

ORIGINAL ARTICLE

Treatment of mice with fenbendazole attenuates allergic airways inflammation and Th2 cytokine production in a model of asthma

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Mouse models have provided a significant insight into the role of T-helper (Th) 2 cytokines such as IL-5 and IL-13 in regulating eosinophilia and other key features of asthma. However, the validity of these models can be compromised by inadvertent infection of experimental mouse colonies with pathogens such as oxyurid parasites (pinworms). While the benzimidazole derivative, fenbendazole (FBZ), is commonly used to treat such outbreaks, the effects of FBZ on mouse models of Th2 disease are largely unknown. In this investigation, we show that mice fed FBZ-supplemented food during the *in utero* and post-weaning period developed attenuated lung eosinophilia, antigen-specific IgG1 and Th2 cytokine responses in a model of asthma. Treatment of the mediastinal lymph node cells from allergic mice with FBZ *in vitro* attenuated cell proliferation, IL-5 and IL-13 production and expression of the early lymphocyte activation marker, CD69 on CD4⁺ T cells and CD19⁺ B cells. In addition, eosinophilia and Th2 responses remained attenuated after a 4-week withholding period in allergic mice treated preweaning with FBZ. Thus, FBZ modulates the amplitude of Th2 responses both *in vivo* and *in vitro*.

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Asthma is a complex inflammatory lung disease that is predominated by CD4⁺ T-helper (Th) 2 lymphocytes, infiltration of eosinophils and mast cells, and in atopic asthmatics, elevated serum IgE.^{1,2} Th2 cytokines, such as IL-5 and IL-13, are thought to underlie the development of key features of asthma, including eosinophil recruitment to the airways, the hypersecretion of mucus, airway wall thickening (remodelling), subepithelial fibrosis and respiratory dysfunction. Mouse models, especially those using gene-targeted and gene-knockout strains have provided significant insight into the mechanisms underlying these cytokine-induced inflammatory processes. However, the validity of experimental data can be severely compromised by the use of experimental mice from colonies that become inadvertently infected with mouse pathogens. For example, although experimental infection of mice with oxyurid parasites (pinworms) has been shown to induce transient Th2 immune responses, helminth infection in conjunction with ovalbumin-induced allergic airways disease attenuates IL-5 responses.³

Pinworms such as *Syphacia obvelata*, *Syphacia muris* and *Aspiculuris tetraptermuri* are common infestations of laboratory mice. Containment of these gastrointestinal nematodes is challenging because of the ease of egg transmission in food, water and bedding. Owing to its low toxicity, treatment of mice by supplementation of food with benzimidazole compounds such as fenbendazole (FBZ: methyl

5-(phenylthio)-1H-benzo[d]imidazol-2-yl carbamate) has gained widespread use for pinworm prophylaxis and infestation in rodent facilities. The efficacy of FBZ as an anthelmintic is attributed to its ability to selectively disturb energy metabolism in helminths and induce neurotoxic effects by nematode-selective inhibition of tubulin polymerisation.⁴ However, a critical issue, especially in the study of the immunopathology that develops in mouse models of disease, is the effect of FBZ on model-associated immune processes. Although limited, studies have suggested that FBZ does not influence cytotoxic responses,⁵ nor affects the development of type I diabetes in non-obese mice.⁶ In direct contrast, others have shown that FBZ treatment of healthy mice attenuates the numbers of splenic CD4⁺ and CD8⁺ T cells.⁷ In other species, treatment of healthy lambs reduces the ability of circulating lymphocytes to proliferate, particularly during secondary immune responses,^{8,9} and FBZ has also been shown to block mitosis of cultured human lymphocytes.¹⁰ These data suggest that FBZ may also affect aspects of mouse immunology.

Although FBZ is widely regarded as a safe and effective anthelmintic treatment for mice, the effects of FBZ on Th2-biased models of disease are largely unknown. The aim of this study is to investigate whether FBZ shows immunomodulatory effects in a mouse model of asthma. We show that FBZ treatment significantly attenuated the development of allergic airways inflammation and Th2 cytokine responses.

In addition, exposure of mice *in utero* and before weaning suppressed Th2 responses in weaned pups for at least 4 weeks after drug withholding, suggesting that FBZ treatment of mice can significantly affect the experimental findings in this disease model even after a significant withholding period.

RESULTS

Treatment of mice with FBZ attenuates allergic airways inflammation

To quantify the effect of FBZ on the development of allergic airways disease, we directly compared allergic inflammation and goblet-cell hyperplasia in wild-type BALB/c mice fed either normal mouse pellets or pellets supplemented with 150 mg kg⁻¹ FBZ, which delivers a minimum dose of 8 mg kg⁻¹ mouse body weight (Teklad TD.01432 data sheet, Harlan Teklad, Madison, WI, USA). The parents of mice used in experiments were fed diets of either unsupplemented food or FBZ-supplemented food when mated. Pups generated from matings were weaned at 4 weeks and fed the same diets as their parent breeders. To induce allergic airways disease, offspring were sensitised with Ova at 8 weeks of age and Ova challenges were started 12 days later. Mice were killed after a further 9 days. Treatment of mice with FBZ-supplemented food continued until their killing. Compared with allergic mice fed unsupplemented pellets, allergic mice fed FBZ-supplemented pellets showed no differences in the relative percentages of blood leukocytes or in goblet cell hyperplasia (Figures 1a and b).

