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NKG2D-dependent activation of dendritic epidermal T cells in contact hypersensitivity

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Abstract

The interaction between keratinocytes (KC) and skin-resident immune cells plays an important role in induction of contact hypersensitivity (CHS). A specific subset of γδ T cells termed dendritic epidermal T cells (DETC) are located in mouse epidermis, and we have recently shown that DETC become activated and produce IL-17 in an IL-1β-dependent manner during CHS. Various receptors on DETC, including NKG2D, are involved in DETC responses against tumors and during wound healing. The ligands for NKG2D (NKG2DL) are stress-induced proteins such as Mult-1, H60, Rae-1 in mice and MICA, MICB and ULBP in humans. Here, we show that allergens up-regulate expression of the NKG2DL Mult-1, H60 and Rae-1 in cultured mouse KC and of MICA in primary human KC. We demonstrate that Mult-1 is expressed in mouse skin exposed to allergen. Furthermore, we find that the vast majority of DETC in murine epidermis and skin-homing cutaneous lymphocyte-associated antigen (CLA) positive γδ T cells in humans express NKG2D. Finally, we demonstrate that blocking of NKG2D partially inhibits allergen-induced DETC activation. These findings demonstrate that NKG2D and NKG2DL are involved in allergen-induced activation of DETC and indicate that the NKG2D/NKG2DL pathway might be a potential target for treatment of CHS.

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Introduction

Contact hypersensitivity (CHS) is a T cell-mediated inflammatory skin disease induced by exposure of the skin to contact allergens. Within the last 20 years it has become clear that keratinocytes (KC) not only are the main targets for allergen-specific T cells, but also directly respond to allergens. Thus, KC exposed to allergens produce a variety of pro-inflammatory cytokines and chemokines (Cumberbatch *et al.*, 1997;Cumberbatch *et al.*, 2001;Enk and Katz, 1992), and the interactions between KC and skin-resident immune cells are central for initiation of the response to contact allergens (Kaplan *et al.*, 2012).

A tissue specific subtype of γδ T cells termed dendritic epidermal T cells (DETC) is found in mouse epidermis (MacLeod and Havran, 2011). Arising from fetal precursors and expressing a canonical Vγ3Vδ 1 TCR (Garman nomenclature) (Garman *et al.*, 1986), DETC are important for wound healing, tumor clearance and tissue homeostasis (Girardi *et al.*, 2001;Jameson *et al.*, 2002;Jameson *et al.*, 2004;Sharp *et al.*, 2005). Furthermore, it has been shown that DETC play an important role in the response to contact allergens (Cruz, Jr. *et al.*, 1989;Dieli *et al.*, 1997;Dieli *et al.*, 1998;Girardi *et al.*, 2002;Huber *et al.*, 1995;Nielsen *et al.*, 2014;Sullivan *et al.*, 1986;Welsh and Kripke, 1990). Interestingly, a previous study showed that allergen-induced DETC activation was mediated via KC by a cell-cell contact-dependent mechanism, and although DETC activation was not MHC-dependent, activation could be partially blocked by treatment with anti-CD3 antibodies (Huber *et al.*, 1995). In accordance, we recently found that allergens do not directly, but rather indirectly, activate DETC by induction of IL-1β production and expression of still unidentified ligands on KC (Nielsen *et al.*, 2014).

DETC express co-stimulatory receptors such as NKG2D, JAML and CD100 (Girardi *et al.*, 2001;Whang *et al.*, 2009;Witherden *et al.*, 2010;Witherden *et al.*, 2012). Several NKG2D ligands (NKG2DL) have been identified in both humans and mice. In mice, these include retinoic acid early inducible-1 (Rae-1)α-ε, histocompatibility (H60)a–c and mouse UL16-binding protein-like transcript 1 (Mult-1) (Carayannopoulos *et al.*, 2002;Cerwenka *et al.*, 2000;Malarkannan *et al.*, 1998;Raulet *et al.*, 2013;Whang *et al.*, 2009). In humans, MHC class I – chain related A and B (MICA and MICB) and UL-16-binding proteins (ULBP) have been identified as NKG2DL (Bauer *et al.*, 1999). Current evidence indicates that NKG2DL are either absent or only weakly expressed by resting cells but become up-regulated as a result of cellular stress, infection or tumorgenesis (Raulet *et al.*, 2013). Both Rae-1 and H60 play critical roles in immune responses against tumors in the skin and during wound healing (Strid *et al.*, 2008;Yoshida *et al.*, 2012). However, a role for Mult-1 in skin immunology has not yet been described.

Evidence of NKG2D-mediated DETC activation was first provided in a study which found that DETC can kill carcinoma cells by an NKG2D-sensitive mechanism (Girardi *et al.*, 2001). In line with this, recent studies found impaired wound healing properties of DETC when NKG2DL were blocked (Jung *et al.*, 2012;Yoshida *et al.*, 2012). Whether NKG2D ligation is sufficient for activation of DETC by it-self or only provides co-stimulatory signals is still a matter of debate (Nitahara *et al.*, 2006;Strid *et al.*, 2008). It has been described that KC-specific up-regulation of Rae-1 has the potential to directly activate

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DETC without an apparent need for TCR engagement (Strid *et al.*, 2008). Whether H60c directly induces DETC activation is still unclear (Strid *et al.*, 2011;Whang *et al.*, 2009). On study found that H60c expressed in damaged skin failed to activate DETC directly but only provided co-stimulatory signals (Whang *et al.*, 2009). In contrast, Strid et al showed that H60c directly induced DETC activation as measured by induction of IL-13 production (Strid *et al.*, 2011). Thus, it might be suggested that different NKG2DL are involved in regulation of DETC responses by different mechanisms depending on the type of stimulation received by the skin. Interestingly, NKG2D^{+} NK cells and NKG2D^{+}CD8^{+} T cells have been shown to be involved in the allergic response in patients with nickel allergy and in a mouse-model for palladium allergy, respectively (Carbone *et al.*, 2010;Kawano *et al.*, 2014). In the mouse model it was found that exposure of the skin to palladium increased expression of H60c in the skin, and that blocking of NKG2D reduced the allergic response to palladium, indicating that H60c plays an important role in activation of NKG2D^{+}CD8^{+} T cells (Kawano *et al.*, 2014). Whether palladium specifically induced H60c expression on KC or other cells of the skin was not determined.

