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

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Abstract

Atopic dermatitis 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 HPV 16 E7 expression in the skin is associated with skin thickening, acanthosis and light spongiosis. Locally, HPV 16 E7 expressing skin secreted high levels of TSLP and contained increased numbers of ILCs. High levels of circulating IgE 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.

Keywords

Human papillomavirus; Atopic Dermatitis; Thymic Stromal Lymphopoitein; innate lymphoid cells; atopic march

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

Supplementary information

Supplementary information is available at Immunology and Cell biology's website

INTRODUCTION

Atopic dermatitis (AD) is a common skin disorder characterized by eczematous lesions which occurs in 15 to 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 six months of life. In ~70% of early-onset atopic dermatitis cases the disease spontaneously regresses before adolescence. Late-onset atopic dermatitis can affect 2 to 10% of adults [1]. Around 70% of patients with atopic dermatitis (also known as extrinsic atopic dermatitis) have high levels of systemic IgE, while 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 HPVs and increased thymic stromal lymphopoitein (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 2 to 3 fold in the last 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 to dizygotic twins, suggesting an underlying genetic predisposition exists, and genes associated with epithelial 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 immunologic 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 over-expression in keratinocytes, is sufficient to trigger atopic dermatitis [16-18]. TSLP expression drives a Th2-biased immune response, an orientation that is also linked to the initial phase of atopic dermatitis [19, 20]. However, the factors leading to the activation of TSLP release are not clearly defined.

The K14.E7 transgenic mouse expresses the E7 oncogene derived from HPV 16 under the control of the K14 promoter, thus forcing expression of the E7 oncoprotein in epithelial cells in the skin [4, 21]. In this study, we show that HPV16 E7 expression in the skin leads to secretion of high levels of TSLP and increased numbers of ILC2 in the skin. E7-associated skin pathology is independent of T-cells, MCs, or the IL-33/IL-33R axis. Naïve 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 to C57 wild-type (wt) controls (Figure 1B), with an overall increase in skin thickness (Figure 1C) [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 [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 induction of Th2 cytokine-mediated inflammation[11, 12], therefore we examined the expression of TSLP in E7 skin. As bone marrow dendritic cells (BMDC) have been described to express TSLP in response to LPS-stimulation[23], we initially confirmed the detection of TSLP in LPS-treated BMDCs (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 (Figure 2B, 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* (Figure 2D, 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 [9, 10]. We observed increased numbers of type 2 ILCs (B220^{-} TCRβ^{--} CD2^{-} CD11b^{-} CD90^{+} CD25^{+} as gated in Figure 3A and C) in E7 skin when compared to C57 wt skin (Figure 3B and C; $p < 0.01$). Altogether, these data show that the expression of HPV 16 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

Together with TSLP and IL-25, the alarmin IL-33, produced by keratinocytes, has been implicated for its involvement in atopic dermatitis [24-26]. Its receptor, ST2, is expressed on many immune cell types including regulatory T cells, NKT and MCs which are present in increased abundance in E7 skin [27, 28]. Therefore, we analyzed IL-33 and ST2 expression in E7 skin. IL-33 expression was lower in unmanipulated E7 skin compared to C57 skin, yet ST2 expression was similar (Supplemental Figure 1A and B). However IL-33 mRNA and protein expression were dramatically increased in E7 skin following treatment with 2,4-Dinitrochlorobenzene (DNCB, not shown), possibly due to IL-33 induction by necrosis [24]. Nevertheless, in transgenic E7.ST2 KO mice where IL-33 signaling is impaired, skin lesions appear similar to E7 skin (Supplemental Figure 1C), also showing increased ear thickness (Supplemental Figure 1D) and acanthosis, suggesting that IL-33 does not contribute to E7-driven skin pathology [9]. Similar to IL-33 expression, IL-25 expression was also lower in unmanipulated E7 skin compared to C57 skin (Supplemental Figure 1E), suggesting that IL-25 does not contribute to E7-driven skin pathology either.

Infiltrating mast cells 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 [22], MCs[28] and other innate immune cells [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 [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 (Figure 4A, B). Furthermore, levels of TSLP in E7.Rag skin were similar to E7 skin (Figure 4C). Therefore, the pathology 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*$^{\text{W-sh/W-sh}}$ 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*$^{\text{W-sh/W-sh}}$ mice lacking MCs display similar ear thickness, hyperkeratosis and acanthosis (Figure 4E and F). Therefore, although MCs contribute in part to the level of TSLP in E7-skin [31] and can respond to TSLP [32], they are not the sole producers of TSLP and do not contribute to the observed skin pathology [30], consistent with their immunosuppressive function in grafted E7 skin[28].

