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ORIGINAL ARTICLE

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Induction of anti-tumor immunity by vaccination with dendritic cells pulsed with anti-CD44 IgG opsonized tumor cells

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Abstract Due to the pivotal role that dendritic cells (DC) play in eliciting and maintaining functional anti-tumor T cell responses, these APC have been exploited against tumors. DC express several receptors for the Fc portion of IgG (Fcγ receptors) that mediate the internalization of antigen-IgG complexes and promote efficient MHC class I and II restricted antigen presentation. In this study, the efficacy of vaccination with DC pulsed with apoptotic B16 melanoma cells opsonized with an anti-CD44 IgG (B16-CD44) was explored. Immature bone marrow derived DC grown in vitro with IL-4 and GM-CSF were pulsed with B16-CD44. After 48 h of pulsing, maturation of DC was demonstrated by production of IL-12 and upregulation of CD80 and CD40 expression. To test the efficacy of vaccination with DC + B16-CD44, mice were vaccinated subcutaneously Lymphocytes from mice vaccinated with DC + B16-CD44 produced IFN-γ in response to B16 melanoma lysates as well as an MHC class I restricted B16 melanoma-associated peptide, indicating B16 specific CD8 T cell activation. Upon challenge with viable B16 cells, all mice vaccinated with DC alone developed tumor compared to 40% of mice vaccinated with DC + B16-CD44; 60% of the latter mice remained tumor free for at least 8 months. In addition, established lung tumors and distant metastases were significantly reduced in mice treated with DC + B16-CD44. Lastly, delayed growth of established subcutaneous tumors was induced by combination therapy with anti-CD44 antibodies followed by DC injection. This study demonstrates the efficacy of targeting tumor antigens to DC via Fcγ receptors.

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Introduction

The frequency of precursor T cells with anti-tumor activity is low in patients with advanced cancers. It is therefore important to activate and expand tumor reactive T-cells as an effective means of immunity. Dendritic cells (DC) are known as the most potent antigen-presenting cells, capable of initiating both primary and memory T cell immune responses and have become an integral player in immunotherapeutic approaches for the treatment of cancer [1–3].

DC loaded with tumor antigens induce T cell responses capable of overcoming active suppression and innate tolerance in murine tumor models [4, 5]. Traditional methods of loading DC with tumor-associated antigens include pulsing with peptides, whole lysates, or apoptotic cells. We, and others, have documented the capacity of tumor lysate- or apoptotic tumor-pulsed DC to elicit potent antitumor T cell responses in mice that result in both protection against tumor challenge and regression of established tumors [4–8]. While initial clinical trials utilizing peptide- or tumor cell- pulsed DC in cancer patients have shown promising immunologic results, clinical responses against established tumors have been rarely seen. It has been suggested that the latter may be related to a lack of induction of robust and long-lasting T cell responses. In an effort to improve DC-based vaccines for immunotherapy, approaches that increase the capacity of DC to stimulate tumor-specific T cells are being explored. These include methods to induce DC maturation and optimization of antigen processing and presentation pathways.

In order for effective anti-tumor immunity to be induced, immature DC must efficiently uptake and process

tumor antigens for presentation to both CD4+ and CD8+ T cells. To enhance the uptake of tumor antigens and hence the T cell priming capacity, targeting of DC cell surface receptors, such as DEC205, mannose, and Fc gamma (Fcγ) receptors, has been examined [9–11]. Targeting antigens to these surface receptors leads to receptor mediated endocytosis and efficient CD4+ T cell priming [12, 13]. In particular, there is strong evidence to support that antibody coating of antigen and targeting Fcγ receptors on DC enhances presentation of antigens by DC resulting in superior T cell activation [14, 15]. In addition to improving CD4+ T cell priming, targeting antigens to DC via Fcγ receptors induces effective cross-presentation of antigen on MHC class I for activation of CD8+ T cells [16–20]. It has been shown that targeting antigen containing liposomes to Fcγ receptors on DC leads to a 1,000–10,000-fold enhancement in MHC class I presentation [21].

Most efficient T cell priming occurs when DC are in the mature state, expressing co-stimulatory molecules such as CD80, CD86, and CD40 as well as producing pro-inflammatory cytokines, such as IL-12 [2, 22]. Adjuvants such as LPS and CpG that target toll-like receptors on the surface of DC are strong inducers of maturation. In both murine and human DC, targeting FcγR on the cell surface also results in the maturation and increased T cell stimulatory properties of DC [14, 16, 23].

To enhance the vaccination efficacy of DC pulsed with whole apoptotic tumor cells, we opsonized B16 melanoma cells with an anti-CD44 antibody for FcγR targeting. CD44 is expressed at low levels on normal tissues and is overexpressed on many tumors, including melanoma, lymphoma, breast, and lung tumor cells [24–26]. Overexpression of CD44 on the surface of tumor cells has been implicated as important in both proliferation and metastases [27–31]. Using a highly aggressive and metastatic model of murine melanoma, we examined the protective and therapeutic efficacy of DC pulsed with anti-CD44 IgG opsonized melanoma cells as well as combination therapy with in vivo monoclonal antibody treatment followed by DC-based immunotherapy.

