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Interleukin-17D Mediates Tumor Rejection through Recruitment of Natural Killer Cells

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SUMMARY

The process of cancer immunoediting generates a repertoire of cancer cells that can persist in immune-competent hosts. In its most complex form, this process begins with the elimination of highly immunogenic unedited tumor cells followed by the escape of less immunogenic edited cells. Although edited tumors can release immunosuppressive factors, it is unknown whether unedited tumors produce cytokines that enhance antitumor function. Utilizing gene microarray analysis, we found the cytokine interleukin 17D (IL-17D) was highly expressed in certain unedited tumors but not in edited mouse tumor cell lines. Moreover, forced expression of IL-17D in edited tumor cells induced rejection by stimulating MCP-1 production from tumor endothelial cells, leading to the recruitment of natural killer (NK) cells. NK cells promoted M1 macrophage development and adaptive immune responses. IL-17D expression was also decreased in certain high-grade and metastatic human tumors, suggesting that it can be targeted for tumor immune therapy.

INTRODUCTION

The cancer immunoediting process involves the initial elimination of highly immunogenic tumor cells from an "unedited" heterogeneous cell repertoire, followed by the eventual escape of poorly immunogenic, "edited" cells (Schreiber et al., 2011; Shankaran et al., 2001). Edited cell lines, which are derived from tumors that develop in wild-type (WT) mice, are termed "progressors" because they are poorly immunogenic and grow progressively when transplanted into syngeneic naive WT mice. Unedited cell lines, which are derived from immune-deficient mice, are often highly immunogenic and are termed "regressors" because they are rejected when transplanted into syngeneic naive WT mice. Immune cells can infiltrate, recognize, become activated, and eliminate regressor, but not progressor, tumor cells (Bui et al., 2006; Flood et al., 1987; Shankaran et al., 2001).

Edited tumors possess antigens (Boon and van der Bruggen, 1996; DuPage et al., 2012) that can concomitantly immunize the host (Vaage, 1971), but the adaptive immune response to edited tumors ultimately fails, leading to cancer progression and death (Schreiber et al., 2011). The failure of the adaptive immune response to control antigenic tumors can involve multiple mechanisms that are intrinsic to the tumor cell, including antigen loss and acquisition of inhibitory ligands, or tumor-cell-extrinsic effects, including immune suppressive cytokines and antigen tolerance (Schreiber et al., 2011; Zitvogel et al., 2006; Zou and Chen, 2008). It is not known to what extent tumor-extrinsic effects or intrinsic escape mechanisms contribute to cancer progression. Nevertheless, it is clear that progressor tumors express cytokines such as transforming growth factor β (TGF-β) that can inhibit antitumor immune responses (Bierie and Moses, 2010). In contrast, it has not been shown whether regressor cells can produce cytokines that serve to activate antitumor immunity. Importantly, cytokine-based immune therapy is a mainstay for treatment of human cancers such as melanoma and renal cell carcinoma (Nicholas and Lesinski, 2011; Rosenblatt and McDermott, 2011). In these diseases, treatment with interleukin-2 (IL-2) and interferon α (IFNα) is associated with severe toxic effects that limit therapeutic efficacy (Garbe et al., 2011; Hutson, 2011). Thus, discovering novel, safe, nontoxic cytokines that can mediate tumor rejection would have a high impact on tumor immune therapy.

The IL-17 family of cytokines is one of the most ancient cytokine families (Paul, 2013) and includes six members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F) identified by homology that possess a putative cysteine-knot structure (Iwakura et al., 2011; Kolls and Lindén, 2004). IL-17A and IL-17F are defining members of the family and are produced by Th-17 cells to mediate immunity against extracellular bacteria and fungi. Recently, IL-17C was shown to have similar activity as IL-17A/IL-17F, although it is expressed by infected epithelial cells and not by T cells (Ramirez-Carrozzi et al., 2011; Song et al., 2011). IL-17D is a cytokine whose function is not well described, although similar to IL-17C, it is known to be expressed outside the immune system and can stimulate human umbilical vein endothelial cells to produce interleukin-6, interleukin-8 (IL-8), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Starnes et al., 2002). It has also been found in rheumatoid

nodules (Müller and Lamprecht, 2008) and is decreased in psoriatic skin (Johansen et al., 2009). Interestingly, IL-17D is considered to be the most ancient cytokine in the IL-17D family (Paul, 2013; Roberts et al., 2008), although there have been no studies addressing the function of IL-17D in cancer or any other disease model system.

In this study, we sought to identify tumor-secreted molecules that can mediate tumor rejection. We found that IL-17D is expressed in some regressor, but not in progressor, cell lines. Importantly, IL-17D is sufficient to induce rejection or growth delay when overexpressed in some progressor cells. We show that the mechanism of action of IL-17D is to stimulate production of monocyte chemotactic protein-1 (MCP-1, aka CCL2), which recruits natural killer (NK) cells to the tumor and leads to M1 macrophage development and productive antitumor adaptive immune responses. These observations identify IL-17D as a cytokine that can promote immune responses via recruitment of NK cells.

RESULTS

IL-17D Is Highly Expressed by Certain Regressor, but Not Progressor, Tumors

To identify genes that could induce tumor rejection, we used a model system whereby edited progressors and unedited regressor methylcholanthrene (MCA)-induced sarcoma cell lines were derived from syngeneic WT and immune-deficient mice (Shankaran et al., 2001; O'Sullivan et al., 2012). We performed gene microarray studies on eight regressor and 16 progressor cell lines (Figure S1A). Among the many gene expression differences detected, we focused on the cytokine IL-17D due to its unknown function in tumor biology. We found that IL-17D was highly upregulated in some regressors but was not expressed in any progressor tumor cell line tested by microarray (Figure 1A), quantitative RT-PCR (qRT-PCR) (Figures 1B and S1B), and intracellular fluorescence-activated cell sorting (FACS) and verified with an independent set of regressors/progressors from another strain (Figures S1C and S1D) (O'Sullivan et al., 2012; Shankaran et al., 2001). Furthermore, treatment of regressor tumor cell lines with protein transport inhibitors doubled the amount of intracellular IL-17D signal (Figure 1C), confirming its secretion.

To assess the expression of *IL-17D* in human cancers, we utilized publicly available National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) data sets to examine *IL-17D* expression in multiple malignant human tissues. Interestingly, *IL-17D* gene expression was decreased in metastatic prostate tumors compared to primary prostate tumors (Figure 1D) and was also suppressed in more advanced, higher-stage gliomas (World Health Organization [WHO] grade III astrocytoma, grade IV glioblastoma multiforme [GBM]) relative to less advanced, lower-stage gliomas (WHO grade II oligodendroglioma) (Figure 1E, left panel). Additional studies confirmed that *IL-17D* expression was suppressed in grade IV GBM when compared to grade III astrocytomas (Figure 1E, right panel) and that high expression of *IL-17D* in tumor biopsy specimens correlated with a greater survival time for a subset of patients with grade IV GBM (Figure 1F).

IL-17D Promotes Progressor Tumor Rejection but Is Not Required for Regressor Tumor Rejection

We then explored whether manipulating IL-17D expression could influence tumor growth. IL-17D was silenced in regressor cell lines by 95%–100% and overexpressed in progressors at approximately 5-fold of control cells, a level similar to the expression in unmanipulated regressors (Figures S2A–S2D). Silencing of IL-17D in regressor tumors led to a slight growth increase and delayed rejection in one regressor tumor (d42m1) while having no measurable effect in another regressor tumor (d30m4) (Figure 2A). In four of the six progressor cell lines tested, the overexpression of IL-17D led to complete rejection (F244 and d30m1) or a significant delay in growth (B16.OVA and LLC) in WT mice (Figure 2B). This effect of IL-17D was due to adaptive immune cells, because in vitro and in vivo growth kinetics (in RAG2$^{-/-}$ mice) remained unchanged (Figures S2F and S2G).

