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Necrotic Tumor Cell Death In Vivo Impairs Tumor-Specific Immune Responses[1]

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The manner in which cells die is believed to have a major impact on the nature of immune responses to their released Ags. In this study, we present the first direct analysis of tumor-specific immune responses to in vivo occurring tumor cell death through apoptosis or necrosis. Mice bearing thymidine kinase-transfected tumors were treated either with ganciclovir to induce tumor cell apoptosis in vivo or a vascular targeting agent, ZD6126, to induce tumor cell necrosis in vivo. In contrast to tumor apoptosis, induction of necrosis reduced the frequency and impaired the function of tumor-specific CD8$^+$ T cells. Adoptive transfer of lymphocytes from mice with apoptotic tumors into tumor-challenged mice resulted in a significant tumor protection, which was absent when splenocytes were transferred from mice with necrotic tumors. Anti-CD40 treatment reversed impaired Ag-specific CD8$^+$ T cell responses in these mice. These observations have not only fundamental importance for the development of immunotherapy protocols but also help to understand the underlying mechanism of in vivo immune responses to tumor cell death. ***The Journal of Immunology,*** **2007, 178: 1573–1580.**

Cell death is an important step in all differentiation processes, which provide normal cellular turnover such as tissue development, viral and bacterial infections, inflammation and injury. Two different types of cell death are known: apoptosis and necrosis. Apoptosis is considered the primary means by which physiologic cell death occurs (1) in contrast to necrosis, which results from cell damage and is characterized by membrane rupture and release of intracellular contents into the extracellular environment inducing an inflammatory response.

The question of cell death and its role in immunity has been of much interest and the subject of numerous studies (2–5). Apoptotic cell death was defined as programmed cell death in the absence of inflammation, and a number of in vitro studies have suggested that apoptotic cell death is an immunologically innocuous event that fails to activate immune responses (6, 7) unless under certain circumstances (8, 9).

This is in contrast to necrotic cell death, which is normally associated with inflammation and shown to induce maturation of dendritic cells (6, 10) and believed to cause strong immune responses (11, 12). It is believed that only necrotic but not apoptotic cell death leads to an effective immune response due to cellular stress (5, 13, 14). However, recently, it has been shown that induction of apoptotic cell death in vitro results in maturation of dendritic cells leading to T cell activation and immunity (4, 9, 15).

Advances in the field of oncology have brought up new therapeutic options for patients with solid tumors such as antiangiogenesis and vascular targeting agents (16). In preclinical models it has been shown that these agents selectively damage the tumor vasculature inducing necrosis within established tumors. One such agent, ZD6126, has been shown to induce pronounced therapeutic effects. ZD6126 is converted in vivo into *N*-acetylcolchinol, which binds to the colchicine-binding site on tubulin, and causes disruption of microtubules. In a number of animal models, ZD6126 selectively induces tumor vascular damage and massive tumor necrosis at well-tolerated doses (17–20), although it has no direct cytotoxic effect on tumor cells.

To compare the effect of different types of cell death occurring in vivo on the immune system and to exclude any immunosuppressive side effects of cytotoxic chemotherapeutics, we have used thymidine kinase (TK)[4]-transfected tumor cells, which have been shown to undergo apoptosis upon ganciclovir (GCV) treatment (21, 22). Using both GCV treatment of TK-transfected tumors and ZD6126, it was possible for the first time to induce tumor apoptosis and necrosis in vivo respectively and investigate the immunological consequences of different types of tumor cell death. This is important, in particular when immunotherapeutic approaches are combined with conventional tumor treatments including chemotherapy as well as vascular targeting therapy (23, 24).

We show that induction of necrosis in vivo causes a significant impairment of Ag-specific CD8$^+$ T cell-mediated immune responses. These data are contradictory to the current view that necrosis induces an effective immune response and is important, when chemotherapy and cancer immunotherapy are combined.

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[4] Abbreviations used in this paper: TK, thymidine kinase; DLN, draining lymph node; GCV, ganciclovir.

