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The role of iNKT cells on the phenotypes of allergic airways in a mouse model

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ABSTRACT

iNKT cells and mast cells have both been implicated in the syndrome of allergic asthma through their activation-induced release of Th2 type cytokines and secretion of histamine and other mediators, respectively, which can promote airways hyperresponsiveness (AHR) to agents such as methacholine. However, a mechanistic link between iNKT cells and mast cell recruitment or activation has never been explored. Our objective was to determine whether iNKT cells are necessary for the recruitment of mast cells and if iNKT cells can influence the acute allergen induced bronchoconstriction (AIB) caused by mast cell mediator release. To do so, we pharmacologically eliminated iNKT cells using a specific antibody (NKT-14) and examined its impact on airway inflammation and physiological phenotype. In mice treated with NKT-14, the elimination of iNKT cells was sufficient to prevent AHR and pulmonary eosinophilic inflammation elicited by administration of the iNKT cell agonist αGalCer. In mice treated with NKT-14 and then sensitized and challenged with house dust mite extract (HDM), eliminating the iNKT cells significantly reduced both AHR and AIB but did not affect pulmonary inflammation, the mast cell population, nor the release of the mast cell mediators mast cell protease-1 and prostaglandin D2. We conclude that while iNKT cells contribute to the phenotype of allergic airways disease through the manifestation of AIB and AHR, their presence is not required for mast cell recruitment and activation, or to generate the characteristic inflammatory response subsequent to allergen challenge.

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1. Introduction

The pathophysiology of asthmatic airways depends to a significant degree on bronchoconstriction, mast cell expansion and mast cell activation [1,2]. There are two distinct physiological hallmark reactions in allergic asthma: allergen induced bronchoconstriction (AIB) of the airway smooth muscles is the manifestation of an immediate allergen-induced activation of mast cells in the asthmatic lung within minutes of exposure, whereas airways hyperresponsiveness (AHR) is a clinical indicator provoked by challenge with agonists such as methacholine that is dominated by peripheral airway closure and that can linger for many days subsequent to allergen exposure or even be a stable phenotype of disease [3–5]. Hence, targeting the expansion and activation of mast cells to achieve adequate treatment of allergic asthma is an important research and therapeutic objective.

Invariant natural killer T (iNKT) cells share characteristics of both the adaptive and innate immune system. iNKT cells are also unique in that they respond to glycolipids, but not protein derived peptide antigens, presented by CD1d-expressing antigen-presenting cells [6,7]. iNKT cell activation is followed, within minutes, by the release of cytokines e.g. IL-4, IL-13, IL-17 and IL-22, which are implicated in asthma [8–10]. However, their release of Th1, Th2, and Th17 type cytokines promotes the function of several leukocyte

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populations bridging the innate and the adaptive immune responses [7,11], making it difficult to predict their role in every circumstance. Previous reports suggest that iNKT cells could function as important cellular mediators in asthma-like inflammatory models, namely the inflammatory response and the process of allergic sensitization suggesting that iNKT cells are required for induction of airway inflammation [6,12,13]. It has also been proposed that iNKT cells might play an important role in human asthma as well as in a mouse model of asthma and indeed iNKT cells have been shown to be increased in asthmatics and absence of iNKT cells can be protective against AHR in mice [6,14,15]. Furthermore, signaling from iNKT cells attracts mast cells [16–18], which has led to the suggestion that interfering with the iNKT cell – mast cell axis might have therapeutic benefits in human asthma [16]. Both mast cells and iNKT cells are stimulated by IL-33, leading to maturation and expansion of mast cells [19–21]. iNKT cells might contribute to the development and maintenance of innate immune responses via the ability of IL-33 to expand iNKT cell numbers and increase their release of IFNγ [22]. In addition, iNKT cells can release IL-9 that has been suggested to be involved in the recruitment and maturation of mast cells [23,24]. On the other hand, it seems like iNKT cell activation by the specific ligand α-GalactosylCeramide (αGalCer) can suppress allergically induced cytokine release and alterations in the breathing pattern [25]. It is, however, not known if iNKT cells are involved in the allergen-driven mast cell expansion.

Studies in animals have suggested that iNKT cells might be important in allergic asthma, which has been supported by findings of their presence in the lung of patients with asthma [6,21,26–29], although some human studies do not support this conclusion [30–32]. In addition, crosstalk between iNKT cells and mast cells has been suggested to regulate inflammation [33]. Uncertainties still remain, however, regarding the specific role of iNKT cells in the context of allergic airways disease, in particular with respect to their relationship to mast cell expansion in asthma. Rather than relying on iNKT cell knock-out mice, some of which have been shown to have issues with respect to their T cell receptor repertoire [34], we used an antibody targeting iNKT cells, allowing for specific depletion of this cell type [35].

We hypothesized that eliminating iNKT cells might reduce AIB, AHR, and mast cell numbers in the allergic lung. This hypothesis is supported by our recently published data showing that mice allergic to ovalbumin display protection against AHR if iNKT cells are eliminated [35]. Because AIB is mast cell dependent we used our house dust mite (HDM) model of asthma [36] to test whether iNKT cells are necessary for the recruitment of mast cells to the lung during allergy and if eliminating iNKT cells would have any effects on the phenotype of AIB in addition to AHR.

2. Methods

2.1. Animals

Female BALB/cJ 6–8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used beginning at 7–9 weeks of age. Housed in a specific pathogen free environment, the mice had free access to standard laboratory chow and water *ad libitum*, and were exposed to 12-h light/dark cycles. All experimental procedures were approved by the University of Vermont Institutional Animal Care and Use Committee.

2.2. Reagents

Pancuronium bromide was purchased from Fisher Scientific (Hampton, NH), HDM extract was purchased from Greer Laboratories (Lenoir, NC), phosphate buffered saline (PBS) was obtained from Life Technologies (Carlsbad, CA), isoflurane was obtained from Vedco Inc. (St Joseph, MO), and Sodium-pentobarbital was purchased from Penro Specialty Compounding (Colchester, VT). Anti-CD16/CD32, anti-CD3-FITC, and anti-CD45-Pacific Orange were purchased from BD Pharmingen (San Jose, CA), anti-cKit-APC and anti-FcεRI-PE were obtained from eBioscience (San Diego, CA). CD1d tetramer loaded with PBS57 was provided by the NIH tetramer facility. The monoclonal antibody targeting iNKT cells, NKT-14 was provided by NKT Therapeutics (Waltham, MA) and the corresponding isotype-control IgG2a antibody was obtained from BioXcell (West Lebanon, NH), α-GalactosylCeramide (αGalCer) was purchased from Funakoshi Co. Ltd., Japan.