To demonstrate the antitumor efficacy of IL-17D on pre-established tumors, we generated a progressor tumor cell line (F244TR17D) that expressed IL-17D upon administration of doxycycline (Figure S2E). Induced expression of IL-17D caused the rejection of 25 mm^2 tumors, but not 100 mm^2 tumors (Figure 2C), indicating IL-17D was most effective in inducing rejection of small tumors. We then tested whether intratumoral injections of recombinant IL-17D could mediate tumor regression of pre-established B16.OVA tumors transplanted into WT mice. Strikingly, intratumoral injections of recombinant IL-17D caused a significant growth delay compared to control-treated tumors, demonstrating the antitumor efficacy of IL-17D (Figure 2D).

IL-17D Expression Enhances Recruitment of NK Cells in Progressor and Regressor Tumors

To define the mechanism of IL-17D-mediated tumor rejection, we characterized tumor-infiltrating immune cells in tumors with high and low levels of IL-17D. We found an approximately 2-fold increase in the amount of NK cells in tumors with high versus low IL-17D (Figures 3A and 3B). These NK cells had similar phenotype to splenic NK cells and did not display markers found in immunoablative NK cells (Terme et al., 2012) or interferon-producing killer dendritic cells (Bonmort et al., 2008) (Figure S3). Notably, NK cells were required for tumor rejection, because mice treated with anti-NK1.1, but not control immunoglobulin G (IgG), failed to reject the IL-17D-overexpressing tumors (d30m1, F244) or showed increased growth (B16.OVA) (Figure 3C). The recruitment of NK cells likely mediates IL-17D's antitumor activity, as we did not observe enhanced numbers of either neutrophils or monocytes in tumors expressing high versus low levels of IL-17D and neutrophils were not required for IL-17D-mediated tumor rejection (data not shown).

Because it is known that NK-dependent tumor rejection can lead to priming of adaptive immune responses (Diefenbach et al., 2001; Kelly et al., 2002), we then tested whether mice that had rejected IL-17D-overexpressing tumors could reject a rechallenge with untransduced progressor tumors. Indeed, we found that parental cells were rejected in primed mice (Figure 3C), confirming that edited tumors possess antigens and that initiating the "correct" innate cell response (via IL-17D) can result in productive antigen-specific antitumor responses.

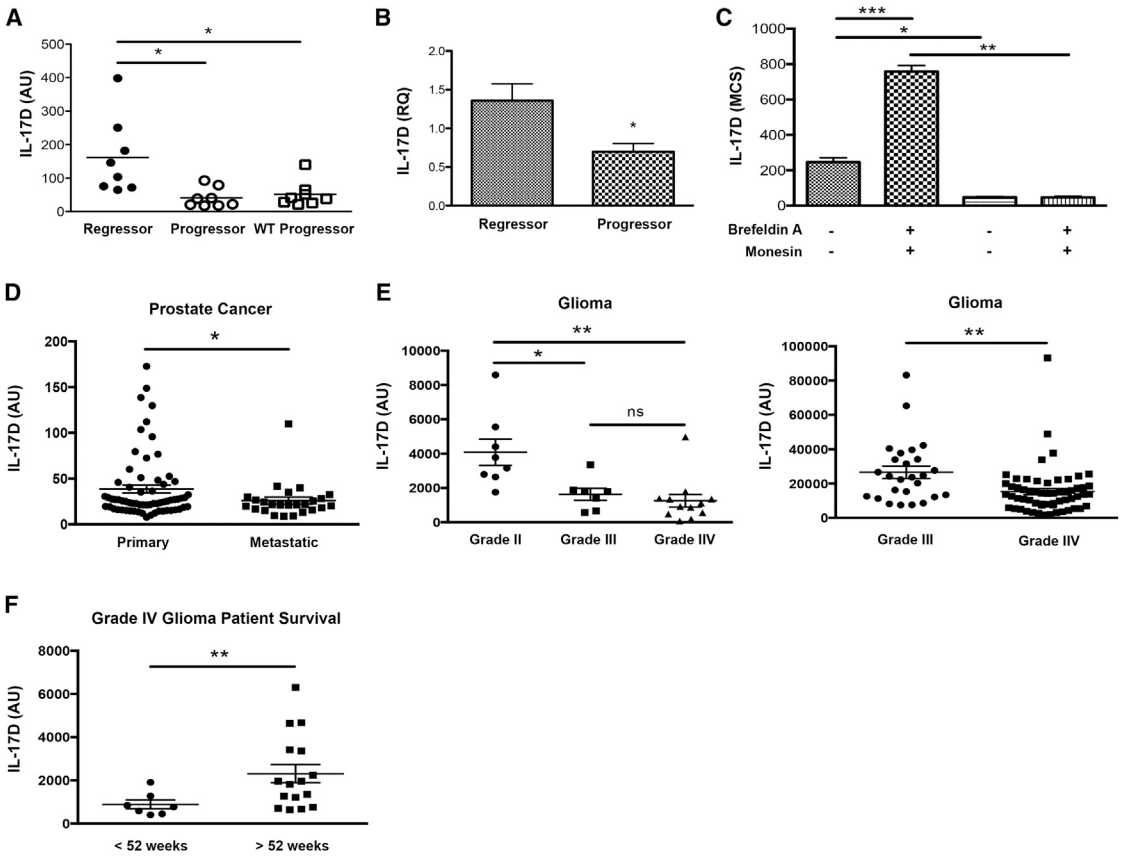

Figure 1. IL-17D Is Highly Expressed in Some Regressor Cell Lines and Is Downregulated in Progressor Tumor Cell Lines and Several Human Cancer Samples

(A) Plotted microarray data of *IL-17D* gene expression of regressor (n = 8) and progressor (n = 16) tumor cell lines.

(B) qRT-PCR analysis of independent regressor (n = 4) and progressor (n = 4) tumor cell lines.

(C) Quantitated IL-17D intracellular protein expression of 129/Sv RAG2$^{-/-}$-derived regressor (n = 3) and progressor (n = 3) tumor cell lines incubated with or without brefeldin A and monensin. IL-17D mean channel shift (MCS) values are calculated by taking the mean florescence of IL-17D intracellular protein signal and subtracting the mean fluorescence signal of the isotype control stain for the same tumor cell line sample.

(D and E) *IL17D* gene expression was evaluated from publicly available NCBI Gene Expression Omnibus data sets (GDS) from studies comparing indicated cancerous and metastatic tissue from human patients.

(F) GDS1816 samples from patients who had been diagnosed with WHO grade IV astrocytomas with necrosis were divided into low or high survival time categories, and *IL17D* gene expression was evaluated. Each point represents an individual patient sample.

Data from (B) and (C) are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's t test with Welch's correction. Error bars are depicted as ±SEM (*p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant).

See also Figure S1.

Previously, we have found a requirement for NK cells and interferon γ (IFNγ) in the accumulation of M1 macrophages in regressor tumors during cancer immunoediting (O'Sullivan et al., 2012). We also observed an approximately 1.5-fold enhancement in the accumulation of M1 macrophages in progressor tumors overexpressing IL-17D (Figure 3D), whereas silencing of IL-17D in regressor tumors reduced M1 macrophages by approximately 2-fold in both WT and RAG2$^{-/-}$, but not RAG2$^{-/-}$ × γc$^{-/-}$, hosts, which are deficient in NK cells (Figure 3D).

