

density and diameter, were approximately twice as large in this study as those of normal skin in previous reports such as Liew et al. [6], in which the body sites studied were not the face. The difference in vessel parameters might be caused by the difference in body site or angiographic methodology, and further studies will be needed to distinguish between these possibilities.

In this pilot study, we demonstrated that OCT angiography can be used to evaluate alterations in facial vasculature with age. The evaluation of dermal vascular plexuses by means of OCT angiography has several advantages. It produces site- and depth-specific images with high-resolution. It is non-invasive and non-destructive. Skin specimens are known to shrink approximately 18% in width immediately after surgical excision [9]. Moreover, it has been suggested that blood vessel diameters measured in histological sections are smaller than those measured in OCT images *in vivo*, because of the dehydration of histological sections and collapse of the blood vessels' structure [10]. Therefore, information about vascular structure obtained by OCT angiography should more accurately reflect *in vivo* biological conditions, compared with conventional histological approaches. On the other hand, the time needed to acquire one angiographic image from time-sequential OCT tomograms is typically in the range of several minutes, because the algorithms used to reconstruct the image are based on the analysis of blood flow. Therefore, skin immobilization is an important consideration, and there are some skin regions that may be difficult to immobilize, for example regions around the nose and eye. Furthermore, it should be noted that OCT angiography reconstructs an image of blood vessels by extraction of moving regions, and so it is difficult to distinguish between veins, arteries and postcapillary venules.

In conclusion, our study demonstrates that OCT angiography is a useful technique to non-invasively address vascular alterations in facial skin. Further development of OCT angiography and the accumulation of *in vivo* data would be beneficial for the diagnosis of skin disease, as well as the validation of medical applications.

Conflict of interest disclosure

YH, TY, KK, CK and KK are employed by Shiseido Co., Ltd., which funded this project.

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Letter to the Editor

Requirement of MHC class I on radioresistant cells for granzyme B expression from CD8⁺ T cells in murine contact hypersensitivity



To the editor,

Allergic contact dermatitis (ACD) is a common cutaneous disorder that affects around 15–20% of the general population

worldwide. Haptens, chemical compounds of molecular weight smaller than 500 Da, are assumed to react with self-peptides or –proteins to serve as pathogenic antigens (Ags) in ACD [1,2]. Ag-presented-CD8⁺ T cells at the elicitation phase produce interferon (IFN)- γ and cytotoxic granules such as granzyme B and perforin, and provoke inflammation [2–4]. Dermal dendritic cells (dDCs) are considered essential for the presentation of these haptenized-Ag to CD8⁺ T cells [2,5,6]. On the other hand, given that haptens can modulate peptides that are attached to major histocompatibility complex (MHC) class I [1], and that MHC class I is expressed in a variety of cells other than dDCs, dDC-independent Ag-presentation by radioresistant cells has been assumed in ACD from some *in-vitro* studies [7–9], however, the actual contribution of which *in vivo* is poorly understood.

Abbreviations: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; Ag, antigen; MHC, major histocompatibility complex; IFN- γ , interferon-gamma; $\beta 2m^{-/-}$, beta-2-microglobulin-deficient.

