

ORIGINAL ARTICLE

Lipocalin2 protects against airway inflammation and hyperresponsiveness in a murine model of allergic airway disease

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Summary

Background Allergen-induced bronchial asthma is a chronic airway disease that involves the interplay of various genes with environmental factors triggering different inflammatory pathways.

Objective The aim of this study was to identify possible mediators of airway inflammation (AI) in a model of allergic AI via microarray comparisons and to analyse one of these mediators, Lipocalin2 (Lcn2), for its role in a murine model of allergic airway disease.

Methods Gene microarrays were used to identify genes with at least a twofold increase in gene expression in the lungs of two separate mouse strains with high and low allergic susceptibility, respectively. Validation of mRNA data was obtained by Western blotting, followed by functional analysis of one of the identified genes, Lcn2, in mice with targeted disruption of specific gene expression. Epithelial cell cultures were undertaken to define induction requirements and possible mechanistic basis of the results observed in the Lcn2 knock-out mice.

Results Lcn2 was up-regulated upon allergen sensitization and airway challenges in lung tissues of both mouse strains and retraced on the protein level in bronchoalveolar lavage fluids. Functional relevance was assessed in mice genetically deficient for Lcn2, which showed enhanced airway resistance and increased AI associated with decreased apoptosis of lung inflammatory cells, compared with wild-type controls. Similarly, application of Lcn2-blocking antibodies before airway challenges resulted in increased inflammation and reduced apoptosis.

Conclusion These data indicate a protective role for Lcn2 in allergic airway disease, suggesting a pro-apoptotic effect as the underlying mechanism.

Keywords adaptive immunity, airway inflammation, allergy, asthma, innate immunity

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Introduction

Allergic asthma is a polygenetic disease that unfolds through the interplay of various genes with environmental factors. Despite the identification of several proteins and pathways involved in this inflammatory process, the necessity for the identification of novel mediators to

improve current and develop new therapeutic strategies remains (reviewed in [1] and [2]). We therefore aimed to identify and validate previously unknown mediators of allergic airway inflammation (AI) in a murine asthma model. We used RNA microarray technology to identify mediators of inflammation whose contribution to allergic AI we assessed via consecutive *in vitro* and *in vivo* studies.

Several recent studies in humans and mice suggest that mechanisms of the innate immune system may play an

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important protective role in the development of atopic diseases [3, 4]. Our approach identified a known participant in innate immune mechanisms, Lipocalin2 (Lcn2) [5, 6], whose role in allergic AI has not been studied to a larger extent. We were able to delineate its possible protective role in a model of allergic AI and identified the possible mediators involved in its regulation in allergic AI.

Lcn2 is a 25 kDa proteins also termed neutrophil gelatinase-associated lipocalin (NGAL) (human and mice), oncogene 24p3 (human) or SIP24 (mice). Lcn2 belongs to the lipocalins, a family of proteins characterized by their common tertiary structure (reviewed in [7]). Lcn2 was originally described as an acute-phase protein in the liver [5] but is also up-regulated in macrophages after lipopolysaccharide (LPS) stimulation [8] and in the lungs of LPS-treated mice [9]. Additional studies have underlined the hypothesis that Lcn2's expression in the lung, a prime tissue for contact with microorganisms and its regulation by pro-inflammatory cytokines, suggests a role in anti-microbial defence and the regulation of inflammation and possibly cell growth [10] in the respiratory tract: human NGAL and mouse Lcn2 have been shown to be expressed in the trachea and lungs under non-inflammatory conditions [11]. Additionally, NGAL has been shown to be up-regulated in lung epithelium through inflammation, comprising expression in goblet cells and type II pneumocytes [12]. NGAL levels in induced sputum were elevated in COPD and asthmatic patients compared with healthy controls [13], where it apparently correlates with bronchial hyperresponsiveness [14]. Furthermore, experiments by Devireddy et al. [15] have shown a role for Lcn2 in the induction of apoptosis of an IL-3-dependent B cell line but also in primary thymocytes, splenocytes, BM cells, neutrophils and lymphocytes. More insight into Lcn2's role in innate immunity and its ligands, enterochelins and iron-chelating protein has been gained very elegantly by knock-out (KO) experiments. Mice lacking Lcn2 show a marked increase in mortality in an *Escherichia coli*-induced sepsis model due to an inability to prevent iron utilization of bacteria. Its expression has been shown to be LPS-induced in this system, pointing towards a connection between LPS-effects and Lcn2 [16], and its induction via IL-9 in lymphocytes points towards a role in diseases characterized by a T-helper type 2 (Th2) preponderance [17].

To further delineate the role of Lcn2 in the context of allergic diseases, we utilized *in vitro* cell cultures and *in vivo* murine models of acute and chronic allergic AI. Our results provide first-time evidence for the direct regulation of Lcn2 via both Th2 and Th1 cytokines and for the pro-apoptotic effects of Lcn2 on murine type II pneumocytes resulting in enhanced inflammation in Lcn KO mice. These data suggest that Lcn2 serves as a regulatory factor in AI by induction of apoptosis and warrant further research of putative preventive or therapeutic applications.

Methods

Animals

Specific-pathogen-free female BALB/c (Charles River, Sulzfeld, Germany) and Lcn2-KO mice [16], kindly provided as a gift from J. B. Cowland, 6–8 weeks of age, were used. Lcn2-KO mice were backcrossed for at least five generations on a C57BL/6 background, all mice were genotyped via tail PCR and WT littermates were used as controls. All experimental procedures were approved by the Berlin Office for health and social policy, Germany.

Sensitization and challenge protocols

Model of acute airway inflammation. Mice were sensitized by an intraperitoneal injection of 20 µg ovalbumin (OVA) (Sigma-Aldrich, München, Germany) in 2 mg of aluminium hydroxide (Sigma-Aldrich) on days 1 and 14. Airways were challenged by a single instillation of 50 µg OVA in 50 µL PBS at day 27 (gene expression experiments, Fig. 1a) or three times with OVA aerosol (1% in PBS) for

