

Research Article

Epidermal NLRP10 contributes to contact hypersensitivity responses in mice

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The nucleotide binding and oligomerization domain-like receptor (NLR) protein NLRP10 is highly expressed in the epidermis and contributes to cell-autonomous responses against invasive bacteria. To investigate the role of NLRP10 in inflammatory responses of the skin we analyzed the effect of full-body and keratinocyte-specific depletion of NLRP10 in croton oil-induced irritant contact dermatitis (ICD) and 1-fluoro-2,4-dinitrobenzene (DNFB)-induced contact hypersensitivity (CHS) in mice. *Nlrp10*^{-/-} mice were phenotypically normal and skin repair after wounding was not affected by lack of NLRP10. Similarly, we did not detect a contribution of NLRP10 to the ICD response induced by croton oil. In contrast, *Nlrp10*^{-/-} mice showed significantly reduced inflammation in the DNFB-induced CHS response as compared to control animals. Microscopic analysis revealed significantly reduced numbers of CD4⁺ and CD8⁺ T cells in the infiltrates of animals lacking NLRP10 expression after CHS challenge. Epidermis-specific deletion of *Nlrp10* by keratin-14 promoter driven Cre-recombinase was sufficient to account for this phenotype, although lymphocyte recruitment seemed to be unaltered in animals lacking NLRP10 expression in keratinocytes. Taken together, we provide evidence that NLRP10 contributes to T-cell-mediated inflammatory responses in the skin and highlight a physiological role of NLRP10 in epidermal keratinocytes.

Keywords: Contact dermatitis · Contact hypersensitivity · NLRP10 · Mouse model



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Introduction

As essential players of innate immune responses nucleotide binding and oligomerization domain-like receptor (NLRs) proteins mediate the rapid detection and removal of microbes [1] as well as responses to endogenous, host-derived danger signals,

so-called danger-associated molecular patterns (DAMPs), which are released in response to tissue injury, stress, or necrotic cell death [2]. Upon sensing of invading microbes and of host-derived DAMPs NLR proteins mediate the activation of intracellular signaling cascades such as NF- κ B and MAPK signaling pathways or the formation of multiprotein complexes known as inflammasomes, leading to the secretion of proinflammatory cytokines [3, 4]. These responses are not only central to facilitate innate immunity, but also crucial for the initiation and orchestration of adaptive immunity (reviewed in [5]). More recently, several studies showed that

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various NLR proteins contribute to the regulation of lymphocytes by generating lineage determining cytokine milieus (reviewed in [6]). NLRs can also affect lymphocytes in a more direct manner as shown for NLRP12 that can control production of cytokines in T cells [7]. Still our knowledge of the contribution of NLR proteins in complex adaptive immune responses is limited. Moreover, some members of the NLR family remain poorly characterized. Among those, NLRP10 is of special interest in the context of a regulator function in immunity as it is the only NLR protein that lacks the prototypical C-terminal leucine-rich repeats (reviewed in [8]). This might imply that NLRP10 rather functions as a signaling modifier than as a classical ligand-binding pattern recognition receptor. NLRP10 positively regulates innate immune responses mediated by NOD1 upon *Shigella flexneri* infection in both epithelial cells and dermal fibroblasts via modulation of p38 and NF- κ B signaling pathways [9]. Work by others further suggested an inhibitory role for NLRP10 in ASC-mediated NF- κ B activation as well as caspase-1 mediated IL-1 β secretion [10]. Recent studies however challenged a contribution of NLRP10 to inflammasome function as bone marrow-derived dendritic cells, bone marrow derived macrophages, and peritoneal macrophages from *Nlrp10* knock-out mice respond normally to inflammasome activation [11, 12]. The same study suggested a critical role for NLRP10 in the initiation of adaptive immunity. NLRP10-deficient mice failed to mount efficient antigen specific immune responses in different models of adaptive immune responses [12] or infection with the fungal pathogen *Candida albicans* [11]. The authors explain this finding by a defect of DCs migration to draining lymph nodes resulting in abolished activation of T cells in *Nlrp10* knock-out animals [12]. However, the molecular mechanism by which NLRP10 regulates DC migration remained elusive. The picture is further complicated by the fact that the used *Nlrp10*^{-/-} mice incidentally showed altered expression of the guanine nucleotide exchange factor DOCK8 (dedicator of cytokinesis 8). Indeed the DC migration phenotype was shown to genetically segregate with a mutation in *Dock8* [13]. Furthermore, expression of murine NLRP10 in DCs has not been analyzed due to the lack of specific antibodies, leaving the question if DCs express physiological relevant levels of NLRP10 protein. Using an antibody specific for human NLRP10 we recently established that NLRP10 is highly expressed in the epidermis of the skin in humans [9]. In comparison with many organs and cell types of murine origin, the epidermis was also found to show the highest expression of *Nlrp10* mRNA [14, 15]. Moreover, a single nucleotide polymorphism in the genomic context of *Nlrp10* has been associated with the susceptibility for atopic dermatitis in humans [16]. Taken together, this strongly suggests a physiological role of NLRP10 in the skin.

Here, we report on the generation of a novel *Nlrp10*^{fl/fl} mouse line and characterization of the effect of full-body and epidermis-specific deletion of NLRP10 in dermatitis models. Our data show that NLRP10 contributes in a positive manner to contact hypersensitivity responses in the skin but not to irritant induced dermatitis. Notably, we found the lack of epidermal expression of NLRP10 to be mainly sufficient for this phenotype.

Results

Generation and characterization of *Nlrp10* knockout mice

To study the role of NLRP10 in inflammatory skin disorders in vivo, we generated transgenic mice with constitutive and conditional knockout of the *Nlrp10* gene. To this end, exons 2 (harboring the translation initiation codon) and 3 of the murine *Nlrp10* gene were flanked by *loxP* sites (*Nlrp10*^{fl/fl}) and germline deletion of *Nlrp10* exons 2 and 3 was obtained by crossing *Nlrp10*^{fl/fl} mice to Cre deleter [17] mice (Fig. 1A). Absence of *Nlrp10* mRNA expression was confirmed by RT-PCR in organs of control *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} mice (Fig. 1B). In accordance with previous findings in human tissues [9], we found the highest levels of *Nlrp10* mRNA in the skin of *Nlrp10*^{fl/fl} mice (Fig. 1B), with no detectable transcripts in tissue from *Nlrp10*^{-/-} mice. Analysis of skin morphology by H&E staining of skin sections revealed no obvious differences between *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} mice (Fig. 1C). The recently described *Nlrp10* knockout mice are deficient in the expression of dedicator of cytokinesis 8 (DOCK8) [13]. To exclude a contribution of DOCK8 in our study, we compared the expression level of DOCK8 protein in murine embryonic fibroblasts (MEFs) derived from *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} animals. This revealed comparable protein levels of DOCK8 protein in MEFs derived from both *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} embryos, irrespective of *Nlrp10* genotype and mRNA expression (Fig. 1D).

NLRP10 is dispensable for tissue repair and irritant contact dermatitis

NLRP10 is highly expressed in the epidermis of both human and murine skin [9, 14, 15] however, the underlying physiological role is unknown. We investigated whether loss of NLRP10 affects essential functions of the skin. We analyzed epidermal wound healing as an important process ensuring reconstitution of skin integrity by the concerted activity of inflammatory, migratory, and proliferative events [18]. To this end, two excisional wounds were generated on the back of both *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} animals and wound closure was monitored over time. Macroscopic analysis of wound closure revealed no signs of altered repair processes in *Nlrp10*^{-/-} animals (Fig. 2A). Measurements of the wound area showed no significant differences in wound closure in *Nlrp10*^{-/-} animals compared to *Nlrp10*^{fl/fl} animals (Fig. 2B).

We previously found NLRP10 to contribute to inflammatory responses in epithelial cells [9]. To elucidate a possible contribution of NLRP10 to irritant contact dermatitis (ICD) we used croton oil as an irritant. *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} mice were challenged by application of a 10 % solution of croton oil in acetone to one ear and vehicle (acetone) to the other. Ear thickness of the challenged ear, compared to the vehicle-exposed ear was measured over time. Croton oil application induced a comparable ear swelling in both *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} animals (Fig. 2C).

