Published in final edited form as:

Vaccine. 2011 November 3; 29(47): 8599–8605. doi:10.1016/j.vaccine.2011.09.022.

Bone Marrow Vaccination: A Novel Approach to Enhance Antigen Specific Antitumor Immunity

Stephanie Fresnay, Xiaoyu Zhang, Scott E. Strome, and Duane A. Sewell

Department of Otorhinolaryngology-Head and Neck Surgery, University of Maryland, MD, USA

Stephanie Fresnay: sfresnay@smail.umaryland.edu; Xiaoyu Zhang: XZhang@smail.umaryland.edu; Duane A. Sewell: dsewell@smail.umaryland.edu

Abstract

Bone marrow (BM) serves as a reservoir for a unique population of memory T cells with strong effector properties that make them ideal target for cancer immunotherapy strategies. However, direct vaccination and priming of T cells within the BM of the host has never been investigated. This study evaluates the specific immune response induced via a new method of direct intra-bone marrow (IBM) vaccinations in an animal model of human papillomavirus-associated cancer. We found that IBM vaccinations with the class I HPV-16 E7 epitope induce large numbers of activated, IFN- γ -producing E7-specific lymphocytes in the BM. In prophylactic tumor challenge experiments, direct intra-BM vaccination was found to be protective against tumor formation for 80% of the mice. In the therapeutic setting, IBM vaccination induced tumor regression in 3 of 10 vaccinated mice and delayed tumor growth in the remaining animals. Finally, adoptive transfer of BM cells from IBM vaccinated mice to naïve animals conferred complete protection against tumor growth. These data demonstrate the capacity of direct IBM vaccination to induce potent antigen-specific immunity resulting in protection from tumor growth in an animal model. Specifically targeting BM T cells with vaccines may improve responses to cancer immunotherapy and offer important clinical advantages, especially in the setting of bone marrow malignancies.

Keywords

Vaccination; Bone Marrow; memory T cells; HPV

1. Introduction

Recent evidence suggests that memory T cells within the bone marrow (BM) have distinct phenotypic and functional properties when compared to memory T cells from other sites [1]. BM derived T cells contain a distinct population of CD8⁺ T_{EM} cells which express high levels of CD27, CD28, CD38, CD69 and HLA-DR [2]. Furthermore, our group has shown

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Corresponding author : Scott E. Strome, MD, Department of Otorhinolaryngology-Head and Neck Surgery, University of Maryland, 16 South Eutaw Street; Suite 500, Baltimore, MD, 21201, USA, Phone: 410-328-6467, Fax: 410-328-5827, sstrom@smail.umaryland.edu.

Disclosure statement

Conflict of Interest: Dr. Strome is a cofounder and major stockholder in Gliknik Inc., a biotechnology company. He also receives royalties through the Mayo clinic college of medicine relating to inventions for the use of 4-1BB and B7-H1.

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these cells exhibit a profound recall response to viral antigens and display unique patterns of perforin and granzyme B regulation in response to TCR stimulation [2]. These unique properties may make BM-derived T cells ideal effectors for cancer immunotherapy.

The use of BM T cells in immunotherapy strategies has been studied previously. One such study showed that adoptive transfer of primed T cells from the BM, could effectively treat autologous breast cancer xenografts in NOD/SCID mice [3]. T cells from the peripheral blood were far less effective [3]. Another study in metastatic breast cancer patients discovered BM T cells which were specific for breast cancer tumor antigens. Adoptive transfer of these cells was feasible and well-tolerated in a pilot clinical trial [4].

In this study, we explore the possibility of generating large numbers of antigen-specific BM T cells by direct injection of a peptide vaccine into the BM. If shown feasible and efficacious, direct BM vaccinations could represent a novel route of vaccination with several advantages. First, BM vaccinations enable the activation of the unique population of T cells found in the BM. Second, BM T cells, unlike T cells in the periphery, have limited exposure to the immunosuppressive milieu of the tumor. This may provide a therapeutic advantage over other types of vaccination strategies. Third, in the setting of BM-related malignancies such as multiple myeloma, although BM constitutes the tumor environment and cannot be considered as a sanctuary for anti-tumor effector T cells, BM vaccinations might facilitate priming/boosting of large numbers of tumor-specific lymphocytes near the primary tumor site. Thus, antigen-specific cells generated via BM vaccinations may offer important clinical advantages for patients undergoing tumor immunotherapy.

