

Expression of a Major Plant Allergen as Membrane-Anchored and Secreted Protein in Human Cells with Preserved T Cell and B Cell Epitopes

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Key Words

IgE-mediated allergy • Recombinant allergen Phl p 5 • Cell-based therapy • Allergenic activity • Surface protein expression

Abstract

Background: Expression of allergens in human cells is a prerequisite for the development of antigen-specific cell therapy in IgE-mediated allergy. We developed a strategy how the clinically relevant major grass pollen allergen Phl p 5 can be efficiently secreted or expressed on the surface of human cells with preserved allergenic activity. **Methods:** The cDNA of Phl p 5 was fused to a leader peptide with or without a transmembrane domain and both constructs were ligated into a mammalian expression vector. Transfection of these plasmids into human cells resulted in a membrane-anchored or secreted version of Phl p 5, respectively, as determined by ELISA or flow cytometric analysis. **Results:** Both the secreted and membrane-anchored Phl p 5 proteins bound IgE from allergic patients in an immunoblot assay and induced specific histamine release and CD203c upregulation in basophils of grass pollen-allergic patients. Proliferation of peripheral

blood mononuclear cells from Phl p 5-allergic individuals was induced upon stimulation with both variants of Phl p 5 expressed in human cells similar to recombinant Phl p 5. **Conclusions:** Secreted and membrane-anchored Phl p 5 expressed in human cells preserved B cell as well as T cell epitopes and may be used to develop and test various cell-based strategies for allergen-specific immunomodulation and to delineate the tolerance mechanisms involved therein.

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Introduction

Allergen-specific immunotherapy is the only causative treatment in IgE-mediated allergy so far, but prevention of allergy is still an unmet goal. Transplantation of hematopoietic stem cells that have been genetically modified to express disease-causing antigens, such as auto- and allo-antigens, is a therapeutic strategy to induce tolerance towards these disease-causing antigens.

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Robust long-term B and T cell tolerance has been demonstrated with this molecular chimerism approach in experimental models including allergy [1, 2]. Therefore, expression of functional disease-causing antigens is a critical prerequisite for the exploration of a chimerism-based tolerance strategy for any specific disorder.

Allergens are highly immunogenic, exposing a number of IgE-binding sites (B cell epitopes) on the surface [3]. T cell epitopes, in contrast, are small peptides resulting from proteolytic processing of allergens and their presentation via MHC molecules. Allergens are antigens derived from a wide range of sources such as plants, mammals, moulds and hymenoptera. The immunologic properties and allergenic activities of allergens may vary dramatically depending on their fold, posttranslational modification, state (i.e. monomeric vs. oligomeric), dose and site of entry into the body [4, 5]. Therefore, allergens expressed in cellular systems must be compared with a reference allergen equaling the natural allergen in order to understand the mechanisms underlying strategies for cell-based allergen-specific immunomodulation and to further develop cell-based therapies.

Phl p 5 is one of the most potent and frequent environmental allergens affecting 80% of grass pollen-allergic individuals. Recombinant (r) Phl p 5 equals the immunological properties and allergenic activity of the corresponding natural Phl p 5 allergen [6].

We constructed fusions of Phl p 5 with a leader peptide for secretion and an additional transmembrane domain for surface anchoring of the allergen for expression in human cells. We studied the allergenic activity of Phl p 5 expressed in human cells determining IgE-binding, mediator release and T cell reactivity.

Materials and Methods

Constructs

cDNA sequence (aa 25–313) of Phl p 5 (accession number X74735) was fused to a signal sequence and a transmembrane domain (TMD) (both pDisplay, Invitrogen, Carlsbad, Calif., USA) by overlapping PCR technique [7]. Primer sequences were used as follows: leader peptide: 5'-GGCGCCATGGAGACAGACACATCCTG-3', 5'-GTAACCGAGATCGGCGTCACCAAGTGA-3', Phl p 5: 5'-ACTGGTGACGCCGATCTCGGTTAC-3', 5'-GCCACAGCGACTTTGTAGCCACC-3', TMD: 5'-TACAAAGTCGCTGTGGGC-3', 5'-GGCGGATCCTAACGTGGCTTCTTC-3'. PCR products were cloned into the vector pcDNA3.1(–) (Invitrogen) under the control of a CMV promoter using EcoRI and BamHI sites resulting in p Phl p 5-TM or p Phl p 5. Positive clones were confirmed by DNA sequencing (VBC Biotech, Vienna).