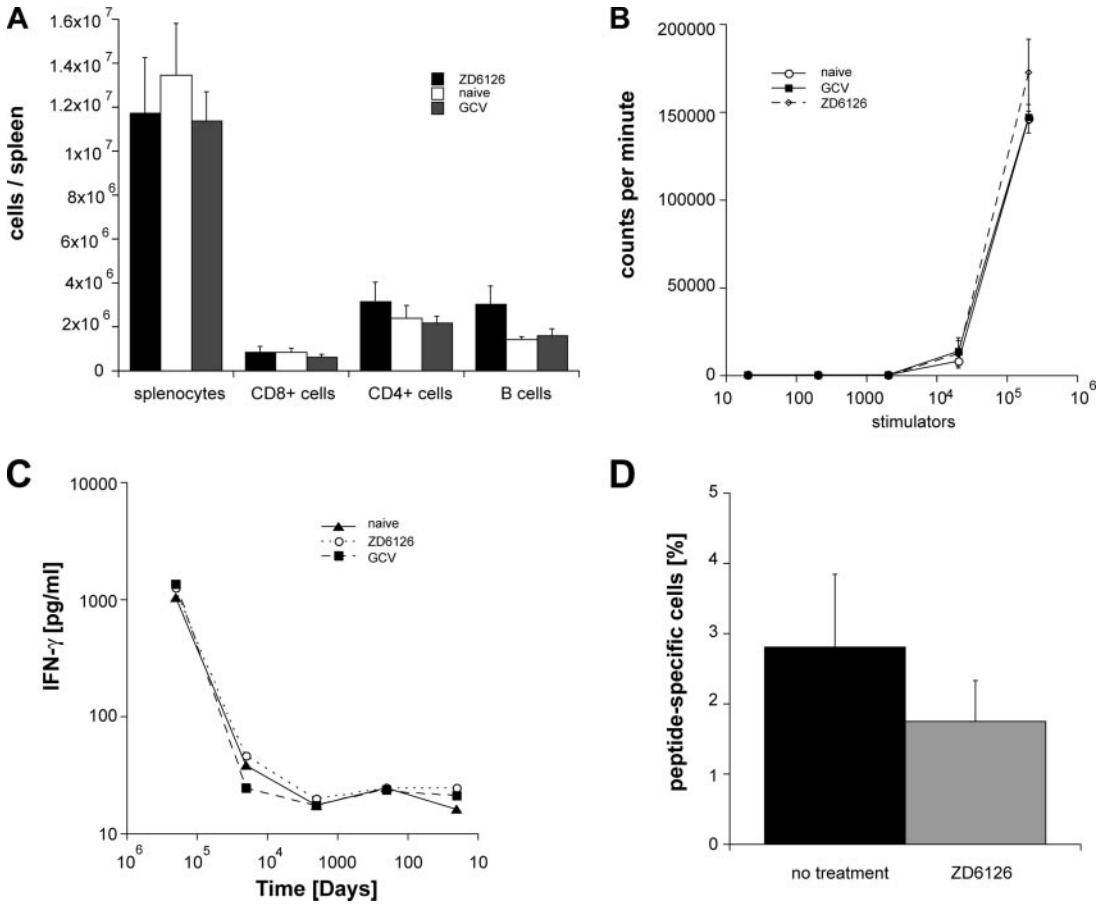

FIGURE 1. Analysis of immune cells in mice treated with GCV and ZD6126. Mice were treated with GCV, ZD6126, or left untreated. *A*, Splenocytes were isolated, and the percentage of $CD4^+$ and $CD8^+$ T cells as well as B cells was analyzed. No differences between the different groups were found. Splenocytes from different mice were isolated after the treatment with ZD6126 or GCV and were nonspecifically stimulated using allogeneic splenocytes in vitro. No differences in cell proliferation (*B*) as well as IFN-γ production (*C*) were observed. *D*, Mice were vaccinated with gamma-irradiated OVA-expressing tumor cells and treated with ZD6126 or PBS. SIINFEKL peptide-specific T cells were detected by intracellular cytokine analysis 3 days after treatment. No differences were observed between ZD6126-treated and control mice.

Materials and Methods

Reagents

GCV for induction of apoptosis was obtained from Hoffmann-La Roche, ZD6126 was provided by AstraZeneca. Cell culture reagents were purchased from Invitrogen Life Technologies.

Mice

Female BALB/c or C57BL/6 mice (6–8 wk old) were obtained from Charles River Laboratories and used according to the institutional guidelines.

Antibodies, fluorescent dyes, and FACS analysis

Abs against mouse CD8α were purchased from BD Pharmingen, CFSE was obtained from Molecular Probes, and propidium iodide was purchased from Sigma-Aldrich. The percentage of H2-Kb/SIINFEKL-specific $CD8^+$ T cells in the spleen and draining lymph nodes (DLN) was determined by flow cytometry using specific tetramers as previously described (25). Flow cytometry was performed using FACScan and data were analyzed with CellQuest software (BD Biosciences).

Generation of OVA and TK-transfected cell lines

B78H1 and CT26 cell lines were retrovirally transduced using HSV-TK-IRES-GFP or OVA-IRES-GFP expressing murine leukemia virus retrovirus according to Klein et al. (26). B78HI-SIIN-K^b cells were transduced using a SIIN-K^b GFP construct, which was generated as previously described (27).

Induction of apoptosis/necrosis in vivo

For induction of apoptosis or necrosis in vivo, GCV and ZD6126 were prepared according to the manufacturer's instructions and injected i.p.

Histone ELISA

Histone ELISA was performed using Cell Death Detection kit (Roche) as previously described (28). Briefly, 10^5 cells were lysed for 30 min. Cell supernatant was mixed with immunoreagent (incubation buffer plus anti-histone-antibody plus anti-DNA-POD) and incubated on streptavidin-coated microtiter plates for 2 h at room temperature. After washing, ABTS solution was added. Absorbance was analyzed at 405 nm reference 490 nm on MR 5000/7000 (Dynatech Laboratories).

DNA laddering

To detect DNA fragmentation, 10^5 tumor cells were harvested and lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, and 20 mM EDTA). DNA was extracted with phenol-chloroform-isoamyl alcohol buffer and precipitated. After washing and treatment with RnaseOne (Promega), samples were loaded on 1.8% agarose gel and analyzed for fragmentation using Gel Doc 2000 (Bio-Rad).

