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

Daniela Ortner \(^a\), Christoph H. Tripp \(^a\), Kerstin Komenda \(^a\), Sandrine Dubrac \(^a\), Bernhard Zelger \(^a\), Martin Hermann \(^b\), Wolfgang Doppler \(^b\), Piotr Z. Tymoszuk \(^c\), Louis Boon \(^d\), Björn E. Clausen \(^e\), and Patrizia Stoitzner \(^f\)

\(^a\)Department of Dermatology, Venereology and Allergology, Medical University of Innsbruck, Innsbruck, Austria; \(^b\)Department of Anesthesiology and Intensive Care Medicine, Medical University of Innsbruck, Innsbruck, Austria; \(^c\)Section for Medical Biochemistry, Medical University of Innsbruck, Innsbruck, Austria; \(^d\)Department of Internal Medicine VI, Infectious Diseases, Immunology, Rheumatology & Pneumology, Medical University of Innsbruck, Innsbruck, Austria; \(^e\)Biocerus, Utrecht, the Netherlands; \(^f\)Institute for Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

**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 TNF\(\alpha\) mainly by LC promoted the recruitment of NK cells into the epidermis. Indeed, the TNF\(\alpha\)-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.

**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-tetradecanoyl-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.\(^4,5\) 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\(^b\) 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...
elucidated, as most studies concentrated on the tumor promotion phase. Thus, we focused on the role of innate immune cells, which are able to rapidly 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<sup>gfp</sup> mice (provided by K. Kotsch and C. Fabritius, Department of Visceral, Transplant and Thoracic Surgery, Center for Operative Medicine, Medical University of Innsbruck, Austria) and Langerin-DTR mice 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 50 µg DMBA (Sigma-Aldrich) in 20 µL acetone (VWR) on the ear skin of 7–12 week old C57BL/6, Langerin-DTR and Ncr1<sup>gfp</sup> mice. Langerin<sup>+</sup> DC were depleted by intraperitoneal injection of DT (Merck) 2 d before the start of experiments. For tumor experiments 100 µg DMBA in 100 µL 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, so we modified the protocol to three applications of 15 µg TPA (Sigma-Aldrich) per week in 100 µL 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<sup>®</sup> 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 TRIzol<sup>®</sup> 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 lens.

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 lens.

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 GHR, GE Healthcare Life Sciences) and gel filtration on Superdex 200 HR (GE Healthcare Life Sciences). For depletion experiments 200 μg 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, 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 Ncr1gfp mice that express green fluorescent protein (gfp) under control of the Ncr1/NKp46 promoter. 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. Moreover, 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 γδ T cells that have been shown to inhibit chemical carcinogenesis can be excluded due to their lack of NK1.1 (Fig. S2B). In the absence of NK cells significantly more γH2AX+ 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, we used Langerin-DTR mice that allow inducible depletion of Langerin+ DC by injection of DT. The administration of DT efficiently ablates Langerin+ DC 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 γH2AX+ 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 dermis and epidermis revealed that in the absence of LC more DNA-damage occurred 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. However, we observed that, in the absence of LC, cultured keratinocytes incubated with DMBA expressed higher mRNA levels of γH2AX and NKG2D-L...
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
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 (ILC1), 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 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 γH2AX+ 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.

