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ARTICLE CD52-targeted depletion by Alemtuzumab ameliorates allergic airway hyperreactivity and lung inflammation

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Allergic asthma is a chronic inflammatory disorder associated with airway hyperreactivity (AHR) whose global prevalence is increasing at an alarming rate. Group 2 innate lymphoid cells (ILC2s) and T helper 2 (T_H2) cells are producers of type 2 cytokines, which may contribute to development of AHR. In this study, we explore the potential of CD52-targeted depletion of type 2 immune cells for treating allergic AHR. Here we show that anti-CD52 therapy can prevent and remarkably reverse established IL-33-induced AHR by reducing airway resistance and alleviating lung inflammation. We further show that CD52 depletion prevents and treats allergic AHR induced by clinically relevant allergens such as *Alternaria alternata* and house dust mite. Importantly, we leverage various humanized mice models of AHR to show new therapeutic applications for Alemtuzumab, an anti-CD52 depletion is a viable therapeutic option for reduction of pulmonary inflammation, abrogation of eosinophilia, improvement of lung function, and thus treatment of allergic AHR. Taken together, our data suggest that anti-CD52 depleting monoclonal antibodies, such as Alemtuzumab, can serve as viable therapeutic drugs for amelioration of T_H2 - and ILC2-dependent AHR.

Mucosal Immunology (2021) 14:899-911; https://doi.org/10.1038/s41385-021-00388-5

INTRODUCTION

Asthma is an atopic and heterogeneous disorder of the airways that is characterized by bronchoconstriction, bronchial hyperresponsiveness, and underlying inflammation.¹ Traditionally, asthma has been categorized as an adaptive T helper 2 (T_H2)associated inflammatory disorder that is perpetuated by type 2 cytokines, such as IL-5 and IL-13.² These type 2 cytokines stimulate downstream myelocytes such as eosinophils, mast cells, and basophils. For instance, IL-5 is essential for recruitment of eosinophils to the lungs from the bone marrow, as well as their respective maturation, growth, activation, and survival.³ On the other hand, IL-13 causes goblet cell hyperplasia and thus augments production of mucus that causes to the narrowing of the bronchioles.⁴ IL-13 can also promote bronchiole smooth muscle contraction by increasing the potency of contractile agonists such as histamine, carbachol, and leukotriene D₄.^{5,6} Although the adaptive response has been classically implicated in asthma immunopathogenesis, with the entrance of the newly discovered ILC2s into the picture, the exact role of the innate immunity in initiating and perpetuating type 2 immune responses remains to be fully elucidated. Under homeostatic conditions, pulmonary ILC2s reside near the basement membrane subjacent to the epithelium layer at a distance of less than 70 µm away from the bronchioles.⁷ Such positioning enables ILC2s to be among the first responders, and thus the earliest inducers of type 2 inflammation in allergic asthma.⁸ Similar to $T_{H}2s$, ILC2s are very rapid and proficient producers of IL-5 and IL-13.⁹ Thus, ILC2s contribute to pathogenesis of type 2 inflammatory in some groups of allergic asthma.⁸⁻¹⁰ Due to the vast heterogeneity of asthma and varied contributions of T_H2 and ILC2s in initiating and/or perpetuating lung inflammation in different patients, there has been a growing effort in the field to identify viable biomarkers that can simultaneously target and dampen both ILC2s and T_H2s responses in future therapeutics.^{8,9,11}

Cluster of differentiation (CD52) is a glycosylphosphatidylinositolanchored cell surface protein that consists of 12 amino acids.¹ Previous reports have suggested that CD52 is highly expressed on effector T and B lymphocytes, and to a slighter lower degree on monocytes, mast cells, myeloid-derived dendritic cells, neutrophils, and eosinophils.¹³⁻²⁰ Importantly, CD52 is not expressed on hematopoietic stem cells (HSCs) and other progenitors that give rise to leukocytes.^{16,21,22} Although its exact physiological function is not yet fully understood, CD52 has shown to be a promising target for several immune system-mediated disease, including multiple sclerosis (MS), graft versus host disease, autoimmune inflammatory neurodegenerative diseases, as well as various lymphomas such as chronic lymphocytic leukemia (CLL) or acute lymphocytic leukemia.²³⁻²⁷ In particular, Alemtuzumab is a US Food and Drug Administration (FDA)-approved recombinant humanized monoclonal immunoglobulin IgG1 kappa that targets CD52, and is utilized to effectively treat patients with CLL or relapsed-refractory MS.^{28–33} The specific role and therapeutic potential of targeting CD52 in allergic asthma has not been previously investigated.

Recently, we reported a genome-wide analysis for 88,486 asthmatic patients and 447,859 healthy controls using data from UK Biobank and the Trans-National Asthma Genetic Consortium.³⁴ We identified several novel asthma-associated human loci and bioinformatically showed that asthma-associated variants of

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Received: 15 September 2020 Revised: 3 February 2021 Accepted: 7 February 2021 Published online: 17 March 2021

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candidate causal genes, such as CD52, are enriched in regions with open chromatin conformations in T and B cells.³⁴ Since asthma is a heterogeneous disease, in this study we evaluate the therapeutic potential of anti-CD52 immunotherapy in preventing and treating various models of allergic asthma. We demonstrate that CD52 depletion can severely dampen a T cell-driven model of airway hyperreactivity (AHR) induced by house dust mite (HDM). We further establish that CD52 is expressed on both murine and human ILC2s, and show that CD52 depletion reduces airway resistance, abrogates eosinophilia and improves dynamic compliance in IL-33-induced AHR in the presence and absence of adaptive immunity. We further validate the therapeutic relevance of these findings by demonstrating the therapeutic efficacy of CD52 depletion in Alternaria alternata-induced AHR. Lastly, we utilize various humanized mice models to suggest new potential therapeutic applications for the FDA-approved drug Alemtuzumab for treatment of allergic asthma and improvement of lung function

