IL-17 Enhances Tumor Development in Carcinogen-Induced Skin Cancer

Lin Wang¹,², Tangsheng Yi², Wang Zhang¹, Drew M. Pardoll³, and Hua Yu¹

Abstract

Inflammatory conditions elicited by extrinsic environmental factors promote malignant cell transformation, tumor growth, and metastasis. Although most attention has been focused on innate immune mechanisms of inflammatory carcinogenesis, more recently the role of T cells in cancer promotion has been examined. Although IFN-dependent Th1 responses that promote Stat1 signaling inhibit tumor growth, the role of T helper type 17 responses, and interleukin-17 (IL-17) in particular, has been controversial. Indeed, IL-17 has been reported to either enhance or inhibit the growth of transplantable tumors, depending on the system. Little is known about the role of IL-17 in de novo carcinogenesis. Using IL-17 knockout mice, we examined the role of IL-17 in the classic DMBA/TPA-induced skin carcinogenesis model. Disruption of IL-17 dramatically reduced tumorigenesis in this model in a manner correlated with diminished Stat3 activation in the tumor microenvironment. IL-17 loss reduced Stat3-associated proliferative and antiapoptotic gene expression along with epidermal cell proliferation and hyperplasia. In addition, IL-17 loss was associated with reduced expression of Stat3-regulated chemokines that attract myeloid cells and a decreased infiltration of myeloid cells into the local tumor microenvironment. Together, our findings point to a critical role of the IL-17–Stat3 pathway in supporting cancer-associated inflammation in the tumor microenvironment. Therapeutic approaches that target this pathway may therefore be effective to inhibit carcinogenesis. Cancer Res; 70(24); 10112-20. ©2010 AACR.

Introduction

Epidemiologic studies identified chronic inflammation as a major risk factor for various types of cancer (1). As a core transcriptional mediator of inflammation, NF-κB activation is a central component of procarcinogenic innate immune responses (2). In a number of systems, Stat3 activation in both epithelial and myeloid cells is a critical downstream mediator of tumorigenesis (3–5). Stat3 activation in epithelial cells drives the transcription of cyclin-dependent kinases, antiapoptotic genes, and proangiogenesis genes, all of which are important for tumor growth (5). Stat3 activation in myeloid cells has been shown to inhibit transcription of the antitumor cytokine interleukin-12 (IL-12) while promoting expression of the procarcinogenic IL-12 family cytokine IL-23 (6). More recently, attention has turned to the role of adaptive immunity, particularly T cell–mediated responses, in tumorigenesis. T-cell immunity can promote or inhibit cancer development and growth (7–11) and it is therefore critical to determine how specific T-cell lineages selectively affect cancer growth. T helper type 1 (Th1) responses promoted by IL-12 seem to mediate antitumor responses via production of IFN-γ and enhancement of antitumor CTLs (12). Stat1 signaling is important in both the induction and effector phases of Th1-type immunity (13). The nature of T-cell responses that promote carcinogenesis and cancer growth is less clear. A number of colon carcinogenesis models have suggested a positive association between the major Th17 cytokine IL-17A (commonly termed IL-17) and cancer development (14). This makes sense, as Stat3 signaling is not only procarcinogenic but also central to Th17 differentiation and function (15). In addition, the Stat3-induced procarcinogenic cytokine IL-23 maintains and mediates expansion of Th17 cells (16). However, the role of IL-17 in growth of established tumors is unclear. Although several reports suggest that certain transplanted tumors grow more slowly in mice lacking either IL-17 or IL-17 receptor (7, 9), other groups have reported increased growth of transplanted tumors in the absence of IL-17, suggesting that the role of IL-17 in cancer is context dependent (17, 18). Moreover, clinically, the presence of Th17 cells in tumors has been associated with both favorable and unfavorable prognoses (19–21). These conflicting observations warrant further investigations into the role of IL-17 in cancer.

