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Obesity lowers the threshold of allergic sensitization and augments airway eosinophilia in a mouse model of asthma

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Abstract

Background: Clinical and epidemiological studies show a close association between obesity and the risk of asthma development. The underlying cause–effect relationship between metabolism, innate and adaptive immunity, and inflammation remains to be elucidated.

Methods: We developed an animal model to study the interaction between metabolic abnormalities and experimentally induced asthma. Obesity-susceptible AKR mice were fed with high-fat diet (HFD) or normal low-fat diet (LFD) and subjected to a protocol of ovalbumin (OVA) sensitization and airway allergen challenges followed by assessment of inflammation and lung function.

Results: AKR mice developed obesity and a prestage of metabolic syndrome following HFD. This phenotype was associated with an increase in proinflammatory macrophages (CD11b+/CD11c+) together with higher serum levels of interleukin 6. Obese mice showed increased susceptibility to allergic sensitization as compared to LFD animals. Anti-ovalbumin IgE antibody titers correlated positively and anti-OVA IgG2a antibodies titers correlated negatively with body weight. Airway eosinophilia showed a positive correlation with body weight, whereas mucus production did not change with obesity.

Conclusions: This obesity model demonstrates that HFD-induced obesity lowers the sensitization threshold in a model of asthma. This finding helps to understand why, particularly during childhood, obesity is a risk factor for the development of allergic asthma.

Obesity as a state of excessive abdominal body fat (1,2) is a risk factor for allergic asthma (3). Both, asthma and obesity are chronic diseases with a high and continuously increasing

impact on the worldwide disease burden (1). Obesity is associated with a higher prevalence of asthma (4–8), as well as an increasing incidence, and worsening of the clinical outcome (9–12). Different pathomechanisms have been discussed how obesity might influence chronic inflammatory disease (13,14). Obesity causes a low-grade systemic inflammatory response (15), characterized by the presence of pro-inflammatory (CD11c+) macrophages in the fat tissue (16) together with elevated serum levels of IL-6 (17–19). Furthermore, saturated fatty acids from the diet might directly activate toll-like receptor (TLR)-2 (20) and TLR-4 (21) signaling pathways and thereby trigger activation of innate immune responses, subsequently supporting a chronic inflammatory state (22).

Animal studies addressing the effect of obesity on the immunological phenotype of experimentally induced asthma showed discrepant results (23). After ozone-induced asthma, obese animals had aggravated lung inflammation and injury compared with lean animals (24–28). In ovalbumin

Abbreviations

CD11b/CD11c, cluster of differentiation 11b/11c; IgE/G1/G2a immunoglobulin E/G1/G2a; BMI body mass index; TH2, T helper cell 2; IL-6/5, interleukin 6/5; TLR, toll-like receptor; OVA, ovalbumin; BAL, broncho-alveolar lavage fluid; HFD, high-fat diet; LFD, low-fat diet; SC subcutaneous injection; PBS, phosphate buffer saline; HE, haematoxylin–eosin; PAS, periodic acid Schiff; CAST, computer-assisted stereological toolbox; IFN- γ , interferon- γ ; ELISA enzyme-linked immunosorbent assay; FC, flow cytometry; MRI, magnet resonance imaging; BSA, bovine serum albumin; TR, repetition time; TE, echo time; FA, flip angle; FoV, field of view; SEM, standard error of the mean; SD, standard deviation; ANOVA, analyses of variance; AHR, airway hyper-responsiveness; C 12-16/17-23, carbon tail length 12-16/17-23; SFA, saturated fatty acids.

(OVA)-induced asthma, one study could not find worsening of the asthma phenotype under obesity (29), but other studies found higher numbers of lung infiltrating eosinophils or higher numbers of leukocytes in the broncho-alveolar lavage (BAL) of obese animals (30,31).

A prerequisite of allergic airway inflammation is the development of an allergen-specific immune response with IgE antibodies (32), referred to as allergic sensitization (33). Higher allergen-specific IgE levels are associated with more severe asthma across a range of clinical and biologic markers and may be indicative of both higher exposure and a greater degree of sensitization, which in turn may result in increased asthma severity (34). However, most of the experimental studies of obesity and asthma have so far concentrated on the phenotype of asthma exacerbation. It therefore remains unclear whether obesity affects the initiation of allergic immune response during sensitization or the elicitation of the clinical phenotype, or both. Therefore, the aim of this study was to develop an animal model that allows to investigate the impact of obesity and concomitant metabolic changes on the sensitization during experimental asthma.

Material and methods

Animal care and metabolic assessment

Female inbred AKR/OlaHsd (Harlan Winkelmann, Borcheln, Germany) mice were housed in a specific pathogen-free facility with a 12-h light/12-h dark cycle and given free access to food and water. All experiments were performed in accordance with German and international guidelines and were approved by local authorities (Regierungspräsidium, Giessen, Germany). From the age of 4 weeks till the end of the 62-day observation period, mice were fed a high-fat diet (HFD) with 40% kcal from fat (32% kcal lard and 8% kcal corn oil). Control mice received a low-fat diet (LFD) with only 11% kcal from fat (both diets from Altromin, Lage, Germany). Total body weight and food intake were measured twice a week. After 62 days of LFD/HFD, the body mass index (BMI) of the mice was calculated by dividing the body weight (g) by the square of the body length (mm), measured from the tip of the nose to the tail base (35).

