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G-CSF mobilizes CD34⁺ regulatory monocytes that inhibit Graft-vs.-Host Disease

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One Sentence Summary: G-CSF-mobilized CD34⁺ monocytes inhibit graft vs. host-disease by the production of nitric oxide and the induction of regulatory T cells

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Abstract:

Granulocyte colony stimulating factor (G-CSF) is routinely used to collect peripheral blood stem cells (PBSC) from healthy donors for allogeneic stem cell transplantation (allo-HSCT). We show that, in both humans and mice, G-CSF mobilizes a subset of CD34⁺ cells with mature monocyte features. These cells, which are phenotypically and functionally conserved in mice and humans, are transcriptionally distinct from myeloid and monocytic precursors but close to mature monocytes and endowed with immunosuppressive properties. In response to IFN- γ released by activated T cells, they produce nitric oxide that induces allogeneic T cell death, both *in vitro* and *in vivo*. Apoptotic T cells are engulfed by macrophages that release TGF- β and promote regulatory T cell expansion. The fraction of CD34⁺ monocytes in peripheral blood CD34⁺ cells inversely correlates with the incidence of acute graft vs. host disease (GVHD) in humans. These G-CSF mobilized cells are an attractive candidate population to be expanded *ex vivo* for cellular therapy against GVHD.

Introduction

The administration of Granulocyte colony stimulating factor (G-CSF) is routinely performed to collect Peripheral Blood Stem Cells (PBSC) from healthy donors for allogeneic hematopoietic stem cell transplantation (allo-HSCT). Despite the higher proportion of T cells in PBSC compared to bone marrow (BM) allografts, the use of PBSC is not associated with an increased incidence of acute graft-vs.-host disease (GVHD) (1). To explain this observation, it was proposed that G-CSF could induce the polarization of alloreactive T cell responses towards a protective Th2 profile (producing IL-4 and IL-10) with a reduced pro-GVHD Th1 response (secreting IL-2 and IFN- γ) (2). G-CSF was also suggested to promote the emergence of tolerogenic dendritic cells (3) and IL-10 producing regulatory T cells (4). It is also recognized that G-CSF can induce and mobilize to the periphery several subsets of granulocytes and monocytes with immunoregulatory properties (5-9). For example, Zavala's group previously reported that G-CSF could mobilize murine CD34⁺ multipotent progenitor precursor (MPP) cells capable of inducing the expansion of regulatory T cells that prevent spontaneous autoimmune type 1 diabetes (10). In the present study, we show that in both humans and mice, G-CSF mobilizes a population of CD34⁺ monocytes endowed with potent immunosuppressive activity. These monocytes strongly inhibit the alloreactive response *in vitro* and *in vivo* in the presence of IFN- γ through nitric oxide (NO) production, and subsequently induce the generation of regulatory T cells (Tregs). This homogeneous subset of monocytes, which is transcriptionally distinct from myeloid and monocytic precursor cells and more closely related to mature inflammatory monocytes, is shown to control GVHD occurrence.

Results

G-CSF mobilizes a subset of CD34⁺ monocytes capable of suppressing alloreactive T lymphocyte proliferation

We studied the CD34⁺ fraction of mobilized hematopoietic stem cells (HSC) in PBSCs from 30 healthy donors. It has been previously reported that human HSC are enriched in the Lin⁻ CD34⁺ CD38⁻ CD90⁺ cell compartment of non-mobilized cord blood and BM cells. By contrast, in mobilized PBSC, the majority of the CD34⁺ cells are CD38⁺ and CD90⁻. Inside the CD34⁺ Lin⁻ fraction of G-CSF mobilized PBSCs, a variable proportion of cells have a monocytic immunophenotype i.e. CD11b⁺ CD33^{hi} CD14^{hi} (Figure 1A). These CD34⁺ monocytes represent a minority of cells within the total CD33⁺ CD11b⁺ CD14⁺ monocytic compartment, which was mainly found in the CD34⁻ fraction and includes classically described HLA-DR⁺ monocytes and HLA-DR^{-/low} monocytes (CD34⁻ DR⁻ Mono). Flow-sorted human CD34⁺ and CD34⁻ Lin⁻HLA-DR⁻ CD33⁺ CD14⁺ G-CSF mobilized cells both revealed features consistent with monocyte differentiation (Figure 1A). The median frequency of CD34⁺ monocytes within the CD34⁺ fraction of the 30 PBSC grafts analyzed was 12% (range 0- 94.3%) (Figure 1B).

By contrast with their CD34⁻ HLA-DR⁺ and CD34⁻ HLA-DR⁻ monocytic (Lin⁻, CD11b⁺, CD14⁺) counterparts, G-CSF mobilized human CD34⁺ monocytes exerted a very strong immunosuppressive effect on T cell activation by either anti-CD3 and -CD28 antibodies or allogeneic dendritic cells in a mixed lymphocyte reaction (MLR) at a stimulator: effector ratio of 1:1 (Figure 2A and Figure S1). In order to evaluate the *in vivo* immunosuppressive effects of these cells in a xenogeneic model of HSCT, we injected irradiated NOD-SCID-IL2R-gamma^{-/-} (NSG) mice with 2.5 x 10⁶ human PBSC. In these experiments, the co-administration of 1x10⁵ human CD34⁺ G-CSF mobilized monocytes reduced weight loss (Figure S1) and improved the overall survival as compared to mice grafted without human CD34⁺ monocytes or with 1x10⁵ human CD34⁻DR⁻ monocytes sorted from the same donor (Figure 2B).

Based on these results, we conducted a clinical prospective study assessing the role of CD34⁺ monocytes contained in HSC grafts on the occurrence of acute GVHD in patients receiving an allogeneic PBSC transplantation. Nineteen recipients (Table 1) were included after providing

their informed consent. The frequency of CD34⁺ monocytes in the CD34⁺ population and the doses of CD34⁺ monocytes injected within the graft were reduced in patients who developed grade II-IV acute GVHD as compared to those who did not (Figure 2C). In addition, the cumulative incidence of acute grade II to IV GVHD on day 100 following allo-HSCT was lower in patients receiving grafts containing CD34⁺ monocyte frequencies above the median (12% of the CD34⁺ population, Figure 1B) than in patients receiving grafts containing CD34⁺ monocyte frequencies below the median (1/13 vs. 5/6, respectively, p=0.007, Kaplan Meyer) (data not shown) (Figure 2D). There were no differences in graft content of CD34⁺ HSCs or in CD34⁺HLA⁻DR⁻ monocytes in patients who did or did not develop acute GVHD (Figure 2E). Furthermore, recipients of grafts containing higher levels of CD34⁺ monocytes had reduced frequencies of proliferating (Ki67⁺) conventional T cells and higher frequencies of CD4⁺CD25^{high} CD127^{low} Treg cells in the peripheral blood on days +15 and +30 following allo-HSCT. (Figures 2F and 2G). Independently of the graft content however, patients who developed grade II-IV acute GVHD had similar frequencies of proliferating T cells and a trend towards lower proportions of Tregs on days +15 and +30 following allo-HSCT in comparison to those who developed grade 0-I acute GVHD (Figure S2).

G-CSF mobilizes a counterpart of the CD34⁺ monocyte population in mice

Based on our observation that G-CSF could mobilize regulatory CD34⁺ monocytes into the periphery in humans, we sought to determine whether such treatment could also generate immunoregulatory monocytes in C57BL/6 (B6) mice. Following G-CSF treatment of B6 mice with human G-CSF for 5 consecutive days, lineage-negative gated cells of the spleen and bone marrow contained a distinct CD34⁺CD11b^{intermediate} population that was absent in non-G-CSF treated mice (Figure 3A and data not shown). Freshly isolated CD34⁺ CD11b^{int} cells had the typical morphology of monocytes (Figure 3A); this population could not be found in the spleen or BM of non-G-CSF treated mice (Figure 3A). Consistent with the concept that the G-CSF-induced CD34⁺ CD11b^{int} cells were of monocytic lineage was their expression of Ly6C and CD115 (CSF-1R), but the lack of expression of dendritic (CD11c, CD135, B220⁻, macrophage (F4/80) and neutrophil (Ly6G) differentiation markers, or of co-stimulation molecules such as CD80, CD86 and MHC class II. CD34⁺ monocytes did not express CD124/IL4-receptor (Figure 3B).

G-CSF mobilized CD34⁺ monocytes strongly inhibited in a dose-dependent manner the proliferation of activated T cells by anti-CD3 and -CD28 antibodies (Figure 3C), while their CD34⁺CD11b⁻ and CD34⁻CD11b⁺ G-CSF mobilized counterparts were devoid of immunosuppressive properties (Figure 3D). G-CSF mobilized CD34⁺ monocytes also strongly inhibited the proliferation of activated T cells by allogeneic dendritic cells (Figure 3E).

To further determine the level of differentiation of CD34⁺ monocytes, we compared them at a transcriptional level to previously well characterized hematopoietic myeloid progenitors (granulocyte macrophage progenitor, monocyte-dendritic precursor, common monocyte precursor and common dendritic progenitor)(11, 12), mature non-inflammatory Ly6C⁻ monocytes, Ly6C⁺ inflammatory monocytes, and monocytic myeloid-derived suppressive cells (mo-MDSC) induced by G-CSF+GM-CSF+IL-13 (13). As shown in the affinity propagation matrix in Figure 3F, G-CSF-mobilized CD34⁺ monocytes were more closely related to mature monocytes than precursor populations or mo-MDSC. However, principal covariance analysis also demonstrated that G-CSF-mobilized CD34⁺ monocytes were distinct from both Ly6C⁺ and Ly6C⁻ monocytes derived from untreated mice (Figure 3G).

Taken together, the phenotypic, transcriptional and functional characteristics of G-CSF mobilized CD34⁺ CD11b^{int} cells indicate that they represent a unique subset of atypical mature monocytes endowed with regulatory functions.

G-CSF induced CD34⁺ monocytes inhibit T cell activation in vitro after their activation by IFN-gamma and through their production of NO

We then explored the mechanisms of the inhibition of T cell activation by G-CSF mobilized CD34⁺ monocytes *in vitro*. We first observed that the inhibition of conventional CD25⁺ T cell activation and proliferation by CD34⁺ monocytes was completely lost in the presence of IFN- γ deficient T cells, thus demonstrating that their immunosuppressive activity required the presence of IFN- γ produced by activated T cells (Figure 4A). Suppressive activity required cell-cell contact (Figure 4B) and was completely reversed in the presence of a selective inhibitor of iNOS (L-NMMA) or when CD34⁺ mobilized monocytes from iNOS^{-/-} B6 mice were used in co-culture (Figure 4C). Of note, transcriptional analysis of iNOS^{-/-} CD34⁺ monocytes showed that they were very similar to their CD34⁺ monocyte counterparts from G-CSF treated wild type mice (Figure S3). Other immunosuppressive mechanisms (arginase, IL-

10, TGF- β , PDL-1, B7-H4) were excluded by adding to the co-cultures a selective inhibitor of arginase (norNOHA) or blocking monoclonal antibodies against IL-10, TGF- β , PDL-1, B7-H4 (Figure 4D). Consistent with other models of NO-mediated immune suppression, G-CSF mobilized CD34⁺ monocytes impaired T cell functions by inhibiting their production of IL-2 (Figure 4E) and by inducing their apoptosis (Figure 4F).

Taken together, these data indicate that IFN- γ secreted by activated T cells induces the production of NO by CD34⁺ monocytes in a contact-dependent manner. In turn, the release of NO impairs the production of IL-2 by T cells leading to an early inhibition of T cell proliferation and to T cell apoptosis.

