$\beta^+$ cells, was ~96%. Our results revealed that anti-IL-2 mAb treatment could significantly reduce the number of CD4$^+$FoxP3$^+$ Treg cells and the expression of *Foxp3* mRNA of culture cellular components (Fig. 10, *B* and *D*), as well as the level of IL-10 in culture supernatants (Fig. 10*E*), compared with IgG isotype mAb treatment ($p < 0.05$ or $p < 0.01$). Collectively, these results indicated that Treg cells induced by α-GalCer–activated iNKT cells from murine asthma may involve in IL-2 *in vitro*.

## Discussion

In this study, our data indicate that intraperitoneal administration of α-GalCer before allergen sensitization could down-

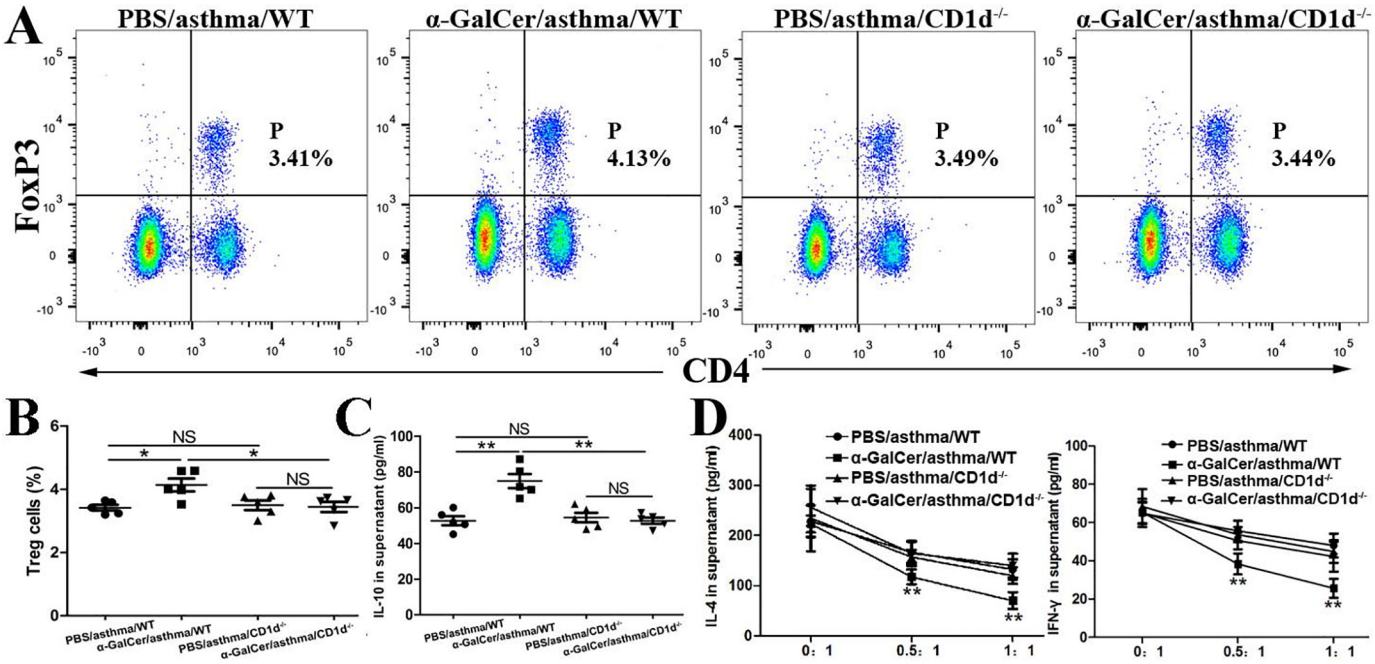


**Figure 6. iNKT cells contribute to the expansion and suppressive activity of Treg cells induced by α-GalCer treatment in asthmatic mice.** WT mice and CD1d$^{-/-}$ mice, immunized and challenged with OVA, were treated with an intraperitoneal injection of α-GalCer or PBS 24 h before the first sensitization. Spleen Treg cells (CD4$^+$CD25$^+$ T cells) were isolated by magnetic microbead selection. CD4$^+$CD25$^-$ T cells were obtained from WT mice by magnetic microbead selection. *A,* lung Treg cells were confirmed by CD4 and FoxP3 staining using flow cytometry. The gating used for Treg cells (CD4$^+$FoxP3$^+$ Treg cells) and the corresponding percentages are shown in each dot plot. *B,* percentages of lung Treg cells. *C,* spleen Treg cells were cultured *in vitro* for 3 days, and then the concentrations of IL-10 in supernatants were determined by ELISA. Data are shown as means ± S.D. of three independent experiments ($n = 5$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$. *NS*, not significant. *D,* CD4$^+$CD25$^-$ T cells from WT mice co-cultured with the indicated ratio of Treg cells for 3 days. The potency of the Treg-mediated suppression was expressed as the relative inhibition of cytokine (IFN-γ and IL-4) production for each Treg/CD4$^+$CD25$^-$ T ratio. Significant differences between the suppression ability of Treg cells from WT asthmatic mice or CD1d$^{-/-}$ asthmatic mice treated with α-GalCer are shown as **, $p < 0.01$.

regulate Th2 cell responses and immunogenic maturation of LDCs in WT mice but not in CD1d$^{-/-}$ mice immunized and challenged with OVA or HDM. Meanwhile, intraperitoneal administration of α-GalCer before allergen sensitization could promote the expansion and suppressive activity of lung CD4$^+$FoxP3$^+$ Treg cells in WT mice, but not in CD1d$^{-/-}$ mice immunized and challenged with OVA. Additionally, our data revealed that functional inactivation of Treg cells could reduce the inhibitory ability of α-GalCer on Th2 cell responses in a murine asthma model. Finally, intraperitoneal administration of α-GalCer could up-regulate production of IL-2 in the lung *in vivo*, and neutralization of IL-2 could reduce the expansion of Treg cells *in vitro*. Taken together, our findings provide evidence that intraperitoneal administration of α-GalCer before the development of asthma disorders, which can induce the generation of lung Treg cells by iNKT cells, may provide a potential therapeutic strategy to prevent allergic asthma.

α-GalCer, a strong and specific activator for iNKT cells, can stimulate iNKT cells to promptly produce large quantities of Th1- and Th2-type cytokines, such as IL-4 and IFN-γ, and thereby regulate a diverse array of immunity (11). Our current results indicated that intraperitoneal administration of α-GalCer before allergen sensitization can down-regulate Th2 cell responses in an OVA- or HDM-induced murine asthma model. Interestingly, our data showed that intraperitoneal administration of α-GalCer before allergen sensitization can reduce the concentrations of IL-4, IL-5, and IL-13 and promote the level of IL-10 in the BALF, suggesting that this α-GalCer therapy can result in a switch of cytokine production profiles in asthma from Th2-like cytokines to an immunosuppressive cytokine IL-10. However, the above results were not observed in CD1d$^{-/-}$ mice sensitized and challenged with OVA. The CD1d$^{-/-}$ mice are short of the MHC class I restricting element needed by the iNKT cell and hence lack functional iNKT cells (30). Therefore, iNKT cells are mainly required for the down-regulation of Th2 cell responses induced by intraperitoneal administration of α-GalCer before allergen sensitization in asthma. Our present results sharply differ from our previous findings showing that intraperitoneal administration of α-GalCer before allergen challenge can promote Th2 cell response by inducing immunogenic maturation of LDCs in murine asthma (18, 19). Ronet *et al.* (16) reported that iNKT cells play a key role in the initiation of the lethal ileitis after *T. gondii* infection, but activation of iNKT cells by intraperitoneal administration of α-GalCer before *T. gondii* infection can enhance the frequency of IL-10 –secreting Treg cells to control the inflammatory intestinal disorder. These data support the idea that the contradictory role of iNKT cells activated by α-GalCer in different pathological conditions may be partially related to the timing of α-GalCer treatment (31).

