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Dendritic Cells Loaded with Stressed Tumor Cells Elicit Long-Lasting Protective Tumor Immunity in Mice Depleted of CD4⁺CD25⁺ Regulatory T Cells¹

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Dendritic cell (DC)-based vaccination represents a promising approach to harness the specificity and potency of the immune system to combat cancer. Finding optimal strategies for tumor Ag preparation and subsequent pulsing of DC, as well as improving the immunogenicity of weak tumor Ags remain among the first challenges of this approach. In this report, we use a prophylactic vaccine consisting of DC loaded with whole, nonmanipulated B16-F10 melanoma cells that had been stressed by heat shock and gamma irradiation. Stressed B16-F10 cells underwent apoptosis and were internalized by bone marrow-derived DC during coculture. Surprisingly, coculture of DC with stressed B16-F10 undergoing apoptosis and necrosis did not induce DC maturation. However, a marked retardation in tumor growth was observed in C57BL/6 mice immunized using DC loaded with stressed B16-F10 cells and subsequently challenged with B16-F10 cells. Growth retardation was further increased by treating DC with LPS before *in vivo* administration. *In vivo* depletion studies revealed that both CD8⁺ and CD4⁺ T cells played a critical role in retarding tumor growth. In addition, treatment with anti-CD25 Ab to deplete CD4⁺CD25⁺ regulatory T cells before DC vaccination considerably improved the effect of the vaccine and allowed the development of long-lived immune responses that were tumor protective. Our results demonstrate that depletion of regulatory T cells is an effective approach to improving the success of DC-based vaccination against weakly immunogenic tumors. Such a strategy can be readily applied to other tumor models and extended to therapeutic vaccination settings. *The Journal of Immunology*, 2005, 174: 90–98.

Dendritic cells (DC)⁴ are potent APC found in trace quantities in most tissues (1). Tissue DC occur in an immature state during which they constantly sample their microenvironment mainly through the process of macropinocytosis. Following maturation, DC become less active at taking up Ag and migrate to the draining lymph node, where they present their antigenic cargo to naive T cells. Mature DC have an enhanced capacity to stimulate naive T cells as a result of increased cell surface expression of MHC and costimulatory molecules. Although maturation and migration appear to be programmed events in the life cycle of DC, they are accelerated and up-regulated by infectious agents and their products such as LPS or proinflammatory cytokines (2).

DC are recognized as powerful activators of immune responses, but their activity can be modulated by different mechanisms such as cytokines or regulatory cells. Recent evidence indicates that DC

can induce the activation and proliferation of CD4⁺CD25⁺ regulatory T cells (T_{reg}) *in vitro* and *in vivo* (3). In turn, the ability of DC to activate CD4⁺ and CD8⁺ T cells is substantially increased by depletion of T_{reg} (4). The susceptibility of DC to the inhibitory function of T_{reg} may vary, depending on the DC maturation status and previous exposure to activating stimuli (5, 6).

The potency of DC as APC has been applied toward the immunotherapy of malignant disease (7). There is now substantial evidence from studies in animal models (8–10) and human clinical trials (11–17), which provides the basis for DC as central players in an immunotherapeutic approach to cancer. A critical issue in increasing the efficacy of DC vaccines is the strategy used for tumor Ag preparation and loading of DC. Most of the initial DC vaccination protocols used DC loaded with tumor-associated peptides obtained through chemical synthesis (8), or by mild acid elution from tumor cells (9). Disadvantages associated with peptide-loaded DC vaccines include the following: the limitation of this strategy to patients with a specific HLA type, a paucity of defined epitopes among most tumors, potential generation of tumor Ag escape variants when immunizing against a single Ag (12), and the presence of a high proportion of non-tumor-specific peptides when using whole tumor cell eluates (18). Furthermore, the weak immunogenicity of many tumors also represents a barrier to the effective induction of antitumor immunity.

The preceding limitations can be circumvented by using whole, stressed tumor cells as the source of a wide array of defined and undefined Ags. Loading of DC with tumor Ags can be facilitated by selective use of a stress regimen that promotes apoptosis, and thus offers the possibility of receptor-mediated uptake of apoptotic material (19, 20). Importantly, uptake of Ag from apoptotic cells has been demonstrated to cross-prime CTL (19, 21–23), recognized as critical mediators of antitumor immunity.

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⁴ Abbreviations used in this paper: DC, dendritic cell; T_{reg}, regulatory T cell; BM-DC, bone marrow-derived DC; cIMDM, complete IMDM; TRP, tyrosine-related protein; FSC/SSC, forward scatter/side scatter; PI, propidium iodide; CTO, Cell Tracker Orange (chloromethyl-benzoyl-aminotetramethyl-rhodamine); HSP, heat shock protein.

In this report, we describe the generation of a prophylactic vaccine using bone marrow-derived DC (BM-DC) loaded with B16-F10 melanoma cells that were stressed by heat shock and gamma irradiation. DC that had taken up stressed B16-F10 cells did not up-regulate expression of maturation markers, but were able to markedly retard tumor growth in immunized C57BL/6 mice subsequently challenged with live B16-F10 melanoma cells. Importantly, we also show that depletion of T_{reg} before vaccination considerably increased the ability of vaccinated mice to survive tumor challenge, and allowed the development of long-lasting tumor protective immunity. We conclude that it is possible to generate effective immunotherapies against weakly immunogenic tumors by combining treatment with DC loaded with apoptotic tumor cells and depletion of T_{reg}.

Materials and Methods

Mice

A colony of C57BL/6 mice, derived from breeding pairs originally obtained from The Jackson Laboratory, was housed and maintained at the Animal Facility of the Wellington School of Medicine. All animal experimental protocols used in this study were approved by the Wellington School of Medicine Animal Ethics Committee, and conducted in accordance with the guidelines of the University of Otago (Dunedin, New Zealand). Mice used in this study were 8–16 wk of age and, where necessary, matched for age and gender.

