



Control of allergic reactions in mice by an active anti-murine IL-4 immunization

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Abstract

Pathogenesis of allergic inflammatory disorders is characterized by allergen-induced IgE stimulated by Th2 cytokines including mainly IL-4 overproduction. To counteract IL-4 effects in sensitized-BALB/c mice, we prepared an IL-4 derivative immunogen, made of KLH and murine IL-4 heterocomplex, termed mIL-4 kinoid. Murine IL-4 kinoid immunized mice produced high titer of anti-IL-4 neutralizing Abs. In contrast to KLH control immunization kinoid immunization reversed the allergic IgE:IgG ratio hallmark in rBet v 1a sensitized mice and reduced pulmonary eosinophil recruitment and bronchial hyperreactivity in Ova-sensitized mice. These data pave the way to alternative therapies to combat allergic conditions.

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1. Introduction

Allergy is a common disease which affects a large proportion of about 15–30% individuals all over the world. Clinical manifestations launched by allergenic compounds in atopic

individuals are multiple. Pollen as well as house dust mites most often may trigger conjunctivitis, rhinitis and contribute to asthma. Food allergens, such as peanuts or eggs, may provoke vomiting, diarrhea and urticaria. Severe forms of allergy which occur particularly following insect stinging or drug injection include Quinck oedema and systemic anaphylactic shock that can result in death.

Pathogenesis of allergic inflammatory disorders results from the exposure of the immune system to allergens that induce specific IgE Abs [1]. These Abs bind to high affinity receptors on mast cells and basophils and subsequent bridging of these IgE by allergens may trigger cell degranulation leading to the release of vasoactive amines such as histamine or serotonin. These mediators are the cause of inflammatory

Abbreviations: 2ME, 2-mercaptoethanol; Ova, ovalbumin; mIL-4, murine Interleukine-4; KLH, keyhole limpet hemocyanin; rBet v 1a, recombinant Betula verrucosa allergen 1a; Ab, antibody; IFA, incomplet Freund's adjuvant; CaHPO₄, calcium phosphate; S.C., subcutaneous; I.M., intramuscular; I.P., intraperitoneal; I.N., intranasal; BAL, bronchoalveolar lavages; Penh, enhanced respiratory pause

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symptoms. B cell switch in Ab response to IgE class is determined by Th2 cytokines. Th2 cytokines involved in allergy include IL-4, and accessorially IL-5 and IL-13 [2–6].

Beside symptomatic medications such as anti-inflammatory corticoid and antihistamine administration, conventional treatments of allergic manifestations consist of desensitizing patients by increasing doses of the involved allergen administered intradermally (I.D.), a process now termed vaccination [7]. Such a vaccination is not always efficacious and represents a long run treatment often to be repeated over the years [8,9]. Recently new treatments have been initiated to specifically target pathogenic factors involved in the disease. Monoclonal Abs to IgE (Omalizumab) [10] are currently available on the market (Xolair®, Novartis) for treating allergic disorders, including allergic rhinitis and conjunctivitis. It has also been used in combination with vaccination against allergen(s) [11]. Also generation of therapeutic antibody responses against IgE through vaccination is currently under experimentation [12]. Monoclonal Abs to Th2 cytokines, including IL-4 [13], IL-5 [14] and IL-13 [15] have also proved experimentally to be beneficial. However, repeated administration of chimeric or humanized mAbs is likely to trigger anti-idiotypic Abs [16]. This limitation prompted us to investigate other strategies to block abnormal IL-4 production.

Using the kinoid technology [17,18], previously initiated to produce innocuous and effective hTNF α and VEGF vaccines to control arthritis [19] and neoangiogenesis [20], respectively, we prepared a murine IL-4 (mIL-4) kinoid to treat experimentally allergic disorders.

We report here that the kinoid, consisting of a KLH–mIL-4 heterocomplex emulsified in Salk adjuvant, when administered to mice, was safe and triggered high neutralizing antibody titers to IL-4. Kinoid immunized mice were further used in two different experimental models. First, when an IL-4 kinoid immunization either combined or not with a rBet v 1a vaccination [7] was carried out in rBet v 1a sensitized BALB/c mice [21,22], the IgE Ab response to a rBet v 1a challenge was negligible compared to that of KLH immunized mice given the same regimen, used as controls. We also showed that, as a result of aerosolized methacholine/ovalbumin-induced anaphylaxis assay [23], following anti-IL-4 kinoid immunization, ovalbumin sensitized BALB/c mice improved their respiratory response, and eosinophilia was lowered in alveolar lavages.

2. Material and methods

2.1. Reagents

KLH was purchased from Pierce (Paris, France), recombinant murine Interleukine-4 (mIL-4) from Cytolab (Rehovot, Israel), mIL-2, anti-mIL-4 mAb from R&D System (Lille, France), Ova (grade V) from Sigma–Aldrich. Recombinant birch pollen major allergen rBet v 1a was from Biomay (Linz,

Austria). ISA51 (SEPPIC, France) was used as Incomplete Freund Adjuvant (IFA).

2.2. Kinoid preparation

mIL-4 kinoid is a KLH–mIL-4 heterocomplex prepared by treating a mixture of KLH and mIL-4, at a ratio of 1:40, with glutaraldehyde (2.25 mM) followed by formaldehyde (66 mM) [19]. It migrates, by isoelectric focusing in a soft 0.8% agarose gel, as a band exhibiting a pI of about 5.8, detected both by Coomassie blue staining and by immunoblotting using specific Abs [24]. As assessed by ELISA, the kinoid exhibits an antigenicity of the same order of magnitude as that of native mIL-4. The IL-4 cytokinetic activity of the mIL-4 kinoid, as evaluated by the standard functional test using the HT-2 cell line [25], is quenched by over 95% in the kinoid. Control samples were prepared by treating KLH, modified KLH, or mIL-4, inactivated mIL-4, with glutaraldehyde and formaldehyde under similar conditions as for kinoid.

2.3. Mice and treatments (Table 1)

Seven-week-old female BALB/c mice were purchased from Charles River (Lyon, France). Mice were randomized for each study. For anti-IL-4 immunizations, mice received either two doses (30 and 15 μ g) of mIL-4 kinoid (experimental group) or corresponding doses of modified KLH or inactivated mIL-4 (control groups) emulsified in ISA51 adjuvant given 3 weeks apart by the I.M. route of administration. Immunized and control mice were used in the three sets of experiments reported in Table 1. Experimental designs for presensitization with rBet v 1a [26] or with Ova [27] are described in Table 1a and c, respectively. Recombinant Bet v 1a vaccination was performed according to the immunizing regimen detailed in Table 1b [28].