2.3. Sensitization with HDM

Mice were sensitized with house dust mite (HDM) *Dermatophagoides pteronyssinus* extract (Greer, Lenoir, NC). HDM was resuspended in PBS at a protein concentration of 2.5 mg/ml. Our previous study demonstrated that this regimen produced not only AHR but also a mast cell-dependent AIB concomitant with lung mast cell expansion and mast cell degranulation products being released in the lung upon allergen exposure [36]. Mice were divided into two groups, experimental and control. Both groups of mice were anesthetized with isoflurane. While the experimental group was sensitized with intranasal instillation of 50 μl of the HDM suspension for 15 days over three consecutive weeks, the control group was sham sensitized with PBS. The animals showed no visible reactions relating to the HDM administration. The amount of lipopolysaccharide (LPS) in the HDM extract varies from batch to batch and we have calculated that the dose of LPS delivered to the mice in our study varied from 4.73 to 31.25 EU at each administration.

2.4. Treatment with NKT-14

Animals were treated with intra peritoneal (i. p.) injections once with NKT-14 or isotype control antibody, a non-specific IgG2a antibody, at a dose of 5 mg/kg. The antibodies were dissolved in PBS and administered three days before the start of HDM sensitization.

2.5. Assessment of acute bronchoconstriction and airways hyperresponsiveness

72 h after the last HDM intra nasal exposure, the mice were anaesthetized with i. p. sodium pentobarbital (90 mg/kg), the trachea cannulated and connected to a computer controlled small animal ventilator (flexiVent™, SCIREQ, Montreal, Canada), and ventilated at 200 breaths/minute with a stroke volume of 0.25 ml, adjusted for gas compression in the tubing resulting in a delivered tidal volume of about 0.20 ml. Next the mice were paralyzed with pancuronium bromide i. p. (0.8 mg/kg). The depth of anesthesia was monitored with EKG throughout the experiment as previously described [3,37,38]. The animals were stabilized over about 10 min of regular ventilation at a positive end-expiratory pressure (PEEP) of 3 cmH_2O. A standard lung volume history was then established by delivering two total lung capacity maneuvers (TLC) to a pressure limit of 30 cmH_2O and holding for 3 s. Next, two baseline measurements of respiratory input impedance (Z_{rs}) were obtained. The mice were then exposed to either nebulized saline, methacholine or HDM via an in-line piezo-electric nebulizer (Aeroneb®) as described previously [36] and detailed in *2.7* and *2.8* below.

2.6. αGalCer induced AHR

Mice were treated with a single 2 μg αGalCer in 50 μl PBS +

0.05% Tween-20 or 50 μl control PBS + 0.05% Tween-20 via oropharyngeal inhalation (o. p.). Mice were either treated with i. p. NKT-14 or isotype control IgG2a three days before the αGalCer administration. The mice were tested for AHR and BALF was collected one day after the αGalCer challenge.

2.7. Assessment of HDM induced bronchoconstriction (AIB)

Seventy-two hours after the last HDM exposure, the mice were connected to the flexiVent as described above and were then tested for AIB. Following stabilization and standard lung volume history as described above, the mice were either exposed to an inhalation of vehicle control dose of PBS over 40 s followed by a Z_{rs} measurements every 10 s for 3 min; or the mice received 40 s of aerosolized HDM (2.5 mg/ml) delivered via the in-line piezo-electric nebulizer. The inhalation was immediately followed by Z_{rs} measurements every 10 s for 3 min. A Z_{rs} measurement takes 3 s; 1 s for exhalation to PEEP and 2 s to deliver the measurement oscillation leaving 7 s for regular ventilation before the next measurement cycle starts. Lungs were harvested and snap-frozen in liquid nitrogen after the AIB assessment and saved for later cytokine measurements.

2.8. Assessment of AHR

Mice were sensitized with HDM and surgically prepared as described above in *2.5*. Following stabilization and standard lung volume history as described above, mice were exposed to an inhalation of aerosolized PBS (vehicle control) for 10 s. Z_{rs} was then measured every 10 s for 3 min. This complete sequence of maneuvers and measurements was then repeated for aerosol exposures to three incremental doses of methacholine (3, 12 and 25 mg/ml) [39]. Lungs were harvested and prepared for flow cytometry.

2.9. Calculation of respiratory mechanics

Z_{rs} was determined using the forced oscillation technique described previously [3,39]. Briefly: Z_{rs} over the frequency range 1–20.5 Hz was determined using a 2 s broadband perturbation in volume applied by the flexiVent™. Each determination of Z_{rs} was fit with the constant phase model of impedance [40] given by

$$Z_{rs}(f) = R_n + i2\pi fI + \frac{G - iH}{(2\pi f)^{\alpha}}$$

where R_n is a frequency independent Newtonian resistance reflecting that of the conducting airways, I is airway gas inertance (in the mouse I is negligible), G characterizes tissue dampening, H characterizes tissue stiffness or elastance, i is the imaginary unit, and f is frequency in Hz [40–42].

2.10. Collection of bronchoalveolar lavage fluid and lung specimens

After the AIB measurement was completed broncho-alveolar lavage fluid (BALF) was collected. Lungs were lavaged with 1 ml of 1X Dulbecco's PBS, and the volume of bronchoalveolar lavage fluid (BALF) collected was measured for each sample, and the total number of leukocytes was counted using an automated cell counter (Advia, Siemens Healthcare Inc.). The cell pellet was separated from the supernatant, re-suspended in 400 μl of PBS, and counted. Differential cell counts were determined by spinning the cells onto glass slides with a Cytospin and staining with hematoxylin and eosin.

