Tumor-Induced Suppression of CTL Expansion and Subjugation by gp96-Ig Vaccination

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Abstract

Established tumors suppress antitumor immune responses and induce tolerance by incompletely characterized mechanisms, and this phenomenon is an important barrier to tumor immunotherapy. Single vaccination with tumor cells expressing gp96-Ig stimulates robust expansion of tumor-specific CTLs in tumor-naïve mice and this expansion is inhibited by established tumors. Interestingly, frequent vaccinations restore antitumor immune responses in the presence of established tumors. Syngeneic EG7 tumor-bearing mice have heterogeneous responses to frequent vaccination with EG7-gp96-Ig, with 32% complete responders and 68% partial responders. Comparison of responders to nonresponders revealed an inverse correlation between tumor-specific CTL expansion in the peripheral blood and tumor size. To identify immune cells and molecules associated with effective antitumor immune responses, reverse transcription-PCR arrays were performed using cells isolated from the vaccination site. ELISAs, cellular phenotyping, and tumor immunohistochemistry were also performed comparing vaccine responders to nonresponders. These data show that up-regulation of T-bet, RORγt, IFNγ, CCL8, CXCL9, and CXCL10 at the vaccination site are associated with vaccine-induced antitumor immunity. These data correlate with increased CTL expansion in the peripheral blood of responders, increased infiltration of responder tumors by CD8+ cells and interleukin-17+ cells, and decreased infiltration of responder tumors by CD11b+Gr-1+ cells and FoxP3+ cells. Furthermore, serum ELISAs revealed a significant elevation of transforming growth factor-β in nonresponders as compared with responders. Interestingly, CD8+ T cells isolated from responders and nonresponders have equivalent cytotoxic activity *in vitro*. Taken together, our data suggest that established tumors may escape immunosurveillance by preventing clonal expansion of tumor-specific CTL without inducing anergy. [Cancer Res 2009;69(5):2026–33]

Introduction

The hypothesis that cancer is controlled by the immune system to varying degrees during the life of the host has received substantial support over the last decade (1). These data have supported the concept that immunotherapy of cancer may provide valuable clinical benefits, but also led to the realization that the evolution of many tumors is accompanied by the acquisition of immunosuppressive properties. Recruitment of regulatory cells by tumors, tumor cell down-regulation of immune costimulatory molecules, or up-regulation of immune negative regulatory molecules, preferential polarization toward Th2 responses, and secretion of immunosuppressive cytokines by tumors and/or their surrounding stroma are all mechanisms that have been proposed to mediate tumor-induced immunosuppression (2–8).

Effective cancer immunotherapy is widely believed to originate with appropriately activated CD8+ CTLs; however, these CTLs will not be capable of providing lasting clinical responses if they are circulating in the presence of multiple systemic and locally immunosuppressive cells and factors. There is a lack of studies which provide information regarding the context in which successful immune responses are generated as compared with failed responses for a given therapy, particularly in the therapeutic setting. Such information would provide important insight into which tumor-induced immunosuppressive mechanisms were most important to target.

We have previously shown that replacing the KDEL ER retention sequence of the heat shock protein gp96 with the Fc portion of the IgG_1 protein results in the expression of a gp96-Ig fusion protein capable of secreting tumor-specific antigens to the extracellular space and effectively stimulating antigen cross-presentation to CTL (9). Using this model, we have shown that immunization with a live tumor cell vaccine expressing gp96-Ig not only leads to CTL-mediated rejection of the vaccine cells themselves, but is capable of rejecting established tumors (9, 10). Importantly, the ability of the vaccine to stimulate CTL expansion is significantly inhibited in the presence of an established tumor (10); however, this study also showed that more frequent vaccinations were sufficient to retard the growth of established tumors. The effect of frequent vaccinations on CTL expansion was not determined.

In the current study, we show that the response of EG7 tumor-bearing mice to frequent vaccinations with gp96-Ig is heterogeneous; with responses ranging from slightly delayed tumor growth to complete tumor rejection. The heterogeneity in antitumor responses allowed comparisons between productive and nonproductive responses. Using a model system with the tumor-specific neoantigen, ovalbumin (ova), we show that there is an inverse correlation between tumor growth and tumor-specific CTL expansion. To elucidate the mechanisms inhibiting CTL expansion, a comparison of immune cells and products was performed at various sites in both responder and nonresponder mice. CTL expansion and tumor rejection coincides with Th1 and Th17 polarizing conditions established at the site of immunization. Importantly, the Th1 polarizing conditions established at the site of immunization correlate with CTL expansion in the peripheral blood and infiltration into a regressing tumor, as well as reduced serum concentrations of CXCL10 and transforming growth factor-β (TGF-β).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Mice. Wild-type mice in the C57BL/6 (B6) background (CD45.2+) were obtained from Charles River Laboratories. CD45.1+ SJL mice were purchased from The Jackson Laboratory. C57BL/6 OT-I mice were obtained from Dr. M. Bevan (University of Washington School of Medicine, Seattle, WA). Green fluorescent protein (GFP) transgenic mice were obtained with permission from the producers (11). All mice were used at 6 to 12 weeks of age.

Cell lines. EG7, the OVA-transfected EL4 lymphoma line, generously provided by Dr. M. Bevan, was further transfected with the vector pCMG-His containing gp96-Ig as described previously (9). NIH 3T3 cells were transfected with OVA in pAC-neo-OVA (generously provided by Dr. M. Bevan) and with pCMG-His containing gp96-Ig. Cell lines were maintained in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 1 μg/mL gentamicin, and the appropriate antibiotics at the indicated concentrations: G418 (1 μg/mL) and histidinol (2 mmol/L).

Antibodies and tumor inoculation. Fluorescent antibodies were purchased from BD PharMingen and eBioscience. EG7 cells were isolated from log-phase cultures and washed in PBS. One million cells were then injected s.c. in the hind-flank of mice in 0.1 mL of PBS.