2.4. Cell cultures

HT-2 cells (ATCC, CRL 1841) were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 0.05 mM 2-mercaptoethanol (2ME), 100 IU/ml IL-2 and 10% fetal bovine serum (FBS). Purified murine splenocytes were resuspended in RPMI medium containing 5% FCS and 0.05 μ M 2ME and incubated in culture medium with or without KLH (3 μ g/ml) and mIL-4 (3 μ g/ml) antigens.

2.5. T cell proliferation assay

Splenocytes from kinoid or KLH (control) immunized mice in presence of IL-4 at varying doses from 0.1 to 10 μ g/ml were cultured for 4 days and then pulsed for 18 h with [^{3} H]-thymidine. Cells were harvested on filter mats, and thymidine incorporation into DNA was quantified by using a beta-counter. Proliferation Index (PI) was expressed as: [(mean

Table 1
Experimental design

(a) Effect of the mIL-4 kinoid immunization on rBet v 1a sensitized BALB/c mice^a

Allergen sensitization rBet v 1a		Kinoid immunization mIL-4 kinoid/KLH		Allergen boost rBet v 1a	
Adjuvant/route	Schedule	Adjuvant/route	Schedule	Adjuvant/route	Schedule
Alum, I.P.	W1–W3	ISA51, I.M.	W6–W9	Alum, I.P.	W11

(b) Effect of combined mIL-4 kinoid immunization and rBet v 1a allergen vaccination on sensitized mice^b

Allergen sensitization rBet v 1a		Kinoid immunization mIL-4 kinoid/KLH		Allergen vaccination rBet v 1a		Allergen boost rBet v 1a	
Adjuvant/route	Schedule	Adjuvant/route	Schedule	Adjuvant/route	Schedule	Adjuvant/route	Schedule
Alum, I.P.	W1–W3	ISA51, I.M.	W6–W9	CaHPO ₄ , I.M.	W11–W14–W17	Alum, I.P.	W19

(c) Effects of mIL-4 kinoid immunization on the bronchospasm and eosinophilia in ovalbumin sensitized mice^c

Allergen sensitization Ova		Kinoid immunization mIL-4 kinoid/KLH		Allergen challenge Ova/NaCl	
Adjuvant/route	Schedule	Adjuvant/route	Schedule	Adjuvant/route	Schedule
Alum, I.P.	W1–W2	ISA51, I.M.	W6–W9	I.N.	W33

^a BALB/c mice ($n = 10$) received 2 μ g rBet v 1a adsorbed onto 2 mg alum at weeks 1 and 3. Twenty-one days afterwards (week 6), rBet v 1a sensitized mice received at 3-week intervals 2 I.M. injections (30 and 15 μ g) of either mIL-4 kinoid (experimental group; $n = 5$) or KLH immunogen (control group; $n = 5$). Two weeks later, both groups were challenged with an I.P. booster injection of 2 μ g rBet v 1a adsorbed onto Alum. Blood samples were taken by retroorbital puncture from each mouse at week 12 to measure serum anti-rBet v 1a (IgG and IgE) titers.

^b BALB/c mice ($n = 10$) received 2 μ g rBet v 1a adsorbed onto 2 mg alum under the volume of 150 μ l at weeks 1 and 3. Twenty-one days afterwards (week 6), rBet v 1a sensitized mice received at 3-week intervals 2 I.M. injections (30 and 15 μ g) of either mIL-4 kinoid (experimental group; $n = 5$) or KLH immunogen (control group; $n = 5$). Two weeks later, both groups of mice received repeated doses of 10 μ g rBet v 1a adsorbed to calcium phosphate microparticles given at weeks 11, 14 and 17. Following vaccination mice were at week 19 challenged with an I.P. booster injection of 2 μ g rBet v 1a adsorbed onto Alum. Blood samples were taken by retroorbital puncture from each mouse at week 18 and week 20 to measure serum anti-rBet v 1a (IgG and IgE) titers.

^c Two separate experiments were carried out to investigate the effects of kinoid immunization in Ova-sensitized BALB/c mice; mice received 2 I.P. injection of 100 μ g Ova adsorbed onto alum (1.6 mg in 400 μ l) administered 1 week apart (weeks 1 and 2). Twenty-eight days after (week 6) Ova-sensitized animals received two immunizing I.M. injections of either mIL-4 kinoid ($n = 3$ in the 1st and $n = 4$ in the 2nd series) or KLH control ($n = 7$ in the 1st and $n = 8$ in the 2nd series) given at 3-week intervals (weeks 6 and 9), of 30 and 15 μ g of immunogen, respectively. Twenty-five weeks after kinoid and KLH control immunized animals in the two experiments were challenged with Ova released I.N. by aerosol. To determine the baseline values of the Penh and of eosinophilia recruitment into BAL and lung, a subgroup of KLH immunized mice ($n = 4$ in each series) were challenged with saline in place of Ova.

of cpm of stimulated cells) – (mean of cpm of unstimulated cells)]/(mean of cpm of unstimulated cells).

2.6. Anti-mIL-4 Ab titer assay

Specific anti-mIL-4 Ab titer in sera of immunized and control mice was determined by a direct ELISA. Precoated ELISA plates with 100 ng/well mIL-4 were incubated with serial dilutions of sera from immunized and control mice. Specific IgG were detected by using peroxidase rabbit anti-mouse IgG (Zymed, Carlsbad, CA). Endpoint titers were expressed as the reciprocal of the highest serum dilution giving an O.D. of 0.3.

2.7. Allergen specific IgG, IgG1, IgG2a and total IgE level quantification

Allergen specific antibody levels in mouse sera were determined by ELISA. Specific IgG Abs were detected using Horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG (Zymed, Carlsbad, CA). Endpoint titers were expressed as the reciprocal of the highest sample dilution giving an O.D. of 0.3. Biotin-conjugated rat anti-mouse γ 1 (clone A85-1), γ 2a (clone R19-15) and streptavidin-HRP (BD PharMingen)

were used for the detection of specific IgG subclasses. For detection of allergen-specific IgE Abs, the IgE were detected with the biotin-conjugated rat anti-mouse IgE (clone R35-118; BD PharMingen) followed by streptavidin-HRP. The antibody levels were expressed in O.D. units. The levels of total IgE Abs were determined using capture and detection antibodies, as well as IgE standard, from the BD OptEIA Set mouse IgE kit (BD Biosciences). Total IgE levels were expressed as ng/ml.

