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IL-17 enhances tumor development in carcinogen-induced skin cancer

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Abstract

Inflammatory conditions elicited by extrinsic environmental factors promote malignant cell transformation, tumor growth and metastasis. Although most attention has focused on innate immune mechanisms of inflammatory carcinogenesis, more recently the role of T cells in cancer promotion has been examined. While IFN- γ dependent Th1 responses that promote Stat1 signaling inhibit tumor growth, the role of Th17 responses, and 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 anti-apoptotic gene expression along with epidermal cell proliferation and hyperplasia. Additionally, IL-17 loss 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.

Keywords

IL-17; Stat3; TPA and skin carcinogenesis

Introduction

Epidemiological studies identified chronic inflammation as a major risk factor for various types of cancer (1). As a core transcriptional mediator of inflammation, NF- κ B activation is a central component of pro-carcinogenic 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, anti-apoptotic genes and pro-angiogenesis genes, all of which are important for tumor growth (5). Stat3 activation in myeloid cells has been shown to inhibit transcription of the anti-tumor cytokine, IL-12, while promoting expression of the pro-carcinogenic 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. Th1 responses promoted by IL-12 appear to mediate anti-tumor responses via production of IFN- γ and enhancement of anti-tumor 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 since Stat3 signaling is not only procarcinogenic but is 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. While 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 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 prognosis (19-21). These conflicting observations warrant further investigations into the role of IL-17 in cancer.

In order 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 two-stage carcinogenesis model, DMBA induces mutations in dermal epithelium at the earliest stage while TPA administration elicits an inflammatory response which 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 Gr-1⁺/CD11b⁺ 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

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, Tokyo, Japan). 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.

DMBA/TPA induced-epithelial carcinogenesis procedure

The two stage skin carcinogenesis was conducted based on peer reports (23). Briefly, the dorsal skin of mice was shaved and painted with DMBA (Sigma) in 200 μ l acetone at 100 μ g per mice once and then treated with TPA in 200 μ l acetone at 30 μ g per mice twice a week.

Mice were evaluated weekly for papilloma development. Only tumors that had attained a size of ≥ 1 mm and were present for ≥ 1 week were counted.

Isolation of mononuclear cells from skin

Mononuclear cells isolation from skin was performed as our previously description (25). Briefly, dorsal skin (3×3 cm²) was cut into small pieces and shaken in RPMI containing 5% fetal bovine serum, 10 mM HEPES (Irvine Scientific, CA), 0.01% DNase (Sigma-Aldrich, MO), 0.27% collagenase type I (Sigma-Aldrich, MO), and 1000 U/ml hyaluronidase (Sigma-Aldrich) at 37°C for 1 h. Skin tissue and suspension will be then intensively washed with PBS containing 2mM EDTA and filtered with a 70µm cell strainer before enrichment with Lympholyte M (Accurate Chemical & Scientific, Westbury, NY).

Monoclonal antibodies and flow cytometric analysis

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

Immunofluorescence Staining

Paraffin-embedded specimens were deparaffinized, hydrated, and baked in antigen unmasking solution (Vector) before stained with antibodies specific to Ki67 (Abcam). Specimens were then detected with secondary antibodies conjugated Alexa Fluor 488 (Invitrogen, CA). After stained 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, Bcl-X_L, Survivin, Cyclin D1, TNF- α , IL-1 β , CXCL2 and Cox2 primers were purchased from SABiosciences. Sequence for CXCL1 primers is: sense 5'-CAAGAACATCCAGAGCTTGAAGGT-3'; antisense 5'-GTGGCTATGACTTCGGTTTGG-3'. Sequence for S100A8 primer is: sense, 5'-CCAATTCTCTGAACAAGTTTTCG-3'; antisense, 5'-TCACCATGCCCTCTACAAGA-3'. Sequence for S100A9 primer is: sense, 5'-GTCCAGGTCTCCATGATGT-3'; antisense, 5'-TCAGACAAATGGTGAAGCA-3'.

