

Human monocyte-derived suppressor cells control graft-versus-host disease by inducing regulatory forkhead box protein 3-positive CD8⁺ T lymphocytes

Nona Janikashvili, PhD,^a Malika Trad, PhD,^a Alexandrine Gautheron, MS,^a Maxime Samson, MD,^{a,b} Baptiste Lamarthée, MS,^a Francis Bonnefoy, PhD,^a Stéphanie Lemaire-Ewing, PhD,^c Marion Ciudad, BS,^a Khatuna Rekhviashvili, MS,^a Famky Seaphanh, MS,^a Béatrice Gaugler, PhD,^a Sylvain Perruche, PhD,^a Andrew Bateman, MD, PhD,^d Laurent Martin, MD, PhD,^{a,e} Sylvain Audia, MD, PhD,^{a,b} Philippe Saas, PhD,^{a,f} Nicolas Larmonier, PhD,^{g*} and Bernard Bonnotte, MD, PhD^{a,b*}
Besançon and Dijon, France, Southampton, United Kingdom, and Tucson, Ariz

Background: Adoptive transfer of immunosuppressive cells has emerged as a promising strategy for the treatment of immune-mediated disorders. However, only a limited number of such cells can be isolated from *in vivo* specimens. Therefore efficient *ex vivo* differentiation and expansion procedures are critically needed to produce a clinically relevant amount of these suppressive cells.

Objective: We sought to develop a novel, clinically relevant, and feasible approach to generate *ex vivo* a subpopulation of human suppressor cells of monocytic origin, referred to as human monocyte-derived suppressive cells (HuMoSCs), which can be used as an efficient therapeutic tool to treat inflammatory disorders.

Methods: HuMoSCs were generated from human monocytes cultured for 7 days with GM-CSF and IL-6. The immune-regulatory properties of HuMoSCs were investigated *in vitro* and *in vivo*. The therapeutic efficacy of HuMoSCs was evaluated by using a graft-versus-host disease (GvHD) model of humanized mice (NOD/SCID/IL-2R $\gamma_c^{-/-}$ [NSG] mice).

Results: CD33⁺ HuMoSCs are highly potent at inhibiting the proliferation and activation of autologous and allogeneic effector T lymphocytes *in vitro* and *in vivo*. The suppressive

activity of these cells depends on signal transducer and activator of transcription 3 activation. Of therapeutic relevance, HuMoSCs induce long-lasting memory forkhead box protein 3-positive CD8⁺ regulatory T lymphocytes and significantly reduce GvHD induced with human PBMCs in NSG mice. **Conclusion:** *Ex vivo*-generated HuMoSCs inhibit effector T lymphocytes, promote the expansion of immunosuppressive forkhead box protein 3-positive CD8⁺ regulatory T cells, and can be used as an efficient therapeutic tool to prevent GvHD. (J Allergy Clin Immunol 2015;■■■■:■■■■-■■■■.)

Key words: Human monocyte-derived suppressor cells, T lymphocytes, regulatory T cells, signal transducer and activator of transcription 3, graft-versus-host disease, inflammation

The induction and maintenance of immune tolerance represent major therapeutic goals in autoimmunity and transplantation. In allogeneic hematopoietic cell transplantation current strategies for controlling lethal graft-versus-host disease (GvHD) are based on administration of immunosuppressive drugs resulting in general immune suppression responsible for severe infections or patient relapse after drug withdrawal.¹ Therefore more targeted approaches are needed. In this context the administration of suppressive (regulatory) immune cells, such as regulatory T (Treg) cells, has been tested as a more refined strategy to inhibit donor T cells with limited adverse side effects.²⁻⁶ However, the implementation of immunosuppressive cells in clinical trials has been limited by their low frequency in human subjects.⁷⁻⁹ Many recent protocols have reported on the possibility of generating or clonally expanding Treg cells *in vitro*.¹⁰⁻¹⁸ However, several challenges have limited the clinical use of *ex vivo*-expanded Treg cells, including maintenance of their survival, regulatory function, alloantigen specificity, and appropriate homing properties in transplant recipients.¹⁹⁻²² In addition to conventional CD4⁺ Treg cells, immunosuppressive CD8⁺ T lymphocytes have been noted for their protective role against GvHD.^{17,23-25} This subset of suppressive T lymphocytes exhibits higher CD8 expression (CD8^{hi}) compared with their effector counterparts and coexpress CD25, CD103, and forkhead box protein 3 (FoxP3).²⁶⁻²⁸ Recent reports have indicated that CD8⁺ Treg cell induction negatively correlates with GvHD development in mice after allogeneic bone marrow transplantation.^{27,29} However, the possible therapeutic advantage of inducing CD8⁺ Treg cells has never been explored in patients undergoing transplantation. Similarly, although studies conducted in animals

From ^aINSERM UMR1098, University of Bourgogne Franche-Comté, EFS Bourgogne Franche-Comté, LabEX LipSTIC, ANR-11-LABX-0021, Besançon; ^bthe Department of Internal Medicine, University Hospital, Dijon; ^cINSERM UMR866, University of Bourgogne, Dijon; ^dCancer Sciences, University of Southampton; ^ethe Department of Pathology and Cytology, University Hospital, Dijon; ^fCHU Besançon, CIC-BT506, FHU INCREASE, Besançon; and ^gthe Department of Pediatrics, Steele Children's Research Center, Department of Immunobiology, BIOS Institute and Arizona Cancer Center, University of Arizona, Tucson.

*These authors share senior authorship.

Supported by the Conseil Régional de Bourgogne 2011-2012, the Agence Nationale de la Recherche (Labex LipSTIC, ANR-11-LABX-0021), and the Conseil Régional de Franche-Comté ("soutien au LabEX LipSTIC" to P.S.).

Disclosure of potential conflict of interest: N. Janikashvili has received research support from Conseil Régional de Franche-Comté and Conseil Régional de Bourgogne and has a patent pending with the University of Burgundy (FR1452286). S. Audia is employed by Amgen and has received payment for lectures from Amgen and GlaxoSmithKline. B. Bonnotte has a patent pending with the University of Burgundy (FR1452286). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 5, 2014; revised October 17, 2014; accepted for publication December 9, 2014.

Corresponding author: Nona Janikashvili, PhD, INSERM UMR 1098, Faculty of Medicine, University of Bourgogne Franche-Comté, 2 bd du mal de Lattre de Tassigny, 21079 Dijon, France. E-mail: Nona.Janikashvili@u-bourgogne.fr.

0091-6749/\$36.00

© 2015 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2014.12.1868>

Abbreviations used

FoxP3: Forkhead box protein 3
 GvHD: Graft-versus-host disease
 HuMoSC: Human monocyte-derived suppressive cell
 MCP-1: Monocyte chemoattractant protein 1
 MSC: Mesenchymal stem cell
 NSG mice: NOD/SCID/IL-2R $\gamma_c^{-/-}$ mice
 STAT: Signal transducer and activator of transcription
 Treg: Regulatory T

and transplant recipients have demonstrated that myeloid-derived suppressor cells can promote transplant tolerance,³⁰⁻³⁵ the therapeutic benefits of *ex vivo*-generated human myeloid suppressor cells have not been investigated.

We have developed an original approach to generate *ex vivo* large numbers of a subpopulation of human suppressor cells of monocytic origin, herein called human monocyte-derived suppressive cells (HuMoSCs). HuMoSCs inhibit the proliferation and activation of both autologous and allogeneic effector CD4⁺ and CD8⁺ T lymphocytes. HuMoSC suppressive function depends on signal transducer and activator of transcription (STAT) 3 signaling and is associated with deprivation of the essential amino acids tryptophan and cysteine. Importantly, using a clinically relevant GvHD model in NOD/SCID/IL-2R $\gamma_c^{-/-}$ (NSG) mice, we demonstrate for the first time that HuMoSCs reduce GvHD symptoms, resulting in prolonged animal survival. HuMoSC-mediated prevention of GvHD positively correlates with induction of memory FoxP3⁺CD8⁺ Treg cells.

METHODS**Generation of HuMoSCs**

Human study was approved by the Establishment Français du Sang (EFS: ENR-B1-051, Besançon, France), with informed consent in compliance with the Declaration of Helsinki. PBMCs were isolated from buffy coats of healthy donors by means of Ficoll density gradient centrifugation. Monocytes were purified from PBMCs by means of Percoll density gradient centrifugation. HuMoSCs were generated by incubating monocytes (5×10^5 cells/mL) in RPMI 1640 (BioWhittaker, Basel, Switzerland) supplemented with 10% FBS and recombinant human GM-CSF (10 ng/mL) and IL-6 (10 ng/mL; both from Miltenyi Biotec, Bergish Gladbach, Germany) for 7 days. The medium was replaced every 3 days.

Magnetic cell isolation and sorting

HuMoSCs, total T cells, naive T cells, and Treg cells were purified by means of magnetic cell sorting with human CD33⁺, Pan-T, naive CD4⁺ T, and CD4⁺CD25⁺ cell isolation kits, respectively, and an autoMACS Pro Separator, according to the manufacturer's instructions (Miltenyi Biotec). CD8⁺CD103⁺ cells were sorted from cocultures by using a FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ).

T-cell proliferation and suppression assays

Total T lymphocytes and CD4⁺CD25⁺ T cells were stained by using Cell Trace Violet (Invitrogen, Cergy Pontoise, France), according to the manufacturer's procedure. Labeled cells were cultured with anti-CD3/CD28-coated T-cell expander beads (Dynabeads; Invitrogen, Carlsbad, Calif) with or without HuMoSCs (T-cell/HuMoSC ratio = 4:1). T-cell division was detected after 4 days by using flow cytometry with an LSRII cytometer (BD Biosciences) and analyzed by using the ModFit software (version 3.0).

In some experiments Treg cell suppressor inspector beads (Miltenyi Biotec) were used for stimulation.

Morphologic analysis

Wright-Giemsa staining (Protocol Hema 3; Fisher Scientific, Kalamazoo, Mich) of CD33⁺ cell cytospin preparations was performed to assess HuMoSC morphology after purification of CD33⁺ cells. Observation, evaluation, and image acquisition were performed with a Carl Zeiss Axiovert 40 CFL inverted microscope (Fisher Scientific).

