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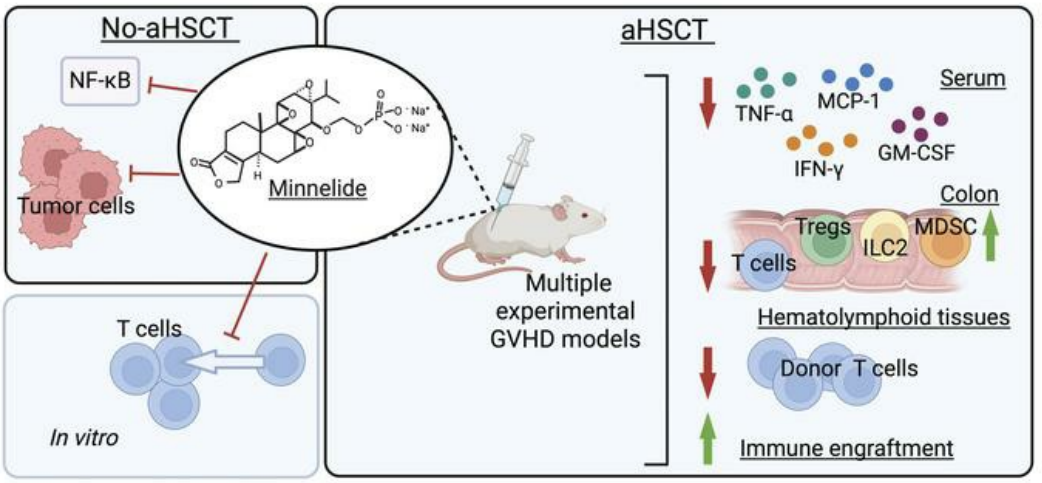
JCI Insight. 2024. https://doi.org/10.1172/jci.insight.165936.

Research In-Press Preview Immunology Transplantation

Graphical abstract



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Minnelide suppresses GVHD and enhances survival while maintaining GVT responses

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Word count for text: 4975

Word count for abstract: 197

Figure/Table count: 8 (+ Supplementary Figures / Tables: 11)

Reference count: 57

Scientific category chosen during submission: Transplantation

Abstract

Allogeneic hematopoietic stem cell transplantation (aHSCT) can cure patients with otherwise fatal leukemias and lymphomas. However, the benefits of aHSCT are limited by graft-versus-host disease (GVHD). Minnelide, a water-soluble analog of triptolide, has demonstrated potent anti-inflammatory and anti-tumor activity in several pre-clinical models and has proven both safe and efficacious in clinical trials for advanced gastro-intestinal malignancies. Here, we tested the effectiveness of Minnelide in preventing acute GVHD as compared to cyclophosphamide post-aHSCT (PTCy). Strikingly, we found Minnelide improved survival, weight loss and clinical scores in an MHC-mismatched model of aHSCT. These benefits were also apparent in minor MHC-matched aHSCT and xenogeneic HSCT models. Minnelide was comparable to PTCy in terms of survival, GVHD clinical score and colonic length. Notably, in addition to decreased donor T cell infiltration early post-HSCT, several regulatory cell populations including Tregs, ILC2s and MDSCs in the colon were increased which together may account for Minnelide's GVHD suppression post-HSCT. Importantly, Minnelide GVHD prevention was accompanied by preservation of graft-versus-tumor (GVT) activity. As Minnelide possesses anti-AML activity and is being applied in clinical trials, together with the present findings, we conclude that this compound might provide a new approach for AML patients undergoing aHSCT.

Introduction

Allogeneic hematopoietic stem cell transplantation (aHSCT) is the preferred curative therapy for high risk and / or relapsed hematological malignancies because of its desirable anti-leukemia / lymphoma (graft-versus-tumor: GVT) effect(1-3). Although GVT is mainly mediated by donor-derived alloreactive T cells, this population is also responsible for the development of graft-versus-host disease (GVHD)(4-6). Despite current prophylactic strategies, GVHD is a major cause of morbidity and mortality following aHSCT, and patients refractory to the first line standard regimens for GVHD treatment have

a poor prognosis. Although the use of post-transplant cyclophosphamide (PTCy) has provided an advance in treatment for aHSCT patients(7-12), new strategies are still needed to improve the complex balance between immune reconstitution and immunosuppression while preserving the beneficial graft-versus-tumor (GVT) effect.

Triptolide is the most potent bioactive compound isolated from the traditional Chinese herb Tripterygium Wilfordii Hook F, that exhibits immunosuppressive, anti-inflammatory, and anti-tumor activities(13-18). Triptolide holds strong promise as an immunosuppressant agent based on several studies that examined its potential therapeutic effect for GVHD prophylaxis and maintenance of GVT activity in transplant recipients(19-27). Therapeutic use of triptolide and many its derivatives is limited by issues with solubility, narrow therapeutic window, and toxicity(28). However, Minnelide (14-*O*-phosphonooxymethyltriptolide disodium salt) is a highly water-soluble prodrug of triptolide converted to its active form from phosphatases present throughout the body including the blood stream(29). Minnelide also has a more favorable toxicity profile than triptolide and is now being tested in preclinical models of multiple solid tumors(29-33) and acute myeloid leukemia (AML)(34). In addition, Minnelide is being pursued in a Phase I clinical trial for patients with AML.

Herein, we present preclinical assessment of Minnelide as a prophylactic therapy for GVHD in multiple murine and humanized models of aHSCT. In a complete MHC-mismatched model, we show that Minnelide effectively prevented GVHD and markedly increased overall survival of recipients with daily treatment of a short regimen of two weeks from day of transplant. Notably, together with decreased donor T cell infiltration early post-HSCT, several adaptive and innate regulatory cell populations were increased following Minnelide treatment. These included Tregs, ILC2s and MDSCs in several target tissues including the spleen, colon and lung. Treatment also promoted hematopoietic engraftment and importantly, GVT responses were effectively maintained. Notably, Minnelide could directly inhibit T cell proliferation and diminished Th1 and CD8 IFN-γ production early post-transplant. Use of NF-κB reporter mice demonstrated that a brief 2-day treatment with Minnelide reduced activation of the NF-κB pathway

consistent with the above findings. Importantly, to directly assess its ability to regulate human T cell mediated GVHD, NSG mice transplanted with mobilized human peripheral blood mononuclear cells (HuPBMC) were treated with Minnelide which ablated *in vivo* human T cell proliferation and prevented xenogeneic GVHD. Additionally, triptolide suppressed human CD4 and CD8 T cell proliferation *in vitro* to alloantigen stimulation. Therefore, direct inhibition of T cells together with an immune regulatory cell network may account for Minnelide suppression of GVHD. In total, these findings support the notion that Minnelide may provide new opportunities for developing translational strategies for the prevention of GVHD while maintaining GVT and potentially providing direct anti-tumor effects in some tumor models.

Results

Minnelide prevents GVHD and promotes hematopoietic engraftment following aHSCT. To determine if the triptolide pro-drug Minnelide affects the immune compartment, in the absence of an inflammatory stimulus, naïve BALB/c mice were treated daily for 30 days with either 0.1 or 0.2 mg/kg of this compound. No alterations in the overall splenic and lymph node CD4, CD8 T cell, regulatory T cells (Treg), and B cell compartments were detected (**Fig. S1).** Transplants were then performed to assess the capacity of Minnelide treatment to ameliorate GVHD. Strikingly we found that daily doses of 0.1 mg/kg of Minnelide from day-1 to day 28 improved weight loss, GVHD clinical score, and overall survival as compared to the untreated group in a complete MHC-mismatched (B6→BALB/c) HSCT model of GVHD (**Fig. 1A-C**). Animals receiving BM only treated with Minnelide exhibited no differences in weight loss, clinical score, lethality, or change in T cell frequency, indicating GVHD in untreated controls resulted primarily from allogeneic donor T cells (**Fig S2A-D**). Given the short half-life of Minnelide, we also tested a dose of 0.05mg/kg twice daily and obtained similar results (**Fig. S3A-C**). Ultimately, a dose of 0.1mg/kg 1x per day was selected for all subsequent experimental treatments. Blood monitoring at two weeks post-transplant showed reduced donor CD4 and CD8 T cells in

Minnelide treated versus non treated animals (**Fig 1D**). At two months post-transplant, CD4 and CD8 T cell analyses demonstrated improved frequency and ratios of these T cell subsets in the spleen similar to the BM only group (**Fig 1E and Fig. S3D**). We also noted thymocyte numbers were elevated and contained a normal number and percentage of DP thymocytes (**Fig 1E, Fig. S3E**). Histological evaluation of Minnelide treated mice indicated a significant decrease in skin involvement as assessed by overall thickening and fibrosis two-months post-aHSCT (**Fig. 1F**). Colon from untread mice exhibited mucosal thickening and severe inflammation with villi distortion. However, colon from Minnelide treated animals showed no disruption of villi architecture, mild inflammation, a marked reduction of infiltrating $CD3^+$ T cells, and improved length (**Fig. 1F-H**). Importantly, all effects on colon pathophysiology were visible as early as one-week post-transplant (**Fig. S3F**).

An independent experiment was performed using B6 congenic donor marrow (CD45.1) and T cells (CD45.2) to assess post-HSCT multi-lineage engraftment. Minnelide treatment augmented splenocyte and thymocyte (as noted above) numbers including higher CD4/CD8 splenic ratios and DP thymocyte numbers 7 weeks post-aHSCT (**Fig. 2A-D**). Immune lineages involved with the pathogenesis of GVHD were then assessed by flow cytometry (CD45.1 / MHC $H2K^b$), including T cells, NK and APCs. Mice receiving Minnelide treatment exhibited CD4 and CD8 T cell engraftment from donor marrow reaching >85% at 7 weeks post-transplant which was indistinguishable from bone marrow only transplanted mice (**Fig. 2E**). Regarding B cells ($CD19^+$), similar results were obtained with overall engraftment and again was comparable to bone marrow alone (**Fig. 2F**). Additionally, CD11b and NK1.1 expressing populations indicated predominant repopulation from donor marrow derived progenitors and the levels were comparable to marrow alone transplanted recipients (**Fig. 2G, H**).

