



Combined vaccination against IL-5 and eotaxin blocks eosinophilia in mice

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ABSTRACT

Interleukin-5 (IL-5) is a cytokine which is essential for the maturation of eosinophils in bone marrow and for their release into the blood. Eotaxin is a CC type chemokine implicated in the recruitment of eosinophils in a variety of inflammatory disorders. Since eosinophil-activity is governed by these two pathways, we targeted both IL-5 and eotaxin by active vaccination to block eosinophilia. We produced two vaccines by chemically cross-linking IL-5 or eotaxin to a virus-like particle (VLP) derived from the bacteriophage Q β , yielding highly repetitive arrays of these cytokines on the VLP surface. Both vaccines overcame self-tolerance and induced high antibody titers against the corresponding self-molecules in mice. Immunization with either of the two vaccines reduced eosinophilic inflammation of the lung in an ovalbumin (OVA) based mouse model of allergic airway inflammation. Animals immunized with the two vaccines at the same time developed high antibody titers against both cytokines and also reduced eosinophil-infiltration of the lung. These data demonstrate that targeting either IL-5 or eotaxin may lower eosinophilia. Simultaneous immunization against IL-5 and eotaxin demonstrates that such a therapeutic approach may be used to treat complex disorders in which multiple mediators are involved.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways in which eosinophils have a prominent role and are present in sputum, bronchoalveolar lavage (BAL) fluid, and mucosal tissue biopsy samples [1]. Eosinophils are multifunctional leukocytes involved in the initiation and propagation of diverse inflammatory responses, as well as the modulation of innate and adaptive immune responses [2]. Important effector molecules of eosinophils are stored in granules and released upon activation. A prominent molecule is major basic protein, which triggers the degranulation of mast cells and basophiles, and increases smooth muscle reactivity. In addition, eosinophils generate large amounts of the cysteinyl leukotrienes [3], which contribute to the development of airway hyper reactivity (AHR). Eosinophils are produced in the bone marrow from pluripotent stem cells and normally circulate in the blood in low numbers (1–2% of blood leukocytes). Three cytokines, IL-3, IL-5 and GM-CSF,

are particularly important in regulating eosinophil development [2,4–6]. Amongst them, IL-5 is responsible for the selective differentiation of eosinophils [7]. IL-5 also stimulates release of eosinophils from the bone marrow into the peripheral circulation and promotes their migration to the lung upon allergen challenge; a key step in the development of lung inflammation [8,9]. In accord with these important roles for IL-5, antibodies that neutralize IL-5 inhibit both allergen-induced blood eosinophilia and the recruitment of eosinophils to the lung in murine models of asthma [10,11].

In addition to IL-5, cytokines from the eotaxin family also stimulate eosinophils to migrate from blood into tissues [12]. There are two variants of murine eotaxin, namely eotaxin 1 (eotaxin) and eotaxin 2 which both belong to the family of CC type chemokines [13,14]. Murine eotaxin has marked synergism with IL-5. Anti-eotaxin and anti-IL-5 antibodies alone and in combination have been shown to reduce OVA-induced airway eosinophilia but failed to inhibit AHR [15].

Importantly, blocking eosinophil-activity in mice prevents allergen induced airway eosinophilia and AHR and results in reduced lung-fibrosis, a severe consequence of asthma [16,17]. For humans, therapeutic intervention strategies aimed at blocking the action of eosinophils have been investigated in various asthma settings and eosinophilic disorders. Blockade of IL-5 with the humanized monoclonal antibody Mepolizumab has reduced circulating and sputum eosinophils and shown evidence for an effect on airway remodelling [17] but, has failed to achieve discernable effects

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on AHR or the late asthmatic response. Recent clinical testing of Mepolizumab in refractory eosinophilic asthma and prednisone dependent asthma has shown decreases in blood and sputum eosinophils and statistically significant decreases in the number of asthma exacerbations [18,19]. Thus, anti-eosinophil strategies may be a promising therapy in asthma subgroups with heavy eosinophilic loads in which conventional anti-inflammatory therapy is only partially effective.

Monoclonal antibodies (mAbs) are highly active molecules that are currently used in a numerous disease indications, including cancer and inflammation. However, due to the high amounts of antibodies required and their generally short half-life, therapies involving monoclonal antibodies are costly. In addition, long-term treatment with mAbs may result in the development of neutralizing anti-antibodies, which may reduce their efficacy or induce adverse effects [20]. Active immunization against self-antigens typically results in relatively long-lived antibody responses and has been viewed as a potential alternative to mAb therapies.

It has previously been shown that highly repetitive antigens displayed on viral surfaces are able to efficiently overcome B cell unresponsiveness [21]. Consequently, self-antigens displayed in this manner are able to induce strong self-specific antibody responses in mice [21–25] and humans [26]. Here we produced two conjugate vaccines, comprising either murine IL-5 or eotaxin covalently coupled to the surface of VLPs derived from the bacteriophage Q β . High titers of neutralizing antibodies against both IL-5 and eotaxin were obtained in mice immunized either singly or with a combination of the two vaccines. Immunization with the vaccines strongly reduced eosinophilia in a model of allergen induced airway inflammation. These results demonstrate that complex disorders regulated by multiple cytokines may possibly be treated with a combination vaccine approach.

2. Materials and methods

2.1. Mice

Female BALB/c mice were purchased from Charles River Laboratories. All mice were maintained under specific pathogen-free conditions and used for experiments according to protocols approved by the Swiss Federal Veterinary Office.

2.2. Cloning, expression and purification of rIL-5

IL-5 was amplified from an ATCC clone (pmIL5-4G; ATCC number: 37562) by PCR. The PCR product was subcloned into a vector derived from pET22b (Novagen, Inc.). The construct comprises a histidine tag, an enterokinase cleavage site and a gamma 3 derived amino acid linker containing a cysteine residue (LEPKPSTPPGSSG-GAPGGCG) and the DNA encoding the mature form of IL-5 protein. The resulting recombinant IL-5 fusion protein (rIL-5) was expressed in *Escherichia coli* BL21 (DE3) cells.

