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Allergic sensitization is associated with inadequate antioxidant responses in mice and men

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4-hydroxynonenal; allergic sensitization; house dust mite; oxidative stress; rodent urinary proteins.

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Abstract

Background: Allergies arise from aberrant Th2 responses to allergens. The processes involved in the genesis of allergic sensitization remain elusive. Some allergens such as derived from house dust mites have proteolytic activity which can induce oxidative stress *in vivo*. A reduced capacity of the host to control oxidative stress might prime for allergic sensitization.
Methods: Two different strains of mice were compared for their antioxidant and immune response to HDM. Protease activity of the HDM extract was reduced to investigate its role in oxidative stress induction in the airways and whether this induction could determine allergic sensitization and inflammation. The role of oxidative stress in allergic sensitization was also investigated in humans. An occupational cohort of animal workers was followed for the development of sensitization to rodent urinary proteins. Levels of oxidative stress in serum and antioxidant responses by PBMCs were determined.
Results: Susceptibility to allergic sensitization to mite allergens in mice was highly dependent on host genetic background and was associated with oxidative stress in the lungs before allergen exposure and poor antioxidant response after allergen exposure. Reduction in mite protease activity limited its capacity to induce oxidative stress and allergic inflammation in mice. We showed that also in human subjects, oxidative stress before allergen exposure and poor antioxidant responses were associated with predisposition to occupational allergy.
Conclusion: Our study indicates that oxidative stress condition before allergen exposure due to an inadequate antioxidant response may prime for allergic Th2 responses.

Allergies arise from aberrant Th2 immune responses to allergens (1). The processes underlying this unwanted response remain elusive. Pattern recognition receptors (PRRs) such as Toll-like receptors are important regulators of immune response to microbial components such as bacterial lipopolysaccharides (LPS) (2). TLR4 triggering by LPS has been found crucial for the initiation of allergen-specific Th2 responses to house dust mite (HDM) in mice (3, 4). However, Th2 responses to inhaled HDM can also be induced in C3H/HeJ, mice with impaired TLR4 signalling (5). The development of Th2 allergic responses to HDM in C3H/HeJ mice, independently of TLR4 signalling, argues strongly for an additional mechanism independent of the LPS–TLR4 pathway.

C3H/HeJ mice have been reported as having a natural reduced antioxidant response to influenza in the lungs, and this is associated with high morbidity to influenza infection (6). An impaired or reduced capacity to regulate oxidative stress could be related to the development of aberrant immune responses including allergies. Oxidative stress has

been described to orchestrate the Th2 response to cysteine proteases such as papain (7). The role of oxidative stress in HDM-induced allergic sensitization is unknown; however, several studies with other allergens support its role in allergic sensitization (7–9). Various HDM derived allergens have distinct biological functions among which proteases (10) that can also induce oxidative stress *in vivo* (7). Oxidative stress happens when reactive oxygen species (ROS) from local and/or environmental sources overwhelm antioxidant responses (11). ROS, during oxidative stress, can activate the immune system (11) and, in the context of allergen exposure, may facilitate allergic sensitization.

We investigated the immune and antioxidant responses to HDM in different strains of mice and whether HDM protease activity was important for allergic sensitization via oxidative stress induction. In a cohort of occupational allergy, we addressed whether oxidative stress was also associated with susceptibility to allergic sensitization to a HDM-unrelated allergen, namely rodent urinary proteins.

Methods

Reagents

Periodic Acid Schiff's, *N*-acetyl-L-cysteine (NAC), Xanthine, Xanthine oxidase and Propidium iodide, Sigma-Aldrich, Corp. St. Louis, MO, USA; rGM-CSF, Thermo Fisher Scientific, Waltham, MA, USA; MHCII-FITC, CD11c-APC, CD86-PE, CD80-PE, CD40-PE, Rat $IgG2_a$, Ham IgG antibodies and ELISA kit Ready-set-go! eBioscience Inc, San Diego, CA, USA IL4, IL-5, IL-13 and IFN-γ, eBioscience Inc via Immunosource, Halle-Zoersel, Belgium; Antibody to FcRγII/III 2.4G2, provided by Louis Boon, Bioceros, Utrecht, the Netherlands; Bicinchoninic acid (BCA) kit, Bio-Rad Laboratories Inc. Hercules, CA, USA; BlueSepharose, Amersham Pharmacia, UK; Antibodies to 4-HNE, Nrf2, HO-1, Santa Cruz CA, USA; Antibody to β-actin, GeneTex Irvine, CA, USA; IgE, IgG_1 and $IgG2_a$, ELISA kit Opteia, BD, San Diego, CA, USA; Trizol, Invitrogen, Life Technologies, USA; First strand cDNA Synthesis Kit, Thermo Fisher Scientific, MA, USA; SYBR Green PCR Master Mix, Applied Biosystems Warrington, UK.

HDM extracts

A spent mite medium extract (Dermatophagoides pteronyssinus, LoToxTM LTN-DPE-4, lot nr. 33019; INDOOR Biotechnologies, Cardiff, UK) and a crushed whole-body house dust mite extract (Dermatophagoides pteronyssinus, XPB82D3A2.5, lot nr. 136401; Greer Laboratories, Lenoir, NC, USA) were used. The first extract will be referred to as low-toxin HDM (LT-HDM) with an endotoxin level of ≤3 EU per 1 mg protein; the second will be referred to as high-toxin HDM (HT-HDM) containing 199 EU per 1 mg protein as determined by a LAL assay. In all experiments, 1 μg Der p 1, which is equivalent to 6.1 μg protein of LT-HDM and 31 μg protein of HT-HDM was used.

Murine studies

Mice

Female mice C3H/HeJ, C3H/HeN (Harlan, Bicester Oxon, UK) and Balb/c (Harlan, Horst, the Netherlands), 6–8 weeks old, were housed under specific pathogen-free conditions at AMC animal facility. All experiments were approved by the AMC animal ethics committee, the Netherlands.

Sensitization

Mice were anaesthetized with isoflurane, and 30 μl of HDM extracts or phosphate-buffered saline (PBS) was administrated as previously described (12). Briefly, mice were exposed intranasally to HDM for 3 cycles of five consecutive days and 2 days' rest. Four weeks after the last cycle, mice were rechallenged three times and killed 2 days later.

Bronchoalveolar lavage fluid (BALF)

Cells from the airway lumen were obtained by three subsequent washes with 1 ml PBS and 0.1 mM EDTA, after intratracheal cannulation. Cell differentiation was determined by FACS as previously described (13).

