

Comprehensive metabolomics identifies the alarmin uric acid as a critical signal for the induction of peanut allergy

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This research is dedicated to Professor Brian McCarry who sadly passed away during the course of this project.

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Abstract

Background: Food allergy, in particular peanut allergy, is a growing concern in Western countries. The prevalence of allergy to peanut, which currently stands at 1.4%, nearly tripled between 1997 and 2008. Allergic sensitization is a particularly difficult process to study as it is clinically silent.

We sought to identify key pathways and mediators critically involved in the induction of allergic sensitization to peanut.

Methods: Comprehensive metabolomics analysis with liquid chromatography–mass spectrometry was used to detect metabolite changes in mice (C57BL/6) undergoing sensitization. Loss-of-function and gain-of-function studies were performed in mice subjected to two models of peanut sensitization and anaphylaxis that involved either oral or epicutaneous sensitization. Flow cytometric analyses on dendritic cells (DCs) *in vitro* and *in vivo* were used to investigate the mechanisms of immune activation.

Results: Elevated levels of uric acid (UA) were detected in mice undergoing sensitization as well as in peanut-allergic children who were not challenged with peanut. In mice, the depletion of UA during sensitization prevented the development of peanut-specific immunoglobulins IgE and IgG1 as well as anaphylaxis while exogenous delivery of UA crystals (monosodium urate, MSU) restored the allergic phenotype. Monosodium urate enhanced CD86 and OX40L expression on DCs, independent of Toll-like receptors 2 and 4, the NLRP3 inflammasome, and IL-1 β , via a PI3K signaling pathway.

Conclusion: Overproduction of the UA alarmin in the local microenvironment plays a critical role in the induction of peanut-allergic sensitization, likely due to its ability to activate DCs. These findings suggest that cellular damage or tissue injury may be an essential requisite for the development of allergic sensitization to foods.

Abbreviations

BMDC, bone marrow-derived dendritic cells; CPE, crude peanut extract; CT, cholera toxin; DAMP, danger-associated molecular pattern; DC, dendritic cell; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; MLN, mesenteric lymph node; MSU, monosodium urate; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; OPLS-DA, orthogonal partial least squares-discriminant analysis; PI3K, phosphoinositide 3-kinase; PN, peanut; PN-IgE, peanut-specific IgE; PN-IgG, peanut-specific IgG1; Syk, tyrosine kinase; TLR, toll-like receptor; UA, uric acid.

Food allergy has become a major healthcare problem in Western countries. In particular, the prevalence of allergy to peanuts and other nuts tripled between 1997 and 2008 (1). Peanut allergy, which has an estimated prevalence of 1–2% in children (2), is of particular concern because it accounts for half to two-thirds of fatal cases of food-induced anaphylaxis (3, 4). In this context, the ontogeny of peanut sensitization remains a major unsolved issue. Uncovering the mechanisms underlying peanut sensitization is a major challenge because this process is clinically silent. In addition,

while experimental models have made significant contributions to elucidate important aspects of peanut sensitization, the conventional hypothesis-driven approach is at risk of bias to evaluate the already-known markers and/or mediators. Here, we used an untargeted, comprehensive metabolomics approach to acquire a global view of all small molecular weight species (<1.5 kDa) present during peanut sensitization. As metabolites represent the total contribution of genomic, transcriptomic, and proteomic activities as well as exogenous factors such as nutritional sources, they represent the chemical process of a living organism in real time (5).

Metabolomics analysis revealed an altered expression of the purine metabolic pathway with an overall increase in the serum level of uric acid (UA) in sensitized mice. Elevated serum levels of UA were also detected in peanut-allergic children compared to healthy controls. Gain-of-function and loss-of-function studies in mice demonstrated that UA is necessary and sufficient for the induction of peanut allergy. In addition, the data showed that UA activates dendritic cells (DCs) *in vitro* as well as *in vivo*, as indicated by the upregulation of MHC II, CD86, CD80, and OX40L. The development of peanut allergy and anaphylaxis remained intact in TLR2, TLR4, caspase-1, and IL-1 β knockout mice, thus suggesting that it was independent of the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome pathway while, *in vitro*, DC activation was prevented by the inhibition of phosphoinositide 3-kinase (PI3K). Collectively, this study shows that alarmins such as UA play a critical role in the development of peanut sensitization and, thus, that cellular stress or tissue damage may be key requisites for the induction of peanut allergy.

Methods

Animals

Female C57BL/6 mice (6–8 weeks old) were purchased from Charles River Laboratory (Ottawa, ON, USA). TLR2-, TLR4-, Caspase-1-, and IL-1 β -deficient mice and their respective age- and sex-matched control animals (C57BL/6 and NOD) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All experiments described were approved by the Animal Research Ethics Board of McMaster University.

Peanut allergy and peanut-induced anaphylaxis models

Gastrointestinal sensitization

Mice were sensitized as previously described (6) with minor modifications. Briefly, 3.75 mg (1 mg protein) of Kraft All Natural™ peanut butter was gavaged with 5 μ g or 2.5 μ g of cholera toxin (CT) (List Biological Laboratories, Campbell, CA, USA) once a week for 4 weeks or for 10 consecutive days, respectively. In gain-of-function studies, CT was replaced with 20 mg of monosodium urate (MSU) (Sigma-Aldrich, Oakville, ON, Canada) and gavaged with peanut butter for three consecutive days per week for 4 weeks.

Epicutaneous sensitization

Twenty microliters of 10 mg/ml crude peanut extract (CPE) (Greer Laboratories, Lenoir, NC, USA) was directly applied onto shaved and tape-stripped skin for 10 consecutive days. For gain-of-function studies, 5 mg of MSU was injected subcutaneously in 500 μ l of PBS 5 min prior to the application of CPE on shaved, but not tape-stripped skin.

Challenge

In all instances, 1 mg of CPE in 500 μ l PBS was injected intraperitoneally (i.p.) 2 weeks after the last sensitization treatment. Rectal body temperature, hematocrit readings, and clinical scores were recorded as described in our previous studies as readouts of anaphylaxis.

Serum collection

Mice were anaesthetized with isoflurane, and peripheral blood was collected via retroorbital bleeding using lime glass Pasteur pipettes (VWR International, Mississauga, ON, Canada) and red-top collection tubes with clot activator (Terumo Capiject; Fisher Scientific, Ottawa, ON, Canada). Samples were incubated at room temperature for 30 min, centrifuged at 16,245 rcf for 10 min at 4°C before sera was collected and stored at –20°C for further analysis.

