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The major allergen of the *Parietaria* pollen contains an LPS-binding region with immuno-modulatory activity

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Abstract

Background: The major allergens in *Parietaria* pollen, Par j 1 and Par j 2, have been identified as lipid transfer proteins. The family of the Par j 1 allergens is composed of two isoforms, which differ by the presence of a 37 amino acid peptide (Par37) exclusive to the Par j 1.0101 isoform. The goal of this study was to elucidate the biological properties of the Par37 peptide.
Methods: *In silico* analysis, spectrofluorimetric experiments and *in vitro* cell culture assays were used to identify the biological properties of Par37. In addition, a mouse model of sensitization was used to study the influence of Par37 in the murine immune response.
Results: *In silico* analysis predicted that Par37 displays characteristics of a host defence peptide. Spectrofluorimetric analysis, real-time PCR and ELISA assays demonstrated that Par37 possesses an LPS-binding activity influencing cell signalling *in vitro*. In RAW264.7 cells, LPS-induced IL-6 and TNF-α transcription and translation were inhibited after preincubation with Par37. Consistent with these data, inhibition of IFN-γ secretion was observed in murine spleen cells and in human PBMC. Finally, mice immunized with the two Par j 1 isoforms differing in the presence or absence of the Par37 peptide showed different immunological behaviours *in vivo*.
Conclusions: This study demonstrates that the Par j 1.0101 allergen displays LPS-binding activity due to the presence of a 37 amino acid COOH-terminal region and that this region is capable of influencing cytokine and antibody responses *in vitro* and *in vivo*.

Type I hypersensitivity is the clinical manifestation of an aberrant immune response against protein molecules, commonly known as allergens, which are potent inducers of immunoglobulin E (IgE) synthesis (1).

Although a large number of allergens have been characterized in recent years, there are little data concerning the functional features that these molecules have in common and could explain their ability to provoke powerful IgE antibody responses (2). For a large number of allergens, their biological functions have been determined (3), showing that some of their activities can contribute to their allergenicity [see (4) for a review]. In the last few years, there has been some evidence that allergenicity may reside in the ability to activate different innate immune pathways at the mucosal surface, suggesting that this early event may represent a crucial step for the pathological onset of the disease (5). One example is the group 2 major house dust mite (HDM) allergens, which show structural homologies to MD-2 (6,7) and the Der p 7 HDM allergen that presents structural homology to another family of innate immune proteins (LPS-binding proteins) that bind various hydrophobic ligands and are involved in the TLR4 pathway (8).

However, the reason why some proteins tend to function as allergens in genetically predisposed subjects is a question that remains fundamentally unanswered and is of paramount

importance for understanding the mechanism involved in the onset of the allergic reaction.

In plants, lipid transfer proteins 1 (LTP1) display a wide range of functional properties (9–13). In the last decade, LTPs from pollen and plant derived foods have attracted new interest as major IgE inducers in humans (see (14) for a review) via the respiratory and gastrointestinal tracts (15). These proteins, together with other plant protein families (cereal prolamins, α-amylase/trypsin inhibitors, 2S albumins, puroindolines, etc.) constitute the prolamin superfamily, the largest group of allergenic proteins in plants (16).

Parietaria (Pj) is one of the major sources of allergens in the Mediterranean area whose allergenic composition has been studied in detail identifying three independent families of proteins with allergenic activity: LTPs (Par j 1 and Par j 2), calcium-binding protein (Par j 4) and profilin (Par j 3) (17). Among them, LTPs represent the major elicitors of the IgE response in humans (18). By molecular cloning, it has been demonstrated that this pollen contains at least three LTP isoforms with allergenic activity (17, 19, 20). In particular, the Par j 1 allergen family is composed of Par j 1.0101, a protein of 139 aa (21), as well as a shorter variant, Par j 1.0201, composed of 102 aa (22). The coding regions of the Par j 1.0102 and Par j 1.0201 isoforms show 95% identity at the amino acid level within the first 97 amino acids. Overall, the two isoforms differ by the presence of a 37 amino acid COOH-terminal tail in the Par j 1.0101 allergen.

