HPV16 E7 expression in skin induces TSLP secretion, type 2 ILC infiltration and atopic dermatitis-like lesions

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Atopic dermatitis (AD) is a common pruritic and inflammatory skin disorder with unknown etiology. Most commonly occurring during early childhood, atopic dermatitis is associated with eczematous lesions and lichenification, in which the epidermis becomes hypertrophied resulting in thickening of the skin. In this study, we report an atopic dermatitis-like pathophysiology results in a murine model following the expression of the high-risk human papillomavirus (HPV) 16 oncoprotein E7 in keratinocytes under the keratin 14 promoter. We show that HPV16 E7 expression in the skin is associated with skin thickening, acanthosis and light spongiosis. Locally, HPV16 E7-expressing skin secreted high levels of thymic stromal lymphopoietin (TSLP) and contained increased numbers of innate lymphoid cells (ILCs). High levels of circulating immunoglobulin E were associated with increased susceptibility to skin allergy in a model of cutaneous challenge, and to airway bronchiolar inflammation, enhanced airway goblet cell metaplasia and mucus production in a model of atopic march. Surprisingly, skin pathology occurred independently of T cells and mast cells. Thus, our findings suggest that the expression of a single HPV oncogene in the skin can drive the onset of atopic dermatitis-like pathology through the induction of TSLP and type 2 ILC infiltration.

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Atopic dermatitis (AD) is a common skin disorder characterized by eczematous lesions, which occurs in 15–30% of children worldwide and is often associated with allergic rhinitis and asthma in adulthood. Progression of atopic dermatitis to allergic disease at other epithelial barrier surfaces is known as ‘atopic march’. Disease onset starts soon after birth, often in the first 6 months of life. In ~70% of early-onset atopic dermatitis cases, the disease spontaneously regresses before adolescence. Late-onset atopic dermatitis can affect 2–10% of adults.1 Around 70% of patients with atopic dermatitis (also known as extrinsic atopic dermatitis) have high levels of systemic immunoglobulin E (IgE), whereas patients with non-atopic dermatitis (also known as intrinsic atopic dermatitis) present without IgE.2 Notably, significant increases in the levels of systemic circulating IgE are also associated with the progression of cervical cancer,3 a disease driven by high-risk human papillomavirus (HPV) infections.4 Recently, a correlation was also reported between infection with high-risk HPV and increased thymic stromal lymphopoietin (TSLP) expression by epithelial cells in women with cervical cancer.5

Often, the causative agents of atopic dermatitis and the consequences of skin barrier dysfunction are difficult to decipher. Environmental and genetic elements, skin barrier defects and immunological factors all need to be considered to define this complex disease and its onset. The prevalence of atopic dermatitis in childhood is less significant in rural areas and has increased by two- to threefold in the past 30 years in developed countries; therefore, roles for both the environment and hygiene in the prevalence of atopic dermatitis have been proposed.6–8 Monozygotic twins have a significantly higher concordance rate of atopic dermatitis compared with dizygotic twins, suggesting that an underlying genetic predisposition exists, and genes associated with epidermal or epidermal structural proteins, regulation of epidermal homeostasis or genes associated with psoriasis (another mostly unrelated skin disorder) have been found to be associated with atopic dermatitis.1

There are several characteristic histological and immunological features of atopic dermatitis. The eczematous lesions are defined by the presence of spongiosis, or epidermal intercellular edema, and acanthosis, or thickening of the skin, together with a high dermal infiltration of lymphocytes, dendritic cells, macrophages, mast cells (MCs) and type 2 innate lymphoid cells (ILCs).9–11 In recent years, a role for the cytokine TSLP as a master switch of atopic dermatitis has been described.12 TSLP is highly secreted by epithelial cells in the skin of atopic dermatitis patients and in the lungs of asthmatic patients.13–15 Several mouse models have shown that the intradermal injection of TSLP, or the induction of TSLP overexpression in keratinocytes, is sufficient to trigger atopic dermatitis.16–18 TSLP expression drives a T helper type 2 (Th2)-biased immune response,

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an orientation that is also linked to the initial phase of atopic dermatitis.\textsuperscript{19,20} However, the factors leading to the activation of TSLP release are not clearly defined.

