BIOMARKERS AND MEDIATORS OF PEANUT ALLERGY AND ANAPHYLAXIS

COMPREHENSIVE METABOLOMIC ANALYSIS IN PEANUT SENSITIZATION AND PEANUT-INDUCED ANAPHYLAXIS: DISCOVERY OF BIOMARKERS AND MEDIATORS

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TITLE: Comprehensive Metabolomic Analysis in Peanut Sensitization and Peanut-Induced Anaphylaxis: Discovery of Biomarkers and Mediators

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ABSTRACT

BACKGROUND: The ontogeny of peanut allergy (PA) is poorly understood, and the treatment of its most severe manifestation, peanut-induced anaphylaxis (PIA), remains limited to rescue epinephrine. We argued that an untargeted metabolomic analysis would be a useful hypothesis-generating tool to identify novel biomarkers, mediators and possibly therapeutic targets in PA and PIA.

METHODS: Models of PA and PIA used in this thesis involved either the oral administration of peanut along with cholera toxin or the topical application of peanut on tape-stripped skin. Liquid-chromatography mass-spectrometry (LC-MS) was performed to identify chemical changes in the serum of mice undergoing sensitization and anaphylaxis. Flow cytometry as well as *in vivo* gain-of-function and loss-of-function immunological studies were used to determine the biological significance of particular molecules in sensitization.

RESULTS: LC-MS followed by multivariant analysis showed that the purine metabolism pathway was altered with elevated levels of uric acid (UA) in sensitized mice. UA depletion using allopurinol and uricase fully prevented the development of the allergic and anaphylactic phenotype. Conversely, administration of UA crystals, instead of cholera toxin or tape stripping along with peanut induced a typical allergic and anaphylactic phenotype. The effects of UA and UA crystals are likely a consequence of effects on the activation of resident dendritic cells. Post-challenge metabolic analysis also revealed a distinct metabolic signature in sensitized mice, highlighted by an increase in several metabolites such as histamine. Likewise, peanut allergic patients display a distinct metabolic profile after oral peanut challenge.

CONCLUSION: We identified UA, released after damage to the mucosa and/or skin, as a critical alarmin that facilitates the development of Th2 immunity, specifically PA and PIA. Metabolomics analyses of either mice undergoing anaphylaxis or peanut allergic children subjected to a peanut oral challenge provided an extensive overview of metabolomic changes underlying these conditions. Further studies may lead to the identification of novel biomarkers and mediators.

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LIST OF ABBREVIATIONS

Ags	Antigens
Alum	Aluminum hydroxide
APC	Antigen presenting cells
CPE	Crude peanut extract
CPPD	Calcium pyrophosphate dihydrate
CRD	Component-resolved diagnostics
DAMP	Danger-associated molecular patterns
DBPCPC	Double blind placebo-controlled oral peanut challenge
DC	Dendritic cells
GC	Gas chromatography
GI	Gastrointestinal
HBSS	Hank's balanced salt solution
HDM	House dust mite
HPLC	High-performance liquid chromatograph
Ig	Immunoglobulin
i.g.	Intragastric
IL-	Interleukin
i.p.	Intraperitoneal
LC	Liquid chromatography
LN	Lamina propria
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MS	Mass spectrometry
MSU	Monosodium urate
NE	Norepinephrine
NE-S	Norepinephrine sulfate
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal partial least squares-discriminant analysis
PA	Peanut allergy
PAMP	Pathogen-associated molecular pattern
PAF	Platelet activating factor
PBS	Phosphate buffered solution
PC	Phosphatidylcholine
PCA	Principle component analysis
PLS	Partial least squares analysis
PI3K	Phosphinositide 3-kinase
PIA	Peanut-induced anaphylaxis

PN	Peanut
SubQ	Subcutaneous
Syk	Spleen tyrosine kinase
TLR	Toll-like receptor
Th	T helper
TOF	Time-of-flight
TS	Tape-stripping
TSLP	Thymic stromal lymphopoietin
UA	Uric acid

DECLARATION OF ACADEMIC ACHIEVEMENT

As the author, I was responsible for designing and executing the experiments. Our laboratory technicians, Tina Walker and Susanna Goncharova, provided me with technical assistance. Metabolomic analysis was performed in collaboration with Dr. Brian McCarry and his Ph.D. student, Kenneth Chalcraft, from the Department of Chemistry (McMaster University). Human sample collections were obtained through collaboration with Dr. Susan Waserman in the Department of Medicine (McMaster University).

CHAPTER 1: INTRODUCTION

The Problem

Peanut allergy (PA) is an aberrant immunological reaction to inherently innocuous peanut antigens (Ags). The prevalence of PA in North America has doubled in the last 10 years, and is currently estimated at approximately 1%.[1,2] It often develops early in childhood but, unlike most food allergies, it is lifelong in ~80% of individuals.[3] PA is the most common cause of severe food-induced allergic reactions.[4] Symptoms range from mild urticaria, wheezing, vomiting, and diarrhea to anaphylaxis, which is characterized by difficulty breathing, hypotension and, eventually, death.[5] Unfortunately, the management of PA is limited to strict avoidance and administration of rescue epinephrine once an anaphylactic reaction has started.[6] Patients with PA are advised to avoid exposure to peanut (PN); however, accidental ingestion of PN has been reported in up to 50% of patients within a 2-year period.[7] The severity of PA combined with its rising prevalence has emerged as a major health concern in dire need of novel preventative and therapeutic strategies.

Diagnosis of Peanut Allergy

In addition to clinical history, the two most commonly used diagnostic methods for PA are the skin-prick test (SPT) and serum PN-specific immunoglobulin E (IgE). Although easy to perform, both tests require a relatively high cutoff value to provide a 95% positive prediction. For SPT and serum PN-specific IgE, values below 8 mm and 13 kU/L respectively are problematic for diagnosing true positivity.[8,9] More recently, component-resolved diagnostics (CRD), which detects IgE specific to different purified or recombinant PN proteins, has been shown to have higher specificity and sensitivity than the traditional IgE test that targets a mixture of PN proteins. However, different studies have reported varying success with CRD, without a consensus on the appropriate cut-off value.[10-12] As a result, double blind placebo-controlled oral peanut challenge (DBPCPC) remains the gold standard for the diagnosis of PA.[13] However, DBPCPC can only be carried out under the supervision of specialized medical personnel prepared to provide emergency treatment if needed. In addition, DBPCPC is not an ideal diagnostic tool, not only due to the dangers and high costs associated with it, but also because the appraisal of clinical symptoms may be sometimes dubious. Thus, there is a need for novel diagnostic tools that can safely, efficiently and effectively differentiate PN allergic from non-allergic individuals.

Phases of Peanut Allergy

The development of PA can be conceptualized within a timeline encompassing two distinct phases: the *sensitization phase*, *i.e.* the process by which the host develops immunoglobulins (Ig) specific for PN Ags, and the *effector phase*, *i.e.* characterized by the clinical and physiological manifestations arising from PN exposure in sensitized patients. Sensitization is thought to develop due to a lack of induction or disruption of oral tolerance, the immune process that generates systemic hyporesponsiveness to ingested Ags. Despite significant progress in recent years, the specific mechanisms that mediate sensitization or oral tolerance to food allergens remain unclear. Likewise, much remains to be elucidated with respect to the effector phase, in particular the identification of the molecules that actually precipitate the anaphylactic reaction. Currently, anaphylaxis is defined as "a serious allergic reaction that is rapid in onset and may cause death," with a list of cumbersome criteria, established to help in the diagnosis.[14] However, its associated signs and symptoms overlap with other diseases and conditions.[15] Partly, this is because anaphylaxis, including PIA, is a syndrome with diverse clinical presentations, including diffuse erythema, pruritus, urticaria, angioedema, bronchospasm, laryngeal edema, hyperperistalysis, hypotension, and cardiac arrhythmias.[5] Among these symptoms, general urticaria and angioedema are the 2 most common manifestations of anaphylaxis, which result from increased plasma leakage of up to 50% into extravascular spaces due to vasodilation and increased capillary permeability.[5,15] As a consequence, fluid loss leads to a decrease in venous return and, eventually, hypotension, vascular collapse and, potentially, death. We suggest that advances in our understanding of the molecular signatures underlying PN sensitization and anaphylaxis will have a direct impact for uncovering biomarkers and/or mediators and, thus, the development of novel diagnostics and therapeutics.

Mechanisms Underlying Peanut Sensitization

Sensitization involves a timely cascade of events that culminates in priming of the immune system. Initially, Ag-presenting cells (APCs), particularly dendritic cells (DCs), take up Ags available in the mucosal environment or the skin, migrate to draining lymph nodes and present them to naïve T cells in the context of major histocompatibility complex (MHC) molecules.[16] Unless DCs are activated via additional signals, the host's immune system will generate either tolerance or ignorance. Conversely, Ag presentation by activated DCs expressing co-stimulatory molecules such as CD80 and CD86 will lead to differentiation of naïve T cells to distinct phenotypes such as T helper (Th)1, Th2, Th17 and Treg. In the case of food allergy, presentation of food Ags in the context of MHC II molecules by DCs is critical to the development of Th2 cells. Furthermore, co-stimulatory molecule expression influences the type of immune activation; specifically,

OX40L plays a key role in the generation of PN-induced Th2 immunity.[17] Several *in vitro* studies have shown that thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 are able to up-regulate the expression of OX40L.[18,19] However, we have shown that only IL-33, but not TSLP or IL-25, signalling is critical for the development of PA and anaphylaxis.[19] Once naïve T cells receive signals from DCs leading to the differentiation into Th2 cells, these cells produce the cytokines IL-4, IL-5, and IL-13 that mediate various hallmarks of Th2 immunity.[20] Among the Th2 cytokines, IL-4 is critical to stimulate B-cell maturation, whereby B cells undergo somatic hypermutation, Ig class-switch and mature into plasma cells to produce and secrete large quantities of Ag-specific IgE and IgG.[21] Ag-specific Igs are essential for the manifestation of PIA. Indeed, we have previously shown that CD40L- or B cell-deficient mice, which are unable to produce PN-specific Igs, were fully protected from PIA.[22]

Mechanism of Peanut-Induced Anaphylaxis

PIA is a type-1 hypersensitivity reaction that is primarily mediated by IgE, FcεRI receptor and mast cells. Mast cells, first identified by Paul Ehrlich in 1878, are found at perivascular sites in tissues readily exposed to the environment such as the skin, mucosa of the lungs and digestive tract. They have been shown to participate in both innate and adaptive immunity as a first line of defense.[23] In the case of PA, peanut Ags interact with peanut-specific IgE bound to high-affinity FcεRI receptors on the surface of mast cells. Receptor cross-linking leads to mast cell degranulation and, thus, the release of bioactive molecules.[24] Interestingly, patients with non-detectable serum IgE levels can also undergo allergic, or even anaphylactic, reactions suggesting the involvement of other cellular and molecular pathways.[25]

Macrophages, first discovered in 1883 by Ilya Mechnikov are present in every tissue and are better known for their role in phagocytosis.[26] However, macrophages can release many products upon activation. In experimental studies of PIA, we demonstrated that macrophages, and to a lesser extent basophils, are also important for the full expression of PIA through an IgG1 and FcγRIII-mediated pathway.[24] Collectively, contents from mast cell and macrophage degranulation or secretion include a wide range of molecules, such as pre-stored vasoactive amines (*i.e.* histamine and serotonin), cytokines (*i.e.* tumor necrosis factor α (TNFα)), arachidonic acid metabolites (*i.e.* leukotrienes and prostaglandins) and lipid molecules (*i.e.* platelet-activating factor (PAF)).[21,27] Some of these molecules, such as histamine, serotonin and PAF, have been reported to have vasoactive properties, increasing vascular permeability and leakage while also inducing other allergic symptoms such as acute bronchoconstriction.[28]

Metabolomics and Analytical Strategies

A major challenge in the discovery of biomarkers and mediators of PA and PIA stems from the inherent bias of conventional research methods. Typically, a hypothesis is stated that identifies the target of interest (*i.e.* the molecule) and its presumed role based on the available literature. As a consequence, the experimental design will inevitably preclude the consideration of other molecules that are unknown and may be important. Although this method has been proven to be effective in many instances, it is not conducive to the discovery of novel targets. In this context, comprehensive metabolomic analysis is a technology well suited to provide an unbiased global view of the molecules present during a given process, such as PN sensitization and PIA, thus providing an array of novel molecules to be further explored as either potential biomarkers or mediators.