IL-17D Recruits Innate Immune Cells in an Air Pouch Model of Inflammation

To show directly whether IL-17D can induce the recruitment of immune cells, we used an in vivo air pouch model of inflammation in WT mice. Sterile air pouches become well vascularized after a period of 7 days (data not shown) and recruit immune cells rapidly after administration of lipopolysaccharide (LPS) (Pelletier et al., 2004). Indeed, we found that LPS, IL-17A, and IL-17D significantly recruited CD45^{+} immune cells into air pouches compared to PBS control (Figure 4A). When we examined the composition of the immune cells, we found that LPS and IL-17A recruited more neutrophils than any other cell type, whereas neutrophils constituted a smaller percentage of cells recruited by IL-17D (Figure 4B). Interestingly, IL-17D recruited significantly more NK cells (Figure 4C), but not monocytes (Figure 4C), neutrophils, or macrophages (Figure S4A), compared to LPS and IL-17A. We found that the IL-17D-recruited NK cells were mostly CD27^{high} (Figure S4B), which could be a semimature population

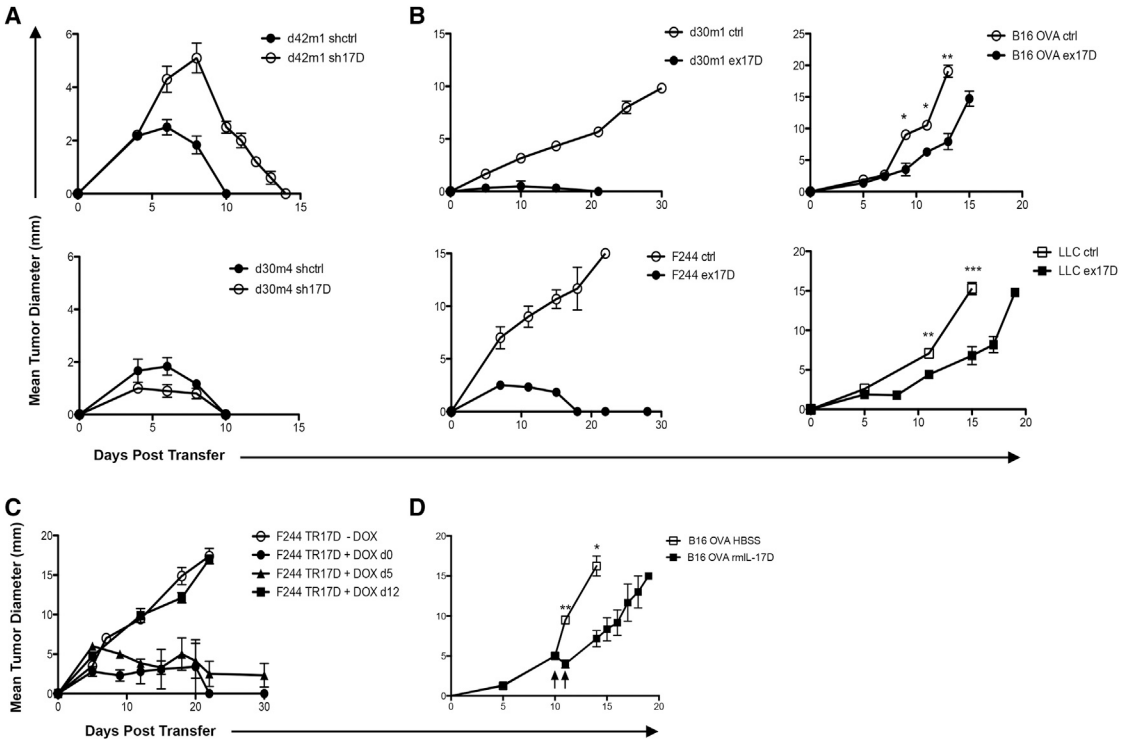

Figure 2. Expression of IL-17D Mediates Progressor Tumor Rejection

(A) Tumor growth of indicated (ctrl, sh17D) regressor tumors transplanted into WT mice (n = 5 for each tumor cell line).

(B) Tumor growth of indicated (ctrl, ex17D) progressor tumors transplanted into WT mice (n = 5 for each tumor cell line).

(C) Tumor growth of inducible IL-17D progressor tumor cell line transplanted into WT mice receiving water or doxycycline continuously from day 0 (n = 5), day 5 (n = 5), or day 12 (n = 5).

(D) Tumor growth of B16.OVA melanoma tumor cell line transplanted into WT mice and receiving intratumoral injections of IL-17D (2 μg) or Hank's balanced salt solution on days 10 and 11. Data from (A)–(D) are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's t test with Welch's correction. Error bars are depicted as ±SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S2.

of NK cells that may participate in IFNγ-dependent T cell priming in lymph nodes (Martín-Fontecha et al., 2004; Watt et al., 2008). Interestingly, IL-17D recruited approximately twice the amount of $CD27^{high}CD11b^{low}$ NK cells as LPS, with no significant recruitment of mature $CD27^{lo}$ NK cells (Figure 4D).

IL-17D Indirectly Recruits NK Cells In Vivo by Stimulating the Production of MCP-1

Because IL-17A is known to induce IL-8 from endothelial cells to recruit neutrophils (Roussel et al., 2010), we examined whether IL-17D utilized a similar mechanism. Indeed, we found that IL-17D induced the expression of MCP-1 in mouse air pouch lavage fluid (Figure 5A). We then repeated air pouch experiments in the presence of blocking antibodies specific for MCP-1 and found that anti-MCP1, but not control IgG, completely inhibited IL-17D-mediated recruitment of NK cells (Figure 5B), monocytes, and neutrophils (Figure S5A). Furthermore, qRT-PCR analysis of purified tumor endothelial cells from two IL-17D-overexpressing tumors (Figure S5B) showed a 4–17 times increase in MCP-1 transcript compared to control tumors, respectively (Figure 5C), while maintaining similar levels of VEGFR1 (Figure S5C). Notably, depletion of MCP-1 led to increased growth of two IL-17D-overexpressing tumors (Figure 5D). These results were likely due to reduced overall numbers of infiltrating NK cells, as MCP-1 depletion reduced the density of tumor-infiltrating NK cells compared to control depletion in tumors overexpressing IL-17D (Figure 5E).

DISCUSSION

The IL-17 family of cytokines promotes immune responses by inducing the expression of proinflammatory cytokines and chemokines, leading to recruitment of neutrophils and other innate immune cells (Pappu et al., 2011). IL-17A and IL-17F are produced by Th-17 cells and are involved in autoimmune disease and host responses to tissue infection. IL-17C may have similar inflammatory functions to IL-17A and IL-17F, although IL-17C is expressed in epithelial cells and is induced by microbial ligands. Our discovery that IL-17D is expressed outside the immune system and functions to recruit NK cells suggests that the IL-17 family may have evolved to evoke distinct arms of the immune response, presumably to deal with specific pathogen insults. We speculate that similar to IL-17C, the expression of IL-17D in nonimmune tissues may represent an early evolutionary adaptation to mediate local antiviral immunity through the recruitment