HPV E7 mice are hyperallergic

Extrinsic atopic dermatitis is associated with a high level of circulating IgE [1, 30]. We found that the sera from naïve 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[33], in a model of delayed-type hypersensitivity[34]. We found that E7 mice generated significantly increased swelling in the skin compared to C57 mice (Figure 5B). In addition, while C57 mice were found to develop inflammation that resolved within 5 days in response to DNCB, E7 mice developed a significantly stronger and sustained response to DNCB that resolved over 10 days (not shown). E7 mice also displayed an early swelling response within the first few hours that was not seen in C57 mice, likely due to the activation of cells from the innate system.

Patients with atopic dermatitis in childhood often develop asthma in a process known as atopic march [35]. Thus, to assess whether E7 mice are similarly prone to the development of bronchial inflammation, we sensitized mice *i.d.* with endotoxin-free OVA in PBS, and 11 days later challenged them *i.n.* with OVA for 4 consecutive days (Figure 5C and D). We hypothesized that high levels of TSLP in E7 skin might act as an adjuvant to promote allergic lung inflammation [17]. Broncho-alveolar lavage fluids (BALFs) from C57 and E7 mice were analyzed for cell content but showed a similar infiltration in terms of siglec-F^+ eosinophils, $CD11b^+$ $Gr1^-$ alveolar macrophages, $CD11b^+$ $Gr1^{high}$ neutrophils, as well as T and B cells (not shown). However, by H&E staining E7 lungs showed infiltration of inflammatory cells surrounding bronchioles and blood vessels (Figure 5C) and enhanced goblet cell metaplasia and mucus production after periodic acid – Schiff staining, while C57 lungs appeared similar to PBS controls (Figure 5D).

In conclusion, these data show that mice expressing HPV E7 in keratinocytes in the skin display atopic dermatitis-like lesions that predispose them to cutaneous allergy and the promotion of 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 [9-11, 30]. The presence of TSLP in skin not only acts as a pruritogen, an itch-inducing stimulus [36], but also aggravates experimental asthma, acting as an adjuvant to promote immune activation [17, 18].

Most likely due to skin barrier defects, viral infections are more common in patients with atopic dermatitis [37]. The most disseminated is 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 (RV) [38, 39], respiratory syncytal virus (RSV) [40-43] and influenza A virus [44, 45], as well as vesicular stomatitis virus (VSV) [46], hepatitis C virus (HCV) [47, 48], and immunodeficiency viruses (HIV, SIV) [49], have been reported to induce TSLP production in humans, macaques, rats, and mice. Although a correlation exists 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 skin, provide a clear link between HPV and TSLP.

Keratinocytes express TLR receptors 1 to 11 and therefore can respond to various stimuli including viral RNA and DNA[50]. Since 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 dsRNA, 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 skin, since in their absence in MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ 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]. Our lab has previously reported that E7 indeed induces pathological skin disorders through Rb-dependent mechanisms[22, 28] that could therefore upregulate TSLP levels in skin.

While 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]. Amongst 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 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 of atopic dermatitis; 1) E7 skin is eczematous with hyperkerathosis, acanthosis and light spongiosis with a large cell infiltrate comprising lymphocytes [22], type 2 ILCs, MCs[28], dendritic cells and other immune cells [27, 29]; 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, HPV18, and low risk HPV6, HPV11, are more generally associated with genital tropism, and HPV’s 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. Favre at 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*. [68] reported HPV38 infection in a patient with a history of atopic dermatitis since childhood. Similarly Fernandez *et al*. [67], 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, since 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 play 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, Australia). HPV16-K14.E7 transgenic C57BL/6 mice (E7), in which the HPV16 E7 oncoprotein is expressed under the K14 promoter, E7 × Rag1$^{-/-}$ 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-*Kit*$^{\text{W-sh/+}}$ mice backcrossed with C57BL/6J mice for 14 generations were used as breeding pairs to produce MC-deficient B6.*Kit*$^{\text{W-sh/W-sh}}$ mice and were maintained at the IMVS Animal Facility (Centre for Cancer Biology, Adelaide, SA, Australia) [69]. B6-*Kit*$^{\text{W-sh/W-sh}}$ mice were crossed with B6-K14.E7 mice to obtain MC-deficient mice expressing the HPV16-E7 oncoprotein (E7. *Kit*$^{\text{W-sh/W-sh}}$ mice). 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 (BMDC) were cultured from C57BL/6 bone marrow in RPMI/20% FBS supplemented with GM-CSF 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 hours with 10^2 Eu/ml Ultrapure LPS (Invivogen) at 37°C. EL4 cells, and HPV16 E7-

