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Title Page

FcRγ promotes contact hypersensitivity to oxazolone without affecting the contact sensitisation process in B6 mice

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Abstract

The process of sensitisation by specific contact allergens is indispensable for the induction of allergic contact dermatitis. Oxazolone is a well-characterised contact allergen. Previous studies suggested that immune cells bearing the FcR γ subunit are essential for oxazolone-induced contact hypersensitivity, but the biological functions of the FcR γ subunit in the process of sensitisation to oxazolone remain unknown. In this study, we show that FcR γ deficiency decreases ear-swelling responses to oxazolone in mice. However, we found that oxazolone-sensitised FcR $\gamma^{-/-}$ mice and oxazolone-sensitised wild-type (WT) mice have comparable numbers of CD11c⁺MHCII^{hi} dendritic cells (DCs) in their draining lymph nodes (LNs). In addition, oxazolone-sensitised LN cells from both FcR $\gamma^{-/-}$ and WT mice showed considerable production of interferon-gamma (IFN γ), interleukin-4 (IL-4), and IL-17A upon oxazolone-Keyhole limpet hemocyanin loading. Consistent with these data, oxazolone-sensitised FcR $\gamma^{-/-}$ and FcR $\gamma^{+/+}$ LN cells conferred contact hypersensitivity to WT naïve mice challenged with the hapten. Our findings clearly indicate that, in an experimental mouse model, the FcR γ subunit positively regulates contact hypersensitivity to oxazolone without affecting the contact sensitisation process.

Key words

Contact hypersensitivity; FcR γ ; IgE

Introduction

Allergic contact dermatitis is a type IV hypersensitivity reaction to contact allergens. The induction of contact hypersensitivity (CHS) results from immune responses that occur in a sensitisation phase and an elicitation phase (1). After the first allergen sensitisation, dermal dendritic cells (DCs) capture allergens and migrate from the skin into the draining lymph nodes (LNs), where they prime naïve T cells (2-4). Allergen-specific T lymphocytes in the LNs are activated upon re-challenge with the same allergen and then infiltrate the site of inflammation (1, 5).

The FcR γ subunit is a common subunit for the cell-surface expression of activating FcRs, such as Fc ϵ RI (6, 7), Fc γ RI (8), Fc γ RIII (8) and Fc γ RIV (9). Thus, FcR γ -deficient mice are recognised as Fc ϵ RI/Fc γ RI/Fc γ RIII/Fc γ RIV knockout mice. FcR γ -deficient (FcR $\gamma^{-/-}$) mice exhibit either suppressed or enhanced features of cutaneous CHS responses, which vary depending on the protocol and the type of contact allergen (10-12). These results suggest that immune cells expressing activating FcRs are essential for the induction of optimal CHS to contact allergens. Oxazolone (Oxa) is a well-known contact sensitizer that is used to study CHS (10, 13-15) and ulcerative colitis (16, 17). FcR γ acts as a positive regulator for the CHS response to Oxa diluted in either acetone/olive oil (10) or ethanol (EtOH) (14). Bryce et al. reported that intact IgE is required for the process of epicutaneous sensitisation to Oxa (14). We recently reported that abrogation of the

IgE-FcεRI-FcRγ axis with a recombinant soluble ectodomain of the human FcεRIα subunit (rsFcεRIα) in the elicitation phase can prevent CHS to Oxa (18). These studies raise the possibility that FcRγ is a crucial element of both the sensitisation phase and the elicitation phase in CHS to Oxa through the IgE-FcεRI-FcRγ axis. However, the role of the FcRγ subunit in the process of contact sensitisation to Oxa remains unknown. To elucidate the mechanisms involved in the cutaneous immune responses regulated by FcRγ and to deepen our understanding of FcRγ function and the CHS, we employed FcRγ^{-/-} mice and rsFcεRIα to address this possibility.

Here, we report that FcRγ is indispensable for the induction of CHS to Oxa but is dispensable for sensitisation to Oxa. Furthermore, we show that rsFcεRIα has no effects on contact sensitisation.

Materials and Methods

Reagents

Oxa was purchased from Sigma (St. Louis, MO, USA). Phycoerythrin (PE)-conjugated anti-mouse I-A/I-E mAb (M5/114.15.2) was purchased from eBioscience (San Diego, CA, USA). Mouse IgE (IgE-3), fluorescein isothiocyanate (FITC)- or PE/Cy5-conjugated anti-mouse CD11c (HL3), and biotin-conjugated anti-mouse IgE (R35-118) mAbs were purchased from BD Biosciences (San Diego, CA, USA). FITC-I and 2,4-Dinitrochlorobenzene (DNCB) were purchased from Dojindo (Kumamoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively.

rsFcεRIα and KLH-conjugated Oxa were prepared in our laboratory as previously described (18, 19).

