

Journal Pre-proof

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PII: S0091-6749(21)00178-0

DOI: <https://doi.org/10.1016/j.jaci.2020.12.654>

Reference: YMAI 14966

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 25 May 2020

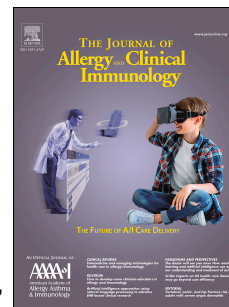
Revised Date: 14 October 2020

Accepted Date: 1 December 2020

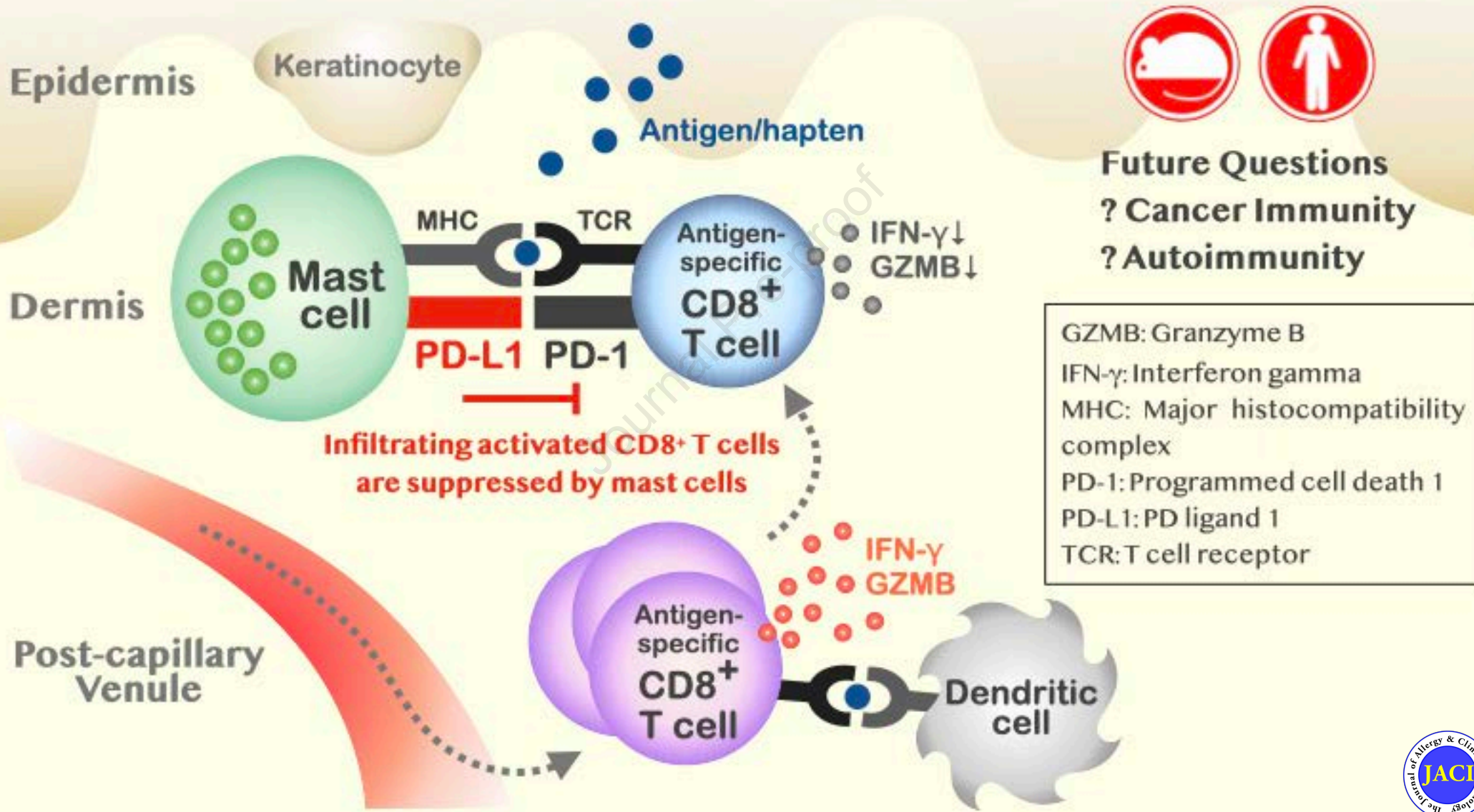
Please cite this article as: Hirano T, Honda T, Kanameishi S, Honda Y, Egawa G, Kitoh A, Nakajima S, Otsuka A, Nomura T, Dainichi T, Yaguchi T, Inozume T, Kataoka TR, Tamada K, Kabashima K, PD-L1 on mast cells suppresses effector CD8⁺ T-cell activation in the skin in murine contact hypersensitivity, *Journal of Allergy and Clinical Immunology* (2021), doi: <https://doi.org/10.1016/j.jaci.2020.12.654>.

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PD-L1 on mast cells negatively suppresses CD8⁺ T-cell activation in the skin



PD-L1 on mast cells suppresses effector CD8⁺ T-cell activation in the skin in murine contact hypersensitivity

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Conflicts of Interest: none

Funding Sources: This work was supported by Japan Society for the Promotion of
Science KAKENHI (JP19K08790, JP15H05906 [T.H.], 263395 [K.K.]), Grants-in-Aid
for Scientific Research (15H05790, 15H1155, 15K15417 [K.K.], JP15H05897), Japan
Agency for Medical Research and Development (AMED) (19ek0410062s0201 [T.H.],
16ek0410011h0003, 16he0902003h0002 [K.K.]), Takeda Science Foundation ([K.K.])
and Advanced Research and Development Programs for Medical Innovation
(AMED-CREST) (JP19gm1210006 [K.K.]).

45 **Word counts: 4318**

46 **Figure number: 5**

47 **Table number: 1**

48 **Supplemental Figure number: 4**

49 **Supplemental Table number: 1**

50

Abstract

Background: The programmed cell death-1 (PD-1)/PD-ligand 1 (PD-L1) pathway is known to inhibit the activation of effector CD8⁺ T cells. However, it remains unclear how this regulatory pathway is involved in the pathophysiology of CD8⁺ T cell-mediated inflammatory skin diseases.

Objective: To elucidate the mechanisms by which the PD-1/PD-L1 pathway exerts its regulatory roles in CD8⁺ T cell-mediated cutaneous immune responses.

Methods: PD-L1 deficient (*Pdl1*^{-/-}) mice were used for the murine contact hypersensitivity (CHS) model. Inflammatory responses such as interferon- γ (IFN- γ) production from CD8⁺ T cells in the skin was evaluated by flow cytometry.

Results: *Pdl1*^{-/-} mice exhibited exacerbated ear swelling and increased number of IFN- γ ⁺ CD8⁺ T cells in the skin compared to wild-type (WT) mice. Adoptive T cell transfer experiments revealed the involvement of the PD-1/PD-L1 pathway in the elicitation phase of CHS. Bone marrow chimera experiments showed that PD-L1 on radio-resistant cells was responsible for this regulatory pathway. Flow cytometric analysis revealed that among the radio-resistant cells in the skin, PD-L1 was most highly expressed on mast cells (MCs) before and after elicitation. Administration of anti-PD-L1 blocking antibody during the elicitation phase significantly enhanced ear swelling responses and increased the number of IFN- γ ⁺ CD8⁺ T cells in the skin of WT mice, while no significant effects were observed in MC-deficient mice (WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J and C57BL/6-*Kit*^{W^{sh}}/*W*^{sh}). High expression level of PD-L1 on human skin MCs was confirmed by database analysis and immunohistochemical

analysis.

Conclusion: PD-L1 on MCs negatively regulates CD8⁺ T-cell activation in the skin.

Key messages: The PD-1/PD-L1 pathway exerts its regulatory roles in the elicitation phase in CHS. PD-L1 on MCs is important for the regulation of effector CD8⁺ T cell activation in the skin.

Capsule summary

MCs regulate excess CD8⁺ T-cell activation in the skin through the PD-1/PD-L1 pathway in contact hypersensitivity. PD-L1 on MCs may play regulatory roles for cutaneous allergic inflammation.

Keywords: PD-1, PD-L1, mast cell, contact hypersensitivity

Abbreviations

APC, Antigen presenting cell; BM, Bone marrow; BMMC, Bone marrow-derived mast cell; CHS, Contact hypersensitivity; CTLA-4, Cytotoxic T-lymphocyte protein 4; DC, Dendritic cell; dLN, Draining lymph node; DNBS, Dinitrobenzene sulfonic acid; DNFB, 2,4-Dinitrofluorobenzene; ELISA, Enzyme-linked Immunosorbent assay; GZMB, Granzyme B; IFN- γ , interferon-gamma; KC, Keratinocyte; LC, Langerhans cell; LN, Lymph node; MART-1, Melanoma antigen recognized by T cells-1; MC, Mast cell;

96 PBMC, Peripheral blood mononuclear cell; PD-1, Programmed cell death 1; PD-L1, PD
97 ligand 1; PMA, Phorbol12-myristate13-acetate; Sash, C57BL/6-*Kit*^{W^{-sh}}/*W*^{-sh}; SD,
98 standard deviation; Treg, regulatory T cell; WT, Wild-type; W/W^v ,
99 WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J.
100

INTRODUCTION

The programmed cell death-1 (PD-1, CD279)/PD-ligand 1 (PD-L1, B7-H1, CD274) pathway is a negative regulator that coordinates the balance between T cell activation and tolerance.^{1,2} PD-1 expressed on T cells binds PD-L1 that can be expressed on various tissue-resident cells as well as on antigen-presenting cells (APCs).^{1,2} Upon ligation with PD-L1, PD-1 transduces signals that inhibit the proliferation of T cells and their effector functions such as interferon- γ (IFN- γ) production and cytotoxicity.¹⁻³ Because PD-1 is expressed on effector T cells but not naïve T cells, it is thought that PD-1 regulates late-phase immune responses such as the effector phase in the peripheral tissues, rather than the early induction phase in the lymphoid organs.^{3,4}

Recent studies have highlighted the importance of this regulatory pathway in various pathological conditions, particularly in tumor immunity, where PD-L1 expressed on tumor cells can inhibit the effector functions of CD8⁺ T cells, leading to the progression of tumors.^{5,6} Based on this understanding, the blockade of the PD-1/PD-L1 pathway is now widely utilized in cancer therapy for enhancing the anti-tumor activity of cytotoxic CD8⁺ T cells.⁷⁻⁹ Recent studies show that PD-L1 on host cells such as dendritic cells (DCs) are also important for this regulation.¹⁰ However, it remains unclear how this regulatory pathway is involved in the pathophysiology of CD8⁺ T cell-mediated inflammatory skin diseases, such as contact dermatitis.

