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IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid derived suppressor cells¹

Donggou He^{*}, Hui Li^{*}, Nabiha Yusuf^{*,†,‡}, Craig A. Elmetts^{*,†,‡}, Jun Li[§], John Mountz^{§,†,‡}, and Hui Xu^{*,†,2}

^{*}Department of Dermatology, University of Alabama at Birmingham

[§]Department of Medicine, University of Alabama at Birmingham

[†]Skin Disease Research Center, University of Alabama at Birmingham

[‡]Birmingham VA Medical Center, Birmingham, Alabama

Abstract

The role of immune responses in tumor development is a central issue for tumor biology and immunology. IL-17 is an important cytokine for inflammatory and autoimmune diseases. Although IL-17 producing cells are detected in cancer patients and tumor bearing mice, the role of IL-17 in tumor development is controversial and mechanisms remain to be fully elucidated. In the current study, we found that the development of tumors was inhibited in IL-17 receptor A (IL-17R) deficient mice. A defect in IFN- γ receptor increased tumor growth whereas tumor growth was inhibited in mice that were deficient in both IL-17R and IFN- γ R compared to wild type animals. Further experiments showed that neutralization of IL-17 by antibodies inhibited tumor growth in wild type mice whereas systemic administration of IL-17 promoted tumor growth. The IL-17R deficiency increased CD8 T cell infiltration whereas it reduced the infiltration of myeloid derived suppressor cells (MDSC) in tumors. In contrast, administration of IL-17 inhibited CD8 T cell infiltration and increased MDSC in tumors. Further analysis indicated that IL-17 was required for the development and tumor promoting activity of MDSC in tumor bearing mice. These data demonstrate that IL-17 mediated responses promote tumor development through the induction of tumor promoting microenvironments at tumor sites. IL-17 mediated regulation of MDSC is a primary mechanism for its tumor promoting effects. The study provides novel insights into the role of IL-17 in tumor development and has major implications for targeting IL-17 in treatment of tumors.

Keywords

IL-17; tumor; myeloid derived suppressor cells; CD8 T cells; IFN- γ

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²The author to whom reprint requests and page proofs should be addressed. Hui Xu, VH566B, 1670 University Blvd., University of Alabama at Birmingham, Birmingham, AL 35294, Tel: 205-9752628, Fax: 205-9345745, xuhui@uab.edu.

DISCLOSURES

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INTRODUCTION

Immune responses have paradoxical roles in tumor development (1, 2). On one side, immune responses play a key role in immune-surveillance for prevention of tumor development. Numerous studies indicate that anti-tumor immune responses are able to prevent and eliminate tumors. On the other side, however, immune responses, especially in a form of chronic inflammation, promote tumor development in many cases (3, 4). A prominent feature of tumor promoting immune responses is the increased number of myeloid derived suppressor cells (MDSC) in the blood, spleen and bone marrow and abundant infiltration of MDSC at the tumor site (5–8). Heavy infiltration of MDSC has been considered as a major cause for immunosuppression at tumor sites (5, 8, 9). MDSC are considered as an immature form of myeloid cells which are mostly identified as CD11b and Gr-1 double positive cells in mice (7, 10). MDSC are able to suppress anti-tumor immune responses and promote tumor growth (5, 8). Recent studies have shown that MDSC are composed of two subpopulations, which suppress T cell responses by different mechanisms (11, 12). Inflammatory cytokines and tumor derived mediators have been reported to regulate MDSC (5–8). However, mechanisms for the development and function of MDSC remain to be fully elucidated.

IL-17 is an inflammatory cytokine secreted by CD4 Th17 and CD8 Tc17 cells (13–17). Six IL-17 family members (IL-17A-F) have been described and the prototype member of the family is IL-17A, often termed IL-17 in literature. The receptor for IL-17A and IL-17F is IL-17RA, generally termed IL-17R which is expressed ubiquitously (14). IL-17 plays an important role in the regulation of leukocyte migration in inflammatory reactions and a defect in IL-17R decreases the expression of cytokines, chemokines and reduces the infiltration of inflammatory cells, especially neutrophils (18–23). The role of IL-17 in inflammatory and autoimmune diseases has been extensively studied (13, 15, 16, 24).

Although IL-17 producing cells are detected in cancer patients and tumor bearing mice (25–28), the role of IL-17 in tumor development is controversial (22, 29–35). Recent reports indicate that tumor growth is increased in IL-17^{-/-} mice and that the mechanism is associated with IFN- γ producing NK and T cells (32, 35). It implicates that IL-17 mediated responses are protective against tumor development. However, another recent report shows that tumor growth is suppressed in IL-17^{-/-} and IL-17/IFN- γ double knockout mice (31). A mechanism is that IL-17 induces the production of IL-6 by tumor cells, which in turn promotes tumor growth in a Stat-3 dependent pathway. Interestingly, the report shows that the production of IFN- γ by tumor infiltrating T cells from IL-17^{-/-} mice is increased (31). Although IFN- γ plays a role in the regulation of anti-tumor immune responses (36–38), CTL activity of tumor specific T cells is an important mechanism for T cell mediated tumor rejection. It is not examined whether IL-17 regulates the CTL activity of tumor specific CD8 T cells. Moreover, it is largely unknown whether IL-17 mediated effects on innate immune cells, such as MDSC, play a role in tumor immune responses.

It is often observed that T cells from tumor patients retain the ability to respond to tumor antigens. However, immune responses in peripherals are not correlated with tumor rejection (39–41). Mechanisms for immunosuppression include failure of immune T cell infiltration into tumors and presence of Treg cells and immune suppressive myeloid cells at tumor sites (8, 42–44). The infiltration of immune T cells in tumors is associated with good prognosis (45) whereas infiltration of MDSC is associated with poor prognosis (5, 8). Although it is well documented that IL-17 is an important cytokine for the regulation of leukocyte infiltration in inflammatory tissues (18–22), it remains to be examined whether IL-17 plays a role in the regulation of immune responses at tumor sites.

In the current study, we examined the effect of endogenous IL-17 on the growth of tumors in mice that were deficient in IL-17 receptor A (IL-17R^{-/-}). Additionally, anti-IL-17 antibodies and exogenous IL-17 cytokine were applied to further confirm the effect of IL-17 on tumor development. We examined IL-17 mediated effects on the infiltration of tumor specific T cells and myeloid cells in tumors and determined the role of IL-17 in the development of MDSC in tumor bearing mice. Our findings indicate that IL-17 promotes tumor development through the induction of tumor promoting responses at tumor sites and enhancement of MDSC.

MATERIALS AND METHODS

Mice

IL-17R^{-/-} mice on C57BL/6 background were provided by Amgen. IFN- γ R^{-/-} (C57BL/6 background) and wild type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). IL-17R^{-/-} mice were cross-bred to IFN- γ R^{-/-} mice to generate double knockout mice in our laboratory. The gene phenotype was routinely confirmed by PCR using specific primers. All animal procedures were performed according to NIH guidelines and the protocols were approved by the Institute Animal Care and Use Committee of the University of Alabama at Birmingham.

Transplanted tumor models: Protocols for inoculation and measurement of tumors in transplanted tumor models were reported previously (46). Mouse lymphoma cell line EL4, E.G7-OVA (EL4 transfected with OVA gene), prostate tumor cell line Tramp-C2 and melanoma cell line B16-F10 were purchased from the ATCC. Tumor cells were injected subcutaneously in mice and tumor growth was monitored every 3 days. Tumor sizes were calculated with the formula: Tumor Size = $L \times S \times H \times \pi/6$ (L: long diameter, S: short diameter, H: height).

To examine the effect of IL-17 on tumor growth, mice were treated intravenously with adenovirus encoding GFP (Ad-GFP) or mouse IL-17A (Ad-IL-17) (10^9 pfu/mouse) (kindly provided by Dr. Jay K. Kolls, University of Pittsburgh) as described (47). Two days later, the mice were inoculated with E.G7-OVA tumor cells.

To examine the effect of neutralizing IL-17 on tumor growth, mice were inoculated subcutaneously with E.G7-OVA tumor cells and treated intraperitoneally with normal rat IgG or a rat anti-mouse IL-17 monoclonal antibody (100 μ g/mouse) (TC11-18H10, anti-IL-17A, Southern Biotechnology Inc., Birmingham, AL) on day 0 (the day of tumor inoculation), 1, 6, 10, and 14.

To examine effects of MDSC on tumor growth, MDSC were purified from spleens of tumor bearing mice by using anti-CD11b antibody coupled magnet beads according to the manufacturer's instructions (MACS system, Miltenyi Biotec, Auburn, CA). The purity of the cells was >90% as assessed by flow cytometry. The cells were co-injected (5×10^6 /mouse) subcutaneously with E.G7-OVA tumor cells (4×10^6 /mouse) in wild type mice. In experiments using B16 tumors, MDSC (2×10^6 /mouse) were co-injected subcutaneously with B16 melanoma cells (1×10^6 /mouse) in wild type mice. Tumor growth was monitored.