Antibodies and flow cytometric analysis

Flow cytometric analyses were performed, as previously reported,³⁶ by using mAbs against CD33, CD1a, CD11c, CD11b, CD14, CD16, CD40, CD80, CD86, HLA-DR, CD123, CD163, CD44, CD31, CD105, CCR5, CCR6, CCR7, CD3, CD20, CD56, CD4, CD8, CD103, CD62 ligand, IFN- γ , granzyme B, and perforin (eBioscience, Paris, France). Treg cells were stained with anti-CD4 (phycoerythrin-Cy5.5), anti-CD25 (phycoerythrin) and anti-Foxp3 (Alexa Fluor 488, Human Treg Flow Kit; BioLegend, San Diego, Calif). Cells were analyzed with an LSRII cytometer (BD Biosciences), and data analysis was performed with FlowJo software (version 5.7.2).

Inhibitors and proteins

Specific inhibitors were used to suppress/neutralize various immunosuppressive enzymes or pathways: nor-NOHA (inhibitor of arginase-1, 50 μ mol/L; Calbiochem, Bad Soden, Germany), L-NMMA (inhibitor of induced nitric oxide synthase, 1 mmol/L; Sigma-Aldrich, St Louis, Mo), FeTPPS (peroxynitrite catabolism accelerator, 50 μ mol/L, Calbiochem), NAC (inhibitor of ROS, 3 mmol/L, Sigma-Aldrich), 1-MT (inhibitor of indoleamine-2,3-dioxygenase, 500 μ mol/L, Sigma-Aldrich), tin protoporphyrin/SnPP (inhibitor of heme oxygenase-1, 50 μ mol/L; Frontier Scientific, Logan, Utah), NS398 (inhibitor of COX-2, 10 μ mol/L; Cayman Chemicals, Ann Arbor, Mich), APCP (antagonist of CD73, 100 μ mol/L, Sigma-Aldrich), and Stattic (STAT3 inhibitor, 10 μ mol/L, Calbiochem). Exogenous L-arginine (400 μ mol/L, Sigma-Aldrich), L-cysteine (100 μ mol/L, Sigma-Aldrich), and recombinant IL-10 (50 μ mol/L, Miltenyi Biotec) were also used. In other experiments β -mercaptoethanol (50 μ mol/L, Sigma-Aldrich) was added to the culture to prevent L-cysteine oxidation.

Cytokine assay

Concentrations of IFN- γ , TNF- α , IL-10, IL-6, IL-5, IL-4, IL-1 β , IL-12p70, CCL2 (monocyte chemoattractant protein 1 [MCP-1]), CCL20, granzyme B, soluble Fas ligand, and TGF- β 1 were determined in cell-culture supernatants by using Multiplex and ELISA kits, according to the manufacturer's procedures (eBioscience).

HPLC assay

JEOL Aminotac 500 (Tokyo, Japan), an automated amino acid analyzer, was used for quantification of all amino acids within cell supernatants. Data acquisition and calculations were made with JEOL Workstation software.

Xenogeneic model of GvHD

NSG mice were purchased from the Jackson Laboratory (Bar Harbor, Me) and housed in specific pathogen-free conditions. Mice were used at 8 to 12 weeks of age. Mice were injected intravenously with human PBMCs (20×10^6 per mouse) with or without autologous HuMoSCs (5×10^6 per mouse). The mix of PBMCs with HuMoSCs was made before injections.

Mice were scored every day for 60 days in a blind fashion for clinical signs of GvHD (weight loss, general appearance of the fur, and mobility). Incidence of GvHD, clinical score, weight, and survival were noted for each mouse. Mice were killed when the clinical end points were reached (>15% weight

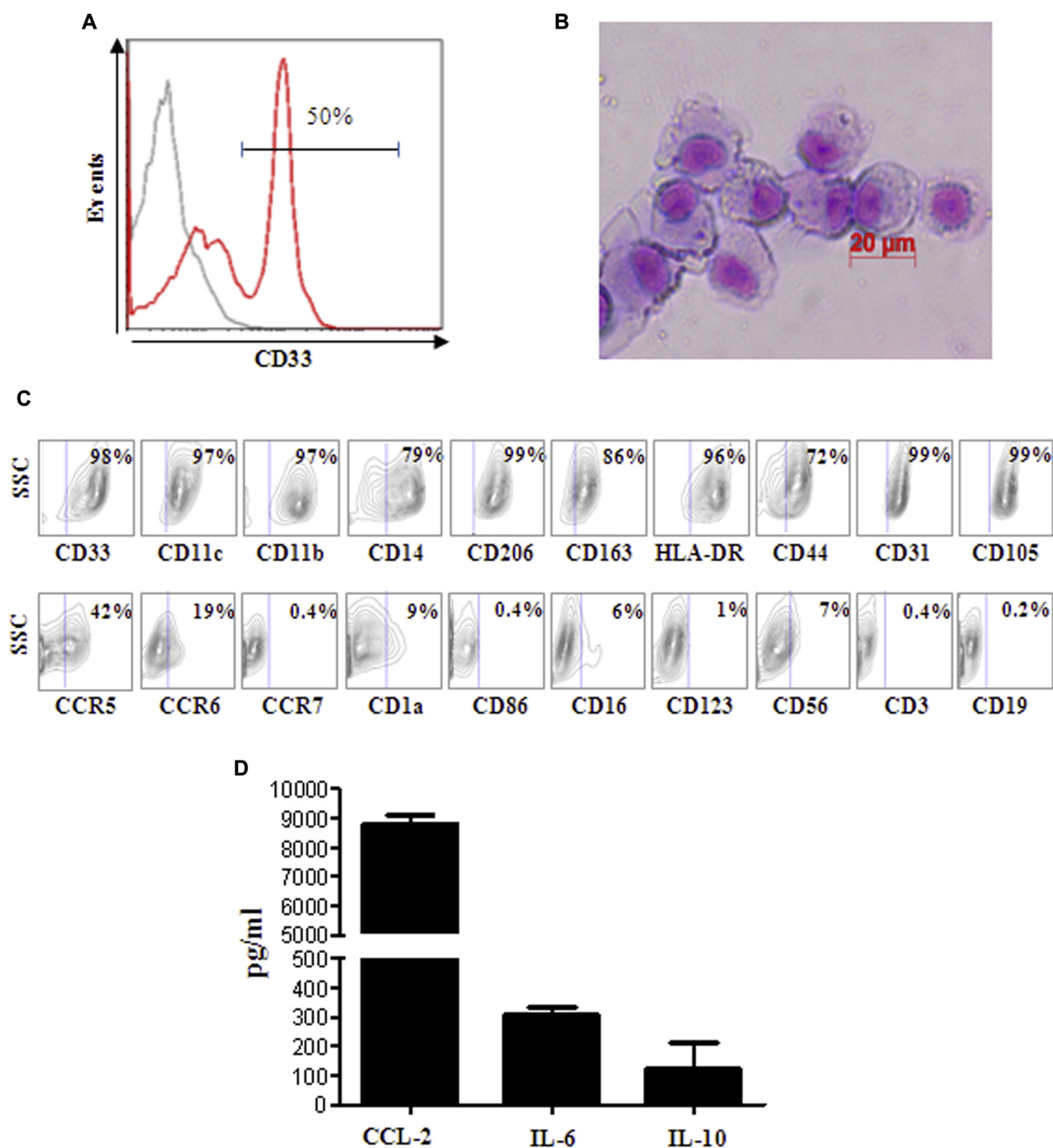


FIG 1. *Ex vivo*-generated HuMoSCs. Peripheral blood monocytes were purified from PBMCs and cultured in complete medium supplemented with recombinant human GM-CSF and IL-6 for 7 days. **A**, Percentage of CD33⁺ cells obtained after 7 days. CD33⁺ cells were then magnetically sorted at the end of the culture and analyzed. **B**, Morphologic analysis of HuMoSCs performed by using Wright-Giemsa staining of cytopsin preparations. **C**, After sorting of CD33⁺ cells, the phenotype of HuMoSCs was determined by means of flow cytometric analysis. Representative results of 10 independent experiments are shown. SSC, Side scatter. **D**, HuMoSC cell-culture medium (48 hours) was collected and analyzed by using Multiplex. Data are shown as means $\pm$ SDs of 3 representative experiments.