The aim of the present study was to examine whether allergens induce NKG2DL expression on mouse and human KC, and to determine the role of NKG2D signaling in CHS. We found that the experimental allergens 2,4-dinitrobenzenesulfonic acid (DNBS)/2,4-dinitrofluorobenzene (DNFB) induce rapid up-regulation of the NKG2DL H60, Rae-1 and Mult-1 in the mouse KC cell line PAM2.12 and of Mult-1 in mouse skin. Likewise, we found that the common allergen nickel induces up-regulation of MICA in primary human KC. We found that DETC are the only cells expressing NKG2D in mouse epidermis and that CLA^{+} skin-homing γδ T cells, CD8^{+} T cells and NK cells in humans all express NKG2D. Finally, we found that anti-NKG2D antibodies block DETC activation both *in vitro* and *in situ*. Thus, our study demonstrates that NKG2DL and NKG2D are involved in allergen-induced interactions between KC and DETC indicating that the NKG2D/NKG2DL pathway might be a potential target for treatment of CHS.

Results

DNBS induces up-regulation of the NKG2DL Mult-1, Rae-1 and H60 on PAM2.12 cells

We have recently shown that DETC play an important role in the allergic response to contact allergen (Nielsen *et al.*, 2014). The allergen-induced DETC activation was indirect and mediated by a combination of IL-1β and activating ligands expressed on allergen-exposed KC (Nielsen *et al.*, 2014). As NKG2DL might activate DETC independently of TCR stimulation, we wanted to investigate if allergens induce the expression of NKG2DL on KC. Consequently, we stimulated PAM2.12 cells with DNBS and determined the expression levels of Mult-1, Rae-1 and H60 by flow cytometry at various time points. DNBS treatment induced up-regulation of all tested NKG2DL on PAM2.12 cells (Figure 1a and b,). Mult-1 and Rae-1 were both rapidly up-regulated as early as 4–6 hours after DNBS exposure (Figure 1a and b). In contrast, H60 expression was not up-regulated until 24 – 48 hours of allergen exposure (Figure 1a and b). Comparable expression levels were fund on medium treated cells stained with either specific NKG2DL antibodies or isotype control (Supplementary Figure S1).

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Next we investigated whether NKG2DL were expressed in allergen-exposed skin. Mice were painted with the indicated concentrations of DNFB in a 1:4 olive oil:acetone (OOA) mixture or the pure OOA mixture (vehicle) on the dorsal side of both ears. 24 hours later the mice were euthanized and proteins were purified from the ears and analyzed by Western blotting using GAPDH as loading control. Equal amounts of protein were applied in each sample. As it was seen that DNFB treatment impaired GAPDH expression, especially in the high concentration, we made direct comparisons of Mult-1 expression in DNFB-treated ears compared to vehicle-treated ears. By this analysis we found a significant 1.5 fold up-regulation of Mult-1 in all DNFB-treated groups when compared to vehicle-treated mice (Figure 1c and d). We could not detect Rae-1 and H60 by Western blot analysis. To further investigate the expression of NKG2DL in allergen-exposed skin, we made a time-course experiment determining the expression of NKG2DL mRNA after treatment with 0.05% DNFB for 6, 12, 24 and 48 hours. We applied this low concentration of DNFB as the Western blot analyses indicated that this concentration did not affect GAPDH expression, which was used as reference in the qPCR experiments. In accordance with the Western blot analyses, the qPCR analyses demonstrated that Mult-1 mRNA was up-regulated after 24 hrs but then became down-regulated after 48 hours (Figure 1e). We could not detect Rae-1 and H60c mRNA by qPCR. To directly determine whether Mult-1 was expressed in the epidermis and/or dermis following exposure of the skin to DNFB, we exposed mice to 0.05% DNFB for 24 hours. The mice were subsequently killed and the ears were split into epidermis and dermis. Mult-1 expression was determined separately in the isolated epidermis and dermis by qPCR. We found that allergen exposure induced Mult-1 expression in the epidermis but not in the dermis (Figure 1f).

DETC are the only cells that express NKG2D in mouse epidermis

Next we wanted to determine the subsets of cells in healthy and allergen-exposed mouse epidermis that express NKG2D. It has been reported that ILC, NKT cells, $CD8^+$ T cells and γδ T cells can express NKG2D. Mice were treated with either 0.15% DNFB or vehicle on their ears for 24 hours and epidermal cell suspensions were subsequently prepared and analyzed by flow cytometry. In both vehicle- and DNFB-treated mice all NKG2D expressing cells were $CD3^+$ (Figure 2a). Of the total $NKG2D^+CD3^+$ population DETC constituted more than 98% of the cells from both vehicle and DNFB-treated mice (Figure 2b). These results demonstrate that DETC constitute the major NKG2D expressing cell subset in both healthy and allergen-exposed mouse epidermis.

