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Antigen Loading of DCs with Irradiated Apoptotic Tumor Cells Induces Improved Anti-Tumor Immunity Compared to Other Approaches

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Abstract

Dendritic cells (DCs) serve as central regulators of adaptive immunity by presenting antigens and providing necessary co-signals. Environmental information received by the DCs determines the co-signals delivered to the responding adaptive cells and, ultimately, the outcome of the interaction. DCs loaded with relevant antigens have been used as therapeutic cellular vaccines, but the optimal antigen loading method has not been determined. We compared different methods to load class I and class II epitopes from the male antigenic complex, HY, onto DCs for the potency of the immune response induced in vivo. Co-incubation of female DCs with HY peptides, RNA or cell lysate from HY expressing tumor induced immune responses equivalent to male DCs. In contrast, female DCs incubated with irradiated, apoptotic HY expressing tumor cells (or male B cells) generated a stronger immune response than male DCs or female DCs loaded using any of the other methods. DC loading with apoptotic tumor resulted in complete protection against high dose HY-expressing tumor challenge whereas 100% lethality was observed in groups receiving DCs that were loaded with peptides, RNA, or lysate. We conclude that signals provided to the DCs by apoptotic cells substantially augment the potency of DC vaccines.

Introduction

Dendritic cells (DCs) orchestrate adaptive immune responses by interpreting signals received by the surrounding environment at the time of antigen presentation and modulating costimulatory signals to shape the outcome of the T cell:DC encounter^{1,2}. Host-derived inflammatory signals, pathogen-derived products and immunosuppressive mediators together provide information to the DC that are capable of initiating or suppressing an immune response to a cognate antigen^{3,4}. DC vaccination can induce potent immune responses in vivo and clinical trials using DC-based immunotherapeutic targeting of tumors are underway⁵. While recent studies of DC vaccines have shown some preliminary activity in the setting of minimal residual disease⁶, little clinical activity has been observed in the setting of established bulky tumors⁷. Thus, although DC vaccination remains a promising approach for the immunotherapy of malignancy, strategies to improve efficacy are needed. Integration of the growing knowledge of DC biology into future DC vaccine trials provides a strategy to achieve this goal.

A number of methods have been used to load tumor associated antigens onto DCs including transfection, dendritic cell:tumor cell fusion, and loading with peptides, tumor RNA, DNA, whole cell lysate and apoptotic cells. While each of these techniques has inherent advantages

and has shown activity in diverse model systems, very few studies have compared techniques for antigen loading in the same model⁸. Given the importance of environmental signals delivered to the DC for adaptive immune response induction, we hypothesized that antigen-loading methods might alter the potency of DC vaccination. To examine this, we utilized immune responses to the model antigen complex HY following DC vaccination using different strategies of antigen loading. Endpoints were quantitative immune responses as measured by tetramer and ELISPOT and protection against the growth of an HY-expressing tumor. Remarkably, antigen loading with apoptotic cells was more potent than all of the other approaches tested, resulting in the highest measured immune responses and the most effective protection from tumor challenge. Therefore, the method used to load antigens onto DCs for vaccination in clinical settings significantly impacts vaccine effectiveness.

Methods

Generation of Bone Marrow Dendritic Cells

Bone marrow was flushed from femurs and tibiae of C57BL/6 mice, filtered through a 70 μ M nylon mesh (BD Falcon), and erythrocytes were lysed by ammonium chloride (BioWhittaker). Cells expressing the lineage specific antigens CD5, CD45R, CD11b, Gr-1 and Ter-119 were depleted by magnetic activated cell sorting (MACS, Lineage Depletion Kit, Miltenyi Biotec), followed by positive selection for sca-1 (Sca-1 Microbeads, Miltenyi Biotec). Sca-1⁺/lin⁻ cells were cultured for seven days in complete media (RPMI, 10% fetal calf serum (FCS, Harlan Technologies), penicillin/streptomycin, Hepes buffer, sodium pyruvate, non-essential amino acids, 2 mercaptoethanol [Life Technologies]) supplemented with GM-CSF 1000 U/mL and IL-4 1000 U/mL (PeproTech, Inc.) and the hematopoietic progenitor cell expansion cytokines Flt-3 ligand (25 ng/mL), SCF (100 ng/mL) and IL-3 (20 ng/mL) based on the method of Jackson et al⁹. The media was changed and additional cytokines were added on days, 3, 5 and 7 of the culture (GM-CSF and IL-4 only on days 3 and 7, all cytokines on day 7). Cultures were maintained at 37°C, 5% CO₂ in tilted T25 flasks.

