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Original article

Hypoallergenic fragment of Par j 2 increases functional expression of Toll-like receptors in atopic children

Background: *Parietaria judaica* (Par j) is one of the main causes of allergy in the Mediterranean countries. The activation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) inhibits nasal inflammation of atopic children.
Objective: To examine, *in vivo* and *in vitro*, the effect of recombinant Par j 2 (rPar j 2) and of its fragments (1–55 and 52–102) on atopic children.
Methods: We used skin prick test for *in vivo* evaluations. We assessed, *in vitro*, in peripheral blood mononuclear cells (PBMC), the effect of rPar j 2 and of the two fragments on neutrophil chemotaxis, on CD45RO, on TLR2 and TLR4 expression, on LPS binding and on interferon (IFN)-γ release, by a micro-chemotaxis chamber, by flow cytometry and by enzyme-linked immunosorbent assay, respectively.
Results: *In vivo* while rPar j 2 induced a positive skin reaction, 1–55 and 52–102 fragments did not. *In vitro*, while rPar j 2 increased both CD45RO expression and neutrophils chemotaxis in PBMC, both Par j 2 fragments did not. 1–55 fragment of Par j 2 upregulated both TLR2 and TLR4 expression and LPS binding, while the rPar j 2 and 52–102 fragment did not. Finally, 1–55 fragment of Par j 2 induced IFNγ release, while the rPar j 2 and 52–102 fragment did not.
Conclusions: Hypoallergenic 1–55 fragment, upregulating innate immunity receptors and increasing IFNγ, might re-orientate, in atopics, the immune system toward a physiologic balance between Th1 and Th2 responses.

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Parietaria judaica, a grassy plant belonging to the family Urticaceae, is one of the main causes of allergy in the Mediterranean area (1). Parietaria flowers from spring to autumn and this long pollen season may account for the prevalence of this pollinosis in this area (2). This pollen contains at least nine allergens, and two of them, named Par j 1 and Par j 2, have been cloned and sequenced (3, 4). The allergeneity of this pollen is crucially dependent by a three-dimensional structure generated by the presence of four disulphide bridges as result of the pairing of the eight cysteine residues (5). The modification of allergen B-cell epitopes represents an approach to obtain hypoallergens that would increase the safety of allergen-specific immunotherapy (SIT; 6).

Several epidemiologic studies shed light on the possible protective influences of natural microbial exposure on atopy and asthma development and has led to the formulation of the hygiene hypothesis (7, 8). The lack of Th1-inducing factors, i.e. the reduction of pathogen-associated molecular patterns (PAMP), which are detected by accessory cells expressing Toll-like receptors (TLR), is associated with the germ-reduced living standard in the cities of the so-called western world (7). This coincides with an increase of Th2-mediated disorders, such as allergic bronchial asthma and atopic dermatitis in the urban population, which are not detected in the rural population (9). Toll-like receptor stimulation on professional antigen-presenting cells (APC) increases the production of interleukins (IL) 12 and 23 and promotes the expansion of APC with enhanced and sustained T helper type 1-polarizing capacity (10). The associated increase in interferon (IFN)γ may steer our immune system away from the allergy-driven type-2 helper T-cell phenotype. According to this hypothesis, a constant Th1 triggering balances the immune system, and the removal of these triggers skews the system toward Th2. As regards, it has been demonstrated that a combination of TLR and IFN-γ receptor stimulation of dendritic cells (DC) results in a shift from a Th2- to a Th1-like response in a coculture system with appropriately pretreated DC and autologous lymphocytes from allergic subjects (11). The present study was performed using both an *in vitro* and an *in vivo* approaches and was aimed to evaluate whether the alteration in the molecular structure of Par j 2 may affect:

Abbreviations: Par j 2, *Parietaria judaica* major allergen 2; MoAb, monoclonal antibody; CM, complete medium; TLR, Toll-like receptors; LPS, lipopolysaccharide; Th, T helper.

(i) the allergenic activity of Par j 2; (ii) the proinflammatory activity of Par j 2; (iii) the expression and the activation of TLRs; and (iv) the release of IFNγ.

