

Received Date : 28-Dec-2015

Revised Date : 25-May-2016

Accepted Date : 18-Jun-2016

Article type : Original Article: Experimental Allergy and Immunology

Oral tolerance inhibits atopic dermatitis-like type 2 inflammation in mice by modulating immune microenvironments

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Running title: Inhibition of atopic inflammation by oral tolerance

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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/all.12960

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Abstract

Background: Oral tolerance is immune unresponsiveness induced by oral administration of innocuous antigens. Oral administration of allergens has been shown to be effective for suppressing IgE production in allergic responses. However, whether oral tolerance has a role in protection from allergic skin inflammation has not been fully investigated. Here, we evaluated the potential protective role of oral tolerance in a murine model of atopic dermatitis (AD) and investigated the underlying immunologic mechanisms.

Methods: Mice were fed with ovalbumin (OVA) in drinking water then epicutaneously sensitized by repeated application of OVA to tape-stripped skin. Skin biopsies were analyzed for immunohistopathologic features. Levels of antibodies in sera and intestinal washes were measured by ELISA. Flow cytometry and real-time PCR analysis of the skin and mesenteric lymph nodes (MLN) were performed to investigate the immunologic effects of oral tolerance in epicutaneous (EC) sensitization-induced allergic responses.

Results: Induction of oral tolerance effectively inhibited inflammatory responses provoked by EC sensitization. Tolerogenic immune mediators were significantly increased in the skin and MLN of EC-sensitized mice following induction of oral tolerance. A marked increase in *Il5* and *Il13* expression and infiltration of eosinophils and type 2 innate lymphoid cells (ILC2) in the skin of EC-sensitized mice were significantly inhibited by oral tolerance.

Conclusions: Oral tolerance plays a protective role in the development of AD in a murine model by modulating immune microenvironments to be more favorable for immune regulation. This modulation involves inhibition of ILC2 infiltration in skin lesions.

Key words: atopic dermatitis, eosinophil, epicutaneous sensitization, oral tolerance, type 2 innate lymphoid cells

Abbreviations

AD: atopic dermatitis

ILC2: type 2 innate lymphoid cells

EC: epicutaneous

OVA: ovalbumin

MBP: major basic protein

MLN: mesenteric lymph node

TSLP: thymic stromal lymphopoietin

Introduction

Atopic dermatitis (AD) is a major allergic disorder of the skin that often begins in infancy and frequently occurs in subjects with a history of atopic disease (1). AD is characterized by chronic dermatitis with severe, long-lasting itching that is accompanied by pathologic histological changes, such as epidermal hyperplasia and immune cell infiltration of the dermis (2). Clinically, eosinophils accumulate in skin lesions of AD patients, often with elevated IgE in serum, suggesting a pathologic Th2-type immune response that contributes to the development of disease (3). Although CD4⁺ Th2 cells are considered producers of Th2 cytokines in AD, type 2 innate lymphoid cells (ILC2) have recently been suggested as important players in the disease based on their ability to produce IL-5 and IL-13 (4, 5). Defective skin barrier function is also a predisposing factor for AD pathogenesis, as indicated by the association of AD with a mutation in the filaggrin gene, which encodes a structural protein expressed in keratinocytes (6). Food and inhaled allergens have been shown to elicit atopic skin lesions, and epicutaneous (EC) application of allergens to the normal skin of sensitized AD patients elicits eczematous reactions (7, 8). Based on the observations that defective epithelial barrier function and EC exposure to allergens play roles in AD, a murine

model of AD, elicited by repeated EC exposures of tape-stripped skin to ovalbumin (OVA), has been established (9, 10).

Immunologic tolerance is a mechanism by which the immune system prevents pathologic reactivity to self-antigens or innocuous foreign antigens (11). Therefore, induction and maintenance of innocuous antigen-specific unresponsiveness is an effective way to prevent a detrimental immune response or to cure immune disorders including allergic diseases (12). Oral tolerance is the state of local and systemic immune unresponsiveness that is induced by oral administration of innocuous antigens such as food proteins (13). Therefore, breakdown of oral tolerance might disrupt immunosuppressive mechanisms, and, accordingly, atopic children are less likely to develop oral tolerance to ingested foods (14, 15). Defective oral tolerance has also been reported in NC/Nga mice, which spontaneously develop dermatitis-like skin lesions (16). Importantly, the skin and intestine share anatomical properties, and these tissues are the first lines of defense in exposure to the external environment. However, whether oral tolerance has a role in protection from allergic skin inflammation has not been fully investigated.

In the present study, using an EC-sensitized murine AD model, we investigated the role of oral tolerance in protection from allergic skin inflammation and the immunologic mechanisms underlying this protection. We found that oral tolerance protects against antigen-induced cutaneous allergic inflammation by modulating local microenvironments to be more favorable for immune regulation. We propose that this modulation involves inhibition of ILC2 infiltration.

Materials and Methods

Mice

Six- to eight-week-old female BALB/c wild-type mice (Orientbio, Gapyeong, Korea) were housed in standard laboratory conditions of temperature and humidity at Gachon University. All of the experiments complied with the institutional guidelines for animal welfare.

Oral tolerance induction and EC sensitization

Oral tolerance to OVA (Sigma-Aldrich, St. Louis, MO, USA) was induced as previously reported (17). Mice were provided 1% OVA in drinking water *ad libitum* for five days, while a control group was given normal drinking water. Two days after the oral treatment, EC sensitization of mice was performed as described previously (10). Briefly, the dorsal skin of anesthetized mice was shaved and tape stripped. OVA (100 µg in 100 µl of normal saline) or placebo (100 µl of normal saline) was placed on a 1cm² sterile gauze patch. Each mouse had a total of three 1-week exposures to OVA, separated by 2-week rest intervals. The mice were sacrificed on day 50, for blood collection, intestinal washes, and tissue harvest.

Histologic analysis

Tissue specimens were obtained from patched skin. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Multiple 4-µm sections were stained with hematoxylin and eosin and analyzed by bright field microscopy. To determine eosinophil infiltration in the skin, sections were stained with rat anti-mouse major basic protein (MBP) monoclonal antibody (mAb) (provided by Dr. James J. Lee; Mayo Clinic, Scottsdale, AZ, USA) overnight at 4°C, followed by biotinylated anti-rat IgG secondary antibody. The sections were then incubated with streptavidin-conjugated alkaline phosphatase (Vector Laboratories,

Burlingame, CA, USA) and developed with permanent red substrate (Dako, Glostrup, Denmark).

ELISA experiment

For measurement of OVA-specific antibody levels, plates were coated with OVA (50 µg/mL) overnight at 4°C and then blocked with PBS/BSA. Serially diluted sera or intestinal washes were incubated with biotinylated anti-mouse IgE (R35-118, BD Bioscience, San Diego, CA, USA), IgG1 (A85-1, BD Bioscience) or IgG2a (R19-15, BD Bioscience). After adding streptavidin-HRP (BD Bioscience), the plates were developed with TMB substrate (KPL, Gaithersburg, MD, USA) and absorbances were read at 450 nm.

Preparation of cell suspensions from skin and mesenteric lymph node (MLN)

Skin sheets were incubated with 0.2% collagenase D (Roche, Mannheim, Germany) and 10 µg/mL DNase I (Roche) in RPMI 1640/10% FCS with continuous stirring at 37°C for 1 hr. EDTA was added (10 mM, final concentration), and cell suspensions were incubated for an additional 5 min at 37°C. After washing, the cells were subjected to density-gradient centrifugation in 40%/75% Percoll. The cells harvested from the interface were washed and used in assays. Single-cell suspensions from MLN were prepared by pressing harvested tissues through a 100-µm nylon mesh.

Flow cytometric analysis

To characterize the cell surface phenotype, cells isolated from the skin and MLN were resuspended in FACS buffer (PBS containing 10% FCS, 20 mM HEPES, and 10 mM EDTA). After Fc receptor blocking with anti-mouse CD16/CD32 (2.4G2, BD Biosciences) for 15 min

at 4°C, the cells were stained for 30 min at 4°C with the following antibodies: mAb against CD103 (M290), CD11c (HL3), SiglecF (E50-2440), and Sca-1, purchased from BD Biosciences; anti-MHC II (M5/114.15.2), CD4 (RM4-5), IgE (23G3), and ST2 (RMST2-2) mAb, purchased from eBioscience (San Diego, CA, USA); CD45 (30-F11), CD3 (145-2C11), c-Kit (ACK2), Gr-1 (RB6-8C5), CD11b (M1/70), and B220 (RA3-6B2) mAb, obtained from BioLegend (San Diego, CA, USA); and anti-CCR3 (83101) mAb, obtained from R&D Systems (Minneapolis, MN, USA). FOXP3 (FJK-16s, eBioscience) staining was performed with a Transcription Factor Buffer Set (BD Bioscience) according to the manufacturer's instructions. To examine intracellular IL-5, cells were fixed and permeabilized using a Cytofix/Cytoperm Kit (BD Biosciences), then stained with anti-mouse IL-5 (TRFK5, BD Biosciences). Each sample was analyzed with a FACSCalibur (BD Biosciences), and the data were processed using FlowJo software (Tree Star, Ashland, OR, USA).

