Heat shock protein 90 inhibition in autoimmunity to type VII collagen: evidence that non-malignant plasma cells are not therapeutic targets

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ABSTRACT

Blocking heat shock protein 90 (Hsp90) induces death of malignant plasma cells by activation of the unfolded protein response (UPR), a signaling pathway activated by accumulation of misfolded proteins within the endoplasmatic reticulum. We hypothesized that non-transformed plasma cells are also hypersensitive to Hsp90 inhibition owing to their high amount of protein biosynthesis. To study this, two different Hsp90 inhibitors, the geldanamycin derivative 17-DMAG or the nontoxic peptide derivative TCBL-145, were applied to mice with experimental epidermolysis bullosa acquisita, an autoimmune bullous disease characterized by autoantibodies against type VII collagen of the dermal-epidermal junction. Both inhibitors ameliorated clinical disease of type VII collagen-immunized mice, suppressed autoantibody production, and reduced dermal neutrophilic infiltrate. Interestingly, total plasma cells numbers, type VII collagen-specific plasma cells and germinal center B cells were unaffected by anti-Hsp90 treatment in vivo. However, T cell proliferation was potently inhibited, as evidenced by reduced response of isolated lymph node cells from immunized mice to in vitro re-stimulation with anti-CD3/CD28 antibody or autoantigen in presence of Hsp90 inhibitors. Our results suggest that Hsp90 blockade has no impact on normal or autoreactive plasma cells in vivo and indentify T cells as targets of anti-Hsp90 treatment in autoimmunity to type VII collagen.
INTRODUCTION

Autoreactive T cells, B cells, and plasma cells have been identified as key players in the pathophysiology of autoimmune diseases. Although much progress has been achieved in revealing the immunological processes in these diseases, their therapy remains challenging and in most cases still consists of conventional unspecific immunosuppressive treatment with corticosteroids and cytostatic agents. The application of these drugs is often limited due to side effects and disease remission can frequently not be achieved.¹

Epidermolysis bullosa acquisita (EBA) is a chronic subepidermal blistering disease characterized by circulating and tissue-bound autoantibodies targeting the non-collagenous domain 1 (NC1) of type VII collagen, a major component of anchoring fibrils of the dermal-epidermal junction (DEJ).²³ The pathogenic relevance of (auto)antibodies against type VII collagen has been conclusively shown ex vivo and in experimental animal models.⁴ In EBA and most other subepidermal autoimmune bullous diseases, autoantibodies alone are not sufficient to induce blisters, but require an Fc-dependent engagement of humoral and cellular inflammatory factors.⁴ In addition, a key role of T cells for initiation of autoimmunity against type VII collagen has recently been demonstrated.⁵ Experimental EBA, which reproduces both the autoimmune response as well as immunopathological, histological, and clinical findings in patients with EBA, can be induced in susceptible mice by immunization with recombinant murine type VII collagen.⁶ Thus, EBA emerges as a model disease to study fundamental, biologically and clinically crucial aspects of antibody-mediated organ-specific autoimmune diseases.

Heat shock proteins (Hsps) are molecular chaperones essential for maintaining cellular functions by preventing misfolding and aggregation of nascent polypeptides and by facilitating protein folding.⁷ Pharmacologic inhibition of the Hsp family member
Hsp90 has been primarily implicated in the context of cancer treatment as this chaperone is used by many cancer cells to facilitate the function of numerous oncoproteins. Several Hsp90 inhibitors with different side effect profiles have been identified, of which new synthesized short peptide-derivatives including TCBL-145 are of particular interest as in vivo toxicity has so far not been recorded.

Recently, Hsp90 has been reported to play important roles in antigen presentation, activation of lymphocytes and macrophages, and activation and maturation of dendritic cells, indicating a potential treatment target of inflammatory diseases, including autoimmune diseases. Indeed, pharmacologic inhibition of Hsp90 has recently been successfully applied in mouse models of autoimmune encephalomyelitis, rheumatoid arthritis, and systemic lupus erythematosus-like autoimmune disease.

