

IL-10 Elicits IFN γ -Dependent Tumor Immune Surveillance

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SUMMARY

Tumor immune surveillance and cancer immunotherapies are thought to depend on the intratumoral infiltration of activated CD8⁺ T cells. Intratumoral CD8⁺ T cells are rare and lack activity. IL-10 is thought to contribute to the underlying immune suppressive microenvironment. Defying those expectations we demonstrate that IL-10 induces several essential mechanisms for effective antitumor immune surveillance: infiltration and activation of intratumoral tumor-specific cytotoxic CD8⁺ T cells, expression of the Th1 cytokine interferon- γ (IFN γ) and granzymes in CD8⁺ T cells, and intratumoral antigen presentation molecules. Consequently, tumor immune surveillance is weakened in mice deficient for IL-10 whereas transgenic overexpression of IL-10 protects mice from carcinogenesis. Treatment with pegylated IL-10 restores tumor-specific intratumoral CD8⁺ T cell function and controls tumor growth.

INTRODUCTION

Human tumor cells display a number of mutations and transcriptional alterations (Wood et al., 2007), distinguishing their transcribed genome from the patient's immunological antigen profile described as immunological self (Segal et al., 2008). Research into the immune system's recognition of tumors over the last 25 years has established that human tumors are immunologically recognized by cytotoxic T cells (Knuth et al., 1984; Lee et al., 1999). CD8⁺ cytotoxic T cells have been shown to recognize point mutations in oncogenes, onco-fetal proteins and cell type-specific genes (Jäger et al., 2003; Van Der Brug-

gen et al., 2002). However, spontaneous immune-mediated remissions are rare. Immune therapies such as tumor vaccines or adoptive T cell transfers frequently aim to increase the number of tumor-specific T cells. However, tumors in patients can progress despite the presence of dramatic vaccine-induced expansion of tumor antigen-specific T cells and the continued expression of the immunogenic antigens in the tumor (Rosenberg et al., 2005). Moreover, the presence of endogenous or vaccine-induced tumor-reactive T cells in systemic circulation does not necessarily improve clinical outcomes (Lee et al., 1999; Rosenberg et al., 2005). In contrast, the presence and activity of CD8⁺ effector T cells in the tumor tissue confers an

Significance

Cancer vaccines and adoptive T cell transfers stimulate an immune response against tumors by increasing the prevalence of tumor-specific cytotoxic T cells. However, the immune suppressive microenvironment of tumors attenuates T cell function and inhibits T cell infiltration into tumors, limiting effectiveness of cancer immunotherapy. We describe how IL-10, previously thought to contribute to the immune suppressive milieu, overcomes immunological obstacles to restore immunological control of tumors. IL-10 induces CD8⁺ T cell infiltration and their cytotoxic activity within pre-established tumors. By induction of IFN γ in CD8⁺ T cells, IL-10 induces expression of intratumoral antigen presenting molecules. This uncovers the cellular and molecular mechanisms to elicit immunological control of tumors, and delineates a strategy for treatment of late stage cancers.

improved prognosis for the patient (Galon et al., 2006; Naito et al., 1998).

Correspondingly, immune evasion of human and mouse tumors can be mediated by mechanisms that reduce tumor cell-specific activity of CD8⁺ T cells. These include loss of antigen expression, reduced tumoral expression of IFN γ receptors, expression of immune suppressive surface molecules or low expression of major histocompatibility complex molecules (MHC-I/II) (Seliger et al., 2002), limiting the presentation of tumor antigens, and recognition by tumor-infiltrating T cells. Expression of MHC molecules on human tumor cells can be restored *in vitro* by IFN γ treatment (Seliger et al., 2002) and correspond with adoptive T cell transfer in responding patients (Dudley et al., 2002).

Seemingly in contrast to the observations described above is the century-old observation that tissue inflammation correlates with an increased local incidence of tumors, a higher progression rate and the decreased survival of cancer patients (Balkwill et al., 2005; Balkwill and Coussens, 2004). Indeed, the microenvironment of human tumors appears to be polarized toward an immune response resembling inflammatory diseases and is characterized by the expression of similar inflammatory cytokines (Langowski et al., 2007; McKenzie et al., 2006). These findings lead us to hypothesize that it is not immune recognition *per se*, but rather the polarization and the effector function of the immune response that might be deregulated by tumors (Mumm and Oft, 2008). Redirection of this immune pathology into cytotoxic effectors is a current topic of significant interest and could present a powerful tool in the arsenal against late stage cancer.

Interleukin 10 (IL-10) can be expressed by most T cells including regulatory T cells (Tregs), antigen presenting cells, macrophages as well as epithelial cells (Moore et al., 2001). IL-10 inhibits secretion of the proinflammatory cytokines IFN γ , tumor necrosis factor α (TNF α), IL-1 β , and IL-6 by CD4⁺ T cells and the expression of several cytokines including IL-12 by antigen presenting cells *in vitro*. IL-10 also inhibits the expression of MHC molecules and costimulatory molecules at several levels (Moore et al., 2001). It has also been demonstrated that IL-10 can impair secondary CD8⁺ T cell responses (Kang and Allen, 2005), whereas viral and tumor clearance can be enhanced in the absence of IL-10 (Brooks et al., 2006; Vicari et al., 2002). In addition, it has been suggested that IL-10 contributes to an immune suppressive tumor microenvironment, because tumor cell lines cultured *in vitro* can express IL-10. Thus, most attention has been devoted to studying the negative, regulatory function of IL-10 in inflammatory diseases or bacterial and parasitic infections where IL-10 expressed by Th1 cells suppresses TNF α - or IL-12-mediated inflammation and attenuates immune-mediated clearance of infections (O'Garra and Vieira, 2007; Trinchieri, 2007).

Clinical studies using recombinant IL-10 to treat patients with inflammatory diseases achieved moderate therapeutic effects (O'Garra et al., 2008). Peripheral blood cells of colitis patients treated with IL-10 showed reduced secretion of TNF α and IL-1 β (Tilg et al., 2002). In humans, treated lipopolysaccharide (LPS) and IL-10 reduced TNF α but, contrary to prevailing dogma, elevated IFN γ and Granzyme B in the serum (Lauw et al., 2000; Pajkrt et al., 1997) IL-10 can also stimulate effector cells of the

humoral and cytotoxic arm of the immune system (Chen and Zlotnik, 1991; Groux et al., 1998). Overexpression of IL-10 in tumor cells transplanted in mice led to tumor rejections implicating CD8⁺ T cells, NK cells or the antiinflammatory properties of IL-10 (Moore et al., 2001; Zheng et al., 1996). Therapeutic administration of recombinant human IL-10-induced antitumor immunity against fibrosarcomas in mice (Berman et al., 1996; Fujii et al., 2001). The explanation of how IL-10 reduces tumor growth despite its immune inhibitory functions on antigen presentation, cytokine expression, and T helper cells remains enigmatic.

When screening a large number of immune modulating cytokines for an influence on the growth of tumors in immune competent mice, we confirmed the strong antitumor activity of IL-10. We therefore set out to analyze the role and mechanism of IL-10 in tumor immune surveillance and rejection of large tumor masses.

RESULTS

IL-10 Deficiency Increases Tumor Incidence and Decreases Immune Surveillance

We subjected wild-type (WT) or IL-10 deficient (IL-10^{-/-}) C57BL/6 mice to a two-step skin carcinogenesis protocol (Langowski et al., 2006). WT mice developed many tumors and invasive carcinomas within 1 year after tumor initiation. Despite their tumor burden, most WT mice have a normal life span. IL-10^{-/-} mice developed increased numbers of tumors and all succumbed to tumors within 1 year after the initiating carcinogen treatment (Figures 1A and 1B). BALB/c mice are less susceptible to chronic inflammation than C57BL/6 mice, but IL-10 deficiency similarly increased the susceptibility of BALB/c mice for skin tumors (Figure 1C).

Due to the central role of IL-10 in controlling inflammation and the established role of inflammation in the promotion of two-step skin carcinogenesis, we analyzed the infiltration of inflammatory cells and the expression of inflammatory molecules in the skins of carcinogen treated WT mice, IL-10^{-/-} mice, or IL-10TG mice that overexpress human IL-10 in all antigen presenting cells under the control of the MHCII promoter (Groux et al., 1999). Remarkably, IL-10TG mice were resistant to tumor induction (Figure 1D) but, surprisingly, given the putative role of IL-10 in inhibiting inflammation, showed a level of infiltration with inflammatory macrophages (CD11b⁺) equivalent to the two other genotypes (Figure 1E). In search of a molecular mechanism for the tumor susceptibility we found that MHC-I molecules were expressed significantly lower in skin lesions from WT or IL-10^{-/-} mice (Figures 1F–1H). Additionally, the mRNA of Granzyme A and Granzyme B, molecules mediating cytotoxic effector and tumor immune surveillance functions, were significantly reduced in IL-10^{-/-} versus WT skins (Figure 1I). We further found that, surprisingly, IL-10TG mice showed increased numbers of skin infiltrating CD8b⁺ T cells and higher expression of the Th1 associated cytokine IFN γ accompanied with increased expression of MHC molecules compared to IL-10^{-/-} mice (Figures 1F and 1J–1L). These data indicated that in the context of transformed tissue, IL-10 not only fails to downregulate CD8⁺ T cell responses, IFN γ , and expression of antigen

