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An Unexpected Role for Uric Acid as an Inducer of T Helper 2 Cell Immunity to Inhaled Antigens and Inflammatory Mediator of Allergic Asthma

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SUMMARY

Although deposition of uric acid (UA) crystals is known as the cause of gout, it is unclear whether UA plays a role in other inflammatory diseases. We here have shown that UA is released in the airways of allergen-challenged asthmatic patients and mice, where it was necessary for mounting T helper 2 (Th2) cell immunity, airway eosinophilia, and bronchial hyperreactivity to inhaled harmless proteins and clinically relevant house dust mite allergen. Conversely, administration of UA crystals together with protein antigen was sufficient to promote Th2 cell immunity and features of asthma. The adjuvant effects of UA did not require the inflammasome (*Nlrp3*, *Pycard*) or the interleukin-1 (*Myd88*, *IL-1r*) axis. UA crystals promoted Th2 cell immunity by activating dendritic cells through spleen tyrosine kinase and PI3-kinase δ signaling. These findings provide further molecular insight into Th2 cell development and identify UA as an essential initiator and amplifier of allergic inflammation.

INTRODUCTION

Allergic asthma is caused by an inappropriate adaptive T helper 2 (Th2) cell-mediated immune response to innocuous antigen, leading to eosinophilic airway inflammation, mucus hypersecretion, structural changes to the airway wall, and variable airway obstruction (Barnes, 2008). The cytokines produced by allergen-specific Th2 cell-type lymphocytes are held largely responsible for orchestrating the many features of asthma. Sensitization results from allergen recognition by structural cells of the airways and innate immune cells like basophils and dendritic cells (DCs) that induce and amplify adaptive Th2 cell-mediated immune responses (Barrett and Austen, 2009; Hammad et al., 2009, 2010; Lambrecht and Hammad, 2009; Sokol et al., 2008). The precise molecular mechanisms involved in allergenicity and Th2 cell response induction by DCs are incompletely understood (Paul and Zhu, 2010). Allergens have a complex molecular structure and often display enzymatic activity that stimulates epithelial and innate immune cells through protease-activated receptors (Hammad and Lambrecht, 2008; Sokol et al., 2008; Tang et al., 2010), whereas others like house dust mite (HDM) contain endotoxin or other pathogen-associated microbial patterns (PAMPS) that trigger epithelial cells and/or DCs in a toll-like receptor 4 (TLR4)- or C-type lectin receptor-dependent manner (Hammad et al., 2009; Nathan et al., 2009; Trompette et al., 2009). Triggering of PAMP receptors on epithelial cells and/or basophils subsequently leads to release of innate pro-Th2 cell cytokines like thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-25, and IL-33 that instruct DCs to promote Th2 cell-type immunity in the mediastinal lymph nodes (Hammad and Lambrecht, 2008; Hammad et al., 2009, 2010; Rank et al., 2009; Sokol et al., 2008; Zhou et al., 2005).

Although contamination of allergens with PAMPs that stimulate pattern recognition receptors provides a good explanation for how immunity can be induced to harmless allergens, another could be that allergens trigger release of damage-associated molecular patterns (DAMPs) that occur intracellularly in homeostasis but are released extracellularly upon physical or metabolic stress (Matzinger, 2002). A well-known DAMP is uric acid (UA) released from dying or stressed cells (Shi et al., 2003; Vorbach et al., 2003). Uric acid crystals potently trigger acute neutrophilic inflammation through stimulation of the NLRP3 inflammasome and release of interleukin-1β (Chen et al., 2006; Martinon et al., 2006). This pathway is at the heart of gouty inflammation, as supported by the absence of UA-induced neutrophilic inflammation in mice lacking the NLRP3, ASC (*Pycard*), IL-1R, or its downstream adaptor MyD88 and by the success of IL-1 receptor antagonist (IL-1RA) treatment for gout. We have reported recently that UA is released in the peritoneal cavity after injection

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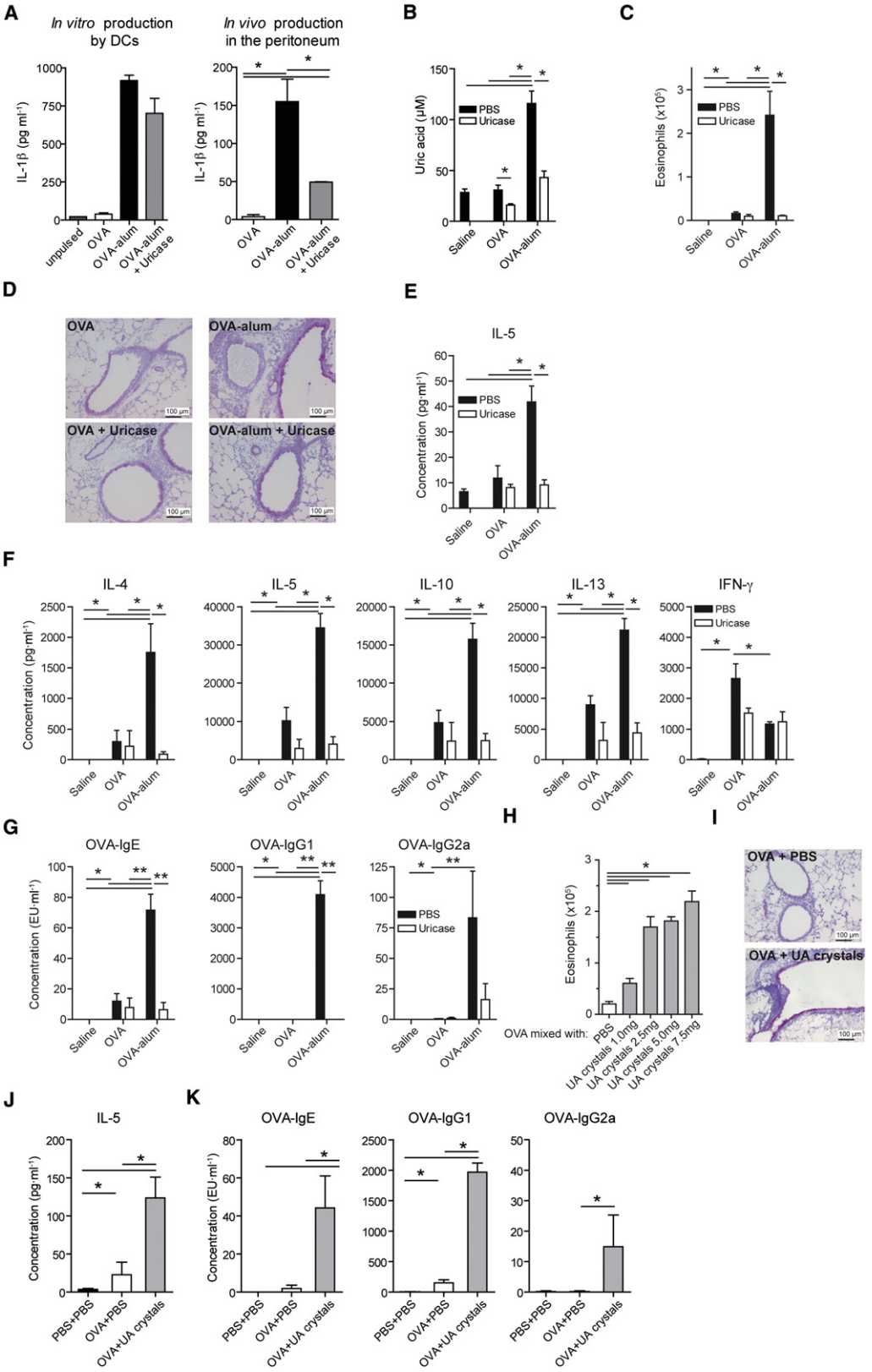

Figure 1. UA Is Necessary and Sufficient for Th2 Cell Response Induction to Ovalbumin

(A) In vitro bone marrow-derived GM-CSF-cultured DCs were stimulated with OVA or OVA+alum in the presence or absence of uricase (0.1 U/ml). In vivo, OVA or OVA+alum with or without 50 U uricase was injected intraperitoneally (i.p.). Bioactive IL-1β was measured in the supernatant or the peritoneal lavage 2 hr after exposure.

of the Th2 cell adjuvant aluminum hydroxide or alum, commonly coinjected with harmless antigens intraperitoneally to induce allergic sensitization and asthma (Kool et al., 2008b). In addition to inducing release of UA, alum can also directly trigger the NLRP3 inflammasome and stimulate secretion of bioactive IL-1β (Eisenbarth et al., 2008; Kool et al., 2008a; Li et al., 2008; McKee et al., 2009). These studies have aroused interest in the UA-inflammasome-IL-1 axis as a trigger of Th2 cell development.

Here, we hypothesized that uric acid is a general inducer and amplifier of Th2 cell immunity. By using models of intraperitoneal injection, as well as airway exposure of HDM allergen in mice and patients, we found that UA is present at sites of Th2 cell development, where it is necessary and sufficient for induction of Th2 cell immunity. Contrary to expectation, this Th2 cell adjuvant effect did not require triggering of the NLRP3-ASC complex, nor signaling through the IL-1R. Rather, UA induced Th2 cell immunity by triggering DC activation in a spleen tyrosine kinase (Syk)- and PI3-kinase δ-dependent manner. These findings identify UA as an unexpected initiator and amplifier of Th2 cell immunity and allergic inflammation.

RESULTS

Uric Acid Is Necessary and Sufficient for Induction of Th2 Cell Immunity to Harmless Antigen

We have reported that UA is released in the peritoneal cavity after injection of alum (Kool et al., 2008b) and is a necessary intermediate for early DC activation in vivo, whereas others argued that the immunostimulatory effects of alum did not require UA (Franchi and Núñez, 2008). As shown in Figure 1A, the degradation of UA by uricase treatment reduced secretion of bioactive IL-1β into the peritoneal cavity, whereas production of IL-1β by DCs in vitro did not require endogenous release of UA. Therefore, the important role of UA in mediating the adjuvant activity of alum is mainly revealed in vivo. The increased UA production after injection of OVA in alum was found to be intact in *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Casp1*$^{-/-}$ mice, excluding an important role for the TLR4 or inflammasome-IL1β axis in its induction (Figure S1 available online).