Materials and methods

Subjects

We selected 10 allergic patients with mild intermittent asthma (age 7–16 years), according to the criteria of the American Thoracic Society. The diagnosis of asthma and the assessment of its severity were performed at study entry according to Global Initiative for Asthma (12). All subjects were monosensitized to *P. judaica* pollen as assessed by skin prick tests including a panel of allergens (Stallergenès) and by evaluation of total and specific immunoglobulin (Ig)E concentrations (CAP System Pharmacia-Upjohn, Uppsala, Sweden). None of the patients received any corticosteroid or antihistamine treatment during the previous 2 weeks. The study fulfilled the criteria of the Ethics Committee of our Hospital and all subjects and/or their parents had given their informed consent. Demographic characteristics of recruited patients are reported in Table 1.

Generation of recombinant Par j 2 and of molecular variants

The cDNA cloning, expression and preparation of *Escherichia coli* cell lysates, purification and sequence analysis of rPar j 2 were performed as previously described (3). The two fragments (patent No. CNR RM 99 A 000737) were generated as previously described (13) including some modifications. In particular, we constructed a fragment containing the first 55 amino acids of Par j 2 (1–55) and a fragment containing the amino acids 52–102. To eliminate disulfide bridges and to disrupt the three-dimensional structure in the 1–55 fragment, as the disulphide bridges are generated between cysteine residues, using a polymerase chain reaction mutagenesis method, four cysteine residues were substituted by alanine residues (Fig. 1). The amino acid exchange was performed at the positions 4, 14, 49 and 52. The sequence of the used oligonucleotides for 1–55 fragment are: 5′: Bam Q gtg-GGA-TCC-gag-gag-gct-**gcc**-ggg-aaa-gtg-gtg-cag-gat-ata-atg-ccg-**gcc**-ctg; 1–55 3′: *Hin*dIII cac-AAG-CTT-**gcg**-cac-tat-**gca**-ctt-gca-ggc-ctc-cct-ctt. Bold and capital letters indicate the mutated nucleotides and sites of restriction enzymes, respectively. All the generated molecules were expressed in a vector (pQE 30) containing a six histidine tag for their affinity purification, did not contain detectable amounts of lipopolysaccharide (LPS) and were >99% pure. The presence of contaminating LPS was assessed by a commercially available kit (Cambrex Corporation, East Rutherfort, NJ) and was below the detection limit of 0.1 EU/ml.

Table 1. Demographic characteristics of patients

Subject number	Age (years)	Sex	IgE (IU/ml)	Eos/ mm^3	RAST Par J (KU/l)	FEV_1 (% prediction)	r-Par j 2 (mm)
1	14	M	1003	600	>100	81	4 × 4
2	16	M	1250	930	>100	93	8 × 5
3	7	F	1571	800	70.65	84	2 × 5
4	16	F	819	811	12.02	107	3 × 4
5	12	M	491	560	>100	100	2 × 4
6	8	F	290	1370	97.76	118	6 × 4
7	13	M	1273	1140	89.40	93	14 × 13
8	11	F	248	300	>100	80	3 × 3
9	12	F	698	690	>100	101	2 × 2
10	7	F	533	790	>100	100	2 × 5

Eos, eosinophils.

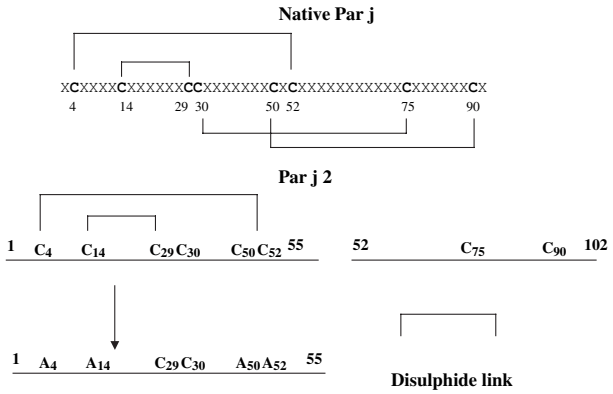

Figure 1. Schematic representation of rPar j 2, of 1–55 and 52–102 fragments.

Skin prick test

Skin prick test on children was performed with rPar j 2 and 1–55 and 52–102 fragments. About 20 μl of rPar j 2 (20 μg/ml) and of molecular variants (10 μg/ml), diluted in sterile water, were applied on forearms of patients and pricked with sterile lancets. The concentration range (from 1 to 20 μg/ml) was previously shown to be optimal in preliminary experiments (data not shown). Reactions were recorded after 20 min by transferring the ballpoint pen-surrounded wheal area with a transparent tape to paper. We measured the maximal longitudinal and transversal diameters of the wheal area.

