



Full length article

CD8⁺ T cells regulated by CD4⁺CD25⁺ regulatory T cells in the early stage exacerbate the development of *Dermatophagoides farinae*-induced skin lesions via increasing mast cell infiltration in mice



Nobuaki Mizutani^{a,*}, Sureeporn Kangsanant^b, Atsunobu Sagara^c, Masayuki Miyazaki^{c,d}, Takeshi Nabe^e

^a Laboratory of Immunology & Allergy, College of Pharmacy, Kinjo Gakuin University, 2-1723 Omori, Moriyamaku, Nagoya, Aichi, 463-8521, Japan

^b Department of Pharmacology, Kobe Pharmaceutical University, 4-19-1 Motoyamakita, Higashinada, Kobe, 658-8558, Japan

^c Department of Hospital Pharmacy, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8560, Japan

^d Department of Neuropsychopharmacology, Nagoya University, Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8560, Japan

^e Laboratory of Immunopharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka, 573-0101, Japan

ARTICLE INFO

Keywords:

Dermatophagoides farinae

Atopic dermatitis

CD4⁺ T cell

CD8⁺ T cell

Treg

Mast cell

ABSTRACT

Atopic dermatitis is a chronic inflammatory skin disease associated with CD4⁺ Th2 cell-shifted immune responses. Although the infiltration of skin lesions by CD8⁺ T cells has been recognized, their roles have not been fully defined. In this study, we examined the relationship between CD4⁺ and CD8⁺ cells in antigen-induced skin lesions of mice. BALB/c mice were repeatedly challenged with *Dermatophagoides farinae* (Der f) applied to the right ear nine times. Pre-treatment with anti-CD4 monoclonal antibody (mAb) during the third to sixth challenges, but not the post-treatment during the sixth to ninth challenges, exacerbated the development of Der f-induced ear swelling; pre-treatment with anti-CD25 mAb, which depletes regulatory T cells (Tregs), also exacerbated the lesions. Furthermore, the number of CD8⁺ T cells in lymph nodes was augmented by these pre-treatments. These findings prompted us to examine the effect of anti-CD8 mAb. Pre-treatment with anti-CD8 mAb, but not post-treatment, strongly inhibited the development of Der f-induced ear swelling; additionally, the epidermal hyperplasia and infiltration of mast cells were inhibited by the pre-treatment. Collectively, we revealed that CD8⁺ T cells regulated by CD4⁺CD25⁺ Tregs in the early stage are key contributors to the development of Der f-induced skin lesions via increasing mast cell infiltration, indicating that CD8⁺ T and Tregs could be potential therapeutic targets for atopic dermatitis.

1. Introduction

Atopic dermatitis is a chronic inflammatory disease of the skin characterized by dry skin, itchy skin, and relapsing eczematous skin lesions with a high prevalence (Bieber, 2008; Eichenfield et al., 2012; Leung and Bieber, 2003). In the development of atopic dermatitis, the house dust mite *Dermatophagoides farinae* (Der f) is considered to be one of the major allergens; in up to 75% of patients with atopic dermatitis, the skin prick test is positive (Darsow and Ring, 2000). Histologically, epidermal and dermal thickening is observed, as well as marked dermal infiltration by inflammatory cells (Bieber, 2008). Furthermore, dysfunction of the skin barrier facilitates antigen penetration (Akiyama, 2010; Palmer et al., 2006), leading to allergic skin inflammation based on skin sensitization to the antigen.

Although the underlying mechanism of atopic dermatitis remains

unclear, it is typically characterized by increased CD4⁺ T cells producing Th1 and Th2 cytokines in skin lesions after repeated exposure to a particular antigen (Leung et al., 2004; Liu et al., 2011; Mamessier and Magnan, 2006; Mekori and Metcalfe, 1999; Nakae et al., 2006). Furthermore, CD8⁺ T cells were isolated from the atopic dermatitis skin lesions (Hennino et al., 2007, 2011), revealing that not only CD4⁺ T cells but also CD8⁺ T cells may contribute to the development of atopic dermatitis. However, the detailed roles of CD8⁺ T cells and relationship between CD4⁺ and CD8⁺ T cells in the development of atopic dermatitis skin lesions remain unclear. Meanwhile, although CD4⁺CD25⁺ regulatory T cells (Tregs), which are a subset of thymus-derived CD4⁺ T cells, contribute to controlling the development of autoimmune diseases (Asano et al., 1996; McHugh and Shevach, 2002; Sakaguchi et al., 1995), their role in atopic dermatitis has not been fully defined.

In the present study, we first tried to establish a model of atopic

* Corresponding author.

E-mail address: n-mizutani@kinjo-u.ac.jp (N. Mizutani).

<https://doi.org/10.1016/j.ejphar.2019.172843>

Received 18 June 2019; Received in revised form 25 November 2019; Accepted 2 December 2019

Available online 09 December 2019

0014-2999/ © 2019 Elsevier B.V. All rights reserved.

dermatitis-like skin lesions induced by multiple antigen (Der f) challenges in the ear skin of mice for three weeks. Secondly, we assessed how CD4⁺ cells contribute to the development of Der f-induced skin lesions using neutralizing monoclonal antibodies (mAbs) in early and late stages. Furthermore, the effects of treatment with anti-CD25 mAb and anti-CD8 mAb on the skin lesions were examined. Thus, we investigated the roles of CD4⁺ and CD8⁺ cells in the development of Der f-induced skin lesions in mice.

2. Materials and methods

2.1. Animals

Male 7-week-old BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). These mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. All of the experimental procedures were approved by the Experimental Animal Research Committee at Kobe Pharmaceutical University and Kinjo Gakuin University.

2.2. Induction of dermatitis by Der f

As shown in Fig. 1A, dermatitis was induced by the repeated application of 40-mg ointment containing 20 µg of Mite- Der f (LG2334) (Cosmo Bio LSL, Tokyo, Japan) or ovalbumin (OVA) (grade V; Sigma-Aldrich, St Louis, MO, USA) with a spatula to the right ear on days 0, 1, 2, 7, 8, 9, 14, 15, and 16. Control mice were painted onto the right ear with 40-mg ointment containing PBS. The mouse skin barrier was disrupted by applying 30 µl of 1% SDS to the ear 1 h before Der f or OVA painting. The ear thickness was evaluated on day 0 and each day after allergen application using a dial thickness gauge (model G-1A; OZAKI MFG CO., LTD, Japan).

2.3. Depletion of CD4⁺, CD8⁺, or Tregs

To evaluate the effects of pre-treatment with anti-CD4 monoclonal

antibody (mAb) (clone: GK1.5) and anti-CD8 mAb (clone: 53-6.72) on Der f-induced dermatitis, the dose (150 µg/mouse) of anti-CD4 mAb or anti-CD8 mAb was intraperitoneally administered 30 min before Der f painting on days 2, 7, 8, and 9 (Mizutani et al., 2012). Furthermore, post-treatment with anti-CD4 mAb or anti-CD8 mAb was performed similarly on days 9, 14, 15, and 16. Based on these experiments, the same amount of rat IgG2a or rat IgG2b was used as a control antibody. To evaluate the effect of the depletion of Tregs on Der f-induced dermatitis, the dose (250 µg/mouse) of anti-CD25 mAb (clone: PC61) was intraperitoneally administered 30 min before Der f painting on day 1, as reported previously (Setiady et al., 2010). With this method, the depletion of Tregs was maintained for 2–3 weeks (Setiady et al., 2010). Based on these experiments, the same amount of rat IgG1 was used as a control antibody.