Nickel induces up-regulation of MICA expression in human keratinocytes

Although no equivalent to DETC is found in human skin, NKG2D expressing γδ T cells are found in both human dermis and epidermis (Bos *et al.*, 1990;Ebert *et al.*, 2006). Therefore, we next investigated the effect of nickel on the expression of the human NKG2DL MICA and MICB on primary human KC. Nickel stimulation induced a 2.5-fold increase of MICA expression while MICB expression was unaffected (Figure 3a and b, data not shown). Next we determined the expression of NKG2D on skin-homing lymphocytes in human peripheral blood from healthy individuals. Blood samples were collected from 7 healthy individuals and PBMC were purified and analyzed by FACS. CLA was used as a marker for skin-homing lymphocytes. γδ ($CD3^+\gamma\delta^+CLA^+$), CD8 ($CD3^+CD8^+CLA^+$), and CD4

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($CD3^+CD4^+CLA^+$) T cells and NK ($CD3^-CD4^-CD8^-\gamma\delta^-CD56^{high}$) cells were analyzed for NKG2D expression (Figure 3c and d). We found that an average of 98% of CLA^+ γδ T cells express NKG2D (Figure 3c and d). In addition, 95% of CLA^+ CD8 T cells and 89% of CLA^+ NK cells also expressed NKG2D (Figure 3c and d). In contrast, only 1% of CLA^+ CD4 T cells were found to express NKG2D (Figure 3c and d). Thus, human γδ T cells, $CD8^+$ T cells and NK cells with skin-homing potential all express NKG2D and have the ability to react with NKG2DL.

Blocking NKG2D signaling down-regulates allergen-induced IFNγ production by DETC

Having observed allergen-induced NKG2DL up-regulation on KC, we next performed co-culture experiments with allergen pre-treated PAM2.12 cells and the DETC cell line 7–17. PAM2.12 cells were pre-treated with various concentrations of DNBS for 24 hours and the DETC 7–17 cells were subsequently added. We found that co-culturing of DETC 7–17 cells with PAM2.12 cells induced an allergen-dependent increase in the number of $IFN\gamma^+$ DETC (Figure 4a and b). Blocking NKG2D signaling with anti-NKG2D antibodies during the co-culture significantly down-regulated the number of $IFN\gamma^+$ DETC (Figure 4b).

Blocking NKG2D in situ partially blocks allergen-induced DETC activation

As a sign of DETC activation, a change in DETC morphology from dendritic to round is observed during wound healing and following allergen exposure (Jameson *et al.*, 2002;Nielsen *et al.*, 2014). To determine if NKG2D signaling is involved in allergen-induced DETC activation in the skin, we treated ear-sheets with either medium, DNBS alone or DNBS in combination with blocking anti-NKG2D antibodies for 24 hours and subsequently studied DETC morphology. Medium-treated ear-sheets mostly contained resting DETC (>2 dendrites), whereas the majority of DETC had a round morphology (0 dendrites) following DNBS treatment (Figure 5a and b). Interestingly, when blocking anti-NKG2D antibody was added together with DNBS, rounding up of DETC was inhibited and the majority of DETC still expressed 1–2 dendrites (Figure 5a and b). Thus, the allergen-induced changes in DETC morphology seem partially driven by NKG2D signaling.

Discussion

In this study we show that allergens induce expression of NKG2DL on keratinocytes from mice and humans, that DETC comprise the majority (98%) of cells in the epidermis that express NKG2D in mice and that the vast majority of human skin-homing γδ T cells, $CD8^+$ T cells and NK cells express NKG2D. Finally, we show that blocking anti-NKG2D antibodies partially inhibit allergen-induced DETC activation as measured by IFNγ production and DETC rounding.

In normal skin, Mult-1, Rae-1 and H60c are all expressed at very low levels (Girardi *et al.*, 2001;Nice *et al.*, 2009;Yoshida *et al.*, 2012). Transcripts encoding Rae-1 and H60c are up-regulated in the skin after 24 hours of treatment with carcinogen (Girardi *et al.*, 2001). Furthermore, the expression of H60c and Rae-1 is up-regulated in wounded skin, whereas Mult-1 is not (Jung *et al.*, 2012;Whang *et al.*, 2009;Yoshida *et al.*, 2012). In the present study, we found that allergens induce up-regulation of Mult-1, Rae-1 and H60 on PAM2.12

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cells in a time-dependent manner. We found that Mult-1 became significantly up-regulated in skin treated with DNFB, whereas we were unable to detect Rae-1 and H60 in both DNFB and vehicle treated skin. Furthermore, we found that Mult-1 is specifically up-regulated in the epidermis and not in the dermis following allergen exposure. Interestingly, while Mult-1 plays a role in the defense against tumors and in the activation of NK cells and macrophages (Diefenbach *et al.*, 2003), a role for Mult-1 in immune responses in the skin has not been described previously. In a mouse model of palladium allergy, it was recently shown that the transcript of H60 is up-regulated in skin 3 hours after palladium exposure, while Rae-1 transcripts were not up-regulated (Kawano *et al.*, 2014). These differences in NKG2DL expression could be explained if different allergens induce expression of different NKG2DL or if the expression of the various NKG2DL is induced with different kinetics depending on the dose and potency of the allergen. However, when we examined the time-course of allergen-induced Mult-1, H60 and Rae-1 expression, we were only able to detect Mult-1 and only after 24 hours of treatment. As we used a low concentration of DNFB in these experiments to avoid affecting GAPDH expression, we cannot rule out that higher doses of DNFB might induce Rae-1 and H60. In contrast to what we observed in the skin, DNBS treatment of PAM2.12 induced expression of all three ligands. This could be due to increased sensibility to DNBS/DNFB of PAM2.12 compared to normal keratinocytes, or it might indicate that Rae-1 and H60 expression actually are induced in normal keratinocytes by treatment of the skin with higher concentrations of DNFB. Further investigations are required to solve this question. Whether different allergens regulate the expression of the NKG2DL differently and thereby induce different types of immune response is unknown. However, just like some contact allergens induce Th1 responses while others induce Th2 responses (Hayashi *et al.*, 2001), we find it likely that the induction of various NKG2DL could be allergen specific.

