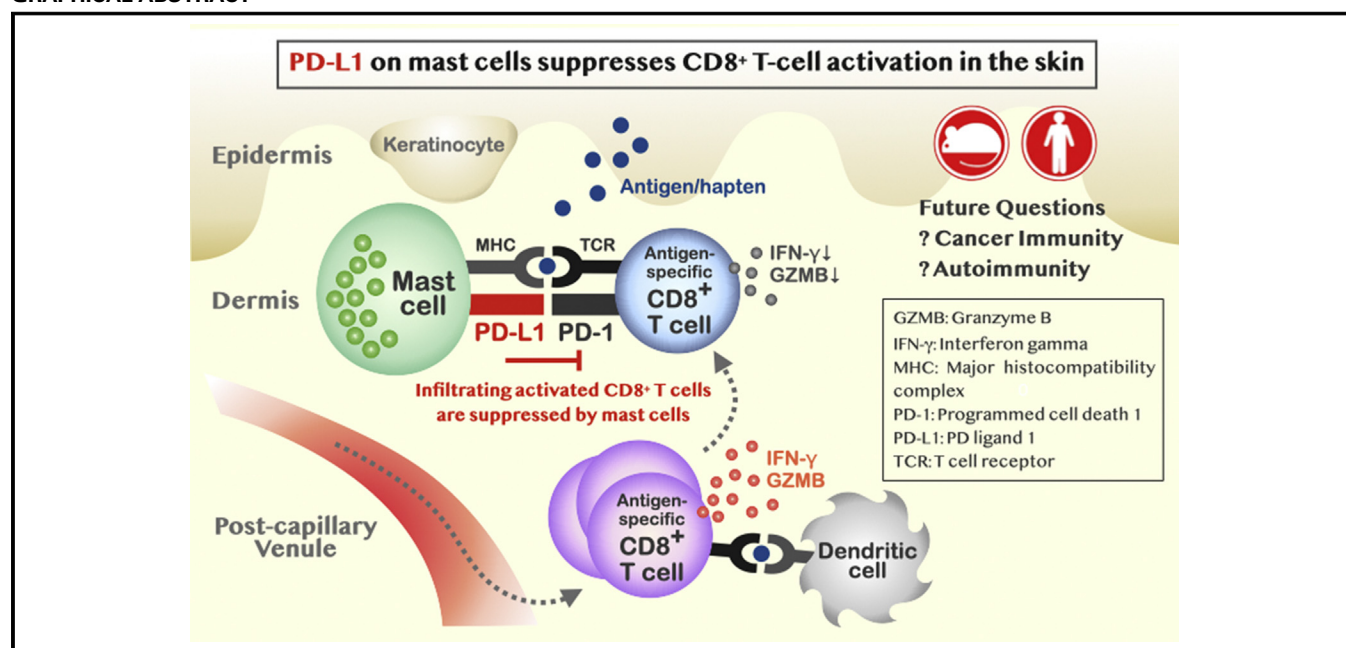


PD-L1 on mast cells suppresses effector CD8⁺ T-cell activation in the skin in murine contact hypersensitivity



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GRAPHICAL ABSTRACT



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

Objective: Our aim was 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 (*Pd1l*^{-/-}) mice were used for the murine contact hypersensitivity model. Inflammatory responses

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

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication May 25, 2020; revised October 14, 2020; accepted for publication December 1, 2020.

Available online February 10, 2021.

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0091-6749/\$36.00

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

such as IFN- γ production from CD8⁺ T cells in the skin was evaluated by flow cytometry.

Results: Compared with wild-type mice, *Pd1*^{-/-} mice exhibited exacerbated ear swelling and increased numbers of IFN- γ ⁺ CD8⁺ T cells in the skin. Adoptive T-cell transfer experiments revealed the involvement of the PD-1/PD-L1 pathway in the elicitation phase of contact hypersensitivity. Bone marrow chimera experiments showed that PD-L1 on radioresistant cells was responsible for this regulatory pathway. Flow cytometric analysis revealed that among the radioresistant 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 wild-type mice, whereas no significant effects were observed in MC-deficient (WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J and C57BL/6-*Kit*^{sh}/*W*^{sh}) mice. The high level of expression 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. (J Allergy Clin Immunol 2021;148:563-73.)

Key words: Contact hypersensitivity, mast cell, PD-1, PD-L1

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, which can be expressed on various tissue-resident cells, as well as on antigen-presenting cells (APCs).^{1,2} Subsequent to ligation with PD-L1, PD-1 transduces signals that inhibit the proliferation of T cells and their effector functions such as IFN- γ production and cytotoxicity.¹⁻³ Because PD-1 is expressed on effector T cells but not on naive 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 pathologic conditions, particularly in tumor immunity, in which PD-L1 expressed on tumor cells can inhibit the effector functions of CD8⁺ T cells, leading to the progression of tumors.^{5,6} On the basis of this understanding, blockade of the PD-1/PD-L1 pathway is now widely utilized in cancer therapy for enhancing the antitumor activity of cytotoxic CD8⁺ T cells.⁷⁻⁹ Recent studies have shown that PD-L1 on host cells such as dendritic cells (DCs) is also important for this regulation.¹⁰ However, exactly how this regulatory pathway is involved in the pathophysiology of CD8⁺ T-cell-mediated inflammatory skin diseases, such as contact dermatitis, remains unclear.

Murine contact hypersensitivity (CHS) is a hapten-induced type IV hypersensitivity reaction in the skin that is mainly mediated by CD8⁺ T cells; it is one of the most frequently used mouse models for analysis of adaptive immune responses in the skin.¹¹ It is also used as a mouse model of contact dermatitis. CHS consists of 2 phases: the sensitization and elicitation phases. In the sensitization phase, naive T cells are presented with antigens in the skin-draining lymph nodes (DLNs) by APCs migrated from the skin, and they differentiate into antigen-specific effector

Abbreviations used

APC:	Antigen presenting cell
BMMC:	Bone marrow-derived mast cell
CHS:	Contact hypersensitivity
DC:	Dendritic cell
DLN:	Draining lymph node
DNFB:	2,4-Dinitrofluorobenzene
GZMB:	Granzyme B
KC:	Keratinocyte
MART-1:	Melanoma antigen recognized by T cells-1
MC:	Mast cell
PD-1:	Programmed cell death 1
PD-L1:	Programmed death ligand 1
Sash:	C57BL/6- <i>Kit</i> ^{sh} / <i>W</i> ^{sh}
Treg:	Regulatory T
WT:	Wild-type
W/W ^v :	WBB6F1/J- <i>Kit</i> ^W / <i>Kit</i> ^{W-v} /J

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 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 wild-type (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 have been 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, whether KCs or DCs play such regulatory roles in physiologic condition is still unclear. Thus, the underlying mechanisms by which the PD-1/PD-L1 pathway regulates immune responses in CHS remain unclear.

