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The NLRP3 Inflammasome Inhibitor, OLT1177[®], Ameliorates Experimental Allergic Asthma in Mice

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To the Editor,

Despite differences, all types of asthma share a complex symptomatology that derives from chronic inflammation of the airways. Since a curative therapy is not yet available, standard of care treatment regimens aim to control symptoms by reducing this inflammation through inhaled corticosteroids. Though this approach has proved to be a reliable and effective treatment option in the majority of patients with mild-to-moderate asthma, patients with severe, neutrophilic, difficult-to-treat or uncontrolled asthma frequently experience acute exacerbations and loss of symptom control despite permanent corticosteroid treatment. A safe and more tolerable treatment option for these patients remains an unmet medical need.¹ Consequently, evaluating selective NLRP3 inhibitor, OLT1177[®] (dapansutrile) on the pathologic features of experimental allergic asthma (EAA) in different mouse models, became the focus of our studies.

Upon activation of the NOD-like Receptor Family Pyrin Domain Containing 3 (NLRP3), the formation of the NLRP3 inflammasome occurs, leading to the release of caspase 1-dependent pro-inflammatory cytokines interleukin (IL) 1 β and IL-18 as well as pyroptosis, which actually

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facilitates clearance of inhaled pathogens. However, overactivation of this axis has been implicated in the development and exacerbation of asthma.²

In our first model, intra-peritoneal treatment with 60 mg/kg body weight (bw) OLT1177[®] of mice with ovalbumin- (OVA-) induced asthma showed reduced NLRP3 expression and caspase-1 activation in lung tissue, and levels of activated IL-1 β in broncho-alveolar lavage fluid (BAL) fluid (Fig. 1A, B, G). All pathophysiologic hallmarks of this Th2-high, eosinophilic asthma endotype were also diminished, such as inflammatory cell counts in BALF and airway tissue, goblet cell hyperplasia, airway hyper-responsiveness (AHR), and BAL levels of T helper 2 (Th2) type (IL-4, IL-5, IL-13) and proinflammatory (IL-6, tumor necrosis factor [TNF]) cytokines (Fig. 1C-G).

Next we evaluated two models that mimic other asthma endotypes or diseases stages, namely HDM-induced EAA and poly(I:C)-triggered exacerbation of EAA. In mice with HDM-induced EAA i.p., OLT1177[®] diminished airway inflammation as evidenced by reduced eosinophil and neutrophil numbers in BAL and inflammatory cell infiltrate in lung tissue and significantly reduced AHR (Fig. 2A-C). Comparable effects of OLT1177[®] were also observed in mice undergoing the induction of an acute exacerbation of OVA-induced EAA.³ Consistent with the previous findings, OLT1177[®] treatment markedly reduced eosinophil and neutrophil numbers in BAL, inflammatory cell infiltrate in lung tissue and lowered AHR (Fig. 2D-F). Since drug administration requiring repeated injection is known to have low compliance in patients, we tested the effectiveness of OLT1177[®] in the oral route of administration in mice with OVA-induced EAA. OLT1177[®] delivered via enriched mouse feed reaching therapeutic exposure confirmed by pharmacokinetic evaluation (Suppl. Fig. 1A), demonstrated therapeutic benefit on the pathophysiologic hallmarks of EAA comparable to the findings using i.p. treatment (Fig. 2G-I).

During the study no signs of negative effects of OLT1177[®] were seen in any study animal. This is consistent with our previous findings from administration of OLT1177[®] in animals⁴, as well as the safety outcomes in all its six human trials to date, including in patients with acute gout flares⁵ or with heart failure⁶.

Taken together, we have shown that systemic treatment, both i.p. and orally, with OLT1177[®] is consistently bioactive and well-tolerated, inhibited NLRP3 and caspase-1 activity *in vivo*, reduced the release of proinflammatory IL-1 β and diminished the pathophysiological hallmarks of three

different mouse models of EAA. These findings not only suggest that NLRP3 is a therapeutic target in asthma, but also highlight the translational potential of OLT1177[®] to be tested in patients with asthma.