Cell Culture and Transfection

HEK (human embryonic kidney) 293 T cells were grown in DMEM media including 10% FCS and antibiotics. 5×10^5 and 2×10^6 cells were grown in 6-well plates or 10-cm dishes (Falcon, Becton Dickinson, Franklin Lakes, N.J., USA) and transfected (1–10 µg) using the Ca_2PO_4 transfection method or transduced resulting in efficient surface expression of Phl p 5 as described previously [8, 9].

Recombinant Allergens, Antibodies

Purified recombinant timothy grass pollen allergen (rPhl p 5) was obtained from BIOMAY (Vienna, Austria). Phl p 5-specific polyclonal antibodies were used as described in [9].

Immunoblotting

Cell extracts, cell culture supernatants of transfected cells and rPhl p 5 were separated on a 16% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. Sera from timothy grass pollen-allergic individuals, non-Phl p 5-allergic donors and healthy donors were diluted 1:10 and used to probe nitrocellulose-blotted extracts and supernatants from Phl p 5-expressing 293 T cells. Extracts and supernatants of 293 T cells transfected with empty vector were used as a negative control (mock). Bound IgE was detected with ^{125}I -labelled anti-human IgE (Phadia, Uppsala, Sweden) [10].

ELISA

Amounts of secreted Phl p 5 in supernatants of p Phl p 5- and p Phl p 5-TM-transfected 293 T cells were quantified by Phl p 5-specific ELISA as described in Focke et al. [11].

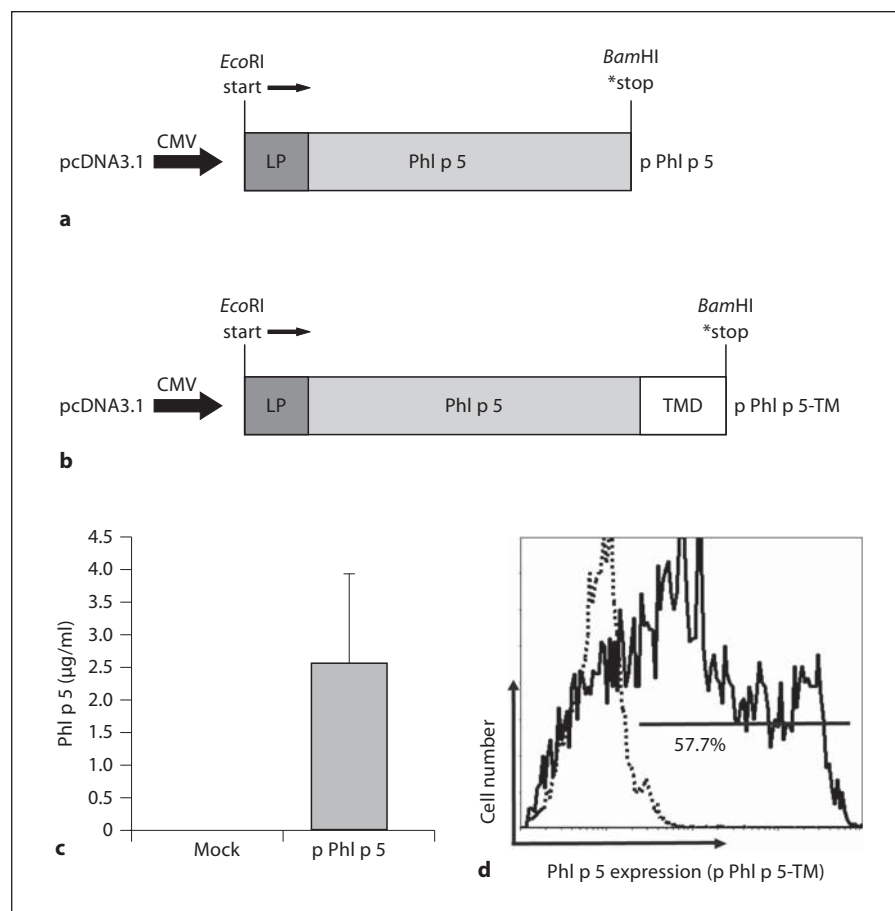
Flow Cytometric Analysis

Biotinylated polyclonal Phl p 5-IgG was used as described in Baranyi et al. [9] and developed with phycoerythrin streptavidin. A Coulter Cytomics FC500 flow cytometer (Coulter, Austria) was used for acquisition and CXP software (Coulter) for analysis of flow cytometric data.

