

ORIGINAL ARTICLE

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Histamine receptor 2 modifies iNKT cell activity within the inflamed lung

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Abstract

Background: Histamine is a key immunoregulatory mediator and can dampen proinflammatory responses via activation of histamine receptor 2 (H₂R). The aim of this study was to determine the role of H₂R in modulating lung inflammatory responses.

Methods: H₂R was blocked using famotidine or activated using dimaprit in both the ovalbumin (OVA) and house dust mite extract (HDM) murine models of respiratory inflammation. H₂R-deficient animals and CD1d/H₂R-deficient animals were utilized to examine the CD1d presentation of lipid antigens (α GalCer or OCH) to invariant natural killer T (iNKT) cells.

Results: Famotidine treatment resulted in more severe airway disease in the OVA model, while dimaprit treatment significantly reduced disease severity. Both OVA and HDM-induced airway diseases were more severe in H₂R-deficient animals. Flow cytometric analysis of lung tissue from H₂R-deficient animals revealed increased numbers of CD1d⁺ dendritic cells and increased numbers of iNKT cells. In vitro, α GalCer-stimulated iNKT cells from H₂R-deficient mice secreted higher levels of IL-4, IL-5, and GM-CSF. In vivo, α GalCer or OCH administration to the lung resulted in enhanced mucus secretion, inflammatory cell recruitment, and cytokine production in H₂R-deficient or famotidine-treated animals, while dimaprit dampened the lung iNKT cell response to α GalCer. Removal of iNKT cells in H₂R-deficient (CD1d^{-/-} H₂R^{-/-}) animals normalized the lung response to HDM.

Conclusion: The deliberate activation of H₂R, or its downstream signaling molecules, may represent a novel therapeutic target for chronic lung inflammatory diseases, especially when CD1d-mediated presentation of lipid antigens to iNKT cells is contributing to the pathology.

KEYWORDS

allergy, CD1d, histamine, inflammation, invariant natural killer T cells

Abbreviations: α -GalCer, alpha-galactosyl ceramide; BAL, bronchoalveolar lavage; cAMP, cyclic adenosine monophosphate; Epac, exchange protein directly activated by cAMP; GPCR, G protein-coupled receptor; HDM, house dust mite extract; H₂R, histamine receptor 2; iNKT, invariant natural killer T cell; IFN- γ , interferon-gamma; IL, interleukin; OVA, ovalbumin; T_H, T helper cell; TLR, toll-like receptor

1 | INTRODUCTION

Histamine (2-[4-imidazolyl]-ethylamine) is a short-acting endogenous amine, which is widely distributed throughout the body. Originally

discovered more than 100 years ago, histamine is well recognized for its effects in the immediate-type hypersensitivity response including essential roles in increasing vascular permeability, smooth muscle contraction, activation of nociceptive nerves, wheal and flare reaction, and itch response.^{1,2} In addition, histamine exerts immunoregulatory effects by influencing multiple cell types including dendritic cells, natural killer cells, epithelial cells, T lymphocytes, and B lymphocytes.^{3,4} The regulatory nature of histamine in immunology is dependent on its binding to four subtypes of histamine receptors, which are G protein-coupled receptors and are named chronologically in order of their discovery—H₁R–H₄R.

Asthma is characterized by recurrent and reversible airflow obstruction with airway inflammation central to its pathogenesis. Exogenous histamine induces bronchoconstriction, while histamine receptor activation on both immune and tissue cells within the lung has been shown to be a component of the disease severity.^{5–7} Deletion of H₁R has been shown to reduce allergic responses in a murine model of asthma, while H₁R receptor antagonists inhibit T_H2 responses, the induction of allergic pulmonary inflammation, and bronchial hyper-responsiveness in murine models.^{8,9} Anti-H₁R therapy alone has modest effects in humans with established bronchial asthma; however, the combination of anti-H₁R with other pharmacological agents, for example, antileukotrienes, is superior to either agent alone.¹⁰ H₄R has also been shown to play a detrimental role in murine models of respiratory inflammatory diseases, but the use of anti-H₄R compounds in asthma patients has not been tested to date.^{11,12}

In contrast to H₁R and H₄R, H₂R activation is associated with regulatory immune responses and in many cases can block the effects of H₁R activation. H₂R can suppress a range of immune system activities such as mast cell degranulation, antibody synthesis, dendritic cell responses to TLR stimulation, T_H1 cytokine production, and T-cell proliferation.^{13–15} H₂R-deficient mice display defects in gastric and immune regulatory functions, while mucosal expression of H₂R is critical for immunomodulatory responses to bacterial-derived histamine and mucosal expression of H₂R increases in inflamed tissue.^{16–19} However, the influence of H₂R on allergic lung inflammation has not been well characterized. In this report, we describe that the blocking or loss of H₂R exacerbates respiratory inflammation, while activation of H₂R suppresses lung inflammatory responses. In addition, we describe for the first time an immunoregulatory role for H₂R in restraining invariant natural killer T (iNKT) cell proinflammatory responses within the lung.

2 | METHODS

2.1 | Animal models

Female BALB/c mice aged 6–8 weeks were obtained from Charles River (Sulzfeld, Germany) and housed at AO Research Institute Davos at least 2 weeks before experiments started. BALB/c H₂R-deficient mice were obtained from Prof. Takeshi Watanabe (Kyoto University, Japan) and were bred at AO Research Institute in Davos. CD1d-deficient mice (strain C.129S2-Cd1tm1Gru/J) were purchased

from Charles River and were bred at the AO Research Institute Davos. H₂R-deficient and CD1d-deficient animals were mated over several generations to obtain H₂R/CD1d-deficient animals. Mice were housed four to six animals per cage in individually ventilated cages in a 12/12-h light/dark cycle with food and water available *ad libitum*. Mice of different genotypes were co-housed for at least 2 weeks before the start of the experiments. All experimental procedures were carried out in accordance with Swiss law.

Mice were sensitized by intraperitoneal (ip) injection of 20 µg of ovalbumin (OVA) grade VI (Sigma-Aldrich, Buchs, Switzerland) emulsified in 500 µg alum (Pierce, Rockford, IL, USA) in 200 µL sterile 0.9% isotonic sodium chloride (NaCl) on days 0, 7, and 21, followed by 20 minutes 1% OVA grade V (Sigma-Aldrich) aerosol exposures on days 26, 27, and 28. Additionally, mice received 5 mg/kg famotidine, 100 mg/kg dimaprit (dissolved in NaCl), or NaCl ip, 30 minutes before each OVA injection and OVA aerosol exposure. Negative control animals received NaCl and alum injections and were exposed to the OVA aerosolization. Analysis of mice occurred 24 hours after the last aerosol challenge. OVA-specific serum IgE levels were measured using a commercially available ELISA kit (Bio-Rad, Puchheim, Germany).

In addition, a house dust mite model of allergic airway inflammation was used. Briefly, mice were sensitized intranasally (in) with 1 µg of HDM extract (Greer Laboratories, Lenoir, NC, USA) on day 0 and challenged with 10 µg of HDM on days 7–11 before they were analyzed on day 14.

α-Galactosylceramide (αGalCer; Abcam, Cambridge, UK) was dissolved in NaCl containing 0.5% Tween and diluted with NaCl. OCH (Caltag-MedSystems Ltd, Buckingham, UK) was dissolved in warm 100% ethanol and diluted with NaCl. Mice were anesthetized by an ip, injection of a mixture of medetomidine, midazolam, and fentanyl. 100 µL containing 2 µg αGalCer or 1 µg OCH (or diluent controls) was applied it before mice were revived by an ip, injection of atipamezole, flumazenil, and naloxone. 24 hours later, mice were analyzed.

