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Dual vaccination against IL-4 and IL-13 protects against chronic allergic asthma in mice

Eva Conde[1,2,3,8], Romain Bertrand[3,8], Bianca Balbino[1,2], Jonathan Bonnefoy[3], Julien Stackowicz[1,2], Noémie Caillot[3], Fabien Colaone[3], Samir Hamdi[3], Raïssa Houmadi[4], Alexia Loste[4], Jasper B. J. Kamphuis[4], François Huetz[1], Laurent Guilleminault[4,5], Nicolas Gaudenzio[4], Aurélie Mougel[4], David Hardy[6], John N. Snouwaert[7], Beverly H. Koller[7], Vincent Serra[3], Pierre Bruhns[1,9], Géraldine Grouard-Vogel[3,9] & Laurent L. Reber[1,4,9]✉

Allergic asthma is characterized by elevated levels of IgE antibodies, type 2 cytokines such as interleukin-4 (IL-4) and IL-13, airway hyperresponsiveness (AHR), mucus hypersecretion and eosinophilia. Approved therapeutic monoclonal antibodies targeting IgE or IL-4/IL-13 reduce asthma symptoms but require costly lifelong administrations. Here, we develop conjugate vaccines against mouse IL-4 and IL-13, and demonstrate their prophylactic and therapeutic efficacy in reducing IgE levels, AHR, eosinophilia and mucus production in mouse models of asthma analyzed up to 15 weeks after initial vaccination. More importantly, we also test similar vaccines specific for human IL-4/IL-13 in mice expressing human IL-4/IL-13 and the related receptor, IL-4Rα, to find efficient neutralization of both cytokines and reduced IgE levels for at least 11 weeks post-vaccination. Our results imply that dual IL-4/IL-13 vaccination may represent a cost-effective, long-term therapeutic strategy for the treatment of allergic asthma as demonstrated in mouse models, although additional studies are warranted to assess its safety and feasibility.

[1] Unit of Antibodies in Therapy and Pathology, Institut Pasteur, UMR 1222 INSERM, Paris, France. [2] Sorbonne University, ED394, Paris, France. [3] Neovacs SA, Paris, France. [4] Toulouse Institute for Infectious and Inflammatory Diseases (Infinity), INSERM UMR1291, CNRS UMR5051, University Toulouse III, Toulouse, France. [5] Department of respiratory medicine, Toulouse University Hospital, Faculty of Medicine, Toulouse, France. [6] Institut Pasteur, Experimental Neuropathology Unit, Paris, France. [7] Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. [8] These authors contributed equally: Eva Conde, Romain Bertrand. [9] These authors jointly supervised this work: Pierre Bruhns, Géraldine Grouard-Vogel, Laurent L. Reber. ✉email: laurent.reber@inserm.fr

Asthma is the most common chronic lung disease, affecting >300 million people worldwide, and with at least 250,000 deaths attributed to the disease each year[1]. An estimate of 20% of asthma patients suffer from uncontrolled, moderate-to-severe asthma[2], presenting with persistent symptoms, with reduced lung functions and recurrent exacerbations, despite the use of high-dose pharmacological therapy[3]. The heterogeneity of asthma phenotypes represents a challenge for adequate assessment and treatment of the disease[4]. However, type 2 inflammation characterized by production of interleukin-4 (IL-4) and IL-13 in the lung, airway eosinophilia, and high levels of IgE antibodies occurs in ~50% of patients with asthma[1,5].

Even though IL-4 and IL-13 present similar structures and share one receptor subunit (IL-4Rα)[6], IL-4 and IL-13 are also thought to have some nonredundant functions in allergy and asthma[7]. In particular, IL-4 is considered to act predominantly in the early phase of asthma development through its role in regulating T cell proliferation and survival, and IgE synthesis[6]. In contrast, IL-13 would predominantly be involved in late phases of allergic reactions, such as airway remodeling and mucus hypersecretion[6].

Phase 3 studies indicated that dupilumab—a monoclonal antibody (mAb) against IL-4Rα that blocks both IL-4 and IL-13 signaling[8]—is efficient at decreasing the rate of severe exacerbations, and at improving lung function in patients with moderate-to-severe asthma[9]. Dupilumab was approved in 2018 as an add-on maintenance treatment in moderate-to-severe asthma with type 2 inflammation. However, use of this (or any other) mAb in chronic asthma is limited by high cost and the need to perform injections over years to lifelong. Therefore, while IL-4 and IL-13 are now clinically validated therapeutic targets for the treatment of asthma, there is a clear need to improve current strategies, with the goal of reaching long-term cost-effective therapeutic effects.

Conjugate vaccines called kinoids can elicit an endogenous, long-lasting neutralizing antibody response against a given cytokine[10], and could be a favorable alternative to therapeutic mAb administration. Vaccination against mouse IL-4 partially reduced IgE levels and eosinophilia with minor effects on mucus hypersecretion in a mouse asthma model[11]. A recombinant mouse IL-13 peptide-based virus-like particle vaccine had significant effects on mucus production without, however, affecting IgE levels[12]. Based on these partial results, and on the superior clinical efficacy in human asthma of targeting both IL-4 and IL-13 signaling (i.e., dupilumab) rather than targeting either IL-4 or IL-13 alone[13–15], we hypothesized that a dual vaccination against IL-4 and IL-13 would be particularly potent at reducing the severity of chronic asthma.

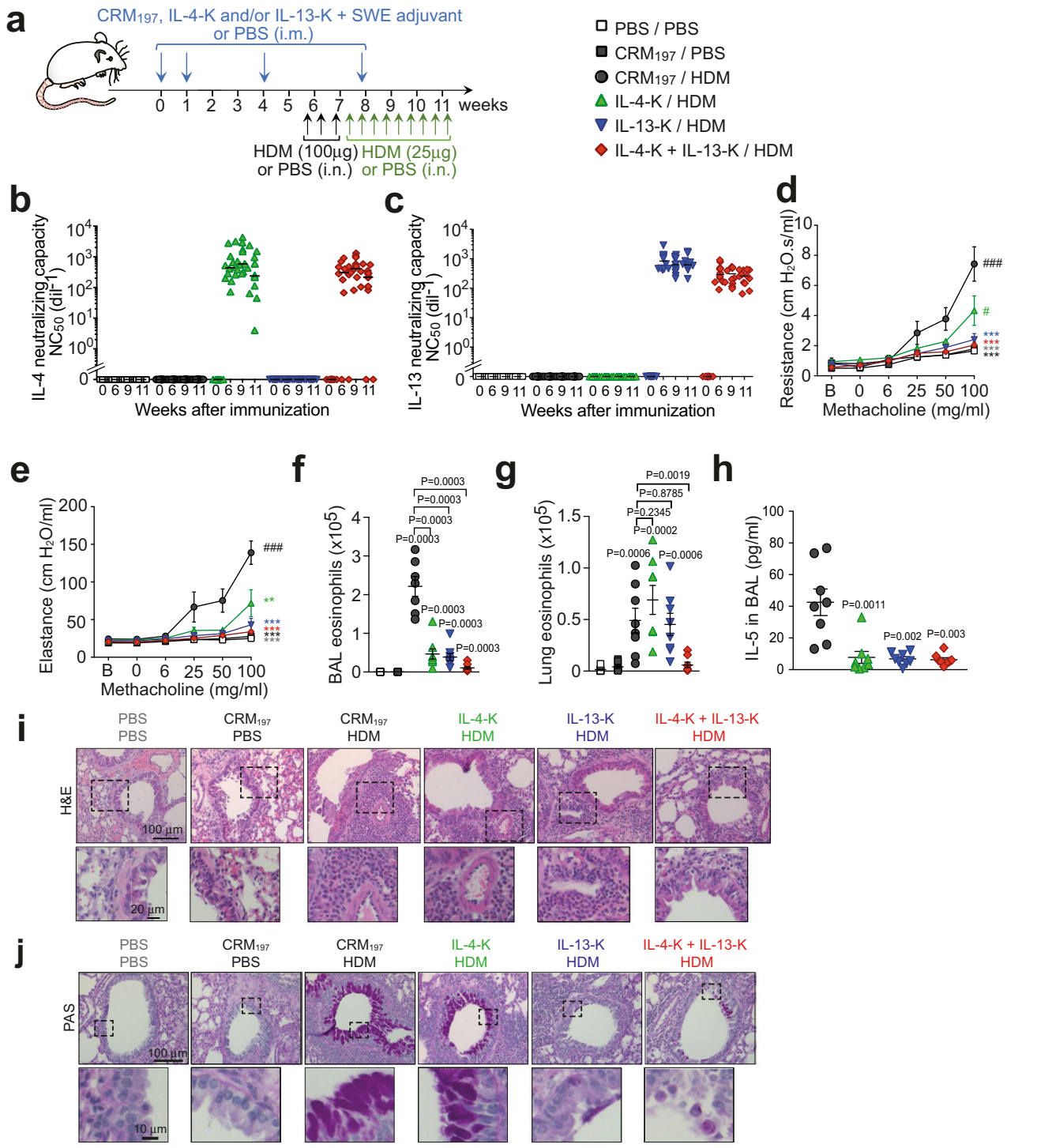
Here, we design conjugate vaccines against IL-4 and IL-13 rather than IL-4Rα to minimize the risk that these vaccines may induce antibodies capable of activating this receptor or inducing antibody-dependent cellular toxicity. We show that prophylactic and therapeutic dual vaccination against mouse IL-4 and IL-13 reduces key features of chronic allergic asthma in mice. We also demonstrate the immunogenicity of a vaccine targeting human IL-4/IL-13 in a novel mouse strain humanized for IL-4, IL-13, and IL-4Rα. Overall, our results suggest that dual IL-4/IL-13 vaccination is a promising long-term therapeutic strategy for allergic asthma, pending further safety and feasibility assessment in additional preclinical models.

Results

Anti-mouse IL-4 and IL-13 kinoids induce potent and long-lasting neutralizing responses. We developed mouse IL-4 and IL-13 kinoids (IL-4-K and IL-13-K), by coupling these cytokines with diphtheria "cross-reactive material 197" (CRM_{197}, a nontoxic mutant of diphtheria toxin used as a carrier protein in a number of approved conjugate vaccines[16]) using a thiol-maleimide conjugation (Supplementary Figs. 1 and 2). Mice were immunized intramuscularly with IL-4-K and IL-13-K alone or in combination (or the carrier protein CRM_{197} alone as a control), combined 1:1 (v:v) with SWE, a squalene oil-in-water emulsion adjuvant[17] (Fig. 1a). We did not observe visible adverse effects of the vaccines, as mice had normal behavior, and vaccination with kinoids had no effect on body weight (Supplementary Fig. 3). Immunization with IL-4-K and/or IL-13-K induced high anti-IL-4 and/or anti-IL-13 antibody titers, respectively, detectable already 6 weeks after primary immunization (Supplementary Fig. 4a, b). As expected, all mice exposed to CRM_{197} or kinoids developed anti-CRM_{197} antibodies (Supplementary Fig. 4c). Importantly, anti-cytokine antibodies generated upon vaccination with the kinoids exhibited strong neutralizing capacities against the respective cytokine in >90% of mice starting 6 weeks after primary immunization (Fig. 1b, c). Such neutralizing capacity could still be detected in >60% of the mice over 1 year after primary immunization (Supplementary Fig. 5). Importantly, conjugation between cytokine and carrier protein was required for potent antibody responses and for neutralizing activity (Supplementary Fig. 6). Altogether, these data indicate that efficient long-term neutralization of both IL-4 and IL-13 can be achieved through vaccination with kinoids.

Combined vaccination against IL-4 and IL-13 protects against chronic asthma in mice. We then tested the prophylactic efficacy of these vaccines in a chronic asthma model. Mice received a total of 12 intranasal (i.n.) administrations of *Dermatophagoides farinae* house dust mite (HDM) extract (one of the major allergens in human asthma[18]) over a period of 6 weeks, a protocol known to reproduce key features of human chronic asthma[19] (Fig. 1a). We assessed airway responses in this asthma model using invasive plethysmography[20]. HDM-treated control mice exhibited marked increase in lung resistance and elastance upon exposure to aerosolized methacholine (a bronchoconstrictor used as part of the diagnostic of asthma in human[21]), as compared to PBS-treated control mice (Fig. 1d, e). These two features were significantly reduced in mice vaccinated with the IL-4-K, and absent in mice vaccinated with the IL-13-K or the combination of IL-4-K and IL-13-K (Fig. 1d, e). The superior benefit of the dual IL-4/IL-13 vaccine on airway hyperresponsiveness (AHR) was even more apparent when using noninvasive whole-body plethysmography[22] (Supplementary Fig. 7). Our data suggest that AHR may be more dependent on IL-13 than IL-4 in this chronic asthma model, and can be prevented upon prophylactic dual vaccination against IL-4 and IL-13.

Next, we assessed the effect of the kinoids on airway eosinophilia and inflammation. HDM-treated mice demonstrated elevated eosinophil numbers in bronchoalveolar lavage (BAL) fluid that were reduced fivefold and sixfold in mice vaccinated with IL-4-K or IL-13-K, respectively, and reduced even further (21-fold) upon dual vaccination (Fig. 1f). The benefit of the dual vaccination was even more evident when assessing eosinophil numbers that reduced eightfold in lung tissue, whereas single vaccination with IL-4-K or IL-13-K alone had no effect on eosinophil numbers (Fig. 1g). Consistent with these results, levels of IL-5—a type 2 cytokine which plays a key role in eosinophilic inflammation[5]—were also markedly reduced in BAL fluid of HDM-treated mice vaccinated with IL-4-K and/or IL-13-K (Fig. 1h). By contrast, we observed similar low levels of $IL\text{-}4^+$ and $IL\text{-}13^+$ $CD4^+$ T cells (Supplementary Fig. 8a, b), as well as similar numbers of $IL\text{-}4^+$ type 2 innate lymphoid cells (ILC2) (Supplementary Fig. 8c, d) in the lungs of HDM-treated controls



and vaccinated mice. Altogether, these results suggest that vaccination with IL-4-K or IL-13-K does not abrogate IL-4 and IL-13 production in this asthma model, but rather induces antibodies that neutralize these cytokines once released.

Neither single nor dual kinoid vaccination, however, altered eosinophil levels in circulation (Supplementary Fig. 9a), indicating that the reduced airway eosinophilia observed after dual vaccination was a consequence of reduced eosinophil recruitment to the lungs rather than systemic effects on eosinophil numbers or progenitors. In addition, while numbers of effector eosinophils were markedly reduced in the lungs of vaccinated mice (Fig. 1f, g), we observed no effect of the vaccine on the number of tissue-resident regulatory eosinophils[23] (Supplementary Fig. 9b, c). The effect of the vaccine on leukocytes appeared to be mostly restricted to effector eosinophils and to a lesser extent B cells, as we observed similar levels of neutrophils, macrophages, and T cells in the BAL fluid of HDM-treated mice vaccinated with IL-4-K and IL-13-K or CRM_{197} as a control (Supplementary Fig. 10).

This HDM-induced asthma model also leads to pronounced peribronchiolar inflammation and mucus production[19]. Single or dual kinoid vaccination reduced peribronchiolar inflammation to a similar extent (Fig. 1i and scoring in Supplementary Fig. 11a), whereas IL-13 vaccination but not IL-4 vaccination was necessary to profoundly reduce mucus production (Fig. 1j and scoring in

Fig. 1 Dual vaccination with IL-4-K and IL-13-K reduces features of chronic asthma. a Protocol outline. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls), combined with the adjuvant SWE. At day 39, mice were sensitized and challenged with HDM or PBS, as indicated. **b**, **c** Anti-IL-4 (**b**) and anti-IL-13 (**c**) neutralizing capacity in sera collected at the indicated time points. Data show values from individual mice ($n = 12$/group) with bars indicating median, from a single experiment representative of three independent experiments. **d**, **e** Lung resistance (**d**) and elastance (**e**) in response to inhaled methacholine 24 h after the last HDM challenge. Data represent mean ± SEM from two independent experiments ($n = 8$ mice in IL-4-K + IL-13-K/HDM group; $n = 9$ mice in PBS/PBS and CRM_{197}/HDM groups; or $n = 10$ mice in CRM_{197}/PBS, IL-4-K/HDM, and IL-13-K/HDM groups). **f**, **g** Numbers of eosinophils in BAL fluid (**f**) and lung tissue (**g**) 24 h after the last HDM challenge. Data show values from individual mice ($n = 7$ mice for HDM/CRM_{197} group and $n = 8$ mice for all other groups) with bars indicating mean ± SEM, from a single experiment representative of two independent experiments. **h** Levels of IL-5 in BAL fluid 24 h after the last challenge. Data show values from individual mice ($n = 8$/group pooled from two independent experiments) with bars indicating mean ± SEM. **i**, **j** Representative lung sections stained with hematoxylin and eosin (H&E; revealing leukocyte infiltration) (**i**), or periodic acid–Schiff (PAS; revealing mucus-producing goblet cells in dark purple) (**j**). Lower panels in **i** and **j** represent magnifications of the dashed areas. Lung sections are representative of each group ($n = 8$ mice/group). *P* values in **d** and **e** were calculated using two-way ANOVA followed by a Tukey posttest. **d**: ### < 0.0001, # = 0.0381, ***: 0.003 for IL-13-K/HDM, 0.0002 for IL-4-K + IL-13-K/HDM, <0.0001 for CRM_{197}/PBS and PBS/PBS; **e**: ### < 0.0001, ** = 0.0019, ***: <0.0001 for all groups. *P* values in **f**–**h** were calculated using two-tailed Mann–Whitney *U* test (**f**–**h**) vs. CRM_{197}/PBS (**f**, **g**), CRM_{197}/HDM group (**h**), or indicated groups. Source data are provided in the Source data file.

Supplementary Fig. 11b), confirming the key role of IL-13 in mucus hypersecretion[6].

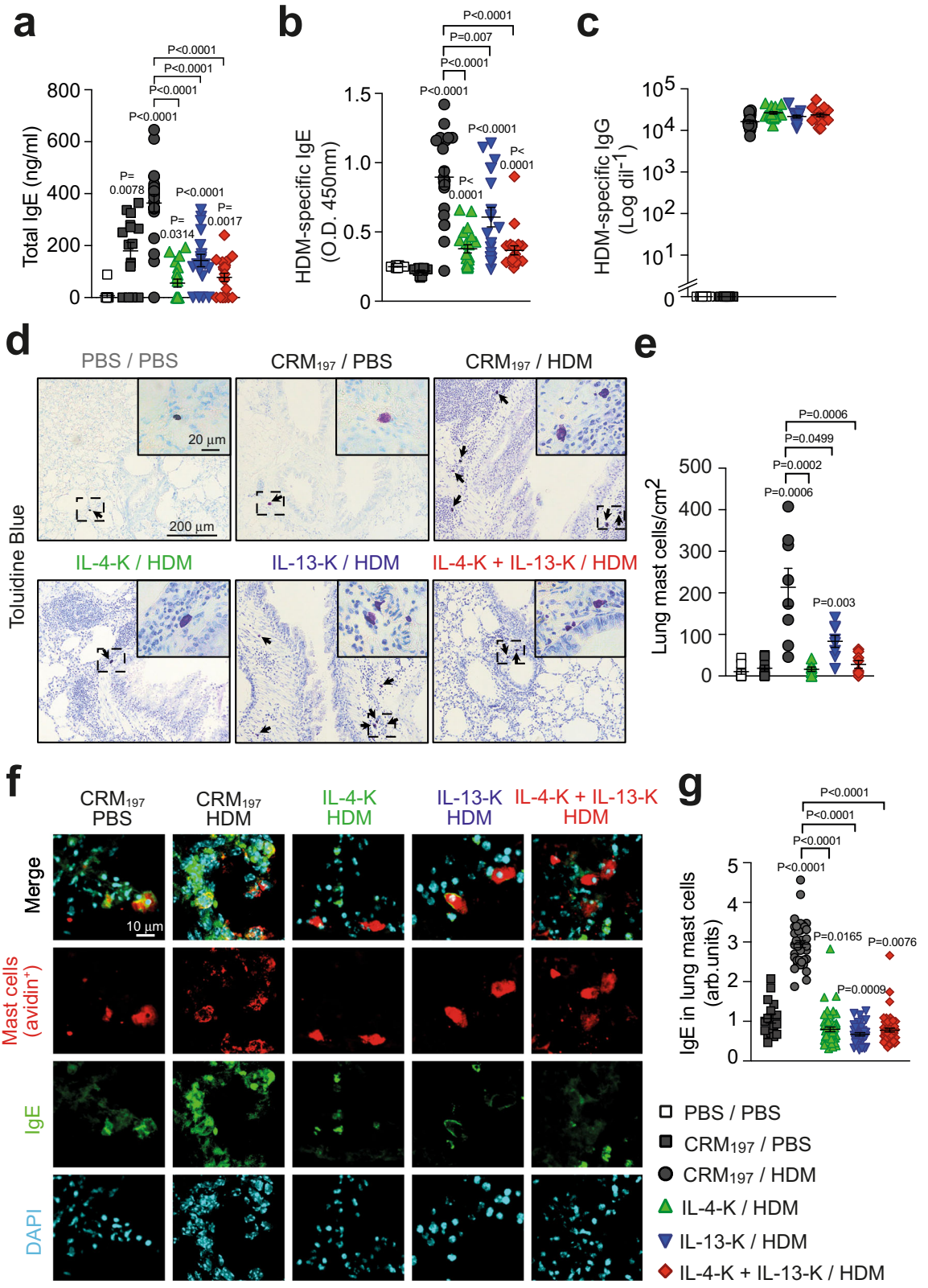
Dual vaccination against IL-4 and IL-13 reduces IgE and mast cell numbers. As IgE antibodies play an important role in allergic asthma[24,25], we next assessed the effects of IL-4-K and IL-13-K on IgE levels in the HDM-induced asthma model. Compared to PBS-treated (naive) mice, control immunization of CRM_{197} in squalene-based adjuvant led to low, but detectable IgE levels in circulation but not, as expected, to detectable HDM-specific IgE (Fig. 2a, b). HDM-treated mice had higher total IgE and significant HDM-specific IgE levels in circulation. Single IL-4 and dual kinoid vaccination markedly reduced total and HDM-specific IgE levels, with more pronounced effects than anti-IL-13 vaccination (Fig. 2a, b), highlighting the prominent role of IL-4 in IgE production[6]. Noteworthy, HDM-treated mice also had elevated levels of HDM-specific IgG antibodies that were not affected by single or dual kinoid vaccination (Fig. 2c and Supplementary Fig. 12).

Mast cells are the main IgE effector cells in the lung[24]. In patients with allergic asthma, inhalation of an aeroallergen leads to crosslinking of membrane-bound allergen-specific IgE, inducing rapid release of mast cell mediators, such as histamine and tryptase[24]. As expected, the chronic i.n. exposure to HDM of the asthma model we use herein resulted in a marked increase in the numbers of lung mast cells, as compared to PBS-treated animals (Fig. 2d, e)[19]. This mast cell recruitment was abolished following IL-4-K or dual kinoid vaccination, and reduced ~2.5-fold following IL-13-K vaccination (Fig. 2d, e). Importantly, in addition to restoring basal numbers of mast cells in the lungs of HDM-treated mice, kinoid vaccination also markedly reduced IgE levels on mast cells (Fig. 2f, g), indicating that both vaccines induce successful mast cell "desensitization". This vaccination-induced desensitization was observed at the systemic level, as membrane-bound IgE levels were also markedly reduced on blood basophils (which also express the high-affinity IgE receptor FcεRI) and peritoneal mast cells upon vaccination with kinoids (Supplementary Fig. 13a, b).

Since dual IL-4-K/IL-13-K prophylactic vaccination prevented or strongly reduced all key features of HDM-induced asthma in mice, whereas single IL-4-K or IL-13-K vaccination affected only a subset of these features (Figs. 1 and 2), we assessed the efficacy of therapeutic vaccination only using dual IL-4-K/IL-13-K vaccination on mice with established asthma (Fig. 3a and Supplementary Fig. 14). Mice were preexposed to HDM for 3 weeks before the first injection of kinoids, and remained exposed to HDM once a week for a total of 15 weeks thereafter (Fig. 3a). Dual vaccination induced high levels of neutralizing antibodies against both IL-4 and IL-13 whether mice were preexposed or not to HDM (Fig. 3b, c and Supplementary Fig. 15), suggestive of potential efficacy in a therapeutic setting. Indeed, dual therapeutic vaccination demonstrated a profound reduction in key features of asthma, including a ~2-fold reduction of total and HDM-specific IgE levels (Fig. 3d, e), of AHR to inhaled methacholine (Fig. 3f, g), of airway eosinophilia (Fig. 3h), and a ~6-fold reduction in mucus production (Fig. 3i, j).

A human IL-4/IL-13 vaccine induces neutralizing responses in humanized mice. Low interspecies similarity of IL-4 (~44%) and IL-13 (~55%) between mice and human would render mouse IL-4-K and IL-13-K highly immunogenic in humans, and less potent to generate neutralizing responses. We therefore developed and characterized kinoids eliciting an immune response against human IL-4 and IL-13 (hIL-4-K and hIL-13-K; Supplementary Fig. 16), and used mice humanized for IL-4, IL-13, and for their common receptor chain IL-4Rα (hIL-4/hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice) by syntenic replacement of two mouse loci: that encoding *Il4/Il13* and the second encoding *Il4ra*, with the corresponding human segment of DNA. These hIL-4/hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice express the human genes in the place of the mouse gene, and thus cytokine receptor interactions in these animals model those of the human proteins (Supplementary Fig. 17). We confirmed that splenocytes from hIL-4/hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice, but not from WT mice, release human IL-4 upon stimulation with PMA and ionomycin (Fig. 4a). We obtained similar results with human IL-13, and further showed that significant levels of human IL-13 can also be detected in BAL fluid from hIL-4/hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice following chronic i.n. sensitization and challenge (Fig. 4b, c). We further confirmed expression of human IL-4Rα (and lack of expression of mouse IL-4Rα) by immunohistology in skin samples from hIL-4/hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice (Fig. 4d, e). Finally, we showed that i.n. challenge with recombinant human IL-4 or human IL-13 leads to eosinophilia, lung inflammation, and mucus production in hIL-4/hIL-13^{KI}; hIL-$4R\alpha^{KI}$ mice (Fig. 4f–j). Altogether, these data demonstrate that this new hIL-4/hIL-13^{KI}; hIL-$4R\alpha^{KI}$ humanized mouse strain both produces and responds to hIL-4 and hIL-13.

Dual hIL-4-K/hIL-13-K vaccination induced neutralizing responses against both human IL-4 and IL-13 in all immunized mice (Fig. 5a–c and Supplementary Fig. 18). Confirming the efficacy of the human dual vaccine, mice showed a >2.5-fold reduction in circulating IgE readily detectable 5 weeks post primary vaccination (Fig. 5d), as well as a decrease in membrane-bound IgE on basophils (Fig. 5e). Mast cell number and IgE level were very low in the lungs of these mice that had not been previously exposed to allergens. However, we could efficiently



detect IgE in most skin mast cells from control hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice, and such IgE levels were reduced ~2.5-fold upon dual vaccination, with hIL-4-K and hIL-13-K (Fig. 5f, g).

