

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING INFORMATION

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SUPPORTING INFORMATION

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Immune microenvironment controls the outcome of PD-1 blockade in cutaneous immune response

To the Editor

The introduction of immune checkpoint inhibitors (ICI) targeting the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1)

pathway or cytotoxic T-lymphocyte antigen 4 (CTLA-4) has brought about a revolution in anticancer therapy by embodying the concept that the host's exhausted pre-existing antitumor immunity can be re-activated. The frequency of ICI treatment-associated skin toxicities

Ogawa and Ishitsuka have equal contribution.

[cutaneous immune-related adverse events (C-irAEs)] is considered to be 40% of the total irAEs.¹ It has been becoming evident that compared with CTLA-4 blockade-associated C-irAEs, the PD-1/PD-L1 pathway blockade-associated C-irAEs manifest as diverse eruptions that mimic

lichen planus, psoriasis, bullous pemphigoid, sarcoidosis, or lupus erythematosus.¹ Because the PD-1/PD-L1 pathway regulates various forms of immunity at the effector phase in the inflamed tissue at the periphery,² we hypothesized that various forms of cutaneous eruptions

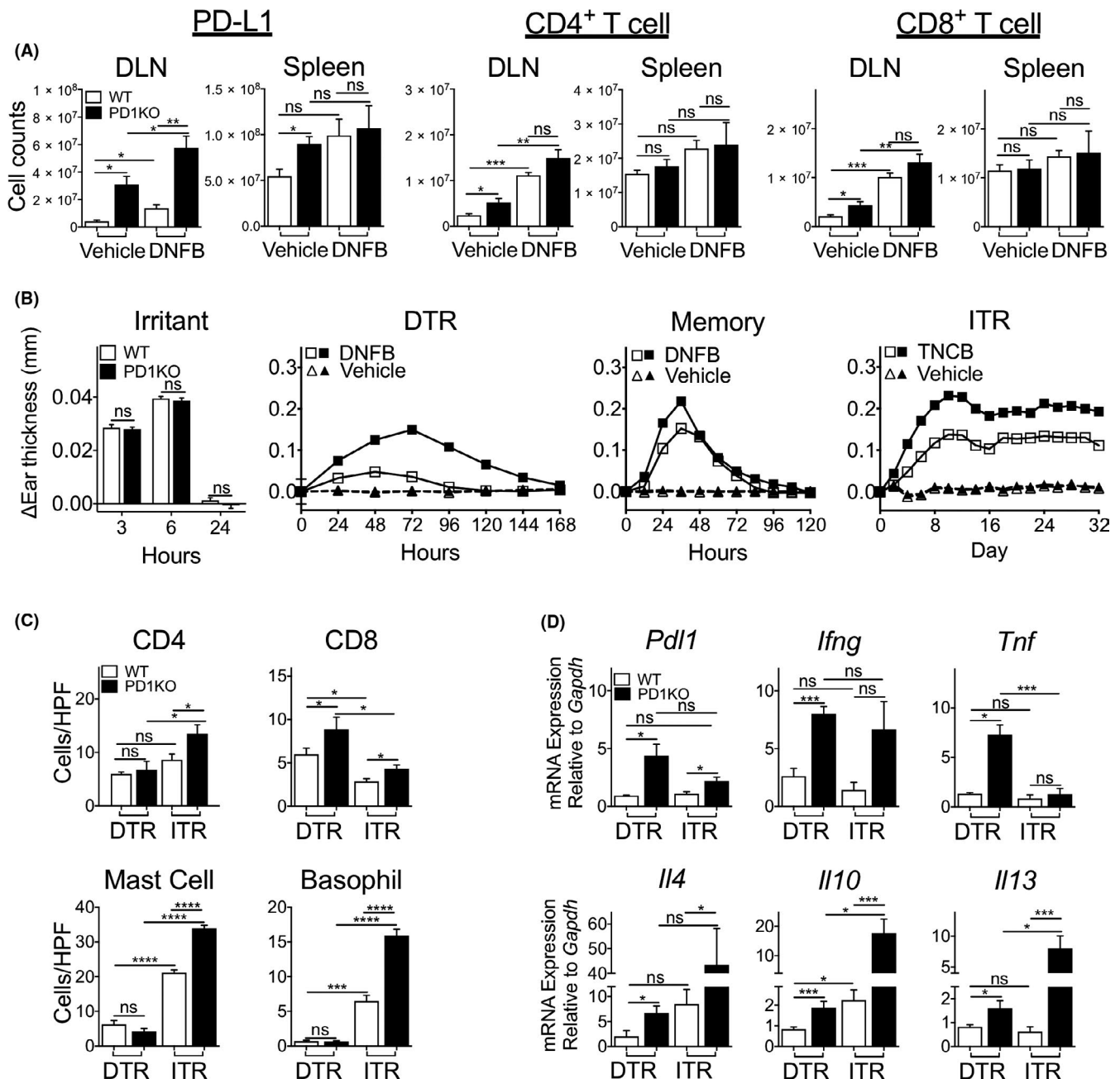


FIGURE 1 Enhanced CHS in the absence of PD-1. A, Enhanced lymphoproliferative response in the draining lymph nodes (DLNs). Single-cell suspensions from DLN or spleen were collected 5 d after single 0.5% 2,4-dinitrofluorobenzene (DNFB) application on the abdominal skin and subjected to flow cytometric analysis. Open bars, wild-type (WT) mice; closed bars, PD-1 knockout (PD1KO) mice; $n = 4$; $*P < 0.05$, $**P < 0.01$, $***P < 0.005$; ns, not significant. B, Ear swelling response after topical chemical application. Irritant dermatitis: single topical croton oil application. Delayed-type reaction (DTR): DNFB challenge after sensitization. Memory response: DNFB rechallenge after 6 wks. Immediate-type response (ITR): repeated 2,4,6-trinitrochlorobenzene (TNCB) application. Open bars, squares, and triangles: WT mice; closed bars, squares, and triangles: PD1KO mice; $n = 6$ for each experimental group. Statistically significant difference between PD1KO and WT mice: DTR (24–144 h), memory (12–48 and 72–120 h), and ITR (2–32 d); ns, not significant. C, Immune effector cell population in CHS-elicited ear skin; CD4 and CD8 (immunohistochemistry; per HPF). Open bars, WT mice; closed bars, PD1KO mice; $n = 5$ or 7 ; $*P < 0.05$, $***P < 0.005$, $****P < 0.001$; ns, not significant. D, Cytokine expression analysis in CHS-elicited ear skin. Open bars, WT mice; closed bars, PD1KO mice; $n = 6$; $*P < 0.05$, $***P < 0.005$; ns, not significant