Uric acid measurement in allergic patients

Analysis in humans was approved by the McMaster Institutional Review Board (IRB). Serum of peanut-allergic and healthy children was collected at McMaster Children's Hospital under the supervision of a clinician and a research nurse. Peanut allergy in patients was confirmed based on a history of significant clinical symptoms within 60 min after the ingestion of peanut, the presence of PN-IgE (CAP-FEIA result of >15 kU/l), and a positive skin prick test to peanut (wheal >3 mm than saline control). Uric acid in human serum was determined using Amplex® Red Uric Acid/Uricase Assay Kit (Molecular Probes, Grand Island, NY, USA) as per their protocol.

Lymph node cell isolation

Mesenteric lymph nodes (MLN) were collected in 1 ml of Hank's buffer salt solution (HBSS), triturated between frosted slides (Corning, Corning, NY, USA) and a 40 μ m cell strainer prior to staining.

Bone marrow-derived dendritic cell stimulation

Bone marrow cells were isolated from the spine or femur in PBS, and washed and resuspended in complete RPMI (10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, and 0.1% β -mercaptoethanol). 2×10^5 cells/ml was cultured in 40 ng/ml GM-CSF complete RPMI for 7–10 days before they were stimulated for 18 h with MSU and analyzed by Information Flow cytometry.

Uric acid depletion

Uric acid was depleted using uricase alone or uricase with allopurinol. Allopurinol in drinking water (200 µg/ml; Sigma-Aldrich) was given starting at 7 days prior to the first sensitization treatment and continued until 7 days post the last sensitization treatment. Average daily consumption of allopurinol was 45 mg/mouse. Uricase (50 units; Sigma-Aldrich) in 500 µl of PBS was administered i.p. or subcutaneously 5 min prior to each sensitization treatment.

Serum peanut-specific immunoglobulins

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

Peanut-specific IgG1

Maxi-Sorp 96-well plates (Nunc; VWR International) were coated with CPE (2 µg/ml) in 50 mM carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich) at 4°C overnight. Coated plates were blocked with BSA (1%) in PBS for 2 h at room temperature. Plates were washed and incubated with serum samples overnight at 4°C. Biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL, USA) was added and incubated with the samples the next day for 2 h. After washing, alkaline-phosphatase-conjugated streptavidin (Sigma-Aldrich) was added to incubate for 1 h at room temperature. *p*-nitrophenyl phosphate tablets were used to develop the assay, and H₂SO₄ (2 M) was added to stop the reaction for absorbance readings taken at 450 nm.

Peanut-specific IgE

Maxi-Sorp 96-well plates were coated with rat anti-mouse IgE Abs (2 µg/ml; BD Biosciences, Mississauga, ON, Canada) in PBS overnight at 4°C. Coated plates were washed and blocked with Tween buffer (10% bovine serum; 1% bovine serum albumin; 0.5% Tween in PBS) for 1 h at 37°C and washed again. Serum samples were then incubated for 2 h at room temperature before CPE-digoxigenin (DIG)-conjugate solution was added for coupling of IgE with CPE. Peroxidase-conjugated anti-DIG was added at 37°C for 1 h before tetramethylbenzidine (0.1 mg/ml) solution was added to develop the color reaction. H₂SO₄ (2 M) was added last to stop the reaction for absorbance reading at 450 nm.

Flow cytometric analysis

Two to four million cells were incubated with anti-CD16/CD32 (2.4G2; eBioscience, San Diego, CA, USA) 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 (San Diego, CA, USA). 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), and 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 (BD, Franklin Lakes, NJ, USA) and analyzed with FLOWJo software (Tree Star, Ashland, OR, USA). Fluorescence minus one controls were used for gating.

Data analysis

Data were analyzed using GRAPHPAD PRISM version 5.0 (Graphpad Software, Inc, La Jolla, CA, USA) 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 was <0.05 (*), 0.01 (**), and 0.001 (***).

Results

Identification of metabolites associated with peanut-allergic sensitization in mice and humans

Liquid chromatography–mass spectrometry was performed to identify chemical changes taking place during allergic sensitization using a previously described mouse model of peanut allergy that involves 4 weekly administrations of peanut along with CT by gavage. Sensitized mice were challenged 2 weeks later with a peanut extract intraperitoneally (Fig. 1A) (7, 8). Serum samples were collected 12 h after the second and the fourth gavages, as well as 2 weeks later immediately before challenge. Treatment groups (*n* = 7 per group) included peanut and cholera toxin (PN + CT), peanut only (PN-only), CT-only, or PBS. To note, only co-administration of PN + CT leads to sensitization.

Orthogonal partial least squares-discriminant analysis (OPLS-DA), a multivariate statistical analysis technique, was utilized to assess the change in metabolomic signatures between groups. At week 2, a clear, yet incomplete, differentiation was observed between treatment groups (Fig. 1B). These separations became prominent after the fourth gavage (Fig. 1B). By the time mice were fully sensitized, as assessed by significantly elevated levels of peanut-specific IgG1 (PN-IgG1) and peanut-specific IgE (PN-IgE) in serum (data not shown) at the sixth week, a clear distinction was observed between sensitized (i.e., PN + CT treated) and nonsensitized mice (i.e., PN-only, CT-only, and PBS treated). Notably, PN-only and CT-only treated groups became indistinguishable (Fig. 1B), indicating that metabolomic changes due to the administration of PN-only or CT-only had subsided by this time.

