Macrophages orchestrate the immune response to tumor cell death


ABSTRACT

The mechanisms by which the immune system distinguishes normal developmental cell death from pathological immunogenic cell killing are central to effective cancer immunotherapy. Using HSVtk suicide gene therapy, we showed that macrophages can distinguish between tumor cells dying through classical apoptosis and tumor cells engineered to die through nonapoptotic mechanisms, resulting in secretion of either immunosuppressive cytokines (interleukin 10 and transforming growth factor β) or inflammatory cytokines (tumor necrosis factor α or interleukin 1β), respectively. Additionally, heat shock protein 70 acts as one component of a bimodal alarm signal that activates macrophages in the presence of stressful, immunogenic tumor cell killing. These differential responses of macrophages can also be used to vaccinate mice against tumor challenge, using adoptive transfer, as well as to cure mice of established tumors.

INTRODUCTION

The majority of cytotoxic therapies, such as chemotherapy and suicide gene therapy, result in apoptosis of the target cells (1, 2). However, it is not sufficient simply to kill tumor cells in situ to raise effective antitumor immunity. Cells that are replaced daily through normal apoptotic processes do not routinely induce autoimmunity. For an immune response to occur, immunogenic cell killing must be a distinct circumstance that can be sensed and distinguished from normal homeostatic processes.

Our own data demonstrated that killing tumor cells in vivo with cytotoxic genes, such as the HSVtk/GCV3 suicide gene/prodrug system could, under certain circumstances, lead to the generation of very effective immunity (3, 4). However, cell killing alone was not sufficient to raise antitumor immunity. The B16 melanoma line, when killed in vivo with HSVtk/GCV, died by mechanisms that were largely nonapoptotic, whereas the colorectal CMT93 line died by classical apoptosis (5, 6). Transfection of the antiapoptotic bcl-2 gene into CMT93tk cells shifted the death mechanism from apoptotic to non-apoptotic, induced increased levels of Hsp 70, and increased the intrinsic immunogenicity of the in vivo killing (3). Similar published work demonstrated that administration of antigen-coupled cells into the eye generated systemic immunity only if the cells died by necrosis. If cell death was via apoptosis, then phagocytes cleared the cells and tolerance to the administered antigen resulted (7). However, it is also clear that apoptosis can both be an effective vaccine modality for activation of antigen-specific immune responses (8–12) and be immunologically “silent” and potentially actively anti-inflammatory (3, 7, 13–16).

Rapid phagocytosis of apoptotic debris ensures that the contents of cells are neatly and safely removed by scavenger cells, preventing the release of proinflammatory noxious materials that are only seen in the tissue milieu in times of pathological tissue destruction (16–19). We show here that macrophages, as one of the principal cell types responsible for clearance of apoptotic cell debris (18), can both sense and respond to different mechanisms of cell killing both in vitro and in vivo. Moreover, we demonstrate that adoptive transfer of macrophages conditioned in vitro with tumor cell lysates derived from nonapoptotic cell killing can lead to significant protection either in a prophylactic manner against tumor rechallenge or as a therapy against small established tumors. These data strongly suggest that macrophages can act as a key cell type in determining the subsequent immune response to in vivo cell death.

MATERIALS AND METHODS

Cell Culture. The CMT93tk and CMT93tk-bcl-2 cell lines used in this study have been described previously (3). Briefly, CMT93tk is a stable clone derived from murine colorectal CMT93 cells by transfection of a plasmid in which the HSVtk gene is expressed from the CMV promoter. CMT93tk-bcl-2 cells were derived from infection of CMT93tk cells by a recombinant retroviral vector expressing the bcl-2 gene. The lines were grown in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% (v/v) FCS (Life Technologies and 1-glutamine (Life Technologies). The murine macrophage cell line IC-21 used in these experiments was grown according to American Type Culture Collection guidelines. All cell lines were monitored routinely and found to be free of Mycoplasma infection. For cell killing in vitro, medium was supplemented with GCV (Cytovene-Roche, Indianapolis, IN) to a final concentration of 5 μg/ml.

