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*J Immunol* published online 5 January 2015
http://www.jimmunol.org/content/early/2015/01/03/jimmunol.1401894

### Supplementary Material
http://www.jimmunol.org/content/suppl/2015/01/03/jimmunol.1401894

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Alkylating Agent Melphalan Augments the Efficacy of Adoptive Immunotherapy Using Tumor-Specific CD4+ T Cells

Xiaoyun Lu,*,†# Zhi-Chun Ding,*,†# Yang Cao,*,‡ Chufeng Liu,*,§ Tsadik Habtetsion,*, Miao Yu,* Henrique Lemos,*, Huda Salman,*, Hongyan Xu, Andrew L. Mellor,*,‖ and Gang Zhou*,¶

In recent years, the immune-potentiating effects of some widely used chemotherapeutic agents have been increasingly appreciated. This provides a rationale for combining conventional chemotherapy with immunotherapy strategies to achieve durable therapeutic benefits. Previous studies have implicated the immunomodulatory effects of melphalan, an alkylating agent commonly used to treat multiple myeloma, but the underlying mechanisms remain obscure. In the present study, we investigated the impact of melphalan on endogenous immune cells as well as adoptively transferred tumor-specific CD4+ T cells in tumor-bearing mice. We showed that melphalan treatment resulted in a rapid burst of inflammatory cytokines and chemokines during the cellular recovery phase after melphalan-induced myelodepletion and leukodepletion. After melphalan treatment, tumor cells exhibited characteristics of immunogenic cell death, including membrane translocation of the endoplasmic reticulum–resident calreticulin and extracellular release of high-mobility group box 1. Additionally, there was enhanced tumor Ag uptake by dendritic cells in the tumor-draining lymph node. Consistent with these immunomodulatory effects, melphalan treatment of tumor-bearing mice led to the activation of the endogenous CD8+ T cells and, more importantly, effectively drove the clonal expansion and effector differentiation of adoptively transferred tumor-specific CD4+ T cells. Notably, the combination of melphalan and CD4+ T cell adoptive cell therapy was more efficacious than either treatment alone in prolonging the survival of mice with advanced B cell lymphomas or colorectal tumors. These findings provide mechanistic insights into melphalan’s immunostimulatory effects and demonstrate the therapeutic potential of combining melphalan with adoptive cell therapy utilizing antitumor CD4+ T cells.

The Journal of Immunology, 2015, 194: 000–000.

Chemotherapy is a major treatment modality for many types of cancer. Even though chemotherapeutic agents are chosen for their cytotoxicity toward cancerous cells, many widely used anticancer drugs have been found to exert immunomodulatory effects (1). It has been shown that some anticancer drugs, including doxorubicin, oxaliplatin, and cyclophosphamide (CTX), 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 (2–5). These studies demonstrate that translocation of CRT from the endoplasmic reticulum to tumor cell surface presents 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 and induce production of the proinflammatory cytokine IL-1β. As a result of enhanced Ag processing and presentation, the endogenous CD8+ T cells are activated and contribute to the antitumor efficacy of chemotherapy. Induction of antitumor immune responses may not translate into therapeutic benefits unless the immunosuppressive mechanisms operative in the tumor microenvironment are antagonized (6). It has been found that regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and various immunosuppressive factors, including IL-10, TGF-β, VEGF, and PGE2, are frequently enriched in the tumor microenvironment and facilitate tumor immune evasion. Some chemotherapeutic agents can potentiate antitumor immune responses by targeting Tregs or MDSCs. Low-dose CTX is capable of depleting cycling CD4+ CD25+ Tregs and inhibiting their suppressive activity (7, 8). Gemcitabine, 5-fluorouracil, sunitinib, doxorubicin, and docetaxel can reduce MDSCs and enhance the antitumor activities of CD8+ T cells and NK cells (9–13). Moreover, some anticancer drugs can create transient lymphopenia and induce a surge of growth factors and proinflammatory cytokines/chemokines, resulting in reprogramming of the tumor immune milieu from immunosuppressive to immunostimulatory (4, 5, 14).