Purification and adoptive transfer of OT-I cells. GFP-marked OT-I cells (or CD45.2+ OT-I cells) were purified by positive selection with anti-CD8 using magnetic separation (≥95% pure, determined by flow cytometry; Miltenyi Biotec). Three days after tumor inoculation, 10^6 GFP-OT-I cells (or Ly5.2+ OT-I cells) were adoptively transferred through tail veins of C57BL/6 mice (or Ly5.1+ SJL mice) in 0.15 mL of HBSS.

Immunization. Two days after adoptive transfer of OT-I or GFP-OT-I (experimental day 5), 10^6 nonirradiated EG7-gp96-Ig cells or control EG7 cells were injected i.p. in 0.1 mL of PBS. For some experiments, mice were immunized i.p. with 3T3-OVA-gp96-Ig. Immunizations were repeated as described above on subsequent days as indicated.

Histology and immunohistochemistry. EG7 tumors were excised between days 10 and 16 in pairs (progressors and regressors), submerged in optimal cutting temperature freezing compound and flash-frozen in liquid nitrogen. Sections were then cut at 5 μm and mounted on glass slides. Sections were fixed and stained as previously described (12). Imaging was performed using a LSM-510 confocal microscope (Zeiss). Acquired images were analyzed using ImageJ (NIH, MacBioPhotonics) software.

Reverse transcription-PCR. Total RNA was harvested from peritoneal exudate cells (PEC) between days 10 and 16 using the RNeasy mini-kit (Qiagen). RNA integrity was analyzed using an Agilent Bioanalyzer 2100; RNA with an integrity value of ≤0.8 was discarded. cDNA was synthesized using the RT^2 First Strand Kit (Superarray). A custom 96-well reverse transcription-PCR (RT-PCR) array was developed (Supplementary Fig. S1), primer sets were synthesized (Sigma), and RT-PCR was performed using the RT^2 SYBR green PCR master mix (Superarray) on an Applied Biosciences 7300 PCR platform.

ELISA. Interleukin (IL)-1β, CXCL10, and TGF-β levels were determined using the Quantikine ELISA kits (R&D Systems) according to the manufacturer's protocol. Antibody pairs for IFNγ, IL-17, and IL-6 were obtained from BD PharMingen and were analyzed in Sandwich ELISA assays by coating plates with capture antibody (1 μg/mL), followed by loading serially diluted serum samples, biotin-conjugated detection antibodies (1 μg/mL), and horseradish peroxidase–streptavidin (0.5 μg/mL, BD Biosciences).

***In vitro* cytotoxicity assays.** Total splenocytes were harvested at the indicated time points and total CD8+ cells were isolated by magnetic bead separation as described above. EG7 target cells were maintained in cell culture according to standard protocols, labeled with PKH2 green fluorescent cell linker (Sigma) according to the manufacturer's protocols and plated in 96-well plates (10^4/well). Purified CD8+ cells were then added to the 96-well plates containing the labeled EG7 cells at the indicated ratios and incubated for 4 h at 37°C. Following incubation, total cells were harvested and labeled with the 7-AAD vital stain (eBioscience) prior to analysis by flow cytometry.

Statistical analysis. Paired comparisons were performed using Student's *t* test, multiple analysis was performed using a one-way ANOVA, *P* values are indicated as necessary.

Results

OT-I expansion correlates with tumor regression. To determine whether frequent vaccinations with gp96-Ig induce CTL expansion in the presence of an established tumor, EG7 tumor-bearing mice were adoptively transferred with GFP+ OT-I cells and subsequently vaccinated with EG7-gp96-Ig or 3T3-ova-gp96-Ig cells (Fig. 1*A*; Supplementary Fig. S2). Peripheral blood was withdrawn from the tail at daily intervals and analyzed by flow cytometry for expression of GFP and CD8 (Fig. 1*B*). Although the overall percentage of CD8+ cells out of the total lymphocyte population remains stable (data not shown), the percentage of OT-I cells out of the total CD8+ cells increases following several vaccinations with EG7-gp96-Ig. The degree of OT-I expansion is variable between mice, with some animals maintaining OT-I levels in the peripheral blood similar to nonvaccinated control mice.

Due to the heterogeneity in OT-I expansion between individual mice, we sought to determine whether there was a correlation between OT-I expansion and overall tumor size. The kinetics of OT-I expansion, illustrated in Fig. 1*B*, suggest that there is a gradual increase in the expansion of OT-I cells following the first three doses of gp96-Ig that peaks near the time of the fourth immunization and then rapidly drops back to baseline by the fifth vaccination. Therefore, to plot the correlation between tumor-specific CTL expansion and tumor size, we plotted the maximum percentage of OT-I cells out of total CD8+ cells achieved between days 10 and 20 on the *Y*-axis and the tumor diameter at day 20 on the *X*-axis (Fig. 1*C*). This analysis shows a significant inverse correlation between maximal OT-I expansion in the peripheral blood and tumor size.

The drop in peripheral blood OT-I cells during the third week of the experiment was unexpected. It was possible that a humoral or cellular anti-GFP response was generated which resulted in the rapid clearance of OT-I cells from the blood. To investigate this possibility, we performed experiments as shown in Fig. 1*A* using CD45.1+ mice as the tumor-bearing mice and adoptively transferred CD45.2+ OT-I cells instead of GFP+ cells. Vaccinations were performed and peripheral blood OT-I cells were monitored by flow cytometry based on the dual expression of CD8 and CD45.2 (Supplementary Fig. S2). GFP-negative, CD45.2+ OT-I cells persist for at least 30 days in a CD45.1+ host in the peripheral blood.

Tumor regression is independent of extrinsic tumor-specific CTLs. An effective anti-EG7 tumor response in the absence of adoptively transferred OT-I cells requires that the host immune response prime and expand tumor-specific T cells against ova or other uncharacterized EG7 tumor antigens. To address this requirement, experiments were performed as described in Fig. 1*A*, in the absence of adoptively transferred OT-I cells. Survival curves comparing vaccination in the absence or presence of OT-I cells are shown in Fig. 2*A*. To comply with Institutional Animal Care and Use Committee regulations, the survival end point for these studies was a tumor area of ≥225 mm^2. Overall survival was not significantly improved for mice receiving OT-I cells in addition to vaccination compared with mice receiving gp96-Ig vaccination alone (42% and 32%, respectively). These data indicate that immunization with gp96-Ig leads to the expansion of endogenous tumor-specific T cell populations capable of mediating tumor rejection.

