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Cannabinoid 1 Receptors in Keratinocytes Modulate Proinflammatory Chemokine Secretion and Attenuate Contact Allergic Inflammation

Evelyn Gaffal,[*,1] Mira Cron,[*,1] Nicole Glodde,[*] Tobias Bald,[*] Rohini Kuner,[†] Andreas Zimmer,[‡] Beat Lutz,[§] and Thomas Tüting[*]

Epidermal keratinocytes (KCs) and cannabinoid (CB) receptors both participate in the regulation of inflammatory responses in a mouse model for allergic contact dermatitis, the contact hypersensitivity (CHS) response to the obligate sensitizer 2,4-dinitro-fluorobenzene. In this study, we investigated the cellular and molecular mechanisms how CB1 receptors attenuate CHS responses to 2,4-dinitrofluorobenzene. We used a conditional gene-targeting approach to identify the relative contribution of CB1 receptors on epidermal KCs for the control of CHS responses. To determine the underlying cellular and molecular mechanisms that regulate inflammatory responses in the effector phase of CHS, we performed further investigations on inflamed ear tissue and primary KC cultures using morphologic, molecular, and immunologic methods. Mice with a KC-specific deletion of CB1 receptors developed increased and prolonged CHS responses. These were associated with enhanced reactive epidermal acanthosis and inflammatory KC hyperproliferation in the effector phase of CHS. In vitro, primary cultures of CB1 receptor–deficient KC released increased amounts of CXCL10 and CCL8 after stimulation with IFN-γ compared with controls. In vivo, contact allergic ear tissue of CB1 receptor–deficient KCs showed enhanced expression of CXCL10 and CCL8 compared with controls. Further investigations established CCL8 as a proinflammatory chemokine regulated by CB1 receptors that promotes immune cell recruitment to allergen-challenged skin. Taken together, these results demonstrate that CB1 receptors are functionally expressed by KCs in vivo and help to limit the secretion of proinflammatory chemokines that regulate T cell–dependent inflammation in the effector phase of CHS. *The Journal of Immunology*, 2013, 190: 4929–4936.

Epidermal keratinocytes (KCs) are important modulators of skin inflammation through the secretion of cytokines, chemokines, arachidonic acid metabolites, and antimicrobial peptides. Depending on the circumstances, they can enhance, inhibit, and modulate T cell–dependent inflammatory diseases (1–3). For example, in the development of allergic contact dermatitis, the application of the hapten induces inflammatory signals that amplify the immune response. In the initiation phase, the production of proinflammatory cytokines such as IL-1 and TNF-α promotes dendritic cell (DC) emigration from the skin into the lymph node, where hapten-specific naive T cells are activated (4). In the early elicitation phase, the production of proinflammatory chemokines such as CXCL2, CXCL10, and CCL2 alerts

effector T cells and neutrophils. The subsequent release of IFN-γ by incoming hapten-specific T cells leads to strong KC activation with additional production of proinflammatory cytokines and chemokines, which further increase the inflammatory response (5, 6). However, KCs also produce immunoregulatory cytokines such as TGF-β or IL-10 that eventually help to downregulate inflammatory responses (7).

The endocannabinoid system has been shown to modulate inflammatory responses in various tissues including brain, gut, and skin (8–10). It consists of cannabinoid (CB) receptors, their lipid ligands (endocannabinoids), and the enzyme system that generates these ligands (11). Both exogenous and endogenous CBs can attenuate inflammatory responses through CB2 receptors, which are primarily expressed by immune cells (12). Somewhat unexpectedly, CB1 receptors, which are primarily expressed in certain neurons in the brain (13), where they control pain perception (14), extinction of aversive memories (15), and protection against excitotoxicity (16), also participate in the regulation of inflammatory responses in peripheral tissues. For example, in a mouse model for colonic inflammation, CB1 receptor signaling suppresses peritoneal inflammation and inhibits inflammation-induced gastrointestinal hypermotility (9).

In a mouse model for allergic contact dermatitis, contact hypersensitivity (CHS) to 2,4-dinitrofluorobenzene (DNFB), we observed strongly enhanced inflammatory responses in CB1 receptor knockout (Cnr1^{−/−}) mice (10). In this article, we analyzed the cellular and molecular mechanisms of how CB1 receptors limit inflammatory responses in the skin. Based on experiments with bone marrow chimeric mice that demonstrated a protective role for CB receptors on radio-resistant cells in contact allergic inflammation, we initially considered a role for CB1 receptors on

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Abbreviations used in this article: CB, cannabinoid; CHS, contact hypersensitivity; DC, dendritic cell; DNFB, 2,4-dinitrobenzene; KC, keratinocyte; qRT-PCR, real-time RT-PCR; WT, wild-type.

peripheral sensory neurons and KCs, because both cell types have been shown to functionally express CB1 receptors (17–19). Using a conditional gene-targeting approach, we found that CB1 receptors are functionally important on KCs but not on sensory neurons. To gain further insight in the molecular mechanisms how CB1 receptors on KCs limit the magnitude and duration of CHS, we combined morphologic, immunologic, and molecular investigations of inflamed tissue in vivo with analyses of primary KC cultures in vitro. Collectively, our results show that CB1 receptors on KCs limit the production of proinflammatory chemokines that regulate the recruitment of effector T cells and myeloid immune cells in the effector phase of CHS.

Materials and Methods

Animals

Mice with a genetic deletion of the Cnr gene (Cnr1$^{-/-}$) (15) and mice with a floxed allele of the mouse Cnr1 gene (Cnr1$^{fl/fl}$ mice) on the C57BL/6 background (16) were bred in our animal facility. K14-cre C57BL/6 mice expressing Cre recombinase under the human keratin 14-promoter (20) were obtained from The Jackson Laboratories and crossed with Cnr1$^{fl/fl}$ mice to generate mice with a tissue-specific deletion of the Cnr1 gene in epidermal KCs (KC-Cnr1$^{-/-}$). SNS-Cnr1$^{-/-}$ C57BL/6 mice with a tissue-specific deletion of the Cnr1 gene in nociceptive sensory neurons were described previously (19). Littermate control mice (Cnr1$^{fl/fl}$ mice) were used in all experiments with KC-Cnr1$^{-/-}$ and SNS-Cnr1$^{-/-}$ to exclude nonspecific genotype effects. All 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 (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany).