Because alum is frequently used to induce allergy in animal models of asthma, we hypothesized that UA is necessary for promoting adaptive Th2 cell immunity to OVA+alum. Injection of OVA+alum into BALB/c mice led to an increased concentration of UA in the peritoneal cavity, which could be neutralized by treatment with uricase (Figure 1B). Uricase treatment during priming to OVA+alum eliminated Th2 cell-dependent bronchoalveolar lavage (BAL) fluid eosinophilia (Figure 1C), peribronchial inflammation and goblet cell hyperplasia (Figure 1D), secretion of IL-5 in the BAL fluid (Figure 1E), production of IL-4, IL-5, IL-10, and IL-13 (but not IFN-γ) in mediastinal LN (MLN) cultures restimulated with OVA antigen for 3 days ex vivo (Figure 1F), and production of serum OVA-specific IgE and IgG_1 (Figure 1G). There was also a trend toward reduced production of IgG_{2a} by uricase treatment. OVA+alum injection also led to release of extracellular ATP, another DAMP known to activate the NLRP3 inflammasome via the P_2X_7 receptor (Mariathasan et al., 2006) and promote Th2 cell immunity (Idzko et al., 2007), yet its neutralization or use of *P2rx7*$^{-/-}$ mice did not reveal a contribution to Th2 cell immunity (Figure S2).

To address whether UA was also sufficient to induce Th2 cell immunity, we injected OVA admixed with UA crystals. Compared with control mice receiving OVA, this treatment dose dependently increased airway eosinophilia (Figures 1H and 1I), goblet cell hyperplasia (Figure 1I), IL-5 in BAL fluid (Figure 1J), and production of OVA-specific IgE, IgG_1, and IgG_{2a} (Figure 1K). Injection of OVA+UA also led to increased production of IL-4, IL-5, and IL-10, but not IFN-γ, in MLN cells (data not shown). These responses were of similar strength as those induced by injection of OVA+alum (Figures 1C–1E). Therefore, UA is necessary and sufficient for Th2 cell response development to intraperitoneal injection of harmless antigen, identifying it as a potent Th2 cell adjuvant.

Endogenous UA Is Necessary for Development of Th2 Cell Immunity to HDM Allergen in the Absence of Adjuvant

We next investigated the involvement of endogenous UA in response to complex and relevant allergens in the airways. A single intranasal HDM administration in naive mice resulted in a rapid increase in UA concentration in BAL fluid and lung (Figure 2A) compared with PBS. Mice sensitized and challenged with HDM developed features of allergic asthma, like BAL fluid and lung eosinophilia, lymphocytosis, goblet cell hyperplasia (Figures 2B and 2D), and airway hyperresponsiveness (AHR) to increasing doses of metacholine (Figure 2C). Intratracheal administration of uricase on day 0 at the time of sensitization neutralized the increase in UA (Figure 2A) and strongly reduced airway lymphocytosis and eosinophilia and AHR (Figures 2A–2D). To explain the mechanism of action, we evaluated the

(B) UA in the peritoneal lavage 2 hr after injection of OVA or OVA+alum in mice treated or not with uricase.
(C) BALB/c mice were treated with PBS or uricase 5 min before sensitization with OVA or OVA+alum i.p. on day 0. On day 7, mice were boosted with OVA i.p. and challenged with OVA aerosols on days 17–19. On day 20, BAL was analyzed for the presence of eosinophils.
(D) Lung sections were stained with periodic acid Schiff (PAS) reagent to visualize goblet cell metaplasia. Scale bars represent 100 μm.
(E) IL-5 concentration in BAL supernatants of mice treated as described in (C).
(F) Cytokine production of in vitro restimulated MLN cells taken on day 20 and cultured for 3 days in vitro in the presence of OVA.
(G) Serum OVA-specific Igs were determined by ELISA.
(H) Mice were sensitized with OVA or OVA mixed with different doses of UA crystals i.p. on day 0. On day 7, all mice were boosted with OVA i.p. and challenged with OVA aerosols on days 17–19. On day 20, the number of eosinophils was assessed in BAL.
(I) Lung sections stained with PAS. Scale bar represents 100 μm.
(J) IL-5 in BAL supernatant.
(K) Serum OVA-specific Igs determined by ELISA.
Data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$, n = 4–8 mice/group. Experiments were repeated twice; data shown are representative of all experiments.

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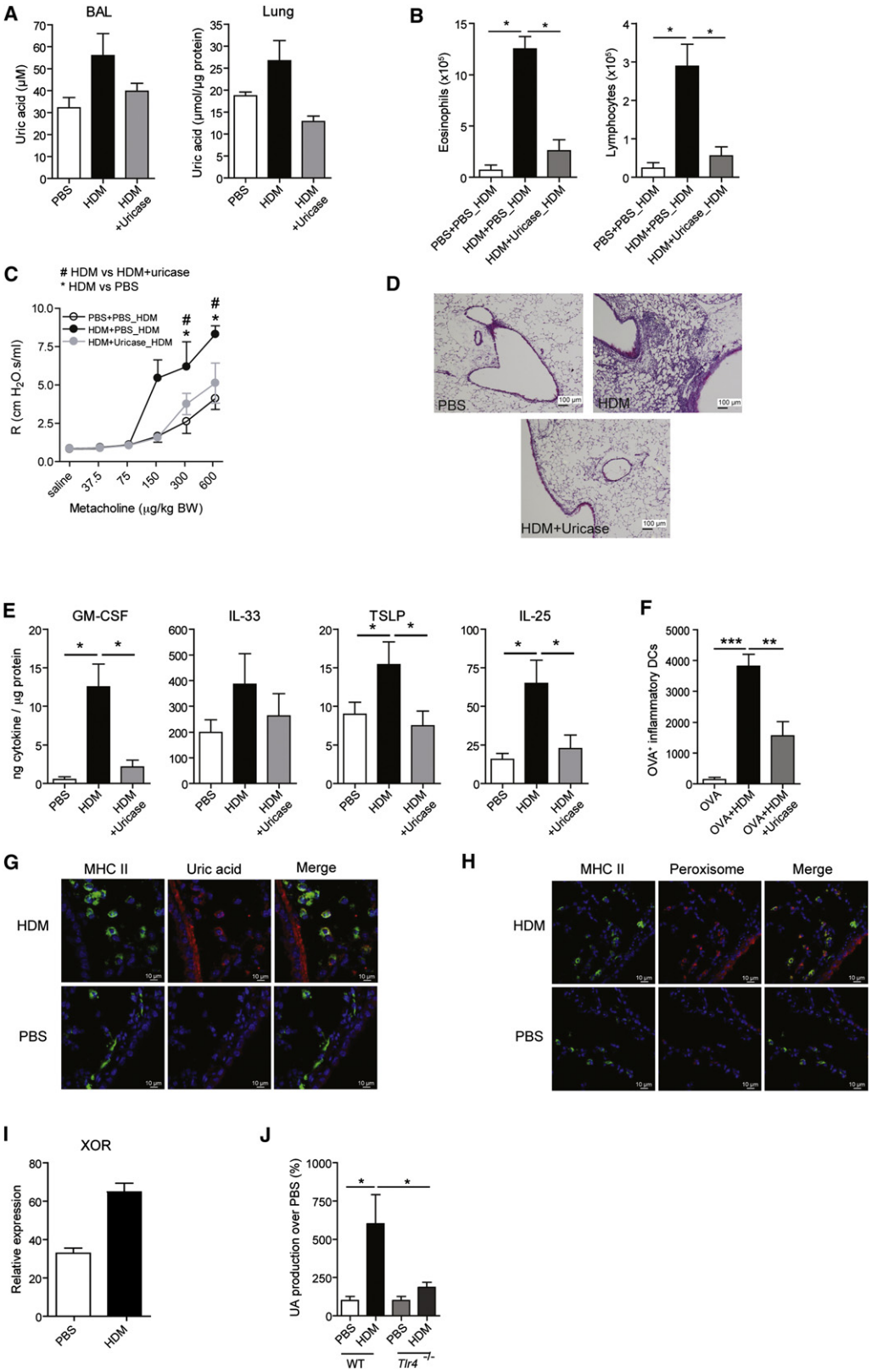

Figure 2. Uric Acid Plays a Key Role in Sensitization to House Dust Mite Allergen via the Airways

(A) C57BL/6 mice were sensitized i.n. with PBS or HDM and treated or not with uricase, and UA concentration was determined 2 hr later in the BAL and lung homogenates.

production of cytokines known to promote eosinophilic inflammation and Th2 cell immunity (Hammad and Lambrecht, 2008; Saenz et al., 2008). As already reported, the concentration of GM-CSF, TSLP, and IL-25 (Figure 2E) were increased in the lungs of animals exposed to a single HDM injection, compared with PBS (Hammad et al., 2009). However, this innate cytokine response was severely reduced in mice treated with uricase. House dust mite-driven Th2 cell development relies on antigen presentation by inflammatory Ly6C^{hi}CD11b^{+} DCs that take up antigen in the lung and migrate to the MLN (Hammad et al., 2010). To address antigen uptake and migration of these inflammatory DCs, we injected fluorescent OVA together with HDM allergen. In mice injected with OVA+HDM, migratory DCs carrying fluorescent cargo were easily identified in the MLN. After uricase treatment, this migration was strongly reduced (Figure 2F).

We next searched for the source of increased UA production. Within 2 hr after HDM administration, there was increased immunoreactivity for UA in airway epithelial cells and major histocompatibility complex II (MHCII)-positive alveolar macrophages and recruited monocytes (Figures 2G) compared to mice administered PBS. Strong UA immunoreactivity was seen along the basement membrane of HDM-exposed mice. Although UA release occurs in response to necrotic cell death, UA is also produced as a pulmonary defense mechanism to oxidative damage, through increased xanthine oxidoreductase (XOR) enzyme activity inside peroxisomes (Vorbach et al., 2003). In bronchial epithelial cells and MHCII-positive cells, HDM exposure induced enhanced staining for the 70 kDa peroxisomal marker PMP70, suggestive of peroxisomal biogenesis (Figure 2H; Imanaka et al., 1999). The expression of XOR was increased in BAL cells of HDM-exposed mice 2 hr after instillation compared with PBS (Figure 2I). We (Hammad et al., 2009) and others (Phipps et al., 2009; Trompette et al., 2009) recently identified TLR4 signaling as a crucial early trigger for the innate pro-Th2 cell immune response to allergens. In contrast to the alum-induced increase of UA in the peritoneal lavage (Figure S1), HDM-driven production of UA was severely reduced in C57BL/6 *Tlr4*$^{-/-}$ mice (Figure 2J), suggesting that triggering of TLR4 by HDM is necessary for activation of XOR and UA production in the airways in response to allergens.

