In vitro-generated MDSCs prevent murine GVHD by inducing type 2 T cells without disabling anti-tumor cytotoxicity

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Key Points

1. MDSC-treatment prevents GVHD by skewing T cells towards type 2 T cells.

2. MDSCs proliferate in vivo, suppress independent of MHC class I expression, and do not impair allogeneic T cell homing and the GVT effect.
Abstract
Myeloid-derived suppressor cells (MDSCs) inhibit T cell expansion and functions by versatile mechanisms such as nutrient depletion, nitrosylation or apoptosis. Since graft-versus-host disease (GVHD) is characterized by the expansion of donor-derived T cells destroying recipient tissue, we analyzed whether MDSCs can be used for GVHD prevention in murine allogeneic bone marrow transplantation models. Transplantation of MDSCs, generated from bone marrow cells by GM-CSF/G-CSF in vitro, inhibited GVHD-induced death and attenuated histological GVHD, while anti-tumor cytotoxicity of alloantigen-specific T cells was maintained. MDSCs expanded in vivo and invaded lymphatic and GVHD target organs. MHC class I expression on MDSCs was dispensable for their suppressive capacity. Inhibition of GVHD required the presence of MDSCs during T cell priming while allogeneic T cell numbers and homing in lymphoid and GVHD target organs were not considerably affected in MDSC-treated mice. However, MDSCs skewed allogeneic T cells towards type 2 T cells up-regulating Th2-specific cytokines. Type 2 T cell induction was indispensable for GVHD prevention since MDSC-treatment failed to prevent GVHD when allogeneic STAT6-deficient T cells, which are unable to differentiate into Th2 cells, were transplanted. MDSC-induced Th2 induction might be applicable for GVHD treatment in clinical settings.
Introduction

Graft-versus-host disease (GVHD) represents the major complication after allogeneic bone marrow transplantation (BMT). Mature T cells in the transplant activated by alloantigens of the host attack and destroy recipient tissue (GVHD) but are also responsible for eradication of residual tumor cells (GVT = graft-versus-tumor effect). Allogeneic T cell activation and proliferation are attempt to be inhibited by routinely given immunosuppressive therapy reducing the GVHD risk but mediating general immunosuppression.\(^1,2\) Preventing recipient tissue destruction while preserving T cell immunity to cope with infections and to destroy tumor cells is a major goal in GVHD-treatment.

Immune responses are modulated by MDSCs, a heterogeneous population of myeloid progenitor cells. In healthy conditions myeloid progenitors quickly differentiate into mature myeloid cells while pathological conditions block myeloid differentiation and support the expansion of MDSCs.\(^3\) Murine MDSCs co-express Gr-1 and CD11b. The two major MDSC subsets are characterized by the different expression of the two Gr-1 epitopes Ly-6C and Ly-6G: granulocytic MDSCs are $\text{CD11b}^+\text{Ly-6G}^+\text{Ly-6C}^{\text{low}}$, while monocytic MDSCs are $\text{CD11b}^+\text{Ly-6G}^+\text{Ly-6C}^{\text{high}}$.\(^4\) Immunosuppressive capacity of both subsets is associated with increased expression of arginase-1 and inducible nitric oxide synthetase (iNOS), enzymes depriving L-arginine from the microenvironment, which is required for T cell proliferation. Nitrosylation of T cell-associated molecules, interference with T cell homing, and induction of T cell apoptosis and Tregs are also reported to be responsible for MDSC-mediated immunosuppression.\(^5\) Whether T cell inhibition by MDSCs requires MHC-mediated antigen presentation or is antigen-unspecific is still not definitely clarified. While MDSCs isolated from tumor bearing mice solely prevent antigen-specific T cell responses\(^6,7\), deficiency in MHC Class I molecules did not impair the suppressive capacity of MDSCs.\(^7\) MDSCs can also influence the cytokine environment. Elevated MDSC numbers in different murine disease
models are associated with an increase of Th2 immunity. While the presence of Th2 cells is advantageous for GVHD-inhibition, the anti-tumor effects of type 2 T cells are less pronounced than for type 1 T cells.

MDSCs can be generated in vitro by various methods using different progenitor cells and cytokine combinations for differentiation and expansion. Usage of MDSCs as cellular therapy was effective for inhibition of experimental autoimmune myasthenia gravis, prolonging allograft survival and for GVHD prevention, clearly indicating that these cells have a therapeutic potential in T cell-mediated diseases.

In the present study we show that MDSCs efficiently prevent clinical GVHD by skewing T cell responses towards type 2 T cell immunity. Since MDSC co-transplantation does not abrogate the GVT effect, these suppressor cells might provide a treatment option in GVHD prevention.
Methods

Cell culture

Cell lines were grown in RPMI-1640, 10% FCS (Lonza, Germany), 2mM L-glutamine, 1mM sodium pyruvat at 37°C, 7.5% CO₂. Primary cells were cultured in α-MEM, 10% FCS, 2mM L-glutamine, 1mM sodium-pyruvate, 100 U/mL Penicillin-Streptomycin and 0.05 mM 2-ME.

MDSC generation

3x10⁵ BM cells/mL were cultured with G-CSF (2000 U/mL) and GM-CSF (250 U/mL) (Peprotech, Germany) for 4 days.