Based on this, the aim of this study was to investigate the biochemical characteristics of the COOH-terminal region of the Par j 1.0101 allergen. The potential of such a region to modulate *in vitro* and *in vivo* immune responses was also explored.

Materials and methods

In silico analysis

Amino acid analysis of the C-terminal region of the Par j 1.0101 allergen (named Par37) was performed using the prediction software of the antimicrobial peptide database (APD) (http://aps.unmc.edu/AP/main.php)(23).

The synthetic peptide covering the carboxyl-terminal region of the Parj1.0101 allergen (from amino acids 103 to 139) used in the present work was prepared by ChemPep (Miami, FL, USA) (Par37 LPVSLRHGPVTGPSDPAHKAR LERPQIRVPPPAPEKA). An unrelated peptide (the NH2 terminal region of Par j 1.0101 from aa 10 to 35) was used as a negative control.

Dansyl polymyxin B binding inhibition experiments

Fluorescence experiments were carried out on a JASCO FP 6500 Spectrofluorimeter. Dansyl polymyxin B (DPX) was purchased from Molecular Probes (INVITROGEN, Milan, Italy). Binding assays were performed by recording the fluorescence emission spectra of samples containing DPX [2.5 mM] and after the addition of LPS [1 μg/ml] (*E. coli,* strain 026:B6, SIGMA, Milan, Italy). Finally, the fluorescence intensity was measured after the addition of increasing amounts (2.5, 5, 12.5, 25 and 50 mM) of Par37. Further details are provided in the supporting information section.

RNA preparation and RT-PCR

The mouse macrophage-like cell line RAW264.7 was cultured in RPMI-1640 medium (Invitrogen-Gibco, Milan, Italy) containing either LPS alone [1 μg/ml] or LPS preincubated with the Par37 peptide [2.5 mM] in 10 μl of medium at R.T for 10 min. The cells were then cultured for 2 h (for transcription assay) or 6 h (for ELISA assay) at 37°C under 5% CO_2. Primers for murine IL-6, TNF-α and HPRT (internal control) were obtained from SABioscience (QIAGEN, Milan, Italy). Details for cell culture, RNA preparation and RT-PCR analysis are provided in the supporting information section.

ELISA

Supernatants from the mouse macrophage-like cell line RAW264.7 cultured as described above were collected and stored at −20°C for cytokine analysis. Levels of IL-6 and TNF-α cytokines were determined by sandwich ELISA kits, according to the manufacturers' instructions (BD Biosciences Pharmingen, San Jose, CA, USA). The sensitivity of each assay was 4 pg/ml (IL-6) and 8 pg/ml (TNF-α).

Recombinant proteins for mouse immunization

Recombinant allergens were expressed as His-tagged proteins (pQE30 vector) in the M15 strain (QIAGEN, Milan, Italy). Induction and purification protocols were conducted as previously described (24). Recombinant proteins were tested for endotoxin content using the Multi-test Limulus Amebocyte Lysate (LAL) pyrogen plus test (Bio-Whittaker, VWR International, Milan, Italy) (the endogenous endotoxin content was $\leq$0.003 ng LPS/μg of recombinant protein). Further details are provided in the Supporting information section.

Immunization protocol

Eight-week-old female Balb/c mice (Charles River, Milan, Italy) (n = 10) were injected intraperitoneally (i.p.) with 2 μg/mouse of purified rPar j 1.0101 or rPar j 1.0201 as previously described (25) Mice were bled from the retro-orbital venous plexus on day 28. Individual sera were stored at −20°C until analysis. On day 35, mice were killed to collect spleen for cell response analysis. Further details are provided in the supporting information section.