Responses to vaccination with gp96-Ig are heterogeneous. The observation that tumor-specific CTL expansion, tumor size, and survival following vaccination with gp96-Ig differs between individual mice suggests that vaccine-induced antitumor responses

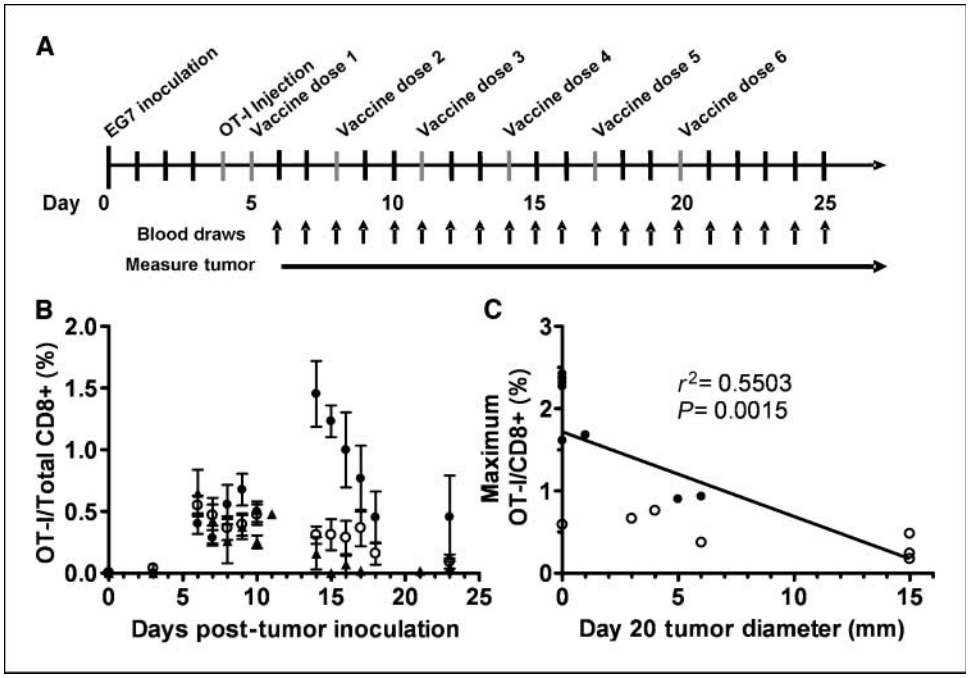

Figure 1. Tumor-specific CTL expansion correlates with tumor regression. *A,* the schedules of EG7 tumor inoculation, OT-I injection, and immunizations. *B,* the percentage of OT-I cells out of the total CD8+ cells in the lymphoid compartment was determined by flow cytometry. ○ and ●, EG7-gp96-Ig immunized mice (seven representative mice per group, divided into two groups based on observed dichotomy in OT-I expansion); ▲, nonvaccinated control mice (two representative mice). *C,* the maximum percentage of OT-I cells out of the total CD8+ lymphoid cells between experimental days 10 and 20 (*Y*-axis); the tumor diameter at experimental day 20 (*X*-axis). ○ and ●, maximum OT-I expansion of mice in *B*. A linear regression analysis was performed and found to be statistically significant with P = 0.0015 and with a correlation coefficient (r^2) of 0.5503 (n = 14).

are heterogeneous. After vaccinating more than 40 mice, it became apparent that the antitumor responses to gp96-Ig were divided into two groups; those that "progressed" after a transient delay in tumor outgrowth and those that "regressed." The determination between mice that progressed or regressed was tracked to ~10 days post-tumor inoculation (Fig. 2*B*). After this time, mice bearing tumors that had decreased in diameter (regressors) would eventually become tumor-free and enjoy long-term disease-free survival. Mice

