

The Role of Proteasome Inhibitor MG132 in 2,4-Dinitrofluorobenzene-Induced Atopic Dermatitis in NC/Nga Mice

Kozo Ohkusu-Tsukada Daiki Ito Kimimasa Takahashi

Department of Veterinary Pathology, Nippon Veterinary and Life Science University (NVLU), Tokyo, Japan

Keywords

Allergic contact dermatitis · Atopic dermatitis · Autoimmunity · Proteasome inhibitor · Th1

Abstract

Background: Although immunosuppressants for therapy of atopic dermatitis (AD) are still being sought, proteasome inhibitors are also potential candidates for the treatment of AD. Proteasome inhibitors exert various effects by blocking proteasomal degradation and help regulate processes such as apoptosis induction via caspase-9, cell cycle progression via cyclins, NF- κ B inactivation via I κ B, and downregulation of antigen cross-presentation. The cells targeted by proteasome inhibitors are therefore activated cells undergoing proliferation or differentiation, and antigen-presenting cells carrying out protein degradation. **Objectives:** This study investigated the therapeutic effects and side effects of a proteasome inhibitor, MG132, on the treatment of AD. **Methods:** AD-like disease in NC/Nga mice housed under specific pathogen-free conditions was induced by repeated application of 2,4-dinitrofluorobenzene (DNFB). Disease progression was evaluated by inflammation score, histopathology, and serum IgE level, and the effects of systemic MG132 ad-

ministration were investigated. The percentages and absolute numbers for each population of Th1, Th2, and Th17 cells in the axillary lymph nodes were analyzed by flow cytometry. **Results:** DNFB application increased the expression of a unique major histocompatibility complex class I mutant molecule D/L^{dm7} in dendritic cells (DCs), and increased Th1 and Th17 cells in NC/Nga mice. In vivo MG132 administration to NC/Nga mice with DNFB-induced dermatitis reduced Th17 cells but maintained the level of Th1 cells, resulting in the alleviation of dermatitis lesions by decreasing both serum IgE hyperproduction and mast cell migration. To understand the mechanisms maintaining Th1 cell levels following in vivo MG132-administration, we focused on the role of proteasomes regulating D/L^{dm7} expression. Interestingly, 20S proteasome activity was higher in NC/Nga DCs than in BALB/c DCs. In vitro MG132 administration partially increased D/L^{dm7} expression in a dose-dependent manner during DC maturation, and induced IFN- γ production from autoreactive CD8⁺ T cells but not from CD4⁺ T cells following coculturing with D/L^{dm7}-upregulated DCs. **Conclusion:**

Edited by: H.-U. Simon, Bern.

Although MG132 administration temporarily alleviated AD pathogenesis in NC/Nga mice, prolonged MG132 treatment may result in immunopathogenesis leading to chronic AD due to its side effect of maintaining Th1 levels via autoreactive CD8+ T cells.

© 2018 S. Karger AG, Basel

Introduction

NC/Nga mice are prone toward spontaneously developing atopic dermatitis (AD)-like skin lesions under normal conditions, but not under specific pathogen-free (SPF) conditions [1]. NC/Nga mice have the unique H-2^{nc} haplotype (K^d, D/L^{dm7}, I-A^k, I-E^k) and are sensitive to house dust-mite allergens under SPF conditions and develop an intense AD-like disease [2, 3]. Sustained application of 2,4-dinitrofluorobenzene (DNFB) on the skin can elicit contact hypersensitivity (type IV allergy) through its role as a hapten [4]; however, while there is susceptibility toward the development of DNFB-induced dermatitis in BALB/c (H-2^d), A/J (H-2^a), and CBA (H-2^k) mice, this has not been observed in C57BL/6 (H-2^b), C57BL/10 (H-2^b), and AB.Y (H-2^b) mice, which implies differing degrees of tolerance toward DNFB [5]. Interestingly, DNFB-induced dermatitis in NC/Nga mice under SPF conditions is associated with the development of AD-like disease (mixed type I and IV allergies) rather than contact dermatitis (type IV allergy) [6]. Because AD-like disease can also develop in DNFB-applied BALB/c mice by the adoptive transfer of IgE that recognizes DNFB as a hapten [7], immunoglobulin class switching to IgE may be associated with the development of AD-like disease. In addition, DNFB-induced development of AD-like disease in NC/Nga mice has been associated with the major histocompatibility complex (MHC) class I type (H-2^{nc}), because signs of contact dermatitis (type IV allergy) have been observed in DNFB-treated H-2^b congenic NC/Nga mice [8].

The proteasome inhibitor MG132 has been shown to inhibit apoptosis through mitochondrial disruption via caspase-9 [9], allow cell cycle progression by inhibition of the proteasomal degradation of cyclin D1 [10], activate cell growth by inhibiting the degradation of the E2F transcription factors [11], and inactivate the transcription factor NF- κ B by inhibiting degradation of I κ B [11]. The efficacy of in vivo MG132 administration has previously been researched for the treatment of inflammatory bowel disease (IBD), for which it is used as an anti-inflammatory agent [12]. The immunoproteasome inhibitor ONX-

0914 and the proteasome inhibitor bortezomib (BTZ) have been shown to affect autoimmune development through the reduction of Th17 cells [13, 14]. BTZ application to the ovalbumin (OVA)-driven AD-developed model in BALB/c mice reduced both the serum IgE level and T cell infiltration into dermatitis lesions [15], and treatment with BTZ alleviated the atopic symptoms of 2,4-dinitrochlorobenzene-induced AD mice by increasing claudin-1 expression [16].

Proteasome inhibitors also target the ubiquitin-proteasome pathway during MHC class I antigen processing. The effects of proteasome inhibitors on MHC class I expression are complicated. MHC class I molecules require 3 elements for correct structural arrangement: the antigen, β_2 -microglobulin (β_2 -m), and the α -chain. The antigenic peptides are generated via cytosolic proteolysis and transported by the transporter associated with antigen processing (TAP) complex into the endoplasmic reticulum, where they bind to MHC class I molecules and are delivered to the cell surface. The loss of some antigenic peptides can result from failed cytosolic proteolysis, in which the 26S proteasome or 20S immunoproteasome performs ubiquitination-dependent or ubiquitination-independent protein degradation. β_2 -m knockouts or mutations in the TAP complex prevent the expression of all MHC class I molecules. Misfolded α -chains result from activation of the standard 20S proteasome for ubiquitination-dependent protein degradation related to translocation from the endoplasmic reticulum into the cytosol [17–20].

In this study, we demonstrated that in vivo MG132 administration to NC/Nga mice alleviated the acute pathogenesis of DNFB-induced dermatitis by decreasing both serum IgE hyperproduction and mast cell migration, and that it maintained Th1 levels via an autoreactive CD8+ T cell response to D/L^{dm7} upregulation in dendritic cells (DCs). We also discuss the immunopathogenesis that leads to chronic AD.

Materials and Methods

Mice

NC/Nga mice (male, H-2^{nc} [2], Mls-1a [21]; Japan SLC, Hamamatsu, Japan) at 10–14 weeks of age were used in all of the experiments. Mice were maintained by breeding in SPF air conditions with microbiological monitoring tests (twice per year) and periodic cage exchange, and with a full-barrier animal facility under a controlled temperature (24–25 °C) and humidity (25–35%), and with a 12-h light/dark regimen. All experiments were approved by the NVLU Institutional Review Board for animal studies, and performed following the guidelines provided by the committee for animal welfare.