Metabolites are small molecules, less than 1.5 kDa, representing the furthest downstream expression of biochemical activity, thus reflecting the total contribution of genomic, transcriptomic, and proteomic activity as well as exogenous factors arising from nutritional sources and environmental stress.[29] As such, they represent the functional readout of the cellular state of a living organism. The study of metabolites, or metabolomics, is generally defined as a comprehensive analysis to identify and quantify the metabolome, understood as the complete collection of metabolites in a biological system.[30] Unlike other "omics" studies such as genomic or proteomic, metabolomics provides *real-time* information on changes in the physiological state of an organism due to both endogenous and exogenous stress.[31] The benefits of metabolomics have been demonstrated in several studies.[32] For example, it has been used to reveal disruptions of metabolic pathways, uncover novel metabolite targets and mechanisms in many diseases including cardiovascular disease,[33,34] liver injury,[35] various cancers,[36-38] and exposure to environmental influences such as ionizing radiation.[35,37,39] To our knowledge, only two limited reports have documented the use of metabolomics in food allergy and anaphylaxis. Peeters *et al.* reported changes in the metabolomic profile of PN allergic patients using nuclear magnetic resonance (NMR).[40] This study reported differences in NMR spectral features such a lactate, creatinine, and glutamine in plasma and saliva between PN-allergic *versus* PN-tolerant subjects.[40] In the second study, Hu *et al.* used gas chromatography-mass spectrometry (GCMS) to study ovalbumin (OVA) and cattle albumin induced anaphylaxis.[41] The investigators found 6 ionic species that were differentially expressed in the animals undergoing anaphylaxis but did not provide a biological explanation for these changes at 1-hour post challenge, the only time point studied.[41] Additional time points for sample collection would have provided a more comprehensive view of the metabolomic changes of an anaphylactic reaction.

Typically, metabolomics research is subdivided into two categories: targeted metabolomics, where selective subsets of the metabolome are quantified, and untargeted/comprehensive metabolomics, where as many metabolites are analyzed as possible. The latter form is a truly unbiased approach that provides unique opportunities for both biomarker and mediator discovery. Comprehensive metabolomics analysis is generally performed using either nuclear magnetic resonance based techniques or by mass spectrometry based techniques.[42] While NMR continues to be popular due to its ability to detect a very wide range of molecules in a single analysis, it is rather insensitive as it detects molecules only in high abundance within a sample; this is a significant weakness for anaphylaxis research since some known mediator molecules such as PAF are found at very low concentrations. Alternatively, recent technological advances in MS instrumentation have enabled the rapid detection of thousands of metabolites in biofluids including those in very low abundance. Numerous configurations of MS analysis have been reported; however, for comprehensive analysis, medium to high resolution MS with a front-end separation platform such as gas chromatography (GC) or liquid chromatography (LC) are most commonly used. This is primarily due to the qualitative information afforded by accurate mass measurement and the increase in quantitative data quality resulting from spatial separation prior to MS ionization. While a number of MS instruments are capable of providing accurate mass determination with medium to high resolution, time-of-flight (TOF) mass spectrometers are often favored due to their excellent performance over a wide mass range.[42] Another strength of TOF-MS is that the instruments are capable of very fast scan rates, which make TOF-MS ideal for use with high performance chromatography. Of the two common chromatography types used with MS, both have been used extensively, however GC analysis requires extensive sample preparation including chemical derivatization and long run times rendering this technique time consuming and costly.[43] LC separation on the other hand can be performed on a wide variety of samples such as blood plasma/serum, urine and tissue extracts with efficient sample preparation protocols. The ability of LC-MS to analyze both polar and non-polar metabolites with low volatility is a critical advantage for anaphylaxis research since previous studies have identified molecules such as the hydrophilic amine histamine and the very hydrophobic PAF as biomarkers and mediators. The large volume of data generated by LC-MS can be analyzed with a number of multivariate statistical analysis such as principle component analysis (PCA) and partial least squares analysis (PLS) to compare total metabolomic profiles or "molecular finger-prints" between samples to identify changes in molecular trends and groupings.[44] Upon the discovery of interesting metabolic features through comprehensive metabolomics, the significance of these molecules can be studied to determine their role and discriminate between biomarkers and mediators

Potential Mediators of Peanut Sensitization

An immune stimulatory signal is required for PN sensitization. In animal models of PIA, CT is required as an exogenous adjuvant for intragastic sensitization; indeed, mice gavaged with PN alone do not become sensitized. In addition, in a model of skin sensitization (Kong & Flader, manuscript in preparation), application of crude peanut extract (CPE) on shaved and tape-stripped skin without CT induced allergic sensitization. These distinct requirements suggest, in light of an identical outcome, that there may be a common signaling pathway that activates the immune system leading to PN sensitization. In this context, CT or tape-stripping may be viewed as external stimuli capable of inducing the release of common endogenous signals that facilitate DC activation. It has been proposed that such endogenous DC activators are the damage-associated molecular patterns (DAMPs) or alarmins, originally described in Matzinger's danger theory.[45] DAMPs are a class of intracellular molecules normally hidden from immune cells. Cell death or stress due to infections, toxins, or trauma can release DAMPs into the extracellular space where they relay danger signals leading to immune cell activation. Arguably, the source of stress in our PIA models may be the toxic effects of CT on intestinal cells or physical disruption or trauma from tape-stripping on skin cells, causing the release of their intracellular contents including DAMPs.

A growing list of DAMPs has been identified including hyaluronan, heat-shock proteins (HSPs), surfactant protein, interferon-α (IFN-α), fibronectin 11, β-defensin, cardiolipin, high mobility group box 1 (HMGB-1), adenosine triphosphate (ATP) and uric acid (UA).[46,47] These molecules have the ability to activate the immune system against pathogens as well as harmless Ags such as food allergens. UA, among other DAMPs is produced by all cell types as the end product of purine metabolism and is excreted through urine in humans. Upon injury or death, cells release UA as a warning of danger to surrounding cells. At normal physiological levels, UA serves as an antioxidant. However, when the concentration of UA in extracellular fluid exceeds the solubility level, crystallizes into monosodium urate (MSU) and becomes a DAMP signal capable of activating DCs and subsequently promoting the development of adaptive immune responses.[48]

Potential Mediators of Anaphylaxis

There is limited knowledge about the identity and significance of molecules released in the course of an allergic/anaphylactic reaction. It is important to note the distinction between biomarkers and mediators. For example, while elevated levels of several molecules including cysteinyl-leukotriene, serotonin, and tryptase are often detected during PIA, functional studies have shown that they do not seem to mediate the

manifestations of PIA; therefore, they are best referred to as biomarkers. In 2008, Vadas *et al.* showed that serum levels of PAF and PAF acetylhydrolase, the enzyme that inactivates PAF, had a direct and an inverse correlation with the severity of anaphylaxis in patients, respectively.[49] In an experimental murine system, we confirmed PAF as a mediator of PIA. Furthermore, we showed that concurrent blockade of PAF and histamine receptor signaling achieved greater, yet, still partial abrogation of anaphylaxis. This suggests the involvement of further unidentified mediators and that full protection will likely require concurrent inhibition of several molecules.[50]

The complex pathophysiological events underlying an anaphylactic reaction likely involve a wide array of molecules, beyond those that have already been identified. In this context, we argue that metabolomics is likely to be a fertile discovery platform. Identification of distinct chemical entities must be followed by appropriate studies, initially in animal experimental models, to determine their biological relevance. In this regard, several criteria may be considered to design a more targeted approach. Since mast cells and phagocytic cells (*i.e.* macrophages) are both necessary for the full expression of PIA,[24] it would be expected that candidate molecules are produced by these cells. In addition, the abrupt onset of PIA makes it more likely for the molecule to be either pre-stored or rapidly synthesized. Such molecules would have the ability to quickly induce hypothermia, hypotension or other symptoms of anaphylaxis.

Thesis Objective

The goal of this thesis is to identify potential biomarkers and mediators of PN allergic sensitization and PIA. First, metabolomic analysis will be used to detect changes in metabolites during the sensitization and the effector phases in our murine models of PIA. Second, some of the molecules that are differentially expressed will be studied in greater detail to determine whether they are biomarkers or mediators of PA. Third, metabolomic analysis will be performed in serum samples from PN allergic patients pre- and post-oral challenge.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River Laboratory (Ottawa, Ontario). The mice were housed in a specific pathogen-free environment and maintained on a 12-hour light-dark cycle. All experiments described were approved by the Animal Research Ethics Board of McMaster University.

2.2 Reagents

Uric acid sodium salt, or MSU was purchased from Sigma-Aldrich (Oakville, Ontario).

2.3 Peanut Allergy and Peanut-Induced Anaphylaxis Models

Gastrointestinal sensitization. Mice were sensitized as previously described (Arias *et al.,* 2011) with minor modifications. Specifically, mice were gavaged with 3.75 mg of Kraft All Natural^{TM} PN butter, containing roughly 1 mg of PN protein, along with 5 μg of cholera toxin (CT) (List Biological Laboratories, Campbell, California) once a week for 4 weeks or once a day for 10 days. In gain-of-function studies in the GI, 20 mg of MSU, instead of CT, were gavaged with 3.75 mg of PN butter in 500 μL PBS for 3 consecutive days per week for 4 weeks.

Skin sensitization. Epicutaneous sensitization was performed as described by Flader (2012).[51] Twenty microlitres of 10 mg/mL CPE (Greer laboratories, Lenoir, North Carolina) were directly applied onto shaved and tape-stripped skin daily for 10 consecutive days. The dorsal hair was removed with a peanut clipper and a mechanical razor (Gilette) on day 1 followed by daily tape-stripping for the subsequent 9 days. For gain-of-function studies in the skin, 5 mg of MSU were injected subcutaneously in 500 μL of PBS 5 minutes prior to application of CPE on shaved, but not tape-stripped skin.

Challenge. In all instances, sensitized mice were challenged with 1 mg of CPE in 500 μL PBS intraperitoneally (i.p.) 2 weeks after the last gavage or application of peanut onto the skin.

2.4 Measurement of Systemic Anaphylaxis

To assess anaphylaxis, rectal body temperature was measured immediately before and after challenge at 10-minute intervals for 40 minutes using a rectal probe (VWR International). Clinical scores were recorded as described in our previous studies (5-point grading scheme: 0 = no clinical signs, 1 = pruritus: repetitive ear scratching and ear canal digging with hind legs, 2 = periorbital/periauricular edema; piloerection, 3 = lethargy/decreased activity; lying prone on stomach. 4 = no response to whisker provocation; 5 = End point (seizures or death).[19,24] Hematocrit readings were taken at the 40-minute time point post challenge and centrifuged at 6000-6200 rpm for 1 min (HemataSTAT-II, Separation Technology Inc.).

2.5 Serum Collection

Mice were anaesthetized with isoflurane and peripheral blood was collected via retroorbital bleeding using lime glass Pasteur pipettes (VWR International).

Approximately 150 µL of whole blood was collected per mouse per time point into red-top collection tubes with clot activator (Terumo Capiject) purchased from Fisher Scientific (Ottawa, Ontario). Collected samples were incubated at room temperature for 30 minutes and were then centrifuged at 13200 rpm for 10 minutes at 4°C for 10 minutes. Supernatants (sera) were then collected and stored at -20°C for further analysis.

2.6 Peritoneal Lavage and Cytospin

Seventy-two hours post challenge, mice were anesthetized with isoflurane and euthanized. Three mL of PBS-EDTA (5 mM) was injected into the peritoneal cavity and the abdomen was gently massaged for 10 seconds. The injected fluid was then retrieved using a 1 mL pipette and immediately kept on ice. 1.85×10^5 cells were then loaded for cytospins that were which was stained 24 hours later with Hema 3 stain set (Fischer scientific, Ottawan, Ontario) and kept for further analysis.

2.7 Splenocyte Cell Isolation and Culture

Spleens were collected in 5 mL of Hank's balanced salt solution (HBSS) and triturated through 40 µm cell strainer (Becton Dickinson) with 3 mL syringe plunger. Samples were then centrifuged at 1200 rpm for 10 minutes at 4°C and supernatants were discarded. Next, red blood cells were lysed with 1 mL of ACK lysis buffer (0.5 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 nM Na_2EDTA, pH 7.2-7.4) for 1 minute before the reaction was stopped with 10 mL of HBSS. The samples were then centrifuged at 1200 rpm for 10 minutes at 4°C and supernatants were discarded.

For culture, samples were resuspended in complete Roswell Park Memorial Institute (RPMI) medium (RPMI supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% β-mercaptoethanol). Counting was done using a hemocytometer in trypan blue. 8×10^5 viable cells were cultured with medium alone or with medium supplemented with CPE (250 µg/mL) in a flat bottom, 96-well plate (BD) in triplicate. Cells were incubated for 5 days at 37 °C. The supernatants were collected and triplicates were pooled and stored at -20 ^oC until cytokine measurements.