Media, Abs, and cell lines

Unless otherwise stated, all cultures were in complete IMDM (cIMDM) (Invitrogen Life Technologies) containing 2 mM glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen Life Technologies), 50 µM 2-ME (Sigma-Aldrich), and 5% FCS (Invitrogen Life Technologies). The synthetic peptides tyrosine-related protein (TRP)-2_{180–188} and gp100_{25–33} were from Chiron Mimotopes.

The anti-CD4, anti-CD8, and anti-CD25 mAb were purified from the supernatants of relevant hybridomas (GK1.5, 2.43, and PC61, respectively) using HyTrap protein G columns (Amersham Biosciences), and concentration was determined from the OD₂₈₀ reading.

The melanoma cell line B16-F10 (24) was obtained from the American Type Culture Collection and maintained in culture in complete medium. Cells were regularly monitored for mycoplasma contamination by PCR.

Culture of BM-DC

C57BL/6 mouse BM-DC were generated as previously described (25). Briefly, bone marrow was harvested from femurs and tibiae and filtered through a Falcon 100-µm nylon cell strainer (BD Labware). Whole bone marrow cells were plated in six-well plates (BD Labware) in cIMDM supplemented with 10 ng/ml recombinant murine GM-CSF and 20 ng/ml recombinant murine IL-4 obtained from the supernatants of transfected cell lines. Cultures were fed every 2–3 days by removing 50% of medium from each well and adding back an equal amount of fresh, growth factor-supplemented cIMDM. Cultures were maintained for 6–8 days and typically contained >90% cells expressing CD11c and MHC class II on the surface, as determined by flow cytometry. After 6–8 days of culture, nonadherent and loosely adherent cells were harvested, washed, and used for *in vitro* and *in vivo* experiments.

Flow cytometry

PE-conjugated mAb against murine CD40, CD80, and CD86 were purchased from BD Pharmingen. The anti-I-A^b (3JP) and anti-CD11c (N418) mAb were affinity purified from hybridoma supernatant and conjugated to FITC (Sigma-Aldrich) and allophycocyanin (Prozyme), respectively, according to the manufacturer's instructions. Cells were preincubated with anti-FcγRII (2.4G2, purified from hybridoma supernatant) and stained with appropriate mAb in PBS containing 2% FCS and 0.01% sodium azide as previously described (26), and analyzed using a FACSort flow cytometer and CellQuest software package (BD Biosciences). Necrotic cells and cellular debris were identified by forward scatter/side scatter (FSC/SSC) profiles and propidium iodide (PI; Sigma-Aldrich) staining, and excluded from analysis.

Induction and analysis of apoptosis in B16-F10 melanoma cells

C57BL/6-derived B16-F10 melanoma cells were subjected to heat shock by incubation at 43°C for 1 h, and then irradiated using 200 Gy from a cesium source. For staining experiments, stressed cells were then placed in an incubator at 37°C and 5% CO₂ for a further 20–24 h to allow apoptosis to occur. Apoptosis was detected by staining cells with annexin V-biotin (Roche Molecular Biochemical) according to the manufacturer's instructions. Following the primary stain, cells were washed, stained with streptavidin-PE (BD Pharmingen) and PI, and analyzed by flow cytometry. For DC-loading experiments, stressed cells were incubated at 37°C and 5% CO₂ for 2 h before coculture with DC.

Detection of tumor uptake by DC

DC were labeled with CFSE (Molecular Probes) as previously described (27). Briefly, DC were harvested, washed, and resuspended at 1×10^6 cells/ml in PBS. An equal volume of 2.5 µM CFSE was added to the cell suspension, immediately vortexed, and incubated for 8 min at room temperature. Unbound dye was quenched by the addition of an equal volume of FCS, and washed with cIMDM. Labeled DC were coincubated in six-well plates at a 1:1 ratio with stressed B16.F10 melanoma cells labeled with Cell Tracker Orange (chloromethyl-benzoyl-aminotetramethyl-rhodamine; CTO; Molecular Probes). B16.F10 cells were labeled with 10 mM CTO in cIMDM by incubating for 8 min at room temperature. CTO-labeled cells were washed three times, stressed by heat shock and gamma irradiation, and then coincubated for 48 h with CFSE-labeled DC. Uptake of tumor material by DC was visualized by flow cytometry by detecting the presence of CFSE-CTO double-positive cells. Dead cells were eliminated from analysis on the basis of PI staining.

Preparation of DC-B16-F10 melanoma vaccine, vaccination, and tumor challenge

DC were harvested on day 7 of culture, and coincubated with stressed B16-F10 melanoma cells for 48 h at a 1:1 ratio. Coincubation was in the plates originally used for DC culture, because the pre-existing adherent monolayer of cells in the original plates prevented substantial DC loss through adherence. In some experiments, 100 ng/ml *Escherichia coli* LPS (Sigma-Aldrich) was added in culture during the last 24 h of incubation. Cells were then harvested, washed three times, counted, and resuspended in incomplete IMDM. On day 0, 5×10^5 tumor-loaded DC were injected s.c. in the right flank of test C57BL/6 mice ($n = 5$). Control groups ($n = 5$ in each group) were left nonimmunized, or immunized with 5×10^5 DC only, or 5×10^5 stressed B16-F10 only. Mice were challenged on day 7 by injecting 5×10^4 B16-F10 cells s.c. in the opposite flank. Mice were thereafter monitored biweekly for tumor growth. Mean tumor area for each group was calculated as the product of bisecting tumor diameters obtained from caliper measurements. Measurements were terminated and mice were sacrificed when the tumor size of one mouse in each group reached or exceeded a threshold of 150 mm², or when mice became visibly unwell, or when the tumor became ulcerated. For survival experiments, tumor-challenged mice in a group were individually monitored until the individual's tumor size reached or exceeded 150 mm². Secondary tumor challenge was with 5×10^4 – 2×10^5 B16-F10 cells injected in the opposite flank to the primary challenge.

