

Opposing effects on immune function and skin barrier regulation by the proteasome inhibitor bortezomib in an allergen-induced eczema model

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Abstract: Proteasome inhibition (PI) has been reported to interfere with antibody-driven autoimmune diseases. The impact of PI on the allergic immune response and on skin diseases like atopic dermatitis (AD) has not been thoroughly explored, however. Here, we examined whether the PI bortezomib interferes with the allergic immune response and the severity of AD by using an established mouse model of allergen-driven dermatitis, to which bortezomib was applied after the establishment of systemic sensitization to ovalbumin. The treatment indeed resulted in a remarkable decrease in total and allergen-specific plasma cells/antibody-secreting cells, as evidenced by flow cytometry and ELISpot, respectively. This was accompanied by rapid reductions in serum antibody titres, including a prominent reduction of the IgE isotype. CD4⁺ and CD8⁺ cells were greatly diminished in lesional skin on immunohistological staining. The impressive effects at the level of immune modulation did not result in any

improvement in the eczema, however. Following up on this unexpected result, we found that the skin itself was susceptible to bortezomib, by which it was instructed to lower the expression of critical skin barrier genes, especially transglutaminase-1 and filaggrin. Together, bortezomib eliminates plasma cells and decreases immunoglobulin responses, including allergenic IgE. Although anti-inflammatory effects are detectable in the skin, counter-regulatory effects from PI on resident skin cells likely undermine improvement in the eczema. These results caution against the therapeutic use of bortezomib for inflammatory skin disorders, which are characterized by inherently impaired barrier function, especially AD.

Key words: antibody-secreting cells – atopic dermatitis – bortezomib (Velcade®) – proteasome inhibition – skin barrier

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Introduction

The ubiquitin-proteasome system (UPS) represents the main protein degradation pathway in human cells (1,2). The network consists of multiple actors dedicated to the identification, targeting, and destruction of unfolded, misfolded, functionally incompetent and otherwise unneeded proteins. These are first marked for degradation by covalent linkage of a chain of ubiquitin moieties in a multistep reaction involving the ubiquitin activase (E1), one of several ubiquitin conjugates (E2s), and one of hundreds of distinct ubiquitin ligases (E3s), the latter, the key determinants of substrate specificity. The polyubiquitinated proteins are recognized by the proteasome, a large barrel-shaped multicatalytic protease complex, and finally, hydrolysed to small peptides. Disruption of proteasome activity causes accumulation of otherwise degradable proteins and eventually blocks proliferation and induces cell death (2–4).

Although the proteasome is a basic structure of all cells, susceptibility to proteasome inhibition (PI) is not equal among cell types. In fact, certain cancer cells display excellent susceptibility, while normal quiescent cells are more resistant (5). This distinction was a prerequisite to the development of bortezomib (Bz; a selective inhibitor of the proteasome) as a drug, which is now an approved treatment for multiple myeloma and mantle cell lymphoma (6–8). The mechanisms behind the selectivity are not entirely understood, but various events likely contribute to the specific anticancer effects, including stabilization of regulatory

proteins (e.g. I κ B, p21 and p53) (1). An additional aspect is that PI sensitivity seems directly coupled to the protein synthesis rate (9). The fact that intense protein synthesis is a hallmark of all antibody-producing cells combined with the observation that clinical responsiveness to Bz is particularly pronounced in the case of multiple myeloma (a malignant plasma cell disorder) paved the way to examine normal, untransformed plasma cells for their sensitivity towards Bz. This appeared even more attractive as plasma cells were found to decrease their proteasomal capacity during terminal differentiation, reaching saturation of the UPS earlier than other cells (10). In fact, several studies conducted in appropriate animal models provided clear evidence that Bz also eliminates normal plasma cells, thereby reducing autoantibody production and alleviating the severity of autoimmune diseases like SLE (lupus), arthritis and myasthenia gravis (11–13).

Like immunoglobulin-driven autoimmune disorders, type-I allergies are characterized by aberrant humoral immune responses, even though the deregulated class is of the IgE isotype and directed against innocuous antigens from the environment. There is limited data available regarding the role of the proteasome and PI in the context of allergy. A recent study reported on modest effects of Bz in a model of chronic asthma where allergen-specific IgE was reduced only after prolonged treatment, while plasma cell depletion could not be detected at all (14), a result somewhat contrasting the observations made in autoimmunity.

To clarify whether PI may have an impact on antibody-producing cells in the context of allergy, we employed a well-defined mouse model of allergen-triggered dermatitis, which is associated with high IgE responses and histological as well as clinical features of human atopic dermatitis (AD) (15,16). Here, PI reduced plasma cells even when applied after established allergic sensitization, and concomitantly decreased allergen-specific immunoglobulins, including IgE. Likewise, PI profoundly decreased skin-infiltrating T cells. Nevertheless, the suppressive effects at the level of immune cells did not translate into clinical improvement of the skin. In seeking to elucidate the basis of this unexpected outcome, we found that the skin itself was targeted by PI, by which it was instructed to decrease the expression of genes specifically involved in skin barrier function. While certainly too early to discard a therapeutic benefit of PI for allergic disorders, effects exerted by PI in the skin caution against its use for disorders characterized by aberrant skin barrier function, including AD.

Materials and methods

AD mouse model

Female BALB/c mice (8–10 weeks old) were obtained from Janvier, Le Genest-Saint-Isle, France. The protocols to perform animal experiments were approved by the local state office of health and social affairs, Germany.

An epicutaneous allergen application patch test method was used to induce eczema (15). The scheme is depicted in Figure S1. After intraperitoneal sensitization with OVA (Sigma-Aldrich, Steinheim, Germany), the skin was shaved and OVA were applied by the patch test method. Each mouse had a total of three 1-week exposures to the same skin site, with patch renewal after 3 days, respectively, and a 2-week interval between each application. Bortezomib (Bz, 0.75 mg/kg body weight) or PBS as a control was administered to these mice intravenously via tail vein as depicted in Figure S1. Because of the short half-life of Bz, a fresh vial was used for each time point (as well as for the *ex vivo* experiment described below). The severity of OVA-induced eczematous skin lesions was assessed by a skin scoring system, which considers typical features of human AD, like erythema, oedema/papules, oozing/crusts, dryness and extension (15).

