

# Long-Term Bortezomib Treatment Reduces Allergen-Specific IgE but Fails to Ameliorate Chronic Asthma in Mice

Michael Wegmann<sup>a</sup> Lars Lunding<sup>a</sup> Zane Orinska<sup>b</sup> David M. Wong<sup>c</sup>  
Rudolf A. Manz<sup>c</sup> Heinz Fehrenbach<sup>a</sup>

<sup>a</sup>Bereich Experimentelle Pneumologie, und <sup>b</sup>Abteilung für Immunologie, Forschungszentrum Borstel, Borstel, und  
<sup>c</sup>Institut für Systemische Entzündungsforschung, Universität Lübeck, Lübeck, Deutschland

## Key Words

Allergic asthma · Animal models · Immunoglobulin E · Therapy · Inhibition of inflammation · B lymphocytes

## Abstract

**Background:** Allergen-specific immunoglobulin (Ig) E initiates the effector cascade of allergic asthma and has been identified as a valuable target for therapeutic treatment of this disease. The proteasome inhibitor bortezomib was previously shown to deplete Ig-secreting plasma cells and to efficiently suppress Ig serum titers. The present study aimed at evaluating the therapeutic potential of the proteasome inhibitor bortezomib in allergic bronchial asthma. **Methods:** To address this question, a chronic experimental asthma mouse model was used in a therapeutic setting. Mice were sensitized to ovalbumin (OVA) and challenged with OVA aerosol for 12 weeks. After 6 weeks of challenge, bortezomib treatment was started and continued for 1 week (short-term) or 6 weeks (long-term) with a dosage of 0.75 mg/kg body weight twice a week. Lung function, lung histology, Ig serum titers and plasma cell numbers were assessed. **Results:** Whereas short-term treatment lowered bronchoalveolar lavage eosinophils, long-term treatment considerably reduced serum titers of anti-OVA IgE in mice with chronic experimental asthma. However, neither short-term nor long-

term treatment significantly reduced plasma cell numbers, anti-OVA IgG1 serum titers or allergic airway inflammation or ablated airway hyperresponsiveness. **Conclusion:** Our results suggest that bortezomib treatment has only limited value as plasma cell-depleting therapy against allergic bronchial asthma.

Copyright © 2011 S. Karger AG, Basel

## Introduction

Immunoglobulin (Ig) E-mediated cellular reactions play critical roles in the formation and chronification of allergic diseases such as bronchial asthma. Inhalation of the respective aeroallergen leads to cross-linking of IgE-Fcε receptor I complexes on the surface of mast cells in the airway mucosa. This event leads to the subsequent release of a plethora of mediators such as histamine and leukotrienes, which provoke a typical inflammatory reaction in the airway mucosa associated with acute broncho-obstruction. Following this early-phase response, T helper 2 cells and eosinophils infiltrate into the airways, establishing an allergic immune response [1]. Additionally, IgE-Fcε receptor II cross-linking by allergens mediates eosinophil effector functions and cytotoxicity [2]. Recurrent inhalation of the respective aeroallergen repeatedly

triggers these cellular reactions, which ultimately leads to chronification of the inflammatory response in the airways and the development of airway hyperresponsiveness (AHR), mucus hypersecretion and airway remodeling [3]. Thus, targeting IgE-mediated reactions represents a promising approach to asthma therapy. The depletion of plasma cells, which continuously produce allergen-specific IgE in atopic patients [4], may be such an option.

Bortezomib is a boronic acid derivative that binds to the proteasome  $\beta$ 2-subunit and therefore reversibly inhibits proteasome functions [5]. This multienzyme complex plays an essential role in degrading defective, misfolded or supernumerous proteins and is further involved in the regulation of several cellular processes such as apoptosis and the cell cycle. Therefore, tumor cells such as myeloma cells, whose survival critically depends on antiapoptotic cell cycle signals, have been demonstrated to be very sensitive to bortezomib treatment [6]. Consequently, bortezomib has been introduced as a third-line therapy in myeloma patients [7]. Recently, a study by Neubert et al. [8] suggested that bortezomib could also be a treatment option in autoantibody-mediated diseases like systemic lupus erythematosus (SLE). This study demonstrated that bortezomib treatment successfully depleted plasma cells in two mouse strains with lupus-like disease, NZB/W F1 and MRL/lpr mice, resulting in diminished autoantibody production, ameliorated glomerulonephritis and prolonged survival [8].

The present study was designed to test the hypothesis that bortezomib treatment has a beneficial therapeutic effect on allergic bronchial asthma. We investigated the effects of bortezomib in a mouse model of chronic experimental asthma that reflects several characteristics of human asthma that are not observed in acute asthma models in mice [9]. The chronic mouse model used in this study exhibits characteristics of chronicity including chronic airway inflammation, stable airflow limitation and airway remodeling; moreover, it has also proven to be a valuable tool for the examination of the B cell response in experimental asthma [9]. In contrast to acute experimental asthma, which can be induced in the absence of IgE in mice [10], a close relationship between chronic airway inflammation and a systemic B cell response could be demonstrated in the chronic mouse model. In this model, asthma pathology is associated with allergen-specific IgE- and IgG-secreting plasma cells in spleen, secondary lymphoid organs and lung [11], and with increased serum titers of allergen-specific IgG, increased longevity of systemic plasma cells and the formation of plasma cell survival niches in airway tissue [12].

Hence, in order to be able to evaluate the potential therapeutic effect of bortezomib-induced plasma cell depletion on allergic asthma, the use of a model of chronic asthma is mandatory. In our study, a therapeutic setting was used with bortezomib treatment starting when the asthmatic phenotype had already been established, thus closely resembling clinical conditions. The following outcome measures were chosen to evaluate the effect of bortezomib on chronic experimental asthma in the mouse: inflammatory cell counts in bronchoalveolar lavage (BAL), lung histology, lung function, determination of Ig serum titers and quantification of plasma cells in spleen.

## Material and Methods

### *Animals*

Female, 6- to 8-week-old BALB/c mice (Harlan Winkelmann, Hannover, Germany) were maintained under specific pathogen-free conditions and received an ovalbumin (OVA)-free diet and water ad libitum. All animal studies were performed with the approval of the local animal ethics committee.

### *Animal Treatment Protocol*

Mice were sensitized to OVA by 3 intraperitoneal injections of 10  $\mu$ g of OVA (OVA grade VI, Sigma, Deisenhofen, Germany) adsorbed to 150  $\mu$ g of aluminum hydroxide (inject alum, Pierce) on days 1, 14 and 21. In order to induce chronic allergic airway inflammation, mice were exposed to an OVA (OVA grade V, Sigma) aerosol (1% wt/vol in PBS) twice a week on 2 consecutive days over a period of 12 weeks, starting on day 26 as previously described [13]. Bortezomib (Velcade®, Janssen-Cilag, Neuss, Germany) treatment was initiated after allergic airway inflammation had already been established on day 60 (fig. 1). Mice received 0.75 mg/kg body weight (BW) bortezomib dissolved in 100  $\mu$ l of sterile PBS intravenously 24 h before the first weekly OVA aerosol challenge and 24 h after the second challenge until the end of the study. Short-term treatment consisted of 2 bortezomib injections, and long-term treatment involved 14 injections. Control animals were sham sensitized to PBS and subsequently challenged with OVA aerosol. The placebo control group was treated intravenously with 100  $\mu$ l of sterile PBS. All animals were sacrificed by cervical dislocation under deep anesthesia on day 106. To exclude any side effects of bortezomib treatment, which have been described in mice and humans, animals receiving short-term or long-term treatment were monitored for signs of physical disorders, including weight loss, scruffy fur or bleeding. We did not observe such indications of adverse side effects in any of the experimental groups studied.

