

Iron administration reduces airway hyperreactivity and eosinophilia in a mouse model of allergic asthma

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Introduction

The prevalence of allergic diseases, such as allergic asthma, hay fever and atopic dermatitis, has increased markedly in children over the past four decades [1]. Many explanations for this increase have been postulated, of which the hygiene hypothesis [2] has gained considerable attention in the last two decades. However, an undisputed causal explanation is still lacking [3]. Associated with the increased prevalence of allergic diseases, during the last decades there has been a steady increase in iron intake by infants in affluent societies [4,5]. Reasons for the increased iron intake by infants are: (i) reduced frequency and duration of exclusive breastfeeding; (ii) increased use of iron-fortified formula milk; and (iii) increasing concentrations of iron in the formula milk to about 20 times the level in human breast milk [6]. In addition, efforts have been made to enhance the uptake of the iron from the formula by switching to the use of highly bioavailable forms of iron, such as ferrous sulphate, and by adding vitamin C. The latter agent stimulates iron uptake by keeping it in its reduced and more bioavailable form [7]. Furthermore, since 1972 there has been a widespread intro-

Summary

The prevalence of allergic diseases has increased dramatically during the last four decades and is paralleled by a striking increase in iron intake by infants in affluent societies. Several studies have suggested a link between increased iron intake and the marked increase in prevalence of allergic diseases. We hypothesized that the increased iron intake by infants offers an explanation for the increased prevalence of allergic disease in industrialized societies during the past four decades. A well-established mouse model of ovalbumin (OVA)-driven allergic asthma was used to test the effects of differences in iron intake and systemic iron levels on the manifestations of allergic asthma. Surprisingly, iron supplementation resulted in a significant decrease in airway eosinophilia, while systemic iron injections lead to a significant suppression of both allergen-induced airway eosinophilia and hyperreactivity compared to placebo. In contrast, mice fed on an iron-deprived diet did not show any difference in developing experimentally induced allergic asthma when compared to those fed on an iron-sufficient control diet. In contrast to our hypothesis, airway manifestations of allergic asthma are suppressed by both increased levels of iron intake and systemic iron administrations in the mouse model.

Keywords: airway hyperreactivity, allergy, asthma, dietary iron, eosinophilia

duction of iron-fortified food in the weaning diet [4,5]. We hypothesized that the marked increase in iron intake by infants offers an explanation for the increased prevalence of allergic diseases during the last decades.

Although an epidemiological study addressing the association between increased levels of dietary iron and the increased prevalence of allergic diseases is still lacking, there is evidence suggesting a link between increased iron intake and the marked increase in prevalence of allergic diseases. The prevalence of asthma is higher in patients with disorders such as thalassaemia minor and sickle cell anaemia, which cause elevated systemic iron levels compared to patients without haemoglobinopathies [8]. Furthermore, it has been observed that asthmatic patients show increased plasma levels of iron compared to healthy controls [9]. Moreover, data from animal models also point to a role for iron in allergic manifestations. It has been shown that administration of an iron chelator significantly decreases the serum levels of immunoglobulin (Ig)E in a rat model of T helper type 2 (Th2)-mediated autoimmunity [10]. Additionally, lactoferrin – a natural iron chelator – plays a protective role against symptoms of experimental allergy in a mouse model

[11]. Furthermore, it has been shown that iron overload strikingly increases serum levels of antigen-specific IgE in experimental candidiasis in mice [12].

Altogether, these data suggest a causal relationship between iron metabolism and the development of allergic diseases. Nevertheless, the effects of dietary iron supplementation on allergic manifestations have not yet been tested experimentally in detail. In the present study, we use a well-established mouse model of allergic asthma to investigate whether increased iron intake augments the manifestations of experimental allergic asthma. We further addressed the impact of elevated level of systemic iron on the symptoms of experimental allergic asthma. Finally, we tested the effects of dietary iron deprivation on the manifestations of experimental allergic asthma. Remarkably, we found that increased serum iron levels significantly reduce allergen-induced airway hyperreactivity (AHR) to methacholine and airway eosinophilia but do not affect allergen-specific IgE levels.

Materials and methods

Animals

Specified pathogen-free (SPF) [according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines] 3-week-old male BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France) and were kept under SPF conditions. All animal experiments were performed in accordance with the guidelines of the institutional animal care and use committee of the University of Groningen.