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 γH2AX+ 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.
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. 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 CD3high T cells, which represent mainly γδ epidermal T cells (Fig. 6C). NK cells can also produce TNFα to activate DC, 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.
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. 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 pre-cancerous 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. 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 occurrence of DNA-damage after topical DMBA treatment. 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. 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. 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 γδ T cells. The role of γδ T cells in the prevention of chemical carcinogenesis is understood; 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. In fact, NKG2D-ligation by NK cells represents a major mechanism for the detection and elimination of pre-malignant cells. 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. NK cells were described in human and murine skin; however, they were mainly found in the dermis and rarely in the epidermal cell layers. Skin infiltration of NK cells with a cytotoxic phenotype occurs in allergic and atop dermatitis as well as psoriasis. 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 ultimately 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. 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 since we and others demonstrate that the metabolization of DMBA by LC is dispensable for cell transformation of keratinocytes. The main difference between the study by Modi et al. and ours is that the former used mice with constitutive absence of LC. In contrast, we employed an inducible model that allowed depletion of LC right before DMBA-mediated initiation of chemical carcinogenesis. 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. One plausible explanation is that the life-long lack of LC in the Langerin-DTA mice most likely causes changes in skin homeostasis. Crosstalk between NK cells and DC is a prerequisite for NK cell proliferation and function. Moreover, human LC can support NK cell survival at least in vitro, 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+CD4+ T cells. Histograms are representative of nine mice, black line isotype control, filled histogram TNFα staining. (D) Ncr1GFP mice were injected intraportaneously 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) Ncr1GFP mice were injected intradermally into ear skin with recombinant TNFα alone or in combination with anti-TNFα mAb intraportaneously 1 d before and during TNFα application. PBS injected animals served as controls. The number of CD3−NK1.1+ 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 intraportaneously 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.
cells are in close contact with DC in atopic dermatitis skin. 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, which are produced by DC including LC. 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. 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. LC were shown to produce low amounts of TNF which can be augmented by stimulation with PMA and lipopolysaccharides. In agreement with these reports, we detected that LC produced the highest levels of TNF in DMBA-treated epidermis. Moreover, TNF 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 TNF is important for the recruitment of NK cells and tumor rejection in the peritoneum. However, this study did not elucidate the underlying mechanism.

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 were determined 12 h later in the epidermis, summary graph of four mice per group is depicted. The numbers of CD3+ GFP+ NK cells were determined 24 h later in the epidermis, summary graph of four mice per group is depicted.

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 were injected intraperitoneally into Ncr1gfp 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 Ncr1gfp 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.
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.

Acknowledgments

We are particularly grateful to Nikolaus Romani for his continuous support. We thank Eva-Maria Putz and Veronika Sxed (Institute for Pharmacology and Toxicology, University of Veterinary Medicine Vienna), Karin Loser (Department of Dermatology, University of Muenster) and Florian Sparber (Institute for Virology, University of Zürich) for their technical advice.

Funding

This work was supported by the Austrian Science Fund (FWF) with grants to PS (FWF-P21487-B13, FWF-P27001-B13), DO (FWF-T-737) and SD (FWF-P28039-B13 and FWF-P21449-B13); the Tyrolean Cancer Society with a grant to PS (Project number 41/2011); the Research Center for Immunotherapy (FZI) Mainz to BEC.

ORCID

Daniela Ortner https://orcid.org/0000-0002-9433-5367
Sandrine Dubrac https://orcid.org/0000-0002-2936-8488
Bernhard Zelger https://orcid.org/0000-0003-0763-0614
Wolfgang Doppler https://orcid.org/0000-0001-6590-6063
Piotr Z. Tymoszuk https://orcid.org/0000-0002-0398-6034
Louis Boon https://orcid.org/0000-0002-0937-9171
Björn E. Clausen https://orcid.org/0000-0002-2484-7842
Patrizia Stoitzner https://orcid.org/0000-0002-8488-6704