In accordance with previous studies, we found that essentially all DETC express NKG2D suggesting that NKG2D and its ligands play an important role in DETC activation (Jamieson *et al.*, 2002;Nitahara *et al.*, 2006). However, whether DETC can be activated solely by NKG2D stimulation or whether simultaneous TCR signaling is required for DETC activation is a matter of debate (Ibusuki *et al.*, 2014;Nitahara *et al.*, 2006;Whang *et al.*, 2009;Yoshida *et al.*, 2012). Using recombinant NKG2DL to elucidate the functional outcome of NKG2D engagement on DETC, a recent study found that all NKG2DL tested triggers DETC degranulation but not cytokine production in the absence of TCR stimulation (Ibusuki *et al.*, 2014). The conflicting data may depend on different cell lines and blocking/ activating anti-NKG2D antibodies used in these studies. Indeed, different anti-NKG2D antibodies have completely different outcomes on DETC activation, ranging from no activation to induction of both degranulation and cytokine production in short term cultured DETC (Ibusuki *et al.*, 2014). In the present study, we found that blocking NKG2D partially inhibited the DNBS-induced rounding and IFNγ production by DETC. This indicates that other factors e.g. TCR ligation and/or cytokines work together with NKG2D to induce full DETC activation following exposure of the skin to allergens. In support of this, we have recently shown that IL-1β and anti-CD3 antibodies work synergistically in inducing IL-17A production by DETC, and that IL-1β enhanced the anti-CD3 antibody-induced IFNγ production by DETC (Nielsen *et al.*, 2014). IL-1β is an important cytokine in the immune

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response to contact allergens (Enk *et al.*, 1993). In our model of CHS we have shown that IL-1β is rapidly produced in the skin and that it can induce partial rounding of DETC (Nielsen *et al.*, 2014). However, the interplay between TCR stimulation, cytokines and NKG2D ligation in DETC activation and the role of these in induction of contact allergy needs further investigation.

Several studies have demonstrated that DETC are required for normal responses to contact allergens (Dieli *et al.*, 1997;Dieli *et al.*, 1998). Accordingly, we have recently shown that lack of γδ T cells results in a 50% reduction in allergen-induced ear-swelling, furthermore supporting that other cells, probably αβ T cells, play an important role in the allergen-induced inflammation (Nielsen *et al.*, 2014;Vocanson *et al.*, 2009). In addition, others have shown that DETC might play an anti-inflammatory role during the response to contact allergens (Girardi *et al.*, 2002). It could therefore be speculated that blocking NKG2D might not only inhibit the allergen-induced inflammation mediated by DETC, but that it might also block a possible anti-inflammatory effect. The present study indicates that blocking of NKG2D would reduce allergen-induced inflammatory responses in situ, but future *in vivo* studies are required to finally answer this question.

Although DETC are not found in human skin, human γδ T cells seem to be involved in immune responses in the skin (Cai *et al.*, 2011;Dyring-Andersen *et al.*, 2013;Laggner *et al.*, 2011;Toulon *et al.*, 2009). Thus, skin-resident γδ T cells are involved in wound healing (Toulon *et al.*, 2009), and human IL-17-producing γδ T cells are involved in the pathogenesis of psoriasis (Cai *et al.*, 2011;Laggner *et al.*, 2011). In addition, we have recently shown that γδ T cells are recruited to the skin of patients with nickel allergy following exposure of their skin to nickel (Dyring-Andersen *et al.*, 2013). The role of γδ T cells in human contact allergy is still not clear but our results indicate that it might be pro-inflammatory by production of IL-17, IL-22 and IFNγ (Dyring-Andersen *et al.*, 2013). Interestingly, in the present study we found that nickel induces up-regulation of MICA in primary human KC. Furthermore, we found that 98% of CLA^+ γδ T cells express NKG2D, suggesting that interactions between NKG2D and NKG2DL might play an important role in allergen-induced γδ T cell activation in man as in mice. In addition, both CLA^+CD8^+ T cells and CLA^+ NK cells express NKG2D and might also be activated by KC expressing MICA like the γδ T cells (Carbone *et al.*, 2010).

In conclusion, we show that allergens induce up-regulation of NKG2DL on KC and that NKG2D signaling plays a role in allergen-induced DETC activation. Thus, blocking the interaction between NKG2D and NKG2DL might be a potential target for future treatment of allergic contact dermatitis.

Materials and Methods

Mice

Female C57Bl/6 mice were purchased from Taconic (Ry, Denmark). Mice were housed in specific pathogen-free facilities at the Department of Experimental Medicine, Faculty of Health and Medical Sciences, University of Copenhagen in accordance with national animal protection guidelines (license number: 2012-2934-00663).

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Cells and culture conditions

The murine keratinocyte cell line, PAM2.12 was maintained as previously described (Nielsen *et al.*, 2014). The 7–17 DETC cell line was cultured in RPMI 1640 (Sigma Aldrich, Brøndby, DK) supplemented with 10% FBS and 20U/ml rIL-2. Medium was changed every other day and cells were split when they were two-thirds confluent. Human primary keratinocytes derived from healthy adult skin (ATCC, Manassa, VA, USA) were cultured in KGM-Gold Medium (Lonza, Slangerup, Denmark). All cells were maintained at 37°C, 5% CO_2. After obtaining written informed consent in accordance with the Helsinki principles for research involving human subjects, peripheral blood mononuclear cells (PBMC) were purified from blood of 7 healthy individuals using Lymphoprep (Nycomed, Oslo, Norway). The study was approved by The Committees of Biomedical Research Ethics for The Capital Region in Denmark (H-4-2011-091)