Iron administration

In the first experiment, mice were maintained on drinking water containing either 250 mg Fe/l as FeSO₄ +1% ascorbic acid (Sigma-Aldrich, Dordrecht, the Netherlands) or only 1% ascorbic acid starting 2 weeks before sensitization until the end of the experiment. Because the concentration of iron in the formula milk is increased to about 20 times the level in human breast milk [6], the dose of supplemented iron in this experiment has been adjusted to 20 times the concentration of iron in mouse milk, which is 12–15 mg/l [13].

For the second experiment, injectable iron dextran (Cosmofer®, Pharmacosmos A/S, Holbaek, Denmark; 60 mg/kg for treatment group) or an equivalent amount of low-density dextran (M_w ≈ 6000 for the control group; Sigma-Aldrich) was injected intraperitoneally (i.p.) on alternate days starting 2 weeks prior to sensitization until the end of the experiment. The dose of injectable iron was determined based on the non-toxic dose of iron used previously by Mencacci *et al.* [12].

In the last experiment mice were fed on casein-based semi-synthetic chow (Research Diet Services B.V., Utrecht, the Netherlands) containing either 7 or 35 parts per million

(p.p.m.) iron. Mice were maintained on the diet from 2 weeks prior to sensitization until the end of the experiment.

Protocol for the induction of experimental allergic asthma

Experimental allergic asthma was induced as described elsewhere [14]. Briefly, mice received two i.p. injections of 10 µg ovalbumin (OVA) (Sigma-Aldrich) + 2.25 mg alum (Pierce, Rockford, IL, USA) in 100 µl of pyrogen-free saline on days 0 and 7. Two weeks later, they were exposed to aerosolized OVA solution in saline (1% w/v) for 20 min three times every third day.

Evaluation of airway responsiveness

Airway responsiveness to inhaled methacholine (Sigma-Aldrich) was measured twice (before and after aerosolized OVA inhalation challenges) in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco; EMKA Technologies, Paris, France), as described in detail previously [14].

Determination of serum levels of OVA-specific IgE

After measuring airway responsiveness, blood was taken and serum samples were prepared and stored at a temperature of –80°C until further analysis. Serum levels of OVA-specific IgE were determined by enzyme-linked immunosorbent assay (ELISA) as described previously and results are expressed as EU/ml [15].

Analyses of the bronchoalveolar lavage (BAL) fluid

BAL was performed as described previously [15]. In brief, animals were lavaged five times through the tracheal cannula with 1 ml aliquots of saline containing a cocktail of protease inhibitors [complete mini tablet (Roche Diagnostics, Almere, the Netherlands) and 1% bovine serum albumin (BSA; Sigma-Aldrich)]. BAL cells were counted, cytopspins were made and stained using Diff-Quik (IMEB, San Marcus, CA, USA). Cells were identified by standard morphology.

Preparation of lung tissue for cytokine measurement

Cardiac lobe of lung was taken, homogenized and used to measure cytokine levels as described previously [16]. Concisely, lung tissue was homogenized in 20% (w/v) Luminex buffer (50 mM Tris-HCl, 150 mM NaCl, 0.002% Tween 20 and protease inhibitor, pH 7.5) on ice. Subsequently, supernatants were collected for cytokine measurement after spinning the lung tissue homogenates for 10 min at 12 000 g.

Measurement of cytokines

Interleukin (IL)-4, IL-5 and IL-13 in the lung tissue were determined by a commercially available ELISA kit according

to the manufacturer's instructions (BD Pharmingen, Franklin Lakes, NJ, USA). The detection limits were 32 pg/ml for IL-5 and 15 pg/ml for IL-4 and IL-13.

Serum iron quantification

Total iron in serum was measured using colorimetric Bathophenanthroline (Sigma-Aldrich) assay and according to the Ramsay method [17], with minor changes. In brief, the optical density (OD) of the reaction resulted from protein-precipitated serum with Bathophenanthroline was read in 96-well half-area plates (Greiner Bio-One B.V.) using an ELx808™ microplate reader. The concentration of serum iron was calculated using standard curves drawn based on measured OD values of known serial dilutions of iron (II) solution. To measure total iron-binding capacity (TIBC), serum was first saturated using iron chloride solution (Sigma-Aldrich) followed by removal of unbound iron. The concentration of total of iron was then measured using the above-mentioned steps.