H&E staining, TUNEL, and caspase-3 staining

For H&E staining, apoptotic, necrotic, or live tumors were fixed in 4% paraformaldehyde overnight and in 70% ethanol afterward. Mayers hematoxylin (Merck) and 0.5% eosin (Merck) were used for staining. TUNEL staining was done using TUNEL kit (Roche) according to manufacturer's protocol. Slides were analyzed using 515- to 565-nm filters on Nikon Eclipse TE300 fluorescence microscope (Nikon). Active caspase-3 was stained with rabbit anti-active caspase-3 Ab (Chemicon International) according to the manufacturer's instructions. H&E-, TUNEL-, and caspase-3-stained slides were analyzed in a blinded fashion and were graded for presence/absence of apoptosis/necrosis.

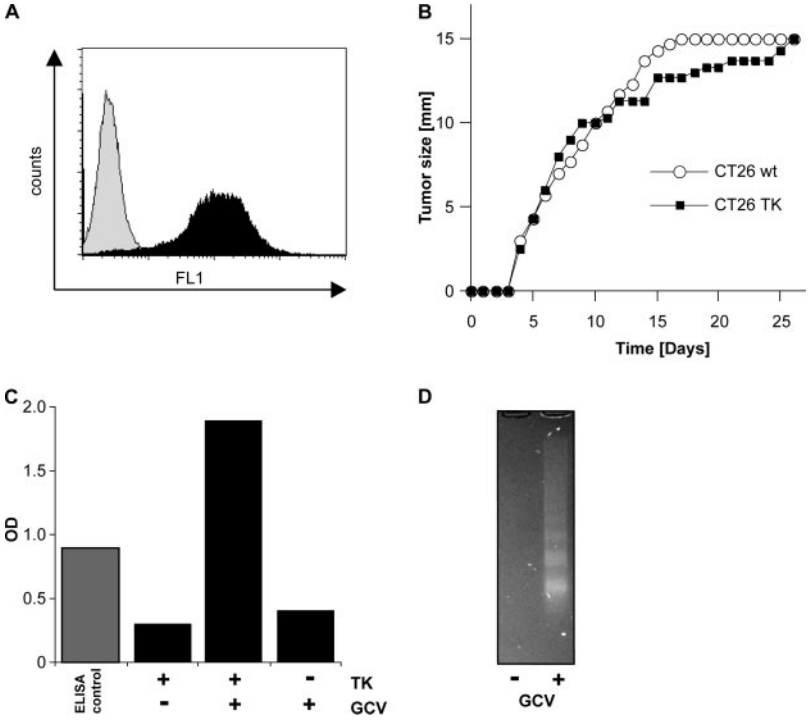

FIGURE 2. TK-transfected cells show regular growth pattern in vivo and die through apoptosis upon GCV treatment in vitro. *A*, Retroviral transduction of CT26 cells with a TK-IRES-GFP construct. *B*, CT26-TK cells (■) showed similar in vivo growth pattern to CT26 wt cells (○). *C*, In vitro treatment with GCV induces apoptotic tumor cell death only in CT26-TK cells but not in nontransfected cells as determined by histone-ELISA. *D*, GCV treatment of CT26-TK cells induces DNA fragmentation.

In vivo CTL assay was performed as described previously (29). Briefly $1–2 \times 10^7$ CFSE-labeled OVA $_{(257–264)}$ (SIINFEKL synthesized by Eurogentec) pulsed and unpulsed target cells were injected in tumor bearing mice i.v. Four hours later they were sacrificed and single-cell suspensions from DLNs as well as spleen were analyzed using FACS. To study the role of CD40, 200 μg of purified FGK 45.5 or isotype control were administered i.v. into necrotic tumor-bearing mice.

T cell transfer experiments

Spleen and draining lymph nodes from apoptotic and necrotic tumor-bearing mice were isolated and transferred into new mice, which were challenged with the tumor. Mice were monitored for tumor-free survival for a minimum of 60 days. $CD8^+$ T cells were purified from tumor-bearing mice using $CD8^+$ T cells isolation kit (Miltenyi Biotec) according to manufacturer's instructions. The purity of the $CD8^+$ T cell-depleted splenocytes was shown to be >95%.

Results

Induction of apoptosis and necrosis in vivo

We chose GCV treatment of TK-transfected tumor cells, because this kind of treatment induced tumor apoptosis without affecting lymphocytes' count (total splenocytes, B cells, $CD4^+$, and $CD8^+$ T cells; Fig. 1*A*) and function (MLR, and peptide-specific immune responses; Fig. 1, *B* and *C*). Although tumor growth of TK-expressing CT26 cells (Fig. 2*A*) was not affected in vivo (Fig. 2*B*), GCV treatment clearly induced apoptosis in these cells in vitro. Incubation of CT26-TK cells in the presence of 10 μM GCV resulted in an increase of histone-complexed DNA fragments as shown by ELISA, which was not observed in GCV-treated wild-type cells or in the absence of GCV (Fig. 2*C*). In addition, clear fragmentation of genomic DNA was only detected in CT26-TK cells after treatment with GCV but not without treatment (Fig. 2*D*).