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Figure legends

Fig. 1: OLT1177 i.p. reduces NLRP3 inflammasome formation and caspase-1 activation and ameliorates allergic inflammation in OVA-induced EAA

(A), Amount of NLRP3 protein in lung tissue homogenate relative to β -actin of healthy (PBS), asthmatic (OVA+vehicle), or OLT1177-treated mice (OVA+OLT1177), n=8. (B), Caspase-1 activity. (C), Numbers of macrophages, lymphocytes, neutrophils, and eosinophils in BALF of healthy (PBS), asthmatic (OVA+vehicle) or OLT1177 treated mice (OVA+OLT1177), n=16. (D),

Inflammatory cell infiltrate in lung tissue per epithelial basal membrane (E.B.M.) area. **(E)**, Area of E.B.M. covered by goblet cells. **(F)**, Airway resistance in response to methacholine inhalation. **(G)**, Cytokine levels in BALF assessed by cytometric bead assay from healthy (PBS), asthmatic (OVA+vehicle) or OLT1177-treated mice (OVA+OLT1177), n=16. Results are presented as mean values $\pm$ SEM. Statistical significance was assessed using ordinary one-way ANOVA and Tukey's multiple comparison post hoc analyses, *P $\leq$ 0.1, **P $\leq$ 0.01, and ****P $\leq$ 0.0001, n.s. = not significant.

Fig. 2: OLT1177 i.p. ameliorates allergic inflammation in HDM-induced EAA and in poly(I:C)-induced exacerbation of EAA. Orally administered OLT1177 ameliorates allergic inflammation in OVA-induced EAA

(A), Numbers of macrophages, lymphocytes, neutrophils, and eosinophils in BALF of healthy (PBS), asthmatic (HDM+vehicle) or OLT1177-treated mice (HDM+OLT1177), n=8. **(B)**, Inflammatory cell infiltrate in lung tissue per E.B.M. area. **(C)**, Airway resistance in response to methacholine challenge. **(D)**, Numbers of macrophages, lymphocytes, neutrophils, and eosinophils in BALF of healthy (PBS), asthmatic (OVA+vehicle), exacerbated (OVA+poly(I:C)+vehicle), or OLT1177-treated mice (OVA+poly(I:C)+OLT1177), n=8. **(E)**, Inflammatory cell infiltrate in lung tissue per E.B.M. area. **(F)**, Airway resistance in response to methacholine inhalation. **(G)**, Numbers of macrophages, lymphocytes, neutrophils, and eosinophils in BALF of healthy (PBS), asthmatic (OVA+std.diet) or OLT1177-treated mice (OVA+OLT1177 diet), n=8. **(H)**, Inflammatory cell infiltrate in lung tissue per E.B.M. area. **(I)**, Airway resistance in response to methacholine challenge. Results are presented as mean values $\pm$ SEM. Statistical significance was assessed using ordinary one-way ANOVA and Tukey's multiple comparison post hoc analyses, *P $\leq$ 0.1, **P $\leq$ 0.01, and ****P $\leq$ 0.0001, n.s. = not significant.