Skin histology and immunohistochemistry

Cryo-sections measuring 5 μ m were prepared and used for staining by the streptavidin–biotin complex method (16). Sections were incubated with rat anti-mouse CD4 (RM4-5), CD8 (53-6.7) or CD11c (HL3; all from BD Pharmingen, Heidelberg, Germany) followed by incubation with biotinylated goat anti-rat IgG (BD). The Dako REAL™ detection system Alkaline Phosphatase/RED (DAKO, Hamburg, Germany) kit was used for the development and papanicolaou dye as a counter stain. Mast cells were stained with 0.1% toluidine blue (Merck, Darmstadt, Germany). Cells were analysed by using Axiovision measuring-tools on the Axio-plan light microscope (Carl Zeiss, Berlin, Germany) at $\times 10$ magnification and expressed as cells per square millimetre.

Flow cytometry

Single-cell suspensions were prepared from bone marrow, spleen and lymph nodes (axillary and inguinal). The flow cytometric analyses were performed using fluorochrome-conjugated monoclonal antibodies against mouse CD3, CD8, CD44, CD21/CD35, CD23, CD19, CD138, CD38 and IgG1 (all from BD) and in house antibodies against mouse CD4, CD62L, B220 and FITC conjugated

OVA (DRFZ, Berlin, Germany). Dead cells were excluded using 1 μ M 7-ADD (PI; Sigma-Aldrich). Plasma cells and OVA-specific plasma cells were identified by staining for surface markers (CD138, B220), followed by intracellular staining with OVA and anti-IgG1. IgG1-specific plasma cells were identified as B220low, CD138+, IgG1+, while OVA-specific IgG1-producing plasma cells stained additionally positive with OVA-FITC. The flow cytometric data obtained are expressed as the total number of cells per spleen or lymph node, calculated on the basis of the total numbers of cells encountered in these organs. All data were analysed using FLOWJO software (Tree Star, Inc., Ashland, OR, USA).

ELISpot assay

ELISpot plates (Millipore, Schwalbach, Germany) were coated with 10 μ g OVA (Sigma-Aldrich) per well (17). For assessment of IgE, IgG1 and IgA antibody-secreting cells (ASCs), serial dilutions of splenocytes and bone marrow cells (starting with 5×10^6 , 1×10^6 and 5×10^5) were plated and incubated overnight. This was followed by incubation with biotinylated secondary-antibody [antimurine IgE-biotin (EM 95.3, kindly provided by Dr Lamers; MPI, Freiburg, Germany), antimurine IgG1-biotin (A85-1; BD) and antimurine IgA-biotin (1040-08; Southern Biotech, Birmingham, AL, USA), respectively]. The reaction was developed with streptavidin-horseradish peroxidase (HRP; R&D system, Wiesbaden, Germany) and peroxidase substrate 33-amino-9-ethyl-carbazole/n, n-dimethylformamide (Sigma-Aldrich). ASCs, appearing as red spots, were analysed with the CTL ImmunoSpot S4 Analyzer (C.T.L. Cellular Technology, Cleveland, OH, USA).

ELISA

OVA-specific and total immunoglobulins (IgE, IgG1, IgA, IgM and IgG2a) were measured by ELISA (18). The concentrations of OVA-specific antibodies were estimated by comparison to a standard curve prepared from pooled sera of OVA-sensitized mice. Data are expressed as laboratory units per millilitre (LU/ml). For OVA-IgG₁, OVA-14 (OVA-specific mouse IgG1; Sigma-Aldrich) was used as standard. To analyse total immunoglobulins, plates (Nunc, Wiesbaden, Germany) were coated with anti-mouse IgE, IgG1 (both from BD), IgA and IgM (all from Southern biotech). After blocking of unspecific binding, detection was performed using biotinylated secondary antibodies and streptavidin-conjugated HRP (R&D system). The colorimetric reaction of peroxidase substrate (TMB; Sigma-Aldrich) was measured at 450 nm after addition of 1 M sulphuric acid. Diluted mouse immunoglobulin was used for standard curve calculation.

Organ culture

Five-millimeter punch skin biopsies were obtained from the belly region of naive Balb/c mice after wet shaving. The punch biopsies were incubated in wells of a 96-well plate in DermaLife K Complete Medium (Lifeline Cell Technology, Frederick, MD, USA), a hydrocortisone free medium. Individual wells were treated with Bz at the concentrations given in the figure legends either without further additives or in the presence of IL-1 β (20 ng/ml) to mimic an inflammatory microenvironment with an increased protein turnover. After 24 h, the samples were snap frozen and kept at -80°C until RNA isolation. All experiments were repeated with at least five mice.

RNA isolation and quantitative RT-PCR

RNA was extracted from skin specimens by Precellys® 24 Homogeniser (PEQLAB, Erlangen, Germany). About 1–2 μ g of total RNA

was transcribed into cDNA using TaqMans reverse transcription reagents (Applied Biosystems, Darmstadt, Germany). Quantitative RT-PCR was performed with the Fast Start DNA Master SYBR Green I and LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Oligonucleotide primers were synthesized by TIB MOLBIOL (Berlin, Germany). Primer pairs for profilaggrin, loricrin and transglutaminase-1 were as described (16); primers for keratin 14 were as follows: forward: AACCTGGAGGAGACCAAAGG and reverse: GGATGACTGAGAGCCAGAGG, and those for jagged-2 forward: CGAGGTCAAGGTG GAAACAG and reverse: CCACCATACGCAGATAACCA. The expression levels of target genes were quantified relative to the expression of the reference gene *Hprt*.

Statistical analysis

The significance of differences between the groups was analysed by Mann–Whitney *t*-test (GraphPad Prism 5.0; GraphPad software, La Jolla, CA, USA). A *P*-value of <0.05 was considered significant.

Results

PI reduces allergen-specific plasma cells and circulating immunoglobulin levels in a mouse allergy model

To identify the impact of PI on the allergic immune response, we first analysed the proportion of plasma cells among splenocytes in Bz-treated mice (Fig. 1a and Figure S2a). PI significantly reduced the total number per spleen of the IgG1-specific plasma cell subset (B220low/IgG1high/CD138high) by 70% and even more potently of the allergen-specific IgG1+ cell subset (identified as B220low/IgG1high/CD138high/anti-OVAhigh), which was reduced by 82%. The IgG1+ subset was selected because of its relatively high abundance among the low frequency of allergen-specific plasma cells (while scarcity of the IgE+ subset precluded an analogous strategy for allergen-specific IgE-switched plasma cells). Conversely, total numbers of B cells, marginal zone B cells (CD19+/CD21high/CD23low) and follicular zone B cells (CD19+/CD21low/CD23high) remained largely stable in the spleen, axillary and inguinal lymph nodes, respectively (Figure S4 and data not shown). Thus, PI preferentially targeted plasma cells but left the B-cell compartments otherwise largely intact.

