T-cell Exhaustion in Multiple Myeloma Relapse after Autotransplant: Optimal Timing of Immunotherapy

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Abstract

Multiple myeloma is the most common indication for high-dose chemotherapy and autologous stem cell transplantation (ASCT), and lenalidomide maintenance after transplant is now standard. Although lenalidomide doubles progression-free survival, almost all patients eventually relapse. Posttransplant immunotherapy to improve outcomes after ASCT therefore has great merit but first requires delineation of the dynamics of immune reconstitution. We evaluated lymphocyte composition and function after ASCT to guide optimal timing of immunotherapy and to identify potential markers of relapse. Regulatory T cells (Treg) decline as CD8+ T cells expand during early lymphocyte recovery after ASCT, markedly reducing the Treg:CD8+ effector T-cell ratio. These CD8+ T cells can respond to autologous dendritic cells presenting tumor antigen in vitro as early as day +12 after transplant, becoming antigen-specific cytolytic T-lymphocyte effectors and thereby demonstrating preservation of cellular reactivity. CD4+ and CD8+ T cells express the negative regulatory molecules, CTLA-4, PD-1, LAG-3, and TIM-3, before and after ASCT. A subpopulation of exhausted/senescent CD8+ T cells, however, downregulates CD28 and upregulates CD57 and PD-1, characterizing immune impairment and relapse after ASCT. Relapsing patients have higher numbers of these cells at +3 months after transplant, but before detection of clinical disease, indicating their applicability in identifying patients at higher risk of relapse. PD-1 blockade also revives the proliferation and cytokine secretion of the hyporesponsive, exhausted/senescent CD8+ T cells in vitro. Collectively, these results identify T-cell exhaustion/senescence as a distinguishing feature of relapse and support early introduction of immunotherapy to stimulate antitumor immunity after ASCT.

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Introduction

High-dose chemotherapy followed by autologous stem cell transplantation (ASCT) can produce complete responses (CR) in up to one third of patients with multiple myeloma (1). Nevertheless, most patients achieving a CR after ASCT eventually relapse, and in patients who fail to attain a CR, progression of disease is inevitable (2). This has remained true even with lenalidomide maintenance therapy, which is now standard after ASCT and extends progression-free survival from approximately 2 to 4 years (3–5).

The immune system participates in the control of multiple myeloma, whereas compromised immunity contributes to its evolution. Myeloma-reactive T cells are present in active disease (6–9) and correlate with disease burden (9). The expansion of T-cell clones after ASCT (10) and the emergence of antigen-specific T cells after allogeneic stem cell transplantation (11, 12) are associated with improved clinical outcomes. The loss of tumor-specific T cells characterizes progression from the benign precursor condition, monoclonal gammopathy of undetermined significance (MGUS), to multiple myeloma (13, 14). Malignant plasma cells themselves also evade immune surveillance by various mechanisms (15–19), including the upregulation of PD-L1 (17–19).

Incorporating immune-based therapies into post-ASCT treatment regimens to induce or restore antitumor immunity offers a promising approach to target residual multiple myeloma. The minimal residual disease state and lymphopenia after ASCT afford a unique platform for promoting antitumor immune responses by limiting tumor-driven immunosuppression (20), eliminating cytokine sinks (21), and transiently depleting regulatory T cells (Treg, ref. 22). Posttransplant reconstitution of immune cell subsets, however, occurs with disparate kinetics that can affect the outcome of immunotherapy. The immunomodulatory effect of lenalidomide maintenance therapy on the dynamics of immune recovery also remains undefined.

The rational development of immunotherapeutic interventions after ASCT, in which relapse remains the primary cause of...
treatment failure, requires a comprehensive understanding of the immunologic milieu. We therefore performed a prospective analysis of immune reconstitution in 55 multiple myeloma patients undergoing ASCT and lenalidomide maintenance therapy to define patterns of lymphocyte recovery and to identify immunologic markers of relapse, which could elucidate potential targets for converting durable responses to long-term cures.

Materials and Methods

Patients

Fifty-five patients were evaluated (Table 1). All patients received pre-ASCT induction regimens that included bortezomib and/or lenalidomide, with 40 (72%) patients receiving both agents. All patients were conditioned for ASCT with high-dose melphalan (140 mg/m² or 200 mg/m², depending on age and comorbidity risk) and received post-ASCT lenalidomide maintenance therapy. Disease status was assessed for response to induction therapy within 30 days before ASCT, at 3 and 12 months after ASCT, and at the time of relapse where applicable. Myeloma response was assessed using International Myeloma Working Group (IMWG) criteria (23).