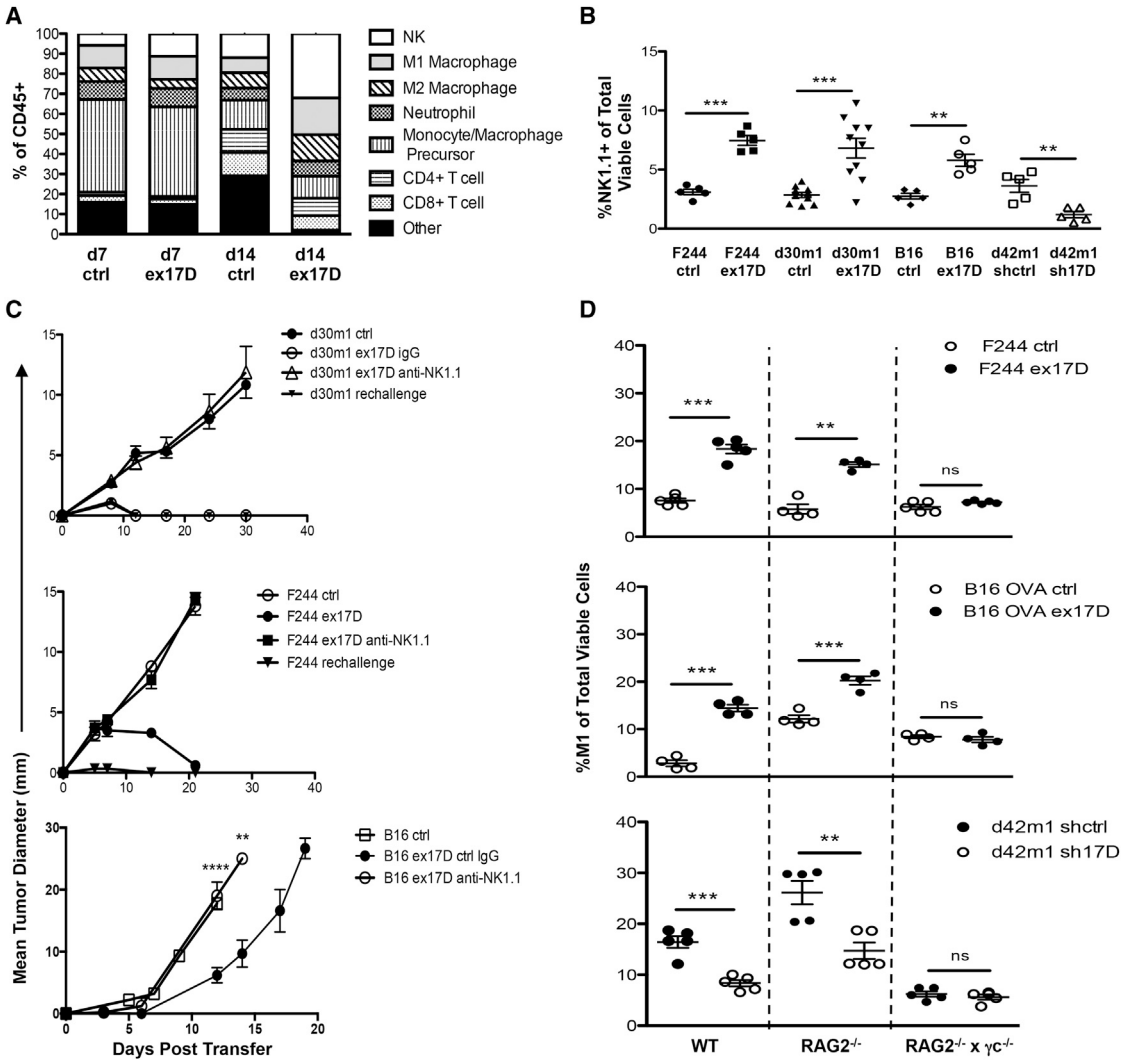

Figure 3. Overexpression of IL-17D in Progressor Tumors Recruits NK Cells that Are Required for Tumor Rejection in WT Mice and Promote M1 Macrophage Infiltration

(A) Percentage of (7AAD$^-$, CD45$^+$, CD3$^-$, NK1.1$^+$) NK cells, (7AAD$^-$, CD45$^+$, CD11b$^+$, Ly6G$^+$, MHCII^{lo}) neutrophils, (7AAD$^-$, CD45$^+$, CD11b$^+$, Ly6C^{hi}) monocytes/macrophage precursors, (7AAD$^-$, CD45$^+$, F4/80$^+$, Ly6C^{lo}, MHCII^{hi}, CD206^{lo}) M1 macrophages, (7AAD$^-$, CD45$^+$, F4/80$^+$, Ly6C^{lo}, MHCII^{lo}, CD206^{hi}) M2 macrophages, (7AAD$^-$, CD45$^+$, CD3$^+$, CD4$^+$, CD8$^-$) CD4$^+$ T cells, and (7AAD$^-$, CD45$^+$, CD3$^+$, CD4$^-$, CD8$^+$) CD8$^+$ T cell-infiltrating immune cells in F244 ctrl or ex17D tumors on days 7 and 14 posttransplantation in WT mice. ("Other" indicates infiltrating Ly6C$^-$MHCII$^-$NK1.1$^-$CD3$^-$ immune cells).

(B) Percent infiltrating NK cells of total viable (7AAD$^-$) cells from transduced regressor and progressor tumors on day 7 posttumor transplant in WT mice.

(C) Tumor growth of IL-17D overexpressing (ex17D) progressor tumors transplanted into WT mice receiving either intraperitoneal injections of anti-NK1.1/control IgG or preimmunized with transplantation of IL-17D overexpressing (ex17D) tumor cell lines.

(D) Percentage of M1 macrophages of total viable cells on day 14 posttumor transplant of progressor tumor cell lines into WT, RAG2$^{-/-}$, or RAG2$^{-/-}$ × γc$^{-/-}$ hosts.

Data from (A)–(D) are representative of two independent experiments. Samples were compared using an unpaired, two-tailed Student's t test with Welch's correction. Error bars are depicted as ±SEM (**p < 0.01, ***p < 0.001, ****p < 0.0001; NS, not significant). See also Figure S3.

of NK cells. Notably, our preliminary studies indeed have found increased IL-17D transcripts in virus-infected skin (R.S.K. and J.D.B., unpublished data). Future studies on the endogenous role of IL-17D in the context of infection, autoimmunity, and cancer and its regulation are certainly warranted.

Our studies have shown that IL-17D is poorly expressed in cancer cells that grow progressively (mouse MCA-induced sarcomas and certain human cancers) but, comparatively, can be more highly expressed in certain immunogenic MCA-induced sarcoma cells and in low-stage tumors. It is not clear what regulates the constitutive expression of IL-17D in certain cells, but it is clear that high expression of IL-17D may not be compatible with tumor progression, because advanced-stage human and edited mouse cancer cells have lower levels of IL-17D and the ectopic expression of IL-17D in progressor cells led to NK dependent tumor rejection. Overexpression of immune-cell-derived

