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IL-12 deficiency suppresses 12-*O*-tetradecanoylphorbol-13-acetate-induced skin tumor development in 7,12-dimethylbenz(*a*)anthracene-initiated mouse skin through inhibition of inflammation

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Interleukin (IL)-12 deficiency exacerbates tumorigenesis in ultraviolet (UV) radiation-induced skin. Here, we assessed the effects of IL-12 deficiency on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in 7,12-dimethylbenz(*a*)anthracene (DMBA)-initiated mouse skin. Using this two-stage chemical carcinogenesis protocol, we found that the development of DMBA/TPA-induced skin tumors was diminished in IL-12p40-knockout mice than in their wild-type counterparts. At the termination of the experiment (at 24 weeks), the skin tumor incidence and tumor multiplicity were significantly lower ($P < 0.005$) in interleukin-12-knockout (IL-12 KO) mice than in their wild-type counterparts, as was the malignant transformation of DMBA/TPA-induced papillomas to carcinomas ($P < 0.01$). Analysis of samples collected at the termination of the experiments for biomarkers of inflammation by immunohistochemical analysis, western blotting, enzyme-linked immunosorbent assay and real-time polymerase chain reaction revealed significantly lower levels of cyclooxygenase-2 (COX-2), prostaglandin (PG) E_2, proliferating cell nuclear antigen, cyclin D1 and the proinflammatory cytokines (tumor necrosis factor-α, IL-1β and IL-6) in the DMBA/TPA-treated tumors and tumor-uninvolved skin of IL-12 KO mice than the skin and tumors of DMBA/TPA-treated wild-type mice. Analysis of the skin 6 h after TPA treatment showed that the TPA-induced promotion of skin edema, inflammatory leukocyte infiltration, COX-2 expression and PGE_2 production was significantly lower in the skin of the IL-12-KO mice than their wild-type counterparts. These results indicate that DMBA/TPA-induced skin tumor development differs from UVB-induced skin tumor development in that endogenous IL-12 acts to inhibit UVB-induced skin tumor development and malignant progression of the skin tumors to carcinoma. In the case of DMBA/TPA-induced skin tumor development, the endogenous IL-12 modulates the tumor promoter stimulation of inflammatory responses.

Introduction

Skin cancer represents a major and growing public health problem (1). The development of skin cancer is a multistage process. In experimental animal models of the disease, this process includes initiation, promotion and progression (2). During the early tumor promotion stage of multistage carcinogenesis, the process is reversible, whereas the initiation stage is irreversible and presumably unavoidable because of continuing exposure to carcinogenic chemicals and physical agents (2). A commonly used two-stage model of skin cancer involves initiation by treatment of the skin with 7,12-dimethylbenz(*a*)anthracene (DMBA) followed by promotion through treatment with skin tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

Interleukin (IL)-12 is a 70 kDa heterodimeric protein composed of two disulfide-bonded chains, the p40 and p35 subunits (3). IL-12 has been shown to possess potent antitumor activity in a wide variety of murine tumor models (4–8) of various histologies, including carcinomas from the colon, kidney and lung (4,9–12). The presence of IL-12 at the tumor site in these models has been shown to be critical for tumor regression (4), and, conversely, the tumor-bearing state is often characterized by a diminished capacity to produce IL-12 and an increase in the immunosuppressive cytokine, IL-10 (13). We have shown that IL-12 deficiency promotes photocarcinogenesis in mouse skin (14); however, the role of IL-12 in chemical carcinogenesis is not clearly understood.

To assess the role of IL-12 in chemical carcinogenesis, we generated IL-12p40-knockout mice on a C3H/HeN background and compared the effects of a DMBA-initiated and TPA-promoted two-stage skin chemical carcinogenesis protocol in the interleukin-12-knockout (IL-12 KO) mice and their wild-type counterparts. Here, we report that IL-12 deficiency is associated with an enhanced resistance to the development of TPA-induced skin tumors in DMBA-initiated mouse skin, which suggests that IL-12 promotes skin tumor development in this model of chemical carcinogenesis. We further show that this resistance to TPA-induced skin tumor promotion in IL-12 KO mice is associated with a reduction in the DMBA/TPA-induced inflammatory responses in the skin and tumors of these mice.

Materials and methods

Animals

IL-12p40-knockout mice on the C3H/HeN background were bred in our Animal Resource Facility at the University of Alabama at Birmingham, as described earlier (14). The IL-12 (−/−) mice carry a germ line disruption of the gene. This mutation removes the p40 chain of the IL-12 protein molecule and therefore completely eliminates the synthesis of biologically active IL-12 protein. Mice that are homozygous for this IL-12p40 deletion mutation are viable and their viability is no lower than that of their wild-type counterparts (C3H/HeN). The 6- to 7-week-old wild-type mice were purchased from Charles River Laboratory (Wilmington, MA) and acclimatized for at least 1 week before the start of experiments. All mice were maintained in our Animal Resource Facility under the following conditions: 12 h dark–12 h light cycle, 24 ± 2°C temperature and 50 ± 10% relative humidity. The mice were fed Purina Teklad diet (Harlan Teklad, Madison, WI) with water *ad libitum*. The experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Antibodies and chemicals

Immunostaining-specific cyclooxygenase-2 (COX-2) antibody and a kit for prostaglandin (PG) E_2 analysis were obtained from Cayman Chemicals (Ann Arbor, MI). The antibodies used to detect proliferating cell nuclear antigen (PCNA), cyclin D1 and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). DMBA, TPA, trypsin, DNase and all other chemicals of analytical grade were purchased from Sigma Chemical Co. (St Louis, MO).