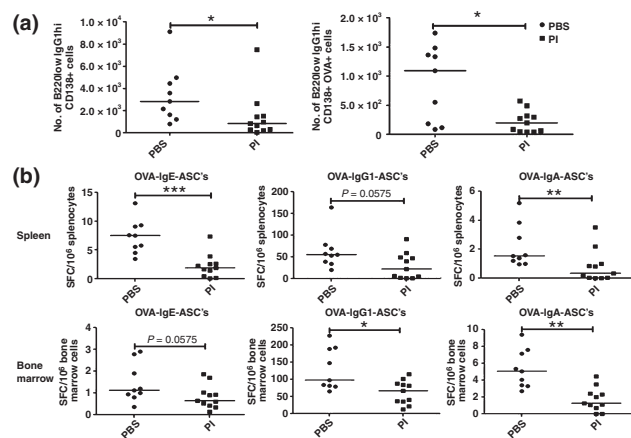


Figure 1. Proteasome inhibition (PI) diminishes plasma cells. (a) Total and OVA-specific plasma cells from individual mice shown as a dot plot. (b) Quantification of the distinct antibody-secreting cells (ASCs) in the spleen and bone marrow, respectively. Values from individual mice are given as dots (bar corresponds to the median). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

The number of antigen-specific ASCs was subsequently analysed *ex vivo*. As evidenced by ELISpot assay, there was a clear reduction in the number of cells secreting antigen-specific immunoglobulins upon PI, both in the spleen (OVA-IgE-ASC's reduction by 75%; OVA-IgG1-ASC's reduction by 59%; OVA-IgA-ASC's reduction by 78%) and in bone marrow (OVA-IgE-ASC's reduction by 43%; OVA-IgG1-ASC's reduction by 32%; and OVA-IgA-ASC's reduction by 68%; Fig. 1b and Figure S2b).

Equally, PI led to a vigorous decrease in OVA-specific and total IgE and IgG1 (Fig. 2) as well as IgM and IgA (Figure S3) in the serum. While there was no difference in Ig titres between the groups before Bz treatment (i.e. on days 0, 21, 36), a potent reduction was noted on days 56 and 71, that is, already 4 days after the first Bz injection. On day 71, OVA-specific immunoglobulins were decreased by 79% (OVA-IgE), 60% (OVA-IgG1), 67% (OVA-IgM) and 82% (OVA-IgA), respectively. The serum concentrations of total immunoglobulins were likewise reduced by PI albeit somewhat less potently if viewed together (IgE reduction by 72%; IgG1 reduction by 42%; IgM reduction by 29% and IgA reduction by 65%). Overall, slight, yet discernible differences among the Ig isotypes could be observed, and a rapid and robust reduction was detected for OVA-IgE (as pointed out above, detectable already 4 days after the first Bz application, Fig. 2). Collectively, the proteasome inhibitor effectively diminished ASCs, leading to substantial reductions in circulating immunoglobulin levels in this model.

PI interferes with T-cell homing to the skin, but does not improve dermatitis

We next concentrated on the local skin inflammatory response. In fact, Bz-treated mice had substantially lower levels of CD4+ and CD8+ T cells in their skin lesions (decrease by 66% and 67%, respectively; Fig. 3a,b), while CD11c+ and mast cell numbers remained comparable between the groups (data not shown). The observed response pattern of the different inflammatory cells perfectly replicates observations made in a model of contact dermatitis upon Bz treatment (19). We also analysed T-cell distribution in the spleen, axillary and inguinal draining lymph nodes, noting that the proportion of both CD4+ and CD8+ T cells decreased in

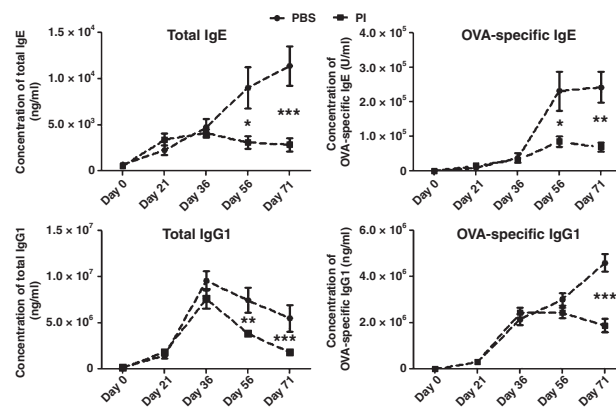


Figure 2. Decreased humoral immune responses after proteasome inhibition (PI). OVA-specific and total IgE and IgG1 were analysed by ELISA from mouse sera at the time points indicated and are depicted as mean $\pm$ SEM from 9 to 11 mice, respectively. Please note that the first injection of the proteasome inhibitor was on day 52. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

the axillary lymph node (Fig. 3d), but that no such tendency could be found in the spleen, where naïve and memory T-cell populations remained unchanged (Figure S4). Taken together, PI selectively counteracts the local accumulation of T cells, but has no systemic effect in the T-cell compartment in this model. Intriguingly, the clinical severity of the dermatitis remained surprisingly unchanged in the PI group despite potent reductions in T cells in the lesions (Fig. 3c). This contrasts the ear swelling response found in the contact dermatitis model, which was effectively countered by Bz (19).

Bortezomib acts directly on the skin to diminish expression of skin barrier genes

A pronounced T-cell infiltrate is a hallmark of AD in mouse and man (20,21), and T cells are an invariable element in AD pathology in mouse models of the disease (22,23). Accordingly, a reduction in the T-cell infiltrate commonly parallels clinical improvement in the skin (15,18,24,25). Therefore, and in the light of the profound changes in lesional lymphocytes observed here, the clinical unresponsiveness of the dermatitis to PI seemed somewhat counterintuitive.

This led us to develop an alternative hypothesis, namely that the unchanged skin score could result from a counter-regulatory mechanism operative outside of the immune system, most likely at the level of the skin itself.

This was tested directly by analysing the impact of Bz on the skin in organ cultures. PI indeed resulted in reduced expression of pro-filaggrin, loricrin (tendency) and, most profoundly, transglutaminase-1 in a dose-dependent manner (Fig. 4). The effects were clearly stronger in the presence of IL-1 β (mimicking an inflammatory micromilieu), which is associated with increased turnover of proteins and adaptation of the proteolytic activity of the proteasome (26). However, even under homeostatic conditions, there was reduced expression of transglutaminase-1. Other genes highly expressed in the skin showed either no effect (keratin 14) or were even upregulated (jagged-2), implying particular susceptibility of genes involved in barrier function to PI-mediated downregulation. Importantly, effects were detectable in a concentration range typi-

cally used for experiments with highly susceptible multiple myeloma cells (27,28), that is, at concentrations below the maximum plasma concentration achieved in therapeutic settings (7).

