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IL-4 receptor α blockade prevents sensitization and alters acute and long-lasting effects of allergen-specific immunotherapy of murine allergic asthma

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Abstract

Background: Allergen-specific immunotherapy (AIT) is the only causal treatment for allergy. However, success rates vary depending on the type of allergy and disease background of the patient. Hence, strategies targeting an increased therapeutic efficacy are urgently needed. Here, the effects of blockade of IL-4 and IL-13 signaling on different phases of AIT were addressed.

Methods: The impact of the recombinantly produced IL-4 and IL-13 antagonist IL-4 mutein (IL-4M) on allergic sensitization and AIT outcome in experimental allergic asthma were analyzed in a murine model. The effects of IL-4M administration were assessed prior/during sensitization, immediately after AIT under allergen challenge, and two weeks post-treatment.

Results: Intervention with IL-4M prior/during sensitization led to strong induction of IgG1, IgG2a, IgG2b, and IgG3, decrease of specific and total IgE, as well as of IL-5 in serum. Similar effects on the serum immunoglobulin levels were observed immediately after IL4M-supplemented AIT during allergen challenge. Additionally, IL4M markedly suppressed type-2 cytokine secretion of splenocytes beyond the effect of AIT alone. These effects were equaled to those of AIT alone two weeks post-treatment. Intriguingly, here, IL-4M induced a sustained decrease of Th2-biased Tregs ($ST2^{+}FOXP3^{+}GATA3^{intermediate}$).

Conclusions: IL-4 and IL-13 blockade during experimental AIT demonstrates beneficial effects on immunological key parameters such as immunoglobulin and cytokine secretion immediately after AIT. Although two weeks later these effects were dropped to those of AIT alone, the number of potentially disease-triggering Th2-biased Tregs was further significantly decreased by IL-4M treatment. Hence, IL-4/IL13-targeting therapies prime the immune memory in therapy success-favoring manner.

Abbreviations: AIT, allergen-specific immunotherapy; Alum, aluminum hydroxide magnesium hydroxide formulation; BAL, bronchoalveolar lavage; CHO, Chinese hamster ovary; FoxP3, forkhead box P3; GATA3, GATA-binding protein 3; HE, hematoxylin and eosin stain; IL-4M, IL-4 mutein, interleukin-4/interleukin-13 antagonist; i.n., intranasal; i.p., intraperitoneal; OVA, ovalbumin; PAS, periodic acid-Schiff stain; s.c, subcutaneous; SD, standard deviation; sIgE, specific IgE; sIgG, specific IgG; ST2, suppression of tumorigenicity 2; tIgE, total IgE; tIgG, total IgG.

Russkamp and Aguilar-Pimentel are contributed equally to this work as first authors.

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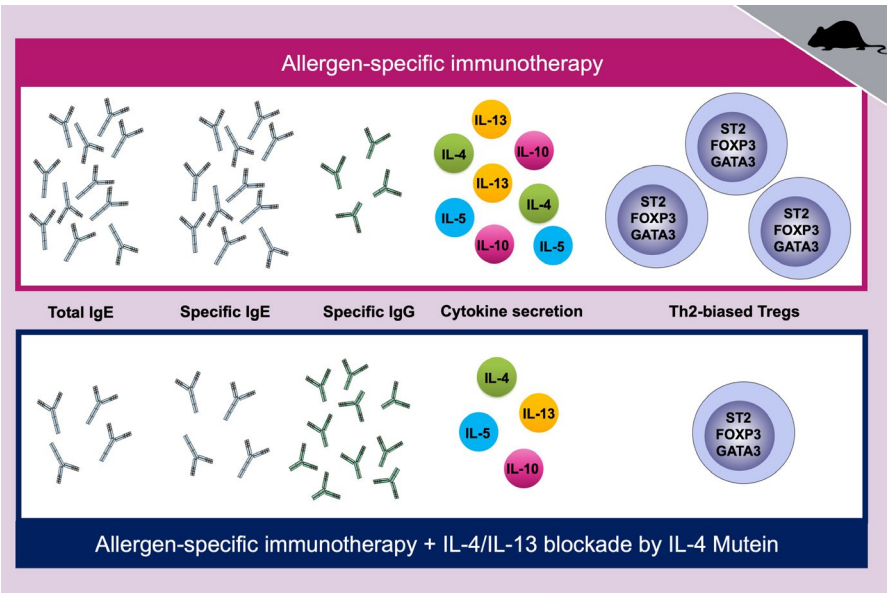
KEYWORDS
allergen-specific immunotherapy, allergic asthma, cytokine antagonist, IL-4-receptor-α, Th2-like regulatory T cells



GRAPHICAL ABSTRACT
Blockade of IL-4 and IL-13 signaling during experimental allergen-specific immunotherapy shows beneficial effects on immunological key parameters such as IgE, IgG and Th2 cytokine secretion as well as significantly decreases the number of potentially disease-triggering Th2-biased Tregs ($ST2^{+}FOXP3^{+}GATA3^{intermediate}$). Hence, Th2 cytokine-inhibiting strategies might be suitable to support allergen-specific immunotherapy in a therapy success-favoring manner. FOXP3: forkhead box P3; GATA3: GATA-binding protein 3; ST2: suppression of tumorigenicity 2

1 | INTRODUCTION

Depending on the type of allergy and the physical condition of the patient, efficiency of allergen-specific immunotherapy (AIT) varies strongly.[1,2] Hence, there is a need for novel advanced strategies for the improvement of therapeutic efficacy of AIT in several disease conditions. In this context, different studies of frontier research focused on the evaluation of immunomodulatory molecules as adjuvant substances during AIT. Toll-like receptor agonists,[3] vitamin D,[4] and JAK inhibitors[5] are only a few exemplary substances whose effect on AIT has been examined recently. The wide variety of action of these candidate substances illustrates that the exact mechanisms of a successful AIT have not been fully understood yet. Although suppression of allergen-specific Th2 cells,[6] regulation of IgE and IgG4,[7] and induction of allergen-specific regulatory T (Treg)[8] and B (Breg) cells[9] are alterations that can be observed under AIT, none of these parameters represents a definite biomarker for positive therapeutic outcome.

Numerous hallmarks of allergic pathogenesis such as sensitization,[10] several allergic symptoms,[11] and the IgE switch in B cells[12] seem to be associated with IL-4/IL-13 receptor signaling. Interestingly, this receptor complex appears to play an important role for the prevention of tolerance induction. More recently, it could be observed that IL-4/IL-13 signaling has several effects on Treg populations. Our previous work showed that IL-4 signaling suppresses FoxP3 expression and that injection of IL-4 reduces the number of induced Tregs in vivo.[13] Furthermore, it was demonstrated that IL-4 signaling is able to promote the differentiation of so-called Ex-FoxP3 Th2 cells during helminthic infections and that these cells are characterized by Th2 cytokine production.[14]

The relevance of IL-4/IL-13 signaling for both, Th2-mediated inflammation and the suppression of Treg induction and stability, makes it an extraordinary interesting target for the treatment of allergic disorders. In recent years, numerous IL-4/IL-13-blocking treatments have been studied[15-18] and are now applied for the treatment of atopic dermatitis[19] and asthma.[20]

In the current study, the effects of IL-4-receptor-α blockade on allergy onset as well as on different AIT treatment phases were investigated. Instead of targeting IL-4/IL-13 signaling using an IL-4-receptor-α specific antibody, which is characterized by a long-lasting serum half-life,[21] a mutant variant of IL-4 (IL-4M) that was developed by Grunewald et al[22] and reached a phase 2 study as inhaled asthma medication[23] was used. It binds to the IL-4-receptor-α chain and prevents binding and signaling of IL-4 and IL-13. The shorter half-life of IL-4M is an ideal characteristic of this molecule for the simultaneous administration during

AIT, since a sustained cytokine inhibition during the extended phase of AIT might be accompanied by unwanted side-effects. Therefore, the presence of the biological AIT adjuvant in the organism is desired to be limited to the presence of the allergen priming phase.

For this study, IL-4M was recombinantly produced for the first time in a mammalian expression system. Its antagonizing abilities were tested in cellular assays and in vivo. In order to assess the importance of IL-4/IL-13 signaling for AIT outcome, the effects of IL-4M administration on experimental AIT of murine allergic asthma with increased systemic levels of IL-4, IL-13, and IgE that closely resembles the situation in allergic patients[24-26] were analyzed.

The understanding of the sequence of immunological events might be a prerequisite for the development of novel therapeutic strategies. Hence, in addition to the typical disease-associated parameters, a special focus of this study was the effect of IL-4/IL-13 receptor inhibition on lung-resident populations of Th2 and Treg cells at different time points of therapy, some of which are hypothesized to represent an intermediate cell population on the edge between allergen reactivity and tolerance loss.[27]

2 | METHODS

2.1 | Animals

Female C57BL/6 and BALB/c mice between 5-7 weeks of age were purchased from Charles River (Sulzfeld, Germany). Reporter mice for Foxp3 (Foxp3^{tm1Flv})[28] and IL-4 transcript (IL4^{tm1Lky})[29] were maintained on a C57BL/6 background and used for in vitro experiments. All experiments were carried out under federal guidelines for the use and care of laboratory animals and approved by the government of the district of upper Bavaria (reference numbers of ethical approvals for all performed animal experiments are 55.2-1-54-2532-50-2017, and 55.2-1-54-2532-104-13).

2.2 | Murine model of allergic sensitization

For allergic sensitization, mice were intraperitoneally (i.p.) injected with 10 μg ovalbumin (OVA) (Grade V, Sigma-Aldrich, Taufkirchen, Germany) and complexed with 2 mg aluminum hydroxide (alum) (Imject Alum, Thermo Fisher Scientific, Waltham, MA) in 200 μL PBS. Mice in the negative control group (nonsensitized) were i.p.-injected with alum in PBS. Mice in the test group (sensitized + IL-4M) were treated with i.p. injections of 100 μg IL-4M in 100 μL PBS 2 hours before and 2 hours after the sensitization. 100 μg IL-4M was further administered i.p. on days 2, 4, 6, and 8. Mice from all groups were euthanized on day 10. Shown are the results of one experiment.

