The IgE-Reactive Autoantigen Hom s 2 Induces Damage of Respiratory Epithelial Cells and Keratinocytes via Induction of IFN-γ

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Hom s 2, the α-chain of the nascent polypeptide-associated complex, is an intracellular autoantigen that has been identified with IgE autoantibodies from atopic dermatitis patients. We investigated the humoral and cellular immune response to purified recombinant Hom s 2 (rHom s 2). rHom s 2 exhibited IgE reactivity comparable to exogenous allergens, but did not induce relevant basophil cell degranulation. The latter may be attributed to the fact that patients recognized single epitopes on Hom s 2 as revealed by IgE epitope mapping with rHom s 2 fragments. In contrast to exogenous allergens, rHom s 2 had the intrinsic ability to induce the release of IFN-γ in cultured peripheral blood mononuclear cells from atopic as well as non-atopic individuals. IFN-γ-containing culture supernatants from Hom s 2-stimulated peripheral blood mononuclear cells caused disintegration of respiratory epithelial cell layers and apoptosis of skin keratinocytes, which could be inhibited with a neutralizing anti-IFN-γ antibody. Our data demonstrate that the Hom s 2 autoantigen can cause IFN-γ-mediated cell damage.

INTRODUCTION

It has been demonstrated that patients suffering from severe manifestations of atopy (for example, atopic dermatitis (AD), chronic asthma) show IgE reactivity to a variety of human antigens (Valenta *et al.*, 1996; Natter *et al.*, 1998; Seiberler *et al.*, 1999a, b; Kortekangas-Savolainen *et al.*, 2004; Schmid-Grendelmeier *et al.*, 2005). Sensitization to autoantigens seems to occur early in life, and several studies have shown that the intensity of IgE autoreactivity is associated with the severity of atopy (Natter *et al.*, 1998; Seiberler *et al.*,

1999a, b; Kinaciyan *et al.*, 2002; Mothes *et al.*, 2005). For example, it has been demonstrated that patients suffering from severe manifestations of AD exhibit stronger IgE auto-reactivity than patients suffering from mild forms of atopy (Natter *et al.*, 1998). Furthermore, it has been demonstrated that patients suffering from exacerbations of atopy increase their IgE autoreactivity (Natter *et al.*, 1998; Seiberler *et al.*, 1999a, b; Kinaciyan *et al.*, 2002).

Human antigens that cross-react with environmental allergens have been identified, and it has been shown that these human antigens can induce basophil degranulation, T-cell proliferation, and immediate- as well as late-phase skin reactions (Valenta *et al.*, 1991; Crameri *et al.*, 1996; Appenzeller *et al.*, 1999; Mayer *et al.*, 1999; Schmid-Grendelmeier *et al.*, 2005). Complementary DNAs (cDNAs) coding for IgE-reactive autoantigens have been isolated from human expression cDNA libraries using serum IgE auto-antibodies from patients with AD and chronic asthma (Natter *et al.*, 1998; Valenta *et al.*, 1998; Mossabeb *et al.*, 2002; Aichberger *et al.*, 2005). Most of the IgE-reactive autoantigens defined so far are intracellular proteins with conserved biological functions. For example, Hom s 2 represents the α-chain of the nascent polypeptide-associated complex, an evolutionarily conserved protein, which is assumed to guide the transport of polypeptides from the ribosome to appropriate cellular locations (Mossabeb *et al.*, 2002). Hom s 3 is identical to an oncogene (Natter *et al.*, 1998) and Hom s 4 is a calcium-binding protein, which despite its reactivity with

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IgE antibodies has been found to induce the secretion of IFN-γ in human peripheral blood mononuclear cell (PBMC) cultures (Aichberger *et al.*, 2005).

Intracellular autoantigens may be released after cell damage, for example, in the course of a preceding allergic tissue inflammation induced by exogenous allergens. In the Th2 environment of atopic patients, IgE antibodies may be formed against autoantigens, which due to their intracellular nature may have escaped tolerance induction.

Interestingly, sensitization of mice with Hom s 2 and the mouse-derived protein not only induced IgE autoreactivity and immediate-type skin reactions but also a mixed pattern of Th2 and Th1 autoreactivity in various tissues (Buender *et al.*, 2004).

In this study, we have analyzed the human immune responses to purified recombinant Hom s 2 (rHom s 2). We mapped the epitope specificity of IgE autoantibodies directed against Hom s 2, exposed basophils to Hom s 2 to study inflammatory mediator release, and analyzed IgG subclass and cytokine responses as well as T-cell proliferations specific for Hom s 2. Surprisingly, we found that Hom s 2 induced strong IFN-γ responses in atopic as well as non-atopic persons and that this cytokine causes damage in human respiratory epithelial cells and skin keratinocytes.

RESULTS

Specific IgE recognition of rHom s 2

rHom s 2 was expressed in *E. coli* cells as His-tagged protein and purified by Ni^{2+}-chelate affinity chromatography. The Coomassie-stained SDS-PAGE shows that the rHom s 2 preparation contains a monomeric protein with a molecular weight of approximately 33 kDa and a polymer of more than 220 kDa (Figure 1). Out of 11 analyzed AD patients, 8 displayed IgE autoreactivity to blotted human epithelial cell extracts, whereas sera from patients with rhinoconjunctivitis (RC) and contact allergy (CA), and non-atopic individuals did not show any IgE autoreactivity (Table 1). Three of the AD patients, but none of the other persons, had Hom s 2-specific IgE antibodies (Table 1). AD and RC patients exhibited IgE reactivity to a variety of exogenous allergen sources (Table 1). Ten of the AD, four of the RC, and none of the other persons had IgE antibodies specific for the recombinant grass pollen allergen, rPhl p 1 (Table 1).

