UNIVERSIDAD POLITÉCNICA DE MADRID

**ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA, ALIMENTARIA Y DE BIOSISTEMAS (02)**

**CENTRO DE BIOTECNOLOGÍA Y GENÓMICA DE PLANTAS
(Departamento de Biotecnología - Biología Vegetal)**

**“Characterization of the Biological Activity of Pru p 3 in Plant and
Intestinal Epithelium”**

TESIS DOCTORAL

NURIA CUBELLS BAEZA

Licenciada en Farmacia

Directora: Araceli Díaz Perales

Madrid, 2017

ABBREVIATIONS

AMP	Antimicrobial protein
APC	Antigen presenting cell
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ESI-qToF	Electrospray ionisation time of flying
FBS	Fetal bovine serum
FcεRI	High affinity IgE receptor
FD4	Fluorescein isothiocyanate-dextran
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal tract
GM-CSF	Granulocyte macrophage-colony stimulating factor
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IEC	Intestinal epithelial cells
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
iNKT	Invariant natural killer T
Ip	Intraperitoneal
KRB	Krebs-Ringer Bicarbonate Buffer
LMPC	Lyso-myristoyl-phosphatidylcholine
LPS	Lipopolysaccharide
M cells	Microfold cells
MALDI-TOF	Matrix-assisted laser desorption ionization
MD	Molecular dynamics
MFI	Median fluorescence intensity
MHC I and II	Major histocompatibility complex I and II
microTOF-Q II	Mass spectrometer
MLN	Mesenteric lymph nodes
moDCs	Monocyte-derived dendritic cells
MS–MS	Tandem mass spectrometry
MW	Molecular weight
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells

NPP	no Peyer's Patches
nsLTP	Non-specific lipid transfer protein
OH-camptothecin	Hydroxycamptothecin
PALM	Pollen-associated lipid mediators
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PE	Anti-phycoerythrin
PFA	Paraformaldehyde
PP	Peyer's Patches
PVDF	Polyvinylidene difluoride
RMSD	Root mean square deviation
RP-HPLC	Reversed-phase high-performance liquid chromatography
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEAP	Secreted embryonic alkaline phosphatase
STR	Strictosidine synthase
Th	T helper
TJ	Tight junction
TLC	Thin layer chromatography
TLR	Toll-like receptors
TLR4	Toll-like receptor 4
TSLP	Thymic stromal lymphopoietin

INDEX

RESUMEN.....	VII
ABSTRACT	IX
1. INTRODUCTION.....	1
1.1 Allergies as a public health problem	3
1.2 Who can be an allergen?	4
1.2.1 Mechanism of allergic sensitization	5
1.3 The sensitization pathway	6
1.3.1 Anatomy of intestinal epithelium	6
1.3.2 Transport through the enterocytes.....	9
1.4. Intestinal epithelium models used in protein permeability/transport studies	9
1.4.1 Tumor cell line models	10
1.4.2 Intestinal organoids	10
1.4.3 <i>Ex vivo</i> models	11
1.5. Lipid transfer proteins as plant allergens.....	12
2. OBJECTIVES	19
3. RESULTS.....	23
3.1 Identification of the ligand of Pru p 3, a peach LTP	25
3.2 Mechanisms underlying induction of allergic sensitization by Pru p 3	45
3.3 The effect of protein transportation through small intestine on the immune resident cells using the InTESTine ^{TM} system. Pru p 3 as a model	69
4. GENERAL DISCUSSION	83
5. CONCLUSIONS	91

RESUMEN

La alergia es un problema social que supone una grave disminución de la calidad de vida del paciente, estando considerada como la mayor enfermedad crónica en Europa en la actualidad. Desde mediados del siglo XX, se ha producido un incremento en la prevalencia de esta enfermedad aunque el origen de este incremento es todavía desconocido. Para prevenir y tratar este tipo de enfermedades es necesario conocer el mecanismo por el cual una proteína se convierte en alergénica.

El objetivo principal de esta Tesis ha sido estudiar los mecanismos moleculares que están implicados en los procesos de sensibilización alérgica. Evidencias crecientes sugieren que los lípidos transportados por determinados alérgenos pueden ser reconocidos por receptores celulares del sistema inmune, pudiendo tener un papel destacado en la sensibilización alérgica. En este trabajo se ha escogido como modelo Pru p 3, alérgeno principal del melocotón y proteína transportadora de lípidos, (*lipid transfer protein*, LTP).

El ligando lipídico de Pru p 3 se ha caracterizado estructuralmente por primera vez empleando ESI-micrOTOF-QII. Así, el ligando se ha identificado como un derivado hidroxilado de camptotecina unida a fitoesfingosina, segmento que forma una cola hidrocarbonada que se inserta en el túnel hidrofóbico de la proteína. La naturaleza química del ligando ha sido confirmada mediante ensayos de inhibición de la topoisomerasa I y emisión de fluorescencia a 254 nm, ambas características típicas de la camptotecina. Por otra parte, combinando diferentes técnicas *in vitro* (como RT-PCR e inmunohistoquímica), se ha caracterizado el posible papel del ligando en planta. La máxima expresión de Pru p 3 se detectó en los estilos de flores polinizadas, en contraste con su ausencia en no polinizados donde su expresión disminuye tras la antítesis. Además, la mayor expresión de Pru p 3 se localizó en piel, más concretamente en los tricomas, pero no en la pulpa. Estos datos, junto con la inhibición de la germinación del polen por parte del ligando, sugieren que Pru p 3 puede inhibir una segunda polinización y mantener alejados los herbívoros hasta la maduración de la semilla.

Igualmente, se ha evaluado la actividad inmunológica del ligando lipídico empleando diferentes modelos *in vitro* (como moDC, PBMCs, THP1 y células epiteliales como Caco2) y un modelo de ratón de anafilaxia. Así se ha determinado que el ligando es capaz de modular el sistema inmune a través de una respuesta Th2, siendo el responsable de la activación de las células presentadoras de antígeno y aumenta la capacidad de sensibilización alérgica a Pru p 3 en el modelo de ratón. Además, el ligando es presentado por el receptor CD1d en las células epiteliales, activando a continuación las células iNKT.

Estos resultados se han confirmado con un empleo del modelo *ex vivo* InTESTine™ con fragmentos de tejido de cerdo. De este modo se ha podido observar que el ligando no afecta al transporte de Pru p 3 a través de la barrera intestinal aunque parece que Pru p 3 queda acumulado en las Placas de Peyer. Por otra parte, se ha observado que la presencia del ligando induce la expresión de citoquinas típicas de una respuesta Th2 como IL4 e IL13. Igualmente se ha confirmado que Pru p 3 co-localiza con el receptor CD1d en presencia del ligando y que aumenta la presencia de células iNKT.

En resumen, este trabajo representa un nuevo enfoque sobre los mecanismos que caracterizan la alergia, aportando resultados de gran utilidad tanto para la mejora del diagnóstico como para nuevas investigaciones sobre esta enfermedad.

ABSTRACT

Allergy is a serious social problem that impairs the quality of life of patients and is currently considered the most prevalent chronic disease in Europe. A large increase in its prevalence has occurred since the mid-twentieth century. However, the underlying causes for this increase and the factors that trigger allergy are still unknown. Knowing why a protein becomes an allergen would be essential in the prevention and treatment of allergy diseases.

The main aim of this doctoral thesis was to study the molecular mechanisms involved in the allergic sensitization processes. Increasing evidence suggests that lipids transported by certain allergens can be recognized by cellular receptors of the immune system, and may play a prominent role in allergic sensitization. In this work, Pru p 3, the major allergen of the peach and a lipid-transfer protein (LTP), has been chosen as model. The lipid ligand of Pru p 3 has been structurally characterized for the first time using ESI-microTOF-QII. The ligand has thus been identified as a hydroxyl derivative of camptothecin bound to phytosphingosine, a hydrocarbon tail which is inserted into the hydrophobic tunnel of the protein. This result was validated by assays of inhibition of topoisomerase I as well as fluorescence emission at 254 nm, both features characteristic of camptothecin. Additionally, different *in vitro* techniques (such as RT-PCR and immunohistochemistry) were combined to characterize the possible role of the ligand in plant. The highest expression of Pru p 3 was detected in pollinated styles by contrast to its absence in non-pollinated flowers where the expression decreased after the antithesis. Besides, the highest expression of Pru p 3 was localized in the trichomes, but not in the pulp. These data, together with the inhibition of pollen germination by the ligand, suggest that Pru p 3 can inhibit a secondary pollination and keep herbivores away until seed maturation.

Additionally, the immunological activity of the lipid ligand was evaluated using different *in vitro* models (moDC, PBMCs, THP1, and Caco2) and a mouse model of anaphylaxis. Thus, it was determined that the ligand was capable of modulating the immune system through a Th2 response, responsible for the activation of antigen presenting cells, and in increasing the capacity of allergic sensitization to Pru p 3 in a mouse model. Furthermore, the ligand was introduced by the CD1d receptor into the epithelial cells to activate iNKT cells. These results have been validated using the *ex vivo* model InTESTine^{TM} with fragments of pig tissue. The ligand did not affect the transport of Pru p 3 through the intestinal barrier, but appears to remain accumulated in Peyer's Patches. The presence of the ligand was also observed to induce the expression of cytokines that are typical of a Th2 response like IL4 and IL13. Moreover, it was

confirmed that Pru p 3 co-localized with the CD1d receptor in the presence of the ligand, as well as increasing the presence of iNKT cells.

In summary, this thesis work represents a novel approach to characterize allergic response mechanisms and presents results which are useful for improving diagnosis and for providing a basis for new investigations related to allergy.

1. INTRODUCTION

1.1 Allergies as a public health problem.

During the last years, an increase of allergic diseases has been reported in many Western countries. It is expected that allergic diseases reach up to four billion people by 2050s¹. The most dramatic increase is seen during childhood. In Europe, one out of four children suffers from some kind of allergy². Allergic diseases have become a huge financial burden for national health systems. In Europe, annual costs range from 55 to 151 billion Euros³. Taking into account all of the above, advances in the knowledge of the mechanisms that underlie the allergic sensitization are essential not only to understand the aforementioned increase but also to try to improve our diagnosis and treatment of allergies.

The majority of IgE-mediated allergic responses is produced in two phases:

- *The sensitization phase*: characterized by the specific production of IgE.

The antigen enters into the body typically through mucus membranes, and is taken up by professional antigen presenting cells (APCs) that process and present it as a small peptide to the major histocompatibility complex II (MHC-II). As a result, differentiation of a specific T helper (Th2) takes place with subsequent production of the cytokines IL4 and IL13 that induce the activation of B-lymphocytes. Afterwards, IgE antibodies are produced and bind to the high-affinity receptor FcεRI on the surface of basophils (circulating cells) and mast cells (tissue cells).

- *The effector phase*: a symptomatic phase.

After a re-exposure to the antigen, the allergen interacts with two IgE cell-bound molecules by cross-linking to produce the degranulation, releasing of mediators such as histamine and leukotrienes that cause the immediate hypersensitivity reaction^{4,5}. These mediators are responsible for the typical symptoms and signs of allergic diseases such as itching, diarrhoea, abdominal pain, and swelling of lips, tongue, face and throat^{6,7}. If this phase is prolonged, the *late phase reactions* could take place. During this, the allergen is presented to memory T cells resulting in the release of IL5 and eosinophil infiltration.

Figure 1: Humoral and cellular mechanisms of food allergy.

Allergens pass the epithelium and are captured by DCs. Epithelial stress, under control of IELs, promotes the transfer of allergen and the release of GM-CSF, IL-25, IL-33 or TSLP, followed by the differentiation of naive allergen specific T cells into Th2 type responses under the influence of eosinophils (via eosinophilic peroxidase) or basophils (via IL-4). Th2 cells promote class switching of B-cells into IgE via IL-4 and IL-13. Mast cell bound IgE induces mast cell degranulation and release of mast cell mediators which induce local and systemic food allergic responses⁸.

1.2. What can be an allergen?

The cause why a specific protein induces an allergenic environment in the sensitization phase has not been clarified even though the immunological recognition of allergens has been extensively studied⁹⁻¹³. The epitopes (sequences recognized by T cells and IgE's) of most allergens are indeed found within highly conserved regions among members of the same protein

family¹⁴⁻¹⁸, despite the fact that the majority of those members lack allergenic activity^{19,20}. Taking into account that the immunologically relevant regions are conserved, other determining factors should play a major role in the induction of allergies. Recently, an increasing amount of evidence suggests that the protein alone is not enough for allergy induction.

Until now, most allergy studies have been focused on the presence of specific IgE antibodies, but little is known about what happens in the first sensitization phase before antibody production. Despite efforts directed towards identifying new allergens, no common biochemical

features have been found. Hence, why a protein can trigger a Th2 response leading to allergic sensitization is still an unanswered question. Finding an answer is essential if we want to prevent this disease and develop definitive treatments²¹.

1.2.1 Mechanism of allergic sensitization

A number of factors have been shown to influence the sensitization process, including the type of allergen, level of exposure and concomitant exposure with adjuvants⁵. For example, ligands of toll-like receptors (TLRs), i.e. lipopolysaccharide (LPS), can promote either Th1- or Th2-like responses depending on the concentration present upon allergen encounter²². Pollen-associated lipid mediators²³ and chitin²⁴ or fungal components²⁵ can also enhance allergic sensitization. The way of exposure of allergens to immune system, e.g. the route of entrance and type of APCs such as dendritic cells (DCs) or mucosal epithelial cells, is also essential. Although APCs have been shown to play a key role in the induction of Th2 immune responses²⁶, there is a considerable gap in our knowledge of the molecular mechanisms underlying these events. APCs may initiate Th2 responses when exposure to the allergen occurs in the presence of adjuvants (i.e. low-dose LPS, lipid ligands) or environmental pathogens (e.g. fungi)²⁷. Adjuvant activity may also be an intrinsic feature of allergenic agents, as reported for allergens from house dust mite²⁷⁻²⁹, cockroach, peanut, dog and mould, which can directly activate DCs, epithelial cells, or basophils resulting in Th2 polarization³⁰.

Studies on the participation of mucosal epithelial cells in the induction of allergic sensitization have demonstrated the essential role of lipid compounds³¹. When purified allergens were added *in vitro* to cultures of mucosal cells in the presence of mixtures of lipids (pollen-associated lipid mediators, PALMs, from pollen and LPS from bacterial cell wall), a clear Th2 (allergic response) was observed, producing a characteristic cytokine profile^{23,31,32}. By contrast, an unexpectedly poor immunological response was found in the absence of lipid compounds. Lipid binding seems to be a quite frequent property of allergens including mite allergens groups 2 and 7. Der p 2 can bind LPS and substitute the homologous human MD-2 thereby promoting Th2 responses in mice via TLR4 signalling. LPS exposure seems to play a variable role in both promoting and inhibiting allergic sensitization³³. Der p 7 showed structural similarity to LPS-binding protein (LBP) and bound weakly to bacterial lipopeptide polymyxin B³⁴. Der p 7 might thus promote Th2 immunity via co-stimulation of TLR2 pathways.

1.3 The sensitization pathway

Classically, three ways have been associated to allergic sensitization: respiratory, epicutaneous, and gastrointestinal. In food allergy, it seems that transport via the gut may lead to different immunological responses as well. Transport of soluble milk allergens via epithelial cells led to anaphylaxis, while transport of the aggregated form of the same milk allergen after heating led to sensitization in a cow's milk mouse model³⁵. These findings suggest that the transport of soluble protein via villous epithelial cells was the main pathway for anaphylactic responses, while transport of the aggregated forms via Peyer's Patches (PP) was needed for sensitization. Furthermore, defects in the integrity of the epithelial barrier have also been reported in food allergy. Clinical studies in children with cow's milk allergy demonstrated that intestinal permeability increased after, but not before the allergen challenge^{35,36}. A recent study based on small intestinal biopsy specimens exposed to food allergens *in vitro*, showed decreased expression of tight junction (TJ) proteins (i.e. occludin, claudin-1, and ZO-1) in tissues obtained from food allergic patients compared to healthy subjects³⁶. Both studies suggest that in sensitized individuals, intestinal permeability and the passage of allergens is enhanced. For that reason, evaluation of protein transport across the intestinal barrier and its effect on epithelial permeability might be a relevant parameter in allergenicity risk assessment³⁷.

In contrast, emerging evidences suggest that the skin may be a highly relevant inductive site for allergic sensitization to food proteins³⁸. Conversely, some routes of exposure have been proposed to be inherently tolerogenic (e.g. oral and sublingual exposure).

1.3.1 Anatomy of intestinal epithelium

The intestinal epithelium is the largest interface between the host and the environment. It regulates fluxes of ions and nutrients and limits host contact with luminal antigens³⁹. The gastrointestinal tract is divided in four layers: mucosa, submucosa, muscularis propria and serosa.

1) Mucosa

1.1) *A single layer of epithelial cells* joined together by tight junctions in their apical side, which prevents the passing of peptides, allergenic proteins, and macromolecular compounds larger than 600 Da⁴⁰. A protective layer of mucus (a mix of water, glycoproteins, lipids, and mineral salts) covers these cells⁴¹. In addition, brush border enzyme activity acts as a defence against pathogens. Furthermore, intestinal epithelial cells

(IECs) transport IgA into the intestinal lumen and contribute to barrier functions by excluding the uptake of antigens and pathogens⁴².

1.2) *Lamina propria* consists of loosely packed connective tissue that forms the platform for the villus, as well as containing the blood supply, lymph drainage and nervous supply for the mucosa⁴³. It is densely populated by immune cells, T effector and regulatory T cells, B cells, macrophages, dendritic cells (DC's), and eosinophil. The lymphoid tissue in the mucosa is organized into *inductive sites* (Peyer's Patches and mesenteric lymph nodes) where antigen-specific cellular and humoral responses are first generated, and *effector sites* (normal intestine mucosa) including B cells, T cells and Ig-producing plasma cells⁴⁴, which are responsible for the induction phase of an immune response such as sensitization^{45,46}.

1.3) The *muscularis mucosae* is a continuous sheet of smooth muscle cells that lie at the base of the lamina propria.

2) The *submucosa* consists of a variety of inflammatory cells, nerve fibres, and ganglion cells, and is a circulation zone for arteries and small veins.

3) The *muscularis propria* comprises smooth muscle cells that propel food through the gut by contractile peristaltic waves.

4) The *serosa* forms a natural barrier against dissemination of inflammatory processes⁴⁷.

Figure 2: Architecture of the gut mucosal wall. Four-layered (mucosa, submucosa, muscularis mucosa, and serosa) organization of the digestive tract⁴⁷.

At the bottom of the crypts of the epithelial cells, the pluripotent stem cells can differentiate into distinct cell types: enterocytes, goblet cells, endocrine cells, and microfold (M) cells.

The enterocytes are the most abundant absorptive cells in the intestinal barrier. Proteins can be endocytosed by vesicles or phagosomes and are digested in lysosomes or left intact⁴⁸.

The endocrine cells secrete numerous hormones that regulate digestive functions. Mucine secretion by goblet cells and antimicrobial proteins (AMPs; defensins, cathelicidins, and histatins), forms a physical and biochemical barrier against microbes⁴⁹. M cells and enterocytes mediate transport of luminal antigens and living bacteria across the epithelial monolayer to the underlying lymphoid cells, such as antigen-presenting cells (e.g. dendritic cells and intestinal macrophages)⁴⁹. The permeability and polarity of the first epithelial layer are maintained by the apical junctional complex, which is composed of TJs, adherent junctions, and the subjacent desmosomes. Permeability depends mainly on the TJs, which are composed of transmembrane proteins such as occludin, claudin, junctional adhesion molecule A, and tricellulin^{39,50}.