2.2 | Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) was performed 24 hours after the last challenge using 1 mL PBS containing 1 × protease inhibitor cocktail (Roche, Mannheim, Germany). Red blood cells were removed by resuspending BAL cells in red blood cell lysis buffer (Sigma-Aldrich) for 2 minutes at room temperature followed by washing in 1 mL PBS. The total number of cells was counted using a Neubauer counting chamber. Cells were centrifuged and the cell-free supernatant was stored at –20°C until cytokines were measured by BioPlex (Bio-Rad, Hercules, CA, USA). For differential cell counts, cytopsin preparations were fixed and stained with Diff-Quick (Merz & Dade AG, Duding, Switzerland). Macrophages, lymphocytes, eosinophils, and neutrophils were identified by standard morphological criteria.

2.3 | Lung cell isolation

To prepare single-cell suspension from lung tissue, the lung dissociation kit for mouse and a gentleMACS™ device (Miltenyi, Bergisch

Gladbach, Germany) were used according to the manufacturer's protocol. Lung-derived single-cell suspensions were plated at a concentration of 1×10^6 cells/mL complete RMPI (Sigma-Aldrich), restimulated with 50 μ g/mL OVA grade VI (Sigma-Aldrich), 1 μ g/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich), 10 μ g/mL ionomycin (Sigma-Aldrich), or 500 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich), and incubated for 48 hours at 37°C and 5% CO₂. Supernatants were frozen at -20°C until the levels of cytokines and chemokines were determined by BioPlex (Bio-Rad, Hercules, USA). All flow cytometric analyses were performed on the Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA). Anti-I-A/I-E, anti-Ly6C, anti-CD11b, anti-CD11c, anti-CD3, anti-CD19, anti-TNF- α , anti-IL-12p40, anti-CD103, and anti-CD4 antibodies were obtained from BioLegend (San Diego, CA, USA). Anti-CD25, anti-Foxp3, anti-IL-10, anti-IL-17A, anti-IFN- γ , and anti-IL-4 antibodies were obtained from eBioscience (Vienna, Austria). Cells were stained by the fixable viability dye eFlour780 (eBioscience). For intracellular cytokine staining, the cells were stimulated with PMA/ionomycin (50 ng/mL and 500 ng/mL) for 4 hours at 37°C and 5% CO in the presence of brefeldin (eBioscience) to stop cytokine secretion. Cells were permeabilized with reagents from eBioscience. Kaluza software (Beckman Coulter) was used for data analysis.

2.4 | Lung histology

Lung tissue was fixed with 4% PFA for 4 hours and stored in 70% isopropanol for a maximum of 1 week at 4°C. Lung tissues were embedded into paraffin and 3- μ m sections were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS).

2.5 | Gene expression analysis

Single-cell suspensions were generated from livers of wild-type and H₂R-deficient mice and were stimulated for 4 hours with α -GalCer/CD1d-loaded beads. Bead loading and stimulation conditions were identical to those previously described.²⁰ RNA was isolated using RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany). Reverse transcription was performed according to the manufacturer's instructions (Thermo Fisher Scientific Kit, Waltham, MA USA) and qPCR was run using the Applied Biosystems 7900 HT Fast Real-Time PCR system with GAPDH as a housekeeping gene. The cycling conditions were as follows: 40 cycles of 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minutes. The mouse DN32.D3 NKT cell hybridoma was kindly provided by Prof. Tonya Webb, University of Maryland School of Medicine, Baltimore, USA.

2.6 | Statistical analysis

Graphing and statistical analysis of normally distributed data were performed using Prism 5 (GraphPad software, San Diego, CA, USA). Data are expressed as mean $\pm$ SEM and are analyzed for significance using Student's *t* test or ANOVA.

3 | RESULTS

3.1 | H₂R activity impacts respiratory inflammation

To investigate the effect of H₂R signaling on respiratory allergic inflammation, we treated OVA-sensitized mice with famotidine (H₂R antagonist) or dimaprit (H₂R agonist). Famotidine treatment significantly increased the number of inflammatory cells in BAL, while in contrast BAL inflammatory cell numbers were significantly reduced with dimaprit treatment (Figure 1A). The differences in BAL cell numbers were primarily driven by increased eosinophils in famotidine-treated animals and decreased eosinophils in dimaprit-treated animals (Figure 1B). Histologic analysis of lung tissue sections revealed increased or decreased mucus production and inflammatory cell recruitment in famotidine- or dimaprit-treated mice, respectively (Figure 1C). Famotidine treatment significantly increased the secretion of the T_H2 cytokines IL-4 and IL-5 from allergen OVA-restimulated lung single-cell suspensions, while dimaprit treatment significantly reduced allergen-stimulated IL-4, IL-5, and IL-13 cytokine secretion (Figure 1D). BAL levels of IL-1 β , IL-4, IL-13, IFN- γ , MIP-1 α , G-CSF, and GM-CSF were elevated in famotidine-treated animals, while BAL levels of IL-1 β , IL-4, IL-5, IL-6, IL-12, IL-13, IFN- γ , G-CSF, GM-CSF, KC, MIP-1 α , MIP-1 β , Rantes, and eotaxin were significantly lower in dimaprit-treated animals (Fig. S1 in this article's Online Repository). However, neither famotidine nor dimaprit influenced the level of circulating OVA-specific IgE (Fig. S2 in this article's Online Repository).

Similar to famotidine-treated mice, increased numbers of inflammatory cells were observed in BALs from H₂R-deficient mice, compared to wild-type animals, in the OVA model (Figure 2A). The differences in BAL cell numbers were primarily driven by increased eosinophils in H₂R-deficient mice (Figure 2B). Interestingly, the heterozygote H₂R^{+/-} animals displayed an intermediate phenotype (Figure 2C). Allergen-restimulated T_H2 cytokine secretion was significantly elevated in OVA-stimulated lung single-cell suspensions from H₂R-deficient animals (Figure 2D). Using the house dust mite (HDM) model, increased numbers of inflammatory cells were observed in BALs from H₂R-deficient mice compared to wild-type animals (Figure 2E), due to increased numbers of both eosinophils and neutrophils (Figure 2F). Thus, H₂R signaling has significant protective effects within the inflamed lung.

3.2 | H₂R protective effect is not mediated by increased T regulatory cell numbers in the lung

One potential mechanism by which H₂R could influence allergic inflammatory responses was hypothesized to be related to decreased T regulatory cell numbers within the lung following H₂R inhibition. As expected, the percentage of CD4⁺CD25⁺Foxp3⁺ lymphocytes within lung tissue increased following allergen challenge (Fig. S3A in this article's Online Repository). However, famotidine treatment did not significantly alter the percentage of CD4⁺CD25⁺Foxp3⁺