This study explores the possibility of generating large numbers of antigen-specific, BM-derived T cells via direct BM vaccinations in an animal model. We show that 1) vaccination with the Class I restricted peptide for tumor antigen HPV-16 E7 results in functional, activated memory T cells in the BM and periphery, 2) that BM vaccination prevents the growth of HPV-associated tumors and 3) that adoptive transfer of BM cells after BM vaccination also leads to prevention of HPV associated tumors. Taken together, these results suggest that stimulation of the BM memory CD8⁺ T cells by direct BM vaccination is feasible to employ in immunotherapeutic strategies.

2. Materials and Methods

2.1 Mice

C57BL/6 (B6, H-2^b) mice were obtained from Charles River Laboratories (Wilmington, Mass). Animals were housed under conventional conditions and used according to the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland Medical School.

2.2 E7 peptide and oligodeoxynucleotides

The H-2b-restricted epitope (RAHYNIVTF) peptide was obtained from Anaspec (San Jose, CA). The purity of the peptides was determined by reverse-phase HPLC and was found to be routinely >90%. The peptides were dissolved in DMSO and phosphate-buffered saline (PBS) to 2mg/ml (the final concentration of DMSO in stock solution is 5% (v/v)) and stored at -20°C. The CpG-containing oligodeoxynucleotides 1826 (5'-TCC ATG ACG TTC CTG ACG TT-3') were synthesized by Invitrogen Corp (Carlsbad, CA). The CpG was brought to a concentration of 5 µg/µl in PBS and stored at -80°C.

2.3 Immunization strategy

C57BL/6 mice were vaccinated SC, IP, or directly into the BM cavity twice at weekly intervals with E7 peptide (200µg/dose) plus CpG (50µg/dose). Direct intra-bone marrow (IBM) injections were conducted based on the technique described by Kushida et al. [5]. Before IBM injection, mice were anesthetized with ketamine (100 mg/kg) plus xylazine (10 mg/kg). The region of the right knee joint was shaved of hair and the knee was flexed to a right angle to allow injection of the vaccine (final volume of 30µl) directly into the BM cavity with U-100 insulin needle (50µl; Becton Dickinson, NJ).

2.4 Isolation of splenocytes and bone marrow cells

Mice were sacrificed at two and eight weeks after the first vaccination. Spleens were collected and single-cell suspensions were made by filtration through a 100-µm cell strainer (BD Bioscience Pharmingen, San Diego, CA). Femurs and tibias were removed and BM was flushed out with RPMI 1640 (BioWhittaker, Verviers, Belgium). Red blood cells were lysed using lysis buffer (containing 1.66% ammonium chloride) and single-cell suspensions were washed three times in PBS, and re-suspended in complete medium.

2.5 Immunostaining and flow cytometry analysis

Cells were stained for cell surface markers with the following anti-mouse monoclonal antibodies (mAb) or isotype control mAb (all obtained from BD Biosciences Pharmingen, San Diego, CA): PerCP-labeled anti-CD8 (clone 53-6.7), APC-labeled anti-CD62L (clone MEL-14) and PE-labeled H2D^b epitope E7 (RAHYNIVTF)-containing tetramers. The H-2D^b restricted immuno-dominant E7 epitope RAHYNIVTF (also called E7/D^b tetramer) was supplied by the NIAID Tetramer Core Facility at Emory University (Atlanta, GA) through the NIH AIDS Research and Reference Reagent Program. Cells were surface stained for 15 min at 4°C and washed with PBS. All samples were acquired using a BD LSRII flow cytometer (BD Biosciences) and analyzed with FACSDiva Software (BD Biosciences).