RESULTS

CD52 depletion ameliorates HDM-induced AHR

We began by asking whether CD52 depletion can prevent the induction of AHR by HDM, one of the most common triggers behind T cell-dependent asthma.^{35,36} We confirmed that CD52 is expressed on T and B cell in our murine model (Supplementary Fig. 1). As shown in timeline, wildtype (WT) mice were intraperitoneally (i.p.) immunized with 100 µg of HDM in 2 mg of aluminum hydroxide and challenged with HDM (50 µg) or phosphate buffered saline (PBS) intranasally (i.n.) on days 8, 9, and 10 (Fig. 1a). In addition, mice were treated with 250 µg of anti-CD52 depleting antibody (aCD52) i.p. or isotype control on day 7. One day after the last *i.n.* challenge, lung function was measured by direct measurement of lung resistance and dynamic compliance in anesthetized tracheostomized mice, in which mice were mechanically ventilated and sequentially challenged with aerosolized increasing doses of methacholine. After measurements of AHR, the bronchial alveolar lavage (BAL) fluid was collected and analyzed by flow cytometry (Supplementary Fig. 2). As expected, i. n. administration of HDM significantly increased lung resistance in isotype-treated group (Fig. 1b); however, lung resistance in aCD52-treated HDM-administered group was significantly reduced, indicating that CD52 depletion prevented HDMinduced AHR. In agreement with lung-resistance findings, the results of dynamic compliance showed an improved response in aCD52-treated HDM-administered group of mice compared to isotype-treated HDM-administered group, but they showed a lower dynamic compliance than their PBS-treated counterparts (Fig. 1c). Moreover, both aCD52-treated and isotype-treated mice that received intranasal PBS had similar lung resistance and dynamic compliance. HDM treatment significantly increased the total numbers of CD45⁺ leukocytes (Fig. 1d), eosinophils (Fig. 1e), CD3⁺ T cells (Fig. 1f), as well as neutrophils (Fig. 1g) in the BAL fluid of isotype-treated control. However, BAL analysis in the aCD52-treated HDM-administered group revealed a significant reduction in the number of these inflammatory cells, indicating that HDM-induced inflammation is impaired via CD52 depletion (Fig. 1d–q). In addition, α CD52-treated and isotype-treated mice that received intranasal PBS both displayed similar BAL cells numbers, although the numbers of CD45⁺ and CD3⁺ were slightly reduced in the aCD52-treated group. Collectively, these results demonstrate that CD52 depletion effectively prevents HDMdependent lung inflammation and AHR.

Next, we asked whether CD52 depletion can reverse HDM-induced AHR. A group of WT mice were *i.p.* immunized with 100 μ g of HDM in 2 mg of aluminum hydroxide. The mice were then challenged with HDM (50 μ g) or PBS *i.n.* on days 8, 9, and 10. Subsequently on day 11, mice were treated with 250 μ g of

anti-CD52 depleting antibody *i.p.* or isotype control. Measurements of lung function and sample acquisition followed on day 13 (Fig. 1h). Consistent with the preventative results, CD52 depletion ameliorated established HDM-induced AHR by decreasing lung resistance (Fig. 1i) and increasing dynamic compliance (Fig. 1j). Furthermore, CD52 depletion effectively abrogated inflammation by reducing the total numbers of CD45⁺ leukocytes (Fig. 1k), eosinophils (Fig. 1l), CD3⁺ T cells (Fig. 1m), as well as neutrophils (Fig. 1n) in the BAL. In concurrence with the reduction of AHR and eosinophilia in BAL fluid, further histological analyses of the lungs revealed that CD52 depletion decreased airway epithelium thickness and the number of infiltrating cells (Fig. 1o). Taken together, these results suggest that anti-CD52 treatment may serve as a novel therapeutic avenue to treat HDM-induced T cell-driven allergic AHR.

CD52 is constitutively expressed on murine ILC2s both at steadystate and under inflammatory conditions

Recently, ILC2s have been recognized for their central role in initiating and perpetuating pulmonary inflammation; therefore, we next inquired whether CD52 is expressed on ILC2s. A group of WT mice was challenged with the alarmin IL-33 (0.5 µg) or PBS i.n. on days 1, 2, and 3 (Fig. 2a). On the fourth day, ILC2s from the lungs were isolated and analyzed by flow cytometry and gated as lineage⁻CD45⁺CD127⁺ST2⁺ (Fig. 2b). Analysis of pulmonary ILC2s revealed that both naïve and IL-33-activated ILC2s (ILC2s stimulated with IL-33) have high expression of CD52 at mRNA (number of transcripts) and protein levels (Fig. 2c, d). Furthermore, expression of CD52 on naïve ILC2s is inducible in vivo by IL-33 (Fig. 2d). In order to determine the kinetics of CD52 induction by IL-33, we next sorted naïve ILC2s from a group of WT mice and cultured them in the presence of IL-2 and IL-7. The pulmonary ILC2s were subsequently ex vivo stimulated with IL-33, and CD52 expression was analyzed over time at 0, 12, 24, and 48 h (Fig. 2e). We observed that CD52 expression was increased over time and reached statistical significance after 48 h of ex vivo IL-33 stimulation. Lastly, we demonstrated that expression of CD52 on ILC2s is the highest among the different groups of innate lymphoid cells (Supplementary Fig. 3). Overall, these results demonstrate that pulmonary murine ILC2s highly express CD52 at both steady-state and under inflammatory conditions.

CD52 depletion ameliorates IL-33-induced AHR in the presence and absence of adaptive immunity

Next, we explored whether CD52 depletion prevent IL-33-induced AHR and lung inflammation by comparing anti-CD52 depleting antibody (α CD52) with isotype-treated control in WT mice. The mice were treated with 250 µg of aCD52 *i.p.* or isotype control on day 1. The mice were then challenged with IL-33 (0.5 µg) or PBS i.n. on days 2, 3, and 4 (Fig. 3a). On the fifth day, lung function was assessed by direct measurement of lung resistance and dynamic compliance in anesthetized tracheostomized mice. After measurements of AHR, the BAL fluid was collected and analyzed by flow cytometry. As anticipated, i.n. administration of IL-33 significantly increased lung resistance in isotype-treated group (Fig. 3b); however, lung resistance in aCD52-treated group was significantly reduced, indicating that CD52 depletion prevented IL-33-induced AHR. In agreement with these findings, the results of dynamic compliance showed an improved response in aCD52-treated group of mice compared to isotype control (Fig. 3c). Furthermore, aCD52-treated and isotype-treated groups that received intranasal PBS displayed similar lung resistance and dynamic compliance. BAL analysis in the aCD52-treated HDM-administered group revealed a significant reduction of inflammatory cells. The total number of leukocytes (Fig. 3d), T cells (Fig. 3e), neutrophils (Fig. 3f), and eosinophils (Fig. 3g) were abrogated in the BAL of aCD52-treated HDM-administered group compared to isotypetreated HDM-administered mice. In addition, aCD52-treated and