Chemotherapy often can reduce the symptoms of cancer, but it is rarely curative. This suggests that the endogenous antitumor im-
munity elicited by chemotherapy may not be sufficient or durable enough to eradicate residual tumors, and hence additional therapeutic interventions are needed to confer better efficacy. Recent advances in adoptive T cell therapy (ACT) have validated it as a viable treatment option for patients with certain types of cancer (15, 16). Although current ACT protocols predominantly use CD8+ T cells to fight against cancer, it has become increasingly clear that CD4+ T cells are critical determinants of effective anti-tumor immune responses. CD4+ T cells can mediate tumor destruction either on their own (17–20) or by cooperating with other tumor-reactive immune cells, including CD8+ T cells (21–29), macrophages (30), and NK cells (31). The allying agent CTX is widely used to potentiate the efficacy of ACT both in animal models and clinical studies. We previously reported that chemotherapy with CTX can reset the immunosuppressive tumor milieu, allowing adoptively transferred tumor-specific CD4+ T cells to undergo robust expansion and differentiate into polyfunctional effector cells (32). Our recent study demonstrated that polyfunctional CD4+ effector cells are essential for tumor eradication in the postchemotherapy window (33). Given the pivotal role of CD4+ T cells in the setting of chemoinmunotherapy, it is desirable to identify additional anticancer drugs with CD4+ T cell–potentiating activities. In this study, we report that melphalan can induce immunogenic tumor cell death as reflected by extracellular HMGB1 release and CRT membrane translocation, and it can enhance the uptake of tumor-associated Ag by DCs in the tumor-draining lymph node (TDNL). Additionally, melphalan resembles CTX in its ability to create transient lymphopenia, reduce Tregs, and induce an inflammatory immune milieu. Notably, conditioning of tumor-bearing hosts with melphalan promoted the clonal expansion and effector differentiation of adoptively transferred tumor-specific CD4+ T cells in multiple murine tumor models. The synergy of melphalan and CD4+ T cell ACT conferred improved therapeutic benefits to mice with advanced B cell lymphoma or colorectal carcinoma. Our study implies that the immunostimulatory effects of melphalan can be exploited to facilitate CD4+ T cell–based immunotherapy.

Materials and Methods

Mice
BALB/c mice (4–6 wk old) were purchased from the National Cancer Institute (Frederick, MD). TCR transgenic mice on a BALB/c background expressing an αβ TCR specific for amino acids 110–120 from influenza hemagglutinin (HA) presented by MHC class II (MHC-II) molecule IE3 were originally generated in the laboratory of Dr. H. von Boehmer (Harvard Medical School, Boston, MA). Thy1.1+ HA-TCR transgenic (Tg) mice were gifts from Dr. Hyam I. Levitsky (Johns Hopkins University School of Medicine, Baltimore, MD). CD45.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Georgia Regents University.

Abs and reagents
The following fluorochrome-conjugated Abs were used for flow cytometry: anti-mouse CD11b-FITC (M1/70), CD44-FITC (IM7), CD11C-PE (N418), IFN-γ–allophycocyanin (XMG1.2), B220-PE (RA3-6B2), CD62L-PE (MEL-14), CD8-PE (55-6.7), CD25-PE (PC61.5), CD40L-PE (MR1), F4/80-PE (Cl:A3-1), Gr1-PE (RB6-8C5), TNF-α-PE (MP6-X22), Ly6C-PE/Cy7 (HK1.4), programmed death 1 (PD-1)–PE (RMP1-30), Ly6G-allophycocyanin/Cy7 (1A8), CD4-allophycocyanin/Cy7 (RM4-5), and control IgG mAbs were purchased from BioLegend. Granzyme B–Alexa Fluor 648 (GB11), Thy1.1-perCP (OX-7), and Ki67-FITC staining set were purchased from Invitrogen. Chemotherapeutic agents were all purchased from Sigma-Aldrich unless otherwise specified. Human G-CSF (Neupogen) was purchased from Amgen.

Tumor cells and animal models
The generation and maintenance of the HA-expressing murine B cell lymphoma cell line A20HA or colorectal tumor cell line CT26HA were described previously (33). The generation of GFP-expressing A20 tumor cells (A20-GFP) was described elsewhere (34). To establish tumor in mice, tumor cells were injected i.v. or s.c. The growth of s.c. tumors was monitored by caliper measurement of the tumor area every 3 d and was expressed as the product of two perpendicular diameters in square millimeters. Mice were euthanized when tumor size reached 400 mm2. For CD4+ T cell adoptive transfer, spleens and LNs from HA-TCR Tg mice were harvested, homogenized, and lysed by ACK lysis buffer to remove RBCs. Cells were enriched for CD4+ T cells by MACS (Miltenyi Biotec) and then reseeded with 2 µl IL-2. The percentage of lymphocytes positive for CD4 and the clonotypic TCR (mAb 6.5 for HA-specific CD4+ T cells) was determined by flow cytometry. A total of 2.5–3 × 10^6 CD4+ TCR+ T cells were injected i.v. into each recipient.

Cell preparation and flow cytometry analysis
Spleen and tumor samples obtained from mice upon euthanization were gently dissociated into single-cell suspensions. RBCs were removed by ACK lysing buffer. After washing with PBS, cells were stained for surface markers for detection of CD45.2+ A20 or CD45.1+ CT26 tumor cells were either untreated or treated with melphalan at 37˚C for 24 h. Tumor cells were then fixed at 4˚C with 0.25% paraformaldehyde in PBS, washed, and stained with rabbit anti-mouse CRT Ab (1:200; Abcam) for 30 min at 4˚C. Cells were washed with PBS and incubated with anti-rabbit Alexa Fluor 488–conjugated secondary Ab (1:500; Molecular Probes) for 30 min at 4˚C. Cells were washed and pellet was resuspended in PBS buffer with addition of DAPI to 0.5 µg/ml before FACS analysis. Isotype-matched IgG Ab was used as a control, and DAPI+ cells were gated for analysis. For intracellular cytokine staining of transferred HA-TCR Thy1.1+CD4+ T cells, 0.5 × 10^6 purified CD4+ T cells were incubated with 1.5 × 10^6 fresh splenocytes from syngeneic mice in the presence of the cognate HA peptide (30 µg/ml) and GolgiStop for 4 h at 37˚C. Cells were harvested and stained for CD4 and Thy1.1 mAbs before intracellular cytokine staining following the manufacturer’s instructions. For endogenous CD8+ T cells, 0.5 × 10^6 purified CD8+ T cells were stimulated for 4 h with 10 ng/ml PMA (Sigma-Aldrich) and 150 nM ionomycin (Sigma-Aldrich) in the presence of GolgiStop. Flow cytometry was performed on an LSR II (BD Biosciences). All data were analyzed using FlowJo software (version 7.6; Tree Star).

