Efficiency of human monocyte-derived suppressor cell-based treatment in graft-versus-disease prevention while preserving graft-versus-leukemia effect

Nona Janikashvili, Claire Gérard, Marine Thébault, Andrea Brazdova, Clovis Boibessot, Claudie Cladière, Marion Ciudad, Hélène Greigert, Séthi Ouandji, Thibault Ghesquière, Maxime Samson, Sylvain Audia, Philippe Saas, and Bernard Bonnotte

ABSTRACT

Background: Immunosuppressive cell-based therapy is a recent strategy for controlling Graft-versus-Host Disease (GvHD). Such cells ought to maintain their suppressive function in inflammatory conditions and in the presence of immunosuppressive agents currently used in allogeneic hematopoietic cell transplantation (allo-HCT). Moreover, these therapies should not diminish the benefits of allo-HCT, the Graft-versus-Leukemia (GvL) effect. We have previously reported on a novel subset of human monocyte-derived suppressor cells (HuMoSC) as a prospective approach for controlling GvHD. Objective: The objective of this study was to explore the therapeutic relevance of the HuMoSC in clinical conditions.

Methods: Immune regulatory functions of HuMoSC were assessed in inflammatory conditions and in the presence of immunosuppressors. The therapeutic efficiency of the association of HuMoSC with immunosuppressors was evaluated in an experimental model of GvHD induced by human PBMC in NOD/SCID/IL2-Rγc−/− (NSG) mice. Interestingly, the inhibitory functions of HuMoSC against T lymphocytes and their ability to polarize Treg are preserved, in vitro, in inflammatory environments and are not affected by immunosuppressive agents. In vivo, the association of HuMoSC-based treatment with an immunosuppressive drug showed a synergistic effect for controlling GvHD. Furthermore, HuMoSC control GvHD while preserving GvL effect in a xenograft-GvHD conditioned mouse model with cell neoplasm (CAL-1). HuMoSC are generated according to good manufacturing practices (GMP) and we demonstrated that these cells tolerate long-term preservation with unaltered phenotype and function. Conclusion: HuMoSC-based therapy represents a promising approach for controlling GvHD and could be quickly implemented in clinical practice.

Introduction

Clinical applications of allogeneic hematopoietic cell transplantation (allo-HCT) are severely limited due to the extremely toxic and often lethal side effect known as graft-versus-host-disease (GvHD), which occurs in up to 50% of patients receiving allo-HCT. Donor T cells are the major effectors of the allogeneic response responsible for both the GvHD and the graft-versus-leukemia (GvL) effect. To prevent the deleterious expansion of pathogenic T cells, steroids and immunosuppressive drugs are generally used in current clinical practice. These commonly cause general immune suppression, resulting in severe infections or patient relapse by limiting the effectiveness of allo-HCT. Therefore, a strategy to induce a sustained state of tolerance with preservation or minimal attenuation of the GvL effect remains the main challenge for allo-HCT.

Immunosuppressive/regulatory immune cell-based therapy is a relatively recent approach for treating inflammatory disorders.2,3 The main mechanisms of action of such cells are directed toward the restoration of dysregulated immune balance rather than the induction of conventional immunodepression, which is an unavoidable problem when using immunosuppressive drugs. However, the implementation of immunosuppressive cell therapy in clinical contexts has been limited by their low frequency in humans.4 The possibility of generating or clonally expanding such cells ex vivo has become a major focus in the field. Moreover, the inflammatory environment with high concentrations of pro-inflammatory cytokines could change the fate of these suppressive cells, and convert them into inflammatory cells, such as Treg into pathogenic T cells5 or myeloid cells into iNOS-producing DC.6 This plasticity of immunosuppressive cells in inflammatory conditions, such as GvHD, could explain the low efficacy of immunosuppressive cell therapies in these contexts.3,6,7

We have previously reported on an original approach to obtain ex vivo large numbers of human myeloid suppressor cells generated from circulating monocytes, and referred to them as human monocyte-derived suppressor cells (HuMoSC).8 HuMoSC are highly potent at inhibiting the...
proliferation and activation of autologous and allogeneic effector T lymphocytes in a STAT3-dependent manner. HuMoSC induce long-lasting memory FoxP3⁺CD8⁺ regulatory T lymphocytes, and significantly reduce GvHD induced by human PBMC in NOD/SCID/IL2-RIc−/− (NSG) mice. Therefore, HuMoSC can be considered an efficient therapeutic tool to prevent GvHD during allogeneic transplantation.