Real-time PCR

RNA from the skin and MLN was extracted using QIAzol lysis reagent (Qiagen, Hilden, Germany) and subsequently column-purified with an RNeasy Mini Kit (Qiagen). RNA (500 ng) was treated with DNase I (New England Biolabs, Ipswich, MA, USA), and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed using an iQ SYBR Green Supermix (Bio-Rad), with a CFX96 Real-Time System (Bio-Rad). Sequences of primers are shown in Table S1.

Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM). When necessary, a two-group comparison was performed using a student's *t*-test. A *P* value < 0.05 was considered statistically significant.

Results

Oral tolerance to OVA decreases skin inflammation and OVA-specific immunoglobulin levels in EC-sensitized mice

To examine whether the development of atopic skin inflammation was altered following oral tolerance induction, mice were fed 1% OVA in drinking water prior to EC sensitization. As shown in Fig. 1B, orally tolerized mice (OT-EC) were protected from inflammatory skin changes of EC-sensitized mice (Ctl-EC), such as papillomatosis, epidermal hyperplasia, and marked dermal infiltration. OT-EC mice also showed a significant decrease in cells isolated from EC-sensitized skin compared to those isolated from mice in the Ctl-EC group (Fig. 1C). In support of these findings, OVA-specific IgE, IgG1, and IgG2a were markedly increased in a time-dependent manner in serum following EC sensitization, and were significantly decreased in sera of OT-EC mice (Fig. 1D,S1). As for intestinal production of OVA-specific antibodies, anti-OVA IgG1 and IgG2a levels were significantly decreased in OT-EC mice relative to levels in the Ctl-EC group (Fig. 1E). OVA-specific intestinal IgE levels were lower in OT-EC mice than in the Ctl-EC group, although no statistically significant difference was observed (Fig. 1E, $P = 0.1171$). Taken together, our observations indicate that induction of oral tolerance effectively inhibits cutaneous, intestinal, and systemic inflammatory responses provoked by EC sensitization.

Regulatory T cell frequency is increased in the MLN of EC-sensitized mice by the induction of oral tolerance

A number of different mechanisms have been implicated in oral tolerance, including clonal deletion or anergy and suppression by regulatory T cells (18, 19) and feeding regimens determine the dominant way of tolerance induction. Multiple low doses of antigens induce T cell-mediated suppression, while a single high dose of antigen favors clonal deletion/anergy

(20). Therefore, we examined the frequency of regulatory T cells in the MLN, where innocuous, orally administered antigens that have been recognized by CD103⁺ dendritic cells in the small intestine are transported and induce expansion of regulatory T cells (21). The frequency of CD103⁺ dendritic cells in the MLN was not affected by either oral tolerance or EC sensitization, implying comparable transport of oral antigen to the MLN among the analyzed groups (Fig. 2A,S2A). However, EC sensitization without OVA feeding resulted in a significant decrease in CD4⁺FOXP3⁺ regulatory T cells (Fig. 2B,S2) and the expression of *Foxp3* in MLN (Fig. 2C); these cells were restored in orally tolerized EC mice (Fig. 2B,S2). Signaling through the γ -chain receptor, primarily mediated by IL-2, and TGF- β signaling are critical for differentiation or expansion of regulatory T cells (22) and we hypothesized that MLN microenvironments that support the expansion of regulatory T cells might be affected by oral tolerance induction. Consistent with this idea, we observed significantly increased *Tgfb1* and *Il2* in the MLN of oral tolerized EC mice (Fig. 2C).

Oral tolerance decreases *Il5* and *Il13* and increases *Tgfb1* in the skin of EC-sensitized mice

The types of antigen and routes of administration critically influence an immune response, and EC sensitization has been reported to induce a Th2-polarized immune response in the skin (10, 23). Therefore, we analyzed the expression levels of innate, Th1-, and Th2-type cytokines in the skin of EC-sensitized mice and checked whether oral tolerance had an influence on the expression of these cytokines. Among the classical Th2-type cytokines examined, including *Il4*, *Il5*, *Il9*, *Il10*, and *Il13*, expression levels of *Il5* and *Il13* were markedly increased in Ctl-EC mice relative to levels in the Ctl-saline group (Fig. 3). However, upregulation of *Il5* and *Il13* following EC sensitization was significantly inhibited in OT-EC mice (Fig. 3). Additionally, expression of *Tgfb1* and *Foxp3* in OT-EC mice was significantly

increased compared to that in the Ctl-EC group. The expression levels of innate cytokines (*Il1b* and *Tnfa*) and Th1-type *Ifng* were not significantly different between any groups of mice, while *Il2* was significantly increased in the Ctl-EC mice relative to that in the OT-EC mice (Fig. 3).

Oral tolerance inhibits eosinophil and mast cell infiltration in EC-sensitized skin

IL-5 and IL-13 are signals that are essential to regulating the activation and migration of eosinophils, the multifunctional proinflammatory leukocytes involved in allergic diseases (24-26). Oral tolerance induction decreased *Il5* and *Il13* levels in EC-sensitized mice, suggesting the inhibition of eosinophil infiltration. This was supported by significantly decreased expression of *Ccr3*, which encodes the G protein-coupled chemokine receptor mediating eosinophil infiltration in allergic tissues (Fig. 4A) (27). Consistent with decreased *Ccr3* expression, the skin of OT-EC mice showed a marked decrease in eosinophil granular MBP, which was prominent in the dermal layer of Ctl-EC mice (Fig. 4B). Infiltration of CCR3⁺SiglecF⁺ eosinophils in the skin was also determined by flow cytometry, and eosinophils were found to be significantly more abundant in Ctl-EC mice than in OT-EC mice (Fig. 4C,S3). Although CCR3 is primarily expressed on eosinophils, it is also expressed on mast cells, which play a role in IgE-mediated atopic skin reactions (28). Accordingly, infiltration of IgE⁺c-Kit⁺ mast cells following EC sensitization was inhibited by OT induction (Fig. 4D,S3). This finding was compatible with decreased chloroacetate esterase reactivity in the skin of OT-EC mice compared to reactivity in the Ctl-EC group (Fig. S4).

Induction of oral tolerance regulates infiltration of ILC2 in the EC-sensitized skin

CD4⁺ Th2 cells have been characterized as producers of IL-5 in inflamed, allergic tissues (29), and ILC2, recently discovered innate immune cells that are lineage marker-deficient, are also important sources of IL-5 in response to allergen exposure (30, 31). To identify the IL-5-producing cells affected by the induction of oral tolerance in EC-sensitized skin, skin cells were subjected to intracellular staining for IL-5 and examined by flow cytometry. The majority of skin cells expressing cytoplasmic IL-5 belonged to the CD4⁺ subset, and OT-EC mice showed a significant decrease in CD4⁺IL-5⁺ cells compared to that in Ctl-EC mice (Fig. 5A,S5). Accordingly, flow cytometric analysis revealed a significant increase in Lin⁻Sca-1⁺ST2⁺ ILC2 in the skin of EC-sensitized mice, and this ILC2 infiltration was efficiently inhibited in OT-EC mice (Fig. 5B,S5). ILC2 express receptors for IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), and stimulation of ILC2 with these molecules triggers the production of IL-5 and IL-13 (31, 32). The increased expression of *Il25* in EC-sensitized skin was significantly reduced by oral tolerance, whereas expression of *Il33* and *Tslp* was not altered either by EC sensitization or oral tolerance induction (Fig. 5C).

Discussion

Although oral administration of allergens has been shown to be effective for suppressing allergic responses (16, 33), whether induction of oral tolerance has a protective role in allergic skin inflammation has not been reported. This study demonstrated that oral tolerance protects against systemic and local Th2-type skin inflammation elicited in a murine AD model of EC sensitization by modulating immune microenvironments, which may contribute to the downregulation of ILC2 infiltration.

