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The mechanistic study behind suppression of GVHD while retaining GVL activities by myeloid-derived suppressor cells.

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Abstract

Graft-versus-host disease (GVHD) is a major barrier to the widespread use of allogeneic hematopoietic stem cell transplantation (allo-HSCT) for treating hematologic malignancies. Myeloid-derived suppressor cells (MDSCs) have been recognized as crucial immunosuppressive cells in various pathologic settings. Here, we investigated whether the unique functional properties of MDSCs could be harnessed to control allo-HSCT-associated GVHD. Using multiple murine GVHD/GVL models including both MHC-mismatched and miHA-mismatched, we demonstrated that treatment with CD115+ MDSCs efficiently suppressed GVHD but did not significantly impair graft-versus-leukemia (GVL) activity, leading to 80% and 67% protection in treated mice in GVHD and GVL models, respectively. The mechanism for this dissociation of GVHD from GVL, specifically the emergence of donor-derived NKG2D$^+$ CD8 T cells with a memory phenotype in MDSC-treated recipient mice, was identified. NKG2D expression on donor T cells was required for eradication of allogeneic lymphoma cells. Furthermore, long-term surviving MDSC recipients that exhibited cytolytic activities against allogeneic leukemia cells had a significantly increased percentage of T regulatory cells and, more importantly, NKG2D$^+$ CD8 T cells. These findings indicate that MDSCs can be used as a novel cell-based therapy to suppress GVHD while maintaining GVL activities through selective induction of NKG2D$^+$ CD8 memory T cells.

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Conflict of Interest

The authors declare no competing interests.

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Keywords

MDSC; GVHD; GVL; NKG2D; HSCT

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative immunotherapy for a variety of hematopoietic malignancies and non-malignancies [1] and has been increasingly used to treat solid tumors [2]. However, utilization and full realization of the therapeutic merit of allo-HSCT procedures has been limited by the occurrence of life-threatening graft-versus-host disease (GVHD) [3, 4]. Despite the involvement of natural killer (NK) cells in certain allo-HSCT settings [5], T cells present in the grafts play a central role in the pathogenesis of GVHD [6, 7]. Furthermore, donor-derived T cells are also the principal effector cells in graft versus leukemia (GVL) [8], the specific anti-tumor activity of allo-HSCT. Due to this dual role for donor T cells, approaches currently employed to diminish GVHD, for example, T-cell depletion and non-specific immunosuppressive drugs, are often associated with increased incidences of graft failure, tumor recurrence, opportunistic infection and/or drug resistance [7, 9, 10]. Thus, developing new strategies to dissociate the suppression of GVHD from GVL remains a major challenge in allo-HSCT.

In recent years, there has been increasing interest in myeloid-derived suppressor cells (MDSCs) owing to accumulating evidence suggesting a critical role for MDSCs in the initiation, progression, and metastasis of tumors as well as in other pathologic conditions, including pathogenic infections, inflammation, and traumatic stress in animal models and human patients [11–19]. MDSCs can function to suppress T-cell responses both *in vitro* and *in vivo* via diverse mechanisms, e.g. production of nitric oxide (NO), reactive oxygen species (ROS), expression of arginase 1 and inducible nitric oxide synthase (iNOS), and/or secretion of IL-10 and TGF-β [18, 20–23].

Although MDSCs may hamper the success of immune-based cancer therapy, multiple immunosuppressive properties of MDSCs, on the other hand, may endow them with great therapeutic potential in the fields of autoimmune disease and transplantation, where immune responses need to be limited. This concept has been supported by recent studies proposing a potential role for MDSCs in the GVHD treatment [24, 25]. However, the effect of MDSCs on GVL activities in allo-HCST recipients remains to be determined. We previously identified a major subset of MDSCs expressing the myeloid markers Gr-1, F4/80, and CD115 in tumor-bearing mice, and demonstrated that, in comparison to MDSCs usually defined as a $Gr\text{-}1^{+}CD11b^{+}$ population, $CD115^{+}Gr\text{-}1^{+}F4/80^{+}$ cells not only display stronger suppressive capabilities but also induce the development of $CD4^{+}CD25^{+}Foxp3^{+}$ T regulatory cells (Tregs) in tumor-bearing mice [26]. In this report, we demonstrate that upon adoptive transfer, $CD115^{+}Gr\text{-}1^{+}F4/80^{+}$ MDSCs freshly isolated from tumor-bearing mice or *in vitro*-generated MDSC could efficiently suppress GVHD while maintaining GVL effects in both MHC-mismatched and miHA-mismatched GVHD/GVL models. The MDSCs mediated induction of NKG2D+ CD8 T cells is the key driver for the retention of GVL activities after allo-HSCT with MDSC treatment.

Materials and Methods

Animals and Cell Isolation

C57BL/6, C3H.SW, BALB/c and congenic (Thy1.1) BALB/c mice were purchased from the National Cancer Institute (Frederick, MD) and the Jackson Laboratory (Bar Harbor, ME). MDSCs from Lewis lung carcinoma-bearing C57BL6 mice were prepared as described [27]. The in vitro derived MDSCs were generated as previously described [28, 29]. Briefly, C57BL/6 bone marrow (BM) cells were cultured in RPMI containing 10% FBS, 40 ng/ml GM-CSF and 40 ng/ml IL-6 (Peprotech) for 5 days. BM cells from naïve C57BL/6 mice underwent negative selection using anti-Thy-1.2 and the Thy-1.2 negative cells were used as T-cell depleted bone marrow (TCDBM). Donor T cells were isolated from splenocytes of naïve C57BL/6 mice using a negative selection kit (R&D systems, Minneapolis, MN).

Mixed lymphocyte reaction and MDSC suppression assay

2×10^5 C57BL/6 splenocytes (responder) were co-cultured with an equal number of irradiated (25 Gy) BALB/c splenocytes or non-irradiated BALB/c splenocytes (stimulator) in the absence or presence of various numbers of $CD115^+$ MDSC isolated from C57BL/6 tumor-bearing mice. [^{3}H]-Thymidine was added for the last 8 hours of 4-day or 5-day culture. For Tregs induction, C57BL/6 and BALB/c splenocytes (5×10^6/well) were cocultured with 1.25×10^6 purified MDSCs for 5 days. Foxp3 expression was measured by flow cytometry.

GVHD and GVL models

The establishment of GVHD models was previously described [30, 31]. Briefly, BALB/c or C3H.SW mice (8–14 weeks old, female) were lethally irradiated and randomly distributed into each group. Within 24 hours after irradiation, recipients were left untreated or reconstituted via tail vein injection with donor-derived cells, C57BL/6 TCDBM (5×10^6/mouse), purified C57BL/6 splenic T cells (5×10^5/mouse for Balb/c recipients and 2×10^6/mouse for C3H.SW recipients) and MDSCs (3×10^6/mouse for Balb/c recipients and 6×10^6/mouse for C3H.SW recipients) isolated from C57BL/6 tumor-bearing mice or from in vitro cultures. Mice that received MDSC on Day 0 were given two additional injections of MDSCs (3 or 6×10^6/mouse) on days 4 and 10 after transplantation. Animals were monitored daily for GVHD symptoms and overall survival. For survival rate analysis, at least 22 mice were used for each *in vivo* experiment. For histopathological analysis, specimens obtained at day 21–30 were fixed in formalin and tissue sections were stained with hematoxylin and eosin. The pathologist was blinded to the group allocation during the analysis.

In the experiments designed for expansion and activation of donor T cells, MDSC-treated recipients were given MDSCs once on day 0, and mice were sacrificed on days 7 or 14 after transplantation. In the GVL experiments, recipients were co-transplanted with A20 cells (1×10^5/mouse) unless otherwise specified. Animals found to have hepatic or lymphoid tumor nodules at postmortem were categorized as death due to tumor. Mice that died without tumors but with clear signs of GVHD were considered deaths due to GVHD.

