

Inhibition of immunoglobulin secretion from peripheral blood mononuclear cells by glucose-regulated protein94 (Grp94) in allergic subjects

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Abstract Glucose-regulated protein94 (Grp94) is the most represented endoplasmic reticulum-resident HSP with the unique property to modulate the immune response. This has opened the way to the use of Grp94 as effective therapeutic agent in both depressed and exaggerated activity of the immune system. We investigated the effect of native Grp94 on peripheral blood mononuclear cells (PBMCs) isolated from blood of two subjects with a different history of bronchial allergic asthma. Whereas in subject 1 an elevated basal level of Ig and altered morphological aspects of PBMCs suggested an intense antigen-driven stimulation of the immune system, subject 2 had an apparently normal basal humoral response. However, Grp94 reduced in a concentration-dependent manner the Ig secretion from PBMCs of both subjects, inhibition being maximal at 100 ng/ml Grp94 after 15 days. The effect was apparently related to inhibition of intra-cellular content of both IgG and IgE, and in subject 1 was still observed a year after the first examination. Dot-blot experiments revealed the presence of anti-Grp94 antibodies in Ig secreted from PBMCs and in plasma of both subjects, confirming the role of Grp94 as antigen responsible for activation of the immune system, even in the absence of clinical signs of asthma.

Anti-Grp94 antibodies significantly decreased after PBMC treatment with Grp94 at 100 ng/ml. Results demonstrate that inhibition of the humoral response by Grp94 crucially depends on being Grp94, the antigen challenging the immune system in these allergic subjects, thus supporting the role of Grp94 as immuno-modulatory agent in pathologies with exaggerated immune response.

Keywords Heat shock proteins · Allergic reaction · Immuno-modulation · Lymphocyte activation

Introduction

Glucose-regulated protein94 (Grp94), the most represented heat shock protein (HSP) of the endoplasmic reticulum, besides its main function to chaperone intra-cellular proteins, is known to play important and unique immuno-modulatory activities [1]. The extra-cellular, non-physiological expression of Grp94 is sensed as immunological danger by the immune system, so that both humoral and cellular immune responses mount against circulating Grp94 and cell-bound Grp94 [2]. The immune reaction can also involve endogenous proteins by a mechanism of molecular mimicry whereby autoimmunity may eventually arise [3, 4]. The role of Grp94 and other HSPs in the pathogenesis of diverse autoimmune diseases has now been firmly established by numerous experimental data [5–7]. Since HSPs are among the most diffuse proteins in the animal and vegetal reign with highly conserved sequence homology among different organisms [8, 9], and since antigenic proteins of many allergens are reported to share extensive sequence similarity with Grp94 and other HSPs [9–11], the hypothesis has been proposed that a pathogenic link exists between allergy and autoimmunity [5, 12]. Immune

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reactivity against exogenous antigens might thus spread to endogenous counterparts, and vice versa. Clinical observations have shown that many allergic patients develop auto-reactivity against endogenous proteins, and reciprocally, mechanisms leading to autoimmunity have been reported to contribute to maintain allergic inflammation even in the absence of exposure to exogenous allergens [12].

In this view, identification of HSPs that are (auto)-antigens in both allergy and auto-immunity represents a fundamental step for using HSPs as therapeutic molecules capable of modulating the immune response, in particular for abating the abnormal immune reactivity that characterizes both allergy and autoimmune diseases [13].

In a recent investigation, we observed that Grp94 caused a time- and dose-dependent stimulation of Ig secretion from peripheral blood mononuclear cells (PBMCs) of normal volunteers. Modulation of the humoral immunity by Grp94 involved an accelerated maturation and differentiation of B-cells by a cytokine-like and cell-mediated mechanism [14]. Among the subjects recruited in that study, there was a woman who presented with a long history of allergic bronchial asthma, a reason for which she was excluded from the group of controls and analyzed separately over a period of a year, testing the effects of Grp94 on PBMCs at distinct time intervals. Another subject with recent mild episodes of allergy was included in the survey.

Results showed that, despite differences in the clinical manifestation of allergy and intensity of the immune system stimulation of the two subjects, both shared Grp94 as the antigen responsible for the activation of a specific immune response, and at variance with what previously observed in non-allergic subjects [14], inhibition of the humoral response was the main effect of Grp94 that significantly reduced the synthesis and secretion of both IgG and IgE antibodies (Abs) from PBMCs.