Clinical chemistry

Serum cholesterol was measured every 2 weeks after 1 h of fasting (Synchron-Systeme; Beckman Coulter, Krefeld, Germany). Fasting triglyceride serum levels (Synchron-Systeme; Beckman Coulter) and postprandial as well as fasting glucose blood levels (Accu-Chek Sensor; Roche, Mannheim, Germany) were measured after 62 days of LFD/HFD according to the manufactures instructions.

Gas chromatography and a flame ionization detector were used to determine the FA composition of diets, erythrocyte membranes, and fat tissue. Erythrocyte membranes were isolated from 500 μ l blood taken by an axillary incision, 500 mg of white adipose tissue was dissected from the

inguinal fat depot, and 500 mg of the diets was taken to prepare and analyze all samples as previously described (36).

Induction of experimental asthma

After 34 days LFD/HFD, animals of both diet groups were randomized to three different protocols to induce experimental asthma (Fig. 1f). For protocol OVA 30 μ g, mice received three subcutaneous injections of 10 μ g OVA (grade VI; Sigma, St Louis, MO, USA) in 200 μ l of PBS, respectively, injections of PBS only as a control, at days 34, 41 and 48. For protocol OVA 20 μ g, mice received two injections of OVA at day 34 and 41 and for protocol OVA 10 μ g only one at day 34. This was followed by 20 min 1% OVA (grade V; Sigma) or PBS aerosol treatments at day 59, 60 and 61, as previously described (37).

Airway reactivity and total lung capacity

Twenty-four hour after the last challenge airway reactivity and total lung capacity were assessed by using a small-animal mechanical ventilator (flexiVent; SCIREQ, Montreal, PQ, Canada) as described previously (38). Airway resistance (R_n) was measured with the Snap Shot 150 perturbation protocol (SCIREQ) in response to progressively increasing concentrations of methacholine (Sigma) in PBS (0–20 mg/ml). Total lung capacity was estimated from the DynPV-P perturbation protocol (SCIREQ); (38).

Broncho-alveolar lavage and lung tissue collection

Broncho-alveolar lavage was performed using 1 ml of PBS containing 1 $\times$ protease inhibitor cocktail (Roche); (39). After BAL, the lungs were inflated with 1 ml of 6% paraformaldehyde (Merck, Darmstadt, Germany) and fixed over night. Lung tissues were embedded into 2% agarose and dissected into 6 mm slices according to the systematic randomized uniform sampling (40). Three-micrometer sections were taken from paraffin-embedded sections and stained with haematoxylin–eosin (HE) or periodic acid Schiff (PAS).

Quantification of tissue eosinophils and goblet cells

Eosinophils and goblet cells were identified by HE and PAS staining, respectively, and examined under the light microscope at $\times 40$ magnification. To estimate the eosinophilic and PAS-positive cell number, a systematic sampling was carried out with the computer-assisted stereological toolbox (CAST; Olympus, Hamburg, Germany). Fifty percent of each lung section was assessed, and eosinophilic cell numbers per square millimeter lung tissue or goblet cells per μ m basal membrane were appraised (41).

Measurements of anti-OVA immunoglobulins, IL-6, IL-5 and IFN- γ

Anti-OVA IgE, IgG1, and IgG2a were measured in the serum by an enzyme-linked immunosorbent assay (ELISA;

