



Lig v 1 structure and the inflammatory response to the Ole e 1 protein family

To the Editor,

Pollen grains of the Oleaceae family are a widespread source of respiratory allergens.¹ The major allergen from these pollen grains is Ole e 1 homologues, including Ole e 1 (olive), Fra e 1 (ash), Lig v 1 (privet) and Syr v 1 (lilac) with identity among them above 83%. Additionally, other species produce members of this protein family with an average 30% identity with Oleaceae allergens, including Pla l 1 (English plantain), Che a 1 (lamb's quarter), Phl p 11 (Timothy grass) and Sal k 5 (Russian thistle).¹⁻³ The Ole e 1 protein family promotes the production of specific IgE causing the development of type I allergies towards various pollen sources thanks to cross-reactivity. To date, 16 Ole e 1-like proteins are reported as allergens.²

We have determined the crystal structure of the major allergen from privet³ Lig v 1 (PDB 6YOA), which unexpectedly forms a homodimer (Figure 1A,B). Recombinant Lig v 1.0102 (rLig v 1) was produced as a glycosylated, non-tagged soluble protein in *Pichia pastoris* with the vector pPICZα/LIG1³; it was purified and characterized before and after enzymatic de-glycosylation with PNGase F. Protein crystals were grown with pure rLig v 1 and analysed with X-rays; additionally, it was immunodetected by WB with anti-*Fraxinus* polyclonal antibodies⁴ and IgE from patients serum (Figure S1). CD spectrophotometry revealed Lig v 1 comprised 40% β-sheet and 60% irregular structure and revealed the protein was folded and thermostable up to 60°C (methods and results at Supporting Information).

The structure of the homologue Pla l 1 (PDB 4Z8W) showed the Ole e 1-like protein family comprises a 7-strand β-barrel fold stabilized by three conserved disulphide bonds.² When the crystal structures of Lig v 1 dimer and Pla l 1 are compared, the β-barrel and three disulphides bonds are conserved (Figure S2). Additionally, rLig v 1 has a C-terminal α-helix and significant differences in the loop structures at the N-terminal end of the β-barrel (Figure 1E) and the dimerization interface is different from that proposed to lead to oligomerization in Pla l 1. This is the first structure of the C-terminal region which is helical, a region shared by the family paradigm Ole e 1 which is a major epitope, as are the N-terminal loops.⁵ The loops 3, 5 and 7 correspond to the connections between β2-β3 (residues 45-54),

β4-β5 (residues 68-76) and β6-β7 (residues 107-119), respectively (Figure 1C,F). Loop 7 is the longest and has the greatest difference in conformation to the corresponding loop in Pla l 1. The determined Lig v 1 structure provides knowledge of both loop conformation and flexibility. Lig v 1 forms a dimer with approximately twofold symmetry in the crystal (Figure 1A,B), but it is unclear if this is of functional significance. Ole e 1 subunits can polymerize and form dimers with higher allergenicity.⁶ Comparison of the structure of the loops between the two copies in the crystal reveals striking divergence in the conformation of the loops, highlighting the high flexibility and conformational heterogeneity present in the loop structures of the Ole e 1 family (Figure 1F,G). Although the residue Asn111 is glycosylated (Figure S1), there is little evidence of glycosylation in the electron density map because this residue is in a highly solvent-exposed position that provides no steric constraint in the crystal on the orientation of flexibility of the carbohydrate.

The biological function of the Ole e 1 family proteins is unknown and the search for similar structures to Lig v 1 using DALI server found Pla l 1 as the most similar. Lower sequence identities were detected with fibrinogen binding protein from *Staphylococcus aureus* (4JDZ), and the collagen adhesion protein from the major pilin subunit of *Bacillus cereus* (3RKP, 3RKT), at 16% and 14%, respectively. The structural similarities include a central barrel connected by loops, consistent with a binding function, supporting the proposal that the Ole e 1 family orchestrate pollen tube assembly. A review of the literature suggests Ole e 1 is involved in pollen tube development and Pla l 1 is proposed to have a role in the modification of the cell wall in the extracellular space. The initiation of the pollen tube development starts with calcium flux into the pollen grain and it is likely that the Ole e 1 proteins interact with carbohydrates in the plant cell and orchestrate pollen tube growth, or they may simply fix the pollen grain in position while the pollen tube grows which is consistent with polymerization proposed for Pla l 1.^{2,6}

Recent studies showed the surfactant activity of Ole e 1 on epithelial cells⁷ suggesting the biology of Ole e 1-proteins could influence the development of allergy. Hence, we aimed to establish the effect of Lig v 1 in the activation of the inflammatory response by the innate immune system in the second part of this work. Peripheral blood mononuclear cells (PBMCs) isolated from randomly selected healthy donors were stimulated with rLig v

Abbreviations: CD spectroscopy, circular dichroism; FACS, fluorescence-activated cell sorter; NF-κβ, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR, Toll-like receptor; TNFα, tumour necrosis factor alpha; TRIF, TIR-domain-containing adapter-inducing interferon-β; WB, Western blot.