Peripheral blood mononuclear cells cultures

Peripheral blood mononuclear cell (PBMC), obtained from 20 ml of heparinized blood from atopic children, were separated by Ficol–Hypaque-gradient centrifugation. Peripheral blood mononuclear cell were incubated in the presence and in the absence of rPar j 2 (20 μg/ml), of 1–55 and of 52–102 fragments (10 μg/ml). The concentration range (from 1 to 40 μg/ml) and incubation times (4, 8 and 18 h) were previously shown to be optimal in preliminary experiments (data not shown). Peripheral blood mononuclear cell were cultured (37°C, 5% CO_2) with the stimuli for 18 h and expression of CD45R0, chemotactic activity, expression of TLR2 and TLR4, LPS binding and IFNγ release were then assessed.

Chemotaxis assay

Neutrophils were purified from atopic children and chemotaxis toward neutrophils was performed as previously described (14), using a microchemotaxis chamber (Costar, Neuro Probe Inc., Cabin John, MD, USA).

Expression of CD45R0

The expression of CD45RO was assessed by flow cytometry by a FITC-conjugated specific mouse monoclonal antibody (Pharmingen, San Diego, CA, USA) using a FACStar Plus (Becton Dickinson, Mountain View, CA, USA) analyzer. Negative controls were

performed using a mouse FITC-conjugated IgG1 (Dako, Glostrup, Denmark). Data are expressed as percentage of positive cells.

Expression of TLR2 and of TLR4 by PBMC

The expression of TLR2 and of TLR4 was evaluated by flow cytometry using specific rabbit monoclonal antibodies anti-TLR2 and anti-TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a FITC-conjugated antirabbit IgG (Dako). Negative controls were performed using rabbit immunoglobulin-negative control (Dako). Data are expressed as geomean fluorescence intensity. The positivity of the cells was evaluated on the gate of monocytes. The specificity for monocytes of the designed gate, was also confirmed by the high percentage of CD14-positive (Dako) cells present within this gate, as assessed by preliminary experiments (data not shown).

Binding of LPS

The binding of LPS was assessed using ALEXA fluor LPS (Molecular Probe, Invitrogen, Carlsband, CA). In particular, PBMC stimulated as described above, were incubated with ALEXA fluor LPS for 30′ and the binding of LPS was evaluated by flow cytometry.

Measurement of IFNγ

The concentration of IFNγ was determined with an enzyme-linked immunosorbent assay (ELISA; Quantikine; R&D Systems, Minneapolis, MN) following the manufacturer's direction.

Statistics

Data are expressed as mean ± SD. Statistical analysis was performed using ANOVA with the Bonferroni test. $P < 0.05$ was accepted as statistically significant.

Results

In vivo effects of rPar j 2 and of 1–55 and 52–102 fragments

We first evaluated whether the structural differences of 1–55 and 52–102 fragments in comparison with rPar j 2, may account for a different allergenicity in skin prick test experiments. The results of the skin prick tests are shown in Table 1. All patients showed pronounced immediate type skin reactions to rPar j 2. The two fragments did not promote any skin reaction (data not shown). All patients displayed immediate and positive reaction to histamine (data not shown).

Effects of rPar j 2 and of 1–55 and 52–102 fragments on neutrophil chemotaxis

Having established that the two fragments of Par j 2 were hypoallergenic, we assessed, using an *in vitro* model, whether PBMC stimulated with rPar j 2 and with both fragments generated a different chemotactic activity toward neutrophils. Interestingly, following stimulation with rPar j 2, PBMC exerted chemotactic activity toward neutrophils. In contrast, PBMC, upon stimulation with the two fragments, were not able to exert this chemotactic activity (Fig. 2).