2.6 ELISPOT

ELISPOT plates (Millipore, Billerica, MA) were coated overnight at 4°C with anti-IFN-γ capture antibody (15µg /ml, MabTech, Cincinnati, OH). After washing with PBS the plates were then blocked with RPMI containing 10% FBS for 1 hour at 37°C. 8×10⁵ splenocytes or BM cells per well were plated in triplicate in complete RPMI and pulsed for 24 hours with E7 peptide (10µg /ml) or with concanavalin A (Con A, 5µg /ml, Sigma, St. Louis, MO) as a positive control. After washing with PBS, biotinylated anti-IFN-γ detection antibody (1µg /ml, MabTech) was added and incubated for 2 hours. After washing, streptavidin-horseradish peroxidase (1:100, Mabtech) was added for 1 hour. The plates were developed by adding 3-amino-9-ethylcarbazole (AEC) substrate kit (BD biosciences), and counted using a computer-assisted ELISPOT image analyzer (T Spot; Cellular Technology, Cleveland, OH).

2.7 Tumor cell line, challenge, and measurement of growth

TC-1 tumor cells are derived from primary lung epithelial cells immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene [6]. Tumors were established by injecting 1×10⁵ TC-1 cells subcutaneously (SC) into the right flank of the mice. Tumor growth was measured regularly using a digital caliper spanning the shortest and longest surface diameters, and the mean diameter were recorded. Mice were euthanized when the tumor diameter reached 20mm or if they showed signs of distress.

2.8 Prophylactic and therapeutic tumor load experiments

In the prophylactic experiments, mice were vaccinated IBM or SC twice at weekly intervals with E7 peptide plus CpG. One week after the last vaccination, mice were challenged with 1×10^5 TC-1 tumor cells. In the therapeutic setting, mice were challenged on day 0 with 1×10^5 TC-1 cells. Seven to ten days later, when palpable tumors formed and reached 1–2 mm in diameter, mice were vaccinated IBM or SC with E7 peptide plus CpG. Mice were boosted seven days after the first vaccination. Control mice were either vaccinated with CpG alone or untreated. Tumor growth was monitored as described above.

2.9 Adoptive transfer experiments

Mice were vaccinated IBM or SC twice at weekly intervals with E7 peptide plus CpG. Ten days after the last immunization, mice were sacrificed and BM cells were collected. After isolation, 2×10^7 total BM cells from the tibias were administered intravenously into naïve mice. One day following transfer, recipient mice were challenged SC with 1×10^5 TC-1 cells into the right flank. Control mice were challenged with TC-1 cells but did not receive BM cells. Tumor growth was monitored as described above.

2.10 Statistical analysis

Statistical analysis was performed using the two-way non-parametric Kruskal-Wallis ANOVA test followed by Dunn's multiple comparison test or using the paired Student's t-test. P values < 0.05 were considered significant.

3. Results

3.1 Direct IBM immunization with E7 peptide induces long-term presence of functional E7-specific cells in the BM

We evaluated the specific immune response induced by vaccination with the immunodominant class I HPV-16 epitope directly into the BM cavity. This new method of IBM vaccination was performed as first described by Kushida et al. and carried out as shown in Figure 1 [5]. The right knee was bent to a right angle, allowing access to the joint surface of the tibia, and the vaccine was injected directly into the BM cavity. C57BL/6 mice were vaccinated IBM, SC, or IP, two times at a weekly interval with E7 peptide (200 µg/dose) using CpG oligonucleotides 1826 as an adjuvant (50 µg/dose). Negative control mice were left untreated (naïve).