DCs were matured with 20 μ g/mL anti-CD40 mAb (R&D Systems, Clone I-C10) and 5 μ g/mL CpG-ODN 1555 (GCTAGACGTTAGCGT, gift of Cindy Leifer, National Cancer Institute). For antigen loading using peptides, DCs were activated 20–22 hours *prior to* peptide pulsing. For antigen loading using RNA, lysate and apoptotic bodies which required antigen processing, DCs were activated 20–22 hours *following* antigen loading.

Dendritic Cell Characterization

Surface Phenotyping—DC surface phenotype on Day 1, 5, 7 and 8 of culture was measured by flow cytometry using fluorescein isothiocyanate (FITC) conjugated MHC Class II (I-A^b) monoclonal antibody, phycoerythrin (PE) conjugated CD11b, CD40, CD54, CD80, and CD86 monoclonal antibody and allophycocyanin (APC) conjugated CD11c, in addition to appropriate isotype (IgG2a) controls (BD Pharmingen). Cells were analyzed using a dual-laser FACS Caliber (BD) with Cellquest® software.

Endocytosis Assay

On Day 7 of culture using the conditions described above, CD11c⁺ DCs were positively selected with magnetic beads (Miltenyi Biotec), then incubated for one hour with 10 μ g of FITC conjugated albumin (Sigma) at either 37°C or 0°C. The uptake reaction was stopped by washing three times with ice-cold FACS staining buffer (PBS plus 2mM EDTA, 10% FCS and 0.1% sodium azide (Life Technologies)). The percent of live, CD11c⁺ DCs incorporating FITC albumin was determined by flow cytometry.

Intracellular IL-12 Expression

Expression of intracellular IL12 was evaluated in activated vs. nonactivated DCs on Day 8 using flow cytometry. Following positive selection for CD11c, protein export was stopped by adding 1 μ g/mL brefeldin A (GolgiPlug, BD Pharmingen) for 10 hours. Cells were fixed and permeabilized (Cytotfix/Cytoperm, PermWash buffer, BD Pharmingen) prior to intracellular staining. DCs stained with the PE-conjugated antibody preincubated with purified IL12 and appropriate PE isotype controls served as negative controls.

Antigen Loading

Peptide Pulsing—Activated female DCs were washed twice in complete media and placed in serum-free media (HL-1 [Cambrex] plus 1% penicillin-streptomycin with glutamine) at 2×10^7 cells/mL, then incubated with 1 μ M HPLC-purified (90–95% pure, prepared from 1000 μ M stock solutions diluted in serum-free media) peptides (Bachem) for two hours at 37° C: Uty (H2-D^b restricted Class I immunodominant, WMHHNMDLI), Smcy (H2-D^b restricted Class I subdominant, KCSRNRQYL), Dby (H2-A^b Class II immunodominant, NAGFNRNRANSSRSS) or irrelevant peptides (for Class I, E7, RAHYNIVTF; for Class II, Trypanosoma cruzii(TC) surface protein, SHNFTLVASVIEEA). Following peptide pulsing, DCs were washed twice in complete media then resuspended at 1×10^6 cells/mL.

Whole Cell Preparations—MB49 a bladder epithelial carcinoma that naturally expresses the male antigenic complex, HY (generously provided by Dr. Edward Latime)¹⁰, was grown to confluence, and cells were harvested by trypsinization. Tumor RNA was isolated from 2×10^6 MB49 by phenol extraction followed by ethanol precipitation. The final concentration of RNA was determined by spectroscopy and 10 μ g of RNA was added to 1×10^6 female dendritic cells in serum-free media for 1 hour at 37° C. MB49 tumor lysates were prepared according to the methods previously described by Mule et al¹¹. Briefly, MB49 cell suspensions of 2×10^6 cells/mL were frozen at –80° C for 20 minutes, thawed at 37° C for 10 minutes. After three freeze-thaw cycles, the lysate was aliquoted and incubated with 1×10^6 DCs (lysate:DC ratio = 3 tumor cells:DCs) overnight at 37° C in complete media. To generate apoptotic bodies, MB49 was harvested by trypsinization, then placed in single cell suspensions at 1×10^7 cells/mL, then exposed to 10,000 cGy gamma irradiation. Following irradiation, cells were washed once, counted and incubated in a 1:1 ratio with 1×10^6 DCs for four hours at 37° C in complete media. Apoptosis was verified 30 minutes post-irradiation by Annexin V staining (BD Pharmingen).

