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Research Article

IFNγ Promotes Papilloma Development by Up-regulating Th17-Associated Inflammation

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Abstract

IFNγ plays a crucial role in immunity against a variety of transplanted tumors and methylcholanthrene-mediated tumorigenesis in mice. However, it is not clear whether and how endogenous IFNγ influences 7,12-dimethylbenz(*a*)anthracene (DMBA)–induced and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced papilloma development. We found here that IFNγ expression was markedly up-regulated shortly after DMBA/TPA application to the skin. Surprisingly, neutralizing IFNγ activity *in vivo* did not increase but rather decreased tumor development. Furthermore, IFNγ receptor–deficient mice were also more resistant to papilloma development than their counterparts were. IFNγ acted mainly in the promotion stage of papilloma development by enhancing TPA-induced leukocyte infiltration and epidermal hyperproliferation. The up-regulation of tumor necrosis factor α, interleukin (IL)-6, and transforming growth factor β was largely dependent on host IFNγ responsiveness. Remarkably, up-regulation of both IL-17 expression in the skin and T helper 17 (Th17) cell number in draining lymph nodes after DMBA/TPA treatment was dependent on IFNγ signaling. Depletion of IL-17 not only decreased the DMBA/TPA–induced inflammation and keratinocyte proliferation but also delayed papilloma development. These results show that IFNγ, under certain conditions, may promote tumor development by enhancing a Th17-associated inflammatory reaction. [Cancer Res 2009;69(5):2010–7]

Introduction

IFNγ is an important cytokine in tumor immunity (1, 2). Many studies have shown that IFNγ plays a critical role in the rejection of transplanted tumors by mechanisms such as inhibition of angiogenesis (3–5), enhancement of cytotoxic responses against tumors (6), and by its direct action on tumor cells (7). IFNγ also shows its inhibitory role during methylcholanthrene (MCA)–induced tumorigenesis (8). Mice deficient in IFNγ, IFNγ receptor (IFNγR), or signal transducer and activator of transcription 1 (STAT1), a critical molecule for IFNγ signaling, or mice treated with IFNγ-neutralizing monoclonal antibodies (mAb) developed more tumors with shorter latency after MCA injection s.c. or i.m. (9, 10). A well-documented theory for the above observations is that IFNγ plays an important part in the "immunoediting" process of tumor development, during which the host immune system can recognize and eliminate emerging malignant cells (11). Another hypothesis distinguishes the roles of IFNγ in inhibition of transplanted tumors and in control of MCA-induced tumorigenesis. It suggests that it is MCA, and not the tumor cells, that is the target of host responses (12); that is, if the carcinogen cannot be removed or degraded effectively *in vivo*, it will be encapsulated by fibroblasts or other fibrotic tissue as a "foreign body" (13). IFNγ enhances this process and therefore limits MCA diffusion and malignant transformation of adjacent normal cells (10).

However, there are also lines of evidence suggesting that IFNγ promotes tumor development. Hanada and colleagues (14) reported that IFNγ was necessary for the spontaneous development of colorectal carcinomas in SOCS1-deficient mice, and Matsuda and colleagues (15) showed that IFNγ was involved in diethylnitrosamine-induced hepatocarcinogenesis by enhancing monocyte/macrophage activation and eventual DNA damage in hepatocytes. Moreover, it has also been shown that exogenous IFNγ can promote or inhibit the development of 7,12-dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced papillomas, depending on the dosage (16). Therefore, the role of IFNγ and its underlying mode of action during tumor development need further investigation, especially considering the complexity of experimental data obtained, to date, from different laboratories using different tumor models in different mouse strains.

Two-stage skin carcinogenesis serves as a well-characterized *in vivo* model for epithelial neoplasia and mimics the multistage nature of human cancer development (17). It involves administration of a single dose of DMBA followed by weekly applications of TPA. DMBA induces mutations in epidermal cells causing initiation of papilloma (18). Promotion requires repeated exposure of the skin to TPA, which boosts tumor development via induction of chronic inflammation (18).

Three lines of evidence encouraged us to study the role of IFNγ in DMBA/TPA–induced two-stage skin carcinogenesis in IFNγR-deficient and competent 129/Sv/Ev mice. (*a*) About 90% of human neoplasm is of epithelial origin (i.e., cancer). Chronic inflammation is thought to be involved in 20% of these cancers (14, 19, 20). (*b*) Painting DMBA/TPA on a fixed area of the skin surface excludes the encapsulation of the carcinogen as a major mechanism against tumor development. (*c*) 129/Sv/Ev mice have already been used to show the antitumor effect of IFNγ during MCA-induced tumorigenesis (2, 10, 12). Surprisingly, we found that the incidence of papilloma did not increase in the absence of IFNγ activity or IFNγR. In contrast, we showed that IFNγ was directly involved in and strongly enhanced papilloma development during the promotion stage by up-regulating several key proinflammatory cytokines and a local T helper 17 (Th17) response.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Animals. IFNγR-deficient and wild-type 129/Sv/Ev mice were inbred strains purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free environment at the Institute of Biophysics, Chinese Academy of Sciences. Sex- and age-matched mice were used with the approval of appropriate authorities.

Skin carcinogenesis. A single application of 25 μg DMBA (Sigma) and, beginning 1 wk later, 4 μg TPA (Sigma) twice a week were administered on a dorsal area of skin 2 cm^2. Tumors were defined as raised lesions of a minimum diameter of 1 mm that had been present for at least 2 wk (21). Mice were sacrificed if tumors reached 1 cm in any diameter or became obviously invasive or ulcerated. Histologic evaluation of the tumor sections was done by experimenters blinded to experimental groups.

Real-time PCR. Total RNA was extracted from mouse skin treated with DMBA and TPA using Trizol (Tiangen Biotech). cDNAs were synthesized using the Reverse Transcription System (Promega). Real-time reverse transcription-PCR was done using a MyiQ real-time PCR detection system (Bio-Rad Laboratories). The level of gene expression in TPA-untreated samples was set as 1 arbitrary unit and used as a baseline to compare expression levels of the same gene in different samples.