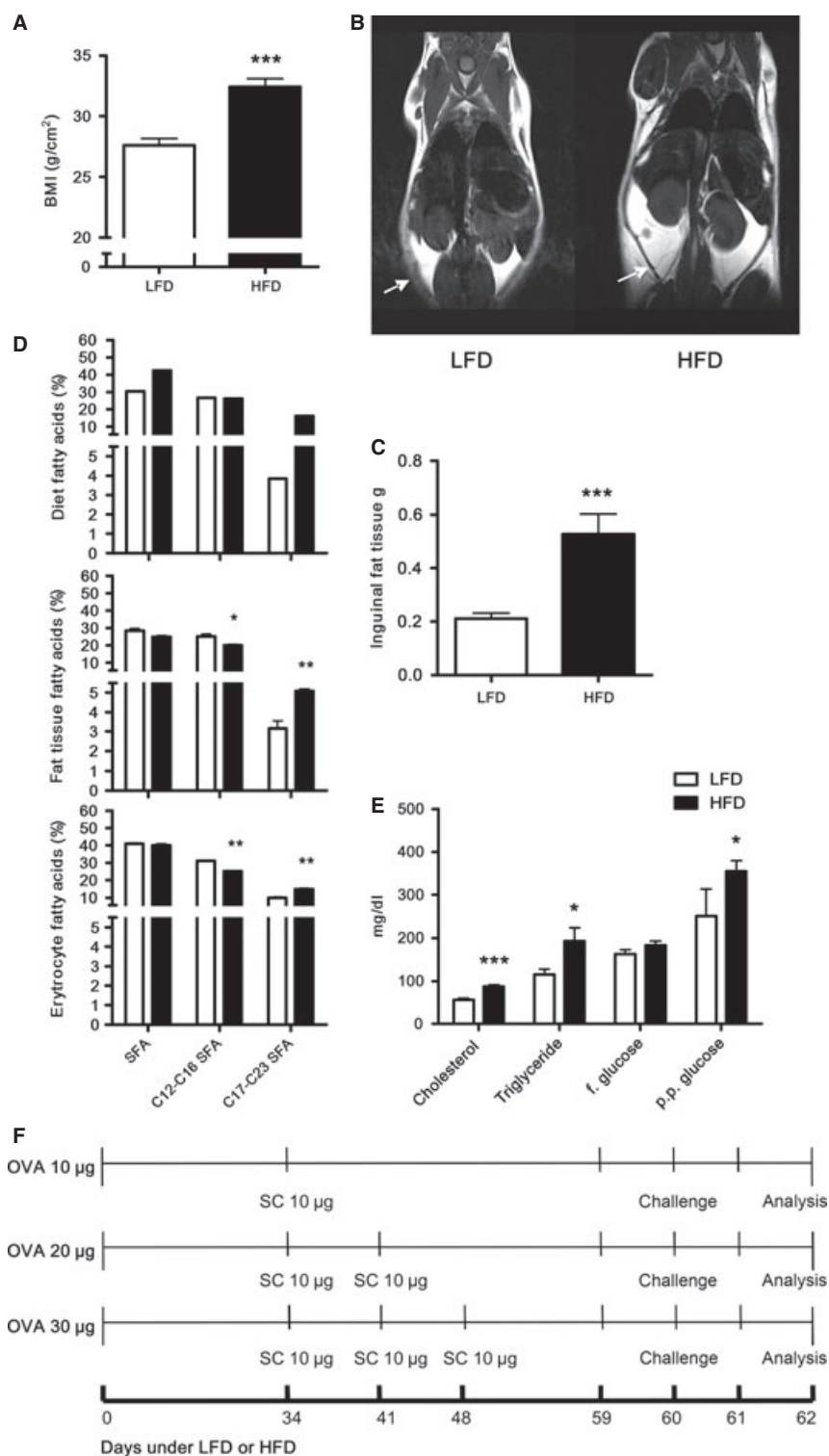


Figure 1 Metabolic assessment of the mice after 62 days of low-fat or high-fat diet feeding. (A), Body mass index ($n = 19$). (B), MRI images of the visceral fat tissue (arrow). (C), Weight of inguinal fat tissue ($n = 21$). (D), Saturated fatty acids (SFA) of different length (C12–C16, C17–C23) were quantified by gas chromatography–mass spectrometry as percentage of total fatty acids ($n = 3$).

(E), Concentration of serum fasting cholesterol, fasting triglycerides and fasting (f.) or postprandial (p.p.) blood glucose ($n = 7$). (F), Protocol to experimentally induce asthma by subcutaneous injections [ovalbumin (OVA) 10–30 µg] of and challenge with Ovalbumin (OVA) under HFD or LFD feeding. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

BD PharMingen, San Jose, CA, USA) as described previously (42). The detection limits were 10 pg/ml.

Serum IL-6 and BAL IL-5 and IFN- γ were measured with ELISA kits (OptEIA™; BD PharMingen) according to manufacturers instructions, the detection limits were 5 pg/ml.

Flow cytometry

Peritoneal macrophages were isolated by lavage with 5 ml of $1\times$ protease inhibitor cocktail in PBS (Roche). Cells from the peritoneal lavage were taken into 1 ml 1% BSA/PBS and stained for F4/80, CD11b and CD11c as described elsewhere (18). Stained cells were suspended in 250 μ l of FC fixation buffer (Cell Fix; BD PharMingen). Appropriate controls with irrelevant isotype immunoglobulins were carried out to ensure the specificity of the labeling. Fluorescence compensation was done as previously described for lung macrophages in our laboratory (43, 44). Small cells or cell debris was excluded by gating on the immune-cell-enriched cell population in the forward and sideward scatter as described elsewhere (45); (Fig. 5a). In this population, CD11b- and F4/80-positive cells were identified, both common markers for macrophages (Fig. 5a). CD11c labeling was then observed in the CD11b, F4/80 double-positive population (Fig. 5b). The percentage of CD11c-positive cells within the CD11b- and F4/80-positive population was calculated.

Magnetic resonance imaging

Animals were anesthetized by continuous isoflurane (2–2.5 ml/l) inhalation (Isoflurane Baxter; Baxter, Unterschleissheim, Germany). MRI was performed on a 7T small animal scanner (ClinScan; Bruker, Ettlingen, Germany) equipped with a mouse body coil of 35 mm diameter (Bruker) and breathing triggered data acquisition (PC Sam; SA instruments, Stony Brook, NY, USA). Coronal images of the abdomen were acquired using three different 3D sequences: a T1-weighted gradient echo sequence (TR: 150 ms, TE: 3.0 ms, FA: 25°, FoV: 25 $\times$ 25 mm, matrix: 128 $\times$ 128, slices: 32, slice thickness: 0.5 mm, voxel size: 0.195 $\times$ 0.195 $\times$ 0.5 mm³, acquisition time: 342 s), a T2-weighted turbo spin echo sequence (TR: 1100 ms, TE: 46 ms, FoV: 30 $\times$ 30 mm, matrix: 320 $\times$ 320, slices: 32, slice thickness: 0.7 mm, voxel size: 0.094 $\times$ 0.094 $\times$ 0.7 mm³, acquisition time: 415 s), and an in-phase/opposed-phase gradient echo sequence (TR: 12 ms, TE: 2.5 and 5 ms, FA: 15° FoV: 42 $\times$ 28 mm, Matrix: 192 $\times$ 192, TA: 306 s) to acquire pure water and pure fat images using the three-point Dixon method (46). The relative fat content of the abdomen was estimated in percent by dividing the pixel intensity of the fat-weighted images by the sum of pixel intensity of the water and fat-weighted images.

Transversal images of the lung to assess the lung volume were acquired during inspiration. A T1-weighted 3D sequence was used (TR 150 ms; TE 3.03 ms; FA 25°; FoV 25 $\times$ 25 mm; slices 32; slice thickness 0.5 mm; voxel size 0.20; acquisition time: 5.42 min). Lung images were analyzed for the lung volume using the MIPAV change fond software (NIH, Bethesda, Maryland, USA) as described previously (47).