Immunohistochemical staining of tissues

Immunohistochemical staining of tissue sections was described in our previous studies (17). Frozen tissue sections (5 μ M) were made for staining of CD8 positive cells. Paraffin embedded sections (6 μ M) were made for staining of PCNA positive cells. The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BD-Biosciences (San Diego, CA). Pictures were taken microscopically (10 $\times$ objective) with a digital camera

(Olympus). Positive cells were counted in 10 fields of each group. Average numbers of positive cells per field were calculated and analyzed statistically.

Flow cytometry analysis

To examine tumor infiltrating cells, tumor tissues were cut into small pieces and digested in RPMI containing collagenase-D 2mg/ml (Sigma), Dnase I 50ug/ml (Sigma), and 10%FCS. To detect MDSC, cell suspensions of tumors and spleens or blood leukocytes were stained with Alexa488 labeled CD11b and allophycocyanin labeled Gr-1 antibodies (BD-Biosciences). The percent of cells which expressed a high level of both CD11b and Gr-1 was analyzed in a flow cytometer (FACScaliber, Becton-Dickinson) as described (17). To detect tumor antigen OVA specific CD8 T cells in tumors, tumor cell suspensions were stained with Alexa488 labeled anti-CD8 antibody and phycoerythrin labeled OVA/MHC class-I tetramers which bind to OVA specific CD8 T cells (iTag MHC Tetramer, Beckman Coulter). CD8 T cells were gated and the percent of the tetramer positive cells was analyzed by flow cytometry.

Assays for T cell functions

CD8 T cells from spleens of tumor bearing mice were purified by using the MACS system according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). In our hands, this procedure results in >90% selection of CD8 T cells. Bone marrow derived dendritic cells (BM-DC) were generated from bone marrows of C57BL/6 as described in our previous report (17) and pulsed with OVA (100 µg/ml) for overnight.

To detect cytokine production by tumor specific T cells, spleen cells of tumor bearing mice were stimulated with OVA-pulsed BM-DC for 4 days (2×10^6 T cells plus 2×10^4 DC/ml). Our preliminary data showed that the stimulation of T cells with OVA-pulsed BM-DC for 4 days induced optimal levels of cytokine producing cells and cytokines in cultures compared to cultures for 1, 2 or 3 days (data not shown). Concentrations of cytokines in supernatants were measured by cytokine specific ELISA as described in our previous studies (23). To detect cytokine producing T cells, cells were harvested 4 days after cultures and stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) in the presence of Golgi Stop (BD Pharmingen) for 4–6 hrs. Intracellular cytokines were stained and cytokine producing cells were analyzed in a flow cytometer as described in our previous report (17).

To examine the CTL activity of tumor specific CD8 T cells, CD8 T cells were purified from spleens of E.G7-OVA tumor bearing mice and stimulated in vitro with OVA-pulsed BM-DC for 5 days (2×10^6 T cells plus 2×10^4 DC/ml). E.G7-OVA tumor cells were labeled with 5 µM CFSE and used as antigen specific target cells. EL4 cells were labeled with 0.5 µM CFSE and used as control cells. E.G7-OVA and EL4 cells were mixed at 1:1 ratio and used as target cells. The CD8 T cells which were harvested after the in vitro stimulation were incubated with mixed target cells (E.G7-OVA/EL4) (1×10^4 /well) at ratios of 100:1, 50:1, 25:1 and 0:1 (control) for 4 hours. Each sample was quadruplicated. The specific lysis of target cells was calculated with the formula as reported (48): %specific cytotoxicity = $100 \times [1 - (E.G7-OVA/EL4)_{\text{experimental}} / (E.G7-OVA/EL4)_{\text{control}}]$.

To examine the effect of MDSC on T cell activation, CD4 T cells were purified from naïve wild type mice by the MACS system and labeled with a fluorescence dye Carboxyfluorescein succinimidyl ester (CFSE, Molecular Probe) at 5 µM for 20 min. The CFSE labeled CD4 T cells were then placed in cultures with purified MDSC from wild type or IL-17R^{-/-} tumor bearing mice (2×10^6 T cells to 1×10^6 MDSC/ml). CD11b⁺ cells from tumor free wild type mice served as controls. The cultures were stimulated with plate-bound anti-CD3 (20 µg, BD Pharmingen) and anti-CD28 (20 µg/ml, BD Pharmingen)

antibodies for 4 days. T cells that were not stimulated with the antibodies served as negative controls. The division of CD4 T cells was analyzed by flow cytometry.

TUNEL assay

Apoptotic cells were detected by TUNEL assay as described in our previous report (46). Briefly, tumor samples were fixed in 10% formalin and sections (5 μ m) were made. TUNEL assay was performed using a commercial apoptosis detection kit according to the manufacturer's instructions (Promega, Madison, WI). Sections were counterstained with DAPI and photographed microscopically with a 10 $\times$ objective. The number of apoptotic cells was counted and results from 10 fields of each group were calculated for statistical analysis.

Real time quantitative RT-PCR

The expression of mRNA was quantified by real time RT-PCR as described in our previous report (23). Briefly, MDSC were purified from spleen of tumor bearing mice by using the MACS system and stimulated with LPS (5 μ g/ml) for overnight. Total RNA was isolated from the cells by using TRIzol Reagent according to the manufacture's instructions (GibcoBRL). Real time TR-PCR was performed with iQ SYBRO Green Supermix Kit in a MyiQ real time qPCR system according to the manufacture's instructions (Bio-Rad). The expression level of cytokines was normalized to the house-keeping gene GAPDH in each sample. The sequences for primers were: Arginase-1: forward, 5'-ATGGAAGAGACCTTCAGCTAC-3', reverse, 5'-GCTGTCTTCCCAAGAGTTGGG-3'; S100A8: forward, 5'-ACAATGCCGTCTGAACTGG-3', reverse, 5'-CTCTGCTACTCCTTGTTGGCTGTCT-3'; S100A9: forward, 5'-TCATCGACACCTTCCATCAA-3', reverse, 5'-GATCAACTTTGCCATCAGCA-3'; MMP9: forward, 5'-CATCGAACTTCGACACTGAC-3', reverse, 5'-AGCCACGACCATACAGATAC-3'; GAPDH: forward, 5'-AATGGTGAAGGTCTGGTGTGAAC-3', reverse, 5'-GAAGATGGTGATGGGCTTCC.

Statistical analysis

All data are presented as means $\pm$ SEM. The two tailed Student's *t*-test was applied for statistical analysis with *P*<0.05 being considered statistically significant.

RESULTS

Tumor growth is inhibited in IL-17R^{-/-} mice

The effect of IL-17 on tumor growth is controversial (22, 30–32). It is not yet reported whether a defect in IL-17R affects tumor growth. To examine it, wild type mice and IL-17R^{-/-} mice which were deficient in response to IL-17A and IL-17F were inoculated subcutaneously with various types of tumor cell lines and tumor growth was monitored. Results showed that the growth of the lymphoma cell line EL4 (2×10^6 /mouse), melanoma cell line B16-F10 (1×10^6 /mouse), and prostate tumor cell line Tramp-C2 (2×10^6 /mouse) was significantly inhibited in IL-17R^{-/-} mice compared to wild type mice (Fig. 1a, b and c).

Literature and our previous studies indicate that IL-17 stimulates IFN- γ production whereas IFN- γ inhibits IL-17 production by T cells (23, 49, 50). However, it is unknown whether the interaction of IL-17 and IFN- γ affects tumor development. To examine the role of IFN- γ in IL-17 mediated regulation of tumor development, we have generated IL-17R/IFN- γ R double knockout mice. Mice were inoculated subcutaneously with a high-immunogenic cell line E.G7-OVA (4×10^6 cells/mouse). Results showed that tumor growth was increased in IFN- γ R^{-/-} mice whereas decreased in IL-17R^{-/-} mice compared to wild type mice (Fig. 1d). In

the double knockout mice that were deficient in both IL-17R and IFN- γ R, tumor growth was significantly reduced compared to wild type mice (Fig. 1d). This suggests that the IL-17R deficiency inhibited the growth of high-immunogenic tumors. Moreover, although the deficiency in IFN- γ R signals enhanced tumor growth, the IL-17R deficiency reversed the susceptibility of IFN- γ R $^{-/-}$ mice to tumor development. In order to examine immunological mechanisms for IL-17 mediated effects on tumor growth, E.G7-OVA tumor cells were applied in all following experiments

To further determine whether IL-17 promoted tumor growth, wild type mice were injected intravenously with adenovirus encoding mouse IL-17 (Ad-IL-17) or GFP (Ad-GFP) (10^9 pfu/mouse) and then inoculated with E.G7-OVA tumor cells. Results showed that the treatment with Ad-IL-17 significantly increased tumor growth compared to control mice that were treated with Ad-GFP or left untreated (Fig. 2a). In contrast, treatment of wild type mice with a neutralizing anti-IL-17 antibody significantly inhibited the growth of E.G7-OVA tumors compared to controls that were treated with rat IgG (Fig. 2b).