Over 2500 metabolite features were detected in fully sensitized animals at the sixth week time point. Table 1 presents the list of molecules that were differentially expressed (*P* < 0.01) and their associated fold changes in allergic mice (i.e., PN + CT treated group) compared to nonallergic mice (i.e., PN-only and CT-only treated groups combined). Seven metabolites were identified with high confidence when compared to authentic chemical standards with an additional five metabo-

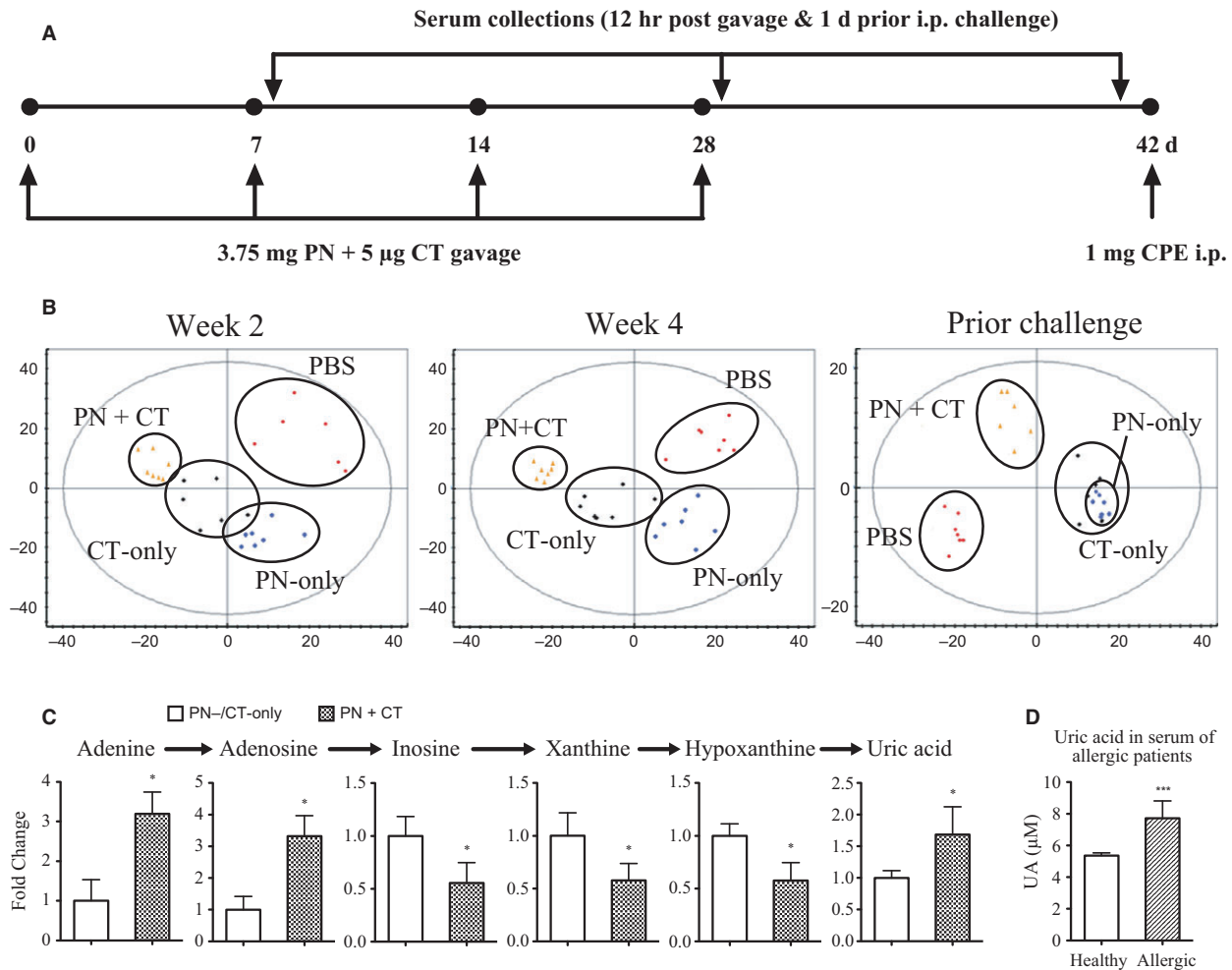


Figure 1 Shifts in metabolomic profiles during peanut sensitization. (A) Model of peanut sensitization and anaphylaxis. (B) OPLS-DA analysis of peanut and cholera toxin (PN + CT), peanut only (PN-only), cholera toxin only (CT-only), and Sham (PBS)-treated mice showing separation in their metabolomic profile after 2 and 4 weeks of sensitization, and 1 day prior challenge. (C) Changes in the purine metabolism pathway in sensitized mice. Fold changes of

purines in serum of sensitized (PN + CT) comparing to nonsensitized (PN-/CT-only) mice at week 6 of the sensitization protocol prior to challenge. (D) Changes in serum levels of UA in peanut-allergic patients (17 allergic patients and 6 healthy controls). Data from mouse studies represent 7 mice per group (B, C) and were repeated twice. (C) and (D) show the mean and SEM. * $P < 0.05$, *** $P < 0.001$ (unpaired t -test).

lites identified by matching with mass spectrum data found in publicly accessible databases (METLIN metabolomics database or Human Metabolome Database). Seven remaining metabolites could not be identified either with in-house standards or from public databases. Six of the 12 identified metabolites, namely adenine, adenosine, inosine, hypoxanthine, xanthine, and UA, are associated with purine metabolism, which is otherwise known as the UA synthesis pathway (Fig. 1C). In agreement with the findings in allergic mice, otherwise healthy peanut-allergic children, who were not challenged with peanut, also exhibited elevated levels of UA in serum compared to nonallergic healthy controls (Fig. 1D). All together, these data suggest that the development of allergy to peanut is associated with the establishment of a distinct metabolic profile, most notably alternations in UA.

Uric acid is necessary and sufficient to induce sensitization to peanut

To assess the impact of UA on peanut sensitization, uricase alone or allopurinol with uricase was used in conjunction to inhibit the production and to deplete UA, respectively, *in vivo*. Mice were sensitized with PN + CT in a shortened 10-day protocol to avoid the development of adaptive immune responses against repeated weekly injections of uricase. Similar to the 4-week protocol, sensitized mice developed PN-IgG1 and PN-IgE 2 weeks after the last gavage and anaphylaxis upon CPE challenge.

Compared to untreated sensitized mice, the serum levels of PN-IgG1 and PN-IgE were drastically reduced in mice that underwent UA depletion using either strategies (Fig. 2A).

