

Counterregulation between thymic stromal lymphopoietin– and IL-23–driven immune axes shapes skin inflammation in mice with epidermal barrier defects

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Background: Epidermal barrier dysfunction has been recognized as a critical factor in the initiation and exacerbation of skin inflammation, particularly in patients with atopic dermatitis (AD) and AD-like congenital disorders, including peeling skin syndrome type B. However, inflammatory responses developed in barrier-defective skin, as well as the underlying mechanisms, remained incompletely understood. **Objective:** We aimed to decipher inflammatory axes and the cytokine network in mouse skin on breakdown of epidermal stratum corneum barrier.

Methods: We generated *Cd32^{iep-/-}* mice with corneodesmosin ablation in keratinocytes selectively in an inducible manner. We characterized inflammatory responses and cytokine expression by using histology, immunohistochemistry, ELISA, and quantitative PCR. We combined mouse genetic tools, antibody-mediated neutralization, signal-blocking reagents, and topical antibiotic treatment to explore the inflammatory axes.

Results: We show that on breakdown of the epidermal stratum corneum barrier, type 2 and type 17 inflammatory responses are developed simultaneously, driven by thymic stromal lymphopoietin (TSLP) and IL-23, respectively. Importantly, we reveal a counterregulation between these 2 inflammatory axes. Furthermore, we show that protease-activated receptor 2

signaling is involved in mediating the TSLP/type 2 axis, whereas skin bacteria are engaged in induction of the IL-23/type 17 axis. Moreover, we find that IL-1 β is induced in skin of *Cd32^{iep-/-}* mice and that blockade of IL-1 signaling suppresses both TSLP and IL-23 expression and ameliorates skin inflammation.

Conclusion: The inflammatory phenotype in barrier-defective skin is shaped by counterregulation between the TSLP/type 2 and IL-23/type 17 axes. Targeting IL-1 signaling could be a promising therapeutic option for controlling skin inflammation in patients with peeling skin syndrome type B and other diseases related to epidermal barrier dysfunction, including AD. (J Allergy Clin Immunol 2016;■■■■:■■■■-■■■■.)

Key words: Epidermal barrier, corneodesmosin, atopic dermatitis, peeling skin syndrome type B, thymic stromal lymphopoietin, IL-23, T_H2, T_H17, IL-1 β , skin inflammation, mouse model

The epidermis functions as a primary defense and biosensor to the external environment. Various abnormalities in the stratum corneum (SC) involving structural proteins, desquamation, or lipid metabolism can lead to epidermal barrier dysfunction, which has emerged as a critical factor in initiating and exacerbating skin inflammation, particularly in patients with atopic dermatitis (AD) and AD-like congenital disorders.¹⁻⁴ Indeed, genetic mutations of key players in the epidermal barrier are associated with or identified for these inflammatory skin diseases, including filaggrin (FLG; for AD), corneodesmosin (CDSN; for generalized peeling skin syndrome type B [PSS-B]), LEKTI (the serine protease inhibitor, for Netherton syndrome [NS]), and desmoglein 1 (for skin dermatitis–multiple severe allergies–metabolic wasting syndrome). Notably, these skin disorders exhibit compromised SC barrier defects and share overlapping clinical features with AD, including eczematoid cutaneous inflammation associated with frequent bacterial colonization or infection in the skin and atopic manifestation with allergies.²⁻⁴ These multiple lines of evidence in human genetics have suggested a causal relationship between primary epidermal barrier dysfunction and an AD-like inflammatory response, which has been supported by a number of studies using mouse models.¹⁻³

Despite these advances, the types of inflammatory responses developed in epidermal barrier–defective skin and the underlying mechanisms remain incompletely understood. Discrepancies and arguments exist, particularly with respect to cytokine expression and inflammatory cell infiltrates. For example, although human AD is generally recognized as type 2 inflammation (eg, expression of T_H2 cytokines with an infiltrate of eosinophils, basophils, and mast cells), evidence of type 17 inflammation (eg, expression

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Abbreviations used

AD:	Atopic dermatitis
ADAM17:	A disintegrin and metalloproteinase 17
CDSN:	Corneodesmosin
FLG:	Filaggrin
IHC:	Immunohistochemistry
MBP:	Major basic protein
MCPT8:	Mast cell protease 8
NS:	Netherton syndrome
PAR2:	Protease-activated receptor 2
PSS-B:	Peeling skin syndrome type B
SC:	Stratum corneum
TSLP:	Thymic stromal lymphopoietin
wka:	Weeks after the first tamoxifen injection

of T_H17 cytokines with an infiltrate of neutrophils) has been provided also.⁵⁻¹⁰ In addition, studies in mouse models also reported varied inflammatory types in barrier-defective skin, which were interpreted as either type 2,¹¹⁻¹³ type 17,¹⁴ or mixed type 2/17 inflammation.^{15,16} These seemingly contradictory reports reflect the complexity and heterogeneity of the inflammation occurring in barrier-defective skin, which remain poorly understood. In view of this, a variety of biological treatments have been or are being developed for interventions of AD and AD-like diseases.¹⁷ Better knowledge of the involved inflammatory axes and underlying cytokine networks, particularly their crosstalk and regulation, is currently required for the mechanistic understanding of the pathophysiology of inflammatory skin diseases related to dysfunction of the epidermal barrier and for defining efficient treatment strategies.

To explore the inflammatory responses and cytokine axes in barrier-defective skin, we generated *Cdsn*^{iep-/-} mice, in which tamoxifen treatment induces disruption of the SC barrier through keratinocyte-selective ablation of the *CDSN* gene. Thus such an inducible model has allowed us to analyze the dynamics of skin inflammation and to dissect the cytokine network underlying the inflammatory process.

METHODS**Mice**

Cdsn^{L2/L2} mice¹⁸ were bred with K14-Cre-ER^{T2(tg0)} mice¹⁹ to obtain K14-Cre-ER^{T2(tg0)/Cdsn^{L2/L2} mice (on a mixed genetic background of 75% C57BL/6J and 25% 129/sv). Eight to 12-week-old K14-Cre-ER^{T2(tg0)/Cdsn^{L2/L2} mice were injected intraperitoneally with tamoxifen (0.1 mg in 100 μ L of sunflower oil for 5 consecutive days) to generate *Cdsn*^{iep-/-} mice. Tamoxifen-injected age- and sex-matched littermate (K14-Cre-ER^{T2(tg0)/Cdsn^{L2/L2}) mice were used as wild-type control mice. *Tslp*^{L2/L2} mice (in a C57BL/6J background) were previously reported.²⁰ *Par2(F2r11)*-null mice (on a C57BL/6N genetic background) were generated at the Institut Clinique de la Souris (Illkirch, France) from the IKMP ES cell clone EPD0592_3_D02, which were homozygous for the knockout-first allele F2r11^{tm1a(EUCOMM)Wtsi}.}}}

Breeding and maintenance of mice were performed under institutional guidelines, and all of the experimental protocols were approved by the animal care and ethics committee of animal experimentation of the Institut de Génétique de Biologie Moléculaire et Cellulaire and Institut Clinique de la Souris.

Histopathology

Mouse ears were fixed overnight at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (3 μ m) were stained with hematoxylin and eosin or toluidine blue.

Immunohistochemistry and immunofluorescence staining

For immunohistochemistry (IHC) staining of major basic protein (MBP), mast cell protease 8 (MCPT8), and IL-23p19, paraffin sections were treated with 0.6% H₂O₂ to block endogenous peroxidase activity before antigen retrieval with either Pepsin (for IHC of MBP; Invitrogen, Carlsbad, Calif) or citric buffer (10 mmol/L citric acid, pH 6, for IHC of MCPT8 or IL-23p19). Slides were then blocked with normal rabbit or goat serum (Vector Laboratories, Burlingame, Calif) and incubated with primary antibody (see Table E1 in this article's Online Repository at www.jacionline.org), followed by incubation with biotinylated rabbit anti-rat IgG or biotinylated goat anti-rabbit IgG and treatment of AB complex (Vector Laboratories). Staining was finally visualized with AEC⁺ high-sensitivity substrate chromogen solution (Dako, Glostrup, Denmark) and counterstained with hematoxylin.

Immunofluorescence staining of thymic stromal lymphopoietin (TSLP) was performed, as previously described,²¹ by using biotinylated TSLP antibody (see Table E1), followed by incubation with CY3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, Pa). For immunofluorescence staining of CD3, CD4, CD8, NIMP14, or *Escherichia coli*, 10- μ m cryosections were fixed in 4% paraformaldehyde, permeabilized with acetone, and blocked with normal goat serum. Slides were then incubated with primary antibody (see Table E1), followed by CY3-conjugated goat anti-rat or donkey anti-rabbit IgG antibody (Jackson ImmunoResearch) and mounted with Vectashield medium (Vector Laboratories) containing 4'-6-diamidino-2-phenylindole dinhydrochloride (for nuclear staining; Invitrogen).

Quantitative RT-PCR

RNA was reverse transcribed by using random oligonucleotide hexamers and amplified by means of quantitative PCR with a LightCycler 480 (Roche Diagnostics, Indianapolis, Ind) and the QuantiTect SYBR Green kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl-transferase as an internal control. For analyses of each set of gene expression, an arbitrary unit of 1 was given to the samples with the highest level, and the remaining samples were plotted relative to this value. Sequences of PCR primers are shown in Table E2 in this article's Online Repository at www.jacionline.org.

ELISA

Mouse skin was chopped and homogenized with a Mixer Mill MM301 (Retsch, Dusseldorf, Germany) in lysis buffer (25 mmol/L Tris [pH 7.8], 2 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche). Protein concentrations of skin extract were quantified by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Calif). TSLP, IL-23, and IL-1 β levels in skin extracts were determined with the DuoSet ELISA Development Kits (R&D Systems, Minneapolis, Minn).

Isolation of CD4⁺ T cells

Ear-draining lymph nodes were mechanically disrupted and filtered through a 70- μ m cell strainer (BD Biosciences, San Jose, Calif). CD4⁺ T cells were isolated with the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany; purity, >96%). Purity was determined by means of flow cytometry with the BD LSR II Flow Cytometer.

In vivo neutralization of IL-23

Mice were injected intraperitoneally with tamoxifen once per day from day 0 to day 4. At days 4, 8, and 12, mice were intradermally injected with 20 μ g of rat anti-mouse IL-23p19 mAb (clone G23-8; eBioscience, San Diego, Calif) into the left ear with the same amount of isotype control antibody rat IgG₁ (clone R3-34, BD Biosciences) into the right ear of the same mouse.