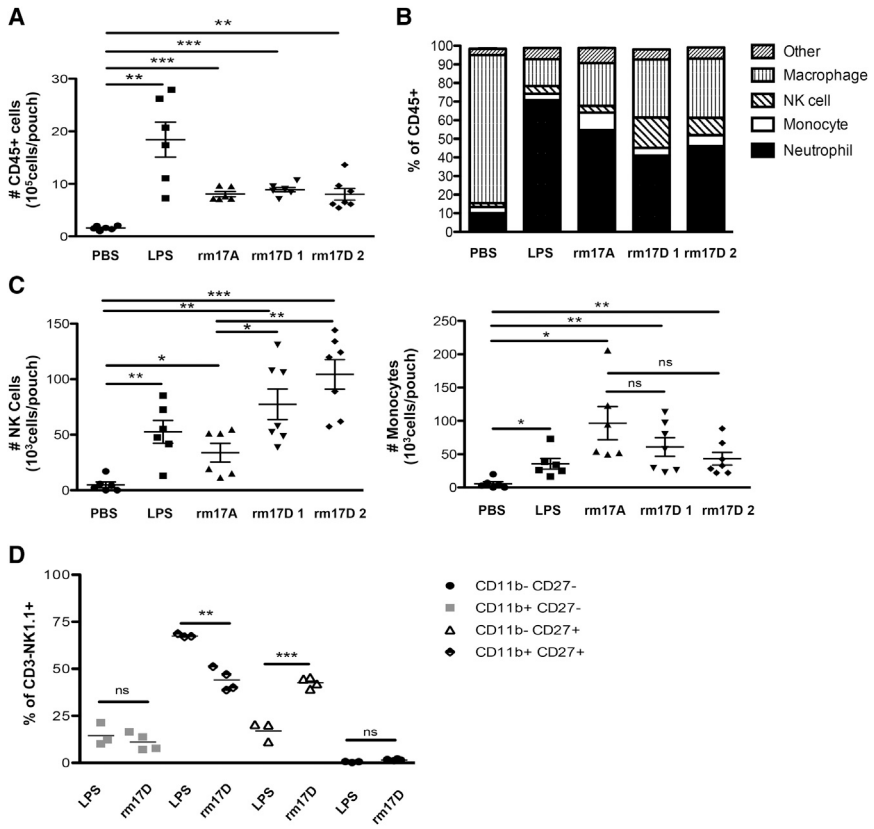

Figure 4. Recombinant Mouse IL-17D Recruits NK Cells in an Air Pouch Inflammation Model

(A) Total number of infiltrating immune cells per air pouch in WT mice receiving intrapouch injections of PBS, LPS, IL-17A, IL-17D-1 (generated from *E. coli*.), or IL17D-2 (generated from *C. reinhardtii*).

(B) Percentages of NK cells, monocytes, neutrophils, and macrophages per air pouch receiving indicated intrapouch injections. (Other indicates $CD4^+$, $CD8^+$ T cells or $Ly6C^-MHCII^-NK1.1^-CD3^-$ recruited immune cells). Cell populations are defined as in Figure 3A.

(C) Total number of NK cells and monocytes per air pouch receiving indicated intrapouch injections.

(D) Immunophenotypic analysis of infiltrating $NK1.1^+CD3^-$ NK cells in mouse air pouches receiving intrapouch injections of LPS or rmIL-17D. Data from (A)–(D) are representative of two independent experiments. Each point represents an individual mouse. Samples were compared using an unpaired, two-tailed Student's t test with Welch's correction. Error bars are depicted as ±SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). See also Figure S4.

chemokines and cytokines such as GM-CSF (Dranoff, 2004; Dranoff et al., 1993) and IL-15 (Liu et al., 2012) have already been demonstrated to have potent antitumor efficacy. Our findings are unique in that IL-17D is tumor expressed (rather than immune cell derived) and thus likely represents an endogenous tumor surveillance activity.

It should be noted that not all regressors have high levels of IL-17D (Figure 1) and that there are multiple genes that are differentially expressed in regressor tumors (Figure S1), thus indicating that IL-17D is one of many genes that could participate in tumor surveillance. This is likely due to the heterogeneity and redundancy that is inherent in our system (and possibly in normal tumor surveillance mechanisms). For example, we have found that some regressors are well recognized by NK cells whereas others are not, and this is not always correlated with NKG2D ligand expression (O'Sullivan et al., 2011), even though NK cells and NKG2D are important for tumor surveillance (Guerra et al., 2008; Smyth et al., 2005). Furthermore, some regressors require type I interferon for their rejection whereas others do not (Dunn et al., 2005), even though $IFNAR^{-/-}$ mice lacking interferon α/β (IFNα/β) responsiveness are more susceptible to cancer (Diamond et al., 2011; Dunn et al., 2005; Fuertes et al., 2011), and IFNα/β is used in the treatment of melanoma (Garbe et al., 2011). We therefore conclude that IL-17D is one of many genes that regressor cells produce that can stimulate antitumor immunity. The identification of other genes that can differentiate regressor from progressor cell lines will involve future studies likely combining proteomic, gene expression, and exome sequencing approaches (Matsushita et al., 2012).

NK cells are known to be integral mediators of tumor surveillance (Bui and Schreiber, 2007; Smyth et al., 2001), but little is known about how they are recruited to sites of stress or transformation. Interestingly, in a model of liver carcinoma, the process of senescence induced MCP-1 and increased NK cell infiltration, leading to tumor suppression (Xue et al., 2007; Iannello et al., 2013), but IL-17D was not measured in this study. A recent study showed that the novel chemokine chemerin can recruit NK cells to mediate tumor surveillance (Pachynski et al., 2012), but it remains unclear what induces chemerin during inflammation. The chemokine receptor CXCR3 is expressed on NK cells (Uppaluri et al., 2008) and it ligands ITAC, MIG, and IP-10 can be induced by interferons during tumor development, but this receptor-ligand axis is not involved in the surveillance of MCA-induced sarcomas (Winkler et al., 2011). On the other hand, CXCR3 is thought to be the receptor that mediates the recruitment of cytokine-secreting $CD27^{high}$ NK cells into lymph nodes (Martín-Fontecha et al., 2004; Watt et al., 2008), and it may be possible that IL-17D can also induce CXCR3 ligands, either directly or indirectly via NK cell production of IFNγ.

Other studies of IL-17 family members in tumor progression have focused on IL-17A and Th-17 cells. These studies have shown both tumor-promoting and tumor-inhibiting roles for IL-17A/Th-17 cells. For instance, transfection of IL-17A can augment the progression of human tumor cell lines transplanted in nude mice by increasing neovascularization (Numasaki et al., 2003, 2005; Tartour et al., 1999), whereas in a mouse syngeneic system, IL-17A promotes tumor rejection by boosting T cell responses (Benchetrit et al., 2002; Hirahara et al., 2001). Th17 cells have also been associated with tumor rejection and good