Phagocytosis Assay. Phagocytosis was assessed by flow cytometry by a variation of the method described in Ronchetti et al. (15). Briefly, 105 tumor cells (CMT93tk or CMT93tk-bcl-2) were labeled with the red dye Cell Tracker Orange [5-(and-6)-(4-chloromethyl)benzoyl]amino]tetramethylrhodamine; Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Separately and simultaneously, 106 macrophages were labeled with the green dye Cell Tracker Green (5′-chloro-methyl-fluorescein diacetate; Molecular Probes). Tumor cells and macrophages were cocultured in the presence or absence of GCV for 24–48 h, after which the populations were measured for fluorescence by FACScan analysis. For confocal analysis, tumor cells were treated for 24 h with GCV, and the nonadherent debris was added to macrophages on glass chamber slides and incubated for an additional 24 h. Slides were then washed thoroughly in PBS, fixed in 4% formaldehyde (v/v), and mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing 2 μg/ml 4′,6-diamidine-2′-phenylindole dihydrochloride (Roche, Indianapolis, IN). Analysis was performed using a LSM510 confocal microscope (Zeiss Inc, Oberkochen, Germany).

Cytokine Detection in Macrophage/Tumor Cell Cocultures. Tumor cells (105; CMT93tk or CMT93tk-bcl-2) were cocultured with 106 macrophages in the presence or absence of GCV for 24 h; the culture supernatant was then removed and assayed by ELISA for IL-10 or TNF-α (PharMingen, San Diego, CA), or IL-1β (R&D Systems, Minneapolis, MN) according to manufacturers’ instructions. Absorbance values were related to standard curves to produce cytokine generated106 macrophages/24 h.

Generation of recombinant virus stocks. The production of retrovirus stocks and infection of target cells have been described previously (20). Briefly, a retroviral vector encoding muHsp 70 was constructed by cloning the Hsp 70 cDNA into the polylinker of the pBabe Puro retroviral vector (21). The vector DNA was transfected into the GP+EnvAM12 amphotropic packaging...
matched for individual experiments. To establish s.c. tumors, 2 min, whereas the lethal heat shock was 45 °C for 20°C water bath. The sublethal heat shock of CMT93tk cells was 42°C PBS, and the cell pellet was transferred 3 times between liquid nitrogen and a

experiments, adherent CMT93tk cells were harvested and washed 3 times in

tumor cell cocultures, as described by Asea et al.

sizes of the established tumors were measured twice per week until they

macrophages, conditions were as described above except that treatment cells

clearing the treatment cells. For treatment with conditioned

cells (CMT93tk or CMT93tk-bcl-2 cells) were injected s.c. on the right flank (treatment tumors). Two days later, mice were treated with 50 mg/kg GCV, eradicating all of the treatment tumors. The data in Fig. 1 show that significantly more mice “vaccinated” with CMT93tk-bcl-2 cells, killed in vivo, reject an established tumor on the other flank compared with mice vaccinated with CMT93tk cells (P < 0.01, stratified log rank test; Fig. 1). These results support our hypothesis that cells dying via nonapoptotic mechanisms are more effective than those dying via apoptotic mechanisms at activating antitumor immunity in vivo (3–5).

Macrophages Take Up More Material from Cells Killed via Apoptosis than from Cells Killed via Necrosis. Tumor cells expressing the bcl-2 gene killed by GCV induce high levels of Hsp 70 (Ref. 3, and data not shown). This in turn enhances tumor immuno-
genicity, induces immune infiltrates into the tumor, and results in expression of Th1-like cytokines, including TNF-α, IFN-γ, and IL-1β in the tumor (3–5). In particular, large numbers of macrophages were seen in Hsp 70-overexpressing tumors that were almost completely absent from parental tumors (4, 5). Therefore, we investigated whether macrophages respond differently when exposed to tumor cells undergoing these alternate mechanisms of cell death. Tumor cells (CMT93tk or CMT93tk-bcl-2) prelabeled with a red membrane dye and macrophages prelabeled with a green dye (see “Materials and Methods”) were mixed in the presence or absence of GCV, and then analyzed by FACS 24–48 h later. When either tumor cell line was mixed with macrophages in the absence of GCV, two largely distinct populations were detected, indicating that the macrophages do not appreciably phagocytose intact tumor cells (Fig. 2a, i and ii). In