The baseline levels derived from the preimmune sera were individually subtracted from the respective immune sera.

2.8. Quantification of IFN- γ

INF- γ in 72-h culture supernatants from antigen activated splenocytes was determined by enzyme-linked immunosorbent assay (ELISA), using commercial kits from R&D (Abingdon, UK). The cytokine detection limit was 5 pg/ml.

2.9. Passive cutaneous anaphylaxis (PCA)

Passive cutaneous anaphylactic reactions were performed in Wistar rats [29]. Pooled sera from mIL-4 kinoid or KLH immunized BALB/c mice were diluted from 1:30 to 1:810

in PBS and intradermally injected in a 50 μ l volume on the back of the rat. Pool of preimmune sera, in a 1:30 dilution, was injected as negative controls. After 24 h, 2 μ g of birch pollen extract in 1 ml 0.5% Evans blue was I.V. injected into each animal. The mast cell degranulation compound 48/80 was intradermally injected as a positive control (50 μ l of 1 mg/ml). The reaction was read 25 min after the antigen challenge. The diameter of the reaction was measured and the area and the color intensity were evaluated on the inside of the skin.

2.10. IL-4 bioassay

IL-4 activity was determined by using the HT-2 cell line [25] in a [^{3} H]-thymidine uptake assay. Cells were washed four times in RPMI 1640 medium and seeded in flat-bottom-96-well plates at 2500 cells per well. Immediately after seeding the cells, various concentrations of mIL-4 and mL-4 kinoid were added to the cultures. Then HT-2 cells were incubated at 37 °C for 36 h. During the last 4 h of culture 1 μ Ci of tritiated thymidine was added. Cells were harvested onto glass fiber filters and the radioactivity was counted. The ability of hyperimmune serum to neutralize mL-4 activity was similarly determined after incubating two-fold serial dilutions of test and control sera with mL-4 (30 ng/ml).

2.11. Plethysmography

Two series of experiments were performed on Ova-sensitized mice immunized either with mL-4 kinoid (experimental) or KLH immunogen (control) and exposed to ovalbumin aerosols. An additional control group of KLH immunized animals received, saline in place of Ova in the aerosol. The response to aerosolized methacholine was investigated at 24 h after the last Ova challenge [23]. Unrestrained conscious mice were placed in whole-body plethysmography chambers (Buxco Electronic, Sharon, CO, USA). BALB/c mice were exposed for 50 s to 100 mM methacholine. The response was measured for 15 min after nebulization.

Enhanced respiratory pause (Penh) measurement can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves; increased phase shift correlates with increased respiratory system resistance. Penh is calculated by the formula $\text{Penh} = (\text{Te}/\text{RT} - 1) \times \text{PEF}/\text{PIF}$, where Te is expiratory time, RT the relaxation time, PEF the peak expiratory flow, and PIF is peak inspiratory flow. For the graphics the Penh mean values are given for –3 to –1 min (baseline) and 14 points or minutes after the methacholine nebulization [27].

2.12. Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) fluids were prepared by washing the lungs four times with 0.5 ml of ice-cold PBS. The cells were sedimented by centrifugation at 400 $\times$ g for

10 min at 4 °C. An aliquot of the cell pellets was stained with Turk's solution, counted, and 200,000 cells centrifuged on microscopic slides (cytospin at 1000 rpm for 10 min, at RT). Air-dried preparations were fixed and stained with Diff-Quik (Merz & Dade A.G., Dudingen, Switzerland). Differential counts were made under oil immersion microscopy. One hundred cells were counted twice for the determination of the relative percentage of each cell type present in the BAL.

2.13. Lung histology

The organs were fixed in 4% buffered formaldehyde overnight and embedded in paraffin as described previously [27]. Lung sections of 3 μ m were stained with hematoxylin and eosin or with periodic acid Schiff reagent (PAS) and examined with a Leica microscope ($\times 40$ and $\times 100$ magnification). Eosinophil peribronchial infiltrates and goblet cell hyperplasia of the bronchiolar epithelium were assessed by a semi-quantitative score (0–5) by two independent observers (SSC, BR).

2.14. Pulmonary eosinophil peroxidase (EPO) activity

EPO activity was determined in order to estimate the recruitment of eosinophils to the lung parenchyma. After BAL lavage and perfusion (see experimental protocol), the lungs were excised, homogenized for 30 s in 1 ml of 0.05 M Tris–HCl buffer pH 8.0 using a Polytron (Kinematic AG, Luzern, Switzerland), and 0.1% (v/v) Triton X-100 added. The homogenate was centrifuged for 15 min at 4 °C at 10,000 $\times$ g. EPO activity in the supernatant was determined as estimated from the oxidation of *O*-phenylenediamine (OPD) by EPO in the presence of hydrogen peroxide (H_2O_2) using the protocol by Van Oosterhout et al. [30]. The substrate solution consisted of 10 mM OPD in 0.05 M Tris-buffer (pH 8) and 4 mM H_2O_2 (BDH, Poole, UK). Substrate solution was added to samples in a 96-wells microplate and incubated at room temperature for 30 min before stopping the reaction

Table 2

IL-4 kinoid-immunization induced inhibition of eosinophil recruitment and mucus hypersecretion to the lung upon Ova challenge

	Eosinophil infiltration	Mucus hypersecretion
KLH + NaCl	1.00 $\pm$ 0.63 (1.00)	1.00 $\pm$ 0.63 (1.00)
KLH + Ova	3.83 $\pm$ 0.75 (4.00)	4.00 $\pm$ 0.89 (4.00)
mIL-4 kinoid + Ova	1.67 $\pm$ 0.52 (2.00)	2.33 $\pm$ 0.82 (2.50)

The histology sections derived from two sets of experiments ($n=6$) were quantified for eosinophil recruitment and mucus hypersecretion, respectively and scored from 0 to 5. The results represent the mean $\pm$ S.D. and median in parenthesis; for eosinophil infiltration experiment, Kruskal–Wallis $P \leq 0.001$ and Mann–Whitney P -value between kinoid and KLH Ova challenge, $P < 0.0001$; between KLH saline and KLH OVA challenge $P < 0.0001$; for mucus hypersecretion experiment, Kruskal–Wallis $P \leq 0.001$ and Mann–Whitney P -value between kinoid and KLH Ova challenge, $P < 0.0001$; between kinoid and KLH saline challenge $P = 0.04$; between KLH saline and KLH OVA challenge $P = 0.002$.