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Fig. 1 CD52 depletion ameliorates HDM-induced airway hyperreactivity (AHR) and abrogates inflammation. a A group of WT mice (n = 6 mice) was intraperitoneally (*i.p.*) immunized with 100 µg of house dust mite (HDM) in 2 mg of aluminum hydroxide (alum) and challenged with HDM (50 µg) or PBS intranasally (*i.n.*) on days 8, 9, and 10. Mice were treated with 250 µg of anti-CD52 depleting antibody (α CD52) *i.p.* or isotype control on day 7. We assessed the lung function and analyzed the BAL fluid on day 11, as shown in the timeline. **b**, **c** Line graphs show lung resistance and dynamic compliance (cDyn) in response to increasing doses of methacholine. Total numbers of CD45⁺ cells (**d**), number of eosinophils (**e**), number of T cells (**f**), and number of nutrophils (**g**) in the BAL fluid have been demonstrated in the bar graphs. **h** WT mice (n = 6 mice) were sensitized on day 1, and challenged intranasally with HDM on days 8, 9, and 10. Subsequently, the mice were treated with 250 µg of α CD52 or isotype control. The lung function and BAL were assessed on day 13. **i** Lung resistance. **j** Dynamic compliance. **k** Total numbers of CD45⁺ cells. **I** Number of T cells. **m** Number of T cells. **n** Number of neutrophils. **o** Representative images (left panel) and quantification (right panel) of hematoxylin and eosin-stained histologic sections of the lungs of mice. Scale bars = 50 µm. Data are shown as means ± SEMs and are representative of three individual experiments. Statistical analysis, two-tailed Student's *t*-test. n.s. not significant, **P* < 0.05, ***P* < 0.01, *****P* < 0.001. *****P* < 0.001. Mouse and lung images are provided with permission from Servier Medical Art.

isotype-treated mice that received intranasal PBS both displayed similar cells numbers, although the numbers of BAL CD45⁺ and CD3⁺ were slightly abrogated in the α CD52-treated group. Importantly, analysis of the lung tissues by flow cytometry

revealed that the number of pulmonary ILC2s is decreased after aCD52 treatment of HDM-administered mice compared to the positive control (Fig. 3h), indicating that IL-33-induced inflammation in the lungs of WT mice is curtailed upon CD52 depletion.





Fig. 2 Murine ILC2s constitutively express CD52, and this expression is inducible by IL-33. a A group of WT mice (n = 6 mice) was challenged with recombinant mouse (rm)-IL-33 (0.5 µg in 50 µL) or PBS intranasally (*i.n.*) on days 1, 2, and 3. The mice were euthanized on day 4 and the lung was isolated, as shown in the timeline. **b** The gating strategy of ILC2s identified as Lin⁻CD45⁺CD127⁺ST2⁺ cells. **c**, **d** mRNA and protein expression levels of CD52 in both naïve (PBS) and IL-33-activated ILC2s in the lungs. Corresponding FACS quantitation of CD52 expression shown as MFI ± SEM, n = 6 mice. mRNA levels obtained from n = 2-3 mice. **e** Naïve pulmonary ILC2s were sorted (n = 6 mice) and subsequently cultured with rm-IL-2 and rm-IL-7 and rm-IL-33 for 12, 24, and 48 h. Freshly isolated ILC2s at 0 h and ex vivo activated ILC2s were analyzed by flow cytometry as indicated in the scheme and the kinetics of CD52 induction by IL-33 is shown. Statistical analysis, two-tailed permission from Servier Medical Art.

Next, we guestioned whether the amelioration of IL-33-induced AHR and lung inflammation via CD52 depletion is independent of the adaptive immunity. We examined the effects of anti-CD52 depleting antibody on IL-33-induced AHR and lung inflammation in Raa2⁻ mice that lacked any mature B and T cells. A group of Rag2^{-/-} mice received either anti-CD52 or isotype-matched control antibody (250 µg per mouse) i.p. on day 1. The mice were then challenged with IL-33 (0.5 µg) or PBS i.n. on days 2, 3, and 4. Measurements of lung function and sample acquisition followed on day 5 (Fig. 3i). Lung function data show that i.n. IL-33 administration increased lung resistance (Fig. 3j) and decreased dynamic compliance (Fig. 3k) in $Rag2^{-/-}$ mice. In contrast, lung resistance in IL-33-treated group that received aCD52 was significantly lower, and dynamic compliance was higher, compared to the IL-33-treated isotype control-treated mice (Fig. 3j, k). Furthermore, IL-33 challenge led to eosinophilia (Fig. 3I), increased ILC2 numbers (Fig. 3m), and thus lung inflammation of the isotype-treated group. However, aCD52-treated group showed significantly abrogated inflammatory eosinophil recruitment, and thus reduced eosinophilia in BAL fluid (Fig. 31). In addition, αCD52treated group showed a significant decrease in the pulmonary ILC2 numbers (Fig. 3m). The secreted amount of IL-5 and IL-13 were similarly decreased in the BAL fluid of aCD52-treated group (Fig. 3n, o). Further histological analysis of the lungs demonstrated that IL-33 challenge led to a thickening of the epithelium and increased inflammatory cells in isotype control but not in the anti-CD52-treated mice (Fig. 3p). Taken together, these results indicate that anti-CD52 therapy ameliorates ILC2-driven AHR and lung inflammation in the absence of adaptive immunity.