CD34⁺ monocytes induce the apoptosis of allogeneic T cells in vivo via iNOS leading to immune tolerance through the induction of Tregs

We then explored the effect of G-CSF mobilized CD34⁺ monocytes *in vivo* by analyzing the impact of their adoptive transfer in a mouse model of GVHD following B6 to BALB/c allo-HSCT. We observed that BALB/c mice receiving B6 bone marrow and B6 T cells had improved survival when B6 G-CSF-mobilized CD34⁺ monocytes were co-transferred (Figure 5A). As expected, the protective effect of CD34⁺ monocytes was lost when they were obtained from iNOS deficient donor mice (Figure 5A). Mice transplanted in the presence of WT CD34⁺ monocytes lost significantly less weight (Figure 5B) and had lower GVHD clinical scores than allogeneic control mice or those receiving iNOS^{-/-} CD34⁺ monocytes by day +35 post allogeneic HSCT (Figure 5C). Recipient mice also had lower GVHD histopathological scores, particularly in the colon, as compared to the other groups, as evaluated by standard criteria (14) (Figure 5D). Following allo-HSCT, we could find the transferred CD34⁺ monocytes in the recipient spleen and lymph nodes until day 15, at which point they demonstrated a similar phenotype to the input cells (CD34⁺ CD11b^{int} CD115⁺ Ly6C⁺ F4/80⁺ CD16/32⁺) and maintained immunosuppressive properties that were again dependent upon iNOS expression (Figure S4).

We further sought to determine the effects of CD34⁺ monocytes on allogeneic donor T cell activation *in vivo*. Following allo-SCT, co-transfer of donor CD34⁺ monocytes did not impair the proliferation of allogeneic T cells as evaluated on day 4 or the production of IFN- γ by

CD4⁺ donor alloreactive T cells on day 8 (Figures 6A and 6B). However, by day 12 following allo-HSCT, splenic and lymph node CD8⁺ and CD4⁺ donor T cells had lower expression of the activation marker CD69 upon co-transfer of wild type but not iNOS^{-/-} CD34⁺ monocytes (Figure 6C).

Because CD34⁺ monocytes induced apoptotic T cells *in vitro*, we also explored the pro-apoptotic properties of CD34⁺ monocytes on activated T cells *in vivo*. At early time points following allo-HSCT (day +4 and +8), there were increased numbers of apoptotic CD3⁺ T cells in spleens of recipients receiving donor T cells and co-transfer of WT CD34⁺ monocytes as compared to those receiving T cells without CD34⁺ monocytes (Figure 6D). Increased apoptosis was dependent upon NO because there was no increase in T cell apoptosis where iNOS^{-/-} CD34⁺ monocytes were co-transferred (Figure 6D). Because apoptotic T cells have been reported to induce Tregs by triggering the production of TGF- β by macrophages (15), we sought to determine whether apoptotic T cells induced by CD34⁺ monocytes could promote the generation of Tregs by the same mechanism. Indeed, *ex vivo* phagocytosis of apoptotic T cell debris by CD11b⁺ F4/80⁺ macrophages from BMT recipients that received CD34⁺ monocytes was observed consistently, whereas this activity was not seen in control recipients (Figure S5). Furthermore, co-transfer of CD34⁺ monocytes induced higher levels of TGF- β in serum of allo-HSCT recipients than the co-transfer of iNOS deficient CD34⁺ monocytes where TGF- β levels were similar to controls (Figure S6). In parallel, the frequency of donor-derived CD45.1⁺ Tregs in the spleens of allo-HSCT recipients was elevated in the CD34⁺ monocyte group but not in the iNOS deficient CD34⁺ monocyte or control groups (Figure 6E). Because donor T cells were depleted of Tregs (CD3⁺CD25⁻) but expressed the donor congenic CD45.1 marker, we reasoned that CD45.1⁺ Tregs were donor-derived induced Tregs. Consistent with the model that NO-mediated T cell apoptosis plays a critical role in the induction of Tregs, the frequency of Tregs was not increased compared to controls in the group of mice co-transplanted with iNOS^{-/-} CD34⁺ monocytes (Figure 6E). Finally, *in vivo* depletion of Tregs by the PC61 antibody after allo-HSCT completely reversed the protective effect of CD34⁺ monocytes on GVHD lethality without affecting the outcome of mice receiving allo-HSCT and donor T cells without CD34⁺ monocytes (Figure 6F).

Taken together, these data indicate that the control of GVHD by CD34⁺ monocytes requires their production of NO that in turn regulates the expansion of induced Tregs in the allo-SCT recipients.

Discussion

We have characterized a previously unidentified monocytic cell population defined by the cell surface expression of CD34. G-CSF induces the expansion of these variant monocytes that inhibit the *in vitro* and *in vivo* alloreactive T cell response both in mice and humans. The G-CSF-mobilized population is found within the Lin⁻ CD33^{hi} CD11b⁺ CD14⁺ monocytic compartment and is characterized by intermediate expression of CD34. This CD34⁺ fraction represents a minority of cells within the monocyte population, but is endowed with potent immunosuppressive properties. At a transcriptional level, the murine monocyte subset is distinct from mature Ly6C⁺ and Ly6C⁻ monocytes and is clearly more differentiated than myeloid and monocytic precursors, including G-CSF+GM-CSF+IL13 induced mo-MDSCs. In humans, G-CSF mobilized CD34⁺ monocytes were CD14^{low} but lacked the expression of HLA-DR, which distinguishes them from inflammatory monocytes. They therefore represent a subset of highly immunosuppressive mature monocytes with expression of CD34.

In mice, we demonstrated that G-CSF-mobilized CD34⁺ monocytes require T cell-mediated IFN- γ to produce NO that inhibits T cell activation and proliferation. We report that CD34⁺ monocyte-derived NO regulates the alloreactive response *in vivo* by inducing T cell apoptosis and subsequently, the induction of regulatory T cells. Although global inhibition of NO generation has been shown to increase GVHD (16), our findings indicate that the effects of NO are context-dependent and are likely to be influenced by the cell source, timing and localization of production. Although high levels of circulating NO may worsen GVHD injury, local production in secondary lymphoid organs by patrolling CD34⁺ monocytes in response to IFN- γ produced by activated T cells has a regulatory effect. Our data also suggest that IFN- γ -activated-CD34⁺ monocytes producing NO induce apoptosis in alloreactive T cells. Uptake of apoptotic T cells by macrophages triggers them to produce high levels of TGF- β that drives the expansion of Tregs and induces immune tolerance. Such a mechanism has also been observed with BM mesenchymal stem cells that induce T cell apoptosis via FAS/FAS-L (17), or in the presence of apoptotic T cells induced by anti-CD3 antibody treatment (15). Our data indicate that this mechanism of immune tolerance can also be induced by a specific CD34⁺ subset of monocytes and reveals a novel requirement for NO. In humans, the immunosuppressive effect of G-CSF mobilized CD34⁺ monocytes on T cell activation and proliferation was also dependent on NO *in vitro* (Figure S7). The correlation between higher frequencies of graft CD34⁺ monocytes and lower rates of acute GVHD, together with the *in*

in vivo reduced T cell proliferation and increased Tregs observed in recipients of higher CD34⁺ monocyte numbers suggest that this population also regulates the alloreactive response in humans by the same mechanism as in mice.

Although previous studies have described G-CSF-mobilized CD11b⁺ myeloid suppressive cells that can induce Tregs and control GVHD in mice, detailed subset characterization has not been performed, and these cells have been assumed to represent immature cells (4, 8). For example, the Hill's group described CD11b⁺Gr1^{dim} cells generated by G-CSF⁺/FLT3-L (termed 'GM cells') that could induce tolerance through the induction of IL-10 producing Tregs (6). Our data indicate that the low frequency CD34⁺ monocytic subset within the CD11b^{+/int} compartment represents the major regulatory subset, capable of inducing the expansion of Tregs via a NO-dependent mechanism and links this subset to a similar population in humans. Monocytic cells with immunoregulatory functions through the production of NO have also been described in chemotherapy- and radiotherapy-treated or tumor-bearing mice (18-20), where they are termed myeloid derived suppressive cells (MDSCs), defined as immature myeloid cells (Gr-1⁺ CD11b⁺ in mice, HLADR^{-/low} CD33⁺ CD11b⁺ in humans) that share the property of suppressing T cell activation (21). Monocytic MDSCs that are Ly6C^{+/high} in mice and CD14⁺ in humans are distinguished from their granulocytic MDSC counterparts by lower expression of Ly6C in mice and or a CD15⁺ phenotype in humans (22). They are believed to derive from myeloid precursors that acquire immunoregulatory properties in the presence of some inflammatory signals within the tumor microenvironment (23). MDSCs can alternatively be induced *ex vivo* in the presence of various reagents and cytokines (24, 25) and have been described to induce tolerance in organ transplantation in mice (26, 27). In the context of allo-HSCT, Blazar's group generated monocytic MDSCs by culturing murine BM cells with G-CSF+GM-CSF+IL13; in this case, cultured cells were CD11b⁺ Ly6G^{low} Ly6C⁺ interleukin-4R α (IL-4R α)⁺ F4/80⁺, and inhibited the allogeneic T-cell proliferation through an Arginase-1-dependent mechanism (13). They were therefore distinct from the CD34⁺ mobilized monocytes in terms of phenotype (F4/80⁺ and IL4-R α ⁺), function (iNOS-independent) and at transcriptional level, where we have shown that they were more similar CoMP. Thus, based on the usual definition of MDSCs as described in the context of cancer, the G-CSF-mobilized CD34⁺ monocytes should be considered as a subtype of monocytic MDSCs distinguished by their features of mature monocytes.

Future clinical application of CD34⁺ monocytes may require strategies to expand this population *ex vivo*. In this regard, we have enriched Lin⁻ CD34⁺ CD11b^{int} cells from the spleen and performed short-term (3 day) cultures in Stemspan media in the presence of SCF, TPO and FLT3L, permitting a 10-fold expansion of cells (Figure S8). The phenotype and functions of CD34⁺ monocytes briefly expanded *ex vivo* were very similar to that of the input cells (Figure S8). We also observed that these cells could be detected in secondary lymphoid organs up to 15 days after adoptive transfer into mice, at which point their immunosuppressive functions were preserved (Figure S4). However, the transcriptional profiles of the expanded population were distinct from the freshly isolated CD34⁺ monocytes and intermediate between the common monocyte progenitor and mature monocytes (Figure S8). These data suggest that the current protocol might selectively expand a distinct cell type that nevertheless shares certain phenotypic and functional features with the original input cells. Future studies will focus on alternate expansion methods based upon identifying G-CSF-responsive progenitor cells that give rise to CD34⁺ monocytes.

In conclusion, we have characterized and described a previously unknown homogeneous population of G-CSF induced CD34⁺ monocytes, with powerful immunosuppressive properties upon murine and human allogeneic T cell activation. This CD34⁺ mobilized regulatory monocyte subset might also be of interest in other conditions such as autoimmunity, chronic inflammatory diseases and organ transplantation. Such tolerogenic monocytes could represent a good candidate for the development of novel immunoregulatory and therapeutic cellular therapies.

Material and Methods

Mice

BALB/cJ (H2^d) and C57BL/6/J (H2^b) mice were purchased from Janvier (Le Genest Saint Isle, France); B6.129P-Nos2^{tm1Lau} (iNOS^{-/-}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6.129S7-Ifng^{tm1Ts}/J (IFN- γ ^{-/-}) were kindly provided by Maria Leite de Moraes (CNRS UMR8147, Paris, France) and NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) were kindly provided by Lucienne Chatenoud (INSERM U1013, Paris, France). All mice were bred and housed in a specific pathogen-free facility in microisolator cages and used at 8 to 12 weeks of age in protocols approved by the local Ethical Committee (CEEA34.0AP.018.11).

Human CD34⁺ monocytes isolation

Peripheral blood samples (10mL in EDTA-tube) from rhG-CSF-treated (filgastrim 10 μ g/kg/day, 5 consecutive days) healthy allogeneic donors (randomly selected at Necker and Saint Antoine Hospitals in Paris) were collected after informed consent. CD34⁺ monocytes were characterized on the Ficoll-purified peripheral blood mononuclear cell (PBMC) fraction, using flow cytometry with the following antibodies: CD3, CD19, CD56, HLA-DR, CD11b, CD33, CD14, CD15, CD34 (eBiosciences) and FACSsorted (FACS Aria II, BD Bioscience).