Materials and methods

Animals

Six to 8-week-old female C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN, USA). Mice were housed at the Animal Research Facility of the H. Lee Moffitt Cancer Center and Research Institute.

Tumor cell lines and medium

Complete medium (CM) was prepared as described previously [32]. The B16 melanoma is a tumor of spontaneous origin that expresses a low level of MHC class I molecules and no detectable MHC class II molecules [33]. A highly metastatic B16 subclone, B16-M, was cultured in our laboratory. Tumor cells were maintained by serial in vitro passage.

Generation of DC

BM cells were harvested from flushed marrow cavities of femurs and tibiae of mice under aseptic conditions and were cultured in CM supplemented with 20 ng/ml GM-CSF and 20 ng/ml IL-4 at 1×10^6 cells/ml (R&D Systems, Minneapolis, MN, USA). DC were harvested from day 5 cultures and enriched by OPTI-prep density gradient separation (Sigma Aldrich, St. Louis, MO, USA). The low-density interface was collected.

Preparation of tumor cells for pulsing of DCs

B16 cells (1×10^6/ml PBS) were exposed to UVB light for 20 min (equal to 200 mJ/cm^2) (Gel Doc 2000; Bio-Rad, Hercules, CA, USA). Cells were washed three times in PBS. For antibody coating, UVB treated B16 cells were incubated for 30 min with 10 µg/ml anti-CD44 antibody (Pharmingen, San Diego, CA, USA), then washed three times. Cells were resuspended in CM at 6×10^6/ml and cultured with 2×10^6 day 5 DC/ml for 24 h. For some experiments, CD11c + DC were purified by MACS sorting (Miltenyi Biotech, Auburn, CA, USA).

Binding assay

B16 cells were labeled with PKH26red according to the supplier's instructions (Sigma). After labeling, the cells were exposed to UVB as above and cultured with day 5 DC at 4 or 37°C. For blocking experiments, DC were incubated with CD16/CD32 antibody (BD Pharmingen) prior to co-culture with PKH26red labeled B16 cells. After 6–24 h, coexpression of MHC class II (IA^b) and PKH26red was measured by FACS analysis with a B-D FACScaliber (BD Biosciences, San Jose, CA, USA).

Cytokine assays

To measure cytokine secretion, day 5 DC were either unpulsed, cultured with UVB treated B16 cells, or cultured with UVB treated B16 cells coated with anti-CD44. After 48 h, culture supernatants were harvested for measurement of cytokine production by standard ELISA (Pharmingen). For measurement of IFN-gamma, lymph node cells were collected from mice 1 week after the 3rd DC vaccination.

Primary immunization

C57BL/6 mice ($n = 5$–8 per group) were vaccinated s.c. three times at 2 week intervals with either PBS, 1×10^6 DC alone, DC pulsed with UVB treated B16, or DC pulsed with UVB treated B16 coated with anti-CD44 antibody. Alternatively, MACS purified CD11c + DC were used for immunizations. Two weeks after the final vaccination, mice were challenged s.c. on the opposite flank with 2×10^5 viable B16 cells. Tumors were palpated weekly. Mice were humanely euthanized when tumor measurements reached 500 mm^2.

Treatment of lung metastases

C57BL/6 mice were injected i.v. with 2×10^5 viable B16 or B16-M cells. One-3 days later, mice were treated i.v. with 1×10^6 DC, B16 coated with anti-CD44, or DC + B16 coated with anti-CD44. Mice received additional treatments on days 3 and 5 after tumor challenge. Lungs were harvested on day 14 and the number of lung metastases was counted, as described previously [34, 35]. The brains and GI tracts of tumor bearing mice were observed for metastases.

Treatment of subcutaneous tumor

C57BL/6 mice were challenged s.c. with 2×10^5 viable B16 cells. Tumors were allowed to grow for approximately 10 days or until the tumor size was 25 mm^2. Four treatments were given at 2 day intervals and mice received intratumoral injections of DC alone, anti-CD44 antibody alone, or a combination of anti-CD44 antibody followed by DC injection 4 h later.

Statistical analysis

A Mann-Whitney test (unpaired) or a Student's paired *t*-test was used to compare between two treatment groups. All statistical evaluations of data were performed using GraphPad Prism software. Statistical significance was achieved at $p < 0.05$.

Results

Induction of apoptosis and overexpression of CD44 on the surface of B16 cells

Apoptosis of B16 cells was induced by exposure to UVB light. Figure 1a shows that, after UVB exposure, 56% of the cells are positive for Annexin V only, indicating apoptosis, while 40% of the cells are positive for both PI and Annexin V, indicating necrosis.

B16 cells express high levels of surface CD44 protein as measured by flow cytometry (Fig. 1b). Due to the fact that, in these experiments, apoptosis of B16 cells was induced by UVB exposure, it was important to determine if expression of CD44 was affected after this exposure. B16 cells were treated for 20 min of UVB irradiation and expression of CD44 was measured. Although the mean fluorescent intensity was decreased overall, CD44 expression was detected on all B16 cells (Fig. 1b).