A new vascular targeting agent, ZD6126, which has no effect on tumor cell growth in vitro (data not shown), was used to induce tumor necrosis in vivo. After systemic effects on T cell function and numbers were excluded upon ZD6126 treatment (Fig. 1), this reagent and GCV were used to analyze their effect on in vivo growing CT26-TK cells. CT26-TK tumors were injected subcutaneously, and GCV or ZD6126 treatment was initiated for 3 days, when palpable tumors reached a size of 5–7 mm. This brief period of treatment resulted in a small but reproducible lag in tumor growth as shown in Fig. 3. In addition, tumor consistency was clearly different between ZD6126 and GCV-treated tumors. Tumors treated with ZD6126 had a soft consistency, which was not observed after GCV administration or without treatment.

Four different methodologies were used to analyze the type of cell death 3 days after beginning of treatment. Untreated tumors

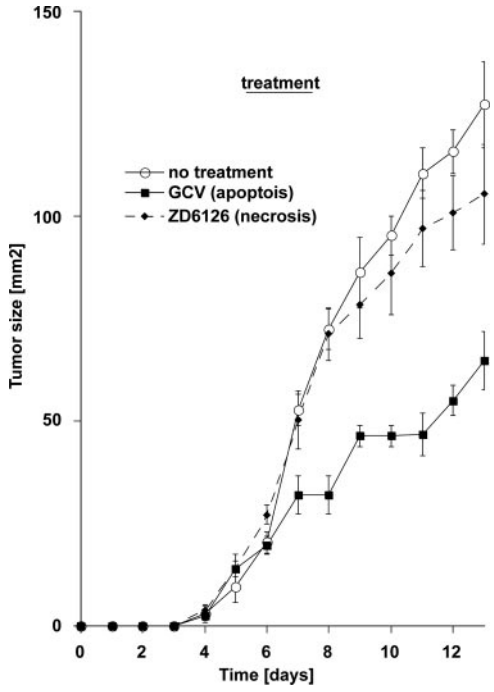

FIGURE 3. Induction of tumor apoptosis and necrosis in vivo. BALB/c mice were challenged with CT26-TK cells. When tumors were clearly palpable (5–7 mm diameter), mice were treated for 3 days with GCV (■), ZD6126 (♦), or were left untreated (○).

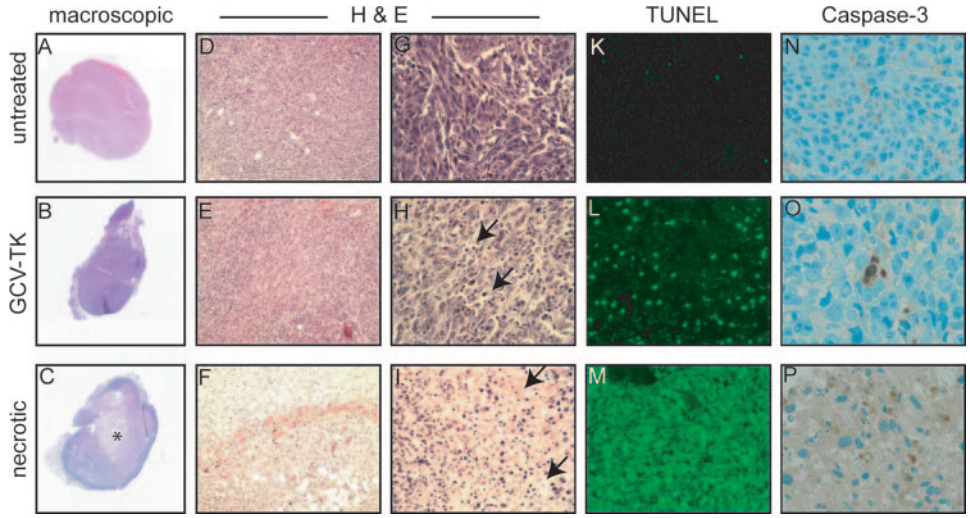

FIGURE 4. Demonstration of tumor cell apoptosis and necrosis in vivo. Macroscopic examination of an untreated tumor (*A*), GCV-treated tumor (*B*), and ZD6126-treated tumor (*C*). The center of the ZD6126-treated tumor (∗) shows a loss of tissue architecture and less intense tissue staining typical for necrosis. Low and high power magnification of H&E-stained sections from untreated tumors (*D* plus *G*), GCV-treated (*E* plus *H*) and ZD6126-treated (*F* plus *I*) tumors. Untreated tumor: The untreated tumor has an overall diffused growth, showing some fascicles and very few fat cells entrapped. No necrosis or obvious apoptotic figures are visible. At high power view, the tumor consists of cohesively growing cells with polygonal or sometimes spindle cell appearance. There is a high nuclear-cytoplasm ratio and the nuclei are hyperchromatic. No prominent necrosis or apoptotic bodies can be detected. GCV-treated tumors: a similar growth pattern can be observed; however, a considerable number of typical apoptotic bodies with condensed cytoplasm and condensed and fragmented nuclei are found (arrow). There is no tissue necrosis or reactive inflammation detectable. ZD6126-treated tumors: Confluent areas of necrosis can be found within the tumor with loss of nuclei and several granulocytes, some of which already show signs of degeneration (arrow). TUNEL staining: Demonstrates the presence of apoptosis indicated by a bright FITC signal only in GCV-treated tumors (*L*), but not in untreated (*K*) or ZD6126-treated tumors (*M*). Caspase-3 staining confirms the presence of tumor cell apoptosis in GCV-treated tumors (*O*) and its absence in untreated tumors (*N*) or ZD6126-treated necrotic tumors (*P*).