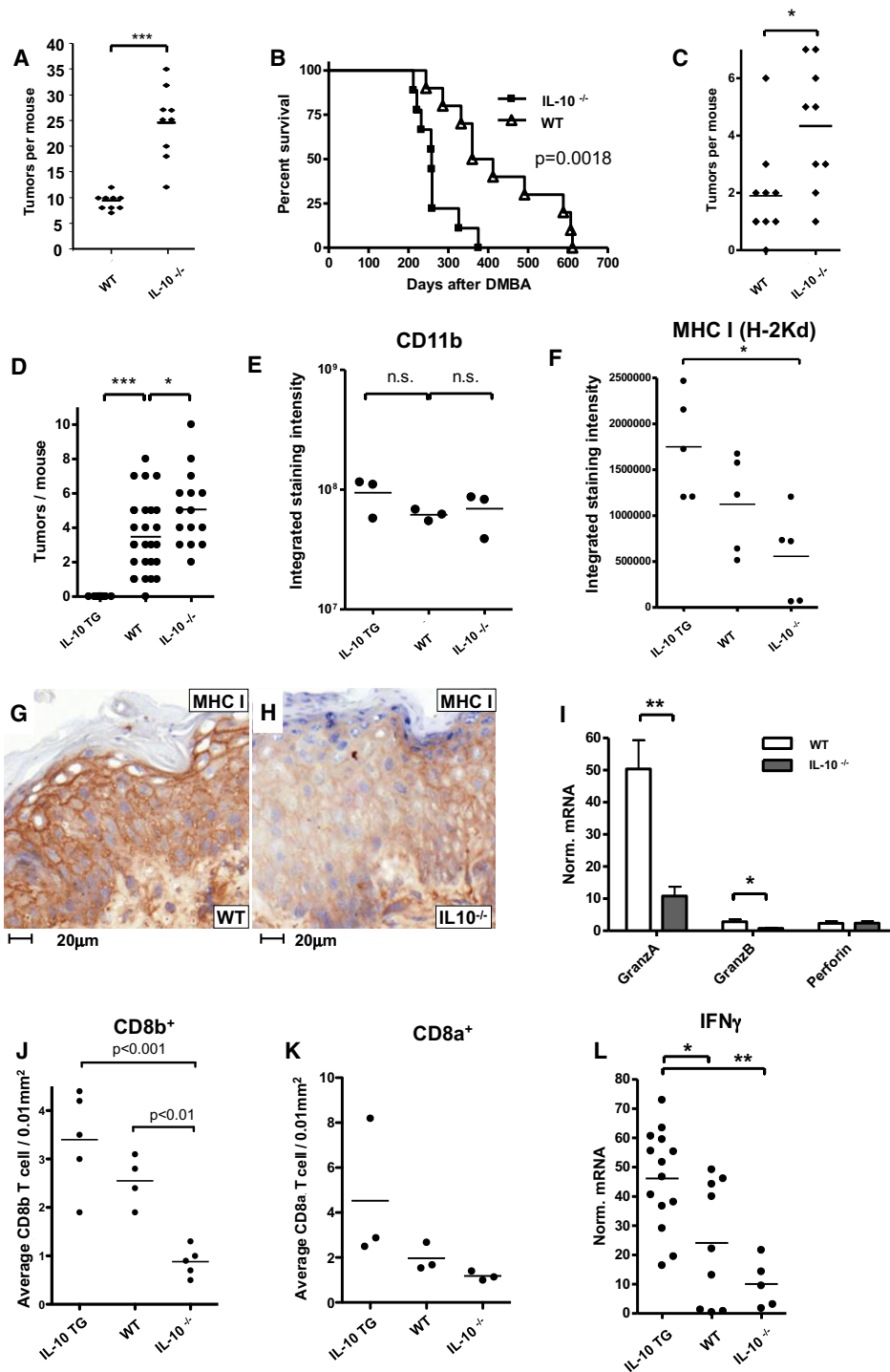
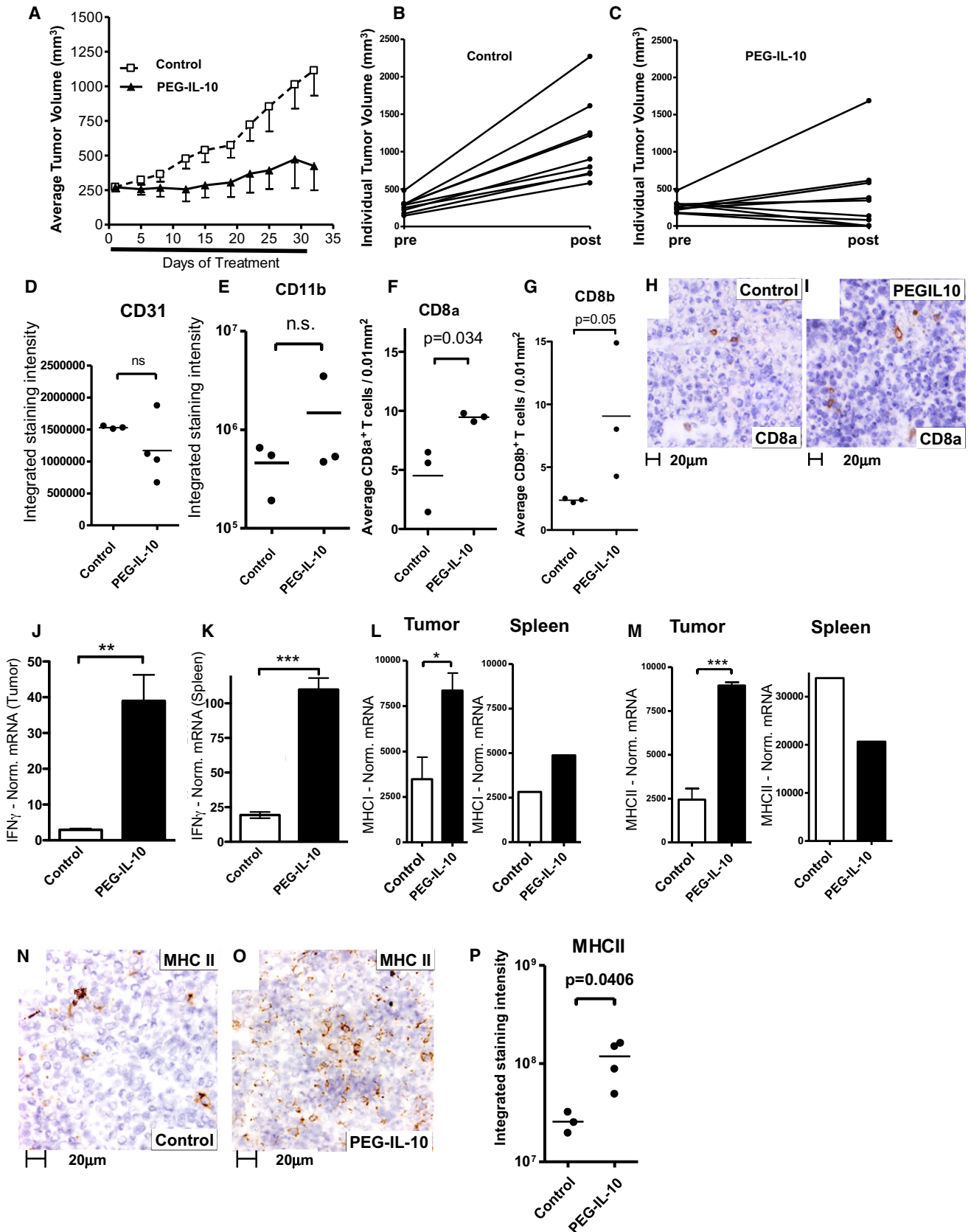


Figure 1. IL-10 Controls Tumor Immune Surveillance against Skin Tumors

(A and B) Tumor incidence 5 months after initiation (A) and mortality (B) after chemical carcinogenesis in IL10^{-/-} and WT C57BL/6 mice. (C) Tumor incidence 5 months after chemical carcinogenesis in IL10^{-/-} and WT BALB/c mice. Data are representative of 8–12 mice per group, performed twice in C57BL/6 and three times in BALB/c. (D) Tumor development after chemical carcinogenesis protocol in IL-10 TG, WT and IL-10^{-/-} mice. (E) Macrophage infiltration into skins in IL-10 TG, WT, and IL-10^{-/-} Balb/c mice (quantified CD11b IHC intensity; light absorption units). (F–H). Quantitation of MHC-I protein expression in hyperplastic skin of IL10^{-/-}, IL-10TG, and WT mice. (I) Quantitative RT-PCR measurement of mRNA levels of cytotoxic molecules in skin of WT and IL10^{-/-} mice. Error bars represent standard error of the mean. (J–L). Infiltration of CD8b⁺ (J) and CD8a⁺ (K) T cells and IFN γ expression (L) in the carcinogen-treated skin of IL10^{-/-}, IL-10TG, and WT mice. Horizontal bars represent mean and each data point (dots) represents one mouse in (A), (C–F), and (J–L). *p < 0.05, **p < 0.01, ***p < 0.001.



presentation molecules, but even appears to promote their induction.

MHC Molecules and Cytotoxic Mediators Are Increased by IL-10 in the Tumor but Not in Secondary Lymphoid Organs

The above findings raised the question whether stimulation of peripheral tumor immune surveillance with IL-10 would inhibit the growth of already established tumors. Recombinant human IL-10 has been used previously to induce rejection of transplanted fibrosarcomas in mice (Berman et al., 1996; Fujii et al., 2001). Recombinant human and mouse IL-10 have short in vivo half lives and human IL-10 is potentially immunogenic in murine models (data not shown). Therefore we utilized a pegylated form of murine IL-10 (PEG-IL-10) with an increased serum half life for most of the animal experiments described in this study. Cancer cell lines used in transplanted tumor models might have acquired mutations during in vitro passage, possibly making their antigenic profile an easier target for cancer immune therapy compared with endogenous tumors. In addition, relatively slow developing endogenous tumors might pose a significantly different challenge to the immune system than rapidly growing transplanted tumors. Moreover, large tumor masses are frequently resistant to intervention with chemotherapies but are even more resistant to immune therapies. Therefore we tested the antitumor effects of PEG-IL-10 in large tumors developing in Her2 transgenic mice (FVB^{MMTV-rtHer2}), an endogenous mouse model for breast cancer, and found that PEG-IL-10 controlled average tumor growth and induced rejection of several well-established large tumors (Figures 2A–2C).