To further assess the role of IL-17 in the carcinogenesis process, we have explored a classic model of inflammation-
induced skin cancer, using 7,12-dimethylbenz[a]anthracene (DMBA) and 12-o-tetradecanoylphorbol-13-acetate (TPA). This model has been widely used to study how an extrinsic chemically induced inflammation initiates epithelial transformation and promotes subsequent papilloma development (22, 23). In this 2-stage carcinogenesis model, DMBA induces mutations in dermal epithelium at the earliest stage whereas TPA administration elicits an inflammatory response that mediates further transformation, resulting in papilloma development.

We show that IL-17 is an important tumor-promoting element in DMBA/TPA skin carcinogenesis. IL-17 induces Stat3 activation, epithelial hyperproliferation, and CD11b+Gr-1+ myeloid cell infiltration at the site of tumor initiation. Our findings support the notion that Th17 responses can enhance carcinogenesis.

Materials and Methods

Animals and animal care

Wild-type (WT) mice were purchased from the National Cancer Institute unless indicated specifically. IFN-γ−/− C57BL/6 mice were purchased from The Jackson Laboratory. The generation of C57BL/6 IL-17−/− mice has been previously reported (24), and the mice were provided by Y. Iwakura (University of Tokyo). Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidance and approved protocols from the Research Animal Care Committee of the City of Hope Medical Center (Duarte, CA).

DMBA/TPA-induced epithelial carcinogenesis procedure

The 2-stage skin carcinogenesis was conducted on the basis of peer reports (23). Briefly, the dorsal skin of mice was shaved and painted with DMBA (Sigma) in 200 μL of acetone at 100 μg per mouse once and then treated with TPA in 200 μL of acetone at 30 μg per mouse twice a week. Mice were evaluated weekly for papilloma development. Only tumors that had attained a size of 1 mm or greater and were present for a week or more were counted.

Isolation of mononuclear cells from skin

Mononuclear cell isolation from skin was performed as previously described (25). Briefly, dorsal skin (3 × 3 cm²) was cut into small pieces and shaken in RPMI containing 5% fetal bovine serum, 10 mM/L of HEPES (Irvine Scientific), 0.01% DNase (Sigma-Aldrich), 0.27% collagenase type I (Sigma-Aldrich), and 1,000 U/mL of hyaluronidase (Sigma-Aldrich) at 37°C for 1 hour. Skin tissue and suspension were then intensively washed with PBS containing 2 mM/L of EDTA and filtered with a 70-μm cell strainer before enrichment with Lympholyte M (Accurate Chemical & Scientific).

Monoclonal antibodies and flow cytometric analysis

Antibodies to mouse CD4, CD8, TCRβ, CD11b, Gr-1, IL-17, and IFN-γ were all purchased from eBioscience. For intracellular staining, cells were stimulated with plate-bound CD3/CD28 for 5 hours, and Brefeldin A (10 μg/mL) was added in the last 2 hours. Cells were then harvested and stained for cytokines. Dead cells were excluded by Fixable Aqua Dead Cell Stain kit (Invitrogen).

Immunofluorescence staining

Paraffin-embedded specimens were deparaffinized, hydrated, and autoclaved in antigen unmasking solution (Vector) before being stained with antibodies specific to Ki67 (Abcam). Specimens were then detected with secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen). After staining with Hoechst 33342 (Invitrogen) to visualize cell nuclei, slides were mounted and analyzed by fluorescence microscopy.

Real-time quantitative PCR

Total RNA from skin sample was extracted by RNeasy Fibrous Tissue kit (Qiagen) and cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad). Mouse IL-17, IL-6, Bel-X₁, survivin, cyclin D1, tumor necrosis factor-α (TNF-α), IL-1β, CXCL2, and cyclooxygenase-2 (COX-2) primers were purchased from SABiosciences. Sequence for CXCL1 primers is as follows: sense, 5'-CAAGAACATCCAGAGCTTGAAGGT-3', antisense, 5'-GTGGCTATGACTTCGGTTTG-3'. Sequence for S100A8 primer is as follows: sense, 5'-CAATTTCTCTGAAAGAATTTCG-3'; antisense, 5'-TACCATGGCCTCTACAAGA-3'. Sequence for S100A9 primer is as follows: sense, 5'-GTCAGGTCTCTCCATGATGT-3'; antisense, 5'-TCAGACAAATGGTGGAAGC-3'.