Collectively, our data suggest that skin barrier function may be directly compromised following PI, providing a rational explanation for the unchanged propensity to develop skin lesions upon proteasomal interference despite manifest signs of reduced inflammation.

Discussion

Normal and malignant plasma cells are critically dependent on unperturbed proteasome function (10,27–29), but studies investigating the consequences of PI on the allergic immune response are limited. In fact, one recent report failed to detect changes in the plasma cell compartment and achieved IgE suppression only upon prolonged treatment with Bz in a model of OVA-driven chronic asthma (14). Therefore, it remained unknown whether allergen-specific plasma cells may be particularly resistant to PI. In fact, IgE-switched B cells possess several peculiarities regarding development, expansion and survival (30). Here, we demonstrate

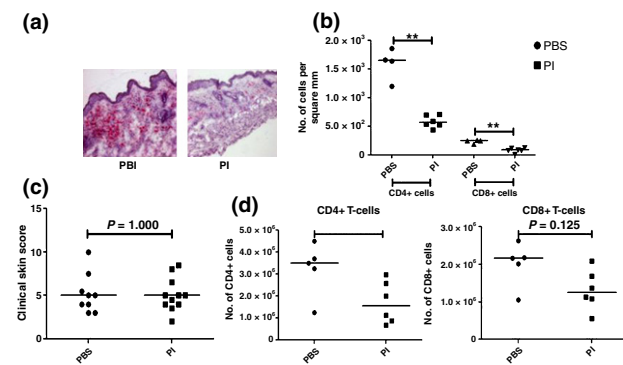


Figure 3. Proteasome inhibition (PI) interferes with T-cell infiltration to the skin and homing to axillary lymph nodes, but does not reduce severity of the eczema. (a) Representative skin sections (5 μ m) stained for CD4 (+) T cells in atopic dermatitis (AD) skin in PI-treated versus PBS-treated control mice. (b) Quantification of T-cell infiltrates (CD4+ and CD8+ T cells) in the skin in all mice. (c) Severity of the eczema (based on a scoring system, as described in Materials and methods). (d) Total numbers of CD4+ and CD8+ T cells in the axillary lymph nodes (lymph nodes were pooled from two mice of the same group to obtain the required cell numbers). The data are shown as a dot plot with the median given as bar. * P < 0.05, ** P < 0.01.

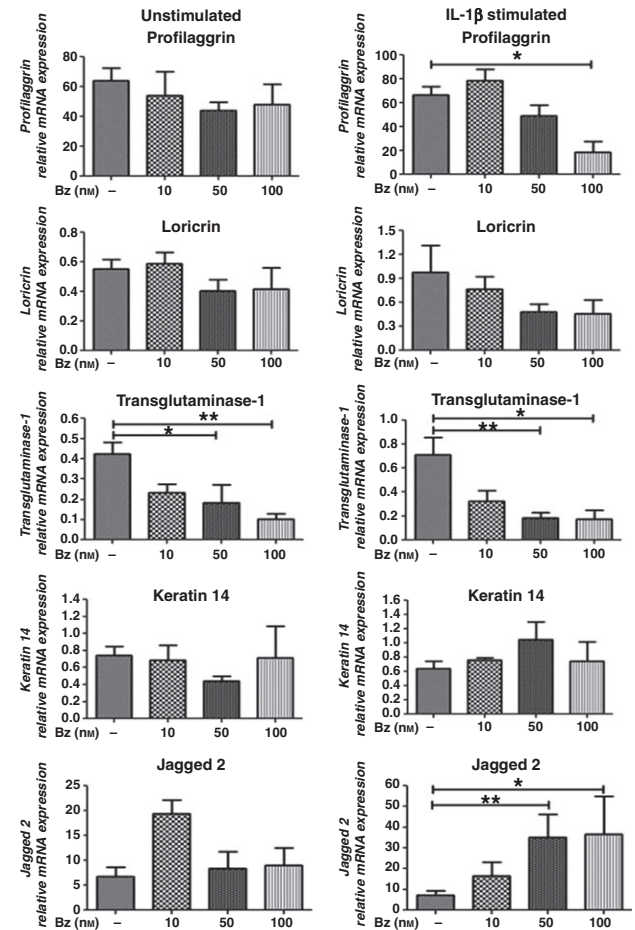


Figure 4. Bortezomib acts directly on the skin to specifically reduce expression of skin barrier genes. Skin organ cultures were subjected to Bz *ex vivo* at the indicated concentrations. After 24 h, biopsies were collected and used for RNA extraction. The expression of profilaggrin, loricrin, transglutaminase-1, keratin-14 and jagged-2 were assessed by quantitative RT-PCR and values normalized to hprt. * P < 0.05; ** P < 0.01.

that plasma cells are susceptible to PI in a mouse allergy model even after a relatively short treatment in a therapeutic setting. Likewise, circulating IgE levels were reduced already 4 days after commencement of treatment and remained low until the end of the experiment. Depletion was at least as effective with regard to IgE-switched ASCs as with the other Ig isotypes (note that IgE ASCs are primarily encountered in spleen, while IgG1 ASCs are mainly in the bone marrow at the time of analysis, Fig. 1b). The relative sensitivity of the IgE-producing subset is well compatible with the theory that IgE+ cells exist predominantly as plasma cells (and not as memory B cells) or have a higher propensity to develop into such cells (30), as plasma cells are the subset that is primarily targeted by PI [(29), and this study]. Interestingly, Wegmann et al. (14) also noted a greater effect of Bz regarding IgE than regarding IgG in their long-term protocol. Overall, our data confirm previous findings obtained in models of autoimmune diseases, but extend them to IgE+ ASCs in the context of allergy, implying that plasma cell depletion is a general consequence of proteasomal interference and not restricted to a particular plasma cell subset or disease, even though there may be subtle differences as for the potency of elimination. Clearly, the consideration of Bz for therapeutic purposes of immune-driven diseases is based on its reported interference not only with plasmablasts/short-lived plasma cells, but also with the long-lived plasma cell subset, which is difficult to eradicate by standard chemotherapy (31). Such long-lived plasma cells have recently been shown by us to contribute to the development of allergic manifestations in mice (32).

Apart from plasma cell depletion, no numerical alterations in the T-cell and B-cell compartments were observed at the systemic level, confirming that Bz at the standard dose applied is not overtly immunosuppressive and that plasma cells are the most susceptible cell subset. Absence of random systemic immunosuppression is certainly a desirable attribute when considering potential therapeutic applications.