RESULTS

CMT93tk Cells Dying by Nonapoptotic Rather than Apoptotic Mechanisms in Vivo Protect against Small, Established Tumors. We previously demonstrated, using a variety of biochemical and morphological assays, that CMT93tk cells when exposed to 5 μg/ml GCV die by predominantly apoptotic mechanisms (3, 5). In contrast, CMT93tk cells stably modified to express bcl-2 when exposed to GCV principally undergo a mechanism of cell death that does not resemble apoptosis (3, 6). The CMT93tk and CMT93tk-bcl-2 cell lines have very similar sensitivities and kinetics of cell death in GCV, and the cultures die (as assessed principally by trypan exclusion) within 72 h of initiation of treatment as described in detail in Melcher et al. (3). For these reasons, we used this model to address the uncertainty relating to the relevance of apoptosis to immune activation in vivo. Vaccination of C57BL/6 mice with irradiated CMT93tk or CMT93tk-bcl-2 cells protected against rechallenge with 106 CMT93 cells in 60–80% of animals in each case (data not shown). This, along with our previous observations, indicated that addition of the bcl-2 gene to these cells does result in enhanced immunogenicity per se (3). To examine the effect of varying the mode of cell death in immune activation, we established parental tumors (106 CMT93) on the left flank of C57BL/6 mice (tumors) and 3 days later injected either CMT93tk or CMT93tk-bcl-2 cells on the right flank (treatment tumors). Two days later, mice were treated with 50 mg/kg GCV, eradicating all of the treatment tumors. The data in Fig. 1 show that significantly more mice “vaccinated” with CMT93tk-bcl-2 cells, killed in vivo, reject an established tumor on the other flank compared with mice vaccinated with CMT93tk cells (P < 0.01, stratified log rank test; Fig. 1). These results support our hypothesis that cells dying via nonapoptotic mechanisms are more effective than those dying via apoptotic mechanisms at activating antitumor immunity in vivo (3–5).

Freeze-Thawing and Heat Shock of Tumor Cells. For freeze-thawing experiments, adherent CMT93tk cells were harvested and washed 3 times in PBS, and the cell pellet was transferred 3 times between liquid nitrogen and a

animals received daily i.p. injection of either GCV (50 mg/kg for treatment) or saline (control) in 100-hg/ml Polybrene. Effective transfer to infect target cells in the presence of 4 μg/ml Polybrene. Effective transfer of vector to target cells was confirmed using Western blotting of infected target cells for Hsp 70 (data not shown).

Virus was expanded from individual plaques, and the identity of the recombinant virus was confirmed by diagnostic restriction enzymes. A positive limiting dilution analysis on 293 cells. Effective transfer of vector to target cells was confirmed using Western blotting of infected target cells for Hsp 70 (data not shown).

Recombinant human Hsp 70 protein was purchased from Sigma Chemical Co. (St. Louis, MO) and used at a concentration of 10 μg/ml in macrophage/tumor cell cocultures, as described by Asea et al. (23).

In Vivo Injection of Tumor Cells. C57BL/6 mice were age- and sex-matched for individual experiments. To establish s.c. tumors, 2 × 106 CMT93tk cells were injected s.c. (100 μl) into the flank region. Animals were examined daily until the tumor became palpable; thereafter the diameter, in two dimensions, was measured three times weekly using calipers. Where appropriate, animals received daily i.p. injection of either GCV (50 mg/kg for treatment) or saline (control) in 100-μl volumes for 5 consecutive days, starting at day 5 after tumor seeding unless otherwise stated. Animals were killed when tumor size was ~1.0 × 1.0 cm in two perpendicular directions.

Freeze-Thawing and Heat Shock of Tumor Cells. For freeze-thawing experiments, adherent CMT93tk cells were harvested and washed 3 times in PBS, and the cell pellet was transferred 3 times between liquid nitrogen and a 37°C water bath. The sublethal heat shock of CMT93tk cells was 42°C for 20 min, whereas the lethal heat shock was 45°C for 1 h. Lactate dehydrogenase release assays indicated that the former treatment produced <5% of maximum lysis and induced Hsp 70 expression by Western blot analysis (data not shown), whereas the latter treatment produced >90% of maximum lysis in addition to Hsp 70 induction.