### *Bronchoalveolar Lavage*

BAL was performed as previously described [13].

### *Lung Histology*

Lungs were fixed ex situ with 4% (wt/vol) paraformaldehyde via the trachea under constant pressure, removed and stored in 4% paraformaldehyde. Lung tissues were embedded in paraffin,

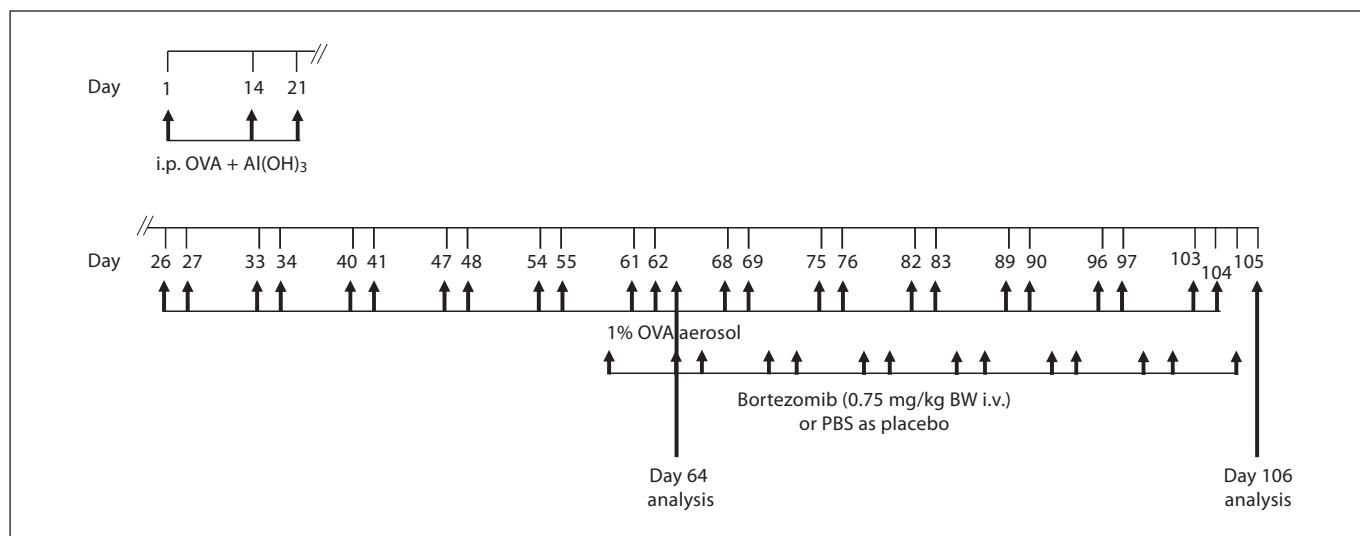


**Fig. 1.** Sensitization, challenge and treatment protocol. BALB/c mice were sensitized to OVA by 3 intraperitoneal (i.p.) injections of OVA adsorbed to aluminium hydroxide [Al(OH)<sub>3</sub>] and consequently challenged with a 1% OVA aerosol to induce local inflammation in the airways. Control animals (n = 8) received PBS intraperitoneally and were similarly challenged. Treatment with

0.75 mg/kg BW bortezomib was performed by intravenous (i.v.) injections 24 h before the first weekly OVA aerosol challenge and 24 h after the second weekly challenge. Short-term treatment consisted of 2 intravenous injections, and long-term treatment included 14 injections (n = 8 in each group). Placebo control animals (n = 8) received sterile PBS intravenously.

and 3- $\mu$ m sections were stained with periodic acid-Schiff (PAS) as described previously [15]. Photomicrographs were recorded by a digital camera (DP-25, Olympus, Japan) attached to a microscope (BX-51, Olympus, Japan) at 100-fold magnification using Olympus cell<sup>^</sup>F software.

#### Assessment of Airway Responsiveness to Methacholine

Airway responsiveness to methacholine (MCh; acetyl- $\beta$ -methylcholine chloride, Sigma) was measured on the basis of the alteration of the midexpiratory airflow in response to gradient concentrations of inhaled MCh aerosol using head-out body plethysmography as described previously [16]. The MCh concentration that caused a 50% reduction in baseline midexpiratory airflow (MCh<sub>50</sub>) was used as a parameter for airway constriction.

#### Quantification of Plasma Cells in Spleen by FACS Analysis

Single-cell suspensions of spleens were filtered through a 70- $\mu$ m cell strainer (BD Falcon, BD Biosciences, Heidelberg, Germany) and washed twice with PBS/0.5% BSA. Subsequently, cells were fixed with 2% formaldehyde in PBS. After repeated washing, cells were resuspended in PBS/0.5% BSA. Unspecific binding of staining antibodies was blocked by preincubation with rat anti-mouse CD16/CD32 (5  $\mu$ g/ml; clone 2.4G2, made in-house) for 5 min on ice. Cells were stained with rat anti-mouse CD138-phycoerythrin (clone 281-2, BD Biosciences), rat anti-mouse Ig  $\kappa$  light chain-Alexa405 (clone 187.1, made in-house) and rat anti-mouse Gr-1-FITC (clone RB6-8C5, made in-house) for 15 min at room temperature in PBS/0.5% BSA containing 0.5% saponin (Sigma). After washing, cells were analyzed by flow cytometry (BD LSRII, BD Biosciences, San Jose, Calif., USA).

#### Measurements of OVA-Specific Ig Titers in Serum Samples

A sandwich enzyme-linked immunosorbent assay (ELISA) was used for OVA-specific Ig measurement in serum, as described previously [17].

#### Statistics

Results are presented as mean values  $\pm$  SEM. Unpaired Student's t test (Microsoft Excel) was used to determine the significance of differences between animal groups.