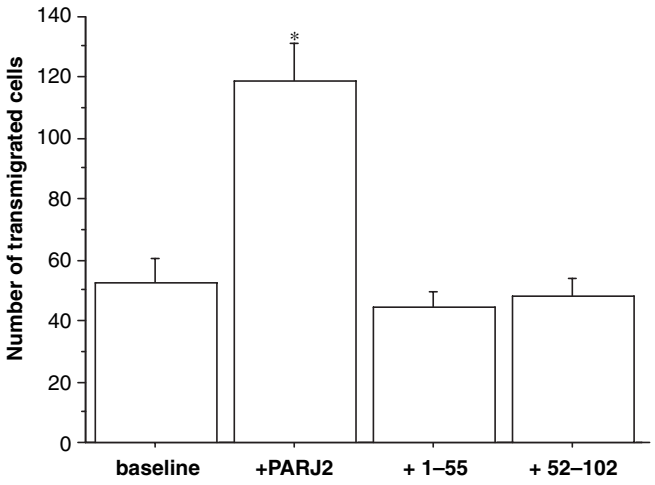

Figure 2. Chemotactic activity toward neutrophils of rPar j 2, 1–55 and 52–102 fragments. Peripheral blood mononuclear cell (PBMC) and peripheral blood neutrophils were isolated from asthmatic and atopic children. PBMC were cultured in the absence or in the presence of rPar j 2, 1–55 and 52–102 fragments for 18 h (see Materials and methods for details). Cell supernatants were collected and were used to stimulate chemotaxis toward neutrophils ($n = 10$) using a microchemotaxis chamber (see Materials and methods for details). Data are expressed as number of transmigrated cells. $*P < 0.001$ compared with the baseline.

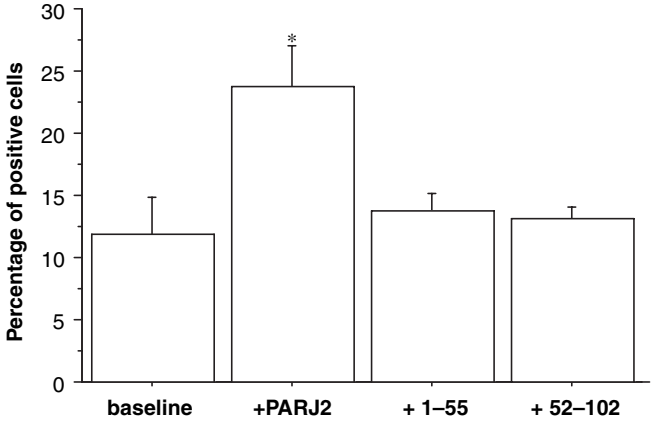

Figure 3. Effect of rPar j 2, 1–55 and 52–102 fragments on the expression of CD45RO by peripheral blood mononuclear cell (PBMC). PBMC isolated from asthmatic and atopic children and cultured in the absence or in the presence of rPar j 2, 1–55 and 52–102 fragments for 18 h, were assessed for CD45RO expression by flow cytometry (see Materials and methods for details). Data are expressed as percentage of positive cells. $*P < 0.004$ compared with the baseline.

Effects of rPar j 2 and of 1–55 and 52–102 fragments on CD45RO expression by PBMC

As in subjects with atopic asthma, following allergen challenge, there is an increase in CD45RO-positive memory T lymphocytes (15), which are responsible of the release of higher concentrations of Th2 cytokines typically present in allergic subjects (16), we evaluated the effects of rPar j 2 and of 1–55 and 52–102 fragments on the expression of CD45RO molecule by PBMC from allergic asthmatic children. Interestingly, while rPar j 2 was able to significantly increase the number of CD45RO-positive cells, both 1–55 and 52–102 fragments did not, confirming that the two fragments of Par j 2 are differently recognized by immune system of the studied patients (Fig. 3).

Effects of rPar j 2 and of 1–55 and 52–102 fragments on TLR2 and TLR4 expression by PBMC

We then evaluated whether the recombinant native allergen and the two fragments differently affected the expression of TLR2 and TLR4. While the recombinant allergen and the 52–102 fragment were not able to significantly increase the expression of TLR2 (Fig. 4) and of TLR4 (Fig. 5), the 1–55 fragment significantly increased the expression of both receptors.

Effects of rPar j 2 and of 1–55 and 52–102 fragments on LPS binding

As the activation of TLR4 receptor preferentially induces Th1 responses, we established whether the expression of TLR4 was functionally active by evaluating the binding of LPS. Interestingly, while rPar j 2 and 52–102 fragment were not able to significantly increase the binding of LPS, 1–55 fragment significantly increased LPS binding (Fig. 6).

Effects of rPar j 2 and of 1–55 and 52–102 fragments on IFNγ release

Finally, we assessed whether the upregulation of functionally active TLR4 receptors was associated with an increased release of IFNγ. Interestingly, the stimulation of PBMC with 1–55 fragment leaded to an increased release of IFNγ. In contrast, rPar j 2 and 52–102 fragment were not able to increase the release of IFNγ (Fig. 7).