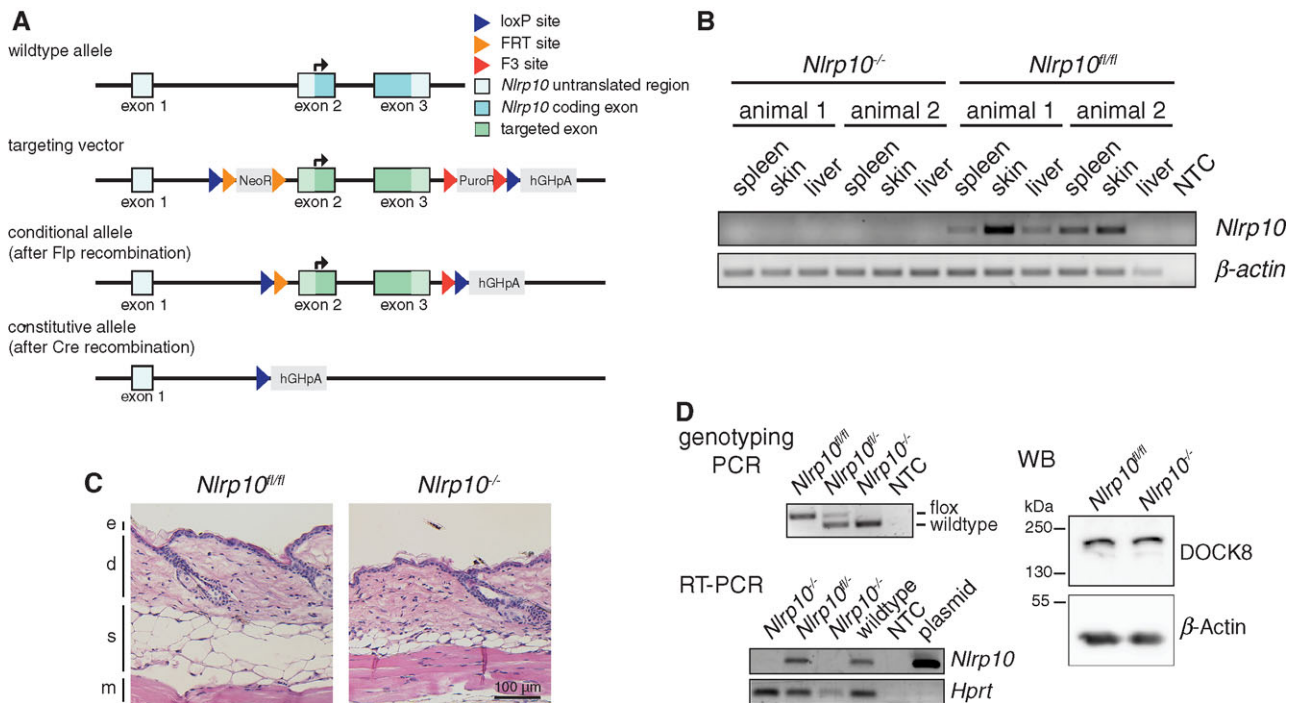


Figure 1. Generation and characterization of *Nlrp10* knockout mice. (A) Targeting strategy for the constitutive and conditional knock-out of *Nlrp10*. (B) Expression of *Nlrp10* mRNA in organs of *Nlrp10*^{-/-} and *Nlrp10*^{fl/fl} mice was analyzed by RT-PCR in two mice per genotype. β-Actin was amplified as a reference. (C) Skin histology was analyzed by H&E staining of 5 μm paraffin sections from mouse skin. Original magnification: ×100. e: epidermis; d: dermis; s: subcutis; m: muscularis. (D) PCR on genomic DNA of tail tissue derived from the indicated mice to show deletion of *Nlrp10* and RT-PCR detecting expression of *Nlrp10* mRNA in MEFs of the indicated genotype (left). Expression of DOCK8 in MEFs of the indicated genetic background is shown by immunoblot (WB, right). Data shown are representative of analyses on five mice from each genotype in (C) and 16 MEF clones in (D). NTC: no template control.

Histological analysis of vehicle and croton oil treated ears sampled 36 h after challenge showed no major differences between *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-} mice. Both groups displayed comparable epidermal hyperplasia and infiltration of inflammatory cells (Fig. 2D). Microscopic analysis of ear thickness in H&E-stained ear sections did not detect any difference in ear swelling 36 h after croton oil challenge (Fig. 2D).

In conclusion, NLRP10 expression is dispensable for tissue repair and irritant contact dermatitis in our in vivo models.

NLRP10 contributes to DNFB-induced contact hypersensitivity

Nlrp10 was mapped to a susceptibility locus for atopic dermatitis [16], a chronic inflammatory disease of the skin characterized by a T-cell-mediated inflammatory response [19]. To investigate a possible contribution of NLRP10 to T-cell-mediated inflammatory responses in the murine skin we analyzed the contact hypersensitivity (CHS) response toward the hapten 1-fluoro-2,4-dinitrobenzene (dinitrofluorobenzene, DNFB) that is a well-established model for allergic contact dermatitis (reviewed in [20]). Mice were sensitized by topical application of DNFB on the shaved abdomen on day 0. Five days later one ear was challenged by a single application of DNFB, whereas the other ear was

treated by vehicle and used as control. Given the dominant expression of NLRP10 in keratinocytes and the important contribution of keratinocytes to inflammatory skin diseases (reviewed in [21]), we also generated an epidermis-specific conditional knock-out of *Nlrp10* (*Nlrp10*^{K14-KO}) by crossing our *Nlrp10*^{fl/fl} mice to mice expressing Cre recombinase under the control of the keratin-14 promoter (K14-Cre) [22]. PCR on gDNA isolated from liver, which does not express K14 [22] confirmed the presence of the floxed and absence of the deleted allele in the liver of *Nlrp10*^{K14-KO} animals (Supporting Information Fig. 1A). In contrast, in tail biopsies of *Nlrp10*^{K14-KO} animals, which contain skin, both the floxed and the knockout alleles were detected (Supporting Information Fig. 1A). RT-PCR confirmed almost complete ablation of *Nlrp10* expression in the epidermis, the residual detectable signal likely derived from nonepithelial cells resident in this compartment (Supporting Information Fig. 1B). Skin morphology of *Nlrp10*^{K14-KO} did not differ from that of *Nlrp10*^{fl/fl} animals (Supporting Information Fig. 1C). Moreover, we observed similar swelling responses in wild-type C57BL/6 mice and our *Nlrp10*^{fl/fl} animals (data not shown). Both *Nlrp10*^{-/-} and *Nlrp10*^{K14-KO} mice showed significantly reduced inflammation compared to *Nlrp10*^{fl/fl} control animals, as measured by ear swelling (Fig. 3A). This difference was visible as early as 8 h after challenge and was more pronounced and highly significant 24 h after DNFB application (Fig. 3A). In contrast to *Nlrp10*^{fl/fl} mice some knockout

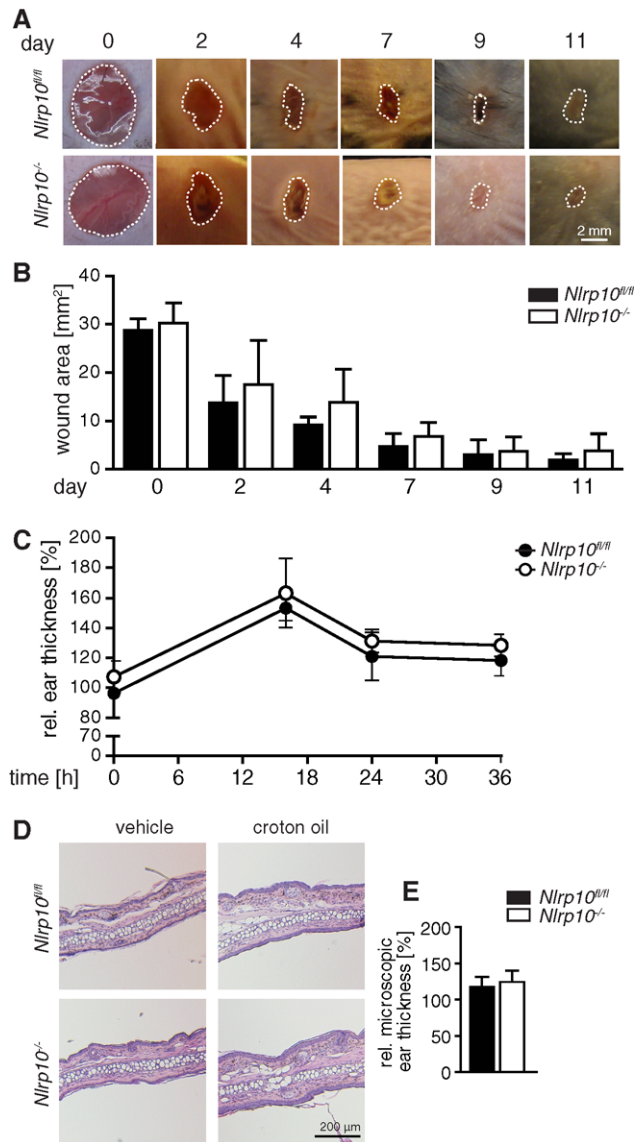


Figure 2. Knockout of *Nlrp10* does not impair cutaneous wound healing and irritant contact dermatitis responses. (A) To analyze wound healing, two 6 mm excisional wounds were generated in the dorsal skin of *Nlrp10^{fl/fl}* and *Nlrp10^{-/-}* mice. Wound closure was documented photographically at 0, 2, 4, 7, 9, 11, and 14 days after wounding. (B) The macroscopic wound area was measured from digital images. (C) To induce irritant contact dermatitis, ears were challenged with croton oil or vehicle only and ear thickness was measured 0, 16, 24, and 36 h after croton oil application. Thickness of croton oil-treated ears was normalized to vehicle-treated ears. (D) H&E staining of 5 μ m paraffin sections from mouse ears 36 h after croton oil application. Original magnification: $\times 50$. (E) Thickness of H&E-stained ear sections 36 h after challenge was measured microscopically at five positions per ear and normalized to vehicle-treated ears. (B–E) Data are expressed as mean $\pm$ SD of $n = 5$ in wound healing and $n = 4$ in ICD, from one experiment.

