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Abstract: 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 gp96-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-TCR. Vaccination of tumor bearing mice is associated with increased cellular recruitment to the vaccine site compared to tumor free mice. However, rejection of established, suppressive tumors required frequent (daily) gp96-vaccination. B cells are known to attenuate Th1 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 CTL 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 anti cancer vaccine-therapy in patients.

Miami, January 17, 2008

RE: JIT-07-158, entitled "Surmounting tumor induced immune suppression by frequent vaccination or immunization in the absence of B cells"

Dear Dr. Rosenberg:

We are submitting the revision of the above named manuscript for your consideration for publication. We have carefully reviewed and responded to the reviewers' comments. Our response is explained in letter attached (below), written in blue font, following the reviewers' comments. The revisions in the manuscript are also in blue font for easy identification.

Many thanks for your consideration and best regards

Eckhard Podack
(for the authors)

Reviewer #1: "Surmounting tumor induced immune suppression by frequent vaccination or immunization in the absence of B cells" by Oizumi et al.

The manuscript describes interesting results on the role of B cells and frequent vaccinations in EL-4 and 3LL animal tumor models. Understanding the failure of the most modern immunotherapeutic protocols is quite important and the authors open new insights into the role of different cell populations in tumor-mediated immunosuppression. Although the findings that established tumors may cause antigen-independent inhibition of CTL generation is not new, the authors claimed that this is due to the vaccine-induced cells which can be isolated from the peritoneal cavity. These are intriguing data, which are, unfortunately, "not shown".

We are actively studying the source of suppression of OT-I in this system. This now indicated in the text without reference to the transfer experiment. While the mechanism of suppression is of great interest, the point of the current paper is to develop vaccine regimens to overcome tumor induced immune suppression (regardless of the mechanism).

Since the authors demonstrated later on that B cells are responsible for CTL down-regulation, it might be interesting to deplete peritoneal cells of B cells prior to their adoptive transfer or to use peritoneal cells from BCDM mice bearing tumors.

Response: We agree with the reviewer that B cell depletion experiments will be valuable for further understanding the role of B cells in cancer immunity. These studies are ongoing in the laboratory using human CD20-transgenic mice and Rituximab for depletion of B cells. These studies will be published separately when completed as mentioned now in the text.

A few statements and conclusions require clarification. The authors state that "in B cell deficient mice, the recruitment of DC was increased (Fig. 4B)". However, as shown in Fig. 4B, the numbers of DC in BCDM mice were equal on Day 2 and 4, while the numbers of DC in B6 mice decreased. This results in the difference between DC in control and B cell deficient mice on Day 4, but does not support the authors' conclusion.

We have now clarified the statement in the text to indicate that DC persist longer in B cell deficient mice than in w.t. mice in the peritoneal cavity after gp96-Ig immunization and that NK cell recruitment is enhanced on day 2 and day 4 in BCDM,

Another important conclusion, which, however, was not supported by any data in the manuscript, is that frequent DC and NK cell activation can overcome tumor-induced immunosuppression in vivo. These results also raise the question about the input of DC vs. NK cells, which can be easily answered using NK cell depletion experiment.

NK depletion and reconstitution has been carried out in {Strbo, 2003 #6}, where we have shown the importance of NK cells and of Perforin for CTL expansion in response to gp96-Ig immunization. This reference is cited in the text along with a sentence indicating the importance of NK cells.

The role of frequent vaccinations is also fascinating, but these studies are not developed further. Involvement of DC and NK cells represent potentially different pathways and the generation of immune memory or long-term animal survival might be a key experiment.

It is clear that mechanistic studies will be necessary and may be very revealing to understand the molecular and cellular consequences of frequent immunization. We have indeed begun a program to study the immunological events ensuing in response to frequent immunization in the presence of large peripheral tumors. These studies are in progress and are expected to clarify the mechanistic aspects and will be published separately.

Minor comments:

- It seems helpful if the authors can add one sentence concluding their findings in the Abstract section

We have added this sentence: Frequent vaccination with cellular vaccines and concurrent B cell depletion may greatly enhance the activity anti cancer vaccines therapy in patients.

- It is not quite right to use t-test for evaluating tumor growth in vivo

We now used a Repeated Measure ANOVA for the analyses and Wilcoxon Signed Rank Test for analysis. Significance values are included in the Figures and in the text

- Appreciating the fact that one tumor might suppress the growth of similar tumors at other sites, Fig.2 experiments should use intact EG7 cells for the i.p. injections instead of irradiated cells. The role of OVA antigen in these studies is unclear since the authors used EG7 cells instead of EL-4 cells. If intact EG7 are used for immunization i.p. tumors will develop in the peritoneal cavity. As we have shown in a previous publication immunization with EG7-gp96-Ig provides immunity against challenge with EL-4 (ref {Yamazaki, 1999 #5}). Ovalbumin antigen this is not necessary to provide anti tumor immunity.

- Some abbreviations are not introduced, including DC and w.t.

Abbreviations have been defined.

- There is no Fig.5C, although it is mentioned and discussed in the text

Reference to Fig 5C was omitted.

- There are no Figures 6 A, B, C, D, E, F, and G although they are discussed in the text
- There is Figure 7 A, B, and C, but they are not discussed in the text.

We apologize for this oversight which occurred at the draft stage of the manuscript. The error has been corrected.

Reviewer #2: In this manuscript, Oizumi et al. discuss the immunosuppressive properties of B cells for tumor bearing mice. This study uses ovalbumin as an antigen with EL4 and Lewis lung carcinoma tumors transfected with ovalbumin as compared to the appropriate wild type mice. In addition, the T cells studied are spleens cells obtained from OT1 mice, which are specific to ovalbumin. The following are comments that the authors might find useful.