Measurements of antigen-specific antibody and cell responses

Antibody responses by individual mice against the two recombinant allergens were monitored by ELISA as previously described (25). Briefly, plates (Greiner Bio-One, Frickenhausen, Germany) were coated with either rPar j 1.0101 or rPar j 1.0201 (5 μg/ml). Serum samples were diluted 1 : 1000 for specific IgG1 and 1 : 50 for specific

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A

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          1                                                                   85
Parj1.0101  QETCGTMVRALMVRALMPCLPFVQGKEKEPSKGCCSGAKRLDGETKTGPQRVHACECIQTAMKTYSDIDGKLVSEVPKHCGIVDS
Parj1.0201  EETCGTVVGALMPCLPFVQGKEKEPSKGCCSGAKRLDGETKTGPQRVHACECIQTAMKTYSDIDGKLVSEVPKHCGIVDSKLPPI

          86                                                  139
Parj1.0101  KLPPIDVNMDCKTVGVV**PRQPQLPVSLRHGPVTGPSDPAHKARLERPQIRVPPPAPEKA**
Parj1.0201  DVNMDCKTLGVLHYKGN
```

B

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1                                   37
LPVSLRHGPVTGPSDPAHKARLERPQIRVPPPAPEKA

Molecular Weight = 3918.614 Dalton
Total hydrophobic ratio = 29 %
Total net charge = +3
Percentage of proline residues = 25 %
Protein-binding Potential (Boman index) is: 2.06 kcal/mol
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Figure 1 *In silico* analysis. Panel A shows the amino acid alignment of the sequences of the Par j 1.0101 and Par j 1.0201 isoforms in single letter code. The sequence in bold within the Par j 1.0101 allergen highlights the COOH-terminal region. Panel B shows the properties of the Par37 peptide detected using the algorithm supplied by the antimicrobial peptide database (http://aps.unmc.edu/AP/about.php).

IgG2a evaluation. Results were expressed as OD measured at 490 nm. Proliferation assays were performed on individual spleen cells as previously reported (25), by adding rPar j 1.0101 or rPar j 1.0201 (10 μg/ml) to cultures. The results were expressed as S.I. Further details are provided in the supporting information section.

IFN-γ inhibition assay

Mouse spleen cells from naïve Balb/c mice and PBMC from normal subjects ($n = 4$) were cultured in the presence (positive control) or absence (negative control) of 1 μg/ml of LPS (*E. coli*, strain 026:B6, SIGMA; Milan, ITALY). IFN-γ secretion in the supernatants was determined by ELISA following the manufacturer's instructions (GE Healthcare, Milan, Italy). Further details are provided in the Supporting information section.

Results

Structural analysis