Materials and reagents

2,4-dinitrofluorobenzene (DNFB), 2,4-dinitrobenzenesulphonic acid (DNBS), trypsin from bovine pancreas, phorbol 12-myristate 13-acetate (PMA), ionomycin, monensin and recombinant murine IL-2 (I0523) were purchased from Sigma Aldrich (Brøndby, Denmark). Anti-mouse-NKG2D (C7), anti-mouse-NKG2D (CX5) and fixable viability dye eFluor780 were purchased from eBioscience (San Diego, CA, USA). Anti-human-CD8 (RPA-T8), anti-human-CD4 (OKT4), anti-human-CD3 (OKT3), anti-human-NKG2D (1D11) and anti-human-CD56 (HCD56) were purchased from Bio-Legend (San Diego, CA, USA). Anti-mouse-CD16/CD32 (2.4G2), anti-mouse-γδ TCR (GL3), anti-mouse-Vγ3 (536), anti-mouse-CD3ε (145-2C11), anti-mouse-IFN-γ (XMG1.2), anti-human γδ TCR (B1.1), anti-human/mouse CLA (HECA-452), streptavidin-APC-Cy7 (554063), Cytofix/Cytoperm (554722) and Perm/Wash buffer (554723) were all purchased from BD Biosciences (Albertslund, DK). Anti-mouse-Rae-1 (237104), anti-mouse-H60 (205326), anti-mouse-Mult-1 (186107), antimouse-NKG2D (191004), anti-human-MICA (159227), anti-human-MICB (236511) and mouse IgG2B isotype control (133303) were purchased from R&D Systems (Abingdon, UK). StemPro Accutase (A11105-01) was purchased from Life Technologies (Nærum, DK).

Sensitization protocol

Mice were painted with 25 μl of DNFB in a 1:4 olive oil:acetone (OOA) mixture on the dorsal side of both ears. The concentrations of DNFB (0.05%, 0.15% and 0.5%) used are indicated in the respective experiments. Vehicle treated mice were painted with OOA. 24 hours later mice were killed and ears were collected for protein purification and subsequent Western blotting or FACS analysis.

Flow cytometry

Epidermal single cell suspensions from ears of C57Bl/6 mice were obtained as previously described (Nielsen *et al.*, 2014). For FACS analysis of epidermal cell suspensions, PAM2.12 cells, co-culture cells and PBMC were stained and analyzed as previously described (Dyring-Andersen *et al.*, 2013;Nielsen *et al.*, 2014).

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Western Blot

Biopsies from ears treated either with various concentrations of DNFB or vehicle for 24 hours were homogenized individually and lysed simultaneously using Precellys technology from Bertin Technologies (Montigny-le-Bretonneux, France). Homogenized ear tissue samples were adjusted to 50 μg protein using Bradford assay, and subjected to SDS-PAGE using pre-casted 10% Bis-Tris acrylamide gel (BioRad, Copenhagen, DK). Proteins were then blotted to a PVDF membrane by wet-transfer and probed for Mult-1, Rae-1, H60 and GAPDH (R&D Systems, Abingdon, UK). Relative Mult-1 expression of treated mice was calculated based on semi-quantification of the Mult-1 bands obtained by Western blotting relative to the control samples from mice treated with vehicle and normalized to the amount of loaded protein.

RNA purification and qPCR analysis

Mice were painted with 25 μl of 0.05% DNFB in OOA for the indicated time. In some experiments the ears were split into epidermis and dermis as previously described (Nielsen *et al.*, 2014). RNA was purified and qPCR analyses were performed as previously described (Bonefeld *et al.*, 2014). The following primer was used: Rae-1 (Mm00558293_g1), Ulbp (Mn01180648_m1), H60c (Mn0424326_m1) and GAPDH (Pre-Developed TaqMan Assay Reagents, Mouse GAPDH (20x) #4352932E) (Life Technologies, Naerum, Denmark).

Keratinocyte stimulation assay

PAM2.12 cells were plated at 2.5×10^5 cells/well in a 6 well plate and incubated overnight at 37°C. The following day cells were stimulated with DNBS for the indicated time and cells were harvested using StemPro Accutase and stained as described above. Primary human keratinocytes were incubated for 24 hours in the absence of hydrocortisone cells and subsequently stimulated with 10 μg/ml nickel chloride for 24 hours or medium control. Cells were loosened with trypsin, stained with anti-bodies to MICA and MICB and analyzed by flow cytometry.

PAM2.12 / DETC co-culture assay

DNBS was added at indicated concentrations to 3×10^4 of PAM2.12 cells in 96 well plates and incubated for 24 hours. The cells were subsequently washed to remove DNBS. One $\times$ 10^5 of 7–17 cells were seeded on the PAM2.12 cells and co-incubated for 4 hours. In some experiments DETC were pre-incubated for 15 min with 50 μg/ml anti-NKG2D (191004) antibodies as indicated.

Stimulation and preparation of ear sheets

Ear sheets were prepared by separating the dorsal and ventral sides of naïve C57Bl/6 ears. Subsequently ear-pieces were floated dermis-side down for 24 hours at 37°C in DMEM medium alone or DMEM medium containing either 0.1% DNBS or 0.1% DNBS plus 40 μg/ml anti-NKG2D (C7). The epidermal sheets were subsequently prepared for immunohistology as previously described (Nielsen *et al.*, 2014). A minimum of 200 cells per slide was counted from two individual experiments with 3 slides per treatment.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of interest

M.M.N is partly employed by LEO Pharma A/S and P.L. is employed by LEO Pharma A/S, which funded part of this project.

Abbreviations

CLA	Cutaneous lymphocyte-associated antigen
CHS	Contact hypersensitivity
DETC	Dendritic epidermal T cells
DNFB	2,4-Dinitrofluorobenzene
DNBS	2,4-Dinitrobenzenesulfonic acid
H60	Histocompatibility 60
ILC	Innate lymphoid cells
KC	Keratinocytes
MICA	MHC class I – chain related A
MICB	MHC class I – chain related B
Mult-1	Mouse UL16-binding protein-like transcript 1
NKG2DL	NKG2D ligand
PBMC	Peripheral blood mononuclear cells
Rae-1	Retinoic acid early inducible-1
ULBP	UL-16-binding protein