served as controls. Confirming our observation of the tumors' consistency, macroscopic examination of the ZD6126-treated tumors showed a central area with a reduced staining of the tissue and patches of destroyed tumor indicative of massive necrosis (Fig. 4*C*), which was not seen in GCV-treated and untreated tumors (Fig. 4, *A* and *B*). Low and high power magnification of H&E staining demonstrated clear apoptosis with typical apoptotic bodies in GCV-treated tumors (Fig. 4, *E* and *H*), confluent areas of necrosis with typical loss of nuclei in the center of ZD6126-treated tumors (Fig. 4, *F* and *I*), and a diffuse tumor cell growth without signs of apoptosis or necrosis in untreated tumors (Fig. 4, *D* and *G*). TUNEL staining confirmed the presence of apoptosis in GCV-treated tumors with bright fluorescent staining indicative for DNA fragmentation (Fig. 4*L*) and absence of apoptosis in untreated tumors (Fig. 4*K*). No specific signal was observed in ZD6126-treated tumors (Fig. 4*M*). Caspase-3 staining was performed to confirm the presence or absence of apoptosis. Again, only in GCV-treated tumors caspase-3 positive cells were detected (Fig. 4*O*). Finally, the degree of tumor cell death through apoptosis and necrosis was analyzed semiquantitatively in a blinded fashion. As summarized in Table I, significant apoptosis was detected in GCV-treated tumors while ZD6126 resulted in substantial tumor necrosis 3 days after treatment.

Table I. *Semiquantitative analysis of tumors for the presence of apoptosis or necrosis*

	H&E			
	Apoptosis	Necrosis	TUNEL	Caspase-3
PBS	0	0	+	+
GCV	+++	0	+++	++
ZD6126	+	+++	+	+

+++, >50%; ++, 25–50%; +, 5–25%; 0, <5%.

In vivo induction of apoptosis protects against subsequent tumor challenge

We next examined the effect of in vivo tumor cell death through apoptosis and necrosis on protection from tumor growth in mice. Mice with s.c. CT26-TK tumors were treated with GCV or ZD6126 to induce tumor apoptosis and necrosis or left untreated. Three days after treatment, when clear apoptosis or necrosis had been observed, splenocytes were isolated and transferred into CT26-TK challenged wild-type mice (Fig. 5*A*). As shown in Fig. 5*B*, 33% of the mice remained tumor free after transfer of lymphocytes from mice with GCV-treated apoptotic tumors up to at least 120 days. No tumor protection was seen after transfer of lymphocytes from mice with necrotic tumors and only 6% of the mice remained tumor free after transfer of splenocytes from mice with untreated tumors.

To analyze which transferred cell type was responsible for tumor protection, splenocytes from mice with GCV-treated tumors were depleted of $CD8^+$ cells and transferred into CT26-TK challenged mice. No protection was seen when CD8 T cell depleted splenocytes were transferred from GCV-treated mice (Fig. 5*C*).

In vivo induction of cell death in OVA expressing MHC class I-negative tumors

To investigate the underlying mechanism of tumor protection seen above in more detail, B78H1, a MHC class I-negative variant of B16 melanoma (30), was retrovirally transduced with TK and the model Ag OVA. Mice were injected with B78H1-OVA-TK tumor cells and treated with GCV and ZD6126 or kept untreated. Three days later, Ag-specific T cell responses were analyzed by tetramer analysis. As shown in Fig. 6*A*, 1.12 ± 0.18% SIINFEKL-specific