1. Immunization is described variously within this manuscript. However, for example, in Figure 1, immunization is by the i.p. injection of 1 or 2 million EL4 cells transfected with JP96IG. It is noted that these are cells that have not been irradiated and as such a substantial tumor burden is being given to the animals. Further, mice that have been immunized are examined 1-5 days later. It is noted that i.p. vaccination is rarely used clinically, with the exception of rabies, but vaccination with a substantive burden of viable non-irradiated cells is unlikely to be considered clinically.

Response: Due to transfection with gp96-Ig, EG7 tumor cells (EG7-gp96-Ig) become immunogenic and are rejected by CD8 T cells. As vaccine cells they do not seem to present a tumor burden. We have shown in previous referenced studies ({Oizumi, 2007 #27; Strbo, 2003 #6}) that the CD8 response is specific for endogenous tumor antigens and for the surrogate antigen ovalbumin as mentioned on page 6 of the manuscript.

We are indeed doing a clinical study currently in lung cancer patients and have completed and published a successful and promising phase I study using 45×10^7 live, transfected lung tumor cells as vaccine in 9 consecutive injections {Raez, 2004 #28}.

Further, studying a T cell response 5 days following primary immunization may be considered premature for the induction of a T cell response. Further, nowhere is this shown to be a T cell response. Adaptive transfer studies with either T cells or CD4 vs. CD8 cells to demonstrate cellular efficacy would be appropriate.

Response: In the manuscripts mentioned above ({Oizumi, 2007 #27; Strbo, 2003 #6}) it was also shown that the cognate CD8 T cell response was maximal on day 4 and 5 post vaccination. The responding cells used as read out were adoptively transferred TCR transgenic CD8 T cells (OT-I), similar to the current study.

2. No statistical analyses are provided throughout this paper. For the survival curves, Kaplan Meyer graphics are recommended and for the tumor growth curves, a repeated measure test might be appropriate. The other studies would most likely be non-parametric test with a Mann-Whitney U test to compare the effect of tumor bearing vs. wt mice, or tumor bearing and wt mice vs. tumor bearing and B cell deficient mice.

We apologize for this omission, statistical analysis was done but not shown. The statistical analysis is now included in the figures and the text and statistical methods suggested were used. This is also stated on page 5.

3. It appears that each study was repeated once with an N of 5. While an N of 5 is likely sufficient in the surrogate studies, survival studies or tumor burden studies would be more rigorous if an N of 10 was used. Further and critically, each study needs to be done at least 2 times, and preferably 3 times, to assess the reproducibility of their observations.

The number of mice is given in the figure legends. Large numbers of mice (15 -19) were used in the frequent immunization experiments. The other experiments were repeated three times as now indicated in the legends. Survival studies in mice in general are not permitted by the animal care and use committee if other methods of evaluation are available. We therefore used tumor progression and growth rate as end points and euthanized mice when the tumor burden began to cause suffering.

4. In several studies, controls are absent. For example, in Figure 6, mice received PBS as the control and the experimental were OT1 and LLC-OVA-GP96-IG. Alternative and appropriate controls would include the use of OT1 cells alone, LLC-OVA-GP96-IG cells alone, OT1 + LLC cells, and potentially LLC cells alone. In some of the other studies, similar comments can be made. Controls were run in all experiments and are now specifically mentioned in the legends.

Title:

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 gp96-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-TCR. Vaccination of tumor bearing mice is associated with increased cellular recruitment to the vaccine site compared to tumor free mice. However, rejection of established, suppressive tumors required frequent (daily) gp96-vaccination. B cells are known to attenuate Th1 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 CTL 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 anti cancer vaccine-therapy in patients.](#)

Introduction:

Anti tumor vaccination is quite effective when administered to naïve, tumor free mice resulting in protection from tumor growth upon subsequent challenge. Protection generally is 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 generally is 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 therapy will depend on

overcoming or neutralizing these tumor induced suppressive effects.

Based on the pioneering work of Srivastava's group (4-7) and Rammensee's group (8-10) who showed that heat shock protein gp96 associated peptides are cross-presented to CD8 cells by dendritic cells, we have developed a vaccination system suitable for anti tumor therapy (11, 12). Transfecting a gp96-IgG1-Fc fusion protein into tumor cells results in secretion of gp96-Ig in complex with chaperoned tumor peptides. Parenteral administration of gp96-Ig secreting tumors triggers robust, antigen specific CD8 CTL expansion combined with activation of the innate immune system (13). Tumor secreted gp96 causes the recruitment of DC and NK cells to the site of gp96 secretion and mediates DC activation via binding to CD91 and TLR2 and TLR4 (14-16). The endocytic uptake of gp96 and its chaperoned peptides triggers peptide cross presentation via 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 use of adoptively transferred TCR transgenic, gfp-marked CD8 T cells (11). Using this test system we now show that in our model system, tumor induced immune suppression is antigen non-specific and can be overcome by frequent immunization or by the absence of B cells.

Material 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 (BCDM) were purchased from The Jackson Laboratory. Gfp (Green fluorescent protein) mice were obtained by kind permission of the producers (17). C57BL/6J OT-I mice (obtained from Dr. M. Bevan, U. of Washington, Seattle) express a transgenic TCR ($V\alpha 2V\beta 5.1.2$) specific for the H-2Kb-restricted chicken ovalbumin derived peptide 257-264 (SIINFEKL). Gfp mice were crossed with OT-I mice to generate gfp-OT-I mice in the animal facility at the University of Miami, according to institutional guidelines. The progeny mice were screened by PCR for the expression of the ova-TCR gene and by fluorescence for gfp. All mice were used at 6-12 week 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 IMDM media (GIBCO, Carlsbad, CA) with 10 % FCS 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-CD3 ϵ (145-2C11), -anti-CD5 (UCHT2), -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-I cells and CD19+ B cells

Pooled single cell suspensions of splenocytes and lymph node cells were obtained from gfp-OT-I mice and were depleted of red blood cells by ammonium chloride lysis. Gfp-OT-I cells were sorted by positive column selection using anti-CD8 α magnetic microbeads and a MACS column (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of isolated OT-I cells was more than 95% as determined by flow cytometric analysis. V α 2 and V β 5.1.2 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, 10⁷ 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 10⁶ gfp-OT-I, immunized two days later by i.p. injection of 1-4 \times 10⁶ non-irradiated EG7-gp96-Ig cells.