Lymph node restimulation

Cells were plated in 96-well round-bottomed plates at 2×10^5 cells per well and restimulated for 4 days with 100 μg/ml HDM extract. Cytokines in supernatants were analysed by ELISA.

Immunoglobulins

Serum IgE, IgG_1 and $IgG2_a$ were analysed by ELISA. For specific immunoglobulin assays, plates were coated overnight with HDM antigens instead of capture antibodies. Standard curves of murine immunoglobulins were used as qualitative reference.

Histology

Frozen lung sections (6 μm) were stained with periodic acid Schiff's. Inflammation and mucus-producing goblet cells were semi-quantified as previously described (14). Briefly, number of goblet cells were determined in the airways and scored according to the percentage of cells per airway (0 = <5%, 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = >75%); airway inflammation was scored as follows: 0 = no inflammation, 1 = cuffing of inflammatory cells at 1 or 2 sides of the airway, 2 = thin (<5 cells thick) layer surrounding the airway, 3 = thick (>5 cells thick) layer surrounding the airway.

Bone marrow-derived dendritic cells (BMDCs)

Bone marrow cells from naïve C3H/HeJ and Balb/c mice were differentiated into dendritic cells *in vitro* as previously described (15). For Nrf2 determination, on day 9 of culture, differentiated DCs were incubated overnight with or without LT-HDM (1 μg Der p 1/ml). For CD40, CD80 and CD86 determination by FACS, on day 9 of culture, cells were incubated with or without 5 mM of NAC at 37°C in 5% CO_2, 30 min prior to overnight incubation with LT-HDM. Propidium iodide was used for cell viability.

Western blots (WB)
Proteins from lung homogenates and BMDCs were extracted with Laemmli buffer: 20% wt/vol sodium dodecyl sulphate (SDS), 30% vol/vol glycerol and 30% vol/vol deionized water in 1 M Tris-base pH 6.8. Proteins were next diluted in 4% wt/vol SDS, 10% vol/vol 2-mercaptoethanol, 20% vol/vol glycerol and 0.004% vol/vol bromophenol blue in 125 mM Tris-HCl pH 6.8 and separated on 13% SDS/PAGE. After transfer to polyvinylidene difluoride membranes and blocking with 5% wt/vol skim milk, blots were incubated with primary polyclonal antibodies to Nrf2 or 4-HNE. Subsequently, they were incubated with IRDye 680LT-conjugated secondary antibodies. Blots were visualized using infrared fluorescence detection Odyssey Imager and software (LI-COR Biosciences, Lincoln, NE, USA). Loading was normalized per β-actin, and relative optical density (Rel.OD) values were used as quantification units.

Real-time PCR
Total lung RNA was extracted with Trizol according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using first-strand cDNA synthesis kit. PCR was performed in a 10 μl reaction volume including 5 μl of SYBR Green, 200 nM of each FW and RV primers, 2 μl of cDNA and nuclease-free water. For each gene, reaction was performed in duplicate. Duplicate standard curves were constructed by serial dilution (1:5) from a concentrated pool of cDNA. All reactions were performed in optical 96-well reaction plates using the ABI Prism 7500 system (Applied Biosystems). mRNA concentrations were calculated based on the standard curve method (16) and normalized to the housekeeping gene HPRT.

Protease activity assay
Heated LT-HDM was serially diluted (1:2) in reaction buffer (0.2 M sodium phosphate, 1 mM EDTA, pH 7.0), mixed with 20% vol/vol 20 mM cysteine substrate OR reaction buffer (50 mM Tris, 20 mM $CaCl_2$, pH 8.2), mixed with 50% vol/vol 2 mM trypsin substrate OR reaction buffer (0.1 M Tris, 0.96 M NaCl, 10 mM $CaCl_2$, pH 8) and mixed with 50% vol/vol chymotrypsin substrate in 96-well NUNC plates. For the standard curves, enzymes were serially diluted (1:2) and mixed with respective substrates. Starting enzymes dilutions: papain, 700 μg/ml; trypsin, 2 μg/ml, and chymotrypsin, 200 μg/ml. Absorbance was measured at 415 nm after the development of colour.

CAP inhibition assay
ImmunoCAP (ThermoFisher Scientific, Thermo Fisher Scientific Inc, Waltham, MA, USA) was performed according the manufacturer's instructions. Prior to incubation in ImmunoCAP, serum from a HDM allergic subject was inhibited for 1 h with LT-HDM or heated LT-HDM. After inhibition, residual IgE binding was measured using CAPs coated with mite extract, Der p 1 or Der p 2. Results were expressed in percentage of inhibition.

Human studies

Study design
Study population consisted of 37 temporary laboratory animal workers from a previous study (17). Briefly, participants were followed for 2 years and occupational allergic sensitization to rodent urinary proteins was monitored. They were seen at the start of their application as animal workers (T0), after 4 months (T4), 1 year (T12) and 2 years (T24) for blood collection and clinical evaluations. Herein, we compared 21 workers who did not develop sensitization to rodents with 16 animal workers who did. 4-HNE-modified proteins and HO-1 were accessed in serum and Nrf2 was accessed in peripheral blood mononuclear cells (PBMCs were available of five '*de-novo*-sensitized' individuals and four 'non-*de-novo*-sensitized' individuals) by WB.

Western blots
Serum samples were treated with BlueSepharose 6B CL to reduce the albumin content. Proteins were treated and blotted as previously described in the murine section. Polyclonal antibodies to Nrf2, HO-1 or 4-HNE were used. Total protein was determined by BCA. Samples were normalized per 50 μg of protein, and optical density (OD) values were used as quantification units.

PBMC
Cells were cultured overnight with xanthine (0.5 mM): xanthine oxidase (50 mU). Nrf2 protein expression was analysed by WB in total cell lysate.

Statistical analysis
Statistical significance was tested with Mann–Whitney *U*-test. Experiments were repeated at least twice unless stated otherwise in Figure Legends. For correlation analysis, Pearson's correlation coefficient was calculated. Significance was established at $P < 0.05$.

