Published in final edited form as:

Crit Rev Immunol. 2016 ; 36(2): 179–191. doi:10.1615/CritRevImmunol.2016017507.

Immunostimulatory Effects of Melphalan and Usefulness in Adoptive Cell Therapy with Antitumor CD4⁺ T Cells

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Abstract

The alkylating agent melphalan is used in the treatment of hematological malignancies, especially multiple myeloma. In the past, the usefulness of melphalan has been solely attributed to its cytotoxicity on fast-growing cancerous cells. Although the immunomodulatory effects of melphalan were suggested many years ago, only recently has this aspect of melphalan's activity begun to be elucidated at the molecular level. Emerging evidence indicates that melphalan can foster an immunogenic microenvironment by inducing immunogenic cell death (ICD) as characterized by membrane translocation of endoplasmic reticulum protein calreticulin (CRT) and by release of chromatin-binding protein high-mobility group box 1 (HMGB1). In addition, the lympho-depletive effect of melphalan can induce the release of pro-inflammatory cytokines and growth factors, deplete regulatory T cells, and create space to facilitate the expansion of infused tumor-reactive T cells. These features suggest that melphalan can be used as a preparative chemotherapy for adoptive T-cell therapy. This notion is supported by our recent work demonstrating that the combination of melphalan and adoptive transfer of tumor-reactive CD4⁺ T cells can mediate potent antitumor effects in animal models. This review summarizes the recent advances in understanding and utilizing the immunomodulatory effects of melphalan.

Keywords

melphalan; CD4; immunostimulation; adoptive cell therapy

I. INTRODUCTION

Chemotherapy is a major treatment modality for many types of cancer. Chemotherapy can often improve the symptoms of cancer initially, but it is rarely curative. Effective treatment of relapsed/refractory cancer remains a major unmet medical need. Recent advances in cancer immunotherapy have offered unprecedented opportunity to harness the power of the immune system to fight against cancer.¹ Interest in combining conventional chemotherapy with immunotherapy to address the problem of cancer recurrence in patients is growing.^{2,3} Therefore, identifying anticancer drugs with immunopotentiating effects can broaden the usefulness of chemotherapy and achieve durable therapeutic benefits.

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In recent years, an increasing number of chemotherapeutic agents have been found to exert immunostimulatory effects.^{4–8} Among these antineoplastic agents, alkylating agent cyclophosphamide (CTX) represents the most well-studied immunopotentiating anticancer drug. Because of its potent immunostimulatory effects, CTX has been widely used both in preclinical studies and clinical settings to enhance the efficacy of cancer immunotherapies.^{9–11} Melphalan is another alkylating agent widely used in cancer chemotherapy. Although melphalan belongs to the same class of anticancer drug as CTX, little attention has been given to its potential immunomodulatory effects. Our recent work provides the first evidence that melphalan resembles CTX in multiple aspects of immunostimulatory properties, justifying its novel use as an immunopotentiating anticancer drug in the context of combinatorial chemo-immunotherapy. Herein, we discuss our current knowledge of melphalan's immunomodulatory effects and its potential usage in adoptive immunotherapy.

II. USE OF MELPHALAN IN CANCER TREATMENT

Melphalan (trade name Alkeran) belongs to the class of nitrogen mustard alkylating agents. Since Gilman and Philips published their initial results of treating neoplasms using mustards,¹² many drugs in this class, including cyclophosphamide (CTX), melphalan, chlorambucil, uramustine, ifosfamide, and bendamustine, have been developed and used in chemotherapy for cancer. Melphalan was synthesized in the early 1950s by Bergel and Stock, and it was subsequently shown that mainly the L-form had biological effect on tumor cells.¹³ Unlike CTX, which is a prodrug that needs to be activated by liver through oxidation of cytochrome P450, melphalan does not require any activation to exert its toxicity. Melphalan enters cells primarily by a neutral amino acid active pathway shared by leucine (and therefore its transport is decreased in the event of high leucine concentration).¹⁴ It has a half-life of 75 minutes *in vivo* and is excreted mostly through urine.¹⁵ Melphalan exerts its cytotoxicity by inducing inter-strand cross-links in DNA,¹⁶ and it may also induce lesions in RNA, proteins, and lipids.¹⁷