B16 melanoma cells coated with anti-CD44 are efficiently endocytosed by DC

To determine if the uptake of B16 cells by DC was enhanced by opsonization with anti-CD44 IgG, B16 cells were labeled with PKH26-red dye and co-cultured with DC for 24 h. The percentage of DC that contained the red dye was determined by FACS. As shown is Fig. 2a, 33% of the DC were labeled with the red dye. An increase to 46% was measured after DC were co-cultured with B16 coated with anti-CD44 (B16-CD44), indicating that opsonization with anti-CD44 IgG enhanced uptake by DC. In additional experiments, receptor mediated endocytosis through FcγR were blocked by pretreating the DC with anti-CD16/CD32 antibodies (Fc block). As shown in Fig. 2b, the percentage of phagocytosis of B16-CD44 was reduced after blocking Fc receptors. No uptake of B16 cells by DC was measured after co-culture at 4°C.

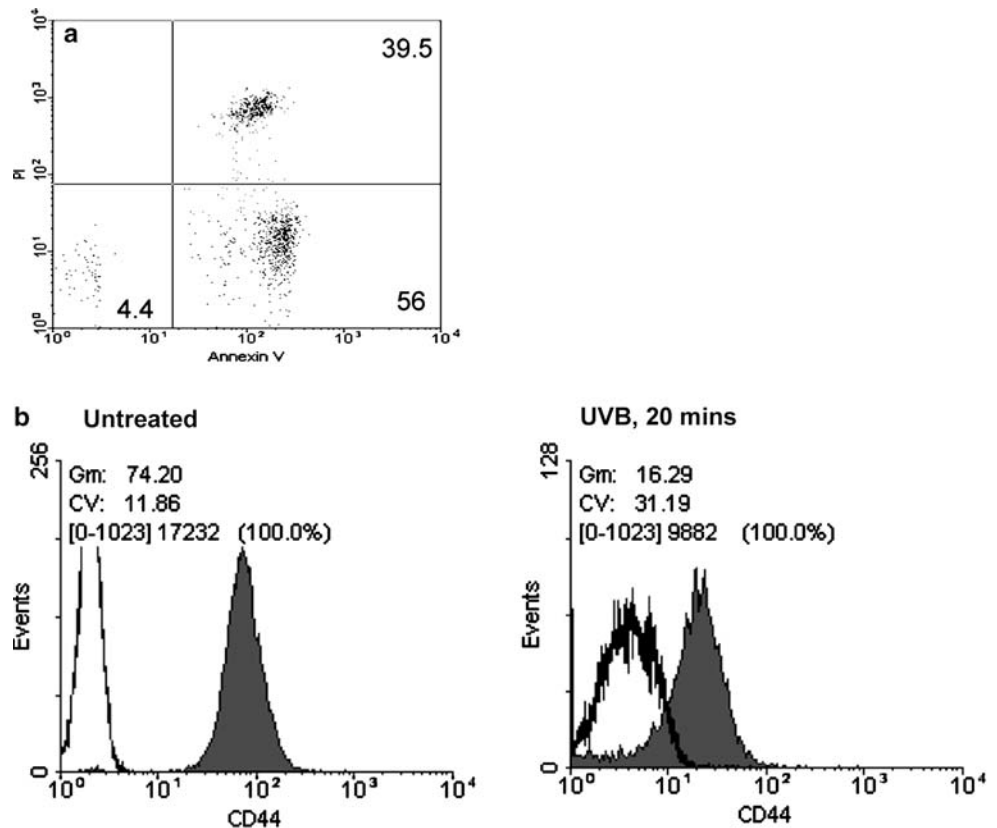
DC demonstrate maturation after uptake of B16 cells coated with anti-CD44 antibody

After 24 h of co-culture of DC with UVB treated B16 cells or B16-CD44, expression of maturation markers was measured by flow cytometry. As indicated in Fig. 3a, increases in CD86, CD80, and CD40 were measured after DC were co-cultured with B16-CD44. DC that had phagocytosed apoptotic B16 cells did not demonstrate increases (black histograms). To determine if these matured DC produced pro-inflammatory cytokines, supernatants were collected and IL-12p70 was measured by ELISA (Fig. 3b). Co-culture of DC with B16-CD44 led to a significant increase in IL-12p70 production (447 ± 10, $p < 0.01$). Supernatants from DC alone or DC co-cultured with B16 cells displayed little IL-12 secretion (103 ± 29 and 113 ± 18, respectively). No IL-10, TNF-(α, or TGF-β secretion was detected in cell supernatants (data not shown).