CD52 depletion ameliorates *Alternaria alternata*-induced AHR and reduces lung inflammation in RAG2-deficient mice

We next investigated whether CD52 depletion prevents AHR and lung inflammation induced by a clinically relevant allergen-Alternaria alternata—that is known to spark a type 2 innate immune response.³⁷ $Rag2^{-/-}$ mice received either α CD52 or isotype control antibody *i.p.* on day 1 (250 µg per mouse). The mice were then challenged i.n. with extract of Alternaria alternata (100 µg per mouse) or PBS on days 2–6, followed by subsequent measurements of lung function and sample acquisition on day 7 (Fig. 4a). Intranasal administration of *Alternaria* induced AHR, as evidenced by increase in lung resistance (Fig. 4b) and decrease in dynamic compliance (Fig. 4c) in isotype-treated mice but not aCD52-treated group. The number of eosinophils was increased in the BAL fluid of Alternaria-administered mice, but it was significantly ablated in aCD52-treated group compared to the isotype-treated control (Fig. 4d). Moreover, the total number of ILC2s in the lungs was significantly lowered in aCD52-treated mice compared to the isotype-treated group (Fig. 4e). Similarly, secreted IL-5 and IL-13 levels were reduced in the α CD52administered group (Fig. 4f, g). In agreement with the aforementioned results, lung histology revealed an increased thickening of

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Fig. 3 CD52 depletion significantly ameliorates IL-33-induced AHR and pulmonary inflammation. a A group of WT mice (n = 6 mice) were treated with 250 µg of anti-CD52 depleting antibody (α CD52) *i.p.* or isotype control on day 1. In addition, the mice were challenged with recombinant mouse (rm)-IL-33 (0.5 µg) or PBS intranasally (*i.n.*) on days 2, 3, and 4. On day 5, we assessed the lung function and acquired the samples, as shown in the timeline. **b**, **c** Line graphs show lung resistance and dynamic compliance (cDyn) in response to increasing doses of methacholine. Total numbers of CD45⁺ cells (**d**), CD3⁺ T cells (**e**), neutrophils (**f**), and eosinophils (**g**) in BAL fluid have been demonstrated in the bar graphs. **h** the numbers of ILC2s in the lungs. **i** *Rag2^{-/-}* mice (n = 6 mice) were treated with 250 µg of α CD52 *i.p.* or isotype control on day 5, **j.** Lung resistance. **k** Dynamic compliance. **I** Number of eosinophils in BAL fluid. **m** Number of ILC2s in lungs. **n** IL-5 levels in BAL. **o** IL-13 levels in BAL. **p** Representative images (left panel) and quantification (right panel) of hematoxylin and eosin-stained histologic sections of the lungs of mice. Scale bars = 50 µm. Data are shown as means ± SEMs and are representative of three individual experiments. Statistical analysis, two-tailed Student's *t*-test; n.s. not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001. Mouse and lung images are provided with permission from Servier Medical Art.

the epithelium layer, as well as an increased number of inflammatory cells in *Alternaria*-treated, isotype-administered mice but not in *Alternaria*-treated, α CD52-administered mice (Fig. 4h). Collectively, these results show that anti-CD52 therapy can prevent *Alternaria alternata*-induced AHR in an ILC2-driven model of allergic asthma.

We next explored whether CD52 depletion can serve as a therapeutic avenue to reverse established *Alternaria* *alternata*-induced AHR. A group of $Rag2^{-/-}$ mice was challenged *i.n.* with extract of *Alternaria alternata* (100 µg per mouse) or PBS on days 1–5. Subsequently, the mice received either α CD52 or isotype control antibody *i.p.* on day 6 (250 µg per mouse). Measurements of lung function and sample acquisition were followed on day 8 (Fig. 4i). α CD52 treatment significantly reduced airway resistance (Fig. 4j) and increased dynamic compliance (Fig. 4k) compared to the isotype control. Furthermore, α CD52

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Fig. 4 CD52 depletion ameliorates *Alternaria alternata*-induced AHR. **a** A group of $Rag2^{-/-}$ mice (n = 6 mice) was treated with 250 µg of anti-CD52 depleting antibody (α CD52) *i.p.* or isotype control on day 1. The mice were then challenged with *Alternaria alternata* (100 µg in 50 µL) or PBS intranasally (*i.n.*) on days 2–6. On day 7, we assessed the lung function, as shown in the timeline. **b**, **c** Line graphs show lung resistance and dynamic compliance (cDyn) in response to increasing doses of methacholine. **d** The numbers of eosinophils in the BAL. **e** The numbers of ILC2s in the lungs. **f**, **g** Bar graphs show secretion levels of IL-5 and IL-13 in BAL. **h** Representative images (left panel) and quantification (right panel) of hematoxylin and eosin-stained histologic sections of the lungs of mice. Scale bars = 50 µm. **i** A group of $Rag2^{-/-}$ mice (n = 6 mice) was challenged with *Alternaria alternata* (100 µg in 50 µL) or PBS intranasally (*i.n.*) on days 1–5. On day 6, the mice were treated with 250 µg of α CD52 *i.p.* or isotype control. The lung function and samples were measured on day 8. **j** Lung resistance. **k** Dynamic compliance. **I** Number of eosinophils in BAL fluid. **m** Number of ILC2s in lungs. **n**, **o** BAL IL-5 and IL-13 levels. Data are shown as means ± SEMs and are representative of 3three individual experiments. Statistical analysis, two-tailed Student's *t*-test. **P < 0.01, ***P < 0.001, ****P < 0.001.

treatment effectively reduced BAL eosinophilia (Fig. 4I), and depleted pulmonary ILC2s (Fig. 4m). In agreement with the aforementioned findings, the secreted IL-5 (Fig. 4n) and IL-13 (Fig. 4o) levels were abrogated in the BAL fluid. Collectively, these results demonstrate that CD52 depletion can ameliorate *Alternaria alternata*-induced AHR.

CD52 is expressed on human ILC2s, and its depletion ameliorates human ILC2s-mediated AHR

In order to further validate the translation potential of our findings, we continued to investigate whether human ILC2s

express CD52. Human ILC2s were freshly purified from healthy human peripheral blood mononuclear cells (PBMCs) and cultured in the presence of both recombinant human IL-2 (rh-IL-2; 10 ng) and IL-7 (rh-IL-7; 10 ng) in the presence or absence of rh-IL-33 (10 ng). Human ILC2s were gated on the basis of the lack of expression of human lineage markers (CD3, CD5, CD14, CD16, CD19, CD20, CD56, CD235a, CD1a, and CD123) and expression of CD45, CRTH2, and CD127 (Fig. 5a). CD52 mRNA levels were analyzed after 9 h of ex vivo IL-33 stimulation and revealed that CD52 is constitutively expressed at a mRNA level (Fig. 5b). Furthermore, flow cytometry analysis confirmed the protein