Recent data have shown that Treg cells have reduced number and impaired function in patients with asthma (3, 4), and the induction of lung Treg cells may be a potential therapeutic protocol for the treatment of asthma (8). IL-10 is a strong immunosuppressive cytokine that plays a fundamental role in the development of peripheral tolerance to allergen (32). It has

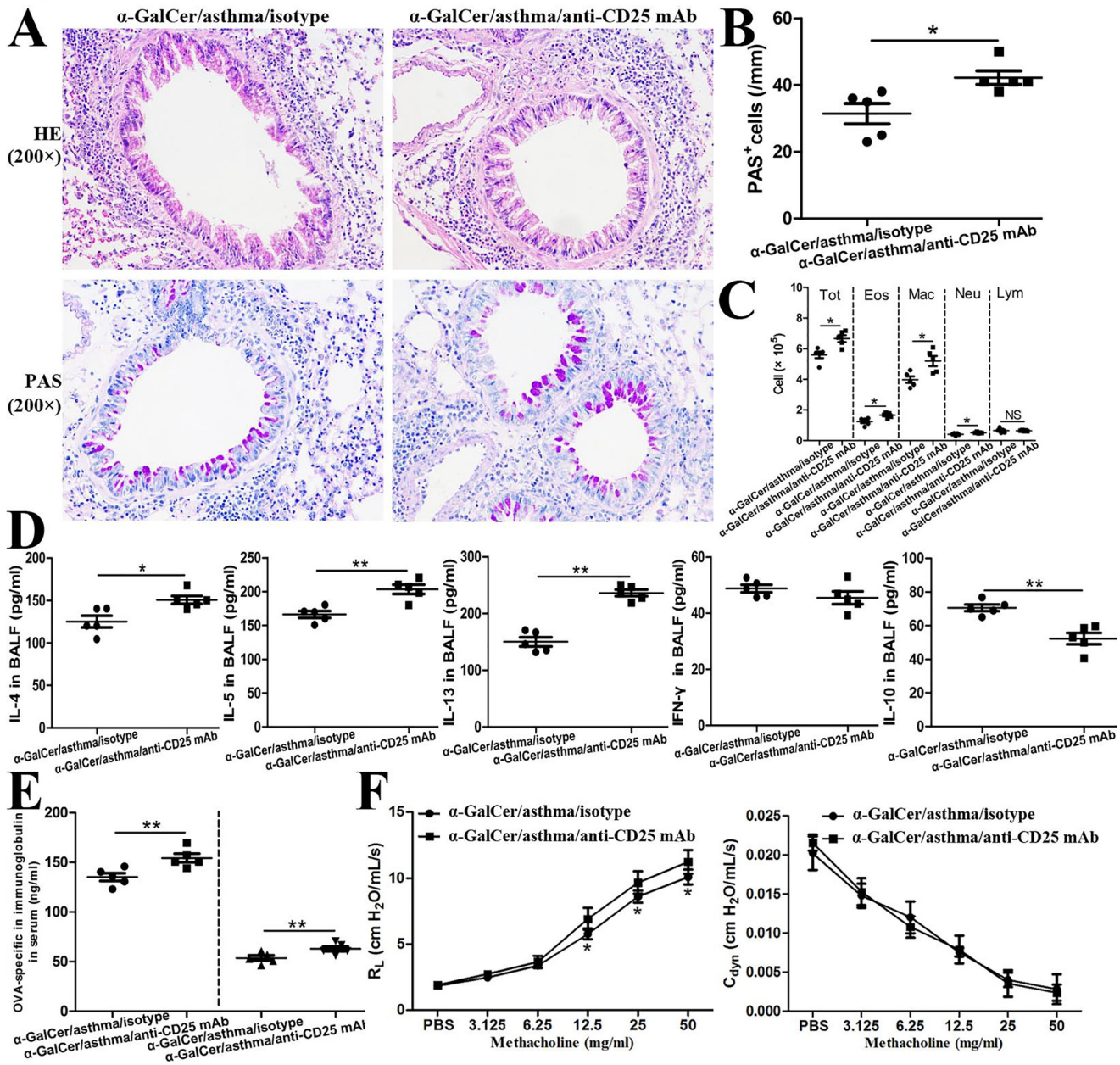


**Figure 7. Treg cells can contribute to the inhibitory effects of α-GalCer treatment on Th2 cell responses in an OVA-induced murine asthma model.** WT mice, intraperitoneally treated with α-GalCer before OVA sensitization, were delivered with anti-CD25 mAb or IgG isotype mAb 24 h before α-GalCer treatment and 48 h before OVA immunization. *A,* histopathological analysis of lung tissue H&E and PAS staining. *B,* lung goblet cell hyperplasia is indicated as the number of PAS-positive cells per unit of length (mm) of the basement membrane. *C,* total and differential cell counting in BALF of mice. *Tot,* total cell counts; *Eos,* eosinophils; *Mac,* macrophages; *Neu*, neutrophils; and *Lym,* lymphocytes. *D,* concentrations of IL-4, IL-5, IL-10, IL-13, and IFN-γ in BALF from mice analyzed by ELISA. *E,* OVA-specific IgE and IgG1 concentrations in the serum. *F,* airway response to increasing concentrations of methacholine was examined. Data are expressed as the means ± S.D. of three independent experiments ($n = 5$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$.

been widely believed that IL-10 released by Treg cells can suppress the production of proinflammatory cytokines and inhibit the production of effector T-cell cytokines, and down-regulate antigen presentation and the expression of major histocompatibility complex II and costimulatory molecules of DCs (8). Actually, allergen immunotherapy can induce the generation of antigen-specific Treg cells that release IL-10 and suppress allergen-specific Th2 cell responses (33), whereas Treg cell-specific depletion of IL-10 can enhance allergic airway inflammation (34). These reports support the notion that IL-10 secreted by Treg cells plays an essential role in the development of immune tolerance in allergic airway diseases. In this study, our findings showed that α-GalCer treatment before allergen sensitization can promote the expansion and suppressive activity of lung Treg cells in WT mice and CD1d$^{-/-}$ mice sensitized and challenged with OVA, indicating that the effects of this α-GalCer treatment on Treg cells mainly required iNKT cells. Furthermore, our data indicated that functional inactivation of CD4$^+$CD25$^+$ Treg cells using injection of anti-CD25 mAb can reverse the inhibitory influence of α-GalCer treatment before allergen sensitization on Th2 cell responses in a murine asthma model, suggesting that Treg cells contribute to the inhibitory

