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Cannabinoid 1 receptors in keratinocytes attenuate FITC-induced mouse atopic-like dermatitis

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Abstract

Atopic dermatitis is a chronic inflammatory disease characterized by an impaired epidermal barrier function combined with a chronic Th2-type inflammatory response and an intense pruritus. Here we used an experimental mouse model for Th2-type contact hypersensitivity (CHS) to fluorescein isothiocyanate (FITC) to investigate the potential role of cannabinoid 1 receptors (CB1) in the pathophysiology of mouse atopic-like dermatitis. Mice lacking CB1 receptors globally ($Cnr1^{-/-}$) or specifically in keratinocytes (KC- $Cnr1^{-/-}$) as well as wild-type (WT) control mice were sensitized and challenged with FITC. We examined ear swelling responses, trans-epidermal water loss, Th2 type skin inflammatory responses and serum IgE levels. Both $Cnr1^{-/-}$ and KC- $Cnr1^{-/-}$ showed enhanced CHS responses to FITC and a delayed epidermal barrier repair when compared with WT mice. mRNA levels for IL-4, thymic stromal lymphopoietin (TSLP) and CCL8, as well as eosinophil activity, were significantly increased in inflamed ear tissue of FITC-challenged $Cnr1^{-/-}$ and KC- $Cnr1^{-/-}$ mice. Importantly, CB1 receptor-deficient keratinocytes secreted increased levels of TSLP, a proinflammatory mediator that drives Th2-type skin inflammation in atopic dermatitis, under basal and Th2-type inflammatory conditions. Taken together, our results demonstrate that CB1 receptors in keratinocytes help to maintain epidermal barrier homeostasis and attenuate Th2-type allergic inflammatory responses. Based on our work we propose that enhanced epidermal allergen penetrance cooperates with increased production of TSLP and CCL8 by epidermal keratinocytes for the induction of Type 2 CD4⁺ T helper cells. Our results place keratinocytes at the cross-roads of outside-in and inside-out pathophysiologic mechanisms of atopic dermatitis.

Key words: Cannabinoid receptors, keratinocyte, T cell, skin barrier, atopic dermatitis

Introduction

Atopic dermatitis (AD) is a chronic relapsing form of skin inflammation characterized by a disturbed epidermal barrier function leading to dry, pruritic skin. A predominance of skin memory T helper cells that express a Th2-type cytokine profile favors IgE-mediated sensitization to food and environmental allergens, increased serum IgE levels, and peripheral blood eosinophilia (1,2). Originally, it was thought, that a genetic predisposition to Th2-type allergic inflammatory responses causes an impairment of epidermal barrier function as a secondary event. This view was supported by early genetic linkage analyses, which revealed an association with polymorphisms in genes encoding immune regulatory proteins which control T and B cell phenotypes as well as IgE synthesis such as IL4 and its receptor. However, geneticists also discovered an association with genes encoding epithelial structural proteins which participate in epidermal barrier functions such as filaggrin or claudin. This suggested that a genetically predetermined epidermal barrier defect can also play an important role in the development of atopic dermatitis (3-6).

Investigations in experimental mouse models provided valuable insights how Th2-type allergic inflammatory responses are regulated in the skin and how they relate to epidermal barrier dysfunction. Several allergens have been shown to induce Th2-type skin inflammation and increased serum IgE levels when applied epicutaneously in mice (7-9). An atopic-like pruritic inflammatory skin disease with high serum IgE was also observed in mice where IL-4, the principle cytokine produced by Th2 cells, was overexpressed as a transgene in keratinocytes (10). It was subsequently shown that the flaky-tail mouse, which represents an experimental model for the epidermal barrier repair defect observed in humans with a null mutation in filaggrin, showed reduced inflammatory thresholds to topical irritants and haptens. These mice develop a severe Th2-type allergic skin inflammation with elevated serum IgE levels and a further deterioration in barrier function (11-15).

The endogenous cannabinoid system consisting of cannabinoid (CB) receptors, their lipid ligands (endocannabinoids) and the enzyme system that generates these ligands participates in the regulation of immune responses (16,17). Cannabinoid-based drugs have been shown to inhibit the production of proinflammatory cytokines and chemokines. In some experimental systems cannabinoids also had an immunomodulatory effect and suppressed Th1 cell activity but enhanced Th2 cell activity (18). In an experimental mouse model of contact hypersensitivity (CHS) we showed that mice lacking CB receptors developed increased allergic immune responses to the obligate contact sensitizer 2,4-dinitrofluorobenzene (DNFB) (19). In particular, CB1 receptors on keratinocytes participated in the regulation of allergic skin inflammation through the regulation of pro-inflammatory chemokine secretion (20). CHS responses to DNFB are known to be mediated primarily by CD8+ cytotoxic T cells and associated with the activation of type 1 CD4+ T helper cells (9). The objective of the present study was to analyse whether CB1 receptors also regulate CHS responses to FITC which share many features with atopic dermatitis including a predominance of IL4-producing, type 2 CD4+ T helper cells, eosinophilic infiltrates and high serum IgE (8,9).