Melphalan has been used in the treatment of lymphoma and leukemia and several types of solid tumor, including neuroblastoma, melanoma, sarcoma, and ovarian cancer.¹⁸ Melphalan is best known for its use in the treatment of multiple myeloma (MM). Melphalan was first used for multiple myeloma in 1962 as a frontline therapy, significantly extending patients' lives.¹⁹ Since prednisone was also shown to have beneficial effect in MM patients, melphalan/prednisone therapy was introduced in 1969; it resulted in increased response rates and median survival of six months over melphalan alone.²⁰ Thalidomide and its derivative lenalidomide, which are immunomodulatory drugs and potent angiogenesis inhibitors,^{21,22} and bortezomib, a proteasome inhibitor, have been introduced into the therapeutic regimens and have been shown to increase the overall survival of MM patients over other combinations used so far.^{23,24} Currently, melphalan is included in regimens like MPB (melphalan-prednisone-bortezomib) for initial treatment of MM,²⁵ or BMPT (bortezomib-melphalan-prednisone-thalidomide) in case of refractory tumor.²⁶ Because of its ablative effect toward bone marrow, melphalan has been used in combination with autologous stem cell transplantation (ASCT). High-dose melphalan (HDM; range: 140–200 mg/m²) plus ASCT currently serves as the standard treatment approach for patients with newly

diagnosed, transplant-eligible multiple myeloma.²⁷ Low-dose melphalan (LDM, range: 100–140 mg/m²) in combination with other drugs, such as prednisone and thalidomide, is used for patients not eligible for stem cells transplantation.^{28,29} Its potent immunosuppressive property is also exploited in allogeneic stem cell transfer to allow graft-versus-tumor effects of the transplanted cells.¹³

Melphalan therapy induces expression of reactive oxygen species that lead to apoptosis through activation of caspase-9, resulting in activation of caspase-3 and subsequent DNA damages that lead to cell death.^{30,31} In addition to acting on fast-growing transformed cells, alkylating agents also have detrimental activities toward normal tissues including intestine, liver, kidneys, and lungs. The gastrointestinal effects of melphalan are common among patients, causing symptoms such as vomiting, nausea, and/or diarrhea.³² High-dose melphalan plus ASCT causes severe mucositis, limiting the dose of melphalan to 200 mg/m².^{33,34} Melphalan also shows toxicity toward liver, kidneys, and lungs, and these side effects need to be accounted for when used in combination with other drugs having similar effects.³⁵ Without ASCT, high-dose melphalan causes severe myelosuppression, which affects cells of the immune origin including T and B lymphocytes and NK cells.^{36,37}

III. IMMUNOMODULATORY EFFECTS OF MELPHALAN

In recent years, it has been realized that in addition to exerting direct cytotoxicity on fast-growing transformed cells, many chemotherapeutic agents can incite antitumor immune responses, which contribute to the efficacy of chemotherapy.^{38–40} It has been shown that some anticancer drugs, including CTX, doxorubicin, and oxaliplatin, can induce immunogenic cell death (ICD), characterized by surface exposure of the endoplasmic reticulum protein calreticulin (CRT), secretion of chromatin-binding protein high-mobility group box 1 (HMGB1), and release of ATP.^{41–44} Translocation of CRT from the endoplasmic reticulum to tumor cell surface causes an “eat-me” signal for phagocytosis by dendritic cells (DCs). HMGB1 released by dying tumor cells can act upon TLR4 on DCs. ATP released by dying tumor cells can trigger purinergic P2RX7 receptors on DCs to activate the NLRP3 inflammasome, thus inducing the production of the proinflammatory cytokine IL-1 β . These events can lead to enhanced antigen processing and presentation, which result in the activation of endogenous CD8⁺ T cells, which in turn contribute to the antitumor efficacy of chemotherapy. In addition, certain chemotherapeutic agents can potentiate antitumor immune responses by mitigating immunosuppression mediated by regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs). Low-dose CTX is capable of depleting cycling CD4⁺CD25⁺ Tregs and inhibiting their suppressive activity.^{45,46} Selective and efficient depletion of Tregs and sparing of effector CD4⁺ cells is highly important because on top of thymic-derived Treg cells, regulatory T cells can arise from the conversion of non-Treg helper cells, especially in tumor microenvironment.⁴⁷ These *de novo* generated Tregs have a suppressive phenotype similar to thymic Tregs, yet they possess an unstable phenotype that depends largely on their context or environment.⁴⁸ Gemcitabine, 5-fluorouracil, sunitinib, doxorubicin, and docetaxel can reduce MDSCs and enhance the antitumor activities of CD8⁺ T cells and NK cells.^{49–53} Furthermore, CTX can induce a surge of growth factors and proinflammatory cytokines/chemokines by removing “cytokine