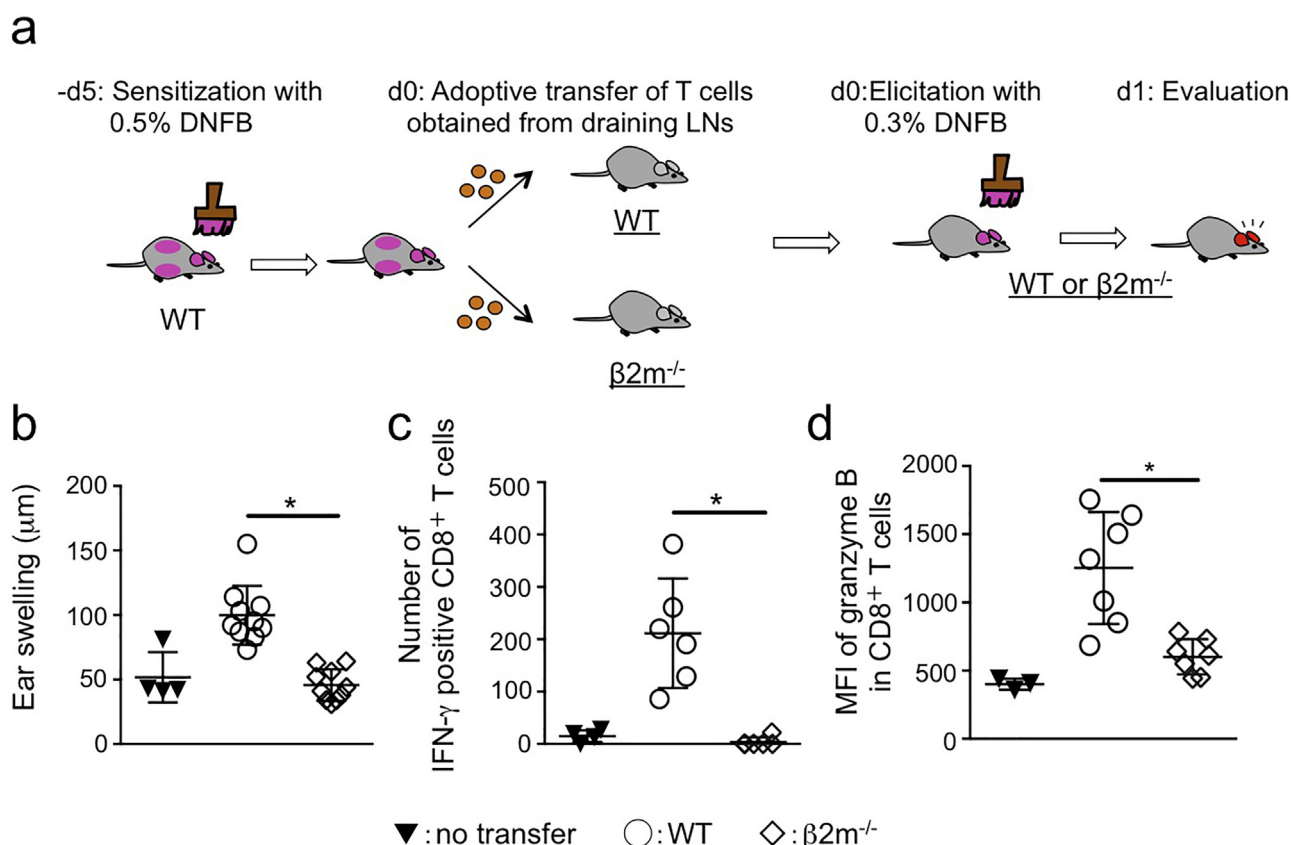


Fig. 1. CHS responses in WT or $\beta 2m^{-/-}$ mice with adoptive transfer of effector T cells. **a**, A schema of CHS in WT or $\beta 2m^{-/-}$ mice with adoptive transfer of T cells from sensitized mice. **b–d**, Ear swelling levels (**b**), the number of IFN- γ positive CD8 $^{+}$ T cells (**c**), and MFI of granzyme B in CD8 $^{+}$ T cells (**d**) in the ear skin of WT and $\beta 2m^{-/-}$ mice transferred with T cells from 0.5% DNFB-sensitized WT mice and elicited with 0.3% DNFB. *, $P < 0.05$. WT mice without adoptive transfer of T cells and elicited with 0.3% DNFB were set as a negative control.

Herein, using a murine contact hypersensitivity (CHS), an animal model of ACD, we attempted to clarify the possibility of dDC-independent Ag-presentation at the elicitation.