Overnight cultures were grown and diluted into TB medium containing 0.1 mg/L ampicillin. IPTG was added to a final concentration of 1.0 mM when an OD₆₀₀ of culture reached 0.7. After 4 h incubation, bacteria were harvested and the pellet re-suspended in PBS. Inclusion bodies were prepared from this suspension and the insoluble rIL-5 solubilized in denaturing buffer (100 mM NaH₂PO₄, 10 mM Tris–HCl, 6.0 M guanidine-hydrochloride, pH 8.0). After centrifugation for 20 min at 20 000 $\times$ g, the supernatant containing soluble rIL-5 was mixed with Ni-NTA resin (Qiagen). The mixture was incubated for 3 h at 4 °C and unbound protein washed away. rIL-5 was eluted from the resin with 100 mM NaH₂PO₄, 10 mM

Tris and 6.0 M guanidine-hydrochloride (pH 4.5). The semi-purified rIL-5 protein was dialysed against 8.0 M urea, 100 mM NaH₂PO₄ and 10 mM Tris–HCl (pH 8.0) at 4 °C. Afterwards, the protein was refolded by sequential dialysis against the following buffers at pH 8.5: buffer 1 (2 M urea, 50 mM NaH₂PO₄, 5 mM glutathione reduced, 0.5 mM glutathione oxidized, 0.5 M arginine and 10% glycerol), buffer 2 (50 mM NaH₂PO₄, 5 mM glutathione reduced, 0.5 mM glutathione oxidized, 0.5 M arginine and 10% glycerol), buffer 3 (50 mM NaH₂PO₄ and 10% glycerol) and buffer 4 (20 mM NaH₂PO₄ and 10% glycerol). Final purification was performed with a Hitrap Q column (Amersham Pharmacia) utilizing an increasing salt gradient (20 mM NaH₂PO₄, 10% glycerol, 2 M NaCl, pH 8.5). Purified rIL-5 protein was dialysed against PBS and the protein concentration estimated by Bradford assay.

2.3. Cell proliferation assay

The bioactivity of rIL-5 was tested in a cell proliferation assay [27]. Briefly, rIL-5 was incubated in flat bottom 96-well plates with 2×10^4 BCL1 cells (a B cell lymphoma line) per well and incubated for 24 h at 37 °C, 5% CO₂. 1 μ Ci of ³H-thymidine (Hartmann Analytic, Switzerland) was added to each well and the plates incubated for 6 h at 37 °C with 5% CO₂. The cells were harvested, washed and the incorporation of thymidine determined by emission-counting with a liquid scintillation counter. Commercial murine IL-5 from R&D systems (cIL-5) was used as a control.

To test the neutralizing activity of serum from Q β -IL-5 vaccinated mice, BCL1 cells (2×10^4 per well) were plated in the presence of 20 ng/ml of rIL-5. Pooled sera from Q β -IL-5 vaccinated or naive mice was titrated with the cells (starting dilution 1/4, titration steps 1/6). After 24 h, 1 μ Ci of ³H-thymidine was added to the cells, which were incubated for 12 h. The incorporation of thymidine was determined by emission-counting with a liquid scintillation counter.

2.4. Cloning, expression and purification of murine eotaxin

Murine eotaxin was expressed as a fusion protein in a vector modified from pET22b. The fusion protein (r-eotaxin) consisted of the mature form of murine eotaxin, a hexa-histidine tag and a cysteine containing linker (GGC) at its C-terminus. Expression of r-eotaxin in *E. coli* BL21 (DE3) was induced with 1 mM IPTG. The soluble fraction of bacterial lysate containing r-eotaxin was mixed with Ni-NTA agarose (Qiagen) in 300 mM NaCl, 50 mM NaH₂PO₄, 0.5% tween 20 and 20 mM imidazole (pH 8). After washing away unbound contaminants, r-eotaxin was eluted with 300 mM NaCl, 50 mM NaCl, tween 20 and 250 mM imidazole (pH 8). Semi-purified r-eotaxin was loaded onto a SP sepharose column (Amersham) in buffer containing 20 mM Tris, 200 mM NaCl (pH 8). After washing r-eotaxin was eluted with an increasing salt gradient (20 mM Tris, 1 M NaCl, pH 8.0).

2.5. Coupling rIL-5 and r-eotaxin to Q β

VLPs derived from the bacteriophage Q β were expressed in *E. coli* containing a expression plasmid pQ10 and purified as described previously [28]. In order to be coupled to IL-5, Q β VLPs were first derivatized with 10-fold excess of a heterobifunctional chemical cross-linker, succinimidyl-6-(β -maleimidopropionamido) hexanoate (SMPH). The unbound SMPH was removed by dialysis against PBS. rIL-5 was reduced for 1 h with an equimolar amount of tri (2-carboxyethyl) phosphine hydrochloride (TCEP) in PBS (pH 8.0). Reduced rIL-5 (80 μ M) was incubated for 4 h at 22 °C with 40 μ M of SMPH derivatized Q β (dQ β). The reaction was dialysed 12 h against PBS pH 8.0.

A slightly different protocol was used to couple r-eotaxin to Q β . Q β VLPs were derivatized with a 2.3-fold molar excess of SMPH. A

1.2–1 molar ratio of TCEP to protein was used to reduce r-eotaxin. Reduced r-eotaxin (20 μ M) was incubated for 1 h at room temperature with 24 μ M of dQ β .

The coupling products (Q β -IL-5 and Q β -Eot) were analyzed by SDS-PAGE and Western blot with anti-His and anti-Q β antibodies. Protein concentration was measured by Bradford. The coupling efficiencies (i.e. mol rIL-5/mol Q β monomer or mol r-eotaxin/mol Q β monomer) were estimated by densitometric analysis of Coomassie blue stained SDS-PAGE gels.

2.6. Immunization of mice with murine Q β -IL-5, Q β -Eot or Q β -IL-5 and Q β -Eot

Eight-week-old female BALB/c mice (5 per group) were vaccinated with either Q β -Eot or Q β -IL-5, or the combination of both without the addition of adjuvant. 50 μ g of total protein of each vaccine was injected subcutaneously on days 0, 21 and 35. Mice were subjected to retro-orbital bleeding on days 0, 21, 35 and 45 and sera analyzed by the use of IL-5 and eotaxin-specific ELISA.