Analysis of IL-17 mediated effects on tumors

To examine effects of the IL-17R deficiency on tumors, tumor tissues from tumor bearing mice were collected and subjected to analysis. Results showed that the number of proliferating cells in tumors, which were stained with anti-proliferating cell nuclear antigen (PCNA) antibody, was significantly reduced in IL-17R $^{-/-}$ mice compared to wild type animals (Fig. 3a). In contrast, the number of apoptotic cells which were detected by TUNEL assay was significantly increased in IL-17R $^{-/-}$ tumors (Fig. 3b). These results suggest that the deficiency in IL-17R inhibits tumor cell proliferation and enhances apoptosis.

The immunity at tumor sites is important for the fate of tumors and the infiltration of T cells is closely associated with prognosis (5, 8, 9, 45). We found that the infiltration of CD8 T cells in tumors, which are major effector cells for tumor rejection, was significantly increased in IL-17R $^{-/-}$ mice (Fig. 3c). The infiltration of CD8 T cells in tumors of IFN- γ R $^{-/-}$ was hardly affected whereas it was significantly increased in the double knockout mice that were deficient in both IL-17R and IFN- γ R. The inhibitory effect of IL-17 on CD8 T cell infiltration in tumors was supported by further experiments showing that the treatment of mice with Ad-IL-17 reduced the infiltration of CD8 T cells in tumors (Fig. 3d). The effect of IL-17 on T cell infiltration appeared to be specific for CD8 T cells since no significant difference was found in CD4 T cell infiltration in tumors of wild type and IL-17R $^{-/-}$ mice (data not shown).

IL-17 mediated effects on tumor specific T cells

Based on the increased infiltration of CD8 T cells in tumors, we further characterized tumor infiltrating CD8 T cells. Results showed that the majority of tumor infiltrating CD8 T cells bound to OVA/MHC class-I tetramers (>70%) (Fig. 4a). There was not a remarkable difference in the percent of tumor specific CD8 T cells between wild type and IL-17R $^{-/-}$ mice although the infiltration of CD8 T cells was significantly increased in IL-17R $^{-/-}$ mice compared to wild type animals (Fig. 4b). Further analysis of tumor infiltrating CD8 T cells did not show a significant change in the expression of CTL related molecules perforin, Fas-L, and granzyme B between wild type and IL-17R $^{-/-}$ tumor bearing mice (data not shown).

To examine whether the IL-17R deficiency had an effect on T cell function, spleen cells of E.G7-OVA tumor bearing wild type and IL-17R $^{-/-}$ mice were collected and stimulated with OVA-pulsed BM-DC for 4 days. Results showed that CD4 and CD8 T cells that produced IFN- γ were reduced whereas IL-17 producing CD4 T cells were increased (Fig. 5a). Furthermore, ELISA assays showed that IL-17 was increased whereas IFN- γ was

reduced in culture supernatants of IL-17R^{-/-} T cells compared to those of wild type T cells (Fig. 5b). However, there was not a significant difference in T cell numbers between wild type and IL-17R^{-/-} mice (data not shown), a result consistent with previous reports (23, 51).

To determine whether the IL-17R^{-/-} deficiency affected the CTL activity of CD8 T cells, CD8 T cells were purified from spleens of E.G7-OVA tumor bearing mice and stimulated with OVA-pulsed BM-DC for 5 days. CTL assays revealed that the CTL activity of CD8 T cells from IL-17R^{-/-} and wild type tumor bearing mice was not significantly different (Fig. 5c). The experiment was repeated three times and similar results were observed. Collectively, the IL-17R^{-/-} deficiency increased the infiltration of CD8 T cells in tumors, inhibited IFN- γ production by T cells but had little effect on the CTL activity of tumor specific CD8 T cells.

IL-17 is required for the development of myeloid derived suppressor cells

An increased number of myeloid derived suppressor cells (MDSC) in spleen, blood and tumors is a hallmark of major immunological abnormalities in cancer patients and tumor bearing animals (5–7). MDSC are considered as an immature form of myeloid cells which are mostly identified as CD11b and Gr-1 double positive cells in mice (7, 10). We found that the percent of CD11b/Gr-1 double positive MDSC in the spleen of IL-17R^{-/-} tumor bearing mice was lower than that of wild type tumor bearing mice (Fig. 6a). The deficiency in IFN- γ R had little effect on MDSC whereas MDSC in the double knockout mice that were deficient in both IL-17R and IFN- γ R was reduced. Further analysis showed that MDSC in the spleen, blood and tumors of IL-17R^{-/-} and the double knockout mice was significantly reduced compared to wild type controls (Fig. 6b).

In further experiments, results showed that spleens from tumor bearing mice that were treated with Ad-IL-17 (as described in Fig. 2a) contained a significantly higher level of MDSC than those from the mice that were treated with Ad-GFP or untreated control (Fig. 6c). In contrast, the number of MDSC in the spleen of tumor bearing mice which were treated with anti-IL-17 antibody (as described in Fig. 2b) was significantly reduced compared to controls that were treated with normal rat IgG (Fig. 6d). The deficiency in IFN- γ R or IL-17R did not have a significant effect on the number of MDSC in tumor free naïve mice (data not shown).

IL-17 regulates the phenotype and function of myeloid derived suppressor cells

The tumor promoting function of MDSC is associated with increased activities of arginase-1 (Arg-1), MMP9, and S100A8/A9 (7, 10, 52). To examine mechanisms for IL-17 mediated effects on the phenotype of MDSC in tumor bearing mice, MDSC were purified from spleens of tumor bearing mice and stimulated with LPS in vitro for overnight. Our initial experiments showed a very low expression level of the molecules by freshly isolated MDSC without the LPS stimulation (data not shown). Results showed that MDSC from IL-17R^{-/-} tumor bearing mice expressed lower levels of Arg-1, MMP9 and S100A8/A9 than those from wild type tumor bearing mice (Fig. 7). Furthermore, MDSC from Ad-IL-17 treated tumor bearing wild type mice expressed a higher level of the molecules than those from mice that were treated with Ad-GFP or left untreated (Fig. 7).

In experiments examining the tumor promoting function of MDSC, results showed that co-injection of MDSC purified from wild type E.G7-OVA tumor bearing mice significantly increased tumor growth whereas co-injection of MDSC from IL-17R^{-/-} tumor bearing mice did not affect tumor growth compared to control mice that were not injected with MDSC (Fig. 8a). In contrast, MDSC from wild type tumor bearing mice that were treated

with Ad-IL-17 promoted tumor growth to a significant greater extent than those from control wild type tumor bearing mice that were treated with Ad-GFP (Fig. 8b). Furthermore, similar results were also observed in mouse B16 melanoma model, which showed a reduced tumor promoting effect of MDSC from IL-17R^{-/-} B16 tumor bearing mice compared to those from wild type counterparts (Fig. 8c). However, the tumor promoting activity of MDSC was dependent on the presence of tumors since co-injection of MDSC cells from naïve tumor free IL-17R^{-/-} or wild type mice did not have a significant effect on tumor growth compared to control animals that were not injected with MDSC (Fig. 8d).

In experiments examining the immunosuppressive effect of MDSC, MDSC from wild type tumor bearing mice significantly inhibited the proliferation of CD4 T cells compared to control CD11b⁺ cells which were purified from tumor free mice (Fig. 8e). In contrast, MDSC from IL-17R^{-/-} tumor bearing mice did not have an inhibitory effect on T cell proliferation compared to the control CD11b⁺ cells. These results indicate that MDSC from IL-17R^{-/-} tumor bearing mice lack the functions in both tumor promotion and immunosuppression.

DISCUSSION

The role of IL-17 in the regulation of tumor immune responses has yet to be fully understood. In the current study, we have used IL-17R^{-/-} and IL-17/IFN- γ R double knockout mice to specifically examine the role of IL-17 mediated immune responses in tumor development. Results indicate that IL-17 promotes tumor growth whereas the blockade of IL-17R inhibits tumor growth and even reverses the susceptibility of IFN- γ R^{-/-} mice to tumor development. IL-17 inhibits the infiltration of CD8 T cells but increases the infiltration of MDSC in tumors, a characteristic of tumor promoting microenvironment at tumor sites. Furthermore, IL-17 is required for MDSC mediated tumor promoting activity but had little effect on the CTL activity of tumor specific CD8 T cells although a defect in IL-17R reduces IFN- γ production by T cells. These data demonstrate that IL-17 mediated responses, especially at tumor sites, promote tumor development and provide insights into novel mechanisms by which Th1 and Th17 responses regulate tumor immunity.