Histamine Release and CD203c Upregulation Experiments

Heparinized peripheral blood samples were obtained from individuals with grass pollen allergy known to react against Phl p 5, from individuals with different allergies (with known non-reactivity to Phl p 5) and healthy individuals. Basophils were enriched from blood of 2 allergic patients by Dextran sedimentation, washed and resuspended in histamine release buffer (HRB; Immunotech, Marseille, France) as described in Valent et al. [12]. Cells ($3 \times 10^6/\text{ml}$) were incubated with various concentrations of rPhl p 5/transfected 293 T cells/supernatants of transfected cells in 96-well microtiter plates (TPP, Trasadingen, Switzerland) for 30 min (37°C). After incubation, cells were centrifuged at 4°C for 10 min. Cell-free supernatants were recovered, and histamine was measured by a commercial radioimmunoassay (RIA; Immunotech). Histamine release was calculated as percentage of total (extracellular and cellular) histamine measured in lysates of cells that were prepared by freeze-thawing. For investigation of CD203c upregulation, blood aliquots were incubated with rPhl p 5 (0.001 and 0.1 µg/ml), cells expressing membrane-anchored Phl p 5 and supernatants containing secreted Phl p 5 produced in 293 T cells. A monoclonal anti-IgE antibody E-124.2.8 (1 µg/ml; Immunotech) and PBS were used as control. Thereafter, cells

Fig. 1. High-level expression of secreted and membrane-anchored Phl p 5 in transfected human cells. **a** Construct for secretion of Phl p 5 (p Phl p 5). **b** Construct for membrane-anchored expression of Phl p 5 (p Phl p 5-TM). Both constructs were cloned into pcDNA3.1 vector under the control of a CMV promoter. LP denotes leader peptide and TM transmembrane domain. The drawing is not to scale. **c** Secretion of Phl p 5 was quantified by Phl p 5-specific ELISA in supernatants of 293T cells transfected with the construct shown in **a**. The mean ($\pm$ SD) of 3 independent transient transfections is shown. As control, supernatant of 293T cells transfected with empty vector (mock) was quantified for soluble Phl p 5. **d** Efficient expression of Phl p 5 on the surface of 293T cells transfected with construct p Phl p 5-TM is shown as determined by flow cytometry. Dotted line indicates mock-transfected 293 T cells (empty vector).



were stained with phycoerythrin-conjugated CD203c mAb 97A6 (Immunotech). After erythrolysis cells were washed and resuspended in PBS and analyzed by 2-color flow cytometry. Upregulation of CD203c was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells and is expressed as stimulation index (SI) ($MFI_{stim}:MFI_{control}$).

T Cell Proliferation Assay

Peripheral blood mononuclear cells (PBMC) were obtained from allergic and nonallergic donors by Ficoll-Hypaque density-gradient centrifugation (Pharmacia Diagnostics, Uppsala, Sweden). PBMC (2×10^5) were cultured for 6 days in triplicates in 96-well plates (Nunc, Nunc, Denmark) in 200 μ l of serum-free Ultra Culture Medium (Bio Whittaker, Walkersville, Md., USA) supplemented with 2 mM/l glutamine and 2×10^{-5} M 2-ME in the presence of rPhl p 5- (1–30 μ g) and p Phl p 5-TM-transfected or transduced cells or supernatants of p Phl p 5-transfected cells like described above. During the last 16 h of culture [^{3} H]-thymidine (0.5 μ Ci per well) was added and the incorporated radioactivity was measured by scintillation counting in counts per minute (cpm). The stimulation index (SI) was calculated as ratio between cpm obtained in T cell cultures plus PBMC plus allergen and cpm obtained in cultures plus PBMC without allergen or as

ratio between cpm obtained in T cell cultures plus PBMC and membrane-anchored or secreted Phl p 5 and cpm obtained in cultures plus PBMC and control cells or supernatants from control cells.

