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Langerhans cells and NK cells cooperate in the inhibition of chemical skin carcinogenesis

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ABSTRACT

Tissue immunosurveillance is an important mechanism to prevent cancer. Skin treatment with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), followed by the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA), is an established murine model for squamous cell carcinoma (SCC). However, the innate immunological events occurring during the initiation of chemical carcinogenesis with DMBA remain elusive. Here, we discovered that natural killer (NK) cells and Langerhans cells (LC) cooperate to impair this oncogenic process in murine skin. The depletion of NK cells or LC caused an accumulation of DNA-damaged, natural killer group 2D-ligand (NKG2D-L) expressing keratinocytes and accelerated tumor growth. Notably, the secretion of TNFα mainly by LC promoted the recruitment of NK cells into the epidermis. Indeed, the TNFα-induced chemokines CCL2 and CXCL10 directed NK cells to DMBA-treated epidermis. Our findings reveal a novel mechanism how innate immune cells cooperate in the inhibition of cutaneous chemical carcinogenesis.

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Introduction

Non-melanoma skin cancer is one of the most prevalent types of neoplasia worldwide caused by accumulating UV-damage in skin cells. In addition, environmental exposure to chemicals such as arsenic and polycyclic aromatic hydrocarbons induces cutaneous carcinoma.[1] An experimental murine model for studying squamous cell carcinoma (SCC) is two-stage chemical carcinogenesis. During the first step, the tumor initiation, topical treatment with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) causes DNA-damage and Hras mutations in keratinocytes. The subsequent weekly administration of tumor-promoting agents like 12-O-tetra-decanoyl-phorbol-13-acetate (TPA) facilitates clonal outgrowth of transformed keratinocytes and inflammation-driven tumorigenesis. The combination of genetic mutations and inflammatory processes leads to the development of papilloma followed by conversion to malignant carcinoma.[2,3] Similar to what has been described in UV-irradiated skin, the first event during chemical carcinogenesis is the induction of DNA-double strand breaks in skin cells, which make the cells permissive for mutations.[2] At the same time, DNA-damaged cells express natural killer group 2D-ligand (NKG2D-L) on their surface allowing recognition of transformed cells by the immune system.[4,5] These cell surface proteins mark cells for clearance by cytotoxic immune cells expressing NKG2D, such as natural killer (NK) cells, $\gamma\delta$ T cells and $CD8^+$ cytotoxic T cells.[6,7]

To date, research on cutaneous chemical carcinogenesis focused mainly on the tumor promotion phase mediated by TPA, and the adaptive immune system proved to be important for tumor control. The main effector cells are $\gamma\delta$ and $\alpha\beta$ T cells that either inhibit tumor development by eliminating transformed cells or promote tumor progression.[8,9] In light of their imminent role in tumor immunity,[10] skin resident dendritic cells (DC) will most likely contribute to the immunological control of the tissue to prevent cutaneous cancer. Several subsets of DC exist in the skin with different context-dependent functional properties.[11] The first indication that Langerhans cells (LC) are involved in chemical carcinogenesis came from studies using transgenic Langerin-DTA mice that lack LC throughout life due to the expression of the toxic diphtheria toxin (DT) subunit A under control of the human *langerin*-promoter.[12] LC were reported to exert a pro-tumorigenic role due to their ability to metabolize DMBA to its carcinogenic form[13] and to drive inflammation during the promotion phase with TPA.[14]

Up to date, the immunological events occurring during tumor initiation with the carcinogen DMBA have not been

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Supplemental data for this article can be accessed on the publisher's website.

elucidated, as most studies concentrated on the tumor promotion phase. Thus, we focused on the role of innate immune cells, which are able to rapidely recognize and eliminate transformed tissue cells. Intriguingly, our findings indicate an essential collaboration between LC and NK cells in the immunosurveillance of the skin to prevent cancer.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Ncr1^{gfp} mice (provided by K. Kotsch and C. Fabritius, Department of Visceral, Transplant and Thoracic Surgery, Center for Operative Medicine, Medical University of Innsbruck, Austria)[15] and Langerin-DTR mice[16] were bred in the animal facility of the Department of Dermatology, Venereology and Allergology at the Medical University of Innsbruck. All experimental protocols were approved by the Austrian Federal Ministry of Science and Research and performed according to institutional guidelines.

Chemical carcinogenesis

The initiation phase of chemical carcinogenesis was investigated after topical application of 50μg DMBA (Sigma-Aldrich) in 20μL acetone (VWR) on the ear skin of 7–12 week old C57BL/6, Langerin-DTR and Ncr1^{gfp} mice. Langerin$^+$ DC were depleted by intraperitoneal injection of DT (Merck) 2 d before the start of experiments.[17] For tumor experiments 100μg DMBA in 100μL acetone was topically applied on mouse back skin one week after shaving followed by depilatory cream (Veet). C57BL/6 mice are quite resistant to TPA promotion during two-step carcinogenesis,[18,19] so we modified the protocol to three applications of 15μg TPA (Sigma-Aldrich) per week in 100μL acetone. Cutaneous papillomas were counted and scored weekly. At the conclusion of experiments, tumors were excised and sections were stained with hematoxylin and eosin (Gatt Koller) for histological analysis and confirmation of conversion to carcinoma.

Quantification of gene expression

Whole skin or enzymatically separated epidermis and dermis (1.2 U/mL Dispase (Roche Diagnostics), 30 min at 37°C) were used for RNA preparation. Total RNA was extracted using TRIzol® Reagent (Gibco) according to the manufacturer's instructions. RNA integrity was analyzed by electrophoresis on 1.5% agarose gels (Sigma-Aldrich) after sample preparation in 2× RNA Loading Dye (Fisher Scientific). Random primed cDNA was prepared (SuperScript® II RNase H-reverse transcriptase, Fisher Scientific) from total RNA. Genomic DNA was removed from samples by DNase treatment (Ambion). Quantitative polymerase chain reaction (PCR) analysis was performed by real-time PCR (detection system CFX96, Bio-Rad) using the Brilliant III Ultra-Fast Quantitative PCR Kit from Agilent technologies. Probes and primers were purchased from Fisher Scientific and are listed in Table S1. Sequences for probes and primers specific for TATA binding protein were selected using Primer Express software (Thermo Fisher Scientific) and synthesized by Microsynth.

Preparation of skin cell suspensions

Murine ear skin was cut into small pieces and digested with 0.15 mg/mL Liberase^{TM} and 0.12 mg/ml DNAse I (both Roche Diagnostics) for 45 min at 37°C and pressed through 100μm cell strainers (BD Biosciences). Epidermal cells were isolated by incubating the skin epidermal side up in 0.6% trypsin (Sigma-Aldrich) for 25 min at 37°C. The epidermis was peeled off, cut into smaller pieces and stirred for 25 min at 37°C in complete R10 medium consisting of RPMI (Lonza), 10% FCS (Pan Biotech), 50μg/mL gentamycin (Gibco) and 2 mM L-glutamine (Lonza). The dermis was further digested with 0.15 mg/mL Liberase^{TM} for 20 min at 37°C. Digested tissues were pressed through 100μm cell strainer to obtain single cell suspensions.