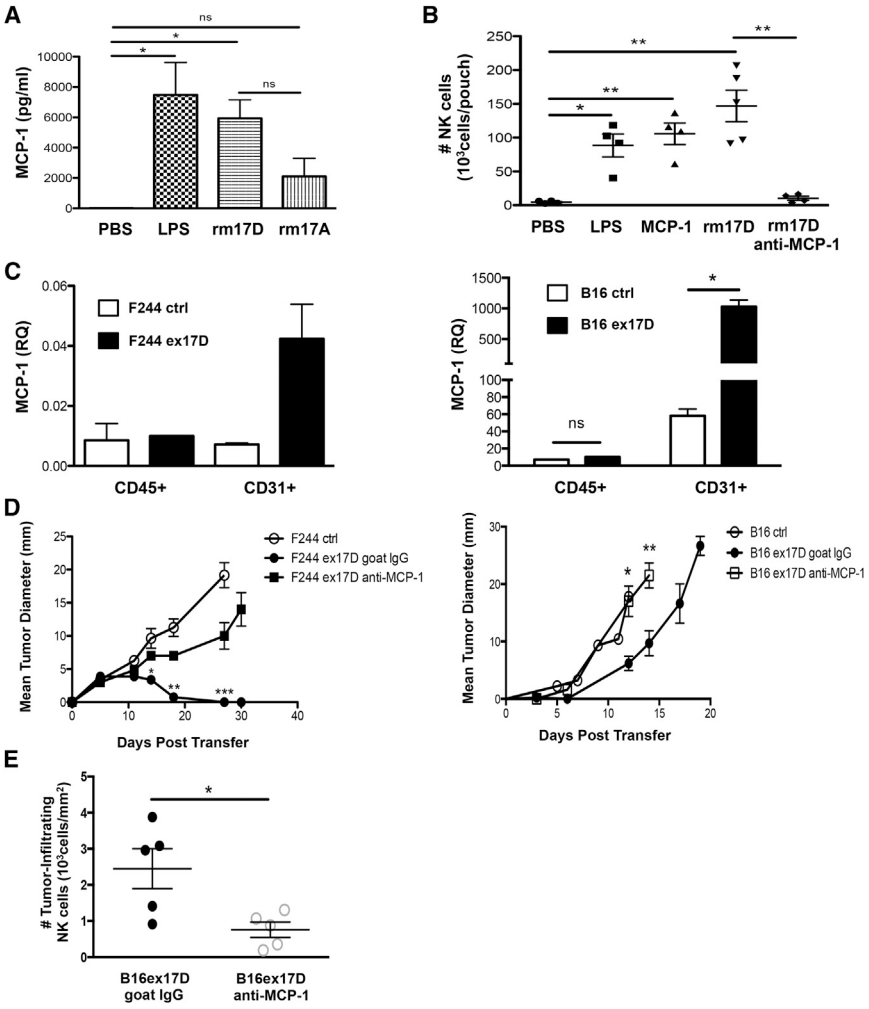

Figure 5. IL-17D Indirectly Recruits NK Cells through Tumor Endothelial Cell Production of MCP-1

(A) Air pouch lavage fluid chemokine levels of MCP-1.

(B) Total number of NK cells per air pouch for WT mice receiving intrapouch injections of PBS, LPS, IL-17A, IL-17D, MCP-1, or IL-17D and anti-MCP-1 monoclonal antibodies.

(C) qRT-PCR analysis of MCP-1 expression from purified tumor leukocytes and endothelial cells harvested from day 7 F244 or B16.OVA control or ex17D tumors.

(D) Tumor growth of F244 or B16 OVA control and ex17D tumors transplanted into WT mice receiving either intraperitoneal (i.p.) injections of goat polyclonal anti-MCP-1 or control goat IgG.

(E) Number of tumor-infiltrating NK cells per square mm of tumor from day 7 B16 OVA ex17D tumors transplanted into WT mice receiving either i.p. injections of goat polyclonal anti-MCP-1 or control goat IgG.

Data are representative of two independent experiments. Each point represents a single mouse. Samples were compared using an unpaired, two-tailed Student's t test with Welch's correction. Error bars are depicted as ±SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; NS, not significant). See also Figure S5.

prognosis in some studies (Kryczek et al., 2007; Muranski et al., 2008), whereas other studies indicate that Th17 cells promote tumor growth (Xiao et al., 2009; Zhang et al., 2008). One potential explanation for these conflicting results is that IL-17A can activate and recruit neutrophils, which recently have been shown to have both tumor-promoting and tumor-inhibiting activities (Fridlender et al., 2009). In contrast, it is well established that NK cells have antitumor activities (Bui and Schreiber, 2007; Smyth et al., 2001) and in fact can promote antitumor T cell (Diefenbach et al., 2001; Kelly et al., 2002) (Figure 2C) and macrophage responses (O'Sullivan et al., 2012) (Figure 3D). Therefore, unlike IL-17A, IL-17D may induce more consistent antitumor responses through NK cell recruitment that could be more effectively translated to cancer immune therapy. On the other hand, because enforced IL-17D expression induced rejection of some, but not all, progressor cell lines, it is likely that IL-17D-based therapy, acting through NK cells, will need to be used in combination with checkpoint blockade or inhibitors of T regulatory cells, which can prevent NK cell activation (Ghiringhelli et al., 2005; Smyth et al., 2006). It is not clear what the total effect enforced IL-17D expression would have on adaptive immunity, but nevertheless, adaptive immunity is required (Figure S2) and induced (Figure 3C) in IL-17D-mediated rejection. Finally, we speculate that the selective expression of IL-17D in neoplastic cells as opposed to immune cells would translate to a more benign side effect profile, because this ancient cytokine may have evolved to mediate early, and clinically silent, innate tissue surveillance of stress, transformation, and/or pathogen infection.

EXPERIMENTAL PROCEDURES

All experiments involving mice were conducted under animal protocols approved by the Washington University Animal Studies Committee and the University of California, San Diego Institutional Animal Care and Use Committee (IACUC protocol #S06201) and were in accordance with their ethical guidelines.

Cell Lines and Mice

MCA sarcoma cell lines are a kind gift from Dr. Robert Schreiber and were generated as described previously (Shankaran et al., 2001). All experiments were done with cells passaged between 4 and 12 cycles. 129/Sv, C57BL/6 × 129/Sv F1, 129/Sv RAG2$^{-/-}$, C57BL/6 RAG2$^{-/-}$, and RAG2$^{-/-}$ × γc$^{-/-}$ mice used were used for tumor transplantation experiments. Cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, nonessential amino acids, sodium pyruvate, sodium bicarbonate, penicillin/streptomycin, and β-mercaptoethanol.

Microarray and Clustering Analysis

Murine Genome U74v2 Set GeneChip Array (Affymetrix) was used for analysis of cDNA generated from regressor and progressor tumor cell lines. Details of RNA preparation, cDNA preparation, microarray setup, and clustering analysis are described in the Supplemental Experimental Procedures.

Human Cancer Microarray Data Analysis

For human clinical samples, *IL-17D* gene expression was evaluated from NCBI GEO data sets from studies comparing primary and metastatic tumors (GDS2546) or low-grade versus high-grade glioma patient samples (GDS4467, GDS1976, and GDS1816) as described previously (Pachynski et al., 2012).

Generation of IL-17D-Deficient and Overexpressing Tumor Cell Lines

Cell lines were generated as described in the Supplemental Experimental Procedures.

Antibodies and FACS Analysis of Tumor Cells

For intracellular staining, cells were either incubated with or without 2 μM monensin (Sigma) and 1 μg/ml Brefeldin A (BD Biosciences) and then harvested by trypsinization, washed once with PBS, stained, and analyzed for intracellular IL-17D signal as described in the Supplemental Experimental Procedures.

Tumor Transplantation and TIL Analysis

Subconfluent tumor cell lines were harvested and injected subcutaneously into syngeneic recipient WT, RAG2$^{-/-}$, or RAG2$^{-/-}$ × γc$^{-/-}$ mice at either 1 × 10^6 cells/mouse (for all growth experiments) or 5–10 × 10^6 cells/mouse (for tumor-infiltrating leukocyte [TIL] analysis), as previously described (Bui et al., 2006). Tumor rechallenge was performed 3 months after mice had rejected transplanted tumors by injecting 1 × 10^6 cells per mouse subcutaneously with parental tumor cell lines. In vivo depletion of various immune subsets, doxycycline administration, and intratumoral injection of IL-17D are described in the Supplemental Experimental Procedures. Tumor growth and immune infiltration were analyzed as described in the Supplemental Experimental Procedures.

Mouse Air Pouch Experiments

C57BL/6 × 129/Sv F1 mice were injected subcutaneously with 3 ml of sterilized air filtered through a 0.2 μm Millipore filter (Bellerica) to form air pouches on day 0 and reinflated again on day 3. On day 7, 1 ml of LPS (1 μg/ml), IL-17A (5 μg/ml) (R&D Systems), IL-17D (5 μg/ml) (R&D Systems), IL-17D (5 μg/ml) (Mayfield Lab), MCP-1 (5 μg/ml) (Peprotech), or IL-17D (5 μg/ml) + anti-MCP-1 polyclonal antibodies (25 μg/ml) (R&D Systems) was injected into mouse air pouches 8 hr before air pouch harvest. Air pouches were lavaged with 2 ml PBS and centrifuged at 1,250 rpm for 5 min at room temperature. Supernatant was harvested and analyzed for chemokine protein levels using the mouse Chemokine FlowCytomix kit from eBioscience. Infiltrating air pouch cells were resuspended in FACS stain buffer, counted on a hemocytometer, and analyzed by cell-surface markers as described in the Supplemental Experimental Procedures.