2.3 | Murine model of AIT during allergen exposure

Nonallergic mice were treated on days 1, 7, and 21 with PBS-alum (i.p.). Subsequently, they received subcutaneous (s.c.) 200 μL injections of PBS on days 35, 38, and 41. Allergic mice were sensitized on days 1, 7, and 21 with OVA-alum (i.p.). Subsequently, they received 200 μL of PBS on days 35, 38, and 41 (s.c.). Allergic-AIT mice were sensitized on days 1, 7, and 21 with OVA-alum (i.p.). Subsequently, they received 500 μg OVA in 200 μL PBS on days 35, 38, and 41 (s.c.). In addition to this treatment, Allergic-AIT + IL-4M mice received daily i.p. injections of 100 μg IL-4M in 100 μL PBS on days 34-42. On days 34, 37, and 40, mice of all groups were challenged for 15 minutes in a challenge chamber with 1% nebulized OVA. Half of the mice were euthanized on day 43. The other half was kept until a second challenge block with 1% nebulized OVA was performed (days 56, 59, and 62). These mice were euthanized on day 63. Shown are the results of one experiment.

2.4 | Recombinant production of IL-4M

Cloning, recombinant production of IL-4M in dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary cells (CHO) and purification by affinity and size exclusion chromatography are described in detail in the Data S1.

2.5 | Cellular in vitro assays

A detailed description of Th2 and Treg polarization assays using naïve T cells from IL-4-GFP and FoxP3-RFP reporter mice, respectively, and OVA-specific restimulation of splenocytes can be found in the Data S1.

2.6 | Isolation of BAL cells and lung-resident lymphocytes

Detailed protocols for the isolation of BAL cells and lymphocytes from the lung are given in the Data S1.

2.7 | Other methods

SDS-PAGE, Western blotting, immunoglobulin- and cytokine-ELISAs, FACS, CTLL2 assay, allergen-specific restimulation of splenocytes, histology, and the HDM mouse model are described in the Data S1

2.8 | Statistical analyses

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 (GraphPad Prism, San Diego, CA). *P*-values of >0.05, ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are shown as ns, *, **, ***, and ****, respectively.

3 | RESULTS

3.1 | CHO-derived IL-4M alters Th2 and Treg differentiation in vitro

The amino acid sequence of IL-4M[22] differs from native IL-4 in only two positions (Q116D, Y119D) (Supporting Information, Figure S1A).

In this study, for the first time, a mammalian expression system (CHO cells) was chosen for recombinant production of IL-4M with high yields (8 mg/L) (Supporting Information, Figure S1B). An IL-4 ELISA kit (OptEIA, BD Biosciences, San Jose, CA) could be utilized for the quantification of correctly folded IL-4M out of the total recombinant IL-4M. Unsurprisingly, the fraction of functionally folded IL-4M was many times higher in CHO cells compared to *E. coli*-derived IL-4M that was used in former studies (Supporting Information, Figure S1C).

In order to quantify the inhibitory capacity of CHO-derived IL-4M, a viability assay using IL-4-dependent CTLL2 cells was performed. IL-4 alone provided the cells with a survival signal; concurrent IL-4M presence resulted in a decreased viability. A 30-fold excess of IL-4M to IL-4 induced half-maximal decrease of viability (Figure 1A)

In a Th2 differentiation assay, naïve T cells from IL-4 reporter mice were polarized to IL-4-expressing Th2 cells by the presence of IL-4. Simultaneous administration of IL-4M abolished Th2 differentiation (Figure 1B).

Additionally, naïve T cells from FoxP3 reporter mice can be skewed to a regulatory phenotype, characterized by FoxP3 expression, by the presence of IL-2 and TGF-β. IL-4 suppressed FoxP3 expression under these conditions, while simultaneous administration of IL-4M rescued FoxP3 expression (Figure 1C).

3.2 | IL-4M prevents allergic sensitization

Sensitization of mice (Figure 2A) with OVA-alum resulted in increased levels of OVA-specific IgE (sIgE), total IgE (tIgE), and total IgG1 (tIgG1) when compared to PBS-alum-treated mice (nonsensitized) (Figure 2B). IL-4M treatment prior/during sensitization (sensitized + IL-4M) suppressed induction of sIgE and tIgE. Residual IgE is very low and may originate from incomplete suppression of IL-4 or from IL-4-independent mechanisms of IgE switching.[30] In contrast, tIgG1was even further increased in IL-4M-treated mice. IgG2a, IgG2b, and IgG3 were not altered by sensitization. However, mice that received IL-4M displayed significantly increased levels of all three isotypes (Figure 2B).

Sensitization induced several changes in serum cytokine levels (Figure 2C). It led to an increased abundance of IL-5, while IL-4M treatment prevented this effect. Sensitization itself did not increase the level of IL-4. However, large amounts of IL-4M were detected in the serum of IL-4M-treated mice. Whereas concentrations of IL-10 and IFNγ were not significantly affected by sensitization or IL-4M treatment, the levels of IL-1β and IL-6 showed a minor but significant decrease after sensitization. IL-4M treatment reversed both effects.

3.3 | Acute effects of IL-4M administration on AIT

The effect of IL-4/IL-13 signal blockade on AIT was assessed in murine allergic asthma (Figure 3A). Mice were sensitized with OVA-alum before OVA-specific AIT. Moreover, mice were repeatedly exposed to nebulized OVA during the phase of AIT, a setting that, in contrast to classical models,[31] induces elevated levels of IL-4, IL-13, and sIgE (Figure S2).

Here, directly after AIT, total BAL cells (Figure 3B) and differential BAL counts (Figure S3A) were not yet significantly affected by AIT or AIT combined with IL-4M treatment. Histological analyses confirmed these results. Lungs of all groups displayed no differences regarding inflammatory infiltration and mucus production (Figure 3C,D)

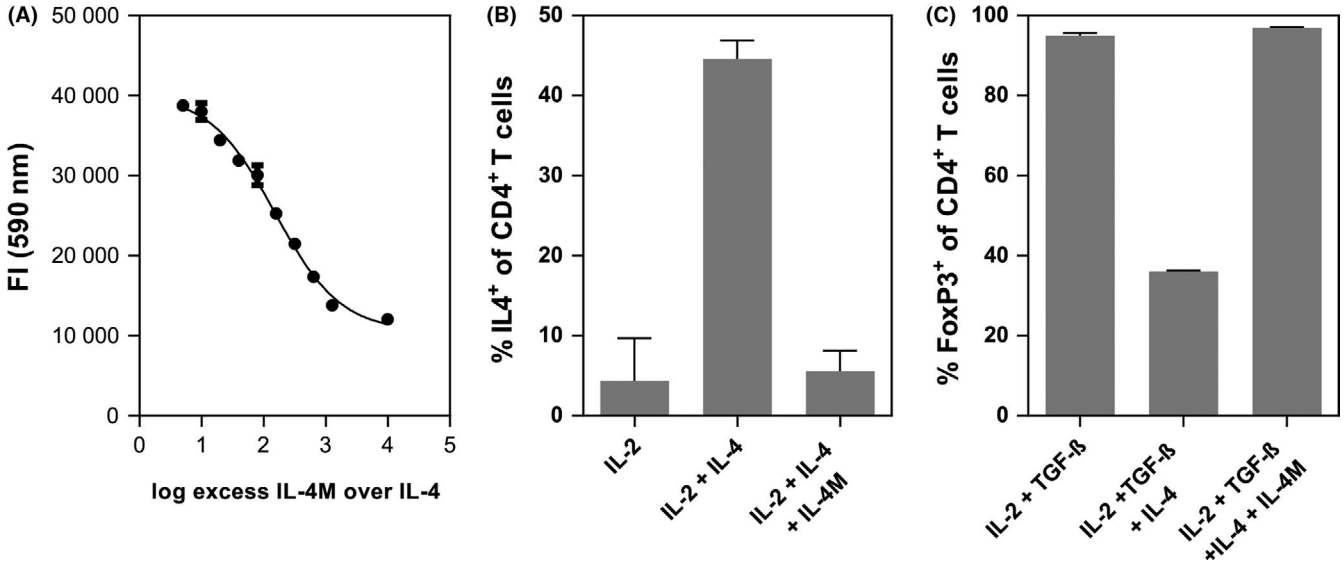


FIGURE 1 In vitro characterization of IL-4M. A, Viability of CTLL2 cells in dependence of the IL-4M to IL-4 ratio. FI, fluorescence intensity. B, Effect of IL-4M on the polarization of naïve T cells from IL-4-reporter mice to IL-4-producing Th2 cells. Presented is one representative experiment performed in duplicates. C, Effect of IL-4 and IL-4M on the polarization of naïve T cells from FoxP3-reporter mice to FoxP3-expressing regulatory T cells. Presented is one representative experiment performed in duplicates. Shown is the mean with SD. FOXP3, forkhead box P3

While AIT alone significantly increased the level of IL-17A in the BAL fluid, the combined therapy abolished this effect completely. Both therapeutic strategies had no further effects on IL-5 and IL-13 levels in the BAL despite the presence of high amounts of IL-4M (Supporting Information, Figure S3B).