We first confirmed the requirement of MHC class I on Ag-presentation in CHS, using $\beta 2$ -microglobulin-deficient ($\beta 2m^{-/-}$) mice that lack MHC class I [10]. To focus on the elicitation phase, we performed an adoptive transfer experiment. T cells from 2,4-dinitro-1-fluorobenzene (DNFB)-sensitized wild-type (WT) mice were adoptively transferred to the recipient WT or $\beta 2m^{-/-}$ mice, which were then applied DNFB to the ear skin for elicitation (Fig. 1a). In $\beta 2m^{-/-}$ mice, the CHS response as estimated by ear swelling levels was significantly abrogated (Fig. 1b). In addition, production of IFN- γ and granzyme B in CD8 $^{+}$ T cells was almost completely abolished in $\beta 2m^{-/-}$ mice (Fig. 1c and d), indicating that MHC class I is actually essential for the elicitation.

Next, we tried to dissect the importance of MHC class I on radioresistant cells and radiosensitive cells using bone marrow chimeras. Bone marrow cells of WT mice or $\beta 2m^{-/-}$ mice were donated into recipient WT mice (WT-to-WT and $\beta 2m^{-/-}$ -to-WT) or $\beta 2m^{-/-}$ mice (WT-to- $\beta 2m^{-/-}$) to establish each chimera. Then, CHS was induced in each chimera by adoptive transfer of T cells from DNFB-sensitized WT mice (Fig. 2a). In WT-to- $\beta 2m^{-/-}$ chimeras, in which radioresistant cells such as Langerhans cells (LCs), keratinocytes, fibroblasts, blood endothelial cells, and lymphatic endothelial cells lack MHC class I, which were confirmed by flow cytometry analysis (Fig. S1), the extent of ear swellings and IFN- γ production from CD8 $^{+}$ T cells were comparable to those of WT-to-WT controls (Fig. 2b and c). The expression of granzyme B in CD8 $^{+}$ T cells, however, was significantly attenuated in WT-to- $\beta 2m^{-/-}$ chimeras (Fig. 2d). These results indicate that MHC class I

on radioresistant cells is dispensable for IFN- γ production, but is required for the granzyme B expression in CD8 $^{+}$ T cells.

We also checked the CHS responses in $\beta 2m^{-/-}$ -to-WT chimeras, in which radiosensitive cells including dDCs were expected to lack MHC class I, therefore, the chimera should exhibit impaired ear swelling responses and IFN- γ production [6]. dDCs in the $\beta 2m^{-/-}$ -to-WT chimera, however, exhibited marginal but significant expression of MHC class I compared to that of $\beta 2m^{-/-}$ mice (Fig. S2). In addition, $\beta 2m^{-/-}$ -to-WT chimeras exhibited normal ear swelling responses and IFN- γ production (Fig. 2b and c), whereas the expression of granzyme B in CD8 $^{+}$ T cells was significantly attenuated (Fig. 2d). These results indicate that, in $\beta 2m^{-/-}$ -to-WT chimeras, dDCs slightly expressed MHC class I, which induced IFN- γ production but was not sufficient to induce granzyme B expression in CD8 $^{+}$ T cells.

Taken together, our data indicate that, in CHS, MHC class I on radioresistant cells mediates granzyme B expression in CD8 $^{+}$ T cells, while MHC class I on radiosensitive cells mediate both IFN- γ and granzyme B production.

Although it remains unknown which radioresistant cells mediate the granzyme B expression from CD8 $^{+}$ T cells in the skin, keratinocytes are one of the candidates considering their capacity to present haptens *in vitro* [7,8]. Given that infiltrative T cells are frequently observed close to blood vessels in CHS and ACD [6], and that there is a report that suggest the contribution of endothelial cells for Ag-presentation [9], blood endothelial cells might also be participated in Ag-presentation and granzyme B expression. LCs may be another candidate population, because LCs possess strong Ag-presentation ability *in vitro*. However, the contribution of LCs seems quite limited, because CHS responses including ear swelling