## Results

#### Short-Term Bortezomib Treatment Has No Therapeutic Effect on Chronic Experimental Asthma

Placebo-treated mice revealed a profound allergic inflammation of the airways as assessed by BAL fluid and lung histology (fig. 2A, B). Whereas the numbers of alveolar macrophages in BAL fluid did not show a significant alteration in comparison with PBS controls, total numbers of eosinophils were significantly elevated, with average numbers of  $1.88 \times 10^5$  cells per BAL (fig. 2A). Neutrophils and lymphocytes were also present in BAL samples of the placebo group, although to a much lesser degree than eosinophils (fig. 2A). The same inflammatory pattern was apparent in lung histology, where an eosinophil-dominant

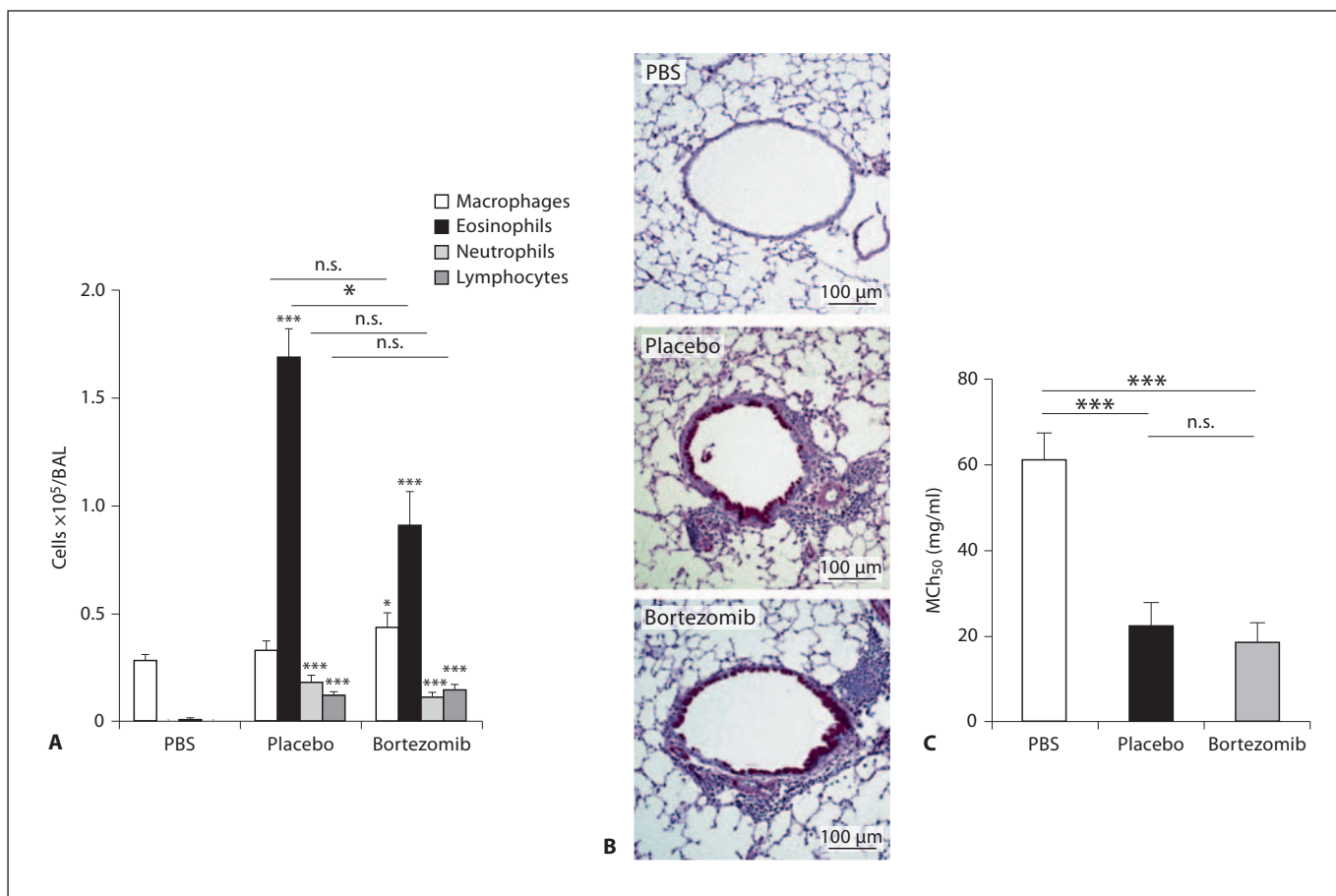


**Fig. 2.** Effects of short-term bortezomib treatment on experimental asthma in mice. **A** Absolute numbers of macrophages, eosinophils, neutrophils and lymphocytes in BAL samples from OVA-sensitized BALB/c mice chronically challenged with aerosolized OVA and given short-term intravenous treatment with either PBS (placebo;  $n = 8$ ) or 0.75 mg/ml bortezomib ( $n = 8$ ) as described in Material and Methods. Control (PBS;  $n = 8$ ) animals were sensitized, challenged and treated with PBS. **B** Lung histology from

BALB/c mice. Representative photomicrographs of PAS-stained airway cross-sections derived from paraffin-embedded whole lung sections are presented. **C** Airway reactivity to MCh. Airway constriction was measured as described in Material and Methods and expressed as MCh<sub>50</sub>. Data are presented as means  $\pm$  SEM. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ : significant difference versus the control group. n.s. = Not significant.

ed infiltrate could be observed along the entire airway tree, which was further associated with a distinctive goblet cell metaplasia (fig. 2B). Short-term treatment with 0.75 mg/kg BW bortezomib resulted in an almost 50% reduction in eosinophils, while the numbers of neutrophils and lymphocytes in BAL remained unchanged. A significant effect on leukocyte accumulation in airway tissues or goblet cell metaplasia could not be observed (fig. 2A, B).

In placebo-treated mice, allergic airway inflammation was further accompanied by the development of AHR, as demonstrated by an average MCh<sub>50</sub> of 22.2 mg/ml as compared with 61.1 mg/ml in the PBS control group (fig. 2C). The bortezomib-treated group showed a 50%

reduction in the MCh<sub>50</sub> at MCh concentrations of 18.4 mg/ml, demonstrating no therapeutic effect of the treatment on AHR. In conclusion, while bortezomib treatment resulted in a significant reduction of the eosinophil influx into the bronchoalveolar lumen, it had no effect on airway responsiveness.

#### *Short-Term Bortezomib Treatment Reduces Allergen-Specific IgE but neither Production of Allergen-Specific IgG nor Plasma Cell Numbers in Spleen*

Whereas OVA-specific antibodies were not detectable in PBS control mice, OVA sensitization and chronic OVA