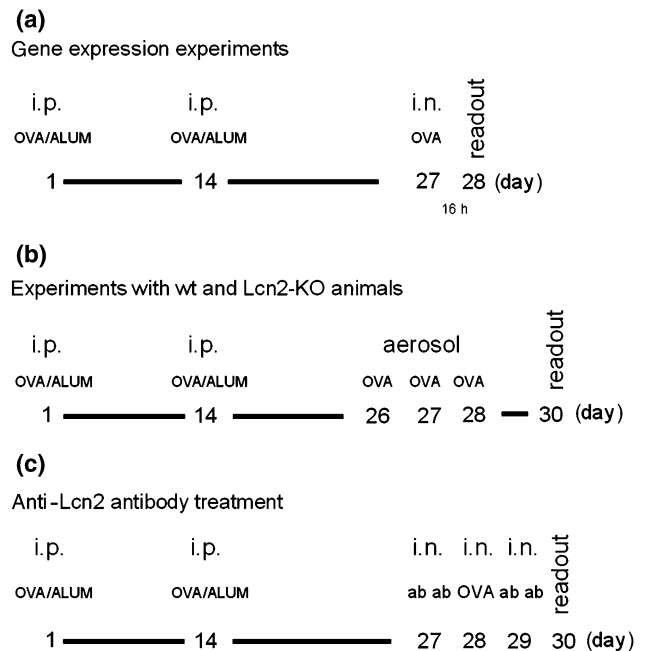


Fig. 1. Experimental protocols. Mice were sensitized by intraperitoneal (i.p.) injections of 20 µg (ovalbumin) OVA in 2 mg of aluminium hydroxide on days 1 and 14. Airways were challenged according to the following protocols: *gene expression experiments*: by a single instillation of 50 µg OVA at day 27, mice were killed 16 h after challenge (a); *knock-out (KO) experiments*: by OVA aerosol once daily at days 26, 27 and 28; mice were killed 48 h after the last challenge (b); *antibody experiments*: by a single instillation of 50 µg OVA at day 28, anti-mouse Lipocalin2 (Lcn2) antibody (ab) was applied 24 and 12 h before and after the challenge; mice were killed 48 h after the challenge (c). In all models, control animals received PBS challenges instead of OVA at the same time-points. As a control antibody, normal goat-IgG was used (c).

20 min at days 26, 27 and 28 (experiments with WT and Lcn2-KO animals, Fig. 1b). Control animals received PBS at the same time-points. Mice were killed 16 h (for array data and RT-PCR data in the validation of array data) or 48 h (all other data) after the last challenge.

Model of chronic airway inflammation. Sensitized mice as described above were repeatedly challenged with OVA aerosol (1% in PBS) on 2 consecutive days per week starting on day 27 for 9 weeks. Mice were analysed 48 h after the last challenge.

Antibody treatment

Anti-mouse NGAL (M-12) was used with an appropriate normal goat-IgG control (both Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Antibody was applied 24 and 12 h before and 12 and 24 h after a single airway challenge via intranasal instillation of OVA in a volume of 125 μ L (equalling 25 μ g of antibody/mouse) (Fig. 1c).

Detection of the allergic sensitization

Phenotypical analysis. Sensitization to OVA was verified by analysis of antibody levels and proliferation of spleen mononuclear cells in response to *in vitro* stimulation with OVA (data not shown). Serum levels of total IgE and OVA-specific IgE, AI in bronchoalveolar lavage (BAL) and non-invasive lung functions measurements were analysed as described previously [18]. Collagen content was determined using Sircol's collagen assay (Biocolor, Carrickfergus, UK) according to the manufacturer's manual.

Invasive lung function measurement in isolated perfused mouse lung

Mouse lungs were prepared, ventilated and analysed as described [19]. After a steady-state period of 30 min, MCh (Sigma-Aldrich) was administered to the perfusate for 30 s at 12-min intervals. Airway resistance values were determined at the end of the steady-state period, as well as at the maximum level of resistance increase. The change in airway resistance was expressed as fold airway resistance.

Preparation of RNA

Total RNA was prepared from mouse lungs and cells using the RNeasy isolation kit (Qiagen, Hilden, Germany). The lungs were perfused with ice-cold PBS to reduce contaminating inflammatory cell influx, snap frozen and homogenized before RNA extraction using a tissue homogenizer. The structural integrity of the RNA (RIN $\geq$ 8 were used) was monitored using an Agilent 2100 Bioanalyzer

(Agilent Technologies, Palo Alto, CA, USA). The A260 nm/280 nm ratios were between 1.8 and 2.0.

Microarray analysis

Total RNA from the lung was converted to cRNA and hybridized to MG-U74Av2 chips (Affymetrix, High Wycombe, UK) according to Affymetrix guidelines. Arrays were scanned at a 3- μ m resolution using a Genechip Scanner with confocal optics made for Affymetrix by Agilent (Böblingen, Germany). In total, 8 lungs were analysed, two treated mice vs. two controls in two different mouse strains (BALB/c and C57BL/6), respectively, 16 h after a single challenge (Fig. 1a). This comparison was repeated once at a different date. Affymetrix's Microarray Suite software 5.0 (MAS5), MicroDB and the Data Mining Tool were used to scan and analyse the relative abundance of each gene based on the intensity of the signal from each probe set. Arrays were scaled by MAS5 to a target value of 150 (scaling factor range 0.33–0.96). The data generated from different mouse strains were analysed separately. We excluded all genes with absent call ($P > 0.06$) in all 4 samples from the initial groups. We obtained a list of 7815 probe sets (BALB/c) (62.6% of the genes present on the MG-74Av2 array) and a list of 7290 probe sets (C57BL/6) (58.4% of the genes present on the MG-74Av2 array), respectively.

Two array (treated vs. control) comparisons were performed, allowing a total of four comparisons (2 $\times$ 2 matrix). For each comparison analysis, MAS5 generates a 'difference call' of no change, marginal increase/decrease or increase/decrease, respectively. Only those genes that were found to be similarly regulated in all four comparisons in two independent experiments were classified as differentially expressed genes. The MAS5 fold-change output of a comparison analysis is the signal log ratio (SLR), which is the fold change presented in log₂. The SLR was converted to a standard, linear scale and the mean fold change of all four comparisons was calculated. Gene categorization was based on the NetAffx database (<http://www.affymetrix.com/>). The output from the microarray analysis was merged with the Unigene or the GenBank descriptor and saved as an Excel data spreadsheet.