Mice

FcRγ^{-/-} male mice (C57BL/6J background) (8) were bred in the animal facility of the Nihon University School of Medicine under specific pathogen-free conditions. Wild-type (WT) C57BL/6J male mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan) to serve as a wild-type control for the FcRγ^{-/-} mice. Following the approval of the animal experimentation committee at Nihon University, all the experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Nihon University.

Sensitisation and elicitation of contact hypersensitivity

CHS to Oxa and DNCB was induced by sensitisation on day 0 by exposing mice to 200 μl of 3% Oxa or EtOH (vehicle) or 100 μl of 2% DNCB or acetone (vehicle) on their shaved abdominal skin as previously described (20). From day 3 or day 5 onward, the sensitised mice were challenged by applying 20 μl of 1% Oxa/EtOH or 0.5% DNCB/acetone to the ear. Twenty-four hours later, ear thickness was measured using a micrometre (Mitutoyo, Tokyo, Japan) as previously described

(18). The net response in ear swelling was calculated as follows: thickness of ears after hapten challenge — thickness of ears before hapten application.

Analysis of DC number

The mice were sensitised with 80 µl of 1% FITC-I or acetone-dibutylphthalate (1:1) (vehicle) or 200 µl of 3% Oxa or EtOH (vehicle) applied on the shaved abdomen. Twenty-four (for FITC) or 72 hours (for Oxa) after hapten treatment, their inguinal LNs were isolated, and their CD11c⁺MHCII^{hi} DCs or FITC⁺CD11c⁺MHCII^{hi} DCs were quantified using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). In some experiments, WT naïve mice were treated with rsFcεRIα 48 hours before sensitisation.

Measurement of IFN γ , IL-4, and IL-17A production

Three days after the mice were sensitised with 3% Oxa on their shaved abdomens, their inguinal LNs were isolated. Then, the LN cells were stimulated with Oxa-Keyhole limpet hemocyanin (KLH) (300 µg/ml) or KLH (300 µg/ml) for 24 hours in 0.2 ml of RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS). The production of interferon-gamma (IFN γ), interleukin-4 (IL-4), and IL-17A

proteins was analysed with specific enzyme-linked immunosorbent assay (ELISA) kits (eBioscience).

Measurement of free IgE in serum

The levels of IgE un-captured with rsFcεRIα in serum were measured using a sandwich ELISA. Briefly, a 96-well plate was coated with rsFcεRIα (1 µg/ml) and then washed three times with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T). Diluted serum samples from C57BL/6J mice received with or without rsFcεRIα were added to the wells and incubated for 2 hours. After washing with TBS-T, biotin-conjugated anti-mouse IgE was added to each well and incubated for 1 hour. Avidin-conjugated horseradish peroxidase (HRP) (BD Bioscience) was added to each well and incubated for 30 min. The reaction was developed with chromogen for 30 min and immediately stopped by the addition of 1 M H₂SO₄. The optical absorbance was measured at a 450 nm wavelength. Mouse IgE (IgE-3) was used as a standard.

Cellular passive transfer

Adoptive transfer of LN cells was performed as previously described (21). In brief, inguinal lymph nodes were prepared three days postsensitisation, and 1×10^7 LN

cells were intravenously injected into naïve recipients that were then immediately challenged with 1% Oxa. In some experiments, WT naïve C57BL/6J mice were treated with rsFcεRIα 48 hours before sensitisation.

Statistical analysis

Statistical analysis was performed using Student's t-test. Probability values of less than 0.05 represent statistically significant differences.

Results

FcRγ is indispensable for optimal CHS to Oxa.

First, we confirmed the CHS response to Oxa in the FcRγ^{-/-} mice, in which immune cells are negative for IgE- and IgG-mediated responses. FcRγ^{-/-} (n = 5 – 7 per group) and WT mice (n = 5 – 7 per group) sensitised with Oxa or EtOH were challenged with the same hapten five days after sensitisation. In accordance with previous reports (10, 14), the FcRγ^{-/-} mice exhibited an attenuated ear swelling response to Oxa compared to the WT mice (Figure 1A). Histological analyses indicated that FcRγ deficiency resulted in reduced leukocytic infiltration of the dermis (Figure 1B). In contrast, the irritant and allergic CHS response to DNCB was increased in FcRγ^{-/-} mice (n = 4 or 5 per group) compared with WT mice (n = 5 per

group) (Figure S1), suggesting that the function of FcR γ in hapten-induced CHS depends on the hapten type. Oxa-reactive T cells are known to appear in the draining LN within three days after sensitisation (18). Therefore, we examined whether the CHS response to Oxa occurs three days after sensitisation. In WT mice, a robust hapten-specific CHS response was observed three days after Oxa sensitisation (Figure 1C). These data suggest that FcR γ is responsible for the induction of optimal CHS responses to Oxa and that the sensitisation of naïve mice to Oxa is nearly complete in 3 days.

