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**Silencing of PD-L2/B7-DC by Topical Application of Small Interfering RNA Inhibits
Elicitation of Contact Hypersensitivity**

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Short title: *PD-L2 Silencing Inhibits Contact Hypersensitivity*

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Abbreviations: *AD*, atopic dermatitis; *Ag*, antigen; *BMDC*, bone marrow-derived DC; *CHS*, contact hypersensitivity; *DC*, dendritic cell; *DKO*, double-knockout; *rLN*, Langerhans cell; *LC*, regional lymph node; *RGMb*, repulsive guidance molecule b; *siRNA*, small interfering RNA

ABSTRACT

PD-L2 is a ligand for the immune checkpoint receptor PD-1, however, its regulatory function is unclear. We previously reported that silencing of CD86 in cutaneous dendritic cells (DCs) by topical siRNA application inhibits the elicitation of contact hypersensitivity (CHS). Here we investigated the effects of topical PD-L2 siRNA application on allergic skin disease. PD-L2 was induced in DCs concurrently with the elevation of MHC class II and CD86 expression. Topical PD-L2 siRNA application inhibited elicitation of CHS by suppressing early proinflammatory cytokine expression and migration of hapten-carrying DCs into lymph nodes. Local injection of neutralizing anti-PD-L2 mAb comparably inhibited CHS. PD-L2 siRNA treatment inhibited the CHS in PD-1/PD-L1 double-knockout mice and in the sensitized T-cell-transferred skin. These results suggest that the effects of PD-L2 silencing are independent of PD-1 but dependent on local memory T cells. Most of the inhibitory effects of PD-L2 and CD86 silencing on CHS were comparable, but PD-L2 siRNA treatment did not inhibit atopic disease-like manifestations and Th2 responses in NC/Nga mice. Our results suggest that PD-L2 in cutaneous DCs acts as a costimulator rather than a regulator. Local PD-L2 silencing by topical siRNA application represents a therapeutic approach for contact allergy.

INTRODUCTION

Dendritic cells (DCs) link innate and adaptive immunity (Steinman and Banchereau, 2007, Steinman and Hemmi, 2006). Production of proinflammatory cytokines and subsequent recruitment of innate lymphocytes greatly influence the maturation of DCs, and induce an adaptive immune response mediated by effector and regulatory T cells and antibodies. The expression of diverse co-signaling molecules is induced in DCs following activation; these modulate the quality and quantity of the immune response. CD86 on DCs is a potent costimulator that interacts with CD28 on T cells to elicit active T-cell responses (Azuma et al., 1993, Inaba et al., 1994). Blockade of CD86-mediated costimulation inhibits the activation of T cells. PD-L1 (B7-H1/CD274) (Dong et al., 1999, Freeman et al., 2000) and PD-L2 (B7-DC/CD273) (Latchman et al., 2001, Tseng et al., 2001) are ligands for the coinhibitory receptor PD-1, which is induced in primed T cells. The PD-1:PD-L1 axis plays a crucial role in maintaining self-tolerance and preventing tissue damages due to an excessive immune response, but the contribution of PD-L2 to PD-1-mediated immune regulation is unclear (Chen, 2004, Francisco et al., 2010). Unlike PD-L1, the expression of which under inflammatory conditions is inducible in non-hematopoietic tissue cells as well as activated immune cells, PD-L2 expression is induced in myeloid DCs and macrophages by certain stimuli together with CD86 (Ritprajak and Azuma, 2015, Tseng et al., 2001, Tsushima et al., 2003, Yamazaki et al., 2002). The powerful costimulatory ability of CD86 obscures the real function of PD-L2 in activated DCs.

In previous studies that have used neutralizing mAbs, PD-L2 was not found to contribute to PD-1-mediated immune regulation. By contrast, PD-L1 is predominantly responsible for the PD-1-mediated regulation in murine models of contact hypersensitivity

(CHS) (Tsushima et al., 2003), autoimmune nonobese diabetes (Fife et al., 2006), fetomaternal and transplantation tolerance (Guleria et al., 2005) (Aramaki et al., 2004), and immune privilege of corneal allograft (Hori et al., 2006). Subsequent to the initial report (Tseng et al., 2001), several studies have suggested opposing costimulatory roles of PD-L2: elevation of T-cell activation by elderly PD-L2⁺ B cells and inhibition of CHS by a higher affinity anti-PD-L2 mAb (Ritprajak et al., 2012, Tomihara et al., 2012). Recently, PD-L2 has been shown to bind another partner, repulsive guidance molecule b (RGMb), which is a co-receptor for bone morphogenic proteins (BMPs). Blockade of the RGMb:PD-L2 interaction impairs the development of respiratory tolerance independently of PD-1 (Xiao et al., 2014). However, a more recent report suggested a costimulatory role for RGMb:PD-L2 interaction in the Th1 response, with reciprocal inhibition of the Th2 response (Nie et al., 2017).

We have shown that silencing of CD86 in cutaneous DCs by topical application of CD86 small interfering RNA (siRNA) efficiently inhibits hapten-induced CHS and mite-induced atopic dermatitis (AD) in NC/Nga mice (Azuma et al., 2010, Ritprajak et al., 2008). Silencing of CD86 in resident DCs decreases the number of additionally recruiting-dermal DCs and the migration of antigen (Ag)-captured DCs to regional lymph nodes (rLNs) in CHS, as well as Th2-mediated allergic responses in NC/Nga mice. In this study, we investigated the role of PD-L2 in skin DCs and the therapeutic potential of PD-L2 siRNA in two models of allergic skin disease.

RESULTS

PD-L2 is induced in activated DCs concomitantly with upregulation of MHC class II and CD86 expression

To confirm the concomitant induction of PD-L2 and CD86 expression, we examined their expression in bone marrow-derived DCs (BMDCs). CD86 and PD-L2 were co-expressed in around one-third of immature BMDCs; moreover, their expression levels were further increased by lipopolysaccharide (LPS) stimulation (Figure 1a). FITC is a hapten-Ag that enables tracing of Ag-carrying DCs from the skin to the rLNs. Based on expression levels of CD11c and MHC class II (MHCII), resident and migratory DCs in the rLNs have been divided (Henri et al., 2010). CD11c^{high}MHCII^{int} resident DCs (Fr-1) barely contained FITC⁺ cells, whereas CD11c^{int-hi}MHCII^{hi} migratory DCs (Fr-2) contained roughly 30% of FITC⁺ cells (Figure 1b). Fr-1 cells expressed CD86, but not PD-L2. FITC⁺ Ag-carrying Fr-3 DCs within Fr-2 cells especially expressed high levels of both CD86 and PD-L2. These results indicate that PD-L2 is induced in activated DCs concomitantly with upregulation of MHC class II and CD86.