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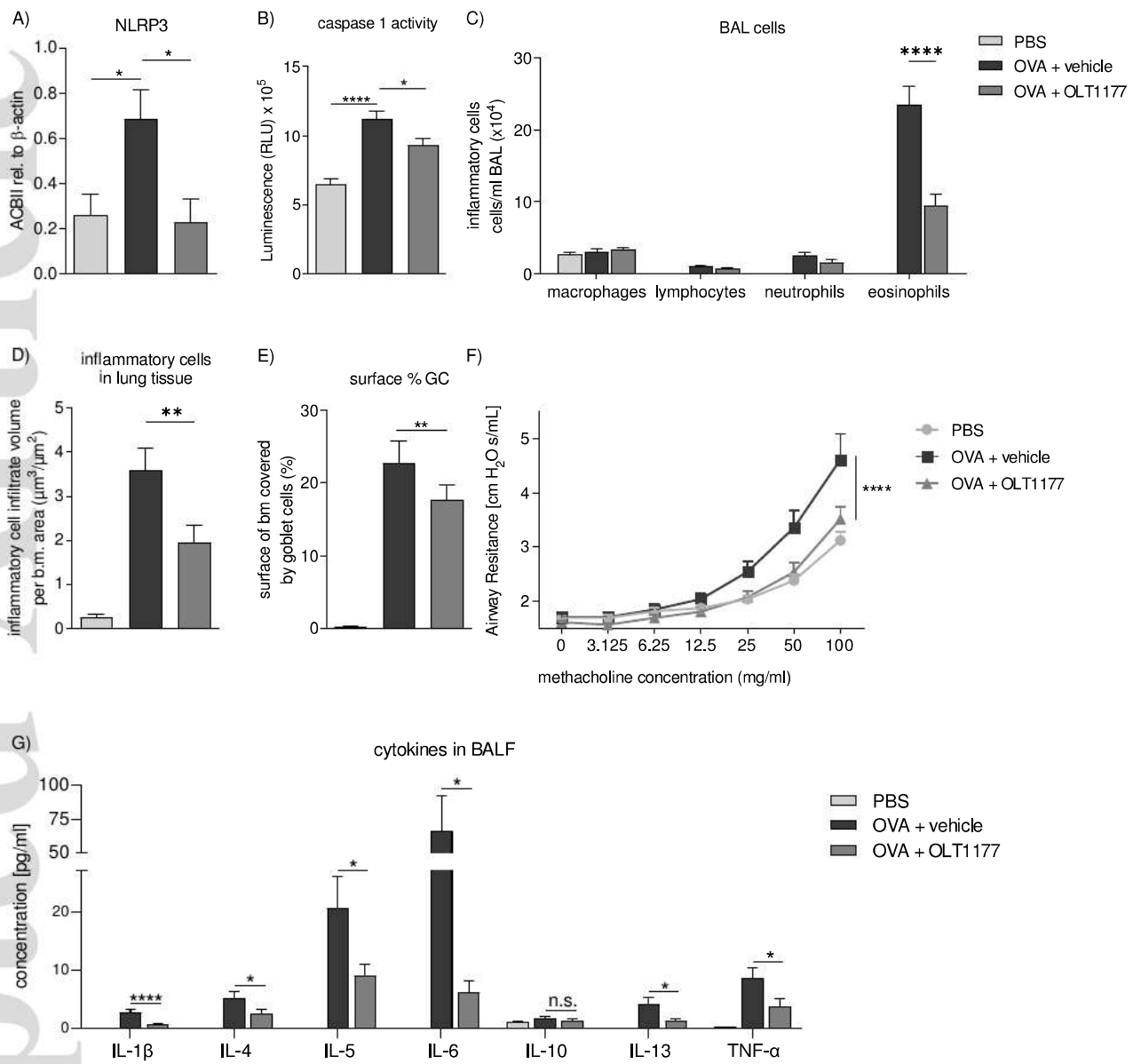
