

Article Type: Regular Article

Histamine suppresses regulatory T cells mediated by TGF- β in murine chronic allergic contact dermatitis

Masahiro Seike¹, Tamio Hagiwara¹, Atsushi Sato², Hiroshi Ohtsu²

1 Department of Food and Nutrition Science, Sagami Women's Junior College, Bunkyo,

Minamiku, Sagamihara, Kanagawa, Japan

2 Department of Quantum Science and Energy Engineering, Graduate School of Engineering,

Tohoku University, Aramaki, Aobaku, Sendai, Miyagi, Japan

Correspondence: Masahiro Seike, MD, PhD, Department of Food and Nutrition Science,

Sagami Women's Junior College, 2-1-1 Bunkyo, Minamiku, Sagamihara, Kanagawa 252-0383,

Japan

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/exd.12644

This article is protected by copyright. All rights reserved.

E-mail: m-seike@star.sagami-wu.ac.jp

Tel: +81-42-749-4784 Fax: +81-42-743-4717

Abstract

Regulatory T cells (Tregs) suppress effector T cells and ameliorate contact hypersensitivity (CH); however, the role of Tregs in chronic allergic contact dermatitis (CACD) has not been assessed. Repeated elicitation of CH has been used to produce CACD models in mice. We previously showed that the presence of histamine facilitates the creation of eczematous lesions in this model by using histidine decarboxylase (HDC) (-/-) mice. Therefore, the effects of histamine on Tregs in the CACD model were investigated in the current study. CACD was developed by repeated epicutaneous application of 2,4,6-trinitro-1-chlorobenzene (TNCB) on HDC (+/+) and HDC (-/-) murine skin to assess the effects of histamine in CACD. Histamine aggravated CACD in the murine model and suppressed the number of Tregs in the skin.

Histamine also suppressed the level of TGF- β 1 in this model. Recombinant TGF- β 1 or anti-TGF- β 1 antibody was injected into the dorsal dermis of HDC (+/+) mice daily just before TNCB challenge to determine the effects of histamine-regulated TGF- β on the Treg population in CACD. Recombinant TGF- β 1 injection promoted the infiltration of Tregs in the skin and the

production of IL-10; however, anti-TGF- β 1 antibody injection suppressed the number of Tregs in the skin and the production of IL-10. Histamine suppresses the number of Tregs in CACD, and this effect is mediated by TGF- β .

Key words: chronic allergic contact dermatitis , histamine, regulatory T cell, TGF- β

Introduction

Contact hypersensitivity (CH) reactions are elicited by single epicutaneous applications of a sensitizing agent in mice previously sensitized with the same agent and are associated with the infiltration of T helper cell type 1 (Th1) cells (1, 2). Repeated elicitation of CH leads to the development of chronic allergic contact dermatitis (CACD) and induces shift of the cutaneous cytokine milieu from Th1 cells to T helper cell type 2 (Th2) cells (3). We have shown that histamine facilitates the development of eczematous lesions in murine CACD after repeated hapten challenges (4). Scratching behavior, which is induced in this CACD model, is mainly mediated by histamine (5). In the CACD model, levels of Th2 cytokines in the skin lesion as well as serum levels of IgE increase in the presence of histamine. These levels are especially controlled through the histamine H4 receptor (6). Combined therapy with histamine H1 and H4

receptor antagonists is effective in treating CACD (7, 8). These studies confirm that histamine is an important factor in the development of CACD. However, the involvement of regulatory T cells (Tregs) in the histamine pathway in CACD has not been investigated.

Tregs are a subset of T cells, which regulate effector T cells and lead to immune tolerance to tumor antigens, reduction of allergic reactions, and play a role in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes (9). Loss of Treg function leads to increased levels of autoantibodies and hyperproduction of immunoglobulin E (10). On the other hand, Tregs suppress CH reactions by blocking an influx of effector T cells into the inflamed skin (11). Tregs express the transcription factor Foxp3 and suppress the activation and proliferation of effector T lymphocytes (12). Introducing Foxp3-positive Tregs into animals with established CH results in suppression of the function and infiltration of mast cells and leads to a decreased production of inflammatory cytokines at the areas of allergic contact (13). Tregs also express and require cytotoxic T lymphocyte antigen 4 (CTLA-4) to suppress immune responses by blocking the ability of antigen-presenting cells to activate other cells (14). CTLA-4 deficiency in Tregs results in spontaneous development of systemic lymphoproliferation and hyperproduction of immunoglobulin E in mice (14). Cutaneous immune responses are prolonged by the depletion of endogenous Tregs (15). These findings suggest that Tregs are one of the most important factors for regulation of CH.