2.4. Detection of CD4⁺ and CD8⁺ T cells by flow cytometry

To evaluate the effect of anti-CD4 mAb or anti-CD8 mAb on the number of CD4⁺ and CD8⁺ T cells, lymphocytes (1×10^6 cells/ml) isolated from the right mandibular lymph nodes (MLNs) on day 18 were used as described previously (Mizutani et al., 2015). Cells were washed twice with 2% heat-inactivated fetal bovine serum (FBS)-phosphate buffered saline (PBS), incubated with the anti-mouse FcγRII/III mAb, washed once with 2% FBS-PBS, and incubated with a PE-Cy5-labelled anti-mouse CD4 mAb (BD Biosciences, San Diego, CA, USA), PE-labelled antimouse CD8 mAb (eBioscience, San Diego, CA, USA), and FITC-labelled anti-mouse CD3 (eBioscience, San Diego, CA, USA). After three washes with 2% FBS-PBS, the cells were fixed with 4% paraformaldehyde. Finally, cells were washed three times with 2% FBS-PBS. Each sample was analyzed using FACSCalibur (BD Biosciences) and CELL QUEST software (version 3.3; BD Biosciences).

The absolute numbers of CD4⁺ or CD8⁺ T cells were calculated by multiplying by the total number of cells and percentages of CD3⁺CD4⁺ or CD3⁺CD8⁺ cells, respectively.

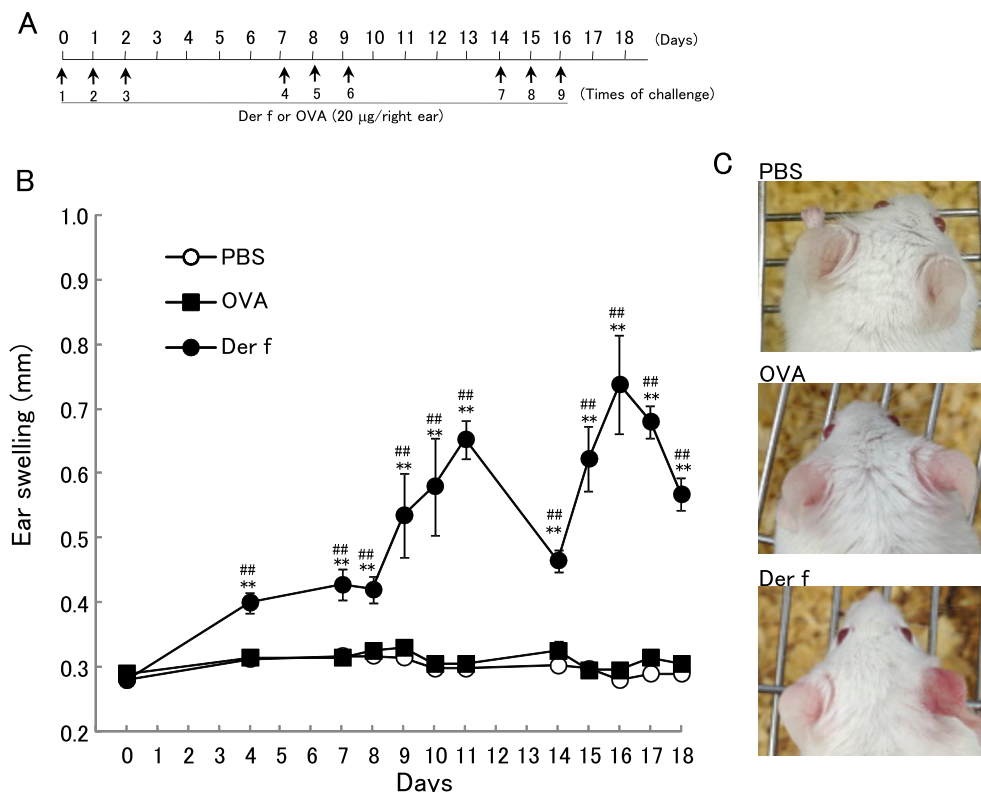


Fig. 1. Multiple Der f challenges induce skin lesions in mice. (A) Experimental protocol for challenge with Der f. (B) Multiple Der f challenges induce ear swelling. (C) Representative clinical picture of Der f-induced skin lesions (day 18). Each value is the mean $\pm$ S.E.M. of 4–6 animals. **P < 0.01; or ##P < 0.01 compared with the PBS or OVA group, respectively.

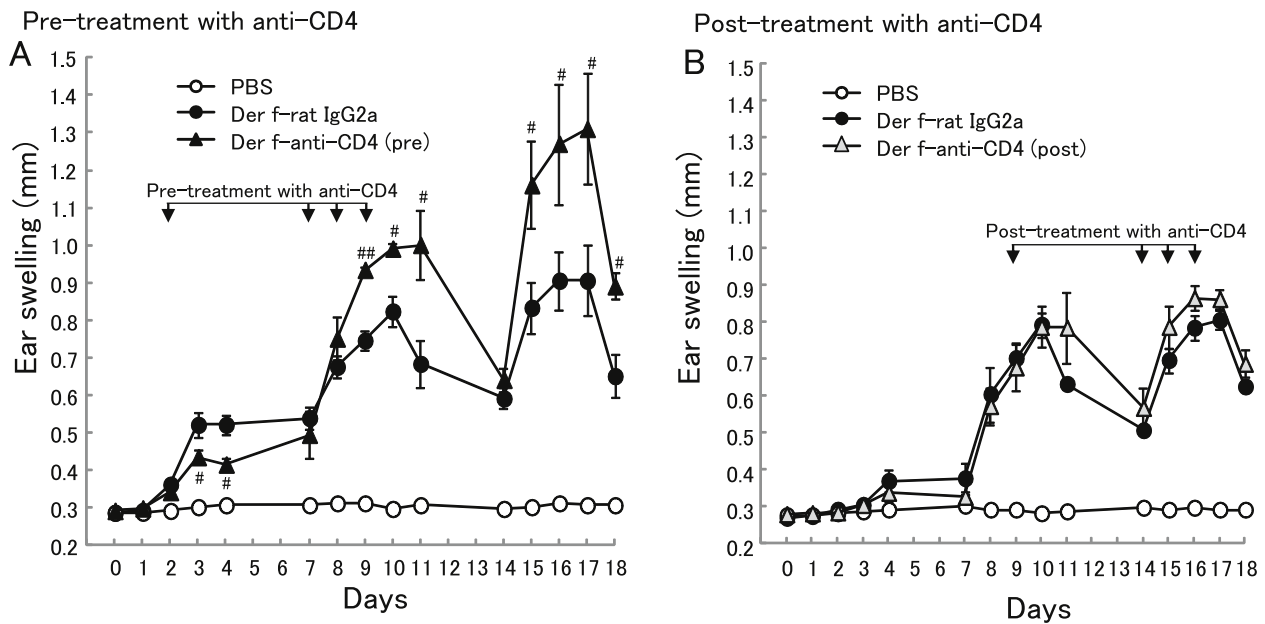


Fig. 2. Effect of treatment with anti-CD4 mAb on the development of Der f-induced skin lesions in mice. (A) Effect of pre-treatment with anti-CD4 mAb during the 3rd to 6th challenges on the development of ear swelling. (B) Effect of post-treatment with anti-CD4 mAb during the 6th to 9th challenges on the development of ear swelling. Each value is the mean $\pm$ S.E.M. of 4–6 animals. * P < 0.05, ** P < 0.01 compared with the Der f-rat IgG2a group.