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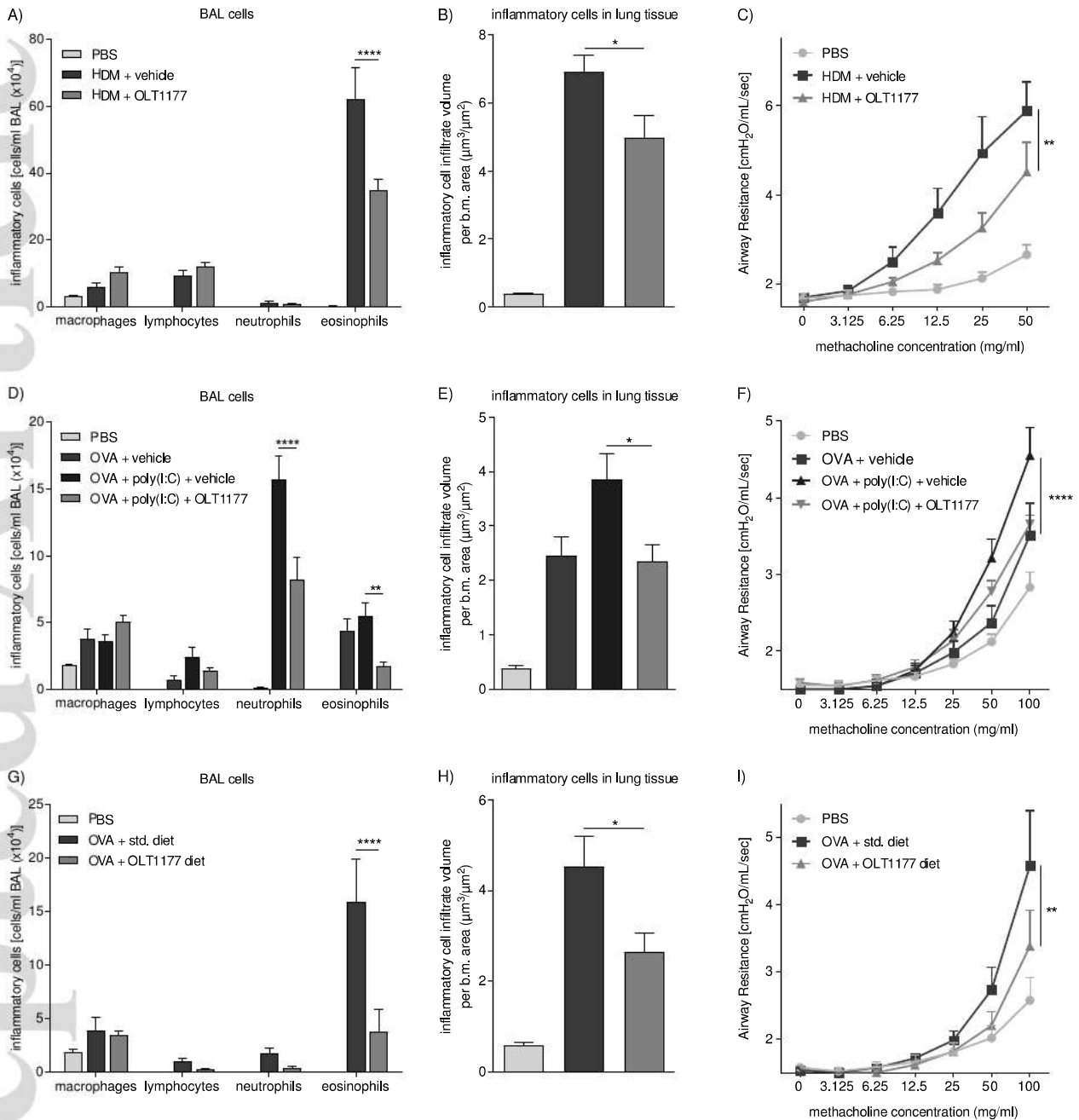
Conflict of interest:

L.P.L., C.V., and M.W. declare to have no conflict of interest. D.B.S. serves as Chairman and Chief Executive Officer of Olatec; C.A.D. serves as Chairman of Olatec's Scientific Advisory Board, is co-Chief Scientific Officer, and has equity in Olatec.



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Figure 2_Lunding et al.

The NLRP3 Inflammasome Inhibitor OLT1177[®] Ameliorates Experimental Allergic Asthma in Mice

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Additional background: With more than 300 million people affected globally, bronchial asthma is the most common chronic inflammatory disease.¹ Characterized by a variable degree of airway obstruction, airway hyperresponsiveness (AHR), productive cough, and respiratory distress, its complex symptomatology leads to the formation of a variety of different asthma pheno- or endotypes. Despite their differences, all types of asthma share a complex symptomatology that arises from a chronic inflammatory reaction in the airways.² Since a curative therapy is not yet available, standard of care treatment regimens aim to control symptoms by reducing local inflammation (e.g., inhaled corticosteroids [CS]).¹ Though this approach has proved to be a reliable and effective treatment option in the majority of patients with mild-to-moderate asthma, there remains a number of serious safety issues that limit its general use; long-term oral treatment of even moderate doses is associated with an increased risk for a plethora of considerable adverse effects including diabetes, osteoporosis, hypertension, thinning of skin and hair, glaucoma, gastritis and gastric ulcer formation.³ Especially patients with severe, neutrophilic, difficult-to-treat or uncontrolled asthma frequently experience acute exacerbations and loss of symptom control despite permanent CS treatment. This necessity of increased and systemic use of CS puts these patients at high risk for the development of the aforementioned side effects. Furthermore, a considerable number of these patients can be described as CS resistant.¹ Consequently, a safe and more tolerable treatment option for patients with bronchial asthma remains an unmet medical need. The NLR family pyrin domain containing protein 3 (NLRP3) inflammasome is a key component of the innate immune response. This high molecular weight protein complex plays a central role in many inflammatory processes since it initiates and regulates IL-1 β and IL-18, two cytokines for combatting infection and tissue damage.^{4,5} Recognition of pathogen-associated molecular patterns (PAMPs) during infection and danger-associated molecular patterns (DAMPs) by pattern-recognition receptors (PRRs) leads to the interaction of NLRP3 with apoptosis-associated speck-like protein containing (ASC) and procaspase-1 to form the inflammasome complex, which, in turn, activates caspase-1.^{4,5} This enzyme then converts pro-IL-1 β and pro-IL-18 into their biologically active forms with multifaceted proinflammatory properties.^{4,5} In fact, NLRP3 is expressed in primary human airway epithelial cells, macrophages, and neutrophils and increased expression of NLRP3 has been found in patients with neutrophil asthma.^{6,7} Furthermore, NLRP3 plays a significant role in the differentiation of T helper (TH) 2 cells and innate lymphoid tissue 3 cells since it e.g. binds to and transactivates the *IL4* promoter.^{8,9} This is of central importance to the pathogenesis of asthma since both cell types orchestrate the allergic immune response (e.g. IgE production, recruitment and maturation of eosinophils), mucus hyperproduction, and airway AHR, which in turn cause the symptoms of the disease.^{10,11} Serum levels of caspase-1 are also increased in patients with perennial allergic asthma.¹² The same is true for IL-1 β , since patients with asthma revealed high expression of IL-1 in bronchial epithelium and elevated IL-1 β levels in broncho-alveolar lavage fluid and serum.^{13–15} Originally studied driving TH1 cell differentiation, IL-18 is found in significantly higher levels in lung tissue, plasma, and sputum of patients with asthma compared to healthy individuals.^{16,17} Activation of NLRP3,¹⁸ or administration of IL-1 β ¹⁹ or IL-18,²⁰ enhances allergic airway inflammation and pathological features of experimental allergic asthma (EAA) in mice, while knock-out of NLRP3 or IL-1 or inhibition of caspase-1 or NLRP3 revealed opposite effects.^{21–26} Thus, we propose that specific blockade of NLRP3 may serve as a putative option for asthma therapy. OLT1177[®] (rINN: dapansutril) is a small molecule, β -sulfonyl nitrile that selectively inhibits the NLRP3 inflammasome.

OLT1177 is orally bioavailable and has been studied in safety and efficacy trials in humans at oral doses up to 2000 mg each day for 14 days, in which no significant clinical, hematological, or organ toxicities were observed.^{27–29}

Animals: Female, 6- to 8-wk-old C57BL/6 wildtype mice (Charles River Laboratories, Sulzfeld, Germany) were housed in single-ventilated cages under specific pathogen-free conditions. They received ovalbumin (OVA)-free diet and water *ad libitum*. All experiments were conducted in accordance with the German Animal Protection Law and were approved by the Animal Research Ethics Board of the Ministry of Environment of Schleswig-Holstein in Kiel, Germany (V241-38961/2017 (78-6/17)). All experiments were carried out under consideration of the national guidelines for animal treatment.