Designed primers were as follows: β-actin, 5′-GAAGTGTGACGTTGA-CATCCGTA-3′ and 5′-CTCAGGAGGAGCAATGATCTTGA-3′; IFNγ, 5′-AAT-CCTGCAGAGCCAGATTATCTC-3′ and 5′-ACCTGTGGGTTGTTGACCTCA-3′; tumor necrosis factor α (TNFα), 5′-CCCAGACCCTCACACTCAGATC-3′ and 5′-CCTCCACTTGGTGGTTTGCTAC-3′; interleukin (IL)-6, 5′-GAGGA-TACCACTCCCAACAGACC-3′ and 5′-CAGAATTGCCATTGCACAACTC-3′; transforming growth factor (TGF)-β, 5′-ATGGTGGACCGCAACAAC-3′ and 5′-TCTGCACGGGACAGCAAT-3′; TGFα, 5′-GAGAAGCCAGCATGTGTCTGC-3′ and 5′-CACAATGGAGACCACCACCAG-3′; IL-17, 5′-GCTTCATCTGT-GTCTCTGAT-3′ and 5′-GGTCTTCATTGCGGTGGAGA-3′; and cyclooxygenase 2 (COX2), 5′-CTCCCTGAAGCCGTACACAT-3′ and 5′-ATGGTGCTC-CAAGCTCTACC-3′.

Western blotting. Total protein from mouse skin was extracted using a total protein extraction kit (Applygen). Protein samples separated on SDS-PAGE were transferred onto polyvinylidene difluoride membranes and incubated with mouse anti-STAT1 (1:1,000; BD Pharmingen), anti-STAT1 PY701 (1:1,000; BD Pharmingen), and rabbit anti-mouse β-actin (1:5,000; Santa Cruz Biotechnology) antibodies. Antimouse or antirabbit antibodies conjugated with horseradish peroxidase were used as secondary antibody and visualized using chemiluminescent substrate (SuperSignal, Pierce Chemical Co.).

Neutralization of IFNγ or IL-17 *in vivo*. To neutralize the IFNγ activity, R46A2, a rat 0.5 mg anti-mouse IFNγ mAb (22), was given every 10 d by i.p. injection. This treatment was effective because 10 d after the antibody application, inoculation of 1×10^5 IFNγ-secreting tumor cells led to tumor growth in the mAb-treated mice but not in the control mice. For IL-17 neutralization experiment, mice were i.p. injected with 0.2 mg of anti–IL-17 mAb (TC11-18H10, Southern Biotechnology) or isotype-matched antibody every 10 d.

Hyperplasia and immunohistochemistry. Preparation of cryostat or paraffin tissue sections and immunostaining were done as described previously (23). The thickness of the epidermis (in micrometers) was measured using an image system (Photoshop) and calculated as follows: actual thickness of epidermis = on-screen measurements of epidermis / magnification (10 fields per section). Sections were stained with anti–proliferating cell nuclear antigen (PCNA; 1:100; Beijing Zhong Shan-Golden Bridge Biological Technology Company), anti-CD11b (1:100; BD Pharmingen), anti-Gr1 (1:100; BD Pharmingen), and biotinylated secondary antibody and subsequently rhodamine-labeled streptavidin and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Immunofluorescence of tissue sections was evaluated on an Olympus FV1000 confocal microscope.

Flow cytometry. Single-cell suspensions of the regional draining lymph nodes were prepared and restimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma), 1 μg/mL ionomycin (Sigma), and brefeldin A solution (Sigma) for 6 h. Cells were first stained with anti-CD4 labeled with peridinin chlorophyll protein (BD Pharmingen). After treatment with Fixation and Permeabilization Buffer (BD Pharmingen), the samples were stained intracellularly with allophycocyanin-conjugated anti–IL-17A (eBioscience) or isotype controls (BD Biosciences). Samples were analyzed on a FACSCalibur cytometer (BD Biosciences) using the CellQuest Pro software.

Preparation of skin homogenates and cytokine analysis. Mice were sacrificed and the dorsally treated skin was removed, weighed, and homogenized in ice-cold Tris-EDTA buffer. The supernatant was determined using a mouse inflammation cytometric bead array kit (BD Pharmingen). The relative amount of a target cytokine = the concentration analyzed by cytometric bead array / the weight of the sample. For each target cytokine assay, the expression levels from TPA-untreated samples were set as 1 arbitrary unit and used as a baseline to compare expression levels of the same cytokine in different samples.

Statistical analysis. Data were analyzed using Fisher's exact test and two-tailed unpaired Student's t test. Means ± SD are presented. $P < 0.05$ was considered statistically significant.

Results

DMBA/TPA stimulated IFNγ expression in the skin. IFNγ is a key regulator not only in the immune response but also in inflammatory reactions (24). To study whether IFNγ is involved in the process of two-stage skin carcinogenesis, we first examined its expression in the skin by real-time PCR before and after DMBA/TPA application. As shown in Fig. 1*A*, the level of IFNγ mRNA in the skin increased >10-fold 3 hours after the first TPA treatment compared with that in untreated skin (0 hour). Although the level of IFNγ was not as high 24 hours after the first TPA application as

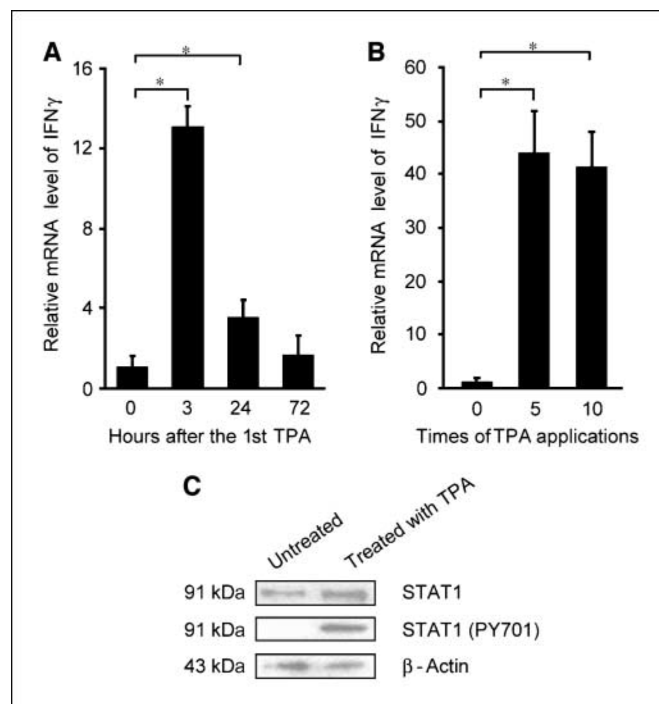

Figure 1. DMBA/TPA treatment up-regulated IFNγ expression and activated its signaling pathway in the skin. *A,* IFNγ expression before and after the first TPA treatment. Groups of wild-type 129/Sv/Ev mice (three to four mice per group) were treated with DMBA/TPA as described in Materials and Methods. Total RNA was extracted from skin 2 h before (0 h) and 3, 24, and 72 h after the first TPA treatment. Relative amounts of IFNγ mRNA were quantified by real-time PCR using β-actin mRNA as an internal standard. *B,* IFNγ expression after a multiple TPA treatment. *, $P < 0.05$. *C,* IFNγ signaling in TPA-treated skin. Total protein was extracted from skin before and 24 h after the first TPA treatment. Western blotting analysis was done to detect the expression and phosphorylation of STAT1. The expression of β-actin was used as a control.