Statistical analyses

All numerical data were expressed as means $\pm$ SEM. Data were analyzed for significance using the Student's unpaired *t*-test. Differences were considered significant at a *P*-value of <0.05 . Levels of significance were indicated as following: **P* ≤ 0.05 , ***P* ≤ 0.01 , ****P* ≤ 0.001 . Linear regression analyses were performed for correlation analyses with the body weight, and an *F*-test was performed to calculate whether the slope significantly deviated from zero. For all linear regression analyses, data were pooled from protocol OVA 30 μ g and OVA 20 μ g (Fig. 1f). Therefore, the mean across the HFD and LFD groups (separately for protocol OVA 30 μ g or OVA 20 μ g) was subtracted from each data value. Thereafter, the result was divided by the corresponding SD. Finally, the data were back-transformed to absolute values, by first multiplying the SD across all original data values, and second, the corresponding mean was added.

Results

Feeding a HFD results in obesity and metabolic syndrome-like changes

To investigate the effect of obesity on asthma, obesity-susceptible AKR mice were subjected to either HFD or normal diet, referred to as LFD. Body mass index, distribution of the white fat tissue and obesity typical metabolic changes were assessed consecutively.

Already within the first 14 days, animals in the HFD group had a significantly (*P* ≤ 0.01) higher body weight as compared with LFD fed animals (HFD 26 $\pm$ 4 g; LFD 22 $\pm$ 4 g, data not shown). This weight difference further increased until day 62 (HFD 37 $\pm$ 4 g; LFD 26 $\pm$ 4 g; data not shown) and resulted in a significantly higher (*P* ≤ 0.001) BMI in the HFD group (HFD 32 $\pm$ 0.7 g/cm²; LFD 28 $\pm$ 0.6 g/cm²; Fig. 1a). It is known that in addition to a high BMI, a particularly visceral and subcutaneously distributed fat tissue predisposes for obesity-related diseases (48,49). To assess its distribution, the fat tissue was quantified by abdominal MRI images (Fig. 1b). Animals in the HFD group had a 35% higher fat tissue/water ratio as compared to animals of the LFD group (*P* ≤ 0.05). Furthermore, the inguinal fat tissue was removed and weighed; this fat depot was also significantly (*P* ≤ 0.001) heavier in the HFD group (HFD 204 $\pm$ 15 mg; LFD 92 $\pm$ 4 mg; Fig. 1c).

Animals fed with HFD had an about 25% higher food intake as compared to LFD (data not shown). The composition of HFD differed from the LFD diet by a ~30% higher fat content with a distinct higher amount of C17–C23 saturated fatty acids (SFA), (Fig. 1d). To evaluate whether this differences in diet composition would lead to an altered body fat constitution, erythrocyte membranes and white fat tissue were analyzed. The high amount of C17–C23 SFA in the HFD was reflected by a significantly higher (*P* ≤ 0.01) percentage of this FA in both compartments (erythrocytes: HFD 15 $\pm$ 0.7%; LFD 10 $\pm$ 0.4%; white fat tissue: HFD 5 $\pm$ 0.1%; LFD 3 $\pm$ 0.4%; Fig. 1d). In contrast, the

percentage of C12–C16 FAs was significantly ($P \leq 0.05$) lower in both compartments for the HFD as compared to the LFD group (erythrocytes: HFD $25 \pm 0.4\%$; LFD $31 \pm 0.1\%$; white fat tissue: HFD $20 \pm 0.7\%$; LFD $25 \pm 1\%$; Fig. 1d). These data indicate that the FA composition of the diet affects the body fat composition.

Fasting cholesterol, triglycerides and blood glucose levels were measured every other week. Metabolic changes were detected from day 28 onwards (data not shown), as shown by a significantly ($P \leq 0.001$) higher serum fasting cholesterol levels in the HFD group (HFD 89 ± 3 mg/dl; LFD 60 ± 2 mg/dl). During the initial 4 weeks of HFD, fasting triglycerides remained unchanged and were significantly ($P \leq 0.05$) higher at day 62 (HFD 193 ± 31 mg/dl; LFD 114 ± 12 mg/dl; Fig. 1e). At this later time point also, increasing fasting glucose blood levels were detected (HFD 183 ± 10 mg/dl; LFD 162 ± 10 mg/dl; Fig. 1e), and together with significantly ($P \leq 0.05$) elevated postprandial blood glucose levels (HFD 355 ± 25 mg/dl; LFD 251 ± 63 mg/dl; Fig. 1e), this indicates proceeding glucose intolerance in the HFD group.

HFD increases the susceptibility to allergic sensitization

To investigate whether obesity affects allergic sensitization an adjuvant-free protocol of sensitization (37) was started at day 28, after the first metabolic changes were present (Fig. 1f).

The protocol included three increasing steps of sensitization doses, starting with a single, two or three subcutaneous injections of OVA. Injections of OVA did neither affect the weight of the animals nor the metabolic changes that were observed under HFD feeding (data not shown).

The level of sensitization was assessed by measuring anti-OVA-IgE, -IgG2a, and -IgG1 serum antibody concentration. Control injections of PBS did not induce any antibody responses in the animals (data not shown). A single dose of OVA was insufficient to induce measurable anti-OVA-IgE, anti-OVA-IgG1 or anti-OVA-IgG2a antibody responses in any study group. After two subcutaneous injections of OVA (OVA 20 μ g), anti-OVA IgE levels were significantly ($P \leq 0.01$) higher in the HFD group (86 ± 11 ng/ml) as compared to the LFD group (20 ± 9 ng/ml). If animals were sensitized three times (OVA 30 μ g), the difference between the diet groups was lost ($P \geq 0.1$), and anti-OVA IgE levels were similarly high in the LFD (71 ± 8 ng/ml) and in the HFD group (94 ± 11 ng/ml, Fig. 2a).