Figure 3. Components of intestinal mucosa. (A) Simplified scheme of intestinal cells and structures in the small intestine. (B) Schematic drawing of polarized epithelial cells with different types of intercellular contacts. (C) Different types of transport through the intestinal epithelium: 1, paracellular transport; 2, passive diffusion; 3, vesicle-mediated transcytosis; 4, carrier-mediated uptake⁵¹.

1.3.2 Transport through the enterocytes

Although most dietary proteins are degraded by digestive enzymes and absorbed as nutrients (amino acids or dipeptides/tripeptides) some can resist low pH and proteolytic hydrolysis of the gastric environment, arriving at the small intestinal lumen. Thus, this may contribute to allergenicity⁵². The transport of these proteins to the intestine may occur either via para-cellular route or via trans-cellular route.

The paracellular pathway is related to the transfer of molecules through the inter-cellular space between cells, which is mainly restricted by tight junctions and a network of transmembrane proteins (e.g. claudins, occludins). These structures allow the diffusion of small hydrophilic compounds (MW < 600 Da) like mannitol and lactulose that are usually used as permeability markers.

The transcellular pathway is related to molecules of MW > 600 Da (such as food antigens and peptides) that are mainly transferred by endocytosis at the apical membrane, and transcytosis facing the lamina propria. During transcytosis the proteins are totally degraded by lysosomal compartments, or partially so in the basolateral side of enterocytes³⁹. Other transcellular routes are passive, involving diffusion and carrier mediated transport of amino acids and di- and tri-peptides⁵³.

1.4. Intestinal epithelium models used in protein permeability/transport studies

An epithelial model used to study protein transport should preferentially include all the components of the intestinal mucosa (e.g. mucus layer and epithelial and M cells). However, in practice this is not always feasible, so several models have been developed. These models differ in complexity and applicability. It is therefore important to define the objective of study (e.g. transport via M cells and/or enterocytes, allergenic activity, mucus effect, permeability) in order to identify the best intestinal model⁵⁴. Irrespective of the cell model chosen, the main readout parameters are the effects on epithelial barrier function, absorption, and trans-epithelial transport of the test compound. The reliability of such studies depends on the uniformity and integrity of the confluent and polarized cell monolayer.

Table 1: Comparison of different types of culture⁵¹.

	Caco cell line	Organoids	Primary culture
High-throughput	High	Low	Low
Expenses	Low	High	High
Redout	Protein/drugs transportation	Protein and drugs metabolism	Transport and metabolism
Advantage	Easy manage	Maintain the integrity of the mucosa, with all the specialized cells represented	Multi-cell system. All intestinal regions can be used Less labor intensive than Using chamber technology
Disadvantages	No mucus, no specialized cell, no 3D structure	The apical side is not accessible	Biological variations Limited viability
Applicability on food allergy	Yes	Yes, specially, interaction with specialized cells	Yes, transport and interaction with specialized cells

1.4.1 Tumor cell line models

Cell lines used to study the transport and absorption of proteins include Caco-2, HT-29, T84, and IPEC-J2. In all cases, cells are grown in a Transwell system to form a monolayer. Monolayers of human colon carcinoma cell lines, the so called Caco-2 cells, have been extensively used over the last 20 years to predict the permeability to proteins of the intestinal mucosa⁵⁵. The polarized monolayer of these well-differentiated columnar absorptive cells expresses a brush border on their apical surface with typical small intestinal enzymes and transporters. The cells differentiate into a polarized apical and basolateral membrane mimicking the luminal and microvilli side (apical) of the intestine and intercellular TJs⁵⁶. Caco-2 exhibit features of enterocytes of the small intestine. Moreover, no mucus layer is produced on the apical side of Caco-2, thus limiting the studies of protein–mucosa interactions. Despite these limitations, the Caco-2 cell line has proved to be the best model to date to study intestinal absorption and toxicity.

1.4.2 Intestinal organoids

Collectively, the intestinal organoid system enables culture and expansion of intestinal stem cells *ex vivo*. Importantly, the resulting epithelial structures faithfully recapitulate the homeostasis and architecture of the functional intestinal epithelium. To date, intestinal organoids have been shown to have multiple applications, including analysis of endogenous stem cell characteristics and gene function, as well as disease modelling⁵⁷. With the recent development of efficient gene-editing tools, it is now possible to rapidly engineer cultured intestinal organoids to generate highly physiological models of human gastrointestinal disease

for use as research tools⁵⁸. Therefore, this system could be an alternative for the study of protein transport and associated diseases. However, the inwards orientation of the epithelial cells (directed to the lumen of the organoids), makes the apical side relatively inaccessible for direct experimental stimulation. This will impede protein transport studies. Furthermore, availability of human tissue is often a bottleneck and hinders their possibilities as a standard approach. To the best of our knowledge, no literature on the use of organoids in allergen transport or permeability studies is available.

1.4.3 *Ex vivo* models

The major drawbacks of the single cell models described above can be overcome by using intestinal tissues. In these models, the asymmetrical distribution of proteins and lipids in the two plasma membrane domains facing the intestinal lumen, the internal milieu, and the presence of highly organized structures (TJs) joining adjacent cells and separating the two membrane domains, enable selective processes of absorption, transport, and secretion to take place across the intestinal mucosa⁵⁹. The maintenance of these characteristics *ex vivo* is particularly important for the study of absorption, metabolism, and toxicity. Traditionally, *ex vivo* intestinal models for intestinal protein transport studies have been based on animal tissue. The techniques used include the everted sac technique⁶⁰ and the Ussing chamber⁶¹. The everted sac is a segment of animal intestine that is everted and used to assess protein transport. In the case of the Ussing chamber, a fresh intestinal segment is mounted into a complex apparatus for measuring protein transport and epithelial membrane properties. Both techniques provide an accurate measurement of intestinal permeability. The most relevant advantage for food allergy is the possibility of studying the effect of sensitization on intestinal protein absorption, using intestinal tissue from sensitized animals^{62,63}. However, both techniques have several limitations. First, tissue viability is rapidly lost (2 h); second, the tissue can be damaged during isolation, which may lead to overestimation of protein transport. On the contrary, the presence of the muscle layer in the everted sac method may lead to underestimation of protein transport⁶⁰. Third, interspecies differences in anatomy, physiology, metabolism, diet, and microbiota complicate data extrapolation to humans^{37,64}. Pigs share more physiological and immunological similarities with humans than rodents, and the use of minipigs is becoming increasingly common in nutritional research⁶⁵.

The recently developed InTESTine^{TM} method is a medium- throughput alternative to the Ussing chamber and is based on intestinal tissue from pigs that is incubated on a rocker platform in a high oxygen incubator⁶⁶. The viability of tissue could be retained for 2 h, paracellular absorption transport resembles that of human intestinal tissue in the Ussing chamber⁶⁷, and the transport of

macromolecular proteins is studied using radioactively labelled proteins⁵³. This method seems to be a good alternative that should be evaluated in future studies on food allergy.

Figure 4: Different types of epithelial models used to study protein transportation. (A) Tumor cell line models, epithelial cells are seeded on plastic structures (Transwell) separating basolateral and apical sides and allowing the polarization of cells. (B) Intestinal epithelial organoids, three-dimensional structures of cultured intestinal cells from isolated intestinal crypts including stem cells, allowing to remake a mini-intestine including all cell types. (C) Everted sac, intestinal segments are cut and inverted so that the apical side of the mucosa is on the outside. (D) Ussing Chamber, an intestine segment placed on an apparatus for measuring epithelial membrane properties in both side and efficiency of transportation⁵¹.

1.5. Lipid transfer proteins as plant allergens

Non-specific lipid transfer proteins (nsLTPs) are basic, small (9 kDa, 92-94 residues) proteins with amino acid sequence identities that range between 25% and 95%. Despite considerable heterogeneity in their primary structure, all nsLTPs share an α -helical compact fold composed of four α -helices connected by short loops together with a long non-structured C-terminal tail. This fold is stabilized by four disulfide bonds from eight conserved cysteine residues⁶⁸. A characteristic tunnel-like cavity aligned to the long axis of the protein is formed by the inner space left within the helices. Hydrophobic amino acid side chains line the inner surface of this cavity, which is large enough to accommodate ligands with long chains. The ability of plant nsLTPs to bind a wide variety of lipids enables them to perform disparate functions⁶⁹.

Despite the abundant literature on these proteins, the debate on their possible functions remains open and new roles for them are still being proposed.

Pru p 3, the major peach allergen and a nsLTP (IPR000528), is the most important allergen in the Mediterranean area ⁷⁰. B- and T-cell epitopes in Pru p 3 and common epitopes present in other nsLTPs^{68,15} have been also characterized. The residues that are relevant to the immune response described in Pru p 3 are highly conserved in the LTP family⁶⁹ although the ability to mediate allergic reactions has been identified in only less than 3% of its members. It seems therefore clear that protein sequences do not suffice to fully account for the allergenicity of some of them. In this regard, the carrier function of Pru p 3 might be linked to its allergenic activity⁶⁹ and, as indicated in other places of this report, the ligand transported by this LTP could play a critical role in the induction of allergic sensitization.

In fact, ligands carried by allergens or exposed together with them might influence host response to a specific allergen^{32,31}. The most widely studied examples of these co-allergenic molecules are pollen-associated lipid mediators (PALMs)²³, which are bioactive and display pro-inflammatory and immunomodulatory effects on immune cells. Accordingly, immunoregulatory lipids could significantly contribute to the allergenicity of several proteins. All these compounds are attractive candidates as non-self molecules recognized by the innate immune system as being capable of inducing Th2-skewed responses that contribute to allergy in susceptible individuals.

References

1. in *Global Atlas of Allergy* (eds. Akdis, C. A. & Agache, I.) (European Academy of Allergy and Clinical Immunology (EAACI), 2014).
2. in *White Book on Allergy* (eds. Pawankar, R., Canonica, G. W., Holgate, S. T. & Lockey, R. F.) (World Allergy Organization (WAO), 2011).
3. *Advocacy Manifesto. Tackling the Allergy Crisis in Europe - Concerted Policy Action Needed.* (The European Academy of Allergy and Clinical Immunology (EAACI), 2015).
4. De Souza Rebouças, J. *et al.* Nanoparticulate adjuvants and delivery systems for allergen immunotherapy. *J. Biomed. Biotechnol.* 2012, 474605 (2012).
5. Galli, S. J., Tsai, M. & Piliponsky, A. M. The development of allergic inflammation. *Nature* 454, 445–454 (2008).
6. Sicherer, S. H. *et al.* Epidemiology of food allergy. *J. Allergy Clin. Immunol.* 127, 594–602 (2011).
7. Bøgh, K. L. *et al.* Current challenges facing the assessment of the allergenic capacity of food allergens in animal models. *Clin. Transl. Allergy* 6, 21 (2016).
8. Smit, J. J., Noti, M. & O'Mahony, L. The use of animal models to discover immunological mechanisms underpinning sensitization to food allergens. *Drug Discov. Today Dis. Model.* 17, 63–69 (2015).
9. Radauer, C., Bublin, M., Wagner, S., Mari, A. & Breiteneder, H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J. Allergy Clin. Immunol.* 121, 847–852.e7 (2008).
10. Hammad, H. & Lambrecht, B. N. Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity* 43, 29–40 (2015).
11. Vacher, G., Niculita-Hirzel, H. & Roger, T. Immune responses to airborne fungi and non-invasive airway diseases. *Semin. Immunopathol.* 37, 83–96 (2015).
12. Lambrecht, B. N. & Hammad, H. Allergens and the airway epithelium response: Gateway to allergic sensitization. *J. Allergy Clin. Immunol.* 134, 499–507 (2014).
13. Escribese, M. M., Gómez-Casado, C., Barber, D. & Diaz-Perales, A. Immune Polarization in Allergic Patients: Role of the Innate Immune System. *J. Investig. Allergol. Clin. Immunol.* 25, 251–8 (2015).
14. Tordesillas, L. *et al.* Molecular basis of allergen cross-reactivity: Non-specific lipid transfer proteins from wheat flour and peach fruit as models. *Mol. Immunol.* 47, 534–540 (2009).
15. Pacios, L. F. *et al.* LocaPep: localization of epitopes on protein surfaces using peptides from phage display libraries. *J. Chem. Inf. Model.* 51, 1465–73 (2011).

16. Palacín, A. *et al.* Graph Based Study of Allergen Cross-Reactivity of Plant Lipid Transfer Proteins (LTPs) Using Microarray in a Multicenter Study. *PLoS One* 7, e50799 (2012).
17. Palacín, A. *et al.* The involvement of thaumatin-like proteins in plant food cross-reactivity: a multicenter study using a specific protein microarray. *PLoS One* 7, e44088 (2012).
18. Tordesillas, L. *et al.* A mutant of the major melon allergen, Cuc m 2, with reduced IgE binding capacity is a good candidate for specific immunotherapy. *Mol. Immunol.* 49, 504–511 (2011).
19. Salcedo, G., Sánchez-Monge, R., Barber, D. & Díaz-Perales, A. Plant non-specific lipid transfer proteins: An interface between plant defence and human allergy. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1771, 781–791 (2007).
20. Tordesillas, L. *et al.* Plant Lipid Transfer Protein Allergens: No Cross-Reactivity between Those from Foods and Olive and *Parietaria* Pollen. *Int. Arch. Allergy Immunol.* 156, 291–296 (2011).
21. Valenta, R. The future of antigen-specific immunotherapy of allergy. *Nat. Rev. Immunol.* 2, 446–53 (2002).
22. Herrick, C. A. & Bottomly, K. To respond or not to respond: T cells in allergic asthma. *Nat. Rev. Immunol.* 3, 405–412 (2003).
23. Gilles, S. *et al.* Pollen allergens do not come alone: pollen associated lipid mediators (PALMS) shift the human immune systems towards a TH2-dominated response. *Allergy, Asthma Clin. Immunol.* 5, 3 (2009).
24. Morgenstern, V. *et al.* Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am. J. Respir. Crit. Care Med.* 177, 1331–1337 (2008).
25. Hammad, H. & Lambrecht, B. N. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 8, 193–204 (2008).
26. Schleimer, R. P., Kato, A., Kern, R., Kuperman, D. & Avila, P. C. Epithelium: At the interface of innate and adaptive immune responses. *J. Allergy Clin. Immunol.* 120, 1279–1284 (2007).
27. Lambrecht, B. N. & Hammad, H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. *Lancet* 376, 835–843 (2010).
28. Barrett, N. A., Maekawa, A., Rahman, O. M., Austen, K. F. & Kanaoka, Y. Dectin-2 Recognition of House Dust Mite Triggers Cysteinyl Leukotriene Generation by Dendritic Cells. *J. Immunol.* 182, (2009).
29. Thomas, W. R., Hales, B. J. & Smith, W.-A. House dust mite allergens in asthma and allergy. *Trends Mol. Med.* 16, 321–328 (2010).

30. Trompette, A. *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457, 585–588 (2009).
31. Thomas, W. R. Allergen Ligands in the Initiation of Allergic Sensitization. *Curr. Allergy Asthma Rep.* 14, 432 (2014).
32. Berin, M. C. & Shreffler, W. G. TH2 adjuvants: Implications for food allergy. *J. Allergy Clin. Immunol.* 121, 1311–1320 (2008).
33. Eisenbarth, S. C. *et al.* Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 196, 1645–51 (2002).
34. Mueller, G. A. *et al.* The structure of the dust mite allergen Der p 7 reveals similarities to innate immune proteins. *J. Allergy Clin. Immunol.* 125, 909–917.e4 (2010).
35. Roth-Walter, F. *et al.* Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy* 63, 882–890 (2008).
36. Pizzuti, D. *et al.* In vitro model for IgE mediated food allergy. *Scand. J. Gastroenterol.* 46, 177–87 (2011).
37. Sjögren, E. *et al.* In vivo methods for drug absorption – Comparative physiologies, model selection, correlations with in vitro methods (IVIVC), and applications for formulation/API/excipient characterization including food effects. *Eur. J. Pharm. Sci.* 57, 99–151 (2014).
38. Tordesillas, L. *et al.* Skin exposure promotes a Th2-dependent sensitization to peanut allergens. *J. Clin. Invest.* 124, 4965–4975 (2014).
39. Ménard, S., Cerf-Bensussan, N. & Heyman, M. Multiple facets of intestinal permeability and epithelial handling of dietary antigens. *Mucosal Immunol.* 3, 247–259 (2010).
40. Heyman, M. Gut barrier dysfunction in food allergy. *Eur. J. Gastroenterol. Hepatol.* 17, 1279–85 (2005).
41. Khanvilkar, K., Donovan, M. D. & Flanagan, D. R. Drug transfer through mucus. *Adv. Drug Deliv. Rev.* 48, 173–93 (2001).
42. Berin, M. C. & Sampson, H. A. Mucosal immunology of food allergy. *Curr. Biol.* 23, R389-400 (2013).
43. Mowat, A. M. & Agace, W. W. Regional specialization within the intestinal immune system. *Nat. Rev. Immunol.* 14, 667–685 (2014).
44. Brandtzaeg, P. Mucosal Immunity: Induction, Dissemination, and Effector Functions. *Scand. J. Immunol.* 70, 505–515 (2009).
45. Hamada, H. *et al.* Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J. Immunol.* 168, 57–64 (2002).
46. Mowat, A. M. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat. Rev. Immunol.* 3, 331–341 (2003).