Contact hypersensitivity

DNFB (Merck) was diluted in acetone/olive oil (4:1). Mice were sensitized by painting 50 μl of 0.2% DNFB on the shaved abdomen on two consecutive days. Controls were treated with 50 μl acetone-olive oil. For elicitation of CHS, ears of mice were painted with 10 μl of 0.3% DNFB. Ear thickness was measured 24, 48, and 72 h after challenge using a spring-loaded caliper (Oditest, Fa. Kroeplin, Germany). Ear swelling was calculated in each mouse as the difference in ear thickness between the unchallenged and challenged ear.

ELISPOT assays

Mice were sensitized with DNFB as described earlier. On day 5, lymphocytes from spleen and draining lymph nodes were isolated. Ag-presenting DCs were cultured from bone marrow precursors in the presence of GM-CSF and IL-4 as previously described (21). Mature DCs were haptenized with 2.5 mM 2,4-dinitrobenzene sulfonic acid (MP Biomedicals), the water-soluble analog of DNFB. Sensitized lymphocytes (1 × 10^5/well) were restimulated with haptenized DCs (1 × 10^4 cells/well) for 48 h in 96-well ELISPOT plates coated with anti-mouse IFN-γ, IL-4, IL-17, or IL-10 capture Abs (BD Biosciences). Secreted cytokines were detected with appropriate biotinylated secondary Abs (BD Biosciences), visualized using the ELISPOT Detection Kit (BD Biosciences), and the number of spots counted with an ELISPOT reader (Bioreader 2000).

Adoptive lymphocyte transfer

For adoptive lymphocyte transfer experiments, groups of wild-type (WT) and Cnr1$^{-/-}$ mice were sensitized with DNFB as described earlier. On day 5, lymphocytes were isolated from spleens and lymph nodes, and transferred i.v. into naive groups of WT or Cnr1$^{-/-}$ mice (5 × 10^7 cells/mouse). On days 1 and 7 after transfer, CHS was elicited and ear swelling was measured 48 h after challenge.

Histology and immunofluorescence

Ear tissue was fixed in a zinc-based fixative (Dako), embedded in paraffin, and routinely stained with H&E. Sections were stained with rat anti-mouse CD45 (BD Biosciences) followed by a biotinylated rabbit anti-rat IgG and development with the LSAB-AP kit (Dako). Immunofluorescence was performed with rabbit polyclonal Abs specific for keratins 1, 5, and 6 (Abcam) followed by an Alexa 488–conjugated secondary Ab. Counterstaining was performed with DAPI. Epidermal thickness was measured in five high-power fields at ×200 magnification (five mice/genotype).

BrdU staining

BrdU was injected i.p. (1 mg/kg) 2 h before harvesting tissue. Sections were treated with 3% H$_2$O$_2$, immersed in 2N HCl followed by neutralization in 0.1 M sodium borate to denature DNA, stained with a biotinylated sheep anti-BrdU polyclonal Ab (Abcam) overnight at 4˚C, followed by streptavidin-HRP and color development with diaminobenzidine (Dako). Counterstaining was performed with hematoxylin.

Primary KC culture

Primary epidermal KCs were isolated from the skin of newborn mice according to standard procedures (22). Cells were resuspended in Keratinocyte Growth Medium 2 with 0.05 mM Ca^{2+} (PromoCell). A total of 5 × 10^6 cells were seeded in 75-cm^2 flasks and cultured until confluent (37˚C, 5% CO$_2$). The medium was changed every 3 d. Confluent primary KC cultures were stimulated with 1000 U/ml rIFN-γ (R&D Systems) or were left untreated. Culture supernatants were collected after 24 h.

RT-PCR

Tissues were harvested and 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 3 μg cDNA and Fast SYBR Green Master Mix (ABI). Relative gene expression was calculated using the 2$^{-\Delta Ct}$ method. Sequences of primers from 5′ to 3′: IL-1b, GGGCCTCAAAGGAAAG-AATC (forward), TTCTTTGGGTATTGCTTGGG (reverse); CCL8, CT-GGGCCAGATAAGGCTCC (forward), CATGGGGCACTGGATATTGT (reverse); CXCL2, CGCTGTCAATGCCTGAAG (forward), GGCGTCA-CACTCAAGCTCT (reverse); CXCL10, GCCGTCATTTTCTGCCTCAT (forward), GCTTCCCTATGGCCCTCATT (reverse); IFN-γ, CTCTTC-TTGGATATCTGGAGG (forward), CCTGATTGTCTTTCAAGACTTC (reverse); Granzyme B, CTCCAATGACATCATGCTGC (forward), TGG-CTTCACATTGACATTGC (reverse); S1008A, GAGTGTCCTCAGTTT-GTGCAG (forward), GCCACACCCACTTTTATCA (reverse); Ubi, AGG-CAAGACCATCACCTTGGACG (forward), CCATCACACCCAAGAA-CAAGCACA (reverse).

ELISA

For protein ELISA, supernatants or tissues were harvested and immediately snap frozen in liquid nitrogen. Frozen tissue was pulverized and protein was isolated using a Protease Inhibitor Cocktail Kit (Thermo Scientific). Protein concentration was determined by a Bradford protein assay. Chemokine levels in supernatants and tissue were then determined using sandwich ELISAs for CXCL2, CXCL10, CCL2, and CCL8 (all from R&D Systems), according to the manufacturer's protocols.