Although inhibitory effects of Hsp90 inhibitors on various immune cells, including T cells, have been reported in autoimmunity, the direct impact of anti-Hsp90 treatment on autoantibody-producing plasma cells has not been studied in vivo. Plasma cells producing high levels of protein (i.e. immunoglobulin) are dependent on the unfolded protein response (UPR), which maintains protein homeostasis within the endoplasmatic reticulum (ER) to ensure cell survival. Activation of the UPR results in a bias of translation toward the synthesis of chaperone proteins involved in protein folding, an increase in the degradation of misfolded proteins via the ubiquitin proteasome pathway, and the delivery of a survival signal. If these objectives are not achieved within a certain time lapse or the disruption is prolonged, an ER stress signal is generated and apoptosis ensues. It has previously been shown that the proteasome inhibitor bortezomib can induce an UPR leading to apoptosis of malignant plasma cells in vitro. Hsp90b1, the ER paralogue of the cytosolic Hsp90, is believed to be one of the key molecular chaperones in controlling the UPR.
Similar to proteasome inhibition, blockade of Hsp90 has been associated with activation of the UPR pathway and apoptosis caused by overload with unfolded monoclonal paraprotein in myeloma plasma cells lines.\textsuperscript{18} Recently, Neubert et al\textsuperscript{19} reported that bortezomib is also capable of depleting normal and autoreactive plasma cells by activation of the UPR and of protecting mice from lupus-like disease. We therefore hypothesized that similar to blocking disposal of misfolded immunoglobulins by bortezomib, disrupting client-chaperone interactions using Hsp90 inhibitors would result in inability to handle immunoglobulin production and death of not only malignant but also autoreactive plasma cells.

Here, we show that pharmacological blockage of Hsp90 by the geldanamycin derivative 17-DMAG (17-desmethoxy-17-N,N-dimethylaminoethylaminogeldanamycin) or the nontoxic peptide derivative TCBL-145 is associated with amelioration of type VII collagen-induced EBA in mice and that T cells, rather than plasma cells, represent targets of anti-Hsp90 treatment in this autoimmune disease.
MATERIAL AND METHODS

Mice

Six- to 8-wk-old SJL mice were purchased from Charles River Laboratories (Sulzfeld, Germany). The experiments were approved by local authorities of the Animal Care and Use Committee (Kiel, Germany; reference number: 6/11f/08) and performed by certified personnel.

Production of autoantigen

Recombinant forms of murine type VII collagen were prepared as previously described. Recombinant tagged fragments GST-mCVIIC and His-mCVIIC were produced using a prokaryotic expression system and purified by glutathione and metallochelate affinity chromatography, respectively.

Induction of EBA and phenotype analysis

Experimental EBA was induced in mice by active immunization as described. Briefly, mice were injected subcutaneously in the hind footpads with a single injection of 100 µl of emulsion containing 60 µg of GST-mCVIIC in TiterMax (Alexis Biochemicals, Lausen, Switzerland). Mice were examined every week for their general condition and cutaneous lesions (i.e., erythema, blisters, erosions, and crusts). Disease severity was calculated as percent of body-surface area affected by skin lesions.

Treatment of mice

We treated mice intraperitoneally with 30 mg/kg of 17-DMAG (InvivoGen, France) or 3 mg/kg of TCBL-145 (produced by the Medical Chemistry Group at University of
Szeged, Szeged, Hungary). We injected control mice with an equivalent volume of the solvent aqua ad injectabilia or aqua ad injectabilia containing 5% ethanol, respectively. For prophylactic treatment, mice received a total of two injections with 17-DMAG or vehicle one day before and one day after immunization or a total of 14 daily injections with TCBL-145 or vehicle starting one day prior to immunization and were each followed over 6 weeks. In a second experimental paradigm, if over 2% of body-surface area was affected by EBA lesions, we analyzed the therapeutic effect of 17-DMAG vs. vehicle three times a week or TCBL-145 vs. vehicle once a day each given over a 6-week treatment period. One day after last injection mice were killed for clinical and immunological evaluation. In a third set of experiments, we gave immunized mice 17-DMAG or vehicle twice with an interval of 36 h. We analyzed mice 48 h after the first injection.