in the PD-1/PD-L1 blockade-associated C-irAEs reflect the nature of the pathway itself. To address this hypothesis, we used the classic model of cell-mediated adaptive immune response contact hypersensitivity (CHS); a single topical application after sensitization in the distant site causes type 1 dominant delayed-type reaction (DTR), whereas repeated topical application of reactive haptens exaggerates type 2 dominant immunological memory and immediate-type response (ITR).

Because expression levels of PD-L1 profoundly affect the immune effector response in both lymphoid and nonlymphoid tissues, we first sought to determine the influence of PD-1 on proliferative response and the regulation of PD-L1 expression in the lymphoid tissue. A single topical application of 2,4-dinitrofluorobenzene (DNFB) not only induced the proliferation of CD4⁺ or CD8⁺ T cells in draining lymph nodes (DLNs) but also resulted in the accumulation of PD-L1⁺ cells, whereas these cell populations in the spleen were not substantially altered in both genotypes (Figure 1A). In line with a previous study in which immune checkpoints were disrupted using monoclonal antibodies (mAbs),³ PD-1 knockout (PD1KO) mice exhibited significantly increased number of CD4⁺ or CD8⁺ T cells compared with wild-type (WT) mice, which was accompanied with significantly increased PD-L1⁺ population that presumably reflects compensatory response (Figure 1A). In line with the fact that the PD-1/PD-L1 pathway primarily affects adaptive immune response, a single topical application of croton oil caused comparable ear swelling between the two genotypes (Figure 1B). In the DTR setting, the absence of PD-1/PD-L1 interaction resulted in significantly increased ear swelling compared with WT mice (Figure 1B and Figure S1) with concomitant increase in CD8⁺ cell numbers in the dermis (Figure 1C). Rechallenge of CHS-elicited PD1KO mice evoked significantly increased memory response compared with WT mice, and repeated topical application of 2,4,6-trinitrochlorobenzene (TNCB) resulted in exaggerated type 2 immune response in the absence of PD-1/PD-L1 interaction (Figure 1B and Figure S1) compared with WT mice. Accordingly, CD4⁺ cells, CD8⁺ cells, mast cells, or basophils in the dermis and type 2 cytokine (*Il4*, *Il10*, and *Il13*) expression levels in the skin were significantly increased in PD1KO mice compared with those in WT mice (Figure 1C,D). PD-1/PD-L1 interaction requirement in the inflamed tissue at the periphery was proven by a series of adoptive transfer experiments, in which the PD1KO recipient constantly exhibited significantly increased ear swelling compared with the WT recipient (Figure 2A). Taken together, these results suggest that genetically disrupted PD-1/PD-L1 interaction exaggerates CHS with distinct effector classes.