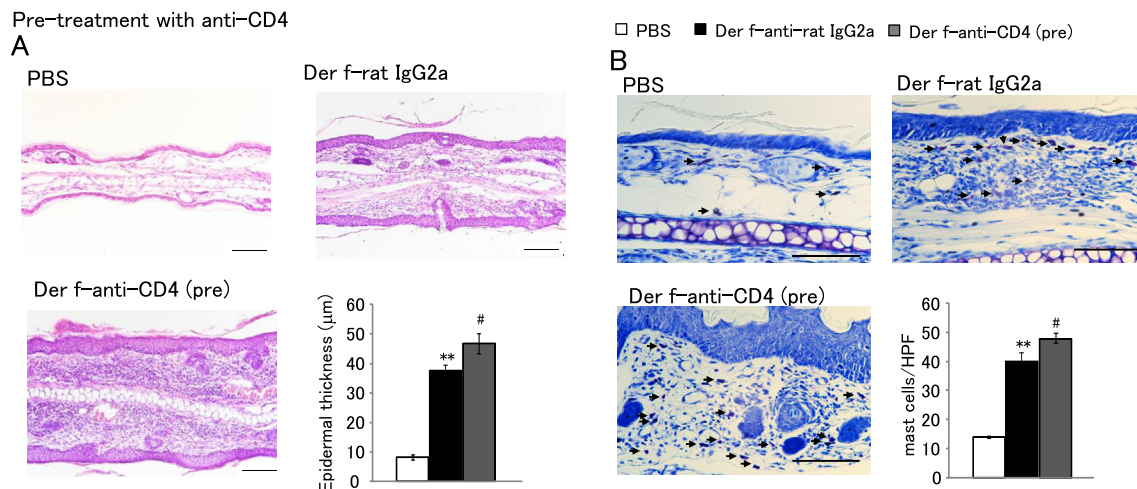


Fig. 3. Effect of pre-treatment with anti-CD4 mAb during the 3rd to 6th challenges on the development of epidermal hyperplasia (A) and mast cell accumulation (B) in the skin of mice. Each value is the mean $\pm$ S.E.M. of 4–6 animals. ** P < 0.01 or # P < 0.05 compared with the PBS or Der f-rat IgG2a group, respectively.

2.5. Histological analysis

The right ear was harvested on day 18, fixed in 10% neutral-buffered formalin for 48 h, embedded in paraffin, and cut into slices 4 μ m thick. Sections were stained with hematoxylin & eosin (H&E) or toluidine blue, as described previously (Mizutani et al., 2015).

Epidermal hyperplasia was quantified in H&E-stained sections by measuring the epidermal thickness from the basal layer to stratum corneum, as described previously (Mizutani et al., 2015). Image acquisition and processing were performed with Nikon Imaging Software NIS Elements D4.30.00 (Nikon Inc., USA). The mean thickness of epidermal layer of at least 6 randomly selected sections was calculated in each group. In terms of the numbers of mast cells (toluidine blue), the mean of at least five randomly selected areas per section in a high-power field (magnification $\times 40$) was counted in each group, as described previously (Mizutani et al., 2015).

2.6. Detection of cytokine production by MLNs

The left and right MLNs were harvested on day 18. In accordance with the previous method, cells (0.5×10^7 cells/ml) isolated from mouse MLNs were incubated with Der f (0–10 μ g/ml) in RPMI-164 medium containing 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin for 72 h at 37 $^{\circ}$ C in a 5% CO_2 atmosphere (Mizutani et al., 2015). The culture supernatants were used for the analysis of cytokine production.

The levels of IL-13, IL-17A, and interferon- γ in the culture supernatants were measured using quantitative colorimetric sandwich ELISA kits (IL-17A and IFN- γ ELISA kits; BioLegend, San Diego, CA, USA, IL-13 ELISA kit: R&D Systems, Minneapolis, MN, USA). The limits of sensitivity for these assays were 2–7 pg/ml (IL-17A), 1–5 pg/ml (IL-13), and 8 pg/ml (IFN- γ). In terms of the detection limits of sensitivity for ELISA, data at lower than these concentrations were considered zero.

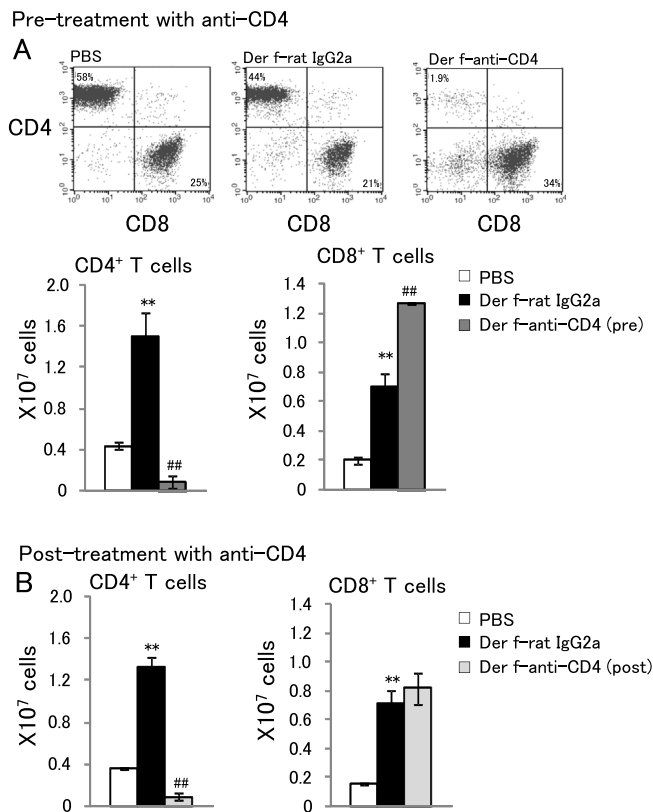


Fig. 4. Effect of treatment with anti-CD4 mAb on the number of CD4⁺ and CD8⁺ T cells. (A) Representative dot plots depicting the percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ in MLNs of PBS, Der f, and anti-CD4 (pre) groups. Effect of pre-treatment with anti-CD4 mAb during the 3rd to 6th challenges on the number of CD4⁺ and CD8⁺ T cells. (B) Effect of post-treatment with anti-CD4 mAb during the 6th to 9th challenges on the number of CD4⁺ and CD8⁺ T cells in MLNs of PBS, Der f, and anti-CD4 (post) groups. Each value is the mean $\pm$ S.E.M. of 4–6 animals. ***p* < 0.01; or ##*p* < 0.01 compared with the PBS or Der f-rat IgG2a group, respectively.