Materials and methods

Case presentation

Subject 1 was a 48-years old woman who had been suffering from allergic asthma for 18 years. The nature of possible allergen(s) responsible for the allergic manifestation was unknown. The subject had no other clinically detectable pathologies, and was in an apparently good state of health. In addition, she did not suffer from any clinically relevant manifestation of bronchial allergic asthma during the entire period of investigation lasting 1 year. She gave her informed consent for blood drawing and treatment of clinical and laboratory data. Blood was drawn for laboratory tests every 3 months and in two separate occasions plasma was

recovered for isolation and purification of PBMCs. At each examination, laboratory findings revealed normal values of red blood cells, platelets, and white blood cells, with a slight increase in the number of eosinophils (7.5–10.7%, normal <7%). Total plasma proteins were in the normal range (74.3–79 g/l) and the electrophoretic profile did not reveal any alteration with the exception of a slight, inconstant elevation of α_2 -globulin fraction (13% of in two of the four tests, normal <12%). Other blood and urine tests were normal and markers for malignancies negative. During the period of investigation she did not take any medication.

Subject 2 was a 25-years old man in whom the first manifestation of allergy to dust allergens had been diagnosed 1.5 year earlier. He had suffered from the last mild asthma attack 3 months before the present investigation. Apparently, he completely recovered from allergy and both routine laboratory tests and clinical examination were negative. At the time of investigation he did not take any medication.

Materials

Native Grp94 was purified from rat hepatocyte microsomal fraction through sequential chromatographic steps, as described [14]. The purified fractions of Grp94 were submitted to ultra-filtration on Amicon Centriplus YM-3 (3,000 MWCO) and to the QCL-1000 chromogenic LAL end-point assay (Cambrex BioScience Inc., Walkersville, MD, USA) to exclude contamination with endotoxin.

Primary Abs used were: anti-CD19 monoclonal (Coulter Electronics, FL, USA), sheep anti-human IgG (whole molecule, The Binding Site, Birmingham U.K.), mouse anti-human IgE (Santa Cruz Biotechnology Inc., CA, USA).

PBMC purification

PBMCs were purified from freshly drawn blood specimens using Ficoll-Paque density gradient [14], and incubated (2×10^6 cell/ml) with Grp94 (final concentrations, 10 and 100 ng/ml) for 3, 7, and 15 days (for 15 days only in subject 2) in duplicate wells of a 24-well plate with 10% FBS, to measure Ig in the medium and cell morphology at the optical microscopy. Controls included treatment with puromycin (100 μ g/ml) used to block the novo protein synthesis and Pokeweed mitogen as the positive control. Aliquots of PBMCs both before and after each incubation time were measured by cytofluorimetric analysis for determining the percentage of CD19⁺ lymphocytes.

Ig measurement

Total Igs were measured by radio immunoassay in the supernatants from PBMC cultures diluted in PBS/BSA and

added to a 96-well flat-bottomed Optiplate coated with goat anti-human Ig Abs, as reported in detail previously [14]. Sheep anti-human Ig Fab₂-I¹²⁵ were added to each well and after incubation of 2 h, the radioactivity was measured in a TOP Count Perkin-Elmer scintillator after the addition of 30 µl of Microscint 20 (Hewlett-Packard).

The concentration of Ig in the supernatant was calculated on the calibration curve obtained by diluting a solution of standard human serum with a known concentration of Ig, and expressed as ng/ml $\times 10^{-5}$ B-cells, as determined by cytofluorimetric analysis with anti-CD19 monoclonal Abs.

Analysis of cell lysates and Western blotting

To measure the expression of Igs in PBMCs, cells were repeatedly washed with PBS and lysed with Laemmli lysis buffer (50 mM Tris-HCl, pH 8.9, 5 mM EDTA, 380 mM glycine, 4% SDS, 10% glycerol) followed by boiling for 3 min. Cell lysates were then analyzed in SDS-PAGE (10% acrylamide gel) and after trans-blot on Immobilon membrane, probed with anti-human IgG polyclonal and IgE monoclonal Abs. Immune detection was obtained with alkaline phosphatase-conjugated, affinity purified IgG. Secondary Abs alone served as control for excluding any false positive reaction.