Mice and BMT

Female C57BL/6 (B6; H-2ᵇ, CD45.2), B6D2F1 (H-2ᵇᵈ, CD45.2) (Janvier, France), B6.129P2-B2m<sup>tm1Unc</sup>/J (Cl I<sup>+</sup>, H-2ᵇ, CD45.2) (Jackson Laboratory, USA), B6.SJL-Ptprc<sup>⁰</sup>Pepc<sup>⁰</sup>/BoyJ (B6.SJL; H-2ᵇ, CD45.1), B6.C-H2-K<sup>bm1</sup>/ByJ (B6.bm1; H-2K<sup>bm1</sup>, CD45.2), and B6.129S2(C)-STAT6<sup>tm1Gru</sup>/J (STAT6<sup>−/−</sup>, H-2ᵇ, CD45.2) (breeding pairs from the Jackson Laboratory and bred at University of Ulm) were used 6 - 12 weeks of age. On day -1 mice were irradiated with 14 Gy split into 2 doses 4 hours apart from a <sup>137</sup>Cs source and were reconstituted on day 0 with 5x10⁶ T cell depleted BM (TCD-BM) intravenously. T cells were depleted by incubation 30-H12 supernatant (anti-Thy-1.2) and subsequent lysis with rabbit complement (Cedarlane, Canada). TCD-BM was co-injected with 2x10⁷ spleen cells and MDSCs. GVHD-scores were determined by analyzing weight, activity, skin, fur ruffling, and posture according to Cook et al. Animals dying during the experiment remained included in the calculation until the end of the experiment with their final GVHD-scores. JM6 (H-2K<sup>bm1</sup>, 5x10⁴/mouse) were injected intravenously at the day of BMT. Regierungspräsidium, Tübingen, Germany approved all animal experiments (1031, 1065, 1084).
Generation of tumor cell line JM6 from B6.bm1 mice

B6.bm1 mice were irradiated 4-times with 1-week intervals with 1.7 Gy.22 Thymomas appearing 6 months later were resected and cultured as single cell suspensions. Outgrowing tumor cells were cloned three times to obtain the stable CD8+ single cell clone JM6.

Histopathology

Organs were fixed in 4% formalin, paraffin embedded, sectioned and stained with H&E. Slides were examined by a pathologist who was blinded for the experimental history. Histopathology of GVHD was graded according to Kaplan et al.23

CFSE-labeling

2x10^6 cells were labeled with 100 µl 50 µM CFSE (eBioscience, Germany) for 10 min at 37°C, washed and immediately used for intravenously injections or in vitro assays.

Mixed lymphocyte reaction (MLR)

2.5x10^5 CFSE-labeled spleen cells were stimulated with 2.5x10^5 irradiated stimulator spleen cells (33 Gy) in the absence or presence of MDSCs. Arginase-1 was inhibited by N-hydroxy-nor-arginine (nor-NOHA) and iNOS by L-N^G-monomethyl-arginine-citrate (L-NMMA) (Merck, Germany). Proliferation was determined by flow cytometry. Percentage suppression = 100-([%proliferating T cells+stimulators+MDSC]/(% proliferating T cells+stimulators)]x100.

Isolation of cells

Information about cell isolation procedures is given in the Method section of supplemental data.
**IL-2 secretion assay and intracellular staining**

Spleen cells were restimulated with medium, PMA (20 ng/mL) plus ionomycin (1 µM) (Calbiochem, Germany) or allogeneic spleen cells (1 : 1 ratio) for 5 h. IL-2 secretion was determined by the IL-2 secretion assay (Miltenyi, Germany). Intracellular cytokines were determined by adding 10 µg/mL BrefeldinA (Sigma, Germany) during the stimulation process. After 5 hours cells were stained for surface markers, fixed with 4% paraformaldehyde, subsequently lysed with 0.1% saponin (Sigma) and stained for cytokine expression. STAT6 was stained with fixation/permeabilization solution kit (BD Biosciences).

**Serum cytokine analysis**

Cytokine serum concentrations were determined using the mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex Kit (eBioscience), measured on LSR II flow cytometer and analyzed by eBioscience’s FlowCytomix software.

**Flow cytometry**

5 x 10^5 cells were stained. Antibodies used are specified in Table S1.

**RNA preparation and qRT-PCR**

RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen, Germany) and cDNA was synthesized using SuperScript II Reverse Transcriptase (Life Technologies, Germany). qRT-PCR was performed with a LightCycler 2.0 using a LightCycler FastStart DNA Master PLUS SYBR Green I kit (Roche Diagnostics, Germany). The qRT-PCR results were normalized using mouse aryl hydrocarbon receptor interacting protein (AIP) as house keeping gene. Primer sets (Thermo Fisher Scientific, Germany) used are listed in Table S2.
Statistics

Survival was analyzed using Kaplan-Meier method and Log Rank statistics while a student $t$ or ANOVA test was applied for the other studies. Results were considered significant if $p \leq 0.5$. 
Results

In vitro-generated MDSCs prevent GVHD when co-injected with the allogeneic transplant

MDSCs were generated from BM cells by GM-CSF/G-CSF incubation. After 4 days 92% of the cells expressed CD11b\(^+\)Gr-1\(^+\) and CD11b\(^+\) cells can be subdivided in monocytic (Ly-6C\(^{\text{high}}\)Ly-6G\(^+\)) and granulocytic MDSCs (Ly-6C\(^{\text{low}}\)Ly-6G\(^+\)) (Fig. 1A). MDSCs expressed CD115 and CD124, markers probably indicative for their suppressive action, co-stimulatory molecules CD80 and CD86 and LFA-1 and CD62L enabling homing into lymphoid organs. Nearly all MDSCs express MHC class I, while only few cells are MHC class II or F4/80 positive (Fig. S1A). Immunosuppressive molecules arginase-1 and iNOS were strongly up-regulated in MDSCs compared to isolated CD11b\(^+\) BM cells (Fig. S1B) while COX2, known to be important for the suppressive capacity of human MDSCs, was not induced (data not shown). Most importantly, MDSCs efficiently prevented the proliferation of CD8\(^+\) CFSE-labeled spleen cells from B6.SJL (H-2K\(^b\)) mice stimulated with irradiated allogeneic spleen cells from B6.bam1 mice (H-2K\(^{\text{bm1}}\)). MDSCs suppressed T cell proliferation preferentially by iNOS, since the iNOS inhibitor L-NMMA abrogated the suppressive effect, while arginase-1 inhibitor nor-NOHA was ineffective (Fig. 1B).