Results

Allergic sensitization to HDM depends on mice genetic background

C3H/HeJ mice have been reported as having a natural reduced antioxidant response in the lungs (6). We investigated whether this characteristic would influence C3H/HeJ immune response to HDM. The composition of HDM extracts can affect the immune response (18); therefore, we used two different commercially available HDM extracts. First, C3H/HeJ and Balb/c mice were exposed to HDM extract with high endotoxin level (HT-HDM). As Balb/c have been described to be dependent on β-glucans/TLR2 and LPS/TLR4 signalling for HDM sensitization (19) and given that β-glucans and LPS are able to induce oxidative stress, we selected a second HDM extract which contains only trace levels of endotoxins (LT-HDM). As expected, inhalation of HT-HDM resulted in a strong Th2-type inflammatory

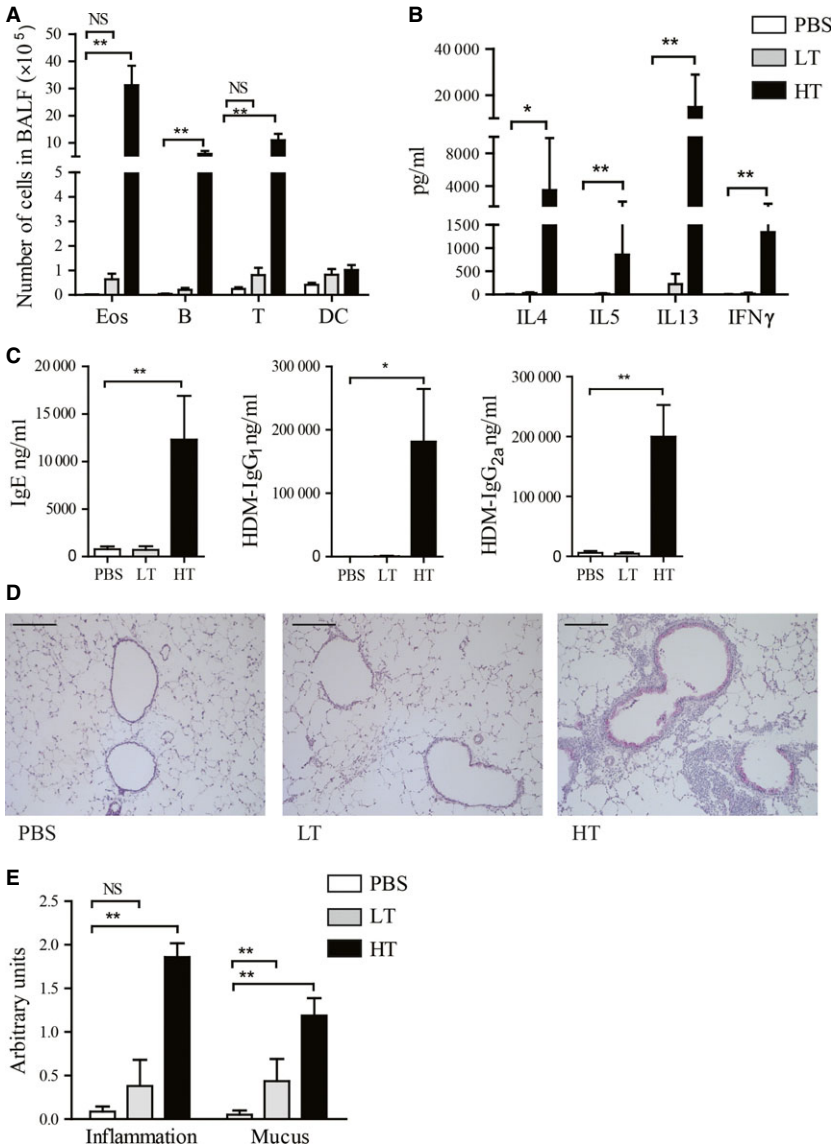

Figure 1 Balb/c immune response to LT-HDM (LT) and HT-HDM (HT). Balb/c mice were intranasally exposed to LT-HDM (LT) or HT-HDM (HT) or PBS as a control. (A) Number of inflammatory cells in bronchoalveolar lavage fluid (BALF). (B) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFN-γ by lung draining lymph node cells. (C) Total IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (D) Peribronchial inflammatory infiltrates and mucus production and (E) quantification. Scale bars in D represent 200 μm. Data are presented as means ± SD, $*P < 0.05$, $**P < 0.01$.

response in Balb/c, as reflected by eosinophil recruitment in the airway lumen, Th2 cytokine production, peribronchial inflammatory infiltrates, goblet cell hyperplasia, total IgE and HDM-specific IgG_1. IFN-γ and HDM-specific IgG_{2a} were also increased (Fig. 1A–E). LT-HDM failed to induce any significant immune response in Balb/c mice, except for a small increase in mucus production (Fig. 1A–E). Despite the mutant nonfunctional TLR4 in C3H/HeJ mice, HT-HDM induced all hallmarks of a robust Th2 inflammation, similar to Balb/c. Also here, IFN-γ and IgG_{2a} were increased. However, in contrast to Balb/c, C3H/HeJ mice also developed a full-blown Th2-type immune response to LT-HDM (Fig. 2A–E). This was not accompanied by an increase in IFN-γ and IgG_{2a}.

To clarify whether the differential responsiveness to LT-HDM between both strains was due to the different genetic background rather than to the mutation in C3H/HeJ mice, we studied C3H/HeN mice the wild-type background of C3H/HeJ. Similar to the mutant mice, C3H/HeN developed a full-blown Th2-mediated airway inflammation in response to LT-HDM without the induction of IFN-γ and IgG_{2a} (Fig. 3A–E). The inflammatory response induced by HT-HDM in C3H/HeN mice was accompanied by the induction of IFN-γ and IgG_{2a} similarly to C3H/HeJ. Together, this