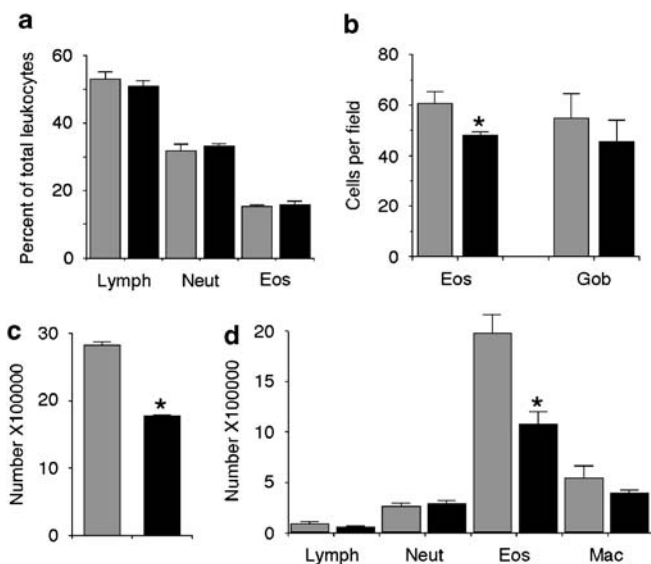


Figure 1 Fenbendazole (FBZ) attenuates eosinophilia in allergic mice. The percentages of blood leukocytes (a) were determined by morphological criteria in May-Grünwald Giemsa-stained smears. Lung sections (b) were either stained with Carbol's chromotrope or with Alcian blue-periodic acid-Schiff and the numbers of eosinophils and goblet cells, respectively, in the peribronchial region were determined in at least ten similar fields of $\times 1000$ magnification per mouse. The total numbers of airway leukocytes (c) and numbers of different cell types (d) were quantified by correlating differential counts of May-Grünwald Giemsa-stained cytopins with the total cell counts in the broncho-alveolar lavage fluid. Lymph, lymphocytes; Neut, neutrophils; Eos, eosinophils; Mac, macrophages; Gob, goblet cells. Values are the mean $\pm$ s.e.m. of five to six mice per group. Similar findings were observed in at least two independent experiments. Grey bars: allergic mice treated throughout with normal food; black bars: allergic mice treated pre- and postweaning with FBZ-supplemented food. * $P < 0.05$ compared with mice fed normal food.

In direct contrast, peribronchial eosinophilia and, in particular, the numbers of eosinophils in the airway lumen (broncho-alveolar lavage fluid (BALF)) were significantly reduced in mice fed FBZ-supplemented pellets (Figures 1b–d). Notably, eosinophilia and goblet-cell hyperplasia shown in Figures 1a–d in both groups of allergic mice were significantly higher relevant to nonallergic controls (data not shown). These data show that FBZ can significantly attenuate the development of eosinophilia in the allergic lung. The effects of this anthelmintic appeared eosinophil specific as no reduction in BALF lymphocytes, neutrophils or macrophages were observed. In addition, no differences were observed in blood eosinophilia, suggesting processes that regulate systemic eosinophilia were unaffected by continuous treatment with FBZ.

FBZ attenuates Th2 cytokine and IgG1 production in allergic mice

To address the mechanisms underlying the ability of FBZ to suppress allergic inflammation, the production of IL-5 and IL-13 by Ova-stimulated mediastinal lymph node (MLN) cells cultured *ex vivo* from allergic mice were quantified in mice fed unsupplemented or FBZ-supplemented pellets. These Th2 cytokines are known to play a major role in eosinophil recruitment to the allergic lung.^{12,14–16} Compared with mice fed unsupplemented pellets, treatment of mice with FBZ-supplemented food significantly inhibited IL-5 and IL-13 production by MLN cultures (Figure 2a). Ova-specific serum IgG1 was also suppressed by FBZ treatment (Figure 2b). In contrast, no significant differences between treatment groups were seen in lung eotaxin-1 levels, Ova-specific serum IgE or respiratory function (resistance and compliance, all data not shown). Collectively, these data show that FBZ inhibits the magnitude of Th2 responses during the development of allergic airways disease.

FBZ suppresses MLN Th2 cytokine and antibody production *in vitro*

We next investigated the influence of FBZ on Th2 immune cell function *in vitro*. MLN were isolated from allergic mice fed a normal diet throughout, and Ova-stimulated MLN cell cultures were treated with varying concentrations of FBZ *in vitro*. Although it is difficult in cell cultures to parallel the exact concentration of FBZ in the MLN of