Methods

Animals

Mice with a genetic deletion of the *Cnr1* gene encoding the CB1 receptor (*Cnr1*^{-/-}) and mice with a tissue-specific deletion of the *Cnr1* gene in epidermal keratinocytes obtained by crossing K14-Cre and *Cnr1*^{fl/fl} mice were described previously (19,20). All mice were maintained on the C57BL/6 background in our animal facility. All mouse experiments were conducted according to the institutional and national guidelines for the care and use of laboratory animals and were approved by the local government authorities (LANUV NRW, Germany).

Induction of Th2 type CHS responses to FITC

Fluorescein isothiocyanate isomer I (FITC) was purchased from Sigma-Aldrich (Munich, Germany) and diluted 1:1 in acetone:dibutylphthalat immediately before use. Mice were sensitized by painting 400µl of 0.5 % FITC on the shaved abdomen repetitively on day 0, 1, 7 and 8. On day 14 mice were challenged by painting 20µl of 0.5 % FITC on both sides of the ear. Control mice were treated with vehicle only. Ear swelling was measured 24h after challenge. Inflammation was determined by the degree of ear swelling of the hapten exposed ear compared with the untreated ear with a spring loaded caliper.

Measurement of epidermal permeability barrier function

Transepidermal water loss (TEWL) was measured using a Tewameter TM300® (CK electronic GmbH, Köln) over a small area of skin (0,5cm²). Analyses were performed either on ear skin immediately before and 24h after FITC challenge or 3h and 6h after acute epidermal barrier disruption on the back skin with an acetone soaked cotton ball for 2min. Data were recorded as mean water loss (in g/h m²) of 5 measurements per ear and time point in at least 10 mice per group.

Histology and immunofluorescence

Ears were harvested 24h after FITC challenge, fixed in a zinc-based fixative (Dako), embedded in paraffin and stained with Wright-Giemsa stain. The number of metachromatic cells was counted (400X magnification, 5 high power fields (HPF) per ear, n=5mice/group). For examination of degranulation status, tissue mast cells (MC) were counted as either resting MC (granules clustered within a cell) or MC undergoing degranulation (granule contents spread throughout the cell). All histological preparations were analysed using a Leica DMLB microscope with a connected digital camera. For penetration studies, back skin was snap frozen 24h after FITC challenge and immediately embedded in TissueTec. Immunofluorescence was performed with a rabbit polyclonal Ab specific for

keratin 6 (Abcam) followed by an Alexa 594–conjugated secondary Ab. Images were acquired with an upright LSM780 confocal laser-scanning microscope (Carl Zeiss Microimaging) (200X magnification).

Measurement of eosinophil peroxidase

Eosinophil peroxidase (EPO) enzyme activity was measured in inflamed ear skin by first homogenizing fresh tissue in ice-cold buffer (0.05 M Tris±HCl pH 8.0 containing 0.1% Triton X-100) followed by centrifugation and harvesting of supernatants. Supernatants were then mixed with EPO substrate solution consisting of 10 mM O-phenylenediamine in 0.05 M Tris±HCl (pH 8.0) and 4 mM hydrogen peroxide, incubated at RT for 60 min and the reaction stopped through addition of 2M sulfuric acid. Absorbance was measured at 490 nm using a microplate reader. Untreated ears served as negative controls. Data were normalized to the value obtained in control groups.

Measurement of serum IgE

Total IgE-levels were determined in serum samples harvested 24h after FITC challenge by ELISA using standard matched antibody pairs (BD Pharmingen) according to the manufacturer protocols.

RT-PCR

For mRNA analyses fresh ear tissue samples were immediately snap-frozen in liquid nitrogen. Total RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel) and was reverse-transcribed using Superscript III (Invitrogen). Quantitative PCR was performed using 3µg cDNA and Fast SYBR Green Master Mix (ABI). Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Sequences of primers from 5' to 3':

Il1b: GGGCCTCAAAGGAAAGAATC (forward), TTCTTTGGGTATTGCTTGGG (reverse),

Ccl8: CTGGGCCAGATAAGGCTCC (forward), CATGGGGCACTGGATATTGT (reverse),

Ccl2: CTCAGCCAGATGCAGTTAACG (forward), GCTGCTGGTGATCCTCTTGT (reverse),

Ifng: CTCTTCTTGATATCTGGAGG (forward), CCTGATTGTCTTTCAAGACTTC (reverse),

Il4: ACAGGAGAAGGGACGCCAT (forward), GAAGCCCTACAGACGAGCTCA (reverse),

Tslp: GGAGCCTCTTCATCCTGCAA (forward), TTGCTCGAACTTAGCCCCTT (reverse),

Flg: ATGTCCGCTCTCCTGGAAAG (forward), TGGATTCTTCAAGACTGCCTGTA (reverse),

Il6: GCCTTCTCCCTCCTGTGAGTTTGTGTTG (forward), AGGTCCCTGAACCACAGCTGGA (reverse), *Krt10*:

GGGGCAGATCCTCACCTGACA (forward), TCTGGCGCAGGGTCACCTCA (reverse), *Lor*:

AACAGTATCAGTGCCAGAGC (forward), TCTGACTGGTCTGCTGAGAG (reverse), *Ubi*:

AGGCAAGACCATCACCTTGGACG (forward), CCATCACACCCAAGAACAAGCACA (reverse).