Atopic dermatitis is a complex skin disease with numerous participants (20,21). While IgE is not indispensable for the disease to develop (22,33), it is believed to modulate its course and to act in an aggravating manner under certain circumstances (34). This is also exemplified by partial effectiveness of anti-IgE therapy in subgroups of AD patients (35–37). Conversely, T cells are viewed as an essential element of AD pathology (20,21), as is also illustrated by their non-redundant nature in mouse AD models (22,23). Consequently, reductions in T cells are associated with a beneficial course of the disease (15,18,24,25). Here, we found a potent reduction in T-cell infiltrates in the skin down to approximately one-third of their density in the control group. This specific reduction in T cells in the inflamed skin perfectly matches previous observations in a contact hypersensitivity model following Bz treatment (19). This specific reduction in T cells in the inflamed skin perfectly matches previous observations in a contact hypersensitivity model following Bz treatment (19). Despite numerous differences between the two models [i.e. typical contact hypersensitivity model with hapten sensitization and short treatments with a maximum length of 9 days, measurement of acute ear swelling responses, Bz first applied after sensitization by Yanaba et al. (19)], the combined data from both studies underscore that local T-cell recruitment to inflamed skin is effectively countered by Bz. Based on the impressive effect at the level of inflammatory cells,

we would have expected an amelioration of the dermatitis, but surprisingly found that this was not the case. However, while contact hypersensitivity is invariably driven by T cells (38), the pathology of AD is more complex, so that a reduction in hapten-specific T cells will likely lead to amelioration of contact dermatitis, but will not necessarily have the same effect in AD. In fact, one hallmark of AD is a defective skin barrier, which predisposes to dry skin (secondary to exaggerated transepidermal water loss) and increased susceptibility to microbial infections and chemical insults (39–42). Various quantitative and qualitative alterations in skin barrier constituents (especially structural proteins and processing enzymes) have been reported in AD. The most prominent example is filaggrin because of its frequent mutation in AD (40,42,43), but other proteins are affected, as well; for example, loricrin was found to be heavily underexpressed in AD skin (44). T cells and their products, especially Th2 cytokines, reportedly interfere with skin barrier function at multiple levels (41,42,45,46), thereby contributing to AD.

The surprising lack of efficiency of Bz therapy in terms of clinical amelioration of the skin, despite the remarkable immunohistological changes, suggested the existence of a counter-regulatory mechanism. In fact, the UPS is essential for normal cellular processes and is active in all cellular subsets, so it is not immediately intuitive why particular cell types should be more susceptible to its inhibition than others (as outlined in Introduction). The clinical unresponsiveness of the skin lesions led us to hypothesize that Bz may exert dual effects that act in opposing directions in AD skin.

To test this directly, Bz was assessed for its ability to influence the expression of critical keratinocyte-specific genes when applied directly to the skin. We found that the skin is indeed highly susceptible to PI and that genes contributing to barrier function are selectively downregulated. Concentrations well below the peak plasma concentration in therapeutic settings were effective (7), and susceptibility of the skin was furthermore illustrated by the similar concentration range typically employed for *in vitro* studies of multiple myeloma cells (27,28). The downregulated genes, as identified here, are implicated in the establishment of the cornified envelope (39), while other genes highly expressed by KCs remained either unchanged (krt14) or experienced regulation to the opposite [like jagged-2 (47)], further underlining the susceptibility of the skin to PI. The barrier-related genes not only show specific perturbations in AD skin, but also their coordinate increase also closely parallels the improvement of dermatitis in this mouse model, as reported recently (16).

The identification of the skin as a significant target of Bz is well compatible with the literature. For example, a recent report demonstrated that the topical application of Bz to the skin caused erythema and skin thickening (characteristics of AD) in a murine model of psoriasis (48). Likewise, proteasome integrity is essentially implicated in preventing misfolded keratins from aggregation, especially in keratin genodermatoses (49), is important for the regulation of p53 levels in keratinocytes (50) and for the maintenance of keratinocyte contacts through stabilization of desmosomes (51). Perhaps, most compelling in this context is the frequency of cutaneous side effects under Bz therapy, with almost 60% of the patients being affected (52). Among these adverse effects, dry skin and eczema are commonly described (>1/100,

<1/10) (53). We provide additional evidence for the sensitivity of the skin to disrupted proteasome function and demonstrate, for the first time, that constituents implicated in skin barrier function are impaired when proteasome function is disrupted directly in the skin.

In summary, this study provides evidence that PI is associated with potent suppression of total and allergen-specific IgE production secondary to plasma cell elimination, as well as local changes in the T-cell compartment in a model of allergen-triggered dermatitis. However, critical processes taking place in the skin itself lead to a disconnect between PI's operation at the level of the exaggerated immune response and the skin, resulting in unchanged severity of the disease. Although PI-mediated plasma cell depletion may prove a successful approach to combat certain types of

IgE-dependent diseases, our study cautions against its consideration for the treatment for skin disorders associated with defective skin barrier function.

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Author contributions

M. Worm designed the study and revised the manuscript. M. Babina contributed to the study design, data interpretation and writing of the manuscript. K. K. Mudnakudu Nagaraju performed the experiments, data analysis and interpretation, and drafted the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

The authors have declared no conflicting interests.