47. Rao, J. N. & Wang, J.-Y. *Regulation of Gastrointestinal Mucosal Growth. Regulation of Gastrointestinal Mucosal Growth* (Morgan & Claypool Life Sciences, 2010).
48. So, A. L. *et al.* Factors Affecting Antigen Uptake by Human Intestinal Epithelial Cell Lines. *Dig. Dis. Sci.* 45, 1130–1137 (2000).
49. Peterson, L. W. & Artis, D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat. Rev. Immunol.* 14, 141–153 (2014).
50. Wells, J. M. & Spence, J. R. How to make an intestine. *Development* 141, 752–60 (2014).
51. Cubells-Baeza, N. *et al.* Applicability of epithelial models in protein permeability/transport studies and food allergy. *Drug Discov. Today Dis. Model.* 17, 13–21 (2015).
52. Astwood, J. D., Leach, J. N. & Fuchs, R. L. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* 14, 1269–1273 (1996).
53. Reitsma, M., Westerhout, J., Wichers, H. J., Wortelboer, H. M. & Verhoeckx, K. C. M. Protein transport across the small intestine in food allergy. *Mol. Nutr. Food Res.* 58, 194–205 (2014).
54. Chopra, D. P., Dombkowski, A. A., Stemmer, P. M. & Parker, G. C. Intestinal Epithelial Cells In Vitro. *Stem Cells Dev.* 19, 131–142 (2010).
55. Navabi, N., McGuckin, M. A., Lindén, S. K., Jr, D. B. & Kurita, Y. Gastrointestinal Cell Lines Form Polarized Epithelia with an Adherent Mucus Layer when Cultured in Semi-Wet Interfaces with Mechanical Stimulation. *PLoS One* 8, e68761 (2013).
56. Sambuy, Y. *et al.* The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* 21, 1–26 (2005).
57. Leushacke, M. & Barker, N. Ex vivo culture of the intestinal epithelium: strategies and applications. *Gut* 63, 1345–1354 (2014).
58. Schwank, G. *et al.* Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* 13, 653–8 (2013).
59. Sambruy, Y., Ferruzza, S., Ranaldi, G. & De Angelis, I. Intestinal cell culture models: applications in toxicology and pharmacology. *Cell Biol. Toxicol.* 17, 301–17 (2001).
60. Alam, M. A., Al-Jenoobi, F. I. & Al-mohizea, A. M. Everted gut sac model as a tool in pharmaceutical research: limitations and applications. *J. Pharm. Pharmacol.* 64, 326–336 (2012).
61. Clarke, L. L. A guide to Ussing chamber studies of mouse intestine. *AJP Gastrointest. Liver Physiol.* 296, G1151–G1166 (2009).
62. Lomasney, K. W. & Hyland, N. P. The application of Ussing chambers for determining

- the impact of microbes and probiotics on intestinal ion transport ¹. *Can. J. Physiol. Pharmacol.* 91, 663–670 (2013).
63. He, L. *et al.* Use of the Ussing chamber technique to study nutrient transport by epithelial tissues. *Front. Biosci. (Landmark Ed.* 18, 1266–74 (2013).
  64. Sjöberg, Å. *et al.* Comprehensive study on regional human intestinal permeability and prediction of fraction absorbed of drugs using the Ussing chamber technique. *Eur. J. Pharm. Sci.* 48, 166–180 (2013).
  65. Patterson, J. K., Lei, X. G. & Miller, D. D. The Pig as an Experimental Model for Elucidating the Mechanisms Governing Dietary Influence on Mineral Absorption. *Exp. Biol. Med.* 233, 651–664 (2008).
  66. Westerhout, J. *et al.* A new approach to predict human intestinal absorption using porcine intestinal tissue and biorelevant matrices. *Eur. J. Pharm. Sci.* 63, 167–177 (2014).
  67. Van Mierlo, L. D., Meiland, F. J. M., Van Hout, H. P. J. & Dröes, R. M. Toward an evidence-based implementation model and checklist for personalized dementia care in the community. *Int. Psychogeriatrics* 28, 801–813 (2016).
  68. Tordesillas, L. *et al.* Molecular basis of allergen cross-reactivity: Non-specific lipid transfer proteins from wheat flour and peach fruit as models. *Mol. Immunol.* 47, 534–540 (2009).
  69. Pacios, L. F. *et al.* Computational study of ligand binding in lipid transfer proteins: Structures, interfaces, and free energies of protein-lipid complexes. *J. Comput. Chem.* 33, 1831–1844 (2012).
  70. Cuesta-Herranz, J. *et al.* Differences among Pollen-Allergic Patients with and without Plant Food Allergy. *Int. Arch. Allergy Immunol.* 153, 182–192 (2010).

2.OBJECTIVES

OBJECTIVES

The principal aim of this PhD project was to characterize the molecular mechanisms involved in the allergic sensitization, and more specifically, the role of its lipid-ligand, taking as a model the principal allergen of Pru p 3. With this purpose, the following objectives were established:

- 1) To identify the lipid ligand carried by Pru p 3 and to characterize the biological role of Pru p 3 in plant.
- 2) To study the role of Pru p 3-ligand on immune and epithelial cells in the induction of allergic response using cell culture model.
- 3) To confirm the effect of Pru p 3, with and without its lipid-ligand, in the mucosa lymphoid system using an *ex vivo* model derived from mini-pig intestine.

3. RESULTS

3.1 Identification of the ligand of Pru p 3, a peach LTP

Published in: Plant Mol Biol. 2017 May;94(1-2):33-44

Nuria Cubells-Baeza¹, Cristina Gómez-Casado², Leticia Tordesillas³, Carmen Ramírez-Castillejo¹, María Garrido-Arandia¹, Pablo González-Melendi¹, María Herrero⁴, Luis F. Pacios^{1,5}, Araceli Díaz-Perales¹

Affiliations

¹Centre for Plant Biotechnology and Genomics (UPM-INIA), and Department of Biotechnology-Plant Biology, ETSIAAB, Technical University of Madrid, Spain

²Department of Experimental Medical Sciences, Lund University, Sweden;

³Icahn School of Medicine at Mount Sinai, New York, USA;

⁴Estación Experimental de Aula Dei (CSIC), Zaragoza, Spain;

⁵Department of Natural Systems and Resources, ETSI Montes, Technical University of Madrid, Spain

The PhD student has done the most of experimental work of the following manuscript. The isolation and biochemical assays of lipid-ligand has been performed by Cubells-Baeza (Figures 5, 6, 7, 9 and 10).

Introduction

Plant non-specific lipid transfer proteins (nsLTPs) are basic proteins with a molecular weight of about 9 kDa (92-94 residues) and amino acid sequence identities ranging from 25% to 95%¹⁻³. Despite the considerable heterogeneity in their primary structure, all LTPs share a compact α -helical fold composed of four α -helices connected by short loops and a non-structured C-terminal tail. This fold is stabilized by eight conserved cysteine residues that form four disulfide bonds^{4,2,5}. A characteristic tunnel-like cavity aligned with the long axis of the protein is formed by the space left within the helices. Hydrophobic side chains coat the inner surface of this cavity, which is large enough to accommodate ligands with long chains⁶⁻⁸. These properties point to a potential role for LTPs in membrane biogenesis, and some members are thought to have a role in defence against pathogens⁹, transport of cutin monomers, and fruit and flower development^{10,2,5}. The ability of plant nsLTPs to bind a wide variety of lipids enables them to perform various functions⁴.

However, given that data on the association between compounds transported by LTPs and their specific functions are scarce in the literature, we expected that time- and tissue-specific expression of LTP genes would shed light on their physiological role in relation to the molecules they carry. Two LTPs, LTP1 (AY792996.1) and LTP2 (AY093699), are expressed in peach (*Prunus persica*)¹¹⁻¹³. Although LTP1 (Pru p 3 [IPR000528]) is expressed in pollinated flowers, it is poorly expressed in the ovary, and its expression is independent of pollination. LTP2 was highly expressed only in the ovary. By contrast, in fruit, only LTP1 mRNA was detected in the peel^{14,12}. Nevertheless, little is known about the physiological role of these LTPs and the specific molecules that they harbour and transport.

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing molecules found in about 20% of plant species. Many of the 16,000 alkaloids, for which structures have been described, participate in the defence of plants against herbivores, microbes, viruses, and competing plants¹⁵. Camptothecin is a cytotoxic quinoline alkaloid that was first isolated from the bark and stem of *Camptotheca acuminata* (Happy tree), a tree that is native to China and is used in traditional Chinese medicine. The biosynthesis of this alkaloid in almost all tissues has been described in many species¹⁶. Camptothecin is synthesized through a modified terpenoid indole alkaloid pathway in which there is a key enzyme: strictosidine synthase (STR), which is involved in the final step in the formation of the strictosidine backbone¹⁶. Gene expression involved in STR synthesis was found in leaves (more active in the young one), flowers (specially in petioles) and fruit and in most plant tissues¹⁷⁻²⁰. Once produced in pre-vacuol compartments before being secreted through glandular trichomes. This mechanism has also been described for other toxic alkaloids such as those in *Cannabis sativa*²¹ and peppermint²².

Although not yet described in peach, the biosynthesis pathway of camptothecin was predicted from the peach genome (<http://pathways.rosaceae.org>), even though evidence for expression of the final steps of the pathway was lacking.

We have characterized the ligand of Pru p 3 as a derivative of camptothecin bound to a long hydrophobic tail identified as phytosphingosine. By monitoring the expression of the *Pru p 3* gene and genes of key enzymes in the biosynthesis pathway of both camptothecin and phytosphingosine at different stages of flower and fruit development, we found that the ligand of Pru p 3 may play a role in those processes. We propose that the Pru p 3–ligand complex could help to prevent double pollination and defend against herbivores until the embryo (seed) has fully matured.

Materials and Methods

Isolation of Pru p 3

Pru p 3 was purified as previously described²³. Briefly, peach peel was separated by cation exchange chromatography on a SepPakR AccellPlus CM cartridge (Waters Corp, Milford, MA, USA) with 20 mM formic acid, pH 4.0. The cationic retained fraction was then eluted with 0.75 mM NaCl in the same buffer (1 ml/min). This fraction was further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Nucleosil 300-C4 column (7 x 250 mm; particle size 5 µm: Tecknokroma, Barcelona, Spain) to obtain the purified HPLC fraction. The fraction was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% for 5 min and 10-100% over 150 min; 1 mL/min).

Protein fractions were quantified using the bicinchoninic acid assay (Pierce, Cheshire, UK), and purity was measured using SDS-PAGE. Replicates were electrotransferred to PVDF membranes, and incubated with Pru p 3 polyclonal antibodies after blocking. The binding was revealed by HRP-chemiluminescence.

N-terminal amino acid sequencing was performed with an Applied Biosystems 477A gas-phase sequencer (Applied Biosystems), and mass spectrophotometric analysis was performed with a Biflex III Spectrometer (Bruker–Franzen Analytik, Bremen, Germany). Fingerprinting analysis was performed using standard methods after trypsin digestion.

Circular dichroism analysis

The samples (cationic retained and HPLC purified fraction) were desalted using MicroSpin G25 Columns (GE Healthcare, United Kingdom). Circular dichroism measurements were performed in water on a J-810 spectropolarimeter (Jasco, Canada) using 1-mm path-length quartz cells equilibrated at 20°C. Spectra were recorded from 190 to 260 nm with 0.5 nm

resolution at a scan speed of 50 nm/min. Final spectra were baseline-corrected by subtracting the corresponding solvent spectra obtained under identical conditions. Data were fitted using the DichroWeb analytical tool. Results were expressed as the mean residue ellipticity (Θ) for a given wavelength.

Isolation of the ligand of Pru p 3

The ligand was separated from the retained cationic fraction by size-exclusion LH-20 chromatography using methanol:water (70:30) as the mobile phase. Fractions were checked using thin layer chromatography on silica gel-coated plates (Merck, USA) and developed with methanol:water (70:30 v/v) in a saturated chromatography chamber for about 20 min. The plates were visualized under UV light (254 nm) and stained with vanillin (1% in ethanol) and dichlorofluorescein (0.1% in ethanol-NaOH 2.5 mM) (Sigma-Aldrich, Germany). Fluorescent fractions were pooled and re-purified by HPLC as previously described²⁴, with slight modifications. Isocratic analytical HPLC was performed using an RP-C18 column (Nucleosil 120 C18, 5 μ m, 250x4mm, Scharlab, Spain). The mobile phase for alkaloid elution was acetonitrile:water (85:15) at a flow rate 0.5 ml/min for 150 μ l of sample. Sample peaks were detected using UV light at 254 nm. All reagents were HPLC grade.

Pru p 3—ligand-binding assays

To study the binding affinity of Pru p 3 for its ligand, increasing amounts of the ligand (0.0001 to 1 μ g) were dotted onto a nitrocellulose membrane. After blocking, the membrane was incubated with a solution of Pru p 3 (1 μ g/ μ l), and binding was revealed by immunodetection using specific antibodies. After extensive washing, dot intensity was calculated using a Bio-Rad Pharos FX^{TM} Plus Molecular Image. Binding affinity was expressed as the dissociation constant $K_d = [\text{Pru p 3}][\text{Ligand}]/[\text{Complex}]$, where [Complex] was measured as the Pru p 3-bound form using a pattern curve with known amounts of the protein. All tests were performed in three independent assays.

Mass spectrometry analysis of the ligand of Pru p 3

The ligand of Pru p 3 was analysed using the HPLC system. Samples were separated by HPLC (Nucleosil 120 C18, 5 μ m, 250x4mm, Tecknokroma) with an acetonitrile gradient of 10-85% for 30 min at 0.5 ml/min. Spectra were recorded at 254 and 280 nm. Commercial camptothecin (Sigma, SL, USA), OH-camptothecin (Santa Cruz, Te, USA) and quercetin (Sigma, SL, USA) were used as standards.

Ionic fragmentation of the ligand of Pru p 3 was performed by diluting the ligand in acetonitrile containing 0.1% formic acid (v/v), and the solution was applied directly to the

MALDI target. The analysis was performed in a micrOTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an ESI source in positive ion mode. Typical instrument settings were as follows: capillary voltage, 4500 V; capillary exit, 130 V; dry gas temperature, 180°C; dry gas flow, 4 l/min. Spectra were obtained in positive ion mode. The mass spectrometer was used to carry out two scans: a full-mass scan between 50 and 1000 m/z at a repetition rate of 2 Hz, and an MS-MS scan of the most abundant ions in the full-mass scan. Argon was used as the collision gas. Collision energy was ramped at between 5 and 25 eV. Mass calibration was performed using sodium formate clusters (10-mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid).

Inhibition assays of topoisomerase I activity

Since camptothecin is known to inhibit topoisomerase I, this activity was examined in the ligand of Pru p 3 using camptothecin assays, as reported elsewhere²⁵. We incubated 200 ng of plasmid pBSK with 1 U of human topoisomerase I (Inspiralis, UK) and increasing amounts of the ligand of Pru p 3 after heating (5 min 90°C) and (S)-(+)-camptothecin (Sigma-Aldrich, Germany) for 30 min at 37°C. The reaction was run on a 0.8% agarose gel at 5-10 V/cm. The gel was then stained with ethidium bromide for 30 min, washed briefly with water, and photographed in a UV transilluminator.

Modelling of structures of the ligand and the Pru p 3–ligand complex

The initial geometry of camptothecin was taken from the X-ray structure of its complex with human DNA topoisomerase I [PDB code 1T8I;²⁵]. The initial geometry with the proper (2S, 3S, 4R) configuration of phytosphingosine was firstly built up with modelling software Chimera 1.10.2²⁶. The ligand model was constructed by binding the two molecules through an amide moiety formed by lactone carbonyl from camptothecin and amine nitrogen from phytosphingosine. Hydroxyl was added to camptothecin C10, and the whole geometry was optimized. Hydrogens were then added and several geometry minimization cycles were run. This modelling was also performed with Chimera 1.10.2²⁶. Phytosphingosine alone was docked in the hydrophobic cavity of the crystal structure of Pru p 3 PDB code 2ALG²⁷ by means of AutoDock Vina calculations²⁸. Three docking results with the lowest affinity energies were used to prepare three initial geometries of the Pru p 3-ligand complex that then underwent 10-ns molecular dynamics (MD) simulations in water to find optimized structures. These calculations were performed with NAMD 1.10²⁹ according to a recently reported procedure³⁰. Force field parameters for the modelled ligand were assigned using SwissParam³¹. One of the three structures showed the best results for stability of ligand atoms (lowest RMSD), protein-ligand interaction Plant Mol Biol energy, and total energy of the complex upon completion of the three

MD simulations. This optimum structure was the final model for the Pru p 3-ligand complex (see below).

Plant material

Tissues from the Super Crimson Gold peach cultivar were used to study gene expression during flower development and fruit set. Pollinated and unpollinated flowers at different time points (–2, –1, 0, 1, 7, 14, 21, 26, 28, 35, 51, 58, 65, 73, 80, 87, 94, 101 and 105 days post-anthesis [dpa]) from anthesis to mature fruit. The anthers, stigma-style, ovary, and pedicel from the flower and seed and peel from the fruit were excised separately, frozen immediately in liquid nitrogen, and stored at –80°C for RNA study.

Pollen was recovered from the same flowers by shaking and stored immediately at –80°C. For testing pollen germination, grains were incubated in 15% glucose in darkness. Pru p 3 (1 µg/ml) and the Pru p 3–ligand complex (0.1 µg/ml) were added to the solution directly.

Real-time PCR in flower and fruit tissues

Total RNA was extracted from the tissues following the method published³². Gel electrophoresis (1% agarose) and spectrophotometry (260/280 nm) were used to determine RNA quality and concentration. The first-strand cDNA was synthesized from 1 µg of DNA-free total RNA treated with DNase I (Roche, Basle, Switzerland) using random primers and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA).

Relative real-time PCR quantification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. Primers 18S-F and 18S-R (5'-GGGCATTCGTATTTTCATAGTCAGAG-3' and 5'-GGTTGAGACTAGGACGGTA TCTGA-3') were used to amplify a 146-bp fragment of the endogenous control 18S from *Prunus persica* (GenBank Accession No. L28749).

Primers Pru p 3-F and Pru p 3-R (5'-TGTGCATGGTTGTGAGCGTG-3' and 5'- GTTGTGACGTTTCCTAATGCCG-3') were designed to amplify a 143-bp fragment of Pru p 3 gene from *P. persica* (GenBank Accession No. AY792996).

The thermal cycling conditions were as follows: 10 min at 95°C, 15 s at 95°C, and 45 s at 60°C (40 cycles). The optimal cDNA and primer concentrations were determined by serial dilution experiments. Finally, 10 ng of cDNA per well were added, and the final concentration of the primers was 1.5 µM. The amount of *Pru p 3*, *STR*, and *S4D* mRNA expression was

normalized using the endogenous control *18S*, and relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$), as previously described³². Changes in gene expression were calculated with reference to anthesis (7 dpa). The products of reactions were separated in 2% agarose gel and stained with ethidium bromide. All PCR experiments were carried out in triplicate, in three independent experiments.

Immunofluorescence and confocal microscopy

To detect the presence of Pru p 3, the samples were fixed with 4% formaldehyde in PBS (pH 7.4) at 4°C overnight. After washing in PBS and sectioning with a razor blade, the tissues were permeabilized (20 freezing/thawing cycles of 5 minutes each). Then, the specimens were washed with PBS and blocked with 5% BSA in PBS for 1 hour. Immunohistochemistry was performed in whole-mount tissue by incubating overnight at 4°C with an anti-Pru p 3 polyclonal antibody (applied 1:50 in PBS;³³) and a secondary antibody, Alexa 488–conjugated anti-rabbit antibody (applied 1:500 in PBS, Molecular Probes). The tissue sections were mounted with glycerol:PBS (1:1) on 10-well slides (FisherScientific Inc., Pittsburgh, USA) and observed with a Leica TCS-SP8 confocal microscope using the laser excitation lines of 488 nm for the localization of Pru p 3 and 405 nm to detect alkaloid autofluorescence. Measurements of fluorescence intensity in ten independent ROIs on Z stack maximal projections were used to compare the immuno-expression of Pru p 3 protein and the alkaloid.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 and Statgraphics Centurion XVI. Gene expression was compared using the Mann-Whitney test. Statistical significance was set at $p < 0.05$ for all analyses. At least three independent experiments were used for all assays.

Results

Pru p 3 carried a lipid compound

Pru p 3 was isolated from peach peel following two chromatographic steps: cationic exchange (cationic-retained) and reversed phase (HPLC purified), as previously described³⁴. The presence of a lipid compound bound to Pru p 3 was confirmed by thin layer chromatography in methanol as the mobile phase and stained with vanillin (Figure 5B). A lipid stain was observed in the cationic-retained fraction, whereas no spot was found in the HPLC-purified protein. The secondary structure of Pru p 3 from the cationic-retained fraction was studied using circular dichroism and revealed an alteration in the far UV region, a feature that is typical of lipid binding (Figure 5C). In contrast, HPLC-purified protein from RP-HPLC showed a circular dichroism spectrum typical of helices with no alteration in this region.