Haemoglobin measurement

Haemoglobin concentration in serum was measured using a commercially available kit (QuantiChrom™ Hemoglobin Assay Kit, BioAssay Systems, Hayward, CA, USA), according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean \pm standard error of the mean (s.e.m.). The airway resistance curves to methacholine were analysed statistically using a general linear model of repeated measurements. All the other data were compared using Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

Results

Dietary iron supplementation reduces airway eosinophilia

To address the question of whether increased iron intake augments the manifestations of experimental allergic asthma, mice were maintained on drinking water supplemented with either 250 mg/l iron +0.1% w/v ascorbic acid or 0.1% w/v ascorbic acid as control throughout the experimental procedure (Fig. 1). According to the protocol, mice were first sensitized by two OVA/alum i.p. injections followed by three inhalation challenges with OVA aerosols 3 weeks later. Airway hyperresponsiveness (AHR) to methacholine was evaluated before and after the airway challenges. We did not observe any significant differences between experimental groups in AHR (Fig. 2a). Nevertheless, we did observe a small but significant decrease in allergen-induced airway eosinophilia in the group

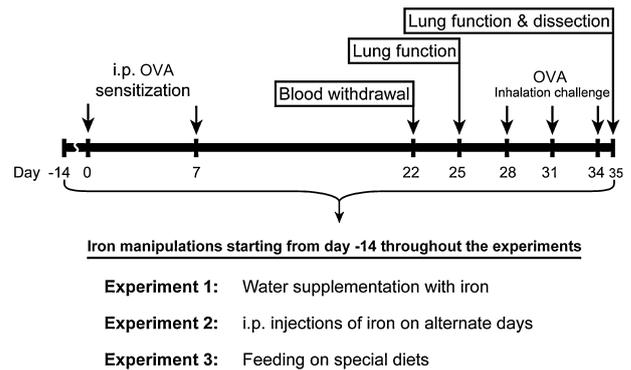


Fig. 1. Experimental layout. A mouse model of ovalbumin (OVA)-driven allergic asthma was used to test the effects of increased levels of oral or systemic iron or decreased levels of dietary iron on the manifestation of experimentally induced allergic asthma. Mice were sensitized intraperitoneally (i.p.) twice at days 0 and 7, followed by three inhalation provocations with aerosolized OVA solution (1% in phosphate-buffered saline) on days 28, 31 and 34 and dissected on day 35. In experiment 1, mice were maintained on drinking water containing either 250 or 35 mg/l iron as FeSO₄ starting 2 weeks before i.p. OVA sensitization until the end of the experiment. In experiment 2, mice received iron injections 60 mg/kg body weight given as iron dextran on alternate days starting at day -14 before i.p. OVA sensitization until the end of the experiment. In experiment 3, mice were fed on iron-deprived diet containing 7 mg/kg food or iron-sufficient diet containing 35 mg/kg food from 14 days before i.p. sensitization throughout the experiment.

receiving increased concentrations of dietary iron in the drinking water compared to the control group ($P < 0.05$, $1.3 \times 10^6 \pm 3.0 \times 10^5$ cells in BAL in iron-supplemented compared to $1.9 \times 10^6 \pm 2.2 \times 10^5$, Fig. 2b). Serum levels of OVA-specific IgE were not different between the two experimental groups (Fig. 2c). Surprisingly, no difference between iron-supplemented and control groups was observed (Fig. 2d) in total iron concentration in serum or in TIBC, which reflects transferrin levels in serum [18]. Body weight gain was monitored constantly during the experiment and no significant differences between groups were observed.

Systemic administration of iron decreases allergen-induced airway eosinophilia and Th2 cytokine release

Because total iron concentration in serum was not increased by iron-supplemented drinking water, we used a more robust strategy to increase the systemic iron levels. To this end, mice received i.p. injections of iron dextran (Cosmofer®; 60 mg/kg Fe) on alternate days throughout the asthma induction protocol (Fig. 1). As expected, total iron concentration in serum and TIBC were increased significantly in the iron treatment group compared to control ($P < 0.05$, $169 \pm 31 \mu\text{mol/l}$ compared to 34 ± 2 and $252 \pm 38 \mu\text{mol/l}$ compared to 97 ± 6 , Fig. 3d). Increased

Fig. 2. Effects of iron supplementation on the manifestation of allergic asthma. Manifestations of experimentally induced allergic asthma in mice receiving iron supplemented drinking water (black and hatched bars) or control water (open and grey bars): (a) airway responsiveness to methacholine before (open and hatched bars) and after inhalation challenge (grey and black bars); (b) numbers of eosinophils, neutrophils and mononuclear cells in bronchoalveolar lavage; (c) levels of OVA-specific serum immunoglobulin (Ig)E before (open bars) and after (black bars) inhalation challenge; (d) total iron and total iron-binding capacity concentrations in serum. Data are shown as mean \pm standard error of the mean, $n = 8$ (* $P < 0.05$; *** $P < 0.005$).