Evidence that Hom s 2-sensitized patients show IgE reactivity to single epitopes on Hom s 2

We expressed an N-terminal (aa 1–79; 8.6 kDa) and a C-terminal fragment (aa 81–167; 10.3 kDa) of Hom s 2 as β-galactosidase fusion protein (Figure 2a). IgE antibodies from atopic patients exhibited reactivity either with the recombinant C-terminal (patient AD1) or the N-terminal Hom s 2 fragment (patient AD2) (Figure 2b, asterisks). Patient AD1 mounted weak and patient AD2 strong IgE reactivity to *E. coli* components of less than 97 kDa (Figure 2b). Neither patient AD1 nor patient AD2 showed IgE reactivity to β-galactosidase (Figure 2b, lane 1).

A rabbit antiserum raised against the rHom s 2 protein also showed exclusive IgG reactivity with one of the two (that is,
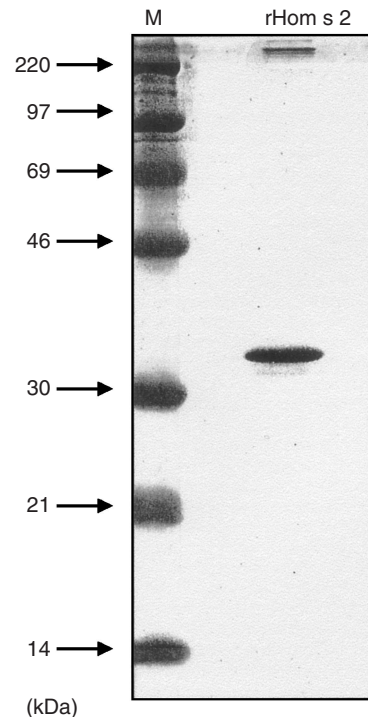
C-terminal) Hom s 2 fragments. No IgG reactivity to any of the blotted components was found for the rabbit preimmune serum (Figure 2b).

In accordance with the IgE epitope mapping results, we found that purified rHom s 2 did not induce relevant release of histamine in basophils of the two atopic patients (Figure 3a and b). Anti-IgE and the major timothy grass pollen allergen, Phl p 1, induced dose-dependent histamine release from the basophils of the two atopic patients, confirming that the cells can respond to IgE-dependent receptor cross-linking (Figure 3a and b).

rHom s 2 induces the secretion of IFN-γ in PBMCs

Next, we measured lymphoproliferative and cytokine responses of human PBMCs to Hom s 2 for all patients characterized in Table 1 and nine non-atopic persons (Figure 4). There was no significant difference in the intensities of lymphoproliferative responses induced by rHom s 2 in PBMCs from atopic and from non-allergic persons when compared to those induced by the major timothy grass pollen allergen, rPhl p 1 (Figure 4a). However, the analyses of Hom s 2- and Phl p 1-specific cytokine levels showed that rHom s 2 induced significantly higher levels of IFN-γ and IL-10 in PBMCs of atopic and non-atopic persons than rPhl p 1 (Figure 4b and c). IL-4 levels were below the detection limit of 3.3 pg ml^{-1} in each of the supernatants. IL-5 levels were generally but not statistically significantly lower in the Hom s 2-stimulated cultures compared to the Phl p 1-stimulated cultures (Figure 4d).

Table 1. Demographic, clinical, and serological characterization of AD, RC, and CA patients

Patient	Sex	Age (years)	Symptoms	EASI	IGA	Total IgE (kU l^{-1})	Allergen sources	IgE autoreactivity	sIgE Hom s 2	sIgE Phl p 1
AD1	M	53	AD, RC, AS	ND	ND	>2,000	Grass, birch, cat, fruits, vegetables, mite	+	+	+
AD2	F	28	AD, RC, AS	15.2	ND	253	Grass, birch, cat, fruits, vegetables, mite	+	+	+
AD3	M	13	AD, RC, AS	17.5	3	545	Grass, birch, rye pollen, mugwort	−	−	+
AD4	M	59	AD, RC, AS	39.6	4	>2,000	Grass, birch, cat, dog, fruits, vegetables, mite	+	−	+
AD5	F	23	AD, RC, AS	39.4	4	>2,000	Grass, birch, mugwort, fruits, vegetables	−	−	+
AD6	M	47	AD, RC, AS	59.8	5	>2,000	Grass, birch, weed, fruits, vegetables, mite	+	−	+
AD7	F	24	AD, RC	4.4	2	67.7	Grass, birch, rye pollen, mite	−	−	+
AD8	M	36	AD, RC, AS	7	2	>2,000	Grass, birch, fruits, vegetables	+	+	+
AD9	F	21	AD, RC, AS	15.2	3	>2,000	Grass, birch, rye pollen, mugwort, mite, cat, dog, cow milk	+	−	+
AD10	M	20	AD, RC, AS	10	3	1,732	Wheat, nuts, fish, crustaceae	+	−	−
AD11	M	27	AD, RC, AS	49.2	5	>2,000	Grass, birch, rye pollen, mugwort, wheat, mite, cat, dog, cow milk	+	−	+
RC1	M	27	RC, AS	—	—	755	Grass, birch, mugwort, cat, fruits, vegetables, nuts, mite	−	−	+
RC2	M	29	RC	—	—	61.9	Grass, weed, wheat flour	−	−	+
RC3	F	32	RC	—	—	187	Birch	−	−	−
RC4	M	26	RC, AS	—	—	101	Mite	−	−	−
RC5	M	31	RC	—	—	>2,000	Grass, birch, rye pollen, fruits	−	−	+
RC6	M	45	RC	—	—	134	Grass, birch, mugwort, fruits, vegetables	−	−	+
RC7	M	44	RC	—	—	153	Grass, birch	−	−	−
CA1	M	63	CA	—	—	<2	Cobalt, formaldehyde	−	−	−
CA2	F	37	CA	—	—	87.7	Nickel	−	−	−
CA3	F	34	CA	—	—	5.49	Nickel	−	−	−
CA4	F	28	CA	—	—	268	Nickel	−	−	−
CA5	F	26	CA	—	—	6.77	Nickel	−	−	−