Elevated serum IgE levels, representing a Th2-polarized immune response, have been reported in both AD patients and murine EC sensitization models (10, 34). In our study, significant increases in OVA-specific IgE and IgG1 levels were observed both in sera and intestinal secretions of EC-sensitized mouse, suggesting the role of cutaneous antigen exposure in inducing a mucosal immune response. A link between immune responses in the skin and gut is supported by the mucosal secretion of protective antibody following administration of a vaccine antigen to the skin surface (35). In current study, the production of antigen-specific IgE and IgG1 was efficiently inhibited by the induction of oral tolerance, further supporting the existence of immunologic cross-talk between the skin and gut. The appearance of IgG2a in EC-sensitized mice might reflect a switching of the antigen-specific Th1-type antibody response at a later stage following antigen exposure (9), although the serum levels of anti-OVA IgG2a were low relative to the levels of anti-OVA IgE and IgG1. Therefore, induction of oral tolerance was effective in suppressing both Th1- and Th2-type antibody responses induced by EC sensitization.

Orally administered antigens are primarily recognized by CD103⁺ dendritic cells in the small intestine. From there, these antigen-loaded CD103⁺ dendritic cells migrate into the draining MLN, driving the selective differentiation of T cells into antigen-specific FOXP3⁺ regulatory T cells (36). The initial differentiation phase of regulatory T cells in the MLN is unlikely affected by oral tolerance, since no significant differences in the frequencies of CD103⁺ dendritic cells between OT-EC and Ctl-EC mice were found in the current study. However, oral tolerance induced a significant increase in the frequency of regulatory T cells and *Foxp3*-expression in the MLN of EC-sensitized mice. Considering that regulatory T cells actively suppress proinflammatory responses (37), it is plausible that the increased regulatory T cells observed in the MLN of orally tolerized mice might contribute to the inhibition of

inflammation in EC-sensitized mice. As for the role of oral tolerance in immune regulation, it is unlikely that the polarization of an immune response in MLN is altered, since the expression of *Ifng*, *Il4*, and *Il5* were comparable in the MLN of OT-EC and Ctl-EC mice (Fig. S6). The essential signals for regulatory T cell differentiation include cytokines, primarily IL-2, that signal through a common γ -chain (38) and TGF- β , which also has a role in the early proliferation of regulatory T cells (22). Therefore, we speculate that oral tolerance contributes to the restoration of an MLN cytokine milieu, affected by EC sensitization, for an environment that is more favorable for regulatory T cell differentiation.

EC-sensitized mice showed a marked increase in *Il5* and *Il13* expression in their skin, and this increase was efficiently inhibited by the induction of oral tolerance. Although IL-4 and IL-13 share receptor components and signaling pathways, differences in the expression and affinity for ligands of these receptors result in distinct functions (39, 40). IL-4 is the major factor driving Th2 differentiation of CD4⁺ T cells and alternative macrophage activation, while IL-13 functions as an effector molecule that mediates eosinophilic inflammation. IL-5 is a major eosinophil-activating cytokine that stimulates the production of eosinophils, promotes the migration of eosinophils out of bone marrow, and enhances the survival of these cells (26). In this regard, our observations suggest that oral tolerance suppresses cytokine signals that are primarily effective in eosinophilic inflammation. In line with this idea, EC-sensitized mice showed remarkable infiltration of eosinophils in their skin, and this was almost completely blocked by induction of oral tolerance. *Il4* expression in the skin was not affected by EC sensitization in this study; however, unidentified technical issues might have influenced the cytokine expression patterns of our experimental mice, as the expression of IL-4 has been reported in other studies with murine models of EC sensitization (9, 10). Significantly increased *Tgfb1* and *Foxp3* in the skin of OT-EC mice supported the role of oral

tolerance in immune regulation mediated by regulatory T cells. Unexpectedly, as for *Il2* expression, we observed the opposite effect of oral tolerance in the MLN and skin; oral tolerance upregulated *Il2* in the MLN, while downregulating this cytokine in the skin. IL-2 has paradoxical functions in immune homeostasis, acting as a potent T cell growth factor during peripheral inflammation, while having a crucial function in the maintenance of tolerance by supporting regulatory T cell development (41). Accordingly, we propose that oral tolerance is an effective way to modulate functionally different immune responses following EC sensitization, which might be affected by tissue-specific immune microenvironments.

Another possible role for oral tolerance in suppressing skin inflammation induced by EC sensitization could be inhibition of cutaneous *Il25* expression and a subsequent decrease in ILC2 infiltration. The skin has been reported to harbor ILC2, which increase significantly after allergen challenge (4). As ILC2 are sources of IL-5 and IL-13, these cells are capable of inducing infiltration of eosinophils in allergic skin and interactions of ILC2 with dermal mast cells have been reported (5). The increased frequency of CD4⁺IL-5⁺ cells and Lin⁻Sca-1⁺ST2⁺ ILC2 in the skin of EC-sensitized mice, which was effectively inhibited by oral tolerance, strongly suggests that ILC2 are key effector cells in the murine AD model, and that oral tolerance regulates allergic skin reactions through inhibition of ILC2 infiltration. Notably, significant expansion of skin-resident ILC2 has been observed in response to IL-2 (5), and TGF- β produced by regulatory T cells has been suggested to be an inhibitor of ILC2 activation. Therefore, the downregulation of *Il2* and upregulation of *Tgfb1* observed in the skin of OT-EC mice might plausibly account for the decrease in ILC2 in their skin. IL-25, IL-33, and TSLP have recently been identified as important initiators of allergic inflammation, and their roles in regulating the recruitment, expansion, and inflammatory function of ILC2

in the skin of mice has been reported (4, 31). Unlike IL-33, which is stored in nuclei, IL-25 is constitutively expressed in the epithelial cells of mucosal tissue, including the intestinal tract (42). These intrinsic properties of ILC2-stimulating cytokines might explain why the expression of *Il25*, in our results, was specifically modulated by the induction of oral tolerance.

Hyposensitization is a strategy used in conventional allergen-specific immunotherapy, and subcutaneous administration of escalating doses of allergens is a common approach in this therapy (43). However, subcutaneous immunotherapy has some disadvantages, such as the potential for anaphylaxis and the need for long-term treatment. Considering that the gut is the primary site of regulatory T cell stimulation in response to antigens (44), induction of oral tolerance is likely the most effective approach for the control of allergic diseases. Recently, oral administration of house dust mites in early childhood was reported to induce both antigen-specific and generalized desensitization effects without any adverse effects (45). By adopting various sources of antigens, our model may be used to investigate therapeutic interventions that promote oral tolerance to treat allergic skin diseases, and results of this study may contribute to elucidating the immunologic cross-talk between skin and the gastrointestinal system.

Acknowledgements

This work was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2013R1A1A2004820), Gachon University Research Fund of 2015 (GCU-2015-5105), and by the Grant Program of Gachon University Gil Medical Center (2013-02 and 2014-17). The authors thank Eun-Hui Lee and Ye-Rang Chung (Gachon University, Korea) for technical assistance.

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Author contributions

J.O.B., J.Y.R., and Y.J. designed the experiments and wrote the manuscript. J.O.B. and Y.J. performed the experiments and analyzed the data.

Conflicts of interest

The authors have no financial conflicts of interest.

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Figure legends

Figure 1. Decreased skin inflammation and ovalbumin (OVA)-specific immunoglobulin levels in epicutaneously sensitized mice following oral tolerance induction to OVA. (A) OVA exposure protocol. Mice were fed with or without 1% OVA in drinking water for 5 days prior to epicutaneous (EC) sensitization. Two days after oral treatment, the dorsal skin of mice was sensitized to 0.1% OVA or saline for 7 days. Each mouse had a total of three 7-day exposures separated by 14-day intervals. (B) Hematoxylin and eosin staining of skin from OVA-fed and EC-sensitized to saline (OT-saline), OVA-fed and EC-sensitized to OVA (OT-EC), EC-sensitized to saline without OVA-fed (Ctl-saline), and EC-sensitized to OVA

without OVA-fed (Ctl-EC). Images are representative of three independent experiments. Original magnification $\times 20$. Scale bar represents 50 μm . (C) Total numbers of cell isolated from EC-sensitized skin. Data are representative of two independent experiments ($n = 3-5$ mice per group). (D & E) OVA-specific IgE, IgG1, and IgG2a titers in sera (D) and intestinal washes (E). Data are representative of three independent experiments ($n = 8-12$ mice per group). Graphs show the mean $\pm$ SEM values. $*P < 0.05$ and $**P < 0.01$ (Student's *t*-test).

Figure 2. Increased frequency of regulatory T cells isolated from the mesenteric lymph nodes (MLN) of EC-sensitized mice following oral tolerance induction to OVA. The frequency of CD103⁺ dendritic cells (A) and regulatory T cells (B) in the MLN of EC-sensitized mice fed with or without OVA. Flow cytometry plots are representative of three independent experiments. (C) Expression levels of *Foxp3*, *Tgfb1*, and *Il2* in the MLN of EC-sensitized mice fed with or without OVA. Data are representative of two independent experiments. Graphs show the mean $\pm$ SEM values. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test).