Multiplex cytokine assay
Naïve BALB/c mice were treated with 9 mg/kg melphalan and sacrificed at the indicated time points. Blood samples were collected from heart and kept on ice. Plasma was collected by centrifuge at 10,000 × g for 10 min at room temperature and stored at −70˚C until the analysis day. A multiplex bio-metric immunoassay was used for cytokine and chemokine measurement according to the manufacturer’s instructions (ProcartaPlex mouse cytokine and chemokine panel 1 [26plex]; EBiScience). Cytokines and chemokines measured were IL-1β, IL-10, IL-12(p70), IL-17A, IL-18, IL-22, IL-23, IL-27, TNF-α, IFN-γ, GM-CSF, MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), CCL5, MIP-1α (CCL3), MIP-1β (CCL4), IFN-γ–inducible protein 10 (CXC10), CXCL1, and Eotaxin. Briefly, 20 µl plasma samples were diluted 1:4 and incubated with Ab-coupled beads. Complexes were washed and then incubated with bio- tinylated detection Ab and, finally, with streptavidin–PE to allow for nicking and chemokine concentrations. Concentrated mouse recombinant cytokines and chemokines were provided by the vendor. A range of 0.74–132,500 pg/ml recombinant cytokines or chemokines was used to establish standard curves and to maximize the sensitivity and the assay dynamic range. Cytokine and chemokine levels were determined using a multiplex array reader from the LumineX instrumentation system (Bio-Plex workstation from Bio-Rad Laboratories). The concentration was calculated using software provided by the manufacturer (Bio-Plex Manager software).

HMGB1 detection by Western blot and ELISA
For Western blot analysis, 1 × 10^6 A20 or CT26 tumor cells were either untreated or treated with melphalan at 37˚C for 24 or 48 h. Supernatants from cultured tumor cells were collected. Tumor cells were incubated for 30 min on ice in lysis buffer (50 mM Tris HCl [pH 8.0], 120 mM NaCl, 0.25% Nonidet P-40, 0.1% SDS) (Sigma-Aldrich) containing the protease inhibitor cocktail (PMSF, aprotinin, leupeptin, and pepstatin A) each at a final concentration of 10 ng/ml, and 1 mM DTT (Sigma-Aldrich). A quantity of 20 µg of each protein sample was loaded onto a 10% SDS-PAGE gel. Loading of supernatants was performed by normalization to cell numbers.
Following separation, proteins were blotted onto a nitrocellulose membrane (Whatman). Membranes were blocked with 5% nonfat dry milk in TBS-0.5% Tween 20 and then probed with rabbit anti-mouse HMGB1 polyclonal Ab (2 mg/ml; Abcam) or anti-mouse β-actin mAb. Serum HMGB1 was quantified using an ELISA kit from Cloud-Clone (Houston, TX) following the manufacturer’s instruction. To prepare mouse serum, whole blood was collected, incubated at room temperature for 20 min, and centrifuged at 3000 × g for 15 min. Then, serum samples were aliquoted and stored at −80°C until further use.

Statistical analysis

Data were analyzed using Prism 4.0 (GraphPad Software). The statistical significance of the results was determined using the Student t test. Data for tumor survival were analyzed using a log-rank (Mantel–Cox) test. Differences in tumor sizes among different treatment groups were analyzed using the Mann–Whitney U test. A p value <0.05 was considered statistically significant.

Results

Melphalan induces myelodepletion and leukodepletion followed by the recovery of various cell types