Blood samples

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Ficoll-Paque PLUS (GE Healthcare) from peripheral blood or leukocyte concentrates from patients and healthy volunteers. Biospecimen sample collection and use adhered to protocols approved by the Institutional Review and Privacy Board of Memorial Hospital, Memorial Sloan Kettering Cancer Center (MSKCC). Leukocyte concentrates (buffy coats) purchased from the Greater New York Blood Center, American Red Cross, were also used as a source of healthy donor cells.

Flow cytometric analysis

PBMCs were incubated with fluorochrome-conjugated mAbs and analyzed on either an FC 500 (Beckman Coulter) or an LSRFortessa (Becton Dickinson) flow cytometer. FITC-, PE-, PE-Texas Red–, ECD-, APC-, PE-Cy7–, PerCP-Cy5.5–, Pacific Blue–, and AF700-conjugated mouse anti-human mAbs included anti-CD3, anti-CD4, anti-CD8, anti-CD11c, anti-CD14, anti-CD16 (clone 3G8), anti-CD19, anti-CD25, anti-CD28, anti-CD45RA, anti-CD45RO, anti-CD80, anti-CD86, anti-CD123, anti–CTLA-4, anti–HLA-DR, anti–IL2, anti–Ki-67 (BD Pharmingen), anti–CD56, anti–CD83 (Beckman Coulter), anti–CD127, anti–IFNγ, anti–IL-2, anti–PD-1, anti–PD-1, anti–TNFα (eBioscience), anti–CCR7, anti–TIM-3 (R&D Systems), and anti–CD57 (BioLegend). Nonreactive isotype-matched antibodies (Becton Dickinson; eBioscience; R&D Systems) were used as controls. The LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies) facilitated exclusion of dead cells. Gates were set for collection and analysis of at least 20,000 live events. Data were analyzed with FlowJo 9.5 software (TreeStar).

Generation of monocyte-derived dendritic cells and isolation of T lymphocytes

Monocyte-derived dendritic cells (MoDC) were generated from PBMCs using media, media supplements, and cytokines, exactly as published (24). Xenogeneic plasma or serum (e.g., FCS) was never used in any cultures. T cells were obtained from tissue culture plastic-nonadherent PBMCs, further purified by elution from nylon wool columns (Polysciences), achieving >90% purity.

Allogeneic mixed leukocyte reactions and PD-1 blockade experiments

MoDCs were added in serial doses (1:30 to 1:3,000, moDC:T) to triplicate wells of 1 × 10⁵ allogeneic T cells in 96 round-well plates. CD28+ and CD28- T cells were primed for 3 days with 1 μg/ml anti-CD3, anti-CD28, pH7.2 PBS, or medium alone. Cells were washed and mixed in a 1:1 ratio with CD28+ or CD28- T cells and restimulated for 72 hours in 96-well plates. The supernatant was assayed for IFNγ and IL-2 activity using cytokine-ELISA kits.

Table 1. Patient characteristics

<table>
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<td>CR</td>
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Abbreviations: ISS, International Staging System; POD, progression of disease; PR, partial response; VGPR, very good partial response.
bottomed well plates (Corning Life Sciences). Final volume was 100 μL/well in RPMI with 10% heat-inactivated, pooled normal human serum. For PD-1 blockade, cells were cultured at a 1:30 moDC:T-cell ratio with nivolumab (10 μg/mL final; Bristol-Myers Squibb) or IgG4 isotype control added at culture initiation. Responder allogeneic T-cell proliferation was measured after 5 days by a colorimetric assay according to the manufacturer’s instructions (CellTiter96 Aqueous One Solution Cell Proliferation Assay MTS; Promega) or by intracellular staining with Ki-67 (BD Pharmingen).

mRNA electroporation of moDCs

WT1 mRNA transcription was performed exactly as published (25, 26). Immature moDCs were electroporated with WT1 mRNA on days 5 to 6. After electroporation, cells were immediately transferred to culture and terminally matured by exposure to inflammatory cytokines for 48 hours (24).