In silico analysis showed that Par37 displays several features common to antimicrobial peptides (23). This domain contains a very high proline content (25%), a net positive charge (+3) and a total hydrophobic ratio of 29%. The protein binding potential of Par37 calculated using the Boman index gave a value of 2,06 kcal/mol (Fig. 1).

Inhibition of Polymyxin B binding

Figure 2 shows the emission spectra obtained from solutions containing DPX+LPS in the presence of increasing amounts of Par37. As shown, the addition of increasing quantities of Par37 in solution caused a gradual quenching of fluorescence, demonstrating that Par37 was capable of displacing the LPS molecules bound to the DPX. At a concentration of Par37 equal to 50 mM, nearly all the LPS molecules were displaced and the intensity was almost restored to its initial value.

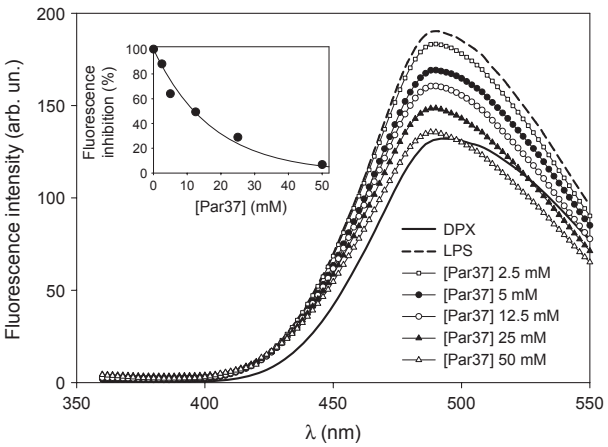

Figure 2 Inhibition of polymyxin B binding. Fluorescence spectra performed on a solution containing 2.5 mM DPX before (solid line) and after (dotted line) addition of LPS (1 μg/ml). Increasing aliquots of Par37 peptide [2.5 mM (empty squares), 5 mM (solid circles), 12.5 mM (empty circles), 25 mM (solid triangles) and 50 mM (empty triangles)] were successively added to the same sample. The percentage of fluorescence intensity inhibition obtained after each addition of Par37 peptide is shown in the inset. The continuous line represents the best fit of the data.

Experiments performed using a control peptide with a different amino acid composition did not shown any decrease in fluorescence intensity as a function of peptide concentration (Figure S1).

Mouse cytokine inhibition assays

Raw 264.7 cells were incubated with LPS and Par37-LPS complexes. Real-time PCR analysis demonstrated that Par37 significantly inhibited LPS-induced IL-6 transcription ($P \leq 0.40$) (Fig. 3 panel A). A similar behaviour was observed for TNF-α expression where a clear trend of inhibition was observed ($P = 0.50$) (Fig. 3 panel B). Using ELISA

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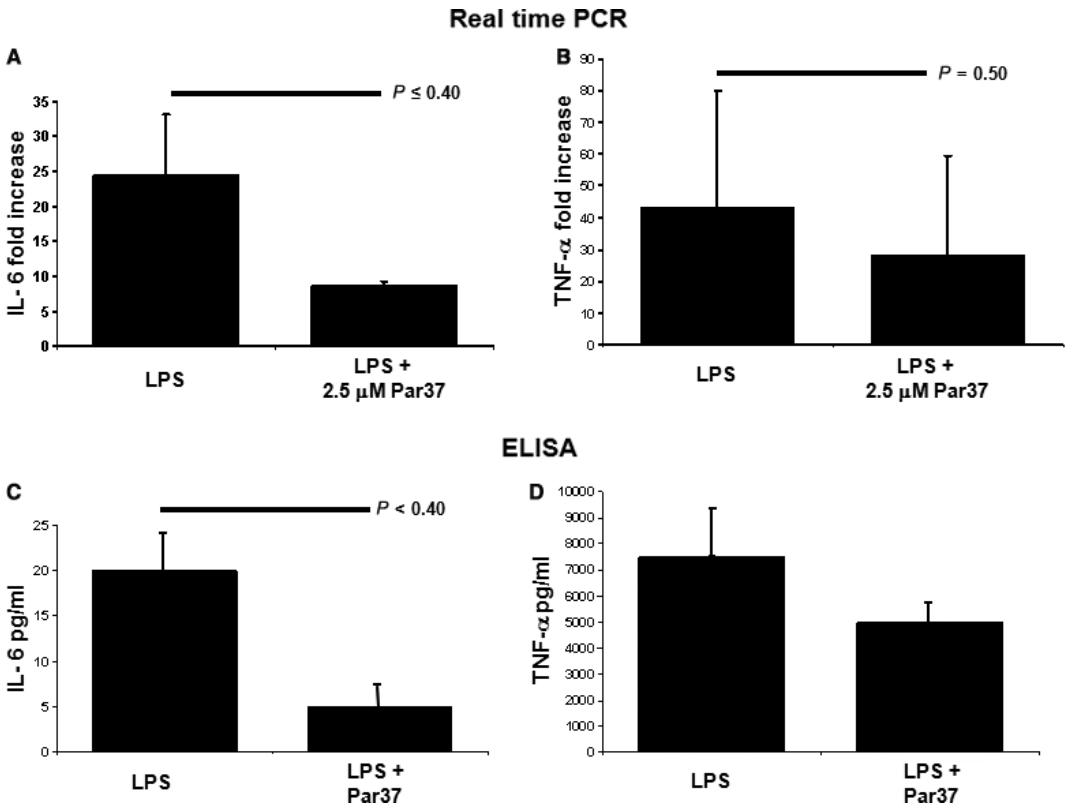

Figure 3 Effects of Par37 on IL-6 and TNF-α transcription and secretion in Raw 264.7 cells. Panels A and B show real-time PCR analysis performed 2 hours after LPS induction. Panels C and D display ELISA assays performed on culture supernatants 6 hours after LPS induction. The values shown are means ± SEM. Statistical analysis was performed comparing the cells treated with 1 ng/ml of LPS versus cells treated with the same concentration of antigen preincubated with LPS-Par37. Bars show statistical significance.

assays, we observed that Par37 preincubation strongly reduced LPS-induced IL-6 production ($P < 0.40$). TNF-α determination also showed reduced production in all the tested samples.

Inhibition of IFN-γ production from human PBMC and murine spleen cells