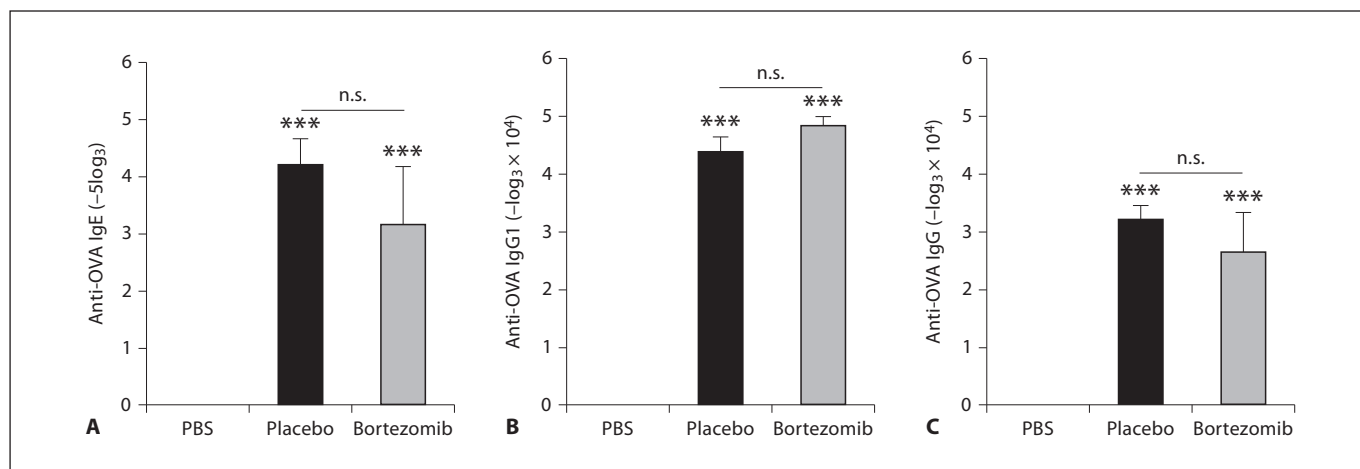


**Fig. 3.** Effects of short-term bortezomib treatment on serum titers of OVA-specific Igs. Anti-OVA Ig serum titers of OVA-sensitized BALB/c mice chronically challenged with aerosolized OVA and given short-term intravenous treatment with either PBS (placebo; n = 8) or 0.75 mg/ml bortezomib (n = 8) as described in Material and Methods were measured. Control (PBS; n = 8) animals were

sensitized, challenged and treated with PBS. Serum titers of anti-OVA IgE (A), anti-OVA IgG1 (B) and anti-OVA IgG (C) were determined by ELISA. Data are presented as means  $\pm$  SEM. \*\*\* p < 0.001: significant difference versus the placebo group. n.s. = Not significant.

aerosol challenge in placebo-treated mice resulted in high titers of anti-OVA IgE, IgG1 and IgG. In mice receiving short-term bortezomib treatment, no reduction of any of these titers could be observed (fig. 3).

Since bortezomib targets plasma cells by inhibiting the proteasome complex, which leads to apoptosis of the respective cells, plasma cell frequencies and numbers in spleen (fig. 4A, B) and bone marrow (fig. 4C, D) were determined by flow cytometry. In placebo-treated animals, the frequency and number of plasma cells in bone marrow was significantly increased. However, short-term bortezomib treatment did not lead to a significant reduction of plasma cell frequency or number in bone marrow or spleen (fig. 4).

#### *Long-Term Bortezomib Treatment Has No Significant Therapeutic Effect on Chronic Experimental Asthma*

In mice undergoing long-term placebo treatment, chronic experimental asthma was characterized by marked inflammation of the airways that predominantly consisted of eosinophils, as demonstrated by high cell numbers in BAL fluid. Additionally, lymphocytes and neutrophils could be detected in BAL fluid, although to a much lesser degree (fig. 5A). A similar leukocytic infiltrate could be observed in airway tissues, which was further associated with a distinct goblet cell metaplasia as assessed in PAS-stained lung sections (fig. 5B). Long-term bortezomib treatment had no significant effect on

the infiltration of eosinophils, neutrophils or lymphocytes into airway tissue or the bronchoalveolar lumen. A reduction of goblet cell metaplasia could not be detected.

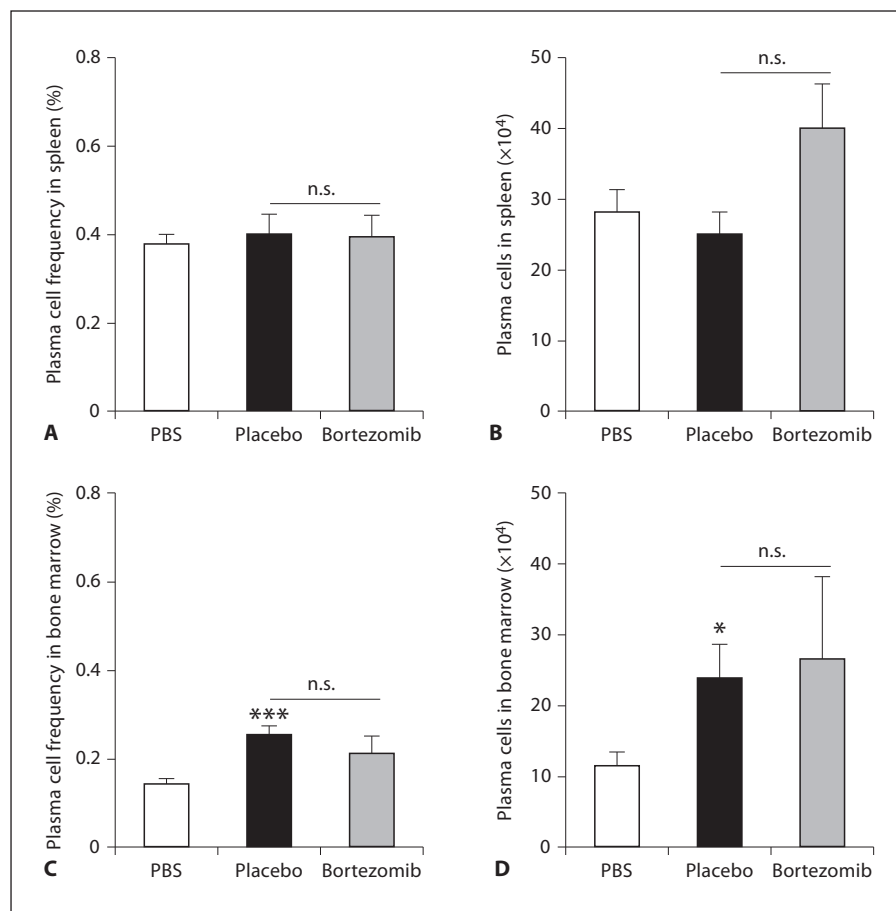
In comparison to healthy mice, long-term placebo-treated mice further revealed pronounced AHR, as demonstrated by an average MCh<sub>50</sub> of 30.5 mg/ml (MCh<sub>50</sub> of the PBS group: 85.9 mg/ml). Long-term bortezomib-treated animals showed an MCh<sub>50</sub> of 27.4 mg/ml, demonstrating no therapeutic effect on airway responsiveness (fig. 5C).

#### *Long-Term Bortezomib Treatment Reduces Allergen-Specific IgE but neither Production of Allergen-Specific IgG nor Plasma Cell Numbers in Spleen*

Animals with experimental asthma undergoing long-term placebo treatment also displayed high titers of anti-OVA IgE, IgG1 and IgG, while in healthy controls, OVA-specific antibodies were not detectable. In contrast, long-term bortezomib treatment lowered anti-OVA IgE titers to one fifth of the level in placebo-treated mice but did not significantly reduce titers of anti-OVA IgG1 or total IgG (fig. 6).