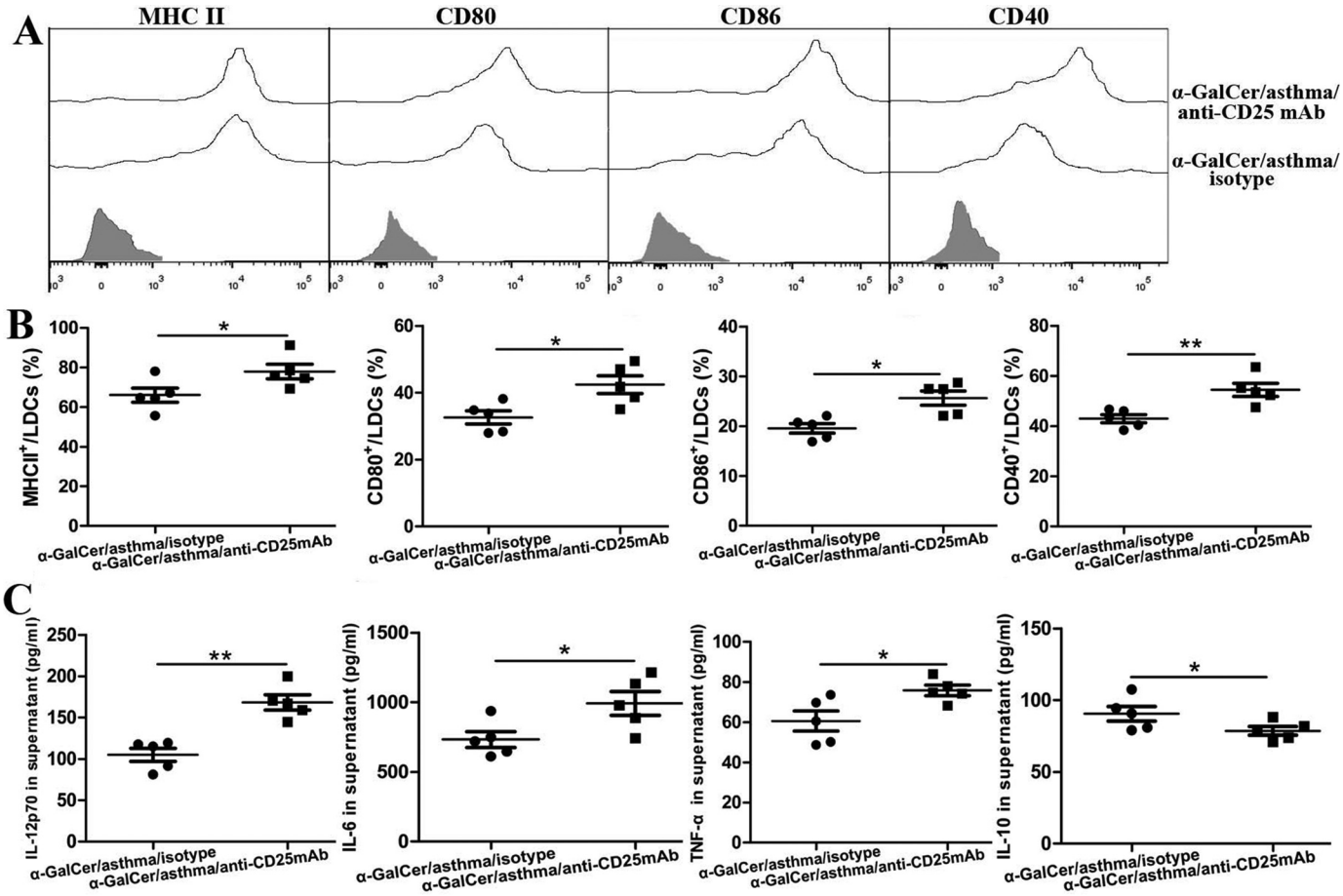


**Figure 8. Treg cells contribute to the inhibitory effects of α-GalCer on immunogenic maturation of LDCs in asthmatic mice.** WT mice, intraperitoneally treated with α-GalCer before OVA sensitization, were delivered with anti-CD25 mAb or IgG isotype mAb 24 h before α-GalCer treatment and 48 h before OVA immunization. Then LDCs ($CD11c^+F4/80^-$) were isolated using a combination of magnetic microbead selection and flow cytometry. LDCs were quantified by CD11c and F4/80 staining using flow cytometry. *A,* expression of MHC II, CD80, CD86, and CD40 was assessed in $CD11c^+F4/80^-$ cells and isotype controls. *B,* expression levels of MHC II, CD80, CD86, and CD40 in $CD11c^+F4/80^-$ cells. *C* LDCs from the two groups of mice were cultured *in vitro* for 72 h. The concentrations of IL-12p70, IL-6, IL-10, and TNF-α in the culture supernatants were determined by ELISA. Data are represented as means ± S.D. from three independent experiments ($n = 5$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$.

effects of α-GalCer on Th2 cell responses. Meanwhile, our findings revealed that α-GalCer treatment before allergen sensitization can promote the release of IL-10 from Treg cells *in vitro*, whereas specific functional inactivation of Treg cells by anti-CD25 mAb can abrogate the production of IL-10 *in vivo*. Our data suggest that Treg cells induced by this α-GalCer therapy may contribute to the production of IL-10 in asthma. Thus, these findings raise a potential mechanism that intraperitoneal administration of α-GalCer before allergen sensitization may be sufficient to protect against asthma through the induction of lung Treg cells by activated iNKT cells. Interestingly, our data showed that α-GalCer treatment before allergen sensitization can down-regulate immunogenic maturation of LDCs in a murine asthma model. Furthermore, in our preliminary study, our data showed that all $CD11c^+$*tnf-*$\alpha^+$ cells, $CD11c^+$*il-12*$^+$ cells, and $CD11c^+$*il-10*$^+$ cells in the lung tissues could be found by immunofluorescence in OVA-induced asthmatic mice treated with α-GalCer or PBS before OVA sensitization, but not in WT mice treated with α-GalCer or PBS. Meanwhile, $CD11c^+$TNF-$\alpha^+$ cell and $CD11c^+$IL-$12^+$ cell infiltration was reduced, but $CD11c^+$IL-$10^+$ cell infiltration was increased in the lung tissues from asthmatic mice treated with α-GalCer before OVA sensitization. Our preliminary results suggested that α-GalCer treatment before allergen sensitization induced the cytokine production profile switch of LDCs from TNFα or IL-12–producing DCs to IL-10–producing DCs, but cannot fully demonstrate that α-GalCer administration prior to allergen sensitization can induce the LDC switch from TNFα or IL-12–producing DCs to later IL-10–secreting DCs, or separate IL-10 only secreting LDC populations exist. Therefore, further study in this area will be required. It has been well-identified that LDCs can direct $CD4^+$ T cells differentiating into effector Th2 cells, inducing and maintaining maladaptive immunological response after allergen exposure in asthma (35, 36). However, Treg cells can result in the development of tolerogenic phenotypes of dendritic cells and also suppress maturation of dendritic cells (37). As such, it is probable that intraperitoneal administration of α-GalCer before allergen sensitization inhibits Th2 cell response through the down-regulation of immunogenic maturation of LDCs mediated by Treg cells in asthma. Our present results partially differ from the previous reports showing that α-GalCer treatment can abrogate AHR and airway inflammation through activation of iNKT cells and IFN-γ production in asthmatic mice (38, 39). The