Statistical analysis

Unpaired *t* test was used to calculate the two-tailed *p*-value. Data were analyzed using Prism software (GraphPad Software, Inc.).

Results

Elevated IL-17 expression and Stat3 activation in DMBA/TPA administrated 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 four-fold three hours after the first TPA administration (Fig. 1A). Consistent with our previous observation that IL-17 can induce IL-6 expression (9), we observed a similar kinetics for IL-6 expression after the TPA administration (Fig. 1A). After the repeated bi-weekly application of TPA for twenty weeks, the expression of IL-17 and IL-6 were 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 the induction of IL-17 and IL-6 in TPA treated skin mediated downstream Stat3 activation. We observed that Stat3 phosphorylation was also markedly increased in skin three hours post TPA administration, but reduced twenty-four 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 DMPA/TPA induced epithelial carcinogenesis**

We next assessed the role of IL-17 in this skin carcinogenesis model using genetic knockout. Sex, age, and background matched WT and *IL-17*^{-/-} mice were administrated with DMPA/TPA. We found that the *IL-17*^{-/-} mice were strongly resistant to DMPA/TPA induced carcinogenesis as compared to WT controls. While about 80% WT mice developed papillomas by 13 weeks post DMBA/TPA administration, none of the *IL-17*^{-/-} mice developed any papillomas (Fig. 2A). After extending TPA administration to 20 weeks, approximately 50% *IL-17*^{-/-} mice remained papilloma-free (Fig. 2A). Those *IL-17*^{-/-} mice that did develop papillomas had significantly fewer and smaller papillomas as compared to WT mice (Fig. 2B, C and 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 only found 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 (Suppl. Fig. 1). Due to genetic background differences among WT mice from NCI and *IL-17*^{-/-}, *IFN- γ* ^{-/-}, *IFN- γ* ^{-/-}*IL-17*^{-/-} mice, we repeated DMBA/TPA treatment using WT mice from Jackson Laboratory. We observed reduced papilloma development in *IL-17*^{-/-} mice relative to WT mice (Suppl. Fig. 2). 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 four percent of CD4⁺ T cells from the skin of DMBA/TPA treated WT mice producing IL-17, while 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 administrated WT vs. *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 to those of WT mice (Fig. 3B). The reduction of IL-6 in *IL-17*^{-/-} mice was associated with down-modulated Stat3 activation in skin samples, as detected by phosphorylated 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 while 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 (Suppl. Fig. 3A). Taken together, these results indicate that the reduced tumor carcinogenesis and papilloma development in *IL-17^{-/-}* mice was associated with reduced local IL-6 production and downregulated Stat3 activation.

IL-17 promotes DMPA/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). Since Stat3 activation was reduced in DMBA/TPA treated skin samples from *IL-17^{-/-}* mice, we reasoned that reduced carcinogenesis in *IL-17^{-/-}* mice may result from downregulated premalignant epidermal hyperproliferation. We therefore compared the histological changes in DMBA/TPA treated skin samples prepared from WT or *IL-17^{-/-}* mice. We observed abnormal epidermal thickening and hyperplasia in DMPA/TPA administrated WT mice, which were dramatically reduced in the skin samples of *IL-17^{-/-}* mice (Fig. 4A). However, we did not observe any phenotypic differences in squamous papilloma between WT and *IL-17^{-/-}* mice. Further in situ Ki67 staining of proliferating cells revealed that there were fewer Ki67⁺ proliferating cells in skin samples prepared from *IL-17^{-/-}* mice as compared to WT control (Fig. 4A). Further evaluation of apoptosis in skin samples from WT and *IL-17^{-/-}* mice was made by staining of cleaved caspase 3. We observed increased cleaved caspase 3 staining in *IL-17^{-/-}* mice as compared to WT mice (Fig. 4A). We also compared expression of several of Stat3-regulated proliferative and anti-apoptotic genes in DMPA/TPA treated skin samples from WT and *IL-17^{-/-}* mice. We found that Stat3 signature oncogenic genes, such as *cyclin D1*, *bcl-x_L* and *survivin*, were markedly reduced in *IL-17^{-/-}* 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.