Next, we compared Minnelide to PTCy treatment, a novel and widely used prophylactic GVHD treatment. Mismatched aHSCT recipients were administered Cy (50 mg/kg) on days 3,4 post-aHSCT(35, 36) or Minnelide as described. Notably, the treatments were virtually identical in preventing GVHD and significantly superior compared to untreated mice as assessed by survival, GVHD clinical

score, colon length, splenic CD4 and CD8 naïve/effector cells and thymic T cells (**Fig. S4A-D**). Additionally, Minnelide was administered over different time periods: twice (D3,4 to mimic post-transplant Cy), one week (D1-7) and 2 weeks. Days 3,4 treatment did have some beneficial effect on the mice survival. Administration for the first week resulted in improved survival and some transient clinical score improvement vs D3,4 (**Fig. S4E,F**). Notably, administration for 14 days resulted in 100% survival and diminished GVHD scores (**Fig. S4E,F**) that correlated with longer GI length (data not shown).

In a model of MHC-matched, minor antigen disparate donors/recipients (C3H.SW→B6), daily Minnelide treatment also reduced GVHD clinical scores and augmented the numbers of lymph node cells in recipients (**Fig. S5A**). However, in a chronic GVHD model (B10.D2→BALB/c) no effect in clinical scores were observed following Minnelide therapeutic administration in the second month post-aHSCT (**Fig. S5B**). In total, these findings demonstrated that Minnelide treatment prevented experimental GVHD in independent models and promoted multi-lineage chimerism from transplanted donor cells.

Minnelide limits donor T cell proliferation and proinflammatory cytokine production during aGVHD. Cellular events within the first 1-2 weeks post-transplant are critical to the development of GVHD. Notably, less $CD3^+$ cell infiltrate in Minnelide vs untreated mice was detected at early time points (one-week post-aHSCT) (**Fig. 3A**). To extend this finding and further investigate the effect by Minnelide on cellular events and signaling pathways involved with early T cell responses. Minnelide requires phosphatase activity leading to activation and passage through cell membranes. Therefore, to examine T cell proliferation *in vitro*, it was necessary to employ triptolide and we identified sharply reduced splenic T cell proliferation in a time and concentration – dependent manner (**Fig. 3B**). *In vivo*, following aHSCT, recipients were administered Minnelide 0.1 mg/kg for up to a week (these animals showed reduced GVHD clinical scores as anticipated, not shown) and the splenic T cell response was

evaluated. After 6h of *in vitro* stimulation with anti-CD3, splenocytes showed a marked diminishment of CD4 (Th1) and $CD8^+$ T cell producing IFNγ (**Fig. 3C, D**), and Th22 cells producing IL-13 (**Fig. 3E**). While Minnelide did not alter Treg frequency and subsets under homeostatic conditions (**Fig. S1D, data not shown),** during the first week post-HSCT in Minnelide treated animals, the frequency of Treg $CD4^+FoxP3^+$ cells was found to be elevated (**Fig. 3F**) and a summary of two independent experiments illustrates these proinflammatory T and Treg cell observations (**Fig. 3G**). Perhaps the Treg increase could result from lymphopenic conditions due to Minnelide's reduction of GVHD, by decreasing T cell expansion early post-HSCT.

Next, to examine the effect of Minnelide on pro-inflammatory cytokine production early post-aHSCT, serum was collected from recipients during the first week after MHC-mismatched transplant. Significant decreases were identified in several inflammatory cytokines at Day 4, specifically TNFα, IFNγ, MCP-1 and GM-CSF (**Fig. 4A-G**). Only TNFα remained decreased at Day 7 (**Fig. 4H-K**). NF-κB is a key signaling pathway involved that induces inflammatory cytokine production in immune cells. Because Minnelide has been shown to suppress NF-κB signaling *in vitro*(37), we next determined whether it could also do so *in vivo*, under inflammatory conditions. Since bacterial products (ex. LPS) leak across the gut wall following conditioning and transplant, NF-κB reporter mice (FVB NGL, see Methods) were injected with LPS 1 mg/kg with or without Minnelide 0.1 mg/kg and 4 hours later luciferase was measured as an indicator of NF-KB activity. Strikingly, we found substantial diminution of NF-kB activation in Minnelide treated mice relative to controls (**Fig. S6**).

Minnelide treatment increases innate and adaptive regulatory cell populations that promote immune suppressive activity in acute GVHD target tissues following MHC-mismatched aHSCT. An independent experiment was performed using a complete MHC-mismatched (B6→BALB/c) HSCT model of GVHD. As shown in Fig 1, daily doses of 0.1 mg/kg of Minnelide improved weight loss and

GVHD clinical score as compared to the untreated group (**Fig. S5C**). Three weeks post-transplant colonic lamina propria from each group of mice were analyzed and we found diminished frequency of $CD11b^+$ cells together with increased levels of regulatory populations including MDSC and ILC2s (**Fig. 5A-C**) which contained substantial levels of KLRG1 and ICOS expression (**Fig. 5D-E**).

Notably, Tregs were found to be significantly increased in the colon in two independent experiments in Minnelide treated animals compared to untreated recipients (**Fig. 5F**). Moreover, a terminally differentiation marker, KLRG1 was also found to be higher in this regulatory population in mice treated with Minnelide (**Fig. 5G**). Moreover, CD4 Th2 ($Gata3^+$) frequency was elevated together with decreased CD4 Tconv and CD8 T cells in the colon at three weeks post-transplant (**Fig. 5H-J**). We also examined another frequent GVHD target tissue, i.e. the lung and found Treg levels were doubled in this compartment as well ~ 3 weeks post-HSCT (**Fig. 5K**). These increases contrast the overall decrease in the frequency of T cells in the colon at this time (**Fig. 3A)** as well as in the spleen (**Fig. 5L)**. Moreover, CD11c expressing cells in the GI tract were examined at several time points (1 week, not shown; 8 weeks, **Fig. S5D**) and the levels of $CD11c^+$ cells were diminished in the Minnelide treated mice (**Fig. S5D**). These new data sets provide insight into regulatory pathways that may underline immune mechanism(s) induced by Minnelide treatment.

Minnelide treatment in recipient mice preserves functional immunity following aHSCT as assessed by effective GVT and skin allograft rejection responses.

To achieve relapse-free survival, amelioration of GVHD must be accompanied by maintenance of potent GVT responses. Here, we report that Minnelide reduces donor T cells and cytokine production in the context of GVHD. However, there is also evidence that Minnelide has anti-tumor cell activity. Therefore, several established mouse tumors used for GVT studies were examined to determine their susceptibility to direct killing by this triptolide pro-drug. First, we tested the effect of triptolide on P815

mastocytoma and A20 B cell lymphoma ($H2^d$) tumor cell lines *in vitro*. Triptolide exhibited potent dose dependent killing activity against both tumor cell lines, beginning at low doses (P815 and A20 IC50: $1.942x10^{-9}$ M and $1.600x10^{-9}$ M, respectively) (**Fig. S7A,B**). Consistent with the *in vitro* findings, non-transplanted mice injected with 2 x 10^6 A20 lymphoma cells showed significant tumor growth ablation in mice treated with Minnelide at GVHD regulation doses (0.1m/kg/day) (**Fig. S7C**). In contrast, Minnelide did not prevent tumor progression in untransplanted BALB/c mice injected with $AF9^{GFP}$ (H2d) cells and therefore could provide a useful preclinical model for a GVT response independent of Minnelide. Next, we assessed whether Minnelide treatment preserved GVT responses in a clinically relevant oncofusion protein induced leukemia. MLL-$AF9^{GFP}$ cells were administered into BALB/c mice together with donor B6 T and BM cells one day after irradiation (8 Gy). Mice were treated with Minnelide 0.1 mg/kg for 30 days. As anticipated, Minnelide treatment reduced GVHD severity, regardless of whether animals were injected with MLL-AF9 leukemia cells (**Fig 6A-C**). Importantly, T-cell mediated GVT activity was preserved as assessed by absence of MLL-$AF9^{GFP}$ tumor cells in peripheral blood three weeks post-aHSCT (**Fig 6D**). Furthermore, when blood, pLN, spleen, and BM were examined six weeks after transplant, we noted that anti-tumor activity in Minnelide treated recipients was as effective as in recipients that were not treated with this drug. (**Fig. S8**).

Next, we investigated the impact of Minnelide on tolerance to donor and recipient antigens placing multiple skin grafts onto individual HSCT recipient mice. Two heterotopic skin grafts, specifically one from B6×BALB/c F1 ($H2^{b/d}$) mice, and one from C3H/HeJ “third-party” ($H2^k$) donors were applied to the trunk of each BALB/c mouse 2 months post-aHSCT (by this time post-HSCT, 100% mortality had occurred in untreated GVHD animals). Strikingly, Minnelide-treated animals transplanted with donor T cells + BM accepted the F1 skin grafts as did recipients transplanted with BM only. These results demonstrated tolerance to donor and host allo-antigens in both groups. In contrast, these same recipient groups rejected complete MHC-mismatched “third party” C3H/HeJ allografts by three weeks post-skin grafting (**Fig 7A-C**). These findings together with the GVT data above provide evidence of

functional immunity in recipients of BM + T cells treated with Minnelide. Moreover, such immunity was accompanied by the establishment of tolerance to donor and host but not “third-party” allo-antigens.

Minnelide diminishes human donor T cell expansion and abrogates xenogeneic GVHD. To assess if Minnelide regulated human T cell responses, we used a xenogeneic GVHD (xGVHD) model involving human peripheral blood mononuclear cells (HuPBMC) from mobilized donors (**Fig. S9**). Recipient NSG mice were irradiated (2Gy) on day-1 and the following day received $6x10^6$ mobilized human PBMC. Groups were either untreated (controls) or treated with Minnelide 0.1 mg/kg for 30 days (**Fig. 8A**). Mobilized human $CD34^+$ cells (without T cells) were also transplanted into separate animals as “negative xGVHD controls”. Once again, we found that Minnelide treatment significantly prevented severe GVHD in this xenograft model as assessed by weight loss, clinical score, and overall survival (**Fig. 8B-E**). Notably, donor human $CD4^+$ and $CD8^+$ T cells were almost completely absent from the blood of Minnelide-treated animals similar to the negative control mice. In contrast, mice undergoing xGVHD contained a marked frequency of human donor cells in the PB (**Fig. 8F**). Interestingly, at this time, increased levels of donor $CD14^+$ cells were identified in these animals consistent with the notion that Minnelide did not directly damage this lineage (**Fig. 8F**). To directly address triptolide regulation of human T cell proliferation, PBMC were stimulated *in vitro* with anti-CD3 mAb and allogeneic stimulator cells (**Fig. 8G**). Both polyclonal and antigen stimulated cultures exhibited dose dependent triptolide inhibition of CD4 and CD8 T cell proliferation. In total, these immune findings demonstrated that Minnelide suppressed xGVHD by specifically targeting human donor T cells.