expressing EL4 cells generated as described[71], were cultured in RPMI/20% FBS. Cell pellets were analysed 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°C. Total serum IgE was measured by ELISA as described [72]. Briefly, An ELISA plate (Nunc MaxiSorp) was coated with purified anti-mouse IgE (BD Pharmingen clone R35-72, 1.25 μg/ml, 100 μl/well) in carbonate buffer pH9.2 at 4°C. Wells were washed and blocked with 200 μl of PBS 10% FCS. Purified mouse IgE isotype control (BD Pharmingen C38-2, 0.5mg/ml) antibody was used to create a standard curve, starting at 1000ng/ml. 100 μl/well of sera was incubated for 2h at room temperature. Biotin rat anti-mouse IgE was used as a detection antibody (BD Pharmingen clone R35-118, 1.25 μg/ml, 100 μl/well) and detected using a BD OptiEA Streptavidin HRP (BD Biosciences cat. 51-9002813, 100μl/well), and BD TMB substrate (BD Biosciences cat. 555214, 100μl/well). The plate was read at 450nm using a Synergy HT multi-mode plate reader (BioTek).

Cell isolation from ear skin

Ears were collected from euthanized 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 hour in 1 mg/ml collagenase D and 20 μg/ml DNase 1 (all from Roche). Tissues were passed through a cell strainer and washed in PBS containing 3% FBS. 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, 1/1000) and antibodies specific for the following markers: anti-TCRβ (clone H57-597, 5.0 μg/ml) from BioLegend; anti-CD45R/B220 (clone RA3-6B2, 1.0 μg/ml), anti-CD2 (clone RM2-5, 0.8 μg/ml), anti-CD25 (clone PC61, 1.0 μg/ml), anti-CD90.2 (clone 53-2.1, 2.5 μg/ml), and anti-CD11b (clone M1/70, 0.3 μg/ml) antibodies from BD Pharmingen; anti-CD45.2 (clone 104, 0.5 μg/ml) from eBioscience. ILC2 were gated as $CD45^+$, $CD2^-$ $TCRb^-$ $B220^-$ $CD11b^-$, $CD90^+$ and $CD25^+$ cells in a similar manner to that described by Roediger et al.[10]. Bronchoalveolar lavage fluid (BALF) cells were additionally labeled using anti-Siglec F (clone E50-2440, 2.0 μg/ml), from BD Pharmingen; anti-Gr-1 (clone RB6-8C5, 0.4 μg/ml), anti-CD8 (clone 53-6.7, 5.0 μg/ml), anti-CD4 (clone GK1.5, 1.0 μg/ml), anti-CD3 (clone 2C11, 1.0 μg/ml) from eBioscience; and anti-CD11c (clone N418, 2.5 μg/ml) from BioLegend.

Ear skin explant culture and ELISA

Ears were collected from C57 or E7 mice in complete DMEM on ice, split into halves and placed dermis side down in 1 ml complete WEHI-conditioned medium at 37°C. Medium was replaced after 1 h and again after 3 h with 600 μl of fresh conditioned medium to reduce cell-death related release of cytokines and danger signals. Ear explants supernatants were collected 20 h later and stored at −80°C until use or used immediately for TSLP ELISA (BD Biosciences).

Cutaneous hypersensitivity to DNCB

For DNCB induced contact hypersensitivity (CHS), mice were sensitized on the shaved abdomen with 50 µl of 5% (w/vol) DNCB (Sigma) in 100% acetone. Five days after sensitization, mice were challenged with 20 µl of vehicle (100% acetone) alone to the left ear and 20ul of 1% (w/vol) DNCB (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).