The K14.E7 transgenic mouse expresses the E7 oncoprotein derived from HPV16 under the control of the K14 promoter, thus forcing expression of the E7 oncoprotein in epithelial cells in the skin.\textsuperscript{4,21} In this study, we show that HPV16 E7 expression in the skin leads to secretion of high levels of TSLP and increased number of ILC2 in the skin. E7-associated skin pathology is independent of T cells, MCs or the interleukin-33 (IL-33)/IL-33 receptor axis. Naive K14.E7 mice display high levels of circulating IgE, increased allergic cutaneous hypersensitivity responses to 2,4-dinitrochlorobenzene (DNCB) and enhanced goblet cell metaplasia and mucus production in a model of atopic march. To our knowledge, this is the first report of an association between the expression of an HPV oncoprotein in the skin and the development of atopic dermatitis-like skin lesions.

**RESULTS**

**HPV E7 mice display an atopic dermatitis-like skin phenotype**

K14.E7 transgenic mice (E7 mice) express the HPV16 E7 oncoprotein in epidermal keratinocytes under the control of the keratin 14 promoter. Histological analysis of ear skin sections show an inflammatory skin pathology (Figure 1a) compared with C57 wild-type (wt) controls (Figure 1b), with an overall increase in skin thickness (Figure 1c).\textsuperscript{22} Pathological features of E7 ear skin include diffuse epidermal hyperplasia with hyperkeratosis, light spongiosis within the stratum spinosum and dermal thickening, as indicated in Figure 1a. The results show that E7 mice develop characteristic pathological features of atopic dermatitis similar to those described previously in other mouse models.\textsuperscript{16,18}

**HPV E7 skin expresses high levels of TSLP and contains increased numbers of type 2 ILCs**

Thymic stromal lymphopoietin (TSLP) may promote atopic dermatitis through the induction of Th2 cytokine-mediated inflammation;\textsuperscript{11,12} therefore, we examined the expression of TSLP in E7 skin. As bone marrow dendritic cells have been described to express TSLP in response to lipopolysaccharide stimulation,\textsuperscript{23} we initially confirmed the detection of TSLP in lipopolysaccharide-treated bone marrow dendritic cells (Figure 2a), and subsequently examined TSLP in E7 skin samples. TSLP mRNA was expressed at higher levels in E7 skin and epidermis than in C57 wt mice (Figures 2b and c; \(P<0.05\)). Similarly, TSLP protein levels were higher in E7 ear skin homogenate, and secreted at higher levels following E7 skin explant culture in vitro (Figures 2d and e; \(P<0.0001\)). To further characterize a direct role for E7 in driving TSLP expression, we compared levels of TSLP expression in EL4 cells with EL4 cells modified to express the E7 gene. As shown in Figure 2f, E7 expression significantly increased TSLP expression (\(P=0.0079\)).

Type 2 ILCs were recently reported to be present in atopic dermatitis skin lesions and promote skin inflammation.\textsuperscript{9,10} We observed increased numbers of type 2 ILCs (B220\(^{-}\) TCR\(\beta^{-}\) CD2\(^{-}\) CD11b\(^{-}\) CD90\(^{-}\) CD25\(^{+}\)) as gated in Figures 3a and c) in E7 skin when compared with C57 wt skin (Figures 3b and c; \(P<0.01\)). Altogether, these data show that the expression of HPV16 E7 in mouse skin results in immunological features characteristic of atopic dermatitis.