Figure 3. Decreased expression of *Il5* and *Il13* and increased expression of *Tgfb1* and *Foxp3* in the skin of EC-sensitized mice following oral tolerance induction to OVA. mRNA expression of innate immune cytokines (*Il1b* and *Tnfa*), Th1-type *Ifng*, Th2-associated cytokines (*Il4*, *Il5*, *Il9*, *Il10*, *Il13*, and *Tgfb1*), *Il2*, and *Foxp3* in the skin of EC-sensitized mice fed with or without OVA. All data are representative of three independent experiments. Graphs show the mean $\pm$ SEM values. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test).

Figure 4. Infiltration of CCR3-expressing cells in the skin of EC-sensitized mice was inhibited by oral tolerance. (A) Expression levels of *Ccr3* in the skin of EC-sensitized mice fed with or without OVA. Data are representative of three independent experiments. (B) Eosinophils in the indicated skin lesions were identified by anti-MBP staining. Images are representative of three independent experiments. Arrows indicate representative MBP⁺ cells. Original magnification × 20, inset magnification × 40. Scale bar represents 50 μm. (C & D) The frequency of eosinophils (C) and mast cells (D) in the skin of EC-sensitized mice fed with or without OVA. Flow cytometry plots are representative of at least three independent experiments. Graphs show the mean ± SEM values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test).

Figure 5. Infiltration of type 2 innate lymphoid cells (ILC2) and *Il25* expression in EC-sensitized skin was inhibited by oral tolerance. (A) The frequency of IL-5⁺ cells in the skin of EC-sensitized mice fed with or without OVA. Flow cytometry plots are representative of two independent experiments. (B) The frequency of ILC2 in the skin of EC-sensitized mice fed with or without OVA. The lineage marker (Lin) was the combination of CD3, B220, CD11b, CD11c, Gr-1, and IgE. Flow cytometry plots are representative of three independent experiments. (C) Expression levels of *Il25*, *Il33*, and thymic stromal lymphoprotein (*Tslp*) in the skin of EC-sensitized mice fed with or without OVA. Data are representative of three independent experiments. Graphs show the mean ± SEM values. **P* < 0.05 (Student's *t*-test).

Supplemental Figure Legends

Figure S1. OVA-specific immunoglobulin levels in the sera of epicutaneously sensitized mice following oral tolerance induction to OVA. OVA-specific IgE, IgG1, and IgG2a titers in the indicated mouse groups were analyzed after the first (A) and second (B) EC sensitization. Data are representative of two independent experiments (n = 4–6 mice per group). Graphs show the mean $\pm$ SEM values. * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test).

Figure S2. Flow cytometry analysis of cells isolated from mesenteric lymph nodes. (A) Strategy used for characterization of dendritic cells and regulatory T cells. Indicated isotype controls were used to define MHCII⁺CD11c⁺CD103⁺ dendritic cells or CD4⁺FOXP3⁺ regulatory T cells in the mesenteric lymph nodes of EC-sensitized mice fed with or without OVA. (B) Mean fluorescence intensity (MFI) and numbers of CD4⁺FOXP3⁺ T cells in the mesenteric lymph nodes. All data are representative of two independent experiments (n = 3–4 mice per group). Graphs show the mean $\pm$ SEM values. * $P < 0.05$ (Student's *t*-test).

Figure S3. Gating strategy for eosinophils and mast cells isolated from skin. The indicated isotype controls were used to define CD45⁺CD3⁺CCR3⁺SiglecF⁺ eosinophils or CD45⁺CD3⁺IgE⁺c-Kit⁺ mast cells in the skin of EC-sensitized mice fed with or without OVA. Flow cytometry plots are representative of two independent experiments (n = 3–4 mice per group).

Figure S4. Mast cells in the indicated skin lesions were identified by chloroacetate esterase staining. Skin sections were stained with naphthol AS-D chloroacetate esterase (Sigma-Aldrich) according to the manufacturer's instructions to detect mast cell infiltration. Images are representative of two independent experiments. Arrows indicate representative

chloroacetate esterase-reactive cells. Original magnification $\times 10$. Scale bar represents 100 μm .

Figure S5. Gating strategy for IL-5⁺ cells and ILC2 isolated from skin. The indicated isotype controls were used to define CD45⁺IL-5⁺ cells or Lin⁻ Sca-1⁺ST2⁺ ILC2 in the skin of EC-sensitized mice fed with or without OVA. Flow cytometry plots are representative of two independent experiments (n = 3–4 mice per group).

Figure S6. The mRNA levels of *Ifng*, *Il4*, and *Il5* in the MLN of EC-sensitized mice fed with or without OVA. Data are representative of three independent experiments. Graphs show the mean $\pm$ SEM values.