Flow cytometric analyses

Skin cell suspensions were analyzed by flow cytometry. Non-specific FcR-mediated antibody staining was blocked with anti-CD16/32 (2.4G2, in-house from hybridoma supernatant or from BD Biosciences) for 15 min at 4°C. Dead cells were excluded with 7AAD (BD Biosciences) or fixable viability dye eFluor® 780 (eBioscience). All antibody incubation steps were performed for 15 min at 4°C, anti-NKp46 mAb was pre-incubated at 37°C for 2 h. For intracellular stainings, cells were fixed, permeabilized and stained using Cytofix/Cytoperm^{TM} (BD Biosciences) or Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Proliferation of NK cells was measured by incorporation of BrdU (BD Biosciences), which was injected intraperitoneally at 2 doses of 1 mg 5 h before and during cytokine administration. The percentages of BrdU$^+$CD3$^-$NK1.1$^+$ NK cells in the epidermis were determined with a BrdU-FITC Flow kit (BD Biosciences) 16 h later. The antibodies used for staining skin cell suspensions are listed in Table S2. Cells were analyzed on a FACS Canto II (BD Biosciences) and with FlowJo software (Tree Star). For analysis, immune cells were always gated on viable CD45$^+$ cells.

Immunofluorescent staining of DNA-damage on skin sections

Paraffin-embedded skin sections were treated with citrate buffer (pH-value 6) for antigen retrieval. Then, sections were stained with an antibody against the phosphorylated form of histone 2A family member X (γH2AX, New England Biolabs) overnight at 4°C, followed by a secondary antibody goat anti-rabbit Alexa-Fluor594 (Invitrogen) for 1 h at room temperature. DAPI (Invitrogen) was used to counterstain cell nuclei. The γH2AX-positive cells were counted on two skin sections per mouse with an Olympus BX60 epifluorescence microscope using a 40× objective lense.

Antibody production and NK cell depletion

Hybridoma cells (clone PK136) were cultured in complete R10 medium followed by culture in serum-free hybridoma medium

(Gibco). The antibody anti-NK1.1 was purified from culture supernatants by protein G affinity chromatography (HiTrap Protein GHP, GE Healthcare Life Sciences) and gel filtration on Superdex 200 HR (GE Healthcare Life Sciences). For depletion experiments 200μg anti-NK1.1 mAb was injected intraperitoneally in 100μL PBS (Gibco).

Quantitative enzyme linked immunosorbent assays (ELISA)

Epidermal cell suspensions from Langerin-DTR mice priorly injected intraperitoneally with PBS, DT or anti-NK1.1 mAb were incubated with 16μg/mL DMBA for 6 h. TNFα protein levels were measured in the culture supernatants with mouse TNFα ELISA Ready-SET-Go!® from Affymetrix (eBioscience), according to the manufacturer's instructions.

Injection of recombinant murine TNFα, anti-TNFα mAb and chemokine receptor antagonists

C57BL/6 mice were injected intradermally into the ear skin with 200 ng recombinant murine TNFα (PeproTech). For neutralization of TNFα we injected intraperitoneally 100μg anti-TNFα mAb (Epirus Biopharmaceuticals) one day before and on the day of DMBA application or intradermal injection of recombinant murine TNFα. For blocking CCR2 and CXCR3 receptors we injected intraperitoneally 50μg of CCR2 antagonist (RS504393, Tocris Biosciences) and 150μg of CXCR3 antagonist (AMG487, R&D Systems) two times, once 12 h before and the second time during intradermal injection of 200 ng recombinant murine TNFα or topical application of 50μg DMBA.

Statistical analysis

Statistical analysis was performed using Prism 5.0 (GraphPad Software). Unpaired Student's *t*-test was used to determine the statistical significance of the mean percentages of cell types between groups. A *p*-value of <0.05 was considered statistically significant (*), <0.01 very significant (**) and <0.001 highly significant (***). The exact numbers of mice used per experiment are indicated in the corresponding figure legends. Error bars represent standard error of the mean.

Results

The carcinogen DMBA induces skin cell transformation and accumulation of NK cells

We first investigated if the application of the carcinogen DMBA onto the skin of C57BL/6 mice triggers DNA-damage and NKG2D-L expression in skin cells. Indeed, a single topical application of DMBA elevated the mRNA expression levels of γH2AX, a marker to measure DNA-damage,[20] within 12 h as compared to the acetone treated control (Fig. 1A). Concurrently, the mRNA expression of the NKG2D-L H60b, H60c, Mult-1 and Rae-1 was increased in DMBA-treated skin (Fig. 1A). Next, we examined whether NK cells are present in DMBA-treated skin and express NKG2D for recognition of transformed cells. Topical application of DMBA significantly elevated the numbers and percentages of $CD3^{-}NK1.1^{+}$ NK cells in the skin (Fig. 1B). To confirm our findings, we used $Ncr1^{gfp}$ mice that express green fluorescent protein (gfp) under control of the *Ncr1/NKp46* promoter.[15] In accordance with our data in C57BL/6 mice, DMBA-treatment led to an accumulation of $CD3^{-}gfp^{+}$ NK cells in the skin (Fig. S1). Phenotypically, NK cells in DMBA-treated skin were CD3-negative and expressed the NK cell markers NK1.1 and NKp46. Morever, NK cells displayed NKG2D on their surface, which enables them to recognize NKG2D-L expressing transformed skin cells (Fig. 1C). These findings demonstrate that the carcinogen DMBA inflicts DNA-damage and transformation of skin cells accompanied by an influx of $NKG2D^{+}$ NK cells into the skin.

NK cells prevent DNA-damage and tumor growth

Our results so far suggested that NK cells might play a hitherto unknown role during the initiation phase of chemical carcinogenesis. To investigate this, we depleted NK cells by intraperitoneal injection of anti-NK1.1 mAb 2 d before DMBA-treatment (Fig. S2A). Notably, simultaneous depletion of $\gamma\delta$ T cells that have been shown to inhibit chemical carcinogenesis[7] can be excluded due to their lack of NK1.1 (Fig. S2B). In the absence of NK cells significantly more $\gamma H2AX^{+}$ cells were counted on skin sections, indicating an accumulation of DNA-damaged cells (Fig. 2A and B). To test the functional consequences of this observation, NK cells were depleted before DMBA application and tumor growth was promoted with TPA for 12 weeks without any further NK cell ablation. Indeed, higher numbers of papillomas developed in the absence of NK cells (Fig. 2C). Thus, NK cells seem to be crucial for the elimination of DNA-damaged keratinocytes during the tumor initiation step of chemical carcinogenesis.

LC play a similar role as NK cells in inhibiting transformation of skin cells

We considered the possibility that other innate immune cell types, such as LC and dermal DC, might be involved in chemical carcinogenesis. To exclude a possible effect on the skin microenvironment by the constitutive lack of LC in Langerin-DTA mice,[12] we used Langerin-DTR mice that allow inducible depletion of $Langerin^{+}$ DC by injection of DT.[16] The administration of DT efficiently ablates $Langerin^{+}$ LC in the epidermis and $Langerin^{+}$ dermal DC within 24 h (Fig. S3). DMBA-treated Langerin-DTR mice depleted of $Langerin^{+}$ skin DC showed an accumulation of DNA-damaged keratinocytes as evaluated by counting $\gamma H2AX^{+}$ cells on skin sections (Fig. 3A and B) and by measuring mRNA expression of γH2AX (Fig. 3C). Concurrently, in the absence of $Langerin^{+}$ DC the expression levels of the various NKG2D-L were increased (Fig. 3C). Notably, separate analysis of epidermis and dermis revealed that in the absence of LC more DNA-damage occured in the epidermis, whereas the loss of $Langerin^{+}$ dermal DC had no effect in the dermis (Fig. 3D). An earlier report claimed that the infliction of DNA-damage in keratinocytes is dependent on the metabolization of DMBA by LC.[13] However, we observed that, in the absence of LC, cultured keratinocytes incubated with DMBA expressed higher mRNA levels of γH2AX and NKG2D-L