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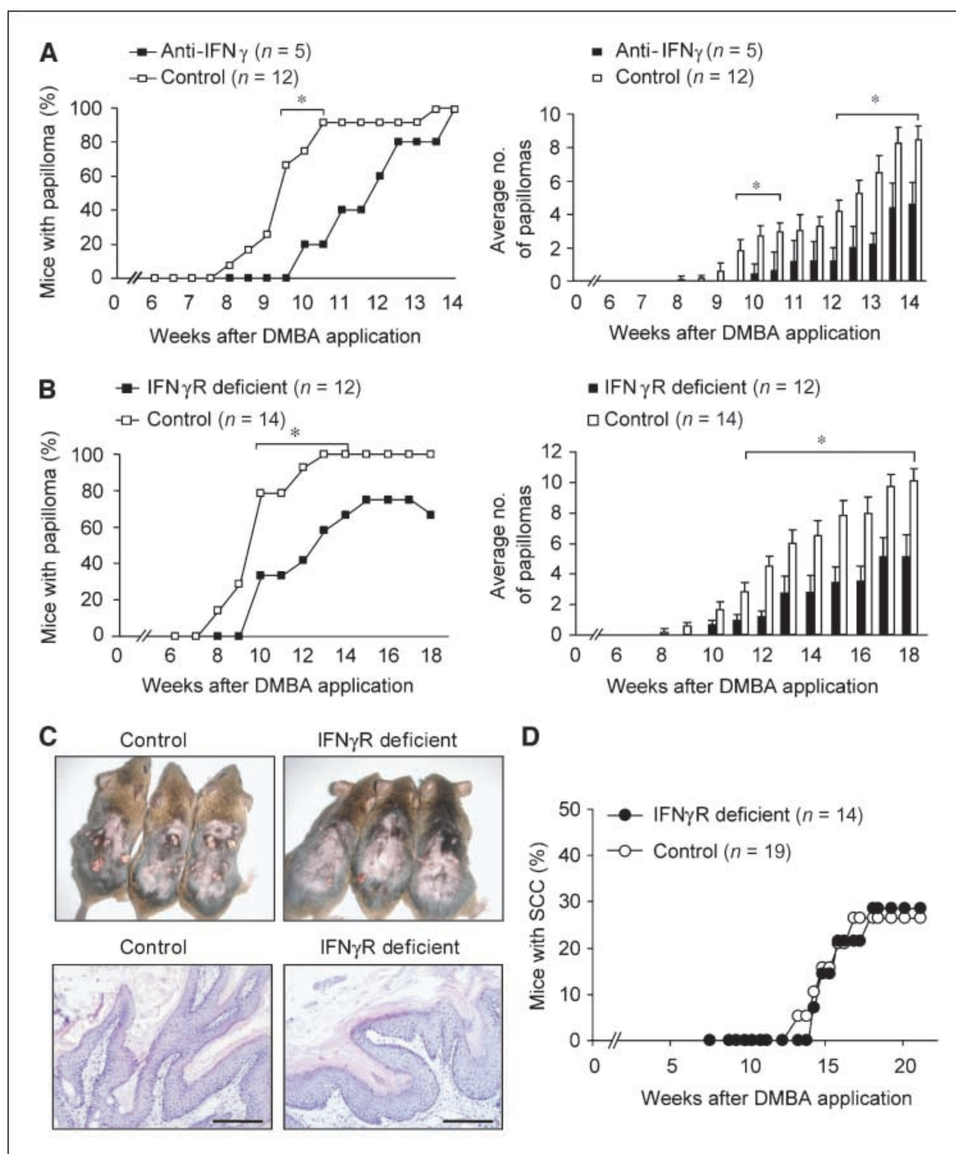

Figure 2. Neutralization of IFNγ *in vivo* or IFNγR deficiency reduced the susceptibility of mice to papilloma development. *A,* groups of wild-type 129/Sv/Ev mice were treated either with an isotype-matched control (*open symbols*; n = 12) or with the neutralizing anti-IFNγ mAb (*closed symbols*; n = 5) every 10 d for 14 wks, starting 2 d before the DMBA treatment. Shown are percentage of mice with papilloma and average number of papillomas per mouse at different time points after DMBA application. *, a significant difference exists between IFNγ-neutralizing and control groups at this period. *B,* groups of IFNγR knockout mice (*closed symbols*; n = 12) and control mice (*open symbols*; n = 14) were treated with DMBA/TPA for 14 wks. The percentage of mice with papilloma and average number of papillomas were presented. *, a significant difference exists between IFNγR knockout and control mice at this period. These experiments were repeated twice with similar results. *C,* representative pictures and H&E staining of papillomas developed on the dorsal skin of three pairs of control and IFNγR-deficient mice at week 14. *Bar,* 50 μm. *D,* groups of IFNγR knockout mice (●; n = 14) and control mice (○; n = 19) were treated with DMBA/TPA for 22 wks. Percentage of mice with squamous cell carcinoma (*SCC*) was evaluated.

it was at 3 hours, it was still significantly higher than at zero hour. With repeated application of TPA, IFNγ expression increased further and maintained at high levels during the promotion stage. For example, the IFNγ mRNA level in the skin was ~40 times higher 3 hours after the 5th or 10th TPA application than that in control skin (Fig. 1*B*).

The IFNγ signal is transduced primarily through Janus-activated kinases and STAT1 (25). To investigate whether up-regulated IFNγ mediates the activation of its downstream gene *STAT1*, we analyzed phosphorylation of STAT1 by Western blotting. As shown in Fig. 1*C*, only constitutive expression of STAT1 existed in untreated skin, whereas an increase in total STAT1 and a phosphorylated form of STAT1 could be detected 24 hours after the first TPA treatment, indicating a strong STAT1 activation. Taken together, these results indicate that the repeated application of TPA induces a long-lasting local expression of IFNγ and activation of its signaling pathway.