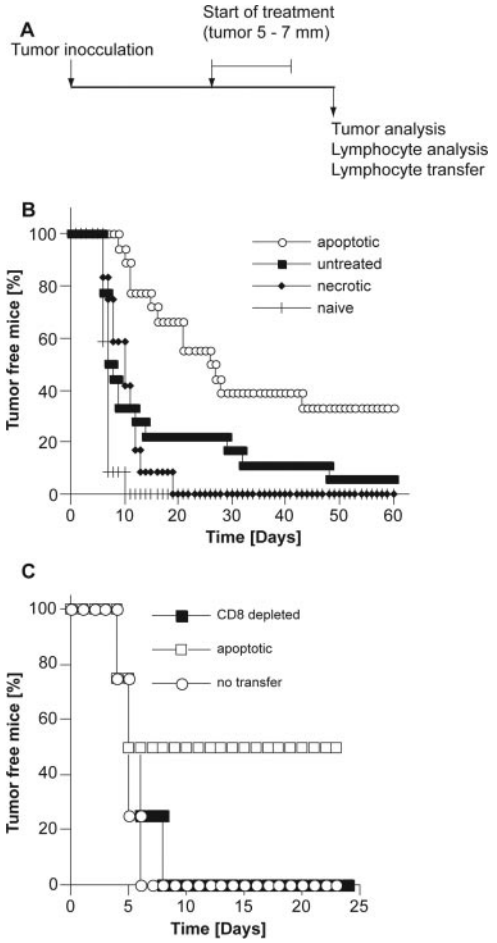

FIGURE 5. Adoptively transferred splenocytes from mice with apoptotic tumors protect from tumor challenge. *A*, The experimental setup for all experiments is shown: Tumors were grown s.c. in mice until they reached a size of 5–7 mm in diameter. Mice were treated for 3 days and tumor-bearing mice were analyzed or splenocytes were transferred into tumor-challenged mice. *B*, CT26-TK grew s.c. until they reached a size of 5–7 mm. No treatment, GCV or ZD6126, was given for 3 days. Splenocytes from tumor bearing mice were isolated and adoptively transferred into CT26-TK-challenged mice. A total of 33% of mice receiving splenocytes from GCV-treated mice (○) remained tumor free in contrast to 6% of mice, which received splenocytes from mice with untreated tumors (■) and 0% mice, which received splenocytes from mice with necrotic tumors (♦). All mice without T cell transfer (cross) developed tumors within 30 days. Combined data from three experiments using a minimum of 18 mice in each group are shown. *C*, Splenocytes from mice with apoptotic tumors were depleted of $CD8^+$ cells (■) and transferred into mice challenged with CT26-TK tumor. Control groups included transfer of lymphocytes from mice with apoptotic tumors (□) or without transfer (○). No tumor protection was observed when splenocytes were depleted of $CD8^+$ T cells. Combined data from two experiments using a minimum of 7 mice in each group are shown.

T cells were detected in spleens from mice with untreated B78HI-OVA-TK tumors. Three days after GCV treatment, mice had a slightly increased number (1.52 ± 0.39%) of SIINFEKL-specific $CD8^+$ T cells in contrast to mice with necrotic tumors, where only 0.66% ± 0.16 specific $CD8^+$ T cells were detected. To analyze the function of T cells in mice with untreated, apoptotic and necrotic tumors, OVA-specific lysis in vivo was measured in spleen and DLN. As shown in Fig. 6*B*, lytic activity was 32 ± 2.7% and 33 ± 3.1% in DLN and 25 ± 1.7% and 24 ± 0.4% in spleens from mice with untreated and apoptotic tumors, respectively, in contrast to 16 ± 0.3% and 8 ± 3.2% lysis in mice with necrotic tumors.

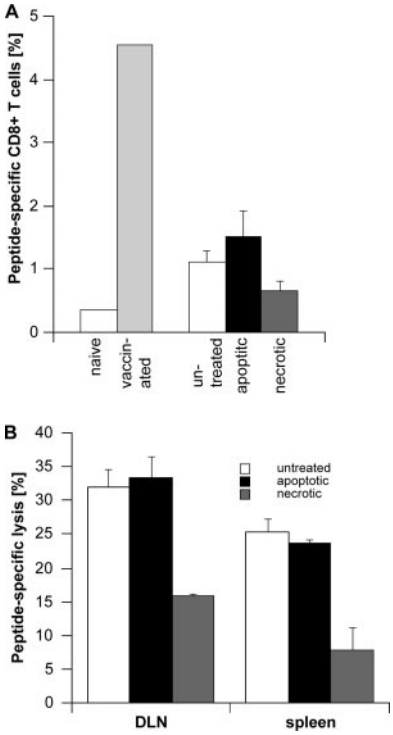

FIGURE 6. Tumor necrosis in vivo reduces the number of tumor-specific $CD8^+$ T cell responses leading to decreased lytic activity in vivo. To analyze Ag-specific $CD8^+$ T cell responses, B78H1-OVA-TK were treated in vivo. Analysis was performed 3 days after induction of apoptosis and necrosis. *A*, SIINFEKL peptide-specific T cells were quantified using H2-Kb/SIINFEKL tetramers. Mice vaccinated with irradiated B78H1-OVA served as positive control; 1.12% SIINFEKL-specific $CD8^+$ T cells were detected in mice with untreated s.c. growing tumors in contrast to 1.52% in mice with apoptotic tumors and 0.66% in mice with necrotic tumors. *B*, In vivo CTL was analyzed in DLN and spleens from mice with untreated, apoptotic, and necrotic tumors; 32, 33, and 16% peptide-specific lysis was observed in DLN from mice with untreated, apoptotic, and necrotic tumors, and 25, 24, and 8% in splenocytes isolated from mice with untreated, apoptotic, and necrotic tumors, respectively. Data shown are representative of three independent experiments.