Topical PD-L2 siRNA application silences skin PD-L2 and inhibits CHS

To investigate the role of PD-L2 in cutaneous DCs, we examined the effects of topical PD-L2 siRNA and CD86 siRNA application on DNFB-induced CHS. Ear swelling (Figure 2a) and histological inflammation and edema (Figure 2b) were clearly inhibited by either of CD86 or PD-L2 siRNA application. Interestingly, the magnitude of the inhibition by both siRNAs were comparable.

To verify specific target silencing by siRNA painting, we examined the mRNA levels of CD86 and PD-L2 in the local ear tissue. The challenge dramatically increased the mRNA levels of CD86 and PD-L2 compared to those of sensitized but unchallenged ear skin, and the increased mRNA levels were significantly inhibited by application of the corresponding siRNA (Figure 2c). Unlike CD86, a substantial PD-L2 expression was not observed in unchallenged ear skin by immunohistochemistry (IHC) (Figure 2d). DNFB-challenge induced PD-L2 expression in the epidermis and PD-L2 siRNA treatment reduced its expression. CD86 signals within the epidermis and dermis were increased by challenge and the increased expression was markedly inhibited by CD86 siRNA treatment. To confirm PD-L2 expression in epidermal Langerhans cells (LCs), we obtained epidermal cell suspensions and performed intracellular staining of PD-L2 by flow cytometry, because cell surface PD-L2 expression was modulated by enzymatic digestion. Although CD326⁺CD11c⁺ LCs from unchallenged ear skin did not express PD-L2, LCs from the DNFB-challenged and control ILTS-treated skin expressed intracellular PD-L2 and this was inhibited by PD-L2 siRNA treatment (Figure 2e). The changes of PD-L2 expression were correlated with MHC class II expression. The signal intensity and the number of positive cells for MHC class II in the epidermis and the dermis by IHC were clearly decreased by the CD86 siRNA application and less inhibitory effects were seen by the PD-L2 siRNA treatment (Figure 2f). mRNA expression levels of four inflammatory cytokines, IL-1 α , IL-1 β , TNF- α , and CCL3 (MIP-1 α) were increased by the challenge as compared to those of sensitized but unchallenged ear skin, and their expression was significantly inhibited by treatment with either of the siRNAs (Figure 2c). These results demonstrated that topical PD-L2 siRNA application decreased the induced PD-L2 expression

in LCs and proinflammatory cytokine expression in the skin, and inhibits elicitation of CHS. CD86 siRNA treatment showed additional effects on the dermal DCs.

PD-L2 siRNA treatment inhibits migration of DCs to rLNs

Next, we assessed the DC and T-cell status in the rLNs. FITC-challenge of the ear skin markedly increased the total cell numbers at 24 hours and its increment was impaired by PD-L2 siRNA treatment. The percentages of FITC⁺ Ag-carrying DCs in CD11c^{int-hi}MHCII^{hi} migratory DCs (Fr-2) were significantly inhibited by both siRNA treatments (Figure 3a). Although CD86 siRNA treatment slightly down-regulated CD86 expression on FITC⁺ migratory DCs, PD-L2 siRNA treatment did not clearly inhibit their PD-L2 expression (Figure 3b). These results indicate that the migration of Ag-carrying skin DCs to the rLNs is inhibited by both siRNAs, but the activation status of DCs that reached the rLNs was not clearly affected, suggesting that CD86 or PD-L2-silenced skin DCs unable to move from the skin. Hapten challenge rapidly induces effector T-cell generation in rLNs, thus, we examined an earlier T-cell status at 12 hours after challenge in a DNFB-induced CHS model. The challenge-induced increment in the total number of cells was significantly inhibited by treatment with either of the siRNAs (Figure 3c). Although the ratios of CD3⁺ T cells, CD8⁺ T cells, Foxp3⁻ conventional CD4⁺ T cells (Tcon), and Foxp3⁺ regulatory T cells were comparable among the three groups (data not shown), IFN- γ expression by CD8⁺ T and CD4⁺ Tcon cells was significantly inhibited by PD-L2 siRNA treatment (Figure 3c). These results indicate that topical PD-L2 siRNA treatment impaired the migration of skin DCs to rLNs, which suppresses generation of effector T cells.

Requirements in the effects of PD-L2 silencing.

To investigate whether the inhibitory effects of PD-L2 siRNA treatment is dependent on cell surface PD-L2, we locally administrated neutralizing anti-CD86 or anti-PD-L2 mAb to the ear skin. Consistent to the effects by siRNA treatments, ear swelling was significantly inhibited by local mAb administration (Figure. 4a). This suggests the involvement of CD86 and PD-L2 in the local cell-cell interactions. Early production of cytokines (IFN- γ and IL-17) from resident memory T cells (T_{RM}) that interact with skin DCs is critical for amplification of CHS elicitation (Gaide et al., 2015, Honda et al., 2013, Jiang et al., 2012, Schmidt et al., 2017). The secreted cytokines further augment inflammatory cytokines by keratinocytes (KCs) and innate immune cells (Enk and Katz, 1992). We first compared IL-1 α and IL-1 β expression in ear tissues at 6 hours after DNFB challenge between wild type and recombination activating gene 2 (Rag2) KO mice lacking T and B cells. The elevation of IL-1 α and IL-1 β expression by DNFB painting was not seen in Rag2 KO mice (Figure 4b). To assess the involvement of sensitized memory T cells in the local response, DNFB-sensitized T cells were directly transferred to the ear skin, followed by siRNA treatment and DNFB challenge. Topical application of either of the siRNAs inhibited ear swelling (Figure 4c). To negate the contribution of endogenous host T cells, we performed similar experiments using Rag2 KO ear skin. Sensitized T cell transfer into the Rag2 KO ears induced a rapid and peak ear swelling during 6 to 24 hours and PD-L2 siRNA treatment significantly inhibit the swelling (Figure 4d), suggesting the involvement of local memory T cells in the inhibitory effect of PD-L2 siRNA.