Furthermore, a positive correlation ($P \leq 0.05$; $r^2 = 0.2$) was found for anti-OVA IgE serum levels, and the body weight of the animals, together with an inverse correlation ($P \leq 0.05$; $r^2 = 0.2$) for the anti-OVA IgG2a isotype (Fig. 2b). IgG2a levels were always lower in animals of the HFD as compared to the LFD group (Fig. 2a). Anti-OVA IgG1 levels increased with increasing sensitization doses. There were no significant differences between the HFD and LFD groups in anti-OVA

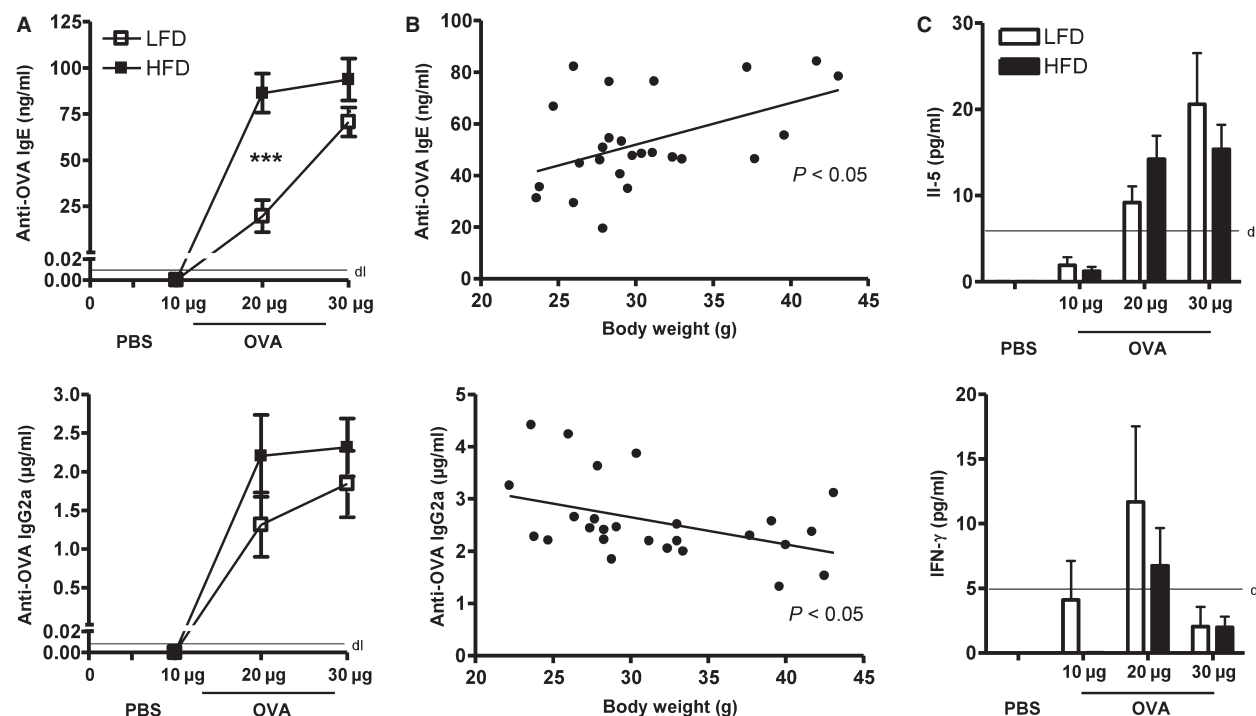


Figure 2 Effects of obesity on allergic sensitization. (A), Anti-ovalbumin (OVA) IgE and anti-OVA IgG2a antibody titers in the serum of mice treated with protocol OVA 10–30 μ g (Fig. 1), ($n = 7$). (B), Correlation of anti-OVA IgE and IgG2a with the body weight. The data were normalized by the mean and the SE. LFD and HFD

animals from OVA 20 μ g and OVA 30 μ g were combined ($n \geq 25$). Dotted lines indicate the 95% confidence interval; $r^2 = 0.2$ for IgE and IgG2a (C), Concentration of IL-5 and IFN- γ in the broncho-alveolar lavage fluid ($n = 7$).

IgG1 levels, and no significant correlation with the body weight was found (Data not shown).

HFD augments allergic airway inflammation but not mucus production

Following OVA sensitization, the asthma phenotype was induced by three consecutive aerosol allergen challenges. IFN- γ and IL-5 production were measured in BAL fluids, as markers of TH1, TH2, and T-cell activation, respectively. IL-5 levels were increased in both diet groups after sensitization to and challenge with OVA, with a trend to higher levels in the HFD group after protocol OVA 20 μ g (Fig. 2c). In contrast, IFN- γ levels were lower in the HFD as

compared to the LFD group, following protocol OVA 20 μ g (Fig. 2c).

Accumulation of eosinophils in the lung and an increasing number of goblet cells in the airway epithelium is characteristic for acute asthma exacerbation. To enumerate eosinophil numbers, lung sections were analyzed by a systematic and randomized approach using the CAST System. After sensitization to and challenge with OVA, eosinophils infiltrated the lung particularly in the peribronchial and perivascular space (Fig. 3a), while there were almost no eosinophils in the lung tissue of PBS-treated mice (0.2 ± 0.15 cells/mm²). After OVA treatment, a significantly ($P \leq 0.05$) higher number of eosinophils was found in the HFD (15.9 ± 1.1 cells/mm²) compared with the LFD group (11.8 ± 1.3 cells/mm²; Fig. 3b).