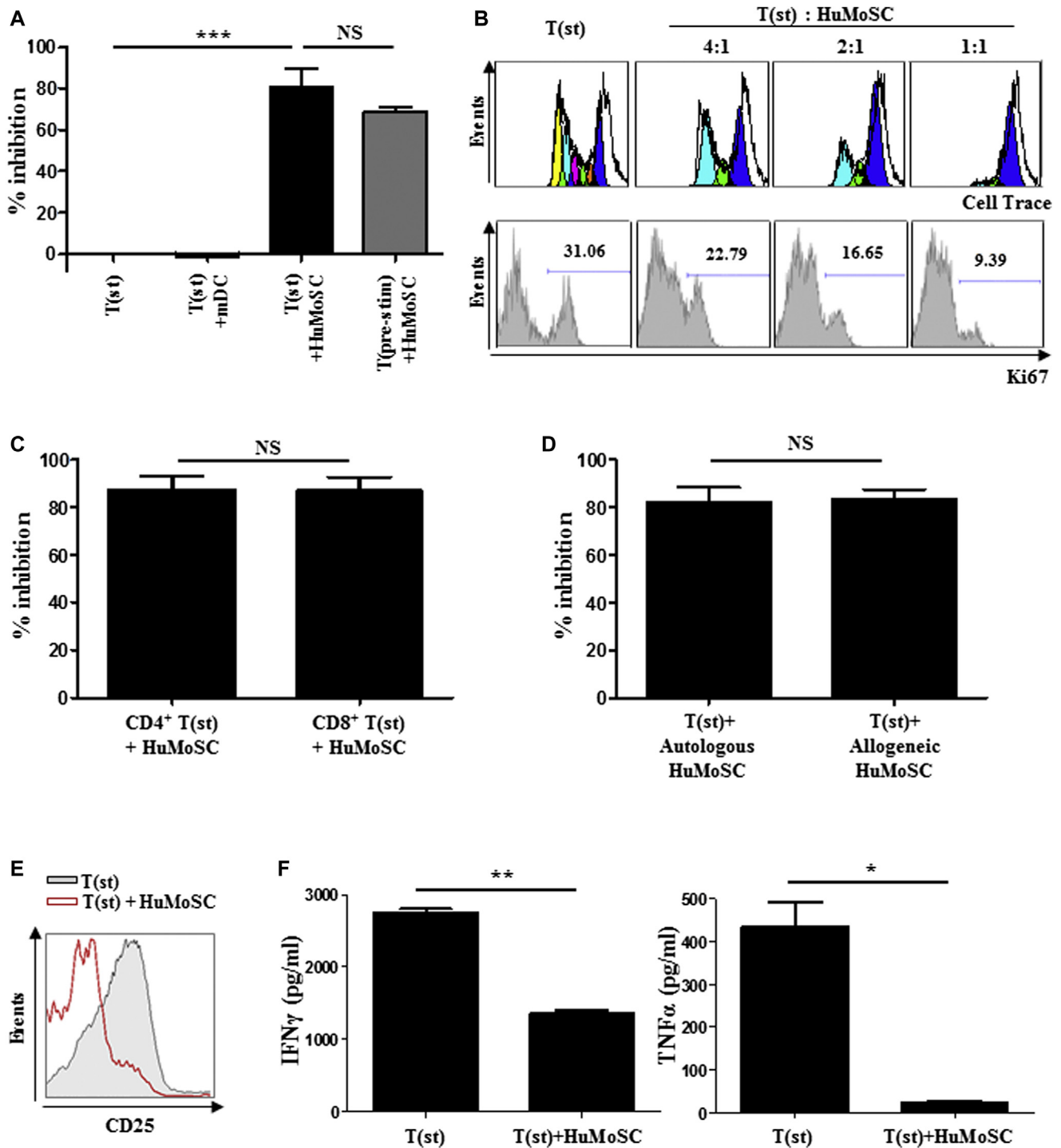


FIG 2. HuMoSCs suppress T-lymphocyte proliferation and activation. **A**, Cell Trace Violet-labeled T lymphocytes stimulated with anti-CD3/CD28 activation beads were cocultured with myeloid dendritic cells as controls or HuMoSCs from day 0 (*T[st]+HuMoSC*) or after 48 hours of stimulation (*T[pre-stim]+HuMoSC*). The T-cell proliferation index was determined after 5 days, and the percentage of T-cell proliferation inhibition was calculated. Results of 10 independent experiments are shown. **B**, Proliferation index (*upper panels*) and Ki67 expression in T cells (*lower panels*) cocultured for 5 days with HuMoSCs at different ratios. Representative results of 5 independent experiments are shown. **C**, Percentage of T-cell proliferation inhibition was determined separately for CD4⁺ and CD8⁺ T-cell populations. Results of 5 independent experiments are shown. **D**, Percentage of T-cell proliferation inhibition was determined separately for autologous or allogeneic T lymphocytes. Results of 5 independent experiments are shown. **E**, CD25 expression by T cells cocultured with HuMoSCs. **F**, IFN- γ and TNF- α concentrations were measured in the culture supernatant from stimulated T cells cocultured with HuMoSCs. Results of 5 independent experiments are shown. Data are shown as means $\pm$ SEMs of representative experiments. *P* values are the result of the Student *t* test: **P* < .05, ***P* < .01, and ****P* < .001. NS, Nonsignificant.

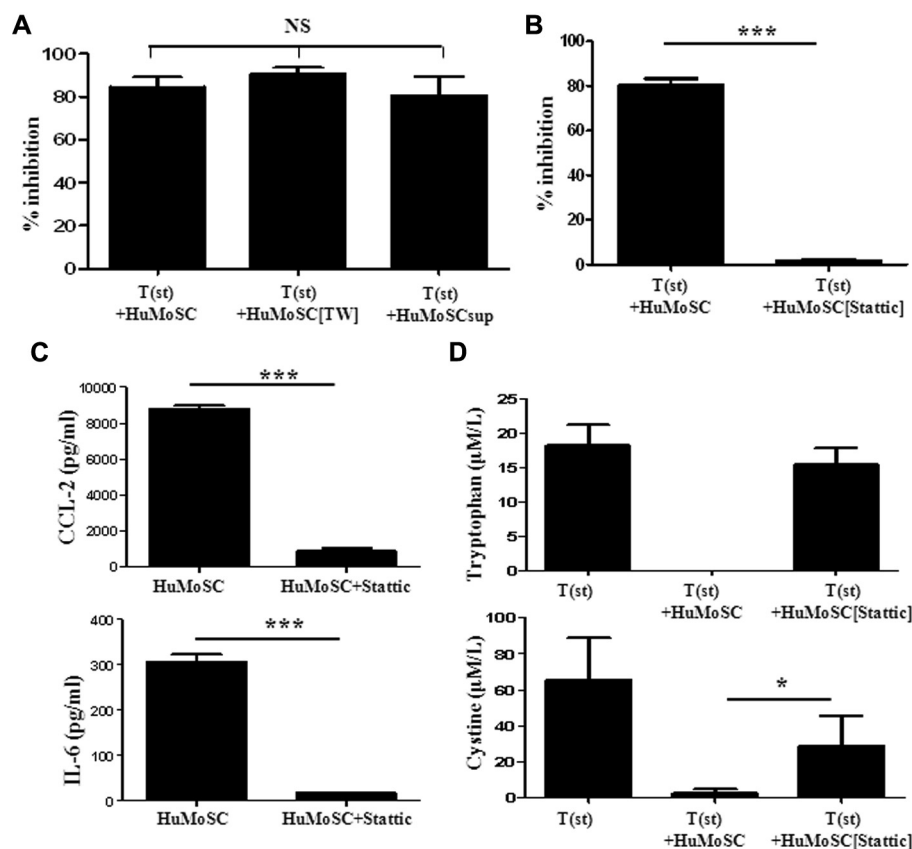


FIG 3. HuMoSC suppressive function depends on STAT3. **A**, HuMoSCs were separated from activated T lymphocytes by a microporous membrane (*HuMoSC[TW]*), and percentages of T-cell proliferation inhibition were compared with those of T cells cultured with HuMoSCs in direct contact. The culture medium from HuMoSCs was collected after 48 hours (*HuMoSCsup*) and added to stimulated T lymphocytes, and the T-cell proliferation index was compared with that of T cells cultured with HuMoSCs. Stimulated T cells cultured alone were used as positive controls for proliferation. Results of 5 independent experiments are shown. Data are presented as means $\pm$ SEMs. One-way ANOVA and the Dunnett test were used. **B**, HuMoSCs pretreated with Stattic for 24 hours were washed and added to activated T lymphocytes. Inhibition of T-cell proliferation was then analyzed. Results of 5 independent experiments are shown. **C**, Culture medium from HuMoSCs treated or not with Stattic was collected and analyzed by using Multiplex. Results of 5 independent experiments are shown. **D**, Concentrations of tryptophan and cysteine were determined in culture supernatants by using HPLC. Results of 3 independent experiments are shown. Data are presented as means $\pm$ SEMs of representative experiments. Student *t* test: **P* < .05 and ****P* < .001. NS, Nonsignificant.

loss, hunched posture, ruffled fur, reduced mobility, and tachypnea). Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Franche-Comté.

Immunophenotypic analysis of transplanted human cells

Blood samples were collected from NSG mice by means of cardiac puncture. Red blood cells were lysed (hemolytic buffer, BD Biosciences), and flow cytometric analysis was performed. Anti-human CD45 staining was used to detect the total count of human cells. Percentages of CD33-, CD3-, CD4-, CD8-, and FoxP3-expressing human cells were determined by using flow cytometry after staining with the appropriate antibodies.

Histologic analysis of GvHD lesions

Mouse livers were fixed in 3.7% formaldehyde immediately after collection and embedded in paraffin. Five-micrometer-thick sections were cut, stained with hematoxylin and eosin, and examined for immune cell infiltration and apoptotic hepatocytes. Trichrome labeling was used to stain collagen and identify fibrosis within hepatic tissues. Perls iron staining was performed to visualize iron overload associated with hemorrhagic lesions. For immunohistochemistry, anti-CD8 (Dako, Glostrup, Denmark) antibodies were

used. Staining was performed with a BenchMark Ultra instrument (Ventana Medical Systems, Roche Diagnostic, Mannheim, Germany). Diaminobenzidine and Ultra Red (Ventana Medical Systems) were used for staining visualization.

Statistical analyses

Analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, Calif). The Student *t* test or paired *t* test was used, where appropriate. One-way ANOVA and the Dunnett multiple comparison test were used when comparing more than 2 groups. Overall survival was calculated as the time from the day of injection to death. Data were censored after 60 days of follow-up. Kaplan-Meier curves were plotted, and differences were evaluated by using the log-rank test. For all statistical analyses, a 2-tailed *P* value of .05 or greater was considered significant.