Antibodies and tumor cells lines

All fluorochrome-labeled and purified mouse antibodies and corresponding isotype controls were purchased from commercial source and listed in Table S1. Flow cytometric surface staining was performed as described [18]. Intracellular staining for Foxp3 and granzyme B was performed per manufacturer's instructions (Mouse Regulatory T cell Staining Kit, eBioscience). For intracellular staining of IFNγ, splenocytes isolated from each group (n = 3) were individually cultured for 6 hours in the presence or absence of PMA (20 ng/ml) and ionomycin (1 μg/ml), with the addition of monensin for the last 4 hours. Data were acquired on a FACSAria II (BD Biosciences) and analyzed using Flowjo software (Tree Star, Inc., Ashland, OR). A20, YAC-1 and EL4 tumor cell lines were purchased from the American Type Culture Collection. L1 (BALB/c line 1 lung carcinoma) and MCA26 (BALB/c-derived colon carcinoma) are maintained in our laboratory. Periodic mycoplasma detection tests were performed in all cell lines used in this study.

Cytotoxic T lymphocyte (CTL) assay

Using Thy1 as the marker, effector T cells were purified following 5-day MLR culture in the absence or presence of MDSC or directly from pooled splenocytes of treated mice (n = 3 mice) then normalized for H-2K^{b+} CD8^{+} T-cell numbers based on FACS data. The purified effector T cells were then co-cultured for 4 hours with target cells (A20, YAC-1, EL4, L1 and MCA-26, 1×10^4/well) at various ratios. Anti-natural-killer group 2, member D protein (NKG2D) and anti-CD3 (5 μg/ml, Biolegend, San Diego, CA) were added during the CTL assays. Supernatants were collected from each well for measurement of lactate dehydrogenase (LDH) release (cytotoxicity assay kit, Promega, Madison, WI). Specific killing was calculated using the following formula: % cytotoxicity = 100 × (experimental release - effector spontaneous release-target spontaneous release)/(total target release - target spontaneous release).

Statistical analysis

Statistical differences in animal survival were analyzed by log-rank test. Differences between two groups were compared using unpaired t-test. Differences between three or more groups were compared using one-way ANOVA. All experiments were repeated at least three times. All the statistical analysis was performed with GraphPad Prism, version 6.05. P <0.05 was considered statistically significant.

Study approval.

All animal studies were approved by the IACUC at the Center for Comparative Medicine and Surgery of the Icahn School of Medicine at Mount Sinai and at Comparative Medicine at Houston Methodist Research Institute.

Results

MDSCs suppress allo-immune response *in vitro*

We first assessed the functional activity of MDSCs in an allogeneic mixed lymphocyte reaction (MLR). As shown in Figure 1A, CD115^{+} MDSCs isolated from tumor-bearing mice

exerted potent suppressive activity in a dose-dependent manner, and almost completely suppressed the allo-specific proliferation of C57BL/6 T cells, when used at high ratios of MDSCs versus C57BL/6 splenocytes (Figure 1A). In contrast, $CD115^{-}$ cells did not show suppressive activity even at a 1:1 ratio. Interestingly, the addition of MDSCs to the MLR cultures at a 1:4 ratio of MDSCs versus C57BL/6 splenocytes led to a marked augmentation of $CD25^{+}Foxp3^{+}$Tregs within the C57BL/6 CD4 T cell compartment (Figure 1B). These results indicate that MDSCs are able to significantly inhibit the proliferation of allo-reactive T cells *in vitro*, a mechanism which may be partially mediated by Treg induction.

MDSCs effectively alleviate GVHD following allo-HSCT

We next sought to determine whether MDSCs would be effective in suppressing the allo-immune response *in vivo*, thus preventing GVHD following allo-HSCT. We adopted a well-characterized murine GVHD model in which MHC-mismatched mouse strains BALB/c ($H\text{-}2^{d}$) and C57BL/6 ($H\text{-}2^{b}$) mice were designated as recipients and donors, respectively. While lethally irradiated mice without treatment died within 10 days, all mice treated with TCDBM alone were healthy and survived for more than 100 days (Figure 1C). As expected, mice that had received TCDBM plus donor-type T cells developed severe signs of GVHD as evidenced by loss of hair, hunched posture, diarrhea, and death in most of the animals between 15 and 25 days after transplantation. In contrast, when given MDSCs derived from tumor-bearing mice, along with TCDBM plus T cells, recipients were largely protected from GVHD lethality and approximately 80% of the animals survived for more than 100 days.

A striking feature of GVHD pathology is the occurrence of inflammation and tissue damage in specific target organs. Histological examination of liver, small intestine, and skin harvested on day 21 after allo-HSCT revealed dense lymphocyte infiltration in all examined tissues obtained from TCDBM plus T cell-treated mice, particularly in the periportal area of the liver and dermis layer of the skin. The intestines from these mice manifested atrophy of villi and mucous membrane and crypt destruction. On the contrary, tissue samples isolated from MDSC-treated mice showed no significant histological changes (Figure 1D and figures S1A), almost indistinguishable from those recovered from TCDBM-only recipients. These observations establish that MDSCs are highly effective in suppressing the development of GVHD, thus resulting in a great improvement in animal survival after allo-HSCT.

To gain insight into the regulation of GVHD by MDSCs, we further investigated the cellular mechanisms underlying this process. It is well established that upon recognition of host antigens, including MHC and miHAs (minor histocompatibility) antigens, donor-derived T lymphocytes become activated and undergo rapid and extensive expansion in the early course of GVHD following allo-HSCT [7]. Accordingly, MDSCs may function to suppress GVHD by restraining the expansion of allo-reactive T cells. To examine this possibility, we performed additional transplantation experiments. On day 7 after adoptive transfer, secondary lymphoid organs and livers were isolated from representative mice in each group and donor-derived T cell subsets were profiled by flow cytometry. As determined by cell percentage (Figure S1B) and absolute cell number (Figure 1E), the proliferation of both donor-derived ($H\text{-}2k^{b}$ positive) CD4 and CD8 T cells was significantly inhibited in MDSC recipients when compared to the group without MDSC treatment. The spleens of these mice

(Figure 1E, left panel) exhibited 46% and 55% decreases in the donor CD4 and CD8 subsets, respectively. The reductions were even more pronounced in mesenteric lymph nodes (66% and 71%, respectively, Figure 1E, middle panel) and liver (69% and 70%, respectively, Figure 1E, right panel). Similar results were also obtained with samples harvested on day 14 after transplantation (Figure S1C). Taken together, these data suggest that upon adoptive transfer, MDSCs remain suppressive *in vivo*, thus causing suppression of allo-reactive T-cell expansion in both lymphoid organs and GVHD target organs.

Since MDSCs induced Treg development *in* MLR (Figure 1B) and Tregs have been shown to be highly effective in suppressing GVHD in murine models [32–37], we evaluated whether Tregs were increased in the GVHD setting. Indeed, as early as day 7 after allo-HSCT, recipients that received MDSCs had at least a 2-fold higher percentage of Tregs in the splenic H-2K^{b+}CD4^{+} population when compared to those mice that had not received MDSCs (Figure 1F). This increase in Treg frequency was even more evident in long-term (day 107) surviving mice that had received MDSCs (Figure S1D). These results suggest that MDSC-induced Tregs may contribute to the long-term suppression of GVHD. We further evaluated the therapeutic effect of *in vitro*-generated MDSCs. Similar to tumor-bearing-mouse-derived MDSCs, *in vitro*-generated MDSCs also ameliorated GVHD severity and mortality in both MHC-mismatched and MHC-matched miHA-mismatched murine GVHD models (Figure 2).

MDSCs preserve allo-reactive T cell cytotoxicity and GVL activity

The profound suppression of GVHD by MDSCs prompted us to examine whether the GVL activity of donor-derived T cells is maintained in the presence of MDSCs. Lethally irradiated BALB/c mice were co-transplanted with A20 cells (a B-cell lymphoma/leukemia cell line, H-2^{d}, 1×10^{5}/mouse) to induce tumor development in the hosts. Consequently, 100% of recipients that received TCDBM plus A20 died before day 30 from progressive leukemia (Figure 3A). Mice that had received TCDBM and A20 plus T cells also died between day 20 and day 51 after transplantation, due to GVHD-related damage rather than tumor growth, as exhibited by the development of severe signs of acute GVHD but no tumor formation as evidenced by the absence of tumor nodules in the liver and lymph nodes of 6 mice examined pre- or post-mortem. Strikingly, 67% of tumor-challenged animals that received TCDBM plus T cells supplemented with tumor-bearing-mouse-derived MDSCs survived over 100 days. These long-term surviving mice displayed neither GVHD nor leukemia symptoms, suggesting that, in the absence of GVHD, donor T cells were able to efficiently eradicate tumor cells. These results indicate that MDSCs can actively suppress GVHD while retaining beneficial GVL activity after allo-HSCT.

