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*J Immunol* published online 17 May 2013
http://www.jimmunol.org/content/early/2013/05/17/jimmunol.1203539

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/05/17/jimmunol.1203539_9.DC1

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Profound Impairment of Adaptive Immune Responses by Alkylating Chemotherapy

Adam J. Litterman,* David M. Zellmer,* Karen L. Grinnen,† Matthew A. Hunt,‡ Arkadiusz Z. Dudek,§ Andres M. Salazar,¶ and John O. Ohlfest*†‡

Overall, cancer vaccines have had a record of failure as an adjuvant therapy for malignancies that are treated with alkylating chemotherapy, and the contribution of standard treatment to that failure remains unclear. Vaccines aim to harness the proliferative potential of the immune system by expanding a small number of tumor-specific lymphocytes into a large number of antitumor effectors. Clinical trials are often conducted after treatment with alkylating chemotherapy, given either as standard therapy or for immunomodulatory effect. There is mounting evidence for synergy between chemotherapy and adoptive immunotherapy or vaccination against self-Ags; however, the impact of chemotherapy on lymphocytes primed against tumor neoantigens remains poorly defined. We report that clinically relevant dosages of standard alkylating chemotherapies, such as temozolomide and cyclophosphamide, significantly inhibit the proliferative abilities of lymphocytes in mice. This proliferative impairment was long-lasting and led to quantitative and qualitative defects in B and T cell responses to neoantigen vaccines. High-affinity responder lymphocytes receiving the strongest proliferative signals from vaccines experienced the greatest DNA damage responses, skewing the response toward lower-affinity responders with inferior functional characteristics. Together, these defects lead to inferior efficiency and overall survival in murine tumor models treated by neoantigen vaccines. These results suggest that clinical protocols for cancer vaccines should be designed to avoid exposing responder lymphocytes to alkylating chemotherapy.

The Journal of Immunology, 2013, 190: 000–000.
such as temozolomide (TMZ) and cyclophosphamide, covalently modify DNA and inflict cytotoxic damage on exposed cells (23). These drugs are commonly used for their antineoplastic effect to treat malignancies that are frequent targets of cancer vaccines, such as glioblastoma multiforme (GBM) (24) and metastatic melanoma (25), as well as to deplete Tregs prior to vaccination (16, 26). Although case reports suggest that individual patients have benefited from cancer vaccines given after standard alkylating chemotherapy for GBM (24, 27), overall, cancer vaccines administered after TMZ have had a record of failure (28). Alkylating chemotherapy has immune inhibitory effects in vitro, specifically via selective toxicity to proliferating lymphocytes (29) and inhibition of differentiation of immune effectors (30). The applicability of these studies to human cancer patients remains unclear: the degree of lymphopenia in TMZ-treated glioblastoma patients is a negative prognostic factor (31), but it also has been associated with greater vaccine-induced Ab responses (32). To examine the impact of clinically relevant doses of alkylating chemotherapy on cancer vaccines, we used controlled animal experiments that minimized the numerous complicating factors encountered in human patients.

Materials and Methods

Cells and culture

GL261 and B16-F10 cells were maintained in DMEM supplemented with 10% FBS. The KM3M14 and O94M2 cell lines were derived from genetically engineered primary murine gliomas and were generated and maintained as described (33). Model Ag-expressing tumors (Quad-GL261 and Quad-KM3M14) were generated by stable transfection with Quad Ag cassette (34), a single coding sequence expressing the OT-I and OT-II epitopes of OVA, as well as human gp100 and mouse Ea. CD8 T cells were isolated to >90% purity using a negative selection kit (Miltenyi). T cells were stimulated with IL-2 (R&D Systems) and CD3/CD28 activator beads (Invitrogen), as previously described (35). Viable cell counts were assessed by trypan blue exclusion periodically after stimulation and are expressed proportionally relative to the number of viable cells at the beginning of the assay.