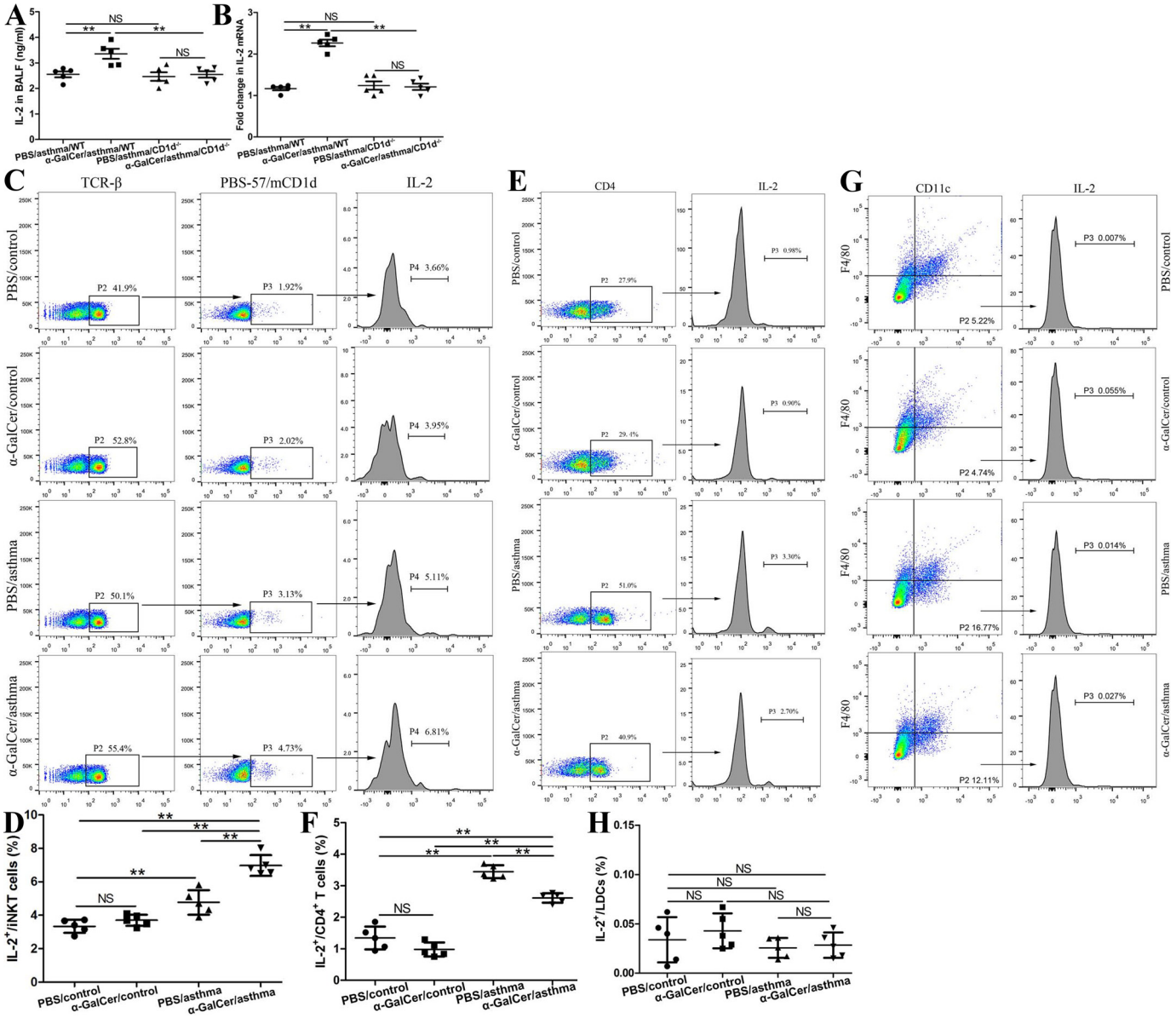


**Figure 9. α-GalCer treatment before allergen sensitization can enhance the level of IL-2 *in vivo*.** WT mice and CD1d$^{-/-}$ mice, immunized and challenged with OVA, were treated with an intraperitoneal injection of α-GalCer or PBS 24 h before the first sensitization. *A*, BALFs were collected from each group of mice, and IL-2 production was analyzed by ELISA. *B*, level of *il-2* mRNA in the lung from each group of mice was measured by quantitative RT-PCR. *C*, lung iNKT cells (PBS-57/mCD1d$^+$ TCR-β$^+$) (gate P3) and lung IL-2$^+$iNKT cells (PBS-57/mCD1d$^+$ TCR-β$^+$ IL-2$^+$) (gate P4) were determined by flow cytometry. The gating for lung iNKT cells and the corresponding percentages are indicated in each dot plot. *D*, percentages of IL-2$^+$iNKT cells in lung iNKT cells were determined. *E*, lung CD4$^+$T cells (CD4$^+$) (gate P2) and lung CD4$^+$IL-2$^+$T cells (CD4$^+$IL-2$^+$) (gate P3) were determined by flow cytometry. The gating for lung CD4$^+$T cells and the corresponding percentages are indicated in each dot plot. *F*, percentages of CD4$^+$IL-2$^+$T cells in lung CD4$^+$T cells were determined. *G*, LDCs (CD11c$^+$F4/80$^-$) (gate P2) and lung IL-2$^+$ LDCs (CD11c$^+$F4/80$^-$IL-2$^+$) (gate P3) were determined by flow cytometry. The gating for LDCs and the corresponding percentages are indicated in each dot plot. *H*, percentages of CD11c$^+$F4/80$^-$IL-2$^+$LDCs in LDCs were determined. Data are represented as means ± S.D. from three independent experiments ($n = 5$), and one representative experiment is indicated. **, $p < 0.01$. *NS*, not significant.

different results may be partially related to the different experimental protocols between their studies and ours, but further study is still needed.

It is widely accepted that immature or partially mature dendritic cells are able to induce the generation of Treg cells, whereas mature dendritic cells possess the capacity to polarize different subsets of effector T cells (40). This study showed that α-GalCer treatment before allergen sensitization can enhance the expansion and suppressive activity of lung Treg cells in asthmatic mice. However, LDCs exhibit the immunogenic maturation status in asthmatic mice treated with α-GalCer before allergen sensitization, albeit at a lower extent than asthmatic mice treated with PBS. Therefore, our findings provided possible evidence that the generation of lung Treg cells induced by α-GalCer treatment before allergen sensitization may not be related to LDCs in asthmatic mice. It is well-established that IL-2 plays a critical role in the generation, activation, and survival of Treg cells (41, 42). Previous studies show some evidence that iNKT cells from murine and humans produce IL-2 to promote the development of Treg cells in the presence or absence of α-GalCer (17, 43). In our present observation, our preliminary data showed that intraperitoneal administration of

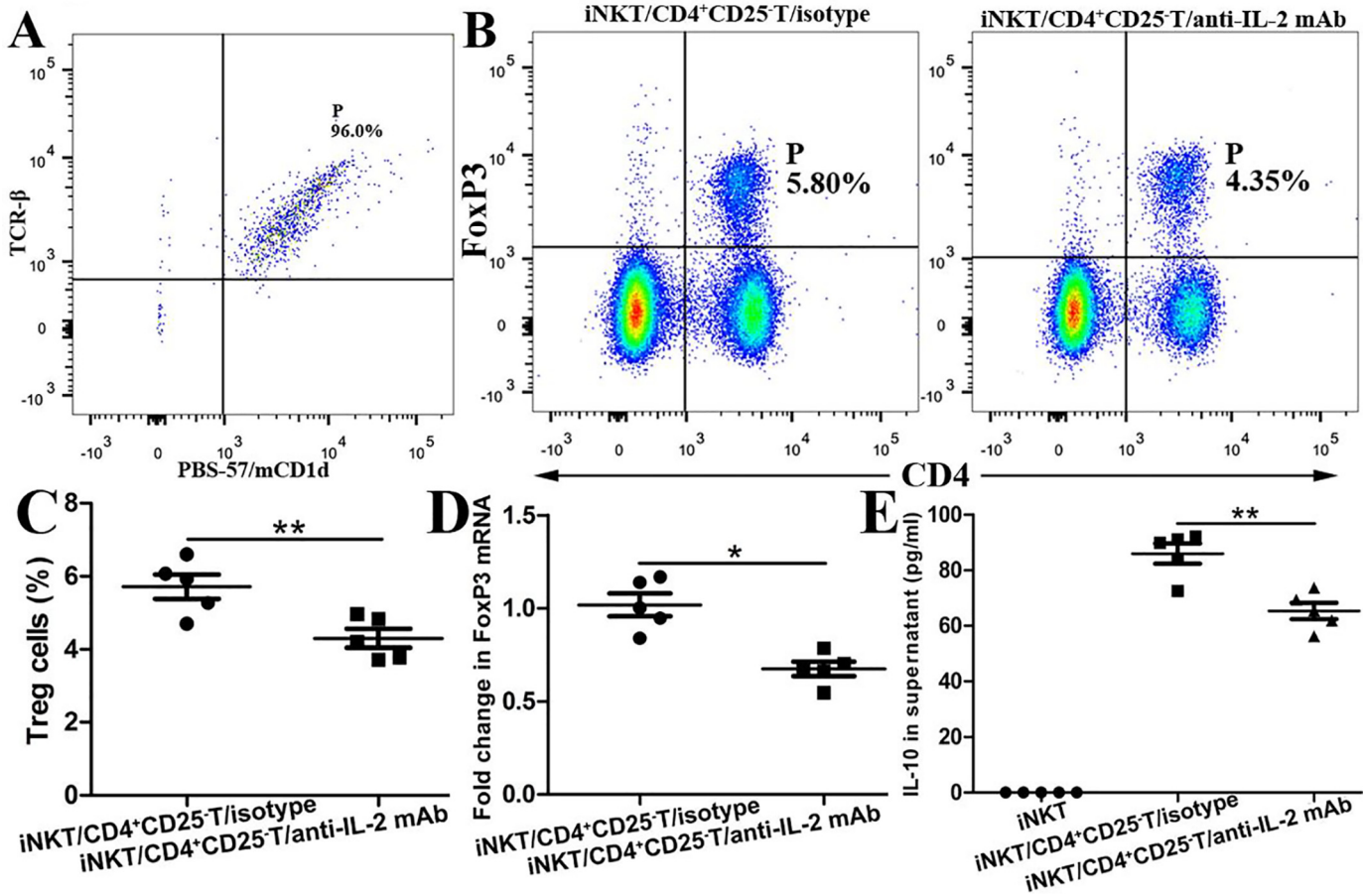


**Figure 10. Treg cells induced by α-GalCer–activated iNKT cells from OVA-induced murine asthma may involve IL-2 *in vitro*.** Lung iNKT cells (PBS-57/mCD1d$^+$TCR-$\beta^+$ iNKT cells) were isolated from OVA-induced asthmatic mice intraperitoneally treated with α-GalCer before the first OVA sensitization, and spleen CD4$^+$CD25$^-$ T cells were isolated from WT mice using magnetic microbead selection. iNKT cells were cultured alone or co-cultured with CD4$^+$CD25$^-$ T cells in the presence of anti-IL-2 mAb or IgG isotype mAb for 72 h. *A,* flow cytometry determined the purity of lung iNKT cells, which were stained with both PBS-57/mCD1d and a mAb against TCR-β (*i.e.* the proportion of iNKT cells was ~96%). *B,* Treg cells of cellular components from culture medium were measured by CD4 and FoxP3 staining using flow cytometry. The gating used for Treg cells (CD4$^+$FoxP3$^+$ Treg cells) and the corresponding percentages are shown in each dot plot. *C,* percentages of Treg cells from cellular components from culture medium. *D,* expression level of *Foxp3* mRNA of cellular components from culture medium was analyzed by quantitative RT-PCR. *E,* concentration of IL-10 in culture supernatants was determined by ELISA. Data are shown as means ± S.D. of three independent experiments ($n = 15$), and one representative experiment is indicated. *, $p < 0.05$; **, $p < 0.01$.