animals displayed a maximal increase in swelling at 48 h after challenge (Fig. 3A) pointing toward a reduction in maximum ear swelling and a retardation of the CHS response in NLRP10-deficient animals. The effect thereby was similar in both *Nlrp10^{-/-}* and *Nlrp10^{K14-KO}* mice. Histological examination of ears sampled

24 h after challenge revealed leukocyte infiltration and edema in the dermis as well as epidermal hyperplasia in animals of all genotypes as expected for CHS (Fig. 3B, Supporting Information Fig. 2A). Measurement of the ear thickness of fixed tissue confirmed, at a microscopical level, significantly reduced ear swelling in *Nlrp10^{-/-}* animals compared to controls at 24 and 48 h post-DNFB challenge (Fig. 3B, Supporting Information Fig. 2B). However, results were not significant for the *Nlrp10^{K14-KO}*, likely due to the lower number of animals and higher technical variability of this method. In histological stainings from *Nlrp10^{K14-KO}* ears taken 24 h after DNFB application similar results were obtained (Fig. 3B) and inflammation was also resolved by 96 h after treatment in these animals (Supporting Information Fig. 3).

Conclusively, this revealed that keratinocyte expressed NLRP10 contributes to CHS responses.

NLRP10 knockout affects immune cell infiltrates and cytokine expression in DNFB-challenged ears

To gain insight into the mechanism underlying the reduced CHS response in *Nlrp10^{-/-}* mice we next analyzed immune cell populations in ear sections collected 24 h after DNFB application, where the most prominent difference in the response was detected. Typical and causative for CHS are lymphocyte infiltrates. Analysis of CD3⁺, CD8⁺, and CD4⁺ and T cells revealed significantly higher numbers of all these cell types in DNFB-treated ears 24 h after challenge as compared to vehicle-treated ears (Fig. 4A and B). Even though these cells were increased in treated ears independently of mice genotype, we detected reduced numbers of CD3⁺ cells as well as CD4⁺ and CD8⁺ cells in the dermis of DNFB challenged ears of *Nlrp10^{-/-}* mice compared to *Nlrp10^{fl/fl}* animals (Fig. 4A and B). Interestingly, upon DNFB challenge CD3⁺, CD8⁺, and CD4⁺ cell numbers in *Nlrp10^{K14-KO}* were comparable to those detected in *Nlrp10^{fl/fl}* animals (Fig. 4A and B). In accordance with reduced ear swelling, we also observed a tendency toward lower numbers of infiltrating CD45⁺ leukocytes and Gr-1⁺ granulocytes in both *Nlrp10^{-/-}* and *Nlrp10^{K14-KO}* mice, albeit the difference was not significant for these cells (Fig. 5A and B). To gain insight if this was correlated with altered cytokine responses, we measured mRNA expression of the key inflammatory cytokines TNF- α and CXCL1 in ears 24 h after DNFB challenge. This showed a reduced, albeit not significantly altered TNF- α and CXCL1 mRNA expression in the skin of *Nlrp10^{-/-}* animals compared to *Nlrp10^{fl/fl}* upon DNFB challenge (Fig. 5C). Notably, virtually the same results were obtained in *Nlrp10^{K14-KO}* mice (Fig. 5D). To substantiate these findings we also measured CXCL1 and CCL3 in homogenates of ears taken 24 h post-DNFB treatment from *Nlrp10^{K14-KO}* animals. Both cytokines were significantly induced by DNFB, however showed no significant difference between the genotypes (Fig. 5D). Due to interindividual variation these results were not statistically significant for the whole cohort.

Analysis of mast cells in the ears 24 h after DNFB challenge revealed a trend toward a reduction of mast cell numbers in *Nlrp10^{K14-KO}* as well as significantly reduced number of

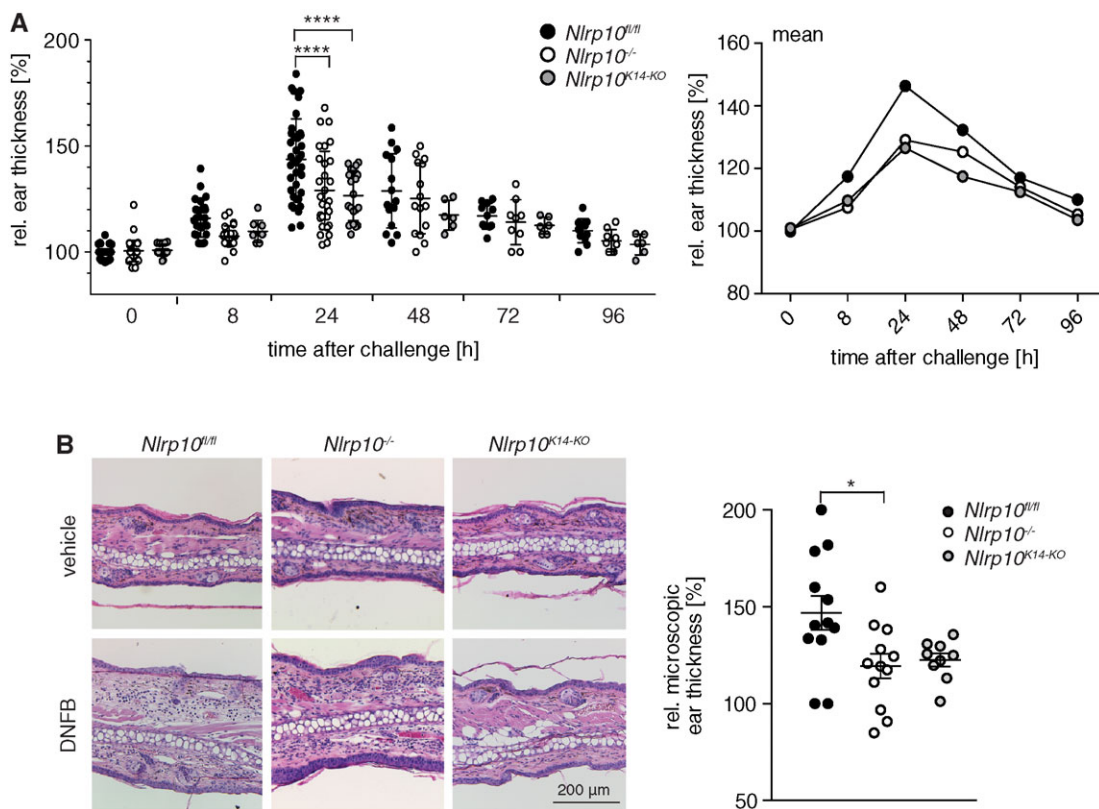


Figure 3. Depletion of *Nlrp10* reduces the contact hypersensitivity response to DNFB. (A) *Nlrp10^{fl/fl}*, *Nlrp10^{-/-}*, and *Nlrp10^{K14-KO}* mice were sensitized by application of DNFB on the shaved abdomen on day 0. On day 5, ears were challenged with DNFB or vehicle only and ear thickness was measured at the time points indicated. Ear thickness of DNFB-treated ears was normalized to vehicle only treated ears and is expressed as % relative thickness. Bars indicate median, each circle corresponds to one individual mouse. Data are pooled from five independent experiments. At 24 h with $n = 37$ (*Nlrp10^{fl/fl}*), $n = 20$ (*Nlrp10^{K14-KO}*), or $n = 29$ (*Nlrp10^{-/-}*). The mean of these experiments is plotted (right). (B) H&E staining of 5 μ m paraffin sections from mouse ears 24 h after DNFB challenge. Original magnification: $\times 100$. Thickness of H&E-stained ear sections of four mice per genotype was measured microscopically at three positions per ear and normalized to vehicle-treated ears (right columns). Data are expressed as scatter dot plot including mean $\pm$ SEM, from a single experiment.

degranulating mast cells in *Nlrp10^{K14-KO}* mice (Fig. 6), supporting a more general role of keratinocyte expressed NLRP10 in regulating various inflammatory mechanisms during CHS. Finally, to address a potential contribution of NLRP10 to migration of skin resident DCs, we quantitatively assessed the number of CD207⁺ Langerhans cells, which are essential regulators in CHS responses. Blinded microscopic analysis showed no significant differences in the number of CD207⁺ Langerhans cells 24 h after DNFB application between the three mouse strains (Supporting Information Fig. 4), suggesting that NLRP10 does not contribute to the emigration of this DC subtype.