Mice and animal models

Mouse experiments were performed in accordance with University of Minnesota Animal Care and Use Committee guidelines. C57BL/6j (B6) mice, C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice, and B6.PL-Thyl1a/ Cdy (Thy1.1+) mice were purchased from The Jackson Laboratory and used at 6–10 wk of age. NutR7tetramer reporter mice (36) were courtesy of K. Hogquist (University of Minnesota). Gliomas were inoculated as described (34). The cell number inoculated was 15,000 for GL261 and 30,000 for O94M2 and KM3M14. A total of 75,000 B16-F10 cells was inoculated in the right flank. Glioma-bearing mice were euthanized when they became symptomatic: B16-F10-bearing mice were euthanized when tumors became >1000 mm3. For adoptive transfer, 2 × 106 OT-I CD8 T cells were transferred into Thy1.1+ mice by retro-orbital injection and allowed to park for 24 h before drug treatment.

Drug treatments

TMZ, carboplatin, doxorubicin, and cyclophosphamide were obtained from Toronto Research Chemicals. Carboplatin, doxorubicin, and cyclophosphamide were dissolved in PBS and administered by i.p. injection. TMZ was well suspended in PBS immediately before being administered via oral gavage. Gamma radiation was administered as a positive control for DNA strand breaks at a dosage of 15 Gy, 30 min before experiments. Mouse dosages of TMZ and cyclophosphamide were chosen to model relevant human pharmacokinetic exposures and were based on a calculated equivalence using published pharmacokinetic exposure data (37, 38) detailed in Supplemental Table I. Dosages of carboplatin and doxorubicin were selected based on the maximum antineoplastic dosages that were previously reported to have an immunostimulatory effect by a Treg depletion–dependent mechanism (9).

Vaccinations

OVA vaccinations were performed with 100 μg whole chicken OVA protein (Fishier) and 10 μg polyinosinic:polycytidylic acid stabilized with poly-l-lysine (polyICLC; gift of A. Salazar, Oncovir). All peptide vaccines were given with 50 μg peptide and 10 μg polyICLC. Vaccinations were administered as s.c. injections at the base of the left hind leg.

Peptides and in vitro stimulation

SINFEKL and variant peptides (Anaspec) were dissolved in sterile water. All other peptides (New England Peptide) were dissolved in minimal DMSO and diluted in sterile water. Splenocytes were incubated with peptides for 8 h (Nur77SH) induction) or 24 h (elaborated IFN-γ). Elaborated IFN-γ was measured by cytokine bead array (BD) and normalized to the number of Ag-specific T cells enumerated with BD counting beads. Leukocytes from 100 μl blood from B16-F10-bearing animals were incubated with 150,000 Ag-pulsed splenocytes for 72 h for IFN-γ elaboration.

The peptide sequences are as follows: GARC-1: RASAALLNKLYAM- GL; B16-F10 mutant peptides: Kif1bb-mutant: SKPSQFEVDWENVSP- PELNSTDQK, Tub3-mutant: RKKAFHWYGMDEMETFIAEASN, Cps3-mutant: FKHEKAFRTAFNCPGMVTPATG, Tap3-mutant: DR- NPQFLDPVLAYLMGLGCEKPLAS, Plod2-mutant: YNTSHLNDVF- QIFENPVDKKEK (5).

Flow cytometry and ELISA

All Abs, except as indicated, were from eBioscience; phosphorylated ataxia telangiectasia mutated (pATM; phosphor-Ser1981) Ab was from Millipore. An isotype control (PE-conjugated murine IgG1 κ isotype control; eBio- science) for pATM staining was included as a control for background staining in non-TMZ-treated lymphocytes. K5-OVA peptide–MHC multimer staining was performed with dextramer (Immudex). PE-conjugated D6–GARC-1 tetramer was synthesized by the National Institutes of Health tetramer core facility (Atlanta, GA). Tetramer fallow assay was performed as described (17), with the following modifications: K5-OVA dextramer was used to stain, and free dextramer was bound with biotinylated anti-K5-OVA mAb. Data were acquired using a BD FACSCanto II and analyzed using Cytobank software. Relative affinity and Ab titer were determined by ELISA, as described (39).