High-dose melphalan is a component of the standard-of-care chemotherapy for patients with multiple myeloma. It is known that high-dose chemotherapy can lead to an immunosuppressive state (35). However, detailed analysis of the impact of melphalan on various subtypes of immune cells has not been reported. In this study, we performed time course experiments to determine the cellular events following melphalan treatment. For comparison purpose, CTX was included in the study because CTX and melphalan belong to the same class of alkylating agent, and the impact of CTX on different cellular compartments has been well characterized (36). Melphalan was lethal to BALB/c mice when used at the dose of 27 mg/kg by single i.p. injection (data not shown). In the following experiments, we chose to use melphalan at 9 mg/kg because this dose was well tolerated and exhibited cytotoxicity comparable to that of 150 mg/kg CTX, the dose at which CTX exhibits immunostimulatory effects. Fig. 1A shows that melphalan and CTX both resulted in acute reduction in overall cellularity, as reflected by a rapid decline in total cell counts in the spleens. After reaching the nadir by day 4, a cell recovery phase began to take place. By day 10, the total spleen cell numbers in CTX-treated mice already fully recovered whereas those in melphalan-treated mice only reached approximately half of the pretreatment level. Complete restoration of cellularity in melphalan-treated mice was seen by day 16, suggesting a delayed cell recovery phase began to take place. By day 10, the total spleen cell numbers in CTX-treated mice already fully recovered whereas those in melphalan-treated mice only reached approximately half of the pretreatment level. Complete restoration of cellularity in melphalan-treated mice was seen by day 16, suggesting a delayed recovery of lymphoid and myeloid cells after melphalan treatment compared with CTX treatment. Indeed, a delayed cell recovery was seen for cells of the adaptive immune system, including B cells, CD8+ T cells, CD4+ T cells, and Tregs (Fig. 1B). Of note, the duration of Treg depletion was longer after melphalan treatment compared with CTX treatment. Consistent with published data (37, 38), we showed that CTX treatment led to an initial reduction followed by rebound and expansion of innate immune cells (Fig. 1C), including monocytes (CD11b+Ly6CintLy6G−), granulocytes/neutrophils (CD11b+Ly6CintLy6Ghi), macrophages (CD11b+MHC-II+), conventional DCs (CD11c’MHC-II’), and plasmacytoid DCs (pDCs; CD11cintB220’PDCA1’). Different from CTX, melphalan had varied impact on innate immune cells. Monocytes, granulocytes/neutrophils, and conventional DCs underwent recovery and expansion after an initial reduction phase. In contrast, there were only modest fluctuations in the numbers of macrophages and pDCs after melphalan treatment.

In the clinic, autologous hematopoietic stem cell transplantation (ASCT) following high-dose chemotherapy is a common treatment option for patients with multiple myeloma and other hematological malignancies. G-CSF is often administered after ASCT to facilitate stem cell engraftment. We examined the impact of G-CSF administration on immune cell recovery following melphalan treatment. G-CSF injections following melphalan resulted in increased cellularity, which was mainly contributed by the increase of innate immune cells, including monocytes, granulocytes/neutrophils, macrophages, and DCs, whereas cells of the adaptive immune cell system, including B cells, CD8+ cells, CD4+ cells, and Tregs, were not significantly affected (Supplemental Fig. 1).

Melphalan induces an inflammatory cytokine/chemokine milieu

To characterize the cytokine milieu in response to melphalan, plasma was collected at different time points and subjected to Bio-Plex cytokine assays. Fig. 2 shows that melphalan treatment induced a surge of inflammatory cytokines and chemokines. In particular, the initial bursts of cytokines, reaching their peaks by day 2, included IFN-γ, a well-known proinflammatory cytokine, and IL-22, an IL-10 superfamily cytokine known to have both inflammatory and anti-inflammatory effects (39). The second wave of cytokines, including IL-5, IL-18, and IL-27, reached their peaks by day 4. Among these cytokines, IL-5 is a key mediator in eosinophil activation (40); IL-18 is a member of the IL-1 superfamily, and its ample induction was indicative of robust inflammatory reactions (41); and IL-27 is a member of IL-12 family known to promote CD4+ T cell Th1 differentiation (42). Additionally, low levels (<10 pg/ml) of IL-1β and TNF-α were detected in serum 2 d after melphalan (data not shown). For chemokines CCL2, CCL3, CCL7, and CXCL1, chemokartactants involved in recruiting monocytes, neutrophils, macrophages, DCs, and activated T cells to the sites of inflammation were promptly induced by day 2 after melphalan. Corresponding to the release of IFN-γ, IFN-γ-inducible chemokine CXCL10 (IFN-γ-inducible protein 10) reached its peak on day 4. Induction of these inflammatory mediators was transient because all cytokine/chemokine levels returned to baseline level by day 7.

Melphalan induces immunogenic tumor cell death

It has been shown that a number of chemotherapeutic agents can induce ICD as characterized by surface expression of CRT and extracellular release of HMGB1, leading to enhanced Ag processing and presentation (2, 3, 5). Earlier studies suggested that melphalan may elicit antitumor immune responses (43); however, whether melphalan can induce ICD in tumor cells has not been addressed. To test whether melphalan treatment led to HMGB1 release from tumor cells, A20 and CT26 tumor cells were treated with melphalan in vitro, and supernatants and cell lysates were collected to detect HMGB1 by immunoblot. Fig. 3A shows that there was an 2.5-fold increase of serum HMGB1 following high-dose melphalan treatment. G-CSF injections following melphalan resulted in increased cellularity, which was mainly contributed by the increase of innate immune cells, including monocytes, granulocytes/neutrophils, macrophages, and DCs, whereas cells of the adaptive immune cell system, including B cells, CD8+ cells, CD4+ cells, and Tregs, were not significantly affected (Supplemental Fig. 1).