**Histopathology and immunofluorescence microscopy**

Biopsies of lesional and perilesional skin were prepared for examination by histopathology and immunofluorescence (IF) microscopy as described previously. Briefly, biopsies collected from experimental animals were fixed in 4% buffered formalin and, subsequently, sections from paraffin-embedded tissues were stained with hematoxylin and eosin. Dermal neutrophil infiltration was assessed semiquantitatively using a score comprising 1, for no infiltration; 2, moderate infiltration; 3, strong infiltration. IgG and C3 deposits were detected by direct IF microscopy on frozen sections prepared from tissue biopsies using 100-fold diluted fluorescein isothiocyanate-labeled antibodies specific to mouse IgG (Dako, Glostrup, Denmark), as well as murine C3 (MP Biomedicals, Solon, OH, U.S.A.). The staining intensity of immunoreactants in the skin of mice was quantified with ImageJ software. Sera were assayed for antibody titers by indirect IF microscopy on cryosections of
normal mouse skin using diluted mouse serum (1:10-1:20,000) and 100-fold diluted fluorescein isothiocyanate-labeled antibodies specific to mouse IgG (Dako, Glostrup, Denmark).

Detection of plasma and germinal center B cells by flow cytometry
Single-cell suspensions were prepared from bone marrows (femora) and spleens as previously described. To detect plasma cells, flow cytometric analyses of splenocytes and bone marrow cells were performed using fluorochrome-conjugated monoclonal antibodies to mouse CD45R (B220, clone RA3.6B2, made in-house) and CD138 (BD Biosciences) as previously described. For analysis of germinal center B cells, peanut hemagglutinin (PNA, Vector) was used. Samples were analyzed on a LSRII flow cytometer (BD Biosciences).

Detection of autoreactive type VII collagen-specific plasma cells by immunohistochemistry
Cryostat sections of draining lymph nodes were fixed in methanol-acetone, incubated with biotinylated recombinant His-mCVIIC and stained with ExtrAvidin-alkaline phosphatase and FastBlue BB salt (Sigma-Aldrich). Secondly, these sections were incubated with rat anti-mouse CD138 (BD Pharmingen) and counterstained with peroxidase-conjugated rabbit anti-rat IgG (H+L) (Jackson ImmunoResearch) and DAB (DakoCytomation). Prior to further analysis, immunohistochemical double staining of mCVIIC-specific CD138⁺ cells was confirmed by immunofluorescence stainings (data not shown). mCVIIC-specific CD138⁺ cells were counted in representative slices of each draining lymph node and numbers were related to the measured area of the respective slice using PALM MicroBeam system (Zeiss).
Assessment of T cell proliferation

T cell proliferation assay was performed as described with minor modifications.\textsuperscript{5} Single-cell suspensions of draining lymph nodes of immunized mice were cultured in RPMI 1640 culture medium supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycine, nonessential amino acids, 50 µM β-mercaptoethanole and 10% FCS. A total of 2.5 x 10\textsuperscript{5} cells were cultured in the presence of either tissue culture plate-bound anti-CD3 mAbs (2.5 µg/ml, BD-Biosciences) with soluble anti-CD28 mAbs (1 µg/ml, BD-Biosciences) or 50 µg/ml of autoantigen (His-mCVIIC) in 96-well plates. Stimulated cells were cultured alone or co-cultured with different concentrations of 17-DMAG (0.1, 0.25 or 2.5 µM) or TCBL-145 (25, 50 or 75 µM). After 96 h, cells were pulsed with BrdU for further 24 h. Cell proliferation was measured using colorimetric cell proliferation BrdU ELISA (Roche). Cell viability was evaluated by trypan blue exclusion.

Statistical analyses

All analyses were performed using t-test or ANOVA (Bonferroni procedure for multiple comparisons). Means are presented ± SEM; P < 0.05 was considered statistically significant.
RESULTS

Prophylactic effects of Hsp90 inhibitors on type VII-collagen-induced EBA

We tested the effects of early administration of 17-DMAG and TCBL-145 on the development of clinical symptoms in mice immunized with GST-mCVIIC. In this EBA model, SJL mice typically show a disease incidence of >90% with clinical signs as early as 2 weeks after immunization, and a clinical disease maintenance for at least 24 weeks.\textsuperscript{4,6} When administered one day before and one day after (17-DMAG) or for 14 days starting one day prior to immunization (TCBL-145), both Hsp90 inhibitors significantly reduced disease development in mice (Figure 1 A and B). The maximum individual scores, defined as percent of body-surface area affected by skin lesions, were 13 in the vehicle-treated and 3 in the Hsp90-treated mice when analyzed 6 weeks after immunization. While scores were always >1 and disease incidence was 100% in both vehicle groups, 75% of 17-DMAG-treated mice showed scores <1 and 67% of mice treated with TCBL-145 were completely free of clinical signs.