2.8 Lamina Propria Cell Isolation, Lymph Nodes Cell Isolation

Small intestine was collected in 20 mL of PBS and cut into 3 mm pieces. Intestine segments were then incubated in HEPES-DTT in PBS at 37°C for 15 minutes. Then, intestines were transferred into HEPES-EDTA in FBS at 37°C for 15 minutes followed by incubation in 10% FBS in RPMI containing collagenase A (0.239 mg/mL) and DNase I (100 µg/mL) at 37°C shaker for 50 minutes. Digested intestine was then triturated with a 40 µm cell strainer in RPMI and spun at 1200 rpm in 4°C for 1- minutes. Pellets were collected in 40% percoll with a under layer of 70% Percoll in RPMI. After spinning at 2400 rmp, lamina propria cells were collected between the 40% and 70% Percoll interface.

Mesenteric lymph nodes (MLN) were collected in 1 mL of HBSS. Triturated between frosted slides (Corning, Massachusetts) and a 40 µm cell strainer prior to counting.

2.9 Flow Cytometry Analysis

Two to 4 million cells were incubated with anti-CD16/CD32 (2.4G2; eBioscience) in FACs buffer (1% BSA; 5 mM EDTA; PBS) for 30 min to minimize nonspecific and Fc receptor-mediated binding and stained. Antibodies were obtained from BD Biosciences, eBioscience and BioLegend. CD40–fluorescein isothiocyanate (FITC), OX40L–biotin, streptavidin–phycoerythrin (PE), CD19–PE-Cy5, CD86–peridinin-chlorophyll-protein complex (PerCP)-Cy5.5, CD11c–PE-Cy7, CD3–brilliant violet 421, CD11b–eFluor 605, MHC II–eFluor 650, CD103–allophycocyanin (APC), CD45–APC-eFluor 780 were used. Propidium iodide (eBioscience) and forward/side scatter were used to gate on live singlet cells. BD CompBeads were used for compensation, except for PI, where cells were used for staining instead. Data were acquired on a BD LSR II and analyzed with FlowJo software (Tree Star, Ashland, Ore). Fluorescence minus one controls were used for gating.

2.10 Metabolomics Analysis

2.10.1 Serum sample preparation

Serum proteins were precipitated by the addition of a 1:1 methanol/ethanol solution, in a 4:1 solvent to serum ratio as described by Bruce *et al*.[37] with internal standards (20μM glycine-Phenylalanine, (Sigma-Aldrich); d8-Phe, (Cambridge Isotope Labs); d3-Met (Cambridge Isotope Labs); d5-Trp (Cambridge Isotope Labs). After centrifugation at 13200 rpm for 1 minute, the resulting supernatant was collected and frozen immediately at -20 ^{o}C.

2.10.2 HPLC-MS Analysis of Serum

Supernatants were thawed and injected into a high-performance liquid chromatograph (HPLC) coupled to a mass spectrometer (Agilent 1200 series HPLC; Brüker micrOTOF II time-of-flight mass spectrometer with ESI source). LC columns used were a HILIC column (SeQuant ZIC HILIC 2.1 x 50 mm, 3.5 μm, Umeå, Sweden) and a reversed phase column (Halo RP-amide, Wilmington DE, USA) connected in series configuration. LC was programmed with a flow rate of 200 μL/min, column temperature at 40 °C, autosampler temperature at 4 °C and an injection volume of 2 μL. Elution conditions were carried out beginning at 95% acetonitrile, holding for 30 seconds, ramped down to 30% over 9.5 minutes, held for 5 minutes, then ramping up to 95% over 2 minutes and finally held for 13 minutes. The accompanying mobile phase solution was 10mM ammonium acetate brought to a pH of 3 by addition of formic acid. MS was performed with a mass range of 75-1000 m/z and an acquisition rate of 2Hz; Drying Gas was N_2 at 6L/min at 250°C; nebulizing pressure of 3.0 bar and capillary voltage of 4.5kV in positive mode and -3.8kV in negative mode. Peaks from the chromatogram were integrated and both retention time and *m/z* of analytes were used as metabolic descriptors for statistical analysis along with peak area.

2.10.3 HPLC-MS Data Processing and Multivariate Analysis

Raw data files were converted to .mzXML by the program Compassxport (Brüker Daltonics). The open source program XCMS with CAMERA addition (Scripps Center for

Metabolomics) was used for data alignment, peak deconvolution, peak picking, peak integration, and adduct identification. Univariate analysis was performed with Excel (Microsoft) and multivariate analysis was performed with SIMPCA-P+ (Umetrics). The unbiased, global identification of analytes of interest wwas determined based on (1) statistical significance between different groups of mice between naïve versus sensitized mice at each time points using a two-sided t-test ($P \leq 0.05$); (2) OPLS analysis S-plot (> 0.1; < -0.1); (3) a minimal peak area of 5,000 (~S/N>10) for any given feature.

2.11 Uric Acid Depletion

Allopurinol added to drinking water (200 µg/mL; Sigma-Aldrich) was given starting 7 days prior to the first gavage until 7 days post the last gavage. Average daily dose of allopurinol intake per mouse was 45 mg/day. Uricase (50 units; Sigma-Aldrich, Oakville, Ontario) in 500 µL of PBS was administered i.p. or subcutaneously 5 min prior to each sensitization treatment.

2.12 Serum Peanut-Specific IgE and IgG1

Peanut specific Igs were measured by sandwich enzyme-linked immunosorbent assay (ELISA).

Peanut specific IgG1. Maxi-Sorp 96-well plate (Nunc; VWR Canlab) was coated with CPE (2 µg/mL) in 50 nM carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich, Oakville, Ontario) at 4 ^{o}C overnight. Coated plated were blocked with BSA (1%) in PBS for 2 hours at room temperature. Plates were washed and incubated with serum samples overnight at 4^{o}C. Biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates) were added and incubated with the samples the next day for 2 hours before washing and a 1 hour incubation with alkaline-phosphatase streptavidin (Sigma-Aldrich, Oakville, Ontario) for 1 hour at room temperature. *P*-nitrophenyl phosphate tablets were used to develop the assay and H_2SO_4 (2 M) was added to stop the reaction before Absorbance readings taken at 450 nm.

Peanut specific IgE. Maxi-Sorp 96-well plate was coated with rat anti-mouse IgE Abs (2 µg/mL; BD Pharmingen) in PBS overnight at 4 ^{o}C. Coated plates were wash and blocked with Tween buffer (10% bovine serum; 1% bovine serum albumin; 0.5% Tween in PBS) for 1 hour at 37^{o}C and washed. Serum samples were then incubated for 2 hours at room temperature before CPE-digoxingenin (DIG) conjugate solution was added for coupling with CPE. Peroxidase-conjugated anti-DIG was added at 37 ^{o}C for 1 hour before tetramethylbenzidine (0.1 mg/mL) solution was added to develop the colour reaction. H_2SO_4 (2 M) was added last to stop the reaction for absorbance reading at 450 nm.

2.13 Oral Peanut Challenge in Peanut Allergic Children

Peanut allergic children, 5 to 10 years old, with a positive skin prick test (wheal size 3 mm larger than that of the saline control) and *in vitro* IgE test [CAP-FEIA] (IgE>15 kU/L) were enrolled into the study. Peanut protein flour was mixed with chocolate pudding and given at increasing amounts (1, 2, 4, 6, 12, 25, 50, 75, 100, 200, 300, 400, 500, 575, 750, 1000 mg) at 30 min intervals until signs or symptoms (*i.e.* erythema, morbilliform rash, urticaria, angioedema, conjunctival swelling, scleral edema, tearing,

nasal congestion, rhinorrhea, sneezing, wheezing, coughing, drop of PEF or FEV1 by >20%, vomiting, diarrhea, abdominal pain and blood pressure dropping by >20%) were observed. Serum samples were collected before challenge and again after the challenge was stopped because of the emergence of symptoms.

2.14 Data Analysis

Data were analyzed using GraphPad Prism version 5.0 and expressed as mean ± SEM. Results were interpreted using either a t-test or a one-way analysis of variance (ANOVA) with a Tukey's or Bonferroni's *post hoc* test. Differences were considered statistically significant when p-value were less than 0.05 (*), 0.01 (**), and 0.001 (***).

CHAPTER 3: BIOMARKERS AND MEDIATORS OF PEANUT ALLERGIC SENSITIZATION

Metabolite Profile of Allergic Mice

Sensitization to peanut is a gradual process. In our model of intragastric (i.g.) sensitization, the number of sensitization gavages positively correlates with the level of circulating peanut-specific Igs as well as the intensity of clinical symptoms upon challenge.[22] To investigate the chemical changes occurring during the process of sensitization, we performed LC-MS analysis in serum of mice undergoing sensitization 12 hours after the second and the fourth gavages as well as 2 weeks later, prior to challenge (Figure 1). With 7 mice per group, mice were either gavaged with PN and CT, PN-only, CT-only or PBS. Of note, only co-administration of PN and CT leads to the production of peanut-specific Igs.

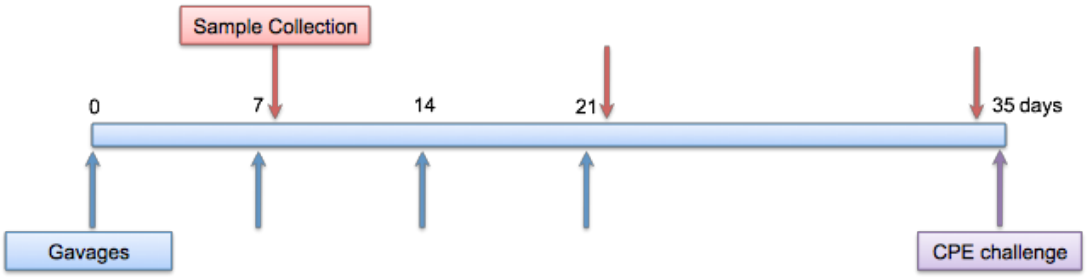

Figure 1. Gastrointestinal model of peanut-induced anaphylaxis in mice. Serum samples were collected 12 hours after the second and fourth sensitization and 2 weeks after the last sensitization.

To qualitatively visualize the metabolomic changes due to sensitization, orthogonal partial least squares-discriminant analysis (OPLS-DA), a multivariate analytical technique, was applied to assess the total degree of metabolomic differences between the treatment and control groups. After 2 weekly gavages, a clear, yet incomplete differentiation was seen in the serum metabolite profile between groups (Figure 2, A). These separations became more apparent after the fourth gavage (Figure 3, B). Prior to challenge, on day 35, when mice were fully sensitized, as indicated by their serum levels of PN-specific IgE and IgG1 (data not shown), a clear separation was seen on the OPLS-DA plot between the sensitized group (*i.e.,* PN and CT treated) and the non-sensitized groups (*i.e.,* PN-only, CT-only and PBS treated). Importantly, PN-only and CT-only treated groups became indistinguishable (Figure 2, C), indicating that metabolomic changes due to PN alone or CT alone had subsided 2 weeks after the last sensitization. Together, the OPLS-DA analysis showed that allergic and non-allergic animals express distinct metabolite profiles.

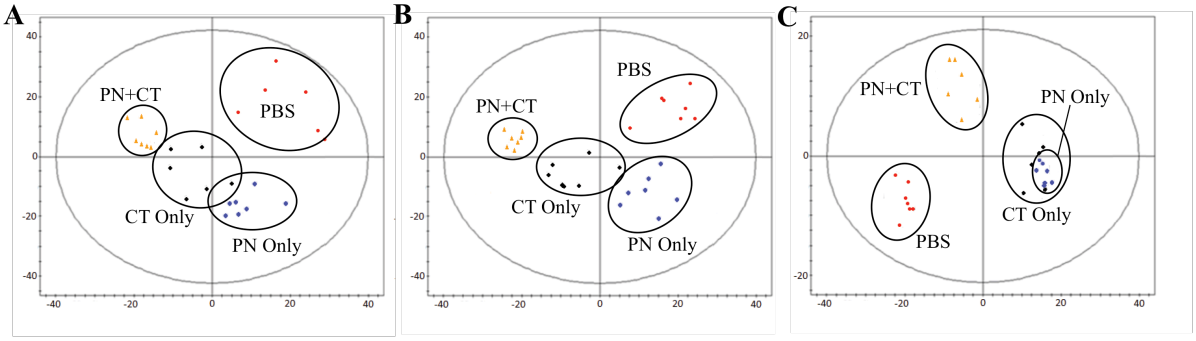

Figure 2. OPLS-DA analysis of PN and CT, PN-only, CT-only and PBS (sham) treated mice showing separation in their metabolomic profile after 2 weeks (**Fig 2, A**) and 4 weeks of sensitization (**Fig 2, B**), and 1 day prior challenge (**Fig 2, C**). Each point on the OPLS-DA plot represents a single mouse.