Results

Construction of Plasmids Coding for Secreted and Membrane-Anchored Phl p 5

For construction of secreted and membrane-anchored Phl p 5, we fused a murine immunoglobulin leader peptide to Phl p 5 [6] replacing the original plant signal peptide for efficient secretion. Additionally, to express Phl p 5 in a membrane-anchored fashion, the transmembrane domain of platelet-derived growth factor receptor, which has demonstrated efficacy in anchoring fusion proteins in human and murine cell lines, was added [9, 13]. The fusion genes were cloned under the control of a CMV promoter into vector pcDNA3.1 known to be a high level expression vector in mammalian cells (fig. 1a, b).

Membrane-Anchored Expression and Secretion of Phl p 5 in Human Cells

293T cells were transiently transfected with vector p Phl p 5 (fig. 1a) and Phl p 5 was quantified in the supernatant of transfected cells by Phl p 5-specific ELISA [11]. In all experiments, we used rPhl p 5, produced and purified from *Escherichia coli*, as a standard reference. Amounts of 1–3 µg/ml allergen secreted by p Phl p 5-transfected human cells were detected in the supernatant (fig. 1c). As a negative control, empty vector pcDNA3.1 was transfected (mock) in 293T cells and supernatants were analyzed by ELISA (fig. 1c). Transient transfection of 293T cells with p Phl p 5-TM (fig. 1b) led to cell surface expression of Phl p 5 (about 60% of cells were Phl p 5+) as quantified by flow cytometry with Phl p 5-specific antibodies [9] (fig. 1d). These cells also liberated Phl p 5 fused to the transmembrane domain (as evident from the supernatant; fig. 2) but at lower quantities than p Phl p 5-transfected cells (about 0.7 µg/ml Phl p 5, as determined in Phl p 5-specific ELISA, data not shown).

Phl p 5-Specific IgE Binding of Phl p 5 Expressed in Human Cells

The IgE-binding capability of Phl p 5 expressed in human cells was assessed with sera of Phl p 5-allergic patients. Secreted and membrane-anchored Phl p 5 of supernatants and extracts of transfected human 293T cells were loaded onto a denatured protein gel and blotted to a nitrocellulose membrane. Membranes were incubated with sera from allergic patients reacting to Phl p 5 (fig. 2a, b), sera from healthy donors as negative control and sera from donors with different allergic history (non-Phl p 5-allergic donors) and bound IgE was detected with labeled anti-IgE. rPhl p 5 was loaded as the positive control. Phl p 5-binding IgE was detected in supernatants and cell extracts of Phl p 5-expressing human 293T cells (fig. 2a, b). Extracts from cells transfected with p Phl p 5-TM showed a higher mobility shift due to the transmembrane domain fusion (additional 50 aa). The IgE reactivity of mammalian cell-expressed Phl p 5 in extracts and supernatants of secreted or membrane-anchored Phl p 5 was comparable to the IgE reactivity of rPhl p 5.

Both Surface-Expressed Phl p 5 and Phl p 5 Secreted by Human Cells Induce Mediator Release

Basophils store a number of vasoactive mediators like histamine in their granules. In allergic individuals, basophils bind IgE via high-affinity receptors (i.e. FcεRI) and release their mediators into the extracellular space upon allergen exposure [14]. The in vitro basophil degranula-

tion assay has been shown to closely reflect the cascade of the allergic effector reaction as a model for the activity of natural and recombinant allergens similar to the in vivo situation [15]. Further, the ectoenzyme CD203c is expressed in basophils (not in other peripheral blood cells) and upregulated on IgE receptor cross-linkage with r allergens in basophils of sensitized individuals [12]. We examined the capacity of secreted and membrane-anchored Phl p 5 to induce degranulation and CD203c upregulation of basophils of Phl p 5-allergic individuals (fig. 3a, b). Basophils of two allergic individuals were incubated with serial dilutions of rPhl p 5 resulting in a typical Phl p 5-dependent bell-shaped peak in dose-response curves described to differ among allergens and allergic individuals [12]. The Phl p 5-specific release varied from about 60% Phl p 5-specific histamine release (fig. 3a1, patient 1) to almost 100% Phl p 5-specific histamine release in basophils of patient 2 (fig. 3a1). Escalating numbers of human cells expressing membrane-anchored Phl p 5 (or mock-transfected cells as control) were co-incubated with basophils of the same patients (fig. 3a2). Membrane-anchored Phl p 5 expressed on human cells efficiently triggered histamine release in a dose-dependent manner in basophils of patient 1 (50–60%). Patient 2-derived high responding basophils yielded a maximum histamine release of about 100% even with the lowest number of transfected cells. Secreted Phl p 5 in supernatants of transfected human cells led to a histamine release comparable to rPhl p 5 (fig. 3a3). Likewise, basophils of a Phl p 5-allergic patient showed upregulation of CD203c with cells expressing membrane-anchored Phl p 5 or secreted Phl p 5 comparable to upregulation with rPhl p 5 (fig. 3b1). On the contrary, basophils of non-Phl p 5-allergic patient and healthy donor showed no CD203c upregulation with rPhl p 5 and membrane-anchored Phl p 5 as well as soluble Phl p 5 incubation (fig. 3b2, b3). These results demonstrate Phl p 5-specific activation of basophils by cross-linking of membrane-anchored and secreted Phl p 5 expressed in human cells by two independent techniques.