Total IgE was significantly increased in mice that were sensitized and challenged (allergic) compared to nonsensitized but challenged mice (nonallergic) (Figure 3E). This acute IgE production stabilizes at lower concentrations in later observation windows (Figure 4E). Whereas the level of tIgE was even further increased in mice that received AIT (allergic-AIT), the combination of AIT with IL-4M suppressed this effect. The concentration of sIgE that was reduced by AIT alone could be further decreased by the combination of AIT with IL-4M (Figure 3E). Interestingly, IgG2b levels that were equally increased in allergic and AIT-treated mice were significantly higher in the allergic-AIT + IL-4M group (Figure 3E). IgG2a levels were not similarly affected (Supporting Information, Figure S4A).

Allergen-specific restimulation of isolated splenocytes showed that AIT alone had no significant effects on the level of secreted cytokines. In contrast, AIT in the presence of IL-4M resulted in decreased levels of IL-4, IL-5, IL-13, and IL-10 (Figure 3F).

3.4 | Long-lasting effects of IL-4M administration on AIT

In order to analyze the long-lasting effects of IL-4/IL-13 signal blockade on AIT outcome, mice were treated as described above but kept for another two weeks before an additional allergen challenge was performed (Figure 4A).

At this time point, total BAL cells (Figure 4B) and BAL cell subsets (Figure S5A) were already significantly decreased in mice that received AIT alone and the combination of AIT with IL-4M treatment showed an equal improvement. IL-4M alone induced a slight reduction of BAL cell numbers. These results were confirmed by histological analyses. Allergic mice exhibited the characteristic inflammatory infiltrate and goblet cell hyperplasia. The lungs of mice that received either IL-4M, AIT alone, or the combination of AIT and IL-4M were characterized by a diminished perivascular and peribronchiolar inflammatory infiltrate and, by trend, mucus hypersecretion compared to allergic mice (Figure 4C,D). The levels of BAL cytokines exhibited no major differences between mice that received AIT alone or in combination with IL-4M (Supporting Information, Figure S5B).

Immunoglobulin titers showed only minor differences in this phase of therapy (Figure 4E, Supporting Information, Figure S4B). Solely, IgG1 was increased in mice that had received AIT in the presence or absence of IL-4M when compared to allergic mice.

Allergen-specific restimulation of splenocytes resulted in slightly increased levels of IL-4, IL-5, IL-13, and IL-10 production in allergic mice. Splenocytes from mice that were treated with AIT alone or in the presence of IL-4M displayed a cytokine secretion pattern similar to nonsensitized mice (Figure 4F).

3.5 | IL-4M administration during AIT alters lung-resident lymphocyte populations

Immediately after AIT in the presence or absence of IL4M, the levels of $GATA3^+ST2^+FoxP3^-$ Th2 cells were not decreased when compared to allergic mice (Figure 5A). Two weeks and an additional challenge phase later, already AIT-treated mice displayed a significant decrease of these Th2 cells. This effect was not further enhanced by the addition of IL-4M (Figure 5B).

The level of $FoxP3^+$ cells among the $CD4^+$ subset was slightly decreased immediately after AIT alone. A further significant reduction could be observed in the group that received AIT and IL-4M (Figure 5A). Two weeks later, this alteration was exclusively sustained in mice that had received the combination of AIT and IL-4M (Figure 5B).

Allergic mice showed higher numbers of $FoxP3^+ST2^+$ among $CD4^+$ cells than nonallergic animals. AIT alone was able to reduce the level of these cells at both analyzed time points. AIT in the presence of IL-4M treatment resulted in an even stronger decrease of this cell population (Figure 5A,B). For the analysis of these cells, $CD3^+CD4^+$ cells were gated for ST2 expression (Figure 5C left). Subsequently, the fraction of $FoxP3^+GATA3^{intermediate}$ cells was analyzed and demonstrated to be highly decreased in mice treated with the combination of AIT and IL-4M compared to allergic mice (Figure 5C middle and right).

To further characterize this cell population, lymphocytes from house dust mite (HDM)-allergic mice (Figure 5D) were isolated from the lung, treated with PMA/ionomycin and brefeldin A, and analyzed by FACS. Here, the fraction of $ST2^+$ $FoxP3^+GATA3^{intermediate}$ cells showed a higher secretion of IL-5 than $ST2^-$ $FoxP3^+GATA3^{intermediate}$ cells (Figure 5E).

4 | DISCUSSION

The current study demonstrates that IL-4 and IL-13 signaling play an important role in the balance of T-cell differentiation and immunoglobulin isotype levels in the context of AIT.

To elucidate the effects of IL-4 and IL-13 blockade on allergic sensitization and different phases of AIT, the IL-4 receptor-α (IL-4Rα) antagonist IL-4 mutein (IL-4M) was used.[22] In this study, IL-4M was recombinantly produced in mammalian cells for the first time and, therefore, exhibits a significantly higher activity than *E. coli*-derived IL-4M.[32] This study underlines the suitability of non-antibody-based cytokine-blocking approaches in atopic diseases. The short half-life of IL-4M[33] makes it an ideal candidate for temporary limited suppression of IL-4 and IL-13 signaling which would be difficult to achieve with IL-4Rα-specific antibodies which are much slower cleared from the system.[34]

A special focus of the study lay on the impact of combining systemic IL-4M administration with AIT on local Treg[13] and Th2[22] cell populations in vivo as previous in vitro experiments revealed a critical role of IL-4R signaling for both cell types. By employing IL-4M in in vitro polarization assays, its capacity to prevent the development of Th2 cells and to rescue the development of Tregs could be

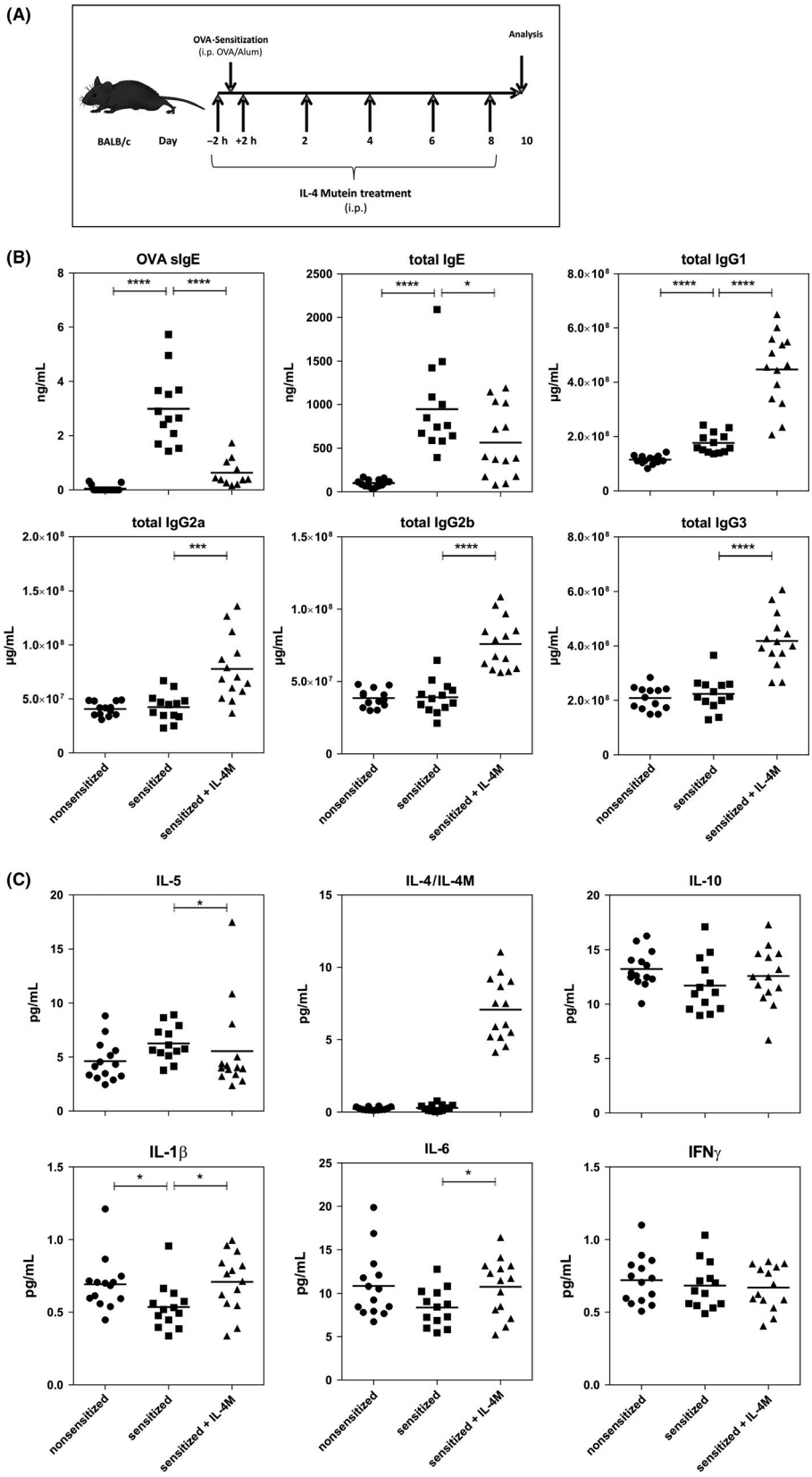


FIGURE 2 IL-4M prevents allergic sensitization. A, Schematic overview of the in vivo treatment scheme (n = 14 in the nonsensitized and sensitized + IL-4M group and n = 13 in the sensitized group). B, Measurement of immunoglobulins in serum samples of mice at the endpoint of the experiment. C, Concentration of cytokines in serum samples of mice at the endpoint of the experiment. Here, the ELISA for the detection of IL-4 also measures the level of IL-4M. Bars indicate the mean. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively