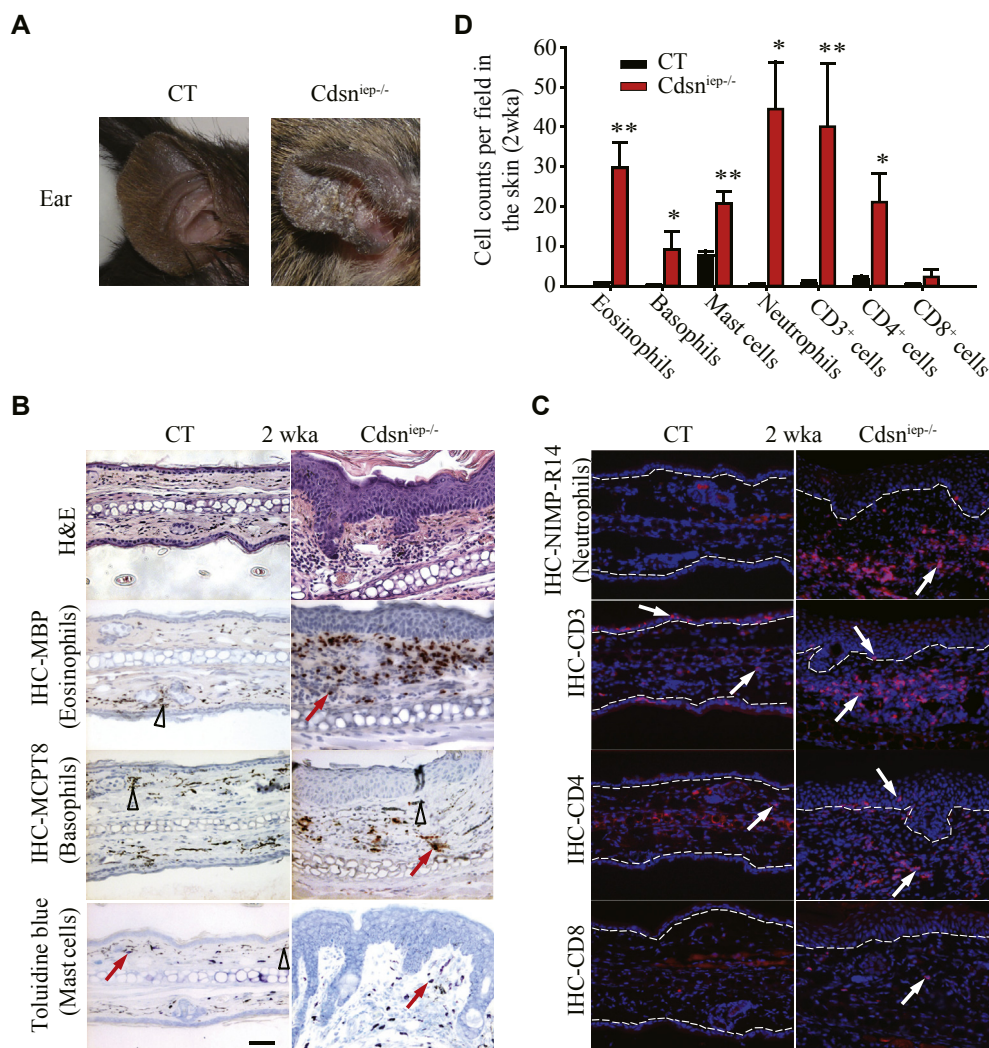


FIG 1. Inflammatory infiltrate in skin of mice with ablation of CDSN in epidermal keratinocytes. **A**, Ear appearance of wild-type control mice (CT) and *Cdsn*^{iep-/-} mice at 2 wka. **B**, Hematoxylin and eosin (H&E) staining, IHC staining, and toluidine blue staining. Red arrows point to one of the positive cells, and empty triangles indicate pigmented melanocytes in dermis (not antibody staining). **C**, Immunofluorescence analyses. Red, Fluorescent staining with antibody (white arrows point to one of the positive cells); blue, 4',6-diamidino-2-phenylindole dihydrochloride staining of nuclei. White dashed lines indicate the dermal/epidermal junction. Bar = 50 μ m. **D**, Summary of cell counts for skin-infiltrating immune cells. Results were obtained by calculating the average number of positive cells per microscopic field (at $\times 200$ magnification; >16 fields were counted per sample). * $P < .05$ and ** $P < .01$, Student *t* test ($n = 4$ for wild-type control mice and $n = 6$ for *Cdsn*^{iep-/-} mice). Data are representative of 4 independent experiments with similar results.

In vivo blockade of IL-1 signaling

Mice were injected intraperitoneally with tamoxifen once per day from day 0 to day 4 and intraperitoneally injected with the IL-1 receptor antagonist anakinra (10 mg/mouse/d; Kineret; Amgen, Thousand Oaks, Calif) or saline (vehicle control) once per 2 days from day 0 to day 12. Samples were analyzed at day 14.

Topical antibiotic treatment

Mice were injected intraperitoneally with tamoxifen from day 0 to day 4. A mixture of broad-spectrum antibiotics containing 25 μ g of ampicillin, 25 μ g of neomycin, 25 μ g of metronidazole, and 12.5 μ g of vancomycin (Sigma, St Louis, Mo) in an aqueous cream (hydrophilic oil in water emulsion; Ultrasicc, Hecht Pharma GmbH, StinStadt, Germany) was topically applied on the left ear and the same quantity of cream was applied on the right ear of the same mouse daily from day 0 to day 13. Samples were analyzed at day 14.

Statistical analysis

Data were analyzed with SigmaStat (Systat Software, Point Richmond, Calif) by using the *t* test or Mann-Whitney rank sum test depending on results from the Kolmogorov-Smirnov test (with Lilliefors correction) for normality and the Levene median test for equal variance. Values shown are means $\pm$ SEMs.

RESULTS

Ablation of CDSN in mouse keratinocytes leads to a complex inflammatory infiltrate in the skin

Mice lacking *CDSN* or with constitutive ablation of *CDSN* in keratinocytes during embryo development die after birth, exhibiting SC detachment caused by defective CDSN formation, resulting in a defective epidermal barrier.^{18,22} We generated

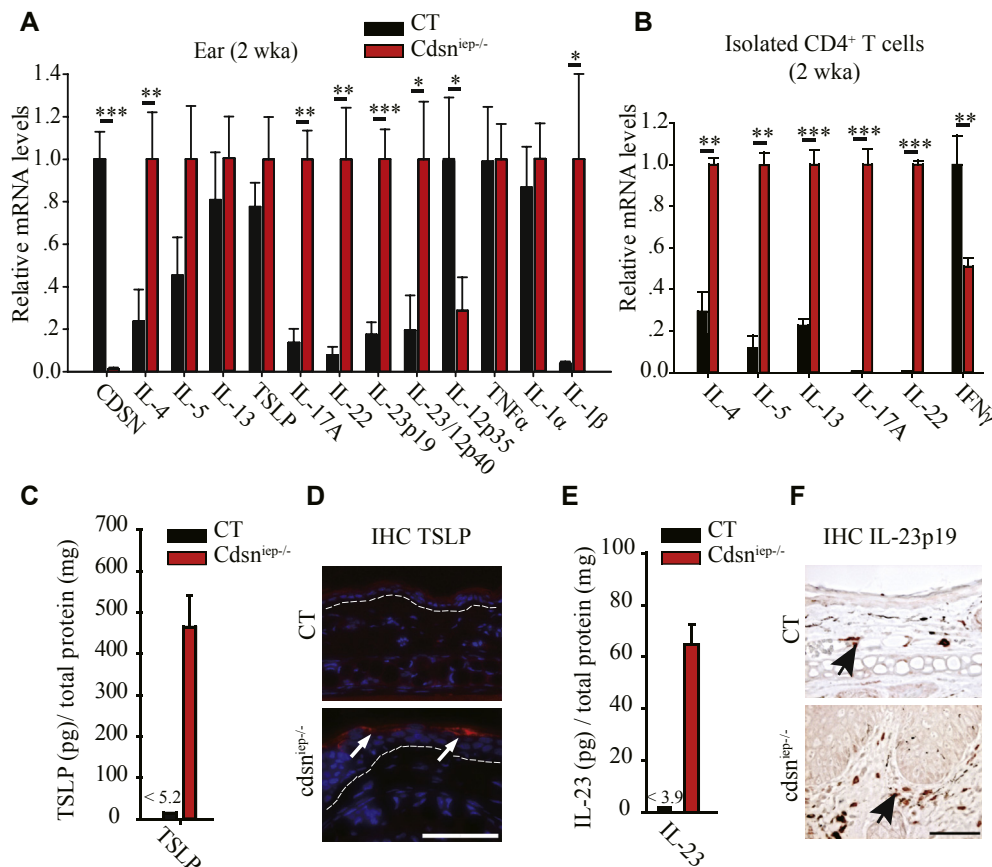


FIG 2. Increased expression of type 2 and type 17 cytokines in *Cdsn^{iep}/-* mouse skin. **A**, Quantitative RT-PCR analysis of cytokines in ears from wild-type control mice (CT) and *Cdsn^{iep}/-* mice at 2 wka. * $P < .05$, ** $P < .01$, and *** $P < .001$, Student t test ($n = 6$). **B**, Quantitative RT-PCR analysis of cytokines in CD4⁺ T cells isolated from ear-draining lymph nodes ($n = 3$). **C**, TSLP protein levels in ears at 2 wka ($n = 6$). **D**, Immunofluorescence staining of TSLP. Red, Staining of anti-TSLP antibody (white arrows point to the positive staining); blue, 4'-6-diamidino-2-phenylindole dihydrochloride staining of nuclei. **E**, IL-23 protein levels in ears at 2 wka ($n = 6$). **F**, IHC staining of IL-23p19. Black arrows point to one of the positive cells. Data are representative of 3 independent experiments with similar results.

K14-Cre-ER^{T2(tg/0)}/*Cdsn^{L2/L2}* mice in which tamoxifen injection induces ablation of the *CDSN* gene in epidermal keratinocytes in adult mice (called *Cdsn^{iep}/-* hereafter). As early as 2 weeks after the first tamoxifen injection (wka), the *Cdsn^{iep}/-* mice exhibited scaly, red, thickened, and inflamed skin at different body sites, including dorsal and ventral sites (see Fig E1, A, in this article's Online Repository at www.jacionline.org), most obviously on the ears (Fig 1, A). As expected, transepidermal water loss was significantly increased in skin of *Cdsn^{iep}/-* mice (see Fig E1, B). In addition, we observed an increase of conductance in skin of *Cdsn^{iep}/-* mice (see Fig E1, C), which is in agreement with the breakdown of SC in these mice. Efficient ablation of the *CDSN* gene was demonstrated by abolished mRNA expression in skin of *Cdsn^{iep}/-* mice (Fig 2, A).

Histologic analyses showed that skin of *Cdsn^{iep}/-* mice at 2 wka exhibited epidermal hyperplasia and inflammatory cell infiltrates in the dermis (hematoxylin and eosin staining; Fig 1, B). IHC revealed increased numbers of eosinophils, basophils, and mast cells (Fig 1, B). Immunofluorescence staining showed a strong infiltrate of neutrophils, as well as CD3⁺ T cells, in the skin (Fig 1, C). We found many more CD4⁺ than CD8⁺ cells in skin of *Cdsn^{iep}/-* mice (Fig 1, C), suggesting a helper T (rather

than cytotoxic T) cell response in skin of *Cdsn^{iep}/-* mice. Cell counts for these infiltrating immune cells are summarized in Fig 1, D. Similar results were obtained from analyses of dorsal or ventral skin (data not shown). Note also that even though results reported here were from *Cdsn^{iep}/-* mice on a mixed genetic background of C57BL/6J and 129/sv, similar results were generated from *Cdsn^{iep}/-* mice on a BALB/c genetic background after backcrossing with BALB/c mice (see Fig E2 in this article's Online Repository at www.jacionline.org). Together, these results indicate that ablation of *CDSN* in epidermal keratinocytes leads to a complex infiltrate of various inflammatory cells into the skin.

Increased expression of T_H2 and T_H17 cytokines in skin of *Cdsn^{iep}/-* mice

Cytokine expression was analyzed in skin at 2 wka by using quantitative RT-PCR to characterize the nature of the inflammatory responses developed in *Cdsn^{iep}/-* mice. We observed a significant increase in levels of the T_H2 cytokine IL-4 (although levels of IL-5 and IL-13 tended to be higher in skin of *Cdsn^{iep}/-* mice than in skin of wild-type control mice, which did not reach statistical significance; Fig 2, A). In addition,