To explore the underlying mechanisms by which MDSCs maintain GVL activity, we examined the activation of donor-derived T cells and their functional activity. Based on the expression levels for a panel of activation markers, including CD69 (Figure S2A), CD44, CD62L (Figure S2B), and CD25 (data not shown), the activation of donor-derived CD4 and CD8 T cells in the spleens or lymph nodes isolated from day 7 MDSC recipients was not significantly altered compared with their counterparts that had not received MDSC treatment. Intracellular staining of re-isolated cells after incubation *in vitro* in the presence

or absence of phorbol myristate acetate (PMA) plus ionomycin stimulation also revealed no substantial differences in IFNγ production as evaluated by the percentages of IFNγ-producing $CD4^+$ and $CD8^+$ cells in the total splenic $CD4^+$ or $CD8^+$ T cells recovered from the two groups (Figure 3B).

The granzyme B/perforin pathway represents one of the primary mechanisms mediating direct T-cell cytotoxicity. Although we have not examined the expression of perforin, no apparent changes in granzyme B expression were detected in the donor-derived T lymphocyte compartment (Figure 3C), suggesting that the cytotoxic capabilities of donor-derived CD8 T cells were unaffected by MDSCs. To verify this, we conducted cytolytic assays using splenic T cells isolated on day 14 after allo-HSCT. As shown in Figure 3D, while CD8 T cells derived from mice that had or had not received MDSCs showed minimal killing against syngeneic tumor targets EL4 ($H\text{-}2^b$), they both exhibited similar cytotoxicity profiles against allogeneic target A20 cells ($H\text{-}2^d$). Indeed, cytotoxicity against allogenic tumor cells was preserved among T cells isolated from MDSC-treated long-term survivors (Figure S2C and D). These data indicate that adoptively transferred MDSCs do not alter the activation or functional activity of donor-derived T cells, which may explain in part why the GVL activity of allo-reactive T cells is retained in the presence of MDSCs.

Up-regulation of NKG2D during allogeneic T cell responses in the presence of MDSCs

Since treatment with MDSCs limited only the expansion, not the activation or effector function of donor T cells, we next determined the cellular composition of MLR in the presence or absence of tumor-bearing-mouse-derived MDSCs. We found that the ratio of responder ($H2K^{b+}$) CD8-to-CD4 T cells in the MLR absence of MDSCs was significantly lower than that in the MLR in the presence of MDSCs (Figure S3A), suggesting that CD4 T cells are more susceptible to suppression by MDSCs. We further confirmed this notion by assessing the proliferation of responder T cells labeled with CFSE. There was a 66% decrease in proliferating CD4 T cells vs. a 48% decrease in proliferating CD8 T cells in MLR in the presence of MDSCs when compared to MLR without MDSCs (Figure S3B). We further analyzed the phenotypes of T cells in MLR. The addition of MDSCs to MLR led to significant increases in the percentages of $CD44^+CD62L^-$ CD4 T cells and $CD44^+CD62L^+$ CD8 T cells (Figure 4A Figure S3C). Strikingly, NKG2D, a crucial co-stimulatory molecule that is involved in anti-tumor immunity of CD8 T cells was also significantly up-regulated when MDSCs were added to the MLR culture (Figure 4A, Figure S3C). We further investigated $NKG2D^+$ T cells in murine GVHD/GVL models. Consistent with the in vitro MLR results, the frequency of $NKG2D^+$ CD8 T cells was significantly increased in mice that had received tumor-bearing-mouse-derived MDSCs treatment at day 30 after allo-HSCT (Figure 4B and 4C). Interestingly, MDSC-treated mice also showed increase of NKG2D expression on CD4 T cells in the GVHD model, indicating that $NKG2D^+$ CD4 T cells may also be involved in preventing and/or inhibiting GVHD pathogenesis after MDSCs treatment. Moreover, we observed a further increase in $CD44^+NKG2D^+$ and $CD44^+CD62L^-$ T cells on day 100 after allo-HSCT (Figure S3D). The results suggest that $NKG2D^+$ CD8 T cells may play a pivotal role in GVL activity after MDSC treatment. Since $NKG2D^+$ T cells were upregulated, we further evaluated whether MDSC express NKG2D ligands. Indeed, MDSCs from tumor-bearing mice expressed a significant level of NKG2D ligands, e.g.

RAE-1 and a certain amount of MULT-1 as detected by flow cytometric analysis (Figure S4).

The cytolytic activity of MDSC-conditioned allo-reactive T cells depends on NKG2D and CD3.

To determine the role of NKG2D in cytotoxicity against tumors mediated by MDSC-conditioned allo-reactive T cells, we performed cytotoxicity assays in the presence of CD3 or NKG2D blocking antibodies. T cells purified from 5-day MLR cultures containing tumor-bearing-mouse-derived MDSCs exhibited stronger cytotoxicity against allogeneic A20 cells and Yac-1 cells than MLR cultures with control non-MDSCs (Figure 5A). A significantly lower level of cytotoxicity against syngeneic EL4 cells was also detected in T cells purified from MLR cultured in the presence or absence of MDSCs (data not shown). The cytotoxicity of T cells purified from control or MDSC-containing MLR was significantly inhibited in the presence of NKG2D or CD3 blocking antibodies. Importantly, purified T cells from MDSC-treated mice exhibited strong cytotoxic activity against allogeneic A20 cells and YAC-1 that was partially blocked by CD3 and NKG2D blocking antibodies (Figure 5A and 5B), indicating that both NKG2D and CD3 are required for the cytotoxicity of donor T cells.

Since NKG2D was upregulated on CD8 T cells in the presence of MDSCs, we further examined the role of the NKG2D ligand RAE1 in T cell-mediated cytolytic activity. We first assessed the expression of RAE-1 on tumor cells (Figure S5). Indeed, not only did A20 tumor cells normally express high levels of RAE-1, but irradiation also increased RAE-1 expression (Figure S5A). However, results from cytotoxicity assays with RAE-1 blockade (Figure S5B) suggested that RAE-1 may not be the only ligands involved in cytotoxicity mediated by NKG2D+ T cells. We further evaluated the role of NKG2D in cytotoxicity of MDSC-conditioned T cells against RAE low-expressing non-hematopoietic tumor cells. As expected, T cells from MLR culture or mice treated with tumor-bearing-mouse-derived MDSC exhibited cytotoxicity against RAE1 low-expressing tumor cells (Figure S5C, S5D and Figure 5C). However, NKG2D blockade did not affect the cytotoxicity against these tumor cells (Figure 5C), suggesting that diverse mechanisms are involved in cytotoxicity of MDSC-conditioned T cells against RAE-1 low-expressing non-hematopoietic malignances and RAE-1 high-expressing hematopoietic malignances.

NKG2D-expressing T cells are essential for maintaining GVL activity after MDSCs treatment.

We next determined whether the NKG2D expression is required for GVL activity after MDSC treatment. As shown in Figure 6A, tumor-bearing-mouse-derived MDSC treatment resulted in significantly prolonged survival of A20-bearing mice that had received allo-HSCT. In mice where the $NKG2D^+$ cell population was depleted using anti-NKG2D antibody (BM+T+A20+MDSC+anti-NKG2D), GVL activity was substantially inhibited compared to mice treated with the corresponding control Ig (BM+T+A20+MDSC+Ig) ($p=0.046$) (Figure 6A). Furthermore, we used NKG2D knockout T cells (KOT) and bone marrow cells (KOBM) as donor cells to clarify whether donor-derived $NKG2D^+$ T cells were required for the GVL activity. MDSC treatment significantly increased survival in mice

receiving wild-type donor cells (BM+T+A20+MDSC) compared to mice that had not received MDSC treatment (BM+T+A20) (p=0.0065). However, MDSC treatment lost its protective effect in mice that received NKG2D-deficient donor cells (KOBM+KOT+MDSC +A20) compared to mice that received wild-type donor cells (p=0.0332) (Figure 6B). These results suggest that NKG2D expression on T cells is crucial for preserving GVL activity after MDSCs treatment.