2.7. ELISA

ELISA plates were coated with mouse rIL-5 or r-eotaxin at a concentration of 5 μ g/ml. Plates were blocked then incubated with serially diluted mouse sera. Bound antibodies were detected with enzymatically labeled anti-mouse IgG antibody. As a control, preimmune serum from the same mice was tested. Antibody titers were calculated as the serum dilution which led to a half-maximal of OD₄₅₀ (OD50%).

2.8. OVA based model of allergic airway inflammation

To induce allergic airway inflammation, female BALB/c mice (5 per group) were injected (i.p.) with 10 μ g of OVA (Grade V, Sigma-Aldrich) mixed with 2 mg of alum (Aluminium Hydroxide Gel Adjuvant, Brenntag Biosector, Denmark). 10 days later, mice were challenged daily with 100 μ g of OVA by intranasal administration for 4 days. 24 hours after the last challenge, BAL and lungs were subjected to histology. Mice injected i.p. with OVA and alum but not challenged intranasally with OVA served as a negative control for disease induction in these experiments.

2.9. Differential cell counting and lung histology

To assay the activity of r-eotaxin, BALB/c mice (5 per group) were immunized i.p. on days 0 and 3 with 100 μ g of OVA mixed with 2 mg of alum. On day 14, mice were injected with either PBS or 0.5 μ g of r-eotaxin i.v. Thirty min after injection, blood samples were collected from each mouse and blood smears were made. The slides were dried in air and stained with Kit RAL 555 (Réactifs RAL) according to manufacture's protocol (a fast-acting variation of May-Grünwald Giemsa staining). The percentage of eosinophils was evaluated with a light microscope.

In the model of allergic airway inflammation, bronchoalveolar cells were collected in successive lavages (BAL) using 0.5 ml aliquots of PBS with 2% BSA at room temperature until the total volume reaches 1.2 ml. The total number of cells in the BAL was counted with a Coulter Counter (Beckman Coulter, Inc.). Cytospins were performed with Shandon Cytospin apparatus (Thermo Fisher Scientific, Inc.) and stained with Kit RAL 555 (Réactifs RAL) according to the manufacture's protocol. Differential cell counts were performed with at least 200 leukocytes. Mouse lungs were removed and fixed in 10% PBS buffered formalin. Paraffin sections were stained with Chromotrope 2R to identify eosinophils [29].

2.10. Statistics

For statistical analysis, Student's *t*-test was used. *p*-Values <0.05 were considered significant.

3. Results

3.1. Production of the vaccines

Recombinant murine IL-5 with an N-terminal hexa-histidine tag, an enterokinase cleavage site and a linker containing a cysteine residue was expressed and purified. IL-5 has been shown to induce the proliferation of the murine B cell tumor line BCL-1 *in vitro* and this assay is used as a standard test for mouse IL-5 activity [27]. In order to verify the bioactivity of the rIL-5 protein and thus the authenticity of the vaccine, we tested the ability of rIL-5 to induce proliferation of BCL-1 cells. As shown in Fig. 1A, rIL-5 induced proliferation of BCL-1 cells in a concentration dependent manner. The highest proliferation rate was induced with 10 ng/ml of rIL-5. This activity was similar to commercially acquired IL-5 (cIL-5). This result demonstrates that rIL-5 was correctly folded and that the His-tag and the Cys-containing linker did not adversely affect the protein.

Murine r-eotaxin 1 with a hexa-histidine tag and a cysteine containing linker at its C-terminus was expressed and purified. It has been previously demonstrated that the number of eosinophils circulating in the blood increases in response to administration of eotaxin and the accumulation of eosinophils in response to eotaxin was more pronounced in mice that had been sensitized with OVA [30]. To verify the bioactivity of r-eotaxin, we tested its chemo-attractant activity towards eosinophils *in vivo*. OVA immunized BALB/c mice (*n*=5) were injected with either PBS or 0.5 μ g of r-eotaxin i.v. The number of eosinophils in the blood was assessed 30 min after the injection. As shown in Fig. 1B, the number of eosinophils in the blood doubled in mice which had been treated with r-eotaxin. This results shows that r-eotaxin was efficient at inducing the accumulation of eosinophils in the blood and was thus expressed in an authentic manner.

In order to produce Q β -IL-5 and Q β -Eot vaccines, rIL-5 and r-eotaxin were both chemically coupled to VLPs derived from bacteriophage Q β via a heterobifunctional cross-linker. The Coomassie-stained SDS-PAGE demonstrates the presence of rIL-5 (lane 2 of the left panel of Fig. 1C), r-eotaxin (lane 4 of the left panel of Fig. 1D), monomeric (14 kDa) and multimeric Q β subunits (lane 3 of the left panel of Fig. 1C and lane 2 of the left panel of Fig. 1D). Coupling products whose molecular masses correspond to rIL-5 or r-eotaxin covalently linked to one or more Q β monomers are shown in lane 4 of the left panel of Fig. 1C and lane 3 of the left panel of Fig. 1D, respectively. Western blot analysis with either anti-His (middle panels of 1C and D) or anti-Q β antibodies (right panels of 1C and D) demonstrated the same bands reacted with both antibodies, confirming the covalent attachment of rIL-5 or r-eotaxin to Q β . In contrast, anti-Q β antibody did not react with either rIL-5 or r-eotaxin (lane 1 of the right panel of Fig. 1C and lane 3 of the right panel of Fig. 1D, respectively). Analysis of the coupling efficiency by densitometry showed that 47% or 15% of Q β monomers were cross-linked to rIL-5 or r-eotaxin, respectively. This corresponds to about 80–90 rIL-5 and 25–30 r-eotaxin molecules displayed per VLP.