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We then asked whether melphalan can induce extracellular release of HMGB1 and tumor cell surface expression of CRT in vivo. To this end, A20 tumor cells (CD45.2+) were s.c. inoculated to CD45.1+ congenic BALB/c mice (Fig. 3C, schema). This setting allowed us to distinguish tumor cells from endogenous B cells. When tumors became palpable, mice were untreated or treated with melphalan. At the indicated time points, serum samples were collected for HMGB1 quantification by ELISA, and tumor nodules were resected for ex vivo CRT analysis by flow cytometry. Fig. 3C shows that there was an ~2.5-fold increase of serum...
HMGB1 in melphalan-treated mice compared with untreated mice. Fig. 3D shows that surface CRT expression on tumor cells was markedly increased 24 h after melphalan treatment and remained evident 48 h later. Altogether, our data provide clear evidence that melphalan is capable of inducing ICD in tumor cells.

Melphalan administration enhances the uptake of tumor-associated Ag by DCs in the draining LN

It has been shown that ICD correlates with enhanced Ag processing and presentation by DCs (2–4). Because melphalan treatment led to immunogenic tumor cell death (Fig. 3), we posited that melphalan administration would promote the uptake of tumor-associated Ags by DCs. To test this, A20 IE-GFP tumor cells, which express GFP in association with MHC-II molecule IEα (34), were s.c. inoculated into CD45.1+ congenic BALB/c mice (Fig. 4, schema). Mice with palpable tumors were either untreated or melphalan treated. Forty-eight hours later, TDLNs and distant nondraining LNs were harvested for FACS analysis to evaluate the uptake of GFP, a surrogate for tumor cell–associated membrane/Ag, by host APCs (DCs). Notably, there was enhanced GFP uptake by host DCs (CD45.1+CD11c+MHC-II+) in the TDLN in melphalan-treated mice compared with untreated mice (Fig. 4A, (+) melphalan versus untreated), and the difference was statistically significant (Fig. 4B). Furthermore, in melphalan-treated mice, only DCs in the TDLN, but not DCs in the distant nondraining LN, had significant uptake of tumor cell–derived GFP (Fig. 4A, TDLN versus LN in the (+)melphalan group), suggesting that the enhancement of tumor Ag presentation following mel-

**FIGURE 1.** The kinetics of immune cell recovery in mice treated with melphalan or CTX. Naive BALB/c mice were treated with melphalan (9 mg/kg) or CTX (150 mg/kg). At the indicated time points, mice were sacrificed for analysis. Spleen cells were enumerated. Aliquots of cells were stained with mAbs and the frequencies of specific cell populations were assessed by flow cytometry. (**A**) Total cell numbers in the spleens after chemotherapy. (**B**) Numbers of cells of the adaptive immune system. The populations examined include B cells, CD8+ T cells, CD4+ T cells, and Tregs. (**C**) Numbers of cells of the innate immune system. The populations examined include monocytes, granulocytes/neutrophils, macrophages (Mφ), conventional DCs (cDC), and pDCs. The absolute numbers of each cell population were calculated as total spleen cell count x frequency of specific cell population. Results are shown as means ± SD of at least three mice per group at each time point.

**FIGURE 2.** Melphalan induces a burst of inflammatory cytokines and chemokines in plasma. Plasma was collected before (day 0) and at the indicated time points after melphalan treatment. The protein levels of cytokines (**A**) and chemokines (**B**) were quantified by a Bio-Plex cytokine assay. Data represent the mean values of the protein levels from two individual mice.
phalan administration is a localized event that occurs predominately in the TDLN.

**Melphalan treatment leads to activation of endogenous CD8\(^{+}\) T cells infiltrating the tumor**

It has been shown that the ability of anticancer drugs, including doxorubicin, oxaliplatin, and CTX, to induce immunogenic tumor cell death is associated with elicitation of host antitumor immune responses (2–5). We therefore hypothesized that melphalan treatment may lead to the activation of host immune cells. To test this, mice with established A20 tumors (~100 mm\(^2\)) were treated with a single dose of melphalan (9 mg/kg). Seven days after treatment, spleen and tumor samples were collected for T cell phenotypic and functional analyses. Fig. 5A shows the representative FACS data for intratumoral CD8\(^{+}\) T cells, and the results are summarized in Fig. 5B. The tumor-infiltrating endogenous CD8\(^{+}\) T cells in melphalan-treated mice were more proliferative, as reflected by increased Ki67 level, than their counterparts in untreated mice (Fig. 5, first column). Additionally, these intratumoral CD8\(^{+}\) T cells had significantly increased expression of a set of markers indicative of immune activation, including CD25, effector memory cell markers (CD44\(^{+}\)CD62L\(^{−}\)), and cytolytic molecule granzyme B (Fig. 5, second, third, and fourth columns, respectively). However, these CD8\(^{+}\) T cells appeared to have higher expression of PD-1, a coinhibitory receptor associated with T cell exhaustion, although the differences between treated and untreated mice were not statistically significant (Fig. 5, fifth column). Furthermore, CD8\(^{+}\) T cells in melphalan-treated mice did not produce significantly more IFN-\(\gamma\) than did their counterparts in untreated mice (Fig. 5, last column). Collectively, these phenotypical and functional changes in CD8\(^{+}\) T cells were indicative of a suboptimal activation. Similar immune profiles were observed in CD8\(^{+}\) T cells from the spleens of melphalan-treated mice, but the extent of activation exhibited by splenic CD8\(^{+}\) T cells was less prominent compared with intratumoral CD8\(^{+}\) T cells (data not shown). Of note, melphalan treatment did not cause significant phenotypical or functional changes in endogenous CD4\(^{+}\) T cells (data not shown).