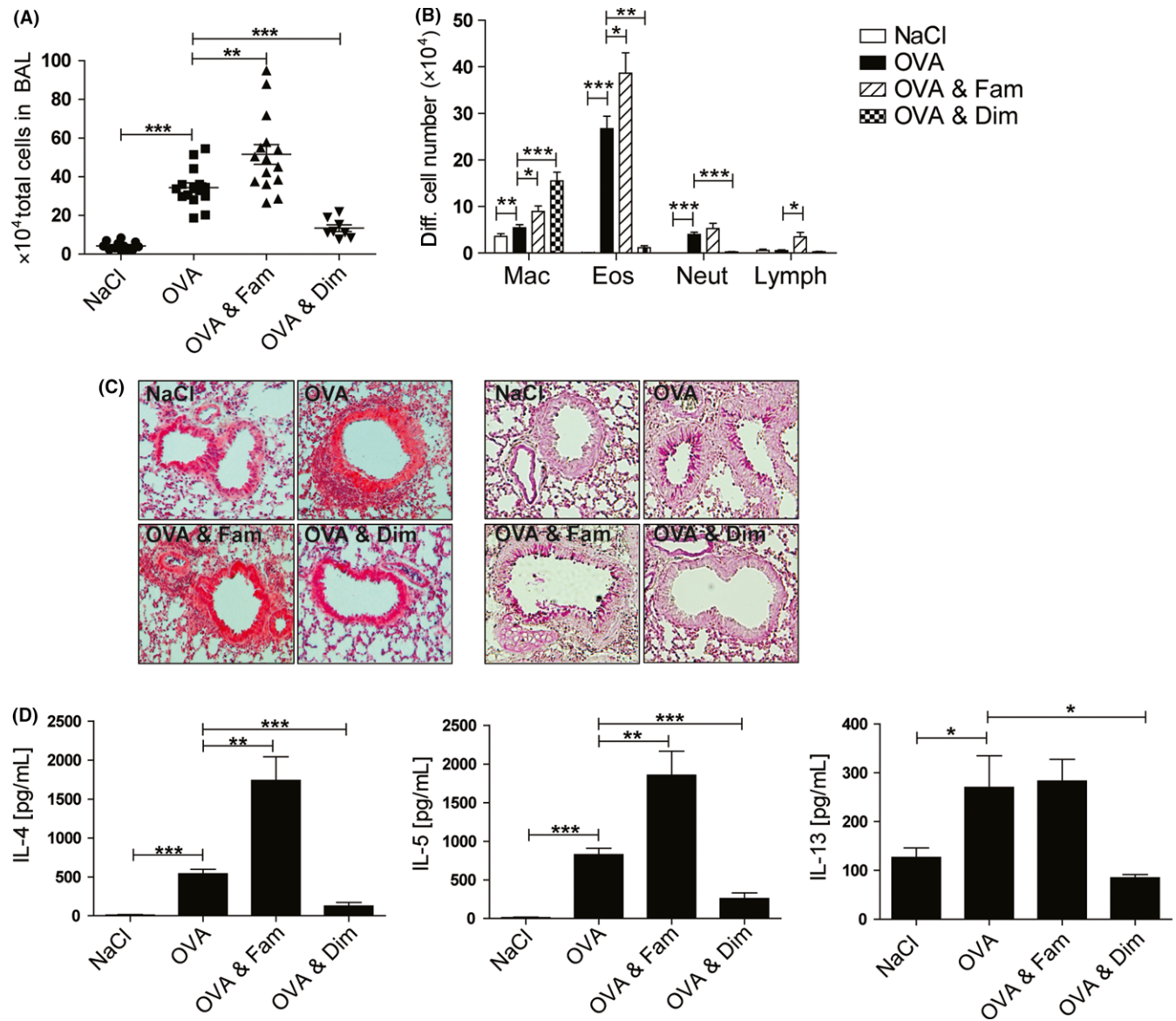


FIGURE 1 H₂R regulates respiratory inflammatory responses. H₂R inhibition by famotidine increases the number of cells in bronchoalveolar lavages, while H₂R activation by dimaprit reduces the number of cells (A). This difference in cell number is primarily driven by altered eosinophil numbers (B). Histological evaluation of lung tissue shows increased mucus and inflammatory cells following famotidine treatment, while dimaprit decreased mucin secretion and inflammatory cell recruitment (C). OVA-restimulated lung lymphocytes secrete more IL-4 and IL-5 in famotidine-treated animals, while dimaprit decreased IL-4, IL-5, and IL-13 secretion (D). **P* < .05, ***P* < .01, ****P* < .001

lymphocytes within lung tissue, while dimaprit treatment was associated with a reduced percentage of CD4⁺CD25⁺Foxp3⁺ cells within the lung tissue. Similarly, the percentage of CD4⁺CD25⁺Foxp3⁺ cells found in the lung draining lymph nodes, lung tissue, and BAL of OVA-challenged H₂R-deficient animals was not reduced compared to wild-type controls (Fig. S3B in this article's Online Repository). Indeed, there was a nonstatistically significant trend for an increased percentage of CD4⁺CD25⁺Foxp3⁺ in the H₂R-deficient animals. The percentage of CD25⁺Foxp3⁺ lymphocytes at other sites, such as the Peyer's patch, mesenteric lymph node, and spleen, was also not reduced in the H₂R-deficient animals (Fig. S3C in this article's Online Repository).

3.3 | H₂R-deficient animals have more CD1d⁺ dendritic cells and iNKT cells within the lung

In order to probe the cellular basis for enhanced lung inflammatory activity in the H₂R-deficient mice, we performed an analysis of dendritic cell phenotypes within lung tissue. Interestingly, the percentage of CD1d⁺ dendritic cells was increased in the lungs of H₂R-deficient animals (Figure 3A). As CD1d presents lipid antigens to invariant NKT (iNKT) cells, we hypothesized that the iNKT cell population may also be altered in these animals. Using alpha-galactosyl ceramide (α -GalCer)-loaded tetramers, we observed a significant increase in the percentage of iNKT cells within lung tissue of