T Cell Proliferation of PBMC of Allergic Donors Stimulated with Phl p 5-Expressing Human Cells

In atopic individuals allergen-specific memory T cells respond to repeated allergen contact leading, for example, to chronic asthma [16]. Stimulation of PBMC with either supernatant containing secreted Phl p 5 or with membrane-anchored Phl p 5 both led to responses similar to rPhl p 5, while nonallergic donors did not respond (table 1).

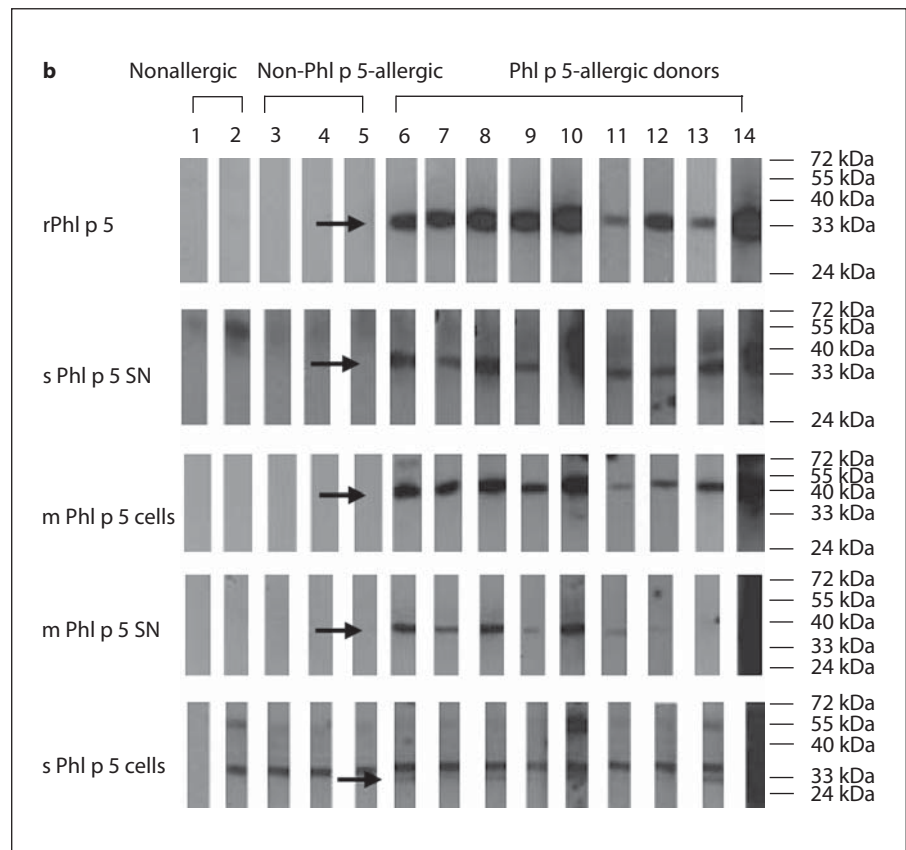
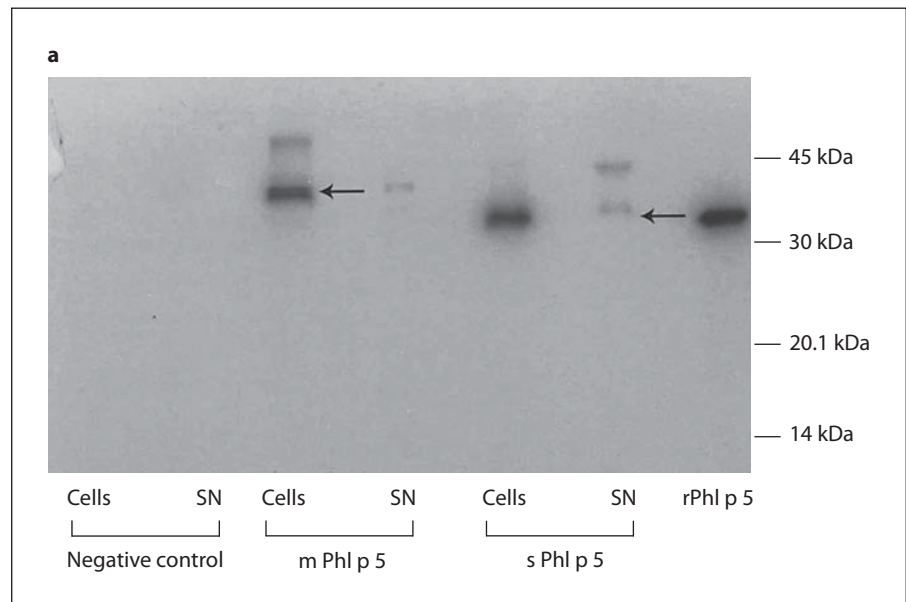