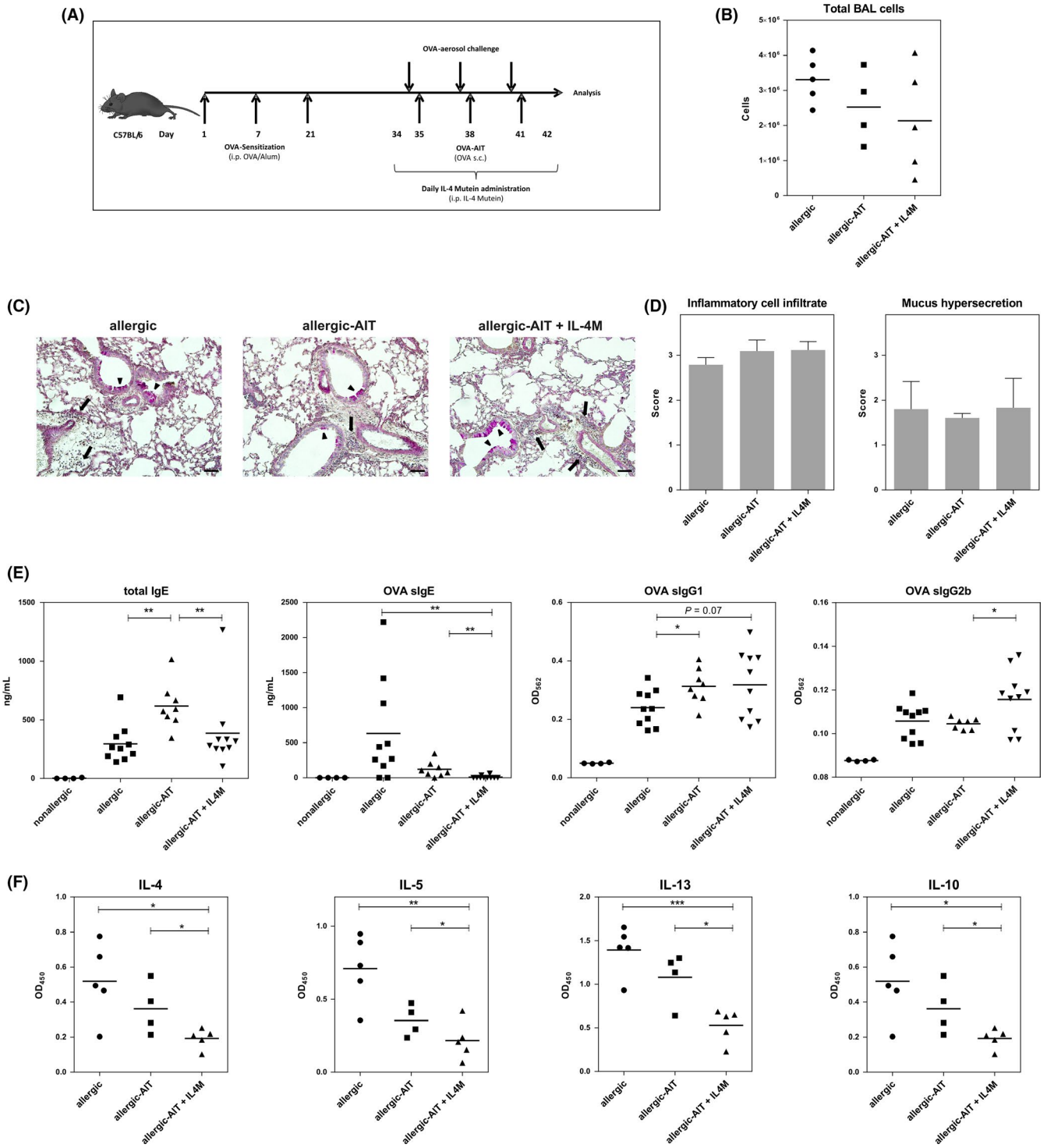


FIGURE 3 Acute effects of IL-4M administration on AIT. A, Schematic overview of the in vivo treatment scheme. B, Counts of total BAL cells. C, Lung histology 2 days after the last AIT and 24 hours after the last IL-4M treatment (periodic acid-Schiff staining). Arrows, inflammatory infiltrate; arrowheads, mucus hypersecretion; scale bar, 50 μm. Representative pictures are shown. D, Inflammatory cell infiltrate and mucus hypersecretion scores of the lung tissue after the last AIT and 24 hours after the last IL-4M treatment. Shown is the mean with SD. E, Measurement of immunoglobulins in serum samples of mice at the endpoint of the experiment. F, Measurement of cytokine levels in culture supernatants of splenocytes after OVA-restimulation in vitro. Bars indicate the mean. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively. Analyses of lung and serum immunoglobulins were performed for n = 12 in the nonallergic, allergic, and allergic + IL-4M group, for n = 9 in the allergic-AIT group and n = 11 in the allergic-AIT + IL-4M group. Splenocyte restimulation assay was performed for n = 5 in the allergic and allergic-AIT+IL-4M group and n = 4 in the allergic-AIT group

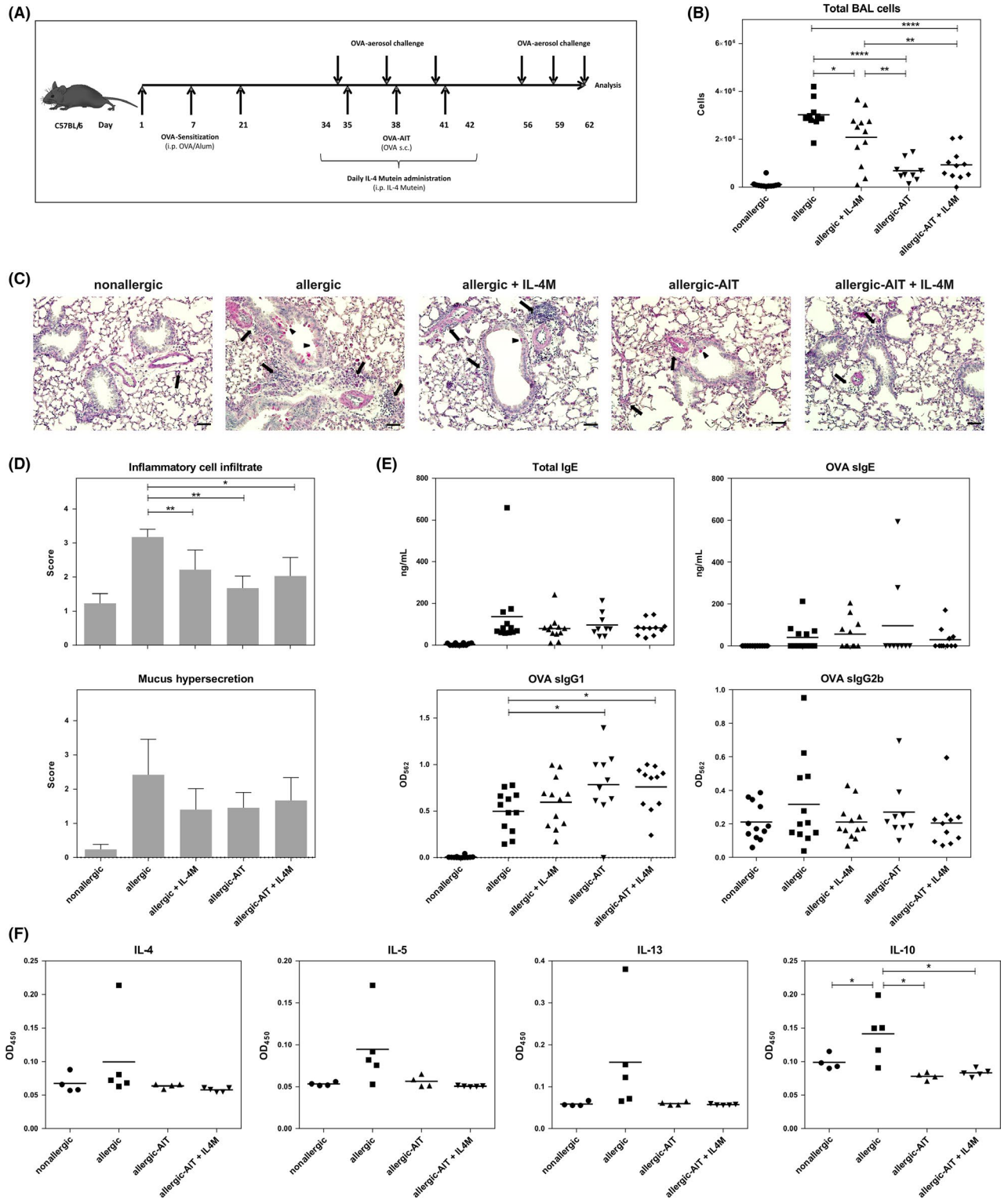


FIGURE 4 Long-lasting effects of IL-4M administration on AIT. A, Schematic overview of the in vivo treatment scheme (n = 9-12). B, Counts of total BAL cells. C, Lung histology 24 hours after the last OVA aerosol challenge (periodic acid-Schiff staining). Arrows, inflammatory infiltrate; arrowheads, mucus hypersecretion; scale bar, 50 μm. Representative pictures are shown. D, Inflammatory cell infiltrate and mucus hypersecretion scores of the lung tissue 24 hours after the last OVA aerosol challenge. Shown is the mean with SD. E, Measurement of immunoglobulins in serum samples at the endpoint of the experiment. F, Measurement of cytokine levels in culture supernatants of splenocytes of n = 4-5 mice after OVA-restimulation in vitro. Bars indicate the mean. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test or Mann-Whitney test, respectively. Analyses of lung and splenocytes were performed for n = 5 in the allergic and allergic-AIT+IL-4M group and n = 4 in the nonallergic and allergic-AIT group. Immunoglobulin levels in serum were measured for n = 4 in the nonallergic group, n = 10 in the allergic and allergic-AIT+IL4M group, and n = 8 in the allergic-AIT group