Results

An intrinsic proliferation defect of lymphocytes exposed to alkylating chemotherapy

To choose clinically relevant doses of TMZ with which to treat mice, we selected doses that yielded an equivalent pharmacokinetic exposure as obtained after oral dosing in humans (40) (J. Gallo, personal communication). Low (25 mg/kg), intermediate (55 mg/kg), and high (80 mg/kg) dosages of TMZ given daily for 5 d led to a transient reduction in lymphocyte counts that were similar in magnitude to that seen in patients treated with TMZ (Supplemental Table I). TMZ significantly inhibited proliferation of splenic T cells (Fig. 1A). This proliferation defect is cell intrinsic, because purified CD8 T cells from TMZ-treated mice demonstrated a similar inhibition of proliferation and activation of the DNA damage response, as assessed by pATM staining (Fig. 1B, 1C).

Quantitative defects in immune responses to cancer vaccines after TMZ treatment

To examine the effects of alkylating chemotherapy on neon- tigen cancer vaccines, we vaccinated mice with the model Ag chicken OVA and polyICLC (18). A cluster of four daily s.c. vaccinations causes a robust CD8 T cell response, with the percentage of K5-OVA–specific CD8 T cells in the blood expanding from essentially undetectable levels to ∼6% of the CD8 compartment in a week (Fig. 2A). This expansion from a precursor frequency ∼1/150,000 (41) to a frequency of 1/20 represents ∼13 doublings. When followed by weekly boosters, similar to many established clinical protocols, a second peak in K5-OVA–specific T cell percentage was observed a week after the third booster (Fig. 2A). The magnitude of the CD8 T cell response was diminished in a dose-de- pendent fashion by TMZ treatment (Fig. 2A). The levels of OVA-specific Ab circulating in the blood of TMZ treated mice were also lower in a dose-dependent fashion (Fig. 2B).
Given that TMZ and other alkylating drugs covalently modify DNA and that some of the directly produced DNA-alkyl adducts (42) or indirectly produced DNA lesions (43, 44) are long-lived, we measured the magnitude of immune responses to vaccines given several weeks after TMZ treatment. B6 mice that were given intermediate-dose TMZ had significantly lower percentages of Kb-OVA–specific T cells elicited by a cluster of four vaccinations that began 80 d after the last dose of TMZ relative to untreated, age-matched controls, with a peak frequency ∼2% versus ∼4.5% (Fig. 2C).

Ag-experienced memory T cells have an intrinsic resistance to DNA-intercalating chemotherapy with daunorubicin (45), and tumor-specific T cell clones may be Ag experienced in cancer patients (46). Therefore, we measured the effect of vaccine-driven T cell expansion in an Ag-experienced memory cell population in mice treated with alkylating chemotherapy prior to vaccination. Mice treated with intermediate- or high-dose TMZ after an initial cluster of vaccines had a significant inhibition of Ag-specific T cell proliferation when vaccinated with a second cluster of vaccines (Fig. 2D). The peak percentage of K\(^\beta\)-OVA–specific CD8 T cells was ∼10-fold lower (∼2% versus ∼20%) in mice treated with intermediate doses of TMZ compared with controls, and the percentage of K\(^\beta\)-OVA–specific CD8 T cells decreased immediately following vaccination in mice treated with a high dose (Fig. 2D).

We next quantified the impact of TMZ on the efficacy of vaccines in tumor-bearing animals using both model Ags and mutated tumor-specific neoantigens. B6 mice were implanted orthotopically with the syngeneic, Ag force–expressing Quad-GL261 glioma line (34). We inoculated B6 mice with the GL261 cell line. This cell line expresses an immunogenic mutant self-protein, GARC-1, and tumor-specific T cell clones may be Ag experienced in cancer patients (46). Therefore, we measured the effect of vaccine-driven T cell expansion in an Ag-experienced memory cell population in mice treated with alkylating chemotherapy prior to vaccination. Mice treated with intermediate- or high-dose TMZ after an initial cluster of vaccines had a significant inhibition of Ag-specific T cell proliferation when vaccinated with a second cluster of vaccines (Fig. 2D). The peak percentage of K\(^\beta\)-OVA–specific CD8 T cells was ∼10-fold lower (∼2% versus ∼20%) in mice treated with intermediate doses of TMZ compared with controls, and the percentage of K\(^\beta\)-OVA–specific CD8 T cells decreased immediately following vaccination in mice treated with a high dose (Fig. 2D).