Discussion

Immunosuppressants with calcineurin inhibitors remain the standard of care for recipients of allogeneic HSCT to prevent GVHD. Unfortunately, these compounds have multiple off-target effects on a variety of tissues. More recently, PTCy has been used as GVHD prophylaxis in matched and mismatched

donors with good GVHD prevention but with its own toxicities(7, 8, 10, 12, 35). GVHD prophylaxis balancing reduction of GVHD and preservation of GVT are still needed. Triptolide compounds have been previously examined for anti-inflammatory activity as well as ability to inhibit immune responses including GVHD; however, it is largely insoluble in aqueous solvents limiting its clinical application(14). Ideally, triptolide derivatives which are water soluble with high efficacy and low toxicity would represent an advance for *in vivo* use to regulate transplant immunity. Minnelide, a water-soluble triptolide pro-drug was shown to exhibit minimal toxicity even after >1 year of *in vivo* administration and it can rapidly convert to triptolide in the presence of phosphatases - which are ubiquitous(29). Preclinical studies reported that Minnelide possesses potent anti-proliferative activity against several cancers including pancreatic and hematologic, dramatically reducing tumor growth, preventing metastasis, and improving overall survival(30, 34, 37). Minnelide is also the most advanced compound clinically for cancer treatment amongst all triptolide analogues(17, 38) (NCT# 03117920). Here, we demonstrated that low doses of Minnelide treatment prevented severe acute GVHD using multiple models of aHSCT with substantial improvement in outcomes without abrogating anti-tumor activity, ie. GVT responses against experimental AML. Overall, in addition to ameliorating GVHD, Minnelide may be effective to eradicate residual disease in some hematological malignancies due to both direct and indirect (via GVT) activities thereby providing a potentially novel therapeutic approach to improve aHSCT.

Recently, Giri et al showed that Minnelide treatment exhibited potent anti-leukemic effects in human AML in vitro and in vivo models, using tumor cell lines and patient derived cells(34). Similarly, in the present study, we found that triptolide induced dose-dependent killing of the P815 mastocytoma and A20 B cell lymphoma tumor cell lines *in vitro* and Minnelide exhibited anti-tumor activity *in vivo* (A20). Since the MLL-AF9^{GFP} cells did not show susceptibility *in vivo* to the Minnelide dose being used, this AML was selected to enable evaluation of GVT activity following HSCT and results showed that anti-tumor response was maintained concurrent with reduction of GVHD. Therefore, we posit that the

potential combination of direct anti-tumor activity concomitant with GVHD-suppression and maintenance of GVT responses make Minnelide an attractive agent for clinical development.

Two decades ago, triptolide and its derivatives were examined in murine haploidentical MHC (B6→F1) and MHC-matched (B10.D2→BALB/c) aHSCT models(19, 20). Using a water soluble triptolide derivative (PG490-88, 0.535 mg/kg for 21 days) Chen *et al.* found amelioration of preclinical GVHD and proposed to involve inhibition of early IL-2 synthesis(19). Because $V\beta3^+$ T cell deletion was incomplete, the authors posited antigen specific tolerance was implicated. *In vitro*, triptolide was previously reported to inhibit NF-kB, NFAT, and diminished IL-2 production in T cells(39). In our studies, Minnelide was employed at substantially lower concentrations (0.1 mg/kg) to investigate its capacity to inhibit initial responses driving GVHD. Indeed, at this low dose, we observed a marked decrease in donor T cell frequency within the peripheral lymphoid compartment following both MHC-mismatched allogeneic (B6→BALB/c) and xenogeneic (huPBMC→NSG) transplant as early as 1-3 weeks post-aHSCT (**Figs 1,5,8**). Consistent with the diminished xenogeneic GVHD findings, triptolide diminished human T cell proliferation to anti-TCR and alloantigen stimulation. Minnelide also markedly diminished proinflammatory effector populations (CD8 and Th1 IFNγ producing cells) and serum levels of proinflammatory cytokines (including TNFα, IFNγ, MCP1, and GM-CSF) (**Fig. 3,4**). These findings support the notion that Minnelide abrogates T cell proliferation and inflammatory responses that promote aGVHD. GVHD prophylaxis for patients often involves calcineurin inhibitor (CNI) based regimens with methotrexate +/- anti-thymocyte globulin for HLA matched transplants(40). Cyclophosphamide after transplant (PTCy) plus tacrolimus +/- mycophenolate mofetil (MMF) has been introduced for haplo-mismatched transplants and shown promise compared to tacrolimus +/- MMF +/- methotrexate based on improvement reported in a number of studies including lower grade 3-4 GVHD and diminished lower GI and chronic GVHD vs CNI(41). Since 2017, several new drugs have been FDA approved for second line defense i.e., Ibrutinib, Belumosudil and Ruxolitinib and the CTLA-4

costimulation blocker abatacept is currently being examined for GVHD treatment (42-45). While CNI (associated toxicity in elderly patients can include nephrotoxicity, hypertension, and electrolyte abnormalities), they are administered typically for several (often 3-6) months and can be tapered(46, 47). Studies here found that 14 days of Minnelide treatment in MHC-mismatched recipients resulted in long lasting effects without loss of anti-tumor activity. As noted above, we also observed that triptolide *in vitro* and Minnelide *in vivo* exhibited anti-tumor activity suggesting the drug could provide additional benefit to patients transplanted due to hematopoietic cancers. Therefore, it may be interesting to consider combining CNI with Minnelide as both inhibit T cells and Minnelide also elevated regulatory populations.

One week after transplant, together with the diminished cytokine levels noted above, a marked reduction in donor T cell infiltrate was found in the colon of Minnelide treated recipients. Strikingly, this effect was maintained eight weeks post-transplant accompanied by conserved villi and overall improved tissue architecture compared to untreated animals. Early damage to the GI tract results in leakage of bacterial products including LPS which plays a key role in driving intestinal inflammation and development of GVHD. Following LPS binding to TLR4, MyD88 signaling leads to NF-kB activation. Along with other transcription factors, these events promote cytokine / chemokine production contributing to initiation and amplification of systemic inflammation resulting in aGVHD that is often associated with morbidity and mortality post-aHSCT(48, 49). We previously reported Minnelide treatment downregulated NF-kB activity in pancreatic tumors(37). Notably, experiments here showed Minnelide significantly inhibited NF-kB activation *in vivo* following LPS administration. Altogether these findings demonstrated that Minnelide prevented GVHD-driven events including GI T cell infiltration, intestinal tissue damage, and inflammatory cytokine production. We propose that the latter process might be mediated by NF-kB signaling inhibition by Minnelide treatment.

Triptolide and its analogs have been found to regulate several immune cell populations in pathologic conditions. For example, in rheumatoid arthritis, triptolide suppressed maturation, migration and differentiation of DC(50, 51). Triptolide was also reported to diminish costimulatory molecules and inflammatory cytokines in monocytes/macrophages(14, 52). These findings, together with the decrease of inflammatory cytokine serum levels in Minnelide-treated animals early post-aHSCT, makes it tempting to speculate that the compound could also affect function and migration of APCs in transplanted mice. In physiologic conditions, we found that Minnelide treatment did not affect B cells, CD4, CD8 and Tregs in naïve mice. Regarding the latter, pSTAT5 expression, which is required for Treg proliferation and function, was not altered by Minnelide treatment in animals undergoing Treg expansion, supporting the notion that Treg IL-2 signaling remained intact (*data not shown*). Interestingly, in the setting of GVHD after donor T cells were transplanted, an increase in $CD4^{+}FoxP3^{+}$ Treg frequency was observed with Minnelide treatment in several GVHD target tissues (ex. spleen, colon, lung). Remarkably, with regard to a regulatory cell mechanism, 3-weeks post-transplant in treated recipients, several innate regulatory populations were also evaluated. Increased levels of MDSCs and ILC2s, the latter population included significant expression of ICOS that reportedly regulates these cells under homeostatic and inflammatory conditions in mice(53) were identified. Thus, we posit that together with direct inhibition of T cell proliferation, Minnelide's induction of an adaptive and innate regulatory cell network may account for GVHD suppression.

In total, findings in the present study demonstrated Minnelide regulation of mouse as well as human T cell immune responses in the aHSCT transplant setting. Notably, this compound is currently in clinical trials for patients with gastric / pancreatic cancers (NCT01927965, NCT03117920) and a study is now recruiting for treatment of non-transplant related relapsed or refractory AML (NCT03760523). Graft-versus-malignancy responses following aHSCT are crucial for the eradication of hematologic malignancies, particularly AML(3). The experimental findings here using a murine leukemia model are encouraging as diminishment of GVHD was accompanied by GVT responses

against this AML. Dependent on the concentration of Minnelide, such responses may benefit from its direct anti-tumor activity in certain cancers. Findings here indicated that in comparison to PTCy application, Minnelide treatment resulted in equivalent reduction in GVHD and improvement of outcomes. Overall, we propose that Minnelide treatment may provide a new translational therapeutic strategy for patients undergoing aHSCT for hematologic cancers to reduce GVHD and maintain GVT.

Methods

Sex as biological variable.

The nature of the experiments involve transplants in which cages must be maintained for extended time periods. The experimental results were therefore obtained using female animals without bias from injuries and hormones due to fighting and aggressive behavior in the cages. We anticipate that results would be the same in males.

Animals

C57BL/6J (B6, Stock: 000664), BALB/c (Stock: 000651), C3H.SW (Stock: 000438), B6-CD45.1 breeder (Stock: 002014), B6-EGFP breeder (Stock: 003291), NGL-NF-κB-GFP-luciferase (Stock: 027529), and NSG (Stock: 005557) mice were purchased from The Jackson Laboratory and maintained in our animal facility. All mice were maintained in specific pathogen-free housing at the University of Miami and given autoclaved food and water ad libitum. Mice were used at 8–16 weeks of age. All animal use procedures were approved by the University of Miami institutional animal care and use committee (IACUC protocol numbers 19–114 and 18–036).