To evaluate the involvement of PD-1 in the effects of PD-L2 silencing, we applied the PD-L2 siRNA to *cd279^{-/-}cd274^{-/-}* double-knockout (PD-1/PD-L1 DKO) mice. We used

DKO mice to avoid the alteration of PD-L2 contribution by Th1/Th17/Th2 skewing (Liang et al., 2006) (Loke and Allison, 2003), autoimmunity (Okazaki and Wang, 2005), and PD-L1:CD80-mediated regulation (Butte et al., 2007) that were observed in PD-1- or PD-L1 KO mice. The induction of PD-L2 expression in the ear skin showed similar magnitude in wild-type and DKO mice (Figure 4e). Ear swelling was generated more rapidly in the PD-1/PD-L1 DKO and was sustained until 96 hours, and topical PD-L2 siRNA treatment inhibited ear swelling and the inflammatory response (Figure 4f and 4g). These results suggest that PD-1 is not involved in the effects of PD-L2 silencing.

Next, we examined the duration of the siRNA-induced inhibition. After the siRNA treatment at the first challenge (day 5), we did the secondary challenge at day 31 or 77. Although rapid and severe ear swelling were induced by the second challenge, the ear swelling was persistently inhibited in the CD86 and PD-L2 siRNA-treated groups (Figure 4h). This suggests that both siRNA treatments influences expansion and activation of memory T cells.

Topical PD-L2 siRNA application is ineffective for AD-prone NC/Nga mice

Repeated mite allergen application induces AD-like skin lesions in NC/Nga mice. We previously reported amelioration of AD by CD86 siRNA application in this model (Ritprajak et al., 2008). In contrast to the marked inhibitory effects of the CD86 siRNA on skin manifestations, the PD-L2 siRNA did not ameliorate the skin lesions (Figure 5a and 5b). Allergen-specific production of IL-4 and IL-5 by rLN cells at 4 weeks after treatment was significantly inhibited by the CD86, but not the PD-L2, siRNA (Figure 5c). The serum titers of IgE and *Dermatophagoides farina* (Df)-specific IgG1 in the control mice were significantly

increased by allergen boosting and control ointment application. The IgE titer was not affected by either of the siRNAs, while the Df-specific IgG1 titer was reduced by the CD86 siRNA (Figure 5d). These results demonstrate that, silencing of PD-L2, but not of CD86, is unable to improve clinical manifestations of AD and to inhibit the Th2-mediated cellular and humoral responses.

DISCUSSION

We demonstrate induction of PD-L2 in activated DCs concurrently with the elevation of MHC class II and CD86 expression. Silencing of PD-L2 by topical siRNA application reduced the induction of PD-L2 in LCs, and inhibited elicitation phase of CHS by suppressing the production of proinflammatory cytokines and migration of hapten-carrying skin DCs into the rLNs. These results are similar to our previous findings on the effects of topical CD86 siRNA application. Our results suggest that PD-L2 induced on epidermal LCs acts as a costimulator in CHS. However, PD-L2 siRNA treatment did not ameliorate AD-like manifestations and pathogenic Th2-mediated responses in NC/Nga mice. Our results show the differential contribution of PD-L2-expressing local DCs between murine models of CHS and AD.

Topical PD-L2 siRNA application resulted in target-specific silencing and marked inhibition of the pro-inflammatory cytokines in ear tissues at an early time point. How does the silencing of PD-L2 in local DCs induce such an early inhibition? Hapten painting induces IL-1 β from LCs, and IL-1 α and TNF- α from KCs at very early time points (Enk and Katz, 1992). Secretion of these proinflammatory cytokines from LCs and KCs plays a crucial role in triggering CHS. Without challenge, considerable numbers of memory T cells are recruited to the skin for scan Ags and hapten challenge rapidly induces cluster formations of DCs and skin T_{RM} (Egawa et al., 2011, Egawa and Kabashima, 2011). We speculate that early interaction with PD-L2-inducing LCs and epidermal resident memory T cells (T_{RM}) may amplify local production of inflammatory responses. Skin CD8⁺ T_{RM} interacting with local DCs greatly contribute to the elicitation phase of CHS (Gaide et al., 2015, Honda et al., 2013, Jiang et al., 2012, Schmidt et al., 2017). IFN- γ and IL-17A produced by CD8⁺ T_{RM} induce IL-1 β by KCs (Schmidt et al., 2017). Consistent inhibition by local PD-L2 silencing in the

T-cell transferred Rag2 KO ears and at secondary challenge further support the involvement of local memory T cells. Our results of similar inhibitory effects by local anti-PD-L2 mAb treatment (Figure 4a) and the PD-L2 silencing-induced inhibitory effects in PD-1/PD-L1 DKO mice (Figure 4f) suggests that cell surface PD-L2 is involved but PD-1 is not involved in the immunoenhancing effects. PD-L2 induced on epidermal LCs seems to act as a costimulatory ligand. The counter-receptor involved in the PD-L2-mediated costimulation remains to be identified. RGMB is a candidate for this receptor as its engagement with recombinant mutant PD-L2 (which is unable to bind to PD-1) costimulates the Th1 response, resulting in inhibition of Th2-mediated asthma (Nie et al., 2017).

Unlike the CD86 siRNA treatment, the PD-L2 siRNA treatment did not inhibit AD, which is considered a Th2-mediated disease. Although CD86 is induced on both epidermal LCs and dermal DCs, we only observed substantial expression of PD-L2 in LCs. The induction of AD in this model requires repeated Ag-application and Th2-mediated humoral responses. Contribution of PD-L2-expressing LCs may differ in this model. In addition, the Th2 response is often inhibited by the action of IFN- γ or NKT-mediated response (Akbari et al., 2010, Matsumoto et al., 2004, van der Werf et al., 2013). Further studies are required to clarify the reason.

In summary, silencing of PD-L2 by a topical siRNA inhibited the elicitation phase of CHS independently of PD-1; in addition, induction of the expression of PD-L2 in the skin promoted the skin inflammatory response. Therefore, local silencing of PD-L2 by topical siRNA application may have therapeutic potential for contact allergy.