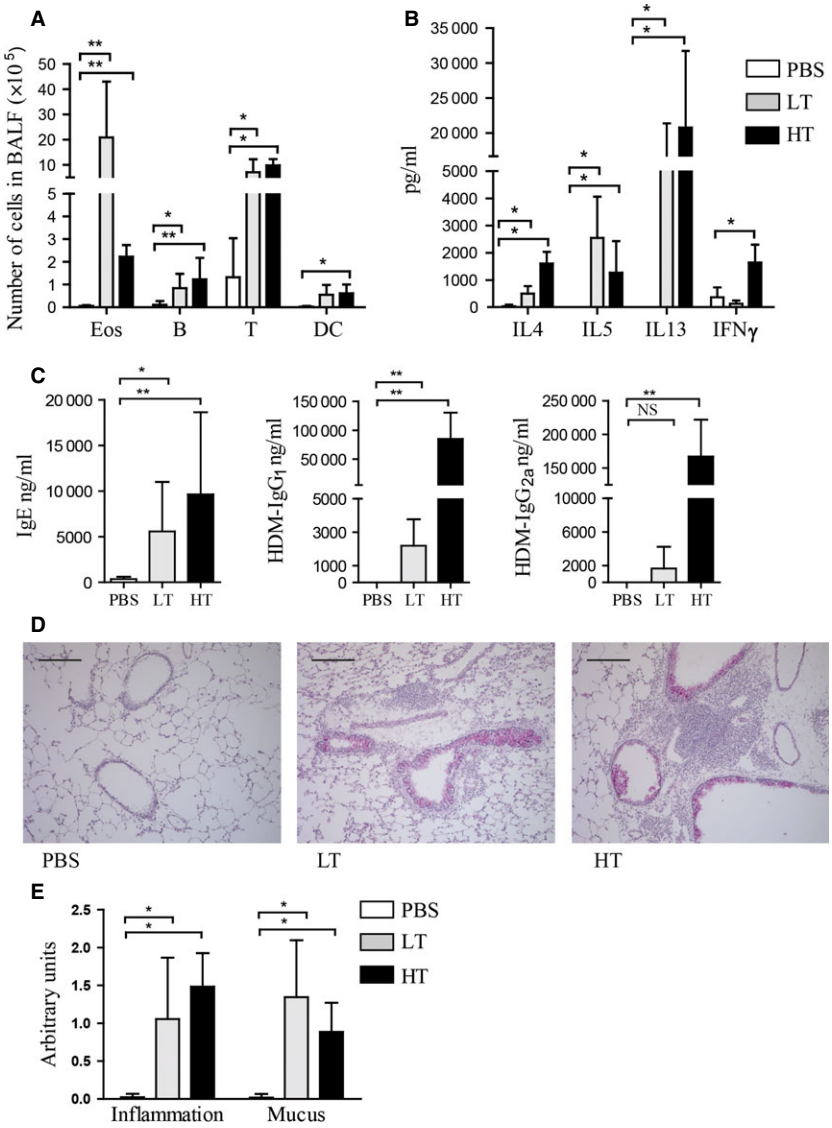

Figure 2 TLR4-mutant mice immune response to LT-HDM (LT) and HT-HDM (HT). C3H/HeJ mice were intranasally exposed to LT-HDM (LT) or HT-HDM (HT) or PBS as a control. (A) Number of inflammatory cells in bronchoalveolar lavage fluid (BALF). (B) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFN-γ by lung draining lymph node cells. (C) Total IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (D) Peribronchial inflammatory infiltrates and mucus production and (E) quantification. Scale bars in D represent 200 μm. Data are presented as means ± SD, $*P < 0.05$, $**P < 0.01$.

indicates that sensitization to HDM depends on mice genetic background and is not strictly dependent on TLR4 signalling.

Oxidative stress and antioxidant protein expression in response to LT-HDM in C3H/HeJ and Balb/c mice

We hypothesized that susceptibility to LT-HDM allergic sensitization in C3H/HeJ mice could be related to a deficient antioxidant response in the lungs. In order to establish the alternative mechanism for HDM allergic sensitization that seems to be at least partly independent of TLR4–LPS pathway, we performed our further experiments with the TLR4-mutant C3H/HeJ mice. We compared the level of oxidative stress before and after a single exposure to LT-HDM in Balb/c and C3H/HeJ mice, resistant and susceptible to LT-HDM sensitization, respectively. Before exposure, the concentration 4-hydroxynonenal-modified proteins (4-HNE), a marker for oxidative stress (20), was markedly lower in lungs of Balb/c mice compared to C3H/HeJ. LT-HDM induced an increase in 4-HNE-modified proteins in the lungs of both strains. However, this increase was significant in Balb/c but not in C3H/HeJ mice, very likely due to its prominent high level of oxidative stress before exposure (Fig. 4A,

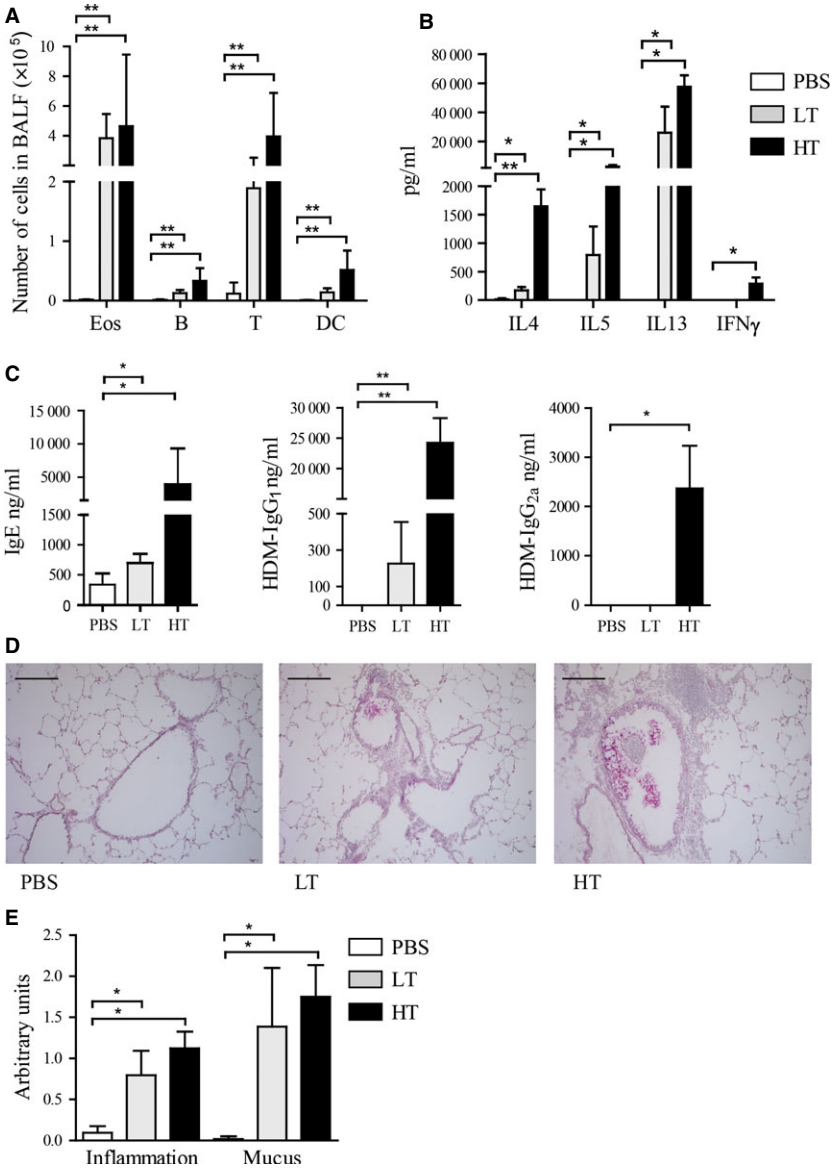

Figure 3 *Wild-type* C3H/HeN immune response to LT-HDM (LT) and HT-HDM (HT). C3H/HeN mice were intranasally exposed to LT-HDM (LT) or HT-HDM (HT) or PBS as a control. (A) Number of inflammatory cells in bronchoalveolar lavage fluid (BALF). (B) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFN-γ by lung draining lymph node cells. (C) Total IgE, HDM-IgG_1 and HDM-IgG_{2a} in serum. (D) Peribronchial inflammatory infiltrates and mucus production and (E) quantification. Scale bars in D represent 200 μm. Experiment performed once with $n = 5$. Data are presented as means ± SD, $*P < 0.05$, $**P < 0.01$.