**Melphalan treatment allows adoptively transferred tumor-specific CD4\(^{+}\) T cells to develop into polyfunctional effector cells**

We previously reported that CTX can create an immunogenic milieu that allows adoptively transferred tumor-specific CD4\(^{+}\) T cells to differentiate into polyfunctional effector cells (32, 33). In this study, we showed that melphalan resembled CTX in provoking ICD, eliminating Tregs, and inducing lymphopenia and cytokine/chemokine release, features known to contribute to CTX’s immune-potentiating effects. These similarities prompted us to examine the impact of melphalan on adoptively transferred tumor-specific CD4\(^{+}\) T cells. We first treated tumor-bearing mice with varying amounts of melphalan to examine the dose effect of melphalan on the expansion of adoptively transferred tumor-specific CD4\(^{+}\) T cells. Melphalan caused severe morbidity in mice at the dose of 12 mg/kg and was lethal at 27 mg/kg (data not shown). Melphalan at 9 mg/kg was well tolerated in mice. At this dose, both the frequency and absolute number of the
transferred CD4^+ T cells increased significantly (Fig. 6A), indicating a robust expansion of the tumor-specific CD4^+ T cells. The immune-potentiating effects on donor CD4^+ T cells were diminished when melphalan was used at ≤3 mg/kg. Based on the results, melphalan was used at 9 mg/kg in subsequent experiments.

Our previous work using the same model system showed that tumor-specific CD4^+ T cells transferred into untreated tumor-bearing mice acquired a dysfunctional phenotype, and CTX can rescue aberrant T cell differentiation and restore a polyfunctional effector phenotype in transferred CD4^+ T cells (32). In the present study, we characterized the phenotype of the tumor-specific CD4^+ T cells transferred into melphalan-treated mice and compared it to that of CD4^+ T cells either transferred into untreated or CTX-treated tumor-bearing mice. Fig. 6B shows that CD4^+ T cells transferred into melphalan-treated hosts displayed a similar phenotype as did those cells in CTX-treated hosts, that is, extensive cell division and clonal expansion, downregulation of PD-1 and Foxp3, upregulation of CD40L, and increased cytokine production. As summarized in Fig. 6C, the differences between the CD4 only group and melphalan plus CD4 group were statistically significant in all parameters measured. Compared to melphalan, CTX appeared to drive stronger cell division, lower PD-1 and Foxp3 expression, and increase CD40L expression and cytokine production, but the differences did not reach statistical significance. Collectively, our data indicate that melphalan resembles CTX in

FIGURE 4. Melphalan administration enhances the uptake of tumor-associated Ag by DCs in the draining LN. As depicted in the schema, A20^EGFP^ tumor cells were s.c. inoculated to the flank of CD45.1^+^ congenic BALB/c mice. Mice with palpable tumors (~50 mm^2^) were either untreated or melphalan treated. Forty-eight hours later, TDLNs and nondraining cervical/axillary LNs were harvested. Cells were stained with mAbs specific for CD45.1, CD11c, and MHC-II and subjected to FACS analysis to evaluate the uptake of tumor cell–derived GFP by host cells. (A) Melphalan treatment leads to enhanced GFP uptake by host DCs in the TDLN. GFP signals in host DCs (CD45.1^+^CD11c^+^MHC-II^+^) under indicated conditions are shown in representative dot plots. Numbers in plots represent the percentage of cells in the indicated region. (B) Summary of percentage of GFP^+^ DCs in the TDLN in untreated versus treated mice. Data are shown as means ± SD of three samples per group.

FIGURE 5. Melphalan treatment results in activation of the endogenous CD8^+^ T cells. Mice with established s.c. A20 tumors were treated or not treated with melphalan. Seven days later, mice were killed and tumor masses were harvested for analysis. (A) Melphalan treatment induces phenotypic and functional changes in tumor-infiltrating CD8^+^ T cells. Representative dot plots are shown to reflect the changes in Ki67, CD25, CD44, CD62L, granzyme B (GzmB), and PD-1 expression profiles in CD8^+^ T cells after melphalan treatment. Cytokine production by host CD8^+^ T cells was determined by intracellular cytokine staining after PMA and ionomycin stimulation in vitro. Numbers in plots indicate the percentage of the gated cells. (B) Summary of results shown in Fig. 4A. Data shown as means ± SD are pooled from two independent experiments with four to six mice per group. *p < 0.05.
promoting the acquisition of a polyfunctional effector phenotype in tumor-specific CD4+ T cells following adoptive transfer.