3.1 Identification of the ligand of Pru p 3

Figure 5. (A) The retained fraction from cationic chromatography (5 μ g; *Cationic retained*) and purified Pru p 3 (5 μ g; *HPLC purified*) were separated by SDS-PAGE and stained with Coomassie R-250 (Coomassie) or incubated with polyclonal antibodies (anti-Pru p 3). (B) Both fractions were also separated in silica gel-coated plates (Merck) using thin layer chromatography (TLC) in methanol:water (7:3 v/v) in a saturated chromatography chamber for about 20 min. The plates were stained with vanillin (Sigma-Aldrich, Steinheim, Germany). (C) Both fractions were used to compare their secondary structure by far-UV (190–260 nm) circular dichroism spectrum in water on a JASCO Spectropolarimeter J-810 (Jasco, Victoria, Canada) using 1-mm path-length quartz cells equilibrated at 20°C. Spectra were recorded from 190 to 260 nm with 0.5 nm resolution at a scan speed of 50 nm/min. Data were fitted using the Dichroweb analytical tool. (D) Increasing amounts of the ligand of Pru p 3 was dot-blotted were incubated onto a nitrocellulose membrane. After blocking, it was incubated with a Pru p 3 solution (1 μ g/mL) and the binding was revealed by specific antibodies

To characterize the interaction between Pru p 3 and this lipid compound, i.e. the ligand of peach LTP, the compound was isolated from the cationic-retained fraction by exclusion chromatography (70% methanol), followed by RP-HPLC in acetonitrile. The lipid fraction was confirmed as the ligand of Pru p 3 by dot-blot binding assay. Increasing amounts of this fraction diluted in PBS were dotted onto a nitrocellulose membrane, and, after blocking, the membrane was incubated with Pru p 3 solution (1 μ g/ μ l). The interaction was revealed by specific antibodies (Figure 5D). These results showed tight binding affinity between Pru p 3 and its ligand, represented by a dissociation constant estimated at $K_d=0.2$ μ M in a 1:1 ratio.

A **B**

Figure 6. (A) The ligand of Pru p 3 was separated by phase reverse-HPLC. Commercial OH-Camptothecin/, camptothecin and quercetin were used as standards. (B) ESI-microTOF-Q II. Deconvolution chromatogram corresponding to the camptothecin obtained from the ligand fraction when it was dissolved in methanol-formic acid.

The ligand of Pru p 3 included a hydroxylated derivative of camptothecin bound to phytosphingosine

The identification of the ligand was analysed using standard compounds and by comparing retention times in HPLC (Figure 6A). The ligand showed a peak overlapping to OH-camptothecin.

The presence of hydroxy-camptothecin and phytosphingosine were identified by ion fragmentation (Figure 6B). Based on the pattern described for ceramides³⁵ and camptothecin²⁰, a fragmentation for the ligand of Pru p 3 was studied. The characteristic camptothecin fragment ions were detected as 364.81 m/z and 202.83 m/z. Phytosphingosine fragment ions were

3.1 Identification of the ligand of Pru p 3

identified as 218.79 m/z and 230.01 m/z. The results also revealed peaks corresponding to both bound compounds, as 380.77 m/z and 424.82 m/z.

The chemical identification of the ligand of Pru p 3 was also confirmed by its capacity to emit blue light when excited with UV radiation (254 nm; Figure 7A), a feature also shown by camptothecin²¹. Moreover, camptothecin is well known for its ability to inhibit the catalytic activity of topoisomerase I, the enzyme responsible for relaxing supercoiling DNA²⁵. Activity assays performed with the ligand of Pru p 3 clearly showed inhibition of topoisomerase I after heating, whereas no inhibition was observed when the ligand was used directly (data not shown) (Figure 7B). The inhibitory activity of topoisomerase I occurs through the closed E-ring of camptothecin (Figure 8A). However, linking to phytosphingosine through the amide bond in the ligand of Pru p 3, forces the open form of the E-ring, thus rendering the ligand inactive in the inhibition of topoisomerase. Given that this α -hydroxy-lactone E-ring is susceptible to hydrolysis upon thermal treatment, the inhibition results are consistent with the chemical nature proposed for the ligand of Pru p 3.

Figure 7: Figure (A) Commercial quercetin (1 μg Sigma), the ligand of Pru p 3 (1 μg; Ligand), commercial camptothecin (1 μg Sigma; CPT) and hydroxy-camptothecin (1 μg Santa Cruz; OH-CPT) were loaded onto a silica gel plate and UV light (354 nm) was applied. **(B)** Inhibition of topoisomerase I activity. Plasmid pBSK (200 ng) was incubated with topoisomerase I (1 u; Inspiralis, UK) for 30 min at 37 °C in the presence of camptothecin (1 μg, 0.5 μg; Sigma, UK) or of the ligand (1 μg, 0.5 μg), heated for 5 min at 90 °C. After the mix was separated on a 0.8% agarose gel at 5–10 V/cm, the gel was stained with ethidium bromide (10 mg/mL) and destained briefly with water. The gel was illuminated with a UV transilluminator.

Modelled structure of the Pru p 3–ligand complex

Phytosphingosine is an 18-carbon chain with a polar head consisting of three hydroxyl groups and one amine group at the first four carbons followed by a 14-carbon saturated

hydrophobic tail (Figure 8A). Camptothecin is composed of a pyrroloquinoline moiety (rings A, B, and C in Figure 8A), a conjugated pyridine ring (D), and a α -hydroxy-lactone ring (E) that is susceptible to hydrolysis and is open in the amide bound to phytosphingosine. In our model structure of the Pru p 3–ligand complex (Figure 8B), which was obtained as explained in Methods, the phytosphingosine tail is inserted into the hydrophobic tunnel of the protein, whereas its polar head lies at the entrance. The camptothecin part of the ligand is located unburied in the large cleft outside the cavity, with rings A-C exposed to the aqueous solvent and the 10-hydroxy group protruding from the structure (Figure 8C).

LTPs are known to bind a wide variety of hydrophobic ligands with polar groups, even in two opposite orientations inside the tunnel⁴. An X-ray structure is available for wheat LTP in a complex with two molecules of a phospholipid (lyso-myristoyl-phosphatidylcholine, LMPC) at opposite orientations with respect to the tunnel axis (PDB code 1BWO;³⁶). Interestingly, the superposition of this wheat LTP–LMPC complex and our modeled Pru p 3–ligand complex places the ligand of Pru p 3 and an LMPC molecule in orientation B⁴ at a remarkably similar position (Figure 8D). Given that this spatial coincidence arises from superposition of proteins only, the finding lends strong support to our model structure.

Figure 8. (A) Structural formula of the modeled ligand of Pru p 3 composed of 10-hydroxy-camptothecin (red) and phytosphingosine (blue). (B) Model structure of the Pru p 3 (ribbon)–ligand (sticks with C, green; N, blue; and O, red) complex. (C) Molecular surface of the Pru p 3–ligand complex structure shown in B. (D) Superposition of proteins in wheat LTP (light orange ribbon)–LMPC (sticks with carbons in yellow) complex (crystal structure, PDB id 1BWO) and Pru p 3 (green ribbon)–ligand (sticks with carbons in green) complex (model structure, present study).

The Pru p 3 gene is expressed mainly in the style of pollinated flowers.

Expression of the *Pru p 3* gene revealed maximum levels in the flower, particularly high in the stigma-style in contrast to that observed in the ovary and pedicel, where the expression was very low (about 1,000-fold less than in the stigma-style; Figure 9A). In the style of non-pollinated flowers, *Pru p 3* expression decreased after the flower opened and continued to decrease after that stage. However, in pollinated flowers, *Pru p 3* expression increased from 0 dpa until 21 dpa.

These results were confirmed by immunofluorescence assays in whole-mount tissue (Figure 10). Pru p 3 was located specifically in the pollinated flower stigma from 7 to 21 dpa, with a maximum expression at 21 dpa, which fell and then finally disappeared at 28 dpa.

The Pru p 3–ligand complex inhibited pollen germination

To gain a deeper understanding of the role of the Pru p 3–ligand complex in pollination, pollen grains were germinated in 15% glucose for 4 hours in the absence of Pru p 3 (Control) and in the presence of 1 µg/ml of Pru p 3 (Pru p 3) or Pru p 3 (1 µg/ml) together with its ligand (0.1 µg/ml) (Pru p 3 + ligand). Germination was essentially inhibited in the presence of the Pru p 3–ligand complex (Figure 9B).

3.1 Identification of the ligand of Pru p 3

Figure 9. (A) Expression profile of *Pru p 3* gene in style, ovary, and pedicel of peach flower collected from -2 to 35 days post-anthesis (dpa), in pollinated and unpollinated flower. All expression values are normalized to the *18S* gene. Relative expression is calibrated on the corresponding gene expression at ovary '0' time. The bars show the standard error. **(B)** Effect of the ligand on the pollen germination process. Pollen grains were grown in 15% glucose for 4 hours, in the absence of Pru p 3 (Control), in the presence of Pru p 3 (1 μ g/ml; + Pru p 3), and in the presence of Pru p 3 and its ligand (0,1 μ g/ml; + Pru p 3 and ligand). The percentage of germinated grains (%) was quantified in both samples. **(C)** Expression profile of *Pru p 3* gene in peach peel and seed fruit collected from 51 to 105 dpa.

The Pru p 3 gene is expressed mainly in the trichomes of peel

In the case of fruit development (Figure 9C), expression of the *Pru p 3* gene was concentrated in the peel, starting at 70 dpa and reaching its maximum at 80 dpa. From that time on, mRNA levels decreased to 105 dpa.

Thus, Pru p 3 protein was also located mainly in the peel of ripe fruits, particularly on trichomes (Figure 10). Interestingly, the alkaloid signal was also localized on peach peel trichomes; this finding is consistent with the kinetics of expression of *Pru p 3* obtained in our study.

Figure 10. (A) Immunolocalization of Pru p 3 in styles from flowers collected at different dpa. v, unpollinated flowers; +, pollinated flowers. Pru p 3 was detected using a specific monoclonal antibody, which showed fluorescence signal in green (Pru p 3 revealed by Alexa 488). (B) Immunolocalization of Pru p 3 in trichomes from mature fruits (105 dpa) detected using specific monoclonal antibody. The fluorescence signal is shown in green (Alexa 488) and alkaloids in blue in (B1); only blue light is observed in (B2); and the face contrast in (B3). x40

Discussion

The present study is the first to characterize the ligand of the peach LTP Pru p 3 as a derivative of camptothecin bound to phytosphingosine. Ligand binding to LTPs is accompanied by slight changes in the unstructured C-terminal tail of the protein, which acts as a lid on the hydrophobic cavity, although the α -helical fold is preserved^{2,5}. Consistent with the behaviour described for LTPs upon ligand binding, the presence of the ligand produced a distortion in the circular dichroism spectrum of Pru p 3 in the vacuum UV region (190-200 nm).

In the mass spectrometry analysis, the ligand of Pru p 3 suggested the presence of two different chemical species, each of which was identified as a monohydroxylated derivative of camptothecin and phytosphingosine. The presence of camptothecin was also confirmed by its autofluorescence properties and its ability to inhibit topoisomerase I activity, two well-known features of this alkaloid. However, given their chemical structures, both molecules barely occupy the hydrophobic central cavity of Pru p 3. We therefore suggest that the two species forming the ligand and are linked through an amide bond that involves the E-ring of camptothecin, which is known to be responsible for the inhibitory activity of topoisomerase I in its closed form²⁵. Since sphingoid molecules are also known to bind disparate moieties as substituents of amino hydrogens, the amide bond seems a reasonable link between alkaloid and sphingoid components of the ligand.

This hypothesis is supported by the data obtained in the mass spectrometry spectra in which the camptothecin ions with the amide bond are identified by the peaks with m/z values of 380.77 and 424.82. The phytosphingosine segment is able to occupy the hydrophobic cavity of Pru p 3 because of the hydrophobic nature of amino acid side chains inside the cavity, which stabilizes binding of the hydrocarbonated nonpolar tail while leaving the polar groups close to the amide bound with camptothecin exposed to the solvent. Thus, given that the E-ring is open when the two segments are bound, the ligand of Pru p 3 lacks the pro-apoptotic activity characteristic of the alkaloid, and the LTP then merely transports an inactive compound causing no plant cell damage. However, if the ligand is released in the apoplast, hydrolysis of the E-ring breaks the bound to phytosphingosine, and camptothecin is released in the closed form of the ring, thus regaining its activity and inhibiting cell division and pollen tube growth. The link can be also broken by extreme conditions such as heat and acid treatment. Therefore, the analysis with high voltage lasers in mass spectrometers could be responsible for splitting both molecules, thus explaining the presence of the two peaks observed.

The fact that the phytosphingosine tail forces the E-ring of camptothecin to remain open could be interpreted as a detoxification mechanism. In many cases, secondary metabolites and

their pathway intermediates are phytotoxic. Plants have evolved several defence mechanisms to avoid self-toxicity from secondary metabolites²¹. A basic strategy to avoid self-toxicity is tight regulation of biosynthesis in terms of temporal/spatial gene expression and localization. In this sense, the highest expression of *Pru p 3* and the Pru p 3–ligand activity in pollinated stigma-styles led us to propose that both features might inhibit double pollination. Moreover, the capacity to inhibit pollen germination, together with timing of expression, also suggests a role in preventing the pollination if one considers that maximum expression has been shown to match fertilization time in peach flowers³⁷.

Similarly, both the timing of *Pru p 3* expression and the presence of the Pru p 3–ligand complex in fruit peel (mainly in trichomes), might also inhibit predator feeding in fruit. The maximum expression of *Pru p 3* coincides with the end of embryo maturation and, thus, with the end of lignin deposition³⁸ and accumulation of *storage* substances (amino acids and sorbitol)³⁹. Protection associated with expression of defence proteins such as LTPs is therefore necessary at that time. LTPs are expressed mainly in the cells of the exocarp in unicellular trichomes secreting alkaloids, as evidenced by immunolocalization of Pru p 3 and ultraviolet fluorescence. Hence, Pru p 3 might prevent ingestion of fruit until the embryo is fully developed.

In summary, Pru p 3 can carry a monohydroxylated derivative of camptothecin bound to phytosphyngosine. This LTP is expressed at two key times of flower and fruit development in peach, namely, during pollination and during embryo development. In both stages, the ligand of Pru p 3 can inhibit cell division, although with two different objectives: first, to avoid pollination, and second, to prevent the plant from herbivores.

References

1. Kader, J.-C. LIPID-TRANSFER PROTEINS IN PLANTS. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47, 627–654 (1996).
2. Salcedo, G., Sánchez-Monge, R., Barber, D. & Díaz-Perales, A. Plant non-specific lipid transfer proteins: An interface between plant defence and human allergy. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1771, 781–791 (2007).
3. Thoma, S., Kaneko, Y. & Somerville, C. A non-specific lipid transfer protein from Arabidopsis is a cell wall protein. *Plant J.* 3, 427–36 (1993).
4. Pacios, L. F. *et al.* Computational study of ligand binding in lipid transfer proteins: Structures, interfaces, and free energies of protein-lipid complexes. *J. Comput. Chem.* 33, 1831–1844 (2012).
5. Salminen, T. A., Blomqvist, K. & Edqvist, J. Lipid transfer proteins: classification, nomenclature, structure, and function. *Planta* 244, 971–997 (2016).
6. Hamilton, J. Fatty acid interactions with proteins: what X-ray crystal and NMR solution structures tell us. *Prog. Lipid Res.* 43, 177–199 (2004).
7. Sossountzov, L. *et al.* Spatial and temporal expression of a maize lipid transfer protein gene. *Plant Cell* 3, 923–33 (1991).
8. Zachowski, A., Guerbette, F., Grosbois, M., Jolliot-Croquin, A. & Kader, J. C. Characterisation of acyl binding by a plant lipid-transfer protein. *Eur. J. Biochem.* 257, 443–8 (1998).
9. Molina, A. & Garcia-Olmedo, F. Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. *Plant J.* 4, 983–991 (1993).
10. Chae, K. *et al.* A multifaceted study of stigma/style cysteine-rich adhesin (SCA)-like Arabidopsis lipid transfer proteins (LTPs) suggests diversified roles for these LTPs in plant growth and reproduction. *J. Exp. Bot.* 61, 4277–4290 (2010).
11. Bonghi, C. *et al.* A microarray approach to identify genes involved in seed-pericarp cross-talk and development in peach. *BMC Plant Biol.* 11, 107 (2011).
12. Botton, A., Andreotti, C., Costa, G. & Ramina, A. Peach (*Prunus persica* L. Batsch) Allergen-Encoding Genes Are Developmentally Regulated and Affected by Fruit Load and Light Radiation. *J. Agric. Food Chem.* 57, 724–734 (2009).
13. Botton, A., Begheldo, M., Rasori, A., Bonghi, C. & Tonutti, P. Differential expression of two lipid transfer protein genes in reproductive organs of peach (*Prunus persica* L. Batsch). *Plant Sci.* 163, 993–1000 (2002).
14. Borges, J.-P. *et al.* The lipid transfer proteins (LTP) essentially concentrate in the skin of Rosaceae fruits as cell surface exposed allergens. *Plant Physiol. Biochem.* 44, 535–542 (2006).

15. Wink, M. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64, 3–19 (2003).
16. Lorence, A. & Nessler, C. L. Camptothecin, over four decades of surprising findings. *Phytochemistry* 65, 2735–2749 (2004).
17. Isah, T. & Mujib, A. Camptothecin from *Nothapodytes nimmoniana*: review on biotechnology applications. *Acta Physiol. Plant.* 37, 106 (2015).
18. Butler, J. E. *et al.* The piglet as a model for B cell and immune system development. *Vet. Immunol. Immunopathol.* 128, 147–70 (2009).
19. Sun, Y. *et al.* Pyrosequencing of the *Camptotheca acuminata* transcriptome reveals putative genes involved in camptothecin biosynthesis and transport. *BMC Genomics* 12, 533 (2011).
20. Zhao, C., Li, C., Wang, L., Zu, Y. & Yang, L. Determination of Camptothecin and 10-Hydroxycamptothecin in *Camptotheca acuminata* by LC-ESI-MS/MS. *Anal. Lett.* 43, 2681–2693 (2010).
21. Sirikantaramas, S. *et al.* Tetrahydrocannabinolic Acid Synthase, the Enzyme Controlling Marijuana Psychoactivity, is Secreted into the Storage Cavity of the Glandular Trichomes. *Plant Cell Physiol.* 46, 1578–1582 (2005).
22. Dudareva, N., Pichersky, E. & Gershenzon, J. Biochemistry of Plant Volatiles. *PLANT Physiol.* 135, 1893–1902 (2004).
23. Diaz-Perales, A. *et al.* cDNA cloning and heterologous expression of the major allergens from peach and apple belonging to the lipid-transfer protein family. *Clin. Exp. Allergy* 32, 87–92 (2002).
24. Malpathak, N., Kulkarni, A., Patwardhan, A. & Lele, U. Production of camptothecin in cultures of *Chonemorpha grandiflora*. *Pharmacognosy Res.* 2, 296 (2010).
25. Wright, C. M., van der Merwe, M., DeBrot, A. H. & Bjornsti, M.-A. DNA Topoisomerase I Domain Interactions Impact Enzyme Activity and Sensitivity to Camptothecin. *J. Biol. Chem.* 290, 12068–12078 (2015).
26. Pettersen, E. F. *et al.* UCSF Chimera?A visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612 (2004).
27. Pasquato, N. *et al.* Crystal Structure of Peach Pru p 3, the Prototypic Member of the Family of Plant Non-specific Lipid Transfer Protein Pan-allergens. *J. Mol. Biol.* 356, 684–694 (2006).
28. Trott, O. & Olson, A. J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, NA-NA (2009).
29. Phillips, J. C. *et al.* Scalable molecular dynamics with NAMD. *J. Comput. Chem.* 26, 1781–1802 (2005).