Cytolytic T lymphocyte assays

Mature WT1 mRNA-electroporated autologous moDCs were added in serial doses to triplicate wells containing 1 × 10^5 T cells in a 96 round-bottomed well plate (Corning Life Sciences). Final volume was 100 μL/well of RPMI-10% heat-inactivated, autologous serum, supplemented with recombinant human IL-15 (10 ng/mL; R&D Systems). After 7 days of moDC-T cell culture, 5 × 10^5 target cells were added directly to each well, and cytolytic activity exerted by responder T lymphocytes was assessed after 4 to 6 hours with a colorimetric cytolytic T lymphocyte (CTL) assay (27). These data represented the total cytolytic activity generated in each culture according to the primary stimulation conditions, rather than per number of effector T cells irrespective of their frequency in the primary cultures. Target cells were 697 cells (HLA-A*0201+, WT1+ cell line). SKYL-16 cells (HLA-A*0201+, WT1* cell line) served as a negative control.

Statistical analysis

Descriptive and graphical measures were used to characterize leukocyte subpopulation patterns both longitudinally and by disease status following ASCT. Unpaired t tests were used to explore mean differences in expression across disease categories, whereas paired t tests were used to compare across time points and treatment conditions. A P value less than 0.05 was considered statistically significant. All statistical analyses were calculated using Prism 6 software (GraphPad).

Results

Kinetics of lymphocyte reconstitution in multiple myeloma patients after ASCT

We evaluated absolute lymphocyte count (ALC) after ASCT to determine the kinetics of lymphocyte reconstitution. ALC nadir occurred at day +5, followed by early recovery at day +12 (Fig. 1A) and complete recovery by day +30 (Fig. 1B). Reconstitution of CD8+ T cells, however, outpaced that of CD4+ T cells, most likely due to the homeostatic proliferation of peripheral T cells that phenotypically resemble memory cells after chemotherapy-induced lymphopenia (28). This resulted in an inverted CD4/CD8 ratio lasting up to 1 year (Fig. 1B). CD4+CD45RO+ memory T cells represented the majority of CD4+ T cells at day +12 (Fig. 1C; 61.11% ± 3.27%), whereas CD4+CD45RA- naive T cells remained low at 1 year (Fig. 1C; 10.13% ± 1.5%). CD8+ CCR7-CD45RO+ effector memory and CD8+CCR7+CD45RO+ central memory cells comprised the majority of CD8+ T cells at day +12 (Fig. 1D; 39.26% ± 2.8% and 35.75% ± 3.15%, respectively), with low levels of CCR7+CD45RO- effector memory (29.21% ± 2.02%) and CCR7+CD45RO- naive CD8+ T cells present at 1 year (Fig. 1D; 8.81% ± 1.79%). Natural killer (NK) cells (CD3negCD56+CD16+ and CD3negCD56negCD16+) exhibited rapid and sustained recovery after ASCT (Fig. 1E). The recovery of CD19+ B cells lagged in comparison with the other lymphocyte subsets but recovered by 3 months (Fig. 1E). Plasmacytoid dendritic cells (CD123+DR+CD11c+CD14-CD16+) were present at similar levels before and after ASCT (Fig. 1E). Subgroup analysis based on 3-month post-ASCT disease response (i.e., partial response vs. very good partial response vs. CR) revealed no statistically significant differences in the pattern of lymphocyte reconstitution between groups (data not shown).

Regulatory T cell-to-CD8+ effector ratio declines in the early post-ASCT period

The balance between Tregs and effector T cells shapes antitumor immune responses and the efficacy of immune-based interventions (29). We compared CD3+CD4+CD25+highCD127low Tregs with CD3+CD8+CD25+ effector T cells after ASCT. As shown in Fig. 1F, the Treg:CD8+ effector T-cell ratio at day +12 (0.59 ± 0.21) was significantly lower than before transplantation (1.04 ± 0.23; P < 0.05) or day +30 after transplant (1.51 ± 0.27; P < 0.001). Tregs therefore decline early after nadir as CD8+ T-cell recovery occurs, resulting in a markedly lower Treg:CD8+ effector T-cell ratio and providing a critical early window for the introduction of immune-based posttransplant consolidation therapies.