Discussion

Recent clinical data highlight the fact that IL-4 and IL-13 are important therapeutic targets in asthma[9]. However, targeting these cytokines or their receptors through the use of therapeutic mAbs is associated with high costs, and the need to perform frequent reinfusions in order to maintain clinical effects. Our current study provides a proof-of-concept that long-term neutralization of IL-4 and IL-13 can be achieved through vaccination with kinoids, which thus could represent a cost-effective alternative to therapeutic mAbs. We demonstrate that vaccination

Fig. 2 Dual prophylactic vaccination with IL-4-K and IL-13-K prevents elevated IgE levels and lung mast cell numbers after HDM challenges. a–c Levels of total IgE (**a**), HDM-specific IgE (**b**), and HDM-specific IgG (**c**) 24 h after the last HDM challenge. Results show values from individual mice with bars indicating mean ± SEM from $n = 12$ mice (PBS/PBS group), $n = 16$ mice (CRM_{197}/PBS group), $n = 19$ mice (IL-13-K/HDM group), or $n = 20$ mice (IL-4-K and IL-4-K + IL-13-K groups) pooled from two independent experiments. **d** Representative lung sections stained with toluidine blue, demonstrating mast cells (arrows) 24 h after the last HDM challenge. Insert represent magnifications of the dashed areas. **e** Quantification of toluidine blue-positive lung mast cells. Results show values from individual mice with bars indicating mean ± SEM from $n = 8$ mice. **f** Representative lung sections stained with avidin (which stains mast cells, red), anti-IgE (green), and DAPI (blue) 24 h after the last HDM challenge. **g** Quantification of IgE levels in avidin-positive lung mast cells. Results show values from individual avidin-positive mast cells analyzed in ear skin sections from $n = 4$ mice/group with bars indicating means ± SEMs. *P* values were calculated using two-tailed Mann–Whitney *U* test vs. PBS/PBS group (in **a**), CRM_{197}/PBS group (in **b**, **e**, and **g**) or vs. indicated groups. Source data are provided in the Source data file.

against IL-4 and IL-13 is well tolerated and protects against key features of chronic asthma in mice, including AHR, eosinophilia, and mucus overproduction, after both prophylactic or therapeutic vaccination protocols.

We observed different effects of prophylactic vaccination against IL-4 or IL-13 on asthma features, highlighting the fact that IL-4 and IL-13 can have important nonoverlapping functions in asthma[15]. In particular, vaccination against IL-4 had more pronounced effects on IgE levels and lung mast cell numbers than vaccination against IL-13. By contrast, AHR and mucus overproduction were reduced to a greater extent by the IL-13 vaccine. These results are in full agreement with previous observations in mice lacking IL-4 or IL-13[6,26–28]. Such nonoverlapping functions could be explained, at least in part, by the different receptor requirement for the two cytokines. The type 1 IL-4 receptor only recognizes IL-4, and is a heterodimer of IL-4Rα and the common gamma chain (γc)[6,29,30]. The type 2 IL-4 receptor binds both IL-4 and IL-13, and is a heterodimer of IL-4Rα and IL-13Rα1[6,29,30]. In addition, IL-13 (but not IL-4) also binds IL-13Rα2, which was long thought to function as a decoy receptor that limits the activity of IL-13[6,29,30]. However, evidence indicates that IL-13 signaling through IL-13Rα2 can induce production of TGF-β1[31].

In line with our data in mice, IL-13 also plays an important role in controlling airway reactivity in human asthma. Indeed, an increase in FEV1 (forced expiratory volume in 1 s) has been observed in clinical trials conducted with anti-IL-13 mAbs[14,32,33], especially when focusing on patients with biomarker evidence of type 2 asthma (e.g., high blood eosinophil counts or periostin concentrations, which is induced by IL-4 and IL-13, ref. [34]). However, LAVOLTA and STRATOS, two large clinical trials, failed to demonstrate an effect of lebrikizumab and tralokinumab (two anti-IL-13 mAbs) on asthma exacerbation[14,35]. Thus, we speculate that combined blockade of IL-4 and IL-13 pathways is probably also required in order to efficiently reduce most features of asthma in human, as we observed here in mouse models with the IL-4 and IL-13 vaccines. This provides a potential explanation for the superior clinical efficiency of dupilumab (which blocks both IL-4 and IL-13 signaling) over various therapeutic anti-IL-4 or IL-13 mAbs in asthma[13–15], and prompted us to focus on the dual vaccine for further evaluation in a therapeutic protocol.

The extent to which the protective effects of anti-IL-4 and IL-13 therapy in asthma reflects local blockade of the cytokines in the lung vs. systemic effects is still not fully understood. Interestingly, local delivery of an anti-IL-13 Fab fragment by nebulization has been tested in a model of allergic asthma in cynomolgus monkeys[36]. This Fab had moderate effects on BAL eosinophilia, but markedly reduced BAL IL-5 levels, which is in full agreement with our data obtained in HDM-exposed mice vaccinated with the IL-13-K. However, even though the anti-IL-13 Fab was delivered by nebulization, significant levels of the antibody fragment could be detected in circulation. By extension one may consider that systemic effects of the anti-IL-13-K therapy are conceivable, even if we did not detect any significant systemic effects.

Levels of IL-4 and IL-13 neutralization obtained upon vaccination with kinoids will likely never reach levels observed directly after injection of high dose of a therapeutic mAb in human, or upon genetic ablation of IL-4 or IL-13 in mice. This was apparent in the therapeutic vaccination protocol in which IgE levels were reduced, but still detectable, in all vaccinated mice. Besides IgE, mice which fully lack IL-4 or IL-4Rα have markedly reduced IgG1 levels[37]. In addition, treatment of mice humanized for IL-4 and IL-4Rα with dupilumab also leads to important decrease in IgG1[38]. However, we found no difference in HDM-specific IgG1 levels between mice vaccinated with IL-4-K and IL-13-K or CRM_{197} alone. This suggests that residual cytokine activity in mice vaccinated with kinoids might sustain IgG production, while reducing the pathogenic functions of IL-4 and IL-13 in asthma. STAT6 activation is a key step in IL-4/IL-13 signaling[6]. However, it is important to note that STAT6 can also be activated by other mediators, including TSLP[39] or cleaved fibrinogen[40]. This could also contribute to the residual allergic inflammation, IgE and IgG1 levels observed in mice vaccinated with IL-4-K/IL-13-K.

We also provided a proof-of-concept of the efficiency of kinoids targeting human IL-4 and IL-13 in mice humanized for these cytokines and their receptor IL-4Rα. These promising results will now need to be confirmed in clinical studies. In this regard, while IL-4 and IL-13 vaccines have never been tested in human, a kinoid targeting interferon alpha (IFN-α) has recently been tested in a phase 2b study in 185 adults with active systemic lupus erythematosus[41]. This IFN-α kinoid induced a neutralizing response against IFN-α in 91% of treated patients, with an acceptable safety profile[41]. In a follow-up study of the phases I/II trial, it was noted that most patients had a ≥10-fold reduction in anti-IFN-α antibody levels 1 year after the first injection of the vaccine[42]. Neutralizing antibodies were still detectable at last follow-up visit in 29% (6/21) patients who received the IFN-α kinoid (range of persistence: 0.45–4.2 years). Although the strength and duration of the antibody response induced by the human IL-4/13 kinoids will need to be determined in clinical trials, they may be similar to the responses observed with the IFN-α kinoids. In our preclinical models, we observed 60% persistence 1 year after primary immunization.

Besides their detrimental role in allergies, IL-4 and IL-13 also play important protective and immunoregulatory functions. In particular, these cytokines can induce host defense responses against helminths infections, and have been implicated in the promotion of anti-inflammatory and tissue repair phenotypes in macrophages[43–45]. Thus, even though we did not observe apparent side effects of the vaccines in a 1-year follow-up study in mice, further work is now required to evaluate whether residual IL-4 and IL-13 activity after vaccination with kinoids is sufficient to sustain protective type 2 immune responses. In particular, it will be important to assess whether separate or combined

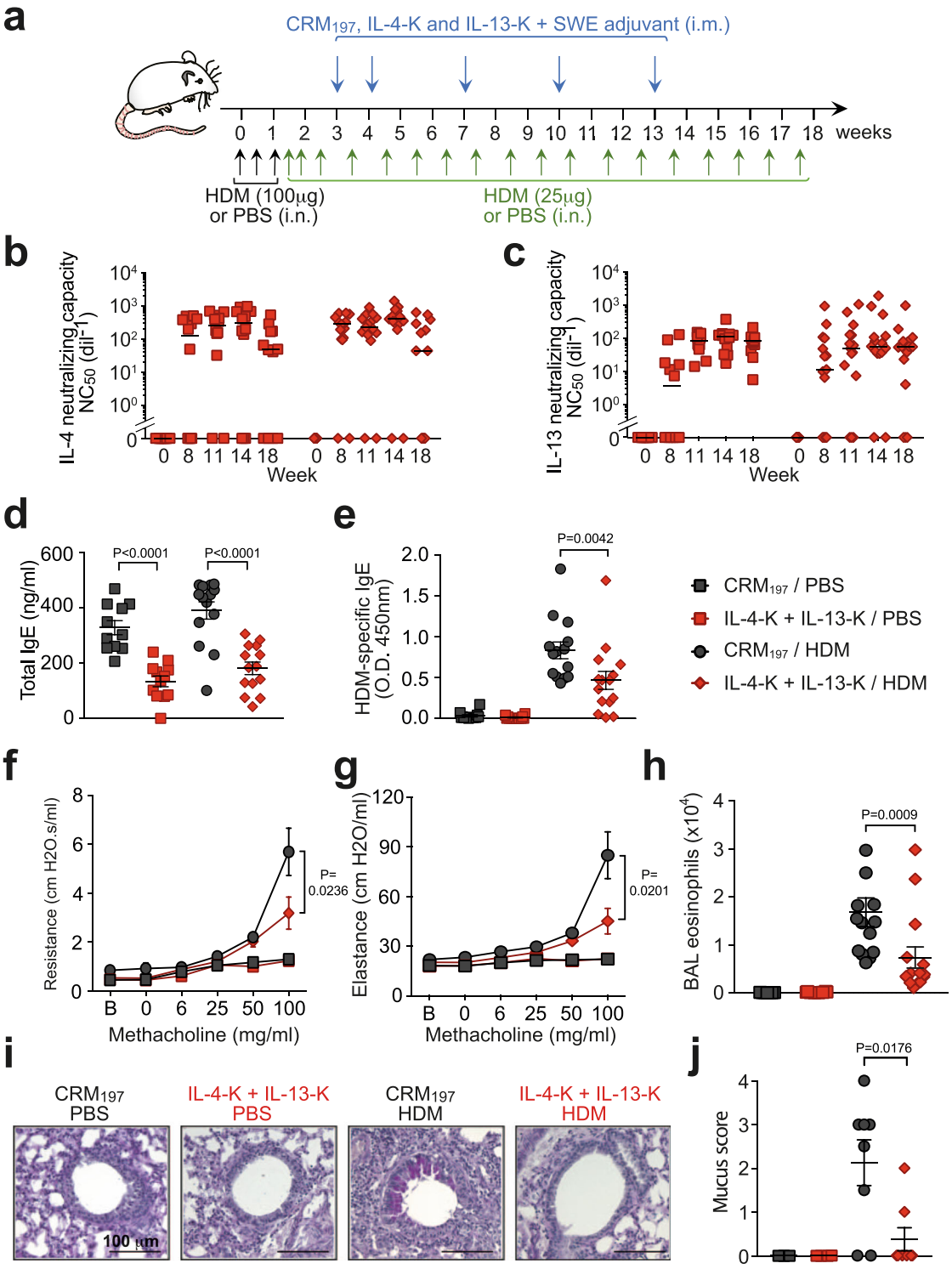
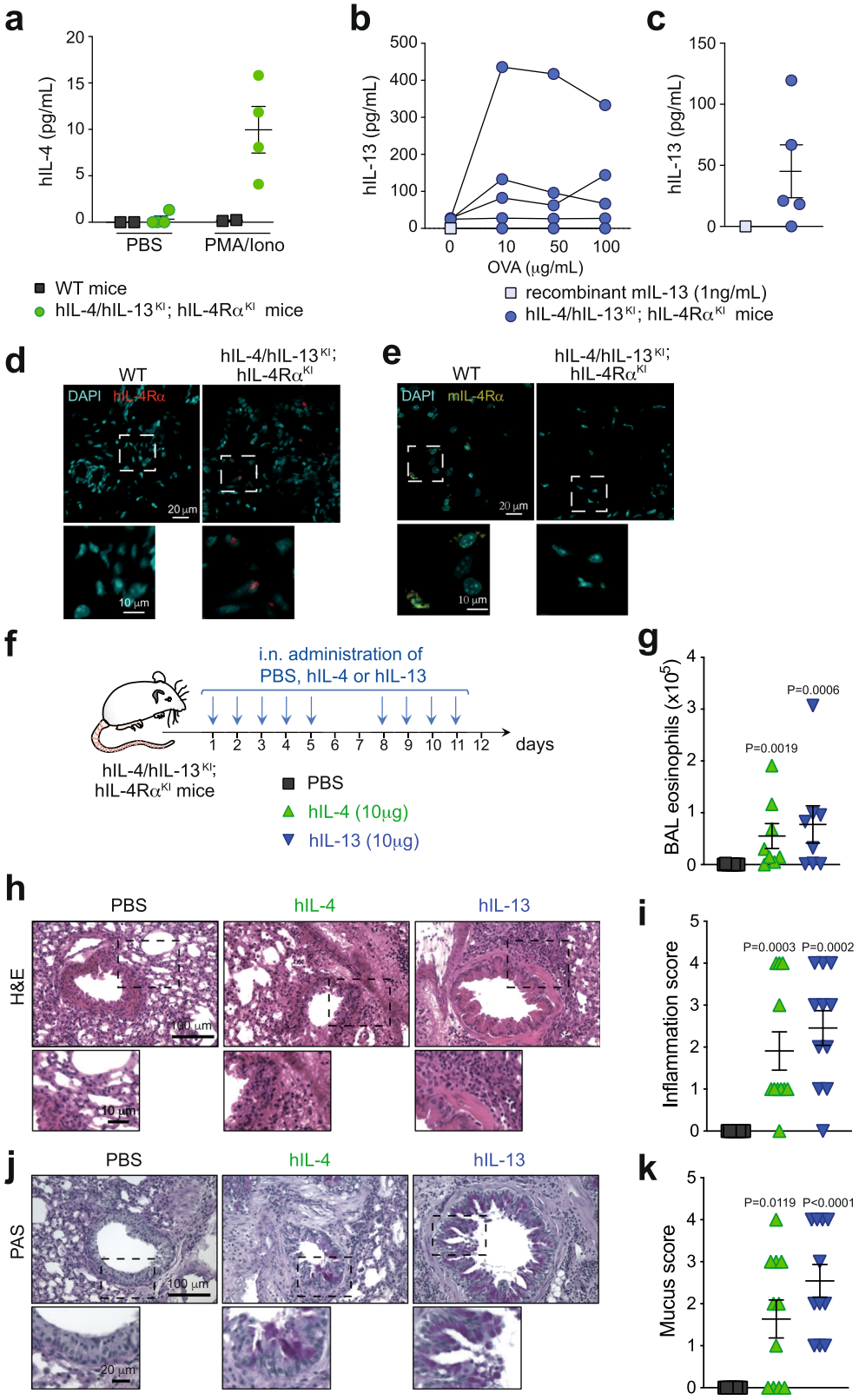


Fig. 3 Dual therapeutic vaccination with IL-4-K and IL-13-K ameliorates chronic asthma. a Protocol outline. Mice were sensitized and challenged with HDM extract (or PBS as a control), as indicated. After the third challenge, mice were vaccinated with IL-4-K and IL-13-K (or CRM_{197} as control), combined with the adjuvant SWE. **b**, **c** Anti-IL-4 (**b**) and anti-IL-13 (**c**) neutralizing capacity in sera collected at the indicated time points. **d**, **e**. Levels of total IgE (**d**) and HDM-specific IgE (**e**) 24 h after the last HDM challenge. **f**, **g** Lung resistance (**f**) and elastance (**g**) in response to inhaled methacholine 24 h after the last HDM challenge. **h** Eosinophil numbers in BAL fluid 24 h after the last HDM challenge. **i** Representative periodic acid-Schiff (PAS) staining of lung sections, demonstrating mucus-producing goblet cells (dark purple). **j** Quantification of mucus-producing goblet cells. Data show median (**b**, **c**) or mean ± SEM (**d**–**h**, **j**) from $n = 4$ (**j**), 7 (**f**, **g**), or 12 (**b**–**e**) mice in the PBS groups, and $n = 8$ (**j**) or 14 (**b**–**e**) mice in the HDM groups pooled from two independent experiments. Each symbol represents individual mice (**b**–**e**, **h**, **j**). *P* values were calculated using two-tailed Mann–Whitney *U* test in **b**–**e**, **h**, and **j**, or two-way ANOVA followed by a Tukey posttest in **f** and **g**. Source data are provided in the Source data file.



vaccination against IL-4 and IL-13 has any effect in models of infection with helminths. This question is particularly important since treatment with IL-4-K/IL-13-K in human may induce a long-lasting antibody response, for which the specific duration will need to be determined through clinical trials, that may not be desirable. Although data on the long-term effects of drugs targeting IL-4, IL-13, or other type 2 cytokines in human are still limited, it is important to note that several large clinical studies are now available for the anti-IL-4Rα mAb with treatment periods from 52 to 96 weeks in both asthma and atopic dermatitis[9,46–50]. Overall, these studies demonstrate a good safety profile, which argues in favor of the feasibility of long-term targeting of IL-4 and IL-13 with a vaccine strategy. Interestingly, the most common side effect noted is conjunctivitis, although this

Fig. 4 Characterization of hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} humanized mice. a hIL-4 levels in the supernatant of splenocytes from $n = 2$ WT or $n = 4$ hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} humanized mice stimulated ex vivo with PMA (20 nM) **b** hIL-13 levels in the supernatant of splenocytes from hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice sensitized with ovalbumin (OVA), and stimulated ex vivo with OVA ($n = 5$ mice). **c** hIL-13 levels in BAL fluid from hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice sensitized and challenged with OVA ($n = 5$ mice). Samples were collected 24 h after the last OVA challenge. Data in **a**–**c** show values from individual mice with bars indicating mean ± SD (in **a** and **c**). **d**, **e** Representative staining of skin samples from WT or hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice with anti-mouse IL-4Rα (**d**) or the anti-human IL-4Rα mAb dupilumab (**e**) and DAPI. Staining skin sample are representative from three WT and six hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice from two separate experiments. **f** Protocol outline. hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice were challenged nine times intranasally with 10 µg recombinant hIL-4, hIL-13, or PBS as a control. **g** Eosinophil numbers in BAL fluid 24 h after the last challenge with hIL-4 or hIL-13. **h** Representative lung sections stained with hematoxylin and eosin (H&E; revealing leukocyte infiltration), 24 h after the last challenge with hIL-4 or hIL-13. **i** Scoring of leukocyte infiltration in H&E-stained lung tissue sections. **j** Representative periodic acid–Schiff (PAS) staining of lung sections, demonstrating mucus-producing goblet cells (dark purple). **k** Quantification of mucus-producing goblet cells. Data in **g**, **i**, and **k** show values from individual mice with bars indicating mean ± SEM from $n = 8$ mice per group, pooled from two independent experiments. *P* values were calculated using two-tailed Mann–Whitney *U* test. Source data are provided in the Source data file.

seems to be restricted to atopic dermatitis patients, as it is not observed for patients with moderate-to-severe asthma[9,46–48,51].

Altogether, our results indicate that long-term neutralization of both mouse and human IL-4 and IL-13 can be achieved through vaccination with kinoids. Dual vaccination could protect against key features of chronic asthma after both prophylactic or therapeutic vaccination protocols. These results pave the way for the clinical development of an efficient long-term vaccine against asthma and other IL-4- and IL-13-mediated allergic diseases, such as food allergies, atopic dermatitis, or chronic urticaria.

Methods

Mice. Female BALB/cJRj mice at 5–6 weeks of age were purchased from Janvier Labs, and maintained in a specific pathogen-free facility at Institut Pasteur or Institut Jacques Monod. The *IL4RA/IL13/IL4* humanized mouse line (named hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} in the manuscript) was generated at University of North Carolina (USA) by syntenic replacement of two mouse loci: that encoding *Il4/Il13* and the second encoding Il4ra, with the corresponding human segment of DNA, as represented in Supplementary Fig. 17. The endogenous mouse *Il4ra* gene was deleted in ES cells with a replacement type targeting vector. Homologous recombination of this vector with the Il4ra locus resulted in deletion of a segment of the Il4ra locus extending from chr7:125,539,693–125,579,740 (GRCm38/mm10). The segment of the human IL4Ra locus extending from chr16:27,309,862–27,372,883 (GRCm38/mm10) human gene was inserted into the deleted locus by Cre-mediated recombination, using previously described methods[52]. Correct insertion of the human DNA segment into the deleted locus was verified by PCR analysis, Southern blot, and sequencing. A similar approach was used to replace the segment of the Il4/Il13 locus in ES cells extending from chr11:53,604,938–53,637,149 with the segment of the human IL-4/IL-13 locus extending from chr5:132,646,866–132,689,719. Mouse lines carrying the individual humanized loci were intercrossed to generate a line homozygous for the humanized loci. All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #170043, and by the French Ministry of Research. The protocol also received the authorization number EU0285 - Institut Jacques Monod PHEA - APAFiS - Autor. APAFiS #165.

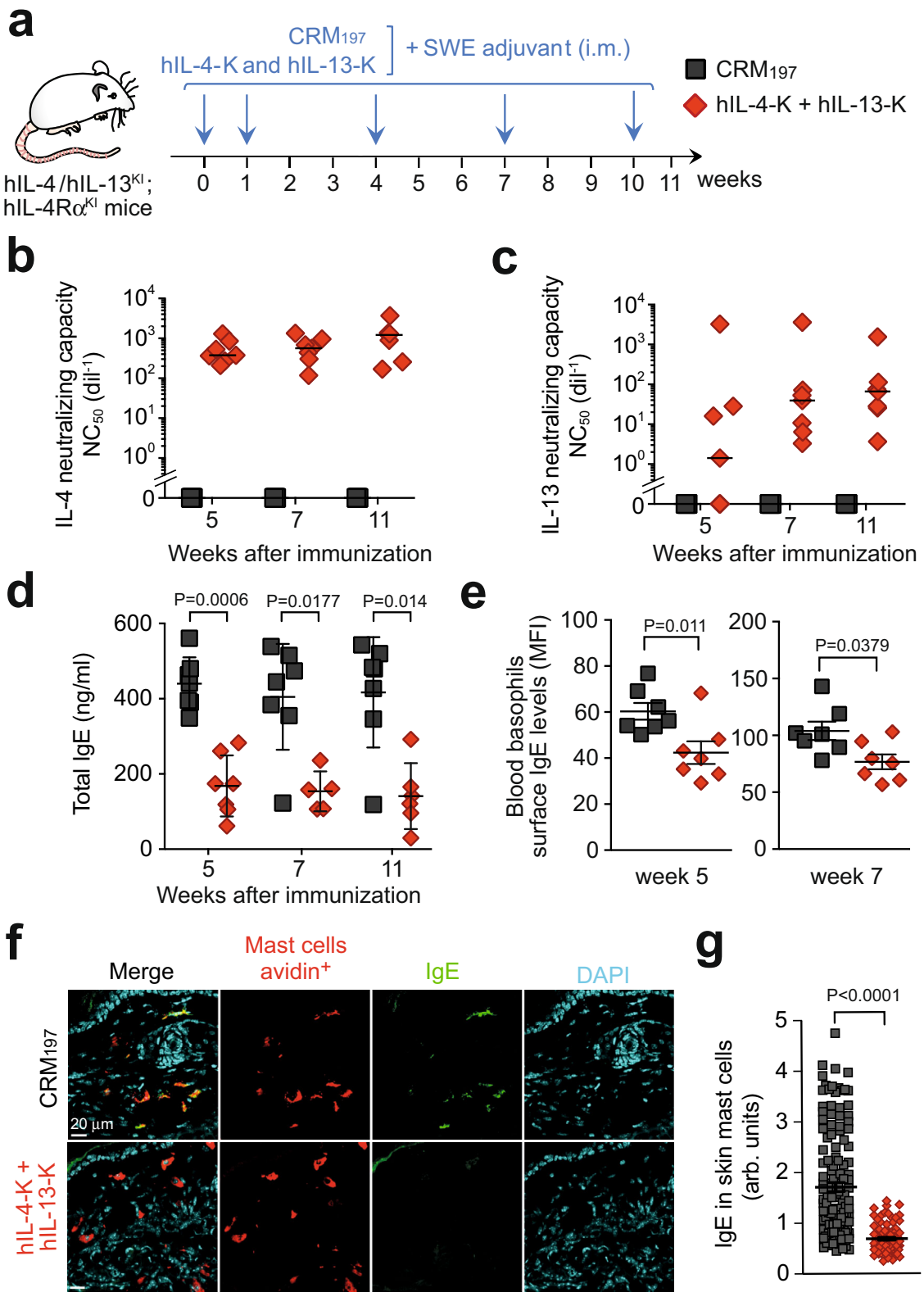
Synthesis and characterization of IL-4 and IL-13 kinoids. Mouse IL-4 (214-14), human IL-4 (200-04), mouse IL-13 (210-13), and human IL-3 (200-13) were purchased from PeproTech. CRM_{197} was purchased from Pfenex. IL-4 and IL-13 were modified with *N*-γ-maleimidobutyryl-oxysuccinimide ester (sGMBS; Thermo Fisher scientific, 22324), a maleimide-containing agent reacting with primary amines. Cytokines were dissolved in modification buffer (70 mM phosphate buffer, 150 mM NaCl, 5 mM EDTA, pH = 7.2) at 1 mg/ml. A solution of 10 mM of sGMBS was prepared and added to the cytokine at a 1:30, 1:20, or 1:10 molar ratio, for human IL-4, mouse IL-4, and human IL-13 or mouse IL-13 modification, respectively, and incubated during 1 h at room temperature (protected from light). Excess sGMBS was removed using a Zeba desalting spin column (Thermo Fisher scientific). Sulfhydryl moieties were introduced on the carrier protein CRM_{197} with SATA (*N*-succinimidyl-*S*-acetylthioacetate; Sigma-Aldrich, A9043). CRM_{197} was diluted in modification buffer at 2 mg/ml and a freshly prepared solution of 100 mM SATA (dissolved in DMSO) was added at a 1:80 molar ratio and incubated 30 min at room temperature (protected from light). Excess SATA was removed and buffer exchanged against modification buffer using a Zeba desalting spin column. SATA-modified CRM_{197} was incubated with a solution of hydroxylamine hydrochloride (Thermo Fisher scientific, 26130) at a 50 mM final concentration, at room temperature for 2 h, protected from light. Excess hydroxylamine was removed and buffer exchanged against modification buffer using a Zeba desalting spin column. After CRM_{197} and IL-4 or IL-13 functionalization, protein content of each preparation was determined by Bradford assay according to manufacturer's instructions (Thermo Fisher scientific).