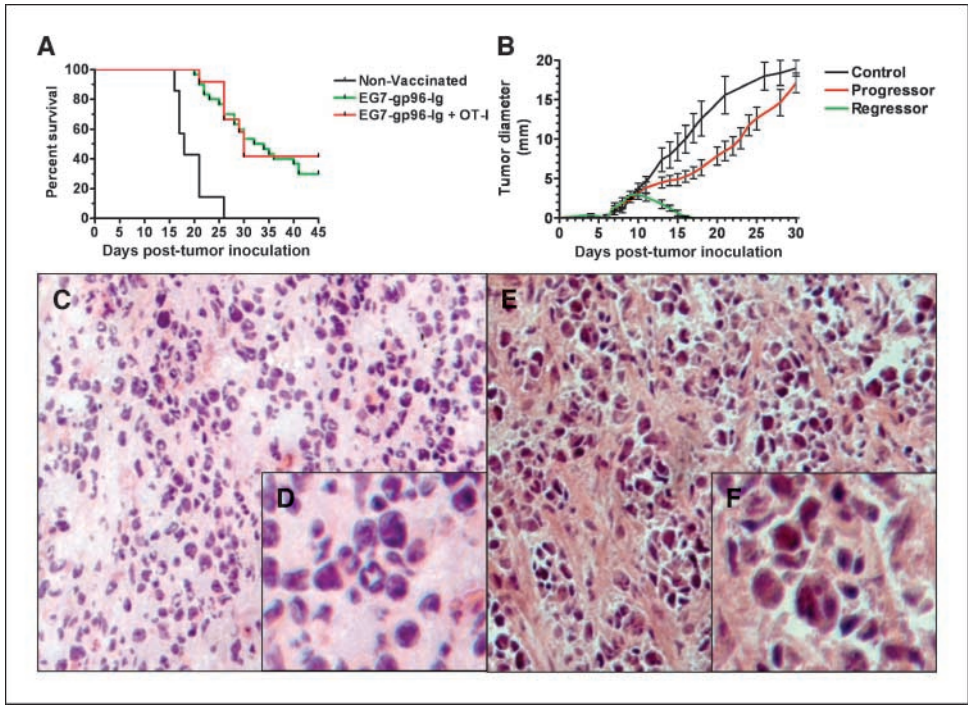

Figure 2. Extrinsic tumor-specific CTLs are not required for long-term tumor-free survival. *A,* wild-type mice were inoculated with EG7 tumor and then either adoptively transferred with OT-I cells and vaccinated with EG7-gp96-Ig (*red line,* n = 12), vaccinated with EG7-gp96-Ig without an OT-I adoptive transfer (*green line,* n = 30), or transferred with OT-I cells but not vaccinated (*black line,* n = 7). Vaccinations were performed according to the schedule outlined in Fig. 1*A*. To comply with Institutional Animal Care and Use Committee protocols, the survival end point for this study was a tumor area of ≥225 mm^2. Median survival for nonvaccinated controls was 18 d, 33 d for EG7-gp96-Ig vaccinated mice, and 30 d for EG7-gp96-Ig vaccinated plus OT-I adoptive transfer mice. Overall survival for the three groups was 0%, 32%, and 42%, respectively. *B,* wild-type mice bearing EG7 peripheral tumors were vaccinated as described in Fig. 1*A*, in the absence of adoptively transferred OT-I cells. Following vaccination, two outcomes were observed, with the first group having rapidly regressing tumors (regressor) and the second with slightly delayed tumor growth as compared with controls (progressor). *C,* progressor tumors were excised, sectioned, and stained with H&E (*D,* close-up of *C*). *E,* regressor tumors were excised, sectioned, and stained with H&E (*F,* close-up of *E*). Tumors for *C* to *F* were excised at day 13 from a paired set of mice in the same experiment.

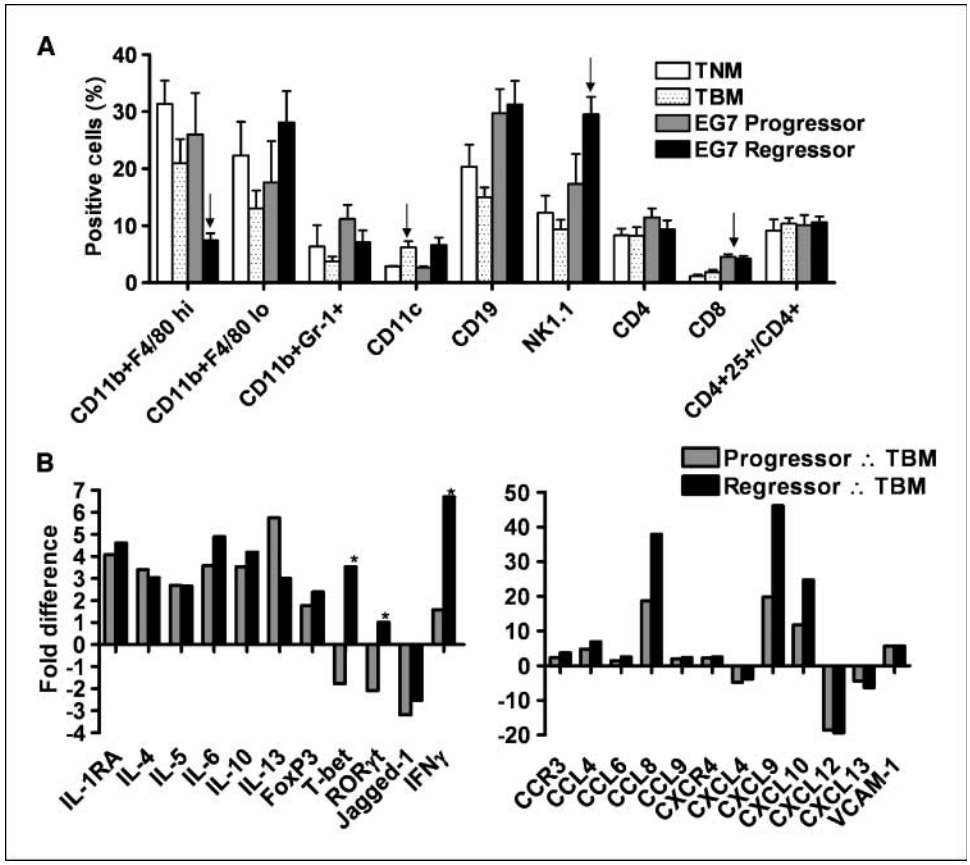

Figure 3. Tumor regression and CTL expansion occur following the expression of Th1 and Th17 polarizing factors at the site of vaccination. *A,* PECs were collected from tumor-naïve mice (*TNM*) or EG7 tumor-bearing mice (*EG7 TBM*) and analyzed by flow cytometry for the indicated surface markers. There were no statistically significant differences between groups. *B,* RT-PCR was performed using a custom 96-well array (Supplementary Fig. S1) to examine differences between EG7 tumor progressors, tumor regressors, and tumor-bearing mice controls. As compared with tumor-bearing mice controls, each of the differences indicated for progressors (*gray columns*) or regressors (*black columns*) reached statistical significance ($P < 0.05$). *, $P < 0.05$, statistically significant differences for the regressors as compared with the progressors. $n \geq 5$ arrays (with a single array per mouse) for each of the four conditions (TNM, TBM, progressor, and regressor).

bearing "progressor" tumors displayed variable degrees of delayed tumor growth as compared with nonvaccinated controls, with responses ranging from transient tumor size stabilization to slightly delayed tumor growth. Due to this heterogeneity, the possibility arose to compare successful and failed gp96-Ig–induced antitumor responses. Tumor masses were excised from mice bearing either progressor and "regressor" tumors at paired time points between days 10 and 16 post-tumor inoculation. Subsequent sectioning and staining of these sections revealed that progressor tumor masses were relatively homogeneous and contained few small lymphocytes (Fig. 2*C* and *D*), whereas regressor tumor masses consisted of focal accumulations of tumor cells and small lymphocytes between large tracts of fibrous extracellular matrix (Fig. 2*E* and *F*).