Measurement of Immune Responses

Tetramer and ELISPOT assays were performed as previously described¹². Briefly, RBC-lysed splenocyte single cell suspensions were incubated for one hour at room temperature with H2-D^b tetramer loaded with peptides derived from Uty, Smcy or the irrelevant H2D^b binding peptide (E7 or TC peptides). Cells were washed in FACS buffer and incubated with anti-CD8, anti-CD44 and anti-CD4 antibodies (BD-Pharmingen) then analyzed. After gating on CD8+/CD4- cells, CD44+/tetramer positive cells were quantified. The total number of specific Uty- and Smcy-reactive cells were calculated following subtraction of E7 binding cells. For the interferon γ ELISPOT assay, 1×10^6 splenocytes were co-incubated with peptide-pulsed stimulators in a 1:1 ratio in 96-well membrane plates (Millipore) that had been pre-coated with purified anti-IFN γ capture antibody (BD-Pharmingen) for 24 hours in a 1:1 ratio with peptide-pulsed stimulators. All samples were run in triplicate. The plates were washed and then incubated for an additional 24 hours and biotinylated anti-IFN γ . Plates were washed again and streptavidin-alkaline phosphatase was added for 2 hr followed by washing and development with substrate solution. Following drying, plates underwent automated counting (CTL).

Specific spots were calculated by subtracting spot numbers in wells stimulated with E7 (for Uty and Smcy) or TC (for Dby).

Tumor Protection Studies

Female C57BL/6 mice were immunized on day 0 with 1×10^5 DCs injected intradermally. On Day 14, MB49 cells were injected subcutaneously at the right flank. Rate of tumor growth and tumor-free survival was monitored every 2–3 days for at least 40 days.

Statistical Analysis

Statistical analysis was performed using Prism (GraphPad Software). Differences between two groups were evaluated using a two-tailed, unpaired Mann-Whitney test. Survival curves were analyzed using the Wilcoxon Rank Sum statistic. P values < 0.05 were considered significant.

Results

Large numbers of mature DCs can be generated from *sca+/lin-* BM derived cells *in vitro*

Bone-marrow derived stem cells can be induced to differentiate into DCs that can potentially induce immune responses in both mice and humans. To study the effects of antigen loading on the induction of immune responses it was necessary to obtain adequate numbers of hematopoietic stem cell derived-DCs at similar levels of maturation. We first expanded *sca+/lin-* bone marrow-derived progenitor cells *in vitro* with IL-4 and GM-CSF (Figure 1a). While homogenous populations of DCs were generated, total expansion was only 5-fold resulting in insufficient numbers for adequate study. However, by combining GM-CSF and IL-4 with Flt3 ligand, Stem cell factor, and IL-3, expansion was 50 fold, generating large numbers of bone marrow-derived DCs at similar levels of maturation from very small numbers of *sca+/lin-* progenitor cells.

Prior to expansion, *sca+/lin-* cells do not express the DC markers CD11c, CD40, CD80 or CD86. By day 5 of culture, the expanding cells express CD11c, but retain an immature DC phenotype characterized by low expression of CD40 and CD86 and no expression of CD80 (Figure 1b). These immature DCs were capable of antigen uptake as evidenced by endocytosis of FITC-conjugated albumin (Figure 1c). Given that immature DCs can induce tolerance¹³, we activated the DCs using anti-CD40 and a CpG oligonucleotide on day 7, resulting in CD80 expression and upregulation of CD40, CD86 and CD11c (figure 1b). Furthermore, the activated DCs showed dendrite formation (Figure 1d), an inability to endocytose FITC conjugated albumin and higher levels of intracellular IL-12 (figure 1e), all features consistent with a mature or activated phenotype. The combination of anti-CD40 and CpG resulted in superior activation when compared to either stimulus alone (data not shown). Thus, this system generated large numbers of immature DCs capable of antigen uptake and processing as well as the reliable DC activation into a mature phenotype associated with characteristic functional changes.