DNFB-Induced Dermatitis Model

The methods in this study have been described previously [6]. AD-like skin lesions were induced by the repeated application of 25 μ L of 0.15% DNFB (Wako Pure Chemicals, Tokyo, Japan) in acetone/olive oil (3:1) to the skin of the ears, calva, and neck on days 0, 3, 5, 7, 9, 11, 13, 15, 17, and 19. The control mice were applied the acetone/olive oil (3:1) alone as the vehicle of DNFB. Mice (body weight approx. 23–25 g) were repeatedly i.p. injected 200 nmol of MG132 (AdooQ BioScience, Irvine, CA, USA) on days 0, 3, 5, 7, 9, 11, 13, 15, 17, and 19. Dermatitis was evaluated by assigning an inflammation score [22]. Briefly, inflammation of the face, ears, and the anterior part of the body was scored as follows: 0, none; 1, mild; 2, moderate, and 3, severe. This scoring was based on the severity of erythema/hemorrhage (e/h), edema (ed), exco-riation/erosion (e/e), and scaling/dryness (s/d), and total points were evaluated as the severity of dermatitis.

Histopathology and Specific Stains

Histopathological analyses were performed on a minimum of 4 animals per experimental group. Tissues were immersion-fixed in 10% buffered formalin and processed by routine methods for paraffin sectioning. Paraffin sections, 6 μ m thick, were stained with hematoxylin and eosin (HE) and toluidine blue (TB) stain, and examined by light microscopy.

ELISA

Blood samples were collected from the orbital sinus in mice using a hematocrit tube, and serum samples were obtained by centrifugation. The total serum IgE was measured by a mouse IgE ELISA kit (Shibayagi Co. Ltd., Gunma, Japan) following the manufacturer's instructions. The IFN- γ , IL-2, and IL-4 in the culture supernatants after stimulation with 10 nM PMA (phorbol 12-myristate 13-acetate; Sigma-Aldrich, St. Louis, MO, USA) and 1 μ M ionomycin (Sigma-Aldrich) for 4 h of CD4 $^{+}$ T cells from axillary lymph nodes (LNs) were measured by mouse Th1/Th2 ELISA Ready-SET-Go! (eBioscience) following the manufacturer's instructions.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from mouse blood using Lymphoprep (ProGen, Heidelberg, Germany). For analysis, PBMCs suspended in PBS were stained by FITC-conjugated anti-Db/Ld mAb (clone 28-14-8; BioLegend), FITC-conjugated anti-Db/Ld mAb (clone 27-11-13S; Southern Biotechnology Associates Inc., Birmingham, AL, USA), PE-conjugated anti-I-Ak mAb (clone 11-5.2), PE-conjugated anti-CCR4 mAb (clone 2G12), biotin-conjugated anti-CCR5 mAb (clone HM-CCR5), biotin-conjugated anti-CCR6 mAb (clone 29-2L17), FITC-conjugated anti-CD4 mAb (clone RM4-5), and PE/Cy5-conjugated streptavidin (BioLegend). One-color, 2-color, or 3-color analysis was conducted by FACS (FACSCalibur, BD, Japan). The population of positive or negative controls in FACS was regulated, and the data of samples simultaneously taken up was analyzed.

Cell Culture

CD11b $^{+}$ cells (1×10^6 /well; >92%) enriched from PBMCs or LNs using anti-mouse CD11b Magnetic Particles-DM (BD Biosciences, San Diego, CA, USA) were cultured for 3 days with 20 ng/mL of recombinant (r)IL-4 (PeproTech, Rocky Hill, NJ, USA) and

20 ng/mL rGM-CSF (PeproTech), or with 0, 0.4, 2, or 10 μ M of proteasome inhibitor MG132 (Cayman Chemical, Ann Arbor, MI, USA) in a 24-well flat-plate. CD4 $^{+}$ T cells (3×10^5 /well) or CD8 $^{+}$ T cells (3×10^5 /well) enriched from LNs using anti-mouse CD4 (GK1.5) or anti-mouse CD8a (53-6.7) Magnetic Particles-DM (BD Biosciences) were cocultured for 72 h at 37 $^{\circ}$ C in 5% CO $_2$ with washed CD11b $^{+}$ cells ($1-3 \times 10^5$ /well) irradiated with a 30-Gy X-ray (CP160, Faxitron, Wheeling, IL, USA). The culture medium was RPMI 1640 containing 10% heat-inactivated FCS, L-glutamine, nonessential amino acids, sodium pyruvate, 2-ME, and penicillin-streptomycin (Wako, Tokyo, Japan).

20S Proteasome Activity

The 20S proteasome activity assay in CD11b $^{+}$ cells purified from LNs was performed according to the manufacturer's instructions using a 20S proteasome assay kit (Cayman Chemical). Briefly, 2 μ M of MG132 was used in the inhibition of 20S proteasome activity. A total of 0.5 μ M of ONX0914 (AdooQ BioScience) was used in selective inhibition of immunoproteasome LMP7. SUC-LLVY-AMC (10 μ L) as a specific substrate for the 20S proteasome was added to 100 μ L of lysate supernatant of CD11b $^{+}$ cells or Jurkat cell lysate supernatant (positive control) or buffer alone (negative control) in a 96-well black plate, and the plate was incubated at 37 $^{\circ}$ C for 1 h. The fluorescent intensity of each well (excitation 360 nm, emission 480 nm) was analyzed with a microplate reader (Bio-Rad Labs, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was performed with analysis of variance (ANOVA) using Excel (Microsoft) and StatPlus (AnalystSoft, Alexandria, VA, USA). A p value <0.05 was considered significant. The ANOVA test was performed after a normal distribution test (Shapiro-Wilk test).

Results

Effects of MG132 Administration on Dermatitis, Serum IgE Levels, and Mast Cells in DNFB-Applied NC/Nga Mice

The efficacy of in vivo administration of MG132 to DNFB-induced dermatitis in NC/Nga mice was investigated. DNFB-induced dermatitis was evaluated from days 3–20 by assigning an inflammation score [22]. The high inflammation scores of the dermatitis lesions and the axillary lymphadenopathy induced in DNFB-applied NC/Nga mice by day 14 were significantly improved by in vivo administration of MG132 (Fig. 1a; online suppl. Fig. S1; see www.karger.com/doi/10.1159/000488155 for all online suppl. material). However, there was no statistically significant difference between treated and untreated mice on day 20 because of a peak in lesion progression at this time. The average number of migrating mast cells per 0.33 mm ^{2} was calculated; it was observed that in vivo administration of MG132 significantly decreased serum IgE