To test, whether MDSCs can be implemented as cellular GVHD therapy in a single MHC class I-disparate BMT model, lethally irradiated B6.bam1 (H-2K\(^{\text{bm1}}\)) mice were reconstituted with TCD-BM and spleen cells from B6 mice (H-2K\(^b\)) and increasing numbers of B6-derived MDSCs. While transplantation of allogeneic BM and spleen cells induced severe clinical GVHD with high GVHD-scores and a lethality of 60%, injection of MDSCs rescued mortality to about 10% independent whether 1, 5 or 10 x 10\(^6\) MDSCs were injected. Lowest GVHD-scores were detected with 1x10\(^7\) MDSCs but were still elevated compared to mice receiving only BM, which do not develop GVHD (Fig. 1C, D). We also analyzed whether MDSCs can prevent GVHD in BMT model with a 50% MHC class I and II disparity. MDSCs prevented
allogeneic proliferation of B6.SJL-derived T cells stimulated with B6D2F1 spleen cells in vitro (Fig. S2A). MDSC-treatment with 5 or 10x10⁶ cells attenuated GVHD-induced death and GVHD scores in B6D2F1 mice (H-2ᵇᵈ) transplanted with B6 (H-2ᵇ) derived TCD-BM and spleen cells from 90% to 30% (Fig. S2B, C). Significant reduction of histological GVHD was only observed, when 1x10⁷ MDSCs were transplanted in both BMT models (Fig. 1E, Fig. S2D). Lower MDSC numbers only improved survival and clinical scores without improving the histological score indicating that mortality and general tissue damage are independently regulated as reported by others 24,25. The following experiments were performed with 1x10⁷ MDSCs. To prevent GVHD, MDSCs need to be co-injected with the allogeneic transplant (day 0) since a therapeutic treatment seven days after BMT (day 7) only slightly improved survival from 38% to 50% (Fig. 1F). All together, we could show that co-transplantation of MDSCs significantly prevent GVHD if MDSCs are transplanted prophylactically.

**MDSC co-transplantation prevents GVHD independent of MHC class I expression and maintains the GVT effect**

To analyze whether MDSC-mediated suppression requires MHC class I expression and antigen presentation26, B6.bm1 mice were transplanted with B6-derived BM cells and spleen cells together with MDSCs derived either from B6 mice (wild type, WT) or β2 microglobulin deficient mice lacking MHC class I expression (Cl I⁻). Interestingly, GVHD-induced death was prevented in about 90% of the mice independent of MHC class I expression on MDSCs, while untreated mice had a survival rate of 45%. Also histological GVHD of intestine and skin was attenuated by Cl I⁻ MDSCs (Fig. 2A, B, C). MDSCs derived from Cl I⁻ mice exhibited a comparable phenotype as wild type MDSCs and suppressed T cell proliferation in vitro in an iNOS-dependent manner (Fig. S3A, B).

To clarify the influence of MDSCs on the GVT effect, B6.bm1 mice were injected with the CD8⁺CD4⁻ syngeneic thymoma cell line JM6 and reconstituted with BM cells alone or BM
cells and spleen cells in the presence or absence of MDSCs. Mice receiving only BM cells died around day 21 from tumor development, reflected by strongly increased spleen and liver weights and increased percentages of CD8+ cells in BM, liver, and spleen. Although mice transplanted with allogeneic BM and spleen cells were tumor free, 70% of the mice died due to GVHD development reflected by increased GVHD-scores (data not shown). Most importantly, mice treated with MDSCs were tumor free (Fig. 2D, E, F). Allogeneic T cells isolated from MDSC-treated and untreated mice exhibited similar expression of granzyme B and perforin known to be the primary cytotoxic molecules of T cell-induced tumor destruction27 (Fig. S4). In the B6→B6D2F1 BMT model primary Bcr-Abl expressing B-ALL cells were also efficiently eradicated in MDSC-treated mice reconstituted with allogeneic transplant (Fig. S5A, B) showing that the GVT effect is maintained after MDSC-treatment in two BMT models.

**MDSCs proliferate in vivo, reduce alloantigen-specific T cell proliferation only insubstantially and do not influence alloantigen-specific T cell homing**

Since a single MDSC injection inhibited GVHD, in vivo proliferation of MDSCs was analyzed by reconstituting B6.bm1 (CD45.2) mice with B6-derived TCD-BM and spleen cells (CD45.2) and CFSE-labeled MDSCs derived from B6.SJL mice (CD45.1). One day after transplantation CD45.1+ MDSCs divided in lymphoid organs and on day 3 extensive proliferation was detected in lymphoid organs and liver (Fig. 3A). Until day 30 transplanted CD45.1+ MDSCs were detectable in the circulation, lymphoid organs and liver. Monocytic MDSCs were the major population in the liver, while granulocytic MDSCs predominated the lymphoid compartment (Fig. S6A, B).