In vivo cytotoxicity assay

In vivo cytotoxicity assays were conducted on mice that had failed to develop melanoma tumors as a result of stressed tumor-loaded DC vaccination. Protected mice were boosted as they were initially vaccinated. After 48 h, boosted mice were injected i.v. with equal numbers of unpulsed or peptide-pulsed, fluorochrome-labeled, syngeneic splenocytes used as target cells. Unpulsed target cells were labeled with CTO, whereas gp100_{25–33}-pulsed or TRP-2_{180–188}-pulsed target cells were differentially labeled with CFSE. Forty-eight to 72 h after target cell administration, the presence of target cells in the lymph nodes of recipient mice was detected by flow cytometry. A numerical value for target cell survival was obtained by calculating the ratio of peptide-pulsed target cells to unpulsed target cells.

In vivo depletion of T cell subsets

Depletion of CD8⁺ or CD4⁺ T cell subsets in naive C57BL/6 mice was achieved by i.p. injections of depleting mAb GK1.5 (anti-CD4) or 2.43 (anti-CD8) on days -3, -2, and -1. A total of 600 µg of mAb was injected per mouse. Optimal conditions for depletion of the target population were determined in a pilot experiment. Mice depleted of either T cell subset were vaccinated as usual on day 0, challenged on day 7, and monitored for tumor development thereafter. No change in tumor growth was

observed in initial experiments where mice were vaccinated with DC and stressed tumor cells, and were treated or not with isotype control Abs. Thus, an isotype control group was omitted from subsequent experiments.

Depletion of T_{reg} was achieved by injecting mice i.p. with 100 μ g of PC61 (anti-CD25) on day -1 before DC immunization. Treatment conditions were determined in a pilot experiment, and were shown to significantly diminish the percentage of CD25⁺ T cells in recipient blood for at least 12 days after the injection of depleting mAb.

Results

Stress by heat shock and gamma irradiation induces apoptosis and necrosis in B16-F10 melanoma cells

It has been previously demonstrated that DC are capable of cross-priming Ag-specific CTLs following engulfment of apoptotic cellular material (19). To generate a source of potentially immunogenic tumor cell Ags derived from apoptotic cells, B16-F10 melanoma cells were stressed by heat shock (43°C for 1 h) and gamma irradiation (200 Gy). Stressed B16-F10 cells were incubated for 20–24 h to allow apoptosis to occur. The presence of apoptotic B16-F10 cells was detected by staining cells with annexin V and PI, and analyzing by flow cytometry. In contrast to unstressed B16-F10 cells, cultures of stressed melanoma cells contained a high proportion of apoptotic and necrotic cells (Fig. 1). Stressed cells displayed reduced cell size and increased granularity, which are consistent with the onset of apoptosis.

Kinetics experiments were conducted to determine the time at which peak levels of apoptosis occurred. The proportion of an-

nexin V⁺ cells in culture slowly increased during the time after heat shock and irradiation, peaking at ~20 h (data not shown).

Stressed B16-F10 melanoma cells have no detectable effect on the maturation status of DC

DC and stressed tumor cells were cocultured to allow the uptake of tumor cell material by DC. Melanoma cells were mixed with DC 2 h after irradiation, to allow uptake of apoptotic cells as soon as they appeared in culture. Optimal ratio and coincubation time were determined in pilot experiments using fluorochrome-labeled DC and tumor cells. Maximal uptake was observed when the cells were mixed at a 1:1 ratio for 48 h. After coincubation in these conditions, ~30% of the DC had taken up fluorescent material from tumor cells, compared with a background level of 2.3% in the control sample containing DC and stressed tumor cells admixed just before analysis (Fig. 2).

The impact of stressed tumor cells or their derivatives on the maturation status of DC remains controversial (23, 28–30). To address this issue, DC coincubated with stressed B16-F10 cells were analyzed by flow cytometry for the up-regulation of selected cell surface markers, including MHC class II, CD40, CD80, and CD86. In control cultures, DC maturation was induced by the addition of 100 ng/ml LPS during the last 24 h of culture. As expected, the presence of LPS provided a robust maturation stimulus to DC, as indicated by marked up-regulation of all the markers

FIGURE 1. Heat stress and gamma irradiation induce apoptosis and necrosis in melanoma cells. B16-F10 melanoma cells were either stressed by heat shock (43°C, 1 h) and gamma irradiation (200 Gy), or left unstressed. Cells were left in culture for 20–24 h to allow apoptosis to occur. To detect the presence of apoptotic tumor cells, cells were stained with annexin V and PI, and analyzed by flow cytometry. Numbers in quadrants indicate the percentage of cells in that quadrant, after gating on the basis of FSC/SSC profile as shown in the *top panels*.