Murine CD34⁺ monocytes generation and expansion

C57BL6/J (B6) or iNOS^{-/-} mice were injected subcutaneously for 5 consecutive days with recombinant human G-CSF (200 μ g/kg/day) (Neupogen) with or without recombinant murine Flt3-L (20 μ g/kg/day) (Immunotools). At day 6, spleen cells labeled with mAbs directed against mature lineage markers (CD4, CD8, Gr-1, CD45R, Ter119) were depleted with anti-rat Ig-coated magnetic beads (DynaL Biotech Invitrogen). The CD34⁺ monocytes were characterized and FACS sorted (FACS Aria II, BD Bioscience) after staining with CD11b and CD34 antibodies (eBiosciences). In some experiments, the CD34⁺ monocytes were expanded from the FACS sorted Lin⁻ fraction cultured in Stemspan SFEM (Stemcell Technologies) + 20ng/mL rmSCF + 20ng/mL rmTPO + 50ng/mL rm FLT3L (Immunotools) in 6 well plate at 2x10⁶ cells/mL concentration and incubated at 37°C 5% CO₂ for 3 days before flow sorting.

Morphology

CD34⁺ monocyte morphology was studied on cytopins after May-Grünwald-Giemsa staining. Photographs were taken using 400X magnification with a 3CCD color camera (Sony) mounted on a DMRB Leica microscope.

Proliferation assays

T cells from B6 mice were purified by negative selection using a pan T cell depletion kit (MiltenyiBiotec). Purity was routinely above 98%. T cell activation (50000 cells/well) was performed in an allogeneic MLR or by non-specific TCR activation in a 96-well culture plate. In MLR, mature dendritic cells were harvested from BALB/cJ mice with a pan-DC selection kit (MiltenyiBiotec). B6 T cells were mixed with BALB/cJ DC stimulators at 1:5 DC:T cell ratio and plated in a 96-well round bottom culture plate (5×10^4 T cells/well). Non-specific T cell activation was performed in a 96 round bottom plate coated with 1 μ g/mL anti-CD3 mAb (clone 145 2C11) and 2.5 μ g/mL anti-CD28 mAb (Biolegend) for 2 hours before the addition of T cells (50000/well). CD34⁺ monocytes were plated at different concentrations: 10^4 /well (1:10 CD34⁺Mono : T), 2.5×10^4 /well (1:5), 5×10^4 /well (1:1). Cells were incubated in custom RPMI 1640 containing physiologic levels of L-arginine (150 μ M) supplemented with 10% fetal calf serum, 50 μ M 2-mercaptoethanol, 10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 1mM sodium pyruvate, and 100 U/mL penicillin-streptomycin (Life Technologies). T cells were stained with 5 μ M carboxyfluoresceinsuccinimidyl ester (CFSE) (Invitrogen). To inhibit IL-10, TGF- β and PDL-1, B7-H4, blocking antibodies were used at 10 to 20 μ g/mL concentration (blocking antibody IL-10, TGF- β from R&D systems and blocking antibody PDL-1, B7-H4 from eBiosciences). To inhibit arginase activity, N^w-hydroxy-nor-arginine (nor-NOHA) (Calbiochem) was used at a concentration of 300 μ M. For inhibition of iNOS, L-N^G-monomethyl-arginine-citrate (L-NMMA) (Calbiochem) was used at a concentration of 300 μ M. To inhibit reactive oxygen species such as hydroxylperoxide, catalase (Sigma-Aldrich) was used at 1000U/mL. In some experiments, CD34⁺ monocytes from B6;129P-*Nos2tm1Lau* (iNOS^{-/-}) were also used.

For human CD34⁺ monocytes, *in vitro* suppressive capacity was tested in MLR involving FACSsorted sub-populations from G-CSF-treated PBSC-donors added as a third-party cell population or issued from the same volunteer donor than T cells. Allogeneic DC were obtained from monocytes of unrelated healthy volunteers cultured in GM-CSF 1000 UI/mL

and IL-4 500 UI/mL (Peprotech) for 6 days. Human MLR were performed with MACS-purified T-cells (pan-T cell isolation kit, MiltenyiBiotec): 50,000 cells/well stimulated in a round bottomed 96-well microculture-plate by allogeneic dendritic cells (DC) (10,000 cells/well) with or without CD34⁺ monocytes at 1:1 DC:T ratio in a final volume of 200 μ L.

Flow cytometry

Murine G-CSF mobilized CD34⁺ monocytes and other splenic monocytes or BM-derived precursor cells were stained with appropriately labeled mAbs to the following antigens: lineage markers (CD4, CD8, Gr-1, CD45R, Ter119), CD34, CD11b, Ly6C, Ly6G, F4/80, CD115, CD117, CD135, CD16/32, CD11c, CD11b (eBiosciences), CX3CR1 (R&D Systems) after incubation with purified anti-CD16/32-mAbs to block non-specific FcR binding. In some experiments, BALB/cJ mice were sacrificed at day 1, 4, 8, 12 and 15 after allogeneic HSC transplantation, to follow T cell apoptosis analyzed by flow cytometry with mAbs directed against CD45.1 and CD3 (eBiosciences) in combination with Annexin V and propidium iodide (BD Biosciences). Allogeneic T cells were studied for their activated phenotype by using mAb to CD69 (eBiosciences). Intracellular cytokine IFN- γ (eBiosciences) staining was performed after a 3-hour incubation of whole splenocytes with PMA (10ng/mL) plus ionomycin (500ng/mL) and Brefeldin A (2mg/mL) at 37°C and 5% CO₂. Macrophages were stained with mAb against CD11b and F4/80 and DCs with mAb against CD11b and CD11c (eBiosciences). In some experiments, BALB/cJ antigen-presenting cells were stained with anti-IL-10 (eBiosciences). Identification of transferred CD34⁺ monocytes from CD45.1 congenic mice to lymph nodes and spleen was analyzed by flow cytometry with the use of anti-CD45.1 mAb (eBiosciences).

Peripheral blood cells from patients were stained with appropriately labeled mAbs against CD34, CD11b, CD33, CD14, CD15, HLA-DR and lineage markers (CD3, CD19, CD56) (eBiosciences, San Diego, CA), Tregs were stained with mAb to CD3, CD4, CD25, CD45RA, CD127, Foxp3, Ki67 mAbs (eBiosciences). Samples were analyzed on a FACS Canto II cytometer (BD Biosciences) using FlowJo software (Treestar).

Microarray Analysis, normalization and validation

Total RNA was extracted from sorted cells using the RNeasy Microkit (Qiagen) and hybridized on Affymetrix Mogene 2.0 arrays at the UCL Cancer Institute core facility. To analyze the data UCL Affymetrix Mogene 2.0 and Immgen Affymetrix Mogene 1.0 were

background corrected, preprocessed and normalized separately using the "oligo" package from Bioconductor(28), specifically the "rma" method(29). Expression data was then filtered to contain only probe sets present on both Mogene 1.0 and 2.0 platforms. They were then combined and renormalized to remove batch effects using the "sva" package from Bioconductor, specifically the "ComBat" method(30). To show the relationship between sample groups we used the R programming language to plot principal components (PCA) of the samples. Further, to objectively show the natural clustering relationship of groups we used affinity propagation clustering(31) (with the R "apcluster" package).

GVHD

BALB/cJ recipients were lethally irradiated using 600 cGy total body irradiation by x-ray on day -1 followed by the intravenous infusion in the caudal vein of 10^7 T cell-depleted B6 donor BM cells, 2×10^6 purified B6 CD25-depleted T cells, and 0.5×10^6 B6 CD34⁺ monocytes on day 0. Control groups were transplanted with BM cells alone or BM + T cells, or BM + T cells and 0.5×10^6 B6 iNOS^{-/-} CD34⁺ monocytes. Mice were monitored for survival, weight and acute GVHD score twice a week. The clinical scoring system was based on 6 parameters: weight loss, posture, activity, fur texture, skin integrity, and diarrhea. A severity scale of 0 to 2 was used for each parameter, with a maximum score of 12. Recipients that had died or had been sacrificed at 3 months after BMT were kept in 10% formalin. Tissues from GVHD target organs (liver, intestine and skin) were embedded in paraffin, sectioned, and stained with hematoxylin, eosin and safran. Photographs were taken using 200x or 400x magnification with a Leica DF295 camera mounted on a Leica DMLB microscope. NSG mice were irradiated using 200cGy total body irradiation by x-ray on day -1 followed by the intravenous infusion in the caudal vein of 2.5×10^6 human PBSC and 1×10^5 CD34⁺ monocytes or 1×10^5 CD34⁻ HLA-DR⁻ monocytes on day 0. Control group was transplanted with only 2.5×10^6 human PBSC on day 0. Mice were monitored for survival and weight twice a week.

Isolation of mono-macrophages and identification of phagocytosis properties

Spleens from allografted BALB/cJ mice were harvested on day 15 after allo-HSCT and were injected with 1 mg of collagenase D (Life Technologies), cut into small pieces and incubated at 37°C for 30 minutes. A single cell suspension was then passed through a 70- μ m cell strainer, washed and incubated with purified anti-CD16 and CD32-mAbs to block non-

specific FcR binding. CD45.1⁺ CD11b⁺ F4/80⁺ mono-macrophages were then purified from the spleen using FACS-Aria II (BD Biosciences). Allogeneic T cells from BALB/cJ BMT recipients with or without CD34⁺ monocytes were FACS sorted based upon their expression of CD45.1 and labeled with 2.5 μ M CFSE (Life Technologies). In phagocytosis assays, macrophages were plated into a 24 well plate (0.1 to 1 x 10⁶ cells/well) and incubated in the presence of CFSE-labeled allogeneic T cells (1:1 ratio) overnight in X-VIVO 20 serum-free milieu (Lonza). Co-incubated cells were then placed on a polylysine slide and stained with PE-conjugated anti-F4/80 mAbs followed by a AF594 goat anti-rat IgG and then analyzed under confocal microscopy (LEICA TCS SP8).

Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood samples (100 μ L) were harvested from mice on days 0, 8 and 15 post-HSCT using micro-hematocrit tubes with heparin (VWR). Plasma was collected after centrifugation of blood samples at 2,000 rpm for 8 min. Measurement of TGF- β 1 was performed using commercially available kits (R&D Systems) according to manufacturers' instructions.

Treg depletion

Allografted BALB/cJ mice were injected intraperitoneously with PC61 (30 μ g/mouse) (Biolegend) on day 4 following allo-HSCT to deplete CD25⁺ cells, and every 10 days thereafter.

Statistical analysis

The results were averaged in each group. The intra-group differences were calculated between the mean values. Comparisons between groups were performed by Student t test or Fisher exact test. Survival curves were compared by log-rank test. A p-value < 0.05 was considered as statistically significant.

Supplementary materials

Fig. S1. Human CD34⁺ monocytes inhibit allogeneic T cell proliferation in vitro and reduce weight loss in the NSG xenogeneic HSCT model

Fig. S2. Post-transplant peripheral blood levels of proliferating CD4⁺ T cells and Tregs in patients who developed grade II-IV acute GVHD and those who did not.

Fig. S3. The transcriptional profile of iNOS^{-/-} CD34⁺ monocytes is very similar to their wild type counterparts.

Fig. S4. CD34⁺ monocytes patrolling in the lymph nodes and spleen 15 days after *in vivo* injection have kept the same phenotype and function as input cells

Fig. S5. Apoptotic T cells are engulfed by splenic macrophages in allo-SCT recipient mice that received CD34⁺ monocytes

Fig. S6. Mice receiving allo-SCT with CD34⁺ monocytes have increased serum TGF- β 1 levels.

Fig. S7. Human CD34⁺ monocytes inhibit allogeneic T cell proliferation in a MLR through iNOS

Fig. S8. The functions and phenotype of *ex vivo* expanded CD34⁺ cells are similar to that of freshly sorted cells

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Author contributions:

MD, MTR, OH designed the experiments, interpreted the data and wrote the manuscript

MD, JR and TC performed the experiments

SS and TM provided the material for the microarray experiments

SH analyzed and interpreted the microarray data

JB performed the histopathology analyses

OAP and MGT provided technical support

FS, MM and MTR took care of the patients

HTN provided the human HSC grafts

FZ, CLB, MM and RC provided a critical review of the data and manuscript.

All authors approved the manuscript.

Competing interests:

The authors declare no financial conflict of interest.