To generate the adenovirus Ad CMV Hsp 70, the shuttle vector pAd2/CMV

Hsp 70 was confirmed using Western blotting of infected target cells for Hsp 70 (data not shown).
contrast, a high proportion (86%) of the CMT93tk and macrophage coculture was double stained in the presence of GCV, demonstrating phagocytosis or at least a physical attachment of tumor cell debris to the macrophages. Conversely, only ~10% of the CMT93tk-bcl-2 and macrophage coculture were double-stained in the presence of GCV. These differences were not attributable to differential sensitivities of the two tumor cell lines to GCV [as described above and in Melcher et al. (3)] and are not attributable to slower kinetics of phagocytosis by macrophages of the CMT93tk-bcl-2 cells because phagocytosis at later time points shows decreased levels of phagocytosis in both cell lines. Confocal analysis of macrophages incubated with cell-free debris from dying cells clearly showed cells staining both green and red predominantly in the presence of apoptotic cell death (Fig. 2b), additionally confirming that the double staining represents phagocytosis, rather than binding, of tumor material by macrophages. These data suggest that the mechanism by which the tumor cells die influences the ability of macrophages to detect the debris and that macrophages are well adapted to recognize and clear apoptotic, as opposed to nonapoptotic, cell death.

Macrophages Respond to Different Mechanisms of Cell Killing with Different Profiles of Cytokine Secretion. Macrophage/tumor cell cocultures, 24 h after administration of GCV, were assayed for cytokines by ELISA for secreted cytokines and RPAs for cytokine mRNA. Neither tumor cells nor macrophages alone secreted detectable levels of cytokines under the assay conditions used. Macrophages cultured with dying CMT93tk cells produced high levels of IL-10.
Macrophages responded to the onset of apoptosis by secreting IL-10 but that this response waned with time. If macrophages were added to dying tumor cells 48 h after initiation of tumor killing, TNF-α secretion occurred. Because apoptotic bodies eventually fragment (secondary necrosis) if not cleared by phagocytosis, these data suggest that the macrophage response to apoptotic cells (IL-10 secretion) was lost as the cells underwent secondary necrosis. Increasingly, necrotic cells dominated the macrophage response, which was characterized by TNF-α secretion.

Hsp 70 Inhibits the Immunosuppressive Response of Macrophages to Apoptotic Cell Killing. Our previous work demonstrated that nonapoptotic HSVtk/GCV killing is associated with increased expression of Hsp 70, which is in part responsible for increased immunogenicity of tumor cell killing (3–5). For this reason, we hypothesized that overexpressed or exogenous Hsp 70 may function by influencing the macrophage response to tumor cell killing. In agreement with Fig. 2, coculture of macrophages with CMT93tk cells in the presence of GCV generated a very high proportion of double-stained cells (95%; Fig. 4i), indicating phagocytosis of the apoptotic debris, whereas dying CMT93tk-bcl-2 cells were only poorly phagocytosed by the macrophages (6.4% double-stained population; Fig. 4ii). However, if the CMT93tk cells were engineered to express Hsp 70 by retroviral infection or the killing occurred in the presence of exogenous recombinant Hsp 70, the proportion of double-stained macrophages was significantly reduced relative to unmodified CMT93tk cocultures (18.8 or 23.8% double-stained populations, respectively, compared with 95%; Fig. 4). We did not see any effect of Hsp 70 transfection/exogenous addition on the phagocytosis of CMT93tk-bcl-2 debris by macrophages.

Similarly, we investigated the effects of Hsp 70 on the cytokine response of macrophages to apoptotic or nonapoptotic tumor cell killing. Macrophages cultured with CMT93tk cells secreted IL-10 in the 24 h after administration of GCV (Figs. 3b and 5). However, IL-10 secretion was reduced when the macrophages were cultured with Hsp 70 (Fig. 4ii). GCV treatment of CMT93tk cells (Fig. 5). The levels of cytokines were counted and presented in Fig. 3c. The cells were cultured with GCV for 48 h (CMT93tk cells), and then the supernatant was assayed by ELISA for IL-10 and TNF-α (data not shown).

In contrast, macrophages mixed with CMT93tk-bcl-2 cells secreted only very low levels of these cytokines at any time point, but produced significant amounts of TNF-α (Fig. 3b) and IL-1β (Fig. 3c) by ELISA and IL-6 by RPA (data not shown). Levels of these cytokines peaked at 24–48 h after initiation of GCV treatment and then fell to low levels at later time points.