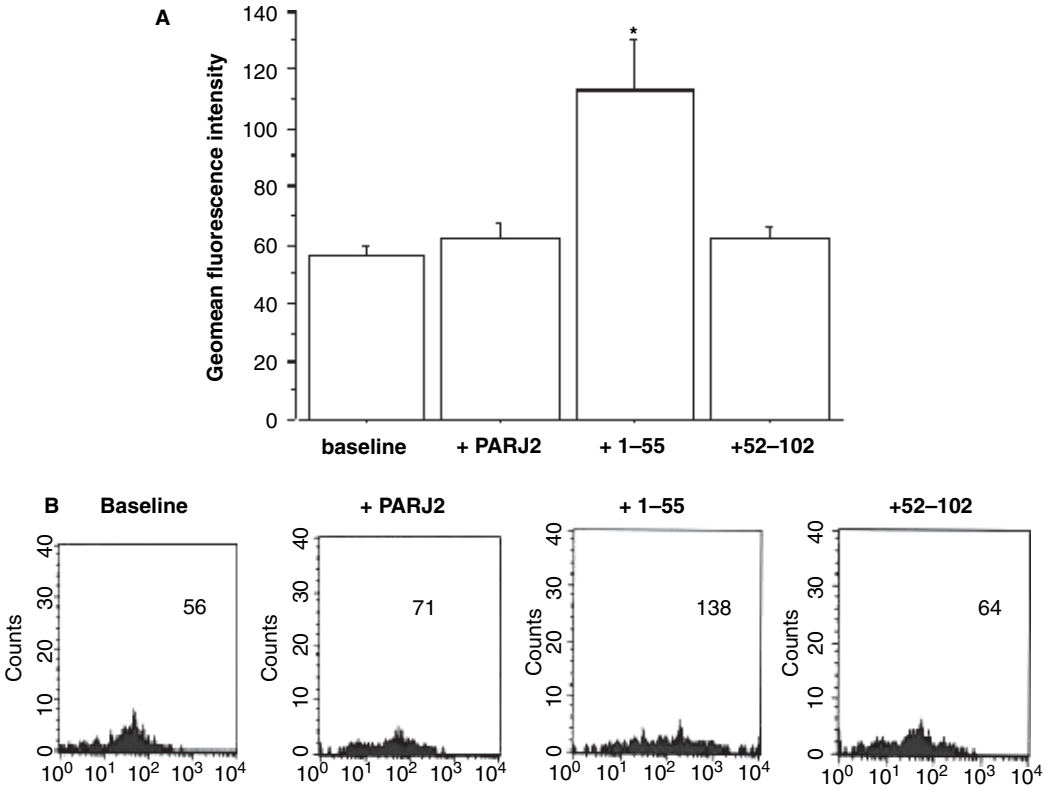

Figure 4. Effect of Par j 2rPar j 2, 1–55 and 52–102 fragments on the expression of Toll-like receptor (TLR)2 by peripheral blood mononuclear cell (PBMC). (A) PBMC isolated from asthmatic and atopic children and cultured in the absence or in the presence of rPar j 2, 1–55 and 52–102 fragments for 18 h, were assessed for TLR2 expression by flow cytometry. Data are expressed as geomean fluorescence intensity. $*P < 0.001$ compared with the baseline. (B) Histograms from a representative experiment.