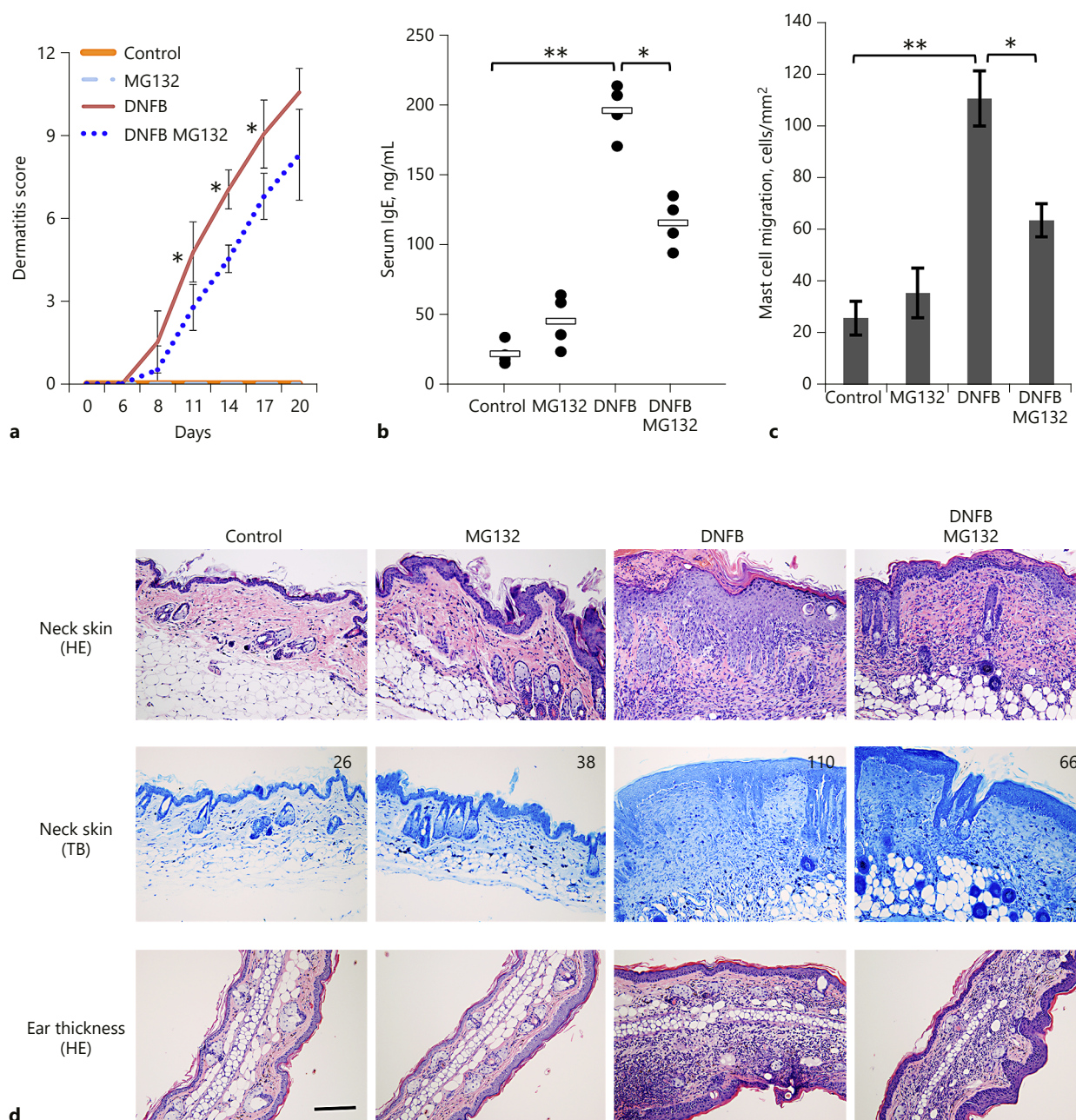


Fig. 1. DNFB-induced dermatitis, serum IgE hyperproduction, and mast cell migration are alleviated due to in vivo MG132 administration in NC/Nga mice. **a** Dermatitis scores evaluated between days 3 and 20 of treatment. The bar graphs indicate the mean $\pm$ SD, $n = 4$ or 5 mice/group. * DNFB versus DNFB with MG132; $p < 0.05$, ANOVA. **b, c** The serum IgE concentration on day 14 was analyzed by ELISA. The number of migrating mast cells

in the neck skin on day 14 was counted over 6 fields at $\times 200$ magnification in tissue sections stained by TB. The bar graphs indicate the mean $\pm$ SD, $n = 4$ or 5 mice/group. * $p < 0.05$, ANOVA; ** $p < 0.01$, ANOVA. **d** On day 14, the degree of acanthosis in the neck skin and the ear thickness were evaluated at $\times 200$ magnification in tissue sections using HE stain. Scale bar, 100 μ m. Data are representative of 2 independent experiments.

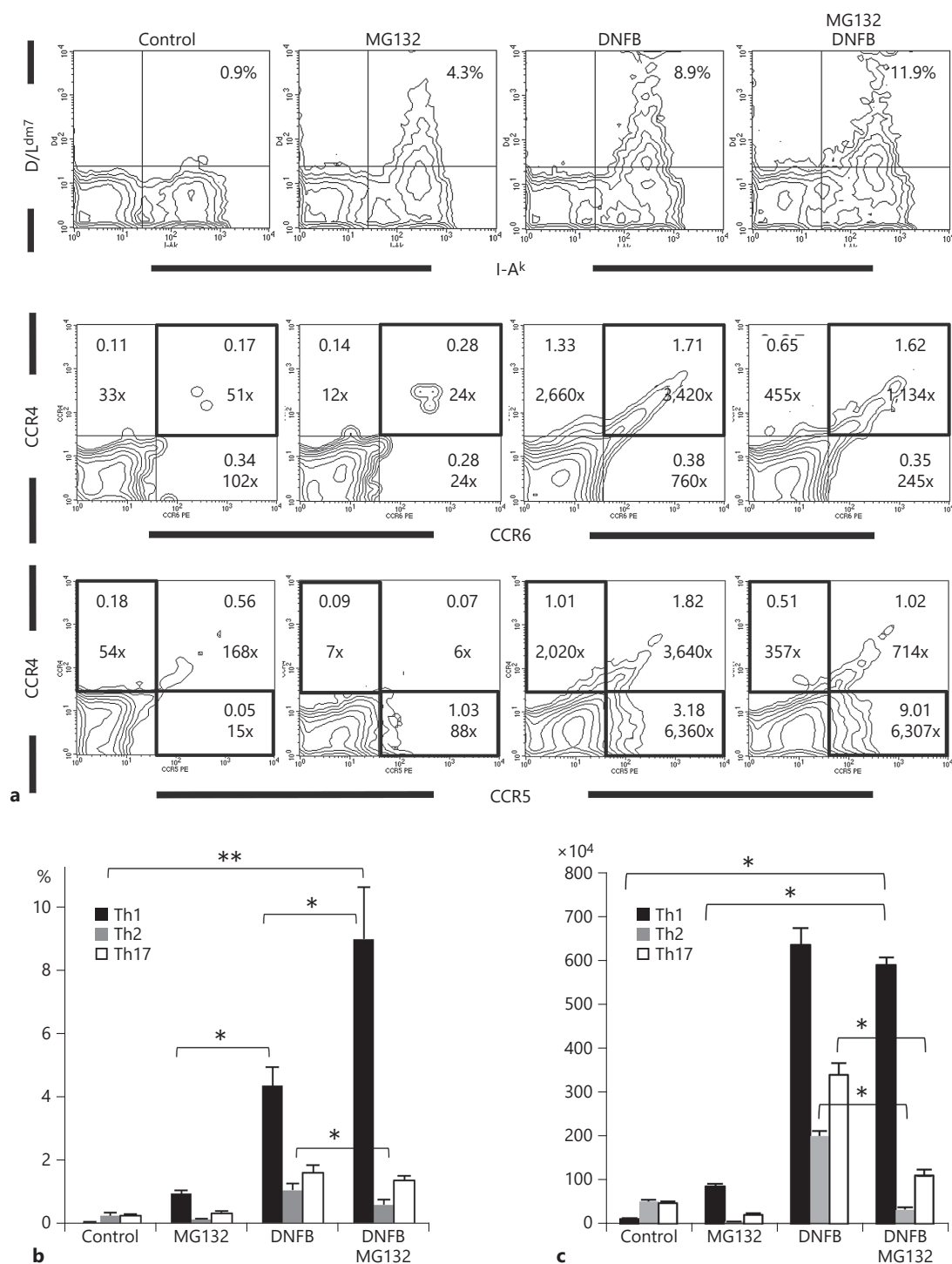


Fig. 2. In vivo MG132 administration induces a Th1 response. **a** D/L^{dm7}-positive monocyte population (I-A^{k+}) from PBMCs on day 10, Th2 (CCR4+CCR5⁻), Th1 (CCR4-CCR5⁺), and Th17 (CCR4+CCR6⁺) populations from axillary LNs on day 14, analyzed by FACS. The number of quadrant regions in the FACS pan-

els is indicated as percentages (upper) and the absolute number $\times 10^{-3}$ (lower). Bar graphs depicting the percentages (**b**) of Th1, Th2, and Th17 and the absolute numbers of Th1, Th2, and Th17 in axillary LNs (**c**) are the mean $\pm$ SD, $n = 4$ or 5 mice/group. * $p < 0.05$, ANOVA; ** $p < 0.01$, ANOVA.

hyperproduction and mast cell migration in DNFB-applied NC/Nga mice (Fig. 1b, c; $p < 0.05$, ANOVA). Histologically, on day 14, the acanthosis and the ear thickening with inflammatory cell infiltration induced by DNFB application were alleviated by in vivo administration of MG132 (Fig. 1d).