Chemokine Secretion Assay

On days 7 and 14 posttransplantation, tumors were harvested and single-cell suspensions were prepared as described for the TIL analysis. Filtered tumor/immune cell suspensions were plated in triplicate wells at 40,000 cells per well in 100 μl for 24 hr at 37°C. Supernatant was analyzed for chemokines using the mouse chemokine flowcytomix kit from eBioscience.

Generation of cDNA and Quantitative PCR

Tumor cell lines were plated in triplicate at 6 × 10^4 cells/well in a six-well plate and incubated for 48 hr at 37°C. Supernatant was aspirated and cells were washed twice with PBS before addition of 1 ml TRIzol reagent (Invitrogen). CD31$^+$ and CD45$^+$ tumor-derived cell populations were washed twice with PBS before addition of 1 ml TRIzol reagent (Invitrogen). Details describing RNA extraction, cDNA preparation, quantitative PCR reactions, and analysis are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.073.

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REFERENCES

Benchetrit, F., Ciree, A., Vives, V., Warnier, G., Gey, A., Sautès-Fridman, C., Fossiez, F., Haicheur, N., Fridman, W.H., and Tartour, E. (2002). Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. Blood *99*, 2114–2121.

Bierie, B., and Moses, H.L. (2010). Transforming growth factor beta (TGF-beta) and inflammation in cancer. Cytokine Growth Factor Rev. *21*, 49–59.

Bonmort, M., Dalod, M., Mignot, G., Ullrich, E., Chaput, N., and Zitvogel, L. (2008). Killer dendritic cells: IKDC and the others. Curr. Opin. Immunol. *20*, 558–565.

Boon, T., and van der Bruggen, P. (1996). Human tumor antigens recognized by T lymphocytes. J. Exp. Med. *183*, 725–729.

Bui, J.D., and Schreiber, R.D. (2007). Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? Curr. Opin. Immunol. *19*, 203–208.

Bui, J.D., Uppaluri, R., Hsieh, C.S., and Schreiber, R.D. (2006). Comparative analysis of regulatory and effector T cells in progressively growing versus rejecting tumors of similar origins. Cancer Res. *66*, 7301–7309.

Diamond, M.S., Kinder, M., Matsushita, H., Mashayekhi, M., Dunn, G.P., Archambault, J.M., Lee, H., Arthur, C.D., White, J.M., Kalinke, U., et al. (2011). Type I interferon is selectively required by dendritic cells for immune rejection of tumors. J. Exp. Med. *208*, 1989–2003.

Diefenbach, A., Jensen, E.R., Jamieson, A.M., and Raulet, D.H. (2001). Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature *413*, 165–171.

Dranoff, G. (2004). Cytokines in cancer pathogenesis and cancer therapy. Nat. Rev. Cancer *4*, 11–22.

Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R.C. (1993). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc. Natl. Acad. Sci. USA *90*, 3539–3543.

Dunn, G.P., Bruce, A.T., Sheehan, K.C., Shankaran, V., Uppaluri, R., Bui, J.D., Diamond, M.S., Koebel, C.M., Arthur, C., White, J.M., and Schreiber, R.D. (2005). A critical function for type I interferons in cancer immunoediting. Nat. Immunol. *6*, 722–729.

DuPage, M., Mazumdar, C., Schmidt, L.M., Cheung, A.F., and Jacks, T. (2012). Expression of tumour-specific antigens underlies cancer immunoediting. Nature *482*, 405–409.

Flood, P.M., Schreiber, H., and Ron, Y. (1987). Protective immunity to progressive tumors can be induced by antigen presented on regressor tumors. J. Immunol. *138*, 3573–3579.

Fridlender, Z.G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., Worthen, G.S., and Albelda, S.M. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell *16*, 183–194.

Fuertes, M.B., Kacha, A.K., Kline, J., Woo, S.R., Kranz, D.M., Murphy, K.M., and Gajewski, T.F. (2011). Host type I IFN signals are required for antitumor

CD8+ T cell responses through CD8alpha+ dendritic cells. J. Exp. Med. *208*, 2005–2016.

Garbe, C., Eigentler, T.K., Keilholz, U., Hauschild, A., and Kirkwood, J.M. (2011). Systematic review of medical treatment in melanoma: current status and future prospects. Oncologist *16*, 5–24.

Ghiringhelli, F., Ménard, C., Terme, M., Flament, C., Taieb, J., Chaput, N., Puig, P.E., Novault, S., Escudier, B., Vivier, E., et al. (2005). CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J. Exp. Med. *202*, 1075–1085.

Guerra, N., Tan, Y.X., Joncker, N.T., Choy, A., Gallardo, F., Xiong, N., Knoblaugh, S., Cado, D., Greenberg, N.M., and Raulet, D.H. (2008). NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity *28*, 571–580.

Hirahara, N., Nio, Y., Sasaki, S., Minari, Y., Takamura, M., Iguchi, C., Dong, M., Yamasawa, K., and Tamura, K. (2001). Inoculation of human interleukin-17 gene-transfected Meth-A fibrosarcoma cells induces T cell-dependent tumor-specific immunity in mice. Oncology *61*, 79–89.

Hutson, T.E. (2011). Targeted therapies for the treatment of metastatic renal cell carcinoma: clinical evidence. Oncologist *16* (*Suppl 2*), 14–22.

Iannello, A., Thompson, T.W., Ardolino, M., Lowe, S.W., and Raulet, D.H. (2013). p53-dependent chemokine production by senescent tumor cells supports NKG2D-dependent tumor elimination by natural killer cells. J. Exp. Med. *210*, 2057–2069.

Iwakura, Y., Ishigame, H., Saijo, S., and Nakae, S. (2011). Functional specialization of interleukin-17 family members. Immunity *34*, 149–162.

Johansen, C., Usher, P.A., Kjellerup, R.B., Lundsgaard, D., Iversen, L., and Kragballe, K. (2009). Characterization of the interleukin-17 isoforms and receptors in lesional psoriatic skin. Br. J. Dermatol. *160*, 319–324.

Kelly, J.M., Darcy, P.K., Markby, J.L., Godfrey, D.I., Takeda, K., Yagita, H., and Smyth, M.J. (2002). Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. Nat. Immunol. *3*, 83–90.

Kolls, J.K., and Lindén, A. (2004). Interleukin-17 family members and inflammation. Immunity *21*, 467–476.

Kryczek, I., Wei, S., Zou, L., Altuwaijri, S., Szeliga, W., Kolls, J., Chang, A., and Zou, W. (2007). Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. J. Immunol. *178*, 6730–6733.

Liu, R.B., Engels, B., Arina, A., Schreiber, K., Hyjek, E., Schietinger, A., Binder, D.C., Butz, E., Krausz, T., Rowley, D.A., et al. (2012). Densely granulated murine NK cells eradicate large solid tumors. Cancer Res. *72*, 1964–1974.

Martín-Fontecha, A., Thomsen, L.L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., and Sallusto, F. (2004). Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. Nat. Immunol. *5*, 1260–1265.

Matsushita, H., Vesely, M.D., Koboldt, D.C., Rickert, C.G., Uppaluri, R., Magrini, V.J., Arthur, C.D., White, J.M., Chen, Y.S., Shea, L.K., et al. (2012). Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature *482*, 400–404.