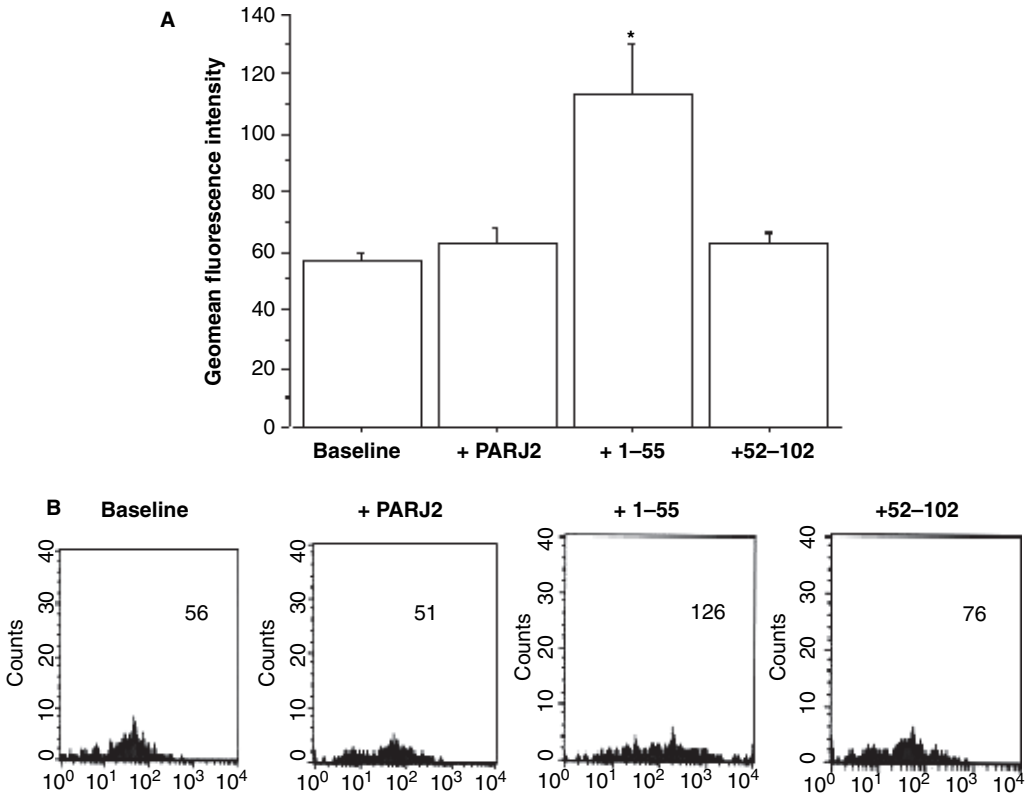

Figure 5. Effect of rPar j 2, 1–55 and 52–102 fragments on the expression of Toll-like receptor (TLR)4 by peripheral blood mononuclear cell (PBMC). (A) PBMC isolated from asthmatic and atopic children and cultured in the absence or in the presence of rPar j 2, 1–55 and 52–102 fragments for 18 h, were assessed for TLR4 expression by flow cytometry. Data are expressed as geomean fluorescence intensity. $*P < 0.001$ compared with the baseline. (B) Histograms from a representative experiment.

Discussion

Parietaria judaica certainly represents one of the main causes of allergy in the Mediterranean countries. One of the most important allergens of *P. judaica* is represented by Par j 2, as it has been previously demonstrated that 83% of the sera from the Mediterranean atopic patients showed IgE reactivity to this allergen (2). Here, we demonstrate for the first time that a hypoallergenic fragment of Par j 2, the 1–55 fragment, missing the anaphylactic and proinflammatory properties of recombinant allergen with native conformation, is able to increase the expression of innate immunity receptors TLR2 and TLR4, the LPS binding and the release of IFNγ by PBMC. These findings support the recent proposed use of modified allergens in the SIT of allergic patients (17) and unveil for the first time some molecular mechanisms potentially responsible of the success of this new approach.

Allergen-specific immunotherapy is an effective treatment for type I allergy modifying the natural course of the disease by preventing the evolution from rhinitis to asthma and the onset of sensitization against new allergens (18). The main problem of conventional SIT is the risk of possible anaphylactic reactions. The three-dimensional structure of an antigen and its recognition by different APC seem to be crucial aspects in the production of Th2 cytokines as well as of IgE antibodies (19). One strategy for the development of molecular variants of Par j with reduced allergenic activities is based on the disruption of the allergen's three-dimensional structure by site-directed mutagenesis targeted to disrupt disulfide bridges (5) and by synthesizing peptides comprising the N-terminal or the C-terminal half of the allergen.

On the basis of these concepts, we disrupted the three-dimensional structure of Par j 2 major allergen by fragmentation and by site-directed mutagenesis. We first assessed whether the generated fragments were, in comparison with recombinant Par j 2 native allergen, hypoallergenic *in vivo*, by skin prick test. rPar j 2 has been shown to possess immunologic properties equivalent to its natural counterpart and its availability represents a fundamental tool for the diagnosis and therapy of *Parietaria* pollen allergy (2). It is well established that, due to the presence of some cross-reactivity with other allergens, the use of rPar j 2 may be useful to unambiguously identify the disease-eliciting allergen sources (2). We confirmed, using rPar j 2, that all the studied patients were genuinely allergic to