Uric Acid Is Increased in Allergen-Challenged Human Asthmatics and Mice

To study whether our findings had any bearing on human asthma, BAL fluid was collected from 21 asthmatic subjects, 10 min and 24 hr after segmental challenge with saline or specific allergens (rye, birch, or HDM). There was no difference in the concentration of UA between saline- and allergen-challenged segments after 10 min. In contrast, 24 hr after allergen provocation, the UA concentration was markedly elevated in the BAL fluid from allergen- compared to saline-challenged segments (Figure 3A). This increase in UA concentration correlated with the influx of eosinophils and lymphocytes into the BAL (Figures 3B and 3C). Subgroup analysis of patients showing a minor rise in UA showed that this was not correlated with the type of allergen used nor the concentration of IgE in the serum, but rather on maintenance inhaled corticosteroid (ICS) therapy up to 7 days prior to allergen challenge (Figure S3).

To test whether UA neutralization would have any therapeutic potential, we again turned to the mouse model of HDM-induced asthma. Mice were first sensitized to HDM and were treated with uricase prior to each allergen challenge. HDM challenge induced a rise in UA in BAL fluid, still measurable 3 days after the last of five challenges (Figure 3D), and uricase treatment during challenge reduced airway eosinophilia (Figure 3E), metacholine-induced AHR, and IL-13 concentration in the BAL fluid (Figures 3F and 3G). Allergen challenge induced high numbers of eosinophils in the BAL fluid, which indicates that cellular death of recruited eosinophils could be a trigger for UA production. Uricase treatment had similar effects in a milder sensitization model in which only 20% of the number of eosinophils was recruited compared with the high dose sensitization model (Figure S4), arguing against the idea that UA plays a role only when there would be massive cellular recruitment and death.

UA Crystals, Alum, and HDM Promote Th2 Cell Immunity in the Absence of NLRP3 Inflammasome Activation

Although UA is the prototypical activator of the NLRP3 inflammasome and several groups (Eisenbarth et al., 2008; Franchi and Núñez, 2008; Hornung et al., 2008; Kool et al., 2008a; McKee et al., 2009) have shown that alum directly triggers NLRP3 in vitro, there is considerable controversy whether the

(B) Mice were sensitized i.n. on day 0 with PBS, HDM, or HDM+uricase and challenged i.n. with HDM on days 7–11. On day 14, BAL was examined for the presence of eosinophils and lymphocytes.

(C) Lung function, as determined by invasive measurement of airway resistance (R) in response to increasing concentrations of metacholine. $^{\#}p < 0.05$ compared with untreated, HDM-sensitized group; $^{*}p < 0.05$ compared with nonsensitized group.

(D) PAS staining of lung sections. Scale bars represent 100 μm.

(E) Production of GM-CSF, IL-33, TSLP, and IL-25 in lung homogenates of mice 2 hr after the i.n. administration of PBS, HDM, or HDM+uricase.

(F) Antigen uptake and migration of inflammatory CD11b^{+}Ly6C^{+} DCs to the mediastinal LN, 24 hr after intranasal injection of OVA-AF647 in the presence or absence of HDM, and effect of treatment with uricase. $^{***}p < 0.005$ compared with OVA; $^{**}p < 0.05$ compared with OVA+HDM.

(G) Fluorescence staining of uric acid (red) and MHCII (green) in lungs of animals exposed for 2 hr to HDM or PBS. Nuclei were counterstained with DAPI (blue). Scale bars represent 10 μm.

(H) Fluorescence staining of the peroxisomal marker PMP70 (red) and MHCII (green) in lungs of animals exposed for 2 hr to HDM or PBS. Nuclei were counterstained with DAPI (blue). Scale bars represent 20 μm.

(I) Expression of XOR mRNA normalized for HPRT by real-time quantitative RT-PCR.

(J) Increased production of UA depends on TLR4 triggering by HDM. In this experiment C57BL/6 WT or *Tlr4*$^{-/-}$ mice received HDM, and UA concentration was measured 2 hr later. Data are shown as mean ± SEM, $^{*}p < 0.05$, n = 4–8 mice/group.

Experiments were repeated twice. Data shown are representative of all experiments.

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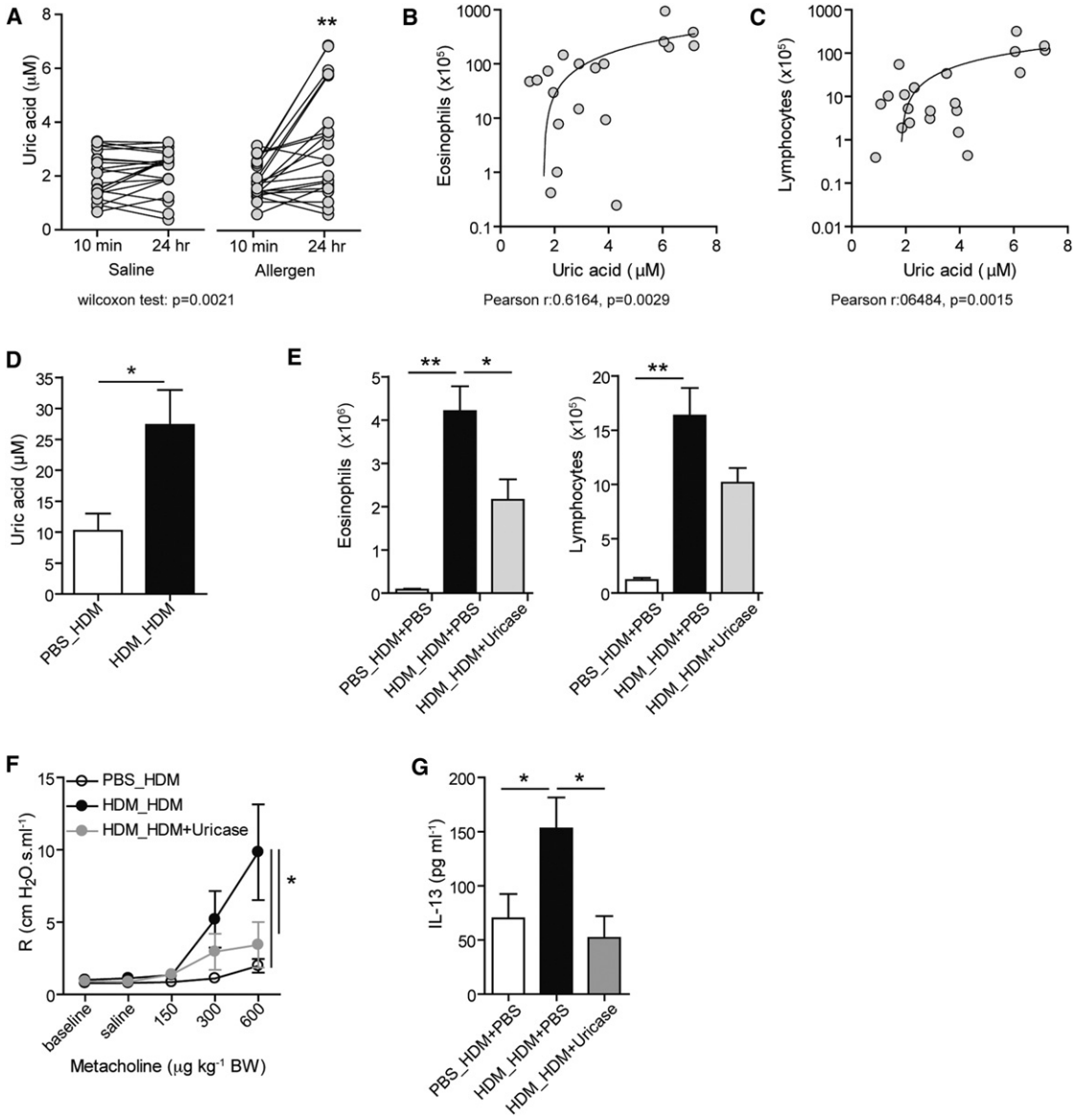

Figure 3. Involvement of UA in Ongoing Allergic Airway Inflammation in Human and Mouse

(A) UA concentration in BAL of asthmatic patients 10 min or 24 hr after segmental bronchial allergen or saline challenge.

(B and C) Correlation of UA concentration with the number of BAL eosinophils (B) or lymphocytes (C) 24 hr after allergen challenge.

(D) Concentration of UA in BAL 3 days after the last of five HDM challenges in HDM-sensitized mice.

(E) Treatment of HDM-sensitized mice with uricase (10 U) concurrent with each allergen challenge reduced airway eosinophilia, measured 3 days after the last challenge.

(F) Effect of uricase treatment prior to allergen challenge on AHR to increasing doses of metacholine.

(G) Concentration of IL-13, a cytokine associated with induction of AHR, in BAL.

Data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$, n = 4–8 mice/group. Experiments were repeated twice. Data shown are representative of all experiments.

inflammasome-IL-1β pathway is necessary for adjuvanticity in vivo (Franchi and Núñez, 2008; Williams et al., 2010). We therefore studied the involvement of this pathway in UA-driven Th2 cell immunity in vivo. Wild-type C57BL/6 mice immunized with OVA+UA showed a marked increase in eosinophilia and lymphocytosis in BAL (Figures 4A–4E), and this response was not affected in *Nlrp3*$^{-/-}$ or *Pycard*$^{-/-}$ mice, lacking essential components of the inflammasome complex (Martinon et al., 2006). Because there might be inflammasome-independent pathways to generate IL-1β, we also immunized mice lacking the IL-1R or mice lacking the critical IL-1R signaling intermediate MyD88. It has also been suggested that UA crystals signal via TLR4, although this could be due to contaminating endotoxin. Strikingly, *Il1r*$^{-/-}$, *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice all developed normal Th2 cell immunity to OVA+UA (Figures 4C–4E). We also studied whether development of Th2 cell immunity and asthma in response to OVA+alum relied on *Nlrp3*. Contrary to a recent report (Eisenbarth et al., 2008), we observed that