Increased D/Ldm7 from MG132 Administration to DNFB-Induced Dermatitis of NC/Nga Mice

Sustained application of DNFB to skin can elicit contact hypersensitivity (type IV allergy) by acting as a hapten. After DCs take up DNFB-binding protein in the skin, MHC expression on the DCs is thought to be upregulated through the acceleration of antigen presentation. Here the efficacy of in vivo administration of MG132 in naïve NC/Nga or dermatitis-induced NC/Nga mice using DNFB under SPF conditions was investigated. D/L^{dm7} expression on monocytes in PBMCs of these mice was observed by sequential sampling. In NC/Nga mice with DNFB-induced dermatitis, D/L^{dm7} expression on monocytes significantly increased on day 14 when treated with MG132 (Fig. 2a).

Effect of in vivo MG132 Administration on Th1/Th2 Balance

CD4⁺ T cell subsets can be roughly identified by the expression patterns of their chemokine receptors: Th1 (CCR4[−]CCR5⁺) [23], Th2 (CCR4⁺CCR5[−]) [24], and Th17 (CCR4⁺CCR6⁺) [25]. The effect after day 14 of in vivo administration of MG132 on DNFB-applied NC/Nga mice under SPF conditions was analyzed using FACS. In axillary LNs, the absolute number of Th1 cells (CCR4[−]CCR5⁺) was unchanged following MG132 administration compared with DNFB application alone ($p = 0.63$, ANOVA). In contrast, the absolute numbers of

Th2 cells (CCR4⁺CCR5[−]) and Th17 cells (CCR4⁺CCR6⁺) were significantly reduced (Fig. 2c; $p < 0.001$, ANOVA). ELISA analysis (online suppl. Fig. S2) of the culture supernatant of CD4⁺ T cells (5×10^5 /well) purified from LNs of DNFB-applied NC/Nga mice after stimulation with a combination of PMA and ionomycin for 4 h showed no marked fluctuation in the levels of IL-4 (a Th2 cytokine), although these levels tended to decrease somewhat following MG132 administration to DNFB-applied NC/Nga mice ($p = 0.132$, ANOVA). However, IFN- γ (Th1 cytokine) and IL-17 (Th17 cytokine) levels in the culture supernatant were greatly increased in DNFB-applied NC/Nga mice ($p < 0.001$, ANOVA), and IFN- γ levels were further increased by MG132 administration to these mice. IL-17 levels were unchanged by MG132 administration after DNFB application. These results correlated with an analysis of the percentage of CCR phenotypes (Fig. 2b; online suppl. Fig. S2). Thus, MG132 administration reduced the populations of Th2/Th17 cells but not the functioning of these cells.

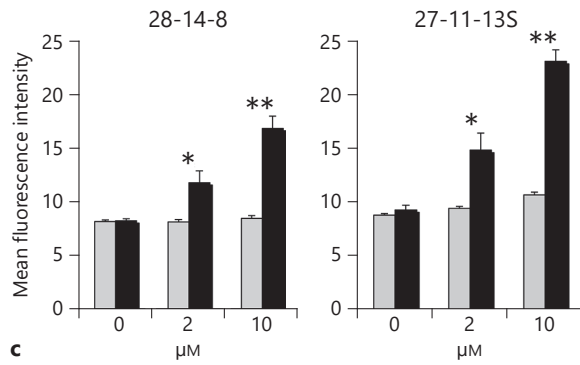
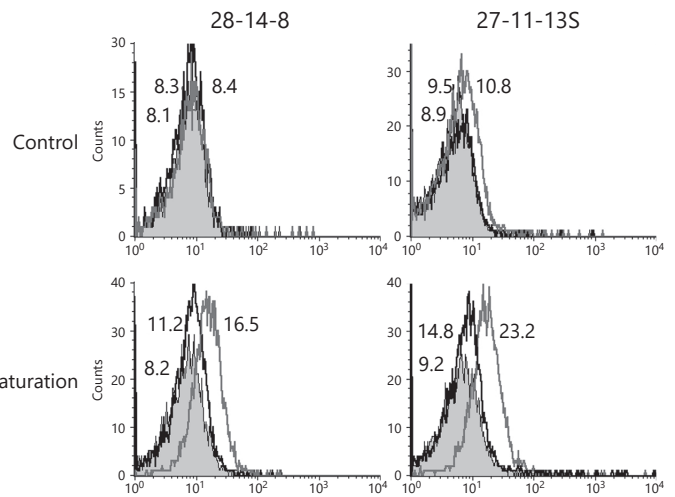
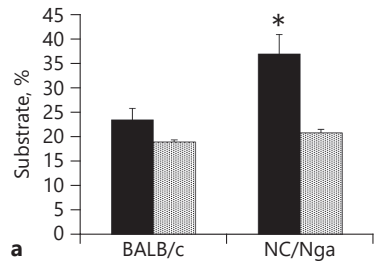
Higher 20S Proteasome Activity in NC/Nga DCs than in BALB/c DCs

To understand the mechanisms of Th1 maintenance in DNFB-applied mice following in vivo MG132 administration, we focused on the role of proteasomes known to regulate D/L^{dm7} expression on antigen-presenting cells. We used CD11b⁺ cells as antigen-presenting cells, because recent research indicated that all classic DCs can be categorized into 2 functional subsets that express either CD103 or CD11b, but not markers such as CD11c or CX3CR1 [26]. Following DNFB application, the partial loss of each MHC class I molecule (the α -chain) likely occurred because of enhanced 20S proteasome activity through posttranscriptional regulation, because 20S