**Development of E7 skin pathology does not require the alarmin IL-33 or IL-25**

Taken together with TSLP and IL-25, the alarmin IL-33, produced by keratinocytes, has been implicated for its involvement in atopic dermatitis.\textsuperscript{24-26} Its receptor, ST2, is expressed on many immune cell types including regulatory T cells, natural killer T cells and MCs, which are present in increased abundance in E7 skin.\textsuperscript{27,28} Therefore, we analyzed IL-33 and ST2 expression in E7 skin. IL-33 expression was lower in unmanipulated E7 skin compared with C57 skin, yet ST2 expression was similar (Supplementary Figures 1A and B). However, IL-33 mRNA and protein expression were markedly increased in E7 skin following treatment with DNCB (not shown), possibly due to IL-33 induction by necrosis.\textsuperscript{24} Nevertheless, in transgenic E7.ST2 knockout (KO) mice, in which IL-33 signaling is impaired, skin lesions appear similar to E7 skin (Supplementary Figure 1C), also showing increased ear thickness (Supplementary Figure 1D) and acanthosis, suggesting that IL-33 does not contribute to E7-driven skin pathology.\textsuperscript{9} Similar to IL-33 expression, IL-25 expression was also lower in unmanipulated E7 skin compared with C57 skin (Supplementary Figure 1E), suggesting that IL-25 does not contribute to E7-driven skin pathology either.

**Infiltrating MCs or T cells do not contribute to E7 skin pathology**

E7 skin has an extensive dermal lymphoid infiltrate characterized by an increased number of T cells,\textsuperscript{22} MCs\textsuperscript{26} and other innate immune cells.\textsuperscript{27,29} Although atopic dermatitis is described as a Th2-mediated disease, a role for T cells and MCs in driving disease is not clear.\textsuperscript{11,30} Using T-cell-deficient Rag1\(^{-/-}\) mice expressing E7 (E7.Rag), we observed that E7-associated skin thickening, hyperkeratosis and acanthosis are all present in the absence of T cells (Figures 4a and b). Furthermore, the levels of TSLP in E7.Rag skin were similar to E7 skin (Figure 4c). Therefore, the pathology

![Figure 1](https://example.com/figure1.png)  
**Figure 1** K14.E7 transgenic mouse skin demonstrates features of atopic dermatitis. Representative histology of E7 (a) and C57 (b) ear skin stained with H&E (scale bar=20 \(\mu\)m). Hyperkeratosis (k), acanthosis (a) and spongiosis (*) are indicated. (c) Ear thickness measured in naive age-matched E7 (\(n=8\)) and C57 (\(n=8\)) mice using a caliper micrometer (***\(P=0.0002\)). Data are pooled from three independent experiments and analyzed using a Mann-Whitney U-test. Bars represent median values.
observed in E7 skin is T-cell-independent, in line with previous observations.11,30 We previously established that degranulated MCs are found within the cellular infiltrate in E7 skin.28 We hypothesized therefore that MCs might contribute to inflammation in E7 skin. MC-deficient skin expressing E7 (E7, Kit<sup>W-sh/W-sh</sup> mice) produced less TSLP than MC-competent E7 skin, but significantly more than C57 skin (Figure 4d). These data demonstrate that MCs are either a source of TSLP or provide a stimulus to induce its production by other cell types. However, E7, Kit<sup>W-sh/W-sh</sup> mice lacking MCs display similar ear thickness, hyperkeratosis and acanthosis (Figures 4e and f). Therefore, although MCs contribute in part to the level of TSLP in E7 skin<sup>31</sup> and

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**Figure 2** K14.E7 skin expresses and secretes increased levels of TSLP. (a) Positive control for TSLP mRNA expression in lipopolysaccharide (LPS)-treated (n=5) or -untreated (n=5) bone marrow dendritic cells (BMDCs) (**P=0.0079). (b-e) Skin samples were isolated and analyzed for TSLP gene expression in (b) full-thickness ear skin (E7, n=7 and C57, n=6; **P=0.005) and (e) epidermal sheets (E7, n=4 and C57, n=4; **P<0.05). (d) TSLP protein present in ear skin homogenates (E7, n=11 and C57, n=11; ****P<0.0001) or (e) secreted following ear skin explant culture (E7, n=12 and C57, n=12; ****P<0.0001). (f) TSLP mRNA expression in EL4 cells±E7 expression (n=5, **P=0.0079). Data are pooled from two to three independent experiments and analyzed using a Mann–Whitney t-test. Bars represent median values. ND, not detected (value of 0).