Identification of Metabolites Associated with Peanut Sensitization

Following this initial study, we proceeded to identify the metabolites that defined the chemical state of PA at week 6. Out of the more than 2,500 chemical features detected, Table 1 presents the list of molecules that were differentially expressed along with their relative change ($p<0.01$), as indicated by the fold change of expression levels in allergic mice compared to non-allergic mice (*i.e.,* PN-only and CT-only treated groups combined). Seven metabolites were identified by standard molecules and 5 molecules were matched with mass spectra found in publicly accessible databases (METLIN metabolomics database or Human Metabolome Database). Seven additional metabolites could not be identified with the in-house standards or the public databases. Collectively, these 19 metabolites represent potential biomarkers or mediators that define the chemical basis of PN sensitization.

Table 1. Metabolites that were significantly changed (p<0.01) in peanut allergic mice (PN and CT treated) compared to non-allergic mice (PN-only and CT-only) one day before challenge. The chemical features are ranked in descending order of their p-values.

m/z	Retention Time (min)	ID	p-value (ANOVA)	Fold Change	Identification Method
(+) 136.073	3.4	Adenine	0.000	3.0	Standards
(+) 137.056	4	Hypoxantine	<0.001	0.5	
(-) 151.026	4	Xanthine	<0.001	0.6	
(-) 167.021	8	Uric acid	0.003	1.7	
(+) 244.099	7.6	Cytidine	<0.001	0.7	
(-) 267.074	6.6	Inosine	0.01	0.7	
(+) 268.115	3.5	Adenosine	<0.001	3.5	
(+) 269.117	3.5	Arabinosylhypoxanthine	<0.001	2.7	Database
(+) 540.43	21.8	Hexacosanoylcarnitine	<0.001	2.0	
(+) 584.454	21.7	LysoPC (18:0)	<0.001	2.4	
(+) 762.568	20.9	PC (34:0)	<0.001	1.5	
(+) 764.536	23	PC (34:0)	0.002	1.6	
(+)275.264	21.7	Unknown	<0.001	1.9	No match
(-)309.002	6.7	Unknown	<0.001	0.6	
(+) 319.289	21.6	Unknown	<0.001	1.7	
(+) 380.342	21.5	Unknown	<0.001	1.6	
(+) 512.422	21.3	Unknown	0.002	1.7	
(+) 614.478	21.5	Unknown	0.001	1.8	
(+) 628.482	21.6	Unknown	<0.001	2.4	

Changes in Purine Metabolism Pathway in Peanut Sensitization

To understand the biological significance of the metabolites detected during sensitization and uncover metabolic pathways that may be involved, we sought to identify biochemical relationships among the metabolites. Our initial analysis revealed that 6 of the 12 identified metabolites, namely adenine, adenosine, inosine, hypoxanthine, xanthine and UA, are associated with purine metabolism, otherwise known as the UA synthesis pathway (Figure 3, A). Thus, we next examined in more detail their expression throughout the course of sensitization. Again, PN-only and CT-only treated groups were combined and used as baseline to determine the fold changes in these metabolites. Serum levels of adenine and adenosine were increased throughout the entire course of sensitization (6 weeks) (Figure 3, B and C). The levels of inosine and hypoxanthine were elevated initially at week 2, but then decreased over the next 4 weeks (Figure 3, D and E). Xanthine levels were consistently lower in mice treated with PN and CT throughout sensitization (Figure 3, F). The level of UA, on the other hand, was indistinguishable between groups at week 2 and began to increase in the sensitized group over the next 4 weeks (Figure 3, G). Overall, aside from adenine and adenosine, serum levels of the three

precursors and intermediate molecules in the pathway decreased steadily during sensitization while the end product, uric acid, increased.

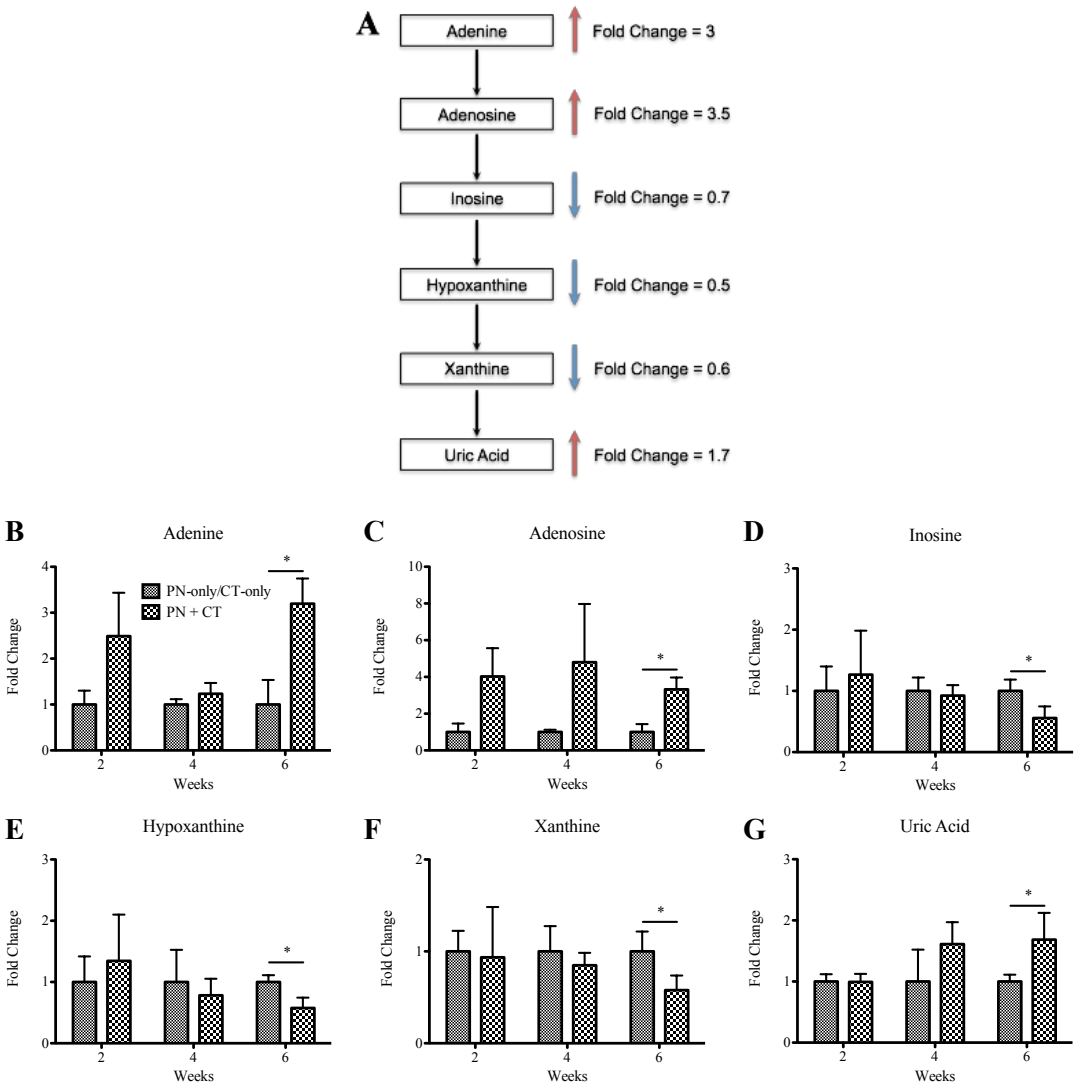

Figure 3. Changes in the level of metabolites in the pathway of purine metabolism during peanut (PN) sensitization. **A**, purine metabolism pathway and fold changes of purines in serum of sensitized mice at week 6 of the sensitization protocol; **B-G**, changes in serum purine levels at week 2, 4, and 6 of PN sensitization. The experiment was repeated once with 5-7 mice per group each time.

Uric Acid is Necessary in Intragastric Sensitization to Peanut

The presence of elevated levels of UA during allergic sensitization to PN was an intriguing observation. To investigate a potential causal relationship between UA levels and PN sensitization, we first employed a combination strategy using allopurinol and uricase to deplete UA during sensitization *in vivo*. Allopurinol and uricase both contribute to the depletion of UA through distinct mechanisms. Allopurinol, an analogue of hypoxanthine, inhibits the activity of xanthine oxidase therefore limiting oxidation of hypoxanthine and xanthine to UA.[52] Uricase, also known as urate oxidase, catalyses the oxidation of UA to allantoin and directly reduces the level of UA.[53]

To investigate the effect of UA depletion during sensitization, allopurinol was given through drinking water starting 1 week prior to the first gavage and continued until 1 week after the last gavage; uricase was injected i.p. 5 minutes prior and 6 hours after each gavage. Unexpectedly, on the third week of sensitization, mice injected with uricase mounted a severe response, marked by a severe drop in body temperature and sometimes, death (data not shown). We attributed this effect to an immune response to uricase itself. Therefore, we utilized a modified 10-day protocol to sensitize mice. Similar to before, allopurinol was given in drinking water starting 1 week prior to the first gavage until 1 week after the last gavage. During the 10 days, repeated uricase treatments were given i.p. prior to each of the 10 daily gavages, in an attempt to avoid the elicitation of an adaptive immune response against uricase.

Two weeks after the last sensitization using the 10-day model, UA depletion resulted in a significant reduction in the levels of PN-specific IgG1 (PN-IgG1) and IgE (PN-IgE) (Figure 4, A and B). After challenge, rectal temperature, hematocrit levels and clinical scores of UA depleted mice were dramatically reduced compared to positive controls and, in fact, indistinguishable from naïve mice, displaying no detectable signs of PIA (Figure 4, C-E). Therefore, UA depletion prevented PN sensitization and fully protected animals from PIA, indicating that UA is, indeed, critical to the development of allergic sensitization in this model.

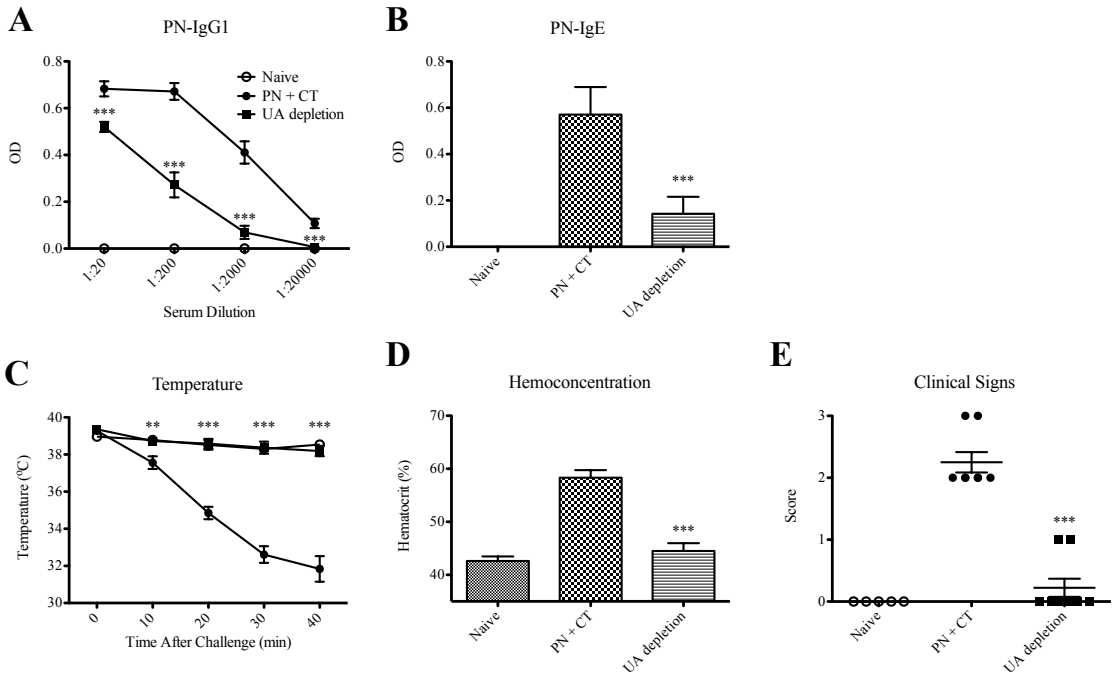

Figure 4. Uric acid is necessary for the development of PA and PIA. Levels of PN-IgG1 (**Fig 4, A**) and PN-IgE (**Fig 4, B**) at 1 day before challenge. Anaphylactic assessment of core body temperatures (**Fig 4, C**), hematocrit levels (**Fig 4, D**) and clinical signs (**Fig 4, E**). The data is pooled from 2 experiments with 5-8 mice per group.

Monosodium Urate Crystals are Sufficient to Induce Peanut Sensitization through the Gut

The loss-of-function study showed that endogenous UA is required for allergic sensitization through the gut. To investigate the role of UA crystals as an adjuvant in GI sensitization, we gavaged mice with PN along with MSU instead of CT. MSU has low solubility in PBS and is difficult to gavage high concentrations through a narrow gavage needle. Therefore, 3 consecutive daily gavages were given per week for 4 weeks to deliver MSU for allergic sensitization.