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Fig. 5 Human ILC2s express CD52, and Alemtuzumab ameliorates human ILC2-mediated AHR. Human peripheral blood ILC2s were freshly sorted (n = 6 donors) and cultured with 10 ng of recombinant human (rh)-IL-2 and rh-IL-7 in the presence or absence of rh-IL-33 (10 ng). CD52 mRNA expression levels were analyzed after 9 h and CD52 protein expression levels were analyzed after 48 h. **a** The gating strategy of human ILC2s identified as Lin⁻CD45⁺CD127⁺CRTH2⁺ cells. **b**, **c** Expression levels of CD52 at a mRNA and protein levels in both naïve (PBS) and IL-33- activated human ILC2s. Corresponding quantitation of CD52 expression shown as MFI ± SEM, n = 6. **d** Human peripheral ILC2s (n = 6 donors) were purified via FACS and cultured with recombinant human (rh)-IL-2 (20 ng/ml) and rh-IL-7 (20 ng/ml) for 48 h, and then adoptively transferred into $Rag2^{-/-II2rg^{-/-}}$ mice that were treated with 250 µg of Alemtuzumab *i.p.* or isotype control on day 1. In addition, mice were challenged with either rh-IL-33 (1 µg) or PBS intranasally (*i.n.*) on days 2, 3, and 4. Measurement of lung function and analysis of BAL followed on day 5, as shown in the timeline. **e**, **f** Line graphs show lung resistance and dynamic compliance (CDyn) in response to increasing doses of methacholine. **g** The numbers of eosinophils in the BAL. **h** The numbers of human ILC2s in the lungs. Data are shown as means ± SEMs and are representative of three individual experiments. Statistical analysis, two-tailed Student's *t*-test or one-way ANOVA followed by Tukey posthoc tests. *P < 0.05, **P < 0.01, ****P < 0.001, ****P < 0.001, and n.s. non-significant. Human, mouse, and lung images are provided with permission from Servier Medical Art.

expression of CD52 after 48 h of ex vivo IL-33 stimulation (Fig. 5c). In agreement with aforementioned murine results, CD52 is highly expressed on human circulating ILC2s.

In order to further confirm the translational potential of our findings, we next explored the efficacy of anti-CD52 treatment in ameliorating human ILC2-mediated AHR. We purified ILC2s from human PBMCs of healthy donors and, after 48 h of ex vivo culture in the presence of rh-IL-2 (20 ng/ml) and rh-IL-7 (20 ng/ml), adoptively transferred the human ILC2s through the tail vein to $Rag2^{-/-}I/2rg^{-}$ mice, which lack T, B, and NK cells and ILCs. The humanized mice were treated with 250 µg of the FDA-approved anti-human CD52 monoclonal antibody Alemtuzumab (Hu116) i.p. or isotype control on day 1, and subsequently challenged with rh-IL-33 (1 µg) or PBS i.n. on days 2, 3, and 4 (Fig. 5d). On the fifth day we measured lung function as described above and assessed the BAL fluid by flow cytometry. Lung function analysis demonstrated that i.n. rh-IL-33 administration increases lung resistance (Fig. 5e) and decreases dynamic compliance (Fig. 5f) in $Rag2^{-/-}Il2rg^{-/-}$ mice. In contrast, lung resistance in rh-IL-33-treated group that received Alemtuzumab was significantly lower, and dynamic compliance higher, compared to the rh-IL-33-treated isotype control-treated mice (Fig. 5e, f). Furthermore, the rh-IL-33 challenge led to eosinophilia, increased human ILC2 numbers and thus lung inflammation in the recipients of isotype control. However, Alemtuzumab-treated group showed significant abrogation of eosinophilia in BAL fluid (Fig. 5g). Moreover, Alemtuzumab-treated group showed significant depletion and decreased presence of human ILC2s in the lungs (Fig. 5h). Taken together, these results indicate that Alemtuzumab depletes human ILC2s in vivo and can potentially serve as a novel therapeutic option for amelioration of human ILC2-mediated lung inflammatory diseases. Moreover, since Alemtuzumab only binds and depletes human CD52, these results further indicate that CD52-mediated depletion of ILC2s is sufficient for abrogation of AHR.

CD52 depletion ameliorates HDM-induced AHR in preventative and therapeutic humanized models

To further validate the translational potential of our findings in a different humanized model of asthma, we next explored the efficacy of anti-CD52 treatment in HDM-mediated AHR. A group of $Rag2^{-/-}Il2rg^{-/-}$ mice were reconstituted with total PBMCs from HDM-allergic patients on day 0. The humanized mice were then sensitized with HDM on days 3 and 4. The mice were treated with Alemtuzumab or the isotype control (250 µg per mouse) on day 8, and subsequently challenged i.n. with HDM on days 9-11. The lung function and BAL fluids were assessed on day 12 (Fig. 6a). Interestingly, preventative treatment of recipients with Alemtuzumab significantly improved AHR (Fig. 6b) and abrogated eosinophilia in the BAL fluid (Fig. 6c). Next, we implemented the Alemtuzumab treatment in a therapeutic humanized model to determine the efficacy of ant-CD52 treatment in reversing established AHR. A group of $Rag2^{-/-} Il2rg^{-/-}$ mice were reconstituted with PBMCs from HDM-allergic patients on day 0 and sensitized i.p. on days 3 and 4 (Fig. 6d). The humanized mice were challenged i.n. with HDM on days 9, 10, and 11. Subsequently, the humanized mice were treated with Alemtuzumab or the isotype control (250 µg per mouse) on day 12. The measurements of lung function and analysis of BAL fluid followed on day 14. In agreement with the preventative model, Alemtuzumab treatment ameliorated AHR (Fig. 6e), and abrogated BAL eosinophilia (Fig. 6f). Taken together, these studies herein demonstrate that CD52 depletion can suppresses AHR in independent models of asthma, and thus crucially suggests a new therapeutic avenue for asthmatic patients.