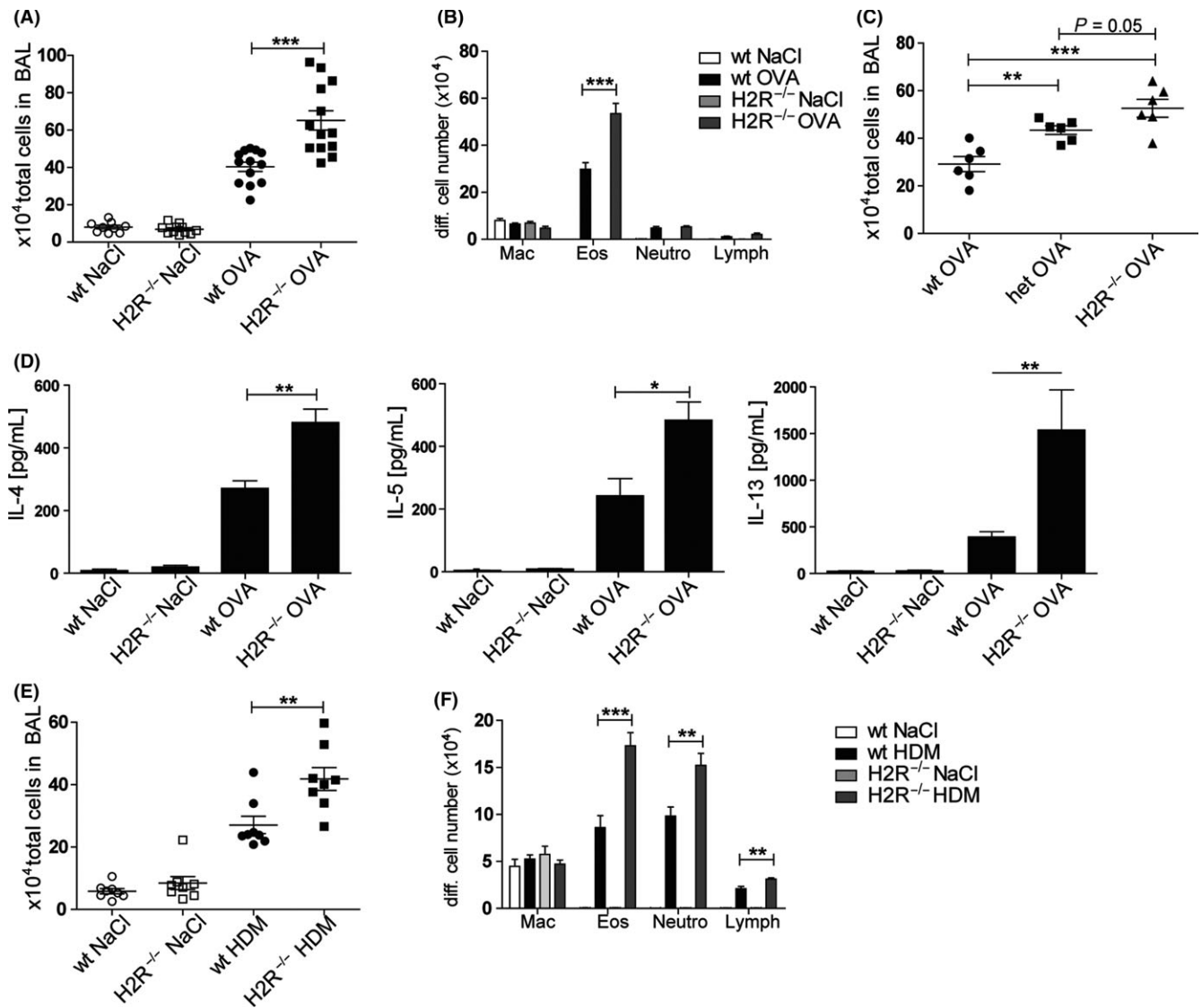


FIGURE 2 H₂R-deficient mice display more severe respiratory inflammatory responses. H₂R-deficient mice have increased numbers of cells in bronchoalveolar lavages in the ovalbumin (OVA) model (A), due to increased numbers of eosinophils (B). There was an increase in bronchoalveolar lavage (BAL) cell numbers for the heterozygote H₂R^{+/−} mice, with a further increase in the homozygous H₂R^{−/−} animals (C). OVA-restimulated H₂R-deficient lung lymphocytes secrete more IL-4, IL-5, and IL-13, compared to OVA-restimulated wild-type lung cells (D). Increased numbers of inflammatory cells were also seen in the BALs of H₂R-deficient animals following challenge with house dust mite extract (E), due to increased numbers of both eosinophils and neutrophils (F). A minimum of eight animals are analyzed per group, representing data from two independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$

H₂R-deficient animals (Figure 3B,C). Ex vivo stimulation of lung-derived single-cell suspensions with α -GalCer resulted in significantly increased secretion of IL-4, IL-5, GM-CSF, and IFN- γ from H₂R-deficient animals (Figure 3D). Similarly, in vitro stimulation with α -GalCer/CD1d-loaded beads for 4 hours upregulated IFN- γ gene expression significantly more in cells from H₂R-deficient animals compared to cells from wild-type animals, with a trend for increased IL-4 gene expression (Figure 3E). As isolation of iNKT cells with 100% purity from BALB/c mice is difficult (due to the lack of NK 1.1 expression in this mouse strain), we quantified H₂R and H₄R gene expression in the murine DN32.D3 NKT cell hybridoma. Both H₂R and H₄R genes were expressed by these NKT cells (Figure 3F).

The specific role of iNKT cells in mediating the exaggerated response observed in H₂R-deficient animals was further investigated by generating double knockout mice that lacked CD1d and H₂R. CD1d-deficient animals lack iNKT cells.²¹ As observed above, administration of HDM to H₂R-deficient animals resulted in a significantly greater influx of inflammatory cells into BAL, compared to wild-type animals (Figure 3G). However, the recruitment of cells into the BAL of CD1d/H₂R-deficient animals was significantly less than that observed in the H₂R-deficient animals, suggesting that iNKT cell responses play a role in the exaggerated response of H₂R-deficient mice to HDM challenge (Figure 3G). CD1d-deficient animals had similar responses to HDM as compared to wild-type animals (Figure 3G).

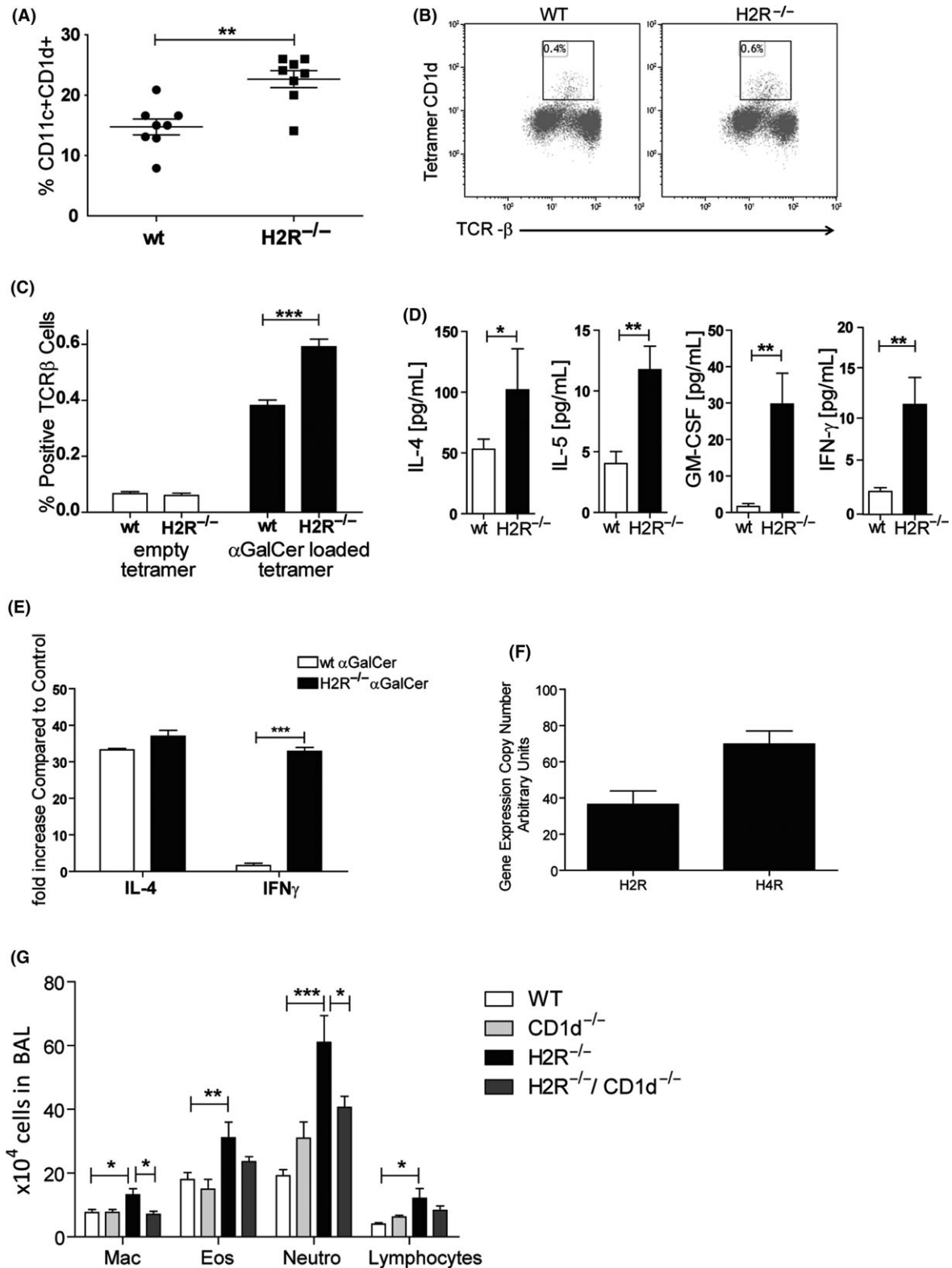


FIGURE 3 invariant natural killer T cell (iNKT) cell numbers and activity are increased in H2R-deficient animals. The percentage of CD1d+ dendritic cells is increased in lung tissue from H2R-deficient mice (A), associated with an increase in iNKT cells (B) and (C), $n = 8$ per group (one of two representative experiments is illustrated). In vitro stimulation of lung single-cell suspensions with α -GalCer resulted in enhanced IL-4, IL-5, GM-CSF, and IFN- γ secretion, $n = 6$ per group (D). In vitro stimulation of isolated cells with α -GalCer-loaded beads resulted in increased IFN- γ and IL-4 gene expression in cells from H2R-deficient mice, $n = 4$ per group (E). Both the H2R and the H4R are expressed by the murine DN32.D3 NKT cell hybridoma (F). Bronchoalveolar lavage (BAL) cell numbers following HDM challenge are illustrated (G). * $P < .05$, ** $P < .01$, *** $P < .001$