Fig. 2. Serum-IgE of allergic patients recognizes Phl p 5 expressed in human cells. **a, b** Nitrocellulose-blotted lysates (cells) and supernatants (SN) of 293T cells transfected with constructs for secreted Phl p 5 (s Phl p 5) or membrane-anchored Phl p 5 (m Phl p 5) were incubated with sera of Phl p 5-allergic donors, donors with a history of different allergic disease and healthy donors. Bound IgE was detected with ^{125} I-labeled anti-human IgE and visualized by autoradiography. rPhl p 5 served as positive control. Negative control denotes equal amounts of supernatants and cells from mock-transfected cells. Predominant proteins are indicated by arrows.

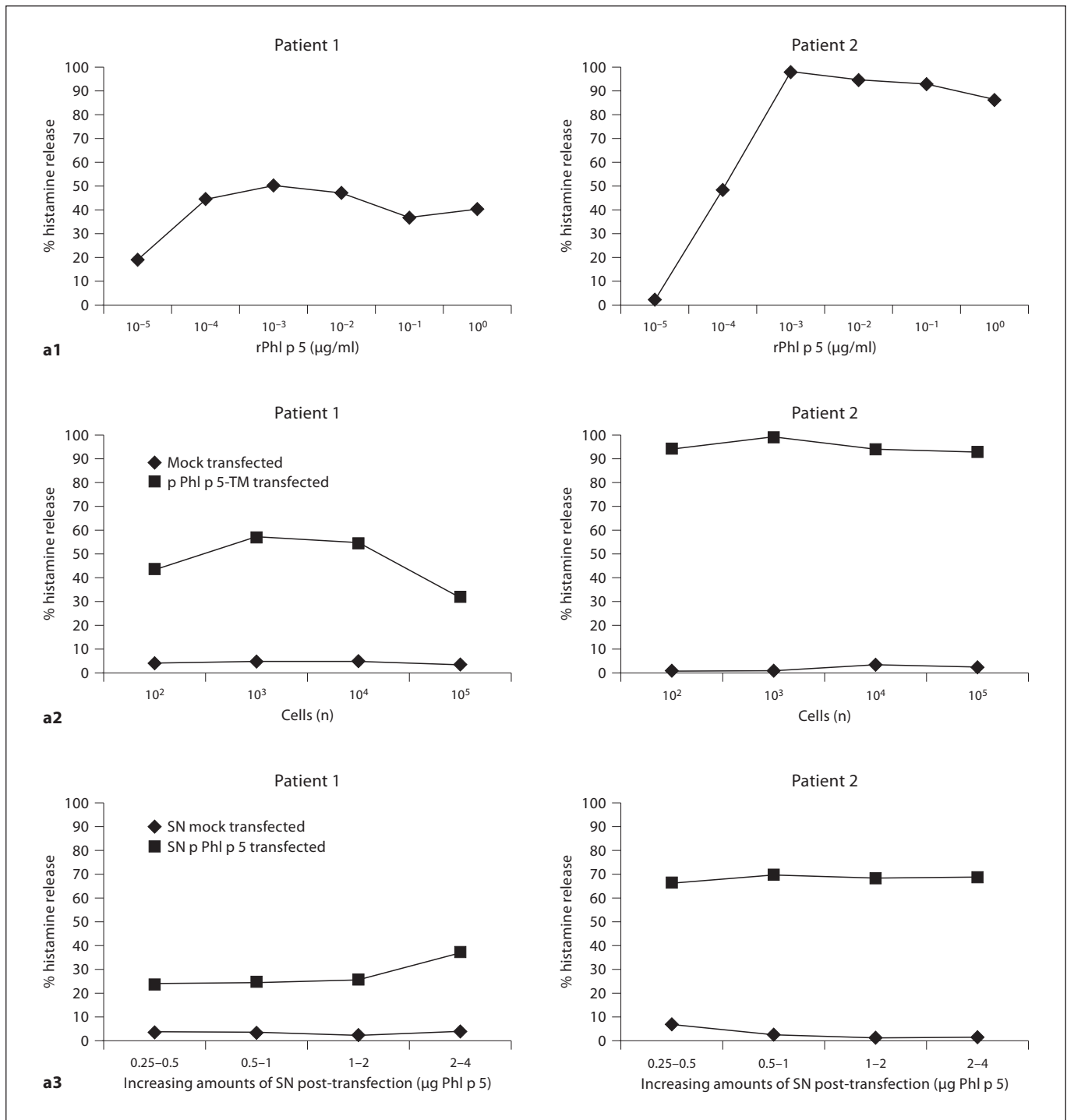


Fig. 3. Membrane-anchored and secreted Phl p 5 lead to histamine release and CD203c upregulation in basophils of allergic patients. Basophils of two allergic patients (patients 1 and 2) were incubated with different concentrations of rPhl p 5 (**a1**), increasing amounts of cells expressing membrane-anchored Phl p 5 or cells transfected with empty vector as control (**a2**), or different amounts of supernatants from cells secreting Phl p 5 (**a3**). Supernatants of mock-transfected cells were used as a negative control. CD203c

upregulation was determined in blood of an allergic donor (**b1**) and a non-Phl p 5 allergic donor (**b2**) as well as in blood of a healthy donor (**b3**). Cells were incubated with rPhl p 5, membrane-anchored Phl p 5-expressing cells (m Phl p 5 cells) and supernatants of p Phl p 5-transfected cells (s Phl p 5). Anti-IgE was used as the positive control. Unstimulated cells (US) were used as the baseline. Mock-transfected cells and supernatants of mock-transfected cells were used as negative controls.