Transwell migration assays

Bone marrow was isolated from femurs and tibias, and resuspended in RPMI 1640 supplemented with fibroblast supernatant. On day 7, cells were starved for 24 h, harvested, and then seeded on polyethylene glycol–coated membrane inserts (8.0-μm pore size) in a 24-well plate at a density of 1 × 10^5 cells/well. DMEM containing 1% FBS and 100 ng/ml Ccl2, Ccl8, or medium conditioned by primary KC cultures was added to the lower chamber of triplicate wells. Twenty-four hours later, cells in the upper chamber were removed by cotton swab. The cells that reached the underside of the membrane were stained with 5 μM CFSE, fixed with 4% paraformaldehyde, and counted in high-power fields using a TE Eclipse microscope (Nikon).

Flow cytometry

Ear tissue was dissociated mechanically before incubation in 1 mg/ml collagenase D +0.02 mg/ml DNAseI (Roche, Germany) in PBS containing 5% FBS (Biochrom, Berlin, Germany) for 30 min at 37˚C. Staining was performed with the fluorochrome-conjugated rat anti-mouse Abs against CD45, CD11b, and Gr-1 (BD Biosciences), and appropriate isotype controls. Gr1$^+$ and CD11b$^+$ cells were analyzed in the CD45$^+$ gate. Fluorescence was measured with a FACSCanto flow cytometer system, and data were analyzed with FlowJo software.

Statistical analyses

Statistically significant differences were calculated with the nonparametric Mann–Whitney U test using SPSS 12 software. In addition, the Bonferroni–Holm method for the correction of the type I error was used. The p values are given as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Conditional gene targeting identifies a functional role for CB1 receptors on KCs in the control of CHS responses to DNFB

In initial experiments, we investigated to what extent the absence of CB1 receptors affects the sensitization of CHS. We exposed groups of five Cnr1$^{-/-}$ and WT mice to the obligate contact sensitizer 1-fluoro-2,4-dinitrobenzene on the shaved abdomen for two consecutive days. Five days later, we isolated lymphocytes from draining lymph nodes and restimulated them in ELISPOT assays with Ag-presenting DCs previously exposed to 2,4-dinitrobenzene sulfonic acid (the water-soluble analog of DNFB). We found similar numbers of T cells capable of producing IFN-γ, IL-4, IL-17, and IL-10 in sensitized Cnr1$^{-/-}$ and WT mice (Fig. 1A), indicating that the absence of CB1 receptors does not alter the induction of Ag-specific T cell responses after exposure to DNFB. Next, we analyzed the role of CB1 receptors in the challenge phase by adoptively transferring lymphocytes from sensitized Cnr1$^{-/-}$ and WT mice donors into naive Cnr1$^{-/-}$ and WT recipients. Cnr1$^{-/-}$ mice injected with donor lymphocytes from sensitized WT mice developed significantly stronger ear-swelling responses to DNFB when compared with recipient WT mice injected with donor lymphocytes from sensitized Cnr1$^{-/-}$ mice (Fig. 1B), demonstrating that the absence of CB1 receptors mainly affects the CHS response in the effector phase. These results were consistent with observations in previous experiments with WT → Cnr1/2$^{-/-}$ and Cnr1/2$^{-/-}$ → WT bone marrow chimeric mice demonstrating that CB receptors on radio-resistant cells in the skin limit contact allergic ear swelling.

Based on our data, we initially hypothesized a role for CB1 receptors on sensory nerve fibers, where they have been shown to control peripheral inflammatory and neuropathic pain using a Cre/LoxP-mediated conditional gene deletion approach (19, 23). We therefore evaluated CHS responses in mice that lack CB1 receptors only on sensory neurons (SNS-Cnr1$^{-/-}$ mice) in direct comparison with littermate controls (Cnr1$^{fl/fl}$) and complete CB1 receptor knockout mice (Cnr1$^{-/-}$ mice). Groups of five animals were sen-

sitized on days 0 and 1 with DNFB on the shaved abdomen and challenged on day 5 and 12 mice with DNFB. We did not find a significant difference in allergic ear swelling in SNS-Cnr1$^{-/-}$ mice when compared with littermate controls (Fig. 1C). As an alternative, we sought to investigate the role of CB1 receptors on KCs, because CB1 receptors are also functionally expressed on these cells (17). Therefore, we generated mice with a tissue-specific knockout of CB1 receptors on KCs (KC-Cnr1$^{-/-}$ mice). These mice developed significantly increased and prolonged allergic ear swelling when compared with littermate controls (Fig. 1D). Contact-allergic ears of KC-Cnr1$^{-/-}$ mice were nearly as thick as those of Cnr1$^{-/-}$ mice, suggesting that the essential CB1 receptor function responsible for the control of allergic inflammation in the skin resides in KCs.

CHS responses in mice lacking CB1 receptors on KCs are characterized by a pronounced reactive epidermal acanthosis associated with an increased inflammatory immune cell infiltrate in the late effector phase