This article is protected by copyright. All rights reserved.

In the present study, we examined the effects of histamine on the levels of Tregs and Tregs-induced cytokines in the murine CACD model. We demonstrate that histamine decreases the numbers of Tregs in CACD, and suppresses TGF- β 1 levels. Introducing TGF- β 1-induced Tregs and then applying anti-TGF- β 1 antibody suppresses Tregs in this model, suggesting that histamine's effect on the number of Tregs in the CACD model is mediated through the action of TGF- β 1.

Methods

Animals and agents

All experiments were performed in accordance with the guidelines for the care and use of experimental animals of the Animal Research Committee of Sagami Women's University. Histamine-deficient mice were generated by disrupting the histidine decarboxylase (HDC) gene (16). All experiments were performed using 9- to 12-week-old female C57BL/6 mice otherwise stated. Mice were housed with soft bedding, a 12-h light/dark cycle, and *ad libitum* access to food and water. 2,4,6-Trinitro-1-chlorobenzene (TNCB) was obtained from Sigma (St. Louis, MO, USA) and dissolved in acetone to obtain a 1% solution. Mouse anti-TGF- β 1 antibody and recombinant mouse TGF- β 1 (R&D Systems, Minneapolis, MN, USA) were diluted with 0.1 M

sodium phosphate buffer solution (PBS). For histological assessment, the optimal cutting temperature (OCT) embedding compound was obtained from Sakura FineTek (Tokyo, Japan), anti-Foxp3 antibody was obtained from Medical & Biological Laboratories (Nagaya, Japan), anti-CTLA-4 antibody from Bioss Inc. (Woburn, MA, USA), anti-TGF- β 1 antibody from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), and anti-IL-10 antibody from LifeSpan BioSciences, Inc. (Seattle, WA, USA).

Sensitization and challenge procedure

Mice were sensitized by painting 40 μ l of 1% TNCB solution on the dorsal skin (day 0). Starting on day 7 after the sensitization, mice were challenged daily for 10 days using 20 μ l of 1% TNCB solution on the same part of the skin (day 7-16). The H1 receptor antagonist olopatadine hydrochloride (olopatadine, 10mg/kg/day) or H4 receptor antagonist JNJ7777120 (100mg/kg/day) was orally administered 30 min before each challenge on BALB/c mice (8). On day 17, 24 h after the final challenge, the skin was collected.

Recombinant TGF- β 1 or anti-TGF- β 1 antibody injection

A hundred microliters of recombinant mouse TGF- β 1 (0.25 μ g/ml) or mouse anti-TGF- β 1 antibody (2.5 μ g/ml) solution was injected daily into the dorsal dermis just before TNCB challenge to determine the effects of TGF- β 1 on the number of Tregs in CACD.

Histological assessment

The skin was biopsied and a part of the sample (4 mm in diameter) was fixed in 4% paraformaldehyde in 0.1M PBS (pH 7.4) for 5 h, immersed in 20% sucrose, and embedded in OCT compound. Frozen 5- μ m sections were thoroughly rinsed and stained with hematoxylin-eosin and toluidine blue. Sections were then immunohistochemically stained with anti-Foxp3, anti-CTLA-4, anti-TGF- β 1, or anti-IL-10 antibody. The number of toluidine blue positive cells, Foxp-3 (+) cells or CTLA-4 (+) cells infiltrating the skin was counted.