3.2. Induction of anti-IL-5 and anti-eotaxin antibodies by active immunization

The immunogenicity of each vaccine was tested by subcutaneously injecting (s.c.) 50 μ g of Q β -IL-5 or Q β -Eot into mice (*n*=5) at days 0, 21 and 35. The generation of anti-IL-5 and anti-eotaxin

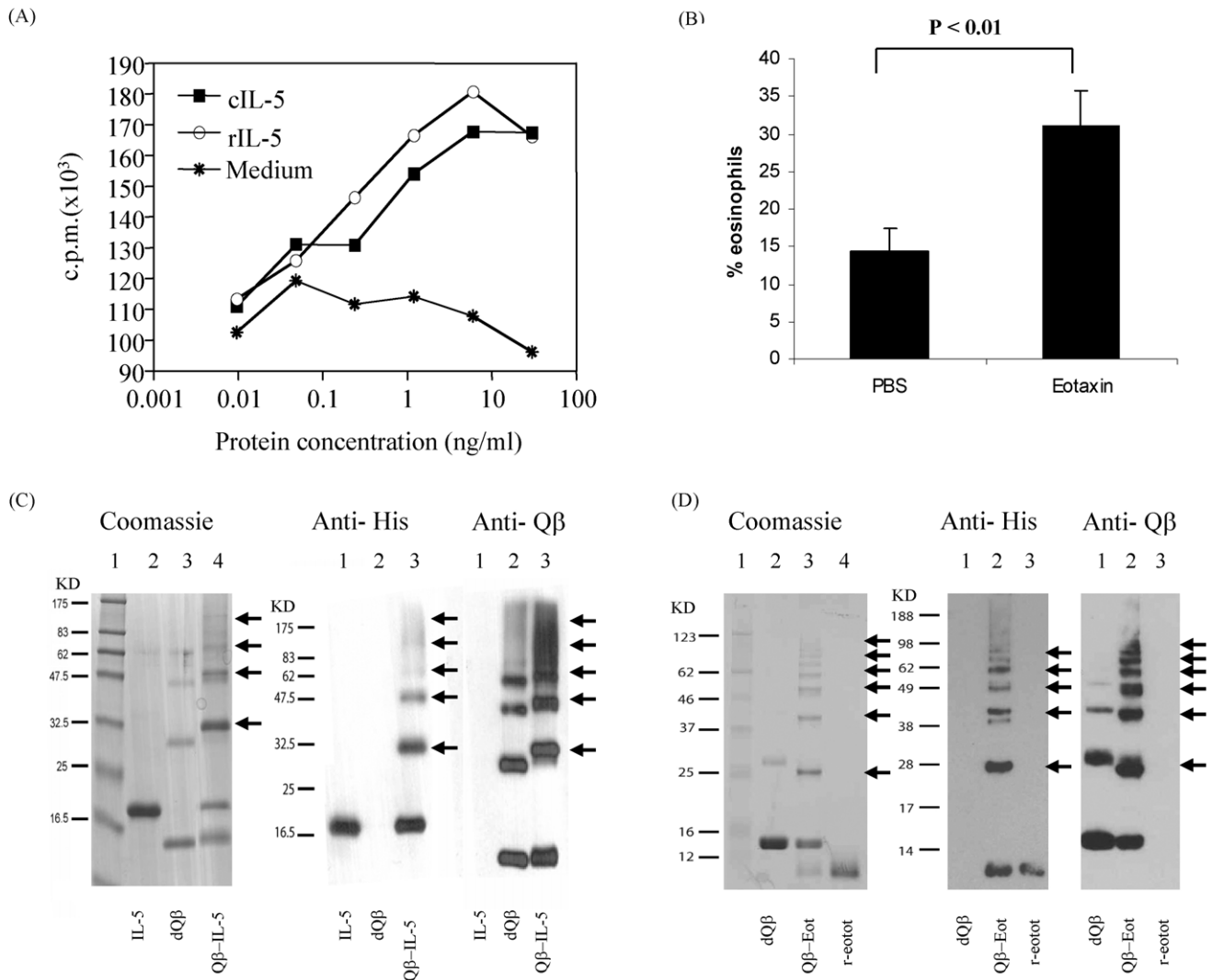


Fig. 1. Production of Qβ-rIL-5 and Qβ-Eot. (A) Measurement of rIL-5 bioactivity. rIL-5 bioactivity was monitored in a cell proliferation assay by measuring the amount of ³H-thymidine incorporated into BCL1 cells after incubation with IL-5. The degree of thymidine incorporation was measured by scintillation counting (determined as counts per minute: c.p.m.). Murine IL-5 from R&D systems was used as a positive control. (B) Measurement of r-eotaxin bioactivity. OVA sensitized mice were injected with either PBS or 0.5 μg of r-eotaxin i.v. 30 min after injection, blood was collected and smears performed. The percentage of eosinophils was evaluated by microscopy. The difference in percentage of eosinophils between eotaxin and PBS group was statistically significant ($p < 0.01$). (C) Coupling rIL-5 to Qβ. Recombinant rIL-5 containing an N-terminal cysteine was cross-linked via SMPH to Qβ VLPs. IL-5, Qβ (after reaction with SMPH) and conjugate vaccine (Qβ-rIL-5) were analyzed by SDS-PAGE (left panel), and α-His and α-Qβ-specific Western blots (middle and right panel, respectively). Bands, with molecular weights higher than rIL-5 (indicated by arrows) which stained both α-Qβ and α-rIL-5 are indicative of successful coupling of rIL-5 to Qβ. (D) Coupling of r-eotaxin to Qβ. Recombinant eotaxin containing a C-terminal cysteine was cross-linked via SMPH to Qβ VLPs. Qβ (after reaction with SMPH), r-eotaxin and conjugate vaccine (Qβ-Eot) were analyzed by SDS-PAGE (left panel), as well as by α-His and α-Qβ-specific Western blots (middle and right panel, respectively). Bands, with molecular weights higher than r-eotaxin (indicated by arrows) which stained both α-Qβ and α-rIL-5 are indicative of successful coupling of r-eotaxin to Qβ.

IgG antibodies was determined by ELISA. As shown in Fig. 2, 21 days after the initial immunization, high antibody titers against either IL-5 or eotaxin were detected. Subsequent immunization further increased the titers. For each antigen, a statistically significant increase in titer from days 21 to 54 was observed ($p < 0.01$). Thus, both vaccines can efficiently overcome B cell unresponsiveness and induce high antibody titers against the displayed auto-antigens.

The immune response to vaccination with both Qβ-IL-5 and Qβ-Eot injected simultaneously was next examined. Following immunization, high levels of auto-antibodies against both IL-5 and eotaxin were induced. The kinetics and magnitude of the response were similar to those observed for immunization with the corresponding single antigen (Fig. 2A and B). Again the increase in titers from days 21 to 54 was statistically significant ($p < 0.01$). These data demonstrate that co-immunization with VLP-based vaccines can simultaneously break tolerance towards more than one

self-antigen and induce high antibody responses against the corresponding molecules.