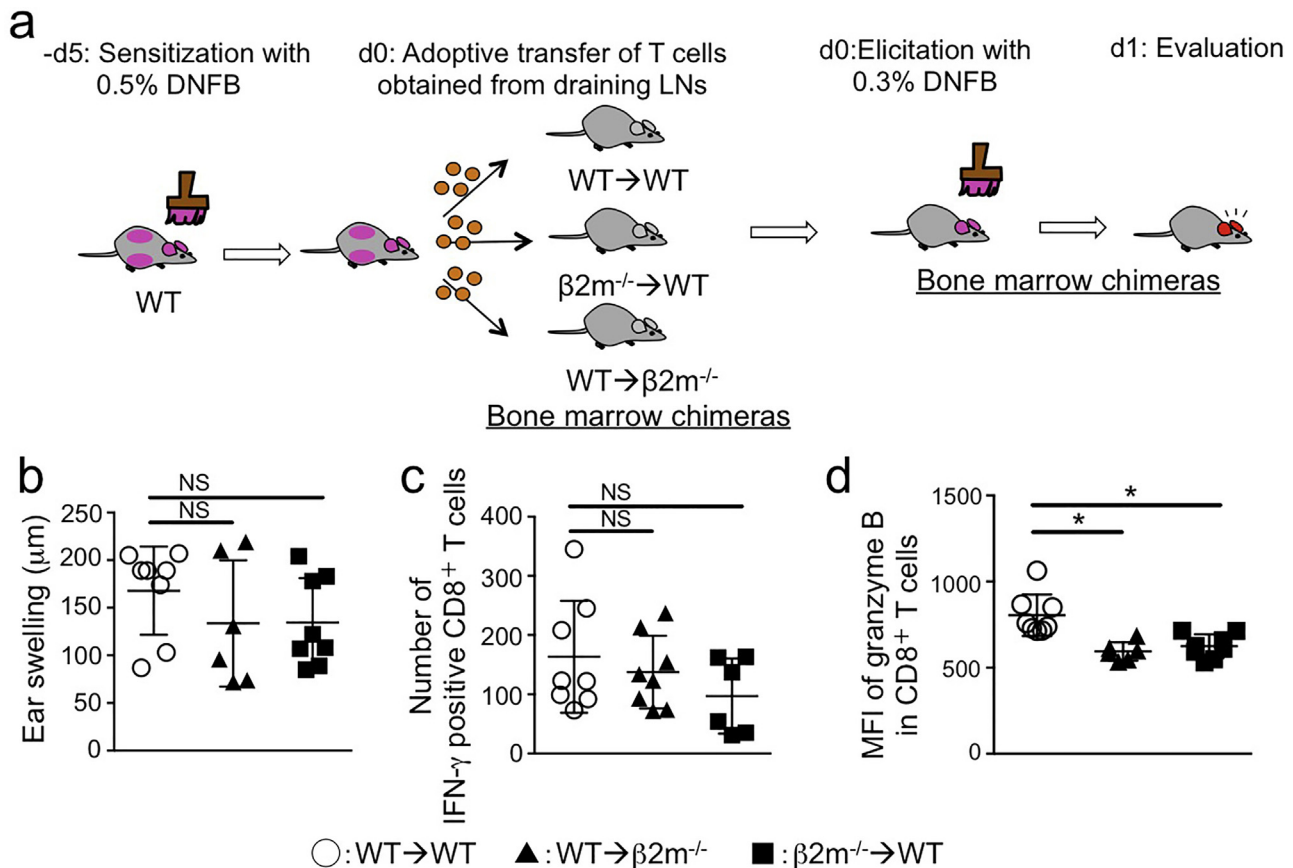


Fig. 2. ChS responses in bone marrow chimeras with adoptive transfer of effector T cells. **a**, A schema of ChS in bone marrow chimeras (WT-to-WT, $\beta 2m^{-/-}$ -to-WT, and WT-to- $\beta 2m^{-/-}$) with adoptive transfer of T cells from sensitized mice. **b–d**, Ear swelling levels (**b**), the number of IFN- γ positive CD8 $^{+}$ T cells (**c**), and MFI of granzyme B (**d**) in CD8 $^{+}$ T cells in the ear skin of chimeras. *, $P < 0.05$. NS, not significant.

responses, IFN- γ production, and granzyme B expression were not decreased under LC-depleted condition using Langerin-diphtheria-toxin-receptor transgenic (DTR) mice (please see supplemental method and Fig. S3a–c).