Concluding, our data show that NLRP10 contributes to different DNFB induced CHS responses in vivo.

Discussion

Here, we describe the generation of a novel conditional *Nlrp10* knockout mouse model. Our data indicate that full-body depletion of NLRP10 does not significantly affect skin repair (Fig. 2A and B)

or irritant contact dermatitis upon croton oil challenge (Fig. 2C and D). By contrast, we found significantly reduced inflammatory responses in DNFB-induced CHS in *Nlrp10^{-/-}* mice when compared to *Nlrp10^{fl/fl}* mice. This was evidenced by reduced oedema, less T cell and granulocyte infiltrates and reduced transcription of proinflammatory cytokines in *Nlrp10^{-/-}* mice.

NLRP10 is highly expressed in the skin [9, 15]. Moreover, we showed that in human epithelial cells NLRP10 contributes positively to bacterial-induced innate immune responses [9]. Independently, a contribution of nonhematopoietic NLRP10 to *Candida albicans* immune responses was suggested [11]. Keratinocytes have a well-established function in the orchestration of skin inflammation (reviewed in [23]). After initial contact with the hapten in the CHS response, these cells produce TNF- α as well as IL-1 β and IL-18 to initiate maturation and migration of DCs from the skin to the lymph nodes, where hapten-specific T cells are activated [24, 25]. Upon elicitation of CHS keratinocyte-derived proinflammatory chemokines such as CXCL1 (GRO α) contribute to the attraction and activation of inflammatory cells as effector T cells and neutrophils [26–28]. In support of a physiological function of NLRP10 in keratinocytes, our data suggest

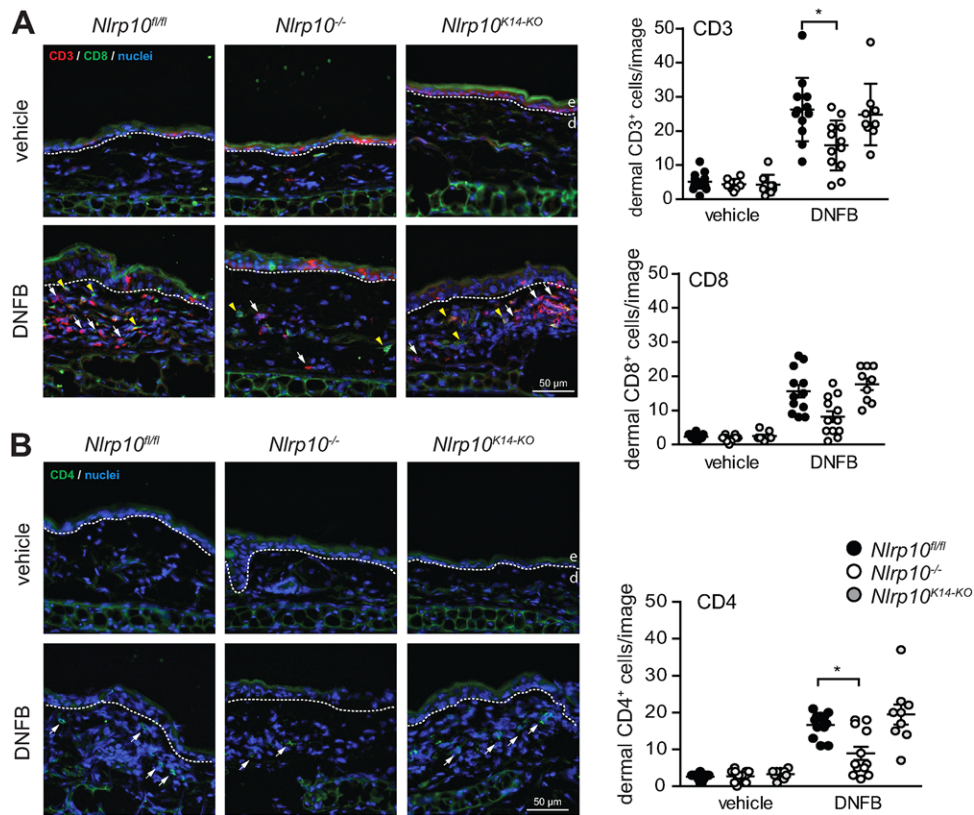


Figure 4. Effect of NLRP10 on lymphocyte recruitment in the contact hypersensitivity response toward DNFB. (A) Quantification of dermal CD3⁺ (red) and CD8⁺ (green) cells by indirect immunofluorescence microscopy in ears 24 h after DNFB challenge in four animals per genotype (three images/ear). Original magnification $\times 200$. White arrows indicate CD3⁺, yellow arrowheads indicate CD8⁺ cells. (B) Dermal CD4⁺ (green) cells were quantified by indirect immunofluorescence microscopy in ears 24 h after DNFB challenge in four animals per genotype (three images/ear). White arrows indicate CD4⁺ cells. All data are expressed as scatter dot plots and bars show mean $\pm$ SEM of ear images and animals from a single experiment. Original magnification $\times 200$, all pictures show representative image details, the dotted line marks dermal-epidermal junction. e: epidermis; d: dermis.

lower numbers of infiltrating Gr-1⁺ as well as CD45⁺ cells in both *Nlrp10^{-/-}* and *Nlrp10^{K14-KO}* animals. Although we did not find a significant reduction of inflammatory mediators in active CHS in animals lacking NLRP10 expression, our data are suggestive for a trend toward reduced expression of the inflammatory mediators TNF- α and CXCL1 in the ears of *Nlrp10^{-/-}* mice. Using *Nlrp10^{K14-KO}* mice that lack NLRP10 expression in keratinocytes, we showed that NLRP10 expression in keratinocytes contributes to the DTH response. *Nlrp10^{K14-KO}* animals also had significantly decreased ear swelling, comparable to that observed in *Nlrp10^{-/-}* mice. We assumed that NLRP10 contributes to CHS by enhancing the expression of inflammatory mediators, including TNF- α , CXCL1, and CCL3 from epidermal cells, as in human epithelial cells NLRP10 was shown to interact with components of the NF- κ B [9] that contributes to the expression of these cytokines. However, we were not able to reveal significant differences in the cytokine responses, maybe due to technical reasons or the fact that subtle differences responsible for the mild phenotype observed might be hard to show.

Although *Nlrp10^{-/-}* and *Nlrp10^{K14-KO}* showed virtually equal defects in the CHS response when compared to *Nlrp10^{fl/fl}* ani-

mals, we found that the numbers of infiltrating T cells was not significantly altered in DNFB challenged ears of *Nlrp10^{K14-KO}* mice. This suggests that NLRP10 might contribute to CHS in multiple ways not exclusively depending on keratinocyte activities. Of note, we also observed a reduced degranulation of mast cells in both *Nlrp10^{-/-}* and *Nlrp10^{K14-KO}* mice. Upon hapten sensitization mast cells secrete TNF- α that induces DC migration, while during elicitation they contribute to endothelial activation and inflammatory cell infiltration via histamine, TNF- α and CXCL1 [29, 30]. NLRP10 thus might have additional roles in mast cells or even cell lineages of the adaptive immune system. It should be noted that a defect in DC migration was recently reported for a *Nlrp10* full body knock-out mouse line [12], although later shown by the same scientists to be provoked by a spontaneous mutation in *DOCK8* in that line [13]. We found that the *Nlrp10^{fl/fl}* and *Nlrp10^{-/-}* mice used in this work expressed equal amounts of DOCK8 protein. Moreover, in accordance with the latter work [13], our results do not support a significant role of NLRP10 in dendritic (CD207⁺ Langerhans) cells. An alternative mechanism how NLRP10 might affect DTH responses is by inhibiting T-cell activation upon sensitization of the mice. However, using the ovalbumin model, Flavell and

