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ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

Immunological effects of adjuvanted low-dose allergoid allergen-specific immunotherapy in experimental murine house dust mite allergy

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Abstract

Background: Native allergen extracts or chemically modified allergoids are routinely used to induce allergen tolerance in allergen-specific immunotherapy (AIT), although mechanistic side-by-side studies are rare. It is paramount to balance optimal dose and allergenicity to achieve efficacy warranting safety. AIT safety and efficacy could be addressed by allergen dose reduction and/or use of allergoids and immunostimulatory adjuvants, respectively. In this study, immunological effects of experimental house dust mite (HDM) AIT were investigated applying high-dose HDM extract and low-dose HDM allergoids with and without the adjuvants microcrystalline tyrosine (MCT) and monophosphoryl lipid A (MPL) in a murine model of HDM allergy.

Methods: Cellular, humoral, and clinical effects of the different AIT strategies were assessed applying a new experimental AIT model of murine allergic asthma based on physiological, adjuvant-free intranasal sensitization followed by subcutaneous AIT.

Results: While low-dose allergoid and high-dose extract AIT demonstrated comparable potency to suppress allergic airway inflammation and Th2-type cytokine secretion of lung-resident lymphocytes and draining lymph node cells, low-dose allergoid AIT was less effective in inducing a potentially protective IgG1 response. Combining

Abbreviations: AIT, allergen-specific immunotherapy; Alum, aluminum hydroxide formulation; BAL, bronchoalveolar lavage; Der f, *Dermatophagoides farinae*; Der p, *Dermatophagoides pteronyssinus*; FoxP3, Forkhead box P3; GATA3, GATA-binding protein 3; HD-extract-AIT, high-dose HDM extract AIT; HDM, house dust mite; i.n., intranasal; i.p., intraperitoneal; LD-allergoid-AIT, low-dose HDM allergoid AIT; LPS, lipopolysaccharide; MCT, microcrystalline tyrosine; MPL, monophosphoryl lipid A; s.c., subcutaneous; sIgE, specific immunoglobulin E; sIgG, specific immunoglobulin G; ST2, suppression of tumorigenicity 2; tIgE, total immunoglobulin E; tIgG, total immunoglobulin G; TLR, Toll-like receptor; Treg, regulatory T cell.

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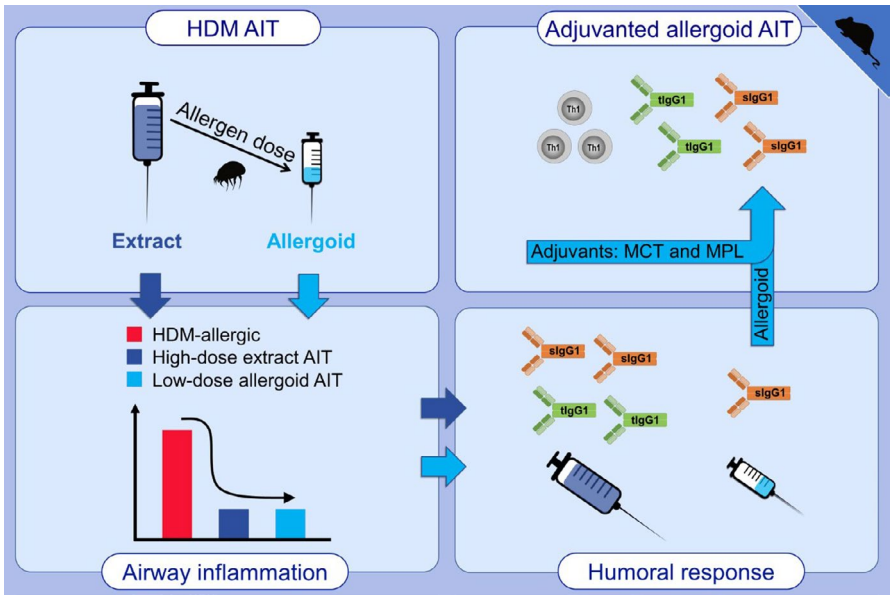
low-dose allergoid AIT with MCT or MCT and dose-adjusted MPL promoted Th1-inducing mechanisms and robust B-cell activation counterbalancing the allergic Th2 immune response.

Conclusion: Low allergen doses induce cellular and humoral mechanisms counteracting Th2-driven inflammation by using allergoids and dose-adjusted adjuvants. In light of safety and efficacy improvement, future therapeutic approaches may use low-dose allergoid strategies to drive cellular tolerance and adjuvants to modulate humoral responses.

KEYWORDS
adjuvant, allergen extract, allergen-specific immunotherapy, allergoid, house dust mite allergy



GRAPHICAL ABSTRACT
In experimental HDM AIT, a 220-fold reduced allergen dose is equally effective in control of allergic inflammation when HDM allergoids instead of extracts are applied. Low-dose nonadjuvanted allergoid AIT is less effective in inducing a potentially protective IgG1 response. Combining low-dose allergoid AIT with the adjuvant MCT or the adjuvant system MCT + MPL promotes Th1-inducing mechanisms and robust B-cell activation.

1 | INTRODUCTION

Allergen-specific immunotherapy (AIT) can restore allergen tolerance and has been introduced over 100 years ago using heat-denatured allergens.[1] Until today, modified allergens (allergoids) with destroyed conformational IgE epitopes, for example, by chemical modification, are well-accepted standard in clinical use. These modifications reduce side effects and, therefore, enhance the utility and safety of AIT.[2-4] However, the tolerogenic potency of allergoids compared to natural allergen extracts is controversially discussed.[5-8]

Mechanisms underlying extract and allergoid immunotherapies have not been explored side by side. However, both therapeutic strategies induce IgG4, which can compete with IgE-binding sites on allergens.[9-11] IgE cross-linking activates FcεR-expressing cells such as mast cells and basophils which degranulate and recruit further pro-inflammatory cells like eosinophils and neutrophils. This inflammatory cascade is a hallmark of allergic airway inflammation.[12] IgE production originally depends on B cells that receive help from IL-4-producing Th2 cells, which also decrease after several years of immunotherapy, while IFN-γ-producing Th1 cells are increasing. These changes are considered to be important mechanisms of AIT and are probably promoted by regulatory T cells (Tregs).[13]

AIT efficacy may be increased by adjuvants,[14] which are immunomodulatory substances that have the potential to enhance antigen-specific responses both on humoral and on cellular level. Two clinically approved adjuvants, addressed in this study, are microcrystalline tyrosine (MCT), a biocompatible and biodegradable depot adjuvant, and monophosphoryl lipid A (MPL), derived from *Salmonella minnesota* LPS with strongly reduced toxicity. Both

MCT and MPL promote Th1 immune responses and IgG-inducing mechanisms.[15-20]

The current study focuses on house dust mites (HDM) as a major perennial allergen source that is linked to allergic asthma and other allergic diseases.[21] As this allergen source is hard to avoid, the design of safe and effective HDM-specific AIT is of great importance.[21] Therefore, the aim was to provide comparative mechanistic insights into the balance between HDM allergens and allergoids as well as adjuvants in relation to changes of humoral and cellular immune responses. Since common murine AIT models are based on intraperitoneal (i.p.) aluminum hydroxide-(alum)-dependent sensitization,[22] a new experimental AIT model was established. Based on more realistic adjuvant-free intranasal (i.n.) sensitization and subsequent subcutaneous (s.c.), AIT this model should bear advantage of mimicking human allergy in a more physiological way. The effects of high-dose HDM extract AIT and low-dose HDM allergoid AIT with and without adjuvants were subjected to an in-depth immunological analysis and revealed tolerogenic effects of low-dose allergoids combined with dose-adjusted adjuvants.

2 | METHODS

2.1 | Animals and reagents

Female C57BL/6J mice between 5 and 6 weeks of age (Charles River, Sulzfeld, Germany) were housed under specific pathogen-free conditions. All experiments were carried out under federal guidelines for the use and care of laboratory animals and were approved by the government of the district of upper Bavaria, Germany (ethical approval: 55.2-1-54-2532-50-2017).

Dermatophagoides pteronyssinus (Der p) and *Dermatophagoides farinae* (Der f) HDM extract, Der p and Der f allergoids, MCT and MPL were provided by Allergy Therapeutics (Worthing, United Kingdom). Details about all reagents are given in Appendix S1.

2.2 | Murine model of HDM AIT

Allergic and AIT-treated mice were i.n.-sensitized with 1 μg (total protein content) HDM extract (1:1, Der p and Der f) in 20 μl PBS on three consecutive days followed by i.n. challenges with 1 μg HDM extract on days 7, 13, and 19 (Figure 1A). Nonallergic mice received 20 μl PBS i.n. AIT was performed with s.c. injections of either 220 μg HDM extract, or 1 μg (total protein content) HDM allergoids (1:1, *Der p* and *Der f*), or 1 μg HDM allergoids combined with 2% (v/v) MCT or 1 μg HDM allergoids combined with 2% (v/v) MCT and 50 μg MPL (or with 12.5 μg, 25 μg, and 100 μg MPL in the experiments addressing dose-dependent effects of MPL) in 200 μl PBS on days 14, 17, and 21. Not AIT-treated allergic and nonallergic mice received s.c. injections of 200 μl PBS. The time points for injections were adapted from existing protocols of murine AIT.[22,23] All mice were challenged i.n. with 10 μg HDM extract on days 29, 30, 31, and 32 and euthanized at day 35 for analysis (day 33 for lung function measurement).

2.3 | Immunological and clinical analysis of mouse phenotype

Allergic airway inflammation was assessed by measuring total and differential BAL (bronchoalveolar lavage) cell counts, including eosinophils, neutrophils, $CD4^+$ and $CD8^+$ T cells, and B2 cells, and by lung histology. Additionally, lung tissue-resident lymphocyte populations were analyzed by FACS. Cytokines released from restimulated lung lymphocytes, cervical lymph node cells, and splenocytes were analyzed by multiplex measurements and total and specific IgE and IgG levels in the serum either by multiplex measurement or by ELISA. Lung function analysis was performed 24 h after last allergen challenge in intubated, mechanically ventilated animals. A detailed description of all methods and statistical analyses is given in the Appendix S1.

3 | RESULTS

3.1 | Effects and mechanisms of high-dose HDM extract and low-dose HDM allergoid AIT in murine allergic asthma

In order to compare immunological and clinical effects of high-dose HDM extract AIT (HD-extract-AIT) and low-dose HDM allergoid AIT (LD-allergoid-AIT) side by side, a new experimental HDM AIT model relying on adjuvant-free i.n. sensitization followed by s.c. AIT (Figure 1A) was established. Additional allergen challenges before and during AIT were introduced to mimic the unavoidable allergen contact of HDM-allergic individuals. Preliminary experiments showed that s.c. AIT with 1 μg HDM extract (Figure S1A) displayed rather pro-inflammatory responses such as increased numbers of total BAL cells, BAL eosinophils, and lung-resident $GATA3^+ST2^+FoxP3^-$ Th2 cells compared with allergic mice (Figure S1B–D). Therefore, the dosage of HDM extract AIT was adapted to a concentration used in other murine studies,[22,24] and the effects of HD-extract-AIT (220 μg extract) and LD-allergoid-AIT (1 μg allergoid) were compared.