3.4 | In vivo activation of iNKT cells within the lung is H₂R dependent

To further investigate the role of H₂R in iNKT cell activation in vivo, we administered a single dose of α -GalCer directly into the lung of nonallergic wild-type mice and assessed lung inflammatory responses 24 hours later. The number of inflammatory cells in BALs was significantly increased following α -GalCer administration, which was significantly greater when H₂R was blocked with famotidine, while activation of H₂R with dimaprit significantly reduced the influx of inflammatory cells in response to iNKT cell activation (Figure 4A).

Similarly, α -GalCer administration to the lungs of nonallergic H₂R-deficient animals resulted in significantly more inflammatory cells in their BALs, compared to wild-type littermate controls (Figure 4B). The inflammatory infiltrate was predominantly composed of macrophages and neutrophils (Figure 4C). Dramatic goblet cell hyperplasia and mucus secretion were observed in the lungs of H₂R-deficient animals 24 hours following challenge with α -GalCer, which was not observed in wild-type animals (Figure 4D). It was previously described that α -GalCer activation of iNKT cells preferentially induces a neutrophilic influx, so we also examined the influence of an α -GalCer analogue named OCH, which contains a truncated

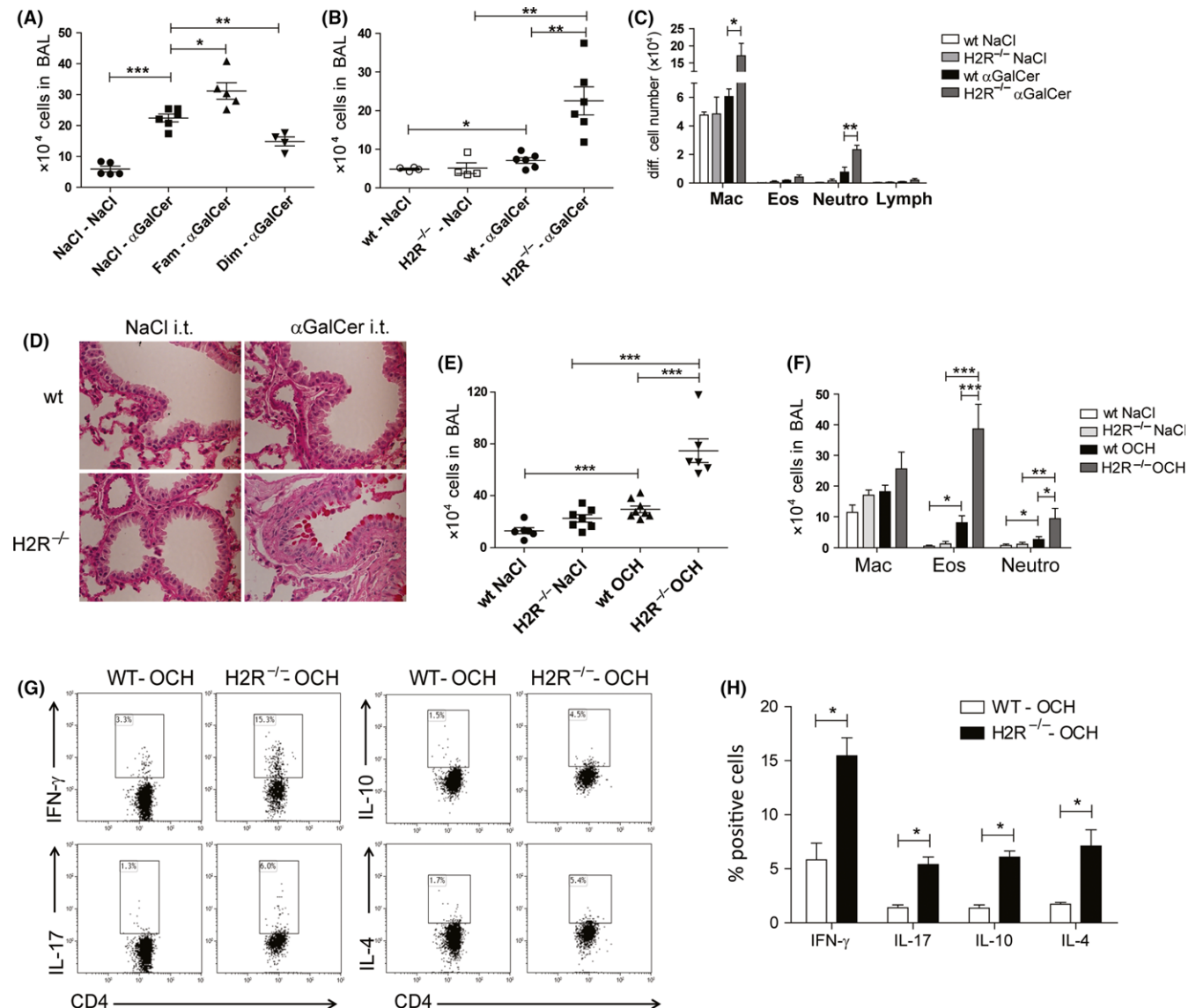


FIGURE 4 H₂R regulates lung invariant natural killer T (iNKT) cell responses. The administration of α -GalCer into the lung results in an increase in bronchoalveolar lavage cell numbers. Famotidine increased cell numbers, while dimaprit decreased this response, $n = 4$ -6 animals per group (A), and H₂R-deficient animals display exaggerated responses, $n = 4$ -6 animals per group (B). Increased cell number is due to an increase in macrophages and neutrophils in the H₂R-deficient animal, $n = 4$ -6 animals per group (C), while goblet cell metaplasia and mucus secretion are significantly enhanced (D). Another iNKT cell ligand, OCH, also induced an exaggerated increase in bronchoalveolar cells in H₂R-deficient animals (E). In contrast to α -GalCer, OCH primarily induced an increase in eosinophils, $n = 6$ -8 animals per group (F). Representative dot plots illustrate an increased percentage of IFN- γ , IL-17, IL-10, and IL-4-positive CD4⁺ lymphocytes within lung tissue of H₂R-deficient mice following exposure to OCH (G), while the grouped data are illustrated in (H). * $P < .05$, ** $P < .01$, *** $P < .001$

sphingosine chain and selectively induces T_H2 cytokines. Similar to α -GalCer, single administration of OCH to the nonallergic lung resulted in increased numbers of inflammatory cells within BAL, which was significantly more elevated in H_2R -deficient animals (Figure 4E). In contrast to α -GalCer, the inflammatory influx was dominated by eosinophils following iNKT cell activation by OCH in vivo (Figure 4F). In addition, increased numbers of IFN- γ , IL-17-, IL-10-, and IL-4-positive T cells were observed in the lung of H_2R -deficient animals exposed to OCH, compared to littermate wild-type controls exposed to OCH (Figure 4G,H). Taken together, these results suggest that H_2R modulates iNKT cell activation within the lung, even in nonallergic animals.