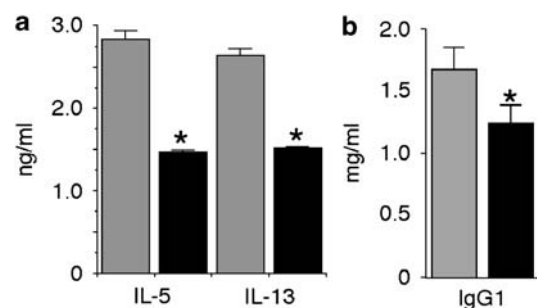


Figure 2 Fenbendazole (FBZ) attenuates Ova-specific Th2 cytokine production and IgG1 in allergic mice. Cytokines produced by Ova-stimulated cultured mediastinal lymph node (MLN) cells (a) and Ova-specific IgG1 in serum (b) from allergic mice treated with normal food or with food supplemented with FBZ were assayed by enzyme-linked immunosorbent assay. Values are the mean $\pm$ s.e.m. for three assays of a pool of MLN from five to six mice per group or serum from four to five mice per group. Similar findings were observed in at least two independent experiments. Grey bars: allergic mice treated with normal food; black bars: allergic mice treated pre- and postweaning with FBZ-supplemented food. * $P < 0.05$ compared with mice fed normal food.

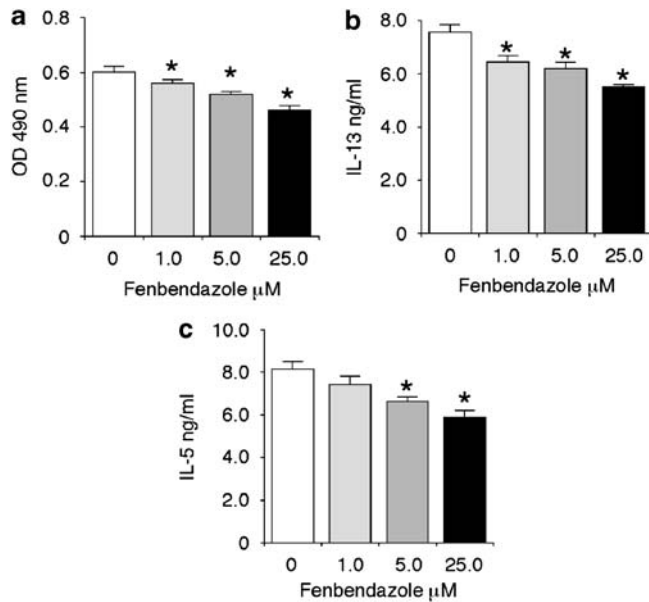


Figure 3 Treatment of mediastinal lymph node (MLN) cultures with fenbendazole (FBZ) *in vitro* inhibits cell proliferation and Th2 cytokine production. Cultures of Ova-stimulated MLN cells from allergic mice fed normal food were incubated with either vehicle (dimethylsulphoxide) or 1, 5 or 25 μ M FBZ for 48 h. Cell proliferation (a) in cultures was determined colorimetrically or IL-13 (b) and IL-5 (c) in cell-free supernatants were determined by enzyme-linked immunosorbent assay. Values are the mean $\pm$ s.e.m. for three assays of MLN from five to six mice per group. * $P < 0.05$ compared with cultures treated with vehicle control. Similar findings were observed in two independent experiments. OD, optical density.

allergic mice fed FBZ-supplemented food, the minimum estimated dose of 8 mg kg⁻¹ mouse body weight (Teklad TD.01432 data sheet) equates to $\sim 38 \mu$ M in the soluble compartment assuming 70% of the body mass is fluid. It is noted that, compared with vehicle-treated cultures, FBZ, even at the lowest dose of 1 μ M significantly inhibited cell proliferation and production of IL-13 (Figures 3a and b). At 5 μ M FBZ, production of IL-5 was also significantly attenuated (Figure 3c). These data suggest that FBZ can directly influence Th2 immune cell function.

FBZ inhibits activation of CD4⁺ T cells and CD19⁺ B cells *in vitro*

To elucidate the process by which FBZ inhibits MLN cell proliferation *in vitro*, fluorescence-activated cell sorting (FACS) analysis was used to quantify the relative numbers and activation state of CD4⁺ T cells and CD19⁺ B cells in Ova-stimulated cultured MLN cells from allergic mice fed a normal diet, and treated with varying concentrations of FBZ *in vitro*. Although no distinct trend was observed in the total number of live CD4⁺ T cells in cultures treated with FBZ (upper panel Figure 4a), fewer live CD19⁺ B cells were observed in cultures treated with the highest dose of FBZ (middle panel Figure 4a). In addition, the numbers of cells expressing CD69, an early marker of lymphocyte activation, was significantly reduced in MLN cultures treated with 5 or 25 μ M FBZ (lower panel Figures 4a and b). When FACS was used to quantify the cell types in which CD69 expression was impaired, it was evident that both CD4⁺ T cells and CD19⁺ B cells showed approximately a 26 and 32% reduction in CD69 expression, respectively, in cultures treated with 25 μ M FBZ compared with that of vehicle control-treated cultures (Figure 4c). In contrast, higher numbers of CD69⁻ CD4⁺ T cells and CD69⁻ CD19⁺ B cells were observed in

cultures treated with 25 μ M FBZ than seen in vehicle control-treated cultures (Figure 4c). These data suggest that FBZ directly attenuates the early activation of CD4⁺ T cells and CD19⁺ B cells.