Both therapies decreased the numbers of total BAL cells (Figure 1B) as well as BAL eosinophils, neutrophils, T cells (Figure 1C), and B-2 cells (Figure S2A) compared to allergic controls. Histological analyses confirmed these results, as lungs of both therapeutic groups displayed significantly decreased peribronchiolar and perivascular inflammatory infiltration. Also, mucus hypersecretion was significantly decreased in both groups, although more pronounced in the LD-allergoid-AIT group (Figure 1D,E). Additionally, the level of IL-5 was significantly decreased in the BAL of both treatment groups compared with allergic mice, while a significant reduction in IL-4 and TNF-α levels was only found in the LD-allergoid-AIT group (Figure 1F and Figure S2B). Moreover, HD-extract-AIT significantly improved lung dynamic compliance, whereas for LD-allergoid-AIT only a trend toward recovery was observed (Figure 1G). However, the increase of lung-resident $GATA3^+ST2^+FoxP3^-$ Th2 cells found in allergic mice

was neither reverted by HD-extract-AIT nor by LD-allergoid-AIT (Figure 1H). In contrast, the number of FoxP3$^+$CD4$^+$ T cells, which was elevated in HDM-allergic mice, was reduced by both treatments (Figure 1H). Furthermore, the frequency of IFN-γ-positive CD4$^+$ T

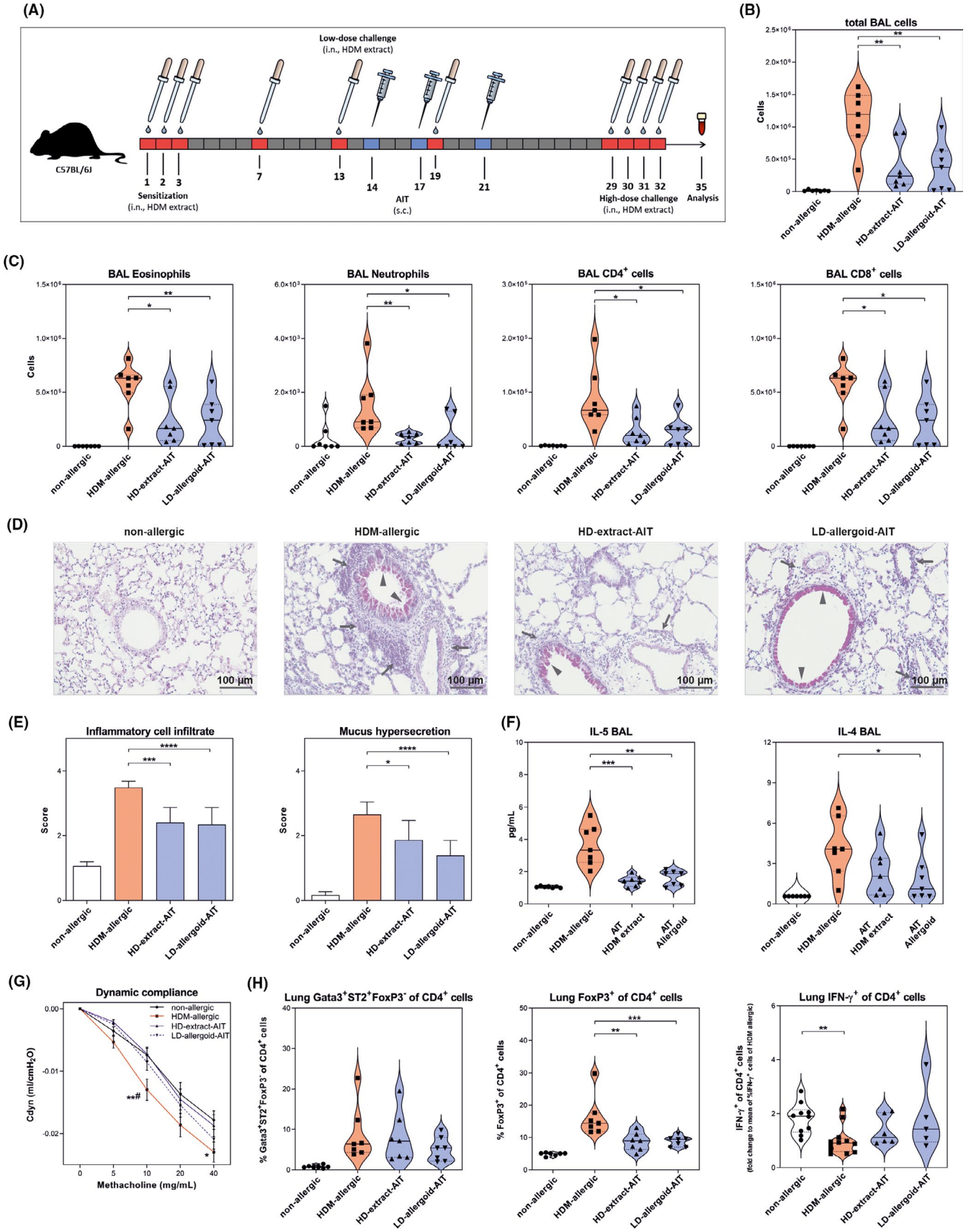


FIGURE 1 Effects of HD-extract-AIT and LD-allergoid-AIT on experimental HDM-allergic airway inflammation. A, Schematic overview of the experimental HDM AIT murine model based on alum-free i.n. sensitization and s.c. AIT. Pipettes and syringes indicate time points of allergen challenges and AIT injections, respectively. i.n, intranasal; s.c., subcutaneous. B, Total BAL cells ($n = 7$). C, Differential counts of eosinophils, neutrophils, $CD4^+$ T cells, and $CD8^+$ T cells from the BAL ($n = 7$). D, Representative lung histology specimen, retrieved 3 days after the last HDM challenge stained with periodic acid-Schiff. Arrows, inflammatory cell infiltrate; arrowheads, mucus hypersecretion. E, Scores of inflammatory cell infiltrate and mucus hypersecretion in lung tissue 3 days after the last HDM challenge ($n = 6$). The scores were analyzed by one-way analysis of variance with Tukey's multiple comparison test. Shown is the mean with SD. F, Levels of IL-5 and IL-4 in the BAL ($n = 7$). G, Measurement of lung dynamic compliance ($n = 6$–12). $^*p < .05$ $^{**}p < .01$ HDM-allergic vs nonallergic; #$p < .05$ HDM-allergic vs AIT HDM extract. Lung function parameters were analyzed by two-way analysis of variance with Tukey's multiple comparison test. Shown is the mean with SEM. H, Analysis of lung-resident lymphocyte populations ($n = 7$) and IFN-γ-producing T cells ($n = 5$–12). In all violin plots, solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired *t* test or Mann–Whitney test, respectively. *p*-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. AIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; HDM, house dust mite

cells was significantly decreased in HDM-allergic mice compared with nonallergic mice. A trend toward reversion of this effect could be achieved by both AIT strategies (Figure 1H).

The levels of secreted Th2-type cytokines IL-4, IL-5, IL-9, and IL-13 were significantly increased in the supernatants of *ex vivo* anti-CD3/anti-CD28-restimulated lung lymphocytes from allergic mice (Figure 2A). Overall, all four cytokines were downregulated by HD-extract-AIT (not significant for IL-9) and LD-allergoid-AIT (not significant for IL-13). Moreover, both AIT regimens significantly reduced the elevated IL-10 levels found in HDM-allergic group (Figure 2A). The levels of cytokines identifying Th17 and Th1 activities (IL-17A/F, IL-22, TNF-α, IFN-γ) were unchanged in both treatment groups (Figure S2C). Also, in the supernatants of restimulated cervical lymph node cells, IL-5 and IL-13 levels were significantly reduced in both AIT groups (Figure 2B). HD-extract-AIT induced slightly, but not significantly, higher levels of IL-4 and slightly lower levels of IL-9 compared to allergic and LD-allergoid-AIT-treated mice. Again, IL-10 secretion was lower in both AIT groups (only significant in the LD-allergoid-AIT group; HD-extract AIT group $p = .053$) (Figure 2B). Furthermore, both AIT strategies reduced the levels of IL-17A, IL-17F, IL-22, TNF-α, and IFN-γ (IFN-γ only significant for LD-allergoid-AIT). Th2-type cytokines were also reduced in the supernatants of restimulated splenocytes following AIT, but its effects were less pronounced. The levels of IL-17A, IL-17F, IL-22, and TNF-α were comparable in all groups of mice (Figure S2D).

HD-extract-AIT and LD-allergoid-AIT had no influence on the elevated total Der p-specific IgE (sIgE) serum levels of HDM-allergic mice, whereas total IgE (tIgE) levels were significantly lower in LD-allergoid-AIT compared with HD-extract-AIT-treated mice (Figure 3A). While HD-extract-AIT induced high levels of total IgG1 (tIgG1), LD-allergoid-AIT failed to induce tIgG1. In contrast, both therapies led to an induction of Der p-specific IgG1 (sIgG1) levels, although changes were only significant in the HD-extract-AIT group (Figure 3B). In addition, Der f-sIgE and Der f-sIgG1 antibodies were detectable in a comparable manner (Figure S3). Moreover, total IgG2c levels were slightly decreased in both AIT groups and total IgG2b levels in the LD-allergoid-AIT group while total IgG3 levels were not affected (Figure S2E).

3.2 | Effects of MCT and MPL on experimental low-dose HDM allergoid AIT

To examine the effects of the adjuvants MCT and MPL on LD-allergoid-AIT, mice received AIT with HDM allergoid alone, HDM allergoid combined with MCT (HDM allergoid + MCT), or HDM allergoid combined with MCT and 50 μg MPL (HDM allergoid + MCT + MPL).

All three AIT regimens had comparable effects on the reduction of total BAL cells (Figure 4A), and BAL eosinophils, neutrophils, $CD4^+$ and $CD8^+$ T cells, and B2 cells (Figure 4A and Figure S4A). Additionally, all three treatment strategies comparably reduced peribronchiolar and perivascular inflammatory infiltration and mucus hypersecretion (Figure 4B and Figure S4B). While IL-5 levels in the BAL were highly reduced in all treatment groups compared to allergic mice, the levels of IL-4 and TNF-α were only significantly reduced in mice treated with allergoid alone or with the combination of allergoid + MCT + MPL (Figure S4C). All AIT groups showed improved lung dynamic compliance compared with HDM-allergic mice, although a significant effect was achieved only in the HDM allergoid + MCT + MPL group (Figure 4C).