In this work, we demonstrate that HuMoSC maintain their immunosuppressive properties in inflammatory settings and in the presence of anti-inflammatory and immunosuppressive agents, such as methylprednisolone, methotrexate, and cyclosporine, currently used in clinical practice in allo-HCT patients. In vivo, the association of HuMoSC-based treatment with an immunosuppressive drug showed a synergistic effect for controlling GvHD. Moreover, HuMoSC preserve the GvL effect in vivo. Of clinical relevance, HuMoSC tolerate long-term preservation with unaltered viability and inhibitory functions. Furthermore, the process has been improved and HuMoSC are now generated according to good manufacturing practices (GMP). These results provide a rationale for the application of HuMoSC to prevent GvHD in future clinical trials.

Methods

Generation of HuMoSC

Peripheral blood cell samples from healthy donors were collected at the French Blood Center (EFS BFC, Besançon, France) after obtaining written informed consent in compliance with the Declaration of Helsinki. Sample collection was approved by the French Ministry of Higher Education and Research (agreement number #AC-2015-2408 of May 22 2015). HuMoSC were generated as described previously.8 Briefly, PBMC were isolated from buffy coats of healthy donors by Ficoll density gradient centrifugation. Monocytes were purified from PBMC by Percoll density gradient centrifugation. HuMoSC were generated by incubating monocytes (1 × 10⁶ 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. GMP grade cytokines were used in the indicated experiments. Finally, the cultured cells were harvested and cryopreserved in complete medium supplemented with 10% DMSO. Cryopreserved HuMoSC were used for all the in vivo experiments.

Magnetic cell isolation and sorting

HuMoSC were purified by magnetic cell sorting using human CD33⁺ isolation kits and an autoMACS-Pro⁺ separator according to the manufacturer’s instructions (Miltenyi Biotec).

Antibodies and flow cytometry analysis

Flow cytometry analyses were performed as previously reported using monoclonal antibodies against CD33, CD11b, CD3, CD4, CD8, CD25, CD39, CD103. Treg were stained with anti-Foxp3 (Alexa 488) (Human Treg Flow Kit, Biolegend). Cells were analyzed using an LSRII cytometer (BD Biosciences) and FlowJo* software (version 10.0.7r2) was used for data analysis.

T cell proliferation and suppression assays

PBMC were stained using Cell Trace Violet according to the manufacturer’s procedure (Cell Trace™, Invitrogen, Cergy Pontoise, France). Labeled cells were cultured with anti-CD3/CD28-coated T cell expander beads (Dynabeads, Invitrogen) with or without HuMoSC (T cell to HuMoSC ratio = 4:1). T cell division was detected after 5 days by Flow Cytometry using an LSRII cytometer (BD Biosciences) and analyzed using ModFit* software (version 5.0). Parent cells are colored in blue and the next generations in other colors. The proliferative fraction is more than 95% in stimulated T cells. The suppressive activity of HuMoSC against responder cells was evaluated in the presence or absence of pro-inflammatory cytokines or TLR ligands, at the indicated concentrations: human recombinant IL-2 (20 UI/ml), IFN-γ (25 ng/ml), TNF-α (25 ng/ml), IL-1β (25 ng/ml), and LPS (50 ng/ml, TLR4 ligand), Pam3Cys-SK4 (50 ng/ml, TLR2 ligand), Poly I:C (50 μg/ml, TLR3 ligand), Flagellin (50 ng/ml, TLR5 ligand).

In the separate experiments, anti-inflammatory and immunosuppressive agents, such as methylprednisolone (MP, 25 ng/ml), methotrexate (MTX, 2.5 ng/ml), and cyclosporine (CsA, 5 ng/ml) were added to stimulated PBMC only or PBMC +HuMoSC co-cultures. Responder cell division was evaluated as described above.