Measurement of cytokines

A skin sample (approximately 200 mg) was homogenized in 500 μ l PBS solution (0.1 M) containing protease inhibitor (CompleteTM; Roche, Mannheim, Germany) for 2 min using the Microtube Homogenizer (Kenis, Osaka, Japan) and centrifuged at 5000 $\times$ g for 5 min. To measure the concentration of interleukin (IL)-2, 4, and 10 and of TGF- β 1 in the supernatant, mouse ELISA kits (R&D Systems) were used in accordance with the manufacturer's instruction. Optical density at 450 nm was measured for each well in a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Values are presented as means $\pm$ standard error. Statistical significance between two groups was estimated using the two-tailed Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

Effect of histamine on chronic allergic contact dermatitis (CACD) induced by repeated hapten challenge

As observed in our previous study with diphenylcyclopropenone ointment (4), repeated application of TNCB ointment also resulted in scaly erythema. Biopsies showed marked epidermal hyperplasia with intercellular edema and remarkable dermal fibrosis with dense cell infiltration in histidine decarboxylase (HDC) (+/+) mice after repeated treatment (Fig. 1b). On the other hand, moderate epidermal hyperplasia and dermal cell infiltration were observed in HDC (-/-) mice (Fig. 1c). Toluidine blue positive mast cells infiltrated the eczematous lesions of HDC (+/+) mice (Fig. 1e, g, i, j) and HDC (-/-) mice (Fig. 1f, h). The number of mast cells in the lesion of HDC (+/+) mice exceeded those of HDC (-/-) mice, however (Fig. 1k). IL-4 levels

were significantly increased in HDC (+/+) mice compared to HDC (-/-) mice (Fig. 1l).

Histamine in this CACD model positively contributed to changes in the skin lesion.

Effect of histamine on the number of Tregs in CACD

The number of Tregs was assessed by staining for Foxp3 and CTLA-4. Foxp3 is a transcription factor that suppresses the activation and proliferation of effector T lymphocytes through the activity of the cell surface protein, cytotoxic T lymphocyte antigen 4 (CTLA-4) (12). Foxp3 (+) cells were very scarce in control skin, while these cells were more prevalent in the dermis of eczematous lesions in HDC (+/+) and HDC (-/-) mice (Fig. 2a-c, g, h). CTLA-4 (+) cells were scarcely observed in control skin (Fig. 2d). CTLA-4 (+) cells were abundant in the dermis of eczematous lesions in the CACD model (Fig. 2e, f, i, j). CTLA-4 was mostly co-expressed with Foxp3 in cells (Fig. 3k-m). The number of Foxp3 (+) or CTLA-4 (+) cells was significantly higher in HDC (-/-) mice compared to HDC (+/+) mice (Fig. 2n, o). These results suggest that histamine suppresses the number of Tregs in CACD model.

Effect of histamine on IL-10 and TGF- β 1 level in CACD

IL-10 levels were higher in HDC (-/-) mice compared to HDC (+/+) mice (Fig. 2p). Since TGF- β is known to be one of the main regulators of Treg recruitment in allergic lesion (17), we

next investigated the effect of histamine on the level of TGF- β 1 in the CACD model. The level of TGF- β 1 in the skin lesions was significantly higher in HDC (-/-) mice compared to HDC (+/+) mice (Fig. 2q). IL-10 was mostly co-expressed in TGF- β 1-positive cells (Fig. 2r-t). Histamine appeared to suppress the level of IL-10 and TGF- β 1 in the skin.

Influence of TGF- β 1 and anti-TGF- β 1 administration on the number of Tregs and the production of IL-10 in CACD

We injected recombinant mouse TGF- β 1 or mouse anti-TGF- β 1 antibody into the dorsal dermis just before each daily challenge of TNCB to assess the effects of TGF- β 1 on Tregs in CACD. Injection of recombinant mouse TGF- β 1 increased the number of Foxp3 (+) or CTLA-4 (+) cells (Fig. 3a, b). On the contrary, the number of Foxp3 (+) or CTLA-4 (+) cells decreased after injection of mouse anti-TGF- β 1 antibody (Fig. 3a, b). IL-10 levels in the eczematous lesions increased and decreased after injection of recombinant mouse TGF- β 1 and mouse anti-TGF- β 1 antibody, respectively (Fig. 3c). The number of mast cells and IL-4 levels in eczematous lesions were not significantly influenced by the injection of recombinant mouse TGF- β 1 or mouse anti-TGF- β 1 antibody (Fig. 3d, e). IL-2 levels tended to increase after injection of recombinant

mouse TGF- β 1 and significantly decreased after mouse anti-TGF- β 1 antibody injection (Fig. 3f).