RESULTS

Generation of HuMoSCs *ex vivo*

We have developed a novel procedure for producing *ex vivo* clinically relevant numbers of suppressor cells of monocytic

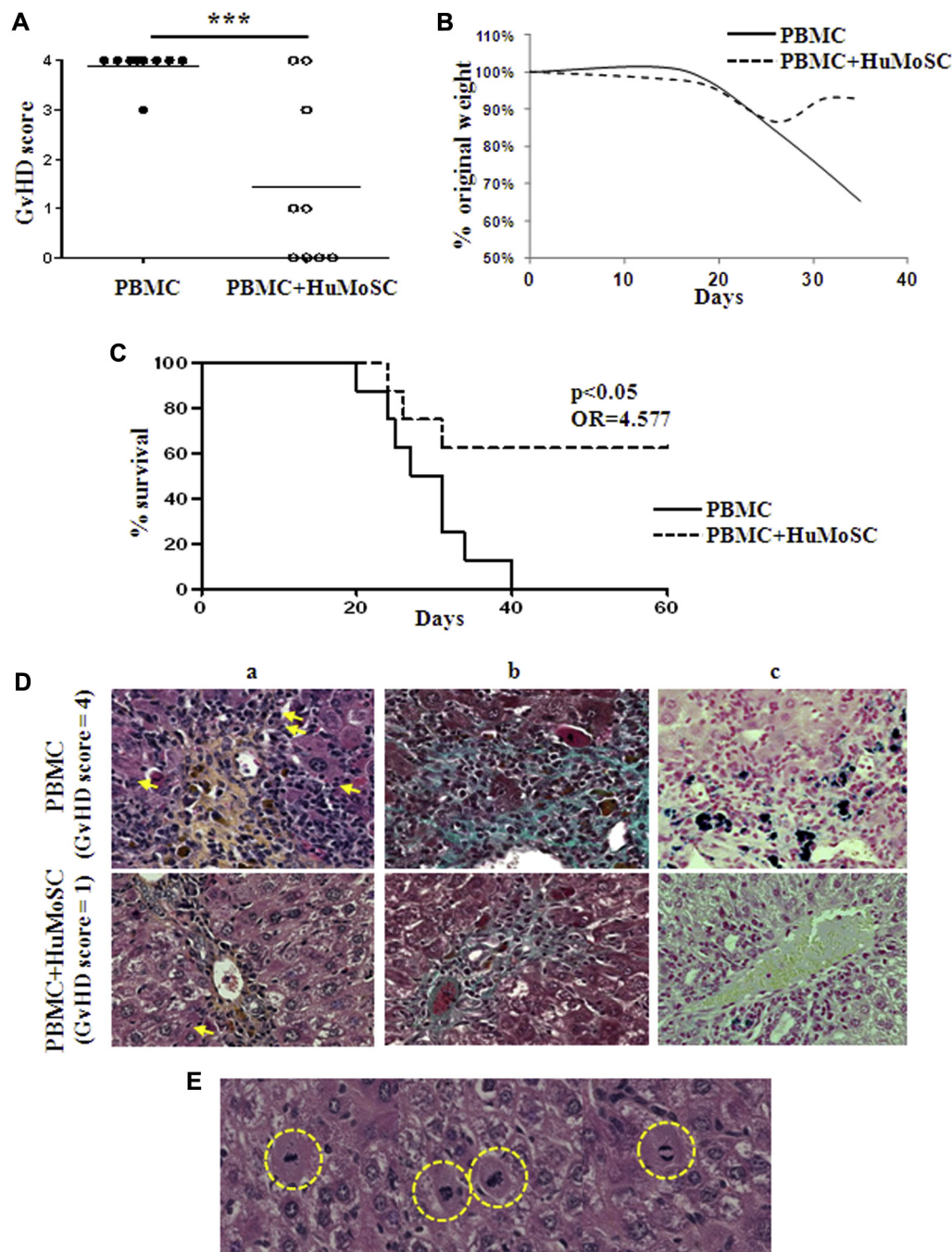


FIG 4. HuMoSCs protect mice from acute GVHD. Human PBMCs were adoptively transferred into NSG mice to induce acute GVHD. Two groups of mice were defined: one receiving PBMCs only and another receiving the same PBMCs mixed with HuMoSCs. **A**, Clinical disease score was graphed at day 50 after injection ($n = 17$). **B** and **C**, Mean percentage of weight variations from original weight was followed (Fig 4, **B**), and mouse survival was assessed (Fig 4, **C**). Data were censored after 60 days of follow-up. The survival curves were compared by using the log-rank test and odds ratio. Pooled data from 2 independent experiments with 8 mice in each group are shown. **D**, Liver histopathology around the portal tract ($\times 40$ magnification). **a**, Dark coloration is indicative of infiltration by human lymphocytes. Apoptotic bodies are indicated by yellow arrows. **b**, Green coloration developed by using trichrome labeling is associated with collagen and indicates fibrosis. **c**, Dark areas indicate hemorrhagic lesions visualized by iron overload. **E**, Regenerative hepatocytes undergoing mitosis are indicated by yellow circles. *** $P < .001$.

origin. Peripheral blood monocytes were purified from PBMCs and cultured in medium supplemented with recombinant human GM-CSF and IL-6 for 7 days. About 50% of the cells express the marker CD33 at the end of culture (Fig 1, **A**). CD33⁺ cells,

referred to as HuMoSCs, were then magnetically sorted and analyzed. These cells encompassed a homogeneous population characterized by large mononuclear cells with basophilic granular cytoplasm (Fig 1, **B**). Phenotypically, HuMoSCs exhibited

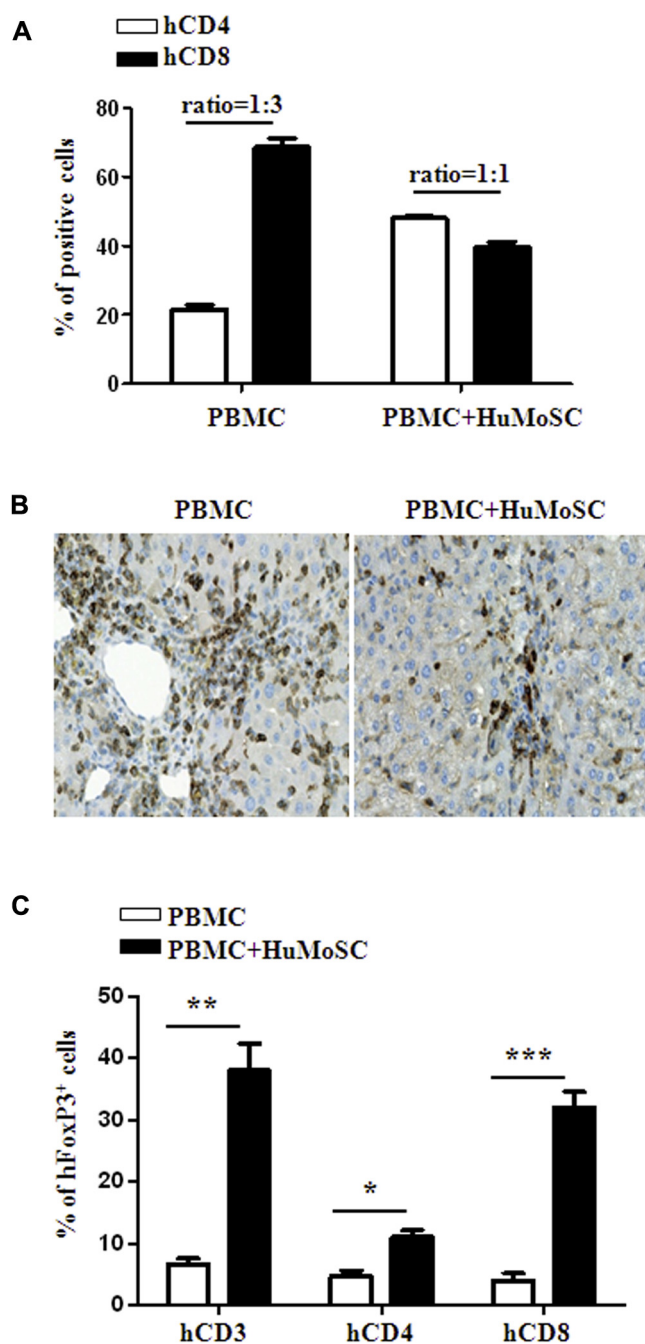


FIG 5. HuMoSCs induce expansion of FoxP3⁺ CD8⁺ T lymphocytes *in vivo*. At day 30 after human PBMC transfer, blood samples were collected by means of cardiac puncture from NSG mice administered PBMCs or PBMCs plus HuMoSCs. **A**, Percentages of CD4⁺ and CD8⁺ T lymphocytes were determined after gating on CD3⁺ cells. CD4/CD8 ratios were compared between groups. **B**, CD8⁺ T lymphocytes were detected in liver sections by using immunohistochemical staining. **C**, Percentages of FoxP3-expressing CD3⁺, CD4⁺, and CD8⁺ lymphocytes were determined after gating on CD3⁺ cells. Representative results of 8 mice are shown. Data are shown as means $\pm$ SEMs of representative experiments. Student *t* test: **P* < .05, ***P* < .01, and ****P* < .001.

features similar to those reported for immunosuppressive/regulatory CD33⁺CD11b⁺CD14⁺CD163⁺CD206⁺HLA-DR⁺ cells, with high expression of the adhesion molecules CD44, CD31, and CD105 (Fig 1, C). The chemokine receptor CCR5

was highly expressed by these cells, whereas CCR6 expression was marginal, and CCR7 was absent (Fig 1, C). HuMoSCs lack the expression of markers defining mature activated dendritic cells (CD1a, CD80, and CD86), granulocytes (CD16), natural killer cells (CD56), and lymphocytes (CD3 and CD19; Fig 1, C, and data not shown). Additionally, these cells produced CCL2 (MCP-1) and IL-6, moderate levels of IL-10 (Fig 1, D), and no IL-4, IL-5, IL-12p70, TNF- α , IL-1 β , CCL20, IFN- γ , granzyme B, soluble Fas ligand, or TGF- β (data not shown).

HuMoSCs suppress T-lymphocyte proliferation and activation

Functionally, HuMoSCs were capable of inhibiting the proliferation of responder T lymphocytes induced with anti-CD3/CD28-coated beads (Fig 2, A). HuMoSCs added from the beginning of the T-cell culture with activation beads or 48 hours later inhibited responder T-lymphocyte proliferation (Fig 2, A, and see Fig E1, A, in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). The efficacy of HuMoSC-mediated suppression of T-lymphocyte proliferation depended on the HuMoSC/T-cell ratio (Fig 2, B). Consistent with these results, HuMoSCs also reduced T-lymphocyte expression of the nuclear protein Ki67, a marker of cell proliferation (Fig 2, B).