It has been shown that IL-10 reduces the expression of antigen presenting molecules in vitro and suppresses the expression of inflammatory cytokines (Moore et al., 2001). IL-10-deficient animals develop autoinflammatory colitis (Kühn et al., 1993). In addition, overexpression of IL-10 peripherally in the brain, but not systemically, reduces inflammatory responses in a mouse model of multiple sclerosis driven by inflammatory Th17 CD4⁺ T cells (Cua et al., 1999). In contrast to IL-10s reported inhibition of angiogenesis, inflammation, and antigen presentation, PEG-IL-10 treatment of Her2 transgenic mice carrying mammary carcinomas did not reduce intratumoral angiogenesis or infiltration of inflammatory macrophages (Figures 2D and 2E). The most striking IL-10-induced change in the tumor microenvironment

was a significant increase of CD8a/b⁺ T cell infiltration (Figures 2F–2I). Contrary to the known anti-inflammatory role of IL-10 however, IL-10 treatment elicited dramatically increased expression of the Th1 cytokine IFN γ predominantly in the tumor, and to a lesser extent in the spleen (Figures 2J and 2K). In addition, PEG-IL-10 significantly induced the intratumoral expression of MHC-I and MHC-II molecules, both at the RNA and protein level (Figures 2L–2P). Interestingly, most of the areas in IL-10-treated tumors showed a significantly higher expression of MHCII than all areas in a control tumor (Figure S1A available online). Despite systemic exposure to IL-10, antigen presentation in the spleen was only moderately regulated (Figures 2L and 2M). To test the importance of CD8⁺ T cell-mediated immune surveillance for IL-10-induced tumor control, we depleted CD8⁺ T cells with antibodies. Depletion of CD8⁺ T cells abrogated the antitumor activity of IL-10 (Figure S1B). Importantly, the significant stimulation of peripheral antitumor immunity by PEG-IL-10 extended to a wide variety of large transplanted tumor types and mouse strains even when the primary tumors were very well established (Figures S1C–S1F).

Activation of CD8⁺ T Cells in the Tumor but Not in Secondary Lymphoid Organs Is Essential to IL-10-Mediated Tumor Immune Surveillance

IL-10 has been suggested to boost immune effectors (Fujii et al., 2001) but derail immunization and inhibit memory responses to tumor antigens (Fujii et al., 2001; Vicari et al., 2002). The observation of peripheral effector T cell activation with minimal systemic immune stimulation could explain the inhibition of long term T cell memory by IL-10 reported previously (Brooks et al., 2006). To test for protective tumor-specific CD8⁺ responses and antitumor immune memory responses we employed a skin squamous carcinoma model (PDV6) syngeneic to C57BL/6 harboring the same Ha-Ras mutation as the chemically-induced tumor model. These tumors grow orthotopically in the skin and metastasize via the lymphnodes to the lung and the bone, similar to human skin or breast carcinomas. Compared to WT mice, the progression of this carcinoma was significantly faster in IL-10^{-/-} mice leading to 50% of the mice succumbing to the tumor and metastasis within 3 months (Figure 3A). When treated with PEG-IL-10, mice carrying PDV6 carcinomas rejected their tumors even if the treatment was initiated at late tumor stages and large tumor masses (Figure 3B). Mice cured by IL-10

Figure 2. IL-10-Mediated Control of Endogenous Her2 Transgenic Tumors Is Accompanied by Infiltration of CD8⁺ T Cells and MHC Expression in the Tumor

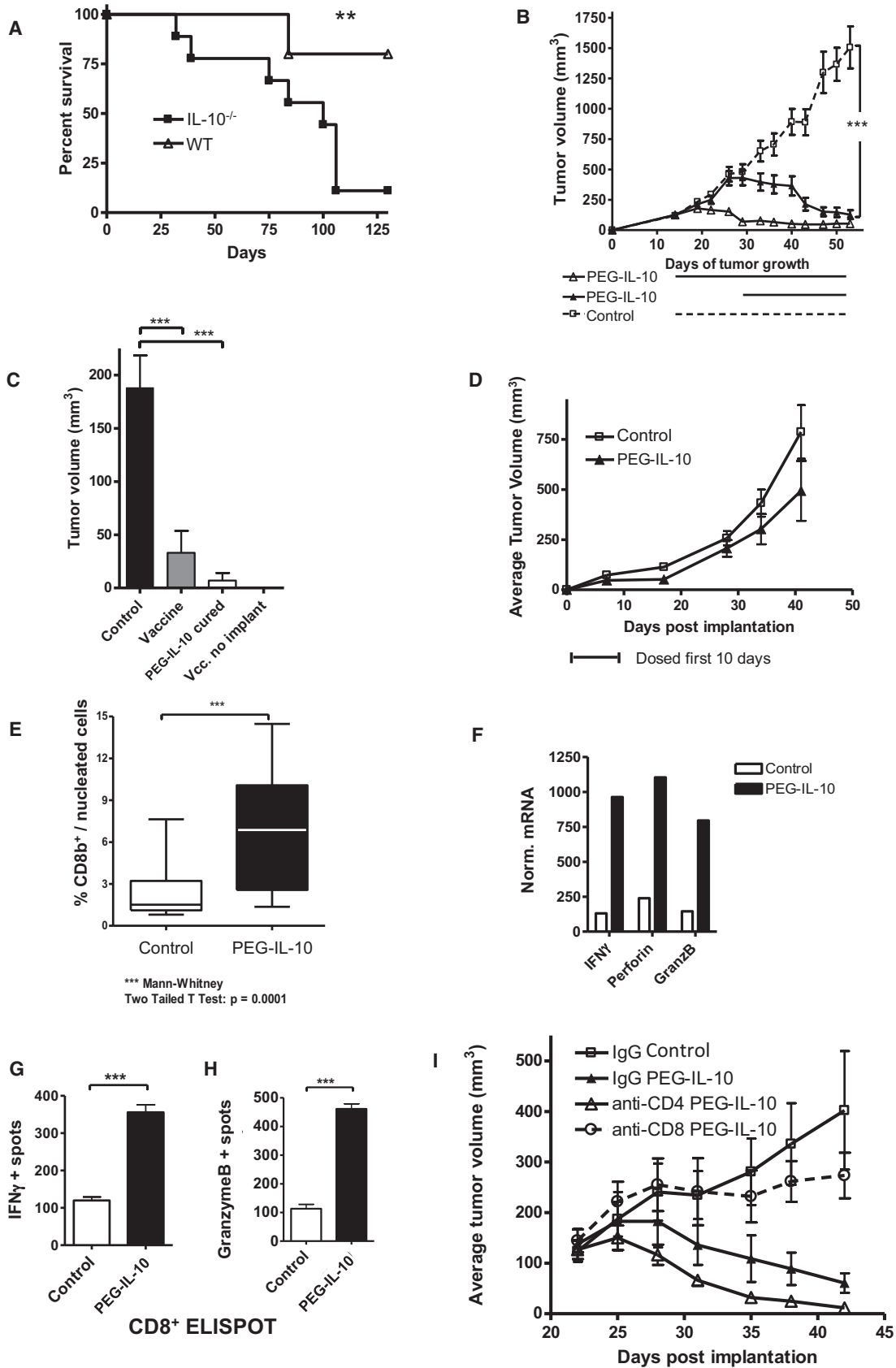
(A–C) PEG-IL10 treatment effect on the growth of endogenous mammary cancer in FVB^{MMTV-rtHer2} mice. Horizontal bar in (A) indicates treatment duration (0.5 mg/kg) n \geq 8/group in three experiments, n \geq 3 mice/group analyzed. Average tumor burden (A) and beginning and ending volumes of each tumor of control (B) and PEG-IL-10-treated (C) mice.

(D–G) Horizontal bars represent the mean and each data point (dots) represents analysis of one mouse. (D) and (E) were performed on three mice selected at random from each group with 20 images quantified per tumor. Data (D–I) is representative of two independent experiments each quantified in this manner. (D) Angiogenesis as measured by CD31⁺ cells. (E) Infiltration of CD11b⁺ inflammatory macrophages.

(F–I) CD8⁺ T cells infiltration in response to IL-10 treatment. Analysis of (F–I) was performed on three to five mice selected at random from each group with 20 images quantified per tumor. Quantitation of CD8a and CD8b IHC results (F and G) and examples for CD8a in control (H) and PEG-IL-10 (I) tumor tissue.

(J–M) Data are representative of three experiments with seven to ten mice/group. Data represent IFN γ mRNA levels in tumor (J) and spleen (K). (L and M) MHC I mRNA levels (L) and MHCII mRNA levels (M) in the tumor and spleen.

(N–P) Tissue from three to four mice/group analyzed from two independent experiments with 20 fields per tumor quantified and in (P) horizontal bars represent the mean and each data point (dots) represents analysis of one mouse. Panels represent immunohistochemistry staining of MHCII in a control (N) or IL-10 treated (O) tumor; quantitation of IHC results (P). IHC or mRNA analysis was performed 3 weeks after treatment initiation. *p < 0.05, **p < 0.01, ***p < 0.001. Error bar(s) represents standard error of the mean for all panels. See also Figure S1.



treatment were resistant to reimplantation of the same tumor, tested up to 8 months after tumor rejection. This protection was comparable to or better than tumor vaccination with freeze-thawed tumor cells (Figure 3C). Clearance of primary tumors by IL-10 treatment was therefore able to stimulate a potent protective long-term immune memory against rechallenge with the same tumor. Similarly, treatment only during the first 10 days after tumor inoculation does not suppress the host immune systems antitumor response (Figure 3D and data not shown). These data indicate that IL-10 induces an effective immunological memory toward tumor antigens and does not derail immunological memory. Similar to overexpression of IL-10 in the chemically-induced tumors, IL-10 treatment of orthotopic squamous carcinomas resulted in a significant increase of CD8⁺ T cells (CD8a/b⁺) detectable in and extractable from tumor tissue (Figure 3E).

Tumor-specific T cells isolated from tumors or the blood of human cancer patients can frequently be restimulated in vitro to proliferate or to produce IFN γ , however, it is their activity inside human tumors that is thought to confer a prognostic advantage (Galon et al., 2006; Naito et al., 1998). We therefore examined the mRNA expression profile and the release of granzymes and IFN γ from tumor-infiltrating CD8⁺ T cells without further in vitro stimulation. The expression of cytotoxic markers such as granzymes and perforin as well as IFN γ was strongly enhanced in CD8⁺ T cells extracted from tumors of PEG-IL-10 treated mice, but only limited changes were observed in secondary lymphoid organs such as the spleen and lymphnodes (Figures 3F, S2A, and data not shown). PEG-IL-10 treatment induced a 3- to 4-fold increase in the spontaneous secretion of Granzyme B and IFN γ by tumor-derived CD8⁺ T cells (Figures 3G and 3H).