While LD-allergoid-AIT alone had only a minor effect on the number of lung-resident $GATA3^+ST2^+FoxP3^-$ Th2 cells, these cells were significantly reduced in mice treated with allergoid + MCT or allergoid + MCT + MPL (Figure 4D). The addition of MPL to the formulation showed no additional effect to the one of MCT. Lung-resident $FoxP3^+CD4^+$ T cells were equally reduced in all treatment groups (Figure 4D). While not effectively restored by HD-extract-AIT or LD-allergoid-AIT alone (Figure 1H), the addition of MCT and MCT + MPL to the LD-allergoid-AIT formulation gradually increased the frequency of IFN-γ-positive lung $CD4^+$ T cells compared to HDM-allergic mice (Figure 4D).

Total IgE and Der p-sIgE levels were not affected by any of the treatment regimens (Figure 4E). While LD-allergoid-AIT alone was not able to induce a significant tIgG1 response, the addition of MCT as well as of MCT + MPL to the formulation iteratively increased tIgG1 levels significantly. Although Der p-sIgG1 levels were elevated in all three treatment groups, changes were only significant in the AIT allergoid + MCT and AIT allergoid + MCT + MPL groups. Here, no additional effect of MPL was visible (Figure 4E). While total

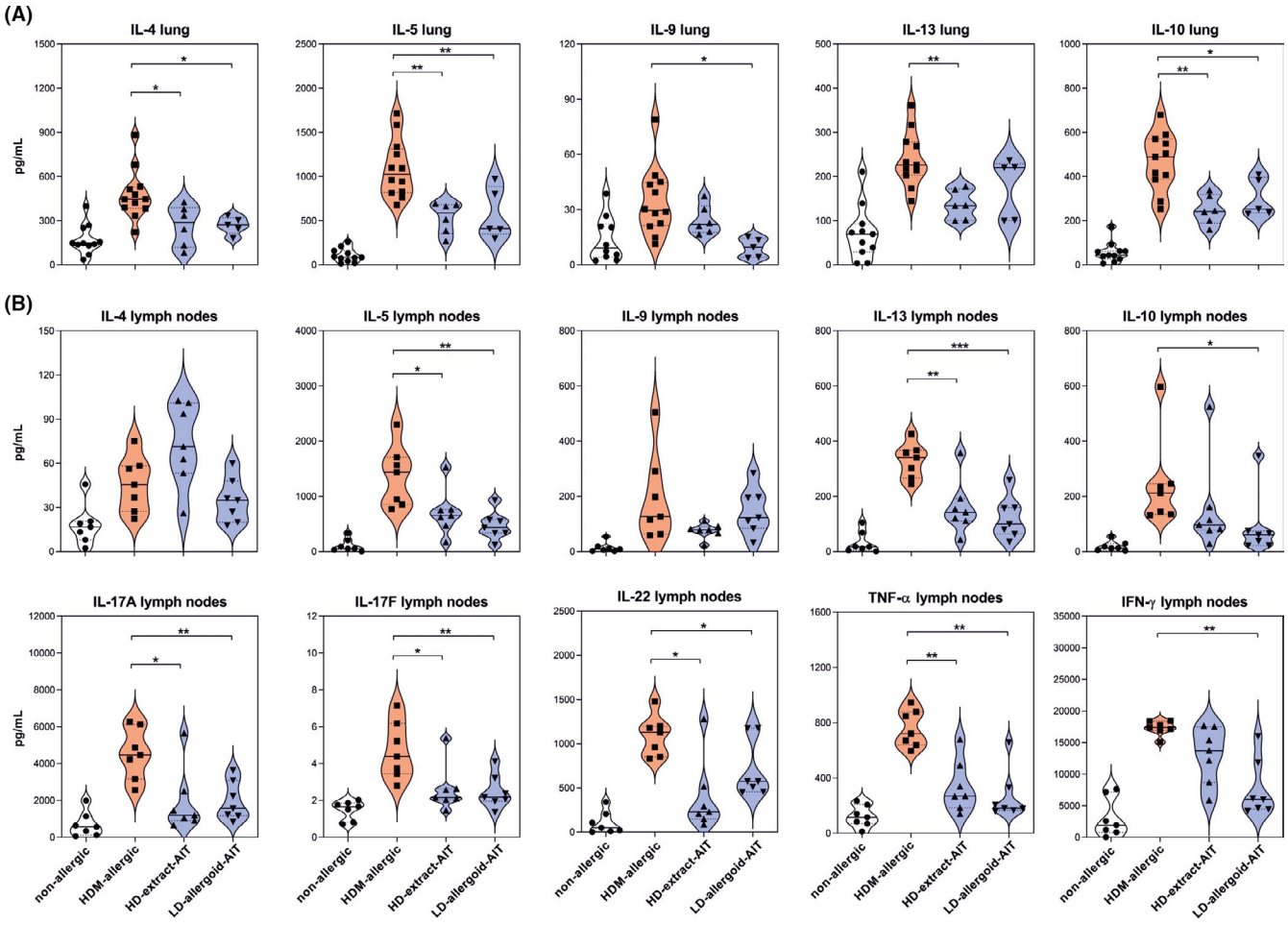


FIGURE 2 Effects of HD-extract-AIT and LD-allergoid-AIT on cytokine release from lung-resident lymphocytes and lymph node cells in experimental HDM allergy. A, Cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro* ($n = 5–12$). B, Analysis of cytokine levels in culture supernatants of cervical lymph node cells after anti-CD3/anti-CD28-restimulation *in vitro* ($n = 7$). Solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired *t* test or Mann–Whitney test, respectively. *p*-values of ≤.05, ≤.01, and ≤.001 are shown as *, **, and ***, respectively. AIT, allergen-specific immunotherapy; HDM, house dust mite

IgG2b levels were gradually increased by MCT and MCT + MPL, total IgG2c levels were comparable in all three treatment groups and lower compared to allergic controls (Figure S4D). Analysis of tIgG3 antibodies revealed a significant induction by the addition of MPL to the formulation (Figure 4E).

All three AIT strategies significantly decreased *ex vivo* secretion of IL-4, IL-5, and IL-10 of anti-CD3/anti-CD28-restimulated lung lymphocytes. Significant reduction of IL-13 secretion was only achieved by adding MCT or MCT + MPL to the formulation. While AIT with HDM allergoid and HDM allergoid + MCT also significantly reduced IL-9 secretion, this effect was reversed by the addition of MPL to the formulation (Figure 4F). Compared to allergic mice, the level of IL-13 in supernatants of restimulated lymph node cells was significantly reduced in all treatment groups. IL-5 levels were significantly lower in the allergoid alone and allergoid + MCT groups and IL-9 levels in the allergoid + MCT group. IL-10 levels were lower in all treatment groups (only significant in the allergoid alone group) while IL-4 levels were unchanged compared to allergic controls. All treatment regimens had comparable effects on the reduction of secreted IL-17A, IL-17F, IL-22, and TNF-α from lymph node cells (Figure 4G), while these cytokines were detectable at equal levels in supernatants of restimulated lung lymphocytes and splenocytes from all groups of mice (Figure S4E,F). The reduced IFN-γ production, observed in LD-allergoid-AIT—compared to HD-extract-AIT-treated mice (Figure 2B), was reverted by the addition of MCT to the AIT formulation with no additional effect of MPL (Figure 4G). Again, the effects of all AIT strategies on Th2-type cytokine secretion from restimulated splenocytes were less pronounced (Figure S4F).

3.3 | Dose-dependent effects of MPL on experimental low-dose HDM allergoid AIT

As not only the allergen, but also the adjuvant dose may have crucial influence on the anti-inflammatory capacity of AIT formulations, the effects of MPL dosage on AIT outcome were addressed. Mice

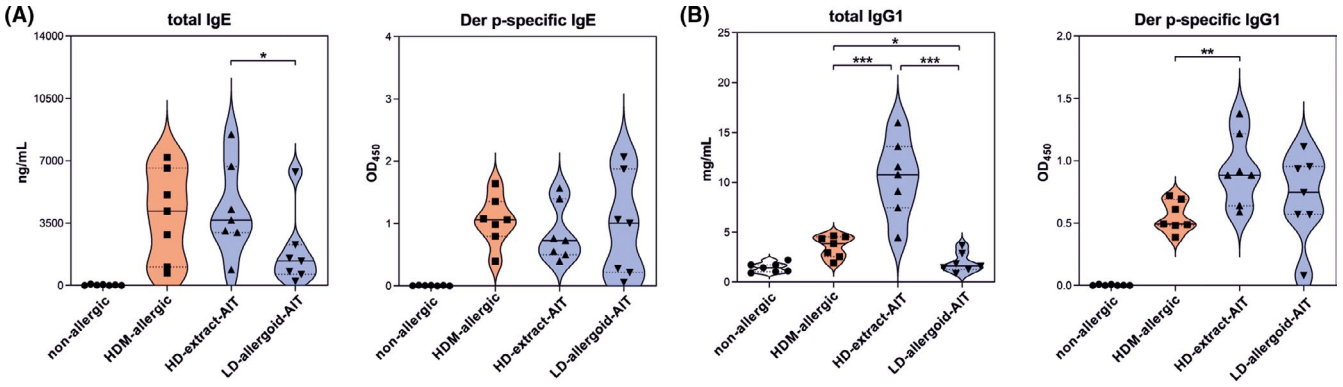


FIGURE 3 Effects of HD-extract-AIT and LD-allergoid-AIT on the humoral immune response in experimental HDM allergy. A, Measurement of total IgE and Der p-specific IgE levels in serum samples at endpoint ($n = 7$). B, Measurement of total IgG1 and Der p-specific IgG1 levels in serum samples at endpoint ($n = 7$). Solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired *t* test or Mann–Whitney test, respectively. *p*-values of ≤.05, ≤.01, and ≤.001 are shown as *, **, and ***, respectively. AIT, allergen-specific immunotherapy; Der p, dermatophagoides pteronyssinus; HDM, house dust mite

received LD-allergoid-AIT + MCT combined with 12.5 μg, 25 μg, 50 μg, or 100 μg MPL per AIT dose. AIT with 12.5 μg, 25 μg, and 50 μg MPL resulted in a significant decrease of total BAL cells, although less pronounced in mice treated with 12.5 μg MPL. This beneficial effect of AIT was nearly completely reversed applying 100 μg MPL (Figure 5A). Comparably, a significant reduction of BAL eosinophils could only be achieved using the 25 μg or 50 μg MPL dose (Figure 5A).

The beneficial effects of adding MCT + MPL to the AIT formulation on the reduction of lung-resident $GATA3^{+}ST2^{+}FoxP3^{-}$ Th2 cells (Figure 4D) were nearly completely reversed using 12.5 μg or 100 μg MPL, while the MPL dose showed no effects on the number $FoxP3^{+}$ Th cells (Figure 5B).

While tIgE and Der p-sIgE levels slightly increased by using lower MPL concentrations (12.5 μg and 25 μg) compared to allergic mice, this effect was reversed applying the two higher concentrations. Most importantly, the use of 25 μg and 50 μg MPL in the formulation worked best for the induction of tIgG1 and Der p-sIgG1 (Figure 5C).