Functionalized CRM_{197} was added to functionalized IL-4 or IL-13 at a molar ratio of 1:2 (for mouse IL-4) or 1:4 (for mouse IL-13, human IL-13, and human IL-4) and a final concentration of 0.4 mg/ml. The mixture was incubated 16 h at 4 °C, protected from light, and subsequently washed with fresh modification buffer using Zeba desalting spin column. Protein content was determined by Bradford assay. Resulting IL-4-K and IL-13-K were then 0.22 µm sterile filtered and stored at 4 °C. Kinoids were characterized using different in vitro methods. To analyze the profiles of the kinoids obtained, SDS–PAGE and western blots were performed with mouse IL-4 (using AF-404-NA as a detection antibody at 0.1 µg/ml, R&D systems), human IL-4 (AF-204-NA at 0.1 µg/ml, R&D systems), mouse IL-13 (AF-413-NA at 0.1 µg/ml, R&D systems), human IL-13 (AF-213-NA at 0.1 µg/ml, R&D systems), and with CRM_{197} (AbD serotec, 3710-0956 at 860 ng/ml). Size-exclusion (SE)-HPLC were performed using a Bio SEC-5 column (2000 Å, 5 µm, 7.8 × 300 mm, Agilent) and a Bio SEC-3 column (300 Å, 3 µm, 7.8 × 300 mm, Agilent) in connected series. SE-HPLC analysis were performed in the isocratic mode at 1 ml/min with column temperature at 25 °C. After filtration (0.22 µm cut-off), samples were injected at 100 µl and analyzed at 280 nm. The total run time was 35 min.

To confirm coupling between the cytokines and the carrier protein, and to evaluate epitope preservation, antigenicity was analyzed by sandwich ELISA. Briefly, capture antibody (mouse monoclonal anti-diphtheria toxin, AbD serotec, 3710-0100) was coated overnight at 1 µg/ml. After each step, plates were washed three times with PBS 0.01% Tween 20. Then plates were blocked with casein 2% dissolved in PBS. Kinoid samples were added at 250 ng/ml and twofold serially diluted in 100 µl final volumes. After incubation, bound kinoids were detected using biotinylated anti-mouse IL-4 antibody (polyclonal goat IgG, R&D systems, BAF-404), biotinylated anti-human IL-4 antibody (polyclonal goat IgG, R&D systems, BAF204), biotinylated anti-mouse IL-13 antibody (polyclonal goat IgG, R&D systems, BAF-413), or biotinylated anti-human IL-13 antibody (polyclonal goat IgG, R&D systems, BAF213) at 250 ng/ml, and then revealed with streptavidin-HRP and an OPD substrate. The reaction was stopped adding 1 M H_2SO_4 after 30 min of OPD incubation at room temperature protected from light, and absorbance was subsequently recorded at 490 nm.

Vaccination with kinoids. Mice were immunized intramuscularly with IL-4-K and/or IL-13-K combined 1:1 (v:v) with SWE, a squalene-in-water emulsion adjuvant (Vaccine Formulation Laboratory, University of Lausanne, Switzerland) in PBS on days indicated in the protocol outlines on Figs. 1, 2, and 5, at two initial doses of 30 µg followed by boosts of 10 µg. As controls, groups of mice were injected with the same schedule with CRM_{197} alone with two initial doses of 15 µg followed by boosts of 5 µg (these doses were defined based on the weight ratio of CRM_{197} used to generate kinoids; as shown in Supplementary Figs. 1, 11, and 13), immunization with CRM_{197} alone induced slightly higher levels of anti-CRM_{197} antibodies than immunization with kinoids combined with SWE, or PBS alone. In the experiments depicted in Supplementary Fig. 5, mouse IL-4 and CRM_{197} or mouse IL-13 and CRM_{197} were also co-injected without prior conjugation, following the same immunization schedule.

Mouse models of house dust mite-induced allergic asthma. In the prophylactic protocol (Figs. 1 and 2), BALB/c mice were immunized with kinoids at days 0, 7, 28, and 55. Starting at day 39, allergic asthma was induced with i.n. exposure to crude HDM extracts (*D. farinae*; lot number: 307244, Greer laboratories). Lightly isoflurane-anesthetized mice were sensitized three times by i.n. exposure with HDM (100 µg in 30 µl PBS), or PBS alone as control, with a 3-day interval between each administration. Lightly isoflurane (3% in air)-anesthetized mice were challenged i.n. with 25 µg of HDM in 30 µl PBS, or PBS alone as a control, twice a week



starting 3 days after the last sensitization, for a total of nine administrations. Mice were sacrificed 24 h after the last challenge with HDM or PBS.

In the therapeutic protocol (Fig. 3), BALB/c mice were sensitized three times with HDM (100 µg in 30 µl PBS), and PBS as control, with a 3-day interval between each administration. Lightly isoflurane (3% in air)-anesthetized mice were then challenged i.n. with 25 µg of HDM in 30 µl PBS, or PBS alone as a control, twice a week for a total of 18 challenges, starting 3 days after the last sensitization. Vaccination with mouse IL-4-K and mouse IL-13-K was initiated 4 days after the third HDM challenge. Mice were sacrificed 24 h after the last challenge with HDM or PBS.

Measurement of airway reactivity to methacholine. Twenty-four hours after the last challenge with HDM or PBS, responses to aerosolized methacholine were measured using whole-body plethysmography (EMKA technologies), using IOX base 8c/RF-8a (0796) software. Responses to inhaled methacholine were assessed

Fig. 5 Efficient vaccination with human IL-4 and IL-13 kinoids in IL-4/IL-13^{KI}; IL-4Rα^{KI} humanized mice. a Vaccination protocol outline. hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice were vaccinated with hIL-4-K and hIL-13-K in combination (or CRM_{197} as control), combined with the adjuvant SWE. **b**, **c** Anti-human IL-4 (**b**) and anti-human IL-13 (**c**) neutralizing capacity in sera collected at the indicated time point. Results show values from individual mice ($n = 7$/group) with bars indicating medians. **d**, **e** Levels of total IgE (**d**) in sera or on the surface of blood basophils (**e**) at the indicated time points. Results show values from individual mice ($n = 7$/group) with bars indicating means ± SEMs. *, **, or ***: $P < 0.05$, 0.01 or 0.001 (two-tailed Mann–Whitney U test). **f** Representative ear skin sections stained with avidin (which stains mast cells, red), anti-IgE (green), and DAPI (blue) 24 h after the last HDM challenge. **g** Quantification of IgE levels in avidin-positive mast cells. Results show values from individual avidin-positive mast cells analyzed in ear skin sections from $n = 7$ mice injected with CRM_{197} and $n = 5$ mice injected with hIL-4-K and hIL-13-K, with bars indicating means ± SEMs. P values were calculated using two-tailed Mann–Whitney U test. Source data are provided in the Source data file.

by recording Penh over 5 min after each dose of aerosolized methacholine (baseline, 0, 3, 5, 7, and 14 mg/ml). Data were analyzed using Datanalyst software (DATA 4238). Invasive measurements were also performed in anesthetized, tracheostomized, mechanically ventilated mice using a FlexiVent software FlexiWare 8.0 (Scireq). Aerosolized methacholine was administered in increasing concentrations (baseline, 0, 6, 25, 50 and 100 mg/ml). Lung resistance (R) and tissue elastance (E) were computed by assuming a constant phase model.

Intranasal challenges with IL-4 and IL-13. hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice were exposed intranasally to 10 µg human IL-4 (Peprotech), 10 µg human IL-13 (Peprotech), or PBS as a control daily for 9 days. Twenty-four hours after the last administration, mice were sacrificed for analysis of BAL eosinophil numbers and lung inflammation.

Flow cytometry analysis of leukocytes in blood, bronchoalveolar lavage fluid, peritoneal lavage, and lung tissue. BALs were performed 24 h after the last challenge with HDM, hIL-4, or hIL-13 in anesthetized mice (187.5 mg/kg ketamine and 18.75 mg/kg xylazine). After semi-excision of the trachea, a plastic canula was inserted, and airspace was washed with 1 ml of PBS containing 2.6 mM EDTA and 2.5% (v/v) FBS. This operation was repeated for a total of three times.

For the analysis of leukocytes in lung tissue, right lung lobes were harvested 24 h after the last challenge with HDM, and transferred into gentleMACS C tubes (Miltenyi) containing lung dissociation kit (Miltenyi). Tubes were attached upside down on a gentleMACS dissociator (Miltenyi). After a washing step, red blood cells were lysed with ammonium chloride potassium (ACK) lysing buffer (Thermo Fisher scientific), and single-cell suspensions were 0.22 µm cut-off filtered. Single-cell suspensions of total right lung tissue and BAL fluid were stained with anti-CD45-FITC (clone # REA737, Miltenyi), anti-Ly6G-PE (clone # 1A8, BD Pharmingen), anti-CD11c-VB (clone # N418, Miltenyi), CD11b-VG (clone # REA592, Miltenyi), anti-Siglec-F-PECy7 (clone # REA798, Miltenyi), anti-B220-APC (clone # RA3-6B2, Miltenyi), and anti-CD3ε-APC (clone # 145-2C11, BD Pharmingen). Macrophages were gated as CD45$^+$, CD11c$^+$, Siglec-F$^+$, CD11b$^+$, B cells as CD45$^+$, CD11c$^-$, B220$^+$, T cells as CD45$^+$, CD11c$^-$, CD3ε$^+$, neutrophils as CD45$^+$, CD11c$^-$, B220$^-$, CD3ε$^-$, Ly6G$^+$, CD11b$^+$, and eosinophils as CD45$^+$, CD11c$^-$, B220$^-$, CD3ε$^-$, Ly6G$^-$, Siglec-F$^+$, SSC^{high}.

For intracellular staining of IL-4 and IL-13, single-cell suspensions from right lung lobes were first permeabilized with Perm/wash buffer (554723, BD Bioscience), according to the manufacturer's instructions. Cells were then stained with anti-CD4-FITC (clone # GK1.5, Miltenyi), anti-IL-4-PE (clone # BVD4-1D11 Miltenyi), and anti-IL-13-PE-Cyanine7 (clone # eBio13A, Fisher Scientific).

Blood was collected on heparin for analysis of eosinophils and basophils. Red blood cells were lysed with ACK lysis buffer (Thermo Fisher scientific). Cells were stained with anti-Siglec-F-PECy7 (clone # REA798, Miltenyi), anti-CD49b-APC (clone Dx5, eBioscience), and anti-IgE-FITC (clone # R35-72, BD Pharmingen). Blood eosinophils were gated as Siglec-F$^+$, SSC^{high}, and blood basophils as CD49b$^+$, IgE$^+$.

For analysis of peritoneal mast cells, 5 ml of PBS were injected into the peritoneal cavity and the abdomen was massaged gently for 20 s. Fluid containing peritoneal cells was collected and cells were stained with anti-c-KIT APC (clone # 2B8, Bioscience) and anti-IgE-FITC (clone # R35-72, BD Pharmingen). Peritoneal mast cells were gated as c-KIT$^+$, IgE$^+$. Samples were acquired on Miltenyi MACSQUANT 10 and 16. Data were analyzed using FlowJo 10.4.2 software. All FACS sequential gating strategies are presented in Supplementary Figs. 19 and 20.

We assessed levels of lung-resident regulatory eosinophils (rEos) in mice vaccinated with IL-4-K and IL-13-K or CRM_{197}. Right lung lobes were harvested and transferred into gentleMACS C tubes (Miltenyi) containing lung dissociation kit (Miltenyi). Tubes were attached upside down on a gentleMACS dissociator (Miltenyi). After a washing step, red blood cells were lysed with ACK lysing buffer (Thermo Fisher scientific). Single-cell suspensions were 0.22 µm cut-off filtered, and stained with anti-CD45-VB (clone # REA737, Miltenyi), CD125-PE (clone # T21, BD Pharmingen), Siglec-F-Alexa 647 (clone # E50-2440, BD Pharmingen), and propidium iodide (PI) solution (Miltenyi). rEos were gated as CD45$^+$, PI$^-$, CD125$^+$, Siglec-F^{int}, as previously described[23] (gating strategy is represented in Supplementary Fig. 9). Samples were acquired on BD LSRFortessa cell analyzer (BD Biosciences). Data were analyzed using FlowJo 10.4.2 software.

Quantification of antibodies against mouse and human IL-4 and IL-13, and CRM_{197}. The immunogenicity of the kinoids was assessed by evaluating antibodies against mouse IL-4, human IL-4, mouse IL-13, human IL-13, and CRM_{197} in sera collected at different time points after vaccination. Mouse IL-4, human IL-4, mouse IL-13, human IL-13, or CRM_{197} were coated and incubated overnight at 4 °C at 1 µg/ml. After each step, plates were washed three times with PBS Tween 20 0.01% (v/v). After blocking with casein 2% (w/v) in PBS, serum samples were added, a twofold serial dilution was conducted starting at 500 dil^{-1} (diluted in PBS, casein 1% (w/v) and Tween 20 0.01% (v/v)). After 90 min of incubation at 37 °C, bound antibodies were detected with HRP-conjugated anti-mouse IgG (Invitrogen), and plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 after 30 min of OPD incubation at room temperature protected from light, and absorbance was subsequently recorded at 490 nm. Samples were analyzed starting at dilution 500 dil^{-1} up to 256,000 dil^{-1}, except for pre-immune sera analyzed only at 500 dil^{-1}. The titers were defined using Microsoft Excel 16.16.23 as the dilution of the serum, where 50% of the ODmax minus OD of corresponding pre-immune sample in the assay was reached. Titers were expressed as serum dilution factors (dil^{-1}). The limit of titer quantification is the lowest dilution tested in the assay: 500 dil^{-1}.

Assessment of the neutralizing capacity against IL-4 and IL-13 in sera from vaccinated mice. Neutralizing capacities of the anti-mouse IL-4 antibodies were evaluated using CTLL-2 cells proliferation assay (ECACC, ref. 93042610, batch number: 12K006.). Cells were grown in presence of human IL-2 (Sigma-Aldrich; 10 ng/ml). For neutralization bioassays, human IL-2 was replaced by mIL-4 (Peprotech; 2 ng/ml). Dilution series of serum samples from mice vaccinated with IL-4 kinoids were mixed with mouse IL-4 (2 ng/ml). After 1 h incubation, 20,000 CTLL-2 cells were added to preincubated samples. After 48 h, cell viability was quantified by MTS/PMS assay (Promega), according to the manufacturer's instructions. Neutralizing capacities of anti-human IL-4, anti-mouse IL-13, and anti-human IL-13 antibodies were evaluated using a HEK-Blue IL-4/IL-13 reporter gene cell line bioassay (InvivoGen, hkb-il413, batch number: X14-37-01), adapted from the manufacturer's instructions. When activated with human IL-4, mouse IL-13, or human IL-13, this cell line produces secreted embryonic alkaline phosphatase, which can be quantified using QUANTI-Blue medium (InvivoGen). Briefly, dilution series of serum samples from mice vaccinated with kinoids were mixed with mouse IL-13, human IL-13 (PeproTech; 2 ng/ml), or human IL-4 (PeproTech; 0.25 ng/ml), and then added to 40,000 HEK-Blue IL-4/IL-13 cells. After 24 h, supernatants were harvested and mixed with QUANTI-Blue. The IL-4/IL-13 neutralizing capacity 50 (NC_{50}) result was expressed as the serum dilution factor (dil^{-1}) neutralizing 50% of IL-4 or IL-13 activity, using Microsoft Excel 16.16.23.

Quantification of total IgE levels and HDM-specific IgE and IgG. Total IgE levels were quantified using a commercial ELISA kit (E90-115; Bethyl Laboratories), according to the manufacturer's instructions. HDM-specific IgE in sera were measured by ELISA, using a protocol adapted from refs. [53,54]. First, HDM was biotinylated using NHS-PEG4-biotin (molar ratio NHS-PEG4-biotin/HDM: 20/1) in phosphate buffer 70 mM, NaCl 150 mM, pH 7.2. The mixture was allowed to react for 30 min, at room temperature with a modification concentration of 0.6 mg/ml. Excess NHS-PEG4-biotin was removed using Zeba desalting spin column. Protein content was determined by Bradford assay. Effective HDM biotinylation was confirmed by direct ELISA: plate was coated with HDM-biotin, detected with poly-HRP streptavidin and revealed using an OPD substrate. HDM-specific IgE were detected by ELISA. Goat polyclonal anti-mouse IgE antibody (STAR110, Bio-rad) was coated and incubated overnight at 4 °C at 2 µg/ml in PBS. After each step, plates were washed three times with PBS Tween 20 0.01% (v/v). After blocking with casein 2% (w/v) in PBS for 90 min at 37 °C, serum samples were added at a 1:50 final dilution (diluted in PBS, casein 1% (w/v), Tween 20 0.01% (v/v)) and incubated for 2 h at 37 °C. Then, HDM-biotin (prepared as described hereabove) was added at a 1:400 final dilution and incubated for 2 h at 37 °C. Bound HDM-specific IgE antibodies were detected with poly-HRP streptavidin (N200; Thermo Fisher scientific; dilution 1:10,000, 60 min at 37 °C incubation) and plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 after 30 min of OPD

incubation protected from light, at room temperature and absorbance was subsequently recorded at 490 nm.

HDM-specific IgG, IgG1, and IgG2a levels in sera were measured by ELISA. HDM was coated in 96-well plates and incubated overnight at 4 °C at 5 µg/ml. After each step, plates were washed three times with PBS Tween 20 0.01%. After blocking with BSA 1% in PBS for 90 min, serum samples were added, a twofold serial dilution was conducted starting at 1:2000 (in PBS, BSA 0.5%, Tween 20 0.01%), 1:4000 (in PBS, BSA 1%), or 1:500 (in PBS, BSA 1%) for IgG, IgG1, or IgG2a respectively. After 90 min of incubation, bound antibodies were detected with HRP-conjugated anti-mouse IgG (Invitrogen) at 1:5000, goat HRP-conjugated anti-mouse IgG1 (Southern Biotech) at 1/8000, or goat HRP-conjugated anti-mouse IgG2a (Southern Biotech) at 1:8000, and plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 after 30 min of incubation at room temperature, and absorbance was subsequently recorded at 490 nm.

Quantification of mouse IL-5, human IL-4, and human IL-13 levels. Mouse IL-5 levels were quantified in BAL fluid collected 24 h after the last challenge with HDM, using MACSPlex Cytokine (Miltenyi), according to the manufacturer's instructions. For quantification of human IL-4 (hIL-4), splenocytes from naive hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice were harvested, and cells were stimulated overnight with PMA/ionomycin (20 nM: 1 µM) or PBS as control at 37 °C. For quantification of hIL-13, hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice were sensitized with 50 µg ovalbumin (OVA) and 2 ml aluminum hydroxide gel (vac-alu-250, Invivogen) at days 0 and 7, and challenged intranasally with 10 µg OVA at days 21–24. BAL fluid was collected 24 h after the last challenge with OVA, as described above. Splenocytes were harvested and stimulated with OVA (10, 50, and 100 µg/ml) for 5 days at 37 °C. hIL-4 and hIL-13 were quantified by ELISA in supernatants from splenocytes or in BAL fluid samples. Mouse anti-hIL-4 (MAB304; clone #3007, R&D systems) or hIL-13 antibody (MAB213; clone #32116, R&D systems) were coated and incubated overnight at 4 °C at 1 µg/ml in PBS. After each step, plates were washed three times with PBS Tween 20 0.01% (v/v). After blocking with BSA 1% (w/v) in PBS for 60 min at 37 °C, serum samples and hIL-4 or hIL-13 as controls starting from 1 ng/ml were added (diluted in RPMI ± 10% FBS or PBS) and incubated for 90 min at room temperature. Then, hIL-4 or hIL-13 were detected with either biotinylated goat polyclonal anti-hIL-4 (BAF204, R&D systems) or hIL-13 (BAF213, R&D systems) antibodies at 1 µg/ml for 90 min at room temperature, followed by Strep-HRP (R&D systems) detection for 60 min at room temperature. Plates were revealed using an OPD substrate. Reaction was stopped with 1 M H_2SO_4 after 30 min of OPD incubation protected from light at room temperature. Absorbance was subsequently recorded at 490 nm.

Lung histology. Left lungs were excised from mice postmortem, fixed with 4% paraformaldehyde (PFA) for 24 h at room temperature, and preserved in 70% ethanol. Longitudinal sections were done and stained with hematoxylin and eosin (H&E; for assessment of leukocyte infiltration), periodic acid–Schiff (PAS) staining (for assessment of goblet cells hyperplasia and mucus production) or toluidine blue (for quantification of mast cell numbers; all from Sigma). The severity of inflammation on H&E-stained lung sections was graded semi-quantitatively in a blind manner for the following features: 0: normal, 1: few cells, 2: a ring of inflammatory cells, 1 cell layer deep, 3: a ring of inflammatory cells 2–4 cells deep, and 4: a ring of inflammatory cells of >4 cells deep (adapted from ref. [55]). The extent of mucus production was also quantified in a blind manner on PAS-stained lung sections by a score according to the percentage of goblet cells in the epithelial cells[56]: 0: no goblet cells, 1: <25%, 2: 25–50%, 3: 50–80%, and 4: >80%. Mast cell quantification on toluidine blue-stained lung sections was performed using Zen Software 3.1 blue edition.

IHC section and acquisition. For lung and ear skin IHC, the bigger lobe from the right lung or the ears were excised postmortem fixed with 1% PFA for 24 h at room temperature. Tissue dehydration was performed in sucrose gradient baths (10, 20 and 30%), then tissues were embedded in OCT compound.

A total of 8-µm thick sections of lung tissue sections were treated using a heat-induced epitope retrieval method, as previously described[57]. Tissue sections were blocked and permeabilized with PBS 0.5% (w/v) BSA (Sigma-Aldrich), 0.3% Triton X-100 (Merck) for 1 h at room temperature. For staining of lung IgE$^+$ mast cells, permeabilized tissue sections incubated with an anti-IgE antibody coupled to AF488 (Biolegend, UK), at 10 µg/ml overnight at 4 °C in the dark. Tissue sections were then washed three times in PBS 0.5% (w/v) BSA, and incubated with DAPI (Thermo Fisher scientific) and avidin-sulforhodamine at 5 µg/ml (Sigma-Aldrich; to stain mast cells[58]) in PBS 0.5% (w/v) BSA, for 2 h in the dark. Finally, samples were mounted in Mowiol medium (Sigma-Aldrich) and sealed with nail polish. A total of 512 × 512 pixels *Z*-stack images were acquired using a confocal microscope SP8 (Leica Microsystems) equipped with a HC PL APO CS2 with 20× NA 0.75 dry or a HC PL APO CS2 60×/NA 1.40 oil objective. In our study, a digital zoom of three or six was applied for 60× or 20× objective, respectively. A maximum intensity projection (MIP) was used for generating 2D images. Individual mean intensity analysis of anti-IgE staining on avidin-sulforhodamine-positive mast cells was performed using ImageJ (NIH). For staining of lung IL-4$^+$ type 2 ILC2, permeabilized skin sections were incubated overnight at 4 °C in the dark AF488-coupled anti-KLRG1 (clone 2F1, BD Pharmingen), APC-coupled anti-IL-4 (clone 11B11, Thermofisher Scientific), and AF532-coupled anti-CD3 (clone 17A2, eBioscience). Sections were then extensively washed, incubated with DAPI for 1 h at room temperature in the dark and mounted in Mowiol mounting medium. Images of 512 × 512 pixels were acquired using Leica Microsystems SP8 confocal laser-scanning microscope equipped with a HC PL APO CS2 with 20× NA 0.75 dry. Images were processed using Zen Software 3.1 blue edition (Zeiss) and ImageJ (1.53c) software. CD3$^-$KLRG1$^+$IL-4$^+$ ILC2 were counted on images of four to six consecutive microscopic fields from each mouse.

A 8-µm thick sections of ear skin sections were blocked and permeabilized with PBS supplemented with 0.5% (w/v) BSA (Sigma-Aldrich) and 0.1% saponin (Sigma-Aldrich), for 30 min at room temperature, and incubated with an anti-IgE antibody coupled to Alexa Fluor (AF) 488 (Biolegend, UK), at 10 µg/ml overnight at 4 °C in the dark. Tissue sections were then washed three times in PBS 0.5% (w/v) BSA and 0.1% saponin, and incubated with DAPI and avidin-sulforhodamine in PBS 0.5% (w/v) BSA and 0.1% saponin, for 2 h at room temperature in the dark. Finally, samples were mounted in Mowiol medium (Sigma-Aldrich). A total of 512 × 512 pixels *Z*-stack images were acquired using a confocal microscope LSM710 (Zeiss) equipped with a HC PL APO 40×/NA 1.30 oil objective. A MIP was used for generating 2D images. Individual mean intensity analysis of anti-IgE staining on avidin-sulforhodamine + mast cells was performed using ImageJ (NIH).

For staining of mouse and human IL-4Rα in wild-type and hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice, skin tissue sections were blocked with PBS supplemented with 1% (w/v) BSA (Sigma-Aldrich), for 1 h at room temperature. Skin sections were then incubated with the anti-human IL-4Rα mAb dupilumab labeled in-house with AF647 (using Alexa Fluor 647 Protein Labeling Kit #A20173, Thermo Fisher, according to manufacturer's instructions) at 20 µg/ml, or a rat-anti-mouse IL-4Rα (clone mIL4R-M1, BD Pharmingen) at 10 µg/ml overnight at 4 °C in the dark. Tissue sections were then washed three times in PBS 1% (w/v) BSA, and incubated with goat anti-rat AF594 (1:150 in PBS 1% (w/v) BSA) for 1.5 h, followed by washing steps again three times in PBS 1% (w/v) BSA. Finally, tissue sections were incubated in 1:5000 with DAPI in PBS 1% (w/v) BSA for 15 min at room temperature in the dark, rinsed in PBS 1% (w/v) BSA, and mounted using DABCO mounting medium and nail polish. To image mouse IL-4Rα marked with AF594; 1524 × 1524 pixel *Z*-stacks were acquired using a LSM710 (Zeiss) confocal microscope equipped with 20×/0.8 plan apo. To image human IL-4Rα marked with AF647; 1024 × 1024 pixel *Z*-stacks were acquired using a SP8 (Leica) confocal microscope equipped with 20×/0.8 plan apo. A MIP was used for generating 2D images in ImageJ 1.53c.