As PEG-IL-10-induced granzymes expression in the tumor and tumor-infiltrating CD8⁺ T cells, we sought to identify the cellular requirements for its tumor inhibition. PEG-IL-10 failed to inhibit growth of syngenic tumors grown in mice deficient for the adaptive immune response (RAG^{-/-} and nude mice; Figure S2B and data not shown). Mice deficient in B cells however responded similarly to WT mice indicating a limited involvement of B cells (data not shown). Antibody-mediated depletion of CD8⁺ T cells but not of CD4⁺ T cells completely abrogated antitumor function of IL-10 indicating that stimulation of CD8⁺ T cell function is absolutely required for IL-10-mediated tumor rejection (Figure 3I). CD8⁺ T cells were also required in other tumor

models like the endogenous breast cancers in HER2 transgenic animals (Figure S1B). Depletion of NK cells did not have a significant effect on IL-10-mediated rejection of PDV6 tumors (Figure S2C). Taken together, these data indicated that the immune stimulation by IL-10 primarily requires the activation of peripheral T cells and CD8⁺ cytotoxic T cells in particular. Importantly, the induction of MHC-I/II by IL-10 was reduced upon antibody-mediated T cell depletion, in particular when CD8⁺ T cells were depleted (Figures S2D and S2E). These data suggested that IL-10-induced activation of CD8⁺ T cells in the tumor microenvironment may lead to T cell-mediated upregulation of antigen presenting molecules.

IL-10 Induces Antigen-Specific CD8⁺ T Cell Responses in Tumor-Bearing Mice and Elevates IFN γ in CD8⁺ T Cells

We next asked if IL-10 alters the frequency of antigen-specific responses to the tumor. CD8⁺ T cells were isolated from the blood or the tumor and in vitro stimulated with soluble anti-CD3 antibodies, the cognate tumor cells (PDV6) or an irrelevant tumor cell line (Figures 4A–4C). Within 4 days, IL-10 treatment increased the number of cells responding to anti-CD3 stimulation with IFN γ secretion in cells isolated from the tumor, indicating broad Tc1 polarized activation of CD8⁺ T cells in IL-10 treated mice. However the frequency of tumor cell-specific responses were low, both in control and IL-10 treated tumors. Interestingly, after allowing polyclonal T cell expansion by treating with IL-10 for 2 weeks, equal numbers of cells responded to anti-CD3 stimulation or to stimulation with the cognate tumor cells (PDV6) but not to irrelevant tumor cells, indicating a high degree of antigen specificity within the IL-10-induced CD8⁺ T cell response (Figure 4B). After 2 weeks in vivo IL-10 exposure, both tumor-derived and blood-derived CD8⁺ T cells secreted IFN γ when restimulated with their cognate tumor in vitro (Figure 4C) indicating a systemic expansion of the tumor-specific CD8⁺ T cells repertoire in the host.

Direct analysis of whole tissue mRNA detected greater IFN γ induction in the tumor, compared to other tissues or the blood of IL-10 treated animals. We therefore isolated CD8⁺ T cells from large tumors or the blood of mice treated with IL-10 and stained for intracellular IFN γ directly without in vitro restimulation. Interestingly, only T cells isolated from the tumors treated with PEG-IL-10 expressed intracellular IFN γ without the requirement for further in vitro restimulation whereas T cells isolated

Figure 3. Increased CD8⁺ T Cell Activity in the Tumor but Not in Secondary Lymphoid Organs Is Necessary for IL-10-Mediated Tumor Control (A and B) Data in (A) reflect two independent experiments with n = 5–10 mice/group, (B) four experiments with n = 10 mice/group. (A) Represents survival of IL-10^{-/-} and WT C57BL/6 mice after PDV6 squamous tumor implantation. (B) PEG-IL-10 therapeutic effect on growth of established PDV6 tumors. (C) Data represents two experiments with n = 10 mice/group, one reimplantation at 5 months and one at 8 months post complete tumor rejection. Panel reflects inhibition of primary tumor growth implanted up to 8 months after rejection of the primary tumor. (D) Two independent experiments (n = 10 mice/group). Data represent analysis of tumor growth after treatment with PEG-IL-10 for 10 days concomitant with tumor implantation (treatment period indicated by horizontal bar below graph). (E) Representative three experiments (n = 10 mice/group). Each tumor was sectioned twice and two independent investigators acquired and analyzed 25 images per section under blinded conditions. Data represent the quantification (blinded) of intratumoral CD8b T cell identified by IHC normalized on all nucleated cells. (F) Three independent experiments with n = 10 mice/group of the mRNA analysis of tumor-infiltrating CD8⁺ T cells for cytotoxic effector molecules. (G and H) Six independent experiments. ELISPOT analysis of purified tumor-infiltrating CD8⁺ T cells (TIL) reporting numbers of cells (5,000/well) secreting IFN γ (G) and Granzyme B (H). (I) Reflects data representative of six independent experiments in different tumor models with n = 5–10 mice/group. Mice were treated with antibodies against CD4 or CD8 to deplete specific cell populations or isotype control to analyze cellular requirement for PEG-IL-10-induced effect on tumor growth. Error bar(s) represent standard error of the mean for all panels. *p < 0.05, ***p < 0.001; 10 mice/group, treated with 0.5–0.1 mg/kg PEG-IL-10. See also Figure S2.

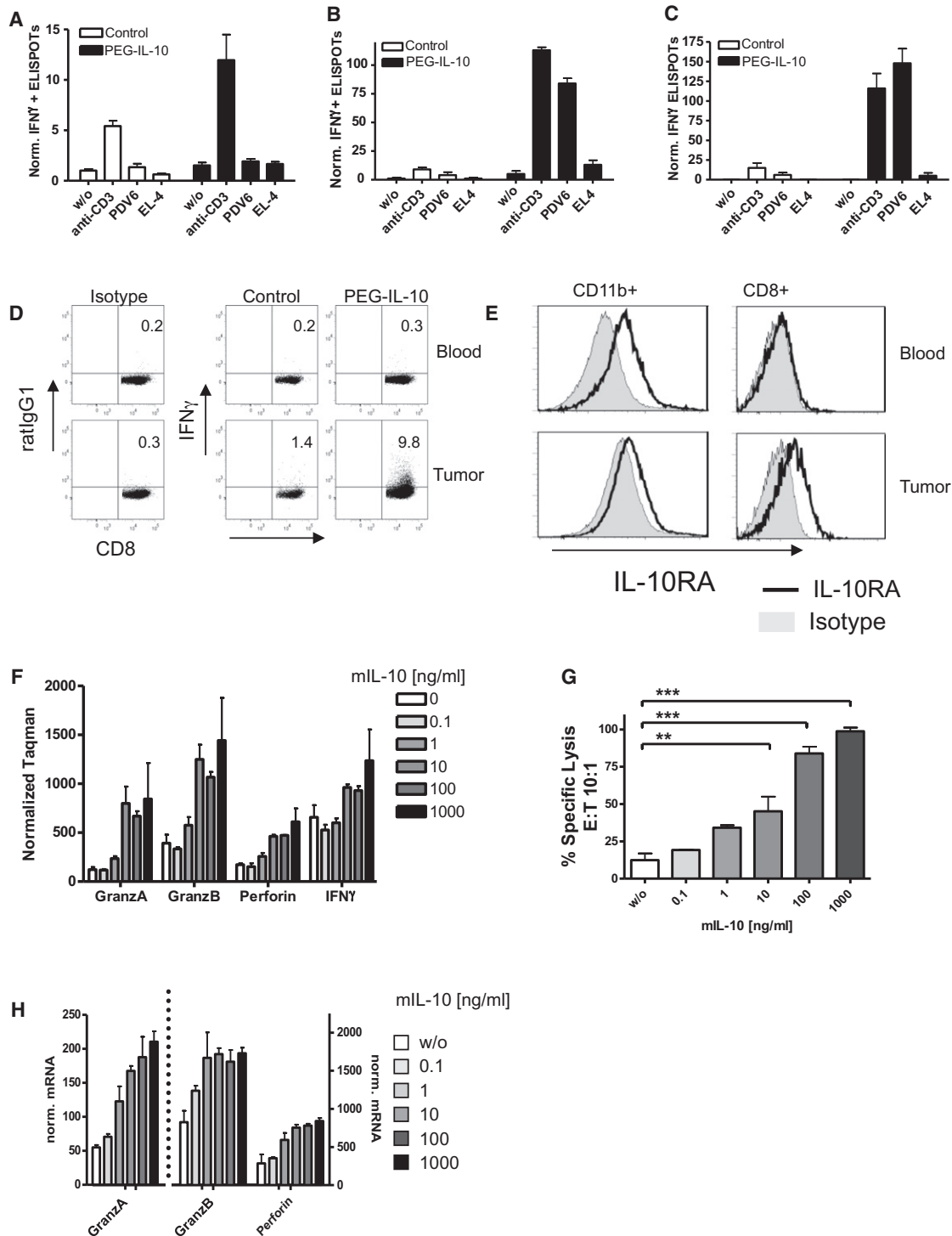


Figure 4. IL-10 Increases Antigen-Specific T Cell Responses, Directly Induces IFN γ in Tumor-Infiltrating IL10R-Positive CD8⁺ T Cells but Does Not Require IFN γ for Induction of Cytotoxicity Effector Molecules

(A–C) Panels are representative of three independent experiments in PDV6 and two independent experiments in CT26 (n = 5–10 mice/group). ELISPOT data for the analysis of antigen-specific CD8⁺ T cells isolated from tumors (TILs) or blood were stimulated with anti-CD3, cognate tumor cells (PDV6), or MHC matched nonprimary control tumor cells (EL-4); IFN γ positive ELISPOTS (1,000 cells/well with E/T of TILs to tumor cells at 10:1) were normalized to untreated nonstimulated controls. TILs were analyzed after 4 days (A), 17 days (B), and blood CD8⁺ T cells analyzed at 17 days (C) of dosing.