Allogeneic HSCT and Minnelide treatment

For MHC-mismatched transplants, BALB/c mice received 7.5-8.5 Gy on day −1 and BM was injected 24 hours later with or without T cells from sex and age-matched B6 (5.5×10^6 TCD BM cells and pooled

splenocytes containing 0.8×10^6 T cells) donors. T cell depletion (TCD) of the BM was performed using HO134 hybridoma supernatant (αThy1.2, 40% of final volume at 25×10^6 cells/ml) and rabbit complement (Cedarlane Labs) immediately prior to transplant. Mice were monitored 3x per week for weight loss and clinical score as previously described(54, 55). In brief, clinical signs of GVHD were scored on a scale from 0 to 2 for 5 parameters: weight loss, diarrhea, fur texture, posture, and alopecia. Mice exhibiting a clinical score greater than 6 were sacrificed and their death was recorded as the next day, in accordance with our animal protocols. Minnelide was dissolved in saline and administered intraperitoneally (i.p.) at a dose of 0.05 mg/kg/2xday or 0.1 mg/kg/day for 30 consecutive days.

Experiments to assess GVT utilized MLL-AF9 GFP^+ BALB/c leukemia cells(56). For these experiments, 5000 leukemia cells were co-transplanted with B6 donor spleen + / - TCD BM cells. Tumor burden was assessed via flow cytometric analysis of blood, spleen, and marrow compartments.

For a description of histopathology and immunostaining, allogeneic heterotopic skin transplantation, flow cytometry, multiplex cytokine array, and T cell proliferation assay, see Supplemental Methods.

Xenogeneic human to mouse transplantation and Minnelide treatment

PBMC were isolated from human mobilized (Filgrastim) peripheral blood by ficoll separation and viable T cells counted (all human cells were obtained from consented donors according to IRB approved (20160363). NSG mice were irradiated (2 Gy, total body irradiation) and transplanted the following day with 6×10^6 PBMC which included 3.6 $x10^6$ T cells and ~$2x10^4$ $CD34^+$ cells. Recipient mice were injected with or without Minnelide 0.1 mg/kg from day -2 to day 28. Mice were monitored 3x per week for GVHD clinical score (as above), weight loss, and survival until 6 weeks post-transplantation. Control recipients were administered positively selected human $CD34^+$ from the same donor to mimic BM only groups in the allogeneic HSCT.

One-way MLR

Fresh blood (30 to 40 mL) from two genetically distinct donors was collected and PBMCs were isolated using Ficoll-Paque following manufacturer's protocol. Triptolide stock, 1 mM in DMSO, was added to complete media to a concentration of 12.5 nM followed by serial dilutions of 2.5, 0.5, and 0.1 nM concentrations. The vehicle was DMSO added to complete media at a concentration of 12.5 nM. Isolated PBMCs were plated at 500,000 (responders) and 1,000,000 irradiated stimulators PBMCs per well. Responder PBMCs were labeled with Cell Trace Violet (CTV) on day 0 of the culture at 2.5 μM CTV final concentration prior to plating. Wells were collected on day 6 for manual counting and CTV proliferation assessment gating CD4 and CD8 lymphocytes. *In vitro* stimulation: PBMCs isolated from one donor were plated in wells with OKT3 (1 μg/well) and rhIL-2 (200 ng/well) at 1,000,000 PBMCs after CTV labeling on day 0 at 2.5 μM CTV final concentration. Wells were collected on day 4 for manual counting and CTV proliferation assessment after gating onto CD8 lymphocytes.

Statistical analysis.

Numbers of animals per group are described in the figure legends. All Figure panels include data sets obtained from individual animals. All graphing and statistical analysis were performed using GraphPad Prism 9 (La Jolla, CA). Significance of differences between two experimental groups were determined using two-tailed unpaired t test. For experiments comparing more than two groups, data were analyzed using a one-way or two-way ANOVA with a post hoc Dunnett's or Tukey's multiple comparisons test. For experiments using multiple t tests over time, P values were adjusted using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli (57). For survival analyses, a Log-rank (Mantel-Cox) test was performed. Statistical tests performed are indicated in the figure legends. Significance indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns=non-significant. Data shown are means ± SEM.

Study Approval

All mice were used at 6 to 12 weeks of age and were maintained in pathogen-free conditions at the University of Miami animal facilities. All animal procedures used were performed under protocols approved by the University of Miami Institutional Animal Care and Use Committee. Gables One Tower, 1320 S Dixie Hwy, Room 650, Coral Gables, Florida 33146-2926.

Data availability

Data can be accessed from the corresponding author (RBL) upon request.

Author Contributions

S.N.C. and V.T.G. designed research studies, discussed, analyzed and interpreted data, conducted experiments, and wrote the manuscript. H.B. and B.M.V. conducted experiments and analyzed data. C.S.B., D.W., B.P. M.G. and A.V. performed research, analyzed and interpreted data, and edited the manuscript. K.V.K., C.L.B. and S.P. provided reagents and edited the paper. A.S. discussed data and supported the research. R.B.L designed experiments, discussed, analyzed and interpreted data, wrote the paper, supervised and supported the research.

Acknowledgements

We are grateful to the staff at the Division of Veterinary Resources at the University of Miami Miller School of Medicine for their outstanding animal maintenance and care of the mice used in these studies. We also thank Dr. Oliver Umland and the Sylvester Comprehensive Cancer Center Flow Cytometry Core for excellent technical assistance. We are thankful to Dr. Robert Negrin (Stanford University, Stanford, CA) for generously providing the $A20^{luc/YFP}$ cell line. The graphical abstract was created using

Biorender.com. **Funding.** This work was supported by funds from the NIH R01EY024484-06, R01EY030283–01, R41AI149916-01, and SRA from Heat Biologics, Inc. and Sylvester Comprehensive Cancer Center (R.B.L); New Investigator Award from ASTCT (S.N.C.); F99CA245728 and K00CA245728(C.S.B); the Applebaum Foundation and Kalish Family Foundation (K.V.K, D.W. and C.L.B); R01CA168814 (S.P.).

Disclosure of Conflicts of Interest

R.B.L. is a compensated consultant/advisory board member for and equity holder in Heat Biologics and a consultant for Kimera Labs, Miramar FL. University of Minnesota has a patent for Minnelide (WO/2010/129918), which has been licensed to Minneamrita Therapeutics LLC. A.K.S. is the cofounder and the CSO of this company. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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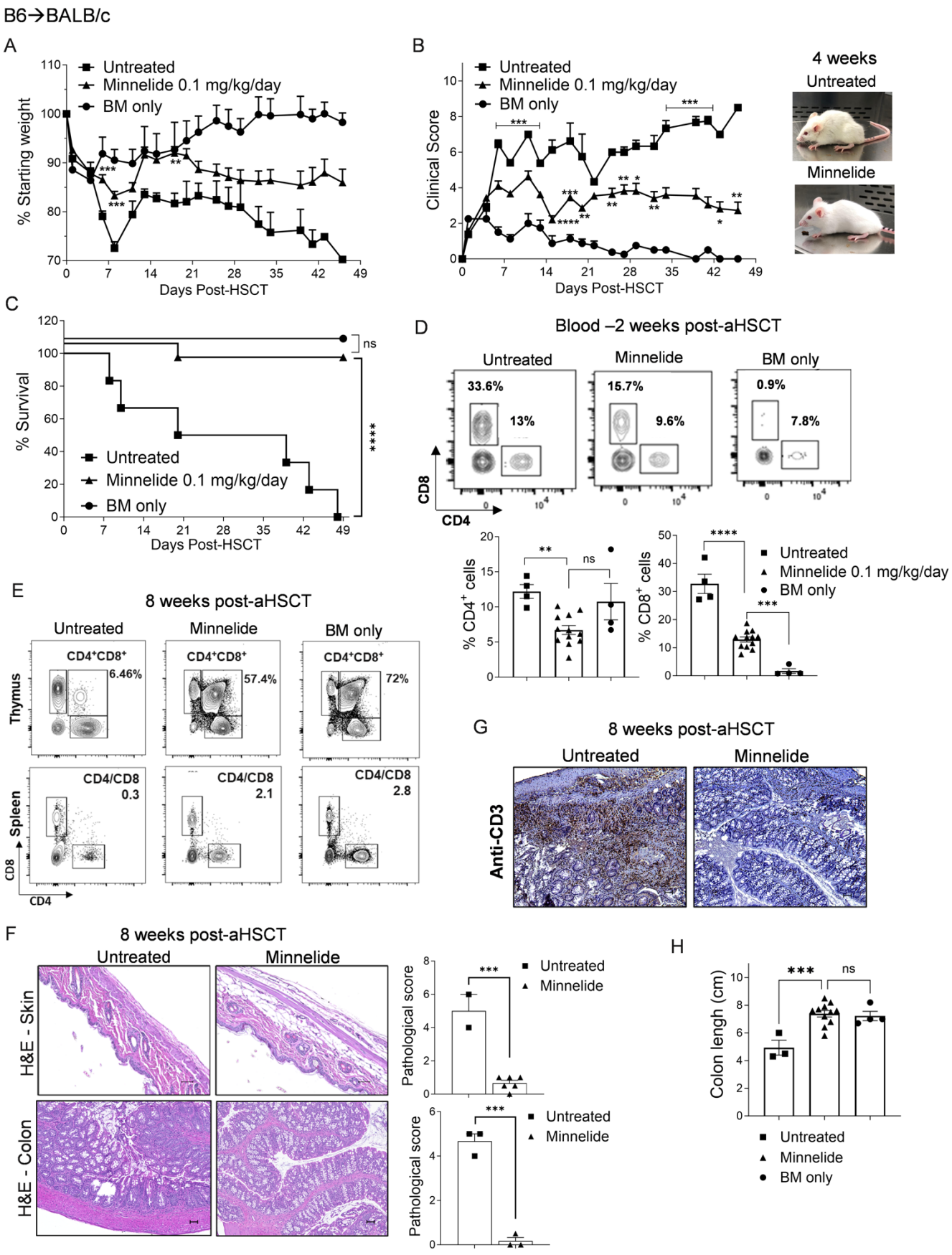