Neutralization of IFNγ protected mice from papilloma development. To clarify the significance of up-regulated IFNγ in DMBA/TPA–treated skin, mice were treated with a neutralizing anti-IFNγ mAb (R46A2) 2 days before DMBA/TPA treatment and then once every 10 days for 14 weeks. As shown in Fig. 2*A*, neutralization of endogenous IFNγ activity led to a 2- to 3-week delay in papilloma development. The first papilloma was observed at week 8 in the control group, but not in mice treated with R46A2 until week 10. At week 10.5, when 91.7% of control mice had at least one papilloma, 80% of R46A2-treated mice remained tumor-free. Besides delaying papilloma development, IFNγ neutralization also significantly reduced papilloma number. At week 14, an average of 8.4 ± 0.8 papillomas was recorded for mice in the control group, whereas only 4.6 ± 1.3 papillomas were recorded for mice treated with R46A2.

IFNγR deficiency correlated with decreased papilloma development. To confirm the results described above, papilloma development was evaluated in IFNγR-deficient mice with syngeneic wild-type 129/Sv/Ev mice as controls. In control mice, the first papilloma was observed 8 weeks after DMBA initiation, and by 13 weeks, 100% of the mice had developed at least one papilloma.

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However, in IFNγR-deficient mice, the first papilloma appeared at week 10, and only 58.3% of the mice had developed papillomas at week 13 (Fig. 2*B*). Furthermore, IFNγR-deficient mice developed significantly fewer papillomas (2.7 ± 1.1 tumors at week 14) compared with control mice (6.5 ± 2.1 tumors at week 14; Fig. 2*B*). There was no obvious difference in the macroscopic form and the pathologic structure between papillomas developed in control and IFNγR-deficient mice (Fig. 2*C*).

The incidence of squamous cell carcinoma was also analyzed. As shown in Fig. 2*D*, there was no significant difference between the wild-type (26% at week 20) and IFNγR-deficient mice (29% at week 20). In both groups, <30% of mice had a malignant tumor at the end of the observation period. Together, these data clearly show that in contrast to the result obtained from the MCA-induced tumorigenesis study (10), IFNγ does not inhibit but rather promotes tumor development in the DMBA/TPA model.

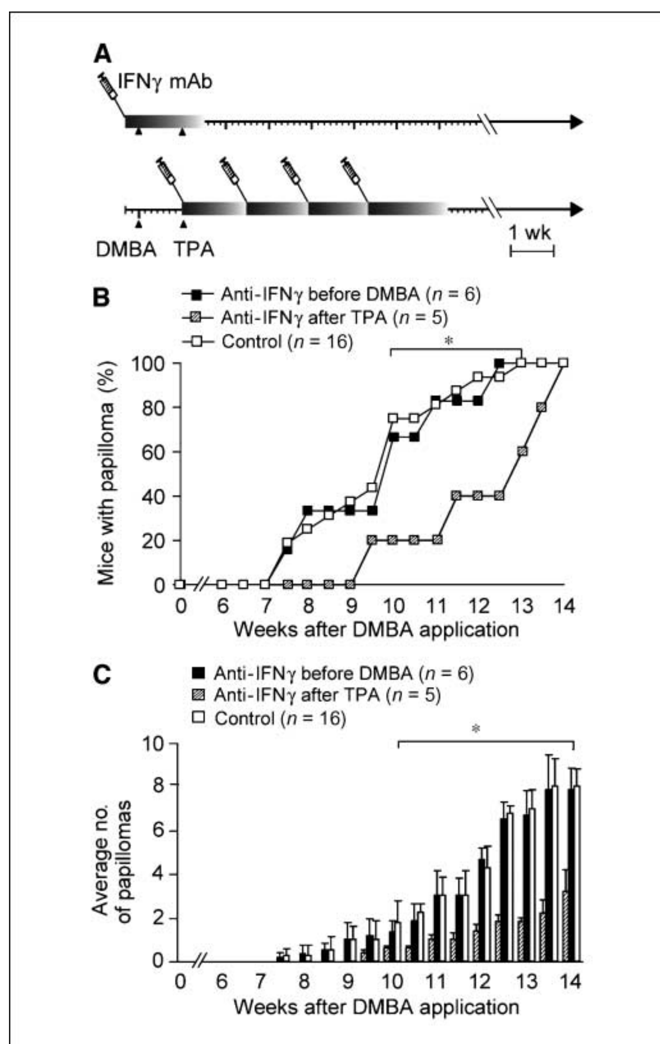

Figure 3. IFNγ acted in the early promotion stage, but not in the initiation stage, of papilloma formation. Groups of 129/Sv/Ev mice were treated with isotype-matched control antibody (*open symbols*; *n* = 16) or with anti-IFNγ mAb given once 2 d before DMBA application (*closed symbols*; *n* = 6) or four times (once every 10 d) after TPA application (*shaded symbols*; *n* = 5), as illustrated in *A*, and papilloma development was induced. Shown are percentage of mice with papilloma (*B*) and average number of papilloma per mouse at different time points after DMBA application (*C*). *, a significant difference exists only between the control and the group with IFNγ neutralization at TPA stage, but not between the control and the group with IFNγ neutralization at DMBA stage at this period.

IFNγ enhanced tumor development during the promotion stage. Next, we investigated in which stage of the tumorigenesis process IFNγ exerted its effect. Initiation is a rapid process characterized by *Ha-ras* mutations here, which can be found in the epidermis 7 days after treatment with DMBA (26). A single treatment with R46A2 that can deplete IFNγ for 10 days is sufficient and necessary to have a specific effect on the initiation process, whereas promotion requires multiple TPA to produce a long-time chronic inflammation. Therefore, IFNγ was depleted at the initiation stage by injecting mice with R46A2 2 days before DMBA treatment or at the promotion stage by injecting mice every 10 days with R46A2 from the start of TPA application for 6 weeks (Fig. 3*A*). A significant decrease in papilloma development was observed when IFNγ was neutralized during the promotion stage. Whereas 81.3% of control mice developed papilloma at week 11, only 20% of mice treated with the antibody had papilloma at week 11 (Fig. 3*B*). At the end of TPA application, an average of 8.0 ± 1.8 papillomas was recorded for control mice, whereas an average of only 3.0 ± 1.0 papillomas was recorded for mice depleted of IFNγ during the promotion stage (Fig. 3*C*). In contrast, there was no significant difference in papilloma development between control group and IFNγ-neutralizing group during the initiation stage. The fact that a short-term IFNγ neutralization in the promotion stage was as effective as IFNγR deficiency or IFNγ neutralization throughout the experiment (Figs. 2 and 3) indicates that IFNγ exerts its tumor-promoting effect primarily in the early promotion stage of papilloma development.