After timed intervals following immunization, cells were harvested from the peritoneal cavity, mesenteric, para-aortic lymph nodes (dLN) and peripheral blood at the indicated times. Red blood cells were removed from samples by ammonium chloride lysis. One million cells were incubated for 10 min at 4°C with anti-CD16/32 mAb in PBS containing 0.5% BSA (PBA) to block FcR binding. Thereafter, cells were incubated with the indicated antibodies for 30min. Samples were analyzed on a FACScan (Becton Dickinson) with CELL Quest software (BD Bioscience). The total number of the indicated immune cells per each tissue was calculated from the percentage of targeted cells and total number of cells in each tissue.

Tumor inoculation and treatment protocol

Non-irradiated EG7, LLC or LLC-ova cells were injected s.c. in 200 µl PBS into the flanks of mice. Five days after the inoculation of LLC-ova cells (day 5), 10⁶ purified gfp-OT-I in a volume of 0.3 ml PBS were injected through tail veins. Two days later, mice were immunized by i.p. injection of 10⁶ non-irradiated 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 two dimensions twice per week for at least 20 days.

Statistical Analysis

Significance was evaluated by Repeated Measures Anova 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 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-I TCR transgenic CD8 cells (19).

Established tumors are known to be suppressive for CTL expansion. To measure CTL responses in the presence and absence of established tumors, we used the TCR transgenic OT-I 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 Lewis lung carcinoma (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 8-12 hours in culture.

After a single i.p. immunization with one million EG7-gp96-Ig-cells, secreting 60-80ng gp96-Ig per 10^6 cells in 24 hours, OT-I CD8 T cells expand from low, pre-immune levels in the CD8 gate ($\sim 0.2\%$) to high frequencies (15-40%) in tumor free mice (Fig. 1A). Administration of irradiated EG7 not secreting gp96-Ig is not able to cause significant OT-I expansion (data not shown, but see ref (11, 13). However, the presence of subcutaneously established EG7 tumors at a distant site in the flank significantly inhibits gp96-vaccine induced expansion of OT-I in the peritoneal cavity (Fig. 1A,B,C) and systemically in spleen and lymph nodes (not shown). EG7 tumors secrete ovalbumin and express K^{b-ova} . It is possible therefore that adoptively transferred OT-I upon recirculation through the tumor bed or tumor draining lymph nodes become anergic due to receiving signals through their K^{b-ova} -specific TCR while not receiving costimulatory signal two. To test this hypothesis, the syngeneic tumors EL4 and LLC, neither expressing ovalbumin, were established subcutaneously at distant sites. Subsequently, OT-I were adoptively transferred i.v. and mice immunized i.p. with EG7-gp96-Ig as before. Established EL4 and LLC were as effective in suppressing OT-I expansion by

secreted gp96-ova as established EG7 indicating that suppression is not dependent on the appropriate TCR antigen, K^{b-ova}, in the tumor (Fig. 1 B, C). While OT-I expansion in the peritoneal cavity and systemically was suppressed by the presence of LLC and EL4 at distant sites, surprisingly, total cell recruitment following immunization into the peritoneal cavity upon EG7-gp96-Ig immunization i.p. was actually increased when compared to tumor free mice (Fig. 1D).

The data indicate that established tumors can cause the induction of antigen non-specific 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.](#)

To overcome antigen non-specific 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

While 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 non-specific 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 60-80ng/10⁶ cells x 24h, were administered intraperitoneally (i.p.) as vaccine and vaccination repeated on day 3, 7, 10 and 14. Compared to mice not receiving therapy, tumor growth is [significantly \(p = 0.0078\)](#) diminished by four EG7-gp96-Ig vaccinations starting on the same day as tumor transplantation (Fig. 2 A). 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 i.p. as vaccine at the identical dose and schedule as EG7-gp96-Ig. When vaccination with EG7-gp96-Ig is started two 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 two days established tumors are more difficult to control by vaccination than tumors that are freshly transplanted.

We next tested whether tumors established for three 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 become established for three to seven days, allowing at least seven 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 i.p. 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 three days (Fig 2B), while 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 Fig. 2 C, D, two 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 intraperitoneal immunizations (day 3, 7, 10, 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.

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

Gp96-mediated DC and NK recruitment and CD8 CTL expansion is enhanced in B cell deficient mice

It has been reported by several groups that Th1 anti tumor responses are enhanced in B cell deficient mice (BCDM) when compared to wild type (w.t.) mice (22-24). We therefore studied the role of B cells in gp96-mediated CTL expansion and anti tumor 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 i.p. immunization with EG7-gp96-Ig the CD5⁻CD19⁺ population increases about five fold by day 4 post immunization, while CD5⁺ B1B cells increase only moderately (Fig. 4A). Gp96-mediated OT-I expansion is maximal on day 4 and 5 post immunization (11). It is preceded by recruitment into and activation of DC 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 B cell deficient mice, the recruitment of DC [into the peritoneal cavity \(the vaccine site\) was similar to recruitment in w.t. mice on day two after vaccination.](#) However, while the DC numbers decreased by day 4 post vaccination by 50% in w.t. mice, DC numbers in B cell deficient remained at the same high frequency (Fig. 4B). [NK cell recruitment in B cell deficient mice was increased on day 2 and day 4 \(Fig. 4B\).](#) The difference did not reach significance but was reproducible in three separate experiments. Adoptive transfer of w.t. B cells to B cell deficient mice abolished increased [retention of DC 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-I CD8 CTL was increased in BCDM in response to gp96-immunization. As shown in Fig. 5, OT-I expansion after gp96-immunization in BCDM was [significantly enhanced on day 5 compared to w.t. mice. Importantly, OT-I persisted at significantly higher frequencies on](#)

day 7 and 12 post immunization in the peritoneal cavity ($p=0.04$) (Fig. 5A) In draining lymph nodes (Fig. 5B) OT1 expansion and retention was also increased without however reaching significance. Adoptive transfer of w.t. B cells to BCDM prior to immunization reduced OT-I expansion to levels at or below those seen in w.t. mice (Fig. 5A, B). The suppression of OT-I expansion by the presence of B cells is not mediated by IL-10 production since IL-10 deficient mice exhibit OT-I expansion similar to w.t. mice rather than the enhanced expansion as seen in BCDM (data not shown).