The presence of E7-specific T cells was evaluated *ex vivo* in the spleen and BM cells of immunized mice 2 and 8 weeks after priming. Cell suspensions were stained with anti-CD8-PerCP, anti-CD62L-APC and E7/Db tetramer-PE. The antigen-specific, effector memory T cell population was defined as those cells which were E7 tetramer positive, CD8 positive and CD62L negative (figure 2A, left panel) [7]. Tibias and femurs from injected legs were analyzed separately in order to distinguish injected bones (tibias) from non-injected bones (femurs). We found that IBM vaccinations resulted in the presence of a very high percentage of E7-specific lymphocytes in the BM of the injected tibia (Figure 2B). Among the activated CD8+CD62L- T cells within the injected bone, $37.3 \pm 5.0\%$ were E7-tetramer positive T cells 2 weeks post-vaccination. Eight weeks post-vaccination, a large memory population was still detected within the BM of vaccinated mice ($17.3 \pm 3.8\%$, figure 2B). Interestingly, after 2 weeks of priming, non-injected femurs contained E7-specific cells ($5.5 \pm 0.9\%$), however to a much lesser extent than the injected tibias. At both time points, although there was an increase in the number of E7-specific T cells in the BM after SC or IP vaccination, this change did not reach statistical significance. We also found that direct IBM vaccination could induce the presence E7-specific T cells in the spleen (Figure 2B). Two weeks after the first vaccination, the average percentage of E7-specific cells found in the spleen of IBM

injected mice was $3.1 \pm 1.8\%$ compared to $0.4 \pm 0.1\%$ in the spleens of naïve, control mice. Despite this trend, the difference was also not statistically significant.

These data demonstrate the efficacy of direct IBM peptide vaccination to induce memory E7-specific T cells populations in the BM. In addition, these T cells seem to have the potential to traffic to non BM sites.

After observing that antigen-specific T cells are found at early and late time points after IBM vaccination, we sought to evaluate their functional ability. We used ELISPOT analysis to determine their ability to secrete the pro-inflammatory cytokine IFN- γ upon re-exposure to the antigen. BM cells from tibias and femurs from injected legs were pooled together for this analysis. Due to the low number of T cells in the BM, we performed the assay with total BM. Eight weeks after vaccination, the number of IFN- γ -producing cells upon E7 re-stimulation was significantly higher in BM of vaccinated mice (208 ± 85 spots) than in BM from naïve animals (20 ± 18) (figure 2C).

3.2 BM vaccination can prevent TC-1 tumor growth

Several studies have demonstrated that the presence of E7-specific T cells correlates with protection against HPV-16 E7-expressing TC-1 tumors [6, 8–11]. After confirming that IBM vaccination induces functional memory Ag-specific BM cells, we analyzed the anti-tumor efficacy of these cells in the TC-1 tumor model. Mice were either untreated or vaccinated IBM or SC twice at a weekly interval with E7 peptide plus CpG. Two weeks after priming, all mice were challenged with 1×10^5 TC-1 tumor cells. Following the challenge, naïve mice started to develop palpable tumors at day 3 which were rapidly progressive (Figure 3). The IBM vaccination was found to be protective against tumor formation for 80% of the mice and not statistically different from SC vaccination. In order to establish if the T cells might be responsible for tumor protection, we investigated their capacity to migrate from the BM to the periphery. Two weeks post-vaccination, antigen-specific T cells were found in the circulating blood (Figure S1, A and B). Eight weeks after immunization, although the number of E7-specific T cells decreased in all organs, their presence was still observed in blood and BM (Figure S1, B). These results illustrate the capacity of direct peptide IBM vaccination to induce a potent immune response and prevent tumor formation in a pre-clinical model of HPV-associated cancer.

3.3 BM vaccination induces regression of some established TC-1 tumors

We next sought to assess IBM vaccinations in the more challenging therapeutic tumor model, and to compare IBM vaccinations with the more standard subcutaneous (SC) route. All mice were first injected subcutaneously with 1×10^5 TC-1 tumor cells. When tumors became palpable (~2mm), mice were treated with either BM vaccinations with E7 plus CpG, BM vaccinated with CpG only, SC vaccinations with E7 with CpG, or left untreated. Mice were boosted seven days later, and tumor growth was measured. As shown in Figure 4A, representing one individual experiment, untreated mice and CpG-vaccinated negative control mice developed rapidly growing tumors after TC-1 challenge. In contrast, 2 mice of 3 IBM vaccinated mice experienced complete regression of the tumor. No relapse occurred until day 70 post-tumor inoculation. Similar results were observed for the subcutaneous vaccination group, in which an early regression of the tumor was observed for 2 of 4 mice.