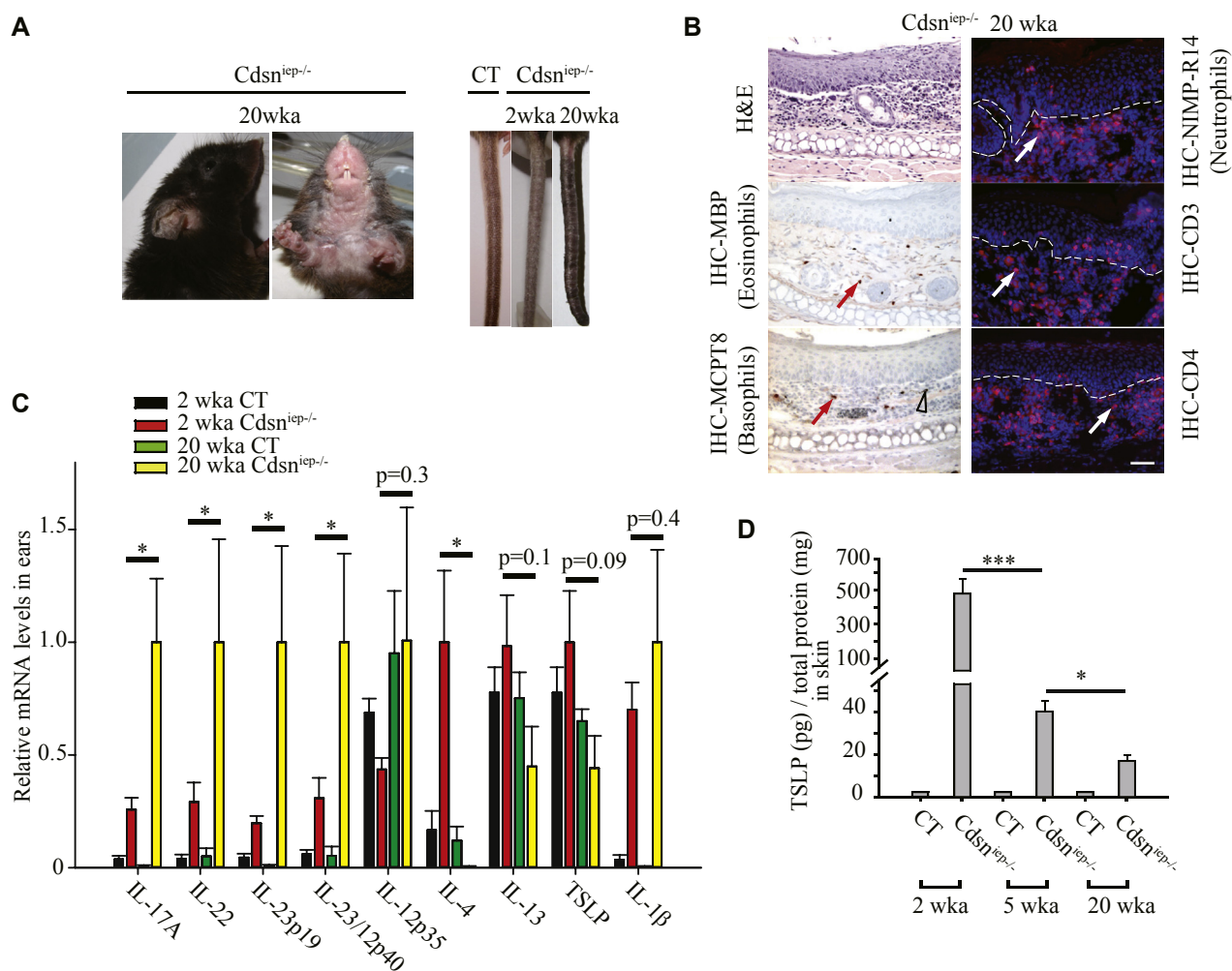


FIG 3. The type 2 response is dominated by the type 17 response in *Cdsn*^{iep-/-} mice over time. **A**, Wild-type control mice (CT) and *Cdsn*^{iep-/-} mice at 20 wka. **B**, Inflammatory infiltrate in skin of *Cdsn*^{iep-/-} mice at 20 wka. Arrows point to one of the positive cells, and empty triangles indicate pigmented melanocytes in dermis (not antibody staining). White dashed lines indicate the dermal/epidermal junction. Bar = 50 μ m. **C**, Relative mRNA levels in skin. **D**, TSLP protein levels in ears. * $P < .05$ and *** $P < .001$, Student *t* test ($n = 6$ for the 2 and 5 wka groups; $n = 4$ for the 20 wka group). Data are representative of 3 independent experiments with similar results.

RNA levels of the T_H17 cytokines IL-17A and IL-22 were both increased in skin of *Cdsn*^{iep-/-} mice (Fig 2, A). In addition, we observed an increase in levels of the proinflammatory cytokine IL-1 β (but not TNF- α or IL-1 α ; Fig 2, A).

Further analyses of ear-draining lymph nodes showed a similar increase in IL-4, IL-17, and IL-22 levels in *Cdsn*^{iep-/-} mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Quantitative RT-PCR was performed with CD4⁺ T cells isolated from ear-draining lymph nodes to examine the expression of these cytokines in T helper cells. Results showed that mRNA levels of IL-4, IL-5, and IL-13, as well as IL-17A and IL-22, were all significantly higher in CD4⁺ T cells from *Cdsn*^{iep-/-} mice (Fig 2, B); in contrast, mRNA levels of IFN- γ were decreased (Fig 2, B), thus indicating an increased T_H2 and T_H17 but decreased T_H1 response in skin of *Cdsn*^{iep-/-} mice. Moreover, in agreement with recent reports,²³ IL-17A was produced not only by T_H17 cells but also by $\gamma\delta$ T cells in dermis of *Cdsn*^{iep-/-} mice (see Fig E4 in this article's Online Repository at www.jacionline.org). This fit well with the observation of more

CD3⁺ T cells than CD4⁺ T cells in dermis of *Cdsn*^{iep-/-} mice (Fig 1, D).

Because TSLP has emerged as a pro- T_H2 cytokine that triggers AD pathogenesis in mice,^{20,24-28} we examined TSLP expression in skin of *Cdsn*^{iep-/-} mice at 2 wka. Although TSLP mRNA levels did not show significant differences between skin from wild-type control and *Cdsn*^{iep-/-} mice (Fig 2, A), ELISA indicated an increase in TSLP protein levels in skin of *Cdsn*^{iep-/-} mice (Fig 2, C). At an earlier time point (1 wka), TSLP protein levels in skin of *Cdsn*^{iep-/-} mice already tended to increase (although no difference was seen for mRNA levels, see Fig E5 in this article's Online Repository at www.jacionline.org). IHC staining detected TSLP in the suprabasal layer of the epidermis of skin of *Cdsn*^{iep-/-} mice but not in wild-type control skin (Fig 2, D), which is consistent with previous reports in skin of human subjects or mice with AD.^{28,29}

We next examined expression of IL-23, a key player for promoting the type 17 immune response.³⁰ The RNA levels of both subunits of IL-23 (ie, IL-23p19 and IL-23/12p40, the subunit

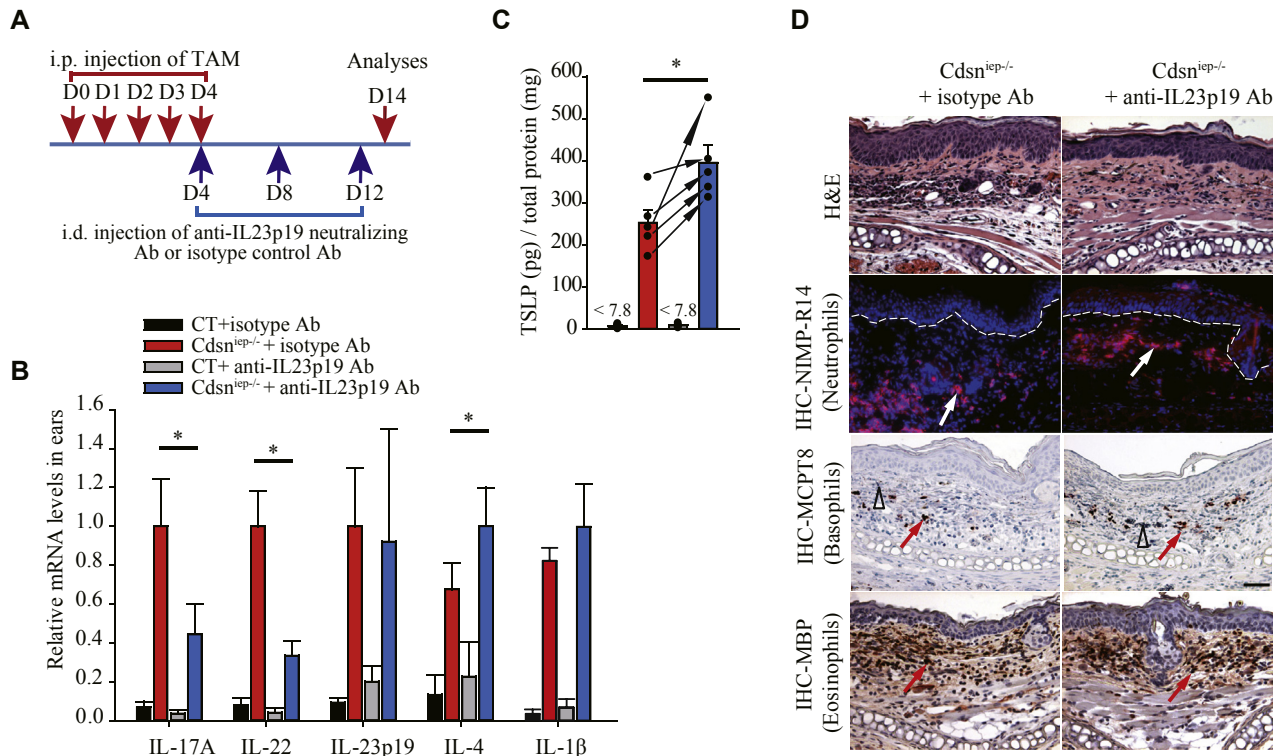


FIG 4. Blockade of IL-23 suppresses the type 17 but enhances the type 2 inflammatory response in *Cdsnip*^{iep-/-} mice. **A**, Experimental protocol. *i.d.*, Intradermal; *i.p.*, intraperitoneal. **B**, Quantitative RT-PCR analysis of cytokines. **C**, TSLP protein levels in ears. **P* < .05, paired *t* test (n = 5). Arrows connect paired samples from the same mouse (one ear treated with isotype antibody and another ear treated with anti-IL-23p19 antibody). **D**, Hematoxylin and eosin (H&E), immunofluorescence, and IHC staining of ear sections. Arrows point to one of the positive cells, empty triangles indicate pigmented melanocytes in dermis (not antibody staining), and white dashed lines indicate the dermal/epidermal junction. Bar = 50 μ m. Data are representative of 3 independent experiments with similar results.

shared with IL-12) were significantly increased in skin of *Cdsnip*^{iep-/-} mice (Fig 2, A). In contrast, expression of IL-12p35 (the specific subunit of the T_H1-promoting cytokine IL-12) was decreased (Fig 2, A), fitting with the decreased T_H1 response (Fig 2, B). ELISA analyses confirmed the increase in IL-23 production in skin of *Cdsnip*^{iep-/-} mice (Fig 2, E). IHC staining showed many more IL-23p19-expressing cells in the dermis of *Cdsnip*^{iep-/-} mice than in wild-type control skin (Fig 2, F). Note that IL-23p19 levels in ear-draining lymph nodes were not different between skin of wild-type control and *Cdsnip*^{iep-/-} mice (see Fig E3), suggesting that induction of IL-23 expression on barrier disruption occurs mainly in the skin.

Taken together, these data suggest that on ablation of CDSN in epidermal keratinocytes, the skin develops a mixed type 2/17 inflammatory response.

The type 2 response is dominated by the type 17 response over time

We observed that with time, ear and body skin (dorsal and ventral) of *Cdsnip*^{iep-/-} mice remained scaly and inflamed, and a progressive dry and scaly phenotype also appeared on tails (Fig 3, A). Comparison of global clinical scores (evaluated in 5 body sites) and, in particular, ear clinical scores between wild-type control and *Cdsnip*^{iep-/-} mice at 2 and 20 wka are shown in Fig E6, A, in this article's Online Repository at

www.jacionline.org. Moreover, we observed that *Cdsnip*^{iep-/-} mice exhibited more frequent colonization by *Staphylococcus aureus*, which was identified by the outgrowth of staphylococci in the selective plate (Mannitol Salt 2 Agar) from ear swab cultures of *Cdsnip*^{iep-/-} but not wild-type control mice (see Fig E7 in this article's Online Repository at www.jacionline.org), which were further confirmed to be *S aureus* by using the Pastorex Staph Plus Kit (data not shown; Bio-Rad Laboratories). Such an observation is in good agreement with the known fact that *S aureus* heavily colonizes the skin of patients with PSS-B,³¹ NS,³² or AD.³³

Histologic analysis of ear samples at 20 wka showed an intense accumulation of neutrophils; however, there were much fewer eosinophils and basophils when compared with 2 wka (Fig 3, B, and see Fig E8, A, in this article's Online Repository at www.jacionline.org, and compare with Fig 1, B-D). The mRNA expression analysis of ears of *Cdsnip*^{iep-/-} mice at 20 wka showed a further increase in IL-17A, IL-22, and IL-23p19 and IL-23/12p40 expression relative to 2 wka (Fig 3, C). In contrast, expression of IL-4 was almost completely abolished (Fig 3, C). Of interest, TSLP levels in skin of *Cdsnip*^{iep-/-} mice exhibited a continuous decrease with time (Fig 3, D). From these dynamic changes in cytokine expression and inflammatory infiltrate in skin of *Cdsnip*^{iep-/-} mice, we reason that the type 17 inflammation dominates the type 2 response and represses TSLP expression over time.

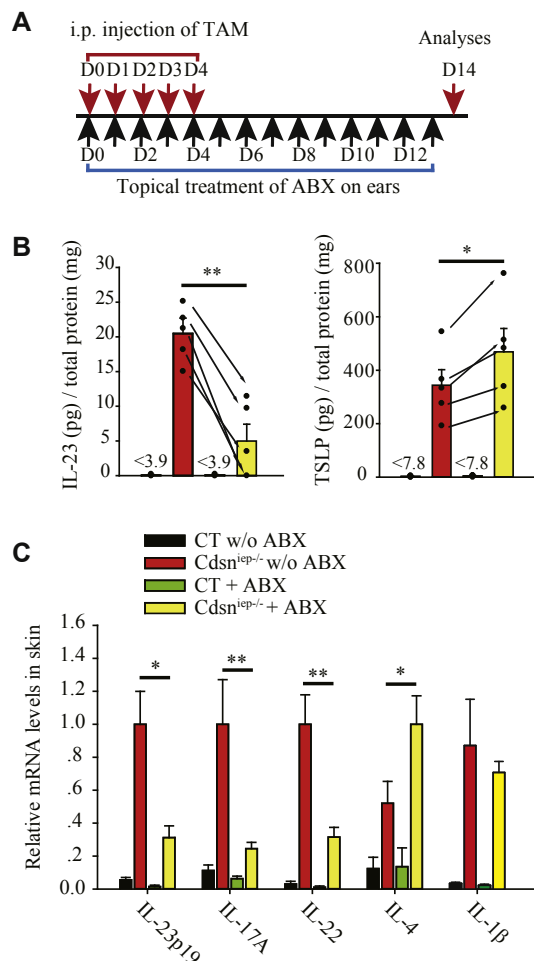


FIG 5. Topical antibiotic (ABX) treatment attenuates the IL-23/IL-17 axis but enhances the expression of TSLP and IL-4 in skin of *Cdsn*^{iep-/-} mice. **A**, Experimental protocol. i.p., Intraperitoneal. **B**, IL-23 and TSLP protein levels in ears. Arrows connect paired samples from the same mouse (one ear without and another ear with ABX treatment). **P* < .05 and ***P* < .01, paired *t* test (*n* = 5). **C**, Quantitative RT-PCR analyses of cytokines in the skin (*n* = 5). Data are representative of 2 independent experiments with similar results.