DMBA-initiated and TPA-promoted two-stage skin tumor protocol

As male mice develop dominance and aggressive behavior with the age compared with female mice, we used female mice in our experiment. Also, based on our previous experience, we did not notice any significant difference in terms of skin tumor development between male and female mice. Two days before the start of the experiments, the dorsal skin area of female mice was

Abbreviations: cDNA, complementary DNA; COX-2, cyclooxygenase-2; C_t, cycle threshold; DMBA, 7,12-dimethylbenz(a)anthracene; IL, interleukin; IL-12 KO, interleukin-12-knockout; MPO, myeloperoxidase; mRNA, messenger RNA; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PG, prostaglandin; TNF-α, tumor necrosis factor-α; TPA, 12-O-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

shaved with electric clippers and depilating skin lotion was applied for 2–3 min, after which the area was washed with water. For tumor initiation, 40 female mice (20 IL-12 KO and 20 wild-type mice) were treated with a single topical application of DMBA (400 nmol/mouse/0.2 ml of acetone). One week later, the tumor promotion protocol was started. The mice were treated topically with TPA (40 nmol/mouse/100 µl acetone) and this treatment was repeated twice weekly throughout the course of the experiment. Negative control groups of mice, which were used to assess spontaneous tumor induction, were IL-12 KO ($n = 10$) and wild-type mice ($n = 10$) treated with vehicle (0.2 ml acetone) alone twice a week. At the termination of the experiment (at the 24th week after application of TPA), mice were killed. Tumors and tumor-uninvolved skin samples were collected for the analysis of biomarkers of interest.

Evaluation of tumor growth

The skin of the mice was examined once a week for the appearance of papillomas or tumors until the yield and size of the tumors had stabilized. Growths that were >1 mm in diameter and that persisted for at least 2 weeks were defined as tumors and recorded.

Malignant conversion of papillomas to carcinomas

To determine the effect of IL-12 on malignant progression of papillomas to carcinomas, the mice were observed for 24 weeks after the start of TPA treatment. Carcinoma development was identified grossly as the number of downward-invading lesions per mouse per week and each lesion was verified histopathologically at the time of termination of the experiment (15,16) or on killing of mice that became moribund prior to the termination of the experiment. At the termination of the experiment or on killing of moribund mice, the dimensions of all the tumors or carcinomas on each mouse were recorded, and all tumors and suspected carcinomas were harvested for histologic verification. The tumor or carcinoma volumes were calculated using the hemiellipsoid model formula: tumor volume = 1/2 (4π/3) (l/2) (w/2) h, where l = length, w = width and h = height, as described earlier (17). The percent malignant conversion of benign skin papillomas into squamous cell carcinoma was calculated by dividing the total number of carcinomas by the total number of papillomas and multiplying by 100 (18).

Pathological evaluation of skin tumors

At the termination of the skin tumor protocol, representative biopsies from all skin tumors were collected, fixed in 10% formaldehyde and embedded in paraffin. Deparaffinized sections (5 µm thick) were stained with hematoxylin and eosin for pathological evaluation by three independent observers who were blinded to the source of the tissues. The specimens were classified as tumors or non-neoplastic lesions according to the following criteria: loss of keratinization or keratinized centers, atypical cells and the presence of horn pearls.

Immunohistochemical detection of COX-2 and PCNA

Five micrometer thick frozen sections were hydrated in phosphate-buffered saline (PBS) and then non-specific binding sites were blocked with 1% bovine serum albumin and 2% goat serum in PBS. The sections were incubated with anti-COX-2 or anti-PCNA antibodies for 2 h at room temperature, washed and then incubated with biotinylated secondary antibody for 45 min followed by horseradish peroxidase-conjugated streptavidin. After washing in PBS, sections were incubated with diaminobenzidine substrate and counterstained with hematoxylin. Representative pictures were taken using an Olympus BX41 microscope fitted with a Q-Color 5 digital camera.

Analysis of PGE_2 by enzyme immunoassay

Skin or tumor samples were homogenized, clarified supernatants prepared and the concentration of PGE_2 determined in the supernatants using the Cayman PGE_2 Enzyme Immunoassay Kit (Cayman Chemicals) following the manufacturer's protocol and reagents.

Western blot analysis

Tumor or epidermal skin samples were washed with cold PBS and homogenized with ice-cold lysis buffer supplemented with protease inhibitors, as detailed previously (19–21). The epidermis or tumor tissue samples were pooled from at least three mice in each group, and three sets of pooled samples from each treatment group were used to prepare lysates. Thus, 10 mice per treatment group were used for sample preparation. For western blotting, proteins (20–35 µg) were resolved on 10% Tris–glycine gels and transferred onto a nitrocellulose membrane. After blocking the non-specific binding sites, the membrane was incubated with the primary antibody at 4°C overnight. The membrane was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and the immunoreactive bands were visualized using the enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). To verify equal protein loading and transfer of proteins from gel to membrane, the blots were stripped and reprobed for β-actin antibody.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from mouse epidermis or tumor samples using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol, as detailed previously (22). The messenger RNA (mRNA) expression of PCNA and cyclin D1 in skin and tumor samples was determined using real-time polymerase chain reaction (PCR). For mRNA quantification, complementary DNA (cDNA) was synthesized using 3 µg RNA through a reverse transcription reaction (iScript™ cDNA Synthesis Kit, Bio-Rad, Hercules, CA). Using SYBR Green/Fluorescein PCR Master Mix (SuperArray Bioscience Corporation, Frederick, MD), cDNA was amplified using real-time PCR with a Bio-Rad MyiQ thermocycler and the SYBR green detection system (Bio-Rad). Samples were run in triplicate to ensure amplification integrity. Manufacturer-supplied (SuperArray, Bioscience Corporation) primer pairs were used to measure the mRNA levels of PCNA and cyclin D1. The standard PCR conditions were as follows: 95°C for 15 min, then 40 cycles at 95°C, 30 s; 55°C, 30 s and 72°C, 30 s, as recommended by the primer's manufacturer. The expression levels of genes were normalized to the expression level of β-actin mRNA in each sample. The threshold for positivity of real-time PCR was determined based on negative controls. For mRNA analysis, the calculations for determining the relative level of gene expression were made using the cycle threshold (C_t) method. The mean C_t values from duplicate measurements were used to calculate the expression of the target gene with normalization to a housekeeping gene used as an internal control (β-actin) and using the $2^{-\Delta C_t}$ formula.