Tumor regression after both BM and SC vaccination was also clearly observed when data from four separate experiments were pooled (Figure 4B). The average tumor size decreased to its lowest value one week after the last immunization, $1.3\text{mm} \pm 0.7$ for BM vaccinated group and $1.6\text{mm} \pm 0.9$ for SC vaccinated group. These differences were highly significant ($p=0.005$) compared to the naïve group (7.0 ± 5.0 , $p<0.05$). The effect of vaccination was

still seen seven weeks after tumor inoculation, since the average tumor size for BM and SC vaccinated mice was still significantly lower than for the control groups (BM 6.6 ± 1.8 ; SC 7.4 ± 1.7 ; Naive 15.9 ± 0.9 ; CpG 13.3 ± 1.7). Moreover, 2/10 SC-vaccinated mice and 3/10 BM-vaccinated mice remained tumor-free (Table 1). These data demonstrate that direct BM vaccination can induce complete tumor regression in some cases and is comparable to standard SC vaccinations. Furthermore, these changes are associated with a higher number of Ag-specific T cells in the tumor of IBM vaccinated mice compare to naïve mice (Figure S2), suggesting, but not proving, a cause effect relationship.

3.4 BM vaccination induces regression of some established TC-1 tumors

In order to show that the protection induced after IBM vaccination resides in the BM cells, we tested the hypothesis that adoptive transfer of BM cells would protect mice from tumor growth. BM cells were isolated from E7 IBM or SC immunized mice and adoptively transferred IV into naïve animals (2×10^7 BM cells / mouse). The next day, the mice were challenged SC with 1×10^5 TC-1 tumor cells. One group of mice did not receive BM cells but were challenged with TC-1 tumor cells as a control. Following tumor challenge, untreated mice and mice that received BM from SC vaccinated mice developed rapidly growing tumors and began to die 27 days after tumor inoculation (Figure 5). In contrast, tumor growth was completely suppressed in all mice that received total BM from BM immunized animals. These mice were still tumor free at day 44 post tumor inoculation. The last protected mouse was re-challenged with 1×10^5 TC-1 tumor cells and was still tumor free 3 months later, suggesting that transfer of E7-specific BM T cells may also induce antigen specific immunologic memory. The complete protection observed in adoptively transferred mice demonstrates the anti-tumor capacity of the BM cells and their ability to exert an immune effect in the periphery.

4. Discussion

Several studies have shown that BM T cells have unique functional and phenotypic properties [2, 3, 12–15]. However, to our knowledge, direct vaccination and priming of T cells within the BM of the host has not been investigated. In this study we show direct BM injections resulted in very high percentages of antigen specific T cells within the BM. Nearly 40% of activated T cells were antigen specific within the BM. This percentage, achieved with only two weekly vaccinations, is much higher than the percentage of Ag-specific cells found in any other organ after peripheral vaccination with the same peptide [16]. This high percentage may be due to proliferation of antigen-specific T cells within the BM. The bone marrow has been shown to be the organ where T cells undergo homeostatic proliferate the most, with the exception of the thymus [17–22]. However, migration of naïve T cells from the periphery followed by priming within the BM can also account for this phenomenon [15, 23]. The fact that a large number of antigen specific BM T cells persisted 8 weeks after vaccination, and were producing IFN- γ as measured by ELISPOT, may indicate antigen specific cells are continually proliferating within the BM long after the vaccinations have ceased. Further studies will be required to elucidate the nature of the BM T cells responsible for the observed anti-tumor effects..