2.7. Statistical analysis

Data are shown as the means $\pm$ S.E.M. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's comparison test. A probability value of *P* < 0.05 was considered significant.

3. Results

3.1. Multiple Der f challenges induce ear swelling

First, we examined whether repeated Der f challenges induce dermatitis in mice. In the present study, we compared two allergen challenge groups: mice treated with Der f or OVA. In Der f-treated mice, significant swelling was observed compared with PBS or OVA-treated mice (Fig. 1B). Furthermore, redness was also observed in Der f-treated mice compared with that in PBS or OVA-treated mice (Fig. 1C). Meanwhile, there were no differences in ear thickness or redness between PBS- and OVA-treated mice (Fig. 1B and C).

3.2. Effect of treatment with anti-CD4 mAb on skin inflammation

Based on the data shown in Fig. 1, we examined the effect of pre-treatment with anti-CD4 mAb during days 2–9 on the development of Der f-induced ear swelling. Pre-treatment with anti-CD4 mAb enhanced the development of ear swelling in the second and third weeks, although post-treatment with anti-CD4 mAb during days 9–16 did not show a change compared with that in the Der f-rat IgG2a group (Fig. 2A

and B). Meanwhile, pre-treatment with anti-CD4 mAb inhibited Der f-induced ear swelling in the first week (Fig. 2A).

Furthermore, multiple Der f challenges induced epidermal hyperplasia and increased the number of mast cells in the skin compared with those in PBS-treated mice. The epidermal hyperplasia and increased number of mast cells were exacerbated by pre-treatment with anti-CD4 mAb (Fig. 3A and B); however, post-treatment with anti-CD4 mAb did not lead to exacerbation compared with the Der f-rat IgG2a group (data not shown).

3.3. Effect of treatment with anti-CD4 mAb on the number of CD4⁺ and CD8⁺ T cells in MLNs

We investigated the effect of pre-treatment with anti-CD4 mAb on the absolute number of CD4⁺ or CD8⁺ T cells in MLNs. Pre-treatment with anti-CD4 mAb inhibited the number of CD4⁺ T cells and enhanced the number of CD8⁺ T cells (Fig. 4A). In addition, the total number of cells in MLNs was $0.76 \pm 0.064 \times 10^7$ cells in the PBS group, $3.57 \pm 0.58 \times 10^7$ cells in the Der f-rat IgG2a group, and $3.29 \pm 0.29 \times 10^7$ cells in the Der f-anti-CD4 (pre) group.

Furthermore, the post-treatment inhibited the number of CD4⁺ T cells, but did not lead to changes in the number of CD8⁺ T cells (Fig. 4B).

3.4. Effect of pre-treatment with anti-CD25 mAb on Der f-induced skin inflammation

Because pre-treatment with anti-CD4 mAb exacerbated the development of Der f-induced skin lesions, we investigated the role of Tregs in Der f-induced skin inflammation using anti-CD25 mAb. Similarly, we found that pre-treatment with anti-CD25 mAb enhanced Der f-induced ear swelling in the second and third weeks; the epidermal hyperplasia and mast cell infiltration were exacerbated (Fig. 5A and C). Additionally, pre-treatment with anti-CD25 mAb enhanced the absolute number of CD8⁺ T cells compared with that of the Der f-rat IgG1 group, but did not show changes in the number of CD4⁺ T cells (Fig. 5B). Meanwhile, pre-treatment with anti-CD25 mAb inhibited Der f-induced ear swelling in the first week (Fig. 5A).

3.5. Effect of treatment with anti-CD8 mAb on Der f-induced skin inflammation

Because pre-treatments with anti-CD4 mAb and anti-CD25 mAb enhanced the number of CD8⁺ T cells associated with the exacerbation of skin lesions, we investigated the role of CD8⁺ T cells in Der f-induced skin inflammation. Pre-treatment with anti-CD8 mAb during days 2–9 strongly inhibited Der f-induced ear swelling during the first to third week; the epidermal hyperplasia and increased number of mast cells in the skin were suppressed by it (Fig. 6A and C).

Meanwhile, post-treatment with anti-CD8 mAb during days 9–16 did not strongly inhibit Der f-induced ear swelling, epidermal hyperplasia, or the increased number of mast cells (Fig. 7). Pre- and post-treatments with anti-CD8 mAb significantly inhibited the absolute number of CD8⁺ T cells, although the number of CD4⁺ T cells was not affected (Figs. 6B and 7B).

3.6. Effect of pre-treatment with anti-CD4 mAb or anti-CD8 mAb on Der f-induced cytokine production in MLNs

The levels of IL-13, IL-17A, and IFN- γ in the right MLNs of the Der f-rat IgG2a or IgG2b group were increased compared with those in the PBS-treated group. Pre-treatment with anti-CD4 mAb inhibited the production of IL-13, IL-17A, and IFN- γ in MLNs (Fig. 8A). Additionally, pre-treatment with anti-CD8 mAb inhibited the production of IFN- γ in MLNs, but not IL-13 or IL-17A (Fig. 8B).