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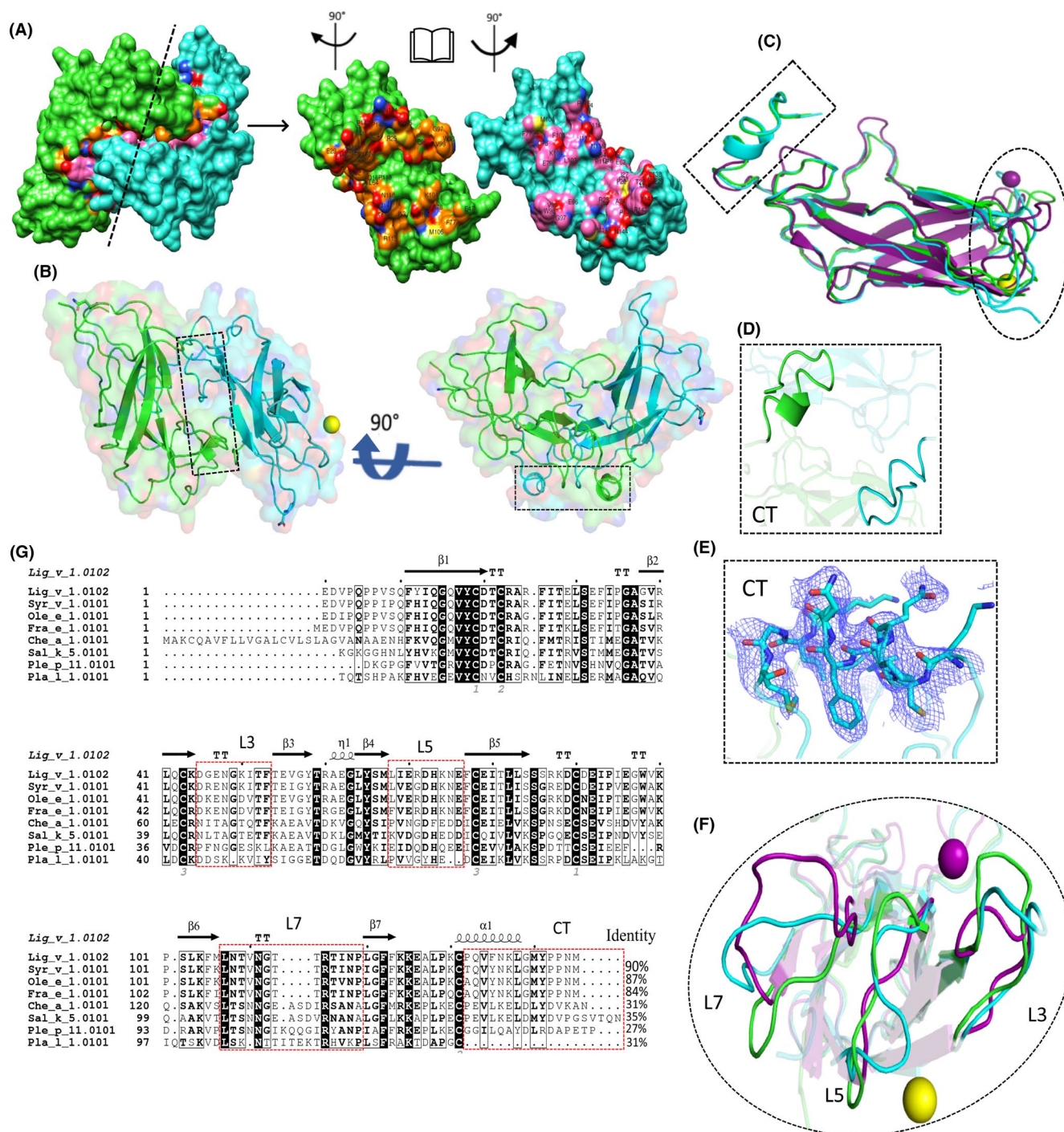
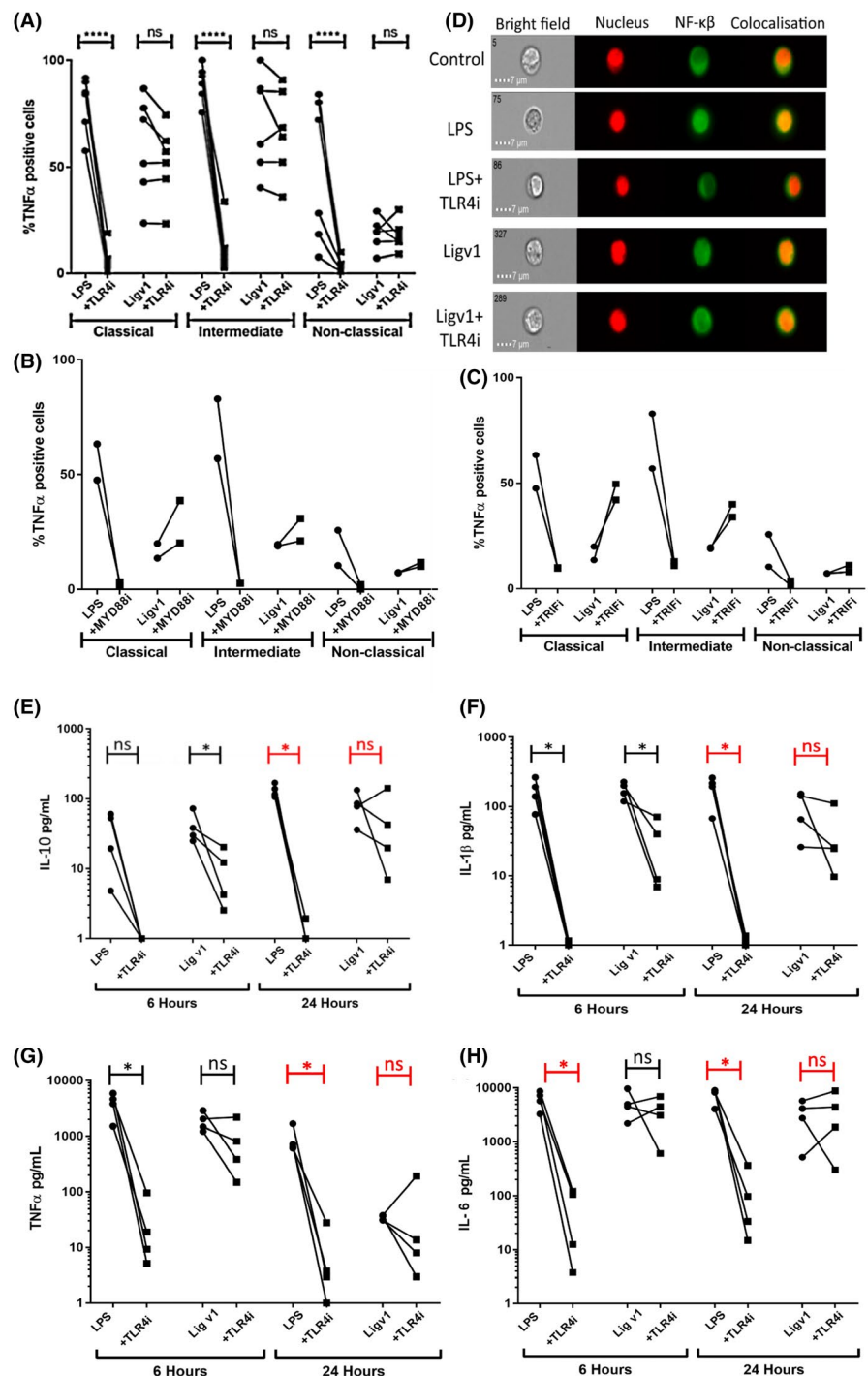