To clarify, how MDSCs prevent GVHD, we first determined whether alloantigen-specific T cell expansion was inhibited by reconstituting B6.bm1 (CD45.2+) mice with B6-BM (CD45.2+) and B6.SJL-spleen cells (CD45.1+) in the presence or absence of B6-MDSCs.
Numbers of CD45.1+ splenic T cells in MDSC-treated mice were reduced at day 1 and 3 after BMT compared to untreated mice, but were comparable in both groups at day 11 (Fig. 3B). Accordingly, alloantigen-specific CD8+ T cells from both groups did not exhibit differences in the development of T_{Eff} cells or the expression of activation markers or homing molecules (Fig. S7). No influence of MDSCs on allogeneic T cell homing was detected since MDSC-treated B6.bm1 mice exhibited no differences in the absolute numbers or percentages of infiltrating CD3+CD45.1+ T cells in spleen, blood, liver or colon at different days after BMT (Fig. 3C). However, MDSC-treatment reduced the amount of IL-2 secreting alloantigen-specific T cells. After restimulation of spleen cells isolated from MDSC-treated B6.bm1 mice with PMA/ionomycin (PMA/iono) or B6.bm1 spleen cells (allo), the percentage of IL-2 secreting CD45.1+CD8+ T cells was significantly reduced compared to untreated mice independent of the activation signal (Fig. 3D). Thus, MDSCs expand in vivo, decrease the amount of IL-2 secreting allogeneic T cells while allogeneic T cell proliferation or homing is not significantly influenced.

**MDSC-treatment induces the induction of type 2 T cells**

Since IL-2 supports Th1 differentiation, serum Th1/Th2 cytokines of transplanted mice were determined. At day 10 after BMT GVHD-associated Th1 cytokines IFN-γ and TNFα were strongly increased in mice developing GVHD. Importantly, co-transplantation of MDSCs significantly decreased both cytokines. Simultaneously, Th2 serum cytokine concentration of IL-5 was strongly elevated in mice treated with MDSCs compared to untreated mice (Fig. 4A). Similar changes in serum cytokines were also detected in the B6→B6D2F1 model (Fig. S2E). To further prove MDSC-mediated type 2 polarization, mRNA expression of alloantigen-specific CD45.1+ T cells isolated from mice reconstituted with allogeneic transplant in the presence or absence of MDSCs were determined (85% CD8+, 15% CD4+). mRNA levels of IL-4, -5 and -13 were strongly up-regulated in T cells from MDSC-treated
mice (Fig. 4B). PMA/iono restimulation of spleen cells confirmed the induction of type 2 T cells since the percentage of IL-5 and -13 producing CD8⁺ T cells increased in MDSC-treated mice (Fig. 4D). Although serum concentrations of IFN-γ and TNFα were decreased in MDSC-treated mice, no differences in mRNA expression and the percentage of cytokine expressing cells were detected. mRNA expression of IL-17 was also not altered (Fig. 4B). Since type 2 T cell responses require IL-4, STAT6, and GATA-3 expression while type 1 T cell responses depend on IL-12, STAT4, and T-bet, mRNA expression of the transcription factors was determined in CD45.1⁺ allogeneic T cells. STAT6 and GATA-3 mRNA expression was up-regulated in the presence of MDSCs while STAT4 and T-bet expression was unchanged (Fig. 4C). STAT6 and GATA-3 protein levels, however, were only marginally increased (data not shown). Most importantly Th2 skewing in MDSC-treated mice was also observed in allogeneic T cells isolated from GVHD target organs lung and liver (Fig. 4E).

To prove that MDSC-mediated T cell polarization is indispensable for GVHD prevention, mice were transplanted with allogeneic STAT6-deficient T cells, which are unable to differentiate into type 2 T cells. Allogeneic STAT6⁻/⁻ T cells induced GVHD with similar kinetics than WT T cells. Most importantly, co-transplantation of MDSCs could not prevent GVHD-induced death and did not attenuate GVHD-scores (Fig. 5A, B). Accordingly, serum levels of IFN-γ and TNFα were not reduced and IL-5 levels not increased in MDSC-treated mice receiving STAT6⁻/⁻ T cells (Fig. 5C). Ineffectiveness of MDSC-treatment in STAT6⁻/⁻-transplanted mice is not due to elevated serum levels of GVHD inducing Th1 cytokines IFN-γ or TNFα or to an altered MDSC expansion or homing compared to WT T cells (Suppl. Fig. 8A, B). Allogeneic T cells isolated from mice transplanted with STAT6⁻/⁻ T cells and MDSCs exhibited no increase in IL-4, -5, and -13 mRNA expression and showed no changes in IFN-γ and TNFα mRNA expression compared to untreated mice. Interestingly, reconstitution with STAT6⁻/⁻ T cells increased IL-17 mRNA, which was reduced after MDSC-treatment (Fig. 5C).
5D). STAT6−/− T cells infiltrated spleen, blood, liver and colon comparable to WT T cells independent of the presence of MDSCs. (Fig. 5E). Thus, MDSC-treatment induces a type 2 T cell response, which is indispensable for GVHD prevention.

A CD11b⁺CD11c⁺Gr-1medCD301b⁻I-Ab⁺ MDSC-subpopulation expands under GVHD conditions in vitro