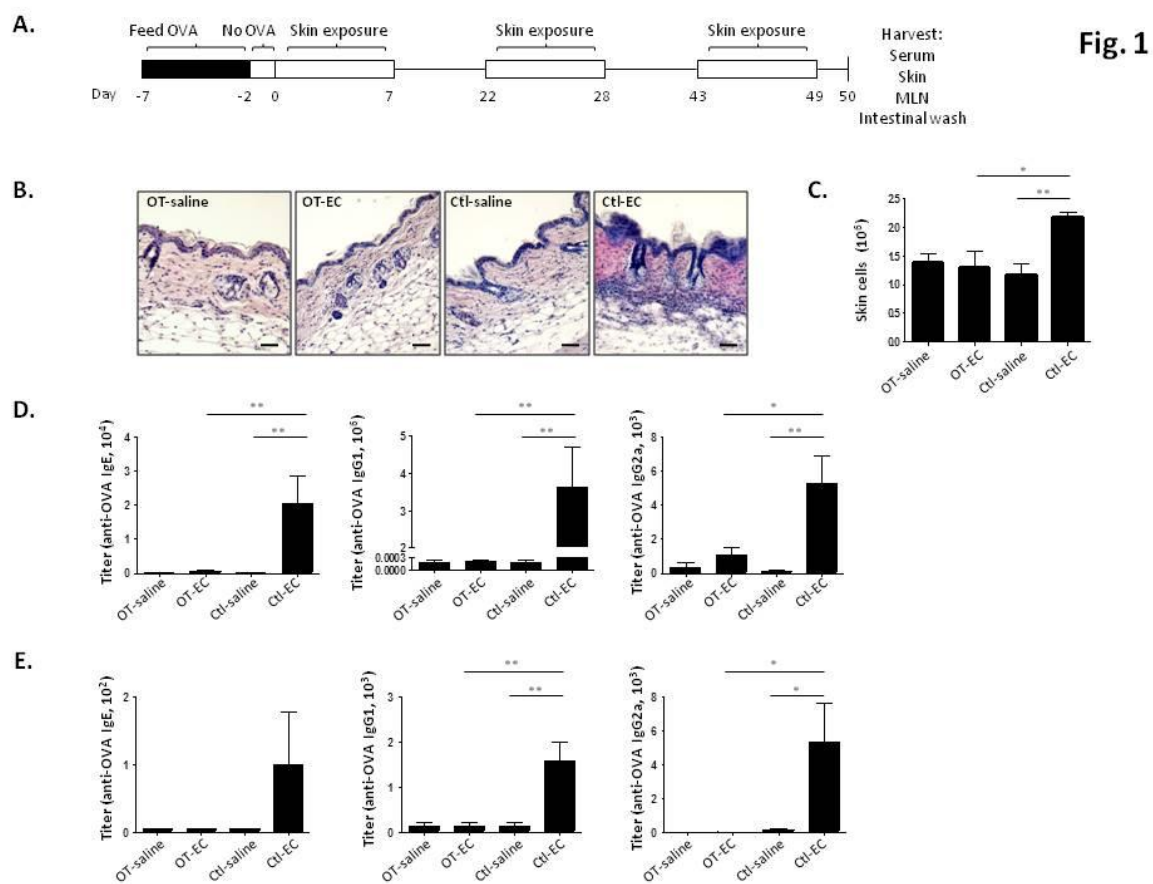
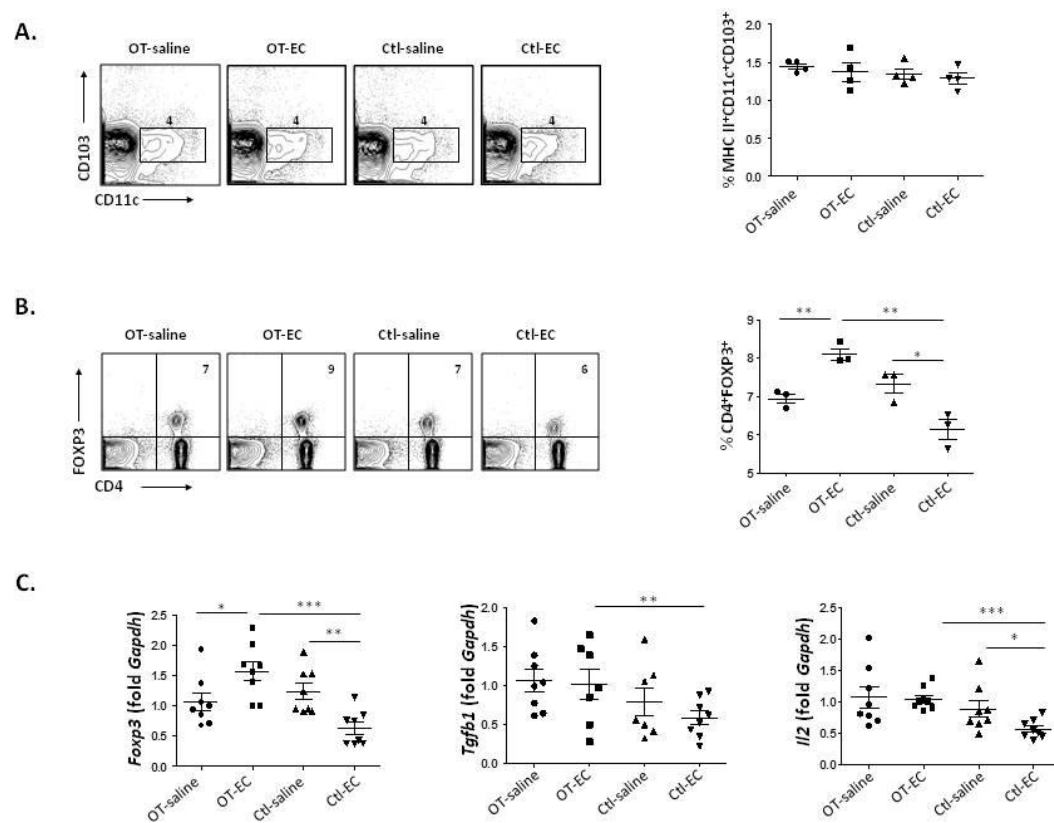
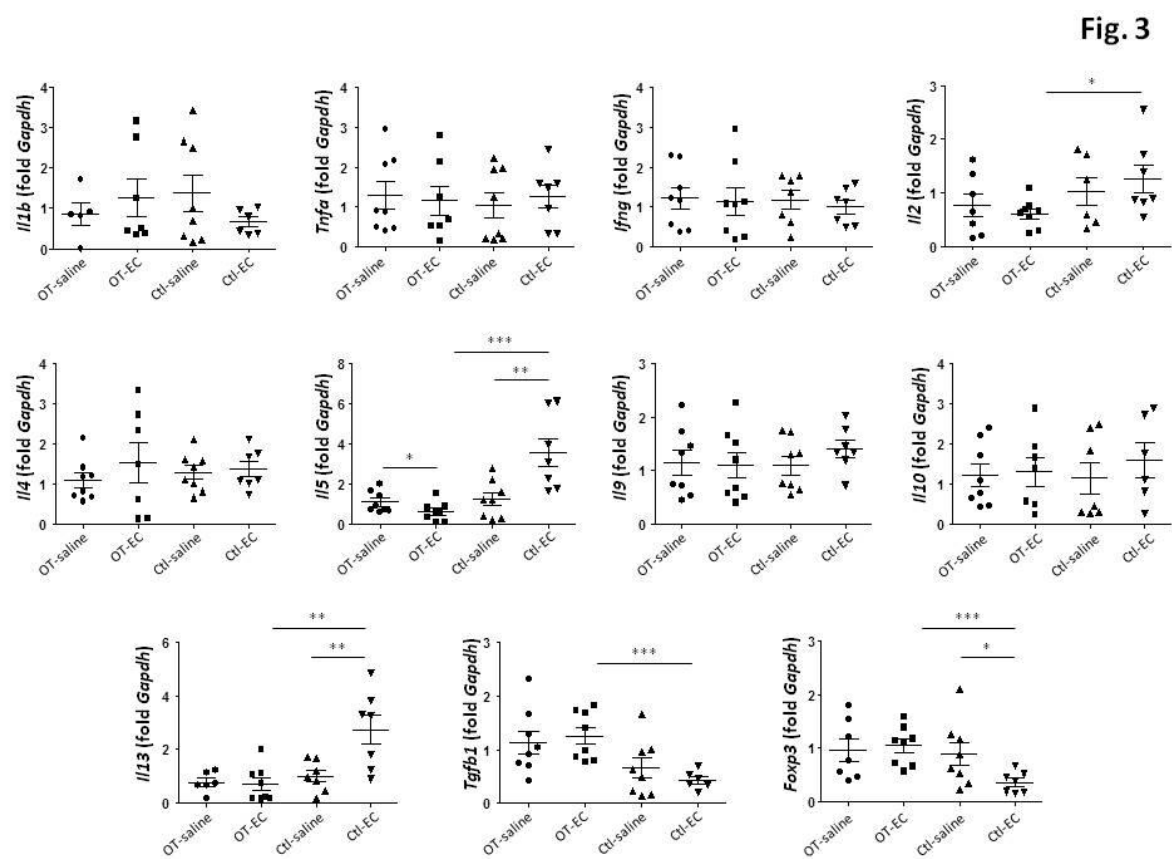