AD, atopic dermatitis; AS, asthma; CA, contact allergy; EASI, eczema area and severity index; F, female; IGA, Investigators Global Assessment Score; kU l^{-1}, kilo units per liter; M, male; ND, not done; RC, rhinoconjunctivitis; +, positive reaction; −, negative reaction.

IgG reactivity of Hom s 2

We detected mainly Hom s 2-specific IgG$_1$ and IgG$_2$ but no IgG$_3$ (data not shown) or IgG$_4$ antibodies in the sera from atopic as well as non-atopic persons (Figure 4e–g). The major timothy grass pollen allergen, Phl p 1, exhibited mainly IgG$_1$ and IgG$_4$, low IgG$_2$, but no relevant IgG$_3$ reactivity (Figure 4).

Culture supernatants from Hom s 2-stimulated PBMCs containing high levels of IFN-γ induce disintegration of respiratory epithelial cell layers

We have recently shown that IFN-γ *per se* induces disintegration of respiratory epithelial cell layers (Reisinger *et al.*, 2005). We therefore asked whether supernatants from Hom s 2-stimulated PBMCs containing high levels of IFN-γ would have similar effects. Figure 5a shows the levels of IFN-γ
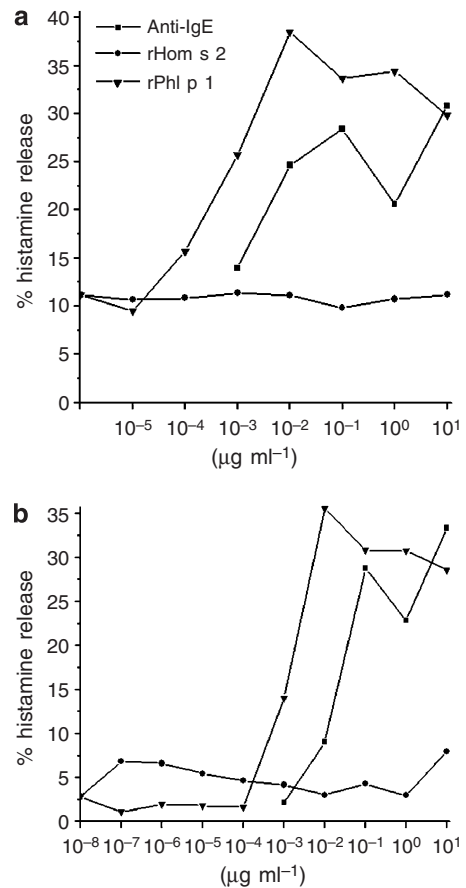
in supernatants from Hom s 2- or Phl p 1-stimulated PBMCs from two atopic patients (AD1, AD2) and four non-atopic individuals (NA1–NA4). These supernatants were added to cultured respiratory epithelial cells, and transepithelial resistance was measured over a period of 120 hours as an indicator for the integrity of the epithelial cell layer. We found that supernatants from Hom s 2-stimulated PBMCs containing high levels of IFN-γ (Figure 5a; AD2: 2,095.65 pg per ml IFN-γ; NA1: 1,280.54 pg per ml IFN-γ, NA2: 2,055.32 pg per ml IFN-γ; NA3: 2,025.965 pg per ml IFN-γ; NA4: 3,221.92 pg per ml IFN-γ) induced the greatest decrease in transepithelial resistance. After 120 hours, the transepithelial resistance decreased to 15.87% of the baseline value for patient AD2, to 19.39% for NA1, to 5.57% for NA2, to 9.28% for NA3, and to 9.60% for NA4 (Figure 5b). A considerably lower decrease in the transepithelial resistance
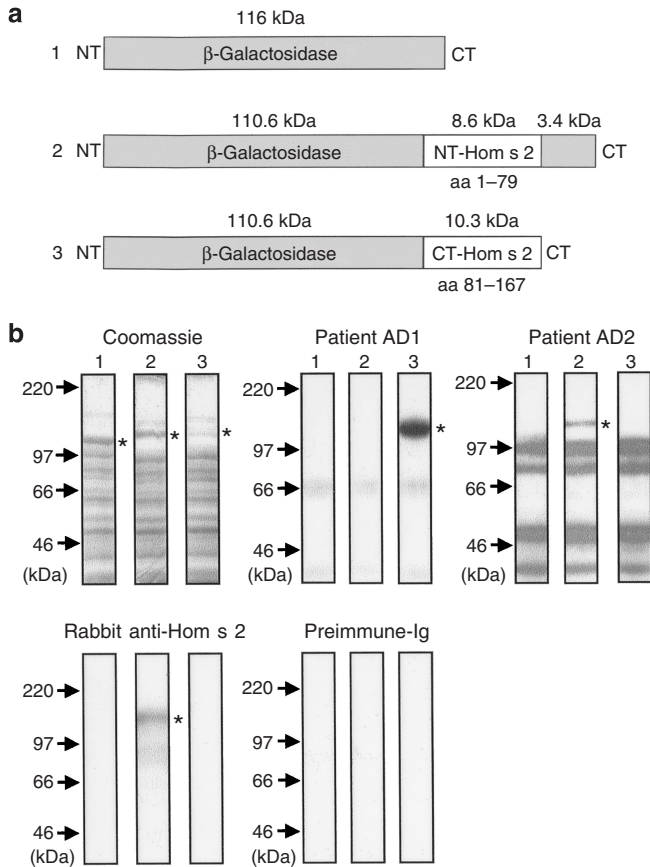
Figure 2. Mapping of IgE and IgG epitopes using rHom s 2 fragments.
(**a**) Schematic diagram of β-galactosidase (1) and Hom s 2-β–galactosidase fusion proteins, containing the N-terminal (2) or C-terminal (3) part of Hom s 2. (**b**) Coomassie-stained SDS-PAGE of *E. coli* extracts containing β-galactosidase (lane 1), the N-terminal (lane 2), or the C-terminal (lane 3) Hom s 2–β-galactosidase fusion proteins. Nitrocellulose membranes containing the blotted extracts were reacted with serum IgE from two Hom s 2-sensitized patients (AD1, AD2), with a rabbit antiserum raised against purified rHom s 2 (rabbit α-Hom s 2) or with the corresponding rabbit preimmune serum (preimmune-Ig). Molecular weights (kDa) and the position of the β-galactosidase fusion proteins (asterisks) are indicated.