References

- Nencioni A, Grünebach F, Patrone F *et al.* Leukemia 2007; **21**: 30–36.
- Dantuma N P, Lindsten K. Cardiovasc Res 2010; **85**: 263–271.
- Kim R, Emi M, Tanabe K *et al.* Apoptosis 2006; **11**: 5–13.
- Obeng E A, Carlson L M, Gutman D M *et al.* Blood 2006; **107**: 4907–4916.
- Chauhan D, Hideshima T, Mitsiades C *et al.* Mol Cancer Ther 2005; **4**: 686–692.
- Mateos M V, San Miguel J F. Best Pract Res Clin Haematol 2007; **20**: 701–715.
- Levêque D, Carvalho M C, Maloisel F. In Vivo 2007; **21**: 273–278.
- Mujtaba T, Dou Q P. Discov Med 2011; **12**: 471–480.
- Cenci S, Oliva L, Cerruti F *et al.* J Leukoc Biol 2012; **92**: 921–931.
- Cenci S, Mezghrani A, Cascio P *et al.* EMBO J 2006; **25**: 1104–1113.
- Neubert K, Meister S, Moser K *et al.* Nat Med 2008; **14**: 748–755.
- Lee S W, Kim J H, Park Y B *et al.* Ann Rheum Dis 2009; **68**: 1761–1767.
- Gomez A M, Vrolix K, Martínez-Martínez P *et al.* J Immunol 2011; **186**: 2503–2513.
- Wegmann M, Lunding L, Orinska Z *et al.* Int Arch Allergy Immunol 2012; **158**: 43–53.
- Dahten A, Koch C, Ernst D *et al.* J Invest Dermatol 2008; **128**: 2211–2218.
- Hartmann B, Riedel R, Jörss K *et al.* J Invest Dermatol 2012; **132**: 330–336.
- Hartmann B, Heine G, Babina M *et al.* Allergy 2011; **66**: 540–548.
- Babina M, Kirn F, Hoser D *et al.* Clin Exp Allergy 2010; **40**: 1256–1265.
- Yanaba K, Yoshizaki A, Muroi E *et al.* J Leukoc Biol 2010; **88**: 117–122.
- Bieber T. Ann Dermatol 2010; **22**: 125–137.
- Leung D Y, Boguniewicz M, Howell M D *et al.* J Clin Invest 2004; **113**: 651–657.
- Woodward A L, Spergel J M, Alenius H *et al.* J Allergy Clin Immunol 2001; **107**: 359–366.
- Hennino A, Vocanson M, Toussaint Y *et al.* J Immunol 2007; **178**: 5571–5577.
- Weise C, Ernst D, van Tol E A *et al.* Pediatr Allergy Immunol 2013; **24**: 361–367.
- Weise C, Zhu Y, Ernst D *et al.* Exp Dermatol 2011; **20**: 805–809.
- Voigt A, Rahnefeld A, Klotzel P M *et al.* Front Physiol 2013; **4**: 42.
- Hideshima T, Richardson P, Chauhan D *et al.* Cancer Res 2001; **61**: 3071–3076.
- Davenport E L, Moore H E, Dunlop A S *et al.* Blood 2007; **110**: 2641–2649.
- Neubert K, Meister S, Moser K *et al.* Nat Med 2008; **14**: 748–755.
- Davies J M, Platts-Mills T A, Aalberse R C. J Allergy Clin Immunol 2013; **131**: 972–976.
- Yoshida T, Mei H, Dörner T *et al.* Immunol Rev 2010; **237**: 117–139.
- Luger E O, Fokuhl V, Wegmann M *et al.* J Allergy Clin Immunol 2009; **124**: 819–826.
- Wüthrich B, Schmid-Grendelmeier P. J Invest Allergol Clin Immunol 2003; **13**: 1–5.
- Liu F T, Goodarzi H, Chen H Y. Clin Rev Allergy Immunol 2011; **41**: 298–310.
- Belloni B, Ziai M, Lim A *et al.* J Allergy Clin Immunol 2007; **120**: 1223–1225.
- Sheinkopf L E, Rafi A W, Do L T *et al.* Allergy Asthma Proc 2008; **29**: 530–537.
- Heil P M, Maurer D, Klein B *et al.* J Dtsch Dermatol Ges 2010; **8**: 990–998.
- Saint-Mezard P, Berard F, Dubois B *et al.* Eur J Dermatol 2004; **14**: 131–138.
- Candi E, Schmidt R, Melino G. Nat Rev Mol Cell Biol 2005; **6**: 328–340.
- Proksch E, Brandner J M, Jensen J M. Exp Dermatol 2008; **17**: 1063–1072.
- Hänel K H, Cornelissen C, Lüscher B *et al.* Int J Mol Sci 2013; **14**: 6720–6745.
- Gutowska-Owsiak D, Ogg G S. Clin Exp Allergy 2013; **43**: 586–598.
- O'Regan G M, Irvine A D. Clin Exp Allergy 2010; **40**: 965–972.
- Guttman-Yassky E, Suárez-Fariñas M, Chiricozzi A *et al.* J Allergy Clin Immunol 2009; **124**: 1235–1244.
- Hatano Y, Adachi Y, Elias P M *et al.* Exp Dermatol 2013; **22**: 30–35.
- Howell M D, Kim B E, Gao P *et al.* J Allergy Clin Immunol 2007; **120**: 150–155.
- Powell B C, Passmore E A, Nesci A *et al.* Mech Dev 1998; **78**: 189–192.
- Tung D, Cheung P H, Kaur P *et al.* Pharmacology 2011; **88**: 100–113.
- Chamcheu J C, Wood G S, Siddiqui I A *et al.* Exp Dermatol 2012; **21**: 481–489.
- Shen S C, Lee W R, Yang L Y *et al.* Dermatology 2012; **21**: 370–375.
- Löffek S, Bruckner-Tuderman L, Magin T M. Exp Dermatol 2012; **21**: 791–793.
- Patrizi A, Venturi M, Dika E *et al.* Cutan Ocul Toxicol 2013; **2**. doi:10.3109/15569527.2013.787086 [Epub ahead of print].
- Van Regenmortel N, Van de Voorde K, De Raeye H *et al.* Haematologica 2005; **90**: 116–117.

Supporting Information

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

Figure S1. Experimental design.

Figure S2. (a) Representative flow cytometry histograms showing CD138+/OVA+ cells, the percentages refer to the parent gate, i.e. the B220low IgG1hi subpopulation; (b) Representative photographs showing the effect of proteasomal inhibition on OVA-IgE and OVA-IgG1-specific antibody secreting cells in the spleen on day 71.

Figure S3. Decreased humoral immune responses after proteasome inhibition.

Figure S4. Numbers of T- and B-cells subsets in the spleen were unaltered upon proteasome inhibition.