(D) Quantitation of intracellular IFN γ by FACS of TILs, reflecting four independent experiments.

(E) Three independent experiments with n = 10 mice/group where cells surface FACS analysis of IL-10RA expression on CD8⁺ T cells isolated from tumors and blood.

from the blood or from nontreated tumors did not (Figure 4D). T cells isolated from secondary lymphoid organs such as the spleen or the tumor draining lymph node also required in vitro restimulation to express IFN γ (data not shown).

IL-10RA Is Highly Expressed on Tumor-Infiltrating CD8⁺ T Cells

These data raised the question why tumor-infiltrating CD8⁺ T cells were uniquely able to respond to IL-10 with IFN γ production. To test the hypothesis that CD8⁺ T cells in different locations differentially express the IL-10R, we stained various cell types for the expression of the IL-10R from different tissues. IL10R was expressed significantly higher on CD8⁺ T cells isolated from tumors but barely detectable on cells derived from blood or secondary lymphoid organs (Figures 4E, S3A, and S3B). This may provide a molecular explanation for the unique activation of peripheral, tumor-infiltrating CD8⁺ T cells by IL-10.

IL-10 Directly Elevates Cytotoxicity and IFN γ Expression in CD8⁺ T Cells

During acute virus infection autocrine IL-10 has been found to limit the activation and the expressions of signature cytokines such as IFN γ in Th1 CD4⁺ T cells (O'Garra and Vieira, 2007). However, IL-10 has previously been shown to enhance IL-2-induced cytotoxic activity of CD8⁺ T cells (Chen and Zlotnik, 1991; Groux et al., 1998). The absolute requirement for IL-2 and the molecular targets of the increased cytotoxic activity remains undefined. Moreover, under physiological conditions IL-2 is not thought to be elevated in tumors. To assess IL-10 capacity to stimulate CD8⁺ T cell cytotoxic function, spleen-derived CD8⁺ T cells were activated in vitro, rested, and then exposed to increasing concentrations of IL-10 in vitro in the absence of exogenous IL-2. IL-10 directly elevated the expression of cytotoxic enzymes and the expression of the Th1 cytokine IFN γ in CD8⁺ T cells, once again contradicting expectations (Figure 4F). Using T cell receptor transgenic CD8⁺ T cells we found that IL-10 alone was also sufficient to enhance their cytotoxic activity toward target cells pulsed with the cognate peptide antigen (Figure 4G). The levels of IL-10 required to activate CD8⁺ T cells in vitro were comparable to serum concentrations achieved in mice treated with therapeutic doses of PEG-IL-10 (data not shown). These data are seemingly in contrast to previously published results that analyzed CD8⁺ T cell responses either in coculture systems with dendritic cells (Kang and Allen, 2005) or with *Listeria*-infected target cells. We hypothesize that under such conditions, IL-10 may antagonize and compete with dendritic cell-derived IL-12. IL-12 induces IFN γ and Th1 polarization of T cells. Expression of IL-12, however, is very limited in the tumor microenvironment (Langowski et al., 2006). Moreover, preliminary data suggest IL-10 induces tumor rejections in both WT and IL-12 deficient animals (data not shown). These findings suggest that IL-10 may represent an alternative pathway to induce IFN γ in tumor-infiltrating CD8⁺ T cells, independent of IL-12 or IL-2.

IL-10 Induces Cytotoxic Enzymes in CD8⁺ T Cells Independently of IFN γ

IFN γ is essential for CD8⁺ T cell-mediated immune surveillance (Dunn et al., 2006). To test if upregulation of IFN γ mediates the cytotoxicity induced by IL-10, we investigated the modulation of cytotoxic molecules in IFN γ ^{-/-} T cells. IFN γ ^{-/-} CD8⁺ T cells were isolated, activated in vitro, rested, and exposed to various concentrations of IL-10. Unexpectedly, IL-10 induced the upregulation of mRNA for granzymes and perforin in IFN γ ^{-/-} CD8⁺ T cells equally to WT CD8⁺ T cells (Figures 4F and 4H). We also asked if exogenous IFN γ would enhance the effect of IL-10 on the expression of those cytotoxic mediators in IFN γ ^{-/-} CD8⁺ T cells. Under these conditions, IFN γ neither directly stimulated nor enhanced IL-10s induction of granzymes or perforin (Figure S3C). These experiments indicate that although IL-10 induces IFN γ in CD8⁺ T cells, the stimulation of cytotoxic molecules in CD8⁺ T cells in vitro by IL-10 is independent of IFN γ . They also suggest that IL-10 is a key regulatory cytokine to induce the expression of cytotoxic enzymes.

IL-10-Mediated Tumor Rejection Depends on IFN γ , but Infiltration of CD8⁺ T Cells and the Intratumoral Induction of Cytotoxic Enzymes Do Not

We next tested the requirement for IFN γ in IL-10-mediated tumor immune rejection using the orthotopic PDV6 model. IL-10 controls tumor growth (Figure 5A) and significantly increased intratumoral CD8⁺ T cell infiltration in this tumor, similar to the DMBA-induced skin tumors (Figure 3E). However, in IFN γ ^{-/-} mice, even though IL-10 treatment induced a similar increase of intratumoral CD8⁺ T cells, it failed to induce tumor rejection (Figure 5B). Consistent with in vitro findings, the induction of cytotoxic molecules were similarly upregulated in the tumors and in CD8⁺ T cells isolated from IL-10 treated WT and IFN γ ^{-/-} mice (Figures 5C–5E). This suggests that both IL-10-mediated intratumoral T cell infiltration and induced expression of cytotoxicity in CD8⁺ T cell in vivo is largely independent of IFN γ . In most experimental antitumor immune therapy approaches, the activation of tumor-infiltrating cytotoxic T cells correlates with the elimination of tumor cells. Our data suggest that CD8⁺ T cell activation by IL-10 in the absence of IFN γ uncouples this correlation, with IL-10 driving efficient infiltration and activation of the CD8⁺ T cells. However, elimination of tumor cells by these granzyme- and perforin-expressing CD8⁺ T cells still required IFN γ (Figures 5A and 5B and data not shown).

IL-10 Induces Upregulation of MHC Molecules through Induction of T Cell-Derived IFN γ

In vitro IL-10 reduces the expression of MHC molecules in antigen presenting cells (Moore et al., 2001). IFN γ has been shown to induce MHC expression in antigen presenting cells and in human, mouse tumor cells, or tumor stroma (Seliger et al., 2002, 2001; Zhang et al., 2008). Our data show that IL-10 induces IFN γ in CD8⁺ T cells. We hypothesized that host-derived IFN γ might therefore be essential to mediate IL-10-induced

(F) Five independent experiments assessing effect of IL-10 on expression of cytotoxic effector molecules and IFN γ in CD8⁺ T cells in vitro.

(G) Analysis of IL-10 effect on OT-1 CD8⁺ T cells cytotoxicity in vitro, representative data from three independent experiments.

(H) Investigation from four independent experiments of IL-10 effect on granzymes and perforin mRNA expression in CD8⁺ T cells from IFN γ ^{-/-} mice in vitro. Error bar(s) represents standard error of the mean for all panels. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Figure S3.

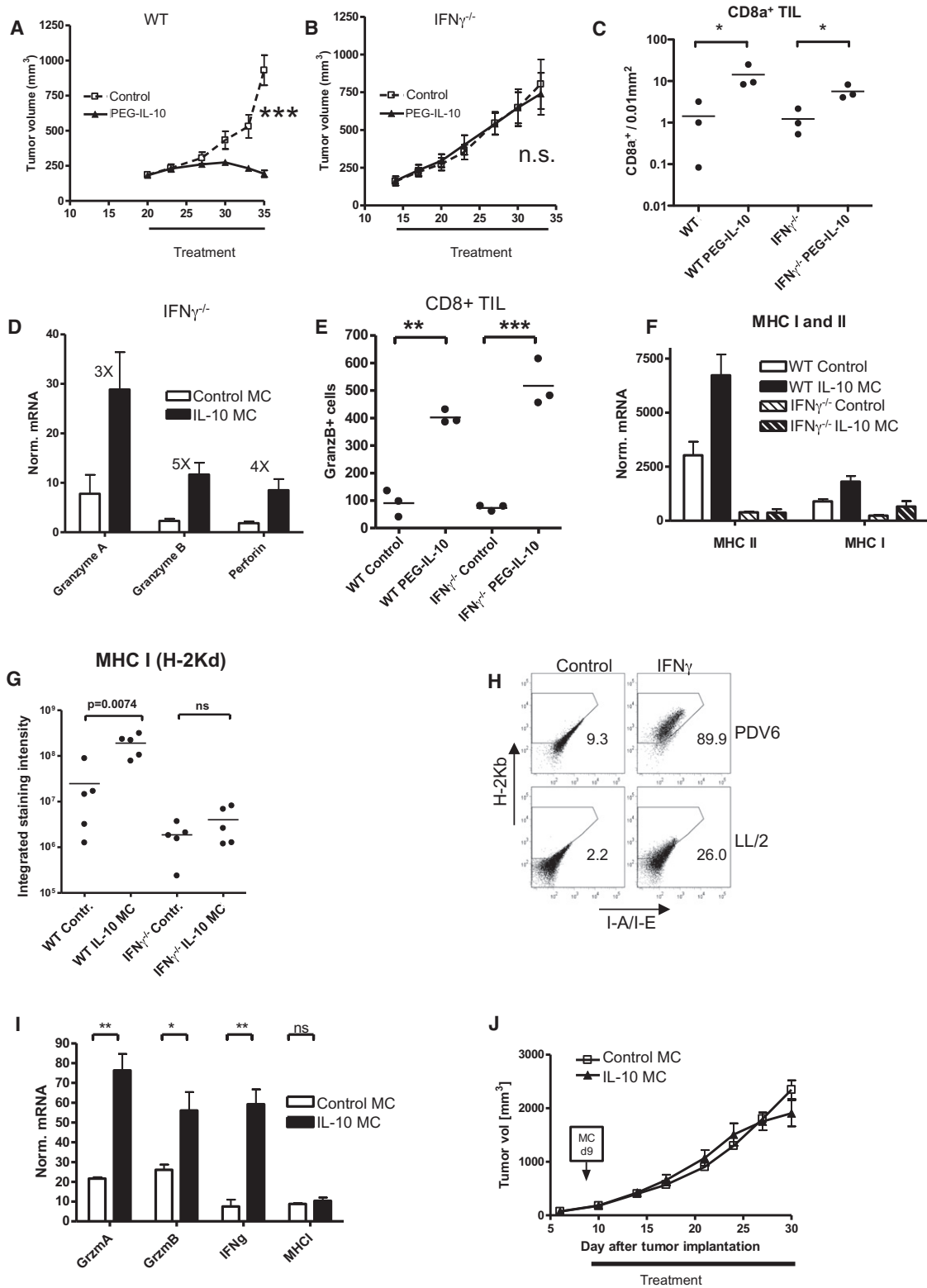


Figure 5. Uncoupling of CD8⁺ T Cell Infiltration and Cytotoxic Activity from MHC I and MHC-II Induction and Tumor Rejection