sinks,” resulting in reprogramming of the tumor immune milieu from immunosuppressive to immunostimulatory.^{43,44,54}

CTX is probably the most well-studied anticancer drug that possesses all of the immunostimulatory properties described above. Compared with CTX, less attention has been given to melphalan in terms of its immunomodulatory effects. Nonetheless, it has been known for years that low-dose melphalan (LDM) induces antitumor immune responses in mouse tumor models. In a series of studies, Mokyr’s group showed that LDM (2.5 mg/kg) therapy of mice bearing plasmacytoma MOPC315 tumors was able to induce robust CD8⁺ T-cell responses that eradicated large tumors.⁵⁵ These tumor-reactive CD8⁺ T cells can originate not only from the spleen but also from the thymus. The same group later showed that low-dose melphalan treatment resulted in upregulation of B7.1 not only in tumor cells but also other nontumor cells in the stroma, likely antigen-presenting cells, which may contribute to improved antitumor effects.⁵⁶

It has been well-established that CTX can exert immunostimulatory effects by promoting the release of pro-inflammatory cytokines (removing “cytokine sinks”), inducing immunogenic cell death, reducing Tregs and MDSCs, and creating space to facilitate T-cell expansion.^{7,42,45,57,58} Although it is reasonable to assume that melphalan has similar immunomodulatory effects as CTX because they belong to the same class of alkylating agent, supporting clinical and experimental evidence has been lacking until recently. It has been shown that myeloma patients had increased plasma level of immune cytokines, including IL-6, IL-7 and IL-15, following HDM and ASCT, and the presence of IL-7 and IL-15 may contribute to the survival and activation of CD3⁺ T cells present in the SCT graft.³⁷ Along this line, the recent report by Dudek-Peri et al.³⁹ showed that limb perfusion of melanoma patients with melphalan led to increased release of IL-1 β , IL-6, and IL-8 in the locoregional sera of patients. In a prophylactic vaccination mouse model, the authors showed that melphalan administration *in vivo* could stimulate a CD8⁺ T-cell-dependent protective antitumor response. Interestingly, although induction of apoptosis in melanoma cells by melphalan *in vitro* did not elicit cell surface expression of CRT, vaccination effect was potentiated in combination with exogenous CRT.

Our recent work showed for the first time that melphalan is able to induce ICD in tumor cells *in vitro* and *in vivo*.⁵⁹ We showed that melphalan treatment led to expression of CRT on the surface of A20 (B-cell lymphoma) and CT26 (colorectal cancer) cells, accompanied by the release of extracellular HMGB1. Notably, the ability of melphalan to induce CRT translocation may be cell dependent: CRT surface expression was not observed in melanoma cells after melphalan treatment even though the treatment triggered inflammatory cell death.⁶⁰ In our study, the emergence of immunogenic danger signals correlated with increased uptake of tumor associated antigens by dendritic cells as well as activation of the endogenous CD8⁺ T cells.⁵⁷ Our study also demonstrated that melphalan is lympho- and myelo-depletive, causing transient reduction of Tregs and MDSCs. In addition, melphalan induces a proinflammatory cytokine/chemokine milieu, resulting in spikes in the level of cytokines including IFN- γ , IL-22, IL-10, IL-5, IL-18, and IL-27, and chemokines including CCL2, CCL7, CXCL1, and CXCL10. These data provide clear evidence that melphalan resembles CTX in immunomodulatory activities.