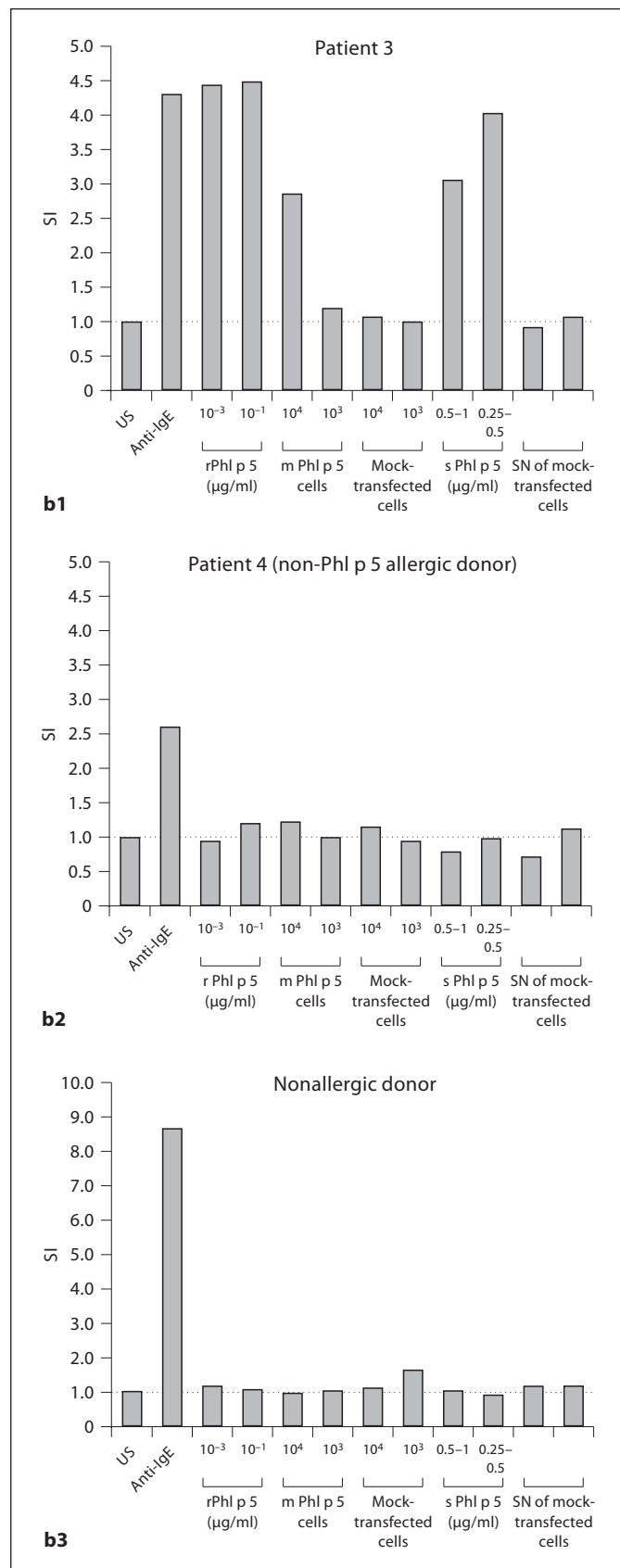


Table 1. Lymphoproliferation (SI) of PBMC derived from three grass pollen-allergic patients (AD) and nonallergic donors (NAD) in response to stimulation with secreted (s) and membrane-bound (m) Phl p 5 derived from transfected human 293T cells

	IL-2	rPhl p 5	s Phl p 5	m Phl p 5
AD 1	22.3	3.3	9.6	4.8
AD 2	59.4	4.3	3.4	1.1
AD 3	29.6	3.5	2.8	6.1
NAD 1	8.1	1.0	0.8	1.1
NAD 2	4.8	1.6	1.6	1.6

As negative controls, nontransfected 293T cells and their supernatants (control cells) were used. For positive control, PBMC were stimulated with rPhl p 5 and r IL-2. Two patients showed no proliferation to the positive control rPhl p 5 in this assay (not shown).

Discussion

Here we demonstrate efficient expression of secreted and membrane-anchored grass pollen allergen Phl p 5 in human cells. The fusion of a leader peptide and/or an additional transmembrane domain leads to the secretion or to the anchoring of Phl p 5 to the surface of human cells.

Saloga and colleagues [17] have previously reported the expression of the full-length allergen Phl p 1 in human dendritic cells by adenoviral transfer. Cytoplasmic expression of this allergen led to stimulation of T cell proliferation in vitro. This approach has been directed to induce T_H1 responses for potential immunotherapy by presentation of T cell peptides of the allergen Phl p 1 for CD8+ T cell proliferation. In contrast, our approach focuses on prophylactic tolerance induction at the T and B cell level and therefore