Next, to evaluate the clinical relevance of the findings above, WT mice on an allergy-prone BALB/c background were administered PD-1 mAb after sensitization and challenged with irrelevant haptens in the DTR or ITR settings as indicated (Figure 2B,C). This not only allowed us to determine whether the activated pre-existing immunity is specific for neoantigen but also explained that immune response outcome primarily depends on the local immune microenvironment rather than the lymphoid tissue from which lymphoproliferative response arises. As expected, PD-1 mAb treatment resulted in significantly enhanced DTR and ITR compared with phosphate-buffered saline (PBS) treatment in a hapten-specific manner, indicating that

the acquired blockade of the PD-1/PD-L1 pathway augments pre-existing adaptive immune effector response (Figure 2B,C).

In line with the fact that the PD-1/PD-L1 pathway promotes T-cell exhaustion at the periphery and thereby prevents pathological consequences in various mouse models of infection or autoimmune diseases,² we have shown the exaggerated allergic response in the skin. After percutaneous immunization, skin mounts various classes of immune responses depending on the peripheral microenvironment that gives rise to distinct effector or memory T-cell subsets. Indeed, it has recently been shown through a parabiotic approach that CHS response outcome is primarily affected by skin-resident T-cell subset (resident memory T [T_{RM}] cells rather than the circulatory counterpart [central memory T cells]) despite shared clonality between the two T-cell subsets.⁴ Thus, our results suggest that a disrupted PD-1/PD-L1 interaction may enhance the effector function or T_{RM} cells and cause exaggeration of CHS response despite the relative abundance of immunosuppressive type 2 cytokines.

Reflecting its primary function as a frontline defense, skin faces constant risks of generating autoreactivity. For instance, viral infection is closely associated with molecular mimicry⁵ and tissue damage potentially causes epitope spreading.⁶ This is a potential explanation for relatively high frequency of C-irAEs compared with irAEs in other organs in patients undergoing ICI treatment (CTLA-4 or PD-1/PD-L1 blockade).⁷ Regarding the diversified C-irAEs associated with PD-1/PD-L1 blockade, along with our results, experimental evidence from nonobese diabetic mice could offer a mechanistic explanation; the CTLA-4 inhibitory pathway only inhibits naive T-cell activation and early disease induction, whereas the PD-1/PD-L1 pathway affects both naive and effector T-cell activation, resulting in severe pathological consequences.⁸ Further evidence from translational perspectives will help determine the true nature of C-irAE development and lead to the development of legitimate management strategies.