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Although TMZ increases median survival in glioma patients (48), benefit from treatment is not uniform, and patients with MGMT promoter unmethylated tumors are less likely to benefit (49). To model this clinical situation, we inoculated B6 mice with a Quad Ag–expressing version of the B6 syngeneic KM3M14 glioma cell line (Quad-KM3M14), which is highly resistant to TMZ treatment in vitro (data not shown). Intermediate-dose TMZ treatment abrogated the magnitude of a K\(^\beta\)-OVA–specific CD8 T cell response to a single cluster of four vaccinations with OVA and polyICLC (Fig. 3C). In addition, a spontaneous K\(^\beta\)-OVA–specific CD8 T cell response observed in untreated mice was abrogated in TMZ-treated mice (Fig. 3C). For this TMZ-insensitive, immunogenic cell line, a single course of vaccination is a largely curative therapy (Fig. 3D). This survival benefit is entirely abrogated by TMZ treatment before vaccination, and median survival for TMZ-treated mice was shorter than for nontreated controls (Fig. 3D). Similarly, we found that inhibition of spontaneous immune responses by TMZ treatment could lead to a failure to reject a highly immunogenic B6 glioma line expressing the SV40 Large T Ag (Supplemental Fig. 1).

To examine the impact of TMZ treatment on mutated self-neoantigens, we inoculated mice with the GL261 cell line. This cell line expresses an immunogenic mutant self-protein, GARC-1, which forms a D\(^\beta\)-binding CD8 T cell epitope based on a single amino acid substitution due to a point mutation (50). We vaccinated using a peptide containing the immunogenic amino acid substitution in glioma-bearing mice, with or without TMZ treatment. A significant reduction in the percentage of activated, D\(^\beta\)-GARC-1–specific CD8 T cells in blood elicited by vaccination was observed (Fig. 3E).

DNA damage response induced by high-intensity TCR stimulation following alkylating chemotherapy

Because we observed activation of the DNA damage response in T cells given a strong stimulation through the TCR (Fig. 1), we hypothesized that the degree of DNA damage response would correlate with TCR signal intensity. To dissect this question, OT-I mice were treated with drugs and then their splenocytes were stimulated with altered peptide ligands that induce varying TCR signal strengths (36). All three peptides induced similar levels of proliferation and minimal DNA damage response in OT-I cells from untreated control animals, as assessed by Ki67 and pATM.
staining, respectively (Fig. 4A). In TMZ-treated animals, the frequencies of Ki67+ OT-I cells were inhibited for all peptides; however, interestingly, the percentage of proliferating cells that had activated the DNA damage response (i.e., were pATM+) increased with increasing strength of TCR stimulation (Fig. 4A, 4B).

To assess the induction of DNA damage response in lymphocytes after treatment with other DNA-damaging cancer therapies, we repeated the above experiments after treatment of OT-I mice with cyclophosphamide, carboplatin, doxorubicin, and gamma radiation. Following treatment with the alkylating chemotherapy cyclophosphamide and gamma radiation, there were significantly more proliferating cells exhibiting DNA damage in response to the strong antigenic peptides SIIQFEKL and SIINFEKL than with the weak antigenic peptide SIIGFEKL or with no peptide (Fig. 4C). The platinum drug carboplatin and the DNA-intercalating agent doxorubicin did not lead to this effect, with similar pATM staining in proliferating cells stimulated with all peptides (Fig. 4D). The induction of the DNA damage response upon stimulation after drug treatment was mirrored by the defect in CD8 T cell responses to vaccines in B6 mice. TMZ and cyclophosphamide led to markedly and significantly lower peak levels of Ag-specific CD8 T cells in the blood following vaccination (Fig. 4E). In contrast, in animals treated with carboplatin and doxorubicin, the percentage of Ag-specific CD8 T cells was incrementally lower and not significantly different from untreated controls. This defect is likely accounted for by responder lymphocytes failing to enter the cell cycle (as in Fig. 4A), as well as undergoing apoptosis as a result of DNA damage response, because a fraction of adoptively transferred OT-I T cells became apoptotic ( Annexin V+7-AAD+) after vaccination with SIINFEKL peptide in TMZ-treated mice (Supplemental Fig. 2).