At first glance, it seems curious that $\beta 2m^{-/-}$ -to-WT chimeras exhibited normal ear swelling responses and IFN- γ production, since this chimera should not express MHC class I on dDCs, and our previous data indicated that dDCs are essential for IFN- γ production from CD8 $^{+}$ T cells at elicitation [6]. Although the reason for this discrepancy remains unclear, our flow cytometry data suggest that intercellular trogocytosis of MHC class I, a phenomena in which $\beta 2m^{+/+}$ cells provides $\beta 2m^{-/-}$ cells with functional MHC class I [Supplemental references, S1 and S2], might have happened in $\beta 2m^{-/-}$ -to-WT chimeras, which might have provided dDCs in $\beta 2m^{-/-}$ -to-WT chimeras with a capacity for Ag-presentation to induce IFN- γ production from CD8 $^{+}$ T cells. The expression level of MHC class I on dDCs in $\beta 2m^{-/-}$ -to-WT chimeras, on the other hand, may not be enough to induce full expression of granzyme B.

Impaired granzyme B expression in WT-to- $\beta 2m^{-/-}$ and $\beta 2m^{-/-}$ -to-WT chimera did not affect ear swelling responses, which is consistent with a previous report that a single defect in granzyme B did not affect the overall ChS reaction in mice [3]. However, the importance of granzyme B seems much more pronounced in human ACD rather than it is in mouse ChS [4]. Therefore, Ag-presentation by radioresistant cells may be an important determinant of the inflammation in ACD, and might have to be considered further to clarify the complex immune responses in ACD.

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Conflict of interest

The authors declare no conflict of interest.

Study approval

All experimental procedures were approved by the institutional Animal Care and Use Committee in Kyoto University Faculty of Medicine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jdermsci.2018.01.013>.

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SUPPLEMENTAL MATERIALS

Methods

Mice

Female C57BL/6-background mice were used in this study, which were purchased from OrientalBioService (Kyoto, Japan). $\beta 2m$ -deficient mice [1], and Langerin-diphtheria-toxin-receptor transgenic (DTR) mice [2] were described previously. To deplete langerin positive cells (Langerhans cells and langerin positive dermal dendritic cells) *in vivo*, 1000 ng of diphtheria toxin was intraperitoneally injected to Langerin-DTR mice a day before elicitation.

Preparation of bone marrow chimeras

To establish bone marrow chimeras, a single-cell suspension generated from the bone marrow cells of femurs and tibias from WT or $\beta 2m^{-/-}$ mice was injected intravenously into irradiated 6-week-old $\beta 2m^{-/-}$ mice (WT-to- $\beta 2m^{-/-}$) or WT ($\beta 2m^{-/-}$ -to-WT) mice, respectively. A bone marrow cell suspension from WT mice was also injected into irradiated 6-week-old WT mice as controls (WT-to-WT). In every experiment, recipient mice were irradiated two doses of 550 rads given 3 h apart. Bone marrow cells were intravenously injected at 5×10^6 cells/mouse just after the final irradiation. Chimeras were incubated for at least 12 weeks before use.

CHS responses with adoptive transfer of T cells

CD45.1 mice were sensitized on shaved abdominal and dorsal skin or the ear skin with 25 μ l (the abdominal and dorsal skin) or 20 μ l (the ear skin) 0.5% (wt/vol) DNFB (1-fluoro-2,4-dinitrofluorobenzene ; Nacalai Tesque) dissolved in acetone and olive oil (at a ratio of 4:1), respectively. For adoptive transfer, T cells were magnetically sorted with an autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) from the draining LNs of sensitized CD45.1 mice five days after sensitization, and then were transferred intravenously (1×10^7 cells) into naive WT mice, $\beta 2m^{-/-}$ mice, or bone marrow chimeras. Just after the transfer of T cells, the ears of recipient mice were elicited with 20 μ l 0.3% DNFB.