2.11. Protein extraction protocol

Frozen lung tissue (from lungs after AIB measurement) was pulverized to a fine powder using a liquid nitrogen-chilled mortar and pestle, transferred to the 100-μl mark of a liquid nitrogen-chilled 1.5-ml Eppendorf tube, and lysed by adding 400 μl of cell extraction buffer [PBS containing 0.5% Triton X-100 and 1 × protease inhibitor cocktail (Sigma, St. Louis, MO)] and allowing the tubes to warm to the temperature of wet ice. The mixture was vortexed and incubated on ice for 30 min with occasional vortexing. The cell lysates were clarified by centrifugation at 13,000 g at 4 °C for 10 min. The clarified cell extracts were transferred to clean microcentrifuge tubes, which were stored at −80 °C until ready for analysis. Protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA), protein concentration was equilibrated using cell extraction buffer, and cell extracts were diluted at least 1:10 in Standard Diluent Buffer before analysis by Luminex. Mast cell protease-1 (mMCP-1) was analyzed using ELISA (eBioscinece, San Diego, CA). The concentration of mMCP-1 in the BALF was related to a standard curve made by pooling samples from four different lungs and diluting in steps creating a standard curve of arbitrary values. The sample concentrations are thus reported as arbitrary units (AU). BALF concentration of prostaglandin D2 (PGD2) was analyzed by ELISA (Cayman, Ann Arbor, MI).

2.12. Enzymatic digestion and mast cell enumeration

Lungs were harvested post AHR and lung cells in freshly prepared enzyme mix (Miltenyi Biotec, Auburn, CA) were mechanically digested using a GentleMACs dissociator (Miltenyi Biotec) using the factory provided onboard programs: four rounds of program m_lung_01 (8 s and 300 total rounds per run), followed by a 15 m incubation at 37°, then two rounds of program "B", a second 15 m incubation at 37° (38 s and 2083 total rounds per run), and, finally, a single round of m_lung_02 (31 s and 553 total rounds per run). A short centrifugation was performed to collect cells, which were then re-suspended in PBS and filtered through a 70 μm filter. After washing the cells in PBS, red blood cells were removed with ACK lysing solution (8024 mg/l NH_4Cl, 1001 mg/l $KHCO_3$, 7.722 mg/l EDTA•$Na_2 \cdot 2H_2O$). Cells were washed, counted with an Advia 120 (Siemens, Munich, Germany) and re-suspended in PBS in preparation for antibody staining and flow cytometric analysis.

2.12.1. Surface staining and flow cytometric analysis

2×10^6 lung cells were stained with UV live/dead discriminator (Invitrogen, Grand Island, NY) and then resuspended in FACS staining buffer (PBS + 2% FCS, 0.2% sodium azide). Fc receptors were blocked using TruStain FcX (Biolegend), followed by staining with anti-CD3-FITC (Biolegend, San Diego, CA), anti-CD45-Pacific Orange (Invitrogen, Grand Island, NY), anti-ckit-APC and anti-FcεRI-PE (eBioscience, San Diego, CA), anti-TCR beta, and CD1d tetramer\PBS57. Data were collected on an LSR II (BD Biosciences), and analysis was performed using FlowJo (Tree Star, Ashland, OR) software. Mast cells were defined as $CD3^-ckit^+Fc\varepsilon RI^+$ cells [43]. Backgating demonstrated that these cells were of intermediate side scatter.

2.13. Data analysis

Origin 8.0 (Origin Lab, Northampton, MA) and Graph Pad Prism 7.01 (GraphPad Software, Inc, LaJolla, CA) were used for analysis, and data are expressed as means ± SEM. ANOVA followed by Tukey's post-hoc test was used to determine statistical significance. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Direct stimulation of iNKT cells

iNKT cells can be stimulated by the specific CD1d ligand αGalCer, thus we decided to first examine whether αGalCer and the iNKT targeting antibody, NKT-14, would have effect on the respiratory phenotype. BALB/c mice pretreated with the isotype control antibody IgG2a or NKT-14 were challenged o. p. with the iNKT cell stimulating glycosphingolipid αGalCer. In Fig. 1A it is shown that αGalCer induced a robust increase of AHR that was reminiscent of the AHR produced by allergen sensitization, i.e. significant increases of R_n, G and H in the IgG2a control treated mice. In the NKT-14 treated mice, the lung mechanics parameters were significantly reduced and comparable to the levels seen in control mice (see Fig. 3A). The BALF displayed an increase of eosinophils that was significantly reduced by NKT-14 (Fig. 1B), although the levels of eosinophils after NKT-14 treatment appeared higher than in non-sensitized mice (see Fig. 3B for comparison). Thus, eliminating the iNKT cells with NKT-14 was sufficient to significantly diminish the AHR and inflammation resulting from the αGalCer challenge.

3.2. iNKT cells depletion

Following sensitization with HDM, we harvested lung tissue to quantify iNKT cells using flow cytometry (Fig. 2). We found that HDM sensitization caused a significant increase in lung iNKT cells and that NKT-14 treatment practically eliminated the lung iNKT cell population while the isotype control antibody had only a partial effect. While NKT-14 significantly reduced the iNKT cell population, we discovered about 10% of tissue-associated iNKT cells remained in the lungs. As a control, we harvested spleens and found that spleen iNKT cells were eliminated to an extent similar to that observed in the lung; the control isotype IgG2a only partially reduced the frequency of iNKT cells in the spleen. These data indicate that the NKT-14 antibody eliminated about 90% of the lung and spleen iNKT cells from HDM-sensitized mice.

3.3. Airways hyperresponsiveness and inflammation

The assessment of AHR was performed using incremental doses of inhaled nebulized methacholine. In Fig. 3A we show that the R_n of PBS control mice increased with methacholine, whereas G and H were virtually unaffected. All mice sensitized with HDM demonstrated a dose dependent response that was elevated compared with PBS controls, but mice pretreated with NKT-14 were significantly less sensitive to methacholine in terms of G and H; hence, NKT-14 reduced the HDM induced AHR.

After the mechanics measurement, BALF was obtained. In Fig. 3B it is shown that cell totals were significantly increased in sensitized mice whether treated with NKT-14, isotype control, or not. Mice that had been sham sensitized or were naïve did not have increased numbers of leukocytes and differential analysis of BALF cells revealed significant increases of eosinophils that were not affected by NKT-14 treatment.