Dot-blot analysis

Dot-blot experiments were performed to detect anti-Grp94 Abs in PBMCs, culture media, and plasma of the two subjects. To this aim, native Grp94 (boiled for 3 min to denature the protein) was used as antigen and deposited on Immobilon membranes (2.5 $\times$ 1.0 cm), previously rinsed in methanol and water, and blocked with 3% BSA for 2 h at room temperature. To detect Abs in cell lysates, Grp94 was deposited in dots of 10 µl (1.2 µg protein) and membranes were incubated with lysates of PBMCs (both control and Grp94-treated) diluted in the buffer solution (1% BSA with 0.1% Tween in TBS) for 4 h at room temperature. Blanks were made with Grp94 incubated with lysis buffer only, diluted in the same buffer solution. To detect Abs in culture media of control and Grp94-treated PBMCs, membranes with dots of native Grp94 (5 µl, 0.1 µg protein) were incubated in culture media diluted in the buffer solution (as above for cell lysates). Blanks were made with the antigen incubated with the culture medium only, diluted in the buffer solution. Plasma of both subjects was dialyzed overnight at 4°C against Tris-HCl buffer (20 mM, pH 7.4), centrifuged at 3,000 rpm for 10 min, and the protein concentration determined at 280 nm. Plasma diluted in the buffer solution (as above) to obtain a final protein concentration of 0.5 mg/ml served as incubation solution

for antigen-coated membranes. Blanks for plasma were made with antigen incubated with FBS containing the final protein concentration of 0.5 mg/ml. Following incubation, antigen-coated membranes of each series of determination (cell lysates, culture media and plasma), were washed repeatedly and incubated overnight with sheep anti-human IgG Abs and then with anti-sheep alkaline phosphatase-conjugated, affinity purified Abs for detecting the immune reaction.

Results and discussion

Both the basal concentration of PBMCs and the percentage of CD19⁺ of the two subjects (Table 1) fell in the range of those previously measured in non-allergic subjects [14]. After incubation of 15 days, the basal concentration of Ig in PBMC culture media of subject 1 was much higher than that found in subject 2, comprised in the range of values measured in non-allergic subjects (range 11–430 ng/ml) [14]. Since the Ig concentration in subject 1 was indicative of an ongoing intense stimulation of the immune system, PBMCs of subject 1 were also cultured at 3 and 7 days to follow in time Ig secretion, and to evaluate cell morphology (Fig. 1). It was thus observed that Ig secretion from control PBMCs followed a linear trend in time, reaching the peak at 15 days incubation, whereas a biphasic pattern characterized the Ig secretion in the presence of Grp94 (Fig. 1a). Indeed, after a transient, dose-dependent stimulation of Ig secretion at 3 days, inhibition followed at 7 and mostly at 15 days with 100 ng/ml Grp94 (Fig. 1a, inhibition of 45%). Also in subject 2, Grp94 caused similar, dose-dependent inhibition of the Ig secretion after 15 days incubation, inhibitions being 16 and 37% at 10 and 100 ng/ml Grp94, respectively, (Fig. 2a). This finding markedly differed from what was observed at the same Grp94 concentrations on PBMCs of non-allergic subjects in whom Grp94 instead induced stimulation of the Ig secretion [14]. At the optical microscopy, control PBMCs of subject 1 appeared as big rounded and elongated cells comprising monocytes, macrophages, and a few dendritic cells with

Table 1 Characteristics of subjects

Subject	Sex	Age (years)	Basal value		
			CD19 ⁺ (%)	PBMCs (no $\times 10^6$ /ml)	Ig secretion (ng/ml $\times 10^{-5}$ B cells)
1	F	48	3.9	1.5	3316
2	M	26	11.6	1.0	210

The percentage of CD19⁺ is calculated on the PBMC value. The number of PBMCs is per ml of whole blood. The concentration of Ig represents the basal value measured after 15 days incubation