Blockade of IL-23 suppresses the type 17 response but enhances TSLP expression and the type 2 inflammatory response

To test whether the type 2 response could be repressed by the type 17 response in skin of *Cdsn*^{iep-/-} mice, we sought to block IL-23 with a neutralizing anti-IL-23p19 antibody (Fig 4, A). Although the clinical score evaluation from the treated ear did not show a significant difference (see Fig E6, B), the *in vivo* blockade of IL-23 led to a significant decrease in IL-17A and IL-22 expression in skin of *Cdsn*^{iep-/-} mice, which is in agreement with the role of IL-23 in promoting the type 17 response (Fig 4, B).³⁰ Interestingly, IL-4 expression (Fig 4, B) and TSLP production (Fig 4, C) were found to both be increased. Consistent with this finding, the skin exhibited a reduced neutrophil infiltrate in the dermis. However, more basophils were observed on the IL-23 blockade, even though eosinophil numbers were not apparently different (Fig 4, D, see Fig E8, B, for cell counts).

Thus these data indicate that IL-23 is the driver for type 17 inflammation in skin of *Cdsn*^{iep-/-} mice and moreover show that blockade of the IL-23/type 17 axis enhances TSLP expression and aggravates type 2 inflammation.

Topical antibiotic treatment reduces the IL-23/type 17 axis but enhances TSLP expression and the type 2 inflammatory response

Breakdown of the epidermal barrier could render the skin more susceptible to the commensal and/or pathogenic microbiome, such as *S aureus*, thereby inducing the IL-23/type 17 axis.³⁴ To examine the involvement of bacterial skin flora in the inflammatory phenotype of *Cdsn*^{iep-/-} mice, we treated mouse ears with broad-spectrum antibiotics³⁵ to eliminate most of the bacteria on the skin surface (Fig 5, A). IHC staining of anti-*E coli*³⁶ indicated an efficient suppression of bacteria in antibiotic-treated ears (see Fig E9, A, in this article's Online Repository at www.jacionline.org), and disc diffusion antibiotic sensitivity tests confirmed that *S aureus* colonized in skin of *Cdsn*^{iep-/-} mice was sensitive to the antibiotic solution (data not shown). On topical treatment with antibiotics, ear clinical scores tended to decrease (Fig E6, C). RNA analyses showed that expression of IL-23, IL-17A, and IL-22 was attenuated on the one hand and that expression of TSLP and IL-4 was enhanced on the other hand (Fig 5, B and C). Consistently, fewer neutrophils but more basophils were observed in skin of *Cdsn*^{iep-/-} mice on antibiotic topical treatment (see Fig E8, C, and E9, B). These results are reminiscent of those observed on IL-23 blockade (Fig 4), which further suggest that the IL-23/type 17 axis represses TSLP expression and type 2 inflammation.

Ablation of TSLP in keratinocytes abolishes the type 2 response but aggravates IL-23 expression and the type 17 response

Next, we examined whether TSLP is the driving factor for the type 2 response in skin of *Cdsn*^{iep-/-} mice. K14-Cre-ER^{T2(tg0)/Cdsn}^{L2/L2} mice were bred with *Tslp*^{L2/L2} mice²⁰ to obtain K14-Cre-ER^{T2(tg0)/Cdsn}^{L2/L2}/*Tslp*^{L2/L2} mice, which generated mutant mice with concomitant ablation of *CDSN* and *TSLP* in keratinocytes on tamoxifen injection (called *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} hereafter). Induction of TSLP was abolished in the skin of *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice, indicating that TSLP is efficiently ablated in keratinocytes (Fig 6, A). Ear clinical scores at 2 wka had a tendency to be lower in *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice (see Fig E6, D). Quantitative RT-PCR analyses at 2 wka found that the increase in IL-4 levels was abolished in skin of *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice (Fig 6, B). In contrast, mRNA levels of IL-17A, IL-22, IL-23p19, and IL-23/12p40 were all further increased in skin of *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice relative to skin of *Cdsn*^{iep-/-} mice (Fig 6, B). Histologic and IHC analyses of ear sections showed a decrease in eosinophil and particularly basophil counts in skin of *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice when compared with skin of *Cdsn*^{iep-/-} mice, whereas the neutrophil infiltrate remained intense in skin of *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice (Fig 6, C, and see Fig E8, D, for cell counts). These results indicate that keratinocytic TSLP is the key driver for the type 2 response in skin of *Cdsn*^{iep-/-} mice and that ablation of TSLP leads to aggravation of IL-23 expression and the type 17 inflammatory phenotype.

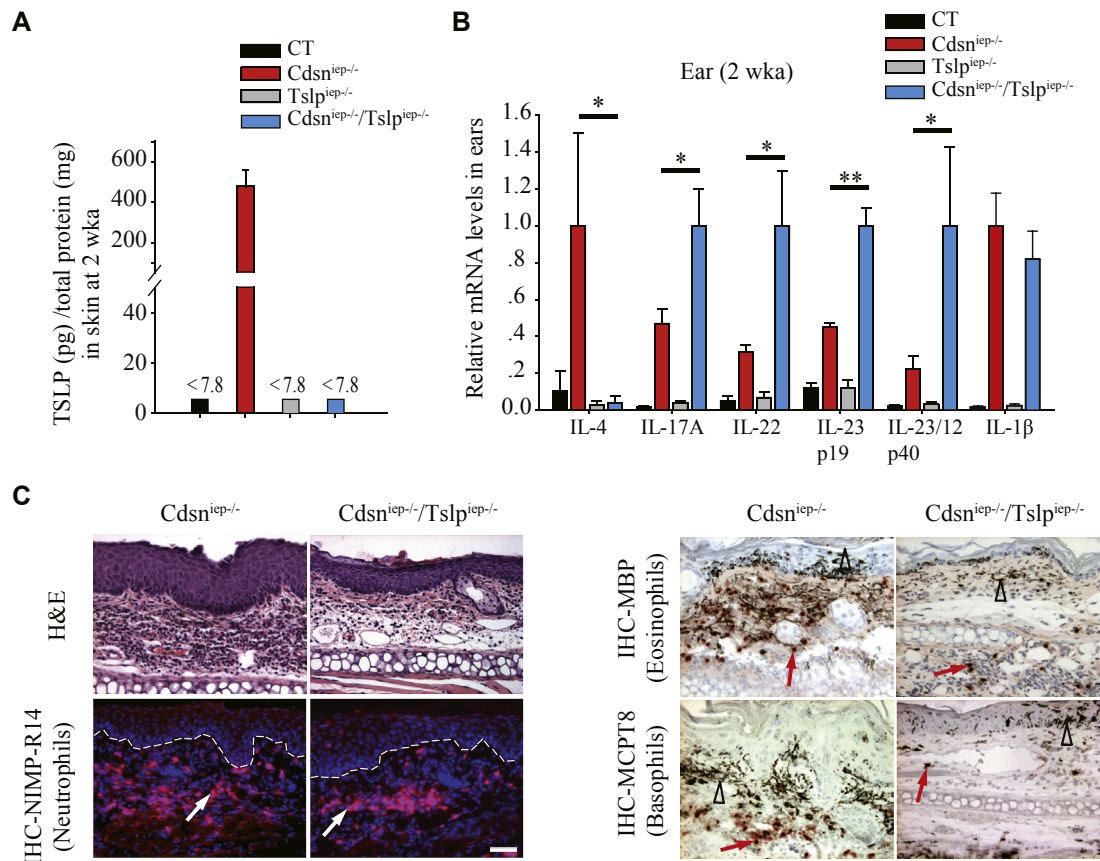


FIG 6. TSLP ablation abolishes type 2 but enhances type 17 inflammatory responses in skin of *Cdsn^{iep-/-}* mice. **A**, TSLP protein levels in ears at 2 wka ($n = 4$). **B**, Quantitative RT-PCR analysis of cytokines at 2 wka. $*P < .05$ and $**P < .01$, Student t test ($n = 6$). **C**, Hematoxylin and eosin (H&E), immunofluorescence, and IHC staining of ear sections at 2 wka. Arrows point to one of the positive cells, empty triangles indicate pigmented melanocytes in dermis (not antibody staining), and white dashed lines indicate the dermal/epidermal junction. Bar = 50 μm. Data are representative of 3 independent experiments with similar results.

Ablation of protease-activated receptor 2 reduces the TSLP/type 2 axis but enhances the IL-23/type 17 axis

Recently, it has been shown that TSLP production in epithelial cells is associated with protease-activated receptor 2 (PAR2) signaling.^{13,37-39} To examine whether PAR2 signaling mediates the TSLP/type 2 axis in skin of *Cdsn^{iep-/-}* mice, K14-Cre-ER^{T2(tg/0)}/*Cdsn^{L2/L2}* mice were crossed with *Par2*-null mice to generate *Cdsn^{iep-/-}/Par2*-null compound mice. Results showed that in these mice the expression of TSLP and IL-4 was greatly diminished (Fig 7) accompanied by a reduced basophil and eosinophil infiltrate (see Fig E10 in this article's Online Repository at www.jacionline.org and see Fig E8, E, for cell counts). Moreover, similar to what was observed in skin of *Cdsn^{iep-/-}/Tslp^{iep-/-}* mice, expression of IL-23, IL-17A and IL-22 was increased in skin of *Cdsn^{iep-/-}/Par2*-null mice (Fig 7, A). These results indicate that PAR2 signaling is involved in induction of the TSLP/type 2 axis in skin of *Cdsn^{iep-/-}* mice and that ablation of PAR2 results in exaggeration of the IL-23/type 17 inflammatory axis.

Inhibition of IL-1 signaling by anakinra suppresses both TSLP and IL-23 expression

Having observed from the cytokine mRNA expression analyses that IL-1β was significantly induced in skin of *Cdsn^{iep-/-}* mice at

2 wka (Fig 2, A) and 20 wka (Fig 3, C), using ELISA, we further confirmed that IL-1β protein levels were highly increased in skin of *Cdsn^{iep-/-}* mice (Fig 8, A). We then tested whether there is a role for IL-1β in skin inflammatory responses of *Cdsn^{iep-/-}* mice. IL-1 signaling was blocked by intraperitoneal injection of anakinra, a recombinant IL-1 receptor antagonist, which has been used clinically for the treatment of a number of inflammatory diseases, such as rheumatoid arthritis (Fig 8, B).⁴⁰ Strikingly, anakinra treatment ameliorated the inflammatory skin phenotype in *Cdsn^{iep-/-}* mice, as shown by significantly decreased ear and global clinical scores (see Fig E6, E). Further analyses showed that anakinra efficiently blocked both the TSLP/IL-4 and IL-23/IL-17/IL-22 cytokine axes (Fig 8, C and D), and correspondingly, it suppressed the infiltrate of eosinophils, basophils, and neutrophils (Fig 8, E, and see Fig E8, F, for cell counts). These data reveal that IL-1β plays a critical role in induction of both TSLP and IL-23 expression and that blockade of IL-1 signaling effectively reduces skin inflammation in *Cdsn^{iep-/-}* mice.