To further prove the effect of in vivo tumor cell death on $CD8^+$ T cell-dependent tumor protection, B78H1 tumor cells were transduced with a β_2-microglobulin-SIINFEKL-H-$2K^b$ (SIIN-K^b) fusion construct as previously described (27). This construct ensures that tumor recognition by $CD8^+$ T cells in recipient mice depends only on SIINFEKL-specific transferred $CD8^+$ T cells, because all K^b molecules harbor this specific peptide on B78H1-SIIN-K^b cells.

C57BL/6 mice were challenged with B78H1-OVA-TK and treated with either GCV or ZD6126 or remained untreated. Splenocytes from tumor bearing mice were transferred into wild-type mice challenged with B78H1-SIIN-K^b 3 days after start of treatment, when clear apoptosis and necrosis was observed (data not shown). Similar to our observations in BALB/c mice, a significant increase in survival was observed, when mice received splenocytes from apoptotic tumor-bearing animals (Fig. 7). No difference in tumor-free survival was observed after transfer of lymphocytes from mice with necrotic tumors or without any cell transfer.

Anti-CD40 Ab treatment reverses impaired tumor-specific $CD8^+$ T cell responses in mice with necrotic tumors

We next investigated, whether an anti-CD40-activating Ab (FGK-45.5) (31) is able to reverse the impaired tumor-specific immune responses after induction of necrosis in mice with necrotic tumors.

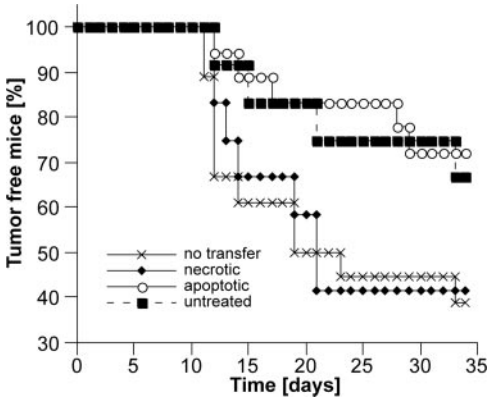

FIGURE 7. Adoptively transferred splenocytes from mice with apoptotic but not with necrotic tumors protect from s.c. injected tumors expressing an MHC class I-restricted tumor Ag. B78H1-OVA-TK grew subcutaneously until they reached a size of 5–7 mm. No treatment, GCV or ZD6126, was given for 3 days. Splenocytes from tumor-bearing mice were isolated and adoptively transferred into mice challenged with B78H1-SIIN-K^b; 42% of mice receiving splenocytes from mice with necrotic tumors remained tumor free (◆) in contrast to 72% of mice, which received splenocytes from mice with apoptotic tumors (○). Control groups included transfer of lymphocytes from mice with untreated tumors (■) or no transfer of cells (stars). Combined data from three experiments using a minimum of 18 mice in each group are shown.

For this, we administered FGK-45.5 or an isotype control Ab in mice with necrotic tumors and determined peptide-specific lysis by in vivo CTL. As shown before, peptide-specific lysis was higher in mice with apoptotic tumors (47 ± 17.0%) than in mice with necrotic tumors (16 ± 2.4%). FGK-45.5 treatment increased lysis in mice with necrotic tumors to 42 ± 3.5%, similar to the amount of lysis seen with apoptotic tumors (Fig. 8).

Discussion

In this study, we provide the first direct comparison of the effect of in vivo tumor cell death through apoptosis vs necrosis on antitumor immune responses. We have investigated tumor-specific immune responses to vascular targeting treatment resulting in tumor necrosis as well as to tumor apoptosis induced by GCV treatment. This experimental approach sheds light not only on the interaction of conventional chemotherapy with immunotherapy, but also answers the more fundamental immunological question as to whether the type of cell death in vivo has an influence on Ag-specific immune responses.

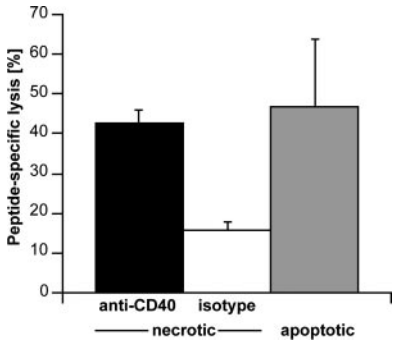

FIGURE 8. Tumor-specific immune responses in mice with necrotic tumors can be enhanced by systemic treatment with an anti-CD40-activating Ab. Tumor-bearing mice were treated with ZD6126 as described above; 200 μg of FGK 45.5 or isotype control was administered i.v. In vivo CTL analysis resulted in 42.5 ± 3.5% lysis in FGK 45.5-treated mice in contrast to 15.62 ± 2.37% of lysis in control mice and 46.9 ± 17.03% in mice with apoptotic tumors. One of two representative experiments with similar results is shown.