Reference List

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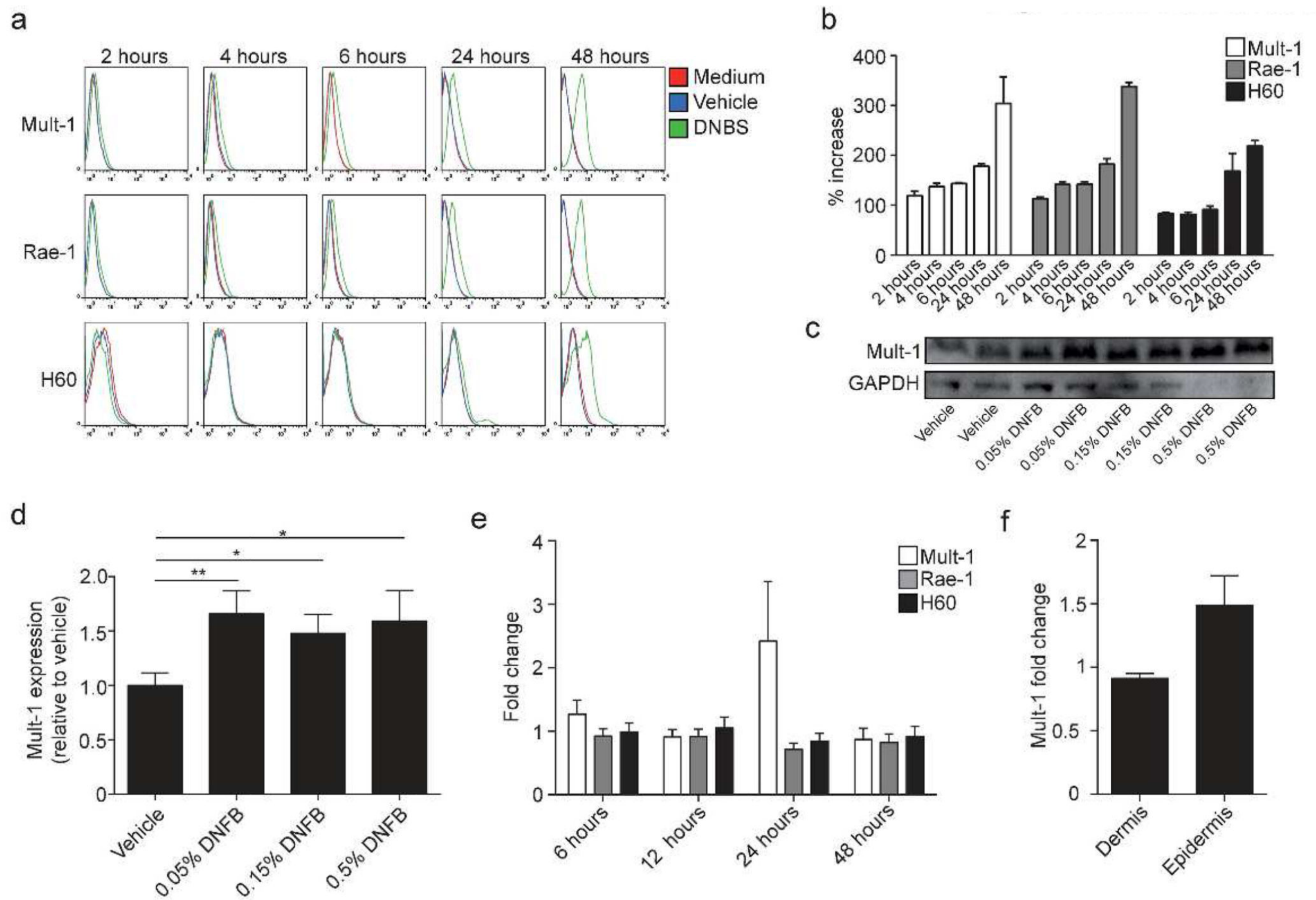

Figure 1. NKG2D ligands are up-regulated following allergen exposure

PAM2.12 cells were treated with vehicle or 0.1% DNBS for the indicated time and the expression levels of the co-stimulatory ligands Mult-1, Rae-1 and H60 were analyzed by FACS. (**a**) Histograms of the expression profile of co-stimulatory ligands. (**b**) Expression of co-stimulatory molecules as $MFI_{DNBS}/MFI_{Vehicle}$ at indicated time points. Values are shown as mean ± SEM for 3 independent experiments. (**c**) Representative Western blots showing Mult-1 and GAPDH expression 24 hours after vehicle or DNFB exposure. (**d**) Relative Mult-1 expression of DNFB compared to vehicle treated ears obtained by semi-quantification of the bands obtained by Western blot normalized to the amount of protein loaded. (**e**) Expression of Mult-1, Rae-1 and H60 normalized to GAPDH expression as measured by qPCR. (**f**) Expression of Mult-1 normalized to GAPDH in epidermis and dermis as measured by qPCR. Data are mean ± SEM of two experiments.* = $p<0.05$, ** = $p<0.01$.

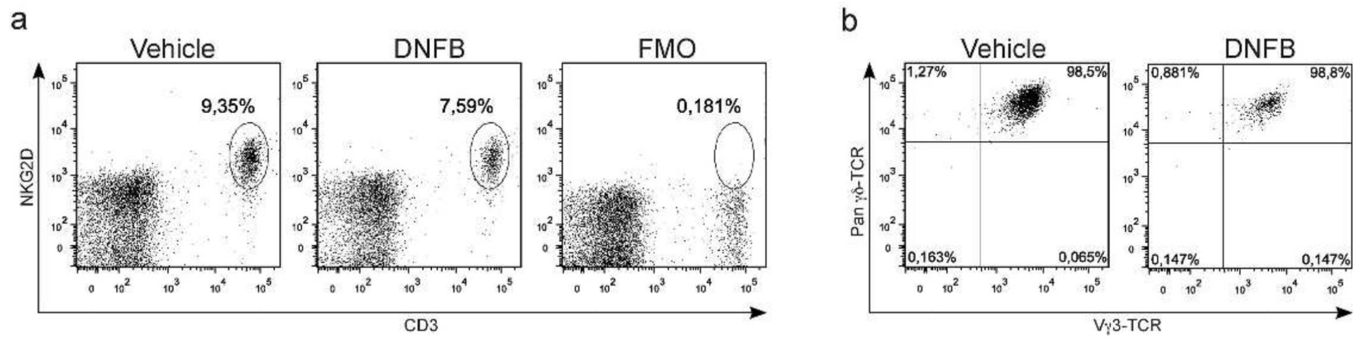

Figure 2. DETC are the only NKG2D expressing cells in resting and sensitized skin