Following 4 weeks of sensitization and an additional 2 weeks of rest period, peanut-specific Abs (PN-Abs) were detected in mice gavaged with PN and MSU, but not in mice gavaged with PN alone (Figure 5, A and B). Furthermore, only mice treated with PN and MSU sensitized developed signs and symptoms of PIA, marked by a substantial drop in rectal body temperatures, rise in hemoconcentration and display of clinical signs (Figure 5, C-E). Together, these findings demonstrated that exogenously delivered MSU is sufficient to induce allergic-sensitization through the GI tract.

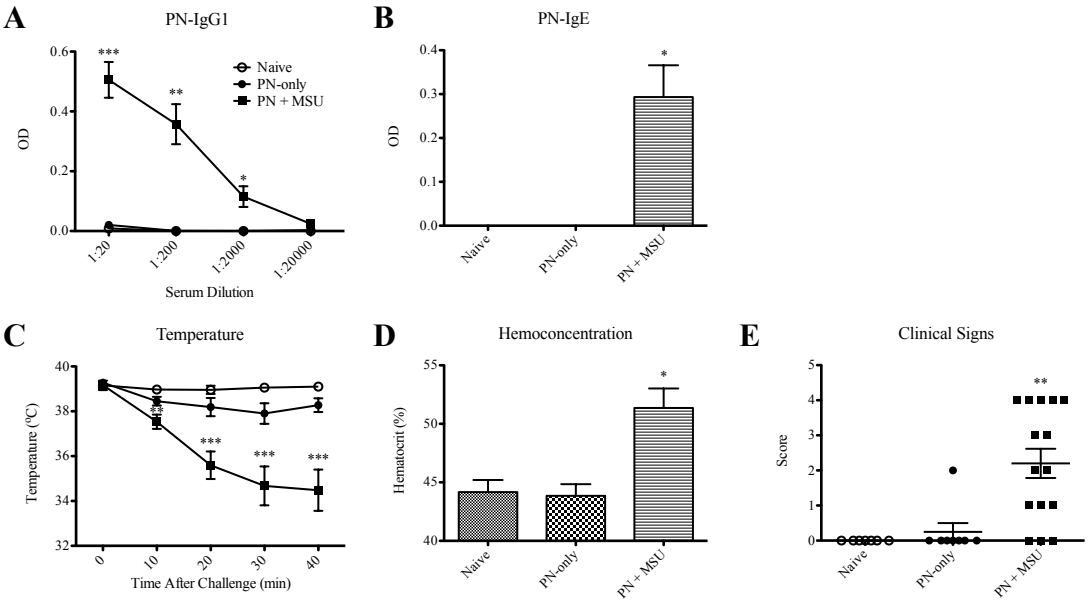

Figure 5. Uric acid crystals induce peanut-allergic sensitization in the gut. Serum levels of PN-IgG1 (**Fig 5, A**) and PN-IgE (**Fig 5, B**) 1 day prior challenge. Anaphylactic assessment by core body temperature (**Fig 5, C**) hematocrit levels (**Fig 5, D**) and clinical signs (**Fig 5, E**). The data represent a pooled collection of 3 separate experiments with 5 mice per group.

Uric Acid is Necessary in Skin Sensitization to Peanut

Data above showed that UA is necessary and sufficient to elicit PN sensitization in a model of oral sensitization driven by CT. To investigate whether the UA requirement is exclusive to this route and adjuvant, we used a model of PIA developed in our laboratory where sensitization is achieved through the skin and does not require CT. In this model, PN sensitization is elicited by the application of PN onto tape-stripped skin.[51] It is important to note that, in this model, PN-IgE, but not IgG1, levels in serum collected before challenge were below the detection limit by our ELISA in this model. UA depletion was accomplished as before through the administration of allopurinol in drinking water and subcutaneously injected uricase adjacent to the site of tape-stripping.

After 10 days of PN application directly onto tape-stripped skin, mice developed significant levels of PN-IgG1 (Figure 6, A). Upon challenge, sensitized mice experienced a severe drop in core body temperature, increase in hematocrit levels and showed clinical signs of anaphylaxis. UA depletion once again inhibited the production of PN-Abs (Figure 6, A & B) and fully abrogated signs and symptoms of PIA (Figure 6, C-E) indicating that the importance of UA is not exclusive to CT driven systems.

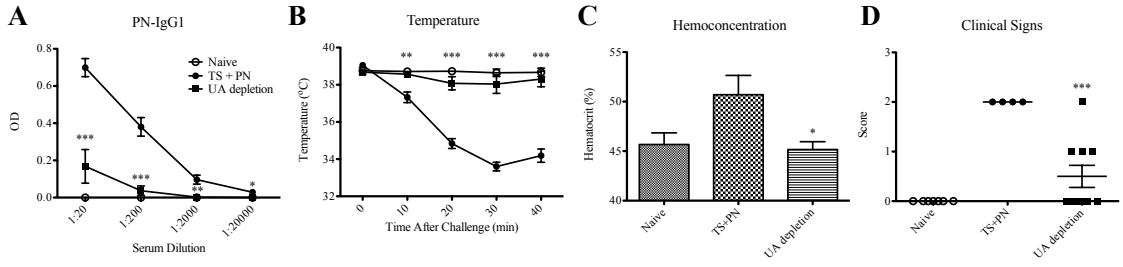

Figure 6. Uric acid depletion during skin sensitization prohibited the development of PA and anaphylaxis. **A,** Serum levels of PN-IgG1. Anaphylactic assessment of core body temperature (**Fig 6, B**), hematocrit levels (**Fig 6, C**) and clinical signs (**Fig 6, D**). The data represent a pooled collection of 2 experiments with 7-10 mice per group.

Monosodium Urate Crystals are Sufficient to Induce Peanut Sensitization through the Skin

Next, we performed a series of studies to investigate if MSU are sufficient to induce sensitization through the skin. In this experiment, we shaved mice to expose a patch of skin and applied PN without tape-stripping. Without tape-stripping, PN application on the skin did not induce PN-IgG1 production (Figure 7, A) or anaphylaxis (Figure 7, B-D). In contrast, when MSU crystals were injected subcutaneously concomitant with epicutaneous application of PN for 10 straight days, mice developed measureable PN-IgG1 2 weeks after the last sensitization treatment and underwent anaphylaxis after PN challenge. These findings are in agreement with those in the model of GI sensitization and, collectively, suggest that UA may serve as a common trigger by which external stresses, such as CT or tape-stripping, activate the immune system.

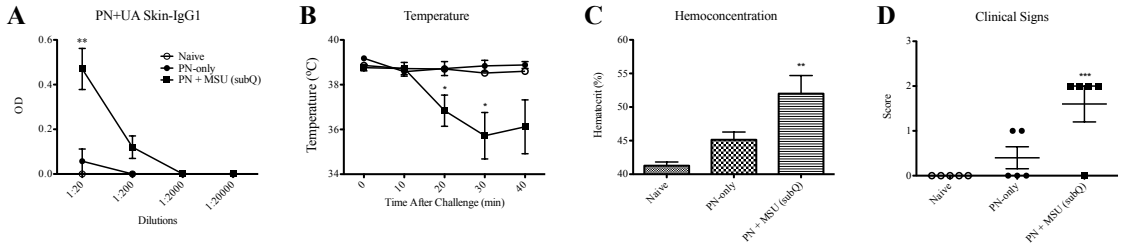

Figure 7. Uric acid crystals induce peanut-allergic sensitization in the skin. **A,** Serum levels of peanut-specific IgG1 1 day prior challenge. Anaphylactic assessment of core body temperature (**Fig 7, B**) hematocrit (Hct) levels (**Fig 7, C**) and clinical signs (**Fig 7, D**). The data was pooled from 2 experiments with 3-5 mice per group. *SubQ*, Subcutaneous injection.

Uric Acid Induces DC Activation in vivo

To understand mechanisms of UA-mediated PA, we examined the DC population in the intestine and the local draining lymph node. Specifically, we isolated cells from the LP and the MLN following one single gavage with PN and CT, with or without UA depletion *in vivo* using the same strategy as before.

In mice that were given a single gavage with PN and CT, an increased proportion of inflammatory DC ($CD11b^+$ $CD11c^+$ MHC II^+) (Fig 8, A) and increased expression of CD86 (Fig 8, B) were observed in the LP 12 hours after gavage. When UA was depleted, both the increase in DCs and their activation status were significantly inhibited (Fig 8, A and B). At the 24-hour time point, the number of DC returned to baseline and was comparable to those in naïve mice. When we examined the effect of UA depletion on migratory DC, *i.e.,* $CD103^+$ DCs, we found that both the positive control (PN + CT) and UA depletion showed similar levels of $CD103^+$ DC at 12 hours post gavage. However, at 24 hours, the levels of $CD103^+$ DC in the intestine LP were significantly increased in mice treated with PN + CT, but this increased was prevented in mice subjected to UA depletion (Fig 8, C).

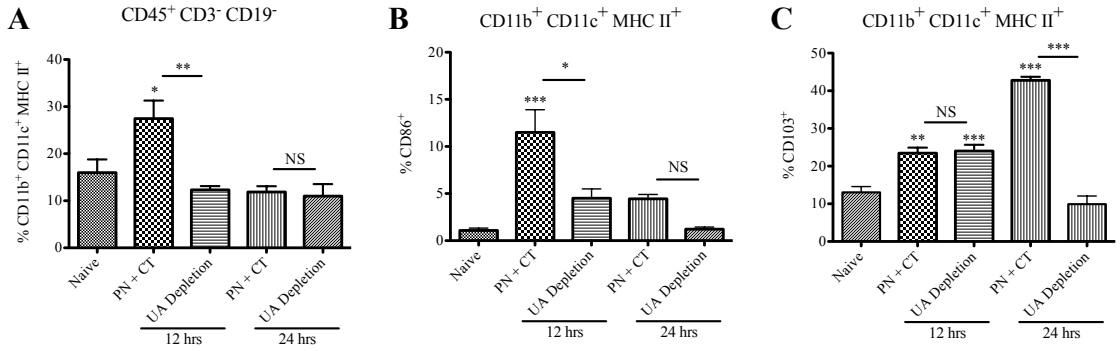

Figure 8. UA depletion prevents recruitment and activation of inflammatory DC ($CD11b^+$ $CD11c^+$ MHC II^+) in the lamina propria (LP) *in vivo*. **A**, DC ($CD11c^+$ MHC II^+) population 12 and 24 hours after oral gavage with peanut and CT (PN + CT). Expression of activation marker CD86 (**Fig 8, B**) and migratory marker CD103 (**Fig 8, C**) on DC in the LP. The experiment was performed twice with 3-7 mice per group each time.

After one single gavage with PN and CT, we observed a gradual increase in the population of inflammatory DC ($CD11b^+$ $CD11c^+$ MHC II^+) in the MLN. This increase reached statistical significance at the 24-hour time point. Interestingly, UA depletion did not prevent this increase (Fig 9, A). However, similar to the observation in the LP, UA depletion inhibited upregulation of CD86 (Fig 9, B). Furthermore, UA depletion greatly reduced the expression of the co-stimulatory molecule OX40L on DC (Fig 9, C). These

findings suggest that UA impacts activation and expression of co-stimulatory molecules but not DC migration to MLN.

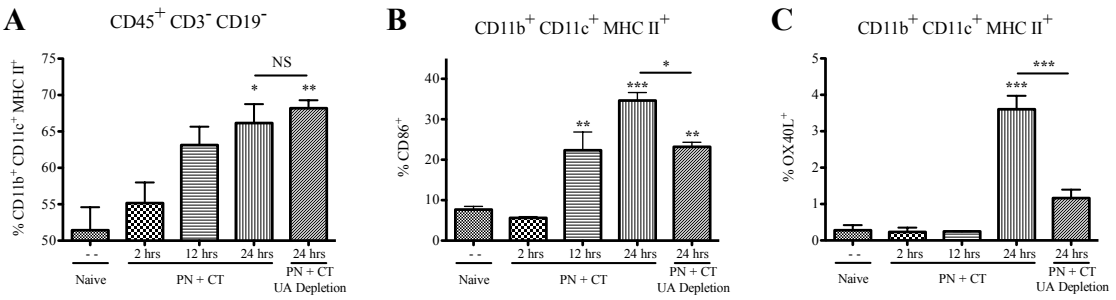

Figure 9. UA depletion prevents DC activation in the MLN after peanut and CT exposure. **A**, DC ($CD11b^+$ $CD11c^+$ MHC II^+) population in the MLN post treatment. Expression of activation makers, CD86 (**Fig 9, B**), and co-stimulatory molecule, OX40L (**Fig 9, C**), on DCs. The experiment was performed twice with 3-7 mice per group each time.

