Surmounting Tumor-induced Immune Suppression by Frequent Vaccination or Immunization in the Absence of B Cells

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Summary: Tumor-induced immune suppression is one of the most difficult obstacles to the success of tumor immunotherapy. Here, we show that established tumors suppress CD8 T cell clonal expansion in vivo, which is normally observed in tumor-free mice upon antigen-specific glycoprotein (gp) 96-chaperone vaccination. Suppression of CD8 T-cell expansion by established tumors is independent of tumor-associated expression of the antigen that is recognized by the CD8–T-cell receptor. Vaccination of tumor-bearing mice is associated with increased cellular recruitment to the vaccine site compared with tumor-free mice. However, rejection of established, suppressive tumors required frequent (daily) gp96 vaccination. B cells are known to attenuate T helper cell-1 responses. We found that in B-cell deficient mice, tumor rejection of established tumors can be achieved by a single vaccination. Accordingly, in tumor-free B-cell deficient mice, cognate CD8 cytotoxic T lymphocyte clonal expansion is enhanced in response to gp96-chaperone vaccination. The data have implications for the study of tumor-induced immune suppression and for translation of tumor immunotherapy into the clinical setting. Frequent vaccination with cellular vaccines and concurrent B-cell depletion may greatly enhance the activity of anticancer vaccine therapy in patients.

Key Words: secreted HSP gp96 vaccine, B-cell deficiency/depletion, tumor-induced immunosuppression, frequent vaccination

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Antitumor vaccination is quite effective when administered to naive, tumor-free mice resulting in protection from tumor growth upon subsequent challenge. Protection is generally long lasting and tumor specific, indicating the participation of the adaptive immune response. This picture changes radically when vaccines are used for the therapeutic treatment of already established tumor. The same dose of vaccine that is able to effectively establish protective immunity is generally unable to provide therapeutic benefit. The reason for this lack of effectiveness of therapeutic vaccination is thought to stem from the induction of tumor-induced suppressor cells,[1] the generation of regulatory cells,[2] the induction of T-cell anergy or tolerance,[3] or a combination of these mechanisms. Whatever the precise mechanisms of tumor-induced immune suppression, the success of vaccine therapy for cancer treatment will depend on overcoming or neutralizing these tumor-induced suppressive effects.

On the basis of the pioneering work of Srivastava's group[4–7] and Rammensee's group,[8–10] who showed that heat shock protein glycoprotein (gp) 96-associated peptides are cross-presented to CD8 cells by dendritic cells, we have developed a vaccination system suitable for antitumor therapy.[11,12] Transfecting a gp96-immunoglobulin (Ig) G1-Fc fusion protein into tumor cells results in the secretion of gp96-Ig in complex with chaperoned tumor peptides. Parenteral administration of gp96-Ig secreting tumors triggers robust, antigen-specific CD8 cytotoxic T lymphocyte expansion combined with activation of the innate immune system.[13] Tumor-secreted gp96 causes the recruitment of dendritic cells (DCs) and natural killer (NK) cells to the site of gp96 secretion and mediates DC activation via binding to CD91 and Toll-like receptor-2 and Toll-like receptor-4.[14–16] The endocytic uptake of gp96 and its chaperoned peptides triggers peptide cross presentation via major histocompatibility complex (MHC) class I and strong, cognate CD8 activation independent of CD4 cells.[12] In this model system, CD8 CTL expansion can be precisely quantitated within 4 to 5 days of vaccination by the use of adoptively transferred, T-cell receptor (TCR) transgenic, green fluorescent protein (GFP)-marked CD8 T cells.[11] Using this test system, we now show that in our model system, tumor-induced immune suppression is antigen nonspecific and can be overcome by frequent immunization or by the absence of B cells.

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MATERIALS AND METHODS

Mice

C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Frederick, MD). Ig-μ-chain–deficient mice having a C57BL/6J background [B-cell deficient mice (BCDM)] were purchased from The Jackson Laboratory.

GFP mice were obtained by kind permission from the producers.[17] C57BL/6J oxytocin-1 (OT-1) mice (obtained from Dr M. Bevan, University of Washington, Seattle, WA) express a transgenic TCR (Vα2Vβ5.1.2) specific for the H-2Kb–restricted chicken ovalbumin-derived peptide 257 to 264 (SIINFEKL). GFP mice were crossed with OT-1 mice to generate GFP–OT-1 mice in the animal facility at the University of Miami, according to institutional guidelines. The progeny mice were screened by polymerase chain reaction for the expression of the ova-TCR gene and by fluorescence for GFP. All mice were used at 6 to 12 weeks of age.

Cell Lines

The EG7 cell line (obtained from M. Bevan) was transfected with the vector pCMG-His containing gp96-Ig as described.[12,18] Control cells were transfected with vector alone. Lewis lung carcinoma (LLC) cells were obtained from the American Tissue Culture Collection and were transfected with ovalbumin in pAC-neo-ova or with both the ovalbumin vector and pCMG-His containing gp96-Ig. All cells were cultured in Iscove modified Dulbecco media (GIBCO, Carlsbad, CA) with 10% fetal calf serum and gentamycin (GIBCO). To maintain transfected cells, antibiotics for selection (G418 or L-Histidinol, Sigma, St Louis, MO) were added to the culture.