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Tables and figures

Table 1: Patients' characteristics

Baseline parameters		Percent
All patients	19	100%
Age of recipient	5.42 (21.4 -66.9)	
Age of donor	48.07 (23.4-60)	
Hematological disease		
AML/ALL	16	84.2%
Lymphoma/myeloma	3	15.8%
Disease status at transplant		
CR	13	68.4%
PR	2	10.6%
PD	4	21%
Donor		
Sibling	13	68.4%
Unrelated 10/10	4	21%
Unrelated 8 or 9/10	2	10.6%
Conditioning regimen		
MAC	7	36.8%
RIC	12	63.2%
In vivo T cell depletion		
yes	16	84.2%
No	3	15.8%

AML: acute myeloid leukemia, ALL: acute lymphoid leukemia, CR: complete remission, PR: partial remission, PD: progressive disease, MAC: myeloablative conditioning, RIC: reduced intensity conditioning.

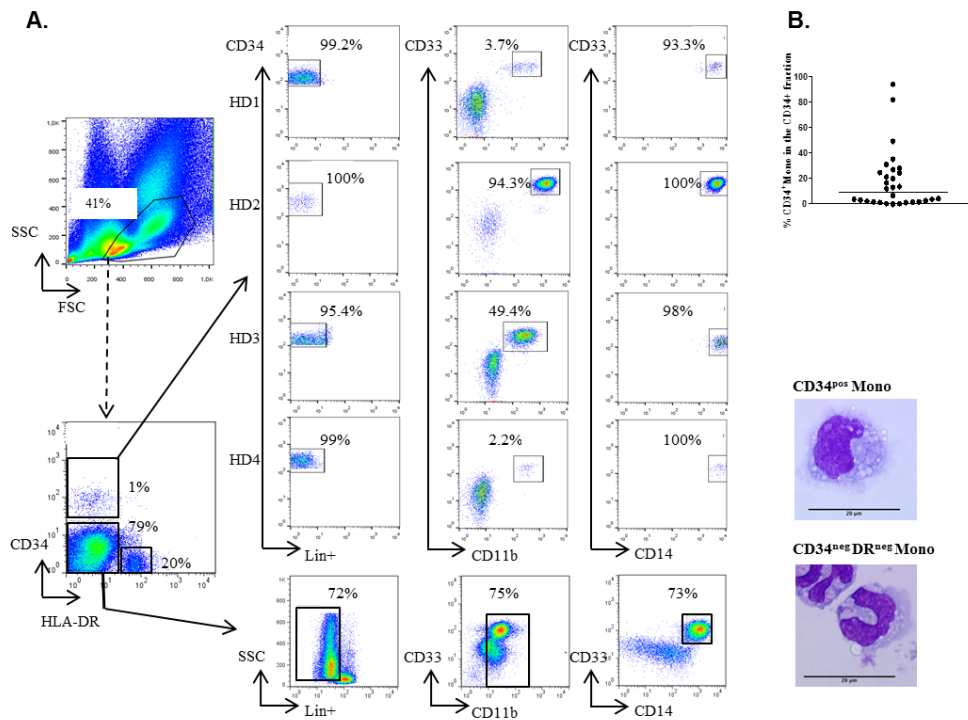


Fig. 1. G-CSF mobilizes a subset of CD34⁺ monocytes in humans

A. Human G-CSF mobilized peripheral blood stem cells (PBSC) were analyzed by flow cytometry. Within the HLA-DR⁻ fraction, we distinguished the CD34⁺ fraction from the CD34⁻ fraction of cells. In the gated HLA-DR⁻ Lin⁻ (CD3⁻CD56⁻CD19⁻) CD34⁺ fraction, we identified a population of cells that homogeneously expressed CD11b, CD14 and CD33, termed CD34⁺ monocytes (CD34⁺ Mono). Analysis of the CD34⁺ fraction of PBSC from 4 different donors is shown (HD1 to 4). Similarly, in the HLA-DR⁻ Lin⁻ CD34⁻ fraction, we identified a subpopulation of cells expressing CD11b, CD33 and CD14 (CD34⁻ DR⁺ Mono). These monocytic populations were distinct from classical Lin⁻ HLA-DR⁺ CD34⁻ CD11b⁺ CD14⁺ monocytes. All gates were based on isotype controls. May-Grumwald-Giemsa cytopsin preparations show morphological features of mononuclear cells in both human CD34⁺ and CD34⁻ HLA-DR⁻ sorted fractions.

B. Proportions of CD34⁺ Mono in the CD34⁺ fraction of the 30 PBSC grafts analyzed (median value = 12%, mean value=17.5%, SEM=4.22%).

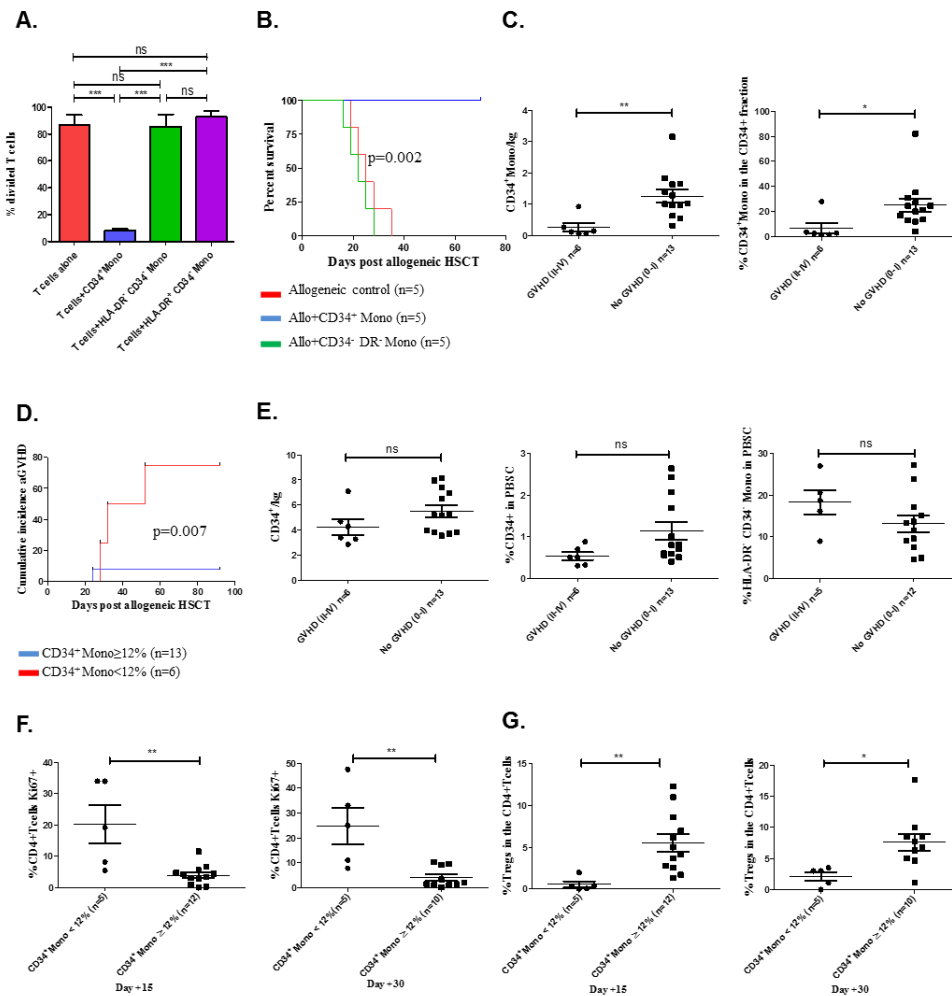


Fig. 2. Human CD34⁺ monocytes are characterized by features of mature monocytes endowed with immunosuppressive properties

A. Anti-CD3 and -CD28 activated T cell proliferation after 4 days of culture with or without CD34⁺ Mono or HLA-DR⁻CD34⁻ Mono or HLA-DR⁺CD34⁻ Mono (percentage of divided T cells = $8.27 \pm 1.12\%$ with CD34⁺ Mono (n=3) vs. $87 \pm 7.4\%$ without CD34⁺ Mono (n=3) (p=0.0005) or vs. HLA-DR⁻CD34⁻ and HLA-DR⁺CD34⁻ monocytes (n=3) (p<0.0001). Data were compared using Student's unpaired t-test (ns: not significant, *: p≤0.05, **: p≤0.01, ***: p≤0.001).

B. Lethally irradiated NSG mice received 2.5×10^6 human PBMC with or without 1×10^5 G-CSF mobilized human CD34⁺ or HLA-DR⁻CD34⁻ monocytes. Median survival in xenografted mice without CD34⁺ Mono (red, n=5) (25 days) was comparable to the median survival in transplanted mice with HLA-DR⁻CD34⁻ Mono (green, n=5) (22 days) (p=0.25). Mice co-transplanted with CD34⁺ Mono (blue, n=5) had improved survival with 100% survival by day 80 post-transplant (p=0.002). Results are derived from 2 pooled independent experiments. Each experiment was performed with PBSC from one donor for all recipients. Results are presented and compared with Kaplan-Meier survival curves.

C. Proportions of CD34⁺ Mono in the total CD34⁺ fraction of patients developing grade II-IV acute GVHD (n=6) and those who did not (n=13) ($6.77 \pm 4.26\%$ vs. $25.04 \pm 5.3\%$, respectively, p=0.04) and numbers of CD34⁺ Mono/kg recipient weight in the same groups of patients ($0.26 \pm 0.14\%$ vs. $1.26 \pm$

0.20%, respectively, $p=0.005$). Data were compared using Student's unpaired t-test (ns: not significant, *: $p\leq 0.05$, **: $p\leq 0.01$).

D. Cumulative incidence of acute grade II to IV GVHD in the group of patients receiving allo-HSCT with a graft containing levels of $CD34^+$ monocytes above 12% of the $CD34^+$ HSCs ($n=13$) compared to patients transplanted with a graft containing $<12\%$ $CD34^+$ monocytes ($n=6$) (at day 100 following allo-HSCT, 1/13 vs. 5/6, respectively, $p=0.007$). Results are compared with Kaplan-Meier curves.

E. *Left-* Numbers of $CD34^+$ /kg in the PBSC graft of patients developing grade II-IV acute GVHD (mean 4.26 ± 0.63 cells/kg, $n=6$) vs. those who did not (5.5 ± 0.47 cells/kg, $n=13$) ($p=0.15$). *Middle-* Proportions of $CD34^+$ cells in the PBSC graft of patients developing grade II-IV acute GVHD ($0.53 \pm 0.09\%$, $n=6$) vs. those who did not ($1.14 \pm 0.22\%$, $n=13$) ($p=0.08$). *Right-* Proportions of HLA-DR⁻ $CD34^+$ Mono in the PBSC graft of patients developing grade II-IV acute GVHD ($18.3 \pm 2.95\%$, $n=6$) vs. those who did not (13.15 ± 2.02 , $n=13$) ($p=0.18$). Data were compared using Student's unpaired t-test (ns: not significant).

~~E-F.~~ Proportions of detectable proliferating ($Ki67^+$) conventional $CD4^+$ T cells in peripheral blood on days 15 and 30 post-transplantation according to the levels of $CD34^+$ Mono contained in the graft. On day 15 following allo-HSCT, $20.11 \pm 6.1\%$ in the $CD34^+$ Mono $<12\%$ group ($n=5$) vs $3.92 \pm 0.9\%$ in the $CD34^+$ Mono $\geq 12\%$ group ($n=12$), ($p=0.001$) and on day 30 following allo-HSCT, $24.86 \pm 7.28\%$ ($n=5$) vs $4.29 \pm 1.39\%$ ($n=10$) in the respective groups ($p=0.001$). Data were compared using Student's t-test (**: $p\leq 0.01$).

~~F-G.~~ Proportions of post-transplant $CD4^+ CD25^{high} CD127^{low}$ peripheral blood regulatory T cells on days 15 and 30 following transplantation according to the levels of $CD34^+$ Mono contained in the HSC graft. On day +15 following allo-HSCT, $0.56 \pm 0.37\%$ in the $CD34^+$ Mono $<12\%$ group ($n=5$) vs $5.52 \pm 1.03\%$ in the $CD34^+$ Mono $\geq 12\%$ group ($n=12$), ($p=0.008$) and on day +30 following allo-HSCT, $2.12 \pm 0.67\%$ ($n=5$) vs $7.37 \pm 1.5\%$ ($n=10$) in the respective groups, ($p=0.03$). Data were compared using Student's unpaired t-test (*: $p\leq 0.05$, **: $p\leq 0.01$).