B). Levels of mRNA for antioxidant enzymes glutathione peroxidase-1 (GPx-1) and heme oxygenase-1 (HO-1) were increased in response to LT-HDM in lungs of Balb/c, but not in C3H/HeJ mice (Fig. 4C). The levels of 4-HNE-modified proteins inversely correlated with the levels of HO-1 mRNA (Fig. 4D).

We compared the capacity to upregulate nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of the antioxidant response among which HO-1 (21), in response to LT-HDM in bone marrow-derived dendritic cells (BMDCs) from both strains. After 24 h of LT-HDM exposure, Nrf2 expression was increased in Balb/c but not in C3H/HeJ BMDCs (Fig. 4E,F).

As DC activation and subsequent migration to lymph nodes is an important step in the initiation of an adaptive response (22) and subsequent induction of specific-allergen type 2 cell differentiation (23), we examined whether LT-HDM induced activation of C3H/HeJ DCs and whether this activation could be inhibited by the antioxidant *N*-acetyl-L-cysteine (NAC), a potent ROS scavenger. LT-HDM exposure

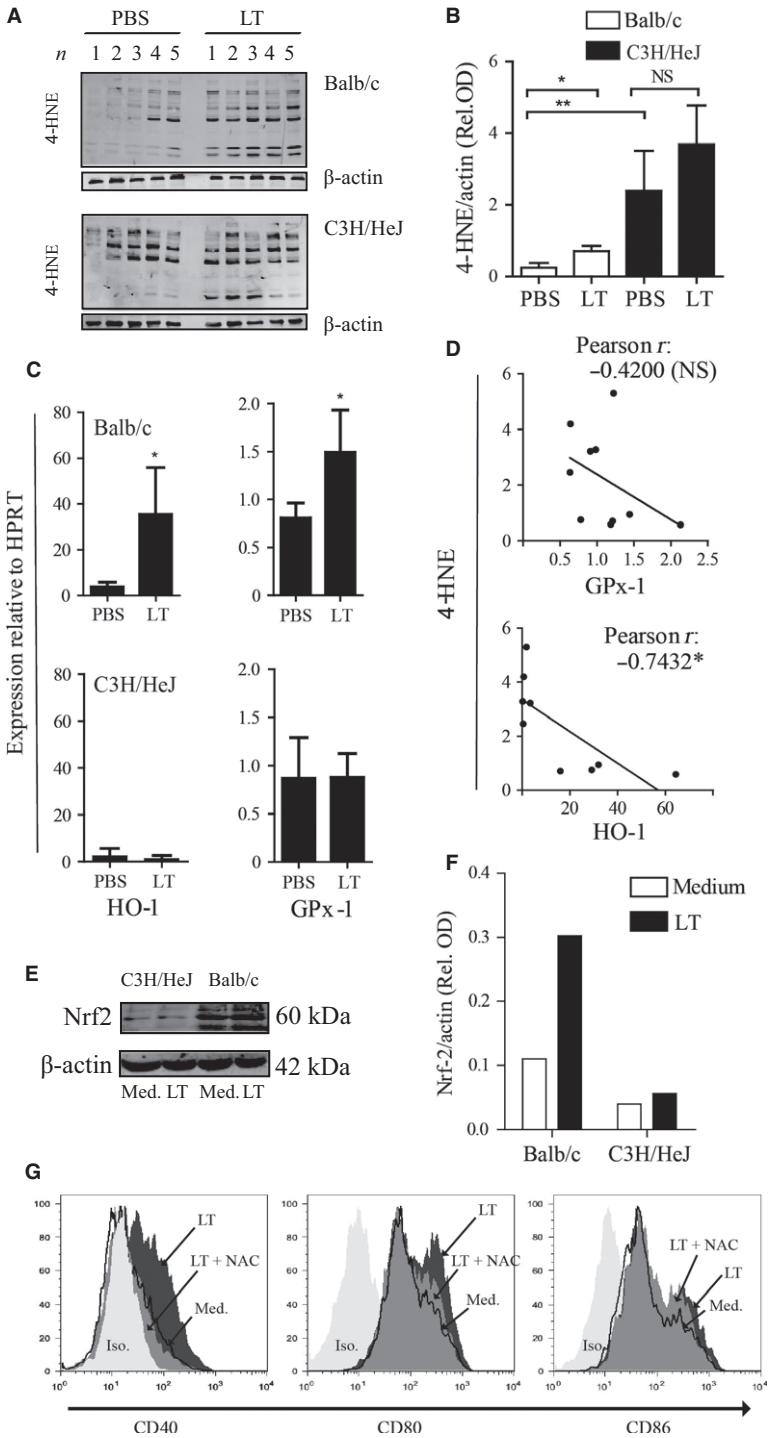

Figure 4 Oxidative stress levels and antioxidant protein expression in mice, after HDM exposure. Balb/c and C3H/HeJ ($n = 5$) were intranasally exposed to LT-HDM (LT) or PBS as a control, and lungs were removed 24 h later for analysis. (A) Immunoblotting of 4-HNE-modified proteins (4-HNE) in lung homogenate and (B) quantification. (C) mRNA expression of antioxidant enzymes hemoxigenase-1 (HO-1) and glutathione peroxidase-1 (GPx-1) in lung tissue. (D) Correlation of 4-HNE-modified proteins (4-HNE) with GPx-1 and HO-1 mRNA expression. (E) Immunoblotting of Nrf2 in total bone marrow-derived dendritic cell lysates (blots are representative of two independent experiments) and (F) quantification of protein expression. (G) Expression of co-stimulatory molecules CD40, CD80 and CD86 on bone marrow-derived dendritic cells from naïve C3H/HeJ mice, cultured with LT-HDM (LT) in the absence or presence of ROS scavenger *N*-acetyl-L-cysteine (NAC). Data are presented as means ± SD, $*P < 0.05$, $**P < 0.01$.