Tumor regression is associated with expression of Th1 and Th17 polarizing factors at the site of immunization. Because prior studies showed that an established peripheral tumor (in the hind flank) suppressed gp96-Ig–induced T cell expansion at the site of vaccination (peritoneal cavity), we compared the transcriptional profiles of PECs from tumor-naïve mice and from EG7 tumor-bearing mice. RT-PCR was performed using total RNA extracted from PECs isolated from tumor-naïve control mice or from mice bearing EG7 tumors at 10 and 16 days post-tumor inoculation. This analysis revealed surprisingly few differences between the two groups, with only an increase in the expression of IL-1RA, CCR3, CXCL13, and RORγt (retinoid-related orphan receptor-γ) reaching statistical significance ($P \leq 0.05$, data not shown). Phenotypic analysis was also performed on PECs from tumor-naïve mice and tumor-bearing mice (Fig. 3*A*), demonstrating that increased fractions of CD11c+ cells are present within the PEC from tumor-bearing mice as compared with TNM. The other cell populations examined seemed relatively unchanged.

Furthermore, because heterogeneous responses to vaccination with gp96-Ig were observed, we compared the transcriptional profiles of PECs from mice that were rejecting the peripheral EG7 tumor (regressors) to those that were not (progressors) following vaccination with gp96-Ig. Because a clonally expanded population of transgenic OT-I cells may artificially alter the total RNA isolated from the PECs, and because they are not necessary for tumor rejection, these experiments were performed in the absence of adoptively transferred OT-I cells. These data (Fig. 3*B*) reveal many vaccination-induced changes in the patterns of chemokines, cytokines, and interleukins between both progressors and regressors to the control tumor-bearing mice. Importantly, regressors produce higher levels of the key Th1 and Th17 transcription factors, T-bet and RORγt, proinflammatory chemokines CCL8, CXCL9, and CXCL10, increased expression of IFNγ, and reduced levels of the pro-Th2 interleukin-13. Regressors were also found to have elevated levels of CXCL10 in peritoneal fluid compared with progressors, 71.90 ± 30.73 and 36.72 ± 4.484 pg/mL, respectively ($n = 5$ per group, peritoneal fluid diluted to 4 mL in PBS). Subsequent phenotypic analysis between progressors and regressors revealed increased peritoneal infiltration by CD11c+ cells ($P = 0.0128$) and NK1.1+ cells ($P = 0.05$), and decreased CD11b+F4/80-hi cells ($P = 0.0215$) in regressors as compared with progressors (Fig. 3*B*). Conversely, there was a gradual increase in the percentage of peritoneal CD11b+F4/80dim cells in regressors. CD8+ T cells were increased in the peritoneal cavity in both groups of vaccinated mice as compared with nonvaccinated tumor bearing and tumor-naïve control mice ($P < 0.0004$).

Tumor regression is associated with increased tumor infiltration by CD8+ and IL-17+ cells and decreased CD11b+Gr-1+ and FoxP3+ cells. Previous data collected from the peritoneal cavity suggested that tumor regression following vaccination with gp96-Ig was associated with the expansion of tumor-specific CTLs in the peritoneal cavity (9, 13) as well as in the peripheral blood (Fig. 1). Furthermore, tumor regression is associated with the increased expression of factors by cells at the site of vaccination with roles in the development of Th1 and Th17 polarized cells (T-bet and RORγt, respectively). To confirm that tumor regression was associated with the recruitment of CTLs within the tumor microenvironment, immunohistochemistry was performed on sections from both progressor and regressor tumors. Intratumoral staining illustrates that regressor tumors recruit significantly more CD8+ cells within the tumor microenvironment as compared with progressor tumors (Fig. 4*A–C*). Interestingly, the number of IL-17+ cells within regressor tumors was greater than within progressor tumors (Fig. 4*D* and *E*; data not shown), and the mean fluorescence intensity for IL-17 increased from 608.6 ± 158.7 in progressors to 2,493 ± 854.6 in regressors (P = 0.0620). The relative differences between intratumoral CD8+ cells or IL-17+ cells and the associated transcription factors were similar (compare Fig. 3*C* to Fig. 4*C* and *F*). Progressor tumors were also found to have a greater fraction of infiltrating FoxP3+ cells to CD8+ cells as compared with regressor tumors (Fig. 4*D–F*).

CD11b+Gr-1+ immature myeloid cells are reported to play an important role in facilitating tumor growth and angiogenesis as well as in suppressing antitumor immunity; however, this activity is controversial (4, 7, 14, 15). Furthermore, immature myeloid cells have been reported to be important for skewing antitumor immunity toward type 2 responses (16). Therefore, based on the observation that type 1 responses are inhibited within the peritoneal cavity of tumor-bearing control mice and progressor mice, we determined the coexpression of CD11b and Gr-1 within the tumor. Intratumoral staining for CD11b and Gr-1 shows that progressor tumors recruit significantly more CD11b+Gr-1+ cells as compared with regressor tumors (Fig. 4*G–I*). Both progressor and regressor tumors recruited equivalent total numbers of CD11b+ cells (Fig. 4*G* and *H*; data not shown).

Tumor progression is associated with elevated levels of serum TGF-β and CXCL10. A number of previous reports have suggested that high levels of TGF-β, produced by an unknown cell type, is associated with tumor progression (17). Furthermore, recent reports have shown that neutralization of TGF-β facilitates tumor rejection (18–20). RT-PCR analysis of PECs from control, tumor-bearing controls, progressors, and regressors revealed no significant differences in the level of TGF-β produced within the peritoneal cavity (data not shown). TGF-β protein was also undetectable in peritoneal fluid (data not shown). The possibility remained, however, that TGF-β may be produced and secreted by the tumor or at another distant site, and exert systemic immunosuppressive effects. Therefore, we collected serum samples from progressor and regressor mice and compared the levels of TGF-β (Fig. 5*A*). Mice bearing progressor tumors were found to have 118.3 ± 10.24 ng/mL TGF-β (n = 8), whereas mice with regressor tumors had 68.41 ± 12.41 ng/mL TGF-β (n = 6, P = 0.0096). Serum TGF-β levels were 91.27 ± 15.91 ng/mL (n = 5) in non–tumor-bearing control mice. Interestingly, whereas an increase in CXCL10 expression and protein within the peritoneal cavity was associated with tumor regression, elevated