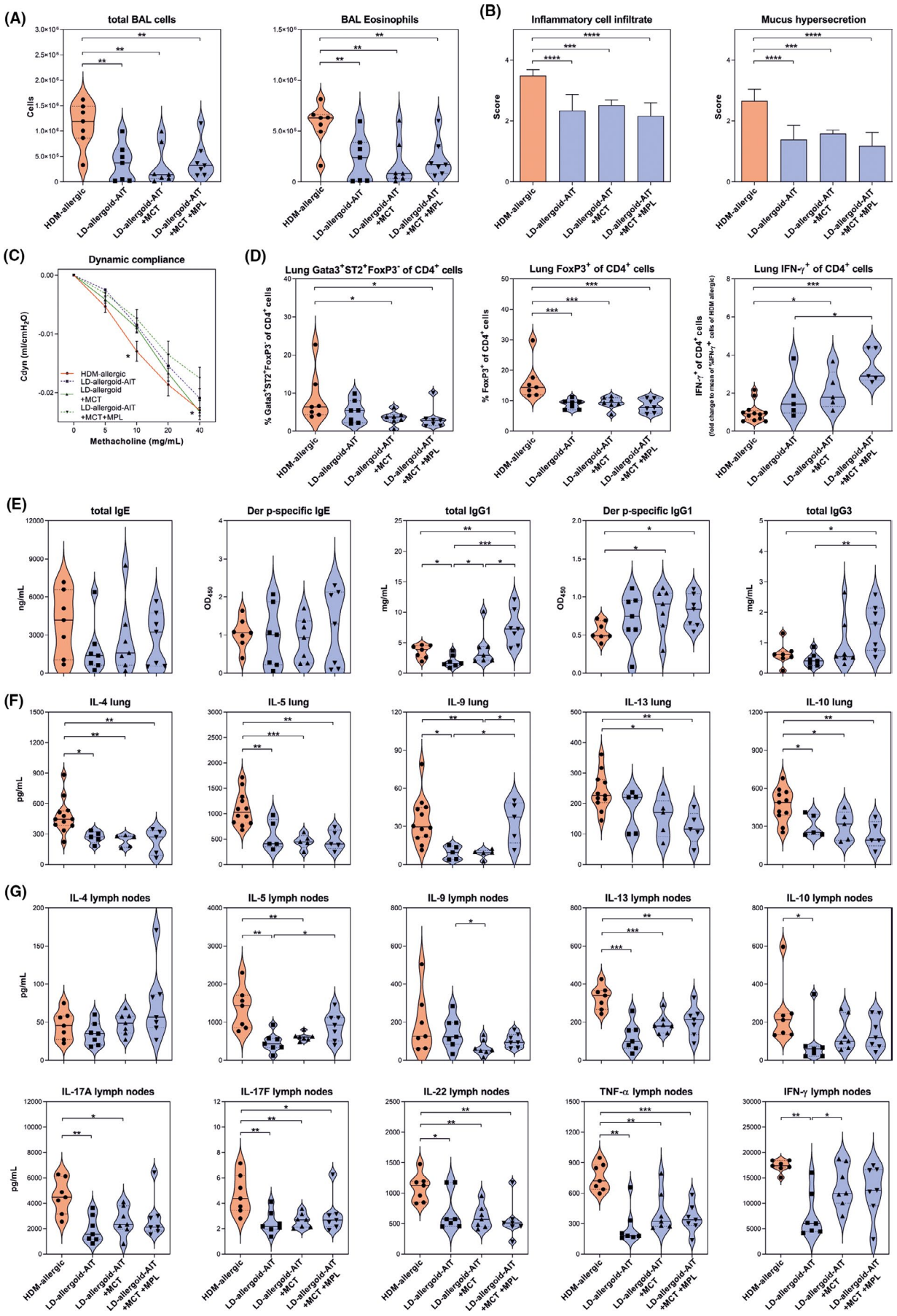
The most prominent effects on the reduction of IL-4, IL-5, IL-9, IL-13, and IL-10 levels in supernatants of *ex vivo* anti-CD3/anti-CD28-restimulated lung lymphocytes were also achieved by using 25 μg or 50 μg in the AIT formulation, while the effects on cytokine levels identifying Th17 and Th1 activities were less pronounced (Figure 5D and Figure S5A). The same concentrations worked best in dampening IL-4, IL-5, IL-9, IL-13, IL-10, and IL-17A secretion by restimulated cervical lymph node cells (Figure 5E and Figure S5B).

4 | DISCUSSION

The current study demonstrates that a 220-fold reduced allergen dose in experimental HDM AIT is comparably effective in controlling allergic inflammation when HDM allergoids are applied instead of extracts. Furthermore, adjuvants MCT[16,25] and MPL[26,27] are shown to modulate humoral responses exceeding the LD-allergoid-AIT effects.

In humans, local allergy-driven and protective AIT mechanisms in the airways and in lymphoid organs are hard to address. Commonly used murine HDM AIT models are based on i.p. sensitization combining the allergen with the adjuvant alum.[22,24,28,29] To avoid broad unspecific alum-mediated immune activation,[30,31] in this study, a novel experimental HDM AIT of murine allergic asthma combining i.n. sensitization and s.c. AIT was established. This model resembles in many aspects human allergic inflammation.[32-37] Typical eosinophilic and Th2 cell infiltration into the lung, decreased lung dynamic compliance, mucus hypersecretion, tIgE, and sIgE induction as well as elevated Th2-type cytokine secretion by lung-resident lymphocytes, lymph node cells, and splenocytes was observed. Similar to human AIT, sIgG antibodies are induced while sIgE levels do not immediately decrease.[11,13,38] In contrast to human AIT, in murine AIT models based on alum-dependent i.p. sensitization, the sIgE response is nearly completely lost during treatment.[39]

This new model allowed the direct comparison of LD-allergoid-AIT with a 220-fold higher HD-extract-AIT regarding efficacy and immunological mechanisms. Allergoids have the potential to improve the safety profile of AIT as they are characterized by reduced IgE-binding capacity but retained immunogenicity.[15,40] Interestingly, here, s.c. LD-allergoid-AIT (1 μg HDM allergoids) revealed control of allergic inflammation comparable to HD-extract-AIT (220 μg HDM extract) while AIT with 1 μg HDM extract even increased inflammation. Earlier studies showed that high allergen doses induce elevated amounts of specific $CD8^{+}$ T cells[41] and Th1 cells in lungs, while low allergen dosage induces Th2 cells and pro-inflammatory mechanisms such as immune cell infiltration.[42,43] The demonstrated comparable control of inflammation of LD-allergoid-AIT and HD-extract-AIT might be explained by the destruction of conformational IgE epitopes. The associated FcεR-mediated uptake of allergoids may be impaired resulting in a decreased Th2 inflammation-promoting immune milieu, while immunogenicity is maintained.[3,5-7,44-47] LD-allergoid-AIT and HD-extract-AIT demonstrated similar capacity to protect against infiltration of pro-inflammatory cells into the lung.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TNF-α lymph nodes
IFN-γ lymph nodes

FIGURE 4 Effects of the adjuvants MCT and MPL on LD-allergoid-AIT in experimental HDM allergy. A, Total BAL cells and differential eosinophil counts ($n = 7$). B, Scores of inflammatory cell infiltrate and mucus hypersecretion in lung tissue 3 days after the last HDM challenge ($n = 6$). The scores were analyzed by one-way analysis of variance with Tukey's multiple comparison test. Shown is the mean with SD. C, Measurement of lung dynamic compliance ($n = 6$–12). *$p < .05$ HDM-allergic vs AIT allergoid + MCT + MPL. Lung function parameters were analyzed by two-way analysis of variance with Tukey's multiple comparison test. Shown is the mean with SEM. D, Analysis of lung-resident lymphocyte populations ($n = 7$) and IFN-γ-producing T cells ($n = 5$–12). E, Measurements of immunoglobulins in serum samples at endpoint ($n = 7$). F, Analysis of cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro* ($n = 5$–12). G, Analysis of cytokine levels in culture supernatants of cervical lymph node cells after anti-CD3/anti-CD28-restimulation *in vitro* ($n = 7$). In all violin plots, solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired *t* test or Mann–Whitney test, respectively. *p*-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. AIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; HDM, house dust mite; MCT, microcrystalline tyrosine; MPL, monophosphoryl lipid A

Furthermore, both treatments restored lung dynamic compliance although HD-extract-AIT was slightly more effective. AIT-mediated control of Th2 cytokines was observed in the BAL as well as in lung lymphocytes, cervical lymph node cells, and to some degree in splenocytes. This reduced Th2-promoting milieu at local and systemic sites has previously been hypothesized to be beneficial for immunological tolerance induction.[12] Additionally, the AIT-mediated reduction of IL-17[13] and TNF-α was previously described to control the recruitment of eosinophils, neutrophils, and T cells into the lung.[48] A difference between the two treatment strategies was the strongly reduced IFN-γ production by LD-allergoid-AIT. This difference may originate from higher amounts of TLR ligands contained in the HD-extract-AIT. Although also allergen-specific restimulation experiments might have provided additional mechanistic insights, in this study, unspecific restimulation was chosen to be able to monitor not only allergen-specific but also bystander and unspecific effects of the different AIT strategies and adjuvants on the cytokine-producing capacity of the addressed immune cells.

The observed decline of $FoxP3^+CD4^+$ T cells in lung tissue as well as the reduced IL-10 secretion by lung lymphocytes and lymph node cells is likely to be a result of the migratory behavior of the cells and, therefore, reflects the inflammatory process rather than the mechanism of tolerance induction.[23,49,50] Increased numbers of Tregs were also observed in asthmatic patients in a phase of active inflammation indicating that rather the quality than the quantity of Tregs is decisive for the anti-inflammatory capacity.[51] Moreover, it was reported that IL-10 is critical for antigen-specific Th2 responses in mice.[50]

Although prior studies have questioned the efficacy of allergoids due to the modification process,[8] this study demonstrates significant effectiveness even at low dose accessing a wide set of biomarkers. Of course, future human studies are needed to confirm these findings. Notably, according to guidelines of the European Academy of Allergy and Clinical Immunology (EAACI), both modified and unmodified allergen extracts are recommended for subcutaneous AIT of allergic rhinoconjunctivitis for short-term benefit.[52] Furthermore, subgroup analyses comparing the combined symptom and medication score (short term) for AIT with modified and unmodified allergen extracts in the context of allergic rhinoconjunctivitis found a clear benefit from allergoids and suggest (but not confirm) a benefit from unmodified preparations.[53]

In contrast to comparable type-2 cellular response to LD-allergoid-AIT and HD-extract-AIT, major differences were observed on humoral level in this study. HD-extract-AIT neither influenced tIgE nor sIgE levels, whereas LD-allergoid-AIT reduced tIgE but not sIgE levels. Reasons for lower tIgE induction properties of HDM allergoids might be the loss of conformational IgE epitopes, reduced amounts of mite body components like chitin and other IgE-inducing factors or serine protease activity.[36,54] Even more striking, HD-extract-AIT led to a robust sIgG1 and tIgG1 response whereas LD-allergoid-AIT led to a slightly lower increase of sIgG1 and no alteration of tIgG1 levels. Murine IgG1 is supposed to be the structural and functional homologue of human IgG4. Like human IgG4, it does not interact with C1q and inhibits the binding of IgG2a, IgG2b, and IgG3 to C1q and, hence, suppresses complement activation. Furthermore, both murine IgG1 and human IgG4 show limited specificity and affinity to activating FcγRs and preferably interact with the classical IgG inhibitory receptor FcγRIIb.[55] In human HDM AIT, induction of sIgG4 is a hallmark of successful AIT, because of its potentially protective role.[56] Hence, the lack of robust tIgG1 immune responses might reflect suboptimal B-cell activation of LD-allergoid-AIT.

