

Alkylating chemotherapy may exert a uniquely deleterious effect upon neo-antigen-targeting anticancer vaccination

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Alkylating chemotherapy exerts both antineoplastic and immunostimulatory effects. However, in addition to depleting regulatory T cells (Treg), alkylating agents also mediate a long lasting antiproliferative effect on responder lymphocytes. Our recent findings indicate that this antiproliferative effect profoundly impairs vaccination-induced immune responses, especially in the case of vaccines that target specific tumor-associated neo-antigens that do not require Treg depletion.

Alkylating Chemotherapy and Immunotherapy

Following the hypothesis that durable responses to conventional treatments in advanced cancer patients may require endogenous immune responses, much interest has been generated by the prospect of combining chemotherapeutic regimens with immunotherapy.¹ Alkylating drugs as well as other cytotoxic chemotherapeutic agents have been reported to mediate immunostimulatory effects by several mechanisms. These include the liberation of inflammatory and homeostatic cytokines in the course of treatment,² the immunostimulatory effect of the death of tumor cells as caused by specific chemotherapeutics,³ as well as the ability of chemotherapy to directly sensitize malignant cells to the cytolytic activity of immune effectors.⁴ Thus, much interest has been attracted by the possibility of combining conventional chemotherapy with active

immunotherapeutic strategies such as anti-cancer vaccines, to try to derive synergy between two complimentary approaches.⁵

This has particularly been the case for a widely used class of chemotherapeutics, namely, alkylating agents. Alkylating chemotherapy involves a class of DNA-damaging compounds that covalently modify DNA by either methylating individual bases or generating inter-strand or intra-strand alkyl crosslinks.^{6,7} These agents include some of the oldest antineoplastic drugs known, such as nitrogen mustards, as well as many compounds that are still commonly employed in the clinic, such as cyclophosphamide, dacarbazine, and temozolomide.⁷ While these drugs act non-specifically and alkylate many chemical species within the cell, their antineoplastic effect is mainly mediated by the accumulation of DNA lesions, particularly in cells that proliferate rapidly, such as lymphocytes or malignant cells.^{8,9} Given their routine use for the treatment of a number of neoplasms, alkylating agents have been used in many clinical protocols of experimental immunotherapy.¹⁰⁻¹² Moreover, investigations into the utility of these drugs as conditioning regimens before the adoptive transfer of immune cells¹³ as well into their immunomodulatory effects¹⁴ have led to considerable interest in combining immunotherapy with alkylating agents.

Previous work has demonstrated that in addition to mediating antineoplastic effects, alkylating agents deplete specific populations of immune cells. In particular,

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Abbreviations: ATM, ataxia telangiectasia mutated; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor; Treg, regulatory T cell

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regulatory T cells (Tregs) seem particularly susceptible to the cytotoxic effects of alkylating chemotherapy,¹⁵ presumably due to the fact that these cells proliferate in response to tolerogenic stimuli in the steady-state.¹⁶ These observations have generated interest in the use of alkylating agents as a conditioning regimen prior to anticancer vaccination. While it has been shown that low-dose alkylating chemotherapy can enhance the immune response to vaccines targeting self antigens, which are normally suppressed by Tregs, it is unclear how generalizable this finding is.^{12,17} Indeed, given the profound proliferative burst that occurs at the initiation of adaptive immune responses,¹⁸ the notion that an antiproliferative drug would exert immunostimulatory effects is counterintuitive. Indeed, we have recently demonstrated that the antiproliferative effects of alkylating chemotherapy exerts an immunosuppressive effect on vaccination by acting in a cell-intrinsic manner in responder lymphocytes.¹⁹ This effect is particularly important in the case of neo-antigens that are derived from mutated self proteins, as these are generally immunogenic even in hosts that harbor normal amounts of Tregs. Here, we summarize the key points of our discovery and put forth a theoretical framework to explain how the quality of the T cells that respond to a given antigen might affect the outcome of anticancer vaccination preceded by alkylating chemotherapy. We conclude by discussing the implications of these data for clinical research, in particular for immunotherapeutic approaches that target patient-specific neo-antigens or based on material from autologous tumors harboring large numbers of mutations, such as melanomas or lung carcinomas.