We also investigated whether the time course of tumor cell killing affected the macrophage response by adding macrophages to tumor cells at varying times after cell death was initiated. Fig. 3d shows that macrophages detectable by ELISA (Fig. 3a) and TGF-β by RPA (data not shown). In contrast, macrophages mixed with CMT93tk-bcl-2 cells secreted only very low levels of these cytokines at any time point, but produced significant amounts of TNF-α (Fig. 3b) and IL-1β (Fig. 3c) by ELISA and IL-6 by RPA (data not shown). Levels of these cytokines peaked at 24–48 h after initiation of GCV treatment and then fell to low levels at later time points.

Fig. 3. Macrophages respond to different mechanisms of cell killing with different profiles of cytokine secretion. Macrophage/tumor cell cocultures, 24 h after administration of GCV, were assayed using ELISA for IL-10 (a), TNF-α (b), and IL-1β (c). Cytokines were determined from CMT93tk cells with macrophages (CMTtk), CMT93tk-bcl-2 cells with macrophages (CMTtk/bcl-2), CMT93tk cells with macrophages and GCV (CMTtk + GCV), or CMT93tk-bcl-2 cells with macrophages and GCV (CMTtk/bcl-2 + GCV). Neither of the cell lines or the untreated macrophages alone reproducibly secreted any of the cytokines shown. Results shown are representative of multiple experiments; bars, SD. a, the production of IL-10 and TNF-α by macrophages (Mø) in response to apoptotic cell killing is dependent on the time after initiation of the killing. CMT93tk cells were seeded 16–24 h before being washed in PBS, followed by the addition of normal medium supplemented with GCV (t = 0 h), and macrophages were added to the cultures at different times thereafter (t = 0, 10, 36, 48, or 60 h). Culture supernatants were assayed by ELISA for IL-10 and TNF-α, 24 h after coculture. Graphs show mean ± SD (bars).
at 45°C. Cells that had been heated at 42°C more physiologically, macrophages were cocultured with tumor cells and macrophages were repeated as described in Fig. 3, with CMT93tk cells previously infected with pBabe Paro Hsp 70 vector (CMTtk/Hsp) or with CMT93tk cells plus recombinant Hsp 70 protein (10 μg/ml) during GCV killing (CMTtk/Hsp). IL-10 or TNF-α release was assayed by ELISA from cocultures of CMT93tk cells with macrophages and GCV (CMTtk), CMTtk/Hsp cells with macrophages and GCV (CMTtk/Hsp), CMT93tk cells plus recombinant Hsp 70 with macrophages and GCV (CMTtk & Hsp), or CMT93tk-bcl-2 cells with macrophages and GCV (CMTtk/b). Results are representative of three separate experiments. Graphs show mean ± SD (bars).

These data suggest that neither cell lysates nor Hsp 70 alone can stimulate TNF-α secretion from macrophages in this system. However, where a “stressful death” occurs, the combination of signal(s) within cell lysates and the presence of stress response proteins are able to activate an inflammatory response.

Adaptive Transfer of Tumor Cell Debris-conditioned Macrophages Is Effective in Both Prophylactic and Treatment Models of Tumor Vaccination. We investigated whether we could use the differential response of macrophages to apoptotic and necrotic forms of tumor killing in a therapeutic manner. One hundred percent of the mice vaccinated with a suspension of either CMT93tk or CMT93tk-bcl-2 cells killed with GCV for 24 h developed tumors. As in the PBS control treatment group, these appeared between 7 and 9 days (0.3 cm in diameter; Table 1). In addition, 100% of mice vaccinated with 10^7 macrophages (which are syngeneic to C57/BL/6 mice) cocultured in vitro with CMT93tk cells and GCV for 24 h developed tumors; however, in two separate experiments, these tumors developed sig-

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Fig. 5. Hsp 70 inhibits macrophage secretion of IL-10 in response to apoptotic cell killing. Cocultures of tumor cells and macrophages were repeated as described in Fig. 3, with CMT93tk cells previously infected with pBabe Paro Hsp 70 vector (CMTtk/Hsp) or with CMT93tk cells plus recombinant Hsp 70 protein (10 μg/ml) during GCV killing (CMTtk/Hsp). IL-10 or TNF-α release was assayed by ELISA from cocultures of CMT93tk cells with macrophages and GCV (CMTtk), CMTtk/Hsp cells with macrophages and GCV (CMTtk/Hsp), CMT93tk cells plus recombinant Hsp 70 with macrophages and GCV (CMTtk & Hsp), or CMT93tk-bcl-2 cells with macrophages and GCV (CMTtk/b). Results are representative of three separate experiments. Graphs show mean ± SD (bars).