Immunomodulation of Th2 responses persists after withholding FBZ

Fenbendazole treatment of mice either prophylactically or for treatment of active helminthic infestation is commonly followed by a withholding period before the mice can be used for experimental purposes. To gain insight into whether the Th2-suppressive effects of FBZ can be alleviated after a withholding period, pups from FBZ-treated breeders were fed a normal diet for 4 weeks postweaning. Control mice consisted of mice fed a normal diet both pre- and postweaning. Mice were sensitised with Ova at 8 weeks (after 4 weeks of normal diet) and then exposed to the Ova aerosol regime 12 days later. Surprisingly, withdrawal of FBZ postweaning had a greater impact in suppressing eosinophilia than seen in mice that had been treated with FBZ both pre- and postweaning. Both tissue and BALF eosinophilia were suppressed despite the 4-week FBZ withholding period before sensitisation (Figures 5b–d). Indeed, the ability of cells to infiltrate the airway lumen was reduced by $\sim 90\%$ after FBZ withholding. In direct contrast to continuous drug-treated mice, eosinophils in the blood, and neutrophils and macrophages in the BALF of drug-withheld mice were also significantly suppressed compared with mice fed a normal diet (Figures 5a and d). The production of IL-5 and IL-13 was also significantly lower in mice from which FBZ had been withdrawn (Figure 5e). To evaluate the effects of FBZ withdrawal on T-cell function, MLN were collected from naive mice treated preweaning with FBZ and these cells were cultured and activated *in vitro* with solid-phase anti-CD3 and soluble anti-CD28 antibodies (Figure 5f). After stimulation, proliferation was significantly higher (1.5-fold) in cells from mice fed a normal diet than in cells from mice fed the FBZ-withheld diet, suggesting inherent changes that occur in immune cell function during *in utero* and pre-weaning exposure to FBZ persisted for at least 4 weeks after FBZ withdrawal. Collectively, these data show that Th2 immune responses are suppressed in mice treated preweaning with FBZ and these effects are not alleviated within a 4-week withholding period. Indeed, withholding amplifies the ability of FBZ to inhibit eosinophil as well as neutrophil and macrophage migration to the airways.

DISCUSSION

Fenbendazole is an anthelmintic commonly administered to cattle, sheep and horses. It is also widely used for treatment of oxyurid parasites in experimental mouse colonies. Although an important consideration for researchers, few studies have addressed the effect of FBZ on the experimental mouse models of disease. Critically, no studies have addressed the effect of FBZ on Th2-biased models of diseases such as asthma. Our findings showed that FBZ significantly affected the production of antigen-specific Th2 cytokines and serum IgG1, and critically attenuated the development of eosinophilic inflammation in the airways. The ability of FBZ to modulate immune cell function persisted for at least 4 weeks after the withdrawal of FBZ.

Compared with mice treated with normal food, mice treated with FBZ *in utero* and postweaning showed significantly reduced peribronchial and airway (cells in lavage fluid) eosinophilia after induction of the allergic airways disease. This reduced eosinophilia correlated with significant attenuation in the capacity of MLN cells to produce the Th2 cytokines, IL-5 and IL-13. These cytokines have well-acknowledged roles in the development of eosinophilic inflammation. Treatment of BALB/c mice with neutralising anti-IL-5 antibody selectively