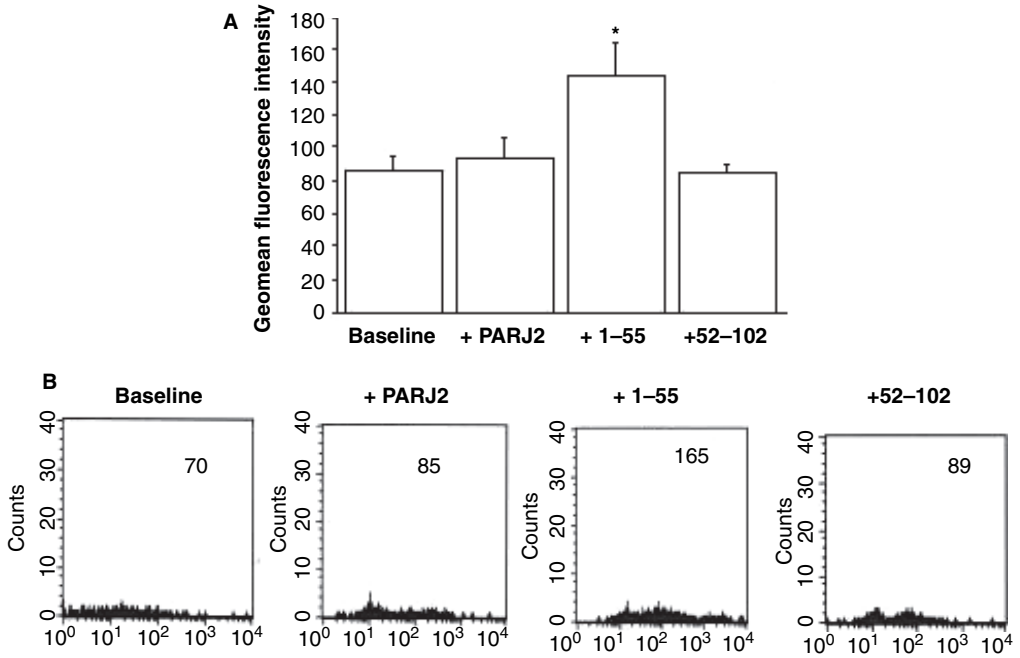

Figure 6. Effect of Par j 2rPar j 2, 1–55 and 52–102 fragments on the binding of lipopolysaccharide (LPS) by peripheral blood mononuclear cell (PBMC). (A) PBMC isolated from asthmatic and atopic children and cultured in the absence or in the presence of rPar j 2, 1–55 and 52–102 fragments for 18 h, were assessed for LPS binding by flow cytometry. Data are expressed as geomean fluorescence intensity. $*P < 0.03$ compared with the baseline. (B) Histograms from a representative experiment.

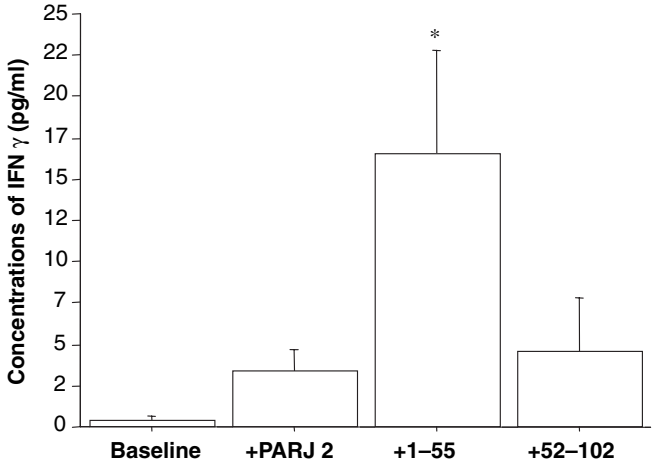

Figure 7. Effect of Par j 2rPar j 2, 1–55 and 52–102 fragments on the release of interferon (IFN)-γ by peripheral blood mononuclear cell (PBMC). PBMC were cultured in the absence or in the presence of rPar j 2, 1–55 and 52–102 fragments for 18 h (see Materials and methods for details). Cell supernatants were collected and IFNγ release was assessed by enzyme-linked immunosorbent assay. Data are expressed as pg/ml of IFNγ and represent mean ± SD of 10 experiments ($*P < 0.002$).

P. judaica pollen. Furthermore, we demonstrated that both 1–55 and 52–102 fragments lost their anaphylactic activity as none of the *Parietaria* monosensitized children showed any positive skin reaction to allergen fragments.

To further characterize the functional differences between rPar j 2 and the two fragments, we evaluated whether all these molecules differently affected the expression of CD45RO+ T cells and the chemotactic activity toward neutrophils by PBMC. We demonstrated that the generated fragments lost the ability to induce a chemotactic activity toward neutrophils as the native counterpart. Furthermore, both fragments (1–55 and 52–102) were differently recognized by immune cells as they did not increase the number of CD45RO+ memory T cells as the rPar j 2 did.