Fig. 1

Fig. 2





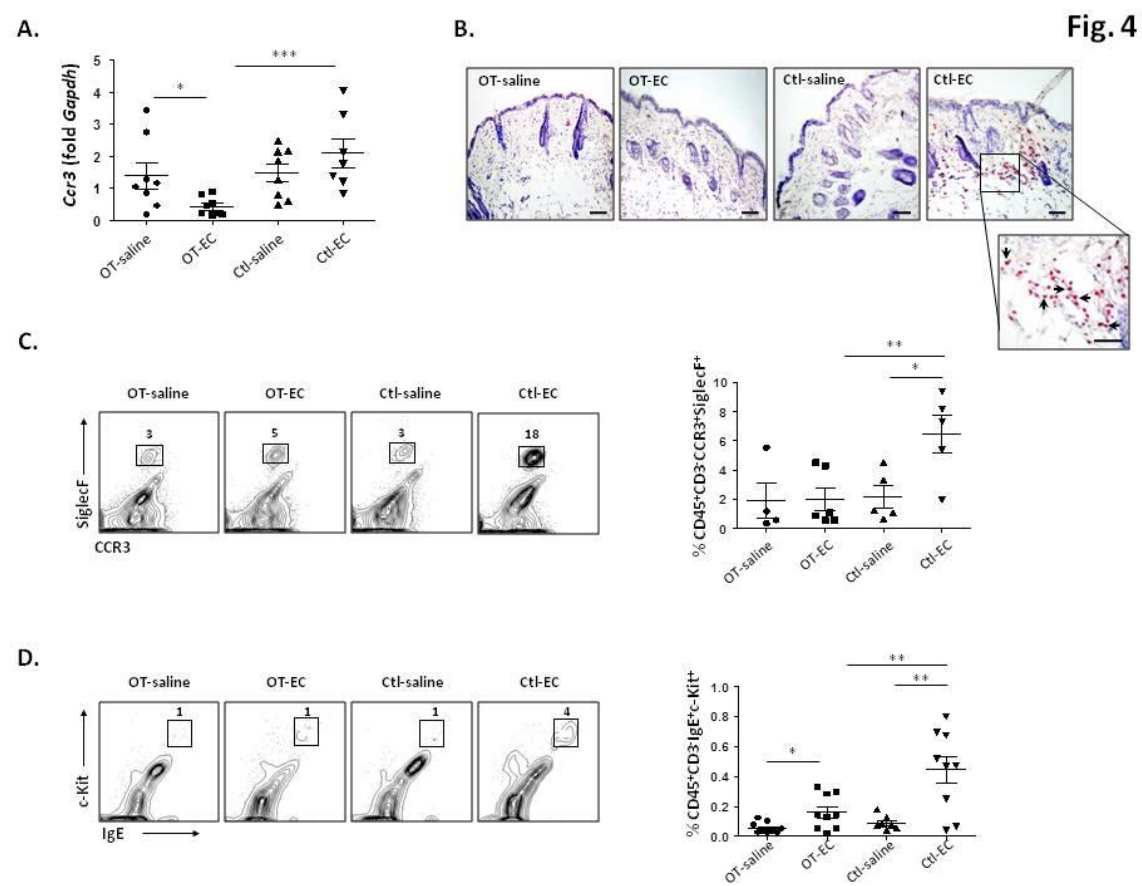


Fig. 5

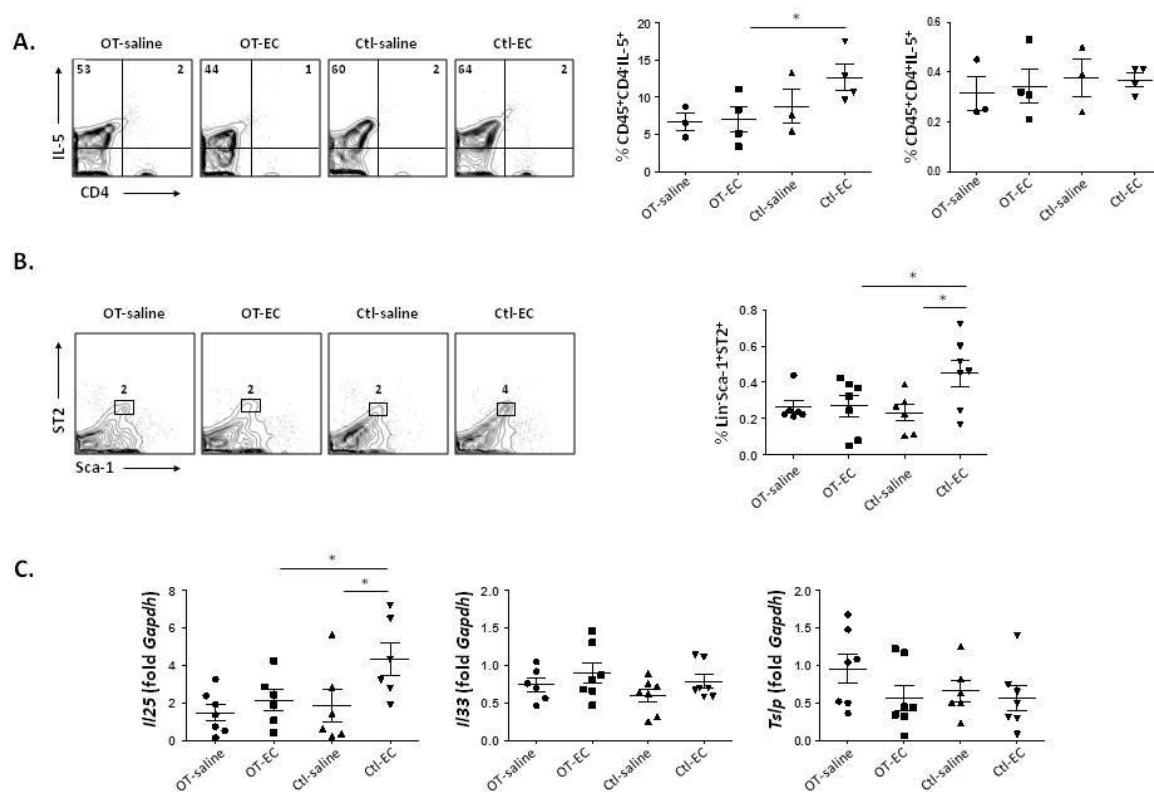
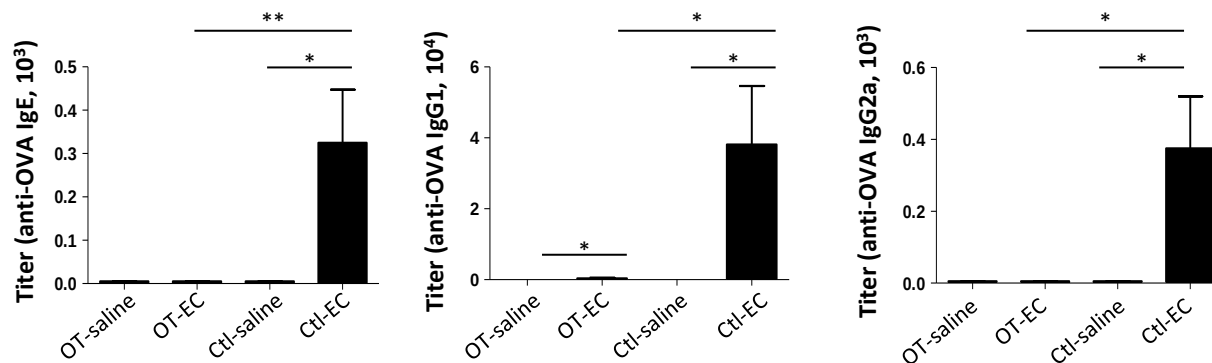


Fig. S1

A.



B.