Murine contact hypersensitivity (CHS) is a hapten-induced type IV hypersensitivity reaction in the skin mainly mediated by CD8⁺ T cells, and one of the most frequently used mouse models for the analysis of adaptive immune responses in the skin.¹¹ It is

also used as a mouse model of contact dermatitis. CHS consists of two phases; the sensitization and elicitation phases. In the sensitization phase, naïve T cells are presented with antigens in the skin draining lymph nodes (dLNs) by APCs migrated from the skin, and differentiate into antigen-specific effector T cells. In the elicitation phase, skin-infiltrating effector T cells are presented with antigens by APCs *in situ*, leading to their activation.^{12,13} The activated effector T cells then produce inflammatory cytokines including IFN- γ and granzyme B (GZMB). Various skin-resident cells, such as mast cells (MCs),¹⁴⁻¹⁶ keratinocytes (KCs)^{17,18} and macrophages,¹² are also involved in the development of CHS.¹¹ Using this model, several studies have investigated the involvement of the PD-1/PD-L1 pathway in murine or human contact dermatitis.¹⁹⁻²³ For instance, exacerbation of CHS responses in PD-1-deficient mice or WT mice treated with an anti-PD-1 blocking antibody has been reported by several groups,^{22,23} suggesting that the PD-1/PD-L1 pathway plays regulatory roles in the context of CHS. However, the phase of CHS (sensitization or elicitation) in which the PD-1/PD-L1 pathway primarily affects CD8⁺ T cells is inconsistent in these previous studies. More importantly, the primary supplier of PD-L1 at the cellular level has not been clarified in these studies. KCs and DCs were proposed as the cell populations that express PD-L1 and play roles in the regulation of CD8⁺ T cell activation in CHS.^{19,20} However, because those conclusions were based on analysis of transgenic mice overexpressing PD-L1 in KCs or DCs, it is still unclear whether KCs or DCs play such regulatory roles in physiological condition. Thus, the underlying mechanisms of how the PD-1/PD-L1 pathway regulates immune responses in CHS remain unclear.

145 Here, we investigated the mechanisms by which the PD-1/PD-L1 pathway regulates
146 CD8⁺ T cells in CHS. We found that MCs play a primary regulatory role in the CD8⁺
147 T-cell activation in the skin during the elicitation phase of CHS. Our results suggest a
148 novel regulatory function of MCs via PD-L1 in cutaneous immunity.
149

METHODS

Mice

All experiments were conducted on 6- to 12-wk-old female mice unless otherwise described. C57BL/6 (B6) N mice, B6N-Tyr^{c-Brd}/BrdCrCrl (B6 Albino) mice and WBB6F1/J-Kit^W/Kit^{W-v}/J (W/W^v) and their littermate mice were purchased from Japan SLC (Kyoto, Japan) or OBS (Kyoto, Japan). PD-L1 (B7-H1)-deficient (*Pdl1*^{-/-}) mice on B6 background were originally generated and kindly provided by Dr. Lieping Chen.^{24,25} C57BL/6-Kit^{W^{sh}}/W^{sh} (Sash) mice were provided by RIKEN BioResource Research Center (Ibaraki, Japan). B6.Cg-Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}/J mice (007908; Jackson Laboratory, Bar Harbor, Maine, USA) were crossbred with EIIa-cre mice (Jackson Laboratory, Bar Harbor, Maine, USA) to generate mice systemically expressing fluorescent proteins (tdTomato mice). All experimental protocols were approved by the Animal Experimentation Committee of Kyoto University and all animal experimental procedures were performed according to the Animal Protection Guidelines of Kyoto University.

CHS

1-Fluoro-2,4-dinitrobenzene (DNFB) (Nacalai Tesque, Kyoto, Japan) was used as hapten. Mice were sensitized with 25 µl of 0.5% (wt/vol) DNFB in 4:1 acetone/olive oil (vol/vol) on their shaved abdomens, and then elicited with 20 µl of 0.3% (wt/vol) DNFB on both sides of each ear 5 days after sensitization. Ear thickness was measured for each mouse before and after elicitation at the indicated time points using a thickness

gauge.

Preparation of single-cell suspensions

The dorsal halves of mouse ears were digested with 0.33 mg/ml of Liberase TL (Roche, Basel, Switzerland) dissolved in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 0.05% DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 70 min at 37 °C. To obtain a single-cell suspension from the epidermis, the dorsal halves were floated on 0.25% trypsin-EDTA for 30 minutes at 37 °C, and then the epidermis was manually collected from the dermis in advance. For intracellular staining of cytokines, ear pinnae were incubated in digestion solution with 10 µg/ml of Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA). After that incubation, the cell suspension was filtered through a 40-µm cell strainer.

Flow cytometric analysis

Non-specific antibody binding was blocked with anti-mouse CD16/CD32 (2.4G2) antibodies (BD Biosciences) for mouse cells or FcR Blocking Reagent, human (Miltenyi Biotec, Bergisch Gladbach, Germany) for human cells before surface staining. After surface staining with antibodies (listed in this article's Online Repository), cells were fixed with Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA, USA) and stained intracellularly. Samples were assessed with a BD LSR Fortessa cell analyzer (BD Biosciences, San Jose, CA, USA) and data were analyzed with FlowJo software (BD Biosciences, San Jose, CA, USA). Numbers of each cell subset were evaluated

using Flow-Count Fluorophores (Beckman Coulter, Brea, CA, USA).

***In vitro* stimulation of hapten-specific T-cells in mouse**

CD8⁺ T cells from dLNs of mice 5 d after sensitization with DNFB were enriched by magnetic cell sorting (Pan T Cell Isolation Kit II, mouse and then CD8a (Ly-2) MicroBeads, mouse; Miltenyi Biotec, Bergisch Gladbach, Germany). These cells were cocultured with bone marrow-derived mast cells (BMMCs) at the indicated ratio with or without 100 µg/ml of dinitrobenzene sulfonic acid (DNBS; Alfa Aesar, Ward Hill, MA, USA) for 72 h, and the supernatants were collected for the measurement of IFN-γ production by enzyme-linked immunosorbent assay (ELISA).

***In vitro* stimulation of antigen-specific T-cells in human**

Human mast cells (LUVA cells)²⁶ were pulsed for 20 minutes with 100 µg /mL of either HLA-A*0201-binding peptide, melanoma antigen recognized by T cells-1 peptides (MART-1, MART-1₂₇₋₃₅: ELAGIGILTV) or control peptides (HIV gag₇₇₋₈₅: SLYNTVATL). Next, 2 × 10⁴ of MART-1-specific T cells (MART-Ts), which were generated by transducing the HLA*0201-restricted MART-1-specific TCR gene into activated PBMC-derived T cells were cocultured with 1 × 10⁵ of the peptide-pulsed LUVA cells in AIM-V medium (ThermoFisher Scientific, Waltham, MA) with 10% human-AB serum (Gemini Bio, West Sacramento, CA, USA) with 10 µg/mL of anti-human PD-L1 blocking antibody or isotype control antibody. The supernatants were collected after a 24-h culture period for the measurement of IFN-γ production by

ELISA.

Research ethics

This study protocol was approved by the ethics committee of the Kyoto University Graduate School of Medicine and participating institutions. Human samples were collected according to the procedure specified in the study protocol. Written informed consent forms were obtained from all patients.

Statistical analysis.

Unless otherwise described, results are presented as means $\pm$ standard deviations (SDs). For a comparison between 2 groups, the two-tailed Mann-Whitney U test was performed. For 2×2 comparison (FIG 4A), two-way ANOVA was performed in order to assess the possible interactive effect of two factors - mouse strain and antibody treatment. For these data, the results of Bonferroni's multiple comparison test within a strain was also shown in order to evaluate the effect of antibody treatment on each strain. A value of $P < 0.05$ was considered to indicate statistical significance. NS indicates not significant. All statistical tests were performed with GraphPad Prism 8 software (GraphPad Software, La Jolla, CA).

Further information on the methods used in this study are provided in the Methods section in this article's Online Repository.

- Antibodies

- 238 • Gating strategies of ear skin cells by Flow Cytometry
- 239 • Histologic examination of murine ear skin
- 240 • Adoptive transfer experiments
- 241 • Generation of BM chimeric mice
- 242 • Generation of BMMCs
- 243 • ELISA
- 244 • Two-photon microscopic analysis
- 245 • Culture of human cells
- 246 • Immunohistochemistry for human skin
- 247 • Database analysis
- 248
- 249

RESULTS

Loss of PD-L1 exacerbates CHS responses

To examine the involvement of the PD-1/PD-L1 pathway in the development of CHS induced by DNFB, we first evaluated the CHS responses in *Pdl1*^{-/-} mice. Compared with wild-type (WT) mice, *Pdl1*^{-/-} mice exhibited significantly increased and prolonged inflammation after elicitation (FIG 1A). Histological analysis revealed more severe swelling in the ear skin of *Pdl1*^{-/-} mice than WT mice 24 h after elicitation (FIG 1B). In addition, flow cytometric analysis showed an increased percentage and number of IFN- γ -producing and GZMB-producing CD8⁺ T cells in the skin of *Pdl1*^{-/-} mice when compared to WT mice (FIG 1C). On the other hand, no significant differences were observed in the ear swelling between WT and *Pdl1*^{-/-} mice in antigen-independent irritant reactions induced by DNFB or phorbol 12-myristate 13-acetate (PMA) (see FIG E1 in this article's Online Repository). Together, these results indicate that the PD-1/PD-L1 pathway negatively regulates antigen-specific immune responses in CHS.