Real-time polymerase chain reaction

One hundred nanograms of total RNA was analysed per sample by one-Step SYBRgreen RT-PCR using an ABI real-time PCR System 7300 (Applied Biosystems, Darmstadt, Germany). The RT step was carried out at 48 °C for 30 min with MMLV-H RT (Promega, Mannheim, Germany); after that, the polymerase (AmpliTaQGold, Applied Biosystems) was activated and cDNA was denatured by a pre-incubation for 10 min at 95 °C; the template was amplified for 40 cycles of denaturation for 15 s at 95 °C,

annealing of primers and extension at 60 °C for 1 min. Fluorescence data were acquired during each extension phase. To determine the presence of contaminating genomic DNA, the RT was excluded as a negative control. Product identity was confirmed by electrophoresis. Specificity was confirmed by a dissociation stage, which was added to the thermal profile after the PCR run. Relative quantification was performed using the comparative $2^{-\Delta\Delta C_T}$ method. β -actin was used as a reference gene. List of primer pairs: β -actin (X03672) 5'-GTTTGAGACCTTCAACACCCCA-3' and 5'-CTGGTCTCCGTATGTCCTGT-3'; Lcn2 (NM_008491) 5'-GGCCTCAAGGACGACAACA-3' and 5'-CCACCCATTGAGTTGCAAT-3'; IL-4 (M25892) 5'-CGCCATGCACGGAGATG and 5'-CGAGCTCACTCTCTGTGGTGT-3'; IL-5 (X06270) 5'-AGCACAGTGGTGAAAGAGACCTT-3' and 5'-CATCGTCTCATTGCTGTCAACA-3'; IL-12 (M86671) 5'-GGTGCAAAGAAACATGGACTTG-3' and 5'-CACATGTCACTGCCCGAGAGT-3'.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blotting

BAL fluids were analysed by SDS-PAGE and Western blot. Lcn2 was detected by a primary anti-NGAL antibody (clone M-12, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a secondary HRP-conjugated antibody (1 : 10 000 dilution, Amersham Pharmacia Biotech, Freiburg, Germany)

Detection of apoptotic cells in lung tissue and murine airway epithelial cells

Murine LA-4 airway epithelial cells (murine alveolar type II cells, LGC Promochem GmbH, Mannheim, Germany) were grown to confluence and incubated with recombinant Lcn2 protein (5 μ M, generously provided as a gift by J. B. Cowland) for 24 h on cell sample slides. Slides of lung tissue sections or LA-4 cells were analysed using the *in situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's description. Quantification of apoptotic cells was performed via light microscopy pictures analysed by ImageJ software (<http://rsb.info.nih.gov/ij/>, National Institutes of Health, Bethesda, MD, USA). Apoptotic cells were discerned by setting a threshold for greyscale intensity determined individually for each slide as the overall greyscale intensity differed in the slides. Above the pre-determined greyscale, a cell was considered terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL⁺) in a defined area. ImageJ calculates the area of TUNEL⁺ cells, which was compared in wild-type (WT) vs. KO lung sections and airway epithelial cell cultures treated with Lcn2 vs. control cultures, respectively.

For cleaved caspase 3 (Asp175, 1 : 200, Cell Signaling, Danvers, MA, USA) labelling biotinylated donkey

anti-rabbit (Dianova, Hamburg, Germany) secondary antibody was used, followed by the streptavidin alkaline phosphatase kit (K5005, Dako, Glostrup, Denmark). Alkaline phosphatase was revealed by Fast Red as chromogen. Negative controls were performed by excluding the primary antibody.

Three sections per animal were chosen according to the size of the bronchi, which were to display medium- to small-sized bronchi as seen in the figures. These sections were evaluated (WT, $n \geq 4$ animals/group, KO, $n = 5$ /group) with five vision fields/section being analysed. For cell culture analysis, three slides were analysed/group (Lcn2 treated vs. sham treated) by an observer blinded to the groups.

Stimulation of LA-4 cell line via different pro-inflammatory cytokines

LA-4 cells were grown to confluence and stimulated with different concentrations of IL-1 β , IL-4, IL-5, IL-10, IL-12p70, IL-13, IFN- γ or TNF- α (all BD Pharmingen, San Jose, CA, USA; LPS content < 0.1 ng/ μ g of cytokine as per the manufacturer, except for IL-1 β with no given information) for 2 or 4 h in 24-well plates. Treatment conditions were set up in triplicate, analysed independently and then combined for error bars. For LPS (Sigma-Adrich) stimulation, cells were stimulated for 12 and 48 h. After stimulation, cells were lysed and total RNA was prepared.

Statistical analysis

Data pertaining to the allergic phenotype were analysed statistically using the Mann-Whitney *U*-test to test for statistic significance and standard deviations (SD).

Microarray data were tested using a non-parametric statistical test (Wilcoxon's signed-rank test). The numbers of animals/group are given in the results section and the figure legends. Unless indicated otherwise, three independent experiments were performed and one representative experiment is shown to represent the results in the figures.

Results

Kinetics of airway inflammation

At 16 h post-challenge (when lungs were obtained for gene expression profiles, Fig. 1a), BAL fluid of BALB/c mice ($n = 5$ /group) contained increased numbers of neutrophils (2.2×10^3 /mL bronchoalveolar lavage fluid in PBS-treated animals vs. 258×10^3 in OVA-sensitized and challenged), but virtually no eosinophils. At this time-point, airway hyperreactivity had not yet developed (data not shown). Forty-eight hours after the last allergen airway challenge (protocol as per Fig. 1b), the BAL showed

lymphocytic (11×10^3 vs. $0.07 \text{ L} \times 10^3/\text{mL}$) and eosinophilic AI (50.4×10^3 vs. $0.1 \times 10^3/\text{mL}$, sensitized and challenged vs. controls).

Identification of the common signature gene of airway inflammation

RNA isolated from whole-lung tissue was used to generate Affymetrix-based gene expression profiles. Lung tissue was obtained at 16 h after a single allergen airway challenge to analyse genes at an early time-point of AI. As shown above 'Kinetics of AI', at this time-point, neither the classical eosinophilic AI nor AHR have developed. We postulated that by investigating this early time-point, we would be able to identify genes involved in the development of the characteristic Th2 phenotype of this model. Gene expression was compared between BALB/c mice and C57BL/6 mice because of their known differences in the development of AI and AHR [20]. While both strains develop significant AI, AHR and systemic sensitization parameters such as allergen-specific IgE are much more pronounced in the BALB/c strain. We postulated that the comparison of these two strains would strengthen our aim ('two identify signature genes of AI') compared with an approach utilizing only one mouse strain, by increasing the probability of identifying genes truly relevant in the development of allergic AI. In total, 8 lungs were analysed, two treated mice vs. two controls in two different mouse strains (BALB/c and C57BL/6), respectively. This comparison was repeated once at a different date. OVA-sensitized and -challenged C57BL/6 mice had an altered expression of 370 probe sets compared with the naive control mice, whereas OVA-challenged BALB/c mice had 2128 probe sets altered in their expression levels. Between these two sets, 95 probe sets were consistently up-regulated in both sensitized and challenged BALB/c and C57BL/6 mice, but only 31 probe sets coding for 27 different genes were up-regulated at least twofold in both mouse strains (Table 1). Among those genes that were up-regulated at least twofold in both mouse strains, we chose to validate the up-regulation of Lcn2. Lcn2's known link to innate immune mechanisms provided an interesting aspect for a gene up-regulated at an early time-point of AI where innate immune signals contribute significantly to the induction of the allergic immune response and its role in allergic AI had not yet been delineated in depth, thus suggesting an interesting candidate for consecutive functional analyses.

