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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 Qβ, 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.

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 Qβ. 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 (LEPKPSTPPGSSGGAPGGCG) 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 OD_{600} 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 NaH_2PO_4, 10 mM Tris–HCl, 6.0 M guanidine-hydrochloride, pH 8.0). After centrifugation for 20 min at 20 000 × *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 NaH_2PO_4, 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 NaH_2PO_4 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 NaH_2PO_4, 5 mM glutathione reduced, 0.5 mM glutathione oxidized, 0.5 M arginine and 10% glycerol), buffer 2 (50 mM NaH_2PO_4, 5 mM glutathione reduced, 0.5 mM glutathione oxidized, 0.5 M arginine and 10% glycerol), buffer 3 (50 mM NaH_2PO_4 and 10% glycerol) and buffer 4 (20 mM NaH_2PO_4 and 10% glycerol). Final purification was performed with a Hitrap Q column (Amersham Pharmacia) utilizing an increasing salt gradient (20 mM NaH_2PO_4, 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% CO_2. 1 μCi of ^{3}H-thymidine (Hartmann Analytic, Switzerland) was added to each well and the plates incubated for 6 h at 37 °C with 5% CO_2. 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 Qβ-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 Qβ-IL-5 vaccinated or naive mice was titrated with the cells (starting dilution 1/4, titration steps 1/6). After 24 h, 1 μCi of ^{3}H-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 NaH_2PO4, 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 Qβ

VLPs derived from the bacteriophage Qβ were expressed in *E. coli* containing a expression plasmid pQ10 and purified as described previously [28]. In order to be coupled to IL-5, Qβ VLPs were first derivatized with 10-fold excess of a heterobifunctional chemical cross-liker, 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 μM) was incubated for 4 h at 22 °C with 40 μM of SMPH derivatized Qβ (dQβ). The reaction was dialysed 12 h against PBS pH 8.0.

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