PD-1/PD-L1 pathway exerts regulatory functions during the elicitation phase of CHS

Next, we examined the phase of CHS in which the PD-1/PD-L1 pathway is primarily involved. First, we checked the number and composition of CD8⁺ T cells in the dLNs of WT and *Pdl1*^{-/-} mice by flow cytometry five days after sensitization with DNFB. There were no significant differences in the number of total CD8⁺ T cells and CD8⁺CD44⁺CD62L⁺ memory T cells between WT and *Pdl1*^{-/-} mice (FIG 2A). In

addition, the amount of IFN- γ production by LN cells re-stimulated with DNBS, a water-soluble analog of DNFB, was similar between WT and *Pdl1*^{-/-} mice (FIG 2B), suggesting that the absence of PD-L1 did not influence the establishment of sensitization. Next, to examine the involvement of the PD-1/PD-L1 pathway in the elicitation phase, we performed an adoptive transfer experiment (see METHODS in this article's Online Repository). We collected cells from the dLNs of DNFB-sensitized WT or *Pdl1*^{-/-} mice 5 days after sensitization, and adoptively transferred the cells to WT or *Pdl1*^{-/-} mice. Then, the recipient mice were elicited with DNFB, and the ear swelling responses were measured. The extent of the ear swelling response was similar between recipient WT mice which were transferred cells from *Pdl1*^{-/-} mice, and recipient WT mice transferred cells from WT mice. On the other hand, the recipient *Pdl1*^{-/-} mice transferred cells from WT mice exhibited significantly increased ear swelling responses compared to recipient WT mice transferred cells from WT mice (FIG 2C), suggesting a regulatory role of the PD-1/PD-L1 pathway in the elicitation phase. Consistently, PD-L1 blockade during the elicitation phase increased the ear swelling response and the number of IFN- γ ⁺ CD8⁺ T cells in the skin of WT mice (FIG 2D). The expression level of PD-1 on CD8⁺ T cells in the skin was significantly upregulated within 24 h after elicitation (FIG 2E). These results indicate that the PD-1/PD-L1 pathway regulates the activation of CD8⁺ T cells in the skin during the elicitation phase of CHS.

We also examined whether PD-L1 blockade during the elicitation phase affects the number and the expression of co-stimulatory/inhibitory molecules (Cytotoxic T-lymphocyte protein 4 (CTLA-4), Inducible T-cell costimulator (ICOS) and PD-1) of

regulatory T cells (Tregs), because Tregs exert anti-inflammatory roles in the elicitation phase of CHS¹¹. We treated mice with either anti-PD-L1 antibody or control isotype antibody 6 h before elicitation, and then analyzed Tregs in the skin by flow cytometry 24 h after elicitation. No significant difference was observed in the number of Tregs and their expression of co-stimulatory/inhibitory molecules (see FIG E2 in this article's Online Repository). These results suggest that the PD-1/PD-L1 pathway regulates the CHS responses during the elicitation phase independent of Tregs.

PD-L1 on radio-resistant cells is responsible for the regulation of CHS responses

We then examined the cell population that expresses PD-L1 and regulates the activation of CD8⁺ T cells in the skin. To investigate this, we created bone marrow chimeras using WT and *Pdl1*^{-/-} mice, and examined the CHS responses of the chimeric mice. The ear swelling responses of the chimera in which *Pdl1*^{-/-} mice were used as recipient were significantly increased and prolonged compared with those of chimera in which WT mice were used as the recipient, irrespective of the genotype of the donor (FIG 3A). These results indicate that PD-L1 on radioresistant cells in the skin, such as KCs and MCs, is essential for PD-L1-dependent regulation of CD8⁺ T cell activation in the skin in CHS.

We next compared the expression level of PD-L1 on radio-resistant cell populations in the skin and performed time course analysis of its upregulation during the elicitation phase in CHS. As the major radio-resistant cell populations in the skin, we

analyzed KCs, LCs, MC, dendritic epidermal T cells, blood/lymphoid endothelial cells and fibroblasts. The expression of PD-L1 was observed in all the radio-resistant cells to various extents in the steady state without any treatment. Among them, MCs had the highest expression of PD-L1 at steady state, and the expression further increased after elicitation (FIG 3B). Two-photon microscopic analysis suggested the colocalization of MCs and CD8⁺ T cells in the skin during the elicitation phase of CHS (FIG 3C). Based on these findings, we next investigated the role of PD-L1 for the regulation of CD8⁺ T cell function in the skin, focusing on MCs.

Lack of PD-1/PD-L1 signaling does not affect the number of skin MCs or their expression of activation markers during the elicitation phase of CHS

Before going into the detailed analysis of PD-L1 on MCs, we checked whether lack of PD-1/PD-L1 signaling affect the number or functions of MCs in the skin. First, we examined the number of skin MCs in WT and *Pd11*^{-/-} mice in the steady state and 24 h after the elicitation, and found no obvious difference between the groups (see FIG E3A in this article's Online Repository). We next examined degranulation of MCs by histology and the expression of MC-activation markers (CD63 and FcεRI), MHC-class I, and co-stimulatory molecules (ICAM1, CD80 and CD86) by flow cytometry 24 h after the elicitation. No apparent differences were observed in the extent of degranulation as well as the expression levels of these markers between the groups (see FIG E3B and E3C in this article's Online Repository). We further evaluated the extent of MC degranulation and their expression of activation markers 1 hour after the elicitation,

when the degranulation of MCs is reported to be induced in CHS¹⁵, following treatment with anti-PD-L1 antibody or isotype control antibody. Degranulation was detected in some MCs in both groups, but again, no apparent differences were observed in the extent of degranulation, their expression of activation markers, or ear swelling response between the groups (see FIG E3D-F in this article's Online Repository). These results suggest that lack of PD-1/PD-L1 signaling does not affect the activation of MCs in CHS.

Lack of MCs diminishes the effects of PD-L1 blockade on CD8⁺ T cell activation during the elicitation phase of CHS

To investigate the importance of PD-L1 on MC in CD8⁺ T cell activation in the elicitation phase of CHS, we examined the effect of PD-L1 blockade on CHS responses without MCs in the skin, using two different mouse strains that lack MCs (WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J (W/W^v) mice and C57BL/6-*Kit*^{W-sh}/*W*^{sh} (Sash) mice). These mice lack MCs due to a mutation of the *Kit* gene. Administration of anti-PD-L1 blocking antibody in the elicitation phase caused enhanced ear swelling responses and increased production of IFN- γ by CD8⁺ T cells in control littermate WT mice, whereas no significant effects of PD-L1 blockade were observed on the CHS responses in W/W^v mice and Sash mice (FIG 4A and 4B in this article's Online Repository, respectively). These results indicate that the effects of PD-L1 blockade on the CHS response during the elicitation phase are dependent on MCs.

PD-L1 on MCs regulates CD8⁺ T cell activation *in vitro*

We next performed an *in vitro* coculture experiment using BMMCs for further confirmation of the role of PD-L1 on MCs in the regulation of CD8⁺ T cell activation. Purified CD8⁺ T cells from DNFB-sensitized WT mice were cocultured with BMMCs generated either from WT or *Pdl1*^{-/-} mice, and the amount of IFN-γ in the supernatant was measured by ELISA. IFN-γ production was significantly higher for CD8⁺ T cells cocultured with *Pdl1*^{-/-} BMMCs, relative to coculture with WT BMMCs (FIG 4C). These results suggest that MCs exert dual roles for the control of CD8⁺ cell activation. On the one hand, MCs induce CD8⁺ T cell activation, possibly with cross-presentation via MHC class I as previously reported²⁷⁻³⁰. On the other hand, as we found in this study, MCs play regulatory roles in CD8⁺ T cell activation via their PD-L1.

Skin MCs express PD-L1 in human.

Finally, we investigated the possible human relevance of our findings. We first checked the expression of *PD-L1* (*CD274*) in MCs in human skin, using a public database provided by the Functional Annotation of the Mammalian Genome (FANTOM5)³¹⁻³³ project, in which the sets of transcripts of various human cells mapped by Cap Analysis of Gene Expression (CAGE) are available. After excluding stimulated or treated cells, based on their sample names (see METHODS in this article's Online Repository), 847 samples remained, including four MC samples that were collected from the skin (see Table E1 in this article's Online Repository). We also compared *PD-L1* expressions among various cell populations commonly detected in the skin (FIG 5A. Note that

samples other than mast cells were not necessarily collected from the skin). The highest, lowest, mean and median *PD-L1* expression levels of each cell populations were shown in Table 1. We also surveyed another set of data provided by a previous publication³⁴, which revealed a unique transcriptional landscape of MCs by comparison with basophils. Among all the evaluated genes, the *PD-L1* expression level in MC was significantly high ($\log_2(\text{fold change}) > 4$ and $-\log_{10}(\text{p-value}) > 10$) than in basophils (FIG 5B and see METHODS in this article's Online Repository). In addition, immunohistochemical analysis of human skin samples from patients with contact dermatitis revealed the expression of PD-L1 on avidin-positive MCs and the co-localization of CD8⁺ T cells and MCs (FIG 5C), supporting a role of PD-L1 on MCs for the regulation of CD8⁺ T cell activation in the skin.

PD-L1 on human MCs regulates CD8⁺ T cell activation *in vitro*

Lastly, we investigated the role of PD-L1 on human mast cells *in vitro*, using two human MC cell lines (LAD2³⁵ and LUVA²⁶). Constitutive expression of PD-L1 was observed on both LAD2 and LUVA cells by flow cytometry (see FIG E4 in this article's Online Repository). MART-1- or control antigen-pulsed LUVA cells were cocultured with human MART-1-specific T cells (MART-Ts), which were generated by transducing the HLA*0201-restricted MART-1-specific TCR gene into activated PBMC-derived T cells in the presence of anti-PD-L1 antibody or isotype control antibody, and 24 h later, IFN- γ levels in the supernatant were measured by ELISA. IFN- γ production was induced only by treatment with the MART-1 antigen (FIG 5D). Furthermore, the IFN- γ

403 levels were marginally but significantly increased by treatment with anti-PD-L1
404 antibody (FIG 5D). These results suggest that human MCs can present antigens to T
405 cells, and that PD-L1 on MCs play regulatory roles for T cell activation, as observed in
406 mouse studies.

407

DISCUSSION

In this study, we demonstrated the involvement of PD-1/PD-L1 signaling in the negative regulation of CD8⁺ T-cell activation in the skin, using CHS as a model. This regulatory pathway was primarily mediated by PD-L1 on radio-resistant cells. Among radio-resistant cells in the skin, MCs showed the highest expression level of PD-L1, and a lack of MCs abolished the effect of PD-L1 blockade on CD8⁺ T-cell activation in the elicitation phase of CHS. These results suggest that PD-L1 on MCs play important roles in the regulation of CD8⁺ T cell-mediated skin inflammation.

The importance of PD-1/PD-L1 signaling in the regulation of CD8⁺ T cell activation in inflammatory skin diseases has been suggested based on the observation that PD-1/PD-L1 inhibitors prescribed for cancer patients frequently cause various types of skin eruption³⁶, including vitiligo, alopecia, lichenoid dermatitis, and rarely, Stevens-Johnson syndrome/toxic epidermal necrolysis, which are considered CD8⁺ T-cell mediated skin diseases.³⁶⁻⁴² In addition, the patients under treatment with PD-1/PD-L1 inhibitors occasionally exhibit exacerbated drug eruption caused by other medications,⁴³ suggesting that the PD-1/PD-L1 pathway is an important negative regulator for CD8⁺ T cell activation in inflammatory skin diseases. However, when and where this regulatory pathway controls CD8⁺ T cell activation have remained unclear. Our results demonstrate that the negative regulation of PD-1/PD-L1 signaling plays a role in the skin during the effector phase of CHS, rather than in the dLNs during the priming phase.