Lipocalin2 is up-regulated in response to acute and chronic allergen challenge in mouse models of airway inflammation

As the sample sizes of our array data were insufficient for statistical analysis, we chose to validate the data

obtained by quantitative RT-PCR in larger sample sizes and repetitive experiments. Quantitative RT-PCR supported the results obtained from the microarray analysis, showing a 2.7-fold induction of Lcn2 mRNA expression in BALB/c ($n = 10/\text{group}$) and a 3.3-fold induction in C57BL/6 ($n = 10/\text{group}$) when comparing sham-treated (PBS) with sensitized and challenged mice (OVA) 16 h after a single challenge in the acute model (Fig. 2a). Increased Lcn2-expression was also detected in a model of chronic AI 48 h after the last challenge after 9 weeks of repeated allergen exposure ($n = 10/\text{group}$), suggesting that Lcn2 may play a role in chronic processes of airway remodelling (Fig. 2b) as well. In this model, OVA-specific IgE reaches a plateau after eight weeks of OVA treatment (supporting information Fig. S1), while inflammatory cell influx and AHR taper off after 9 weeks of treatment (supporting information Fig. S1). The pulmonary collagen content remains elevated at 12 weeks (supporting information Fig. S1), modelling one aspect of the remodelling process found in chronic asthma.

Western blotting of BAL fluids showed that increased mRNA expression was translated into increased production of secreted protein in the BALB/c mouse strain ($n = 2/\text{group}$) 48 h after the last challenge in the acute model (Fig. 2c).

Lipocalin2 deficiency enhances allergic airway inflammation

In order to evaluate the functional significance of Lcn2, we evaluated the consequences of Lcn2 absence or blockade in the context of acute allergen-induced AI by utilizing mice genetically deficient for Lcn2 expression [16] or Lcn2-specific antibodies. We chose to evaluate the effects of Lcn2 deficiency after three allergen challenges. At this time-point, AI of this acute model reaches its maximum. We postulated that if we were to observe differences between WT and KO due to differences in Lcn2 expression leading to changes in the pathways involved in the development of AI, these would be most pronounced at this time-point. As shown in Fig. 3, a direct comparison of Lcn2-KO ($n = 8$) mice with WT ($n = 11$) littermates revealed higher levels of lymphocytes and eosinophils in Lcn2-KO mice (Fig. 3a), whereas OVA-specific Total IgE levels remained similar in both strains (Figs 3b and c). Similar results of enhanced AI were obtained when anti-Lcn2 antibodies were administered to sensitized WT mice before allergen airway challenges, indicating that the results in the KO mice were not due to developmental defects in the lungs or the immune system and not unique to the C57BL/6 background strain of the KO animals (Fig. 3e, anti-Lcn2, $n = 5$, sham-treated, $n = 5$).

In order to evaluate the functional consequences of Lcn2 deficiency, we analysed airway resistance after methacholine provocation in isolated and perfused lungs

Table 1. List of up-regulated genes after ovalbumin (OVA) challenge

Identifier	Gene title	Gene symbol	BALB/c (fold change)	C57BL/6 (fold change)
EDL04793	Arginase 1, liver	Arg1	66.2±15.8	13.4±1.6
BE200119	Small chemokine (C–C motif) ligand 11	Ccl11	14.2±6.5	2.8±0.4
BC145867	Chemokine (C–C motif) ligand 2	Ccl2	9.9±0.4	11.9±1.9
L04694	Chemokine (C–C motif) ligand 7	Ccl7	39.4±6.2	20.4±7.6
AAF22536	Chemokine (C–C motif) ligand 9	Ccl9	3.5±0.6	2.8±0.2
AAH39919	Cholesterol 25-hydroxylase	Ch25h	3.8±0.2	3.3±0.5
U56900	Chitinase 3-like 3	Chi3l3	4.8±0.5	2.6±0.4
BC011134	Chitinase, acidic	Chia	35.8±13.9	3.2±0.1
BC116319	Chloride channel calcium activated 3	Clca3	65.8±35.0	32.5±1.1
AAD15798	Cytokine-responsive protein CR6	CR6	4.0±0.7	2.1±0.1
AK180750	Esterase D/formylglutathione hydrolase	Esd	2.4±0.1	2.5±0.2
AK170282	Coagulation factor X	F10	2.3±0.4	2.3±0.3
BG087011	Glycine amidinotransferase	Gatm	2.4±0.2	2.7±0.5
BC010823	Glutathione peroxidase 2	Gpx2	7.9±2.9	2.6±0.2
BAE27469	Glutathione S-transferase ω 1	Gsto1	4.0±0.3	2.5±0.2
AK085689	Potassium inwardly rectifying channel	Kcnj15	180.3±54.7	2.1±0.3
<u>AAI32070</u>	<u>Lipocalin 2</u>	<u>Lcn2</u>	<u>9.8±0.5</u>	<u>3.4±0.3</u>
BAE42486	Matrix metalloproteinase 12	Mmp12	4.2±0.8	4.5±1.1
AAH61139	Regenerating islet-derived 3 gamma	Reg3g	45.7±15.7	2.6±0.3
AAH55885	Serum amyloid A 3	Saa3	10.9±0.6	57.1±6.9
AAA40130	Serine peptidase inhibitor, member 3N	Serpina3n	5.8±0.6	3.3±0.5
AF208031	Solute carrier family 5, member 1	Slc5a1	19.7±17.4	2.7±0.2
CAM19105	Suppressor of cytokine signalling 3	Socs3	2.3±0.1	2.1±0.3
AK007633	Small proline-rich protein 2A	Sprp2a	8.9±1.8	2.5±0.7
AK007296	Sulfiredoxin 1 homologue	Srxn1	9.4±3.0	2.1±0.1
BC008107	Tissue inhibitor of metalloproteinase 1	Timp1	5.7±1.2	2.5±0.3
BC004676	Upstream binding transcription factor	Ubtf	5.3±0.7	2.3±0.1

The lungs of two different mouse strains (BALB/c and C57BL/6) were analysed in sensitized vs. control mice, 16 h after a single allergen airway challenge (as shown in Fig. 1a). Lipocalin 2, the gene of interest, is underlined.

from WT and KO. Here, we observed significantly increased airway resistance in sensitized and challenged KO mice in direct comparison with corresponding WT mice (Fig. 3d, WT, $n = 6$, KO, $n = 6$).