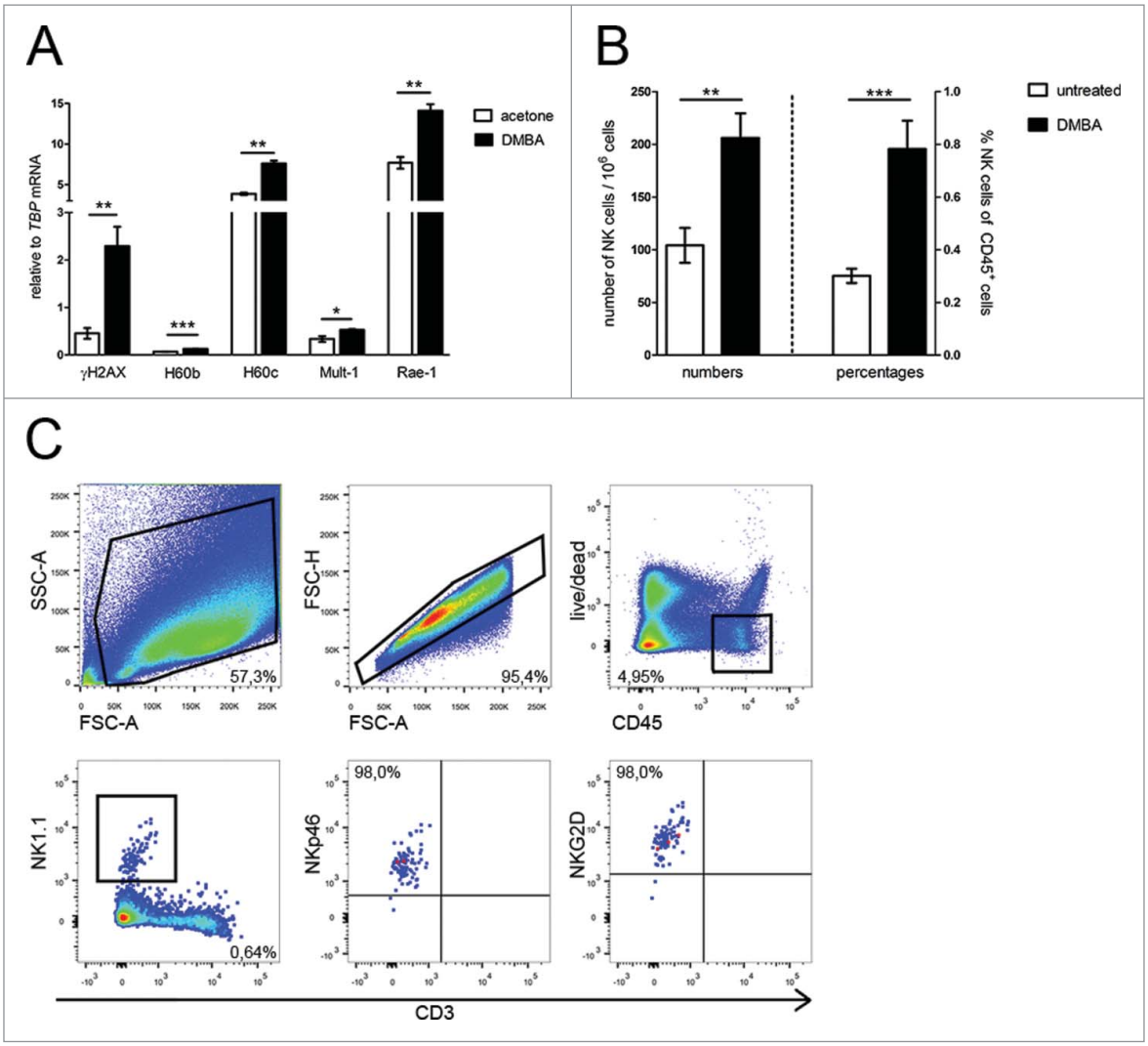

Figure 1. The carcinogen DMBA induces skin cell transformation and accumulation of NK cells. (A) C57BL/6 mice were treated on the ear skin with DMBA or acetone. The mRNA expression levels for γH2AX and NKG2D-L (H60b, H60c, Mult-1 and Rae-1) in the skin were analyzed 12 h later ($n = 3–5$ mice per group). (B) C57BL/6 mice were treated on the ear skin with DMBA. Skin cell suspensions were analyzed 24 h after DMBA-treatment for the numbers and percentages of $CD3^{-}NK1.1^{+}$ NK cells and compared to untreated skin. Summary graph of nine mice per group is shown. (C) Flow cytometric analysis of NK cells from DMBA-treated ear skin of one representative of nine mice is depicted.

(Fig. S4A) and more NKG2D-L on their surface (Fig. S4B). In conclusion, our data etablish that—besides NK cells—LC are crucial for the inhibition of DNA-damage and cell transformation in keratinocytes during chemical carcinogenesis.

The absence of Langerin^{+} DC accelerates tumor development in the skin

Next, we sought to prove that the accumulation of DNA-damaged keratinocytes in Langerin^{+} DC-depleted mice affects tumor development. Langerin-DTR mice were injected once with DT 2 d before topical application of DMBA, followed by weekly tumor promotion with TPA without any further depletion of Langerin^{+} DC. The absence of Langerin^{+} DC accelerated growth of papilloma (Fig. 4A and B), and the tumor area was significantly enlarged 12 weeks after tumor initiation (Fig. 4C). Most of these papilloma converted to carcinoma as judged by histological examination of tumor sections (Fig. 4D) and more carcinoma occured in DT-treated mice (Fig. 4E). These data support our observation that LC play an antitumorigenic role in the epidermis during the initiation of chemical carcinogenesis.

NK cell accumulation in carcinogen-treated epidermis depends on LC

Crosstalk between DC and NK cells is important for the survival, proliferation and function of the latter.[21] Therefore, we wondered whether the accumulation of NK cells in DMBA-treated skin is dependent on the presence of LC. We depleted Langerin^{+} DC in Langerin-DTR mice before topical application of DMBA and separately analyzed epidermal and dermal cell suspensions. Indeed, the accumulation of NK cells in the epidermis was dependent on LC, whereas NK cell numbers in the dermis were unaltered by the depletion of Langerin^{+} dermal DC (Fig. 5A). We phenotypically characterized NK cells in DMBA-treated epidermis. $CD3^{-}NK1.1^{+}$ NK cells in epidermal cell suspensions expressed NKp46, NKG2D and granzyme B indicative of