References

1. Alam M, Ratner D. Cutaneous squamous-cell carcinoma. N Engl J Med 2001; 344:975-83; PMID:11274625; http://dx.doi.org/10.1056/NEJM20010329344134
2. Yuspa SH. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. J Dermatol Sci 1998; 17:1-7; PMID:9651822; http://dx.doi.org/10.1016/S0923-1811(97)00071-6
3. Rundhaug JE, Fischer SM. Molecular mechanisms of mouse skin tumor promotion. Cancers 2010; 2:436-82; PMID:21297902; http://dx.doi.org/10.3390/cancers2020436
4. Cervenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH, Lanier LL. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity 2000; 12:721-7; PMID:10894171; http://dx.doi.org/10.1016/S1074-7613(00)08222-8
5. Rauel DH, Guerra N. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. Nat Rev Immunol 2009; 9:568-80; PMID:19629084; http://dx.doi.org/10.1038/nri2604
6. Diefenbach A, Jensen ER, Jamieson AM, Rauel DH. Rael and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature 2001; 413:165-71; PMID:11557981; http://dx.doi.org/10.1038/35093109
7. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobbs P, Sutton B, Tigelaar RE, Haydac AC. Regulation of cutaneous malignancy by gammadelta T cells. Science 2001; 294:605-9; PMID:11567106; http://dx.doi.org/10.1126/science.1063916
8. Strid J, Roberts SJ, Filler RB, Lewis JM, Kwong BY, Schpero W, Kaplan DH, Haydac AC, Girardi M. Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. Nat Immunol 2008; 9:146-54; PMID:18176566; http://dx.doi.org/10.1038/nii1556
9. Antiseforova M, Huber M, Meyer M, Pwko-Czuchra A, Ramad T, MacLeod AS, Havran WL, Dummer R, Hohl D, Werner S. Activin enhances skin tumourigenesis and malignant progression by inducing a pro-tumourigenic immune cell response. Nat Commun 2011; 2:576; PMID:22146395; http://dx.doi.org/10.1038/ncomms1585
10. Steinman RM. Decisions about dendritic cells: past, present, and future. Ann Rev Immunol 2012; 30:1-22; PMID:22136168; http://dx.doi.org/10.1146/annurev-immunol-100311-102839
11. Clausen BE, Stoitzner P. Functional specialization of skin dendritic cell subsets in regulating T cell responses. Front Immunol 2015; 6; 5:334; PMID:26557117; http://dx.doi.org/10.3389/fimmu.2015.00534
12. Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. Immunity 2005; 23:611-20; PMID:16356859; http://dx.doi.org/10.1016/j.immuni.2005.10.008
13. Modi BG, Neustadter J, Binda E, Lewis J, Filler RB, Roberts SJ, Kwong BY, Reddy S, Overton JD, Galan A et al. Langerhans cells facilitate epithelial DNA damage and squamous cell carcinoma. Science 2012; 335:104-8; PMID:22223807; http://dx.doi.org/10.1126/science.1211600
14. Lewis JM, Burgler CD, Fraser JA, Liao H, Goblets K, Kucher CL, Zhao PY, Filler RB, Tigelaar RE, Girardi M. Mechanisms of chemical cooperative carcinogenesis by epidermal langerhans cells. J Invest Dermatol 2015; 135:1405-14; PMID:25233073; http://dx.doi.org/10.1038/jid.2014.411
15. Gazit R, Grada R, Elboim M, Arnon TI, Katz G, Achdout H, Hanna J, Qimron U, Landau G, Greenbaum E et al. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. Nat Immunol 2006; 7:517-23; PMID:16565719; http://dx.doi.org/10.1038/ni1322
16. Bennet CL, van Rijn E, Jung S, Inaba K, Steinman RM, Kampsenberg ML, Clausen BE. Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. J Cell Biol 2005; 169:569-76; PMID:15897263; http://dx.doi.org/10.1083/jcb.200501071
17. Noordegraaf M, Flacher V, Stoitzner P, Clausen BE. Functional redundancy of Langerhans cells and Langerin+ dermal dendritic cells in contact hypersensitivity. J Invest Dermatol 2010; 130:2752-9; PMID:20703247; http://dx.doi.org/10.1038/jid.2010.223
18. Woodworth CD, Michael E, Smith L, Vijayachandra K, Glick A, Hennings H, Yuspa SH. Strain-dependent differences in malignant conversion of mouse skin tumors is an inherent property of the epidermal keratinocyte. Carcinogenesis 2004; 25:1771-8; PMID:15105299; http://dx.doi.org/10.1093/carcin/bgh170
19. Yusuf N, Timares L, Seibert MD, Xu H, Elmets CA. Acquired and innate immunity to polyaromatic hydrocarbons. Toxicol Appl Pharmacol 2007; 224:308-12; PMID:17258781; http://dx.doi.org/10.1016/j.taap.2006.12.009
20. Sedelnikova OA, Pilch DR, Redon C, Bonner WM. Histone H2AX in DNA damage and repair. Cancer Biol Ther 2003; 2:233-5; PMID:12878854; http://dx.doi.org/10.4161/cbt.2.3.373
21. Walzer T, Dalol M, Robbins SH, Zitvogel L, Vivier E. Natural-killer cell subsets in regulating T cell responses. Front Immunol 2015; 6:534; PMID:26203671; http://dx.doi.org/10.3389/fimmu.2015.00621
22. Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. Immunol Rev 2014; 259:354-65; PMID:25238093; http://dx.doi.org/10.1111/imr.12345
23. Fernandez NC, Lozier A, Flamment C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 1999; 5:405-11; PMID:10202929; http://dx.doi.org/10.1038/7403
28. Cipolat S, Hoste E, Natsuga K, Quist SR, Watt FM. Epidermal barrier defect induces atopic dermatitis with altered skin cancer susceptibility. eLife 2014; 3:e01888; http://dx.doi.org/10.7554/eLife.01888