MATERIALS AND METHODS

Mice

Female 6-week-old BALB/c and NC/Nga mice were purchased from Japan SLC (Hamamatsu, Japan). PD-1/PD-L1 DKO and Rag2 KO mice on the BALB/c background were generated as described previously (Kang et al., 2016). All mice except for NC/Nga mice were maintained under specific-pathogen-free conditions and NC/Nga mice were maintained under the conventional conditions. All experimental procedures were reviewed and approved by the Animal Care and Use Committee of Tokyo Medical and Dental University (0160309A, 0170344A, A2018-262C).

Preparation of PD-L2 and CD86 siRNA ointments

siRNA oligonucleotides for mouse PD-L2 were designed and synthesized by Qiagen (Valencia, CA), and the most efficient was selected by co-transfection of the PD-L2 expression vector (pCAG/PD-L2) (Ritprajak et al., 2012) and the respective PD-L2 siRNA duplex into 293T cells as described previously (Ritprajak et al., 2008). The sequences of the siRNA duplexes for CD86 and PD-L2 are shown in Supplementary Table. The CD86 and PD-L2 siRNA duplexes were obtained from JBioS (Asaka, Saitama, Japan). Ionic liquid transdermal system (ILTS) by MEDRx (Kagawa, Japan) (Miwa et al., 2016) was performed to increase skin permeability to oligonucleotides. Briefly, ionic liquid-siRNA formulations were prepared by mixing a prepared ionic liquid and hydrocarbon gel and used as the siRNA/ILTS ointment for topical application.

Induction of CHS and treatment with siRNAs or mAbs

CHS to DNFB (Sigma-Aldrich) or FITC was induced as described previously (Azuma et al., 2010, Nuriya et al., 2001, Ritprajak et al., 2008). Briefly, 20 µl of 0.5% DNFB were applied to the shaved abdominal skin on days 0 and 1 for sensitization, and 10 µl of 0.2% DNFB was applied to the ear skin on day 5 for challenge. For FITC-induced CHS, 400 µl of 0.5% FITC dissolved in a 1:1 vol/vol mixture of acetone: dibutylphthalate were applied on day 0 for sensitization, followed by challenge with 20 µl of 0.5% FITC on day 5. For siRNA treatment, control ILTS without siRNA, PD-L2 siRNA/ILTS, or CD86 siRNA/ILTS ointment (250 pmol siRNA duplex/3.3 mg ointment/ear) that contained amounts of the siRNA duplexes identical to those in a prior study (Ritprajak et al., 2008) was applied to the ear skin one hour before challenge. Ear thickness was measured before and at the indicated time points after the challenge. For re-challenge experiments, after siRNA treatment at the first challenge, the secondary challenge was performed on day 31 or 77. For mAb administration, either rat IgG, anti-CD86 (PO3), or anti-PD-L2 (MIH37) mAb (10 µg/20 µl/ear) was administrated into the ear skin one hour before challenge.

For local T-cell transfer experiments, rLN cells were collected from DNFB-sensitized mice on day 5 and T cells were isolated by negative selection using magnetic beads as described previously (Ritprajak et al., 2010). A >90% purity of CD3⁺ cells was confirmed by flow cytometry. Isolated LN T cells (1×10^6) were subcutaneously administrated into the ear skin of intact mice one hour before, and then treatments with ILTS ointment and DNFB challenge were performed as described above.

Generation of activated DCs *in vitro* and *in vivo*

BMDCs were generated from the bone marrow cells of BALB/c mice cultured with GM-CSF as described previously (Ritprajak et al., 2008). To induce maturation, DCs were stimulated with LPS from *Salmonella enterica* (10 ng/ml, Sigma-Aldrich) for 24 hours. To quantify Ag-bearing DCs in the rLNs, 30 µl of 0.5% FITC solution were applied to the ear skin (Chalermarp and Azuma, 2009), and cervical LNs were collected after 24 hours. LN cells were isolated as described previously (Chalermarp and Azuma, 2009).

mAbs and flow cytometry

mAbs against the following Ags were used: CD11c (N418), CD11b (M1/70), PD-L2 (TY-25), MHC class II (M5/114), CD86 (PO3), CD3 (17A2), CD4 (RM4-5), CD8 (53-6.72), CD45 (30-F11), CD45R (B220, RA3-6B2), CD326 (EpCAM, G8.8), Foxp3 (FJK-165), and IFN-γ (XMG1.2). All mAbs were obtained from eBioscience (San Diego, CA) or BD-Biosciences (San Diego, CA). Samples were preincubated with anti-CD16/32 (2.4G2) mAb to block FcγR and subsequently subjected to multicolor staining of cell surface and intracellular molecules. Stained cells were analyzed using a FACSVerse (BD Bioscience) and FlowJo software (Tree Star, Ashland, OR).

Immunohistochemistry

Cryostat sections of ear tissues were subjected to H&E or enzymatic IHC as described previously (Chalermarp and Azuma, 2009, Ritprajak et al., 2008). The sections were first incubated with mAbs against PD-L2 (TY-25), CD86 (PO3), or control rat IgG, and subsequently with a biotinylated anti-rat IgG (Vector Lab., Burlingame, CA). For MHC class II staining, a biotinylated anti-MHC class II (M5/114) mAb was used. Antibody binding was

detected using avidin–biotin–peroxidase complex system (Vectastain; Vector Lab.), visualized with the substrate diaminobenzidine, and counterstained with hematoxylin. Quantitative analyses for MHC class II expression was performed in four continuous high-power fields ($\times 200$) that contained approximately 1.2 mm of epithelium. The number of positive cells was counted and verified by two independent investigators.

Preparation of epidermal cell suspensions

Epidermal sheets were separated from the dermis after incubation with Dispase II (1 U/ml, Sigma-Aldrich) and trypsin (0.05%) in PBS for 30 min at 37°C, and epidermal cell suspensions were obtained by digestion with collagenase type I and DNase I. Isolated cells were subjected to cell surface and intracellular multicolor staining for flow cytometry.