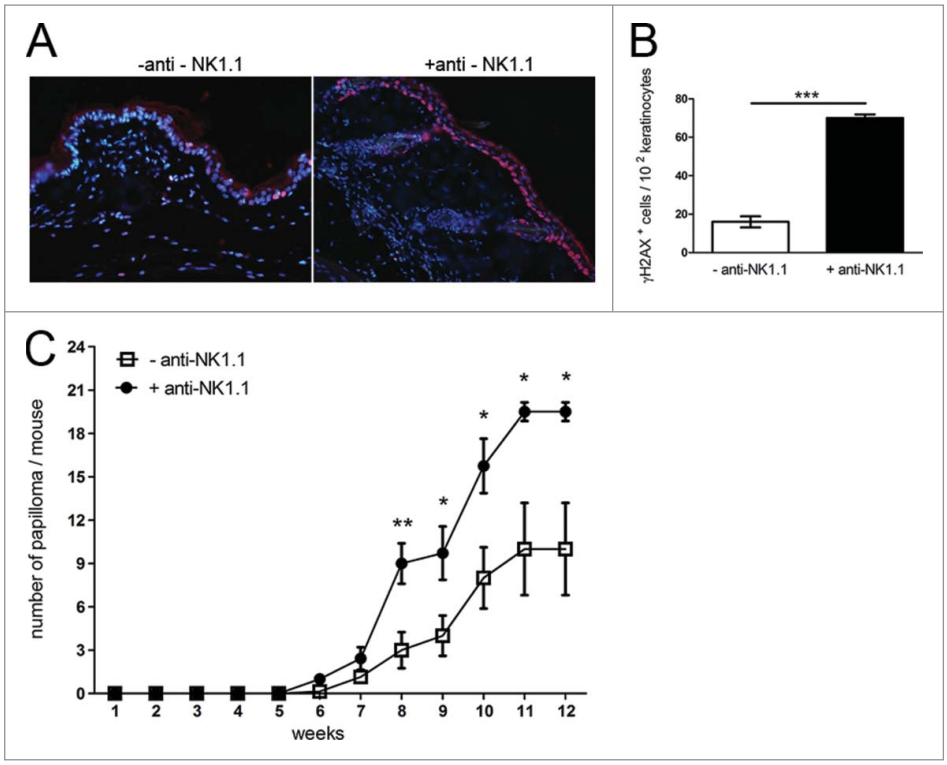

Figure 2. NK cells prevent DNA-damage and tumor growth. (A, B) C57BL/6 mice were injected intraperitoneally with anti-NK1.1 mAb 2 d before and 1 d after DMBA was applied on the ear skin. Skin sections were stained 48 h later with anti-γH2AX mAb (red fluorescence) and DAPI (blue fluorescence). (A) Representative examples of skin section stainings. (B) Numbers of $\gamma H2AX^+$ keratinocytes were counted on skin sections from four mice per group. (C) C57BL/6 mice were injected with anti-NK1.1 mAb 2 d before and 1 d after treatment with DMBA on the back skin, followed by three applications of TPA per week on the same skin site without further depletion of NK cells. Time course with the number of papillomas for eight mice per group is depicted.

cytotoxic capacity. Moreover, they displayed a mature phenotype as determined by expression of CD11b and absence of CD27. Due to their close relationship to innate lymphoid cells (ILC)1, we also analyzed markers that allow discrimination between both cell types.[22] As expected, the ILC1-marker IL-7R/CD127 was absent, whereas the transcription factor Eomes was detected in NK cells in DMBA-treated epidermis (Fig. 5B). Thus, we describe here a LC-dependent accumulation of cytotoxic NK cells in the epidermis in response to carcinogen-treatment.

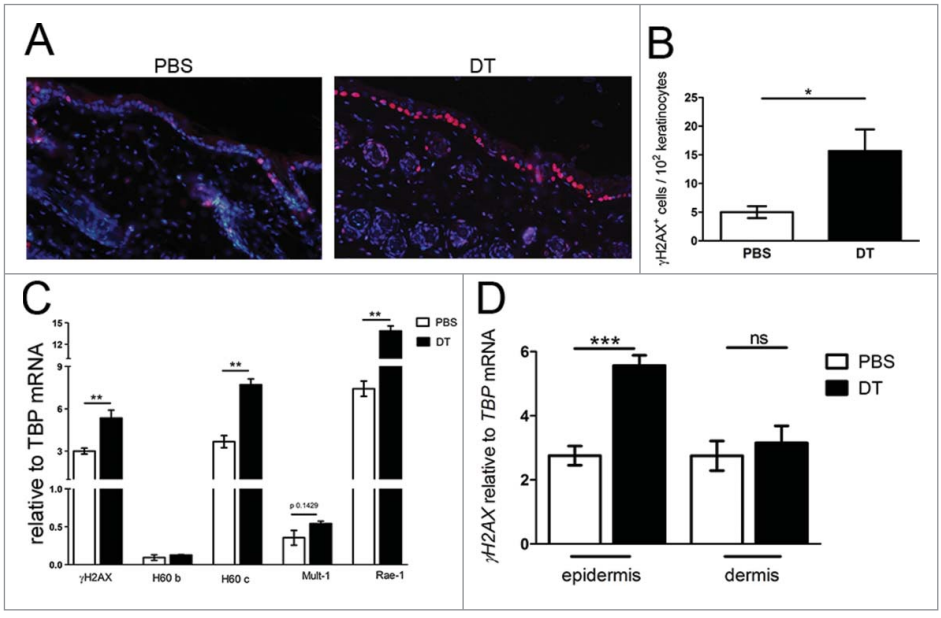

Figure 3. LC play a similar role than NK cells in inhibiting transformation of skin cells. Langerin-DTR mice were injected with either PBS or DT 2 d before DMBA was applied to the ear skin. (A, B) Skin sections were stained 48 h later with anti-γH2AX mAb (red fluorescence) and DAPI (blue fluorescence). Representative examples of skin section stainings are shown in A, and the numbers of $\gamma H2AX^+$ keratinocytes from six mice per group are depicted in (B). (C) The mRNA expression levels of γH2AX and NKG2D-L were measured in the ear skin 24 h after DMBA-treatment. Summary graph of 3–6 mice per group is shown. (D) Enzymatically separated epidermis and dermis were analyzed for mRNA levels of γH2AX 24 h after DMBA-treatment. Summary graph of six mice per group is presented.