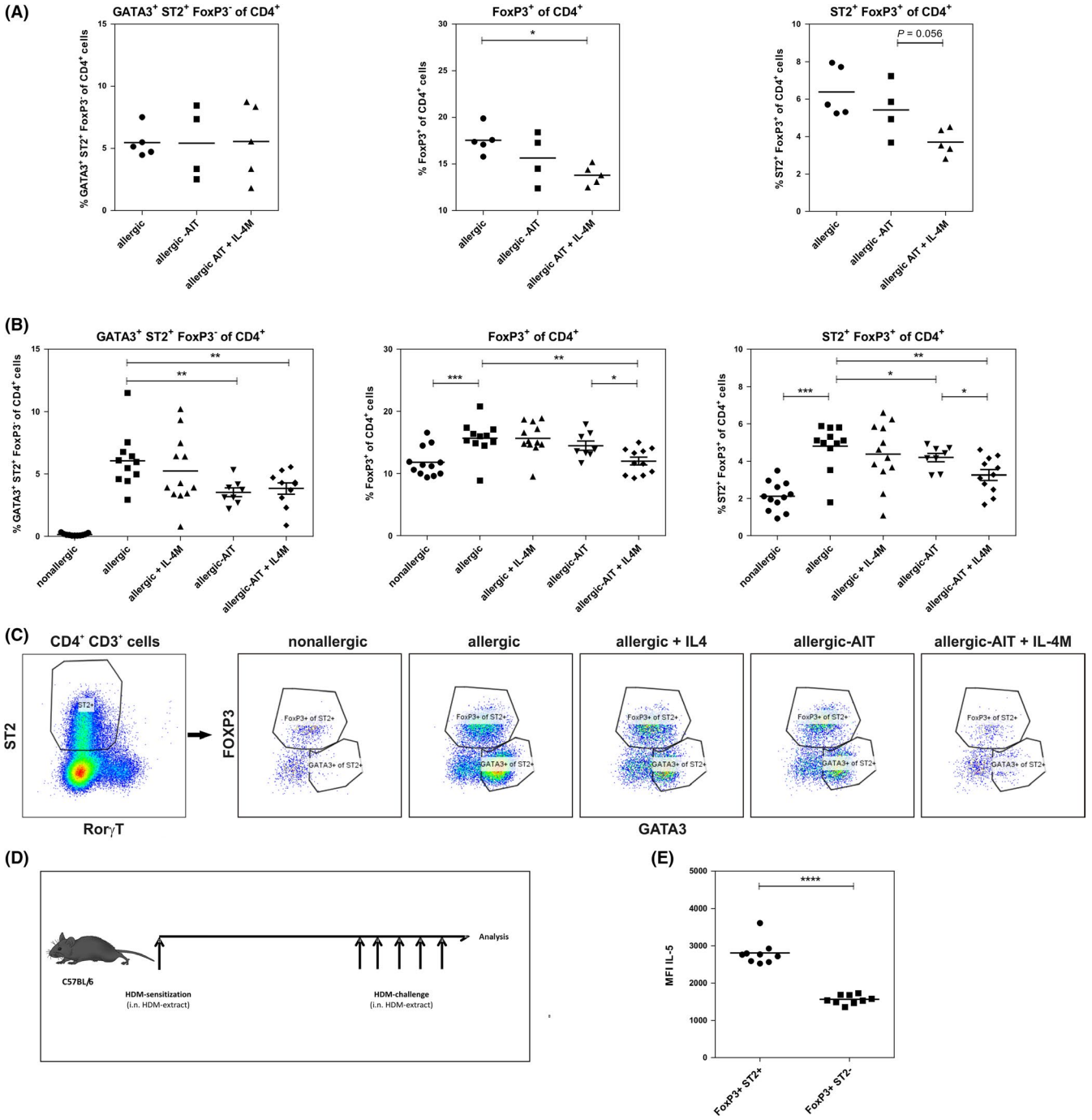


FIGURE 5 IL-4M administration during AIT alters lung-resident lymphocyte populations. A, Analysis of lung-resident lymphocyte populations immediately after AIT (n = 4-5). B, Analysis of lung-resident lymphocyte populations two weeks and an additional challenge block after AIT (n = 8-12). The complete gating strategy is shown in Figure S6. C, Gating strategy for the identification of $FoxP3^{+}ST2^{+}GATA3^{intermediate}$ cells and their abundance in the different treatment groups. D, Schematic overview of the in vivo treatment scheme for the induction of house dust mite allergy (n = 9). i.n., intranasally. E, IL-5 production of $FoxP3^{+}ST2^{+}$ and $FoxP3^{+}ST2^{-}$ T cells. MFI, mean fluorescence intensity; FOXP3, forkhead box P3; GATA3, GATA-binding protein 3 [Colorfigure can be viewed at wileyonlinelibrary.com]

confirmed. Similar effects on T-cell populations have been observed in patients undergoing regular AIT.[6,35]

Administration of IL-4M prior/during allergic sensitization induced several serological changes that are, according to recent studies, also seen after successful AIT. Besides the suppression of IgE,[12] enhanced induction of the blocking antibodies IgG2a and IgG2b could be detected. Similar observations could be made in a study using IL-4-deficient mice.[36] The observed IgG2b-inducing effect of IL-4M is in concordance with a study that observed an inhibitory effect of IL-4 on TGF-β-induced IgG2b secretion.[37] Moreover, IL-4M reversed the OVA-alum-induced changes in cytokine level back to the levels of healthy mice. It can be hypothesized

that the restored cytokine status is beneficial for immunological tolerance.

Via repeated allergen exposure during the phase of AIT, this study aimed at adjusting the model of experimental AIT to the situation that patients are exposed to during the allergen season. Indeed, this approach resulted in an increase of IL-4, IL-13, and IgE as observed in patients suffering from allergic asthma.[24-26] Additionally, the IL-4M dosage was adjusted to the short serum half-life in vivo.[38]

Immediately after AIT and simultaneous exposure to allergen, IL-4M induced similar changes of the IgE and IgG2b level that were also visible after IL-4M administration during the sensitization phase. While tIgE is generally not affected by AIT in man, the AIT-mediated induction of sIgE was also observed in grass-specific AIT in humans.[27] IL-4M administration prevented this IgE-inducing effect: sIgE and tIgE were significantly decreased in mice treated with AIT and IL-4M. However, the diminished levels of IgE in combination with increased abundance of IgG2b did not result in attenuated lung inflammation at this phase, what is likely to be related to the limited role of IgE for inflammation in the lung tissue of mice.[39]

Two weeks after AIT, the IL-4M-mediated effects on immunoglobulin levels were not detectable. Here, the immunoglobulin levels of all groups did not display major differences. Still, the IgG1 titer was significantly increased by AIT in the presence or absence of IL-4M.

Allergen-specific immunotherapy alone or in the presence of IL-4M resulted in an immediate and sustained decrease of IL-13 levels in the BAL. Interestingly, co-administration of IL-4M prevented the increase of IL-17A in the BAL that was induced by AIT alone. A recent study observed that AIT is followed by increased numbers of IL-17A-producing T cells in nasal scrapings of allergic rhinitis patients.[27] The fact that IL-4M prevented this AIT-mediated effect illustrates that IL-4/IL-13 blockade does not only affect type-2 immune responses but also alters other types of inflammation.

Following these lines, AIT in the presence of IL-4M exhibited numerous remarkable effects on T-cell populations and thus on allergen memory. Allergen-specific restimulation of splenocytes isolated from IL-4M-treated mice immediately after AIT exhibited a reduced production of Th2 cytokines when compared to splenocytes from mice that had received AIT alone. This reduction in Th2 cytokine production is most likely caused by the suppressive effect of IL-4M on type-2 differentiation. This effect was not present two weeks later as all treatment groups returned to the cytokine levels observed in nonallergic mice. Unfortunately, the effect of IL-4M treatment on the IL-4 level cannot be assessed as it is not possible to discriminate between IL-4 and IL-4M in an in vivo setting.

Other IL-4M-induced changes in the T-cell level turned out to be sustained. One of these long-lasting effects is the decrease of $FoxP3^+$ T cells at the site of inflammation. This observation is likely to be a result of the migratory behavior of the cells and, therefore, reflects the inflammatory process rather than the process of tolerance induction. Increased numbers of Tregs were also observed in asthmatic patients in a phase of active inflammation[40] indicating that rather the quality than the quantity of Tregs is decisive for the anti-inflammatory capacity of Tregs.

Whereas AIT in the presence and absence of IL-4M did not result in an immediate decrease of Th2 cells, both treatments induced a similar reduction two weeks later. The fact that IL-4M did not affect the level of Th2 cells implies that IL-4R signaling is rather important for the differentiation of Th2 and has only minor effects on already differentiated Th2 cells.[41,42]

Enhanced type-2 disease susceptibility can be caused by intensified IL-4R signaling activity which poises Tregs to a Th2-like phenotype that causes impaired tolerance induction and contributes to disease pathogenesis such as food allergy.[43] Therefore, plastic Treg subpopulations are thought to be involved in the balance between tolerance and immunity. We demonstrate that IL-4M treatment prevents the type-2 skewing of a plastic lung-resident Treg cell population that is characterized by the co-expression of FoxP3 and GATA3 as well as the IL-33 receptor ST2. This population is abundant in lungs of allergic, untreated mice. AIT in combination with IL-4M treatment prevented the expansion of this population even two weeks after treatment when immunoglobulin levels already returned to baseline. These lung-resident type-2-poised Tregs were described to represent hyperactivated Tregs that are homing to nonlymphoid tissues, efficiently suppress T-cell responses, independently of IL-10 and TGF-β, but which can respond to IL-33 with IL-5 and IL-13 secretion.[44,45] It appears that they can escape their role as suppressive cells in response to epithelial-derived IL-33. The existence of these cells in man remains unclear; however, it was demonstrated that house dust mite allergens in vitro induce $GATA3^+FoxP3^+$ T cells that contribute to the polarization of the Th2-associated response.[46] Future experiments need to clarify the role of these cells in AIT and their suppressive capacity.