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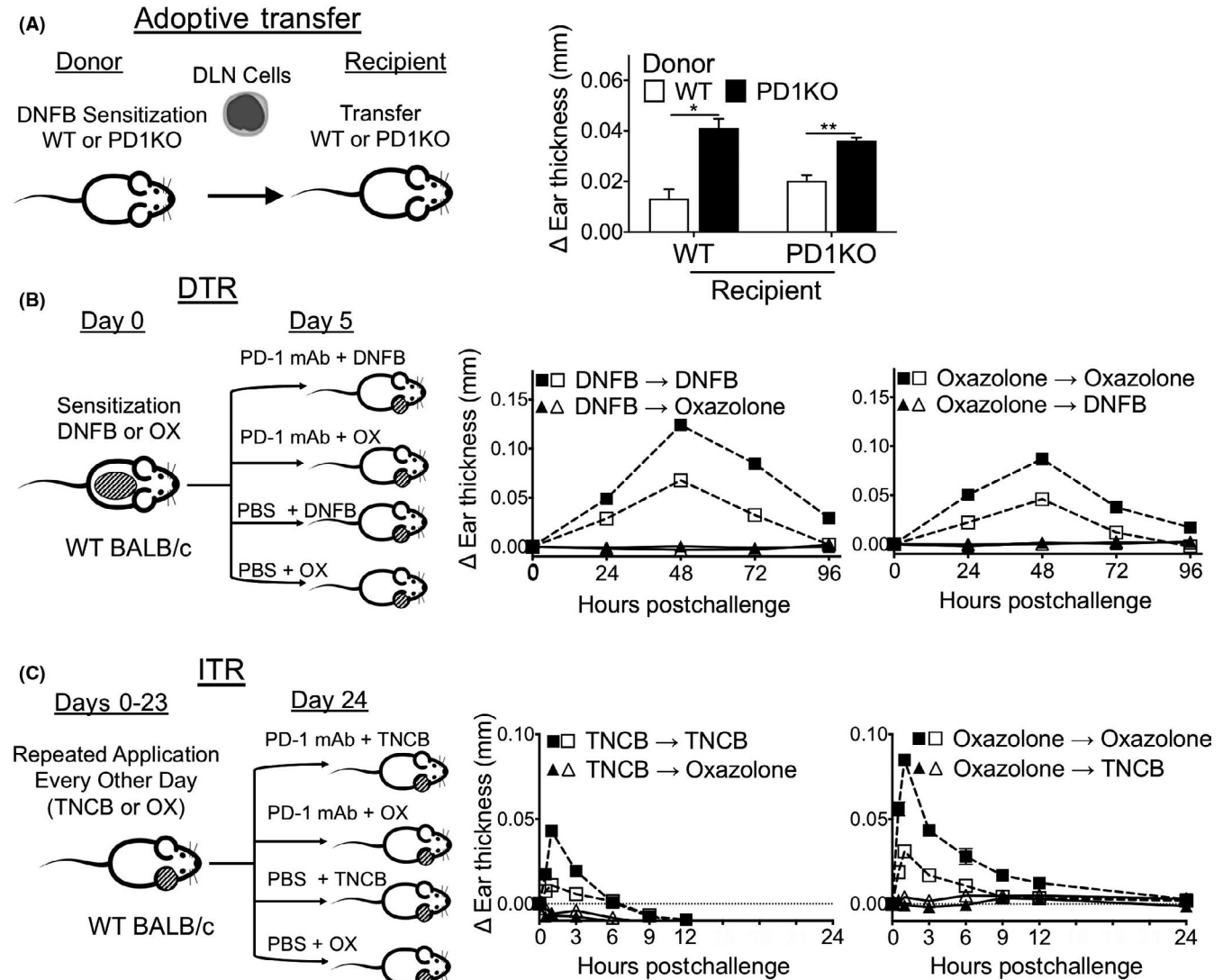


FIGURE 2 Hapten-specific effector phase regulation by the PD-1/PD-L1 pathway. A, DNFB-sensitized DLN cells were intravenously injected, CHS was elicited, and ear swelling response was measured after 24 h. $n = 4$; * $P < 0.05$, ** $P < 0.01$. (B and C) WT BALB/c mice were sensitized before PD-1 mAb administration, and CHS was elicited using unrelated haptens. Open squares or triangles, PBS-treated control mice; closed squares or triangles, PD-1 mAb-treated mice; $n = 4$ for each experimental group

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Respiratory allergies with no associated food allergy disrupt oral mucosa integrity

To the Editor,

Respiratory allergy is the most common allergy worldwide. Studies in Europe have shown that up to 30% of the population suffers from allergic rhinoconjunctivitis, while up to 20% suffer from asthma. This group of allergic diseases is characterized by an aberrant immune response to aeroallergens. The prevalence of aeroallergens varies in different regions depending on the climate.¹ Patients overexposed to grass pollen are frequently sensitized to the pan-allergen profilin. In fact, we have recently demonstrated that a fraction of these patients develops severe profilin-mediated food reactions that are associated with intense oral mucosa remodeling,² and with unique metabolic and transcriptomic signatures.³

In the present study, we aim to investigate whether oral mucosa is compromised in the absence of food allergy. We formulated the hypothesis that an intense systemic inflammation could induce structural changes in the oral mucosa after respiratory sensitization.