FIGURE 1 Three-dimensional structure of Lig v 1 dimer at a nominal resolution of 2.8 Å. A, Lig v 1 dimer with chain A (green) and B (teal). Residues involved in dimer formation are identified in “open book” representation with residues in chains A and B in orange and pink, respectively. Side-chain atoms are coloured with oxygen, nitrogen and sulphur, red, blue and yellow, respectively. B, Lig v 1 cartoon with Chain A (green) and B (teal) C-terminus α -helix framed, and glycosylated residues are represented as sticks (Asn111). C, Structural alignment of Lig v 1 Chain A (green), B (teal) with bound nickel-ion (yellow sphere) and Pla I 1 (purple) bound to Zn²⁺ (purple sphere), (D) Lig v 1 C-terminus close-up. E, Lig v 1 C-terminus Chain B fitted in the density map σ 1.0. F, Structural alignment of Lig v 1 monomers and Pla I 1 loops 3(L3), 5(L5) and 7(L7). G, Sequence alignment of Lig v 1 and other Ole e 1 protein family members, the red squares show the sequences for loops L3, L5, L7 and C-terminus in the sequences

1 for 3 hours, detailed methods and analyses are described in Supporting Information. In brief, cells were stained with fluorochrome-conjugated monoclonal antibodies to surface molecular markers (HLA-DR, CD14, and CD16), and intracellularly stained

for TNF α (flow cytometry experiments) or NF- κ B (co-localization experiments). FACS data were analysed with WinList software (version 7.1; Verity Software House), and NF- κ B was analysed with IDEAS software (Amnis Co.), using the co-localization wizard.

FIGURE 2 Proinflammatory response activated by Lig v 1. PBMCs stimulation with 1.5 $\mu\text{g/mL}$ Lig v 1 or 0.1 ng/mL LPS in medium alone or in the presence of inhibitors of TLR4 (A), MyD88 (B) or TRIF (C). Intracellular TNF α production by classical, intermediate and non-classical monocytes was determined by flow cytometry. D, Trans-localization of NF- κB to the nucleus of non-classical monocytes, after 3 hours stimulation with 3 $\mu\text{g/mL}$ Lig v 1 or 0.1 ng/mL LPS. Co-localization of the nucleus (red) and NF- κB (green) showing NF- κB translocation by Lig v 1 even in the presence of the TLR4 inhibitor. Cells were gated into classical, intermediate and non-classical subsets in all the experiments. The cytokines IL-10 (E), IL-1 β (F), TNF α (G) and IL-6 (H) were detected in cultured media after 6 and 24 hours stimulation with Lig v 1 or LPS. Paired t tests (black) for normally distributed data and Mann-Whitney U tests (red) for non-normally distributed data were used to determine statistical significance: $P < .05$ (*), $P < .01$ (****) and not statistically significant (ns). Each spot represents an experiment, which varied depending on the donor's availability