The observed potential B-cell activation deficit may be compensated by higher allergoid doses or adjuvants. Hence, the immunological effects of the adjuvants MCT and MPL on LD-allergoid-AIT were addressed. In addition to the effects of the well-established depot adjuvant MCT[15,40] alone the synergistic effects of the adjuvant system combining MCT with MPL were of interest as such adjuvant systems are already in clinically approved.[45] The adsorption of allergoids and MPL to MCT has been previously characterized.[26] The current study revealed that allergoid + MCT and allergoid + MCT + MPL AIT formulations showed similar effects on the reduction of BAL cell infiltration compared to LD-allergoid-AIT alone. However, the allergoid + MCT + MPL formulation was slightly more effective in restoring lung dynamic compliance. In contrast to allergoid alone, adding MCT to the formulation led to significant reduction of the percentage of lung-resident Th2 cells, whereas MPL showed no additional effect. Strikingly, LD-allergoid-AIT treatment combined with MCT and MCT + MPL led to a gradual increase of the number of IFN-γ-producing lymphocytes of the lower airways as well as to higher levels of IFN-γ secretion by cervial lymph node

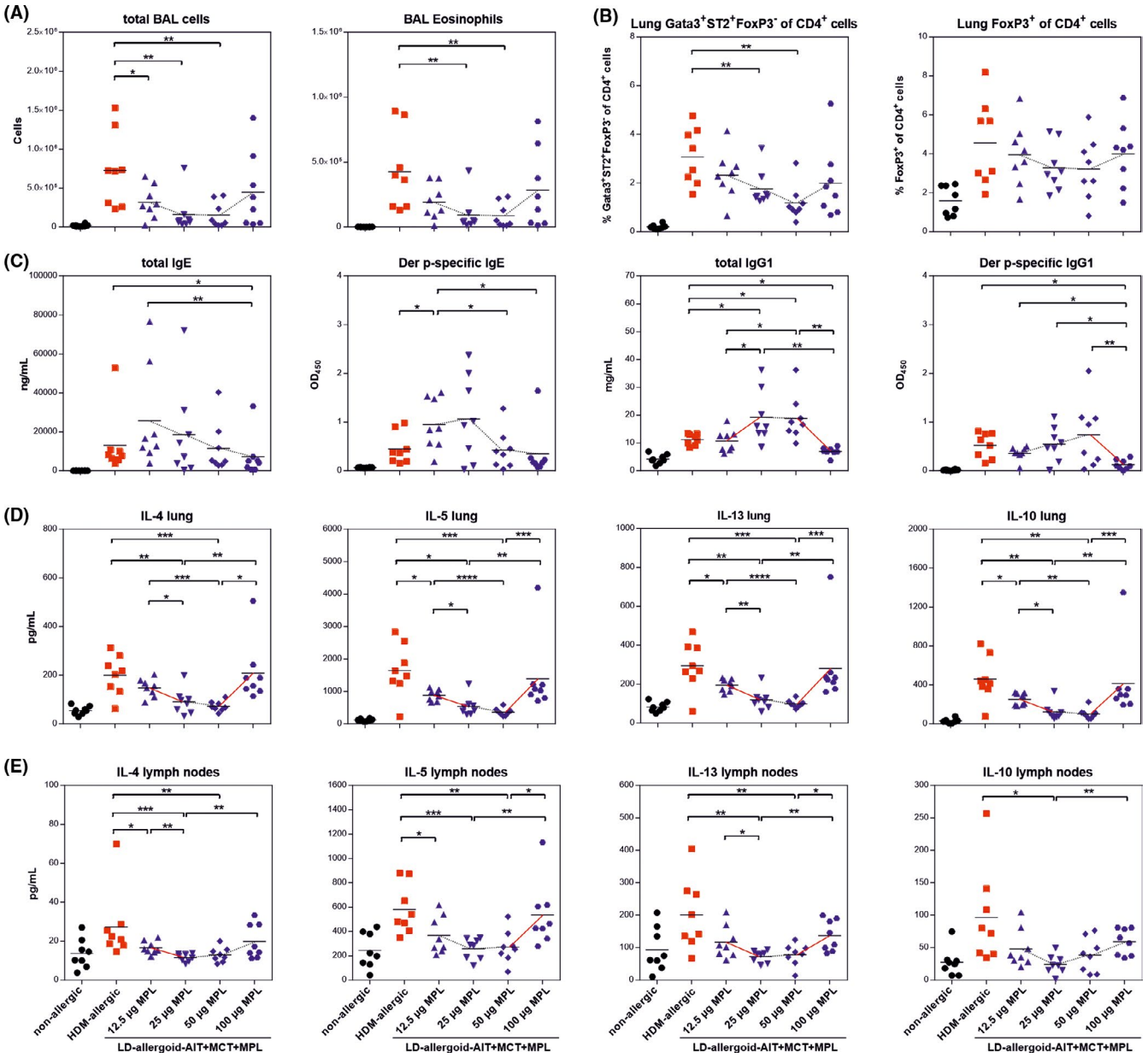

FIGURE 5 Dose-dependent effects of increasing MPL concentrations on LD-allergoid-AIT in experimental HDM allergy. A, Total BAL cells and differential eosinophil counts ($n = 8$). B, Analysis of lung-resident lymphocyte populations ($n = 8$). C, Measurements of immunoglobulins in serum samples of mice at endpoint ($n = 8$). D, Analysis of cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro* ($n = 8$). E, Analysis of cytokine levels in culture supernatants of cervical lymph node cells after anti-CD3/anti-CD28-restimulation *in vitro* ($n = 8$). Bars indicate the mean. Red solid and black dashed lines are intended to visualize the dose-dependent effects of the different MPL concentrations. Red solid and black dashed lines indicate significant and not significant differences between neighboring groups, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired *t* test or Mann–Whitney test, respectively. *p*-values of ≤.05, ≤.01, ≤.001, and ≤.0001 are shown as *, **, ***, and ****, respectively. AIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; HDM, house dust mite; MCT, microcrystalline tyrosine; MPL, monophosphoryl lipid A

cells. In fact, previous murine studies showed that MCT triggers Th1-associated immune response mechanisms more efficiently than alum, by modulating the recruitment of DCs, $CD8^+$ as well as $CD4^+$ T cells accompanied with IFN-γ and TNF-α production.[16] Furthermore, MPL significantly increases IFN-γ production of *in vitro* restimulated peripheral blood mononuclear cells in context of grass pollen allergy, postulating the induction of Th1-promoting mechanisms as a protection for allergy.[27]

Importantly, combining LD-allergoid-AIT formulation with MCT and MCT + MPL also enhanced the humoral response and gradually increased tIgG1 and tIgG2b levels. Moreover, both adjuvant-based therapies equally increased sIgG1 levels. These effects on

IgG production rely on the adjuvant-induced Th1-biased immune milieu.[16,57] Significant levels of tIgG3 were only induced by MPL. This is in line with a study showing that LPS is able to induce B-cell switching to IgG3 via TLR4 signaling.[58] Importantly and in contrast to alum-based adjuvants, MCT and MPL showed no IgE-inducing properties, as demonstrated previously.[16,59] Taken together, these findings demonstrate that adding the adjuvant MCT alone, or in combination with MPL to the AIT formulation has the potential to promote Th1-inducing mechanisms and robust B-cell activation of LD-allergoid-AIT counterbalancing the allergic Th2 immune response.

Not only the allergen, but also the adjuvant dose may have crucial influence on the anti-inflammatory capacity of AIT formulations.[60,61] Hence, the effects of different MPL concentrations on the efficacy of LD-allergoid-AIT were addressed. The doses of 25 μg and 50 μg were most effective in reducing Th2 and eosinophilic infiltration and Th2-cytokine production as well as in inducing tIgG1 and sIgG1 responses. Most of these effects were almost completely reversed by using the 100 μg dose. While the 12.5 μg dose seems to be too low for effective adjuvant effects, the 100 μg dose had rather adverse effects on AIT outcome. These might be explained by altered TLR4 signaling mechanisms. While MPL acts via TRAM/TRIF-biased stimulation of TLR4 along with selective activation of p38 signaling followed by induction of adaptive immune responses and TRIF-dependent endotoxin tolerance, LPS-mediated TLR4 stimulation causes MAL/MyD88, and TRAM/TRIF-dependent signaling events, which are followed by pro-inflammatory responses. Nevertheless, MPL is not completely devoid of MyD88 involvement, which might explain the observed dose-response effects.[18] Of note, the 50 μg dose is also used in vaccines for human use.[26]

In summary, this study provides a side-by-side comparison of high-dose extract- and low-dose allergoid-based AIT and demonstrates that low allergen doses can induce cellular and humoral mechanisms counteracting Th2-driven inflammation by using allergoids and dose-adjusted adjuvants. Future therapeutic approaches may use low-dose allergoid strategies to drive cellular tolerance and adjuvants to modulate humoral, potentially protective responses.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

AH performed experiments, analyzed data, and wrote the manuscript. FA, DR, SH, and BS performed experiments, analyzed data, and revised the final version of the manuscript. AC, MAS, and MFK discussed the data and revised the final version of the manuscript; JM, TLCV, and MDH prepared and characterized HDM extracts and allergoids, discussed the data, and revised the final version of the manuscript. UMZ discussed the data and wrote the manuscript; CBS-W initiated and supervised the study, contributed to the interpretation of data and wrote the manuscript. SB initiated and supervised the study, analyzed the data, created the figures, and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Figure S1. Adverse effects of low-dose HDM extract AIT on the allergic status. A, Schematic overview of the murine HDM AIT model using 1µg HDM extract for AIT injections. Pipettes and syringes indicate time points of allergen challenges and AIT injections, respectively. i.n, intranasal; s.c., subcutaneous. B, Total BAL cells (n=8). C, Differential counts of BAL eosinophils (n=8). D, Analysis of lung-resident Gata3^{+}ST2^{+}FoxP3^{-}CD4^{+} T-cells (n=8). Solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively**.** P-values of ≤0.01 and ≤0.001 are shown as ** and ***, respectively. AIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; HDM, house dust mite.