High serum titers of OVA-specific Igs were further associated with significantly increased frequency of plasma cells in the spleen as detected by staining against CD138 and intracellular Ig  $\kappa$  chain (fig. 7). An increased plasma cell frequency in spleen could also be observed in animals

**Fig. 4.** Effects of short-term bortezomib treatment on plasma cell numbers in spleen and bone marrow. Plasma cell frequencies (**A, C**) and plasma cell numbers (**B, D**) in spleens (**A, B**) and bone marrow (**C, D**) of OVA-sensitized BALB/c mice chronically challenged with aerosolized OVA and given short-term intravenous treatment with either PBS (placebo;  $n = 8$ ) or 0.75 mg/ml bortezomib ( $n = 8$ ) were assessed by FACS analysis as described in Material and Methods. Control (PBS;  $n = 8$ ) animals were sensitized, challenged and treated with PBS. Data are presented as means  $\pm$  SEM. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ : significant difference versus the placebo group. n.s. = Not significant.



that received long-term bortezomib treatment. Interestingly, the bortezomib-treated group even revealed a slightly albeit not significantly increased frequency and total number of plasma cells as compared with the placebo group. However, placebo-treated animals showed only a minute and insignificant increase in plasma cell numbers as compared with the PBS control group, while bortezomib-treated mice revealed a nearly 3-fold increase.

## Discussion

The present study aimed to evaluate the therapeutic potential of the proteasome inhibitor bortezomib in allergic bronchial asthma. Bortezomib treatment was assessed in a mouse model of chronic experimental asthma, which mimics human asthma more closely than the widely used acute murine asthma models [18]. Moreover, a therapeutic setting was chosen, with long-term borte-

zomib treatment being initiated 4 weeks after the onset of allergen challenges. Neither the short-term nor the long-term bortezomib treatment significantly reduced any of the characteristic features of asthma, including AHR, in this experimental model. Titers of OVA-specific IgE were significantly decreased in bortezomib-treated animals, whereas reductions in OVA-specific IgG1 and OVA-specific IgG titers did not reach statistical significance. Furthermore, the frequency and total numbers of plasma cells in spleen did not show any decrease as compared with the placebo-treated group.

Blocking the proteasome stabilizes many cell cycle-inhibitory proteins and inhibits the antiapoptotic transcription factor nuclear factor- $\kappa$ B, leading to cell cycle arrest and apoptosis [19]. Additionally, another mechanism leads to exceptional sensitivity to proteasome inhibition in myeloma cells. Extensive Ig synthesis as observed in myeloma cells is compulsorily associated with the production of unfolded protein, inclusive of defective ribosomal products. Inhibition of the proteasome leads to

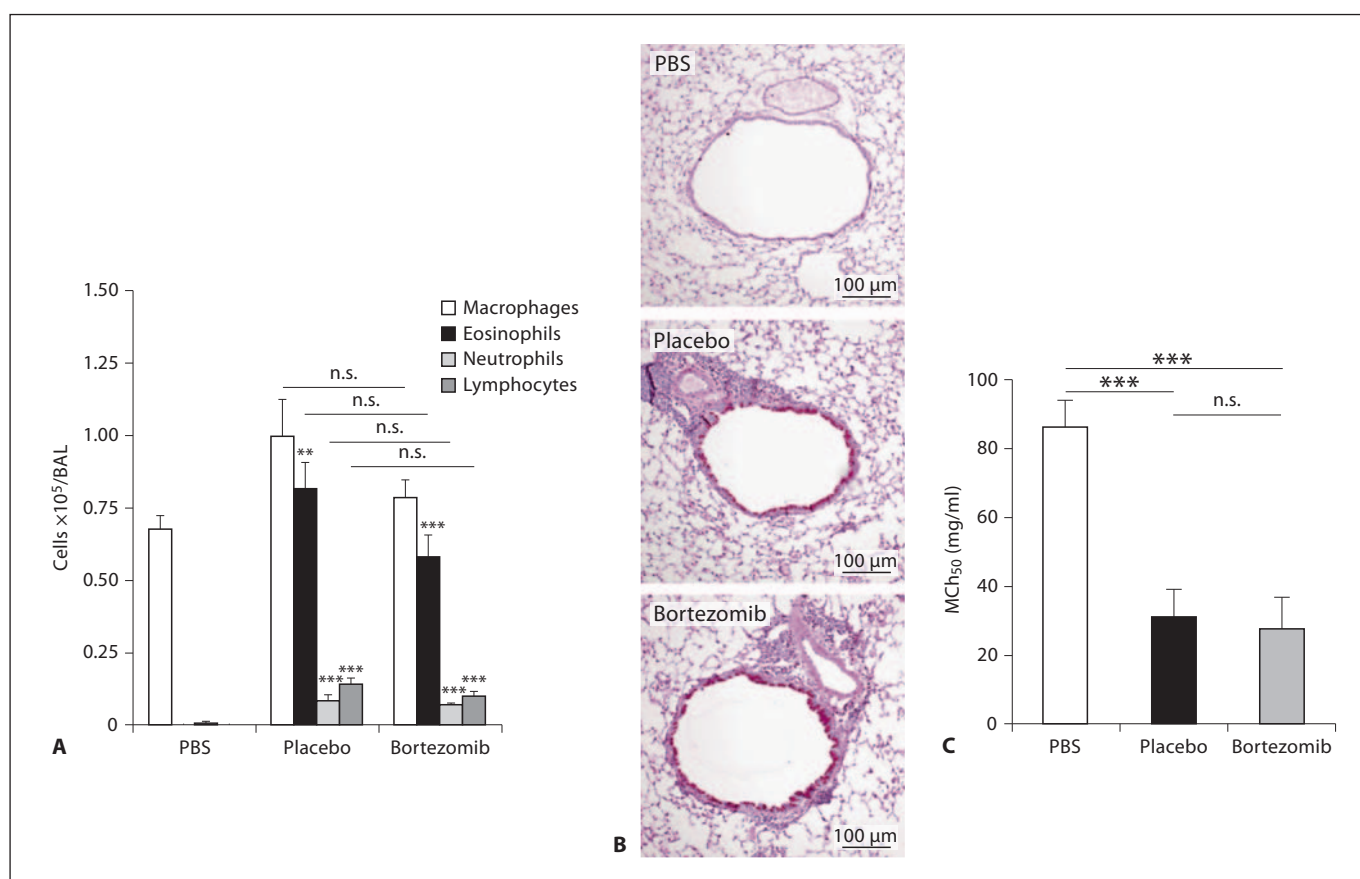


**Fig. 5.** Effects of long-term bortezomib treatment on experimental asthma in mice. **A** Absolute numbers of macrophages, eosinophils, neutrophils and lymphocytes in BAL samples from OVA-sensitized BALB/c mice chronically challenged with aerosolized OVA and given long-term intravenous treatment with either PBS (placebo;  $n = 8$ ) or 0.75 mg/ml bortezomib ( $n = 8$ ) as described in Material and Methods. Control (PBS;  $n = 8$ ) animals were sensitized, challenged and treated with PBS. **B** Lung histology from

BALB/c mice. Representative photomicrographs of PAS-stained airway cross-sections derived from paraffin-embedded whole lung sections are presented. **C** Airway reactivity to MCh. Airway constriction was measured as described in Material and Methods and expressed as MCh<sub>50</sub>. Data are presented as means  $\pm$  SEM. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ : significant difference versus the control group. n.s. = Not significant.

accumulation of unfolded proteins in the endoplasmic reticulum and cytoplasm, which induces proapoptotic signals and reduces the survival signal Bcl-2, ultimately leading to apoptosis of the respective cells [6]. This observation led to the question of whether bortezomib could be a treatment option in diseases arising from pathologic antibody production such as systemic SLE, rheumatoid arthritis and asthma [4, 20].