Antibodies

The following antibodies were used for staining; anti-CD16/32 (2.4G2), CyChrome–anti-CD3ε (145-2C11), CyChrome–anti-CD5 (UCHT2), CyChrome–anti-CD8a (53-6.7), PE-CD19 (4G7), PE or FITC–anti-NK1.1 (PK136), and PE or FITC–anti-CD11c (HL3) were purchased from BD PharMingen (San Diego, CA).

Purification and Adoptive Transfer of GFP–OT-1 Cells and CD19$^+$ B Cells

Pooled single cell suspensions of splenocytes and lymph node cells were obtained from GFP–OT-1 mice and were depleted of red blood cells by ammonium chloride lysis. GFP–OT-1 cells were sorted by positive column selection using anti-CD8α magnetic microbeads and a MACS column (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. The purity of isolated OT-1 cells was more than 95% as determined by flow cytometric analysis. Vα2 and Vβ5.1.2 expression on purified cells was quantified by flow cytometry. For purification of B cells, CD19$^+$ cells were purified with anti-CD19 microbeads (Miltenyi Biotec, Auburn, CA). To reconstitute B cells in BCDM mice, 10^7 purified cells were adoptively transferred through tail veins 2 days before transplantation of tumor cells.

Analysis of in Vivo CD8 CTL Expansion

To measure CD8$^+$ CTL expansion, mice were adoptively transferred with 10^6 GFP–OT-1 and immunized 2 days later by IP injection of 1×10^6 to 4×10^6 nonirradiated EG7–gp96-Ig cells. After timed intervals following immunization, cells were harvested from the peritoneal cavity, mesenteric, para-aortic lymph nodes [draining lymph nodes (dLN)], and peripheral blood at the indicated time. Red blood cells were removed from samples by ammonium chloride lysis. One million cells were incubated for 10 minutes at 4°C with anti-CD16/32 monoclonal antibodies in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (phenyl boric acid) to block FcR binding. Thereafter, cells were incubated with the indicated antibodies for 30 minutes. Samples were analyzed on a FACScan (Becton Dickinson) with CELL Quest software (BD Bioscience). The total number of the indicated immune cells per tissue was calculated from the percentage of targeted cells and total number of cells in each tissue.

Tumor Inoculation and Treatment Protocol

Nonirradiated EG7, LLC, or LLC-ova cells were injected SC in 200-μL PBS into the flanks of mice. Five days after the inoculation of LLC-ova cells (day 5), 10^6 purified GFP–OT-1 in a volume of 0.3-mL PBS were injected through tail veins. Two days later, mice were immunized by IP injection of 10^6 nonirradiated LLC-ova–gp96-Ig or EG7–gp96-Ig cells in a volume of 0.5-mL PBS according to the schedule indicated in the graphs. Control mice were treated with PBS or with EG7 or LLC-ova. The size of tumors in the flank was measured in 2 dimensions twice per week for at least 20 days.

Statistical Analysis

Significance was evaluated by repeated measures analysis of variance and by Wilcoxon signed rank test. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Established Tumors Suppress Gp96-mediated CD8 CTL Expansion Independent of TCR Specificity

Transfection of heat shock fusion protein gp96-Ig into tumor cells results in the secretion of gp96-Ig along with gp96-chaperoned peptides.[12] Gp96-Ig is a fusion protein generated by the replacement of the endoplasmic reticulum retention signal (KDEL) of gp96 with the Fc portion of IgG1. Injection of mice with gp96-Ig–secreting tumor cells results in the induction of tumor-specific immunity and memory and protection from subsequent challenge with the same, but untransfected tumor. Tumor immunity generated by secreted gp96-Ig is specific for gp96-chaperoned peptides, including peptides derived

from tumor endogenous antigens, such as EL4-specific antigens,[12] and for surrogate antigens, such as ovalbumin transfected into EL4 (EG7) or LLC (LLC-ova).[11] The ovalbumin surrogate antigen offers a method to accurately determine CD8 CTL expansion in vivo via adoptive transfer of ovalbumin-specific, OT-1 TCR transgenic CD8 cells.[19]

Established tumors are known to be suppressive for CTL expansion. To measure CTL responses in the presence or absence of established tumors, we used the TCR transgenic OT-1 system in which transgenic CD8 CTL respond to ovalbumin-transfected syngeneic or allogeneic tumors secreting gp96-Ig–ova.[11,19] As transplantable tumor models, we used EG7, derived from the EL4 by ovalbumin transfection, which is classified as immunogenic and highly tumorigenic. In addition, we also used the LLC and LLC-ova, which is considered less immunogenic and highly tumorigenic. The division rate of both cell lines is very rapid with a doubling time of 8 to 12 hours in culture.