To test this hypothesis, we took advantage of respiratory phenotypes associated with olive pollen (seasonal allergy) and mites (perennial allergy), previously described.^{4,5} We recruited patients with respiratory allergy to olive pollen from Cordoba, Spain (n = 5), defined by clinical history and with positive skin prick test (SPT) to olive allergens Ole e1 and/or Ole e7, and patients with allergy to house dust mites (HDM) from Gran Canaria, Spain (n = 6), who experienced urticaria, respiratory distress or pruritus after ingestion of mite-contaminated flours. Clinical and demographic data of allergic participants are summarized in Table 1. Oral mucosa biopsies were obtained from the cheek lining of the subjects by using a scalpel or punch and used for histological and immunohistochemical analyses (AppendixS1). We analyzed structural components and cell infiltrate in oral mucosal samples from these patients compared with a control group of nonallergic subjects (n = 5).

First, we studied whether the oral mucosal barrier integrity was compromised. For this purpose, we determined tight junction (TJ) and adherens junction (AJ) protein expression. We observed a significant and similar decrease in occludin and E-cadherin expression in both groups of allergic patients in accordance with previous results²

SUPPORTING INFORMATION

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(Figure 1A-D). However, we could not find significant differences in claudin-1 expression (Figure 1E,F) as previously reported.² Next, we studied fibrosis in the oral connective tissue of these patients. Augmented fibrosis has been previously associated with epithelial disruption.^{2,6} We observed increased collagen deposition in olive pollen and HDM-allergic patients (Figure 1G,H). An affected epithelial barrier together with increased fibrosis of the oral mucosa suggest that extensive structural changes are taking place in respiratory phenotypes associated with olive pollen and mites.

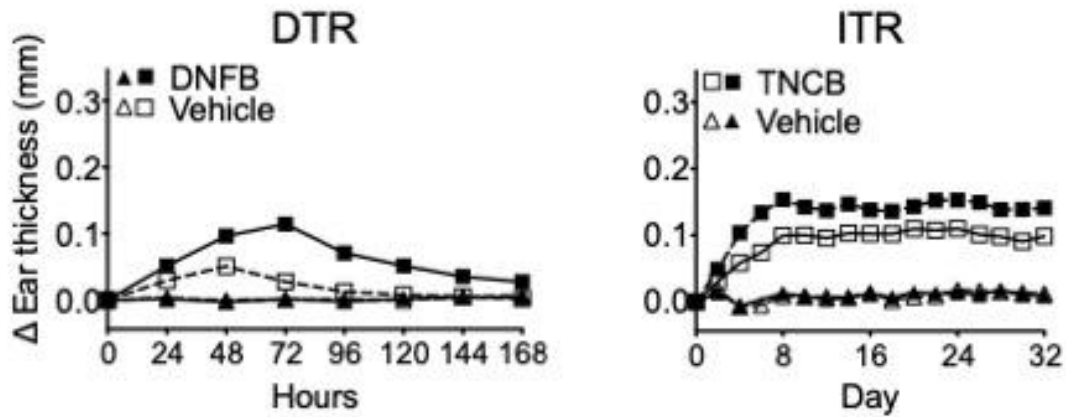
Increased angiogenesis has been previously described in the oral mucosa of patients with inflammatory pathologies.⁷ No significant differences were found in this study for the angiogenic factor VEGFa (data not shown). However, when measuring capillary density, an increased number of blood vessels was observed in HDM-allergic patients (Figure 1I). Regarding immune recruitment to the oral mucosal tissue, we could not find major differences in immune cell counts when comparing allergic groups with nonallergic controls. We focused on phagocytes, mast cells, innate lymphoid cells and lymphocytes. For this purpose, the expression of langerin, MCT, FcεRI, CRTH2, CD3, CD4 and FoxP3 was determined. Numbers of langerin+ cells among the experimental groups were similar; however, FcεRI expression in these cells tended to be higher in the oral epithelium of allergic patients (data not shown). Regarding the connective tissue, MCT+ cells represented almost 100% of the FcεRI-expressing cells in nonallergic subjects. In contrast, FcεRI/MCT ratio revealed that other cell types were also FcεRI+ in the allergic populations (Figure 1J). CD3+ and CD4+ counts were higher in the connective tissue of nonallergic subjects (data not shown). The majority of CD3+ cells were also CD4+ (Figure 1K). Among the CD3+ cells, HDM patients showed a significantly increased proportion of regulatory T cells (Tregs). Although not significant, this trend was also found for olive pollen allergic patients (Figure 1L). We also stained for eosinophils (Figure 1M) and neutrophils (Figure 1N) but found hardly any positively stained cell.