Vaccination with DC pulsed with B16-IgG Induces B16 specific CD8 T cells

To test the induction of B16 specific T cells, mice were vaccinated three times at 2 week intervals with

Fig. 1 Phenotype and viability of B16 melanoma after UVB exposure. **a** B16 cells (1×10^6/ml PBS) were exposed to UVB light for 20 min. Cells were stained with Annexin V and PI for determination of apoptotic and necrotic cell population by FACS, **b** CD44 expression on B16 cells was measured by FACS prior to and after UVB exposure. *Filled histograms* indicate CD44 positive cells while the negative control is shown as *empty histograms*



DC + B16, or DC + B16-CD44. Two weeks after the final vaccination, lymph node cells were collected. Cells were restimulated with DC pulsed with B16 cell lysate. Supernatants were collected after 48 hours and IFN-γ was measured by ELISA. Lymph node cells from naïve mice produced very little IFN-γ (147 ± 20 pg/ml). In contrast, mice vaccinated with either DC + B16 or DC + B16-CD44 produced IFN-γ in response to B16 lysate (Fig. 4a: $1{,}629 \pm 238$ and $1{,}891 \pm 458$ pg/ml, respectively, $p < 0.05$ compared to naïve).

It has been shown that antigen loading through the Fc gamma receptors leads to enhanced presentation to CD8 T cells. To test this hypothesis, splenocytes from vaccinated mice were restimulated for 48 h with DC pulsed with the MHC class I restricted melanoma peptide, Trp-2. In Fig. 4b, mice vaccinated with DC + B16 produced IFN-γ in response to this peptide ($1{,}293 \pm 30$, $p < 0.01$ compared to naive). A significant enhancement in IFN-γ secretion was detected in mice vaccinated with DC + B16-CD44 ($2{,}413 \pm 231$, $p < 0.01$ compared to DC + B16 vaccinated mice).

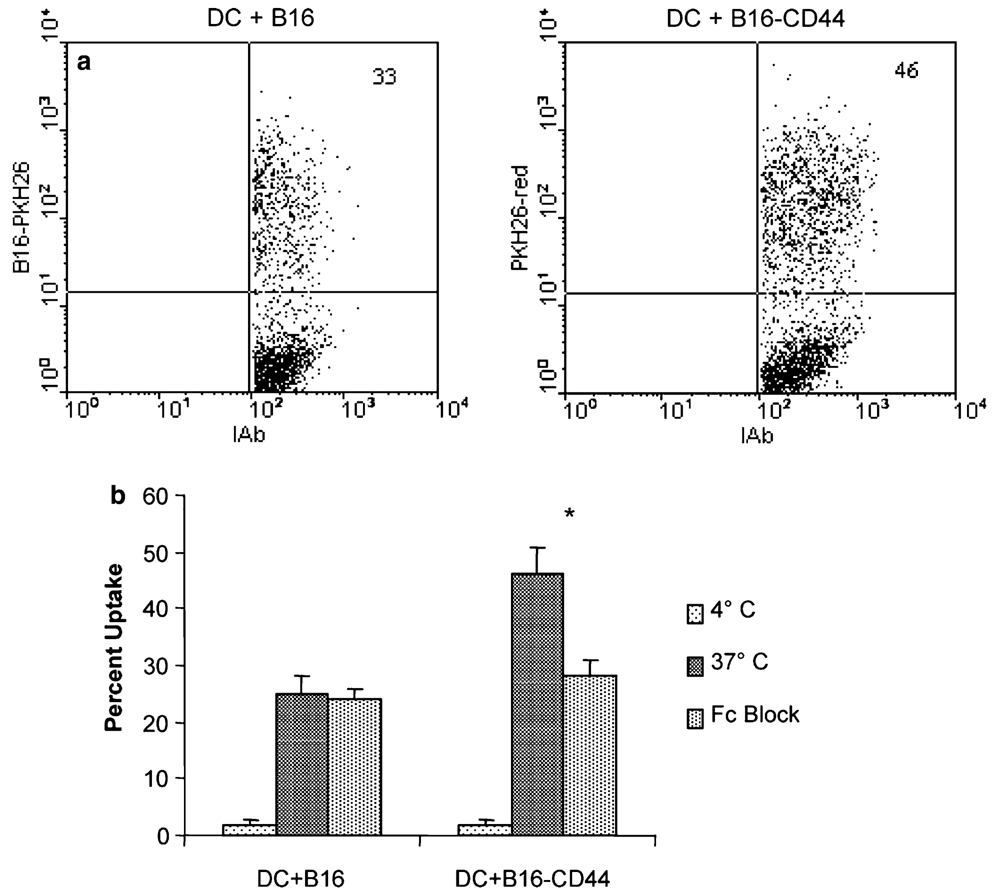
Vaccination with DC + B16-IgG induces effective anti-B16 tumor immunity

To determine if vaccination with DC + B16-CD44 induced an enhanced anti-tumor immunity and protection, mice were challenged s.c. with B16 cells 2 weeks after the third vaccination with either DC, DC + B16, or DC + B16-CD44. While vaccination with PBS alone, DC alone, or DC + B16 was ineffective at inducing protection against B16 tumor challenge, 60% of mice vaccinated with DC + B16-CD44 were protected (Fig. 5a). This protection was long-lasting as mice were tumor free at 8 months. To exclude the possibility that B16 cells opsonized with anti-CD44 antibody were inducing an anti-tumor immune response by themselves, CD11c + DC were purified after co-culture with B16-CD44. Again, mice vaccinated with $CD11c^+$ DC + B16-CD44 were protected to a greater extent than mice vaccinated with $CD11c^+$ DC + B16 (Fig. 5b).