To further investigate the Th2-inducing capacity of MDSCs, we incubated in vitro-generated MDSCs (day4-MDSCs) for 3 days with medium supplemented with 2,5% serum derived from GVHD mice (GVHD-MDSC) or with serum derived from mice transplanted with BM cells alone (BM-MDSC). Day4-MDSCs exhibited a mixed phenotype of CD11b⁺CD11c⁻ (81%) and CD11b⁺CD11c⁺ (16%) cells. After incubation with GVHD-medium, percentage and total numbers of CD11b⁺CD11c⁺ cells increased and the percentage of CD11b⁺CD11c⁻ cells decreased compared to BM-MDSCs (Fig. 6A). CD11b⁺CD11c⁺ cells of GVHD-MDSCs expressed CD301b, F4/80, I-Ab and Gr-1med, while CD11b⁺CD11c⁻ cells were CD301b⁻, F4/80⁻, I-Ablow, and Gr-1hi (Fig. 6B). Surface molecule expression of subpopulations is identical in BM-MDSCs and day4-MDSCs (data not shown). Since CD301b expression on DCs is associated with Th2 induction30,31, the expression of transcription factors associated with Th2 (IRF4, Klf4)32,33 and Th1 (Batf3)34,35 induction was determined. CD11b⁺CD11c⁻ cells always express less Klf4 and IRF4 than CD11b⁺CD11c⁺ cells in day4-MDSCs and GVHD-MDSCs. Interestingly, Th1-associated Batf3 expression in CD11b⁺CD11c⁺ cells is significantly lower in GVHD-MDSCs compared to day4-MDSCs (Fig. 6C). These data suggest, that under GVHD conditions an APC-like MDSC-subpopulation is developing, which might be responsible for Th2 polarization.
Discussion

GVHD development is characterized by the expansion and the deleterious functions of donor-derived T cells destroying recipient target tissues. To our knowledge we show for the first time that co-transplantation of in vitro-generated MDSCs abrogates destructive alloantigen-specific T cell functions by skewing T cells into type 2 T cells thereby preventing GVHD while not abrogating the GVT effect.

MDSCs generated in vitro by incubating BM cells with GM-CSF/G-CSF for 4 days exhibited a CD11b+Gr-1+ phenotype and suppressed T cell proliferation in vitro in an iNOS-dependent manner. MDSC-treatment induced nearly 100% survival in the B6→B6.bml1 model (difference in one MHC class I molecule) and increased survival from 10% to 70% in the B6→B6D2F1 model (differences in 50% of MHC Class I and II molecules). Efficiency of MDSC-treatment might have two explanations. First, success of MDSC-treatment might be controlled by the GVHD severity induced, which is dependent on the amount of disparate MHC molecules. Second, CD8+ T cells might be more efficiently suppressed than CD4+ T cells since GVHD in the B6→B6.bml1 model is dependent on CD8+ T cell expansion, while CD4+ T cells are the GVHD inducers in the B6→B6D2F1 model36,37. Preferential blockade of CD8 functions might be due to the high expression of MHC class I on MDSCs compared to class II, which would point to antigen-specific suppression involving antigen up-take, MHC presentation and interaction with antigen-specific T cells. Antigen-dependent suppression was reported for MDSCs isolated from tumor bearing mice blocking T cell responses to MHC class I specific peptides but failed to inhibit T cell responses induced by Con A or a MHC class II-specific peptide. Also masking MHC class I molecules on MDSCs abrogated immune suppression by MDSCs6. However, MHC Cl I- MDSCs arising from tumors grown in β2-microglobulin-deficient mice did not exhibit any impairment in inhibiting T cell proliferation in vitro7. Since transplantation of MHC Cl I- MDSCs did prevent GVHD, MDSC...
suppression is independent of MHC class I-mediated antigen presentation and depends rather on GVHD severity. MDSC-treatment requires prophylactic co-injection with the allogeneic transplant. Multiple MDSC injections after GVHD establishment will show, whether MDSCs can also be used therapeutically. Although MDSC function is often associated with activation of regulatory T cells (Treg)\(^{38,39}\), Tregs were not increased in MDSC-treated mice (data not shown).

Of note, transplanted MDSCs further expanded after transplantation in lymphoid and GVHD target organs. These results are surprising since apoptosis of CD95\(^+\) MDSCs by CD95L-expressing T cells or myeloid cells was reported in murine tumor models\(^{40,41}\), suggesting that transplanted MDSCs become apoptosis resistant or do not have direct contact with T cells, which express CD95L constitutively. This is further supported by the observation that no significant reduction of alloantigen-specific T cells is detected in mice treated with MDSCs. However, T cells exhibited a different T cell polarization in the presence of MDSCs. MDSC-treatment increased the amount of type 2 T cells and Th2-specific cytokines, although the induction of type 1 T cells was not completely inhibited. Impairing Th2 differentiation by transplanting STAT6\(^+\) allogeneic T cells abrogated the therapeutic effect of MDSCs completely. Association between MDSC induction and the prevalence of Th2 cells was also reported in a sepsis model, in influenza virus infected mice\(^{8,9}\) and patients with esophageal, pancreatic or gastric cancer\(^{42,43}\). In murine asthma models, however, MDSCs shift the balance towards Th1 by blunting the ability of lung DCs to induce re-activation of effector Th2 cells\(^{44,45}\). Crosstalk of tumor-derived MDSCs with macrophages on the other hand induces a Th2 response by decreasing macrophage-produced IL-12\(^{46}\) indicating that the source of MDSCs used and the inflammatory status of the target organ might influence the type of T cell response initiated. Medium supplemented with serum from GVHD developing mice induced the outgrowth of a CD11b\(^+\)CD11c\(^+\)Gr-1\(^+\) MDSC-subpopulation expressing surface molecule CD301b and transcriptions factors IRF4 and Klf4, whose expression was mandatory.
on dendritic cells for Th2 responses.\textsuperscript{30-33} CD11b\textsuperscript{+}CD11c\textsuperscript{+} DCs were also described to exhibit immunosuppressive and tolerogenic characteristics.\textsuperscript{47,48} Advanced experiments are required to define, whether a CD11b\textsuperscript{+}CD11c\textsuperscript{+}Gr-1\textsuperscript{+} MDSC-subpopulation develops in BM-transplanted animals after MDSC-treatment in vivo and to determine its possible role in initiating a Th2 response.