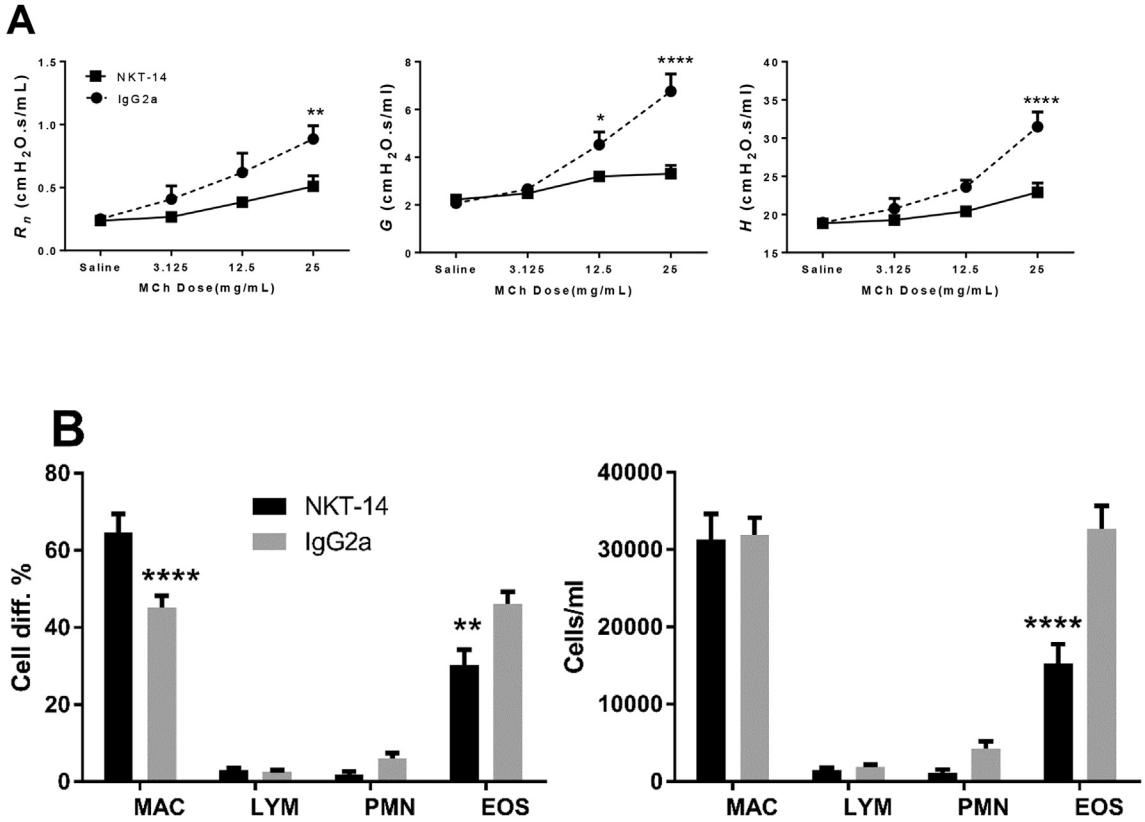

Fig. 1. Effects of NKT-14 on iNKT dependent AHR. Mice were treated with NKT-14 or control IgG2a and three days later challenged with a single dose of oropharyngeal inhalation of αGalCer (2 μg in 50 μl PBS) and 24 h later AHR was assessed with incrementing doses of inhaled methacholine. **A**) NKT-14 significantly inhibited the AHR all lung function parameters R_n, G and H induced by iNKT cell activation with the specific ligand αGalCer. **B**) Cell differentials in BALF demonstrated elevated numbers of eosinophils that were partially reversed by NKT-14. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

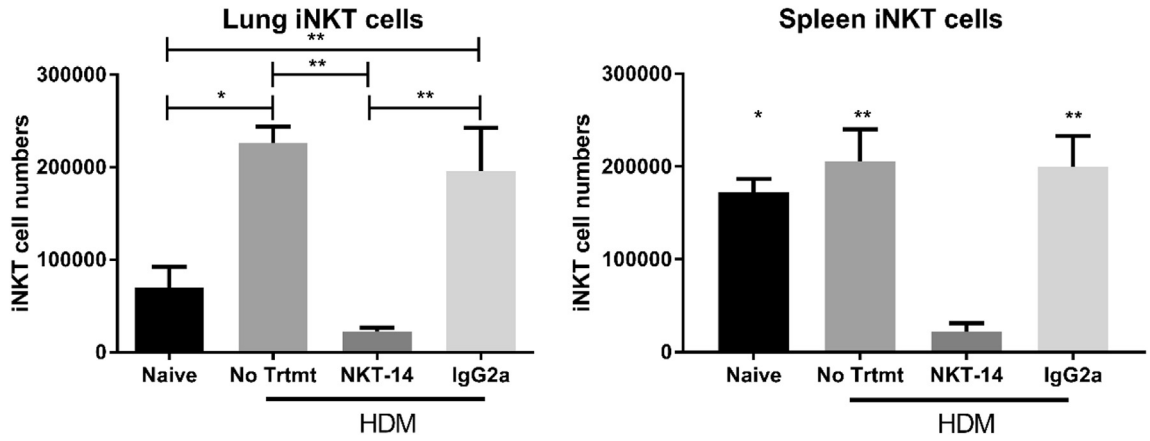

Fig. 2. iNKT cells in lungs and spleen. Mice were treated i. p. with NKT-14, IgG2a (control Ab) or no treatment and then sensitized with HDM. Lungs and spleens were harvested and processed for flow cytometry. NKT-14 significantly reduced the iNKT in both lung and spleen. *p < 0.05, **p < 0.01.

3.4. Mast cell population and lung cytokines

Mast cells are key players in generating the physiological lung phenotype as they are rapid responders to inhaled allergens. We hypothesized that the lung mast cell population would be dependent on iNKT cells; hence, we determined the number of mast cells using flow cytometry in mice sensitized with HDM following NKT-14 treatment (Fig. 3C). We found, however, no evidence of reduced mast cell numbers in NKT-14 treated mice. The isotype control IgG2a was also without effect on the mast cell number. Analysis of the total numbers of lung tissue leukocytes showed that mice sensitized with HDM had increased numbers of lung tissue leukocytes but that NKT-14 was without effect on the cell numbers (Fig. 3C). We confirmed our previous findings [36] that naïve mice have almost no lung mast cells.