A line of evidence indicates that IL-17 has tumor promoting effects, especially in the context of inflammations (29, 53, 54). A recent report shows that the growth of tumors is inhibited in IL-17^{-/-} and IFN- γ /IL-17 double knockout mice (31). However, conflict recent reports show that tumor growth is increased in IL-17^{-/-} mice (32, 35). In the report by Wang et al, IL-17 induces IL-6 production by tumor cells and stromal cells that express IL-17R. IL-6 promotes tumor growth in a Stat-3 dependent pathway (31). In the report by Kryczek et al, IFN- γ producing NK and T cells are reduced, which is considered to be a mechanism for the increased tumor growth in IL-17^{-/-} mice (32). Martin-Orozco et al showed that application of Th17 cells increased IFN- γ producing CD8 T cells in lungs with metastatic B16 tumors (35). Interestingly, Wang et al show that the infiltration of CD8 T cells in tumors and IFN- γ production by tumor infiltrating T cells are increased in IL-17^{-/-} mice (31). The report by Wang et al (31) and our studies used the mouse melanoma cell line B16 and found similar results in IL-17^{-/-} and IL-17R^{-/-} mice whereas Kryczek et al used MC38 sarcoma and Martin-Orozco et al used B16 metastasis model (32, 35). It is possible that IL-17 may have different roles in different tumors and tumor models. Additionally, different mouse models (IL-17^{-/-} vs. IL-17R^{-/-}) may also contribute to the discrepancy. Notably, the studies reporting protective roles of IL-17 in tumor immunity imply that the stimulation of IFN- γ production by IL-17 is important for IL-17 mediated anti-tumor immunity and the reduction of IFN- γ production and IFN- γ producing cells is a primary mechanism for the deficient anti-tumor immunity in IL-17^{-/-} mice (31, 35). Our results suggests that IFN- γ appears to play a minor role in IL-17 mediated regulation of tumor development since tumor growth is

inhibited in the IL-17R/IFN- γ R double knockout mice (Fig. 1d). This result is in consistent to Wang's report showing that tumor growth is inhibited in IL-17/IFN- γ double knockout mice (31). Our studies implicate that IL-17 mediated tumor promotion is associated with the inhibition of CD8 T cell infiltration in tumors and enhancement of MDSC development and function. Importantly, the CTL activity of tumor specific CD8 T cells is not significantly affected by the IL-17R deficiency although IFN- γ production is inhibited (Fig. 5). The inhibition of MDSC is a critical mechanism for the suppression of tumor growth in IL-17R $^{-/-}$ and IL-17R/IFN- γ R double knockout mice. Certainly, additional studies are required to further determine why IL-17 has different effects on modulating tumor development in different tumor models. An important issue is the effect of IL-17 responses on the function of tumor infiltrating CD4 and CD8 T cells, which is under our on-going studies.

MDSC are considered as an immature form of myeloid cells, which are present in the blood, spleen and bone marrow of normal mice and are increased in infectious diseases and tumors (5–8, 10). MDSC play important roles in the suppression of immune responses and promotion of tumor growth (5, 6, 8). Although IL-17 production is increased in inflammatory reactions and considered as an inflammatory cytokine promoting tumor development (29, 53, 54), it was not known whether IL-17 had an effect on MDSC in tumor bearing hosts. Our studies have implicated that IL-17 is required for the development of MDSC in tumor bearing mice. A defect in IL-17R reduces the number of MDSC in the blood, spleen and tumors. This is further supported by our data showing that the administration of exogenous IL-17 increases the number of MDSC in wild type tumor bearing mice whereas neutralization of IL-17 in wild type tumor bearing mice reduced the number of MDSC. It will be very interesting to examine whether IL-23 mediated promotion of tumor development, which is associated with an increased level of IL-17 and myeloid cell infiltration in tumors (29), is linked to IL-17 mediated regulation of MDSC.

MDSC inhibit immune responses and promote tumor growth by complexes of mechanisms (6, 55). We show that MDSC from wild type tumor bearing mice exhibit high levels of Arg-I, A100/A8/A9 and MMP9 molecules which are known to be mediators for MDSC mediated immunosuppression and tumor promotion (5, 7, 8, 52). However, MDSC from IL-17R $^{-/-}$ tumor bearing mice express a low level of the molecules, which is almost comparable to that of MDSC from tumor free naïve mice. Accordingly, co-application of MDSC from IL-17R $^{-/-}$ tumor bearing mice does not have a significant effect on tumor growth. In contrast, the treatment with exogenous IL-17 increases the expression level of the molecules and enhances the tumor promoting activity of MDSC. Furthermore, MDSC from IL-17R $^{-/-}$ tumor bearing mice do not have a significant immunosuppressive effect on T cell activation compared to the wild type counterparts. These data demonstrate that IL-17 signals are required not only for the development but also for the tumor promoting and immunosuppressive activity of MDSC.

Heavy infiltration of MDSC has been considered as a major cause for immunosuppression at tumor sites (5, 8, 9). The infiltration of immune T cells in tumors is associated with good prognosis (45) whereas infiltration of MDSC is associated with poor prognosis (5, 8). Our data show that although IL-17 does not have inhibitory effects on tumor specific T cells or even stimulates IFN- γ production by T cells, its opposite effects on the infiltration of CD8 T cells and MDSC in tumors imply an important mechanism for IL-17 mediated tumor promoting microenvironments at tumor sites. This is supported by our data showing that proliferation is inhibited whereas apoptosis is increased in the tumors of IL-17R $^{-/-}$ mice (Fig. 3). It is to note that the tumor growth is increased whereas the number of MDSC is not significantly affected in IFN- γ R $^{-/-}$ mice. Since IFN- γ signals are known to have antitumor activities (36–38), the deficiency in IFN- γ mediated anti-tumor immunity may lead to the increased tumor growth. However, the reduction of MDSC provides an interpretation for the

suppression of E.G7-OVA tumor growth in IL-17R/IFN- γ R $^{-/-}$ compared IFN- γ R $^{-/-}$ mice. A recent report shows that MDSC are able to inhibit the migration of T cells by down regulating the expression of L-selectin by T cells (56). Further experiments are required to determine whether IL-17 mediated opposite effects on CD8 and MDSC infiltration is dependent on the interaction of MDSC with CD8 T cells or the induction of specific chemokines for the cells.

In summary, our studies have demonstrated that IL-17 promotes tumor growth. Moreover, a defect in IL-17 mediated responses can reverse the susceptibility to tumor development in mice which are deficient in IFN- γ mediated anti-tumor responses. IL-17 induces tumor promoting microenvironments at tumor sites and its effects on MDSC represent an important mechanism for the tumor promoting effect. Our studies provide insights into novel mechanisms by which IL-17 mediates tumor promoting immune responses at tumor sites. Targeting IL-17 may be developed to new immunotherapeutic strategies for treatment of tumors.

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REFERENCE

1. Vakkila J, Lotze MT. Inflammation and necrosis promote tumour growth. *Nat Rev Immunol.* 2004; 4:641–648. [PubMed: 15286730]
2. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer.* 2006; 6:24–37. [PubMed: 16397525]
3. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell.* 2005; 7:211–217. [PubMed: 15766659]
4. Clevers H. At the Crossroads of Inflammation and Cancer. *Cell.* 2004; 118:671–674. [PubMed: 15369667]
5. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009; 9:162–174. [PubMed: 19197294]
6. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. *Immunological Reviews.* 2008; 222:162–179. [PubMed: 18364001]
7. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: Recruitment, phenotype, properties, and mechanisms of immune suppression. *Seminars in Cancer Biology.* 2006; 16:53–65. [PubMed: 16168663]
8. Ostrand-Rosenberg S, Sinha P. Myeloid-Derived Suppressor Cells: Linking Inflammation and Cancer. *J Immunol.* 2009; 182:4499–4506. [PubMed: 19342621]
9. Nagaraj S, Gabrilovich DI. Tumor Escape Mechanism Governed by Myeloid-Derived Suppressor Cells. *Cancer Res.* 2008; 68:2561–2563. [PubMed: 18413722]
10. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol.* 2005; 5:641–654. [PubMed: 16056256]
11. Movahedi K, Guillemins M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood.* 2008; 111:4233–4244. [PubMed: 18272812]
12. Youn J-I, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of Myeloid-Derived Suppressor Cells in Tumor-Bearing Mice. *J Immunol.* 2008; 181:5791–5802. [PubMed: 18832739]
13. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 Family Cytokines and the Expanding Diversity of Effector T Cell Lineages. *Annual Review of Immunology.* 2007; 25:821–852.