Effective treatment of lung metastases with DC + B16-IgG

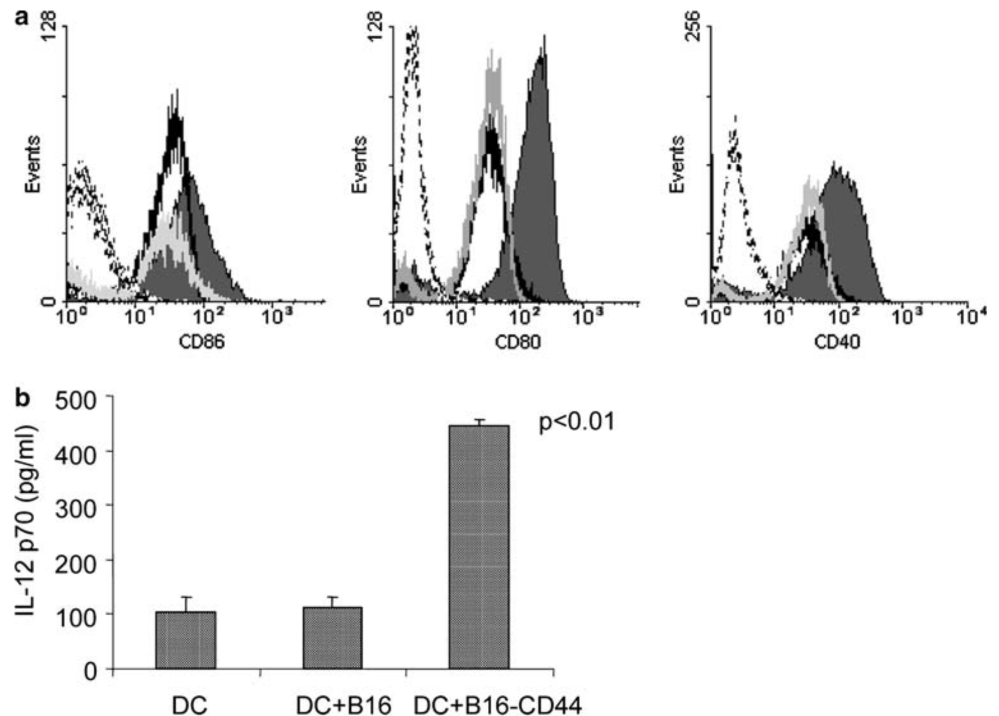
Next, we tested the ability of DC + B16-CD44 to induce rejection of lung metastases. One day after i.v. injection of B16 cells, mice received PBS alone, DC alone, B16-CD44, or DC + B16-CD44 with two additional treatments at 2 day intervals. Fourteen days after tumor injection, lung metastases were enumerated. As shown in Fig. 6, PBS treated mice had greater than 250 lung metastases with a similar number in mice that received B16-CD44 (197 ± 49). Mice that had received DC + B16-CD44 displayed a significant decrease in the overall number of metastases (91 ± 59, $p < 0.01$).

Fig. 2 Binding of B16 cells by DC via Fcγ Receptors. **a** B16 cells were labeled with PKH26 red dye and treated for 20 min with UVB irradiation. Cells were coated with normal rat IgG or anti-CD44 antibodies prior to a 24 h incubation with day 5 DC. DC were stained for MHC class II expression. Two-color analysis revealed cells that were positive for both PKH26 red dye and MHC class II, indicative of DC that have taken up tumor cells, **b** Prior to co-culture with UVB treated, PKH26 *red labeled* B16 cells, DC were pretreated with anti-CD16/CD32 antibodies (Fc *block*) to block Fcγ receptors, * indicates $p < 0.05$



Using a highly metastatic B16 melanoma clone (B16-M), the efficacy of DC + B16-CD44 treatment to prevent metastatic disease was also tested. B16-M is a clone that, after i.v. injection, frequently metastasizes to additional sites. As shown in Table 1, 100% of the mice treated with PBS alone demonstrated grossly visible,

Fig. 3 Maturation of DC after uptake of B16 cells. DC were co-cultured for 24 h with UVB treated B16 cells stained with normal rat IgG (DC + B16) or anti-CD44 antibodies (DC + B16-CD44). **a** Expression of CD86, CD80, and CD40 was measured on the surface of MHC class II positive cells by FACS. Dotted histogram = negative control, gray histogram = DC alone, black histogram = DC + B16, filled histogram = DC + B16-CD44, **b** Supernatants were collected and IL-12p70 was measured by standard ELISA