Müller, A., and Lamprecht, P. (2008). [Interleukin-17 in chronic inflammatory and autoimmune diseases: rheumatoid arthritis, Crohn's disease and Wegener's granulomatosis]. Z. Rheumatol. *67*, 72–74.

Muranski, P., Boni, A., Antony, P.A., Cassard, L., Irvine, K.R., Kaiser, A., Paulos, C.M., Palmer, D.C., Touloukian, C.E., Ptak, K., et al. (2008). Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood *112*, 362–373.

Nicholas, C., and Lesinski, G.B. (2011). Immunomodulatory cytokines as therapeutic agents for melanoma. Immunotherapy *3*, 673–690.

Numasaki, M., Fukushi, J., Ono, M., Narula, S.K., Zavodny, P.J., Kudo, T., Robbins, P.D., Tahara, H., and Lotze, M.T. (2003). Interleukin-17 promotes angiogenesis and tumor growth. Blood *101*, 2620–2627.

Numasaki, M., Watanabe, M., Suzuki, T., Takahashi, H., Nakamura, A., McAllister, F., Hishinuma, T., Goto, J., Lotze, M.T., Kolls, J.K., and Sasaki, H. (2005). 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. *175*, 6177–6189.

O'Sullivan, T., Dunn, G.P., Lacoursiere, D.Y., Schreiber, R.D., and Bui, J.D. (2011). Cancer immunoediting of the NK group 2D ligand H60a. J. Immunol. *187*, 3538–3545.

O'Sullivan, T., Saddawi-Konefka, R., Vermi, W., Koebel, C.M., Arthur, C., White, J.M., Uppaluri, R., Andrews, D.M., Ngiow, S.F., Teng, M.W., et al. (2012). Cancer immunoediting by the innate immune system in the absence of adaptive immunity. J. Exp. Med. *209*, 1869–1882.

Pachynski, R.K., Zabel, B.A., Kohrt, H.E., Tejeda, N.M., Monnier, J., Swanson, C.D., Holzer, A.K., Gentles, A.J., Sperinde, G.V., Edalati, A., et al. (2012). The chemoattractant chemerin suppresses melanoma by recruiting natural killer cell antitumor defenses. J. Exp. Med. *209*, 1427–1435.

Pappu, R., Ramirez-Carrozzi, V., and Sambandam, A. (2011). The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases. Immunology *134*, 8–16.

Paul, W. (2013). Fundamental Immunology, Seventh Edition (Philadelphia: Lippincott Williams & Wilkins).

Pelletier, M., Bouchard, A., and Girard, D. (2004). In vivo and in vitro roles of IL-21 in inflammation. J. Immunol. *173*, 7521–7530.

Ramirez-Carrozzi, V., Sambandam, A., Luis, E., Lin, Z., Jeet, S., Lesch, J., Hackney, J., Kim, J., Zhou, M., Lai, J., et al. (2011). IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. Nat. Immunol. *12*, 1159–1166.

Roberts, S., Gueguen, Y., de Lorgeril, J., and Goetz, F. (2008). Rapid accumulation of an interleukin 17 homolog transcript in Crassostrea gigas hemocytes following bacterial exposure. Dev. Comp. Immunol. *32*, 1099–1104.

Rosenblatt, J., and McDermott, D.F. (2011). Immunotherapy for renal cell carcinoma. Hematol. Oncol. Clin. North Am. *25*, 793–812.

Roussel, L., Houle, F., Chan, C., Yao, Y., Bérubé, J., Olivenstein, R., Martin, J.G., Huot, J., Hamid, Q., Ferri, L., and Rousseau, S. (2010). IL-17 promotes p38 MAPK-dependent endothelial activation enhancing neutrophil recruitment to sites of inflammation. J. Immunol. *184*, 4531–4537.

Schreiber, R.D., Old, L.J., and Smyth, M.J. (2011). Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science *331*, 1565–1570.

Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J., and Schreiber, R.D. (2001). IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature *410*, 1107–1111.

Smyth, M.J., Godfrey, D.I., and Trapani, J.A. (2001). A fresh look at tumor immunosurveillance and immunotherapy. Nat. Immunol. *2*, 293–299.

Smyth, M.J., Swann, J., Cretney, E., Zerafa, N., Yokoyama, W.M., and Hayakawa, Y. (2005). NKG2D function protects the host from tumor initiation. J. Exp. Med. *202*, 583–588.

Smyth, M.J., Teng, M.W., Swann, J., Kyparissoudis, K., Godfrey, D.I., and Hayakawa, Y. (2006). CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. J. Immunol. *176*, 1582–1587.

Song, X., Zhu, S., Shi, P., Liu, Y., Shi, Y., Levin, S.D., and Qian, Y. (2011). IL-17RE is the functional receptor for IL-17C and mediates mucosal immunity to infection with intestinal pathogens. Nat. Immunol. *12*, 1151–1158.

Starnes, T., Broxmeyer, H.E., Robertson, M.J., and Hromas, R. (2002). Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis. J. Immunol. *169*, 642–646.

Tartour, E., Fossiez, F., Joyeux, I., Galinha, A., Gey, A., Claret, E., Sastre-Garau, X., Couturier, J., Mosseri, V., Vives, V., et al. (1999). Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice. Cancer Res. *59*, 3698–3704.

Terme, M., Ullrich, E., Aymeric, L., Meinhardt, K., Coudert, J.D., Desbois, M., Ghiringhelli, F., Viaud, S., Ryffel, B., Yagita, H., et al. (2012). Cancer-induced

immunosuppression: IL-18-elicited immunoablative NK cells. Cancer Res. *72*, 2757–2767.

Uppaluri, R., Sheehan, K.C., Wang, L., Bui, J.D., Brotman, J.J., Lu, B., Gerard, C., Hancock, W.W., and Schreiber, R.D. (2008). Prolongation of cardiac and islet allograft survival by a blocking hamster anti-mouse CXCR3 monoclonal antibody. Transplantation *86*, 137–147.

Vaage, J. (1971). Concomitant immunity and specific depression of immunity by residual or reinjected syngeneic tumor tissue. Cancer Res. *31*, 1655–1662.

Watt, S.V., Andrews, D.M., Takeda, K., Smyth, M.J., and Hayakawa, Y. (2008). IFN-gamma-dependent recruitment of mature CD27(high) NK cells to lymph nodes primed by dendritic cells. J. Immunol. *181*, 5323–5330.

Winkler, A.E., Brotman, J.J., Pittman, M.E., Judd, N.P., Lewis, J.S., Jr., Schreiber, R.D., and Uppaluri, R. (2011). CXCR3 enhances a T-cell-dependent epidermal proliferative response and promotes skin tumorigenesis. Cancer Res. *71*, 5707–5716.

Xiao, M., Wang, C., Zhang, J., Li, Z., Zhao, X., and Qin, Z. (2009). IFNgamma promotes papilloma development by up-regulating Th17-associated inflammation. Cancer Res. *69*, 2010–2017.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656–660.

Zhang, B., Rong, G., Wei, H., Zhang, M., Bi, J., Ma, L., Xue, X., Wei, G., Liu, X., and Fang, G. (2008). The prevalence of Th17 cells in patients with gastric cancer. Biochem. Biophys. Res. Commun. *374*, 533–537.

Zitvogel, L., Tesniere, A., and Kroemer, G. (2006). Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat. Rev. Immunol. *6*, 715–727.

Zou, W., and Chen, L. (2008). Inhibitory B7-family molecules in the tumour microenvironment. Nat. Rev. Immunol. *8*, 467–477.