Table 1 Identification of altered metabolites during allergic sensitization

<i>m/z</i>	Retention time (min)	ID	<i>P</i> -value (ANOVA)	Fold change	Identification confidence
(+136.073)	3.4	Adenine	<0.001	3.0	Compared to authentic standards
(+268.115)	3.5	Adenosine	<0.001	3.5	
(+244.099)	7.6	Cytidine	<0.001	0.7	
(−)151.026	4	Xanthine	<0.001	0.6	
(+137.056)	4	Hypoxanthine	<0.001	0.5	
(−)267.074	6.6	Inosine	0.01	0.7	
(−)167.021	8	Uric acid	0.003	1.7	Matched with database
(+269.117)	3.5	Arabinosylhypoxanthine	<0.001	2.7	
(+584.454)	21.7	LysoPC (18:0)	<0.001	2.4	
(+540.430)	21.8	Hexacosanoylcarnitine	<0.001	2.0	No match
(+762.568)	20.9	Phosphatidylcholine (34:0)	<0.001	1.5	
(+764.536)	23	Phosphatidylcholine (34:0)	0.002	1.6	
(+628.482)	21.6	Unknown	<0.001	2.4	
(+275.264)	21.7	Unknown	<0.001	1.9	
(+319.289)	21.6	Unknown	<0.001	1.7	
(+380.342)	21.5	Unknown	<0.001	1.6	
(−)309.002	6.7	Unknown	<0.001	0.6	
(+614.478)	21.5	Unknown	0.001	1.8	
(+512.422)	21.3	Unknown	0.002	1.7	

m/z, mass to charge ratio.

Metabolites that were significantly changed ($P < 0.01$) in peanut-allergic mice (PN + CT) compared to non-allergic mice (PN-only/CT-only) 1 day before challenge. The chemical features are ranked in descending order of their *P*-values.

When challenged, these mice were fully protected from anaphylaxis, as demonstrated by no changes in rectal temperature or hematocrit and minimal clinical signs (Fig. 2B). Thus, UA is required to induce allergy to peanut.

To determine the immunogenic properties of UA in the gut, we gavaged mice with peanut and MSU crystals instead of CT. Following a 4-week sensitization regime and a 2-week rest period, significantly increased levels of PN-IgG1 and PN-IgE were detected in peanut and MSU-treated mice but not in mice given peanut only (Fig. 2C). Furthermore, only mice treated with peanut and MSU developed severe anaphylaxis upon challenge (Fig. 2D). Together, these results demonstrated that endogenously produced UA and exogenously delivered MSU are required and capable, respectively, of inducing allergic sensitization to peanut.

Peanut allergy is independent of IL-1 β and caspase-1

Two distinct pathways of UA-mediated immunity have been proposed (9). One pathway involves Toll-like receptors (TLR) 2 and 4 signaling, activation of the NLRP3 inflammasome, and secretion of mature IL-1 β (10, 11). The other is dependent on Syk and PI3K δ kinase signaling through direct crystal membrane lipid interaction in a receptor-independent manner (12). To examine the potential involvement of the first pathway in the development of peanut allergy, TLR2- and TLR4-deficient mice, caspase-1-deficient mice that are not able to form NLRP3, and IL-1 β -deficient mice were sensitized with PN + CT with the 10-day protocol as previously described. Our data showed that all of these knockout mice could become fully sensitized and developed severe

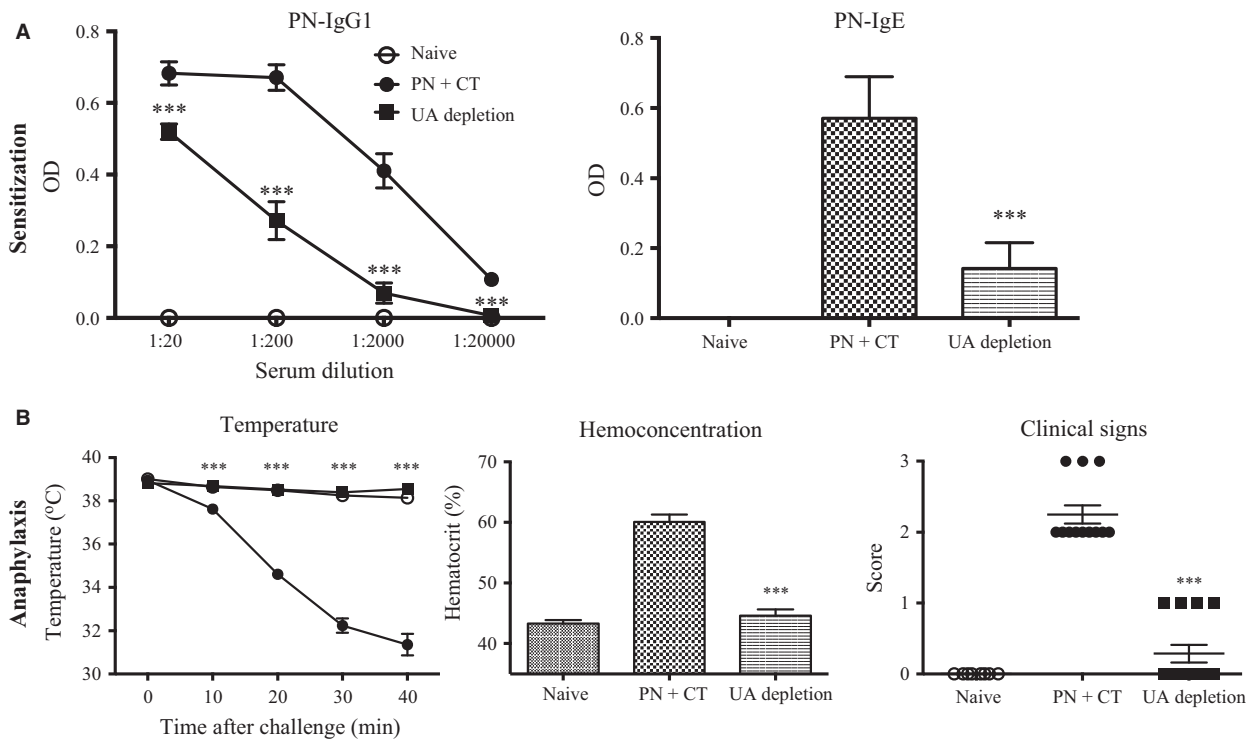
anaphylaxis (Fig. 3A–C), thus indicating that UA-mediated sensitization in the gut is not dependent on the NLRP3 inflammasome.