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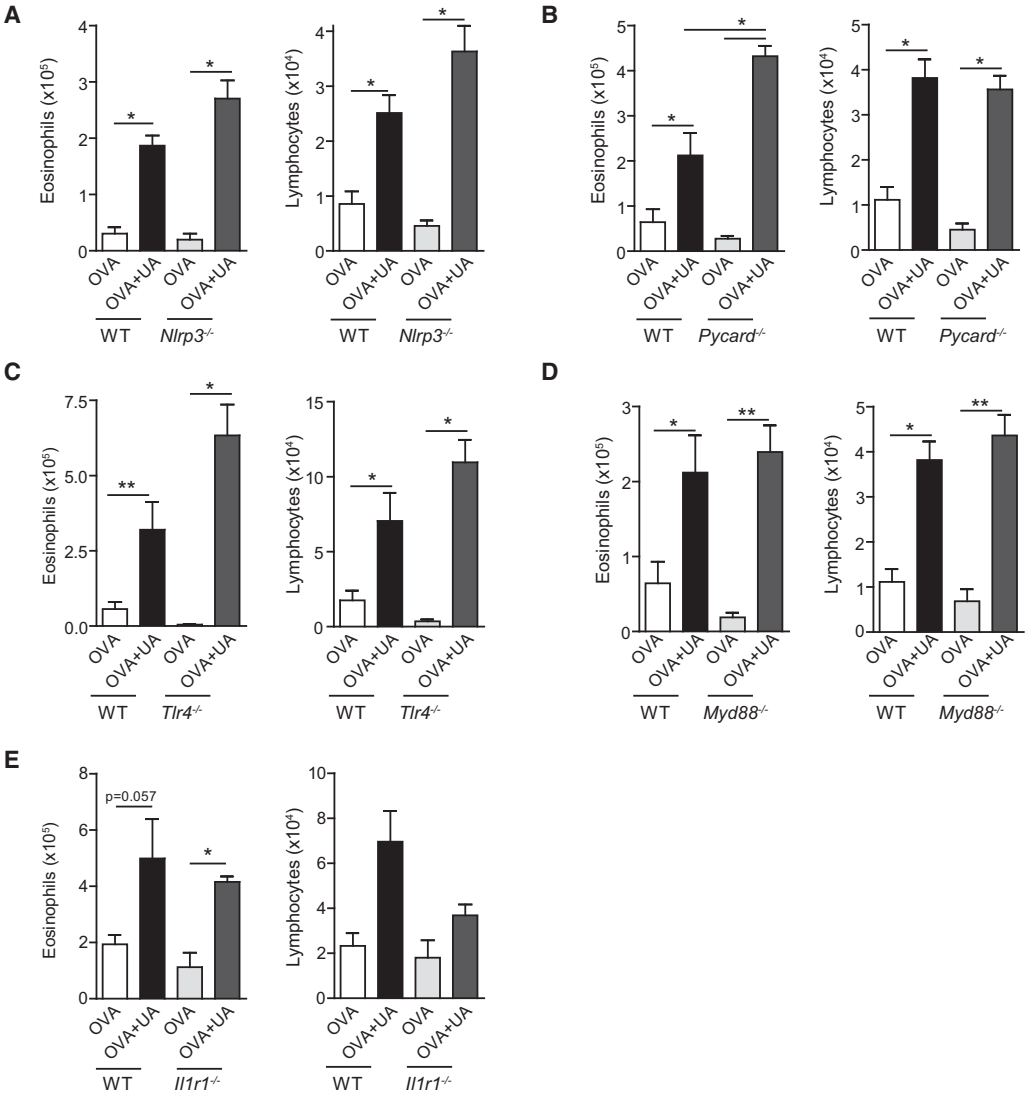

Figure 4. Uric Acid-Mediated Adaptive Th2 Cell Responses Do Not Require the IL-1-Inflammasome Axis

C57BL/6 mice were administered OVA mixed or not with UA crystals (UA) on day 0, boosted on day 7 with OVA intraperitoneally (i.p.), and challenged with OVA aerosols on days 17–19.

(A) Cellular composition in the BAL of WT and *Nlrp3*$^{-/-}$ mice.

(B) Cellular composition in the BAL fluid (BALf) of WT and *Pycard*$^{-/-}$ mice.

(C) Cellular composition in the BALf of WT and *Tlr4*$^{-/-}$ mice.

(D) Cellular composition in the BALf of WT and *Myd88*$^{-/-}$ mice.

(E) Cellular composition in the BAL of WT and *Il-1r*$^{-/-}$ mice.

Data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$, n = 4–6 mice/group. Experiments were repeated 2–3 times. Data shown are representative of all experiments.

Th2 cell-dependent asthmatic airway inflammation developed normally in *Nlrp3*$^{-/-}$ mice immunized with OVA+alum (Figure S5). To address whether HDM-driven asthma was dependent on these pathways, *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, and *P2rx7*$^{-/-}$ mice were sensitized and challenged with HDM via the airways but showed no reduction in Th2 cell-dependent eosinophilia (Figure S6). As already reported, *Myd88*$^{-/-}$ and *Tlr4*$^{-/-}$ mice had reduced HDM-driven airway inflammation (data not shown; Hammad et al., 2009, 2010). In conclusion, the prototypical Th2 cell stimuli alum and HDM, like UA crystals, do not rely on the NLRP3 inflammasome to induce Th2 cell-adaptive immunity leading to allergic airway inflammation.

UA Crystals Recruit Inflammatory DCs that Are Necessary and Sufficient for Th2 Cell Immunity

Twenty-four hours after injection of OVA-Alexa Fluor 647+UA, there was a strong increase in the number of inflammatory Ly6C^{hi}CD11b^{+} monocytes and inflammatory Ly6C^{+}CD11b^{+}

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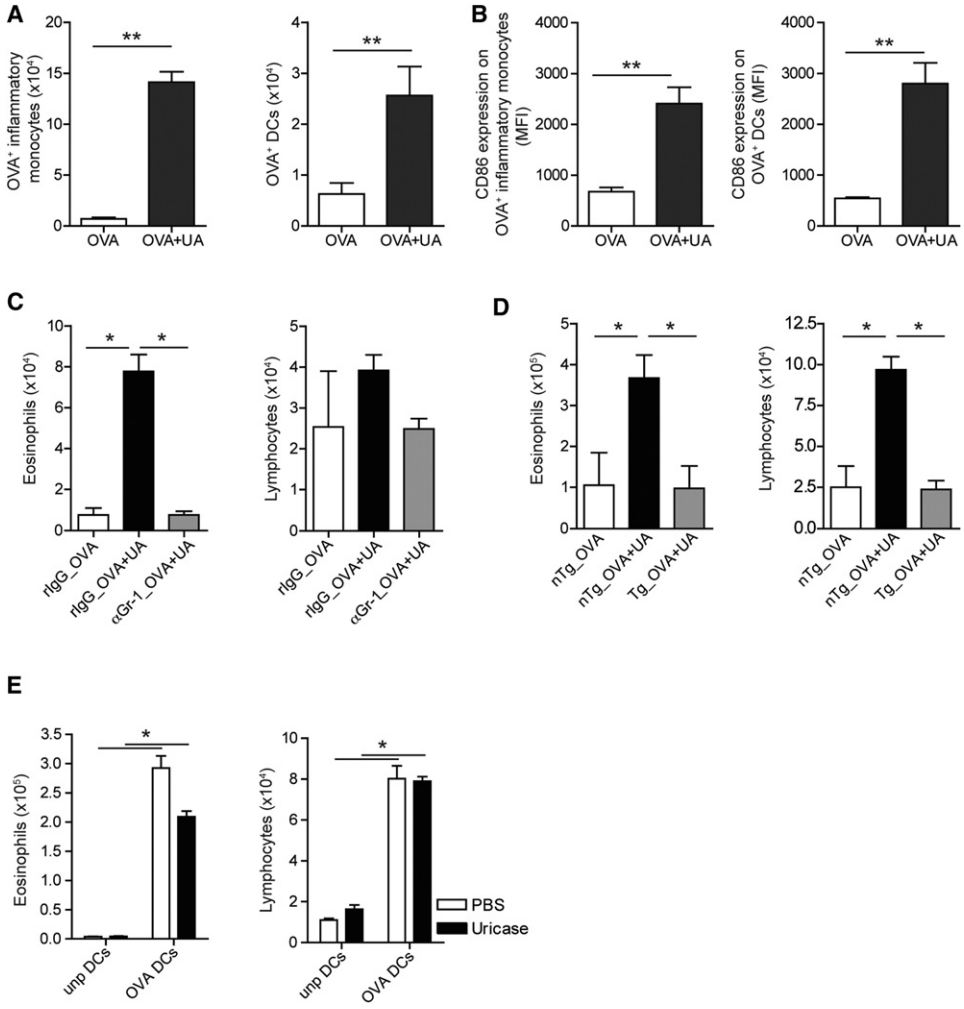

Figure 5. Inflammatory Monocytes and Dendritic Cells Are Critical for the Uric Acid-Mediated Adaptive Th2 Cell Responses

(A) C57BL/6 mice received OVA-AF647 mixed or not with UA crystals intraperitoneally (i.p.), and 24 hr later the peritoneal lavage was examined for the presence of $OVA^+CD11b^+Ly6C^{hi}$ inflammatory monocytes and DCs.

(B) OVA^+ inflammatory monocytes and DCs were analyzed for the expression of CD86.

(C) C57BL/6 mice were treated with Gr-1 or control antibodies (500 μg/mouse) on day −1, 0, 1, 2. On day 0, mice were treated with OVA mixed or not with UA crystals (UA), boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17–19. Cellular composition in the BALf is shown.

(D) C57BL/6 *Cd11c*-DTR mice were treated with diphtheria toxin (100 ng/mouse i.p.) on day −1. On day 0, mice were treated with OVA mixed or not with UA crystals, boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17–19. Cellular composition in the BALf was determined by flow cytometry.

(E) BALB/c mice were sensitized by intratracheal instillation of 1×10^6 unpulsed or OVA-pulsed bone marrow-derived GM-CSF DCs with or without uricase. On days 10–12, mice were challenged with OVA aerosols. Cellular composition in the BAL is shown.

Data are shown as mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$, n = 4–6 mice/group. Experiments were repeated 2–3 times. Data shown are representative of all experiments.

DCs that had ingested OVA in the peritoneal cavity compared with mice injected with OVA-AF647 alone (Figure 5A), and these recruited cells had higher expression of the costimulatory molecule CD86 (Figure 5B). Although recruitment and activation of DCs suggests a functional role for these cells in the UA effect, we still wanted to address whether DCs are really necessary for mediating Th2 cell immunity, because recent work suggested that DCs are poor inducers of Th2 cell immunity, whereas basophils readily are (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). We therefore injected OVA+UA in *Cd11c*-DTR C57BL/6 mice, together with diphteria toxin (DT) to deplete DCs (Jung et al., 2002). DT injection in Tg mice abolished airway eosinophilia induced by OVA+UA (Figure 5D). This effect was also seen when we injected an antibody to Ly6C/G (Gr1) that depletes inflammatory monocytes (Figure 5C), but in this setting one cannot exclude that neutrophils play an additional role. To finally demonstrate that inflammatory DCs are sufficient to induce Th2 cell immunity, we injected GM-CSF-cultured OVA-pulsed DCs (a model to generate inflammatory type DCs) into the peritoneal cavity, followed by OVA challenge to the lung. Intraperitoneal injection of OVA-pulsed DCs, but not unpulsed DCs, was able to prime for an eosinophilic Th2 cell response

(Figure 5E). However, treatment with uricase did not inhibit Th2 cell development induced by DC injection, demonstrating that the immunostimulatory effects of UA are upstream of DC activation in vivo. These experiments also demonstrate that uricase treatment does not have a generalized off-target suppressive effect on Th2 cell polarization, but rather inhibits inflammatory-type DC recruitment and activation.