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

METHODS

Mice

All experiments were conducted on 6- to 12-week-old female mice unless otherwise described. C57BL/6 (B6) N mice, B6N-*Tyr*^{c-Brd}/BrdCrCl (B6 Albino) mice, and WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J (W/W^v) and their littermate control mice were purchased from Japan SLC (Kyoto, Japan) or OBS (Kyoto, Japan). PD-L1 (B7-H1)-deficient (*Pd1*^{-/-}) mice on a B6 background were originally generated and kindly provided by Dr Lieping

Chen (Yale School of Medicine).^{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 (catalog no. 007908; Jackson Laboratory, Bar Harbor, Me) were crossbred with EIIa-cre mice (Jackson Laboratory) 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. The mice were sensitized with 25 μ L of 0.5% (wt/vol) DNFB in 4:1 acetone–olive oil (vol/vol) on their shaved abdomens and then elicited with 20 μ L of 0.3% (wt/vol) DNFB on both sides of each of their ears 5 days after sensitization. Each mouse's ear thickness was measured by using a thickness gauge before and after elicitation at the indicated time points.

Preparation of single-cell suspensions

The dorsal halves of the 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 and 0.05% DNase I (Sigma-Aldrich, St Louis, Mo) for 70 minutes 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, after which the epidermis was manually collected from the dermis in advance. For intracellular staining of cytokines, ear pinnae were incubated in digestion solution with 10 μ g/mL of brefeldin A (Sigma-Aldrich, St. Louis, Mo). After that incubation, the cell suspension was filtered through a 40- μ m cell strainer.

Flow cytometric analysis

Nonspecific antibody binding was blocked with anti-mouse CD16/CD32 (2.4G2) antibodies (BD Biosciences, San Jose, Calif) for mouse cells or FcR blocking reagent and human antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) for human cells before surface staining. After surface staining with antibodies (listed in this article's Online Repository at www.jacionline.org), cells were fixed with Cytofix/Cytoperm solution (BD Biosciences) and stained intracellularly. Samples were assessed with a BD LSR Fortessa cell analyzer (BD Biosciences) and data were analyzed with FlowJo software (BD Biosciences). The numbers of cells in each cell subset were evaluated by using Flow-Count fluorophores (Beckman Coulter, Brea, Calif).

In vitro stimulation of hapten-specific T cells in mice

CD8⁺ T cells from DLNs of mice 5 days 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 MCs (BMMCs) at the indicated ratio with or without 100 μ g/mL of dinitrobenzene sulfonic acid (Alfa Aesar, Ward Hill, Mass) for 72 hours, after which the supernatants were collected for measurement of IFN- γ production by ELISA.

In vitro stimulation of antigen-specific T cells in humans

Human MCs (LUVA cells)²⁶ were pulsed for 20 minutes with 100 μ g/mL of either the HLA-A*0201-binding peptide melanoma antigen recognized by T cells-1 peptides (melanoma antigen recognized by T cells-1 [MART-1] and MART-1₂₇₋₃₅; ELAGIGILTV) or control peptides (HIV gag₇₇₋₈₅; SLYNTVATL). Next, 2×10^4 of MART-1-specific T cells, which were generated by transducing the HLA*0201-restricted MART-1-specific TCR gene into activated PBMC-derived T cells, were cocultured with 1×10^5 of

the peptide-pulsed LUVA cells in AIM-V medium (ThermoFisher Scientific, Waltham, Mass) with 10% human-AB serum (Gemini Bio, West Sacramento, Calif) with 10 μ g/mL of anti-human PD-L1 blocking antibody or isotype control antibody. The supernatants were collected after a 24-hour culture period for 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 $\pm$ SDs. For comparison between 2 groups, the 2-tailed Mann-Whitney *U* test was performed. For 2×2 comparison, 2-way ANOVA was performed to assess the possible interactive effect of 2 factors: mouse strain and antibody treatment. For these data, the results of the Bonferroni multiple comparison test within a strain were also shown to evaluate the effect of antibody treatment on each strain. A value *P* less than .05 was considered to indicate statistical significance. All statistical tests were performed with GraphPad Prism 8 software (GraphPad Software, La Jolla, Calif).

Further information on the methods used in this study is provided in the Methods section in this article's Online Repository (available at www.jacionline.org), which covers the following topics: antibodies, gating strategies of ear skin cells by flow cytometry, histologic examination of murine ear skin, adoptive transfer experiments, generation of bone marrow–chimeric mice, generation of BMMCs, ELISA, 2-photon microscopic analysis, culture of human cells, immunohistochemistry for human skin, and database analysis.

RESULTS

Loss of PD-L1 exacerbates CHS responses

To examine involvement of the PD-1/PD-L1 pathway in the development of CHS induced by DNFB, we first evaluated the CHS responses in *Pd1l*^{−/−} mice. Compared with WT mice, *Pd1l*^{−/−} mice exhibited significantly increased and prolonged inflammation after elicitation (Fig 1, A). Histologic analysis revealed more severe swelling in the ear skin of *Pd1l*^{−/−} mice than in that of WT mice 24 hours after elicitation (Fig 1, B). In addition, flow cytometric analysis showed increased percentages and numbers of IFN- γ -producing and GZMB-producing CD8⁺ T cells in the skin of *Pd1l*^{−/−} mice when compared with the respective percentages and numbers in the skin of WT mice (Fig 1, C). On the other hand, no significant differences were observed in the ear swelling between WT and *Pd1l*^{−/−} mice in antigen-independent irritant reactions induced by DNFB or phorbol 12-myristate 13-acetate (see Fig E1 in this article's Online Repository at www.jacionline.org). Together, these results indicate that the PD-1/PD-L1 pathway negatively regulates antigen-specific immune responses in CHS.

The 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 numbers and composition of CD8⁺ T cells in the DLNs of WT and *Pd1l*^{−/−} mice by flow cytometry 5 days after sensitization with DNFB. There were no significant differences in the total number of CD8⁺ T cells and number of CD8⁺CD44⁺CD62L⁺ memory