Therapeutic effects of Hsp90 inhibitors on type VII-collagen-induced EBA

To investigate therapeutic efficacy of Hsp90 inhibitors in EBA and to study their effects on immunological parameters of this disease, 17-DMAG and TCBL-145 were given to mice during ongoing disease. Immunized mice were allowed to reach moderate clinical severity (primarily individual scores of $\geq 2$), and were then administered 17-DMAG or TCBL-145 for 6 weeks. While disease activity progressed in the vehicle-treated groups, average clinical score significantly dropped from 4.1 ± 0.6 to 0.9 ± 0.3 in the 17-DMAG-injected mice and from 3.3 ± 0.4 to 1.7 ± 0.8 in the TCBL-145-treated animals by the end of the study (Figure 2 A-C). No signs of toxicity, weight loss or mortality were found during treatment with either Hsp90 inhibitor.
Histological examination of lesional skin biopsies from vehicle-treated mice revealed characteristic dermal-epidermal separation accompanied by dense inflammatory infiltrates that were dominated by neutrophils. Dermal neutrophil infiltration was significantly reduced in 17-DMAG- and TCBL-145-treated mice compared to vehicle-injected animals (Figure 3 A-C).

Generation of circulating IgG autoantibodies against the DEJ was sufficiently suppressed by 17-DMAG or TCBL-145 resulting in significantly lower serum autoantibody levels compared to vehicle-injected animals at the end of the observation period as detected by indirect IF microscopy (Figure 4 A and B). However, no substantial differences in signal intensities for basal membrane-bound anti-type VII collagen IgG and C3 were found by direct IF microscopy between the treatment groups (data not shown).

**Effects of Hsp90 inhibitors on plasma and germinal center B cells**

To investigate whether Hsp90 inhibitors eliminate not only malignant, but also normal plasma cells, B220− CD138+ plasma cells from spleen and bone marrow were investigated by flow cytometry 24 h after last injection with 17-DMAG and TCBL-145 given over 6 weeks. Cytofluorometric quantification revealed no difference in the number of splenic and bone marrow plasma cells in vehicle- and 17-DMAG-treated mice (Figure 5 A-D). B220+PNA+ germinal center B cells in spleen were also not significantly affected (Figure 5 E-F). To rule out compensatory mechanisms of plasma cells after long-term treatment with Hsp90 inhibitors, splenic plasma cells were also analyzed already 48 h after the start of treatment with 17-DMAG. Again, no depletion of plasma cells was achieved after short-term treatment (data not shown). Comparable results were obtained with TCBL-145 (data not shown).
Next, we investigated if Hsp90 inhibitors may have an impact on autoreactive plasma cells. Similar to normal plasma cells, immunohistochemical studies revealed that the numbers of CD138+ mCVIIC-specific plasma cells from draining lymph nodes were not significantly altered by 17-DMAG after long-term (6 weeks) (Figure 5 G and H) or short-term treatment (48 h) or treatment with TCBL-145 (data not shown).

**Effects of Hsp90 inhibitors on T cell proliferation**

To test whether 17-DMAG or TCBL-145 affected T cell proliferation, we isolated draining lymph node cells from mice 6-8 weeks after immunization with GST-mCVIIC and stimulated them with anti-CD3/CD28 antibody or autoantigen in the absence or presence of different amounts of 17-DMAG or TCBL-145. Cells from immunized mice proliferated in response to ex vivo activation with the stimulants. The addition of 17-DMAG or TCBL-145 significantly abolished (autoreactive) T cell proliferation partly in a dose-dependent manner (Figure 6 A-D). Cell viability after treatment with either Hsp90 inhibitor was >80% as measured by the amount of trypan blue-negative (i.e., living) cells.
DISCUSSION

The results of our work indicate that in contrast to malignant plasma cells, normal or autoreactive plasma cells do not present targets of Hsp90 inhibitors in vivo and that efficacy of anti-Hsp90 treatment is at least in part mediated by immunosuppressive functions on T cell responses in autoimmunity to type VII collagen.