Cytokine assay by enzyme-linked immunosorbent assay

Epidermal or tumor homogenates were used for the analysis of cytokines, including tumor necrosis factor-α (TNF-α), IL-1β and IL-6, using enzyme-linked immunosorbent assay kits (BioSource International, Camarillo, CA) following the manufacturer's protocol.

Short-term in vivo experiments

To verify that IL-12-deficient mice are resistant to TPA-induced skin inflammation as compared with their wild-type counterparts, short-term *in vivo* experiments were performed. For this purpose, IL-12 KO and wild-type mice were treated with single or multiple applications of TPA and killed 6 h after the last TPA treatment. Treated skin areas were collected and analyzed for biomarkers of inflammation, including TPA-induced edema, infiltration of leukocytes and the levels of myeloperoxidase (MPO), COX-2 and PGE_2.

Leukocyte infiltration

TPA-induced leukocyte infiltration and the levels of MPO were assessed in skin samples obtained from mice subjected to the short-term *in vivo* protocol. MPO was measured as a marker of tissue infiltration in skin homogenate samples, as described previously (21).

Statistical analysis

The susceptibility of IL-12 KO mice to tumors and carcinomas caused by DMBA/TPA treatment was compared with the susceptibility of wild-type, C3H/HeN, mice. The four outcome variables used in this comparison were tumor incidence, tumor multiplicity, carcinoma incidence and carcinoma multiplicity. The data were collected in an aggregate form over a period of 24 weeks. A linear model was fit to each of the outcome variables. The independent variables in the model were group, time and a group–time interaction term. The interaction term helps to test if the rate of change in the outcome variables over time is a function of the group. To compare the levels of COX-2-positive and PCNA-positive cells in treatment groups, at least five to six different fields from each section were selected. A simple analysis of variance followed by Tukey's post-hoc test was used to calculate statistical significance of the data obtained from tumor or carcinoma volume and the levels of MPO, PCNA, cyclin D1, PGE_2 and cytokines. A P value <0.05 was considered significant.

Results

IL-12-deficient mice are resistant to TPA-induced tumor promotion in DMBA-initiated mouse skin

Following a two-stage chemical carcinogenesis skin tumor protocol, we found that TPA-induced tumor development occurred 4 weeks earlier in C3H/HeN (wild-type) mice than IL-12 KO mice (Figure 1A). Throughout the carcinogenesis protocol, the percentage of wild-type mice with tumors was higher than the percentage of IL-12 KO mice with tumors. This effect persisted through the termination of the experiment but did not attain statistical significance under the conditions used in this study (Figure 1A). In terms of tumor multiplicity, the total number of tumors in the group of wild-type mice remained higher than the total number of tumors in the group of IL-12

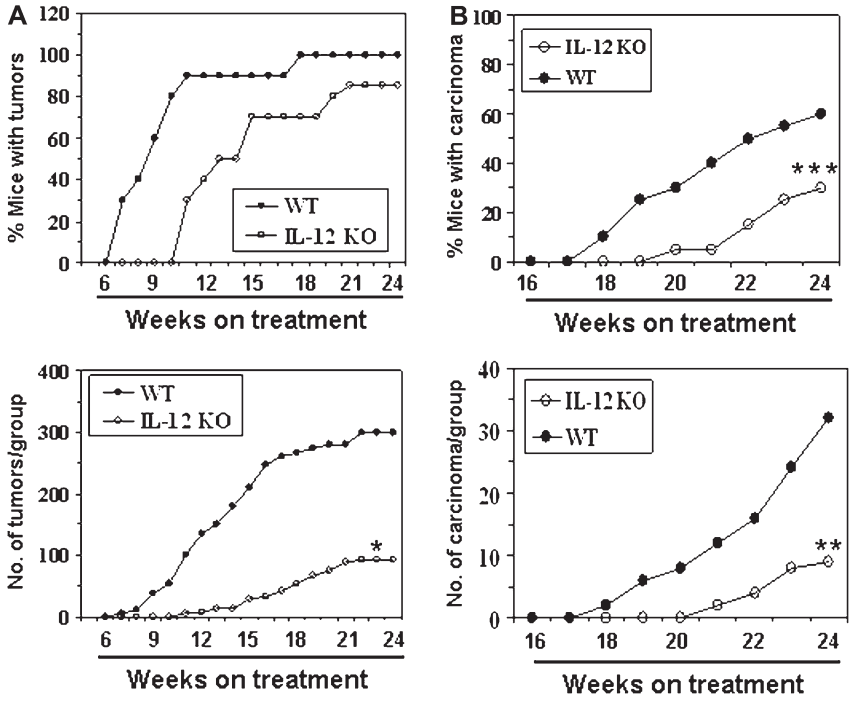

Fig. 1. Effect of IL-12 deficiency on TPA-induced tumor and carcinoma growth in DMBA-initiated mouse skin. (**A**) IL-12-deficient mice are resistant to TPA-induced tumor promotion in DMBA-initiated mouse skin compared with wild-type (WT) mice. The percentage of mice with tumors (A) and the total number of tumors per group are plotted as a function of the number of weeks on treatment ($n = 20$). (**B**) IL-12-deficient mice are resistant to TPA-induced malignant transformation of papillomas to carcinomas in DMBA-initiated mouse skin compared with their wild-type mice. The percentage of mice with carcinoma and total number of carcinomas per group are plotted as a function of the number of weeks on treatment (20 mice per treatment group, $n = 20$). Significant difference versus wild-type mice, $^{*}P < 0.005$; $^{**}P < 0.01$; $^{***}P < 0.05$.

KO mice throughout the experimental protocol (Figure 1A and B). At the termination of the experiment, the total number of tumors was significantly higher in the group of wild-type mice than the group of IL-12 KO mice (299 versus 92, $P < 0.005$). Taken together, these tumor data indicate that IL-12-deficient mice are more resistant to TPA-induced tumor development in DMBA-initiated mouse skin than their wild-type counterparts.