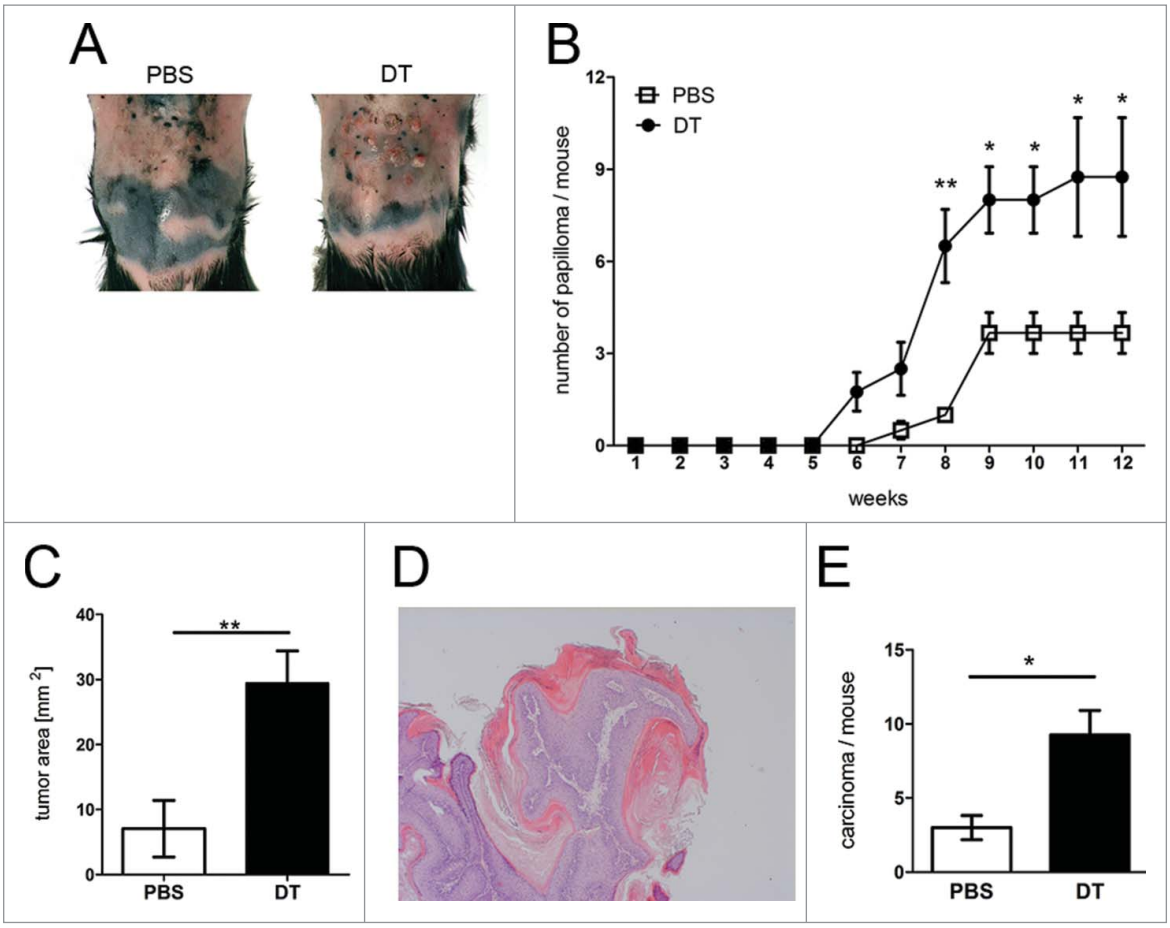

Figure 4. The absence of Langerin$^+$ DC accelerates tumor development in the skin. Langerin-DTR mice were injected with either PBS or DT and 2 d later treated on back skin with DMBA. Tumor promotion was performed with three applications of TPA per week on the same skin site without further depletion of Langerin$^+$ DC. (A) Representative photographs of mouse back skin with tumors 12 weeks after tumor initiation. (B) Time course with the number of papillomas for seven mice per group is shown. (C) Tumor area was measured at week 12, summary graph for seven mice per group. (D) Histologic picture of a carcinoma at week 12. (E) The number of carcinoma at week 12 was evaluated by histologic examination, summary graph for seven mice per group.

TNFα drives NK cell accumulation in DMBA-treated epidermis

Our subsequent experiments focused on identifying factors mediating the cooperation between NK cells and LC in the skin. We screened the epidermis for cytokines important for DC-NK cell interaction, like IL-12, IL-15, IL-18 and TNFα[23,24]. In the absence of LC, the mRNA expression levels of IL-12, IL-15 and IL-18 were unaltered in DMBA-treated epidermis (Fig. S5). In contrast, DMBA-induced TNFα mRNA expression was abrogated when LC were depleted (Fig. 6A). To confirm TNFα on the protein level, epidermal cells from Langerin-DTR mice injected with PBS or DT 2 d earlier were incubated with DMBA. In line with the mRNA expression data, supernatants from cultures with DMBA contained significantly more TNFα than controls. Moreover, protein secretion was lower in the absence of LC, suggesting LC as a source of TNFα (Fig. 6B). Flow cytometric analysis of cells prepared from DMBA-treated skin confirmed that LC produced higher levels of TNFα compared to CD45$^-$ skin cells and CD3^{high} T cells, which represent mainly γδ epidermal T cells (Fig. 6C). NK cells can also produce TNFα to activate DC,[25] but epidermal cell suspensions from mice depleted of NK cells showed unchanged TNFα levels (Fig. S6).

In the next set of experiments, we analyzed the effect of TNFα on NK cell numbers and prevention of DNA-damage. When TNFα was neutralized *in vivo* by intradermal injection of anti-TNFα mAb before DMBA application, NK cell numbers were decreased in the epidermis (Fig. 6D). In contrast, intradermal injection of recombinant TNFα elevated NK cell numbers in the epidermis (Fig. 6E). NK cell recruitment was abrogated when TNFα and neutralizing antibody were injected simultaneously (Fig. 6E). In regard to DNA-damage, more γH2AX$^+$ cells were counted on skin sections after injection of anti-TNFα mAb (Fig. 6F and G). Taken together, these findings demonstrate that TNFα is an essential factor for the prevention of DNA-damage and induces an influx of NK cells into the epidermis.

TNFα-induced chemokines CCL2 and CXCL10 recruit NK cells to the epidermis

We finally sought to investigate whether NK cells proliferate locally in response to TNFα or whether they are recruited to the epidermis by chemokines. To examine the former, we intraperitoneally injected BrdU together with TNFα. Less than 10% BrdU$^+$ NK cells were detected in the epidermis