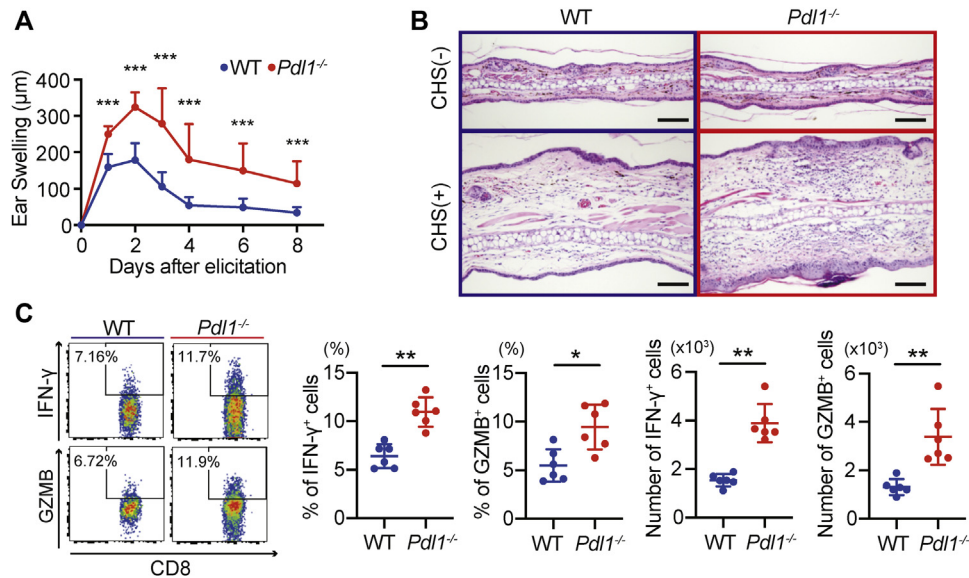


FIG 1. PD-L1-deficient (*Pdl1*^{-/-}) mice exhibit exacerbated CHS responses. **A**, Changes in ear thickness after elicitation in CHS. WT C57BL/6N mice and *Pdl1*^{-/-} mice (*n* = 5) were sensitized with 0.5% DNFB (wt/vol); ear thickness was measured at each time point after elicitation with 0.3% DNFB (wt/vol). **B**, Hematoxylin and eosin staining of the ear skin of WT and *Pdl1*^{-/-} mice at steady state and 24 hours after the elicitation with DNFB. Scale bar = 100 μm. **C**, Representative flow cytometric plots of CD45⁺TCRβ⁺CD8⁺ cells in the ear skin (*left*). The proportion of IFN-γ or GZMB-positive cells out of total CD8⁺ cells and the number of IFN-γ- or GZMB-positive CD8⁺ cells per ear are shown (*middle and right, respectively*). Cell suspensions were prepared from the dorsal side of the ears 18 hours after elicited and were then subjected to flow cytometric analysis. Each dot represents an individual mouse, except in line graphs. Results are expressed as means ± SDs. Data are representative of at least 3 independent experiments. **P* < .05; ***P* < .01; and ****P* < .001.

T cells between WT and *Pdl1*^{-/-} mice (Fig 2, A). In addition, the amount of IFN-γ production by lymph node cells restimulated with dinitrobenzene sulfonic acid, a water-soluble analog of DNFB, was similar between WT and *Pdl1*^{-/-} mice (Fig 2, B), suggesting that the absence of PD-L1 did not influence the establishment of sensitization. Next, to examine involvement of the PD-1/PD-L1 pathway in the elicitation phase, we performed an adoptive transfer experiment (see the [Methods](#) section in the 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 their ear swelling responses were measured. The extent of the ear swelling response was similar between recipient WT mice into which cells from *Pdl1*^{-/-} mice had been transferred and recipient WT mice into which cells from WT mice had been transferred. On the other hand, the recipient *Pdl1*^{-/-} mice into which cells from WT mice had been transferred exhibited significantly increased ear swelling responses compared with recipient WT mice into which cells from WT mice had been transferred (Fig 2, C), 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 2, D). The expression level of PD-1 on CD8⁺ T cells in the skin was significantly upregulated within 24 hours after elicitation (Fig 2, E). 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 costimulatory/inhibitory molecules (cytotoxic T-lymphocyte protein-4 [CTLA-4], inducible T-cell costimulator [ICOS], and PD-1) of regulatory T (Treg) cells, because Treg cells exert anti-inflammatory effects in the elicitation phase of CHS.¹¹ We treated the mice with either anti-PD-L1 antibody or control isotype antibody 6 hours before elicitation and then analyzed the Treg cells in their skin by flow cytometry 24 hours after elicitation. No significant differences were observed in the number of Treg cells and their expression of costimulatory/inhibitory molecules (see Fig E2 in this article's Online Repository at www.jacionline.org). These results suggest that the PD-1/PD-L1 pathway regulates the CHS responses during the elicitation phase independently of Treg cells.

PD-L1 on radioresistant 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 by 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 recipients were significantly increased and prolonged compared with those of chimera in which WT mice were used as the recipients, irrespective of the genotype of the donor (Fig 3, A). These results indicate that PD-L1 on radioresistant cells in the skin, such as KCs