Statistical analysis

Unpaired t test was used to calculate the 2-tailed P value. Data were analyzed using Prism software (GraphPad Software, Inc.). For real-time reverse transcription (RT)-PCR, data before normalization were used for t-test analysis.

Results

Elevated IL-17 expression and Stat3 activation in DMBA/TPA-administered mice

To study the role of IL-17 in skin carcinogenesis, we first examined the expression of IL-17 in skin after DMBA/TPA administration. We found that the expression of IL-17 was induced approximately 4-fold after 3 hours of the first TPA administration (Fig. 1A). Consistent with our previous observation that IL-17 can induce IL-6 expression (9), we observed similar kinetics for IL-6 expression after the TPA administration. We found that the expression of IL-17 was further increased and constitutively maintained at a high level in skin of TPA-treated mice (Fig. 1A). These results indicate that DMBA/TPA administration can elicit a strong IL-17 response, with associated inflammation in the local epidermal environment.

We next investigated whether TPA-induced IL-17 and IL-6 production would mediate Stat3 activation. We observed that Stat3 phosphorylation was also markedly increased in skin 3 hours post–TPA administration but reduced 24 hours...
post–TPA administration (Fig. 1B). Stat3 activation was maintained at a high level after long-term TPA treatment (Fig. 1B). Taken together, these results indicate that DMBA/TPA administration elicits a strong induction of IL-17 in epidermal local environment, which is associated with constitutively activated Stat3 signaling.

**IL-17**/−mice are resistant to the DMBA/TPA-induced epithelial carcinogenesis

We next assessed the role of IL-17 in this skin carcinogenesis model using genetic knockout. Sex-, age-, and matched WT and IL-17−/−mice were administered with DMBA/TPA. We found that the IL-17−/−mice were strongly resistant to DMBA/TPA-induced carcinogenesis as compared to WT controls. While about 80% of WT mice developed papillomas by 13 weeks post–DMBA/TPA administration, only 10% of the IL-17−/−mice developed any papillomas (Fig. 2A). After extending TPA administration to 20 weeks, approximately 50% of IL-17−/−mice remained papilloma-free (Fig. 2A). Those IL-17−/−mice that did develop papillomas had significantly fewer and smaller papillomas than WT mice (Fig. 2B–D). Because Th1 cytokine IFN-γ exerts reciprocal regulation and distinct effector functions relative to IL-17 in vivo, we also tested the skin carcinogenesis with TPA/DMBA treatments in IFN-γ−/−and IFN-γ−/−IL-17−/−mice. Although we found only a small change in papilloma development in the absence of IFN-γ, papilloma development in IFN-γ−/−IL-17−/−mice was dramatically reduced as relative to IFN-γ−/−mice (Supplementary Fig. S1). Because of genetic background differences among WT mice from the National Cancer Institute and IL-17−/−, IFN-γ−/−, and IFN-γ−/−IL-17−/−mice, we repeated DMBA/TPA treatment by using WT mice from The Jackson Laboratory. We observed reduced papilloma development in IL-17−/−mice relative to WT mice (Supplementary Fig. S2). These results emphasize the pivotal role played by IL-17 in skin carcinogenesis in this system.

### IL-17 ablation leads to reduced Stat3 activation in the tumor microenvironment

To determine the mechanism(s) by which IL-17 promotes papilloma development, we next performed intracellular cytokine staining to measure IL-17 production in the inflammatory skin. There were approximately 4% of CD4+ T cells from the skin of DMBA/TPA-treated WT mice producing IL-17, whereas IL-17 secreting cells were not detectable in the IL-17−/−mice (Fig. 3A, left). The lack of IL-17 expression in IL-17−/−mice was confirmed by real-time PCR (Fig. 3A, right). We did not detect any IL-17 production in non–T cells by intracellular staining (data not shown). These results indicate that DMBA/TPA administration stimulates classic Th17 cell production of IL-17, which exerts an important role in promoting carcinogenesis-associated inflammation.