Figure 3. Basophil histamine release experiments. Basophils from patients (**a**) AD1 and (**b**) AD2 containing rHom s 2-specific IgE antibodies were exposed to increasing concentrations of rHom s 2, anti-IgE, or the grass pollen allergen, rPhl p 1 (*x* axis). The percentages of total histamine release are displayed on the *y* axis.

was observed when the supernatants from Phl p 1-stimulated PBMCs were added to the epithelial cell layers. After 120 hours, a decrease in the transepithelial resistance to 74.24% of the baseline value for patient AD2, to 71.36% for NA1, to 46.91% for NA2, to 25.47% for NA3, and to 29.25% for NA4 was noted (Figure 5c). The magnitude of transepithelial resistance decrease induced with the supernatants from Phl p 1-stimulated PBMC cultures was also associated with the levels of IFN-γ in the supernatants.

Further support for the assumption that IFN-γ plays an important role in this process came from experiments performed with a neutralizing anti-IFN-γ antibody. Addition of supernatants from Hom s 2-stimulated cultures containing an isotype-matched control antibody decreased the resistance to 70% (that is, $706 \, \Omega \, cm^{-2}$) of the value obtained with medium alone (that is, $999 \, \Omega \, cm^{-2}$), and the addition of a neutralizing anti-IFN-γ antibody almost completely reverted the effect (that is, $933 \, \Omega \, cm^{-2}$) (Figure 6a).

Culture supernatants from Hom s 2-stimulated PBMCs induce keratinocyte apoptosis

HaCat keratinocytes were exposed to supernatants from Hom s 2-stimulated PBMCs from three individuals (Figure 6b). The addition of a medium (complete RPMI) left 75% of the keratinocytes alive, whereas the addition of Hom s 2-stimulated supernatants containing an isotype-matched control antibody reduced keratinocyte survival to 49–52%. The addition of a neutralizing anti-IFN-γ antibody could restore the keratinocyte survival up to 60–68%, demonstrating a key role of IFN-γ in keratinocyte apoptosis (Figure 6b).