Discussion

The data presented here strongly suggest that MDSC treatment might represent a cell-based tolerogenic therapy that is capable of preventing lethal GVHD while retaining sufficient GVL activity to eradicate leukemia cells. Co-transplantation with MDSCs resulted in suppressed development of lethal acute GVHD but did not jeopardize the GVL activity of allo-reactive T cells or the long-term immunity of treated mice, making MDSC administration an attractive approach to GVHD prophylaxis.

In a recent report, in-vitro-generated MDSCs have been shown to prevent GVHD by inducing T helper 2 (Th2) cells while preserving the cytotoxic activity of donor T cells against H-2K mismatched thymoma (JM6) cells [38]. In contrast to our study, Tregs were not increased in MDSC-treated group. This may be attributed to the functional differences between MDSCs isolated from tumor-bearing mice and in-vitro generated MDSCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF.

In this study, we have shown that MDSCs exhibit a potent immuno-suppressive activity, effectively suppressing allo-specific immune responses and, remarkably, augmenting regulatory T cells ($CD4^+CD25^+Foxp3^+$). We also observed that MDSC treatment favored the development of a central and effector memory T cell and $NKG2D^+$ T cell populations *in vitro* and *in vivo*, which may be partially attributed to the expression of NKG2D ligands on MDSC (Figure S4) and on tumor cells (Figure S5). Mechanistically, both CD3 and NKG2D played a role in T cell-mediated cytolytic activity against tumor cells in vitro. To assessing the contribution of NKG2D to GVL activities by using NKG2D depleting Ab and NKG2D deficient mice as the source of bone marrow and T cells, we demonstrated that donor-derived NKG2D positive T cells play an important role in the GVL effect observed in allo-HSCT mice treated with MDSC.

$NKG2D^+$ CD8 T cells have been shown to mediate GVHD and GVL responses [39]. Since NKG2D ligands were transiently induced on normal tissues of allo-HSCT mice, transient NKG2D blockade from days 0 to 6 after transplantation was able to attenuate GVHD while preserving GVL effect of $NKG2D^+$ CD8 T cells. In our GVHD/GVL model, the acute allo-responses were suppressed by MDSCs, thereby preventing the attack on normal cells that transiently expressed NKG2D ligands by $NKG2D^+$ CD8 T cells, which depends on both CD3 and NKG2D (Figure 5). At later stages of GVHD, NKG2D induction in normal tissues of recipient mice and the suppressive activities of MDSC waned thereby allowing donor $NKG2D^+$ CD8 T cells to regain GVL function and eradicate allogeneic tumor cells without negatively affecting normal tissues in recipient mice.

Based on our findings in the present study, we propose the model in Figure 7 that MDSC can prevent GVHD by suppressing the early expansion of donor-derived T cells and inducing Tregs. Furthermore, MDSC can augment GVL activity by favoring the development and activation of donor-derived NKG2D$^+$ CD8 T cells while retaining their effector function, such as tumor cytolytic activity as well as IFNγ production. In conclusion, we have demonstrated that NKG2D expression on T cells can be induced by MDSCs during allogenic immune responses and its expression is essential for maintaining GVL activity after allo-HSCT. Our findings may provide new insight into understanding the mechanism of how MDSCs control GVHD while preserving GVL activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Aschan J Allogeneic haematopoietic stem cell transplantation: current status and future outlook. British medical bulletin 2006; 77–78: 23–36.
2. Demirer T, Barkholt L, Blaise D, Pedrazzoli P, Aglietta M, Carella AM, et al. Transplantation of allogeneic hematopoietic stem cells: an emerging treatment modality for solid tumors. Nature clinical practice Oncology 2008 5; 5(5): 256–267.
3. Shlomchik WD. Graft-versus-host disease. Nature reviews Immunology 2007 5; 7(5): 340–352.
4. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. Nature reviews Immunology 2012 6; 12(6): 443–458.
5. Ruggeri L, Mancusi A, Burchielli E, Aversa F, Martelli MF, Velardi A. Natural killer cell alloreactivity in allogeneic hematopoietic transplantation. Current opinion in oncology 2007 3; 19(2): 142–147. [PubMed: 17272987]
6. Korngold R, Sprent J. Variable capacity of L3T4+ T cells to cause lethal graft-versus-host disease across minor histocompatibility barriers in mice. The Journal of experimental medicine 1987 6 1; 165(6): 1552–1564. [PubMed: 3108446]
7. Vogelsang GB, Lee L, Bensen-Kennedy DM. Pathogenesis and treatment of graft-versus-host disease after bone marrow transplant. Annual review of medicine 2003; 54: 29–52.
8. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nature reviews Cancer 2004 5; 4(5): 371–380. [PubMed: 15122208]
9. Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. Blood 2001 12 1; 98(12): 3192–3204. [PubMed: 11719354]
10. Storb R, Deeg HJ, Whitehead J, Appelbaum F, Beatty P, Bensinger W, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. The New England journal of medicine 1986 3 20; 314(12): 729–735. [PubMed: 3513012]
11. Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, et al. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature 2007 12 6; 450(7171): 825–831. [PubMed: 18064003]
12. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunological reviews 2008 4; 222: 162–179. [PubMed: 18364001]

13. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. The Journal of clinical investigation 2007 5; 117(5): 1155–1166. [PubMed: 17476345]
14. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, et al. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer cell 2004 10; 6(4): 409–421. [PubMed: 15488763]
15. Young MR, Wright MA, Lozano Y, Prechel MM, Benefield J, Leonetti JP, et al. Increased recurrence and metastasis in patients whose primary head and neck squamous cell carcinomas secreted granulocyte-macrophage colony-stimulating factor and contained CD34+ natural suppressor cells. International journal of cancer Journal international du cancer 1997 2 20; 74(1): 69–74. [PubMed: 9036872]
16. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. Journal of immunology 2007 7 15; 179(2): 977–983.
17. Gabrilovich DI, Bronte V, Chen SH, Colombo MP, Ochoa A, Ostrand-Rosenberg S, et al. The terminology issue for myeloid-derived suppressor cells. Cancer research 2007 1 1; 67(1): 425; author reply 426. [PubMed: 17210725]
18. Pan PY, Ozao J, Zhou Z, Chen SH. Advancements in immune tolerance. Adv Drug Deliv Rev 2008 1 14; 60(2): 91–105. [PubMed: 17976856]
19. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Seminars in cancer biology 2006 2; 16(1): 53–65. [PubMed: 16168663]
20. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nature reviews Immunology 2005 8; 5(8): 641–654.
21. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nature medicine 2007 7; 13(7): 828–835.
22. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. The Journal of experimental medicine 2003 12 1; 198(11): 1741–1752. [PubMed: 14657224]
23. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunological reviews 2008 4; 222: 180–191. [PubMed: 18364002]
24. Paraiso KH, Ghansah T, Costello A, Engelman RW, Kerr WG. Induced SHIP deficiency expands myeloid regulatory cells and abrogates graft-versus-host disease. Journal of immunology 2007 3 1; 178(5): 2893–2900.
25. Billiau AD, Fevery S, Rutgeerts O, Landuyt W, Waer M. Transient expansion of Mac1+Ly6-G +Ly6-C+ early myeloid cells with suppressor activity in spleens of murine radiation marrow chimeras: possible implications for the graft-versus-host and graft-versus-leukemia reactivity of donor lymphocyte infusions. Blood 2003 7 15; 102(2): 740–748. [PubMed: 12676788]
26. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res 2006 1 15; 66(2): 1123–1131. [PubMed: 16424049]
27. Pan PY, Wang GX, Yin B, Ozao J, Ku T, Divino CM, et al. Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. Blood 2008 1 1; 111(1): 219–228. [PubMed: 17885078]
28. Haverkamp JM, Smith AM, Weinlich R, Dillon CP, Qualls JE, Neale G, et al. Myeloid-derived suppressor activity is mediated by monocytic lineages maintained by continuous inhibition of extrinsic and intrinsic death pathways. Immunity 2014 12 18; 41(6): 947–959. [PubMed: 25500368]