Quantitative real-time PCR

Total RNA was extracted from ear tissues using NucleoSpin RNA II (Macherey-Nagel, Duren, Germany). cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio, Kusatsu, Japan) and real-time PCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Premix Ex *Taq* II (Takara Bio). The primers sequences used are shown in Supplementary Table. Data are presented as the ratios to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Induction of AD and siRNA treatment

AD-like lesions were induced in NC/Nga mice by repeated mite antigen application as described previously (Ritprajak et al., 2008), with minor modifications. Mice were treated

with 150 µl of 4% sodium dodecyl sulfate on the ear and the shaved upper back skin to disrupt skin barrier function (Nakagawa et al., 2011). One hour later, 20 µg mite extract (*Dermataphagoides farina*, [Df], LSL, Tokyo, Japan) was topically applied to the above areas three times per week. The skin scores of the ears and the upper back were separately monitored, and siRNA treatment was started when the score of one site became greater than 5. Mice were randomly divided into three groups and the allergen-applied areas were treated with the control/ILTS, PD-L2 siRNA/ILTS, or CD86 siRNA/ILTS ointment (500 pmol siRNA duplex/6.6 mg of 0.05% ointment) daily for 4 weeks. Mite allergen application was continued even after the start of treatment. Skin scores were evaluated as described previously (Azuma et al., 2010, Ritprajak et al., 2008).

Measurement of serum antibodies and Th2-cytokine production

Sera were collected before and 4 weeks after treatment, and the total IgE and Df-specific IgG1 (Df-IgG1) levels were measured by ELISA as described previously (Azuma et al., 2010, Ritprajak et al., 2008). For assessment of Df-IgG1, the titer of the pooled standard serum was defined as 1,000 units and the levels in individual samples are expressed as unit values. To measure Df-specific cytokine production, cervical LN cells at 4 weeks after treatment were cultured in the presence or absence of Df antigen (10 µg/ml) for 4 days, and the IL-4 and IL-5 levels in the supernatants were measured using ELISA.

Statistics

Statistical analyses were performed by Mann–Whitney U-test using the Prism 6 software (Graphpad). A value of $p < 0.05$ was considered to indicate significance.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information file.

CONFLICT OF INTEREST

K.K. and H.H. are employee of MEDRx (Kagawa, Japan). Other authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS

E.F., S.N., H.Y. and M.A. contributed to conception and design of the research, interpretation and wrote the paper. E.F., T.O., T.N. and T.K. performed the experiments, data acquisition and analysis. K.K. and H.H. contributed to the preparation of materials. M.M. contributed to the critical discussion of the research and manuscript preparation.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2018.08.020>.

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FIGURE LEGENDS

Figure 1. PD-L2 and CD86 are co-expressed in activated DCs.

(a) Immature and mature BMDCs were stained with fluorochrome-conjugated anti-CD11c, anti-PD-L2, and anti-CD86 mAbs. An electronic gate was placed on CD11c⁺ lymphocytes, and the expressions of PD-L2 and CD86 are shown. (b) Regional LN cells 24 hours after FITC application to the ear skin were stained with fluorochrome-conjugated anti-CD86, anti-PD-L2, anti-CD11c, anti-CD45R, and anti-MHC class II (MHCII) mAbs or appropriate control Abs. Gating strategies were shown in the left two panels. An electronic gate was placed on either CD11c^{hi}MHCII^{int} (Fr-1), CD11c^{int-hi}MHCII^{hi} (Fr-2) in CD45R⁺ alive cells, or FITC⁺ cells within Fr-2 (Fr-3), and then expressions of FITC and MHCII are displayed as dotted plots. The region gate was placed on FITC⁺ cells. Values are the percentages of FITC⁺ cells. The lower three panels show expression of CD86 and PD-L2 in each fraction. Markers were positioned to include >98% of cells stained with control Igs in the lower left quadrant. Representative profiles from three mice are shown.

Figure 2. Topical application of PD-L2 siRNA inhibits CHS responses.

CHS against DNFB was induced as described in the Methods. One hour before challenge, control/ILTS, CD86 siRNA/ILTS, or PD-L2 siRNA/ILTS ointment was applied topically to the right and left ears. (a) The ear thickness was evaluated before and 24, 48, and 72 hours after challenge. The values shown are the mean ear thickness changes $\pm$ SD (n = 6). Data are from two independent experiments. **p* < 0.05 vs. the control/ILTS group. (b) H&E-stained sections of ear tissues at 48 h after challenge. Scale bar = 50 μ m. (c, d, f) Sensitized but

unchallenged-, or sensitized and challenged-ear tissues at 6 hours were subjected to quantitative real-time PCR (c) and IHC (d and f). Dotted lines in c, mean relative expressions from the sensitized but unchallenged ear tissues. Values are means $\pm$ SD (n = 8-12) from 2-3 independent experiments. * $p < 0.05$. Expressions of CD86 and PD-L2 (d), and MHC class II (f) are shown. Scale bar = 50 μ m. The numbers of MHC class II⁺ cells within the epidermis and the dermis in four high power field (hpf) were counted in f. Values are means $\pm$ SD (n = 6) of two independent experiments. * $p < 0.05$. (e) Epidermal cell suspensions from the ear skins were stained with fluorochrome-conjugated anti-CD45, anti-CD11c, anti-CD326, and anti-MHCII mAbs, or appropriate control Abs. An electronic gate was placed on CD11c⁺CD326⁺ in CD45⁺ leukocytes, and then expressions of MHCII and PD-L2 are displayed as dotted plots. Markers were positioned to include >98% of cells stained with control Igs in the lower left quadrant. The values are the mean fluorescence intensity. Data are representative from two independent experiments.

Figure 3. Topical PD-L2 siRNA treatment inhibits migration of DCs and activation of regional LN T cells.