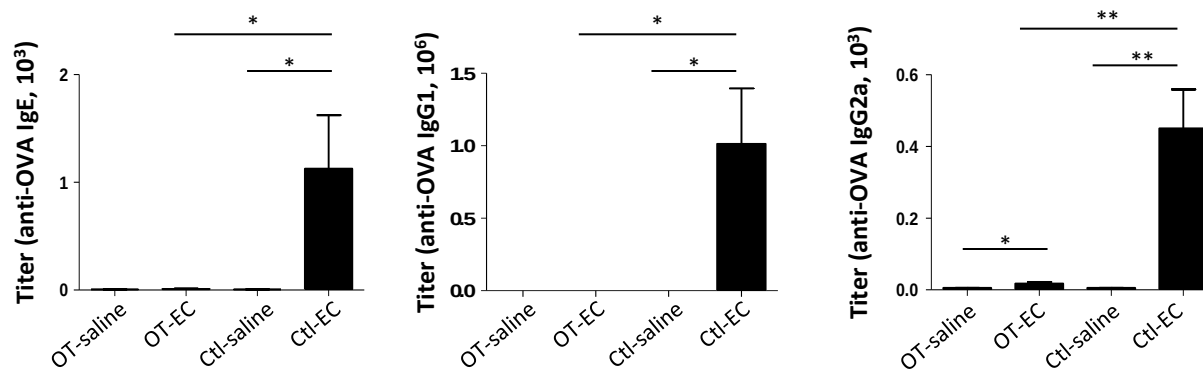


Fig. S2

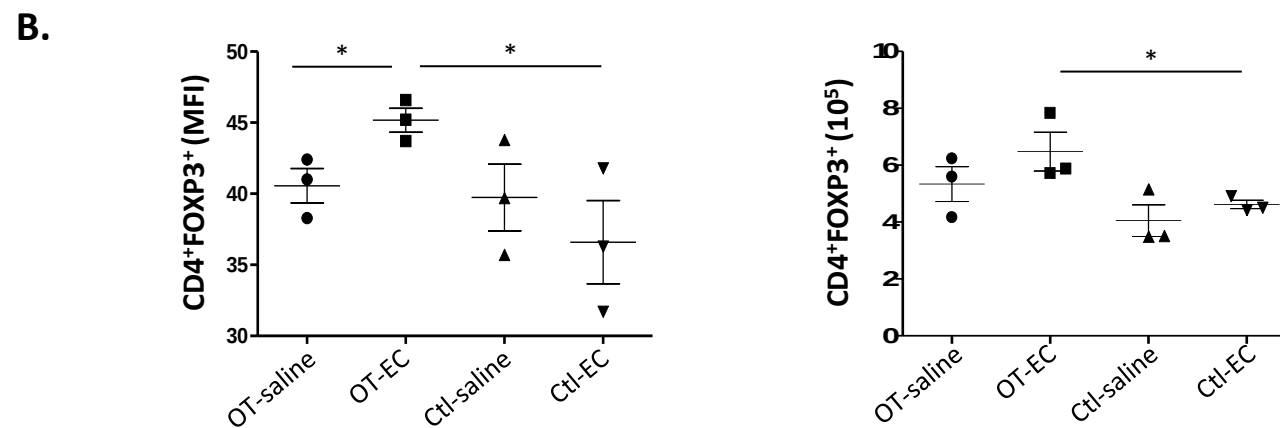
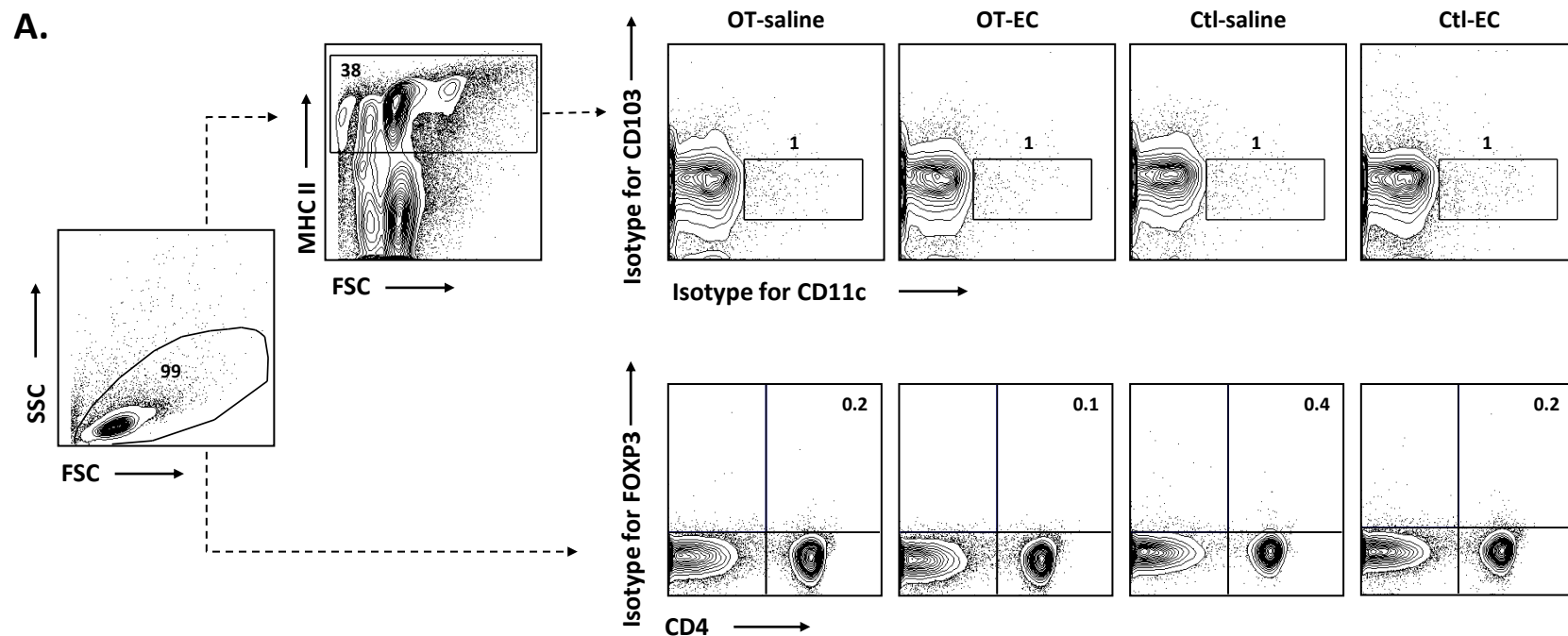


Fig. S3

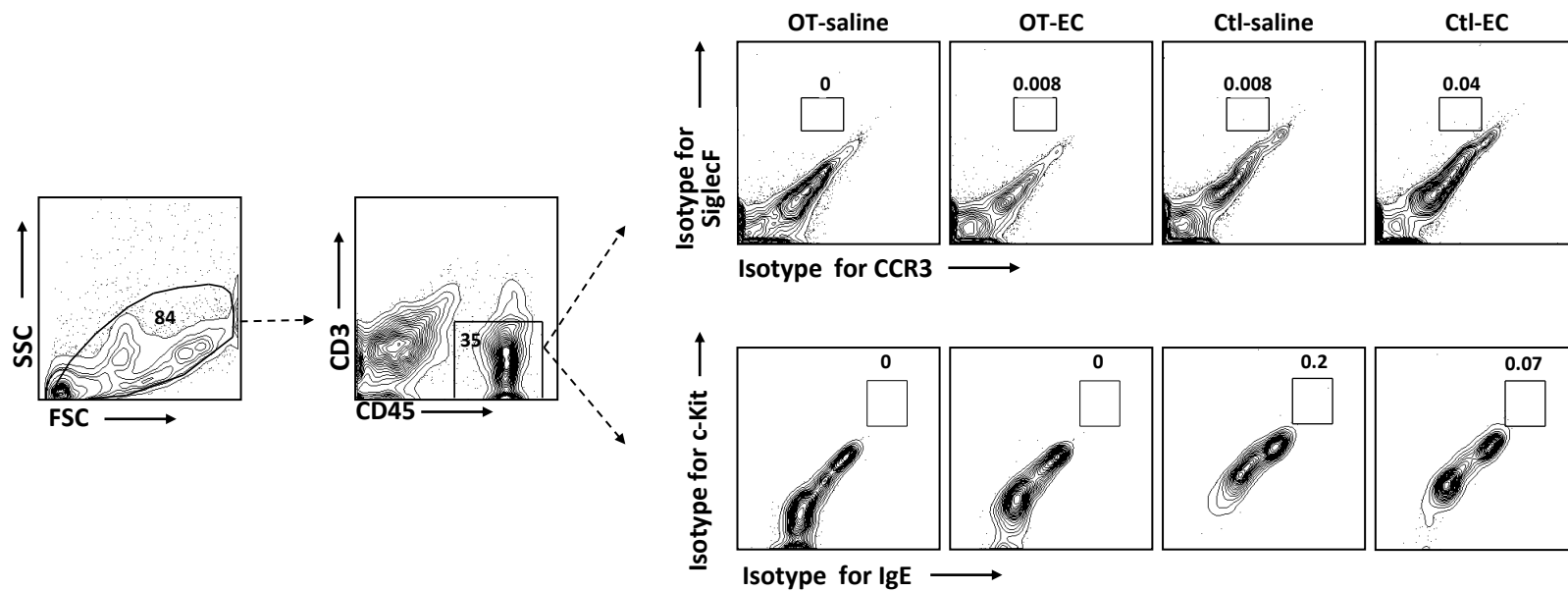


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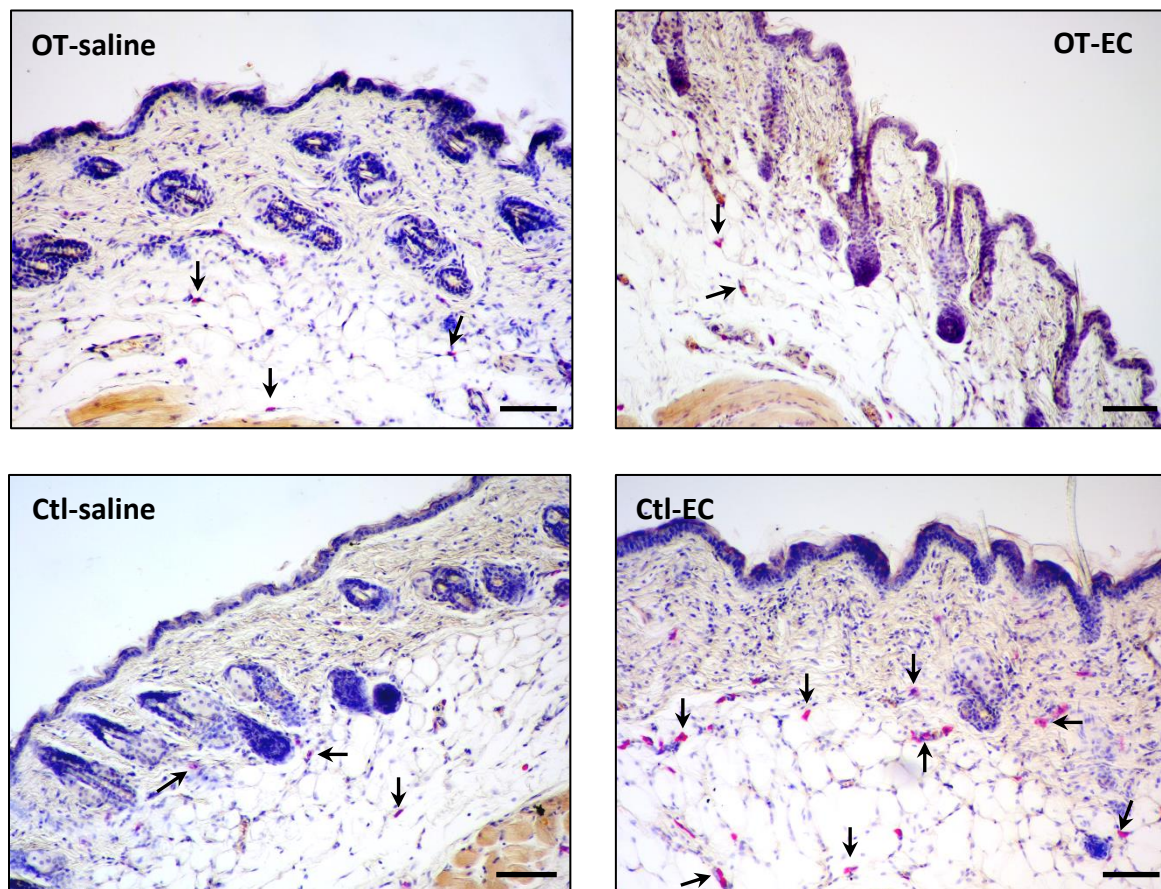