Animal treatment protocols: In order to induce ovalbumin- (OVA-) induced EAA, mice were sensitized to OVA by three intra-peritoneal (i.p.) injections of 10 µg OVA (OVA grade VI; Sigma-Aldrich, Deisenhofen, Germany) adsorbed to 150 µg aluminum hydroxide (Imject Alum Adjuvant; Thermo Fisher Scientific, Waltham, MA, USA) on days 1, 14, and 21. Acute allergic airway inflammation was elicited by three 20 minute exposures to an OVA (OVA grade V; Sigma-Aldrich) aerosol (1% w/v in PBS) on days 26, 27, and 28.³⁰ OLT1177 was administered intraperitoneally or orally. For the i.p. route, mice received OLT1177 (60 mg/kg body weight [bw]) i.p. on days 25, 26, 27, and 28. For oral administration, mice were fed fortified feed pellets containing 7.5 g OLT1177[®] per kg feed from day 22 to day 28. The dose level was selected to target daily exposure equivalent to the body weight (adjusted to a 25-gram mouse and an estimated food intake of 4 g daily per mouse). A control group received a matching unfortified diet with no OLT1177 in the food pellets (0 mg/kg OLT1177[®]). Mice were monitored during the study for any changes in physiological parameters including irregular weight gain or loss. Analyses were performed 24 hours after the last OVA challenge on day 29. Mice with OVA-induced EAA received 200 µg poly(I:C) (Sigma-Aldrich) dissolved in 50 µl sterile PBS intra-nasally under sevoflurane anesthesia 1 h after the last OVA aerosol challenge to trigger acute exacerbation of the disease.³¹ Control mice received PBS (i.p.), were challenged with OVA aerosol, and received PBS (i.n.). Analyses were performed 24 hours later on day 29. In order to elicit house dust mite- (HDM-) induced EAA, mice were sensitized to HDM by one intranasal (i.n.) application of 1 µg HDM extract (Greer Laboratories, Lenoir, NC, USA) in 40 µl PBS under sevoflurane anesthesia on day 1. Acute allergic airway inflammation was triggered by five consecutive i.n. challenges with 10 µg HDM extract (Greer Laboratories) in 40 µl PBS under sevoflurane anesthesia on days 8, 9, 10, 11, and 12. OLT1177[®] (60 mg/kg bw) was applied i.p. on days 8, 9, 10, 11, and 12. Control mice received PBS i.n. and i.p. Analyses were performed 48 hours later on day 14. All animals were sacrificed by cervical dislocation under deep anesthesia.

Assessment of airway responsiveness in response to methacholine: Airway reactivity was assessed by methacholine (MCh) provocation testing using invasive lung function assessment (FinePointe RC units; Data Sciences International, St. Paul, MN, USA) as previously described.³¹

Bronchoalveolar lavage and inflammatory cell count: Bronchoalveolar lavage (BAL) fluids were collected and lungs were isolated and instilled with 1 ml ice-cold PBS containing protease inhibitor (Complete, Roche, Basel, Switzerland) via a tracheal cannula. Total number of immune cells was determined using a Neubauer cell counting chamber. Aliquots of BAL fluids (50 µl) were cytopspinned and stained with Diff-Quick (Medion Diagnostics, Miami, FL, USA), and leucocyte subpopulations were identified by light microscopy using morphologic criteria.

Lung histology: Left lung lobe was dissected from mice and snap-frozen in liquid nitrogen. Remaining lung lobes histologically analyzed and mucus production was quantified as previously described.^{31,32}

Western blot and caspase-1 activity assay: Western blot analysis was performed on deep frozen lung tissue cells. Lung tissue was homogenized with mortar and pestle. Lung powder was transferred into RLT buffer and proteins were isolated, separated on SDS-PAGE (Mini Protean TGX Stain Free 12%; Biorad, Hercules, CA, USA), and transferred to PVDF membrane (mini 0.2 µM; Biorad).