In our study, 17-DMAG and TCBL-145 effectively suppressed development of EBA disease when administered before the appearance of clinical signs and induced clinical recovery when applied to mice that were already diseased. In comparison with control mice, animals treated with Hsp90 inhibitors showed a reduced dermal inflammatory infiltrate at the DEJ and lower levels of circulating autoantibodies against the basal membrane zone. Our data is in accordance with previous observations from animal models of other autoimmune diseases, including autoimmune encephalomyelitis, rheumatoid arthritis, and systemic lupus erythematosus-like autoimmune disease, in which inhibitors of Hsp90 affected inflammatory disease pathways and efficiently improved the clinical course. The mechanisms of action by which Hsp90 inhibitors led to clinical improvement in these mouse models included reduction of maturation of dendritic cells, populations of antigen presenting cells, activated T and B cells, and cytokine production. However, effects of anti-Hsp90 treatment on autoantibody-producing plasma cells have not yet been studied. This is an important question to address considering that Hsp90b1 is believed to be one of the key downstream chaperones in the ER that controls the ER UPR and considering the increasing recognition of ER UPR in modulating plasma cell function.

In vitro, both proteasome and Hsp90 inhibition have been linked to UPR-mediated death of malignant plasma cells in multiple myeloma caused by the build-up of misfolded immunoglobulins within the ER. In addition, it has later been shown...
that normal plasma cells are also hypersensitive to proteasome inhibition owing to their extremely high amount of protein biosynthesis. Bortezomib depleted normal and autoimmune plasma cells from bone marrow and spleen in vivo via activation of the UPR and protected mice with lupus-like disease from nephritis.\(^{19}\) In contrast, we found no effect of anti-Hsp90 treatment on survival of normal or autoreactive plasma cells in vivo. Numbers of B220\(^{-}\) CD138\(^{+}\) plasma cells from spleen and bone marrow as well as type VII collagen-specific plasma cells from draining lymph nodes after the 6-week treatment with either 17-DMAG or TCBL-145 were comparable to plasma cell counts of mice treated with vehicle. Splenic plasma cells were also not depleted after a 48 h short-term treatment with 17-DMAG, ruling out possible compensatory mechanisms that may had occurred during long-term treatment. Apart from plasma cells, germinal center B cells were also not affected by Hsp90 inhibitors in vivo.

In contrast to the findings obtained with Hsp90 inhibition in transformed plasma cells,\(^{18}\) our data imply that in vivo anti-Hsp90 treatment does not lead to sufficient ER stress for initiation of the UPR and subsequent apoptosis of non-malignant plasma cells. It is possible that Hsp90 exhibits diverse susceptibility towards its inhibitors in malignant and non-malignant plasma cells. In fact, previous studies have established that Hsp90 produced by cancer cells is found within a multi-chaperone complex associated with high ATPase activity, which has a 100-fold higher affinity for inhibitors than its free, uncomplexed form expressed in normal cells.\(^{24}\) Another assumption is that immunoglobulin is not the true client protein of Hsp90b1 or the immunoglobulin-chaperoning function is redundant and can be compensated by other immunoglobulin-interacting ER chaperones such as GRP78\(^{25}\) and GRP170\(^{26}\).

Similarly, a recent study found no intrinsic defect with B cells from B cell-specific Hsp90b1-null mice in terms of B cell receptor expression, proximal signalling, immunoglobulin assembly and production, and plasma cell differentiation.\(^{27}\) Our data
extends these previous observations and indicates that targeting Hsp90 has no impact on survival of normal or autoreactive plasma cells in vivo.

Since T cells have recently been described to be required for both production of autoantibodies and blistering in experimental EBA, we investigated whether these cells are affected by Hsp90 inhibition. Isolated draining lymph node cells from immunized mice stimulated with the T cell-activating anti-CD3/CD28 antibody revealed a partially dose-dependent reduction of proliferation index when co-cultured with Hsp90 inhibitors. Importantly, re-stimulation with recombinant type VII collagen was also suppressed, indicating that autoreactive T cells were targets of anti-Hsp90 treatment. These findings are in line with previous reports which showed an inhibitory potential of Hsp90 blockade on activated T cells, including those of the autoreactive and alloreactive type.

Although autoreactive plasma cells were not depleted by anti-Hsp90 treatment, we found a suppressed serum autoantibody production in anti-Hsp90-treated mice. There are two mutually non-exclusive explanations for this observation. The first is linked to the inhibitory effects of Hsp90 inhibitors on T cells, which are known to provide help to B cells. The second is based on the observation that generation of T cell-dependent antigen-specific antibody responses requires activation of Toll-like receptors in B cells and the increasing appreciation of the importance of these receptors in the pathophysiology of autoimmune diseases. As HSP90b1 ablation in B cells has been recently shown to be associated with an attenuated antibody production in the context of Toll-like receptor stimulation, disruption of chaperoning Toll-like receptors rather than immunoglobulin assembly might have additionally accounted for this suppressed autoantibody response following Hsp90 treatment.