Fig. 6. Macrophage activation requires signals released from dying cells concomitant with evidence of a stress response. a. TNF-α release was assayed by ELISA from supernatants of macrophages exposed to lysates of 10^5 CMT93tk cells that had undergone three cycles of freeze-thawing (CMTtk F/T), from macrophages exposed to 10 μg/ml recombinant human heat shock protein (Hsp70), or from macrophages exposed to the freeze-thawed lysate plus 10 μg/ml recombinant Hsp 70 (CMTtk F/T & Hsp), b, the experiment shown in a was repeated, but instead of exogenous Hsp 70, CMT93tk cells were preinfected 48 h earlier with Ad-CMV-Hsp 70 at a multiplicity of infection of either 1 or 10 (CMTtk/hsp (1) F/T or CMTtk/hsp (10) F/T). c, TNF-α release from macrophages was also measured when incubated with 10^7 CMT93tk cells that had been treated immediately before coculture with either sublethal or lethal heat shock. Results are representative of two separate experiments. Graphs show mean ± SD (bars).
A total of 10^6 live CMT93tk cells were injected into the left flank of C57BL/6 mice. When tumors were palpable (typically 3 days later), mice received injections of fresh peritoneal macrophages (12.5%), and three of eight mice treated with macrophages alone (25%), one of eight mice treated with macrophages alone (12.5%), and three of eight mice treated with macrophages conditioned with apoptotic debris (37.5%) did not grow after the treatment (Table 2). In contrast to the control groups, none of the eight animals treated with macrophages conditioned with CMT93tk-bcl-2/GCV-killed debris progressed beyond 0.3 cm in diameter, and all tumors regressed completely within 7 days of the treatment (Table 2).

Taken together, these data show that macrophages conditioned with GCV-killed tumor cell debris can be therapeutically effective in both prophylactic and treatment models of tumor growth, provided that the conditioned debris comes from cells dying via nonapoptotic mechanisms. In addition, in the prophylactic model, animals vaccinated with apoptotic debris-conditioned macrophages consistently developed tumors more quickly than controls.

**DISCUSSION**

The experiments reported here show that macrophages may be one of the key cell types that both sense and direct the immune response to different physiological types of cell death in vivo. We demonstrated that tumor cells can be killed through different biochemical processes and that the apoptotic pathway is less immunogenic in vivo (Fig. 1). Apoptotic tumor cell death provokes high levels of phagocytosis by macrophages and induces secretion of immunosuppressive cytokines (Figs. 2 and 3). These data are consistent with other reports showing that the process of apoptotic removal may be actively anti-inflammatory (18, 24–26) and that this facet of apoptotic cell clearance is key for avoidance of autoimmunity during normal tissue remodeling processes (18). Thus, although scavenger macrophages involved in apoptotic cell clearance can present ingested antigens to T cells (12), in the absence of activating cytokine environments the antigens are presented in a nonpermissive setting (15). In sharp contrast, nonapoptotic cell death of CMT93tk-bcl-2 cells leads to secretion of immunostimulatory cytokines (Fig. 3). Importantly, these in vitro assays were supported by our observations of Th1-type cytokine expression in tumors dying by nonapoptotic mechanisms in vivo (4, 5). Although markers of apoptosis are reduced or absent during death of most (but not all) CMT93tk-bcl-2 cells, such nonapoptotic cell death is not instantaneous and involves active transcription of stress-related and probably other genes (3, 5, 27). This form of cell death is distinct from nonphysiological death, such as osmotic shock or freeze-thaw rupture, which is unlikely to be relevant in vivo (14, 27). Nonapoptotic cell death is accompanied by release of highly inflammatory intracellular contents (17), such as intracellular sugars (16, 28), caspase-processed neoantigens (29), oligonucleosomes (30), or other noxious substances (19). Thus, one might expect this to be a more immunogenic way to kill tumor cells. Our data obtained with the IC21 macrophage cell line, which were paralleled by similar experiments using fresh peritoneal macrophages, suggest that the macrophage response to nonapoptotic cell death significantly amplifies the immunostimulatory effect of such killing and may provide a powerful adjuvant for subsequent immune responses.