DISCUSSION

We have shown that on breakdown of the epidermal SC barrier, the skin develops simultaneous type 2 and type 17 inflammatory responses driven by TSLP and IL-23, respectively. We have revealed a counterregulation mechanism showing that

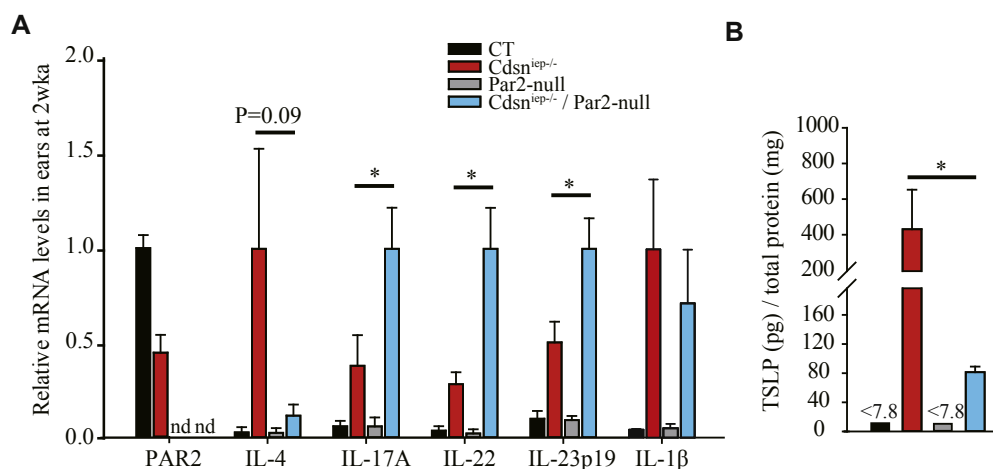


FIG 7. PAR2 is involved in induction of the TSLP/IL-4 axis in skin of *Cdsn*^{ie^{-/-}} mice. **A**, Quantitative RT-PCR analyses of ears at 2 wka. *nd*, Not detected. **B**, TSLP protein levels in ears. **P* < .05, Student *t* test (*n* = 4 for the *Par2*-null group and *n* = 5 for the other groups). Data are representative of 2 independent experiments with similar results.

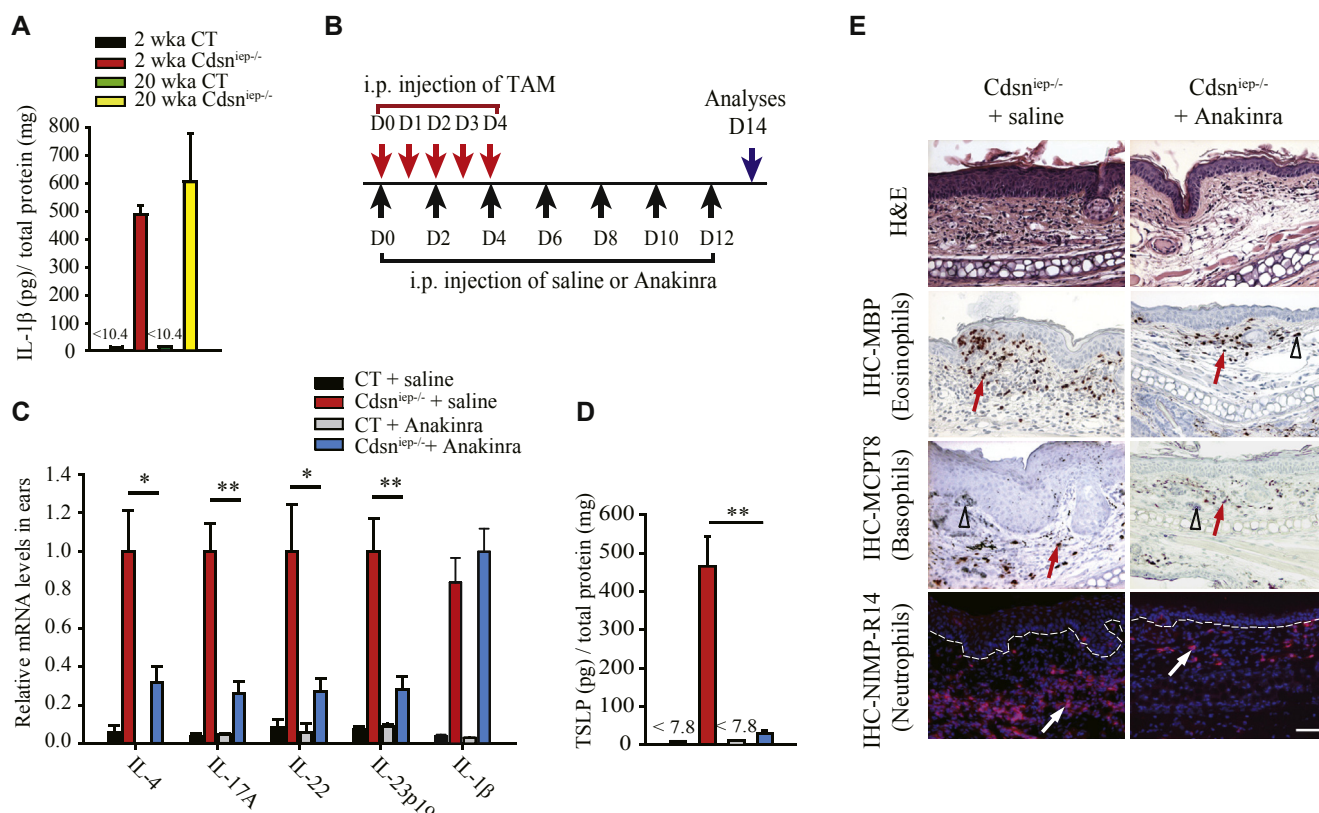


FIG 8. Inhibition of IL-1 signaling diminishes both TSLP/IL-4 and IL-23/IL-17 inflammatory axes in skin of *Cdsn*^{ie^{-/-}} mice. **A**, IL-1β protein levels in ears at 2 and 20 wka (*n* = 6 for 2 wka groups and *n* = 4 for 20 wka groups). **B**, Experimental protocol. *i.p.*, Intraperitoneal. **C**, Quantitative RT-PCR analyses of cytokines in skin. **P* < .05 and ***P* < .01, Student *t* test (*n* = 4). **D**, TSLP protein levels in ears (*n* = 4). **E**, Hematoxylin and eosin (H&E), immunofluorescence, and IHC staining of ear sections at 2 wka. *Arrows* point to one of the positive cells, *empty triangles* indicate pigmented melanocytes in dermis (not antibody staining), and *white dashed lines* indicate the dermal/epidermal junction. *Bar* = 50 μm. Data are representative of 3 independent experiments with similar results.

the IL-23/type 17 axis represses TSLP on one side while the TSLP/type 2 axis represses IL-23 on the other side, and provided further evidence that PAR2 signaling is involved in mediating the TSLP/type 2 axis while skin bacteria are engaged

in induction of the IL-23/type 17 axis. Finally, we have found that IL-1β crucially regulates both TSLP and IL-23 expression and shown that blockade of IL-1 signaling ameliorates skin inflammation.

Our study suggests that the outcome of the inflammatory phenotype in barrier-defective skin is shaped by crosstalk between the TSLP/type 2 and IL-23/type 17 immune axes. This should help to resolve the controversies on the T_H2 versus T_H17 inflammation from mouse and human studies on AD and AD-like congenital diseases, thus broadening our understanding of the complexity and heterogeneity of inflammatory responses in barrier-defective skin. Although our study is based on a mouse model with CDSN deficiency (a cause for human PSS-B),³¹ such a counterregulation between TSLP- and IL-23–driven axes might represent a common mechanism in various mouse models related to epidermal barrier dysfunction. Supporting this speculation, in an AD mouse model (flaky tail mice) dominant T_H2 and T_H17 features were reported at an early and later stage, respectively^{12,14}; in addition, both T_H2 and T_H17 features have been characterized recently in an NS mouse model.¹⁶

We have identified *S aureus* colonization in skin of *Cdsn*^{iep-/-} mice, as universally occurs in human patients with PSS-B, NS, and AD. Our data showed that topical administration of antibiotics that eliminate skin bacteria, including *S aureus*, suppresses the IL-23/type 17 axis, which is in agreement with the role of *S aureus* in induction of the IL-23/type 17 inflammatory axis.³⁴ Interestingly, antibiotic treatment led to enhancement of the TSLP/type 2 response, which differs from previous reports that *S aureus* promoted T_H2-type inflammation through TSLP⁴¹ or mast cell activation.⁴² Rather, our results suggest that factors other than skin bacteria (eg, endogenous or exogenous proteases) contribute to induction of the TSLP/IL-4 axis, although we do not rule out the possibility that *S aureus* might somehow induce TSLP or IL-4. It will be interesting to study further the dysbiotic pattern in skin of *Cdsn*^{iep-/-} mice and to define the contribution of *S aureus* and other bacterial flora to skin inflammatory pathogenesis, such as by using antibiotics specific for bacterial species or gnotobiotic mice.

Nevertheless, our data suggest a potential mechanism for the hygiene hypothesis, which posits that improved hygiene is associated with a reduction in type 17 immune responses but an increased risk of type 2–related diseases, such as AD. This is supported by a previous study showing that the absence of microbiota under germ-free conditions enhanced TSLP expression in mice with loss of Notch signaling.⁴³ One might thus assume that interventions, such as strict asepsis or topical antibiotic treatment, which are used for patients with PSS-B, NS, and AD because of recurrent bacterial colonization and superinfection,³¹⁻³³ might risk enhancement of the TSLP/type 2 response.

One question raised by our study is how TSLP is induced in barrier-defective skin. We showed that PAR2-mediated signaling represents a major, if not the only, trigger of TSLP expression in skin of *Cdsn*^{iep-/-} mice, which is in keeping with recent reports in mouse models of NS³⁷ or AD.⁴⁴ It is recognized that mutation of the *SPINK5* gene in patients with NS leads to uncontrolled protease activity and thus directly activates PAR2.^{13,37} In addition, an increase in skin pH, a feature in human AD with *FLG* mutations and in a murine model of *FLG* deficiency, might also contribute to activate SC proteases.⁴⁵ But how could PAR2 signaling be activated in skin of *Cdsn*^{iep-/-} mice? We observed that kallikrein 14, one of the endogenous ligands of PAR2, exhibits higher expression in skin of *Cdsn*^{iep-/-} mice (see Fig E11 in this article's Online Repository at www.jacionline.org), further supporting that TSLP induction could be due to endogenous PAR2 activation. Whatever the mechanism, we believe that PAR2/TSLP/IL-4 is a cascade commonly

involved in promoting the type 2 response in human subjects or mice with inflammatory diseases related to skin barrier defects.

However, this differs from a recent report showing that the T_H2 response was driven by *Corynebacterium bovis* colonization in a mouse model with Sox9Cre-mediated ablation of a disintegrin and metalloproteinase 17 (ADAM17).⁴⁶ In those mice antibiotic treatment reduced both T_H2 and T_H17 cell numbers. In our case *C bovis* colonization was not detected from ear swabs of *Cdsn*^{iep-/-} mice (performed by IDEXX BioResearch, et Med Labor GmbH, Ludwigsburg, Germany; data not shown). It remains unclear whether colonization of *C bovis* is a feature related in particular to ADAM17 deficiency and whether the T_H2 response in ADAM17-deficient mice is mediated by TSLP. The differences in *Cdsn*^{iep-/-} and ADAM17-deficient mice could be due to the different nature of their skin barrier defects: a primary defect in the SC barrier in the former versus a possible secondary barrier defect in the later (eg, resulting from defective release of TNF-α).

Our finding that TSLP production in keratinocytes is negatively regulated by the IL-23/type 17 inflammatory axis adds a new regulating pathway between type 17 and type 2 responses. It remains yet to be defined in *Cdsn*^{iep-/-} mice which component of the IL-23/type 17 axis represses TSLP production and how it does so. Interestingly, a recent study reported that IL-17A inhibited TSLP production in human skin explants.⁴⁷ Thus a similar mechanism might exist in both mice and human subjects, but this needs to be further explored at the molecular level.

On the other hand, TSLP negatively regulates the IL-23/type 17 axis. There can be several interpretations.

First, given that skin bacteria play an important role in induction of the IL-23/IL-17 response, the aggravated IL-23/type 17 axis in *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice might reflect a higher susceptibility to certain skin bacteria. Indeed, we observed more frequent colonization of *S aureus* in the skin of *Cdsn*^{iep-/-}/*Tslp*^{iep-/-} mice (data not shown). Interestingly, human TSLP was reported to exert antimicrobial activity,^{48,49} but whether mouse TSLP has such a feature remains to be explored.

Second, TSLP might directly inhibit IL-23 expression in dermal dendritic cells/macrophages.⁵⁰

Third, TSLP downstream signals, such as IL-4, might inhibit IL-23 expression. This is supported by recent reports that IL-4 mediated IL-23/T_H17 silencing in mice⁵¹ and that IL-4 inhibits IL-23 in human primary dendritic cells.⁵²

The fact that blockade of one of the TSLP/type 2 and IL-23/type 17 axes leads to aggravation of the other suggests that application of the cytokine-based targeting strategies in patients with inflammatory skin diseases, including anti-TSLP therapy in patients with AD⁵³ or anti-IL-23/IL-17 in patients with AD and psoriasis,^{53,54} deserve more careful evaluation. In addition, it will be important to explore the counterregulatory mechanism in other barrier epithelia, such as the airways and intestine,^{55,56} where TSLP- and IL-23–driven pathways are involved in homeostasis and inflammatory conditions and anti-TSLP or anti-IL-23 therapies have been developed or are under development for treating a number of inflammatory diseases, including asthma and inflammatory bowel disease.^{57,58} We found that blockade of IL-1 signaling by anakinra significantly ameliorates the inflammatory skin phenotype in *Cdsn*^{iep-/-} mice, with reduced expression of both TSLP and IL-23 and diminished type 2/17 inflammation. Thus this finding provides encouraging insight into the hypothesis that blocking IL-1 signaling could be an efficient approach for treating skin

inflammation in patients with PSS-B, which is advantageous than targeting the individual TSLP/type 2 or IL-23/type 17 axis.