Treg Depletion Can Be Beneficial for the Immunization Against Self Antigens

The immunological rationale for combining alkylating chemotherapy with vaccination relies upon the effects of alkylating agents on Treg populations. Such effects have been reported for both conventional and metronomic dosing schedules in rodents, and have also been observed in patients.^{12,15,17,20} While at high

doses alkylating agents often cause a generalized leukopenia that is associated with an increased susceptibility to opportunistic infections²¹ (for instance, due to profound neutropenia), at commonly used clinical doses these drugs have a relatively benign safety profile and are rarely associated with lymphopenia as a dose-limiting toxicity.²² We and others have observed that Tregs are semi-selectively depleted upon the administration of alkylating chemotherapy, decreasing both in absolute number and in relative proportion to other lymphocytes.^{17,19} We speculate that this might reflect the fact that Treg are more likely to actively proliferate in steady-state conditions than naïve T cells. Recent work examining the effect of tolerogenic antigen-presenting cells on Tregs in the steady-state appears to support this idea. In the skin of healthy individuals, for instance, the majority of cycling T cells are Tregs, which proliferate in an antigen-specific manner upon interaction with local antigen-presenting cells, presumably in response to self antigens.¹⁶

The results of experiments involving murine tumors that overexpress tolerized self antigens may explain why the depletion of Tregs by alkylating chemotherapy may have a beneficial effect in this context. Using transgenic mice overexpressing human *v-erb-b2* avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2, best known as HER2) as hosts for a tumor driven by human HER2, Jaffee and colleagues found that diverse chemotherapeutics can synergize with a vaccination, if administered before.¹⁴ This effect appeared to be mediated by an enhanced priming of tumoricidal T cells. Subsequent work demonstrated that such an enhanced priming resulted from the depletion of Tregs, allowing for the recruitment into the immune response of self-specific T cells that were not activated in the presence of Tregs.¹⁷ Interestingly, the beneficial effect of chemotherapy-driven Treg depletion appears to be specific for self antigens, as only HER2-transgenic mice benefited from the administration of chemotherapy before vaccination, whereas wild-type mice were able to generate anti-HER2 CD8⁺ T-cell responses in the presence of normal amounts of Tregs.¹⁷ This

finding is in line not only with the fact that immune responses to pathogens routinely occur in Treg-replete hosts, but also with the lymphoproliferative and autoimmune phenotype of individuals that harbor an impaired Treg compartment.^{23,24} It has also been posited that Tregs can act as a sink for immunostimulatory cytokines that are produced in response to the antigenic stimulation of T cells, such as interleukin-2 (IL-2). By sequestering the small amounts of IL-2 generated in response to weak self antigens, Tregs may therefore raise the threshold for mounting a proficient T-cell response, hence delineating a sharp, all-or-none boundary between typically ineffective, weak (self) antigens and inherently immunogenic, strong (non-self) antigens.²⁴ Interestingly, a recent clinical trial seems to provide experimental evidence in man supporting the hypothesis that the administration of alkylating chemotherapy can promote immune responses from a normally non-reactive, latent population of T cells specific for self antigens. In an early phase clinical trial testing a vaccine that consisted in a pool of highly-expressed HLA-A2-restricted tumor-associated self peptides, Walter and colleagues reported enhanced immune responses and increased overall survival as a result of the administration of low-dose cyclophosphamide prior to vaccination.¹²

Alkylating Chemotherapy has an Antiproliferative Effect on Responder Lymphocytes

In addition to exerting Treg-depleting effects, alkylating agents affect all exposed host cells, including any potentially tumor-reactive lymphocytes that would be expanded upon vaccination. For antigens that are inherently immunogenic, the influence of Treg depletion on the efficacy of vaccination is limited. Therefore, we sought to understand the responder cell-intrinsic effect of alkylating chemotherapy on immune responses raised against this class of non-self antigens.¹⁷ We did indeed identify a surprisingly prolonged antiproliferative effect of alkylating chemotherapy on responder lymphocytes, with both the magnitude and the quality of immune responses