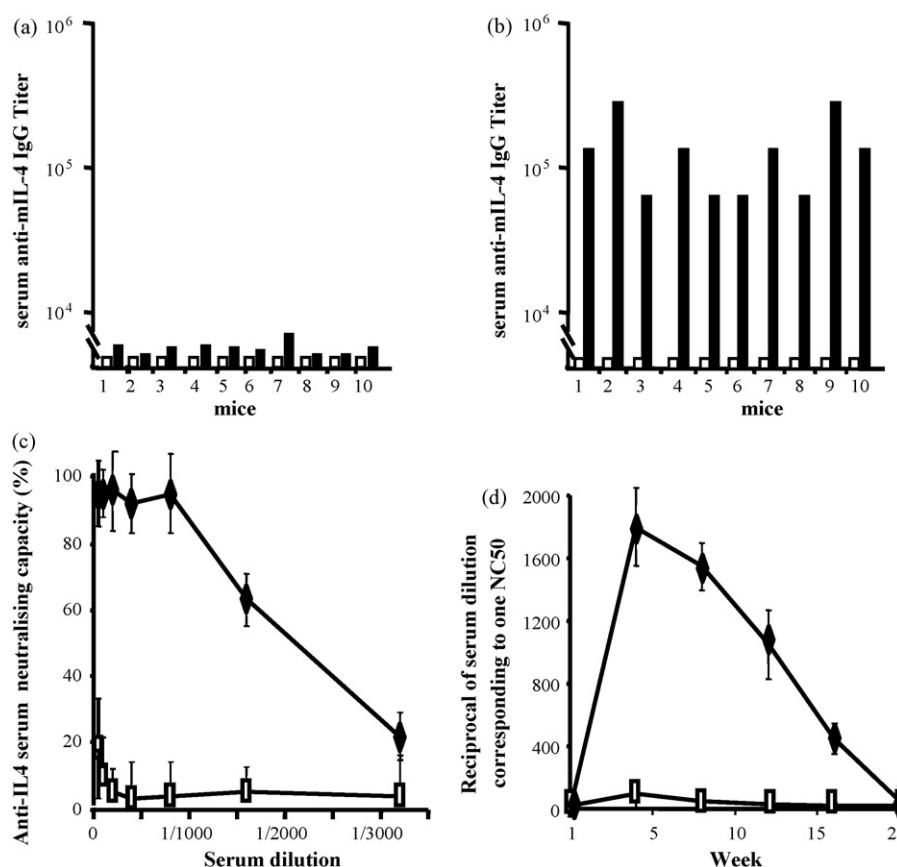


Fig. 1. Ab response to mIL-4 immunization. Open bars: preimmunization and dark bars: post-immunization. Two groups of 10 BALB/c mice were immunized either with inactivated mIL-4 in (a) or with mIL-4 kinoid in (b). IgG Ab titer is expressed as the reciprocal of serum dilution. In (c), neutralizing capacity (NC) was performed on a pool of sera originating from the kinoid immunized mice and in (d), kinetics of NC₅₀. Results are expressed as mean $\pm$ S.D. in (c and d).

by addition of 4 M sulfuric acid. Duplicate incubations were carried out in the absence and presence of the EPO inhibitor 3-amino-1,2,4-triazole (AMT, 2 mmol/l). The absorbance was then measured at 492 nm (Flow Labs, Irvine, UK). Results are expressed as O.D. 492 nm and were corrected for the activity of other peroxidases, which were not inhibited by AMT.

2.15. Statistical analysis

The data are presented as the mean $\pm$ S.D. or $\pm$ S.E.M. In Figs. 3 and 4, the significance of differences between groups was determined by Student *T*-test (two-tailed). In Table 2 and Fig 5, comparisons between groups were assessed by Kruskal–Wallis test followed by a Mann–Whitney test

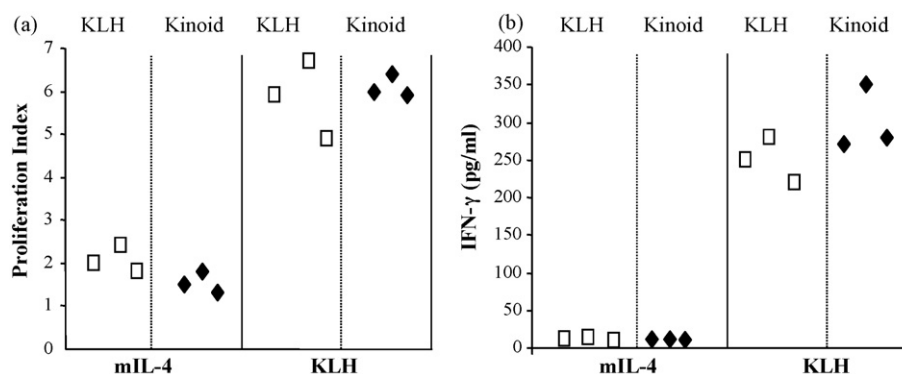


Fig. 2. Cellular immune response to native mIL-4. Splenocytes from KLH (open square: control, $n=3$) and mIL-4 kinoid (closed losange: experimental, $n=3$) immunized mice were cultured in presence of mIL-4 (3 μ g/ml) or KLH (3 μ g/ml). T cell activation was evaluated by T-cell proliferation measured by [^{3} H]-thymidine incorporation and expressed as proliferation index in (a) and by the detection of IFN- γ in splenocyte culture supernatant using ELISA in (b). These results are representative of at least three separate experiments carried out with varying doses of mIL-4 ranging from 0.1 to 10 μ g/ml.

because of the non-parametric distribution of the data. Statistical significance was reported if $P \leq 0.05$ was achieved.

3. Results

3.1. Immune reaction to mIL-4

3.1.1. Induction of Ab response

While low anti-mIL-4 Abs titers ($<10^4$) were detected in serum of BALB/c mice immunized with inactivated mIL-4 (Fig. 1a), mIL-4 kinoid immunization elicited high anti-IL-4 Ab titers ranging from 10^5 to 5×10^5 (Fig. 1b). The IgGs represent the prevalent class of Ig Abs. Of importance, the neutralizing capacity of hyperimmune sera was high with a 50% neutralizing capacity (NC50) equal to 1600^{-1} dilution (Fig. 1c), and it declined after about 10 weeks (Fig. 1d).