Statistical analysis. Statistical significance was determined using the unpaired Student's *t* test (unpaired Mann–Whitney *U* test) or test two-way ANOVA followed by a Tukey posttest. $P \leq 0.05$ was considered statistically significant. Calculations were performed using the Prism® 7.0 software program (GraphPad 7.0 Software).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary information files). Source data are provided with this paper.

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Author contributions

Experimental design, E.C., R.B, G.G.-V., and L.L.R; investigation, E.C., R.B., B.B., J.B., J.S., R.H., J.B.J.K., N.C., F.C., S.H., D.H., L.G., A.M., A.L., F.H., N.G., and L.L.R.; generation of

hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice, J.N.S. and B.K; formal analysis, E.C., R.B., P.B., N.C., F.C., S.H., V.S., R.H., J.K., N.G., G.G.-V., and L.L.R.; writing (original draft), E.C. and L.L.R.; and writing (review and editing), all authors.

Competing interests

E.C., R.B., P.B., N.C., V.S., G.G.-V., and L.L.R. are inventors on patent related to this work (WO2019228674 (A1)), and/or related to Kinoid technology. E.C., R.B., J.B., N.C., F.C., S.H., V.S., and G.G.-V. are currently or were previously employees of NEOVACS and company stocks owners. The other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.L.R.

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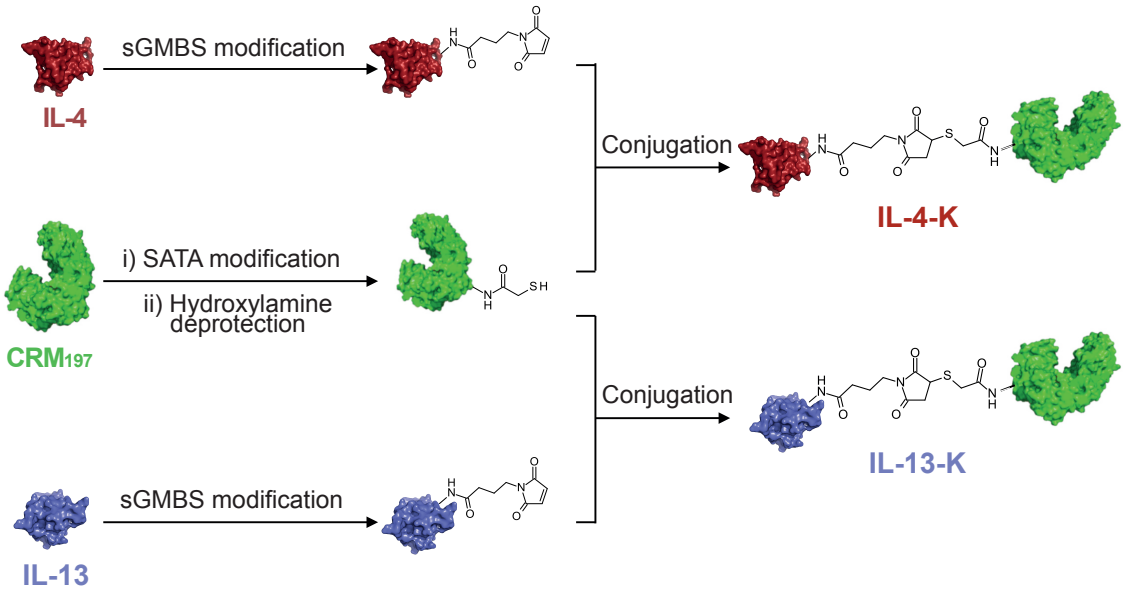
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Dual vaccination against IL-4 and IL-13 protects against chronic allergic asthma in mice

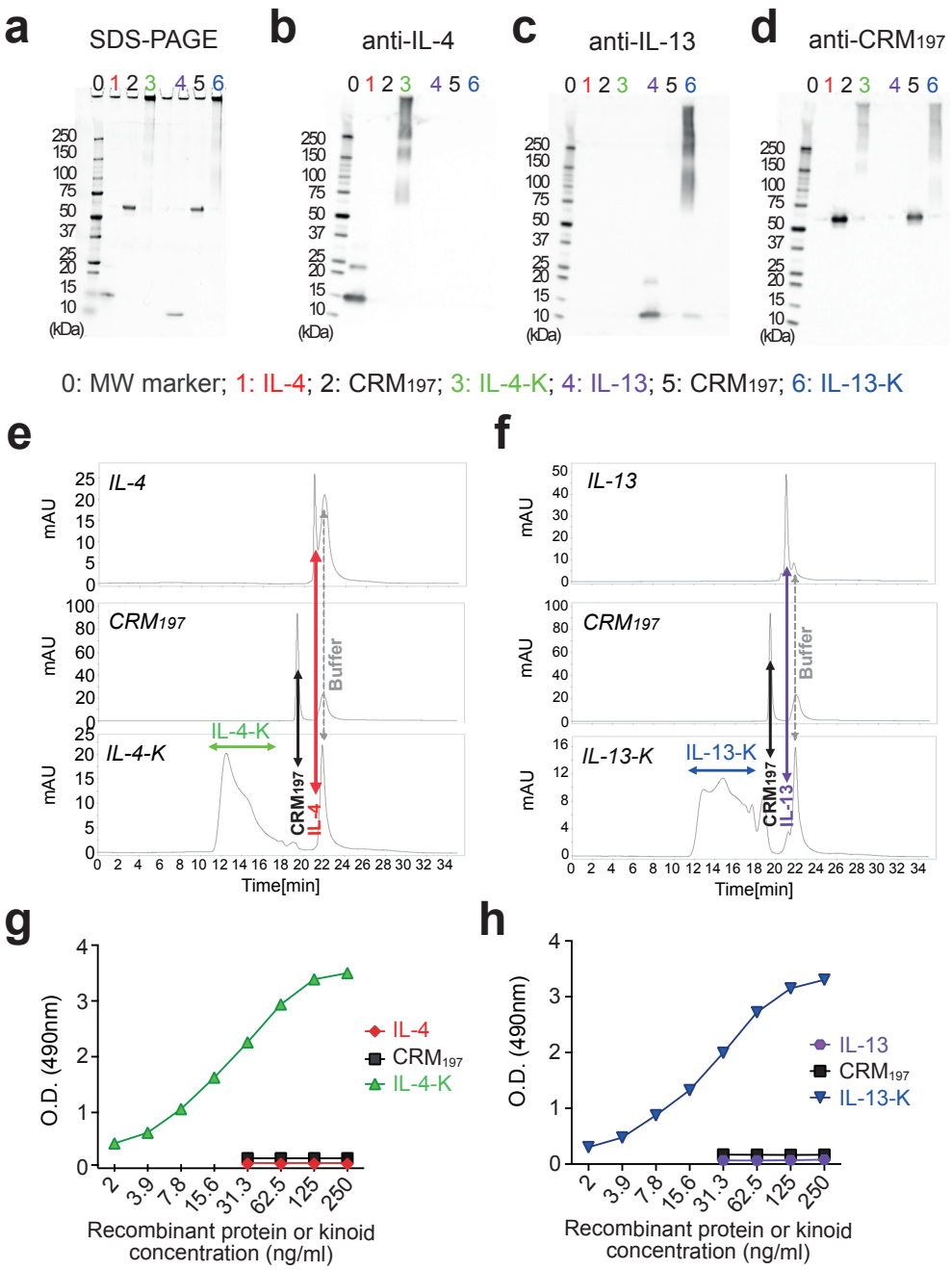
Eva Conde[1, 2, 3, †], Romain Bertrand[3, †], Bianca Balbino[1, 2], Jonathan Bonnefoy[3], Julien Stackowicz[1, 2], Noémie Caillot[3], Fabien Colaone[3], Samir Hamdi[3], Raïssa Houmadi[4], Alexia Loste[4], Jasper B.J. Kamphuis[4], François Huetz[1], Laurent Guilleminault[4, 5], Nicolas Gaudenzio[4], Aurélie Mougel[4], David Hardy[6], John N. Snouwaert[7], Beverly H. Koller[7], Vincent Serra[3], Pierre Bruhns[1, §], Géraldine Grouard-Vogel[3, §], Laurent L. Reber[1, 4, §, *]

[1]Unit of Antibodies in Therapy and Pathology, Institut Pasteur, UMR 1222 INSERM, F-75015 Paris, France. [2]Sorbonne University, ED394, F-75005 Paris, France. [3]Neovacs SA, Paris, France. [4]Toulouse Institute for Infectious and Inflammatory Diseases (Infinity), INSERM UMR1291, CNRS UMR5051, University Toulouse III, 31024 Toulouse, France. [5] Department of respiratory medicine, Toulouse University Hospital, Faculty of Medicine, Toulouse, France. [6]Institut Pasteur, Experimental Neuropathology Unit, Paris, 75015, France. [7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA. [†]These authors contributed equally. [§]These authors jointly supervised this work. [*]Corresponding author.

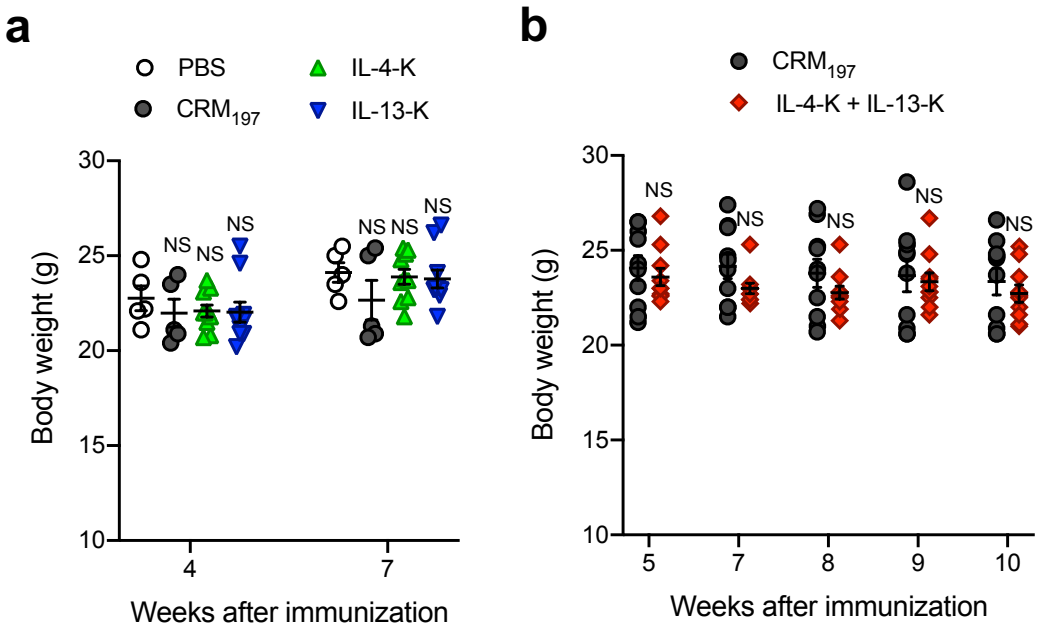
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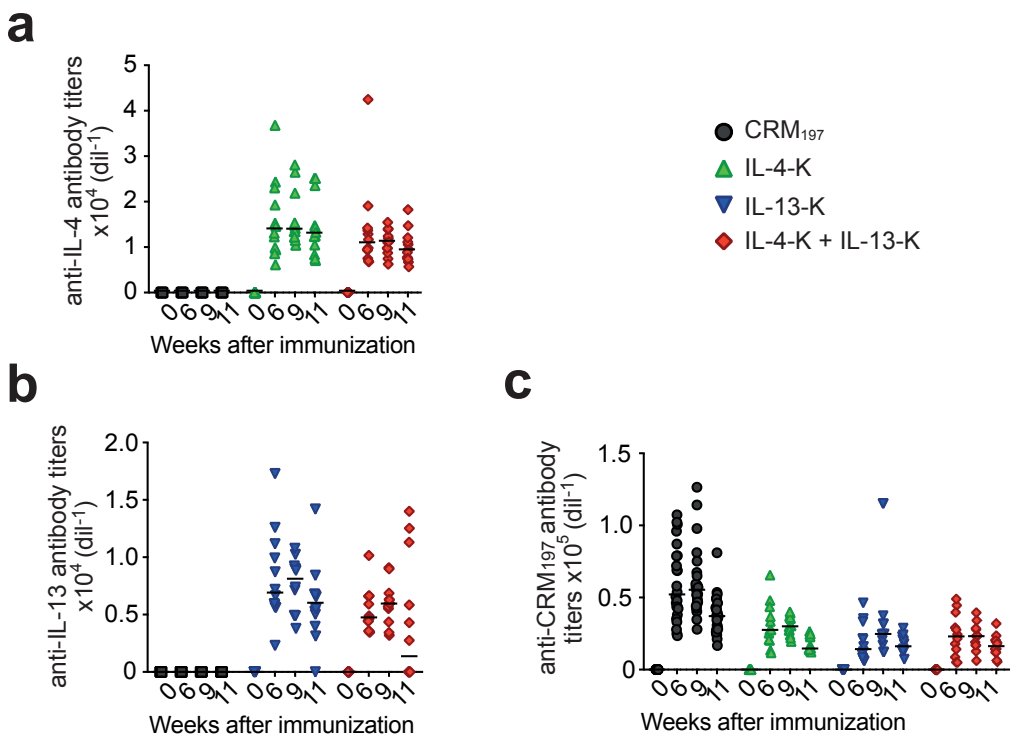
Supplementary Figure 1. Synthesis of IL-4-K and IL-13-K using a thiol-maleimide conjugation. Recombinant mouse IL-4 and IL-13 were modified with N-γ-maleimidobutyryl-oxysuccinimide ester (sGMBS), a maleimide-containing agent reacting with primary amines. Sulfhydryl moieties were introduced on the carrier protein, CRM_{197}, with SATA (N-succinimidyl-S-acetylthioacetate). Functionalized CRM_{197} was added to functionalized IL-4 or IL-13 at a molar ratio of 1:2 (CRM_{197}-IL-4) or 1:4 (CRM_{197}-IL-13), to generate the IL-4 kinoid (IL-4-K) and IL-13-K, respectively.



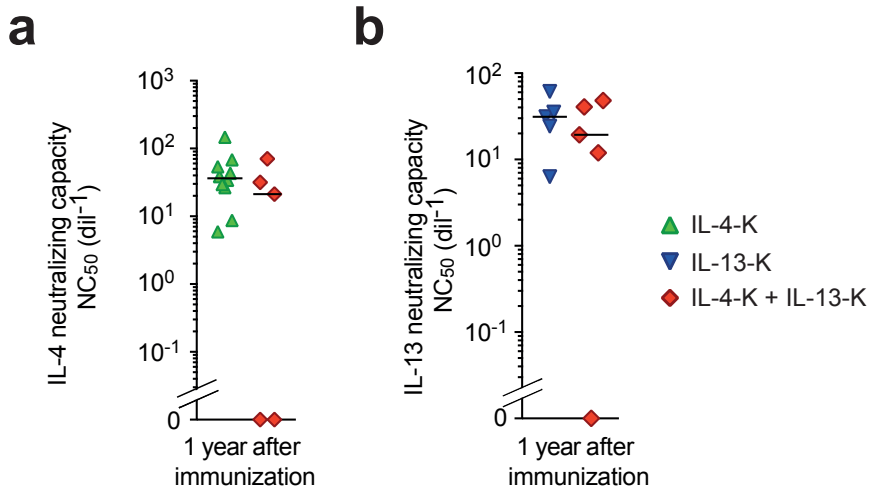
Supplementary Figure 2. Characterization of mouse IL-4-K and IL-13-K. Generation of high molecular weight kinoids upon conjugation of IL-4 or IL-13 to CRM_{197} was confirmed using SDS-PAGE (**a**), western blots against IL-4 (**b**), IL-13 (**c**) or CRM_{197} (**d**), and size exclusion (SE)-HPLC (**e** and **f**). Epitope preservation and antigenicity of IL-4-K and IL-13-K was analyzed by sandwich ELISA using plates coated with anti-CRM_{197} antibodies and revealed with anti-IL-4 (**g**) or anti-IL-13 (**h**) antibodies. Data from a, b, c and d are representative of five independent experiments. Source data are provided in the Source Data file.



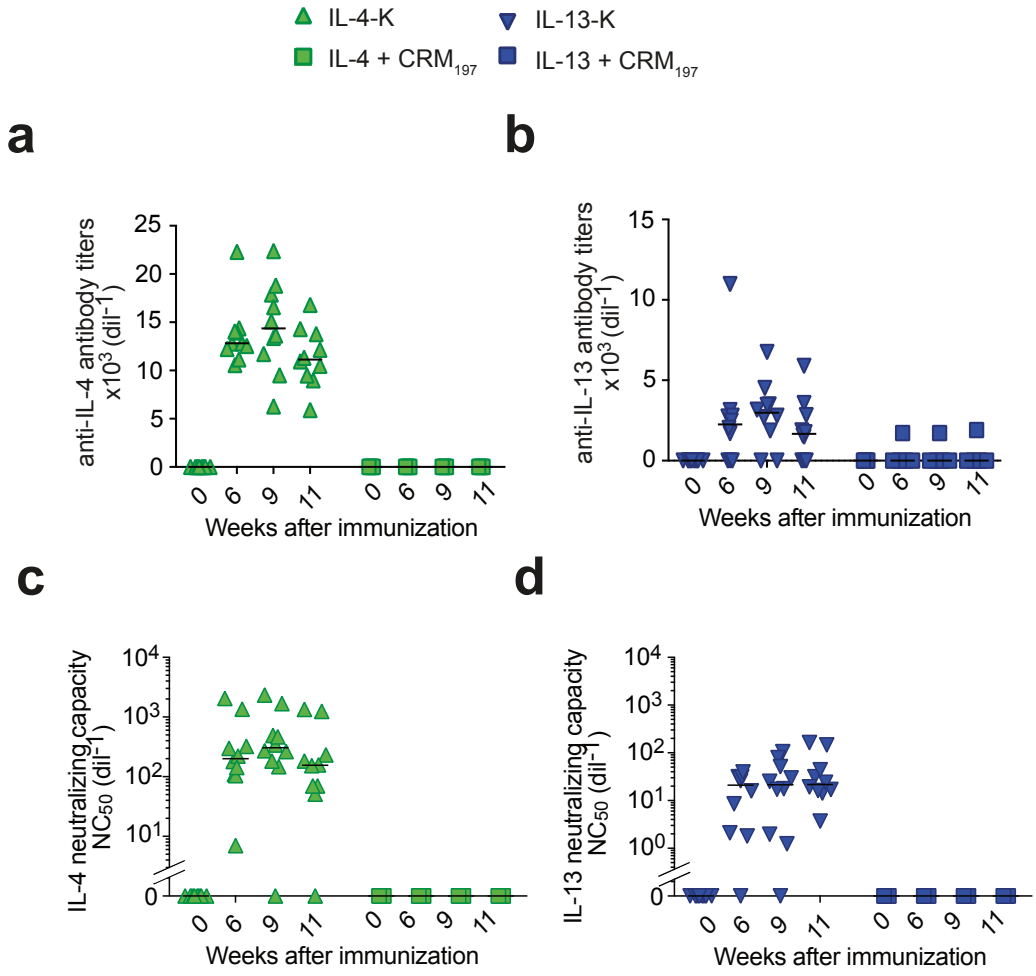
Supplementary Figure 3. Changes in body weight following vaccination with kinoids. Mice were immunized with IL-4-K, IL-13-K, CRM_{197} or PBS as control (**a**), or IL-4-K and IL-13-K in combination or CRM_{197} (**b**), as outlined in Figure 1A. Body weight was measured at the indicated time points after first injection of kinoids. Results show values from individual mice with bars indicating mean values ± SEMs (n=5 mice in PBS and CRM_{197} control groups from (a) or n=10 mice in IL-4-K, IL-13-K, IL-4-K+IL-13-K and CRM_{197} groups), and are pooled from two independent experiments. NS: not significant (P>0.05) *vs*. PBS group in (a) and *vs*. CRM_{197} group in (b). (Mann-Whitney U test). Source data are provided in the Source Data file.



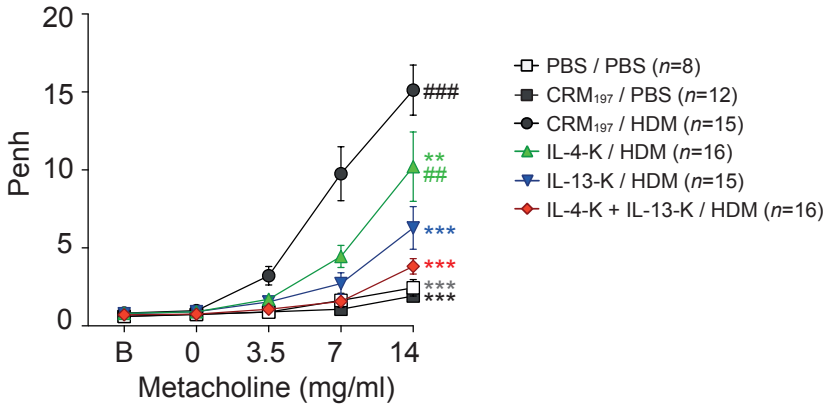
Supplementary Figure 4. Anti-IL-4, anti-IL-13 and anti-CRM$_{197}$ antibody titers in mice vaccinated with kinoids. a-c. Anti-IL-4 (**a**), anti-IL-13 (**b**) and anti-CRM$_{197}$ (**c**) antibody titers in sera at 6, 9 and 11 weeks after first injection of IL-4-K and IL-13-K alone or in combination, or CRM$_{197}$ as a control. Results show values from individual mice with bars indicating medians. Data are from a single experiment with n=12 mice per group, representative of two independent experiments. Source data are provided in the Source Data file.



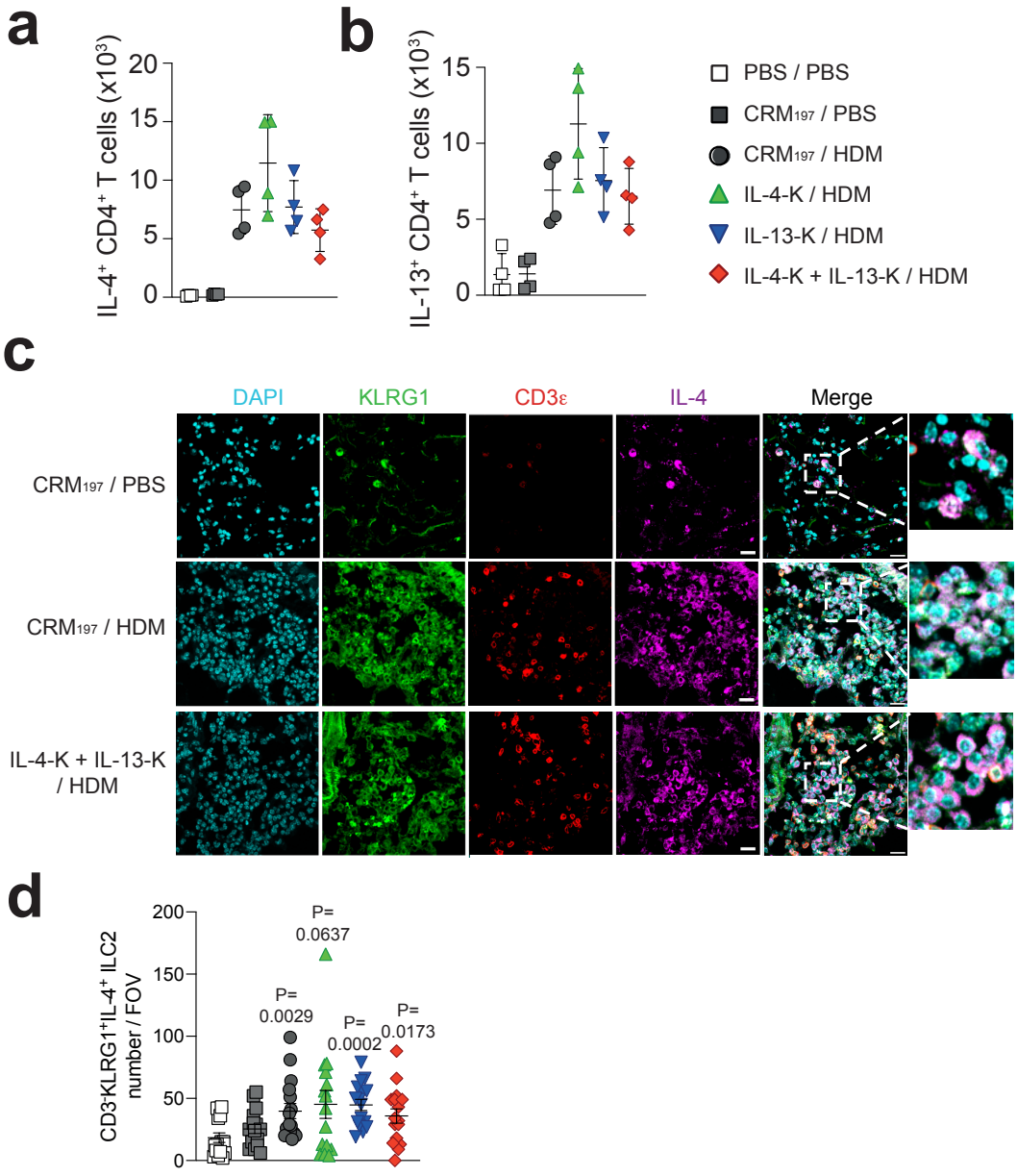
Supplementary Figure 5. Anti-IL-4 and anti-IL-13 neutralizing capacity one year after vaccination. Anti-IL-4 (**a**) and anti-IL-13 (**b**) neutralizing capacity in sera collected one year after the first injection of IL-4-K and/or IL-13-K. Results show values from individual mice with bars indicating medians, and are expressed as the serum dilution factor (dil^{-1}) neutralizing 50 % of IL-4 or IL-13 activity (0 indicates no neutralizing capacity). Data are from a single experiment (n=5 m mice in IL-13-K and IL-4-K+IL-13-K groups and n=10 mice in IL-4-K group). Source data are provided in the Source Data file.