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 (Suppl. Fig. 3B). The percentage CD8⁺ T cells and skin infiltrating CD8⁺IFN- γ ⁺ T cells in *IL-17^{-/-}* mice was increased as compared with WT controls (Fig. 5B), although granzyme B expression among CD8⁺ T cells from WT and *IL-17^{-/-}* mice was similar (Suppl. Fig. 3D). We also analyzed immune cell infiltration in skin samples after acute TPA treatments. We observed reduced F4/80⁺ macrophage and moderate reduced CD11b⁺ myeloid cell infiltration in *IL-17^{-/-}* mice (Suppl. Fig. 3C).

While CD8⁺IFN- γ ⁺ T cells have been associated with anti-tumor 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 β , CXCL1, CXCL2, S100A8, S100A9, Cox-2 and TNF- α was significantly reduced in skin samples from *IL-17^{-/-}* mice as compared with WT controls (Fig. 5C). All of these cytokines and chemokines have been reported to promote myeloid-derived suppressor cell generation and recruitment into the inflammatory environment (27,29-31). In contrast, we did not observe reduced expression of T cell attracting chemokines, such as CXCL9-11, but reduced expression of MMP9, IL-17F and IL-21 (Suppl. Fig. 4A,B&C).

Discussion

We have demonstrated that, in DMPA/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-17R does not appear 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). While over-activation 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 (VEGF and FGF2) 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 report, we demonstrated 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 (enterotoxogenic *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 in tumor-associated inflammatory conditions. However, our results do not address the role of IL-17 in the initiation stage of papilloma development. Using IL-17 neutralization antibody before DMBA treatment may provide important information on whether IL-17 is critical for papilloma development at early stages.

Previous studies have demonstrated a critical role of TNF- α /NF- κ B axis in DMBA/TPA skin carcinogenesis model (35). It has been shown that TNF- α contributed to tumor progression by increasing myeloid cell recruitment in IL-17-dependent way (36). In our study, we observed reduction of TNF- α in *IL-17^{-/-}* mice. At this point, it remains to be clarified whether Stat3 signaling inhibits TNF- α indirectly or directly. Several independent studies have shown that NF- κ B-induced tumorigenesis is Stat3 dependent (37).

While consistent with several recent publications on the role of IL-17 in promoting growth of transplanted tumor growth (7,9) and ETBF induced colon tumorigenesis in Min mice (14), these findings contradict with other reports suggesting that IL-17 can provide an anti-tumor effect against certain transplanted tumors (17,18). Additionally, a recent report demonstrated that lung metastases from polyoma middle T driven breast cancer involved a Th2-dependent mechanism (38). It is therefore possible that cytokine requirements for growth vs. inhibition of established tumors or metastases may be diverse depending on tumor type and expression of cytokine receptors by tumor cells themselves. These

requirements are likely quite different from de novo carcinogenesis, which appears to be enhanced by IL-17 and Stat3 signaling. Further analysis of additional examples of transplanted tumors, natural metastases and de novo carcinogenesis will be necessary to define whether there are common or distinct immunologic responses for these processes.

The IL-17-Stat3 pathway can promote epithelial carcinogenesis at multiple levels. First, as an oncogenic transcription factor, Stat3 activation in epithelial cells is associated with cell proliferation and anti-apoptosis. We observed Stat3 is constitutively activated in DMBA/TPA treated skin, which is associated with elevated expression Stat3-regulated proliferation and anti-apoptosis genes. Down-modulation Stat3 activation in *IL-17^{-/-}* mice resulted in reduced epithelial cell hyperproliferation and gland hyperplasia, and downregulated oncogenic gene expression. Secondly, 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. IL-17-Stat3 pathway augments the recruitment of myeloid cells but not CD8⁺ T cells into the local skin environment. Stat3-regulated inflammatory mediator expression, including Cox-2, CXCL1, CXCL2, S100A8, S100A9, and IL-1 β , has been reported to be critical for the generation of myeloid suppressive cells and granulocyte cells into the inflammatory environment (27,29-31). Lastly, 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 (Suppl. Fig. 4B).