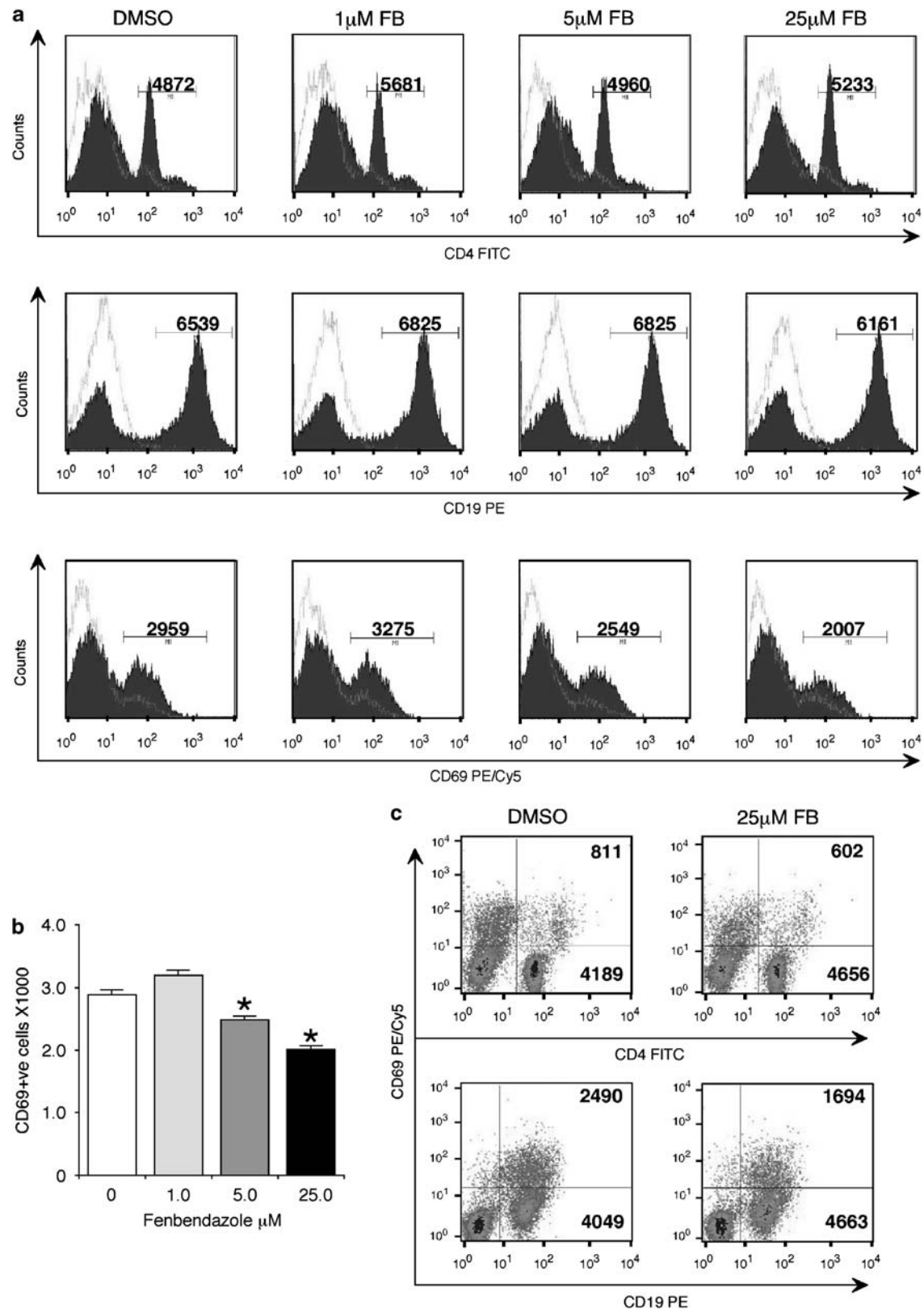


Figure 4 Treatment of mediastinal lymph node (MLN) cultures with fenbendazole (FBZ) *in vitro* inhibits expression of CD69 on CD4⁺ T cells and CD19⁺ B cells. Cultures of Ova-stimulated MLN cells from allergic mice fed normal food were incubated with either vehicle (dimethylsulphoxide (DMSO)) or 1, 5 or 25 μ M FBZ for 48 h. Numbers of live CD4⁺, CD19⁺ (a) and CD69⁺ cells (a and b) were quantified by fluorescence-activated cell sorting by single staining, and then CD69 expression on CD4⁺ T cells and CD19⁺ B cells were determined by double staining (c). * $P < 0.05$ for 5 and 25 μ M FBZ treatment compared with vehicle of three cultures per concentration. Similar findings were observed in two independent experiments. FB, fenbendazole; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

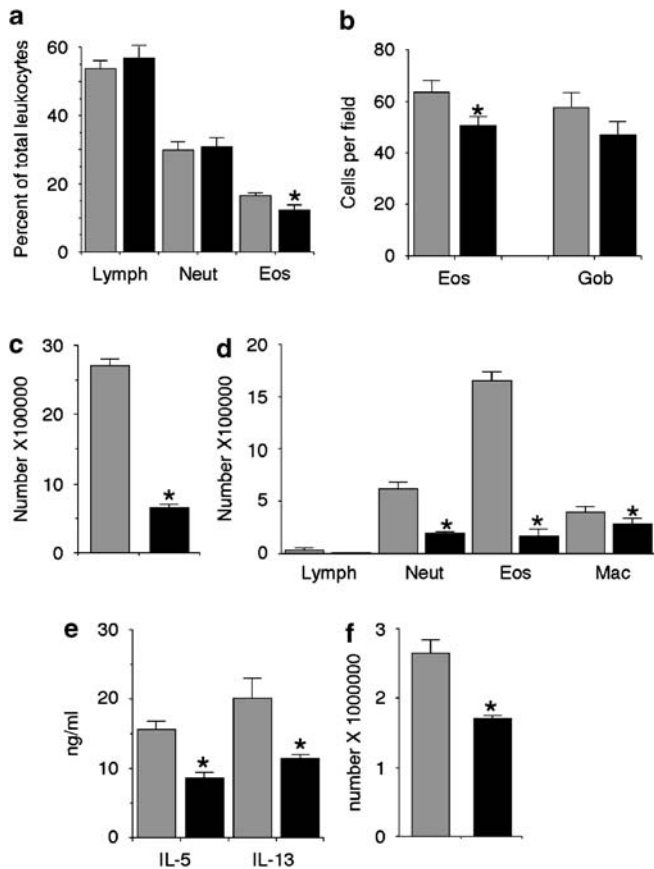


Figure 5 Treatment of mice *in utero* and preweaning with fenbendazole (FBZ) attenuates allergic inflammation in mice. The percentages of blood leukocytes (a), the numbers of eosinophils and goblet cells in the peribronchial region (b), the total numbers of airway leukocytes (c) and numbers of different cell types (d) and mediastinal lymph node (MLN) cytokine production (e) were quantified as described in the legend to Figures 1 and 2. Lymph, lymphocytes; Neut, neutrophils; Eos, eosinophils; Mac, macrophages; Gob, goblet cells. (f) Naive MLN cell proliferation after *in vitro* stimulation with anti-CD3 and CD28. Values are the mean $\pm$ s.e.m. of five to six mice per group (a–e) or from a pool of MLN cells from four mice per group (f). Similar findings were observed in at least two independent experiments. Grey bars: allergic mice treated throughout with normal food; black bars: allergic mice treated *in utero* and preweaning with FBZ-supplemented food.