Immunological consequences of cell death have been of much interest in the past. It has been a matter of debate whether dying cells induce or suppress immune responses (4, 32, 33). A number of in vitro studies have analyzed the impact of cell death through apoptosis and necrosis on Ag-specific immune responses with different results. Initially, it was suggested that apoptotic cell death as a physiological death, fails to induce a potent immune response unless under certain circumstances (8). It was thought that uptake of apoptotic cells by dendritic cells fails to mature them, resulting in cross-tolerance (3). In contrast, uptake of necrotic cells was shown to mature dendritic cells, which promoted the generation of Ag-specific T cell responses in vitro (6). However, recent data suggest that apoptotic cells can deliver Ags to dendritic cells for cross-presentation leading to a potent immune response (4, 34–36). In addition, apoptotic tumor cell death induced by irradiation enhances MHC class I expression and modulates the peptide repertoire inducing a potent antitumor immune response (37). There has also been accumulating evidence from our laboratory and others that in vitro generated apoptotic cells, when injected into mice, can confer protection upon tumor challenge in vivo (9, 28, 38). So far, due to the lack of sufficient methodologies to induce apoptosis or necrosis in vivo, it has been impossible to investigate and compare the impact of these types of cell death in vivo on tumor-specific immune responses.

We have developed a tumor model, which allowed for the induction of necrosis and apoptosis in vivo, without affecting lymphocyte function. However, it is obvious that in the context of in vivo tumor cell death, the boundaries between apoptosis and necrosis are not so clearly defined. Over time, an increasing number of apoptotic cells can develop to secondary necrosis (39). Therefore, the results obtained depend primarily on the extent of apoptosis and necrosis in the tumors in vivo. We have used four different methodologies to demonstrate and confirm that the majority of the tumor cells were either apoptotic or necrotic in our experimental setting. We have also shown similar results in two different mouse strains using different transplantable tumor models. It is important to note that we performed all assays shortly after initiation of GCV/ZD6126 treatment (day 3) to obtain a more "clean" apoptosis/necrosis and to minimize the effect of secondary necrosis in our experiments. GCV- and ZD61626-treated tumors were also analyzed 7 days after initiation of treatment. At this time, significant secondary necrosis was observed in mice with GCV-treated tumors and tumor-specific T cell responses were impaired in these mice compared with PBS-treated control mice with live tumors supporting our observation that necrotic tumor cell death impairs tumor-specific immune responses (data not shown).

Recently, treatment options for patients with solid tumors have increased through the development of new targeted therapies including vascular targeting agents (40, 41). It has been shown in vivo that these therapies can lead to tumor cell death through necrosis (18, 42) in contrast to conventional chemotherapy, which leads in most cases to apoptosis (43). Although chemotherapy and immunotherapy have initially been thought to be antagonistic, a number of studies have begun to analyze the relation between these types of treatments (43, 44).

Our study indicates that in contrast to apoptosis, necrotic tumor death impairs tumor-specific immune responses. To exclude that tumor-specific T cells are trapped in necrotic tumors or that ZD6126 treatment inhibited the access of immune cells trafficking into the tumor, we have transferred CSFE-labeled SIINFEKL-specific $CD8^+$ T cell from TCR transgenic OT-I mice into mice with

necrotic and apoptotic tumors. Here we saw similar numbers of proliferated OT-I cells in the spleen after 72 h (data not shown) indicating that tumor-specific $CD8^+$ T cells circulate in the periphery of mice with necrotic tumors.

In vivo studies of dying tumor cells are hampered by the fact that it is technically impossible to characterize these cells by flow cytometric analysis due to nonspecific binding of Abs to dead cells as well as difficulties arising from the isolation of RNA samples. Therefore, we have started in vitro experiments to investigate the possible mechanism, how necrotic but not apoptotic tumor cells impair $CD8^+$ T cell responses. Uric acid (45, 46), heat shock proteins (47, 48), pentraxins (49, 50), and phosphatidyl-serine expression on apoptotic cells (51) have been suggested as possible links between abrupt cell death and immune responses generation (4). We have not been able to analyze these factors in our experimental set up, however, we have shown that anti-CD40 treatment reversed the impairment seen in mice with necrotic tumors. The impaired T cell responses observed in mice with necrotic tumors could be due to several factors. Insufficient recruitment of APCs at the tumor site, lack of T cell costimulation, absence of inflammatory signals or inhibitory signals generated by tumor necrosis could all result in reduced T cell responses as we have observed in necrotic tumors.

Our data clearly suggest that in the context of treatment-induced tumor cell death in vivo, necrosis suppresses potent antitumor immune response; however, we have also observed a small but reproducible enhancement of tumor-specific immune responses when mice with untreated and apoptotic tumors were compared. This observation might be unexpected because necrosis has been considered to be a typical danger signal (14) leading to a potent T cell response. However, results from several in vitro as well as in vivo experiments suggest that under different conditions, apoptotic tumor cell death might enhance tumor-specific T cell responses (9, 23, 36, 37). Therefore, we propose that under nonphysiological conditions that occur upon tumor treatment, apoptotic but not necrotic tumor cell death provides the necessary stimuli needed to induce a potent antitumor immune response.

In summary, our study provides clear evidence that in the context of tumor cell death through the induction of apoptosis or necrosis, necrosis abrogates a robust tumor-specific immune response. This observation is not only important for current understanding of cell death-dependent immune responses but will also help in improving the efficiency of combination of conventional chemotherapy with immunotherapy.

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Disclosures

The authors have no financial conflict of interest.

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