B6→BALB/c
A
Untreated
Minnelide 0.1 mg/kg/day
BM only
% Starting weight
Days Post-HSCT
B
Clinical Score
4 weeks
Untreated
Minnelide
C
% Survival
ns
****
D
Blood –2 weeks post-aHSCT
33.6%
13%
15.7%
9.6%
0.9%
7.8%
CD8
CD4
% CD4+ cells
% CD8+ cells
E
8 weeks post-aHSCT
CD4+CD8+
6.46%
57.4%
72%
Thymus
Spleen
CD4/CD8
0.3
2.1
2.8
G
8 weeks post-aHSCT
Anti-CD3
F
8 weeks post-aHSCT
H&E - Skin
H&E - Colon
Pathological score
H
Colon lengh (cm)

Figure 1. Recipients treated with Minnelide exhibited diminished acute GVHD after MHC-mismatched aHSCT. An aHSCT was performed utilizing a B6→ BALB/c donor and recipient mouse model (Day -1: 7.5-8.5 Gy; Day 0: 5.5 $\times 10^6$ TCD BM cells and pooled splenocytes containing 0.8×10^6 T cells) and recipients were treated with Minnelide 0.1 mg/kg from day −2 to 28 after transplant (**A-C**). Weight loss (**A**), clinical scores (**B**), and GVHD survival (**C**) are presented (n=8 mice untreated, n=16 mice Minnelide and n=4 mice BM only group). (**D**) Representative flow cytometry contour plots and frequency of $CD4^+$ and $CD8^+$ cells in the blood day 14 post-aHSCT. (**E**) Eight weeks post-aHSCT thymic and splenic T cell populations were evaluated and representative flow cytometry plots are shown. (**F**) Representative H&E staining and pathology scores (on the right) (n=2-6) from the skin and colon eight weeks post-aHSCT. Magnification 200x and 100x, respectively. (**G**) Representative photographs of colon anti-CD3 staining eight weeks after transplant. Magnification 100x. (**H**) Increased colon length in recipients of Minnelide treatment eight weeks post-aHSCT. These data are representative of 4 independent HSCT experiments in this model. Clinical scores were compared using multiple t tests over time. Groups were compared using one-way ANOVA Tukey's multiple comparison test and log-rank was used for survival analyses. *p<0.05, **p<0.01, *** p<0.001 and **** p<0.0001. Data are means ± SEM.

B6→BALB/c

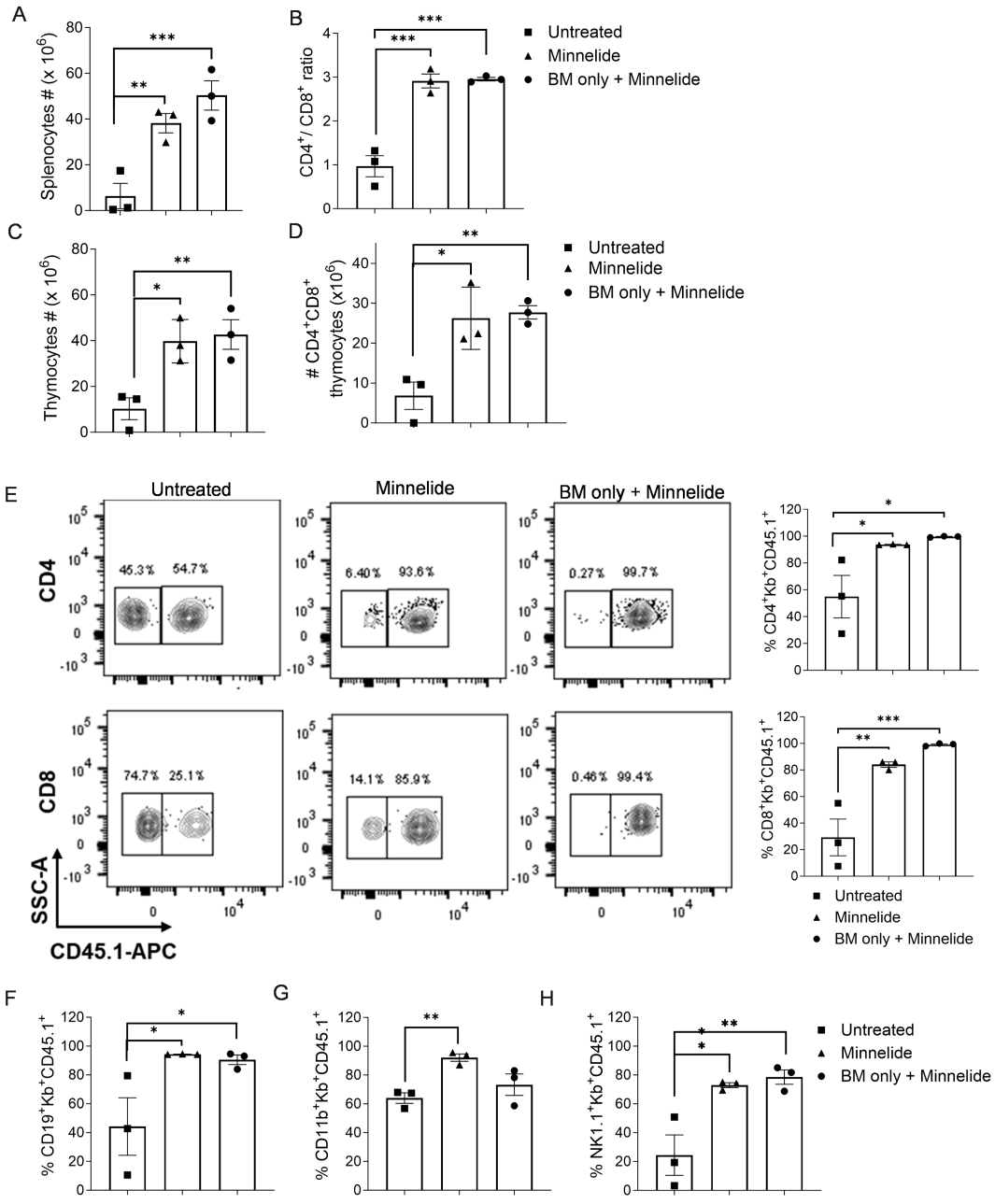

A
Splenocytes # (x 10^6)
B
CD4+ / CD8+ ratio
Untreated
Minnelide
BM only + Minnelide
C
Thymocytes # (x 10^6)
D
CD4+CD8+ thymocytes (x10^6)
E
Untreated
Minnelide
BM only + Minnelide
CD4
CD8
SSC-A
CD45.1-APC
45.3% 54.7%
6.40% 93.6%
0.27% 99.7%
74.7% 25.1%
14.1% 85.9%
0.46% 99.4%
% CD4+Kb+CD45.1+
% CD8+Kb+CD45.1+
F
% CD19+Kb+CD45.1+
G
% CD11b+Kb+CD45.1+
H
% NK1.1+Kb+CD45.1+

Figure 2. Minnelide treatment promotes lymphoid engraftment after MHC-mismatched aHSCT. An aHSCT was performed utilizing the same model as Figure 1 (B6 → BALB/c donor and recipient strain combination) but transplanted bone marrow was derived from congenic B6-CD45.1 donors and T cells from B6-CD45.2 donors. Recipients were treated with Minnelide 0.1 mg/kg from day −2 to 28 after transplant (**A-H**). Seven weeks after aHSCT splenic and thymic tissues were analyzed for total cell numbers (**A, C**), splenic CD4/CD8 ratio (**B**), and $CD4^+CD8^+$ DP thymocytes (**D**). Representative flow cytometry contour plots and frequency of donor (transplanted hematopoietic progenitors) bone marrow-derived (Kb+CD45.1+) CD4 and CD8 cells in the spleen seven weeks post-aHSCT are shown (**E**). Frequency of splenic $CD19^+$ **(F),** $CD11b^+$ **(G)** and $NK1.1^+$ **(H)** derived from donor bone marrow seven weeks after transplant. GVHD, n=3; Minnelide, n=3; BM only+Minnelide, n=3. Groups compared using one-way ANOVA Dunnett's multiple comparison test. *p<0.05, **p<0.01, and *** p<0.001. Data are means ± SEM.