IV. THE USE OF MELPHALAN IN CANCER IMMUNOTHERAPY

A. Combination of Melphalan-Based Care and Adoptive T-Cell Therapy for Multiple Myeloma

Commonly, chemotherapy can reduce the symptoms of cancer initially, but refractory cancers develop and become resistant to further treatment.⁶¹ This phenomenon suggests that the endogenous antitumor immune responses incited by chemotherapy are insufficient to mediate durable antitumor effects. Therefore, additional therapeutic interventions are needed to confer long-term, curative efficacy. Recent advances in immune checkpoint blockade, therapeutic cancer vaccines, and adoptive T-cell therapy (ACT) have validated immunotherapy as a viable treatment option for patients with cancer.^{1,62–64} There is increasing interest in combining immunotherapy with conventional cancer therapies to improve patient outcomes. Because chemotherapy is the stand of care for many types of cancer, the concept of combining chemotherapy and immunotherapy for synergistic effect has gained considerable traction and has begun to be tested.^{2,3,6,65} In this context, melphalan has been used in combination with adoptive T-cell therapy and cancer vaccines in the treatment of multiple myeloma. Rapoport et al. reported that in myeloma patients treated with HDM and ASCT, infusion of *in vivo* vaccine-primed, *ex vivo* costimulated autologous T cells followed by post-transplant vaccines improved the severe immunodeficiency associated with high-dose chemotherapy and led to enhanced memory T-cell responses.^{66–68} In an attempt to generate marrow-tropic and tumor-specific T-cell populations suitable for ACT, Noonan et al. established a protocol in which *ex vivo*-activated and –expanded marrow-infiltrating lymphocytes were used for adoptive immunotherapy of multiple myeloma.⁶⁹ Their recent work has further demonstrated the feasibility and efficacy of marrow-infiltrating lymphocyte-based ACT following HDM and ASCT.⁷⁰

In recent years, adoptive T-cell therapy using T cells engineered to express selected TCRs or chimeric antigen receptors (CARs) has emerged as a promising cancer treatment option.^{63,64} Fueled by the encouraging outcomes of ACT in melanoma and B-cell malignancies, these approaches have been applied to multiple myeloma. In two recent clinical trials, the HDM and ASCT regimen was used in combination with infusion of autologous T cells engineered to express NY-ESO-1-specific TCR or CD19-targeting chimeric antigen receptor (CAR).^{71,72} In both studies, the engineered T cells were safe and able to traffic to marrow and exhibited clinical activity against myeloma.

B. Potential of Melphalan as Preparative Chemotherapy for Adoptive Immunotherapy

In the aforementioned clinical studies, melphalan, as part of the standard care protocol, has been integrated with cancer immunotherapies. Its potential immune-potentiating effect has not been a factor considered in the research design, even though it might contribute to the therapeutic outcome. The immunostimulatory features of melphalan unraveled by our study support the notion that melphalan can be used as an immune-potentiating anticancer drug similar to CTX.⁵⁹ We tested this in animal models in which tumor-specific CD4⁺ T cells were transferred to tumor-bearing mice following melphalan or CTX pre-conditioning. We found that when mice were pre-treated with melphalan at the dose that causes lymphodepletion, immunogenic cell death and cytokine/chemokine surge, the transferred