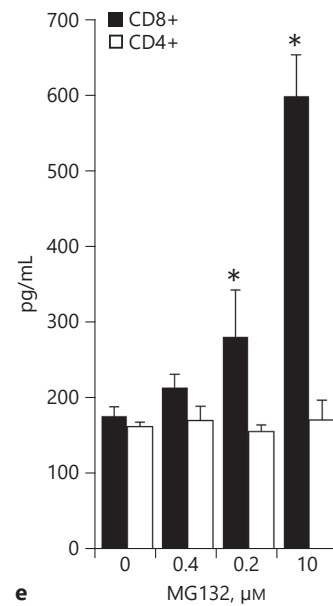
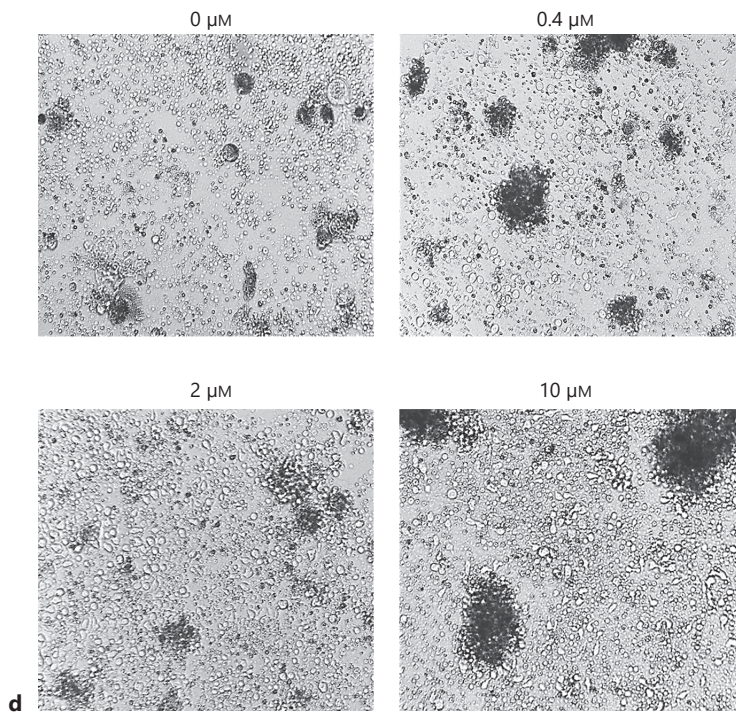
Fig. 3. D/L^{dm7} expression on antigen-presenting cells increased by in vitro MG132 administration induces autoreactive CD8⁺ T cells in NC/Nga mice. **a** 20S proteasome activity in lysed CD11b⁺ cells purified from the LNs (black bars) of NC/Nga and BALB/c mice and analyzed using a 20S proteasome assay kit (Cayman). Jurkat cell lysate supernatant was used as a positive control (100% substrate). The effects of MG132 administration (gray bars) are indicated. Data are representative of 2 independent experiments. The bar graphs indicate the mean $\pm$ SD. * NC/Nga (black bar) versus BALB/c (black bar), $p < 0.05$, ANOVA. **b** D/L^{dm7} expression (anti-L^d mAb; clone 28-14-8 and clone 27-11-13S) on the cell surface of CD11b⁺ cells after control culture (upper) or maturation (lower) with administration of 0 μ M (gray fill), 2 μ M (no fill, black line), and 10 μ M (no fill, gray line) of MG132, as analyzed by FACS. The

numbers indicated in the panel show the mean fluorescence intensity. **c** Bar graphs representing the mean fluorescence intensity of D/L^{dm7} expression in CD11b⁺ cells after control culture (gray fill) or maturation (black fill) with administration of 0, 2, and 10 μ M of MG132, depicted as the mean $\pm$ SD, $n = 3$ mice/group. * 10 μ M versus 0 μ M, $p < 0.05$, ANOVA; ** 10 μ M versus 2 μ M, $p < 0.05$, ANOVA. Data are representative of 3 independent experiments. **d** Microscope images of CD8⁺ T cells cocultured with irradiated D/L^{dm7}-upregulated CD11b⁺ cells after 0, 0.4, 2, or 10 μ M MG132 administration. **e** IFN- γ produced by CD8⁺ or CD4⁺ T cells in the supernatants of cocultures with irradiated D/L^{dm7}-upregulated CD11b⁺ cells after 0, 0.4, 2, or 10 μ M MG132 administration. * $p < 0.05$, ANOVA. Data are representative of 2 independent experiments.

(For figure see next page.)



Response of CD8+ T cells



proteasome activity in CD11b+ cells purified from the LNs of NC/Nga mice was enhanced compared with that from BALB/c mice (Fig. 3a). Chemical inhibitors of proteasomes are widely used in research on proteasome function. MG132 (peptide aldehyde type; Z-Leu-Leu-Leu-CHO) when used as a general proteasome inhibitor has a more sensitive effect on proteasome inhibition than ALLN (N-acetyl-Leu-Leu-norleucinal) [27]. The 20S proteasome activity in both NC/Nga and BALB/c mouse strains was significantly decreased by MG132 administration (Fig. 3a, gray bar). A selective chemical inhibitor such as ONX0914 can specifically inhibit the function of the immunoproteasome subunit LMP7 [28]. The 20S proteasome activity in NC/Nga mice was unaffected by immunoproteasome-specific inhibitor ONX0914 administration (data not shown). These results indicated that the 20S proteasome activated in NC/Nga mice following DNFB application is likely a standard 20S proteasome rather than a 20S immunoproteasome.

Upregulation of D/Ldm7 Expression in NC/Nga DCs by MG132 Administration

The H2-haplotype of NC/Nga mice comprises a unique genotype (K^d , D/L^{dm7} , $I-A^k$, $I-E^k$), in which D/L^{dm7} expression is lost at the protein level [2]. To investigate whether D/L^{dm7} expression in NC/Nga mice is upregulated by the inhibition of protein cleavage, we researched the effect of the proteasome inhibitor MG132 on D/L^{dm7} expression in CD11b+ cells under maturation conditions. The expression of D/L^{dm7} after in vitro MG132 administration, as detected with 2 commercial mAbs (28-14-8 and 27-11-13S) specific to the $\alpha 3$ domain of L^d [2], was slightly increased under maturation conditions, but not under the control culture conditions, in an MG132 concentration-dependent manner (0, 2, and 10 μ M; Fig. 3b, c). Although administration of rIFN- γ and rTNF- α are reported to increase MHC class I expression by reversing antigen-dependent downregulation [29], in our study their in vitro administration did not increase D/L^{dm7} expression on NC/Nga CD11b+ cells under maturation conditions (data not shown).

IFN- γ Production from CD8+ T-Cells, but Not CD4+ T-Cells, in Coculture with MG132-Administered DCs

To understand which cells are activated by D/L^{dm7} -upregulated matured CD11b+ cells, the IFN- γ production of CD8+ T cells and CD4+ T cells purified from the LNs of NC/Nga mice was analyzed by coculturing with irradiated D/L^{dm7} -upregulated CD11b+ cells following in vitro MG132 administration under maturation condi-

tions. D/L^{dm7} -upregulated DCs were inactivated by irradiation at 30 Gy before coculture. Autoreactive activation of CD8+ T cells, but not CD4+ T cells, was detected by coculturing with irradiated DCs after the administration of 2 or 10 μ M of MG132 (Fig. 3d). IFN- γ produced from CD8+ T cells, but not CD4+ T cells, exhibited a significant increase in the supernatant of cocultures with irradiated DCs after the administration of 2 or 10 μ M of MG132, as determined by ELISA (Fig. 3e).

Discussion

Nonpharmacologic interventions and pharmacologic topical therapies are available for the treatment of AD. Most AD patients rely on pharmacologic topical therapies. As a maintenance regimen, 1.25% hydrocortisone cream applied to the acid mantle of the skin as a steroid-based emollient is both effective and safe for longer periods (e.g., months) to prevent acute flares, while stronger steroids are used for the rapid treatment of acute flares [30]. However, because AD patients often gradually acquire drug resistance, there is documented risk, particularly with systemic corticosteroid use. For patients with severe AD, particularly in adults, phototherapy, methotrexate, azathioprine, cyclosporine, and mycophenolate mofetil have been successfully used instead of corticosteroids [31]. A drug that can be safely used as a systemic immunosuppressant is sought for the treatment of severe AD.