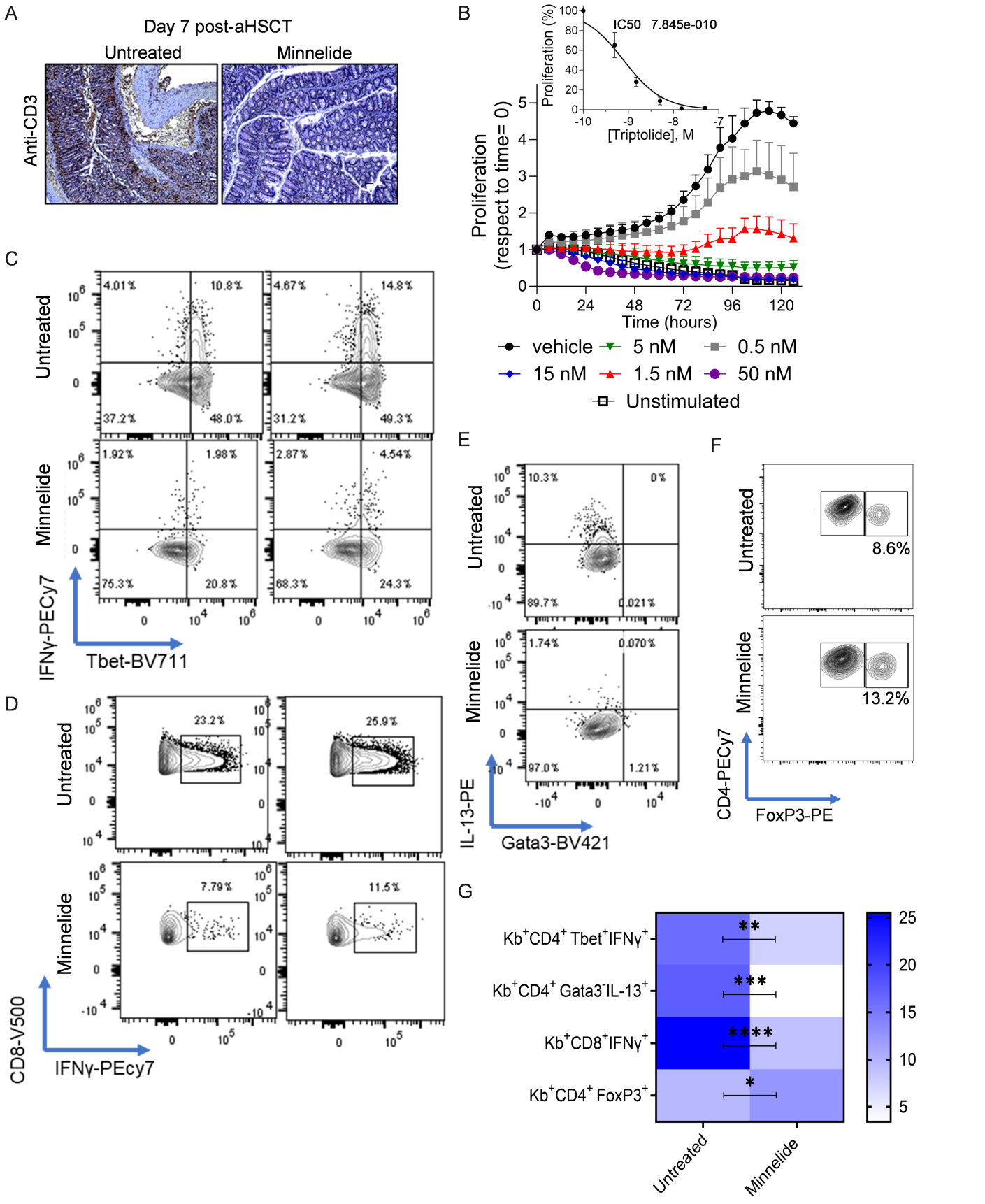
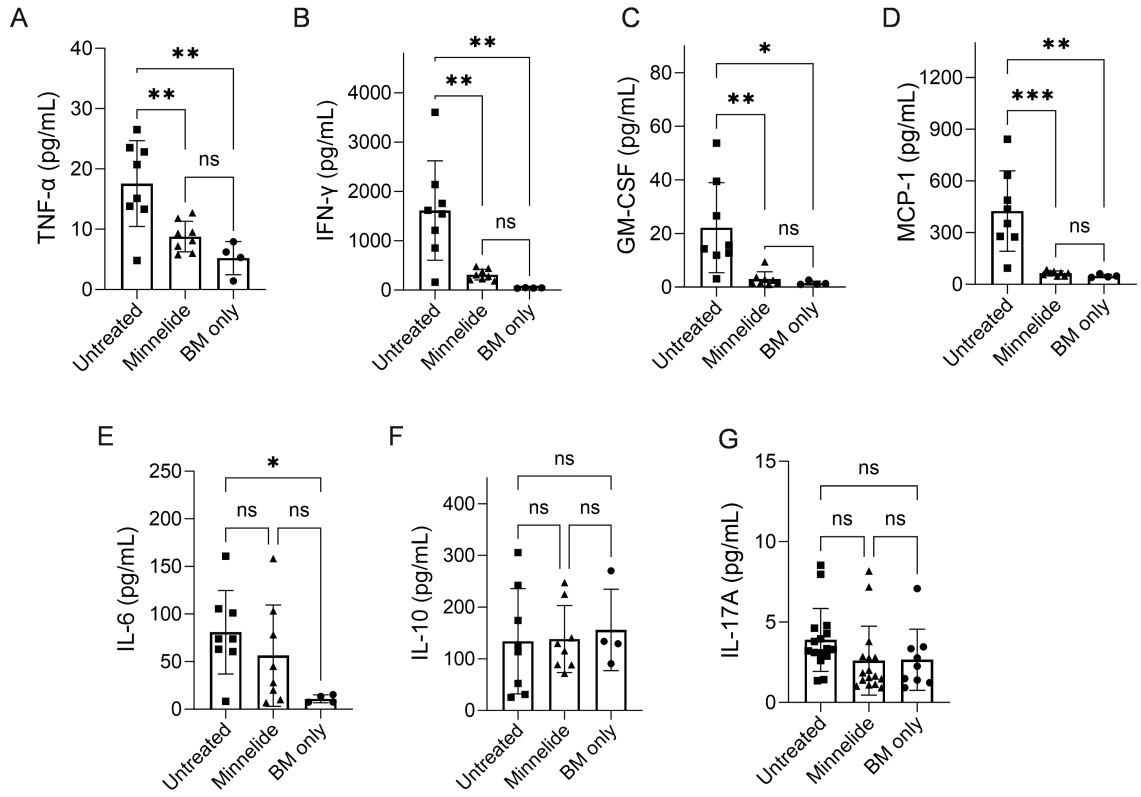

A
Day 7 post-aHSCT
Untreated
Minnelide
Anti-CD3
B
IC50 7.845e-010
Proliferation (%)
[Triptolide], M
Proliferation (respect to time= 0)
Time (hours)
vehicle
5 nM
0.5 nM
15 nM
1.5 nM
50 nM
Unstimulated
C
Untreated
Minnelide
4.01%
10.8%
37.2%
48.0%
4.67%
14.8%
31.2%
49.3%
1.92%
1.98%
75.3%
20.8%
2.87%
4.54%
68.3%
24.3%
IFNγ-PECy7
Tbet-BV711
D
Untreated
Minnelide
23.2%
25.9%
7.79%
11.5%
CD8-V500
IFNγ-PEcy7
E
Untreated
Minnelide
10.3%
0%
89.7%
0.021%
1.74%
0.070%
97.0%
1.21%
IL-13-PE
Gata3-BV421
F
Untreated
Minnelide
8.6%
13.2%
CD4-PECy7
FoxP3-PE
G
Kb+CD4+ Tbet+IFNγ+
Kb+CD4+ Gata3-IL-13+
Kb+CD8+IFNγ+
Kb+CD4+ FoxP3+
**
***
****
*
Untreated
Minnelide

Figure 3. Minnelide can inhibit T cell proliferation and decreases T cell proinflammatory cytokines early after MHC-mismatched aHSCT. Recipients were treated with Minnelide 0.1 mg/kg from day −2 to 7 after transplant and on day 7 colon and spleen were evaluated. (**A**) Representative photographs of colon anti-CD3 staining showed a marked decrease in $CD3^+$ cell infiltrate. Magnification 100x. (**B**) Purified T cells were seeded in 96-well plates and treated with different doses of triptolide for 120h. A dose-dependent decrease in proliferation was observed in response to Triptolide 0.5 – 50 nM. A significant and lasting reduction in proliferation was observed in response to treatment. (**C-G**) On day 7 post-aHSCT spleen was analyzed for frequency of donor (**C**) Th1 cells producing IFN-γ, (**D**) $CD8^+$ producing IFN-γ, (**E**) Th22 producing IL-13, and (**F**) $CD4^+FoxP3^+$Tregs. (**G**) Summary data of the frequency of the indicated donor (Kb^+) cell populations. These data were pooled from two independent HSCT experiments Untreated, n=5; Minnelide, n=5. Groups were compared using unpaired t test. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. Data are means ± SEM.

B6→BALB/c

Day 4 post-aHSCT



Day 7 post-aHSCT

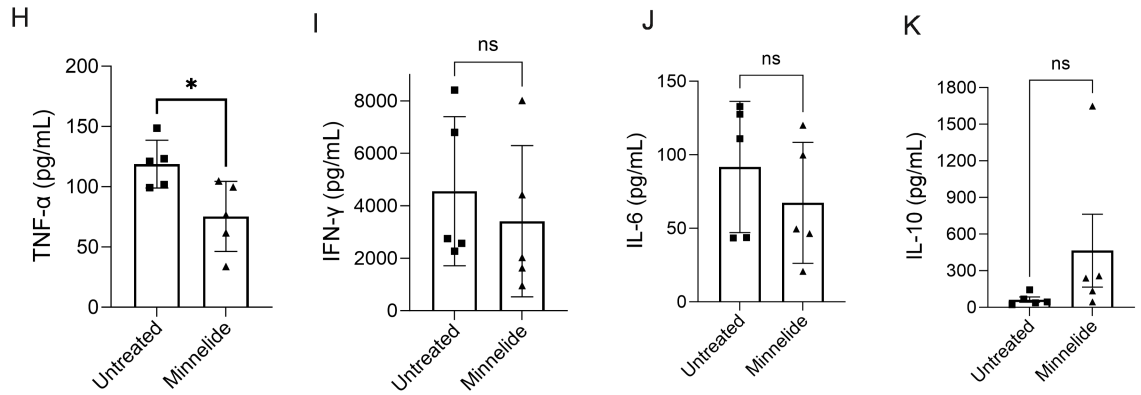


Figure 4. Minnelide decreases cytokine storm in recipient mice following MHC-mismatched aHSCT. **(A**) Using the major BMT model described in Fig 1, recipients were treated with Minnelide 0.1 mg/kg from day −2 to 7 after transplant and on day 4 (**A-G**) and 7 (**H-K**) serum was collected via cardiac puncture for cytokine quantification using LEGENDplex™ Mouse Inflammation Panel. (**A-K**) The specific cytokines evaluated are indicated in the "y-axis" of each graph. Groups were compared using one-way ANOVA Tukey's multiple comparison test. *p<0.05, **p<0.01, *** and p<0.001. Data are means ± SEM.