Following euthanasia, the upper right-hand lobe of the lung was collected, snap-frozen in liquid nitrogen, and later analyzed for selected cytokines. In Fig. 4 it is shown that only IL-33 was significantly increased by HDM exposure whereas IL-10 was significantly decreased. However, neither NKT-14 nor the isotype control IgG2a antibody had any detectable effects on these or any other lung tissue cytokine.

3.5. Allergen induced bronchoconstriction

Next, we determined the effects of NKT-14 on AIB during allergy. These studies were performed in HDM sensitized mice who received an inhalation of nebulized HDM while being ventilated and lung mechanics immediately measured. As presented in Fig. 5A HDM sensitized mice responded with an increase in R_n that we interpret as a constriction of the conducting airways caused by mast cell mediator release, as previously demonstrated [36]. NKT-14 limited the R_n increase; however, we saw a similar effect with the isotype control antibody. The negative control mice (sham sensitized with PBS) did not respond to inhaled HDM. We also observed an increase in *H*, reflecting the stiffness of the lung. We found an increase in *G* in sensitized mice that was partially inhibited by NKT-14 but not by the isotype control antibody. The BALF from mice tested for AIB was also analyzed for mMCP-1 and PGD2 as indicators of mast cell mediator release. Both mMCP-1 and PGD2 were significantly increased by HDM inhalation but neither were significantly affected by iNKT cell elimination (Fig. 5B).

4. Discussion

We have recently developed a mast cell dependent model of allergic asthma in mice that in addition to the commonly studied AHR, Th2 cytokine profile, and cellular airway inflammation, also displays allergen-inducible mast cell-dependent bronchoconstriction [36], emulating what might be observed in the airways of a patient with allergic asthma. In the present study, we show that NKT-14, an antibody specifically targeting iNKT cells, effectively eliminated iNKT cells from the lung. In our previous study we demonstrated that the HDM induced bronchoconstriction was indeed mast cells dependent because mast cell deficient mice did not demonstrate any AIB [36].

We have also demonstrated that the elimination of iNKT cells using NKT-14 was effective, and that it did not affect the total number of leukocytes in lung tissue, supporting the notion that NKT-14 is specific for iNKT cells [35,44]. In this study, we show that targeting iNKT cells with NKT-14 also translated to an elimination of αGalCer induced AHR, further illustrating the specificity and impact of NKT-14. In this context we noticed that αGalCer induced an increase of eosinophils in BALF which was significantly reduced by NKT-14, further supporting the notion that iNKT cells could be responsible for recruitment of other inflammatory cells. NKT-14 did not return the eosinophil numbers back to baseline and we speculate that this could be due to incomplete elimination of iNKT cells. iNKT cells have previously been suggested to be important for AHR in the context of allergy [35], and our experiment using αGalCer illustrates that iNKT cells are capable of generating an AHR with strong similarities with allergically generated AHR in terms of the pattern of the parameters R_n, *G* and *H*. This observation of similarity should, however, not be interpreted to mean that iNKT cells are critical for generating AHR or asthma. We found that while eliminating iNKT cells reduced AHR in HDM allergic mice (*G* and *H*, but not R_n), AHR did not return to baseline (Fig. 3). Hence, iNKT cells might play a partial role for AHR during allergy, or this observation could suggests that the small number of iNKT cells that appeared to remain after NKT-14 treatment (about 10%) were sufficient to induce AHR. In a similar manner, we tested allergic mice for AIB (Fig. 5), and again, elimination of iNKT cells offered partial protection. In this model, the AIB response is mast cell driven and typically dominated by a response of the conducting airways, i.e. R_n, as we previously demonstrated [36]. The AIB is significant for being primarily a response in the conducting airways and as such demonstrated by an increase in R_n upon stimulation with an