FcR γ is dispensable for the hapten-specific production of IFN γ , IL-4, and IL-17A in Oxa-sensitised LN cells

To examine the involvement of FcR γ in the sensitisation phase, we sensitised FcR γ ^{-/-} (n = 8 per group) and WT mice (n = 6 per group) on their abdomens and analysed the draining LN cells three days later. Figure 2A shows that the number of CD11c⁺MHCII^{hi} DCs in each draining LN was comparable. Experiments using FITC indicated that dendritic cells migrated from the skin sensitisation site to the inguinal LNs (Figure S2). Furthermore, we examined the cellular responses of the LN cells from FcR γ ^{-/-} (n = 7 per group) and WT mice (n = 7 per group) sensitised with Oxa. Oxa-sensitised LN cells were stimulated with Oxa-KLH. As shown in Figure 2B, the Oxa-dependent production of cytokines (IFN γ , IL-4, and IL-17A) was not diminished

in Oxa-sensitised $\text{FcR}\gamma^{-/-}$ LN cells. Unsensitised $\text{FcR}\gamma^{-/-}$ and WT LN cells did not produce any cytokines.

Oxa-sensitised $\text{FcR}\gamma^{-/-}$ LN cells can transfer CHS response to naïve WT mice

Figure 2 raise the possibility that $\text{FcR}\gamma$ is dispensable for the contact sensitisation process to generate Oxa-reactive immune cells. Therefore, we investigated whether Oxa-sensitised draining LN cells can transfer CHS to naïve WT recipient mice. As shown in Figure 3A, sensitised LN cells from both $\text{FcR}\gamma^{-/-}$ and WT mice conferred ear-swelling responses to naïve WT recipients ($n = 5$ or 7 per group) following an Oxa challenge. Transferring sensitised or unsensitised LN cells from WT mice into naïve $\text{FcR}\gamma^{-/-}$ recipients ($n = 5$ or 7 per group) failed to induce a significant CHS response to Oxa (Figure S3). Collectively, these results suggest that $\text{FcR}\gamma$ may play an important role at the elicitation phase.

Application of rsFcεRIα does not alter the process of contact sensitisation to Oxa

A previous study employing congenital $\text{IgE}^{-/-}$ mice suggested that the IgE - $\text{Fc}\epsilon\text{RI}$ - $\text{FcR}\gamma$ axis plays an important role in the process of sensitisation to Oxa (14). However, as we demonstrated above, the process of contact sensitisation to Oxa was not altered in the presence or absence of the $\text{FcR}\gamma$ subunit. Therefore, we

re-evaluated the possibility that IgE is responsible for optimal contact sensitisation to Oxa under our experimental conditions. To test this hypothesis, we prepared rsFcεRIα that can capture murine IgE (22-24). Figure 4A shows that pre-incubation of the purified rsFcεRIα (0.25 μg) prevented murine IgE (1 μg) from binding to FcεRI in a rat basophilic leukaemia cell line (RBL-2H3). As shown in Figure 4B, free IgE in the serum was effectively decreased 48 hours after intravenous administration of rsFcεRIα. Thus, we injected rsFcεRIα (4 μg) or PBS into WT mice (n = 4 – 6 per group) 48 hours before elicitation (Figure 4C) or sensitisation (Figures 4D and E). Administering rsFcεRIα at the elicitation phase decreased the ear-swelling response to Oxa challenge (Figure 4C). On the other hand, Figure 4D shows that rsFcεRIα administration prior to sensitisation had no effect on the numbers of CD11c⁺MHCII^{hi} DCs in the draining LNs. Consistent with this observation, the passive transfer of the CHS response to naïve WT mice was the same in the presence or absence of rsFcεRIα treatment (Figure 4E).

Discussion

In this study, we have shown that FcRγ^{-/-} mice exhibit severe defects in the CHS response to Oxa under our experimental conditions (Figure 1). However, neither the numbers of CD11c⁺MHCII^{hi} DCs in draining LNs nor the hapten-reactive cytokine production in the LN cells were altered in the Oxa-sensitised FcRγ^{-/-} mice (Figure 2).