HLA-DR $^{+}$ cells were gated into classical (CD14 $^{+}$ CD16 $^{-}$), intermediate (CD14 $^{+}$ CD16 $^{+}$) and non-classical (CD14 $^{+/-}$ CD16 $^{+}$) monocyte subsets. Production of TNF α and NF- κB localization were assessed for each monocyte population. Cell incubation with Lig v 1 resulted in translocation of NF- κB into the nucleus (Figure 2D) and the production of TNF α when compared to non-stimulated cells. Importantly, TNF α production was maintained or enhanced in the presence of the specific TLR4 inhibitor CLI-095 (Figure 2A), inhibitors MyD88-dependent TLR signalling (Figure 2B), or TRIF-dependent TLR signalling (Figure 2C) after rLig v 1 stimulation. In contrast, TNF α production stimulated by the TLR4 agonist

Escherichia coli LPS (endotoxin) was completely blocked by both inhibitors as expected (Figure 2). Therefore, monocyte activation by rLig v 1 is TLR independent and cannot be attributed to endotoxin contamination. Activation with Lig v 1 also induced secretion of IL-10 (Figure 2E), IL-1 β (Figure 2F), TNF α (Figure 2G) and IL-6 (Figure 2H) after stimulation of PBMCs with Lig v 1 for 6 or 24 hours, although IL-33 and IL-12p(70) were not detected (Supporting Information). The activation of innate immune cells independently of TLRs has been previously reported for other allergens,^{8,9} but this is the first time this pathway has been explored with the Ole e 1 family proteins.

These results show for the first time the ability of Lig v 1 to activate innate immune cells. To understand better the contribution of these effects to allergy, it would be interesting in future to compare Lig v 1 with other Ole e 1-like proteins and to analyse responses from allergic individuals as well as healthy donors.

KEYWORDS

activation of innate immune system, crystal structure, Lig v 1, Ole e 1 allergy, Ole e 1-like proteins

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING INFORMATION

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Tania Robledo Retana¹ 
Jack Bradley-Clarke¹ 
Tristan Croll² 
Ruth Rose³ 
Inva Hoti⁴ 
Andrew J. Stagg⁴ 
Mayte Villalba⁵ 
Richard W. Pickersgill¹ 

¹Department of Biochemistry, School of Biological and Chemical Sciences, Queen Mary University of London, London, UK

²Cambridge Institute for Medical Research, Cambridge, UK

³Protein Production Facility, School of Biological and Chemical Sciences, Queen Mary University of London, London, UK

⁴Centre for Immunobiology, Blizard Institute, Queen Mary University of London, London, UK

⁵Biochemistry and Molecular Biology I, Complutense University of Madrid Faculty of Chemical Sciences, Madrid, Spain

Correspondence

Richard W. Pickersgill, School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK.

Email: r.w.pickersgill@qmul.ac.uk

ORCID

Tania Robledo Retana  <https://orcid.org/0000-0002-9879-101X>
Jack Bradley-Clarke  <https://orcid.org/0000-0001-8015-4779>
Tristan Croll  <https://orcid.org/0000-0002-3514-8377>
Ruth Rose  <https://orcid.org/0000-0001-6406-137X>
Inva Hoti  <https://orcid.org/0000-0002-4685-5712>
Andrew J. Stagg  <https://orcid.org/0000-0001-6588-0844>
Mayte Villalba  <https://orcid.org/0000-0002-0042-9953>
Richard W. Pickersgill  <https://orcid.org/0000-0001-6600-2381>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

On-line Materials and Methods

Production of Lig v 1: Lig v 1.0102 insert with clone L10 was excised from the vector PUC/LIG1¹ and re-cloned into pPICz α . The resulting vector was used to transform *E. coli* DH5 α and the vector pPICz α /LIG1 vector was purified using the Maxiprep protocol (Qiagen). *Pichia pastoris* cells strain KMH17 (Thermofisher) were transformed with linearized pPICz α /LIG1 by electroporation with a gene pulser Xcell (BioRad)¹. Cells were screened for protein expression as recommended². Finally, soluble Lig v 1 was secreted into the media after 48 hrs induction with 5% methanol at 28°C and 250 RPM.

Lig v 1 purification: Media containing Lig v 1 was dialysed against 20 mM ammonium bicarbonate pH 8 overnight and separated by size exclusion chromatography with a S200 column attached to an ÄKTA FPLC machine (GE healthcare). The immunodetection of rLig v 1 was performed by WB after transferring the proteins into a PVDF membrane (GE Healthcare) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad), according to the user's manual with a current of 25 V applied for 30 minutes. The membrane was blocked with 5% skimmed milk-TBS. Washes were performed with TBS-T between incubations, rLig v 1 was detected with polyclonal rabbit-anti-*Fraxinus*³ and goat-anti-rabbit IgG conjugated to AP (Thermofisher) as secondary antibody, and revealed with Novagen substrate (Fig. S1 C1). Furthermore, Lig v 1 glycosylated and non-glycosylated were detected with a pooled serum from mono-sensitized patients to *Ligustrum* as the primary antibody (kindly donated by Dr. Terán's group from the National Institute for Respiratory Diseases in Mexico), and rabbit-anti-IgE conjugated to streptavidin as secondary antibody, revealed with HRP (Pierce) as shown in Figure S1 C2-3.

A final purification was performed with endotoxin removal columns (Pierce), and purified Lig v 1 was tested for endotoxin contamination with Etodate kit (Sigma), by following the vendor's instructions before its use in cell activation experiments.