To determine whether addition of the Par37 peptide has any effect on LPS-induced IFN-γ production, human PBMC and murine spleen cells were stimulated with different premixed LPS-Par37 solutions. Preincubation of Par37 with LPS significantly reduced IFN-γ production in a dose dependent manner when compared to a culture control with LPS alone (Figs 4 and 5). In addition, Par37 alone had no effects on IFN-γ production by mouse spleen cells and human PBMC. These data provide evidence that Par 37 acts efficiently as an inhibitor of the human and mouse immune response to endotoxins.

Mouse antibody and cell responses to rPar j 1 isoforms

Different groups of Balb/c mice were immunized with either the rPar j 1.0101 or rPar j 1.0201 isoforms. Serum IgG subclasses and spleen cell responses were evaluated against both Par j 1 isoforms. Immunization with rPar j 1.0101 induced strong IgG1 and IgG2a responses, which bound both rPar j 1.0101 and rPar j 1.0201 with comparable efficiency in ELISA assays. On the other hand, when mice were immunized with the shorter isoform lacking Par37 (Par j 1.0201), very low levels of IgG1 antibodies and barely detectable IgG2a antibodies were induced, which were in all cases at significantly lower levels than those obtained after rPar j 1.0101 immunization ($P < 0.01$). This highly significant difference was observed for both IgG1 and IgG2a subclasses, and regardless of the antigen used in the ELISA assay (Fig. 6, panels A and B). Slight differences in spleen cell responses were found depending on the antigen used in the proliferation assay. In particular, when rPar j 1.0101 was added to *in vitro* re-stimulated immune spleen cells, significantly higher S.I. values ($P < 0.05$) were observed in rPar j 1.0101-immunized mice compared with rPar j 1.0201-immunized animals (Fig. 6, panel C). Conversely, re-stimulation with the shorter rPar j 1.0201 isoform generally induced lower S.I. levels, but they were comparable in both groups of mice (Fig. 6, panel C).

Discussion

The mechanisms by which a subject becomes sensitized to certain proteins rely on a series of interactions within the immune

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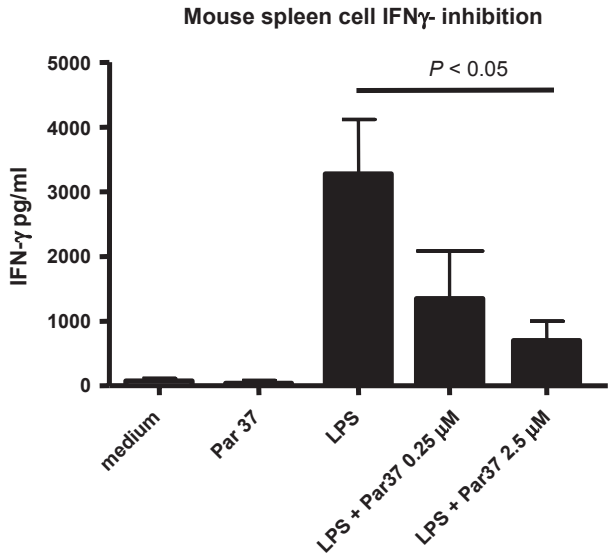

Figure 4 Mouse cytokine inhibition assays. INF-γ cytokine release by spleen cells from naïve Balb/c mice after *in vitro* stimulation under different conditions: no stimulus, Par37 alone, LPS alone and LPS after preincubation with increasing concentrations (0.25 and 2.5 μM) of the Par37 synthetic peptide. Preincubation with the highest concentration of Par37 (2.5 μM) significantly inhibited IFN-γ release ($P < 0.05$). Bars represent mean ± SEM of data from four independent experimental replicates.