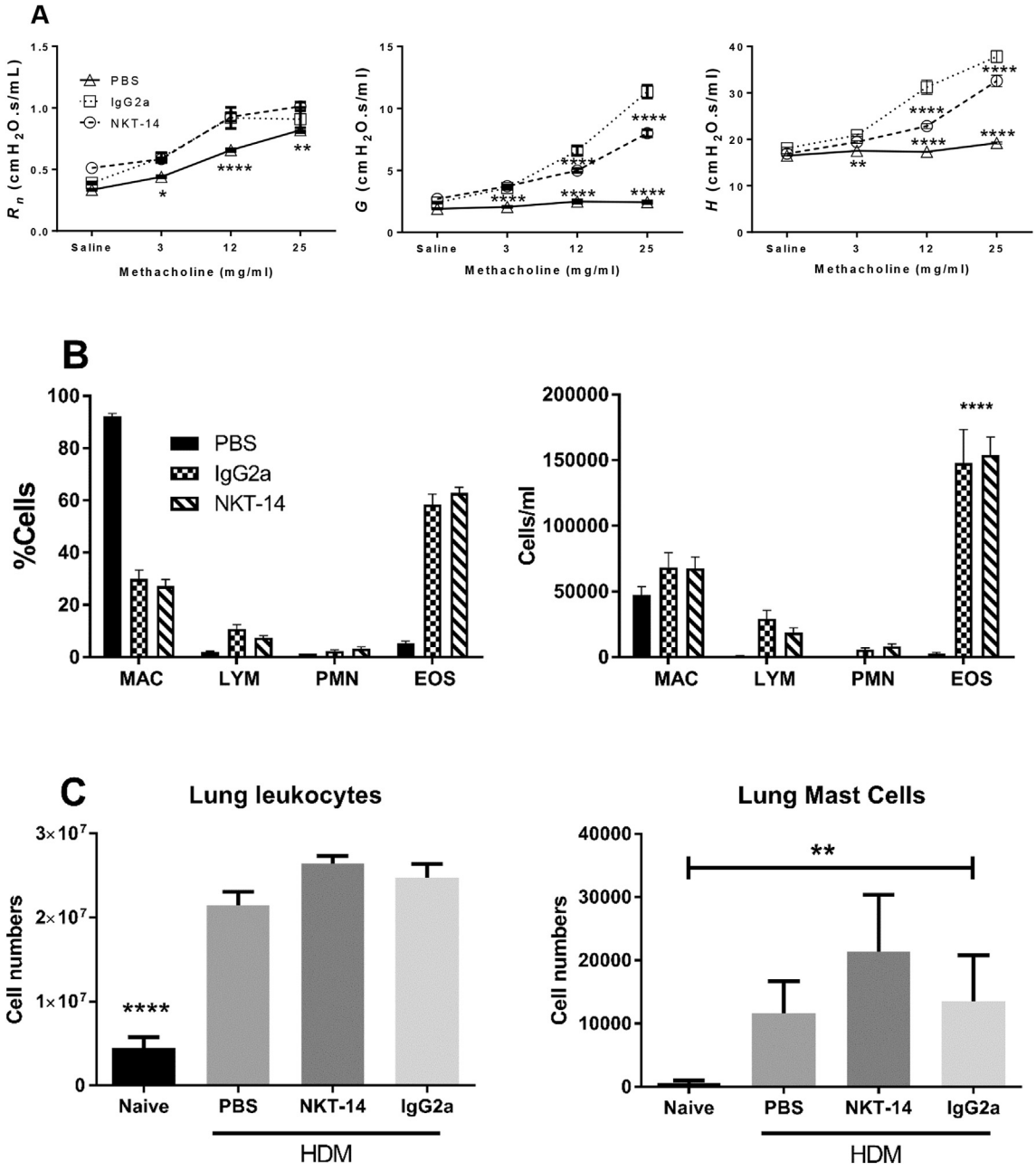

Fig. 3. Effects of NKT-14 on HDM induced AHR and BALF cells. Mice were treated i. p. with NKT-14, IgG2a (control Ab) or no treatment and then sensitized with i. n. HDM; one group received sham sensitization with i. n. PBS. 72 h later, AHR was assessed with incrementing doses of inhaled methacholine. **A**) Both groups treated with IgG2a and NKT-14 had significantly increased responses compared with control PBS, however NKT-14 treated mice had significantly lower responses in *G* and *H* compared with IgG2a treated mice. **B**) After euthanasia, mice were lavaged with PBS. HDM sensitization significantly increased the number of eosinophils (EOS) but this was not affected by neither NKT-14 nor IgG2a treatment. **C)** Following the AHR assessment lung tissue was harvested and prepared for flow cytometry. Total lung leukocytes were significantly increased by sensitization with HDM and neither NKT-14 nor IgG2a had any significant effects on lung tissue leukocyte numbers. Lung tissue mast cells were increased by sensitization with HDM but neither NKT-14 nor IgG2a had any effect on the mast cell numbers. A totally naïve group of mice, not exposed to methacholine, was also included to determine baseline lung leukocytes and mast cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

allergen. Our data demonstrate that this happens with HDM and is reduced by elimination of the iNKT cells. Depletion of 90% of iNKT cells using NKT-14 did not influence mast cell recruitment to the lung, suggesting that iNKT cells exhibit little influence over mast cell recruitment. However, we cannot rule out the possibility that the small number of iNKT cells remaining after depletion was sufficient to mediate an effect on mast cell recruitment. Additional experiments would be needed to rule out this possibility.

The cytokine release in allergic models often displays a Th2 profile, i.e. characterized by a release of IL-4, IL-5 and IL-13, all of which have been connected with the development of AHR. In contrast with other studies using B6. Jα18$^{-/-}$ mice (deficient in type I NKT cells but not type II NKT cells) or CD1d$^{-/-}$ mice [14,28], we did not detect any effects of iNKT depletion on lung cytokines. It should be noted, however, that there has been issues identified with the B6. Jα18$^{-/-}$ mice because of their impaired TCRα diversity, suggesting that results obtained with this particular strain of mice must be interpreted with a great deal of caution or even reevaluated as