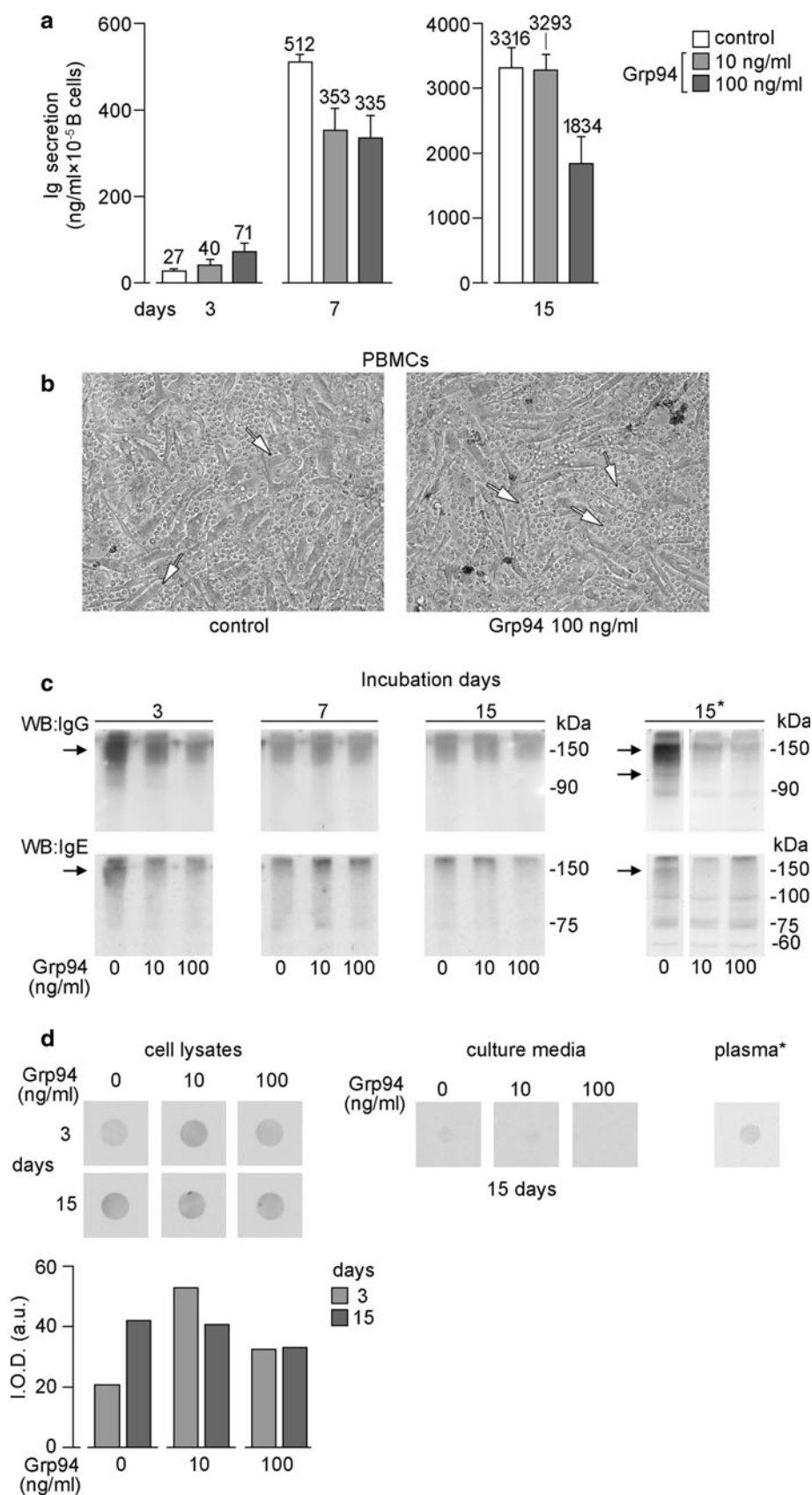


Fig. 1 Grp94-induced effects on morphology and Ig content and secretion from PBMCs in subject 1. **a** Ig concentration in the medium of PBMC cultures at different incubation times. Ig concentration was measured in the supernatants of cell cultures as specified in the text, and expressed as $\text{ng/ml} \times 10^{-5}$ B cell. Histograms are the mean ($\pm$ SD) of three independent measurements, made in duplicate. **b** PBMC morphology in both the absence and presence of Grp94. Cultures of PBMCs were analyzed at the optical microscopy at each incubation time. Representative pictures of several others are shown of control and Grp94-treated cells at 7 days. Arrows in control PBMCs mark some cells of big dimensions that almost entirely replace normal lymphocyte, whereas in treated cells arrows indicate areas of normal lymphocytes. **c** Time- and Grp94-dependent changes in the cell content of both IgG and IgE. The IgG and IgE expression was measured in cell lysates at different incubation times, as specified in the text, by loading the same protein quantity (in non-reducing conditions of samples) in the gel for SDS-PAGE followed by Western blotting with anti-human IgG polyclonal and IgE monoclonal antibodies. Arrows mark bands of IgG and IgE that decrease in intensity in time under the effect of Grp94. A representative blotting of three others for each type of Igs is shown on left, whereas representative Western blotting of three others made on lysates of PBMCs (at 15 days incubation only) purified a year after the first investigation is shown on right (asterisk). **d** Anti-Grp94 antibodies in cell lysates, culture media, and plasma. Dot-blot experiments were performed using native Grp94 as antigen, as specified in the text. A representative dot-blot image of three experiments made on different occasions is shown for each condition. Dots are obtained after subtraction of corresponding blanks. Below dot-blot in cell lysates is the densitometric analysis of dots expressed in arbitrary units. Dot-blot experiments on plasma are made a year after the first examination (asterisk)

rare normal lymphocytes (Fig. 1b, arrows). This picture was consistent with an intense antigen-driven cell differentiation and sharply contrasted with that of normal PBMCs as those characterizing PBMCs of subject 2 that for the most part were formed with small lymphocytes of regular morphology (data not shown).