(A and B) Data represents four experiments (n = 7–10 mice/group). PEG-IL-10 antitumor function in WT (A) and IFN γ ^{-/-} (B) mice. (C) Analysis of PEG-IL-10 effect on intratumoral CD8⁺ T cell infiltration in both WT and IFN γ ^{-/-} mice (IHC CD8a⁺ cell quantitation, bars represent mean for all similar panels), where data represents two independent experiments (n = 3–5 mice per group). A total of 20–25 images/tumor – IHC quantified. One 0.2 mg/kg PEG-IL-10 daily subcutaneous injection, one minicircle IL-10.

upregulation of antigen presenting molecules in the tumor. Therapeutic administration of PEG-IL-10 induced a potent upregulation of mRNAs for MHC-I and II molecules in WT tumors in WT hosts (Figures S4A–S4D), but the same WT tumors failed to express MHC molecules in IFN γ ^{-/-} hosts irrespective of IL-10 treatment (Figure 5F). Similarly, IL-10 induced MHC I and II protein expression was detected only in tumors in IFN γ competent animals (Figures 5G and S4E–S4H). Contrary to our current understanding of the immune regulatory function of IL-10, these data suggest not only that IL-10 induces the Th1 cytokine IFN γ in intratumoral CD8⁺ T cells but also that the IFN γ -mediated induction of intratumoral antigen presentation machinery is essential for the antitumor function of IL-10.

The majority of human tumor cells upregulate antigen presentation upon IFN γ treatment in vitro, but tumor cells can escape immune recognition by altering their MHC and antigen processing machinery (Seliger et al., 2002). We therefore tested if IL-10 could induce rejection of tumors with reduced IFN γ responsiveness. We screened several syngeneic tumor cell lines for IFN γ -induced upregulation of antigen presentation and expression of MHC I in particular. The lung carcinoma cell line LL/2 is largely resistant to upregulation of cell surface MHC I when exposed to IFN γ in vitro (Figure 5H). We therefore tested if IL-10 treatment would still suppress growth of LL/2 tumors. Therapeutic administration of IL-10 induced the mRNA expression of cytotoxic enzymes and IFN γ in LL/2 tumors (Figure 5I and data not shown). As predicted by the proposed model, IL-10 treatment or more specifically IL-10-induced-IFN γ failed, however, to upregulate intratumoral expression of MHC molecules and correspondingly failed to suppress tumor growth (Figures 5I and 5J).

Human Inflammatory Diseases but Not Cancer Exhibit Elevated IL-10 Expression

IL-10 is thought to be primarily antiinflammatory and immune suppressive. Consequently, IL-10 is suspected to induce tumor-associated immunosuppression (Nieder Korn, 2008). Chronic inflammatory diseases like ulcerative colitis (UC) are statistically significantly associated with an increased risk of cancer incidence. Inflammatory cytokines like IL-23 are expressed highly in inflammatory lesions of such patients and may also contribute to tumor-promoting inflammation (Langowski et al., 2006 and data not shown). We therefore evaluated whether IL-10 expression may be lower in inflammatory bowel disease and higher in colon tumors. On the contrary, IL-10 was significantly elevated in ulcerative colitis and Crohn's disease (Figure 6A), very similar to proinflammatory cytokines (data not shown). More-

over, IL-10 was expressed lower in colonic polyps compared to normal tissues and there was no difference of IL-10 mRNA when colorectal cancer tissue was compared to normal adjacent tissue (Figure 6B). Similarly, IL-10 mRNA expression within breast cancer tissue is equal to normal, noninflamed adjacent tissue controls (Figure 6C) and the IL-10 concentration in the serum of breast cancer patients and normal volunteers was similar (Figure 6D). Thus, contrary to the prevailing expectations, IL-10 mRNA expression was not increased in any human carcinoma type investigated, but was significantly upregulated under inflammatory conditions.

IL-10 Directly Induces Cytotoxicity of Human CD8⁺ T Cells and Its Expression Correlates with the Expression of Cytotoxic Enzymes

We next confirmed that IL-10 regulates cytotoxic enzymes and the Th1 cytokine IFN γ also in human CD8⁺ T cells (Figure 6E). These data suggest that IL-10 regulation of cytotoxic activity and IFN γ expression is similar in CD8⁺ T cells from mice and man. The direct activation of cytotoxicity led us to investigate the nature of such CD8⁺ T cell activation. mRNA of granzymes and perforin was strongly induced within a few hours of IL-10 stimulation (Figures S5A–S5C) suggesting a direct transcriptional activation by IL-10-induced signaling. IL-10 is known to induce phosphorylation and activation of Stat3. Indeed, addition of a Stat3 inhibitor to CD8⁺ T cell could partially abrogate IL-10-mediated induction of cytotoxic enzymes (Figure 6F), supporting involvement of Stat3 in the induction of cytotoxic enzymes by IL-10.

Expression of IL-10 in human tissue and tumors might correlate with the expression of cytotoxic enzymes if IL-10 directly induces the expression of cytotoxic enzymes. Indeed, IL-10 and Granzyme B expression were highly correlated in melanoma samples (Figures 6G and 6I). IL-12 (IL12p35), stimulates Granzyme B expression in CD8⁺ T cells and its expression also correlated with Granzyme B expression in melanoma tissues (Figures 6H and 6I).

Given the antitumor function in mouse carcinomas of IL-10, we further analyzed if the expression of IL-10 would also correlate with the expression of cytotoxic enzymes, the expression of antigen presenting molecule, and the expression of IFN γ in human colorectal cancers (CRC) and normal adjacent tissue. In support of an induction of immune surveillance molecules by IL-10, expression of all three classes of molecules mediating cytotoxicity and immune recognition correlated very strongly with intratumoral IL-10 expression (Figures 6J–6L, S5D, and

(D) Quantitation of mRNA for cytotoxic effector molecules from tumors of PEG-IL-10 or control treated WT or IFN γ ^{-/-} mice. Analysis representative of two experiments (n = 3–10 mice/group), one PEG-IL10 and one minicircle.

(E) Comparison of CD8⁺ TILs secretion of Granzyme B (ELISPOT) upon PEG-IL-10 treatment in WT and IFN γ ^{-/-} mice. Data representative of two experiments, TILs of ten mice per treatment pooled and analyzed.

(F and G) PEG-IL-10 regulation of MHC molecules expression in tumors grown in WT and IFN γ ^{-/-} mice as determined by mRNA quantitation (F). (G) IHC quantitation of intratumoral MHC I, panel data representative of two experiments, three to five tumors taken per treatment group, 20–25 images per tumor analyzed.

(H) FACS analysis of MHC I regulation after in vitro stimulation of tumor cells with IFN γ , LL/2 compared with PDV6 tumor cells, data representative of three experiments.

(I) Experimental data from three experiments, n = 3–10 mice/group where data is the analysis of LL/2 tumor mRNA from IL-10 versus control treated mice.

(J) Effect IL-10 treatment exerts on LL/2 tumor growth in vivo, where data is representative of three independent experiments, once with PEG-IL-10, twice with IL-10 derived from minicircle. Error bar(s) represents standard error of the mean for all panels. *p < 0.05, **p < 0.01, ***p < 0.001, horizontal bars indicate duration of increased serum IL-10, average tumor sizes plotted. See also Figure S4.