29. Diefenbach A, Jamieson AM, Liu TD, Shastri N, Raulet DH. Ligands for the murine NKGD2 receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol 2000; 1:119-26; PMID:11248803; http://dx.doi.org/10.1038/77793

30. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid receptor (RAR) prevents NK cell-induced rejection of a MHC class I-bearing tumor in vivo. Proc Natl Acad Sci USA 2001; 98:11521-6; PMID:11562472; http://dx.doi.org/10.1073/pnas.200121282

31. Girardi M, Glusac E, Filler RB, Roberts SJ, Propperova I, Lewis J, Tige-laar RE, Hayday AC. The distinct contributions of murine T cell receptor (TCR)gammadelta and TCRalphabeta T cells to different stages of chemically induced skin cancer. J Exp Med 2003; 198:747-55; PMID:12953094; http://dx.doi.org/10.1084/jem.20021282

32. Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. J Immunol 2006; 176:1517-24; PMID:16424180; http://dx.doi.org/10.4099/jimmunol.176.3.1517

33. Luci C, Reynolds A, Ivanov II, Cognet C, Chiche L, Chasson L, Hardwigen J, Anguiano E, Banchereau J, Chassaclé D et al. Influence of the transcription factor RORgammat on the development of NKp46+ NK cell populations in gut and skin. Nat immunol 2009; 10:75-82; PMID:19029904; http://dx.doi.org/10.1038/ni.1681

34. Buentke E, Heffler LC, Wilson JL, Wallin RP, Lofman C, Chambers BJ, Ljunggren HG, Scheunis A. Natural killer and dendritic cell contact in lesional atopic dermatitis skin—Malassezia-influenced cell interaction. J Invest Dermatol 2002; 119:850-7; PMID:12406330; http://dx.doi.org/10.1046/j.1523-1747.2002.00132.x

35. Ottaviani C, Nasorri F, Bedini C, de Pita O, Girolomoni G, Cavani A. CD56brightCD16−/− NK cells accumulate in psoriatric skin in response to CXCL10 and CCL5 and exacerbate skin inflammation. Eur J Immunol 2006; 36:118-28; PMID:16323244; http://dx.doi.org/10.1002/eji.20053523

36. Carbone T, Nasorri F, Pennino D, Eyerich K, Foerster S, Cifaldi L, Traidl-Hoffman C, Behrendt H, Cavani A. CD56brightCD16+CD62L− NK cells accumulate in allergic contact dermatitis and contribute to the expression of allergic responses. J Immunol 2010; 184:1102-10; PMID:20008290; http://dx.doi.org/10.4049/jimmunol.0902518

37. Smyth MJ, Crowe NY, Godfrey DI. NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int Immunol 2001; 13:459-63; PMID:11282985; http://dx.doi.org/10.1093/intimm/13.4.459

38. Swanson HI. Cytochrome P450 expression in human keratinocytes: an aryl hydrocarbon receptor perspective. Chem Biol Interact 2004; 149:69-79; PMID:15501429; http://dx.doi.org/10.1016/j.chbi.2004.08.006