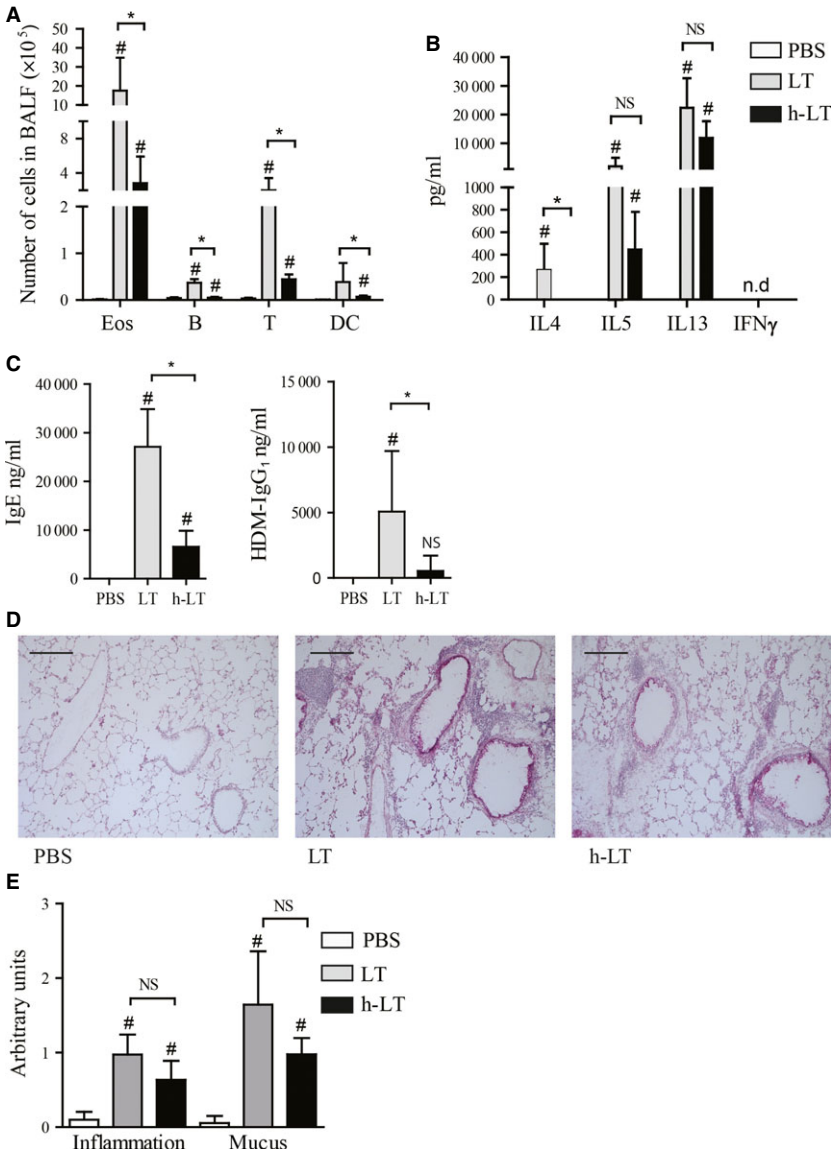

Figure 5 Effect of HDM protease activity inhibition *in vivo*. C3H/HeJ mice were intranasally exposed to LT-HDM (LT) or heated LT-HDM (h-LT) or PBS as a control. (A) Number of inflammatory cells in bronchoalveolar lavage fluid (BALF). (B) Production of Th2 cytokines IL-4, IL-5, IL-13 and IFN-γ by lung draining lymph node cells. (C) Total IgE and HDM-IgG_1 in serum. (D) Peribronchial inflammatory infiltrates and mucus production and (E) quantification. Scale bars in D represent 200 μm. Experiment performed once. Data are presented as means ± SD, $^{\#}P$ *vs* PBS, $^{\#,*}P < 0.05$.

upregulated CD40, CD80 and CD86 compared to unexposed cells. Treatment of DCs with NAC inhibited upregulation of CD40 and CD80 after LT-HDM exposure (Fig. 4G).

Partial inhibition of protease activity in HDM extract reduced HDM-allergic inflammation in mice

Next, we verified whether the induction of oxidative stress was dependent on the protease activity in LT-HDM. Protease activity was attenuated by heating (30 min at 65°C). In heated LT-HDM, there was significant inhibition of its trypsin and chymotrypsin protease activity, while cysteine protease activity was not affected (see Figure S1). Importantly, heating did not compromise the allergenic potency as expressed by unaltered IgE recognition of heated mite allergens (see Figure S2 and S3). Heated LT-HDM induced markedly less 4-HNE-modified proteins in C3H/HeJ BMDCs, compared to control LT-HDM (see Figure S4A,B). To confirm this finding *in vivo*, mice were exposed to PBS, LT-HDM or heated LT-HDM and the level of 4-HNE-modified proteins in lung was determined after 24 h. In concordance with the *in vitro* assay, heated LT-HDM induced less oxidative stress compared to LT-HDM (see Fig. S4C,D). Next, we examined the effect of the reduced oxidative capac-

ity of heated LT-HDM *in vivo*. Allergic characteristics were significantly decreased in mice exposed to heated LT-HDM in comparison with LT-HDM. Heat treatment significantly reduced the recruitment of eosinophils, dendritic cells and T and B lymphocytes to the airways, IL-4, total IgE and HDM-specific IgG_1 production (Fig. 5A–E). Peribronchial inflammatory infiltrates, goblet cell hyperplasia, IL-5 and IL-13 showed a small decrease as well but did not reach statistical significance. This suggests that a reduced capacity to induce allergic inflammation by heated LT-HDM could be attributed to its reduced capacity to generate oxidative stress.