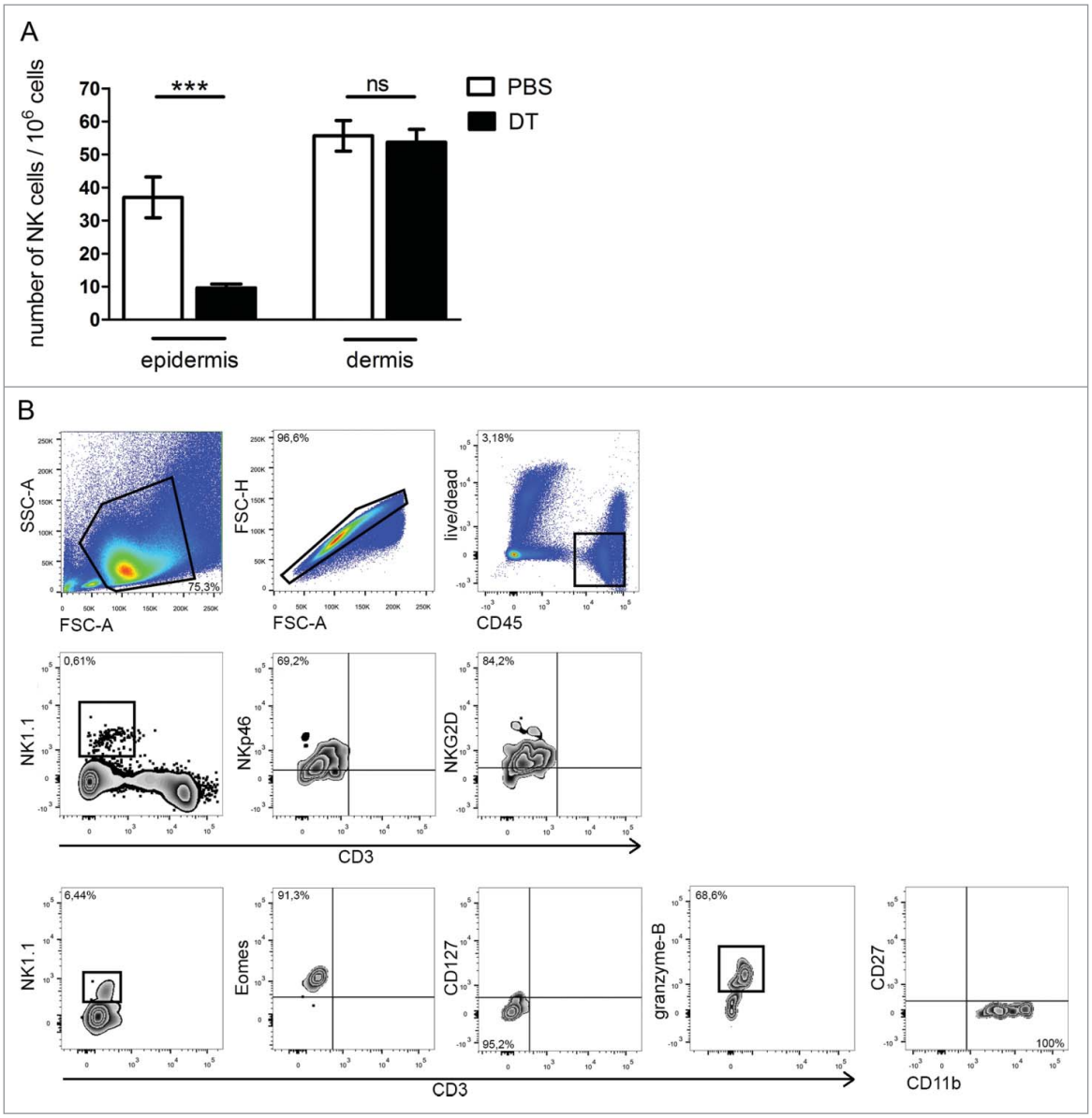

Figure 5. NK cell accumulation in carcinogen-treated epidermis depends on LC. Langerin-DTR mice were injected with either PBS or DT 2 d before DMBA was applied on the ear skin. $CD3^{-}NK1.1^{+}$ NK cells were analyzed in enzymatically separated epidermis and dermis. (A) Numbers of $CD3^{-}NK1.1^{+}$ NK cells in epidermal and dermal cell suspensions were determined 24 h later. Summary graph for seven mice per group is shown. (B) Phenotype of NK cells in DMBA-treated epidermis. Results are representative of three experiments.

16 h later, which cannot fully explain the rapid increase in NK cell numbers (Fig. S7). Next, we tested the hypothesis that NK cells are recruited to the skin by TNFα-inducible chemokines. Recombinant TNFα was injected and mRNA expression levels of CCL2–CCL5 as well as CXCL9–CXCL11 were measured in the epidermis. From these only CCL2 and CXCL10 were significantly elevated in response to TNFα (Fig. 7A). In accordance, mRNA levels for CCL2 and CXCL10 were higher in DMBA-treated epidermis when compared to untreated skin (Fig. 7B). To prove that these two chemokines are responsible for the recruitment of NK cells into the epidermis we blocked chemokine receptor binding with a mix of antagonists for CCR2 (RS504393) and CXCR3 (AMG487). These antagonists abrogated the recruitment of NK cells in response to intradermal injection of recombinant TNFα (Fig. 7C) as well as topical DMBA application (Fig. 7D), confirming the importance of CCL2 and CXCL10 for NK cell recruitment to the epidermis. Thus, TNFα induced chemokines recruit NK cells to the epidermis in response to DMBA to prevent chemical carcinogenesis.

Discussion

Two-stage chemical carcinogenesis in murine skin serves as a well-established model for epithelial neoplasia and mimics the multistage nature of human skin cancer development. Tumor initiation with a single topical exposure to the carcinogen DMBA is followed by weekly applications of TPA that trigger inflammation and hyperplasia. As a consequence papilloma develops that can convert into invasive carcinoma. Evolution of human SCC has many similarities with murine chemically

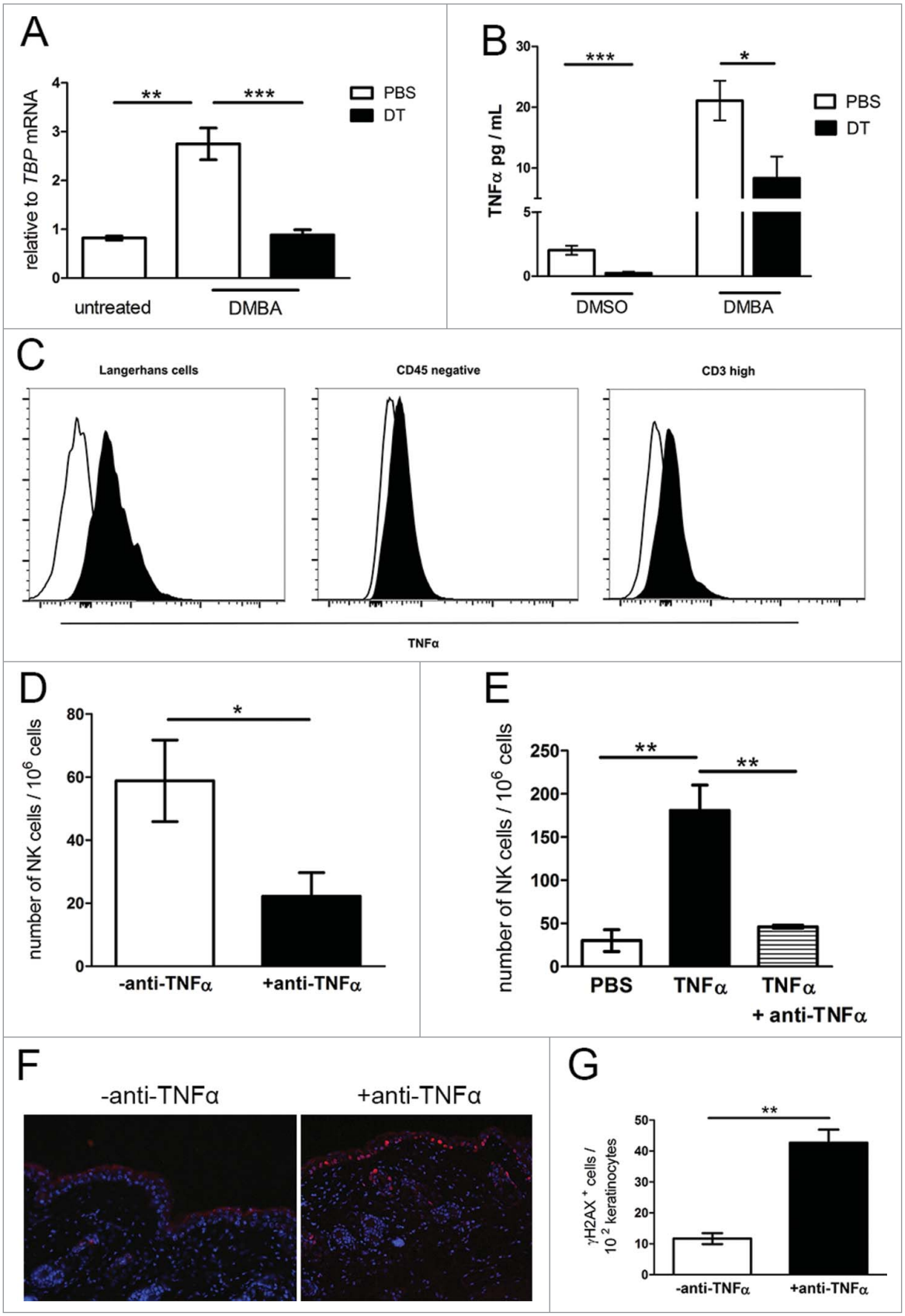

Figure 6. (For figure legend, see page 9.)

induced skin tumors.[26] The immunological events occurring during TPA-driven promotion of tumors are fairly well understood. In contrast, the innate immune response during tumor initiation with DMBA remains elusive. Hence, the focus of our study was to dissect the early innate immune events after application of the carcinogen DMBA to murine skin.