Here, the effects of systemic administration of the proteasome inhibitor MG132 were researched using the AD-like disease model, NC/Nga mice. In vivo MG132 treatment of DNFB-induced dermatitis reduced Th2 and Th17 cell numbers but had no effect on Th1 cells, resulting in the alleviation of dermatitis lesions by decreasing both serum IgE hyperproduction and mast cell migration. An interesting effect of MG132 was the partial upregulation of D/L^{dm7} . Fromm et al. [32] also reported that MG132 administration in vitro increased the cell surface expression of MHC class I heavy chains. This study indicated that the Th1 response via autoreactive CD8+ T cells activated by D/L^{dm7} -upregulated DCs can alleviate Th2-dependent type I allergic dermatitis by shifting the ratio of Th1/Th2 [33]. In addition, some reports have indicated that blockage of the ubiquitination-dependent protein degradation pathway induces a Th1 response. The administration of USP10 (ubiquitin-specific protease 10), which can remove ubiquitin conjugated to a target protein T-box transcription factor (T-bet), rescues Th1 im-

mune response [34]. It was also reported that a novel N-amidinopiperidine-based proteasome inhibitor can rescue DC differentiation from the immunosuppressive environment of a tumor, consequently inducing Th1 effector differentiation [35].

In recent research, these immunologic responses by Th1 and Th17 have been suggested to be part of the immunopathogenesis that leads to chronic AD [36, 37], and these responses are also detected in various autoimmune diseases. The autoallergens Hom s 1–5 were identified by screening a human epithelial cDNA library for IgE binding of sera from AD patients [38–40]. Hom s 2 corresponds to the α -chain of the nascent polypeptide-associated complex (α -NAC), and α -NAC-specific autoreactive CD8+ T cells were identified as secreting both IL-4 and IFN- γ in AD patients [41]. However, autoreactive CD4+ T cells were not generated from PBMCs in AD patients by α -NAC peptides [42]. In this study, autoreactive activation of CD8+ T cells but not of CD4+ T cells was also detected by coculturing with irradiated D/L^{dm7}-upregu-

lated CD11b+ cells treated with MG132. This suggests an avenue for further investigation as a condition similar to dermatitis in AD patients was observed in our MG132-treated AD model of NC/Nga mice. This study indicated that the side effects of the proteasome inhibitor MG132 may induce the immunopathogenesis that leads to chronic AD.

Acknowledgments

We thank Dr. Tohei for microbiological monitoring tests of the mice, Dr. Hakamata for management of the full-barrier animal facility, and Dr. Yokosuka for the welfare of the laboratory animals used in the research.

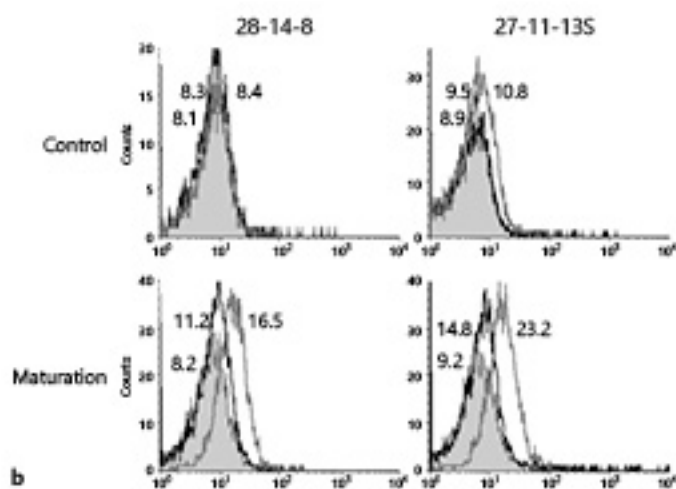
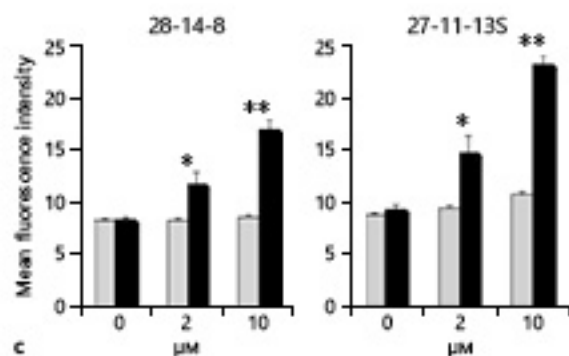
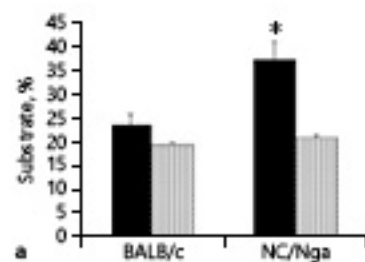
Disclosure Statement

No author has any financial interest or conflict of interest to disclose.

References

- ▶ 1 Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, Matsumoto M, Ushio H, Saito S, Askenase PW, Ra C: Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997;9:461–466.
- ▶ 2 Ohkusu-Tsukada K, Yamashita T, Tsukada T, Takahashi K: Low expression of a D^{dm7}/L^{dm7}-hybrid mutant (D/L^{dm7}) in the novel haplotype H-2^{nc} identified in atopic dermatitis model NC/Nga mice. *Genes Immun* 2017, Epub ahead of print.
- ▶ 3 Heishi M, Imai Y, Katayama H, Hashida R, Ito M, Shinagawa A, Sugita Y: Gene expression analysis of atopic dermatitis-like skin lesions induced in NC/Nga mice by mite antigen stimulation under specific pathogen-free conditions. *Int Arch Allergy Immunol* 2003; 132:355–363.
- ▶ 4 Miller JF, Vadas MA, Whitelaw A, Gamble J: H-2 gene complex restricts transfer of delayed-type hypersensitivity in mice. *Proc Natl Acad Sci USA* 1975;72:5095–5098.
- ▶ 5 Sauder DN, Katz SI: Strain variation in the induction of tolerance by epicutaneous application of trinitrochlorobenzene. *J Invest Dermatol* 1983;80:383–386.
- ▶ 6 Tomimori Y, Tanaka Y, Goto M, Fukuda Y: Repeated topical challenge with chemical antigen elicits sustained dermatitis in NC/Nga mice in specific-pathogen-free condition. *J Invest Dermatol* 2005;124:119–124.
- ▶ 7 Katayama I, Tanei R, Yokozeki H, Nishioka K, Dohi Y: Induction of eczematous skin reaction in experimentally induced hyperplastic skin of Balb/c mice by monoclonal anti-DNP IgE antibody: possible implications for skin lesion formation in atopic dermatitis. *Int Arch Allergy Immunol* 1990;93:148–154.
- ▶ 8 Ohkusu-Tsukada K, Ito D, Okuno Y, Tsukada T, Takahashi K: Signs of atopic dermatitis and contact dermatitis affected by distinct H2-haplotype in the NC/Nga genetic background. *Sci Rep* 2018;8:2586.
- ▶ 9 Nencioni A, Garuti A, Schwarzenberg K, Cirmena G, Dal Bello G, Rocco I, Barbieri E, Brossart P, Patrone F, Ballestrero A: Proteasome inhibitor-induced apoptosis in human monocyte-derived dendritic cells. *Eur J Immunol* 2006;36:681–689.
- ▶ 10 Stewart AG, Harris T, Fernandes DJ, Schachte LC, Koutsoubos V, Guida E, Ravenhall CE, Vadeveloo P, Wilson JW: β 2-adrenergic receptor agonists and cAMP arrest human cultured airway smooth muscle cells in the G₁ phase of the cell cycle: role of proteasome degradation of cyclin D1. *Mol Pharmacol* 1999;56:1079–1086.
- ▶ 11 Lee DH, Goldberg AL: Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998;8:397–403.
- ▶ 12 Fritsch Fredin M, Vidal A, Götling YY, Wilén R, Jansson L, Hultgren Hörnquist E, Melgar S: The application and relevance of ex vivo culture systems for assessment of IBD treatment in murine models of colitis. *Pharmacol Res* 2008;58:222–231.
- ▶ 13 Xiao F, Lin X, Tian J, Wang X, Chen Q, Rui K, Ma J, Wang S, Wang Q, Wang X, Liu D, Sun L, Lu L: Proteasome inhibition suppresses Th17 cell generation and ameliorates autoimmune development in experimental Sjögren's syndrome. *Cell Mol Immunol* 2017, Epub ahead of print.
- ▶ 14 Liu RT, Zhang P, Yang CL, Pang Y, Zhang M, Zhang N, Yue LT, Li XL, Li H, Duan RS: ONX-0914, a selective inhibitor of immunoproteasome, ameliorates experimental autoimmune myasthenia gravis by modulating humoral response. *J Neuroimmunol* 2017; 311:71–78.
- ▶ 15 Mudnakudu Nagaraju KK, Babina M, Worm M: Opposing effects on immune function and skin barrier regulation by the proteasome inhibitor bortezomib in an allergen-induced eczema model. *Exp Dermatol* 2013;22:742–727.
- ▶ 16 Kim YE, Cho N, Cheon S, Kim KK: Bortezomib, a proteasome inhibitor, alleviates atopic dermatitis by increasing claudin 1 protein expression. *Biochem Biophys Res Commun* 2017;493:744–750.
- ▶ 17 Hughes T, Coit P, Adler A, Yilmaz V, Aksu K, Düzgün N, Keser G, Cefle A, Yazici A, Ergen A, Alpsoy E, Salvarani C, Casali B, Köttler I, Gutierrez-Achury J, Wijmenga C, Direskeneli H, Saruhan-Direskeneli G, Sawalha AH: Identification of multiple independent susceptibility loci in the HLA region in Behçet's disease. *Nat Genet* 2013;45:319–324.