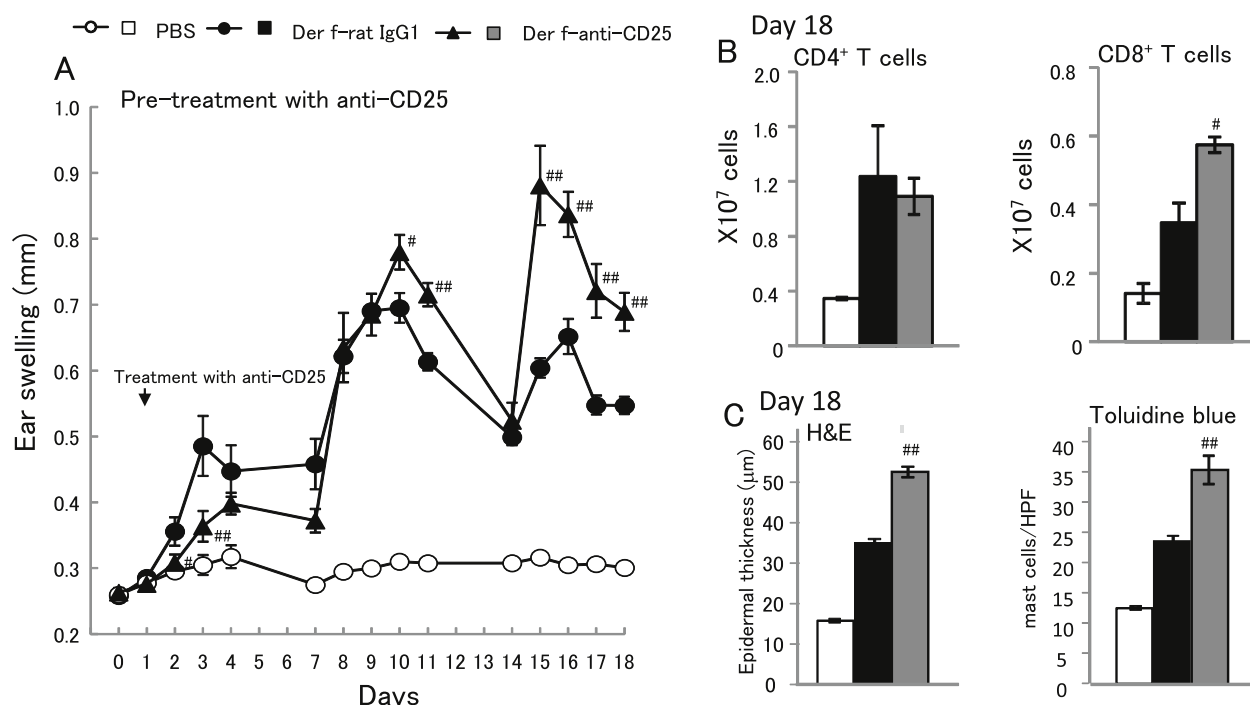


Fig. 5. Effect of pre-treatment with anti-CD25 mAb on day 1 on the development of Der f-induced skin lesions in mice. (A) Effect of pre-treatment with anti-CD25 mAb on the development of ear swelling. (B) Effect of pre-treatment with anti-CD25 mAb on the number of CD4⁺ and CD8⁺ T cells in MLNs of PBS, Der f, and anti-CD25 groups. (C) Effect of pre-treatment with anti-CD25 mAb on the development of epidermal hyperplasia and mast cell accumulation in mice. Each value is the mean $\pm$ S.E.M. of 4–6 animals. #P < 0.05, ##P < 0.01 compared with the Der f-rat IgG1 group.

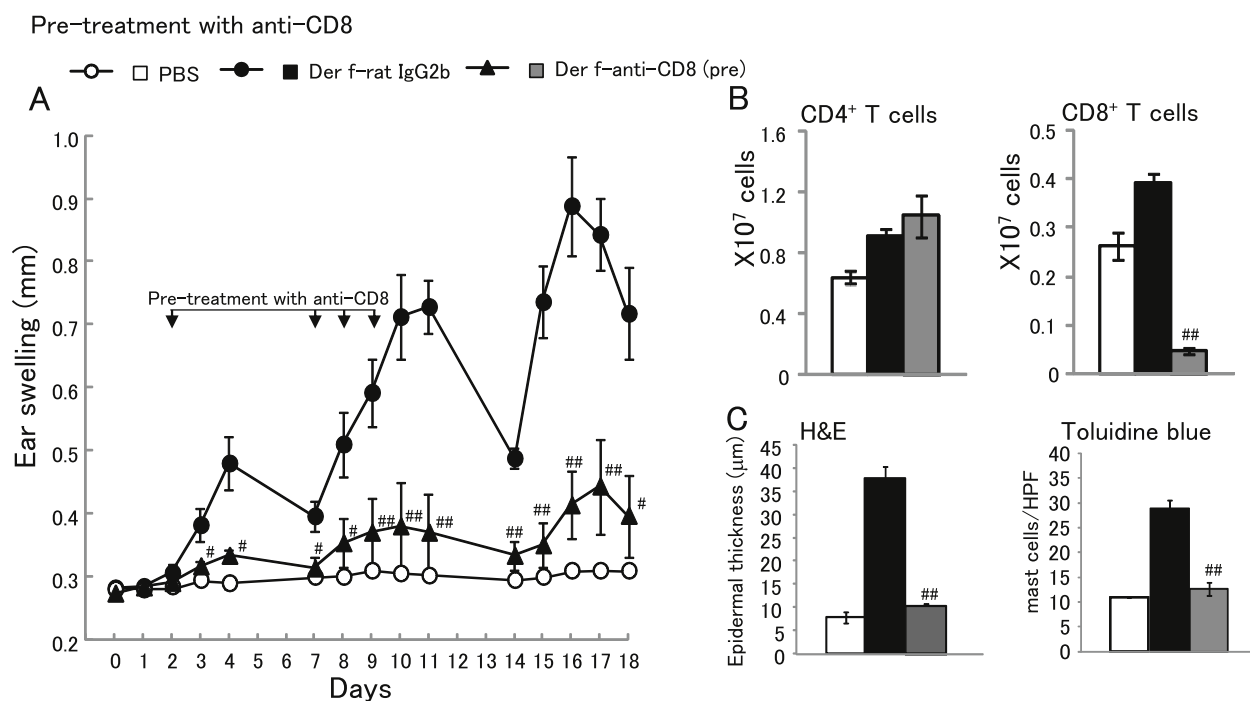


Fig. 6. Effect of pre-treatment with anti-CD8 mAb during the 3rd to 6th challenges on the development of Der f-induced skin lesions in mice. (A) Effect of pre-treatment with anti-CD8 mAb on the development of ear swelling. (B) Effect of pre-treatment with anti-CD8 mAb during the 3rd to 6th challenges on the number of CD4⁺ and CD8⁺ T cells in MLNs of PBS, Der f, and anti-CD8 (pre) groups. (C) Effect of pre-treatment with anti-CD8 mAb on the development of epidermal hyperplasia and mast cell accumulation in mice. Each value is the mean $\pm$ S.E.M. of 4–6 animals. #P < 0.05, ##P < 0.01 compared with the Der f-rat IgG2b group.

4. Discussion

It has been generally accepted that CD4⁺ T cells producing Th2 and Th1 cytokines play an important role in the development of atopic

dermatitis (Bieber, 2008). Furthermore, we reported that IgE-mediated skin lesions were suppressed by treatment with anti-CD4 mAb (Mizutani et al., 2015). Therefore, we examined the effect of treatment with anti-CD4 mAb on Der f-induced skin lesions in the present study.