Dendritic cells from multiple myeloma patients after ASCT, irrespective of disease status, induce autologous antigen-specific CTLs comparable with those stimulated by healthy donor dendritic cells

In the nontransplant setting, there are reports of defective dendritic cell (DC) function in multiple myeloma (30, 31). To evaluate the integrity of DCs from patients after transplant, moDCs were generated from PBMCs (24) from patients in CR 3 months after transplant, patients who relapsed after transplant, and healthy donors. DCs were assessed by flow cytometry for the upregulation of the prototypical DC maturation marker, CD83 (32). Neither posttransplant status nor disease relapse impaired terminal, inflammatory cytokine–induced maturation (Fig. 2A). We then evaluated the functional capacity of these moDCs in the allogeneic mixed leukocyte reaction (alloMLR), which is a standard assay of overall DC immunogenicity (24). MoDCs from each group stimulated comparably vigorous proliferation of allogeneic T cells (Fig. 2B).

We also assessed the generation of antigen-specific CTLs by mRNA-electroporated moDCs from patients who had undergone ASCT for multiple myeloma. MoDCs were electroporated with WT1 mRNA and then matured and activated with inflammatory cytokines for 48 hours (25, 26). The resulting mature moDCs were added in serial 3-fold dilutions to a fixed number of purified autologous T cells obtained before and after ASCT (days +12, 30, and 90). Cultures were supplemented with recombinant human IL-15, because unlike CD34+ HPC-derived Langerhans-type DCs, moDCs do not provide sufficient endogenous IL-15 for CTL stimulation (25). Autologous WT1 mRNA-electroporated moDCs plus IL-15 stimulated potent antigen-specific CTLs after
only 7 days' stimulation, demonstrating that active cellular immune responses can be elicited in vitro as early as day +12 from multiple myeloma patients after ASCT (Fig. 2C). We therefore conclude that the DC compartment causes no alteration in immune responsiveness in multiple myeloma patients after ASCT.

T cells retain immune checkpoint receptor expression after ASCT

Immune checkpoint pathways can impede antitumor immune responses and contribute to persistent and/or relapsed malignancy (33). To assess this key regulatory axis in the setting of ASCT, we compared T cells before and after ASCT for expression of the inhibitory receptors CTLA-4 (Fig. 3A), PD-1 (Fig. 3B), LAG-3 (Fig. 3C), and TIM-3 (Fig. 3D). CD4+ and CD8+ T cells maintained the expression of each inhibitory receptor at 3 and 12 months after transplant, with CTLA-4 and PD-1 showing higher overall expression than LAG-3 and TIM-3. These findings provide a rationale for the inclusion of checkpoint inhibition to augment T-cell responses after ASCT.

Increased Tregs and decreased NK cells are associated with relapse after ASCT

Treg expansion has been implicated in multiple myeloma pathogenesis (34, 35) and is associated with inferior survival (36). Early NK-cell reconstitution correlates with improved progression-free survival after ASCT (37), and impaired NK-cell function correlates with multiple myeloma progression (38). We compared patterns of Treg and NK-cell content in patients who remained in a continuous CR 1 year after ASCT with a subset of patients who initially achieved a CR at 3 months but subsequently relapsed compared with nonrelapsed patients, 1.65 ± 0.25 and 0.25 ± 0.13, respectively (Fig. 4A). Treg:CD8+ effector ratio was also increased in relapsed compared with nonrelapsed patients, but did not reach statistical significance (data not shown). The fold increase in NK cells was greater in patients who remained in a continuous CR compared with relapsed patients, 1.65 ± 0.25 and 0.94 ± 0.13, respectively (Fig. 4B).