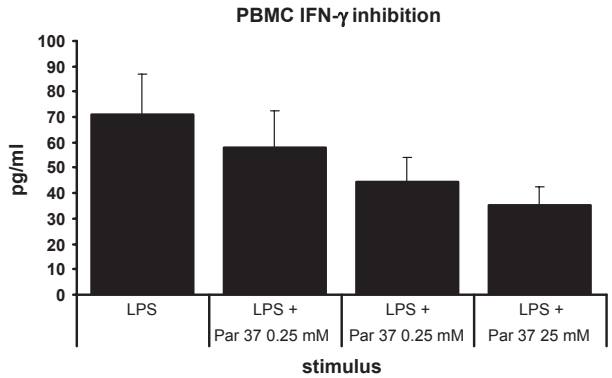

Figure 5 Human IFN-γ cytokine inhibition assay. ELISA assay inhibition of IFN-γ cytokine release by PBMC from healthy subjects after stimulation with LPS or LPS preincubated with increasing concentrations of the Par37 synthetic peptide. The values on the *x*-axis indicate the relative concentrations of the antigens used. Values on the *y*-axis indicate the amount of cytokine released in response to the treatments. Bars show the limits of statistical significance.

system, which are not well understood. In addition to genetic factors, different triggering agents in the environment may be responsible for the induction of the allergic reactions.

In this study, we focused our attention on the characterization of a region of the major *Parietaria* Par j 1.0101 allergen, which has not been found in any other plant LTP. This COOH-terminal region (named Par37) presents biochemical features of peptides involved in host defence including a high percentage of proline residues, a high ratio hydrophobic residues and a net positive charge. When isolated from the full-length allergen and used as a synthetic peptide, the Par37 peptide showed LPS-binding activity and was capable of influencing LPS-mediated signalling *in vitro*. By means of real-time PCR and ELISA assays, we were able to show that LPS-induced IL-6 and TNF-α expression were inhibited at both the transcriptional and translational level in the RAW264.7 cell line after preincubation with Par37. This inhibitory effect was confirmed when spleen cells from naïve Balb/c mice and human PBMC were examined for their ability to secrete IFN-γ after LPS induction. In these experimental settings, the Par37 peptide showed an ability to reduce cytokine production in a dose responsive manner. No cytokine production was induced by the peptide *per se* either in the murine or in the human assays. Taken together, these data demonstrated that the Par 37 peptide can bind and sequester LPS interfering with the physiological response to endotoxin.

The identification of an LPS-binding region within the Par j 1.0101 allergen raises the question whether this binding can modulate the immune response. For instance, a recently published paper reported that the presence of a very low level of contaminating LPS can influence the immune response to HDM (26). To further understand the immunological properties of the Par37 peptide, Balb/c mice were injected i.p. with either of the two isoforms of the Par j 1 allergen characterized by the presence (Par j 1.0101) or absence (Par j 1.0201) of the Par37 peptide. Immunization studies demonstrated that the Par j 1.0201 