**Figure 3** K14.E7 ear skin contains increased number of type 2 ILCs. Ears were collected from E7 (n=5) and C57 (n=5) mice and analyzed by flow cytometry by gating for type 2 ILCs. (a) Representative gating strategy for live type 2 ILCs; TCR<sup>β−</sup> B220<sup>−</sup> CD2<sup>−</sup> CD11b<sup>−</sup> CD90+. (b) Data show the percentage (left) and absolute numbers (right, **P<0.01) of Lin<sup>−</sup> CD90<sup>+</sup> type 2 ILCs in ear skin. (c) Plots show the expression of CD25 in Lin<sup>−</sup> CD90<sup>+</sup> type 2 ILCs. Data are pooled from three independent experiments and analyzed using a Mann–Whitney t-test. Bars represent median values.
can respond to TSLP, they are not the sole producers of TSLP and do not contribute to the observed skin pathology, consistent with their immunosuppressive function in grafted E7 skin.

**HPV E7 mice are hyperallergic**

Extrinsic atopic dermatitis is associated with a high level of circulating IgE. We found that the sera from naive E7 mice contained significantly increased levels of IgE compared with sera from naive C57 wt mice (Figure 5a). To test the predisposition of E7 mice to allergic responses, we examined cutaneous challenge to a chemical sensitizer, DNCB, in a model of delayed-type hypersensitivity. We hypothesized that high levels of TSLP in E7 skin might act as an adjuvant to promote allergic lung inflammation in a model of atopic march.

**DISCUSSION**

Atopic dermatitis is an inflammatory and itchy skin disorder characterized by epidermal proliferation. The targeted expression of TSLP in skin keratinocytes has been shown to result in the appearance of atopic dermatitis-like lesions with clinical, histological and cellular characteristics in keeping with eczematous skin lesions, including the dermal infiltration of high numbers of both innate and adaptive immune cells, a strong Th2-associated bias and increased systemic IgE levels. The presence of TSLP in skin not only acts as a pruritogen, an itch-inducing stimulus, but also aggravates experimental asthma, acting as an adjuvant to promote immune activation. Most likely due to skin barrier defects, viral infections are more common in patients with atopic dermatitis. The most disseminated herpes simplex virus, which triggers eczema herpeticum, the molluscum contagiosum virus triggers eczema molluscatum and the rarest but most life-threatening viral association is due to vaccinia.
virus, which triggers eczema vaccinatum. Moreover, a few viruses have been shown to induce the production of TSLP by various epithelial cells, described as the master switch of atopic dermatitis. 12 Viruses from the respiratory tract, such as the rhinovirus, 38,39 respiratory syncytial virus, 40-43 and influenza A virus, 44,45 as well as vesicular stomatitis virus, 46 hepatitis C virus 47,48 and immunodeficiency viruses (HIV, SIV), 49 have been reported to induce TSLP production in humans, macaques, rats and mice. Although a correlation between infection with high-risk HPVs and increased TSLP expression, 5 whether HPVs induce TSLP production is currently unknown. Our data, using a mouse model where HPV protein is expressed in the skin, provide a clear link between HPV and TSLP.

Keratinocytes express Toll-like receptors (TLR) 1–11 and therefore can respond to various stimuli including viral RNA and DNA. 50 As the HPV16 K14.E7 mouse model only expresses the oncoprotein E7 as a transgene, and not HPV dsDNA, it is highly unlikely that the direct activation of a TLR contributes to the expression of TSLP in this model. Nevertheless, keratinocytes also express many functional cytokine receptors. As shown by Kato et al., 38 TSLP mRNA is upregulated in human airway epithelial cells through the activation of TLR3 by double-stranded RNA but also by Th2 cytokines such as IL-4 and IL-13. In our model, we previously detected Th2 cytokines, 33 which could partially contribute to TSLP expression in the skin. Our current findings also suggest that MCs could contribute to the production of TSLP in the skin, since in their absence in MC-deficient KitWΔ/WΔ mice, TSLP levels slightly but significantly decrease (Figure 4). TSLP production by MCs may be linked to IgE stimulation. 51,52 Finally, skin barrier injury and disturbance of epidermal homeostasis may also be involved in TSLP production. 52