To understand how the absence of CB1 receptors only on KCs in the epidermis can alter the CHS response to DNFB, we comparatively investigated the morphologic changes in the epidermis after the first allergen challenge in Cnr1$^{-/-}$, KC-Cnr1$^{-/-}$, and WT mice. A careful review of H&E-stained sections of contact-allergic ear tissue over time revealed that the reactive epidermal acanthosis was increased and prolonged in both Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice when compared with WT mice (Fig. 2A). Comprehensive measurements of epidermal thickness confirmed a statistically significant increase at 96 and 168 h (Fig. 2B). We next examined the proliferative activity of KCs, which represents a key mechanism to restore epidermal integrity by replenishing cells lost because of T cell–mediated apoptosis. BrdU incorporation assays revealed a significantly increased proliferation of epidermal KCs 48 and 96 h after allergen exposure in both Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice when compared with WT mice (Fig. 2C, 2D). Thus, CB1 receptor–deficient KCs show hyperproliferation leading to pronounced reactive epidermal acanthosis in the late effector phase of CHS.
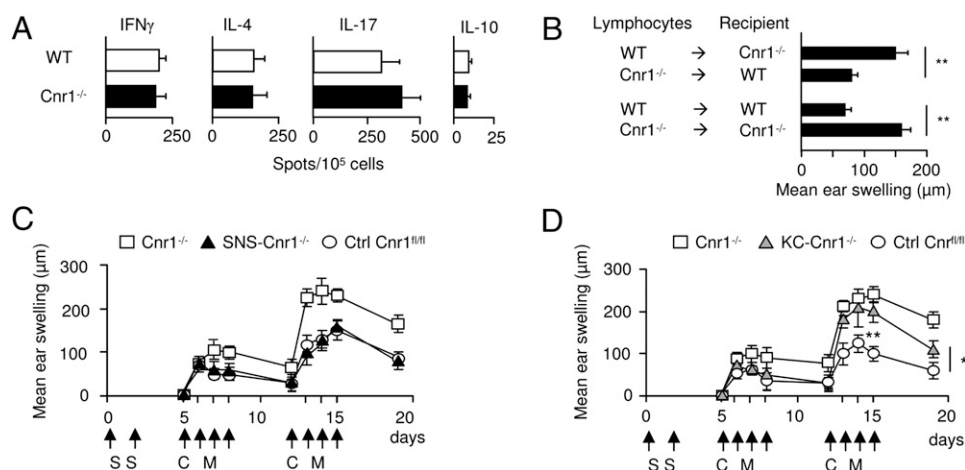
FIGURE 1. Enhanced CHS responses to DNFB in mice that lack CB1 receptors on KCs. (**A**) Ag-specific restimulation of sensitized lymphocytes in ELISPOT assays for IFN-γ, IL-4, IL-17, and IL-10. Results are expressed as spots per 10^5 cells (*n* = 5/group). Similar results were obtained in three independent experiments. (**B**) Lymphocytes isolated from sensitized WT and Cnr1$^{-/-}$ mice were adoptively transferred into WT and Cnr1$^{-/-}$ recipients. One and seven days after transfer, mice were challenged with DNFB. Shown is the ear swelling on day 9 after adoptive transfer of sensitized lymphocytes (*n* = 5/group ± SEM). Similar results were obtained in three independent experiments. (**C**) Groups of mice lacking CB1 receptors on sensory neurons (SNS-Cnr1$^{-/-}$, *n* = 10/group ± SEM) and (**D**) on KCs (KC-Cnr1$^{-/-}$, *n* = 10/group ± SEM) were sensitized (S) with 0.2% DNFB on days 0 and 1. On day 5 after sensitization, mice were challenged (C) on the ears with 0.3% DNFB and the increase in ear thickness was measured (M) 24, 48, and 96 h later. A second challenge was performed on day 12. Shown is the mean ear swelling ± SEM. Experiments were repeated two times with a total of 30 mice/group. *$p < 0.05$, **$p < 0.01$.
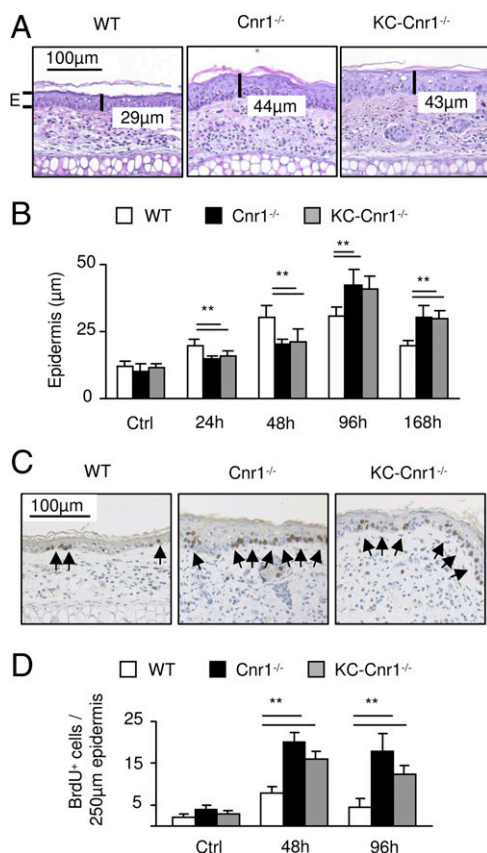
FIGURE 2. Increased reactive epidermal acanthosis and KC hyperproliferation in the late effector phase of CHS in $Cnr1^{-/-}$ and $KC-Cnr1^{-/-}$ mice. (**A**) Representative H&E-stained sections of contact-allergic ear tissue in WT, $Cnr1^{-/-}$, and $KC-Cnr1^{-/-}$ animals 96 h after the first challenge. Vertical bars indicate thickness of epidermis (E) (original magnification ×200). (**B**) Epidermal thickness over time ($n = 5$/group, ± SEM). (**C**) Representative BrdU-stained sections of contact-allergic ear tissue in WT, $Cnr1^{-/-}$, and $KC-Cnr1^{-/-}$ animals 96 h after the first challenge. Arrows indicate BrdU-positive KCs. Original magnification ×200. (**D**) Number of BrdU+ cells per 250-μm epidermis over time ($n = 5$/group, ± SEM). **$p < 0.01$.