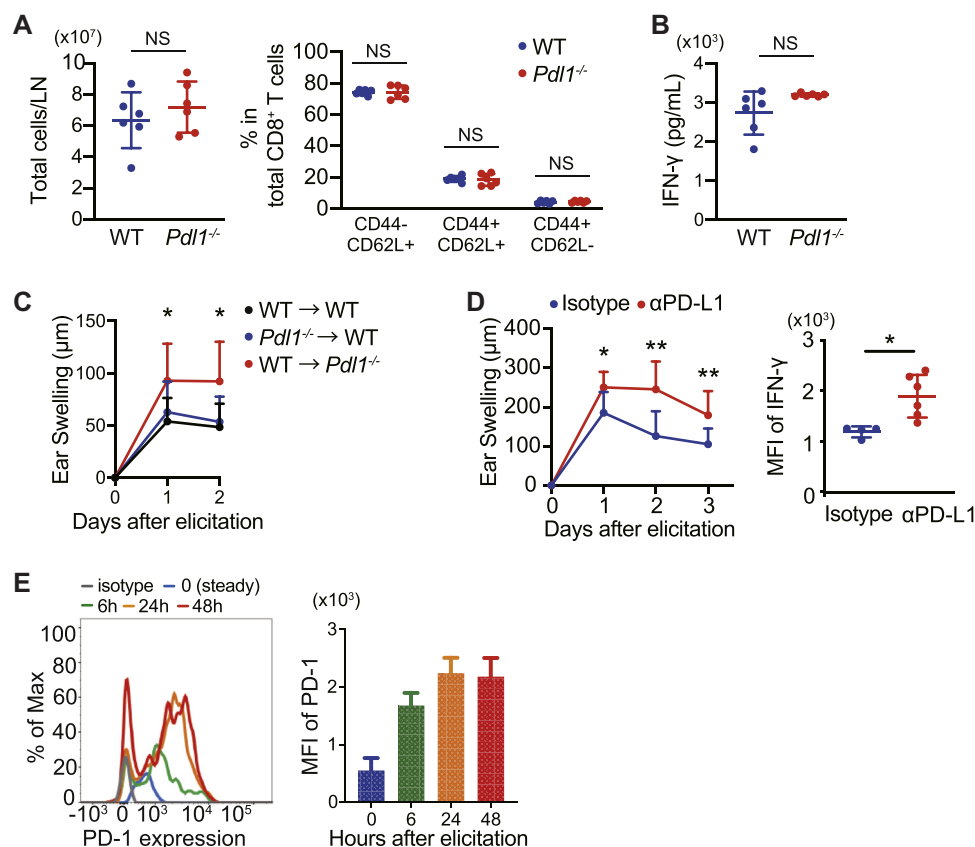


FIG 2. PD-1/PD-L1 signaling negatively regulates CHS responses during the elicitation phase. **A**, The total lymphoid cell numbers (*left*) and the proportions of CD8⁺ T cells (*right*) in the DLNs 5 days after the sensitization. **B**, IFN-γ levels in the culture supernatants. Cell suspensions from the DLNs of DNFB-sensitized mice were stimulated with dinitrobenzene sulfonic acid for 72 hours. **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 hours after the elicitation (*right*). Anti-PD-L1 mAbs (200 μg/mouse) were administered 6 hours before and every 24 hours 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**), and (**D**) [*right*]. Results are expressed as the means ± SDs. Data are representative of 2 (**A** and **B**) or more than 3 (**C-E**) independent experiments. **P* < .05; ***P* < .01. LN, Lymph node; Max, maximum; NS, not significant.

and MCs, is essential for PD-L1-dependent regulation of CD8⁺ T-cell activation in the skin in CHS.

We next compared the level of expression of PD-L1 on radioresistant cell populations in the skin and performed time course analysis of its upregulation during the elicitation phase in CHS. As the major radioresistant cell populations in the skin, we analyzed KCs, Langerhans cells, MCs, dendritic epidermal T cells, blood/lymphoid endothelial cells, and fibroblasts. Expression of PD-L1 was observed in all the radioresistant cells to various extents in the steady state without any treatment. Among them, the MCs had the highest expression of PD-L1 at steady state, and the expression further increased after elicitation (Fig 3, B). Two-photon microscopic analysis suggested the colocalization of MCs and CD8⁺ T cells in the skin during the elicitation phase of CHS (Fig 3, C). On the basis of these findings, we next investigated the role of PD-L1 in 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 affects the number or functions of MCs in the skin. First, we examined the number of skin MCs in WT and *Pdl1*^{-/-} mice in the steady state and 24 hours after the elicitation and found no obvious difference between the groups (see Fig E3, A in this article's Online Repository at www.jacionline.org). We next examined degranulation of MCs by histology and expression of MC-activation markers (CD63 and FcεRI), MHC class I, and costimulatory molecules (ICAM1, CD80, and CD86) by flow cytometry 24 hours after the elicitation. No apparent differences were observed in the extent of degranulation or in the levels of expression of these markers between the groups (see Fig E3, B and C). We further

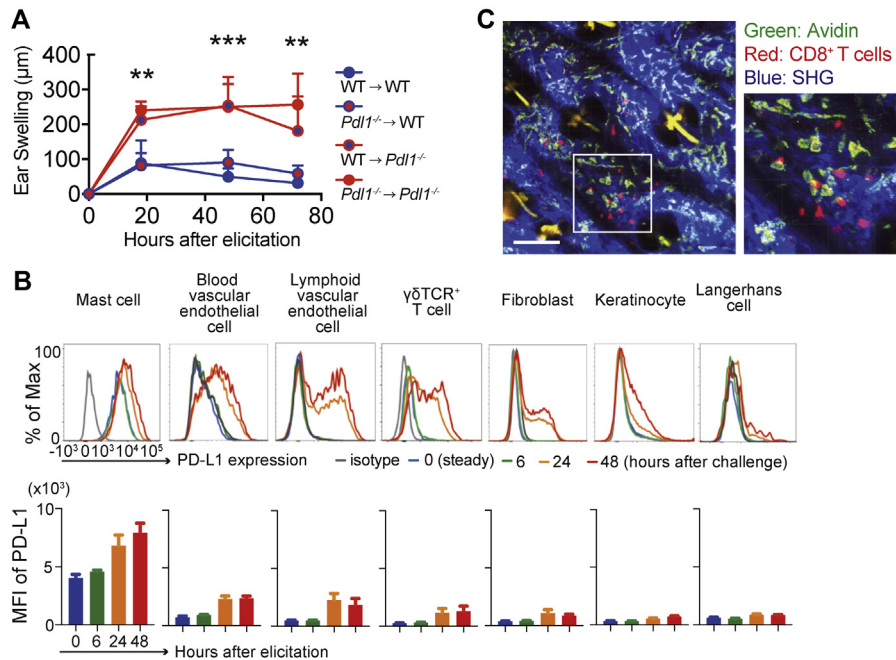


FIG 3. PD-L1 on radioresistant cells negatively regulates CHS responses. **A**, Kinetics of ear swelling after the elicitation in bone marrow (BM)-chimeric mice. BM cells from WT or *Pdl1*^{-/-} mice were transferred into lethally irradiated WT or *Pdl1*^{-/-} mice (n = 2 to 4). Statistical differences between *Pdl1*^{-/-} mice → WT mice and WT mice → *Pdl1*^{-/-} mice are shown. **B**, Histogram of flow cytometric analysis for PD-L1 expression on radioresistant 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 [Methods](#) section. **C**, Two-photon microscopic analysis of the ear skin of a recipient mouse into which isolated CD8⁺ T cells from DNFB-sensitized TdTomato mice were adoptively transferred. Image was taken 24 hours after the elicitation. Red indicates TdTomato (CD8⁺ T cells), green indicates fluorescein isothiocyanate-avidin (MCs), blue indicates 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 means ± SDs. Data are representative of 2 (**B** and **C**) or more than 3 (**A**) independent experiments. ***P* < .01; ****P* < .001. MFI, Mean fluorescence intensity; Max, maximum; SHG, second harmonic generation.

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, expression of activation markers, or ear swelling response between the groups (see [Fig E3, D-F](#)). 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 MCs 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 by using 2 different mouse strains that lack MCs (*W/W^v* mice and *Sash* mice). These mice lack MCs because of 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 on the CHS responses in *W/W^v* mice and *Sash* mice were observed ([Fig 4, A and B](#)). 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 BMDCs 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 BMDCs generated from either 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*^{-/-} BMDCs than for those cocultured with WT BMDCs ([Fig 4, C](#)). 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 reported.²⁷⁻³⁰ On the other hand, as we found in this study, MCs play regulatory roles in CD8⁺ T-cell activation via their PD-L1.