Finally, our findings strongly urge clinical studies. First, it remains to be examined whether a complex type 2/type 17 inflammatory phenotype is a feature of patients with PSS-B, although this has been reported in patients with AD or NS,⁵⁻¹⁰ who all share SC barrier defects and overlapping clinical features.

Second, it will be interesting to perform kinetic studies during the disease course to investigate how various environmental factors (eg, skin flora, antibiotic treatment, and protease allergens) affect the inflammatory phenotype, as suggested by our mouse data.

Third, it will be important to examine IL-1 β expression in patients with PSS-B and to test the effects of anakinra and/or other IL-1–blocking reagents in treating the skin inflammation of these patients. Notably, anakinra has been used clinically for the treatment of a number of inflammatory diseases, such as rheumatoid arthritis.⁴⁰ However, there is not yet a clinical study reported on anakinra efficacy in patients with AD and AD-like congenital disorders.¹⁷ Actually, the increased IL-1 β expression has been observed in human patients with NS¹³ and AD.⁴⁵ Thus we propose that targeting IL-1 might have broad potential for the treatment of various inflammatory skin diseases related to epidermal barrier dysfunction.

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Key messages

- Type 2 and type 17 inflammatory responses are induced simultaneously in mouse skin on breakdown of the epidermal SC barrier.
- TSLP and IL-23 drive type 2 and 17 inflammatory responses, respectively.
- PAR2 signaling is involved in mediating the TSLP/type 2 axis, whereas skin bacteria are engaged in induction of the IL-23/type 17 axis.
- The TSLP/type 2 and IL-23/type 17 axes counterregulate each other.
- Blockade of IL-1 signaling suppresses both TSLP and IL-23 expression and ameliorates skin inflammation.

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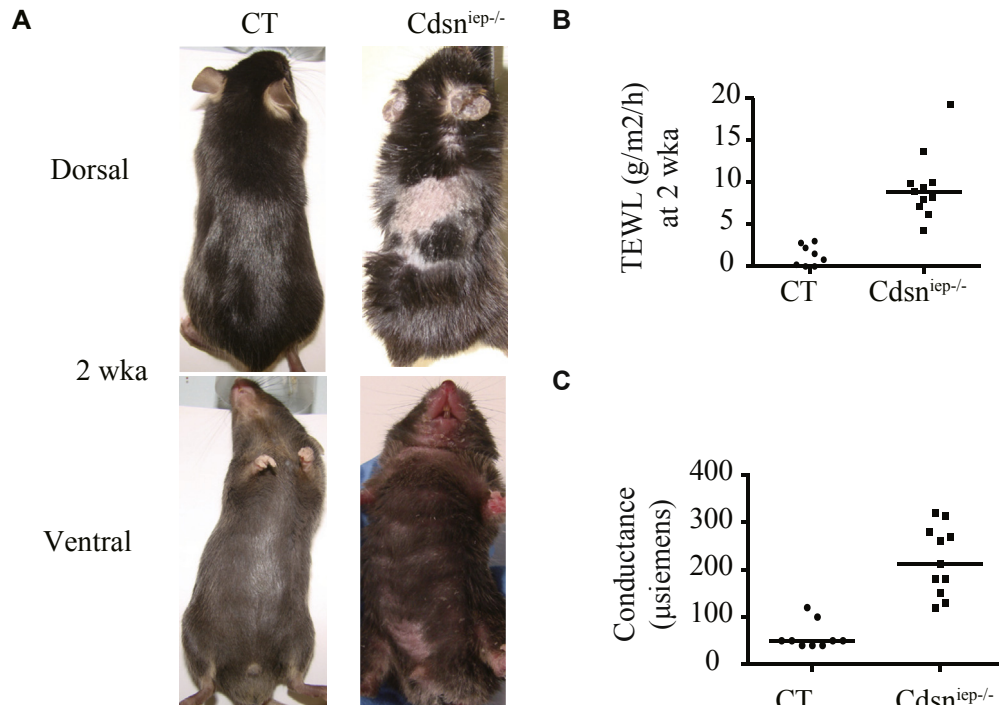


FIG E1. **A**, Appearance of wild-type control mice (CT) and $Cdsn^{iep-/-}$ mice at 2 wka. **B**, Transepidermal water loss (TEWL) of skin on the dorsal area, as determined by using a DermaLab (Cortex Technology, Hadsund, Denmark). Values recorded were means over the last 8 cycles of measurement. Measurements were performed at an ambient temperature of 21°C to 22°C and humidity of 50% ± 5%. **C**, Skin superficial hydration measured with Moisture Module of DermaLab (Cortex Technology) on mouse ears. Values recorded were means of the last 3 measurements in the measurement sequence. Results show increased conductance in skin of $Cdsn^{iep-/-}$ mice in agreement with breakdown of the SC in these mice.

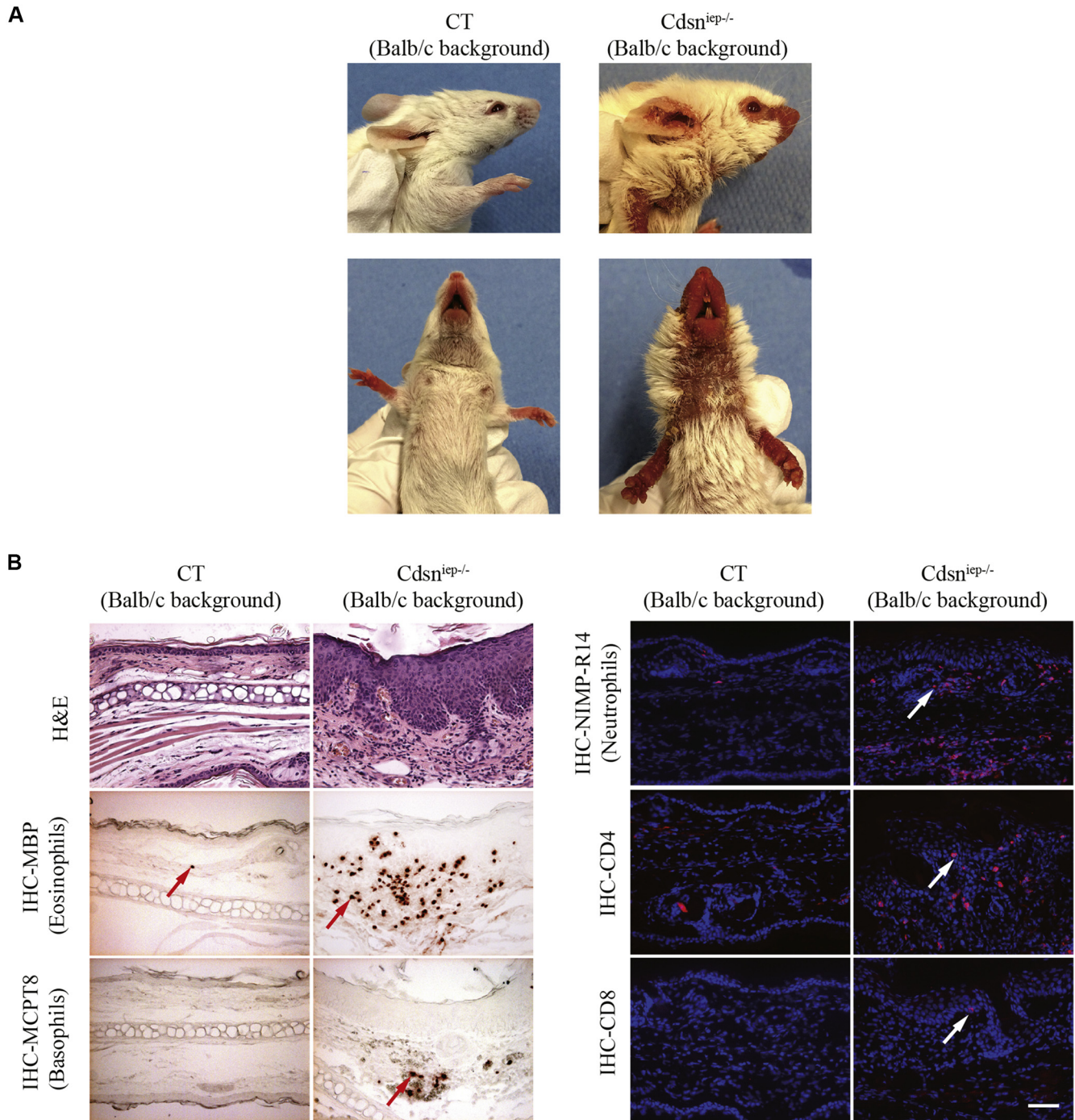


FIG E2. A, Appearance of wild-type control mice (CT) and $Cdsn^{iep-/-}$ mice on a BALB/c genetic background at 2 wka. **B**, Hematoxylin and eosin (H&E) staining, IHC staining with MBP (for eosinophils) or MCPT8 (for basophils), and immunofluorescence analyses of NIMP-R14 (for neutrophils), CD4, or CD8 on ear sections. For MBP and MCPT8 IHC, dark red corresponds to staining with antibody (red arrows point to one of the positive cells); for immunofluorescence staining, red corresponds to fluorescent staining with antibody (white arrows point to one of the positive cells), and blue corresponds to 4'-6-diamidino-2-phenylindole dihydrochloride staining of nuclei. Bar = 50 μ m. Data are representative of 3 independent experiments with similar results.

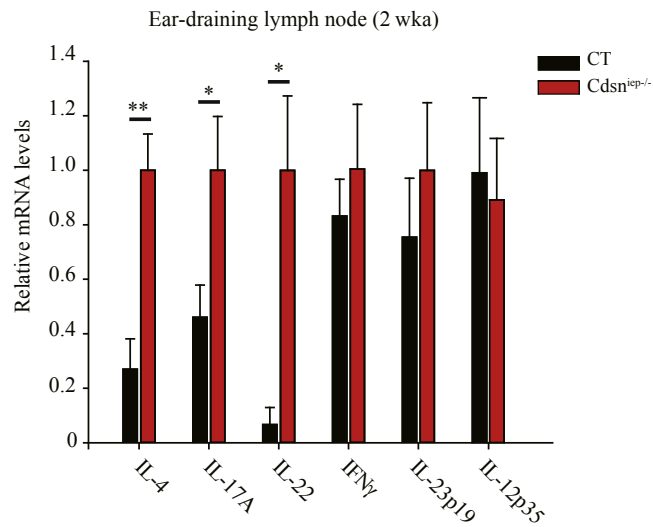


FIG E3. Quantitative RT-PCR analysis of cytokine expression in ear-draining lymph nodes from wild-type control mice (CT) and *Cdsn*^{ie^{-/-}} mice at 2 wka. * $P < .05$ and ** $P < .01$, Student t test. Values are means $\pm$ SEMs ($n = 6$ mice per group). Data are representative of 3 independent experiments with similar results.

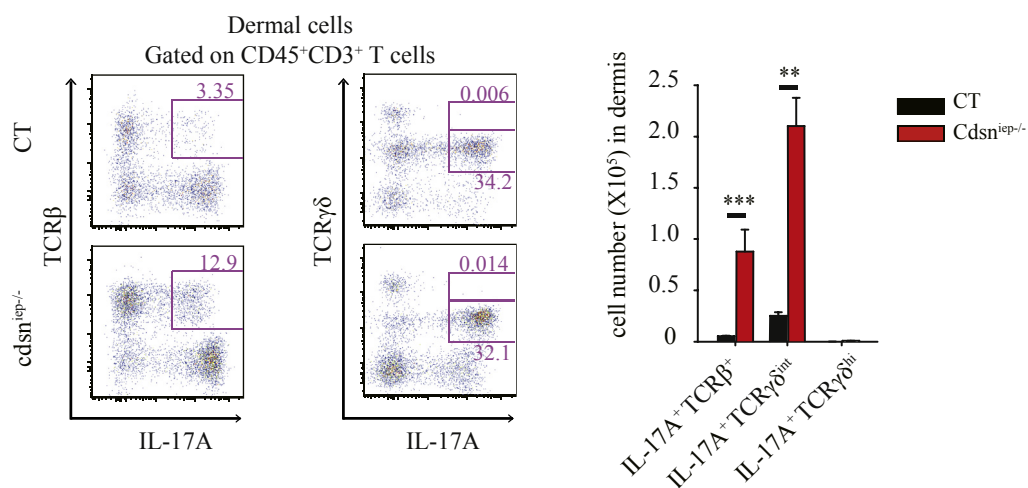


FIG E4. Intracellular staining with IL-17A antibody of dermal cells from wild-type control mice (*CT*) and *Cdsn^{iep/-}* mice at 2 wka. Frequency (*left panel*) and number (*right panel*) of IL-17A⁺ T-cell receptor β ⁺, IL-17A⁺ T-cell receptor $\gamma\delta$ ^{int}, and IL-17A⁺ T-cell receptor $\gamma\delta$ ^{hi} T cells in dermis are shown. ***P* < .01 and ****P* < .001, Student *t* test. Values in the *right panel* are means $\pm$ SEMs (*n* = 3 per group).