The inflammatory reaction was impaired in the absence of IFNγR. To further investigate the role of IFNγ during the promotion stage, we carried out a series of histologic analysis of DMBA/TPA–treated skin from IFNγR knockout and wild-type mice. Epidermal thickening was significantly impaired in IFNγR knockout mice (32.0 ± 11.5 μm) compared with control mice (92.0 ± 21.6 μm) at week 3 (i.e., 24 hours after the fourth TPA treatment; Fig. 4*A*; Supplementary Fig. S1). Accordingly, there were fewer proliferating cells in the absence of IFNγR than in controls as detected by the PCNA staining (Fig. 4*B*). Detection of leukocyte infiltration in the skin on TPA treatment was followed, showing that the number of $CD11b^+$ and $Gr1^+$ cells decreased significantly in the absence of IFNγR (Fig. 4*C*).

Cytokines are important mediators of inflammation (27). The mRNA expression of TNFα, IL-6, TGFβ, and TGFα was investigated. With the exception of TGFα, all the cytokines analyzed were expressed at significantly higher levels in IFNγR-competent mice compared with IFNγR-deficient mice 3 hours after the first TPA application. Although the difference between the two groups was less clear or even disappeared 24 hours after the first TPA application, it became significant again when TPA was reapplied to the skin (Fig. 4*D*; data not shown), indicating the importance of continuous use of TPA for maintaining a chronic inflammation. Taken together, these results show that IFNγ plays a critical part in up-regulating TPA-induced cell proliferation, hyperplasia, and chronic inflammation in the skin and that reduced inflammation coincided with decreased papilloma development in the absence of IFNγR.

The Th17 response was impaired in IFNγR-deficient mice. IL-17 plays a critical role in maintaining chronic inflammation (28), and it is often induced by IL-6 and TGFβ (29). Because both of these cytokines were down-regulated in the absence of IFNγ

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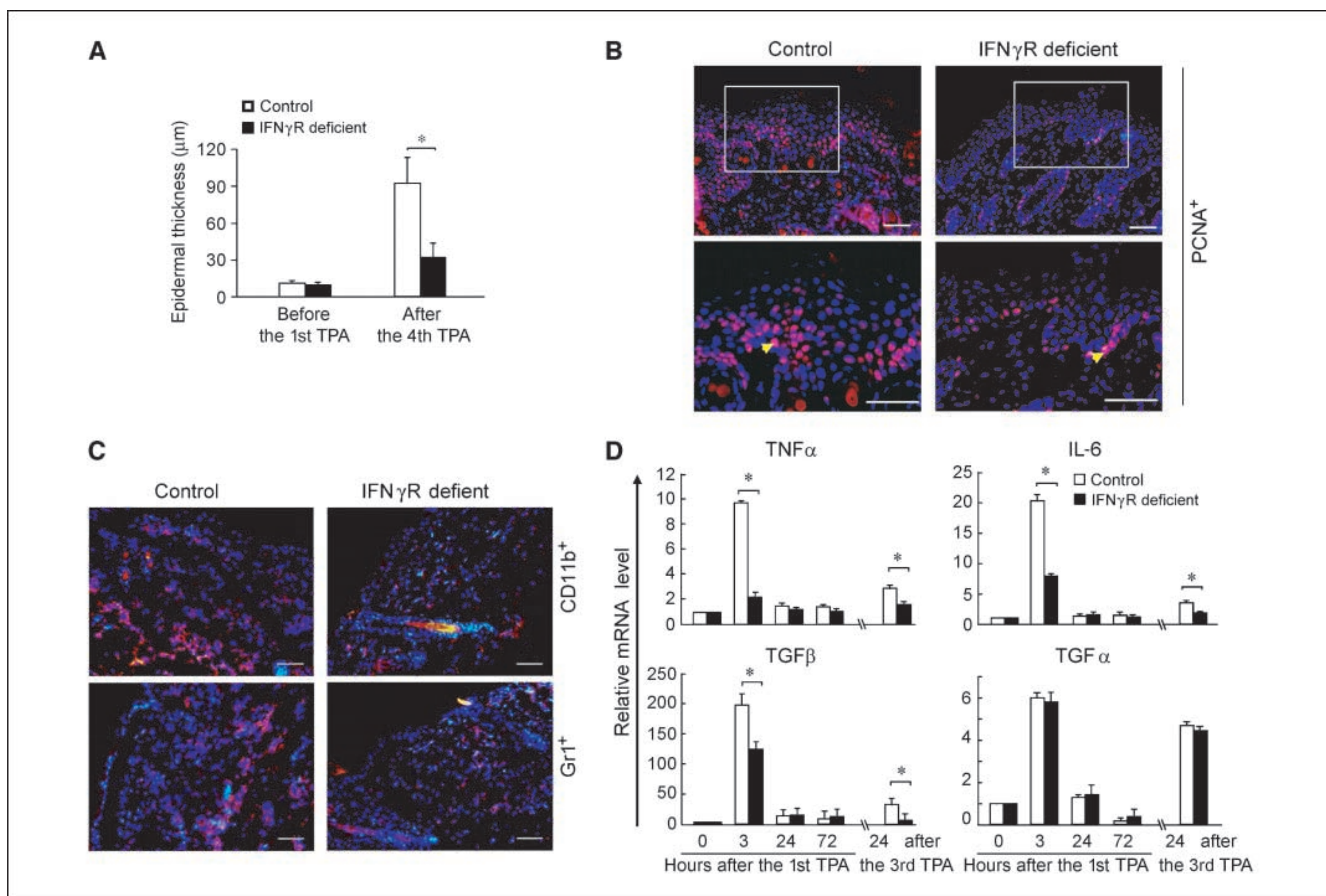