Figure S1

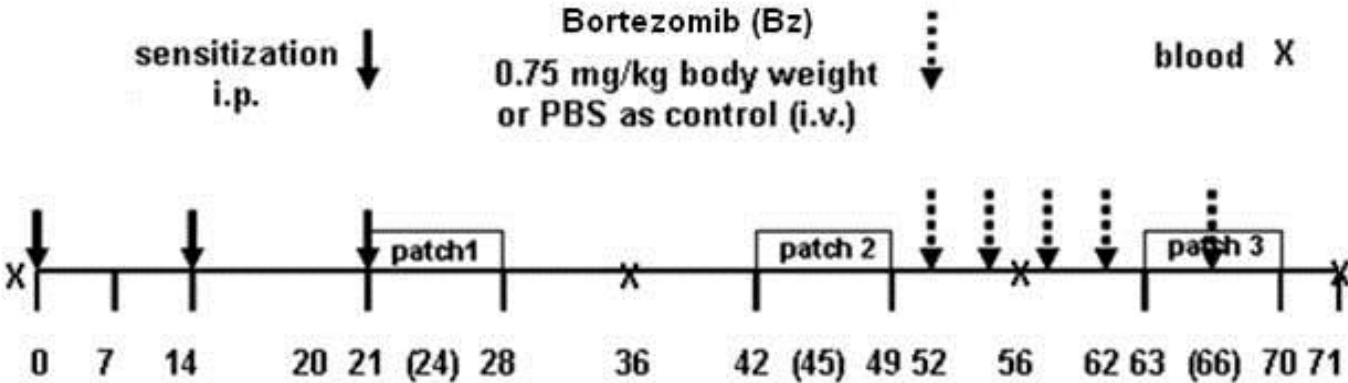


Figure S2

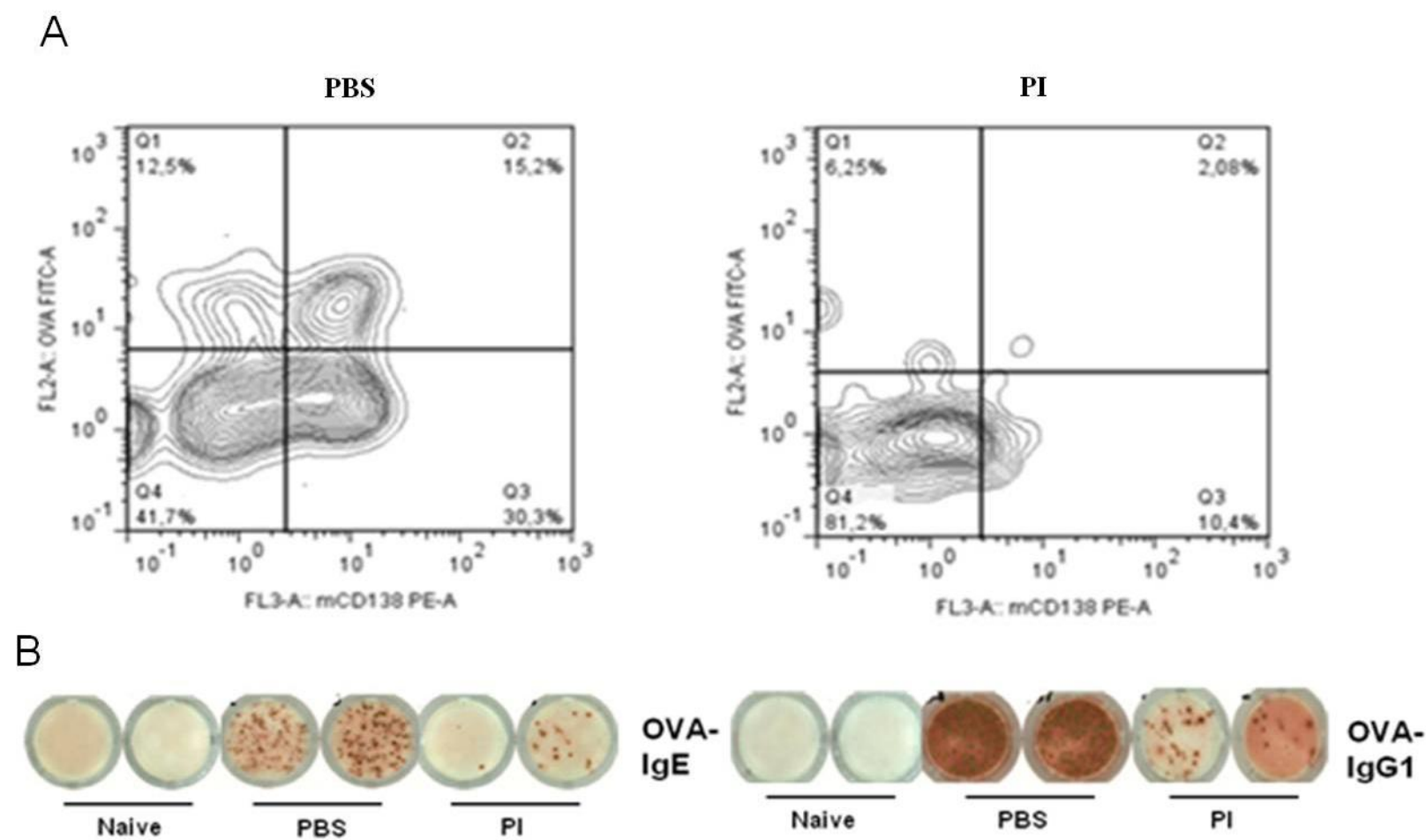


Figure S3

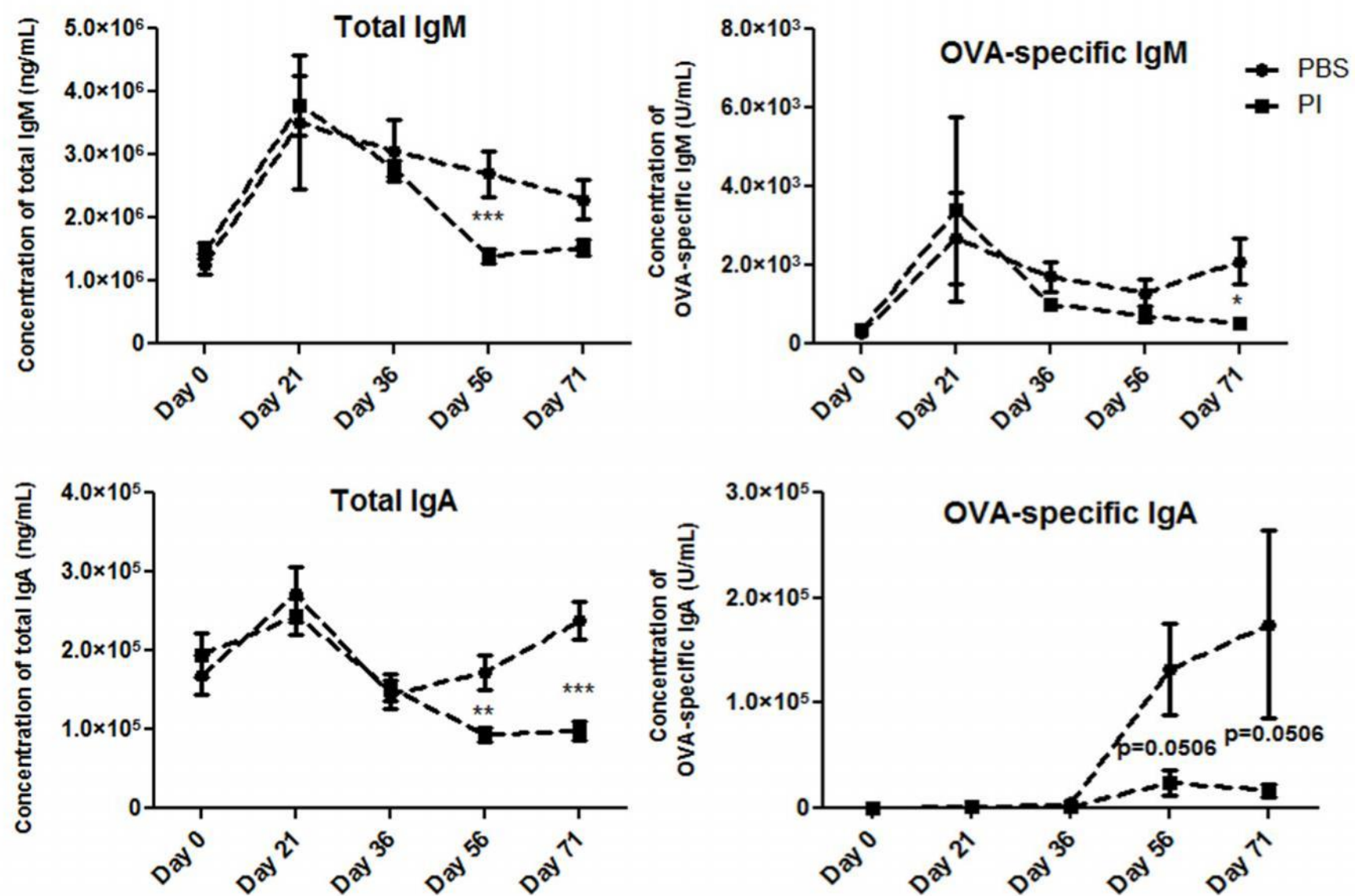
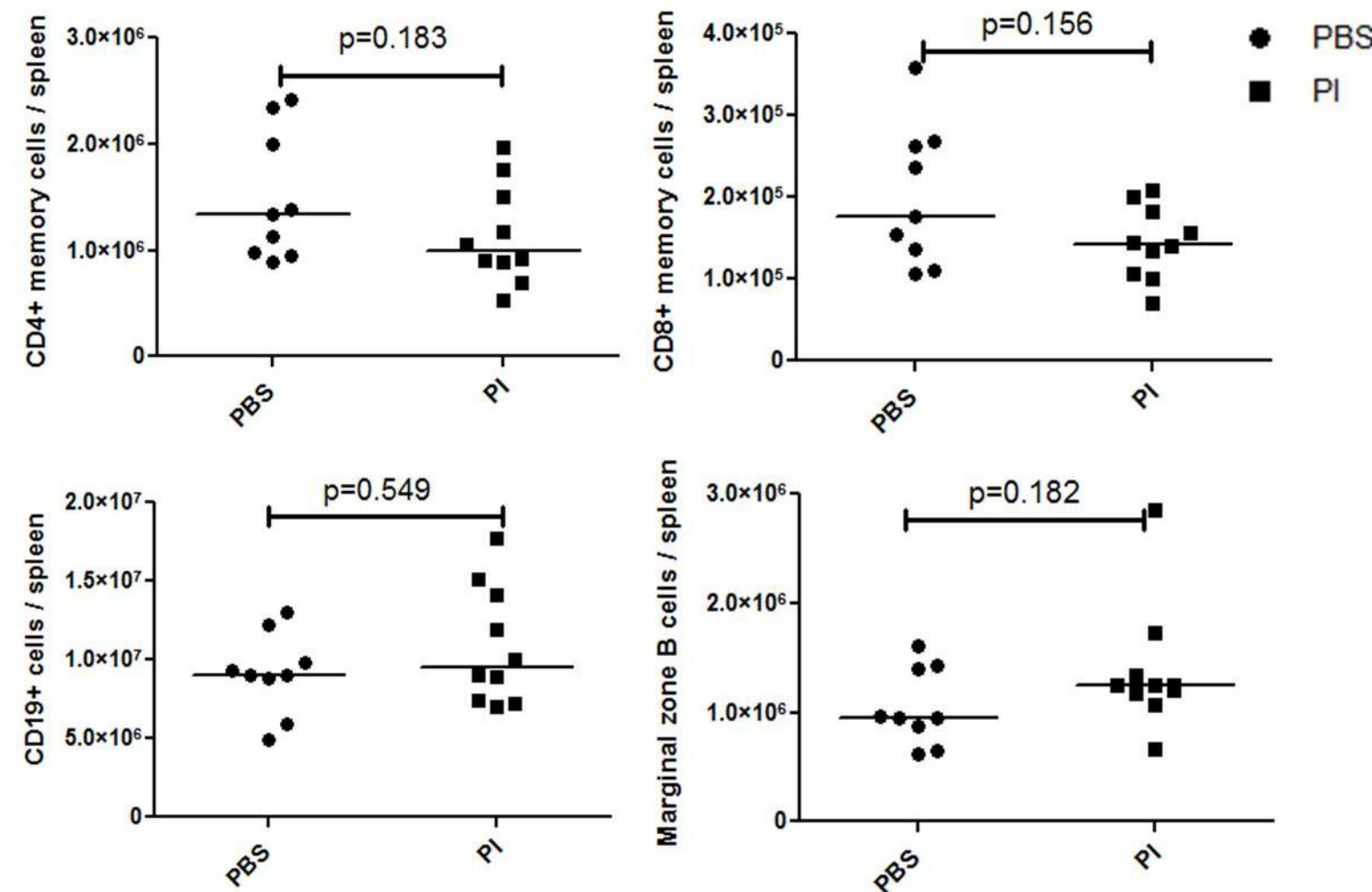


Figure S4



Supporting Information

Supplementary figure legends

Figure S1: Experimental design. Eczema was induced in mice as previously described (15, 16).

Figure S2: (A) Representative flow cytometry histograms showing CD138⁺/OVA⁺ cells, the percentages refer to the parent gate, i.e. the B220^{low} IgG1^{hi} subpopulation; (B) Representative photographs showing the effect of proteasomal inhibition on OVA-IgE and OVA-IgG1-specific antibody secreting cells in the spleen on day 71.

Figure S3: Decreased humoral immune responses after proteasome inhibition. The OVA-specific and total immunoglobulin subtypes – IgM and IgA were analyzed by ELISA from mouse sera at the time points given and are displayed as mean±SEM, respectively (in analogy to figure 2). Please note that the first injection of the proteasome inhibitor was on day 52. * p < 0.05, ** p < 0.01, *** p < 0.001

Figure S4: Numbers of T- and B-cells subsets in the spleen were unaltered upon proteasome inhibition. Values from individual mice are shown as a dot plot with the median given as bar. The p-values are given as well.