C57Bl/6 mice were exposed to vehicle (OOA) or DNFB on the dorsal side of both ears. Epidermal single cell suspensions were prepared and NKG2D expression was analyzed by FACS. (**a**) Representative FACS plots of cells stained with antibodies against CD3 and NKG2D. Fluorescent minus one (FMO) shows cells stained with all antibodies except the anti-NKG2D antibody. (**b**) Representative FACS plots of the $NKG2D^{+}CD3^{+}$ cells (gates shown in **a**) stained with antibodies against Vγ3 and pan-γδ (GL3). Data represent two individual experiments with 4 mice per group.

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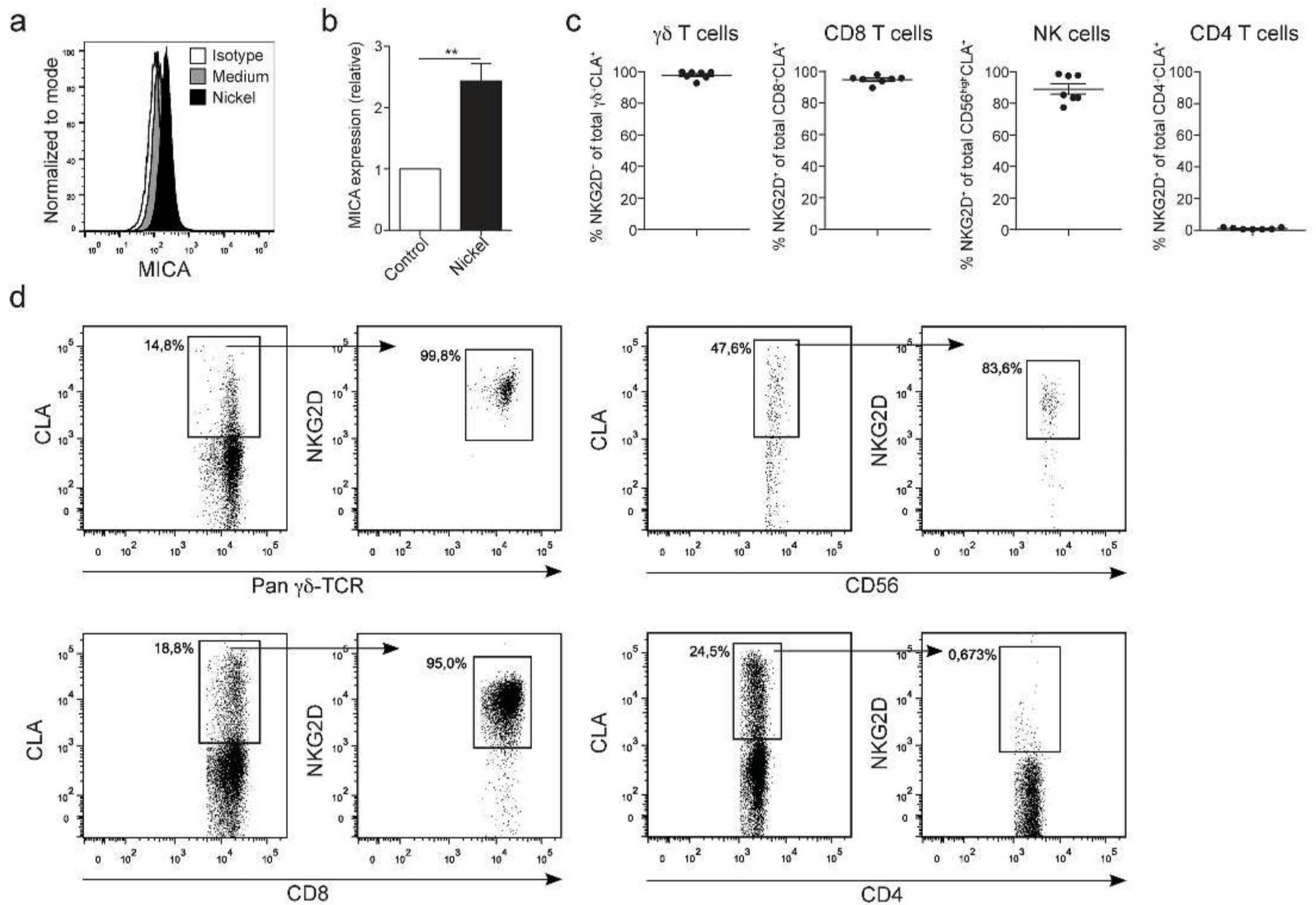

Figure 3. MICA expression on human KC following nickel stimulation and NKG2D expression profile on skin homing lymphocytes

Human primary KC were treated with 10 μg/ml nickel chloride for 24 hours and the expression level of MICA was determined as mean fluorescent intensity (MFI) using FACS. (**a**) Histogram of MICA expression. (**b**) Fold increase expression of MICA on nickel treated KC compared to vehicle treated KC. Data are mean ± SEM from 4 individual donors. (**b**) The percentage of NKG2D$^+$ cells of each CLA$^+$ subset from 7 healthy individuals. (**c**) Representative FACS plots of skin-homing (CLA$^+$) γδ$^+$, CD8$^+$, CD4$^+$ T cells and NK cells, and the NKG2D expressing percentage of each CLA$^+$ subset. Data are mean ± SEM.