Because CB1 receptors have been shown to affect the differentiation of human KCs in vitro (17, 18), we also studied the keratin expression pattern in contact-allergic ear tissue by immunofluorescence staining. Ninety-six hours after the first DNFB challenge, we observed expression of keratin 5 in the undifferentiated basal epidermis, expression of keratin 1 in the differentiated suprabasal epidermis, and prominent expression of keratin 6 in all epidermal layers of $Cnr1^{-/-}$ and $KC-Cnr1^{-/-}$ mice (Fig. 3A). We hypothesized that the expression of keratin 6 may be driven by the proinflammatory cytokine IL-1β, a potent KC activator that is known to be strongly induced during contact allergic inflammation (24, 25). Real-time RT-PCR (qRT-PCR) analyses demonstrated significantly increased expression of IL-1β in contact-allergic ear tissue of $Cnr1^{-/-}$ and $KC-Cnr1^{-/-}$ mice compared with WT mice after the first DNFB challenge (Fig. 3B, *left panel*). This observation was confirmed by measurements of the chemokine CXCL2 (the mouse homolog of human IL-8/CXCL8) and of the proinflammatory mediator S100A8, which are also known to be strongly upregulated in contact allergic inflammation (Fig. 3B, *middle* and *right panels*). Taken together, the data support the notion that mice that lack CB1 receptors on KCs show a persistent inflammatory response in the late effector phase of CHS.
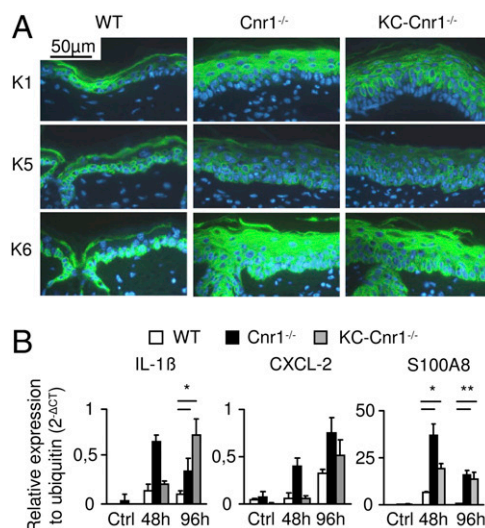

FIGURE 3. Inflammatory keratin expression pattern in the late effector phase of CHS in $Cnr1^{-/-}$ and $KC-Cnr1^{-/-}$ mice. (**A**) Shown are representative immunofluorescence stains for keratins 1, 5, and 6 of contact-allergic ear skin harvested 96 h after the first allergen challenge from WT, $Cnr1^{-/-}$, and $KC-Cnr1^{-/-}$ mice (original magnification ×400). (**B**) qRT-PCR analyses for IL-1β, CXCL-2, and S1008A. Shown is the mean expression relative to ubiquitin ($2^{-\Delta CT}$) in ear tissue of three mice (± SEM) 48 and 96 h after challenge versus naive controls. *$p < 0.05$, **$p < 0.01$.

Myeloid immune cells that are known to infiltrate contact-allergic ear tissue during the effector phase strongly express the cytokine IL-1β and the chemokine CXCL2. We therefore considered the possibility that the increased expression of these proinflammatory mediators in contact-allergic ear tissue of $Cnr1^{-/-}$ and $KC-Cnr1^{-/-}$ mice reflects an increased infiltration with these cells. Immunohistologic analyses indeed revealed enhanced infiltration with CD45+ immune cells 96 h after the first challenge in contact-allergic ears of $Cnr1^{-/-}$ and $KC-Cnr1^{-/-}$ mice when compared with controls (Fig. 4A). Detailed flow cytometric quantifications confirmed significantly larger numbers of infiltrating CD45+ immune cells at this time point in $Cnr1^{-/-}$ and $KC-Cnr1^{-/-}$ mice (Fig. 4B). These cells could be quantified as Gr1+/CD11b+ cells in the CD45+ gate by flow cytometry (Fig. 4C). Thus, mice that lack CB1 receptors on KCs show a strongly increased inflammatory infiltrate with myeloid cells in the late effector phase of the CHS response.

CB1 receptor–deficient KCs proliferate and differentiate normally but secrete increased amounts of CXCL10 and CCL8 after stimulation with IFN-γ

Our observations suggested that mice that lack CB1 receptors on KCs fail to resolve the contact-allergic inflammatory response induced by repetitive application of the obligate contact sensitizer DNFB. To further investigate the mechanistic basis of this phenomenon, we carefully compared primary in vitro cultures of CB1 receptor–deficient and WT KCs. Initially, we hypothesized that CB1 receptor–deficient KCs show increased proliferation and impaired differentiation. However, in a series of >15 primary cultures of epidermal cells isolated from newborn skin of $Cnr1^{-/-}$ or $KC-Cnr1^{-/-}$ mice, we routinely expanded similar numbers of KCs when compared with cultures from WT or littermate control mice (Fig. 5A). All cultured KCs were morphologically indistinguishable and showed the same differentiation pattern with strong expression of the keratins 5 and 6, as well as patchy expression of the keratin 1 (Fig. 5B). An increase of calcium levels in the culture
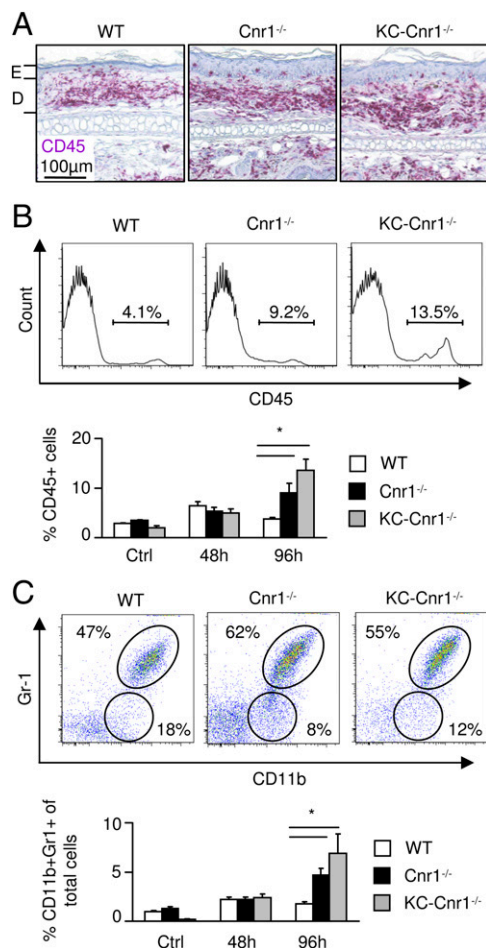
FIGURE 4. Increased myeloid immune cell infiltration in the late effector phase of CHS in Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice. (**A**) Mice were sensitized with DNFB on days 0 and +1, and challenged on both ears with 0.3% DNFB on day 5. Ear specimens were taken 96 h after challenge and processed for histologic analyses. Representative immunohistochemical stains for CD45 (red) 96 h after challenge are shown. D, dermis; E, epidermis. (**B**) Ear specimens were taken 96 h after challenge, digested to prepare single-cell suspensions of the tissue, and stained with fluorescent Abs to identify infiltrating immune cells. (*Top panel*) Flow cytometric histograms quantifying CD45$^+$ immune cells in inflamed ear tissue 96 h after challenge. (*Bottom panel*) Time-course analyses of infiltrating CD45$^+$ cells ($n = 10$/group, ± SEM). (**C**) (*Top panel*) Flow cytometric dot plots for Gr1 and CD11b on CD45$^+$ immune cells in inflamed ear tissue 96 h after challenge. (*Bottom panel*) Time-course analyses of infiltrating Gr1$^+$/CD11b$^+$ immune cells ($n = 10$/group, ± SEM). *$p < 0.05$.