Thus, we conclude that FcR γ mainly works to enhance CHS responses to Oxa at the elicitation phase. To our knowledge, this report is the first addressing the biological function of the FcR γ subunit in contact sensitisation to Oxa. Suto et al. reported that FcR γ deficiency results in a decrease in the CHS response to FITC without reducing the migration of FITC⁺CD11c⁺MHCII^{hi} DCs into the draining LNs (11). Although it is unclear whether the generation of FITC-reactive T cells in the draining LNs is diminished in FITC-sensitised FcR γ ^{-/-} mice and whether the LN cells from FITC-sensitised FcR γ ^{-/-} mice can transfer a CHS response to naïve recipients, we speculate that FcR γ may be dispensable for contact sensitisation to FITC. Unlike Oxa and FITC, DNCB application resulted in enhanced CHS responses in FcR γ ^{-/-} mice (Figure S1). In addition, rsFc ϵ RI α administration at the effector phase increased allergic ear-swelling responses to DNCB (Figure S4). These findings strongly suggest that the positive or negative role of FcR γ in CHS depends on the type of hapten. However, we could not elucidate whether the loss of FcR γ function in contact sensitisation is responsible for enhanced CHS responses to DNCB. Therefore, we are currently investigating the role of FcR γ in the contact sensitisation phase to DNCB.

Murine mast cells and basophils express two FcR γ -associated receptors (Fc ϵ RI and Fc γ RIII) (25). A recent study employing novel mice that were deficient in mast cells (Mas-TRECK mice) revealed that dermal mast cells, but not basophils, contribute to contact sensitisation to dinitrofluorobenzene (DNFB) or Oxa in mice (26). In

addition, Bryce et al. reported that, in a mouse model, IgE, but not IgG, is responsible for contact sensitisation to Oxa (14). These studies suggest that dermal mast cells and IgE cooperate to regulate the primary immune response to Oxa. However, our results of rsFcεRIα administration were not consistent with the above hypothesis. The IgE dependency of immune responses differs between mouse strains (27). For example, high IgE-responding mice require IgE to eliminate T spirals, but low IgE-responding mice can exclude the same parasite in the absence of IgE (28). The following reasons might explain this discrepancy: (i) the FcRγ^{-/-} (C57BL/6J background) and C57BL/6J mice used in this study are well-known low IgE-responding mice, and (ii) the Mas-TRECK mice are also generated on a C57BL/6J background (26), but (iii) the IgE^{-/-} mice (BALB/c background) used in Bryce's report are high IgE-responding mice. Thus, we cannot exclude the possibility that the IgE-FcεRI-FcRγ axis plays an important role in contact sensitisation to Oxa in high IgE-responding mice.

Importantly, in naïve mice with a C57BL/6J background, cutaneous mast cells do not need the IgE-FcεRI-FcRγ axis to promote immune responses after the first Oxa sensitisation. Dudeck et al. reported that the first challenge with a contact allergen triggers an immediate swelling response in the ears of naïve mice with a C57BL/6J background, resulting from the degranulation of dermal mast cells (29). However, it is unclear how the sensitisation of naïve mice with hapten induces rapid mast cell

activation independently of the IgE-FcεRI-FcRγ axis. More recently, in WT and FcRγ^{-/-} bone marrow-derived DCs, various haptens (including Oxa) elicited a rapid activation of the DAP12-Syk-Card9/H₂O₂ signalling pathway, leading to IL-1α/β secretion (30). As murine mast cells also express functional DAP12 (31), Syk (32), and Card9 (33, 34) molecules, DAP12-Syk-Card9/H₂O₂ signalling may explain why Oxa sensitisation induces IgE-FcεRI-FcRγ axis-independent mast cell activation.

In conclusion, we addressed the biological actions of the FcRγ subunit in immune responses to Oxa at the sensitisation phase, and our findings in this study provide useful information for understanding the pathogenesis of allergic dermatitis to contact allergens.

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Author contributions

Satoshi Nunomura and Mino Ohtsubo-Yoshioka performed the experiments and analysed the data; Satoshi Nunomura and Chisei Ra designed the research study;

Satoshi Nunomura wrote the paper; Tadashi Terui and Yoshimichi Okayama analysed the data.

Conflict of Interest

The authors have declared no conflicting interests.

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Legends for illustrations

Figure 1. CHS to Oxa is diminished in $\text{Fc}\gamma\text{R}^{-/-}$ mice.

$\text{Fc}\gamma\text{R}^{-/-}$ and WT mice were sensitised with 3% Oxa or vehicle (EtOH) on their shaved abdomens. Five (A and B) or three (C) days later, these hapten-sensitised mice were challenged with 1% Oxa or vehicle (EtOH) on the ear. Twenty-four hours later, the ear thickness was evaluated with a dial micrometre. In (A) and (C), the numbers in each group show the total number of mice from three independent experiments. The data shown are the means $\pm$ SEM. *, $p < 0.05$ (Student's t-test). (B) Representative haematoxylin and eosin (H&E) staining of the ear tissues. (Scale bar = 100 μm).