isoform did not give rise to detectable levels of IgG antibodies capable of recognizing both rPar j 1 isoforms. Accordingly, only immunization with the rPar j 1.0101 (encompassing the Par37 sequence) was able to induce an antibody response and spleen cell proliferation in response to *in vitro* re-stimulation with the same antigen. Starting from the observation that both allergens were tested for LPS contamination, we cannot address the question whether this behaviour is associated with the Par37's biochemical characteristics or its ability to bind tiny undetectable amounts of LPS. Another future question to elucidate will be to understand whether the Par j 1.0101 isoform may have immunological effects different from those induced by Par j 1.0201 in clinical settings.

In conclusion, the data reported in this study demonstrate that one isoform of the major *Parietaria* allergen (Par j 1.0101) displayed LPS-binding activity due to the presence of a short COOH-terminal domain and that this region showed immune modulatory activity influencing cytokine and antibody responses *in vitro* and *in vivo*.

Conflict of interest

The authors have not received funding or sponsorship that impacts on this study. There is no conflict of interest.

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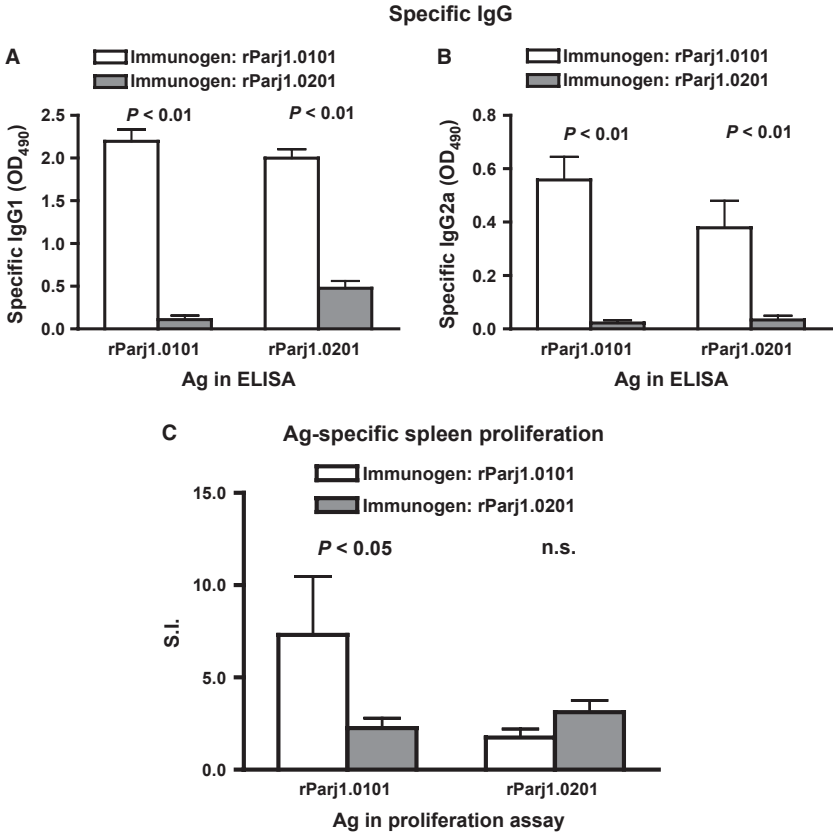

Figure 6 Mouse antibody and cell responses to rPar j 1 isoforms. Panels A–B: Specific IgG1 and IgG2a responses in sera from mice immunized with rPar j 1.0101 (white histograms) or rPar j 1.0201 (grey histograms), evaluated by ELISA assays to rPar j 1.0101 and rPar j 1.0201 at the time of peak response (d. 28). Results are expressed as mean OD_{490} values of individual sera (mice $n = 8$) ± SEM. Statistical significance is indicated by *P* values in the Figure. Panel C: Proliferation of spleen cells from mice immunized with rPar j 1.0101 (white histograms) or rPar j 1.0201 (grey histograms), stimulated *in vitro* with rPar j 1.0101 and rPar j 1.0201. Results are expressed as mean stimulation index (S. I.) ± SEM, obtained from eight individual mice. Statistical significance is indicated by *P* values in the Figure.

Author contributions

PC and AB designed and performed the *in vitro* experiments; GDF, SC and ES performed the experiments with mice; DG performed the fluorescence experiments; FG contributed to the experimental design; PC and GDF wrote the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Inhibition of the Polymyxin B binding. Solid lines: DPX alone; dashed lines: DPX with LPS; empty and full triangles: control PEP3 peptide at the concentrations of 25 and 50 mM, respectively.

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