For the CHS in Langerin-DTR mice, Langerin-DTR mice were sensitized on shaved abdominal skin with 25 μ l 0.5% DNFB five days before elicitation, treated either with

1000 ng of DT or vehicle a day before elicitation, and elicited on the ears with 20 μ l 0.5% DNFB. Langerin-DTR mice without sensitization were set as negative controls.

In every experiment, the ear swelling levels and the production of IFN- γ and granzyme B in CD45.1⁺ (or CD45⁺) CD8⁺ T cells were evaluated 18 h after elicitation.

Cell isolation and flow cytometry

To obtain a single-cell suspension for the T cell analysis, the dorsal halves of mouse ears were digested with 0.33 mg/ml liberase TL (Roche, Basel, Switzerland) and 0.05% DNase I (Sigma-Aldrich, St. Louis) dissolved in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS) and 10 μ g/ml of brefeldin A (Sigma-Aldrich) for 60 min at 37°C and filtered through 70- μ m nylon mesh.

To obtain a single-cell suspension for the analysis of keratinocytes, Langerhans cells, dermal dendritic cells, and dermal structural cells (fibroblasts and blood and lymphatic endothelial cells), the dorsal halves of mouse ears were first floated on 5 mg/ml dispase II (Godo Shusei Co., LTD, Tokyo, Japan) dissolved in RPMI 1640 containing 10% FCS for 30 min at 37°C. Subsequently, the epidermis was manually separated from the dermis by fine tweezers. To obtain an epidermal cell suspension, the epidermis was floated on 0.25% trypsin-EDTA for 8 min at 37°C and filtered through 70- μ m nylon mesh. To obtain a dermal cell suspension, the dermis was digested with 0.33 mg/ml liberase TL dissolved in RPMI 1640 containing 10% FCS for 60 min at 37°C and filtered through 70- μ m nylon mesh.

Cells were stained with the following: anti-mouse antibody to CD4 (GK1.5), CD8 (53-6.7), CD11c (N418), MHC class I (AF6-88.5), MHC class II (M5/114.15.2), Epcam (G8.8), IFN- γ (XMG1.2), CD16/CD32 (2.4G2), CD31 (MEC13.3), PDGFR β (APB5), and gp38 (8.1.1) were purchased from BD Biosciences (San Jose, CA); anti-mouse antibody to CD45 (30-F11), CD45.1 (A20), TCR β (H57-597), E-cadherin (36/E-Cadherin), and Ter119 (TER-119) were purchased from BioLegend (San Diego, CA); anti-mouse antibody to granzyme B (NGZB) was purchased from eBioscience (San Diego, CA). Fixable viability dye (eBioscience) was used to gate out dead cells.

T cells were detected as CD45⁺(or CD45.1⁺) TCR β ⁺CD4⁻CD8⁺ fraction in the suspension obtained from the dorsal halves of the ears. For analysis of intracellular cytokine production, T cells were fixed with Cytofix Buffer and permeabilized with Perm/Wash Buffer according to the manufacturer's protocol (BD Biosciences).

Keratinocytes were detected as CD45⁻E-cadherin⁺ fraction and LCs were detected as CD45⁺Epcam⁺ fraction in the epidermal cell suspension. In the dermal cell suspension, dermal dendritic cells were detected as CD45⁺CD11c⁺MHC classII⁺ fraction, fibroblasts as CD45⁻CD31⁻gp38⁻PDGFR β ⁺ fraction, blood endothelial cells as CD45⁻CD31⁺gp38⁻Ter119⁻ fraction, and lymphatic endothelial cells as CD45⁻CD31⁺gp38⁺Ter119⁻ fraction.

Flow cytometry was performed using LSR Fortessa (BD Bioscience) and analyzed by FlowJo software (Tree Star, Ashland, OR).