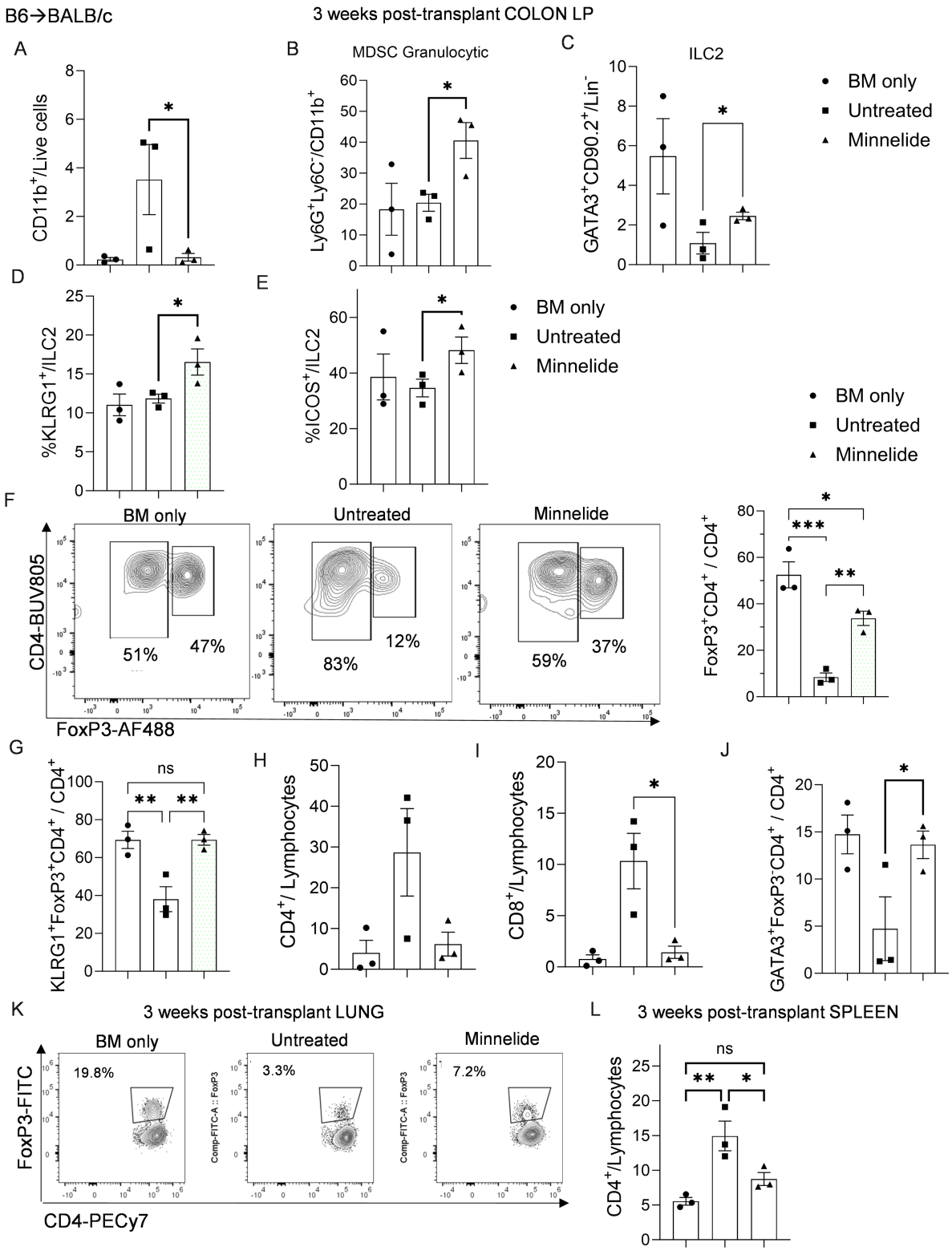

B6→BALB/c
3 weeks post-transplant COLON LP
A
CD11b+/Live cells
B
MDSC Granulocytic
Ly6G+Ly6C-/CD11b+
C
ILC2
GATA3+CD90.2+/Lin-
BM only
Untreated
Minnelide
D
%KLRG1+/ILC2
E
%ICOS+/ILC2
F
BM only
Untreated
Minnelide
CD4-BUV805
FoxP3-AF488
51%
47%
12%
83%
59%
37%
FoxP3+CD4+ / CD4+
G
KLRG1+FoxP3+CD4+ / CD4+
ns
H
CD4+ / Lymphocytes
I
CD8+/Lymphocytes
J
GATA3+FoxP3-CD4+ / CD4+
K
3 weeks post-transplant LUNG
FoxP3-FITC
CD4-PECy7
19.8%
3.3%
7.2%
L
3 weeks post-transplant SPLEEN
CD4+/Lymphocytes

Figure 5. Minnelide treatment increases innate and adaptive regulatory cell populations that promote immune suppressive activity in GVHD target tissues following MHC-mismatched aHSCT. Using the major BMT model, B6→BALB/c (described in Fig 1), recipients were treated with Minnelide 0.1 mg/kg from day −2 to day +20 after transplant. On day 20 post-aHSCT, lamina propria (LP) from colon (**A-J**) as well as lung lymphocytes (**K**) were evaluated. Data is presented as frequency of colonic $CD11b^+$ cells (**A**); frequency of MDSCs ($Ly6G^+Ly6C^-/CD11b^+$) **(B);** frequency of ILC2 ($GATA3^+CD90.2^+Lin^-$) **(C);** frequency within the ILC2 population of $KLRG1^+$cells **(D)** and $ICOS^+$ cells **(E).** Representative flow cytometry contour plots and frequency of Tregs ($CD4^+FoxP3^+$) in colonic LP from a BM only, Untreated (received BM+T), Minnelide (received BM+T) (n=3) **(F)**. Frequency of LP T cell subsets, $CD4^+FoxP3^+KLRG1^+$ **(G),** total $CD4^+$ **(H),** total $CD8^+$ **(I),** and TH2 ($CD4^+FoxP3^-GATA3^+$) **(J).** Representative flow cytometric contour plots of Treg cells in the lung ($CD4^+FoxP3^+$ / $CD4^+$) **(K).** Frequency of CD4 and CD8 cells in the spleen 3 weeks post-aHSCT (**L**). Groups were compared using one-way ANOVA Tukey's multiple comparison test *p<0.05, **p<0.01, *** p<0.001.

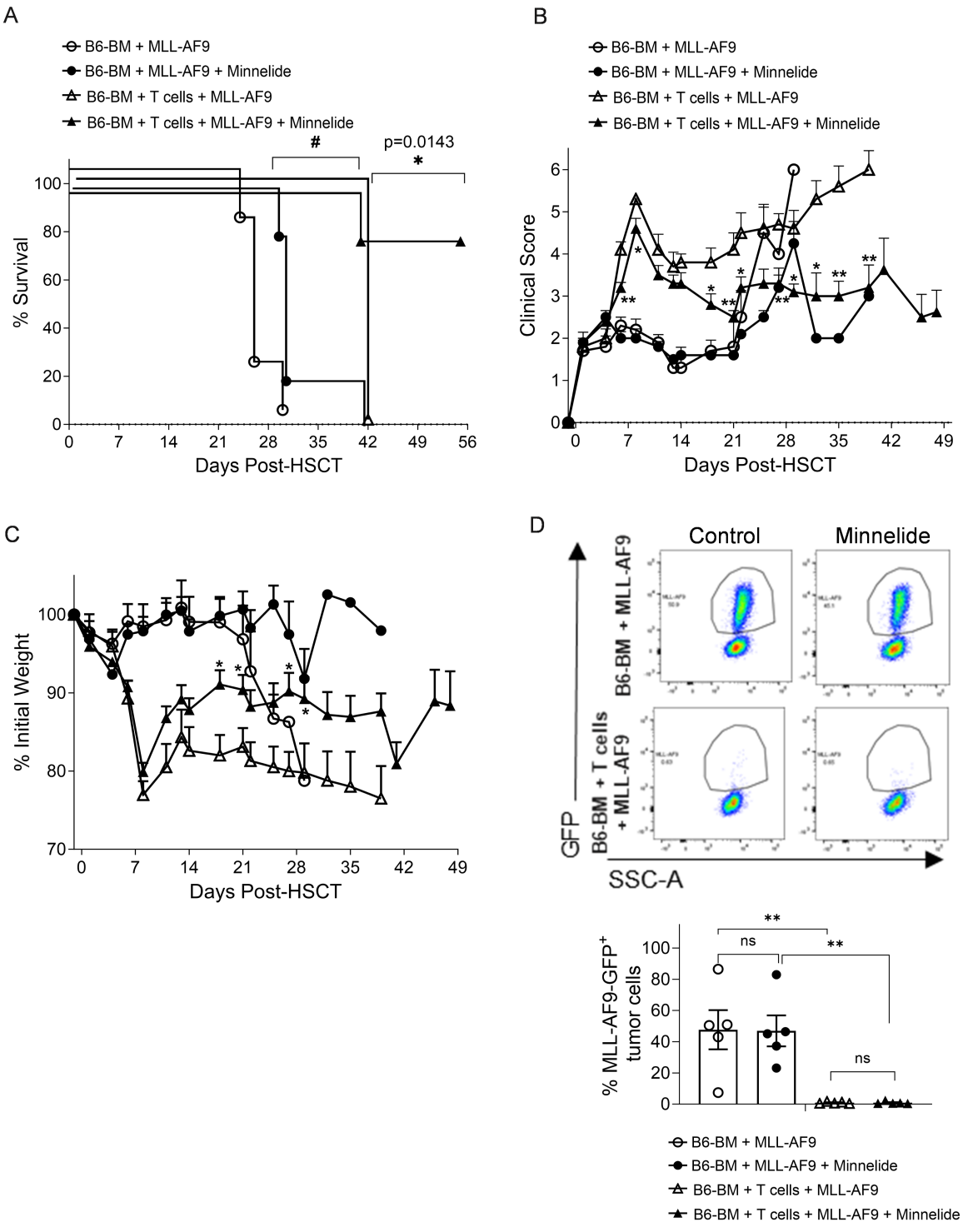
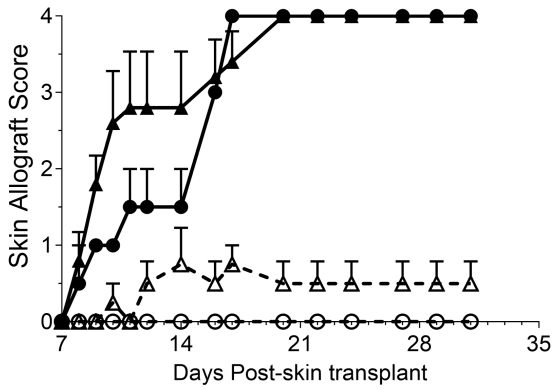
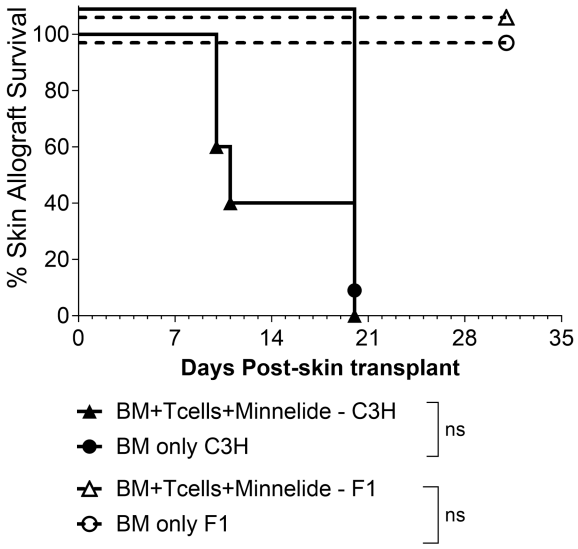