UA Activates DCs and Th2 Cell Immunity in a Syk- and PI3-Kinase δ-Dependent Manner

The above data implied that DCs are a crucial intermediate cell type in mediating the Th2 cell immune response to OVA+UA (Figure 5) but also that the NLRP3-ASC-IL1-R axis were not involved in this process in vivo (Figure 4). UA crystals mixed with OVA in vitro were able to induce the secretion of bioactive cleaved IL-1β in inflammatory DCs, and this effect was completely absent in cells of *Nlrp3*$^{-/-}$ mice, essentially showing that UA does trigger this inflammasome pathway in DCs. However, OVA+UA also induced secretion of IL-6 and TNF-α in inflammatory DCs and these responses were not abolished in *Nlrp3*$^{-/-}$ mice. Strikingly, OVA+UA did not induce production of the Th1 cell instructive cytokine IL-12. Recently, via atomic force microscopy, UA crystals were shown to directly engage cholesterol-rich cellular membranes of DCs in a receptor-independent manner, leading to recruitment and activation of spleen tyrosine kinase (Syk) on membrane-associated immunoreceptor tyrosine activation motifs (Ng et al., 2008). Other studies have also identified Syk signaling and downstream PI3-kinase activation as an essential intermediate of UA-induced human neutrophil activation (Desaulniers et al., 2001; Popa-Nita et al., 2007). When the in vitro Syk inhibitor piceattanol was added, UA+OVA no longer induced IL-1β, IL-6, or TNF-α production in inflammatory DCs, suggesting that this pathway was more broadly involved in inducing cytokine production in DCs (Figure 6A). Oral administration of the Syk inhibitor BAY61-3606 reduced influx of OVA-positive inflammatory Ly6C^{+}CD11b^{+} monocytes in the peritoneum 24 hr later (Figure 6B). Signaling downstream of Syk has been shown to involve interaction of p85 with p110δ subunits of PI3-kinase (Popa-Nita et al., 2007). The number of OVA^{+}Ly6C^{+}CD11b^{+} monocytes 24 hr after injection of OVA+UA was strongly reduced in mice expressing a catalytically inactive form of the p110δ subunit of PI3-kinase (D910A) (Figure 6C). We finally addressed whether Syk and downstream PI3-kinase δ signaling was important for development of adaptive Th2 cell immunity to OVA+UA. Oral administration of BAY61-3606 compound strongly reduced airway lymphocytosis and eosinophilia (Figure 6D). Accordingly, OVA+UA did not induce Th2 cell immunity in *Pik3cd*^{D910A} mice (Figure 6E), although this finding could also reflect effects on other cells like mast cells (Ali et al., 2004). To address whether the inibition of Syk was specifically acting at the level of DCs, we took a genetic approach and immunized *Cd11c*Cre × *Sykb*$^{fl/fl}$ mice specifically lacking Syk in CD11c^{hi} cells (unpublished observations) or control mice with OVA or OVA+UA crystals. Upon OVA challenge, the degree of reduction in airway eosinophilia in these *Sykb*$^{-/-}$ DC mice was of similar magnitude as those treated with a pharmacological Syk antagonist, suggesting indeed that Syk triggering in DCs is crucial for mounting Th2 cell immunity to UA (Figure 6F).

DISCUSSION

Although UA is a known trigger for acute neutrophilic inflammation (Martinon et al., 2006), we here identified its role as a promotor of Th2 cell-dependent allergic inflammation. The release of UA was seen in patients with allergic asthma challenged with relevant allergen, in naive and sensitized mice exposed to the prototypical Th2 cell inducer and relevant HDM allergen, and after injection of the prototypical Th2 cell adjuvant alum.

The Th2 cell-inducing capacity of UA occurred independently from its actions on the NLRP3-ASC-IL-1-MyD88 axis. UA is released upon alum administration in vivo, but not in vitro, so these findings could explain the controversy that has arisen as to whether triggering NLRP3 inflammasome activation is crucial for alum adjuvanticity (Marrack et al., 2009). All authors that have studied the involvement of NLRP3 agree that NLRP3, PYCARD (ASC), and caspase-1 are crucial for alum-induced IL-1β secretion in vitro and in vivo, but this does not automatically imply that all adjuvant effects of alum must therefore occur via this pathway (Franchi and Núñez, 2008; Li et al., 2007, 2008; McKee et al., 2009). Our findings suggest that UA released upon alum injection in vivo is by itself sufficient to induce Th2 cell adjuvant effects that do not depend on the inflammasome-IL-1R pathway. It has indeed long been known that alum-induced asthma models do not rely on IL-1R signaling (Piggott et al., 2005; Schmitz et al., 2003) nor on the downstream essential adaptor molecule MyD88 (Piggott et al., 2005). One report has suggested that *Nlrp3* is absolutely required for alum to induce Th2 cell immunity to OVA and subsequent development of allergic type inflammation of the lung (Eisenbarth et al., 2008), at odds with findings in the current report. These authors, however, also demonstrated that the effects of alum were MyD88 independent, arguing against a role for IL-1R signaling, given the crucial role of MyD88 as a signaling intermediate of the IL-1R pathway. These differences could be explained best by subtle differences in antigens used, type of alum used, and timing of analysis. Also we have described previously that NLRP3, IL-1, and MyD88 are involved in the early innate response of DCs to alum injection in the peritoneal cavity, but clearly these reactions are redundant with other pathways to induce Th2 cell immunity to OVA (Figure 4), and their elimination never leads to complete abolition of DC activation or subsequent T cell activation (Kool et al., 2008a, 2008b).

The most striking finding of our paper is that UA is also released upon primary exposure of the lung to HDM, as well as upon allergen challenge in already sensitized human asthmatics and mice. Experiments with the UA-degrading enzyme uricase furthermore showed that endogenously released UA promotes Th2 cell sensitization by amplifying the production of innate pro-Th2 cytokines that instruct DCs to induce Th2 cell immunity, like TSLP, GM-CSF, and IL-25 (Barrett and Austen, 2009; Lambrecht and Hammad, 2009), as well as stimulating the activation of inflammatory DCs. Many reported that this innate pro-Th2 cytokine release and DC activation depends on TLR4 triggering on lung epithelial cells via endotoxin contained in the HDM allergen (Hammad et al., 2009, 2010; Trompette et al., 2009). We now show that TLR4 triggering is functionally connected to the induction of UA production in epithelial cells and macrophages. Most probably this occurs via induction of the enzymatic

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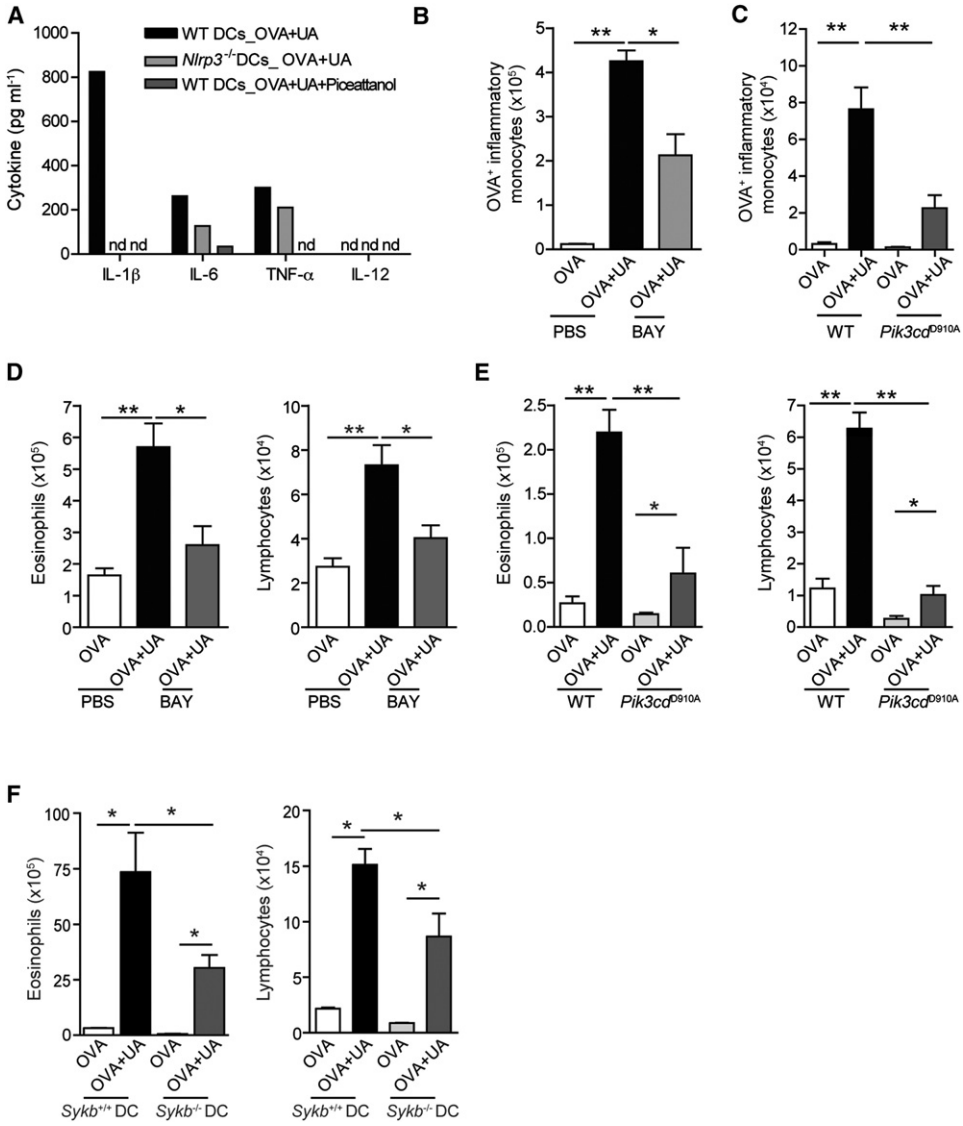

Figure 6. UA Crystals Induce Th2 Cell Immunity through Spleen Tyrosine Kinase and PI3-Kinase δ

(A) Bone marrow-derived DCs were cultured in GM-CSF from WT and *Nlrp3*$^{-/-}$ mice and preincubated with the Syk inhibitor piceatennol for 30 min (50 μM), then pulsed with OVA (100 μg/ml) with or without UA crystals (250 μg/ml) for 3 hr, after which cytokine concentration was determined in the cell supernatant.