Effect of histamine H1 or H4 receptor antagonist on Tregs in CACD

IL-4 levels in eczematous lesions in BALB/c mice were significantly decreased by treatment with histamine H1 or H4 receptor antagonist (Fig. 4a). On the other hand, the number of Foxp3 (+) cells and IL-10 and TGF- β 1 levels were significantly increased by treatment with histamine H1 or H4 antagonist (Fig. 4b-d).

Discussion

Allergic contact dermatitis is one of the common skin diseases. Repeated applications of sensitizing agents have been reported to cause evolution of the lesion into the chronic phase, leading to a model of chronic allergic contact dermatitis (CACD) (18-20). Tregs are essential for the maintenance of immune homeostasis and for the prevention of autoimmunity. Tregs also suppress effector T cells and are reported to ameliorate CH (11, 15, 21). However, the effects of Tregs on CACD have not been investigated.

In HDC (-/-) mice, no plasma extravasation reaction is observed after passive cutaneous anaphylaxis test (22). In contrast to immediate-type responses, CH (delayed-type responses, observed as a thickening of ear skin) shows no difference between HDC (+/+) and HDC (-/-) mice (22). However, using this CACD model in HDC (-/-) mice, we found that histamine aggravates CACD eczematous lesions (4). Histamine appears to be one of the mediators of CACD. Histamine receptor blocker(s) is one of the candidate(s) for the treatment of CACD (7, 8). In the present study, a number of Foxp3 (+) or CTLA-4 (+) cells, which are markers of Tregs, infiltrated CACD eczematous lesions induced by repeated exposure to TNCB (sensitizing agent). Foxp3, along with other transcription factors, upregulates CTLA-4 (9). CTLA-4 is one of the surface proteins of Tregs, which are postulated to suppress effector T-cell proliferation (23). The suppressive activity of Tregs is also supported by the fact that mutant mice with selective deletions of CTLA-4 develop systemic autoimmune disease (14). This body of evidence led us to investigate the involvement of Tregs using a CACD model. We found that histamine controlled the level of Tregs, probably through the action of TGF- β 1. Since CD4+CD25+ naïve T cells directly convert to CD4+CD25+ Tregs with *Foxp3* expression by TGF- β 1 (24), histamine might control Tregs through the conversion of T cells and the infiltration of Tregs to the skin sites.

In the present study, HDC (+/+) mice had a lower number of Foxp3 (+) and CTLA-4 (+) cells and lower levels of IL-10 compared to those in HDC (-/-) mice with CACD. The transcription factor Foxp3 seems to be an important regulator of inflammation because loss-of-function mutations follow an intense multi-organ inflammatory response associated with allergic airway inflammation, striking hyperimmunoglobulinemia E, eosinophilia, and dysregulated Th1 and Th2 cytokine production (25). From such observations, we hypothesize that histamine might lead to the development of CACD by suppressing Tregs. It has been reported that injection of Tregs into animals with established CH suppresses the infiltration and functions of mast cells and leads to decreased production of inflammatory cytokines at the contact site (13). Assuming that the role of Tregs in CH as a reaction suppressor is similar in CACD, an increase of Treg activity appears to suppress, at least in part, the infiltration of mast cells and decrease IL-4 levels in CACD eczematous lesions in HDC (-/-) mice.