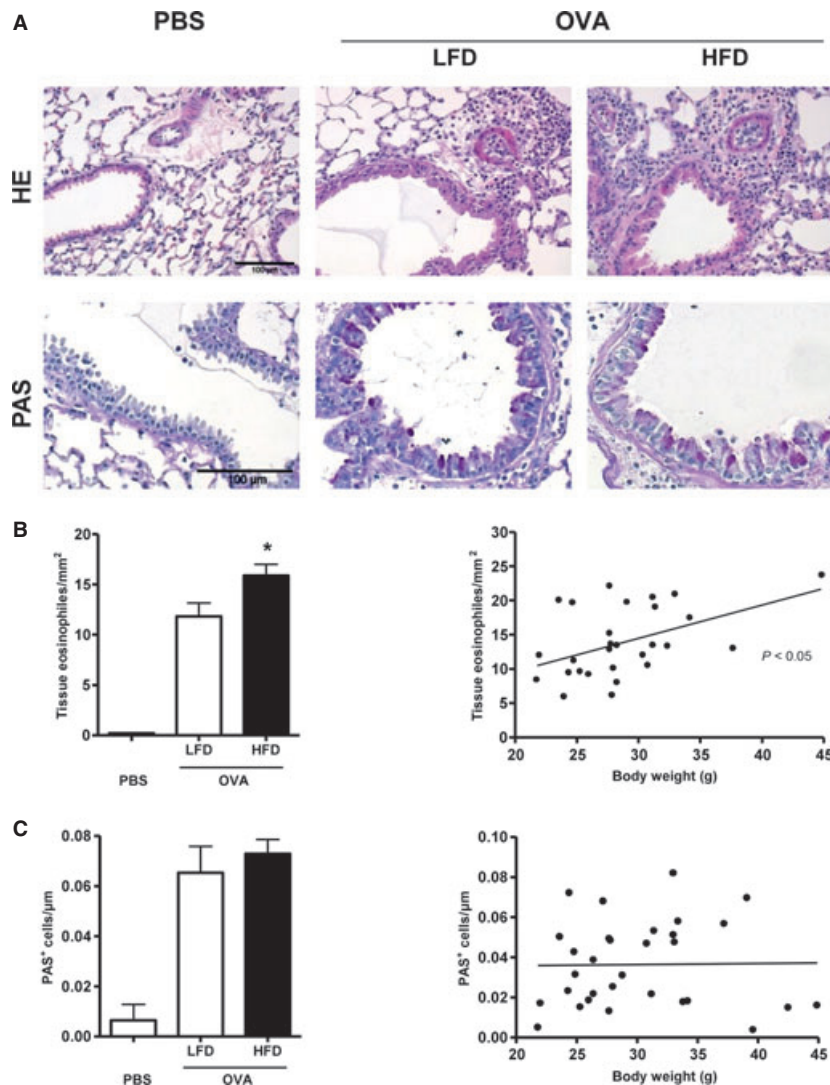


Figure 3 Effect of high-fat diet on the allergic airway inflammation. (A), Following protocol ovalbumin (OVA) 30 μ g, lung sections were stained with hematoxylin–eosin (HE) to identify lung infiltrating leukocytes or periodic acid shift (PAS)-positive cells to identify goblet cells. (B, C), numbers of eosinophils and goblet cells were

correlated with body weight. The data were normalized by the mean and the SE. LFD and HFD animals from OVA 20 μ g and OVA 30 μ g were combined ($n \geq 28$) eosinophils $r^2 = 0.2$; goblet cells $r^2 = 0.0003$.

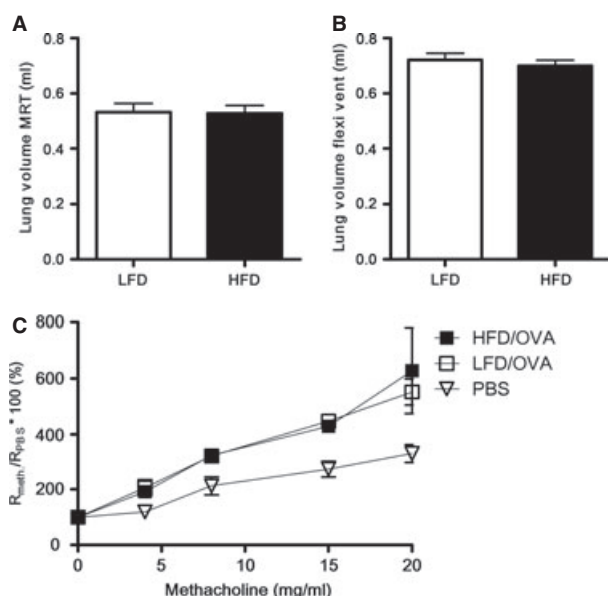


Figure 4 Lung physiology in low-fat diet and high-fat diet fed animals. (A), Lung volume after spontaneous inspiration calculated from MRI images, ($n = 7$). (B), total lung capacity measured in the flexi-vent ($n = 7$). (C), airway responsiveness defined as the resistance after inhalation of increasing amounts of methacholine (R_{meth}), divided by the baseline resistance before methacholine (R_{PBS}), ($n = 7$).