DISCUSSION

In the present study, we established the therapeutic potential of anti-CD52 immunotherapy in both preventing and reversing AHR

and allergic asthma, independent of initiation mechanism. We established that CD52 is expressed on T and B lymphocytes in our AHR model and demonstrated that CD52 depletion can severely lower a T cell-driven model of AHR induced by HDM. We further established that CD52 is constitutively expressed by murine and human ILC2s under steady-state and inflammatory conditions. Notably, IL-33 enhances CD52 expression in murine ILC2s, but not in human ILC2s. Such dichotomy could be due to the reported biological differences among murine and human ILC2s.^{38,39} Our data also suggest that CD52 expression is not inducible on other murine cell types such as T cells and B cells. This observation could be attributed to the unique and distinct biological identities of these immune populations. Subsequently, we established the high efficacy of CD52 depletion for amelioration of IL-33-induced AHR. We utilized an IL-33-based model because both IL-33 and IL-25 have been previously shown to induce ILC2-mediated AHR and lung inflammation, although IL-33 was more potent than IL-25. In order to exclude the effects of adaptive immune system cells in amelioration of AHR, we utilized $Rag2^{-/-}$ mice that lacked any mature B and T cells. 41,42

Since alarmins, such as IL-33, are not naturally found in the pulmonary environment, we subsequently examined the potential of anti-CD52 treatment on lung inflammation and AHR induced by clinically relevant allergens. In addition to HDM, we utilized *Alternaria alternata* as this allergen has been shown to induce severe allergic AHR in humans.⁴³ Moreover, Alternaria *alternata* has been reported to cause allergic inflammation in mice independent of adaptive immunity, making it an ideal model to study ILC2-dependent asthma.^{44,45} Collectively, our result shows that depletion of ILC2s and T cells by targeting CD52 improves lung function and curtails lung inflammation following exposure to different principal allergenic agents.

In addition to the CD52-targeted approaches presented in this study, our group and others have previously explored various other tactics for treating allergic asthma by modulation of ILC2 and T cell responses.^{46–53} For example, previous studies have recommended direct depletion of CD4 $^+$ T_Hs and CD8 $^+$ CTLs as a therapeutic avenue for treating asthma, while others have instead suggested targeted depletion of CRTH2⁺ cells to reduce pulmonary inflammation.^{54,55}These approaches are similar to our presented data as they all result in depletion of T_H2s and ILC2s. Since degranulation of eosinophils is also a key exacerbating event during the autoinflammatory process, others have raised the potential of directly targeting and depleting eosinophils for management of severe asthma.⁵⁶ Our results collectively demonstrate that CD52targeted approach efficiently reduces eosinophilia. Similarly, other groups have highlighted the pathological roles of neutrophils and mast cells, suggesting these cells should be targeted for treatment of asthma.57-59 Our study further establishes that CD52 depletion can abrogate the number of neutrophils in the BAL. Myeloid-derived dendritic cells are yet another important population in asthma immunopathogenesis.^{60,61} Therefore, depletion of this population by a CD52-targeted approach or other similar strategies can have severe therapeutic implications.

Asthma is a complex and heterogeneous disease with different phenotypes and variable manifestations due to participations of multiple types of proinflammatory cells.^{62,63} As a result, targeting a single cell type may not be sufficient to restore pulmonary tolerance and abrogation of underlying inflammation. There has been a dire need for a viable biological marker, like CD52, that effectively targets all of the pathogenic type 2 populations simultaneously and creates "space in immunity" for reestablishment of peripheral immune tolerance. CD52 is advantageously expressed by all of the aforementioned type 2 immune cells and our results now firmly establish that CD52-based therapeutics can ameliorate allergic AHR.^{16,21,22} Since HSCs do not express CD52, depletion of CD52⁺ cells has been shown to be subsequently followed by a gradual repopulation and reprogramming of the

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Fig. 6 Alemtuzumab ameliorates HDM-induced AHR in preventative and therapeutic humanized mice models. a Total PBMCs from HDM-positive patients (5 million) were adoptively intravenously transferred into $Rag2^{-/-1}l2rg^{-/-}$ mice that were sensitized on days 3 and 4 (n = 6 donors). At day 8, mice received an intraperitoneal injection of Alemtuzumab or control isotype (250 µg). Subsequently, the humanized mice were challenges with HDM (50 µg) or PBS i.n. on days 9, 10, and 11. Measurement of lung function and inflammation were performed on day 14. **b** Lung resistance measured in anesthetized, tracheostomized, and mechanically ventilated mice that were exposed to increasing concentrations of methacholine. **c** Number of eosinophils in BAL. **d** Five million total PBMCs from HDM-positive patients (n = 6 donors) were adoptively intravenously transferred into $Rag2^{-/-1}l2rg^{-/-}$ mice that were then sensitized on days 3 and 4. The humanized mice were then challenges with HDM (50 µg) or PBS i.n. on days 9, 10, and 11. On day 12, mice received an intraperitoneal injection of Alemtuzumab or control isotype (250 µg). Measurement of lung function and inflammation were performed on day 14. **e** Lung resistance. **f** Number of eosinophils in BAL. Statistical analysis, two-tailed Student's *t*-test. ****P* < 0.001, and *****P* < 0.0001. Human, mouse, and lung images are provided with permission from Servier Medical Art.

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immune cells that arise from these stem cell precursors.^{8,9,64,65} Such edited immune profile has been shown to ultimately result in a shift of the immunological networks toward a more tolerogenic state, as evident by the reportedly fast rebound kinetics of regulatory cells such as Tregs.⁶⁶ Unlike our CD52-based approach, other asthma therapeutics that target the ubiquitously expressed CD45 are not ideal because they lead to depletion of the HSCs in the bone marrow and thus prevent reconstitution of the immune repertoire without undergoing a bone marrow transplantation.^{67,68} In addition to such therapeutic advantages, our results here introduce powerful amelioration of AHR by CD52targeted depletion in various models of asthma. Collectively, our results establish depletion of CD52⁺ cells as a novel strategy for targeting the pathophysiological roots of asthma, ameliorating pulmonary inflammation and AHR, and restoring peripheral immune tolerance to potential treat patients.