General experimental design and statistical analysis

For animal experiments, a sample size of three to eight mice per group was used on the basis of past experience in generating statistical significance. Mice were randomly assigned to study groups and no specific randomization or blinding protocol was used.

Data are presented as means $\pm$ SDs and each data point is representative of three independent experiments. Prism software (GraphPad) was used for statistical analyses. Normal distribution was assumed a priori for all samples. An unpaired parametric t-test was used for comparison of data sets. *P* values of less than 0.05 were considered significant.

Study approval

All experimental procedures were approved by the institutional Animal Care and Use Committee in Kyoto University Faculty of Medicine.

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Supplemental figure legends

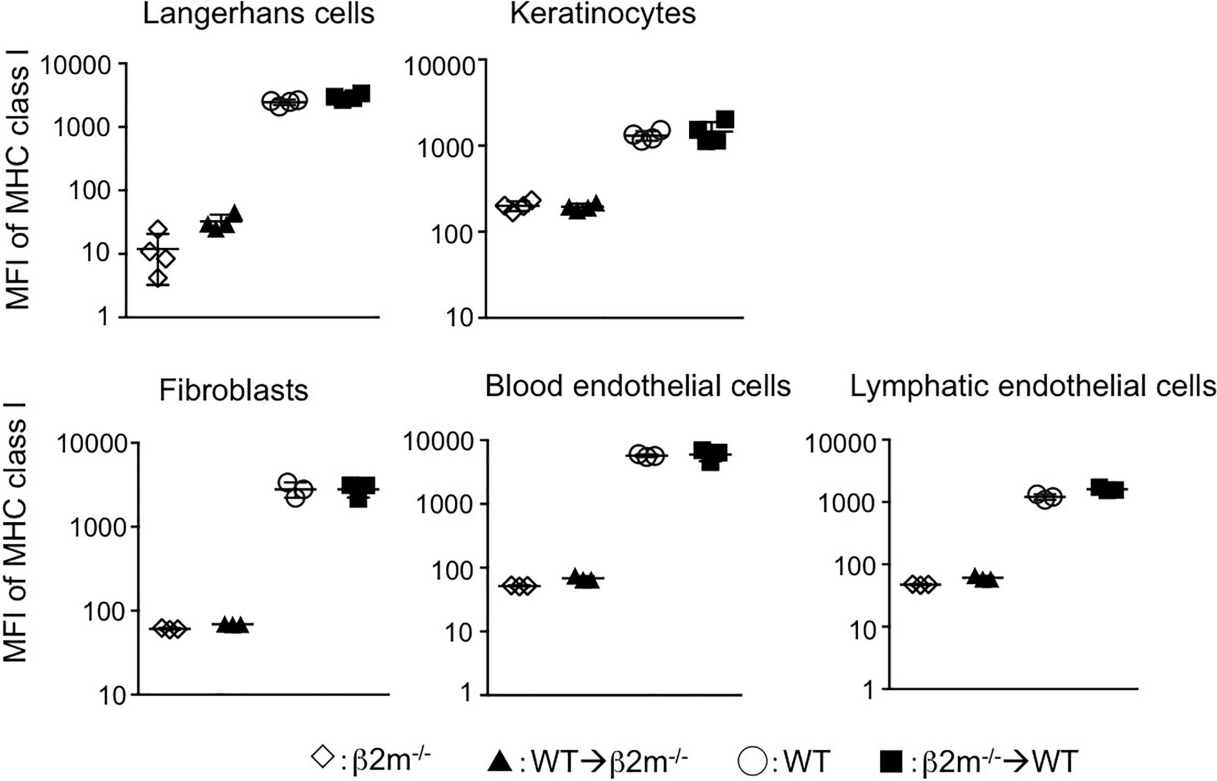
Figure S1. MHC class I expression of cutaneous radioresistant cells in bone marrow chimeras. MFI of MHC class I on structural cells, such as Langerhans cells, keratinocytes, fibroblasts, blood endothelial cells, and lymphatic endothelial cells in $\beta 2m^{-/-}$ mice, WT mice, and chimeras.