Lower affinity for Ag of vaccine responder lymphocytes after TMZ treatment

As a result of the greater DNA damage response that we observed in OT-I T cells stimulated with stronger antigenic peptides, we hypothesized that in vivo vaccine responder cells would be skewed toward lymphocytes with AgRs with lower affinity for cognate Ag. We found that the median fluorescent intensity of peptide–MHC multimer staining of memory CD8 T cells elicited by vaccination was lower in TMZ-treated mice than in controls (Fig. 5A, 5B). The higher initial rate of decay of staining in a multimer falloff assay (17) in TMZ-treated animals also suggested a lower avidity of vaccine-elicited CD8 T cells for Ag (Fig. 5C). Similarly, we measured a lower relative affinity of anti-OV A serum Ig in TMZ-treated animals than in controls (Fig. 5D).

Inferior functional characteristics of vaccine-responder lymphocytes after TMZ treatment

The lower affinity of responder lymphocytes for Ag after alkylating chemotherapy suggests that, post-TMZ, vaccine-responder cells

FIGURE 2. TMZ exposure leads to a dose-dependent inhibition of adaptive-immune responses to vaccination. (A) C57BL/6 mice (n = 7–8/group) were treated with the indicated dosages of TMZ for 5 d; 2 d after the last dose, mice were vaccinated daily for 4 d with OVA and polyICLC, followed by weekly booster vaccinations, as indicated. Kb-OVA–specific CD8 T cells in the blood were assessed by flow cytometry. Error bars indicate SEM. One representative experiment is shown; this experiment was performed independently three times with similar results. (B) The mice in (A) were terminally bled 70 d after the first vaccination, and anti-OVA IgG/IgM was quantified by ELISA. Error bars indicate SEM. (C) C57BL6 mice (n = 9/group) were treated with TMZ at the indicated dosages for 5 d; 80 d after the last dose of TMZ, mice were vaccinated daily for 4 d, with Ag-specific CD8 T cells in blood assessed as above. Error bars indicate SEM. The experiment shown is representative of two independent experiments with similar results. (D) C57BL/6 mice (n = 5/group) were vaccinated daily for 4 d, and immunological memory was allowed to establish for 1 mo, at which point TMZ was administered daily for 5 d at the indicated dosages. The mice were again vaccinated daily for 4 d and given booster vaccinations weekly, as indicated, and Ag-specific CD8 T cells in blood were assessed by flow cytometry throughout the experiment. Error bars indicate SEM. Data are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
are less sensitive toward antigenic targets. We hypothesized that these cells would receive lower-intensity proliferative signals in TMZ-treated mice than controls and display inferior effector function upon stimulation. Using the Nur77GFP TCR signal strength reporter mouse (36), we directly tested this hypothesis by measuring GFP fluorescence intensity in vaccine-expanded CD8 T cells upon antigenic stimulation. Vaccine-expanded Kb-OVA–specific CD8 T cells from untreated control mice displayed high-intensity GFP fluorescence upon culture with SIINFEKL peptide (Fig. 6A, 6B). In TMZ-pretreated mice, Ag-specific GFP fluorescence upon stimulation was significantly lower (Fig. 6B). We next measured IFN-γ elaboration upon antigenic stimulation in culture and enumerated Ag-specific cells/well. We calculated that, in TMZ-pretreated animals, vaccine-activated CD8 T cells elaborated 10-fold fewer IFN-γ molecules on a per-cell basis (Fig. 6C).