30. Garrido-Arandia, M., Gómez-Casado, C., Díaz-Perales, A. & Pacios, L. F. Molecular Dynamics of Major Allergens from *Alternaria*, Birch Pollen and Peach. *Mol. Inform.* 33, 682–694 (2014).
31. Zoete, V., Cuendet, M. A., Grosdidier, A. & Michielin, O. SwissParam: A fast force field generation tool for small organic molecules. *J. Comput. Chem.* 32, 2359–2368 (2011).
32. Tordesillas, L. *et al.* Transport of Pru p 3 across gastrointestinal epithelium - an essential step towards the induction of food allergy? *Clin. Exp. Allergy* 43, 1374–1383 (2013).
33. Palacín, A. *et al.* Graph Based Study of Allergen Cross-Reactivity of Plant Lipid Transfer Proteins (LTPs) Using Microarray in a Multicenter Study. *PLoS One* 7, e50799 (2012).
34. Díaz-Perales, A. *et al.* Lipid-transfer proteins as potential plant panallergens: cross-reactivity among proteins of *Artemisia* pollen, *Castanea* nut and *Rosaceae* fruits, with different IgE-binding capacities. *Clin. Exp. Allergy* 30, 1403–10 (2000).
35. Shin, J.-H. *et al.* A lipidomic platform establishment for structural identification of skin ceramides with non-hydroxyacyl chains. *Anal. Bioanal. Chem.* 406, 1917–1932 (2014).
36. Charvolin, D., Douliez, J. P., Marion, D., Cohen-Addad, C. & Pebay-Peyroula, E. The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. *Eur. J. Biochem.* 264, 562–8 (1999).
37. Herrero, M. & Arbeloa, A. Influence of the Pistil on Pollen Tube Kinetics in Peach (*Prunus persica*). *Am. J. Bot.* 76, 1441 (1989).
38. Dardick, C. D. *et al.* Stone formation in peach fruit exhibits spatial coordination of the lignin and flavonoid pathways and similarity to *Arabidopsis* dehiscence. *BMC Biol.* 8, 13 (2010).
39. Lombardo, V. A. *et al.* Metabolic Profiling during Peach Fruit Development and Ripening Reveals the Metabolic Networks That Underpin Each Developmental Stage. *PLANT Physiol.* 157, 1696–1710 (2011).

3.1 Identification of the ligand of Pru p 3

3.2 Mechanisms underlying induction of allergic sensitization by Pru p 3

Published in: Clin Exp Allergy (2017). 10.1111/cea.12962

Leticia Tordesillas^{*a}, Nuria Cubells-Baeza^{*b}, Cristina Gómez-Casado^c, Cecilia Berin^a, Vanesa Esteban^d, Weronika Barcik^e, Liam O'Mahony^e, Carmen Ramirez-Castillejo^{b,f}, Luis F. Pacios^{b,g}, María Garrido-Arandia^b, Araceli Díaz-Perales PhD^{b, f}

*These authors contributed equally to this work.

^aIcahn School of Medicine at Mount Sinai, New York, USA; ^bCentre for Plant Genomics and Biotechnology (UPM-INIA), Pozuelo de Alarcon, Madrid, Spain; ^c Institute of Applied Molecular Medicine (IMMA), CEU San Pablo University, Spain; ^d Fundacion Jimenez Díaz; ^eSwiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland; ^f Department of Biotechnology and Plant Biology, ETSI Agronómica, Alimentaria y de Biosistemas, Technical University of Madrid, Madrid, Spain; ^gDepartment of Natural Systems and Resources, ETSI Montes, Technical University of Madrid, Madrid, Spain.

The PhD student has done the most of experimental work, in collaboration to Dr Tordesillas, of the following manuscript. All experiments with immune cells has been performed by Cubells-Baeza (Figure 11, 13, 14 and 15).

Introduction

The prevalence of allergy is increasing in Europe where it has been predicted that over 50% of the population will have some form of allergy by 2050^{1,2}, with a most dramatic increase in children whose quality of life is severely affected³. The annual cost of allergic diseases for national health systems is as high as for seasonal influenza⁴, and it has been estimated that the annual cost of food allergy exceeds thousands of millions of euros in Europe alone⁵.

Even though the role of proteins in allergic sensitization has been extensively studied^{6,7}, the reasons why a specific protein may have allergenic activity are not yet well understood. Several hundred protein allergens have been described (www.allergen.org) and a large number of their epitopes identified⁸. However, only 1% of known protein families include allergens and besides, not all the members of those families are allergenic⁶⁻⁸. Although no biochemical feature common to allergenic proteins has been found, more than 50% of allergens have been shown to be lipid carriers, including Bet v 1 (the major allergen of birch pollen) and 2S allergens, which contain a large hydrophobic pocket able to harbour and store many lipid compounds and flavonoids as well⁸⁻¹⁰. The ligand of Pru p 3, the peach allergenic lipid transfer protein (LTP), has been identified as a derivative of the alkaloid camptothecin bound to phytosphingosine (Figure 11a), a lipidtail which is inserted into the Pru p 3 cavity⁹. Phytosphingosine is a structural component in membranes¹⁰, while camptothecin is widely used as a chemotherapeutic agent for its ability to inhibit the enzymatic activity of topoisomerase I¹¹.

Lipid ligands carried by allergens, or exposed together, might influence the host response to the specific allergen. Accordingly, immune-regulatory lipids could significantly contribute to the allergenicity of several proteins¹². All of these molecules are attractive candidates as non-self structures recognized by the innate immune system as being capable of inducing Th2-skewed responses that contribute to allergy in susceptible individuals¹³. Lipids from the pollen coat and furry animals as well as the so-called pollen-associated lipid mediators are co-delivered together with the allergens and can modulate the immune responses of predisposed subjects by interacting with the innate immune system and invariant natural killer T (iNKT) cells¹³.

iNKT cells are innate-like T lymphocytes with important immunomodulatory properties that can be damaging (e.g. allergic inflammation) or protective (e.g. maintenance of transplant tolerance, inhibition of autoimmunity)¹⁴. They express glycolipid-reactive $\alpha\beta$ T cell receptors (TCRs) together with several characteristic markers of NK cells and they are “CD1-restricted”¹⁵. The CD1-receptor family includes monomorphic major histocompatibility complex (MHC) class I-like glycoproteins that can present lipid antigens. The CD1 family in

humans has five members, namely CD1a–e, while rodents only express CD1d¹⁶. Given the nonpolar nature of lipids, their mobilization across an aqueous endocytic environment likely requires extraction from membranes and, in most of the cases, transport across aqueous biological buffers into the lipid-binding grooves of CD1 molecules.

The results presented in the current report suggest that the lipid-ligand carried by the peach allergen Pru p 3 could act as an adjuvant with the ability to modulate the immune system towards a Th2 response. Pru p 3 could release its lipid ligand to CD1 receptors present in the epithelial and dendritic cells, which in turn would present it to iNKT cells, ultimately resulting in activation of the immune response. Activation of the mucosal immune system by allergen-associated lipids may provide an important missing link in our understanding about the genesis of food allergic responses.

Materials and Methods

Isolation of the lipid-ligand of Pru p 3.

To carry out the assays in this work, as Pru p 3 was used the recombinant protein (Pru p 3). In this way, the absence of pollutants (residues of its lipid-ligand) in the purified protein was ensured. As complex, it referred the mix of Pru p 3 and its purified ligand (10:1). The ratio was determined by binding assays between Pru p 3 and the lipid ligand as previously described⁹.

The recombinant Pru p 3 was produced in *P. Pastoris* and isolated using the methods describe by Diaz-Perales et al. (2002)¹⁷. The presence of endotoxin in the protein-samples was measured using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, USA), following manufacturer's instructions.

The lipid-ligand of Pru p 3 was extracted from peach peel. Briefly, peach peel extract was performed in PBS with 0.5 M NaCl and separated by cation-exchange chromatography on a Waters AccellTM Plus CM SepPakR cartridge (Waters Corp, Milford, MA, USA) with 20 mM formic acid, pH 4.0. The cationic retained fraction was separated by size-exclusion LH-20 chromatography using methanol:water (70:30) as the mobile phase. Fractions were checked using thin layer chromatography (TLC) on silica gel-coated plates (Merck, USA), and developed with methanol:water (70:30 v/v) in a saturated chromatography chamber for about 20 min. The plates were visualized under UV light (254 nm) and stained with vanillin (1% in ethanol) and dichlorofluorescein (0.1% in ethanol-NaOH 2.5 mM) (Sigma-Aldrich, Germany). Fractions containing fluorescence were pooled and re-purified by HPLC as previously described¹⁸ with slight modifications. Isocratic analytical HPLC was performed using an RP-C18 column Nucleosil 120 C18 5 µm 250x4mm (Scharlab, Spain). The mobile phase for

alkaloid elution was acetonitrile: water (85:15) at a flow rate 0.5 ml/min for 150 µl of sample. Sample peaks were detected using UV light at 254 nm. The standard was prepared using methanol 80% further diluted in 20% acetonitrile. All reagents were HPLC grade.

Lymphocyte transformation test (LTI) with peripheral blood mononuclear cells (PMBCs)

Buffy coat (cell-enriched plasma) samples were obtained from 8 healthy donors at the Transfusion Centre (Madrid, Spain) for isolation of PBMCs. The study was approved by the ethics committees of the Transfusion Centre (PO.DLS.09; Madrid, Spain) and the Technical University of Madrid (PIC 52-2013; Madrid, Spain).

PBMCs isolated using a density gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway) were stained with CFSE following the manufacturer's instructions (Bdbioscience, CA, USA). Then, cultures (5×10^5 cells per well) were established in the presence of 5 µg/ml of Pru p 3 or Complex (Pru p 3 with its ligand) in triplicate in 96-well plates (Costar, NY, USA) for proliferation analysis in a total volume of 200 µl of RPMI media (Invitrogen), supplemented with 10% (v/v) of fetal calf serum (Invitrogen), 0.02 mM mercaptoethanol, 2 mM of glutamine, and 10 mM HEPES, for 5 days at 37°C, in a 5% CO₂ humidified atmosphere. The stimulation index (SI) was calculated as the ratio between counts of antigen-stimulated cultures and counts without any activator. Supernatants were obtained to measure cytokines.

The significant statistically differences were analysed by GraphPad6 using Mann-Whitney test. P-values < 0.05 were considered significant for all assays.

Quantification of the cytokines produced by PMBCs

To measure the PMBCs cytokines, Bio-Plex Pro™ Assays (Bio-Rad 10014905) for 27 different proteins detection powered by Luminex xMAP technology was used following the instructions at www.bio-rad.com/bio-plex. The final analysis was performed with the Bio-Plex Manager™ Software using xMAP technology for analysis of multiplex data. Cytokines were quantified according to a calibration curve in pg/mL (Table 2). The statistical analysis was performed using the two-way ANOVA with corrections for multiple comparisons. P values < 0.001 were considered significant.

Those, cytokines with statistical differences were also represented as fold change as result of the coefficient of amount of cytokines present in the culture with stimulus, between the cytokine content of the control without stimulation.

Generation of human antigen-presenting cells (moDC)

Monocytes were purified from human PBMCs by positive selection using CD14 Dynabeads (Invitrogen, Norway), following the manufacturer's protocol. Purity was assessed by flow cytometry (BD Accuri Cytometers, USA) and found to be higher than 95%. Dendritic cells (DCs) were derived from monocytes (moDC) by culturing the CD14⁺ fraction in complete medium RPMI (Invitrogen, Norway) with L-glutamine (brand, country), 10% heat-inactivated fetal bovine serum (FBS, Lonza, Germany), 100 mg/ml antibiotics (streptomycin and penicillin; Invitrogen, Norway), 200 ng/ml rhIL4, and 100 ng/ml rhGM-CSF (Immunotools, Germany) for 4-5 days at 5% CO₂ and 37°C, as described previously¹⁹. Immature DCs (iDCs) derived from monocytes were incubated in complete medium at 5 x 10⁵ cells/ml in 24-well plates (Falcon BD Labware, France) with Pru p 3 at 20 µg/ml, with or without lipid-ligand (2 µg/ml). LPS at 10 µg/ml (Sigma-Aldrich, USA) was used as a positive control. After 72 h stimulation at 37°C in 5% CO₂, treated DCs were recovered, and maturation was assessed by up-regulation of CD80 and CD86 (Immunotools, Germany) in an Accuri cytometer (BD Accuri Cytometers, USA). Untreated DCs were used as a control. In order to determine the receptor involved in molecular recognition, cells were previously incubated with 1 µg of polyclonal antibodies anti TLR2, TLR4 (Invivogen, France) and anti CD1d (Thermo Fisher). Results were expressed as the activation of CD80 or/and C86 and the maturation index was calculated as the ratio between stimulated and non-stimulated DCs.

The statistical analysis was performed using the Kruskal-Wallis test, and p-values <0.05 were considered positive.

NF-κB/AP-1 activation

The transfected cell line THP1-XBlue™ (Invivogen, France) derived from the human monocytic THP-1 cell line was used according to the manufacturer's instructions. In brief, THP1-XBlue cells were seeded at a density of 1x10⁶ cells/ml in cRPMI medium (RPMI 1640, 2 mM L-glutamine, 10 mM HEPES, 10% heat-inactivated FBS, PenStrep cocktail (penicillin 50 µg/ml and streptomycin 50 µg/ml) and 200 µg/ml of Zeocin. One microgram of sample/per well (5 µg/µl) was added to the cells for 24 h. LPS (Sigma-Aldrich, USA) and PBS were used as positive and negative controls, respectively. Twenty microliters of cell suspension (~200,000 cells) per well was added to a flat-bottom 96-well plate containing 180 µl of QUANTI-Blue™ per well and incubated for 18-24 h. Soluble embryonic alkaline phosphatase (SEAP) levels were determined using a spectrophotometer at 620 nm. The statistical analysis was performed using the Kruskal-Wallis test, and p-values <0.05 were considered positive.

Epicutaneous sensitization of mice and Pru p 3 challenge.

C3H/HeOuJ mice, which present normal TLR4, were obtained from Jackson Laboratories (Bar Harbor, ME) and used with the permission of the Institutional Animal Care and Use Committee (IACUC) (number LA11-00273). Six- to eight-week-old female mice were anesthetized, and abdominal fur was removed with depilatory cream (Veet, Reckitt Benckiser, Parsippany, NJ), immediately followed by exposure to Pru p 3 (100 ug), with or without lipid-ligand (10ug) in 50 ul of PBS spread on the abdominal skin to dry.

Mice were exposed weekly for a total of 6 exposures and challenged a week after the last exposure. Mice were challenged by intraperitoneal injection with increasing doses of Pru p 3. Body temperature was measured before and 30 minutes after challenge by rectal thermometer (WPI Instruments, Sarasota, FL).

Antibody measurement

Pru p 3-specific IgE and IgG1 were measured in serum obtained prior to challenge, by directly coating Pru p 3 on the plate and detecting with biotinylated anti-IgE or IgG1 (BD Biosciences, Franklin Lakes, NJ), followed by Avidin-HRP and TMB reagent (eBiosciences, San Diego, CA). A SpectraMax plate reader with background subtraction was used to measure absorbance (450 nm). The statistical analysis was performed using SPSS 17.0 and two ways ANOVA with corrections for multiple comparisons. P values < 0.05 or p < 0.01 were considered positives.

Basophil activation tests

Basophil activation tests were performed as previously described²⁰. Blood was diluted in RPMI and incubated at 37°C for 90 minutes with Pru p 3 at different concentrations. Red blood cells were lysed, and cells were stained with CD49b and IgE to detect the population of basophils, after gating out T and B cells with CD3/CD19 staining. CD200R was used as basophil activation marker (all antibodies were from eBioscience).

The statistical analysis was performed using SPSS 17.0 and two ways ANOVA with corrections for multiple comparisons. P values < 0.05 or < 0.01 were considered positives.

Epithelial cell culture

The Caco-2 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown following the manufacturer's instructions. Cells were seeded in 24-well Transwell® culture plates (0.4 mm pore diameter, Corning Inc., NY, USA) at a

density of 8×10^4 cells per well and grown for 18-21 days, replacing the medium every 2 days. The integrity of the Caco-2 cell monolayer was checked by measuring the transepithelial electrical resistance (TEER) using a Millicell-ERS device (Millipore, Bedford, MA, USA). Cell monolayers were used in transport studies when values of TEER reached a plateau exceeding 300 Wcm^2 .

Immunolocalization of Pru p 3 and innate immune receptors (TLR2, TLR4 and CD1d).

Monolayers of Caco 2 cells were cultured as described above. Pru p 3, without or with its lipid-ligand (Complex), was added in the apical chamber during short times (5 min, 10 min). Cells were then fixed with 4% formaldehyde in PBS, pH 7.4, at 4°C for 15 min. After washing with PBS, samples were permeabilized by freeze-thaw cycles. After additional washing with PBS, specific anti-Pru p 3 (1:50; Jimenez Díaz Foundation²¹), anti-TLR2 (Invivogen, France), anti-TLR4 (Invivogen, France) and anti-CD1d antibodies (Thermo Fisher) were incubated overnight at 4°C. The binding was revealed using secondary antibodies produced in the appropriate host: anti-rabbit Alexa 647-conjugated antibodies for detected Pru p 3 and anti-mouse Alexa 488-conjugated antibodies for detected the innate receptors (Molecular Probes). The specimens were mounted with glycerol:PBS 1:1 and observed with a Leica TCS-SP8 confocal microscope using the 488-nm/567-nm laser excitation.

Generation of CD1d-based Artificial Antigen Presenting cell (aAPC)

Dynabeads® M-450 epoxy beads (Invitrogen) were coated with dimeric murine CD1d:Ig fusion Protein (BD Biosciences) and with Leaf Tm purified anti-mouse CD28 clone CD28.2 (Biolegend) as costimulatory molecule at 4°C with rotation during 48 h. Subsequently, 5×10^7 beads/ml were incubated with α -Galactosylceramide (5 ug; Sigma) as a positive control, the complex (Pru p 3+lipid-ligand; 5 μ g) or lipid-ligand alone (0.5 ug) during 48 h at 37 °C. Then, beads were co-cultured with a murine iNKT cell line (DN32.D3 NKT cell hybridoma was kindly provided by Prof. Tonya Webb, University of Maryland School of Medicine, Baltimore, USA.) in 1:1 ratio in a 96 well flat bottom. The supernatant was collected after 24 h and the production of IL-2 was measured by ELISA assays.

CD1d-Dextramer loaded with the lipid-ligand of Pru p 3.

A standard protocol recommended by the company including some modifications according to the stability of the lipid-ligand was used to load the CD1d/unloaded Dextramers®(Immudex TF1037.01) with the lipid-ligand of Pru p 3. The lipid was dissolved in methanol at 1 mg/ml and dissolved completely at room temperature. Dextramer with the lipid was always kept at 2-8°C in the dark until its use with cultured cells. The binding to

experimental cells was performed at 37°C for 10 min, and then analysed by flow cytometry (BD-Accuri C6, BD-Biosciences, USA) or confocal microscopy (Leica TCS-SP8)

Statistical analysis

The statistical analysis was performed using SPSS 17.0 and GraphPad 6. The *t* test, Kruskal-Wallis test, one-way ANOVA, and two-way ANOVA with corrections for multiple comparisons were used when applicable. *p*-values < 0.05 were considered significant for all assays.