(a and b) Regional LN (rLN) cells from FITC-sensitized and FITC-challenged mice were counted and stained with fluorochrome-conjugated anti-CD86, anti-PD-L2, anti-CD11c, and anti-MHC class II mAbs. An electronic gate was placed as described in Figure 1b. The percentages of CD11c^{int-hi}MHCII^{hi} (Fr-2) in alive cells and FITC⁺ cells (Fr-3) in Fr-2 cells were determined in a. An electronic gate was placed on CD11c^{int-hi}MHCII^{hi} cells, and then expression of indicated Ags with FITC are shown as dotted plots. Data shown are representative from each group of three mice. (c) rLN cells from DNFB-sensitized and

DNFB-challenged mice were analyzed 12 hours after challenge. Cells were stained with fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8, anti-IFN- γ and anti-Foxp3 mAbs. Electronic gates were placed on CD8⁺CD3⁺ (CD8⁺ T) or CD4⁺Foxp3⁻CD3⁺ (CD4⁺ Tcon) lymphocytes, and then the percentages of IFN- γ ⁺ cells were determined. Values are means $\pm$ SD (n = 3). Data are representative of two independent experiments with similar results. * p < 0.05. Dotted lines in a and c, mean values of the sensitized mice before challenge.

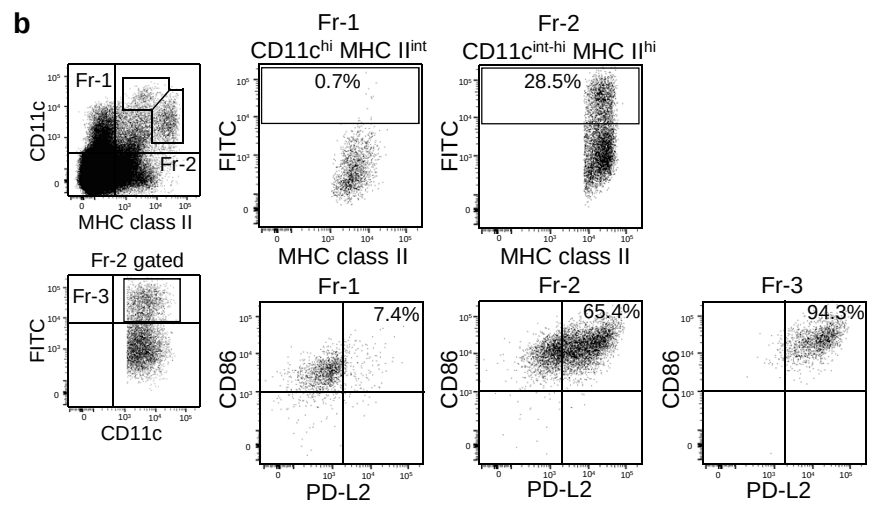
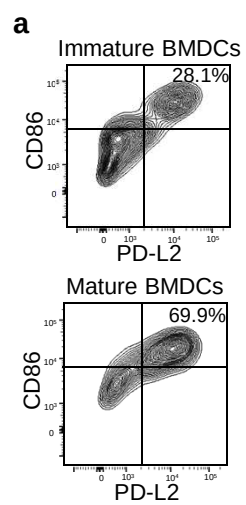
Figure 4. Requirements for the effects of topical PD-L2 siRNA treatment.

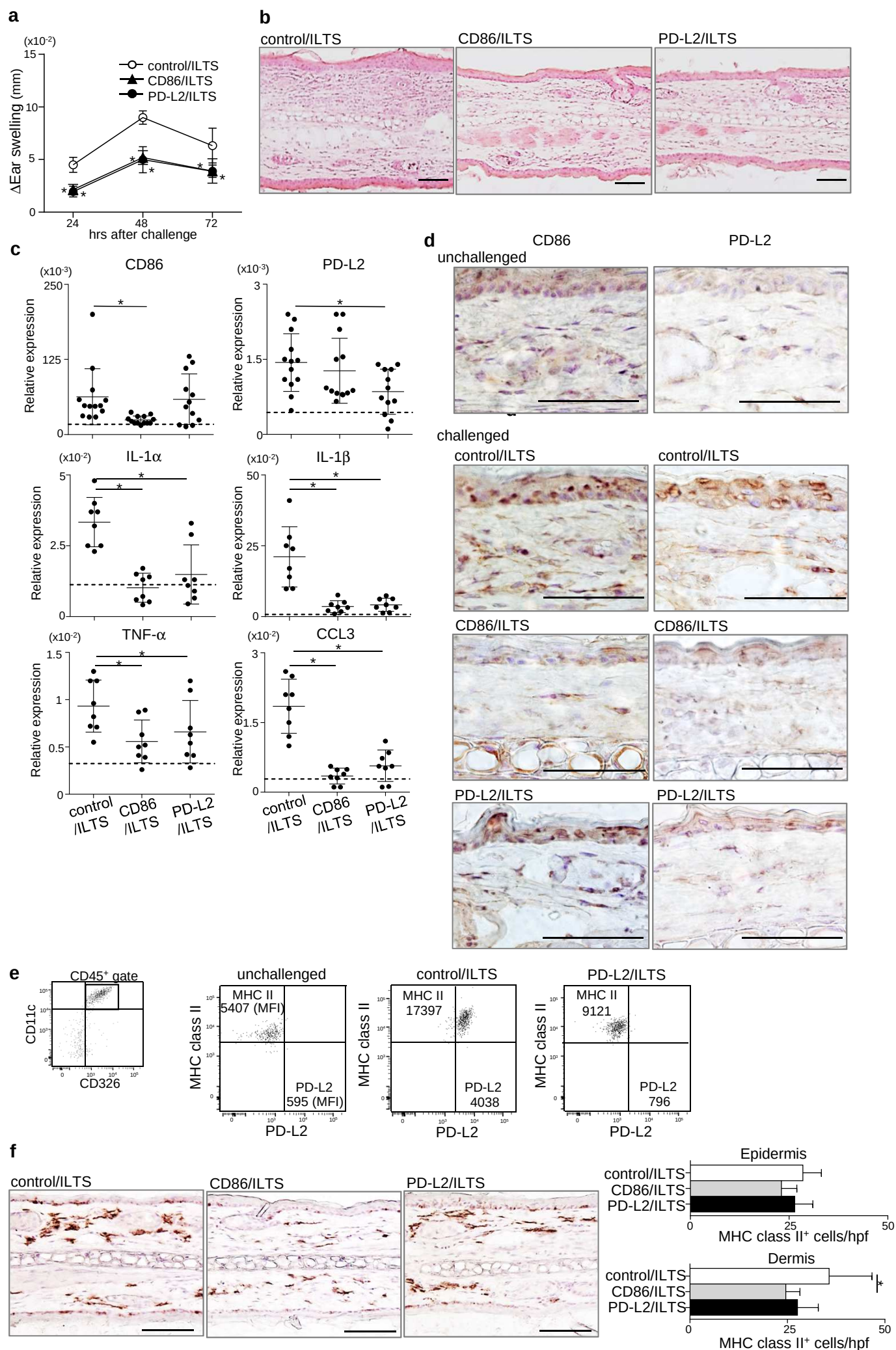
(a) Either control rat IgG, anti-CD86 (PO3) mAb, or anti-PD-L2 (MIH37) mAb was administrated into the ear skin one hour before DNFB-challenge, and the ear thickness was evaluated. The values shown are the mean ear thickness changes $\pm$ SD (n = 6). Data are from two independent experiments. * p < 0.05 vs. the control group. (b) Ear skins of the indicated conditions from sensitized-wild type (WT) and RAG2 KO mice were subjected for IL-1 α and IL-1 β mRNA expression by quantitative real-time PCR. Values are means $\pm$ SD (n = 4). * p < 0.05. (c and d) DNFB-sensitized T cells were transferred to the intact ears of wild type (c) and RAG2 KO (d) mice. After one hour, control/ILTS, CD86/ILTS, or PD-L2/ILTS was applied to the ear skin, and then DNFB was painted. The ear thickness changes were measured. Values are mean ear thickness changes $\pm$ SD (n = 3). * p < 0.05 vs. the control/ILTS group. (e, f, and g) WT and PD-1/PD-L1 DKO BALB/c mice were sensitized with DNFB, and control/ILTS or PD-L2/ILTS was topically applied one hour before challenge. Control/ILTS treated ear tissues at 6 hours after challenge were collected and subjected to IHC for PD-L2 (e). Scale bar = 50 μ m. Ear thickness were measured in f. Values are means $\pm$ SD (n = 6) of two independent experiments. * p < 0.05. Representative ear skin histology at 48