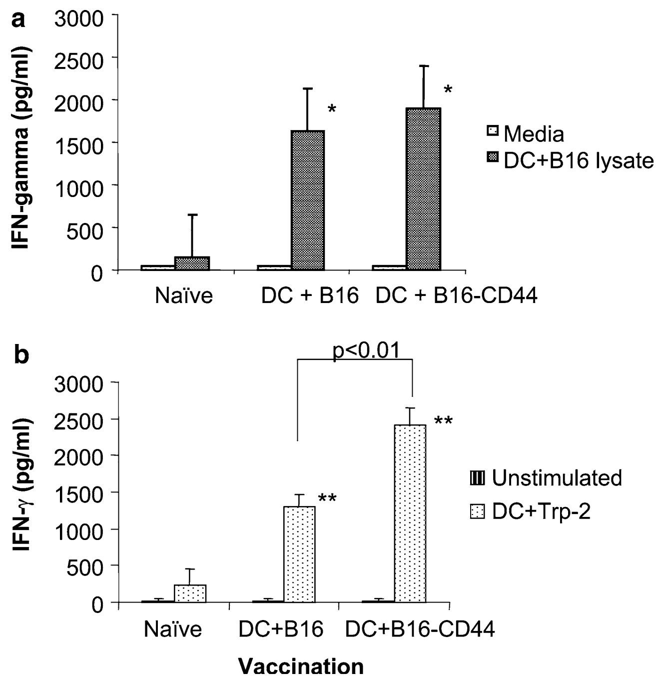


Fig. 4 IFN-gamma production in response to B16 lysate and peptide. Mice were vaccinated three times at 2 week intervals with DC + B16 or DC + B16-CD44. One week after the final vaccination, lymph node cells were collected and co-cultured for 48 h with **a** DC + B16 lysate or **b** Trp-2 peptide. IFN-g production was measured by standard ELISA. Data are shown as mean ± SEM of two independent experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to naïve lymph node cells

diffuse lung and gastrointestinal (GI) metastases. Half of these mice also developed grossly visible lesions in the brain. Mice treated with DC alone demonstrated an overall decrease in the incidence of lung and GI metastases (75%) with 50% of the mice developing brain metastases. All mice treated with DC + B16 presented with both lung and GI metastases with 75% also developing brain lesions. In contrast, none of the mice treated with DC + B16-CD44 showed grossly visible lung metastasis while only 25% of the mice grew tumors in the GI tract and brain.

Combination therapy with anti-CD44 monoclonal antibodies and DC

We next examined the efficacy of intratumoral injection with anti-CD44 antibodies followed by DC immunotherapy for the treatment of established s.c. B16 melanoma. Ten days after s.c. injection with B16 cells, when the tumor size was approximately 25 mm^2, mice were treated by intratumoral injection of anti-CD44 antibodies alone, DC alone, or a combination of anti-CD44 antibody followed by DC injection. A total of four treatments were given every 2 days. As shown in Fig. 7a, mice receiving a combination of both anti-CD44 antibodies and DC displayed a significant delay in tumor growth ($p < 0.05$ compared to all other groups). While the mice in all other groups had succumbed to tumor by day 25, mice receiving the combination therapy survived to day 40. To examine the contribution of CD8 T cells, one group received specific monoclonal antibodies to deplete CD8+ T cells. As shown in Fig. 7b, mice receiving the combination therapy were unable to delay tumor growth after CD8+ T cell depletion, indicating that CD8+ T cells are contributing to the observed anti-tumor immunity.

Discussion

This study demonstrates the feasibility of loading DC with apoptotic tumors via cell surface receptors. It has been previously described that targeting antigen to Fcγ receptors leads to enhanced antigen uptake, upregulation of DC maturation markers and secretion of pro-inflammatory cytokines[14, 16, 19]. In this study, by targeting a ubiquitous antigen, CD44, on the surface of apoptotic B16 melanoma cells, enhanced uptake by bone-marrow derived DC was demonstrated. This increase was selectively blocked by initial treatment of DC with antibodies that block the Fcγ receptors. In addition, upregulation of CD80 and CD40 expression were detectable in DC pulsed with anti-CD44 opsonized B16 cells, but not in DC pulsed with B16 alone. Secretion of the pro-inflammatory cytokine IL-12p70 was detected in supernatants from DC pulsed with B16-CD44 but not in the supernatants of DC alone or DC pulsed with apoptotic B16 cells. Taken together, our data confirms enhanced uptake and induction of DC maturation status after targeting tumor cells to Fcγ receptors.

Prior studies utilizing model tumor antigens such as OVA have demonstrated that receptor mediated endocytosis through Fcγ receptors leads to processing and presentation of antigen to both CD4 and CD8 T cells [10, 20]. In our protection model, while we have not demonstrated directly that T cells are participating in the observed immunity against B16 tumor in vivo, activation of CD8+ T cells against B16 tumor rejection antigens has been measured in vitro. Vaccination with DC loaded with B16 tumor opsonized with anti-CD44 antibodies led to T cell specific IFN-γ production to both B16 tumor lysates as well as to Trp-2 peptide, a distinct MHC class I restricted peptide expressed by B16 tumor. Importantly, we have shown a significant enhancement in the CD8 T cell response to the Trp-2 peptide after vaccination with DC + B16-CD44, indicating that loading the B16 cell through the Fcγ receptors has led to enhanced cross-presentation of antigens by DC and CD8 T cell activity. Reactivity to another B16 specific peptide, p15e, was also measured and IFN-gamma production was only detectable in mice vaccinated with DC + B16-CD44 (data not shown).