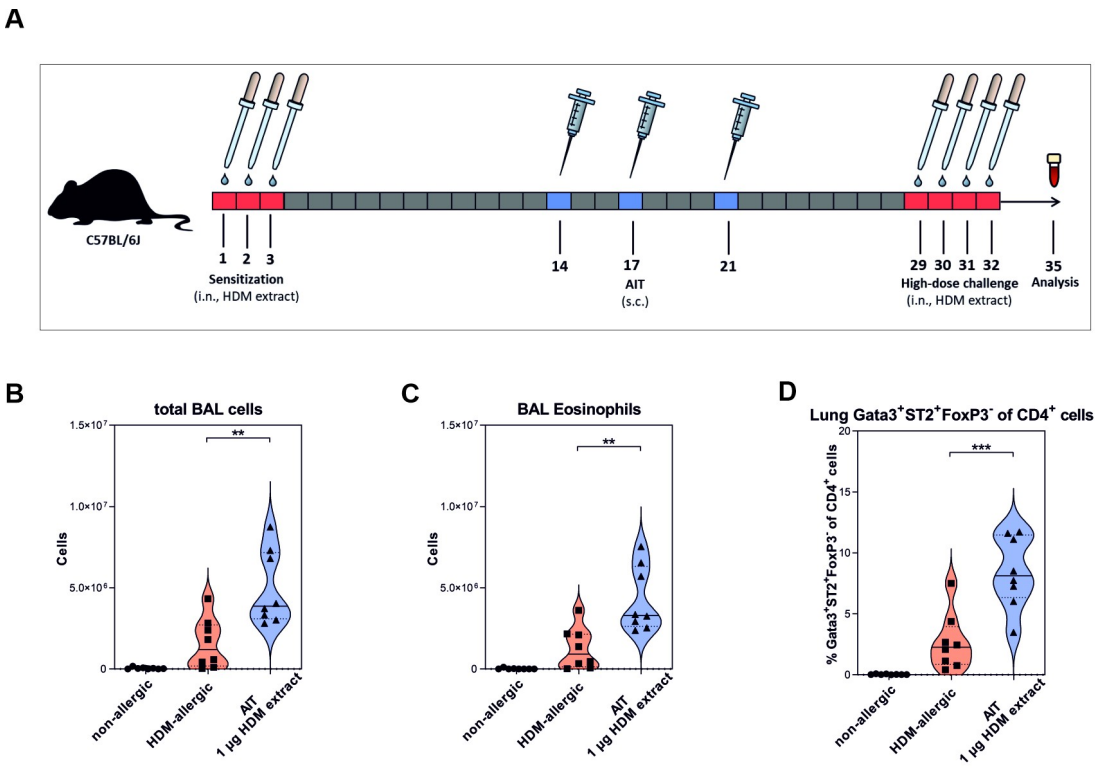
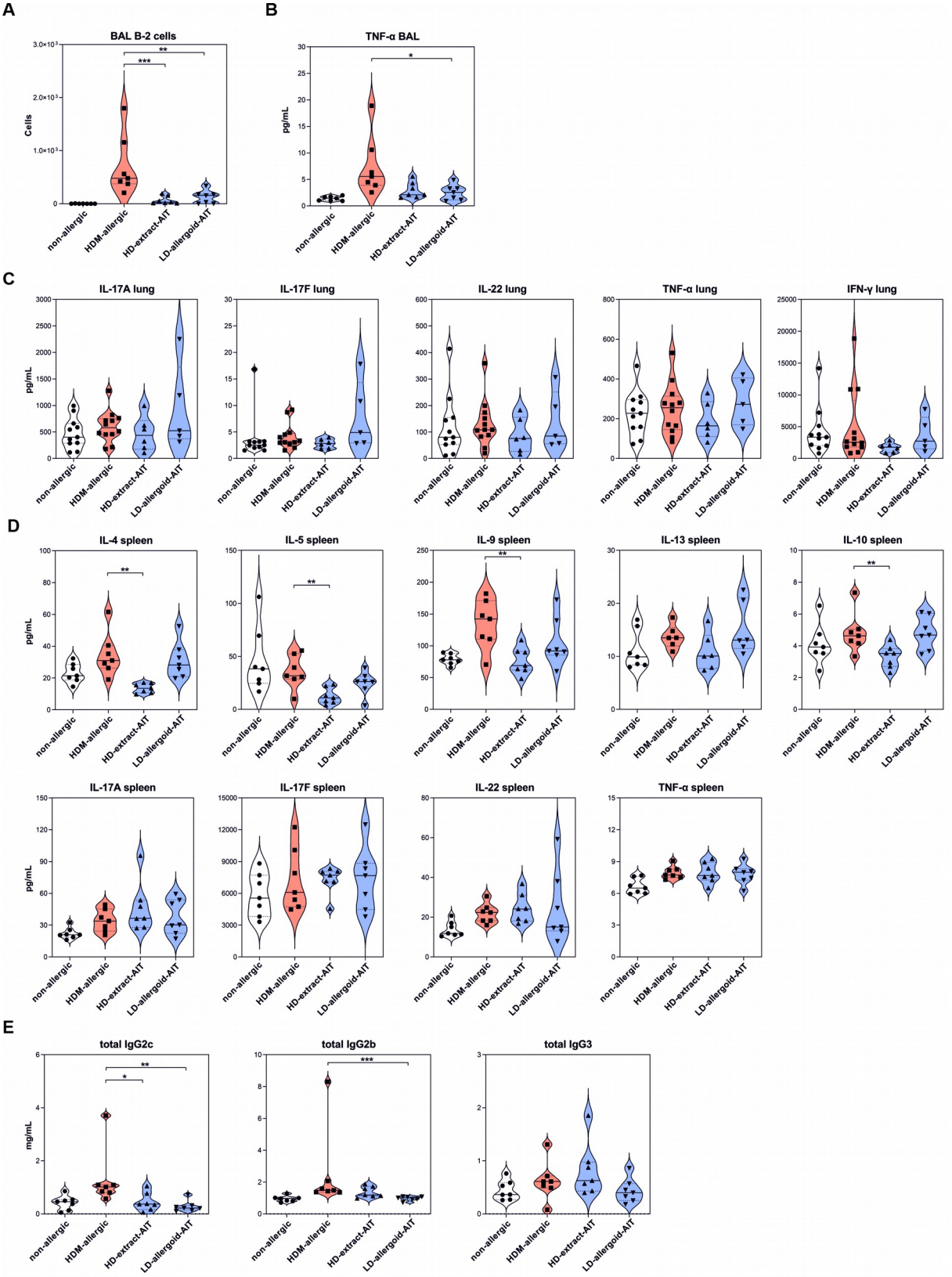


Figure S2. Effects of HD-extract-AIT and LD-allergoid-AIT on experimental HDM allergy. A, Differential count of BAL-B2-cells (n=7). B, Level of TNF-α in in the BAL (n=7). C, Cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro* (n=5-12). D, Analysis of cytokine release from splenocytes after anti-CD3/anti-CD28-restimulation *in vitro* (n=7). E, Measurement of total IgG2c, IgG2b and IgG3 in serum samples at endpoint (n=7). Solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively**.** P-values of ≤0.05, ≤0.01 and ≤0.001 are shown as *, ** and ***, respectively. AIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; HDM, house dust mite.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TNF-α lung
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IL-4 spleen
IL-5 spleen
IL-9 spleen
IL-13 spleen
IL-10 spleen
IL-17A spleen
IL-17F spleen
IL-22 spleen
TNF-α spleen
E
total IgG2c
total IgG2b
total IgG3
mg/mL
non-allergic
HDM-allergic
HD-extract-AIT
LD-allergoid-AIT

Figure S3. Comparison of Der p-specific and Der f-specific IgE and IgG1 levels (n=6). Solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively**.** AIT, allergen-specific immunotherapy; HDM, house dust mite; Der f, *Dermatophagoides farinae*; Der p, *Dermatophagoides pteronyssinus*.

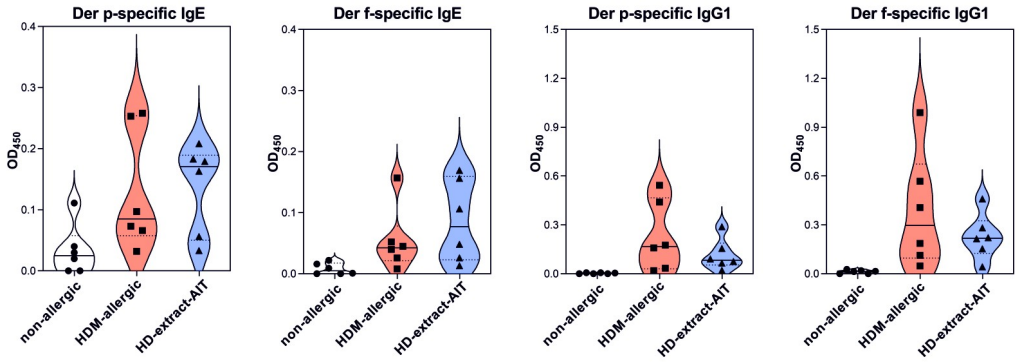


Figure S4. Effects of the adjuvants MCT and MPL on LD-allergoid-AIT in experimental HDM allergy. A, Differential counts of BAL neutrophils, $CD4^+$-cells, $CD8^+$-cells and B-2-cells (n=7). B, Representative lung histology specimen, retrieved 3 days after the last HDM challenge stained with periodic acid-Schiff. Arrows, inflammatory cell infiltrate; arrowheads, mucus hypersecretion. C, Levels of IL-5, IL-4 and TNF-α in the BAL (n=7). D, Measurement of total IgG2c and IgG2b levels in serum samples at endpoint (n=7). E, Cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro* (n=5-12). F, Analysis of cytokine release from splenocytes after anti-CD3/anti-CD28-restimulation *in vitro* (n=7). Solid and dashed bars indicate the median and quartiles, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively**.** P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are shown as *, **, ***, and ****, respectively. AIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; HDM, house dust mite; MCT, MicroCrystalline Tyrosine; MPL, monophosphoryl lipid A.