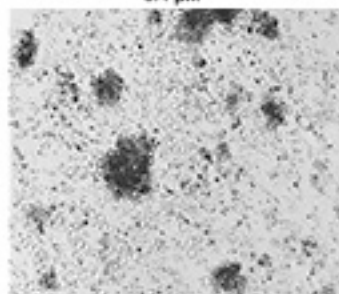
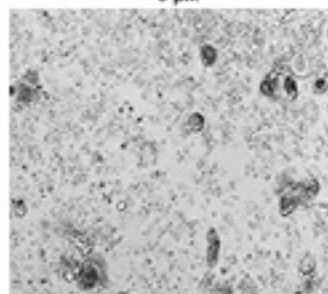
- ▶ 18 Randow F, Lehner PJ: Viral avoidance and exploitation of the ubiquitin system. *Nat Cell Biol* 2009;11:527–534.
- ▶ 19 Ben-Nissan G, Sharon M: Regulating the 20S proteasome ubiquitin-independent degradation pathway. *Biomolecules* 2014;4:862–884.
- ▶ 20 Kisselev AF, Groettrup M: Subunit specific inhibitors of proteasomes and their potential for immunomodulation. *Curr Opin Chem Biol* 2014;23:16–22.
- ▶ 21 Ohkusu-Tsukada K, Tsukada T, Takahashi K: Clonal deletion of T cell repertoires with specific T cell receptor V β chains by two endogenous superantigens in NC/Nga mice. *Biosci Biotechnol Biochem* 2017;81:2160–2163.
- ▶ 22 Leung DY, Hirsch RL, Schneider L, Moody C, Takaoka R, Li SH, Meyerson LA, Mariam SG, Goldstein G, Hanifin JM: Thymopentin therapy reduces the clinical severity of atopic dermatitis. *J Allergy Clin Immunol* 1990;85:927–933.
- ▶ 23 Loetscher P, Uguccioni M, Bordoli L, Baggiolini M, Moser B, Chizzolini C, Dayer JM: CCR5 is characteristic of Th1 lymphocytes. *Nature* 1998;391:344–345.
- ▶ 24 Andrew DP, Ruffing N, Kim CH, Miao W, Heath H, Li Y: Murphy K, Campbell JJ, Butcher EC, Wu L: C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential. *J Immunol* 2001;166:103–111.
- ▶ 25 Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G: Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007;8:639–646.
- ▶ 26 Brähler S, Zinselmeyer BH, Raju S, Nitschke M, Suleiman H, Saunders BT, Johnson MW, Böhner AMC, Viehmann SF, Theisen DJ, Kretzer NM, Briseño CG, Zaitsev K, Ornatsky O, Chang Q, Carrero JA, Kopp JB, Artyomov MN, Kurts C, Murphy KM, Miner JH, Shaw AS: Opposing roles of dendritic cell subsets in experimental GN. *J Am Soc Nephrol* 2018;29:138–154.
- ▶ 27 Li SZ, Song Y, Zhang HH, Jin BX, Liu Y, Liu WB, Zhang XD, Du RL: UbcH10 overexpression increases carcinogenesis and blocks ALLN susceptibility in colorectal cancer. *Sci Rep* 2014;4:6910.
- ▶ 28 Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW, Lauer C, Sylvain C, Ring ER, Shields J, Jiang J, Shwonek P, Parlati F, Demo SD, Bennett MK, Kirk CJ, Groettrup M: A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* 2009;15:781–787.
- ▶ 29 Seliger B, Pfizenmaier K: Post-transcriptional downregulation of MHC class I expression in oncogene-transformed cells is reverted by IFN- γ and TNF- α . *J Immunogenet* 1989;16:315–320.
- ▶ 30 Eichenfield LF, Tom WL, Berger TG, Krol A, Paller AS, Schwarzenberger K, Bergman JN, Chamlin SL, Cohen DE, Cooper KD, Cordoro KM, Davis DM, Feldman SR, Hanifin JM, Margolis DJ, Silverman RA, Simpson EL, Williams HC, Elmetts CA, Block J, Harrod CG, Smith Begolka W, Sidbury R: Guidelines of care for the management of atopic dermatitis: section 2. Management and treatment of atopic dermatitis with topical therapies. *J Am Acad Dermatol* 2014;71:116–132.
- ▶ 31 Sidbury R, Davis DM, Cohen DE, Cordoro KM, Berger TG, Bergman JN, Chamlin SL, Cooper KD, Feldman SR, Hanifin JM, Krol A, Margolis DJ, Paller AS, Schwarzenberger K, Silverman RA, Simpson EL, Tom WL, Williams HC, Elmetts CA, Block J, Harrod CG, Begolka WS, Eichenfield LF: Guidelines of care for the management of atopic dermatitis: section 3. Management and treatment with phototherapy and systemic agents. *J Am Acad Dermatol* 2014;71:327–349.
- ▶ 32 Fromm SV, Mey-Tal SW, Coligan JE, Schechter C, Ehrlich R: MHC class I heavy chain mRNA must exceed a threshold level for the reconstitution of cell surface expression of class I MHC complexes in cells transformed by the highly oncogenic adenovirus 12. *J Biol Chem* 1998;273:15209–15216.
- ▶ 33 Rook GA, Hernandez-Pando R, Lightman SL: Hormones, peripherally activated prohormones and regulation of the Th1/Th2 balance. *Immunol Today* 1994;15:301–303.
- ▶ 34 Pan L, Chen Z, Wang L, Chen C, Li D, Wan H, Li B, Shi G: Deubiquitination and stabilization of T-bet by USP10. *Biochem Biophys Res Commun* 2014;449:289–294.
- ▶ 35 Švajger U, Gobec M, Obreza A, Mlinarič-Raščan I: Novel N-amidinopiperidine-based proteasome inhibitor preserves dendritic cell functionality and rescues their Th1-polarizing capacity in Ramos-conditioned tumor environment. *Cancer Immunol Immunother* 2015;64:15–27.
- ▶ 36 Carmi-Levy I, Homey B, Soumelis V: A modular view of cytokine networks in atopic dermatitis. *Clin Rev Allergy Immunol* 2011;41:245–253.
- ▶ 37 Boguniewicz M, Leung DY: Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* 2011;242:233–246.
- ▶ 38 Natter S, Seiberler S, Hufnagl P, Binder BR, Hirschl AM, Ring J, Abeck D, Schmidt T, Valent P, Valenta R: Isolation of cDNA clones coding for IgE autoantigens with serum IgE from atopic dermatitis patients. *FASEB J* 1998;12:1559–1569.
- ▶ 39 Valenta R, Natter S, Seiberler S, Wichlas S, Maurer D, Hess M, Pavelka M, Grote M, Ferreira F, Szepefalusi Z, Valent P, Stingl G: Molecular characterization of an autoallergen, Hom s 1, identified by serum IgE from atopic dermatitis patients. *J Invest Dermatol* 1998;111:1178–1183.
- ▶ 40 Hradetzky S, Werfel T, Rösner LM: Autoallergy in atopic dermatitis. *Allergo J Int* 2015;24:16–22.
- ▶ 41 Roesner LM, Heratizadeh A, Wieschowski S, Mittermann I, Valenta R, Eiz-Vesper B, Hennig C, Hansen G, Falk CS, Werfel T: α -NAC-specific autoreactive CD8 $^{+}$ T cells in atopic dermatitis are of an effector memory type and secrete IL-4 and IFN- γ . *J Immunol* 2016;196:3245–3252.
- ▶ 42 Hradetzky S, Roesner LM, Balaji H, Heratizadeh A, Mittermann I, Valenta R, Werfel T: Cytokine effects induced by the human autoallergen α -NAC. *J Invest Dermatol* 2014;134:1570–1578.