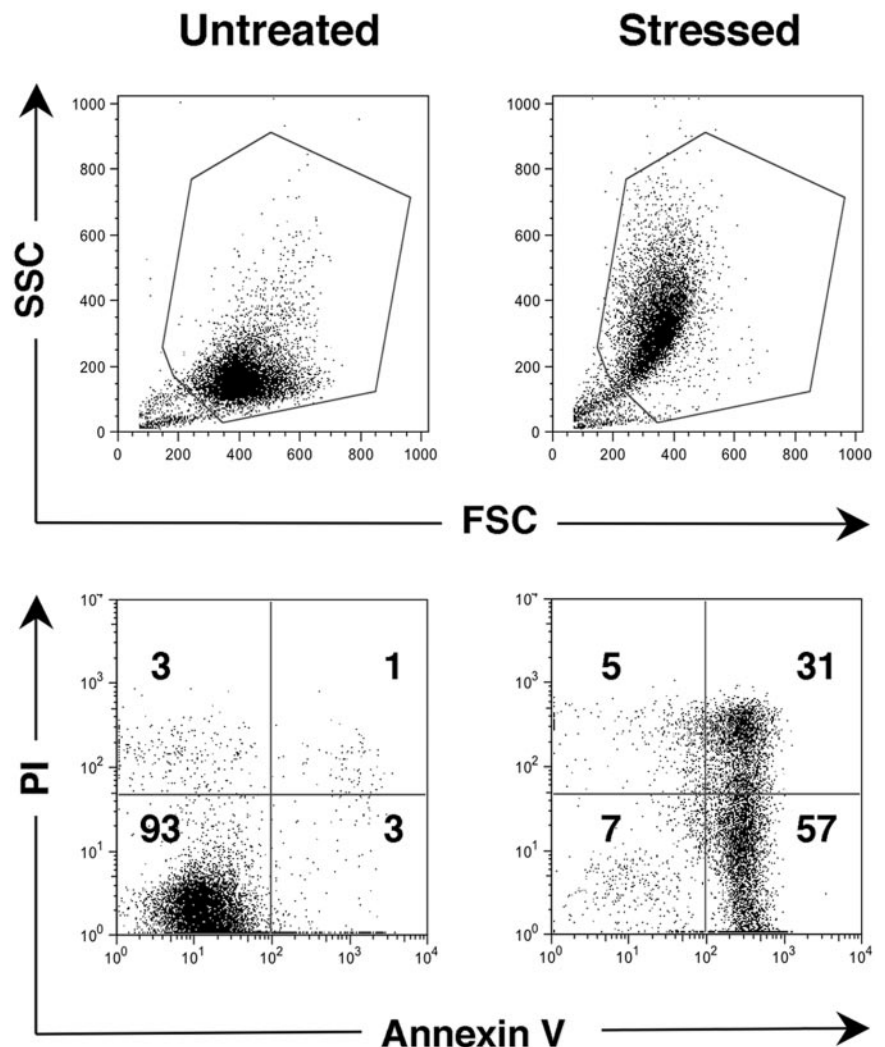
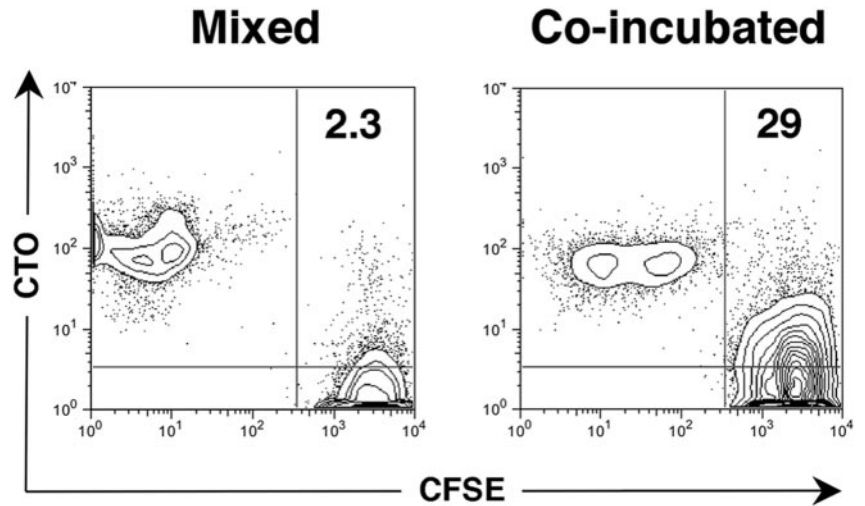


FIGURE 2. DC take up stressed melanoma cells. BM-DC were labeled with CFSE and co-incubated for 48 h, at a 1:1 ratio, with stressed B16.F10 melanoma cells that had been labeled with CTO. As a control, labeled DC and stressed labeled melanoma cells admixed immediately before analysis are shown. Uptake of stressed tumor material by DC is revealed as an increase in the mean CTO fluorescence of the DC population. Numbers in the *top-right quadrants* indicate the percentage of CTO-positive DC after exclusion of debris and dying cells by PI staining and FSC/SSC profile.



examined (Fig. 3). In contrast, stressed tumor cells had no detectable effect on the expression of any of the DC maturation markers examined, and expression levels remained essentially identical with those seen on mock-treated DC.

DC loaded with stressed B16-F10 melanoma cells elicit CD4⁺ and CD8⁺ T cell-mediated immune responses that retard tumor growth

We next investigated the efficacy and mechanism of the stressed tumor cell-loaded DC vaccine on tumor development in a prophylactic vaccination setting. Test groups of C57BL/6 mice were vaccinated on day 0 with 5 × 10⁵ stressed tumor-loaded DC by s.c. injection on the right flank, and subsequently challenged with 5 × 10⁴ B16-F10 cells on day 7 by s.c. injection on the left flank. Test groups were either left intact or were depleted of CD8⁺ or CD4⁺ T cells by i.p. injections of 2.43 or GK1.5 mAb, respectively. Treatment with anti-CD8 mAb does not affect the numbers of CD8⁺ DC in the spleens and lymph nodes of recipient mice (J.

Kirman, unpublished observations). Control groups of mice were challenged after being left unvaccinated, or vaccinated with DC only, or stressed tumor cells only (not shown in figure, as tumor growth kinetics in this group were comparable to the unvaccinated group). Tumor growth in mice vaccinated with DC loaded with stressed tumor was markedly retarded compared with tumor growth in control groups (Fig. 4). This retardation in tumor growth was dependent on the presence of both CD8⁺ and CD4⁺ T cells, as evidenced by similar tumor growth kinetics in the DC-only vaccinated group and in the DC- and stressed tumor cell-vaccinated groups depleted of either T cell subset. Mice in negative control groups and groups vaccinated in the absence of the relevant T cell subset were all sacrificed by day 25 after tumor challenge, because the mean tumor area had either reached or exceeded 150 mm². In contrast, all mice in the group vaccinated with DC loaded with stressed tumor cells survived beyond day 25 after tumor challenge and were tracked individually to generate survival data. Importantly, 60% of the mice in the normal vaccinated group