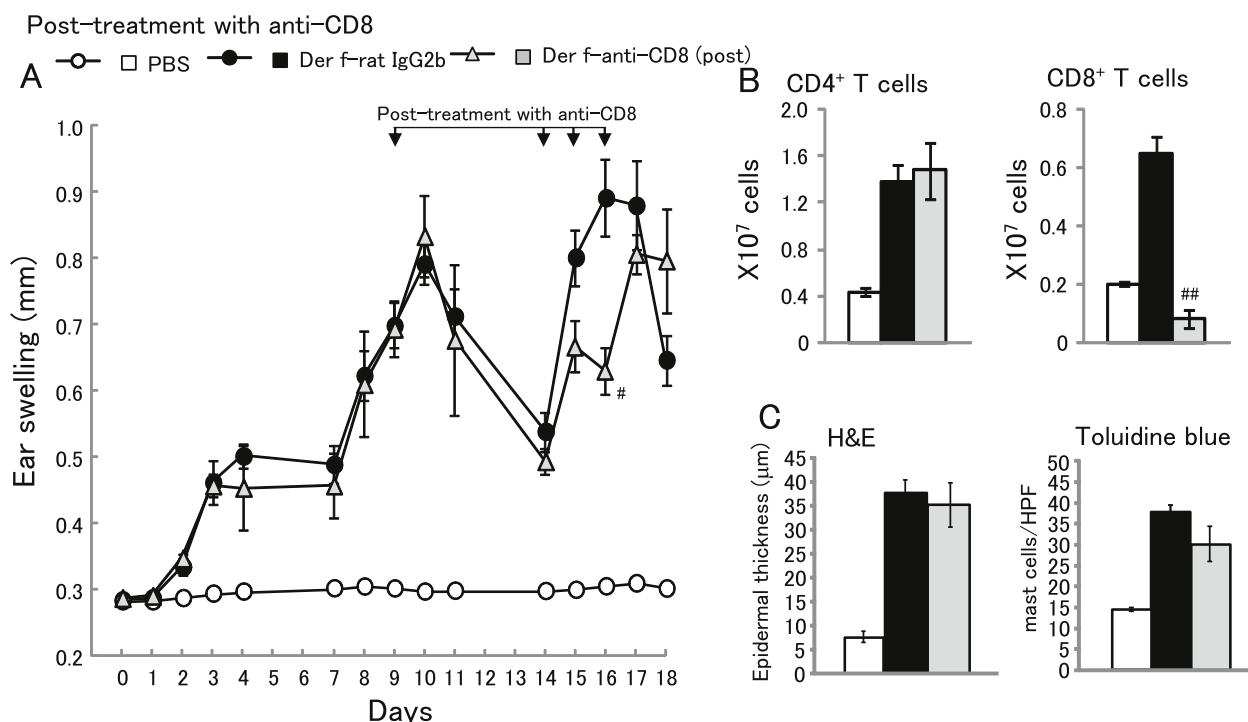


Fig. 7. Effect of post-treatment with anti-CD8 mAb during the 6th to 9th challenges on the development of Der f-induced skin lesions in mice. (A) Effect of post-treatment with anti-CD8 mAb on the development of ear swelling. (B) Effect of post-treatment with anti-CD8 mAb during the 3rd to 6th challenges on the number of CD4⁺ and CD8⁺ T cells in MLNs of PBS, Der f, and anti-CD8 (post) groups. (C) Effect of post-treatment with anti-CD8 mAb on the development of epidermal hyperplasia and mast cell accumulation in mice. Each value is the mean $\pm$ S.E.M. of 4–6 animals. * P < 0.05, ** P < 0.01 compared with the Der f-rat IgG2b group.

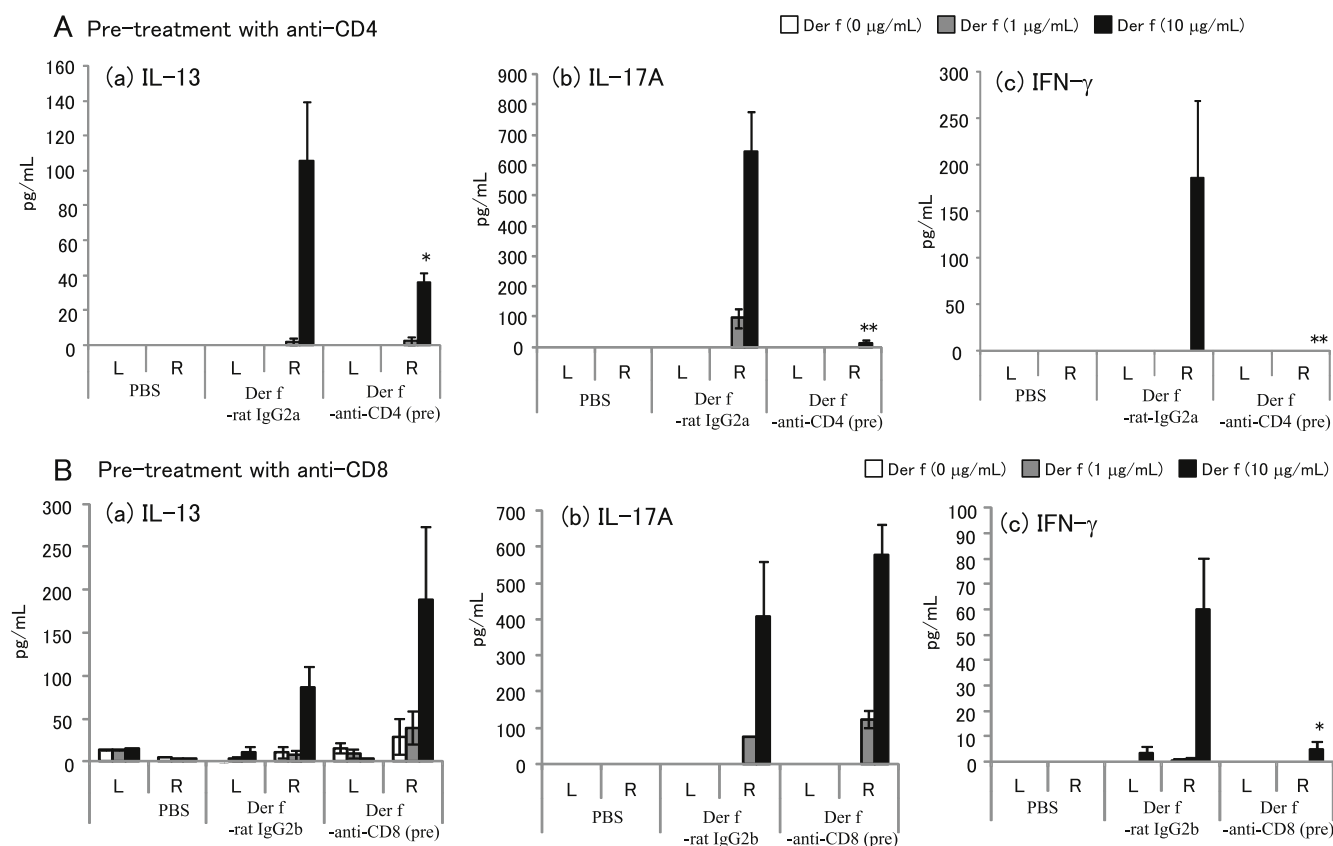


Fig. 8. Effect of pre-treatment with anti-CD4 mAb or anti-CD8 mAb on cytokine production in MLNs. Effects of anti-CD4mAb (A) and anti-CD8 mAb (B) on the production of IL-13 (a), IL-17A (b), and IFN- γ (c). Each value is the mean $\pm$ S.E.M. of 4–6 animals. * P < 0.05, ** P < 0.01 compared with the Der f-rat IgG2a or IgG2b group. L, left MLNs; R, right MLNs.

Pre-treatment with anti-CD4 mAb, but not post-treatment, exacerbated the development of Der f-induced skin lesions in the second and third weeks via increasing the number of CD8⁺ T cells in MLN, although the pre-treatment suppressed the skin lesions in the first week, indicating that there are different mechanisms between the early and late stages in Der f-induced skin lesions. Furthermore, pre-treatment with anti-CD8 mAb, but not post-treatment, suppressed the skin lesions via decreasing the number of mast cells. Based on these data, we concluded that CD8⁺ T cells regulated by CD4⁺ cells in the early stage contributed to the development of Der f-induced skin lesions in the late stage.