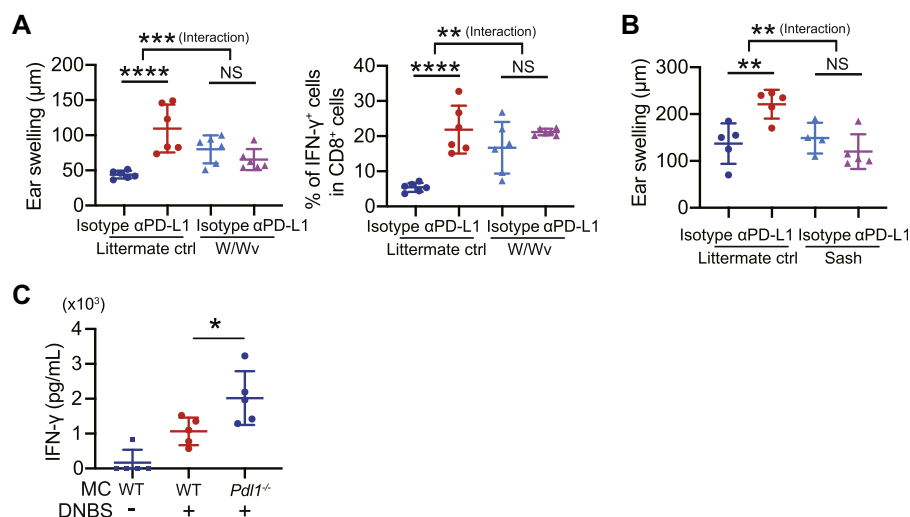


FIG 4. Lack of MCs abolishes the effects of anti-PD-L1 antibody during the elicitation phase in CHS. **A**, Ear swelling (left) and the percentage of IFN-γ⁺ CD8⁺ T cells (middle) of W/W^v mice 24 hours after the elicitation. Anti-PD-L1 mAb (300 μg/mouse) or its isotype-matched Ctrl (control) antibody were administered 6 hours before the elicitation. **B**, Ear swelling of Sash mice 24 hours after the elicitation. Anti-PD-L1 mAb (300 μg/mouse) or its isotype-matched Ctrl antibody were administered 6 hours 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 BMDCs from WT or *Pd1*^{-/-} mice were cocultured at a 1:1 ratio for 3 days with or without 100 μg/mL of dinitrobenzene sulfonic acid (quintuplicate). Results are expressed as the means ± SDs. Data are representative of 3 (A and C) or 2 (B) independent experiments. In (A), 2-way ANOVA was performed for analysis of interaction effect between mouse strain and antibody treatment. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. NS, Not significant.

Skin MCs express PD-L1 in humans

Finally, we investigated the possible human relevance of our findings. We first checked the expression of *PD-L1* (*CD274*) in MCs in human skin by using a public database provided by the Functional Annotation of the Mammalian Genome³¹⁻³³ project, in which the sets of transcripts of various human cells mapped by Cap Analysis of Gene Expression are available. After excluding stimulated or treated cells on the basis of their sample names (see the **Methods** section in the Online Repository), 847 samples remained, including 4 MC samples that were collected from the skin (see **Table E1** in this article's Online Repository). We also compared levels of *PD-L1* expression among various cell populations commonly detected in the skin (**Fig 5, A**; note that samples other than those of MCs were not necessarily collected from the skin). The highest, lowest, mean, and median levels of *PD-L1* expression of each cell population are shown in **Table I**. We also surveyed another set of data provided by a previous publication,³⁴ which revealed a unique transcriptional landscape of MCs by comparison with basophils. Among all the evaluated genes, the level of *PD-L1* expression in MCs was significantly higher ($\log_2(\text{fold change}) > 4$ and $-\log_{10}(P \text{ value}) > 10$) than in basophils (**Fig 5, B** and see the **Methods** section in the 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 colocalization of CD8⁺ T cells and MCs (**Fig 5, C**), 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 MCs *in vitro* by using 2 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 at www.jacionline.org). MART-1- or control antigen-pulsed LUVA cells were cocultured with human MART-1-specific T cells, 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; 24 hours 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 5, D**). Furthermore, the IFN-γ levels were marginally but significantly increased by treatment with anti-PD-L1 antibody (**Fig 5, D**). These results suggest that human MCs can present antigens to T cells, and that PD-L1 on MCs play regulatory roles for T-cell activation, as observed in mouse studies.

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 by using CHS as a model. This regulatory pathway was primarily mediated by PD-L1 on radioresistant cells. Among the radioresistant 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