29. Marigo I, Bosio E, Solito S, Mesa C, Fernandez A, Dolcetti L, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. Immunity 2010 6 25; 32(6): 790–802. [PubMed: 20605485]
30. Zhou Z, French DL, Ma G, Eisenstein S, Chen Y, Divino CM, et al. Development and function of myeloid-derived suppressor cells generated from mouse embryonic and hematopoietic stem cells. Stem Cells 2010 3 31; 28(3): 620–632. [PubMed: 20073041]
31. Zhang J, Ramadan AM, Griesenauer B, Li W, Turner MJ, Liu C, et al. ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease. Sci Transl Med 2015 10 7; 7(308): 308ra160.
32. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood 2002 5 15; 99(10): 3493–3499. [PubMed: 11986199]
33. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. The Journal of experimental medicine 2002 8 5; 196(3): 389–399. [PubMed: 12163567]
34. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. The Journal of experimental medicine 2002 8 5; 196(3): 401–406. [PubMed: 12163568]
35. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nature medicine 2003 9; 9(9): 1144–1150.
36. Zhang C, Todorov I, Zhang Z, Liu Y, Kandeel F, Forman S, et al. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. Blood 2006 4 1; 107(7): 2993–3001. [PubMed: 16352808]
37. Zhao D, Zhang C, Yi T, Lin CL, Todorov I, Kandeel F, et al. In vivo-activated CD103+CD4+ regulatory T cells ameliorate ongoing chronic graft-versus-host disease. Blood 2008 9 1; 112(5): 2129–2138. [PubMed: 18550852]
38. Messmann JJ, Reisser T, Leithauser F, Lutz MB, Debatin KM, Strauss G. In vitro-generated MDSCs prevent murine GVHD by inducing type 2 T cells without disabling antitumor cytotoxicity. Blood 2015 8 27; 126(9): 1138–1148. [PubMed: 26185131]
39. Karimi MA, Bryson JL, Richman LP, Fesnak AD, Leichner TM, Satake A, et al. NKG2D expression by CD8+ T cells contributes to GVHD and GVT effects in a murine model of allogeneic HSCT. Blood 2015 6 4; 125(23): 3655–3663. [PubMed: 25788701]

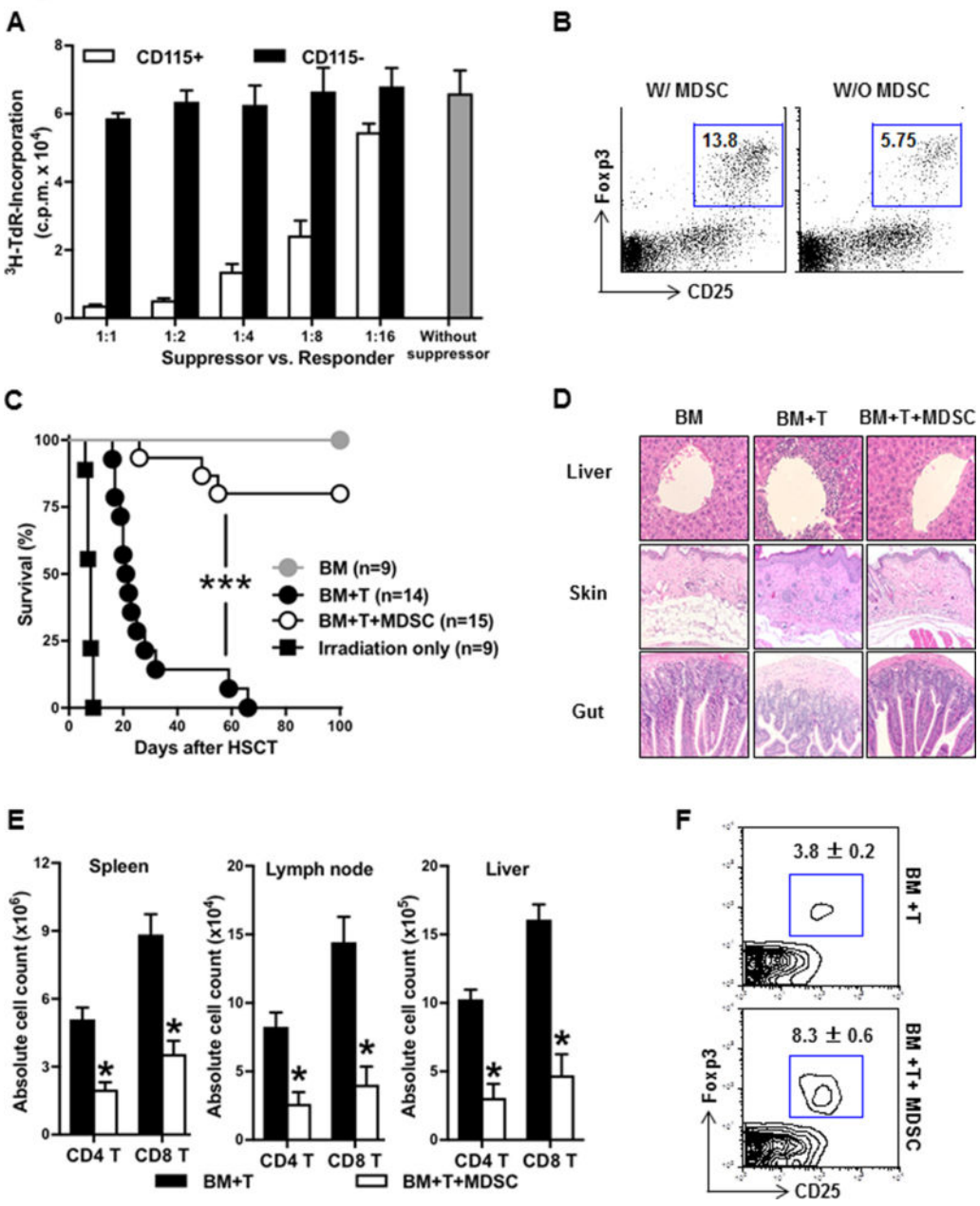

Figure 1. MDSCs induce Tregs and suppress allogeneic T cell responses *in vitro* and *in vivo*.

(A) C57BL/6 splenocytes (responders, 2×10^5/well) were co-cultured with an equal number of irradiated BALB/c splenocytes in the absence or presence of indicated ratios of $CD115^+$ (suppressors) or $CD115^-$ (control) cells from tumor-bearing C57BL/6 mice. T cell proliferation was determined by measuring [^{3}H]-thymidine incorporation during the last 8 hours of a 4-day culture. **(B)** C57BL/6 and BALB/c splenocytes (5×10^6/well) were cultured in the presence or absence of MDSCs isolated from tumor-bearing mice (1.5×10^6/well) for 5 days. Treg population ($CD4^+CD25^+Foxp3^+$) was measured by flow cytometry. **(C)** The Balb/c GVHD model and MDSCs treatment was described in Methods. Survival curve of recipient mice with indicated treatments. *** $P< 0.0001$, BM+T+MDSC versus BM+T

recipients. Data shown were combined from three independent experiments with reproducible results. **(D)** Histopathological analyses of GVHD target tissues. Liver, gut and skin tissues from the mice received BM, BM+T or BM+T+MDSC were collected on day 21 after transplantation and hematoxylin and eosin staining of tissue sections was performed for histopathological analysis. Representative photomicrographs are shown. **(E)** Absolute numbers of CD4 and CD8 T cells in spleen, lymph node and liver at day 7 post-treatment. Data are presented as mean ± S.D. ($n = 3$). * $P < 0.05$. **(F)** Splenic Treg populations in indicated groups were analyzed by gating on $H2K^{b+}CD4^{+}$ cells on day 7 post-treatment. Data are presented as mean ± S.D. ($n = 3$ mice).