After a single IP immunization with one million EG7–gp96-Ig cells, secreting 60 to 80-ng gp96-Ig/10^6 cells in 24 hours, OT-1 CD8 T cells expand from low, preimmune levels in the CD8 gate ($\sim$0.2%) to high frequencies (15% to 40%) in tumor-free mice (Fig. 1A). Administration of irradiated EG7 not secreting gp96-Ig is not able to cause significant OT-1 expansion (data not shown, but see Refs. 11 and 13). However, the presence of subcutaneously established EG7 tumors at a distant site in the flank significantly inhibits gp96-vaccine–induced expansion of OT-1 in the peritoneal cavity (Figs. 1A–C) and systemically in spleen and lymph nodes (not shown). EG7 tumors secrete ovalbumin and express $K^{b\text{-}ova}$. It is possible, therefore, that adoptively transferred OT-1, upon recirculation through the tumor bed or tumor dLN, become anergic due to receiving signals through their $K^{b\text{-}ova}$-specific TCR while not receiving costimulatory signal 2. To test this hypothesis, the syngeneic tumors EL4 and LLC, neither expressing ovalbumin, were established subcutaneously at distant sites. Subsequently, OT-1 were adoptively transferred IV and mice were immunized IP with EG7–gp96-Ig as before. Established EL4 and LLC were as effective in suppressing OT-1 expansion by secreted gp96-ova as established EG7 indicating that suppression is not dependent on the appropriate TCR antigen, $K^{b\text{-}ova}$, in the tumor (Figs. 1B, C). Although OT-1 expansion in the peritoneal cavity and systemically was suppressed by the presence of LLC and EL4 at distant sites, surprisingly, total cell recruitment after immunization into the peritoneal cavity upon EG7–gp96-Ig immunization IP was actually increased when compared with tumor-free mice (Fig. 1D).

The data indicate that established tumors can cause the induction of antigen nonspecific suppression of CTL expansion, as has also been reported by others.[20,21] This induction of suppression correlates with increased cellular recruitment to the vaccine site in the peritoneal cavity. Whether this increased cellular recruitment is responsible for the suppression of CD8 T cells is under investigation.

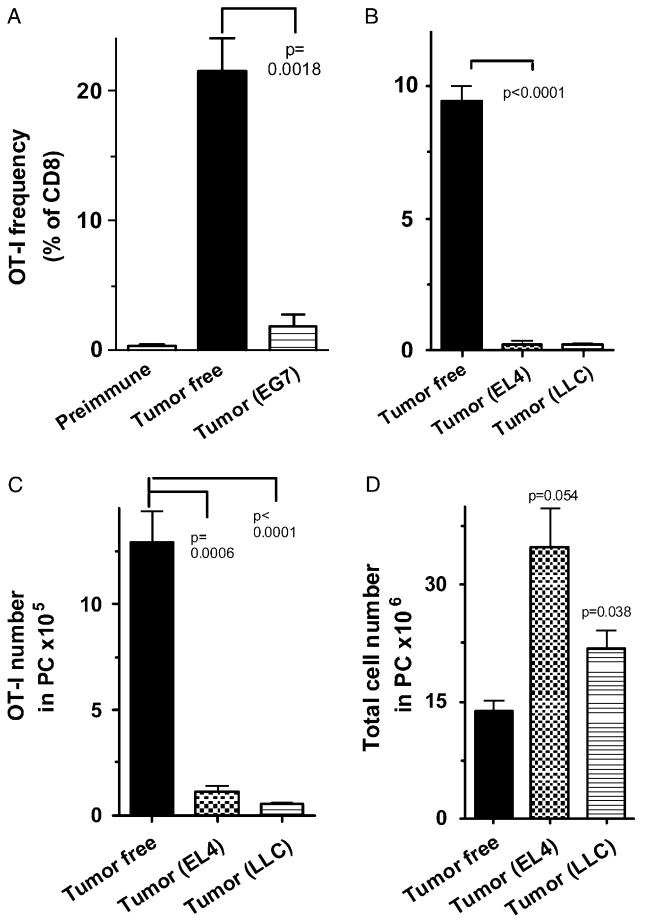

FIGURE 1. Antigen nonspecific suppression of OT-1 CTL expansion by distant, established tumors. A, Comparison of OT-1 CD8 CTL frequency in the peritoneal cavity in unimmunized mice; in immunized, tumor-free mice; and in immunized, EG7–tumor-bearing mice. One million EG7 tumor cells were transplanted subcutaneously in the flank and allowed to be established for 5 days before immunization with EG7–gp96-Ig. One million OT-1 CD8 T cells were adoptively transferred IV 2 days before immunization. Mice were immunized with 2 million EG7–gp96-Ig IP. Peritoneal cells were analyzed 5 days later by flow cytometry. B, Suppression of OT-1 expansion by established tumors is antigen nonspecific. EL4 and LLC, not expressing ovalbumin, were established for 5 days in place of EG7. OT-1 adoptive transfer and vaccination was carried out as in A. C, Absolute numbers of OT-1 accumulating in the peritoneal cavity, the vaccination site, in the absence or presence of established tumors (same experiment as B). D, The total cell number recruited to the peritoneal cavity by EG7–gp96-Ig immunization is increased in the presence of established tumors. A representative experiment of 3 or more individual experiments is shown. n = 3 to 5 mice in each group. Significance values indicated in the figure were calculated by *t* test. Negative controls are unimmunized mice (preimmune) and positive controls are mice without peripheral tumor in the flank. CTL indicates cytotoxic T lymphocytes; gp, glycoprotein; Ig, immunoglobulin; LLC, Lewis lung carcinoma.