We demonstrated that the high percentage of memory E7-specific T cells populations induced in the BM was associated with protection against tumor development. Direct BM vaccinations resulted in complete protection from tumor growth for 80% of the mice in our pre-clinical model of HPV-associated cancer. In a more challenging experiment in which BM vaccination was performed to treat established tumors, we observed complete regression in 30% of tumors and delayed tumor growth in the rest of the mice. Overall, direct BM vaccination was comparable to standard SC vaccinations, and we observed slightly more complete tumor regressions with BM vaccinations. These results were

particularly encouraging because we observed an anti-tumor effect despite the fact that only modest numbers of the activated antigen specific T cells escape the BM and reached the periphery after vaccination. Furthermore, the persistence of these cells in the BM and the fact that these animals were resistant to tumor rechallenge, suggests that this approach may provide long-lasting immunologic memory.

We hypothesized that a therapeutic effect could be attained via adoptive transfer of activated BM derived T cells, since the migration of the E7-specific T cells to the tumor site is critical to mediate contact-dependent effector functions. Our data showed that adoptive transfer of BM derived T cells can completely protect mice from growth of HPV associated tumors. The efficacy of the adoptive transfer was especially interesting in respect to the actual number of antigen specific T cells transferred. In our adoptive transfer model, only $0.4\text{--}1.4 \times 10^5$ activated specific T cells are transferred, without any further *ex vivo* reactivation prior to the transfer. Other studies have shown success with adoptive transfer in murine tumor model, but with larger numbers of BM T cells (around 5×10^6) that were restimulated *ex vivo* prior to the transfer [4, 13, 24]. Moreover, BM transfer in our study was performed without prior myeloablative regimen. These findings are particularly interesting since most studies indicate that adoptive cell transfer of lymphocytes is not effective in the absence of precondition [25].

Although there was only a small increase in efficacy of direct IBM vaccination over subcutaneous vaccinations, our data show that direct BM vaccination – a completely novel route of vaccine administration – is feasible and efficacious in a mouse model. Furthermore, our model, while clinically relevant, does not account for the peripheral immune tolerance seen in cancer patients. While SC vaccinations largely lose efficacy in the face of tolerance seen in clinical settings, BM vaccinations may be able to partially overcome tolerance. BM cells exhibit a profound recall response to viral antigens and display unique patterns of perforin and granzyme B regulation in response to TCR stimulation [2]. When translating pre-clinical findings to clinical responses, accessing the unique, activated T cell population within the BM may result in improved outcomes in a clinical trial setting.

Valid questions can be raised regarding the feasibility of this strategy in a clinical setting. However, BM aspirations and biopsies are routinely performed for the diagnosis of a number of malignancies. In addition, BM injection recently has received attention as a rapid and safe alternate route for fluid and drug administration in emergency medicine. There are few contra-indications or complications associated with this particular procedure [26, 27]. In fact, specialized medical devices are now available which facilitate intra-osseous infusion of drugs in adults [27]. A clinical trial can be designed in which peptide vaccines are injected into the BM, then BM harvested and antigen specific T cells selected and expanded *ex vivo*. Cells could then be re-infused as part of an adoptive transfer strategy. We have demonstrated that a small number of these highly activated cells may have a therapeutic effect. Furthermore, this method addresses one of the most serious drawbacks of adoptive cell transfer (ACT) – the frequent inability to generate tumor reactive T cell cultures because of insufficient tumor samples. Even more interestingly, trials can be designed for the treatment of multiple myeloma with direct BM vaccinations of a peptide vaccine against the cancer testis antigen MAGE A3/6, which was found to be expressed in 70% of MM specimens [28].

In summary, our data show that direct IBM peptide vaccination can induce a high memory E7-specific T cells populations in the BM and result in protection from tumor growth in an animal model of cancer. The protection from tumor growth induced by direct BM vaccination and by adoptive transfer of BM cells constitutes a proof of principle of the potential role of the BM in immunological memory and tumor protective immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Role of the funding source

This work was supported in part by NIH grant R21DE017159 and by a generous contribution from the Orakawa Foundation.

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Highlights

- We evaluated the specific immune response induced by direct intra-bone marrow (IBM) vaccinations.
- IBM vaccinations with E7 peptide induce activated, IFN- γ -producing, E7-specific lymphocytes in the BM.
- IBM vaccinations can prevent and treat human papillomavirus-associated tumors.