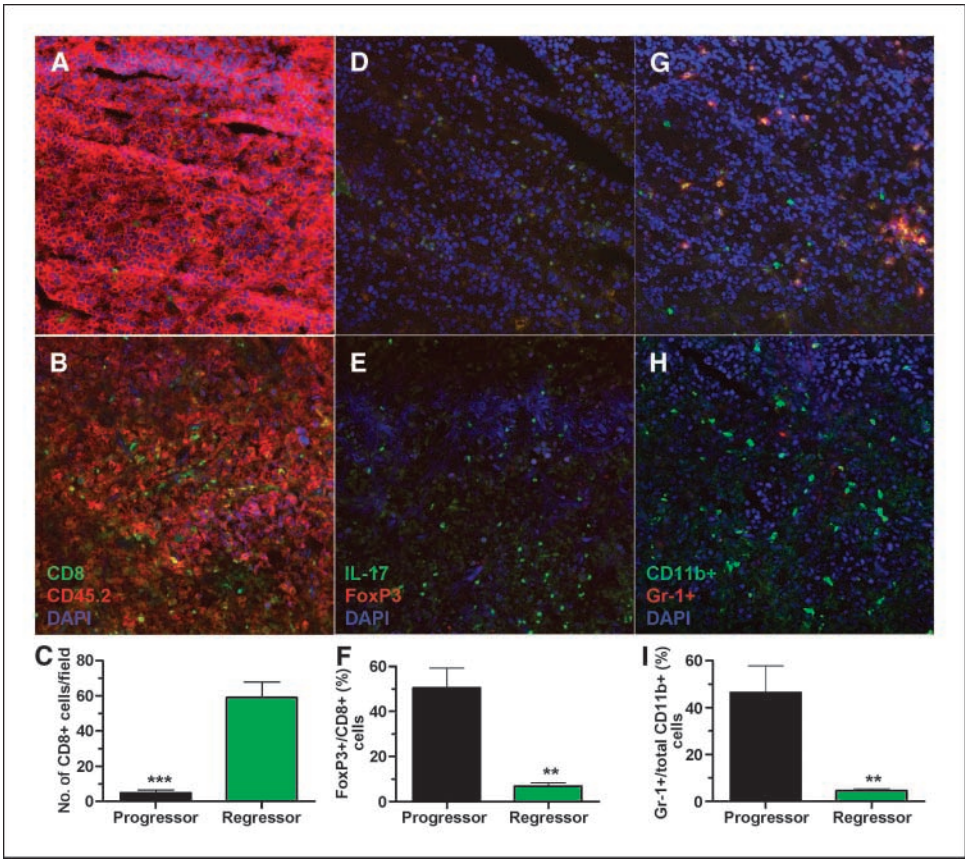

Figure 4. Tumor regression is correlated with increased intratumoral effector cells and decreased immunosuppressive cells. Flash-frozen sections from EG7 tumors (CD45.2+) harvested from EG7-gp96-Ig vaccinated, CD45.1+ progressor (*A*) mice, or EG7-gp96-Ig vaccinated CD45.1+ regressor (*B*) mice were stained with anti-CD8 and anti-CD45.2 (quantitated in *C*) or anti–IL-17 and FoxP3 (*D* progressor and *E* regressor and quantitated in *F*) or anti-CD11b and anti–Gr-1 (*G* progressor and *H* regressor and quantitated in *I*) and counterstained with 4′,6-diamidino-2-phenylindole (*A–H*). The number of positive cells was determined using ImageJ software and is plotted in *C, F,* and *I* (**, $P < 0.01$; ***, $P < 0.001$). Tumor sections were quantitated for ≥2 mice per group with a minimum of two sections per tumor for each staining condition, with five fields per section used for counting.

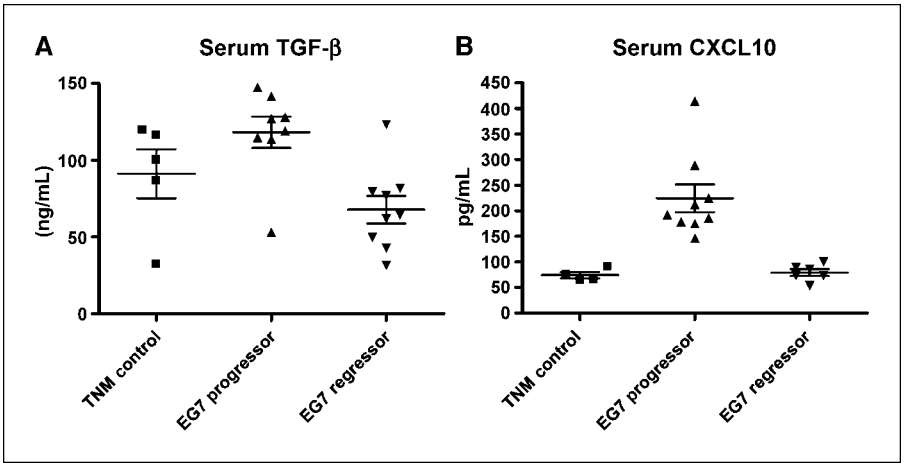

Figure 5. Tumor progression is correlated with increased serum TGF-β and CXCL10. Blood samples were collected from progressor, regressor, or tumor-naïve control mice (*TNM*) and serum was collected as described in Materials and Methods. *A,* serum levels of TGF-β in tumor-naïve control mice were 91.27 ± 15.91 (n = 5), 118.3 ± 10.24 (n = 8) for progressor mice, and 67.99 ± 9.001 ng/mL (n = 9) for regressor mice (P = 0.0021). *B,* serum levels of CXCL10 in tumor-naïve mice were 73.94 ± 6.149 (n = 4), 224.3 ± 27.27 (n = 9) for progressors, and 79.08 ± 6.589 (n = 6) for regressors, with P < 0.001 between progressors and either tumor-naïve mice or regressors.

serum CXCL10 associates with tumor progressors. Serum CXCL10 was found to be 73.94 ± 6.149 pg/mL (n = 4), 224.3 ± 27.27 pg/mL (n = 9), and 79.08 ± 6.589 pg/mL (n = 6) for tumor-naïve mice, progressors, and regressors, respectively (Fig. 5*B*). There is no difference between tumor-naïve mice and regressors, however, expression of CXCL10 by both groups is lower than in progressors (P = 0.0002). No difference was detected in the level of serum IL-1β, IFNγ, IL-6, or IL-17 between progressors or regressors and tumor-naïve control mice by ELISA (data not shown).