To overcome antigen nonspecific immune suppression, we tested whether frequently repeated antigen-specific stimulation of CD8 CTL by vaccination could counteract the suppressive activity found in tumor-bearing mice.

Rejection of Established Tumors Requires Frequent Gp96-Ig Immunizations

Although many vaccination strategies, including secreted gp96-Ig, are able to establish protective immunity in mice against tumors and tumor antigens, it is more difficult to reject already established tumors by therapeutic vaccination. Given the observation of antigen nonspecific suppression of CD8 expansion, we analyzed how different vaccination schedules affected tumor rejection and/or tumor growth.

We initially analyzed the effect of therapeutic vaccination by beginning vaccination on the same day as tumor transplantation. One million EG7 tumor cells were transplanted subcutaneously in the flank of syngeneic mice. On the same day (day 0), one million gp96-Ig–secreting EG7 vaccine cells (EG7–gp96-Ig), secreting gp96-Ig at a rate of 60 to 80 ng/10^6 cells × 24 hours were administered IP as vaccine, and vaccination was repeated on day 3, 7, 10, and 14. Compared with mice not receiving therapy, tumor growth is significantly ($P = 0.0078$) diminished by 4 EG7–gp96-Ig vaccinations starting on the same day as tumor transplantation (Fig. 2A). The therapeutic effect is gp96 and antigen dependent. Irradiated EG7, not secreting gp96-Ig (Fig. 2A), or LLC–gp96-Ig (Fig. 2B), not expressing EG7 antigens but secreting gp96-Ig at the same rate as EG7–gp96-Ig, are unable to retard tumor growth when administered IP as vaccine at the identical dose and schedule as EG7–gp96-Ig. When vaccination with EG7–gp96-Ig is started 2 days or later after EG7 inoculation, the therapeutic effect using the same vaccination schedule is substantially diminished (Fig. 2A). These data demonstrate that even after 2 days, established tumors are more difficult to control by vaccination than tumors that are freshly transplanted.

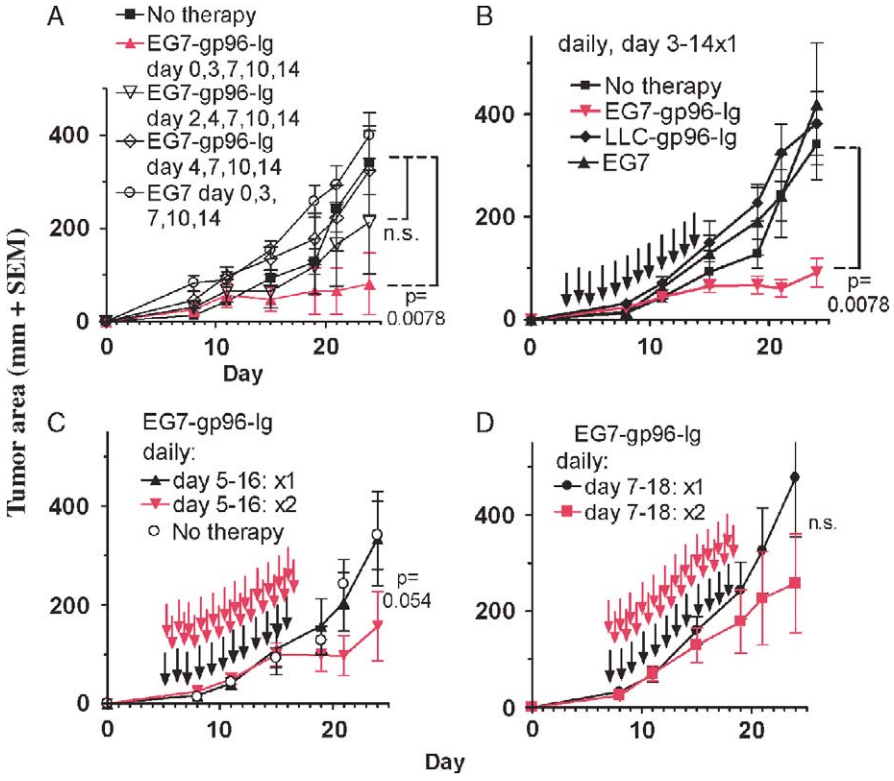

FIGURE 2. Frequent gp96 immunizations can overcome tumor-induced immune suppression. A, One million EG7 tumor cells were transplanted subcutaneously in the flank. Immunization by IP administration of one million EG7–gp96-Ig or irradiated EG7 was started on the same day or 2 or 4 days after tumor transplantation. Negative controls—no therapy, n = 17; irradiated EG7 immunization, n = 15. Immunization with EG7–gp96-Ig at different schedules, n = 15. B, Same as in A except that IP immunization was started on day 3 and was repeated daily until day 14 (black arrows). One million EG7–gp96-Ig (n = 17) or one million LLC–gp96-Ig (n = 5) or irradiated EG7 (negative control, n = 5) or no therapy (negative control, n = 19). C, Tumors were established for 5 days and then immunization IP with one million EG7–gp96-Ig was given once (black arrows) or twice daily (red arrows) from day 5 to 16; n = 5 in each group. D, Tumors were established for 7 days and then immunization IP with one million EG7–gp96-Ig was given once (black arrows) or twice daily (red arrows) once or twice daily from day 7 to 18; n = 5 per group. The significance values of differences in tumor growth are indicated in the individual graphs. gp indicates glycoprotein; Ig, immunoglobulin; LLC, Lewis lung carcinoma; ns, not significant.