14. Gaffen SL. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol.* 2009; 9:556–567. [PubMed: 19575028]
15. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity.* 2004; 21:467–476. [PubMed: 15485625]
16. Dong C. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nat Rev Immunol.* 2006; 6:329–334. [PubMed: 16557264]
17. He D, Wu L, Kim HK, Li H, Elmets CA, Xu H. CD8+ IL-17-Producing T Cells Are Important in Effector Functions for the Elicitation of Contact Hypersensitivity Responses. *J Immunol.* 2006; 177:6852–6858. [PubMed: 17082599]
18. Kelly MN, Kolls JK, Happel K, Schwartzman JD, Schwarzenberger P, Combe C, Moretto M, Khan IA. Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against *Toxoplasma gondii* infection. *Infect Immun.* 2005; 73:617–621. [PubMed: 15618203]
19. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, Shellito JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med.* 2001; 194:519–527. [PubMed: 11514607]
20. Kolls JK, Linden A. Interleukin-17 Family Members and Inflammation. *Immunity.* 2004; 21:467–476. [PubMed: 15485625]
21. Koenders MI, Kolls JK, Oppers-Walgreen B, van den Bersselaar L, Joosten LA, Schurr JR, Schwarzenberger P, van den Berg WB, Lubberts E. Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis Rheum.* 2005; 52:3239–3247. [PubMed: 16200598]
22. Numasaki M, Watanabe M, Suzuki T, Takahashi H, Nakamura A, McAllister F, Hishinuma T, Goto J, Lotze MT, Kolls JK, Sasaki H. IL-17 Enhances the Net Angiogenic Activity and In Vivo Growth of Human Non-Small Cell Lung Cancer in SCID Mice through Promoting CXCR-2-Dependent Angiogenesis. *J Immunol.* 2005; 175:6177–6189. [PubMed: 16237115]
23. He D, Wu L, Kim HK, Li H, Elmets CA, Xu H. IL-17 and IFN- γ Mediate the Elicitation of Contact Hypersensitivity Responses by Different Mechanisms and Both Are Required for Optimal Responses. *J Immunol.* 2009; 183:1463–1470. [PubMed: 19553527]
24. McGeachy MJ, Cua DJ. Th17 Cell Differentiation: The Long and Winding Road. *Immunity.* 2008; 28:445–453. [PubMed: 18400187]
25. Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, Kolls J, Chang A, Zou W. Cutting Edge: Th17 and Regulatory T Cell Dynamics and the Regulation by IL-2 in the Tumor Microenvironment. *J Immunol.* 2007; 178:6730–6733. [PubMed: 17513719]
26. Nam JS, Terabe M, Kang MJ, Chae H, Voong N, Yang YA, Laurence A, Michalowska A, Mamura M, Lonning S, Berzofsky JA, Wakefield LM. Transforming growth factor beta subverts the immune system into directly promoting tumor growth through interleukin-17. *Cancer Research.* 2008; 68:3915–3923. [PubMed: 18483277]
27. Miyahara Y, Odunsi K, Chen W, Peng G, Matsuzaki J, Wang R-F. Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer. *Proceedings of the National Academy of Sciences.* 2008; 105:15505–15510.
28. Yusuf N, Nasti TH, Katiyar SK, Jacobs MK, Seibert MD, Ginsburg AC, Timares L, Xu H, Elmets CA. Antagonistic Roles of CD4+ and CD8+ T-Cells in 7,12-Dimethylbenz(a)anthracene Cutaneous Carcinogenesis. *Cancer Res.* 2008; 68:3924–3930. [PubMed: 18483278]
29. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, Basham B, McClanahan T, Kastelein RA, Oft M. IL-23 promotes tumour incidence and growth. *Nature.* 2006; 442:461. [PubMed: 16688182]
30. Honorati MC, Neri S, Cattini L, Facchini A. IL-17 enhances the susceptibility of U-2 OS osteosarcoma cells to NK cell lysis. *Clin Exp Immunol.* 2003; 133:344–349. [PubMed: 12930359]

31. Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J. Exp. Med.* 2009; 206:1457–1464. [PubMed: 19564351]
32. Kryczek I, Wei S, Szeliga W, Vatan L, Zou W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood.* 2009; 114:357–359. [PubMed: 19289853]
33. Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, Gattinoni L, Wrzesinski C, Hinrichs CS, Kerstann KW, Feigenbaum L, Chan C-C, Restifo NP. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood.* 2008; 112:362–373. [PubMed: 18354038]
34. Hinrichs CS, Kaiser A, Paulos CM, Cassard L, Sanchez-Perez L, Heemskerk B, Wrzesinski C, Borman ZA, Muranski P, Restifo NP. Type 17 CD8+ T cells display enhanced anti-tumor immunity. *Blood.* 2009 blood-2009-2002-203935.
35. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, Hwu P, Restifo NP, Overwijk WW, Dong C. T Helper 17 Cells Promote Cytotoxic T Cell Activation in Tumor Immunity. *Immunity.* 2009; 31:787. [PubMed: 19879162]
36. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol.* 2006; 6:836. [PubMed: 17063185]
37. Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, Anichini A. Interleukin-12: Biological Properties and Clinical Application. *Clin Cancer Res.* 2007; 13:4677–4685. [PubMed: 17699845]
38. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol.* 2003; 3:133. [PubMed: 12563297]
39. Frey AB, Monu N. Effector-phase tolerance: another mechanism of how cancer escapes antitumor immune response. *J Leukoc Biol.* 2006; 79:652–662. [PubMed: 16415165]
40. Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J, Harlin H. Immune resistance orchestrated by the tumor microenvironment. *Immunological Reviews.* 2006; 213:131–145. [PubMed: 16972901]
41. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene.* 2008; 27:5904–5912. [PubMed: 18836471]
42. Wang HY, Wang R-F. Regulatory T cells and cancer. *Current Opinion in Immunology.* 2007; 19:217–223. [PubMed: 17306521]
43. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* 2009; 9:239–252. [PubMed: 19279573]
44. Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol.* 2006; 6:295–307. [PubMed: 16557261]
45. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Page C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc P-H, Trajanoski Z, Fridman W-H, Pages F. Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. *Science.* 2006; 313:1960–1964. [PubMed: 17008531]
46. Kim HK, Zhang H, Li H, Wu ZT, Swisher S, He D, Wu L, Xu J, Elmetts CA, Athar M, Xu XC, Xu H. Slit2 Inhibits Growth and Metastasis of Fibrosarcoma and Squamous Cell Carcinoma. *Neoplasia.* 2008; 10:1411–1420. [PubMed: 19048120]
47. Hsu H-C, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, Le T-vL, Lorenz RG, Xu H, Kolls JK, Carter RH, Chaplin DD, Williams RW, Mountz JD. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat Immunol.* 2008; 9:166–175. [PubMed: 18157131]
48. Kapp JA, Honjo K, Kapp LM, Xu Xy, Cozier A, Bucy RP. TCR transgenic CD8+ T cells activated in the presence of TGF{beta} express FoxP3 and mediate linked suppression of primary immune responses and cardiac allograft rejection. *Int. Immunol.* 2006; 18:1549–1562. [PubMed: 16966495]
49. Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I, Sekikawa K, Asano M, Iwakura Y. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity.* 2002; 17:375–387. [PubMed: 12354389]

50. Bettelli E, Oukka M, Kuchroo VK. TH-17 cells in the circle of immunity and autoimmunity. *Nat Immunol.* 2007; 8:345–350. [PubMed: 17375096]
51. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, Shellito JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *Journal of Experimental Medicine.* 2001; 194:519. [PubMed: 11514607]
52. Gebhardt C, Németh J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. *Biochemical Pharmacology.* 2006; 72:1622–1631. [PubMed: 16846592]
53. Charles KA, Kulbe H, Soper R, Escorcio-Correia M, Lawrence T, Schultheis A, Chakravarty P, Thompson RG, Kollias G, Smyth JF, Balkwill FR, Hagemann T. The tumor-promoting actions of TNF- α involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *The Journal of Clinical Investigation.* 2009; 119:3011. [PubMed: 19741298]
54. Wu S, Rhee K-J, Albesiano E, Rabizadeh S, Wu X, Yen H-R, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, Sears CL. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med.* 2009; 15:1016. [PubMed: 19701202]
55. Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: The role of tumor-associated macrophages. *Critical Reviews in Oncology/Hematology.* 2008; 66:1–9. [PubMed: 17913510]
56. Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S. Myeloid-Derived Suppressor Cells Down-Regulate L-Selectin Expression on CD4⁺ and CD8⁺ T Cells. *J Immunol.* 2009; 183:937–944. [PubMed: 19553533]