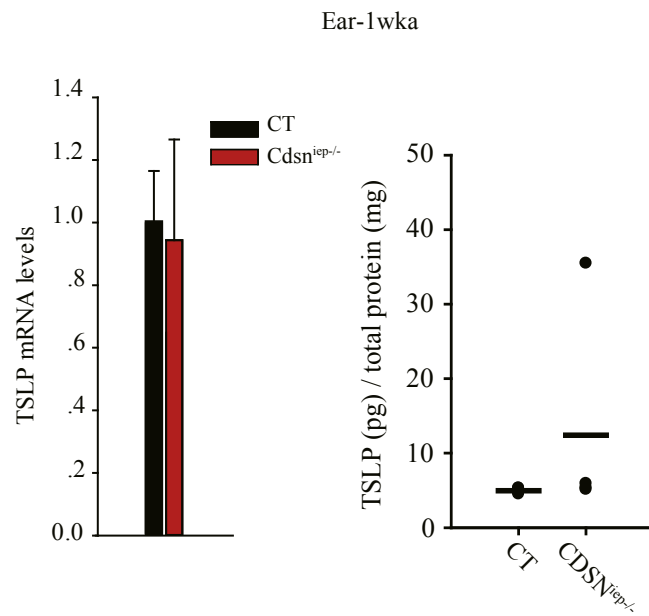


FIG E5. TSLP RNA (*left*) and protein (*right*) levels in ears from wild-type control mice (CT) and *Cdsn^{iep-/-}* mice at 1 wka (n = 3 per group). Data are representative of 2 independent experiments with similar results.

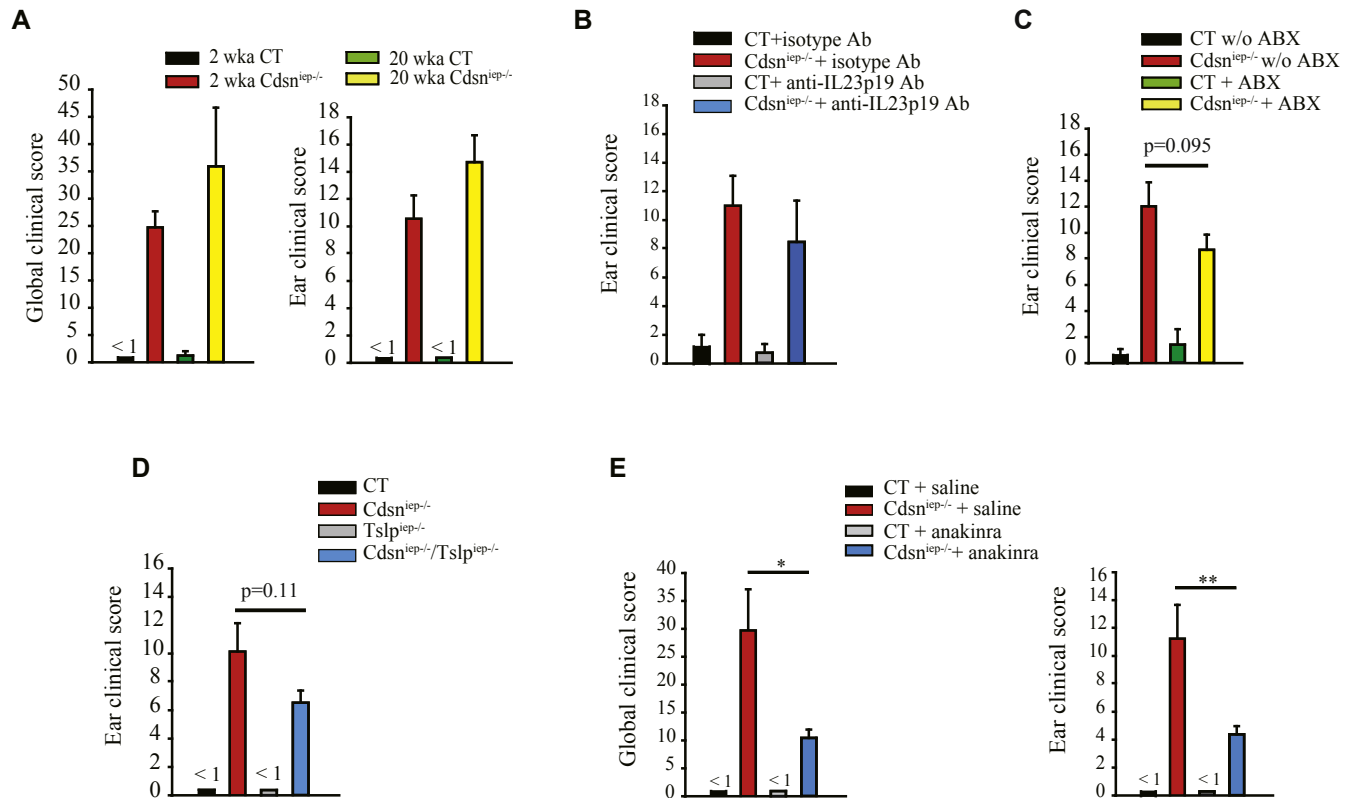


FIG E6. Comparison of global clinical scores (evaluated in 5 body sites) and ear clinical scores in different experimental settings. Global clinical scores of mice were measured by assessing the severity and extent of lesions (see Plant JD, Gortel K, Kovalik M, Polissar NL, Neradilek MB. Development and validation of the Canine Atopic Dermatitis Lesion Index, a scale for the rapid scoring of lesion severity in canine atopic dermatitis. *Vet Dermatol* 2012;23:515-e103), according to 2 parameters, parameter 1 (including erythema, excoriation, and erosion) and parameter 2 (including alopecia, lichenification, and hyperpigmentation), at 5 different body sites: the head and ear, forefeet, hind feet, ventral thorax and axillae, and ventral abdomen and inguinal region. The severity and extent of lesions of the 2 parameters at each body site were graded as 0 (none), 1 (mild), 2 or 3 (moderate), or 4 or 5 (severe and extensive lesions), with a maximum score of 50. The ear clinical score was evaluated by using 5 parameters (modified from Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. *Dermatology* 1993;186:23-31): redness, swelling, oozing/crusting, scratch marks, and skin thickening. The intensity of each parameter was given as none (0), mild (1), moderate (2), or severe (3), with a maximum score was 15. Values shown are means $\pm$ SEMs (A: $n = 6$ for 2 wka groups and $n = 4$ for 20 wka groups; B and C: $n = 5$; D: $n = 6$; E: $n = 4$). * $P < .05$ and ** $P < .01$.

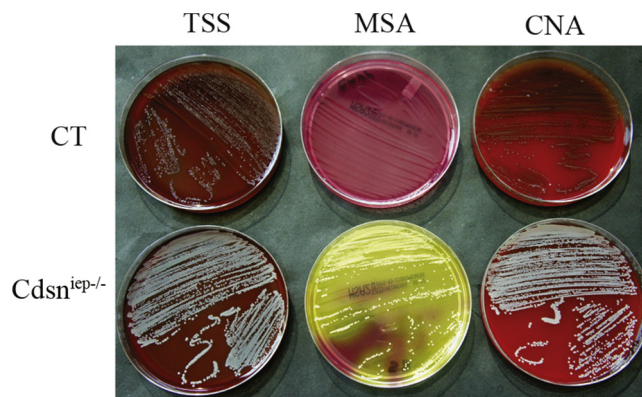


FIG E7. Identification of *Staphylococcus aureus* from ear swabs of *Cdsn^{iep-/-}* mice. Cultures obtained with ears swabs of wild-type control mice (CT) and *Cdsn^{iep-/-}* mice were plated on Tryptic Soy Agar (TSS) plus 5% sheep's blood (nonselective for bacteria; Biomerieux, Marcy l'Etoile, France), Mannitol Salt 2 Agar (MSA; selective isolation of staphylococci; Biomerieux), or Columbia CNA Agar (CNA) plus 5% sheep's blood (selective for gram-positive bacteria; Biomerieux). Streptococci were identified as small, white to grayish colonies grown in TSS and CNA plates but not grown in MSA plates; staphylococci were identified as colonies selectively grown in MSA plates, as well as large and white colonies grown in TSS and CNA plates. Results show the outgrowth of streptococci from wild-type control mice and staphylococci from *Cdsn^{iep-/-}* cultures.

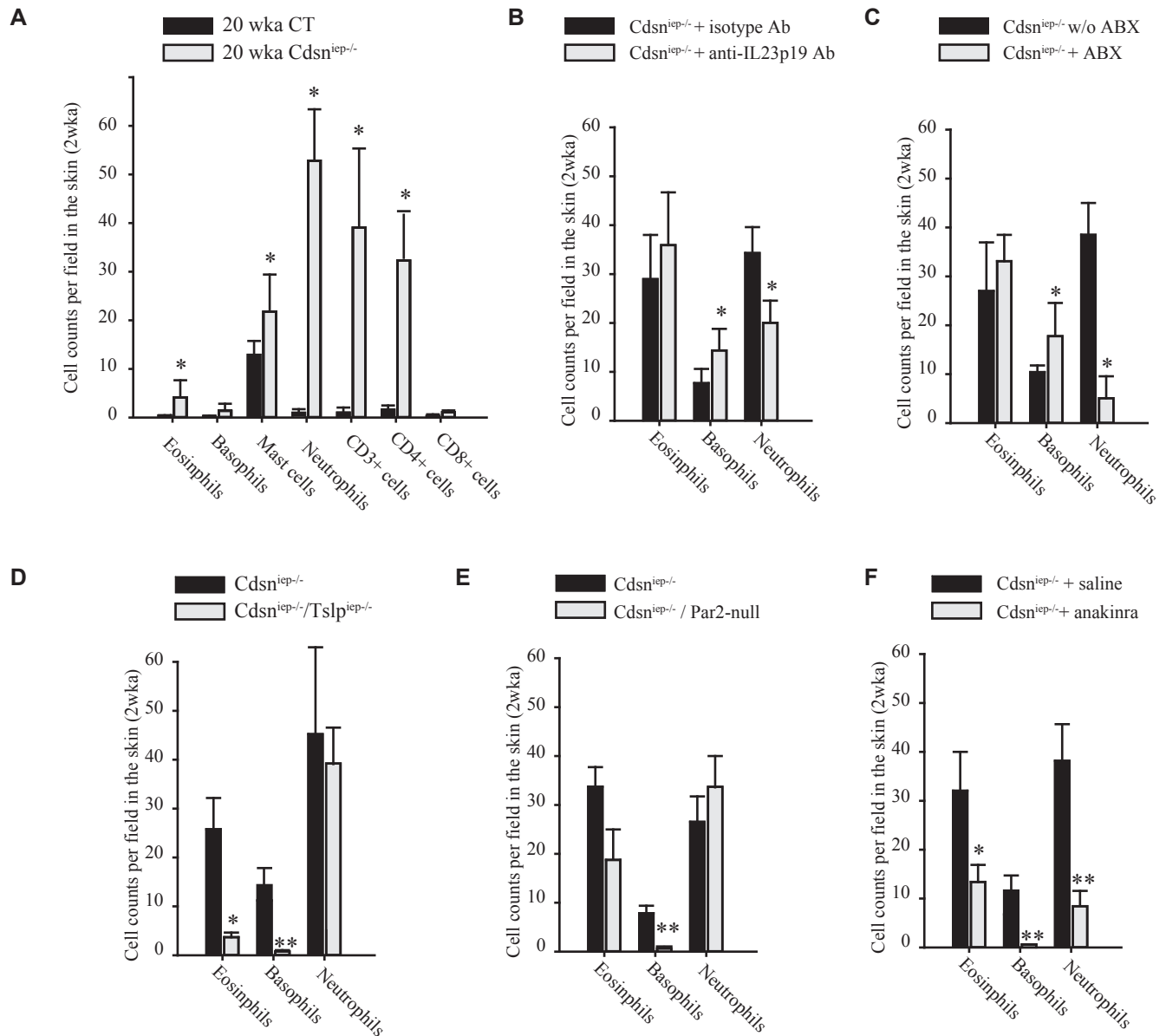


FIG E8. Comparison of counts for skin-infiltrating immune cells in different experimental settings. Results were obtained by calculating the average number of positive cells per microscopic field (at $\times 200$ magnification; >12 fields were counted per sample). $*P < .05$ and $**P < .01$, Student *t* test. Values are means $\pm$ SEMs (A and F: $n = 4$ per group; B, C, and E: $n = 5$ per group; D: $n = 6$ per group). Data are representative of 3 independent experiments with similar results.