Eosinophil cell numbers furthermore correlated positively ($P \leq 0.05$; $r^2 = 0.2$) with the body weight of the animals (Fig. 3b). PAS-positive goblet cells were quantified in the airway epithelium of the mice. After OVA treatment, the number of PAS-positive cells was about tenfold higher (HFD 0.07 ± 0.01 cells/ μm ; LFD 0.07 ± 0.01 cells/ μm) compared with the PBS group (0.006 ± 0.01 cells/ μm). However, goblet cell numbers were not affected by the diet nor did the body weight of the animals correlate ($P \geq 0.05$; $r^2 = 0.0003$) with the number of PAS-positive cells (Fig. 3c).

No differences in lung function between obese and lean animals

To test whether increasing body fat might have restrictive effects on the anatomical or physiological lung volume, static lung volume with forced inspiration was measured in the flexi-vent system and dynamic lung volume in the MRI during normal breathing of the mice. Both methods confirmed that the lung volume remained unaffected by the diet or the body weight of the animals (Fig. 4a,b). As worsening of lung inflammation might be associated with increasing methacholine-induced airway hyper-responsiveness (AHR), invasive lung function measurements were performed in the animals. In both diet groups, methacholine-induced AHR was clearly increased in mice challenged with OVA (OVA $30 \mu\text{g}$) as compared to mice treated with PBS; however, there was no difference in the AHR between the two diet groups (Fig. 4c).

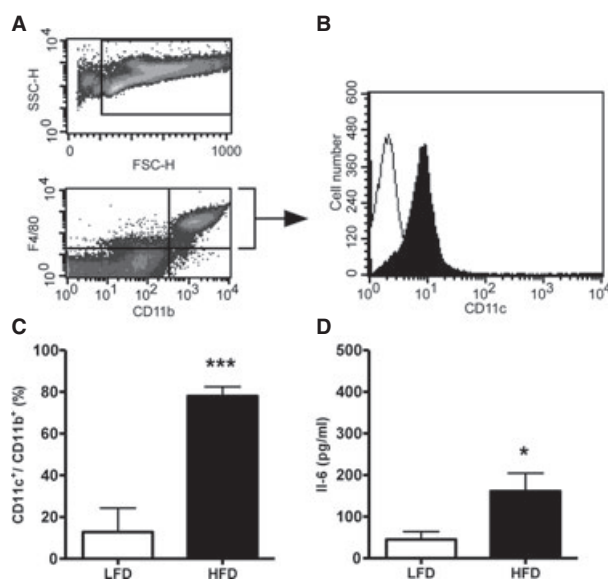


Figure 5 Immunological changes after high-fat diet. (A), Representative flow cytometry to identify macrophages by their expression of CD11b and F4-80. (B), CD11b⁺/F4-80⁺ cells were analyzed for CD11c expression. (C), Percentage of CD11c⁺ cells on CD11b⁺/F4-80⁺ macrophages in the peritoneal lavage of nonasthmatic mice ($n = 5$). (D), IL-6 in the serum of nonasthmatic animals ($n = 7$).

Obesity is associated with a systemic inflammatory response

It is well established that obesity induces inflammatory immune responses that are characterized by the increased frequency and activation of a pro-inflammatory, IL-6-producing macrophage subtype in the fat tissue (18,50). These fat tissue macrophages are distinguished by CD11c expression compared with CD11c-negative, IL-10-producing anti-inflammatory macrophages. (18,50). Macrophages from the peritoneal lavage were characterized by CD11b and F4-80 expression via FC at day 62 (Fig. 5a). In this population, a high percentage of CD11c-positive macrophages was found in the HFD group (HFD $78 \pm 5\%$; LFD $13 \pm 11\%$; Fig. 5b,c). In the LFD group, most macrophages were CD11c negative (Fig. 5b,c). In parallel, IL-6 was measured in serum samples as a marker of systemic inflammation. A significant ($P \leq 0.05$) increase in IL-6 serum levels (HFD 162 ± 43 pg/ml; LFD 45 ± 19 pg/ml) was detected in the HFD group (Fig. 5d).

Discussion

A mouse model of obesity with profound metabolic alterations was established to investigate the impact on allergic sensitization and lung inflammation in experimentally induced asthma. It was found, for the first time, that obesity lowered the threshold of allergic sensitization, as smaller amounts of allergen were sufficient to induce a comparable level of allergen-specific antibodies as compared to lean mice.

Allergen-specific IgE levels correlated positively with the body weight of the animals. Importantly, an increasing body weight was also correlated with worsened eosinophilic airway inflammation.

A higher prevalence of asthma in obese and overweight adults and children has been demonstrated by several clinical studies (3,51,52). The concept that there exists a cause–effect relationship between obesity and asthma development is supported by the observation that obesity precedes asthma (3,51,52).

To further explore underlying mechanisms, a variety of genetically engineered animals have been employed, including obese *ob/ob* and *db/db* mice, Cpe fat mice, carboxypeptidase E $-/-$ mice, and others (26–28). However, genetically engineered models only allow the assessment of that particular gene on the development of phenotype. In contrast, our wt approach allows the assessment of the complex cellular and molecular nature of phenotype development independent of the genetic background.