Airway sensitization to OVA

Mice were anaesthetized by isoflurane inhalation and sensitized intradermally (*id*) in the ventral and dorsal surfaces of the ear with 20µl PBS or 20µl PBS containing 10µg endotoxin free OVA (Invivogen). At day 11, for 4 consecutive days, mice were challenged intranasally (*i.n.*) with 50µg endotoxin free OVA in 50µl PBS. At day 15, mice were culled and bronchoalveolar lavage (BAL) performed as described previously [73]. Cell counts in BAL fluid were performed using a haemocytometer, cell analysis performed by flow cytometry, and cytokine analysis by ELISA. Lungs were used for histology; H&E and periodic acid-Schiff (PAS) 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 4-6 µm in thickness sections. Ear skin sections were then stained with hematoxylin/eosin. Images of each coded samples were taken with a 20x microscope objective (Nikon Brightfield, final magnification, ×200). Field lengths (µm) were determined using NIS-Element software (Nikon).

mRNA extraction and semi-quantitative Real-Time PCR

At collection, samples were snap-frozen in dry ice and stored at −80oC until mRNA extraction. Total RNA extraction (under Trizol®, Sigma), retro-transcription (Applied BioSystems) and semi-quantitative Real-Time PCR (Takara) were performed as per manufacturer recommendations and as detailed previously[28]. Primers were designed using IDT (Integrated DNA Technologies): TSLP Fw 5′-CCTGACTGGAGATTTGAAAGGG-3′ and Rev 5′-AGCCAGGGATAGGATTGAGAG-3′ (TSLP mRNA NM_021367); RPL32 Fw 5′-AAGCGAAACTGGCGGAAAC-3′ and Rev 5′-TAACCGATGTTGGGCATCAG-3′ (RPL32 mRNA NM_172086); IL-33 Fw 5′-TCCTTGCTTGGCAGTATCCA-3′ and Rev 5′-TGCTCAATGTGTCAACAGACG-3′ (IL-33 mRNA NM_ 001164724); IL-25 Fw 5′-GGAGCTCTGCATCTGTGTC-3′ and Rev 5′-CGATTCAAGTCCCTGTCCAAC-3′ (IL-25 mRNA NM_080729).

Statistics

A non-parametric 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 less than 0.05. Prism (GraphPad Software, La Jolla, CA) software was used to prepare graphs and for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ASB conceived the work, carried out most of the experiments, analyzed and interpreted the data, and wrote the article. NM helped conceived the work, carried out experiments, and helped analyze and interpret the data. LST and DM helped conceive the work and carried out experiments. JA and RJS provided expertise in airway challenge, and contributed to the writing of the article. MAG provided the expertise in mast cell biology, helped conceive the work, contributed to the writing of the article, and approved the final manuscript. IHF helped conceive the work, contributed to experimental design and data analysis, reviewed the article critically, and approved the final manuscript. JWW helped conceive the project, contributed to experimental design and data analysis, contributed to writing of the article, and approved the final manuscript.

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Abbreviations

(HPV)	Human papillomavirus
(AD)	Atopic Dermatitis
(TSLP)	Thymic Stromal Lymphopoitein
(ILC)	innate lymphoid cell
(MC)	mast cell

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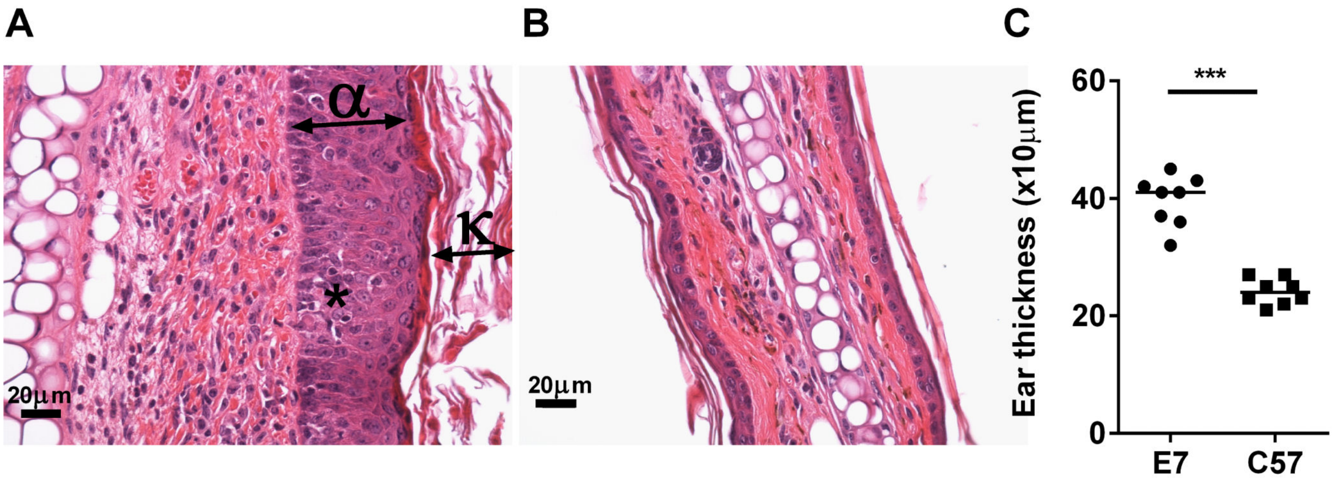