Primary keratinocyte culture

Primary epidermal keratinocytes were isolated from the skin of newborn mice according to standard procedures as described previously (20), resuspended in Keratinocyte Growth Medium 2 with 0,05mM Ca^{2+} (PromoCell) and 5×10^6 cells were seeded in 75cm² flasks. The medium was changed every 3 days. Confluent primary keratinocyte cultures were stimulated with 1000U/ml recombinant TNF α , 10ng/ml recombinant IL-4 (PeproTech) or were left unstimulated. Culture supernatants were collected after 24 hours and TSLP levels were determined using a commercial sandwich ELISA (R&D Systems) according to the manufacturer's protocol.

Statistical evaluation

The statistical significance of differences in ear swelling measurements were calculated with the non-parametric Wilcoxon-Mann-Whitney U test. The statistical significance of differences in TSLP protein levels, EPO enzyme activities and qRT-PCR mRNA expression values were analyzed with parametric Student's T-test. P values are given as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

CB1 receptor-deficient mice show enhanced CHS responses to FITC and a delayed epidermal barrier repair.

To examine the role of CB1 receptors for the induction of Th2-type CHS responses to FITC we sensitized and challenged $Cnr1^{-/-}$ and wild-type (WT) mice as shown in Fig. 1a. We observed significantly increased contact allergic ear swellings 24h after challenge with FITC in $Cnr1^{-/-}$ compared to WT mice (Fig. 1a, left). Impaired epidermal barrier functions are a hallmark of atopic dermatitis. Accordingly, we measured strongly increased trans-epidermal water loss in inflamed ear skin of $Cnr1^{-/-}$ mice (Fig. 1a, right). Interestingly, we also observed a delayed repair response following acute epidermal barrier disruption with acetone in $Cnr1^{-/-}$ compared to WT mice (Fig. 1b). Confocal microscopic analyses of skin sections 24 hours after FITC challenge in sensitized mice revealed increased allergen penetration into the skin of $Cnr1^{-/-}$ mice when compared to WT control mice (Fig. 1c). Furthermore, mRNA expression levels coding for the epidermal differentiation proteins filaggrin, involucrin and keratin 10 were significantly reduced in FITC-challenged ear tissues of $Cnr1^{-/-}$ mice when compared to those of WT mice (Fig. 1d). These results not only provided evidence that CB1 receptors attenuate CHS responses to FITC but also indicated that they participate in the homeostatic regulation of epidermal permeability barrier functions.

FITC-challenged CB1 receptor-deficient mice show increased Th2-type allergic skin inflammation with features of atopic dermatitis.

To gain insights in the underlying mechanisms, we further characterized the cytokine expression pattern in FITC-challenged, inflamed ear skin of $Cnr1^{-/-}$ and WT mice. Consistent with previous observations (8,9), we observed higher mRNA levels for IL4 than for IFN γ , which were dramatically increased in $Cnr1^{-/-}$ mice when compared to WT mice (Fig. 2a). We also detected increased mRNA levels of thymic stromal lymphopoietin (TSLP) and CCL8, two proinflammatory mediators that are produced by epidermal keratinocytes and have been associated with Th2-type skin inflammation in atopic dermatitis (Fig. 2b) (21,22). Furthermore, we found enhanced eosinophilic peroxidase (EPO) enzyme activity as an index for eosinophil accumulation in inflamed ear tissue (Fig. 2c) and elevated serum IgE levels (Fig. 2d) in $Cnr1^{-/-}$ compared to WT mice. The number and degranulation status of dermal mast cells in FITC challenged ear tissue of $Cnr1^{-/-}$ WT mice was similar (Fig. 2e). Together, these data support the notion that CB1 receptors participate in the regulation of Th2-type allergic inflammatory responses.

Mice lacking CB1 receptors exclusively in keratinocytes also show increased Th2-type allergic skin inflammatory responses and a delayed epidermal barrier repair.

We previously observed a role of CB1 receptors specifically in keratinocytes for the regulation of Th1-type CHS responses to DNFB (20). To investigate whether CB1 receptors in keratinocytes also regulate Th2-type CHS responses to FITC we repeated our experiments in KC- $Cnr1^{-/-}$ mice and WT litter mate control mice. We also observed increased contact allergic ear swellings in KC- $Cnr1^{-/-}$ mice sensitized and challenged with FITC compared to WT litter mate controls (Fig. 3a, left). This was associated with strongly increased trans-epidermal water loss in inflamed ear skin (Fig. 3a, middle). We also observed a delayed repair response following acute epidermal barrier disruption with acetone (Fig. 3a, right).