medium led to typical morphologic changes in both CB1 receptor–deficient and WT KCs, with individual cells becoming larger and losing their distinct phase-dense outlines, indicating initiation of a terminal differentiation program. Thus, CB1 receptor–deficient KCs proliferated normally and reached a similar state of activation and differentiation in vitro when compared with WT KCs.

In our previous work, we identified the chemokine CCL8 as one of the most significant differentially expressed genes in contact-allergic ear tissue of Cnr1/Cnr2 double-knockout mice compared with WT mice (10). We therefore considered the possibility that the absence of CB1 receptors on KCs might affect the chemokine production profile of these cells. We first measured the secretion of CXCL2 and CCL2, which are known to be constitutively produced by KCs in the culture supernatant. In addition, we determined the secretion of CXCL10 and CCL8 after stimu-
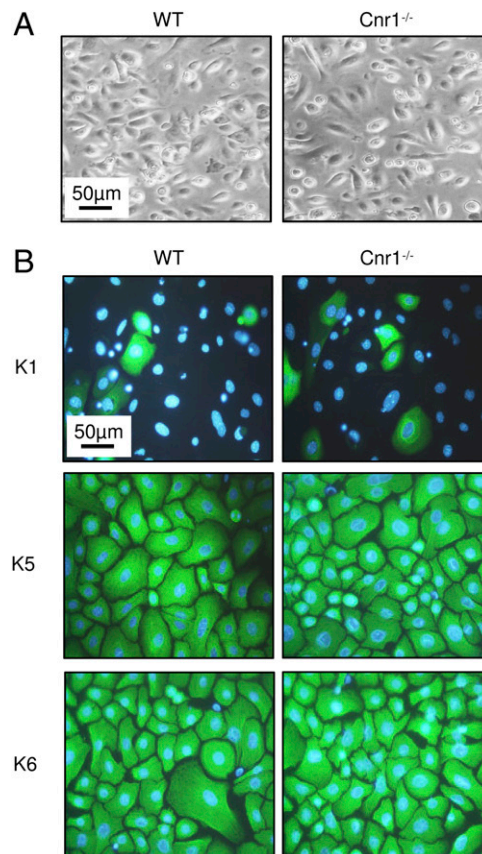

FIGURE 5. Normal differentiation of CB1 receptor–deficient KCs in vitro. (**A**) Cells were isolated from the epidermis of newborn mouse pups and grown to confluence in low-calcium medium. Shown are representative phase-contrast images from WT and Cnr1$^{-/-}$ KCs after 7 d of culture (original magnification ×200). (**B**) Corresponding immunofluorescence images of primary KCs stained for keratins 1, 5, and 6 (blue represents DAPI, green represents keratin). Original magnification in (A) and (B) ×200.

lation with IFN-γ, TNF-α, IL-10, or IL-17, cytokines abundantly produced by hapten-specific effector T cells and other immune cells in the elicitation phase of CHS in vivo. We consistently found slightly lower levels of CXCL2 and slightly higher levels of CCL2 in the supernatants of near-confluent primary CB1 receptor–deficient KC cultures when compared with WT cultures, whereas CXCL10 and CCL8 were not detectable (Fig. 6A, 6B). After stimulation with IFN-γ, but not with TNF-α, IL-10, or IL-17, we consistently observed increased levels of CXCL10 and CCL8 in CB1 receptor–deficient KC cultures when compared with WT cultures (Fig. 6C, 6D). Collectively, these results indicated that the absence of CB1 receptors on KCs inhibits chemokines produced under inflammatory conditions in the presence of IFN-γ.

CHS responses in mice that lack CB1 receptors on KCs show increased expression levels of the IFN-γ–inducible chemokines CXCL10 and CCL8 in vivo