Peanut Allergy is Independent of IL-1β and Caspase-1

To further investigate the mechanism of UA-mediated PN sensitization, we examined molecules that are typically associated with the UA pathway, namely IL-1β and caspase-1. In IL-1β deficient mice, PN sensitization and anaphylaxis remained intact in both the GI (Fig 10, A-D) and the skin model (Fig 10, E-H), as shown by elevated levels of PN-IgG1 prior to challenge and the display of anaphylactic signs and symptoms after PN challenge. In fact, caspase-1 deficient mice also became sensitized to PN through the skin (Fig 11, A-D). In the experiments involving caspase-1 deficient mice through intragastric gavages, we observed that all mice (n=5) succumbed when subjected to the 10-day protocol, possibly because mice in BALB/c or NOD backgrounds were more sensitive to gavaged-induced stress and damage compared to their C57BL/6 counterparts, which resulted in their death. Nevertheless, these findings showed that IL-1β is dispensable in PN sensitization and that the process is independent of the NLRP3 inflammasome pathway.

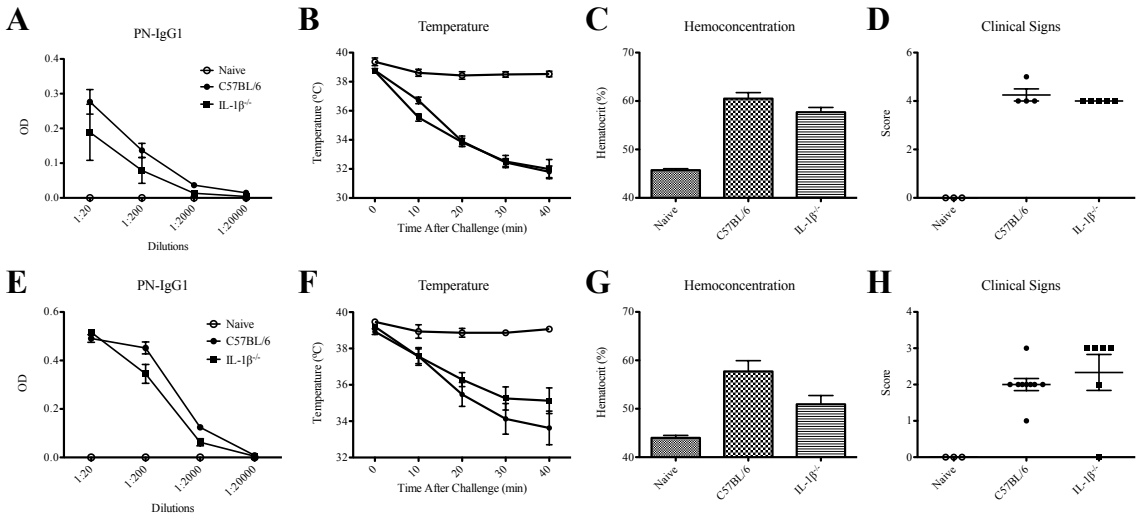

Figure 10. IL-1β is dispensable in peanut sensitization. The study was performed both in the gut sensitization model (**A-D**) and the skin sensitization model (**E-H**). **A** and **E,** Serum levels of peanut-specific IgG1 (PN-IgG1) 1 day prior challenge. Anaphylactic assessment of core body temperature (**Fig 10, B** and **F**) hematocrit (Hct) levels (**Fig 10, C** and **G**) and clinical signs (**Fig 10, D** and **H)**. For these experiments, each group contained 3-5 mice.

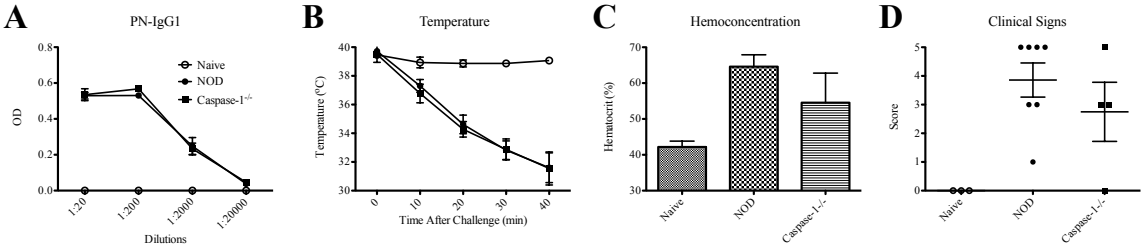

Figure 11. Peanut sensitization through the skin is not dependent on caspase-1. **A,** Serum levels of peanut-specific IgG1 (PN-IgG1) 1 day prior challenge. Anaphylactic assessment of core body temperature (**Fig 11, B**) hematocrit (Hct) levels (**Fig 11, C**) and clinical signs (**Fig 11, D**). The experiment was performed with 3-7 mice per group.

CHAPTER 4: BIOMARKERS OF PEANUT-INDUCED ANAPHYLAXIS IN MICE AND HUMANS WITH PEANUT ALLERGY

Metabolite Profile of Allergic Mice Undergoing Anaphylaxis

The aim of this study was to identify potential biomarkers or mediators of PIA, which could have either prognostic or therapeutic value, respectively. In this study, mice were sensitized with our conventional 4-week long protocol followed by a 2-week rest period. After challenge, sensitized mice responded quickly to intraperitoneal injection of CPE, as indicated by a rapid drop in rectal temperatures along with a drastic increase in hematocrit levels (Fig 12, A & B). Each mouse was only bled once in this experiment to avoid introducing additional variables to the analysis due to repeated bleeding. Separate groups of mice (n=5-8) were used for each time point. From the analysis, a noticeable difference was seen in the metabolomic profile of allergic mice in a OPLS-DA plot beginning at 1 minute after CPE challenge when compared to their healthy counterparts prior to challenge (Fig 12, C). As the severity of the disease increased, the separation in the metabolite profiles between allergic and non-allergic mice became more pronounced (Fig 12 A-C). This initial analysis showed that anaphylaxis is associated with a change in the metabolomic profile.

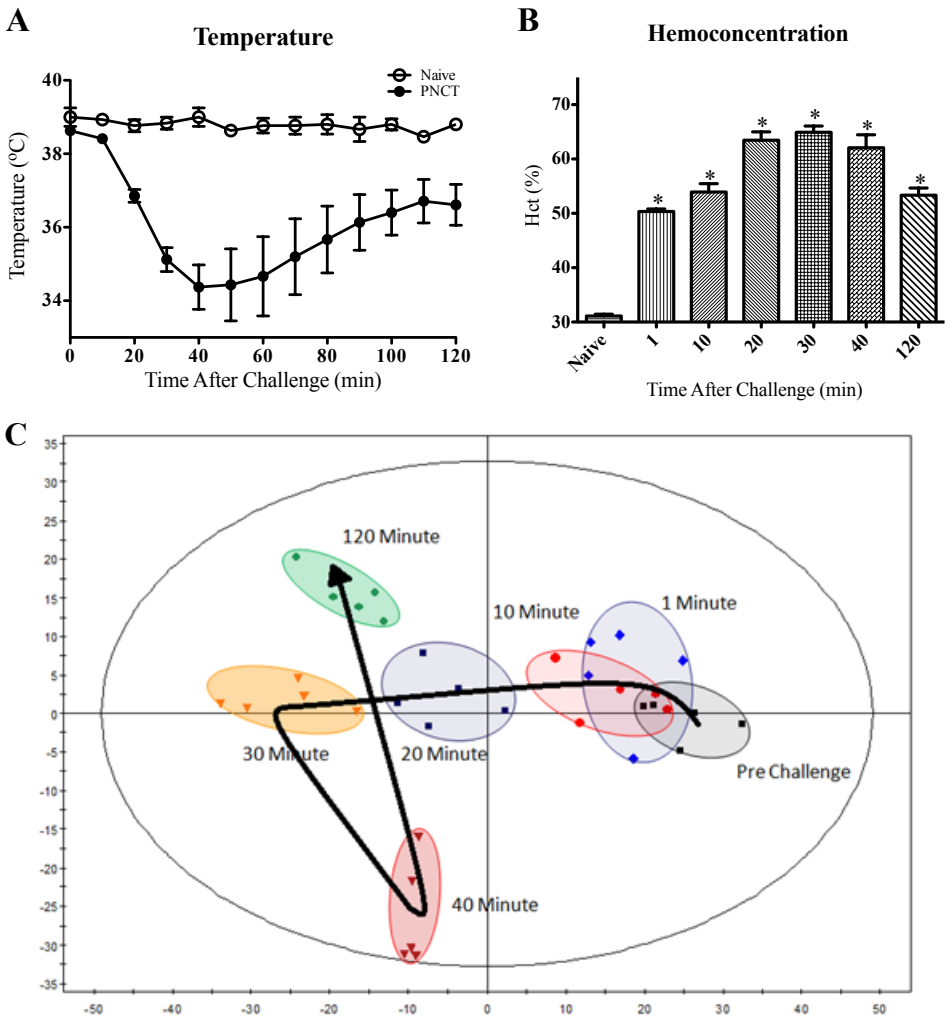

Figure 12. Metabolomic changes in mice undergoing PIA. Anaphylactic assessment of core body temperature (**Fig 12, A**) and hematocrit levels (**Fig 12, B**). **C**, OPLS-DA of mice undergoing anaphylaxis over a 2-hour period. Each point represents a single mouse.

Identification of Metabolites Associated with Peanut-Induced Anaphylaxis

Over 3,500 mass spectral features were detected in the serum at 1 minute after challenge. At this time, we identified 9 molecules that were significantly elevated or reduced (Table 2). The identity of the first 3 molecules was confirmed by matching their spectra features with that of standard molecules; 3 additional molecules were identified by matching their mass spectra with online databases as mentioned in Chapter 3; the 3 remaining molecules were unidentifiable with our in-house standards or the online databases. As expected, histamine was detected in high abundance in mice undergoing anaphylaxis, while levels of histamine were below the detection limit in naïve mice that were also challenged. As a result, the lower detection limit was used as the reference level of histamine in naïve,

challenged mice to calculate the fold change of histamine, indicated as >100, in allergic animals. Other molecules detected include 2 phosphatidylcholine (PC) lipids and methylhistamine, which were elevated in allergic animals; while metanephrine and allantoin were reduced.

At 10, 20 and 30 minutes, an increasing number of spectral features were detected, but due to the lack of time-matched controls for these time points; metabolites unique to these time points were not examined. Nonetheless, we detected 12 molecules, whose levels were significantly changed at 40 minutes after challenge (Table 3). Of these molecules, 7 were identified by standards, 3 by online databases, and 2 were unidentified. Serum levels of taurine, uridine, creatinine, creatine, 4-aminobutyric acid (GABA), norepinephrine sulfate (NE-S), and methylhistamine were elevated. Levels of ascorbic acid, acetylcarnitine, and erythroxanthin, on the other hand, were reduced during anaphylaxis. A positive correlation was observed between the number of metabolites detected and the severity of PIA. The identification of these metabolites provides a list of potential biomarkers and mediators.

Table 2. Metabolites that were significantly changed (p<0.01) in mice undergoing PIA in comparison to healthy mice at one minute after challenge. The chemical features are ranked in descending order of their p-values.

m/z	Retention Time (min)	ID	*p*-value (t-test)	Fold change	Identification Method
(+) 112.0871	13.6	Histamine	<0.001	>100	Standards
(-) 198.0987	8.5	Metanephrine	<0.001	0.73	
(+) 157.0362	7.8	Allantoin	0.005	0.78	
(+) 832.5767	27.2	PC(40:7)	0.02	1.2	Database
(+) 126.1025	13.2	Methylhistamine	0.036	1.3	
(+) 806.5629	27.3	PC(38:6)	0.039	1.2	
(-) 163.0614	8.5	$C_{10}H_{11}O_2$	0.002	0.65	No match
(-) 243.0635	7.3	$C_9H_{11}N_2O_6$	0.003	0.76	
(+) 582.4254	8.7	Unknown	0.025	1.3	

Table 3. Metabolites that were significantly changed (p<0.01) in mice undergoing PIA in comparison to healthy mice at 40 minute after challenge. The chemical features are ranked in descending order of their p-values.

m/z	Retention Time (min)	ID	*p*-value (t-test)	Fold change
(-) 124.005	9.4	Taurine	0.001	1.5
(-) 243.062	4.3	Uridine	0.002	1.7
(+) 114.068	6.5	Creatinine	0.005	1.6
(-) 175.023	3.1	Ascorbic Acid	0.006	0.4
(+) 132.077	9.8	Creatine	0.007	1.6
(+) 104.109	8.5	4-aminobutyric acid	0.008	1.7
(+) 204.123	9.6	Acetylcarnitine	0.009	0.7
(-) 250.044	4.8	Norepinephrine Sulfate	0.001	2.9
(+) 170.093	12.4	Methylhistidine	0.004	1.6
(-) 599.38	7.7	Erythroxanthin	0.01	0.5
(+) 166.018	6.4	$C_4H_7NO_4S$	0.001	0.5
(+) 399.271	2.2	$C_{44}H_{79}O_{10}P$ (z=2)	0.001	0.2

Metabolomic Profile of Allergic Reactions to Peanut in Children

Next, to determine the metabolic changes in allergic patients during an allergic reaction, we analyzed the metabolomics profile of PN allergic children undergoing oral PN challenge. Serum samples were collected before the ingestion of PN and, again, after signs or symptoms of an allergic reaction were reported. It is important to acknowledge that, unlike in the mouse model, PN was given in small increments over time to avoid the onset of overt anaphylaxis and that challenges were stopped at the earliest indication of a clinical response. In this preliminary study with 9 patients, the OPLS-DA plot demonstrated that, comparable to what was observed in the mouse study, there was a distinct separation between the metabolite profile before and after challenge (Fig 13). These data illustrate that even a relatively mild reaction can lead to a detectable change in the chemical profile in human serum.