tumor-specific CD4⁺ T cells underwent robust clonal expansion and effector differentiation, giving rise to polyfunctional CD4⁺ effector cells. Notably, the combination of melphalan and CD4⁺ T-cell ACT was more efficacious than either treatment alone in prolonging the survival of mice with advanced B-cell lymphomas or colorectal tumors. Notably, although the majority of current ACT protocols use CD8⁺ T cells or unfractionated total T cells, mounting evidence indicates that CD4⁺ T cells can be a good cellular source of ACT.^{73–77} In fact, the use of tumor-reactive CD4⁺ T cells for ACT has become a reality in the clinic and has demonstrated efficacy in patients.^{78,79} Therefore, our demonstration of the efficacy of melphalan and CD4⁺ T-cell ACT is clinically relevant. Even though our study focused on the use of CD4⁺ T cells for ACT, it is conceivable that CD8⁺ T-cell ACT will benefit from melphalan pre-conditioning as well. In summary, our study results imply that the immunomodulatory effects of melphalan can be exploited to potentiate the efficacy of adoptive T-cell therapy.

V. COUNTER-REGULATION MECHANISMS ASSOCIATED WITH MELPHALAN

A. Chemotherapy-Induced MDSCs Attenuate Antitumor Immune Responses

It has now been well-established that in addition to exerting direct cytotoxicity on fast growing tumor cells, many antineoplastic agents can incite antitumor immune responses. These anticancer drugs exert immunostimulatory effects through a variety of mechanisms.^{7,80} On the other hand, it also has been well-documented in animal models that chemotherapy can exert unwanted “opposite effects” that promote the growth of the residual tumors.^{81,82} This paradoxical feature of chemotherapy is exemplified by the alkylating agent CTX. With its well-recognized immunostimulatory effects, CTX has been widely used both in preclinical studies and clinical settings to enhance the efficacy of cancer immunotherapy such as adoptive T-cell therapy.^{9–11} Meanwhile, it has been shown that CTX is capable of promoting tumor metastasis,^{83,84} and inducing myeloid cells with immunosuppressive activities.^{85–90} We recently reported that CTX-induced myeloid cells can attenuate the efficacy of adoptive T-cell therapy in a PD1/PDL1-dependent fashion in a murine lymphoma model.⁹¹ Although the induction of inflammatory myeloid cells is not unique to CTX, a number of widely used anticancer drugs, including doxorubicin and paclitaxel, have been found to have similar effects.^{91–94} The induction of immunosuppressive MDSC-like myeloid cells by chemotherapy is not limited to animal models; chemotherapy-driven MDSC expansion has been observed in cancer patients. In a study conducted in patients with breast cancer, circulating MDSC numbers were significantly increased in patients receiving doxorubicin-CTX chemotherapy, and the results correlated with clinical cancer stage and metastatic tumor burden.⁹⁵ Our recent study indicated that melphalan can also induce the expansion of inflammatory myeloid cells.^{91–94} Given that CTX-induced myeloid cells can be depleted or functionally blocked by low-dose gemcitabine, α CCR2 monoclonal antibody, or PD1/PDL1 blockade,⁹¹ the same approaches may be applicable to melphalan to inhibit its MDSC-promoting effect while preserving its immunostimulatory effects.

B. B7.1/CD28 Interaction Promotes the Survival of Myeloma

It has been shown that low-dose melphalan can induce B7.1 in tumor and stromal cells.⁵⁶ Although this effect is considered immunogenic, it may also simultaneously contribute to tumor survival because myeloma cells express B7 receptor molecule CD28 on their surface. Upon ligation of CD28 on MM cells with B7 molecules on DCs (or by agonistic antibodies), tumor cells exhibited decreased apoptosis in response to chemotherapy or serum starvation.⁹⁶ The interaction between MM and DCs triggers the production of IL-6 and indolamine-2,3-dioxygenase (IDO) by DCs.⁹⁷ IL-6 can improve MM survival,⁹⁸ whereas IDO promotes a tolerogenic milieu by suppressing effector T cells and activating regulatory T cells (Tregs).⁹⁹ It is possible that melphalan induces B7.1 in tumor cells and stroma cells, which then engages CD28 on MM and hence promotes the survival and persistence of myeloma cells after chemotherapy. This notion is supported by the observation that CD28 expression on myeloma cells correlates with poor prognosis.¹⁰⁰ Thus, it is conceivable that disrupting CD28-B7.1 interaction after melphalan treatment would be beneficial. Indeed, this was demonstrated by a recent study showing that blockade of CD28/B7 using CTLA4-Ig resulted in sensitization of myeloma cells to melphalan.¹⁰¹