Circular Dichroism: Tests were performed in 1 mm path length fused silica cuvette with a Jasco J-715 spectropolarimeter and a PTC-348WI temperature controller. The data obtained for 3 readings was averaged and corrected according to the baseline by using Chiriscan platform (Applied Photophysics Ltd.), resulting data were analysed and deconvoluted with CAPITO software⁴.

Mass-spec: Lig v 1 sugar was removed with PNGase F (Promega) for 2 hrs and analysed by SDS-PAGE, the band was excised from the gel and sent to Cambridge Centre for Proteomics for sequencing. It was trypsin digested and analysed with MALDI-TOF. Analysis with Mascot (Matrix Science, London, UK; version 2.5.0) and Scaffold (version Scaffold 4.3.0, Proteome Software Inc., Portland, OR) was performed to validate MS/MS-based protein identifications.

Crystallisation and crystal analysis: Lig v 1 crystals were grown at 17 °C for 7 months with the hanging-drop vapour equilibration technique. Using 3 µl drops including 1.5 µl Lig v 1 (20 mg/ml) and 1.5 µl of reservoir containing 0.1 M Tris pH 8.5, 1 mM NiCl₂, 25% PEG MME 2000, equilibrated with 500 µl of reservoir.

Lig v 1 crystals showed a very asymmetric shape with 0.1- 0.2 mm on edge and 3 mm in length, stored with 40 % glycerol as cryoprotectant and vitrified by plunging into liquid nitrogen. Crystals were stored in liquid nitrogen before transfer to a dry shipper sent to the Diamond synchrotron, UK. Using the DLS beamline IO3, data collection and reduction with Xia2-DIALS, molecular replacement was performed using a Lig v 1 structure predicted by Phyre2⁵ and Phenix⁶ software. Structure refinement was achieved with Phenix, COOT, and ISOLDE⁶⁻⁸; structure data and refinement statistics are shown in Table E1.

The PDBePISA analysis from the EMBL-EBI website, showed the two protein chains in the asymmetric unit show a solvent content of 58% with Matthew's coefficient of V_m of 2.93. The packing of these two chains in the asymmetric unit suggests that Lig v 1 may be a homodimer. The residue interactions between the two chains that form the dimer with a surface area of 14110 Å² and a buried area of 3360 Å² suggests the dimer is stable in solution.

Chain B was used to predict models of Ole e 1 (Olive) and Fra e 1 (Ash) with Phyre 2⁵, as they are considered major allergens from the Oleaceae plant family. Showing Lig v 1 could be a better model to predict the structure of Ole e 1 and Fra e 1 allergens (Fig S4).

Peripheral Blood Mononuclear Cells (PBMCs) isolation: Blood was obtained from randomly selected healthy subjects who gave informed written consent, into 10 ml vacutainers with heparin. PBMCs were isolated by density gradient centrifugation with Ficoll-paque as previously described⁹. Cell incubations were performed at approximately 2 million cells/ml with complete Dutch media including modification of RPMI-1640 (Sigma-7638), 10 % heat-inactivated Fetal calf serum (Sigma), 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin.

PBMCs stimulation: cells were incubated in 24-well plates (Corning) at 37 °C in a CO₂ incubator. For the intracellular detection of TNFα or NF-κβ experiments, the sheddase ADAM17 was inhibited by adding 3 µM monensin and 50 µM TMI-005 to all the wells and thereby maintain CD16 expression. Inhibitors (5 µM CLI-095 TLR4 inhibitor, 50 µM Pepinh-MYD88, or 50 µM Pepinh-TRIF) were added to relevant wells 30 min before stimulation. Control cultures were incubated without stimulants, stimulation was performed for 3 hours with 1.5 µg- (TNFα experiments), 3 µg- (NF-κβ co-localisation) -Lig v 1 or 0.1 ng/ml LPS¹⁰. Each point in the graph represents different patients, and the different numbers of patients in the experiments are related to the donor's availability.

For the cytokine expression, cells were incubated with 5 μ M CLI-069 TLR4 inhibitor in specified wells and stimulated with 1.5 μ g/ml Lig v 1 or 0.1 ng/ml LPS for 6 and 24 hrs. Each point of the graph represents a repetition of the experiment with 2 different donors.

Antibody labelling and Flow cytometry: Activated cells were stained for extracellular markers: anti-CD16-PerCP-Cy5.5, anti-CD14-Pacific Blue, and anti-HLA-DR-APC (BD Biosciences) and fixed with Leucoperm A (AbD Serotec). Cells were incubated with anti-TNF α -PE (BD Biosciences) in the presence of the permeabilization agent Leucoperm B (AbD Serotec)^{10,11}.

TNF α identification: Samples were acquired using the Fluorescent Activated Cell Sorter (FACS) Canto II (BD Biosciences) and the FACS data was analysed with WinList software. HLA-DR⁺ cells were gated into classical (CD14⁺ CD16⁻), intermediate (CD14⁺ CD16⁺) and non-classical (CD14⁺/CD16⁺) monocyte subsets. Cell activation was estimated for each subset, with the comparison of control (non-activated) cells and stimulated ones¹⁰ as shown in Figure S3.