Further analyses indicated that the proliferation of both CD4⁺ and CD8⁺ T lymphocytes was suppressed by HuMoSCs to a comparable extent (Fig 2, C). Furthermore, HuMoSCs were not only capable of suppressing autologous T-lymphocyte proliferative responses but also blocked allogeneic T-cell proliferation (Fig 2, D, and see Fig E1, B). Additionally, HuMoSCs reduced the expression of the activation marker CD25 (Fig 2, E) and impaired IFN- γ and TNF- α production by responder T lymphocytes (Fig 2, F). It is noteworthy that T cells did not lose their viability throughout culture with HuMoSCs (data not shown).

HuMoSC suppressive function depends on STAT3

Further analysis indicated that the suppressive activity of HuMoSCs did not depend on direct cell-cell contact but required a soluble factor or factors (Fig 3, A, and see Fig E2). Interestingly, HuMoSC inhibitory function was not affected by blockade of the most commonly reported mechanisms of immunosuppression, including arginase-1, inducible nitric oxide synthase, COX2, heme oxygenase11, indoleamine-2,3-dioxygenase, adenosine, or IL-10 (see Fig E3, A, in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). Of note, control T-cell proliferation was not modified by the tested inhibitors, thus excluding a possible direct cytotoxic or cytostatic effect of these molecules on responder T cells (see Fig E3, B). However, the transcription factor STAT3 was required for HuMoSC inhibitory function because pretreatment of HuMoSCs with a specific inhibitor of phosphorylated STAT3, Stattic, led to the abrogation of their suppressive activity (Fig 3, B) and impaired secretion of CCL2 and IL-6 (Fig 3, C) without affecting HuMoSC viability (data not shown). Interestingly, levels of the essential amino acids tryptophan and cysteine were significantly reduced by the presence of HuMoSCs in the coculture (Fig 3, D). Importantly, this effect was not observed when HuMoSCs were pretreated with Stattic (Fig 3, D). Therefore HuMoSC suppressive activity depends on STAT3 and is associated with the deprivation of tryptophan and cysteine.

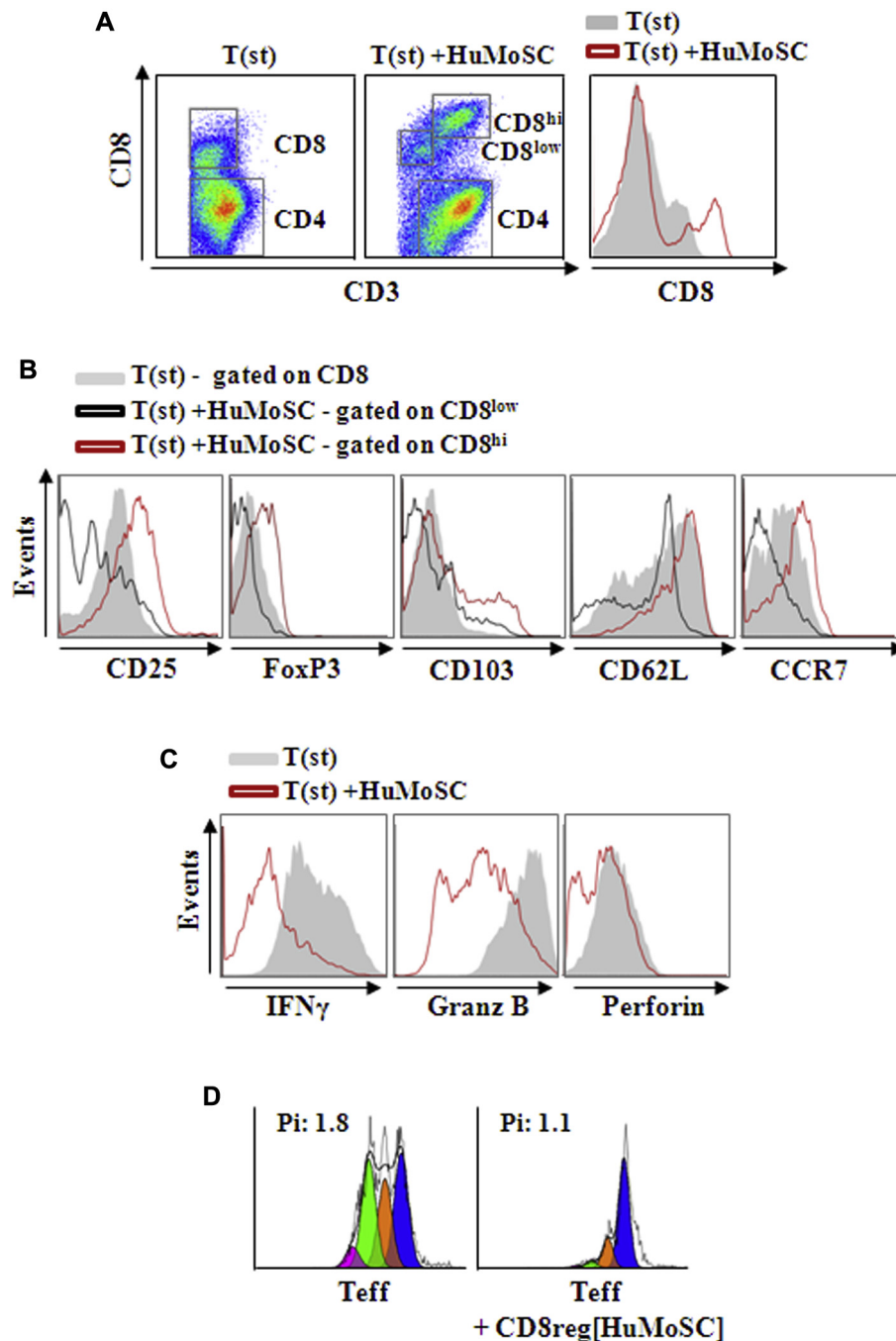


FIG 6. HuMoSCs promote FoxP3⁺CD8⁺ T-lymphocyte development *in vitro*. **A**, Stimulated total T lymphocytes were cocultured with HuMoSCs, and expression of CD8 was detected within CD3⁺ cells. **B**, Expression of CD25, FoxP3, CD103, CD62 ligand, and CCR7 was examined within the CD8^{hi} and CD8^{low} T-cell populations after gating on CD3⁺ cells. Representative results of 4 independent experiments are shown. **C**, IFN- γ , granzyme B, and perforin expression by T cells gated on the CD8⁺ population was evaluated. Representative results of 4 independent experiments are shown. **D**, Stimulated total T lymphocytes were cocultured with HuMoSCs, and CD8^{hi}CD103⁺ T cells were sorted and cocultured with Cell Trace Violet-labeled CD4⁺CD25⁻ (Teff) cells. The proliferation index (Pi) was determined within Cell Trace Violet-positive Teff cells. Representative results of 4 independent experiments are shown.

HuMoSCs prevent acute GvHD induced by human PBMCs in immunocompromised mice

The therapeutic potential of HuMoSCs was next explored by using a clinically relevant model of GvHD.³⁷⁻⁴⁰ Human PBMCs administered to NSG mice induce GvHD (weight loss, hunched

posture, ruffled fur, reduced mobility, skin integrity, and diarrhea).⁴¹ We demonstrated that the coadministration of HuMoSCs with PBMCs efficiently prevented the development of GvHD in NSG mice (Fig 4, A and B), resulting in significantly improved long-term survival (odds ratio, 4.577; 95% CI, 1.3-16.4;

$P = .0196$; Fig 4, C). In addition to clinical symptoms and outcome, histologic analysis of GvHD severity in the liver demonstrated that HuMoSCs significantly decreased immune cell infiltration and reduced the numbers of apoptotic bodies around the portal tract, as identified by using hematoxylin and eosin staining (Fig 4, D, a). Trichrome labeling of collagen further emphasized the protective effect of HuMoSC treatment on liver fibrosis (Fig 4, D, b). In addition, HuMoSC-treated mice were protected from hemorrhagic lesions in the liver, as indicated by Perls iron staining (Fig 4, D, c). Interestingly, the same liver sections contained regenerative hepatocytes that were not present in the PBMC control group (Fig 4, E). Therefore these results indicate that HuMoSCs are potent therapeutic tools to relieve symptoms and promote overall survival in a clinically relevant mouse model of human PBMC-induced GvHD.

HuMoSCs induce expansion of immunosuppressive FoxP3⁺CD8⁺ T lymphocytes

Treg cells represent a major population of suppressive cells capable of promoting alloantigen-specific tolerance.^{42,43} CD8⁺ Treg cells have been reported recently as a new regulatory cell population capable of controlling GvHD in mouse^{27,29} and human acute GvHD models.¹⁷ Our current results indicate that HuMoSC administration promotes the development of a CD8⁺ T-lymphocyte subset expressing FoxP3. In NSG mice administered human PBMCs, a significant decrease in the CD4/CD8 ratio was observed within human circulating CD3⁺ T lymphocytes (CD4/CD8 ratio = 1:3; Fig 5, A, and see Fig E4, A, in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). Consistently, CD8⁺ T cells were detected in higher numbers within liver-infiltrating lymphocytes in untreated mice compared with those seen in HuMoSC-treated mice (Fig 5, B). It is worth noting that CD33⁺ cells were not detectable in the circulation at day 30 (data not shown). Importantly, HuMoSC coadministration resulted in a substantial increase in the frequency of CD3⁺ T cells expressing the transcription factor FoxP3 and particularly of FoxP3-expressing CD8⁺ T lymphocytes (Fig 5, C, and see Fig E4, B). A less significant increase in the number of FoxP3-expressing CD4⁺ T cells was detected (Fig 5, C, and see Fig E4, B).