In contrast to our results, one study reported negative regulation by PD-1/PD-L1

signaling in the sensitization phase of CHS.²² The study reported that mice treated with anti-PD-1 or anti-PD-L1 blocking antibody during the sensitization phase exhibited an increased ear swelling response compared with mice administered with isotype antibody. It remains unclear what caused such discrepant results, however, differences in mouse strains (BALB/c background in the previous study and C57BL/6 background in our study) might be a possible reason, because mouse strain significantly impact the phenotype of CHS.¹¹ In addition, the different extent of skin inflammation might be another possible reason. In the previous study, the increase in ear thickness induced by DNFB was quite small (less than 20 μm), whereas it was around 100-200 μm in our study. Thus, differences in several experimental conditions might be reasons for the discrepancy. Another recent study also reported the involvement of the PD-1/PD-L1 pathway in the sensitization phase in CHS, in which PD-1-deficient (*Pd1*^{-/-}) mice exhibited enhanced CHS responses.²³ However, the numbers of CD4⁺ and CD8⁺ T cells in the dLNs in *Pd1*^{-/-} mice were significantly increased even at steady state. These data suggest that the baseline immune status of *Pd1*^{-/-} mice was activated,^{44,45} which might have affected the phenotype of CHS in these mice.

The expression of PD-L1 on skin MCs have previously been reported in both mice^{1,46} and humans.⁴⁷ In a human study, the low expression of PD-L1 on MCs in patients with alopecia areata was observed by immunohistochemistry analysis⁴⁷, suggesting a suppressive potential of MCs on pathogenic CD8⁺ T cells; however, its physiological significance has remained unclear. Here, we showed that PD-L1 on MCs is involved in the regulation of CD8⁺ T cell activation in the skin, at least in the context

of CHS. In addition to the pro-inflammatory roles in the elicitation phase of CHS,^{15,29} MCs may exert anti-inflammatory functions through the PD-1/PD-L1 pathway to finetune the extent of CD8⁺ T cell activation. In support of our results, a recent study has shown a possible immunosuppressive role of PD-L1 on MCs in tumor immunity, in which high expression of PD-L1 was observed on MCs in gastric cancer. Consistently, increased number of MCs significantly correlated with poor prognosis.⁴⁸ Thus, PD-L1 on MCs may play important roles for the regulation of CD8⁺ T cell activation in various organs, including the skin.

Another study reported a possible regulatory role of PD-L1 on keratinocytes (KCs) during the effector phase of CHS, using transgenic mice overexpressing PD-L1 on KCs.²⁰ In our study as well, PD-L1 was expressed on KCs during the effector phase in CHS, suggesting a possible contribution of KCs to PD-1/PD-L1-mediated regulation. However, anti-PD-L1 antibody had no significant effect in the absence of MCs, suggesting MCs, not KCs, are the primary population contributing to the PD-L1-mediated suppression of CD8⁺ T cells in CHS. In CHS, because effector T cells infiltrate in the skin through post-capillary venules and are activated in the dermis^{49,50}, where MCs but not KCs exist; therefore, the activation status of effector T cells may be finetuned by PD-L1 on MCs first. The regulatory functions of PD-L1 on KCs may be important in the late stage of CHS or in conditions where T cells are activated in the epidermis, such as graft-versus host disease, as previously reported⁵¹.

Although we showed the importance of PD-L1 on MCs, there are several unresolved issues and limitations in our study. For example, we cannot completely exclude the

possibility of the involvement of other PD-L1 expressing cells in the skin for the regulation of CD8⁺ T cell activation in the skin. Establishment of MC-specific PD-L1 depletion system *in vivo* will be required to clarify this point. The relation of this MC-PD-L1-dependent regulatory system to other regulatory systems in CHS, such as the Treg-mediated regulatory system, is another important issue that needs further investigation, because Tregs also exert regulatory roles in the elicitation phase of CHS.¹¹ Investigation of the effects of anti-PD-L1 antibody in the absence of Tregs may provide an answer to this issue.

In summary, we revealed a novel regulatory mechanism which is dependent on PD-L1 on MCs in cutaneous immunity. Our results may bring new insights to our understanding of complex pathophysiology of inflammatory skin diseases.

Acknowledgements

We appreciate Prof. Lieping Chen, Yale School of Medicine, for providing B7-H1 (PD-L1)-deficient mice, and Dr. Arnold S. Kirshenbaum, National Institute of Allergy and Infectious Diseases, for providing LAD2 cells. We also thank the lab members at Kyoto University, in particular, Ms. Hiromi Doi, Ms. Kaori Tomari and Ms. Natsuki Ishizawa for technical assistance, Dr. Sho Hanakawa for technical advice, Dr. Yoshihiro Ishida for reviewing data analysis and Ms. Rosemary Menzies for helpful proofreading.

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Table 1. Comparison of the mRNA expression levels of PD-L1 in various human cell populations from a public data base (FANTOM 5).

p1@CD274 (TSS)	Top	Bottom	Mean	Median	
Mast cells	129.56	10.83	64.33	58.46	
Basophils	8.26	1.70	4.86	4.61	
Melanocytes	1.43	0.00	0.59	0.76	
Keratinocyte	21.31	6.74	15.89	17.76	
Dendritic cells	143.59	0.37	68.90	66.42	
All	197.42	0.00	9.29	2.89	[TPM]

TSS: transcriptional start sites

TPM: tags per million

Figure Legends

FIG 1. Programmed cell death ligand 1 (PD-L1)-deficient (*Pdl1*^{-/-}) mice exhibit exacerbated contact hypersensitivity (CHS) responses

(A) Changes in ear thickness after the elicitation in CHS. Wild-type (WT) C57BL/6N mice and *Pdl1*^{-/-} mice (n = 5) were sensitized with 0.5% (w/v) 1-Fluoro-2,4-dinitrobenzene (DNFB), and the ear thickness was measured at each time point after the elicitation with 0.3% (w/v) DNFB. (B) Hematoxylin and eosin staining of the ear skin of WT and *Pdl1*^{-/-} mice at steady state and 24 h after the elicitation with DNFB. Bar = 100 μ m. (C) Representative flow cytometric plots of CD45⁺ TCR β ⁺ CD8⁺ cells in the ear skin (left). The proportion of interferon- γ (IFN- γ) or granzyme B (GZMB) positive cells out of total CD8⁺ cells and the number of IFN- γ or GZMB positive CD8⁺ cells/ear are shown (middle and right, respectively). Cell suspensions were prepared from the dorsal side of the ears 18 h after elicited and subjected to flow cytometric analysis. Each dot represents an individual mouse except line graphs. Results are expressed as the mean + or $\pm$ standard deviation (SD). Data are representative of at least three independent experiments. *, p < 0.05, **, p < 0.01 and ***, p < 0.001.

FIG 2. PD-1/PD-L1 signaling negatively regulates CHS responses during the elicitation phase

(A) The total lymphoid cell numbers (left) and the proportions of CD8⁺ T cells (right) in the draining lymph nodes (dLNs) 5 days after the sensitization. (B) IFN- γ levels in the

culture supernatants. Cell suspensions from the dLNs of DNFB-sensitized mice were stimulated with DNBS for 72 h. (C) CHS responses after adoptive transfer. T cells from DNFB-sensitized mice were adoptively transferred to recipient mice ($n = 3$ or 4). (D) Kinetics of ear swelling after the elicitation (left) and IFN- γ production from CD8 $^{+}$ T cells in the skin 18 h after the elicitation (right). Anti-PD-L1 mAb ($200 \mu\text{g/body}$) were administered 6 h before and every 24 h after the elicitation ($n = 4$ or 5). (E) Representative histogram (left) and mean fluorescence intensity (MFI) of PD-1 expression on CD8 $^{+}$ T cells at the indicated time point after the elicitation ($n = 5$). Each dot represents an individual mouse ((A), (B), (D) right). Results are expressed as the mean $\pm$ standard deviation (SD). Data are representative of two (A, B) or more than three (C-E) independent experiments. *, $p < 0.05$ and **, $p < 0.01$. NS, not significant.

FIG 3. PD-L1 on radio-resistant cells negatively regulates CHS responses

(A) Kinetics of ear swelling after the elicitation in bone marrow (BM) chimeric mice. BM cells from WT or *Pdl1* $^{-/-}$ mice were transferred to lethally irradiated WT or *Pdl1* $^{-/-}$ mice ($n = 2$ to 4). Statistical differences between *Pdl1* $^{-/-} \rightarrow$ WT and WT $\rightarrow$ *Pdl1* $^{-/-}$ are shown. (B) Histogram of flow cytometric analysis for PD-L1 expression on radio-resistant cells at the steady state and indicated time point after the elicitation ($n = 5$). The gating strategy for each cell population is indicated in the Method. (C) Two-photon microscopic analysis of the ear skin of a donor mouse adoptively transferred with isolated CD8 $^{+}$ T cells from DNFB-sensitized-TdTomato-mice. Image

was taken 24 h after the elicitation. Red = TdTomato (CD8⁺ T cells), Green = FITC-avidin (MCs), Blue = second harmonic generation (collagen fibers). The right figure indicates the zoomed area in the white square of the left figure. Results are expressed as the mean + SD. Data are representative of two (B, C) or more than three (A) independent experiments. **, $p < 0.01$ and ***, $p < 0.001$.

FIG 4. Lack of MCs abolishes the effects of anti-PD-L1 ab during the elicitation phase in CHS

(A) Ear swelling (left) and the percentage of IFN- γ ⁺ CD8⁺ T cells (middle) of W/W^v mice 24 h after the elicitation. Anti-PD-L1 mAb (300 μ g/body) or its isotype matched control Ab were administered 6 h before the elicitation. (B) Ear swelling of C57BL/6-KitW-sh/W-sh (Sash) mice 24 h after the elicitation. Anti-PD-L1 mAb (300 μ g/body) or its isotype matched control Ab were administered 6 h before the elicitation. (C) The concentrations of IFN- γ in the culture supernatants. CD8⁺ T cells from the dLNs of DNFB-sensitized WT mice and bone marrow derived MCs from WT or *Pdl1*^{-/-} mice were cocultured at 1:1 for 3 d with or without 100 μ g/ml DNBS (quintuplicate). Results are expressed as the mean $\pm$ SD. Data are representative of three (A and C) or two (B) independent experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ and ****, $p < 0.0001$. NS, not significant. In A, two-way ANOVA was performed for analysis of interaction effect between mouse strain and antibody treatment.