Figure 4. DMBA/TPA–induced cell proliferation and inflammatory reaction was reduced in the absence of IFNγR. *A* and *B,* groups of control and IFNγR knockout mice were left untreated (before the first TPA) or treated with DMBA/TPA for 3 wks (after the fourth TPA). Skin sections were analyzed for cell proliferation by the mean epidermal thickness of control mice (*white columns*) and IFNγR knockout mice (*black columns*; *A*) and PCNA staining (*B*). *B, bottom,* labeled regions of the top images, respectively, with enhanced magnification. *Arrows,* examples of positively stained nuclei by PCNA antibody (*pink*). *Blue,* cell nucleus stained with DAPI. *Red,* spontaneous fluorescence generated by the hair follicle. *Bar,* 50 μm. *C,* immunohistologic analysis was carried out to detect leukocyte infiltration in the skin 24 h after the first application of TPA. Note the reduced number of $CD11b^+$ and $Gr1^+$ cells in IFNγR knockout mice (*right*) compared with IFNγR-competent mice (*left*). *Bar,* 50 μm. *D,* relative mRNA expression of TNFα, IL-6, TGFβ, and TGFα from the skin of control (*white columns*) and IFNγR knockout mice (*black columns*) before (0 h) and at 3, 24, and 72 h after the first TPA treatment and 24 h after the third TPA treatment. Shown are folds of increase in the expression over that of zero-hour group. *, $P < 0.05$. Three mice per group were analyzed and representative results from indicated groups are shown.

signaling (Fig. 4*D*), we investigated Th17 cells in the draining lymph nodes in the DMBA/TPA model. No significant increase in the percentage of Th17 cells was observed 24 hours after the first TPA treatment in both IFNγR-deficient and control groups. However, 24 hours after the fifth TPA application, significant induction of Th17 cells was found in IFNγR-competent, but not in IFNγR-deficient, mice (Fig. 5*A*). As shown in Fig. 5*B*, whereas 4.24% of $CD4^+$ T cells from the draining lymph nodes of control mice stained positive for IL-17, only 1.53% of $CD4^+$ T cells from IFNγR-deficient mice did so. Twenty-four hours after the 10th application of TPA, 4.04% of cells found in lymph nodes of IFNγR-competent mice were Th17 cells, whereas only 1.36% of cells from IFNγR-deficient mice were such cells (Fig. 5*A*). In addition, the absolute number of Th17-producing cells from IFNγR-deficient mice was also decreased in comparison with that from wild-type mice after TPA treatment (data not shown).

The IL-17 mRNA in DMBA/TPA–treated skin was also up-regulated 3 weeks after TPA treatment. It increased 11.8-fold 24 hours after the fifth TPA treatment in the presence of IFNγR; however, it increased only 7-fold in the absence of IFNγR (Fig. 5*C*). The difference in IL-17 expression between these groups remained significant 24 hours after the 10th TPA application. The above results indicate that the Th17 response may take an important part in the DMBA/TPA model and the decreased IL-17 may contribute to the resistance to the papilloma development in IFNγR-deficient mice.

Depleting IL-17 led to decreased inflammation in IFNγR-competent, but not in IFNγR-deficient, mice. Because the up-regulation of Th17 response was observed ∼3 weeks after the DMBA/TPA treatment, we analyzed the relationship between IL-17 and IFNγ responsiveness by depleting this cytokine with a specific mAb in mice 2 days before the fifth TPA treatment. The expression of several key inflammatory mediators in skin was analyzed. As shown in Fig. 6*A* and Supplementary Fig. S2*A*, TPA treatment markedly increased skin expression of IL-6 and TNFα at both mRNA and protein levels in wild-type mice. When IL-17 was neutralized, the induction of IL-6 was obviously impaired in comparison with the controls ($P < 0.05$). Although not affected in the mRNA level, the up-regulation of TNFα protein was almost totally eliminated by IL-17 neutralization ($P < 0.05$). In

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IFNγR-deficient mice, however, depletion of IL-17 activity had no significant effect on the induction of IL-6 and TNFα. COX2, induced by inflammatory cytokines, can mediate prostaglandin accumulation, which is critical for epithelial carcinogenesis (30). Similar results were obtained when the mRNA expression of COX2 was analyzed (Fig. 6*B*).

Furthermore, IL-17 neutralization diminished TPA-stimulated keratinocyte proliferation in wild-type mice (epidermal thickness, 96.6 ± 13.6 to 35.3 ± 6.1 μm) but not in IFNγR knockout mice (35.6 ± 9.3 to 37.1 ± 3.6 μm; Fig. 6*C* and *D*). The papilloma development in the absence of IL-17 was also observed. We found one papilloma at week 8, two at week 9, and four at week 10 in the three wild-type mice without IL-17 neutralization (Supplementary Fig. S2*B*). However, at the same time, no papilloma appeared in IFNγR-competent mice injected with IL-17 mAb or in IFNγR-deficient mice no matter whether injected with IL-17 mAb or not. Collectively, when IL-17 activity was neutralized, the IFNγ-mediated inflammation and keratinocyte proliferation on DMBA/TPA induction was reduced and papilloma development was delayed. These data indicate that Th17 cells play an important role in IFNγ-mediated enhancement of inflammation during papilloma development.