Figure 2. $\text{Fc}\gamma\text{R}$ is dispensable for the Oxa-induced migration of DCs and the generation of Oxa-reactive cells

(A) $\text{Fc}\gamma\text{R}^{-/-}$ and WT mice were sensitised with 3% Oxa on their shaved abdomens. As a control, $\text{Fc}\gamma\text{R}^{-/-}$ and WT mice were sensitised with EtOH. Three days later, their inguinal LNs were isolated. The numbers of total LN cells, the percentages of the total LN cells that were $\text{CD11c}^+\text{MHCII}^{\text{hi}}$ DCs, and the total numbers of

CD11c⁺MHCII^{hi} DCs were analysed. (B) The IFN γ , IL-4, and IL-17A production from inguinal LN cells is shown. FcR $\gamma^{-/-}$ and WT mice were sensitised with 3% Oxa as in A. The Oxa-sensitised inguinal LN cells (1×10^6 /ml) were stimulated with Oxa-KLH (300 μ g/ml) or KLH (300 μ g/ml) for 24 hours. In (A) and (B), the numbers in each group show the total number of mice from three independent experiments. The data shown are the means $\pm$ SEM.

Figure 3. Oxa-sensitised FcR $\gamma^{-/-}$ LN cells can transfer CHS to naïve recipients.

FcR $\gamma^{-/-}$ and WT mice were sensitised with 3% Oxa as in Figure 2A. As a control, FcR $\gamma^{-/-}$ and WT mice were sensitised with EtOH. The Oxa-sensitised or unsensitised inguinal LN cells (1×10^7) were injected into naïve WT mice. The mice that received LN cells were challenged with 1% Oxa or EtOH. Twenty-four hours later, the ear thickness was evaluated as in Figure 1A. The numbers in each group show the total number of mice from three independent experiments. The data shown are the means $\pm$ SEM. (B) Representative H&E staining of the ear tissues. (Scale bar = 100 μ m).

Figure 4. The effects of rsFc ϵ RI α administration on the process of sensitisation to Oxa.

(A) Mouse IgE (1 μ g/ml) was incubated with or without rsFc ϵ RI α (0.25 μ g/ml) for 30 min. RBL-2H3 cells (1×10^6 /ml) were incubated with the reaction product for 30 min. After washing with PBS, the cells were stained with 1 μ g/ml of

FITC-conjugated anti-mouse IgE mAb. The amount of surface-bound IgE was analysed using a flow cytometer. The results shown are representative of three independent experiments with similar results. (B) rsFcεRIα was intravenously injected into naïve WT mice. Forty-eight hours later, sera were collected from both mice treated with rsFcεRIα and untreated mice. IgE uncaptured with rsFcεRIα was measured as described in the Materials and Methods. (C) rsFcεRIα (4 µg) or PBS was intravenously injected into Oxa-sensitised or unsensitised WT mice. Forty-eight hours later, these mice were challenged with 1% Oxa or vehicle (EtOH). Twenty-four hours later, the ear thickness was evaluated as in Figure 1A. rsFcεRIα (4 µg) or PBS was intravenously injected into naïve WT mice. Forty-eight hours later, these mice were sensitised with 3% Oxa, and then (D) the number of CD11c⁺MHCII^{hi} DCs in the draining LNs and (E) the transfer of the CHS response to Oxa by the LN cells were analysed as in Figure 2A and Figure 3A, respectively. In (B) - (E), the numbers in each group show the total number of mice from three independent experiments. The data shown are the means ± SEM. *, $p < 0.05$ (Student's t-test).