Structural analyses

For Pru p 3, the chain B of the crystal structure PDB id. 2B5S²² was used. For saposins, the crystal structures from the following PDB id's were selected: 2DOB (saposin A), 1N69 (saposin B), 2GTG (saposin C), and 2RB3 (saposin D). Identification of secondary structure in Pru p 3 and saposins was performed with DSSP²³. Three different methods to determine structural alignments were employed to compare the structures of Pru p 3 and saposins: (i) the rigid (no twists) version of FATCAT (Flexible structure AlignmenT by Chaining Aligned fragment pairs allowing Twists) algorithm²⁴, (ii) TM (Template Modelling)-align superposition algorithm²⁵ and (iii) CE (Combinatorial Extension) algorithm²⁶. According to the prescriptions given in the references for these methods, aligned structures with FATCAT are considered to have structural relationship if the alignment *p*-value is < 0.01, with lower values indicating higher similarity. Structural alignments obtained with TM-align are scored with a “TM-score” in the (0.0-1.0) scale, with values closer to 1.0 indicating better similarities and values between 0.40 and 0.50 suggesting structural relationship. For CE superpositions, *Z*-scores between 3.0 and 4.0 suggest some structural similarity and values > 4.0 indicate a stronger structural resemblance.

All the molecular graphics were prepared and rendered with PyMOL 1.8.2 (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Results

The lipid-ligand of Pru p 3 is responsible for the activation of antigen-presenting cells

The immunological activity of Pru p 3 with or without its lipid-ligand was investigated using human PBMCs obtained from four healthy donors. PBMCs were stained with CFSE and after that, Pru p 3 and the complex were added. On the 5th day, PBMCs proliferation was quantified by flow cytometer, with strong activation in the presence of the ligand (Figure 11b;

$p \leq 0.001$). This activation was also measured as the production of pro-inflammatory cytokines (Table 2). Significant differences were only observed in the production of IL8, IL10, $\text{INF}\alpha$, but especially in the production of IL13 in the presence of ligand (Figure 11c).

In the same way, moDCs derived from peripheral blood of the same donors were also incubated with Pru p 3 and its ligand. As shown in Figure 1d, the presence of the lipid-ligand gave rise to significant induction of the maturation markers CD80 and CD86, while no changes were observed when only Pru p 3 was utilized ($p \leq 0.001$).

Moreover, the immunological activity of the lipid-ligand was also confirmed using THP1-XBlue™ cells (human monocyte cell line). The lipid-ligand, alone or in complex with Pru p 3, was able to stimulate NF- κ B/AP-1 activation in these cells (Figure 11e; $p=0.01$). To identify the fraction of the lipid-ligand that had immunological activity, THP1-XBlue™ cells were again incubated in the presence of increasing amounts of the lipid-ligand, camptothecin and phytosphingosine. Thus, it was phytosphingosine the only one to be capable of inducing a similar response as that produced by the lipid-ligand (Figure 11f), indicating that the immunological activity was associated to the phytosphingosine hydrophobic moiety and not to the camptothecin polar head of the lipid-ligand (Figure 11a).

Figure 11. Characterization of immunogenic activity of the lipid-ligand of Pru p 3. (A) Structural formula of the lipid-ligand of Pru p 3 (10-hydroxy-camptothecin linked to phytosphingosine) (B) Proliferation assays of human PBMCs (n=6) stained with CFSE were incubated with Pru p 3 and Complex (Pru p 3; 10 μg/ml and the lipid-ligand 1 μg/ml). After 5 days, the proliferation of PBMCs was measured by loss of CFSE staining. Stimulation index (SI) was calculated as (% CFSE^{neg}-PBMCs + antigen) / (% CFSE^{neg}-PBMCs – antigen). Means and SE (bars) are shown. *p < 0.05. (C) Cytokine produced by PMBCs by Bioplex system. The results are expressed as the fold change between the amount (pg/mL) produced in the presence of the stimulus (Pru p 3, Complex), and the amount without stimulus. Means and SE (bars) are shown. *p < 0.001. (d) Phenotype of human monocyte-derived DCs (moDC) induced by Pru p 3 and its lipid-ligand. Changes in the expression of costimulatory molecules (CD80 and CD86) in monocyte-derived DCs from healthy donors, after stimulation with Pru p 3 alone (Pru p 3; 10 μg/mL), with the lipid-ligand (Complex; 10 μg/ml), and with the lipid-ligand only (Ligand; 1 μg/mL). Maturation indices of CD80 and CD86 expression are shown (p<0.05). (E,F) NF-κB/AP-1 activation using the transfected cell line THP1-XBlue™. Cells (1×10⁶ cells/ml) were incubated with Pru p 3 (Pru p 3; 5 μg/ml), Pru p 3 with lipid-ligand (Complex; 5 μg/ml) and only lipid-ligand (Ligand; 1 μg/ml) in (e), or with the lipid-ligand (Ligand), phytosphingosine (Phytosphingosine) and camptothecin (CPT) in (F). In both, LPS was used as positive control. Activation of NF-κB/AP-1 was revealed with QUANTI-Blue™. *p < 0.05 **p < 0.01 ***p < 0.001

Table 2: Cytokine production of PBMCs from healthy donors.

Cytokine (pg/mL)	Control	Complex	Pru p 3
IL-1b	8,36 ± 11,48	11,58 ± 19,24	6,81 ± 10,08
IL-10	62,06 ± 13,24	66,39 ± 2,86	50,42 ± 13,27
IL-1ra	999,36 ± 1147,53	1105,20 ± 1168,56	585,15 ± 304,86
IL-2	ND	ND	ND
IL4	24,53 ± 30,58	33,53 ± 29,71	27,08 ± 24,33
IL5	ND	ND	ND
IL6	ND	ND	ND
IL7	279,67 ± 116,58	262,05 ± 71,76	250,69 ± 84,20
IL8	6781,23 ± 7082,21	12150,06 ± 11266,12	9315,71 ± 9241,23
IL10	97,98 ± 129,32	105,32 ± 136,90	81,40 ± 98,49
IL12	694,87 ± 122,99	740,14 ± 95,20	632,33 ± 176,93
IL13	28,86 ± 15,64	39,65 ± 11,53	28,81 ± 8,36
IL15	ND	ND	ND
IL17	33,81 ± 50,81	33,11 ± 46,27	37,42 ± 52,35
Eotaxin	331,16 ± 241,30	365,23 ± 163,45	309,94 ± 103,64
FGF basic	111,18 ± 55,47	79,77 ± 20,76	104,38 ± 58,08
G-CSF	57,58 ± 61,06	81,99 ± 60,49	121,83 ± 100,20
GM-CSF	ND	ND	ND
IFN-γ	277,27 ± 286,14	360,44 ± 182,14	285,65 ± 121,41
IP10	8938,96 ± 7215,37	15385,28 ± 11246,47	12686,54 ± 10118,47
MCP-1	1221,35 ± 2442,71	1288,37 ± 2576,75	ND 0,00
MIP-1a	27,85 ± 52,99	18,77 ± 34,45	7,15 ± 11,66
PDGF-bb	7906,00 ± 13282,38	7444,10 ± 12840,43	7394,45 ± 13354,97
MIP-1b	112,93 ± 172,62	75,04 ± 103,32	22,49 ± 20,20
Rantes	58940,23 ± 34494,60	64969,43 ± 34954,12	59924,48 ± 34279,81
TNF-α	9,64 ± 19,27	5,19 ± 9,01	2,00 ± 3,99
VEGF	15288,22 ±	16459,71 ±	13460,43 ±

*ND, non detected.

The lipid-ligand of Pru p 3 increases the capacity to sensitize mice through the epicutaneous route

To determine the role of the lipid-ligand of Pru p 3 in inducing allergic sensitization, mice were epicutaneously exposed to Pru p 3, with or without its lipid-ligand, once a week for six weeks, following a previously described protocol²⁷ (Figure 12a). Briefly, hair was removed with depilatory cream, but no tape stripping was performed and the antigen was added topically in PBS without occlusive bandages. After six weeks of sensitization, blood was obtained for basophil activation test and specific immunoglobulin measurement. Mice exposed to Pru p 3 plus its lipid-ligand (*Complex* in Figure 12b) developed significantly more Pru p 3-specific IgE than Pru p 3 alone (*Pru p 3*, Figure 12b), while IgG1 levels were similar in both cases. Similarly, basophil activation was increased in mice sensitized with the complex, as reflected by a higher increase in CD200R MFI (Figure 12c). Despite the differences in IgE induction, when mice were intraperitoneally challenged with Pru p 3, no differences were observed in terms of drop in body temperature during anaphylactic responses (Figure 12d).

Figure 12. Impact of the lipid-ligand of Pru p 3 on peach allergy. (A) Mice were left naïve or topically exposed to 100 ug of Pru p 3 alone (*Pru p 3* label) or Pru p 3 together with 10 ug of its lipid-ligand (*Complex*) once per week for six weeks. After sensitization, intraperitoneal challenge was performed with Pru p 3. (B) Antigen-specific IgE and IgG were measured prior to allergen challenge. Data are mean $\pm$ SEM. (C) Pru p 3-induced basophil activation was measured by up-regulation of CD200R. Change in MFI (median fluorescence intensity) was calculated with respect to the stimulation with media alone. Data are mean $\pm$ SEM. (D) Mice were challenged with increasing doses of Pru p 3 by intraperitoneal challenge. Body temperature prior to the challenge and 30 minutes after each ip injection are shown. $n = 5$ mice per group. * $p < 0.05$, ** $p < 0.01$.

Pru p 3 is bound by CD1d in the presence of the lipid-ligand.

To determine which receptor mediated the recognition of the lipid-ligand, specific antibodies to TLR2, TLR4 and CD1d (receptors that recognize lipid ligands) were added to Pru p 3-moDCs cultures and DC maturation was studied. Maturation of human moDC could be only inhibited in presence of anti-CD1d (Figure 13a), but not with polyclonal antibodies anti-TLR2 or anti-TLR4 ($p < 0.001$).

In the same way, polarized epithelial monolayers (Caco2 cells, Figure 13b) were also incubated with Pru p 3 (*Pru p 3*) and complex (*Complex*), during short times (5 min). After fixing cells, immune receptors (PRR) such as *TLR2*, *TLR4* and *CD1d* were localized using specific antibodies. In the presence of the ligand, Pru p 3 colocalized with CD1d-type receptors (Figure 13c) but not when Pru p 3 was alone (Figure 13d). In the case of TLR2 and TLR4, no differences in localization were observed in the presence of absence of the lipid-ligand (Figure 14).

Figure 13. (A) Inhibitory effect in the activated phenotype (positive CD80 and/or CD86) of moDCs, after stimulation with Pru p 3 (Pru p 3; 10 µg/mL) or Pru p 3 with its lipid-ligand (Complex; 10 µg/ml) and specific antibodies against TLR2, TLR4 and CD1d. (*p < 0.05). (B-I) Immunolocalization of Pru p 3 (Red) or Complex (Red), and CD1d surface proteins (Green) in polarized CaCo 2 monolayer after 5 minutes incubation. (B) DAPI staining (E) Phase contrast. (C, D) Co-localization was just found between CD1d protein expression and Pru p 3. Bar 20 microns. 190x254mm (300 x 300 DPI)

Fig 14 Immunolocalization of Caco 2 monolayer after 5 minutes of incubation with Pru p 3 or Complex. Pru p 3 was detected by polyclonal antibodies in red (Alexa 647) and TLR2 (B) and TLR4 (A) were detected in green (Alexa 488) . From left to right: TLR2 (green), Pru p 3 (red), DAPI, phase contrast, triple combination (TLR/Pru p63/DAPI) and colocalization of Pru p 3 and TLR. Bar 20 microns.

The lipid-ligand presented by CD1d could stimulate iNKTs.

According to the results above described, we studied the role of the lipid-ligand in the activation of iNKT cells through CD1d receptor. iNKTs have been described as specialized cells in the recognitions of glycolipid antigens. Using a CD1d-based artificial antigen-presenting cell system (aAPC)²⁸ and the murine DN32.D3 iNKT cell hybridoma, a clear increase in the secretion of IL-2 was observed in the presence of the lipid-ligand (*Ligand*) and the positive control α -Galactosylceramide (*Positive*), indicating the activation of iNKTs (Figure 14a).

To further demonstrate that the lipid-ligand could interact with iNKTs through the CD1d receptor, the lipid-ligand was incubated with a CD1d unloaded human dextramer according to the manufacturer's instructions. This probe is capable of binding to the TCR present on the surface of iNKTs provided it has the appropriate lipid. As can be seen in

Figure 14b, the fluorescent probe loaded with the lipid-ligand (labelled with APC) was able to interact with iNKT cells (labelled with FITC). In fact, most of the lipid-ligand-CD1d probes bound specifically to iNKTs (Figure 15b). In the figure 15c, it could be observed two examples of iNKT cell surrounded by CD1d-tetramers-lipid-ligand probe labelled with APC (red) .

Figure 15. (A) CD1d-based artificial antigen-presenting cells (aAPC) were incubated with a positive control (α -Galactosylceramide; 5 μ g), and with the lipid-ligand (0.5 μ g) during 48h at 37 °C. After that, aAPC were co-cultured with iNKT cells at a 1:1 ratio in a 96 well flat bottom. The supernatant was collected at 24h and the production of IL-2 was measured by ELISA assays. (B) Cyc5-labeled CD1d-tetramers (red) were loaded with the lipid-ligand of Pru p 3 and incubated with healthy volunteer's PBMCs during 2 h. iNKTs were detected with a monoclonal antibody specific for the TCR of iNKTs labelled FITC (Va24-Ja18 combined with V β 11). (C) Confocal microscopy image of CD1d-tetramers-lipid-ligand probe (red) and TCR iNKTs (green) and phase contrast image of two different examples.

Pru p 3 could play a putative role as an external saposin.

The experimental structure of Pru p 3 shows similar features to those of saposins, small non-enzymatic lipid transfer proteins that assist the loading of lipids onto CD1d. There are four saposins A, B, C, and D with pairwise sequence identity not higher than 39% that show a highly conserved structure with four α -helices and three disulphide bridges. Although not essential for their activity, they have a conserved N-glycosylation site (Figure 16a). The spatial arrangement of the four α -helices is nearly identical (Figure 16b). However, while in the absence of lipids,

saposins adopt a compact form (saposins A, C and D in Figure 16b), in the presence of lipids they undergo a conformational change adopting an open form (saposin B in figure 16b). The architecture of saposins also present some particular features: (i) the longest helix has a highly localized kink associated with a local disruption of two main-chain hydrogen bonds around a conserved Tyr54 residue which is not the classic example of disruption caused by a proline, (ii) the shortest $\alpha 4$ helix starts at a conserved proline, and (iii) with the exception of saposin C, they also have short 3_{10} helices (Figure 16a and 16b).

The structure of Pru p 3 (Figure 16c) presents significant structural similarities to saposins. Not only Pru p 3 has a similar architecture with four α helices, one 3_{10} helix and disulfide bonds (four instead of three in saposins), but it also displays particular structural features analogous to those indicated above for saposins. In fact, Pru p 3 presents a kink in the longest α helix (although the disruption of main-chain hydrogen bonds is caused by a proline), its shortest $\alpha 4$ helix starts also at a proline and it exhibits also a three-residue 3_{10} helix (Figure 16a and 16d). Besides these structural characteristics, Pru p 3 is similar to saposins in size, existence of an inner cavity and lipid transfer abilities that are known to span diverse lipidligands²⁹. Three procedures used to obtain structural alignments based on different approaches (FATCAT, TM-Align, and CE: see Materials and Methods) found that the best structural similarity is actually that existing between Pru p 3 and saposin A (Figure 15e). Superposition scores for these three methods were the following. FATCAT (rigid algorithm) significance p-value score was 0.00196 for Pru p 3/saposin A while it ranged between 0.00348 and 0.0101 for the remaining saposins. TM-Align TM-score was 0.403 for Pru p 3/saposin A while it ranged between 0.358 and 0.392 for the remaining saposins and CE significance Z-score was 4.07 for Pru p 3/saposin A while it ranged between 1.99 and 3.50 for the remaining saposins. Within the confidence levels conveyed by those scores (see Materials and Methods), it can be concluded that Pru p 3 and saposin A have a significant structural relationship.

Figure 16. Structures of saposins and Pru p 3. (A) Alignment of sequences of saposins A-D and sequence of Pru p 3. Colours labelling the residues that form α and 3_{10} helices are also used in (B)-(D) below. Black lines connecting marked cysteines represent disulphide bridges. Red box indicates conserved N-linked glycosylation sites and green box indicates conserved tyrosines at the kink of helix $\alpha 3$ shown in (D) for saposin A. (B) X-ray crystal structures of saposins A-D. (C) X-ray crystal structure of Pru p 3. (D) Kink in the longest helix $\alpha 1$ in Pru p 3 and $\alpha 3$ in saposin A. Dashed lines in these helices represent main chain hydrogen bonds. (E) Structural superposition of saposin A (blue) and Pru p 3 (orange) computed with FATCAT rigid.

Discussion

According to our own results and evidence reported in the literature in recent years, we hypothesized that allergic sensitization is enhanced by the recognition of lipid-ligands carried by some allergens. These lipid-ligands could be exposed to immune cells at the time of first contact, inducing the production of specific IgE against the protein carrier associated with the ligands. In a later encounter, the interaction between the allergen and specific IgE bound to the high-affinity receptor (FcεRI) on the surface of effector cells (mast cells and basophils) would induce the symptoms associated with allergy.

In the case of Pru p 3, our results reported here suggest that the lipid-ligand of this LTP is a critical collaborator for activation of dendritic cells, CD1 presentation and iNKT cells, associated with enhanced induction of antigen-specific IgE in mouse models.

The lipid-ligand carried by Pru p 3 seems to act as an adjuvant with the ability to modulate the immune system toward a Th2 response associated with class-switching towards IgE production, which is characteristic of allergy. Although Pru p 3 in the absence of the lipid-ligand was able to sensitize mice, the Pru p 3-lipid-ligand complex induced a much more robust IgE production. This result suggests that the presence of the lipid-ligand could increase susceptibility to peach allergy and decrease the exposure level to Pru p 3 required to induce sensitization to this allergen, thus acting as an adjuvant. In addition, we have shown for the first time that Pru p 3 is able to sensitize through the skin in a mouse model. The results presented here and the fact that the majority of peach allergic patients experience their first allergic reaction the first time they consume this food, together suggest that epicutaneous exposure is the main route of sensitization to peach, as it has been proposed for other allergens^{27,30,31}.

In this sense, Pru p 3 in the presence of its lipid-ligand was presented by CD1d receptors in epithelial and dendritic cells. CD1 proteins are expressed on professional APCs such as Langerhans cells, dendritic cells, monocytes, B lymphocytes, intestinal epithelium, smooth muscle and endothelial blood vessels. They can present lipids and glycolipids to T-cell subpopulations, which in turn mediate effector functions including cytokine secretion^{16,32}. CD1 lipid loading seems to occur in endosomal compartments of many antigen presenting cells such as epithelial mucosa. In their trafficking and binding to lipid antigens for recognition of T-cell receptors, CD1 molecules are assisted by saposins, small lipid transfer proteins that facilitate non-enzymatically the hydrolysis of a variety of glycosphingolipids in lysosomes^{33,34}, although the mechanism of this transfer and whether there is a direct CD1-saposin binding is still unknown. As CD1 molecules recycle through cellular

compartments, they encounter in addition to saposins other proteins such as Niemann-Pick type C2 protein³⁵ or apolipoprotein E³⁶ that are also involved in the loading of lipids onto CD1.

Based upon their similar structural features, we propose that there could also be a functional relationship between Pru p 3 and saposins. There are four homologous saposins A, B, C, and D that derive from a unique prosaposin precursor protein upon endosomal proteolytic cleavage. Saposins are cysteine-rich proteins with four α -helices that exist in both isolated soluble and lipid-bound states. Although their sequences have pairwise identities not higher than 39%³⁷, their topology is conserved and their architecture is rather similar. Structural alignments determined with three different algorithms reveal a significant relationship between Pru p 3 and saposin A. Given these structural resemblances together with the similarities in size, cavity and lipid transfer abilities, we conjecture that Pru p 3 could play a role in the trafficking of molecules with lipidmoieties that are eventually loaded onto CD1d receptors.