Gp96-mediated rejection of established non-immunogenic tumors is enhanced in the absence of B cells.

As shown above, growth control of established EG7 tumors in w.t. 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 w.t. mice (24). LLC-ova was established subcutaneously in the flank for 5 days in BCDM and in w.t. mice. OT-I were adoptively transferred i.v. and two days later one million LLC-ova-gp96-Ig was administered as a single dose i.p. and tumor growth in the flank monitored. In w.t. 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, seven day LLC-ova tumors in three mice and significant tumor shrinking in two (Fig 6B). In the absence of treatment, LLC-ova grows progressively in BCDM (Fig. 6B) albeit at a slower rate than in w.t. mice (Fig. 6A). B cell reconstitution of BCDM (Fig. 7C) rendered the effect of vaccination similar to that seen in w.t. 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) appear 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-I) and on antigen specific immunization (LLC-ova-gp96-Ig). In BCDM the presence of one million adoptively transferred OT-I without gp96-immunization 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 anti tumor 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 non-specific myeloid-suppressor cells and T regulatory cells have been implicated in suppression of anti tumor immunity (20, 21, 25). Our studies show that suppression of CTL responses in vivo can be achieved by established tumors through antigen independent pathways. OT-I 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.

While the OT-I response to gp96-ova immunization is strongly inhibited in the presence of established tumors, it is not totally blocked, suggesting that there is balance between immune suppression by the established tumor and vaccine induced CD8-CTL activation through antigen cross presentation by activated DC stimulated by secreted gp96-ova. We have shown previously that in tumor naïve mice gp96-ova results in the recruitment and activation of NK and DC followed by OT-I expansion. Established tumors, while actually enhancing recruitment of cells into the peritoneal cavity by LLC-gp96-Ig vaccination, inhibit OT-I 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 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, daily 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 were sufficient and daily immunization were 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-I response to i.p. 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 anti tumor immunity prompting the question as to their role in gp96 mediated OT-I expansion. Using B cell deficient mice, it became clear immediately both NK and DC recruitment and retention in the peritoneal cavity was increased and OT-I expansion enhanced following gp96-ova immunization. B cell reconstituted BCDM responded like w.t. mice to gp96-ova mediated OT-I 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-I expansion but also strongly enhanced tumor rejection of seven 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 appear 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 anti tumor immune responses (data not shown). It remains to be determined how OX40-L expressed on B cells mediates suppression of anti tumor 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 anti tumor 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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Figure legends:

Fig. 1: Antigen non-specific suppression of OT-I CTL expansion by distant, established tumors

A: Comparison of OT-I CD8 CTL frequency in the peritoneal cavity in unimmunized mice, in immunized, tumor free and in immunized, EG7-tumor bearing mice. One million EG7 tumor cells were transplanted subcutaneously in the flank and allowed to become established for 5 days prior to immunization with EG7-gp96-Ig. One million OT-I CD8 T cells were adoptively transferred i.v. two days before immunization. Mice were immunized with two million EG7-gp96-Ig i.p. Peritoneal cells were analyzed five days later by flow-cytometry.

B. Suppression of OT-I expansion by established tumors is antigen non-specific. EL4 and LLC, not expressing ovalbumin, were established for five days in place of EG7. OT-I adoptive transfer and vaccination was carried out as in A.

C. Absolute numbers of OT-I accumulating in the peritoneal cavity, the vaccination site, in the absence and 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 three or more individual experiments is shown. N = 3-5 mice in each group. Significance values indicated in the figure were calculated by t-test. Negative controls are unimmunized mice \(preimmune\), positive controls are mice without peripheral tumor in the flank.](#)

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 i.p. administration of one million EG7-gp96-Ig or irradiated EG7 was started on the same day or two or four 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 i.p. 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 i.p. 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 i.p. 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; n.s. – not significant.

Figure 3:

Frequent immunizations cause tumor growth retardation of established LLC

LLC (10^5) were transplanted subcutaneously in the flank and allowed to become established for three 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.

Figure 4:

B cells inhibit gp96 mediated recruitment of NK into and retention of dendritic cells (DC) 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 i.p. Accumulation of CD5 positive and CD5 negative B cells was determined daily thereafter by flow cytometry. Representative of more than three experiments.

B: Increased recruitment of NK cells and retention of NK and DC and in B cell deficient mice (BCDM) and its reversal by adoptive transfer of B cells. Wild type and B cell deficient mice were immunized i.p. with two million EG7-gp96-Ig and cells harvested from the peritoneal cavity two and four days later and analyzed by flow cytometry. B cell reconstitution was done by i.v. adoptive transfer of 10^7 w.t. B cells two days prior to immunization with EG7-gp96-Ig. Representative of three experiments.

Figure 5:

Gp96-mediated OT-I CD8 CTL expansion is increased and sustained in the absence of B cells

Wild type mice and B cell deficient mice received one million gfp-OT-I, B cell reconstituted mice in addition received ten million w.t. B cells by i.v. adoptive transfer. The mice were immunized two days later with four million EG7-gp96-Ig and analyzed on the days indicated by harvesting cells from the peritoneal cavity (upper panel) and mesenteric and para-aortic lymph nodes (dLN) lower panel. * $p = 0.04$ by repeated measures Anova. Four mice in each group, representative of three experiments.