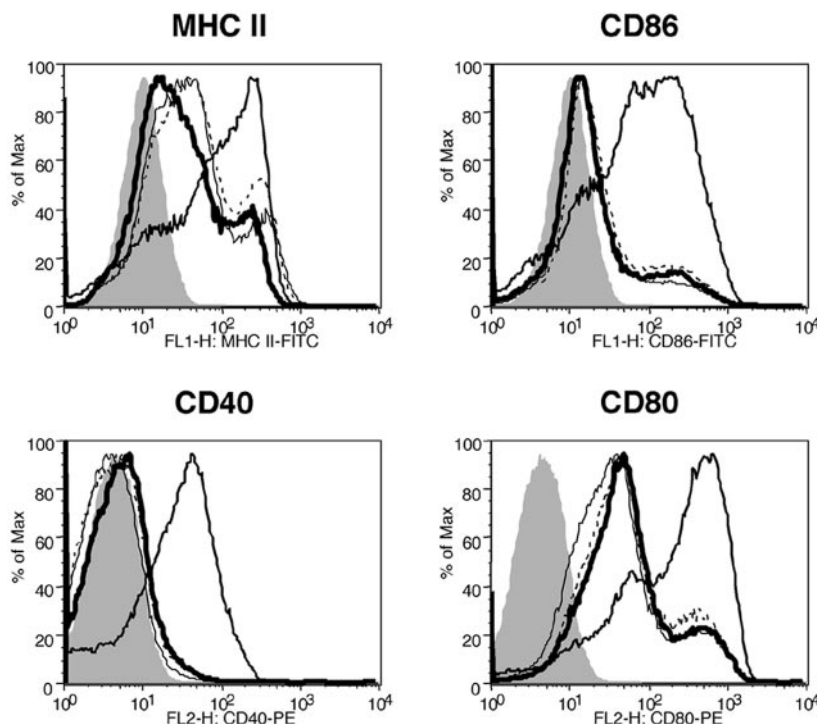


FIGURE 3. Exposure to stressed melanoma cells does not induce DC maturation. DC from bone marrow cultures were subcultured for 48 h into their original plates with or without addition of stressed B16-F10 cells (sB16). Control DC were left untreated, or matured in the presence of 100 ng/ml LPS during the last 24 h of culture. DC from each culture condition were harvested, preincubated with 2.4G2, and either left unstained or stained with a mixture of mAb against MHC class II and CD11c, and a third mAb against either CD40, or CD80, or CD86. The effect of culture conditions on DC surface marker expression was analyzed by flow cytometry. Necrotic cells and cellular debris were identified by FSC/SSC profiles and PI staining, and excluded from analysis. Histograms show expression of the indicated markers on CD11c⁺ cells. Unstained cells are shown as shaded histograms for each culture condition; thin line, untreated DC; dashed line, mock-treated DC; medium line, LPS-treated DC; thick line, DC cocultured with stressed B16-F10 melanoma cells.

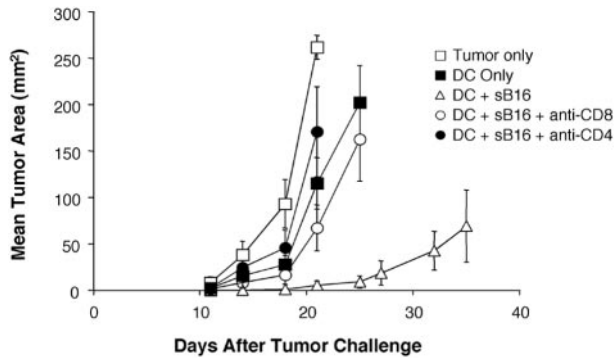


FIGURE 4. Retardation of tumor growth following vaccination with DC and stressed B16-F10 melanoma cells requires both CD8⁺ and CD4⁺ T cells. On day 0, groups of mice were left untreated, or vaccinated with 5×10^5 DC only, or 5×10^5 stressed B16.F10 (sB16) melanoma cells only, or 5×10^5 DC loaded with stressed B16.F10 cells. CD4⁺ or CD8⁺ T cells were depleted from mice that were to be vaccinated with DC and stressed tumor by i.p. injections of the mAb GK1.5 or 2.43, respectively, on days -3 , -2 , and -1 . Each mouse received a total of $600 \mu\text{g}$ of mAb. Seven days after vaccination, mice were challenged with 5×10^4 B16-F10 tumor cells, and monitored for tumor development thereafter. Data shown represent mean tumor area (in square millimeters) \pm SEM. Results are representative of two independent experiments.

survived until day 46 after tumor challenge. Thus, stressed tumor-loaded DC retarded tumor growth in a T cell-dependent manner.

Given that exposure to stressed tumor cells did not alter the DC's maturation status, it was possible that exposure of DC to agents that induce their maturation would also improve their ability to induce antitumor immune responses. Thus, DC were cocultured with stressed tumor cells and treated with 100 ng/ml LPS during the last 24 h of coculture. As shown in Fig. 5, both untreated and LPS-treated DC were able to induce tumor retardation in recipient mice, but LPS-treated DC were superior to untreated DC ($p = 0.045$ at day 28). Again, this suggests that coculture with stressed tumor cells was not by itself sufficient to induce DC maturation.

Because CD8⁺ T cells were clearly critical to the antitumor effect induced by DC loaded with apoptotic melanoma cells, the presence of CTL specific for known melanoma Ags such as TRP-2₁₈₁₋₁₈₈ or gp100₂₅₋₃₃ was tested in mice that had been vaccinated and remained tumor free, using a sensitive in vivo cytotoxicity assay (31). Before the cytotoxicity assay, mice were again injected with DC and stressed melanoma cells to boost the response. As shown in Fig. 6, some cytotoxic activity could be demonstrated in only one of three mice tested. In additional experiments, no cytotoxic activity could be demonstrated in mice that had been vaccinated only, but had not been challenged with tumor, or in mice that had been vaccinated and had rejected tumor challenge (not shown). We conclude that CTL specific for the TRP-2₁₈₁₋₁₈₈ and gp100₂₅₋₃₃ epitopes were probably not involved in tumor protection, and that the tumor-protective CD8⁺ T cells elicited by the DC and stressed tumor cell vaccine were possibly directed to melanoma Ags other than TRP-2₁₈₁₋₁₈₈ or gp100₂₅₋₃₃.