Response of CD8+ T cells

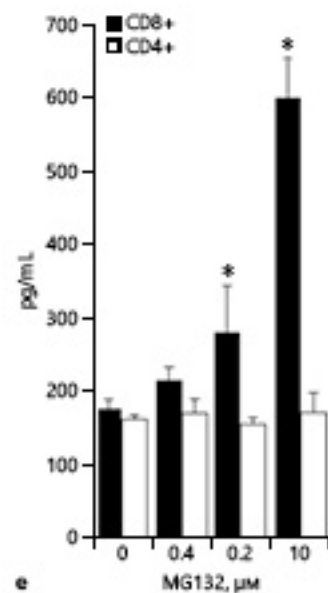
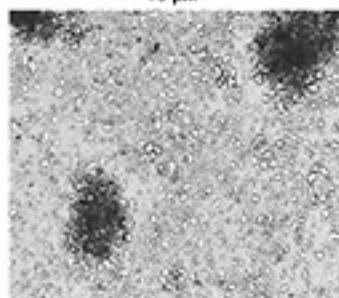
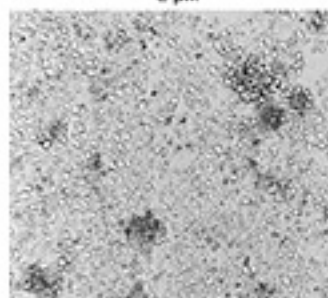
0 μM

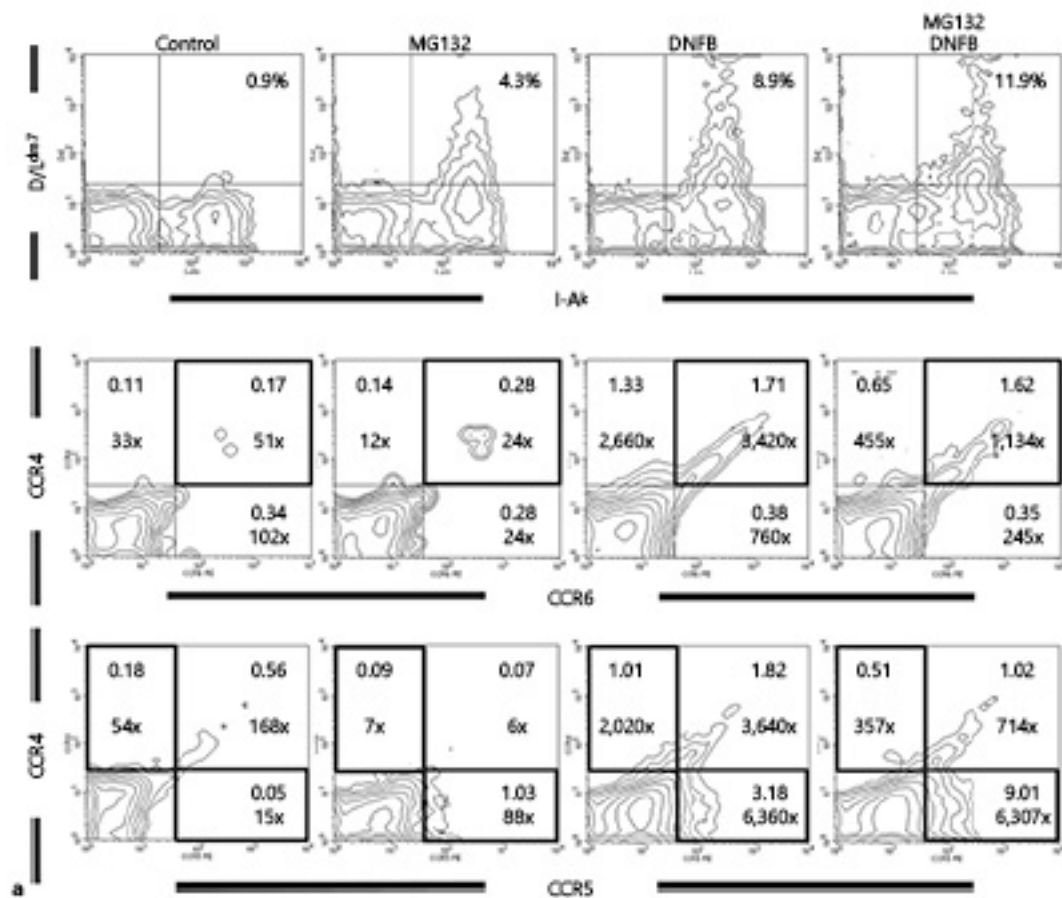
0.4 μM



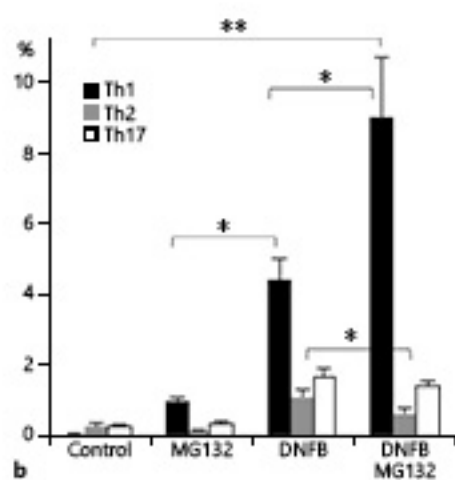
2 μM

10 μM

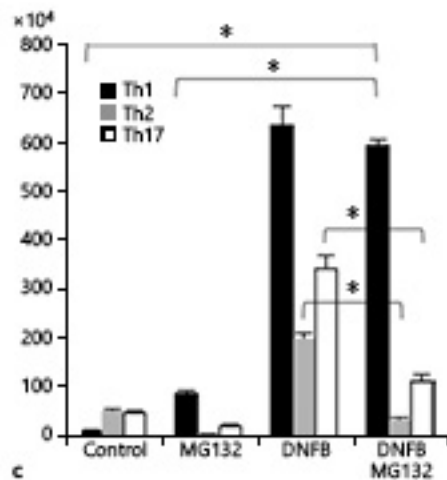




a



b



c