DISCUSSION

Hom s 2 is one of the few IgE-reactive autoantigens that have been directly isolated with serum IgE from a patient suffering from severe AD and chronic asthma by screening of an expression cDNA library made from human cells (Natter *et al.*, 1998; Mossabeb *et al.*, 2002). Here, we attempted to analyze if and how Hom s 2 may be involved in the induction and maintenance of inflammatory responses. Although Hom s 2 showed highly specific IgE reactivity with comparable intensity to regular environmental allergens, it did not induce
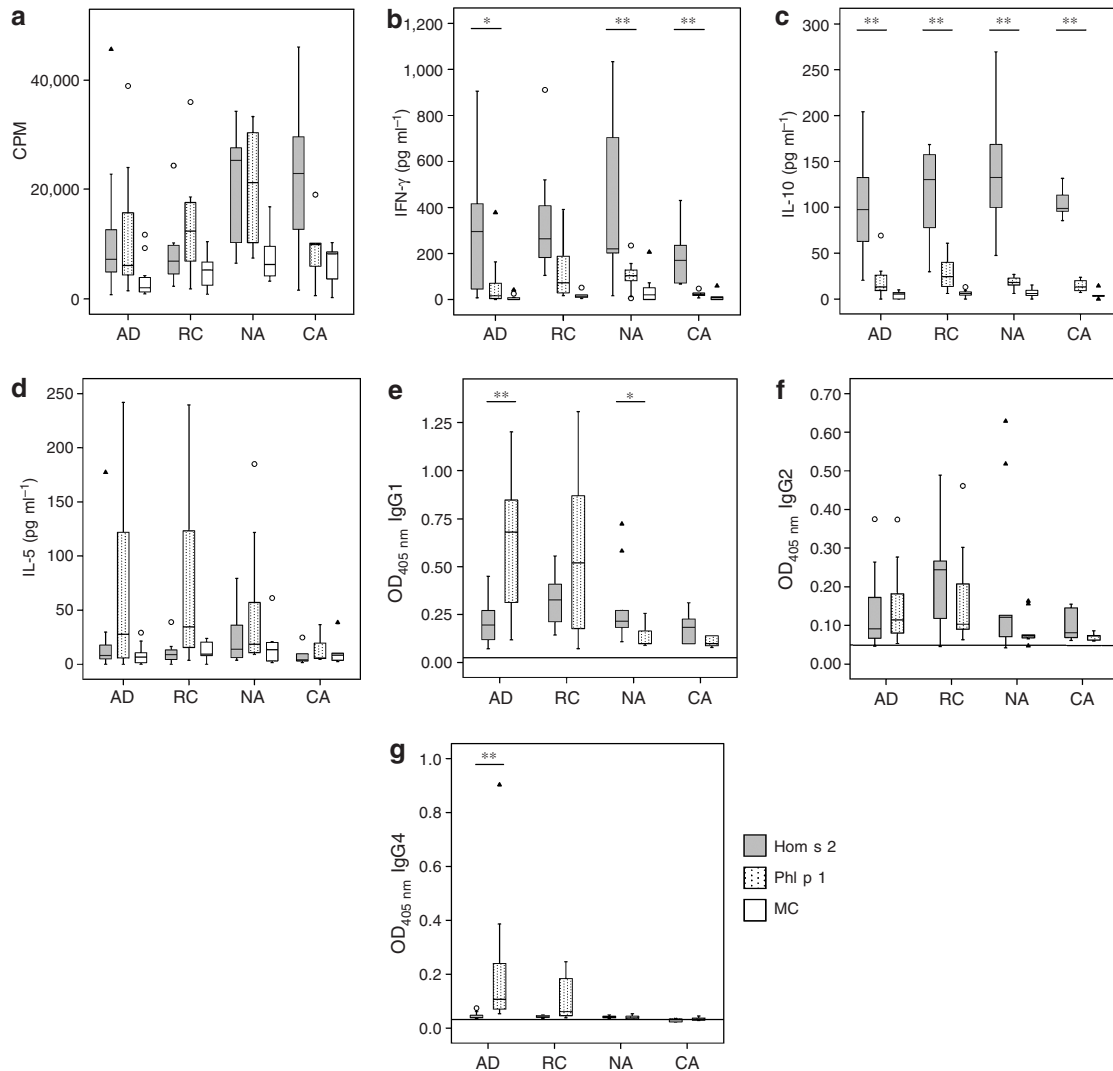
Figure 4. Cellular, cytokine, and humoral immune responses to rHom s 2 and rPhl p 1. Box plots showing (**a**) lymphoproliferative responses (*y* axis: mean counts per minute (c.p.m.)) of AD patients, RC patients, non-atopic individuals (NA), or CA patients to rHom s 2 (gray boxes), rPhl p 1 (dotted boxes), or medium control (MC) (white boxes). (**b**) INF-γ, (**c**) IL-10, and (**d**) IL-5 levels (*y* axis: pg ml^{-1}) as determined in the cultures. Statistically significant differences are indicated (*$P < 0.05$ or **$P < 0.01$). (**e**) IgG$_1$, (**f**), IgG$_2$, and (**g**) IgG$_4$ subclass reactivity to Hom s 2 and to the grass pollen allergen Phl p 1. Optical density (OD) values corresponding to the amount of bound antibodies are displayed. The cutoff levels (median ODs for HSA) of the ELISAs are displayed as horizontal lines. Open dots represent outliers and triangles represent extreme values. HSA, human serum albumin.

immediate inflammatory responses as shown in basophil histamine release experiments. Similar results have been reported for IgE-reactive carbohydrate moieties on environmental allergens (Van Ree, 2002) and recombinant allergen fragments containing only one binding site for IgE (Ball *et al.*, 1994).

rHom s 2 used in our study has been obtained by expression in *E. coli*, and it is not glycosylated. The weak induction of basophil degranulation can therefore not be attributed to the presence of IgE-reactive carbohydrates on the protein. We therefore analyzed the IgE recognition of Hom s 2 with rHom s 2 fragments. Similar to earlier studies, we used β-galactosidase-fused fragments for the IgE epitope mapping, because we observed in these studies that IgE epitopes get better displayed for IgE recognition when expres-

sed as fusion proteins than as isolated peptides (Ball *et al.*, 1994, 1999). The epitope mapping studies performed with rHom s 2 fragments showed that the patients who had failed to mount a basophil histamine release in response to rHom s 2 exhibited IgE reactivity only with single IgE epitopes on Hom s 2. It is therefore likely that the poor induction of histamine release by Hom s 2 is due to monovalent IgE recognition, which causes insufficient or no cross-linking of basophil-bound IgE.