(B) C57BL/6 mice were treated twice a day on days −1 and −2 orally with the specific Syk inhibitor BAY61-3606 (Yamamoto et al., 2003) and injected on day 0 with OVA-AF647 mixed or not with UA crystals intraperitoneally (i.p.). 24 hr later, the peritoneal lavage was examined for the presence of OVA^{+} inflammatory monocytes.

(C) *Pik3cd*^{D910A} or WT C57BL/6 mice were injected with OVA-AF647 mixed or not with UA crystals (UA) i.p., and 24 hr later the peritoneal lavage was examined for the presence of OVA^{+} inflammatory monocytes.

(D) C57BL/6 mice were treated twice a day on days −1 and −2 orally with the specific Syk inhibitor BAY61-3606 . On day 0, mice were treated with OVA mixed or not with UA crystals, boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17–19. Cellular composition in the BAL is shown.

(E) Cellular composition in the BALf of WT and *Pik3cd*^{D910A} mice, which have been sensitized with OVA+UA crystals and challenged with OVA aerosol as decribed in (D), is depicted.

(F) Cellular composition in the BAL of *Sykb*$^{-/-}$ DC and *Sykb*$^{+/+}$ DC chimeric mice, which were sensitized with OVA+UA crystals and challenged with OVA as described in (D).

Data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$, n = 4–6 mice/group, representative of at least two experiments per panel.

activity of the rate-limiting uric acid-synthesizing enzyme xanthine oxidoreductase, leading to production of UA inside peroxisomes. Alternatively, increased UA concentration could also result from reduced catabolism from urate oxidase. Although we have not formally investigated the mechanisms of TLR4-driven induction of UA production, it is known that UA is found in the lining fluid of the airway epithelial layer in vivo and is induced in response to various types of oxidative stress like ozone exposure or respiratory viral infection (Akaike et al., 1990; Papi et al., 2008). The xanthine oxidoreductase gene is

induced as part of an innate immune response to various stimuli like LPS, tissue hypoxia, and early innate cytokines like IL-1, and the promotor region contains an NF-κB control element (Hassoun et al., 1998; Vorbach et al., 2003). How exactly locally released UA affects the innate cytokine production by epithelial cells is at present unclear, nor do we have any indication that UA concentration would be so high that UA crystals are formed in the lung lining fluid. Because of physicochemical constraints, we have been unable to transfer UA crystals to the lungs of mice to directly probe for effects on epithelial cells without mortality of the mice. One possiblility that needs further exploration is the fact that the antioxidant properties of UA in the lung lining fluid affect the potential of bronchial epithelial cells to synthesize, correctly fold, and secrete proteins, processes sensitive to oxidative stress (Santos et al., 2009).

We have also reported previously that ATP is released in the airways of OVA-challenged sensitized mice, as well as allergen-challenged patients, indicating that a DAMP can contribute to asthma pathogenesis (Idzko et al., 2007). Because ATP is a well-known activator of the NLRP3 inflammasome (Franchi et al., 2007), acting via the P2X7 purinergic receptor that is a potassium efflux channel, it made sense to address whether either alum-induced or HDM-induced asthma relied on P2X7. Experiments in *P2rx7*$^{-/-}$ mice did not, however, reveal a crucial role of this receptor in asthma development, again arguing against a predominant role of NLRP3 in asthma. However, there are many ways by which ATP can activate innate and structural cells of the lung to promote asthma and Th2 cell development, because the purinergic receptor family is very broad and widely expressed among others on eosinophils (Kobayashi et al., 2010).

Recently, several groups studying Th2 cell response induction to the protease allergen papain or to gastrointestinal parasites have reported that DCs are poor inducers of Th2 cell immunity that are neither sufficient nor required for Th2 lymphocyte differentiation, basophils being clearly superior (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). However, it was recently also shown that for HDM sensitization, inflammatory DCs are the antigen-presenting cells that provide the first instruction to Th2 cell development, whereas basophils act to enhance these responses (Hammad et al., 2010). Given the fact that the relative contribution of DCs versus basophils seems to depend on the type of Th2 cell stimulus or the route of injection (Tang et al., 2010), it was therefore of interest to study the involvement of DCs in mediating the Th2 cell adjuvant activities of UA injected into the peritoneal cavity. Employing the logic of Koch's postulates, we first found that inflammatory type DCs and their monocytic precursors were recruited to the site of UA injection, that depletion of CD11c^{hi} dendritic cells abolished UA-driven Th2 cell responses, and that adoptive transfer of inflammatory DCs to the peritoneal cavity was sufficient to induce Th2 cell immunity. Moreover, because we did not observe basophil recruitment to the draining lymph nodes of the peritoneal cavity (data not shown), we believe that these experiments are sufficient proof that DCs are the cells driving Th2 cell immunity to OVA+UA.

Recently, UA crystals were shown to directly engage cholesterol-rich cellular membranes of DCs in a receptor-independent manner, leading to activation of Syk and downstream PI3K signaling (Ng et al., 2008). Experiments with in vitro and in vivo Syk inhibitors, as well as kinase-dead *Pik3cd*^{D910A} mice, revealed that this pathway was relevant for early in vivo recruitment of inflammatory DCs to the site of injection and in vivo development of Th2 cell immunity to OVA+UA. Although these manipulations could also affect other cells, the use of mice specifically lacking Syk expression in DCs allowed us to conclude that at least the DC is a predominant sensor of UA crystals. How exactly DCs exposed to UA subsequently polarize Th2 cell immune responses is more speculative, but again probably involves Syk signaling. We observed that DCs exposed to OVA+UA produced IL-1β, IL-6, and TNF-α in the absence of the polarizing cytokine IL-12, in a Syk-dependent manner. We and others have previously reported that absence of IL-12 production is a prerequisite for inflammatory DCs to be able to induce Th2 cell immunity, because retroviral overexpression of IL-12 abolishes DC-driven Th2 cell development and subsequent asthma (Eisenbarth et al., 2002; Kuipers et al., 2004), whereas others reported that IL-6 and TNF-α are all involved in promoting DC-driven Th2 cell development in asthma (Dodge et al., 2003; Ebeling et al., 2007; Eisenbarth et al., 2002; Rincón et al., 1997). One report demonstrated that Syk signaling in DCs is crucial for Th2 cell-dependent asthma development driven by adoptive transfer of DCs, but it is unclear how this relates to polarizing cytokine secretion (Matsubara et al., 2006). Further definitive experiments will involve neutralization of individual cytokines to address these issues in detail.

In conclusion, we have identified an unexpected role for UA as a crucial initiator and amplifier of Th2 cell immunity and allergic inflammation in the mouse by activating inflammatory DCs. At the same time we have identified a pathway of immune activation by UA crystals that is independent of their well-known capacity to trigger the NLRP3 inflammasome. The fact that neutralization of uric acid at the time of allergen challenge reduces the salient features of asthma, and the fact that increased UA concentrations are found in patients after allergen challenge, open up the prospects that this pathway could be used for novel therapeutics in asthma.

EXPERIMENTAL PROCEDURES

Human Subjects

Twenty-one nonsmoking patients with mild allergic asthma (Table S1) were recruited based on airway hyperresponsiveness, positive allergen skin prick tests, elevated total or specific IgE concentrations, and a dual reaction after allergen challenge (Lommatzsch et al., 2006). The calculation of the individual provocation dose was performed based on responsiveness to inhalation allergen challenge (Lommatzsch et al., 2006). Inhaled and segmental allergen challenges were separated by at least 4 weeks. Segmental allergen challenge was performed as described (Lommatzsch et al., 2006). Corticosteroids were withdrawn at least 7 days before challenge. Patients gave their written informed consent, and he study was approved by the local ethics committee of the University Hospital Rostock, Germany.

Mice

BALB/c mice and C57BL/6 mice (6–9 weeks old) were purchased from Harlan (Zeist, The Netherlands). *Nalp3*$^{-/-}$, *Pycard*$^{-/-}$, *Tlr4*$^{-/-}$, *P2rx7*$^{-/-}$, *Myd88*$^{-/-}$, *Il1r*$^{-/-}$, and WT C57BL/6 mice (6–10 weeks old) were kindly provided by J. Tschopp (University of Lausanne, Switzerland) and B. Ryffel (University of Orleans, France). *Pik3cd*^{D910A} were kindly provided by B. Vanhaesebrouck (Queen Mary University London). To generate mice lacking Syk specifically in DCs, *Cd11c*Cre mice were crossed to *Sykb*$^{fl/fl}$ mice (unpublished

observations). Bone marrow of these mice was used to generate chimeric mice, by sublethally irriaditing C57BL/6 mice (5Gy), followed 4 hr later by intravenous (i.v.) injection of 2×10^6 BM cells. Chimeric mice were used 10 weeks after hematopoietic reconstitution. All experiments were approved by the animal ethics committee at Ghent University and Erasmus University Medical Center.

Monosodium Urate Crystal Preparation

MSU crystals were prepared as described (Chen et al., 2006). In brief, saturated uric acid (5 mg/mL, Sigma) in 0.1 M borate buffer (pH 8.5) was incubated at room temperature for 48 hr. After harvesting the crystals, they were washed with alcohol and acetone and air-dried for at least 2 days. Crystals were between 5 and 25 μm long and contained <0.005 EU/mL endotoxin.

Treatment with Uricase

For intraperitoneal injections, mice received 50 U of uricase (Sigma-Aldrich) or rasburicase (Sanofi-Synthelabo) in 100 μl PBS 5 min prior to injection of OVA+alum. For intrapulmonary administration, HDM was mixed with 10 U uricase and injected intranasally into 40 μl.

Induction of Allergic Airway Inflammation

To test the adjuvant properties of MSU crystals, mice were injected intraperitoneally (i.p.) with 10 μg of OVA (Worthington) mixed with increasing amounts of MSU crystals (1–7.5 mg in 500 μl). Mice were boosted i.p. on day 7 with OVA and challenged with OVA aerosols (Sigma, 1% for 30 min) on days 17–19. On day 20, mice were analyzed for the presence of Th2 cell-dependent eosinophilic airway inflammation.