IL-12 deficiency suppresses the malignant progression of papillomas to carcinomas

Histopathologic examination of the mice at the termination of the experiment (week 24) further revealed that there were 9 squamous cell carcinomas, 80 squamous cell papillomas and 3 keratoacanthomas in the group of DMBA/TPA-treated IL-12 KO mice. In contrast, there were 32 squamous cell carcinomas, 261 squamous cell papillomas and 6 keratoacanthomas in the group of DMBA/TPA-treated wild-type mice. Sixty percent of mice in the wild-type group developed at least one carcinoma, whereas only 30% ($P < 0.05$) of mice in the IL-12 KO group developed at least one carcinoma at the termination of the experiment. The malignant progression of papillomas to carcinoma also was recorded on weekly basis and the kinetics of malignant conversion of papillomas into carcinomas in IL-12 KO mice was also significantly lower compared with wild-type mice (Figure 1B; Fisher–Irwin Exact test; $P < 0.01$). These data suggest that IL-12-deficient mice are not only resistant to DMBA/TPA-induced skin tumor development but also resistant to malignant progression of papillomas to carcinomas.

IL-12 deficiency modulates DMBA/TPA-induced epidermal COX-2 expression and PGE_2 production

Skin tumor promoters induce COX-2 expression resulting in a subsequent increase in the production of PG metabolites in the skin and enhanced COX-2 and PG metabolite expression is a characteristic response of keratinocytes following acute or chronic exposure to tumor promoters. The increased expression of COX-2 and PG metabolites has been observed in squamous and basal cell carcinomas of the skin (23,24). Therefore, we examined and compared the levels of COX-2 and PGE_2 in the DMBA/TPA-treated skin and tumor samples from IL-12 KO mice and their wild-type counterparts. Skin and tumor samples were collected at the termination of the chemical carcinogenesis protocol. Immunostaining of COX-2 indicated that treatment of the skin with TPA resulted in higher expression of COX-2 in the skin tumors of both IL-12 KO and wild-type mice as compared with the expression in non-TPA-treated normal mouse skin. Microscopic examination indicated that staining of the COX-2-positive cells in the skin tumors of the wild-type mice was greater than in the skin tumors of IL-12 KO mice (Figure 2A). Western blot analysis confirmed higher expression of COX-2 protein in the skin and tumors of DMBA/TPA-treated wild-type mice than the skin and tumors of IL-12 KO mice, as shown in terms of the fold differences calculated from densitometric analyses of the immunoblots (Figure 2B). As increased expression of COX-2 results in greater synthesis of PG metabolites, we determined the levels of PG metabolites in the tumors and tumor-uninvolved skin of IL-12 KO and wild-type mice with particular emphasis on PGE_2 because PGE_2 is known to play a pivotal role in tumor promotion. As shown in Figure 2C, the levels of PGE_2 were significantly higher in the DMBA/TPA-treated skin of both IL-12 KO and wild-type mice than in non-DMBA/TPA-treated epidermis; however, the levels of epidermal PGE_2 were significantly lower in the DMBA/TPA-treated skin of IL-12 KO mice than the DMBA/TPA-treated skin of wild-type mice. Similar results were observed when the levels of PGE_2 were determined in skin tumor samples of IL-12 KO mice and compared with the tumor samples of wild-type mice (Figure 2C).

IL-12 deficiency modulates DMBA/TPA-induced proliferation of epidermal cells and skin tumors

As tumor promoters, including TPA, have been shown to stimulate proliferation of skin cells, we estimated the proliferation potential of

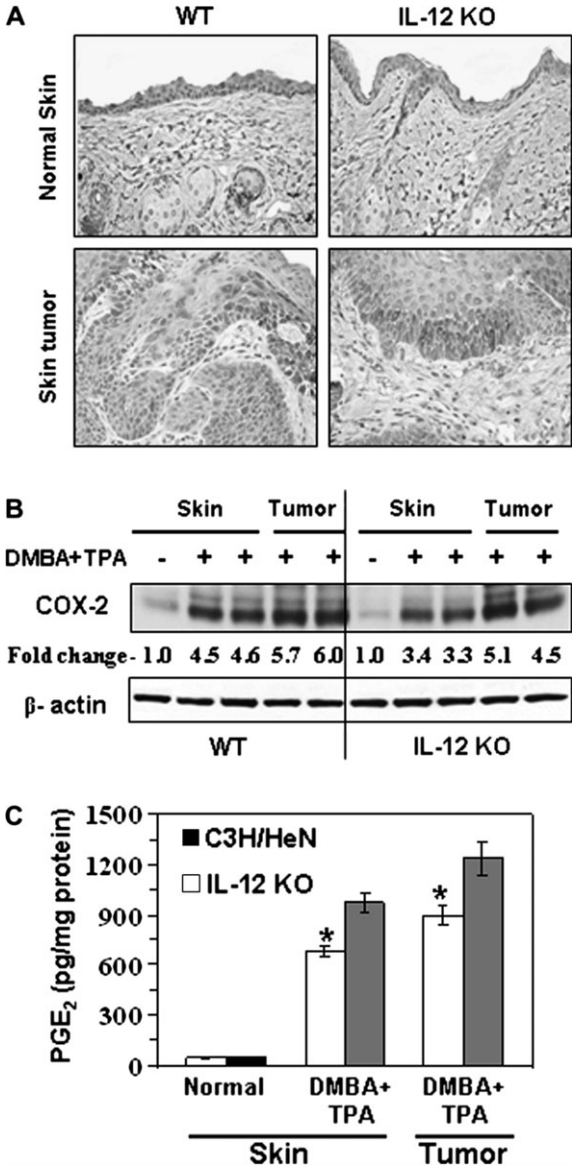

Fig. 2. IL-12 deficiency suppresses TPA-induced inflammatory responses in terms of COX-2 expression and PGE_2 production in the skin and tumors of DMBA-initiated mouse skin compared with their wild-type counterparts. (**A**) Immunohistochemical detection of COX-2 in skin tumor samples from IL-12 KO and WT mice and data were compared with normal skin of the mice. Tumor-uninvolved skin and skin tumor samples were collected at the termination of the carcinogenesis experiment and processed for the analysis of epidermal COX-2 expression using western blotting (**B**) and the levels of PGE_2 production (**C**) were determined in the homogenates of epidermal skin or tumor lysates by an enzyme immunoassay. The concentration of PGE_2 was expressed in terms picogram per milligram protein as mean ± SD (10 mice per group; $n = 10$). Significantly less than WT mice, $^*P < 0.05$.