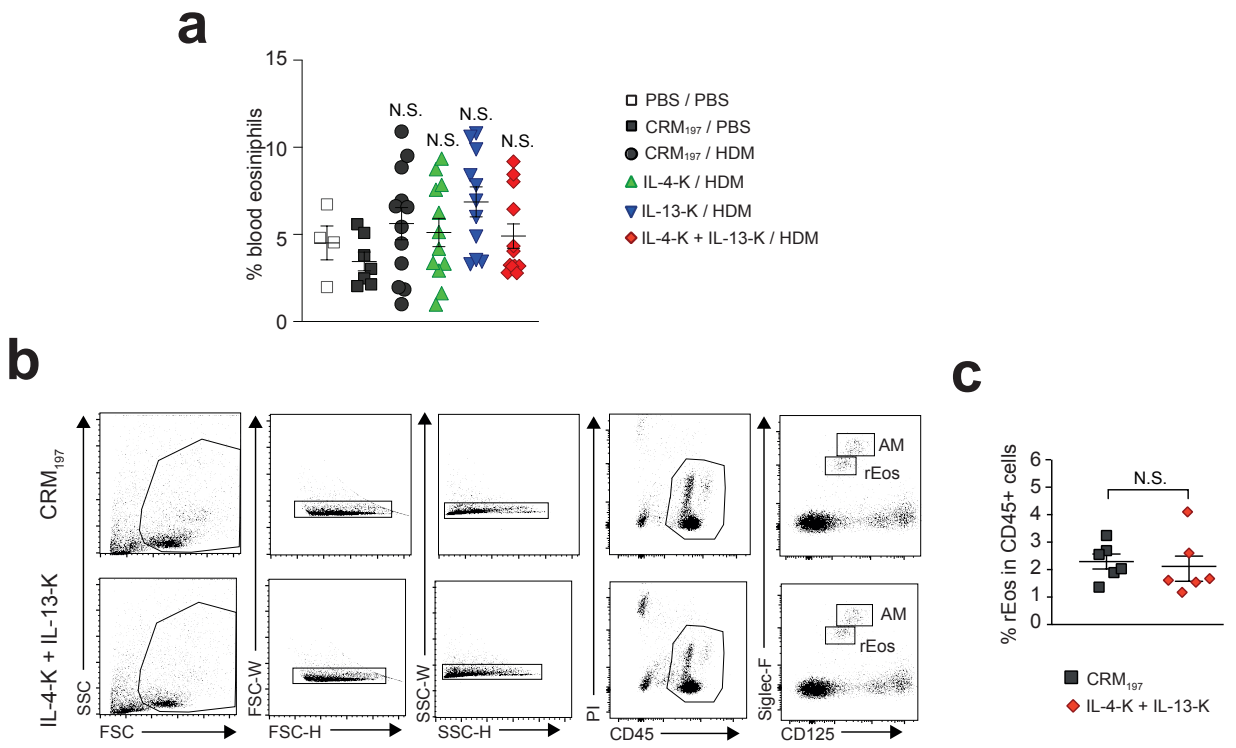
Supplementary Figure 6. Conjugation between IL-4/IL-13 and CRM_{197} is mandatory to mount a potent antibody response. a. Anti-IL-4 antibody titers in sera from mice immunized with IL-4-K or with a mixture of soluble IL-4 and CRM_{197} without prior conjugation. **b**. Anti-IL-13 antibody titers in sera from mice immunized with IL-13-K or with a mixture of soluble IL-13 and CRM_{197} without prior conjugation. Results in **a** and **b** show values from individual mice with bars indicating medians from *n*=10 (IL-4-K or IL-13-K) or *n*=5 (IL-4 + CRM_{197} or IL-13 + CRM_{197}) mice per group. **c**. Anti-IL-4 neutralizing capacity in sera from mice immunized with IL-4-K or with a mixture of soluble IL-4 and CRM_{197} without prior conjugation. **d**. Anti-IL-13 neutralizing capacity in sera from mice immunized with IL-13-K or with a mixture of soluble IL-13 and CRM_{197} without prior conjugation. Results in **c** and **d** show values from individual mice with bars indicating medians from *n*=10 (IL-4-K or IL-13-K) or *n*=5 (IL-4 + CRM_{197} or IL-13 + CRM_{197}) mice per group, and are expressed as the serum dilution factor (dil^{-1}) neutralizing 50 % of IL-4 or IL-13 activity (0 indicates no neutralizing capacity). Source data are provided in the Source Data file.



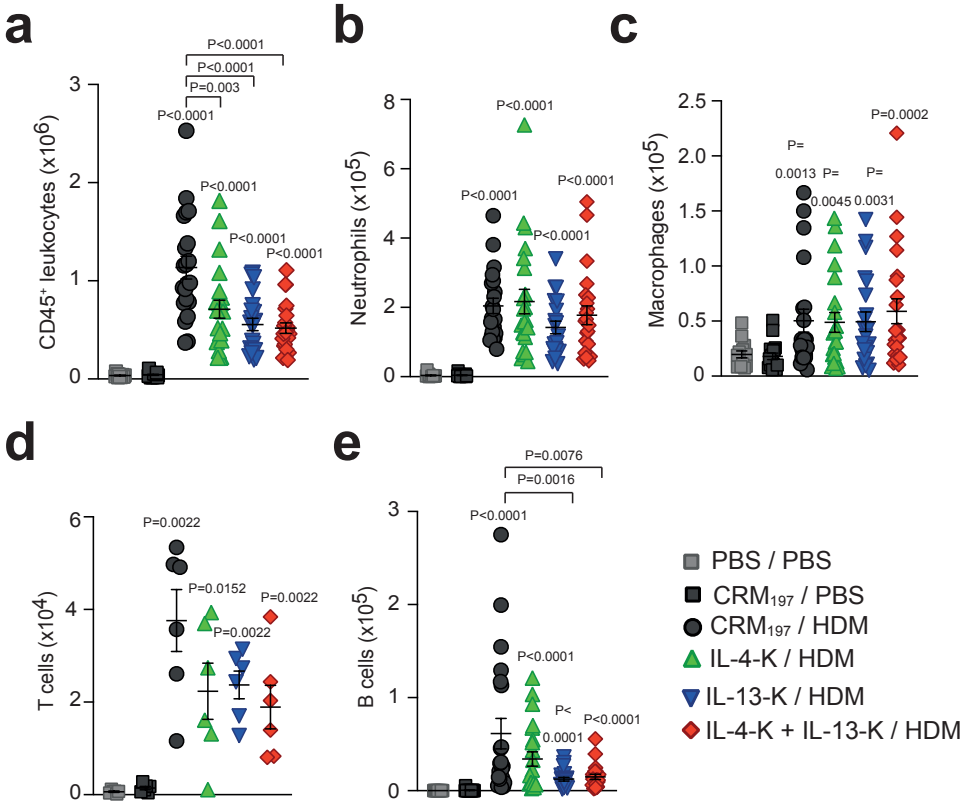
Supplementary Figure 7. Effects of IL-4-K and/or IL-13-K on HDM-induced airway hyperresponsiveness measured using non-invasive plethysmography. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenges with HDM (or PBS as a control), as outlined in Figure 1A. Penh responses to increasing doses of methacholine was measured using non-invasive plethysmography 24 hours after the last challenge. Data represent mean values ± SEMs and are pooled from two independent experiments. Adjusted *P*-values calculated using a 2-way ANOVA test followed by a Tukey posttest: ###: $P<0.0001$ and ##: $P=0.0019$ *vs*. CRM_{197}/PBS group; **: $P=0.0027$, ***: $P<0.0001$ *vs*. CRM_{197}/HDM group. Source data are provided in the Source Data file.



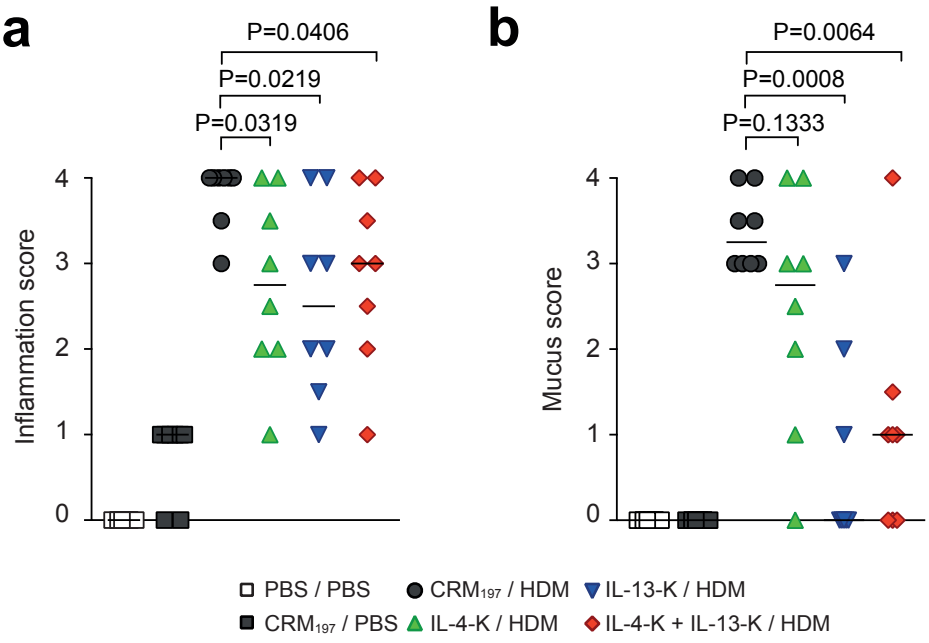
Supplementary Figure 8. Effects of IL-4-K and/or IL-13-K on IL-4$^+$ and IL-13$^+$ CD4 T cells and on IL-4+ ILC2. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM$_{197}$ as controls) followed by sensitization and challenges with HDM (or PBS as a control), as outlined in Figure 1A. **a-b.** Number of IL-4$^+$ CD4$^+$ T cells (**a**) IL-13$^+$ CD4$^+$ T cells (**b**) in the lung. Data show values from individual mice with bars indicating mean ± SEM, from a single experiment (*n*=4/group). **c.** Representative lung sections stained with anti-KLRG1 antibodies (green), anti-CD3ε (red), anti-IL-4 (purple) and DAPI (blue), 24 h after the last HDM challenge. Right panels represent magnification of the dashed areas. Scale bars: 30 µm. **d**. Number of ILC2 (identified as KLRG1$^+$CD3ε$^-$ cells) expressing IL-4. Results show values from individual field of view (FOV), with 4 FOV analyzed in each of 4 mice/group. Bars indicate means ± SEMs. *P* values *vs*. PBS/PBS group were calculated using two-tailed Mann-Whitney *U* test. Source data are provided in the Source Data file.



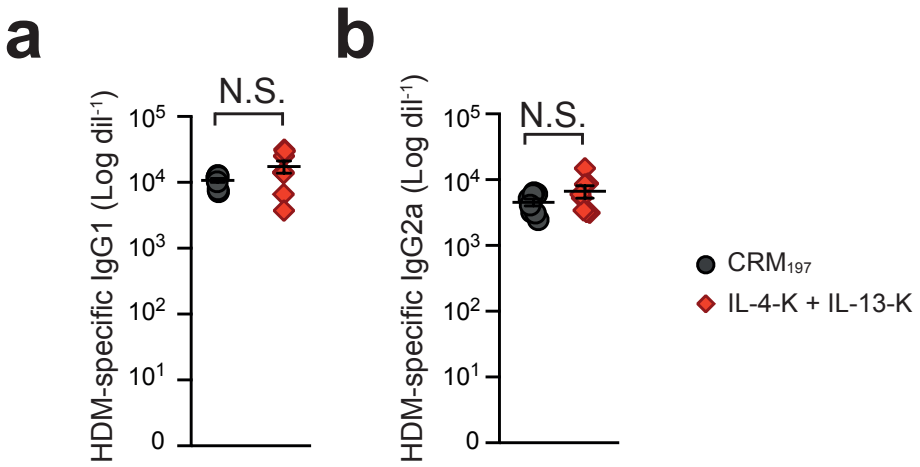
Supplementary Figure 9. Effect of vaccination with IL-4-K and/or IL-13-K on blood eosinophils and lung-resident regulatory eosinophils. a. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenge with HDM (or PBS as a control), as outlined in Figure 1A. Percentage of SSC^{high} Siglec-F^{+} blood eosinophils 24 h after the last HDM challenge. Results show values from individual mice with bars indicating means ± SEMs from *n*=4 mice (PBS/PBS control group) or *n*=7 mice (CRM_{197}/PBS control group) or *n*=11 (IL-13-K/HDM group) or *n*=12 mice (CRM_{197}/HDM, IL-4-K/HDM and IL-4-K+IL-13-K/HDM groups) pooled from two independent experiments. **b.** Dot plot of lung leukocytes showing strategy to gate regulatory eosinophils (rEos) according to Siglec-F and CD125 expression (as described previously[23]). AM: alveolar macrophages; FSC, forward scatter; SSC, side scatter; PI: propidium iodide. **c**. Percentage of regulatory eosinophils among resident $CD45^{+}$ lung leukocytes in mice vaccinated with IL-4-K and IL-13-K or CRM_{197} as a control. In a and c, results show values from individual mice with bars indicating means ± SEMs from *n*=6 mice per group pooled from two independent experiments. N.S.: not significant; $P > 0.05$ using two-tailed Mann-Whitney *U* test. Source data are provided in the Source Data file.



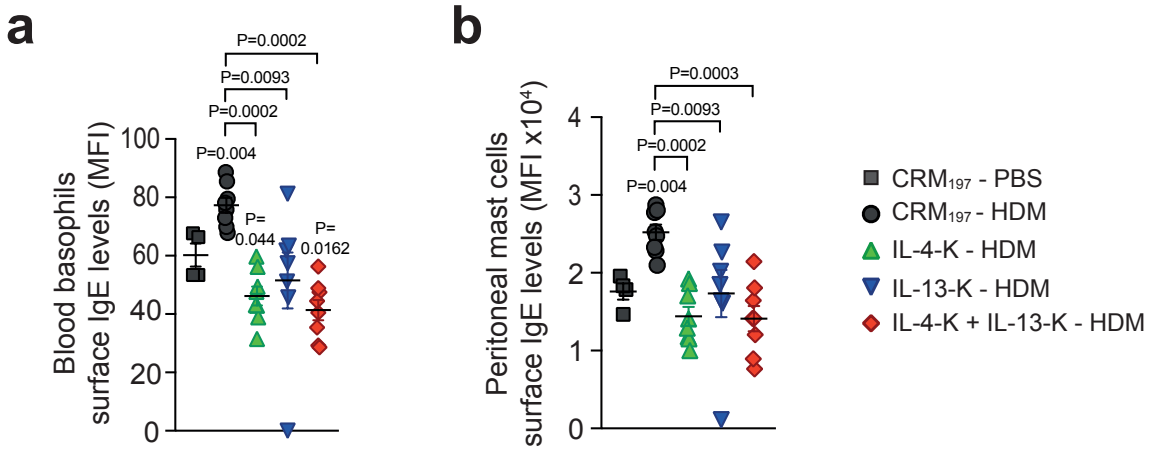
Supplementary Figure 10. Effects of IL-4-K and/or IL-13-K on BAL leukocytes in an asthma model. a-e. Numbers of $CD45^+$ total leukocytes (**a**), $CD45^+$, $CD11c^-$, $B220^-$, $CD3\varepsilon^-$, $Ly6G^+$, $CD11b^+$ neutrophils (**b**), $CD45^+$, $CD11c^+$, Siglec-F^+, $CD11b^+$ macrophages (**c**), $CD45^+$, $CD11c^-$, $CD3\varepsilon^+$ T cells (**d**) and $CD45^+$, $CD11c^-$, $B220^+$ B cells (**e**) in BAL fluid 24 h after the last HDM challenge. Data show values from individual mice with bars indicating mean ± SEM, from a single experiment (in d; *n*=6/group) or pooled from three independent experiments. In a-c and e, *n*=12 mice (PBS/PBS group), *n*=16 mice (CRM/PBS group), *n*=19 mice (IL-13-K/HDM group) or *n*=20 mice (IL-4-K and IL-4-K+IL-13-K groups. *P* values were calculated using two-tailed Mann-Whitney *U* test *vs*. CRM/PBS group or *vs*. indicated group. Source data are provided in the Source Data file.



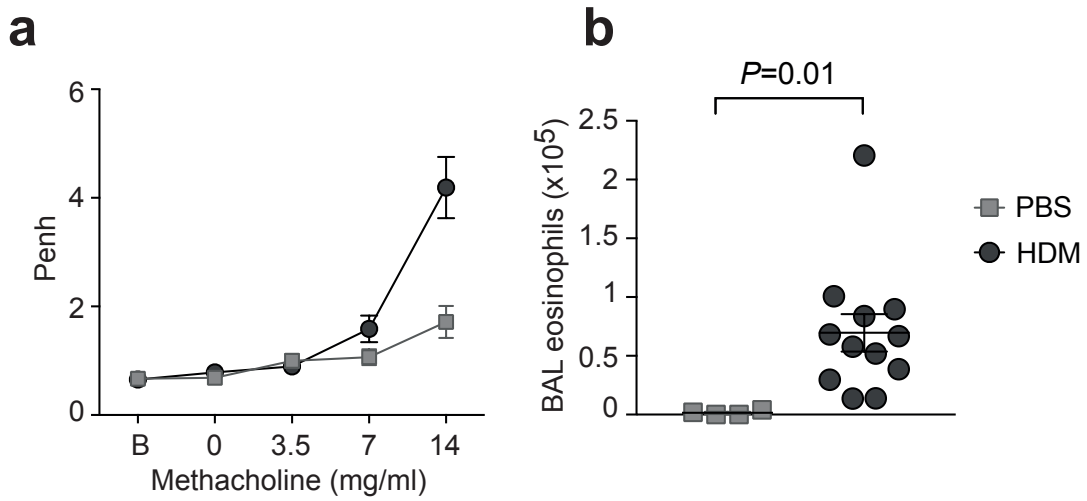
Supplementary Figure 11. Effects of IL-4-K and/or IL-13-K on lung inflammation and mucus production in an asthma model. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenge with HDM (or PBS as a control), as outlined in Figure 1a. **a**. The extent of leukocyte infiltration was scored in the H&E-stained lung tissue sections shown in Fig. 1h obtained 24 h after the last challenge with HDM. **b**. The extent of mucus production was scored according to the percentage of goblet cells in bronchial epithelium from the PAS-stained lung tissue sections shown in Fig. 1i obtained 24 h after the last challenge with HDM. Data show values from individual mice with bars indicating median, from a single experiment with n=8 mice per group. P values vs. CRM_{197}/HDM group were calculated using two-tailed Mann-Whitney U test. Source data are provided in the Source Data file.



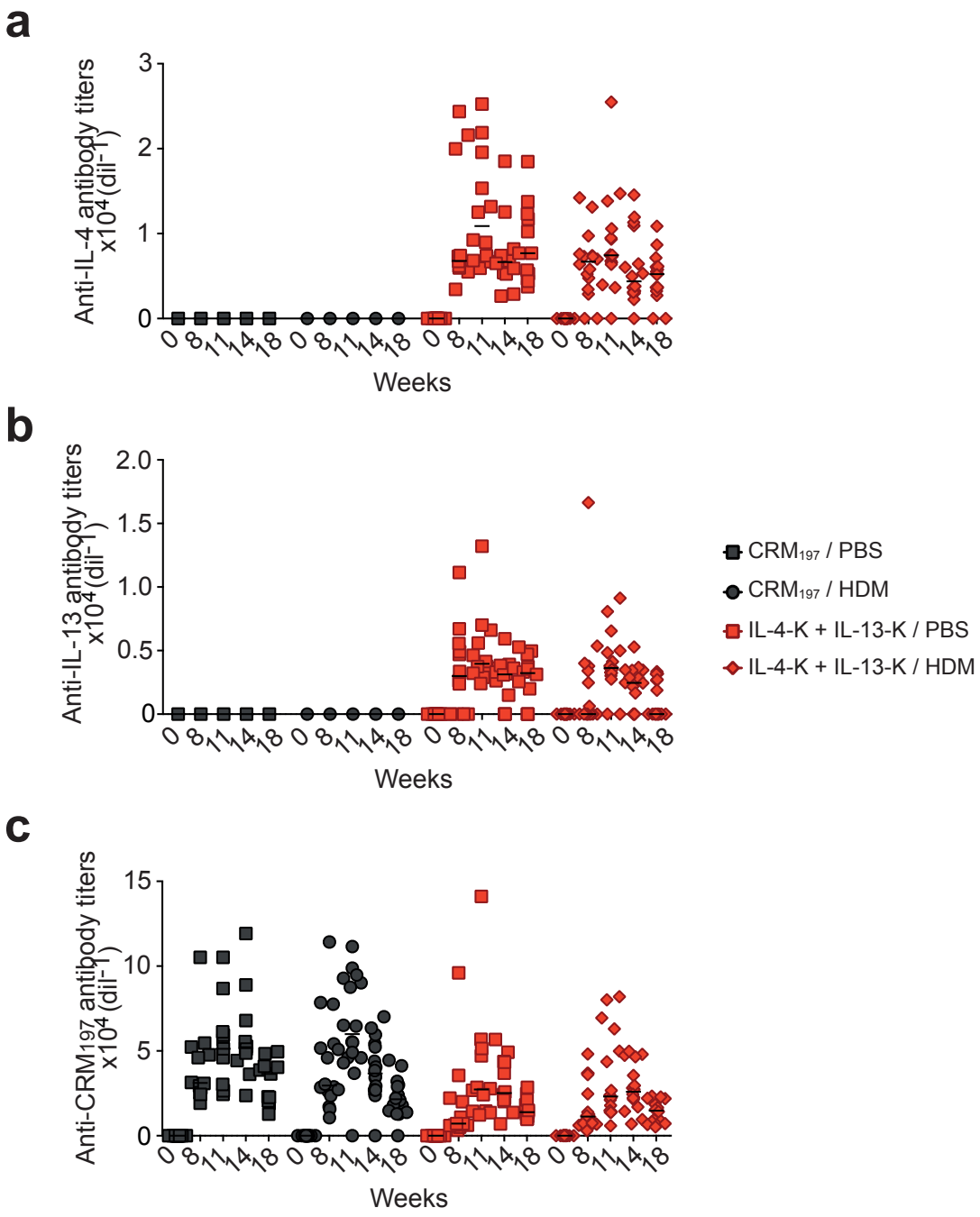
Supplementary Figure 12. Levels of HDM-specific IgG1 and IgG2a in mice vaccinated with IL-4-K and IL-13-K. Levels of HDM-specific IgG1 (**a**) and HDM-specific IgG2a (**b**) 24 h after the last HDM challenge in mice vaccinated with IL-4-K and IL-13-K, or CRM_{197} alone as a control. Results show values from individual mice with bars indicating mean ± SEM. Data are from a single experiment with n=8 mice per group, representative of two independent experiments. N.S.: not significant ($P > 0.05$ using two-tailed Mann-Whitney U test). Source data are provided in the Source Data file.



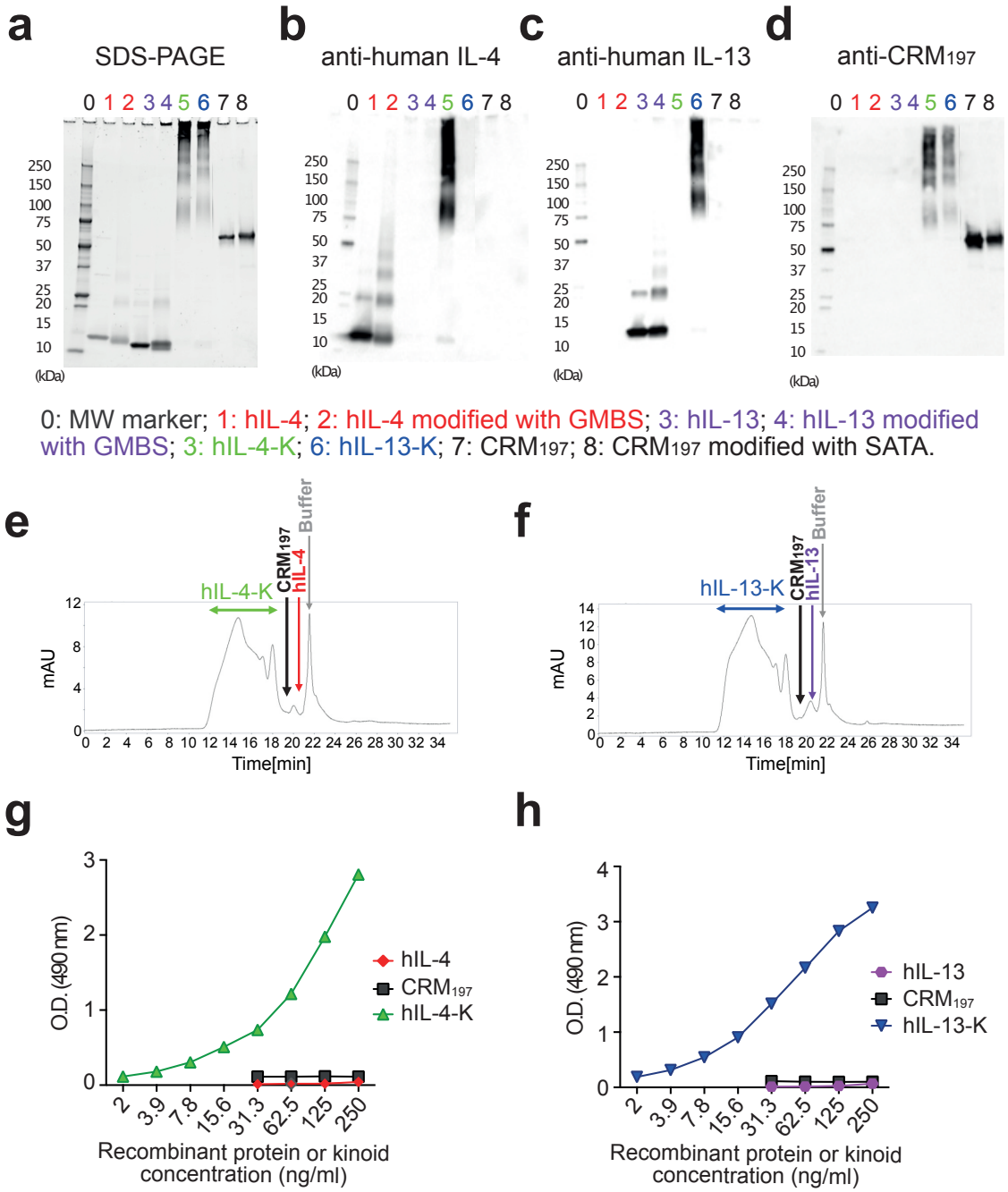
Supplementary Figure 13. Effects of IL-4-K and/or IL-13-K on surface IgE levels in blood basophils and peritoneal mast cells. Mice were vaccinated with IL-4-K and/or IL-13-K (or PBS or CRM_{197} as controls) followed by sensitization and challenge with HDM (or PBS as a control), as outlined in Figure 1**a**. Levels of IgE on the surface of IgE^+ $CD49b^+$ blood basophils (MFI: mean fluorescence intensity) (a), and levels of IgE on the surface of $CD117^+$ peritoneal mast cells (MFI) (b) 24 h after the last HDM challenge. Results show values from individual mice with bars indicating means ± SEMs from *n*=4 mice (CRM_{197}/PBS control group), *n*=7 mice (IL-13-K/HDM) or *n*=8 mice (IL-4-K/HDM, IL-4-K+IL-13-K/HDM) pooled from two independent experiments. *P* values vs. CRM_{197}/PBS group or *vs*. indicated group were calculate using two-tailed Mann-Whitney U test. Source data are provided in the Source Data file.