Presumably, the clinical effects of Hsp90 inhibitors observed in our experiments do not exclusively depend on inhibition of T cells. Inhibitory effects on dendritic cells
might have also played a role as there is increasing evidence of Hsp90 inhibitors interfering with dendritic cell function and autoantigen presentation.\textsuperscript{11,14} Neutrophils, pro-inflammatory cytokines and proteases, all of which are important pathogenic factors in EBA,\textsuperscript{4} might have been additional targets. In fact, we found reduced numbers of neutrophils in the skin following anti-Hsp90 treatment in comparison with control mice.

In conclusion, our results suggest that in contrast to killing malignant plasma cells, Hsp90 inhibitors do not affect normal or autoimmune plasma cells in vivo, but exhibit suppressive effects on T cell function. Therefore, the therapeutic approach to selectively deplete autoimmune plasma cells remains confined to the proteasome inhibitor bortezomib, although its clinical use may be limited due to significant toxicity.\textsuperscript{32} The strong in vivo efficacy observed with anti-Hsp90 treatment in experimental EBA supports the introduction of Hsp90 inhibitors, especially the nontoxic TCBL-145, into the clinical setting for the treatment of autoimmune disorders like autoimmune blistering skin diseases.
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AUTHORSHIP

Contribution: M.K. conceived the idea, designed experiments, performed research, analyzed data, and wrote the paper. R.Ma. designed, performed and analyzed the experiments in Figure 5A-F; C.M.H. and J.W. designed, performed and analyzed the experiments in Figure 5G-H; R.Mü., M.M. and E.S. designed, performed and analyzed the experiments in Figure 6; S.C. produced TCBL-145; A.O. sold idea and together with R.J.L. and D.Z. analyzed the research, performed statistical analyses and contributed to composition of the paper. All authors critically revised the manuscript.

Conflict-of-interest disclosure: All authors declare no competing financial interests.

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REFERENCES


FIGURE LEGENDS

Figure 1 Early administration of Hsp90 inhibitors suppresses type VII collagen-induced development of EBA clinical phenotype. (A) Vehicle or 17-DMAG (30 mg/kg i.p.) was administered at one day before and one day after immunization of SJL mice with 60µg GST-mCVIIC. (B) Vehicle or TCBL-145 (3 mg/kg i.p.) was given daily for 14 days staring one day prior to immunization. Data are presented as average clinical scores defined as percent of body-surface area affected by skin lesions 6 weeks after immunization. Values are mean ± SEM of ≥ 6 mice per group. *P < .05.

Figure 2 Hsp90 inhibitors exert therapeutic activity in type VII collagen-induced EBA. Mice were immunized with 60µg GST-mCVIIC and disease was allowed to progress until moderate average clinical scores (primarily ≥2) were observed, at which time (A) vehicle vs. 17-DMAG (30 mg/kg i.p.; 3 times a week) or (B) vehicle vs. TCBL-145 (3 mg/kg i.p.; daily) were administered over a 6-week treatment period. Data are presented as average clinical scores defined as percent of body-surface area affected by skin lesions at the end of the treatment period. Values are mean ± SEM of ≥ 6 mice per group. *P < .05. (C) Representative clinical presentations of vehicle- and 17-DMAG-treated mice at the end of the treatment period. Vehicle-treated mice show erythema, erosions and crusts predominantly located on the ears and around the eyes, while 17-DMAG-injected mice present with significantly less severe disease.

Figure 3 Hsp90 inhibitors reduce dermal neutrophil infiltration. Semi-quantitative scoring of dermal neutrophil infiltration ranging from 0 (no infiltration) to 3 (severe
infiltration) in mice treated with (A) vehicle vs. 17-DMAG or (B) vehicle vs. TCBL-145 at the end of the 6-week treatment period. Values are mean ± SEM of ≥ 6 mice per group. *P < .05. (C) Representative histopathology specimens obtained from the ears of mice injected with vehicle or 17-DMAG by the end of the treatment period. In contrast to 17-DMAG-treated mice, vehicle-injected mice show dermal-epidermal separation and stronger dermal inflammatory infiltrate dominated by neutrophils (magnification, x400).