CD8+ T-cell exhaustion and/or senescence distinguish relapse after ASCT

T-cell exhaustion and senescence occur under conditions of chronic antigen stimulation and contribute to impaired immune responses (39, 40). Although investigators have not fully distinguished the molecular characteristics of exhaustion and senescence (41), cell surface markers can be used to segregate these T-cell states phenotypically. Downregulation of the costimulatory
Figure 2.
DCs generated from multiple myeloma patients after ASCT induce autologous antigen-specific CTLs. A, cytokine-matured, moDCs generated from PBMCs from healthy donors, patients in CR after ASCT, and patients who relapsed after ASCT were compared for expression of the maturation marker, CD83. Representative dot plots of mature moDCs from each group are shown, along with pooled data (mean ± SD, n = 3 independent experiments). B, mature moDCs generated from these same 3 groups were added as stimulators in graded doses to a fixed number of allogeneic T-cell responders from healthy volunteers (alloMLRs). DC:T-cell ratios ranged from 1:30 to 1:3,000. T-cell proliferation was measured by a flow cytometry-based colorimetric assay (triplicate mean ± SEM, n = 3 independent experiments). C, MoDCs generated from peripheral blood obtained on day +90 after ASCT were electroporated with WT1 mRNA, terminally matured and activated by a combination of inflammatory cytokines (24), and then added in serial doses to triplicate microwells each containing 1 x 10^5 autologous T cells obtained before and after ASCT (days +12, 30, and 90), in the presence of exogenous recombinant human IL15 for 7 days. Antigen-specific target cell lysis by CTLs stimulated by these WT1 mRNA-electroporated moDCs was evaluated using a flow cytometry-based assay. Target cells were 697 cells (HLA-A^*0201^+, WT1^+^ cell line). SKLY-16 cells (HLA-A^*0201^+, WT1^neg^ cell line) served as a negative control. Specific lysis is plotted against the y axis, comparing the lysis activity of T cells from the indicated time points before and after ASCT, after stimulation by WT1 mRNA-electroporated moDCs (triplicate mean ± SEM, n = 3 independent experiments).

Figure 3.
ASCT does not alter T-cell expression of inhibitory receptors. PBMCs were analyzed by flow cytometry for expression of the inhibitory receptors CTLA-4 (A), PD-1 (B), LAG-3 (C), and TIM-3 (D) on CD4^+^ and CD8^+^ T cells before (white bar) and 3 months (gray bar) and 12 months (black bar) after ASCT. Pooled data (mean ± SD) from 10 patients are shown.
marker, CD28, is a feature of both exhaustion and senescence (41). Upregulation of the glycoprotein CD57 can be used to identify T cells with low proliferative capacity (42). Senescent cells can also express PD-1, a prototypical marker of exhaustion (39, 40). Using these markers, we assessed the pattern of T-cell exhaustion/senescent cells at the time of relapse than did patients remaining in a continuous CR at 12 months. Of note, patients in the relapsed group had higher baseline levels of CD8+CD28negPD-1+ T cells at the time of relapse than did patients remaining in a continuous CR at 12 months. These findings indicate that impaired immune function due to exhaustion and/or senescence may contribute to relapse after ASCT. The senescence/exhaustion T-cell phenotype could also provide a predictive marker supporting earlier intervention to prevent relapse.

**PD-1 blockade activates exhausted/senescent CD8+ T cells in vitro**

Using cells from patients in continuous CR 1 year after ASCT and from patients who initially achieved CR but relapsed beyond 3 months after ASCT, we tested the capacity of CD8+CD28negPD-1+ T cells to respond to stimulation by moDCs derived from healthy donors in alloMLRs as a generic response assay, with and without the PD-1 inhibitor, nivolumab. In all cases, PD-1 inhibition increased CD8+CD28negPD-1+ T-cell proliferation (Fig. 6A; 61.33% ± 10.58% vs. 27.82% ± 6.78% for isotype control; P < 0.01). Nivolumab treatment also induced a concomitant increase in the secretion of the cytokines IFNγ, IL2, and TNFα. These findings demonstrate augmented responsiveness of a significant fraction of cells in the setting of PD-1 inhibition, thus underscoring the potential benefit of checkpoint blockade to enhance/restore T-cell responses in this patient population.

**Discussion**

This study identifies several key immunologic parameters of immune reconstitution and relapse in multiple myeloma after ASCT. Lymphocyte recovery from high-dose chemotherapy-induced lymphopenia relies primarily on the peripheral expansion of memory cells with only a minor contribution from their thymic-derived naïve counterparts. The early posttransplant period is marked by a transient decline in the Treg-to-effector T-cell ratio, which reverts to pretransplant levels by 1 month. DCs are functionally intact, comparable with those from healthy donors, and induce autologous antigen-specific T cells with robust lytic activity in vitro. CD4+ and CD8+ T cells retain immune-inhibitory receptor expression after transplant, however. A significantly increased subset of these T cells with an exhausted/senescent phenotype and reversible hyporesponsiveness to PD-1 inhibition characterizes patients who relapse after transplant. Increased Tregs and decreased NK cells further characterize immune dysfunction in these patients.