Uric acid induces dendritic cell activation *in vitro* and *in vivo*

Of note, PI3K signaling has been reported to mediate DCs activation. Because the activation of DCs and their migration to the MLN are essential events for the initiation of peanut sensitization (13, 14), we first investigated the impact of UA on DCs *in vitro* and *in vivo* with or without inhibition of PI3K signaling. Bone marrow-derived dendritic cells (BMDC) stimulated with 0.5, 1.0, or 2.0 mg/ml of MSU exhibited a dose-dependent increased expression of the activation markers, MHC II, CD86, and CD80, in addition to the Th2 costimulatory marker, OX40L (Fig. 4A). Upregulation of these molecules was inhibited by pretreatment with wortmannin, a PI3K inhibitor, at 2.0, 20, and 200 nM prior to MSU stimulation, indicating that BMDC activation by MSU is dependent on PI3K signaling (Fig. 4B).

We next sought to evaluate whether this UA-PI3K pathway mediated DC activation *in vivo*. To test this, mice were exposed to PN + CT with or without concomitant UA depletion, and subsequently evaluated for DC activation status within the MLN. We observed an increase in the proportion of DCs at 24 h post gavage with PN + CT in the MLN, which was accompanied by increased expression of both CD86 and OX40L (Fig. 4C). In agreement with the *in vitro* data, DC activation *in vivo* was dependent on UA, since UA depletion prevented upregulation of CD86 and OX40L. These data showed that UA plays a critical

Depletion of endogenous UA



Administration of exogenous MSU crystals

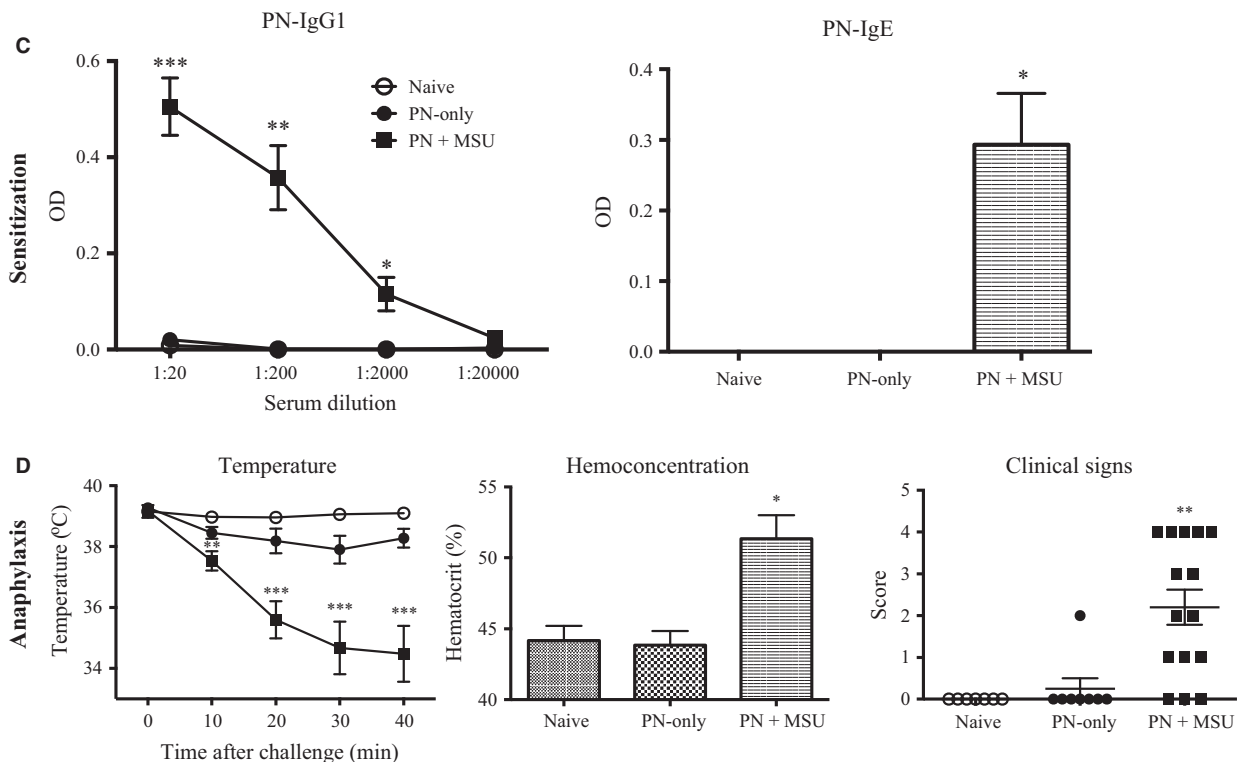


Figure 2 Uric acid is necessary (A, B) and sufficient (C, D) for the induction of PA and PIA. Serum levels of peanut-specific IgG1 (PN-IgG1) and IgE (PN-IgE) (A, C) at 1 day before challenge. Anaphylactic assessment of core body temperature, hematocrit

levels, and clinical sign (B, D). Data are pooled from two independent experiments with total of $n = 6-15$ mice per group (mean and SEM). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ (one-way ANOVA).

role in the activation of DCs during the development of peanut allergy.

Uric acid is necessary and sufficient in skin sensitization to peanut

To assess whether the role of UA in allergic sensitization was dependent on the GI route or the type of adjuvant used, we established an epicutaneous model of peanut sensitization that does not require the use of CT. This model relies on the application of peanut protein for 10 consecutive days directly onto tape-stripped skin. This technique leads to the removal of *stratum corneum* (not shown). With this protocol, mice developed significant levels of PN-IgG1 and developed anaphylaxis upon challenge (Fig. 5A). Application of peanut without tape-stripping does not induce peanut sensitization or anaphylaxis (Fig. 5B). As in the GI model, UA depletion during sensitization fully prevented the development of peanut allergy. Similarly, with subcutaneous injections of MSU crystals, but not PBS, epicutaneous application of peanut in the absence of tape-stripping induced an allergic phenotype (Fig. 5B). In addition, caspase-1 and IL-1 β were not essential for sensitization via the skin (Fig. 5C,D). Thus, the UA pathway mediates

sensitization to peanut regardless of whether the route of induction is through the GI tract or skin.

Discussion

The mechanisms underlying the development of Th2 immunity remain a subject of intense investigation. In the case of food allergy, this challenge is magnified because foods are innocuous antigens and, as such, the default immunological response to food exposure is tolerance. Consequently, it is increasingly recognized that the initiation of food allergy may require perturbations in the local microenvironment that prevent the induction of tolerance. Experimental models support this point insofar as tissue damage, such as that facilitated by the oral administration of CT or tape-stripping of the skin, is required to elicit peanut-allergic sensitization. In this context, the identification of signals produced as a result of tissue damage or cell injury may provide important insights into the conditions required for the development of allergic sensitization. Chief among these are alarmins or danger-associated molecular patterns (DAMPs), intracellular molecules present at abnormally high concentrations in the extracellular environment as a result of cell stress or injury.