Abbreviations

MDSC	myeloid derived suppressor cells
BM-DC	Bone marrow derived dendritic cells

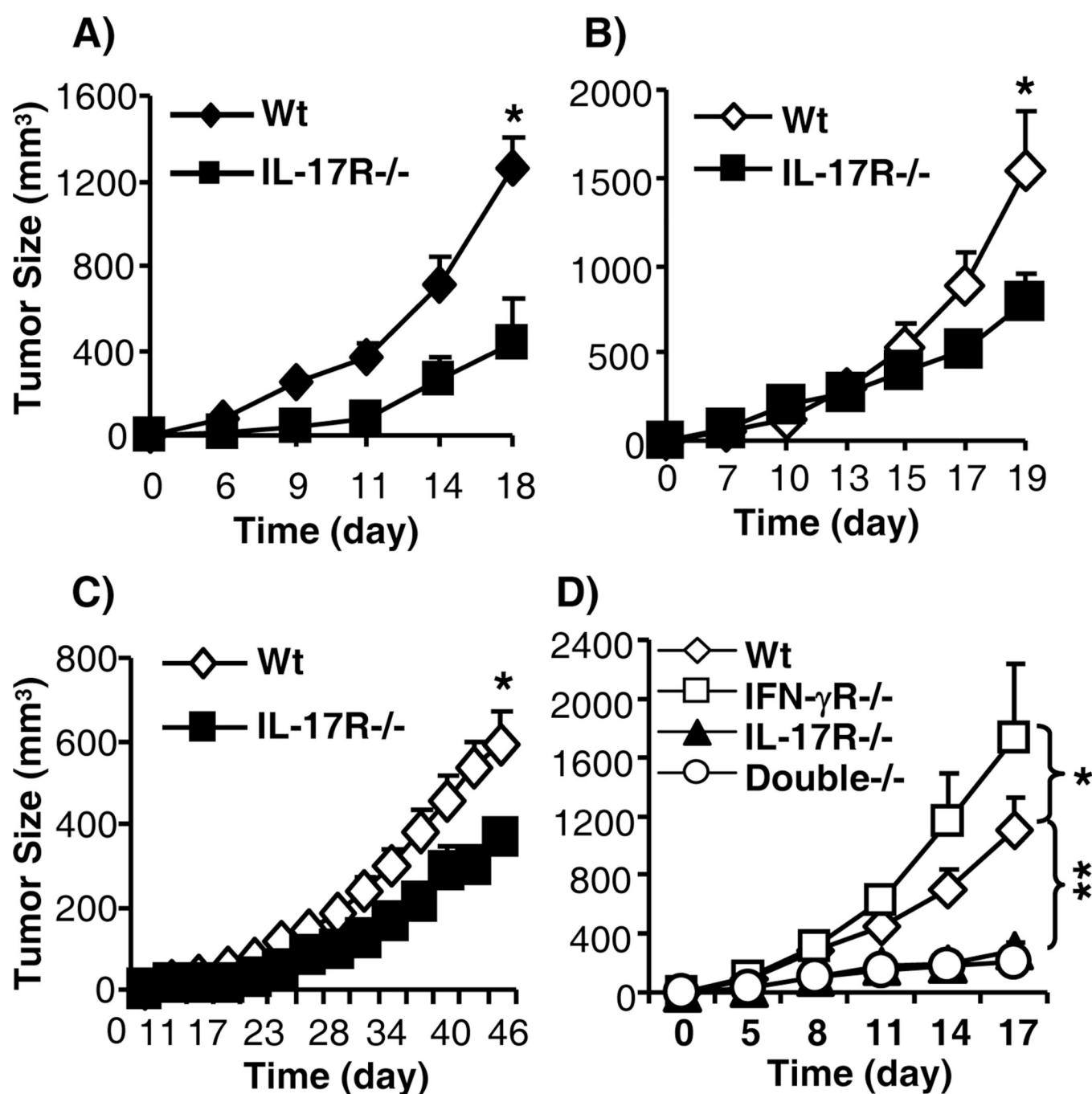


Figure 1. A defect in IL-17R inhibits tumor development

Mice were inoculated subcutaneously with tumor cells and tumor sizes were monitored. **A).** Lymphoma cell line EL4, 2×10^6 cells/mouse. (n=5). **B).** Melanoma cell line B16-F10, 1×10^6 cells/mouse. (n=5). **C).** Prostate tumor cell line Tramp-C2, 2×10^6 cells/mouse. (n=5). **D).** E.G7-OVA tumor cells, 4×10^6 cells/mouse (n=5). Data are presented as the mean tumor size $\pm$ SEM and are representative of 2–4 independent experiments. * P<0.05; ** P<0.01.

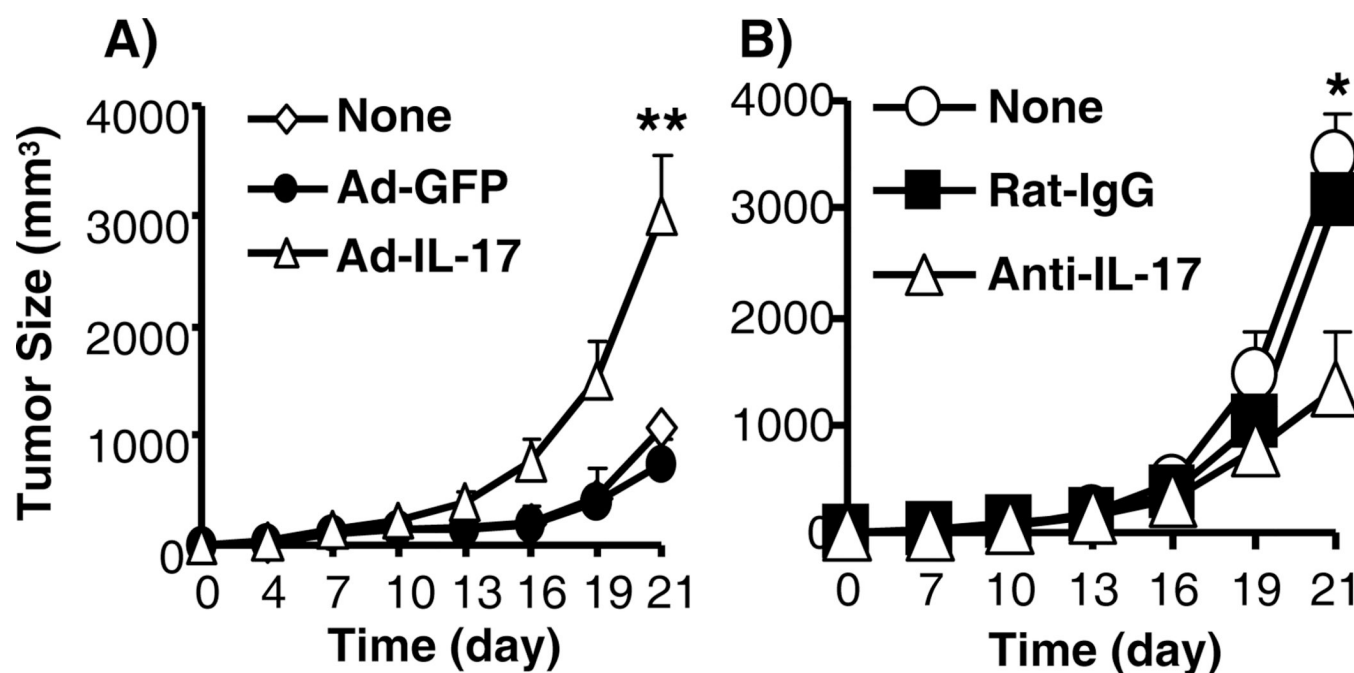


Figure 2. Administration of IL-17 promotes whereas neutralization of IL-17 inhibits tumor growth in wild type mice

A). Wild type mice were treated intravenously with adenovirus encoding IL-17 (Ad-IL-17) or green fluorescence protein (Ad-GFP) (10^9 pfu/mouse) or left untreated (None). Two days later, the mice were inoculated with E.G7-OVA tumor cells and tumor growth was monitored ($n=5$, * $P<0.01$). **B).** Wild type mice were inoculated with E.G7-OVA tumor cells and injected intraperitoneally with normal rat-IgG or a rat anti-mouse IL-17 monoclonal antibody ($100\mu\text{g}/\text{mouse}$) on day 0, 1, 6, 10, and 14 ($n=4$, * $P=0.01$). Control mice were left untreated (None). The data show means $\pm$ SEM of tumor size and are representative of 2–3 independent experiments.