Peptide pulsed DCs expand functional antigen specific T cells to the same magnitude as DCs endogenously expressing the same antigens

To compare the functional capacity of activated bone marrow-derived DC endogenously expressing antigen vs. those loaded exogenously with peptide antigen, normal B6 female mice were injected intradermally on Day 1 and Day 14 with 1×10^5 of either anti-CD40/CpG-activated male DCs or activated female DCs pulsed with the dominant class I antigen derived from the Uty, the subdominant class I antigen derived from Smcy and the dominant class II antigen derived from Dby¹⁴. As seen in Figure 2a, T cells binding MHC class I tetramers conjugated to the Uty peptide epitope do not expand in mice immunized with female DCs or with female DCs pulsed with irrelevant peptides (data not shown), whereas immunization with

male DCs expands HY-reactive T cells such that approximately 1% of the total CD8+ in the spleen recognize Uty two weeks after the second immunization. Pulsing of Uty peptides onto DCs also expanded Uty-reactive T cells, as evidenced by a statistically significant increase when compared to female DC-immunized mice. However, the magnitude of expansion induced by peptide-pulsed DCs failed to reach that observed in male DC-immunized mice (Figure 2b). When the frequency of T cells that produce interferon γ in response to Uty peptide stimulation was measured using ELISPOT, there was no difference between peptide-pulsed DC immunization and male DC immunization (Figure 2c). Similar patterns were seen with Smcy-reactive CD8+ T cells and Dby-reactive CD4+ T cell expansion. These data indicates that both peptide-pulsed DCs and DCs endogenously expressing the same antigens can expand functional antigen-specific T cells to approximately the same magnitude with similar results observed for dominant and subdominant CD8+ responses and dominant CD4+ responses.

HY-expressing tumor cell RNA and tumor lysate loading of DCs expands HY reactive T cells with a potency equivalent to peptide-pulsed DCs, but DCs loaded with irradiated apoptotic tumor cells elicit stronger immune responses

One of the disadvantages associated with the clinical translation of peptide-pulsed DC vaccines is that specific epitopes for the targeted antigens must be identified for each MHC allele, requiring extensive preclinical characterization of each epitope. For this reason, peptide-based immunization has primarily targeted common Class I HLA alleles and often does not incorporate Class II epitopes, which are difficult to identify. To overcome this, alternative strategies have been employed to load antigens derived from whole tumor cells which are then processed and presented according to the MHC molecules present on the DC. Such techniques offer the advantage of not requiring knowledge of specific antigens, but it remains unclear whether these strategies efficiently load tumor antigens and whether these antigens can successfully compete with the self antigen repertoire present in both the DC and on the tumor itself. To explore this in our model, we used cells from MB49, a bladder epithelial carcinoma that expresses HY antigens, to prepare RNA, lysate and irradiated apoptotic cells using standard techniques. These sources for HY antigen were then added to cultures containing immature bone marrow-derived DCs. Based upon the ability for these DCs to take up FITC albumin, we inferred that they would also be capable of lysate and/or RNA uptake. To optimize presentation, the DCs were activated with anti-CD40/CpG 20–22 hours following coincubation with lysate or RNA. Antigen-loaded DCs were then injected intradermally using the same day 0 and day 14 schedule employed for the peptide-loaded and male DCs. As seen in Figure 2, HY-expressing tumor lysate or tumor RNA-loaded DCs elicited quantitatively similar CD8+ and CD4+ T cell expansion to HY peptide-pulsed DCs and male DCs. Thus the magnitude of the vaccine response induced by peptide-pulsed DCs, DCs endogenously expressing HY, tumor lysate-pulsed DCs, and RNA-pulsed DCs are equivalent in this model system. Remarkably however, immunization with DCs loaded with apoptotic HY-expressing tumor cells resulted in a statistically significant increase in CD8+ dominant, CD8+ subdominant and CD4+ responses when compared to all other methods of antigen loading. Furthermore, this method was also superior to the responses induced by male DCs endogenously expressing the HY antigen complex. This was not unique to tumor cells as a similar magnitude of expansion was seen when irradiated male spleen cells were used to load HY antigens into DCs (data not shown).