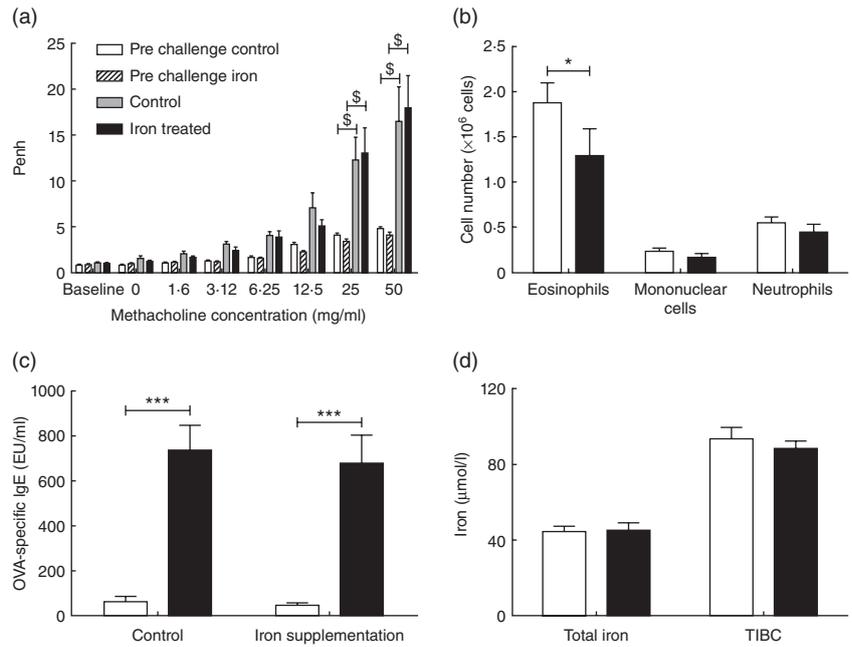
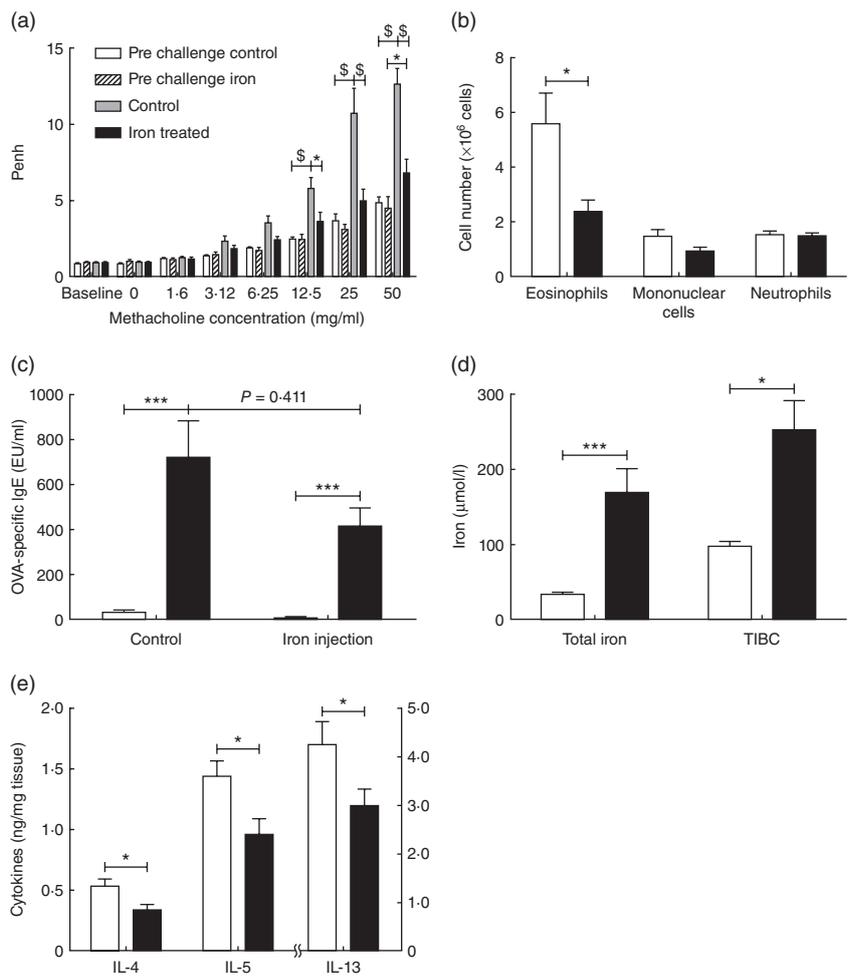