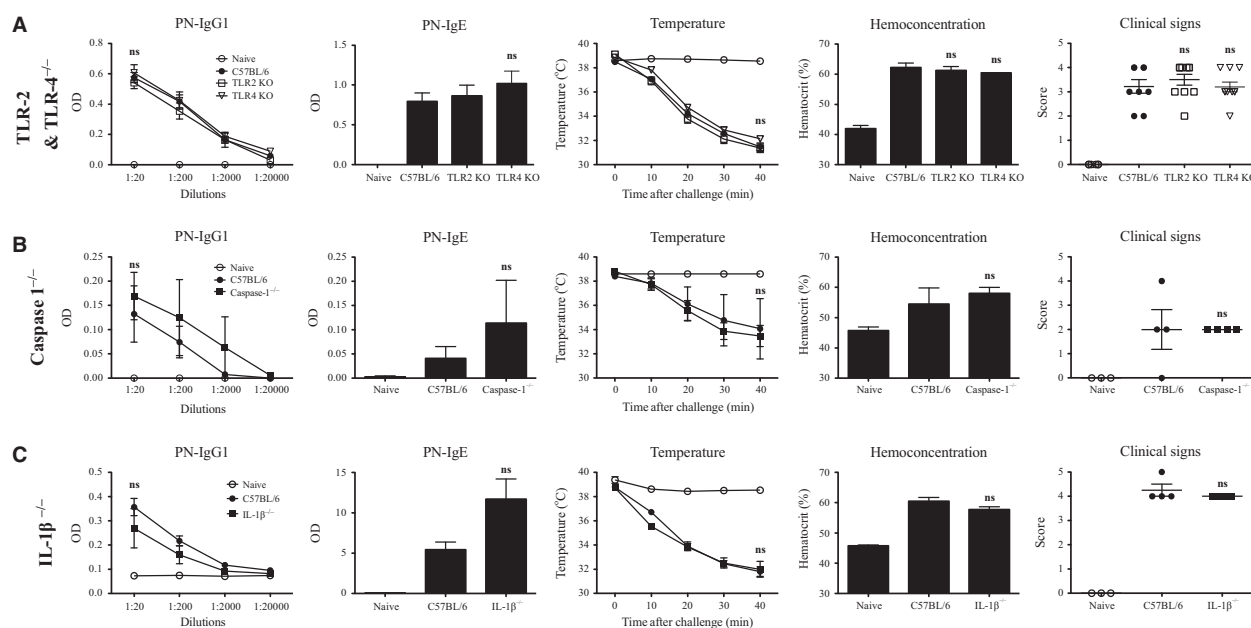


Figure 3 TLR-2, TLR-4, caspase-1, and IL-1 β are dispensable in peanut sensitization through the gut. Peanut allergy is intact in TLR-2-deficient (A), TLR-4-deficient (A), caspase-1-deficient (B), and IL-1 β -deficient mice (C). The readout of sensitization is peanut-specific IgG1 (PN-IgG1) and IgE (PN-IgE). Anaphylaxis is assessed by

the drop in core body temperature rise in hemoconcentration, and expression of clinical signs. 'A' is pooled from two independent experiments with a total $n = 6-10$ mice per group (mean and SEM). (B) and (C) include $n = 3-5$ mice per group (mean and SEM). ns, not significant (one-way ANOVA).

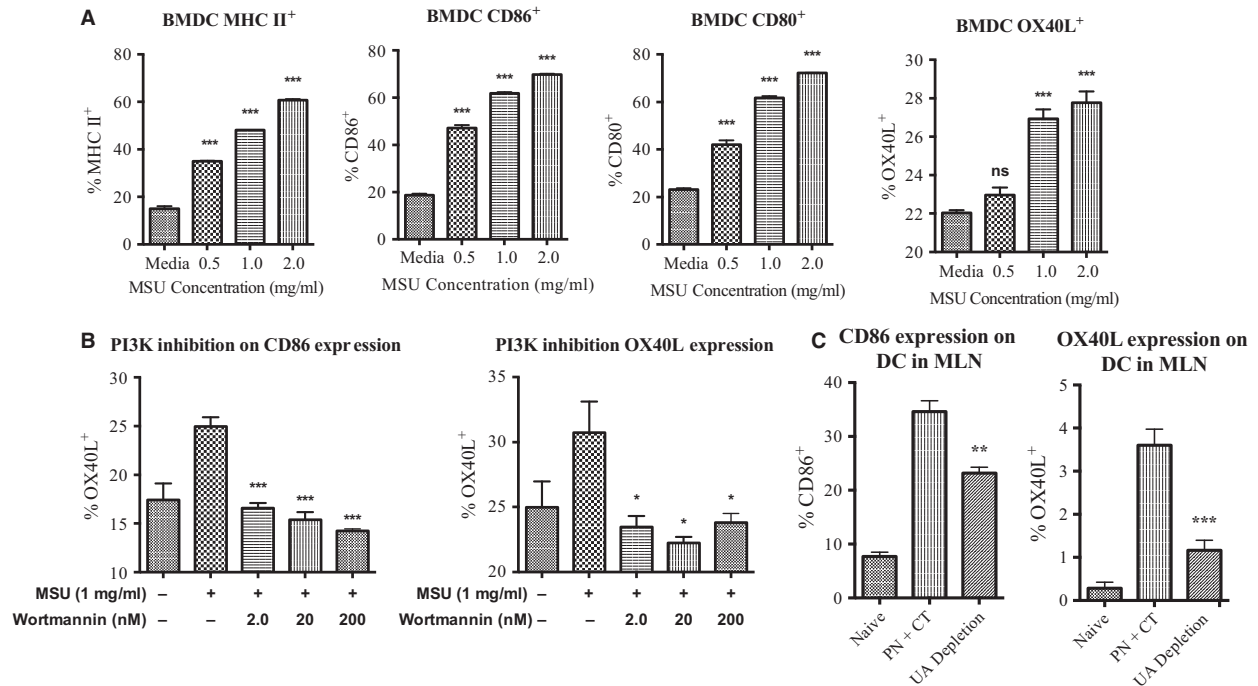


Figure 4 (A) and (B) Monosodium urate crystal (MSU) activates bone marrow-derived dendritic cells *in vitro*. Expression of activation markers MHC II, CD86, CD80, and OX40L following 18 h of MSU stimulation (A). (B) Inhibition of PI3K signaling prevents dendritic cells (DC) activation and expression of CD86 and OX40L *in vitro*. (C) Uric acid depletion *in vivo* prevents activation of DC

(CD11c⁺ MHC II⁺) in the mesenteric lymph nodes (MLN) following a single exposure to peanut and CT. (A) and (B) is a representative of three independent experiments each performed in triplicate (mean and SEM). (C) represents three independent experiments with $n = 3-5$ per group (mean and SEM). ns, not significant; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ (one-way ANOVA).