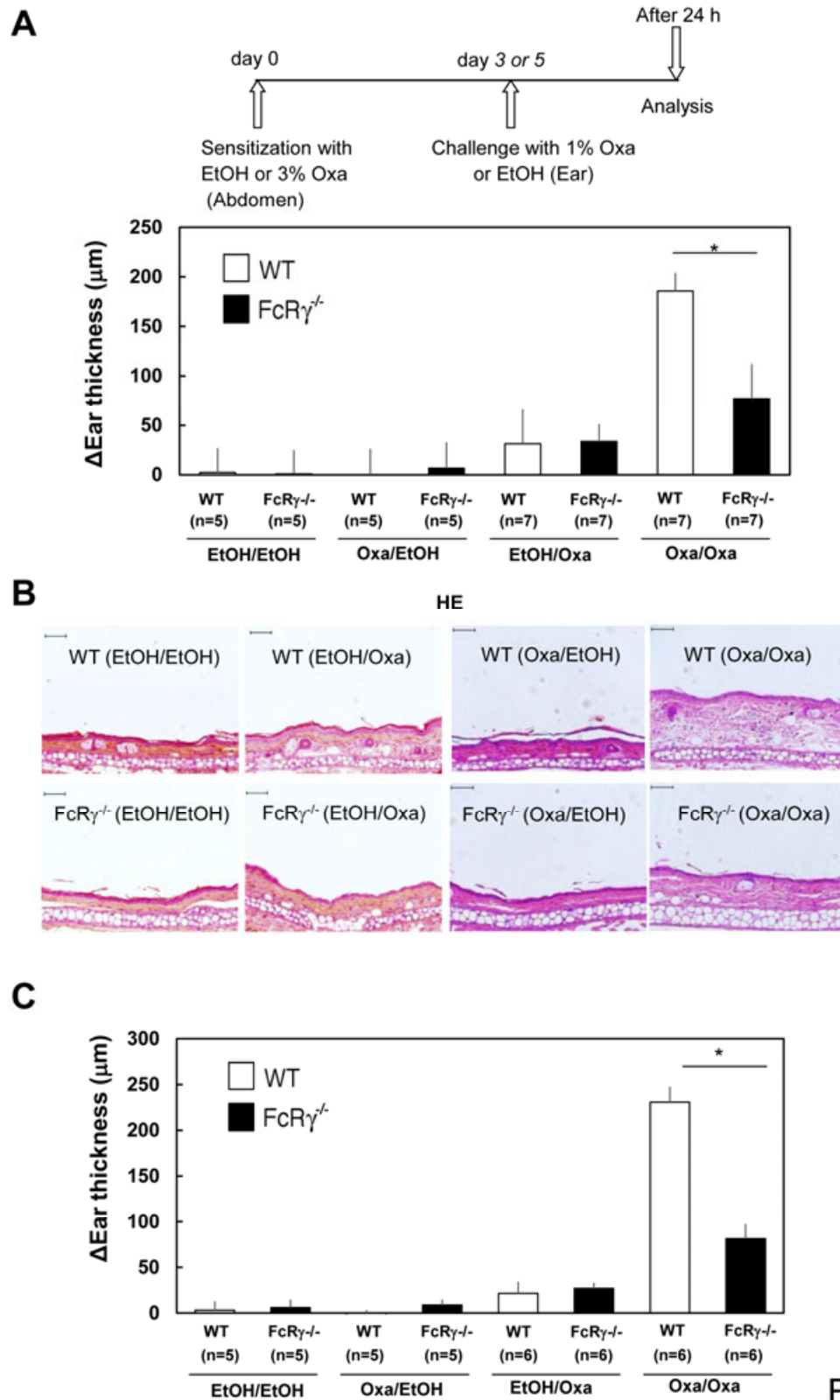
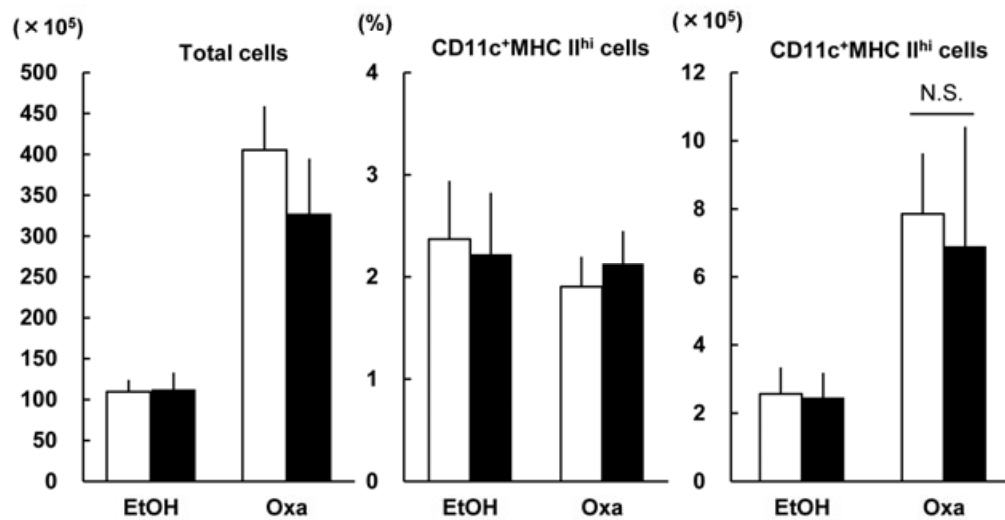


Figure1

A

□ WT (n = 6)
 ■ FcR γ ^{-/-} (n = 8)