3.1.2. Absence of cellular immune response

As previously reported for other kinoid immunizations against self cytokines including TNF α [19] and VEGF [20], cultured splenocytes from mIL-4 kinoid immunized mice did not trigger an increased response to mIL-4, compared to that of KLH immunization as assessed by T-cell proliferation test (Fig. 2a) and IFN- γ release in 72 h culture supernatants (Fig. 2b). In contrast, a cell-mediated immune response was observed when splenocytes from both KLH and mIL-4 kinoid immunized mice were cultured in the presence of KLH.

3.2. Effect of mIL-4 kinoid immunization on rBet v 1a sensitized BALB/c mice

As described in Table 1a, BALB/c mice were first sensitized by I.P. injections given 2 weeks apart of rBet v 1a adsorbed onto alum. All mice developed specific IgE one week after the last injection (data not shown). Then mice were immunized with either mIL-4 kinoid (experimental, $n=5$) or KLH (control, $n=5$) before being challenged 2 weeks afterwards with a rBet v 1a boost (Table 1a). Following challenge, whereas all control mice mounted a strong IgE Ab response to rBet v 1a, all experimental mice did not do so ($P<0.0001$) (Fig. 3a). Furthermore IgG1 antibody levels were slightly higher in the KLH immunized mice than in kinoid immunized mice ($P<0.01$). IgG2a antibody levels were low in both groups, with a stronger response in the kinoid immunized group ($P<0.0001$). Noteworthy, passive cutaneous anaphylaxis carried out in Wistar rats using sera from kinoid immunized or control mice confirmed the presence of high titers of IgE Abs in control but not in kinoid immunized mice.

3.3. Effect of combined mIL-4 kinoid immunization and rBet v 1a vaccination on rBet v 1a sensitized mice

In another series of experiments, rBet v 1a sensitized mice, immunized either with mIL-4 kinoid ($n=5$) or KLH ($n=5$),

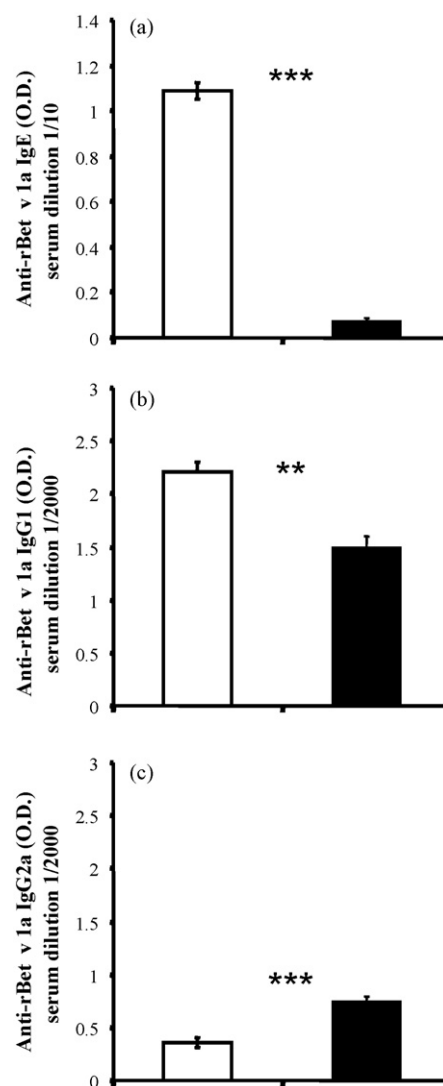


Fig. 3. Effect of the kinoid immunization on rBet v 1a sensitized BALB/c mice. IgE (a), IgG1 (b) and IgG2a (c) antibody response to rBet v 1a in sera originating from sensitized mice with rBet v 1a, immunised with KLH (open bars) or kinoid (closed bars) and challenged with a booster of rBet v 1a. Sera diluted at 1/10 for IgE, 1/2000 for IgG1 and IgG2a were tested by ELISA and results expressed as mean of O.D. values $\pm$ S.E.M. P -values calculated with Student T -test were indicated as ** $P<0.01$; *** $P<0.001$. The baseline levels derived from the preimmune sera values were individually subtracted from the respective immune sera values.

were given a rBet v 1a vaccination. In response to this combined immunization and before a challenge with a rBet v 1a booster I.P. injection (Table 1b), total IgE as well as specific anti-rBet v 1a IgE Abs were higher in control mice than in kinoid immunized mice sera ($P<0.01$ for total IgE; not significant for specific IgE), while anti-rBet v 1a IgG Ab titers were comparable (Fig. 4 pre-boost). One week after challenge with rBet v 1a boost, serum anti rBet v 1a Abs titers increased in both groups. However the IgE Ab response in kinoid immunized mice remained low compared to that in KLH immunized control ($P<0.05$ for total IgE; $P<0.01$ for specific IgE) (Fig. 4b₂), while the IgG Ab response was