We previously reported that IL-17 is essential for the activation of Stat3 in an IL-6–dependent manner in transplantable tumor models (9). We next tested whether loss of IL-17 also led to reduced IL-6 expression and Stat3 activity in inflammation-driven skin carcinogenesis. IL-6 expression in the skin samples from TPA-administered WT versus IL-17−/−mice was compared by real-time PCR. We found that IL-6 expression was reduced in the skin samples prepared from IL-17−/−mice as compared with those of WT mice (Fig. 3B). The reduction of IL-6 in IL-17−/−mice was associated with downmodulated Stat3 activation in skin samples, as detected by phosphorylated Stat3 (p-Stat3; Fig. 3C). p-Stat3 activity was also assessed by immunofluorescence staining. Stat3 was activated in epidermal layer, outer root sheath of hair follicle, and epidermal- and dermal-infiltrating cells in WT skin samples, whereas its activation was reduced in skin from IL-17−/−mice (Fig. 3D). To specify which cell type displays decreased p-Stat3 level, we performed flow analysis and observed that skin-infiltrating CD11b+Gr-1+ myeloid cell and F4/80+ macrophages had reduced Stat3 activity (Supplementary Fig. S3A). Taken together, these results indicate that the
reduced tumor carcinogenesis and papilloma development in IL-17/C0/C0 mice were associated with reduced local IL-6 production and downregulated Stat3 activation.

**IL-17 promotes DMBA/TPA-mediated epidermal hyperproliferation and Stat3-regulated oncogenic gene expression**

As an oncogenic transcriptional factor in tumor, Stat3 activation mediates the proliferation of malignant cells and their escape from apoptosis (5). Treatment with a Stat3 antagonist suppresses DMBA/TPA-induced epithelial hyperproliferation and subsequently carcinogenesis process (26). Because Stat3 activation was reduced in DMBA/TPA-treated skin samples from IL-17/C0/C0 mice, we reasoned that reduced carcinogenesis in IL-17/C0/C0 mice may result from downregulated premalignant epidermal hyperproliferation. We therefore compared the histologic changes in DMBA/TPA-treated skin samples prepared from WT or IL-17/C0/C0 mice. We observed abnormal epidermal thickening and hyperplasia in DMBA/TPA-administrated WT mice, which were dramatically reduced in the skin samples of IL-17/C0/C0 mice (Fig. 4A). However, we did not observe any phenotypic differences in squamous papilloma between WT and IL-17/C0/C0 mice. Further *in situ* Ki67 staining of proliferating cells revealed that there were fewer Ki67+/proliferating cells in skin samples prepared from IL-17/C0/C0 mice than from WT control (Fig. 4A). Further evaluation of apoptosis in skin samples from WT and IL-17/C0/C0 mice was made by staining of cleaved caspase 3. We observed increased cleaved caspase 3 staining in IL-17/C0/C0 mice as compared with WT mice (Fig. 4A). We also compared expression of several of Stat3-regulated proliferative and antiapoptotic genes in DMBA/TPA-treated skin samples from WT and IL-17/C0/C0 mice. We found that Stat3 signature oncogenic genes, such as cyclin D1, bcl-xL, and survivin, were markedly reduced in IL-17/C0/C0 mice as compared with WT control (Fig. 4B and C). Taken together, these results suggest that DMBA/TPA-induced IL-17 plays a critical role in promoting epithelial cell hyperproliferation through the induction of Stat3 and its regulated oncogenic gene expression.