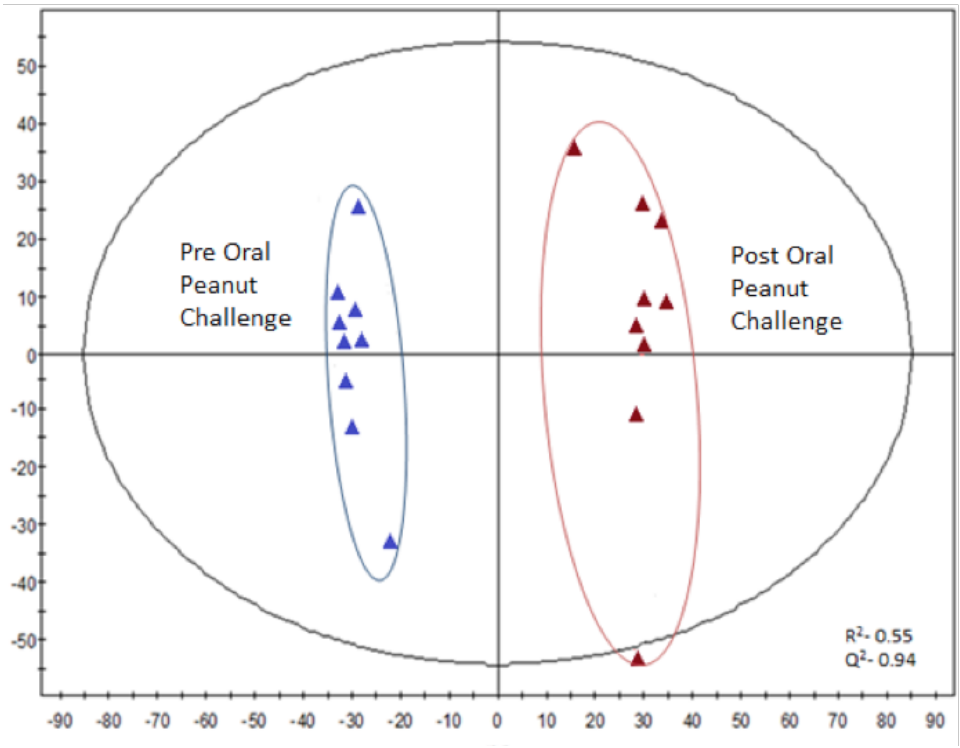

Figure 13. OPLS-DA of serum samples from allergic patients before and after oral peanut challenge.

Out of the 5,500 chemical features detected, approximately 200 features appeared only in serum after challenge. Furthermore, another 70 and 43 additional features were significantly elevated and reduced, respectively, after challenge as shown in Table 4. Presently, we are unable to provide the specific chemical structure to the molecular formulas we have identified using solely LC-MS. More robust chemical analytical techniques such as tandem-MS will be required. Each point on the plot represents a patient.

Table 4. Metabolites that were significantly changed (p<0.01) in 9 peanut allergic children after oral peanut challenge. The chemical features are ranked in descending order of their p-values. Fold change for features present only in post challenged samples were estimated by dividing the signal to noise (S/N) of the peak by the quantitation limit (S/N=10).

m/z	Identification	Retention Time (min)	Fold Change	t-test
(+) 377.264	$C_{20}H_{33}N_4O_3$	2.1	10	1.80E-07
(+) 555.356	PG(21:0)	7.1	10	2.30E-07
(+) 511.331	$C_{18}H_{43}N_{10}O_7$	6.7	15	3.30E-07
(+) 606.391	LysoPC(24:1)	6.6	20	8.50E-07
(+) 465.310	PA(20:1)	2.9	400	1.20E-06
(-) 740.391	$C_{25}H_{59}N_9O_{14}P$	3.3	150	1.20E-06
(+) 467.305	$C_{16}H_{39}N_{10}O_6$	5.8	20	1.40E-06
(+) 562.364	LysoPE(24:2)	5.8	120	1.50E-06
(+) 406.253	$C_{14}H_{32}N_9O_5$	4.5	20	1.90E-06
(-) 635.352	$C_{23}H_{51}N_6O_{14}$	3.1	5	1.90E-06
(+) 421.285	$C_{20}H_{42}N_2O_5P$	2.1	60	2.00E-06
(+) 452.305	$C_{18}H_{42}N_7O_4S$	3.2	9	2.00E-06
(+) 518.340	LysoPC(18:3)	4.5	40	2.10E-06
(+) 540.354	LysoPS(20:0)	5.1	50	2.30E-06
(-) 723.400	PI(26:1)	3.3	20	2.30E-06
(+) 520.342	LysoPC(18:2)	2.3	150	2.80E-06
(+) 566.413	LysoPE(24:0)	2.5	10	2.80E-06
(+) 362.226	$C_{12}H_{28}N_9O4$	3.6	30	2.90E-06
(+) 476.318	LysoPC(O-16:0)	5.5	24000	3.10E-06
(+) 482.315	LysoPC(O-16:3)	2.2	35	3.10E-06
(+) 401.266	LysoPE(24:1)	2.5	50	3.20E-06
(+) 564.372	$C_{23}H_{38}O_4(Na^+)$	4	6	3.20E-06
(+) 466.331	$C_{31}H_{52}N_8O_7P$	3.8	14.5	3.50E-06
(-) 679.372	PE(18:0)	2.9	7	3.50E-06
(-) 713.373	$C_{26}H_{54}N_{10}O_{11}P$	3.4	10	3.60E-06
(+) 693.455	LysoPA(36:6)	2	40	3.70E-06
(+) 608.403	LysoPC(24:0)	2.7	65	4.00E-06
(+) 388.260	$C_{22}H_{34}N_3O_3$	2	15	5.60E-06
(-) 625.323	$C_{33}H_{46}N_4O_6P$	3.2	60	7.50E-06
(+) 379.253	Neuroprostane	3.5	40	8.90E-06

m/z	Identification	Retention Time (min)	Fold Change	t-test
(-) 581.294	$C_{31}H_{42}N_4O_5P$	2.9	15	1.00E-05
(-) 669.344	PI(22:0)	3	15	1.00E-05
(+) 524.343	LysoPC(18:0)	2.8	2500	1.10E-05
(+) 126.023	Taurine	9.3	0.55	1.20E-05
(-) 124.007	Taurine	9.3	0.54	2.20E-05
(+) 402.237	$C_{14}H_{36}N_5O_6S$	5.6	3	3.00E-05
(+) 313.158	Phe-Phe	6.8	0.31	4.10E-05
(+) 314.184	$C_{13}H_{24}N_5O_4$	4.5	3	5.20E-05
(+) 311.140	Phe-Phe	6.9	0.31	6.40E-05
(-) 151.025	Xanthine	4.3	0.48	8.90E-05
(+) 335.114	$C_{10}H_{21}N_6O_3P_2$	4.5	3	1.10E-04
(+) 110.036	$C_4H_4N_3O$	4.1	0.43	1.40E-04
(+) 275.121	$C_7H_{15}N_8O_4$	4.2	4	1.70E-04
(+) 153.043	Xanthine	4.2	0.48	2.10E-04
(+) 539.270	$C_{20}H_{40}N_6O_9P$	2.4	4	2.30E-04
(+) 253.133	$C_{16}H_{17}N_2O$	4.2	2	2.60E-04
(-) 233.014	Phenylglycol-3-O-sulfate	4.5	10	3.70E-04
(+) 274.194	Val-Arg	11.9	0.48	4.20E-04
(-) 161.037	$C_6H_9O_5$	2.5	8	5.80E-04
(+) 712.509	LysoPE(34:4)	2.2	0.42	5.90E-04
(+) 231.087	$C_6H_{11}N_6O_4$	4.1	2.49	6.60E-04
(+) 309.238	$C_{17}H_{34}O_3Na$	2	10	7.90E-04
(-) 154.049	$C_5H_6N_4O_2$	2.8	15	9.20E-04
(-) 232.027	Dopamine Sulfate	3.4	700	1.30E-03
(-) 246.991	$C_4H_7O_{12}$	3.9	5	1.30E-03
(+) 659.505	LysoPA(P-34:1)	2.5	0.33	2.50E-03
(-) 175.075	$C_{24}H_{32}N_4O_5$	3.7	3	1.00E-02
(-) 455.232	$C_{11}H_{11}O_2$	10.3	0.57	1.00E-02
(+) 580.420	LysoPC(22:0)	2	0.38	1.00E-02

CHAPTER 5: DISCUSSION, FUTURE DIRECTION AND CONCLUSION

Biomarkers and Mediators of Peanut-Allergic Sensitization

PA has become an increasing health concern. Indeed, the prevalence of PA has virtually doubled over the last decade, and PIA, the most lethal manifestation of PA, remains responsible for half to two-thirds of fatalities due to food-induced anaphylaxis in the United States.[4] Against this background, the ontogeny of PN sensitization as well as the molecular basis of PIA remain largely unknown. Regarding PA, it has been generally assumed that the most prominent route of sensitization occurs through the gut epithelium.[54] However, recent evidence from a number of studies, including studies in humans,[55,56] suggest that PN sensitization can also occur through the skin, particularly when its integrity has been compromised.[51,57] Breaching the integrity of the epithelial barrier at either the gut mucosa or the skin allows for Ags to be taken up by resident DCs and be carried to the draining lymph nodes where DCs will interact with naïve T cells. It is then clear that a key checkpoint in the generation of immunity is the acquisition of DCs in the mucosa or skin of an appropriate instructive program that includes the expression of activating co-stimulatory and migratory molecules. From this perspective, elucidating the incipient signals generated in the mucosa and/or skin that are capable of triggering DC activation is critical to clarify the ontogeny of PA.

Janeway described, in his Pattern Recognition Theory, signals (*i.e.* lipopolysaccharide), referred to as Pathogen-Associated Molecular Patterns (PAMP), expressed by pathogenic microbes capable of activating the immune system, notably DCs, through pattern-recognition receptors (*i.e.* toll-like receptors (TLR)).[58] However, this theory falls short to explain how non-microbial antigens such as food allergens or self antigens that do not mimic microbial epitopes might trigger immune responses.[59] In this context, Matzinger proposed the Danger Theory to explain how non-microbial entities may elicit detrimental immune responses.[60] In essence, the theory proposes that certain typically intracellular molecules, referred to as DAMPs released from damaged or dying cells become immuno-stimulatory once they are exposed to the extracellular environment. We would argue that the Danger Theory is well suited to explain the development of harmful immune responses to innocuous food allergens, and further suggest that the development of PA may, in fact, be largely dependent on the concurrent existence of danger signals released as a result of tissue damage at the time of allergen exposure. That the development of PA in the experimental models we used in this thesis definitely requires the elicitation of tissue damaged, either by the administration of CT in the GI tract or tape-stripping in the skin, supports this contention.

Using metabolomics as an unbiased discovery method, we identified a metabolic profile unique to mice that were sensitized to PN through the GI route. The principal signature of this profile was a change in the purine metabolism pathway, primarily manifested by an increase in the overall level of its end product, UA. Using a colorimetric analysis, we measured the UA concentration in serum at several time points within 24 hours, rather than at 2 weeks, after a single sensitization gavage. Our results showed a gradual increase in the UA levels starting at 12 hours after the gavage, which continued to rise over 24 hours; these changes however did not reach statistical significance. We suspect that it is because the level of UA in serum is tightly regulated by the kidney under normal physiological conditions. Therefore, increasing the number of gavages or allowing additional time to pass before taking the measurements may be required for the difference to reach statistical significance

For over 50 years, several studies have examined the inflammatory effects of crystals in human diseases, including MSU in gout, calcium pyrophosphate dihydrate (CPPD) in pseudogout, silicon dioxide in silicosis of the lung and cholesterol crystals in atherosclerosis.[61,62] Notably, in the last decade, MSU crystals were recognized as potent alarmins capable of stimulating the immune system.[48,63-65] With respect to allergy, Kool *et al.*[66] recently identified UA as a critical mediator of allergic asthma in both an OVA-aluminum hydroxide (alum) model and a house dust mite model (HDM). These investigators showed that the adjuvant effect of alum and HDM stem from their ability to induce UA accumulation in the lung. In regards to food allergy, we found that UA is an essential mediator to facilitate sensitization to PN in two distinct models of sensitization. Indeed, depletion of UA prevented the development of PA and anaphylaxis, and administration of UA instead of CT or tape-stripping reconstituted the phenotype, including the production of PN-IgE and PN-IgG1. The generation of PN-Abs induced by UA administration is in agreement with Behren *et al.*'s findings where UA increased IgG1-based humoral immunity.[64] It is known that tape-stripping injures the skin, and we have evidence that the administration of CT induces intestinal epithelial and subepithelial damage including cell death and abundant debris (Chu *et al.,* manuscript submitted).[48] Therefore, the release of UA from damaged tissue may be viewed as a common immune trigger in these models.