We next tested whether tumors established for 3 or more days could be controlled by more frequent vaccination schedules. One million EG7 tumor cells were transplanted subcutaneously in the flank and allowed to be established for 3 to 7 days, allowing at least 7 or more tumor cell doublings. During this period, vascularization of the tumor nodule occurs, which is detectable visually (not shown). Mice were then vaccinated daily IP with one million EG7–gp96-Ig cells or, in specificity controls, with the same schedule and dose of LLC–gp96-Ig cells, or irradiated EG7 cells, or left unvaccinated. Daily vaccination with EG7–gp96-Ig significantly ($P = 0.0078$) and effectively controlled growth of EG7 that had been established for 3 days (Fig. 2B), whereas daily vaccination with irradiated EG7 or with LLC–gp96-Ig had no effect on growth of established EG7 (Fig. 2B). In further studies, we allowed the transplanted EG7 tumors to become established for 5 and 7 days before starting vaccination with EG7–gp96-Ig. As shown in Figures 2C and D, 2 vaccinations every day were required to retard tumor growth at this later stage of tumor establishment. The data show that frequent immunization can check tumor growth for a period of 24 days in mice. Further studies will be needed to determine whether continued long-term vaccination schedules can completely eradicate tumors.

To validate the data obtained with the immunogenic EG7 lymphoma, experiments were repeated with less immunogenic, established LLC (Fig. 3). Repeated IP immunizations (day 3, 7, 10, and 14) with LLC–gp96-Ig beginning on the third day after tumor transplantation resulted in significant ($P = 0.0234$) retardation of tumor progression of LLC. Daily immunizations for LLC were not more effective in tumor retardation. The effect of immunization was tumor specific as EG7–gp96-Ig vaccination was unable to control LLC tumor growth. Tumor growth control also could not be achieved by irradiated LLC, but was dependent on gp96-Ig secretion.

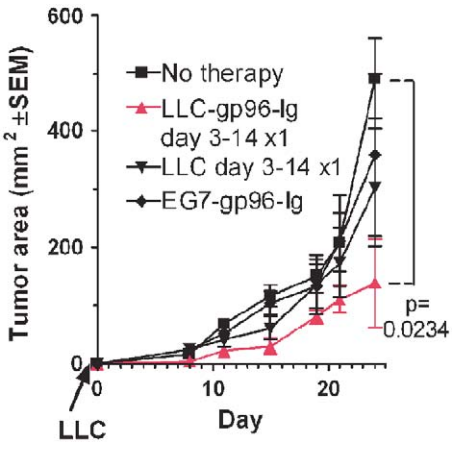

FIGURE 3. Frequent immunizations cause tumor growth retardation of established LLC. LLC (10^5) were transplanted subcutaneously in the flank and allowed to be established for 3 days. Immunization with one million LLC–gp96-Ig (n = 15), EG7–gp96-Ig (n = 5) or irradiated LLC (n = 5), or no therapy (n = 19) was started on day 3 and repeated on day 7, 10, and 14. The significance of the difference between 19 untreated and 15 treated tumor-bearing mice ($P = 0.0234$) is shown. gp indicates glycoprotein; Ig, immunoglobulin; LLC, Lewis lung carcinoma.

These data suggest that frequent DC and NK cell activation, combined with antigen cross presentation by secreted gp96-Ig and its chaperoned peptides, can overcome established tumor-induced, antigen nonspecific immune suppression.

Gp96-mediated DC and NK Cell Recruitment and CD8 CTL Expansion is Enhanced in BCDM

It has been reported by several groups that T helper cell-1 antitumor responses are enhanced in BCDM when compared with wild type (WT) mice.[22–24] We, therefore, studied the role of B cells in gp96-mediated CTL expansion and antitumor immunity. The peritoneal cavity is populated by $CD5^-CD19^+$ B cells and by $CD5^+CD19^+$ B1-B cells, the latter producing IgM antibody and not undergoing isotype switching upon activation (Fig. 4A). Upon IP immunization with EG7–gp96-Ig, the $CD5^-CD19^+$ population increases about 5-fold by day 4 postimmunization, whereas $CD5^+$ B1-B cells increase only moderately (Fig. 4A). Gp96-mediated OT-1 expansion is maximal on day 4 and 5 postimmunization.[11] It is preceded by recruitment into and activation of DCs and NK cells in the peritoneal cavity, the site of vaccination. NK cells are important facilitators of gp96-Ig mediated CD8 CTL expansion as shown previously.[11] In BCDM, the recruitment of DCs into the peritoneal cavity (the vaccine site) was similar to recruitment in WT mice on day 2 after vaccination. However, although the DC numbers decreased by day 4 postvaccination by 50% in WT mice, DC numbers in B-cell deficient mice remained at the same high frequency (Fig. 4B). NK cell recruitment in BCDM was increased on day 2 and day 4 (Fig. 4B). The difference did not reach significance but was reproducible in 3 separate experiments. Adoptive transfer of WT B cells to BCDM abolished increased retention of DCs and recruitment of NK cells. The finding suggests that B cells influence gp96-induced recruitment of innate immune cells and suggest that B cells may also be involved in regulating or suppressing CD8 CTL expansion.