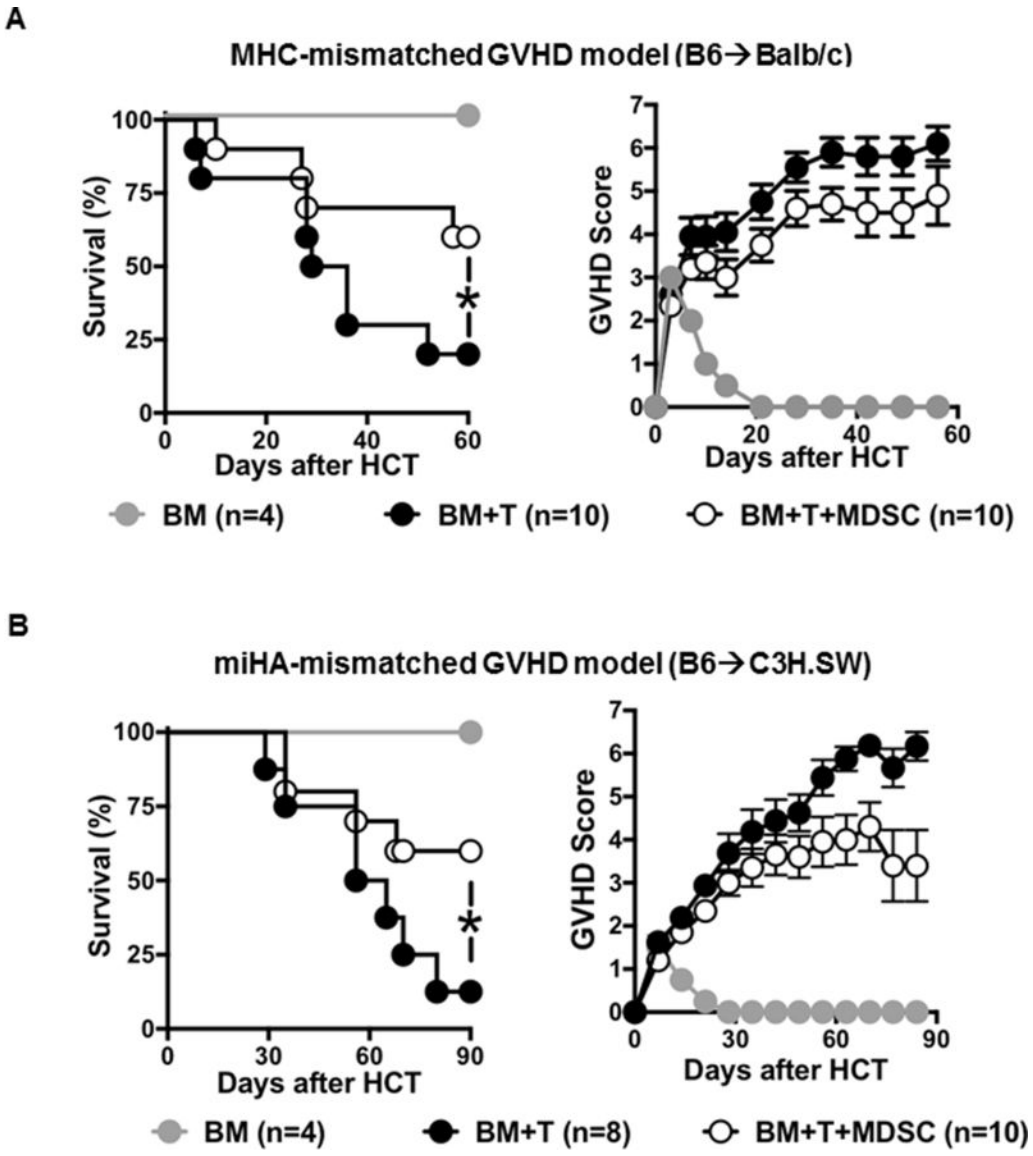

Figure 2. Treatment of in-vitro-derived MDSCs alleviates GVHD in multiple murine models.

GVHD models and MDSC treatment was described in Methods. Survival curves and GVHD scores for MHC-mismatched GVHD model **(A)** and miHA-mismatched GVHD model **(B)** * $P < 0.05$, data are presented as mean ± S.D.

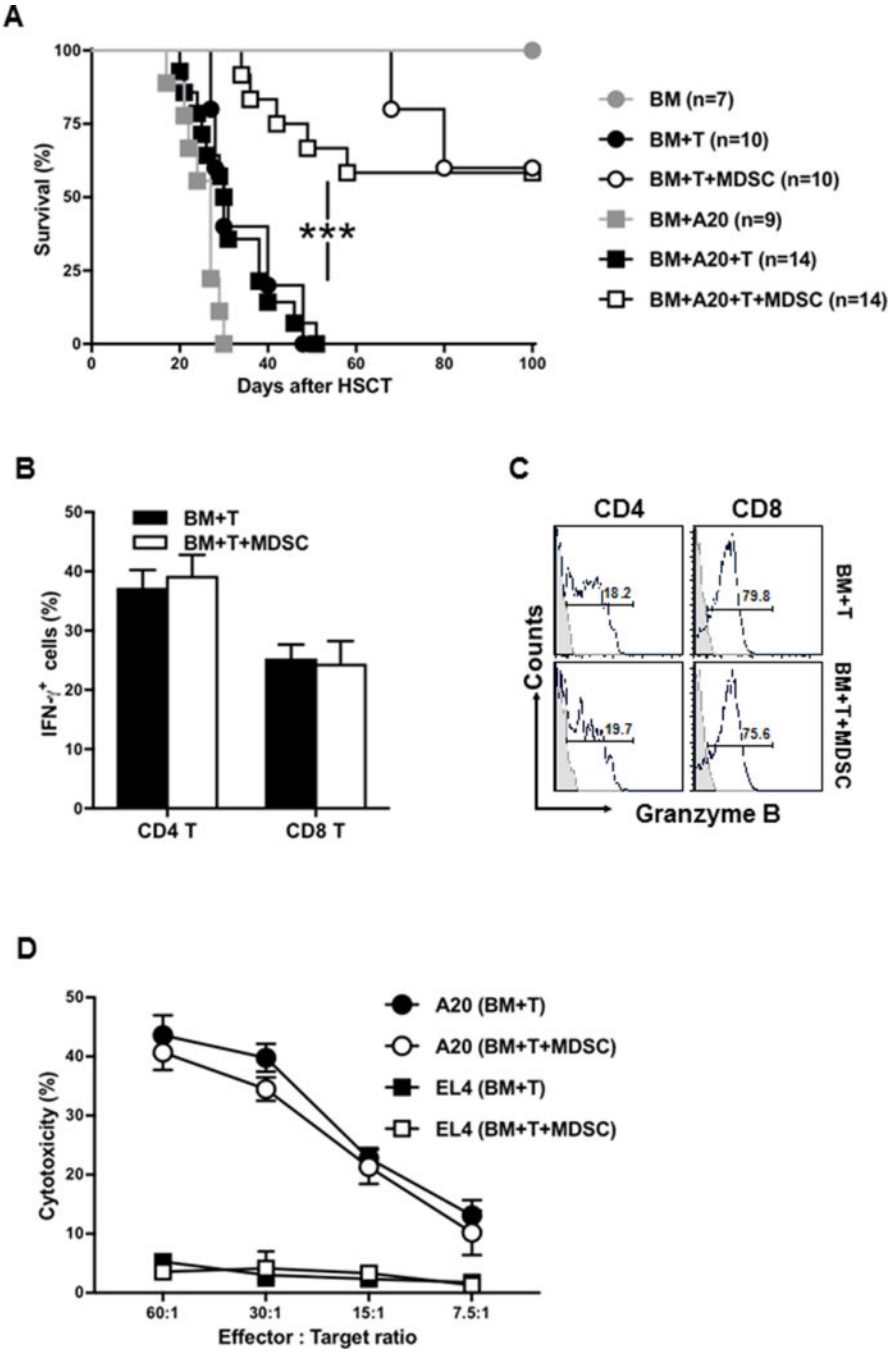

Figure 3. Treatment of tumor-bearing-mouse-derived MDSCs preserves GVL activity.

The GVHD/GVL model with Balb/c recipients and MDSC treatment was described in Methods. **(A)** Survival curve. ***$P < 0.001$, BM+A20+T+MDSC versus BM+A20+T. Data shown are combined from three independent experiments. **(B)** The frequency of donor-derived IFNγ-producing CD4 and CD8 splenic T cells. **(C)** Granzyme B expression in donor-derived splenic T cell subsets (BM+T and BM+T+MDSC). Gray line indicates Isotype. **(D)** Cytolytic activity of donor CD8 T cells isolated from mice that had received allo-HSCT. Splenic T cells isolated on day 14 after allo-HSCT were co-cultured with tumor

cells, A20 (H-2K^{d}) or EL4 (H-2K^{b}) for 4 hours at indicated ratios. The extent of cytotoxicity was determined by LDH release. Data are presented as mean ± S.D., $n = 3$.