To assess the functional consequences of vaccination strategies on tumor growth, female mice were vaccinated as described above on day 0 and day 14, followed by challenge with MB49 on Day 28. As shown in Figure 3a, an inoculum (3×10^6 cells) of MB49 is rapidly lethal in female mice. Tumor lysate-pulsed, tumor RNA-pulsed and HY peptide-pulsed DC immunization protected approximately 40% of mice from tumor development whereas male DCs were superior in mediating tumor protection and resulted in tumor growth prevention in

80% of the mice. Remarkably, DC loading with apoptotic MB49 cells resulted in a statistically significant improvement in tumor protection when compared to all other DC loading strategies except male DC with prevention of tumor growth in all mice treated. Increasing the tumor dose to 5×10^6 cells resulted in 100% lethality in mice immunized with all DC loading methods except apoptotic MB49 which resulted in protection of all mice receiving this higher dose tumor challenge (Figure 3b).

Discussion

A number of reports have compared DC loading strategies, but these have generally used only two loading methods and the majority did not quantitate immune responses to specific epitopes (reviewed in ⁸). We report a comparison of quantitative (including class I dominant and subdominant and class II responses) and functional immunity to a model tumor antigen system following a diverse array of antigen loading methods that encompass many of the common DC loading methods in current clinical use. Although all methods induced CD4 and CD8 T cell expansion, DC loading with irradiated tumor cells induced higher quantitative responses to the three epitopes studied which also translated into improved antitumor activity. While a technical explanation based upon improved protein stability/recovery following irradiation as compared to the other methods could be invoked to explain the superiority of the irradiated cells compared to peptide, RNA or lysate loading, the fact that apoptotic cell loading also afforded enhanced protection over male DCs endogenously expressing HY provides evidence that protein integrity does not entirely explain the data presented here. Rather, the results suggest that apoptotic tumor cells provide additional signals to DCs that optimize antigen presentation beyond that conferred by the maturational stimuli already provided by anti-CD40 in this model system.

These results are remarkable in light of the substantial data in the literature demonstrating that apoptotic cell death is associated with tolerogenic immune signals^{15,16}. Numerous studies have demonstrated that naturally-occurring apoptosis in normal development, is interpreted as immunologically “bland” resulting in tolerogenic DCs which appear to contribute to the maintenance self-tolerance^{17–20}. In contrast, necrotic cell death, associated with tissue destruction *in vivo* activates immunity via DCs, presumably as a result of endogenous and/or exogenous cofactors associated with the tissue damage. This paradigm does not predict that apoptotic tumor cells delivered to the dendritic cell would enhance anti-tumor reactivity. However, conclusions drawn from non-inflammatory models may not be entirely relevant to a model system in which exogenous stimuli are simultaneously provided to the DCs and induce IL-12 production and DC maturation. In such settings, substantial data has demonstrated that the tolerogenic impact of apoptotic cells can be overridden by the immune activating stimuli provided by DCs. Consistent with these findings, several studies have demonstrated that apoptotic cells are immune activating when provided in the context of tumors^{21,22} or infection²³ *in vivo* or *ex vivo* if they are presented via activated mature DCs^{24–26}. It is important to note that the method used to induce apoptosis is critical in determining whether the cell death is immunogenic or tolerogenic^{27,28}. Thus, the results presented here are consistent with previous data demonstrating that activated mature DCs induce immune activation when apoptotic cells or apoptotic cell fragments are released. However, this is the first data to demonstrate that apoptotic cells actually enhance immune activation beyond that provided by anti-CD40 alone. It remains unclear whether this results from more effective access of epitopes acquired via phagocytosis of apoptotic cells as compared to those acquired via other means or whether this reflects improved antigen presentation by cells that have ingested apoptotic bodies. The fact that gamma irradiation-induced apoptosis has been shown to be immunogenic further supports the notion that the irradiated cells used in this model provided additional activation signals beyond the anti-CD40 and CpG rather than simply improving antigen loading²⁷.