Confocal microscopy also revealed increased allergen penetration into the skin of KC-Cnr1^{-/-} mice (Fig. 3b) and qPCR analyses demonstrated significantly reduced mRNA expression levels coding for filaggrin and involucrin in FITC-challenged ear tissues of KC-Cnr1^{-/-} mice when compared to controls (Fig. 3c). Enhanced allergic inflammation in ear tissues of FITC-challenged KC-Cnr1^{-/-} mice was also associated with increased mRNA levels for IL4, IFN γ , TSLP and CCL8 (Fig. 3d,e), as well as with enhanced EPO enzyme activity (Fig. 3f) when compared to WT control mice. Interestingly, KC-Cnr1^{-/-} mice did not show elevated serum IgE levels (Fig. 3g). Again, the number and degranulation status of dermal mast cells in FITC challenged ear tissue of Cnr1^{-/-} WT mice was similar (Fig. 3h). These results demonstrated that CB1 receptors in keratinocytes also attenuate Th2-type allergic inflammatory responses to FITC and suggested that CB1 receptors on another cell type additionally contributes to the deregulated IgE production.

CB1 receptor-deficient keratinocytes secrete increased amounts of TSLP under basal and Th2-type inflammatory conditions.

TSLP, which is mainly produced by epithelial cells including keratinocytes, has recently emerged as a critical regulator of Th2-type inflammatory responses in the skin (23,24). We previously found that CB1 receptor-deficient keratinocytes produce increased levels of CCL8 (20). CCL8 was shown to mediate the recruitment of inflammatory Th2-type CD4⁺ T helper cells (22). We therefore considered the possibility that CB1 receptor-deficient keratinocytes also produce increased levels of TSLP. Indeed, we consistently observed increased levels of TSLP in the supernatants of near confluent primary CB1 receptor-deficient keratinocyte cultures when compared to WT keratinocyte cultures (Fig. 4). These differences became even more pronounced when keratinocytes were stimulated with TNF or IL4, two cytokines abundantly produced by allergen-specific Th2-type CD4⁺ T helper cells (Fig. 4). These results indicated that CB1 receptors in keratinocytes inhibit the production of TSLP, an effect that is particularly prominent under Th2-type inflammatory conditions.

Discussion

In our work we show that CB1 receptors not only participate in the regulation of Th1-type but also in the regulation of Th2-type CHS responses. This demonstrates that CB1 receptors have a more general role in attenuating allergic skin inflammation. We observed strongly increased trans-epidermal water loss in inflamed skin of CB1 receptor-deficient mice. In addition, a physiological skin stress test for the stratum corneum permeability barrier function also revealed a delayed repair response in CB1 receptor-deficient mice compared to WT controls. This was associated with increased penetration of the allergen FITC and reduced expression of epidermal differentiation proteins such as filaggrin, involucrin and keratin 10 in inflamed skin. These findings are consistent with the notion that mice lacking CB1 receptors in keratinocytes show an impaired epidermal barrier response due to an altered regulation of epidermal barrier proteins under inflammatory conditions. Our results agree with the observations reported by Roelandt et al. (25) that mice lacking CB1 receptors show an altered epidermal barrier repair response following tape-stripping which was associated with reduced expression of filaggrin and involucrin in the epidermis. Electron microscopic studies revealed abnormal lipid bilayer structures in CB1 receptor-deficient mice under basal conditions that were enhanced following barrier perturbation, providing first insights in the ultrastructural basis of the observed functional abnormalities. The fact that endogenous cannabinoids influence the expression of epidermal differentiation related genes in human keratinocytes in a CB1 receptor-dependent manner (26) further supports an important role of CB1 receptors in the regulation of epidermal barrier functions.

Our results confirm a specific role for CB1 receptors in keratinocytes, as mice with a specific CB1 receptor deletion in this cell type largely recapitulated the phenotype of global CB1 receptor gene knockout mice. Consistent with our previous observations, we found that inflamed ear tissue of CB1 receptor-deficient mice shows increased levels of CCL8, a chemokine which is produced in large

amounts by epidermal keratinocytes and contributes to the recruitment of Th2-type CD4⁺ T helper cells (19,20,22). In addition, we now discovered that inflamed ear tissue of CB1 receptor-deficient also show increased levels of TSLP and that CB1 receptor-deficient keratinocytes secrete increased amounts of this cytokine under basal and under Th2-type inflammatory conditions. Interestingly, we found that the proinflammatory cytokine TNF strongly induced TSLP production by keratinocytes while IL-4 had no effect. The ability of TNF to promote TSLP production was also described in the human system (27). A recent report in mice also demonstrated that TNF critically affects epidermal barrier function and induces atopic dermatitis like features on epidermal differentiation (28). TSLP is highly expressed in acute and chronic skin lesions of patients with atopic dermatitis (21). It is upregulated in the skin of mice following epidermal barrier disruption (29) and is strongly expressed in several models of Th2-type allergic skin inflammation (23,24,30). Furthermore, keratinocyte-specific overexpression of TSLP results in a Th2 type inflammatory response with features of atopic dermatitis including enhanced expression of IL4, IL5 and IL13, increased infiltration with eosinophils and elevated IgE levels (31). Importantly, genetic or pharmacologic blockade of TSLP activity decreases Th2-type inflammatory responses (23,28). TSLP was also shown to specifically activate Stat5 in dermal dendritic cells, which is required for the induction of Th2 T helper cell responses (32).