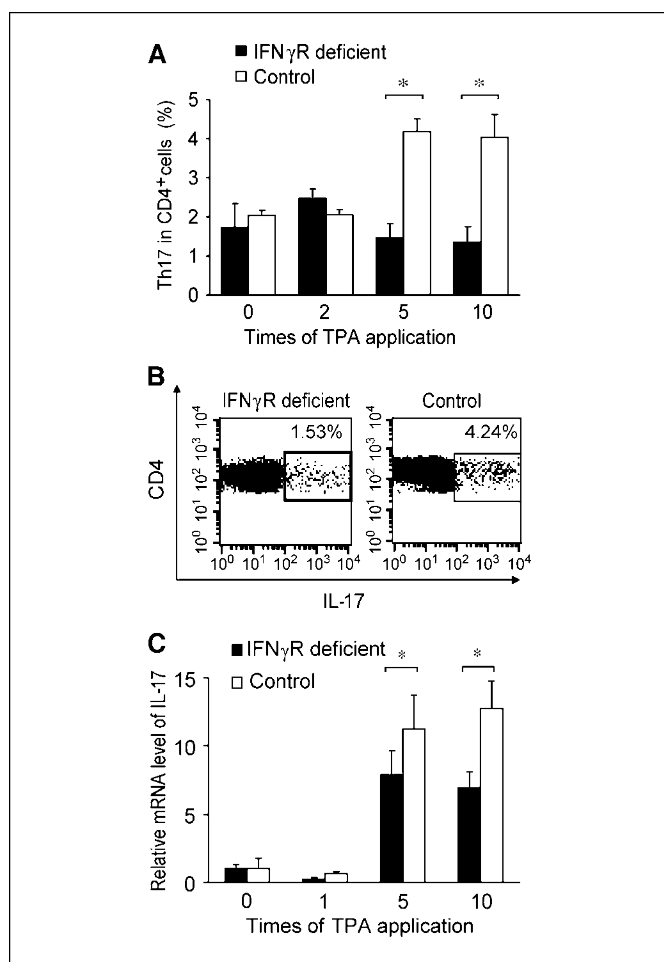

Figure 5. IFNγR was required for the up-regulation of Th17 response after DMBA/TPA treatment. *A,* Th17 cell numbers in draining lymph nodes. Groups of control mice (*white columns*) and IFNγR-deficient mice (*black columns*) were treated with DMBA and then TPA for 0, 2, 5, or 10 times as described in Materials and Methods. The single-cell suspensions of draining lymph nodes were prepared for flow cytometry. Shown is percentage of IL-17–positive cells among the gated $CD4^+$ T cells. At least three mice were analyzed in one group and the representative data are shown. *, $P < 0.05$. *B,* a representative result of the flow cytometric analysis at 24 h after the fifth application of TPA as described in *A. C,* IL-17 expression in skin. Mice were treated as described in *A* and the relative IL-17 mRNA expression in skin was determined by real-time PCR. Shown is the comparison of IL-17 expression in control and IFNγR-deficient mice at different times after TPA application. *, $P < 0.05$.

Discussion

In this study, we have shown that endogenous IFNγ does not inhibit but effectively promotes the development of papillomas in DMBA/TPA–induced two-stage skin carcinogenesis. This is in sharp contrast to previous results indicating that IFNγ inhibits MCA-induced fibrosarcomas in the same strains of mice (9, 10).

Both DMBA and MCA belong to a group of well-analyzed chemical carcinogens, the polycyclic aromatic hydrocarbons (18). Although DMBA is often used, MCA in combination with TPA has also been shown to induce papilloma and eventually squamous cell carcinoma if it is applied on the skin surface (18, 31). One explanation for the contradiction in results from these two tumorigenesis models is that IFNγ-mediated immune surveillance controls the development of most types of tumors, including fibrosarcoma, but does not control papilloma. Another likely explanation for the difference between these results and previous reports is that the carcinogens were administered at different positions. MCA is administered s.c. or i.m., whereas DMBA is administered on the skin surface. The local microenvironment under these two circumstances is obviously different in at least the following aspects: (*a*) Types of cells. In the MCA model, the microenvironment is complex because, in addition to fibroblasts, there are many types of resident or infiltrating cells, such as adipocytes, endothelial cells, muscular cells, granulocytes, macrophages, and lymphocytes, present around the carcinogen (10). In the DMBA/TPA model, other than keratinocytes, there are only a few dendritic cells in the epidermal tissue (32). (*b*) Tissue structure. The s.c. layer below the dermis consists of loose connective tissue, and the injected carcinogen can diffuse easily (10). However, the epidermis is formed by an ordered arrangement of keratinocytes closely linked together with tight junctions in an overlapping fashion (32). Thus, carcinogens painted on the skin surface are limited to a fixed area. (*c*) Characters of local inflammation. In the MCA model, inflammation leads to the encapsulation of s.c. MCA. IFNγ promotes this process and therefore limits the exposure range of the carcinogen (10). In the DMBA/TPA model, there is an epidermal basement membrane separating the proliferating papilloma cells from the underlying inflammatory cells. These cells are activated polymorph nuclear leukocytes, macrophages, or mast cells that release inflammatory cytokines, including IFNγ, which promote tumor growth (33). Therefore, the two contradictory effects of IFNγ on tumorigenesis may result from differences in local microenvironment.

In agreement with our findings here, exogenous IFNγ has been found to enhance tumor development if injected at a relatively low dose (16). The tumor-promoting effect of endogenous IFNγ has also been shown in other models. Interestingly, tumors in these models are all derived from epithelial origins (i.e., they are cancers of the skin, colon, and liver; refs. 14, 15). Whether IFNγ promotes the development of cancers but inhibits nonepithelial-derived tumors is not clear. In addition, a novel population of tumor-promoting T cells has been recently revealed in DMBA/TPA model

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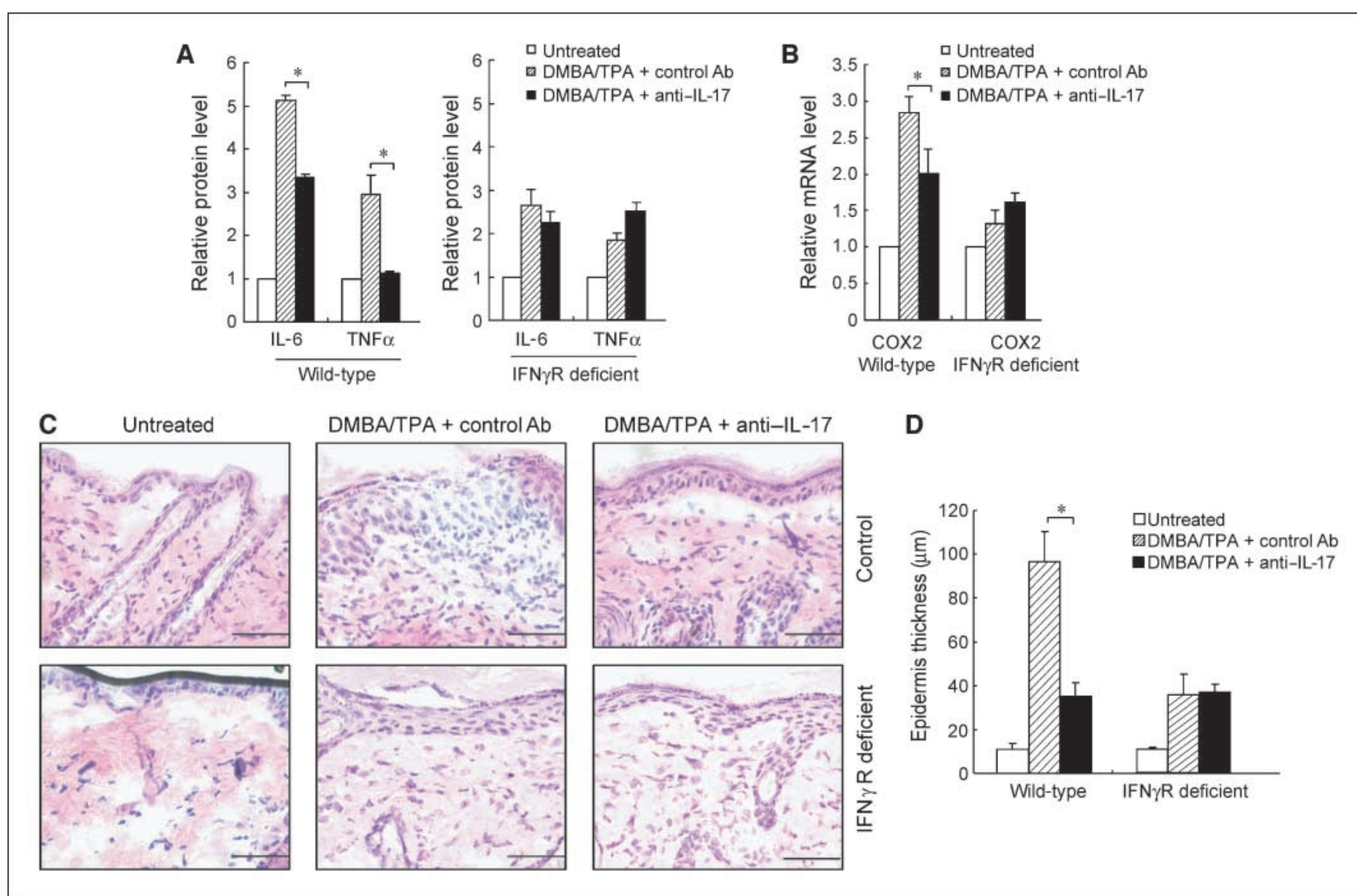