α-GalCer before allergen sensitization could promote the expression of *il-2* gene and *il-2* protein in the lung from asthmatic WT mice, but not asthmatic CD1d$^{-/-}$ mice. It has been well-demonstrated that the CD1d$^{-/-}$ mice are devoid of the MHC class I restricting element required by iNKT cells and thereby lack iNKT cells (30). Meanwhile, our data show that the lung IL-2$^+$ iNKT cells were markedly elevated, whereas lung CD4$^+$IL-2$^+$ T cells were significantly reduced in asthmatic mice treated with α-GalCer before OVA sensitization. Also, the number of IL-2$^+$ LDCs was very limited in asthmatic mice treated with α-GalCer before OVA sensitization. These results provided the evidence that the elevated production of IL-2 induced by α-GalCer is mainly dependent on lung iNKT cells, but not lung CD4$^+$T cells and LDCs. Furthermore, neutralization of IL-2 could reduce the production of IL-10 in culture supernatant, the expression of *Foxp3* mRNA of cellular components, and the number of CD4$^+$FoxP3$^+$ Treg cells *in vitro*. Taken together, our preliminary findings revealed that α-GalCer treatment before antigen sensitization could promote the level of IL-2 in the airways through activation of lung iNKT cells, which is likely to play a potential role in the generation of lung Treg cells in asthma.

In summary, our present findings demonstrated that α-GalCer treatment before antigen sensitization can promote iNKT cell-mediated induction of lung Treg cells, preventing Th2 cell responses in murine asthma. Presently, the mostly important treatments utilized to control asthma are inhaled glucocorticoids, with or without β2 agonists, which possess broad-scale anti-inflammatory ability (44). However, these therapy strategies merely ameliorate the symptoms, rather than abrogating the underlying mechanism, and thereby fail to control the disorders in marked proportion of patients with asthma. Treg cells can directly or indirectly suppress the initiation of allergic inflammation, mucus overproduction, IgE release, and AHR through modulating antigen-presenting cells, Th2 and Th17 cells, inflammatory cells, and B cells in asthma (8). Because CD1d molecules are extremely conserved in mammals, and TCRs expressed by murine and human iNKT cells have structural and functional similarity (45, 46), α-GalCer can bind human CD1d molecules and activate iNKT cells in

humans (47). Additionally, α-GalCer appears to be well-tolerated over a broad range of doses by human patients (48). Meanwhile, our present observations that α-GalCer treatment before antigen sensitization can inhibit Th2 cell responses in an OVA- or HDM-induced murine asthma model indicate that this therapy may be effective for multiple antigens. Thus, our data raise a possibility that α-GalCer treatment before the development of asthma symptoms induces the generation of lung Treg cells via activated iNKT cells and may provide a potential therapeutic strategy to prevent allergic asthma for humans.

## Experimental procedures

### Mice

Female WT BALB/c mice (6–8 weeks old) were obtained from the Animal Biosafety Level 3 Laboratory, the Center for Animal Experiment, Wuhan University (Wuhan, China). $CD1d^{-/-}$ mice (BALB/c background) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred under specific pathogen-free and controlled environmental conditions (22 °C, 12-h light/12-h dark cycle). All procedures were approved by the Institutional Ethics Committee of Wuhan University.

### Animal experimental models

WT BALB/c mice or $CD1d^{-/-}$ BALB/c mice were intraperitoneally immunized on days 0 and 14 with 20 μg of chicken OVA (grade V, Sigma) absorbed in 2 mg of aluminum hydroxide (ThermoFisher Scientific Pierce) in 200 μl of PBS. Intranasal OVA challenges (100 μg/50 μl in PBS) were followed on days 21–23. Mice were sacrificed 24 h after the final challenge to further study. In some experiments, WT mice were sensitized intratracheally on day 0 with 1 μg of HDM extracts (*Dermatophagoides pteronyssinus*, Greer Laboratories) and were subsequently intranasally challenged from days 6 to 10 with 10 μg of HDM extracts (49). Mice were euthanized 3 days after the final challenge to further analyze. Sex- and age-matched mice were similarly sensitized and challenged with PBS alone as control.

### In vivo administration of α-GalCer

α-GalCer (Enzo Life Sciences, Ann Arbor, MI) was dissolved in PBS containing 20% dimethyl sulfoxide (DMSO) at 0.01 mg/ml as a stock solution and stored at −20 °C. The intraperitoneal injection was used as the route of administration of α-GalCer, as reported previously (50). Mice received a single intraperitoneal injection of 2 μg of α-GalCer 1 day before the first sensitization with OVA or HDM. Control mice received intraperitoneal administration of an identical volume of vehicle solution (PBS/20% DMSO).

### In vivo Ab administration

For functional inactivation of $CD4^+CD25^+$ Treg cells, mice were intravenously delivered with 500 μg of anti-CD25 mAb (clone PC61; BD Pharmingen) or IgG isotype mAb (Sigma), rested for 1 day, followed by *in vivo* administration of α-GalCer, then rested for 1 day, and subsequently sensitized and challenged with OVA.

### RNA extraction and real-time quantitative PCR

To assess the expression of *Foxp3* mRNA and *il-2* mRNA, total RNA was isolated with TRIzol (Invitrogen) following the manufacturer's manuals. cDNA preparation was conducted using a Revertaid first-strand cDNA synthesis kit (ShineGene, Shanghai, China) on the basis of the manufacturer's instructions. Quantitative PCR was performed with a SYBR Premix Ex Taq™ kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. The primer sequences are as follows: *Foxp3*, forward, 5′-GAAGAATGCCATCCGCCACAAC-3′ and reverse, 5′-ATTCATCTACGGTCCACACTGCTC-3′; *il-2*, forward, 5′-TGAACTTGGACCTCTGCG-3′ and reverse, 5′-ATTGAGGGCTTGTTGAGA-3′; *GAPDH*, forward, 5′-AGGGGGGCTAAGCAGTTGGT-3′ and reverse, 5′-AGGAGCGAGACCCCACTAACA-3′. Data were normalized according to expression levels of GAPDH.

### Isolation of LDCs and cytokine production

LDCs were harvested by magnetic bead purification combined with fluorescence-activated cell sorting (FACS). WT mice and $CD1d^{-/-}$ mice were sacrificed 24 h after the last OVA challenge. Lungs were excised, and single cell suspensions were prepared, as described previously (21). Subsequently, $CD11c^+$ cells were selected using CD11c microbeads (Miltenyi Biotec, Auburn, CA) and MASC columns (Miltenyi Biotec). A previous study has demonstrated that LDCs express the DC marker CD11c, but not the macrophage marker F4/80 (51). Thus, LDCs were gated as $CD11c^+F4/80^-$ cells and isolated with a BD FACSAria III cell sorter (BD Biosciences). The purity of the sorted LDCs was measured by flow cytometry for APC-cy7–conjugated anti-CD11c–positive and PE-cy5–conjugated anti-F4/80–negative cells.

LDCs isolated from different groups of mice were cultured (37 °C, 5% $CO_2$) in complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% streptomycin/penicillin, 0.1 mM nonessential amino acids, and 50 μM mercaptoethanol (all from Invitrogen, Karlsruhe, Germany) in 96-well plates at $2 \times 10^5$ cells/well for 72 h in the presence of OVA (100 μg/ml) or vehicle. Supernatants were obtained to determine the concentrations of IL-10, IL-12p70, IL-6, and TNFα by ELISA (eBioscience, San Diego, CA) on the basis of the manufacturer's instructions.