Lipocalin2 deficiency reduces apoptosis in vivo

As Lcn2 has been described to induce apoptosis [15], a mechanism with an interesting role in allergic AI [21, 22], we compared TUNEL-staining of lung tissues from Lcn2-KO mice ($n = 5$) subjected to allergen sensitization and challenge to samples of lung tissue from WT littermates ($n = 4$). TUNEL⁺ cells occurred at a significantly higher frequency in WT mice than in the Lcn2-KO mice with localization to epithelial and peri-epithelial areas (Fig. 4a), suggesting direct staining of epithelial cells and/or of the peribronchial leucocyte infiltrates. Results from the TUNEL assay were supported by cleaved caspase 3 staining indicating apoptotic and not necrotic effects in the analysed lung tissues (Fig. 4b).

Quantitative morphometry revealed that the lack of Lcn2 (KO) resulted in an approximately threefold reduc-

tion in TUNEL⁺ cells compared with WT mice (Fig. 4c). These results suggested a protective role for Lcn2 in allergic AI, possibly conferred through a pro-apoptotic effect.

Lipocalin2 induces apoptosis in murine epithelial cells in vitro

In the consecutive step, we investigated whether this pro-apoptotic effect *in vivo* might be due to a pro-apoptotic activity of Lcn2 on murine pulmonary epithelial cells. To this end, LA-4 cells, a murine type II pneumocyte cell line, were treated with recombinant Lcn2 received as a gift from our cooperation partner J. B. Cowland [23] for 24 h, and TUNEL staining was used to detect DNA fragmentation accompanying apoptosis. Comparing Lcn2-treated with untreated control LA-4 cells, we observed a significant, almost twofold increase in the apoptosis counts (Fig. 5). Three slides were analysed/group (Lcn2 treated vs. sham treated) by an observer blinded to the groups and three independent repeats were performed of this experiment.

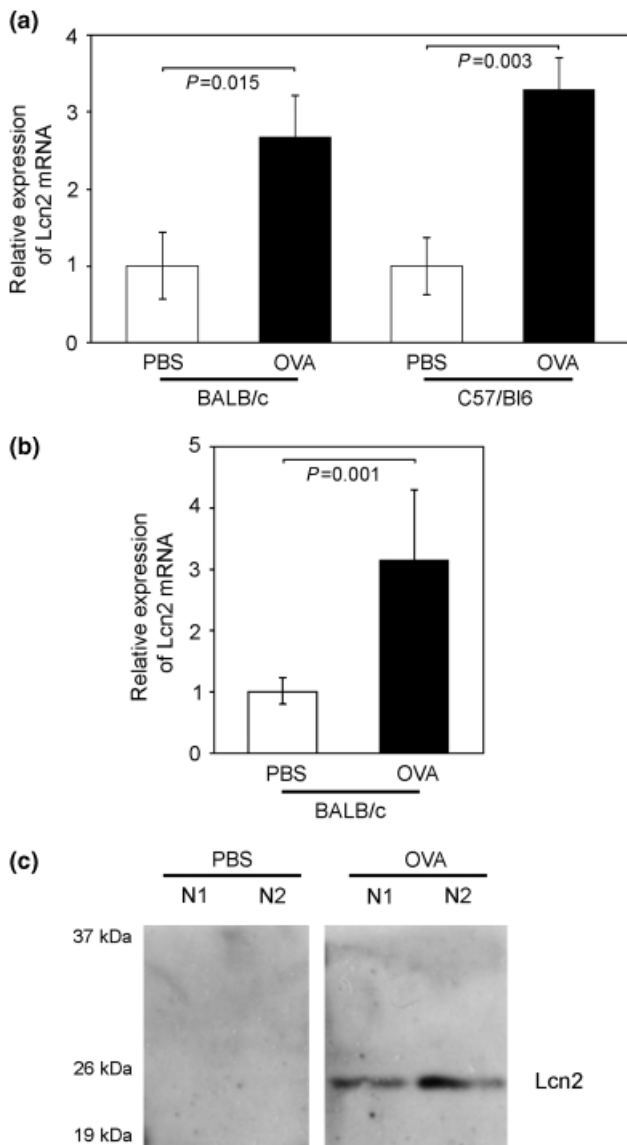


Fig. 2. Lipocalin2 (Lcn2) is up-regulated in response to airway inflammation in mouse models of acute and chronic allergen challenge. Comparison of mRNA levels (mean $\pm$ SD) in lung tissues of allergic ovalbumin (OVA, $n = 10$) and sham-treated animals (PBS, $n = 10$) revealed higher expression in allergic mice in the model of acute (a) and chronic airway allergen challenge (b) (for a description of the protocols, see Fig. 1). Lcn2 protein levels in bronchoalveolar lavage fluid of allergic BALB/c mice (OVA) were increased compared with the levels in sham-treated animals (PBS) (acute model) (c).

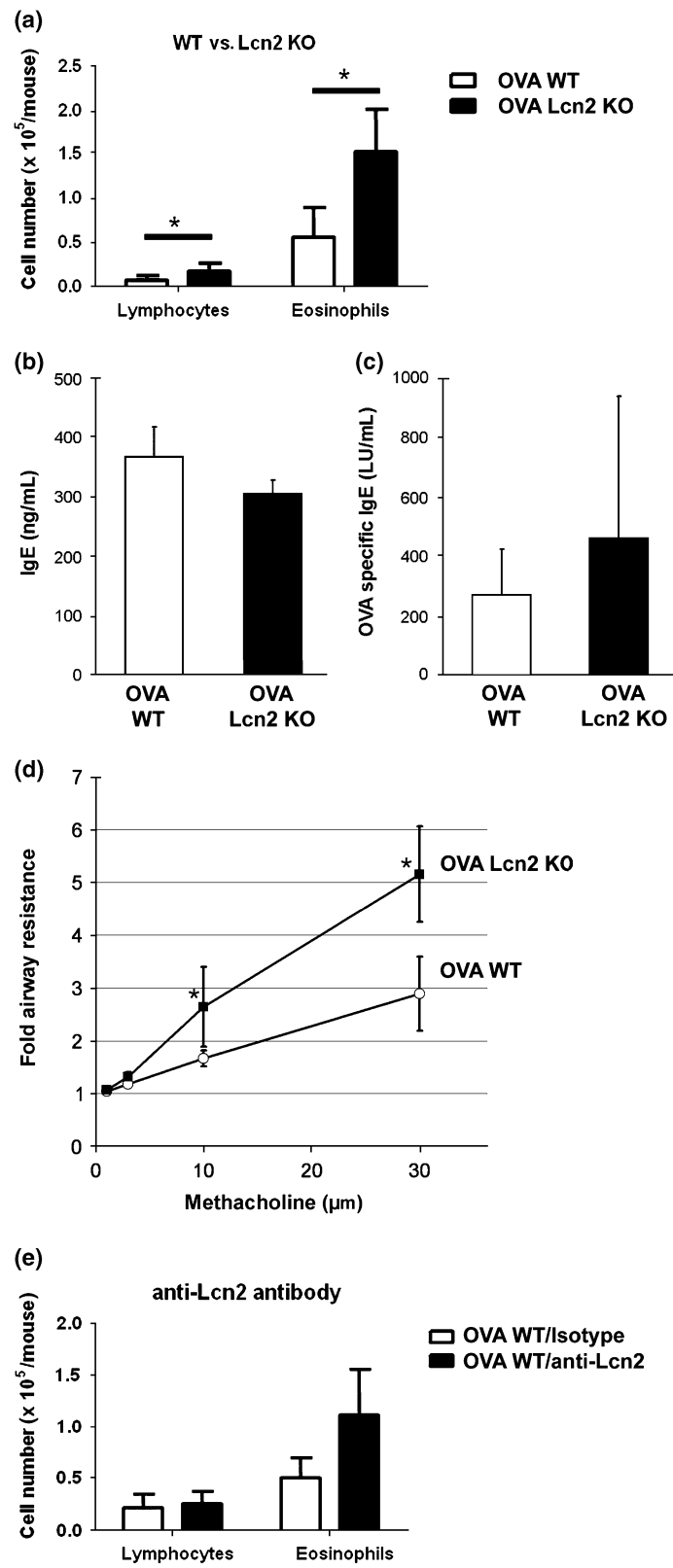
Lipocalin2 is regulated in murine epithelial cells via different pro-inflammatory signals