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**Figure 2. IL-17−/− mice are resistant to the DMBA/TPA-induced epithelial carcinogenesis.** WT and IL-17−/− mice were administered with DMBA/TPA for 20 weeks. Mice were monitored for papilloma development every week. A, time course of papilloma occurrence in WT and IL-17−/− mice. Shown is the percentage of mice without papilloma. B, average number of papilloma per mouse in WT and IL-17−/− mice following treatment. C, percentage of mice with indicated numbers of papillomas is shown (WT, n = 19; IL-17−/−, n = 11). D, representative photographs of WT and IL-17−/− mice with papillomas. **, P < 0.01; ***, P < 0.001.
IL-17 augments myeloid cell recruitment and Stat3-associated local inflammation

The skin carcinogenesis induced by DMBA/TPA administration has been recognized as an example of inflammation-driven tumorigenesis. Infiltration of immune cells and the local production of inflammatory cytokines and mediators are essential for this carcinogenesis process. To test the role of IL-17 in the tumor-associated inflammation, we compared immune cells in DMBA/TPA-treated skin from WT and IL-17−/− mice. We found that the percentage and yield of CD11b+ Gr-1+ myeloid cells were significantly reduced in skin of IL-17−/− mice as compared with WT controls (Fig. 5A). Similar reduction of skin infiltration of CD11b+ myeloid cell was also observed using immunofluorescence staining (Supplementary Fig. S3B). The percentage of CD8+ T cells and skin-infiltrating CD8+ IFN-γ+ T cells in IL-17−/− mice was

**Figure 3.** IL-17 ablation leads to reduced Stat3 activation in the tumor microenvironment. WT and IL-17−/− mice were administered with DMBA/TPA for 20 weeks. A, intracellular IL-17 staining of enriched mononuclear cells from skin of DMBA/TPA-treated WT and IL-17−/− mice (left). Gated CD4+ T cells were shown as CD4 versus IL-17. B, mean ± SE of mRNA expression of IL-6 in skin. Right, mean ± SE of mRNA expression of IL-17 in dorsal skin from treated WT and IL-17−/− mice, as determined by real-time RT-PCR; n = 4. C, Western blotting analysis of Stat3 and p-Stat3; β-actin serves as the loading control. Two representative samples from each group of mice are shown. All experiments were repeated at least 3 times. D, immunofluorescence staining of p-Stat3 in WT and IL-17−/− skin samples. One representative photograph of 3 skin sections is shown ND, not detectable. **, P < 0.01.
Figure 4. IL-17 promotes DMBA/TPA-mediated epidermal hyperproliferation and Stat3-regulated oncogenic gene expression. WT and IL-17−/− mice were administered with DMBA/TPA for 20 weeks. A, hematoxylin and eosin (H&E), Ki67, and cleaved caspase 3 staining of skin section collected 20 weeks after DMBA/TPA administration. One representative photograph of 4 skin sections is shown. Ki67 and cleaved caspase 3 staining was quantified by counting the number of positive cells in the field, n = 4. B, mean ± SE of mRNA level of Bcl-XL, survivin, and cyclin D1 in skin of DMBA/TPA-treated mice; n = 4. C, 2 representative samples of Western blotting analysis of Bcl-XL, survivin, and cyclin D1 in skin of DMBA/TPA-treated mice are shown. These experiments were repeated with similar results. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
increased as compared with WT controls (Fig. 5B), although granzyme B expression among CD8\(^{+}\) T cells from WT and IL-17\(^{-/-}\) mice was similar (Supplementary Fig. S3D). We also analyzed immune cell infiltration in skin samples after acute TPA treatments. We observed reduced F4/80\(^{+}\) macrophage and moderately reduced CD11b\(^{+}\) myeloid cell infiltration in IL-17\(^{-/-}\) mice (Supplementary Fig. 3C).