Co-localisation of NF-k β in monocytes: Activated cells were stained for extracellular molecular markers as above. Intracellular NF-k β was stained with the Amnis® NF-kB Translocation kit (Merck) according to the vendor's protocol. Data were acquired with the Amnis imagestream MKII flow cytometer and analysed with the IDEAS software (Merk). HLA-DR cells were gated into classical, intermediate and non-classical as before, and images were produced using the co-localization wizard.

Multiplex ELISA: Medium from PBMCs cultures stimulated with Lig v 1 or LPS were analysed with a Legendplex (BioLegend) Mix and Match panel designed to measure levels of IL-1 β , IL-6, IL-12p(70), IL-10, IL-33, and TNF α detection following the vendor's instructions. Data were acquired with the LRSII flow cytometer and analysed with the Legendplex (BioLegend) software provided with the kit¹².

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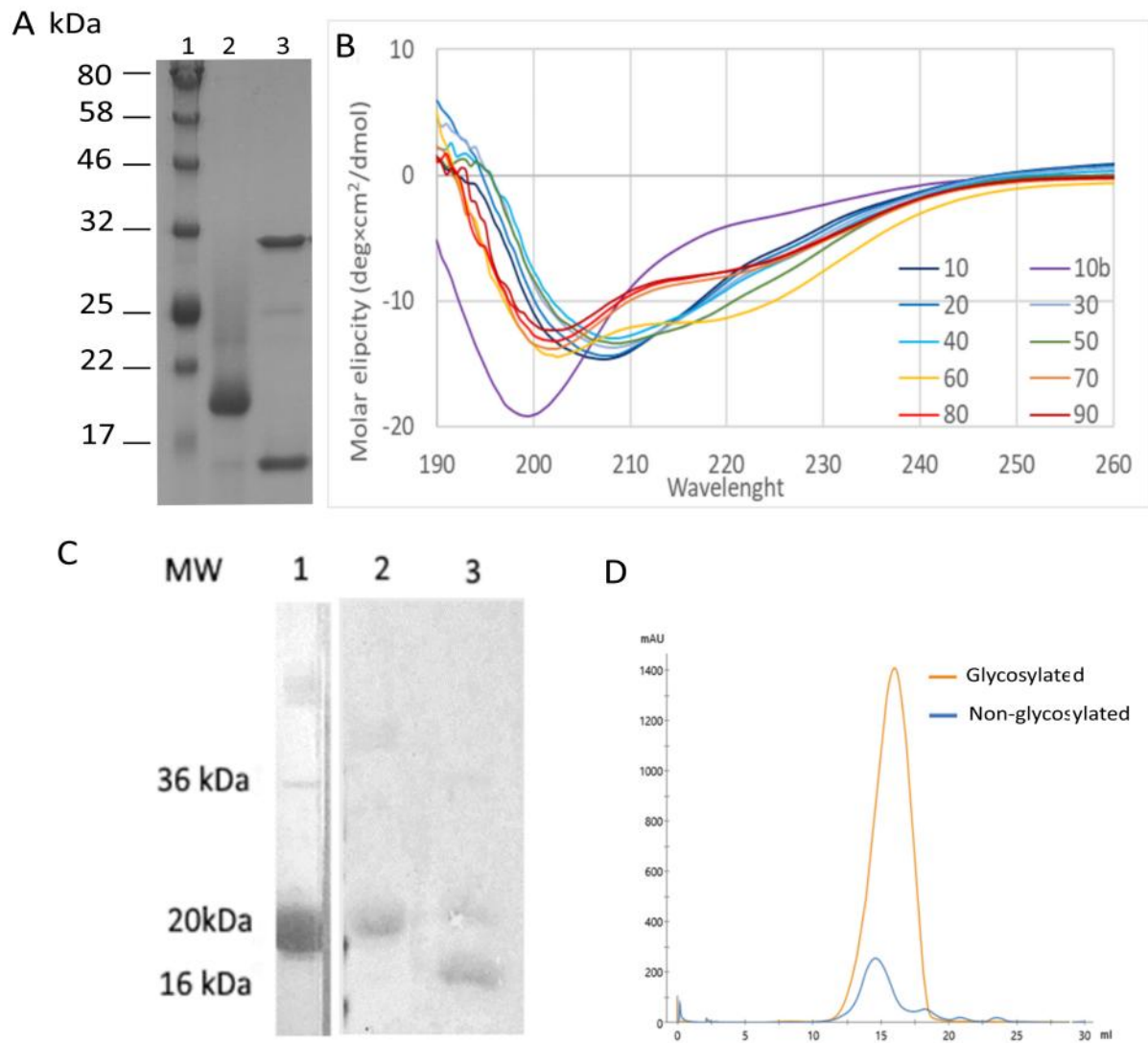


Figure S1. rLig v 1 characterisation. A) SDS-PAGE. Lanes 1) Ladder. 2) Glycosylated rLig v 1 (20 kDa), 2) Non-glycosylated rLig v 1 (16 kDa) and PNGase F (30kDa). B) CD spectra from 260- 190 nm at increasing temperatures (10-90°C) and second spectra recorded at 10°C. Each spectrum is the average of three readings. C) Lig v 1 identified by Western Blot. Lanes: MW) molecular weight. 1) Detection with rabbit polyclonal anti-*Fraxinus* and goat anti-rabbit IgG as secondary antibody. 2-3) rLig v 1 detected with serum from mono-sensitised patients to *Ligustrum* and anti-human IgE as

secondary antibody; for glycosylated (2) and de-glycosylated (3) forms. D) Chromatogram of glycosylated (orange) and non-glycosylated (blue) Lig v 1.