FIG 5. PD-L1 is expressed on human MCs

(A) PD-L1 expressions of 847 various human cells available in a public data base, FANTOM5. Four skin MC samples and other interested cells are labeled with different colors. (B) Comparison of the mRNA expression levels between MCs and basophils from a published data (Supplemental Table 8 in Motakis et.al.³⁴). X axis indicates $-\log_{10}$ (fold change), and Y axis indicates $\log_2$ (BH p-value). Fold changes and BH p-values were already calculated and provided in the publication. A red circle and black circles represent CD274 (PD-L1) and well-known mast cell markers, respectively. (C) A representative immunostaining of MCs (FITC-avidin), PD-L1 (PE), CD8 (APC) and DAPI in the skin of patients with contact dermatitis on the face. White squares in the left bottom in each panel indicate the areas of high magnification pictures at the white squares in the center in each panel. (D) IFN- γ levels in the culture supernatants. Melanoma antigen recognized by T cells-1 (MART-1)-specific T cells (MART-Ts) were cocultured with HLA-A*0201-binding MART-1 peptide- or HIV (control) peptide-pulsed human mast cells (LUVA cells) for 24 h in the presence of anti-PD-L1 antibody or isotype control antibody. Data are representative of three (D) independent experiments. *, $p < 0.05$.

1 Online Repository - Table

2 Table E1. The PD-L1 expression in human skin mast cell samples from a public
3 database, FANTOM5.

Sample ID	p1@CD274 (TSS) [TPM]	Rank out of 847
Mast cell, donor3.CNhs12593.11566-120D9	129.56	8
Mast cell, donor2.CNhs12594.11565-120D8	89.15	16
Mast cell, donor4.CNhs12592.11567-120E1	27.77	66
Mast cell, donor1.CNhs12566.11563-120D6	10.83	179
Average	64.33	67.75

TSS: transcriptional start sites

TPM: tags per million

4

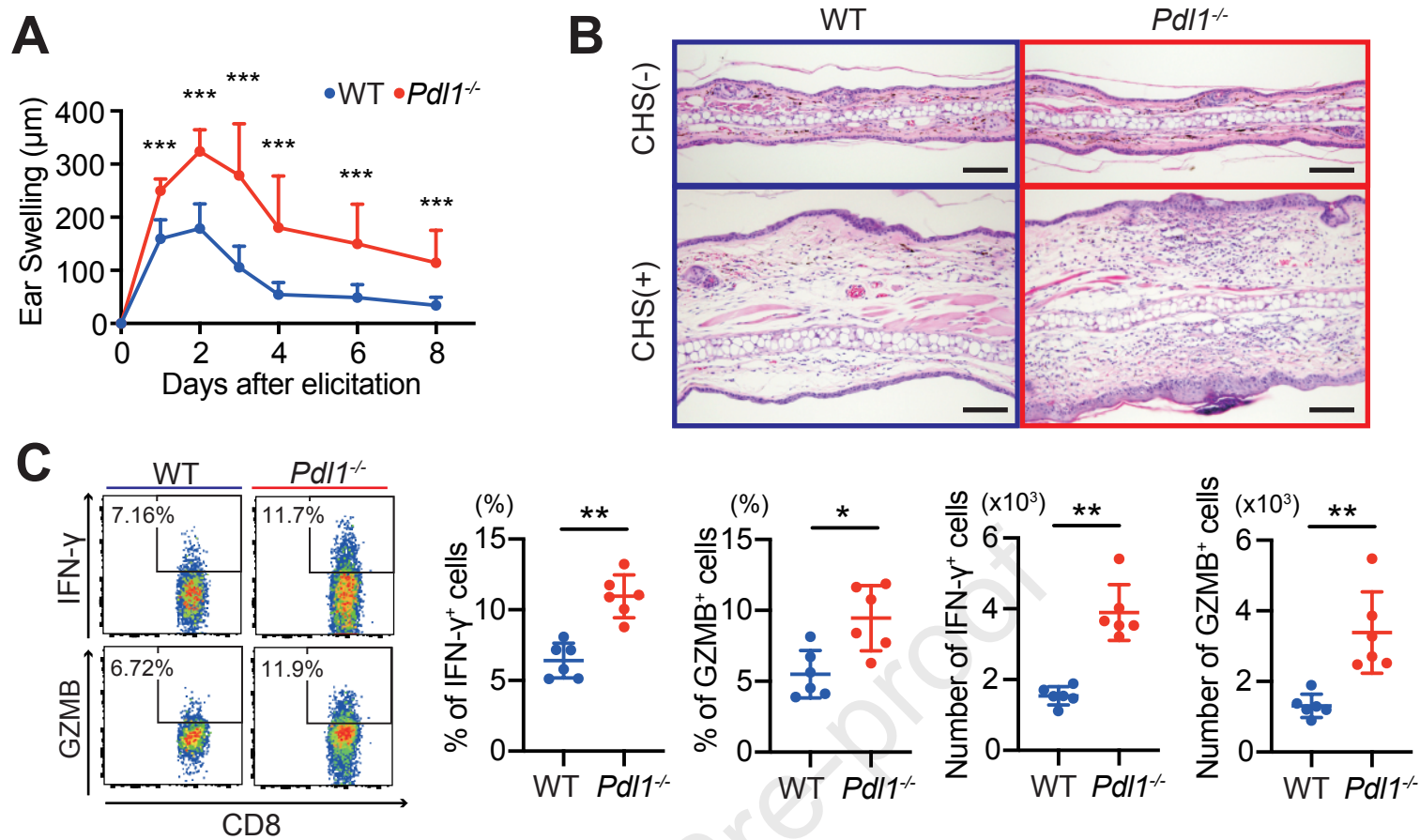
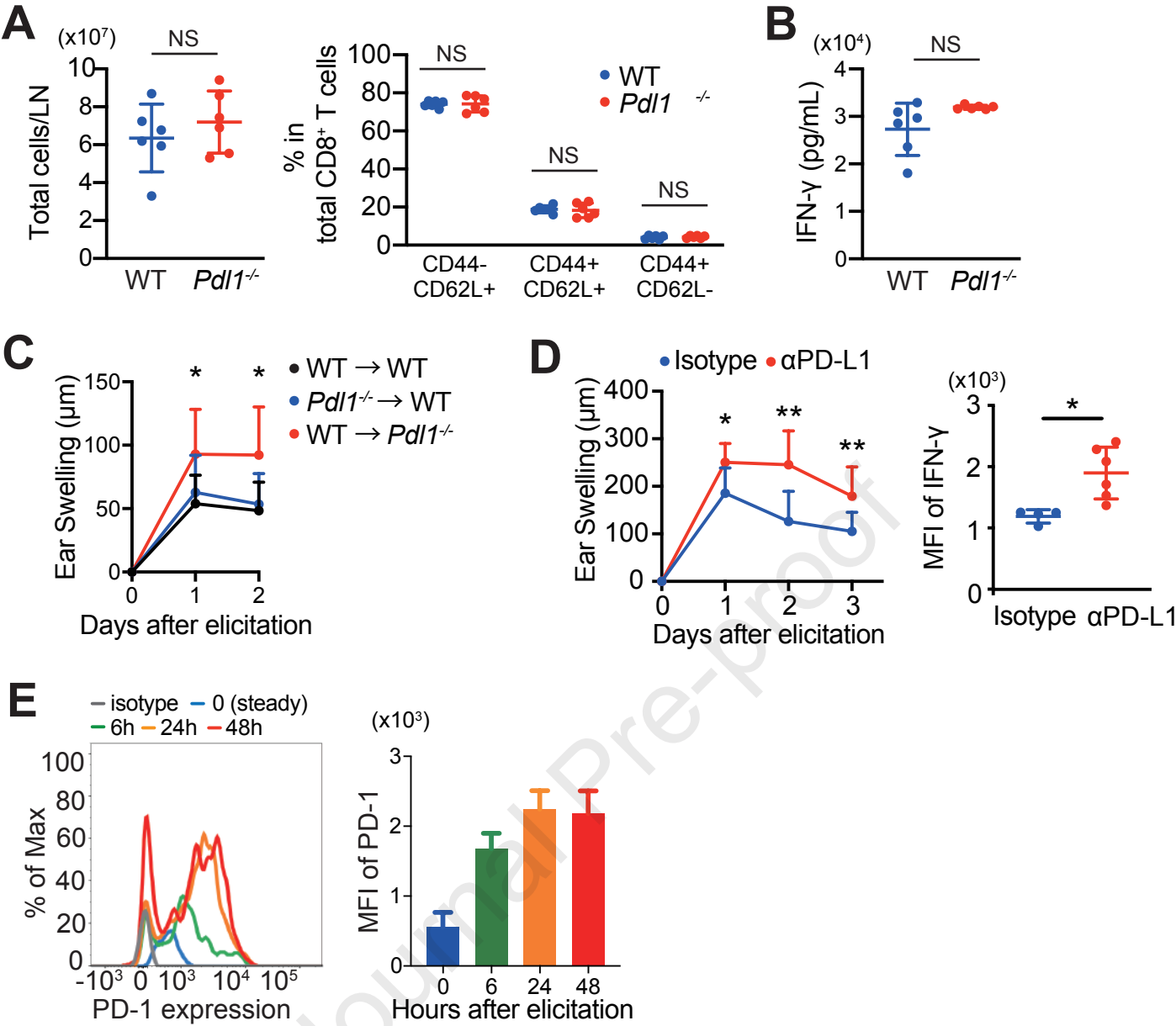