Although CD8\(^{+}\)IFN-\(\gamma\) T cells have been associated with antitumor immune responses, CD11b\(^{+}\)Gr-1\(^{+}\) myeloid cells in the tumor microenvironment are cancer promoting and Stat3 is required for the generation and recruitment of myeloid cells into the tumor environment (27, 28). We next compared expression of IL-6/Stat3-regulated inflammatory mediators in skin from DMBA/TPA-treated WT and IL-17\(^{-/-}\) mice. We found that the expression of IL-1\(\beta\), CXCL1, CXCL2, S100A8, S100A9, and Cox-2 in mRNA level in skin from DMBA/TPA-treated mice; \(n = 4\). P < 0.01 for all panels.
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Discussion

We have shown that, in the DMBA/TPA-induced skin carcinogenesis model, local IL-17 production by CD4+ T cells plays a critical role in papilloma genesis and development. TPA-induced IL-17 production induced persistently activated oncogenic Stat3 and promoted subsequent epidermal cell proliferation and hyperplasia. In contrast, mice lacking IL-17 exhibited diminished Stat3 activation, reduced Stat3-associated oncogenic and inflammatory gene expression, and reduced tumorigenesis. Our findings therefore revealed a pivotal role of IL-17–Stat3 pathway in tumor carcinogenesis process. Even though the IL-17 receptor does not seem to signal directly through Stat3, it does activate Stat3 indirectly through IL-6 (9, 32).

Constitutive activation of Stat3 in tumor microenvironment has been observed in many mouse tumors and human cancers (33). Although overactivation of various tyrosine kinases induces persistent Stat3 activation in transformed cells, Stat3 activation in the tumor microenvironment is mediated by many extrinsic mediators, including cytokines (e.g., IL-6, IL-10, and IL-23) and growth factors (vascular endothelial growth factor and fibroblast growth factor 2) whose receptors are known to signal through Stat3 (6, 34). We previously reported that in the tumor progression stage, IL-17 is a potent activator of Stat3 signaling through stimulating IL-6 production (9). In the current study, we showed that IL-17 triggered by the extrinsically administered chemical TPA played a critical role in elevating Stat3 signaling and its associated gene transcription in the tumor initiation stage, which subsequently promotes malignant cell proliferation and formation of papillomas. This observation is in agreement with a recent report showing that a human colonic commensal [enterotoxigenic Bacteroides fragilis (ETBF)] promotes colon tumorigenesis in a Th17–Stat3-dependent manner (14). These studies emphasize an important role of IL-17 in activating Stat3 signaling to facilitate the tumor angiogenesis. In agreement with this notion, we observed that lack of IL-17 is associated with diminished Stat3 activation, reduced Stat3-associated oncogenic Stat3 and promoted subsequent epidermal cell proliferation and antiapoptosis. We observed that Stat3 is constitutively activated in DMBA/TPA-treated skin, which is associated with elevated expression of Stat3-regulated proliferation and antiapoptosis genes. Downmodulated Stat3 activation in IL-17−/− mice resulted in reduced epithelial cell hyperproliferation and gland hyperplasia and downregulated oncogenic gene expression. Second, Stat3 activation can result in dysregulated immune surveillance against tumors. Tumor-infiltrating myeloid cells are reported to be immunosuppressive and can facilitate malignant cells escaping from the immune surveillance. The IL-17–Stat3 pathway augments the recruitment of myeloid cells but not of CD8+ T cells into the local skin environment. Stat3-regulated inflammatory mediator expression, including Cox-2, CXCL1, CXCL2, S100A8, S100A9, and IL-1β, has been reported to be critical for the generation of myeloid suppressive cells and granulocyte cells into the inflammatory environment (27, 29–31). Finally, IL-17–Stat3 signaling can upregulate MMP9 expression to facilitate the tumor angiogenesis. In agreement with this notion, we observed that lack of IL-17 is associated with reduced MMP9 expression (Supplementary Fig. S4B).

In summary, our study has emphasized an important role of IL-17–Stat3 pathway in promoting epithelial carcinogenesis. Therapies that target IL-17 and Stat3 may be developed as potential therapeutic approaches to inhibit carcinogenesis.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were reported.

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