It is important to note that our results have explored the viability of CD52-targeted therapy for acute models of allergic asthma that do not exhibit lung remodeling. Thus, future investigations are necessary to explore the role and therapeutic effect of anti-CD52 therapy on lung remodeling in chronic models of asthma. Furthermore, we observed a reduction in the number of CD45⁺ and CD3⁺ cells in healthy murine models without lung inflammation. Since temporary reduction of these cells can result in short-term partial immune suppression, healthy individuals may be at increased risk of infections and malignancies.⁶⁵ Nonetheless, there may still be a population of severe asthmatics with limited therapeutic options,⁶⁹ such as those with corticosteroid-resistant or eosinophilic asthma, in whom the long-term clinical benefits of anti-CD52 drugs, such as the FDA-approved Alemtuzumab, may justify its potential adverse effects. The generally favorable longterm outcomes observed with Alemtuzumab in small numbers of patients with hypereosinophilic syndrome^{70,71} further uphold CD52-targeted treatment as viable concept worthy of further consideration. Moreover, the adverse effects of anti-CD52 treatment could be minimized through careful dosing strategies, such as those used for MS compared to leukemia, and development of other α CD52 antibodies that may be more efficacious but less immunogenic.72,7

In order to further assess the clinical relevance of our findings, we developed various preventative and therapeutic humanized mouse models in which human peripheral ILC2s from healthy donors or PBMCs from HDM-allergic patients are adoptively transferred to $Rag2^{-/-} II2rg^{-/-}$ mice followed by intranasal administration of either recombinant human IL-33 or HDM to induce AHR and inflammation. Importantly, human IL-5 has been reported to activate murine eosinophils, underscoring the feasibility of using humanized mice in eosinophilic inflammatory studies.^{74,75} As a result, these humanized mouse models provide a unique platform for investigating the contribution of immune cells to human asthma and assessing the efficacy of potential therapeutic targets in preclinical studies. Utilizing this model, we demonstrated that the aforementioned FDA-approved anti-CD52 monoclonal antibody Alemtuzumab efficiently ameliorates both human ILC2-driven and human T_H2-driven AHR by improving lung functions and reducing lung inflammation. Furthermore, our humanized models provided additional insights into the mechanism of anti-CD52 treatment by excluding the direct targeting effect of other murine cells such as CD52⁺ neutrophils and eosinophils. Alemtuzumab specifically binds and depletes human CD52⁺ cells only. Thus, our humanized mice results demonstrate that specific CD52target depletion of human ILC2s in vivo is sufficient for amelioration of AHR and reduction of mouse eosinophilia. Overall, our humanized models further emphasize the therapeutic potential and translatability of our findings to future clinical studies of asthma.

METHODS

Mice

WT BALB/cByJ, recombination-activating gene 2-deficient ($Rag2^{-/-}$), recombination-activating gene 2-deficient gamma-chain-deficient ($Rag2^{-/-}Il2rg^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility at the University of Southern California. Mice were maintained at macroenvironmental temperature of 21–22 °C, humidity (48–52%), in a conventional 12:12 light/dark cycle with lights on at 6:00 a.m. and off at 6:00 p.m. We used 5- to 8-week-old age-matched female mice in our studies. All animal studies were approved by the USC institutional Animal Care and Use Committee and conducted in accordance with the USC Department of Animal Resources' guidelines.

Induction and measurement of AHR and collection of bronchoalveolar lavage fluid

The mice were *i.n.* challenged via rm-IL-33 (BioLegend, San Diego, CA), Alternaria alternata extracts (Greer Laboratories, Lenoir, NC), HDM (Greer Laboratories, Lenoir, NC), as shown in the experimental schemes. For CD52 depletion, anti-murine CD52 monoclonal antibody (BTG-2G, MBL Laboratories) and the corresponding isotypes were used. Lung function was measured by direct measurement of lung resistance and dynamic compliance in anesthetized tracheostomized mice, in which mice were mechanically ventilated via the FinePointe RC system (Buxco Research Systems, Wilmington, NC) and sequentially challenged with aerosolized increasing doses of methacholine. After measurements of AHR, the trachea was cannulated and the BAL fluid was collected as described before.⁷⁶ Briefly, we tracheostomized and intubated the mice and then washed the airways three times with 1 mL of PBS each time, followed by centrifuging at 400 g for 7 min and harvesting the cells. Data were analyzed with FlowJo software (TreeStar, Ashland, OR). The levels of IL-5 and IL-13 were measured in BAL using Legendplex multiplex kits (BioLegend) and data were analyzed via the LEGENDplex data analysis software v8.0. The absolute cell numbers in BAL fluid were calculated by means of flow cytometry by staining the cells with phycoerythrin (PE)-anti-Siglec-F (BD Biosciences, San Jose, CA), fluorescein isothiocyanate (FITC)-anti-CD19, peridinin-chlorophyll-protein complex (PerCP)/Cy5.5-anti-CD3ε, allophycocyanin (APC)-anti-Gr-1, PE/Cy7-anti-CD45, APC/Cy7-anti-CD11c (BioLegend, San Diego, CA), and eFluor450-anti-CD11b (eBioscience, San Diego, CA) in the presence of anti-mouse FC-block (BioXcell, West Lebanon, NH). We used CountBright Absolute Count Beads (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. At least 10⁵ CD45⁺ cells were acquired on a BD FACSCanto II (BD Biosciences).

Tissue preparation for flow cytometry

Utilizing fine surgical scissors, murine lungs were surgically removed and minced in a sterile environment subsequently incubated in type IV collagenase (1.6 mg/mL; Worthington Biochemicals, Lakewood, NJ) at 37 °C for 60 min. After digestion, murine lung fragments were then pressed through a 70-µm nylon cell strainer, using the rubber end of a sterile 10 mL syringe plunger, in order to create a single cell suspension. In order to terminate the enzymatic reaction of collagenase, the cells were washed with 1× PBS by centrifugation at $400 \times g$ for 7 min at 4 °C. In order to exclude and lyse the red blood cells (RBCs), the cell pellet was subsequently resuspended in $1 \times RBC$ lysis buffer (Biolegend[®], San Diego, CA) and incubated at room temperature for 5 min. In order to terminate the chemical reaction, the cells were subsequently washed and centrifugedat $400 \times q$ for 7 min at 4 °C—with 1× PBS. The remaining pellet was then further prepared for flow cytometry. The absolute cell numbers in lung tissue were calculated by means of flow cytometry. Murine ILC2s were defined based on the lack of expression of classical lineage markers (CD3e, CD5, CD45R, Gr-1, DX5, CD11c, CD11b, Ter119, NK1.1, TCR-y\delta, and FCERI) and expression of CD45, ST2, and CD127.41 Murine ILC1s were defined as lineage negative, ST2 negative, c-Kit negative, CD45 and CD127 positive. Murine ILC3s were defined as lineage negative, ST2 negative, CD45, CD127, and c-Kit positive. All cells were stained with Biotinylated anti-mouse lineage (CD3e (145-2C11), CD5 (53-7.3), CD45R (RA3-3B2), Gr-1 (RB6-8C5), DX5 (DX5), CD11c (N418), CD11b (M1/70), Ter119 (TER-119), NK1.1 (PK136), TCR-b (H57 597), TCR-γδ (GL3), and FcεRla (MAR-1)), FITC-anti-streptavidin, PE-anti-CD52 (MBL International, Woburn, MA), APC/Cy7-anti-CD45, PE/Cy7-anti-CD127 (BioLe-Diego, CA), PerCP/Cy5.5-anti-ST2 (Thermo aend San Fisher Scientific, Waltham, MA), and APC anti-mouse c-Kit (BioLegend, San Diego, CA). We used CountBright Absolute Count Beads (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. At least 10⁵ CD45⁺ cells were acquired on a BD FACSCanto II (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR).