**Discussion**

We found that alkylating chemotherapy has a long-lasting anti-proliferative effect on lymphocytes in mice, and this effect leads to inferior responses to cancer vaccines targeting mutated self-Ags. Animals pretreated with alkylating chemotherapeutic drugs had lower peak numbers of vaccine-responding CD8 T cells and lower Ab titers. This impairment corresponds to the activation of DNA damage responses in proliferating cells, and this activation of DNA damage responses is greatest in responder cells receiving the strongest TCR signals from the vaccine. In turn, this selective toxicity in the cells with the highest affinity for cognate Ag leads to impairment of CD8 T cell and Ab responses. These responses consist of lymphocytes with, on average, lower-affinity AgRs that have inferior effector function. Importantly, these effects were observed even at low, Treg-depleting doses of alkylating chemotherapeutics (9).
The defects that we observed are likely general to all populations of rapidly proliferating immune responder cells expanded by vaccination (e.g., CD4 T cells, B cells). Activated lymphocytes implement a metabolic and antiapoptotic program that allows for sustained synthesis of macromolecules and cell division (51), dividing up to twice a day during the peak of adaptive-immune responses (17). Alkylating chemotherapy covalently modifies DNA with methyl adducts for methylating drugs, like TMZ or dacarbazine (43), or inter- and intrastrand alkyl cross-links for nitrogen mustard derivatives, like cyclophosphamide (52). These lesions cause stalling of replication forks and dsDNA breaks in proliferating cells (53). This DNA damage is detected by proteins, such as ATM, which binds to dsDNA breaks and autophosphorylates, in turn activating numerous downstream effectors involved in cell cycle arrest and apoptosis, such as Chk2 kinase (54) and p53 (55). Therefore, proliferation-driven toxicity in vaccine-responder cells is a side effect of alkylating chemotherapy that must be balanced against its reported immunomodulatory effects. In the case of neoantigen vaccines for which Treg depletion is not required for efficacy, our data suggest that the negative antiproliferative effect of chemotherapy is dominant over the immunomodulatory effect.

We observed that the immune-inhibitory effect of alkylating chemotherapy was long-lived, with significant defects in CD8 T cell priming persisting for 10 wk after cessation of TMZ treatment (Fig. 2C). The persistence of this effect could be due to the fact that DNA repair is induced by proliferative signals (56), so quiescent naive lymphocytes may not fully repair DNA damage. This damage is then "activated" by replication fork read through during DNA synthesis in response to proliferative signals, like vaccines.

Numerous studies examined the effect of alkylating chemotherapy on immunotherapeutic modalities. The predominant finding reported is depletion of Tregs and induction of lymphopenia (11, 57), although high doses have been associated with peripheral Treg expansion in rodents (58) and humans (32). Several studies...
that reported an immunostimulatory effect of alkylating chemotherapy as a result of Treg depletion were conducted using transferred cells not exposed to drug (59–61). The clinical application of this strategy is complicated by the difficulty in generating large numbers of tumor-specific lymphocytes ex vivo for human patients (62), and cancer vaccines are typically administered after standard chemotherapies (7). Conversely, other studies of endogenous antitumor immune responses following Treg depletion focused on self-Ags for which breaking tolerance is required (9, 10, 16, 63) or did not directly compare immune responses in exposed and non-exposed lymphocytes (58, 64).

We focused on tumor-specific neoantigens derived from mutated self-proteins, as well as exogenous model Ags, both of which are inherently immunogenic (i.e., can readily be targeted by vaccination without additional therapy to break tolerance). Such neoantigens are technically challenging to predict from patient tumor samples, but they have been retrospectively identified in several studies in clinically responding patients using tumor cell lines and patient lymphocytes (3). Similarly, clinical experience from vaccination with idiotypic Ig for lymphoma suggests that nongermline-encoded epitopes from hypervariable regions are more immunogenic and stimulate CD4 and CD8 cells preferentially over framework regions (65). Because of their generation de novo in neoplastic cells, such mutant antigenic targets are less likely to cause autoimmune side effects and are not subjected to central tolerance that can cause negative selection of high-avidity T cells (66). Recent advances in bioinformatics have made prospective identification of immunogenic mutations possible and development of this technology to generate personalized cancer vaccines is an active area of research (5). However, the experience of adoptive immunotherapy suggests that the proliferative potential of effector cells is a critical variable (19). For personalized cancer vaccines targeting tumor-specific mutations to be successful, they should be administered in a protocol designed to maximize the quality and proliferative ability of responder lymphocytes.