CTLs from progressor and regressor mice have equivalent cytotoxic activity. Because expression of Th1-type cytokines and chemokines within the peritoneal cavity, peripheral expansion of tumor-antigen–specific CTLs, and tumor infiltration by CD8+ T cells all correlate with tumor regression, we hypothesized that CD8+ cells isolated from regressor mice would have enhanced killing activity of EG7 target cells as compared with CD8+ cells isolated from progressor mice. We performed 4-hour *in vitro* cytotoxicity assays with freshly isolated CD8+ splenocytes from progressor and regressor mice and found no difference in their ability to kill EG7 target cells (Fig. 6). Furthermore, no qualitative differences were found in the expression of IFNγ or granzyme-B between CD8+ cells from regressor and progressor mice (data not shown).

Discussion

The clinical utility of therapeutic anticancer vaccines is dependent on the expression of tumor-specific or tumor-associated antigens by individual patient tumors. It has been reported that malignant human tumors contain between 40 and 100 mutations that may result in the presentation of a tumor neoantigen (21–23) in addition to the expression of developmental, unmutated antigens. CD8+ CTLs reacting to these antigens are thought to play a primary role in immune-mediated control and rejection of established tumors (24). An obstacle to following the clonal expansion of a population of tumor-specific CTLs is that the dominant antigens to which those CTLs are reacting are rarely known. To better track the expansion of tumor-specific CTLs, we have used the ovalbumin-expressing EG7 tumor cell line as both the primary tumor bolus and the vaccine cells secreting gp96-Ig. This model system is artificial in that the tumor neoantigen, ovalbumin, is not representative of a true tumor-specific or tumor-associated antigen, but is nonetheless instructive for monitoring antitumor immune responses. In animals adoptively transferred with the TCR-transgenic ovalbumin-specific T-cell, OT-I, it was possible to monitor the proliferation of T cells responding to the tumor neoantigen, ova.

Using this model system, we first advanced previous studies (10) by showing that more frequent vaccinations with gp96-Ig led to a dose-dependent increase in peripheral expansion of tumor antigen–specific CTLs. These studies also illustrated that not all mice respond equally well to the vaccine, with ~68% progressing slower than controls but without regression, and 32% rejecting the established tumor and advancing to long-term disease-free survival. The group of mice capable of rejecting the peripheral tumor was the same group that exhibited robust CTL expansion, providing significant evidence for a correlation between tumor-specific CTL expansion and tumor regression. Importantly, vaccination with gp96-Ig in the absence of adoptively transferred OT-I cells led to similar antitumor responses seen in the presence of OT-I cells, suggesting that the expansion of tumor-specific CTL with other specificities is responsible for tumor rejection. The experiments therefore validate the use of OT-I expansion as a surrogate marker for CTL expansion upon vaccination without influencing the outcome of the therapeutic effect.

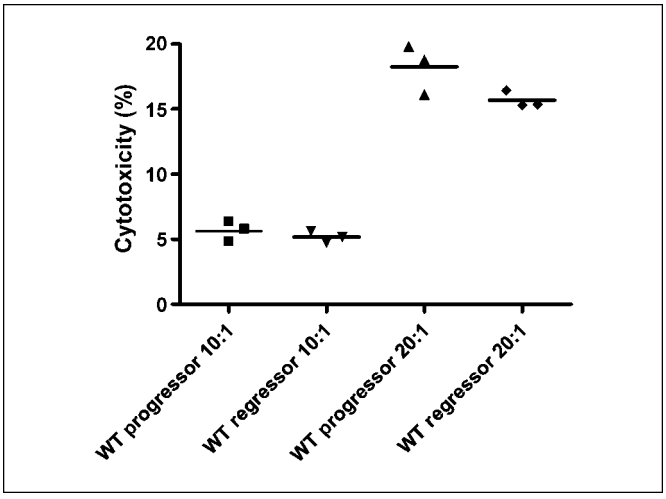

Figure 6. Equivalent *in vitro* cytotoxicity between progressor and regressor CTLs. CD8+ splenocytes were isolated by magnetic bead separation as described and immediately incubated for 4 h with EG7 target cells at the indicated effector/target ratios. No significant difference exists between the ability of progressor or regressor CTLs to kill EG7 targets *in vitro* (n = 3 per group).

The reason is unclear as to why syngeneic, age-, and sex-matched mice bearing similar-sized tumors respond heterogeneously to identical doses of the same batch of a vaccine. One potential reason is likely to be a function of the tumor-specific antigen concentration within the cytosol of the administered vaccine cells. Because gp96 binds peptides nonspecifically, it is likely to be a rare event that an appropriate tumor-specific antigen encounters gp96 before it binds other cellular proteins or is secreted from the tumor cell (25, 26). This may also be one reason why cell-based gp96 vaccines, capable of continuously secreting gp96, are superior to a single bolus of purified gp96 or gp96-Ig (9, 13). Other potential reasons for the heterogeneity in vaccine responses may include the availability of CD91+ dendritic cells or activated natural killer (NK) cells at the appropriate time within the site of immunization. Recent clinical studies with purified autologous gp96 have also shown similar heterogeneity in human patients, suggesting a conserved phenomena for gp96-induced antitumor immune responses (27, 28).