We report here that the depletion of two innate immune cell types, namely NK cells and LC, during DMBA-mediated tumor initiation compromised the elimination of DNA-damaged, NKG2D-L expressing keratinocytes and subsequently caused higher tumor burden. Mechanistically, we identified an influx of NK cells into the epidermis that was dependent on LC and TNFα. In particular, the TNFα-triggered chemokines CCL2 and CXCL10 mediated the recruitment of NK cells into the epidermis, where most of the DNA-damaged cells were localized. These findings outline a cascade of immunological events in carcinogen-treated skin that ensures the rapid clearance of precancerous cells. Therefore, our study reveals a novel and hitherto unknown cellular cooperation between NK cells and LC in the skin that inhibits chemical carcinogenesis.

One of the earliest events after oncogenic insult is the induction of DNA-damage and transformation of cells.[27] We verified that the carcinogen DMBA inflicts DNA-damage to keratinocytes, which leads to cell transformation. The DNA-damaged cells were localized preferentially in the uppermost layer of the skin, i.e. the epidermis. These findings are in line with previous reports demonstrating the occurence of DNA-damage after topical DMBA treatment.[13,28] DNA-damage, as induced by UV-radiation, chemical carcinogens or chemotherapeutics, leads to expression of NKG2D-L, molecules minimally expressed on healthy tissue cells. These proteins, namely H60, Mult-1 and Rae-1, indicate cell transformation and mark cells for clearance by the immune system.[4,5,27] The increased expression of the two NKG2D-L H60 and Rae-1 has been described in pre-cancerous skin as well as papilloma and carcinoma.[7,28] We confirmed and extended these findings, as our study shows that already after the application of DMBA the mRNA levels of the two H60 isoforms H60b and H60c as well as Rae-1 were elevated. In addition, we observed an increased expression of Mult-1 that had not been described before in chemical carcinogenesis.

NKG2D-L are recognized by NKG2D present on NK cells, CD8$^+$ T cells and $\gamma\delta$ T cells.[7,29,30] The role of $\gamma\delta$ T cells in the prevention of chemical carcinogenesis is understood;[7,31] however, the possible involvement of NK cells in this process has not been investigated so far. For this reason, we focused our analysis on NK cells and their role in early chemical carcinogenesis. The carcinogen DMBA increased the frequency of CD3$^-$NK1.1$^+$ NK cells in the epidermis that expressed NKp46, NKG2D and granzyme B, endowing them with the ability to recognize and eliminate transformed cells in the skin.[5] In fact, NKG2D-ligation by NK cells represents a major mechanism for the detection and elimination of pre-malignant cells.[6] Moreover, NK cells displayed a mature phenotype in the epidermis since they were CD11b$^+$CD27$^-$, a phenotype also exhibited by NK cells in other peripheral tissues.[32] NK cells were described in human and murine skin; however, they were mainly found in the dermis and rarely in the epidermal cell layers.[33] Skin infiltration of NK cells with a cytotoxic phenotype occurs in allergic and atopic dermatitis as well as psoriasis.[34-36] Our study proposes the novel concept that NK cells infiltrate the skin in the case of chemical carcinogenesis. The depletion of NK cells caused an accumulation of DNA-damaged, transformed keratinocytes in the epidermis ultimatively resulting in a higher tumor burden. This is in line with an earlier report on another tumor model demonstrating that mice were more susceptible to chemically induced sarcoma when NK cells were ablated.[37] Thus, our work describes for the first time a mature NK cell population with cytotoxic ability in the epidermis after initiation of chemical carcinogenesis. Moreover, these NK cells exert a critical and novel role in the clearance of DNA-damaged cells to prevent cutaneous chemical carcinogenesis.

Besides NK cells LC are crucial for the immunosurveillance of carcinogen-treated epidermis. Our findings indicate that LC are mandatory for the clearance of transformed skin cells. We proved this antitumorigenic role of LC in chemical carcinogenesis by depletion experiments in Langerin-DTR mice that exhibited more DNA-damage and higher tumor burden in the absence of LC. These findings contradict the reported pro-tumorigenic role of LC as metabolizers of DMBA[13] since we and others demonstrate that the metabolization of DMBA by LC is dispensable for cell transformation of keratinocytes.[38] The main difference between the study by Modi et al. and ours is that the former used mice with constitutive absence of LC.[12] In contrast, we employed an inducible model that allowed depletion of LC right before DMBA-mediated initiation of chemical carcinogenesis.[16] Conflicting results from constitutive versus inducible depletion of LC in mouse models have been reported before, e.g., in contact hypersensitivity, and the reasons for this remain obscure.[39,40] One plausible explanation is that the life-long lack of LC in the Langerin-DTA mice most likely causes changes in skin homeostasis.[11] Crosstalk between NK cells and DC is a prerequisite for NK cell proliferation and function.[21,23] Moreover, human LC can support NK cell survival at least *in vitro*,[41] and NK

Figure 6. (see previous page) TNFα drives NK cell accumulation in DMBA-treated epidermis. Langerin-DTR mice were injected with either PBS or DT 2 d before DMBA was applied to the ear skin. (A) The mRNA level of TNFα was determined 24 h later in the epidermis and compared to untreated skin ($n = 4$–6 mice per group). (B) Epidermal cell suspensions were prepared from Langerin-DTR mice injected with either PBS or DT 2 d earlier and incubated with DMSO as a control or DMBA for 6 h. TNFα protein levels were measured in the culture supernatants by ELISA, summary graph of six mice per group. (C) Skin cell suspensions were analyzed for production of TNFα by Langerin$^+$CD103$^-$ LC, CD45$^-$ skin cells, and CD3^{high} T cells. Histograms are representative of nine mice, black line isotype control, filled histogram TNFα staining. (D) Ncr1^{gfp} mice were injected intraperitoneally with anti-TNFα mAb 1 d before and during DMBA application. The numbers of CD3$^-$GFP$^+$ NK cells were determined in the epidermis 24 h later. Cells were gated on viable CD45$^+$ cells, summary graph of four mice per group. (E) Ncr1^{gfp} mice were injected intradermally into ear skin with recombinant TNFα alone or in combination with anti-TNFα mAb intraperitoneally 1 d before and during TNFα application. PBS injected animals served as controls. The number of CD3$^-$gfp$^+$ NK cells in the epidermis was determined 12 h later. Cells were gated on viable CD45$^+$ cells, summary graph of six mice per group. (F, G) C57BL/6 mice were injected intraperitoneally with anti-TNFα mAb 1 d before and during DMBA treatment. Skin sections were stained with anti-γH2AX mAb (red fluorescence) and DAPI (blue fluorescence) 48 h later. (F) Representative examples of skin section stainings are shown. (G) Numbers of γH2AX$^+$ keratinocytes were determined on skin sections, summary graph of six mice per group.