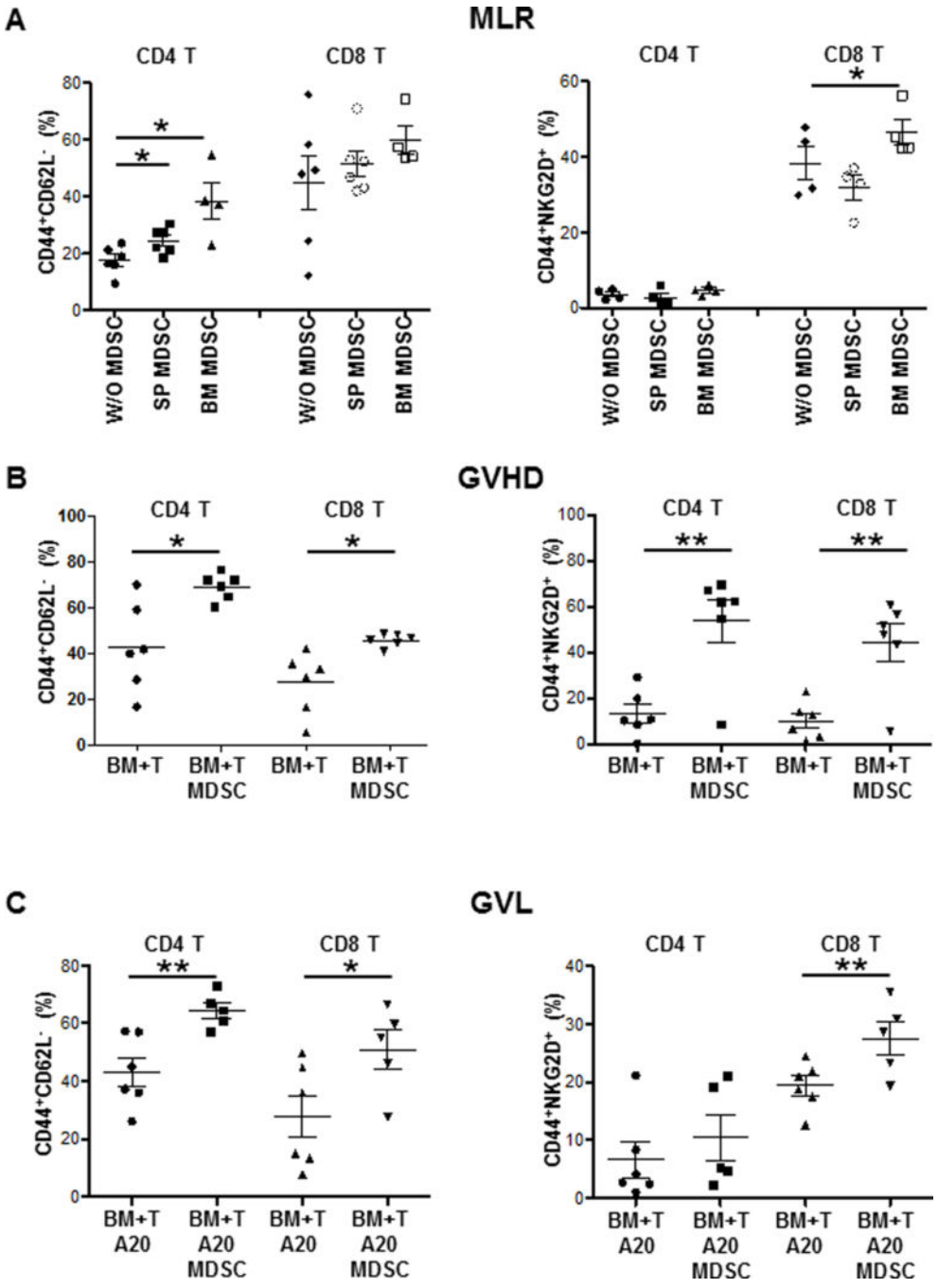

Figure 4. Tumor-bearing-mouse-derived MDSCs induce NKG2D$^+$ memory T cells.

Flow cytometric analysis of CD44$^+$CD62L$^-$ and CD44$^+$NKG2D$^+$ splenic T cell populations in 5-days MLR **(A)**, GVHD **(B)** and GVL models **(C)** at day 30 after allo-HSCT and MDSC treatment. SP MDSC and BM MDSC represent MDSCs isolated from spleen and bone marrow of tumor-bearing mice, respectively. *p<0.05, ** p<0.01, n=6. The data are from three independent experiments.

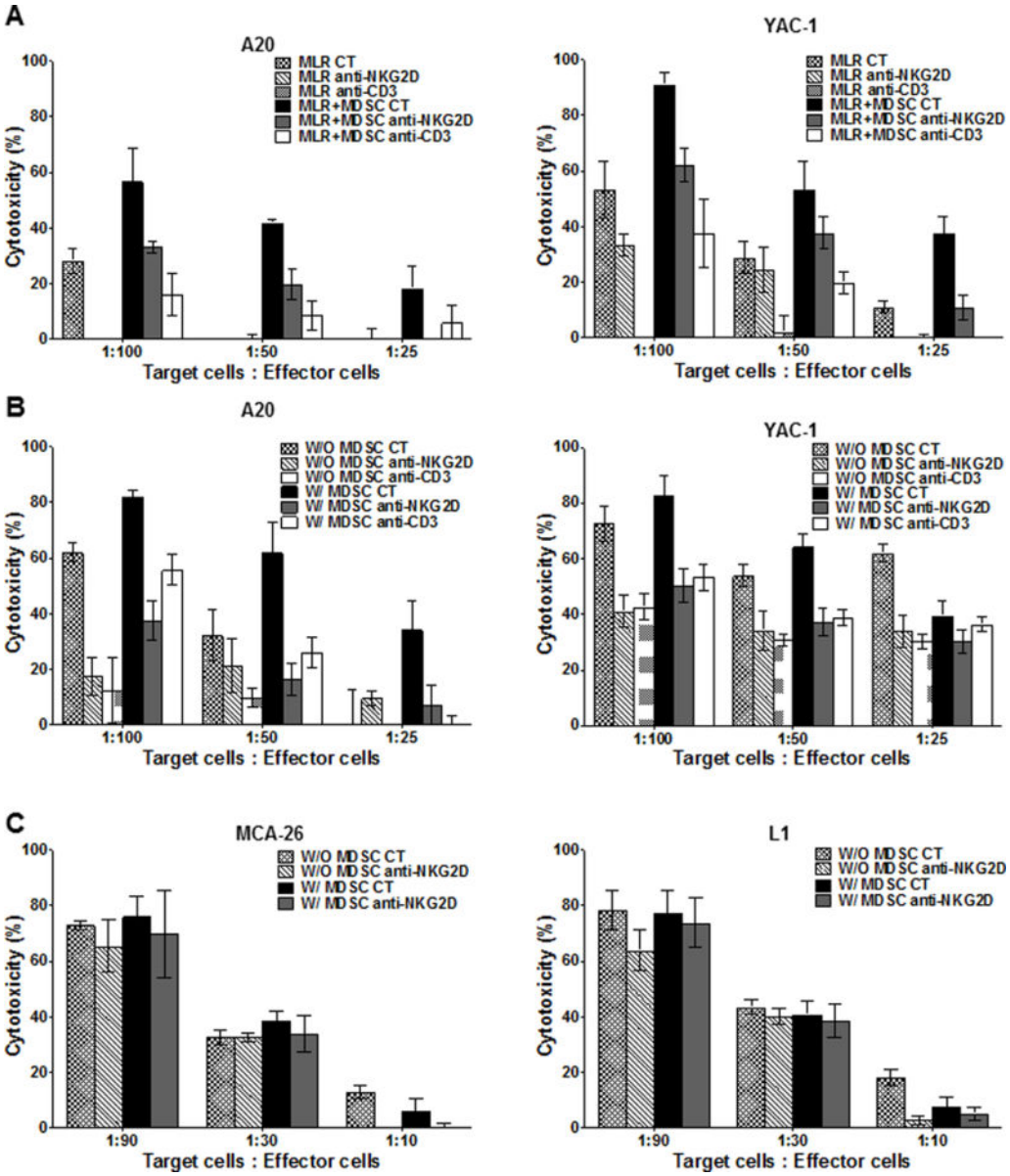

Figure 5. NKG2D and CD3 are essential for MDSC-conditioned T mediated tumor lysis.