Depletion of T_{reg} enhances the potency of the DC and stressed tumor cell vaccine and allows the establishment of tumor-protective memory

Although the DC and stressed melanoma cell vaccine was clearly effective in retarding tumor growth, a large proportion of vaccinated mice still developed tumors. To determine whether the efficacy of our DC vaccine could be further increased by depletion

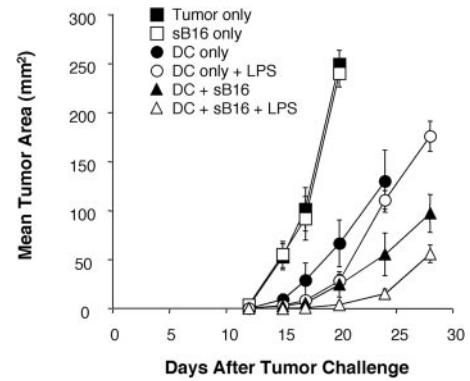


FIGURE 5. Pretreatment of DC with LPS improves the effect of vaccination with DC and stressed B16.F10 melanoma cells. The experimental setup was as described in the legend to Fig. 4, with the only exception that some mice received DC that had been cocultured with stressed melanoma cells, and then treated with 100 ng/ml LPS for the last 24 h before in vivo injection. Seven days after vaccination, mice were challenged with 5×10^4 B16-F10 tumor cells, and monitored for tumor development thereafter. Data shown represent mean tumor area (in square millimeters) \pm SEM of five mice per group.

of T_{reg}, mice were treated with one i.p. injection of the anti-CD25 mAb PC61 on day -1 relative to vaccination. Fig. 7 shows cumulative data from three independent experiments examining the effect of T_{reg} depletion on tumor immunity induced by the DC vaccine. Each line refers to a group of 9–15 mice. Overall, 50% of mice that had been depleted of T_{reg} and immunized with DC and stressed tumor cells remained tumor-free for >60 days after tumor challenge. At this time point, the incidence of tumor-free mice in both the DC and stressed tumor cell group, and in the DC only and anti-CD25 control groups was only $\sim 10\%$. When all tumor-free mice were challenged again with tumor on day 90, most of the mice in the T_{reg}-depleted group remained tumor-free. In contrast, all mice in other groups eventually succumbed to tumor. Thus, depletion of T_{reg} enhances the effects of vaccination with DC and

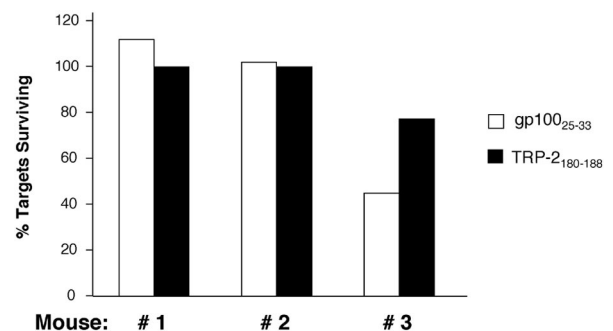


FIGURE 6. Vaccination with DC and stressed melanoma cells fails to reproducibly elicit CTL specific for melanocyte differentiation Ag. In vivo cytotoxicity assay was performed on mice that had been vaccinated with DC and stressed melanoma cells, and had rejected a challenge with B16-F10 melanoma cells. All mice were boosted as they were initially vaccinated 3 days before the assay. In vivo cytotoxicity was tested by i.v. injection of fluorochrome-labeled syngeneic target cells as indicated. Unpulsed target cells were labeled with CTO, whereas gp100₂₅₋₃₃-pulsed or TRP-2₁₈₀₋₁₈₈-pulsed target cells were differentially labeled with CFSE. Forty-eight hours following target cell administration, the presence of target cells in the lymph nodes of recipient mice was detected by flow cytometry. A numerical value for target cell survival was obtained by calculating the ratio of peptide-pulsed to unpulsed target cells, and comparing it to the ratio in control C57BL/6 mice, which was adjusted to 100%. Each bar refers to an individual mouse; thus, no error bars are shown.