Consistent with previous reports (28), disruption of the normal balance of immune cells with a bias toward T-cell memory phenotypes, protracted diminution of naïve T-cell output from the thymus, and inversion of CD4+/CD8+ T-cell ratios characterize the immune reconstitution in our study population. Other groups have shown that T-cell repertoire diversity of expanding lymphocytes after lymphopenia is limited in the absence of adequate thymopoiesis (43, 44). These changes contribute to a state of prolonged posttransplant immune deficiency and predispose patients to infections and compromised antimicrobial immunity.

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**Figure 4.**

Treg and NK-cell trends in nonrelapsed and relapsed patients after ASCT. A and B, PBMCs from patients who remained in a continuous CR 1 year after ASCT (CCR; n = 15) and patients who initially achieved a CR but relapsed beyond 3 months after ASCT (relapsed; n = 14) were compared for Treg (CD3+CD4+CD25brightCD127neg) and NK-cell (CD3−CD56dimCD16neg) content. The percentage of Tregs or NK cells was determined by flow cytometry at 3 and 12 months after ASCT, or at the time of relapse. Interval fold change was calculated by comparing Treg (A) or NK-cell (B) numbers at the 12-month or relapse time point with values from the 3-month time point when all patients were in a CR. Pooled data (mean ± SD) are shown. *, P < 0.05.
surveillance (28), but the specific dynamics of T-cell recovery in the context of our current understanding of exhausted/senescent T cells and checkpoint inhibitors have not been defined.

During initial lymphocyte recovery, the Treg:CD8<sup>+</sup> effector T-cell ratio is significantly reduced. This shift provides an opportunity to stimulate maximal antitumor responses in the
absence of Treg-mediated suppression. DC-based vaccination in this setting offers one approach to redirect recovering T cells toward specific MHC-restricted antigen. Our data show the induction of potent autologous antigen-specific CTLs by moDCs supplemented with IL15 at day +12 after transplant, thus confirming preservation of DC and T-cell function in vitro. Non–DC-based pre-ASCT vaccination supplemented with the adoptive transfer of ex vivo–expanded T cells promotes accelerated reconstitution of immune responses against both microbial and multiple myeloma tumor antigens in vivo (45, 46), supporting the concept of immunotherapy in the setting of posttransplant lymphopenia. We are testing the feasibility of early posttransplant vaccination using autologous Langerhans-type DCs (LC) electroporated with mRNA encoding three multiple myeloma–associated antigens in a phase I clinical trial (NCT01995708). LCs provide sufficient endogenous IL15, obviating the need for IL15 supplementation that moDCs require to induce CTLs (25). Adoptive T-cell infusions should not be necessary in the setting of DC-based vaccines, unless perhaps used in a prime-boost sequence.

Inhibitory receptor blockade augments vaccine-induced T-cell responses (19, 47), reviving antigen-reactive T cells from their exhausted state (39). Our data demonstrate the persistent expression of CTLA-4, PD-1, LAG-3, and TIM-3 by T cells after ASCT, providing a rationale for early inhibition of checkpoint blockade in posttransplant treatment strategies. Such intervention should promote the recognition of multiple myeloma–associated antigens, boost antigen-specific T-cell activation and

Figure 6. PD-1 inhibition stimulates the proliferation and cytokine secretion of exhausted/senescent CD8+ T cells in vitro. A–D, mature moDCs generated from PBMCs from healthy donors were added as stimulators to T cells from patients in a continuous CR (CCR) 1 year after ASCT or T cells from patients who initially achieved CR but relapsed beyond 3 months after ASCT, at a 1:30 ratio with nivolumab or IgG4 isotype control in alloMLRs. After 5 days, cells were harvested, and CD8+CD28negPD-1+ T cells were assessed by flow cytometry for (A) proliferation by Ki-67 expression, and secretion of (B) IFNγ, (C) IL2, and (D) TNFα. Representative dot plots from 1 patient are shown, with pooled data (mean ± SD) from 6 patients, 3 in CCR (○) and 3 relapsed (■), in the far right column for each parameter. **, P < 0.01.
proliferation, enhance immune reconstitution, expand anti-
multiple myeloma immune responses, and counteract relapse
from minimal residual disease. Using the alloMLR as a proxy
assay for robust immune reactivity, our data in fact demonstrate
a role for PD-1 blockade in reversing the hyporesponsiveness of
exhausted/senescent CD8\(^+\) T cells \textit{in vitro}.