hours after challenge (g). Scale bar = 50 μ m. (h) The siRNA/ILTS treatments at the first challenge was performed at day 5. The secondary challenge was performed at day 31 or 77, and the ear thickness changes were measured. Values are means $\pm$ SD (n = 3). Data are representative of two independent experiments with similar results.

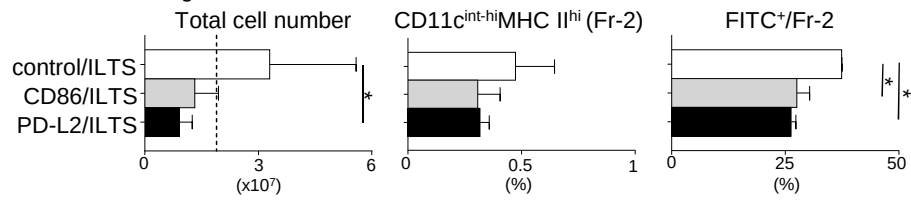
Figure 5. PD-L2 siRNA treatment shows marginal effects in AD-prone NC/Nga mice.

AD-like lesions were induced in NC/Nga mice. Treatment was started when the skin score of the ears or the upper back became greater than 5. (a) Mean skin score $\pm$ SD (n = 8) from each group at the indicated time points (b) Representative images of two mice from each group at 4 weeks after treatment. (c) IL-4 and IL-5 production by rLN cells stimulated with Df-Ag determined by ELISA. Values are means $\pm$ SD (n = 4-5). (d) Serum titers of total IgE and Df-specific IgG1. Values are means $\pm$ SD (n = 8). * $p < 0.05$.