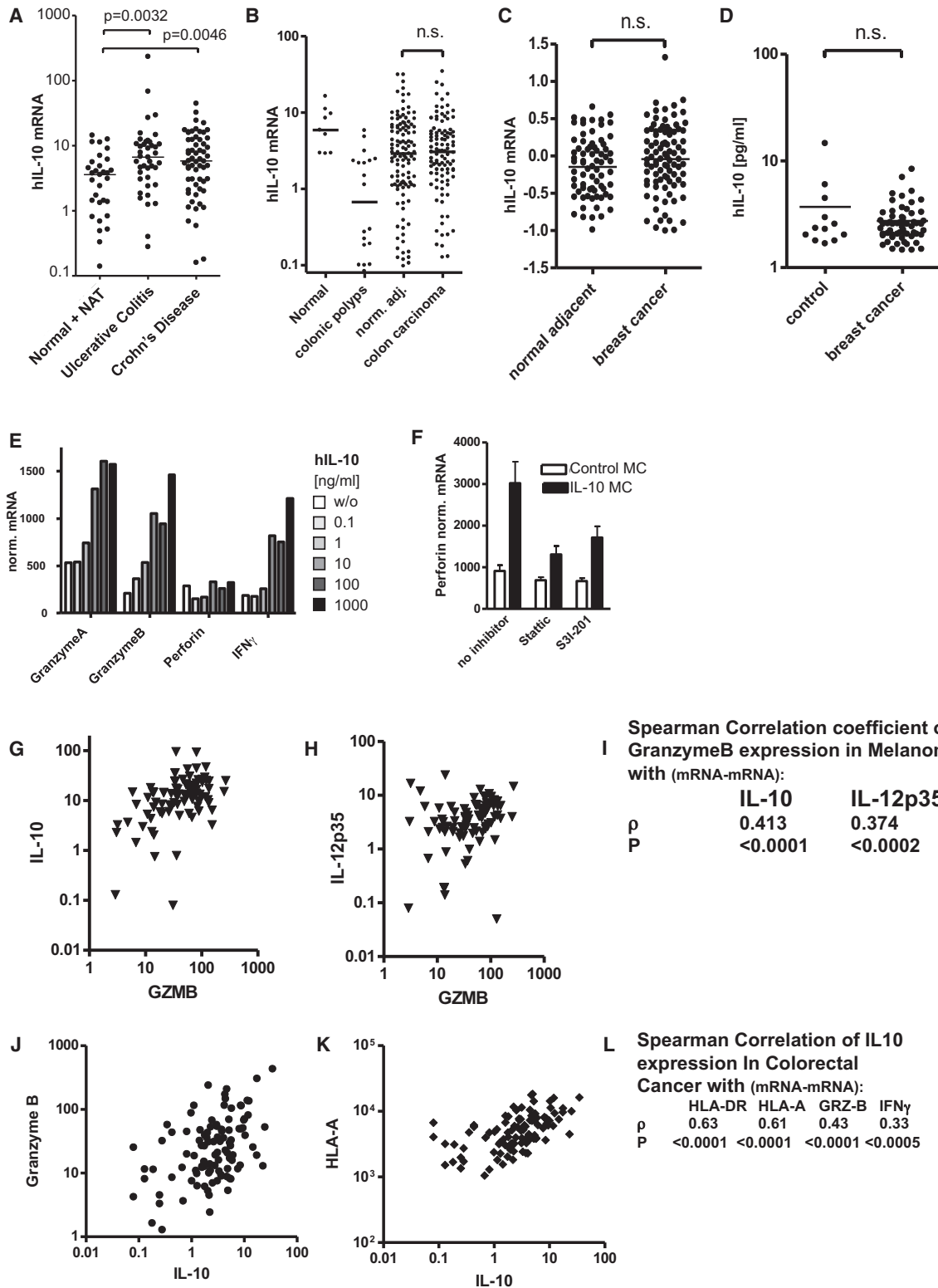


Figure 6. IL-10 Expression in Chronic Inflammation but Not in Tumor-Associated Inflammation -IL-10 Directly Correlates with Granzyme Expression in Human Tumors

(A–D) Inflammatory and cancer associated IL-10 expression. Association of hIL-10 mRNA expression with inflammation in human ulcerative colitis and Crohn's disease (A), colon cancer (B), human breast cancer (C), and IL-10 protein in serum of breast cancer patients (D).

(E) IL-10 regulation of cytotoxic enzymes mRNA in human CD8⁺ T cells (data representative of 15 independent experiments) in vitro.

S5E; data for normal tissues not shown). Surprisingly, the expression of MHC I and MHC II correlated with IL-10, but not with IL-12 in the tested human CRC (Figures S5F–S5H). These data may suggest that IL-10 is more important for the regulation of CD8⁺ activity and antigen presentation in human tumors than previously anticipated.

DISCUSSION

The data presented here challenge our current perception of how the immune system is regulated during tumor immune surveillance and the role of IL-10 therein. First, IL-10 is required for efficient immune surveillance against the incidence and progression of endogenously arising skin tumors. Second, IL-10 induces the expression of MHC I and IFN γ in tumors, two key molecules for immune surveillance against tumors. Third, our data suggest IL-10 induces the production of cytotoxic enzymes and IFN γ primarily in tumor-infiltrating CD8⁺ T cells by virtue of their high IL-10R expression.

The specific delivery of cytotoxic molecules to antigen expressing tumor cells by IL-10 requires the IFN γ -mediated upregulation of MHC molecules on the target cell. In human cancer patients, both MHC expression and CD8⁺ T cell activity in the tumor are very low but the presence of both have been correlated to improved survival of cancer patients (Galon et al., 2006). Interestingly, IL-10 expression in colorectal cancers correlated not only with the expression of granzymes, but also with the expression of IFN γ and antigen presenting molecules such as HLA-DR. Most experimental tumor immune therapies aim to rapidly increase the number of tumor-specific CD8⁺ T cells in the patient or the experimental animal. Unfortunately, the efficacy of such experimental immunological interventions is even in experimental tumor systems frequently limited to the early phases of tumor growth and to particularly immune-sensitive tumor types. Such rapid CD8⁺ T cell expansions require involvement of secondary lymphoid organs as the site of antigen presentation and a strong stimulation of CD4⁺ T helper cells. Although IL-10 has been shown to interfere with the activity and polarization Th1 and IFN γ production of CD4⁺ T cells in response to bacterial products (O'Garra and Vieira, 2007), we find that IL-10 directly activates and expands the antigen-specific, IFN γ producing CD8⁺ effector T cell pool in the tumor, leading to enhanced expression of intratumoral antigen presenting molecules. The direct activation of CD8⁺ T cells appears to circumvent the absolute requirement for CD4⁺ T cell help. Aside from indirect effects on antigen presentation, specific delivery of IFN γ to the tumor cells may also increase the sensitivity to apoptosis in tumor cells.

IL-10 is thought of as a broadly immune inhibitory cytokine, but the immune suppressive effects of IL-10 are most prominently uncovered in experimental and physiologic settings dependent on TNF α , such as Toll like receptor (TLR)-mediated immuniza-

tions with bacterial cell wall products and bacterial oligonucleotides or T cell costimulation such as with anti-CD40. In stark contrast, treatment with PEG-IL-10 exerts a very potent activation of the tumor-infiltrating effector T cell pool despite suppression of systemic TNF α and very limited systemic T cell responses. The therapeutic levels of IL-10 used in our studies are similar to endogenous high levels of IL-10 experienced under physiological stress conditions such as sepsis or pneumonia (Deng et al., 2006; Sawa et al., 1997; Steinhauser et al., 1999) (data not shown). TNF α is rate limiting for chemically-induced skin carcinomas and TNF α neutralizing antibodies are in clinical trials for treating several tumor types (Szlosarek et al., 2006). It is possible that suppression of TNF α production contributes to the antitumor effect of IL-10 by limiting tumor-promoting innate inflammation.

Chronic inflammation is associated with increased incidence of cancer. Inflammatory cytokines like IL-1 β and TNF α , in turn, are associated with decreased survival of human cancer patients (Mumm and Oft, 2008). Many of these cytokines are expressed in human tumor tissue and in chronic inflammatory lesions at similarly high levels. Surprisingly, we found that IL-10 is not elevated in human tumor tissue, despite the presence of inflammatory cells and the prominent expression of other inflammatory cytokines such as IL-23 (Langowski et al., 2006). Moreover, expression of IL-10 correlated with the expression of cytotoxic mediators such as Granzyme B suggesting that endogenous IL-10 regulates cytotoxic activity of the immune response within human tumors similar to the mouse models analyzed in this study. These data also support the concept that tumor-associated inflammation may share many characteristics with chronic inflammatory lesions such as the expression of proinflammatory cytokines, but not the expression of IL-10 or cytotoxic enzymes. Tumors, therefore, appear to thrive in an inflamed environment poor in cytotoxic effector cells and low in IL-10.

In many experimental settings in vitro, IL-10 has been shown to antagonize the expression and function of the Th1 cytokine IFN γ , with primary CD4⁺ T cell proliferation and Th1 polarized T cell cultures being especially sensitive to IL-10 (Moore et al., 2001). Overexpression of IL-10 in mice can inhibit experimental autoimmune encephalitis (EAE), a disease mediated by proinflammatory IL-17 producing CD4⁺ T cells (Cua et al., 1999; McKenzie et al., 2006). Acute systemic inflammatory responses and rapid systemic expansions of CD8⁺ T cells in secondary lymphoid organs are dependent on TNF α and IL-12 and enhanced when IL-10 or the IL-10 receptor is inhibited during acute infection models (Brooks et al., 2006). In contrast, therapeutically elevated levels of IL-10 specifically enhanced expression of IFN γ and granzymes peripherally in tumor-infiltrating CD8⁺ T cells experiencing antigen. IL-10 stimulation of expression and secretion of IFN γ by CD8⁺ T cells in vivo and in the tumor appears to be direct. Moreover, based on studies in cytokine knockout mice IL-10-mediated antitumor function appears to be independent of TNF α and IL-12 (data not shown).

(F) Effect of blockade of STAT3 phosphorylation on regulation of perforin expression by IL-10 (six independent experiments). Error bar(s) represents standard error of the mean.

(G–I) Correlation plot between the mRNA expression of IL-10 with Granzyme B (G) or IL-12p35 expression in human melanoma samples (H), and Spearman correlation with significance (I).

(J–L) Correlation between IL-10 and MHC I (HLA-A) (J) or Granzyme B (K) mRNA expression in human colon cancer samples and Spearman correlation with significance for IL-10 and MHCII (HLA-DR), MHC I (HLA-A), Granzyme B, and IFN γ (L). *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S5.

We also found that IL-10 directly induces the expression and secretion of cytotoxic molecules in CD8⁺ T cells *in vitro* and *in vivo*. However, increased expression and release of cytotoxic molecules from tumor-infiltrating T cells was not sufficient to achieve significant antitumor function. Antitumor function and tumor cell killing was dependent on IFN γ secretion by IL-10 activated tumor-infiltrating CD8⁺ T cells. IFN γ was required for the upregulation of intratumoral MHC-I and MHC-II molecules but not for cytotoxic enzymes or T cell infiltration. The expression of MHC molecules in human cancers is typically very low and thought to contribute to the immune evasion of human cancers (Seliger *et al.*, 2002). *In vitro* treatment of human and mouse cancer cells with IFN γ can restore expression of MHC molecules and the antigen processing pathways, indicating that these pathways are functional in tumor cells and are not genetically lost (Seliger *et al.*, 2002).