### Isolation of $CD4^+$ T cells, $CD4^+$ $CD25^+$ T cells, and $CD4^+$ $CD25^-$ T cells

$CD4^+CD25^+$ T cells were isolated from spleens of WT or $CD1d^{-/-}$ mice treated with intraperitoneal injection of α-GalCer or PBS before OVA sensitization, whereas $CD4^+$ T cells and $CD4^+CD25^-$ T cells were isolated from spleens of WT mice. $CD4^+$ T cells were enriched from single spleen cell suspensions using R&D $CD4^+$ subset enrichment columns (R&D Systems, Minneapolis, MN). For obtaining $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells, $CD4^+$ T cells were labeled with anti-CD25–PE, and then the $CD4^+$ T-cell fraction was loaded onto a MACS column placed in the magnetic field of a MACS separator (Miltenyi Biotec). Magnetically-labeled $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells were obtained following the manufacturer's procedures. The purity of the sorted $CD4^+CD25^+$ T cells was examined by flow cytometry

using anti-CD4–FITC and anti-CD25–PE (eBioscience). $CD4^+$ $CD25^-$ T cells, used as responder T cells, and $CD4^+CD25^+$ T cells were harvested for further analysis.

### Culture and suppressive activity of $CD4^+$ $CD25^+$ Treg cell in vitro

$CD4^+$ $CD25^+$ T cells, defined as Treg cells (52), were seeded at a density of $1 \times 10^5$ cells/well in round-bottom 96-well plates pre-bound with anti-CD3 (2 μg/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% streptomycin/penicillin, 2 mM L-glutamine, 5 mM Hepes, 1 mM sodium pyruvate, and anti-CD28 (2 μg/ml) (all from Invitrogen, Karlsruhe, Germany). After 72 h, culture supernatants were collected to detect the level of IL-10 by ELISA according to the manufacturer's protocol (eBioscience).

$CD4^+CD25^-$ T cells, used as responder T cells, co-cultured (37 °C, 5% $CO_2$) with $CD4^+CD25^+$ T cells at different ratios (0:1, 0.5:1, and 1:1) of $CD4^+CD25^+$/$CD4^+CD25^-$ T in 96-well plates pre-coated with anti-CD3 (2 μg/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% streptomycin/penicillin, 2 mM L-glutamine, 5 mM Hepes, 1 mM sodium pyruvate, and anti-CD28 (2 μg/ml) (all from Invitrogen, Karlsruhe, Germany) for 3 days. The suppressive activity of Treg cells was assessed by the concentrations of IL-4 and IFN-γ in culture supernatants by ELISA following the manufacturer's protocols (eBioscience).

### Co-culture of iNKT cells and $CD4^+CD25^-$ T cells

To sorted lung iNKT cells, WT mice treated with intraperitoneal injection of α-GalCer before OVA immunization were sacrificed 24 h after the final OVA challenge. The lung single cell suspensions were obtained. iNKT cells were enriched using magnetic bead purification following the manufacturer's instruction (Miltenyi Biotec). The purity of the sorted iNKT cells was examined by flow cytometry for FITC-TCR-β and PE-PBS57/mCD1d tetramer double-positive cells. iNKT cells ($1.5 \times 10^5$/well) were cultured alone or with the indicated numbers of $CD4^+CD25^-$ T cells from WT mice in round-bottom 96-well plates pre-bound with anti-CD3 (2 μg/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% streptomycin/penicillin, 2 mM L-glutamine, 5 mM Hepes, and 1 mM sodium pyruvate and anti-CD28 (2 μg/ml) (all from Invitrogen, Karlsruhe, Germany) and restimulated with α-GalCer (100 ng/ml) in the presence of anti-IL-2 mAb (0.2 μg/ml) (IgG2a, clone S4B6; BD Pharmingen) or IgG isotype mAb (Sigma-Aldrich, Tauf-kirchen, Germany). After 3 days, the cellular components were harvested, and the frequency of $CD4^+FoxP3^+$ Treg cells and the expression of Foxp3 mRNA were analyzed by flow cytometry and RT-PCR, respectively. The level of IL-10 in culture supernatants was measured by ELISA on the basis of the manufacturer's protocol (eBioscience).

### Flow cytometric analysis

To examine the frequency of LDCs, lung MNCs were resuspended in FACS buffer, and then the cells were incubated with an anti-CD16/CD32 antibody (BD Biosciences) for 1 h to reduce nonspecific binding. After two washes, isotype controls or PE–cy5-conjugated anti-F4/80 and APC–cy7-conjugated anti-CD11c (eBioscience) were labeled. Forward scatter and side scatter were used to exclude dead cells. To further assess the expression of different surface markers, freshly sorted LDCs from the different experimental groups were labeled with anti-CD86–PE, anti-CD80–PE, anti-CD40–PE, anti-MHC II–PE, or respective isotype controls (eBioscience).

To assess the effect of α-GalCer treatment before allergen sensitization on the number of lung $CD4^+$T cells or Treg cells, lung MNCs were harvested and resuspended in the FACS buffer at a level of $1–2 \times 10^6$ cells/ml. The lung MNCs were first blocked using an anti-CD16/CD32 antibody (BD Biosciences) to abate nonspecific binding and then labeled with isotype controls or the antibodies as follows: anti-CD4-FITC and anti-FoxP3-PeCy7 (eBioscience). Intracellular staining for FoxP3 was performed with Fix/Perm buffer reagents (eBioscience) according to the manufacturer's procedure. Lung Treg cells were identified as CD4 and Foxp3 double-positive cells.

The lung MNCs were labeled with isotype controls or PeCy5–TCR-β (eBioscience) and PE–PBS-57/mCD1d tetramer (gifted by the Natural Institutes of Health tetramer core facility) to determine the number of lung iNKT cells. In addition, to examine the secretion of IL-2 by lung MNCs, intracellular cytokine staining of BV421–IL-2 (BD Biosciences) was performed following the manufacturer's procedures (eBioscience).

All staining reactions were conducted at 4 °C. The cells were measured by flow cytometry (Epics Altra; Beckman, Seattle, WA), and the acquired data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

### Examination of AHR

AHR was examined by invasive examination of airway resistance, as reported previously (53). WT mice and $CD1d^{-/-}$ mice intraperitoneally treated with α-GalCer or PBS before OVA or HDM sensitization or asthmatic WT mice intravenously injected with anti-CD25 mAb or IgG isotype mAb were anesthetized 24 or 72 h after the last allergen challenge, and then the mice were inserted with a 20-gauge polyethylene catheter. Subsequently, mechanical ventilation was performed. Dynamic compliance (Cdyn) and airway resistance (RL) to increasing concentrations of aerosolized methacholine (Mch), ranging from 3.12 to 50 mg/ml in PBS, for 3 min were measured by the FinePointe RC system (Wilmington, NC) to analyze AHR. Nonspecific airway responsiveness was measured on mice that were exposed to aerosolized PBS to decide their baseline value.

### Bronchoalveolar fluid and lung histopathology

After the measurement of AHR, the lungs were lavaged using a total volume of 1.5 ml of PBS with 1 mM sodium EDTA, and subsequently, the lungs were resected, as mentioned previously (21). The supernatants of BALF were harvested and stored at −80 °C to examine the levels of IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-γ by ELISA on the basis of the manufacturer's procedures (eBioscience). Cells in the BALF were collected to stain with May-Grunswald Giemsa (Jiancheng, Nanjing, China) for differential cell counting. The frequencies of eosinophils, neu-

trophils, lymphocytes, and macrophages were expressed as the absolute numbers on the basis of the total cell counts.

The left lungs were fixed in 4% buffered paraformaldehyde immediately after bronchoalveolar lavage. Then the samples were dehydrated and embedded in paraffin. Lung tissue sections were stained using hematoxylin-eosin (H&E) staining to evaluate airway inflammation and PAS staining (Baso, Taiwan, China) to assess goblet cell hyperplasia using light microscopy. Goblet cell hyperplasia was measured by counting the frequency of PAS-positive cells in the epithelium of the central respiratory tract with a digital image. The circumferences of the respiratory tract at the basement membrane were determined using ImageJ software (National Institutes of Health, Bethesda). Goblet cell hyperplasias were expressed as the frequency of PAS-positive cells per unit length (mm) of the basement membrane.