displays allergens containing B and T cell epitopes on the cell surface or as secreted product. Both forms, membrane-anchored and secreted Phl p 5, recognize IgE obtained from a Phl p 5-allergic individual. Furthermore, basophil mediator release and T cell proliferation suggest correct expression of B cell epitopes and presentation of T cell epitopes of the released and membrane-anchored allergen Phl p 5 expressed by human cells.

Allergenic activity similar to the wild-type allergen is successfully retained in both instances.

In a murine model, we successfully induced long-term tolerance towards Phl p 5 by transplantation of hematopoietic stem cells expressing membrane-anchored Phl p

5 into syngeneic recipients. No Phl p 5-specific IgE, IgA and IgG subtypes were detectable in these mice after numerous immunizations with rPhl p 5 [9]. Similar results were observed after tolerization with the membrane-anchored major birch pollen allergen Bet v 1 expressed in hematopoietic stem cells [M.G. and T.W., unpubl. data]. Therefore, it appears likely that this approach can be extended to a variety of allergens. Although several impediments have to be overcome, molecular chimerism is a promising strategy for tolerization type I allergic immune responses [2].

Here we show the successful expression of two forms of Phl p 5 in human cells offering establishment of cell-based therapy approaches for prophylaxis and treatment of allergy.

Acknowledgments

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