In subject 1, measurement of the cellular content of both IgG and IgE at 3, 7, and 15 days revealed a higher content of these Ig in the control PBMCs at 3 days (Fig. 1c, blotting on left) followed by a significant time-dependent decrease in the presence of Grp94, especially at 100 ng/ml. Interestingly, overlapping inhibitory effect of the cellular content of both IgG and IgE was observed in PBMCs of the same subject analyzed a year after the first examination (Fig. 1c, blotting on right with asterisk). Also in subject 2, inhibition of Ig secretion was accompanied by a marked reduction in the cellular content of IgG and IgE (Fig. 2b). Results were consistent with an inhibitory effect of Grp94 on the Ig production in PBMCs of both subjects that thus displayed similar responses to the challenge with Grp94.

After incubation with Grp94 (100 ng/ml), the big, antigen-transformed cells which were the hallmark of control PBMCs of subject 1, were slightly reduced in the number and partially replaced by lymphocytes of normal dimension and morphology (Fig. 1b, arrows). The result was consistent with the capacity of Grp94 to modulate the antigen-

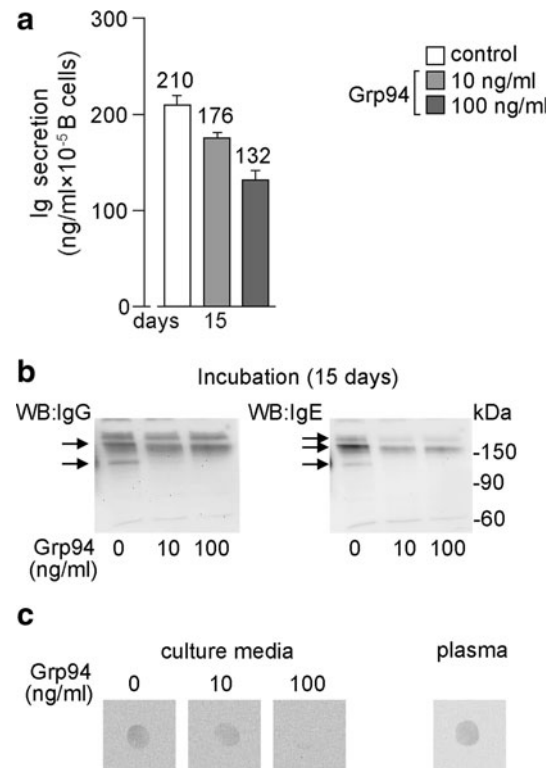


Fig. 2 Grp94-induced effects on Ig content and secretion from PBMCs in subject 2. **a** Ig concentration in the medium of PBMCs cultured for 15 days. Ig concentration was measured in the supernatants of cell cultures as specified in the text, and expressed as $\text{ng/ml} \times 10^{-5}$ B cell. Other indications are as in the legend to Fig. 1. **b** Grp94-dependent changes in the cell content of both IgG and IgE measured in cell lysates as specified in the text, by loading the same protein quantity (in non-reducing conditions of samples) in the gel for SDS-PAGE followed by Western blotting with anti-human IgG polyclonal and IgE monoclonal antibodies. Arrows mark bands of IgG and IgE that decrease in time under the effect of Grp94. A representative blotting of three others for each type of Ig is shown. **c** Dot-blot for detection of anti-Grp94 Abs in culture media and in plasma following the method described in the text. Representative dots of two experiments made on separate occasions are shown after subtraction of corresponding blanks