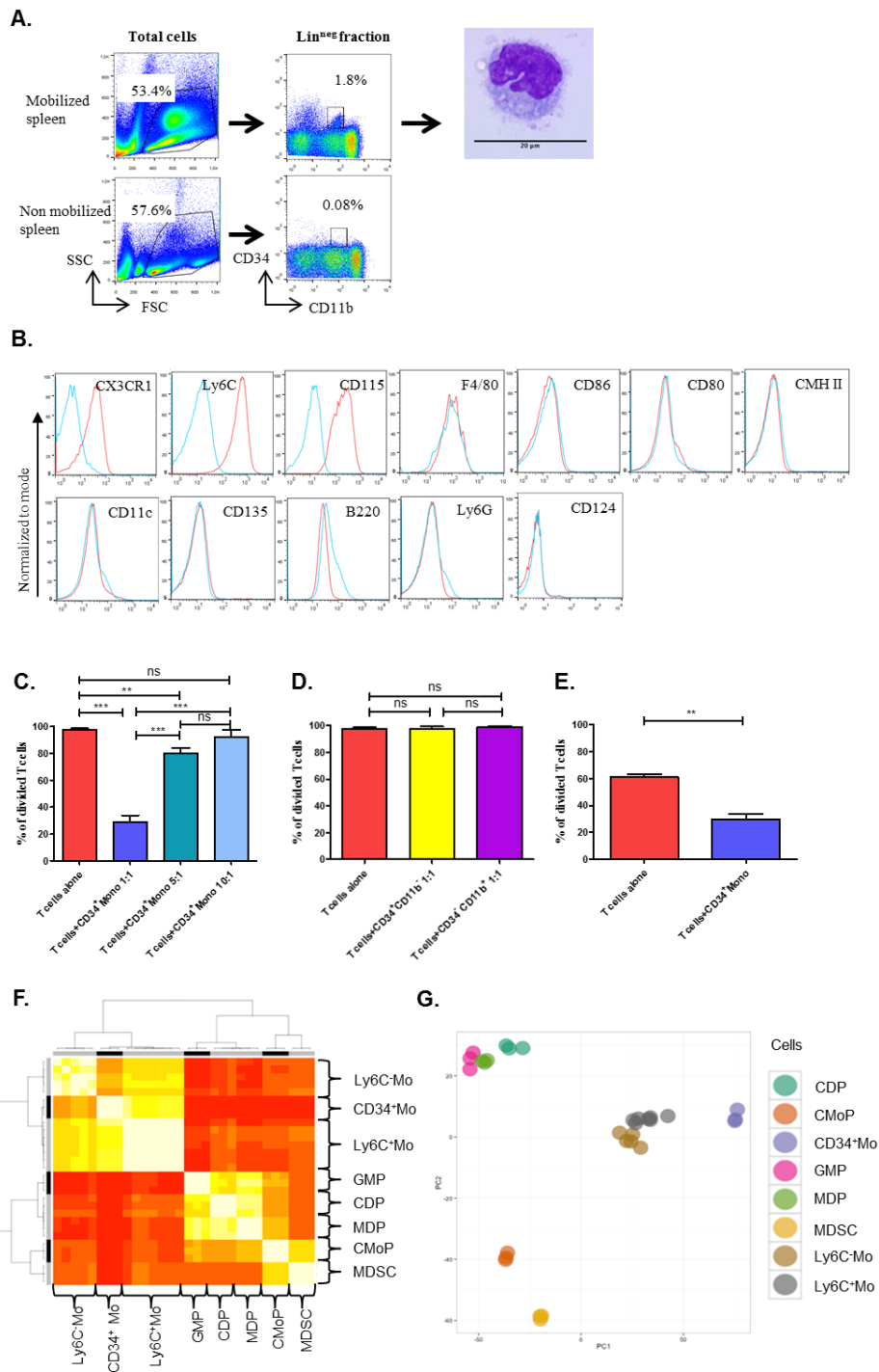


Fig. 3. G-CSF mobilizes murine CD34⁺ monocytes with immunosuppressive properties

A. Within the Lin⁻ (CD3⁻CD56⁻CD19⁻) fraction of G-CSF mobilized splenic cells from B6 mice, a CD11b^{int} CD34⁺ subset was identified. This CD11b^{int} CD34⁺ subset of cells could not be identified in non-mobilized spleen cells from B6 mice. All gates were based on isotype controls.

B. Freshly sorted Lin⁻CD34⁺CD11b^{int} cells had a homogeneous monocyte-like morphology and a monocyte cell surface phenotype (CD115⁺, CD11b⁺ Ly6C⁺, Ly6G⁻, F4/80⁻) expression pattern, with markers associated with myeloid progenitors including CX3CR1, CD16/32 and CD34 but lacking expression of co-stimulatory molecules CD80, CD86 or MHC Class II (red line). All gates were based on isotype controls (blue line).

C. B6 T cells activated by anti-CD3 and anti-CD28 mAbs were cultured alone (red) or with CD34⁺ Mono and T cells in a 1:1 ratio (dark blue), 5:1 ratio (green) and 10 :1 ratio (light blue). Percentage of divided T cells in control without CD34⁺ Mono = 97.33 ± 1.2% vs. 28.97 ± 4.9% at 1:1 ratio, p<0.001; vs. 80.1 ± 4.16% at a 5:1 ratio, p<0.01; vs. 92.33 ± 5.17% at a 10:1 ratio, p=0.39. Data were compared using Student's unpaired t-test (ns: not significant, **: p≤0.01, ***: p≤0.001).

D. B6 T cells activated by anti-CD3 and anti-CD28 mAbs were cultured alone (red) or with CD34⁺ CD11b⁻ (yellow) or with CD34⁻CD11b⁺ (purple) cells from G-CSF mobilized spleens in a 1:1 ratio. Percentage of divided T cells in control (n=3) = 97.33 ± 1.2% vs. 97.67 ± 1.6% with CD34⁺CD11b⁻ (n=3) and vs. 99 ± 0.57% with CD34⁻CD11b⁺ (n=3). Data were compared using Student's unpaired t-test (ns: not significant).

E. B6 T cells activated by mature DCs from BALB/c mice were cultured without (red) or with CD34⁺ Mono (blue). After 4 days, percentage of control without CD34⁺ Mono divided T cells 61.33 ± 1.85% (n=3) vs. 29.5 ± 3.9% in the presence of CD34⁺ Mono (n=3), p=0.002. Data were compared using Student's unpaired t-test (**: p≤0.01).

F. Splenic G-CSF mobilized Lin⁻CD34⁺CD11b⁺ monocytes were sorted to high purity and analyzed by hybridization to an Affymetrix microarray (n=3). Gene expression was compared to non-mobilized Lin⁻CD11b⁺CD115^{high}Ly6C⁻ monocytes (n=5) and, Lin⁻CD115^{high}Ly6C⁺ inflammatory monocytes from spleen (n=7), Lin⁻CD117⁺CD115⁺CD135⁻Ly6C⁺CD11b⁻ common monocyte progenitors from bone marrow (n=3), and Lin⁻CD11b⁺Ly6C⁺ monocytic MDSCs (n=3) sorted from bone marrow cells cultured in the presence of G-CSF+ GM-CSF + IL-13 as described elsewhere (13). For comparison, we included transcriptional profiling data available from Immgen for Granulocyte-Macrophage Precursors (GMP), Common Dendritic Precursors (CDP) and Macrophage-Dendritic Precursors (MDP). Transcriptional profiling is summarized as an affinity propagation matrix, where intensity of red color is inversely proportional to similarity of the transcriptional profiles.

G. Principal Component Analysis comparing the transcriptional profile of G-CSF mobilized CD34⁺ monocytes to other precursors and monocyte populations as described above.

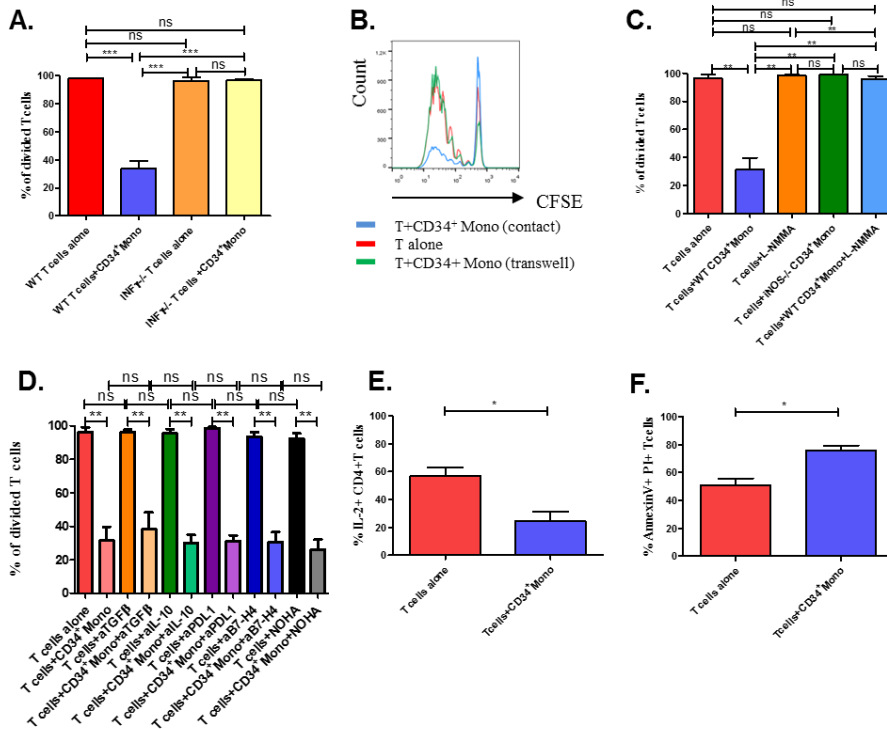


Fig. 4. CD34⁺ monocytes regulate allogeneic T cell activation through iNOS

A. T cells from WT B6 or IFN- γ ^{-/-} B6 mice were stimulated by anti-CD3 and anti-CD28 antibodies without (red and orange, respectively) or with CD34⁺ Mono (blue and yellow, respectively). After 4 days, T cell proliferation was analyzed by CFSE dilution. Percentage of divided T cells in controls without CD34⁺ Mono = 98 ± 1.34% (n=3) vs. 33.6 ± 5.88% with CD34⁺ Mono at a 1:1 T cell: CD34⁺ Mono ratio (n=3) (p=0.0004), and 96.4 ± 2.75% without (n=3) vs. 96.4 ± 0.9% with CD34⁺ Mono a 1:1 T cell: CD34⁺ Mono ratio (n=3) in the presence of IFN- γ ^{-/-} T cells (p=0.87). Data were compared using Student's unpaired t-test (ns: not significant, ***: p≤0.001). Results are representative of 4 independent experiments.

B. WT B6 T cells activated by anti-CD3 and anti-CD28 mAbs were cultured alone (red) or with CD34⁺ Mono in the same (blue) or in different compartments of Transwell plates (green). Similar results were obtained in 4 independent experiments.

C. WT B6 T cells were cultured for 4 days with anti-CD3 and anti-CD28 mAbs. Activated B6 T cells were cultured alone (red) or in the presence of 1:1 T cell: WT CD34⁺ Mono (dark blue) ratio or at the same ratio with iNOS^{-/-} CD34⁺ Mono (green). L-NMMA was added to activated T cells alone (orange) or to activated T cells cultured with WT CD34⁺ Mono (light blue). Proliferation was measured by CFSE dilution. Percentage of divided T cells in control T cells = 96.2 ± 3 % (n=3) vs. 31.57 ± 8.23% with WT CD34⁺ Mono at a 1:1 ratio (n=3) (p=0.0018); vs. 98.57 ± 0.41% in T cells with LNMMA (n=3)(p=0.48); vs. 98.87 ± 0.46% with iNOS^{-/-} CD34⁺ Mono (n=3) (p=0.43); vs. 95.6 ± 2.52%, with WT CD34⁺ Mono and L-NMMA (n=3) (p=0.88). Data were compared using Student's unpaired t-test (ns: not significant, **: p≤0.01). Similar results were obtained in 6 independent experiments.