In any event, we have shown in this work that the lipid-ligand of Pru p 3, perhaps especially its phytosphingosin domain, is presented to iNKT cells by CD1 receptors. Although it is likely that this is not the only innate immune pathway involved in the recognition of Pru p 3, our results reveal that it is active in moDC and epithelial mucosa. This is not the first report associating iNKTs to allergen activation. iNKTs can be activated by phospholipids, specially phosphatidylcholine and phosphatidylethanolamine present on the surface of cypress nuts¹⁶. A recent study analyzed the general ability of olive pollen lipids to activate DCs and stimulate iNKT cells and demonstrated that polar lipids isolated from olive pollen grains upregulated CD1d on DCs, which then activated iNKT cells in co-cultures³⁸. In the same way, the 2S albumin from Brazil nut, Ber e 1, was not sufficient to cause IgE or IgG production in mice³⁹. In contrast, the co-administration of the allergen with polar lipid fractions isolated from the same nut induced the production of Ber e 1-specific IgE and IgG1. In the absence of iNKT cells, the specific IgE levels were lower⁴⁰. Lastly, it has been suggested that the activation of iNKT cells induce Th2-mediated inflammation to cow's milk-derived sphingolipids⁴¹.

In summary, we have shown that the lipid-ligand of Pru p 3 enhances the allergic activity and participates actively in the triggering of antigen-presenting cells. Pru p 3 would tend to be targeted by innate immune response because of its auto-adjuvant lipid-ligand. Moreover, Pru p 3 has structural features that support its possible involvement in trafficking and loading of lipidcompounds onto CD1d receptors. The fact that other allergens have been described as lipid binding proteins, suggest that intrinsic adjuvant activity by such proteins and their accompanying lipid cargo could be a general essential feature of the mechanisms underlying the phenomenon of allergenicity.

References

1. Pawankar, R., Canonica, G. W., Holgate, S. T. & Lockey, R. F. Allergic diseases and asthma. *Curr. Opin. Allergy Clin. Immunol.* 12, 39–41 (2012).
2. Pawankar, R. The unmet global health need of severe and complex allergies: meeting the challenge. *World Allergy Organ. J.* 5, 20–1 (2012).
3. Hedlin, G. *et al.* Problematic severe asthma in children, not one problem but many: a GA2LEN initiative. *Eur. Respir. J.* 36, (2010).
4. Muraro, A. *et al.* The management of the allergic child at school: EAACI/GA2LEN Task Force on the allergic child at school. *Allergy* 65, 681–689 (2010).
5. Fox, M. *et al.* Health sector costs of self-reported food allergy in Europe: a patient-based cost of illness study. *Eur. J. Public Health* 23, 757–762 (2013).
6. Breiteneder, H. *et al.* A classification of plant food allergens. *J. Allergy Clin. Immunol.* 113, 821–30; quiz 831 (2004).
7. Hoffmann-Sommergruber, K. Plant allergens and pathogenesis-related proteins. What do they have in common? *Int. Arch. Allergy Immunol.* 122, 155–66 (2000).
8. Aalberse, R. C. & Cramer, R. IgE-binding epitopes: a reappraisal. *Allergy* 66, 1261–1274 (2011).
9. Cubells-Baeza, N. *et al.* Identification of the ligand of Pru p 3, a peach LTP. *Plant Mol. Biol.* 94, 33–44 (2017).
10. Fischer, C. L. *et al.* The roles of cutaneous lipids in host defense. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1841, 319–322 (2014).
11. Lorence, A. & Nessler, C. L. Camptothecin, over four decades of surprising findings. *Phytochemistry* 65, 2735–2749 (2004).
12. Thomas, W. R. Allergen Ligands in the Initiation of Allergic Sensitization. *Curr. Allergy Asthma Rep.* 14, 432 (2014).
13. Bublin, M., Eiwegger, T. & Breiteneder, H. Do lipids influence the allergic sensitization process? *J. Allergy Clin. Immunol.* 134, 521–9 (2014).
14. Szabo, P. A., Anantha, R. V., Shaler, C. R., McCormick, J. K. & Haeryfar, S. M. M. CD1d- and MR1-Restricted T Cells in Sepsis. *Front. Immunol.* 6, 401 (2015).
15. Hofmann, S. C. *et al.* Invariant natural killer T cells are enriched at the site of cutaneous inflammation in lupus erythematosus. *J. Dermatol. Sci.* 71, 22–28 (2013).
16. Agea, E. *et al.* Human CD1-restricted T cell recognition of lipids from pollens. *J. Exp. Med.* 202, 295–308 (2005).
17. Diaz-Perales, A. *et al.* cDNA cloning and heterologous expression of the major allergens from peach and apple belonging to the lipid-transfer protein family. *Clin. Exp. Allergy*

- 32, 87–92 (2002).
18. Malpathak, N., Kulkarni, A., Patwardhan, A. & Lele, U. Production of camptothecin in cultures of *Chonemorpha grandiflora*. *Pharmacognosy Res.* 2, 296 (2010).
  19. Tordesillas, L. *et al.* Transport of Pru p 3 across gastrointestinal epithelium - an essential step towards the induction of food allergy? *Clin. Exp. Allergy* 43, 1374–1383 (2013).
  20. Leonard, S. A. *et al.* Dietary baked egg accelerates resolution of egg allergy in children. *J. Allergy Clin. Immunol.* 130, 473–480.e1 (2012).
  21. Palacín, A. *et al.* Graph Based Study of Allergen Cross-Reactivity of Plant Lipid Transfer Proteins (LTPs) Using Microarray in a Multicenter Study. *PLoS One* 7, e50799 (2012).
  22. Pasquato, N. *et al.* Crystal Structure of Peach Pru p 3, the Prototypic Member of the Family of Plant Non-specific Lipid Transfer Protein Pan-allergens. *J. Mol. Biol.* 356, 684–694 (2006).
  23. Kabsch, W. & Sander, C. Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22, 2577–2637 (1983).
  24. Ye, Y. & Godzik, A. Flexible structure alignment by chaining aligned fragment pairs allowing twists. *Bioinformatics* 19 Suppl 2, ii246-55 (2003).
  25. Zhang, Y. & Skolnick, J. TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Res.* 33, 2302–2309 (2005).
  26. Shindyalov, I. N. & Bourne, P. E. Protein structure alignment by incremental combinatorial extension (CE) of the optimal path. *Protein Eng.* 11, 739–47 (1998).
  27. Tordesillas, L. *et al.* Skin exposure promotes a Th2-dependent sensitization to peanut allergens. *J. Clin. Invest.* 124, 4965–4975 (2014).
  28. East, J. E., Sun, W. & Webb, T. J. Artificial antigen presenting cell (aAPC) mediated activation and expansion of natural killer T cells. *J. Vis. Exp.* (2012). doi:10.3791/4333
  29. Pacios, L. F. *et al.* Computational study of ligand binding in lipid transfer proteins: Structures, interfaces, and free energies of protein-lipid complexes. *J. Comput. Chem.* 33, 1831–1844 (2012).
  30. Benedé, S., Blázquez, A. B., Chiang, D., Tordesillas, L. & Berin, M. C. The rise of food allergy: Environmental factors and emerging treatments. *EBioMedicine* 7, 27–34 (2016).
  31. Noti, M. *et al.* Exposure to food allergens through inflamed skin promotes intestinal food allergy through the thymic stromal lymphopoietin–basophil axis. *J. Allergy Clin. Immunol.* 133, 1390–1399.e6 (2014).
  32. Stenger, S. *et al.* Differential effects of cytolytic T cell subsets on intracellular infection. *Science* 276, 1684–7 (1997).
  33. Leon, L. *et al.* Saposins utilize two strategies for lipid transfer and CD1 antigen

- p presentation.
- Proc. Natl. Acad. Sci.*
- 109, 4357–4364 (2012).
34. Vartabedian, V. F., Savage, P. B. & Teyton, L. The processing and presentation of lipids and glycolipids to the immune system. *Immunol. Rev.* 272, 109–119 (2016).
  35. Barral, D. C. & Brenner, M. B. CD1 antigen presentation: how it works. *Nat. Rev. Immunol.* 7, 929–941 (2007).
  36. Zajonc, D. M. & Girardi, E. Recognition of Microbial Glycolipids by Natural Killer T Cells. *Front. Immunol.* 6, 400 (2015).
  37. Ahn, V. E., Leyko, P., Alattia, J.-R., Chen, L. & Privé, G. G. Crystal structures of saposins A and C. *Protein Sci.* 15, 1849–57 (2006).
  38. Abós-Gracia, B. *et al.* Olea europaea pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J. Allergy Clin. Immunol.* 131, 1393–9.e5 (2013).
  39. Dearman, R. J., Alcocer, M. J. C. & Kimber, I. Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1. *Clin. Exp. Allergy* 37, 582–591 (2007).
  40. Mirotti, L. *et al.* Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. *Allergy* 68, 74–83 (2013).
  41. Jyonouchi, S. *et al.* Invariant natural killer T cells in children with eosinophilic esophagitis. *Clin. Exp. Allergy* 44, 58–68 (2014).

3.3 The effect of protein transportation through small intestine on the immune resident cells using the InTESTine™ system. Prup 3 as a model

Nuria Cubells-Baeza¹, Carmen Ramirez-Castillejo¹, Maria Garrido-Arandia¹, Luis F. Pacios^{1,2}, K.C.M. Verhoeckx³, Araceli Díaz-Perales¹.

¹Center for Plant Biotechnology and Genomics (UPM-INIA), Pozuelo de Alarcon, Madrid, Spain

²Department of Natural Systems and Resources, ETSI Montes, Technical University of Madrid, Madrid, Spain

³TNO, Zeist, The Netherlands

The PhD student has done the experimental work and collaborated in the writing of the following manuscript. The samples were obtained in the TNO (Utrecht, Holland) at one of her stays during this thesis work. The process and analysis of the samples were conducted in the UPM.

Introduction

The transport of food proteins across the gastrointestinal barrier should be necessary to induce allergic sensitisation, making that process an important factor in allergy research. However, the knowledge on how proteins are transported and which immune cells are induced in the intestinal mucosa is still far from being complete¹. The gastrointestinal barrier has different roles to fulfill such as absorbing and processing nutrients. The intestinal tract is composed of different layers such as glycocalyx, luminal mucosa (made of a monolayer of epithelial cells), lamina propria (connective tissue scaffold with blood and lymphatic vessels), and the muscularis mucosa (muscle layer)². The lamina propria is highly folded to form villi, increasing the intestinal surface. This surface is covered by a heterogeneous population of epithelial cells, including absorptive intestinal epithelial cells (enterocytes), enteroendocrine cells (L-cells), mucin-secreting goblet cells and microfold cells (M cells)³. The epithelial cells are joined at their apical side by tight junctions and other integral membrane proteins (e.g. claudins and occludins) that prevent the passage of macromolecular compounds over 600 Da^{4,5}. Mucus, a mixture consisting mainly of water, glycoproteins (e.g. mucin), lipids and proteins, forms a protective layer of 50–450 µm on top of the intestinal epithelial cell layer⁶.

After the consumption of food, the majority of proteins are degraded by the action of gastric, pancreatic and small intestinal brush border proteases (such as pepsin, trypsin and chymotrypsin), resulting in a mixture of free amino acids, di- and tri-peptides, intact proteins and other products of proteolysis. The majority of intact proteins, absorbed by enterocytic cells, are degraded by intra-cellular enzymes⁷. So, only the 10% of the non-degraded proteins are able to cross the intestinal barrier intact, as previously demonstrated^{8–10}. These intact proteins should be the responsible for induction of the immune response mediated by resident cells.

The absorption of food proteins is regulated by intestinal permeability, which is a critical feature of the gastrointestinal epithelium. Permeability allows the efficient absorption of nutrients, and restricts the entry of large molecules so that the appropriate immune responses against food antigens can occur¹². However, a small proportion of intact antigens can reach the lamina propria, the intestinal lymphatic system, and subsequently enter the circulation. These antigens are taken up by antigen-presenting cells in the lamina propria, and then processed and presented to the surrounding T cells or T cells in mesenteric lymph nodes (MLN)¹². Nonetheless, little is known of the immune system cells that are related to allergenic proteins, and where that initial response takes place.

Several *in vitro* methods have been intensely applied to study intestinal protein absorption, in particular by means of polarized cell lines (e.g. Caco-2)¹³. Using cell lines as the object of study, the intestinal transport of proteins is the relatively high throughput at which different proteins can be studied. In contrast, due to the lack of a physiologically relevant

environment (e.g. interactions with other cell-types, intestinal fluid composition and mucus presence), prediction of the immunological role of allergens might be prone to mistakes. In this context, the InTESTine™ system has been recently developed. This system uses different parts of intestinal tissue from pigs, incubated on a rocker platform in a high oxygen incubator. An advantage of using animal *ex vivo* tissue is the possibility to investigate the sensitisation effect on the intestinal absorption of proteins by immunising the tissue *in vivo*¹⁴. Pigs share many physiological and immunological similarities with humans, more than rodents, and the use of (mini) pigs is becoming increasingly common in nutritional research¹⁵. In the InTESTine™ system, the viability of tissue can be assured for 2 h¹⁶ and the transport of macromolecules can be studied using labelled proteins¹.

On the other side, increasing evidences suggest that the allergic sensitization might be due to a variety of compounds such as lipid ligands transported by the allergens¹⁷. This is the case of Pru p 3, a lipid transfer protein (LTP) and the principal allergen of peach¹⁸. We aimed to investigate the immune response induced by the ligand of Pru p 3 in the InTESTine™ system, using two different parts of the intestinal mucosa: jejunum, non-Peyer's Patches (NPP), and ileum, Peyer's Patches (PP). To this end, the transport of the allergen through mucosa, cytokine profiles and identification of the resident immune cells were analysed by means of different techniques.

Materials and methods

Porcine intestinal tissue collection

Two healthy domestic pigs (*Sus scrofa domesticus*, female, bodyweight 65-67 kg) from GDL (Gemeenschappelijk Dieren Laboratorium, Utrecht University) were used for acquisition of fresh porcine tissue. The InTESTine™ system was performed in the TNO laboratory facilities (Zeist, Utrecht) using the methodology previously described by Westerhout, J. *et al*¹⁶. Briefly, jejunal and ileum were excised and stored in cold Krebs-Ringer Bicarbonate Buffer (Sigma) pH 7.4 (10 mM glucose, 2.5 mM calcium chloride, 15 mM sodium bicarbonate, 25 mM HEPES) and perfused with carbogen (95% O₂, 5% CO₂) for 120 min. After removing muscularis propria, 2 cm segments having an exposed surface area of 0.7 cm² were collected and placed in the InTESTine™ system. In the apical side of the chamber, Pru p 3 and the complex (Pru p 3 together with its ligand) previously labelled with Alexa 647 dye (Thermo Fisher Scientific) were added in 700 µl of KRB buffer with a final concentration of 47 µg/ml of Pru p 3 and 4.7 µg/ml of the lipid ligand (t=0). Basolateral medium (7.5 ml) containing KRB buffer + 0.1 % BSA were collected at different time points.

The segments were incubated at 37°C with 90% O₂, 5% CO₂ and continuous slight shaking. In the case of NPP segments, collection times were 20, 45, 105, and 165 min whereas for PPs segments, times were 20 and 45 min. The transport of proteins was measured from freeze-dried basolateral mediums on a white 96 well plate. The fluorescence was measured with a fluorescence microplate reader TECAN (Männedorf, Switzerland) at 612/670 nm. The amount the protein was calculated according a standard curve.

The integrity of the intestinal barrier during all the experiment was tested by measuring the leakage with FD4 (fluorescein isothiocyanate-dextran) at final concentration of 50 µM on the apical side and the linearity of transport with [¹⁴C] Caffeine (9 µM) and [³H] Mannitol (10µM). Less than 0.5 % FD4 and P^{app} caffeine/ P^{app} mannitol over 3 was obtained, which was considered optimal conditions. The transport of Pru p 3 and LTP1 was quantified by measuring the fluorescence of the supernatants recovered from the basolateral or apical side, using a fluorescence microplate reader (Spectrafluor Fluorometer TECAN; TECAN group, Männedorf, Switzerland) at 485/535 nm. All tests were performed in four independent assays.

Immunohistochemistry

Pig tissues were placed into a cassette and fixed in paraformaldehyde 4 % in PBS overnight. Then, they were transferred to a 30% sucrose solution in PBS for 48 hours completely submerged. Samples were put in cryomolds with cryocompound (Klinipath) and frozen using 2-methylbutane (Sigma) cooled with dry ice. 15 µm sections were cut with a microtome at -20°C (Leica CM1860 UV) and put onto glass slides and stored in the freezer. Immunohistochemistry was performed in whole-mount tissue by incubating overnight at 4 °C with specific antibodies (1:100) against CD1d (Thermo Fisher Scientific), iNKT (MACS), CD4, CD8, CD5 and CD19 (Immunotools). Tissue sections were mounted with PBS on microscope slides (Thermo Fisher Scientific) and observed with a Leica TCS-SP8 confocal microscope using appropriate laser excitation lines.

Cytokine profile expression

Total RNA was extracted from the tissues with Nucleospin® RNA (Macherey-Nagel 740955.250) according to the manufacturer's recommendations. Spectrophotometry (260/280 nm) was used to determine RNA quality and concentration. The first-strand cDNA was synthesized from 2 µg of total RNA treated with DNase I (Roche, Basle, Switzerland) using the primers detailed in Table I and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). Relative real-time PCR quantification was performed using Power

SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations.

Primer name	Sequence 5'-3'	Source	Tm
GAPDH forward GAPDH reverse	ACACTCACTCTTCTACCTTTG CAAATTCATTGTCGTACCAG	(Nygard, Jørgensen <i>et al.</i> 2007)	58°C
IL 25 forward IL 25 reverse	GGGAACCCACACCTTCCATTT GCATTCTGCTTCAGCCACTC	NCBI	58°C
TSLP forward TSLP reverse	CCTCCAGCTTTGTGGCAGTT AGTTCTCCTCCTCCTACACTTTT	NCBI	60°C
IL 33 forward IL 33 reverse	GCAGCCCTCCAAATGTGTTTC CTCAGGGTGTCCCCTAGTT	NCBI	60°C
IL 10 forward IL 10 reverse	AATGCCGAAGGCAGAGAGTG ACGGCCTTGCTCTTGTTTTT	NCBI	59°C
IL 4 forward IL 4 reverse	CTCCCAACTGATCCCAACCC TGCACGAGTTCTTTCTCGCT	NCBI	60°C
IL 8 forward IL 8 reverse	GGCCAGCATTCACAAGTCTTC AAGGTAGGATGGGGGCTGAA	NCBI	58°C
IFN- γ forward IFN- γ reverse	GGCCATTCAAAGGAGCATGG AGTTCATGATGGCTTTGCG	NCBI	59°C
IL13 forward IL13 reverse	CAAGCACATCCGAGACACCA CTGAACCTACCCGTGGCGAAA	NCBI	59°C
IL12 forward IL12 reverse	GTCCATCCATCTGCGTCCAG CCTGCCCAAACCTGAGATGGT	NCBI	59°C
IL2 forward IL2 reverse	TGCACTAACCCCTTGCACTCA GCATCCTGGAGAGATCAGCA	NCBI	58°C
V α 24 forward V α 24 reverse	ACACAAAGTCGAACGGAAG GATTTAGAGTCTCTCAGCTG	(Peterfalvi, Gomori <i>et al.</i> 2008)	60°C

Thermal cycling conditions were as follows: 10 min at 95 °C, 15 s at 95 °C, and 45 s at (58-60 °C), 40 cycles. Optimal cDNA and primer concentrations were determined by serial dilution experiments. Finally, 20 ng of cDNA per well were added, and the final concentration of the primers was 1.5 μ M. The amount of mRNA expression was normalized using the endogenous control GAPDH, and relative quantification was performed using the comparative threshold cycle method ($2^{-\Delta\Delta C_t}$), as previously described¹⁹.