To investigate which cytokines were responsible for the up-regulation of Lcn2 production by murine type II pneumocytes in the course of allergic AI, we measured Lcn2 mRNA expression 2 and 4 h after treatment of LA-4 cells with different cytokines (Fig. 6). We chose to depict the results for IL-1 β as a positive control because it is

known to induce Lcn2 [12]. Additionally, the results for two cytokines known to be highly secreted in models of TH2-polarized allergic AI (TNF- α [24]) and IL-4 [25] and a classical Th1 cytokine (IL-12) as an antagonist of allergic AI are shown. Additionally, we tested the effects of IFN- γ , IL-5, -10 and -13 but these did not induce the up-regulation of Lcn2 (data not shown). Finally, we demonstrate the effect of LPS on the secretion of Lcn2, because it has been described to contribute to allergic AI under certain circumstances via the contamination of the model allergen OVA, which we also used [24]. Treatment conditions were set up in triplicate, analysed independently and then combined for error bars. Similar to data by Cowland et al. [12], we observed a dose-dependent up-regulation of Lcn2 by IL-1 β (Fig. 6a). Moreover, TNF- α (Fig. 6b), IL-4 (Fig. 6c) and IL-12 (Fig. 6d) induced significant Lcn2 transcription. Given the known contamination of commercially available OVA with LPS [24], we were interested in analysing whether induction of Lcn2 in epithelial cells is a possible consequence of LPS. Treatment of LA-4 cells with LPS led to a significant up-regulation of Lcn2 message in a dose-dependent fashion (Fig. 6e). In summary, these results indicate that several cytokines and inflammatory markers may induce Lcn2 production, suggesting that Lcn2 expression critically depends on the micro-milieu during an inflammatory process.

Discussion

Utilizing microarray technology in a murine asthma model, we compared the expression profiles in two different mouse strains and identified common inflammatory genes, among these Lcn2. We further investigated the functional role of Lcn2 in allergic airway disease because its contribution to this kind of disorder had not been analysed in detail. Here, we present data providing support for a role of Lcn2 in the development of allergic AI. Our results show that the inflammatory response in the murine model of allergic airway disease was associated with significant up-regulation of Lcn2 at mRNA and protein levels in lung tissues and BAL fluid. This up-regulation was found not only in the early stages of acute AI (acute model) but also after chronic allergen exposure of sensitized animals (chronic model), suggesting that Lcn2 might also play a role in sustained alterations linked to chronic asthma such as repair and remodelling. The functional relevance of Lcn2 was supported by experiments utilizing genetically deficient animals and antibody blocking studies, which showed that airway resistance and AI were significantly increased when Lcn2 is lacking during the development of allergic AI. We further showed that Lcn2 is produced by murine alveolar type II cells in response to various pro-inflammatory cytokines and LPS and exerts pro-apoptotic effects on pulmonary cells *in vitro* and *in vivo*. In conclusion, these