The results of the present study clearly demonstrate that short-term as well as long-term treatment with a high dose of 0.75 mg/kg BW bortezomib are not sufficient to ablate the strong humoral immune response characteristic of the chronic murine asthma model. While short-term treatment did not show any significant effects on the

production of OVA-specific Igs, long-term bortezomib treatment resulted in a significant reduction of OVA-specific IgE serum titers. This demonstrates that bortezomib actually had an effect in this model, although plasma cell frequencies in spleen and OVA-specific IgG or IgG1 titers could not be reduced. One could speculate that bortezomib depleted plasma cells, as has been shown in other models, but that this effect was negated by continuous formation of new plasma cells in response to the chronic OVA challenge. Such temporary plasma cell depletion would have a much more pronounced effect on IgE than on IgG levels, because serum IgE has a much shorter half-life than IgG, i.e. 12 h compared to about 1 week [21]. Therefore, when Ig-secreting plasma cells are depleted by

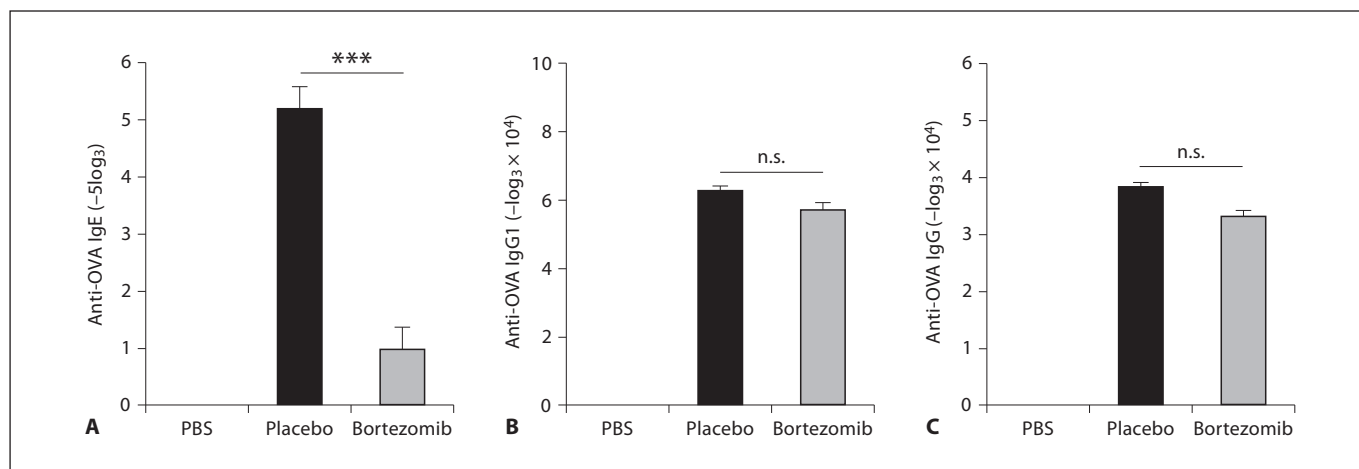
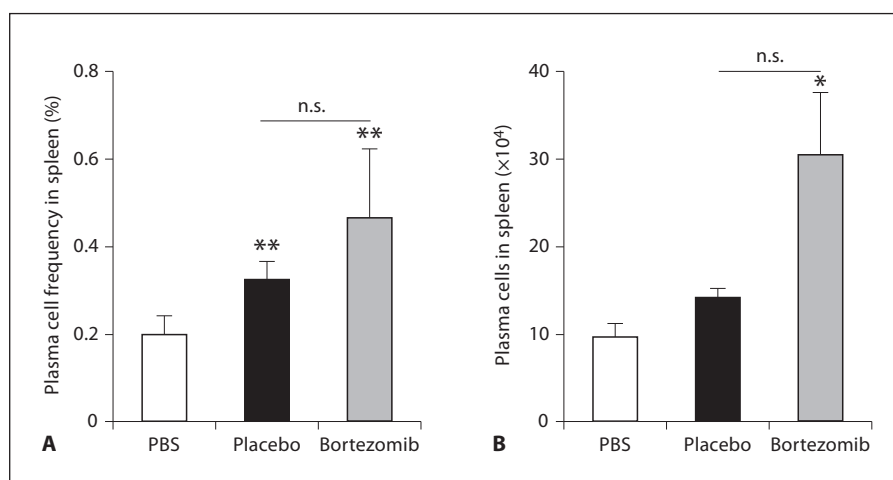


**Fig. 6.** Effects of long-term bortezomib treatment on serum titers of OVA-specific Igs. Anti-OVA Ig serum titers of OVA-sensitized BALB/c mice chronically challenged with aerosolized OVA and given long-term intravenous treatment with either PBS (placebo;  $n = 8$ ) or 0.75 mg/ml bortezomib ( $n = 8$ ) as described in Material and Methods were measured. Control (PBS;  $n = 8$ ) animals were

sensitized, challenged and treated with PBS. Serum titers of anti-OVA IgE (A), anti-OVA IgG1 (B) and anti-OVA IgG (C) were determined by ELISA. Data are presented as means  $\pm$  SEM. \*\*\*  $p < 0.001$ : significant difference versus the placebo group. n.s. = Not significant.

**Fig. 7.** Effects of short-term bortezomib treatment on plasma cell numbers in spleen. Plasma cell frequencies (A) and plasma cell numbers (B) in spleens of OVA-sensitized BALB/c mice chronically challenged with aerosolized OVA and given short-term intravenous treatment with either PBS (placebo;  $n = 8$ ) or 0.75 mg/ml bortezomib ( $n = 8$ ) were assessed by FACS analysis as described in Material and Methods. Control (PBS;  $n = 8$ ) animals were sensitized, challenged and treated with PBS. Data are presented as means  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ : significant difference versus the placebo group. n.s. = Not significant.



bortezomib treatment, the existing IgE titer drops immediately, while IgG titers would drop only with time. This difference between IgE and IgG is critical when the results are considered in the context of bortezomib pharmacology and the time points of serum titer measurement. The mean elimination half-life of bortezomib in humans has been estimated to be 9–15 h, with proteasome inhibition peaking 24 h after application [22]. Thus, it is reasonable to assume that at the time point of analysis, e.g. 24 h after bortezomib application, the protea-

some-inhibitory effect of bortezomib led to decreased serum titers of anti-OVA IgE but not IgG. Since in mice, in contrast to humans, IgG1 is also able to mediate mast cell degranulation after allergen contact [23], the subsequent inflammatory reactions that ultimately lead to formation of the asthmatic phenotype are not ablated. In conclusion, in accordance with previous observations, the data presented here indicate that bortezomib can reduce pathogenic antibodies. However, due its short-lived effect it may not be very effective in human allergic asthma,

where allergen exposure could be very frequent, as mimicked by the mouse model used in the present study.