Statistical analyses

Statistical analyses were performed using GraphPad 6. Two-way ANOVA with corrections for multiple comparisons were used, when applicable. Values $p < 0.05$ were considered to be significant.

Results

Pru p 3 was transported through the epithelial barrier, but preferentially in the non Peyer's Patches place.

The InTESTine™ model was performed as previously described¹⁶. Alexa 647-labelled Pru p 3 (43 µg/ml) without (Prup 3) and with its ligand (Complex; 43 µg/ml: 4.3 µg/ml) were added in the apical side, and basolateral media were collected at different times in NPP (20, 45, 105 and 165 min) and in PP (20 and 45 min). As seen in Figure 17, the transport of Pru p 3, alone or with its ligand (Complex) could be observed in the NPP samples at the same range. In contrast, no transport was noticed in the PP samples. Although this result could be due to the presence of a lesser number of time points, the rate of transportation was similar in both kinds of samples.

In NPP model, the complex co-localized with iNKT and CD1d-cells

To identify immune cells which might interact with Pru p 3 and the complex in the pig tissue, different immune chemistry were performed as previously described. To this aim, specific antibodies markers were utilized. In NPP model, we could observe the presence of iNKT that overloaded in the presence of the complex but not in the presence of Pru p 3 alone (Fig 18). This co-localization was also confirmed by using anti-CD1d specific antibodies (Fig 19). Both results confirm the data reported in the chapter 3.2 where it has been described that the lipid-ligand of Pru p 3 co-localized to CD1d in an epithelial cell model (Caco2) and could activate iNKT by interaction with CD1d tetramers.

B

	% Transported dose			
Time (min)	20	45	105	165
Pru p 3	0	0,02895943	0,68375199	1,88855263
Complex	0	0,01721114	0,33834054	1,19671899

Figure 17 Curve of transport kinetics of Pru p 3 and its Complex (Pru p 3+ligand) in NPPs and PPs (**A**). Pru p 3 labelled with Alexa 647 was added to the apical side with and without its ligand, and the basolateral medium was recovered at different times. Accumulated transport of the allergen in the basolateral medium was measured by fluorescence on a reader plate. The results correspond to two independent experiments in triplicate. Means and SD are shown. (**B**) % Transported dose over time points.

Figure 18: Immunolocalization of Pru p 3 and the Complex with its ligand in pig tissue with iNKT: Pru p 3 labelled with Alexa 647 showed fluorescence signal in green. iNKT detected using a specific monoclonal antibody labelled with Alexa 488 showed the signal in red.

Figure 19: Immunolocalization of Pru p 3 and the Complex with its ligand in pig tissue with CD1d: Pru p 3 labelled with Alexa 647 showed fluorescence signal in green. CD1d detected using a specific monoclonal antibody labelled with Alexa 488 showed signal in red.

The ligand of Pru p 3 activated a Th2 response

The expression of mRNA cytokines, in the presence or in the absence of the ligand of Pru p 3, was measured by q-PCR. In NPP model, a strong stimulation of IFN- γ , IL4, IL10 and IL13 was observed in the presence of the complex but not with Pru p 3 alone (Figure 20). We can hypothesize that the ligand of Pru p 3 is able to modulate the immune system towards a Th2 response. In contrast, no noticeable increase in the mRNA expression of IL8, IL12 and IL22 cytokines was observed. Likewise, no differential expression of mRNA from epithelial cytokines such as IL25, IL33 and TSLP was detected neither. However, IL33 expression produced by the complex has been observed by ELISA (data not shown)

Figure 20: Cytokines expression profile at NPPs model. All expression values are normalized to the GAPDH gene. Relative expression was calculated respect to the untreated tissues. All amplifications were performed in quadruplicate and two independent assays. Means and SD (bars) are shown.

Discussion

In the present study, we have been able to confirm that the presence of a ligand of an allergen protein is determinant for interaction with cells of the innate immune system such as iNKT cells. This interaction appears to occur mainly in the NPP zone. Our results show that the Pru p 3-ligand is transported through the intestinal mucosa in NPP without disturbing its structure in NPPs. Confirming data previously reported¹³, the peach allergen is transported by a transcellular route. In the case of PP, it is probable that more time points would be needed²⁰. But this suggests that the allergen is being accumulated in the inductive sites, which means lymph tissue.

On the other hand, Pru p 3 in the presence of its ligand was co-localized with invariant NKT (iNKT) cells and CD1d receptors as evidenced by immunochemistry. CD1d proteins are MHC-I class-like molecules that present lipid antigens to the iNKT (lipid specific T cells) and are mainly expressed by APCs such as dendritic cells (DCs), B cells²¹ and the majority of intestinal epithelial cells²². After TCR stimulation, iNKT cells expand the innate immune system by producing cytokines such as INF- γ and IL4²³. These data support the hypothesis that the ligand of Pru p 3 enhances the allergic activity by being loaded onto CD1d receptors and presented to iNKT cells (chapter 3.2). This allergenic response was completed with the expression of IL4, IFN- γ , IL10, and IL13 Th2-associated cytokines²⁸, as seen in NPP in the presence of the complex.

In summary, we have shown that the lipid ligand of Pru p 3 is transported through the intestinal barrier in a minipig *ex vivo* model and increase the allergic activity mainly through a Th2 response in the no-lymphoid part of the gut. Furthermore, the ligand activates the iNKT cells by being presented by the CD1d molecule, a result that strongly supports the theory that the lipids carried by some proteins could play an essential role in the allergenic process.

References

1. Reitsma, M., Westerhout, J., Wichers, H. J., Wortelboer, H. M. & Verhoeckx, K. C. M. Protein transport across the small intestine in food allergy. *Mol. Nutr. Food Res.* 58, 194–205 (2014).
2. Reitsma, S., Slaaf, D. W., Vink, H., van Zandvoort, M. A. M. J. & oude Egbrink, M. G. A. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch.* 454, 345–59 (2007).
3. Hillgren, K. M., Kato, A. & Borchardt, R. T. *In vitro* systems for studying intestinal drug absorption. *Med. Res. Rev.* 15, 83–109 (1995).
4. Heyman, M. Gut barrier dysfunction in food allergy. *Eur. J. Gastroenterol. Hepatol.* 17, 1279–85 (2005).
5. Kondoh, M., Takahashi, A. & Yagi, K. Spiral progression in the development of absorption enhancers based on the biology of tight junctions. *Adv. Drug Deliv. Rev.* 64, 515–522 (2012).
6. Khanvilkar, K., Donovan, M. D. & Flanagan, D. R. Drug transfer through mucus. *Adv. Drug Deliv. Rev.* 48, 173–93 (2001).
7. Shimizu, M. Food-derived peptides and intestinal functions. *BioFactors* 21, 43–47 (2004).
8. Husby, S., Jensenius, J. C. & Svehag, S. E. Passage of undegraded dietary antigen into the blood of healthy adults. Further characterization of the kinetics of uptake and the size distribution of the antigen. *Scand. J. Immunol.* 24, 447–55 (1986).
9. VAN ALSTYNE, E. V. N. THE ABSORPTION OF PROTEIN WITHOUT DIGESTION. *Arch. Intern. Med.* XII, 372 (1913).
10. Smith, P. L., Wall, D. A., Gochoco, C. H. & Wilson, G. (D) Routes of delivery: Case studies. *Adv. Drug Deliv. Rev.* 8, 253–290 (1992).
11. Burks, A. W. *et al.* ICON: food allergy. *J. Allergy Clin. Immunol.* 129, 906–20 (2012).
12. Perrier, C. & Corthésy, B. Gut permeability and food allergies. *Clin. Exp. Allergy* 41, 20–28 (2011).
13. Tordesillas, L. *et al.* Transport of Pru p 3 across gastrointestinal epithelium - an essential step towards the induction of food allergy? *Clin. Exp. Allergy* 43, 1374–1383 (2013).
14. Walker, W. A., Isselbacher, K. J. & Bloch, K. J. Immunologic control of soluble protein absorption from the small intestine: a gut-surface phenomenon. *Am. J. Clin. Nutr.* 27, 1434–40 (1974).
15. Patterson, J. K., Lei, X. G. & Miller, D. D. The Pig as an Experimental Model for Elucidating the Mechanisms Governing Dietary Influence on Mineral Absorption. *Exp. Biol. Med.* 233, 651–664 (2008).

16. Westerhout, J. *et al.* A new approach to predict human intestinal absorption using porcine intestinal tissue and biorelevant matrices. *Eur. J. Pharm. Sci.* 63, 167–177 (2014).
17. Bublin, M., Eiwegger, T. & Breiteneder, H. Do lipids influence the allergic sensitization process? *J. Allergy Clin. Immunol.* 134, 521–9 (2014).
18. Salcedo, G., Sanchez-Monge, R., Diaz-Perales, A., Garcia-Casado, G. & Barber, D. Plant non-specific lipid transfer proteins as food and pollen allergens. *Clin. Exp. Allergy* 34, 1336–1341 (2004).
19. Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* 25, 402–408 (2001).
20. Gebert, A., Steinmetz, I., Fassbender, S. & Wendlandt, K.-H. Antigen transport into Peyer's patches: increased uptake by constant numbers of M cells. *Am. J. Pathol.* 164, 65–72 (2004).
21. Darmoise, A., Maschmeyer, P. & Winau, F. in *Advances in immunology* 105, 25–62 (2010).
22. Blumberg, R. S. *et al.* Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J. Immunol.* 147, 2518–24 (1991).
23. Barral, D. C. & Brenner, M. B. CD1 antigen presentation: how it works. *Nat. Rev. Immunol.* 7, 929–941 (2007).
24. Appleyard, G. D. & Wilkie, B. N. Characterization of porcine CD5 and CD5+ B cells. *Clin. Exp. Immunol.* 111, 225–30 (1998).
25. Watanabe, R. *et al.* CD19 expression in B cells is important for suppression of contact hypersensitivity. *Am. J. Pathol.* 171, 560–70 (2007).
26. Dasu, T., Sindhava, V., Clarke, S. H. & Bondada, S. CD19 signaling is impaired in murine peritoneal and splenic B-1 B lymphocytes. *Mol. Immunol.* 46, 2655–65 (2009).
27. Bamias, G. & Cominelli, F. Role of type 2 immunity in intestinal inflammation. *Curr. Opin. Gastroenterol.* 31, 471–6 (2015).
28. Paul, W. E. & Zhu, J. How are TH2-type immune responses initiated and amplified? *Nat. Rev. Immunol.* 10, 225–235 (2010).

4. GENERAL DISCUSSION

GENERAL DISCUSSION

Food allergy is a serious health problem that affects millions people and can greatly impair the quality of life of patients. For decades, no significant progress has been made in discovering the reasons why a protein can trigger an allergic reaction. The main drawback has been that no common biochemical features among different allergens have been found. Moreover, even the biological activity of many of the allergens is unknown. This has been the case of Pru p 3, the principal allergen of peach.

In this project, we have confirmed the lipidic nature of the ligand of Pru p 3 which has been characterized as a hydroxylated derivative of camptothecin bound to phytosphingosine. According to our structural model of the Pru p 3-ligand complex, the phytosphingosine tail inserts into the hydrophobic tunnel of the protein whereas the polar head composed of the amide bond and polar groups in the camptothecin moiety are located in the surface exposed to the aqueous solvent. With these results in mind, we found that Pru p 3 shows activity at two different points of flowering maturation: during the fertilization avoiding the secondary pollination, and during fruit development, expressing mainly in trichomas and preventing the feeding before embryo maturity. In natural systems, the expression of Pru p 3 was induced in the stigma, when the first pollen grain reached the ovary. Pru p 3 transported the ligand to the apoplast this way. Once there, the amide bond linking phytosphingosine and camptothecin segments of the ligand hydrolyzes as a consequence of the low pH in the medium. Upon hydrolysis, camptothecin adopts the chemical form (lactone ring closed) needed for its inhibitory features, becomes active and is able to inhibit pollen tube growth. A similar mechanism happened in the trichomas.

In a different context, the ligand of Pru p 3 is essential to allergenic sensitization. Our results demonstrated that it was the ligand, not the protein alone, which activates dendritic cells and iNKT cells¹ mediated by CD1 presentation. In this regard, many major allergens such as Pru p 3, Bet v 1², and 2S³ have been described as lipid carriers. Recently, lipid carriers have been linked to allergenic activity, suggesting that sensitization could be based on the recognition of lipid ligands by receptors on cells of immune system⁴. The presentation of lipidic compounds is known to depend on CD1 receptors throughout a process that requires endosome compartments, acidic pHs and the loading of lipids onto CD1 molecules by means of small non-enzymatic lipid transfer proteins called saposins⁵. Our *in silico* studies demonstrated the structural similarities between Pru p 3 and saposins. They not only share a same fold composed of four α -helices held by four (in Pru p 3) or three (in saposins) disulphide bonds but both Pru p 3 and saposins also have similar sizes and hydrophobic cavities as well as the capability to present conformational changes in their interaction with receptors. Although the details of these interactions still remain to be elucidated, it is usually accepted that these changes take place in

the loading of lipids onto CD1d receptors. It is thus highly likely that Pru p 3 could act through a similar mechanism to saposins in the process of loading its lipid-ligand onto CD1d receptors thus inducing the activation of iNKT cells⁶⁻¹⁰. Although this mechanism could occur in all mucosa, our results suggest that the lipid-ligand increased the susceptibility to peach allergy by the epicutaneous route. This way, skin exposure might be a main route of sensitization to peach allergy as it has been previously proposed for other allergens¹¹⁻¹³.

Model of Pru p 3 sensitization. (Modified from Cuenca. J, 2017)

Taking into account all the results reported, we propose the following model. Pru p 3 can cross through the mucosa, but CD1d receptors are activated only in the presence of its ligand. In the late endosome which is characterized by a low pH ~ 5.5 , the protein-ligand complex would dissociate and the released phytosphingosine segment of the ligand could be loaded onto CD1d receptors. In contrast, the uncomplexed protein Pru p 3 would be processed and its resulting peptides loaded onto MHCII (conventional antigen-presenting DCs that play a critical role in generating type-2 responses)¹⁴. Both loaded CD1d and MHCII receptors should be exposed in the basolateral size being recognized by different types of immune cells, iNKT and dendritic cells. In parallel, epithelial cells should produce large amount of IL33 and IL25 in the short and in the long range time leading to a Th2 response. After that, iNKT activated cells would expand the Th2 response secreting also cytokines such as IL4, IL5 and IL13.

In the case of the Pru p 3 epitopes, they would be recognized by conventional T-cell receptors (TCR, complex of integral membrane proteins) leading to CD4+ cells switching to a Th2 type. This type of effectors would be the responsible for activation of the production of IgE antibodies by B cells.

References:

1. Kimber, I., Griffiths, C. E. M., Basketter, D. A., McFadden, J. P. & Dearman, R. J. Epicutaneous exposure to proteins and skin immune function. *doi.org* 24, 10–14
2. Radauer, C., Lackner, P. & Breiteneder, H. The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol. Biol.* 8, 286 (2008).
3. Moreno, F. J. & Clemente, A. 2S Albumin Storage Proteins: What Makes them Food Allergens? *Open Biochem. J.* 2, 16–28 (2008).
4. Bublin, M., Eiwegger, T. & Breiteneder, H. Do lipids influence the allergic sensitization process? *J. Allergy Clin. Immunol.* 134, 521–9 (2014).
5. Vartabedian, V. F., Savage, P. B. & Teyton, L. The processing and presentation of lipids and glycolipids to the immune system. *Immunol. Rev.* 272, 109–119 (2016).
6. Agea, E. *et al.* Human CD1-restricted T cell recognition of lipids from pollens. *J. Exp. Med.* 202, 295–308 (2005).
7. Abós-Gracia, B. *et al.* Olea europaea pollen lipids activate invariant natural killer T cells by upregulating CD1d expression on dendritic cells. *J. Allergy Clin. Immunol.* 131, 1393–9.e5 (2013).
8. Dearman, R. J., Alcocer, M. J. C. & Kimber, I. Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1. *Clin. Exp. Allergy* 37, 582–591 (2007).
9. Mirotti, L. *et al.* Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. *Allergy* 68, 74–83 (2013).
10. Jyonouchi, S. *et al.* Invariant natural killer T cells in children with eosinophilic esophagitis. *Clin. Exp. Allergy* 44, 58–68 (2014).
11. Tordesillas, L. *et al.* Skin exposure promotes a Th2-dependent sensitization to peanut allergens. *J. Clin. Invest.* 124, 4965–4975 (2014).
12. Benedé, S., Blázquez, A. B., Chiang, D., Tordesillas, L. & Berin, M. C. The rise of food allergy: Environmental factors and emerging treatments. *EBioMedicine* 7, 27–34 (2016).
13. Noti, M. *et al.* Exposure to food allergens through inflamed skin promotes intestinal food allergy through the thymic stromal lymphopoietin–basophil axis. *J. Allergy Clin. Immunol.* 133, 1390–1399.e6 (2014).
14. Oliphant, C. J. *et al.* MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* 41, 283–95 (2014).

5. CONCLUSIONS

CONCLUSIONS

- 1) The lipid-ligand of Pru p 3 has been identified by ESI-qToF as a monohydroxylated derivative of camptothecin bound to phytosphingosine.
- 2) In flowering, the highest mRNA expression of *Pru p 3* was observed in pollinated stigma-styles, matching with fertilization. Since the lipid-ligand can inhibit the pollen germination, its presence might be implicated in avoiding the double pollination that would provoke fruit disruption.
- 3) In fruit development, the maximum mRNA expression of Pru p 3 took place during the last stage of embryo maturation, mainly localized in peel trichomas. Thus, the lipid-ligand might avoid predator feeding in fruit until the embryo maturation.
- 4) The lipid-ligand, and not the protein alone, could induce the activation of antigen presenting cells such as dendritic and monocyte cells. In this way, the lipid-ligand carried by Pru p 3 played a role as an immune adjuvant with the ability to modulate the immune system toward a Th2 response associated with class-switching leading to IgE production which is characteristic of allergy.
- 5) In the epithelial cells, the lipid-ligand of Pru p 3 did not interfere with the transport of the allergen, but it rather favoured co-localization with CD1d receptors. This finding supports the putative involvement of the ligand in the trafficking and loading of lipid compounds onto CD1d receptors, stimulating iNKT cells.
- 6) The lipid-ligand of Pru p 3 increases the capacity to sensitize mice through the epicutaneous route.
- 7) Pru p 3 and saposins (particularly saposin A) showed significant structural relationships and lipid-transport abilities that suggest similar processes of loading lipids onto CD1 receptors. The Pru p 3-ligand complex dissociates at the acidic pH in the endosome and the released ligand could be loaded onto CD1d in a similar way to that done by saposins. Loaded CD1d was exposed on the basolateral side of the epithelial monolayer. There, the interaction with iNKT cells could stimulate production of Th2 cytokine profiles.

- 8) By using intestinal fragments of minipigs in the InTESTine™ system, the presence of Pru p 3 ligand showed no influence in the protein transport across the intestinal epithelium in non-Peyer's Patches plaques. The complex could modulate the immune system presumably toward a Th2 response in non-Peyer's Patches.