### Immunofluorescence histology

Immunostaining was performed on 25-μm sections. Paraffin-embedded lung tissue sections were blocked and permeabilized in 4% bovine serum albumin in 0.1 M PBS with 0.3% Triton X-100, incubated with anti-rabbit CD11c at 1:100 (Cell Signaling Technology, Danvers, MA) doubled with anti-goat *tnf-α*, anti-goat *il-12/il-35 p35*, or anti-goat *il-10*, respectively, at 1:100 (all from RND, Nasdaq) at 4 °C overnight. Then, the sections were incubated with Alexa Fluor 594–conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) and Alexa Fluor 488–conjugated donkey anti-goat IgG fluorescent secondary antibodies (Absin, Shanghai, China) for 1 h at room temperature in the dark. Finally, the images were acquired using an Olympus IX73 fluorescence microscope (Olympus, Tokyo, Japan).

### Analysis of OVA- or HDM-specific IgE and IgG1 in the serum

For immunoglobulin analysis, 24 h after the final OVA challenge or 3 days after the last HDM challenge, the whole blood was harvested to determine the levels of OVA-specific IgE and IgG1 or HDM-specific IgE and IgG1 by means of ELISA (eBioscience) following the manufacturer's instructions. Serum samples were diluted for total IgE or total IgG according to the instructions, respectively, in PBS containing 1% BSA (w/v) (Invitrogen, Karlsruhe, Germany). The OVA-specific IgE and IgG1 or HDM-specific IgE and IgG1 levels in serum samples were assessed by an indirect ELISA method. Briefly, the 96-well plates (ThermoFisher Scientific) were coated with 200 g/ml OVA or 10 μg/ml HDM extract diluted in PBS, and blocked with 3% BSA (w/v) in PBS overnight at 4 °C. Serum samples (50 μl/well) were incubated overnight at 4 °C. Biotin-conjugated goat anti-mouse IgE or IgG1 (Biolegend, San Diego) were added to the wells used as secondary detection antibodies and incubated for 1 h at room temperature. Avidin-horseradish peroxidase (HRP) (eBioscience) and 3,3′,5,5-tetramethylbenzidine (TMB) (ThermoFisher Scientific) were used to develop the colorimetric reaction. Reaction was stopped by 2 M $H_2SO_4$, and the absorbance was recorded using a BioTek Synergy 4 microplate reader (BioTek).

### Statistical analyses

All data are expressed as the mean ± S.D. Statistical analyses were performed with a Student's unpaired *t* test or one-way analysis of variance using GraphPad Prism 5 (GraphPad Software Inc., San Diego) software. $p < 0.05$ was considered statistically significant.

*Author contributions*—Q. C., S. C., and H. N. resources; Q. C., X. G., N. D., S. C., R. L., and H. N. data curation; Q. C., N. D., L. L., S. C., and H. N. formal analysis; Q. C., X. G., and H. N. investigation; Q. C., X. G., L. L., S. C., A. W., R. L., Y. H., X. D., H. Y., S. H., and H. N. methodology; Q. C. and H. N. writing-original draft; Q. C., X. G., A. W., R. L., Y. H., X. D., H. Y., S. H., and H. N. writing-review and editing; N. D., L. L., and S. C. software; N. D. and H. N. project administration; H. N. funding acquisition.

*Acknowledgments—We are grateful to Dale Long and colleagues at the Tetramer Core Facility at the National Institutes of Health for kindly providing PE-labeled PBS-57/mCD1d tetramer.*

## References


1. Afshar, R., Medoff, B. D., and Luster, A. D. (2008) Allergic asthma: a tale of many T cells. *Clin. Exp. Allergy* **38,** 1847–1857 Medline
2. Lloyd, C. M., and Hawrylowicz, C. M. (2009) Regulatory T cells in asthma. *Immunity* **31,** 438–449 CrossRef Medline
3. Lee, J. H., Yu, H. H., Wang, L. C., Yang, Y. H., Lin, Y. T., and Chiang, B. L. (2007) The levels of $CD4^+CD25^+$ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clin. Exp. Immunol.* **148,** 53–63 Medline
4. Grindebacke, H., Wing, K., Andersson, A. C., Suri-Payer, E., Rak, S., and Rudin, A. (2004) Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergies during birch pollen season. *Clin. Exp. Allergy* **34,** 1364–1372 Medline
5. Kearley, J., Barker, J. E., Robinson, D. S., and Lloyd, C. M. (2005) Resolution of airway inflammation and hyperreactivity after *in vivo* transfer of $CD4^+CD25^+$ regulatory T cells is interleukin 10 dependent. *J. Exp. Med.* **202,** 1539–1547 CrossRef Medline
6. Strickland, D. H., Stumbles, P. A., Zosky, G. R., Subrata, L. S., Thomas, J. A., Turner, D. J., Sly, P. D., and Holt, P. G. (2006) Reversal of airway hyperresponsiveness by induction of airway mucosal $CD4^+CD25^+$ regulatory T cells. *J. Exp. Med.* **203,** 2649–2660 CrossRef Medline
7. Kearley, J., Robinson, D. S., and Lloyd, C. M. (2008) $CD4^+CD25^+$ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *J. Allergy Clin. Immunol.* **122,** 617–624.e6 CrossRef Medline
8. Thorburn, A. N., and Hansbro, P. M. (2010) Harnessing regulatory T cells to suppress asthma: from potential to therapy. *Am. J. Respir. Cell Mol. Biol.* **43,** 511–519 CrossRef Medline
9. Brigl, M., and Brenner, M. B. (2004) CD1: antigen presentation and T cell function. *Annu. Rev. Immunol.* **22,** 817–890 CrossRef Medline
10. Matsuda, J. L., Naidenko, O. V., Gapin, L., Nakayama, T., Taniguchi, M., Wang, C. R., Koezuka, Y., and Kronenberg, M. (2000) Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* **192,** 741–754 CrossRef Medline
11. Bendelac, A., Savage, P. B., and Teyton, L. (2007) The biology of NKT cells. *Annu. Rev. Immunol.* **25,** 297–336 CrossRef Medline
12. Berzins, S. P., Smyth, M. J., and Baxter, A. G. (2011) Presumed guilty: natural killer T cell defects and human disease. *Nat. Rev. Immunol.* **11,** 131–142 CrossRef Medline
13. Matsuda, J. L., Mallevaey, T., Scott-Browne, J., and Gapin, L. (2008) CD1d restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr. Opin. Immunol.* **20,** 358–368 CrossRef Medline
14. Ly, D., Mi, Q. S., Hussain, S., and Delovitch, T. L. (2006) Protection from type 1 diabetes by invariant NKT cells required the activity of