We demonstrated that the generation of T cell responses against mutated self-proteins by cancer vaccines was inhibited by TMZ in a mouse model of glioma (Fig. 3E), as well as by cyclophosphamide in a mouse model of melanoma (Fig. 7A). In addition, using the model Ag OVA, we found that T cell clones that expanded after alkylating chemotherapy had lower affinity for cognate Ag, as well as lower TCR signal strength and inferior effector function upon antigenic stimulation (Figs. 5, 6). These differences seem sufficient to account for the loss of survival benefit from vaccination that we observed in both TMZ- and cyclophosphamide-treated mice (Figs. 3B, 3D, 7B).
In conclusion, we found that vaccine-driven and spontaneous adaptive antitumor immune responses were inhibited by the direct antiproliferative effect of alkylating chemotherapy. These findings are particularly noteworthy, because alkylating chemotherapy is a standard treatment for several malignancies that have been the target of vaccine immunotherapy, including TMZ for GBM (48) and dacarbazine for metastatic melanoma (25). These findings suggest that easily implemented modifications in conventional clinical protocols for cancer vaccine trials, such as banking unexposed PBMCs prior to chemotherapy for use in later immunotherapy, could yield improved results. It was reported, for instance, that 500 ml of blood contains sufficient numbers of naive precursor CD8 T cells to allow large numbers of T cells specific to multiple tumor and viral Ags to be expanded in vitro (67). Thus, easily extracted quantities of lymphocytes could be frozen and stored, either as source material for the in vitro expansion of antitumor T cells or as a banked pool of nondrug-exposed naive T cells to be infused prior to vaccination.

Furthermore, future trials of immune therapy could use such prognostic markers to stratify patients based on their relative likelihood to benefit from conventional alkylating chemotherapy versus cancer vaccines, as well as prioritize immune therapy over chemotherapy in those most likely to benefit. MGMT promoter methylation status in glioblastoma is prognostic of response to TMZ and is widely measured clinically (49), whereas tumors with the mesenchymal gene-expression pattern have a poor survival prognosis but appear to be more sensitive to active immune therapy than do glioblastomas with other gene-expression patterns.

**FIGURE 6.** CD8 T cells expanded by vaccines following TMZ have inferior functional characteristics. (A) Nur77GFP TCR signal strength reporter mice (n = 4/group) were treated with TMZ, as indicated, and vaccinated daily for 4 d with OVA and polyICLC. Seven days later, splenocytes were plated with the indicated amount of SIINFEKL peptide for 8 h. Ag-specific T cells were identified by staining for Kb-OVA and CD8, and GFP intensity of Ag-specific cells was assessed by flow cytometry. (B) Aggregate data for experiments performed as described in (A). Error bars indicate SEM. Data shown are pooled from two independent experiments with similar results. (C) C57BL/6 mice (n = 3–4/group) were treated with TMZ, as indicated, and vaccinated daily for 4 d with OVA and polyICLC. Seven days later, mice splenocytes were plated for 24 h with the indicated amount of SIINFEKL peptide. Elaborated IFN-γ was measured by cytokine bead array and normalized to Ag-specific T cells, as indicated in Materials and Methods. Error bars indicate SEM. Data shown are pooled data from two independent experiments with similar results. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 7.** Cyclophosphamide pretreatment is associated with less survival benefit from melanoma vaccines. (A) C57BL/6 mice (n = 9/group) were implanted s.c. with 7.5 × 10^5 B16-F10 melanoma cells. Mice were given cyclophosphamide, as indicated, on day 2 after tumor inoculation and vaccinated with B16-F10 cell line mutant peptides and polyICLC on days 3–6 and 17–20 after tumor inoculation. On day 10 after tumor inoculation, leukocytes from 100 μl blood were incubated with splenocyte APCs pulsed either with irrelevant (OVA-derived) peptides or B16-F10 mutant peptide mixture. Elaborated IFN-γ was measured by cytokine bead array for all nine mice/group for vaccinated groups and four mice/group for nonvaccinated groups. Error bars indicate SEM. *p < 0.05. (B) Survival of mice in (A). Mice were sacrificed when tumors reached >1000 mm^3 in volume; survival is depicted by Kaplan–Meier plot. *p < 0.05, log-rank test. ns, Not significant (p > 0.05).
(24). Altering clinical protocols and basing patient treatment on known prognostic indicators of treatment response could minimize harm of conventional therapies to cancer vaccines and maximize efficacy, leading to improved outcomes for patients treated with these vaccines.