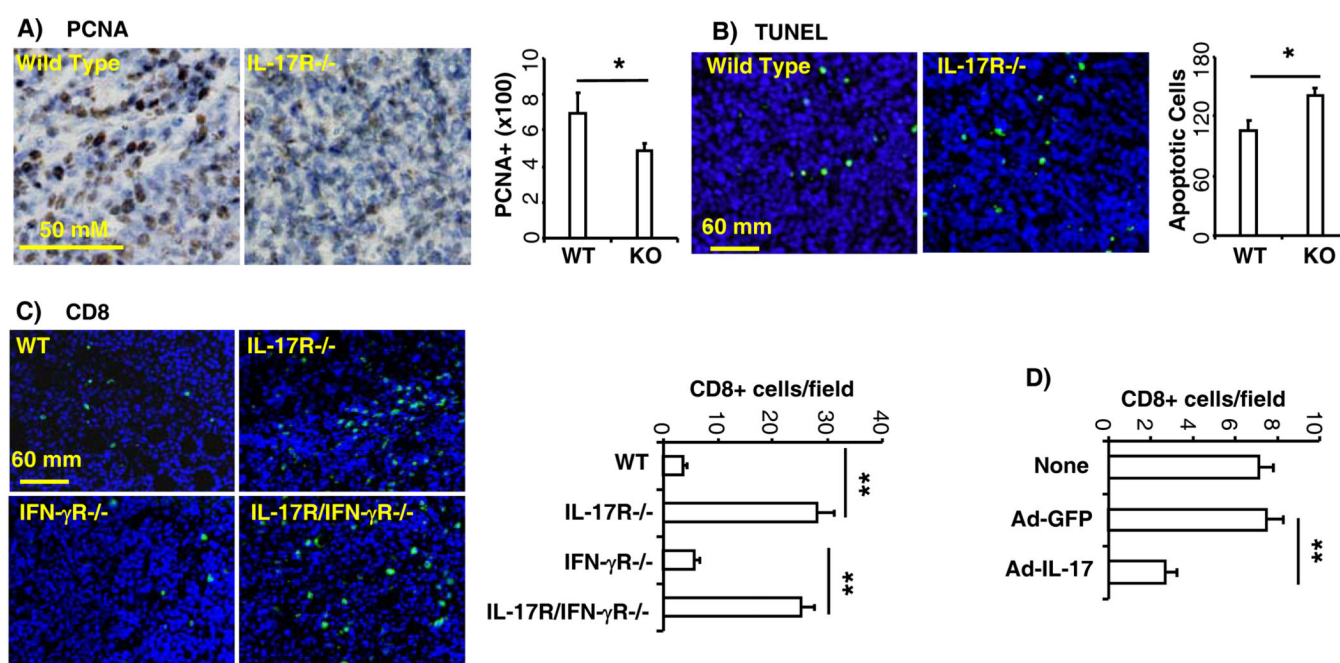


Figure 3. IL-17 mediated responses at tumor sites

E.G7-OVA tumor cells were inoculated subcutaneously and tumor tissues were harvested for analysis. **A).** Paraffin sections were prepared and stained with anti-PCNA antibodies. Samples were counter-stained with hematoxylin (blue) and PCNA positive cells (brown) were counted microscopically. **B).** Paraffin sections were applied for the TUNEL assay and counter-stained with DAPI (blue). Apoptotic cells (green) were counted microscopically. **C).** Frozen tissue sections were stained with Alexa488 labeled anti-CD8 antibody (green) and counterstained with DAPI (blue). The number of CD8 T cells was counted microscopically. **D).** Wild type mice were treated with Ad-IL-17, Ad-GFP or left untreated and then inoculated with E.G7-OVA tumor cells as described in Fig. 2a. Frozen tumor sections were stained with anti-CD8 antibody and the number of CD8 T cells was counted microscopically as described above. The data show means $\pm$ SEM of positive cells/field ($n=10$, * $P<0.05$; ** $P<0.01$) and are representative of 2–3 independent experiments.

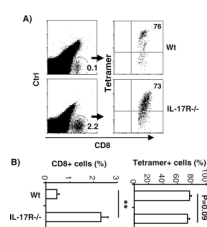


Figure 4. IL-17 mediated effects on infiltrating CD8 T cells in tumors

A). Tumor cell suspensions were stained with anti-CD8 antibody and MHC class-I/OVA tetramers. The left panel shows CD8 T cells in tumor suspensions. The right panel shows the percent of tetramer positive cells in the gated CD8+ cells. **B).** Statistical analysis shows a significant increase of tumor infiltrating CD8 T cells in IL-17R^{-/-} mice (n=5, **P<0.01). The majority of tumor infiltrating CD8 T cells are specific for OVA/MHC class-I tetramers. There is not a significance between wild type and IL-17R^{-/-} mice (n=4). The data show means ± SEM and are representative of 2 independent experiments.

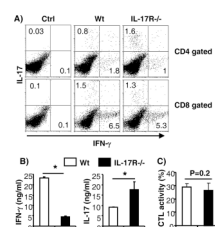


Figure 5. IL-17 mediated effects on the development of tumor specific T cells

T cells were purified from spleens of E.G7-OVA tumor bearing mice and stimulated with OVA-pulsed BM-DC for 4 days. **A).** The cells were stained with anti-CD4, CD8, IL-17 and IFN- γ antibodies. CD4 and CD8 T cells were gated and the percent of IL-17 or IFN- γ producing cells was analyzed. **B).** Concentrations of cytokines in the culture supernatants were measured by ELISA (n=4, * P<0.05). **C).** CD8 T cells were purified from spleens of E.G7-OVA tumor bearing mice and stimulated with OVA-pulsed BM-DC for 5 days. The CTL activity of cultured CD8 T cells was assessed as described in the text (n=4). The data show means $\pm$ SEM and are representative of 2–3 independent experiments.

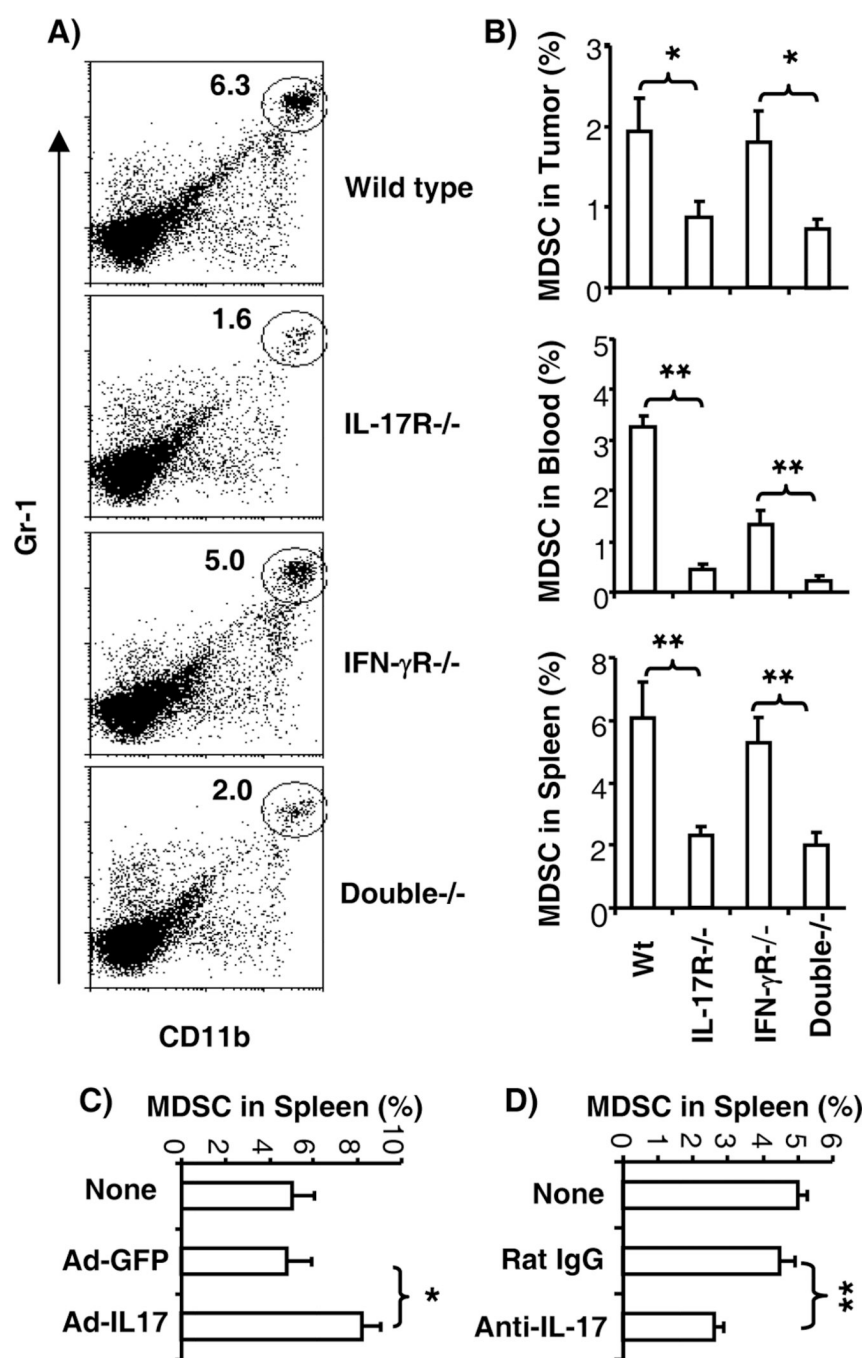


Figure 6. IL-17 is required for the development of myeloid derived suppressor cells (MDSC) in tumor bearing mice

A). spleen cells from E.G7-OVA tumor bearing mice were stained with anti-CD11b and Gr-1 antibodies. MDSC were identified as CD11b/Gr-1 double positive cells (circled) and analyzed. **B).** MDSC in the spleen, blood and tumor samples of E.G7-OVA tumor bearing mice (n=8). **C).** Wild type mice were inoculated with E.G7-OVA tumor cells and treated intraperitoneally with normal rat-IgG or rat anti-mouse IL-17 antibodies as described in Fig. 2b. MDSC from spleens of tumor bearing mice was analyzed (n=5). **D).** Wild type mice were treated intravenously with Ad-IL-17, Ad-GFP or left untreated (None) and then inoculated with E.G7-OVA tumor cells as described in Fig. 2a. MDSC from spleens of

tumor bearing mice was analyzed (n=5). The data show means $\pm$ SEM and are representative of 2–3 independent experiments. * $P < 0.05$; ** $P < 0.01$.