To test whether melphalan can potentiate CD4+ T cell ACT in other tumor models, the melphalan plus CD4 regimen was applied to mice bearing established HA-expressing CT26 colorectal tumors (CT26HA). Inoculation of CT26HA cells via tail vein led to the outgrowth of tumors exclusively in the lungs. Analogous to the results seen in the A20HA tumor model, melphalan treatment led to extensive proliferation of the transferred CD4+ T cells in the lungs and relatively mild proliferation in the spleen (Fig. 7A). Melphalan also promoted the generation of polyfunctional effector cells, marked by elevated levels of CD40L and the ability to simultaneously produce multiple proinflammatory cytokines (Fig. 7B). Similar results were also obtained in the renal cell carcinoma model (Renca) and 4T1 mammary carcinoma model (data not shown). The results suggest that the immune-potentiating effects of melphalan on CD4+ T cells are not restricted to hematologic malignancies.

The combination of melphalan and CD4+ T cell ACT exhibits synergistic antitumor effects

We showed that melphalan as monotherapy can elicit immune responses in endogenous T cells, but the immune activation was suboptimal and restricted to CD8+ T cells (Fig. 5). The results suggested that the antitumor immunity elicited by chemotherapy alone may not be sufficient to induce regression of advanced tumors, and additional immunological maneuvers are needed to achieve durable therapeutic outcome. Given that melphalan treatment allowed adoptively transferred tumor-specific CD4+ T cells to undergo robust expansion and develop into polyfunctional effector cells (Figs. 6, 7), we posited that the combination of melphalan and CD4+ T cell ACT may confer enhanced antitumor effects compared with either treatment alone. To test this, mice with advanced A20HA tumors (∼100 mm³) were subjected to one of four conditions: no treatment, CD4+ T cell ACT only, melphalan only, and the combination of melphalan and CD4+ T cell ACT. Fig. 8A shows that CD4+ T cell ACT only had a negligible effect on tumor growth;
melphalan only retarded tumor growth initially but tumors regrew rapidly thereafter. In contrast, the combination of melphalan and CD4 ACT led to significant tumor regression and marked prolongation of mouse survival. The enhanced antitumor effect of the melphalan plus CD4 ACT regimen was recapitulated in the CT26HA tumor model. Fig. 8B shows that CT26HA tumor growth was not altered by CD4 ACT, and it was only modestly delayed by melphalan treatment but was significantly inhibited by the combination of melphalan and CD4 ACT. Taken together, the data suggest that melphalan can effectively facilitate CD4⁺ T cell adoptive immunotherapy, and the synergy can result in augmented efficacy against advanced cancer.

**Discussion**

High-dose melphalan followed by ASCT has improved the overall survival of patients with multiple myeloma (44) and is now regarded as a standard treatment for this disease. High-dose chemotherapy induces severe and persistent immunosuppression characterized by delayed T cell recovery, restricted T cell repertoire, and impaired T cell functions (35, 45). Although high-dose
melphalan has been perceived as an immunosuppressant, melphalan used at a low dose (0.75 mg/kg in mice) appeared to exert immune-potentiating effects as evidenced by its ability to upregulate B7.1 and B7.2 costimulatory molecules on tumor cells and APCs, leading to the acquisition of potent CD8+ T cell–mediated antitumor immunity capable of eradicating large established tumors (46–49). The present study provides novel evidence that at relatively high dose (9 mg/kg in mice), melphalan can induce ICD, enhance tumor Ag uptake by DCs, eliminate Tregs, and induce transient lymphopenia along with a burst of proinflammatory cytokines/chemokines, and that these immunomodulatory effects can be exploited to potentiate adoptive immunotherapy using CD4+ T cells.

Our results reveal that in many aspects melphalan resembles CTX, one of the most studied anticancer drugs known to possess immunomodulatory properties. At very high doses (>300 mg/kg for CTX, >20 mg/kg for melphalan), both drugs cause severe immunodeficiency that is fatal to mice. At medium–high doses (100–300 mg/kg for CTX, 6–12 mg/kg for melphalan), the two drugs induce myelodepletion and leukodepletion followed by homeostatic cell recovery, which coincides with ICD induction, Treg reduction, and cytokine/chemokine release (“cytokine storm”), fostering an immunogenic milieu that favors adoptive T cell immunotherapy. At metronomic doses (10–40 mg/kg for CTX, 0.75 mg/kg for melphalan), CTX selectively depletes Tregs (50) whereas melphalan increases the immunogenicity of tumor cells by inducing B7.1/B7.2 costimulatory molecules (46, 47). It is not surprising that melphalan and CTX share these similarities, because they both are nitrogen mustard agents and thus have the same mechanism of action. However, there are differences in their immunomodulatory activities. Compared to CTX, melphalan appeared to induce longer duration of lymphodepletion as reflected by a slower recovery of B cells and T cells (Fig. 1B). Moreover, melphalan had a modest effect on macrophages and pDCs whereas CTX led to eventual expansion of these two populations (Fig. 1C). Furthermore, consistent with the fact that pDCs are major producers of type I IFNs, type I IFNs were amply induced after CTX (51), but not upregulated after melphalan treatment. Note that although both melphalan and CTX are alkylating agents, several other alkylating agents, including bendamustine, chlorambucil, carmustine, and temozolomide, were not effective because they both are nitrogen mustard agents and thus have the same mechanism of action. However, there are differences in their immunomodulatory activities. Compared to CTX, melphalan appeared to induce longer duration of lymphodepletion as reflected by a slower recovery of B cells and T cells (Fig. 1B). Moreover, melphalan had a modest effect on macrophages and pDCs whereas CTX led to eventual expansion of these two populations (Fig. 1C). Furthermore, consistent with the fact that pDCs are major producers of type I IFNs, type I IFNs were amply induced after CTX (51), but not upregulated after melphalan treatment. Note that although both melphalan and CTX are alkylating agents, several other alkylating agents, including bendamustine, chlorambucil, carmustine, and temozolomide, were not effective in potentiating CD4 ACT in our model system (data not shown). Therefore, the ability of an anticancer drug to potentiate adoptive immunotherapy may not be a common feature of alkylating agents, but rather is pertinent to the unique combination of certain immunomodulatory effects exerted by the drug, that is, induction of ICD, transient lymphopenia, and cytokine release.