We, therefore, tested whether expansion of GFP-marked OT-1 CD8 CTL was increased in BCDM in response to gp96 immunization. As shown in Figure 5, OT-1 expansion after gp96 immunization in BCDM was significantly enhanced on day 5 compared with WT mice. Importantly, OT-1 persisted at significantly higher frequencies on day 7 and 12 postimmunization in the peritoneal cavity ($P = 0.04$) (Fig. 5A) In dLN (Fig. 5B), OT-1 expansion and retention was also increased without, however, reaching significance. Adoptive transfer of WT B cells to BCDM before immunization reduced OT-1 expansion to levels at or below those seen in WT mice (Figs. 5A, B). The suppression of OT-1 expansion by the presence of B cells is not mediated by interleukin (IL)-10

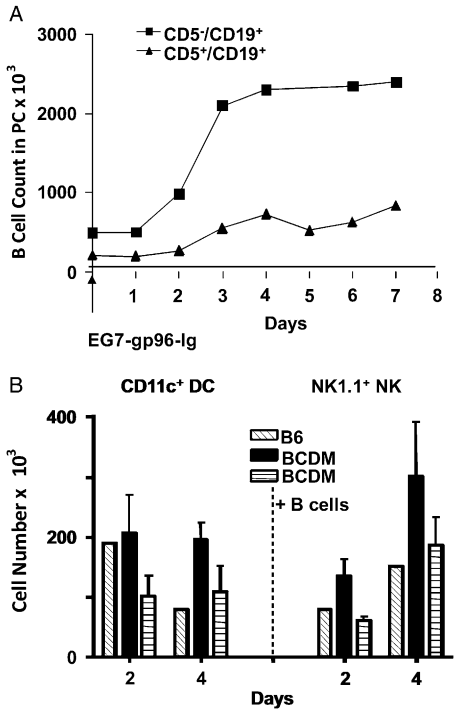

FIGURE 4. B cells inhibit gp96-mediated recruitment of NK cells into and retention of DCs in the peritoneal cavity. A, Recruitment of B cells, but only modest $CD5^+$ B cells, into the peritoneal cavity by EG7–gp96-Ig immunization. Tumor-free mice received one million EG7–gp96-Ig IP Accumulation of $CD5^+$ and $CD5^-$ B cells was determined daily thereafter by flow cytometrty. Representative of more than 3 experiments. B, Increased recruitment of NK cells and retention of NK cells and DCs and in BCDM and its reversal by adoptive transfer of B cells. WT and BCDM were immunized IP with 2 million EG7–gp96-Ig and cells harvested from the peritoneal cavity 2 and 4 days later and analyzed by flow cytometry. B-cell reconstitution was carried out by IV adoptive transfer of 10^7 WT B cells 2 days before immunization with EG7–gp96-Ig. Representative of 3 experiments. BCDM indicates B-cell deficient mice, DC, dendritic cell, gp, glycoprotein; Ig, immunoglobulin; NK, natural killer, WT, wild type.

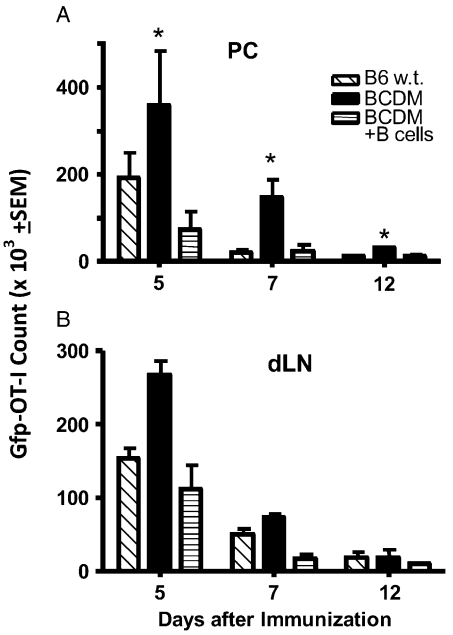

FIGURE 5. Gp96-mediated OT-1 CD8 CTL expansion is increased and sustained in the absence of B cells. WT mice and B-cell deficient mice received one million GFP–OT-1, B-cell reconstituted mice in addition received 10 million WT B cells by IV adoptive transfer. The mice were immunized 2 days later with 4 million EG7–gp96-Ig and were analyzed on the days indicated by harvesting cells from the peritoneal cavity (A) and mesenteric and para-aortic lymph nodes (dLN) (B). $*P = 0.04$ by repeated measures ANOVA. Four mice in each group, representative of 3 experiments. ANOVA indicates analysis of variance; CTL, cytotoxic T lymphocytes; dLN, draining lymph nodes; GFP green fluorescent protein; gp, glycoprotein; Ig, immunoglobulin; WT, wild type.

production because IL-10–deficient mice exhibit OT-1 expansion similar to WT mice rather than the enhanced expansion as seen in BCDM (data not shown).