Interestingly, it was recently reported that the secretion of TSLP by primary keratinocytes derived from filaggrin-deficient flaky tail mice is also constitutively increased (24). Based on our work we propose that increased production of TSLP by epidermal keratinocytes cooperates with increased allergen penetrance for the induction of Th2-type inflammatory responses in filaggrin-deficient and CB1 receptor-deficient mice. In this way keratinocytes dysfunction connects the “outside-in” and the “inside-out” hypotheses in the pathophysiology of atopic dermatitis (33). This concept is further supported by observations in mice genetically lacking the cathelicidin gene, which encodes the cathelin-related antimicrobial peptide (CRAMP). These mice also show abnormal epidermal barrier

functions, including decreased antimicrobial skin defense and delayed epidermal permeability barrier repair, in combination with enhanced contact allergic sensitization (34-36).

Taken together, our work shows that abnormal epidermal barrier functions and increased production of cytokines and chemokines are mechanistically linked in epidermal keratinocytes, which highlights their important role for the regulation of allergic immune response (2,37). This includes the communication with cutaneous sensory neurons for the induction of pruritus, which has recently been shown to also involve TSLP (38). The highly complex interplay between skin barrier abnormalities, immunologic dysfunction, and chronic itch, which has recently been proposed as a “trinity” in the pathophysiology of atopic dermatitis (39), remains to be fully understood. Because the endocannabinoid system, and in particular CB1 receptors, are also involved in the regulation of peripheral sensory functions in the nervous systems (40), we propose that treatment strategies targeting the endocannabinoid system (18,41) have the potential to simultaneously interfere with all three interrelated pathophysiologic aspects of atopic dermatitis: They may help to restore defective epidermal barrier function, limit allergic inflammation and alleviate itch.

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Authors contributions

E.G. performed all mouse experiments *in vivo* including EPO assays. N.G. and M.C. performed mRNA analyses and ELISA. T.B. performed confocal microscopic analyses. E.G. and T.T. conceived and

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supervised all aspects of the project, designed experiments, interpreted the data and wrote the manuscript.

Conflict of interest statement

The authors declare no conflict of interests.

References

1. Bieber T. Atopic dermatitis. *N Engl J Med* 2008; **358**: 1483-1494.
2. Werfel T. The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J Invest Dermatol* 2009; **129**: 1878-1891.
3. Barnes K C. An update on the genetics of atopic dermatitis: scratching the surface in 2009. *J Allergy Clin Immunol* 2010; **125**: 16-29.
4. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. *Allergy* 2013; **68**: 974-982.
5. Kypriotou M, Huber M, Hohl D. The human epidermal differentiation complex: cornified envelope precursors, S100 proteins and the 'fused genes' family. *Exp Dermatol* 2012; **21**: 643-9.
6. Hansmann B, Ahrens K, Wu Z, Proksch E, et al. Murine filaggrin-2 is involved in epithelial barrier function and down-regulated in metabolically induced skin barrier dysfunction. *Exp Dermatol*. 2012; **21**: 271-6.
7. Spergel J M, Mizoguchi E, Oettgen H *et al*. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest* 1999; **103**: 1103-1111.
8. Dearman R J, Kimber I. Role of CD4(+) T helper 2-type cells in cutaneous inflammatory responses induced by fluorescein isothiocyanate. *Immunology* 2000; **101**: 442-451.

9. Takeshita K, Yamasaki T, Akira S *et al.* Essential role of MHC II-independent CD4+ T cells, IL-4 and STAT6 in contact hypersensitivity induced by fluorescein isothiocyanate in the mouse. *Int Immunol* 2004; **16**: 685-695.
10. Chan L S, Robinson N, Xu L. Expression of interleukin-4 in the epidermis of transgenic mice results in a pruritic inflammatory skin disease: an experimental animal model to study atopic dermatitis. *J Invest Dermatol* 2001; **117**: 977-983.
11. Fallon P G, Sasaki T, Sandilands A *et al.* A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. *Nat Genet* 2009; **41**: 602-608.
12. Oyoshi M K, Murphy G F, Geha R S. Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. *J Allergy Clin Immunol* 2009; **124**: 485-493.
13. Scharschmidt T C, Man M Q, Hatano Y *et al.* Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants and haptens. *J Allergy Clin Immunol* 2009; **124**: 496-506.
14. Moniaga C S, Egawa G, Kawasaki H *et al.* Flaky tail mouse denotes human atopic dermatitis in the steady state and by topical application with *Dermatophagoides pteronyssinus* extract. *Am J Pathol* 2010; **176**: 2385-2393.
15. Kawasaki H, Nagao K, Kubo A *et al.* Altered stratum corneum barrier and enhanced percutaneous immune responses in filaggrin-null mice. *J Allergy Clin Immunol* 2012; **129**: 1538-1546.
16. Piomelli, D. The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci* 2003; **4**: 873-884.
17. Kupczyk P, Reich A, Szepietowski J C. Cannabinoid system in the skin – a possible target for future therapies in dermatology. *Exp Derm* 2009; **18**: 669-679.
18. Klein T W. Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nat Rev Immunol* 2005; **5**: 400-411.