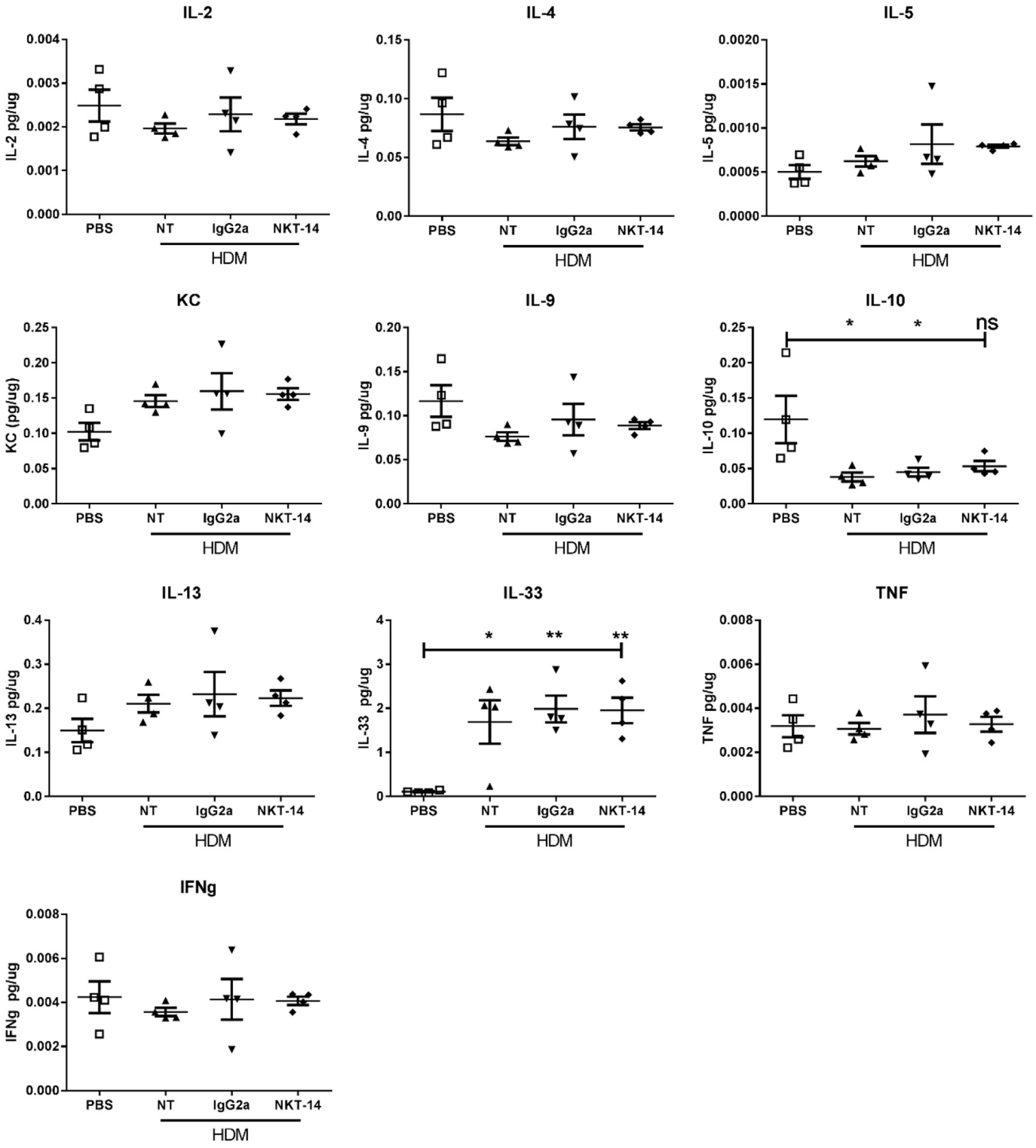

Fig. 4. Effects of NKT-14 on lung tissue cytokines. Mice were treated i. p. with NKT-14, IgG2a (control Ab) or no treatment (NT) and then sensitized with i. n. HDM; one group received sham sensitization with i. n. PBS. 72 h later mice were challenged with HDM, AIB measured and then lungs were harvested and cytokines measured. HDM sensitization and challenge significantly increased tissue IL-33 and also decreased IL-10. All other changes in cytokine levels were insignificant. $^{*}p < 0.05$, $^{**}p < 0.01$, non-significant (ns).

suggested by Bedel et al. [34]. Bedel et al. showed that the genetic modifications in the B6. J$\alpha 18^{-/-}$ mice were not limited to the iNKT cells but had widespread consequences on the T cell population in general. In this context, it is important to note that NKT-14 is specific for iNKT cells but does not affect the type II NKT cells [44]. The possibility to use a specific agent like NKT-14 therapeutically to deplete a cell type in a model is significantly different from studying a model in which the cell type never existed due to a gene knockout. By using a specific antibody targeting iNKT cells we avoided the issues of life-long depletion and also avoided the non-

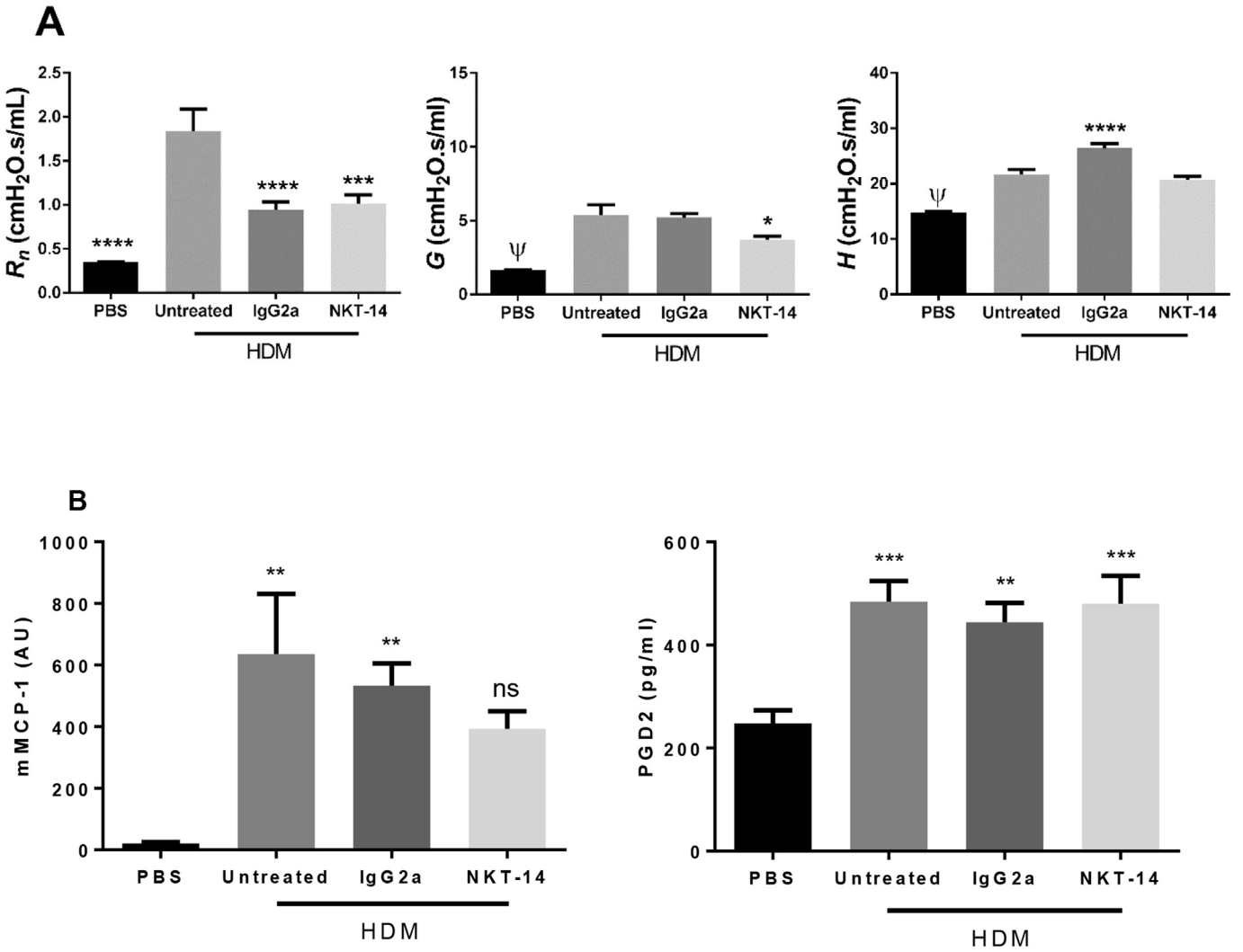

Fig. 5. Effects of NKT-14 on AIB and mast cell mediators. A) Mice were treated i. p. with NKT-14, IgG2a (control Ab) or no treatment and then sensitized with i. n. HDM; one group received sham sensitization with i. n. PBS. 72 h later, mice were given inhaled nebulized HDM and the resulting AIB was measured. The HDM challenge induced a significant R_n increase in HDM sensitized mice over control PBS, interpreted as a bronchoconstriction, this was significantly reduced by NKT-14 and IgG2a. Likewise HDM induced significant increases in *G* and *H*. NKT-14 partly but significantly reduced the response in *G*, but did not affect *H*. **B**) The levels of mMCP-1 and PGD2 in BALF were measured with Elisa. Mice sensitized with HDM expressed significantly elevated release of mMCP-1 in untreated and control treated (IgG2a) mice but not in NKT-14 treated animals. Elimination of the iNKT cells with NKT-14 was without significant effect. PGD2 levels were significantly increased in all groups and NKT-14 was without effect $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$, $^{\psi}p<0.0001$ (PBS vs. Untreated), non-significant (ns).