Based on these results, we conclude that oral mucosal integrity is compromised in respiratory phenotypes in the absence of food allergy. Moreover, oral mucosa structural changes take place with no associated local recruitment of inflammatory infiltrate, which is presumably directed to the airway mucosa, but higher numbers of

Abbreviations: AJ, adherens junction; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; FFPE, formalin-fixed paraffin embedded; HDM, house dust mites; ILC, innate lymphoid cell; MCT, mast cell tryptase; S1P, sphingosine-1-phosphate; SPT, skin prick test; TJ, tight junction; Treg, regulatory T cell; VEGFa, vascular endothelial growth factor A.

1 Supplemental material

2 Figure S1. Enhanced CHS in the absence of PD-L1



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4 DTR and ITR were induced as described before. Open squares and triangles, WT mice; closed
5 squares and triangles, PDL1KO mice; $n = 4$ for each experimental group. Statistically significant
6 difference between PDL1KO and WT mice: DTR (24–144 h) and ITR (2–32 days).

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8 **Methods**

9 **Mice**

10 C57BL/6 and BALB/c mice (8–12 weeks of age) were purchased from Charles River
11 Laboratories, and programmed death-1 (PD-1)-deficient (PD1KO) mice were kindly provided by
12 Dr. T. Honjo¹. Programmed death-ligand 1 (PD-L1)-deficient (PDL1KO) mice were generated at
13 the Laboratory Animal Resource Center, University of Tsukuba.

14

15 **Flow cytometric analysis**

16 Abdominal skin of the mice was shaved and painted with 25 μ L of 0.5% 2,4-
17 dinitrofluorobenzene (DNFB) (70-34-8; Tokyo Chemical Industry, Tokyo, Japan) in 4:1
18 acetone:olive oil, and draining lymph node (DLNs; axillary, brachial, popliteal, and inguinal)
19 cells and splenocytes were collected after 5 days. Single-cell suspension was prepared and
20 stained with the following monoclonal antibodies (mAbs) purchased from BioLegend (San
21 Diego, CA): phycoerythrin (PE)-conjugated anti-CD274 (10F.9G2), allophycocyanin-conjugated
22 anti-CD3 (17A2), fluorescein isothiocyanate-conjugated anti-CD4 (GK1.5), and PE-conjugated
23 anti-CD8 (53-6.7). Isotype-matched antibodies were used as controls. A LIVE/DEAD[®] Fixable
24 Violet Dead Cell Stain Kit (Life Technologies, Gaithersburg, MD) was used to detect dead cells.
25 Flow cytometric analysis was performed using a Gallios cytofluorometer (Beckman Coulter,
26 Fullerton, CA) and FlowJo platform (FlowJo LLC, Ashland, OR).

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Irritant dermatitis

Ten microliters of 2% croton oil (Tokyo Chemical Industry, Tokyo, Japan) in 4:1 acetone:olive oil were painted on the mouse ear. Ear thickness was measured using a digital micrometer (MDC-25-MX; Mitsutoyo, Kawasaki, Japan) before challenge and at 3, 6, and 24 h after challenge.

CHS reaction

Abdominal skin of the mice was shaved and painted with 25 μ L of 0.5% DNFB. Five days later, the mice were challenged on the dorsal surface of the ears with 10 μ L of 0.3% DNFB. Ear thickness was measured using a digital micrometer before challenge and up to 7 days at 1-day intervals as described previously². For memory response, the mice were rechallenged with 10 μ L of 0.3% DNFB 6 weeks after the first challenge. For the chronic model³, the mice were sensitized by applying 20 μ L of 1% 2,4,6-trinitro-1-chlorobenzene (TNCB) (Tokyo Chemical Industry, Tokyo, Japan) on the dorsum of the ear, and the reaction was elicited with repeated 1% TNCB (20 μ L) application for up to 32 days every other day.