19. Karsak M, Gaffal E, Date R *et al.* Attenuation of allergic contact dermatitis through the endocannabinoid system. *Science* 2007: **316**: 1494-1497.
20. Gaffal E, Cron M, Glodde N *et al.* Cannabinoid 1 receptors in keratinocytes modulate proinflammatory chemokine secretion and attenuate contact allergic inflammation. *J Immunol* 2013: **190**: 4929-4936.
21. Soumelis V, Reche P A, Kanzler H *et al.* Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002: **3**: 673-680.
22. Islam S A, Chang D S, Colvin R A *et al.* Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells. *Nat Immunol* 2011: **12**: 167-177.
23. Larson R P, Zimmerli S C, Comeau M R *et al.* Dibutyl phthalate-induced thymic stromal lymphopoietin is required for Th2 contact hypersensitivity responses. *J Immunol* 2010: **184**: 2974-2984.
24. Moniaga C S, Jeong S K, Egawa G *et al.* Protease activity enhances production of thymic stromal lymphopoietin and basophil accumulation in flaky tail mice. *Am J Pathol* 2013: **182**: 841-851.
25. Roelandt T, Heughebaert C, Bredif S *et al.* Cannabinoid receptors 1 and 2 oppositely regulate epidermal permeability barrier status and differentiation. *Exp Dermatol* 2012: **21**: 688-693.
26. Pasquariello N, Oddi S, Malaponti M *et al.* Regulation of gene transcription and keratinocyte differentiation by anandamide. *Vitam Horm* 2009: **81**: 441-467.
27. Bogiatzi S I, Fernandez I, Bichet J C, *et al.* Proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. *J Immunol* 2007: **178**: 3373-7.
28. Danso M O, van Drongelen V, Mulder A, *et al.* TNF- α and Th2 Cytokines Induce Atopic Dermatitis like Features on Epidermal Differentiation Proteins and Stratum Corneum Lipids in Human Skin Equivalents. *J Invest Dermatol*. 2014: Epub Feb 11. doi: 10.1038/jid.2014.83.

29. Oyoshi M K, Larson R P, Ziegler S F *et al.* Mechanical injury polarizes skin dendritic cells to elicit a T(H)2 response by inducing cutaneous thymic stromal lymphopoietin expression. *J Allergy Clin Immunol* 2010; **126**: 976-984.
30. He R, Oyoshi M K, Garibyan L *et al.* TSLP acts on infiltrating effector T cells to drive allergic skin inflammation. *Proc Natl Acad Sci U S A* 2008; **19**: 11875-11880.
31. Yoo J, Omori M, Gyarmati D *et al.* Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med* 2005; **202**: 541-549.
32. Bell B D, Kitajima M, Larson R P *et al.* The transcription factor STAT5 is critical in dendritic cells for the development of TH2 but not TH1 responses. *Nat Immunol* 2013; **14**: 364-371.
33. Elias P M, Hatano Y, Williams M L. Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J Allergy Clin Immunol* 2008; **121**:1337-1343.
34. Nizet V, Ohtake T, Lauth X *et al.* Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 2001; **414**: 454-457.
35. Di N A, Braff M H, Taylor K R *et al.* Cathelicidin antimicrobial peptides block dendritic cell TLR4 activation and allergic contact sensitization. *J Immunol* 2007; **178**: 1829-1834.
36. Aberg K M, Man M Q, Gallo R L *et al.* Co-regulation and interdependence of the mammalian epidermal permeability and antimicrobial barriers. *J Invest Dermatol* 2008; **128**: 917-925.
37. Albanesi C. Keratinocytes in allergic skin diseases. *Curr Opin Allergy Clin Immunol* 2010; **10**: 452-6.
38. Wilson S R, The L, Batia L M *et al.* The Epithelial Cell-Derived Atopic Dermatitis Cytokine TSLP Activates Neurons to Induce Itch. *Cell* 2013; **155**: 285-295.
39. Kabashima K. New concept of the pathogenesis of atopic dermatitis: interplay among the barrier, allergy, and pruritus as a trinity. *J Dermatol Sci* 2013; **70**: 3-11.
40. Agarwal N, Pacher P, Tegeder I *et al.* Cannabinoids mediate analgesia largely via peripheral type 1 cannabinoid receptors in nociceptors. *Nat Neurosci* 2007; **10**: 870-879.

41. Gaffal E, Cron M, Glodde N *et al.* Anti-inflammatory activity of topical THC in DNFB-mediated mouse allergic contact dermatitis independent of CB1 and CB2 receptors. *Allergy* 2013; **68**: 994-1000.