specific effects that could arise from using a genetically modified mouse strain. In this context we also note that our model using high doses of HDM produced a very different phenotype characterized by the physiological response (AIB) and mast cell recruitment, but also the conspicuous lack of several cytokines compared with the traditional OVA model, as exemplified by Akbari et al. [14]. While we do not know what might be the mechanism behind these differences, one could speculate that iNKT cells could work via a mechanism that is independent of e.g. IL-5 and IL-13.

We did observe significant increases of IL-33 in the lung lysates from all sensitized groups. We analyzed the cytokines in homogenized lung tissue and we speculate that the IL-33 levels reflect both secreted as well as internally stored IL-33 that was released during tissue processing. Nevertheless, eliminating iNKT cells did not have any effect on the production of IL-33 illustrating that iNKT cells are likely not essential for the production of this cytokine in the lung, at least not as a consequence of allergic sensitization and challenge. In this study, we did not observe any significant changes in the traditional Th2 cytokines. It is, however, possible that the harvest at 72 h post the last allergen challenge could be too late to recover any of these induced cytokines.

We analyzed mMCP-1 and PGD2 to determine if HDM inhalation acutely released mast cell products into the airways. While mMCP-1 and PGD2 were significantly elevated in sensitized mice, we conclude that the influence of iNKT cells was minimal. We have previously shown that HDM triggers the release of mMCP-1 and histamine in lungs with expanded mast cells and that this coincided with bronchoconstriction [36]. Hence this finding illustrates that our model behaved as expected with the additional observation that PGD2 release is similarly affected by HDM sensitization and challenge. What appears to be a contrast between the physiological effect (reduced AIB) and mast cell mediator release (mMCP-1 and PGD2) might be a reflection of the fact that NKT-14 did only partially, albeit significantly, reverse the HDM induced AIB. It is also possible that iNKT signaling to the mast cells only affects a limited set of mast cell derived mediators and that mMCP-

1 and PGD2 are not among those affected.

We acknowledge the limitations with our study. The mice received a single injection of NKT-14 and while the flow cytometry analysis showed a significant reduction of iNKT cells, there was a remaining small population that could potentially be iNKT cells, suggesting that NKT-14 did not fully eliminate these cells. In our previous study [35], we administered a second dose at day 14 but this did not further diminish lung iNKT cell numbers. Consequently, we decided to commence with a single dose of NKT-14 in this study. Whatever the nature of these remaining 10% of cells, it is unlikely that such a small remnant population would be responsible for the lack of efficacy in regard to IL-33 and mast cell response observed in this study. In other studies of inflammatory models, we have found reduction of iNKT cells in target tissue as low as 70% has had therapeutic efficacy (unpublished observations).

In this study, we also found that the isotype IgG2a had some effects on the outcome variables. While we do not have any explanation for this, we feel that it would be beyond the scope of the present study to further elucidate the IgG2a effects. That being said, our observations raise a general question about how to best identify a proper control antibody in *in vivo* models. Interestingly, the effects of the IgG2a were limited to the respiratory mechanics but did not affect the lung lymphocyte population, iNKT cells or the cytokines.

We recently published that iNKT cell elimination with NKT-14, in a traditional model using ovalbumin (OVA) as the sensitizing allergen, can reduce AHR if NKT-14 was administered before allergen exposure. However, we also found that iNKT cells were not essential for AHR in mice with an established allergy. Briefly, when the mice were first sensitized and then later had iNKT cells eliminated, the AHR was largely unaffected [35]. Despite the effects on AHR in pretreated mice, we did not observe any effects on cellular inflammation nor did the treatment with NKT-14 affect lung cytokines. The effects of NKT-14 on the lung function parameters in the present study were not as pronounced as observed in the OVA-model, raising a couple of possibilities on the role of iNKT cells. One possibility is that allergy is not the only factor affecting the development of AHR. It has been shown that LPS can induce AHR in the context of allergic sensitization [45] and because LPS is present in HDM extract, this is certainly a possibility, although the LPS levels are relatively low. Another possibility is that the robustness of the HDM induced inflammation (long-term and high dose) overrides any effects that removal of iNKT cells might have had. HDM extract is a complex cocktail from the entire house dust mite and its fecal matter, which includes not only protein epitopes but also contains various enzymes, chitin and lipopolysaccharides, all with the potential to affect the airways; whereas OVA is a purified protein antigen that has been shown to produce a well-defined Th2 response [42]. Indeed, it has been shown that HDM can facilitate OVA sensitization, probably involving a GM-CSF mechanism [46–48]. Thus, it is possible that the effects of iNKT cell elimination on the physiological outcomes were varied due on the sensitization model used (e.g. HDM or OVA) and a possible effect of adjuvant cannot be excluded either. One could speculate that AHR caused by HDM extract and similar complex allergens depends on mechanisms partly unrelated to allergy and Th2, e.g. by affecting the epithelial lining leading to increased permeability, which we have shown can lead to increased AHR by simply altering the kinetics of the methacholine-smooth muscle interaction [49]. Indeed, the primary source for IL-33 in the lung is likely the epithelial cells [50] and HDM extract has been shown to induce an asthma-like phenotype via Toll-like receptor 4 [51]. If the same is true in human asthma, it could help in our understanding of why some asthma is refractory to steroid treatment, because steroids would likely have limited effects on epithelial integrity damage not caused by an immunological response.

In summary, we have demonstrated that NKT-14 effectively eliminated iNKT cells in the lung and that this significantly reduced AHR and AIB. However, we also conclude that iNKT cells do not influence mast cell recruitment to the lung. Consequently, the effects of iNKT cells on the AIB is likely via either direct effects on the smooth muscle or via modifying signaling to the mast cells.

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