inhibits allergy-induced pulmonary eosinophilia,^{17,18} and allergic BALB/c mice genetically deficient in IL-13 display attenuated eosinophilia¹⁹ that is associated with reduced expression of the eotaxins.^{20,21} Our observed reduction in Th2 cytokines paralleled the levels of Ova-specific IgG1, suggesting that the influence of FBZ was not limited to T cell production of Th2 cytokines, but extended to the capacity of B cells to produce the Th2-associated antibody, IgG1.

To investigate the possible mechanisms underlying the ability of FBZ to suppress production of antigen-specific Th2 cytokines and serum IgG1, we examined the effects of culturing MLN cells from allergic mice with varying concentrations of FBZ *in vitro*. FBZ significantly attenuated Ova-induced proliferation of cells and suppressed IL-5 and IL-13 production dose dependently. Although there was some variation in the total numbers of live CD4⁺ T cells in cultures treated with different concentrations of FBZ, only live CD19⁺ B cells showed an apparent trend toward reduced numbers with the highest concentration of FBZ. In addition, compared with vehicle

control, the expression of CD69 by CD4⁺ T cells and CD19⁺ B cells was significantly reduced with increasing concentrations of FBZ. CD69 is the earliest induced cell surface glycoprotein acquired during lymphocyte activation and is involved in lymphocyte proliferation.²² The higher number of CD69⁺ CD4⁺ T cells and CD69⁺ CD19⁺ B cells in FBZ-treated cultures suggested that FBZ might not be cytotoxic *per se* but that it attenuates the early activation of T and B cells. As CD69 is also associated with cell proliferation, this observation may explain why FBZ-treated cultures showed reduced proliferation compared with vehicle-treated cultures. Collectively, these data show that FBZ blocks the proliferation of activated CD4⁺ T cells and CD19⁺ B cells in MLN cultures.

Many mouse facilities that use FBZ to either eliminate pinworm infestation or to treat mice prophylactically use an appropriate withholding period before the treated mice can be used experimentally. Therefore, we also investigated whether withholding FBZ treatment for 4 weeks postweaning of mice from breeders treated with FBZ eliminated the Th2-suppressive effects of this drug. It is noted that this treatment regime extended the range of FBZ effects to include suppression of blood eosinophilia, and also suppression of neutrophil and macrophage recruitment to the airways of allergic mice. In addition, compared with continuous drug exposure, the ability of FBZ to suppress cellular recruitment to the airways was amplified in drug-withheld mice. Therefore, in contrast to mice continuously exposed to FBZ, withdrawal of this drug seemed to generate a different pattern of immunosuppression that extended beyond eosinophil recruitment. Although we have shown that continuous feeding of mice and direct application of FBZ to MLN cultures reduces Th2 cytokine production, the observation that withdrawal of FBZ generates a different profile of immune suppression suggests that FBZ may also influence alternative processes of immune cell function beyond the immediate effects of FBZ. At this stage it is unclear how withdrawal of FBZ extends and amplifies its suppressive effects. However, it is possible that epigenetic mechanisms imprinted during *in utero* exposure to FBZ could reduce responsiveness to Th2-inducing agents, and once imprinted, this reduced responsiveness may be amplified after the influence of FBZ is removed postweaning. This concept is supported by the observation that proliferation of naive MLN cells stimulated *in vitro* was significantly reduced in cells from mice fed a FBZ-withdrawn diet when compared with mice fed a normal diet.

The mechanism by which benzimidazole analogues function as anthelmintics is attributed to the ability of these compounds to selectively bind nematode tubulin and prevent microtubule assembly, thus disrupting mitotic spindle formation. Importantly, this process is a prerequisite for the correct segregation of chromosomes during cell division. The efficacy of benzimidazole compounds as nematode-selective agents is thought to rely on their ability to preferentially bind nematode rather than mammalian tubulin.²³ However, of a number of benzimidazole compounds tested, FBZ was shown to be the most active in also inhibiting mammalian microtubule assembly.⁴ Further, evaluation of benzimidazole analogues in a human lymphocyte culture system showed that FBZ induced mitotic arrest, supporting the concept that these agents can interfere with mitotic spindle formation in mammalian cells.¹⁰ It is noted that analogues such as thiabendazole showed very low binding to mammalian microtubules and this correlated with an inability of thiabendazole to induce mitotic arrest in mammalian lymphocytes.^{4,10}

Collectively, our data show that continuous treatment of mice with FBZ attenuated the development of eosinophilic inflammation, Th2 cytokine production and Ova-specific IgG1 in a mouse model of asthma. The ability of FBZ to inhibit the early activation of CD4⁺

T cells and CD19⁺ B cells (CD69 expression) and attenuate Th2 cytokine production *in vitro* parallels the ability of FBZ to attenuate Th2 responses *in vivo*. In addition, mice exposed to FBZ *in utero* and preweaning are also immunosuppressed. It is likely that these phenomena are related to the previously observed capacity of FBZ to inhibit mammalian microtubule formation⁴ and induce mitotic arrest in human lymphocytes.¹⁰ As other benzimidazole derivatives have weaker affinities for mammalian microtubulin, they should be considered when assessing treatment for pinworms in experimental mice.