D. WT B6 T cells were cultured for 4 days with anti-CD3 and anti-CD28 mAbs. Activated B6 T cells were cultured alone (dark red) or in the presence of WT CD34⁺ Mono at a 1:1 T cell: CD34⁺ Mono ratio (light red) with or without mentioned blocking antibodies. Percentage of divided T cells alone (96.2 ± 3 %, n=3) was reduced in the presence of WT CD34⁺ Mono (31.57 ± 8.23% (n=3)),

($p=0.0018$). The respective percentages of divided T cells alone or with WT CD34⁺ Mono (1:1 ratio) in the presence of neutralizing TGF- β antibody = $96.4 \pm 1.36\%$ ($n=3$) vs. $31.57 \pm 8.23\%$ ($n=3$), $p<0.001$, (dark and light orange, respectively); with neutralizing IL-10 antibody = $95.6 \pm 2.52\%$ ($n=3$) vs. $30.2 \pm 4.65\%$ ($n=3$), $p<0.001$, (dark and light green respectively); with blocking PD-L1 antibody = $98.77 \pm 0.66\%$ ($n=3$) vs. $31.37 \pm 3.27\%$ ($n=3$), $p<0.001$, (dark and light purple respectively); with blocking B7-H4 antibody = $93.6 \pm 2.75\%$ ($n=3$) vs. $30.67 \pm 5.92\%$ ($n=3$), $p<0.001$, (dark and light blue respectively); and with NOHA blocking arginase = $92.33 \pm 3.33\%$ ($n=3$) vs. $26 \pm 6.11\%$ ($n=3$), $p<0.001$, (black and grey respectively,). Data were compared using Student's unpaired t-test. (ns: not significant, ***: $p\leq 0.001$).

~~D~~-E. B6 T cells were cultured with anti-CD3 and anti-CD28 mAbs without (red) or with CD34⁺ Mono (blue) for 4 days. Percentage of IL-2⁺ CD4⁺ T cells in control without CD34⁺ Mono = $57 \pm 5.9\%$ ($n=3$) vs. $24.7 \pm 6.4\%$ with CD34⁺ Mono at a 1:1 ratio ($n=3$), ($p=0.02$). Data were compared using Student's unpaired t-test (*: $p\leq 0.05$).

~~E~~-F. B6 T cells were cultured with anti-CD3 and anti-CD28 mAbs without (red) or with CD34⁺ Mono (blue) for 4 days. Percentage of AnnexinV⁺PI⁺ apoptotic T cells in control without CD34⁺ Mono = $51.17 \pm 4.62\%$ ($n=3$) vs. $76.23 \pm 3.12\%$ with CD34⁺ Mono at a 1:1 ratio ($n=3$), ($p=0.01$). Data were compared using Student's unpaired t-test (*: $p\leq 0.05$). Results are representative of 3 independent experiments.

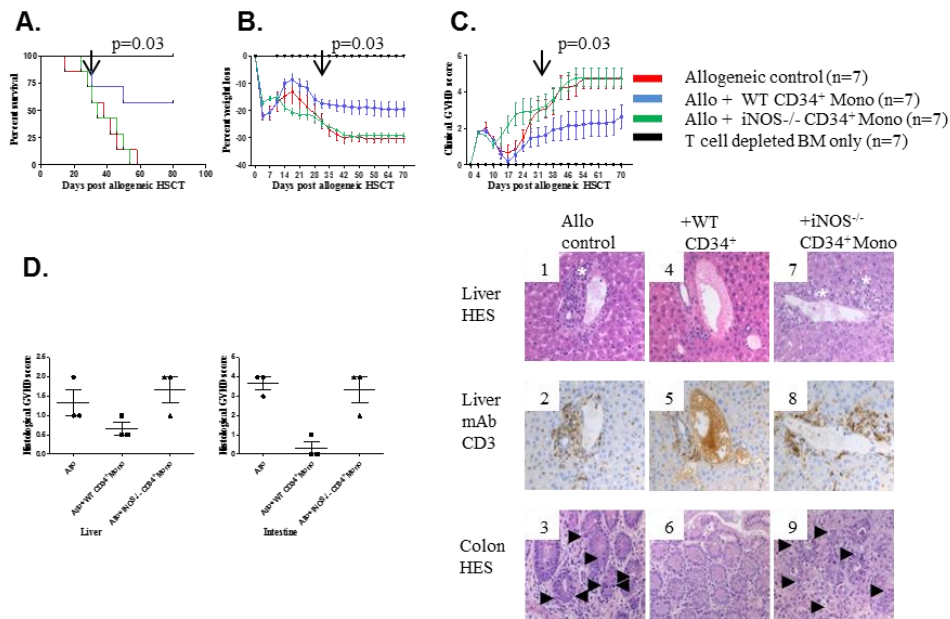


Fig. 5. CD34⁺ monocytes prevent GVHD by regulating allogeneic T cell activation without impairing proliferation

A. Lethally irradiated (6 Gy) BALB/cJ recipients received 1×10^7 B6 T cell depleted BM cells alone (black, n=7) or with 2×10^6 purified B6 CD25⁻ T cells (allogeneic control, red, n=7). Additional groups received CD25⁻ T cells with co-transfer of 0.5×10^6 B6-derived CD34⁺ Mono (green, n=7) or iNOS^{-/-} B6-derived CD34⁺ Mono (blue, n=7) on day 0. Median survival in mice receiving co-transfer of WT CD34⁺ Mono was not reached by day 80 vs. 38 days without WT CD34⁺ Mono (p=0.03) vs. 31 days in mice receiving co-transfer of iNOS^{-/-} CD34⁺ Mono (p=0.03). Data shown represent pooled results from 2 independent experiments. Results were compared with Kaplan-Meier survival curves.

B. Weight loss of BALB/cJ allo-HSCT recipients described above. Percent weight loss on day 70 post-HSCT: $23.1 \pm 2.57\%$ in mice co-transferred with CD34⁺ Mono (n=7) vs. $30.2 \pm 1.03\%$ in allogeneic control (n=7) p=0.009, vs. $29 \pm 0.75\%$, in mice co-transferred with iNOS^{-/-} CD34⁺ Mono (n=7) p=0.04. Data were compared using Student's unpaired t-test.

C. Clinical GVHD scores in recipient mice receiving allo-HSCT as described above. Clinical score on day 70 post-allogeneic HSCT was 2.64 ± 0.63 in mice with co-transfer of CD34⁺ Mono (n=7) vs. 4.71 ± 0.56 in allogeneic controls (n=7), (p=0.03) and 4.78 ± 0.52 in mice with co-transfer of iNOS^{-/-} CD34⁺ Mono (n=7) (p=0.002). Data were compared using Mann-Whitney's test.

D. Representative histological analyses of GVHD target organs in each group. Points correspond to histopathological scores of individual mice. 1-3. Control allo-HSCT mice receiving T cells demonstrated histopathological signs of intestinal GVHD mainly in the colon (3) (apoptotic epithelial cells in the glands or gland destruction with focal inflammatory infiltration) and the liver (1-2) (portal inflammation with diffuse or nodular infiltrate of CD3⁺ T cells, and endothelitis). 4.-6. Mice receiving allo-HSCT with T cells and WT CD34⁺ Mono did not have histopathological evidence of GVHD, either in the liver (4-5), or in the colon. 7-9. Allo-HSCT mice receiving T cells with iNOS^{-/-} CD34⁺ Mono had histopathological evidence of GVHD mainly in the colon (9). White stars: hepatocyte necrosis. Black arrows: epithelial necrosis.

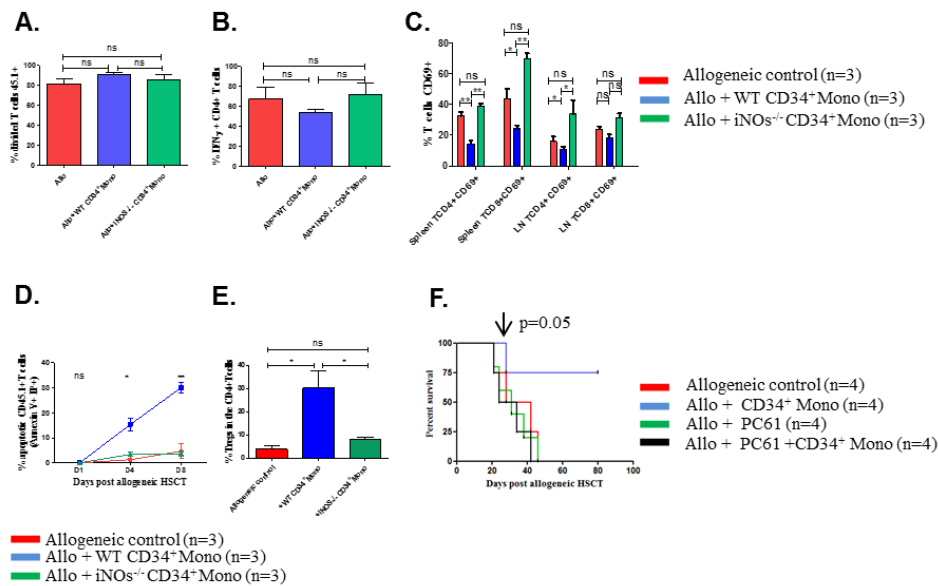


Fig. 6. CD34⁺ monocytes regulate allogeneic T cell activation by inducing their apoptosis and triggering the generation of Tregs

A. Lethally irradiated BALB/cJ mice were transplanted with 2×10^6 CFSE-labeled CD25-depleted B6 Ly5.1⁺ T cells alone or with 0.5×10^6 purified CD34⁺ Mono. On day 4, proliferation was analyzed by CFSE dilution in gated CD45.1⁺ T cells. Percentage of dividing cells was $90.33 \pm 2.9\%$ in mice with co-transfer of CD34⁺ Mono (n=3) vs. $81.77 \pm 5.2\%$, in allografted mice without CD34⁺ Mono (n=3) (p=0.22) and $85.67 \pm 4.9\%$, in mice with co-transfer of iNOS^{-/-} CD34⁺ Mono (n=3) (p=0.46). Data were compared using Student's unpaired t-test (ns: not significant).

B. IFN- γ intracellular staining was performed on day 8, on lymph node and splenic CD4 T cells of allografted mice with or without co-transfer of WT or iNOS^{-/-} CD34⁺ Mono. Percentage of T cells producing IFN- γ were $67.53 \pm 11.91\%$ in allografted controls without CD34⁺ Mono (n=3) vs. $53.57 \pm 3.58\%$ with co-transfer of WT CD34⁺ Mono (n=3), (p=0.32) vs. $71.7 \pm 11.74\%$ with co-transfer of iNOS^{-/-} CD34⁺ Mono (n=3) (p=0.81). Data were compared using Student's unpaired t-test (ns: not significant).

C. Expression of the common activation marker CD69 on splenic and lymph node CD4⁺ and CD8⁺ T cells in recipient mice. For splenic CD4⁺ T cells, the percentage that were CD69⁺ cells was $14.13 \pm 2.51\%$ in group receiving co-transfer of CD34⁺ Mono (n=3) vs. $30.6 \pm 2.44\%$ in the group without co-transfer of WT CD34⁺ Mono (n=3), (p=0.006), or vs. $38.9 \pm 1.9\%$, in the group with co-transfer of iNOS^{-/-} CD34⁺ Mono (n=3), (p=0.001). For splenic CD8⁺ T cells, the percentage of CD69⁺ cells was $23.7 \pm 2.17\%$ in group receiving co-transfer of CD34⁺ Mono vs. $43.6 \pm 6.64\%$ in the group without co-transfer of WT CD34⁺ Mono (p=0.04) or vs. $64.57 \pm 5.4\%$ in the group with co-transfer of iNOS^{-/-} CD34⁺ Mono (p=0.002). In lymph node CD4⁺ T cells, percentages of CD69⁺ T cells were: $10.99 \pm 1.62\%$ vs. $26 \pm 4.7\%$ (p=0.04) or vs. $33.83 \pm 8.99\%$ (p=0.07) respectively. In lymph node CD8⁺ T cells, percentages of CD69⁺ T cells were not statistically different between the different groups. Data were compared using Student's unpaired t-test (ns: not significant, *: p<0.05, **: p<0.01).