Figure 1. K14.E7 transgenic mouse skin demonstrates features of atopic dermatitis

Representative histology of E7 **(A)** and C57 **(B)** ear skin stained with H&E (scale bar = 20μm). Hyperkeratosis (κ), acanthosis (α), spongiosis (*) are indicated. **C)** Ear skin 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 3 independent experiments and analyzed using a Mann-Whitney t-test. Bars represent median values.

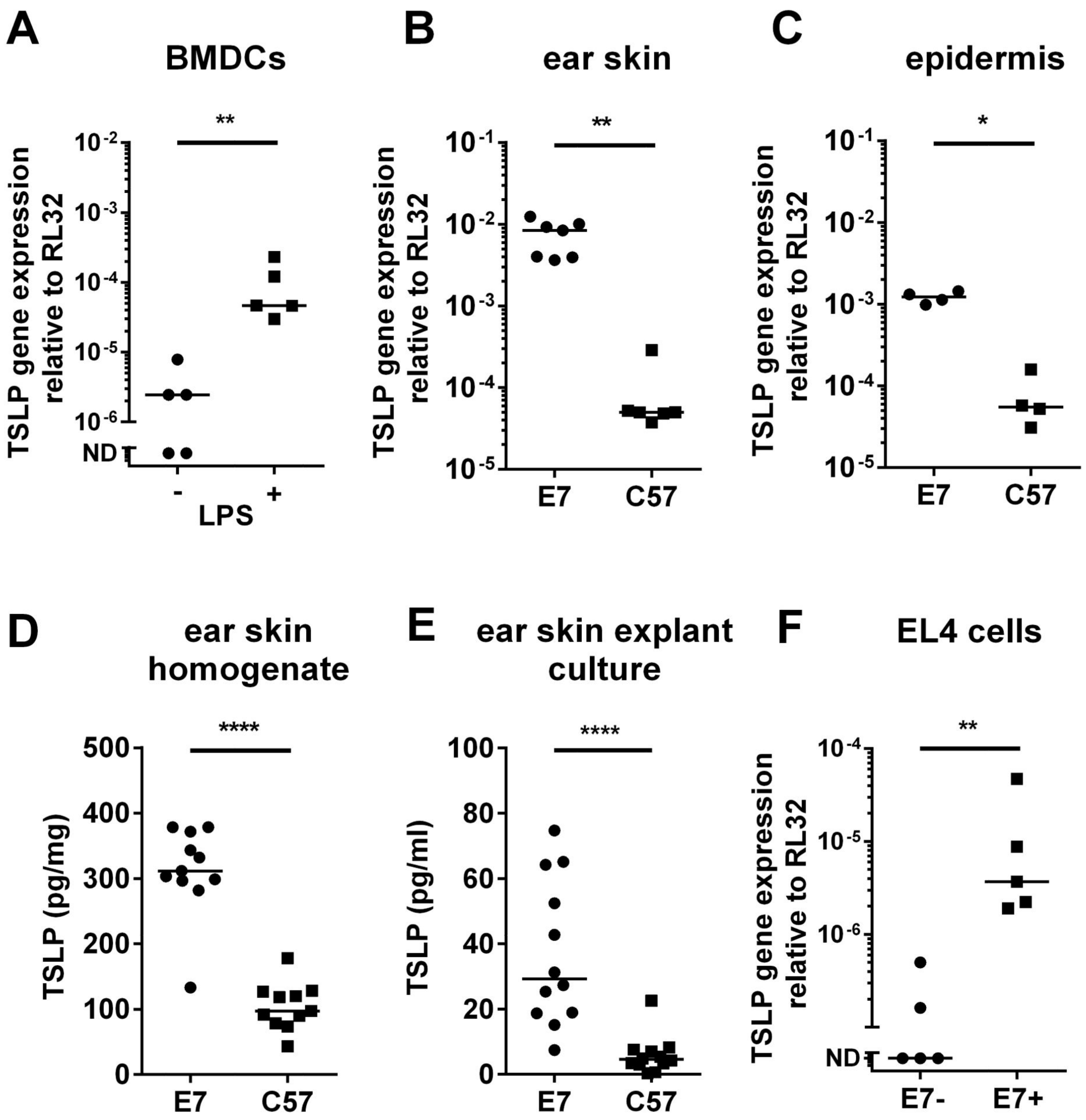

Figure 2. K14.E7-skin expresses and secretes increased levels of TSLP

(A) Positive control for TSLP mRNA expression in LPS-treated (n=5) or untreated (n=5) 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 **(C)** 