In addition to the Treg-stabilizing effect caused by IL-4M as co-administered adjuvant during AIT, also the IL-4M treatment alone reduced the immune cell infiltration into the lung as well as the IL-4 level in the BAL in a long-lasting manner, possibly due to a decreased type-2 differentiation of T cells.

In conclusion, inhibition of IL-4/IL-13 receptor signaling during experimental AIT of allergic asthma mediated acute changes in immunoglobulin levels and cytokine production exceeding the impact of AIT alone. Moreover, the observed long-lasting changes in a recently identified plastic or transitory type-2-poised Treg population indicate that IL-4Rα blockade during AIT could be of value in the human allergic situation.

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AUTHOR CONTRIBUTIONS

DR performed the experiments, analyzed the data, and wrote the manuscript. AA-P performed the experiments and analyzed the

data. FA performed the experiments and analyzed the histological data. VG-D planned experiments and discussed the data. HF coordinated mouse experiments and discussed the data. CO contributed to in vitro experiments and interpretation of data. AC, MHdA, and MO contributed to the interpretation of data and revised the final version of the manuscript. CS-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, designed the figures, and wrote the manuscript.

CONFLICTS OF INTEREST

AC has consultant arrangements via Technical University Munich with Allergopharma, ALK-Abello, HAL Allergy, Mundipharma, and Lofarma; has conducted clinical studies and received research grants via Technical University Munich from Allergopharma, Novartis, the German Federal Environmental Agency, Bencard/Allergy Therapeutics, ASIT Biotech, GlaxoSmithKline, Roche and Zeller AG; and has received payment via Technical University Munich for lectures from Allergopharma, ALK-Abello, and GlaxoSmithKline, outside the submitted work. MO reports personal fees and/or nonfinancial support from Thermo Fisher Scientific and Siemens Healthcare, consultancy fees as scientific advisor on the advisory board of Bencard Allergie GmbH and of Hycor Diagnostics, outside the submitted work. CS-W reports grants and personal fees from Bencard, grants from Leti Pharma, grants and personal fees from Allergopharma, grants and personal fees from PLS-Design, outside the submitted work. In addition, CS-W has a patent on diagnostic success prediction in AIT, which is pending. SB reports nonfinancial support from ALK-Abelló; grants, personal fees, and nonfinancial support from Bencard Allergie GmbH; personal fees from Teomed AG; personal fees from Thermo Fisher Scientific; grants from Allergy Therapeutics, outside the submitted work. The other authors have nothing to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Figure S1: Recombinant expression of IL-4M. A, Amino acid sequence alignment of IL-4M and mouse IL-4. B, Coomassie staining and Western blot analysis with an anti-IL-4 antibody of purified IL-4M. C, Percentage of correctly folded out of total IL-4M recombinantly produced either in CHO cells or *E. coli* as assessed by sandwich ELISA (detects only folded IL-4).

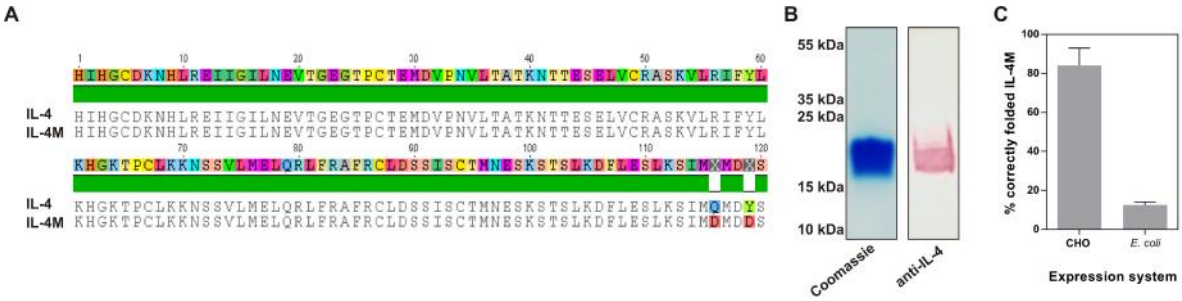


Figure S2: Levels of TH2 markers in unchallenged and challenged mice A, Levels of IL-4 and IL-13 in the BAL fluid of OVA-sensitized mice before and after OVA aerosol challenge. B, Serum level of OVA-specific IgE of OVA-sensitized mice before and after OVA aerosol challenge.

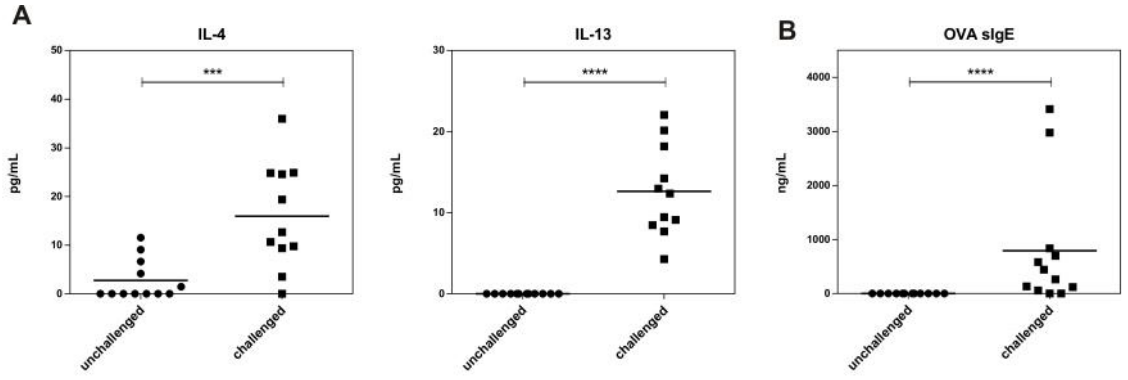


Figure S3: IL-4/IL13 blockade during AIT: Immediate effects on inflammation. A, Differential cell count of BAL cells. B, Cytokine levels in the BAL fluid. Here the ELISA for the detection of IL-4 also measures the level of IL-4M. Bars indicate the mean.

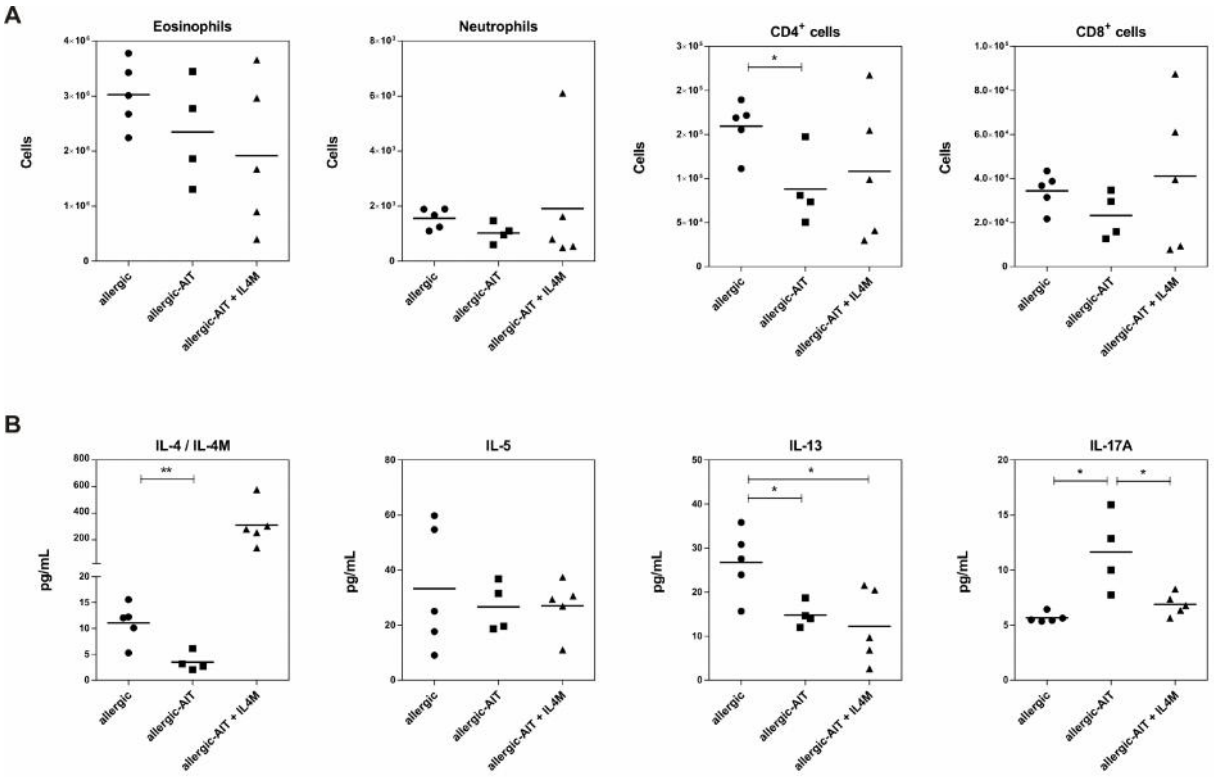


Figure S4: Serum levels of OVA-specific IgG2a . A, IgG2a levels immediately after AIT. B, IgG2a levels 2 weeks after AIT and an additional OVA challenge block. Bars indicate the mean.