B

□ WT (n = 7)
 ■ FcR γ ^{-/-} (n = 7)

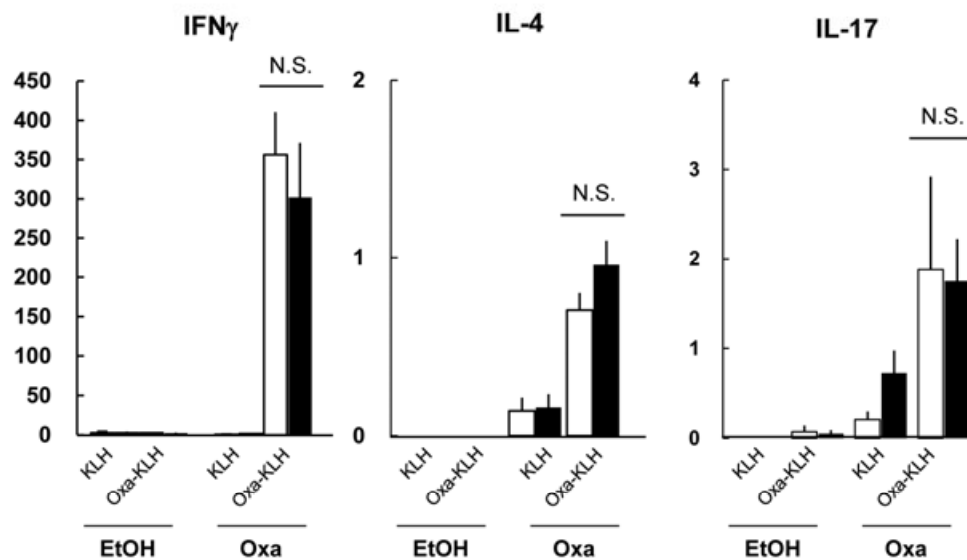
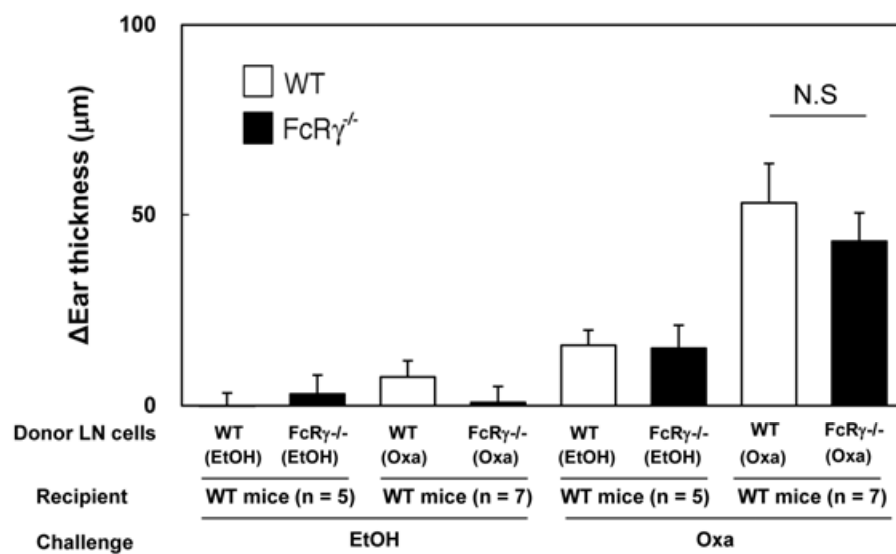