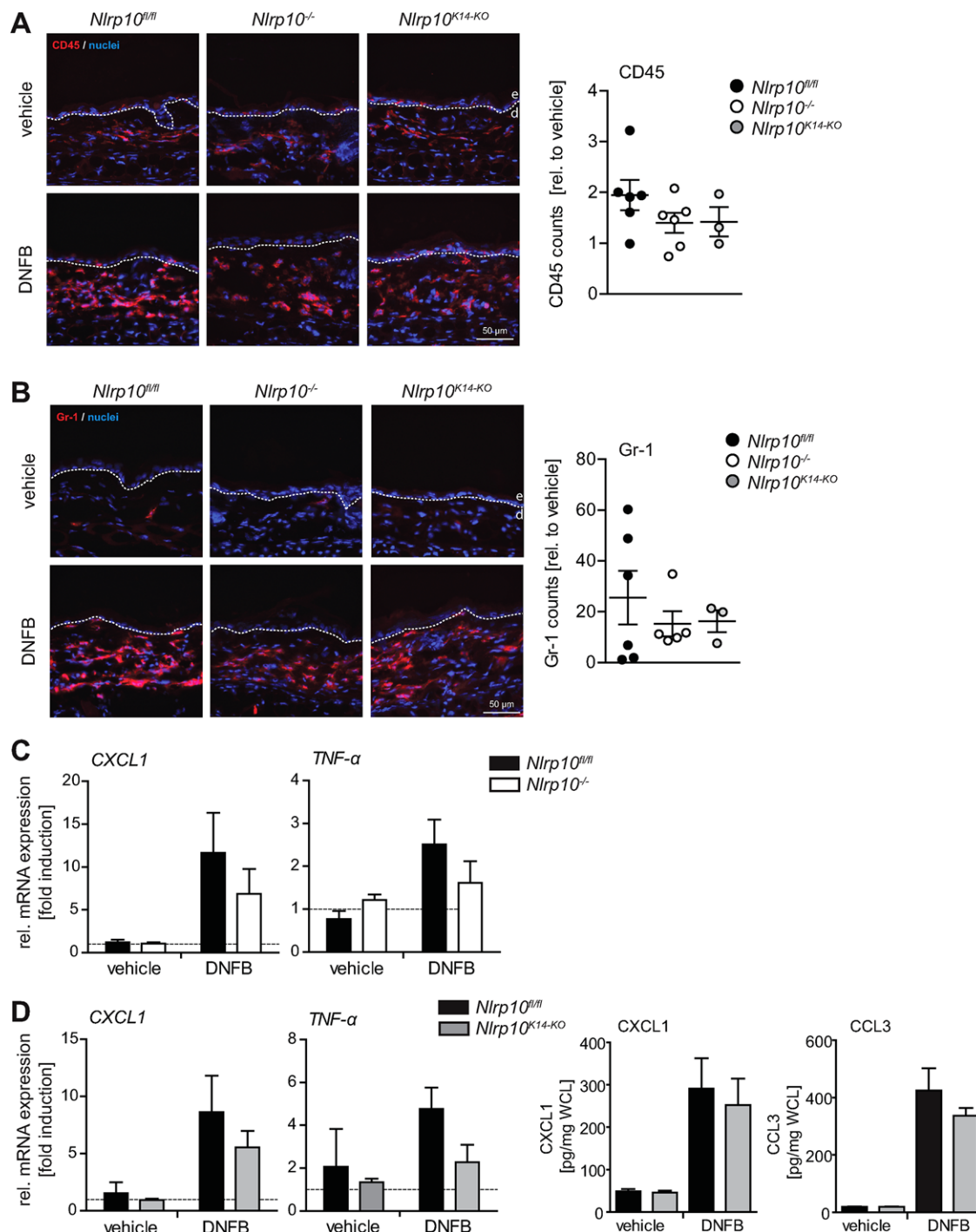


Figure 5. Effects of NLRP10 on leukocyte recruitment and cytokine responses in the contact hypersensitivity response toward DNFB. (A, B) Quantification of (A) CD45⁺ and (B) Gr-1⁺ cells in ears 24 h after DNFB challenge by indirect immunofluorescence. Data were normalized to vehicle-treated ears. Each symbol represents an individual mouse and bars represent mean $\pm$ SEM of $n = 6$ for *Nlrp10^{fl/fl}* and *Nlrp10^{-/-}*, $n = 3$ for *Nlrp10^{K14-KO}*. Original magnification $\times 200$. Shown are representative images. Dotted line marks dermal-epidermal junction. (C) mRNA expression of TNF- α and CXCL1 in the ears 24 h after DNFB application was determined by quantitative real-time PCR. Data are calculated as fold-induction compared to the mean of vehicle-treated ears and expressed as mean $\pm$ SEM of $n = 6$ animals per genotype for CXCL1, $n = 5$ for TNF- α . (D) mRNA expression in the ears 24 h after DNFB application was determined as in (C) and expressed as mean $\pm$ SEM of $n = 3$ animals per genotype (left). CXCL1 and CCL3 levels were measured by ELISA from whole cell lysates (WCL) of ears taken 24 h post-DNFB treatment (right). Data are shown as mean $\pm$ SEM of $n = 4$ animals per genotype, samples were arbitrarily chosen for lysate preparation from two DTH experiments performed with 4–8 animals per group.

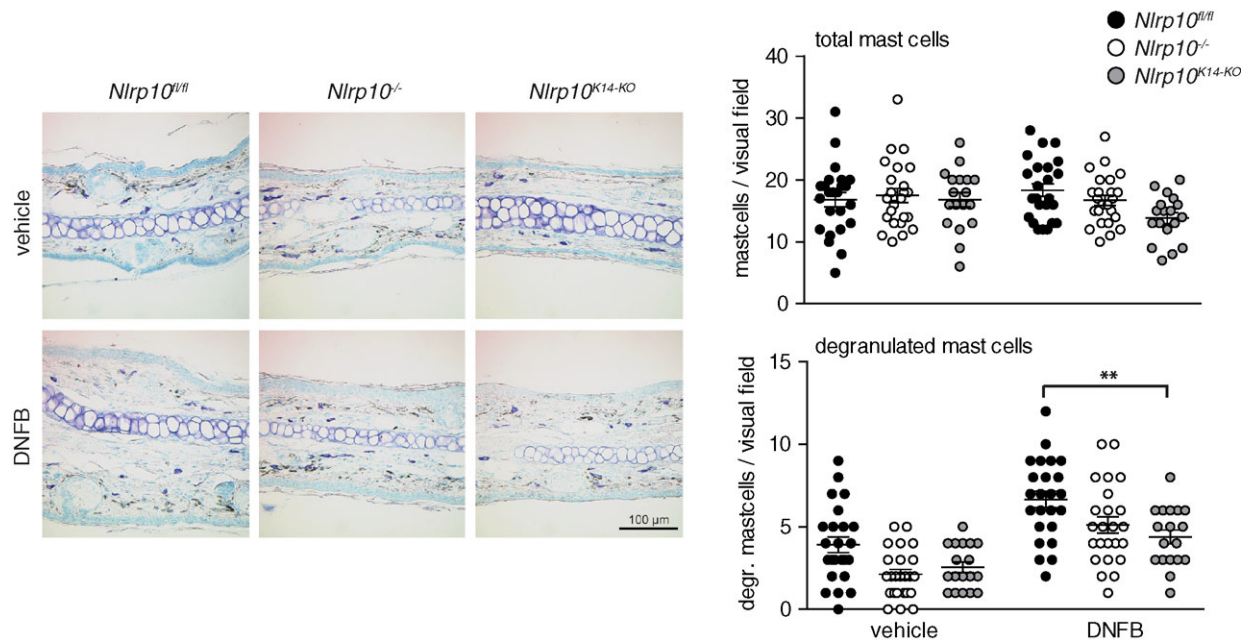


Figure 6. Characterization of the effects of NLRP10 on mast cells. Mast cells in ears sampled 24 h after DNFB challenge were stained by toluidin blue and total numbers as well as numbers of degranulated mast cells were quantified microscopically. Data are expressed as scatter dot plots and show mean $\pm$ SEM of $n = 4$ for *Nlrp10*^{fl/fl} and *Nlrp10*^{-/-}, $n = 3$ for *Nlrp10*^{K14-KO} (five positions per ear) from a single experiment.

colleagues showed that T-cell activation in vitro and proliferation of CD8⁺ T cells in vivo is not altered in *Nlrp10*^{-/-} animals [12]. In addition, our finding that epidermal-specific deletion of *Nlrp10* in mice is sufficient for a reduced DTH response argues against an intrinsic defect in T cells. Notably, NLRP10 is not the only NLRP protein with a role in CHS. A recent study showed that depletion of NLRP12 also results in reduced inflammation upon FITC and oxazolone-induced CHS [31]. However, for NLRP12 this effect was tracked to defects in migration of myeloid and dendritic cells rather than an effect in nonhaematopoietic cells [31], suggesting that both NLRs contribute differently to CHS.