D. Annexin V⁺ PI⁺ apoptotic splenic donor-derived CD3⁺ T cells on days +4 and +8 following allo-HSCT in mice as described above. On day +4, the percentages of apoptotic CD45.1⁺ T cells were $15.42 \pm 2.53\%$ in mice receiving co-transfer of CD34⁺ Mono (n=3) vs. $1.39 \pm 0.45\%$ in allogeneic

controls (n=3), (p=0.005) vs. $3.37 \pm 0.93\%$ in mice with co-transfer of iNOS^{-/-} CD34⁺ Mono (n=3), (p=0.01). On day +8 post-HSCT, the percentages of apoptotic CD45.1 T cells were respectively $30.14 \pm 2.01\%$ vs. $4.7 \pm 3.01\%$ (p=0.002) and vs. $3.72 \pm 1.1\%$, (p=0.0003). Data were compared using Student's unpaired t-test (ns: not significant, *: p≤0.05, **: p≤0.01).

E. Percentage of donor-derived CD45.1⁺ CD4⁺ Foxp3⁺ splenic T cells in mice receiving allo-HSCT as described above on days +8 and +15 post-HSCT. On day +15, the percentage of splenic Foxp3⁺ T cells in the CD4⁺ T cell fraction was $30.37 \pm 7.23\%$ in mice with co-transfer of CD34⁺ Mono (n=3) vs. $4.1 \pm 1.37\%$ in allogeneic controls (n=3) (p<0.05) vs. $8.3 \pm 1.04\%$ in mice with co-transfer of iNOS^{-/-} CD34⁺ Mono (n=3) (p<0.05). Data were compared using Student's unpaired t-test (ns: not significant, *: p≤0.05).

F. Lethally irradiated 8 BALB/cJ recipients were given 1×10^7 B6 T cell depleted BM cells plus 2×10^6 purified CD25-depleted T cells injected by intra-peritoneal injection together with subcutaneous administration of the anti-CD25-depleting mAb PC61 in red (n=4) or the IgG isotype control mAb in green (n=4) or PC61 antibody plus 0.5×10^6 CD34⁺ Mono (black) (n=4) or IgG isotype antibody plus 0.5×10^6 CD34⁺ Mono (blue) (n=4). Median survival of mice receiving allo-HSCT with co-transfer of WT CD34⁺ Mono without Treg depletion was not reached on day +80 vs. 35 days in allogeneic controls without CD34⁺ Mono with IgG control vs. 31 days with PC61 treatment (p=0.05) vs. 29 days in allo-HSCT recipients with co-transfer of CD34⁺ Mono and also receiving PC61 (p=0.05). Results were compared with Kaplan-Meier survival curves.

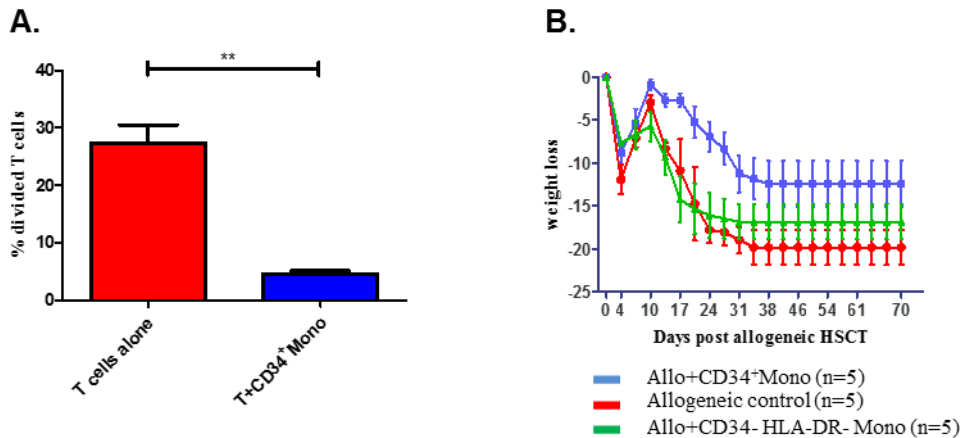


Fig. S1. Human CD34⁺ monocytes inhibit allogeneic T cell proliferation in vitro and reduce weight loss in the NSG xenogeneic HSCT model

A. A mixed lymphocyte reaction was performed by culturing purified CFSE labeled human T cells with allogeneic human monocyte-derived dendritic cells stimulators (5:1 ratio) with or without human CD34⁺ Mono from the same donor as T cells (1:1 ratio DC:T cell ratio). After 4 days, T cell proliferation was analyzed by CFSE dilution. Percentage of divided T cells in control without CD34⁺ Mono was $27.37 \pm 3.22\%$ (n=3) vs. $4.61 \pm 0.5\%$ with CD34⁺ Mono at a 1:1 T cell:CD34⁺ Mono ratio (n=3) ($p < 0.001$). Data were compared using Student's unpaired t-test (***: $p \leq 0.001$). Results are representative of 5 independent experiments.

B. Lethally irradiated NSG mice received 2.5×10^6 human PBMC with or without 1×10^5 G-CSF mobilized human CD34⁺ or HLA-DR⁻CD34⁻ monocytes. Percent weight loss on day 70 post-HSCT was $19.85 \pm 2\%$ in xenografted mice without CD34⁺ Mono (red, n=5), vs. $16.86 \pm 2.9\%$ in mice co-transplanted with HLA-DR⁻CD34⁻ Mono (green, n=5) ($p = 0.33$) vs. $12.46 \pm 2.64\%$ in mice co-transplanted with CD34⁺ Mono (blue, n=5) ($p = 0.05$). Results are pooled from 2 independent experiments. Each experiment was performed with PBSC from one donor for all recipients. Data were compared using Student's unpaired t-test.

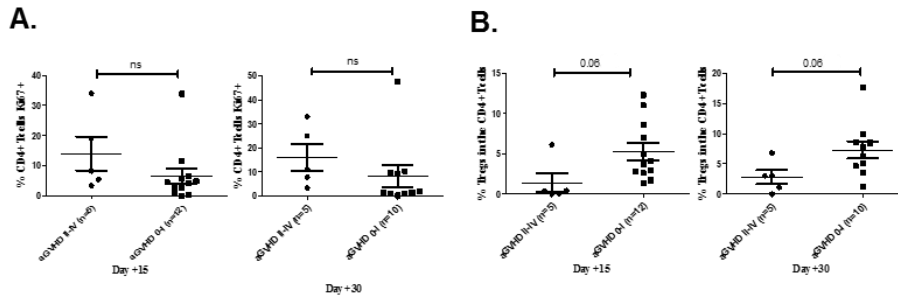


Fig. S2. Post-transplant peripheral blood levels of proliferating CD4⁺ T cells and Tregs in patients who developed grade II-IV acute GVHD and those who did not.

A. Proportions of proliferating conventional CD4⁺ T cells in peripheral blood on days 15 and 30 following allo-HSCT according to the incidence of severe aGVHD. On day 15 post HSCT, the percentage of CD4⁺Ki-67⁺ T cells was 14 ± 5.69% in the aGVHD (II-IV) group (n=5) vs. 6.47 ± 2.65% in the aGVHD (0-I) group (n=12) (p=0.18) and on day 30 was 16.03 ± 5.58% vs 8.42 ± 4.52% in the respective groups (p=0.33). Data were compared using Student's unpaired t-test (ns: not significant).

B. Proportions of post-transplant CD4⁺CD25^{high}CD127^{low} peripheral Treg on days 15 and 30 following allo-HSCT according to the incidence of acute GVHD. On day +15, the percentage of CD4⁺CD25^{high}CD127^{low} T cells was 1.39 ± 1.89% in the aGVHD (II-IV) group (n=5) vs 5.25 ± 1.06% in the aGVHD (0-I) group (n=12) (p=0.06) and on day 30 was 2.8 ± 1.16% (n=5) vs. 7.3 ± 1.42% (n=10) in the respective groups (p=0.06). Data were compared using Student's unpaired t-test (ns: not significant).

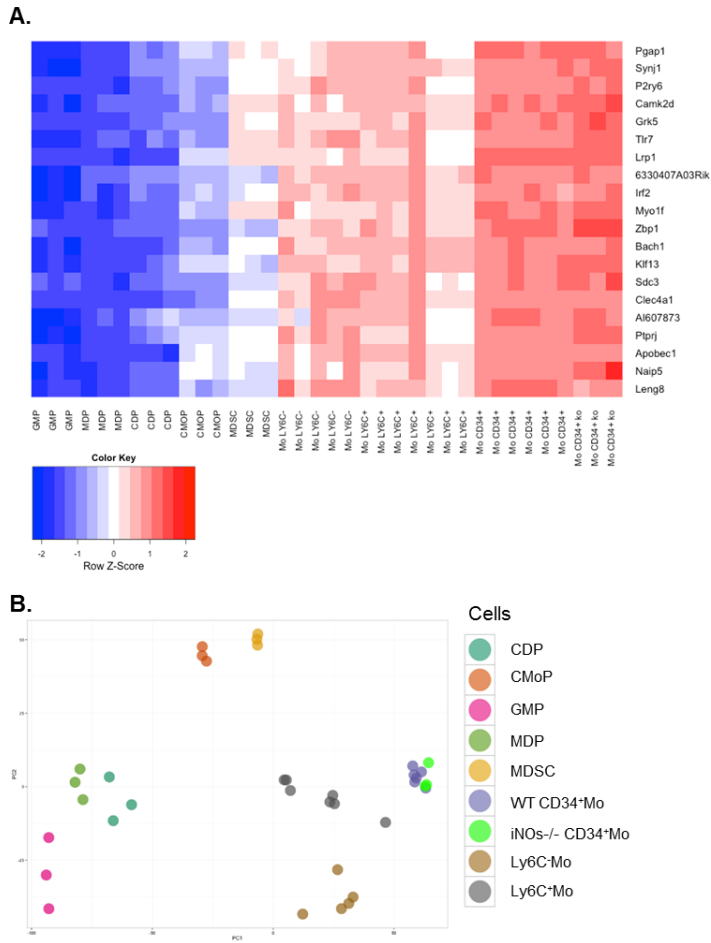


Fig. S3. The transcriptional profile of iNOS^{-/-} CD34⁺ monocytes is very similar to their wild type counterparts.

G-CSF mobilized Lin⁻CD34⁺CD11b⁺ monocytes from wt B6 or iNOS deficient B6 mice were flow sorted to high purity and analyzed by hybridization to an Affymetrix microarray (n=3 in each group).

A. Transcriptional profiling is summarized as a Principal Component Analysis comparing CD34⁺ monocyte populations from WT or iNOS deficient mice to other mature or precursor cells within the mononuclear phagocyte system.

B. Relative expression of genes that define Principal Component 1 in the above analysis are displayed as a heat map.

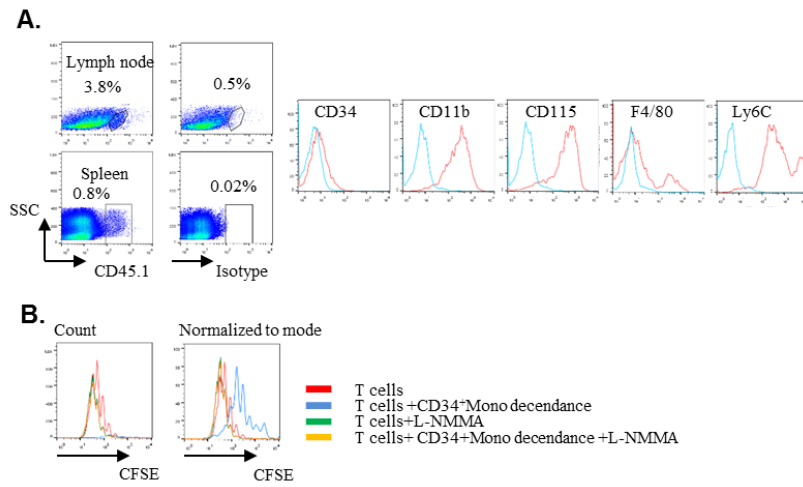


Fig. S4. CD34⁺ monocytes patrolling in the lymph nodes and spleen 15 days after *in vivo* injection have kept the same phenotype and function as input cells

A. Purified CD34⁺ monocytes expressing the CD45.1 congenic marker were administrated *in vivo* to WT B6 CD45.2 mice. Fifteen days after adoptive transfer, CD45.1⁺ cells detected in lymph nodes and spleens expressed low levels of CD34 and F4/80 but were still CD11b⁺ CD115⁺ and Ly6C⁺ (red line). All gates were based on isotype controls (blue line).