Gp96-mediated Rejection of Established Nonimmunogenic Tumors is Enhanced in the Absence of B Cells

As shown above, growth control of established EG7 tumors in WT mice minimally requires daily gp96 immunization. Similarly, LLC progression can be retarded by frequent immunizations. EG7 and EL4 cells are rejected in BCDM and do not establish tumors; however, LLC and LLC-ova can be established in BCDM although they grow at a slower rate than in WT mice.[24] LLC-ova was established subcutaneously in the flank for 5 days in BCDM and in WT mice. OT-1 were adoptively transferred IV and, 2 days later, one million LLC-ova–gp96-Ig were administered as a single dose IP and tumor growth in the flank monitored. In WT mice, a single immunization with LLC-ova–gp96-Ig caused significant retardation of tumor progression in the flank, but failed to reject tumors (Fig. 6A). In contrast, in BCDM, a single immunization resulted in complete rejection of established, 7-day LLC-ova tumors in 3 mice and significant tumor shrinking in 2 (Fig. 6B). In the absence of treatment, LLC-ova grow progressively in BCDM (Fig. 6B) albeit at a slower rate than in WT mice (Fig. 6A). B-cell reconstitution of BCDM (Fig. 7C) rendered the effect of vaccination similar to that seen in WT mice (Fig. 6A), namely retardation of progression. It will be of interest to determine whether complete or partial B-cell depletion by antibody will have similar effects as B-cell deficiency. Ongoing preliminary studies (unpublished) seem to support this approach.

Optimal tumor control of established LLC in BCDM by a single immunization is dependent on sufficiently high numbers of tumor-specific CTL precursors (OT-1) and on antigen-specific immunization (LLC-ova–gp96-Ig). In BCDM, the presence of one million adoptively transferred OT-1 without gp96 immunization

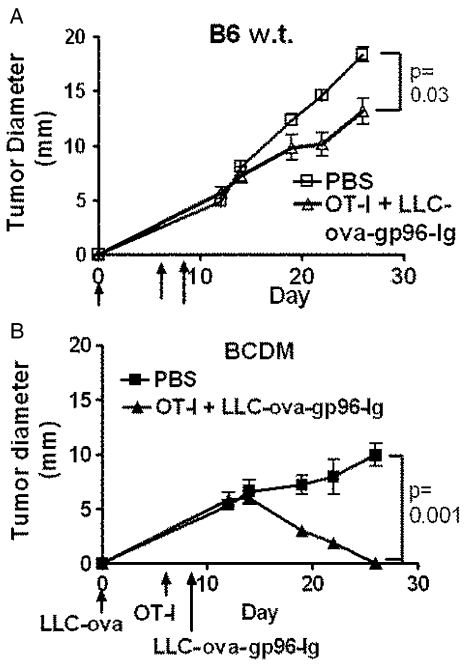

FIGURE 6. Gp96-mediated tumor rejection is enhanced in BCDM and abrogated by B-cell reconstitution. A, Wild type mice. B, BCDM. One million LLC-ova cells in 0.2-mL PBS were transplanted into the flank. Five days later, one million OT-1 were given IV. Seven days after tumor transplantation, mice were immunized IP with one million LLC-ova–gp96-Ig. Tumor size was measured with a caliper in 2 dimensions. n = 5 in each group, representative of 3 experiments. BCDM indicates B-cell deficient mice; gp, glycoprotein; Ig, immunoglobulin; LLC; Lewis lung carcinoma; PBS, phosphate-buffered saline.

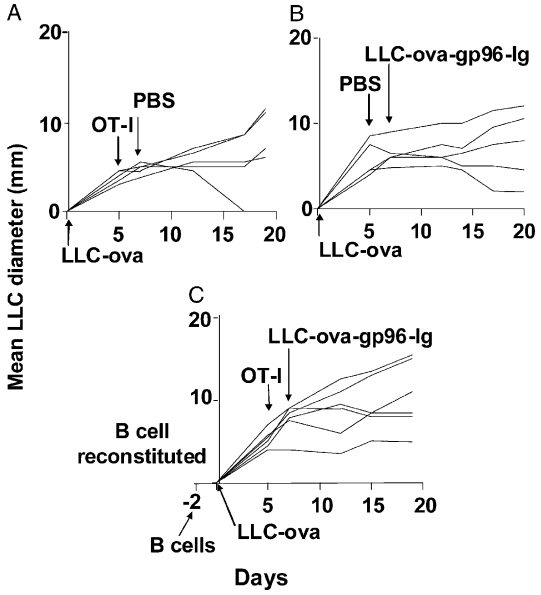

FIGURE 7. High CTL precursor frequency and immunization enhance tumor rejection by gp96 vaccines in BCDM. A, BCDM were treated as in Figure 6 except that vaccination with LLC-ova–gp96-Ig was omitted. B, As in Figure 6. Omitting OT-1 transfer. C. As in Figure 6, except that BCDM mice were reconstituted with 10 million B cells before tumor (LLC-ova) transplantation. n = 5 to 6 mice in each group, representative of 2 experiments. BCDM indicates B-cell deficient mice; CTL, cytotoxic T lymphocytes; gp, glycoprotein; Ig, immunoglobulin; LLC; Lewis lung carcinoma.

does not result in tumor rejection in the majority of mice (Fig. 7A). Likewise, gp96 immunization alone without OT-1 transfer is less effective than the combination (Fig. 7B).