TGF- β is reported to increase Tregs through Foxp3 induction (17). Tregs inhibit established CH by suppressing the activity of effector T-cells in mice (11). The suppressing effect of Tregs might be transmitted through the cell surface molecule CTLA-4, because effector T-cell proliferation is suppressed by anti-CTLA-4 treatment in mice (23). In humans, in individuals who are not allergic to nickel, Tregs were able to inhibit effector T cells activations, while allergic individuals were unable to suppress nickel-specific effector T cell activation *in vitro*,

supporting the conclusion that Tregs are involved in allergic contact dermatitis suppression and hapten tolerance (26). However, the effects of TGF- β on Tregs in CACD were not reported until now. In the present study, injection of recombinant TGF- β 1 increased the number of Foxp3 (+) cells and the levels of IL-10 in CACD eczematous lesions. On the contrary, injection of anti-TGF- β 1 antibody decreased them. Since these results indicate that Tregs are induced by TGF- β 1, histamine might suppress the number of Tregs in CACD by decreasing the levels of TGF- β 1. However, we need to further identify the target cell(s) of the action of histamine to understand the mechanism that mediates the interaction between histamine and TGF- β 1.

Tregs induced by injection of recombinant TGF- β 1 did not significantly decrease production of IL-4, which is one of the representative cytokine of Th2 cells, in CACD. Conversely, Tregs suppressed by injection of anti-TGF- β 1 antibody did not significantly increase production of IL-4 in CACD. Taken together, since histamine increased production of IL-4 in this model as a whole, the influence of suppressed Tregs on IL-4 production might be negated by some other mechanism(s) of TGF- β 1 action.

The levels of IL-10 in skin tissue increased after CACD and were exaggerated in HDC (-/-) mice. This cytokine might contribute to the reduction of the skin CACD thickening reaction if

CACD has a pathological mechanism similar to that in bronchial allergic reaction, in which Tregs suppress Th2 cell-driven allergic reactions through IL-10 production (27). IL-2 is essential for TGF- β -mediated induction of Tregs (28). We observed that levels of IL-2 in eczematous lesions tended to increase after injection of recombinant TGF- β 1. Injection of anti-TGF- β 1 antibody decreased the level of IL-2 in eczematous lesions. Therefore, TGF- β 1 appears to positively control the production of IL-2 and both cytokines might cooperatively induce Tregs to suppress the effector T cells in CACD.

In this study, we found that Tregs are important regulators in CACD and that histamine is one of the mediators controlling Tregs in a CACD reaction. We also report that TGF- β 1 is controlled by histamine, which influences the CACD reaction through Tregs. Histamine has been shown to be an important mediator of the effector site in allergic reaction; however, here again our data support the fact that its action is not limited to the effector site, but is modulated more upstream. Histamine suppresses Tregs in murine CACD presumably through histamine H1 and H4 receptors by inducing Tregs in the present study. Further studies are warranted to clarify the signaling mechanism underlying the effect of histamine action on the induction of Tregs in CACD.

Acknowledgements

Masahiro Seike and Tamio Hagiwara performed the research. Masahiro Seike, Tamio Hagiwara, Atsushi Sato and Hiroshi Ohtsu designed the research study and analysed the data. Masahiro Seike and Hiroshi Ohtsu wrote the paper. We thank Kyowa Hakko Kirin for providing olopatadine and Johnson & Johnson Pharmaceutical Research & Development for donating JNJ7777120. This study was supported by grants from Sagami Women's University.

Conflict of interest

The authors have declared no conflicting interests.

References

1. Park JM, Je JH, Wu WH *et al.* Attenuation of contact hypersensitivity by cell-permeable heat shock protein 70 in BALB/c mouse model. *Exp Dermatol* 2012; **21**: 969-971.
2. Nakajima S, Honda T, Sakata D *et al.* Prostaglandin I₂-IP signaling promotes Th1 differentiation in a mouse model of contact hypersensitivity. *J Immunol* 2010; **184**: 5595-5603.