It is also clear that in the context of killing of tumor cells in vivo, the boundaries between apoptosis and necrosis are far from clearly defined. Once the mechanisms for controlled clearance of apoptotic cells are overwhelmed in vivo, for example, by large amounts of cell killing over a short period, the apoptotic debris will not be cleared. As a result, increasing numbers of apoptotic cells progress to secondary necrosis (31) and an apoptotic, immune suppressive environment becomes a necrotic, immune stimulatory environment (as supported by the time course data in Fig. 3d). Therefore, the balance of immunogenicity achieved by apoptotic cell killing is heavily dependent on the levels of cell death relative to the local phagocytic capacity (31). This is consistent with data showing that the antigen load, the balance of immune cells available to clear dying cells, and the presence of tissue destruction are critical in generating immunogenicity through apoptotic killing (15, 31–33).

The induction of nonapoptotic killing in the CMT93tk-bcl-2 and other systems is also associated with induction of Hsp 70 expression (3–5). We showed here that Hsp 70 markedly suppresses secretion of IL-10 and phagocytosis of cell debris by macrophages exposed to cells dying by apoptosis. In our system, Hsp 70 alone was unable to generate the immunostimulatory phenotype of macrophages; however, when supplied in the context of cell killing, Hsp 70 was clearly a key molecule that signaled activation of macrophages (Figs. 4, 5, and 6). These results suggest that there exists at least a bimodal alarm signal that indicates the presence of physiologically relevant killing to macrophages. A potential first signal may be the release of intracellular contents that are never normally seen outside the cell and that are normally packaged safely away in apoptotic bodies for efficient clearance by scavenger macrophages (18). The second signal could be overt signs of stress during the killing, such as Hsp 70 and other stress proteins, which would indicate to the immune system that the cell death was not controlled by standard internal mechanisms. That is, we believe that for tumor cell killing to be maximally immunostimulatory, it should proceed via a stressful death. Heat shock proteins have been described to function as chaperones of immunogenic peptides (34–37), as cytokines (23), as immunogens (38, 39), and as a potent

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**Table 1** Adaptove transfer of lysate-conditioned macrophages in a prophylactic model of tumor vaccination

C57BL/6 mice were vaccinated with three injections, 7 days apart, of 10^5 macrophages conditioned in vitro in various different ways (Vaccine). Fourteen days after the final vaccination, 10^5 live CMT93tk cells were injected into the opposite flank, and the development of tumors was followed with time until the experiment was terminated at 60 days. Animals in which the tumor grew >0.3 cm in diameter were scored as tumor positive.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Animals developing tumors (Days until all animals developed tumors)</th>
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<tbody>
<tr>
<td>Mo&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10/10 (7 days)</td>
</tr>
<tr>
<td>CMTtk + GCV</td>
<td>10/10 (7 days)</td>
</tr>
<tr>
<td>CMTtk/b + GCV</td>
<td>10/10 (9 days)</td>
</tr>
<tr>
<td>Mo + CMTtk + GCV</td>
<td>10/10 (3 days)</td>
</tr>
<tr>
<td>Mo + CMTtk/b + GCV</td>
<td>7/10 (21 days)</td>
</tr>
</tbody>
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<sup>a</sup> Mo, macrophages.

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**Table 2** Adaptove transfer of lysate-conditioned macrophages in a treatment model of tumor vaccination

A total of 10^6 live CMT93tk cells were injected into the left flank of C57BL/6 mice. When tumors were palpable (typically 3 days later), mice received injections, on the contralateral flank, of 10^5 macrophages treated as shown (Treatment). Growth of the established tumor was followed with time until the experiment was terminated at 60 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animals cured of tumor, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>2/8 (25)</td>
</tr>
<tr>
<td>Mo&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/8 (12.5)</td>
</tr>
<tr>
<td>Mo + CMTtk + GCV</td>
<td>3/8 (37.5)</td>
</tr>
<tr>
<td>Mo + CMTtk/b + GCV</td>
<td>8/8 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mo, macrophages.
adjuvants (40, 41). Our experiments suggest that heat shock proteins also provide one signal to macrophages that counteracts uptake of apoptotic bodies. Blocking of macrophage phagocytosis by carrageenan had an effect similar to that produced by the addition of Hsp 70; however, antibodies blocking CD14 did not affect the action of Hsp 70. Although Hsp 70 has been shown to bind to human macrophages (23), it is not yet clear whether the Hsp 70 action here was through direct competition by Hsp 70 for a phagocytosis receptor on macrophages. Identification of specific receptors for Hsp 70 (23) and other heat shock proteins (42, 43) will help elucidate the observed role of Hsp 70 in down-regulating the immunosuppressive response of macrophages to apoptotic cells.