C. IL-6 Signaling Pathway Promotes Tumor Drug Resistance

Multiple lines of evidence, from both animal and human studies, indicate that IL-6 is one of the cytokines markedly induced after melphalan treatment.^{102,103} It is now greatly appreciated that enhanced IL-6/STAT3 signaling pathway is a common feature of human cancer leading to progression of the malignancy.^{104–106} IL-6 plays a role in hematopoiesis, immune regulation, and oncogenesis and influences cancer cell migration, invasion, metastatic potential, proliferation, angiogenesis, and apoptosis.¹⁰⁷ IL-6 stimulates the growth of a number of cancers including MM, breast cancer, and lung cancer.^{108,109} Elevated levels of circulating IL-6 have been correlated with poor prognosis and multidrug resistance of tumor cells.¹¹⁰ IL-6 activates STAT3-dependent pathway and the signal mediated by IL-6/STAT3 is terminated by SOCS3.¹¹¹ Upon translocation to the nucleus, pSTAT3 recognizes its DNA sequence called GAS (IFN- γ -activated sequence), which initiates changes in the expression of antiapoptotic molecules like Bcl2, Bcl-xL, c-Myc, or Fas. STAT3 also activates Ras, MEK, MAPK, Cox, and PI3K/Akt pathways.¹¹² In MM, IL-6 can activate tumor cells in two ways: Akt independently (through RAS) and Akt dependently through PI3K p85/STAT3.¹¹³ IL-6 has also been attributed to the development of stem cell-like properties of tumor cells (cancer stem cells, CSC);¹¹⁴ IL-6 was shown to induce CSC sphere formation of MM cells by upregulation of Notch-dependent Jagged-1 in a STAT3-dependent manner.¹¹⁵ Therefore, disrupting the IL-6 signaling pathway represents an attractive strategy for cancer treatment.¹¹⁶ In line with this hypothesis, the combination of melphalan and siltuximab was able to overcome protective effect of bone marrow stroma on MM cells by inhibiting the PI3K/Akt pathway responsible for myeloma resistance to melphalan.¹¹⁷ Moreover, blockade of interleukin-6 signaling with siltuximab enhanced melphalan cytotoxicity in preclinical models of multiple myeloma.¹¹⁸ The combination of α IL-6 monoclonal antibody with MM standard care is currently being evaluated clinically.^{119–121}

VI. CANCER CELLS ADAPT METABOLICALLY IN RESPONSE TO CHEMOTHERAPY

It has been well established that cancer cells can reprogram their metabolisms to meet the biosynthetic needs of rapid proliferation.^{122,123} There is emerging evidence that cancer cells can adapt metabolically in response to the stress elicited by chemical insults.^{124–126} Using transcriptomic and proteomic approaches, Zub et al. observed that in melphalan-resistant myeloma cell lines, there is a metabolic switch conforming to the Warburg effect (aerobic glycolysis) and an elevated oxidative stress response, resulting in enhanced survival and proliferation partially mediated by VEGF/IL-8 signaling.¹²⁷ Specifically, in melphalan-resistant myeloma cell line RPMI8226, most of the glycolytic and pentose phosphate–pathway enzymes were upregulated, whereas the tricarboxylic acid cycle and electron transport chain proteins were downregulated. The switch of metabolic dependence to aerobic glycolysis implicates the glycolytic pathway as a potential target for therapeutic intervention to improve the efficacy of chemotherapy. Supporting this notion, inhibitors of selected metabolic and oxidative stress response enzymes displayed a selective cytotoxicity against melphalan-resistant myeloma cells. It was recently shown that metabolic competition, especially for glucose (and perhaps for other metabolites) in the tumor microenvironment, renders T cells dysfunctional even in the face of antigenic stimulation.¹²⁸ Glucose consumption by tumor cells leads to glucose deprivation and inhibition of mTOR pathway in T cells, blocking T-cell effector differentiation.¹²⁸ At the time of activation, CD28 signaling in T cells not only serves as a co-stimulation signal to fully activate T cells but also functions to increase T-cell glucose uptake and glycolysis during an immune response.¹²⁹ On the other hand, immunosuppressive Tregs and M2 macrophages preferentially use fatty acids, rather than glucose, as an energy source. Therefore, in the tumor microenvironment, immunoregulatory cells are less likely to be affected by a tumor's glucose consumption, leaving effector T cells most vulnerable to the reduced availability of glucose.^{130,131} Interestingly, Chang et al. showed that in immune checkpoint blockade targeting CTLA-4, PD1 and PDL1 can relieve the nutrient restriction on T effector cells and restore glucose availability in the tumor tissue,¹²⁸ suggesting that the efficacy of immune checkpoint blockade is at least partially attributable to metabolic normalization of the tumor microenvironment.