3. Kitagaki H, Ono N, Hayakawa K *et al.* Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a T helper cell type 2 profile. *J Immunol* 1997; **159**: 2484-2491.
4. Seike M, Takata T, Ikeda M *et al.* Histamine helps development of eczematous lesions in experimental contact dermatitis in mice. *Arch Dermatol Res* 2005; **297**: 68-74.
5. Seike M, Ikeda M, Kodama H *et al.* Inhibition of scratching behaviour caused by contact dermatitis in histidine decarboxylase gene knockout mice. *Exp Dermatol* 2005; **14**: 169-175.
6. Seike M, Furuya K, Omura M *et al.* Histamine H4 receptor antagonist ameliorates chronic allergic contact dermatitis induced by repeated challenge. *Allergy* 2010; **65**: 319-326.
7. Ohsawa Y, Hirasawa N. The antagonism of histamine H1 and H4 receptors ameliorates chronic allergic dermatitis via anti-pruritic and anti-inflammatory effects in NC/Nga mice. *Allergy* 2012; **67**: 1014-1022.
8. Matsushita A, Seike M, Okawa H *et al.* Advantages of histamine H4 receptor antagonist usage with H1 receptor antagonist for the treatment of murine allergic contact dermatitis. *Exp Dermatol* 2012; **21**: 714-715.

9. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057-1061.
10. Wing JB, Sakaguchi S. Foxp3+ T(reg) cells in humoral immunity. *Int Immunol* 2014; **26**: 61-69.
11. Ring S, Schäfer SC, Mahnke K *et al.* CD4+D25+ regulatory T cells suppress contact hypersensitivity reactions by blocking influx of effector T cells into inflamed tissue. *Eur J Immunol* 2006; **36**: 2981-2992.
12. Sakaguchi S, Wing K, Yamaguchi T. Dynamic of peripheral tolerance and immune regulation mediated by Treg. *Eur J Immunol* 2009; **39**: 2331-2336.
13. Su W, Fan H, Chen M *et al.* Induced CD4+ forkhead box protein-positive T cells inhibit mast cell function and established contact hypersensitivity through TGF- β 1. *J Allergy Clin Immunol* 2012; **130**: 444-452.
14. Wing K, Onishi Y, Prieto-Martin P *et al.* CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 2008; **322**: 271-275.

15. Tomura M, Honda T, Tanizaki H *et al.* Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. *J Clin Invest* 2010; **120**: 883-893.
16. Ohtsu H, Tanaka S, Terui T *et al.* Mice lacking histidine decarboxylase exhibit abnormal mast cells. *FEBS Lett* 2001; **502**: 53-56.
17. Fantini MC, Becker C, Monteleone G *et al.* Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004; **172**: 5149-5153.
18. Wang G, Savinko T, Wolff H *et al.* Repeated epicutaneous exposures to ovalbumin progressively induce atopic dermatitis-like lesions in mice. *Clin Exp Allergy* 2007; **37**: 151-161.
19. Tamura T, Matsubara M, Takada C. Effects of olopatadine hydrochloride, an antihistamine drug, on skin inflammation induced by repeated topical application of oxazolone in mice. *Br J Dermatol* 2004; **151**: 1133-1142.
20. Wang LF, Lin JY, Hsieh KH *et al.* Epicutaneous exposure of protein antigen induces a predominant Th2-like response with high IgE production in mice. *J Immunol* 1996; **156**: 4077-4082.

21. Honda T, Otsuka A, Tanizaki H *et al.* Enhanced murine contact hypersensitivity by depletion of endogenous regulatory T cells in the sensitization phase. *J Dermatol Sci* 2011; **61**: 144-147.
22. Ohtsu H, Kuramasu A, Tanaka S *et al.* Plasma extravasation induced by dietary supplemented histamine in histamine-free mice. *Eur J Immunol* 2002; **32**: 1698-1708.
23. Thorton AM, Piccirillo CA, Shevach EM. Activation requirements for the induction of CD4+CD25+ T cell suppressor function. *Eur J Immunol* 2004; **34**: 366-376.
24. Chen W, Jin W, Hardegen N *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875-1886.
25. Lin W, Truong N, Grossman WJ *et al.* Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. *J Allergy Clin Immunol* 2005; **116**: 1106-1115.
26. Cavani A, Nasorri F, Ottaviani C *et al.* Human CD25+ regulatory T cells maintain immune tolerance to nickel in healthy, nonallergic individuals. *J Immunol* 2003; **171**: 5760-5768.