Figure 6. Depleting IL-17 led to decreased inflammation and keratinocyte proliferation in IFNγR-competent, but not in IFNγR-deficient, mice. Groups of wild-type and IFNγR-deficient mice (three mice per group) were left untreated or injected with either control or anti–IL-17 mAb at a 10-d interval 2 d before the fifth TPA application. Total RNAs and proteins were prepared from skin 24 h after the fifth TPA treatment. The indicated cytokines were detected as described in Materials and Methods. *A,* protein levels of IL-6 and TNFα in wild-type and IFNγR-deficient mice, untreated (*white columns*) or DMBA/TPA treated with (*black columns*) or without (*shaded columns*) IL-17 neutralization. *, $P < 0.05$. *B,* mRNA expression of COX2 in both groups of mice. *, $P < 0.05$. *C,* mice were sacrificed 24 h after the 10th TPA application. Skin sections were analyzed by H&E staining. *Bar,* 50 μm. *D,* the epidermal thickness was evaluated. *, $P < 0.05$.

(34). These cells are notable for local production of substantial amounts of IFNγ, indicating that chronic, local production of IFNγ may result in a variety of protumor effects.

IFNγ may act mainly in the early promotion stage of skin tumor development by enhancing the inflammatory reaction (Fig. 4). It has been suspected for a long time that inflammation contributes to cancer development (35, 36). As an important inflammatory cytokine, TNFα is involved in skin carcinogenesis (21), colorectal carcinogenesis associated with chronic colitis (37), and epithelial ovarian cancer (38). Both IFNγ and TNFα are key proinflammatory cytokines and they often act synergistically (39, 40). Here, we found that the up-regulation of TNFα mRNA by DMBA/TPA was partly dependent on IFNγ (Fig. 4). Actually, in TNF receptor-1 and -2 double knockout mice, TPA-induced up-regulation of IFNγ expression was inhibited completely (data not shown). These results indicate a reciprocal regulation of IFNγ and TNFα during TPA-induced inflammation.

Keratinocytes contact TPA at the first line and they can secrete IFNγ (41). Moreover, we found that IFNγ in newborn murine keratinocytes from wild-type 129/Sv/Ev mice was induced rapidly after treatment with TPA *in vitro* (data not shown). Therefore, we tend to consider keratinocytes as an origin of IFNγ here. However, we cannot exclude further possibilities because we have known that IFNγ, essential for tumor immunity, is mainly produced by hemopoietic cells (42). In addition, the leukocytic infiltration of the dermis can be detected within 24 hours after TPA application and persists for long if TPA treatment is repeated (18). Therefore, the leukocytes may be also important origins of IFNγ here.

Th17 cells, a novel subset of T-helper cells, were found to participate in TPA-induced inflammation. This population of cells secretes several proinflammatory cytokines, including IL-17 (43). Recent studies showed that IL-6, TGFβ, and TNFα are important for optimal production of IL-17 (29). Given that IL-17 was also found to be expressed at high levels in the hyperplastic skin in the DMBA/TPA model (44) and that increased levels of local IL-6, TGFβ, and TNFα were observed here (Fig. 4), our results strongly suggest that Th17 plays an active role in papilloma development here. The results shown in Fig. 6 are consistent with a lot of the experimental and clinical evidence to date, which suggests a role for IL-17 family members in amplifying and sustaining chronic inflammatory responses and promoting tumor development (28, 45).

Furthermore, we found that the effect of IFNγ here was Th17 dependent, at least partly (Fig. 6). Although several investigators have shown that inhibition of IFNγ signaling could enhance the development of pathogenic Th17 response (46), other studies have indicated that IL-17 has a synergistic effect with IFNγ *in vitro*,

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stimulating normal human keratinocytes to increase the secretion of proinflammatory cytokines (47). In our model, IFNγ may promote local inflammatory reaction via Th17. Whether the observed difference in the relationship between IFNγ and Th17 arises from the local environment is not clear.

It is important to note that in clinical trials, IFNγ treatment of patients with melanoma, colorectal, or other cancers has no major therapeutic role (48, 49) and sometimes has severe detrimental effects, including metastasis (50). Our finding that IFNγ promotes DMBA/TPA–induced papilloma development by Th17-associated inflammation not only sheds new light on understanding the complicated actions of IFNγ during tumor development but also gives an important indication for cancer therapy especially where inflammation is involved.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Correction

Correction: Article on Th17 for IFNγ-Mediated Papilloma Promotion

In the article on Th17 for IFNγ-mediated papilloma promotion in the March 1, 2009 issue of *Cancer Research* (1), the Acknowledgments section should have included the following:

We sincerely express our gratitude to Professor Thomas Blankenstein for his help on this article. Some of the experiments reported in the article (Fig. 2*D*) were performed at the Institute of Immunology, Charite Universitätsmedizin Berlin, Berlin, Germany under the supervision of Prof. Blankenstein. Experiments described in Fig. 2*D* were supported by the Deutsche Forschungsgemeinschaft by a grant to Prof. Blankenstein as Principal Investigator and Z. Qin as Co-Investigator (BL288-1).

The authors apologize for having omitted this fact.

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