Fig. 3. Effects of parenteral iron administration on the manifestation of allergic asthma. Manifestations of allergic asthma in mice receiving iron injections (black and hatched bars) or control injections (open and grey bars): (a) airway responsiveness to methacholine before (open and hatched columns) and after inhalation challenges (grey and black-filled columns); (b) numbers of eosinophils, neutrophils and mononuclear cells in bronchoalveolar lavage; (c) levels of OVA-specific serum immunoglobulin (Ig)E before (open bars) and after (black bars) inhalation challenge; (d) total iron concentration in serum and total iron and total iron-binding capacity; (e) cytokines levels in the lung tissue. Data are shown as mean \pm standard error of the mean, $n = 8$ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$).



total iron in serum and TIBC indicate that current iron administration method is suitable for dissecting the effects of increased serum levels of iron on the manifestations of allergic asthma in our mouse model.

We measured AHR to methacholine before and after airway challenges, followed by dissecting the mice, counting airway eosinophils and measuring Th2 cytokines in lung and allergen-specific IgE levels in serum. As presented in Fig. 3b, airway eosinophilia was decreased markedly and significantly in iron-treated mice compared to dextran-treated controls ($P < 0.05$, 2.0×10^6 cells compared to 5.4×10^6). Surprisingly, parenteral iron treatment almost completely inhibited the allergen-induced AHR compared to dextran-treated control mice ($P < 0.05$, e.g. at dose 50 mg/ml 6.8 ± 0.9 Penh compared to 12.6 ± 1.0 Fig. 3a). However, iron treatment did not affect serum levels of OVA-specific IgE ($P = 0.12$, Fig. 3c). Because, in this experiment, total iron concentration in serum and TIBC were clearly increased and a significant suppressive effect was observed for iron administration on airway manifestation of allergic asthma, we investigated the T cell response in the lung tissue by measuring IL-4, IL-5, IL-13, interferon (IFN)- γ and IL-10 in the lung tissue. Levels of the Th2 cytokines IL-4, IL-5 and IL-13 in lung tissue were reduced significantly in iron-treated mice compared to dextran-treated controls ($P < 0.05$, 342 ± 36 compared to 534 ± 38 , 961 ± 126 compared to 1437 ± 127 and 2997 ± 335 compared to 4248 ± 471 , respectively, Fig. 3e). Neither IFN- γ nor IL-10 levels in the lung tissue were affected by iron treatment (data not shown). These data show that differences in serum levels of iron have a direct effect on manifestations of allergic asthma in the mouse model, with a marked reduction of AHR and airway eosinophilia in mice with increased levels of serum iron and TIBC.

Low dietary iron does not affect the development of allergic asthma

In contrast to our initial hypothesis, we found that increased iron intake and high systemic iron administration suppress the manifestations of allergic asthma in the mouse model. Therefore, we asked the question of whether restricting dietary iron causes augmented allergic response. It is recommended that mice are maintained on diets containing at least 35 p.p.m. iron to prevent iron deficiency [19]. However, lower concentrations of iron have been considered sufficient and used previously in other studies without causing deficiencies [20]. Therefore, we examined the effect of a low-iron diet (7 p.p.m. iron) compared to iron-sufficient control diet (35 p.p.m. iron) in our mouse model of allergic asthma. As presented in Fig. 1, mice were fed on diets starting 2 weeks prior to the first i.p. sensitization and continued throughout the experiment. The total iron concentration in serum at the end of the experiment showed a trend towards a decrease ($P = 0.067$, 26.8 ± 3.3 compared to 35.5 ± 2.4 , Fig. 4d) in the low iron diet group compared to the iron-sufficient

control group. Nevertheless, the mice maintained on a low dietary iron diet did not show any difference in allergen-induced airway eosinophilia, AHR and serum levels of OVA-specific IgE compared to mice fed on a regular iron diet (Fig. 4a–c). Haemoglobin concentration was measured in this experiment as an indicator of iron body status, and showed no difference between mice fed on low iron containing chow compared to control-treated mice (Fig. 4e).