A polarization towards type 2 T cell immunity is positively associated with attenuated murine GVHD\textsuperscript{10,49}. Mice transplanted with allogeneic STAT6\textsuperscript{-/-} T cells and therefore unable to develop Th2 cells exhibit accelerated GVHD development compared to mice reconstituted with STAT4\textsuperscript{-/-} T cells, which are defective in Th1 development.\textsuperscript{50} Also the transfer of in vitro or in vivo polarized type 2 alloreactive CD4\textsuperscript{+} (Th2) and CD8\textsuperscript{+} (Tc2) donor T cells attenuates GVHD.\textsuperscript{12,51} In human GVHD, Th1 and Th2 subsets are described to be equally induced in acute and chronic GVHD and decreased when GVHD resolved.\textsuperscript{52} However, treatment with the JAK 1/2 selective inhibitor ruxolitinib impairs Th1/17 differentiation and thereby strongly reduces steroid-refractory human GVHD.\textsuperscript{53} Dampered Th1/17 responses are also observed in patients receiving extracorporeal photopheresis, which strongly induces MDSC expansion.\textsuperscript{54} Anti-tumor cytotoxicity, however, is not abrogated in the presence of Tc2 cells or in mice defective for Th1/Th17 development.\textsuperscript{13,49} Likewise, anti-tumor cytotoxicity towards thymomas or Bcr-Abl transduced primary B-ALL cells was maintained in MDSC-treated mice.

Highfill et al.\textsuperscript{16} identify L-arginine depletion and subsequent reduction of alloantigen-specific T cell numbers as the major mechanism of MDSC-induced GVHD prevention. In addition to GM-CSF/G-CSF they use IL-13 known to increase arginase-1 expression\textsuperscript{55} for MDSC generation, indicating that the cytokines used for MDSC establishment might influence the mode by which MDSCs execute their immunosuppressive function. Alloantigen-specific T cell proliferation and allogeneic T cell phenotype in vivo, however, is only determined until day 4 after BMT, while later time points and T cell polarization are not analyzed. Comparable
to our results, the transplanted MDSC population contains a small proportion of about 12% of cells expressing Gr-1, CD11b and CD11c indicating that our specific MDSC culturing conditions might not be causative responsible for the appearance of the CD11b⁺CD11c⁺Gr-1⁺ MDSC-subpopulation.

In summary, we show that MDSCs might be useful as cellular therapy in GVHD prevention since their ability to skew the immune response towards type 2 immunity abrogates GVHD while maintaining tumor cytotoxicity.
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Authorship Contribution
J.J. M performed experiments and analyzed data.
T.R. performed intracellular stainings and homing experiments.
F.L. performed histological analysis and reviewed the data.
M.B.L. gave technical advice and edited the manuscript.
K-M.D. contributed to the experimental design and edited the manuscript.
G.S. conceived experiments and wrote the paper.

Disclosure of Conflicts of Interests
The authors declare no competing financial interests.
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Figure legends

Figure 1. MDSCs prevent GVHD in a MHC class I mismatched B6 → B6.bm1 BMT model when co-injected with the transplant

A) MDSCs were generated in vitro from BM cells of B6 mice in the presence of GM-CSF and G-CSF. After 4 days cells were stained for CD11b and Gr-1 expression or the distribution of MDSC subsets was defined on CD11b+ cells by the expression of Ly-6C and Ly-6G. Data show one representative FACS staining from three independent experiments performed. B) CFSE-labeled B6.SJL-derived spleen cells (CD45.1+, H-2Kb+) were stimulated with medium or irradiated B6.bm1 (CD45.2+, H-2K^{bm1}) allogeneic spleen cells in the presence or absence of B6-derived MDSCs. iNOS inhibitor L-NMMA or arginase-1 inhibitor nor-NOHA was added. After 4 days cells were stained for CD3, CD4, and CD8. Proliferation of CD45.1+CD3+CD8+ T cells was analyzed and suppression of proliferation was calculated. Data represents the mean of triplicates ±SD of one representative experiment out of three experiments performed. C-E) Lethally irradiated B6.bm1 recipient mice were reconstituted with TCD-BM from B6 mice without or with B6-derived spleen cells (SC) and co-injected with 1x10^6, 5x10^6 or 1x10^7 B6-derived MDSCs on the day of transplantation. Survival (C) and GVHD-scores (D) were determined. C): TCD-BM+SC vs. TCD-BM+SC+1x10^6 MDSCs, p≤0.05*; vs. TCD-BM+SC+5x10^6 MDSCs, p≤0.05*; vs. TCD-BM+SC+1x10^7 MDSCs, p≤0.01**. Surviving animals / total animals treated are indicated in brackets. Error bars indicate SEM. Paraffin sections of ileum and colon, skin and liver of 5-9 animals / treatment group were analyzed for histological signs of GVHD on the day mice were sacrificed due to their moribund state or at the end of the experiment, p≤0.05*, p≤0.01**, n.s. = not significant (E). F) Lethally irradiated B6.bm1 recipient mice were reconstituted with TCD-BM and SC from B6 mice. 1x10^7 B6-derived MDSCs were injected either on the day of transplantation (day 0) or 7 days after BMT (day 7) and survival was determined. Surviving animals / total
animals treated are indicated in brackets. TCD-BM+SC vs. TCD-BM+SC+1×10⁷ MDSCs day 0, p≤0.05*; vs. TCD-BM+SC+1×10⁷ MDSCs day 7, p=0.49 not significant.