Further analysis of immunoreactivity showed that Hom s 2 induced significantly stronger secretion of IFN-γ than a major exogenous allergen (that is, Phl p 1). In addition, we found that Hom s 2 induced significantly higher levels of IL-10 and lesser of IL-5 than Phl p 1. The preferential induction of an IFN-γ response was observed in PBMC cultures of atopic
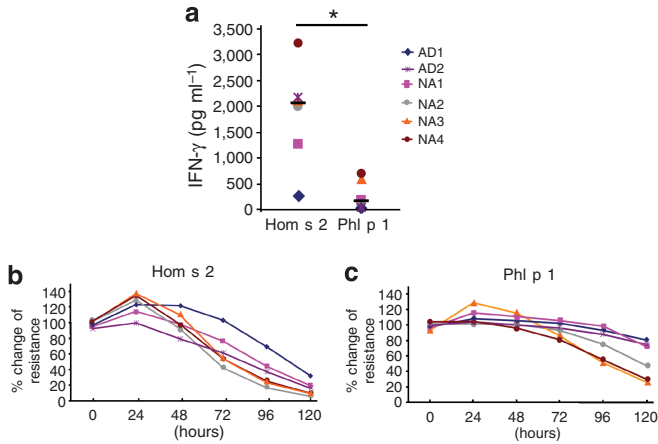
Figure 5. **Culture supernatants from Hom s 2-stimulated PBMCs decrease transepithelial resistance in respiratory epithelial cells.** (**a**) INF-γ levels (*y* axis: pg ml^{-1}) determined in supernatants of PBMC cultures stimulated with rHom s 2 or rPhl p 1 from AD and non-atopic (NA) individuals. Statistically significant differences are indicated (*P<0.05). Change of transepithelial resistance (*y* axis: % change of resistance) over time (*x* axis) in epithelial cell layers exposed to culture supernatants from (**b**) Hom s 2- or (**c**) Phl p 1-stimulated PBMCs is shown.
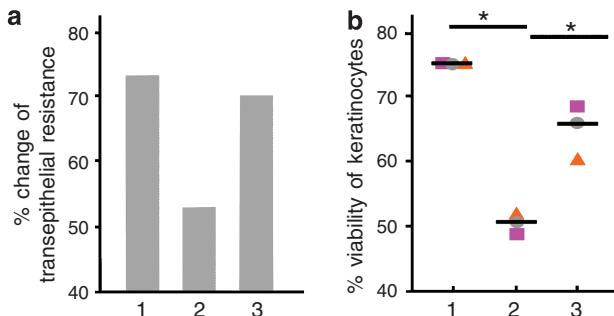
Figure 6. **Culture supernatants from Hom 2-stimulated PBMCs induce IFN-γ-dependent (a) disintegration of respiratory epithelial cell layers and (b) apoptosis in keratinocytes.** (**a**) Respiratory epithelial cells or (**b**) HaCat keratinocytes were incubated with medium (1) or supernatants of Hom s 2-stimulated PBMCs with an isotype antibody control (2) or with a neutralizing anti-IFN-γ mAb (3). (**a**) The percentage of the change of transepithelial resistance and (**b**) the percentages of annexin-V- and/or 7AAD-negative viable keratinocytes are shown.

and non-atopic individuals, suggesting that Hom s 2 has an intrinsic property to induce a Th1 response. In agreement with the cytokine data, we found that most individuals tested showed lymphoproliferative responses as well as IgG$_1$ and IgG$_2$ antibody responses to Hom s 2.

In fact, we have made a similar observation for another IgE-reactive autoantigen, a calcium-binding human protein, designated Hom s 4, which had also induced a strong secretion of IFN-γ in PBMCs (Aichberger *et al.*, 2005).

Hom s 2 and Hom s 4 may therefore be considered as examples of Th1-driving autoantigens that have escaped tolerance, possibly because of their intracellular nature. If such antigens are released due to cell damage, as it may occur in

the course of allergic inflammation or in the course of other inflammatory processes (for example, infection, physical trauma) also in non-allergic persons, they may induce autoantibody formation and Th1 responses. We have no evidence that Hom s 2-specific antibody responses mediate cell damage, but we demonstrate that culture supernatants from Hom s 2-stimulated PBMCs induce epithelial cell damage in an IFN-γ-dependent manner. It is possible that other factors also may contribute, but experiments performed with neutralizing anti-IFN-γ antibodies demonstrate the importance of IFN-γ. The high levels of IL-10 found in the Hom s 2-induced PBMC cultures may be interpreted as a counter-regulatory response, because IL-10 has been described as an anti-inflammatory cytokine that inhibits activation and cytokine production of Th1 cells (Moore *et al.*, 2001; O'Garra and Vieira, 2007).

Since Hom s 2 has been isolated with IgE autoantibodies from AD patients, it is possible that it contributes to the Th1 responses accompanied by IFN-γ production, which are detectable in eczematous chronic skin lesions and old (48–72 h) patch test reaction sites in AD patients. In fact, these lesions are strongly reminiscent of type IV-like contact dermatitis (Grewe *et al.*, 1994, 1995; Thepen *et al.*, 1996; Werfel *et al.*, 1996; Chen *et al.*, 2004).

Several other findings suggest the importance of IFN-γ for chronic allergic diseases. First, IFN-γ induces keratinocyte apoptosis (Trautmann *et al.*, 2000, 2001; Konur *et al.*, 2005) and the production of chemokines, which in turn promotes the further infiltration of T cells into the epidermis of AD patients (Klunker *et al.*, 2003). Second, it has been shown that allergen-specific Th1 cells cause severe airway inflammation (Hansen *et al.*, 1999). Third, it has recently been shown that IFN-γ can reduce the barrier function of respiratory epithelial cells and may enhance the penetration of exogenous allergens (Reisinger *et al.*, 2005).

However, at the moment we cannot prove that Hom s 2 indeed contributes to inflammatory lesions in patients with AD. We can only demonstrate that Hom s 2 can induce Th1 immune responses in atopic as well as non-atopic persons, which are capable of damaging epithelial cells. Nevertheless, Hom s 2 may serve as a paradigmatic tool to study mechanisms underlying chronic inflammation, which ultimately may result in the development of novel therapeutic strategies for the treatment of such chronic inflammatory processes.