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 2-3 independent experiments and analyzed using a Mann-Whitney t-test. ND = Not Detected (value of 0). Bars represent median values.

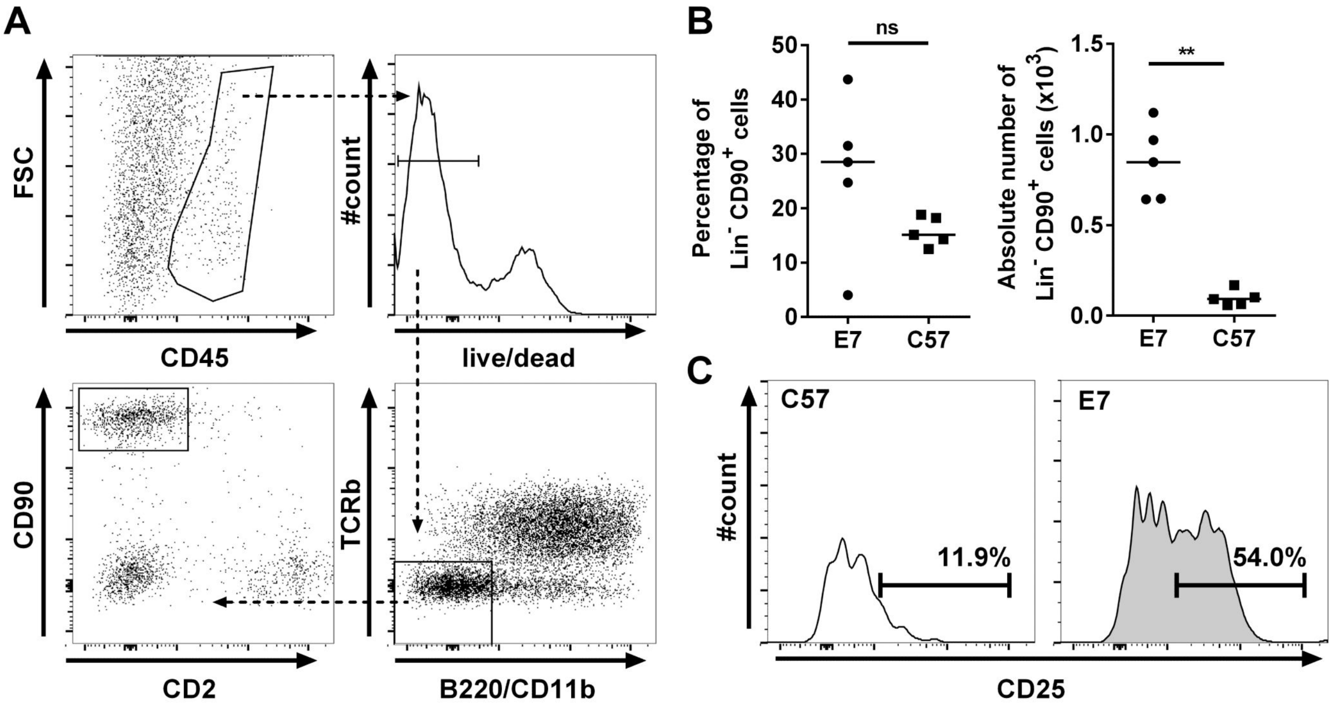

Figure 3. K14.E7 ear skin contains increased numbers of type 2 ILCs

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; TCRβ$^-$ B220$^-$ CD2$^-$ CD11b$^-$ CD90$^+$. **(B)** Data show the percentage (**left**) and absolute numbers (**right**, ** p<0.01) of Lin$^-$ CD90$^+$ type 2 ILCs in ear skin. **(C)** Plots show the expression of CD25 in Lin$^-$ CD90$^+$ type 2 ILCs. Data are pooled from 3 independent experiments and analyzed using a Mann-Whitney t-test. Bars represent median values.