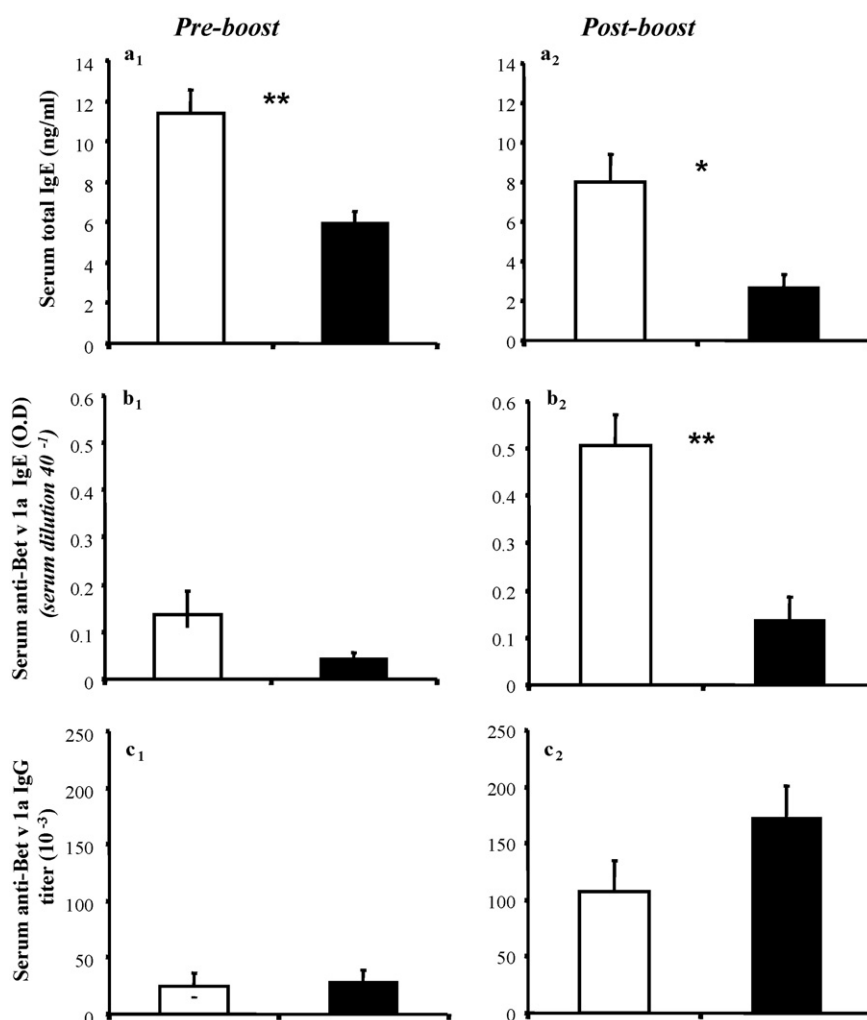


Fig. 4. Effect of combined kinoid immunization and rBet v 1a vaccination in rBet v 1a-sensitized mice. Total IgE in a₁ and a₂, specific IgE Abs in b₁ and b₂ and IgG Abs in c₁ and c₂ titers in serum from sensitized mice immunized either with KLH (open bars) or mIL-4 kinoid (closed bars) followed by a rBet v 1a vaccination before and after rBet v 1a booster challenge (Table 1b). Results in (a₁) and (a₂) are expressed as ng/ml for total IgE, in (b₁) and (b₂) as O.D. values $\pm$ S.E.M. units at 490 nm for specific IgE in 40 times diluted serum and in (c₁) and (c₂) as Ab titers for specific IgG (see Section 2). *P*-values calculated with Student *T*-test were indicated as: * *P* < 0.05; ** *P* < 0.01.

more increased in kinoid immunized mice than in controls (Fig. 4c₂).

3.4. mIL-4 immunization reduces airway hyperactivity as well as eosinophil recruitment and inflammation upon allergen-methacholine challenge

In two experiments carried out separately according to the protocols described in Table 1c, BALB/c mice, sensitized by two S.C. injections given at 2 weeks apart of Ova adsorbed onto alum, developed specific IgE one week after the last injection (data not shown). Ova-sensitized mice challenged intranasally with Ova but not saline, developed a robust response to aerosolized methacholine, as measured in Penh values, which provide an estimate for airway obstruction and may indicate airways hyperreactivity and inflammation [27]. In either of these experiments, the group of mice initially

Ova-sensitized and immunized with KLH (control animals) exhibited a marked airways hyperactivity following Ova challenge but not following saline challenge (Fig. 5a₁ and b₁). In Ova-sensitized and kinoid-immunized mice, Penh values following Ova challenge were higher than baseline with saline challenge. However compared to those in the control group, these values were reduced, significantly in the first experiment (Mann–Whitney test *P* < 0.0001 between groups, with *n* = 3–4 per group) (Fig. 5a₁ and a₂), but not in the second one (Fig. 5b₁). Even though the number of animals used per group for each session of plethysmography was low, these results point to a reduction of Ova-challenge-induced airway hyperreactivity in mIL-4 kinoid immunized mice.

We also investigated in one of these experiments the effect of mIL-4 kinoid immunization on antigen-induced lung inflammation. Twenty-four hours after methacholine response analysis, eosinophil recruitment into the lung tissue