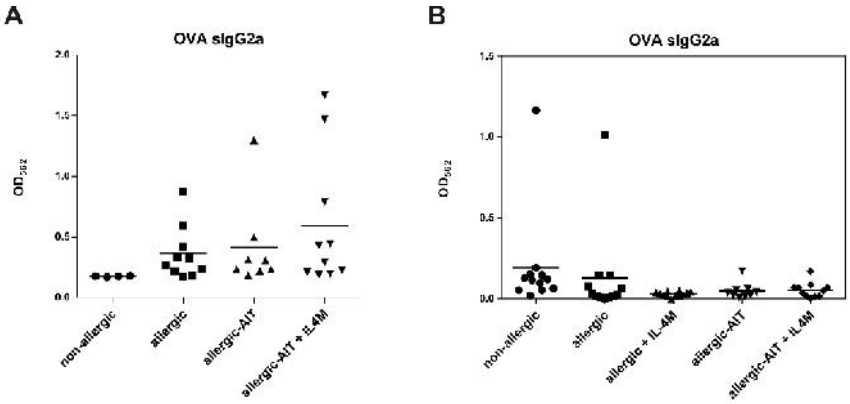


Figure S5: IL-4/IL13 blockade during AIT: Long term effects on inflammation. A, Differential cell count of BAL cells. B, Cytokine levels in the BAL fluid. Here the ELISA for the detection of IL-4 also measures the level of IL-4M. Bars indicate the mean.

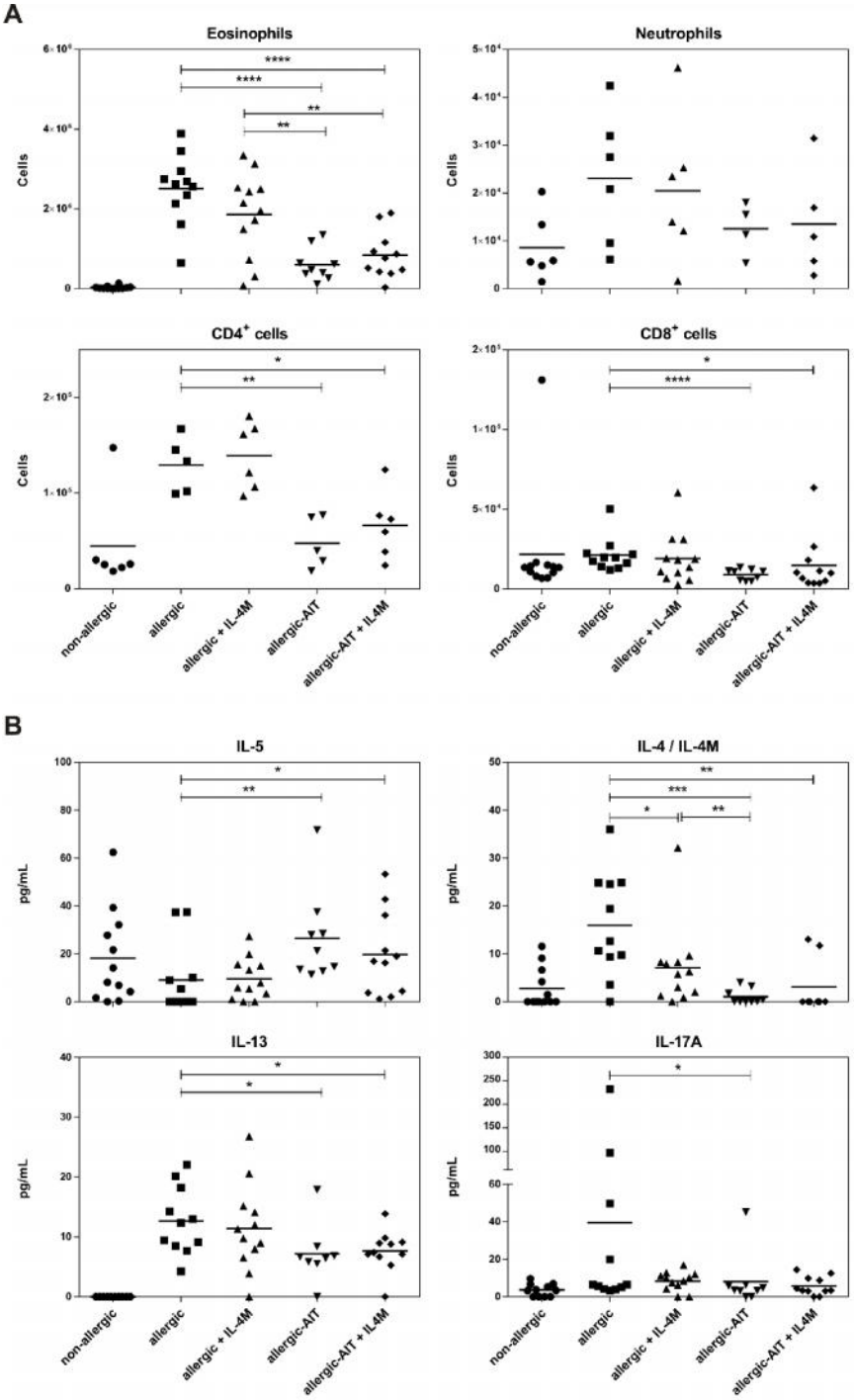
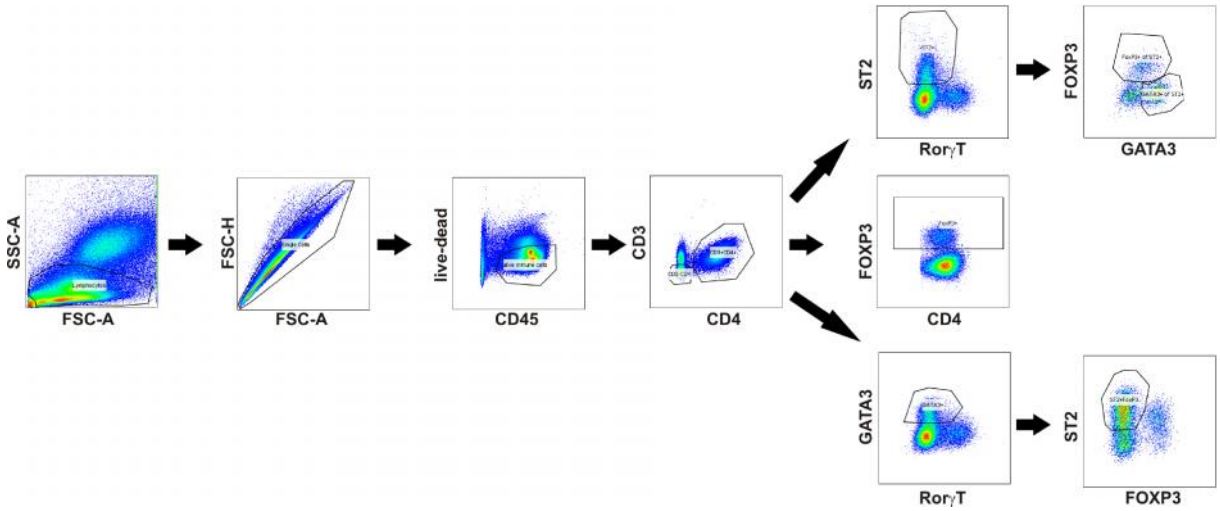


Figure S6: Gating strategy for the characterization of lung-resident lymphocyte populations. Shown is one representative gating.



Data S1: Supplemental methods

Cloning of IL-4M

The coding sequence of IL-4M attached to the serum albumin signal peptide and a 10-fold his tag was designed using Geneious8.1.5 (1). The construct was ordered with *Not*I and *Bam*HI restriction sites at GeneArt® Strings (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The expression vector pIRES-DHFR (vector system in which the gene of interest is coupled via an internal ribosomal entry site to the selection marker dihydrofolate reductase) (2) and the IL-4M construct were digested in a total reaction volume of 20 µL containing FastDigest Buffer green, DNAse free water, alkaline phosphatase and the restriction enzymes *Bam*HI and *Not*I (all Thermo Fisher Scientific). The digested DNA fragments were purified via gelelectrophoresis in a 1% agarose gel and the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) according to the protocol of the manufacturer. A vector to insert ratio of 1:3 was used to ligate both fragments for 30 min at 22 °C using T4 DNA Ligase (Thermo Fisher Scientific). The ligated plasmid was used to transform chemically competent NEB 5-alpha *E. coli* (Ipswich, Massachusetts, USA) by heat shock (42° C, 1 min). The GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) was used for plasmid purification from selected clones. The correct sequence of purified plasmids was verified by sequencing (GATC Biotech AG, Konstanz, Germany) using the primer pBR1 (GATC Biotech AG).

Recombinant production and purification of IL-4M

Dehydrofolate reductase (DHFR)-deficient Chinese hamster ovary cells (CHO) (ATCC CRL 9096) were transfected by electroporation with the pIRES-DHFR vector system containing the coding sequence of IL-4M and the siglal peptide of murine serum albumin coupled by an internal ribosomal entry site (IRES) to the gene coding for DHFR. In order to increase the IL-4M production, several rounds of genomic amplification by culturing the cells

with increasing concentrations of the DHFR inhibitor methotrexate (Mtx) (Sigma-Aldrich) were performed (2). For large scale production, CHO cells were cultured in OptiCHO medium (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 8% CO_2 in a humidified atmosphere in 1450 cm^2 roller bottles (Corning, Corning, NY, USA) until maximal density was reached. Cells were removed from the protein containing supernatant via centrifugation (20 min, 4 °C, 400 g). The supernatant was sterile filtered and purified using an ÄKTApure chromatography system (GE Healthcare Life Sciences, Freiburg, Germany): First, the supernatant was a nity-purified using an HisTrap Excel column (GE Healthcare Life Sciences). Subsequently, protein containing fractions were concentrated using an Amicon Ultra Centrifugal Filter Unit with a molecular weight cut-off of 3000 Da (Merck Millipore, Burlington, Massachusetts, USA) and applied to a HiLoad 16/600 Superdex 75pg column (GE Healthcare Life Sciences). IL-4M-containing fractions were identified via immunoblot and concentrated using Amicon Ultra Centrifugal Filter Units. Next, the protein-containing fluid was sterile filtered using 0.22 µm syringe filter units (Merck KGaA, Darmstadt, Germany), aliquoted and stored at -20 .