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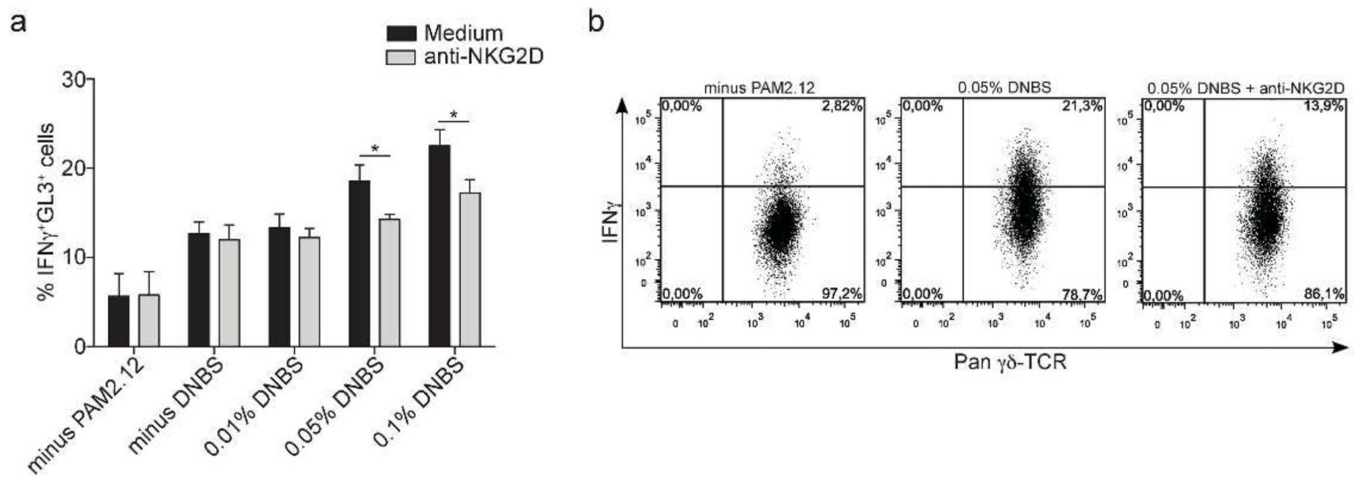

Figure 4. Allergen-induced PAM2.12-mediated DETC activation is significantly downregulated when NKG2D signaling is abrogated

(**a**) PAM2.12 cells were pre-treated with indicated concentrations of DNBS for 24 hours and 7–17 cells were subsequently seeded on the PAM2.12 cells in the presence (grey bars) or absence (black bars) of anti-NKG2D antibodies. (**b**) Representative FACS plots of IFNγ producing DETC following the indicated treatment. Data show mean ± SEM of three individual experiments. * = $p<0.05$.

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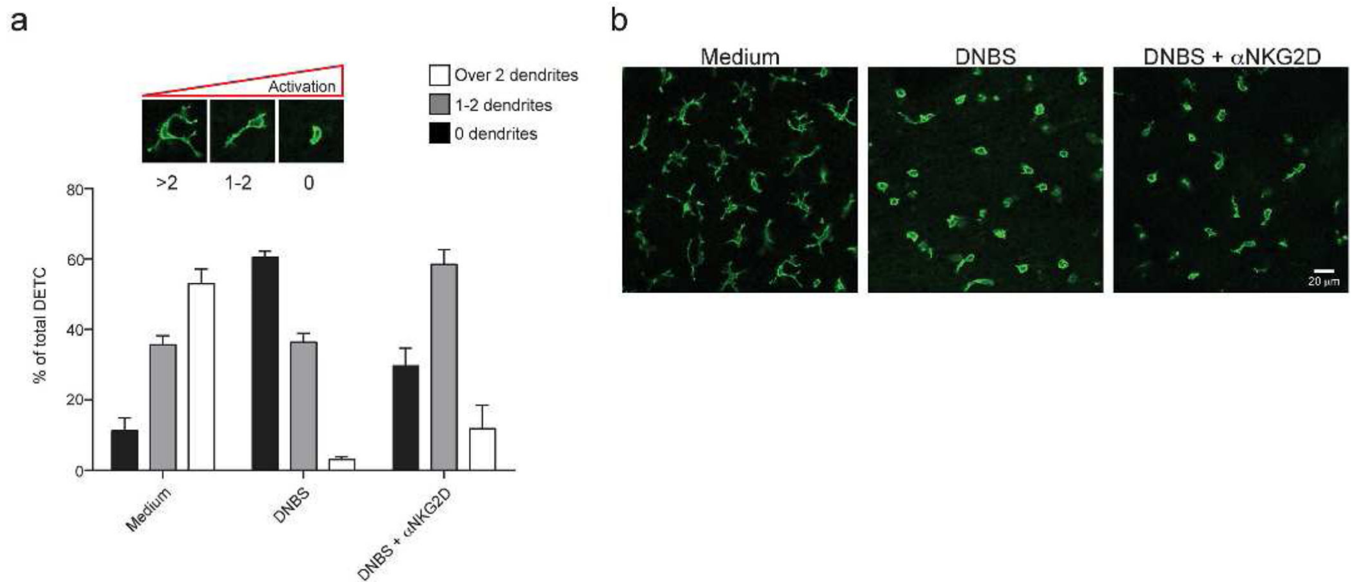

Figure 5. Anti-NKG2D partially blocks *in situ* DETC activation in response to DNBS exposure

Ear-sheets were treated with DNBS or DNBS + anti-NKG2D antibodies for 24 hours and DETC morphology was subsequently assessed by confocal microscopy. (**a**) Insert show examples of confocal microscopy images of DETC stained with anti-γδ TCR (green). Changes in DETC morphology (>2 dendrites = resting; 1–2 dendrites = partially activated; 0 dendrites = activated) was assessed in a blinded manner. (**b**) Representative images from each ear-sheet treatment, scale bar is 20μm. A minimum of 200 cells was counted from two individual experiments with 3 slides pr. treatment. Data are presented as mean ± SEM.