In irritant contact dermatitis neutrophils are recruited by keratinocyte derived cytokines and importantly contribute to the local inflammation, and in both NLRP10 mice we did observed reduced infiltration of neutrophils in CHS. However, why NLRP10 does not affect croton oil induced inflammation remains elusive. A plausible explanation might be that NLRP10 differentially affects different PRR signals, as we showed recently for NOD1- and TNFR-mediated responses [9]. Our data however, do not clearly point to a defined functional mechanism for the contribution of NLRP10 to CHS. Future studies, analysing the kinetic of cytokine responses and in-depth flow-cytometric assessment of Th cell populations could help to define how NLRP10 expression affects cytokine profiles in keratinocytes and unravel the molecular details of the contribution of NLRP10 to inflammatory responses of the skin.

Concluding, this study identifies a novel role of NLRP10 in contact hypersensitivity responses. We show that loss of NLRP10 is associated with reduced CHS responses and provide evidence that epidermal expressed NLRP10 is of particular importance in this

model. NLRP10 thus is a protein critically involved in the regulation of both innate and adaptive immune processes in CHS. Given the potential involvement of the *Nlrp10* gene locus in hereditary forms of dermatitis [16], NLRP10 might be an interesting target for the therapeutic intervention in dermatitis. The availability of the herein described mouse strains, will allow further work to elucidate the molecular details underlying the function of NLRP10 in inflammatory responses in the skin in further detail.

Materials and methods

Mice

Nlrp10^{fl/fl} mice were generated in collaboration with Taconic Artemis GmbH (Cologne, Germany). The targeting vector was designed to have exons 2 and 3 of *Nlrp10* flanked by loxP sites to result in loss of function of NLRP10 by deleting the complete open reading frame. To obtain the constitutive knockout allele, *Nlrp10*^{fl/fl} animals were crossed to Cre-Deleter mice [17]. Offspring was in-bred to outcross the Cre allele and to obtain animals homozygous for the knockout allele (*Nlrp10*^{-/-}). By crossing of *Nlrp10*^{fl/fl} animals to animals expressing Cre recombinase under the keratin-14 promoter (K14-Cre) [22] we generated mice with an epidermis specific knock-out of *Nlrp10* (*Nlrp10*^{K14-KO}). Genotyping was performed on genomic DNA isolated from tail biopsies using the DNeasy mini kit (Qiagen, Hilden, Germany) and the following PCR protocols:

PCR A for the detection of wild-type (219 bp) and floxed allele (338 bp) using primers 5'-GACTATTTTGAGCACCTCCATACG-3' and 5'-CTCATAGGGTCTCTAGGTTAGGTAAGG-3'; PCR B for the additional detection of the knock-out allele (302 bp) using primers as in PCR A and 5'-CCCAAGGCACACAAAAACC-3'; and PCR C to detect Cre (~600 bp) using primers 5'-GACATGTTTCAGGGATCGCCAGGCG-3' and 5'-GACGGAAATCCATCGCTCGACCAG-3'. Mice were bred and kept under SPF conditions in the central animal facility of the CMMC Cologne. The use of mice and all following treatments (see below) were performed according to institutional guidelines and approval by the local authorities of the state North Rhine-Westphalia, in accordance with the animal protection law of Germany under the licence number Az 84-02.04.2013.A022.

Wound healing

9 to 10 weeks old *Nlrp10^{fl/fl}* and *Nlrp10^{-/-}* mice were anesthetized by i.p. injection of ketamine (Ketanest S; Parke Davis, Karlsruhe, Germany) and xylazine (Rompun 2 %; Bayer, Leverkusen, Germany). The animals' back was epilated using Veet Haarentfernungs-Crème Sensitive (Reckitt Benckiser, Mannheim, Germany). Subsequently skin was extensively washed with PBS and two full-thickness excisional wounds (6 mm in diameter) were generated on the back of each animal. The wounded area was left to dry and form a scab. Animals were sacrificed 14 days after wounding. Wound closure was documented using a Canon PowerShot A620 camera and wound area was determined in digital image using Fiji software (Life-Line version, November 25, 2014) [32].

Irritant dermatitis

Ears of *Nlrp10^{fl/fl}* and *Nlrp10^{-/-}* animals (9 weeks old) were challenged by application of 10 µL of 10 % v/v croton oil (Sigma-Aldrich, Munich, Germany) in acetone on one ear or acetone as the vehicle on the other ear. Ear thickness was measured over time using the Quickmini 700-121 PK-1025 Caliper (Mitutoyo Europe, Neuss, Germany) and calculated as relative ear thickness in % of vehicle. Animals were sacrificed 36 h after challenge and ears were cut, bisected, and fixed over night at 4°C in 4 % paraformaldehyde for paraffin embedding or directly embedded in OCT compound (Sakura Finetek Germany, Heppenheim, Germany) for cryo sectioning.

Contact hypersensitivity

Nine to 11 weeks old control (*Nlrp10^{fl/fl}*), constitutive knock-out (*Nlrp10^{-/-}*) as well as epidermis-specific knock-out (*Nlrp10^{K14-KO}*) mice were sensitized by application of 25 µL of 1 % v/v 1-fluoro-2,4-dinitrobenzene (dinitrofluorobenzene, DNFB, Sigma-Aldrich, Munich, Germany) in acetone/olive oil (4:1) (Sigma-

Aldrich, Munich, Germany) on the shaved abdomen on day 0. Five days later, the CHS response was elicited by application of 10 µL of 0.4 % DNFB in acetone/olive oil on one ear of the animals. The second ear was treated with the vehicle acetone/olive oil alone. Ear thickness was measured over time using the Quickmini 700-121 PK-1025 Caliper (Mitutoyo Europe, Neuss, Germany) and calculated as relative ear thickness in % of vehicle. Depending on the experimental setup, animals were sacrificed 8, 24, 48, or 96 h after challenge. Ears were bisected and fixed over night at 4°C in 4 % paraformaldehyde for paraffin embedding or directly embedded in OCT compound (Sakura Finetek Germany, Heppenheim, Germany) for cryo sectioning.

Histology and indirect immunofluorescence

Histological analysis was performed on 5 µm cryo- or paraffin-sections unless otherwise indicated. H&E and Toluidin blue staining for mast cell quantification were performed in paraffin-embedded skin and ear sections according to the manufacturer's recommendations (Thermo Scientific, UK). Images were acquired and ear thickness measurements were performed on a DM4000B microscope (Leica Microsystems, Wetzlar, Germany) using the Diskus software version 4.50 (Diskus, Königswinter, Germany). The number of toluidine blue stained mast cells fully retaining or partly releasing granules (degranulating) were counted in each section. For indirect immunofluorescence staining, 5 µm cryo sections were fixed in acetone, blocked in 10 % normal goat serum and incubated with the following antibodies: rat α CD207 (clone eBioRMUL.2, eBioscience, Frankfurt, Germany), rabbit α collagen type IV (CL50451, Cedarlane, Burlington, NC, USA), rabbit α CD3, rat α CD4, rat α CD8 (ab5690, ab25475 and ab22378, Abcam, Cambridge, UK), rat α CD45 (Serotec, Düsseldorf, Germany) and rat α Gr-1 (BD Bioscience, Heidelberg, Germany). Alexa-488 and Alexa-594-labelled secondary antibodies were purchased from Life technologies (Darmstadt, Germany), nuclei were counterstained using DAPI. Pictures were acquired on a Keyence BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) and for CD207 stainings on an Olympus IX81 microscope using the Cell^R software version 3.2 (Olympus, Düsseldorf, Germany). CD207⁺ cells in the ear epidermis or CD3⁺, CD4⁺, or CD8⁺ cells in the dermis were counted from digital image using Fiji software (Life-Line version, November 25, 2014) [32]. CD45⁺ and Gr-1⁺ cells were quantified in 3–5 images per specimen using ImageJ software [33]. Results obtained for DNFB-treated ears were normalized to vehicle-treated ears when indicated.