To induce HDM-driven eosinophilic airway inflammation, anesthetized mice were administered intranasaly (i.n.) with 40 μl of house dust mite extracts (100 μg, Greer Laboratories) on day 0 and challenged on days 7–11 with HDM. On day 14, mice were sacrificed and different parameters were analyzed. Assessment of bronchial hyperreactivity and histological analysis of goblet cell hyperplasia was measured as described previously (Hammad et al., 2009).

Immune Analysis

To determine the degree of airway eosinophilia, bronchoalveolar lavages (BAL) were performed by injecting 1 ml of PBS containing 0.01 mM EDTA. Cells were stained with FITC anti-I-A^d and I-E^d, PE-labeled anti-Siglec F, PerCp-labeled anti-CD3 and anti-CD19, and APC-labeled anti-CD11c (all from BD Biosciences). Peritoneal inflammatory cells were stained as described (Kool et al., 2008b). Data were collected on a FACS Aria II (Becton Dickinson) and were analyzed with FlowJo software (Treestar, Inc.).

The concentration of UA (Amplex Red Uric Acid; Uricase assay, Invitrogen), GM-CSF, IL-33, TSLP, and IL-25 (all R&D Systems) was measured in BAL fluid and lung homogenates by ELISA 2 and 24 hr after HDM administration. The concentration of Th2 cytokines in BAL fluids was measured 24 and 72 hr after the last challenge, respectively.

OVA-specific IgG_1, IgE, and IgG_{2a} were measured in sera of mice sensitized and challenged with OVA by ELISA (antibodies from R&D Systems).

Uric Acid Staining in the Lung

Two hours after the i.n. administration of PBS or HDM, lungs were inflated with PBS and OCT and frozen at −80°C. Ten μm thick sections were fixed with 4% formaline for 10 min and stained with rabbit anti-uric acid antibodies (Abcam) followed by Cy3-labeled goat anti-rabbit antibodies (Jackson Laboratories), blocking with rabbit serum, biotin-labeled anti-peroxisomal membrane protein 70 (Genetex), and FITC-labeled anti-MHCII (BD Bioscience), followed by streptavidin Alexa Fluor 647. Nuclei were counterstained with 4′,6′diamidino-2-phenylindole (DAPI). Images were collected on a Zeiss LSM 710 equipped with a Ar 488 nm laser, a He-Ne 561 nm laser, a He-Ne 633 nm laser, and a Mai Tai femtosecond-pulsed laser tuned at 800 nm to excite DAPI. Images were analyzed with Imaris (Bitplane).

qPCR on Xanthine Oxido Reductase

RNA was isolated from BAL cells with High Pure RNA Isolation Kit (Roche) with on-column DNase I treatment. 100 ng RNA was reverse transcribed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Quantitative PCR was performed with LightCycler 480 Probes Master (Roche) and primers (xanthine oxidoreductase; forward, AGGGATTCCGGACCTTTG; reverse, GCAGCAGTTTGGGTTGTTTC) and probe mixes (probe 69; Roche). PCR conditions were 10 min at 95°C, followed by 45 cycles of 10 s at 95°C and 50°C for 1 min with a LightCycler 480 Instrument (Roche). PCR amplification of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was performed during each run for each sample to allow normalization between samples.

Statistical Analysis

For all experiments unless stated otherwise, the difference between groups was calculated with the Mann-Whitney U test for unpaired data (GraphPad Prism version 4.0; GraphPad, San Diego, CA). Comparison of the human BAL fluid UA concentration was performed with the Wilcoxon-signed rank test. Correlations between the human data were tested with Pearson's correlation test. Differences were considered significant when $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.005$ (***).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at doi:10.1016/j.immuni.2011.03.015.

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Immunity, Volume *34*

Supplemental Information

An Unexpected Role for Uric Acid as an Inducer of T Helper 2 Cell Immunity to Inhaled Antigens and Inflammatory Mediator of Allergic Asthma

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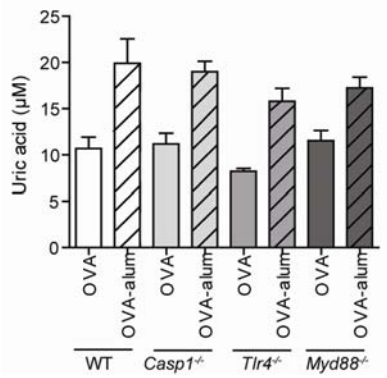

Figure S1: OVA-alum induces UA independent of the TLR/IL-1R axis.
Casp$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$ mice and their control littermates were injected with OVA-alum or OVA alone. 2hrs later the peritoneal lavage was taken and UA levels were determined.

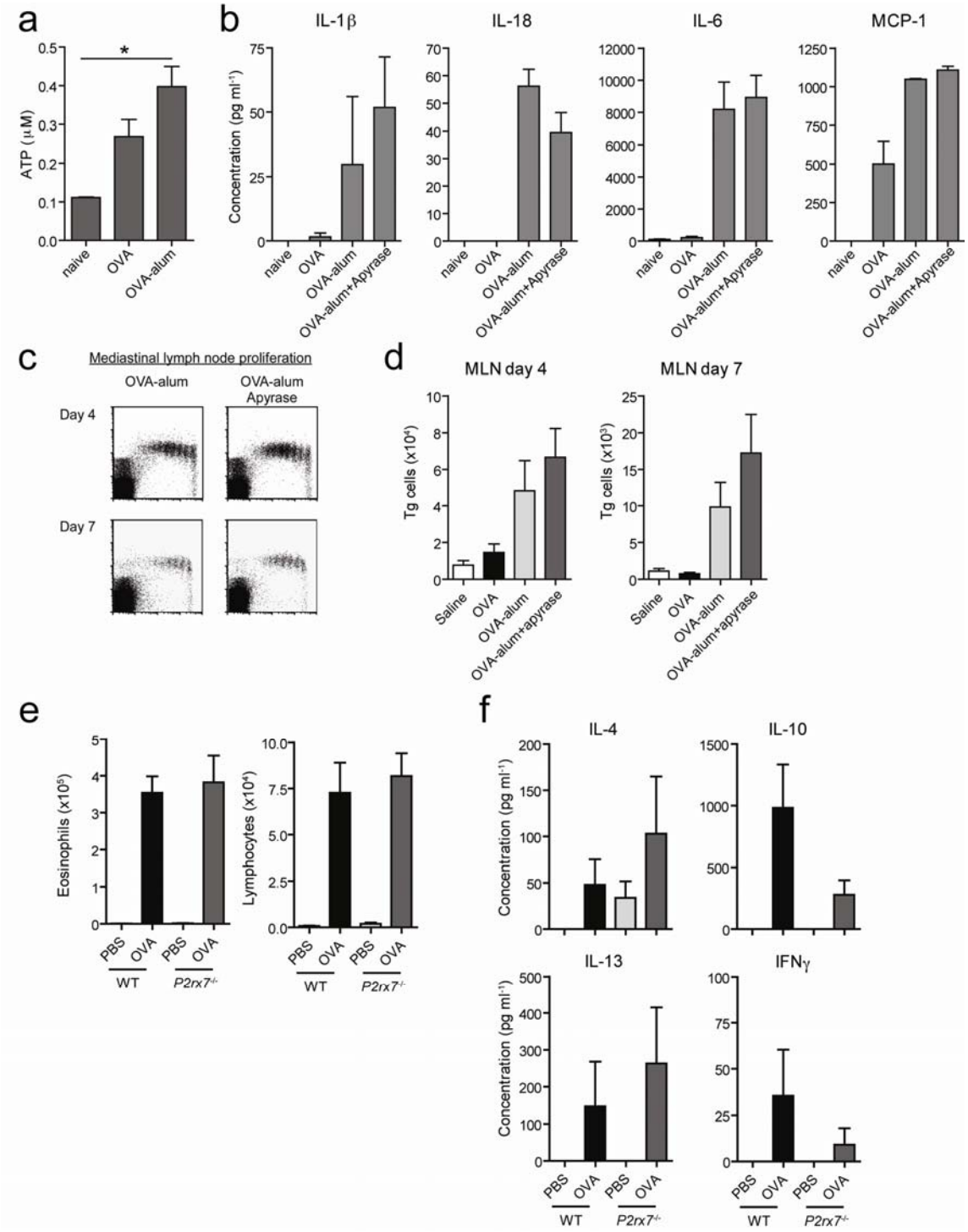

Figure S2: OVA-alum responses are independent of ATP or P2X7.
(a) BALB/c mice were injected i.p. with OVA or OVA-alum and 2 hrs the levels of ATP were determined in the peritoneal lavage. **(b)** The peritoneal lavage was also examined for the levels of IL-1β, IL-6, IL-18, and MCP-1 by ELISA. **(c)** $2.5*10^6$ CFSE-labeled DO11.10 OVA-TCR Tg $CD4^+$ T cells were given to BALB/c mice one day prior to OVA-alum. Mice were treated 5 min before OVA-alum with apyrase. Four and 7 days later the mediastinal lymphnode cells were collected and examined by flowcytometry for divisions. **(d)** Total numbers of OVA-TCR Tg cells are depicted. **(e)** WT or *P2rx7*$^{-/-}$ mice were sensitized on day 0

with alum (PBS) or OVA-alum (OVA), boosted on day 7 with OVA i.p., and challenged with OVA aerosols on days 17-19. Mice were sacrificed on day 20, and the cellular composition in the BALf is shown. **(f)** On day 20 the mediastinal lymphnode cells were restimulated with OVA (10 µg/ml) for four days. Cytokines were determined in the supernatant by ELISA. Data are shown as mean ± SEM, * $p<0.05$, n=4-6 mice/group.

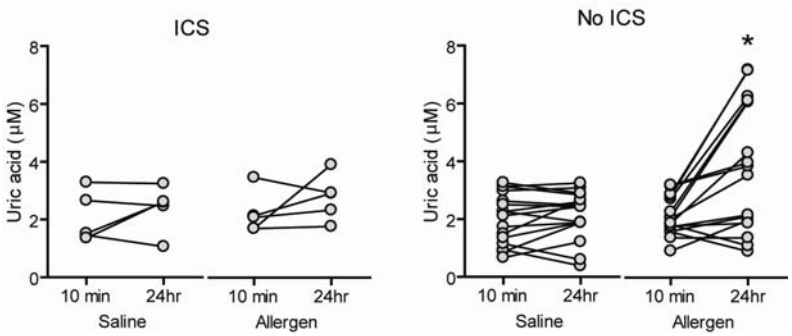

Figure S3: Uric acid levels are only elevated in patients not receiving inhaled corticosteroids.
Uric acid levels in BALf of asthmatic patients 10 min or 24h after segmental bronchial allergen or saline challenge, defined as patients on ICS (inhaled corticosteroids) prior to segmental challenge or patients not on ICS.