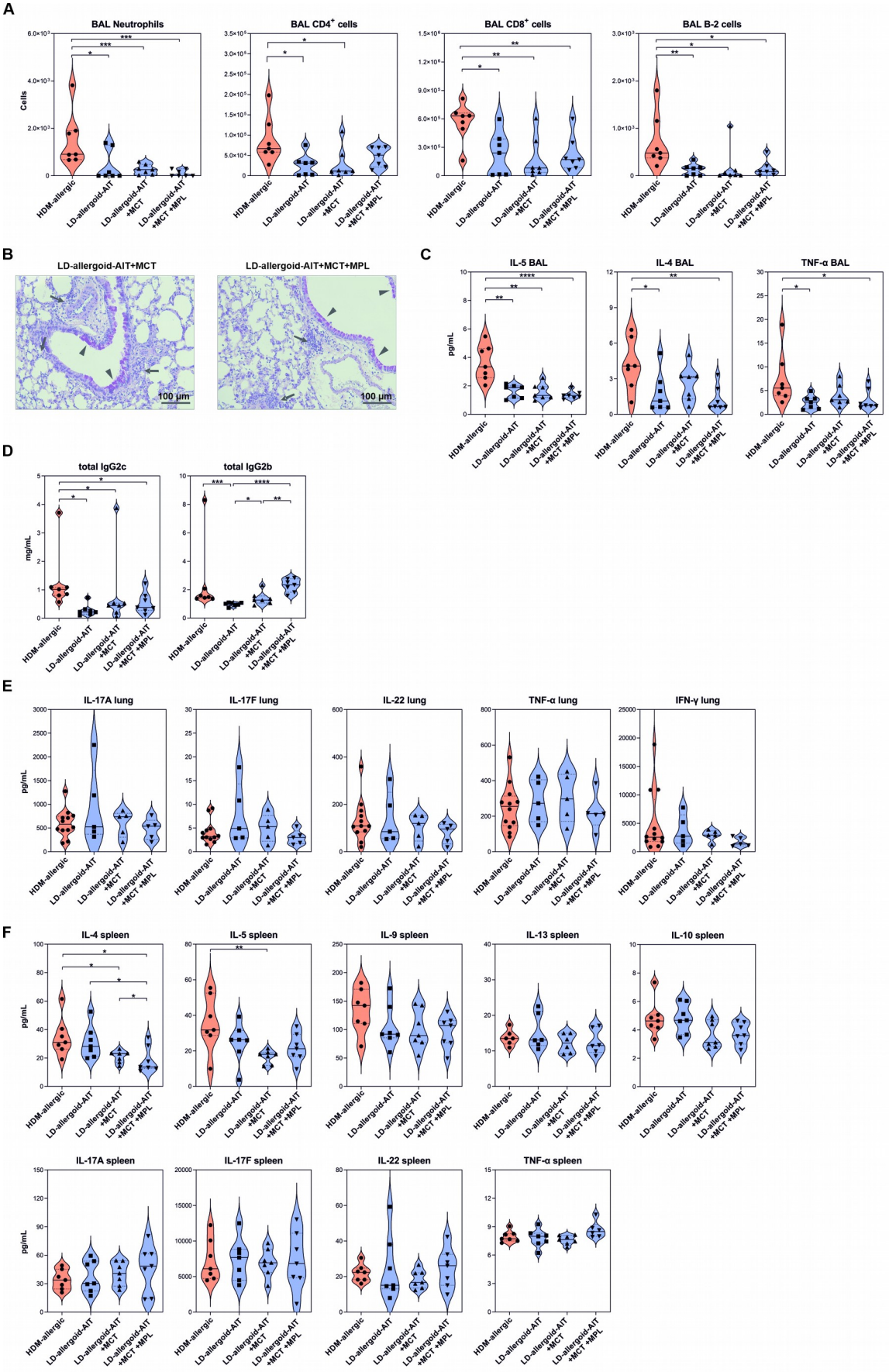
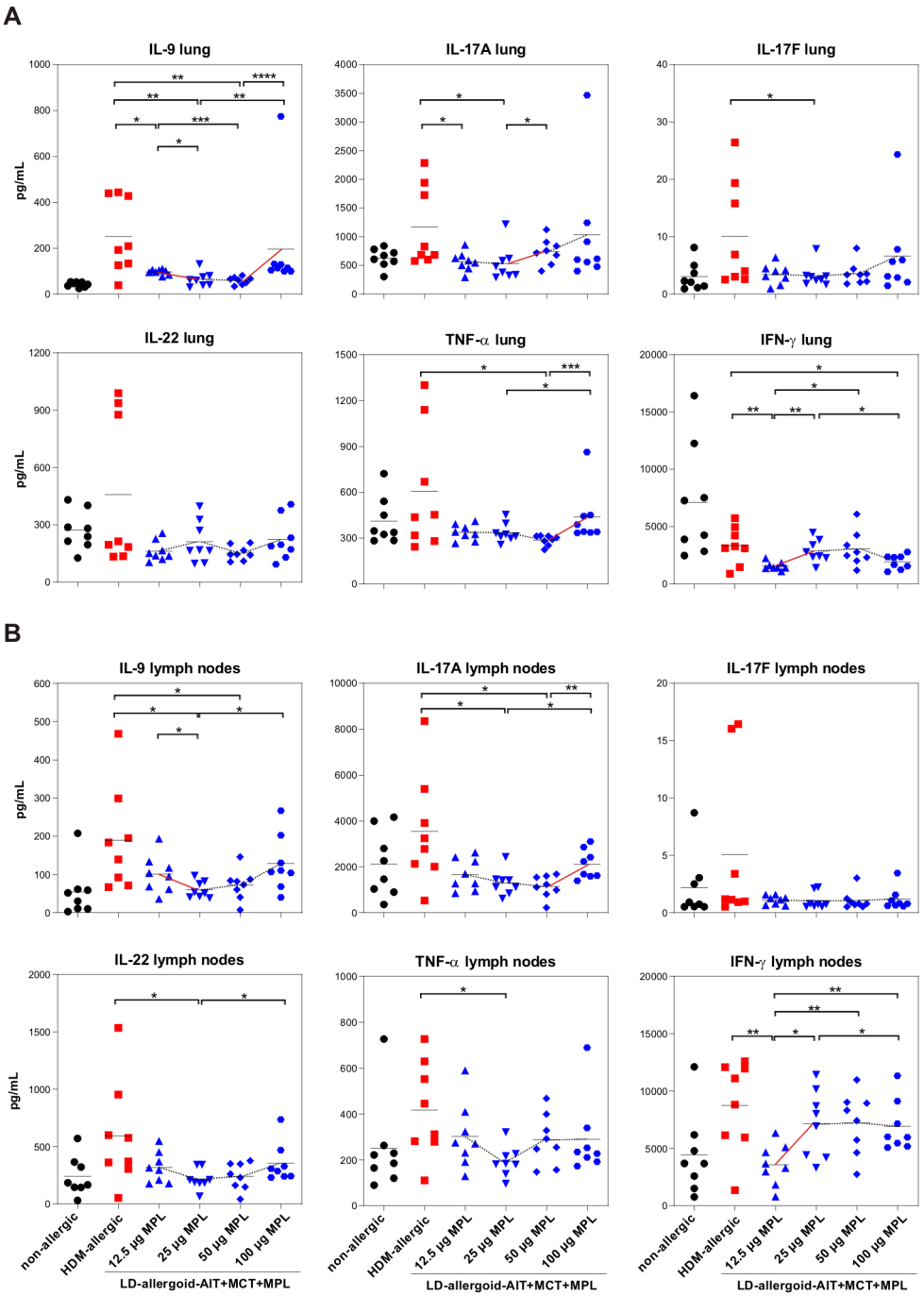

A
BAL Neutrophils
BAL CD4+ cells
BAL CD8+ cells
BAL B-2 cells
Cells
B
LD-allergoid-AIT+MCT
LD-allergoid-AIT+MCT+MPL
100 µm
C
IL-5 BAL
IL-4 BAL
TNF-α BAL
pg/mL
D
total IgG2c
total IgG2b
mg/mL
E
IL-17A lung
IL-17F lung
IL-22 lung
TNF-α lung
IFN-γ lung
pg/mL
F
IL-4 spleen
IL-5 spleen
IL-9 spleen
IL-13 spleen
IL-10 spleen
IL-17A spleen
IL-17F spleen
IL-22 spleen
TNF-α spleen
pg/mL
HDM-allergic
LD-allergoid-AIT
LD-allergoid-AIT +MCT
LD-allergoid-AIT +MCT +MPL

Figure S5. Dose-dependent effects of increasing MPL concentrations on LD-allergoid-AIT in experimental HDM allergy. A, Analysis of cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro* (n=8). B, Analysis of cytokine levels in culture supernatants of cervical lymph node cells after anti-CD3/anti-CD28-restimulation *in vitro* (n=8). Bars indicate the mean. Red solid and black dashed lines are intended to visualize the dose-dependent effects of the different MPL concentrations. Red solid and black dashed lines indicate significant and not significant differences between neighboring groups, respectively. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively**.** P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are shown as *, **, ***, and ****, respectively. AIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; HDM, house dust mite; MCT, MicroCrystalline Tyrosine; MPL, monophosphoryl lipid A.


A
IL-9 lung
IL-17A lung
IL-17F lung
IL-22 lung
TNF-α lung
IFN-γ lung
pg/mL
B
IL-9 lymph nodes
IL-17A lymph nodes
IL-17F lymph nodes
IL-22 lymph nodes
TNF-α lymph nodes
IFN-γ lymph nodes
non-allergic
HDM-allergic
12.5 µg MPL
25 µg MPL
50 µg MPL
100 µg MPL
LD-allergoid-AIT+MCT+MPL

Data S1: Supplemental methods

Allergen extracts and adjuvants

HDM extracts were supplied by Allergy Therapeutics (Worthing, United Kingdom). For extract preparation, 7.5 g of raw material (>99% purity, GMP-grade) of freeze-dried *D. farinae* and *D. pteronyssinus* (bodies and faeces) were subjected to media extraction for 24 hours and purification by clarification using a 0.2 µm syringe filter and then analyzed..

GMP-grade modified allergen extracts (allergoids) were supplied by Allergy Therapeutics. Following purification by clarification, the extract was diafiltered, modified with glutaraldehyde, diafiltered, sterile filtered, and then filled and analyzed.

Unmodified and modified extracts are derived from the same source material and the modification process has no influence on the allergen composition. Biochemical characterization of extracts included total protein content, total allergenicity and individual allergen content measured against biologically standardized in-house reference preparations (Quality Assurance Units/vial).

GMP-manufactured allergoids + 2% MCT (Acarovac Plus®) and allergoids + 2% MCT + 50 µg MPL (Acarovac MPL®) were supplied by Allergy Therapeutics.

4% (w/v) MicroCrystalline Tyrosine® (40 mg /mL) was supplied by Allergy Therapeutics, used to formulate vaccines at point of use, targeting a 2% (w/v) final concentration for all formulations. A sterile aqueous form of 1mg/mL MPL® was supplied by Allergy Therapeutics, manufactured in accordance to the Ph. Eu. monograph 2537. These were used to formulate allergoid+MCT+MPL formulations to explore the dose-dependent effects of MPL (12.5, 25, 50 and 100 µg).