In murine models of SLE and rheumatoid arthritis, bortezomib treatment suppressed the production of pathogenic IgG antibodies and attenuated the respective disease pattern [8, 24]. In the present study, bortezomib-treated mice exhibited a considerable reduction in allergen-specific IgE titers but showed no reduction of IgG-positive plasma cells in spleen or allergen-specific IgG titers. Consequently, bortezomib did not affect the asthmatic phenotype. Since in all three therapeutic studies of bortezomib previously carried out in mice, the applied dosage of bortezomib was in a comparable range (between 0.75 and 1 mg/kg BW), the different outcome in our study is unlikely to be an issue of dosage. Long-term treatment with a dose of more than 0.75 mg/kg BW has so far not been carried out in animals, because of potential toxic side effects as have already been described in numerous studies in patients as well as in animals [25, 26]. In turn, the poor effect of bortezomib observed in the present study may be explained by differences in the pathogenetic mechanisms that are active in the different disease models used. The model of collagen-induced arthritis utilized by Lee et al. [24] triggers a systemic immune response by means of 2 intradermal injections of bovine type II collagen emulsified in complete Freund's adjuvant. In the course of the subsequent protocol, the animals do not receive any further exogenous antigen trigger. Neubert et al. [8] used the NZB/W F1 and MRL/lpr mouse models, which spontaneously develop lupus-like disease. In both cases, female mice exhibit production of autoimmune IgG against nuclear antigens that increases over time, ultimately leading to severe immune complex-mediated glomerulonephritis and lethal renal failure in virtually all animals by 12 months of age [25]. Exogenous antigen exposure is not used in these models to further trigger the autoimmune response. Interestingly, IgG autoantibodies (anti-DNA) are completely suppressed by bortezomib treatment in lupus mouse models, whereas total IgG is reduced to only about 50% and quickly rises again after bortezomib treatment [8]. In contrast to the above-mentioned models, in the present study a mouse model of chronic experimental asthma was used in which the phenotype is achieved by repetitive, daily, long-term antigen exposure to the model allergen OVA [9]. Because such a treatment mimics the repeated allergen contact typical of asthmatic patients sensitized to airborne allergens, such models have already been proven to be a valuable tool for the assessment of new therapeutic strategies in asthma [13, 26–28]. This

model closely reflects the hallmarks of human bronchial asthma, including chronic allergic airway inflammation, mucus hypersecretion, development of AHR, chronic airflow limitation and airway remodeling [9]. By reflecting chronic aspects of the human disease, this model is superior to the 'classic' OVA model, which utilized only short-term allergen exposure and is still the subject of lively discussion with regard to modeling asthma in mice [29]. Typical for the chronic model used in our study is a close relationship between the allergen-triggered local inflammatory reaction and the systemic humoral immune response. In a previous study, we could demonstrate that chronic allergen exposure results in increased numbers of long-lived plasma cells in secondary lymphoid organs, producing allergen-specific IgE and IgG1 [11]. Furthermore, upon chronic allergen exposure, inflamed airway tissues created survival niches for plasma cells, leading to a local humoral immune response [12]. Thus, chronic allergen exposure via the airway tract elicits a humoral immune response in this model which is likely to be different from the one seen in mouse models of human SLE and collagen-induced arthritis. Hence, different effects of bortezomib in these models are not unlikely.

We observed that short-term bortezomib treatment significantly reduced eosinophil numbers in BAL fluid. Whether this effect is due to direct effects of bortezomib on eosinophils or an indirect effect, e.g. on eosinophil recruitment, remains elusive, especially since the reduction is only gradual and not associated with an improved asthmatic phenotype in mice. However, long-term bortezomib treatment with the same dosage did not decrease the numbers of eosinophils in BAL fluid.

The failure to reduce plasma cells in spleen in the present study appears not to be an issue of administration route, since bortezomib was applied systemically, but could be due to mechanisms of resistance developed during long-term treatment. In a clinical phase II trial with 202 multiple myeloma patients, Richardson et al. [31] reported that only 35% of the cohort responded to bortezomib treatment, with 10% achieving complete remission, whereas 65% did not show any response. This observation suggested that myeloma cells develop mechanisms of resistance to bortezomib treatment. In an *in vitro* study, Oerlemans et al. [32] demonstrated that the proteasome subunit  $\beta 5$  (PSMB5) plays a critical role in the development of resistance to bortezomib treatment. Both overexpression of the PSMB5 gene and an Ala49Thr mutation residing in a highly conserved bortezomib-binding pocket of PSMB5 provided protection against proteasome inhibition [32]. Since this study was carried out in human

cells, its transferability to the murine system is limited, but it does indicate the possibility of resistance development. Different mouse strains are likely to exhibit individual kinetics for the development of resistance to long-term treatment, thus offering an additional option to explain the differential efficiency of bortezomib in distinct experimental disease models.

In conclusion, long-term bortezomib treatment successfully reduced OVA-specific IgE titers in mice with established chronic experimental asthma. However, plasma cell numbers in spleen and in particular OVA-specific IgG1 titers remained largely unchanged. Therefore, the subsequent inflammatory cascade leading to the formation of experimental asthma, which depends on IgG1 and

mast cells, remained unaffected. Because the chronicity and frequency of allergen exposure in the studied model closely resembles the conditions of severe asthma patients, our results suggest that bortezomib treatment has only limited value as a therapy against allergic bronchial asthma.

## Acknowledgements

We thank Franziska Ganzert and Kathleen Kurwahn for their excellent technical assistance. The study was supported by the Deutsche Forschungsgemeinschaft (Cluster of Excellence 'Inflammation at Interfaces' EXC 306).