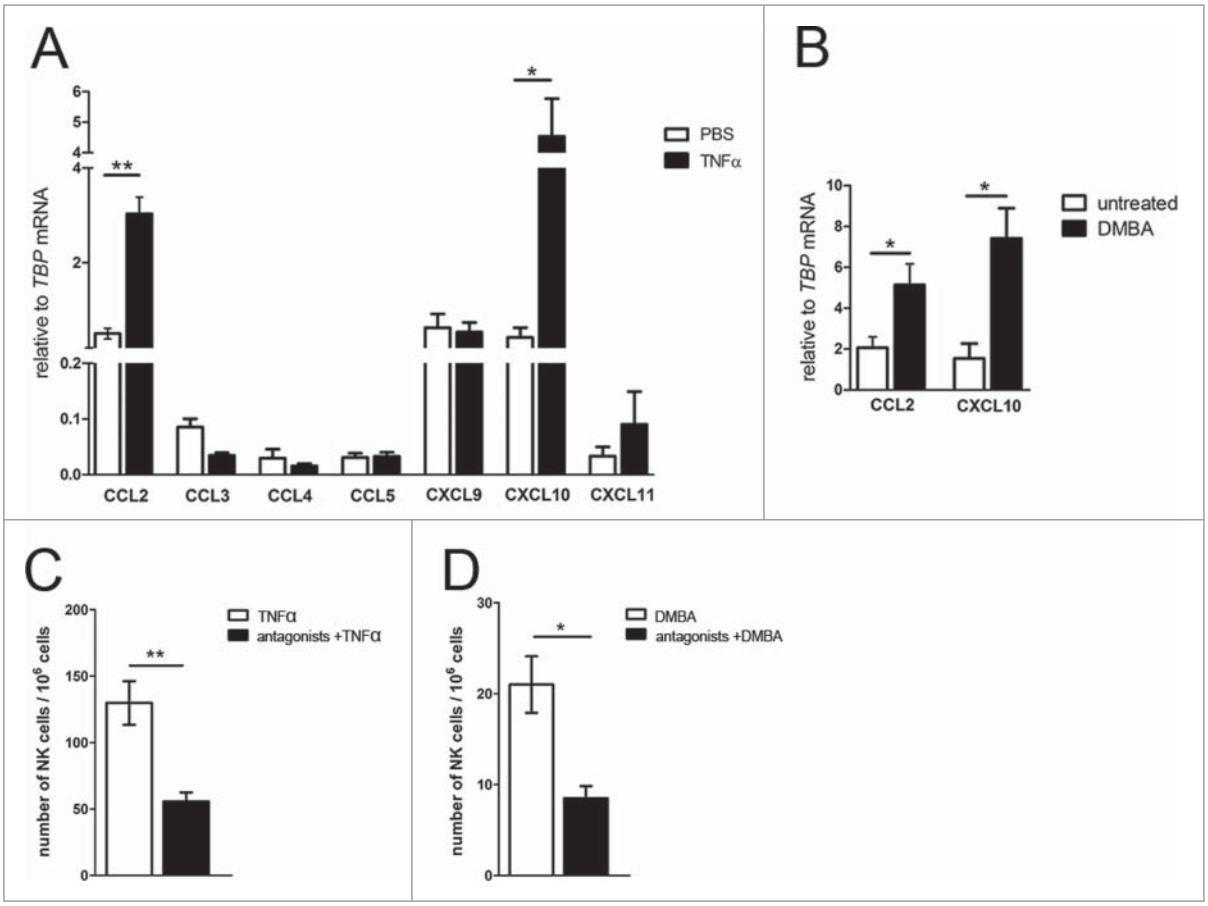

Figure 7. TNFα-induced chemokines CCL2 and CXCL10 recruit NK cells to the epidermis. (A) C57BL/6 mice were injected intradermally with recombinant TNFα and 5 h later epidermis was analyzed for mRNA expression levels of chemokines, summary graph of four mice per group is shown. (B) C57BL/6 mice were treated with DMBA on the ear skin. After 24 h, the mRNA expression levels of CCL2 and CXCL10 were analyzed and compared to untreated epidermis, summary graph of four mice per group is depicted. (C) A mix of the CCR2 antagonist RS504393 and the CXCR3 antagonist AMG487 was injected intraperitoneally into Ncr1^{gfp} mice 12 h before and during intradermal injection of TNFα. The numbers of CD3$^-$GFP$^+$ NK cells were determined 12 h later in the epidermis. Summary graph of five mice per group is shown. (D) A mix of the CCR2 antagonist RS504393 and the CXCR3 antagonist AMG487 were injected intraperitoneally into Ncr1^{gfp} mice right before and 12 h after topical application of DMBA on the ear skin. The numbers of CD3$^-$GFP$^+$ NK cells were determined 24 h later in the epidermis, summary graph of four mice per group is depicted.

cells are in close contact with DC in atopic dermatitis skin.[34] Hence, we hypothesized that NK cell accumulation in the epidermis during chemical carcinogenesis may depend on LC. Intriguingly, NK cells failed to accumulate in DMBA-treated epidermis after depletion of LC, whereas absence of Langerin$^+$ dermal DC did not affect NK cell numbers in the dermis. This not only points toward a unique role of LC in modulating NK cells in the epidermis, we also provide evidence for a cooperation of LC and NK cells in the rapid elimination of transformed keratinocytes.

Several cytokines are important for NK cell survival, proliferation and function,[21] which are produced by DC including LC.[42-44] For example, IL-12 and IL-18 are pivotal for cytokine production and cytotoxicity, whereas IL-15 affects primarily the survival and proliferation of NK cells.[41,45,46] However, all of these cytokines were unaltered by LC depletion. On the other hand, there was evidence that DC and NK cells can interact via TNF-family members.[47] LC were shown to produce low amounts of TNFα which can be augmented by stimulation with PMA and lipopolysaccharides.[48,49] In agreement with these reports, we detected that LC produced the highest levels of TNFα in DMBA-treated epidermis. Moreover, TNFα proved to be crucial for the DMBA-induced accumulation of NK cells in the epidermis and the prevention of DNA-damage in the skin. These results are in line with a transplantable tumor mouse model demonstrating that TNFα is important for the recruitment of NK cells and tumor rejection in the peritoneum.[50] However, this study did not elucidate the underlying mechnism.

To this aim, we investigated whether the NK cell accumulation in the epidermis was due to recruitment of cells and/or their local proliferation. Following DMBA treatment, only a minor part of the NK cells in the epidermis incorporated BrdU, excluding a major contribution of locally proliferating NK cells. On the other hand, TNFα can induce expression of chemokines that can attract NK cells.[51] In psoriatic skin NK cells were chemotactically recruited to the dermis and epidermis by the chemokines CCL5 and CXCL10[35] and in liver carcinoma CCL2 and CXCL10 mediated NK cell recruitment.[52] Indeed, both intradermal injection of TNFα and topial application of DMBA, elevated mRNA levels of CCL2 and CXCL10 in the epidermis. To confirm the role of CCL2 and CXCL10 in guiding NK cells to the epidermis, we employed antagonists of the corresponding receptors for CCR2 and CXCL10. A combination of both antagonists significantly reduced the number of NK cells in the epidermis in response to both TNFα injection and topical treatment with DMBA.

In conclusion, our study proposes a novel concept how innate immune cells surveil the epidermis for DNA-damaged, transformed cells in order to prevent chemical carcinogenesis. The topical exposure to the carcinogen DMBA recruits NK cells to the epidermis, a process dependent on LC and TNFα-induced chemokines CCL2 and CXCL10. This cooperation of NK cells and LC in the prevention of tumor formation in the epidermis underlines the relevance of the innate immune system in the immunosurveillance of the skin during chemical carcinogenesis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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