We next checked the neutralizing ability of anti-IL-5 serum in a cell (BCL1 cells) proliferation assay cell. As shown in Fig. 2C, anti-IL-5 antiserum inhibited the proliferation of BCL1 cells induced by IL-5 in a concentration dependant manner. We further investigated the neutralizing ability of the anti-IL-5 antibodies induced by Qβ-IL-5 by counting blood eosinophils after immunization. Fig. 2D shows that relative to mice immunized with a control Qβ vaccine, the number of peripheral blood eosinophils in Qβ-IL-5 immunized mice was reduced by 87% ($p < 0.01$). There was no statistically significant difference between unvaccinated animals and those receiving control Qβ vaccine demonstrating anti-Qβ antibodies do not neutralize IL-5. These results show the anti-IL-5 antibodies induced by immunization with Qβ-IL-5 neutralize the activity of IL-5 *in vitro* and *in vivo*.

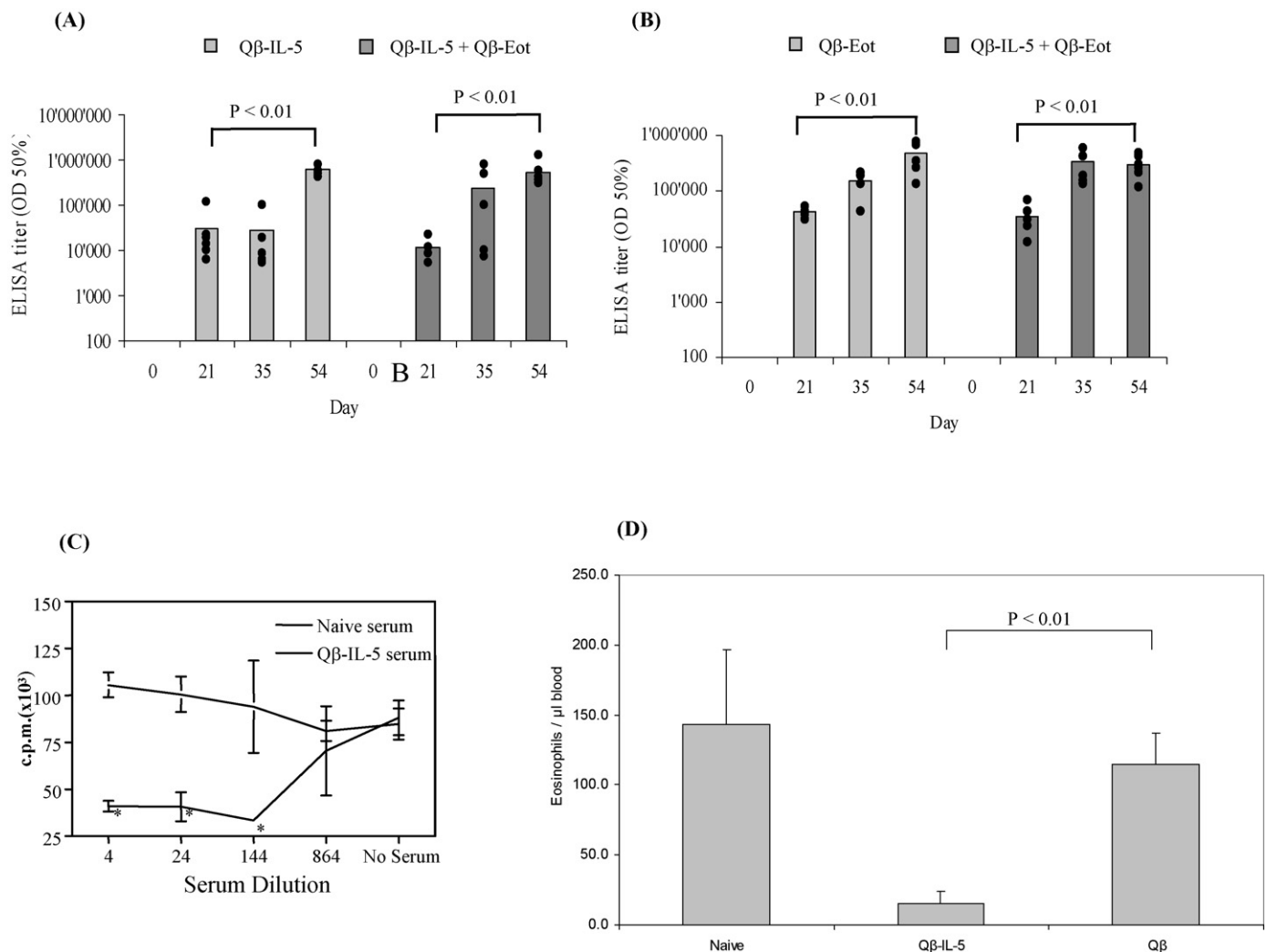


Fig. 2. Immune response to immunization with Qβ-IL-5, Qβ-Eot or combination of Qβ-IL-5 and Qβ-Eot. BALB/c mice (5 per group) were vaccinated s.c. with either Qβ-IL-5, Qβ-Eot or both Qβ-IL-5 and Qβ-Eot without the addition of adjuvant. 50 μg of each vaccine was injected subcutaneously on days 0, 21 and 35. Sera from days 0, 21, 35 and 54 were analyzed by IL-5 or eotaxin-specific ELISA. (A) Anti-IL-5 IgG antibody response to vaccination with Qβ-IL-5 or Qβ-IL-5 and Qβ-Eot. (B) Anti-eotaxin IgG antibody response to vaccination with Qβ-Eot or Qβ-IL-5 and Qβ-Eot. Values for individual animals are represented by dots. Student's *t*-test was used to compare titers from day 21 with days 35 or 54 and statistically significant differences are indicated. (C) Anti-IL-5 serum inhibits IL-5 induced BCL1 cell proliferation. BCL1 cells (2×10^4 per well) were plated in the presence of 20 ng/ml cIL-5. Serum of from Qβ-IL-5 vaccinated (filled circles) or naive mice (open circles) was titrated onto the cells. After incubation, 1 μCi of ³H-thymidine was added to the cells. The incorporation of thymidine was by scintillation counting (measured as counts per minute: c.p.m.). Error bars indicate $\pm$ SD of triplicate wells. (D) BALB/c mice (3–4 per group) were repeatedly vaccinated with Qβ-IL-5 or a Qβ control vaccine. Two weeks after final immunization, blood was collected and eosinophil numbers measured with an automated analyser. Unvaccinated mice were used as a control to determine basal levels of eosinophils. Student's *t*-test was used to compare eosinophil counts for different groups. Error bars indicate standard error of the mean.