To better understand how a peripheral tumor growing subcutaneously in the flank conditions the host immune system such that it is unable to respond to a single dose of the gp96-Ig vaccine, we analyzed the phenotype and expression profile of PECs of tumor-naïve and tumor-bearing mice. The peritoneal cavity was selected because we have shown previously that peripheral tumors, regardless of whether they contained ovalbumin, interfered with OT-I expansion upon i.p. immunization with gp96-Ig-(10). This finding suggested that peripheral tumors exerted long-distance effects on peritoneal cells, which we decided to analyze. This analysis revealed significant elevations in the expression of IL-1RA, CCR3, CXCL13, and RORγt in peritoneal lavage cells. IL-1RA is the natural competitive inhibitor to IL-1β. IL-1β is known to be found at high concentrations within many tumors, and has a wide range of opposing effects on tumorigenesis involving tumor growth and invasiveness, as well as in enhancing tumor/immune interactions (29). CCR3 is one of the more promiscuous chemokine receptors, and is expressed by a wide range of leukocytes including eosinophils, monocytes, Th2-polarized T cells, neutrophils, and dendritic cells. CXCL13, by comparison, binds only to CXCR5 and is primarily associated with B cell homing to lymphoid tissue, and especially recruitment of autoreactive B1 B cells (30). These findings may offer some evidence toward a Th1-adverse environment maintained by the peripheral tumor; however, the majority of cytokines and chemokines analyzed showed no significant change between tumor-bearing and control naïve mice. Further analysis is required to understand the antigen nonspecific mechanism of tumor-induced immunosuppression by peripheral tumors.

The results of the analysis of peritoneal cells of vaccinated, tumor-bearing mice revealed interesting differences between mice with progressing as compared with regressing tumors. These studies were performed with the expectation that because CTL expansion is inhibited in progressors despite receiving identical treatment with the vaccine, those factors critical in driving CTL expansion and overcoming tumor-induced immunosuppression would be found to be different between the two cohorts. Importantly, the expression of T-bet, RORγt, and IFNγ was opposite between regressors and progressors, with regressors expressing significantly higher levels of each of these factors. Furthermore, regressors also produced more CCL8, CXCL9, and CXCL10. In addition to being induced by IFNγ, CXCL9 and CXCL10 are important NK and Th1-polarized cell chemoattractants; the increases in peritoneal NK1.1-positive cells correlate with the increases in these chemokines (Fig. 3*A* and *B*) and NK cells were previously shown to be critical for gp96-Ig–mediated CTL expansion (13). Importantly, regressors were found to have increased levels of T-bet and IFNγ at the site of vaccination, increased frequencies of tumor-specific CTLs in the peripheral blood and increased infiltration of CD8+ cells within a regressing tumor. These observations provide a logical continuum of events between immunization with gp96-Ig and the rejection of the established tumor.

The observation that tumor-bearing mice as well as tumor regressors produced more RORγt than tumor-naïve mice or tumor progressors is incompletely understood. These findings are additionally supported by an increased frequency of IL-17+ cells, and increased IL-17 expression by those cells, within the tumor microenvironment in tumor regressors as compared with tumor progressors. The role of CD8+ CTLs in antitumor immunity has long been established, and a few recent reports have suggested that Th17 cells play an important role in antitumor immunity (31, 32); however, there continues to be controversy in this developing area of research (9, 24, 33–37). Previous studies with gp96-Ig have clearly shown that CTL expansion is independent of CD4+ cells (9, 13), therefore, the contribution of gp96-Ig toward the development of IL-17–producing cells may or may not be a result of a direct interaction, and is under further investigation.

Recently, the role of TGF-β in suppressing antitumor immune responses has been more firmly established by experiments demonstrating that antibody blockade of TGF-β leads to tumor rejection (18, 20). The source of TGF-β in these studies is unknown, however, it has been reported that TGF-β is produced by both tumor cells and their surrounding stroma (38). Therefore, although we saw no difference in TGF-β production by peritoneal cells (data not shown), we analyzed the levels of TGF-β present in the serum of both progressor and regressor mice and found reduced levels of TGF-β to be associated with tumor rejection (Fig. 5). In addition, tumor progressors were found to have elevated levels of serum CXCL10, despite having reduced CXCL10 message and protein within the peritoneal cavity. The effect of this finding is under further investigation, however, it is tempting to speculate that elevated levels of Th1-attracting chemokines within the serum may abolish a tissue-favored gradient of those chemokines and reduce the ability of Th1-polarized cells to extravasate to inflamed tissues by chemokine receptor desensitization (39). Other recent findings have suggested that defects in effector cell trafficking to tumors is a dominant mechanism of tumor-induced immunosuppression (40).

Surprisingly, no difference was found between the ability of CD8+ splenocytes harvested from progressor or regressor mice to kill EG7 target cells *in vitro*. This observation suggests that tumor-specific CTLs are not necessarily tolerant of the "progressing" tumor, but that the dominant mechanism of tumor-induced immunosuppression may be through increased serum levels of Th1-inhibitory chemokines and cytokines such as TGF-β and potentially by regulatory cells that are excluded from an *in vitro* killing assay. This view is supported by recent data demonstrating that a reagent capable of bringing CTLs into close proximity with tumor targets is sufficient to induce lysis of those tumor cells, indicating that no intrinsic cytotoxic defect exists in those CTLs (41).

Taken together, these data provide evidence that helps to explain the multiple mechanisms by which established tumors may dampen an effective antitumor immune response. Although the source remains unknown, elevated serum levels of suppressive cytokines such as TGF-β and Th1 chemoattractants such as

CXCL10 may have the dual effect of suppressing T-effector function and recruitment to target tissues in an antigen-nonspecific fashion. Importantly, Th1-polarizing vaccine strategies, such as the one used herein with gp96-Ig, may overcome both local and systemic tumor-induced immunosuppression to recapitulate an effective antitumor immune response leading to long-term disease-free survival. Our data supports the view that suppression of CTL expansion and subsequent tumor infiltration are major mechanisms of tumor immune evasion, without necessarily inducing T-cell anergic tolerance. Future studies currently underway will seek to clarify the relative roles of CTLs, IL-17–producing cells, and NK cells to tumor rejection, as well as identify secondary interventions that may facilitate the conversion of progressors to regressors, including *in vivo* blockade of serum TGF-β.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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