Based on the in vitro data, we hypothesized that CB1 receptor–deficient KCs also produce increased levels of CXCL10 and CCL8 in response to IFN-γ secreted by hapten-specific T cells in the effector phase of CHS in vivo. qRT-PCR analyses indeed showed significantly elevated expression levels for CXCL10 and CCL8 in contact-allergic ear tissue samples from Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice compared with WT mice (Fig. 7A, 7B). Interestingly, we also found increased expression levels for IFN-γ, as well as the cytotoxic effector molecule granzyme B (Fig. 7C, 7D). This
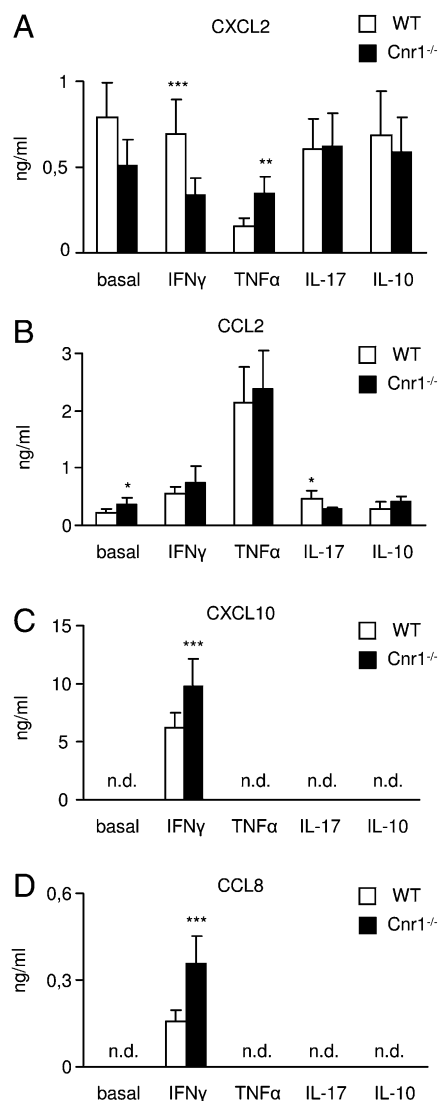
FIGURE 6. Enhanced IFN-γ–dependent secretion of CXCL10 and CCL8 by CB1 receptor–deficient KCs in vitro. Confluent primary WT and Cnr1$^{-/-}$ KC cultures ($n = 15$) were stimulated with rIFN-γ (1000 U/ml), rTNF-α (1000 U/ml), rIL-17 (50 ng/ml), rIL-10 (50 ng/ml), or were left untreated. Shown are protein levels of (**A**) CXCL2, (**B**) CCL2, (**C**) CXCL10, and (**D**) CCL8 measured by ELISA in the cell culture medium collected after 24 h (± SEM). *$p < 0.1$, **$p < 0.05$, ***$p < 0.01$. n.d., Not detected.

FIGURE 7. Elevated expression levels of CXCL10, CCL8, IFN-γ, and Granzyme B (GrB) in contact-allergic ear tissue of Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice in vivo. (**A–D**) qRT-PCR analyses for CXCL10, CCL8, IFN-γ, and GrB. Shown is the mean expression relative to ubiquitin ($2^{-\Delta CT}$) in ear tissue ($n = 3$/group, ± SEM) 48 and 96 h after challenge versus naive controls. *$p < 0.1$, **$p < 0.05$.

suggests that the increased amount of IFN-γ–induced CXCL10 released by CB1 receptor–deficient KCs enhances the recruitment of CXCR3-expressing effector T cells, and thereby amplifies the CHS response.

To further investigate the role of CCL8, we also determined the protein levels in tissue lysates by ELISA. In agreement with the qRT-PCR results, we detected significantly increased amounts of CCL8 protein in contact-allergic ears of Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice compared with WT mice 48 and 96 h after the first DNFB challenge (Fig. 8A). CCL8 was originally identified as a monocyte chemotactic protein (26). In subsequent experiments, we could confirm that CCL8 can induce the migration of bone marrow–derived macrophages as efficiently as the positive reference control CCL2 (Fig. 8B). Thus, the increased amounts of CCL8 additionally drive the inflammatory response by recruiting CCR2-expressing myeloid immune cells. These results are supported by

experiments that showed increased migration of macrophages toward culture medium conditioned by IFN-γ–activated CB1$^{-/-}$ KCs when compared with medium conditioned by IFN-γ–activated WT KCs (Fig. 8C).

Discussion

In our work, we established for the first time, to our knowledge, an in vivo function of CB1 receptors on epidermal KCs in a mouse model for contact-allergic inflammation using a conditional gene targeting approach. Mice that lack CB1 receptors specifically in KCs show strongly increased and persistent ear-swelling responses to the obligate contact sensitizer DNFB that closely resembled those in mice with global deletion of CB1 receptors in terms of magnitude and duration. These observations demonstrate that the CB1 receptor, a G-coupled–receptor that was initially discovered in rat brain tissue where it governs behavior (13), is also expressed at lower levels by KCs in the skin where it limits the inflammation caused by hapten-specific T cells in vivo. A similar observation has been reported in a mouse model for multiple sclerosis, experimental autoimmune encephalitis, where CB1 receptors on neurons attenuate the inflammation induced by encephalitogenic T cells in the brain (8). Taken together, these observations suggest that CB1 receptors expressed by tissue resident nonimmune cells may generally provide protection against excessive T cell–dependent inflammatory responses on an organismal level.

Careful time-course analyses revealed increased and prolonged reactive epidermal acanthosis in association with an enhanced inflammatory infiltrate during the effector phase of CHS in Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice when compared with controls. We reasoned that inflammatory immune cells drive KC hyperproliferation through the production of proinflammatory cytokines and chemokines. This hypothesis was supported by gene expression analyses of IL-1β and CXCL2, although increased mRNA levels corresponded to higher numbers of infiltrating CD45$^+$ cells only in the late effector phase 96 h after hapten challenge. One explanation for this finding might be that skin resident cell types contribute to the expression levels of these mediators. For example, mast cells have been shown to be important producers of CXCL2 during contact-allergic inflammation (27). In addition, some CD45$^+$ immune cell subpopulation cells could, on average, produce increased mRNA
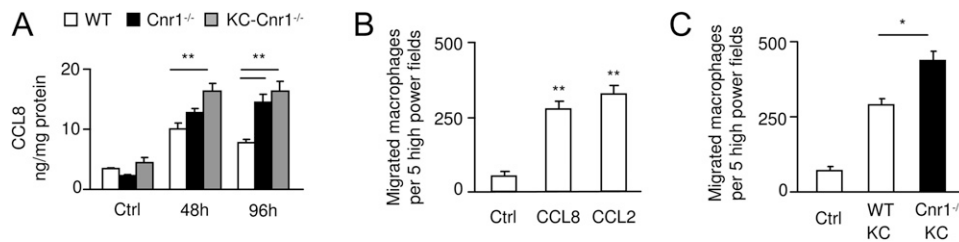
FIGURE 8. Increased amount of CCL8 protein in contact-allergic ear tissue of Cnr1$^{-/-}$ and KC-Cnr1$^{-/-}$ mice in vivo and CCL8-dependent migration of myeloid cells. (**A**) CCL8 protein levels determined by ELISA in ear tissue lysates 48 and 96 h after challenge ($n = 6$/group, $\pm$ SEM). (**B**) Migration of bone marrow–derived macrophages in response to CCL8 and CCL2, and (**C**) in response to culture medium conditioned by IFN-γ–stimulated WT and CB1$^{-/-}$ KCs in transwell assays. Shown is the number of migrated macrophages per five high-power fields ($\pm$ SEM). *$p < 0.1$, **$p < 0.05$.

levels. For example, Langerhans cells and other DCs express strongly increased levels of IL-1β upon activation after exposure to contact allergens (28).