Discussion

In the present study we demonstrate that in contrast to our initial hypothesis, increased iron intake does not exacerbate the manifestations of experimental allergic asthma. Conversely, high iron intake induces a small but significant decrease in allergen-induced airway eosinophilia, while serum allergen-specific IgE levels and AHR are not affected. Moreover, we show that elevated levels of systemic iron due to parenteral iron administration impacts strongly upon the symptoms of experimental allergic asthma by completely inhibiting AHR and largely suppressing airway eosinophilia as well as Th2 cytokines in lung tissue.

Remarkably, our data show that supplementation of drinking water with iron does not increase the total iron concentration in serum compared to control mice. This is due most probably to a phenomenon known as mucosal blockade of iron absorption, which leads to a rapid reduction in iron absorption from the gut subsequent to a high-dose iron intake [21]. Interestingly, it has also been shown that iron supplementation does not increase iron absorption in infants under the age of 6 months, while it increases iron absorption in older infants [22]. Despite the absence of increased serum iron levels, we observed a significant reduction of BAL eosinophil numbers in mice receiving iron supplementation in drinking water. Reduced number of eosinophils in the BAL can be an indirect consequence of several iron-induced regulatory mechanisms. It has been shown that increased dietary iron enhances the expression of lactoferrin, L-ferritin and hepcidin to reduce iron absorption and increase iron storage capacity [23–25]. Interestingly, Kruzel and colleagues showed that lactoferrin decreases pollen-induced airway eosinophilia by decreasing the formation of reactive oxygen species in a murine model of asthma [11]. Therefore, we speculate that enhanced expression of lactoferrin as a result of increased iron intake may explain the reduction of BAL eosinophilia in mice receiving iron-supplemented drinking water.

As serum iron levels were not increased after iron supplementation, we studied the effect of parenteral iron administration in the mouse model of allergic asthma. Our data clearly demonstrate that elevated systemic levels of iron due to parenteral iron administration inhibit allergen-induced AHR completely, and largely suppresses airway eosinophilia. These suppressive effects are associated with reduced levels of Th2 cytokines in lung tissue. The observed inhibition in AHR and eosinophilia could be caused by increased expres-