3.3. Active immunization against IL-5 and eotaxin reduced airway inflammation

The ability of the vaccines either singly or in combination to induce neutralizing antibodies *in vivo* in an inflammatory setting was assessed by the use of an OVA-based mouse model of allergic airway inflammation. BALB/c mice ($n=5$) were either not vaccinated (injected with PBS) or vaccinated with 50 μg of Qβ-IL-5 or Qβ-Eot singly or with both vaccines simultaneously (a total of 100 μg of vaccine corresponding to 50 μg of Qβ-IL-5 and 50 μg of Qβ-Eot) on days 0, 21 and 35. A three-dose regimen was chosen in order to rapidly establish high antibody titers. After anti-IL-5 and eotaxin antibody titers were confirmed by ELISA, airway inflammation was induced by intraperitoneal (i.p.) and intranasal (i.n.) injection of OVA as described. One day after the final i.n. challenge, mice were sacrificed and assessed for one of the key pathological features of asthma, namely lung inflammation. The total cell numbers in BAL fluid of OVA sensitized and challenged mice increased

over 10-fold to 650 000 compared with those in OVA sensitized but not challenged mice (57 000) indicating severe pulmonary inflammation in these animals. Interestingly, mice which received either Qβ-Eot or Qβ-IL-5 showed reduced inflammation in the airways (Fig. 3A). Specifically, the total number of infiltrating cells in Qβ-Eot immunized mice reached 250 000 and Qβ-IL-5 immunized mice reached 200 000. A further reduction in infiltrating cell number (140 000) was achieved by the combined vaccination of both vaccines.

Since eosinophils are the main effector cells during airway inflammation, we quantified their numbers in BAL fluid by differential cell staining (Fig. 3B). OVA sensitized and challenged mice vaccinated with Qβ-IL-5 had 97% ($p=0.012$) fewer eosinophils relative, while Qβ-Eot vaccinated mice had an 80% reduction in eosinophil numbers ($p=0.031$) relative to animals that were OVA sensitized and challenged, but not vaccinated. This result demonstrates that active immunization against either IL-5 or eotaxin efficiently reduces eosinophilic airway inflammation in a mouse

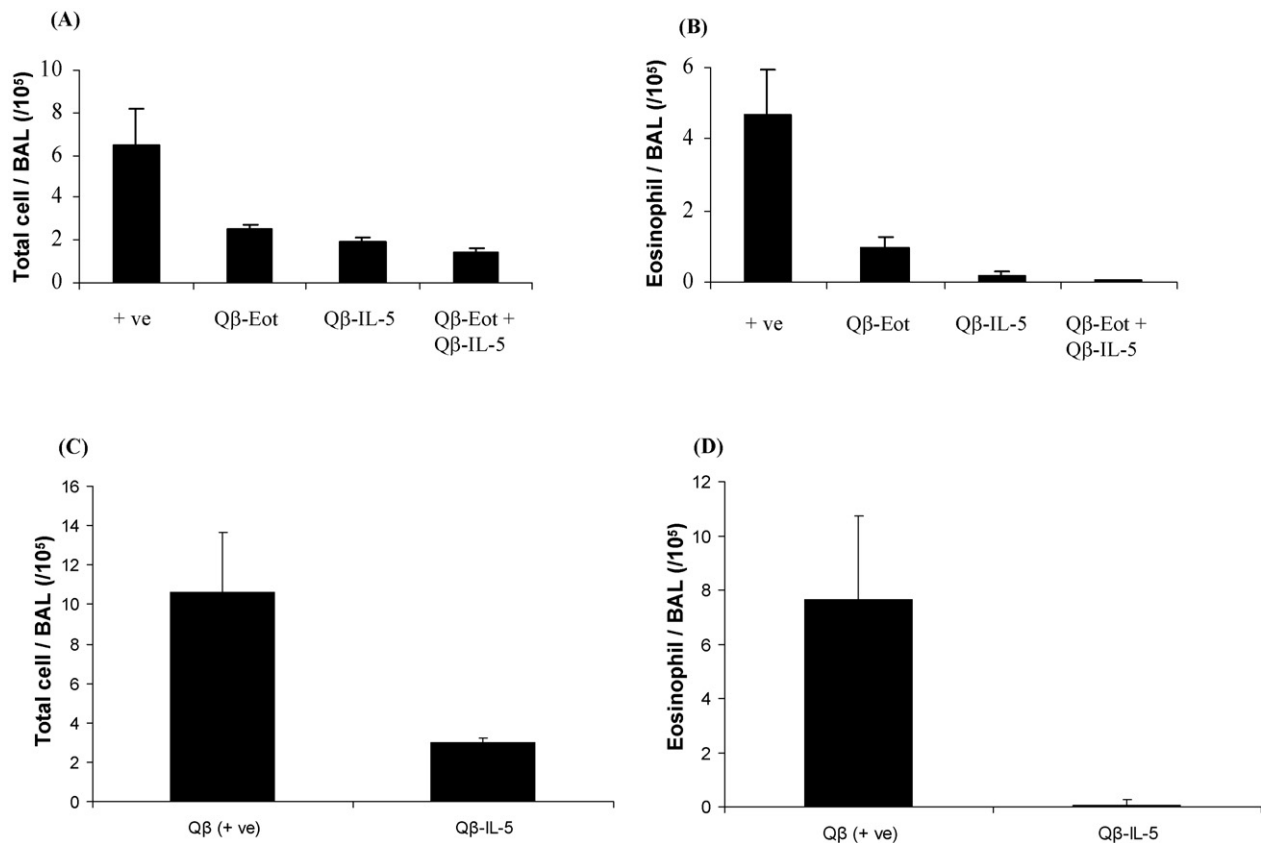


Fig. 3. Qβ-IL-5 and/or Qβ-Eot vaccination reduces numbers of infiltrating cells in BAL. For A and B mice (5 per group) were vaccinated s.c. with either PBS, Qβ-IL-5, Qβ-Eot, or a combination of both vaccines without the addition of adjuvant. Groups vaccinated with vehicle (PBS) only served as positive control for disease induction. For C and D mice (5 per group) were immunized with either Qβ or Qβ-IL-5. Mice immunized with Qβ served as positive control for disease induction. On day 53, all mice were injected i.p. with 10 μg of OVA mixed with 2 mg of alum. Ten days later all mice were challenged daily with 100 μg of OVA by intranasal administration for 4 days. BALF was collected 24 h after the last challenge and cells counted. (A and C) Shows total cells in BAL and (B and D) shows the number of eosinophils. Error bars indicate standard error of the mean.