VII. CONCLUSIONS

Melphalan has been used in the clinic for its potent antineoplastic activity. High-dose melphalan followed by autologous hematopoietic stem cell transplantation is regarded as a standard treatment for patients with multiple myeloma. Melphalan is also the mainstay of various chemotherapy regimens used for transplant-ineligible MM patients. In recent years, there have been multiple clinical trials in which melphalan was used in combination with adoptive T-cell therapy for refractory myeloma.^{65–67,69–71} This form of chemo-immunotherapy essentially applies immunotherapy on the platform of conventional chemotherapy in which melphalan is a major component. In these complex clinical studies, it is difficult to assess the contribution of the immunopotentiating effects of melphalan. Our animal study manifests the immunostimulatory effects of melphalan in a physiologically

relevant setting.⁷² The ability of melphalan to augment CD4⁺ T-cell adoptive therapy provides a strong rationale for exploiting the immunopotentiating effects of melphalan in future clinical studies. Our study presents clear evidence that melphalan can induce immunogenic cell death, enhance tumor antigen uptake by DCs, eliminate Tregs, and induce transient lymphopenia along with a burst of proinflammatory cytokines/chemokines. Future studies should determine how each of these activities contribute to the enhanced efficacy of antitumor CD4⁺ T cells. Notably, the immunomodulatory effects of melphalan are not always beneficial or desirable. For example, melphalan-induced IL-6 and immunosuppressive myeloid cells can serve as counter-regulation mechanisms that attenuate the efficacy of immunotherapy. Thus, it will be important to take the necessary measures, such as IL-6 neutralization and PD1 blockade, to overcome the unfavorable tumor-promoting effects associated with melphalan. The results from our study support a scenario in which melphalan-induced HMGB1 attracts immature DCs into the draining lymph nodes; meanwhile, surface CRT expression on tumor cells presents an “eat-me” signal for phagocytosis by immature DCs, and the pro-inflammatory milieu induced by melphalan enhances DC maturation and antigen presentation. These events collectively contribute to robust CD4⁺ T-cell activation and effector differentiation. Once activated, polyfunctional CD4⁺ effector cells can mount immune responses against tumor cells through multiple mechanisms.¹³² In summary, our study identifies melphalan as an anticancer drug with potent immunopotentiating properties, and our results support its usefulness beyond its direct tumoricidal activity in the setting of chemo-immunotherapy for synergistic therapeutic outcomes.

Acknowledgments

The authors thank members of the Zhou Laboratory for insightful discussions. This work is funded by National Institutes of Health grant R01CA158202 and the American Cancer Society Research Scholar Grant (RSG-12-169-01-LIB) to G.Z.

ABBREVIATIONS

ACT	adoptive T-cell therapy
ASCT	autologous stem cell transplantation
CRT	calreticulin
CTX	cyclophosphamide
DC	dendritic cells
HDM	high dose melphalan
ICD	immunogenic cell death
LDM	low dose melphalan
MDCs	myeloid derived suppressor cells
MM	multiple myeloma

Tregs regulatory T cells

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