The ability of HuMoSCs to promote FoxP3⁺CD8⁺ T-lymphocyte development *in vitro* was next assessed. Total T lymphocytes exposed to HuMoSCs *in vitro* were analyzed for the expression of CD8⁺ regulatory cell markers, including CD25, FoxP3, and CD103 (integrin $\alpha_E\beta_7$). After gating on CD3⁺ cells, 2 subpopulations of CD8⁺ T cells were detected in total T-lymphocyte/HuMoSC cocultures, which were distinguished based on CD8 expression levels: CD8^{hi} and CD8^{low} (Fig 6, A). CD8^{hi}CD3⁺ T lymphocytes were induced by HuMoSCs and were not detected in cultures of lymphocytes performed in the absence of HuMoSCs. After gating on CD3⁺ T cells, CD8^{hi} and CD8^{low} cells were then gated separately and analyzed further. CD8^{hi} T cells expressed high levels of CD25, FoxP3, and CD103 compared with CD8^{low} cells from the same samples (Fig 6, B). Expression of the homing marker CD62 ligand and the chemokine receptor CCR7 by CD8^{hi} T lymphocytes was increased, indicating a central memory phenotype (Fig 6, B). Furthermore, the expression of IFN- γ , granzyme B, and perforin by CD8^{hi} T cells in T lymphocytes plus HuMoSC cocultures was lower compared with that by CD8⁺ T cells in T lymphocytes cultured in the absence of HuMoSCs (Fig 6, C).

More importantly, CD8⁺CD103⁺ T cells induced in the presence of HuMoSCs were potent suppressors of CD4⁺CD25⁺ T-lymphocyte proliferation induced with activation beads (Fig 6, D), demonstrating that these cells were endowed with immunosuppressive properties.

DISCUSSION

Immunosuppressive/regulatory immune cell-based therapy is a relatively recent approach that might allow reduction of the doses of immunosuppressive drugs being administered to patients with autoimmune diseases, patients having undergone allogeneic transplantation, or both. The evaluation of these cells has highlighted their potential in preclinical studies,^{6,10,20,44-48} but their therapeutic efficacy in human subjects remains unclear. A major pitfall precluding clinical studies with suppressive cells of both myeloid and lymphoid origin is their low frequency in human subjects.^{8,9,49} Therefore strategies aimed at promoting their expansion and enhancing their suppressive function *ex vivo* are critically needed. The benefit of targeted strategies to promote Treg cell expansion, function, or both *in vivo* in transplant recipients has been demonstrated.²⁰ Several pharmacologic agents (immunosuppressive drugs and mAbs) currently used in human transplantation are being assessed for their effects on Treg cells in experimental and clinical studies.^{50,51} Because they target global T-cell activation pathways (eg, blockade of IL-2 signaling with cyclosporine, tacrolimus, anti-CD25 antibodies, and others), these molecules also impair Treg cell-mediated regulatory responses to donor alloantigens. In addition, all these studies have only focused on conventional CD4⁺ Treg cells, and the possibility of harnessing the suppressive potential of CD8⁺ Treg cells has not been examined. Mesenchymal stem cells (MSCs) have also been considered an alternative to immunosuppressive drugs for GvHD.^{52,53} However, MSCs are not constitutively immunosuppressive, and their inhibitory function highly depends on the environmental proinflammatory milieu.⁵⁴ This explains why the efficacy of MSCs to prevent GvHD development remains unclear.

In the current study we report on an original and clinically applicable method to generate human suppressor cells of monocytic origin *ex vivo* (HuMoSCs). Two studies have reported on the *ex vivo* generation and expansion of human CD33⁺ or CD11b⁺ myeloid-derived suppressor cells from total PBMCs by using various cytokine cocktails (IL-1 β , IL-6, vascular endothelial growth factor, TNF- α , and GM-CSF), tumor-derived factors, or tumor cell lines,^{55,56} conditions that restrict the clinical application of these cells. Of clinical importance, in our study HuMoSCs were generated without exposure to cancer cells or tumor-derived factors. In addition, HuMoSCs were phenotypically and functionally different from the cells described in the studies mentioned above^{55,56} because they exhibited high CD14 expression and lacked the granulocytic marker CD66b. As an additional difference, the suppressive activity of HuMoSCs did not require direct cell-cell contact.

HuMoSCs are capable of inhibiting both autologous and allogeneic T lymphocytes. This is an important observation because T cell-mediated alloresponses represent a main impediment in transplantation. Donor alloantigens (major and minor histocompatibility antigens) result in alloreactive effector T-cell responses, leading to graft rejection.^{20,57} Therefore controlling these alloreactive T cells is of paramount importance

for the regulation of immune responses after transplantation. Therefore the ability of HuMoSCs to suppress both autologous and allogeneic T lymphocytes makes these cells ideal candidates for therapeutic interventions aimed at regulating alloreactive responses in the clinic. One might propose to use donor-derived monocytes to generate HuMoSCs. Several therapeutic schemes can be proposed, as already reported in clinical trials with Treg therapy.^{6,18} Donor-derived HuMoSCs can be infused to prevent GvHD and to reduce immunosuppressive drug use at the time of hematopoietic cell transplantation. Moreover, as performed in a clinical trial,⁶ HuMoSCs can be administered after a myeloablative conditioning regimen and before a CD34-selected hematopoietic stem cell graft together with a defined number of donor T cells without posttransplantation immunosuppressive drugs. This has the advantage of using a defined donor T-cell/HuMoSC ratio comparable with those used in our study (ie, 4/1). On the other hand, HuMoSCs can be generated from donor monocytes, frozen, and then thawed to treat ongoing corticoid-resistant acute GvHD. Overall, HuMoSC therapy can be developed in allogeneic hematopoietic cell transplantation by taking into account other cell-based therapeutic strategies.

We demonstrate that HuMoSCs are potent therapeutic tools to mitigate the symptoms of GvHD and promote overall survival in a humanized mouse model of GvHD. Importantly, we demonstrate that HuMoSCs administered *in vivo* promote the development of a CD8⁺ T-lymphocyte subset expressing FoxP3, which likely represents a critical event in the establishment of a sustained state of specific tolerance. CD8⁺ Treg cells have recently been reported as a new regulatory population with potential to mitigate GvHD symptoms.^{17,27,29} These cells can impair alloreactive T-lymphocyte infiltration in target organs. However, the possibility of successfully inducing and expanding these cells *in vivo* has not been reported. Therefore our study, by highlighting HuMoSCs as major inducers of CD8⁺ Treg cells, provides important new data to address this aspect. Further underlying the therapeutic interest of HuMoSCs, our results demonstrate that these cells were also capable of promoting classical CD4⁺ Treg cell suppressive function (not shown).

Several chemokines and their receptors have been reported as having roles in regulatory immune cell accumulation and anti-inflammatory responses *in vivo*. For instance, 2 recent publications described the role of CCR5 in the recruitment of Treg cells by myeloid-derived suppressive cells to the graft⁵⁸ and tumor environment,⁵⁹ where they play a key role in blockade of effector immune responses. It is interesting to note that HuMoSCs produce high concentrations of MCP-1 (CCL2), a CCR5 ligand, while expressing CCR5. This mechanism might represent an autocrine amplification loop for HuMoSC accumulation and for recruitment of Treg cells.

In summary, for the first time, we report on a clinically relevant and feasible approach to generate *ex vivo* a new population of HuMoSCs endowed with the peculiar ability to promote expansion of immunosuppressive FoxP3⁺CD8⁺ Treg cells that can be used as an efficient therapeutic tool to prevent GvHD, transplant rejection, and autoimmune diseases.

Flow cytometric experiments were performed and analyzed in Plateforme de Cytométrie, Institut Fédératif de Recherche 100, Université de Bourgogne. We thank Jessica Borgeot for the technical help.

Key messages

- HuMoSCs can be routinely generated *ex vivo*.
- HuMoSCs induce FoxP3⁺CD8⁺ Treg lymphocytes.
- HuMoSCs prevent GvHD development.

REFERENCES

1. Ho VT, Soiffer RJ. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood* 2001;98:3192-204.
2. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008;133:775-87.
3. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 2003;9:1144-50.
4. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* 2002;196:389-99.
5. Nguyen VH, Shashidhar S, Chang DS, Ho L, Kambham N, Bachmann M, et al. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood* 2008;111:945-53.
6. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* 2011;117:3921-8.
7. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000;101:455-8.
8. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2002;2:389-400.
9. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 2005;6:331-7.
10. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. *J Exp Med* 2002;196:401-6.
11. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001;193:1295-302.
12. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 2002;99:3493-9.
13. Golshayan D, Jiang S, Tsang J, Garin MI, Mottet C, Lechler RI. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* 2007;109:827-35.
14. Di Ianni M, Del Papa B, Cecchini D, Bonifacio E, Moretti L, Zei T, et al. Immunomagnetic isolation of CD4+CD25+FoxP3+ natural T regulatory lymphocytes for clinical applications. *Clin Exp Immunol* 2009;156:246-53.
15. Hippen KL, Merkel SC, Schirm DK, Nelson C, Tennis NC, Riley JL, et al. Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. *Am J Transplant* 2011;11:1148-57.
16. Putnam AL, Safinia N, Medvec A, Laszkowska M, Wray M, Mintz MA, et al. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant* 2013;13:3010-20.
17. Zheng J, Liu Y, Liu M, Xiang Z, Lam KT, Lewis DB, et al. Human CD8+ regulatory T cells inhibit GVHD and preserve general immunity in humanized mice. *Sci Transl Med* 2013;5:168ra9.
18. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 2011;117:1061-70.
19. Hippen KL, Merkel SC, Schirm DK, Sieben CM, Sumstad D, Kadidlo DM, et al. Massive ex vivo expansion of human natural regulatory T cells (Tregs) with minimal loss of in vivo functional activity. *Sci Transl Med* 2011;3:83ra41.
20. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* 2003;3:199-210.
21. Sagoo P, Lombardi G, Lechler RI. Regulatory T cells as therapeutic cells. *Curr Opin Organ Transplant* 2008;13:645-53.
22. Tang Q, Bluestone JA, Kang SM. CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. *J Mol Cell Biol* 2012;4:11-21.