SDS-PAGE and Western Blot

The supernatant of IL-4M-producing cells was harvested via centrifugation. A 5x loading buffer (non-reducing) was added, proteins denatured for 10 min at 80 °C for 10 min, loaded on a 10% acrylamid gel and separated for 1.5 h at 150 V. Subsequently, the gel was either stained with a colloidal Coomassie solution or proteins transferred to a polyvinylidenfluorid (PVDF) membrane. In order to block unspecific binding sites, the membrane was incubated for 1 h at RT in 5% non-fat dry milk powder in PBS. Subsequently, the membrane was incubated at 4 °C over night with the primary antibody diluted in 2.5% non-fat dry milk powder in PBS. After washing, the membrane was incubated for 1 h at RT

with the alkaline phosphatase-coupled secondary antibody. After further washing steps, bound antibodies were visualized with a para-nitrophenylphosphate-containing substrate solution.

Analysis of the biological activity of IL-4M using CTLL2 cells

CTLL-2 cells (ATCC® TIB-214.) were cultured in the presence of IL-2 (20 ng/ml), 20.000 cells per well plated in flat bottom 96-well plates (Nunc delta surface, Sigma-Aldrich, Taufkirchen, Germany) and stimulated with 5 ng/ml IL-4 as well as different concentrations of IL-4M. After 48 h, the number of metabolically active cells was determined according to the manual of the CellTiter-Blue® Cell Viability Assay (Promega, Madison, Wisconsin, USA). Fluorescence intensity was measured at 560/590 nm.

In vitro T cell polarization assays

Flat bottom 96-well plates (Nunc delta surface, Sigma-Aldrich, Taufkirchen, Germany) were coated with anti-CD3 (BD Biosciences, San Jose, CA, USA) and anti-CD28 (BD Biosciences) antibodies overnight at 4 . Naive T cells from IL-4-EGFP reporter mice ($IL4^{tm1Lky}$) (3) or FoxP3-RFP reporter mice ($Foxp3^{tm1Flv}$) (4) were isolated from splenocytes according to the MACS manual of the Naive $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Living cells were counted using trypan blue staining in a haemacytometer and 200.000 cells plated per well. TH2 polarization of naive $CD4^+$ T cells was achieved by addition of 10 ng/mL IL-4 (PreproTech, Rocky Hill, NJ, USA) and 3.4 µg/mL anti-IFNγ (BioLegend, San Diego, CA, USA). Treg polarization of naive $CD4^+$ T cells was achieved by addition of 80 ng/mL IL-2 (ImmunoTools GmbH, 26169, Friesoythe, Germany), 2.4 ng/mL TGF-β (PreproTech) and 3.4 µg/mL anti-IFNγ (BioLegend). In the FoxP3 rescue assay, 800 ng IL-4M were used. Cells were cultured at 37 °C, 5% CO_2 and a relative humidity of 95% for five days and subsequently analyzed via FACS.

Restimulation of primary splenocytes with OVA

Splenocytes were plated out at a densitiy of $2x10^5$ cells per well in 200 µl RPMI (Thermo Fisher Scientific) in flat bottom 96-well plates (Sigma-Aldrich). For antigen-specific restimulation, a final concentration of 10 µg/mL OVA grade V (Sigma-Aldrich) was added to the splenocytes. Cells were cultured for 72 h at 37 °C, 5% CO_2 in a humidified atmosphere. After 72 h supernatants were collected and cytokine concentrations were quantified by ELISA.

Restimulation of lymphocytes with PMA/Ionomycin

Lymphocytes isolated from lung tissue were cultured in RPMI in round bottom 96 well plates (Sigma-Aldrich) at 37 °C, 5% CO_2 in a humidified atmosphere for 4 h in the presence of 1 µg Ionomycin (Sigma-Aldrich) and 20 ng phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). After 2 h of cultivation, 5 µg Brefeldin A (Biolegend) were added. Cells were cultured for another 2 h before they were surface-stained and fixed/permeabilized for intracellular staining.

Isolation of BAL cells and lung-resident lymphocytes

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 and BAL cells analyzed via FACS.

For isolation of tissue resident lymphocytes, lungs were removed, chopped into approximately 1 mm x 1 mm sized pieces and digested in 3 mL medium containing 1mg/mL collagenase (Collagenase IV from *Clostridium histolyticum*, Sigma-Aldrich) and 100 µg/mL DNAse (Deoxyribonuclease I from bovine pancreas, Sigma-Aldrich). Digestion was stopped by the addition of 15 µL 0.5 M EDTA (Sigma-Aldrich). Digested lungs were mashed trough a 70 µm cell strainer. Next, cells were pelleted (400 g, 4 °C, 5 min), resuspended in 5 mL 40%

percoll in RPMI (v/v) solution, underlayered with 5 mL 80% percoll solution and centrifuged (1600 g, RT, 15 min). Lymphocytes were collected from the interphase and washed with PBS.

Measurement of immunoglobulins and cytokines

Cytokine levels (IL-4, IL-5, IL-10, IL-13, IL-17A, IFNγ, IL-1β and IL-6) in the BAL fluid, culture supernatants and plasma were measured either by ELISA (BD Opt EIA sets, BD Biosciences) according to the recommendations of the manufacturer or by an electrochemiluminescence multiplex assay (Meso Scale Discovery, Rockville, MD USA).

The detection of immunoglobulins in the serum of mice from the two AIT experiments was performed using different ELISA kits: For the detection of tIgG1 plates were coated with clone A85-3 (BD.Pharmingen). Immunoglobulins were detected with biotinylated A85-1 (BD-Pharmingen). For quantification of tIgE plates were coated with clone R35-72 (BD-Pharmingen) and detected with clone R35-118 (BD-Pharmingen). Quantification of OVA-specific IgE, IgG2a and IgG2b was performed after coating with OVA and subsequent detection using following antibodies: 05775 for sIgE (Biozol), R19-15 for sIgG2a (BD-Pharmingen) and R12-3 for sIgG2b (BD-Pharmingen). Sera were diluted 1:10 with PBS for the measurement of tIgE and sIgE and 1:100.000 for the detection of sIgG1, sIgG2a and sIgG2b.

The determination of antibody levels in the murine model of allergic inflammation was performed as follows: For the measurement of tIgE, plasma samples and standards (mouse IgE clone C38-2; BD Pharmingen, Heidelberg, Germany) were transferred to microtiter plates, coated with 10 µg/mL monoclonal rat anti-mouse-IgE (IgG, clone-PC 284; The Binding Site, Schwetzingen, Germany). For OVA-specific IgE measurement, plasma samples and standards (mouse IgE anti-Ovalbumin clone: 2C6; Biozol, Eching, Germany) were transferred to microtiter plates coated with 2 µg/mL of OVA. As secondary antibody, 2.5 µg/mL of biotinylated monoclonal rat anti-mouse IgE (clone R35-118; BD Pharmingen)

were used, followed by incubation with BD OptEIA Reagent Set B (BD Pharmingen). Signal intensities were measured at 450 nm. The determination of further immunoglobulin isotype levels was performed with a combined electrochemiluminescence multiplexed assay system (Meso Scale Discovery).

Murine model of house dust mite (HDM)-induced allergic airway inflammation

Mice in the HDM-allergic group were sensitized on day 1 by intranasal (i.n.) administration of 1 µg HDM extract (Stallergenes Greer, London, UK). Mice were challenged on days 7, 8, 9, 10 and 11 with 10 µg HDM extract (i.n.). Mice from the non-allergic group were treated on day 1 with 10 µl PBS (i.n.) and on days 7, 8, 9, 10 and 11 with 10 µl PBS.

Flow Cytometry

Single cell suspensions of unfixed cells were prepared in FACS buffer (PBS, 1 % BSA). Thereafter, cells were pelleted (400 g, 5 min, 4 °C) and resuspended in Fc-block (anti-CD16/32) (Thermo Fisher Scientific) in order to prevent unspecific binding of immunoglobulins and incubated at 4 °C for 20 min. Next, cells were washed twice with FACS buffer and stained with 40 µL of a master mix of fluorochrome-conjugated antibodies (Tables S1 and S2). Cells were incubated in the dark for 30 min at 4 °C. After washing, cells were resuspended in 65 µL FACS buffer, filtered through a cell strainer and analyzed using a BD LSRFortessa™flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA). For intracellular staining of cytokines and transcription factors, a fixation/permeabilization kit was used (BD Biosciences). Cells were fixed in the dark at 4° C over night with 200 µL buffer after surface staining. Subsequently, cells were washed twice with 200 µL fixation/permeabilization buffer. Finally, cells were resuspended in PBS and analyzed via FACS. Data were acquired using a BD LSRFortessa™(BD Bioscience) and further analyzed with FACSDiva software (BD Biosciences) and FlowJo V.7.2.2 (Tree star, Ashland, USA).

Table S1Antibodies for FACS analysis of lymphocytes isolated from lung

MARKER	FLUOROCHROME	CLONE	MANUFACTURER
CD3ε	FITC	145-2C11	BDPharmingen
CD4	BV711	RM4-5	BioLegend
CD45	APCeF780	30-F11	eBiosciences
FoxP3	PerCP-Cy5.5	FJK-16s	eBiosciences
GATA3	eF660	TWAJ	eBiosciences
RorγT	PE	AFKJS-9	eBiosciences
ST2	BV421	DIH9	MDBioproducts
Live/dead aqua fixable cell stain	405nm	—	ThermoFisher

Table 2Antibodies 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

Histological analysis

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). Mucus hypersecretion and inflammatory cell infiltration were graded on a scale from 0 to 4 in a blind fashion as previously described (5).

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