Table E1. Data collection and refinement statistics

Lig v 1	
Data collection	
Space group	P 31 2 1
Cell dimensions	
a, b, c (Å)	76.4842 76.4842 113.498
α, β, γ (°)	90 90 120
Resolution (Å)	38.24 - 2.83 (2.93 - 2.83)
R_{merge}	0.04182 (0.3236)
$I / \sigma I$	6.66 (1.22)
Completeness (%)	99.72 (99.68)
Redundancy	2.0 (2.0)
Refinement	
Resolution (Å)	2.8
No. reflections	9565 (937)
$R_{\text{work}} / R_{\text{free}}$	0.2615 (0.396)/ 0.3193 (0.4886)
No. atoms	2194
Protein	2193
Ligand/ion	1 Nickel ion
Water	
B -factors	86.42
Protein	86.41
Ligand/ion	91.47
R.m.s. deviations	

Bond lengths (Å)	0.009
Bond angles (°)	0.97

These data were recorded from a single crystal.

Values in parentheses are for the highest-resolution shell.

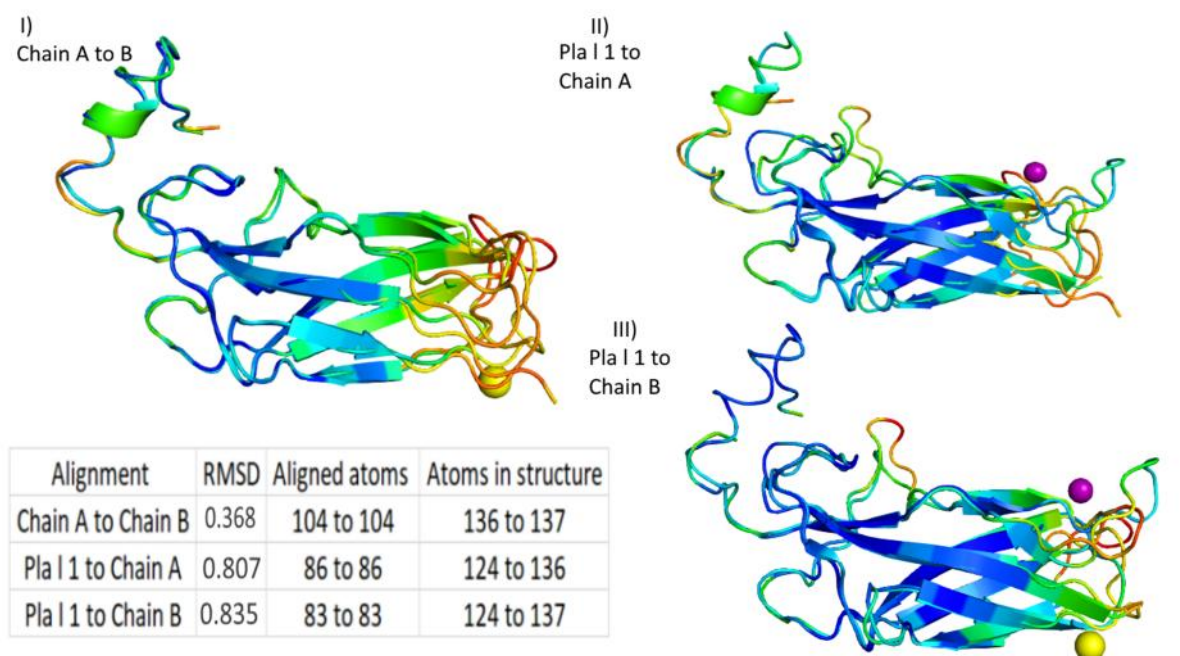


Figure S2. Structural alignment of Lig v 1 monomeric units to Pla l 1. Molecules are coloured by RMSD, with the conserved structures in blue and rainbow gradient with the greatest differences coloured in red. Nickel bound to chain B (yellow) and Zinc bound to Pla l 1 (purple). I) Alignment of Lig v 1 Chains A and B. II) Pla l 1 to Lig v 1 Chain A. III) Alignment of Pla l 1 and Lig v 1 Chain B.