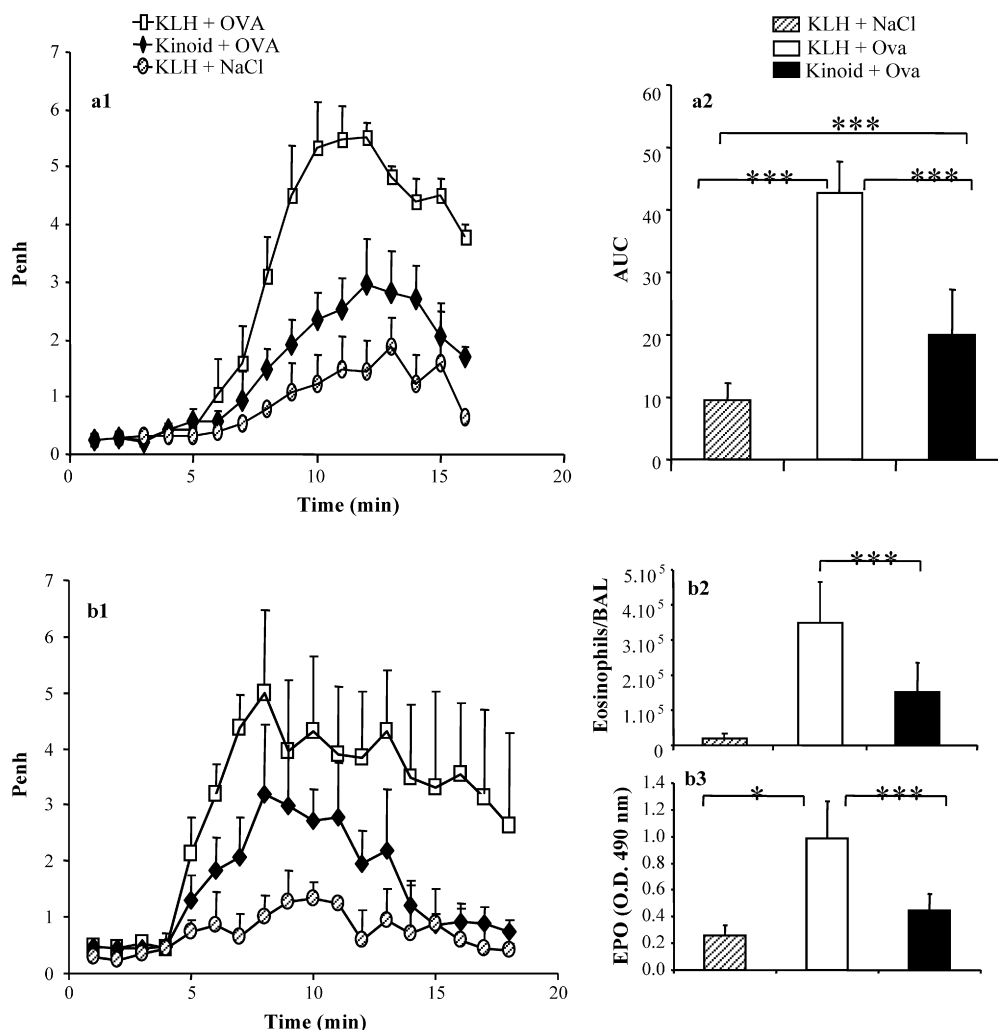


Fig. 5. mIL-4 kinoid immunization inhibits methacholine response and eosinophil recruitment in BAL and lung upon allergen challenge. *Plethysmography (a1 and b1)*: In two separate experiments, Ova-sensitized BALB/c mice were immunized with either mIL-4 kinoid or KLH (control). Mice were then challenged I.N. with either Ova or saline alone (Table 1c). Twenty-four hour post-challenge, the methacholine response was measured using whole body plethysmography showing the time course of Penh in mIL-4 kinoid Ova-challenge ($n = 3$ in a1 and $n = 4$ in b1), KLH Ova-challenge ($n = 3$ in each series) and KLH saline-challenge ($n = 4$ in a1 and $n = 2$ in b1). The area under each curve (AUC) of a1 was computed and represented in a2 (Kruskal–Wallis test, $P = 0.018$; with Mann–Whitney test, KLH Ova challenge vs. KLH NaCl challenge groups, KLH Ova challenge vs. Kinoid Ova challenge groups, or Kinoid Ova challenge vs. KLH NaCl challenge groups: $P < 0.0001$ indicated by ***). *Eosinophilia (b2 and b3)*: In the second experimental series, eosinophil cells from the bronchoalveolar lavage (BAL) were counted (Mann–Whitney test between KLH Ova challenge and mIL-4 kinoid Ova challenge groups P -value < 0.0001) (b2) and the eosinophil peroxidase activity (EPO) in the lung tissue homogenates was determined (b3) in mIL-4 kinoid Ova-challenge ($n = 4$), KLH Ova-challenge ($n = 4$) and KLH NaCl-challenge mice ($n = 4$) (Kruskal–Wallis $P < 0.01$; Mann–Whitney test between KLH Ova challenge vs. Kinoid Ova challenge groups or KLH Ova challenge vs. KLH NaCl challenge groups $P < 0.0001$ and indicated by ***, between Kinoid Ova challenge vs. KLH NaCl challenge groups: $P = 0.03$ indicated by *). The results represent the mean $\pm$ S.D. Stripped and open symbols represent KLH immunized mice exposed to NaCl- or Ova-challenge, respectively, and closed symbols represent mIL-4 kinoid immunized mice exposed to Ova-challenge.

and the alveolar space was analyzed (Fig. 5b2 and b3). The eosinophil counts in BAL upon Ova challenge was reduced in mIL-4 kinoid immunized mice as compared to KLH immunized mice (Fig. 5b2). Eosinophil recruitment into lung parenchyma assessed as eosinophil peroxidase activity (EPO) was significantly reduced in mIL-4 kinoid immunized mice (Mann–Whitney test between KLH Ova challenge and mIL-4 kinoid $P < 0.0001$) (Fig. 5b3). Microscopically the lung tissue of Ova-challenged mice showed peribronchial cell recruitment together with hyperplasia of bronchial smooth muscle. IL-4 kinoid immunization reduced peribronchial eosinophil

infiltration and the mucus hypersecretion and hyperplasia of goblet cells (Fig. 6) from 4 to 2 using scoring scale from 0 to 5 (Table 2).

4. Discussion

IL-4 is a pivotal cytokine in the pathogenesis of allergic disorders and it has been therapeutically targeted by passive anti-IL-4 Ab administration to BALB/c mice infected with *Leishmania major* (Lm) [31]. Whereas BALB/c mice