Patients who initially achieved a CR at 3 months but subse-
sequently relapsed after ASCT were assessed for immune markers of
relapse. Analysis of Tregs showed a relative increase among the
relapsed cohort, a finding that is consistent with recent reports
showing greater risk for disease progression with increased Tregs
(35) and an association of long-term disease control with
decreased Tregs (36). NK cells exhibit rapid recovery after ASC,
but are less abundant in patients who relapse, thus supporting
their role in conferring an anti–multiple myeloma benefit after
ASCT as well (37, 38).

T-cell dysfunction, including exhaustion and/or senescence,
contributes to tumor persistence and progression (39, 40). Our
studies define a T-cell exhaustion/senescent phenotype associ-
ated with post-ASCT relapse, which is most pronounced in the
CD8\(^+\) T-cell compartment. These cells downregulate CD28, an
essential costimulatory receptor involved in the activation and
modulation of key cellular functions (48), upregulate CD57, a
marker of low proliferative capacity (42), and display increased
PD-1 expression. Patients with relapsed multiple myeloma had
the highest levels of CD8\(^+\)CD28\(^{lo}\), CD8\(^+\)CD28\(^{hi}\)CD57\(^+\), and
CD8\(^+\)CD28\(^{lo}\)PD-1\(^+\) T cells. Patients in the continuous CR group,
however, also had higher levels of exhausted/senescent T cells
compared with healthy controls, possibly due to chronic malign-
nancy and related treatments.

An intriguing finding is that patients in the relapsed group
had higher baseline levels of exhausted/senescent T cells at the
3-month mark before the detection of clinical relapse, sug-
gesting the utility of these immune biomarkers for identifying
patients at higher risk of relapse who are candidates for early
immunotherapy. Because T cells become exhausted/senescent
from chronic antigen stimulation, one might ask whether
patients who eventually relapse have enough minimal residual
disease persistence, despite high-dose chemotherapy, to pro-
scribe sufficient chronic antigen stimulation after ASCT to cause
T-cell exhaustion/senescence. Alternatively, although it is not a
standard practice to characterize the cell composition of auto-
grafts, apart from their CD34\(^+\) content, our findings lend
sufficient rationale to evaluating whether exhausted/senescent
T cells, as well as Tregs, are over-represented in the autografts of
patients more likely to relapse.

Results from many studies suggest that exhaustion and
senescence are mechanistically distinct, but the underlying
molecular pathways differentiating the two have yet to be fully
elicitated (41). Distinguishing T-cell exhaustion from senes-
cence and delineating the plasticity and function of these cells
remain important unknowns. Exhausted T cells can be reacti-
vated by blocking the PD-1 pathway (49). Senescent T cells can
transiently upregulate telomerase activity and proliferate (40),
and blockade of p38 MAPK reverses senescence via an mTOR-
independent mechanism (50). In this study, PD-1 inhibition
significantly enhanced the proliferation and cytokine secretion
of CD8\(^+\)CD28\(^{lo}\)PD-1\(^+\) T cells, demonstrating the potential to
revive these hyporesponsive cells with checkpoint blockade.
Additional studies of this T-cell subpopulation, including the
contribution of other inhibitory receptors and immunosup-
pressive factors, are warranted to gain a more complete under-
standing of the biology of these cells with regard to post-ASCT
disease status and immunotherapy.

These results provide a rationale for the early introduction
of immunotherapeutic modalities like vaccines and check-
point blockade agents to induce antitumor immunity after
ASCT. Our findings also underscore the contribution of
immune dysregulation to disease relapse, with T-cell exhaust-
ion/senescence as an immune biomarker that deserves vali-
dation by prospective testing because of its potential value in
identifying candidates for early therapeutic intervention. The
reservoir of DC immunostimulatory function and the poten-
tial reactivity of T cells and NK cells comprise an untapped
resource to alter the natural history of multiple myeloma in
the setting of ASCT.

Disclosure of Potential Conflicts of Interest

A.M. Lesokhin has received speakers bureau honoraria from and is a con-
sultant/advisory board member for Bristol Myers-Squibb and Janssen. No
potential conflicts of interest were disclosed by the other authors.

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