A
B6-BM + MLL-AF9
B6-BM + MLL-AF9 + Minnelide
B6-BM + T cells + MLL-AF9
B6-BM + T cells + MLL-AF9 + Minnelide
#
p=0.0143
*
% Survival
Days Post-HSCT
B
Clinical Score
Days Post-HSCT
C
% Initial Weight
Days Post-HSCT
D
Control
Minnelide
B6-BM + MLL-AF9
B6-BM + T cells + MLL-AF9
GFP
SSC-A
% MLL-AF9-GFP+ tumor cells
ns
**

Figure 6. Recipients treated with Minnelide exhibited maintained GVT while GVHD was ameliorated after MHC-mismatched aHSCT. aHSCT was performed utilizing a B6 → BALB/c donor and recipient mouse model and recipients were treated with Minnelide 0.1 mg/kg from day −2 to 28 after transplant (**A-C**). GVHD survival (**A**), clinical scores (**B**), and weight loss (**C**) are presented (n=8 mice GVHD, n=16 mice Minnelide and n=4 mice BM only group). (**D**) Tumor burden in recipient blood on day 22 post-BMT. The data are from two independent experiments. Clinical scores were compared using multiple t tests over time. Groups were compared using one-way ANOVA Tukey's multiple comparison test or log-rank for survival analyses. *p<0.05 B6-BM+Tcells+MLL-AF9 vs B6-BM+Tcells+MLL-AF9+Minnelide, $^{\#}$p<0.05 B6-BM+MLL-AF9 vs B6-BM+MLL-AF9+Minnelide, **p<0.01, *** p<0.001. Data are means ± SEM.

A


Skin Allograft Score
0
1
2
3
4
7
14
21
28
35
Days Post-skin transplant

B


% Skin Allograft Survival
0
20
40
60
80
100
0
7
14
21
28
35
Days Post-skin transplant
BM+Tcells+Minnelide - C3H
BM only C3H
ns
BM+Tcells+Minnelide - F1
BM only F1
ns

C

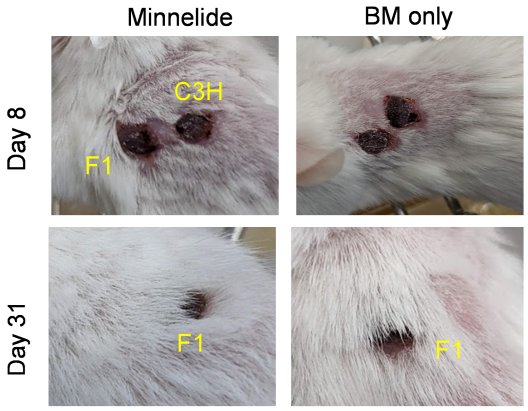

Minnelide
BM only
Day 8
Day 31
C3H
F1
F1
F1

Figure 7. Functional immunity is intact in Minnelide-treated recipients. Two months post-aHSCT recipients received 2 skin grafts, applied on the trunk of each mouse, 1 from B6×BALB/c F1 ($H2^{b/d}$) mice and 1 from C3H/HeJ third-party ($H2^{k}$) donors. Grafts were assessed and scored on the indicated days (Minnelide *n*= 5; BM only n=2). (**A**) Allograft score, graft scoring was performed as follows: 0, intact graft and healthy appearance; 1, inflamed graft, but without signs of necrosis observed; 2, inflamed graft and less than 25% necrosis observed; 3, inflamed graft and between 25% and 75% necrosis observed; and 4, greater than 75% necrosis detected or loss of graft. (**B**) Allograft survival, all mice accepted the F1 (B6×BALB/c) skin grafts, whereas all C3H/HeJ grafts were rejected in both Minnelide and BM only transplant recipients by day 21 (**C**) Representative photographs of skin grafts present on recipient mice on days 8 and 31 from both groups. Groups were compared using log-rank for survival analyses. Data are means ± SEM.

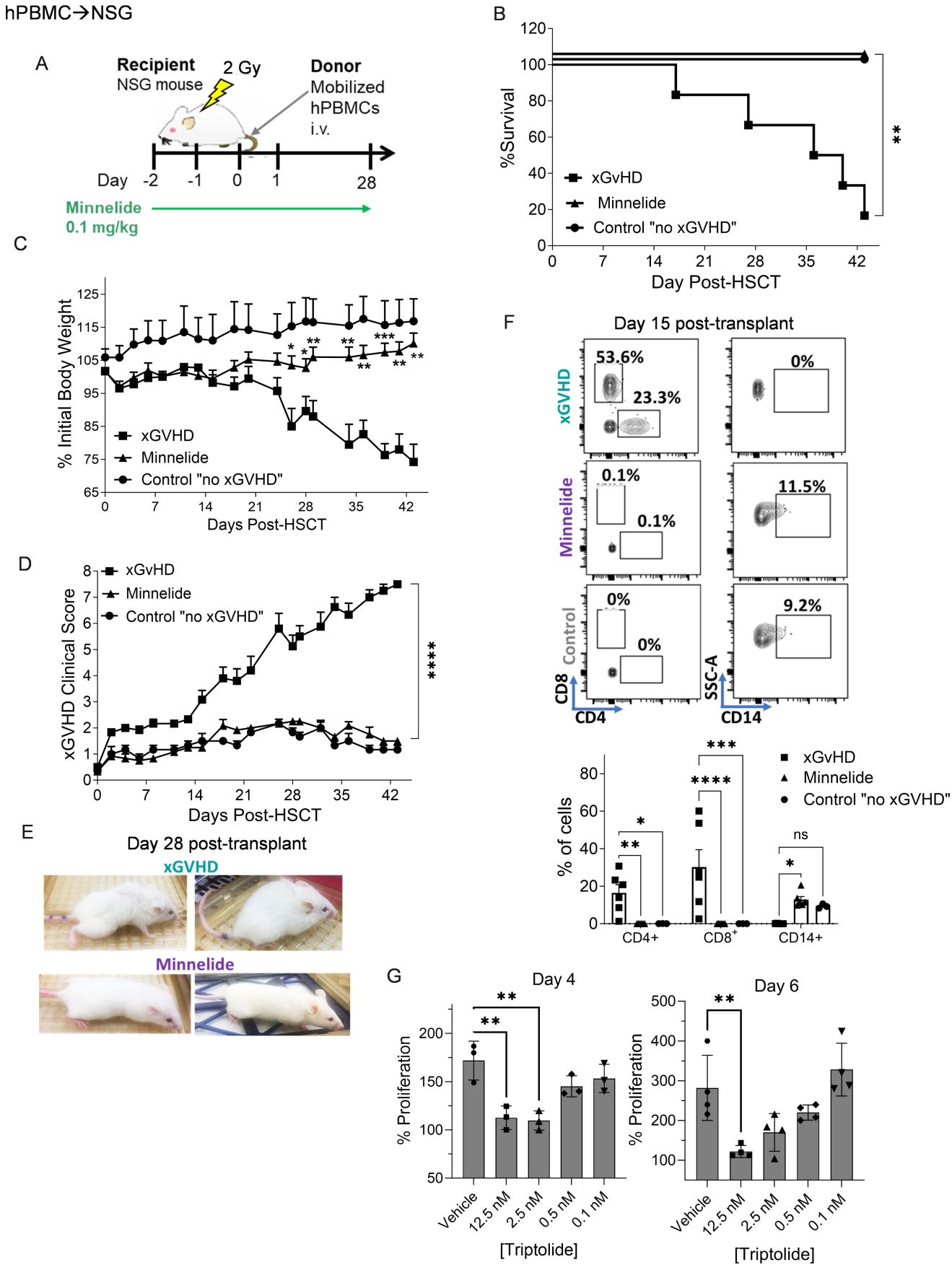

hPBMC→NSG
A
Recipient
NSG mouse
2 Gy
Donor
Mobilized
hPBMCs
i.v.
Day -2 -1 0 1 28
Minnelide
0.1 mg/kg
B
%Survival
xGvHD
Minnelide
Control "no xGVHD"
Day Post-HSCT
C
% Initial Body Weight
xGVHD
Minnelide
Control "no xGVHD"
Days Post-HSCT
D
xGVHD Clinical Score
xGvHD
Minnelide
Control "no xGVHD"
Days Post-HSCT
E
Day 28 post-transplant
xGVHD
Minnelide
F
Day 15 post-transplant
xGVHD
Minnelide
Control
53.6%
23.3%
0%
0.1%
0.1%
11.5%
0%
0%
9.2%
CD8
CD4
SSC-A
CD14
% of cells
CD4+
CD8+
CD14+
ns
G
Day 4
Day 6
% Proliferation
Vehicle
12.5 nM
2.5 nM
0.5 nM
0.1 nM
[Triptolide]

Figure 8. Treatment with Minnelide reduced xenogeneic GVHD. NSG recipient mice were irradiated (2Gy) and the following day underwent transplantation with human mobilized PBMC ($6x10^6$). Recipients were treated with Minnelide 0.1 mg/kg from day −2 to 28 after transplant (**A-F**). Schematic of experimental outline (**A**), overall survival (**B**), weight loss (**C**), and xGVHD clinical scores (**D**) are presented (n=6 mice xGVHD or Minnelide and n=3 mice Control "no xGVHD"). Representative photographs of xGVHD and Minnelide treated recipients four weeks after transplant (**E**). Flow cytometry contour plots and frequency of human $CD4^+$, $CD8^+$, $CD14^+$ cells in the blood two weeks after transplantation (**F**). These data are representative of 2 independent (n=6/group) human-to-mouse HSCT. Clinical scores were compared using multiple t tests over time. Proliferation assays of Cell Trace Violet labeled human PBMCs stimulated by either anti-CD3 (OKT3 mAb) *in vitro* for 4 days and a one-way MLR 6 days (**G**). Groups compared using two-way ANOVA with Dunnett's multiple comparison test or log-rank for survival analyses. *p<0.05, **p<0.01, *** p<0.001 and **** p<0.0001. Data are means ± SEM.