There is evidence that macrophages can act as both positive and negative regulators of the immune response to tumors and that tumors can even redirect macrophage responses to promote tumorigenesis (44). For these reasons, the use of macrophages as therapeutics for cancer patients is controversial. Similarly, several groups have now shown that dendritic cells can also ingest apoptotic cells and that uptake of such cells by dendritic cells leads to MHC class I cross-presentation of peptides and generation of class I-restricted CD8+ T cells (8, 9, 45). Hence, both necrotic (46) and apoptotic (8–10) cell death can serve as the physical source of antigen for uptake by those antigen-presenting cells. However, the combination of available antigen, together with either inflammatory or anti-inflammatory cytokines in the tumor environment, would be potent determinants of whether dendritic cells could traffic to lymph nodes and initiate antigen-specific immune responses (15). We show here that an understanding of how macrophages respond to different methods of tumor killing can be used to enhance antitumor vaccination in vivo (Tables 1 and 2). At present, we are investigating the mechanism of macrophage therapy in vivo in response to apoptotic or nonapoptotic tumor cell killing. In summary, our data support the model in which macrophages act as one of the major cell types that both sense and respond to cell death in vivo (18). Controlled apoptosis is not immunogenic because of active phagocytosis and the immunosuppressive phenotype of the macrophages at the site of cell death. In contrast, when a stressful form of death occurs, or even uncontainable levels of apoptosis, a proinflammatory environment is created. In addition, our data indicate that manipulating the mechanism of cell death and the resultant macrophage response can be used as an effective therapeutic tool for tumor vaccination. Thus, gene transfer or other modalities, such as radiation therapy, may be very useful in manipulating the way that tumor cells die and inducing the appropriate in vivo immune responses to enhance tumor vaccination.

**ACKNOWLEDGMENTS**

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**REFERENCES**


7. G quantitative studies of annexin V binding, canan had an effect similar to that produced by the addition of Hsp 70; however, antibodies blocking CD14 did not affect the action of Hsp 70. Although Hsp 70 has been shown to bind to human macrophages (23), it is not yet clear whether the Hsp 70 action here was through direct competition by Hsp 70 for a phagocytosis receptor on macrophages. Identification of specific receptors for Hsp 70 (23) and other heat shock proteins (42, 43) will help elucidate the observed role of Hsp 70 in down-regulating the immunosuppressive response of macrophages to apoptotic cells.

There is evidence that macrophages can act as both positive and negative regulators of the immune response to tumors and that tumors can even redirect macrophage responses to promote tumorigenesis (44). For these reasons, the use of macrophages as therapeutics for cancer patients is controversial. Similarly, several groups have now shown that dendritic cells can also ingest apoptotic cells and that uptake of such cells by dendritic cells leads to MHC class I cross-presentation of peptides and generation of class I-restricted CD8+ T cells (8, 9, 45). Hence, both necrotic (46) and apoptotic (8–10) cell death can serve as the physical source of antigen for uptake by those antigen-presenting cells. However, the combination of available antigen, together with either inflammatory or anti-inflammatory cytokines in the tumor environment, would be potent determinants of whether dendritic cells could traffic to lymph nodes and initiate antigen-specific immune responses (15). We show here that an understanding of how macrophages respond to different methods of tumor killing can be used to enhance antitumor vaccination in vivo (Tables 1 and 2). At present, we are investigating the mechanism of macrophage therapy in vivo in response to apoptotic or nonapoptotic tumor cell killing.

In summary, our data support the model in which macrophages act as one of the major cell types that both sense and respond to cell death in vivo (18). Controlled apoptosis is not immunogenic because of active phagocytosis and the immunosuppressive phenotype of the macrophages at the site of cell death. In contrast, when a stressful form of death occurs, or even uncontainable levels of apoptosis, a proinflammatory environment is created. In addition, our data indicate that manipulating the mechanism of cell death and the resultant macrophage response can be used as an effective therapeutic tool for tumor vaccination. Thus, gene transfer or other modalities, such as radiation therapy, may be very useful in manipulating the way that tumor cells die and inducing the appropriate in vivo immune responses to enhance tumor vaccination.

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