The hygiene hypothesis is considered the most reasonable explanation for the allergy epidemics in western countries that has occurred over the past few decades (8, 9). The immunologic mechanisms on the basis of the hygiene hypothesis are a missing immune deviation of allergen-specific responses from a Th2 to a type Th1 profile and a reduced activity of T-regulatory cells (20). As regards, it has been demonstrated that TLR-4 signaling may promote the induction of IL-10-secreting type 1 regulatory T cells (21). We evaluated the IL-10 production by stimulated PBMC in order to explore the effects of the rPar j 2 and of the fragments on the activity of T-regulatory cells. We did not observe any significantly increased release of IL-10 following molecular variants exposure (data not shown). This phenomenon was probably due to the fact that our model of *in vitro* stimulation includes a single antigen exposure while the regulatory cell number appears to arise following repeated antigen stimulation, either *in vitro* or *in vivo* (22).

A reduced activation (11) or expression (23) of specific TLRs may account for a missing immune deviation of allergen-specific responses from a Th2 to a Th1 profile. On the basis of these concepts, we evaluated whether the recombinant allergen with native conformation and the two fragments differently affected the expression of TLR2 and TLR4 innate immune receptors. We demonstrated that the 1–55 fragment significantly increased the expression of both TLR2 and TLR4 in PBMC from recruited allergic children. In contrast, the native allergen and the 52–102 fragment did not. Consequently, the 1–55 fragment via the upregulation of innate immune receptors may have the potential to ameliorate the Th2 immune deviation of allergic subjects. To assess whether the expression of these receptors, and in particular of TLR4, was functionally active, we evaluated the binding of LPS. The potential of Th1 inducers, like LPS and other microbial exposures, to prevent or to mitigate allergy and asthma is supported by the findings that the treatment with LPS reduces allergic responses in atopics (24) and that higher house dust endotoxin levels correlated with increased proportions of IFNγ producing cells in the peripheral blood of infants (25). In this context, several epidemiologic studies reported the reduced prevalence of allergy and asthma in rural areas in comparison with urban areas (26) as well as the findings that LPS levels were inversely related to hay fever, atopic asthma and atopic sensitization but not to nonatopic asthma in school-age children (9). However, a recent study, evaluating an adult population, has reported that LPS exposure is detrimental for asthma (27). To reconcile this paradox, it is possible to argue that the timing of the LPS exposure, the concentrations of LPS and the presence of atopy determine the beneficial or the detrimental effects of endotoxin (7, 9). As regards, it has been demonstrated that LPS inhibits nasal allergic inflammation, by skewing local immune responses from Th2 to Th1 and by upregulating production of IL-10. All these beneficial effects are mediated by TLR4 and are present in atopic children but not in adults (24). In the present study, we demonstrate for the first time that the exposure to the 1–55 hypoallergenic Par j 2 fragments increased, differently to the native allergen, the LPS binding of PBMC from allergic children with asthma. These findings allowed to identify new advantages to use these compounds for the therapy of allergic disorders in children. Moreover, innate immune cells when stimulated by LPS via the activation of recognition receptors for LPS (i.e. TLR4–CD14 complex) trigger nitric oxide synthase activity (28) and induce a Th1-type T-lymphocyte response (with the preferential production of IFNγ) that promotes IgG production generating a positive feedback loop that, in turn, limits the Th2-mediated responses and the IgE production (22). Here, we provide data concerning the ability of the 1–55 fragment to increase the release of IFNγ by stimulated PBMC. Predominant Th1 responses, via the production of IFNγ, might divert the development of atopy as well as of pathologic remodeling processes in asthma. As regards, it has been demonstrated that IFNγ has the potential to provide a milieu for airway repair without fibrosis (26). While Th2 cytokines (IL-4 and IL-13) stimulate fibroblasts to proliferate and to produce collagen *in vitro* (29), IFNγ counteracts the collagen synthesis (30), and antagonizes transforming growth factor-β activities (31).

Furthermore, we pointed out that between the two tested variants of Par j 2, only the 1–55 fragment has the potential to divert the immune system of Par j 2 allergic children toward a more balanced equilibrium between Th2 and Th1 responses. It is possible to speculate that the epitopes of the 1–55 fragment are preferentially recognized by professional APCs which, in turn, upregulate the expression of innate immunity receptors and increase the LPS binding and IFNγ release. All these intrinsic properties of this fragment may be clinically relevant to improve the safe and the success of SIT and to re-orientate, in atopics, the immune system toward a physiologic balance between Th1 and Th2 responses.

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