Although the direct contribution of IgE to atopic dermatitis remains uncertain, the beneficial effect of anti-IgE therapy has been demonstrated in a number of clinical studies. 53 The high systemic level of IgE is hypothesized to cause immunological sensitization, leading to increased local inflammation in the skin. 1 Among others, food allergens have been proposed as triggers and can lead to skin allergic reactions in 40% of children with atopic dermatitis. 54,55 Atopic dermatitis patients are also more susceptible to aeroallergens, and skin lesions can appear following their inhalation. 56,57 Most IgE in the sera of atopic dermatitis patients are speciﬁc to self-proteins (autoallergens) 58 and a thin border between atopic dermatitis and autoimmunity has been suggested. Cases of bacterial infection with Staphylococcus aureus are also frequently reported and some IgE against staphylococcal superantigens can be found in the sera of atopic dermatitis patients. 59 We show in this study that mice expressing HPV in the skin have a high level of circulating IgE and are indeed more susceptible to skin and airway allergy.

In the E7 transgenic mouse, HPV16 E7 is expressed under the K14 promoter, driving expression of E7 in epithelial cells, including the skin. We show here that the skin of these mice display all the features

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**Figure 5** K14.E7 mice develop enhanced inflammatory responses. (a) E7 mice (n = 11) and non-transgenic littermates (n = 8) were bled and sera collected and tested by ELISA. Data show all optical density (OD) values (left, *P < 0.05) and total IgE concentration (right, *P < 0.05). (b) Cutaneous hypersensitivity reaction to DNCB. C57 (n = 8) and E7 (n = 14) were sensitized on the shaved abdomen with 5% DNCB at day 0 and challenged at day 0 with 1% DNCB on the dorsal and ventral surface of the ear. The ear thickness was measured over time using a micrometer gauge. (c and d) E7 or C57 mice were sensitized intradermally at day 0 with endotoxin-free OVA (nine mice E7 and C57) or PBS (five mice E7 and C57) and all mice were challenged at days 11, 12, 13 and 14 with OVA intranasally. Representative lung tissue cross-sections stained with H&E (c) to visualize mucus-producing goblet cells and airway inflammation. Scale bar = 200 μm. Data are expressed as median (a) or mean ± s.e.m. (b), pooled from two to three independent experiments and analyzed using a Mann–Whitney t-test. br, Bronchioles; v, vessels.
of atopic dermatitis: (1) E7 skin is eczematous with hyperkeratosis, acanthosis and light spongiosis with a large-cell infiltrate comprising lymphocytes, type 2 ILCs, M\(\text{C}s\), and dendritic cells and other immune cells;\(^{27,28}\) (2) E7 skin produces high levels of TSLP, the master switch of atopic dermatitis;\(^{60}\) (3) E7 sera from naïve animals contain detectable levels of total IgE; (4) E7 mice develop strong allergic reactions and experimental allergic lung inflammation following skin sensitization; (5) the production of Th2-associated cytokines, notably IL-4, IL-5 and IL-13, can be detected in lesional and non-lesional skin during the acute phase of disease;\(^{11,60}\) and has also been shown in E7 skin.\(^{61}\) The E7 mouse model therefore reconciles with numerous features attributed to atopic dermatitis.