Figure Legends

Figure 1. Enhanced CHS responses to FITC and a delayed epidermal barrier repair in *Cnr1*^{-/-} mice. (a) Top: Experimental protocol for the induction of CHS responses. *Cnr1*^{-/-} and WT mice were sensitized (S) with FITC on day 0,1,7 and 8. On day 14, mice were challenged (C) on one ear and the increase of ear swelling was measured (M) 24h later. Bottom left: CHS response to FITC 24h after challenge. Shown is the increase in ear swelling (mean±SEM, n=5/group). Experiments were repeated three times with similar results. Bottom right: Transepidermal water loss in inflamed ear tissue before and 24h after FITC challenge (mean±SEM, n=10/group). (b) Top: Experimental protocol for the induction of barrier disruption with acetone. Bottom: Kinetics of epidermal barrier repair responses in *Cnr1*^{-/-} and WT mice. Shown is the percent reduction of the maximal increase in transepidermal water loss 3h and 6h after barrier disruption (mean±SEM, n=10/group). (c) Confocal microscopic images of skin sections showing the allergen FITC (green) and an immunofluorescence stain for keratin 6 (red). E= epidermis, D= dermis (scale bar=100µm). (d) Quantitative RT-PCR analyses for filaggrin (Flg), involucrin (Ivl), keratin 10 (Krt10) and loricrin (Lor). Shown is the mean expression relative to ubiquitin ($2^{-\Delta Ct}$) in ear tissue of five sensitized mice (±SEM) 24h after challenge vs. naïve controls. ** $p<0.01$, *** $p<0.001$.

Figure 2. Elevated mRNA levels for Th2-associated cytokines and increased eosinophil activity in inflamed ear tissue associated with increased serum IgE in FITC challenged *Cnr1*^{-/-} mice. (a,b) qRT-PCR analyses. Shown are the mean mRNA expression levels of the indicated genes relative to ubiquitin ($2^{-\Delta Ct}$) in inflamed ear tissue of *Cnr1*^{-/-} and WT mice 24h after challenge with FITC compared to normal control ear tissue (n=5/group, ±SEM). (c) Eosinophil peroxidase activity in inflamed ear tissue of *Cnr1*^{-/-} and WT mice 24h after FITC challenge compared to normal control ear tissue

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(mean±SEM, n=10/group). (d) Mean levels of total IgE in the serum of FITC-challenged Cnr1^{-/-} and WT mice compared to serum of normal control mice (mean±SEM, n=10/group). $p<0.01$, *** $p<0.001$. (e) Number of resting and degranulated mast cells in inflamed ear tissue of Cnr1^{-/-} and WT mice 24h after FITC challenge (mean±SEM, n=5/group) compared to normal control ear tissue. n.s.= not significant.

Figure 3. Enhanced CHS responses to FITC, impaired epidermal barrier repair, elevated mRNA levels for Th2-associated cytokines and increased eosinophil activity in mice lacking CB1 receptors specifically in keratinocytes. (a) Left: CHS response to FITC 24h after challenge. Shown is the increase in ear swelling (mean±SEM, n=5/group). Experiments were performed three times with similar results. Middle: Transepidermal water loss in inflamed ear tissue before and 24h after FITC challenge (mean±SEM, n=10/group). Right: Kinetics of epidermal barrier repair responses in KC-Cnr1^{-/-} and WT mice. Shown is the percent reduction of the maximal increase in transepidermal water loss 3h and 6h after barrier disruption with acetone (mean±SEM, n=10/group). ** $p<0.01$, *** $p<0.001$. (b) Confocal microscopic image for the allergen FITC (green) and an immunofluorescence stain for keratin 6 (red) (scale bar=100µm). (c) qRT-PCR analyses. Shown are the mean mRNA expression levels of the indicated genes relative to ubiquitin ($2^{-\Delta Ct}$) in inflamed ear tissue of KC-Cnr1^{-/-} and WT mice 24h after challenge compared to normal control ear tissue (n=5/group, ±SEM). (d,e) Mean mRNA expression levels of the indicated genes relative to ubiquitin ($2^{-\Delta Ct}$) in inflamed ear tissue of KC-Cnr1^{-/-} and WT mice 24h after challenge compared to normal control ear tissue (n=5/group, ±SEM). (f) Eosinophil peroxidase activity in inflamed ear tissue of KC-Cnr1^{-/-} and WT mice 24h after FITC challenge compared to normal control ear tissue (mean±SEM, n=10/group). (g) Mean levels of total IgE in the serum of KC-Cnr1^{-/-} and WT mice at this time compared to serum of normal control mice (mean±SEM, n=10/group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (h) Number of resting and degranulated mast cells in inflamed ear tissue of Cnr1^{-/-} and WT mice 24h after FITC challenge (mean±SEM, n=5/group). n.s.= not significant.

Figure 4. Enhanced secretion of TSLP by CB1 receptor-deficient keratinocytes under basal and Th2-type inflammatory conditions. TSLP protein levels determined by ELISA in supernatants of near confluent primary keratinocyte cultures from *Cnr1*^{-/-} and WT mice under basal conditions and 24h after stimulation with recombinant TNF α and IL4. Shown are mean levels of 5 independent cultures $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$.