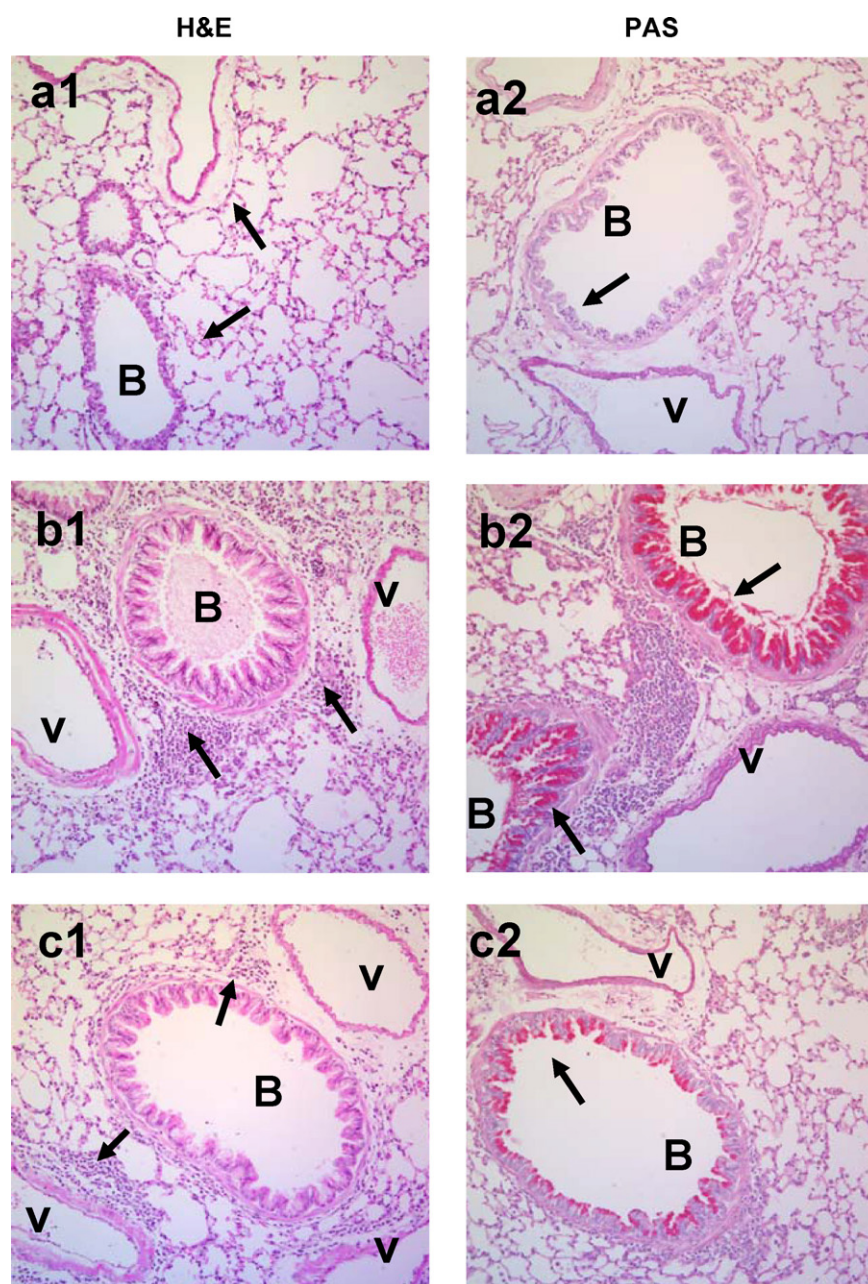


Fig. 6. mIL-4 kinoid immunization inhibits cell recruitment to the lungs and mucus hypersecretion. Lung sections of mIL-4 kinoid vaccinated and Ova-sensitized BALB/c mice sacrificed 24 h after saline or Ova challenge are shown. The formalin fixed tissue sections were stained with hematoxylin and eosin to visualize cell recruitment (a1–c1) and with periodic acid Schiff reagent (PAS) to visualize mucus (a2–c2). (a and b) KLH immunized mice exposed to NaCl or Ova, respectively; (c) kinoid immunized mice exposed to Ova. Magnification is $\times 20$. B: Bronchi; V: pulmonary alveola.

receiving Lm by I.P. exhibited strong inflammatory reactions leading to death within 10 days, animals given anti-IL-4 mAbs neutralizing IL-4 survived. In the present report we show that blocking IL-4 can also be safely achieved by high affinity polyclonal Abs raised following active I.M. immunization with an IL-4 derivative devoid of biological activity but still immunogenic, termed IL-4 kinoid. Murine IL-4 kinoid is a heterocomplex formed by aldehyde coupling of mIL-4 to KLH according to the described procedure [18] initially applied to TNF α [19] and VEGF [20] kinoids. In

this study, we showed that, as other kinoids [19,20], mIL-4 kinoid appropriately adjuvanted in IFA triggered in mice a strong, though transient, immune B but not T cell response to IL-4. While indeed specific B cells directed toward self antigens, including self cytokines, are physiologically dormant [32], they may however be reactivated under experimental instances such as in presence of CFA or of CpG [33] or, as here, following kinoid immunization. We assumed that the normal mechanisms of B cell tolerance to self IL-4 were broken by the conjugation of the foreign T cell help induced

by the KLH partner and the highly concentrated and conformationally conserved IL-4 epitopes crosslinking specific B cell receptors [17]. The failure to induce a specific cellular immune response to the cytokine, following mIL-4 kinoid immunization, also observed following TNF α [19] and VEGF [20] kinoid vaccinations, is not surprising considering the negative T cell clonal selection to self antigens in the thymus and the regulatory T cells controlling self antigens in the periphery. Given the short life of B cell memory in the absence of specific T cell help [34], the lack of specific immune T cell to self IL-4 could also account for the rapid decline of an effective neutralizing Ab response to the cytokine (Fig. 1d), as reported for kinoid immunizations of other specificities [19,20].

As to safety concern, in mIL-4 kinoid immunized mice, the absence of IL-4 specific auto-immune disorders anticipated by the lack of immune T cell response to IL-4, is in accordance with the good tolerance to kinoid immunization [19,20], as also stressed by normal growth and long term survival (over 18 months) of immunized mice without occurrence of any adverse effect (not shown).

Efficacy of the kinoid immunization was ascertained in both rBet v 1a- and Ova-sensitized BALB/c mice models. By eliciting high titers of anti-IL-4 neutralizing Abs, kinoid immunization resulted in the blocking of allergen-induced specific IgE Abs. When rBet v 1a sensitized BALB/c mice were immunized with either mIL-4 kinoid or KLH as control, without other treatment (Fig. 3) or in combination with an anti-rBet v 1a vaccination (Fig. 4), a much lower anti-rBet v 1a IgE response was elicited in kinoid-immunized animals than in control mice. By contrast, the anti-rBet v 1a IgG response was nearly similar in both groups (Figs. 3 and 4).

We also report in the present study that in the classical anaphylactic murine asthma model induced in mice by Ovalbumin sensitization, kinoid immunization may be as beneficial as soluble IL-4 receptor [35], by reducing following Ova challenge eosinophilia (number and peroxidase activity) and to some extent bronchospasm (Fig. 5). Therefore we assume that reducing the IL-4 level by IL-4 kinoid immunization, as shown in this study, should prevent/reduce allergen-induced inflammatory manifestations in humans. Applied to allergic patients as a combined treatment, IL-4 kinoid immunization initiated before conventional vaccination should reinforce the desensitization efficacy by promoting an IgG instead of an IgE response (Fig. 4) and make the cure more comfortable. Given that IL-4 kinoid immunization reduces the production of total IgE, it is safe to predict that kinoid immunization will be beneficial also to polyallergic subjects. In this prospective, we prepared a human IL-4 kinoid, which induced in BALB/c mice high titers of anti-human IL-4 neutralizing Abs (not shown). This immunization also proved to be safe in mice without any apparent adverse effects over 18 months and at sacrifice no histological abnormalities of organs were observed. Immunization with this human IL-4 kinoid preparation preceding a conventional desensitization with an allergen is still to

be clinically tested in human to evaluate its therapeutic value.

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Contributors: Hélène Le Buanec has performed research, Bernhard Ryffel performed research and analyzed data, and Bernard Bizzini designed biochemical aspects. Daniel Zagury, Robert Gallo and Gabriel Peltre designed research and wrote the paper.

Disclosure: HLB is scientist; P.L. is VP Drug Development, RG consultant and DZ Director of the Scientific and Research board of Neovacs S.A.

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