The E7 model, however, does not reflect the natural epidemiology for HPV skin infection. Although HPV16 has reportedly been found in a small proportion of non-melanoma skin cancers,\(^{62}\) high-risk HPV16 and HPV18 and low-risk HPV6 and HPV11 are more generally associated with genital tropism, and HPVs such as HPV5, HPV8, HPV38, HPV1 and HPV2 are more generally associated with skin tropism.\(^{63}\) Nevertheless, E7 proteins are conserved/related across HPV subtypes in their protein sequences and in their function \(in vivo\). As an example, E7 from HPV5, HPV8, HPV16 and HPV18 can all bind to the main E7 target, the retinoblastoma protein Rb, and transform cells. However, only HPV16 and 18 can immortalize cells.\(^{64}\) Some HPV subtypes, found at early stages of disease, may contribute to the pathogenesis of psoriasis.\(^{65}\) Similar to psoriasis, we propose that HPV could contribute to atopic dermatitis. Favor et al.\(^{66}\) found HPV DNA in 35.5% of atopic dermatitis lesions. Moreover, two recent studies highlight a direct link between atopic dermatitis and HPV.\(^{67,68}\) Slodkowska et al.\(^{69}\) reported HPV38 infection in a patient with a history of atopic dermatitis since childhood. Similarly, Fernandez et al.\(^{70}\) reported a patient with atopic dermatitis developing epidermodysplasia verruciformis (EV) due to HPV5 infection in skin lesions. It is unclear, however, whether these associations with HPV infection arise as a consequence of underlying atopic dermatitis lesions or whether they may be driving the disease. HPV infections in children could easily be passed from a mother to her child at birth, as most women are infected with various HPV subtypes, including genital HPV such as HPV16. Clinically, this could be tested using a skin swab at birth, and later correlated with the occurrence of atopic dermatitis in childhood.

In conclusion, our results demonstrate that the expression of the HPV16 E7 protein in the skin can drive the production of TSLP and the development of atopic dermatitis-like skin lesions. Our data raise the question as to whether viruses such as HPV could have a role in the onset of atopic dermatitis through the induction of TSLP and type 2 ILC infiltration.

**METHODS**

**Mice**

C57BL/6 mice (C57) were obtained from the Animal Resources Centre (ARC, Perth, WA, Australia). HPV16 K14.E7 transgenic C57BL/6 mice (E7), in which the HPV16 E7 oncoprotein, is expressed under the K14 promoter, \(E7\times Rq1^{-/-}\) KO mice (E7/Rag), ST2 KO mice (ST2) and E7×ST2 KO mice (E7/ST2) were maintained locally at the Princess Alexandra Hospital Biological Research Facility (Brisbane, QLD, Australia) under specific pathogen-free conditions. Genetically, c-kit mutant MC-deficient B6-Ki\(\text{c}\)\(\text{w}^{-/-}\) mice backcrossed with C57BL/6 mice for 14 generations were used as breeding pairs to produce MC-deficient B6-Ki\(\text{c}\)\(\text{w}^{-/-}\) mice and were maintained at the IMVS Animal Facility (Centre for Cancer Biology, Adelaide, SA, Australia).\(^{69}\) B6-Ki\(\text{c}\)\(\text{w}^{-/-}\) mice were crossed with B6-K14.E7 mice to obtain MC-deficient E7 mice deficient in expression of the HPV16 E7 oncoprotein (E7.Ki\(\text{c}\)\(\text{w}^{-/-}\)). All mice were sex matched for all experiments and were used at 10 to 16 weeks of age. Experiments were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia, with approval from the University of Queensland Animal Ethics Committee and SA Pathology/CALHN Animal Ethics Committee, South Australia.

**Cells**

Bone marrow-derived dendritic cells were cultured from C57BL/6 bone marrow in RPMI/20% fetal bovine serum supplemented with granulocyte-macrophage colony-stimulating factor and IL-4 as described.\(^{70}\) After 7 days, the non-adherent cells were harvested and split into two aliquots, one of which was stimulated for 3 h with \(10^{2}\) Eu ml\(^{-1}\) Ultrapure lipopolysaccharide (Invivogen, San Diego, CA, USA) at 37°C. E14 cells, and HPV16 E7-expressing E14 cells generated as described,\(^{71}\) were cultured in RPMI/20% fetal bovine serum. Cell pellets were analyzed by real-time PCR as described below.