Th17 cytokines were increased in patients with atopic dermatitis and Th17 cells, which are CD4⁺ T cells as a cellular source of IL-17A, induced atopic dermatitis (Koga et al., 2008; Ong and Leung, 2006; Toda et al., 2003). Consistent with this, we reported that IgE-mediated skin lesions were inhibited by the depletion of CD4⁺ cells associated with decreased levels of IL-17A (Mizutani et al., 2015) and treatment with anti-IL-17A mAb inhibited the skin lesions, revealing that Th17 cells are important for the development of IgE-mediated dermatitis. However, in the present study, pre-treatment with anti-CD4 mAb exacerbated Der f-induced skin lesions in the second and third weeks, showing that CD4⁺ cells inhibited the lesions. This discrepancy in the data using the two models may be related to the level of antigen-specific IgE because Der f-specific IgE and IgG1 in serum of the Der f-treated group, which were detected by ELISA using plates coated with Der f, did not show increased levels compared with those of the PBS-treated group (data not shown), suggesting that the development of Der f-induced skin lesion may not be associated with IgE.

Meanwhile, pre-treatment with anti-CD4 mAb enhanced Der f-induced dermatitis in the second and third weeks via increasing the number of CD8⁺ T cells in MLNs, suggesting that CD4⁺ cells regulate CD8⁺ T cells. It was reported that Tregs, which are CD4⁺ T cells expressing CD25, acted as suppressor cells (Laidlaw et al., 2016); furthermore, Tregs can modulate the CD8⁺ T-cell response following immunization by suppressing the maturation state of dendritic cells (Laidlaw et al., 2016). Based on these data, we hypothesized that Tregs suppressed the development of Der f-induced dermatitis via decreasing CD8⁺ T cells. Therefore, we examined the effect of anti-CD25 mAb on Der f-induced dermatitis. Consistent with pre-treatment with anti-CD4 mAb, pre-treatment with anti-CD25 mAb enhanced the development of lesions associated with the increased number of CD8⁺ T cells. Thus, the present study suggests that CD4⁺CD25⁺ Tregs contribute to the suppression of Der f-induced dermatitis via suppressing CD8⁺ T cells. Meanwhile, it has been reported that treatment with anti-CD25 mAb inhibits the effector T cells expressing CD25 along with CD4⁺CD25⁺ Tregs (Couper et al., 2009). In the Der f model, the pre-treatment with not only anti-CD4 mAb but also anti-CD25 mAb suppressed the skin lesions in the first week, suggesting that the suppression of ear swelling in first week by treatment with anti-CD25 mAb contributes to the effector T-cell inhibition.

In patients with atopic dermatitis, CD8⁺ T cells infiltrated the skin (Hennino et al., 2007, 2011), showing that CD8⁺ cells are important for the development of the disease. Furthermore, pre-treatment with anti-CD8 mAb, but not post-treatment, strongly inhibited the development of Der f-induced dermatitis, suggesting that CD8⁺ cells in the early stage contributed to the development of Der f-induced skin lesions in the late stage. Additionally, mast cells are best known as effector cells for allergic diseases (Liu et al., 2011; Mekori and Metcalfe, 1999; Nakae et al., 2006). Furthermore, it has been reported that mast cells play a critical role in adoptive immunity, contributing to the process of inflammation via activating various T cells (Dudeck et al., 2011). In the present study, the number of mast cells increased in the skin of Der f-treated mice. Therefore, we investigated the effect of pre-treatment with anti-CD8 mAb or anti-CD4 mAb on the increased number of mast cells. Pre-treatment with anti-CD8 mAb inhibited the increased infiltration by mast cells associated with suppressing Der f-induced skin lesions; furthermore, the exacerbation of Der f-induced skin lesions by

pre-treatment with anti-CD4 mAb or anti-CD25 mAb was dependent on the enhancement of CD8⁺ T cells and mast cell infiltration, suggesting that CD8⁺ T cell-mediated mast cells, which show these responses as effector cells and adoptive immunity, may contribute to the development of Der f-induced skin lesions. Furthermore, it has been reported that mast cells mediated CD8⁺ T cells (Stelekati et al., 2009). Thus, the relationship between CD8⁺ T cells and mast cells may be critical for the exacerbation of skin lesions by repeated antigen challenges.

To investigate the involvement of Th2 (IL-13) and Th17 (IL-17A) cytokine production in the exacerbation of skin lesions in the second and third weeks by pre-treatment with anti-CD4 mAb, we measured the cytokines produced in MLNs using ELISA. As a result, their production was suppressed by pre-treatment with anti-CD4 mAb, although the skin lesions were not improved. Conversely, although pre-treatment with anti-CD8 mAb did not inhibit the production of Th2 and Th17 cytokines, the skin lesions were reduced, indicating that these cytokines did not play an important role in the development of Der f-induced skin lesions. Furthermore, it has been reported that IFN- γ (Th1), with CD8⁺ T cells being a major producer, plays a crucial role in the development of atopic dermatitis (Hennino et al., 2007). In the present study, pre-treatment with anti-CD8 mAb significantly suppressed the increased levels of IFN- γ ; however, pre-treatment with anti-CD4 mAb also inhibited the production, suggesting that the cytokine may not be involved in the development of Der f-induced dermatitis. Meanwhile, pre-treatment with anti-CD4 mAb inhibited the skin lesions in the first week associated with reducing IL-17A production in MLNs; furthermore, pre-treatment with anti-IL-17A mAb reduced the development of Der f-induced skin lesions in the first week (data not shown), suggesting that IL-17A contributes to the skin lesions in the early stage. Thus, there are different mechanisms for the development of Der f-induced skin lesions in the early and late stages.

It has been reported that perforin and granzyme B, which are cytotoxic molecules produced by CD8⁺ T cells, are expressed in the lesional skin of atopic dermatitis patients (Kehren et al., 1999; Yawalkar et al., 2001). Thus, CD8⁺ cell-induced molecules might be associated with the development of Der f-induced dermatitis.

We showed that CD8⁺ T cells and Tregs could be therapeutic targets for atopic dermatitis. Regarding the function of CD8⁺ T cells, it has been reported that histone deacetylase 6 (HDAC6) is highly expressed on CD8⁺ T cells and HDAC6 inhibitors suppressed the contact hypersensitivity of a CD8⁺ T-cell-related skin disease model (Tsuji et al., 2015), indicating that HDAC6 inhibitors, which modulate CD8⁺ T cell activation, might be therapeutic agents for atopic dermatitis associated with CD8⁺ T-cell activation. Meanwhile, it has been reported that the mammalian target of rapamycin (mTOR) inhibitor everolimus enhanced Treg conversion in addition to inhibiting immune responses (Daniel et al., 2010), suggesting that mTOR inhibitors have an inhibitory effect on the development of atopic dermatitis. Thus, CD8⁺ T cells and Tregs may represent new targets in therapeutics for atopic dermatitis.

5. Conclusion

Our findings suggest that CD8⁺ T cells in the early stage contribute to the development of repeated Der f challenge-induced dermatitis via mast cell infiltration of the skin; CD8⁺ T cells are regulated by CD4⁺CD25⁺ Tregs. Thus, CD8⁺ T cells and Tregs may be potential therapeutic targets for atopic dermatitis.