DISCUSSION

It is well appreciated that established tumors suppress antitumor immunity. As shown by Sotomayor et al,[3] tumor-specific T cells become anergic in the presence of established tumors. Anergy to the B-cell lymphoma used in that study was antigen specific, MHC restricted, and dependent on the presence of MHC-matched bone marrow-derived antigen presenting cells. In other studies, antigen nonspecific myeloid-suppressor cells and T-regulatory cells have been implicated in suppression of antitumor immunity.[20,21,25] Our studies show that the suppression of CTL responses in vivo can be achieved by established tumors through antigen independent pathways. OT-1 CD8 CTL expansion in response to gp96-ova vaccination is inhibited by established tumors independent of the expression of ovalbumin by the tumors. This type of suppression may be achieved by T-regulatory cells or by other suppressor cells such as myeloid-suppressor cells or M2 macrophages. In accord with this hypothesis, the suppressive activity, in preliminary experiments, is transferable to tumor-free mice by the transfer of peritoneal cells elicited in tumor-bearing mice by gp96 vaccination.

Although the OT-1 response to gp96-ova immunization is strongly inhibited in the presence of established tumors, it is not totally blocked, suggesting that there is a balance between immune suppression by the established tumor and vaccine-induced CD8 CTL activation through antigen cross presentation by activated DCs stimulated by secreted gp96-ova. We have shown previously that in tumor, naive mice gp96-ova results in the recruitment and activation of NK cells and DCs followed by OT-1 expansion. Established tumors, although actually enhancing recruitment of cells into the peritoneal cavity by LLC–gp96-Ig vaccination, inhibit OT-1 expansion, suggesting that in the presence of established tumors, many of the recruited cells are likely to be suppressor cells. This hypothesis predicts that frequent immunizations with gp96-ova may overcome the suppressive activity by shifting the balance from suppression to increased immune activation through repeated gp96-mediated DC and NK cell stimulation, increased antigen cross presentation, and CTL priming. Indeed, frequent immunizations have significant effects on retardation of tumor progression. In the case of established EG7, once or twice daily vaccinations were much more effective in stopping

tumor progression than vaccination every second or third day. For LLC, immunization every other or every third day was sufficient and daily immunization was not more effective (data not shown). These tumor-specific differences may be related to the rate by which suppressor cells are generated by the presence of the peripheral tumor. Alternatively, it may depend on the mechanism by which tumors mediate the induction of suppressor cells or the nature of the suppressor cells that have been induced. These questions are currently under study.

By studying the OT-1 response to IP immunization with tumor–secreted gp96-ova, we noticed that large numbers of B cells are recruited into the peritoneal cavity, which is the vaccine site. B cells have been reported to be inhibitory for antitumor immunity, prompting the question as to their role in gp96-mediated OT-1 expansion. Using BCDM, it became immediately clear that both NK cell and DC recruitment and retention in the peritoneal cavity were increased and OT-1 expansion was enhanced after gp96-ova immunization. B-cell reconstituted BCDM responded like WT mice to gp96-ova–mediated OT-1 expansion, ruling out the possibility that B-cell deficiency had modified the responsiveness of BCDM to gp96-ova immunization in a manner unrelated to the absence of B cells. B-cell deficiency not only caused enhanced OT-1 expansion but also strongly enhanced tumor rejection of 7-day established LLC-ova tumors after a single gp96-Ig immunization. The data suggest that tumor-mediated induction of suppressor cells is greatly diminished in the absence of B cells or that B cells themselves act as "suppressor cells." Whether B cells participate in the induction of suppressor cells or whether B cells themselves are immunosuppressive for CTL responses needs further study; IL-10, however, does not seem to be involved in B-cell mediated suppression of tumor immunity. In ongoing studies, we have found that OX40-L–deficient B cells show reduced ability to suppress antitumor immune responses (data not shown). It remains to be determined how OX40-L expressed on B cells mediates suppression of antitumor immunity and CTL expansion by gp96.

Our studies provide a model by which antigen-independent immune suppression can be studied and further defined. The role of B cells in particular in this process will be of great interest. In addition, our studies point to ways in which antitumor vaccines can be made more effective. Depletion of B cells with antibodies and subsequent frequent vaccination, for instance with tumor-secreted gp96 vaccines, may result in more efficient control of tumor growth than that seen with conventional vaccination methods. Studies are under way to test these hypotheses.

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