3.5 | Activation of iNKT cells exacerbates inflammatory disease in the H_2R -deficient mouse

As H_2R seems critical in controlling lung iNKT cell activity within a previously noninflamed lung, we asked whether a similar effect could be observed during the induction of allergic airway inflammation. Following sensitization with OVA, mice were administered α -GalCer or diluent control into the lung 24 hours before OVA aerosol challenge. As described above, significantly more cells were present in the H_2R -deficient lung following OVA challenge. However, in combination with α -GalCer, the number of inflammatory cells in BAL was significantly increased only in the H_2R -deficient lung, compared to OVA alone (Figure 5A). The increased BAL cell number was primarily due to increased numbers of neutrophils and macrophages (Figure 5B). Within the lung tissue, a significant increase in iNKT cells was observed following OVA challenge and α -GalCer administration in the H_2R -deficient animals (Fig. S4 in this article's Online Repository). Associated with the increased number of inflammatory cells, G-CSF, KC, MCP-1, MIP-1 α , MIP-1 β , and Rantes were all increased in the BAL of α -GalCer-treated H_2R -deficient animals, while IL-5 and eotaxin levels were unaltered (Fig. S5 in this article's Online Repository). In addition, BAL IL-4, IL-17, and IFN- γ cytokine levels were strongly elevated in the H_2R -deficient mouse (Figure 5C). In order to better define the cellular source of these cytokines, we quantified IL-4-, IL-17-, and IFN- γ -positive $CD3^+CD4^+$ and $CD3^+CD4^-$ lymphocytes from lung single-cell suspensions. The percentage of IFN- γ -positive $CD3^+CD4^+$ lymphocytes was significantly increased in H_2R -deficient animals following α -GalCer and OVA challenge, compared to OVA alone (Figure 5D). Challenge with OVA alone already increased the percentage of IL-4 $^+$ $CD3^+CD4^+$ lymphocytes in H_2R -deficient animals and this did not increase further when combined with α -GalCer, while the percentage of IL-17 $^+$ $CD3^+CD4^+$

lymphocytes was unaltered (Figure 5D). In contrast, an increased percentage of IL-4- and IL-17-positive cells was observed in the $CD3^+CD4^-$ lymphocyte population in H_2R -deficient animals challenged with both α -GalCer and OVA, compared to OVA alone (Figure 5E). These data suggest that $CD4^+$ lymphocytes contribute to increased BAL IFN- γ levels, while $CD4^-$ lymphocytes contribute to increased BAL IL-4 and IL-17 levels, following exposure to α -GalCer in the H_2R -deficient animals. In conclusion, these data demonstrate that lung-associated iNKT cells potentially exaggerate inflammatory responses following allergen challenge, but only when H_2R is absent.

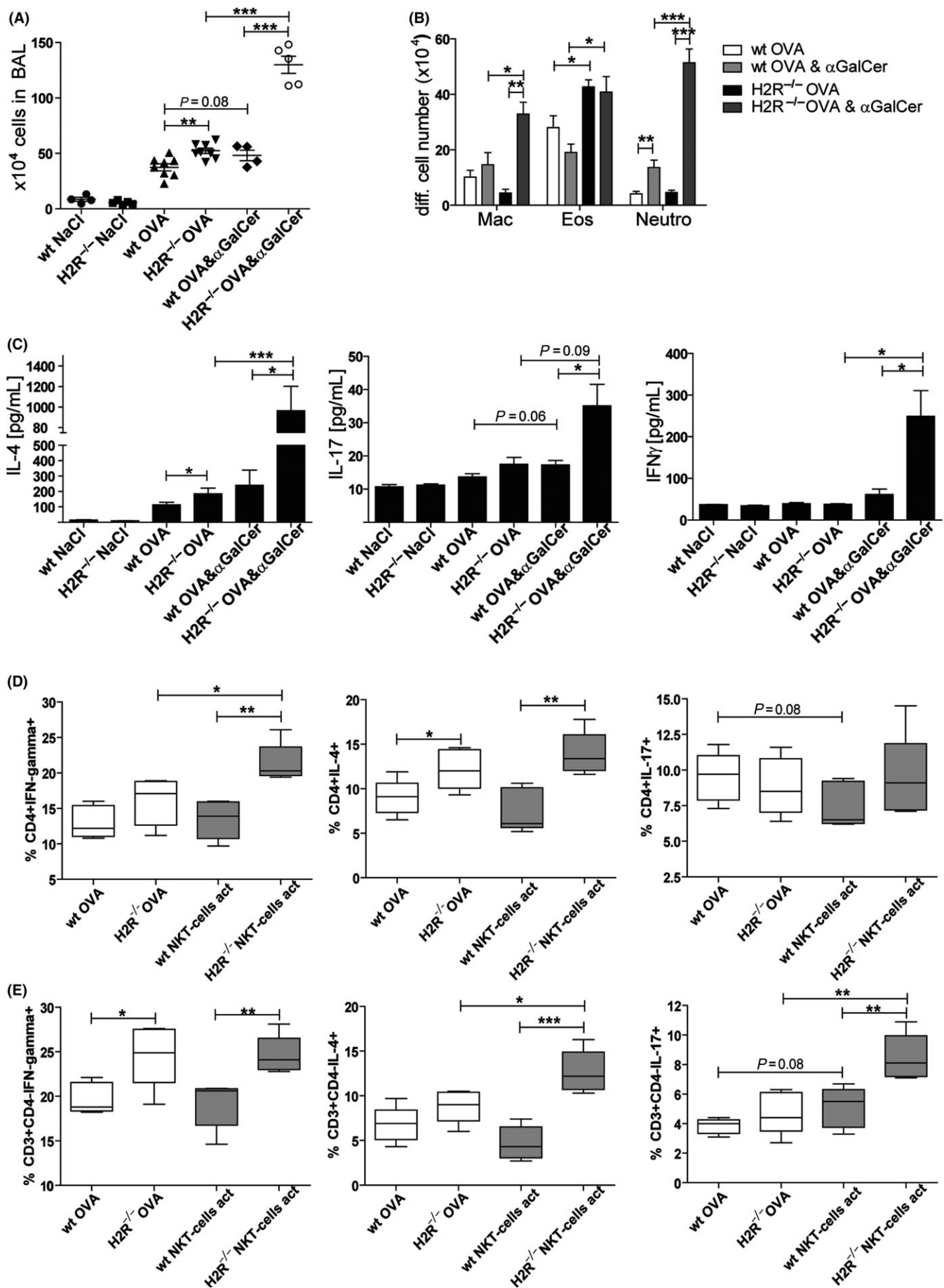
4 | DISCUSSION

In this report, we describe that H_2R signaling is required to restrain iNKT cell-associated inflammatory responses within the lung. Pharmacological inhibition of H_2R leads to exaggerated inflammatory responses, while pharmacological activation of H_2R inhibits inflammatory responses. The H_2R -deficient mice display increased respiratory inflammatory activity in both the OVA and house dust mite models. iNKT cell activation is greatly exaggerated, in vitro and in vivo, by pharmacological inhibition of H_2R and in the H_2R -deficient mice, while pharmacological activation of H_2R dampens the iNKT cell response within the lung.