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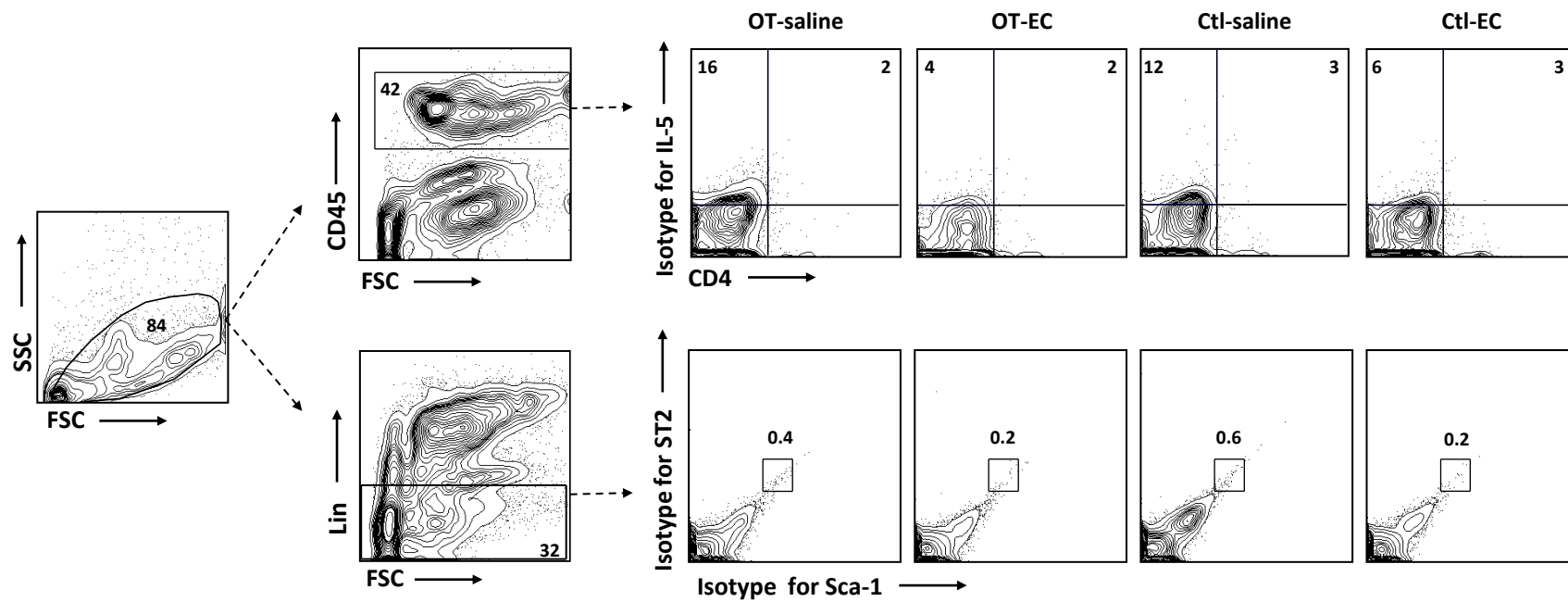


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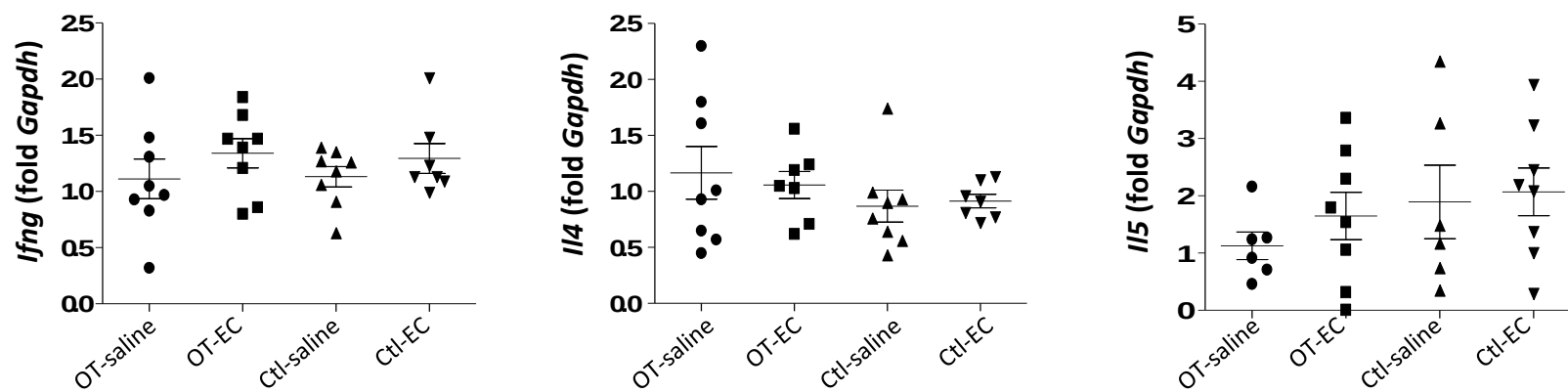


Table S1. Primer sequences for real-time PCR

Gene	Primer sequence
<i>Foxp3</i>	Forward: 5'-CAC CTA TGC CAC CCT TAT CCG-3' Reverse: 5' –CAT GCG AGT AAA CCA ATG GTA GA-3'
<i>Tgfb1</i>	Forward: 5'-CGA GAG CGC CGA CCC AGA G-3' Reverse: 5'-GGG TTC GGG CAC TGC TTC C-3'
<i>Il1b</i>	Forward: 5'-TGG CAG GAG CTA CTT CCC T-3' Reverse: 5'-TCC AGT CTT TTC TGA GCC TGT-3'
<i>Tnfa</i>	Forward: 5'-CCT GTA GCC CAC CTC GTA G-3' Reverse: 5'-GGG AGT AGA CAA GGT ACA ACC C-3'
<i>Ifng</i>	Forward: 5'-ATG AAC ACT ACA CAC TGC ATC-3' Reverse: 5'-CCA TCC TTT TGC CAG TTC CTC-3'
<i>Il4</i>	Forward: 5'-GGTCTCAACCCCCAGCTAGT-3' Reverse: 5'-GCCGATGATCTCTCTCAAGTGAT-3'
<i>Il5</i>	Forward: 5'-ACA AGC AAT GAG ACG ATG AG-3' Reverse: 5'-CCA CGG ACA GTT TGA TTC TT-3'
<i>Il9</i>	Forward: 5'-CTC TCC GTC CCA ACT GAT-3' Reverse: 5'-CTT GAT TTC TGT GTG GCA TTG-3'
<i>Il10</i>	Forward: 5'-GCT CTT ACT GAC TGG CAT GAG-3' Reverse: 5'-CGC AGC TCT AGG AGC ATG TG-3'
<i>Il13</i>	Forward: 5'-CCT GGC TCT TGC TTG CCT T-3' Reverse: 5'-GGT CTT GTG TGA TGT TGC TCA-3'
<i>Ccr3</i>	Forward: 5'-CAG TGC TTT TGG GTG TTT GTC-3' Reverse: 5'-GAT TTC TAG GGT CTG TGT GCC-3'
<i>Il25</i>	Forward: 5'-TAT GAG TTG GAC AGG GAC TTG A-3' Reverse: 5'-TGG TAA AGT GGG ACG GAG TTG-3'
<i>Il33</i>	Forward: 5'-TCC AAC TCC AAG ATT TCC CCG-3' Reverse: 5'-CAT GCA GTA GAC ATG GCA GAA-3'
<i>Tslp</i>	Forward: 5'-ACG GAT GGG GCT AAC TTA CAA-3' Reverse: 5'-AGT CCT CGA TTT GCT CGA ACT-3'
<i>Il2</i>	Forward: 5'-TGA GCA GGA TGG AGA ATT ACA GG-3' Reverse: 5'-GTC CAA GTT CAT CTT CTA GGC AC-3'
<i>Il7</i>	Forward: 5'-TTC CTC CAC TGA TCC TTG TTC T-3' Reverse: 5'-AGC AGC TTC CTT TGT ATC ATC AC-3'
<i>Gapdh</i>	Forward: 5'-CTG GTA TGA CAA TGA ATA CGG-3' Reverse: 5'-GCA GCG AAC TTT ATT GAT GG-3'