27. Kearley J, Barker JE, Robinson DS *et al.* Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* 2005; **202**: 1539-1547.
28. Davidson TS, DiPaolo RJ, Andersson J *et al.* Cutting edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol* 2007; **178**: 4022-4026.

Figure legends

Figure 1. Phenotypic changes in the skin lesions of HDC (+/+) and HDC (-/-) CACD model mice

Mice were sensitized using TNCB. CACD was induced by daily challenge of TNCB on the skin for 10 days (day 7-16) starting from day 7 after sensitization. Skin biopsies were performed a day after these repeated challenges (day 17). (a-c) Hematoxyline and eosin staining. (d-j) Toluidine blue staining. (a, d) TNCB-untreated HDC (+/+) mice. (b, e) TNCB-treated HDC (+/+) mice. (c, f) TNCB-treated HDC (-/-) mice. (g, h) Sectional enlargement of the quadrangle of (e, f), respectively. Arrows are toluidine blue positive cells. (i, j) High magnification images of toluidine blue positive cells of HDC (+/+) CACD model mice. (k) Number of toluidine blue

positive mast cells. (l) Levels of IL-4 in the skin. Description of the abscissa in each graph:

HDC; histidine decarboxylase, TNCB; 2,4,6-trinitro-1-chlorobenzene. Data are expressed as mean $\pm$ SEM (n = 6). *P<0.05, **P<0.01.

Figure 2. Tregs in HDC (+/+) and HDC (-/-) CACD model mice

CACD was induced in the same procedure as described in Fig. 1. (a-c) Foxp3 staining. (d-f) CTLA-4 staining. (a, d) TNCB-untreated HDC (+/+) mice. (b, e) TNCB-treated HDC (+/+) mice. (c, f) TNCB-treated HDC (-/-) mice. Arrows indicate Foxp3 (+) (b, c) and CTLA-4 (+) (e, f) cells. (g, h, i, j) Sectional enlargement of the quadrangle of (b, c, e, f), respectively. (k) Foxp3 staining. (l) CTLA-4 staining. (m) Merged image. (n, o) Total number of Foxp3 (+) and CTLA-4 (+) cells in the section. Levels of IL-10 (p) and TGF- β 1 (q) in the skin in HDC (+/+) and HDC (-/-) mice. Description of the abscissa in each graph: HDC; histidine decarboxylase, TNCB; 2,4,6-trinitro-1-chlorobenzene. Data are expressed as mean $\pm$ SEM (n = 6). *P<0.05, **P<0.01. (r) IL-10 staining. (s) TGF- β 1 staining. (t) Merged image.

Figure 3. Assessment of TGF- β 1 activity in the CACD model

Recombinant TGF- β 1 or anti-TGF- β 1 antibody was injected into the dorsal skin of C57BL/6 mice every day during repeated challenges just before the TNCB challenge. The number of Foxp3 (+) (a), CTLA-4 (+) (b), and mast cells (d). The levels of IL-10 (c), IL-4 (e), and IL-2 (f)

in the skin. Description of the abscissa in each graph: Control; TNCB-untreated and PBS injected, TNCB+Veh; TNCB-treated and PBS injected, TNCB+TGF- β 1; TNCB-treated and recombinant TGF- β 1 injected, and TNCB+anti-TGF- β 1; TNCB-treated and anti-TGF- β 1 antibody injected mice. Data are expressed as mean $\pm$ SEM (n = 8). *P<0.05, **P<0.01, n.s., not significant.

Figure 4. Effect of H1 and H4 receptor antagonist on Treg induction in the CACD model

Histamine H1 or H4 receptor antagonist was orally administrated every day during repeated challenges just before the TNCB challenge. The levels of IL-4 (a), TGF- β 1 (c) and IL-10 (d) in the skin. (b) The number of Foxp3 (+) cells. Description of the abscissa in each graph: Control; TNCB-untreated and solvent administrated, TNCB+Veh; TNCB-treated and solvent administrated, TNCB+Olo; TNCB-treated and olopatadine administrated, and TNCB+JNJ; TNCB-treated and JNJ777120 administrated mice. Data are expressed as mean $\pm$ SEM (n = 8). *P<0.05, **P<0.01.