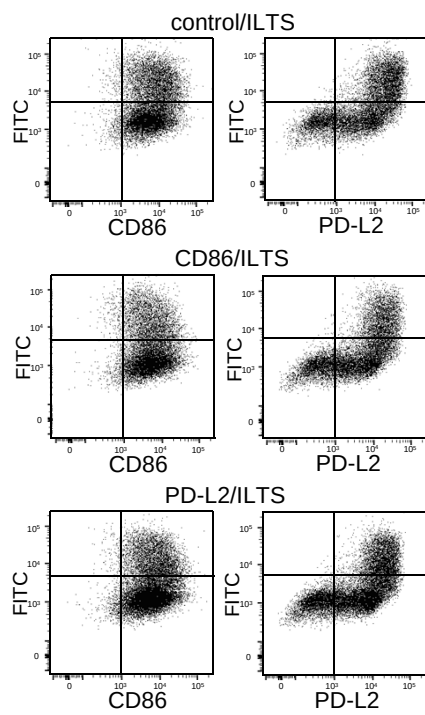




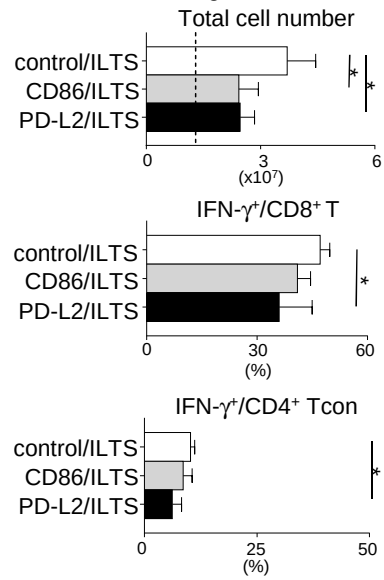
a FITC challenge/24h

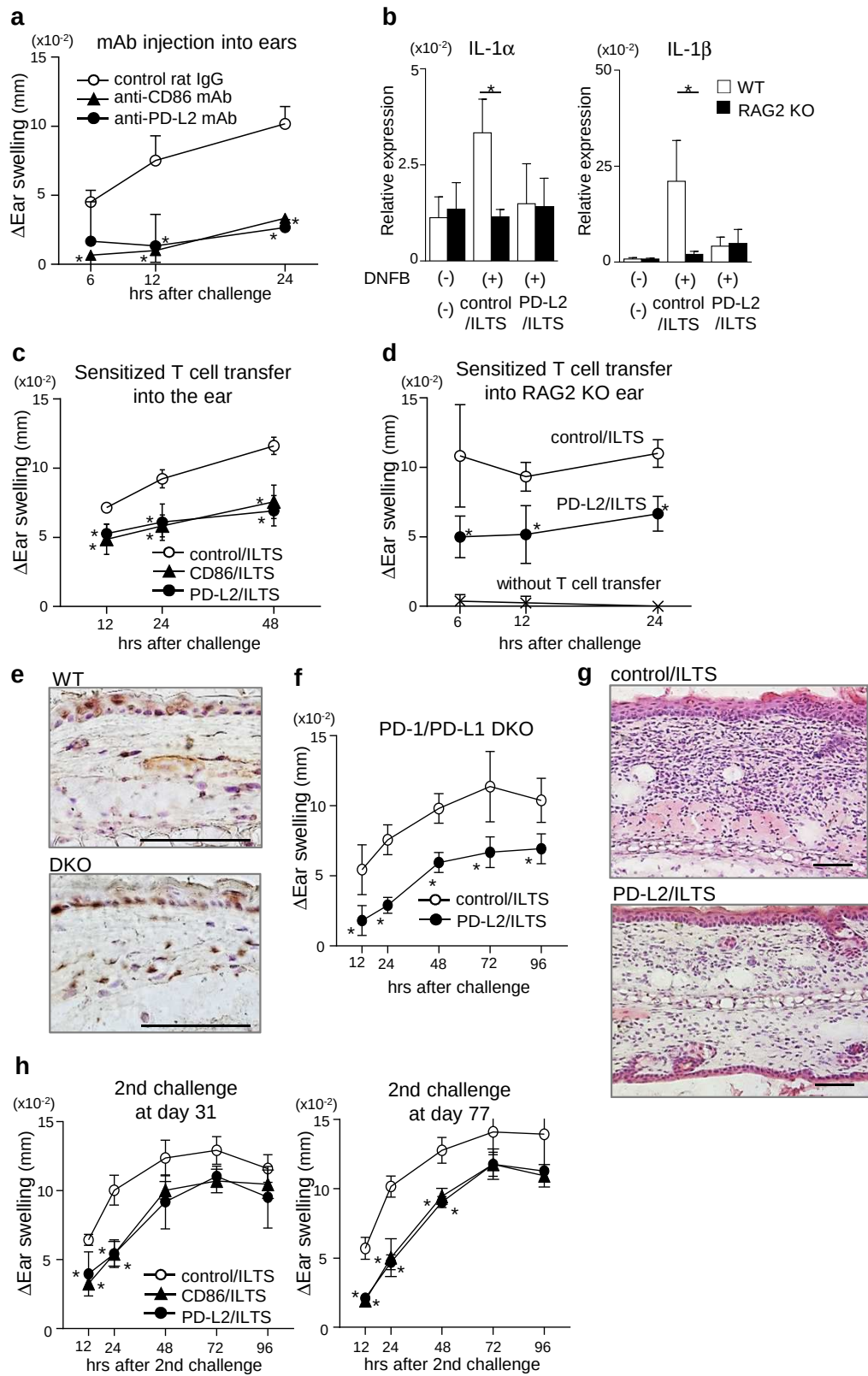


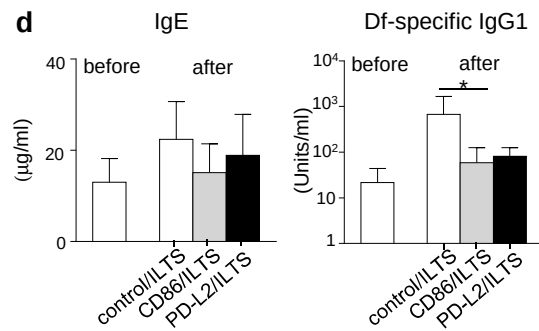
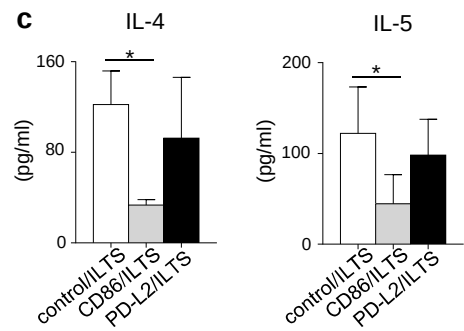
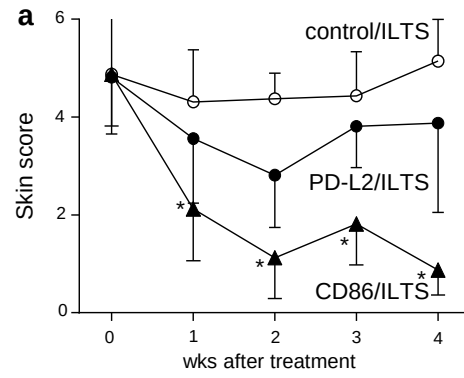
b FITC challenge/24h



c DNFB challenge/12h







Supplementary Table

Sequences of siRNAs

siRNA	Sequence	
PD-L2	Sense	5'-CCUCUGAAGUUCUAAUUAATT-3'
	Anti-sense	3'-TCGGAGACUUCAAGAUUAAUU-5'
CD86	Sense	5'-CGUUGUGUGUGUUCUGGAATT-3'
	Anti-sense	3'-TTGCAACACACACAAGACCUU-5'

Primer sequences for quantitative real-time PCR

Gene	Sequence	
CD86	Forward	GACCGTTGTGTGTGTTCTGG
	Reverse	GATGAGCAGCATCACAAGGA
PD-L2	Forward	CTGCTGCCGATACTGAACCT
	Reverse	CTGCGGTCAAATCGCACTC
IL-1 α	Forward	GCCCGTGTTGCTGAAGGAGT
	Reverse	TGGATAAGCAGCTGATGTGAAGTA
IL-1 β	Forward	GTGACGTTCCCATTAGACAGC
	Reverse	CCCAAGGCCACAGGTATTT
TNF- α	Forward	CACCACGCTCTTCTGTCTA
	Reverse	ATGGGCTCATACCAGGGTT
CCL3	Forward	CCTCTGTCACCTGCTCAACA
	Reverse	GTGAACAACCTGGGAGGGAGA
GAPDH	Forward	GCATGGCCTTCCGTGTTCT
	Reverse	GGTCCTCAGTGTAGCCCAAGATGC