To determine the mechanisms how CB1 receptors on KCs regulate inflammatory responses in the skin, we studied in vitro cultures of primary KCs from Cnr1$^{-/-}$ or KC-Cnr1$^{-/-}$ mice in direct comparison with WT or littermate control mice. Cultures of CB1 receptor–deficient KCs expanded and differentiated normally under standard culture conditions in vitro and did not recapitulate the increased proliferative activity observed in the effector phase of CHS in vivo. Furthermore, exposure of primary mouse KCs to CB1 agonists did not alter their proliferation or their differentiation response after exposure to high calcium medium. Thus, our experiments with mouse KCs did not recapitulate previously reported results with the immortalized human KC cell line, showing that CB1 receptor–dependent effects impair their differentiation capacity in vitro (17, 18). However, primary mouse KCs express high levels of keratin 5, a marker that in vivo is mostly expressed by basal KCs, but only low levels of keratin 1, a marker for differentiated corneocytes in the upper layer of the epidermis, even after increase of calcium levels in the culture medium. It is therefore conceivable that the absence of CB1 receptor signaling affects terminal differentiation and cornification of KCs in a manner that is impossible to study with mouse KCs in vitro.

Several in vivo and in vitro studies have reported that endogenous and exogenous CBs modulate the production of proinflammatory cytokines and chemokines (12, 29). In one study it was shown that CB1 receptor signaling also affects the release of chemokines by human HaCaT epithelial cells and primary KCs (30). Consistent with these observations, we found that CB1 receptor–deficient KCs produce increased amounts of CXCL10 and CCL8 in response to IFN-γ released by hapten-specific T cells in the effector phase of CHS in vitro. In contrast, stimulation with TNF-α, IL-10, or IL-17 did not induce detectable changes in CXCL10 and CCL8 levels. Subsequent studies in vivo supported the notion that increased expression of CXCL10 promotes the recruitment of CXCR3-expressing effector T cells and CCL8 the recruitment of CCR2-expressing myeloid immune cells, which amplifies the resulting CHS response. The increased IFN-γ sensitivity of CB1 receptor–deficient KCs is compatible with the prevailing concept that CBs generally suppress a Th1-biased immune response.

We previously identified CCL8 as a differentially expressed gene in a whole-genome expression analysis of contact-allergic ear tissue from Cnr1/Cnr2 double-knockout mice compared with WT mice. In situ hybridization experiments suggested that epidermal KCs are the major source of CCL8 (10). In this article, we show that KCs indeed produce CCL8 after stimulation with IFN-γ, but not TNF-α, IL-10, or IL-17 in vitro, and that the IFN-γ–induced

secretion of CCL8 is increased in KCs that lack CB1 receptors. qRT-PCR and ELISA measurements confirm enhanced expression of CCL8 on the mRNA and protein level in contact-allergic ear tissue of mice that lack CB1 receptors only on KCs when compared with controls. CCL8, initially identified as monocyte chemotactic protein 2, has long been known as an IFN-γ–inducible chemokine (31). Similar to CXCL10, it can also be produced by stromal fibroblasts and macrophages in an IFN-γ–dependent manner. We confirmed the previously reported ability of CCL8 to recruit cells of the myeloid lineage in a transwell migration assay with bone marrow–derived macrophages (32). The importance of CCL8 produced by KCs is also highlighted by a recent report describing its role in a mouse model of atopic dermatitis (33). Taken together, these results establish CCL8 as an important player in T cell–dependent inflammatory diseases of the skin.

Our results provide more evidence for the important role that KCs play in the regulation of T cell–dependent immune responses in the skin, a topic that has recently received much attention. We were impressed by the fact that a seemingly marginal genetic alteration only in KCs leading to deregulated production of proinflammatory chemokines can cause considerable perturbations in the control of contact-allergic inflammation. This observation fits well with the emerging "outside-inside" pathogenetic concept of allergic inflammation, which postulates that a primary dysfunction of KCs can subsequently promote immunologic abnormalities (34). We expect that our results in an experimental model for allergic contact dermatitis will also be relevant to understand the role of KCs in other T cell–dependent inflammatory diseases including atopic dermatitis and psoriasis.

As a future perspective, it will be interesting to investigate the role of endocannabinoids like 2-arachidonoylglycerol and of CB2 receptors for the regulation of contact-allergic inflammation. Endogenous CB levels are increased in inflamed ear tissue (10, 35, 36). KCs are capable of producing endocannabinoids in vitro (34) and also express CB2 receptors (35, 37). The generation and investigation of mice carrying a KC-specific deletion of CB2 receptors and experiments with CB2 receptor inverse agonists in KC cultures may help to understand the potential synergy or antagonism of the two CB receptor subtypes.

Based on the work presented in this article, we propose that peripheral CB1 receptors on KCs limit the production of proinflammatory chemokines that regulate the T cell–dependent recruitment of immune cells in the effector phase of allergic contact dermatitis. Novel pharmaceutical strategies targeting receptors or enzymes of the endocannabinoid system may help to develop alternative therapies for the treatment of allergic inflammation.

Disclosures

The authors have no financial conflicts of interest.

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