Allergic sensitization to rodent proteins in humans is associated with reduced capacity to express HO-1 and Nrf2

Next, we investigated whether an insufficient capacity to cope with oxidative stress also correlates with allergic sensitization in humans. Previously, we have determined sensitization to rodents urinary proteins in a cohort of atopic individuals up to 2 years after occupational exposure (17). Sixteen of 37 atopic individuals became *de novo*-sensitized to rodents urinary proteins during this period as determined by allergic symptoms, the development of allergen-specific IgE and allergen-induced IL-4 production (17). We accessed 4-HNE-modified proteins in serum collected before occupational exposure started (T0) and after 4 months (T4), 1 year (T12) and 2 years (T24). Those who became *de novo*-sensitized (S) to laboratory animals showed significantly higher levels of 4-HNE-modified proteins in serum before exposure started (T0) and in all subsequent time points, compared to control individuals that did not develop *de novo* sensitization (Non-S) (Fig. 6A,B). Expression of HO-1 in serum was significantly lower in sensitized group (S), indicative of a reduced antioxidant capacity (Fig 6C,D). At T0, T4 and T12, a significant inverse correlation was observed between 4-HNE-modified proteins and HO-1 expression (Fig. 6E), although this correlation attenuated over time and was lost after 2 years. In order to analyse the capacity to upregulate Nrf2 during oxidative stress induced by xanthine/xanthine oxidase, which induces ROS peroxide, we evaluated PBMCs collected at T0 from *de novo*-sensitized ($n = 5$) and not sensitized subjects ($n = 4$). Nrf2 expression in PBMCs from *de novo*-sensitized individuals (S) was lower than controls (Non-S). Although this did not reach significance possibly due to the limited number of available samples (Fig. 6F,G), the relevance of the differences in Nrf2 expression between both groups was reflected by the strong negative correlation with the level of oxidative stress (4-HNE-modified proteins) (Fig. 6H). Overall, our human data are in support of our murine studies, in which the development of allergic sensitization was associated with an inadequate antioxidant response.

Discussion

What makes individuals more susceptible than others to allergic sensitization is considered a multifactorial process that involves among others, genetics and types of allergens. In the present study, we show that inadequate antioxidant responses are strongly associated with allergic sensitization.

In our study, we showed that C3H/HeJ mice which have a natural reduced antioxidant response were susceptible to sensitization to LT-HDM, while Balb/c mice with a better antioxidant response were resistant. In an occupational cohort of animal laboratory workers, we observed that individuals with higher levels of oxidative stress before allergen exposure were prone to develop *de novo* sensitization to urinary proteins. In addition, PBMCs of sensitized individuals showed a reduced capacity to upregulate the major regulator of antioxidant responses Nrf-2, when subjected to oxidative stress condition *in vitro*. These findings indicate that exposure to allergenic proteins combined with host inadequate antioxidant response dramatically increased the likelihood for the development of allergic sensitization.

The exact mechanism by which ROS, in the context of oxidative stress, can initiate adaptive immune responses to an allergen is unknown. ROS at a relatively low concentration serve as essential second messenger mediating cellular responses to many physiological stimuli for example, by regulating the redox status of transcription factors (24, 25). However, excessive ROS production can contribute to an enhanced immune response (25). ROS are known for stimulating Th2-like responses (7, 26) and to induce maturation and antigen presentation by DCs (27–29), which is an important step in the initiation of adaptive immunity including allergy. In concordance, treatment of DCs with antioxidant NAC decreased LT-HDM induced expression of costimulatory molecules CD40 and CD80. ROS is able to decrease the thresholds for CD28 activation on T cells by enhancing IL-2 and IL-2R expression (30, 31). ROS can affect DCs also indirectly. Under oxidative stress condition, damaged, dead or activated structural cells, which can release danger-associated molecular patterns (DAMPs) and cytokines, are able to promote DC maturation (32–34).

To address whether HDM protease activity was involved in the induction of oxidative stress and allergic inflammation, we reduced protease activity of HDM allergens using mild heating. The use of heat has the advantage of specific intervention, targeting the allergen alone in contrast to other intervention methods. The systemic use of antioxidants could also affect oxidative stress induced by the inflammatory process itself and the baseline redox status of the host. Protease inhibitors can inhibit endogenous proteases activated during HDM-induced inflammation which would overestimate the effect of protease inhibition. Mild heating of HDM reduced its proteolytic activity without affecting IgE-binding potency. This led to a clear inhibition of oxidative stress in dendritic cells *in vitro* and in lungs *in vivo* (Figure S4) and significant attenuation of allergen sensitization and Th2 inflammation *in vivo*. This suggested that the level of allergic airway inflammation was dependent on the level of oxidative stress induced by HDM proteases. However, when the antioxidant response was sufficient to counter HDM-induced oxidative stress, as observed in Balb/c exposed to LT-HDM, no inflammatory response was induced. In Balb/c, TLR4 signalling via LPS present in the HT-HDM extract was likely strong enough to

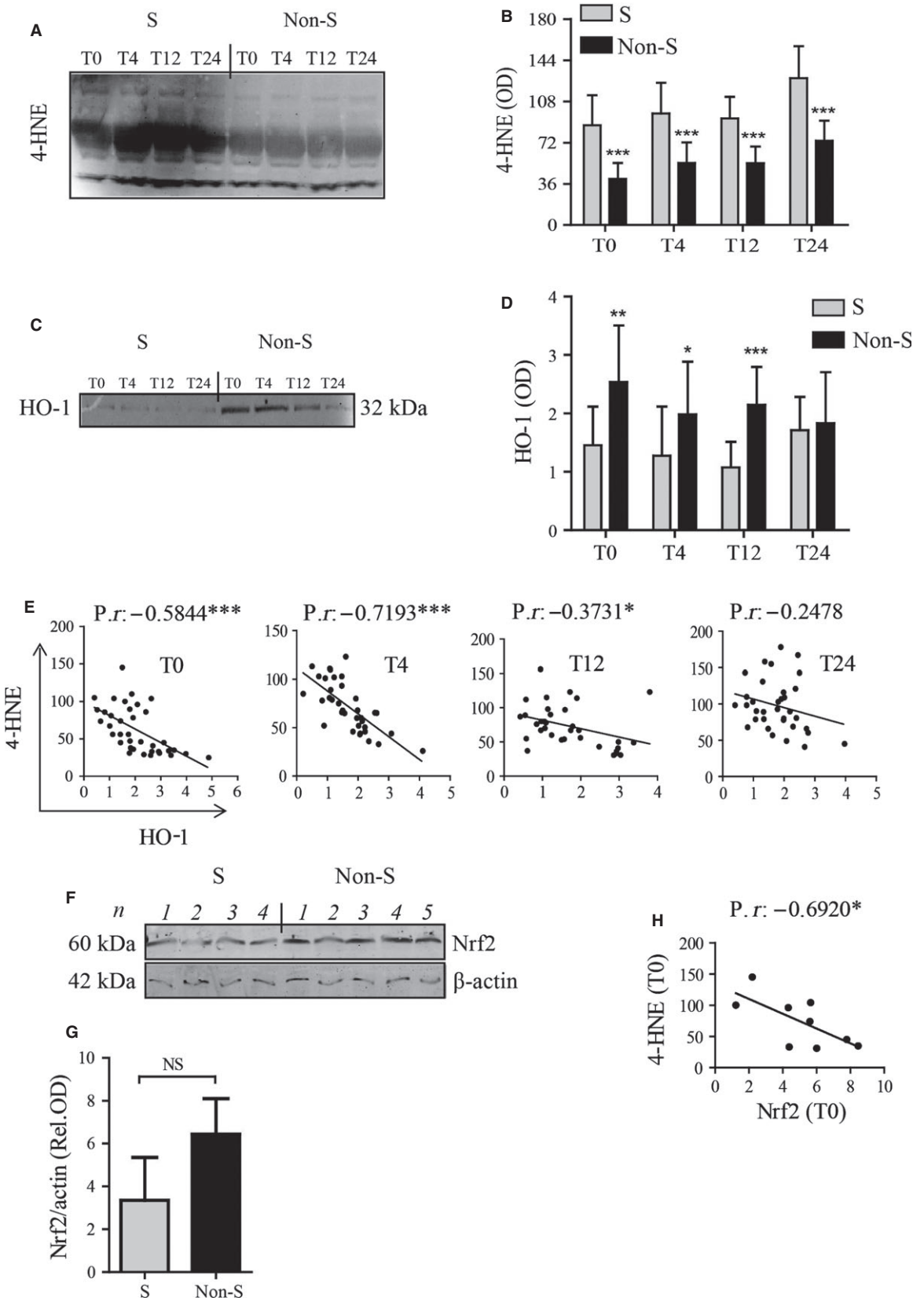
A
S
Non-S
T0 T4 T12 T24 T0 T4 T12 T24
4-HNE
B
S
Non-S
4-HNE (OD)
180
144
108
72
36
0
***
T0 T4 T12 T24
C
S
Non-S
T0 T4 T12 T24 T0 T4 T12 T24
HO-1
32 kDa
D
S
Non-S
HO-1 (OD)
**
*
***
T0 T4 T12 T24
E
P.r: −0.5844***
P.r: −0.7193***
P.r: −0.3731*
P.r: −0.2478
T0
T4
T12
T24
4-HNE
HO-1
F
S
Non-S
n 1 2 3 4 1 2 3 4 5
60 kDa
Nrf2
42 kDa
β-actin
G
NS
Nrf2/actin (Rel.OD)
S
Non-S
H
P. r: −0.6920*
4-HNE (T0)
Nrf2 (T0)