Figure 2



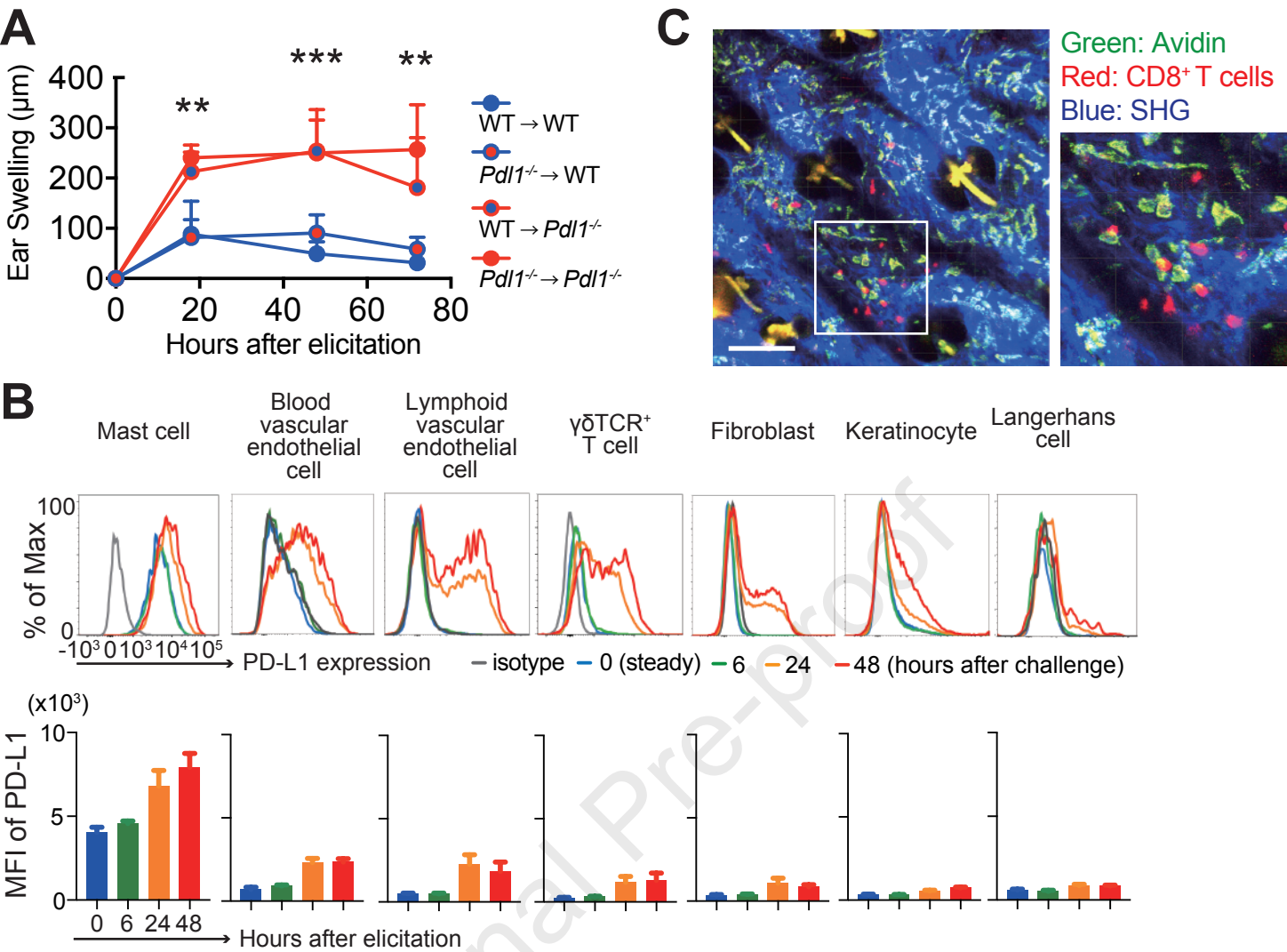
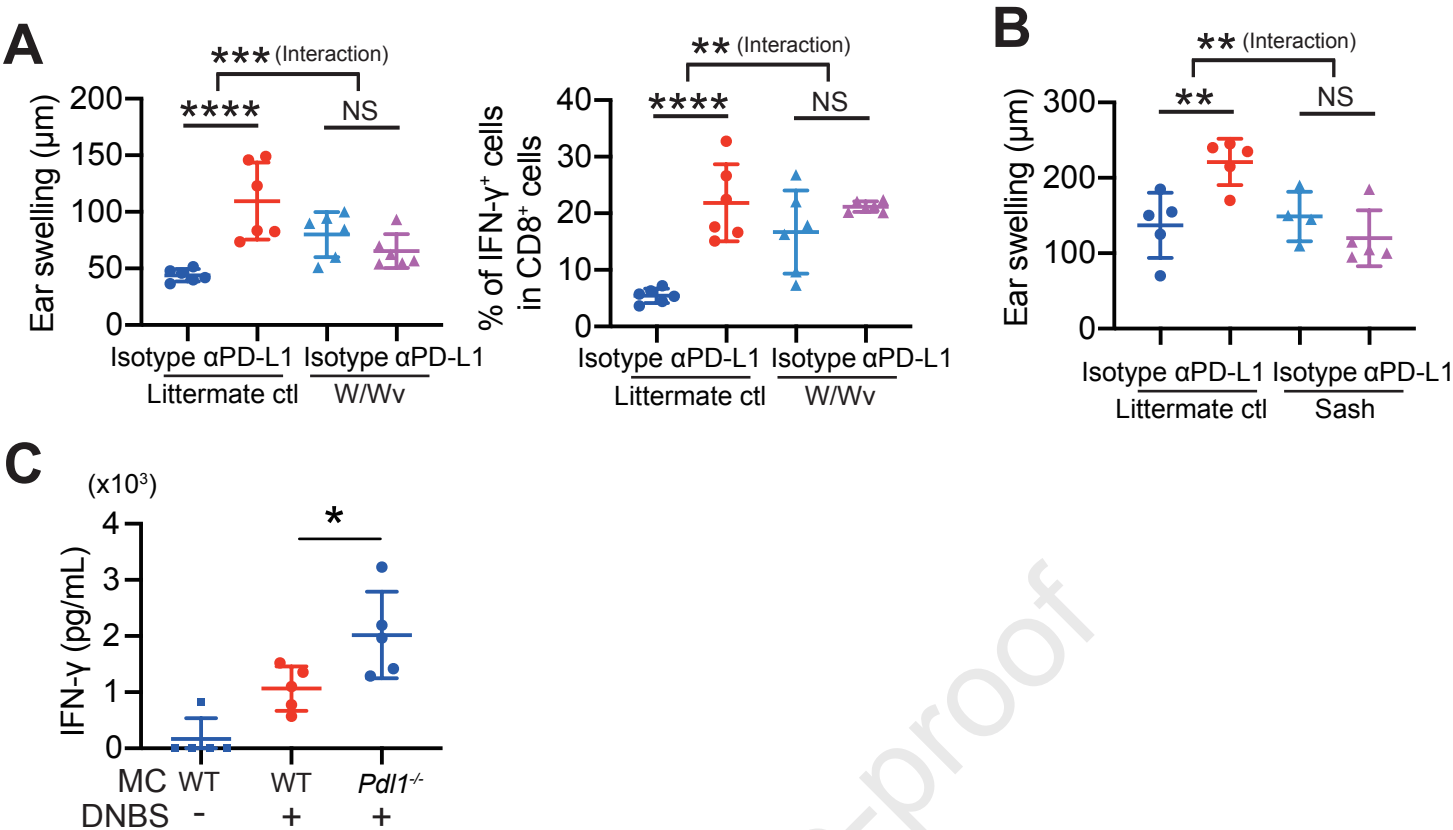