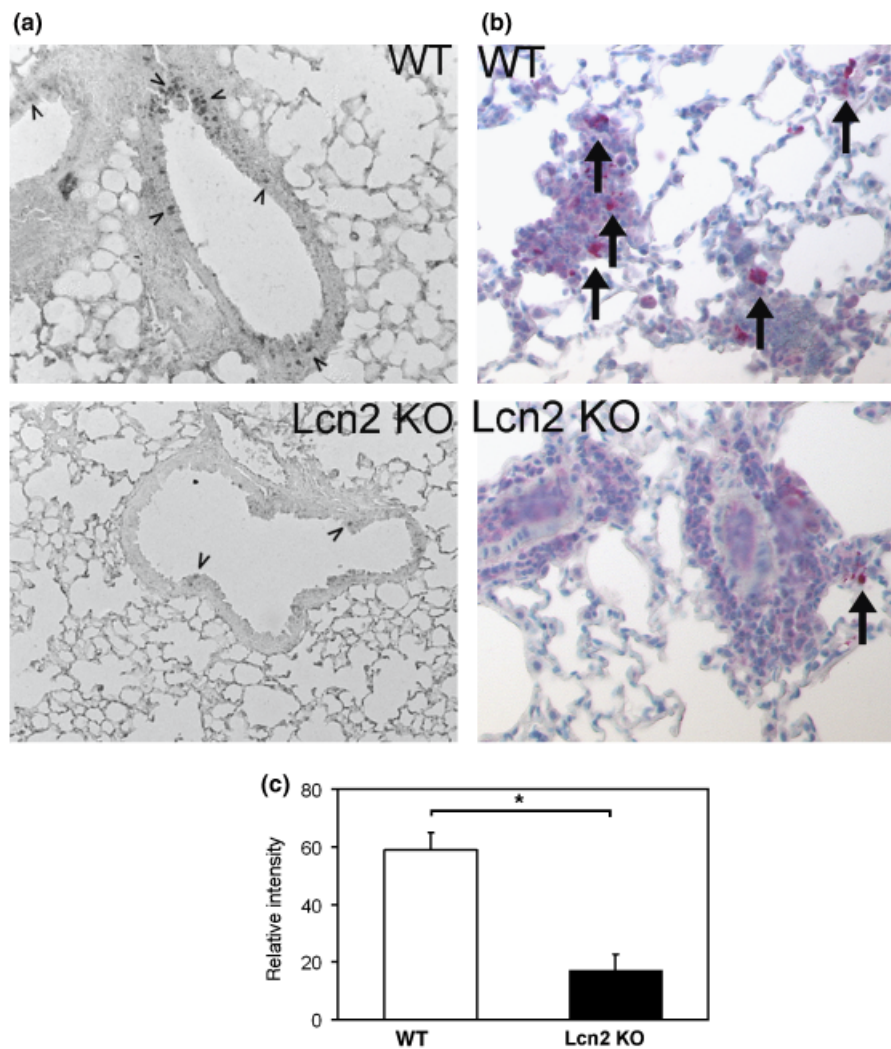


Fig. 4. Lipocalin2 (Lcn2) deficiency reduces pulmonary apoptosis in *in vivo*. In the acute model of airway inflammation (AI) (see Fig. 1 for details), TUNEL⁺ cells (a) as well as caspase 3⁺ cells (b) occurred at a higher frequency in lung tissues of wild-type (WT; $n = 4$) mice compared with Lcn2-deficient mice knock out (KO, $n = 5$) with staining concentrating in (peri)-epithelial areas. Statistical analysis of the TUNEL⁺ areas showed a significant reduction of apoptosis in KO compared with WT mice (c). * P -value of $P < 0.05$. Time-points for analyses were identical to Fig. 2. TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

data suggest a protective effect of Lcn2 in the development of airway hyperreactivity and inflammation.

The finding of increased Lcn2 production by epithelial cells after stimulation with IL-1 β , TNF- α and IL-4 does not fully correspond to previous publications from human cells [12]. This might be best explained by differences in the experimental design and/or species [23]. Especially,

IL-4-dependent up-regulation of Lcn2 mRNA is of interest and warrants further investigation. Given the pivotal role of this cytokine in the early phases of allergen-induced AI [25], our results indicate that the pro-inflammatory milieu found at the early stages of AI – but also the exaggerated Th2 environment governed by IL-4 – may be crucial for the up-regulation of Lcn2 in our model, reflecting some

Fig. 3. Lipocalin2 (Lcn2) deficiency augments the asthmatic phenotype in the model of acute airway inflammation. Compared with wild-type (WT) littermates (WT, $n = 11$), Lcn2-deficient mice knock out (KO, $n = 8$) showed increased airway inflammation, due to an increased influx of eosinophils and lymphocytes after sensitization and challenge with antigen. (a) Total IgE (b) and OVA-specific (c) IgE levels were similar in both strains. Airway resistance in isolated perfused lungs of Lcn2-deficient mice (KO, $n = 6$) was significantly higher in KO compared with WT mice ($n = 6$) after sensitization and challenge (d). Treatment of BALB/c mice with anti-Lcn antibody ($n = 6$) resulted in higher numbers of eosinophils and lymphocytes compared with sham-treated controls ($n = 6$) (e). * $P < 0.05$. Analyses were performed 48 h after the first airway challenge in the acute model, as depicted in Fig. 1b.

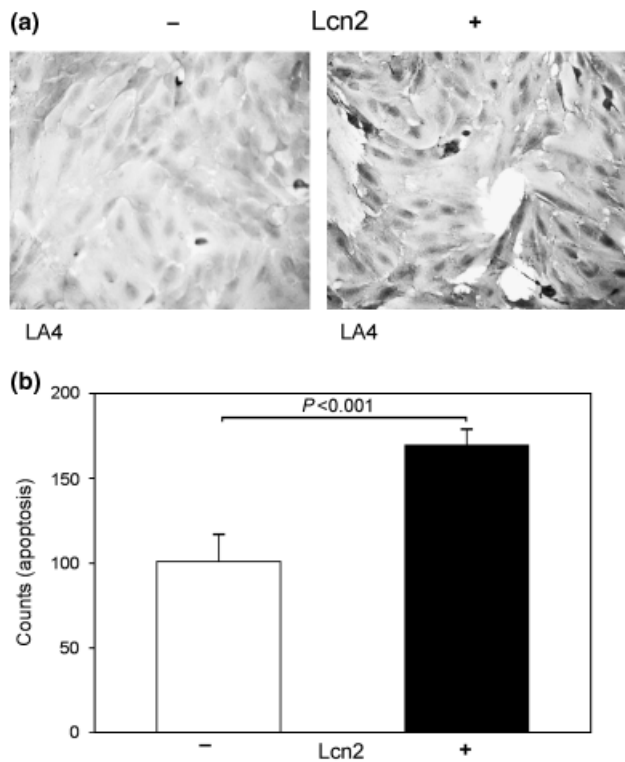
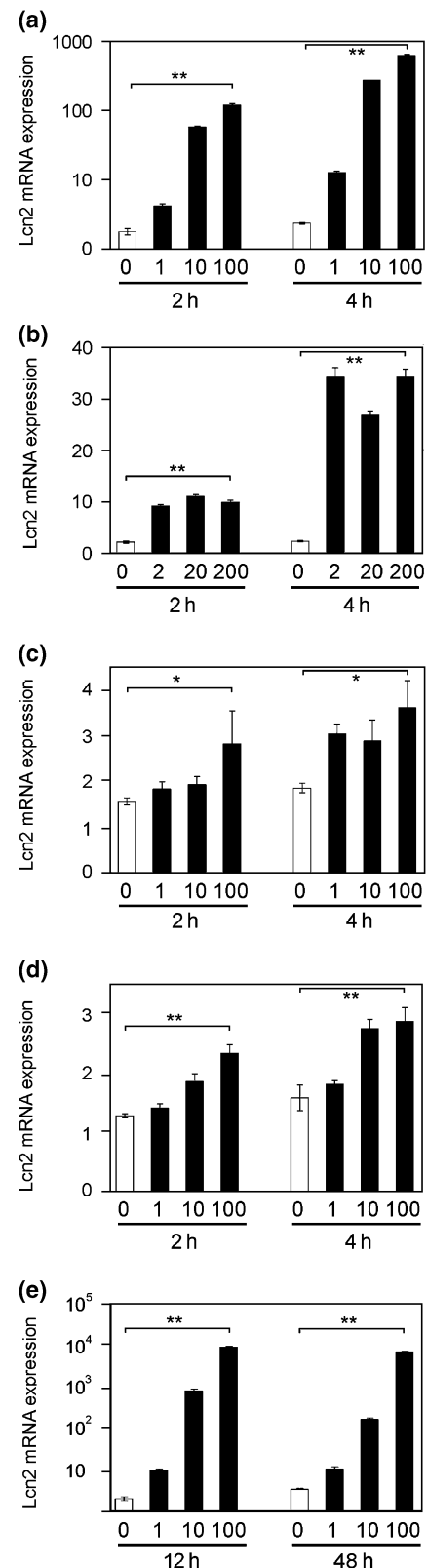