CHS adoptive transfer

For the adoptive transfer of CHS reaction, PD1KO and WT mice were sensitized with 25 μ L of 0.5% DNFB applied to their abdomen as well as 10 μ L to each paw. After 5 days, lymph node cells were collected from DLN (axillary, brachial, popliteal, and inguinal) cells by physical disruption and filtered through a 40- μ m screen. Groups of unsensitized PD1KO and WT mice were injected intravenously with 5×10^7 cells. Two hours later, baseline ear thickness was

measured, and the mice were challenged with 10 μ L of 0.3% DNFB. Following this, ear thickness was measured 24 h later.

Antibody-mediated blockade of PD-1 in CHS reactions

Ten days after DNFB sensitization, WT BALB/c mice were challenged with either DNFB or 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OX) (Santa Cruz, Dallas, TX). During the experimental course, mice received intraperitoneal injections of anti-PD-1 mAb (500 μ g/mouse; Ono Pharmaceutical, Osaka, Japan) on day 0 and 250 μ g/mouse every other day from day 2 onward. PBS injection was performed as a mock procedure. For ITR, mice were subjected to repeated TNCB or OX application every other day and challenged with either TNCB or OX. During the experiment course, mice received PD-1mAb from day 14 (500 μ g/mouse) to day 24 (250 μ g/mouse) every other day, and PBS injection was performed as a mock procedure.

Histopathological analysis

Ear skin was fixed with 10% buffered formalin. After paraffin embedding, 3- μ m sections were subjected to staining. Using a digital microscope (Olympus, Tokyo, Japan) and Flovel filing system (Flovel, Tokyo, Japan), immunohistochemistry was performed as described previously⁴. The following primary antibodies were used: anti-CD4 antibody (1 μ g/mL; Invitrogen, Carlsbad, CA) and anti-CD8 antibody (1 μ g/mL; Invitrogen). The numbers of infiltrating mast cells and basophils were quantified by counting toluidine blue- and Alcian blue-positive cells, respectively, in the dermis per 20 random high-power fields (HPF). The results are expressed as

the mean number of stained cells $\pm$ standard error of the mean (SEM) per HPF obtained from four mice.

Quantitative real-time PCR (QRT-PCR)

Pieces of full-thickness ear skin from PD1KO and WT mice, sensitized with 0.5% DNFB and challenged with 0.3% DNFB, were taken 24 h later and homogenized in TRIzol reagent (15596-026; Thermo Fisher, Waltham, MA) and total RNA was isolated. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher).

Primers specific for each target with double-quenched probes were purchased from the IDT Primetime Predesigned Library (IDT, Coralville, IA), and primers for *Gapdh* (Thermo Fisher) were used for internal reference. All data were analyzed using an QuantStudio 5 Real-Time PCR System (Thermo Fisher). The interpretation of QRT-PCR data was performed using the comparative Ct method ($\Delta\Delta CT$ method).

Statistical analysis

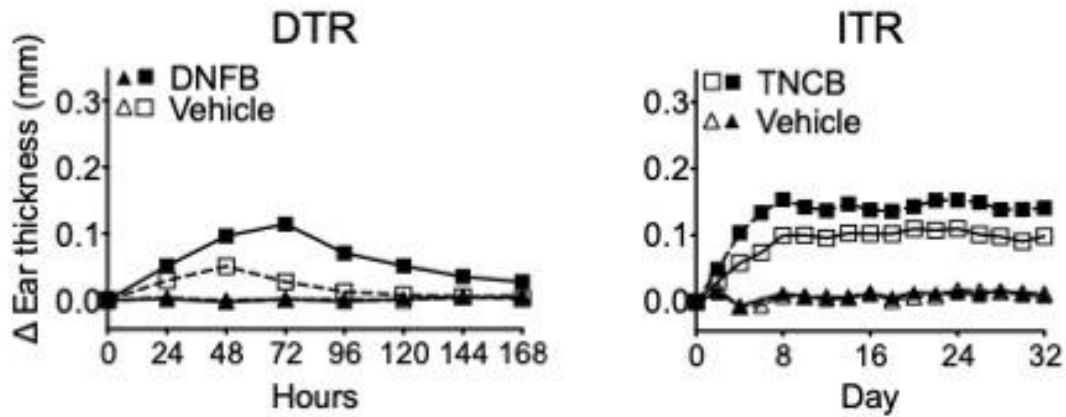
Data are expressed as means $\pm$ SEM. Comparisons were performed using unpaired *t* test with Welch's correction (between two groups) or two-way analysis of variance (time course experiments) using Prism 6 software (GraphPad, La Jolla, CA). For all analyses, significance was set at $p < 0.05$.