Figure 4



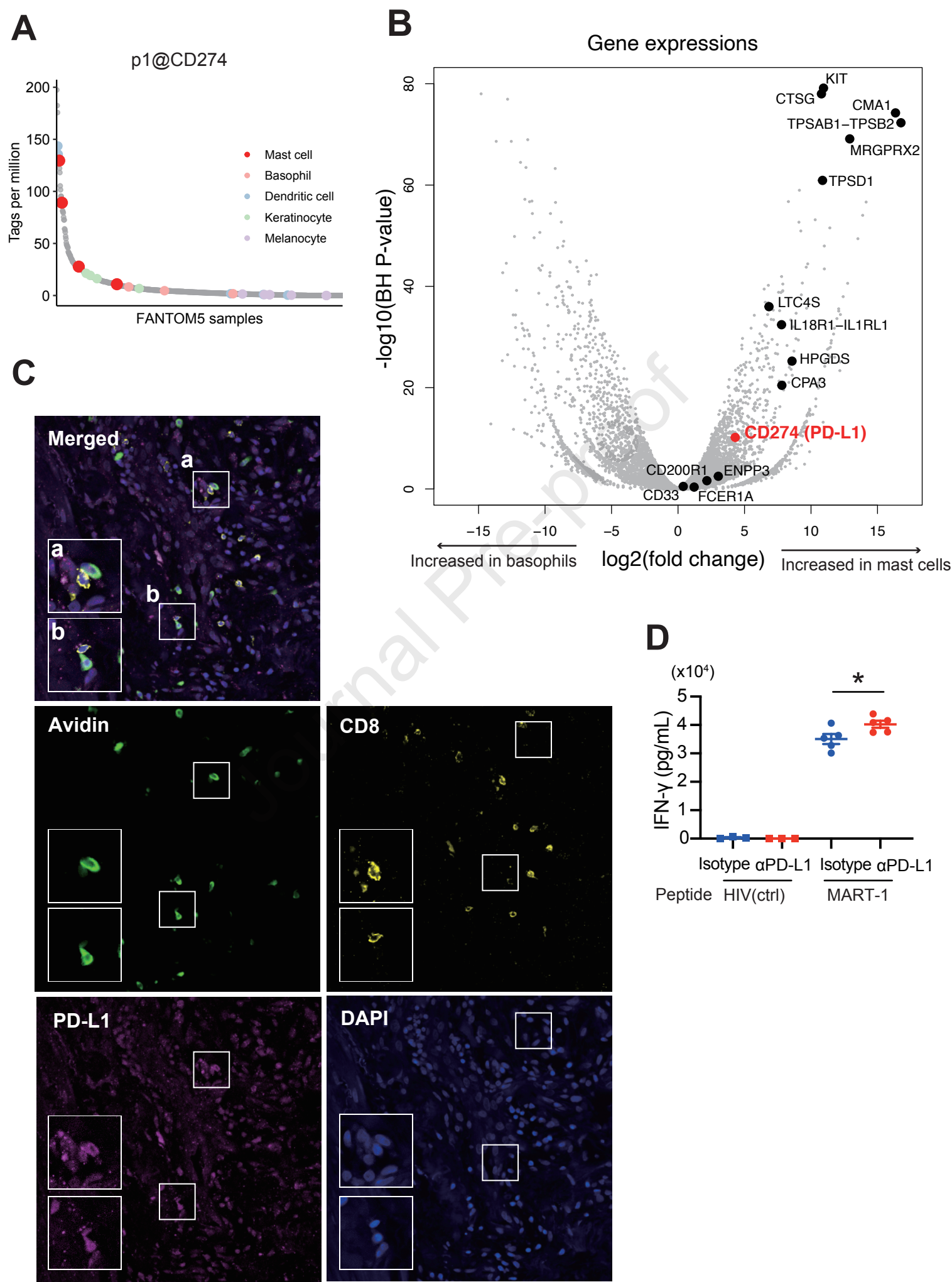
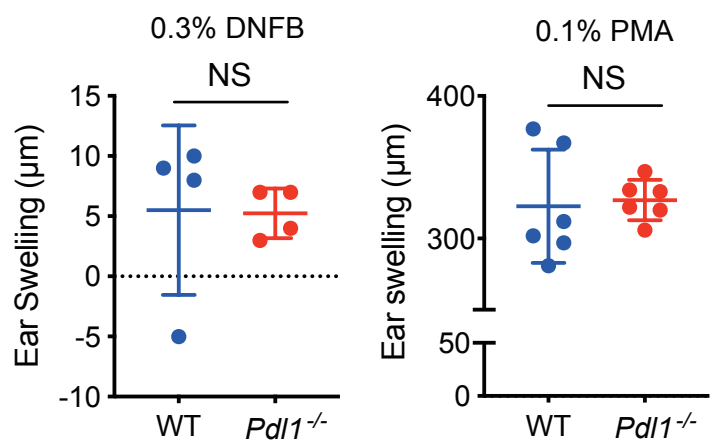
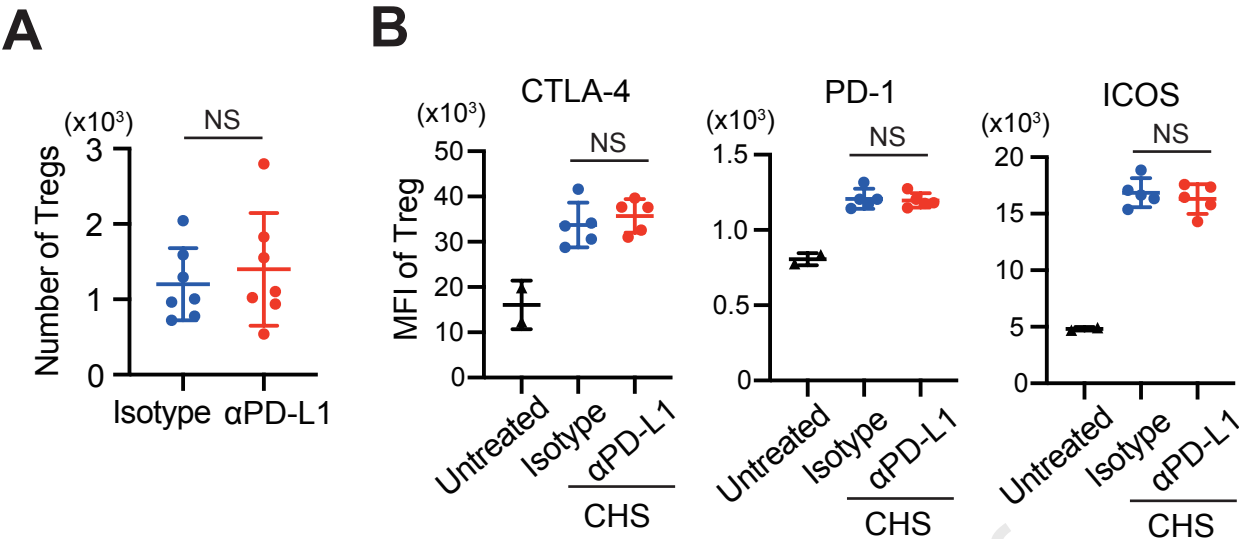
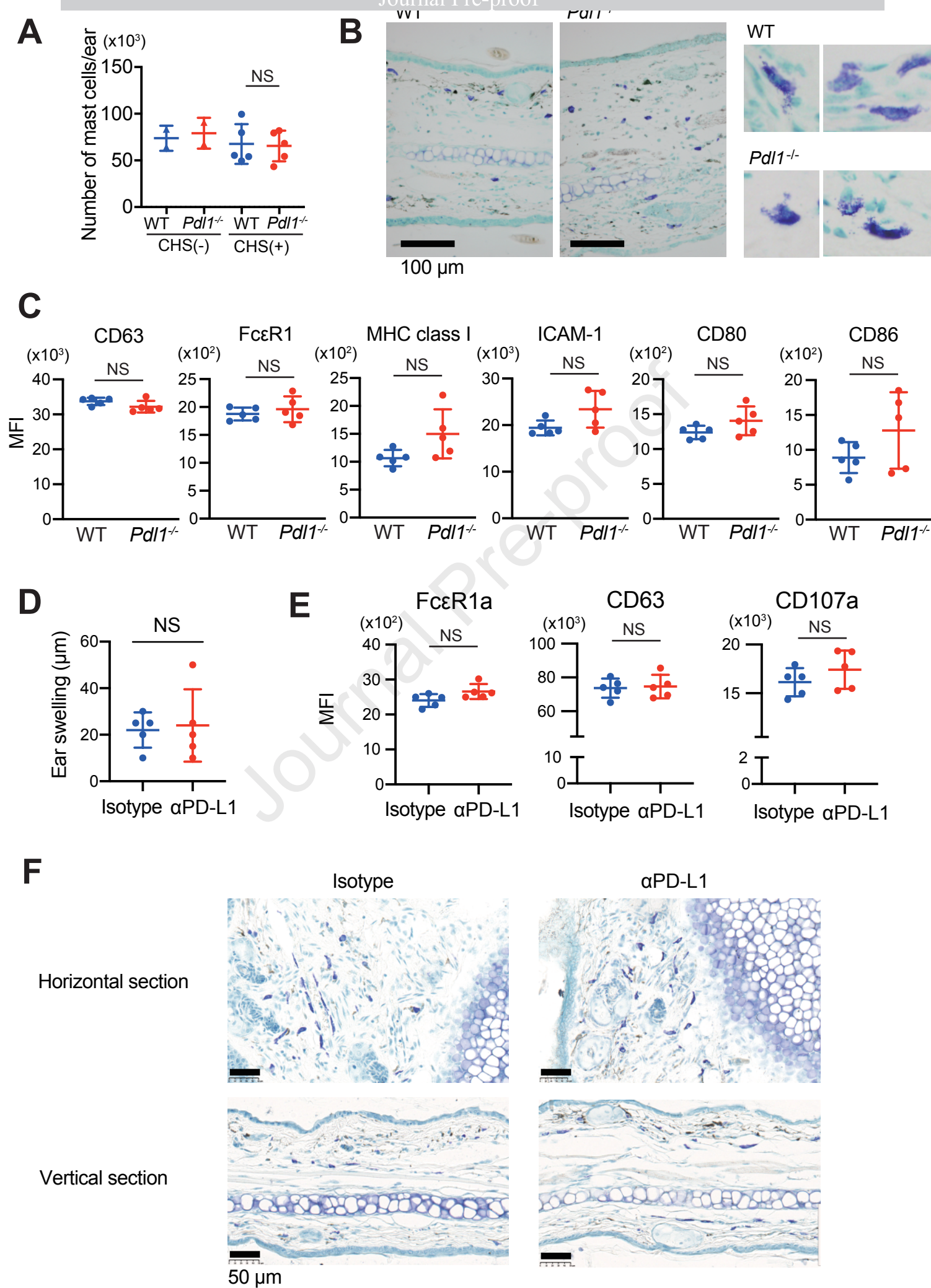
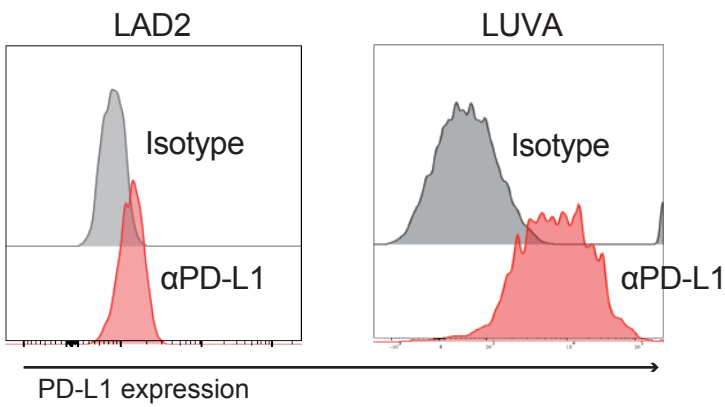


Figure E1









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PD-L1 on mast cells suppresses effector CD8⁺ T-cell activation in the skin in murine contact hypersensitivity

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METHODS

Antibodies

For flow cytometric analysis, FITC-conjugated anti-Granzyme B (GB11), anti-MHC class II (M5/114.15.2), anti-CD49f (eBioGoH3), anti- $\gamma\delta$ TCR (GL3), anti-TCR β , anti-CD279 (RMP1-30), anti-FC ϵ R1 α (MAR-1) and avidin; PE-conjugated anti-CD11c (N418), anti-CD200R3 (Ba13), anti-gp38 (8.1.1), anti-XCR1 (ZET) anti-CD152 (UC10-4B9) and anti-ICOS (7E.17G9); PE/Dazzle-conjugated anti-CD8b (YTS156.7.7); PerCP-Cy5.5-conjugated anti-Ter119 (TER-119) and anti-CD3 (145-2C11), PerCP-eFluor710-conjugated anti-CD8b (H35-17.2); PE/Cy7-conjugated anti-TCR β (H57-597), anti-CD31 (390), anti-FC ϵ R1 α (MAR-1), anti-CD11c (N418), anti-CD8b (eBioH35-17.2) and anti-CD11b (M1/70); APC-conjugated anti-IFN- γ (XMG1.2), anti-CD117 (2B8), anti-E-cadherin (DECMA-1), anti-EpCAM (G8.8), anti-CD279 (RMP1-30), anti-CD63 (NVG-2), anti-CD107a (1D4B), anti-Foxp3 (FJK-16s) and anti-human CD274 (29E.2A3); Pacific Blue-conjugated anti-CD8b (H35-17.2), anti-FC ϵ R1 α (MAR-1) and anti-TCR γ/δ (GL3); Brilliant Violet 421-conjugated anti-CD274 (MIH1) and anti-CD117 (2B8); V450-conjugated anti-CD4 (RM4-5); Brilliant Violet 605-conjugated anti-CD4 (RM4-5) and anti-CD11b (M1/70); BUV395-conjugated anti-CD45 (30-F11) antibodies and Fixable Viability Dye eFluor 780 were purchased from abcam (Cambridge, UK), BD Bioscience (San Jose, CA, USA), Biolegend (San Diego, CA, USA), eBioscience (San Diego, CA, USA) or Invitrogen (Carlsbad, CA, USA).