Effectiveness of immunotherapy is frequently limited by unintended side effects, such as systemic autoimmunity, presumably due to the reduction in signaling threshold of the T cell receptor complex. PEG-IL-10-treated animals showed limited systemic responses in primary and secondary lymphatic organs with activation of T cells predominantly in the periphery and at the tumor site (Figure 2 and data not shown). Infiltration of monocytes and lymphocytes was found in many glandular organs such as the parotid gland, the liver, and the pancreas in mice treated with PEG-IL-10. The cellular components of this infiltrate were similar in type and activation to infiltrates documented in the tumors of such animals and lead in some organs to varying degrees of immunopathology characterized by apoptosis of epithelial cells. It remains unclear if the CD8⁺ T cells within these parenchymal lesions are autoreactive or whether this represents a not antigen-specific bystander effect. Although mice subjected to a curative treatment schedule of PEG-IL-10 did not show weight loss over 5%, this immune-mediated toxicity could lead to dose limiting toxicities if observed in human cancer patients treated with PEG-IL-10.

The lack of T cell infiltration, their diminished intratumoral activity, and deficient MHC expression in tumors are prominent mechanisms for tumor evasion in both mice and men. The data presented here suggest that IL-10 can induce the minimal immunological circuit required to overcome all three mechanisms at once. By stimulating the activity of peripheral CD8⁺ T cells in the tumor, IL-10 delivers cytotoxic mediators and IFN γ to the tumor microenvironment without further activation of central immune mechanisms. IL-10-induced elevation of the Th1 cytokine IFN γ locally in tumor-infiltrating CD8⁺ T cells may facilitate chemokine cascades inducing further T cell infiltration and increased antigen presentation primarily localized to the tumor microenvironment. These data suggest that the use of pegylated IL-10 as a monotherapy could elicit an immune biology missing from most cytokine or vaccine therapies employed to date, and may therefore represent a beneficial treatment for human cancer patients.

EXPERIMENTAL PROCEDURES

Tumor Models

Chemical induction of skin tumors was performed as previously described (Langowski *et al.*, 2006). Three-month-old C57BL/6 or Balb/c mice were

treated with 100 μ g DMBA/mouse and then treated twice weekly with 30 μ g TPA for a duration of 5 months. For targeting IL-10 to antigen-presenting cells, IL-10 transgenic mice expressing hIL-10 under the control of the MHCII promoter were used (Groux *et al.*, 1999).

Breast carcinomas in FVB^{MMTV-rtHer2} transgenic mice were monitored until the tumor size reached between 100 and 2,000 mm³. Animals were allowed to have up to two such large tumors. The mice were separated in two groups, normalized for tumor size and total tumor burden per animal, and subjected to PEG-IL-10 (0.5 mg/kg two times a day subcutaneously) or control treatment.

In tumor transplantation studies, tumor cell lines were mixed with 50% growth-factor-reduced Matrigel (GIBCO) or resuspended in PBS and injected subcutaneously or intradermally. Squamous carcinoma cells (PDV6 [Langowski *et al.*, 2006]; DNAX) are derived from the DMBA transformed keratinocyte cell line PDV (C57BL/6) and were orthotopically injected into C57BL/6 mice (Langowski *et al.*, 2006). CT-26, 4T1 (colon, breast cancer; Balb/c) CM3 (melanoma; DBA) were received from ATCC and subcutaneously implanted. Typically, tumors were left untreated for at least 2 weeks and until they reached a size of 100–250 mm³. For tumor rechallenge, mice were treated until complete regression of the tumor, left untreated for up to 8 months, and reinjected with the original tumor cell line. In the vaccine control arm, a freeze-thaw preparation of the same tumor cell line was injected subcutaneously three times several weeks before tumor rechallenge.

All animal experiments were performed under the guidance and approved by the Institutional Animal Care and Use Committee of Schering-Plough Biopharma, Palo Alto and in its Association for Assessment and Accreditation of Laboratory Animal Care-approved facility.

Cytokine Treatment and Cell Depletion

PEG-IL-10 was injected at the indicated doses, typically 0.1 mg/kg or 0.5 mg/kg daily or twice daily for the times indicated by a black bar below the x axis. Tumor growth was followed for an observation period of up to 3 months. The pegylated mouse IL-10 used in the studies has a serum T1/2 of 3.7 hr after subcutaneous injection. No anti-mIL-10 antibodies were detected in mice after repeated dosing.

For cell ablation studies, 0.35 mg per mouse of anti-CD8 (53-6.7) 0.25 mg per mouse anti-CD4 (GK1.5), or anti-NK1-1 (PK136) antibody at 0.3 mg per mouse (ebioscience) was injected intraperitoneally once per week. For depletion studies, animals were not dosed with PEG-IL-10 on the days they received the antibodies to limit anti-antibody responses. Antibodies were dosed 3 days and 1 day prior to initiation of dosing with PEG-IL-10 and every 7 days thereafter.

Tumor-Infiltrating Immune Cell Analysis

Mouse tumor biopsies were harvested snap-frozen, fixed, and subjected to immunohistochemistry (IHC) and counterstained with hematoxylin. Antibodies used include MHC I, MHC II, CD3, CD31, CD8a, CD8b, CD4, CD11b, and CD11c (Biolegend). Images of IHC for tumor-infiltrating cells were acquired using Zeiss Mirax scanner and software. Randomly selected fields were quantified either by manual counting or by using Zeiss Axiovision software (Carl Zeiss, Jena, Germany) to measure the staining intensity or the number of positive events. Where intensity measurements were taken, the reduction in light intensity per pixel in the respective color spectrum was recorded. The sum of the light intensity reductions of all pixels in an identical region of interest was calculated and plotted for all samples in one measurement group. Some studies were counted blindly using random selected fields (number set derived from <http://www.random.org>) and plotted against the treatment groups after unblinding. For cell isolation, subcutaneous tumor masses were dissected and digested with 2 mg/ml collagenase and 0.5 mg/ml DNase (Sigma). Single-cell suspensions were created and live cells collected by centrifugation over a Histopaque (Sigma) gradient. Similar isolations from naive spleen were performed for comparison. Collected cells were positively selected for CD8 by magnetic selection (Miltenyi Biotec).

Cytotoxicity Assay

Spleens and lymph nodes from OT1 mice were isolated and stimulated in IMDM media supplemented with 5% FCS, containing 1 μ g/ml SIINFEKL peptide and 25 U/ml mL-2 for 3 days *in vitro*. Activated CD8⁺ T cells were

double purified using anti-CD8 Macsbeads (Miltenyi, Auburn, CA, USA), cultured at 1×10^6 cells/ml in 48-well plates for 24 hr in the presence of IL-10. Cells were counted, washed, and mixed with peptide pulsed target cells (1 μ g/ml 2 hr) or EG-7 target cells for 4 hr in U-96 well plates at an E/T of 10:1. Cell supernatants were harvested and Cytotox 96 assays (Promega) were performed following the manufacturers instructions.

ELISA and ELISPOT Analysis

ELISA assays from R&D Labs (Minneapolis, MN, USA) were performed according to the manufacturer's guidelines. Tumor-infiltrating (CD8⁺) T cells were purified from tumor-bearing mice treated for 7 days with 0.1 mg/kg PEG-IL-10 bid subcutaneously. Single cell suspensions of double purified CD8⁺ T cells were plated at 10,000 and 25,000 cells/IFN- γ or Granzyme B ELISPOT well in triplicate, nonstimulated, or treated with PMA 20 ng/ml, anti-CD3 soluble at 20 μ g/ml, anti-CD3 beads 20 μ g/ml, or Sepharose-Pro-A beads. Isolated and purified TILs were exposed to these conditions for 24 hr on ELISPOT membranes. ELISPOTs were developed following manufactures instructions, counted, and quantified with Immunospot 3 (Cellular Technology Limited, Cleveland). Antigen specificity was performed similar. Briefly, TILs were isolated from individual tumors, five tumors in parallel, plated at 1,000, 2,000, and 5,000 cells/well in triplicate and exposed to media, 1 μ g/ml anti-CD3, or 10:1 E/T of cognate tumor or irrelevant tumor cells. Tumor cells were exposed to 10 ng/ml IFN γ for 24 hr prior to plating with TILs to ensure MHC I expression that was verified by FACS analysis.

In Vitro CD8⁺ T Cell Assay

CD8⁺ T cells were isolated from tissue or peripheral blood using Miltenyi Beads for enrichment. Cells were exposed 3 days to immobilized anti-CD3/anti-CD28 at 10 and 2 μ g/ml, respectively in AIM V, (human) or cRPMI containing 1 μ M β -mercaptoethanol and 10% FCS. Cells were washed into AIM V and replated. IL-10 exposures ranged from 0.1 to 1000 ng/ml for 1 hr up to 3 days.

Human Tissue Samples

All human tissues were obtained under Institutional Review Board (IRB)-approved protocols. Depending on the collection protocols established at each site, tissues were acquired either under IRB-approved waiver of consent (National Disease Research Interchange [NDRI] and Cooperative Human Tissue Network [CHTN]) or by informed consent (Princess Alexandra Tumour Tissue Bank [PAH], Zoion, Ardaís, and Anatomical Gift Foundation [AGF]). All samples have been de-identified or anonymized, so investigators have no ability to identify the patients. Paired human tumor and normal adjacent tissues were obtained from patients undergoing routine therapeutic surgery from the following sources: Dr. David Gotley of the PAH in Woolloongabba (Queensland Australia), Zoion Diagnostics (Hawthorne, NY, USA), and NDRI (Philadelphia, PA, USA). Normal human tissue and inflammatory bowel disease tissue were obtained from patients undergoing routine surgery (NDRI, Ardaís Corporation [Lexington, MA, USA] and CHTN [Nashville, TN, USA]), from short hour autopsy (≤ 5 hr, Zoion Diagnostics), and from transplant donors (AGF, Hanover, MD, USA). All surgical samples were frozen as quickly as possible, typically within 1 hr of excision. The majority of samples were accompanied by demographic information and pathology reports.

Gene Expression Analysis and Statistical Methods

All mRNA analysis was performed using quantitative RT-PCR. Briefly, total RNA was isolated by standard methodologies and reverse transcribed. Expression analysis for marker-specific mRNAs was measured using real-time quantitative PCR (ABI 5700) with SYBR Green PCR Mastermix (Applied Biosystems). Ubiquitin levels were measured in a separate reaction and used to normalize the data by the $\Delta - \Delta C_t$ method, using the mean cycle threshold (C_t) value for ubiquitin and the gene of interest for each sample. Statistical analysis was performed using GraphPad Prism software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.ccr.2011.11.003.

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