## References

- Broide D: New perspectives on mechanisms underlying chronic allergic inflammation and asthma in 2007. *J Allergy Clin Immunol* 2008;122:475–480.
- Matsumoto K, Tamari M, Saito H: Involvement of eosinophils in the onset of asthma. *J Allergy Clin Immunol* 2008;121:26–27.
- Fanta CH: Asthma. *N Engl J Med* 2009;360:1002–1014.
- Luger EO, Wegmann M, Achatz G, Worm M, Renz H, Radbruch A: Allergy for a lifetime? *Allergol Int* 2010;59:1–8.
- Teicher BA, Ara G, Herbst R, Palombella VJ, Adams J: The proteasome inhibitor PS-341 in cancer therapy. *Clin Cancer Res* 1999;5:2638–2645.
- Meister S, Schubert U, Neubert K, Herrmann K, Burger R, Gramatzki M, Hahn S, Schreiber S, Wilhelm S, Herrmann M, Jäck HM, Voll RE: Extensive immunoglobulin production sensitizes myeloma cells for proteasome inhibition. *Cancer Res* 2007;67:1783–1792.
- Katodritou E, Kartsios C, Gastari V, Verrou E, Mihou D, Banti A, Lazaraki G, Lazaridou A, Kaloutsis V, Zervas K: Successful treatment of extramedullary gastric plasmacytoma with the combination of bortezomib and dexamethasone: first reported case. *Leuk Res* 2008;32:339–341.
- Neubert K, Meister S, Moser K, Weisel F, Masada D, Amann K, Wiethe C, Winkler TH, Kalden JR, Manz RA, Voll RE: The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. *Nat Med* 2008;14:748–755.
- Wegmann M, Fehrenbach H, Fehrenbach A, Held T, Schramm C, Garn H, Renz H: Involvement of distal airways in a chronic model of experimental asthma. *Clin Exp Allergy* 2005;35:1263–1271.
- Mehlhof PD, van de Rijn M, Goldberg AB, Brewer JP, Kurup VP, Martin TR, Oettgen HC: Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc Natl Acad Sci USA* 1997;94:1344–1349.
- Luger EO, Fokuhl V, Wegmann M, Abram M, Tillack K, Achatz G, Manz RA, Worm M, Radbruch A, Renz H: Induction of long-lived allergen-specific plasma cells by mucosal allergen challenge. *J Allergy Clin Immunol* 2009;124:819–826.
- Abram M, Wegmann M, Fokuhl V, Sonar S, Luger EO, Kerzel S, Radbruch A, Renz H, Zemlin M: Nerve growth factor and neurotrophin-3 mediate survival of pulmonary plasma cells during the allergic airway inflammation. *J Immunol* 2009;182:4705–4712.
- Wegmann M, Göggel R, Sel S, Sel S, Erb KJ, Kalkbrenner F, Renz H, Garn H: Effects of a low-molecular-weight CCR-3 antagonist on chronic experimental asthma. *Am J Respir Cell Mol Biol* 2007;36:61–67.
- Lonial S, Waller EK, Richardson PG, Jagannath S, Orłowski RZ, Giver CR, Jaye DL, Francis D, Giusti S, Torre C, Barlogie B, Berenson JR, Singhal S, Schenkein DP, Esseltine DL, Anderson J, Xiao H, Heffner LT, Anderson KC; SUMMIT/CREST Investigators: Risk factors and kinetics of thrombocytopenia associated with bortezomib for relapsed, refractory multiple myeloma. *Blood* 2005;106:3777–3784.
- Sel S, Wegmann M, Sel S, Bauer S, Garn H, Alber G, Renz H: Immunomodulatory effects of viral TLR ligands on experimental asthma depend on the additive effects of IL-12 and IL-10. *J Immunol* 2007;178:7805–7813.
- Sel S, Wegmann M, Dicke T, Sel S, Henke W, Yildirim AO, Renz H, Garn H: Effective prevention and therapy of experimental allergic asthma using a GATA-3-specific DNase. *J Allergy Clin Immunol* 2008;121:910–916.
- Orinska Z, Osiak A, Löhler J, Bulanova E, Budagian V, Horak I, Bulfone-Paus S: Novel B cell population producing functional IgG in the absence of membrane IgM expression. *Eur J Immunol* 2002;32:3472–3480.
- Fulkerson PC, Rothenberg ME, Hogan SP: Building a better mouse model: experimental models of chronic asthma. *Clin Exp Allergy* 2005;35:1251–1253.
- Hershko A: Roles of ubiquitin-mediated proteolysis in cell cycle control. *Curr Opin Cell Biol* 1997;9:788–799.
- van der Vlag J, Berden JH: Proteasome inhibition: a new therapeutic option in lupus nephritis? *Nephrol Dial Transplant* 2008;23:3771–3772.
- Vieira P, Rajewsky K: The half-lives of serum immunoglobulins in adult mice. *Eur J Immunol* 1988;18:313–316.
- Dubey S, Schiller JH: Three emerging new drugs for NSCLC: pemetrexed, bortezomib, and cetuximab. *Oncologist* 2005;10:282–291.
- Herz U, Lumpp U, Daser A, Gelfand EW, Renz H: Murine animal models to study the central role of T cells in immediate-type hypersensitivity responses. *Adv Exp Med Biol* 1996;409:25–32.
- Lee SW, Kim JH, Park YB, Lee SK: Bortezomib attenuates murine collagen-induced arthritis. *Ann Rheum Dis* 2009;68:1761–1767.
- Schiff D, Wen PY, van den Bent MJ: Neurological adverse effects caused by cytotoxic and targeted therapies. *Nat Rev Clin Oncol* 2009;6:596–603.

- 26 Argyriou AA, Iconomou G, Kalofonos HP: Bortezomib-induced peripheral neuropathy in multiple myeloma: a comprehensive review of the literature. *Blood* 2008;112:1593–1599.
- 27 Theofilopoulos AN, Dixon FJ: Murine models of systemic lupus erythematosus. *Adv Immunol* 1985;37:269–390.
- 28 Henderson WR Jr, Chiang GK, Tien YT, Chi EY: Reversal of allergen-induced airway remodeling by CysLT1 receptor blockade. *Am J Respir Crit Care Med* 2006;173:718–728.
- 29 López E, del Pozo V, Miguel T, Sastre B, Seoane C, Civantos E, Llanes E, Baeza ML, Palomino P, Cárdbaba B, Gallardo S, Manzarbeitia F, Zubeldia JM, Lahoz C: Inhibition of chronic airway inflammation and remodeling by galectin-3 gene therapy in a murine model. *J Immunol* 2006;176:1943–1950.
- 30 Munitz A, Bachelet I, Levi-Schaffer F: Reversal of airway inflammation and remodeling in asthma by a bispecific antibody fragment linking CCR3 to CD300a. *J Allergy Clin Immunol* 2006;118:1082–1089.
- 31 Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, Rajkumar SV, Srkalovic G, Alsina M, Alexanian R, Siegel D, Orlowski RZ, Kuter D, Limentani SA, Lee S, Hideshima T, Esseltine DL, Kauffman M, Adams J, Schenkein DP, Anderson KC: A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med* 2003;348:2609–2617.
- 32 Oerlemans R, Franke NE, Assaraf YG, Cloos J, van Zantwijk I, Berkers CR, Scheffer GL, Debipersad K, Vojtekova K, Lemos C, van der Heijden JW, Ylstra B, Peters GJ, Kaspers GL, Dijkmans BA, Scheper RJ, Jansen G: Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood* 2008;112:2489–2499.
- 33 Bates JH, Rincon M, Irvin CG: Animal models of asthma. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L401–L410.
- 34 Rokudai A, Terui Y, Kuniyoshi R, Mishima Y, Mishima Y, Aizu-Yokota E, Sonoda Y, Kasahara T, Hatake K: Differential regulation of eotaxin-1/CCL11 and eotaxin-3/CCL26 production by the TNF-alpha and IL-4 stimulated human lung fibroblast. *Biol Pharm Bull* 2006;29:1102–1109.