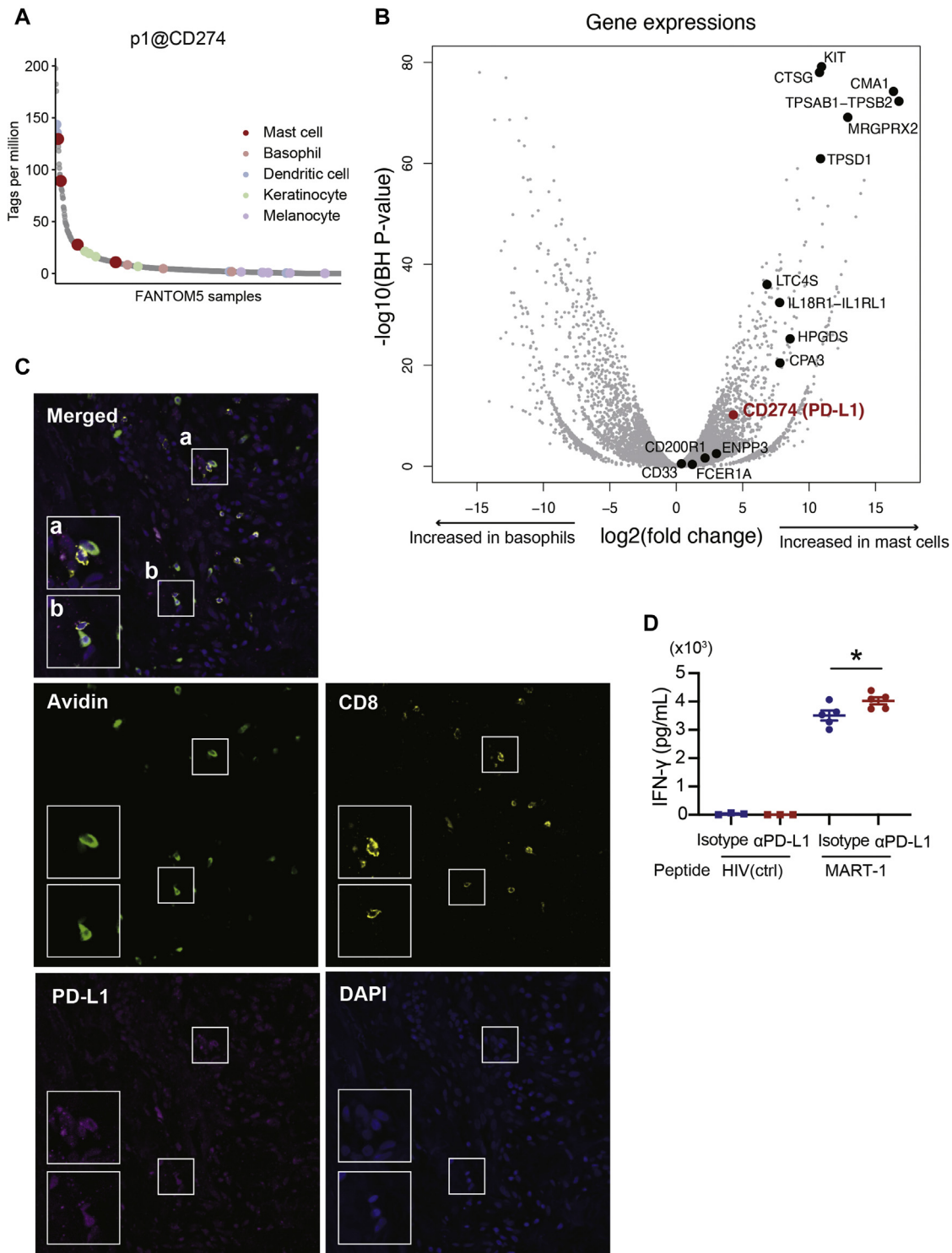


FIG 5. PD-L1 is expressed on human MCs. **A**, Levels of PD-L1 expression of 847 various human cells available in the public database Functional Annotation of the Mammalian Genome 5 (FANTOM5). Four skin MC samples and other cells of interest are labeled with different colors. **B**, Comparison of the mRNA expression levels between MCs and basophils from published data (see Supplemental Table 8 in Motakis et al³⁴). The x-axis indicates $-\log_{10}$ (fold change), and the y-axis indicates $\log_2$ (Benjamini-Hochberg [BH] P value). Fold changes and BH P values were already calculated and provided in the publication. Red and black circles represent CD274 (PD-L1) and well-known MC markers, respectively. **C**, A representative immunostaining of MCs (fluorescein isothiocyanate-avidin), PD-L1 (phycoerythrin), CD8 (allophycocyanin), and 4',6-diamino-2-phenylindole (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. MART-1-specific T cells were cocultured with HLA-A*0201-binding MART-1 peptide- or HIV (control [ctrl]) peptide-pulsed human MCs (LUVa cells) for 24 hours in the presence of anti-PD-L1 antibody or isotype control antibody. Data are representative of 3 (**D**) independent experiments. * $P < .05$.

TABLE I. 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 (TPM)	Bottom (TPM)	Mean (TPM)	Median (TPM)
MCs	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
Keratinocytes	21.31	6.74	15.89	17.76
DCs	143.59	0.37	68.90	66.42
All	197.42	0.00	9.29	2.89

TPM, Tags per million; TSS, transcriptional start sites.

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 on the basis of the observation that PD-1/PD-L1 inhibitors prescribed for patients with cancer frequently cause various types of skin eruption,³⁶ 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 undergoing 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, 1 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 isotype antibody. What caused such discrepant results remains unclear; 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 the mouse strain significantly affects the phenotype of CHS.¹¹ In addition, the different extents of skin inflammation might be another possible reason. In the previous study, the increase in ear thickness induced by DNFB was quite small (<20 μ m), whereas it was around 100 to 200 μ m in our study. Thus, differences in several experimental conditions might be reasons for the discrepancy. Another recent study also reported 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 of *Pd1*^{-/-} mice were significantly increased even at the 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 has 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 analysis,⁴⁷ suggesting a suppressive potential of MCs on pathogenic CD8⁺ T cells; however, its physiologic significance has remained unclear. Here, we have shown 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 their proinflammatory role in the elicitation phase of CHS,^{15,29} MCs may exert anti-inflammatory functions through the PD-1/PD-L1 pathway to fine-tune the extent of CD8⁺ T-cell activation. In support of our results, a recent study has shown a possible immunosuppressive effect of PD-L1 on MCs in tumor immunity, in which high expression of PD-L1 was observed on MCs in patients with 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 using transgenic mice overexpressing PD-L1 on KCs reported a possible regulatory role of PD-L1 on KCs during the effector phase of CHS.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 did not have a significant effect in the absence of MCs, suggesting that MCs and not KCs are the primary population contributing to the PD-L1-mediated suppression of CD8⁺ T cells in CHS. In CHS, effector T cells infiltrate in the skin through postcapillary 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 fine-tuned by PD-L1 on MCs first. The regulatory functions of PD-L1 on KCs may be important in the late stage of CHS or under conditions in which T cells are activated in the epidermis, such as during graft-versus host disease, as previously reported.⁵¹

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

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

We appreciate Lieping Chen, MD, PhD, 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 laboratory 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.

Key messages

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

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METHODS

Antibodies

For flow cytometric analysis, fluorescein isothiocyanate (FITC)-conjugated anti-GZMB (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-FCeR1 α (MAR-1) and avidin; phycoerythrin (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-CD3e (145-2C11), PerCP-eFluor710-conjugated anti-CD8b (H35-17.2); PE/Cy7-conjugated anti-TCR β (H57-597), anti-CD31 (390), anti-FCeR1 α (MAR-1), anti-CD11c (N418), anti-CD8b (H35-17.2) and anti-CD11b (M1/70); allophycocyanin-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-FCeR1 α (MAR-1) and anti-TCR γ/δ (GL3); Brilliant Violet 421-conjugated anti-CD274 (MIH5) and anti-CD117 (2B8); V450-conjugated anti-CD4 (RM4-5); Brilliant Violet 605-conjugated anti-CD4 (RM4-5) and anti-CD11b (M1/70); and BUV395-conjugated anti-CD45 (30-F11) antibodies and Fixable Viability Dye eFluor 780 were purchased from Abcam (Cambridge, United Kingdom), BD Bioscience, Biolegend (San Diego, Calif), eBioscience (San Diego, Calif) or Invitrogen (Carlsbad, Calif).

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). Anti-human PD-L1 blocking mAb (29E.2A3) and mouse IgG2b (MPC-11) isotype control mAb were purchased from Biolegend.

For immunohistochemistry, anti-CD8 (144B, Abcam) and anti-PD-L1 (SP142) were purchased from Abcam (Cambridge, United Kingdom) and Spring Bioscience (Pleasanton, Calif), 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⁺(FCeR1⁺) 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 Treg cells as CD45⁺TCR β ⁺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 μ m 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).

Adoptive transfer experiments

For adoptive transfer, axillary and inguinal DLNs of each donor mouse were collected 5 days after the sensitization with 0.5% DNFB, and single-cell suspensions were prepared by mincing with a 40- μ m cell strainer. Cells were injected intravenously into each recipient mouse (2×10^7 cells per mouse, diluted in PBS). After 6 hours, the recipient mice were elicited with 20 μ L of 0.5% DNFB on both sides of each ear.