epidermal cells as another marker of the TPA-induced inflammatory reaction in the skin and skin tumors. For this purpose, we determined the levels of PCNA through immunohistochemical detection of PCNA-positive cells, western blotting and real-time PCR analysis of mRNA expression. The immunostaining of PCNA-positive cells (the dark brown staining in Figure 3A) in DMBA/TPA-treated skin was intense in the skin of both IL-12 KO and wild-type mice as compared with the skin of non-DMBA/TPA-treated mice; however, the PCNA-positive cells in the DMBA/TPA-treated skin of wild-type mice exhibited higher intensity staining than the PCNA-positive cells in the DMBA/TPA-treated skin of IL-12 KO mice (Figure 3A) suggesting that the proliferation potential of epidermal cells in TPA-treated wild-type mouse skin is higher than the proliferation potential of epidermal cells in TPA-treated IL-12 KO mouse skin. A similar difference in staining intensity also was noted in the tissue sections of tumor samples from wild-type mice as compared with the tumor samples of IL-12 KO mice (Figure 3A, blue circled areas). The microscopic observations also indicated that there were a greater number of infiltrating leukocytes in the tumors of wild-type mice than the tumors of IL-12 KO mice (circled by the red line). A greater influx of infiltrating leukocytes may increase the levels of reactive oxygen species and proinflammatory cytokines and thus may promote the growth of tumors. The higher level of DMBA/TPA-induced PCNA in wild-type mouse skin and tumors was further confirmed by western blot analysis (protein level, Figure 3B) and real-time PCR (mRNA expression, Figure 3C), which revealed that the levels of PCNA were higher in DMBA/TPA-treated mouse skin and tumors than non-DMBA/TPA-treated normal mouse skin and the levels of PCNA in DMBA/TPA-treated IL-12-deficient mouse skin and tumors were lower (mRNA level, $P < 0.05$) than the skin and tumors of DMBA/TPA-treated wild-type mice. The mRNA levels, as estimated by real-time PCR, of cyclin D1 were also higher in the tumors of IL-12 KO and wild-type mice than the age-matched normal mouse skin, with the levels of cyclin D1 being markedly lower in the tumors of IL-12 KO mice than the tumors of wild-type mice.

Tumors of IL-12 KO mice express lower levels of proinflammatory cytokines than wild-type mice

It is well known that treatment of skin with tumor promoters, including the phorbol esters, induces inflammatory responses, including the induction of proinflammatory cytokines (2). Therefore, we examined and compared the effects of chronic TPA application on the induction of proinflammatory cytokines in tumors of both IL-12 KO and their wild-type counterparts. The analysis of tumor homogenates by enzyme-linked immunosorbent assay revealed that the levels of the proinflammatory cytokines TNF-α (48%), IL-1β (42%) and IL-6 (53%) were significantly lower in the tumors of IL-12 KO mice than the tumors of wild-type mice (Figure 4).

IL-12-deficient mice are more resistant to tumor promoter-induced inflammatory reactions than wild-type mice

To further verify that IL-12 deficiency is associated with resistance to TPA-induced inflammatory responses in mouse skin, we performed short-term *in vivo* experiments. The edema induced by tumor promoters is used as a marker of the inflammatory responses in the skin (2). The punch weight, that is the weight of a 1 cm diameter punch of the skin, indicates the accumulation of edema. Treatment of the skin of IL-12 KO or wild-type mice with an acute (single) TPA application resulted in a significantly higher skin punch weight (≈50%, $P < 0.01$) compared with normal non-TPA-treated mouse skin. However, the punch weight of the skin from IL-12 KO mice was lower (21%, $P < 0.05$) than the punch weight of wild-type mice. Similar results were obtained after the multiple treatments with TPA (3×) of both IL-12 KO and wild-type mouse skin. The effect of another skin tumor promoter, mezerein, was also determined. Acute application of mezerein on the IL-12 KO and wild-type mice skin resulted in a significantly higher skin punch weight as compared with non-mezerein-treated normal mice skin. However, the punch weight of the skin after mezerein treatment of IL-12 KO mice skin was lower ($P < 0.05$) than the skin punch weight of wild-type mice. To further verify the difference in the TPA-induced edema between IL-12 KO and wild-type mice, we measured the bi-fold skin thickness before and 6 h after acute TPA or mezerein application of the dorsal skin. As shown in Table I, the bi-fold skin thickness was increased significantly after TPA or mezerein treatments ($P < 0.05$) in both IL-12 KO mice and wild-type mice compared with non-TPA- or non-mezerein-treated normal mouse skin; however, the bi-fold skin thickness of the skin of IL-12 KO mice after treatment with the tumor promoters was less than the bi-fold skin thickness of the wild-type mice (Table I).