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

TPA treatment induces IL-17 expression and activates Stat3 signaling. WT mice were first treated with DMBA and then TPA one week later. Skin samples were collected at 3 h, 24 h after first TPA treatment. Skin samples were also prepared from mice that were under long-term TPA administration. Mice that were treated with DMBA only serve as controls. *A*, IL-17 and IL-6 expression at mRNA level in skin from mice at 0 h, 3 h, 24 h after first TPA administration, or 20 weeks after TPA administration. Data shown are real-time PCR; Mean ($\pm$ SE); $n = 4$. *B*, Western blotting analysis of phosphorylated Stat3 protein in skin from mice at 0 h, 3 h, 24 h, 12 weeks or 20 weeks after TPA administration; $n = 4$. One representative of three replicated experiments is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. *IL-17*^{-/-} mice are resistant to the DMBA/TPA induced epithelial carcinogenesis. WT and *IL-17*^{-/-} mice were administrated 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 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 photos of WT and *IL-17*^{-/-} mice with papillomas. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Figure 3.

IL-17 ablation leads to reduced Stat3 activation in the tumor microenvironment. WT and *IL-17*^{-/-} mice were administrated with DMBA/TPA for 20 weeks. **A**, Left panel: Intracellular IL-17 staining of enriched mononuclear cells from skin of DMBA/TPA treated WT and *IL-17*^{-/-} mice. Gated CD4⁺ T cells were shown as CD4 versus IL-17. **B**, Mean (± SE) of mRNA expression of IL-6 in skin. Right panel: 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, phosphorylated Stat3; β-actin serves as loading control. Two representative samples from each group of mice are shown. All experiments are repeated at least three times. **D**, Immunofluorescence staining of p-Stat3 in WT and *IL-17*^{-/-} skin samples. One representative photo of three skin sections is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Figure 4.

IL-17 promotes DMBA/TPA mediated epidermal hyperproliferation and Stat3-regulated oncogenic gene expression. WT and $IL-17^{-/-}$ mice were administrated with DMBA/TPA for 20 weeks. **A**, H.E. staining, Ki67 staining and Cleaved Caspase 3 staining of skin section collected 20 weeks after DMBA/TPA administration. One representative photo of four skin sections is shown. Ki67 staining and Cleaved Caspase 3 staining was quantified by counting the number of positive cells in the field, $n=4$. **B**, Mean ($\pm$ SE) of mRNA level of Bcl- x_L , Survivin, and Cyclin D1 in skin of DMBA/TPA treated mice; $n=4$. **C**, Two representative samples of western blotting analysis of Bcl- x_L , Survivin, and Cyclin D1 in skin of DMBA/TPA treated mice are shown. These experiments were repeated with similar results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Figure 5.

IL-17 augments myeloid cell recruitment and Stat3-associated local inflammation. WT and *IL-17^{-/-}* mice were administrated with DMBA/TPA for 20 weeks. Cutaneous infiltration of CD11b⁺Gr-1⁺ myeloid cells, CD8⁺ T cells and CD8⁺IFN γ ⁺ T cells was analyzed by flow cytometry. **A**, Shown is mean ($\pm$ SE) of percentage and yield of CD11b⁺Gr-1⁺ myeloid cells in skin samples from DMBA/TPA treated WT and *IL-17^{-/-}* mice; $n = 4$. **B**, Shown is mean ($\pm$ SE) of percentage of CD8⁺ T cells and yield of CD8⁺IFN γ ⁺ T cells in skin samples from DMBA/TPA treated WT and *IL-17^{-/-}* mice; $n = 4$. **C**, Cutaneous expression of TNF- α , IL-1 β , CXCL1, CXCL2, S100A8, S100A9, and Cox-2 in mRNA level in skin from DMBA/TPA treated mice; $n = 4$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.