For neutralization, anti-mouse PD-L1 blocking mAb (10F.9G2) and rat IgG2b isotype control mAb (LTF-2) were purchased from Bio X Cell (Lebanon, NH, USA). Anti-human PD-L1 blocking mAb (29E.2A3) and mouse IgG2b (MPC-11) isotype control mAb were purchased from Biolegend (San Diego, CA, USA). For immunohistochemistry, anti-CD8 (144B, abcam) and anti-PD-L1 (SP142) were purchased from abcam (Cambridge, UK) and Spring Bioscience (Pleasanton, CA, USA), respectively.

Gating strategies of ear skin cells by flow cytometry

Before the gating, dead cells were excluded by the viability dye. KCs were gated as CD45⁻CD49f⁺ epidermal cells, Langerhans cells as CD45⁺CD11c⁺MHC class II⁺EpCAM⁺ epidermal cells, $\gamma\delta$ TCR⁺ T cells as CD45⁺CD3⁺ $\gamma\delta$ TCR⁺ epidermal cells, MCs as CD45⁺(CD11c⁻)(CD11b⁻)CD200R3⁺c-kit⁺(FcεR1⁺) cells, blood endothelial cells as CD45⁻CD31⁺gp38⁻ dermal cells, lymph endothelial cells as CD45⁻CD31⁺gp38⁺ dermal cells, fibroblasts as CD45⁻CD31⁻Ter119⁻ dermal cells and Tregs as CD45⁺TCRβ⁺CD4⁺Foxp3⁺ cells.

Histologic examination of murine ear skin

Skin tissues were fixed with 10% formalin and embedded in paraffin. Sections with a thickness of 5 μm were prepared, stained with hematoxylin and eosin or toluidine blue, and examined by using a light microscopy or a slide scanner (NanoZoomer S60, Hamamatsu Photonics, Hamamatsu, Japan).

80

81 **Adoptive transfer experiments**

82 For adoptive transfer, axillary and inguinal dLNs of each donor mouse were collected 5
83 days after the sensitization with 0.5% DNFB, and single cell suspensions were prepared
84 by mincing with a 40- μ m cell strainer. Cells were injected intravenously into each
85 recipient mouse (2×10^7 cells per body, diluted in phosphate-buffered saline (PBS)).
86 After 6 h, the recipient mice were elicited with 20 μ l of 0.5% DNFB on both sides of
87 each ear.

88

89 **Generation of BM chimeric mice**

90 Recipient mice were lethally irradiated with 9.5 Gy by a gamma irradiation device and
91 received 5×10^6 bone marrow (BM) cells from donor mice intravenously through the
92 tail vein. The chimeric mice were subjected to the indicated experiments over 2 months
93 after the reconstitution.

94

95 **Generation of BMMCs**

96 For the generation of bone marrow-derived mast cells (BMMCs), 1×10^6 BM cells
97 from WT and *Pdl1*^{-/-} mice were cultured in 20 ml of complete RPMI (RPMI 1640;
98 Gibco®; Thermo Fisher Scientific, Waltham, MA, USA), containing 10%
99 heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA, USA), 50 μ M
100 2-mercaptoethanol, 1 mM nonessential amino acids, 1 mM sodium pyruvate and 100
101 U/ml penicillin/streptomycin supplemented with 10 ng/ml of recombinant murine stem

cell factor and Interleukin 3 (PeproTech, London, UK) using 10-cm non-coated culture dishes or flasks for floating cells (Nunc Non-treated EasYFlasks; Thermo Fisher Scientific, Waltham, MA, USA). The culture medium was basically changed every week based on the cell growth. After 3 weeks of culture, cells were utilized for experiments (>95% of living cells were c-kit⁺ and CD200R3⁺).

ELISA

The murine IFN- γ concentration of culture supernatants was measured by Mouse IFN- γ ELISA Set (RUO) (555138, BD Biosciences, CA, USA). The human IFN- γ concentration of culture supernatants was measured by using anti-human IFN- γ Antibody (M700A, Invitrogen, Carlsbad, CA, USA) and anti-human IFN- γ biotinylated antibody (M701B, Invitrogen, Carlsbad, CA, USA).

Two-photon microscopic analysis

CD8⁺ T cells were sorted by magnetic cell sorting (CD8a (Ly-2) MicroBeads, mouse; Miltenyi Biotec, Bergisch Gladbach, Germany) from the dLNs of DNFB-sensitized tdTomato mice, according to the manufacturer's protocols. Twenty-millions of these cells were intravenously injected to a B6 Albino mouse, which was elicited with 0.5% DNFB 24 h before the analysis. To visualize cutaneous MCs, 20 μ L of avidin-FITC (1:200 in PBS) was intradermally injected to the ear right before the analysis. Two-photon microscopic analysis was performed as previously described,^{E1} with some modifications. In brief, mice were anesthetized with isoflurane

and were positioned on the heating plate on the stage of a two-photon microscope (IX-81; Olympus), with their earlobes fixed beneath coverslips with a single drop of immersion oil. Images were further processed by using Imaris software (Bitplane, Zurich, Switzerland).

Culture of human cells

Laboratory of Allergic Diseases 2 (LAD2)³⁵, which derives from a human mast cell sarcoma/leukemia was kindly provided by Dr. Arnold S. Kirshenbaum. Cells were cultured in StemPro-34 serum-free medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin/streptomycin, 100 U/mL glutamine and 100 ng/mL recombinant human stem cell factor (PeproTech, London, UK), which was half replaced every week or two weeks based on the cell growth.

Another human mast cell line, LUVA²⁶, was commercially purchased (Kerafast, Boston, MA, USA). Cells were cultured in StemPro-34 serum-free medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin/streptomycin and 100 U/mL glutamine and 100 ng/mL with or without recombinant human stem cell factor (PeproTech, London, UK), which was changed every few days based on the cell growth.

MART-specific T cells (MART-Ts) were generated as previously described.^{E2, E3} Briefly, the plasmid encoding the HLA-A*0201-restricted MART-1-specific TCR was a gift from Dr. Ken-ichi Hanada (Surgery Branch, National Cancer Institute, National Institutes of Health, USA). PBMCs from healthy donors were stimulated with soluble

OKT-3 (50 ng/ml) and recombinant human IL-2 (500 IU/ml; Novartis, East Hanover, NJ, USA) for 2 d before transduction. The stimulated cells were then transfected with a retrovirus expressing the MART-1-specific TCR gene. These cells were expanded in AIM-V medium (ThermoFisher Scientific, Waltham, MA) with 10% human-AB serum (Gemini Bio, West Sacramento, CA, USA) and recombinant human IL-2 (500 IU/ml; Novartis, East Hanover, NJ, USA), and used for experiments after more than 7 days of expansion.

Immunohistochemistry for human skin

Biopsied skin samples from patients clinically diagnosed with allergic contact dermatitis (n=5, 40s-70s, 2 males and 3 females) were used. Paraffin-embedded tissue samples were deparaffinized and were incubated with FITC-conjugated avidin overnight at 4 °C. Then, samples were antigen retrieved using antigen retrieval buffer pH 9.0 in a microwave at 95 °C for 15 minutes. Tissue was incubated with anti-CD8 primary antibody (1:500) for 30 minutes, with HRP polymer conjugated anti-mouse antibody for 10 minutes, and then with Opal 570 (1:500) for 10 minutes. After washed, samples were retrieved using antigen retrieval buffer pH 6.0 in a decloaking chamber for 20 min. Tissue was incubated with anti-PD-L1 primary antibody (1:300) for 30 min, with HRP polymer conjugated anti-rabbit antibody for 10 minutes, and then with Opal 690 (1:500) for 10 minutes. Stained samples were mounted with Prolong Gold Antifade Mountant with DAPI (ThermoFisher Scientific, Waltham, MA). Fluorescence images were obtained using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan).

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169 **Database analysis**

170 A list of PD-L1 mRNA expression levels of various human cell samples was obtained
 171 from a database service, the Functional Annotation of Mammalian Genome 5
 172 (FANTOM5) project, Semantic catalog of Samples, Transcription initiation And
 173 Regulators (SSTAR)³¹⁻³³. The data set of mapping transcription start site (TSS)
 174 expressions of human CD274 (EntrezGene:29126, p1@CD274) were exported. Among
 175 889 entries (available registered samples), 847 entries remained by excluding samples
 176 whose name includes the word “stimulated” or “treated (but except untreated)”. Each
 177 cell population was extracted based on the sample name. For the comparison between
 178 MCs and basophils, the data were obtained from a previous report (Supplemental Table
 179 8 in Motakis et.al.³⁴). In the data, log fold changes and BH p-values of the 16,533 genes
 180 were provided. Graphs were drawn with published R packages including
 181 EnhancedVolcano^{E4} and ggplot2.

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198
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Figure Legends

FIG E1. Programmed cell death ligand 1 (PD-L1)-deficient (*Pdl1*^{-/-}) mice exhibit equivalent ear swelling responses in irritant dermatitis.

Ear thickness changes 24 h after the application of 0.3% DNFB (left) and 6 h after the application of 0.1% phorbol 12-myristate 13-acetate (PMA) (right) in WT and *Pdl1*^{-/-} mice. Data are representative of two independent experiments. Results are expressed as the mean ± SD. NS, not significant.

FIG E2. No significant difference was observed in the number and surface markers of Treg in the skin under the blockade of PD-1/PD-L1 signaling.

(A) The number and (B) surface markers of Tregs in the ear skin analyzed by flow cytometry 24 h after the elicitation in mice treated with anti-PD-L1 mAb (300 µg/body) or its isotype matched control Ab. Results are expressed as the mean ± SD. Data are representative of two independent experiments. NS, not significant.

FIG E3. Lack of PD-1/PD-L1 signaling does not affect the number and the expression of activation markers in skin MCs during the elicitation phase of CHS

(A) The number of MCs in the ear skin analyzed by flow cytometry 24 h after the elicitation of CHS in WT and *Pdl1*^{-/-} mice. (B) Representative toluidine blue staining of the ear skin 24 h after elicitation. Right panels: High magnification of degranulated MCs. (C) Activation markers of MCs in WT and *Pdl1*^{-/-} mice 24 h after elicitation analyzed by flow cytometry. (D-F) Ear swelling (D), activation markers of MCs (E) and

representative toluidine blue staining of the ear skin (F) 1 h after the elicitation of CHS.

Mice were treated with anti-PD-L1 mAb (300 µg/body) or its isotype matched control

Ab 6 h before the elicitation. The horizontal section and vertical section are shown for

each sample in (F). Results are expressed as the mean ± SD. Data are representative of

three (A) or two (C-E) independent experiments. NS, not significant.

FIG E4. Human MC lines constitutively expresses PD-L1

Flow cytometric analysis of PD-L1 expression on LAD2 cells and LUVA cells. Gray

peak represents a control stained with isotype IgG.