Generation of BM-chimeric mice

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

Generation of BMMCs

For the generation of BMMCs, 1×10^6 BM cells from WT and *Pd1*^{-/-} mice were cultured in 20 mL of complete RPMI medium (RPMI 1640 medium; Gibco; Thermo Fisher Scientific, Waltham, Mass), containing 10% fetal calf serum, 50 μ M 2-mercaptoethanol, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 100 U/mL of penicillin/streptomycin supplemented with 5 ng/mL of recombinant murine stem cell factor and IL-3 (PeproTech, London, United Kingdom) using 10-cm noncoated culture dishes or flasks for floating cells (Nunc Non-treated EasYFlasks; Thermo Fisher Scientific). Basically, the culture medium was changed every week depending on cell growth. After 3 weeks of culture, the 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 using a Mouse IFN- γ ELISA Set (RUO) (catalog no. 555138; BD Bioscience). The human IFN- γ concentration of culture supernatants was measured by using anti-human IFN- γ antibody (M700A, Invitrogen) and anti-human IFN- γ biotinylated antibody (M701B, Invitrogen).

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 million of these cells were intravenously injected into a B6 albino mouse, which was elicited with 0.5% DNFB 24 hours before the analysis. To visualize cutaneous MCs, 20 μ L of avidin-FITC (1:200 in PBS) was intradermally injected into the ear right before the analysis. Two-photon microscopic analysis was performed as previously described,^{E1} with some modifications. In brief, the mice were anesthetized with isoflurane and positioned on the heating plate on the stage of a 2-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) cells,^{E2} which derive from a human MC sarcoma/leukemia were kindly provided by Dr Arnold S. Kirshenbaum (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md). Cells were cultured in StemPro-34 serum-free medium (Invitrogen) supplemented with 100 U/mL of penicillin/streptomycin, 100 U/mL of glutamine, and 100 ng/mL of recombinant human stem cell factor (PeproTech, London, United Kingdom), which was half replaced every week or 2 weeks depending on cell growth.

Another human MC line, LUVA,^{E3} was commercially purchased (Kerafast, Boston, Mass). Cells were cultured in StemPro-34 serum-free medium (Invitrogen) supplemented with 100 U/mL of penicillin/streptomycin and 100 U/mL of glutamine with or without 100 ng/mL recombinant human stem cell factor (PeproTech, London, United Kingdom), which was changed every few days based on the cell growth.

MART-1-specific T cells were generated as previously described.^{E4,E5} 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). 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) for 2 days 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) with 10% human-AB serum (Gemini Bio, West Sacramento, Calif) and recombinant human IL-2 (500 IU/mL; Novartis) and used for experiments after more than 7 days of expansion.

Immunohistochemistry for human skin

Skin biopsy samples from patients clinically diagnosed with allergic contact dermatitis (n = 5; 40s-70s; 2 males and 3 females) were analyzed. Paraffin-embedded tissue samples were deparaffinized and incubated with FITC-conjugated avidin overnight at 4°C. Antigen retrieval using pH 9.0 buffer was performed in a microwave oven at 95°C for 15 minutes. Tissue samples were incubated with anti-CD8 primary antibody (1:500) for 30 minutes, with horseradish peroxidase polymer-conjugated anti-mouse antibody for 10 minutes, and then with Opal 570 (1:500) for 10 minutes, followed by washing. Antigen retrieval using pH 6.0 buffer was then performed in a decloaking chamber for 20 minutes. The tissue samples were incubated with anti-PD-L1 primary antibody (1:300) for 30 minutes, with horseradish peroxidase polymer-conjugated anti-rabbit antibody for 10 minutes, and then with Opal 690 (1:500) for 10 minutes. Coverslips were mounted with Prolong Gold Antifade Mountant with 4',6-diamino-2-phenylindole (ThermoFisher Scientific). Fluorescence images were obtained using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan).

Database analysis

A list of PD-L1 mRNA expression levels of various human cell samples was obtained from a database service, the Functional Annotation of Mammalian Genome 5 (FANTOM5) project, Semantic Catalog of Samples, Transcription Initiation and Regulators (SSTAR).^{E6-E8} The data set of mapping transcription start site expression of human CD274 (EntrezGene:29126, p1@CD274) was exported. Of 889 entries (available registered samples), 847 entries remained after exclusion of samples whose name included the words *stimulated* or *treated* (but *except untreated*). Each cell population was extracted on the basis of the sample name. For the comparison between MCs and basophils, the data were obtained from a previous report (see Supplemental Table 8 in Motakis et al.^{E9}). In the data, log fold changes and Benjamini-Hochberg *P* values of the 16,533 genes were provided. Graphs were drawn by using published R packages, including EnhancedVolcano^{E10} and ggplot2.

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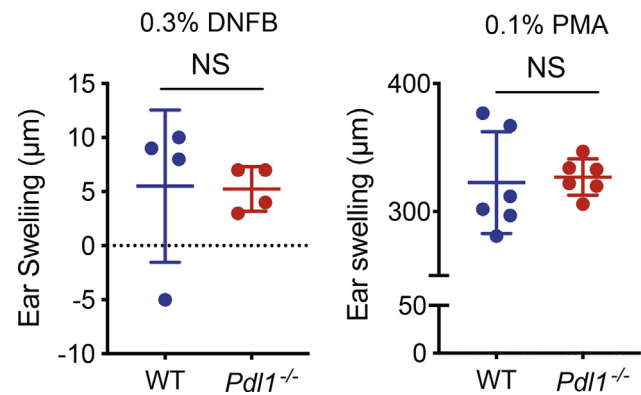


FIG E1. PD-L1-deficient (*Pdl1*^{-/-}) mice exhibit equivalent ear swelling responses in irritant dermatitis. Ear thickness changes 24 hours after the application of 0.3% DNFB (*left*) and 6 hours after the application of 0.1% phorbol 12-myristate 13-acetate (*right*) in WT and *Pdl1*^{-/-} mice. Data are representative of 2 independent experiments. Results are expressed as the means ± SDs. *PMA*, Phorbol 12-myristate 13-acetate; *NS*, not significant.

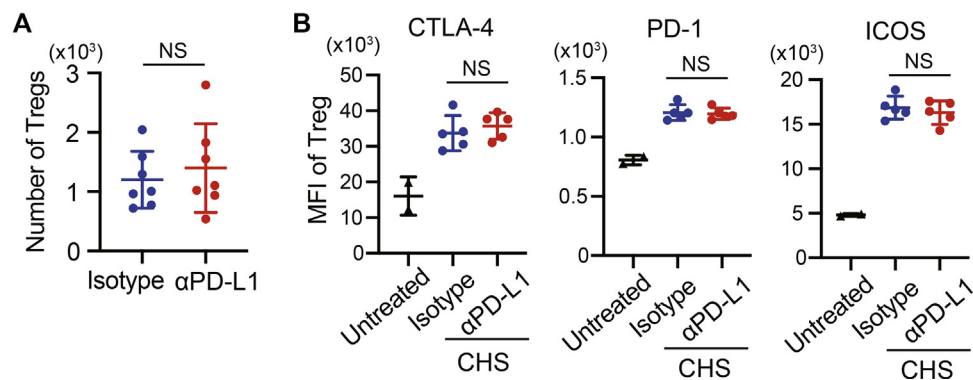


FIG E2. No significant difference was observed in the number or surface markers of Treg cells in the skin under the blockade of PD-1/PD-L1 signaling. Numbers (**A**) and surface markers (**B**) of Treg cells in the ear skin, as analyzed by flow cytometry 24 hours after the elicitation in mice treated with anti-PD-L1 mAb (300 μ g/mouse) or its isotype-matched control antibody. Results are expressed as the means $\pm$ SDs. Data are representative of 2 independent experiments. *CTLA-4*, cytotoxic T-lymphocyte protein-4; *MFI*, mean fluorescence intensity; *NS*, not significant.