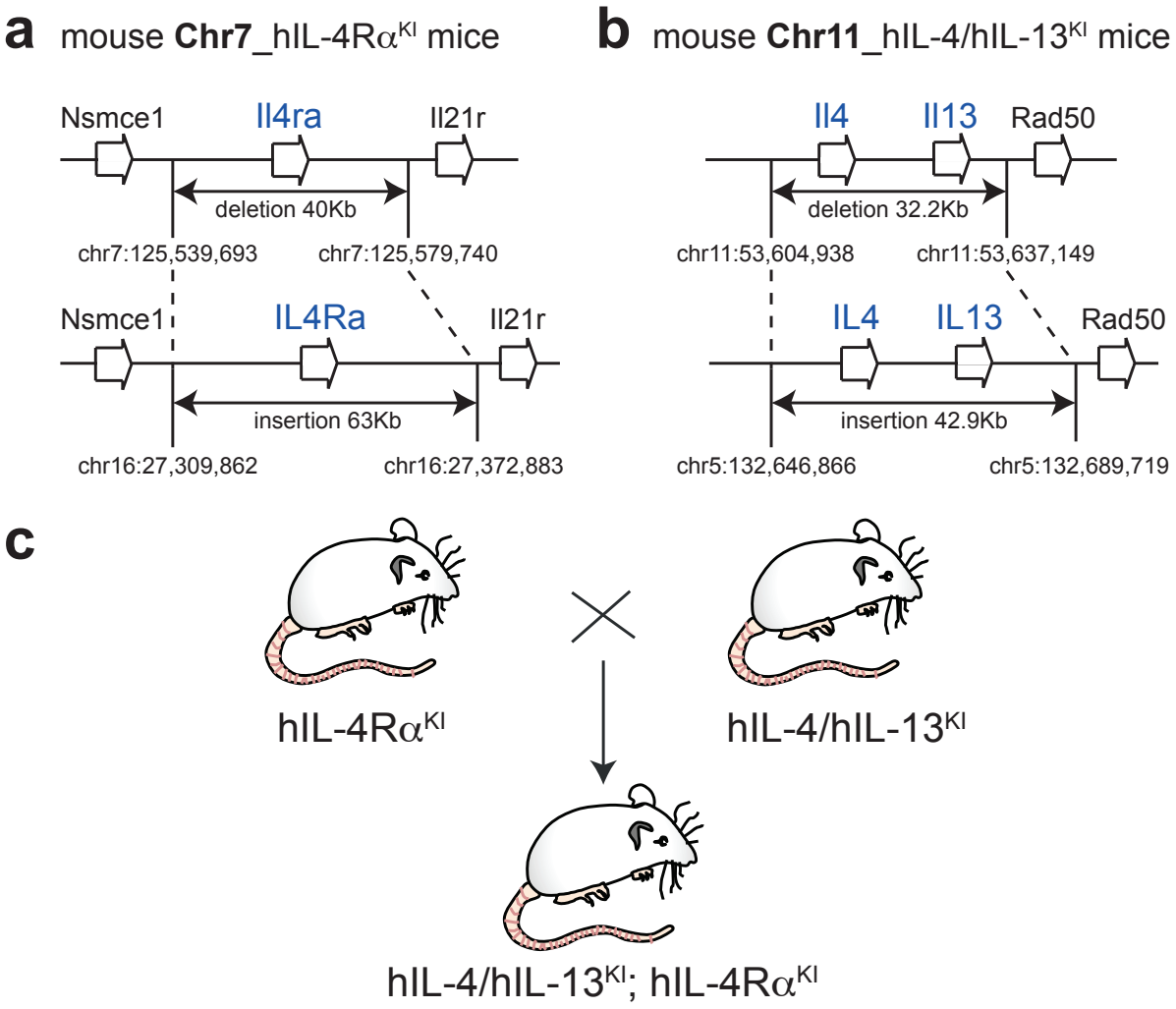
Supplementary Figure 14. Evidence of established airway hyperreactivity and eosinophilia before the start of therapeutic vaccination. Mice were sensitized and challenged with HDM or PBS as a control, as outlined in Figure 3A. Penh responses to increasing doses of methacholine measured using non-invasive plethysmography (**a**), and numbers of eosinophils in BAL fluid (**b**) 24 hours after the third challenge (*i.e.* immediately before vaccination with IL-4-K and IL-13-K). Data represent mean values ± SEMs from n=4 mice (PBS group) or n=12 mice (HDM group) pooled from two independent experiments. P values were calculated using two-tailed Mann-Whitney U test. Source data are provided in the Source Data file.



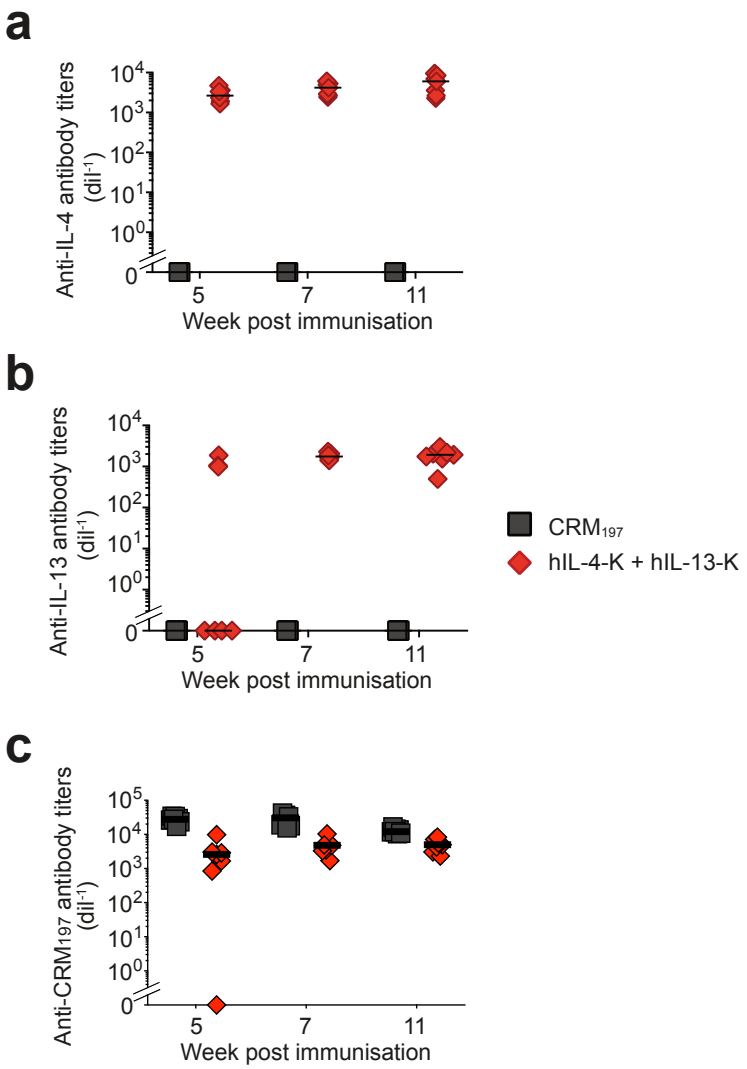
Supplementary Figure 15. Anti-IL-4, anti-IL-13 and anti-CRM$_{197}$ antibody titers upon therapeutic dual vaccination with IL-4-K and IL-13-K. Mice were sensitized and challenged with HDM extract (or PBS as a control). After the third challenge, mice were vaccinated with IL-4-K and IL-13-K in combination (or CRM$_{197}$ as control), as outlined in Figure 3A. Anti-IL-4 (**a**), anti-IL-13 (**b**) and anti-CRM$_{197}$ antibody titers (**c**) in sera from mice at indicated time points. Results show values from individual mice pooled from two independent experiments with bars indicating median (n=12 for all PBS groups and n=16 for all HDM groups). Source data are provided in the Source Data file.



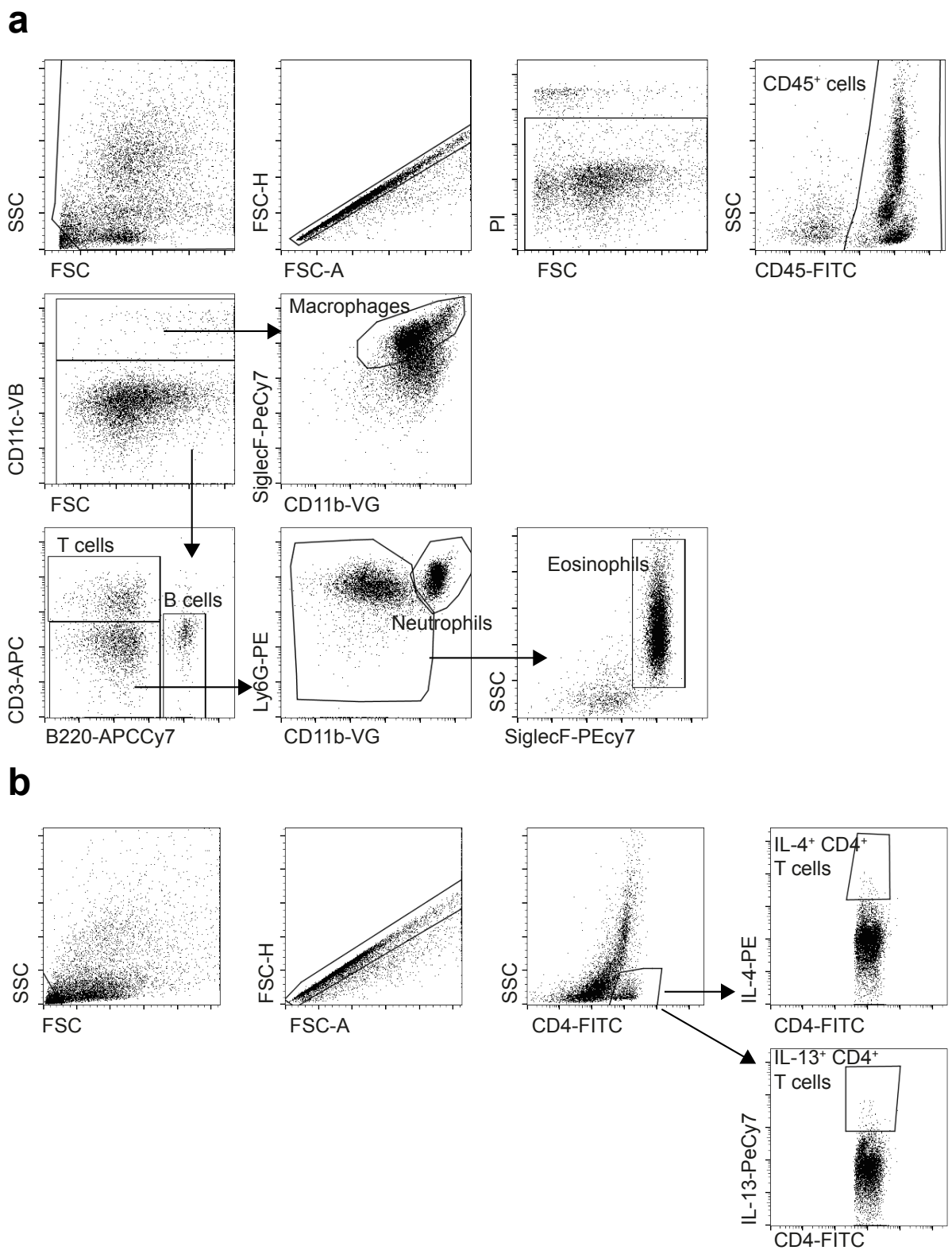
Supplementary Figure 16. Characterization of human IL-4-K and IL-13-K. Generation of high molecular weight kinoids upon conjugation of human IL-4 or human IL-13 to CRM_{197} was confirmed using SDS-PAGE (**a**), western blots against human IL-4 (**b**), human IL-13 (**c**) or CRM_{197} (**d**), and size exclusion (SE)-HPLC (**e** and **f**). Epitope preservation and antigenicity of human IL-4-K (hIL-4-K) and hIL-13-K was analyzed by sandwich ELISA using plates coated with anti-CRM_{197} antibodies and revealed with anti-human IL-4 (**g**) or anti-human IL-13 (**h**) antibodies. Data from a-d are representative of 3 independent experiments. Source data are provided in the Source Data file.



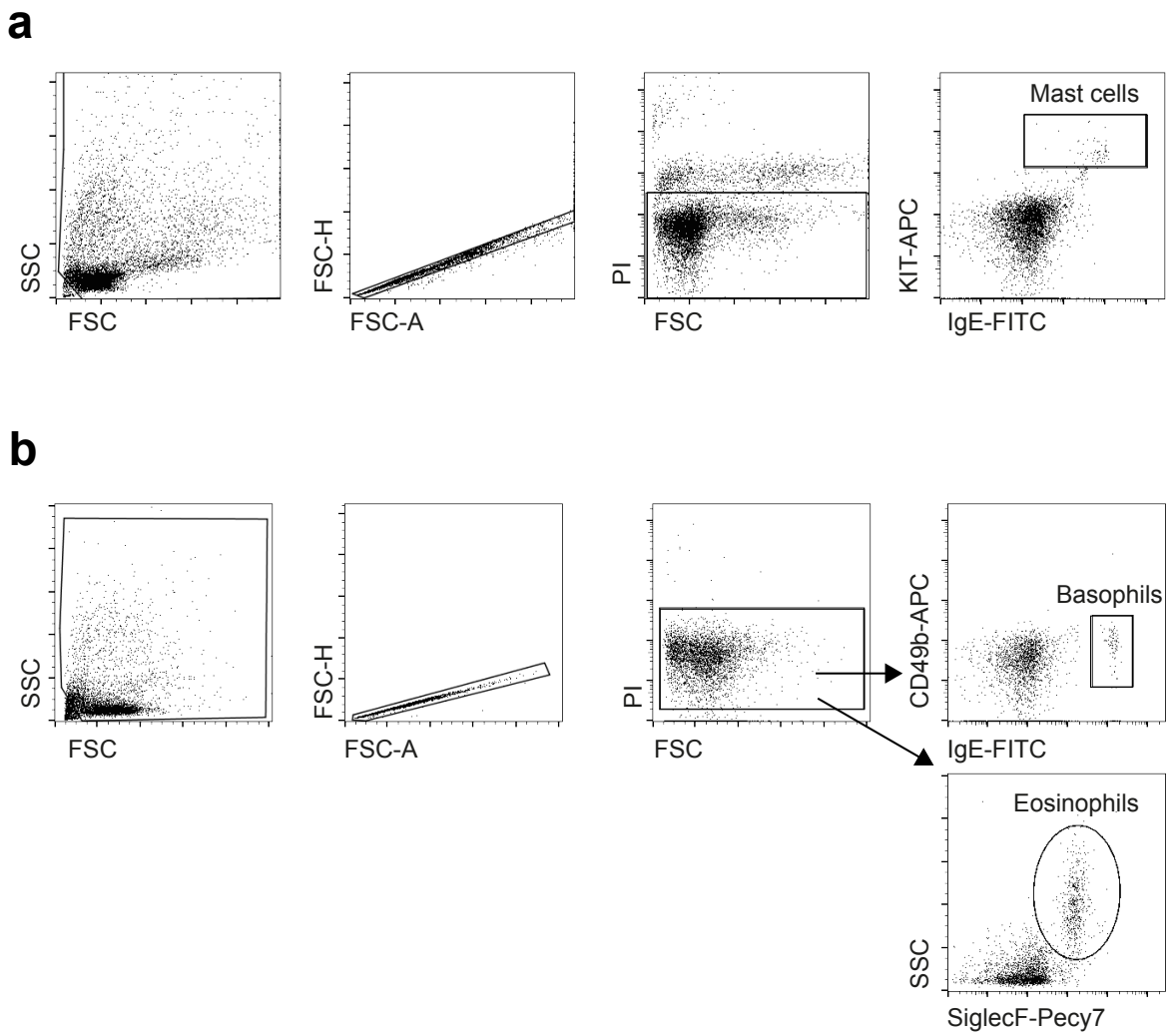
Supplementary Figure 17. Generation of hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice. a-b. Humanization of the mouse Il4ra locus (**a**) and il4/il13 locus (**b**). Representations are not drawn to scale. **c**. Breeding scheme to obtain hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice.



Supplementary Figure 18. Anti-hIL-4, anti-hIL-13 and anti-CRM$_{197}$ antibody titers upon dual vaccination with human IL-4 and IL-13 kinoids in IL-4/IL-13^{KI}; IL-4Rα^{KI} humanized mice. IL-4/IL-13^{KI}; IL-4Rα^{KI} humanized mice were vaccinated with hIL-4-K and hIL-13-K in combination (or CRM$_{197}$ as control), as described in Fig. 4a. Anti-human IL-4 (**a**), anti-human IL-13 (**b**) and anti-CRM$_{197}$ (**c**) antibody titers in sera at the indicated time-points. Results show values from individual mice with bars indicating medians (n=7 mice per group). Source data are provided in the Source Data file.



Supplementary Figure 19. Gating strategy for the analysis of leukocyte populations in BAL and lung cell suspensions (**a**) and intracellular IL-4 and IL-13 levels in lung CD4+ T cells (**b**) by flow cytometry.



Supplementary Figure 20. Gating strategy for the analysis of peritoneal mast cells (**a**) and blood basophils and eosinophils (**b**) by flow cytometry.

ANTIBODY	SOURCE	CLONE	IDENTIFIER	Dilution
anti-mouse IL-4 polyclonal goat IgG	R&D systems	n/a	AF-404-NA	0.1 µg/ml
anti-human IL-4 polyclonal goat IgG	R&D systems	n/a	AF-204-NA	0.1 µg/ml
anti-mouse IL-13 polyclonal goat IgG	R&D systems	n/a	AF-413-NA	0.1 µg/ml
anti-human IL-13 polyclonal goat IgG	R&D systems	n/a	AF-213-NA	0.1 µg/ml
anti-diphtheria toxin polyclonal goat IgG	AbD serotec	n/a	3710-0956	860 ng/ml
monoclonal mouse anti-diphtheria toxin IgG1	AbD serotec	8G1	3710-0100	1 µg/ml
anti-human IL-4 monoclonal mouse IgG1	R&D systems	3007	MAB304	1 µg/ml
anti-human IL-13 monoclonal mouse IgG1	R&D systems	32116	MAB213	1 µg/ml
biotinylated anti-mouse IL-4 polyclonal goat IgG	R&D systems	n/a	BAF-404	250 ng/ml
biotinylated anti-human IL-4 polyclonal goat IgG	R&D systems	n/a	BAF-204	250 ng/ml
biotinylated anti-mouse IL-13 polyclonal goat IgG	R&D systems	n/a	BAF-413	250 ng/ml
biotinylated anti-human IL-13 polyclonal goat IgG	R&D systems	n/a	BAF-213	250 ng/ml
anti-Ly6G-PE rat IgG_{2a}	BD Pharmingen	1A8	561104	1:100
anti-CD3-APC armenian hamster IgG_1	BD Pharmingen	145-2C11	561826	1:100
anti-CD4-FITC Rat IgG2bk	Miltenyi Biotec	GK1.5	130-120-819	1:50
anti-IL-4-PE rat IgG2b	Miltenyi Biotec	BVD4-1D11	130-103-016	1:20
anti-mouse IL-4-APC Rat IgG1k	eBioscience	11B11	53-7041-82	10 µg/ml
anti-IL-13-PE-Cyanine7 Rat IgG1	Fisher Scientific	eBio13A	15538636	1:200
anti-KLRG1-AF488 Hamster $IgG_2\kappa$	BD Pharmingen	2F1	562190	10 µg/ml
anti-CD3-AF532	eBioscience	17A2	58-0032-82	2 µg/ml
anti-CD45-FITC human IgG_1	Miltenyi Biotec	REA737	130-110-658	1:100
anti-CD45-VB human IgG1	Miltenyi Biotec	REA737	130-110-802	1:200
anti-Siglec-F-PECy7 human IgG_1	Miltenyi Biotec	REA798	130-112-334	1:100
anti-CD11b-VG recombinant human IgG_1	Miltenyi Biotec	REA713	130-110-559	1:100
anti-B220-APC rat IgG_{2a}	Miltenyi Biotec	RA3-6B2	130-102-259	1:100
anti-CD11c-VB, hamster IgG	Miltenyi Biotec	N418	130-102-797	1:100
anti-CD49b-APC, Rat IgM	eBioscience	DX5	17-5971-82	1:100
anti-IgE-FITC, Rat IgG_1	BD Pharmingen	R35-72	553415	1:50

anti-CD125-PE, Rat IgG1	BD Pharmingen	T21	558488	1:100
Purified NA/LE Rat Anti-Mouse CD124	BD Pharmingen	mIL4R-M1	552288	10 µg/mL
anti-Siglec-F-Alexa647, Rat IgG2a	BD Pharmingen	E50-2440	562680	1:100
anti-c-KIT APC	eBioscience	2B8	17-1171-82	1:200
rabbit polyclonal HRP-conjugated anti-mouse IgG	Invitrogen	n/a	61-6520	1:5000
Goat polyclonal Anti-Mouse IgG1 Human ads-HRP	Southern Biotech	n/a	1070-05	1:4000
Goat anti-Rat IgG (H+L), Alexa Fluor 594	Invitrogen	n/a	A11007	1:150
Goat polyclonal Anti-Mouse IgG2a Human ads-HRP	Southern Biotech	n/a	1080-05	1:4000
goat polyclonal anti-mouse IgE antibody	Bio-rad	n/a	STAR110	2 µg/mL
goat polyclonal HRP-conjugated anti-mouse IgG	Bethyl Laboratories	n/a	A90-131P	1:5000
anti-IgE antibody coupled to AF488, Rat IgG1, κ	Biolegend	RME-1	406910	10 µg/ml

Supplementary Table 1. List of antibodies used in this study.

REVIEWER COMMENTS

Reviewer #1 (Airway inflammation, type 2 immunity) (Remarks to the Author):

The authors demonstrate convincingly that vaccination against IL-4 and IL-13 can be achieved in wild type mice that are tolerized to these cytokines and furthermore that such vaccination is highly effective at preventing and attenuating HDM-mediated allergic airway disease. The results furthermore indicate that neutralizing antibody levels are quite durable over periods of up to a year. The experimental design, data description and analysis, and conclusions are outstanding. The figures are also of outstanding quality.

Major remaining questions and concerns:
1. How well can humans in general be vaccinated against these cytokines?
2. Will the vaccination approach be effective in persons at the extremes of age-young children and the elderly?
3. What is the long-term effect of having no IL-4/13 and no means of readily correcting this deficiency if needed?
Only human studies can of course answer these questions, but the discussion is quite brief and could be expanded to consider these issues not already addressed.

Other questions/issues possibly useful for considering in either the introduction or discussion:
1. Why did the authors not choose a simpler vaccination approach by choosing IL-4Rα as the antigen?
2. While the approach may be very helpful in asthma, it is likely to be equally or even vastly more useful in other conditions such as chronic urticaria; severe allergic rhinitis; chronic idiopathic anaphylaxis; food allergies; and chronic rhinosinusitis.
3. The approach ultimately works because it inhibits activation (by IL-4 and IL-13) of the transcription factor STAT6. Limiting the effectiveness of the approach-same concern with dupilumab-is that there are other soluble factors distinct from IL-4/13 that activate STAT6 in the allergic context, including TSLP (Han et al., J Invest Derm 2014) and cleaved fibrinogen (FCPs; Millien et al., Science 2013)

Other comments:

1. Line 191: humans, not men.
2. Line 56: though, not tough

David B. Corry, M.D.

Reviewer #2 (Allergy, asthma) (Remarks to the Author):

The work by Conde and colleagues reports on the effects of autovaccination for IL-4 and/or IL-13 in a murine model of allergic asthma to house dust mite. Using this elegant methodology, they were able to demonstrate the efficacy in this preclinical model that is relevant to targeted therapy of type 2 asthma, in particular with allergic phenotype, with the approved biologic dupilumab (anti-IL-4Ra mAb). Ony partly overlapping were observed when comparing IL-4 and IL-13 (and dual) blockade, consistently with previous studies. The study was very well designed, using a revelant exerimental model and readouts. Some issues could however be addressed to further elucidate the mechanisms of action.

Specific comments.
1. It is quite clear from the data that downstream targets of IL-4 and/or IL-13 are effectively altered following the vaccination protocol. It should be studied whether it also affected the functionality of type 2 T cells, either Th2 cells or ILC2 as well as the production of IL-5 which is shared by those cells and mediates tissue influx and activation of eosinophils. In addition, it xould be interesting to look whether it targets mainly effector eosinophils and not the recently reported regulatory eosinophils in the lung.

2. The authors documented by invasive lung function tests that the protocol affected AHR, at least in the prophylactic setting. therefore, in addition to mucus production, it should be confirmed whether the treatment approach (in particular targeting IL-13 or both IL-4/IL-13) did alter smooth muscle hypertrphy that is classically seen (at least in human asthma, but also in chronic experimental asthma).

3. It would be extremely interesting to see whether this strategy is applicable in human asthma patients, altough the reviewer aknowledge this will be the next step through an early phase trial. It could be however important, in order to get some translational applicability, to discuss the contrast between the efficacy of IL-13 vaccination in mice and the failure of anti-IL-13 mAbs in human (severe) asthma. Similarly, it should be discussed to what extent findings could be extrapolated to type 2 non allergic asthma (which may be also very eosinophilic, as some cases asssociated with nasal polyposis), notably because dupilumab efficacy does not rely on the allergic nature of asthma. Could the Authors comment on those translational aspects?
Charles Pilette, MD PhD.

Reviewer #3 (Asthma, allergy) (Remarks to the Author):

The manuscript by Conde et al. describes the effects of a dual vaccination against IL-4 and IL-13 in a mouse model of allergic asthma. As worded by the authors, the rationale for this work is that "dupilumab - a monoclonal antibody (mAb) against IL-4Ra that blocks both IL-4 and IL-13 signaling - is efficient at decreasing the rate of severe exacerbations, and at improving lung function in patients with moderate-to-severe asthma. However, use of this (or any other) mAb in chronic asthma is limited by high cost and the need to perform injections overs years to lifelong." This is a somewhat biased view. Costs are currently high but there is no reason they could not be lowered in the future. Most importantly, though, the need for lifelong therapy has the positive flipside that it makes it possible to stop treatment as needed – an option that does not appear to be available in the authors' approach. Type 2 immunity (both innate and adaptive) has protective, tissue repair-inducing properties, not to mention its protective effects against parasites and possibly viruses such as SARS-CoV-2. In this context, long-lasting elimination or even just suppression of type 2 responses by vaccination is a questionable, concerning goal. A more nuanced and practical alternative to addressing the asthma problem may be to prevent its inception by engaging natural regulatory/balancing mechanisms.