Importantly, alarmins are capable of activating surveillance tissue resident cells, notably DCs, to launch an adaptive immune response (15).

Here, an unbiased analytical approach, comprehensive metabolomics, detected serum metabolites during the course of peanut sensitization. Specifically, it identified an altered profile of metabolites belonging to the purine pathway, notably an increase in the level of UA. Under normal physiological conditions, UA can be beneficial as a potent antioxidant (16). Altered UA levels in human, however, have been associated with various diseases including gout, kidney disease, hypertension, cardiovascular disease, multiple sclerosis, Parkinson's disease, Alzheimer's disease, and cancer (17). During tissue damage and cell necrosis, release of poorly soluble UA in the extracellular compartment leads to the formation of MSU crystals, which are capable of activating the immune system. Our principal analysis was carried out in a model of oral sensitization that involves the administration of peanut along with CT, an AB5 type of toxin, which induces tissue damage and is typically used in experimental models to break tolerance and elicit sensitization (13). Loss-of-function (i.e., UA depletion) and gain-of-function (i.e., MSU administration instead of CT) studies remarkably prevented or fully generated, respectively, the allergic phenotype. These findings are not restricted to the use of CT since similar prevention-reconstitution of this phenotype was observed in a model of

skin sensitization where tissue damage was caused by tape-stripping. Evidence for the role of UA (18, 19), IL-33 (8, 20), ATP (21), and HMGB1 (22) in allergic airway inflammation, food allergy, and allergic rhinitis argue for the critical importance of tissue damage and the pervasive role of alarmins in the initiation of allergic sensitization and, ultimately, Th2 immunity. In a model of oral sensitization, such as the one used here, we have shown that IL-33 plays a similarly critical role (8) and, more recently, we have demonstrated that eosinophils and, specifically, eosinophil peroxidase (EPO) are essential for the development of peanut allergy (13). How these signals interact with each other is not yet known but, clearly, deciphering the intricacies of this complex network is a fertile venue for future investigations. Lastly, it is particularly interesting that serum levels of UA were increased in peanut-allergic children. Whether this reflects a state of sub-clinical tissue damage, substantially different dietary habits, or altered metabolism is not known and will require further investigation.

The conventional mechanism of UA-mediated immune activation, such as in gout, proposes that UA crystals engage TLR2 and TLR4 receptors on macrophages to induce IL-1 β production through the NLRP3-ASC-caspase-1 inflammasome pathway (9, 11, 23-25). IL-1 β then signals through IL-1 receptors on neutrophils and parenchymal cells to elicit local inflammation (26). Our data indicate that the