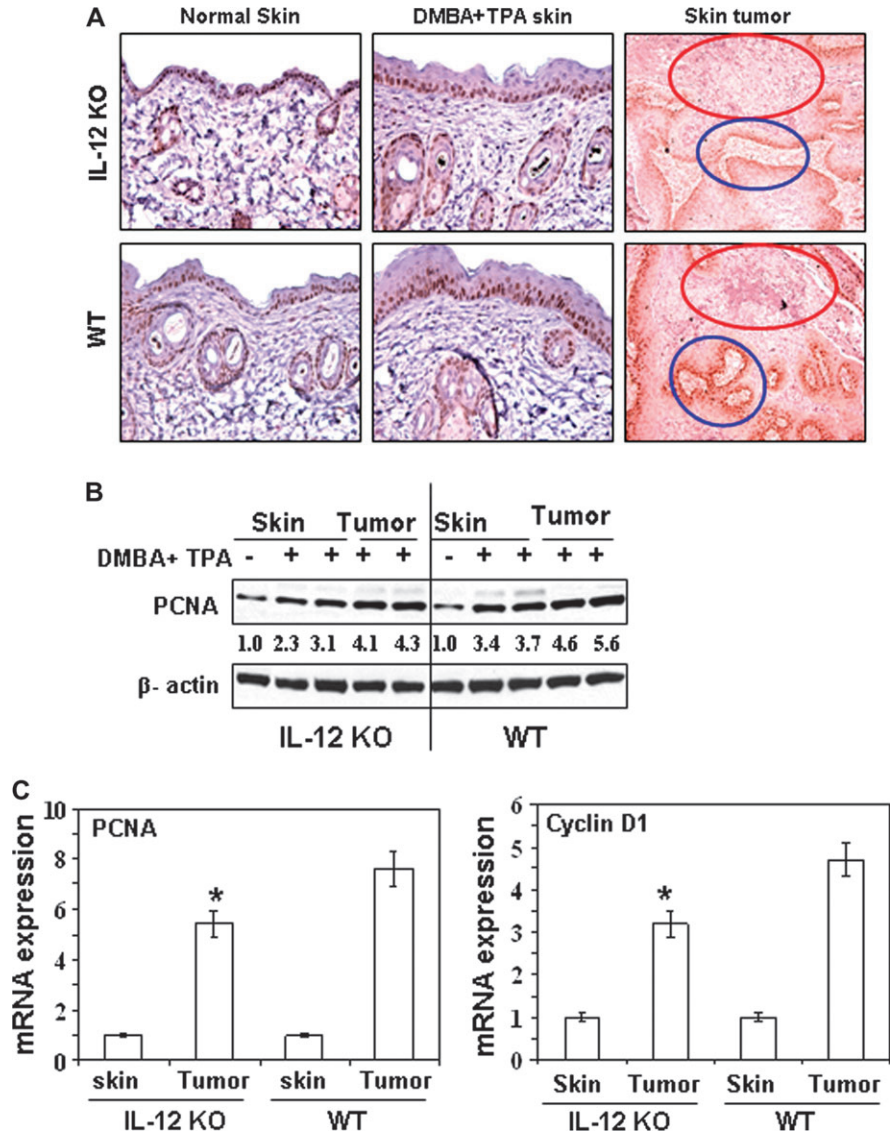

Fig. 3. IL-12 deficiency suppresses the levels of PCNA in TPA-promoted and DMBA-initiated mouse skin and skin tumors. (**A**) Immunohistochemical detection of PCNA expression in skin and tumor samples of IL-12 KO and WT mice. Representative photomicrographs of areas with high numbers of infiltrating cells and intensely PCNA$^+$ cells are shown in tumors of WT and IL-12 KO mice. The areas encircled with the red line illustrate the differences in the levels of cellular infiltration, whereas the areas encircled with the blue line illustrate the differences in the intensity of PCNA$^+$ cells between the IL-12 KO and WT mice, $n = 6$. (**B**) PCNA expression was analyzed in the skin and tumor samples of IL-12 KO mice and their wild-type counterparts using western blot analysis, as described under Materials and Methods. A representative blot is shown from three independent experiments with identical observations, and the relative density of each band (arbitrary) is shown under the immunoblot. In each experiment, the epidermis or tumor samples were pooled from at least three mice. (**C**) The results of mRNA expression of PCNA and cyclin D1 are presented after normalization to β-actin using the C_t method. Significantly less versus wild-type mice, $^*P < 0.05$ (10 mice per group were used for data analysis, $n = 10$).

Infiltrating leukocytes are considered to be a major source of inflammatory reactions and oxidative stress (2,23,25). Routine hematoxylin and eosin staining of skin sections revealed that treatment of the skin with TPA induces infiltration of inflammatory leukocytes (activated monocytes/macrophages and neutrophils), which peaks around 6 h post-TPA application (Figure 5A). At 6 h post-TPA treatment, the acute TPA application-induced infiltration of leukocytes was lower in the skin of IL-12 KO mice compared with wild-type mice in the treated skin sites at 6 h post-TPA treatment. We also found an increase in the levels of MPO in skin samples after treatment with TPA (Figure 5B), suggesting an influx of leukocytes into the inflamed skin, with the levels of MPO were significantly lower ($P < 0.05$) in the skin of IL-12 KO mice than the skin of wild-type mice both after acute (Figure 5B) and multiple treatments (data not shown) of the skin with TPA. These data suggest that the skin of IL-12 KO mice is more resistant to TPA-induced inflammatory reactions than the skin of wild-type mice.

Using short-term experiments, we further determined whether IL-12 deficiency inhibits TPA-induced COX-2 expression and thereby PGE_2 production. COX-2-specific immunostaining revealed that treatment of the mouse skin with TPA resulted in higher levels of COX-2 expression as compared with non-TPA-treated normal mouse skin (Figure 5C). However, the levels of TPA-induced COX-2 expression in terms of intensity of immunostaining were lower after an acute application of TPA in the skin of IL-12 KO mice than the skin of wild-type mice. Similarly, enzyme immunoassay data indicated that TPA-induced increases in the levels of PGE_2 were higher in the skin of wild-type and IL-12 KO mice than non-TPA-treated normal mouse skin and, further, that the levels of TPA-induced PGE_2 were significantly lower ($P < 0.05$) in the skin of IL-12 KO mice than the skin of wild-type mice.