Fig. 1. Schematic of intra-bone marrow injection technique. C57Bl6 mice were anesthetized and shaved at the knee. The joint was then bent to a right angle, allowing access to the surface of the tibia. The vaccine was injected directly into the bone marrow cavity of the tibia.

Fig. 2.

Direct IBM vaccination with E7 peptide induces long term presence of functional E7-specific cells in the BM. C57BL/6 mice were vaccinated IBM, SC, or IP, two times at a weekly interval with E7 peptides plus CpG. Control mice were left untreated (naïve). Two and eight weeks after priming, splenocytes and BM cells were stained with anti-CD8-PerCP, anti-CD62L-APC and E7/Db tetramer-PE and analyzed by flow cytometry. A, Cells were gated on CD8+CD62L- T cells, representing activated memory T cells as shown in Figure A. The right panel shows the percentage of E7-specific CD8+ cells gated on the CD8+CD62L- in the spleen and bone marrow detected for one mouse per group. Tibias and femurs from injected legs were collected separately in order to separate injected bone (tibias) from non-injected bones (femurs). B, The mean and SEM of the percentages of E7-specific CD8+ cells gated on the CD8+CD62L- cells are represented for each group (n = 3). C, IFN- γ ELISPOT of splenocytes and BM cells upon stimulation with E7 peptide. BM cells from tibias and femur from the injected leg were pooled together for analysis. The mean and SEM of the number of IFN- γ for each group (n = 3) at eight weeks after priming. These data are representative of five independent experiments. Statistical analysis was performed by two-way non-parametric Kruskal-Wallis ANOVA test followed by a Dunn's multiple comparison test. (*, $p < 0.05$, **, $p < 0.01$).

Fig. 3.

IBM vaccination can prevent TC-1 tumor growth. Mice were either untreated or immunized IBM or SC with E7 peptide plus CpG at day 0 and day 7. At day 14, all mice were challenged with 1×10^5 TC-1 tumor cells. The mice were regularly monitored for evidence of tumor growth by palpation and measurement. The values represent the mean and SEM of the tumor diameter for each group of mice ($n = 5$) plotted against days post tumor inoculation. This represents a pool of two separate experiments. Statistical analysis was performed by Student's *t*-test. (*, $p < 0.05$ **, $p < 0.01$).

Fig. 4.

IBM vaccination slowed tumor growth of established TC-1 tumors. On day 0, 1×10^5 TC-1 tumor cells were implanted subcutaneously into mice. When tumor size reached 1–2mm, mice were immunized IBM or subcutaneously (SC) with E7 peptide plus CpG and boosted seven days later. Control groups were either IBM immunized with CpG alone or left untreated. A, Tumor growth is represented for each individual mouse in one representative experiment.

B, The mean and SEM of the tumor diameter for a total of four separate experiments are represented for each group of mice (Naïve $n = 10$, CpG $n = 3$, IBM $n = 10$, SC $n = 10$) over time. Statistical analysis was performed by Student's *t*-test. (*, $p < 0.05$. **, $p < 0.01$).

Fig. 5.

Adoptive transfer of BM cells from IBM immunized mice protects naïve mice from tumor development. C57BL/6 mice were vaccinated IBM or SC at day 0 and day 7 with E7 peptides plus CpG. Two weeks after priming, BM cells from vaccinated mice were isolated and adoptively transferred into naïve mice (2×10^7 BM cells / mouse). The day after the adoptive transfer, animals were challenged with 1×10^5 TC-1 tumor cells. A group of naïve mice did not receive BM cells but were challenged with TC-1 tumor cells as control (untreated). The values represent the average and SEM of tumor diameter of each group ($n = 3$) plotted against days post-tumor inoculation. These data are representative of three independent experiments. statistical analysis was performed by Student's *t*-test (**, $p < 0.01$).

Table 1

Number of fully protected mice/number of mice challenged with tumor

Immunization group	surviving mice
Naïve	0/10
CpG	0/3
BM	3/10
SC	2/10