Our study suggests that melphalan can be used, as an alternative to CTX, in combination with adoptive immunotherapy, especially in settings where melphalan is the current standard of care. Supporting this notion, Condomines et al. (52) indicated that the early lymphodepletion period after high-dose melphalan and ASCT in myeloma patients represents a window for adoptive immunotherapy. Along this line, it has been shown that posttransplant infusion of in vivo vaccine-primed and ex vivo–costimulated autologous T cells improved CD4+ and CD8+ T cell counts and led to the induction of clinically relevant immunity in myeloma patients (53, 54). We showed in the present study that although melphalan monotherapy only resulted in activation of CD8+ but not CD4+ T cells in the endogenous repertoire, it fostered an immune milieu highly favorable for adoptive transfer of tumor-specific CD4+ T cells. Note that although the melphalan plus CD4 ACT regimen exhibited strong antitumor effects initially (Fig. 8), nearly all mice had relapse eventually. We recently reported that CTX can induce the expansion of immunosuppressive monocytes (CD11b+Ly6C+Ly6G−) and that depletion/inactivation of these monocytes can augment the efficacy of CTX plus CD4 ACT treatment (55). We confirmed in the present study that both melphalan and CTX can induce the expansion of monocytes (Fig. 1C). It is conceivable that durable antitumor effects can be achieved when monocyte-targeting strategies, such as low-dose gemcitabine, anti-CCR2 mAb, or PD-1 blockade, are applied following melphalan plus CD4 ACT.

It is noteworthy that melphalan’s immune-potentiating effect on CD4 ACT is not limited to B cell lymphoma, but it is also applicable to carcinomas, including CT26, renal cell carcinoma (Renca), and 4T1 (Figs. 7, 8 and data not shown). Because CT26HA tumor cells are unable to express MHC-II molecules (33), and thus activation of the transferred HA-specific CD4+ T cells must be mediated through the host APCs, most likely DCs. In the present study, we provide evidence that melphalan administration induces extracellular release of HMGB1, membrane translocation of CRT on tumor cells, and enhances the uptake of tumor-associated Ag by DCs. These data support a scenario whereby melphalan-induced HMGB1 attracts immature DCs into the draining LN; meanwhile, surface CRT expression on tumor cells presents an eat-me signal for phagocytosis by immature DCs, and the proinflammatory milieu induced by melphalan enhances DC maturation and Ag 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 MHC-II+ tumors through multiple mechanisms (56). Our results imply that the melphalan plus CD4 ACT treatment strategy may have broader applicability beyond hematological malignancies.

In summary, our study demonstrates that melphalan at cytotoxic doses can exert multifaceted immunomodulatory effects, which correlate with its ability to potentiate the efficacy of adoptive immunotherapy using antitumor CD4+ T cells. Our data provide a preclinical rationale for the use of melphalan in the conditioning regimen for adoptive immunotherapy and underscore the therapeutically promising potential of this combination strategy. These findings may have implications for the treatment of hematological malignancies as well as solid tumors.

Acknowledgments
We thank W. King at the Georgia Regents University Cancer Center Flow Cytometry Core Facility for technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References
MELPHALAN POTENTIATES CD4+ T CELL ADOPTIVE THERAPY


SUPPLEMENTAL FIGURE 1. The impact of G-CSF on immune cell recovery after melphalan treatment. Naïve BALB/c mice were treated with melphalan (9 mg/kg) on day 0. On day 1, half of the mice received G-CSF (6 µg) i.p. injection. G-CSF was administered every other day thereafter. Mice were sacrificed for analysis on day 10. Spleen cells were enumerated. Aliquots of cells were stained with mAbs and the frequencies of specific cell populations were assessed by flow cytometry. Bar graphs summarize total cell numbers in the spleens and numbers of the indicated immune cell population. The absolute numbers of each cell population were calculated as: total spleen cell count x frequency of specific cell population. Results are shown as mean ±SD of at least 3 mice each group.