These findings have a number of clinical implications for DC vaccination-based strategies. First, while acquisition of apoptotic tumor cells from solid tumors may be technically challenging in the clinical setting, leukemic blasts can be readily harvested and exposed to gamma irradiation *ex vivo* to generate apoptotic bodies. Our results suggest that apoptotic tumor cells, generated via irradiation, should be tested in the context of DC vaccines targeting leukemia and solid tumors where possible, since apoptotic bodies may be more effective than antigen loading via tumor lysates, peptides or tumor RNA. Second, even if loading of DCs with apoptotic cells is not possible, or if a more restricted immune response is desired (such as might be the case following allogeneic stem cell transplantation for neoplastic disease where a broad repertoire of recipient antigens on tumor vaccines could induce graft versus host disease), future work should seek to identify the mechanism by which apoptotic bodies enhance the efficiency of immune priming in this system. The ability to mimic such signals in the context of alternative loading strategies could potentially improve the efficacy of DC vaccines. Finally, these results emphasize that strategies seeking to generate potent antigen-specific adaptive immunity must optimize the signals delivered by the innate immune system in order to reap the greatest possible clinical benefit since the anti-tumor effects resulting from a DC vaccine differed substantially based solely upon the method used to antigen load the DC. Future work should focus upon optimizing signals received by DCs in the context of tumor vaccines in an effort to improve the effectiveness of DC-based cellular vaccination for cancer or infection.

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Figure 1. Marked expansion of dendritic cells from *sca*⁺/*lin*⁻ bone marrow cells

(a) Purified *sca*⁺/*lin*⁻ cells expand 50-fold when cultured with Flt-3 ligand (25 ng/mL), IL-3 (20 ng/mL) and stem cell factor (100 ng/mL), GM-CSF (1000U/mL) and IL-4 (1000 U/mL). This is a 5-fold increase in yield over cells cultured in GM-CSF and IL-4 alone (dotted line).

(b) Analysis surface phenotype on Days 1, 5, and 8 (post-activation) of culture indicates that these conditions expand a population of stem cells without a committed lineage that later differentiate into dendritic cells expressing high levels of CD11c, CD40, CD80 and CD86 compared to isotype controls. DCs that are activated (solid) on Day 7 with aCD40 monoclonal antibody (20 ug/mL) and CpG ODN 1555 (5 ug/mL) show an upregulation of CD40, CD80 and CD86 (solid histogram) compared to nonactivated DCs (- -) and isotype controls (—).

(c) DCs on Day 7 retain an immature phenotype, as indicated by their ability to endocytose FITC-conjugated albumin at 37° (solid histogram), a phenomenon that disappears at 0° (open histogram). Following activation, DCs lose the ability to take up FITC protein at 37.

(d) Morphology of cultured DCs on day 5(pre-activation) and day 8 (post-activation) showing the acquisition of dendrites. **(e)** Activated DCs (solid histogram) produce significantly higher levels of intracellular IL12 than nonactivated DCs (light grey line). Isotype control staining of permeabilized cells is represented by the black line.

Figure 2. Expansion of HY-reactive T cells by DCs loaded with apoptotic cells induced by irradiation is greater than that induced by other antigen loading methods or endogenously expressing DCs

Naïve female C57BL/6 mice were primed (Day 0) and boosted (Day 14) with DCs endogenously expressing male antigens or loaded with male antigens using various methods as outlined in materials and methods. **(a)** Representative contour plots showing the frequency of Uty tetramer-binding T cells in the spleens of immunized animals on day 28. **(b)** Antigen loading of bone marrow-derived DCs results in expansion of Uty tetramer-binding cells. N=7/group. **-p<0.05, ***p<0.01 compared to apoptotic cell-loaded DCs. **(c)** The number of splenocytes producing interferon- γ in response to peptide-specific stimulation is significantly increased following immunization with apoptotic cell-loaded DCs when compared to all other methods. Uty represents the dominant CD8 HY epitope, Smcy the subdominant CD8 epitope and Dby the dominant CD4 epitope.

Figure 3. Immunization with apoptotic cell-loaded DCs results in complete protection against a lethal tumor challenge

(a) Tumor-free survival in C57BL/6 female mice given a DC vaccine intraperitoneally on days 0 and 14 followed by subcutaneous challenge with 3×10^6 HY-expressing MB49 cells on day 28. All DC loading methods were statistically superior to female DCs ($p < 0.02$). Immunization with male DCs resulted in protection of 80% percent of the mice. However, loading DCs with apoptotic cells resulted in statistically improved protection when compared to all other methods ($p < 0.001$) except male DC vaccination and 100% survival. (b) With higher dose tumor challenge (5×10^6 HY-expressing MB49 cells on day 28), immunization with DCs loaded with apoptotic cells still resulted in complete protection whereas 100% tumor-induced lethality occurred in all other vaccinated groups ($p < 0.002$).