Other studies that induced obesity by HFD feeding used C57BL/6 mice (29,30,53). As these mice poorly develop AHR in OVA-induced asthma models (54), we chose AKR mice, which are prone to a phenotype of AHR and obesity (55,56). Long-term HFD feeding caused a prestage of metabolic syndrome (57) and a particularly visceral distribution of body fat; both are established predictors for the development of chronic diseases (48,49,58). In contrast, previous studies of HFD feeding induced a rather low (29) or no (53) body weight increase. Following allergen sensitization and challenge, AKR mice developed a robust TH2-related inflammatory response with IgE and IL-5 production in our model. Obese individuals are characterized by the presence of a low-grade chronic systemic inflammatory response as defined by increased circulating leukocytes, increased serum concentrations of certain cytokines (adipocines); (15,59), cytokine receptors, and acute-phase protein (60–62). A link between obesity and (systemic) inflammation has been recently supported by studies in patients, showing decreased responses to steroids in obese asthmatics (63) and a higher percentage of neutrophils in induced sputum together with increase in TLR-4 gene expression after a high-fat meal (64). Innate immunity plays an important role during asthma development, IL-6 (65–67) and activation of TLR-2 and TLR-4 (68) are known to facilitate TH2 differentiation. Previously, the pro-inflammatory state in obesity was linked with an increased number and activation state of pro-inflammatory macrophages, whereas in lean animals, the fat tissue contains only a few rather anti-inflammatory macrophages (69). These pro-inflammatory macrophages are characterized by the expression of CD11c on their surface in addition to common macrophage markers such as CD11b and F4/80 (69). Furthermore, these macrophages show a strong activation by free fatty acids and certain saturated fatty acids (70–72) via TLR 2 and 4 (20,21) to produce cytokines including IL-1 β , IL-6, MCP-1, and TNF- α (73), whereas CD11c-negative macrophages produced more IL-10 (18,69,74). Importantly, TLR 2 and 4 activation and subsequent cytokine expression is associated with increased severity of asthma exacerbation (75).

These observations are in line with our findings, linking metabolic changes with increased concentrations of saturated fatty acids, increased influx, and activation of CD11c-positive macrophages, systemic low-grade inflammation and an increased state of allergic sensitization with an up-regulated TH2-immune response, whereas most previous animal studies of HFD feeding did not take inflammatory changes into account (29–31,53).

As clinical studies show that particularly childhood obesity predisposes for higher incidence and severity of adult asthma (76), we wanted to investigate whether obesity also influences the development of allergic sensitization. Previous animal studies focused on the investigation of acute exacerbation phase (29,30,53) and can therefore not explain why the asthma prevalence increases in obese patients. In the current study, it was found that the TH2-related anti-OVA IgE antibody response was positively and the TH1-related anti-OVA IgG2a antibody response was negatively correlated with the weight of the animals. Previous studies compared diet groups instead of assessing individual mice in correlation with their weight (29,53). Therefore, they might have missed an association between these two variables. The increase in IgE levels under HFD supports previous clinical studies showing elevated IgE antibody levels in obese asthmatics (77,78). Moreover, a lower dose of allergen was required in the obese animals to induce comparable IgE antibody titers as in non-obese mice. Interestingly, only the protocol with a medium cumulative allergen dose (OVA 20 μ g) caused a significant difference in IgE levels between the obese and lean mice. We suggest that there is a dose window that allows the observation of obesity effects on allergic immune responses in asthma. If a certain critical allergen dose is exceeded, the obesity effects on the asthmatic phenotype are overwhelmed by a saturated antibody response to the allergen.

Furthermore, we examined whether the observed changes affect the asthmatic phenotype. A positive correlation of lung eosinophilia and the body weight was found.

Surprisingly, no effects on the animals' lung function were apparent. This could be explained by the genetic AKR background that predisposes for an overreaction to methacholine-induced AHR and might therefore prevent the detection of minor changes in AHR (41). The relationship between inflammation and AHR is still not entirely clear. We and others have previously observed in some models that AHR develops in the absence of airway inflammation (79–81). Conversely, even in the presence of airway inflammation, AHR does not always develop in parallel (54). Therefore, the proposed concept is that there are certain inflammatory pathways that may alter AHR, whereas other inflammatory pathways do not have an effect on airway contractility. Also, increased airway contractility may develop in the absence of airway inflammation, indicating that AHR might be the common phenotype of different signaling pathways. Applying this concept to this study, it clearly shows that the state of inflammation induced in the obese mice has no effect on AHR. This is also in line with several clinical studies showing that obesity does not predispose for AHR (82,83) but rather predispose for a higher incidence of asthma and a 'worse to

treat' phenotype with more frequent asthma exacerbation (9–12). In fact a most recent study even shows a protective effect of overweight on AHR but still negative effects on other symptoms as wheezing (84).

In summary, here it was shown that obesity leads to severe and sustained metabolic and inflammatory changes. These system alterations might predispose for a TH2-biased immune response, with lower allergen threshold as well as increased IgE and suppresses IgG2a antibody responses. In turn, this could lead to aggravated asthma exacerbation with increased release of TH2 cytokines and airway eosinophilia.

Conclusions

The presented study supports the concept of a cause–effect relationship between obesity and asthma. In particular, childhood obesity is a strong risk factor for the later development of allergic asthma. Our findings provide strong evidence that this could be due to a decrease in sensitization threshold in obese individuals. This increases the likelihood of allergic sensitization to even small doses of allergen and a concurrently more severe effector phase.

Obesity is strongly related to immunological changes causing a low-grade systemic inflammation. Most likely, this

immune modulation predispose for stronger allergen sensitivity and TH2 differentiation (85). Markers of inflammation might be even used as predictors for later development of asthma and are known as risk factors for asthma exacerbation (86). It is therefore important that animal models to study obesity effects on allergy closely mimic the metabolic phenotype of obese patients and strongly control for obesity-related immunological changes. With respect to our findings, it seems relevant for further studies to rather focus on the pathology of sensitization than exacerbation of obesity-associated asthma.

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Conflict of interest

None.

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