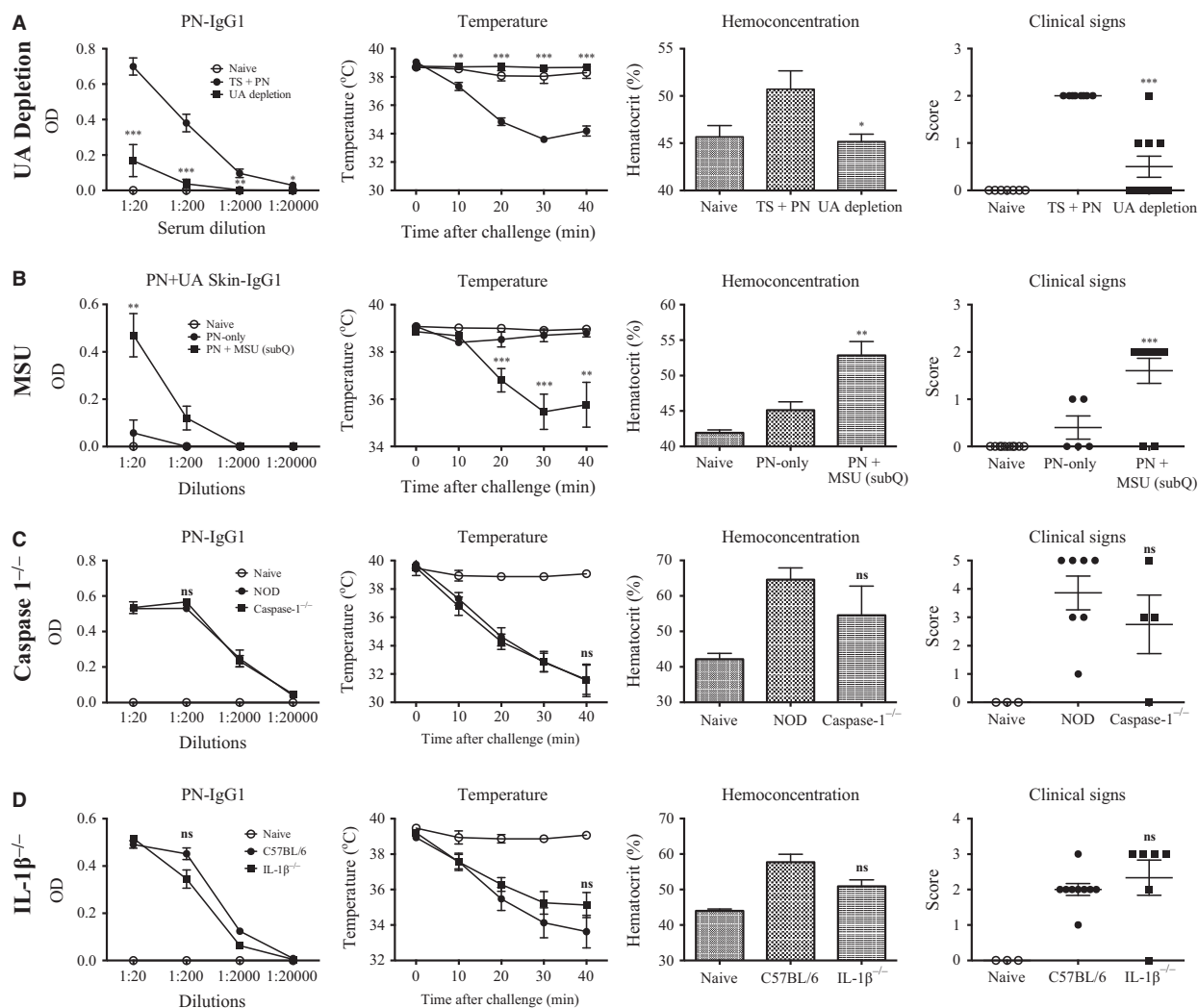


Figure 5 Uric acid (UA) is necessary and sufficient in epicutaneous sensitization to peanut in a caspase-1- and IL-1 β -independent pathway. (A) UA depletion during epicutaneous sensitization prevented the development of peanut allergy. (B) Monosodium urate (MSU) induces peanut sensitization through the skin in the absence of tape-stripping. (C) and (D) epicutaneous sensitization to peanut

does not require caspase-1 or IL-1 β . Peanut allergy is assessed by peanut-specific IgG1 and anaphylaxis as previously described. SubQ, subcutaneous injection. Each data represent 2–3 independent experiments with $n = 3–8$ mice per group (mean and SEM). ns, not significant; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ (one-way ANOVA).

development of peanut allergy is not dependent on TLR2 or TLR4 signaling, nor caspase-1 or IL-1 β , arguing for the involvement of an alternative pathway of UA-mediated inflammation in peanut allergy. In this regard, Ng et al. (12) provided support for a receptor-independent pathway, at least *in vitro*, whereby UA crystals interact directly with cholesterol-rich regions on DC cell membrane via tyrosine kinase (Syk) and PI3K signaling resulting in DC activation. The precise role of Syk and PI3K in peanut allergy and anaphylaxis *in vivo* is difficult to determine as these molecules have pleiotropic effects including clonal expansion, differentiation, and effector functions of B and T cells (27), as well as IgE production (28) and mast cell degranulation (29). However,

we showed, *in vitro*, that PI3K signaling was critical for the activation of BMDC by UA crystals.

Activation of mucosal DCs, and subsequent migration to draining lymph nodes, is an essential event in the initiation of adaptive immunity to peanut (13). Thus, elucidating the incipient signals instructing DCs is critical to clarify the development of peanut allergy. Our *in vitro* studies showed that MSU stimulation increased the expression of the costimulatory molecules CD86, CD80, and OX40L on BMDC. The increased levels of CD86 and CD80 indicate an overall activated status of DCs (30) while OX40L has been shown to be critical for the development of Th2 immunity (31). *In vivo*, UA depletion prevented DC activation in the MLN (i.e.,

reduced CD86 and OX40L expression) following intragastric exposure to PN + CT.

In conclusion, we have used a comprehensive metabolomic analysis as an unbiased, hypothesis-generating tool to identify metabolites relevant to the development of peanut allergy. We report for the first time that UA, which was elevated in both allergic mice and peanut-allergic children, is a critical mediator of allergic sensitization to peanut likely through its effects on mucosal DCs. These findings support the notion that the local production of signals generated as a result of tissue damage or cell injury is an essential requisite for the induction of allergic sensitization.

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Author contributions

Joshua Kong, Kenneth Chalcraft, and Manel Jordana designed the research study, analyzed and interpreted the

data, and wrote the manuscript. Talveer S. Mandur, Rodrigo Jimenez-Saiz, Tina D. Walker, Susanna Goncharova, Melissa E. Gordon, and Leen Naji provided technical help with experiments. Manel Jordana, Brian McCarty, Susan Wasserman, Derek K. Chu, Mark Larché, and Kristin Flader discussed the data and revised the article critically.

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Conflicts of interest

The authors have no conflict of interest.

Supporting Information

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

Methods S1. Supplementary methods.

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1 **Supplementary**

2 Methods

3 ***LC-MS Configuration***

4 An Agilent 1200 RR series LC was programmed with a flow rate of 200 μ L/min, column
5 temperature at 50°C, autosampler temperature at 4°C and an injection volume of 2 μ L. LC
6 columns used were a SeQuant ZIC HILIC (50x2.1mm, 3 μ m) and a HALO RP amide (50x2.1mm,
7 2.7 μ m) coupled in series. Elution conditions were carried out beginning at 95% acetonitrile,
8 holding for 30 sec, ramped down to 30% over 9.5 min, held for 5 min, then ramping up to 95%
9 over 2 min and finally held for 13 min. The accompanying mobile phase solution was 10mM
10 ammonium acetate brought to a pH of 3 by addition of formic acid. MS analysis was performed
11 on a Bruker micrOTOF II with a mass range of 75-1000 m/z and an acquisition rate of 2Hz;
12 Drying Gas was N₂ at 6L/min at 250°C; nebulizing pressure of 3.0 bar and capillary voltage of
13 4.5kV in positive mode and -3.8kV in negative mode. Injections for positive mode and negative
14 mode were done individually. Peaks from the chromatogram were deconvoluted, aligned, and
15 annotated by the open source programs XCMS and CAMERA. Integrated peaks aligned by both
16 retention time and *m/z* of analytes were used as metabolic descriptors for statistical analysis
17 along with peak area.

18 For all OPLS-DA analyses unit variance scaling with a 1/square root block weight was applied to
19 the normalized peak areas for each metabolite feature defined by a unique retention time/mass
20 value after the initial PCA model generation. Student's t-test calculations assumed a 2-tail
21 distribution with heteroscedastic scattering. ANOVA was done with 2-factor (2-way) with
22 replication.

23 ***XCMS and CAMERA Operational Parameters***

```
24  sleep=0.0001
25  family="s", plotype="m"
26  bw = 30, minfrac = 0.5, minsamp= 1, mzwid = 0.25, max = 5, sleep = 0
27  xsa <- xsAnnotate(xset3)
28  groupFWHM(xsa, perfwhm=0.6)
29  xsaFI <- findIsotopes(xsaF, mzabs=0.01, maxcharge=3, maxiso=3, ppm=5)
30  xsaC <- groupCorr(xsaFI, cor_eic_th=0.75, polarity="")
```