Isolation of mRNA, cDNA synthesis, RT-, and quantitative real-time PCR

Isolation of mRNA from 50 µm ear sections prepared from OCT blocks or organs snap-frozen in liquid nitrogen was performed using Trizol® reagent (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions after

tissue homogenisation using the Precellys® Tissue homogenizator (Peqlab, Erlangen, Germany). Genomic DNA in RNA samples was digested using DNase I and RNA was transcribed into cDNA using the First strand cDNA synthesis kit (Thermo Scientific). Semiquantitative RT-PCR on mRNA isolated from spleen, liver and skin was performed using the following primers: NLRP10: 5'-GGAGCTGTAGACTACCTCA-3' and 5'-AAAGTCTCCACATCGACAGG-3'; β -actin: 5'-TTGCTGACAGGATGCAAG-3' and 5'-ACATCTGCTGGAAGGTGGAC-3'. Quantitative real-time PCR on ear samples was performed on a 96-well-plate multicolour real-time PCR detection system (CFX96 Touch™, Bio-Rad) using iQ™ SYBR® Green Supermix (Biorad) and the following primer sequences: β -actin: 5'-TTGCTGACAGGATGCAAG-3' and 5'-ACATCTGCTGGAAGGTGGAC-3'; TNF- α : 5'-AGAACTCCAGGCGGTGC-3' and 5'-AGGGTCTGGGCCATAGAACT-3'; CXCL1: 5'-TGGCTGGGATTACCTCAAG-3' and 5'-AGTGTGGCTATGACTTCGGTT-3'. Ct values obtained were analyzed according to the Pfaffl method [34], using β -actin as a reference gene.

ELISA

For ELISA measurements, ears were homogenized in a buffer containing 100 mM Tris pH = 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease, and phosphatase inhibitors using the Precellys® Tissue homogenizator (Peqlab, Erlangen, Germany). After homogenization Triton X100 was added to a final concentration of 0.5 %. After incubation at 4°C and clarification of the samples by centrifugation CXCL1 and CCL3 were quantified by ELISA kits from Bio-technie according to the manufacturer's instructions. Cytokine concentrations were normalized to protein concentration of the lysates.

Statistical analysis

Statistical analysis was performed using GraphPad Prism5 (GraphPad Software, Inc., San Diego, CA, USA). Data were analysed using ANOVA with a post-hoc Bonferroni test. Differences between two groups were considered statistically significant when $p < 0.05$. Only p -values < 0.05 are indicated in the figures or figure legends. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

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- Abbreviations:** CHS: contact hypersensitivity · DNFB: 1-fluoro-2,4-dinitrobenzene · ICD: irritant contact dermatitis · MEF: murine embryonic fibroblast · NLR: nucleotide binding and oligomerization domain-like receptor
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**Supporting Information
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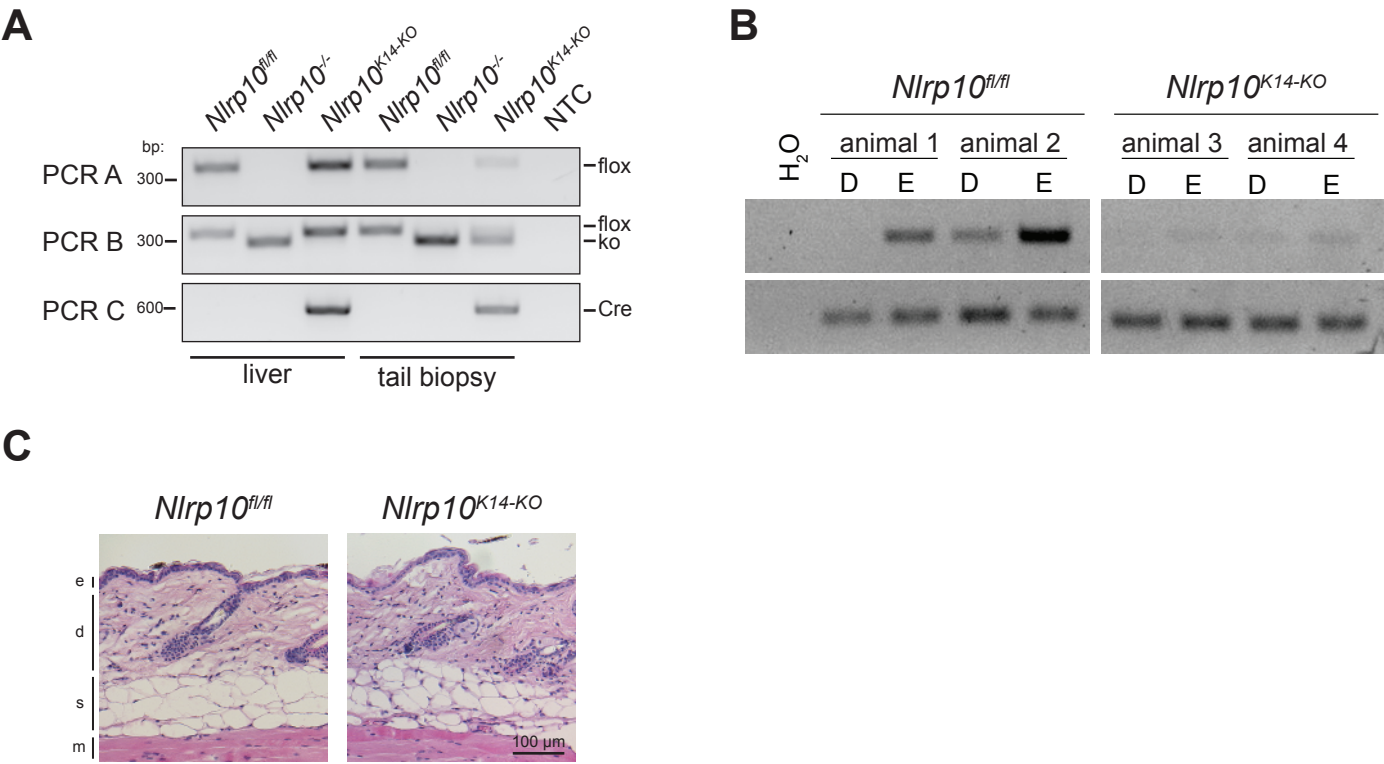
Epidermal NLRP10 contributes to contact hypersensitivity responses in mice

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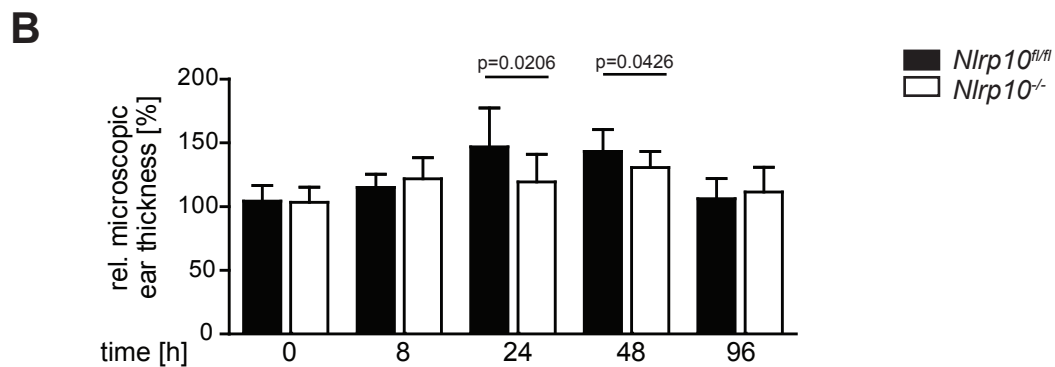
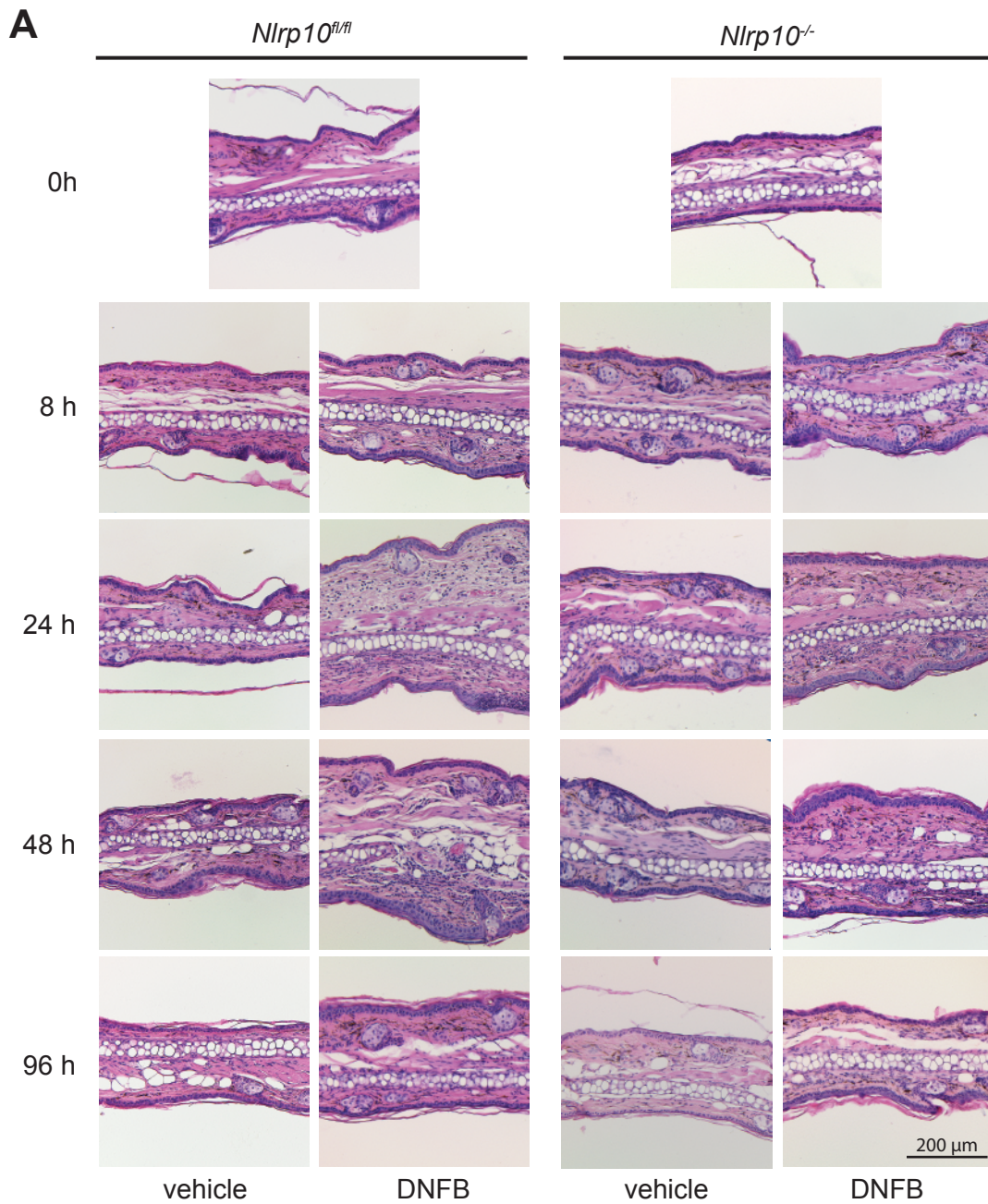
Anna Damm, Nives Giebeler, Jan Zamek, Paola Zigrino, Thomas A. Kufer