Kotera et al. demonstrated that vaccination with DC pulsed with tumor lysates or purified apoptotic tumor cells induce equal protective and therapeutic efficacy [6].

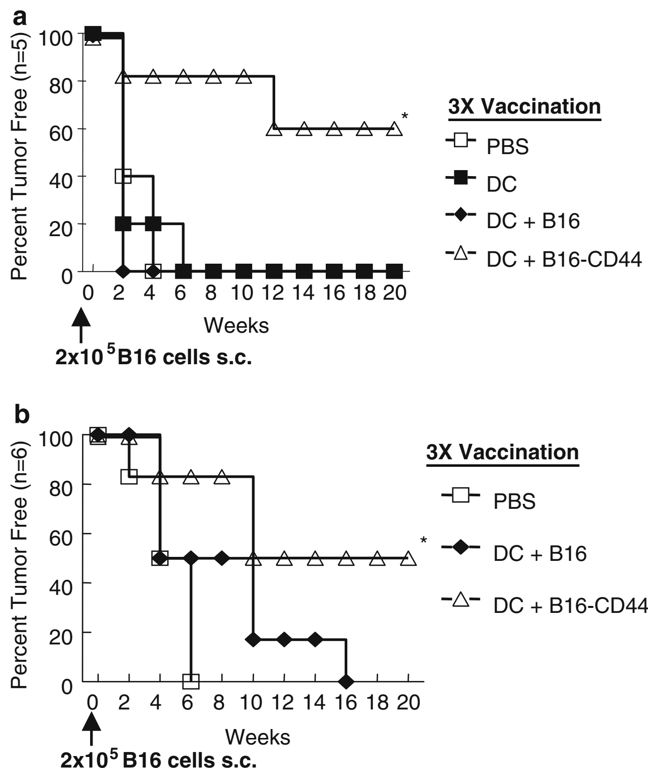


Fig. 5 Protection against B16 tumor. **a** Mice ($n=5$) were vaccinated s.c. three times at 2 week intervals with PBS alone, DC alone, or DC pulsed for 24 h with UVB treated B16 cells coated with normal rat IgG (DC + B16) or anti-CD44 antibodies (DC + B16-CD44). Two weeks after the final vaccination, mice were challenged with 2×10^5 B16 cells in the opposite flank. The results of three experiments were very similar and are therefore combined in this figure, **b** Mice ($n=6$) were vaccinated s.c. three times at 2 week intervals with PBS alone, or DC pulsed for 24 h with UVB treated B16 cells coated with normal rat IgG (DC + B16) or anti-CD44 antibodies (DC + B16-CD44). Prior to injection, $CD11c^+$ DC were purified by MACS separation. Two weeks after the final vaccination, mice were challenged with 2×10^5 B16 cells in the opposite flank. Tumors were palpated weekly. * indicates $p<0.01$

Here, we demonstrate that vaccination with DC pulsed with UVB treated tumor cells can be enhanced by opsonizing the tumor cells first with antibodies to a cell surface protein. In a melanoma protection model, 60% of mice vaccinated with DC + B16-CD44 were able to reject tumor and remain tumor free for 8 months whereas all mice vaccinated with DC + B16 alone died within 4 months. In addition, mice treated with DC + B16-CD44 were able to reject lung metastasis and prevent the formation of distant metastases.

The treatment of established s.c. B16 tumors with anti-CD44 antibodies followed by intratumoral injection of unpulsed DC led to a significant delay in tumor growth and an increase in the overall survival time. It is possible that in vivo coating of the tumor with antibodies followed by injection of immature DC led to uptake of the tumor antigen via Fcγ receptors on the DC in vivo. Others have demonstrated that DC directly

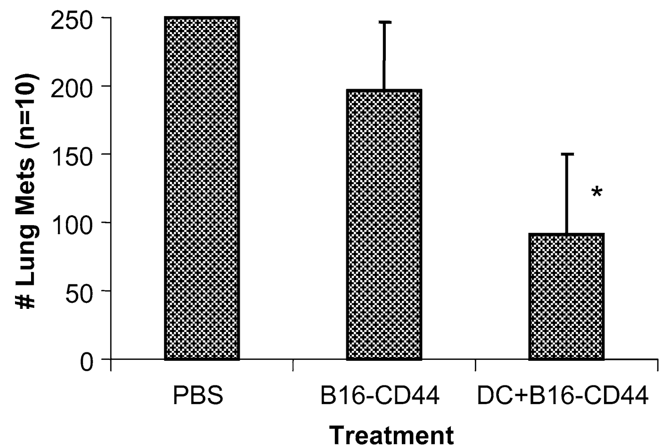


Fig. 6 Regression of metastatic B16 tumors. Mice ($n=10$) were injected with i.v. with 2×10^5 B16 cells. One day later, mice were treated with PBS, 1×10^6 B16 coated with anti-CD44, or DC + B16-CD44. Mice received additional treatments on days 3 and 5 after tumor challenge. Lungs were harvested on day 14 and the number of lung metastases was counted. * indicates $p<0.05$ compared to PBS treated mice. Data shown is the combination of two independent experiments