Fig. 5. Lipocalin2 (Lcn2) induces apoptosis in murine epithelial cells *in vitro*. LA-4 cells were treated with Lcn2 for 24 h, and TUNEL⁺ cells were compared with untreated controls (a). Statistical analysis of the TUNEL⁺ areas showed significantly increased apoptosis in LA-4 cells compared with untreated controls (b). Three slides per group were analysed by an observer blinded to the groups; the experiment was repeated three times. TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

kind of inhibitory feedback mechanism for self-limitation of allergen-induced AI.

A decrease in apoptosis has been postulated to play an important role in allergic diseases [21, 22]. Our results identify the pro-apoptotic effects of Lcn2 on pulmonary cells *in vitro* and within the airways *in vivo* and support and extrapolate the results of Devireddy *et al.* [15], who initially identified Lcn2's pro-apoptotic effects. Our

Fig. 6. Lipocalin2 (Lcn2) is regulated in murine epithelial cells via different pro-inflammatory cytokines and LPS. LA-4 cells were treated with different cytokines and concentrations (0, 1, 10, 100 and 100 ng/mL) or lipopolysaccharide (LPS) (0, 1, 10 and 100 ng/mL). Lcn2 mRNA expression was analysed via RT-PCR at indicated time-points. We observed a dose-dependent up-regulation of Lcn2 by IL-1 β (a), a strong induction of Lcn2 by TNF- α (b) and a small increase of Lcn2 by the Th2 cytokine IL-4 (c) and the Th1 cytokine IL-12 (d). Treatment with LPS induced a significant dose-dependent up-regulation of Lcn2 (e). * P -value of $P < 0.05$ and ** $P < 0.001$, respectively. Treatment conditions were set up in triplicate and independently analysed. RT-PCR results of triplicates were combined for error bars.



in vitro results suggest that Lcn2's has a direct pro-apoptotic effect on epithelial cells; morphologically, the *in vivo* data also seem to indicate staining of epithelial cells; however, as we did not include double stainings to clearly identify epithelial cells, other cell types (e.g. immune cells) might also be affected by the pro-apoptotic effects of Lcn2 [15]. Apoptosis is a likely mechanism regulating the development and resolution of AI via induced cell death of important effector cells [22]. In our model, the relative lack of apoptosis that we observed in the Lcn2-KO mice might contribute to the increased AI and airway responsiveness in those mice due to the failure to abrogate the inflammatory cell influx. In summary, these data showed decreased apoptosis in the KO animals to be associated with an increased inflammatory response.

Our experiments affirm that Lcn2 is yet another protein that originates from the innate immune system [16] to be associated with allergic AI but they also link this pathway to the adaptive immune responses of allergic airway disease. This hypothesis is supported by our observation that Lcn2 was up-regulated *in vitro* after stimulation with LPS. Standard OVA-preparations are 'contaminated' with trace amounts of LPS that might be necessary for the induction of allergic Th2 responses [24]. Lcn2 might be responsive to LPS induction in two ways: either directly via TLR4, as other *in vivo* studies suggested [16, 26, 27], and/or indirectly via LPS-induced pro-inflammatory cytokines, such as IL-1 β , TNF- α or even IL-4, as suggested by our *in vitro* data. 'Danger-sensing' via Lcn2 induction after contact with bacterial components such as LPS might thus initiate an innate immune response necessary for the induction of an ensuing adaptive immune response against invading pathogens [16, 27]. Yet, as we and others show, Lcn2 may also play a protective role against the deleterious (adaptive) immune responses observed in different inflammatory settings [28] such as allergic airway disease. In this context, it is tempting to speculate that protection from allergic AI by LPS, as observed in experimental models of airway disease [3], might at least be enhanced by LPS-driven up-regulation of Lcn2. To this end, the induction of Lcn2 via the Th2 cytokine IL-4, as we demonstrated above (Fig. 6), or IL-9 [17] might constitute an important negative feedback loop where initial Lcn2 induction by LPS or pro-inflammatory cytokines is followed by sustained up-regulation of Lcn2 by ongoing Th2 inflammation serving to protect against an excess of inflammation. Yet, induction of this protective molecule does not seem to confer sufficient protection to prevent AI in our experimental model. The reasons for this are unclear. One possibility is that other pro-inflammatory molecules override its effect. Another possibility is that the amounts of Lcn2 are simply not high enough to confer protection. In renal injury, Lcn2 was also first described as a marker for acute injury [29]. Increasing the amount of Lcn2 by an intravenous injection of the protein, however,

markedly improved acute ischaemic kidney injury [29]. It is an exciting possibility that Lcn2 treatment could be used in a similar fashion in AI. Lcn2 size and protein nature make it a prime candidate for inhalational treatment, much preferable to intravenous injections applied in renal disease [29].

We are only slowly beginning to understand the interplay between innate and adaptive immunity in allergic airway diseases. Lcn2 is one novel candidate that may help to further delineate the insight into the underlying mechanisms and interactions, ultimately leading to a better understanding of the pathophysiology that governs one of the most prevalent and costly chronic diseases in industrialized countries.

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Supporting Information

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

Figure S1. Comparison of three different models of airway inflammation: acute AI (4 weeks), semi chronic AI (8 weeks) and chronic AI (14 weeks). Sensitized BALB/c mice were repeatedly challenged with OVA aerosol (1% in PBS) on two consecutive days per week starting on day 27. Mice were analysed 48 h after the last challenge. Control animals received PBS at the same time points. Expressed are the time kinetics of inflammatory cell influx in BALF (A), allergen-specific IgE secretion (B), collagen deposition (C) and airway reactivity (D).

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Supplement Fig. 1: Comparison of three different models of airway inflammation: acute AI (4 weeks), semi chronic AI (8 weeks) and chronic AI (14 weeks).

Sensitized BALB/c mice were repeatedly challenged with OVA aerosol (1% in PBS) on two consecutive days per week starting on day 27. Mice were analysed 48h after the last challenge. Control animals received PBS at the same time points. Expressed are the time kinetics of inflammatory cell influx in BALF (A), allergen-specific IgE secretion (B), collagen deposition (C) and airway reactivity (D).