Supporting Information

Figures 1-4.

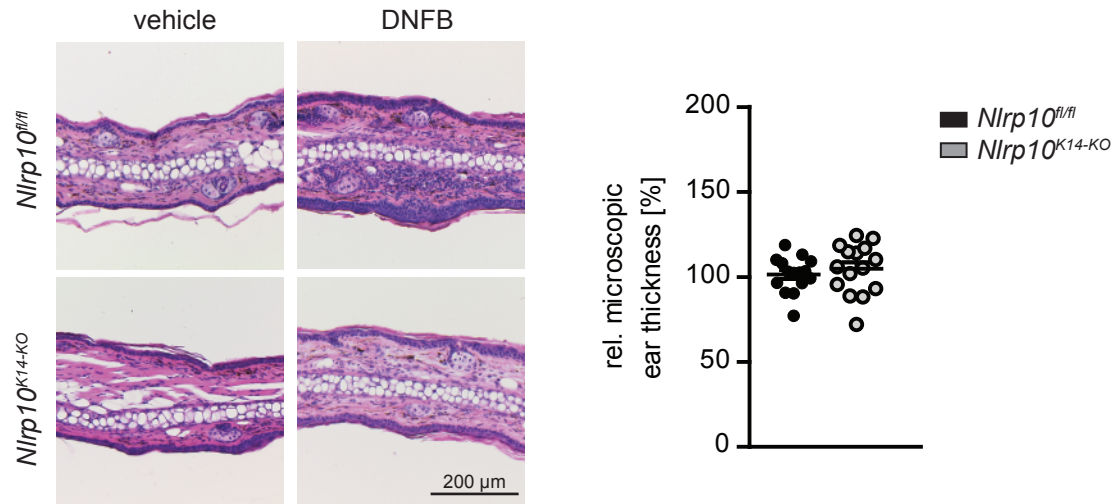


Supporting Information Fig. 1. Characterization of *Nlrp10*^{K14-KO} mice.
(A) gDNA was isolated from liver and tail biopsies from *Nlrp10*^{fl/fl}, *Nlrp10*^{-/-} and *Nlrp10*^{K14-KO} mice and analyzed by genotyping PCRs. (B) RT-PCR for *Nlrp10* and *actin* in samples of the dermis and epidermis of the indicated animals. (D:dermis, E: epidermis) (C) H&E staining of 5 µm skin sections. Original magnification x100. e: epidermis; d: dermis; s: subcutis; m: muscularis



Supporting Information Fig. 2. Histological analysis of vehicle and DNFB treated ears of *Nlrp10^{fl/fl}* and *Nlrp10^{-/-}* mice.

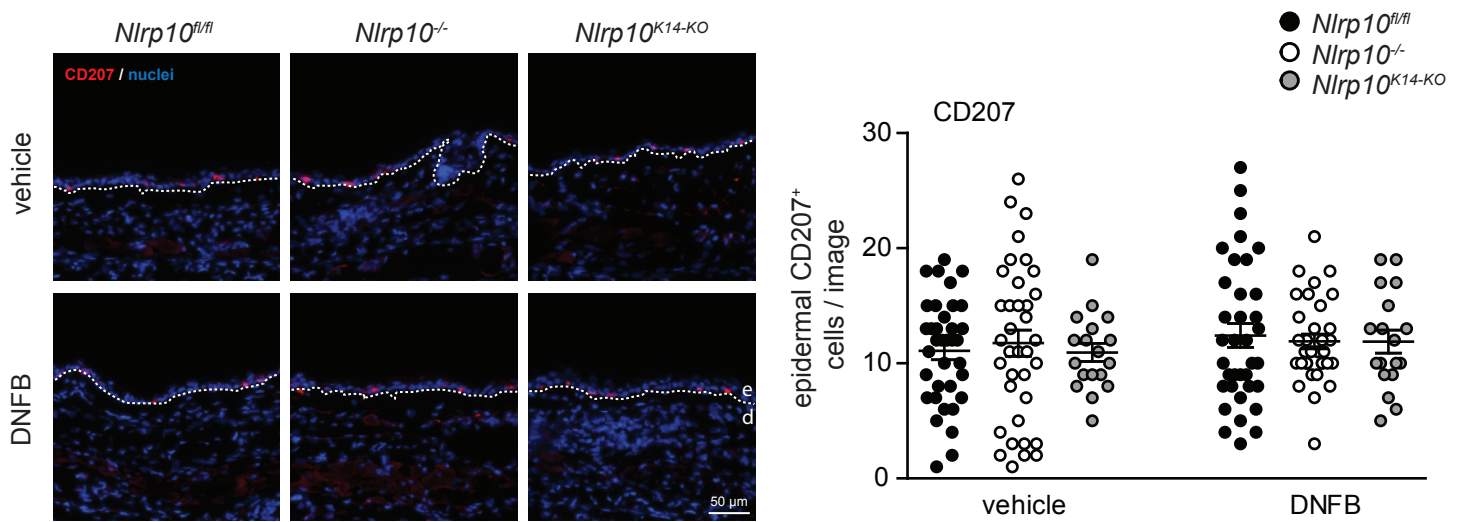
(A) 5 μ m sections of paraffin embedded ears of vehicle or DNFB treated ears from the indicated strain were stained with H&E. Original magnification x100. (B) Thickness of ears was measured in digital images of 4 mice per genotype at 3 positions per ear. Thickness of DNFB treated ears was normalized to the corresponding vehicle treated ear. Data are expressed as mean $\pm$ SD.



Supporting Information Fig. 3. Resolution of DNFB induced DTH in *Nlrp10^{K14-KO}* mice.

5 μ m sections of paraffin embedded ears of vehicle or DNFB treated ears sampled 96 h after challenge were stained with H&E. Thickness of ears was measured in digital image of 4 mice per genotype at 3 positions per ear. Thickness of DNFB treated ears was normalized to the corresponding vehicle treated ear. Data are expressed as mean $\pm$ SEM.

Original magnification x100. e: epidermis; d: dermis; s: subcutis; m: muscularis



Supporting Information Fig. 4. Characterization of the effect of NLRP10 on CD207⁺ langerhans cells. Quantification of epidermal CD207⁺ cells in ears sampled 24 h after DNFB challenge in 3 sections per ear. Dotted line marks dermal-epidermal junction. n=6 for *Nlrp10^{fl/fl}* and *Nlrp10^{-/-}*, n=3 for *Nlrp10^{K14-KO}*. Data represent mean ± SEM.