METHODS

Mice

Wild-type BALB/c mice were supplied by the Animal Services Division, JCSMR. Eight-week-old male and female mice from the same litter were distributed to individually ventilated cages (Tecniplast, Varese, Italy) and provided with either unsupplemented Gordon's mouse pellets (Gordon's Specialty Feeds, Yanderra, New South Wales, Australia: 23% protein, 6% fat, 5% fibre) or pellets supplemented with 150 mg kg⁻¹ FBZ. Pups from FBZ-treated parents were weaned at 4 weeks of age when they were transferred to a diet of FBZ-supplemented food or in some experiments unsupplemented food. Environmental conditions were maintained at 19–21 °C, 12-h light/dark cycles, with sterilised water, bedding and cages. No pinworm infection was detected (by faecal floatation) in the mouse colony from which mice used in these experiments were sourced.

Induction of allergic airways disease

Allergic airways inflammation was induced in sex-matched 8-week-old mice by intraperitoneal sensitisation (day 0) with 50 µg of ovalbumin (Ova, Fraction V: Sigma, St Louis, MO, USA) mixed with 20 µl of Rehydrogel (Reheis, Berkely Heights, NJ, USA) made up to a total volume of 200 µl with 0.9% sterile saline. Control mice received the same inoculum without Ova. On days 12, 14, 16 and 19, all mice were challenged with an aerosol of 10 mg ml⁻¹ Ova in 0.9% saline for 3 × 30 min per day with 30-min breaks between aerosols as previously described.¹¹ At 24 h after the last challenge, mice were killed by cervical dislocation and the lungs were either removed for histology, or BALF was obtained by cannulating the trachea and gently flushing the airways with two 1 ml volumes of phosphate-buffered saline. All mice were treated according to the Australian National University Animal Welfare guidelines (Protocol JMB32/07).

Quantification of eosinophils and goblet cells

Lungs were fixed in 10% phosphate-buffered formalin, sectioned and then stained with Carbol's chromotrope-hematoxylin, and the number of eosinophils in similar peribronchial regions were counted. The sections were also stained with Alcian blue–periodic acid–Schiff for enumeration of mucin-secreting cells. Leukocytes in blood smears and BALF cytopspins were stained with May–Grünwald Giemsa and identified by morphological criteria and quantified as previously described.¹²

Cytokine and antibody production

Cells from the MLNs from allergic mice were isolated, washed in mixed lymphocyte culture (MLC) medium¹³ and then stimulated with 1 mg ml⁻¹ Ova in MLC medium for 68 h at 37 °C and 5% CO₂ in 96-well plates with 2 × 10⁶ cells per well. The concentrations of IL-5 and IL-13 in the cell-free supernatants from cultured MLN were measured by enzyme-linked immunosorbent assay (ELISA) as described elsewhere.¹² The paired capture and detection IL-5 antibodies were from Pharmingen (San Diego, CA, USA), and the IL-13 antibodies were from R & D Systems (Minneapolis, MN, USA). The sensitivity of detection was 6–24 pg ml⁻¹. Ova-specific IgE in serum was measured with an ELISA capture assay. Essentially, total serum IgE was bound by solid-phase rat anti-mouse IgE (Zymed, San Francisco, CA, USA). The bound Ova-specific IgE was then detected with biotinylated Ova and streptavidin-APase (Amersham, Pharmacia Biotech, Rydalmere, NSW, Australia). Data were expressed as ELISA units, which was the product of the dilution and the optical density. Ova-specific IgG1 in serum was determined by ELISA

using solid-phase Ova and isotype-specific detection antibodies (Southern Biotechnologies, Birmingham, AL, USA).

Proliferation of MLN cells activated *in vitro*

Cells from MLN from naive mice were collected, cultured *in vitro* as described above and activated with solid-phase anti-CD3 antibody (clone 2C11: 10 µg ml⁻¹) and anti-CD28 (clone 37.51: 5 µg ml⁻¹) for 36 h. Proliferation was determined by counting the total number of cells after activation or bioreduction of (3-(4-5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to formazan.

FBZ treatment of MLN

Mediastinal lymph node cells were cultured as described above, and varying concentrations of FBZ or vehicle control (dimethylsulphoxide) were added to cell cultures, which were then incubated for 48 h at 37 °C and 5% CO₂ in 96-well plates with 2 × 10⁶ cells per well. Cytokine analysis was carried out on cell-free culture supernatants as described above and cells were analysed by FACS (BD FACS Calibur) for expression of CD4 (BD, Franklin Lakes, NJ, USA, clone GK1.5), CD19 (BD clone ID3) and CD69 (BD clone HL2F3). In some experiments at the 48 h time point, proliferation was assessed colorimetrically by quantifying bioreduction of MTS.