The intragastric administration of CT with Ag promotes the migration of $CD11c^+$ DC to the MLN and, ultimately, the development of Th2 immunity that is characterized by the production of IL-4, IL-5 and IL-13 cytokines along with the generation of Ag-specific Igs, namely IgE and IgG1. We have previously shown that the co-stimulatory molecule OX40L is an important contributing factor to this process.[17] Others have shown that UA

crystals can upregulate the activation marker CD86 on DCs *in vitro*.[48,65-67] In our current study, we demonstrated that the expression of both CD86 and OX40L are dependent on the presence of UA. Also, we showed that after a single gavage with PN and CT, there was a transient increase in the MHC II^+ $CD11c^+$ DC population in the lamina propria at 12 hours, which returned to baseline levels at 24 hours. This change was accompanied by a gradual increase in the population of MHC II^+ $CD11c^+$ DCs in the MLN that was not affected by the allopurinol and uricase treatment. These data suggest that DCs were recruited to the lamina propria at 12 hours after the gavage before they migrated to the MLN at 24 hours in a UA independent manner. The fact that the population of 103^+ DCs, which are known for their migratory role,[17,68] in the lamina propria at 12 hours were not affected by allopurinol and uricase treatments supports this hypothesis. Selective upregulation of DC markers has been shown in the past when MSU induced expression of CD86, but not CD80, in DC cultures.[48] Speculatively, the remarkable effect of UA in preventing the generation of Th2 immunity and anaphylaxis may be the result of migration of "unarmed DCs" to the regional lymph nodes, thus suggesting that signals, other than UA, are responsible for mediating the migration of DCs from the lamina propria to the regional lymph nodes.

A well-established pathway of UA-mediated inflammation, particularly in gout, involves the recruitment of caspase-1, activation of the NLRP3 inflammasome and production of IL-1β through TLR-2 and TLR-4 signaling on macrophages.[69-71] The NLRP3 inflammasome can be activated by various self-derived (*i.e.* ATP, cholesterol crystals, MSU/CPPD crystals, glucose, Amyloid β and hyaluronan) or environmentally-derived (*i.e.* alum, asbestos, silica, alloy particles, UV radiation, skin irritants) stimuli.[71] Surprisingly, we found that the development of PA was not dependent on either caspase-1 or IL-1β. Likewise, TLR-2 and TLR-4 were dispensable for PN sensitization (unpublished data). These findings suggest the existence of an alternative pathway. One possibility is that other inflammasomes such as NLRP1, 2, 6, 12, NLRC4, or AIM2 may play a role. However, since the activation of these inflammasomes ultimately leads to the production of IL-1β, their involvement in an IL-1β independent fashion becomes less likely. Another possibility is direct physical contact in a receptor independent manner between UA crystals and the cholesterol-rich region on DC's cellular membrane. Recent *in vitro* studies have provided support for this hypothesis by showing that MSU crystals can engage DC in this manner through tyrosine kinase (Syk) and phosphinositide 3-kinase (PI3K) signaling.[67] Unfortunately, it is difficult, if not impossible, to investigate this pathway in our model of PIA *in vivo* because Syk and PI3K are involved in clonal expansion, differentiation, and effector functions of B and T cells,[72] as well as IgE production[73] and mast cell degranulation.[74] Consequently, inhibition of Syk and PI3K for

the full duration of our model would not allow us to ascertain the effect of Syk and PI3K on, specifically, MSU-induced DC activation. To overcome this problem, we plan to inhibit these molecules and assess their effect on DC activation and migration using the single gavage model described earlier. Also, we will also examine the expression of different activation markers of DC *in vitro* after direct stimulation with MSU while blocking Syk and PI3K. These studies can provide evidence to support the involvement of the alternative pathway in MSU-mediated peanut sensitization.

Biomarkers of Peanut-Induced Anaphylaxis in Mice and Peanut Allergy in Human

Food-induced anaphylaxis is a precipitous event, arguably resulting from a massive release of potent biologically active mediators that target various organs involved in this process.[75] Although anaphylaxis has been recognized for over 160 years,[76] knowledge about the mediators involved is rather limited. To better understand PIA at the molecular level, the second half of our study attempted to identify metabolites that were significantly altered in animals undergoing PIA.

Soon after challenge, sensitized mice experienced a drastic drop in core body temperature and exhibited a marked increase in hemoconcentration, indicative of overall illness and the extent of vascular leakage in mice, respectively. Metabolic profiles from the OPLS-DA plot showed that disease severity corresponded to an increasing deviation from the naïve, pre-challenged profile. This drift indicates either an overall increase in the number of metabolites that were differentially expressed and/or an increase in the magnitude of the change associated with each individual molecule.

The rapid onset of PIA suggests that mediators are released promptly following exposure to the allergen. Indeed, our analysis detected several molecules elevated at 1 minute after challenge. These include histamine, methylhistamine, and 2 PC lipids. The considerable increase in histamine, which was virtually undetectable in healthy animals, served to validate the use of untargeted metabolomic analysis as a discovery tool since histamine is a well-known biomarker and mediator of allergic reactions.[77] In the pathway of histidine metabolism, methylhistamine is a metabolite of histamine. Intriguingly, methylhistamine is also a potent agonist for H3 and H4 receptors, which can induce vasodilation, reduce blood pressure and heart rate in animals,[78-80] therefore possibly contributing to the pathophysiology of PIA. The identity of the 2 PC lipids could not be determined with our current method of analysis; however, their presence was interesting. PAF, also known as 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine, is an ether analogue of PC and its involvement in the manifestation of anaphylaxis has been well documented.[50,81-83] Whether these unknown PCs are precursors of PAF or they contribute directly to the

outcome of PIA, in a similar fashion as PAF, requires further experimentation. Overall, the identification of these molecules and their relation to PAF will be the prime focus of our future studies.

Previously, our targeted immunological studies identified PAF and histamine to be 2 important mediators of PIA. In that study, pharmacologic interventions were used to inhibit PAF receptor as well as histamine H1 and H2 receptor signaling.[50] It was therefore difficult to assess whether the remaining allergic responses were a consequence of an incomplete blockade of the receptors, involvement of H3 and H4 receptors signaling, or that additional mediators remain to be identified. Presently, using an unbiased approach, we detected histamine, its metabolite, methylhistamine, and 2 PC lipids that are possibly associated with PAF. Taking into account that in an unbiased, comprehensive analysis, these are the only molecules detected raises the hypothesis that PAF, histamine and methylhistamine may in fact be the key mediators of PIA. It remains to be confirmed whether in a setting where there is a complete absence of these molecules or their receptors, possibly in a knockout animal, animals are fully protected from PIA.

Taurine, uridine, creatinine, creatine, GABA, and NE-S were elevated at 40 minutes after the challenge when anaphylaxis was most severe. Taurine is known for its vasoactive properties and its hypotensive effect.[84] It is an organic acid that has many essential biological roles, such as cell volume regulation, bile-salt conjugation, regulation of intracellular free-calcium concentration, and osmoregulation. Deficiency in this molecule is associated with cardiomyopathy, renal dysfunction, developmental abnormalities, and retinal neuron damage.[85] Taurine is widely distributed in animal tissues, including mast cells.[86] Intracisternal and intracerebral ventricular injections of taurine induced an acute and transient drop in body temperature and blood pressure in rats.[87] Additionally, our unpublished data showed that intravenous injection of taurine in naïve mice also induces drops in rectal temperature and blood pressure. Taken together, these studies suggest an active role for taurine in PIA and prompts for further investigations. Next on the list, uridine nucleoside is an important building block of ribonucleic acid. Two recent studies showed a direct correlation between the serum levels of uridine and the degree of purine nucleotides breakdown due to exercise-induced hypoxia.[88,89] The reason for its elevation in anaphylaxis remains to be confirmed; however, reduced blood flow into tissues leads to hypoxia, impaired oxidative resynthesis of ATP and consequently increase degradation of pyrimidine nucleotides.[89] Creatinine, the end product of creatine metabolism and creatine is a source of energy reservoir for conversion of ADP to ATP.[90] The presence of creatine and creatinine in serum of mice undergoing anaphylaxis may be related to ischemia. One report found that elevated levels of creatine in serum correlated with myocardial ischemia

in patients.[91] This observation may explain its presence in PIA because increased vascular leakage reduces mean arterial pressure. Compromised delivery of blood supply to tissues then leads to the release of creatine. Next, GABA is a inhibitory neurotransmitter that has been shown to dampen the effect of delayed-type hypersensitivity reactions,[92] ameliorate experimental autoimmune encephalomyelitis (EAE)[93,94] and inhibit the development of inflammatory responses in type-1 diabetic model in mice.[95] Delayed release of GABA may serve to counteract and modulate the inflammatory response of anaphylaxis. Similarly, elevated levels of NE-S, produced from sulfonconjugation of plasma norepinephrine (NE), can also act as a compensatory molecule. In humans, uptake and efflux of NE and NE-S by platelets has been a major site of NE sulfonconjugation.[96] NE, like epinephrine, is a stress hormone that increases heart rate, vascular tone and blood pressure. The elevated levels of NE-S may be a consequence of homeostatic response by the body to compensate for reduced blood pressure induced by anaphylaxis. Future goals might involve performing functional studies on the biological significance of these molecules.

For the last part of our study, we analyzed the serum of PN allergic children before and after an oral challenge. This preliminary study revealed an impressive number of metabolites, surely representing the complexity of biochemical pathways involved in the allergic reaction. For most of these metabolites, it was difficulty to accurately identify their structural formula due to the high mass to charge (m/z) values, giving them a myriad of possible structures. Through matching of spectral peaks, online databases identified neuroprostane, phenylglycol-3-O-sulfate and dopamine sulfate as significantly increased. Neuroprostanes consists of 8 structural isomers derived from non-enzymatic lipid peroxidation of docosahexaenoic acid. F_4-neuroprostane, in particular, is a sensitive marker of brain-related oxidative stress in human,[97] but whether their presence is an indication of oxidative stress in allergic children is unclear. Minimal information has been published on the biological pathways and functions of phenylglycol-3-O-sulfate. Metlin Metabolite and Tandem MS Database provided by Scripps Center for Metabolomics identified it as a metabolite of phenylnephrine, which is an α-adrenoceptor agonist for vasoconstriction and decongestion. Over 95% of dopamine in the circulation is in the form of dopamine sulfate, derived from sulfoconjugation of dopamine in the circulation. Dopamine sulfate levels increase in blood from ingestion of food and metabolism of endogenously formed dopamine.[98] The increase in circulating dopamine sulfate in after challenge may reflect the consumption of PN flour and chocolate pudding or increased dopamine. For the unidentified metabolites, additional techniques such as lipidomics and tandem-MS are required to differentiate the lipid compounds and to provide structural formula of the molecules.

Conclusion

The first part of our study identified a metabolic signature for PA in mice during sensitization. From this, we then generated a hypothesis to investigate the role of UA in the initiation of allergic sensitization. The results showed that UA and MSU crystals are essential and sufficient, respectively, to the process of sensitization. Specifically, they are important for DC activation, characterized by increased expression of CD86 and OX40L, possibly via an unconventional pathway that is independent of the NLRP3 inflammasome. The second part of our study detected 21 molecules that were differentially expressed during the immediate and late phases of PIA. Based on the kinetics of the allergic reaction after exposure to the antigen, molecules detected at the early time point, such as histamine, methylhistamine and PC lipids, possibly PAF precursors, were more likely to mediate symptoms of anaphylaxis. The majority of molecules detected at later time points are likely markers of the complex pathophysiology of anaphylaxis or have compensatory roles for restoring homeostasis. Overall, our data highlight the biochemical processes in animals undergoing PA and anaphylaxis and provide potential target molecules for future functional studies. The final part of our study examined the sera of patients experiencing allergic reactions to PN. This is a preliminary study and the vast majority of molecules remain unidentified at this point. However, it serves as a foundation for future human metabolomics, and lipidomics, studies, involving more in-depth and specific chemical analysis. Ultimately, the goal is to use these data to generate and test hypothesis to improve our current mechanistic understanding of PA and anaphylaxis.

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