23. Chess L, Jiang H. Resurrecting CD8⁺ suppressor T cells. *Nat Immunol* 2004;5: 469-71.
24. Lerret NM, Houlihan JL, Kheradmand T, Pothoven KL, Zhang ZJ, Luo X. Donor-specific CD8⁺ Foxp3⁺ T cells protect skin allografts and facilitate induction of conventional CD4⁺ Foxp3⁺ regulatory T cells. *Am J Transplant* 2012;12:2335-47.
25. Van Kaer L. Comeback kids: CD8(+) suppressor T cells are back in the game. *J Clin Invest* 2010;120:3432-4.
26. Zheng J, Liu Y, Qin G, Chan PL, Mao H, Lam KT, et al. Efficient induction and expansion of human alloantigen-specific CD8 regulatory T cells from naive precursors by CD40-activated B cells. *J Immunol* 2009;183:3742-50.
27. Robb RJ, Lineburg KE, Kuns RD, Wilson YA, Raffelt NC, Olver SD, et al. Identification and expansion of highly suppressive CD8(+)FoxP3(-) regulatory T cells after experimental allogeneic bone marrow transplantation. *Blood* 2012; 119:5898-908.
28. Bisikirska B, Colgan J, Luban J, Bluestone JA, Herold KC. TCR stimulation with modified anti-CD3 mAb expands CD8⁺ T cell population and induces CD8⁺CD25⁺ Tregs. *J Clin Invest* 2005;115:2904-13.
29. Beres AJ, Haribhai D, Chadwick AC, Gonyo PJ, Williams CB, Drobyski WR. CD8⁺ Foxp3⁺ regulatory T cells are induced during graft-versus-host disease and mitigate disease severity. *J Immunol* 2012;189:464-74.
30. Boros P, Ochando JC, Chen SH, Bromberg JS. Myeloid-derived suppressor cells: natural regulators for transplant tolerance. *Hum Immunol* 2010;71:1061-6.
31. Dugast AS, Vanhove B. Immune regulation by non-lymphoid cells in transplantation. *Clin Exp Immunol* 2009;156:25-34.
32. Lees JR, Azimzadeh AM, Bromberg JS. Myeloid derived suppressor cells in transplantation. *Curr Opin Immunol* 2011;23:692-7.
33. Natarajan S, Thomson AW. Tolerogenic dendritic cells and myeloid-derived suppressor cells: potential for regulation and therapy of liver auto- and alloimmunity. *Immunobiology* 2010;215:698-703.
34. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood* 2010;116:5738-47.
35. Luan Y, Mosheir E, Menon MC, Wilson D, Woytovich C, Ochando J, et al. Monocytic myeloid-derived suppressor cells accumulate in renal transplant patients and mediate CD4(+) Foxp3(+) Treg expansion. *Am J Transplant* 2013;13:3123-31.
36. Lakomy D, Janikashvili N, Fraszczak J, Trad M, Audia S, Samson M, et al. Cytotoxic dendritic cells generated from cancer patients. *J Immunol* 2011;187: 2775-82.
37. Hippen KL, Bucher C, Schirm DK, Bearl AM, Brender T, Mink KA, et al. Blocking IL-21 signaling ameliorates xenogeneic GVHD induced by human lymphocytes. *Blood* 2012;119:619-28.
38. Gregoire-Gauthier J, Durrieu L, Duval A, Fontaine F, Dieng MM, Bourgey M, et al. Use of immunoglobulins in the prevention of GvHD in a xenogeneic NOD/SCID/gammac-mouse model. *Bone Marrow Transplant* 2012;47:439-50.
39. King MA, Covassin L, Brehm MA, Racki W, Pearson T, Leif J, et al. Human peripheral blood leucocyte non-obese diabetic-severe combined immuno-deficiency interleukin-2 receptor gamma chain gene mouse model of xenogeneic graft-versus-host-like disease and the role of host major histocompatibility complex. *Clin Exp Immunol* 2009;157:104-18.
40. Ali N, Flutter B, Sanchez Rodriguez R, Sharif-Paghalah E, Barber LD, Lombardi G, et al. Xenogeneic graft-versus-host-disease in NOD-scid IL-2Rgamma(null) mice display a T-effector memory phenotype. *PLoS One* 2012;7:e44219.
41. Cooke KR, Kobzik L, Martin TR, Brewer J, Delmonte J Jr, Crawford JM, et al. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. the roles of minor H antigens and endotoxin. *Blood* 1996;88: 3230-9.
42. Janikashvili N, LaCasse CJ, Larmonier C, Trad M, Herrell A, Bustamante S, et al. Allogeneic effector/memory Th-1 cells impair FoxP3⁺ regulatory T lymphocytes and synergize with chaperone-rich cell lysate vaccine to treat leukemia. *Blood* 2011;117:1555-64.
43. Audia S, Samson M, Guy J, Janikashvili N, Fraszczak J, Trad M, et al. Immunologic effects of rituximab on the human spleen in immune thrombocytopenia. *Blood* 2011;118:4394-400.
44. Taylor PA, Noelle RJ, Blazar BR. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med* 2001;193:1311-8.
45. Nadig SN, Wiekiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med* 2010;16:809-13.
46. Issa F, Hester J, Goto R, Nadig SN, Goodacre TE, Wood K. Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. *Transplantation* 2010;90:1321-7.
47. Stenger EO, Turnquist HR, Mapara MY, Thomson AW. Dendritic cells and regulation of graft-versus-host disease and graft-versus-leukemia activity. *Blood* 2012;119:5088-103.
48. Janikashvili N, Bonnotte B, Katsanis E, Larmonier N. The dendritic cell-regulatory T lymphocyte crosstalk contributes to tumor-induced tolerance. *Clin Dev Immunol* 2011;2011:430394.
49. Gabilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;9:162-74.
50. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* 2002;195:603-16.
51. Gregori S, Casorati M, Amuchastegui S, Smirardo S, Davalli AM, Adorini L. Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J Immunol* 2001;167:1945-53.
52. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;363:1439-41.
53. Le Blanc K, Frasson F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008;371:1579-86.
54. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;24:386-98.
55. Lechner MG, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *J Immunol* 2010;185:2273-84.
56. Lechner MG, Megiel C, Russell SM, Bingham B, Arger N, Woo T, et al. Functional characterization of human CD33⁺ and CD11b⁺ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines. *J Transl Med* 2011;9:90.
57. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* 1999;285:412-5.
58. Dilek N, Poirier N, Usal C, Martinet B, Blanco G, Vanhove B. Control of transplant tolerance and intra-graft regulatory T cell localization by myeloid-derived suppressor cells and CCL5. *J Immunol* 2012;188:4209-16.
59. Schlecker E, Stojanovic A, Eisen C, Quack C, Falk CS, Umansky V, et al. Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J Immunol* 2012;189:5602-11.

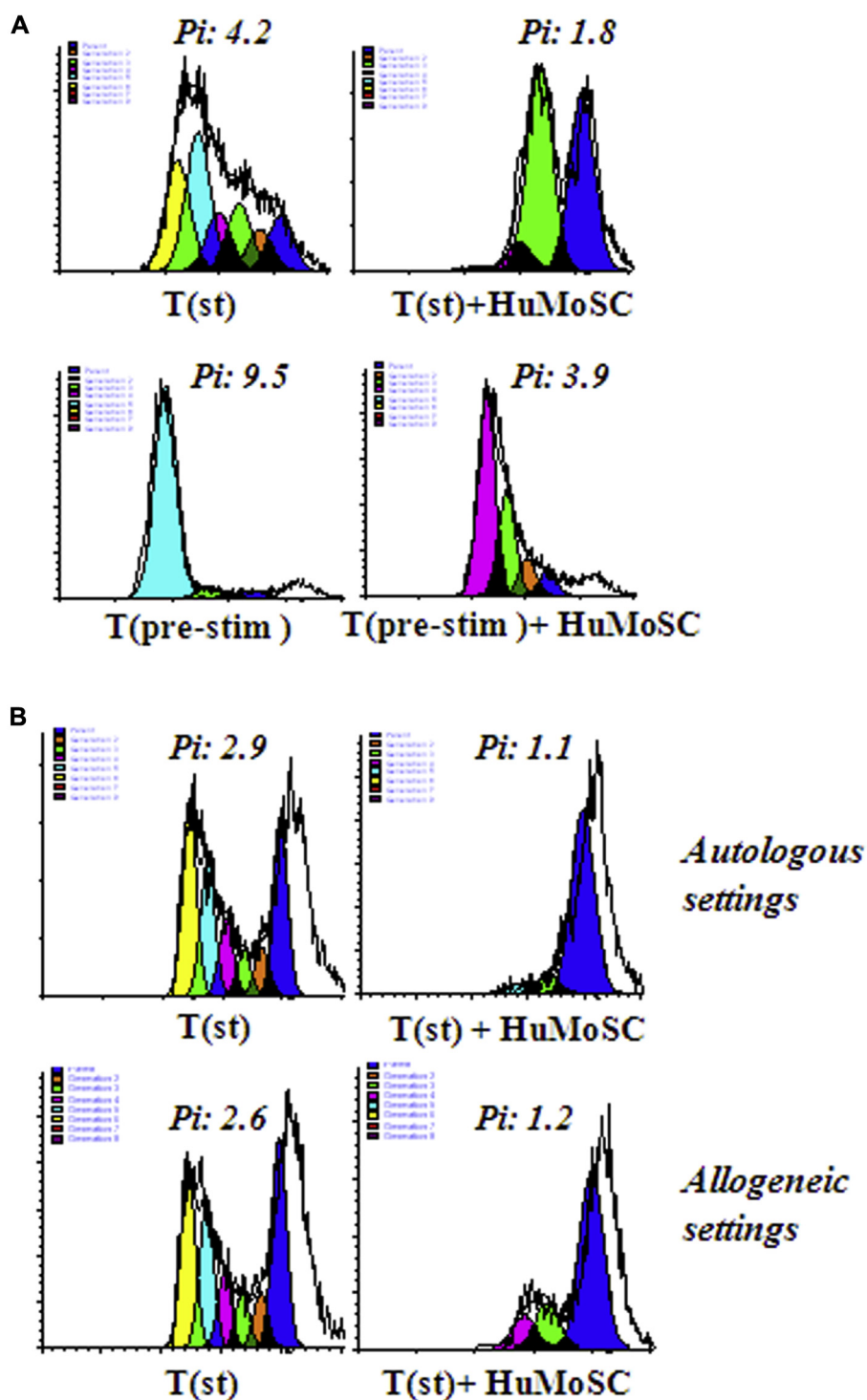


FIG E1. Inhibition of T-cell proliferation by autologous and allogeneic HuMoSCs. **A**, Proliferation of T cells cocultured with activation beads and HuMoSCs added at day 0 (*T[st]+HuMoSC*) or after 48 hours of prestimulation (*T[pre-stim]+HuMoSC*). **B**, Proliferation of T cells cocultured with autologous or allogeneic HuMoSCs. Representative ModFit analyses are shown. *Pi*, Proliferation index.