In humans, acid suppression with H_2R antagonists has been shown to promote IgE responses to dietary antigens.²² However, H_2R inhibition in our respiratory allergy model did not influence sensitization; rather, the loss of H_2R signaling amplified innate inflammatory responses. Increased IL-5 secretion, but not eotaxin secretion, was associated with increased numbers of eosinophils following blockade of H_2R or H_2R deficiency. Further studies are required to determine whether the increase in eosinophil numbers was due to IL-5.

Our report adds to a growing body of literature, which suggests that histamine signaling via H_2R exerts anti-inflammatory effects on many different cell types in multiple experimental models.³ H_2R modulation of immune responses is 3',5'-cyclic adenosine monophosphate (cAMP) dependent. cAMP is an important second messenger, which can activate protein kinase A, exchange protein activated by cAMP (Epac), cAMP response element-binding (CREB), and cyclic nucleotide-gated channels.²³ Activation of both innate and adaptive immune cells is suppressed by cAMP and drugs targeting cAMP-degrading enzymes, for example, PDE4, have shown beneficial effects in patients with chronic obstructive pulmonary disease (COPD), while clinical studies in asthma patients are ongoing.^{13,24-27}

FIGURE 5 Activation of invariant natural killer T (iNKT) cells exacerbates allergic airway inflammation in H_2R -deficient animals. Bronchoalveolar lavage cell numbers are significantly increased in OVA-sensitized and OVA-challenged H_2R -deficient animals, when iNKT cells are simultaneously activated by α -GalCer (A). The increase in cell number was due to an increase in macrophages and neutrophils, $n = 4-8$ animals per group (B). IL-4, IL-17, and IFN- γ secretion was significantly increased in H_2R -deficient mice, $n = 4-8$ animals per group (C). IFN- γ -positive $CD4^+$ lymphocytes are increased in α -GalCer-exposed, OVA-challenged H_2R -deficient animals, compared to OVA challenge alone (D). IL-4 $^+$ and IL-17 $^+$ $CD4^-$ lymphocytes are increased in α -GalCer-exposed, OVA-challenged H_2R -deficient animals, compared to OVA challenge alone (E), $n = 4-8$ animals per group. * $P < .05$, ** $P < .01$, *** $P < .001$



The cAMP pathway has also been shown to suppress iNKT cell proliferation and cytokine secretion, which may explain in part the effects we observed on iNKT cell activity in the absence of H₂R signaling.²⁸

A hallmark of iNKT cells is their capacity to rapidly (within hours) release a wide range of cytokines and thereby influence the activation of dendritic cells, NK cells, B cells, and conventional T cells.²⁹ Consequently, iNKT cell activation can strongly impact innate and adaptive immune responses and iNKT cells have been implicated in immune responses against infectious agents, tumors, and tissue grafts and in regulating a variety of autoimmune and inflammatory diseases.³⁰ Histamine has been previously demonstrated to exert significant effects on murine iNKT cells as histamine-free histidine decarboxylase-deficient mice displayed numerical and functional deficits in the iNKT cell population.³¹ Histamine injection upregulated IL-4 and IFN- γ production by iNKT cells activated in vivo, which was H₄R dependent. In line with our data showing H₂R and H₄R expression by iNKT cells, a recent report on iNKT cell gene expression profiles also showed that both H₂R and H₄R genes are expressed by iNKT cells.³² Thus, iNKT cell activity may be significantly influenced by the balance between H₂R and H₄R signaling, with the H₂R suppressing iNKT cell activation and the H₄R promoting iNKT cell activation following exposure to histamine. It was recently demonstrated that there are increased numbers of histamine-secreting microbes in the gut of asthma patients; however, it is not yet known whether bacterial-derived histamine can activate H₂R or H₄R on mucosal iNKT cells.³³ Indeed, changes in iNKT cell histamine receptor expression or activity have yet to be examined in asthma patients.

The specific contribution of iNKT cells in asthma has been intensively debated due to differing results published on the number of iNKT cells in the lungs of patients with asthma.^{34–37} However, it has been consistently demonstrated that iNKT cells play an important role in the development of airway inflammation in experimental models and several environmental substances, including bacterial and fungal glycolipids, have been shown to activate iNKT cells.^{38–41} In addition, age-dependent contact with commensal microbes has been shown in murine models to be important in establishing mucosal iNKT cell tolerance to later environmental exposures in both the gut and the lung.⁴² Our results identify another factor in the control of lung iNKT cell responses, the H₂R. The enhanced inflammatory response in H₂R-deficient animals to both α -GalCer and OCH suggests that H₂R signaling is required to suppress iNKT cell-induced neutrophilic and eosinophilic responses. Interestingly, H₂R-deficient iNKT cell exposure to α -GalCer/CD1d-loaded beads upregulated IFN- γ gene expression, but not IL-4. Host cells, such as CD1d dendritic cells, were required to see enhanced IFN- γ , IL-4, and IL-5 secretion, suggesting that H₂R deficiency on host cells other than iNKT cells also contributes to the exaggerated cytokine response.

The recent developments in histamine research suggest an important role for histamine in controlling innate and adaptive immune responses. Histamine and its four receptors represent a complex system of immunoregulation with distinct effects dependent

on receptor subtype expression. The role for differential expression or activation of histamine receptors on immunocompetent cells in chronic inflammatory diseases is surprisingly poorly described to date, and further examination of this potent immunoregulatory network will likely lead to a better understanding of these disorders. The deliberate activation of H₂R, or its downstream signaling molecules, is a novel therapeutic target for inflammatory diseases.

CONFLICTS OF INTEREST

Liam O'Mahony has received research support from GSK and consulted for Alimentary Health Ltd. Cezmi Akdis has received research support from Novartis and Stallergenes and consulted for Actelion, Aventis, and Allergopharma. David Groeger is an employee of Alimentary Health Pharma Davos, while all other authors have no potential conflicts of interest. The authors have no additional financial interests.

AUTHOR CONTRIBUTIONS

LOM, SZ, RL, and CA designed the studies and interpreted the data; RFe, RFr, WB, ES, KW, MZ, NR-P, DG, PK, and DN performed the murine experiments and laboratory procedures. All authors contributed to the preparation and review of the manuscript.