Acknowledgments

We thank D.A. Largaespada, C.A. Pennell, T.S. Griffith, and B.M. Andersen for editorial assistance and critical comments.

Disclosures

The authors have no financial conflicts of interests.

References


**Figure S1. Spontaneous anti-tumor immune responses are inhibited by temozolomide treatment.** (A) C57/BL6 mice (n=5 per group) were implanted with 30,000 cells of the immunogenic syngeneic glioma cell line O94M2 which express the SV40 Large T antigen. Mice were treated as indicated with temozolomide on days 3-7 after tumor implantation and tumor growth was tracked with bioluminescent imaging. (B) Survival of mice in (A) is indicated with Kaplan Meier plot. P values shown are for log rank test. **, p<0.01.

**Figure S2. Adoptively transferred OT-I undergo apoptosis following temozolomide exposure and vaccination.** (A) 2x10^6 Thy1.2+ OT-I CD8 T cells were transferred into Thy1.1+ host mice (n=3 per group). Mice were given the indicated treatment and then vaccinated in the left leg with SIINFEKL and polyICLC. 24 hrs later both the vaccinated (left) and opposite (right) inguinal lymph nodes were stained for Thy1.2, CD8, Annexin V and viability (7-AAD). Representative flow plots gated on CD8+Thy1.2+ (OT-I) or CD8+Thy1.2- (bulk CD8) are shown.
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<td>21 days / cycle</td>
<td>2100 mg/m²</td>
<td>n/a</td>
<td>10-20%</td>
<td>Sampson et al., 2011</td>
</tr>
<tr>
<td>TMZ</td>
<td>Mouse / High</td>
<td>5 days</td>
<td>3000 mg/m² equiv.</td>
<td>80 mg/kg</td>
<td>10-15%</td>
<td>This study</td>
</tr>
<tr>
<td>TMZ</td>
<td>Mouse / Medium</td>
<td>5 days</td>
<td>2100 mg/m² equiv.</td>
<td>55 mg/kg</td>
<td>10-50%</td>
<td>This study</td>
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<td>TMZ</td>
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<td>1000 mg/m² equiv.</td>
<td>25 mg/kg</td>
<td>55-65%</td>
<td>This study</td>
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<tr>
<td>Cy</td>
<td>Human / i.v.</td>
<td>Once</td>
<td>300 mg/m²</td>
<td>n/a</td>
<td>n/a</td>
<td>Dudek et al., 2008</td>
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<tr>
<td>Cy</td>
<td>Mouse / i.p.</td>
<td>Once</td>
<td>600 mg/m² equiv.</td>
<td>150 mg/kg</td>
<td>n/a</td>
<td>Genka et al., 1990 Struck et al., 1987</td>
</tr>
</tbody>
</table>
Figure S1.

A

Biomembrane signal (photons/s/cm²)

Days after tumor inoculation

Limit of detection

B

Percent of mice surviving

Days after tumor inoculation
Figure S2.

<table>
<thead>
<tr>
<th>0 mg/kg TMZ</th>
<th>55 mg/kg TMZ</th>
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<td>OT-I</td>
<td>OT-I</td>
</tr>
<tr>
<td>bulk CD8</td>
<td>bulk CD8</td>
</tr>
</tbody>
</table>

Vaccinated LN

Opposite LN

7-AAD

Annexin V