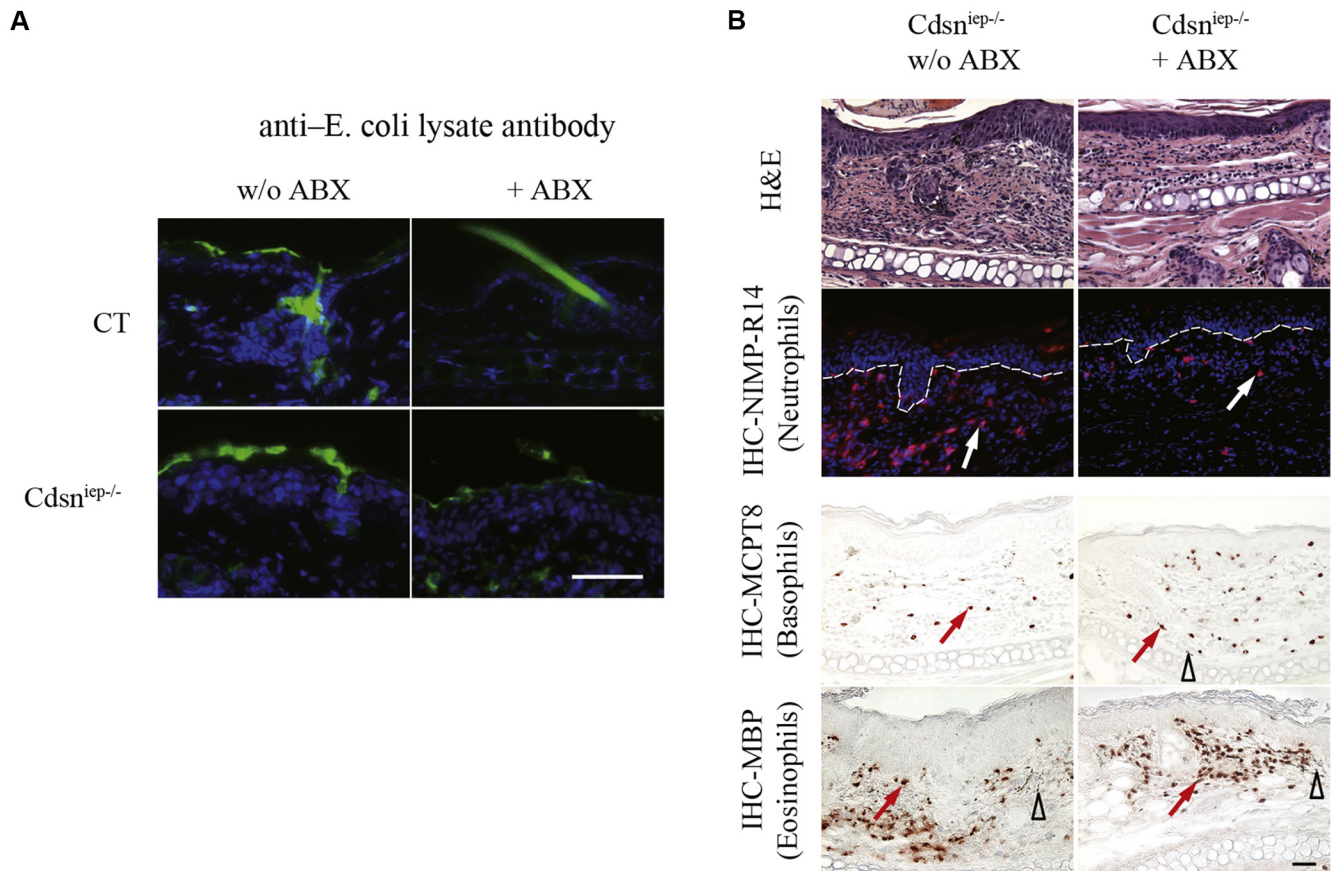


FIG E9. A, Efficient suppression of bacteria in antibiotic (ABX)-treated skin, as shown by means of immunofluorescence staining of bacterial products with an anti-*E. coli* lysate antibody on ear sections from wild-type control mice (CT) and *Cdsn*^{iep-/-} mice treated with (+) or without (w/o) ABX. **B**, Fewer neutrophils but more basophils were found in skin of *Cdsn*^{iep-/-} mice on ABX topical treatment. In immunofluorescence staining red corresponds to staining with antibody (white arrows point to one of the neutrophils), whereas blue corresponds to 4'-6-diamidino-2-phenylindole dihydrochloride staining of nuclei. White dashed lines indicate the dermal/epidermal junction. In IHC staining dark red corresponds to staining with antibody (red arrows point to one of the positive cells), and empty triangles indicate pigmented melanocytes in dermis (not antibody staining). Bar = 50 μ m. Data are representative of 2 independent experiments with similar results.

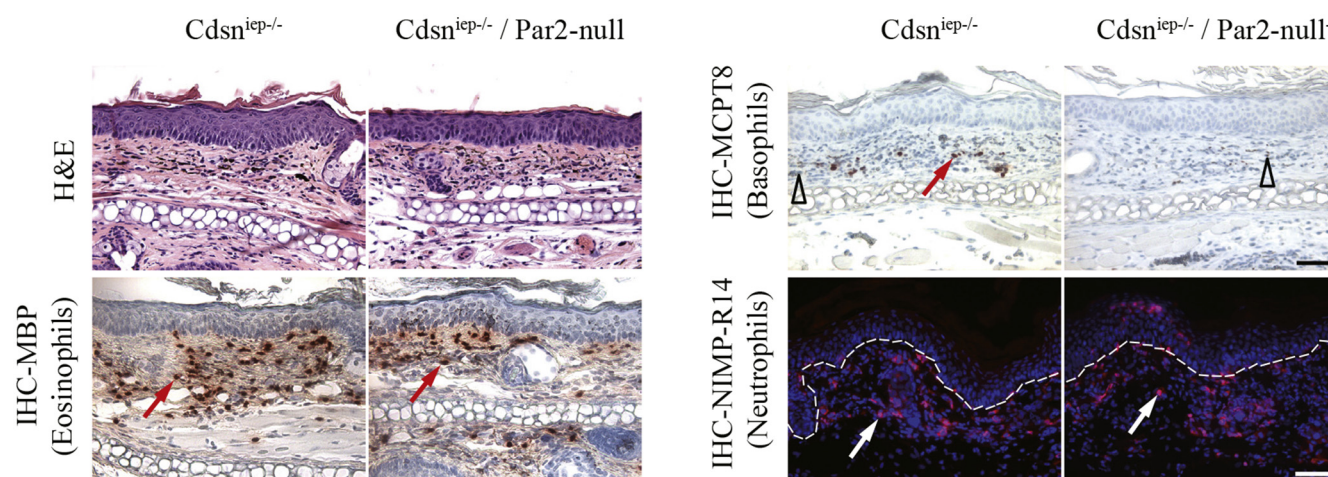


FIG E10. Reduced basophil and eosinophil infiltrate, but not neutrophil infiltrate, in *Cdsn^{iep-/-} Par2-null* mice at 2 wka. In IHC staining *red arrows* point to one of eosinophils or basophils (*dark red* corresponds to staining with antibody), and *empty triangles* indicate pigmented melanocytes in dermis. In immunofluorescence staining *white arrows* point to one of the neutrophils (*red* corresponds to fluorescent staining with antibody, and *blue* corresponds to 4'-6-diamidino-2-phenylindole dihydrochloride staining of nuclei). *White dashed lines* indicate the dermal/epidermal junction. *Bar* = 50 μ m. Data are representative of 2 independent experiments with similar results.

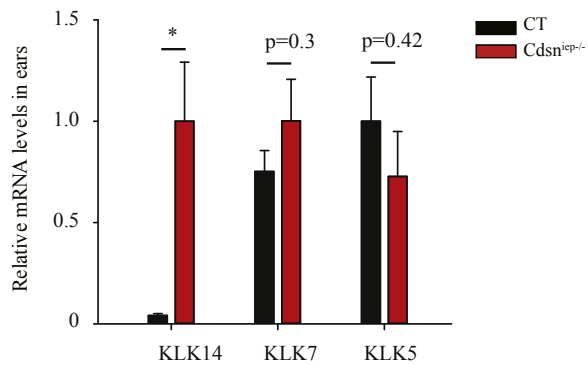


FIG E11. Quantitative RT-PCR analysis of kallikrein (*KLK*) 14, KLK7, and KLK5 expression in ears from wild-type control mice (*CT*) and *Cdsn^{iep-/-}* mice at 2 wka, showing a significant increase in KLK14 expression in skin of *Cdsn^{iep-/-}* mice. **P* < .05, Student *t* test. Values are means $\pm$ SEMs (*n* = 4 mice for the CT group; *n* = 6 for the *Cdsn^{iep-/-}* group). Data are representative of 3 independent experiments with similar results.

TABLE E1. Antibodies used for IHC analysis: Primary antibodies

Antibody	Vendor	Clone	Isotype
CD3	BD Biosciences	Clone 17A2	IgG _{2b}
CD4	BD Biosciences	Clone GK1.5	IgG _{2b}
CD8	BD Biosciences	Clone 53-6.7	IgG _{2a}
NIMP-R14	Abcam, Cambridge, United Kingdom	NIMP-R14	IgG _{2b}
Anti- <i>E coli</i>	Dako	Rabbit polyclonal	—
TSLP	R&D Systems	Biotinylated goat polyclonal	—
MBP	Provided by Dr James J. Lee, Mayo Clinic, Rochester, Minn	Rat anti-mouse mAb	IgG ₁
MCPT8	BioLegend, San Diego, Calif	Clone TUG8	IgG _{2a}
IL-23p19	Abcam	Rabbit polyclonal	—

TABLE E2. Sequences of the primers used for quantitative RT-PCR analysis

Gene name	Sequence (5' to 3')
<i>HPRT</i> (164 bp)	
F	TGGATACAGGCCAGACTTTG
R	GATTCAACTTGCGCTCATCTTA
<i>CDSN</i> (233 bp)	
F	GCCCATGAGCTTCTGTCACT
R	GCTGTTGGAGCCAGTCTTC
<i>IL4</i> (132 bp)	
F	GGCATTTTGAACGAGGTCAC
R	AAATATGCGAAGCACCTTGG
<i>IL5</i> (117 bp)	
F	AGCACAGTGGTGAAAGAGACCTT
R	TCCAATGCATAGCTGGTGATT
<i>IL13</i> (194 bp)	
F	GGAGCTGAGCAACATCACACA
R	GGTCCTGTAGATGGCATTGCA
<i>IL17A</i> (239 bp)	
F	CCAGGGAGAGCTTCATCTGT
R	ACGTGGAACGGTTGAGGTAG
<i>IL22</i> (166 bp)	
F	CCGAGGAGTCAGTGCTAAGG
R	GCTGATGTGACAGGAGCTGA
<i>IL23p19</i> (213 bp)	
F	AATAATGTGCCCCGTATCCA
R	CTGGAGGAGTTGGCTGAGTC
<i>IL23/12p40</i> (176 bp)	
F	CCTGAAGTGTGAAGCACCAA
R	AGTCCCTTTGGTCCAGTGTG
<i>IL12p35</i> (179 bp)	
F	CATCTTCTCAAAATTCGAGTGACAA
R	TGGGAGTAGACAAGGTACAACCC
<i>TNFA</i> (175 bp)	
F	CATCTTCTCAAAATTCGAGTGACAA
R	TGGGAGTAGACAAGGTACAACCC
<i>IL1B</i> (230 bp)	
F	GCCCATCCTCTGTGACTCAT
R	AGGCCACAGGTATTTTGTCTG
<i>IL1A</i> (220 bp)	
F	TCGGGAGGAGACGACTCTAA
R	TGGCAATAAACAGCTCTGGA
<i>IFNG</i> (238 bp)	
F	AACGCTACACACTGCATCTTGG
R	GACTTCAAAGAGTCTGAGG
<i>PAR2</i> (230 bp)	
F	TCAGAGTAGGGCTCCGAGTT
R	GAACCCCTTTCCAGTGATT
<i>TSLP</i> (194 bp)	
F	AGCTTGTCTCCTGAAAATCGAG
R	AGGTTTGATTGAGGCAGATGTT

F, Forward; *HPRT*, hypoxanthine phosphoribosyltransferase; R, reverse.