**Sera collection and anti-IgE ELISA**

K14.E7 mice and non-transgenic littermates were bled at 14–16 weeks of age. Sera were collected and stored at \(-20^\circ \text{C}\). Total serum IgE was measured by enzyme-linked immunosorbent assay (ELISA) as described.\(^{72}\) Briefly, an ELISA plate (Nunc Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA) was coated with purified anti-mouse IgE (BD Biosciences, Franklin Lakes, NJ, USA; clone R35-72; 1.25 \(\mu\)g ml\(^{-1}\); 100 \(\mu\)l per well) in carbonate buffer (pH 9.2) at 4°C. Wells were washed and blocked with 200 \(\mu\)l of PBS 10% fetal calf serum. Purified mouse IgE: isotype control (BD Biosciences; C38-2; 0.5 mg ml\(^{-1}\)) was used to create a standard curve, starting at 1000 ng ml\(^{-1}\). Hundred microliters per well of sera was incubated for 2 h at room temperature. Biotin rat anti-mouse IgE was used as a detection antibody (BD Biosciences; clone B33-118, 1.25 \(\mu\)g ml\(^{-1}\); 100 \(\mu\)l per well) and detected using a BD OptiEA Streptavidin HRP (BD Biosciences; cat. no. 51-9002813; 100 \(\mu\)l per well) and BD TMB substrate (BD Biosciences; cat. no. 555214; 100 \(\mu\)l per well). The plate was read at 450 nm using a Synergy HT multimode plate reader (BioTek, Winooski, VT, USA).

**Cell isolation from ear skin**

Ears were collected from killed mice. Ear skin was split into halves using forceps and incubated epidermis down in 1.2 mg Dispase II (Roche) at 37°C. After an hour, the epidermal layer was peeled off the dermis. Alternatively, ear skin was torn into small fragments using curved forceps and left for 1 h in 1 mg ml\(^{-1}\) collagenase D and 20 \(\mu\)g ml\(^{-1}\) DNase I (all from Roche). Tissues were passed through a cell strainer and washed in PBS containing 3% fetal bovine serum. Isolated cells were then stained for flow cytometry.

**Flow cytometry**

Cells were analyzed by flow cytometry following staining with live/dead aqua dyes (Live/Dead Fixable Aqua Dead Cell Stain Kit; Invitrogen; I/1000, Life Technologies Australia, Mulgrave, VIC, Australia) and antibodies specific for the following markers: anti-TCRβ (clone H57-597; 5.0 \(\mu\)g ml\(^{-1}\)) from BioLegend, San Diego, CA, USA; anti-CD45RB/B220 (clone RA3-6B2; 1.0 \(\mu\)g ml\(^{-1}\)), anti-CD2 (clone RM2-5; 0.8 \(\mu\)g ml\(^{-1}\)), anti-CD3 (clone PC61; 1.0 \(\mu\)g ml\(^{-1}\)), anti-CD90.2 (clone M1/70; 0.2 \(\mu\)g ml\(^{-1}\)) antibodies from BD Biosciences; anti-CD45.2 (clone 104, 0.5 \(\mu\)g ml\(^{-1}\)) from eBioscience, San Diego, CA, USA. ILC2 were gated as CD45\(^{+}\), CD2\(^{-}\) TCRβ\(^{-}\) B220\(^{-}\) CD11b\(^{-}\), CD90\(^{-}\) and CD25\(^{+}\) cells in a similar manner to that described by Roediger et al.\(^{10}\) Bronchoalveolar lavage fluid cells were additionally labeled using anti-Siglec-F (clone E50-2440; 2.0 \(\mu\)g ml\(^{-1}\)) from BD Biosciences; anti-Gr-1 (clone RB6-8C5; 0.4 \(\mu\)g ml\(^{-1}\)), anti-CD8 (clone 53-6.7, 5.0 \(\mu\)g ml\(^{-1}\)), anti-CD4 (clone GK1.5, 1.0 \(\mu\)g ml\(^{-1}\)), anti-CD3 (clone 2C11, 1.0 \(\mu\)g ml\(^{-1}\)) from BioLegend; and anti-CD11c (clone N418, 2.5 \(\mu\)g ml\(^{-1}\)) from BioLegend.