MATERIALS AND METHODS
Phage, *E. coli* strains, plasmids, allergens, antibodies
Lambda gt11 phage and *E. coli* strains Y1090 (hsd (r$_k^-$m$_k^+$) *lac* U169, *Pro*A$^+$, Ion$^-$, *ara*D 139, *Str*A, Sup F*trp*C22:Tn10(pMC9)), Y1098 (hsd (r$_k^-$m$_k^+$) *lac* U169, *Pro*A$^+$, Ion$^-$, *ara*D 139, *Str*A, *hfl*A 150 chr:Tn10 (pMC9)) and XL-1 Blue: *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* (F'proAB *lacIq*ZEM15 Tn10(Tetr))c and BL21 (DE3): F$^-$, *omp*T r$_B^-$m$_B^-$(DE) were purchased from Stratagene (La Jolla, CA). Plasmid pUC19 used for subcloning was obtained from Boehringer (Mannheim, Germany).

Purified recombinant timothy grass pollen allergen, rPhl p 1, which had been expressed in *E. coli*, was obtained from Biomay

(Vienna, Austria; www.biomay.com). rHom s 2 (isoform Hom s 2.01; accession number: NP_954984) was expressed in *E. coli* and purified by Nickel affinity chromatography as described (Mossabeb *et al.*, 2002, Buender *et al.*, 2004). Recombinant protein preparations were subjected to affinity chromatography using immobilized polymyxin (BIORAD Affi-Prep Polymyxin Matrix) to reduce endotoxin contaminations. The endotoxin contents in the purified recombinant protein preparations were determined with the Limulus-Amebocyte-Lysate assay (BioWhittaker, Walkersville, MD) and are expressed in endotoxin U (EU ml^{-1}) (rHom s 2: 27–36.5 EU ml^{-1}, rPhl p 1: 24.5–33 EU ml^{-1}). Purity of proteins was checked by SDS-PAGE and Coomassie blue staining (Fling and Gregerson, 1986). The protein concentrations were determined by Micro BCA Protein Assay (Pierce, Rockford, IL). A rabbit anti-rHom s 2 antiserum was raised by immunizing New Zealand white rabbits with purified rHom s 2.01 using complete and incomplete Freund's adjuvant (Charles River, Kisslegg, Germany).

Patients

The demographic, clinical, and serological characterization of AD patients (*n* = 11), RC patients (*n* = 7), and CA patients (*n* = 5) is shown in Table 1. Nine non-allergic individuals were included in the antibody and cellular experiments. Eczema area and severity index and Investigators Global Assessment Score were assessed for the AD patients at the time of bleeding (Hanifin *et al.*, 2001; Eichenfield *et al.*, 2005). Sensitization to exogenous allergen sources was diagnosed based on case history, skin prick testing, and specific IgE measurements (Phadia, Uppsala, Sweden) (Aichberger *et al.*, 2005). IgE autoantibodies in sera were detected using nitrocellulose-blotted extracts of the human epithelial cell line A431 as described (Valenta *et al.*, 1996). IgE reactivity to rHom s 2 and rPhl p 1 was determined by dot blotting (Aichberger *et al.*, 2005). None of the patients or control persons had received systemic immunosuppressive treatments at the time of bleeding. Serum samples were obtained from subjects who had given written informed consent with the approval of the local ethics committee of the Medical University of Vienna. The study was conducted according to the Declaration of Helsinki Principles.

Expression of rHom s 2 fragments

A cDNA (nt 1–237) coding for an N-terminal Hom s 2.01 fragment (aa 1–79) was obtained by PCR amplification using synthetic oligonucleotide primers (MWG-Biotech AG, Ebersberg Germany): forward: 5′-CGGAATTCATGCCGGGCGAAGCCACAGAAAC-3′ and reversed: 5′-CGGAATTCAGCCTTCCGTGCCCTCTTTTCAC-3′ (*Eco*RI sites are underlined) and the Hom s 2.01 cDNA as template (Mossabeb *et al.*, 2002) (accession number: AJ278883). The PCR product was cut with *Eco*RI and ligated into lambda gt11 arms (Stratagene) (Ball *et al.*, 1994, 1999). Phage DNA was *in vitro* packaged using *in vitro* packaging extracts (Stratagene), and lambda gt11 phages containing the DNA insert were identified by plaque hybridization with the ^{32}P-labeled forward primer (Sambrook *et al.*, 1989). The correct insertion and sequence of the construct was confirmed by double-strand DNA sequencing of positive phage clones (MWG-Biotech AG). For this purpose, the inserted DNA was PCR amplified with lambda gt11-specific primers flanking the *Eco*RI site (lambda forward: 5′-CG GGATCCCGGTTTCCATATGGGGATTGGTGGC-3′; lambda reversed: 5′-CGCGGATCCCGTTGACACCAGACCAACTGGTAATG-3′; *Bam*HI

sites are underlined), subcloned into the *Bam*HI site of plasmid pUC 19 (Boehringer), and subjected to DNA sequencing (MWG-Biotech AG). The N-terminal Hom s 2 fragment (aa 1–79) and an IgE-reactive C-terminal Hom s 2 fragment (Hom s 2.02: aa 81–167) described earlier (Natter *et al.*, 1998, Mossabeb *et al.*, 2002) were expressed as β-galactosidase fusion proteins in *E. coli* Y1089 (Natter *et al.*, 1998).

IgE reactivity of rHom s 2 fragments

Comparable amounts of *E. coli* Y1089 extracts containing the β-galactosidase-fused N- and C-terminal Hom s 2 fragments or β-galactosidase alone were subjected to 8% SDS-PAGE to obtain high resolution of proteins > 60 kDa (Fling and Gregerson, 1986) and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) (Towbin *et al.*, 1979). IgE reactivity to nitrocellulose-blotted rHom s 2 fragments was detected with ^{125}I-labeled anti-human IgE antibodies (Phadia, Uppsala, Sweden) and visualized by autoradiography for AD patients AD1 and AD2 (Table 1). The reactivity of rabbit anti-rHom s 2-specific IgG antibodies with the blotted rHom s 2 fragments was determined as described (Valenta *et al.*, 1992).