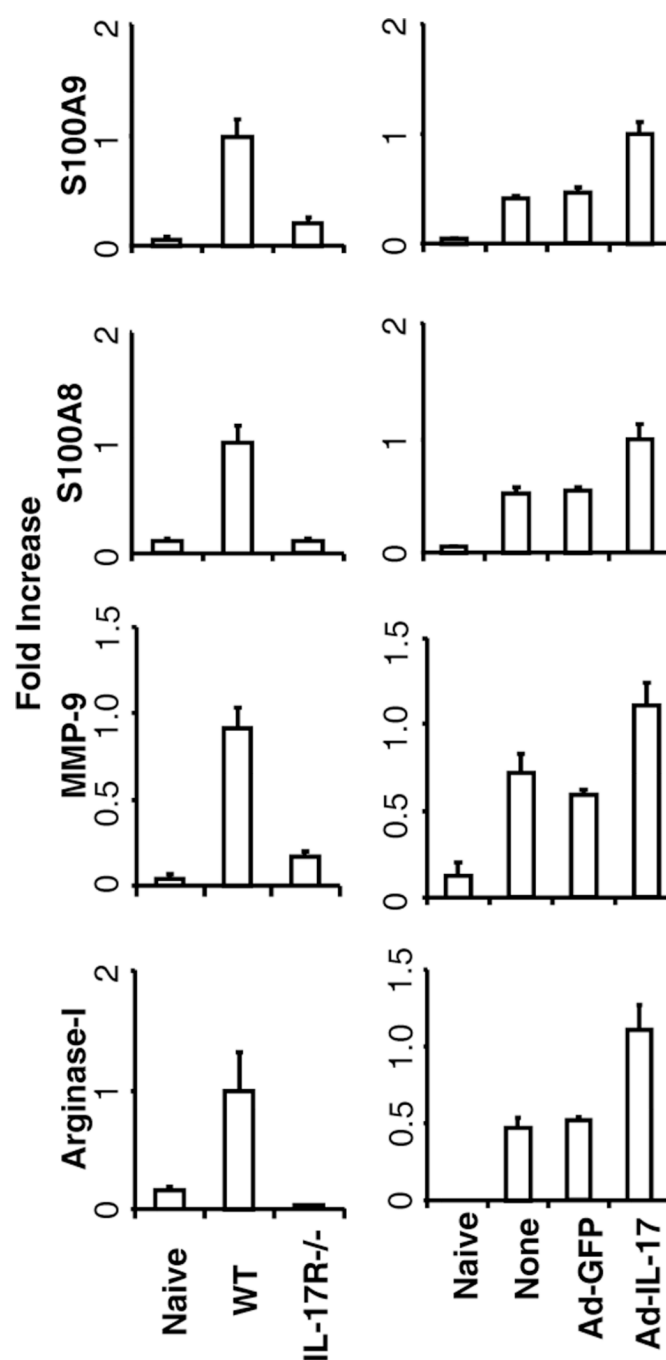


Figure 7. IL-17 regulates the phenotype of MDSC from tumor bearing mice

MDSC were purified from spleens of E.G7-OVA tumor bearing mice and stimulated overnight with LPS. CD11b⁺ cells from naïve tumor free mice were treated with LPS and served as controls. mRNA levels of the indicated molecules were determined by real time RT-PCR and normalized to the house-keeping gene GAPDH in each sample (n=5).

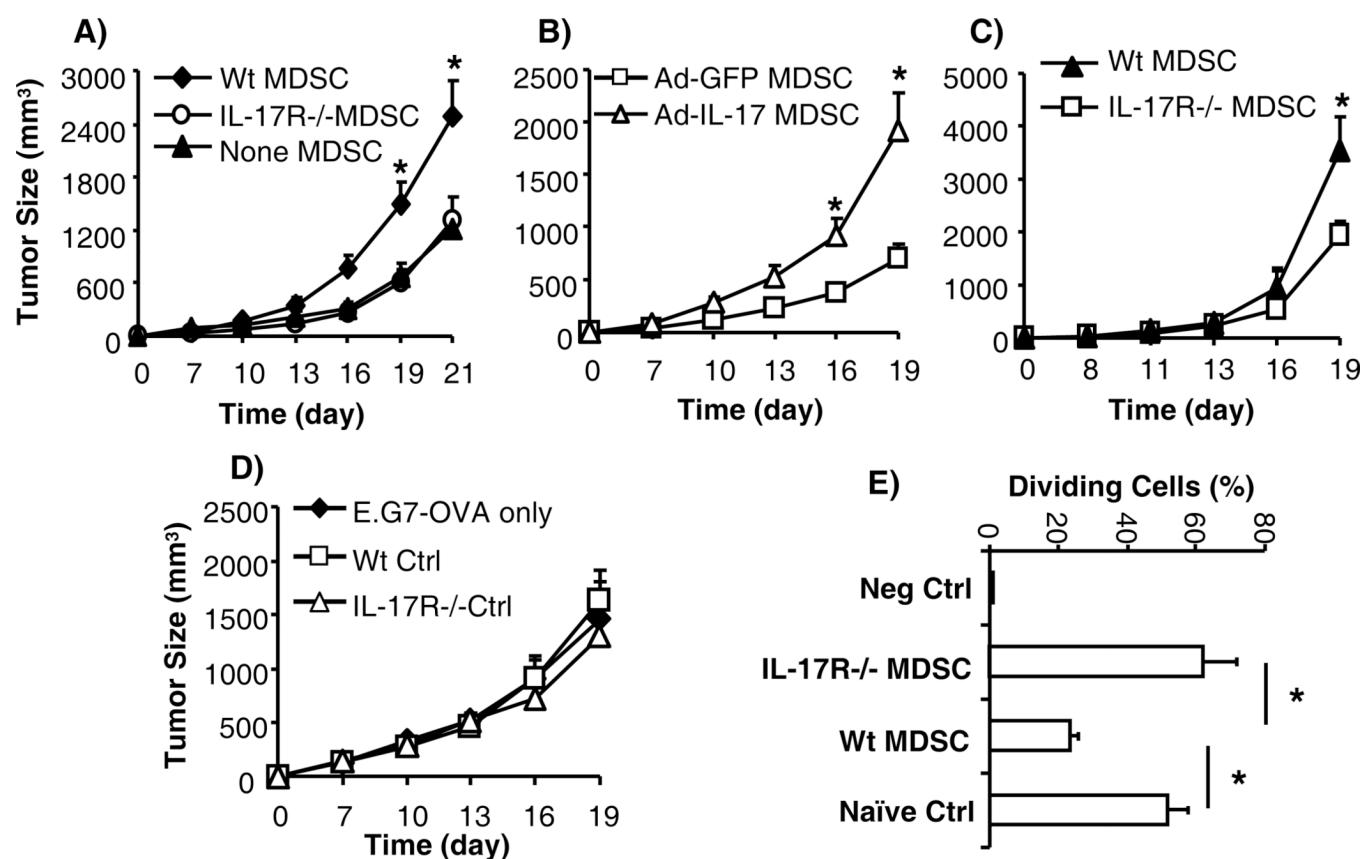


Figure 8. IL-17 regulates the tumor promoting and immunosuppressive activity of MDSC from tumor bearing mice

A). MDSC were purified from spleens of E.G7-OVA tumor bearing mice and co-injected subcutaneously with E.G7-OVA tumor cells in wild type mice. Mice that were injected with tumor cells only served as controls (None MDSC) (n=5). **B).** Wild type mice were treated intravenously with Ad-IL-17 or Ad-GFP and then inoculated with E.G7-OVA tumor cells as described in Fig. 2a. MDSC were purified from spleens of tumor bearing mice and co-injected with E.G7-OVA tumor cells in wild type mice as described above (n=5). **C).** MDSC were purified from spleens of B16 tumor bearing mice and co-injected subcutaneously with B16 tumor cells in wild type mice (n=5). **D).** CD11b⁺ cells were purified from spleens of naïve tumor free wild type or IL-17R^{-/-} mice and co-injected with E.G7-OVA tumor cells in wild type mice as described above. Mice which were injected only with tumor cells served as controls (n=5). **E).** CD4 T cells were purified from naïve wild type mice, labeled with a fluorescence dye CFSE. The T cells were placed in cultures with purified MDSC from wild type or IL-17R^{-/-} tumor bearing mice or CD11b⁺ cells from tumor free wild type mice (Naïve Ctrl). The cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 4 days. T cells which were not stimulated with the antibodies served as controls (Neg Ctrl). The percent of dividing CD4 T cells was analyzed by flow cytometry (n=4). The data show means $\pm$ SEM and are representative of 2–3 independent experiments. * P<0.05.