Figure 6:

Gp96-mediated tumor rejection is enhanced in B cell deficient mice and abrogated by B cell reconstitution

Upper panel: wild type mice. Lower panel: B cell deficient mice (BCDM). One million LLC-ova cells in 0.2 ml PBS were transplanted into the flank. Five days later one million OT-I were given i.v.. Seven days after tumor transplantation, mice were immunized i.p. with one million LLC-ova-gp96-Ig. Tumor size was measured with a caliper in two dimensions. N = 5 in each group, representative of three experiments.

Figure 7:

High CTL precursor frequency and immunization enhance tumor rejection by gp96 vaccines in B cell deficient mice

A: BCDM were treated as in Fig. 6 except that vaccination with LLC-ova-gp96-Ig was omitted. B. As in Fig. 6, omitting OT-I transfer. C. As in Fig 6, except that BCDM mice were reconstituted with 10 million B cells prior to tumor (LLC-ova) transplantation. N =5 to 6 mice in each group, representative of two experiments.

Fig. 1

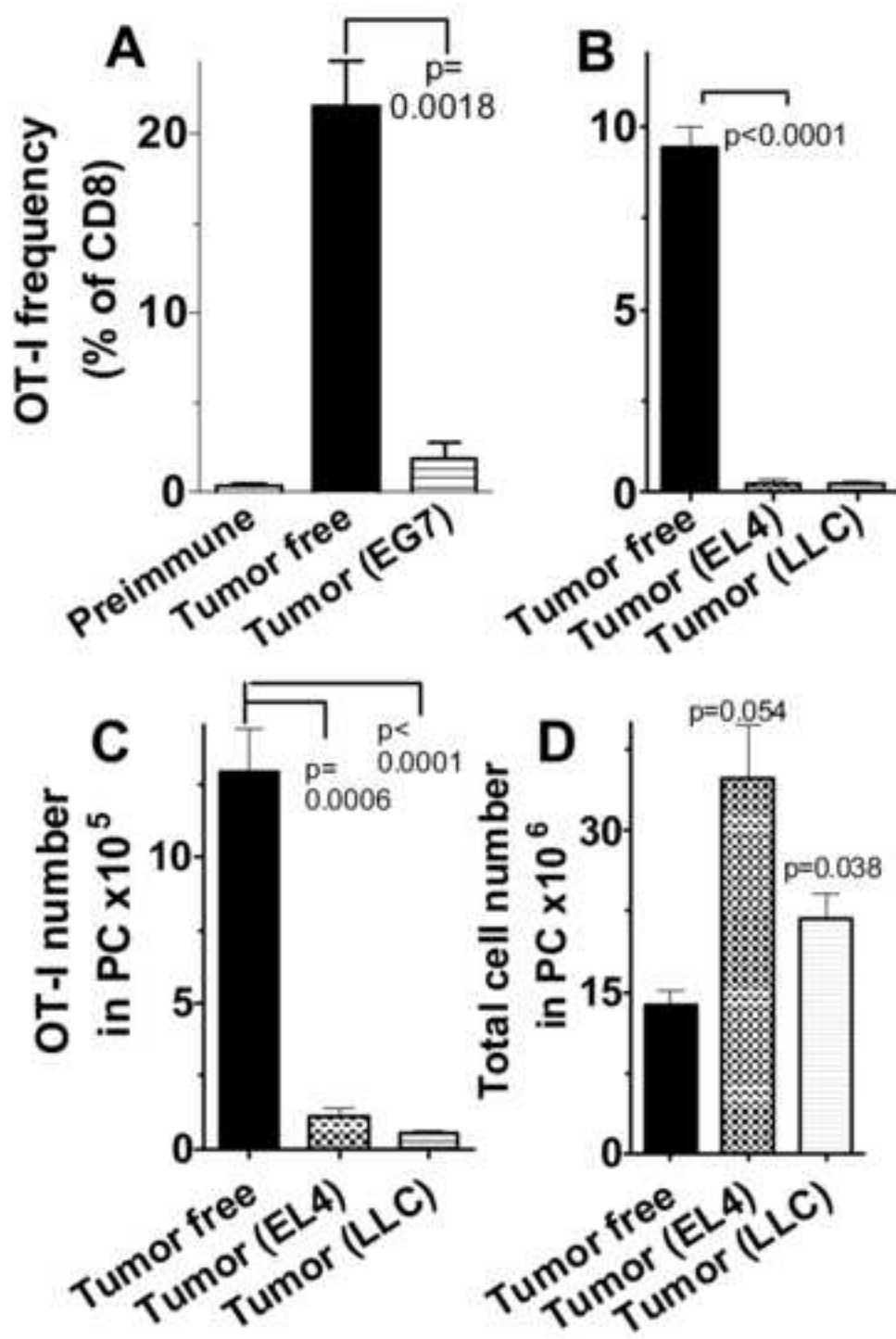


Figure 2

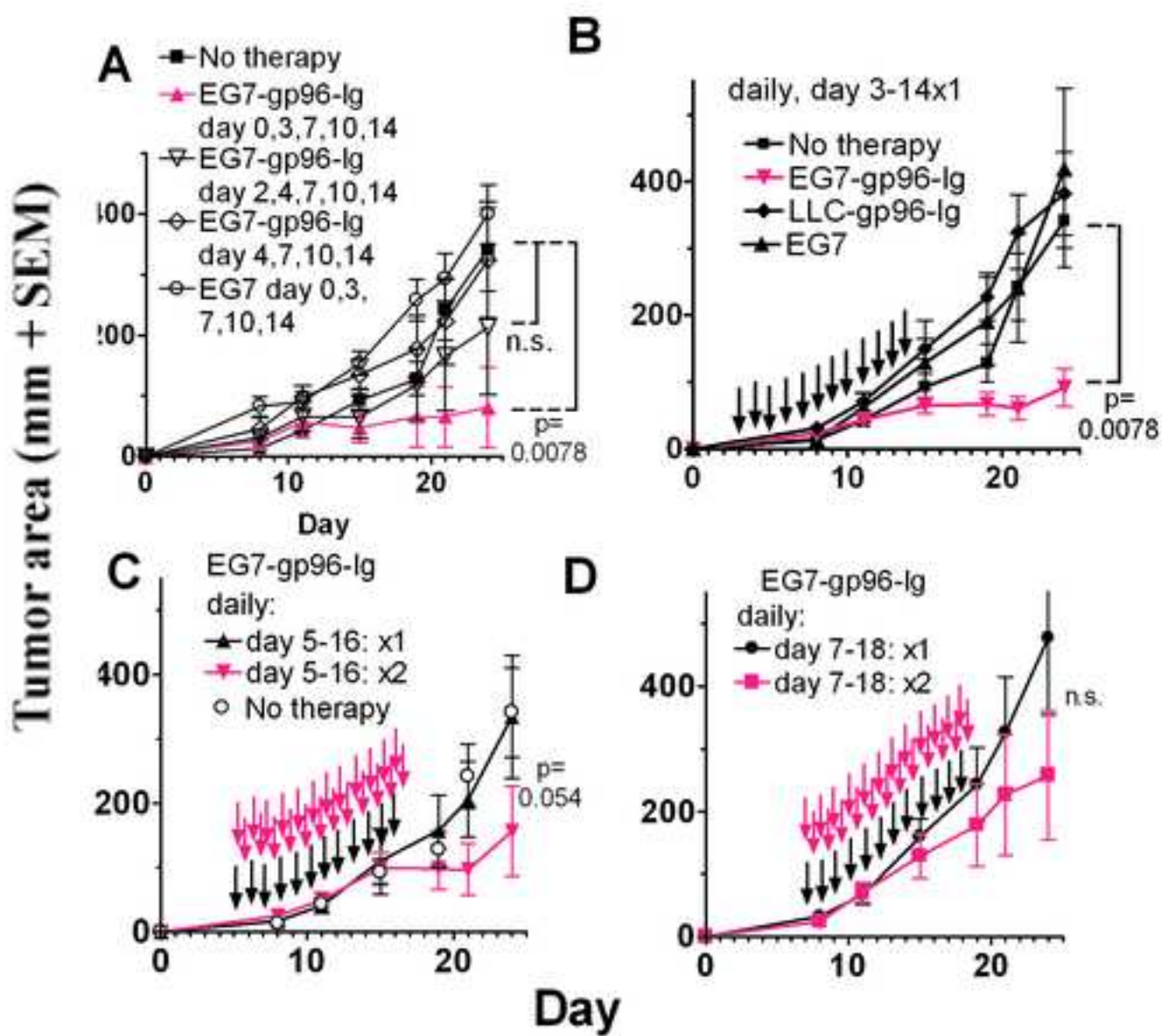


Figure 3

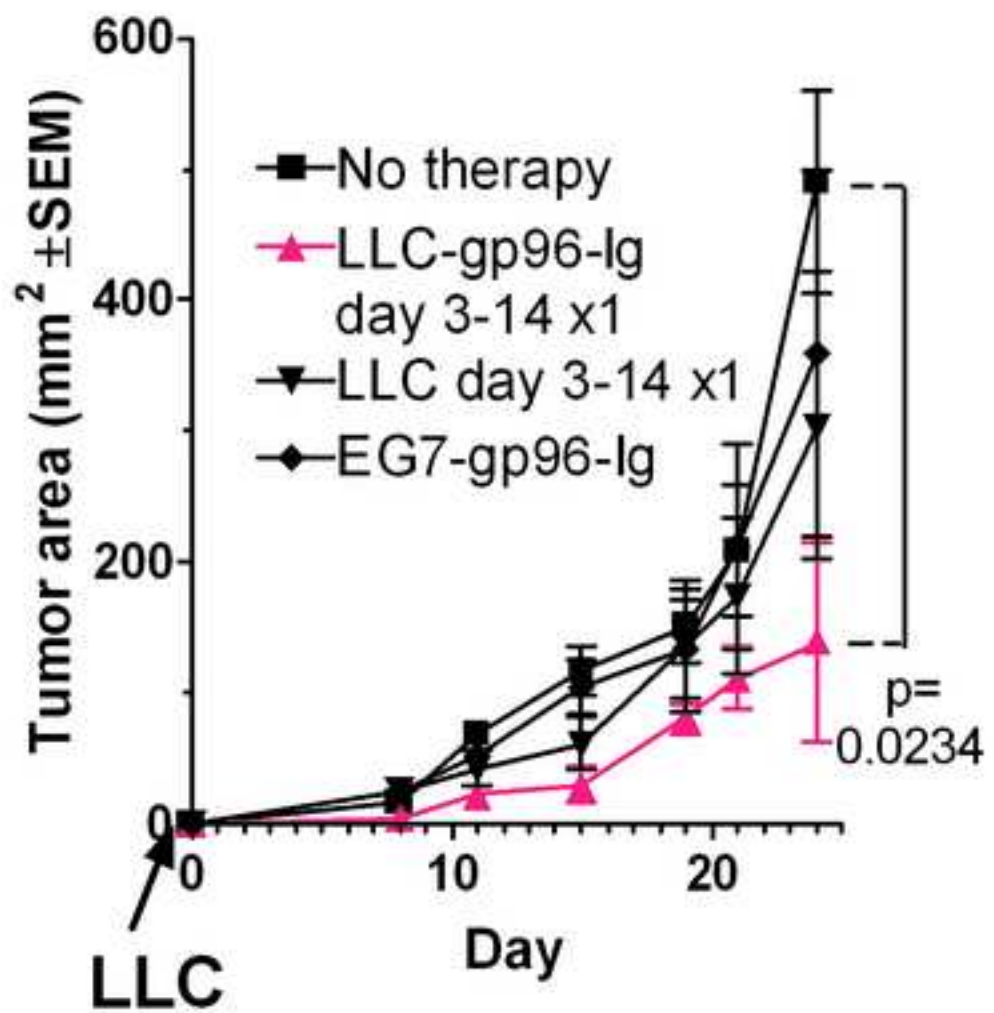


Figure 4

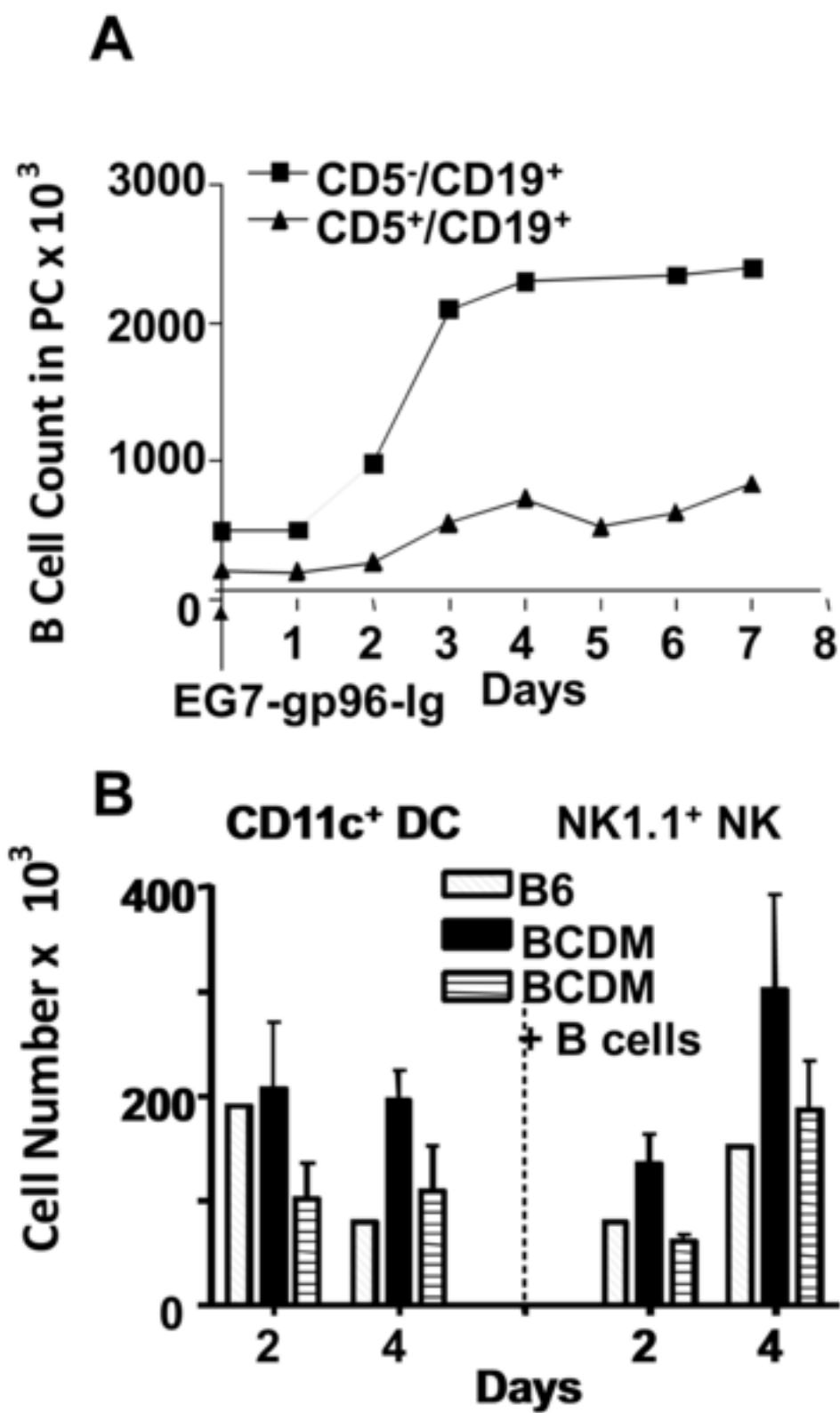


Figure 5

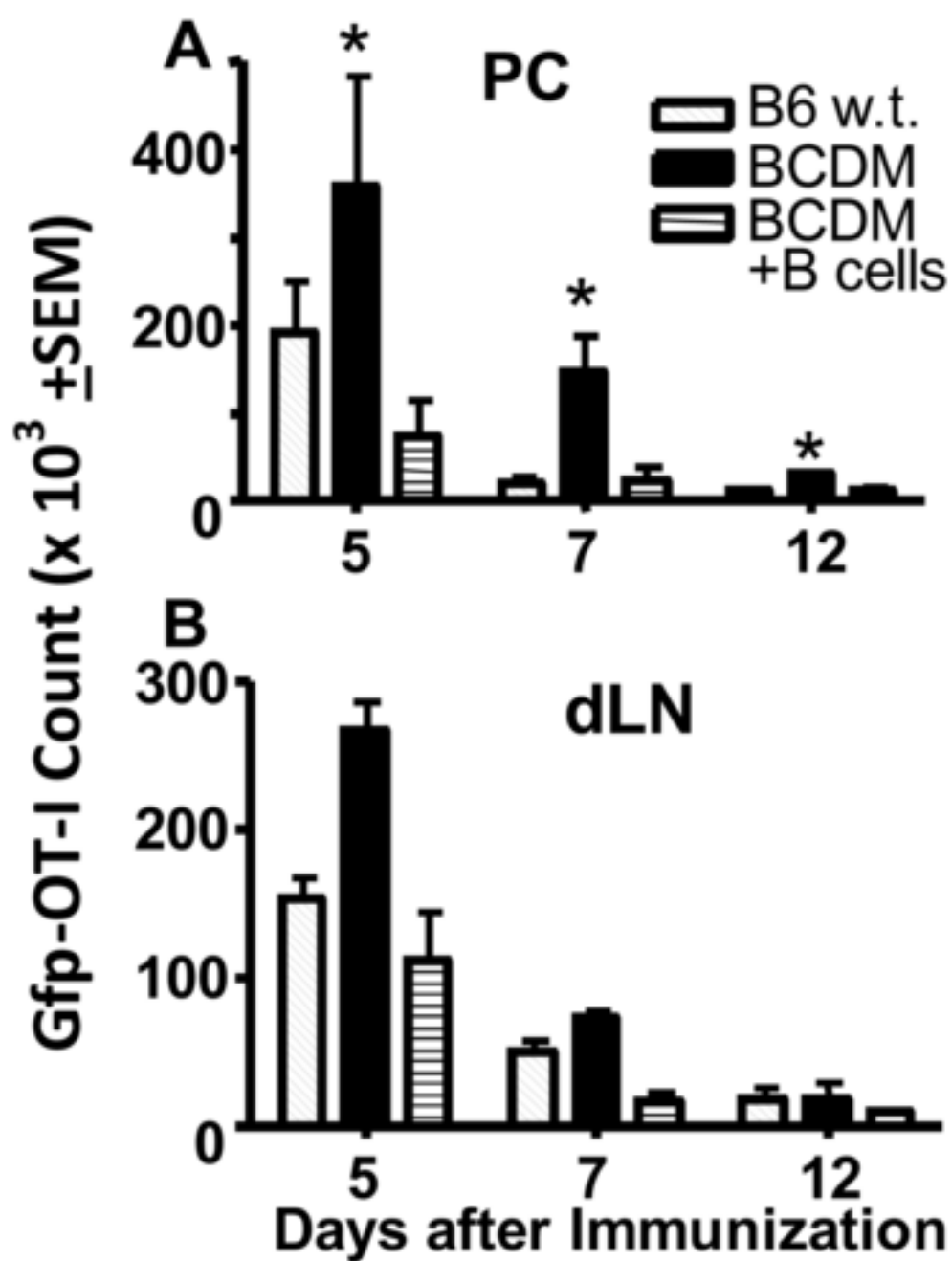


Figure 6

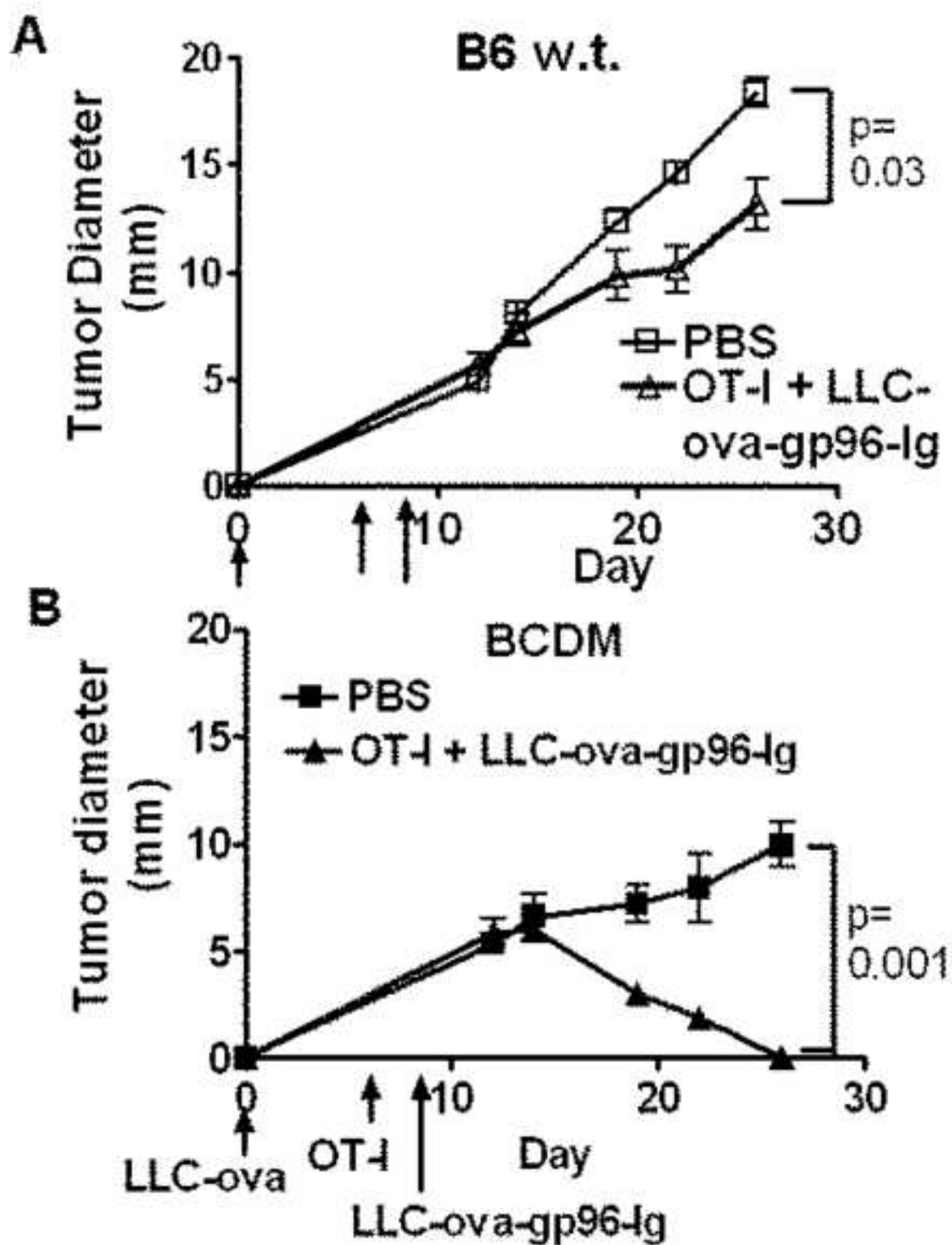


Figure 7