Basophil histamine release experiments

Granulocytes were obtained from the peripheral blood of AD patients containing IgE antibodies reactive with complete rHom s 2 and rHom s 2 fragments (Table 1: AD1, AD2). Granulocytes were isolated by dextran sedimentation (Valent *et al.*, 1989) and exposed to different concentrations of rHom s 2, rPhl p 1, or anti-IgE antibodies. Histamine released into the culture supernatants was determined by radioimmunoassay (Immunotech, Marseille, France) as described (Valent *et al.*, 1989). All experiments were performed in duplicates.

Lymphoproliferation assays

PBMCs from each of the AD, RC, and CA patients (Table 1) and from nine non-atopic persons were isolated by Ficoll density-gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). PBMCs (2 × 10^5 cells per well) were cultured in triplicates in 96-well plates (Nunclone; Nalgen Nunc International, Roskilde, Denmark) in 200 μl serum-free Ultra Culture medium (BioWhittaker, Rockland, ME) supplemented with 2 mm L-glutamine (Sigma, St Louis, MO), 50 μM β-mercaptoethanol (Sigma), and 0.1 mg gentamicin per ml (Sigma) at 37 °C using 5% CO$_2$ in a humidified atmosphere. Cells were stimulated with different concentrations (0.5–2.5 μg per well) of rHom s 2, with rPhl p 1, 4 U IL-2 per well (Boehringer), or medium alone. After 6 days, 0.5 μCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK) was added to each well, and 16 h thereafter incorporated radioactivity was measured by liquid scintillation counting as counts per minute (c.p.m.; means of triplicates) using a microbeta scintillation counter (Wallac ADL, Freiburg, Germany). The stimulation index was calculated as quotient of c.p.m. with agonists and the medium control. For most individuals, proliferation experiments were repeated at least twice.

Antibody measurements and quantification of cytokine levels

Hom s 2- and Phl p 1-specific IgE, IgG$_1$, IgG$_2$, IgG$_3$, and IgG$_4$ levels in sera from each of the AD, RC, and CA patients (Table 1) and from nine non-atopic persons were measured by ELISA (Vrtala *et al.*, 1996; Stern *et al.*, 2007). Cytokine levels (IL-4, IL-5, IL-10, and

IFN-γ) were measured in supernatants collected from PBMC cultures at day 6 of culture using ELISA assays (Aichberger *et al.*, 2005) or the xMAP Luminex fluorescent bead-based technology (Luminex Corp., Austin, TX). The assays were performed according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany), and fluorescent signals were read on a Luminex 100 system (Luminex Corp.). The limits of detection were $<3.3 \, \mathrm{pg \, ml^{-1}}$ for IL-4, $<1.8 \, \mathrm{pg \, ml^{-1}}$ for IL-5, $<3.66 \, \mathrm{pg \, ml^{-1}}$ for IL-10, and $<2.6 \, \mathrm{pg \, ml^{-1}}$ for IFN-γ. Statistical analysis of results was performed by using box–plot analysis and the Mann–Whitney U-test (SPSS 14; SPSS Inc., Chicago, IL), and statistically significant results were indicated by $*P<0.05$ or $**P<0.01$.

Influence of PBMC supernatants on respiratory epithelial cell layers

Human respiratory epithelial cell line, 16HBE14o (Cozens *et al.*, 1994; Wan *et al.*, 2000), was grown in minimal essential medium (Gibco, Carlsbad, CA) containing 10% fetal calf serum, $100 \, \mathrm{U \, ml^{-1}}$ penicillin (Gibco), $100 \, \mathrm{\mu g \, ml^{-1}}$ streptomycin (Gibco), and 2 mM glutamine (Gibco) and then incubated with rHom s 2- or rPhl p 1-stimulated PBMC supernatants. The transepithelial resistance reflecting the coherence of the epithelial layer was quantified with an ohm voltmeter as described (Reisinger *et al.*, 2005). Neutralization experiments were performed with supernatants from three persons by adding an anti-IFN-γ antibody (PeproTech, Rocky Hill, NJ) or an isotype-matched control antibody to the supernatants. Results are means of duplicate experiments with a less than 5% variation.

Influence of PBMC supernatants on keratinocyte viability and apoptosis

The human keratinocytes cell line HaCat (a gift from N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany) was grown in keratinocyte growth medium containing 2.5 μg per 500 ml human recombinant epidermal growth factor, 25 mg per 500 ml bovine pituitary extract, 5 ml per 500 ml L-glutamine 200 μM (all from Gibco, Luzern, Switzerland), and 5 ml per 500 ml antibiotic–antimycotic solution (Sigma, Buchs, Switzerland). To the medium, either 50% of complete RPMI or 50% of Hom s 2 supernatant with isotype control mAb IgG1 (Chemicon, Dietikon, Switzerland) or α-IFN-γ mAb 45-15 (Novartis Pharmaceuticals, Basel, Switzerland) was added. The viability was measured by flow cytometry after staining with 7-amino-actinomycin (7AAD) and annexin-V (Beckman Coulter, Nyon, Switzerland). Both early (annexin-V-positive cells) and late (annexin-V- and 7AAD-positive cells) apoptotic cells and necrotic cells (7AAD-positive) were excluded for the determination of viable keratinocytes.

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