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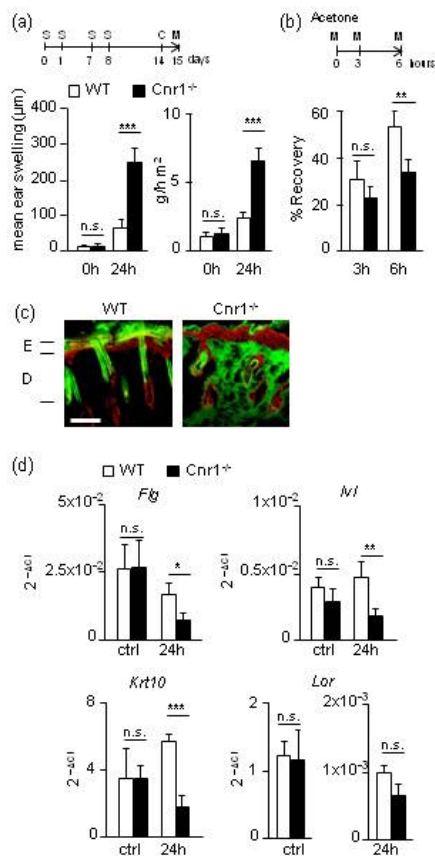


Figure 2 Gaffal et al.

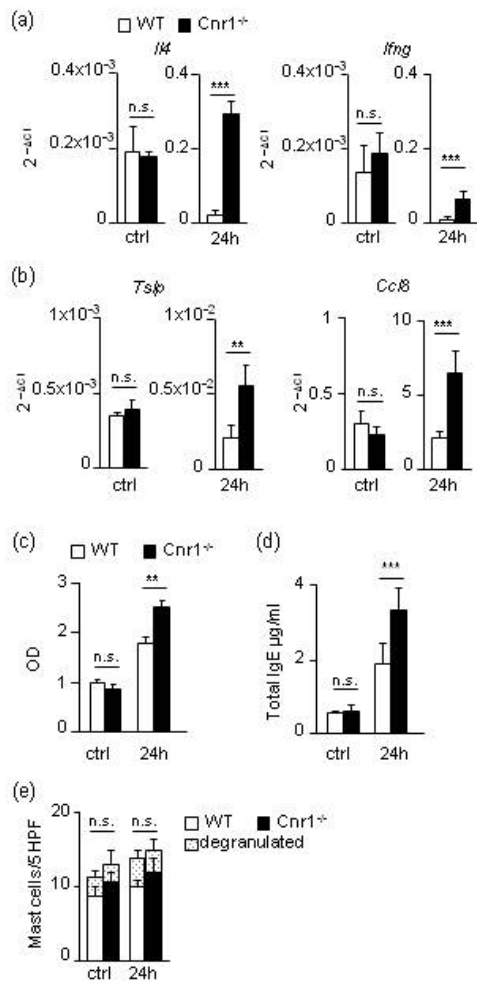


Figure 3 Gaffal et al.

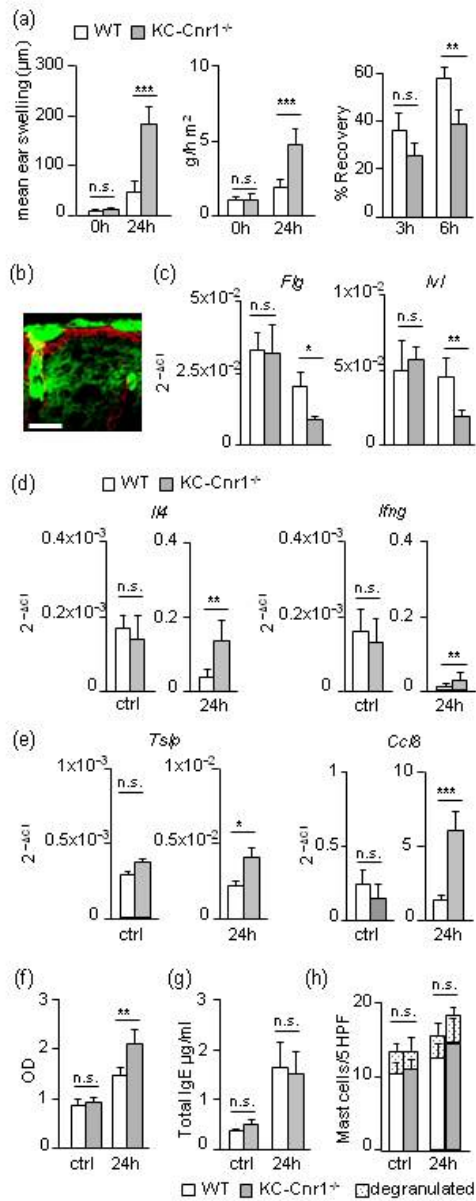


Figure 4 Gaffal et al.