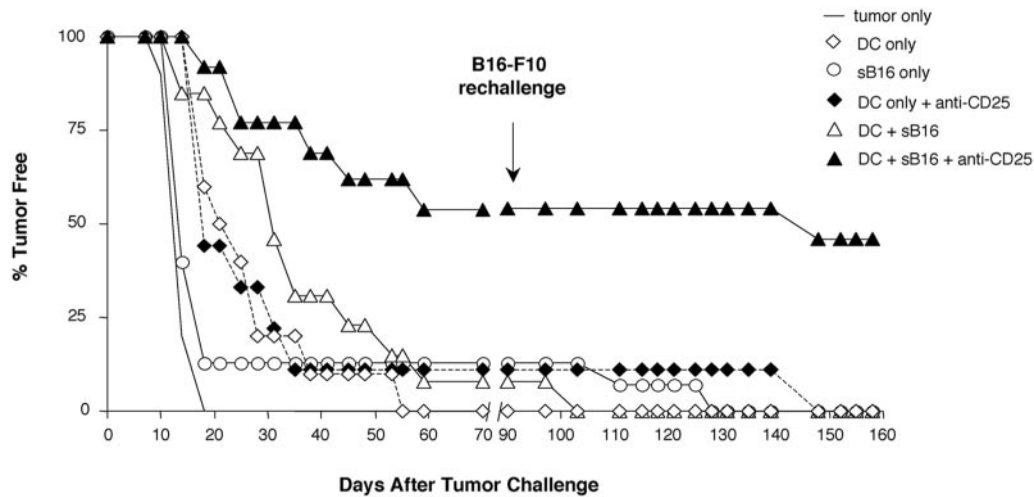


FIGURE 7. The protective effect of the DC and stressed tumor cell vaccine is enhanced by prior depletion of $CD4^+CD25^+ T_{reg}$. On day 0, mice were left untreated, or vaccinated with 5×10^5 DC only, or 5×10^5 stressed melanoma cells only, or 5×10^5 DC loaded with stressed melanoma cells. In some of those mice, $CD25^+ T_{reg}$ had been previously depleted by i.p. injection of $100 \mu\text{g}$ of the mAb PC61 on day -1 . Seven days after vaccination, mice were challenged with 5×10^4 B16-F10 tumor cells, and monitored for tumor development thereafter. Mice were scored as tumor positive when their tumors became $>3 \text{ mm}^2$. Mice that had remained tumor-free after the first tumor challenge received a second challenge of B16-F10 tumor cells on day 90, and were monitored for tumor development as above. The graph shows cumulative data from three independent experiments, including a total of 9–15 mice in each experimental group.

stressed tumor cells and allows the induction of long-lived tumor protective immunity.

Induction of strong immune responses to melanoma Ags has been associated in some cases with the onset of autoimmune symptoms such as vitiligo (32). Because it was possible that the simultaneous depletion of T_{reg} would allow the activation of autoreactive cells in response to the DC and melanoma vaccine, mice were regularly monitored for signs of hair discoloration. However, no such signs were observed at any time point, in any group of mice. In addition, mice that had been depleted of T_{reg} and vaccinated with DC and stressed tumor cells were again examined for *in vivo* CTL responses to the melanoma Ags TRP-2_{180–188} and gp100_{25–33}, which have been implicated in autoimmune vitiligo. Again, no detectable responses to these Ags could be demonstrated (Fig. 8). Thus, depletion of T_{reg} allows the induction of powerful tumor-protective memory T cell responses by the DC and stressed tumor cell vaccine, without detectably increasing the risk of autoimmune side effects.

Discussion

In this report, we show that DC loaded with stressed tumor cells elicit T cell-mediated retardation of tumor growth in a prophylactic vaccination setting. DC could be readily loaded with Ags derived from syngeneic, weakly immunogenic melanoma cells that had been stressed by heat shock and gamma irradiation. Upon injection into syngeneic hosts, the DC and melanoma cell vaccine activated tumor-protective $CD8^+$ and $CD4^+$ T cells that retarded tumor growth. The potency of the vaccine was increased by treatment of DC with LPS. In addition, the concomitant depletion of T_{reg} led to complete tumor rejection and establishment of long-lasting tumor immunity in a considerable proportion of the tumor-inoculated mice.

We have attempted to address critical issues concerning tumor Ag preparation and loading of DC by using whole, stressed tumor cells as a potential source of a broad array of tumor Ags. We reasoned that loading of DC with tumor Ags would be facilitated by the use of a stress regimen that promoted apoptosis, and thus offered the possibility of receptor-mediated uptake of apoptotic material. Necrotic cells were also present in our stressed cell preparations, and could in turn act as potential sources of tumor Ag.

Substantial evidence indicates that DC can internalize apoptotic material through a number of receptors, including CD36 and the $\alpha_v\beta_5$ integrin (33), the phosphatidylserine receptor (20), and the common receptor for heat shock proteins (HSP) (34). Apoptotic material is then cross-presented to specific CTL (19, 21–23), which are recognized as critical mediators of antitumor immunity (35, 36). Incorporating heat shock as part of the stress regimen had the potential of rendering DC vaccines more immunogenic by providing DC with a maturation stimulus through up-regulated HSP (37), and increasing the opportunities for presentation of HSP-chaperoned epitopes to T cells (38).

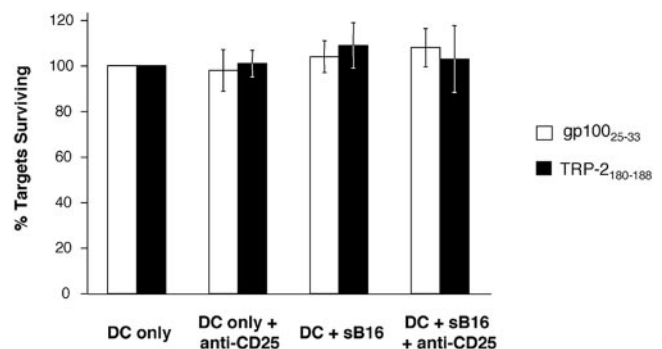


FIGURE 8. The depletion of T_{reg} *in vivo* does not reveal the activity of CTL specific for melanocyte differentiation Ags in mice vaccinated with DC and stressed melanoma cells. An *in vivo* cytotoxicity assay was performed on mice that had been injected with saline or with $100 \mu\text{g}$ of the anti-CD25 mAb PC61 on day -1 , and were vaccinated with DC and stressed melanoma cells, or DC only, on day 0. On day 7, *in vivo* cytotoxicity was tested by i.v. injection of fluorochrome-labeled syngeneic target cells as described in Fig. 6. Three days later, the presence of target cells in the lymph nodes of recipient mice was detected by flow cytometry. A numerical value for target cell survival was obtained by calculating the ratio of peptide-pulsed to unpulsed target cells, and comparing it to the ratio in DC-only-immunized mice, which was adjusted to 100%. Each bar refers to a group of four mice; error bars indicate SD.

There is conflicting evidence in the literature on the effect of apoptotic/necrotic cells and their derivatives on DC maturation (23, 28–30). Although HSP have been shown to confer a maturation stimulus to DC (39, 40), recent evidence suggests that such an effect could be attributable to trace quantities of endotoxin contamination in HSP preparations (41). Furthermore, maturation stimuli provided by necrotic/apoptotic cells to DC were shown to be a consequence of infection with mycoplasma (42). The B16-F10 melanoma cells used in this study were regularly monitored and found free of mycoplasma contamination. Even after heat shock and irradiation, these B16-F10 melanoma cells were consistently unable to induce up-regulation of costimulatory molecules on DC, whereas stressed mycoplasma-infected B16-F10 cells reproducibly induced DC maturation (data not shown).