Figure2

A



B

HE

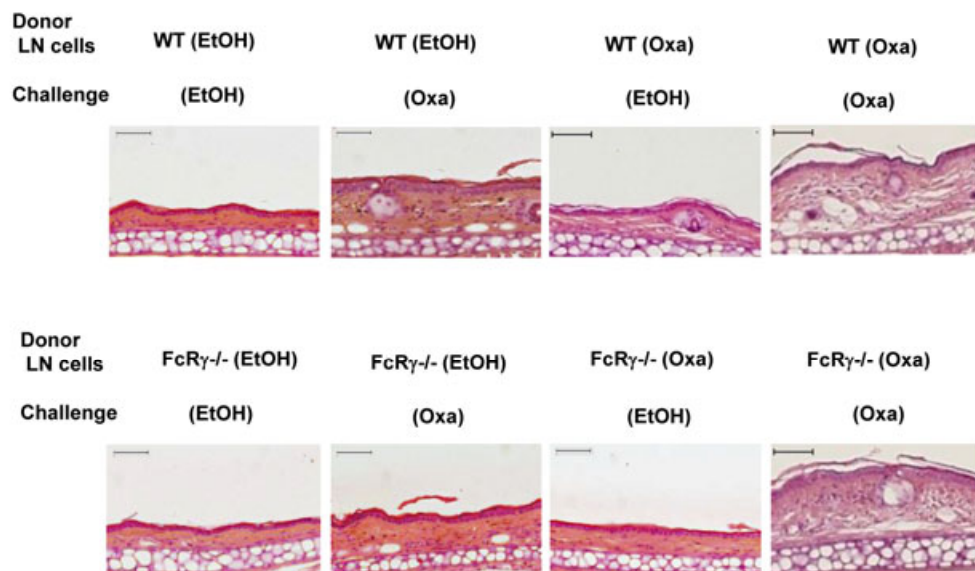


Figure3

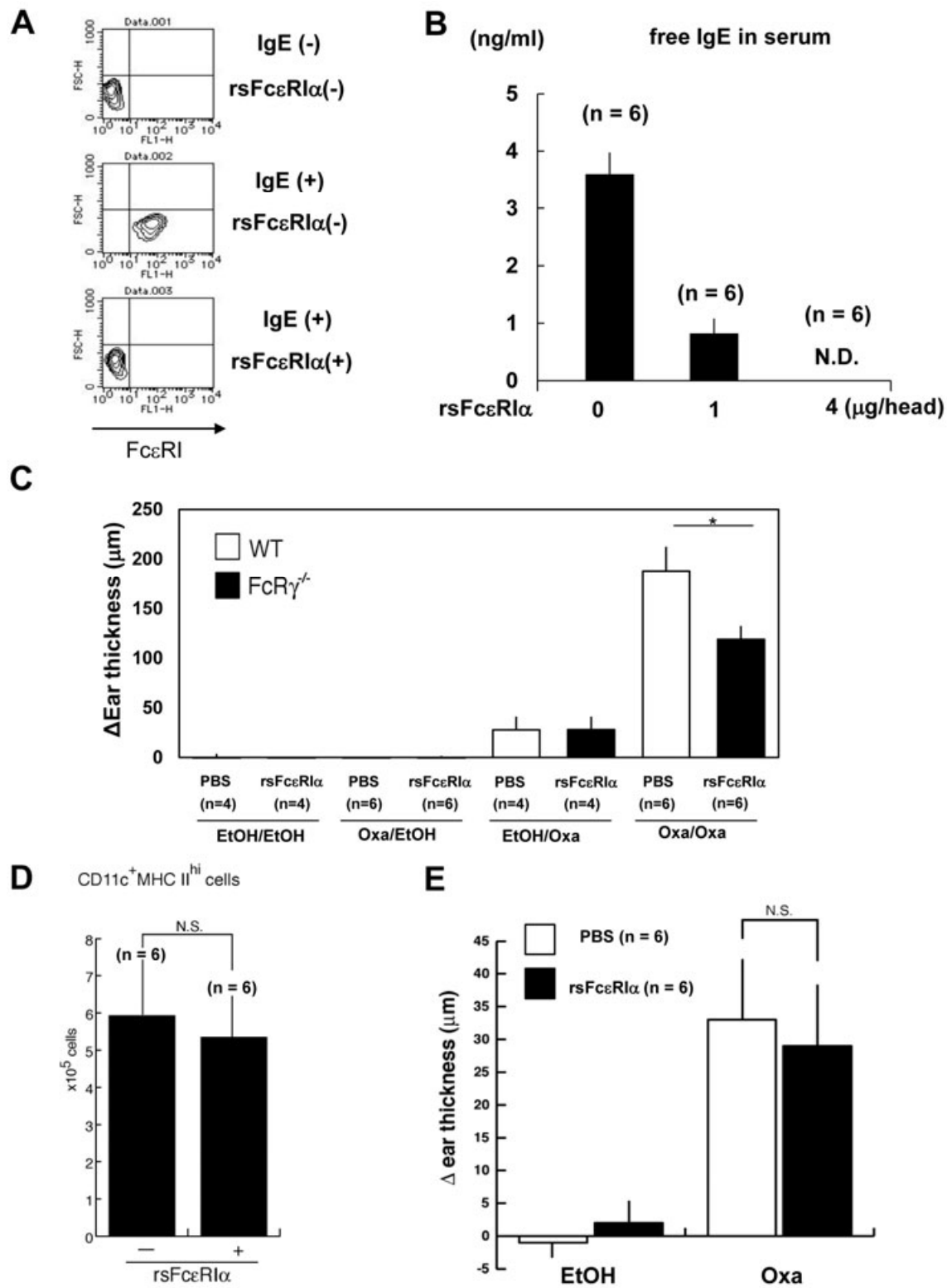


Figure4