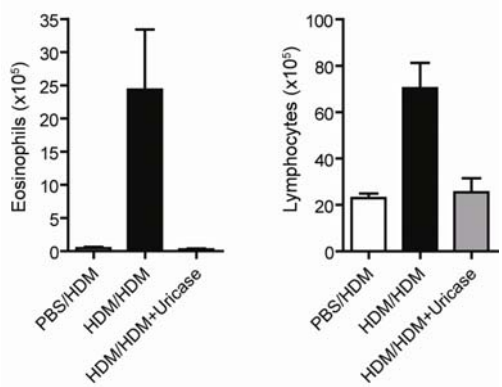

Figure S4: Uricase treatment during challenge in a milder HDM model.
Mice were sensitized i.n. on day 0 with PBS or 1 µg HDM. During challenge (day 7-11) with 10 µg HDM mice were treated with uricase. On day 14, BALf was examined for the presence of eosinophils and lymphocytes.

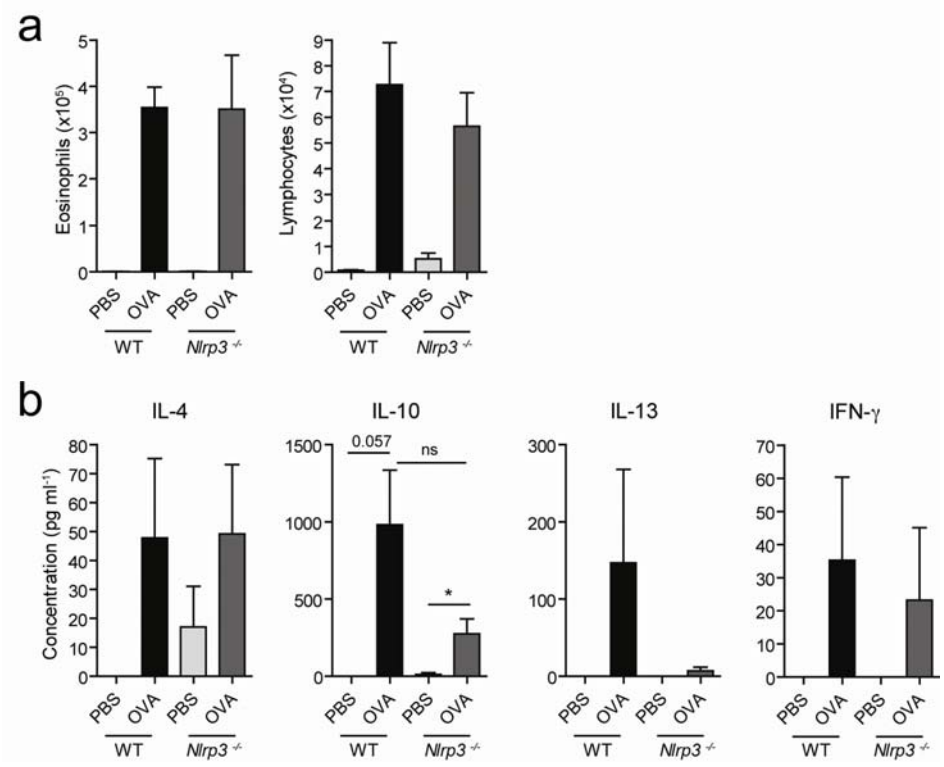

Figure S5: OVA-alum driven Th2 development in a model of asthma develops normally in *Nlrp3*$^{-/-}$ mice.

(a) WT C57BL/6 or *Nlrp3*$^{-/-}$ mice were immunized with OVA-alum i.p. on day 0. On day 7, mice were boosted with OVA i.p. and challenged with OVA aerosols on days 17-19. On day 20, BALf was analyzed for the presence of eosinophils and lymphocytes. **(b)** MLN cytokine production was measured as in Supplementary Fig. 2. Data are shown as mean ± SEM, * $p<0.05$, ** $p<0.01$, n=4-6 mice/group.

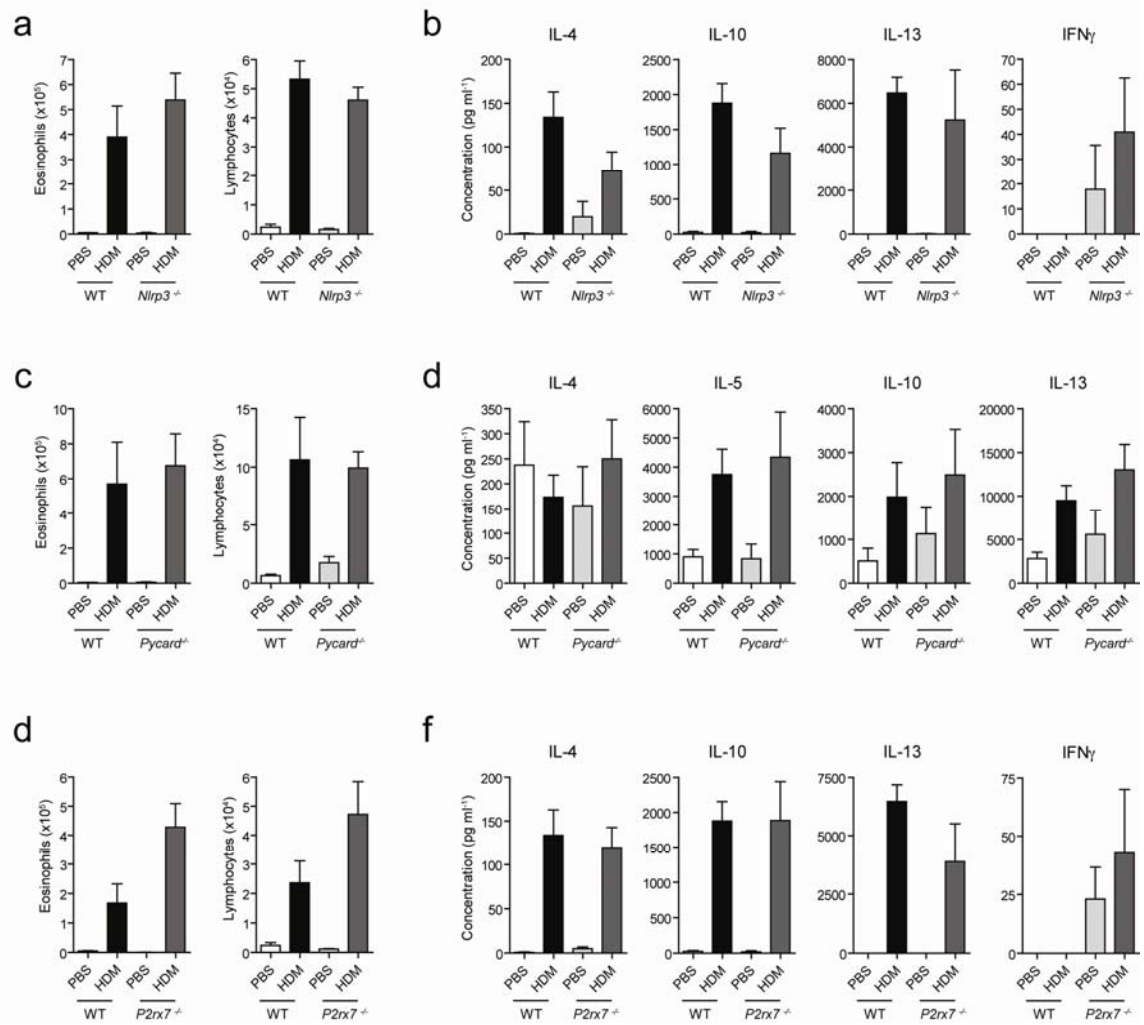

Figure S6: HDM driven asthma develops normally in *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, and *P2rx7*$^{-/-}$ mice

WT C57BL/6 or *Nlrp3*$^{-/-}$ **(a, b)**, *Pycard*$^{-/-}$ **(c,d)** or *P2rx7*$^{-/-}$ **(e,f)** mice were sensitized i.n on day 0 with PBS or HDM, and challenged i.n with HDM on days 7-11. On day 14, BALf was examined for the presence of eosinophils and lymphocytes **(a, c, e)**. MLN cytokine production was measured as in Supplementary Fig. 2 **(b,d,f).**

Table S1: Clinical characteristics of asthmatic patients

	Sex	Age (y)	FEV1 (% pred)	Total IgE (kU l^{-1})	Specific IgE (kU l^{-1})	Asthma duration (y)	Allergen	Allergen dose (AU)
1	f	23	114	246	5.5	17	Birch	110
2	f	20	112	2047	27.5	14	Birch	53
3	m	23	95	345	11.8	5	Birch	400
4	m	23	77	548	26.1	5	Birch	76
5	f	25	105	64	16.9	2	HDM	1.8
6	f	24	102	155	5.21	21	Rye	4.25
7	m	20	114	141	18.7	13	Rye	1150
8	f	28	105	22	6.85	10	Birch	64
9	m	34	114	172	30.7	10	Rye	46
10	m	24	94	368	66.3	17	HDM	49
11	m	24	105	104	8.3	4	HDM	71
12	f	22	110	134	39.6	2	Rye	340
13	m	24	97	1335	>100	16	Birch	123
14	m	23	107	622	46.8	4	HDM	2180
15	f	30	100	298	45.1	12	HDM	51
16	m	31	120	129	22.4	6	Birch	1450
17	m	27	83	430	44.8	20	HDM	5.3
18	m	39	97	770	29.7	26	HDM	2.86
19	m	25	112	57	14.7	13	HDM	1340
20	m	25	80	226	37.0	4	Birch	0.056
21	f	20	98	504	66.9	16	HDM	1460

The table displays sex and age (in years) of the patients, the prebronchodilator forced expiratory volume in the first second (FEV1, % predicted), the serum levels of total (normal range: < 100 kU l^{-1}) and allergen-specific (normal range: < 0.7 kU l^{-1}) Immunoglobulin E (IgE) levels, the asthma duration (in years), the allergen, and the dose (in allergen units, AU) used for segmental allergen challenge. Median age of the patients was 25 years old, range 20-31.