Discussion

The two-stage chemical carcinogenesis experiments conducted in IL-12 KO mice and their wild-type counterparts indicated that IL-12 deficiency promotes resistance to tumor development in terms of tumor incidence and tumor multiplicity. Additionally, the present study indicates that mice that are deficient in IL-12 are also resistant to malignant transformation of papillomas to carcinomas. These data clearly suggest that endogenous IL-12 promotes TPA-induced tumor development in DMBA-initiated mouse skin. In contrast, we have found previously that endogenous IL-12 plays a crucial role in inhibition of photocarcinogenesis in mice (14).

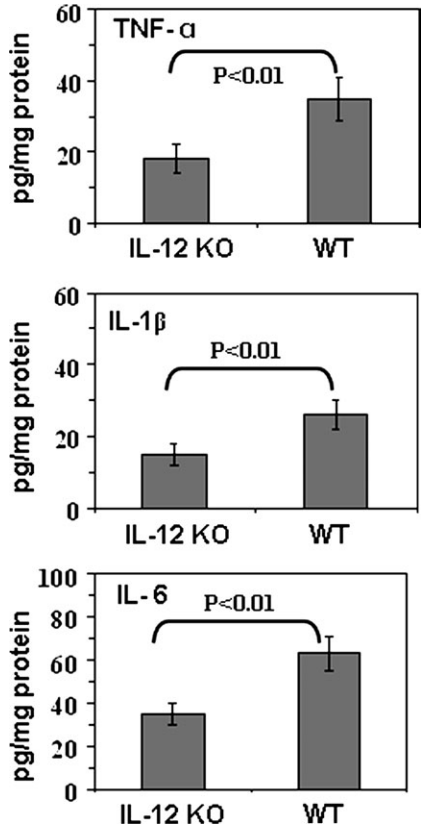

Fig. 4. Effect of DMBA/TPA treatment on the proinflammatory cytokines in skin tumors. Tumors arising in WT after treatment with DMBA/TPA exhibited relatively higher levels of proinflammatory cytokines than tumors of similarly treated IL-12 KO mice. At the termination of the experiment, tumor samples were collected and homogenates were prepared for the analysis of the levels of TNF-α, IL-1β and IL-6 using enzyme-linked immunosorbent assay. The concentration of each cytokine is reported in terms of picogram per milligram protein as a mean ± SD (8–10 mice per treatment group were used, $n = 8–10$).

Treatment of mouse skin with various tumor promoters has been shown to induce inflammatory responses and chronic inflammation has been associated with the development of skin tumors (2,23). Therefore, tumor promoter-induced inflammation and the mediators of this inflammation are considered to be potent regulators of skin tumor promotion. We had found that IL-12 deficiency-induced enhancement of ultraviolet (UV) radiation-induced skin tumor development was mediated through exacerbation of the UV radiation-induced skin inflammatory reactions (26). In contrast, the current studies indicate that IL-12 deficiency inhibits TPA-induced inflammatory responses and that this is a contributing factor in promoting resistance to the development of skin tumors in DMBA-initiated mouse skin.

One of the most important enzymes in the process of inflammation and tumor development is inducible COX-2. COX-2 is a rate-limiting enzyme for the generation of PG metabolites from arachidonic acid (27). The overexpression of COX-2 has been linked to the pathophysiology of inflammation and cancer (28) due to enhanced synthesis of PG metabolites, which have been shown to be potential contributing factors in chemical carcinogenesis (29,30). A number of studies have demonstrated the involvement of COX-2 overexpression in two-stage chemical carcinogenesis, as well as transformation of premalignant lesions to squamous cell carcinomas (31). A role of COX-2 in chemical carcinogenesis is also supported by several studies, demonstrating that inhibition of COX-2 activity by specific inhibitors can partially block or inhibit skin tumor development in DMBA-initiated mouse skin (32,33). In this study, we found that IL-12-deficient mice expressed lower levels of COX-2 expression and less production of PGE_2 than wild-type mice after TPA treatment. This may have contributed to delayed tumor development and the inhibitory effect on tumor growth in IL-12 KO mice compared with wild-type mice. The decreased proliferating potential of epidermal keratinocytes as indicated by the lower expression of PCNA protein and mRNA may also be a contributing factor to the delayed and lower tumor burden in DMBA/TPA-treated IL-12-deficient mice than wild-type mice.

Chronic induction of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, also has been associated with the promotion of skin tumor growth (23,34,35). Human and murine squamous cell

Table I. IL-12 deficiency is associated with resistance to tumor promoter-induced skin edema in mice[a]

Treatments	Dose of tumor promoter (μg)	Skin punch weight (mg)	% lower	Bi-fold skin thickness (mm)	% lower
Acetone (WT)		20.3 ± 1.0[b]		0.82 ± 0.08	
Acetone (IL-12 KO)		20.0 ± 1.0		0.80 ± 0.09	
Acute TPA (1×)					
WT	5	29.2 ± 1.3		1.13 ± 0.10	
IL-12 KO		27.0 ± 1.3[c]	21	1.00 ± 0.10[c]	35
Multiple TPA (3×)					
WT	5	36.6 ± 1.4		1.26 ± 0.09	
IL-12 KO		32.5 ± 1.3[c]	23	1.11 ± 0.08[c]	29
Acute mezerein (1×)					
WT	5	26.3 ± 2.0		1.05 ± 0.08	
IL-12 KO		24.5 ± 1.5[c]	25	0.94 ± 0.09[c]	38

WT, wild-type.

[a]Tumor promoter-induced skin edema was determined by weighing 1.0 cm diameter punch biopsies of treated skin sites that were obtained 6 h after tumor promoter treatment. Bi-fold skin thickness of treated areas of the skin was measured 6 h after tumor promoter application. Mice were topically treated with either TPA or mezerein in 0.2 ml acetone per mouse at the indicated doses of tumor promoters. In the case of TPA, the effect was also evaluated after multiple applications (3×) of TPA. Control mice were treated with vehicle (acetone, 0.2 ml acetone per mouse) alone.