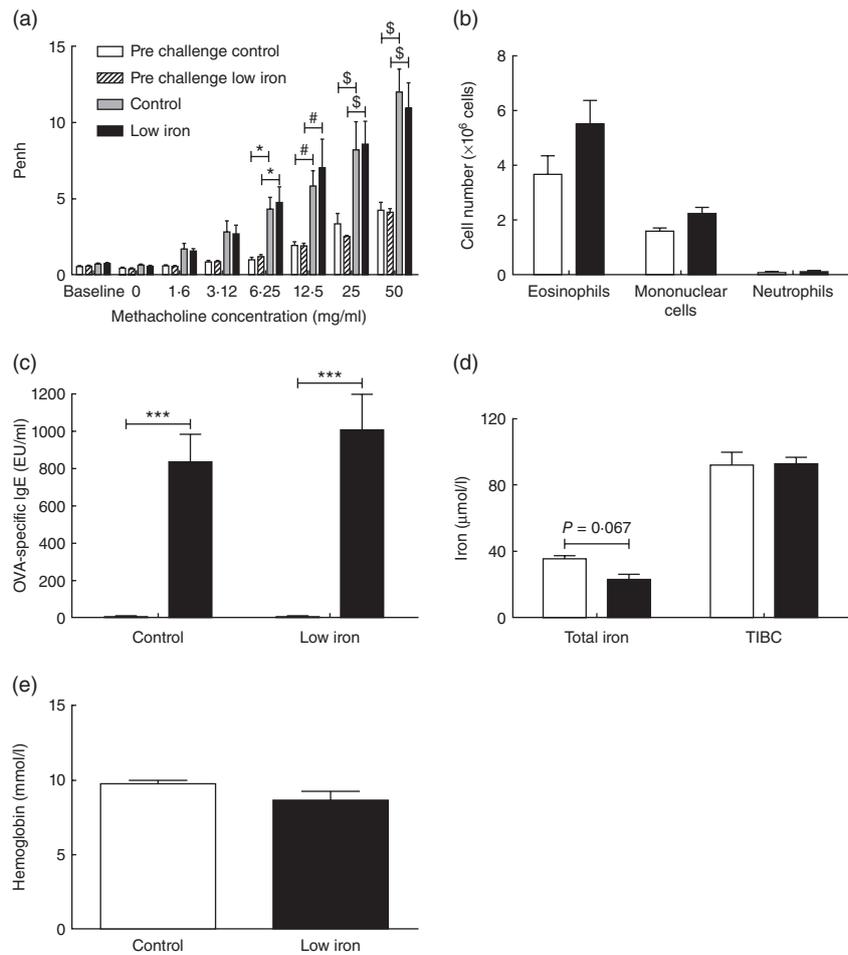


Fig. 4. Effects of iron deprivation on the manifestation of allergic asthma. Manifestations of experimentally induced allergic asthma in mice fed on iron-deprived diet (black and hatched bars) or iron-sufficient diet (open and grey bars): (a) airway responsiveness to methacholine before (open and hatched columns) and after inhalation challenge (grey and black columns); (b) numbers of eosinophils, mononuclear cells and neutrophils in bronchoalveolar lavage; (c) OVA-specific serum immunoglobulin (IgE) before (open columns) and after inhalation challenge (black columns); (d) total iron concentration in serum and total iron and total iron-binding capacity; (e) haemoglobin concentration in blood. Data are shown as mean \pm standard error of the mean, $n = 8$ (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$).

sion of haem oxygenase-1 (HO-1) as a result of the elevated iron levels in serum. HO-1 is an enzyme involved in haem metabolism that has been demonstrated to be increased, particularly in lungs, by iron overload [26]. Interestingly, Xia and colleagues have demonstrated that induction of HO-1 by haemin or Sn-protoporphyrin was able to suppress allergen induced AHR, eosinophilia and antigen-specific serum IgE in a mouse model of allergic asthma [27]. However, these data are not completely in line with our observations, as we did not observe an effect on serum IgE levels.

Our data also reveal that OVA-specific IgE level in serum was not influenced either by high iron intake or by parenteral iron administration. This appears to be in sharp contrast to the study of Mencacci *et al.*, who showed that iron overload as a result of i.p. iron administration induces high levels of antigen-specific IgE in serum [12]. However, it should be noted that they used an infection model using *Candida albicans* which is fundamentally different from our model of allergic asthma. Candidiasis induces Th1-dominated immune responses for protection [28], while our asthma model is dominated by a Th2 response. Moreover, *Candida* infection is associated with abundant production of pathogen recognition pattern molecules triggering innate

and adaptive immunity through different pattern recognition receptors [29], whereas in our model OVA lacks intrinsic danger signals.

Considering the suppressive effects on airway manifestation of allergic asthma in our model after oral or parenteral iron administration, we were interested in whether deprivation of dietary iron causes the opposite effect, e.g. exaggerated allergic responses. However, we demonstrate that there is no difference between mice maintained on a diet containing 7 p.p.m. *versus* those maintained on a diet containing 35 p.p.m. iron in developing the manifestation of experimental allergic asthma.

We used a well-established mouse model of allergic asthma under the situation of oral and systemic iron manipulations to find evidence supporting our hypothesis that the marked increase in iron intake by infants offers an explanation for the increased prevalence of allergic diseases during the last decades. Using inbred animals with a single genetic background, we did not observe augmented manifestations of allergic diseases due to increased oral or systemic iron administrations. Genetic background may be an important determinant of the outcome of increased iron intake on allergic diseases. Therefore, at this point it cannot be excluded that

increased consumption of dietary iron by infants may lead to an increased prevalence of allergic diseases, including asthma in human populations. Hence, examining the effects of high iron intake in different mouse strains would be an informative experiment to perform along with comprehensive retrospective epidemiological and genetic studies on the association between increased dietary iron and the prevalence of allergic diseases. Overall, we demonstrate that, in contrast to our hypothesis, allergen-induced airway manifestations of allergic asthma in the mouse can be inhibited by parenteral iron administration.

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Disclosure

The authors confirm that there are no conflicts of interest to disclose.

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