39. Romani N, Clausen BE, Stoitzner P. Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. Immunol Rev 2010; 234:120-41; PMID:20193016; http://dx.doi.org/10.1111/j.1600-0625.2009.00866.x

40. Bennett CL, Clausen BE. DC ablation in mice: promises, pitfalls, and challenges. Trends Immunol 2007; 28:325-31; PMID:17964853; http://dx.doi.org/10.1016/j.it.2007.08.011

41. Munz C, Diao T, Ferlazzo G, de Cos MA, Goodman K, Young JW. Mature myeloid dendritic cell subsets have distinct roles for activation and viability of circulating human natural killer cells. Blood 2005; 105:266-73; PMID:15331446; http://dx.doi.org/10.1182/blood-2004-06-2492

42. Heufer C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, Enk A, Steinman RM, Romani N, Schuler G. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur J Immunol 1996; 26:659-68; PMID:8605935; http://dx.doi.org/10.1002/eji.1830260323

43. Stoll S, Jonuleit H, Schmitt E, Muller G, Yamahauchi H, Kurimoto M, Knop J, Enk AH. Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent T cell development. Eur J Immunol 1998; 28:3231-9; PMID:9808192; http://dx.doi.org/10.1002/(SICI)1521-4141(199810)28:10%3c3231::AID-IMMU321%3e3.0.CO;2-Q

44. Romano E, Cotari JW, Barreira da Silva R, Betts BC, Chung DJ, Avogadri F, Fink MJ, St Angelo ET, Mehrara B, Heller G et al. Human Langerhans cells use an IL-15R-alpha/IL-15/pSTAT5-dependent mechanism to break T-cell tolerance against the self-differentiation tumor antigen WT1. Blood 2012; 119:5182-90; PMID:22510877; http://dx.doi.org/10.1182/blood-2011-09-382200

45. Andrews DM, Scalzo AA, Yokoyama WM, Smyth MJ, Degli-Esposti MA. Functional interactions between dendritic cells and NK cells during viral infection. Nat Immunol 2003; 4:175-81; PMID:12496964; http://dx.doi.org/10.1038/nature03880

46. Ferlazzo G, Pack M, Thomas D, Paludan S, Schmid D, Strowig T, Bougras M, Muller WA, Moretta L, Münz C. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc Natl Acad Sci USA 2004; 101:16606-11; PMID:15536127; http://dx.doi.org/10.1073/pnas.0407522101

47. Makarenkova V, Chakrabarti AK, Liberator JA, Popovic P, Lu G, Watkins S, Vujanovic NL. Dendritic cell and natural killer cells interact via multiple TNF family molecules. J Leukoc Biol 2005; 77:408-13; PMID:15604121; http://dx.doi.org/10.1189/jlb.1104675

48. Larrick JW, Morhenn V, Chiang LY, Shi T. Activated Langerhans cells release tumor necrosis factor. J Leukoc Biol 1989; 45:429-33; PMID:2708913.

49. Schreiber S, Kilgus O, Payer E, Kutil R, Elbe A, Mueller C, Stingl G. Cytokine pattern of Langerhans cells isolated from murine epidermal cell cultures. J Immunol 1992; 149:3524-34; PMID:1431123

50. Smyth MJ, Kelly JM, Baxter AG, Korner H, Sedgwick JD. An essential mechanism to break T-cell tolerance against the self-differentiation tumor antigen WT1. Blood 2005; 104:2402-11; PMID:15995699; http://dx.doi.org/10.1182/blood-2004-06-2492

51. Munz C, Diao T, Ferlazzo G, de Cos MA, Goodman K, Young JW. Mature myeloid dendritic cell subsets have distinct roles for activation and viability of circulating human natural killer cells. Blood 2005; 105:266-73; PMID:15331446; http://dx.doi.org/10.1182/blood-2004-06-2492

52. Heufer C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, Enk A, Steinman RM, Romani N, Schuler G. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur J Immunol 1996; 26:659-68; PMID:8605935; http://dx.doi.org/10.1002/eji.1830260323