Statistical analysis

Significance of differences between experimental groups was analysed using the Student's unpaired *t*-test. Values are the mean ± s.e.m. Differences in means were considered significant if *P* < 0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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- 1 Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM *et al*. Predominant Th2-like bronchoalveolar lavage T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; **326**: 298–304.
- 2 Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow Jr JC. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992; **146**: 109–115.
- 3 Michels C, Goyal P, Nieuwenhuizen N, Brombacher F. Infection with *Syphacia obvelata* (pinworm) induces protective Th2 immune responses and influences ovalbumin-induced allergic reactions. *Infect Immun* 2006; **74**: 5926–5932.
- 4 Dawson PJ, Gutteridge WE, Gull K. A comparison of the interaction of anthelmintic benzimidazoles with tubulin isolated from mammalian tissue and the parasitic nematode *Ascaridia galli*. *Biochem Pharmacol* 1984; **33**: 1069–1074.
- 5 Reiss CS, Herrman JM, Hopkins RE. Effects of antihelminth treatment on the immune response of mice. *Lab Anim Sci* 1987; **37**: 773–775.
- 6 Franke DD, Shirwan H. Prophylactic fenbendazole therapy does not affect the incidence and onset of type 1 diabetes in non-obese diabetic mice. *Int Immunol* 2006; **18**: 453–458.
- 7 Dvoroznakova E, Boroskova Z, Dubunsky P, Velebny S, Tomasovicova O, Machnicka B. Changes in cellular immunity of mice treated for laval toxocarosis with fenbendazole. *Helminthologica* 1998; **35**: 189–195.
- 8 Stankiewicz M, Cabaj W, Jonas WE, Moore LG, Chie WN. Oxfendazole treatment of non-parasitized lambs and its effect on the immune system. *Vet Res Commun* 1994; **18**: 7–18.
- 9 Cabaj W, Stankiewicz M, Jonas WE, Moore LG. Fenbendazole and its effect on the immune system of the sheep. *NZ Vet J* 1994; **42**: 216–220.
- 10 Holden HE, Crider PA, Wahrenberg MG. Mitotic arrest by benzimidazole analogs in human lymphocyte cultures. *Environ Mutagen* 1980; **2**: 67–73.
- 11 Webb DC, Mahalingam S, Cai Y, Matthaai KI, Donaldson DD, Foster PS. Antigen-specific production of interleukin (IL)-13 and IL-5 cooperate to mediate IL-4/alpha-independent airway hyperreactivity. *Eur J Immunol* 2003; **33**: 3377–3385.
- 12 Webb DC, McKenzie ANJ, Koskinen AML, Yang M, Mattes J, Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol* 2000; **165**: 108–113.

- 13 Webb DC, Cai Y, Matthaei KI, Foster PS. Comparative roles of IL-4, IL-13, and IL-4 α in dendritic cell maturation and CD4⁺ Th2 cell function. *J Immunol* 2007; **178**: 219–227.
- 14 Yamaguchi Y, Hayashi Y, Sugama Y, Miura Y, Kasahara T, Kitamura S *et al*. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs *in vitro* survival: IL-5 as an eosinophil chemotactic factor. *J Exp Med* 1988; **167**: 1737–1742.
- 15 Hogan SP, Koskinen A, Matthaei KI, Young IG, Foster PS. Interleukin-5-producing CD4⁺ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice. *Am J Respir Care Med* 1998; **157**: 210–218.
- 16 Horie S, Okubo Y, Hossain M, Sato E, Nomura H, Koyama S *et al*. Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. *Intern Med* 1997; **36**: 179–185.
- 17 Hamelmann E, Oshiba A, Loader J, Larsen GL, Gleich G, Lee J *et al*. Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am J Respir Crit Care Med* 1997; **155**: 819–825.
- 18 Hogan SP, Koskinen A, Foster PS. Interleukin-5 and eosinophils induce airway damage and bronchial hyperreactivity during allergic airway inflammation in BALB/c mice. *Immunol Cell Biol* 1997; **75**: 284–288.
- 19 Webb DC, Matthaei KI, Cai Y, McKenzie AN, Foster PS. Polymorphisms in IL-4 α correlate with airways hyperreactivity, eosinophilia, and Ym protein expression in allergic IL-13(-/-) mice. *J Immunol* 2004; **172**: 1092–1098.
- 20 Pope SM, Brandt EB, Mishra A, Hogan SP, Zimmerman N, Matthaei KI *et al*. IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J Allergy Clin Immunol* 2001; **108**: 594–601.
- 21 Pope SM, Fulkerson PC, Blanchard C, Akei HS, Nikolaidis NM, Zimmermann N *et al*. Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation. *J Biol Chem* 2005; **280**: 13952–13961.
- 22 Ziegler SF, Ramsdell F, Alderson MR. The activation antigen CD69. *Stem Cells* 1994; **12**: 456–465.
- 23 Friedman PA, Platzer EG. Interaction of anthelmintic benzimidazoles with *Ascaris suum* embryonic tubulin. *Biochim Biophys Acta* 1980; **630**: 271–278.