Acknowledgements

Part of this work was supported by JSPS KAKENHI Grant Number 16K08289 (to NM).

References

- Akiyama, M., 2010. FLG mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics. *Br. J. Dermatol.* 162, 472–477.
- Asano, M., Toda, M., Sakaguchi, N., Sakaguchi, S., 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184, 387–396.
- Bieber, T., 2008. Atopic dermatitis. *N. Engl. J. Med.* 358, 1483–1494.
- Couper, K.N., Lanthier, P.A., Perona-Wright, G., Kummer, L.W., Chen, W., Smiley, S.T., Mohrs, M., Johnson, L.L., 2009. Anti-CD25 antibody-mediated depletion of effector T cell populations enhances susceptibility of mice to acute but not chronic *Toxoplasma gondii* infection. *J. Immunol.* 182, 3985–3994.
- Daniel, C., Wennhold, K., Kim, H.J., von Boehmer, H., 2010. Enhancement of antigen-specific Treg vaccination in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16246–16251.
- Darsow, U., Ring, J., 2000. Airborne and dietary allergens in atopic eczema: a comprehensive review of diagnostic tests. *Clin. Exp. Dermatol.* 25, 544–551.
- Dudeck, A., Dudeck, J., Scholten, J., Petzold, A., Surianarayanan, S., Köhler, A., Peschke, K., Vöhringer, D., Waskow, C., Krieg, T., Müller, W., Waisman, A., Hartmann, K., Gunzer, M., Roers, A., 2011. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity* 34, 973–984.
- Eichenfield, L.F., Ellis, C.N., Mancini, A.J., Paller, A.S., Simpson, E.L., 2012. Atopic dermatitis: epidemiology and pathogenesis update. *Semin. Cutan. Med. Surg.* 31, S3–S5.
- Hennino, A., Jean-Decoster, C., Giordano-Labadie, F., Debeer, S., Vanbervliet, B., Rozières, A., Schmitt, A.M., Nicolas, J.F., 2011. CD8⁺ T cells are recruited early to allergen exposure sites in atopy patch test reactions in human atopic dermatitis. *J. Allergy Clin. Immunol.* 127, 1064–1067.
- Hennino, A., Vocanson, M., Toussaint, Y., Rodet, K., Benetiere, J., Schmitt, A.M., Aries, M.F., Berard, F., Rozières, A., Nicolas, J.F., 2007. Skin-infiltrating CD8⁺ T cells initiate atopic dermatitis lesions. *J. Immunol.* 178, 5571–5577.
- Kehren, J., Desvignes, C., Krasteva, M., Ducluzeau, M.T., Assossou, O., Horand, F., Hahne, M., Kagi, D., Kaiserlian, D., Nicolas, J.F., 1999. Cytotoxicity is mandatory for CD8⁺ T cell-mediated contact hypersensitivity. *J. Exp. Med.* 189, 779–786.
- Koga, C., Kabashima, K., Shiraishi, N., Kobayashi, M., Tokura, Y., 2008. Possible pathogenic role of Th17 cells for atopic dermatitis. *J. Investig. Dermatol.* 128, 2625–2630.
- Laidlaw, B.J., Craft, J.E., Kaech, S.M., 2016. The multifaceted role of CD4⁺ T cells in CD8⁺ T cell memory. *Nat. Rev. Immunol.* 16, 102–111.
- Leung, D.Y., Bieber, T., 2003. Atopic dermatitis. *Lancet* 361, 151–160.
- Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I., Hamid, Q.A., 2004. New insights into atopic dermatitis. *J. Clin. Invest.* 113, 651–657.
- Liu, F.T., Goodarzi, H., Chen, H.Y., 2011. IgE, mast cells, and eosinophils in atopic dermatitis. *Clin. Rev. Allergy Immunol.* 41, 298–310.
- Mamessier, E., Magnan, A., 2006. Cytokines in atopic diseases: revisiting the Th2 dogma. *Eur. J. Dermatol.* 16, 103–113.
- McHugh, R.S., Shevach, E.M., 2002. Cutting edge: depletion of CD4⁺CD25⁺ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J. Immunol.* 168, 5979–5983.
- Mekori, Y.A., Metcalfe, D.D., 1999. Mast cell-T cell interactions. *J. Allergy Clin. Immunol.* 104, 517–523.
- Mizutani, N., Goshima, H., Nabe, T., Yoshino, S., 2012. Complement C3a-induced IL-17 plays a critical role in an IgE-mediated late-phase asthmatic response and airway hyperresponsiveness via neutrophilic inflammation in mice. *J. Immunol.* 188, 5694–5705.
- Mizutani, N., Sae-Wong, C., Kongsanant, S., Nabe, T., Yoshino, S., 2015. Thymic stromal lymphopoietin-induced interleukin-17A is involved in the development of IgE-mediated atopic dermatitis-like skin lesions in mice. *Immunology* 146, 568–581.
- Nakae, S., Suto, H., Iikura, M., Kakurai, M., Sedgwick, J.D., Tsai, M., Galli, S.J., 2006. Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. *J. Immunol.* 176, 2238–2248.
- Ong, P.Y., Leung, D.Y., 2006. Immune dysregulation in atopic dermatitis. *Curr. Allergy Asthma Rep.* 6, 384–389.
- Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J., O'Regan, G.M., Watson, R.M., Cecil, J.E., Bale, S.J., Compton, J.G., DiGiovanna, J.J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C.S., El Houate, B., McElreavey, K., Halkjaer, L.B., Bisgaard, H., Mukhopadhyay, S., McLean, W.H., 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat. Genet.* 38, 441–446.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., Toda, M., 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164.
- Setiady, Y.Y., Coccia, J.A., Park, P.U., 2010. In vivo depletion of CD4⁺FOXP3⁺ Treg cells by the PC61 anti-CD25 monoclonal antibody is mediated by FcγRIIIb⁺ phagocytes. *Eur. J. Immunol.* 40, 780–786.
- Stelekati, E., Bahri, R., D'Orlando, O., Orinska, Z., Mittrücker, H.W., Langenhaun, R., Glatzel, M., Bollinger, A., Paus, R., Bulfone-Paus, S., 2009. Mast cell-mediated antigen presentation regulates CD8⁺ T cell effector functions. *Immunity* 31, 665–676.
- Toda, M., Leung, D.Y., Molet, S., Boguniewicz, M., Taha, R., Christodoulouopoulos, P., Fukuda, T., Elias, J.A., Hamid, Q.A., 2003. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. *J. Allergy Clin. Immunol.* 111, 875–881.
- Tsuji, G., Okiyama, N., Villarroel, V.A., Katz, S.I., 2015. Histone deacetylase 6 inhibition impairs effector CD8 T-cell functions during skin inflammation. *J. Allergy Clin. Immunol.* 135, 1228–1239.
- Yawalkar, N., Schmid, S., Braathen, L.R., Pichler, W.J., 2001. Perforin and granzyme B may contribute to skin inflammation in atopic dermatitis and psoriasis. *Br. J. Dermatol.* 144, 1133–1139.