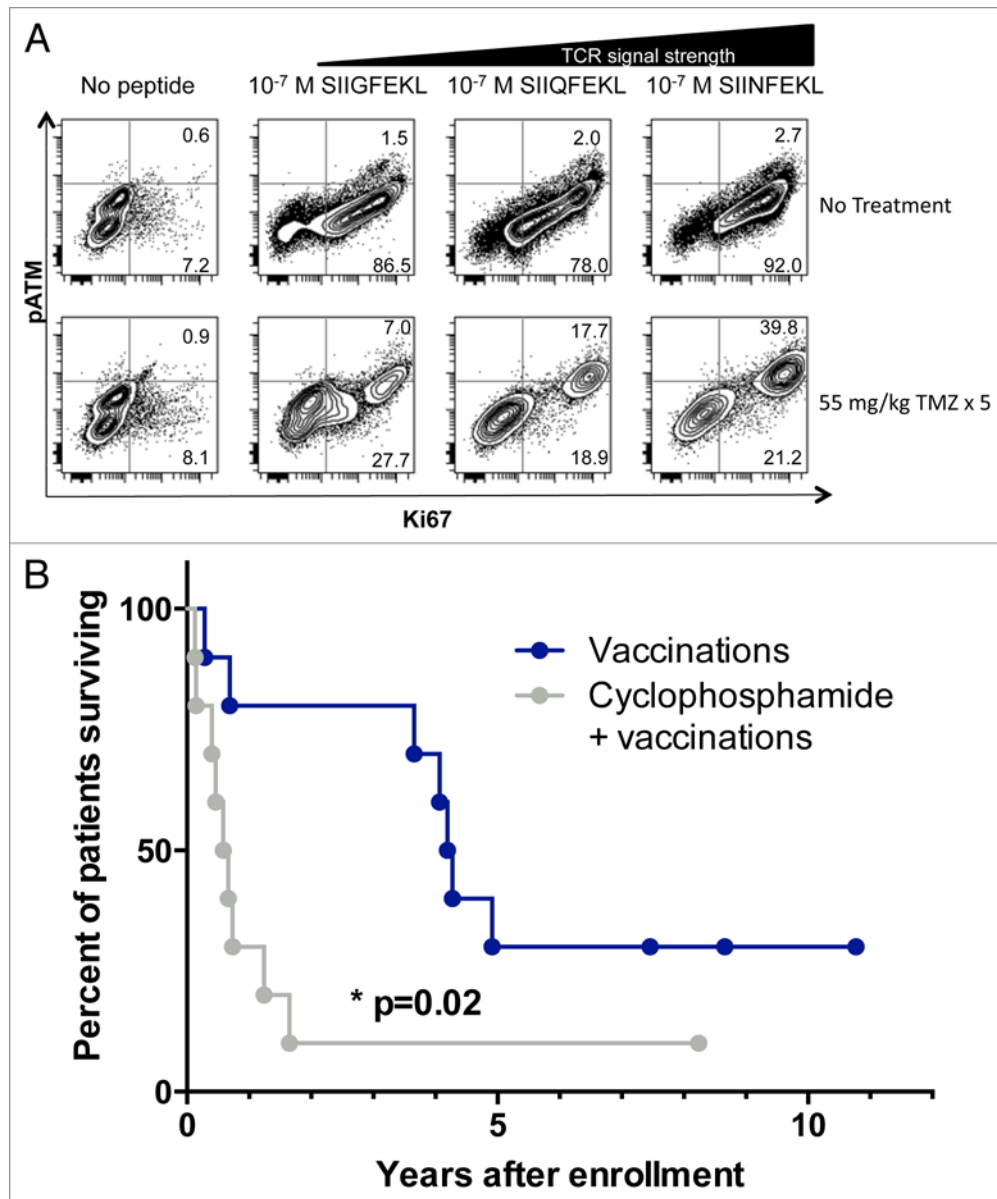


Figure 1. Effects of alkylating chemotherapy on vaccine-induced immune responses. **(A)** Robust TCR signaling leads to DNA damage in lymphocytes previously exposed to alkylating chemotherapy. OT-I mice were treated with temozolomide or left untreated, and their splenocytes were stimulated with the indicated variants of the chicken ovalbumin-derived peptide SIINFEKL. Strongly immunogenic peptides (such as SIINFEKL, SIIQFEKL) led to a considerable accumulation of DNA double strand breaks (measured with an antibody against phosphorylated ATM) in proliferating (Ki67⁺) cells as compared with no antigenic stimulation or weak peptides (such as SIIGFEKL). **(B)** Compromised overall survival of metastatic melanoma patients upon the administration of an autologous vaccine alone or upon pre-treatment with cyclophosphamide. Patients enrolled in a clinical trial testing a large autologous multivalent vaccine were either treated with 300 mg/m² cyclophosphamide or left untreated, and subjected to vaccination one week later. Overall survival is depicted (n = 10 patients/group).