model of allergic airway inflammation. Mice vaccinated with both vaccines showed a 99% reduction in infiltrating eosinophils relative to the positive control ($p = 0.005$). Nonetheless, a small population of eosinophils remained in the BAL. In contrast no change in the numbers of macrophages, neutrophils and lymphocytes could be observed in these vaccinated mice (data not shown). While the use of two vaccines in combination was numerically better than either eotaxin Qβ or IL-5-Qβ vaccine alone, the result did not achieve statistical significance when analyzed by 4-way ANOVA.

In a separate experiment we compared the total number of cells and eosinophils in BAL obtained from mice immunized with Qβ or IL-5 Qβ (Fig. 3C and D). For Qβ immunized mice, the total number of cells and eosinophils in the BALF were in a comparable range to those for the unvaccinated animals in the experiment described above (see Fig. 3A and B). For the group immunized with Qβ-IL-5 there was a 72% reduction in the number of total cells in the BALF ($p = 0.038$) and a 97% reduction in the number of eosinophils ($p = 0.008$).

To determine if the reduction in inflammatory cells and eosinophils in the BAL following immunization reflected cellular changes in the lung, H&E (data not shown) and Lendrum staining of lung sections were also performed (Fig. 4). Histological analysis indicated that mice which were sensitized but not challenged with OVA had no (or only minimal) histopathological lesions (Fig. 4A). In contrast, OVA sensitized and challenged mice developed histopathological lesions typical of those described for this model of allergic airway inflammation. In these mice, eosinophils, lymphocytes, macrophages and plasma cells infiltrated the lung with eosinophilic granulocytes representing the dominant cell type

(Fig. 4C). The infiltrates were mainly located in perivascular and peribronchial areas (Fig. 4B). However, for mice immunized with Qβ-Eot, Qβ-IL-5 or a combination of both, lung inflammation was substantially reduced (Fig. 4D–F). It was also evident that the eosinophilic component of the lung-infiltrates of vaccinated mice was markedly reduced. Indeed, eosinophils no longer represented the major infiltrating cell type. H&E staining supported these observations.

4. Discussion

IL-5 has been shown to be important for the development of eosinophils in the bone marrow and for their release into the peripheral circulation [7–9]. Furthermore, eotaxin together with IL-5 are important for the distribution of eosinophils into the tissues [12]. Consequently, inhibiting the biological activity of either one of these key molecules by administration of anti-IL-5 or anti-eotaxin monoclonal antibodies diminished eosinophilia in response to antigen inhalation in mouse models of asthma [15]. Although therapies with monoclonal antibodies are highly effective, they may have some limitations, including high costs, immunogenicity of mAbs and poor pharmacokinetics [31–33]. In some cases, active vaccination strategies might offer a valuable alternative [34]. In a recent preclinical study, active immunization with a DNA vaccine against IL-5 was shown to bypass immunological tolerance, induce neutralizing antibodies and reduce airways inflammation and eosinophilia. However, at least four injections were needed to obtain a 100% response and long lasting effects of this vaccine