Ex vivo stimulation of ILC2s

All cells were purified by flow cytometry using BD FACS ARIA III (BD biosciences, San Jose, CA) with a purity of >95%. The isolated ILC2s were cultured (at least 5×10^3 cells/well) in 96-well round-bottom plates with GibcoTM Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, Waltham, MA) that was supplemented with 10% fetal bovine serum, 2% antibiotics (penicillin and streptomycin), and 0.05 mM b-mercaptoethanol. The cells were maintained in a 37 °C incubator with 5% CO₂.⁷⁷ All murine ILC2s were cultured in the presence of recombinant mouse rm-IL-2 (10 ng/mL), rm-IL-7 (10 ng/mL), and/or rm-IL-33 (10 ng/mL). All human ILC2s were cultured in the presence of rh-IL-2 (10 ng/mL), rh-IL-7 (10 ng/mL), and/or rh-IL-33 (10 ng/mL).

Isolation of human PBMCs and humanized mice models

All human studies were approved by USC Institutional review board and conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all human participants. Human blood samples were obtained from male and female healthy donors (age 18-65). In few experiments, blood was collected from allergic patients having positive skin tests to HDM antigen. Informed consent was obtained from all subjects according to our approved IRB protocols. PBMCs from donors were first isolated from fresh blood by diluting the blood 1:1 in PBS then adding to SepMateTM-50 separation tubes (STEMCELL Technologies Inc, Vancouver, Canada) prefilled with 15-mL LymphoprepTM each (Axis-Shield, Oslo, Norway) and centrifugation at $1200 \times g$ for 15 min. Human PBMCs were then stained and purified using BD FACS ARIA III (BD Biosciences, San Jose, CA) with a purity of 95%. Human ILC2s were defined based on the lack of expression of classical human lineage markers and expression of CRTH2, CD127, and CD45. The cells were stained with FITC-anti-human lineage (CD3, CD5, CD14, CD16, CD19, CD20, CD56, CD235a, CD1a, CD123), APC/Cy7-anti-CD45, PE-anti-CD294 (CRTH2), PE/Cy7-anti-CD127, APC-anti-CD52 (BioLegend, San Diego, CA). We used CountBright Absolute Count Beads (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. At least 10⁵ CD45⁺ cells were acquired on a BD FACSCanto II (BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, OR). For some experiments, purified human ILC2s were cultured with recombinant human (rh)-IL-2 (20 ng.mL⁻¹) and rh-IL-7 (20 ng.mL⁻¹) for 48 h, then adoptively transferred to $Rag2^{-/-1/2rg^{-/-}}$ milico (2.5 × 10⁵ ILC2s per mouse). For some experiments, 5 million PBMCs were adoptively transferred to $Rag2^{-/-1/2rg^{-/-}}$ mice.⁷⁸ The humanized mice were challenged i.n. via 1 µg of rh-IL-33 (BioLegend, San Diego, CA) or HDM (Greer Laboratories, Lenoir, NC). Alemtuzumab (Hu116) or the isotype control were purchased from R&D Systems.

Histological analysis of the lungs

The lungs were injected and fixed with 10% paraformaldehyde in PBS until inflated as described before.⁷⁹ After overnight fixation, the lungs were processed for histology as described before.⁷⁹ The lung tissue was embedded in paraffin, cut into 4-µm sections and stained with H&E according to standard protocols.^{49,51} Sections were scanned using light microscope for inflammation. Images of hematoxylin and eosin-stained tissue slides were acquired with a KeyenceBZ-9000 microscope (Keyence, Itasca, IL) and assembled into multipanel figures using Adobe Illustrator software (version 22.1). Histologic images were analyzed per user guide with the ImageJ Analysis Application (NIH & LOCI, University of Wisconsin) and quantified results were presented as demonstrated before.49,51 Briefly, acquired Images of hematoxylin and eosinstained tissue were saved as high-resolution TIFF files. Subsequently, we measured the epithelium thickness of each individual airway in eight randomly selected segments. This process was repeated for six different airways in each animal and the representative epithelial thickness for each mouse was then quantified by averaging these obtained measurements. For quantification of the cell number, eight random representative sites of the same area (250 µm²) for each mouse were selected, cells were enumerated in each site, and the average of the number of cells per site was calculated and presented as the representative value for each mouse.

Statistical analysis

All data are expressed as mean \pm standard error of the mean. Comparisons between study groups were analyzed by Student's *t*tests and one-way ANOVA by Tukey post-hoc tests. *P* values of <0.05 were considered to be statistically significant. Statistical analyses were performed using the GraphPad Prism 7 software (La Jolla, CA).

ACKNOWLEDGEMENTS

This article was financially supported by National Institutes of Health Public Health Service grants R01 HL133169, R01 HL148110 (H. A.), R01 ES025786, R01 ES021801, R01 HL151493, R01 HL144790, R01 Al145813, and R21 Al132084 (O. A.).

AUTHOR CONTRIBUTIONS

P. S.-J. designed, performed, and analyzed all experiments and wrote the manuscript. D. G. H., B. P. H., L. G.-T., E. H., C. Q., J. D. P., M. F., and R. L. helped perform experiments and provided animal husbandry for experiments. H. A. assisted with design and interpretation of data and edited the manuscript. O. A. supervised, designed the experiments, conceptualized, interpreted the data, and finalized the manuscript. All authors helped with reviewing and editing the manuscript.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41385-021-00388-5.

Competing interests: The authors declare no competing interests.

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