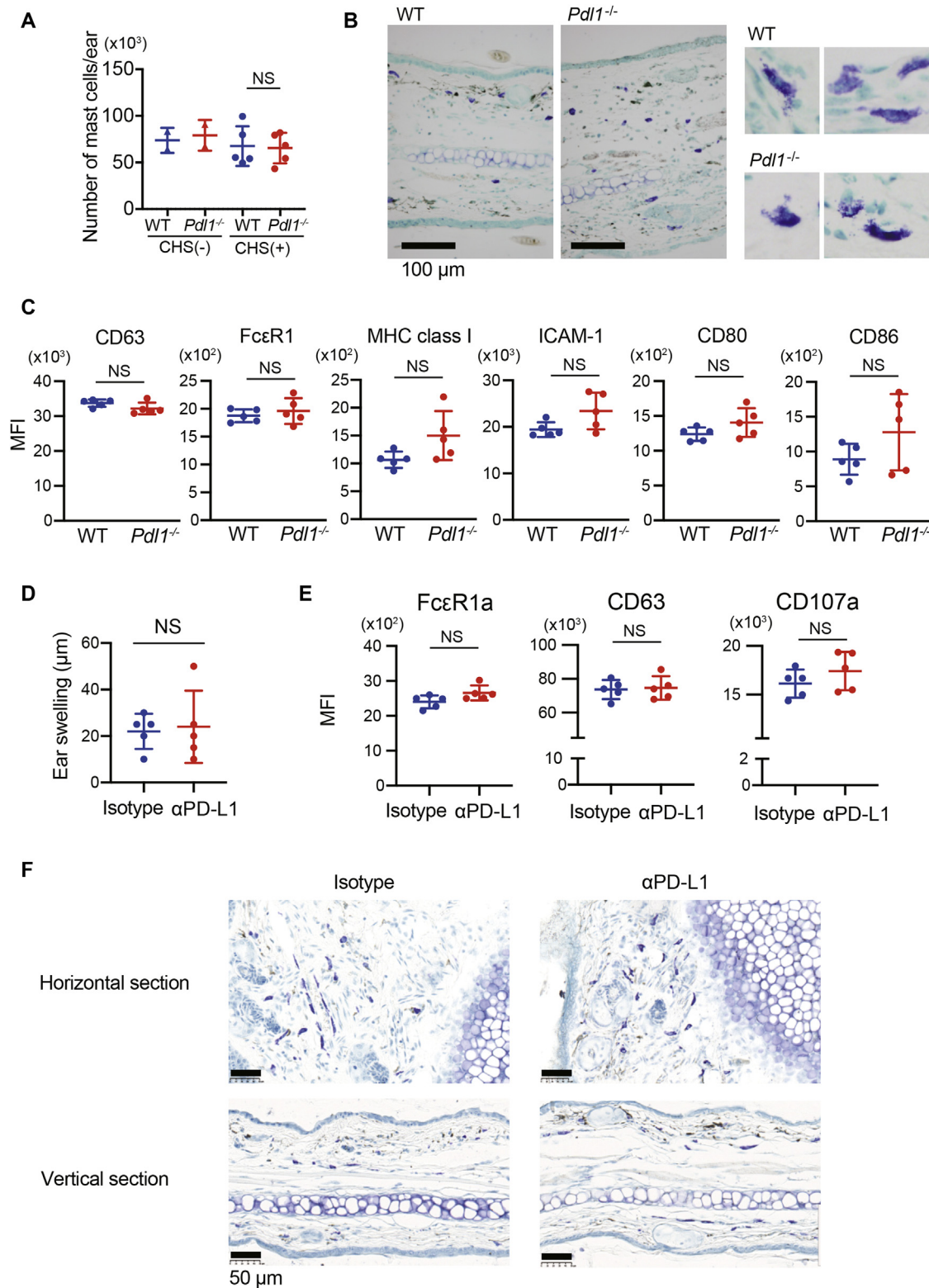


FIG E3. Lack of PD-1/PD-L1 signaling does not affect the numbers or expression of activation markers in skin MCs during the elicitation phase of CHS. **A**, The number of MCs in the ear skin, as analyzed by flow cytometry 24 hours after the elicitation of CHS in WT and $Pd1^{-/-}$ mice. **B**, Representative toluidine blue staining of the ear skin 24 hours after elicitation. (Right) High magnification of degranulated MCs. **C**, Activation markers of MCs in WT and $Pd1^{-/-}$ mice 24 hours after elicitation, as 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 hour after the elicitation of CHS. Mice were treated with anti-PD-L1 mAb (300 μ g/mouse) or its isotype-matched control antibody 6 hours before the elicitation. The horizontal and vertical sections are shown for each sample in (**F**). Results are expressed as means $\pm$ SDs. Data are representative of 3 (**A**) or 2 (**C-E**) independent experiments. *MFI*, Mean fluorescence intensity; *NS*, not significant.

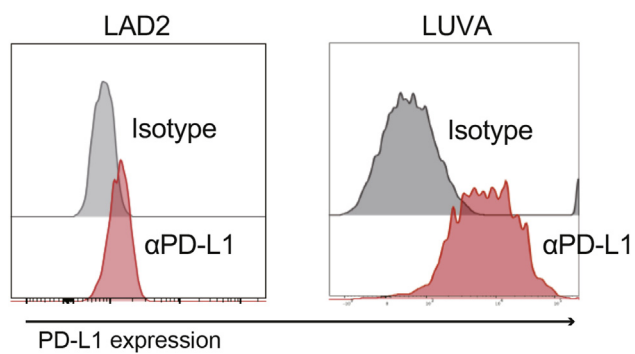


FIG E4. Human MC lines constitutively express PD-L1. Flow cytometric analysis of PD-L1 expression on LAD2 cells and LUVA cells. Gray peak represents a control stained with isotype IgG.

TABLE E1. PD-L1 expression in human skin MC samples from the public database Functional Annotation of the Mammalian Genome 5 (FANTOM5)

Sample ID	p1@CD274 (TSS) (TPM)	Rank (among a total 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

ID, Identifier; TPM, tags per million; TSS, transcriptional start site.