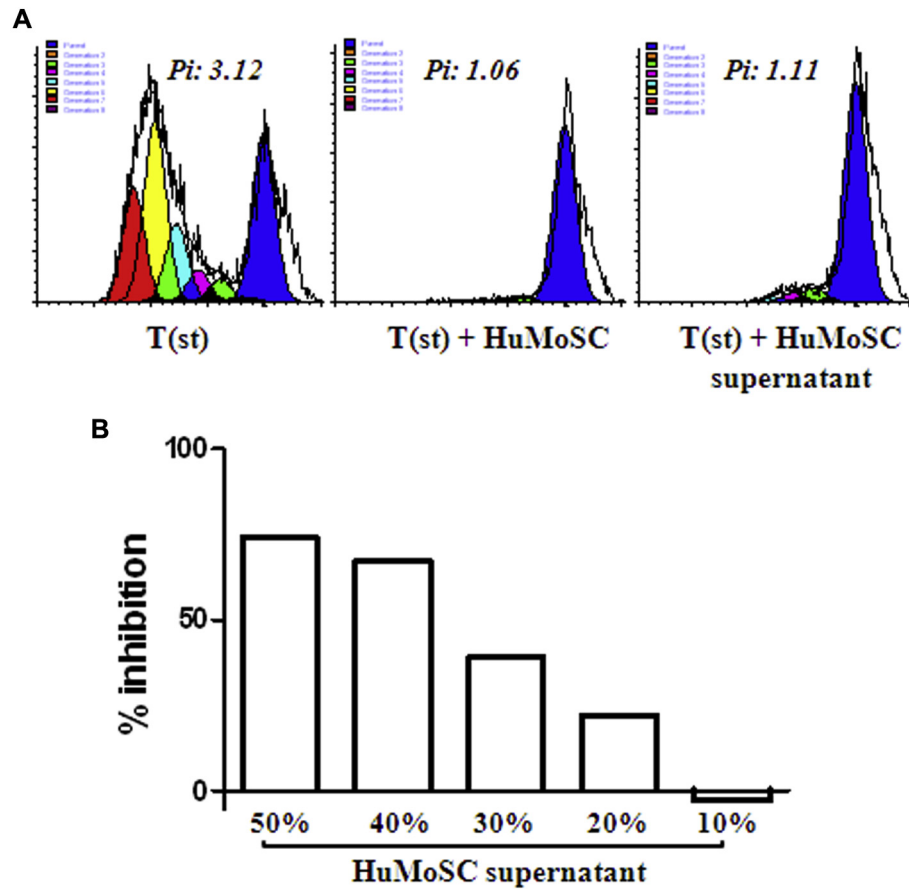


FIG E2. Inhibition of T-cell proliferation by soluble factors produced by HuMoSCs. **A**, Proliferation of T cells cultured with activation beads and HuMoSCs or with the supernatant of HuMoSC cultures, as indicated. *Pi*, Proliferation index. **B**, Percentage of inhibition of T-cell proliferation in the presence of HuMoSC culture supernatants added at different volumes.

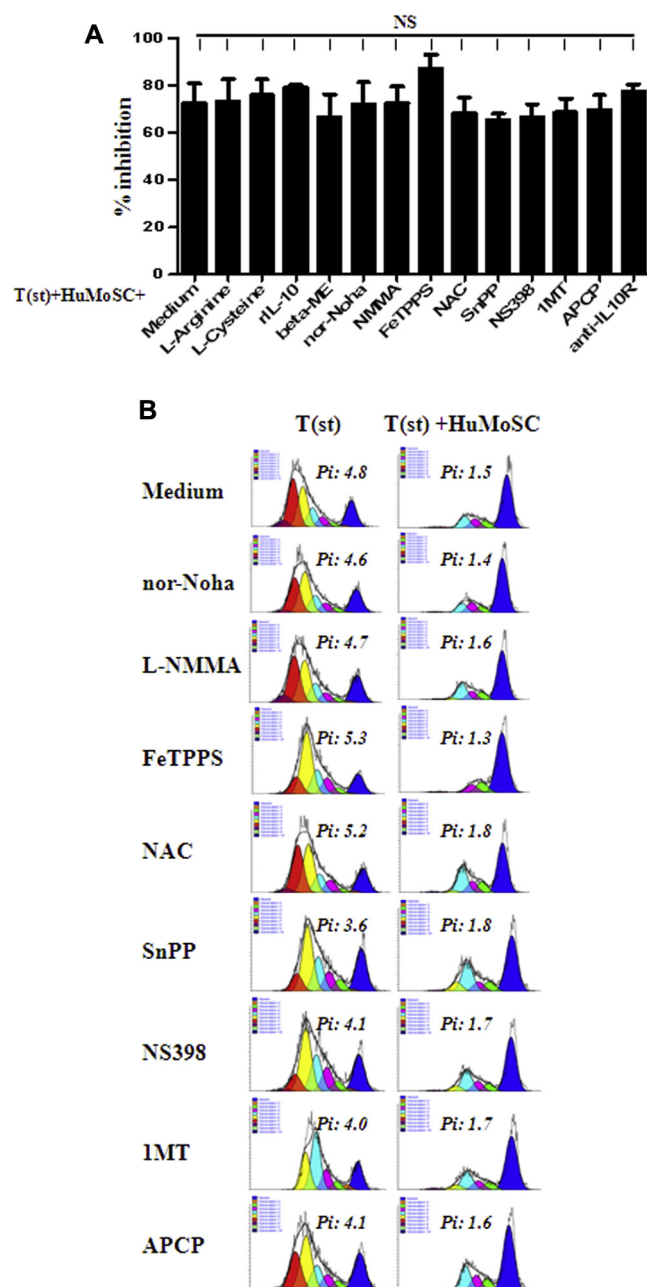


FIG E3. Inhibition of T-cell proliferation by HuMoSCs after blockade of different inhibitory pathways. **A**, Inhibition of T-cell proliferation by HuMoSCs in the presence of the indicated inhibitory agents, blocking antibodies, or exogenous metabolites. Activated T cells cultured alone and treated with the same agents were used as controls to calculate percentages of proliferation inhibition for each sample. Results of 3 independent experiments are shown. Data are presented as means $\pm$ SEMs. One-way ANOVA and the Dunnett test were used. **B**, Proliferation of T cells in the presence of HuMoSCs and the indicated inhibitory agents. Representative results of 3 independent experiments are shown. *Pi*, Proliferation index.

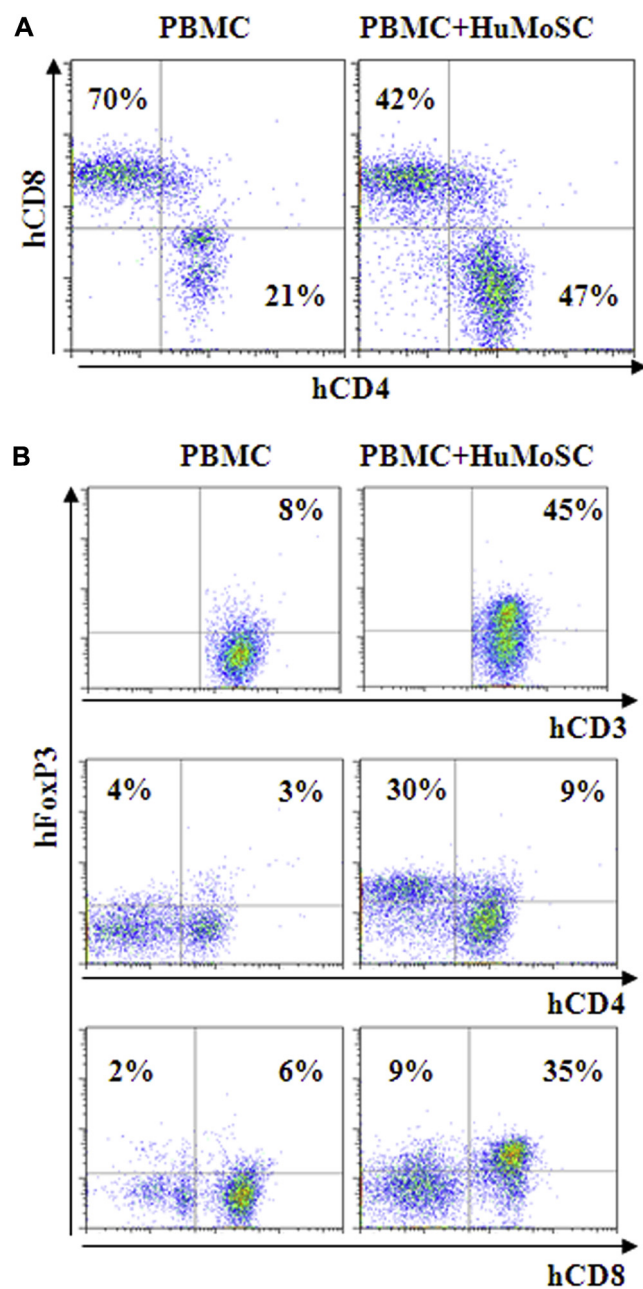


FIG E4. Induction of FoxP3⁺CD8⁺ T lymphocytes by HuMoSCs. **A**, Total PBMCs were cultured alone or in the presence of HuMoSCs. Percentages of CD4⁺ and CD8⁺ T lymphocytes were determined after gating on CD3⁺ cells. **B**, Frequency of FoxP3-expressing CD3⁺, CD4⁺, or CD8⁺ T lymphocytes was assessed after gating on CD3⁺ cells.