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Abdominal skin of the mice was shaved and painted with 25 μ L of 0.5% DNFB. Five days later, the mice were challenged on the dorsal surface of the ears with 10 μ L of 0.3% DNFB. Ear thickness was measured using a digital micrometer before challenge and up to 7 days at 1-day intervals as described previously². For memory response, the mice were rechallenged with 10 μ L of 0.3% DNFB 6 weeks after the first challenge. For the chronic model³, the mice were sensitized by applying 20 μ L of 1% 2,4,6-trinitro-1-chlorobenzene (TNCB) (Tokyo Chemical Industry, Tokyo, Japan) on the dorsum of the ear, and the reaction was elicited with repeated 1% TNCB (20 μ L) application for up to 32 days every other day.

CHS adoptive transfer

For the adoptive transfer of CHS reaction, PD1KO and WT mice were sensitized with 25 μ L of 0.5% DNFB applied to their abdomen as well as 10 μ L to each paw. After 5 days, lymph node cells were collected from DLN (axillary, brachial, popliteal, and inguinal) cells by physical disruption and filtered through a 40- μ m screen. Groups of unsensitized PD1KO and WT mice were injected intravenously with 5×10^7 cells. Two hours later, baseline ear thickness was

measured, and the mice were challenged with 10 μ L of 0.3% DNFB. Following this, ear thickness was measured 24 h later.

Antibody-mediated blockade of PD-1 in CHS reactions

Ten days after DNFB sensitization, WT BALB/c mice were challenged with either DNFB or 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (OX) (Santa Cruz, Dallas, TX). During the experimental course, mice received intraperitoneal injections of anti-PD-1 mAb (500 μ g/mouse; Ono Pharmaceutical, Osaka, Japan) on day 0 and 250 μ g/mouse every other day from day 2 onward. PBS injection was performed as a mock procedure. For ITR, mice were subjected to repeated TNCB or OX application every other day and challenged with either TNCB or OX. During the experiment course, mice received PD-1mAb from day 14 (500 μ g/mouse) to day 24 (250 μ g/mouse) every other day, and PBS injection was performed as a mock procedure.

Histopathological analysis

Ear skin was fixed with 10% buffered formalin. After paraffin embedding, 3- μ m sections were subjected to staining. Using a digital microscope (Olympus, Tokyo, Japan) and Flovel filing system (Flovel, Tokyo, Japan), immunohistochemistry was performed as described previously⁴. The following primary antibodies were used: anti-CD4 antibody (1 μ g/mL; Invitrogen, Carlsbad, CA) and anti-CD8 antibody (1 μ g/mL; Invitrogen). The numbers of infiltrating mast cells and basophils were quantified by counting toluidine blue- and Alcian blue-positive cells, respectively, in the dermis per 20 random high-power fields (HPF). The results are expressed as

the mean number of stained cells $\pm$ standard error of the mean (SEM) per HPF obtained from four mice.

Quantitative real-time PCR (QRT-PCR)

Pieces of full-thickness ear skin from PD1KO and WT mice, sensitized with 0.5% DNFB and challenged with 0.3% DNFB, were taken 24 h later and homogenized in TRIzol reagent (15596-026; Thermo Fisher, Waltham, MA) and total RNA was isolated. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher).

Primers specific for each target with double-quenched probes were purchased from the IDT Primetime Predesigned Library (IDT, Coralville, IA), and primers for *Gapdh* (Thermo Fisher) were used for internal reference. All data were analyzed using an QuantStudio 5 Real-Time PCR System (Thermo Fisher). The interpretation of QRT-PCR data was performed using the comparative Ct method ($\Delta\Delta$ CT method).

Statistical analysis

Data are expressed as means $\pm$ SEM. Comparisons were performed using unpaired *t* test with Welch's correction (between two groups) or two-way analysis of variance (time course experiments) using Prism 6 software (GraphPad, La Jolla, CA). For all analyses, significance was set at $p < 0.05$.

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