driven immune response by interfering in the mechanisms of Ab production by B-cells. Although our results do not permit to establish whether effects of Grp94 on B-cells are mediated by a direct or indirect mechanism, the intense proliferative stimulus induced in a dose-dependent manner by Grp94 on PBMCs of subject 1 especially at shorter incubation times (data not shown) rather favors the possibility that the inhibition of Ab secretion from B-cells is a T cell-mediated effect.

Since inhibition of the humoral response by Grp94 could be explained by considering that Grp94 was the antigen responsible for activation of the immune system in both these subjects, we searched for anti-Grp94 Abs in culture media of PBMCs after 15 days incubation, and in the plasma of both subjects (Figs. 1d, 2c). In subject 1, anti-Grp94 Abs

were also searched in cell lysates of PBMCs at different incubation times (Fig. 1d). These last experiments showed that anti-Grp94 Abs were present in control PBMCs already at 3 days and rose in time, in accord with the observed increase in Ig secretion that parallels in time the differentiation of B-cells (Fig. 1a). Instead, in the presence of Grp94, after a transient rise in anti-Grp94 Abs at 3 days, Ab secretion dropped, and at 15 days the cellular content of anti-Grp94 Abs was lower than that measured in control PBMCs (Fig. 1d). Thus, a latency period seems to be necessary to fully activate the mechanism(s) leading to inhibition of Ab production, a result that supports a T cell-mediated rather than a direct effect of Grp94 on B-cells. The reduction in anti-Grp94 Abs measured in PBMC lysates of subject 1 after 15 days was also mirrored in the culture media in which the content of anti-Grp94 Abs in the presence of 100 ng/ml Grp94 was much lower than that measured in the control and also in PBMCs treated with 10 ng/ml Grp94 (Fig. 1d). A clear-cut effect of inhibition of anti-Grp94 Abs secretion was also observed in culture media of PBMCs of subject 2 treated with 100 ng/ml Grp94. Thus, in both cases, the inhibition of Ig secretion at longer incubation time was related to a significant reduction in the specific antigen-driven Ab production. The intense positivity for anti-Grp94 Abs detected in the plasma of both subjects (Figs. 1d, 2c) proved that activation of the immune system by Grp94 has been lasting for long time.

In this respect, the finding that in subject 1 Grp94 induced the same effects on PBMCs isolated after a year from the first examination, was a proof for the persistence of the same antigenic stimulus. However, since in this period the subject did not suffer from any clinically overt manifestation of allergy, the above result raises the question of whether immune reactivity was directed against truly exogenous Grp94-containing allergens or other, so far undetected, endogenous Grp94-like molecule(s). If the latter possibility were true, then, inhibition of the humoral response by Grp94 would occur by the same mechanisms invoked to occur in inflammatory conditions and autoimmune diseases in which there is an over-expression and/or extra-cellular liberation of HSPs and the administration of the HSPs responsible for the immune stimulation can induce the appearance of regulatory, HSP-specific T cells with protective anti-inflammatory activity [15]. The same considerations might apply to subject 2 who at the time of investigation apparently recovered completely from mild asthma attacks, and both the clinical and laboratory examination testified a status of good health. Thus, in both subjects, despite the significant difference in the clinical course of allergy and intensity of the immune response, Grp94 appears to be the antigen responsible for the immune system stimulation that extends beyond the phase of overt clinical manifestations of allergy.

Identification of Grp94 as antigen is the prerequisite for using Grp94 as therapeutic agent to induce a specific immunization that breaks down the unwanted exaggerated immune response. Although further studies are necessary to confirm the role of Grp94 as antigen in allergy, our results offer the ex vivo experimental proof in support of the capacity of Grp94 to modulate the immune response depending on the existing activity of the immune system [14, 15].

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