Membranes were then incubated with anti-caspase-1 p20 (AdipoGen, San Diego, CA, USA), NLRP3 (R&D Systems, Minneapolis, MN, USA), or β -actin (mouse monoclonal, Sigma-Aldrich) at 1/1000, 1/250, and 1/1000 dilution, respectively. A 1/5000 dilution of HRP conjugated goat anti-mouse IgG1 (Abcam, Cambridge, UK), rat IgG (R&D Systems), and goat anti-rabbit IgG (AdipoGen) served as secondary antibodies. Immunoreactive proteins were visualized using the ECL western blotting detection system (ChemiDoc; Biorad). ACBII was calculated with ImageJ (NIH). Lung powder was transferred into PBS (50 mg/ml), and Caspase-1 activity in lung tissue isolates was measured using Caspase-Glo 1 Inflammasome Assay (Promega, Madison, WI, USA) according to the manufacturer's guidelines.

Assessment of cytokines in bronchoalveolar lavage fluid: Cytokine levels in BAL fluid were assessed using enhanced sensitivity cytometric bead array (Flex Set Kits; BD Biosciences, Franklin Lakes, NJ, USA) on a BD Accuri C6 flow cytometer (BD Bioscience) according to the manufacturer's guidelines. Analysis was performed using the FCAP Array™ v3.0 Software (BD Bioscience).

Isolation and stimulation of murine mononuclear cells from spleen: Spleens were isolated from OVA-sensitized mice and ground through a 40 μ m-cell strainer (BD Biosciences) followed by density gradient centrifugation using Lympholyte M (Cedarlane, Burlington, ON, Canada). Mononuclear cells (MNC) were subsequently washed, counted, and resuspended in DMEM containing 10% FCS and 1% Penicillin/Streptomycin. Spleen MNCs were stimulated with 50 μ g/mL OVA Grade V and 10 μ M OLT1177 (Olatec, New York, NY, USA) for 24 hours at 37°C. Cell-free supernatants were collected and analyzed for IL-1 β by Meso Scale Diagnostics (MSD, Rockville, MD, USA) according to manufacturer's protocol.

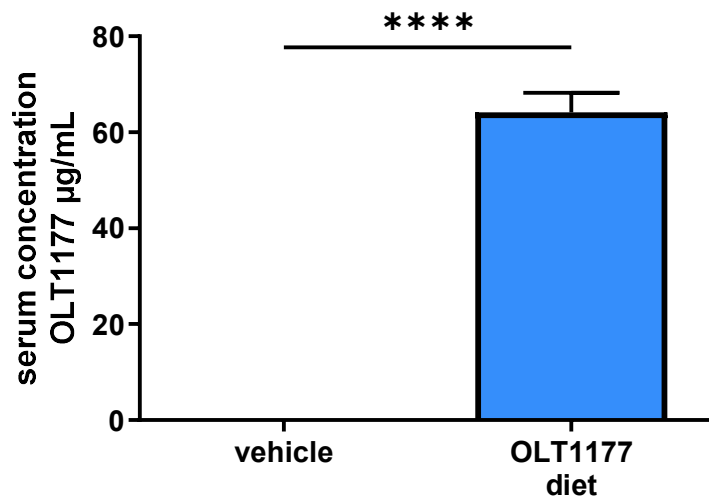
Statistical analysis: All data are presented as mean $\pm$ SEM. The results were analyzed using Student's *t* test or one-way ANOVA with subsequent Tukey's test in GraphPad Prism (GraphPad Software, San Diego, CA, USA). Levels of significance were indicated by **P* $\leq$ 0.05, ***P* $\leq$ 0.01, ****P* $\leq$ 0.001 and *****P* $\leq$ 0.0001. Animal groups consisted of n=8, if not stated otherwise.

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Supplementary Figure 1: Feeding of OLT1177 containing diet leads to high bioavailability of OLT1177 in serum

(A), comparison of serum concentration of OLT1177 in mice after feeding. Results are presented as mean values $\pm$ SEM. Statistical significance was assessed using unpaired t-test, **** $P \leq 0.0001$.