(A) Cytotoxicity of T cells from MLR in the presence or absence of tumor-bearing-mouse-derived MDSCs. T cells were purified from MLR and their cytotoxicity against allogeneic A20 cells and Yac-1 cells was determined at various effector: target ratios in the presence or absence of CD3 or NKG2D blocking antibodies. **(B)** Cytotoxicity of donor T cells purified from Balb/c mice at day 14 after allo-HSCT with or without treatment of tumor-bearing-mouse-derived MDSC. The cytotoxicity against allogeneic A20 cells and Yac-1 cells at various effector: target ratios was assessed in the presence of CD3 or NKG2D blocking antibodies or isotype control. **(C)** Cytotoxicity of donor T cells against RAE1 low-expression tumor cells, L1 and MCA-26, in the presence of NKG2D blocking antibodies. Splenic donor T cells were purified from Balb/c mice at day 14 after allo-HSCT with or

without treatment of tumor-bearing-mouse-derived MDSCs. Data are presented as mean ± S.D., $n = 3$.

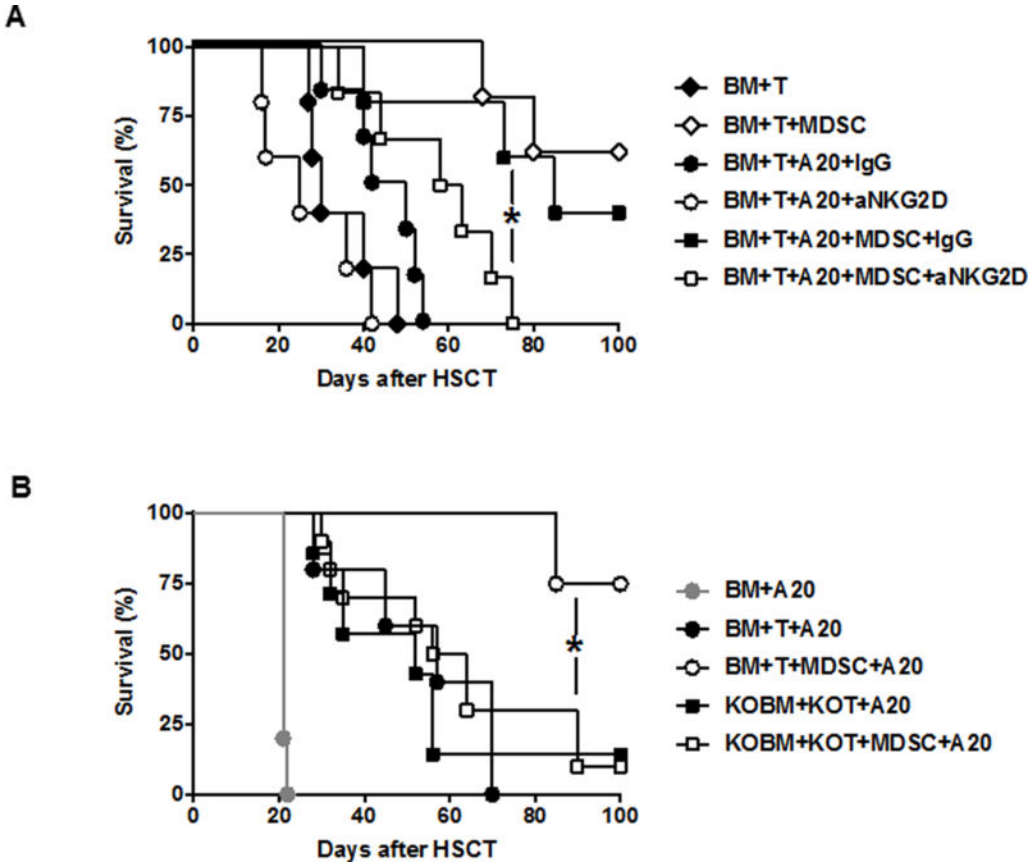

Figure 6. NKG2D is required for maintaining GVL activity in MDSCs-treated mice after allo-HSCT. (A) NKG2D blocking antibodies abrogate GVL effect in allo-HSCT mice treated with MDSC.

Lethally irradiated BALB/c mice were grafted with C57BL6 TCDBM and T cells, tumor-bearing-mouse-derived MDSC, and A20. MDSC recipients were given MDSCs on days 0, 4 and 10 after transplantation. For depletion of NKG2D population, mice were continuously injected with anti-NKG2D blocking antibodies (100 μg/mouse/day) for 10 days starting on day 14 post-transplantation. *$p<0.05$, n = 5 to 6 per group. **(B)** NKG2D deficient T cells fail to mediate GVL effect in allo-HSCT mice treated with MDSCs. BM and T cells were isolated from NKG2D deficient or wild-type C57BL6 mice then transplanted into lethally irradiated BALB/c mice that were injected with A20. MDSC recipients received tumor-bearing-mouse-derived MDSCs on days 0, 4 and 10 post-transplantation. Significantly prolonged survival is observed in the BM/T/A20/MDSC versus KOBM/KOT/A20/MDSC group, *$p<0.05$, n = 5 to 10 per group.

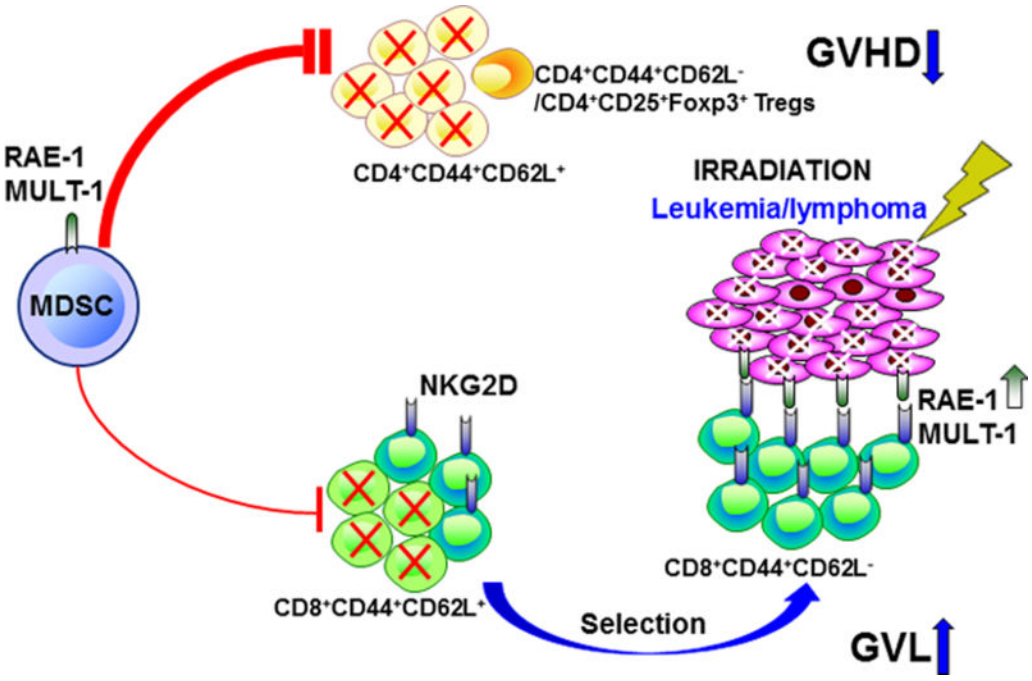

Figure 7. Proposed mechanisms by which MDSCs differentially regulate GVHD and GVL effect.

NKG2D ligand-expressing MDSCs favor Tregs expansion that prevent GVHD, while preserving GVL activity by selection expansion of NKG2D^{+} memory CD8 T cells. RAE-1, Retinoic acid early inducible 1; MULT-1, mouse UL16-binding protein-like Transcript 1; NKG2D, Natural killer group 2, member D; Tregs, regulatory T cells.