Figure 2. MDSC-mediated GVHD suppression is independent of MHC class I expression and does not interfere with the GVT effect

A-C) Lethally irradiated B6.bm1 recipient mice were reconstituted with TCD-BM from B6-mice without or with B6-derived spleen cells (SC) and co-injected with 1×10⁷ B6-derived WT or MHC Cl I⁺ (Cl I⁺) MDSCs. Survival (A) and GVHD-scores (B) were determined and surviving animals / total animals treated are indicated in the survival curve in brackets. A) TCD-BM+SC vs. TCD-BM+SC+1×10⁷ WT MDSC, p≤0.05*; TCD-BM+SC vs. TCD-BM+SC+1×10⁷ Cl I⁺ MDSC, p≤0.05*. Error bars indicate SEM. Paraffin sections of ileum and colon, liver and skin of 8-10 animals / treatment group were analyzed for histological signs of GVHD on the day mice were sacrificed due to their moribund state or at the end of the experiment, p≤0.05*, n.s. = not significant (C). D-F) Lethally irradiated B6.bm1 mice were transplanted with B6-derived TCD-BM in the presence or absence of B6-derived spleen cells (SC) and 1×10⁷ B6-derived MDSCs. CD8⁺ JM6-thymoma cells (H-2Kbm1) were co-injected. Mice were analyzed for survival and surviving animals / total animals treated are indicated in brackets (D). D): TCD-BM+SC vs. TCD-BM+SC+1×10⁷ MDSC, p≤0.001***. Spleen and liver weights were determined either the day mice were killed due to their moribund state or at the end of the experiment (p≤0.001***). Presence of tumor cells in bone marrow, liver, and spleen was determined by the expression of CD8. CD4 and CD8 expression was compared to a non-transplanted B6.bm1 mice (untreated) (F). FACS analysis is shown for one representative mouse out of at least five mice analyzed at the end of the experiment or the day mice were killed due to their moribund state.
Figure 3. In vitro-generated MDSCs proliferate in vivo, reduce alloantigen-specific T cell proliferation only early after transplantation and do not interfere with allogeneic T cell homing

A) Lethally irradiated B6.bm1 recipient mice (H-2K<sup>bm1</sup>, CD45.2<sup>+</sup>) were reconstituted with BM and spleen cells (SC) from B6 mice (H-2K<sup>b</sup>, CD45.2<sup>+</sup>) and co-injected with 1x10<sup>7</sup> CFSE labeled B6.SJL-derived MDSCs (H-2K<sup>b</sup>, CD45.1<sup>+</sup>). Different days after transplantation, mice were sacrificed, stained for CD45.1, and proliferation of transplanted CD45.1<sup>+</sup> MDSCs was analyzed by CFSE dilution in BM, spleen and liver cells. B-D) Lethally irradiated B6.bm1 recipient mice (H-2K<sup>bm1</sup>, CD45.2<sup>+</sup>) were reconstituted with BM from B6 mice (H-2K<sup>b</sup>, CD45.2<sup>+</sup>) plus B6.SJL-derived spleen cells (H-2K<sup>b</sup>, CD45.1<sup>+</sup>) in the presence or absence of 1x10<sup>7</sup> B6-derived MDSCs (H-2K<sup>b</sup>, CD45.2<sup>+</sup>). 1, 3, and 11 days after BMT mice were sacrificed, spleen cells stained for CD45.1 and CD3, and numbers of CD45.1<sup>+</sup>CD3<sup>+</sup> T cells were determined (B). Different days after transplantation spleen, blood, liver, and colon cells were stained for CD45.1 and CD3 and numbers of infiltrating CD45.1<sup>+</sup>CD3<sup>+</sup> T cells were determined in spleen and liver while the percentage of CD45.1<sup>+</sup>CD3<sup>+</sup> T cells was calculated in blood and colon (C). Spleen cells of mice receiving allogeneic BM and SC in the presence or absence of MDSCs were isolated 7 days after transplantation and restimulated in vitro with medium, PMA/ionomycin (PMA/ino) or allogeneic B6.bm1 spleen cells (allo). After 5 h percentage of IL-2 secreting CD45.1<sup>+</sup> T cells was determined (D). FACS diagrams are representative for one mouse out of five analyzed (A). Data represent the mean value ± SD of 5 mice analyzed in (B), of 3 mice analyzed in (C) and of triplicates from spleen cells, which were pooled from spleens of 7 mice treated in (D); p≤0.05*, p≤0.01**, n.s. = not significant; no significant statistical differences were detected in T cell numbers between TCD-BM + SC versus TCD-BM + SC + 1x10<sup>7</sup> MDSCs in (C).
Figure 4. MDSC co-transplantation skews T cells towards a type 2 phenotype

A) Lethally irradiated B6.bm1 recipient mice were reconstituted with TCD-BM from B6 mice with or without B6-derived spleen cells in the presence or absence of 1x10⁷ B6-derived MDSCs. TNFα, IFN-γ and IL-5 concentrations were determined in the serum of transplanted animals 10 days after BMT. B-E) Lethally irradiated B6.bm1 recipient mice (H-2K<sup>bml</sup>, CD45.2⁺) were reconstituted with TCD-BM from B6 mice (H-2K<sup>b</sup>, CD45.2⁺) plus B6.SJL-derived spleen cells (H-2K<sup>b</sup>, CD45.1⁺) in the presence or absence of 1x10⁷ B6-derived MDSCs (H-2K<sup>b</sup>, CD45.2⁺). 10 days after transplantation mice were sacrificed and CD45.1⁺ allogeneic T cells were isolated. qRTPCRs for the expression of cytokines (B) and transcription factors (C) in allogeneic T cells were performed and relative expression to AIP was calculated. 10 days after BMT spleen cells from mice reconstituted with allogeneic BM and spleen cells in the presence or absence of MDSCs were restimulated with PMA/iono. Cells were stained for surface markers CD45.1, CD3, CD4 and CD8. Percentage of CD45.1⁺CD3⁺CD8⁺ T cells expressing IL-5, IL-13, IFN-γ, and TNF-α was determined by intracellular stainings (D). 10 days after transplantation mice were sacrificed and CD45.1⁺ allogeneic T cells were isolated from lung and liver. qRTPCRs for the expression of IL-4, -5, -13, IFN-γ, TNF-α, STAT6, GATA-3, STAT4, and T-bet were performed and relative expression to AIP was calculated (E). Data represent the mean value ±SD of triplicates from spleens of at least 5 mice pooled (B, C). (D) shows the mean value ±SD of 3 experiments with spleen cells pooled from 3 mice. (E) shows the mean value ±SD of 2 experiments with lung and liver cells pooled from at least 6 mice. p≤0.05*; p≤0.01**, p≤0.001***; n.s. = not significant.
Figure 5. Type 2 induction of T cells is required for MDSC-mediated GVHD suppression