**Figure 4 Hsp90 inhibitors suppress autoantibody production.** Mean relative titer changes of autoantibodies directed against the DEJ as detected by indirect IF microscopy using normal murine skin as a substrate after the 6-week treatment period with (A) vehicle vs. 17-DMAG or (B) vehicle vs. TCBL-145. Values are mean ± SEM of ≥ 6 mice per group. *P < .05.

**Figure 5 Plasma cells are not affected by Hsp90 inhibitors in vivo.** (A) Bar graph of flow cytometric analysis of the frequency of B220− CD138+ plasma cells in spleen after 6 weeks of treatment with vehicle or 17-DMAG. Values are mean ± SEM of ≥ 6 mice per group. P > .05. (B) Representative corresponding flow cytometric analysis with numbers in the top panels representing percentages of plasma cells with respect to total cell numbers. (C) Bar graph of flow cytometric analysis of the frequency of B220− CD138+ plasma cells in bone marrow after 6 weeks of treatment with vehicle or 17-DMAG. Values are mean ± SEM of ≥ 6 mice per group. P > .05. (D) Representative corresponding flow cytometric analysis with numbers in the top panels representing percentages of plasma cells with respect to total cell numbers. (E) Bar graph of flow cytometric analysis of the frequency of B220+PNA+ germinal center B cells in spleen after 6 weeks of treatment with vehicle or 17-DMAG. Values
are mean ± SEM of ≥ 6 mice per group. *P < .05. (F) Representative corresponding flow cytometric analysis with numbers in the top panels representing percentages of plasma cells with respect to total cell numbers. (G) Bar graph of immunohistochemic analysis of the frequency of CD138+ recombinant type VII collagen (mCVIIC)-specific autoreactive plasma cells from draining lymph nodes after 6 weeks of treatment with vehicle or 17-DMAG. Values are mean ± SEM of ≥ 6 mice per group. *P < .05. (H) Representative immunohistochemical specimens obtained from draining lymph nodes of mice injected with vehicle or 17-DMAG illustrating comparable numbers of autoreactive plasma cells (doublestained in blue and brown) by the end of the treatment period.

**Figure 6 Anti-Hsp90 treatment leads to inhibition of T cell proliferation ex vivo.** Draining lymph node cells were isolated from GST-mCVIIC-immunized mice 6-8 weeks after immunization and re-activated with anti-CD3 (2.5 µg/ml) and anti-CD28 (1 µg/ml) antibody or with the recombinant autoantigen His-mCVIIC (50 µg/ml). Incubations were performed in the absence or presence of different amounts of (A and B) 17-DMAG or (C and D) TCBL-145 and T cell proliferation was determined by BrdU ELISA. Dotted lines represent the values obtained from cells incubated with medium alone. Values are mean ± SEM of 6 mice per group. *P < .05.
(a) Clinical score

Vehicle  17-DMAG

(b) Clinical score

Vehicle  TCBL-145

Kasperkiewicz et al, Fig 1 a, b
Kasperkiewicz et al, Fig 2 a, b
(c)

Vehicle

17-DMAG

Kasperkiewicz et al, Fig 2 c
Kasperkiewicz et al, Fig 3 a, b
Kasperkiewicz et al, Fig 3 c
Kasperkiewicz et al, Fig 4 a, b
Kasperkiewicz et al, Fig 5 a, b
% of plasma cells in bone marrow

Vehicle 17-DMAG

Kasperkiewicz et al, Fig 5 c, d
Vehicle 17-DMAG

% of germinal center B cells in spleen

Vehicle 17-DMAG

Kasperkiewicz et al, Fig 5 e, f
mCVII-specific CD138+ plasma cells/1000μm² in draining lymph nodes

Kasperkiewicz et al, Fig 5g
Kasperkiewicz et al, Fig 5 h
Kasperkiewicz et al, Fig 6 a

Anti-CD3/CD28

Proliferation index

17-DMAG (µM)

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Kasperkiewicz et al, Fig 6 b
Kasperkiewicz et al, Fig 6 c
Kasperkiewicz et al, Fig 6d
Heat shock protein 90 inhibition in autoimmunity to type VII collagen: evidence that non-malignant plasma cells are not therapeutic targets

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