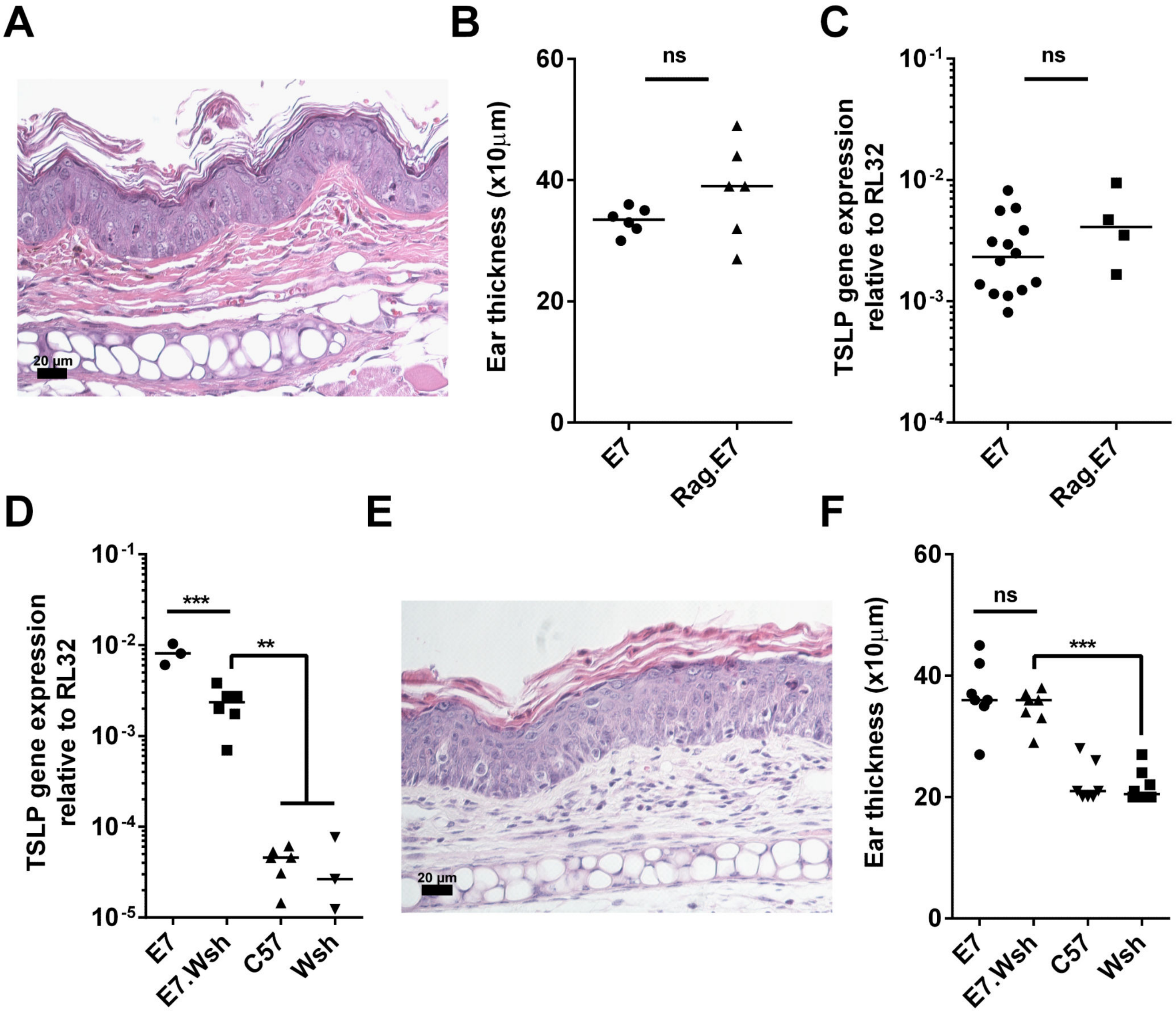

Figure 4. Skin lesions occur independently of T cells and Mast cells

(A-C) Lesions are independent of T cells. **(A)** Representative images E7.Rag ear skin tissue stained with H&E (scale 20 µm). **(B)** Ear skin thickness for E7 (n=6) and E7.Rag (n=6) mice, ns = not significant. **(C)** TSLP gene expression in E7 (n=14) and E7.Rag (n=4) total skin, ns. **(D-G)** Lesions are independent of MCs. **(D)** TSLP gene expression in E7 (n=3), E7.*Kit*$^{W\text{-}sh/W\text{-}sh}$ (E7.Wsh, n=6), C57 (n=5), and *Kit*$^{W\text{-}sh/W\text{-}sh}$ (Wsh, n=3) skin (** $p<0.01$ and *** $p<0.001$). **(E)** Representative image of MC-deficient E7.Wsh ear skin tissue stained with H&E (scale 20 µm). **(F)** Ear skin thickness for K14.E7 (n=7), E7.Wsh (n=7), C57 (n=7) and Wsh (n=7) mice (*** $p<0.005$). Data are pooled from 2-3 independent experiments and analyzed using a Mann-Whitney t-test. Bars represent median values.

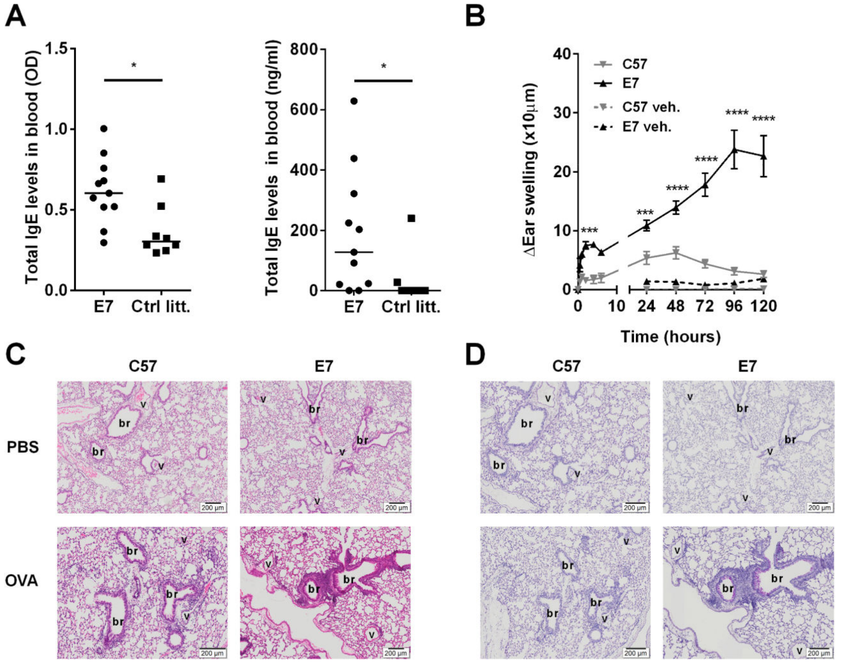

Figure 5. K14.E7 mice develop enhanced inflammatory responses

(A) E7 mice (n=11) and non-transgenic littermates (n=8) were bled and sera collected and tested by ELISA. Data show all 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) mice were sensitized on the shaved abdomen with 5% DNCB at day -5 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. ΔEar swelling is the difference in thickness from baseline on day 0 (*** $p=0.0001$ and **** $p<0.0001$ between E7 and C57 treated groups).**(C-D)** E7 or C57 mice were sensitized *i.d.* at day 0 with endotoxin-free OVA (9 mice E7 and C57) or PBS (5 mice E7 and C57) and all mice were challenged at days 11, 12, 13 and 14 with OVA *i.n.* Representative lung tissue cross sections stained with H&E **(C)** to visualize cells infiltrates and **(D)** periodic acid – Schiff stain to visualize mucus producing goblet cells and airway inflammation; br, bronchioles; v, vessels; Scale bar = 200 μm. Data are expressed as median (**A**) or mean +/− SEM (**B**), pooled from 2-3 independent experiments and analyzed using a Mann-Whitney t-test.