A-D) Lethally irradiated B6.bm1 recipient mice were reconstituted with TCD-BM from B6 mice without or with B6-derived spleen cells (SC) or SC derived from STAT6<sup>−/−</sup> animals in the absence or presence of 1x10<sup>7</sup> B6-derived MDSCs. Survival (A) and GVHD-scores (B) were determined. A): TCD-BM+SC(B6) vs. TCD-BM+SC(B6)+1x10<sup>7</sup> MDSCs, p≤0.05*; TCD-BM+SC(STAT6<sup>−/−</sup>) vs. TCD-BM+SC(STAT6<sup>−/−</sup>)+1x10<sup>7</sup> MDSCs, p=0.94 n.s.. Surviving animals / total animals treated are indicated in brackets. Error bars indicate SEM. TNFα, IFN-γ and IL-5 concentrations were determined in the serum of transplanted animals 10 days after BMT (C). 10 days after transplantation mice were sacrificed and CD3<sup>+</sup> T cells were isolated. qRTPCRs for the expression of cytokines IL-4, -5, -13, -17, TNFα, and IFN-γ were performed and relative expression to AIP was calculated (D). Lethally irradiated B6.bm1 recipient mice were reconstituted with TCD-BM from B6 mice together with B6.SJL-derived SC or SC derived from STAT6<sup>−/−</sup> animals in the absence or presence of 1x10<sup>7</sup> B6-derived MDSCs. Different days after BMT spleen, blood, liver, and colon cells were analyzed for numbers of infiltrating CD3<sup>+</sup> T cells in spleen and liver while the percentage of CD3<sup>+</sup> T cells was calculated in blood and colon. B6.SJL-derived allogeneic T cells were detected by gating on CD45.1<sup>+</sup>CD3<sup>+</sup> T cells, while STAT6<sup>−/−</sup>-derived allogeneic T cells were detected by gating on STAT6<sup>−/−</sup>CD3<sup>+</sup> T cells (E). Data represent the mean value ±SD analyzed of triplicates from spleens of at least 5 mice pooled (D) or of 3 mice analyzed on each time point (E). p≤0.05*; p≤0.01**; n.s.= not significant; no significant statistical differences were detected in T cell numbers between the different treatment groups (E).
Figure 6. A CD11b+CD11c+Gr-1medCD301b’I-A h+ MDSC-subpopulation expands under GVHD conditions in vitro

A-C) B6-derived MDSCs were generated in vitro in the presence of GM-CSF and G-CSF. After 4 days in vitro-generated MDSCs (day4-MDSCs) were incubated for 3 days with medium supplemented with 2.5% serum from mice receiving either TCD-BM alone (BM-MDSCs) or TCD-BM plus spleen cells (GVHD-MDSCs). Cells were stained for CD11b and CD11c expression before and after serum incubation and percentage and absolute numbers of CD11b+CD11c- and CD11b+CD11c+ cells were determined. Data represents the mean of triplicates ± SD of one representative experiment out of three experiments performed (A). Cells were stained for CD11b and CD11c and the expression of CD301b, F4/80, I-A h and Gr-1 was determined on CD11b+CD11c- and CD11b+CD11c+ subpopulations 3 days after serum incubation. Data show one representative FACS staining from three independent experiments performed. Isotype staining is shown for the CD11b+CD11c+ population but was identical in the CD11b+CD11c- population (B). CD11b+CD11c- and CD11b+CD11c+ cells were isolated from day4-MDSCs and GVHD-MDSCs and mRNA expression of Klf4, IRF4 and Batf3 was determined and relative expression to AIP was calculated. Data represents the mean ± SD of three independent experiments performed (C). p ≤ 0.05*; p ≤ 0.01**; p ≤ 0.001***; n.s. = not significant.
Figure 1

A

B

CD8

C

D

E

F

Survival

Survival

Survival

Survival

Survival

Survival

Survival
Figure 2

A. Survival of mice in different treatment groups over time.

B. Comparison of spleen weight [g] among different treatment groups.

C. Histological score of ileum + colon in different treatment groups.

D. Survival of mice in different treatment groups with JM6 treatment.

E. Comparison of spleen and liver weight [g] among different treatment groups.

F. Bone marrow, liver, and spleen CD4 and CD8+ T-cell counts for different treatment groups.

Legend:
- TCD-BM (9/9)
- TCD-BM + SC (5/11)
- TCD-BM + SC + 1x10^7 Cl I-/- MDSC (8/9)
- TCD-BM + SC + 1x10^7 WT MDSC (9/10)

*Significant difference
**Highly significant difference
***Very highly significant difference
n.s. Non-significant difference
Figure 6
In vitro-generated MDSCs prevent murine GVHD by inducing type 2 T cells without disabling anti-tumor cytotoxicity

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