**Ear skin explant culture and ELISA**

Ears were collected from C57 or E7 mice in complete Dulbecco’s modified Eagle’s medium on ice, split into halves and placed dermis side down in 1 ml complete WEHI-conditioned medium at 37°C. The medium was replaced after 1 h and again after 3 h with 600 \(\mu\)l of fresh conditioned medium to reduce cell-death-related release of cytokines and danger signals. Ear explant supernatants were collected 20 h later and stored at \(-80^\circ\text{C}\) until use or used immediately for TSLP ELISA (BD Biosciences).
Cutaneous hypersensitivity to DNBC

For DNBC-induced contact hypersensitivity, mice were sensitized on the shaved abdomen with 50 μl of 5% (w vol\(^{-1}\)) DNBC (Sigma-Aldrich, Sydney, NSW, Australia) in 100% acetone. Five days after sensitization, mice were challenged with 20 μl of vehicle (100% acetone) alone to the left ear and 20 μl of 1% (w vol\(^{-1}\)) DNBC (in 100% acetone) to the right ear (10 μl to the dorsal and 10 μl to the ventral surface of the ear pinnae). Ear thickness was measured at the indicated intervals after hapten challenge with a micrometer gauge (Ozaki MFG, Tokyo, Japan).

Airway sensitization to OVA

Mice were anesthetized by isoflurane inhalation and sensitized intradermally in the ventral and dorsal surfaces of the ear with 20 μl PBS or 20 μl PBS containing 10 μg endotoxin-free OVA (Invivogen). At day 11, for 4 consecutive days, mice were challenged intranasally with 50 μg endotoxin-free OVA in 50 μl PBS. At day 15, mice were culled and bronchoalveolar lavage was performed as described previously. Cell counts in the bronchoalveolar lavage fluid were performed using a hemocytometer, cell analysis was performed by flow cytometry and cytokine analysis was carried out using ELISA. Lungs were used for histology: H&E and periodic acid-Schiff staining.

Histology on ear skin tissues

Mice were culled by CO\(_2\) inhalation and samples of ear pinnae were fixed in 4% formalin. Samples were coded using a serial number, so the evaluator was not aware of their identity and sent to the histology facility to be embedded in paraffin (ensuring a cross-sectional orientation) and cut as 4- to 6-μm-thick sections. Ear skin sections were then stained with H&E. Images of each coded sample were taken with a ×20 microscope objective (Nikon Brightfield, final magnification, ×200). Field lengths (μm) were determined using NIS-Element software (Nikon, Tokyo, Japan).

mRNA extraction and semiquantitative real-time PCR

At collection, samples were snap frozen in dry ice and stored at −80°C until mRNA extraction. Total RNA extraction (under Trizol; Sigma), retrotranscription (Applied Biosystems) and semiquantitative real-time PCR (Takara) were performed. Total RNA extraction (mRNA extraction). Total RNA extraction (under Trizol; Sigma), retrotranscription (Applied Biosystems) and semiquantitative real-time PCR (Takara) were performed as per the manufacturer’s recommendations and as detailed previously. Primers were designed using IDT (Integrated DNA Technologies): TSLP Fw, 5′-CCTGACTGGAAGTTGAAAGG-3′ and Rev, 5′-AGGCGAGGATAGATTGGAG-3′ (TSLP mRNA NM_021367); RPL23 Fw, 5′-AACCTGGGGGAAAC-3′ and Rev, 5′-TAAAGCAGATGGGCAGCATG-3′ (RPL23 mRNA NM_172086); IL-33 Fw, 5′-CTCCGGCTGGCCAGATATCCA-3′ and Rev, 5′-GTGCAATGTTGGCAGACG-3′ (IL-33 mRNA NM_0016472); IL-25 Fw, 5′-GGACGCTCTGCACTTGTC-3′ and Rev, 5′-GGATTAAGTGTCGTGCCA-3′ (IL-25 mRNA NM_008729).

Statistics

A nonparametric Mann-Whitney t-test was used as indicated for assessment of differences between groups. Differences were considered to be significant when the P-value was <0.05. Prism (GraphPad Software, La Jolla, CA, USA) software was used to prepare graphs and for statistical analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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