Although the effects of exposure to stressed tumor cells should be detectable on DC within the 48 h of *in vitro* treatment, it was still possible that further DC maturation could occur *in vivo* after injection. We tried to address this point by determining expression of maturation markers on DC that had or had not been cocultured with stressed tumor cells, and had migrated to the draining lymph node, but the numbers of DC recovered were insufficient for a meaningful comparison. Data obtained with LPS-treated DC showed that these were better able to induce antitumor immune responses than DC that had been exposed to stressed tumor cells only, indicating that exposure to stressed tumor cells had not been sufficient to induce full DC activation. Together, these data suggest that incubation with stressed melanoma cells did not induce DC maturation *in vitro* or *in vivo*. It is unclear why our results differ from those of other authors (43), who were able to show DC maturation after loading with irradiated, non-mycoplasma-infected B16 melanoma cells. Differences in the DC culture conditions, source of melanoma cells, and protocols for apoptosis induction and DC loading could each contribute to this discrepancy.

We show in this paper that DC that had been exposed to stressed melanoma cells and LPS induced stronger tumor protective responses than DC exposed to stressed melanoma cells only. We hypothesize that these stronger immune responses are due to the DC's higher expression of costimulatory molecules and better ability to migrate to the draining lymph node after injection (S. Huck, J. Yang, and F. Ronchese, manuscript in preparation) and the consequent enhanced interaction with Ag-specific T cells. Notably, DC that had not been exposed to LPS were still able to induce effective immune responses, indicating that, even in the absence of stimuli such as LPS, those DC were sufficiently mature to initiate tumor-protective immune responses. This is consistent with the expression of moderate to high levels of MHC II, CD80, and CD86 in a proportion of these DC populations.

The role of T cells in mediating antitumor immunity is clearly established (35, 36). Tumor retardation in our prophylactic vaccination model clearly required the presence of both CD8⁺ and CD4⁺ T cells. CD8⁺ CTL are recognized as the dominant effector cells that mediate tumor destruction through perforin-dependent lysis and/or IFN- γ secretion (44). In contrast, CD4⁺ T cells are thought to have a broader role in providing help to CTL as well as a direct role in tumor rejection (45). Consistent with our finding, an important role of CD4⁺ T cells has been reported in previous studies using vaccination with DC loaded with peptides eluted from whole tumor cells (46) or with irradiated, whole tumor vaccines (45, 47).

Although CD8⁺ T cells were critical to tumor retardation, we found no evidence of CTL reactive against TRP-2_{180–188} or gp100_{25–33} in our vaccinated mice. The TRP-2_{180–188} and gp100_{25–33} epitopes are expressed in melanomas and normal melanocytes, and activation of T cells specific for these epitopes has

been documented both in melanoma immunity (43, 48–51) and in autoimmune situations such as vitiligo (48). Because the signs of vitiligo in our vaccinated mice were at best weak and infrequent, we hypothesize that the tumor-reactive CD8⁺ T cells elicited by our vaccine may be directed against additional melanoma epitopes that are not shared with normal melanocytes. No experimental evidence is presently available to support or disprove this hypothesis. Alternatively, lack of vitiligo may be due to inadequate activation of tumor-loaded DC, and consequent suboptimal activation of specific CD8⁺ T cells. Recent evidence suggests that DC must receive a strong stimulus, mediated through the IL-1R or through Toll-R, to activate autoreactive T cells and initiate autoimmunity (52, 53). It is possible that such signals, if used in our protocol, would greatly increase the capacity of our vaccine to activate both anti-tumor and autoreactive T cells.

We report here that the depletion of T_{reg} in mice treated with DC and stressed melanoma cells considerably increased the potency of the DC vaccine, and induced long-term tumor immunity. Several reports have documented that depletion of T_{reg} can enhance spontaneous immune responses to tumors (54–56), and increase the antitumor effect of different immunotherapeutic procedures (57–59). In addition, tumor-specific T_{reg} have been isolated from melanoma lesions in humans (60). Those cells could be demonstrated to be specific for the nonmutated tumor Ag LAGE, thus suggesting the possibility that the use of self-Ags in immunotherapy may lead to preferential activation of T_{reg} and suppression of the antitumor immune response. The DC vaccine used in our study included a preparation of stressed melanoma cells, which was likely to contain a significant proportion of self-Ags. Hence, it is possible that depletion of T_{reg} may have been necessary to allow an active immune response against self-Ags in the vaccine. However, other authors (4) have also reported that depletion of T_{reg} will allow the generation of stronger Th1 and CTL responses after immunization with DC and a non-self-Ag such as OVA. Thus, it appears more likely that the inhibitory effect of T_{reg} is not limited to responses involving self-Ags, but is a general phenomenon that affects responses to both self and non-self-Ags.

Again, it is interesting that, in our system, the depletion of T_{reg} did not result in the activation of CTL reactive to melanocyte Ags, or in a more frequent or more severe occurrence of vitiligo (data not shown). Depletion of T_{reg} in mice that are immunized with self-Ags has been shown to result in increased susceptibility to autoimmune disease in a model of autoimmune gastritis (61), and in more frequent occurrence of vitiligo in mice receiving modified melanoma cells and anti-CTLA-4 treatment (59). Whether the lack of vitiligo in our system is due to insufficient activation of T cells, or to the activation of T cells of different specificity, will require further investigation.

In conclusion, our results demonstrate that DC-based vaccination against melanoma generates effective T cell-mediated antitumor immunity with essentially no autoimmune complications. Depletion of T_{reg} further enhances the effects of vaccination without increasing the incidence of autoimmunity. Such a vaccination strategy can be readily applied to other tumor models and extended into a therapeutic vaccination setting.

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