against neo-antigens being impaired for at least 10 weeks after the administration of temozolomide.¹⁹ The peak of both B- and T-cell responses had a reduced magnitude, and both the antibodies and T-cell receptors (TCRs) of such responses exhibited a low affinity for cognate antigens. All these defects appeared to stem directly from the DNA-damaging nature

of the chemotherapy. Indeed, while temozolomide-treated murine splenocytes do not stain positively for phosphorylated ataxia telangiectasia mutated (ATM, a key sensor of the DNA damage response) directly after isolation, a robust increase in the amount of phosphorylated ATM is observed upon TCR stimulation ex vivo. Our key finding (Fig. 1A) was that such

a proliferation-induced DNA damage resulting from alkylating chemotherapy is dependent on the strength of antigenic stimulation. Indeed, whereas only a modest accumulation of phosphorylated ATM was observed with a weak antigen-TCR pair (the mutant ovalbumin-derived peptide SIIGFEKL and the OT-I TCR, respectively), a robust DNA damage

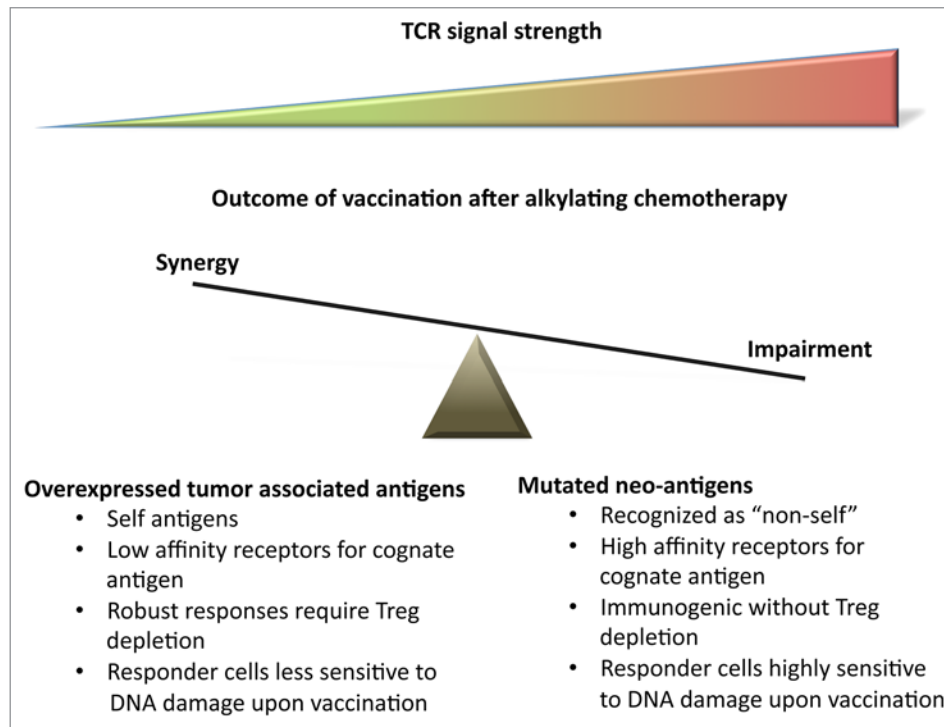


Figure 2. The outcome of vaccination upon alkylating chemotherapy may depend upon the type of antigenic target. The generation of robust immune responses against overexpressed self antigens is likely require to depletion of regulatory T cells (Tregs), providing a rationale for the use of immunostimulatory doses of alkylating chemotherapy. Conversely, mutated neo-antigens are recognized by T cells harboring high-affinity T-cell receptors in spite of the presence of normal amounts of Tregs. In this case, the DNA-damaging potential of alkylating chemotherapy is deleterious for the efficiency of vaccination.

response was elicited with a high affinity antigen-receptor pair (the wild-type peptide SIINFEKL and OT-I).

We hypothesized that alkylating chemotherapy would mostly impair adaptive immune responses against non-self antigens, against which precursor B- and T-cell populations bearing high affinity receptors are present. We found this to be the case for both a model antigen (chicken ovalbumin) and for previously published neo-antigens identified in syngeneic mouse models of melanoma and glioma. Upon low-dose cyclophosphamide or temozolomide pre-treatment, respectively, and peptide vaccination, neo-antigen-reactive cells were undetectable in tumor-bearing animals.¹⁹