[b]Mean ± SD of three individual values from each mouse (five mice per group).

[c]Significantly less versus WT mice, $P < 0.05$.

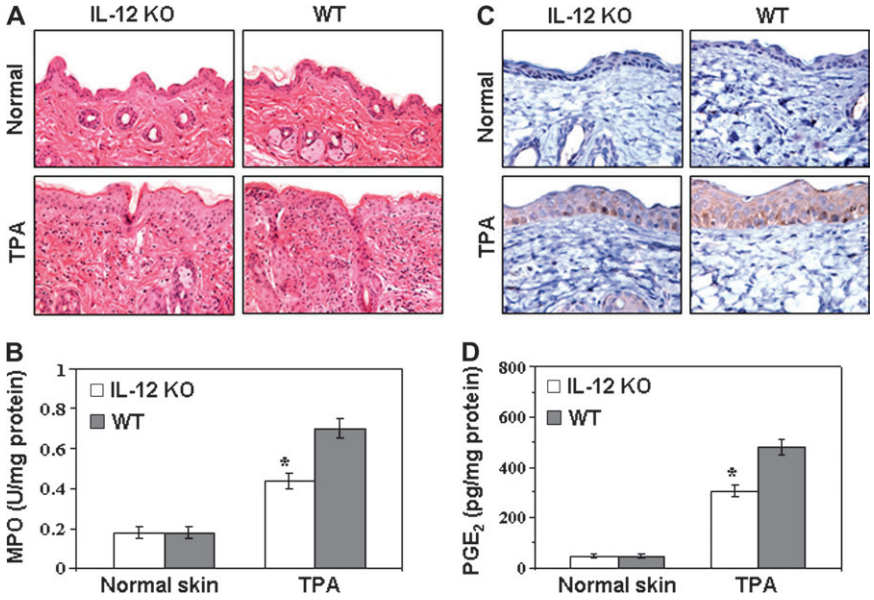

Fig. 5. *In vivo* short-term experiments. IL-12-deficient mice exhibit reduced TPA-induced inflammatory reactions in the skin as compared with their wild-type counterparts. TPA (40 nmol) was topically applied only once (acute effect) to the shaved dorsal skin of either wild-type or IL-12 KO mice. The mice were killed 6 h later, and the treated and non-treated control skin samples were collected. (**A**) Skin samples were processed for standard hematoxylin and eosin staining to examine cellular infiltration. Representative micrographs from each treatment group are shown, $n = 5$. (**B**) MPO was determined as a marker of tissue infiltration in skin homogenates. MPO data are reported as the mean ± SD of unit per milligram protein ($n = 5$). (**C**) Frozen skin sections were processed for immunostaining for the detection of COX-2 expression, as described under Materials and Methods. COX-2 staining is shown as dark brown, $n = 5$. (**D**) The levels of PGE_2 were determined in the epidermal homogenate samples using Enzyme Immunoassay Kit following the manufacturer's protocol, and the data are expressed in terms of picogram per milligram protein as mean ± SD. Significant difference versus wild-types, $^*P < 0.05$. Five mice per treatment group were used for data analysis ($n = 5$).

carcinomas have been reported to produce proinflammatory cytokines, including TNF-α, IL-1β and IL-6, and the production of these cytokines promotes a suitable microenvironment for tumor progression and angiogenesis in a variety of neoplasms (36). In our study, the levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) were lower in DMBA/TPA-treated tumors of IL-12 KO mice than wild-type mice. The lower levels of these inflammatory cytokines in IL-12-deficient mice may contribute to the inhibition of skin tumor growth and thus, the development of tumors may be slower and less in DMBA/TPA-treated skin (Figure 1). These data suggest a positive relationship between TPA-induced inflammation and increased skin tumorigenesis in wild-type mice. Thus in the presence of higher levels of PGE_2, the risk of tumor promoter-induced skin tumor development would increase in wild-type mice compared with IL-12-deficient mice. Proinflammatory cytokines can induce COX-2 expression (37), which in turn may increase the production of PG metabolites that would stimulate tumor growth. Thus, these reports suggest that IL-12 deficiency inhibits tumor promoter-induced inflammatory responses in mouse skin compared with their IL-12-proficient wild-type counterparts and that may be a contributing factor for the inhibition of skin tumor development in DMBA/TPA-treated skin of IL-12-deficient mice. However, reverse effects of IL-12 were observed in UVB-irradiated mouse skin. In photocarcinogenesis mouse model, IL-12 deficiency enhances inflammatory responses, such as induction of COX-2 expression and increase in the levels of PGE_2 and proinflammatory cytokines, in UVB-exposed skin and that may have contributed to the enhanced growth and multiplicity of skin tumors (38).

It is desirable to discuss the role of p40 subunit. It is well known that IL-12 and IL-23 share a common p40 subunit that is covalently linked either to a p35 subunit to form IL-12 or to a p19 subunit to form IL-23. Despite similarities in p40 chain, there is increasing evidence that IL-12 and IL-23 drive divergent immunological pathways. Whereas IL-12 leads to development of interferon-γ-producing Th1 cells and enhances cytotoxic and antitumor responses and IL-23 drives a pathway that leads to the generation of IL-17-producing CD4+ T cells. The induction of IL-23-derived processes leads to recruitment of a range of inflammatory cells as well as T_HIL-17 T cells and has been shown to be crucial to the pathogenesis of a number of inflammatory diseases (39,40). As the p40 subunit is depleted in our mouse model, we assume that the loss of p40 may affect the functions of both IL-12 as well as IL-23. Also there are evidences that p40 homodimer is immunosuppressive in nature (41), and some reports indicate p40 homodimer as a 'so-called' biologically inactive molecule and has a role in inflammation in microglia (42). Further studies are required to address some more unexplained issues.

Collectively, the data from this study suggest that a reduction in the levels of endogenous IL-12 may be considered as an effective strategy for the prevention of chemical tumor promoter-induced skin tumor development in chemical carcinogen-initiated skin.

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