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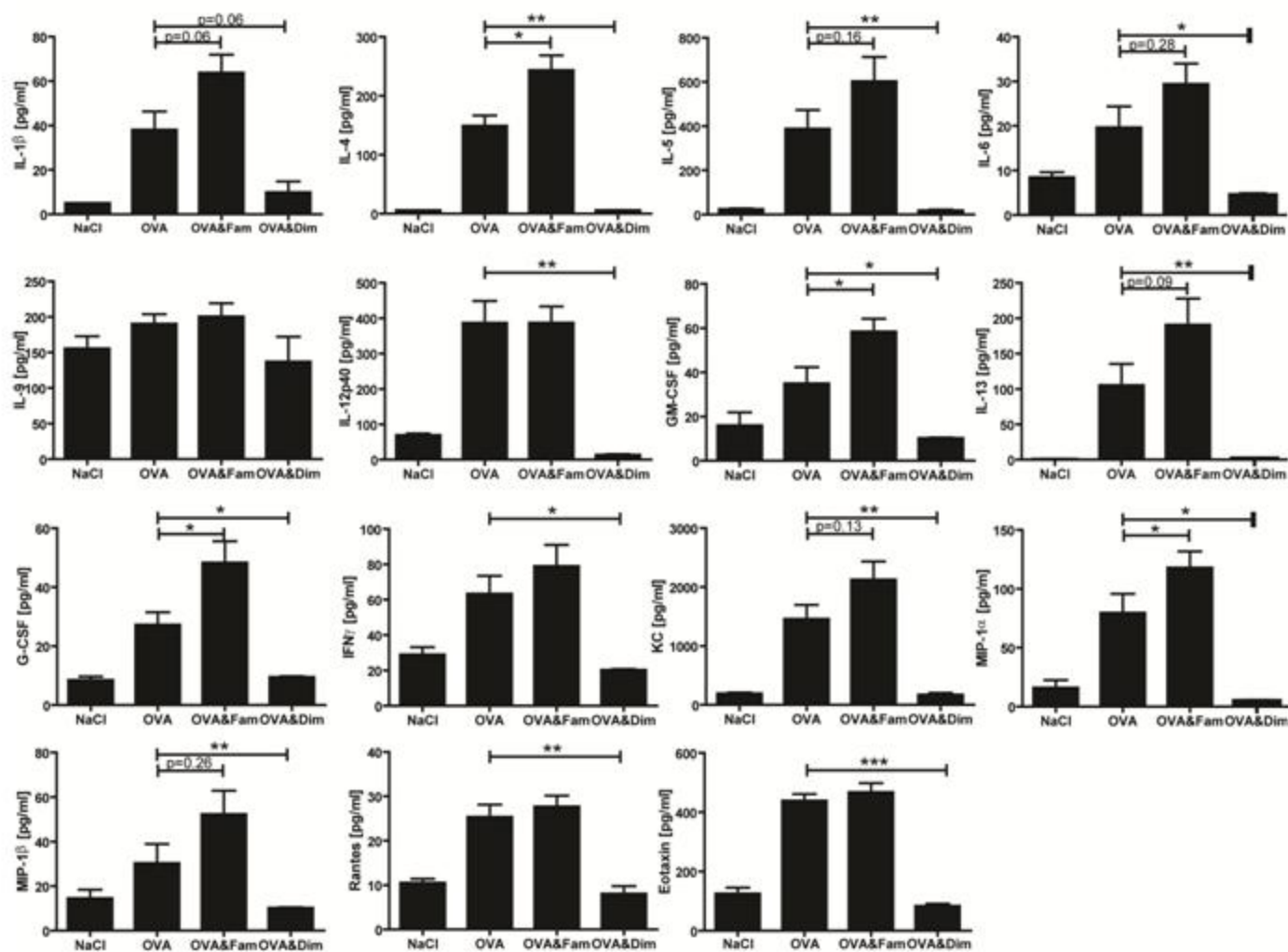
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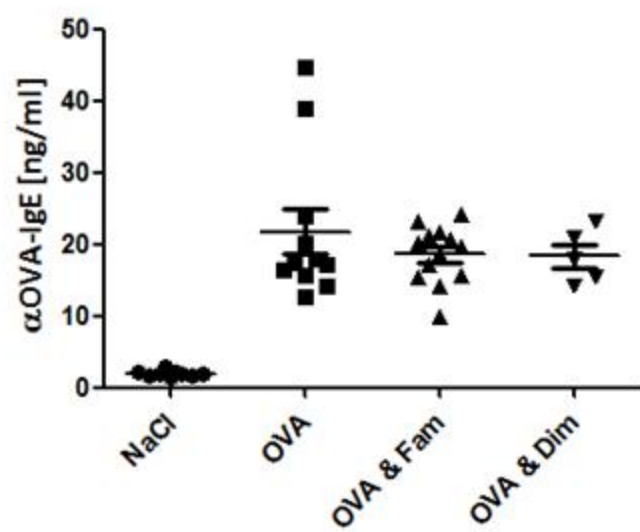
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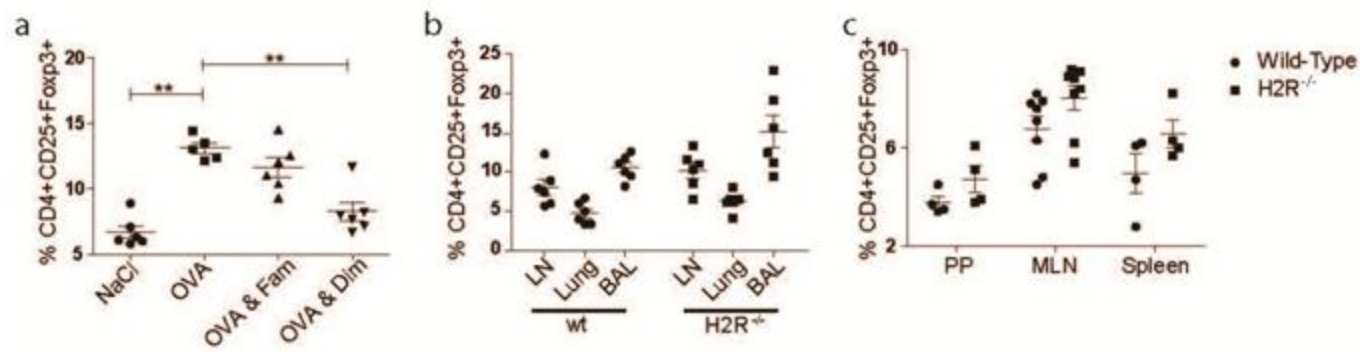
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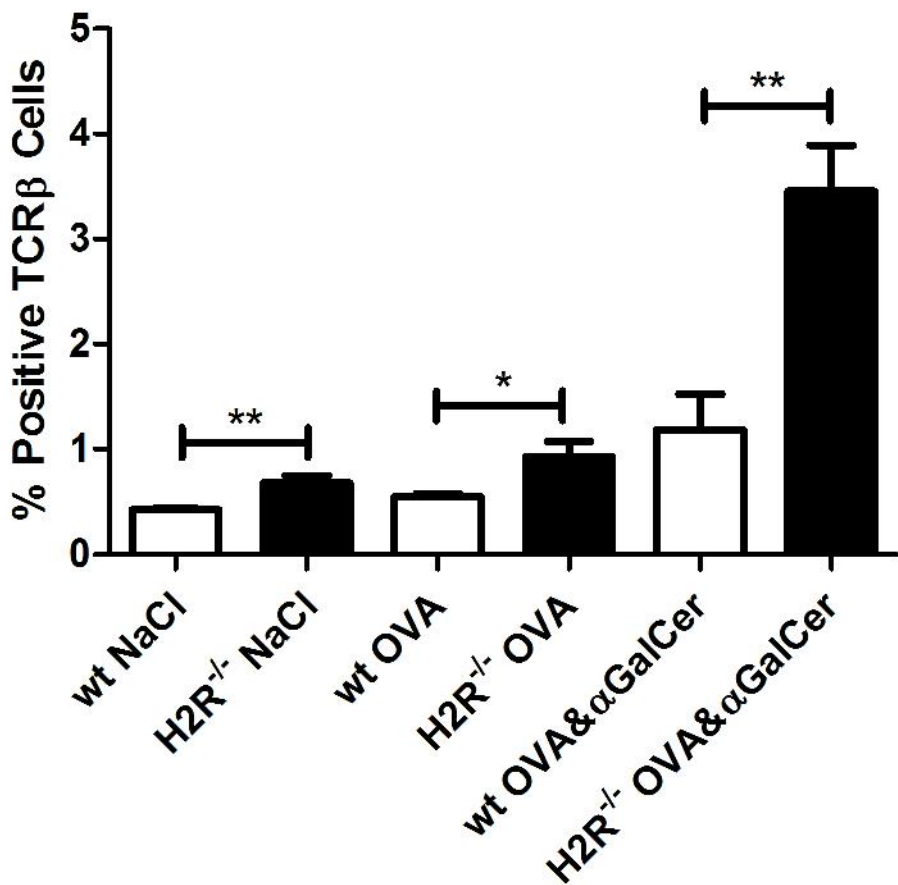
Supplementary Figure 1







Supplementary Figure 4



Supplementary Figure 5