Some specific issues:
- The statement that "type 2 inflammation characterized by high levels of cytokines such as interleukin-4 (IL-4) and IL-13, high levels of IgE antibodies, and airway eosinophilia occurs in approximately 50 % of patients with asthma" is overall true but incomplete. Except for high IgE, these responses are essentially tissue-specific, not systemic. Therefore, it is not clear that a systemic suppressive approach would be optimal.
- Given the topic of this work, the authors may want to discuss the ligand/receptor interaction patterns that underpin the differential asthma-promoting effects of IL-4 and IL-13 despite their shared receptor.

- As mentioned above, reading that "Such neutralizing capacity could still be detected in more than 60 % of the mice over one year after primary immunization" is perplexing, given the life span of a mouse – especially because there is no switch off mechanism for this approach.
- For the reasons repeatedly mentioned above, the proposed vaccination strategy might be translationally significant (perhaps!) only as a treatment, not as prevention. However, the effects of dual vaccination in the therapeutic model are tenuous, especially for AHR.
- The authors should directly investigate, not just discuss, how their vaccination strategy affects protective type 2 responses, both innate and adaptive.
- The dissection of the differential effects of IL-4 and IL-13 in asthma pathogenesis, while interesting, is not novel.
- The humanized mice used in these experiments need to be described in some detail. In particular, it is important to specify whether they are knock-ins (as their denomination acronym might indicate) or they still carry the endogenous mouse genes. Result interpretation will be deeply affected in the latter case.

REVIEWER COMMENTS

Reviewer #1 (Airway inflammation, type 2 immunity) (Remarks to the Author):

The authors demonstrate convincingly that vaccination against IL-4 and IL-13 can be achieved in wild type mice that are tolerized to these cytokines and furthermore that such vaccination is highly effective at preventing and attenuating HDM-mediated allergic airway disease. The results furthermore indicate that neutralizing antibody levels are quite durable over periods of up to a year. The experimental design, data description and analysis, and conclusions are outstanding. The figures are also of outstanding quality.

Response: We thank Reviewer #1 for the positive comments about our work.

Major remaining questions and concerns:

1. How well can humans in general be vaccinated against these cytokines?

Response: We provide here a proof-of-concept of the efficacy of IL-4/IL-13 kinoids using mouse models, including humanized mice in which we tested a vaccine targeting the human cytokines. However, next steps of the development of this vaccine will include a toxicity study in cynomolgus monkeys, followed by an early phase trial in human to prove that this vaccine strategy can be applied in humans.

While this question can only be answered with a clinical study, it is important to note that a similar vaccination strategy has already been tested in human: a phase 2b trial recently assessed the effect of a kinoid targeting IFN-α (IFN-K) in *n*=185 adults with active systemic lupus erythematosus (SLE). This vaccine strategy has proven to be safe and to induce a neutralizing antibody response against IFN-α in 91% of treated patients (Houssiau *et al*. *Ann Rheum Dis*, 2020 Mar;79(3):347-355). We are thus confident that the kinoid strategy could also be efficient against IL-4 and IL-13 in humans, and are pursuing the development of this vaccine in order to start clinical trials within two years. In a follow-up study of the phase I/II trial with the IFN-K (Ducreux *et al. Rheumatology*, 2016), it was noted that most patients had a ≥ 10-fold reduction in anti-IFN-α antibody levels one year after the first injection of the vaccine. Neutralizing antibodies were still detectable at last follow-up visit in 29% (6/21) patients who received IFN-K (range of persistence: 0.45–4.2 years). Although the strength and duration of the antibody response induced by the IL-4/13 kinoids will need to be determined in trials, they may be similar to the responses observed with the IFN-α kinoid. In our pre-clinical models, we observed 60 % persistence one year after primary immunization. We now added this information in the discussion part:

Pages 14-15 lines 303-316 "We also provided a proof-of-concept of the efficiency of kinoids targeting human IL-4 and IL-13 in mice humanized for these cytokines and their receptor IL-4Rα. These promising results will now need to be confirmed in clinical studies. In this regard, while IL-4 and IL-13 vaccines have never been tested in human, a kinoid targeting interferon alpha (IFN-α) has recently been tested in a phase 2b study in 185 adults with active systemic lupus erythematosus[41]. This IFN-α kinoid induced a neutralizing response against IFN-α in 91% of treated patients, with an acceptable safety profile[41]. In a follow-up study of the phase I/II trial, it was noted that most patients had a ≥ 10-fold reduction in anti-IFN-α antibody levels one year after the first injection of the vaccine[42]. Neutralizing antibodies were still detectable at last follow-up visit in 29 % (6/21) patients who received the IFN-α kinoid (range of persistence: 0.45-4.2 years). Although the strength and duration of the antibody response induced by the human IL-4/13 kinoids will need to be determined in clinical trials, they may be similar to the responses observed with the IFN-α kinoids. In our pre-clinical models, we observed 60 % persistence one year after primary immunization".

2. Will the vaccination approach be effective in persons at the extremes of age-young children and the elderly?

Response: We thank Reviewer #1 for this important comment. IL-4/13 kinoids are conjugate vaccines similar to polysaccharidic vaccines in which T cell help is provided by the carrier protein. For polysaccharidic vaccines, the clear benefits of conjugate vaccines in improving the protective responses of the immature immune systems of young infants, adults and the senescent immune systems of the elderly have been made clear (Pichichero, *Hum Vaccin Immunother*, 2013, 9:12, 2505–2523; Jackson *et al. Vaccine*, 2013 Aug 2;31(35):3577-84). It is thus possible that we would observe similar effect for IL-4/13 kinoids, and in any case clinical trial will need be conducted to demonstrate immunogenicity in these populations.

3. What is the long-term effect of having no IL-4/13 and no means of readily correcting this deficiency if needed? Only human studies can of course answer these questions, but the discussion is quite brief and could be expanded to consider these issues not already addressed.

Response: We thank Reviewer #1 for this comment. Long-term targeting of IL-4 and IL-13 could in theory affect many innate and adaptive immune responses. In particular, a key role for both cytokines has been demonstrated in mouse models of host defense against helminths. Regarding risks of helminth infection in humans, more than 1.5 billion people are currently infected with helminths worldwide (According to the World Health Organization [https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections]). However, to the best of our knowledge no clear data are reported on the effect of the therapeutic anti-IL-4Rα mAb dupilumab on the incidence and severity of helminths infections. This is likely due, at least in part, to the fact that dupilumab is typically administered in countries with low helminth infection rates. More "long-term" safety data are now available for dupilumab in both atopic dermatitis (AD) and asthma with several large clinical studies with treatment periods from 52 to 96 weeks. Overall, these studies demonstrate a good safety profile, which argues in favor of the feasibility of long-term targeting of IL-4 and IL-13. We now added this information in the discussion part:

Pages 15-16 lines 318-333: "Besides their detrimental role in allergies, IL-4 and IL-13 also play important protective and immunoregulatory functions. In particular, these cytokines can induce host defense responses against helminths infections, and have been implicated in the promotion of anti-inflammatory and tissue repair phenotypes in macrophages[43-45]. Thus, even though we did not observe apparent side effects of the vaccines in a one-year follow-up study in mice, further work is required to evaluate whether residual IL-4 and IL-13 activity after vaccination with kinoids is sufficient to sustain protective type 2 immune responses. This question is particularly important since treatment with IL-4/IL-13 kinoids in human would induce an antibody response which would likely last several months. Although data on the long-term effects of drugs targeting IL-4, IL-13 or other type 2 cytokines in human are still limited, it is important to note that several large clinical studies are now available for the anti-IL-4Rα mAb with treatment periods from 52 to 96 weeks in both asthma and atopic dermatitis[9,46-50]. Overall, these studies demonstrate a good safety profile, which argues in favor of the feasibility of long-term targeting of IL-4 and IL-13 with a vaccine strategy. Interestingly, the most common side effect noted is conjunctivitis, although this seems to be restricted to atopic dermatitis patients, as it is not observed for patients with moderate-to-severe asthma[9,46-48,51]".

Other questions/issues possibly useful for considering in either the introduction or discussion:

1. Why did the authors not choose a simpler vaccination approach by choosing IL-4Rα as the antigen?

Response: We decided to target IL-4 and IL-13 rather than IL-4Rα for three main reasons. First, this dual vaccination strategy leaves open the possibility to target only IL-4 or only IL-13 in other diseases, shall we find that such pathology is mainly driven by one of the cytokines. Also - and most importantly - dupilumab is produced as an IgG4 which has no functional effector interactions with FcγRs nor complement. This antibody format was chosen in order to reduce risks of antibody-dependent cellular toxicity (ADCC). In the case of the vaccine, a polyclonal antibody response against IL-4Rα would likely induce ADCC against a range of cells expressing IL-4Rα. In addition, such polyclonal response against the receptor could also be at risk of inducing antibodies which activate IL-4Rα. We thus decided to rather target the soluble cytokines in order to mitigate these risks. We now discuss this at the end of the introduction part:

Page 4 lines 78-84: "Based on these partial results, and on the superior clinical efficacy in human asthma of targeting both IL-4 and IL-13 signaling (i.e. dupilumab) rather than targeting either IL-4 or IL-13 alone[13-15], we hypothesized that a dual vaccination against IL-4 and IL-13 would be particularly potent at reducing the severity of chronic asthma. We decided to target IL-4 and IL-13 rather than IL-4Rα to minimize risks that the vaccine would induce antibodies which could activate this receptor and/or induce antibody-dependent cellular toxicity (ADCC)".

2. While the approach may be very helpful in asthma, it is likely to be equally or even vastly more useful in other conditions such as chronic urticaria; severe allergic rhinitis; chronic idiopathic anaphylaxis; food allergies; and chronic rhinosinusitis.

Response: We fully agree with Reviewer #1 that this vaccine approach could be useful in many conditions, not only asthma. Given the fact that dupilumab is already approved for asthma, we decided to first demonstrate the efficacy of the vaccine using validated asthma models. However, our laboratories have several studies that are now planned to further assess the efficacy of the vaccine in models of atopic dermatitis, food allergy, anaphylaxis, etc… We now acknowledge the fact that the use of this anti-IL-4/IL-13 vaccine could be extended to many other conditions in the last sentence of the discussion part:

Page 16 lines 338-340: "These results pave the way for the clinical development of an efficient long-term vaccine against asthma and other IL-4- and IL-13-mediated diseases, such as food allergies, atopic dermatitis or chronic urticaria".

3. The approach ultimately works because it inhibits activation (by IL-4 and IL-13) of the transcription factor STAT6. Limiting the effectiveness of the approach-same concern with dupilumab-is that there are other soluble factors distinct from IL-4/13 that activate STAT6 in the allergic context, including TSLP (Han et al., J Invest Derm 2014) and cleaved fibrinogen (FCPs; Millien et al., Science 2013)

Response: We thank Reviewer #1 for highlighting the fact that other factors could drive STAT6 activation in individuals vaccinated with IL4/13 kinoids. As stated by the Reviewer, this is also the case with dupilumab, which nevertheless is efficient in a majority of treated patients. However, this could explain why we observed residual allergic inflammation, low levels of IgE, and normal IgG1 levels in mice vaccinated with IL-4/13 kinoids. We now added this information in the discussion part:

Page 14 lines 304-307: "STAT6 activation is a key step in IL-4/IL-13 signaling[6]. However, it is important to note that STAT6 can also be activated by other mediators, including TSLP[39] or cleaved fibrinogen[40]. This could also contribute to the residual allergic inflammation, IgE and IgG1 levels observed in mice vaccinated with IL-4/IL-13 kinoids".

Other comments:

1. Line 191: humans, not men.

Response: We now corrected this mistake.

2. Line 56: though, not tough

Response: We now corrected this mistake.

Reviewer #2 (Allergy, asthma) (Remarks to the Author):

The work by Conde and colleagues reports on the effects of autovaccination for IL-4 and/or IL-13 in a murine model of allergic asthma to house dust mite. Using this elegant methodology, they were able to demonstrate the efficacy in this preclinical model that is relevant to targeted therapy of type 2 asthma, in particular with allergic phenotype, with the approved biologic dupilumab (anti-IL-4Ra mAb). Ony partly overlapping were observed when comparing IL-4 and IL-13 (and dual) blockade, consistently with previous studies. The study was very well designed, using a revelant exerimental model and readouts. Some issues could however be addressed to further elucidate the mechanisms of action.

Response: We wish to thank Reviewer #2 for the positive assessment of our work, and these helpful comments.

Specific comments.

1. It is quite clear from the data that downstream targets of IL-4 and/or IL-13 are effectively altered following the vaccination protocol. It should be studied whether it also affected the functionality of type 2 T cells, either Th2 cells or ILC2 as well as the production of IL-5 which is shared by those cells and mediates tissue influx and activation of eosinophils. In addition, it xould be interesting to look whether it targets mainly effector eosinophils and not the recently reported regulatory eosinophils in the

lung.

Response: We agree with Reviewer #2 that these are important aspects which were not included in the first version of our manuscript. We have now quantified IL-5 in bronchoalveolar lavage (BAL) fluid collected 24h after the last challenge with HDM. Our data indicate that vaccination against IL-4 and IL-13 markedly reduces levels of IL-5 in BAL fluid, which is in full agreement with the reduced eosinophilia in vaccinated mice. These new data are now presented in **Fig 1h**, and discussed in the main text:

Page 7 lines 134-136: "Consistent with these results, levels of IL-5 - a type 2 cytokine which plays a key role in eosinophilic inflammation[5] - were also markedly reduced in BAL fluid of HDM-treated mice vaccinated with IL-4-K and/or IL-13-K (**Fig. 1h**)."

In addition, we assessed intracellular levels of IL-4 and IL-13 in $CD4^+$ T cells by flow cytometry in single cell suspensions of lung tissues collected 24h after the last HDM challenge. Our data, which are presented in **Supplementary Fig. 8a and 8b**, indicate that the vaccine does not abrogate the ability of these cells to produce IL-4 and IL-13. We also quantified the number of $IL\text{-}4^+$ ILC2 (identified as $CD3^-KLRG1^+$ cells) in lung tissue by immunofluorescence. Similar to our data on CD4 T cells, we observed a slight elevation of $IL\text{-}4^+$ ILC2 in the lungs of HDM-treated mice as compared to PBS controls. However, $IL\text{-}4^+$ ILC2 numbers were not reduced in vaccinated mice. These new data are presented in **Supplementary Fig. 8c and 8d**. Altogether, these results suggest that vaccination against IL-4 and IL-13 does not abrogate the ability of T_H2 and ILC2 to produce these cytokines, but rather induces antibodies which neutralize these cytokines once released. We now discuss this in the main text of our manuscript:

Page 7 lines 136-142: "By contrast, we observed similar low levels of $IL\text{-}4^+$ and $IL\text{-}13^+$ $CD4^+$ T cells (**Supplementary Fig. 8, a and b**), as well as similar numbers of $IL\text{-}4^+$ type 2 innate lymphoid cells (ILC2) (**Supplementary Fig. 8, c and d**) in the lungs of HDM-treated controls and vaccinated mice. Altogether, these results suggest that vaccination with IL-4-K or IL-13-K does not abrogate IL-4 and IL-13 production in this asthma model, but rather induces antibodies which neutralize these cytokines once released".

Finally, as suggested by the Reviewer, we quantified numbers of tissue-resident eosinophils in the lungs of control mice and mice vaccinated against IL-4 and IL-13. We found no difference between both groups, indicating that the vaccine has no effect on the number of regulatory eosinophils in the lung at baseline. We discussed with Dr. Thomas Marichal, corresponding author of the paper describing these regulatory eosinophils (Mesnil *et al. J Clin Invest* 2016), who validated our gating strategy (we now thank him in the acknowledgement section). These data are presented in **Supplementary Fig. 9b and 9c**, and discussed in the main text of our manuscript:

Page 7 lines 147-150: "In addition, while numbers of effector eosinophils were markedly reduced in the lungs of vaccinated mice (**Fig. 1, f and g**), we observed no effect of the vaccine on the number of tissue-resident regulatory eosinophils[23] (**Supplementary Fig. 9, b and c**)".

In addition, we found that the vaccine mainly targets effector eosinophils and, to a lesser extent, B cells, but that numbers of neutrophils, macrophages and T cells were

similar in the BAL fluid of controls and vaccinated HDM-treated mice. These data are presented in a novel **Supplementary Fig. 10**, and discussed in the main text of our manuscript:

Page 7 lines 150-153: "The effects of the vaccine on leukocytes appeared to be mostly restricted to effector eosinophils and to a lesser extent B cells, as we observed similar levels of neutrophils, macrophages and T cells in the BAL fluid of HDM-treated mice vaccinated with IL-4-K and IL-13-K or CRM_{197} as a control (**Supplementary Fig. 10**)".

2. The authors documented by invasive lung function tests that the protocol affected AHR, at least in the prophylactic setting. therefore, in addition to mucus production, it should be confirmed whether the treatment approach (in particular targeting IL-13 or both IL-4/IL-13) did alter smooth muscle hypertrophy that is classically seen (at least in human asthma, but also in chronic experimental asthma).

Response: We thank Reviewer #2 for this suggestion. We now stained alpha smooth muscle actin (α–SMA) in lung tissue of all groups of mice collected 24h after the last challenge with HDM, in order to quantify smooth muscle hypertrophy in our model. Although the staining of α–SMA gave a clear peribronchial signal, we did not observe significant differences between PBS controls and HDM-treated mice (please see **Fig. A**, below). Thus, unfortunately the asthma model we used, although leading to clear AHR, eosinophilia and mucus production, does not permit us to assess the effect of the vaccine on smooth muscle hypertrophy.

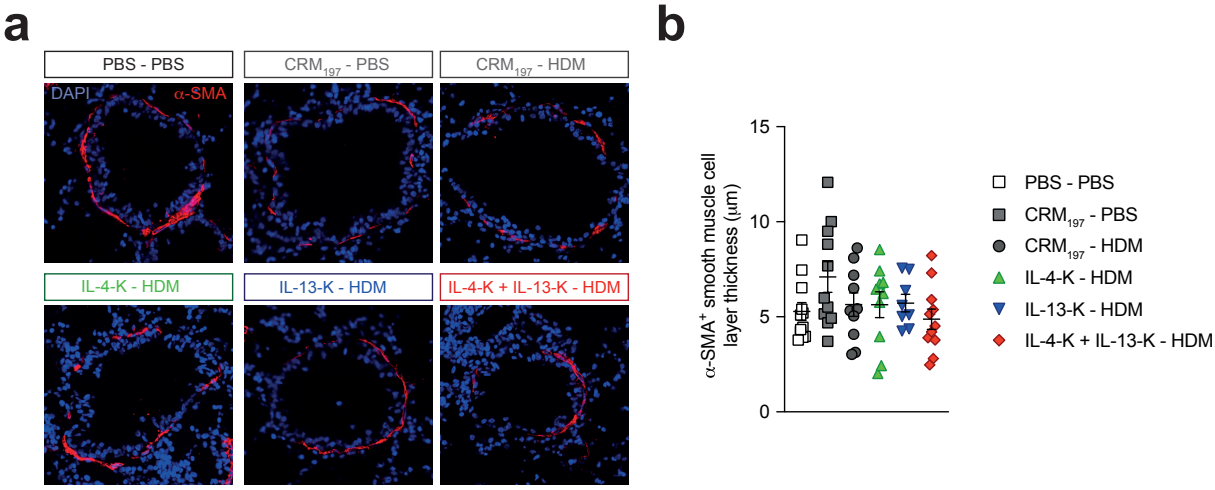


Fig A. Effect of HDM-treatment and vaccination with IL-4/IL-13 kinoids on alpha-smooth muscle actin in the lung. Mice vaccinated with IL-4-K and/or IL-13-K (or CRM_{197} as a control) were sensitized and challenged intranasally with HDM as described in Fig. 1a. **a**. Representative lung sections stained with anti-alpha smooth muscle actin (α-SMA) antibodies (which stains the peri-bronchial smooth muscle cell layer, red) and DAPI (which stains nucleus, blue) 24 h after the last challenge. **b**. Quantification of the thickness of the peribronchial α-SMA^+ smooth muscle cell layer. Results show mean values from individual bronchi (8-12 bronchi were analyzed from a total of 4 mice/group). Bars indicate means ± SEMs.

3. It would be extremely interesting to see whether this strategy is applicable in human asthma patients, altough the reviewer aknowledge this will be the next step through an early phase trial. It could be however important, in order to get some translational applicability, to discuss the contrast between the efficacy of IL-13 vaccination in mice

and the failure of anti-IL-13 mAbs in human (severe) asthma. Similarly, it should be discussed to what extent findings could be extrapolated to type 2 non allergic asthma (which may be also very eosinophilic, as some cases asssociated with nasal polyposis), notably because dupilumab efficacy does not rely on the allergic nature of asthma. Could the Authors comment on those translational aspects?

Response: Thank you for giving us the opportunity to discuss the translational applicability of the vaccine. We think that the contrast between the beneficial effect of the anti-IL-13 vaccine in mice and the failure of anti-IL-13 treatments in clinical trials is likely to be due to the difference in terms of outcomes analyzed. In fact, LAVOLTA and STRATOS, two large clinical trials, failed to demonstrate an effect of lebrikizumab and tralokinumab (two anti-IL-13 mAbs) on the reduction of asthma exacerbation (Hanania *et al. Lancet Respir Med*, 2016; Panettieri Jr *et al. Lancet Respir Med*, 2018). The mechanisms of asthma exacerbation are not fully understood, and are likely to rely on a combination of complex parameters, including eosinophilic (and/or neutrophilic) airway inflammation, IgE effector mechanisms and airflow obstruction. In our mouse study, some of these features were reduced upon vaccination against IL-13 (e.g. AHR and mucus production), while some others required combined vaccination against IL IL-4 and IL-13 (e.g. IgE production and lung eosinophilia).

In agreement with the effect of IL-13 vaccination on AHR in our mouse study, an increase in FEV1 (forced expiratory volume in 1 second) has been observed in clinical trials conducted with anti-IL-13 mAbs, especially when focusing on patients with biomarker evidence of type 2 asthma (high blood eosinophil counts or periostin concentrations, which is induced by IL-4 and IL-13) (Piper *et al. Eur Respir J*, 2013; Brightling *et al. Lancet Respir Med*, 2015 ; Hanania *et al. Lancet Respir Med*, 2016).

We speculate that combined blockade of IL-4 and IL-13 pathways is probably also required in order to efficiently reduce most features of asthma in human, leading to reduced asthma exacerbation. This would provide a potential explanation for the superior clinical efficiency of dupilumab over various therapeutic anti-IL-4 or IL-13 mAbs in asthma. As highlighted by the Reviewer, dupilumab has a beneficial effect on severe eosinophilic asthma regardless of the allergic status in humans. It is thus tempting to speculate that the IL-4/IL-13 vaccine could also have beneficial effects in type 2 non-allergic asthma (but of course this will need to be proven through clinical trials).

We now discuss these points in the discussion part:

Page 13 lines 268-281 "In line with our data in mice, IL-13 also plays an important role in controlling airway reactivity in human asthma. Indeed, an increase in FEV1 (forced expiratory volume in 1 second) has been observed in clinical trials conducted with anti-IL-13 mAbs[14,32,33], especially when focusing on patients with biomarker evidence of type 2 asthma (e.g., high blood eosinophil counts or periostin concentrations, which is induced by IL-4 and IL-13[34]). However, LAVOLTA and STRATOS, two large clinical trials, failed to demonstrate an effect of lebrikizumab and tralokinumab (two anti-IL-13 mAbs) on asthma exacerbation[14,35]. Thus, we speculate that combined blockade of IL-4 and IL-13 pathways is probably also required in order to efficiently reduce most features of asthma in human, as we observed here in mouse models with the IL-4 and IL-13 vaccines. This provides a potential explanation for the superior clinical efficiency

of dupilumab (which blocks both IL-4 and IL-13 signaling) over various therapeutic anti-IL-4 or IL-13 mAbs in asthma[13-15], and prompted us to focus on the dual vaccine for further evaluation in a therapeutic protocol".

Reviewer #3 (Asthma, allergy) (Remarks to the Author):

The manuscript by Conde et al. describes the effects of a dual vaccination against IL-4 and IL-13 in a mouse model of allergic asthma. As worded by the authors, the rationale for this work is that "dupilumab - a monoclonal antibody (mAb) against IL-4Ra that blocks both IL-4 and IL-13 signaling - is efficient at decreasing the rate of severe exacerbations, and at improving lung function in patients with moderate-to-severe asthma. However, use of this (or any other) mAb in chronic asthma is limited by high cost and the need to perform injections overs years to lifelong." This is a somewhat biased view. Costs are currently high but there is no reason they could not be lowered in the future.

Response: We thank Reviewer #3 for these comments. We certainly hope that the price of dupilumab (and other mAbs) will be lowered in the future. However, there is no sign of such trend at this time. Indeed, dupilumab is protected by patents until October 2029, which clearly blocks development of similar mAbs, as exemplified by the recent decision of the European Patent Office to invalidate Immunex European patent claiming mAbs that target IL-4R (http://sitepilot.firmseek.com/client/mckool2/www/media/news/159_2019%2002%2014%2096%20Final%20Written%20Decision.pdf). In addition, discounts for originator biologics facing biosimilar competition remain low (below 20%) compared to generic, and will not happen before all patents expire.

Current cost per year is around $37,000 for dupilumab. So even if prices were to be lowered in the future, this certainly would still represent substantial costs linked to manufacturing costs and repeated injections, as compared to a vaccine approach. Finally, the emergence of additional strategies to treat allergic diseases, such as a vaccine approach, could help lowering prices of therapeutic mAbs, including dupilumab, in the future.

Most importantly, though, the need for lifelong therapy has the positive flipside that it makes it possible to stop treatment as needed – an option that does not appear to be available in the authors' approach. Type 2 immunity (both innate and adaptive) has protective, tissue repair-inducing properties, not to mention its protective effects against parasites and possibly viruses such as SARS-CoV-2. In this context, long-lasting elimination or even just suppression of type 2 responses by vaccination is a questionable, concerning goal. A more nuanced and practical alternative to addressing the asthma problem may be to prevent its inception by engaging natural regulatory/balancing mechanisms.

Response: We fully agree with the Reviewer that a very important point for future clinical development of this vaccine would be its safety with regards to the ever-growing list of potential protective type 2 immune responses. This can include tissue repair, metabolism, neuroinflammation, cancer, helminth infection, etc. We fully

acknowledge the importance and concern of targeting such protective pathways, and this will be one of the main focus of our work in future years. As suggested by the Editor, and Reviewers #1 and #2, we have now added an extensive discussion on the implication, caveats, and potential long-term adverse effects in human application, as well as a deeper characterization of the effect of the vaccine on key innate cells including ILC2, neutrophils and macrophages, as well as T_H2 cells and the newly-described lung-resident regulatory eosinophils. In addition, we found that IgG antibody responses are intact in vaccinated mice, which is important in the current vision that COVID pandemic may last several years, or may be a recurrent epidemic. All these points are detailed in our responses to your specific comments, below, and in the response to the specific comment 1 from Reviewer #2 (pages 6-8, above).