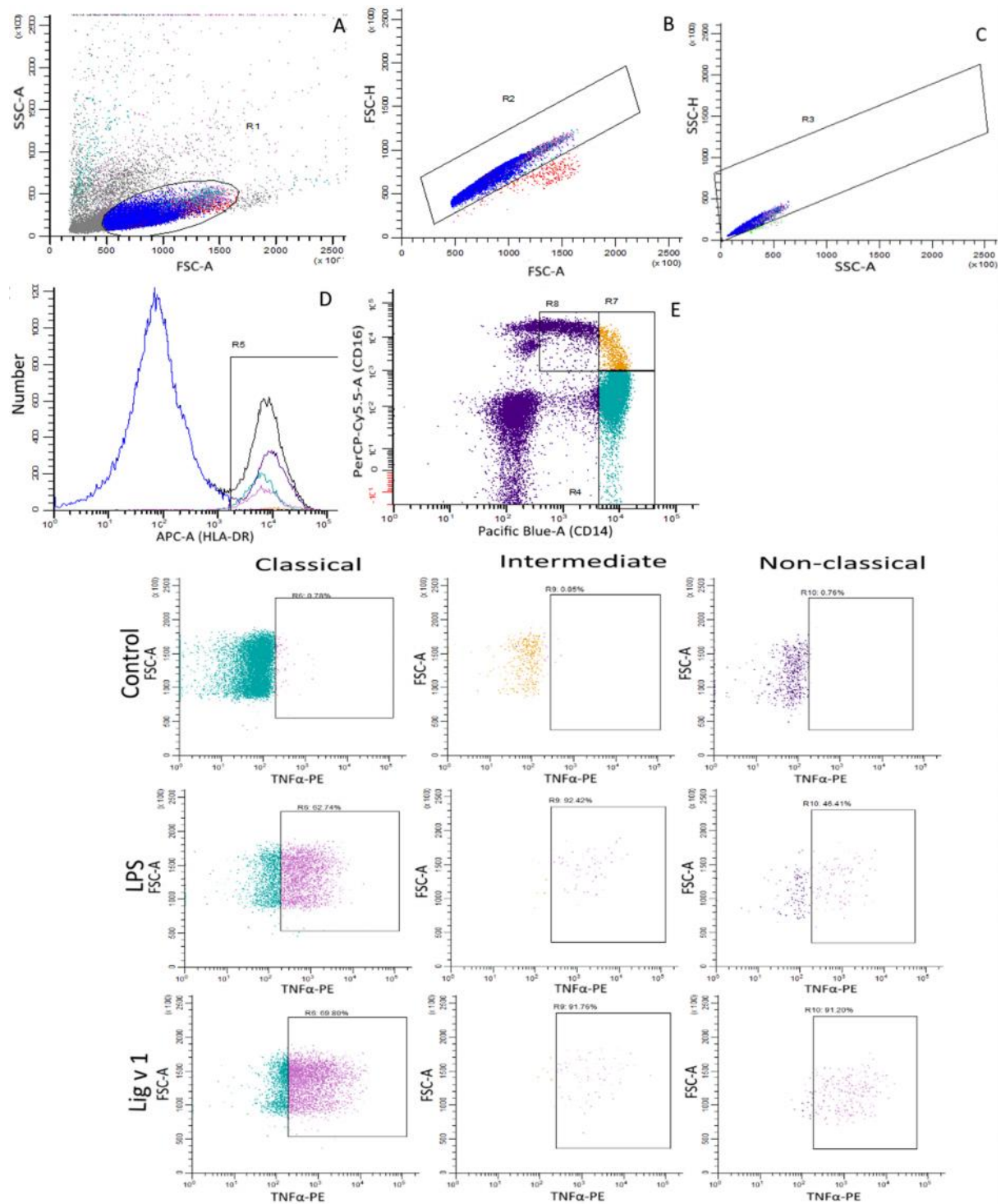


Figure S3. Gating strategy for monocyte identification and detection of intracellular TNF α by FACS. A-C) Size separation. D) HLA-DR (APC) histogram. E) Monocytes subsets identified with Pacific Blue (CD14) vs PerCP-Cy5.5 (CD16). TNF α detection on the different subsets F) Classical (CD14⁺⁺ CD16), G) Intermediate (CD14⁺⁺ CD16⁺), H) Non-classical (CD14⁺ CD16⁺⁺). I) Percentage of activated cells Control (non-stimulated or baseline), LPS and Lig v 1 experiments

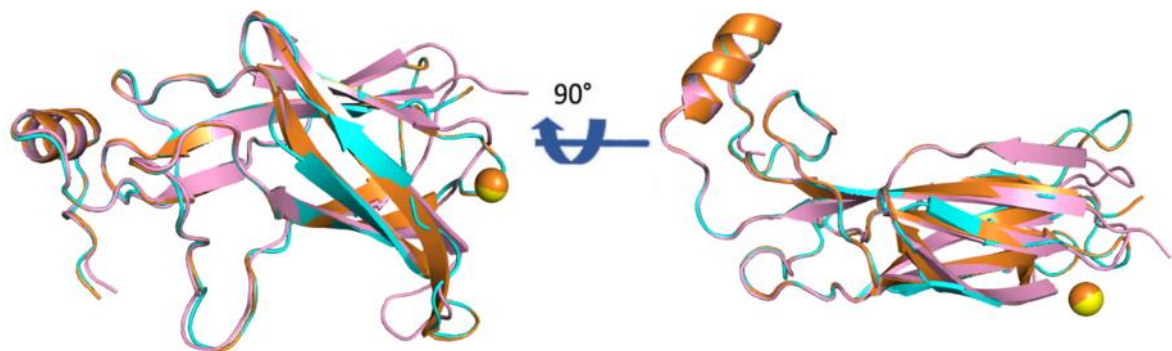


Figure S4. Structural alignment of Ole e 1 protein members. Alignment of Lig v 1 monomer (teal) to predicted models of Ole e 1 (pink) and Fra e 1 (orange). Nickel is bound to Lig v 1 (yellow) and Fra e 1 (orange). Showing Lig v 1 is an excellent model for the family paradigm, Ole e 1.