B. Purified splenic CD45.1⁺ cells sorted on day 15 after *in vivo* transfer inhibited the proliferation of T cells by anti-CD3 and anti-CD28 via a NO-dependent mechanism.

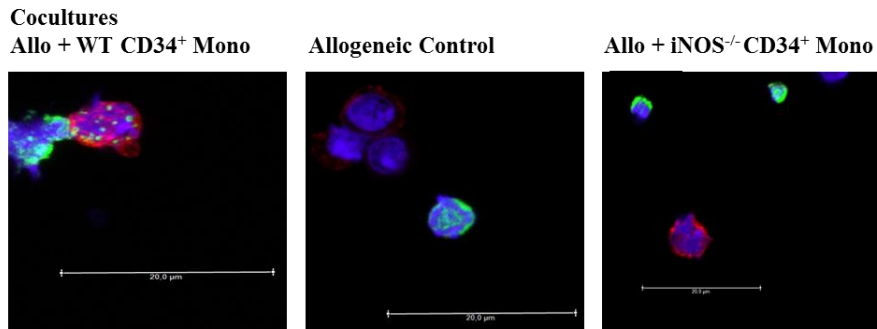


Fig. S5. Apoptotic T cells are engulfed by splenic macrophages in allo-SCT recipient mice that received CD34⁺ monocytes

Splenic allogeneic CD45.1⁺ CD3⁺ T cells (green) were sorted on day 15 post-HSCT and co-cultured for 24 hours with CD45.1⁺ CD11b⁺ F4/80⁺ macrophages (red) sorted from the same spleen. Allogeneic T cells were engulfed by macrophages derived from BMT-recipient mice allografted with WT CD34⁺ Mono (CD45.2) but not by macrophages from allogeneic control recipients, nor from Allo+iNOS^{-/-} CD34⁺ Mono recipients. Results from one representative experiment of 3 performed are shown.

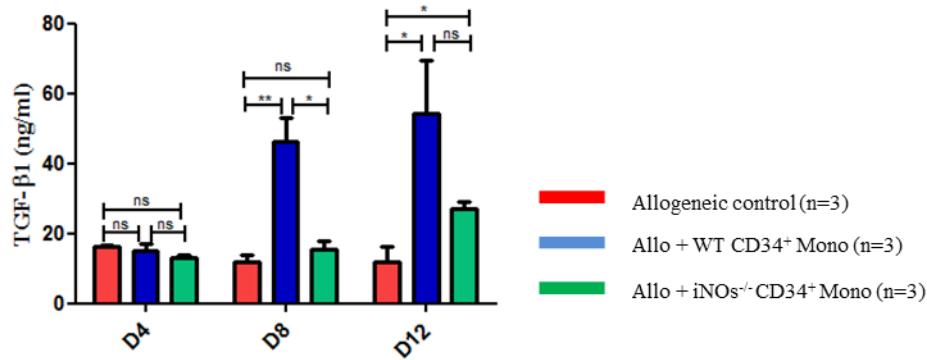


Fig. S6. Mice receiving allo-SCT with CD34⁺ monocytes have increased serum TGF- β 1 levels.

Serum TGF- β 1 levels were increased in mice receiving allo-SCT with WT CD34⁺ Mono (n=3) on day 8 post-HSCT as compared to allo-SCT control mice not receiving CD34⁺ Mono (n=3) or receiving iNOS^{-/-} CD34⁺ Mono (n=3) (day 8 mean TGF- β 1 serum levels were 46.28 ± 6.7 ng/mL vs. 11.75 ± 2.1 ng/mL, $p=0.008$ and vs. 15.28 ± 2.55 ng/mL, $p=0.012$, respectively). Day 15 mean TGF- β 1 serum levels were 54.28 ± 15.28 ng/mL vs. 12.04 ± 4.09 ng/mL, $p=0.05$ and vs. 27.2 ± 1.8 ng/mL, $p=0.15$, respectively). Data were compared using Student's unpaired t-test (ns: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$).

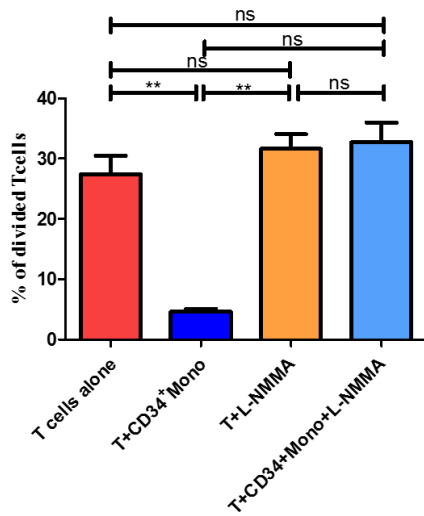


Fig. S7. Human CD34⁺ monocytes inhibit allogeneic T cell proliferation in a MLR through iNOS

Mixed lymphocyte reactions were performed by culturing purified CFSE-labeled human T cells with allogeneic human monocyte-derived dendritic cells stimulators (5:1 ratio) with or without human CD34⁺ Mono from the same donor as T cells (1:1 CD34⁺ Mono:T cell ratio) in the presence or absence of L-NMMA. After 4 days, T cell proliferation was analyzed by CFSE dilution. Percentage of divided T cells in control alloactivated T cells without CD34⁺ Mono = 27.37 ± 3.22% (n=3) vs. 4.6 ± 0.49% with CD34⁺ Mono (n=3), (p=0.002) vs. 31.63 ± 2.43% with L-NMMA (n=3), (p=0.61) ; vs. 32.7 ± 3.8% in the presence of CD34⁺ Mono and L-NMMA (n=3), (p=0.8). Data were compared using Student's unpaired t-test (ns: not significant, **: p≤0.01). Results are representative of 10 independent experiments.

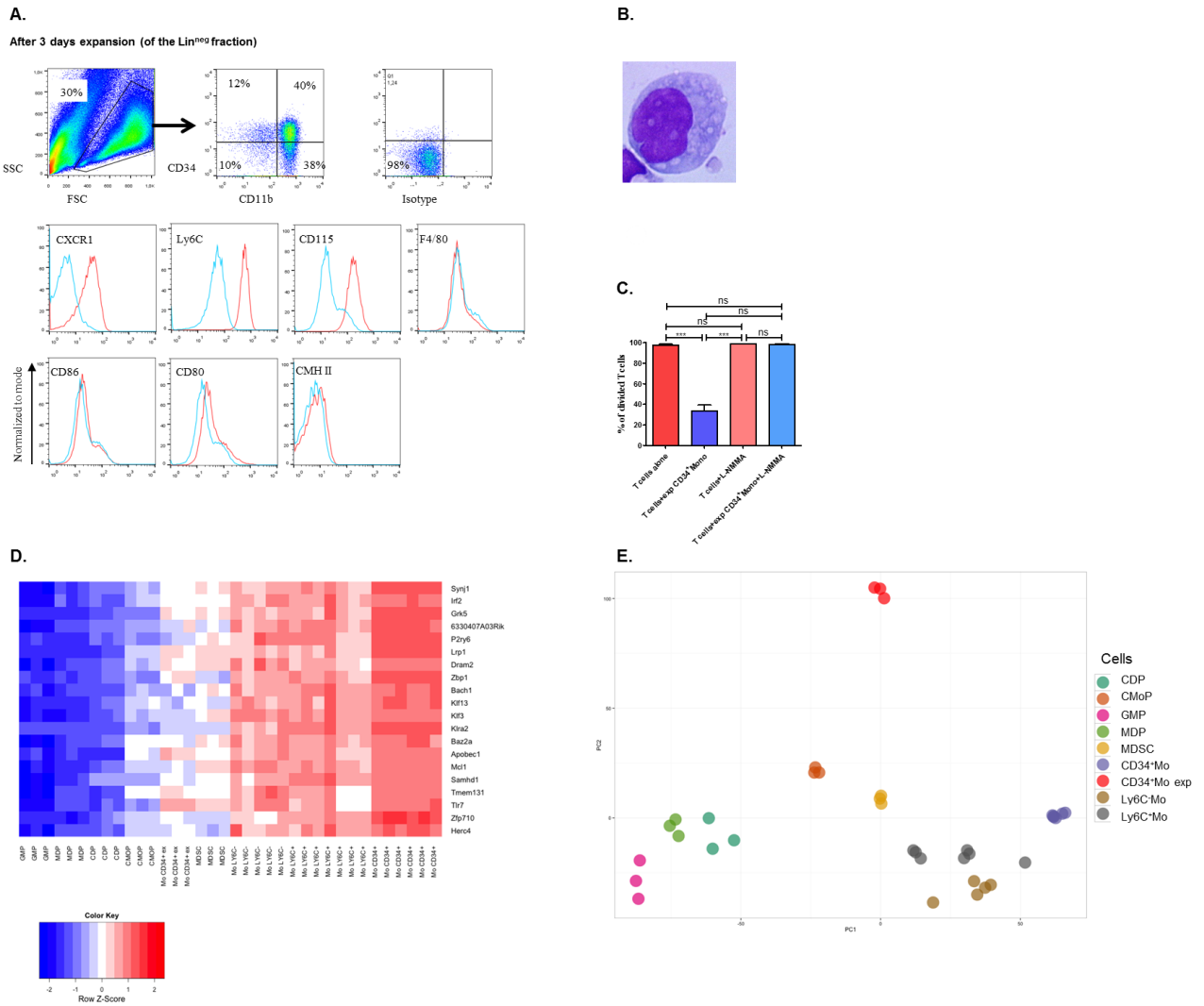


Fig. S8. The functions and phenotype of *ex vivo* expanded CD34⁺ cells are similar to that of freshly sorted cells

A. FACS sorted and expanded Lin⁻CD34⁺CD11b⁺ cells had a monocyte cell surface phenotype (CD115⁺, CD11b⁺ Ly6C⁺, F4/80⁻) with markers associated with myeloid-lineage progenitors including CX3CR1 and CD34, but lacked expression of co-stimulatory molecules CD80, CD86 or MHC Class II (red line). All gates were based on isotype controls (blue line).

B. Expanded Lin⁻CD34⁺CD11b⁺ cells had monocyte morphological features.

C. T cells from B6 were stimulated with anti-CD3 and anti-CD28 antibodies with or without L-NMMA (light red and red, respectively) or with expanded CD34⁺ Mono (blue and light blue, respectively). After 4 days, T cell proliferation was analyzed by CFSE dilution. Percentage of divided T cells in controls without CD34⁺ Mono = 97.3 ± 1.2% (n=3) vs. 33.6 ± 5.7% with CD34⁺ Mono at a 1:1 T cell: CD34⁺ Mono ratio (n=3), (p≤0.001), and 98.7 ± 0.3% (n=3) vs. 98.33 ± 0.7% with CD34⁺ Mono at a 1:1 ratio (n=3) in the presence of L-NMMA. Data were compared using Student's unpaired t-test (ns: not significant, ***: p≤0.001)

D. Freshly G-CSF mobilized Lin⁻CD34⁺CD11b⁺ monocytes (Mono CD34+) or expanded (Mono CD34+ ex or exp) from WT B6 mice were flow sorted in to high purity and analyzed by hybridization to an Affymetrix microarray (n=3 in each group). Transcriptional profiling is summarized as a Principal Component Analysis comparing CD34⁺ monocyte populations before or after expansion to other mature or precursor cells within the mononuclear phagocyte system.

E. Relative expression of genes that define Principal Component 1 in the above analysis are displayed as a heat map.