Some specific issues:

1. The statement that "type 2 inflammation characterized by high levels of cytokines such as interleukin-4 (IL-4) and IL-13, high levels of IgE antibodies, and airway eosinophilia occurs in approximately 50 % of patients with asthma" is overall true but incomplete. Except for high IgE, these responses are essentially tissue-specific, not systemic. Therefore, it is not clear that a systemic suppressive approach would be optimal.

Response: We thank Reviewer #3 for this comment. We have now revised our sentence to reflect the fact that IL-4 and IL-13 responses and eosinophilia are mostly tissue-specific.

Page 3 lines 51-54: "type 2 inflammation characterized by production of interleukin-4 (IL-4) and IL-13 in the lung, airway eosinophilia and high levels of IgE antibodies occurs in approximately 50 % of patients with asthma[1,5]".

We agree that it would be very interesting in the future to develop a strategy to selectively block IL-4 and IL-13 locally in the airways. However, while local delivery is efficient for small molecules, and is currently under investigation for antibodies (especially small antibody fragments such as Fab fragments, nanobodies or ScFv), such approach is to date not applicable for a vaccine strategy. Interestingly, an anti-IL-13 Fab fragment has been tested in a model of allergic asthma in cynomolgus monkeys (Lightwood *et al. Am J Respir Crit Care Med*, 2018). The Fab fragment had moderate effects on BAL eosinophils and markedly reduced BAL IL-5, which is in agreement with our data in mice obtained with the IL-13 kinoid. However, even though the Fab fragment was delivered by nebulization, significant levels of the antibody fragment could be detected in the blood. Therefore, even with this approach, systemic effects of the anti-IL-13 therapy cannot be totally ruled out. We now discuss this in the discussion part of the manuscript:

Pages 13-14 lines 277-286: "The extent to which the protective effects of anti-IL-4 and IL-13 therapy in asthma reflects local blockade of the cytokines in the lung *vs*. systemic effects is still not fully understood. Interestingly, local delivery of an anti-IL-13 Fab fragment by nebulization has been tested in a model of allergic asthma in cynomolgus monkeys[36]. This Fab had moderate effects on BAL eosinophilia but markedly reduced BAL IL-5 levels, which is in full agreement with our data obtained in HDM-exposed mice vaccinated with the IL-13 kinoid. However, even though the anti-IL-13 Fab was

delivered by nebulization, significant levels of the antibody fragment could be detected in circulation. By extension one may consider that systemic effects of the anti-IL-13 kinoid therapy are conceivable, even if we did not detect any significant systemic effects".

2. Given the topic of this work, the authors may want to discuss the ligand/receptor interaction patterns that underpin the differential asthma-promoting effects of IL-4 and IL-13 despite their shared receptor.

Response: We thank Reviewer #3 for this suggestion. We now added a discussion of the ligand/receptor interaction patterns in the discussion part of our manuscript:

Pages 12-13 lines 254-261: "Such non-overlapping functions could be explained, at least in part, by the different receptor requirement for the two cytokines. The type 1 IL-4 receptor only recognizes IL-4, and is a heterodimer of IL-4Rα and the common gamma chain (γc)[6,29,30]. The type 2 IL-4 receptor binds both IL-4 and IL-13, and is a heterodimer of IL-4Rα and IL-13Rα1[6,29,30]. In addition, IL-13 (but not IL-4) also binds IL-13Rα2, which was long thought to function as a decoy receptor that limits the activity of IL-13[6,29,30]. However, evidence indicates that IL-13 signaling through IL-13Rα2 can induce production of TGF-β1[31]".

3. As mentioned above, reading that "Such neutralizing capacity could still be detected in more than 60 % of the mice over one year after primary immunization" is perplexing, given the life span of a mouse – especially because there is no switch off mechanism for this approach.

Response: While the lifespan of a laboratory mouse is around two years, we think that the duration of an immune response in mice cannot be extrapolated to human only based on the lifespan expectancy. In addition, in our mouse models we used 4 (in WT mice) or 5 (in humanized mice) injections of IL-4/13 kinoids in a relatively short period of time. Both the doses and timing of injections in human will need to be defined based on a dose-range-finding study in monkeys followed by clinical trials.

A kinoid directed against IFN-α (IFN-K) has already been tested in patients with systemic lupus erythematosus (SLE). A phase 2b trial recently conducted in *n*=185 adults showed that the vaccine strategy is safe and induces a neutralizing antibody response against IFN-α in 91% of treated patients (Houssiau *et al*. *Ann Rheum Dis*, 2020 Mar;79(3):347-355). In a follow-up study of the phase I/II trial with the IFN-K (Ducreux *et al. Rheumatology*, 2016), it was noted that most patients had a ≥ 10-fold reduction in anti-IFN-α antibody levels one year after the first injection of the vaccine. Neutralizing antibodies were still detectable at last follow-up visit in 29% (6/21) patients who received IFN-K (range of persistence: 0.45–4.2 years). Although the strength and duration of the antibody response induced by the IL-4/13 kinoids will need to be determined in clinical trials, they may be similar to the responses observed with the IFN-α kinoid (i.e. only be transient in the absence of booster doses of the vaccine). We now added this information in the discussion part:

Pages 14-15 Lines 303-316: "We also provided a proof-of-concept of the efficiency of kinoids targeting human IL-4 and IL-13 in mice humanized for these cytokines and their

receptor IL-4Rα. These promising results will now need to be confirmed in clinical studies. In this regard, while IL-4 and IL-13 vaccines have never been tested in human, a kinoid targeting interferon alpha (IFN-α) has recently been tested in a phase 2b study in 185 adults with active systemic lupus erythematosus[41]. This IFN-α kinoid induced a neutralizing response against IFN-α in 91% of treated patients, with an acceptable safety profile[41]. In a follow-up study of the phase I/II trial, it was noted that most patients had a ≥ 10-fold reduction in anti-IFN-a antibody levels one year after the first injection of the vaccine[42]. Neutralizing antibodies were still detectable at last follow-up visit in 29 % (6/21) patients who received the IFN-α kinoid (range of persistence: 0.45-4.2 years). Although the strength and duration of the antibody response induced by the human IL-4/13 kinoids will need to be determined in clinical trials, they may be similar to the responses observed with the IFN-α kinoids. In our pre-clinical models, we observed 60 % persistence one year after primary immunization".

4. For the reasons repeatedly mentioned above, the proposed vaccination strategy might be translationally significant (perhaps!) only as a treatment, not as prevention. However, the effects of dual vaccination in the therapeutic model are tenuous, especially for AHR.

Response: We agree that this vaccine should first be considered as a treatment, not as a prevention strategy. This is the reason why we also chose to test the vaccine using a therapeutic protocol in mice. We agree that the effect of the vaccine on AHR in this model is somehow less evident than in the prophylactic protocol, however both airway resistance and elastance were significantly reduced by the vaccine. Importantly, in this long model, mice were exposed to high doses of HDM twice a week for a total of 18 weeks, which permitted us to initiate the injection of the vaccine once significant AHR and eosinophilia had developed (as shown in **Supplementary Fig 14**). However, the repeated HDM injections over such a long period of time probably also initiated resolution pathways, as the overall AHR (in HDM-treated non-vaccinated mice) was lower in this model than in the shorter prophylactic protocol. This might, at least in part, account for the less evident effect of the vaccine on AHR in the therapeutic *vs*. prophylactic protocol. The therapeutic effects of the vaccine were however more evident when analyzing levels of IgE, airway eosinophilia and mucus production, which overall demonstrates the efficiency of the vaccine in this setting.

5. The authors should directly investigate, not just discuss, how their vaccination strategy affects protective type 2 responses, both innate and adaptive.

Response: As stated by the Reviewer, targeted therapies against IL-4/IL-13 could affect many innate and adaptive immune responses, and assessing the safety/potential long-term effects of the vaccine is our main priority prior to any clinical development of such vaccine. While we are planning to assess the effect of the vaccine in various models of innate immune responses, and in host defense against parasites, these models require new ethic protocol approval, and will require substantial time to assess the effects of the vaccine in an unbiased manner (assessing effects at different time-points after vaccination; comparing results to that obtained with IL-4Rα KO mice, or IL-4/13 KO mice; comparing responses of hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} humanized mice vaccinated with hIL-4/hIL-13 kinoids *vs*. treatment with dupilumab).

Regarding risks of helminth infection, more than 1.5 billion people are currently infected with helminths worldwide (According to the World Health Organization [https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections]). However, to the best of our knowledge no clear data are reported on the effect of dupilumab therapy (or other drugs targeting type 2 cytokines) on the incidence and severity of helminths infections. This is likely due, at least in part, to the fact that dupilumab is most often administered in countries with low helminth infection rates. More "long-term" safety data are now available for dupilumab in both atopic dermatitis (AD) and asthma with several large clinical studies with a 52-week treatment period in both asthma and atopic dermatitis (AD). Overall, these studies demonstrate a good safety profile, which argues in favor of the feasibility of long-term targeting of IL-4 and IL-13. We now added this information in the discussion part:

Pages 15-16 lines 318-333: "Besides their detrimental role in allergies, IL-4 and IL-13 also play important protective and immunoregulatory functions. In particular, these cytokines can induce host defense responses against helminths infections, and have been implicated in the promotion of anti-inflammatory and tissue repair phenotypes in macrophages[43-45]. Thus, even though we did not observe apparent side effects of the vaccines in a one-year follow-up study in mice, further work is required to evaluate whether residual IL-4 and IL-13 activity after vaccination with kinoids is sufficient to sustain protective type 2 immune responses. This question is particularly important since treatment with IL-4/IL-13 kinoids in human would induce an antibody response which would likely last several months. Although data on the long-term effects of drugs targeting IL-4, IL-13 or other type 2 cytokines in human are still limited, it is important to note that several large clinical studies are now available for the anti-IL-4Rα mAb with treatment periods from 52 to 96 weeks in both asthma and atopic dermatitis[9,46-50]. Overall, these studies demonstrate a good safety profile, which argues in favor of the feasibility of long-term targeting of IL-4 and IL-13 with a vaccine strategy. Interestingly, the most common side effect noted is conjunctivitis, although this seems to be restricted to atopic dermatitis patients, as it is not observed for patients with moderate-to-severe asthma[9,46-48,51]".

6. The dissection of the differential effects of IL-4 and IL-13 in asthma pathogenesis, while interesting, is not novel.

Response: We are glad that the Reviewer considers that our dissection of the differential effects of IL-4 and IL-13 through separate vaccination is interesting. We agree that such differential effects are not novel, and we cite and discuss previous findings in both human and mouse models on this topic. However, we consider that it was essential to show that such results can be reproduced through a vaccine strategy, and to demonstrate that optimal protective effects of the vaccine can only be obtained upon dual targeting of IL-4 and IL-13.

7. The humanized mice used in these experiments need to be described in some detail. In particular, it is important to specify whether they are knock-ins (as their denomination acronym might indicate) or they still carry the endogenous mouse genes. Result interpretation will be deeply affected in the latter case.

Response: We thank Reviewer #3 for this very important comment. We generated hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice by syntenic replacement of two mouse loci: that

encoding *Il4/Il13* and the second encoding *Il4ra*, with the corresponding human segment of DNA. Therefore, these hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice express the human genes in the place of the mouse gene, and thus cytokine receptor interactions in these animals model those of the human proteins. We now added a scheme (new **Supplementary Fig. 17**) describing the strategy chosen to replace mouse IL-4/IL-13 and IL-4Rα by the human proteins in these mice. We now further characterized this novel humanized mouse strain, and results of these experiments are presented in a novel **Figure 4**. In particular, we confirmed by ELISA that splenocytes from these mice (but not from WT mice) can release human IL-4. We obtained similar results with human IL-13, and further showed that significant levels of human IL-13 can be detected in BAL fluid from hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice following chronic intranasal sensitization and challenge.

Page 10 lines 206-225: "Low interspecies similarity of IL-4 (~44 %) and IL-13 (~55 %) between mice and human would render mouse IL-4-K and IL-13-K highly immunogenic in humans, and less potent to generate neutralizing responses. We therefore developed and characterized kinoids eliciting an immune response against human IL-4 and IL-13 (hIL-4-K and hIL-13-K) (**Supplementary Fig. 16**), and used mice humanized for IL-4, IL-13 and for their common receptor chain IL-4Rα (hIL-4^{KI}; hIL-13^{KI}; hIL-4Rα^{KI} mice) by syntenic replacement of two mouse loci: that encoding *Il4/Il13* and the second encoding *Il4ra*, with the corresponding human segment of DNA. These hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice express the human genes in the place of the mouse gene, and thus cytokine receptor interactions in these animals model those of the human proteins (**Supplementary Fig. 17**). We confirmed that splenocytes from hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice, but not from WT mice, release human IL-4 upon stimulation with PMA and ionomycin (**Fig. 4a**). We obtained similar results with human IL-13, and further showed that significant levels of human IL-13 can also be detected in BAL fluid from hIL-4/hIL-13^{KI}; hIL-4Rα^{KI} mice following chronic intranasal sensitization and challenge (**Fig. 4, b and c**). We further confirmed expression of human IL-4Ra (and lack of expression of mouse IL-4Ra) by immunohistology in skin samples from hIL-4^{KI}; hIL-13^{KI}; hIL-4Rα^{KI} mice (**Fig. 4, d and e**). Finally, we showed that intranasal challenge with recombinant human IL-4 or human IL-13 leads to eosinophilia, lung inflammation and mucus production in hIL-4^{KI}; hIL-13^{KI}; hIL-4Rα^{KI} mice (**Fig. 4, f-j**). Altogether, these data demonstrate that this new hIL-4^{KI}; hIL-13^{KI}; hIL-4Rα^{KI} humanized mouse strain both produces and responds to hIL-4 and hIL-13".

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

All concerns adequately addressed; no further concerns.

Reviewer #2 (Remarks to the Author):

The Authors have appropriately addressed my concerns/remarks.

Reviewer #3 (Remarks to the Author):

Thank you for the opportunity to read the revised version of Dr. Reber's manuscript describing the effects of dual vaccination against IL-4 and IL-13 in chronic allergic experimental asthma models. As I stated in my initial review, the experiments are overall well done and well described, and the latest data provide further mechanistic details. I remain unimpressed by the results obtained using the vaccine as a treatment, but this is a relatively minor concern at this stage. My major concern is that I still cannot agree with the rationale and premise of this work – not because I think this vaccine may fail to suppress Th2 responses, but because it may succeed. The authors have not convinced me that disabling for a long, undefined period of time an entire arm of the immune system - one that likely evolved NOT to make us allergic or asthmatic but to protect the integrity of our mucosal tissues - is a sound, valid strategy against asthma. In fact, the Authors' arguments in this respect are rather lame (allergic asthma and active systemic lupus erythematosus are in distinct leagues of severity, and parasitic infections are only one of several conditions raising concern).
I realize that decisions about manuscripts ultimately rest with the Editors, whom we reviewers can only advise, and I am tempted to "hide" by providing a purely technical review of methodologies and figures, all of which are of good quality. The problem is, this work is framed explicitly as a preclinical study, and its publication would provide a steppingstone towards clinical trials. Therefore, I cannot in good conscience endorse it.

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Response: We thank Reviewer #3 for the thoughtful review of our manuscript, and for stating that our experiments are overall well done and well described. We do hear the argument of the Reviewer that targeting IL-4 and IL-13 might on the long term reduce protective type 2 immune responses. The goal of this first pre-clinical work was to provide a proof-of-concept of the efficacy of this vaccine approach in a model of asthma in mice.
In line with the concerns raised by this Reviewer, we discuss the potential long-term effects of targeting IL-4/IL-13 in response to parasite infections and beyond, and the need for further work to define these potential long-term effects, as well as to better define the duration of the immune response induced by the vaccine. In an effort to present a balanced/unbiased discussion, we also discuss the overall good safety profile of the anti-IL-4R mAb dupilumab in asthma patients, even in some studies where continuous treatment was given for up to 96 weeks. No major adverse effects were reported and a clear health and quality of life benefit resulted from this long treatment period. We cannot speculate on the duration of anti-IL-4 and anti-IL-13 vaccination in humans for now, but immune responses to other conjugate vaccines wane with time and require boosters to maintain efficacy. Further studies are required to evaluate the duration of our proposed therapeutic vaccine regimen, but it may not

deprive patients from these TH2 cytokines more than dupilumab long treatments have done so far, which did not reveal major susceptibility to other diseases or infections.
While type 2 immune responses undoubtedly have protective effects, it is also clear that more and more people develop aberrant type 2 responses that can lead to severe chronic diseases, and that need to be reduced on the long term.

nature research

Corresponding author(s): Laurent L. Reber

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	IOX base 8c/RF-8a (0796) software, FlexiVent software FlexiWare 8.0
Data analysis	FlowJo 10.4.2, GraphPad Prism 7.0, Microsoft Excel 16.16.23, Zen Software, ImageJ (1.53c), Datanalyst (DATA 4238)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary information files). Source data are provided with this paper.

Field-specific reporting

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size for each experiment is indicated in the legend. No statistical tests were used to pre-determine sample size. Sample size was chosen based on previous experiments published by some of the co-authors (Sibilano et al Nat Commun 2016) and comparable studies in literature.
Data exclusions	No data were excluded in this study
Replication	All attemps at replication were successful. Each animal experiment presented in the paper was repeated in multiple animals and the number of replications was stated in the figure legends. Experiments from figure 1 and 2 were repeated three times. Experiments from figure 3 were repeated two times. Experiments from figure 4 were repeated two times Experiments from figure 5 were done once
Randomization	Mice were randomly atributed to a treatment or control group. Allocation was done randomly. All the experiments were done with samples coming from the mice randomly atributed to a treatment or control group.
Blinding	No blinding was possible because experiments required repeated injections of different vaccines. For histology studies, quantification of staining and cell population were determined by an individual unaware of the sample's identity.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

ANTIBODY SOURCE CLONE IDENTIFIER Dilution
anti-mouse IL-4 polyclonal goat IgG R&D systems n/a AF-404-NA 0.1 µg/ml
anti-human IL-4 polyclonal goat IgG R&D systems n/a AF-204-NA 0.1 µg/ml
anti-mouse IL-13 polyclonal goat IgG R&D systems n/a AF-413-NA 0.1 µg/ml
anti-human IL-13 polyclonal goat IgG R&D systems n/a AF-213-NA 0.1 µg/ml
anti-diphtheria toxin polyclonal goat IgG AbD serotec n/a 3710-0956 860 ng/ml
monoclonal mouse anti-diphtheria toxin IgG1 AbD serotec 8G1 3710-0100 1 µg/ml
anti-human IL-4 monoclonal mouse IgG1 R&D systems 3007 MAB304 1 µg/ml
anti-human IL-13 monoclonal mouse IgG1 R&D systems 32116 MAB213 1 µg/ml
biotinylated anti-mouse IL-4 polyclonal goat IgG R&D systems n/a BAF-404 250 ng/ml
biotinylated anti-human IL-4 polyclonal goat IgG R&D systems n/a BAF-204 250 ng/ml
biotinylated anti-mouse IL-13 polyclonal goat IgG R&D systems n/a BAF-413 250 ng/ml
biotinylated anti-human IL-13 polyclonal goat IgG R&D systems n/a BAF-213 250 ng/ml
anti-Ly6G-PE rat IgG2a BD Pharmingen 1A8 561104 1:100
anti-CD3-APC armenian hamster IgG1 BD Pharmingen 145-2C11 561826 1:100
anti-CD4-FITC Rat IgG2bk Miltenyi Biotec GK1.5 130-120-819 1:50
anti-IL-4-PE rat IgG2b Miltenyi Biotec BVD4-1D11 130-103-016 1:20
anti-mouse IL-4-APC Rat IgG1k eBioscience 11B11 53-7041-82 10 µg/ml
anti-IL-13-PE-Cyanine7 Rat IgG1 Fisher Scientific eBio13A 15538636 1:200
anti-KLRG1-AF488 Hamster IgG2κ BD Pharmingen 2F1 562190 10 µg/ml
anti-CD3-AF532 eBioscience 17A2 58-0032-82 2 µg/ml
anti-CD45-FITC human IgG1 Miltenyi Biotec REA737 130-110-658 1:100

anti-CD45-VB human IgG1 Miltenyi Biotec REA737 130-110-802 1:200
anti-Siglec-F-PECy7 human IgG1 Miltenyi Biotec REA798 130-112-334 1:100
anti-CD11b-VG recombinant human IgG1 Miltenyi Biotec REA713 130-110-559 1:100
anti-B220-APC rat IgG2a Miltenyi Biotec RA3-6B2 130-102-259 1:100
anti-CD11c-VB, hamster IgG Miltenyi Biotec N418 130-102-797 1:100
anti-CD49b- BV421, Rat IgM BD Horizon Dx5 563063 1:50
anti-IgE-FITC, Rat IgG1 BD Pharmingen R35-72 553415 1:50
anti-CD125-PE, Rat IgG1 BD Pharmingen T21 558488 1:100
Purified NA/LE Rat Anti-Mouse CD124 BD Pharmingen mIL4R-M1 552288 10 µg/mL
anti-Siglec-F-Alexa647, Rat IgG2a BD Pharmingen E50-2440 562680 1:100
anti-CD131-PE Miltenyi Biotec REA193 130-118-456 1:100
anti-c-KIT APC eBioscience 2B8 17-1171-82 1:200
rabbit polyclonal HRP-conjugated anti-mouse IgG Invitrogen n/a 61-6520 1:5000
Goat polyclonal Anti-Mouse IgG1 Human ads-HRP Southern Biotech n/a 1070-05 1:4000
Goat anti-Rat IgG (H+L), Alexa Fluor 594 Invitrogen n/a A11007 1:150
Goat polyclonal Anti-Mouse IgG2a Human ads-HRP Southern Biotech n/a 1080-05 1:4000
goat polyclonal anti-mouse IgE antibody Bio-rad n/a STAR110 2 µg/mL
goat polyclonal HRP-conjugated anti-mouse IgG Bethyl Laboratories n/a A90-131P 1:5000
anti-IgE antibody coupled to AF488,
Rat IgG1, κ Biolegend RME-1 406910 10 µg/ml

Validation

Antibody validation was deferred to the manufacturers (including evaluation of purity, sensitivity, F/P ratio, specificity and lot-to-lot consistency).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	CTLL-2 cells (ECACC, Ref. 93042610, batch number: 12K006) HEK-Blue™ IL-4/IL-13 reporter gene cell line (InvivoGen, hkb-il413, batch number: X14-37-01)
Authentication	None of the cell lines were autheticated.
Mycoplasma contamination	Cell lines were not tested for mycoplasma contamination but no indication of contamination was observed.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Female BALB/cJRj mice at 5-6 weeks of age were purchased from Janvier Labs, and maintained in a specific pathogen-free facility at Institut Pasteur or Institut Jacques Monod. IL4RA/IL13/IL4 humanized mice were generated at University of North Carolina, USA and maintained in a specific pathogen-free facility at Institut Pasteur, France. Full description of the generation and characterization of IL4RA/IL13/IL4 mice are provided in the supporting information file, Figure 4 and Supplementary Figure 17. Mice will be housed at Institut Pasteur in specific pathogen free conditions ensuring an appropriate lighting. Fluorescent light is provided with 14:10 h light:dark cycle (6:30 AM to 8:30 PM). Animal facility has central air conditioning equipment which maintains constant temperature of 22 ± 2°C. Air is renewed at least 20 times per hour in animal rooms. Mice will receive food and water ad libitum.
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve samples collected from the field.
Ethics oversight	All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #170043, and by the French Ministry of Research. The protocol also received the authorization number EU0285 - Institut Jacques Monod PHEA - APAFiS - Autor. APAFiS #165.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Bronchoalveolar lavages (BALs) were performed 24 h after the last challenge with HDM in anesthetized mice (187.5 mg/kg ketamine and 18.75 mg/kg xylazine). After semi-excision of the trachea, a plastic canula was inserted, and airspace was washed with 1 ml of PBS containing 2.6 mM EDTA and 2.5 % (v/v) FBS. This operation was repeated for a total of 3 times. For the analysis of leukocytes in lung tissue, right lung lobes were harvested 24 h after the last challenge with HDM, and transferred into gentleMACS™ C tubes (Miltenyi) containing lung dissociation kit (Miltenyi). Tubes were attached upside down on a gentleMACS dissociator (Miltenyi). After a washing step, red blood cells were lysed with Ammonium Chloride Potassium (ACK) lysing buffer (Thermo Fisher scientific), and single cell suspensions were 0.22 µm-cut-off filtered. Blood was collected on heparin. Red blood cells were lysed with ACK lysis buffer (Thermo Fisher scientific). Cells were stained with anti-SiglecF-PECy7 (clone # REA798, Miltenyi), anti-CD49b-BV421 (clone Dx5, eBioscience), anti-IgE-FITC (clone # R35-72, BD Pharmingen) and anti-CD131-PE (clone #REA193, Miltenyi). Blood eosinophils were gated as SiglecF+, SSChigh, and blood basophils as CD49b+, CD131+, IgE+. To harvest peritoneal cells, 5 ml of PBS were injected into the peritoneal cavity and the abdomen was massaged gently for 20 seconds. Fluid containing peritoneal cells was collected.
Instrument	Data were acquired using Miltenyi MACSQUANT 10 and 16 or BD LSRFortessa™ cell analyzer (BD Biosciences).
Software	Data were analysed using FlowJo software (TreeStar).
Cell population abundance	All cell populations in bronchoalveolar lavage (BAL) fluid, lung single cell suspensions or blood leukocytes are represented as percentage of total leukocytes or numbers (with total leukocyte numbers provided)
Gating strategy	All gating strategies are detailed in the Supporting Information file. A figure exemplifying the gating strategy for lung-resident regulatory eosinophils is provided in the supporting information file. Macrophages were gated as CD45+, CD11c+, Siglec-F+, CD11b+, B cells as CD45+, CD11c-, B220+, T cells as CD45+, CD11c-, CD3+, neutrophils as CD45+, CD11c-, B220-, CD3-, Ly6G+, CD11b+ and eosinophils as CD45+, CD11c-, B220-, CD3-, Ly6G-, Siglec-F+, SSChigh. Blood eosinophils were gated as Siglec-F+, SSChigh, and blood basophils as CD49b+, CD131+, IgE+.). Peritoneal mast cells were gated as c-KIT+, IgE+. Regulatory eosinophils were gated as CD45+, PI-, CD125+, Siglec-F int.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.