In light of these new preclinical data, we performed a retrospective analysis of overall survival among metastatic melanoma patients that had been enrolled in an early phase clinical trial testing the safety and therapeutic profile of an autologous, large multivalent vaccine.¹¹ In this setting, cell surface proteins were extracted from autologous tumor material and were

adsorbed onto cell-sized silica beads. Interestingly, the study was designed to involved two cohorts of 10 patients receiving an identically prepared vaccine, either as a standalone intervention or one week after the administration of low-dose cyclophosphamide. Upon the analysis of long-term overall survival data, we observed an intriguing difference: the median survival of patients receiving the vaccine alone was > 4 y, while that of patients pre-treated with cyclophosphamide was ~7 mo (Fig. 1B). Of note, immunomonitoring conducted at the time of the trial revealed minimal responses against several common overexpressed tumor-associated antigens in vaccinated patients.¹¹ While it is difficult to test this hypothesis, we speculate that the long-term survival of the patients who received the vaccine only might reflect the elicitation of immune responses against patient specific neo-antigens, a process that was abrogated in patients that received cyclophosphamide pre-vaccination. Multiple investigators have found that immune responses targeting patient-specific neo-antigens are

prevalent among the lymphocytes that infiltrate metastatic melanoma lesions, suggesting these peptides may be the most immunogenic antigens present.^{25,26} In addition, in a follow-up Phase II clinical trial, an autologous large multivalent vaccine using an HLA-transfected allogeneic melanoma cell line as the antigen source failed to demonstrate the same long term survival of vaccinated patients,²⁷ providing additional circumstantial evidence that private mutations were the source of the responses seen in the first study, which were inhibited by cyclophosphamide pre-treatment.

A Hierarchy of Tumor-Associated Antigens and Differential Susceptibilities of Immune Responses to Alkylating Chemotherapy

Recent reviews summarizing new findings gleaned from genome-wide analyses posit a hierarchy of tumor antigens: overexpressed self antigens are widely shared among different patients but minimally

immunogenic, whereas neo-antigens are highly immunogenic but also very unlikely to be shared.^{28,29} In addition, we propose that differences in the nature of the T-cell repertoire reactive to these types of tumor-associated antigens results in a spectrum of susceptibilities to the immunosuppressive effects of alkylating chemotherapy (Fig. 2). At one end of the spectrum are normal self proteins that are expressed in the thymus during T-cell development as well as in the periphery in steady-state conditions. The T cells specific for these proteins are minimally reactive as they bear low-affinity TCRs that cannot be activated in the presence of Tregs. The low-intensity proliferative signals that such cells receive upon antigenic stimulation and the fact that their efficient activation cannot be achieved in the presence of normal amounts of Tregs may lead to a situation in which Treg-depleting alkylating chemotherapy and vaccination synergize in the elicitation of immune responses against self proteins. At the other end of the spectrum are non-self proteins like model antigens or tumor-specific neo-antigens. Immune responses against these antigens can be generated in Treg-replete hosts and result in the delivery of high intensity proliferative signals to responder lymphocytes. Such responses are impaired by DNA damage, such as

that induced by alkylating chemotherapy followed by vaccination.

Recent clinical trials using chimeric antigen receptor transduced autologous T cells show the kinds of dramatic responses that are achievable when an overwhelming immune response targets every cell in the body that expresses a tumor antigen.³⁰ Given their high immunogenicity and restricted expression by tumor cells, neo-antigens represent extremely attractive targets for active immunotherapy. Recent advances in bioinformatics make the targeting of these antigens possible in principle, and are driving further research toward the implementation of this strategy as a clinical reality. One of the potential pitfalls in this context is that conventional or immunomodulatory alkylating chemotherapy is routinely used for several malignancies that would constitute desirable targets for vaccination including metastatic melanoma, lung carcinoma and glioblastoma. For cancers for which it is not feasible or preferable to dispense with this chemotherapy altogether, clinical protocols might perhaps be modified to spare responder lymphocytes from cytotoxic effects and hence obtain a synergy between chemotherapy and immunotherapy. This would entail isolating large numbers of peripheral blood mononuclear cells before chemotherapy

and either expanding neo-antigen-specific cells in culture or transducing them with an artificial neo-antigen specificity, perhaps using a TCR cloned from immunized HLA-transgenic mice.¹³ More simply, re-infusing naïve lymphocytes that have not been exposed to chemotherapy prior to peptide- or vector-based vaccination might also yield synergistic effects. Furthermore, at least in the case of some malignancies, drugs that have a reduced impact on T cells (such as different classes of chemotherapeutics or targeted agents) can be employed, allowing for the elicitation of efficient immune responses upon vaccination. For instance, we examined anti-neoplastic doses of both a DNA-intercalating agent (doxorubicin) and a platinum derivative (carboplatin) and observed significantly smaller inhibitory effects on T cell responses upon vaccination relative to alkylating chemotherapy.¹⁹ Targeted therapeutic agents, such as sorafenib or vemurafenib, should also be studied in this context. By developing clinical strategies that allow chemotherapy and immune therapy to synergize, improved results of experimental immune therapy trials may be achieved.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest or financial interests to disclose.

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