Figure 6 Oxidative stress levels and antioxidant protein expression in animal laboratory workers. Human serum samples from 16 *de novo*-sensitized (S) and 21 non-*de-novo*-sensitized (Non-S) individuals were analysed for 4-HNE-modified proteins (4-HNE) and HO-1 expression in different time points (T0 = before occupational exposure and T4, T12 and T24 = 4, 12 and 24 months, respectively, after occupational exposure). (A) Representative immunoblotting of 4-HNE-modified proteins (4-HNE) from one individual per group and (B) quantification of protein expression. (C) Immunoblotting of heme oxigenase-1 and (D) quantification of protein expression. (E) Correlation of 4-HNE-modified proteins (4-HNE) with HO-1 expression in different time points (Pearson's r: T0 = −0.5844***, T4 = −0.7193***, T12 = −0.3731* and T24 = −0.2478). (F) Immunoblotting of Nrf2 in PBMCs from S ($n = 4$) and Non-S ($n = 5$) individuals and (G) quantification of protein expression. (H) Correlation of 4-HNE-modified proteins (4-HNE) with Nrf2 expression (Pearson's r: −0.6920*). Data are presented as means ± SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

outcompete the antioxidant capacity in Balb/c mice resulting in sensitization. Recently, Hammad et al. (3) demonstrated elegantly the dependence on TLR4 for HDM sensitization in Balb/c, herein we show that C3H/HeJ mice with an impaired TLR4 signalling were able to mount an immune response to inhaled HT-HDM. Our results indicate that their natural reduced antioxidant response might have bypassed the need for TLR4 signalling. Oxidative stress is known to preferentially lead to the induction of type 2 cytokines (IL-4, IL-13) in $CD4^+$ T cells in favour of type 1 cytokines (IFN-γ) (7, 26). Interestingly, LT-HDM induced a polarized type 2 response, with IL-4, IL-5 and IL-13 production, but not IFN-γ or IgG2a in wild-type C3H/HeN and mutant C3H/HeJ mice, suggestive of a response induced by oxidative stress. HT-HDM, however, additionally induced IFN-γ and IgG2a in TLR4-mutant C3H/HeJ mice, suggesting that another stimulus provided by HT-HDM, such as LPS and β-glucans able to trigger TLR2, was needed for this mixed Th1/Th2 response without the need of TLR4 signalling.

There is increasing evidence that a deficient antioxidant system may contribute to allergy development. Nrf2 and HO-1 deficiency predisposes mice to more severe allergic inflammation (35–37). In humans, polymorphisms in genes coding for enzymes that play a role in scavenging ROS have been associated with an increased risk for the development of atopic disorders (38, 39). Antioxidant proteins not only provide protection against oxidant injury but are also involved in immune modulation. For example, HO-1 suppresses T-cell function and proliferation *in vitro* and *in vivo* (40), and its expression in DCs is involved in the induction of $CD4^+CD25^+$ T regulatory cells (41). Antioxidant mechanisms are crucial in the regulation of cellular redox homeostasis. Deficiency of key antioxidant components (such as Nrf2) perturbs intracellular redox status, increasing the basal levels of intracellular ROS (26, 42) affecting DC phenotype and function (27, 28).

In conclusion, our findings indicate that oxidative stress before allergen exposure, due to inadequate antioxidant response, primes for allergic Th2 responses. The inability to cope with oxidative stress might represent an underlying mechanism of why certain proteins act as an allergen in some individuals and are harmless to others. More studies are necessary to understand the mechanisms by which oxidative stress initiates a Th2 response to allergens.

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None.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

LU, CF, LvR, RL and RvR performed conception and design, and LU, CF, JA, LvR, AL, MP, JZ and EK performed acquisition of data.

Supporting Information

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

Figure S1. Protease activity of LT-HDM (LT) and heated LT-HDM (h-LT).

Figure S2. Heating of LT-HDM extract did not compromise antigen recognition by mite allergen specific IgE as determined by immunoblotting.

Figure S3. Heating of LT-HDM did not compromise antigen recognition by mite allergen specific IgE as determined by CAP inhibition assay.

Figure S4. Heat treatment of LT-HDM reduced its capacity to induce oxidative stress *in vitro* and *in vivo*.

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