Figure S2. MHC class I expression of dermal dendritic cells in bone marrow chimeras. Representative plots of MHC class I expression (**a**) and MFI of MHC class I (**b**) on dDCs extracted from murine ear dermis as evaluated by flow cytometry. *, $P < 0.05$.

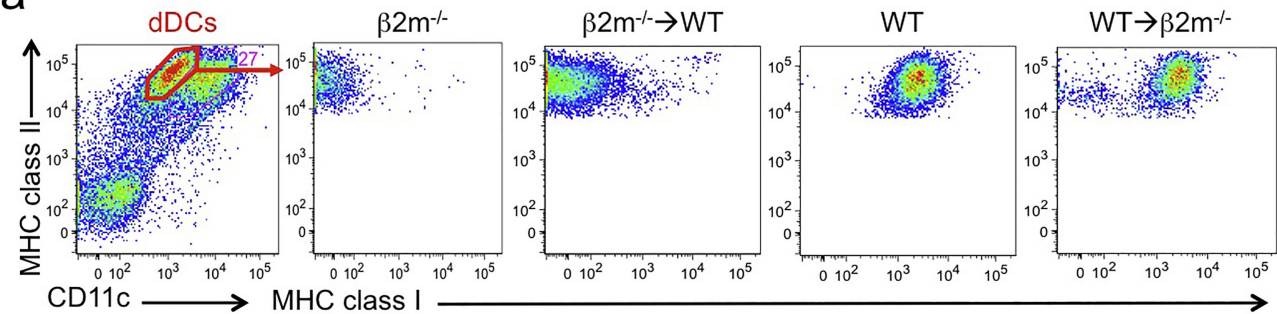
Figure S3. CHS responses in Langerin-DTR mice. a-c, Ear swelling levels (**a**), the number of IFN- γ positive cells (**b**), and MFI of granzyme B (**c**) in CD8⁺ T cells in the ear skin of Langerin-DTR mice after CHS responses. Langerin-DTR mice without sensitization were set as negative controls.

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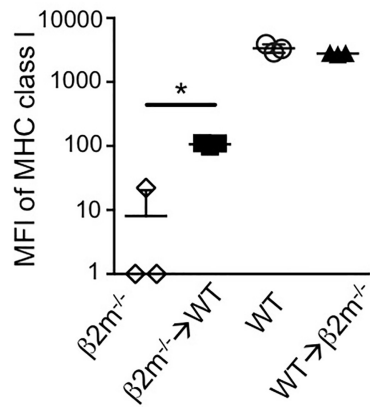
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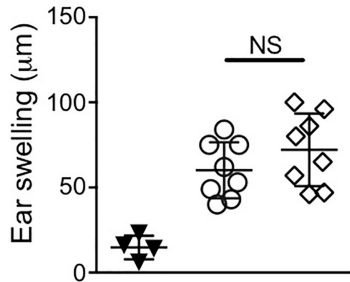


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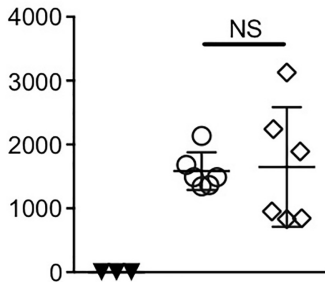


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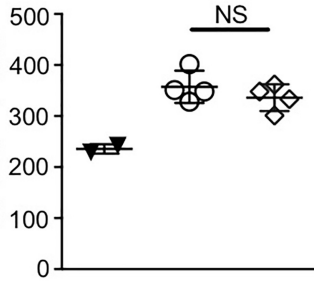


a**b**

Number of
IFN- γ positive CD8⁺ T cells

**c**

MFI of granzyme B
in CD8⁺ T cells



▼ : no sensitization

○ : vehicle i.p.

◇ : DT i.p.