administered to a growing tumor mass are capable of inducing potent anti-tumor responses in the absence of ex vivo antigen pulsing [36, 37]. Kirk et al. demonstrated the ability of DC injected directly into tumor masses to prime anti-tumor specific T cells [38]. Our results further support the hypothesis that DC are capable of engulfing necrotic or dying cells within tumors, processing tumor specific antigens, and presenting these antigens to T cells. In our study, tumor bearing mice treated with anti-CD44 antibody followed by DC injections were unable to delay tumor growth after depletion of CD8+ T cells, indicating that tumor specific CD8 T cells are intimately involved in tumor rejection.

For in vivo coating of tumor cells, the antibody must target a protein that is highly expressed on tumor yet expressed at low levels by normal tissues. In this study, CD44 was chosen as the targeted antigen. CD44 is a cell surface receptor responsible for metabolizing soluble hyaluronic acid (HA), a major component of extracellular matrix. While it is expressed at low levels on normal tissues, it is overexpressed on B16 melanoma as well as many human tumors, including melanoma, lymphoma, breast, and lung cancers [24–28]. Eliaz et al. have demonstrated that anti-cancer drugs could be targeted to CD44 overexpressing tumors, but not cells

Table 1 Treatment of 3 day established B16 lung metastases

Site of metastasis (% positive)			
Treatment ($n=8$ mice)	Lung	Gut	Brain
PBS	100	100	50
DC	75	75	50
DC + B16	100	100	75
DC + B16-CD44	0	25	25

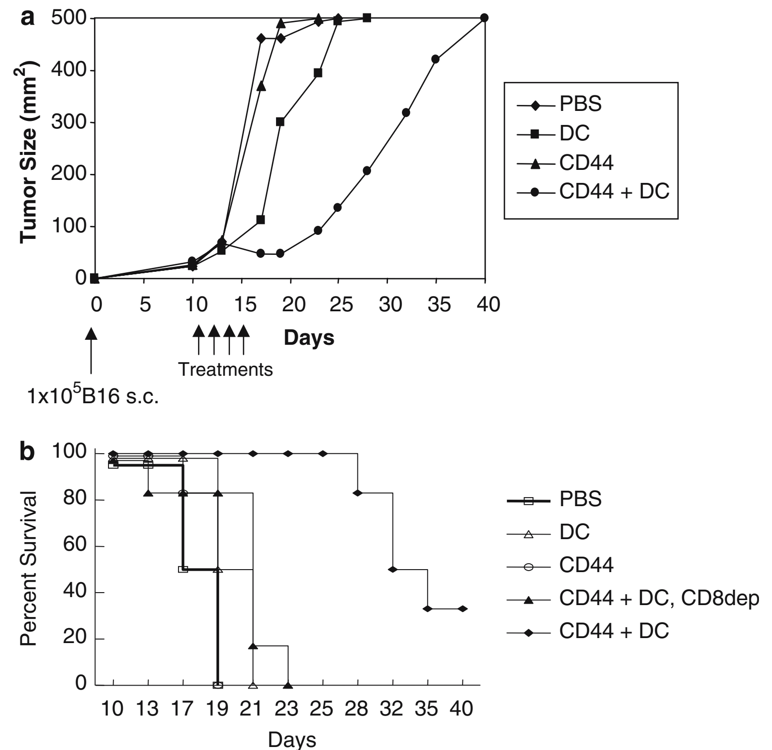


Fig. 7 Combination therapy of established s.c. B16 tumors with anti-CD44 antibody and DC. C57BL/6 mice were challenged s.c. with 1×10^5 B16 cells. Tumors were allowed to grow for 10 days. **a** Treatments were given on days 10, 12, 14, and 16 and mice received intratumoral injections of DC alone, anti-CD44 antibody alone, or a combination of anti-CD44 antibody followed by DC injection 4 h later, **b** Mice received intumoral injections of DC alone, anti-CD44 antibody alone, or a combination of anti-CD44 antibody followed by DC injection 4 h later. To deplete CD8+ T cells, mice received 200 μg of 2.43 monoclonal antibodies i.p. every 3 days starting 1 day after injection of B16 cells. Two experiments with similar results were combined

expressing low levels of CD44, via HA containing liposomes, supporting the use of CD44 as a potential target for anti-tumor therapies [39].

Collectively, our data illustrate the successful induction of anti-tumor immunity by the administration of DC loaded with opsonized apoptotic tumor cells, a potential approach to improve current DC immunotherapy. In tumor-bearing patients, vaccination with DC pulsed with opsonized tumor cells may enhance the expansion of low frequency tumor reactive CD8+ T cells leading to robust and durable anti-tumor immune responses. The feasibility of combination therapy of anti-tumor monoclonal antibodies followed by intratumoral DC injection was also demonstrated and raises the potential for such an approach in patients.

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