$CD4^+CD25^+$ regulatory T cells. *J. Immunol.* **177,** 3695–3704 CrossRef Medline
15. Liu, R., La Cava, A., Bai, X. F., Jee, Y., Price, M., Campagnolo, D. I., Christadoss, P., Vollmer, T. L., Van Kaer, L., and Shi, F. D. (2005) Cooperation of invariant NKT cells and $CD4^+CD25^+$ regulatory T cells in the prevention of autoimmune myasthenia. *J. Immunol.* **175,** 7898–7904 CrossRef Medline
16. Ronet, C., Darche, S., Leite de Moraes, M., Miyake, S., Yamamura, T., Louis, J. A., Kasper, L. H., and Buzoni-Gatel, D. (2005) NKT cells are critical for the initiation of an inflammatory bowel response against *Toxoplasma gondii*. *J. Immunol.* **175,** 899–908 CrossRef Medline
17. Jiang, S., Game, D. S., Davies, D., Lombardi, G., and Lechler, R. I. (2005) Activated CD1d-restricted natural killer T cells secrete IL-2: innate help for CD4+CD25+ regulatory T cells? *Eur. J. Immunol.* **35,** 1193–1200 CrossRef Medline
18. Nie, H., Yang, Q., Zhang, G., Wang, A., He, Q., Liu, M., Li, P., Yang, J., Huang, Y., Ding, X., Yu, H., and Hu, S. (2015) Invariant NKT cells act as an adjuvant to enhance Th2 inflammatory response in an OVA-induced mouse model of asthma. *PLoS ONE* **10,** e0119901 CrossRef Medline
19. He, Q., Liu, L., Yang, Q., Wang, A., Chen, S., Li, R., Huang, Y., Zhang, G., Ding, X., Yu, H., Hu, S., and Nie, H. (2017) Invariant natural killer T cells promote immunogenic maturation of lung dendritic cells in mouse models of asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **313,** L973–L990 CrossRef Medline
20. Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., Hammad, H., and Lambrecht, B. N. (2013) Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* **38,** 322–335 CrossRef Medline
21. Zhang, G., Nie, H., Yang, J., Ding, X., Huang, Y., Yu, H., Li, R., Yuan, Z., and Hu, S. (2011) Sulfatide-activated type II NKT cells prevent allergic airway inflammation by inhibiting type I NKT cell function in a mouse model of asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **301,** L975–L984 CrossRef Medline
22. Crowe, N. Y., Smyth, M. J., and Godfrey, D. I. (2002) A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J. Exp. Med.* **196,** 119–127 CrossRef Medline
23. Novak, J., and Lehuen, A. (2011) Mechanism of regulation of autoimmunity by iNKT cells. *Cytokine* **53,** 263–270 CrossRef Medline
24. Dalod, M., Chelbi, R., Malissen, B., and Lawrence, T. (2014) Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J.* **33,** 1104–1116 CrossRef Medline
25. Lutz, M. B., and Schuler, G. (2002) Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol.* **23,** 445–449 CrossRef Medline
26. Gregori, S., Tomasoni, D., Pacciani, V., Scirpoli, M., Battaglia, M., Magnani, C. F., Hauben, E., and Roncarolo, M. G. (2010) Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10–dependent ILT4/HLA-G pathway. *Blood* **116,** 935–944 CrossRef Medline
27. Kohm, A. P., McMahon, J. S., Podojil, J. R., Begolka, W. S., DeGutes, M., Kasprowicz, D. J., Ziegler, S. F., and Miller, S. D. (2006) Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J. Immunol.* **176,** 3301–3305 CrossRef Medline
28. Pham, M. N., von Herrath, M. G., and Vela, J. L. (2016) Antigen-specific regulatory T cells and low dose of IL-2 in treatment of type 1 diabetes. *Front. Immunol.* **6,** 651 CrossRef Medline
29. Klatzmann, D., and Abbas, A. K. (2015) The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat. Rev. Immunol.* **15,** 283–294 CrossRef Medline
30. Akbari, O., Stock, P., Meyer, E., Kronenberg, M., Sidobre, S., Nakayama, T., Taniguchi, M., Grusby, M. J., DeKruyff, R. H., and Umetsu, D. T. (2003) Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* **9,** 582–588 CrossRef Medline
31. Caielli, S., Conforti-Andreoni, C., Di Pietro, C., Usuelli, V., Badami, E., Malosio, M. L., and Falcone, M. (2010) On/off TLR signaling decides pro-inflammatory or tolerogenic dendritic cell maturation upon CD1d-mediated interaction with invariant NKT cells. *J. Immunol.* **185,** 7317–7329 CrossRef Medline
32. Palomares, O., Martín-Fontecha, M., Lauener, R., Traidl-Hoffmann, C., Cavkaytar, O., Akdis, M., and Akdis, C. A. (2014) Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β. *Genes Immun.* **15,** 511–520 CrossRef Medline
33. Akdis, M., and Akdis, C. A. (2014) Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J. Allergy Clin. Immunol.* **133,** 621–631 CrossRef Medline
34. Durant, L., Watford, W. T., Ramos, H. L., Laurence, A., Vahedi, G., Wei, L., Takahashi, H., Sun, H. W., Kanno, Y., Powrie, F., and O'Shea, J. J. (2010) Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity* **32,** 605–615 CrossRef Medline
35. Hoffmann, F., Ender, F., Schmudde, I., Lewkowich, I. P., Köhl, J., König, P., and Laumonnier, Y. (2016) Origin, localization, and immunoregulatory properties of pulmonary phagocytes in allergic asthma. *Front. Immunol.* **7,** 107 Medline
36. Lambrecht, B. N., and Hammad, H. (2009) Biology of lung dendritic cells at the origin of asthma. *Immunity* **31,** 412–424 CrossRef Medline
37. Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008) CTLA-4 control over Foxp3+ regulatory T cell function. *Science* **322,** 271–275 CrossRef Medline
38. Matsuda, H., Suda, T., Sato, J., Nagata, T., Koide, Y., Chida, K., and Nakamura, H. (2005) α-Galactosylceramide, a ligand of natural killer T cells, inhibits allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* **33,** 22–31 CrossRef Medline
39. Hachem, P., Lisbonne, M., Michel, M. L., Diem, S., Roongapinun, S., Lefort, J., Marchal, G., Herbelin, A., Askenase, P. W., Dy, M., and Leite-de-Moraes, M. C. (2005) α-Galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: role of IFN-γ. *Eur. J. Immunol.* **35,** 2793–2802 CrossRef Medline
40. Akbari, O., DeKruyff, R. H., and Umetsu, D. T. (2001) Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* **2,** 725–731 CrossRef Medline
41. Malek, T. R., and Castro, I. (2010) Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity* **33,** 153–165 CrossRef Medline
42. Barron, L., Dooms, H., Hoyer, K. K., Kuswanto, W., Hofmann, J., O'Gorman, W. E., and Abbas, A. K. (2010) Cutting edge: mechanisms of IL-2-dependent maintenance of functional regulatory T cells. *J. Immunol.* **185,** 6426–6430 CrossRef Medline
43. Setoguchi, R., Hori, S., Takahashi, T., and Sakaguchi, S. (2005) Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* **201,** 723–735 CrossRef Medline
44. Bateman, E. D., Hurd, S. S., Barnes, P. J., Bousquet, J., Drazen, J. M., FitzGerald, J. M., Gibson, P., Ohta, K., O'Byrne, P., Pedersen, S. E., Pizzichini, E., Sullivan, S. D., Wenzel, S. E., and Zar, H. J. (2008) Global strategy for asthma management and prevention: GINA executive summary. *Eur. Respir. J.* **31,** 143–178 CrossRef Medline
45. Dascher, C. C., and Brenner, M. B. (2003) Evolutionary constrains on CD1 structure: insights from comparative genomic analysis. *Trends Immunol.* **24,** 412–418 CrossRef Medline
46. Brossay, L., Chioda, M., Burdin, N., Koezuka, Y., Casorati, G., Dellabona, P., and Kronenberg, M. (1998) CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J. Exp. Med.* **188,** 1521–1528 CrossRef Medline
47. Kim, S., Lalani, S., Parekh, V. V., Wu, L., and Van Kaer, L. (2008) Glycolipid ligands of invariant natural killer T cells as vaccine adjuvants. *Expert Rev. Vaccines* **7,** 1519–1532 CrossRef Medline
48. Giaccone, G., Punt, C. J., Ando, Y., Ruijter, R., Nishi, N., Peters, M., von Blomberg, B. M., Scheper, R. J., van der Vliet, H. J., van den Eertwegh, A. J., Roelvink, M., Beijnen, J., Zwierzina, H., and Pinedo, H. M. (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 Medline

49. Gregory, L. G., and Lloyd, C. M. (2011) Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* **32,** 402–411 CrossRef Medline
50. Tomura, M., Yu, W. G., Ahn, H. J., Yamashita, M., Yang, Y. F., Ono, S., Hamaoka, T., Kawano, T., Taniguchi, M., Koezuka, Y., and Fujiwara, H. (1999) A novel function of $V\alpha14^+CD4^+$ NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J. Immunol.* **163,** 93–101 Medline
51. Bedoret, D., Wallemacq, H., Marichal, T., Desmet, C., Quesada Calvo, F., Henry, E., Closset, R., Dewals, B., Thielen, C., Gustin, P., de Leval, L., Van Rooijen, N., Le Moine, A., Vanderplasschen, A., Cataldo, D., *et al.* (2009) Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J. Clin. Invest.* **119,** 3723–3738 CrossRef Medline
52. Sakaguchi, S. (2004) Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* **22,** 531–562 CrossRef Medline
53. Li, P., Gao, Y., Cao, J., Wang, W., Chen, Y., Zhang, G., Robson, S. C., Wu, Y., and Yang, J. (2015) $CD39^+$ regulatory T cells attenuate allergic airway inflammation. *Clin. Exp. Allergy* **45,** 1126–1137 CrossRef Medline