Lung function analysis

Dynamic compliance after methacholine (Mch, Sigma-Aldrich, Taufkirchen, Germany) challenge was measured 24 h after the last HDM challenge in intubated,

mechanically ventilated animals (Data Sciences International (DSI), New Brighton, MN, USA), as previously described (1). Briefly, animals were anesthetized by an intraperitoneal injection of ketamine (100mg/kg) and xylazine (5mg/kg) in PBS. After cannulation of the trachea and starting mechanical ventilation, the animals were challenged with increasing methacholine concentrations, using an in-line nebulizer (5 µl Mch solution in PBS delivered for 30 seconds at the following concentrations: 0, 5, 10, 20 and 40 mg/ml). Data were recorded using the FinePoint software v2.4.6 (DSI). The lowest values of dynamic compliance were recorded every 5 seconds during the data recording interval set at 3 min after each Mch level. The mean value of this parameter measured during each recording interval was then calculated. The heart rate of each animal was continuously monitored using an electrocardiography device connected with three subcutaneous electrodes throughout the entire experiment (DSI). Respiratory system resistance was not monitored due to the known lack of response in C57BL/6 mice (2).

Isolation of BAL cells

For isolation of bronchoalveolar lavage (BAL) cells, the chest of euthanized mice was opened, the trachea cannulated, airways lavaged five times with 0.8 mL PBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and BAL cells analyzed via FACS.

Isolation of lung-resident lymphocytes

For isolation of tissue-resident lymphocytes, lungs were removed, chopped into approximately 1 mm x 1 mm sized pieces and digested in 3 mL RPMI medium (Thermo Fisher Scientific) containing 1 mg/mL collagenase (Collagenase A from *Clostridium histolyticum*, Sigma-Aldrich, Taufkirchen, Germany) and 100 µg/mL DNAse (Deoxyribonuclease I from bovine pancreas, Sigma-Aldrich). Digestion at 37 °C was stopped after 30 minutes by the addition of 15 µL 0.5 M EDTA (Sigma-Aldrich). Digested lungs were

mashed through a 70 µm cell strainer (Greiner Bio-One, Frickenhausen, Germany). Next, cells were pelleted (400 g, 4 °C, 5 min), resuspended in 5 mL 40% percoll (GE Healthcare, Chicago, Illinois, USA) in RPMI (v/v) solution (Thermo Fisher Scientific), underlayered with 5 mL 80% percoll solution and centrifuged (1600 g, RT, 15 min, without brake). Lymphocytes were collected from the interphase, washed with PBS and stored on ice.

Isolation of lymph node cells

Cervical lymph nodes were removed from mice and collected in a 15 mL tube with 5 ml RPMI. Lymph nodes were mashed through a 70 µm cell strainer (Greiner Bio-One) into a 50 mL tube, washed with 5 mL RPMI (400 g, 4 °C, 5 min) and stored on ice.

Isolation of splenocytes

Spleens were removed from mice and collected in a 15 mL tube with 5 ml RPMI. Spleens were mashed through a 70 µm cell strainer (Greiner Bio-One) into a 50 mL tube, washed with 10 mL PBS and pelleted (400 g, 4 °C, 5 min). For removing erythrocytes, spleen cells were resuspended in 1 mL ACK-lysis buffer (1.5 M NH4Cl (Sigma-Aldrich, St. Louis, Missouri, USA), 100 mM KHCO3, 10 mM Na2EDTA (both Merck, Darmstadt, Germany), ddH2O, pH 7.4) and incubated at room temperature for 2 minutes. 7 mL PBS were added and cells filtered through a 70 µm cell strainer. Cells were pelleted (400 g, 4 °C, 5 min) and resuspended in 3 mL RPMI complete (RPMI, 10% FCS (Sigma-Aldrich), 1% glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% Na-pyruvat (all from Thermo Fisher)). After adding 10 mL PBS, splenocytes cells were washed twice with PBS and stored on ice.

Counting of cells

Cell numbers were determined using a BD Accuri C6 flow cytometer (BD Bioscience,

Heidelberg, Germany). Cells from BAL fluid and lymph nodes were counted without dilution and cells from spleen and lung were diluted 1:10. Cells in a final volume of 50 µL PBS were transferred to FACS tubes and 1 µL cell counting master mix was added (0.5 µL anti-CD45-FITC (eBioscience, San Diego, California, USA), 0.5 µL propidium iodide (diluted 1:10 with PBS) (Sigma-Aldrich). After incubation for 5 minutes at 4 °C, cells were counted and diluted for *ex vivo* restimulation assays.

Ex vivo restimulation of lung lymphocytes, lymph node cells and splenocytes

Flat bottom 96-well plates (Sigma-Aldrich) were coated with 2 µg/ml anti-CD3 and 2 µg/ml anti-CD28 (both BD Bioscience) in PBS and incubated at 4 °C overnight. Wells were washed with PBS. Lymph node cells and splenocytes were plated out in duplicates at a density of $2x10^5$ and lung lymphocytes at a density of $1x10^5$ cells per well in 200 µl RPMI complete. Cells were incubated for 72 h at 37 °C, 5% CO2 in a humidified atmosphere. Supernatants were collected and stored at -80 °C for further analysis.

Measurement of immunoglobulins

For total IgE quantification, plates were coated with IgE ELISA MAX™ Capture Antibody (200X) (BioLegend, San Diego, California, USA) and detected with Mouse IgE ELISA MAX™ detection antibody and ELISA MAX™ Avidin-HRP (BioLegend) according to manufacturers' protocol. Sera were diluted 1:600 with PBS for the measurement of tIgE.

Measurement of Der p- and Der f-specific IgE and IgG1 levels was done by coating the plates with mouse IgE ELISA MAX™ capture antibody (BioLegend), or 50 µl of 12.5 µg/ml polyclonal anti-mouse IgG1 antibody (Citeq Biologics, Groningen, Netherlands) and detection with 50 µl biotinylated *D. pteronyssinus* or *D. farinae* HDM extract (both Citeq Biologics, concentration according to manufacturers' protocol). Detection was done with ELISA MAX™ Avidin-HRP (BioLegend). Sera were diluted 1:10 with PBS for the

measurement of sIgE and 1:300 for the detection of sIgG1. Analyses of total IgG1, IgG2a, IgG2b and IgG3 were done with Legendplex Immunoglobulin Isotyping Panel (BioLegend) according the manufacturers' protocol and analyzed by BD LSRFortessa™ flow cytometer (BD Bioscience).

Measurement of cytokines

Measurement of cytokine levels in the BAL as well as in supernatants of anti-CD3 and anti-CD28 restimulated cells from lungs, lymph nodes and spleens was performed using BioLegend's LEGENDplex T Helper Cytokine Panel according the manufacturers' protocol and analyzed by BD LSRFortessa™ flow cytometer (BD Bioscience).

Flow cytometric analysis of total BAL cells and lung lymphocytes

Single cell suspension of isolated lung cells were stained with live/dead aqua (ThermoFisher) in PBS for 10 min, washed with FACS buffer (PBS, 1 % FCS) (centrifuged at 450 g, 5 min, 4 °C), stained for 20 min with extracellular antibody mix (Table S1) and washed twice with FACS buffer (centrifuged at 450 g, 5 min, 4 °C). Cells were fixed with eBioscience™ FoxP3/Transcription kit (Thermo Fisher Scientific) and washed twice in fixation/permeabilization kit buffer (centrifuged 980 g, 5 min, 4 °C). Fixed cells were stained for 30 min with intracellular GATA3/FoxP3 antibody mix (Table S1), washed with fixation/permeabilization kit buffer (centrifuged at 980 g, 5 min, 4 °C), washed with FACS buffer (PBS, 1 % FCS) (centrifuged at 980 g, 5 min, 4 °C) and analyzed using a BD LSRFortessa™ flow cytometer, FACSDiva software (BD Biosciences) and FlowJo V.7.2.2 (Tree star, Ashland, USA).

For intracellular IFN-γ staining, single cell suspension of isolated lung cells were stimulated with 20 ng/ml phorbol myristate acetate (Sigma-Aldrich) and 1 µg/ml ionomycin (Biomol GmbH, Hamburg, Germany)(1 h, 37 °C, 5% CO_2) followed by addition of 5 µg/µl

Brefeldin A (Sigma-Aldrich)(3 h, 37 °C, 5% CO_2). Cells were washed twice with PBS (at 450 g, 5 min, 4 °C) and then live/dead aqua (ThermoFisher), extracellular and intracellular IFN-γ staining were performed (described above).

BAL cell suspension was washed twice with PBS (at 450 g, 5 min, 4 °C), stained with 40 µL of a master mix of fluorochrome-conjugated antibodies for 30 min (Tables S2) and after washing twice (at 450 g, 5 min, 4 °C) with FACS buffer (PBS, 1 % FCS) analyzed using a BD LSRFortessa™ flow cytometer, FACSDiva software (BD Biosciences) and FlowJo V.7.2.2 (Tree star).

Table S1: Antibodies for FACS analysis of lymphocytes isolated from lung

MARKER	FLUOROCHROME	CLONE	MANUFACTURER
EXTRACELLULAR			
CD3ε	FITC	145-2C11	BD Pharmingen
CD4	AF700	RM4-5	BioLegend
CD45	APCeF780	30-F11	eBiosciences
ST2	BV421	DIH9	MDBioproducts
INTRACELLULAR			
FoxP3	PerCP-Cy5.5	FJK-16s	eBiosciences
GATA3	eF660	TWAJ	eBiosciences
IFN-γ	PE-Cy7	XMG1.2	eBiosciences

Table S2: Antibodies for FACS analysis of BAL cells

MARKER	FLUOROCHROME	CLONE	MANUFACTURER
CD4	A700	RM4-5	BioLegend
CD8	FITC	53-6.7	BDPharmingen
CD11b	BV711	M1/70	BioLegend
CD11c	PE-Cy7	N418	Biolegend
CD45	PerCP-Cy5.5	30-F11	BioLegend
CD206	PE-Dazzle594	C068C2	BioLegend
F4/80	APC-e780	BM8	eBiosciences
NK1.1	APC	PK136	eBiosciences
Ly-6G	PacificBlue	1A8	Biolegend
Siglec-F	PE	E50-2440	BDPharmingen
Live/dead aqua fixable cell stain	405nm	—	ThermoFisher

Histology

For the histological analysis, after bronchoalveolar lavage (BAL) the lungs were excised and the left lobe fixed in 4% buffered formalin and embedded in paraffin. Sections of 4 µm thickness were stained with hematoxylin-eosin (H&E) and periodic acid Schiff (PAS). Scoring of the inflammatory cell infiltrate and mucus hypersecretion was performed as described previously (3).

Statistical analysis

Gaussian distribution was tested by D'Agostino & Pearson omnibus normality test. Gaussian and non-Gaussian distributed results were further analyzed by unpaired t-test or Mann-Whitney test, respectively. Lung function parameters were analyzed by two-way analysis of variance with Tukey's multiple comparison test. Scores of inflammatory cell infiltrate and mucus hypersecretion in the lung were analyzed by one-way analysis of variance with Tukey's multiple comparison test (GraphPad Prism, San Diego, CA). P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are shown as *, **, ***, and ****, respectively.

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