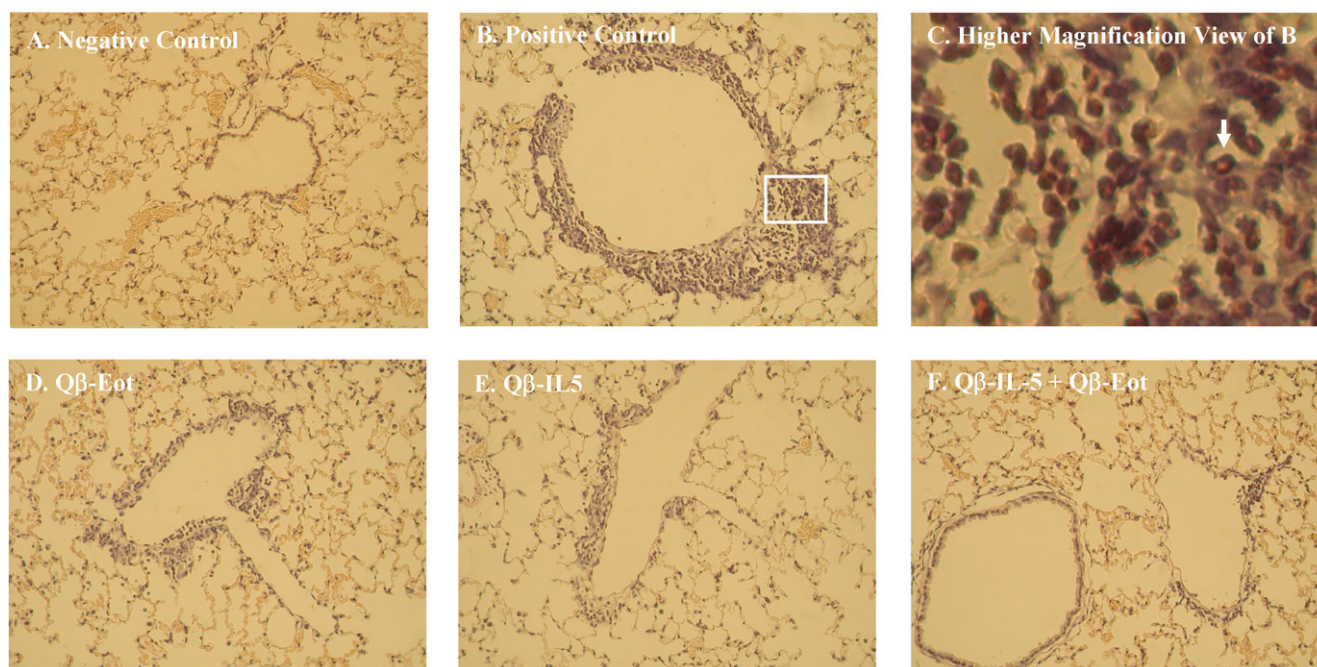


Fig. 4. Q β -IL-5 and/or Q β -Eot vaccination reduces numbers of infiltrating cells in Lung. Lendrum stain of lung sections. (A) Lungs from OVA sensitized but not challenged mice had no or very mild histopathological lesions. (B) Mice which were sensitized and challenged with OVA (positive control) had large numbers of infiltrating cells in the lung. (C) Higher magnification view of (B) to show that most infiltrating cells were Chromotrope 2R positive (arrow) eosinophils. (D) Q β -Eot treated mice had slightly reduced numbers of infiltrating eosinophils in the lung. (E) The percentage of eosinophils in infiltrating cells from Q β -IL-5 vaccinated mice was markedly reduced in comparison with the positive control. (F) Q β -IL-5 and Q β -Eot double vaccinated mice also demonstrate reduction of eosinophils in the lung.

have not yet been demonstrated [35]. Furthermore, DNA vaccination has proven to be unsuccessful at inducing antibody responses in humans. In contrast, a number of studies in mice [21–25,36] and humans [37–40] with VLP-based vaccines have shown that highly repetitive display of antigens on VLPs results in potent antibody responses. Indeed, self-specific antibody responses with clinically meaningful efficacy have been achieved with such vaccines [26]. Antibodies induced by active immunization with VLP-based vaccines decline relatively slowly over time with a estimated half-life of 2–3 months [26,37] and titers can be boosted or at least maintained by additional immunizations making it an attractive strategy to treat chronic disease. In this study, we have shown that a single immunization with Q β -IL-5 or Q β -Eot resulted in a 100% responder rate in the absence of adjuvant. Furthermore, by using a combined vaccination strategy, neutralizing antibodies against IL-5 and eotaxin could be simultaneously induced and maintained.

In murine models of asthma, inhibition or lack of IL-5 consistently suppresses pulmonary eosinophilia in response to antigen inhalation; however, this effect does not always correlate with improved lung function [41]. This may be due to the different mouse strains used in these studies and/or to different degrees of residual tissue eosinophilia present in these mice after allergen inhalation [42,43]. Similarly in a short-term clinical study, treatment of patients with severe persistent asthma with the monoclonal antibody Mepolizumab showed a dramatic depletion of blood eosinophils but no appreciable effect on bronchial mucosal staining of eosinophil major basic protein [44,45]. Other clinical studies have not demonstrated appreciable effects on late asthmatic reactions, airway hyper-responsiveness or other clinical outcomes including lung function but indirect evidence for an effect on airway remodelling has been reported. Interestingly, blocking IL-5 resulted in reduced airway remodelling in mice [46], a finding consistent with the observation in mice that selective removal of eosinophils by genetic means also resulted in reduced fibrosis of the lung [47,48]. Recent clinical data has shown that in

refractory eosinophilic asthma and prednisone dependent asthma, Mepolizumab not only decreased eosinophils in blood and sputum eosinophils but also decreased the number of asthma exacerbations [18,19].

Our studies showed that although eosinophils in BAL were largely reduced in Q β -IL-5 vaccinated mice which were then sensitized and challenged with OVA, some eosinophils were still present in lung tissues. This result was not completely unexpected, since eosinophils may also be recruited by chemokines like eotaxin. Vaccination with Q β -Eot alone also resulted in a reduction of eosinophils in the airways of OVA sensitized and challenged mice, even though the effect was less pronounced than in Q β -IL-5 vaccinated mice. As a caveat, it should be noted that, in order to establish effective neutralizing titers, Q β -IL-5 and Q β -Eot vaccines were administered prior to OVA sensitization. Such prophylactic use of the vaccines was necessary due to the limited time-span between the sensitization and challenge phases employed in the model. Hence it is possible that a reduction of eosinophils may have interfered with the induction of the allergic response prior to sensitization which could have inhibited the effector phase during the challenge [9]. However it has been shown in murine and guinea pig models of allergic asthma that administering neutralizing anti-IL-5 monoclonal antibodies after antigen sensitization reduces lung eosinophilia [49,50]. It is also likely that if vaccination could be employed therapeutically in these models it would have a similar effect.

One approach to developing effective vaccines which may ameliorate the disease symptoms would be to target both molecules simultaneously. We therefore targeted eotaxin in addition to IL-5. As expected, there was only minimal number of eosinophils in BAL of mice immunized with both Q β -IL-5 and Q β -Eot. Since immunization with Q β alone had no effect on eosinophilia in this animal model, the reduction of infiltrating cells in Q β -IL-5 and/or Q β -Eot immunized mice must be the result of high antibody titers against IL-5 and/or eotaxin. This means that the antibodies induced by Q β -

IL-5 and Q β -Eot are neutralizing antibodies and they can block the bioactivity of the corresponding cytokines *in vivo*. We also noticed that low numbers of eosinophils in lung tissue were still present. The pathological role of these eosinophils should be further investigated. In order to completely block the eosinophilia in the lung, a combination of vaccines against eotaxin, eotaxin-2 and IL-5 may be beneficial.

The reduction of eosinophilia may not only have a role in the abrogation of acute processes but also in events further down stream such as repair and remodelling caused by chronic eosinophilic inflammation. As discussed above, a recent study in man has shown that even modest eosinophil depletion by anti-IL-5 was associated with significant reductions in tenascin and lumican deposition in the bronchial reticular basement membrane, two markers of airway remodelling [17]. Therefore, combined vaccination against IL-5 and eotaxin using VLP-based vaccines which induce high and lasting auto-antibody titers against the corresponding molecules, abrogating eosinophilia, may prevent lung remodelling.

To our knowledge, this is the first report which describes active vaccination simultaneously targeting more than one self-antigen. The result shown here is of potential consequence for our continuously aging society. According to the World Health Organization, in the industrialized world, as many as 25% of 65–69-year olds and 50% of 80–84-year olds are affected by two or more chronic health conditions. Combined vaccination against more than one self-antigen opens the possibility to target chronic diseases in which multiple factors are involved. Moreover, this strategy could be used to target more than one disease at the same time.

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