Leukemia model

Mice were bred and maintained according to both the Federation of Laboratory Animal Science Associations and the Animal Experimental Ethics Committee guidelines (University of Burgundy; Dijon, France). NOD/SCID/IL2-RIc−/− (NSG) male mice were purchased from the Jackson Laboratory and housed in specific pathogen-free conditions and were used at 6 weeks of age. To establish the leukemia model, different numbers, ranging from 1 to 10 × 10⁶ plasmacytoid dendritic cell (pDC) neoplasm, CAL-1 cells (kindly provided by Prof. T. Maeda [Nagasaki University, Japan] via Prof. P. Saas) per mouse were inoculated intraperitoneally. Mice were scored every day for two weeks for clinical signs of leukemia (reduced mobility, red spots on the skin, paralysis). Once the tumor established, the mice were given low-dose irradiation (2 Gy) and engrafted with human PBMC as described below.

Xenogeneic mouse model of GvL/GvHD

For xeno-GvHD induction, mice were irradiated (2 Gy) six hours before the injection of 10 × 10⁶ human PBMC with or without 2.5 × 10⁶ HuMoSC followed or not by one intraperitoneal injection of cyclophosphamide (CTX) at the
notified dose. PBMC were mixed with HuMoSC before the injections. Each mice were scored twice a week in a blinded fashion for clinical signs of GvHD (weight loss, general appearance of the fur and mobility). Mice were euthanized when the clinical endpoints were reached (>15% weight loss, hunched posture, ruffled fur, reduced mobility, tachypnea). Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Burgundy.

**Statistical analysis**

GraphPad Prism® 7 (GraphPad Software) was used for the analyses. Student's t-test was used, when appropriate. One-way ANOVA and Dunnett's multiple comparison test were used when comparing more than two groups. Overall survival was calculated as the time from the day of injection of PBMC or tumor cells to death. Kaplan-Meier curves were plotted and differences were evaluated using the log-rank test. For all statistical analyses, a 2-tailed p-value of less than 0.05 was considered significant.

**Results**

**HuMoSC maintain suppressive potential in different inflammatory environments**

We have previously reported on a novel procedure for producing *ex vivo* clinically applicable suppressor cells of monocytic origin, referred to as HuMoSC. HuMoSC exhibit features of immunosuppressive/regulatory CD33<sup>+</sup>CD11b<sup>+</sup>CD14<sup>+</sup>CD163<sup>+</sup>CD206<sup>+</sup>HLA-DR<sup>+</sup> cells. Our recent data also indicate that HuMoSC are able to inhibit the proliferation of responder T lymphocytes and to regulate inflammatory responses. Before using these cells as therapy for controlling flares of autoimmune diseases or GvHD, the ability of these cells to maintain their immunosuppressive properties, such as inhibition of T cell proliferation and induction of Treg differentiation, in inflammatory environments had to be demonstrated. To identify HuMoSC suppressive potential in these situations, HuMoSC co-cultured with stimulated PBMC were exposed to several pro-inflammatory cytokines or different TLR ligands to mimic inflammatory conditions. The addition of pro-inflammatory cytokines, such as IL-2, IFN-γ, TNF-α, IL-1 (Figure 1a) or TLR ligands, such as LPS, Pam3Cys-SK4, Poly I:C, Flagellin, used at high doses solely or as a mixture (Figure 1b), did not impair the ability of HuMoSC to inhibit T cell proliferation. Furthermore, the ability of HuMoSC to induce Treg differentiation, either CD4 Treg or CD8 Treg, was not diminished when inflammatory cytokines (Figure 1c, E) or TLR ligands (Figure 1d, F) were added to the culture. Moreover, CD103 and CD39 expression by CD8 Treg induced by co-culture with HuMoSC was not decreased in inflammatory environments, for example, in the presence of inflammatory cytokines or TLR ligands (data not shown).

**HuMoSC treatment allows dose reduction of immunosuppressive drugs currently used to control GvHD**

Patients suffering from autoimmune or inflammatory diseases and patients undergoing allo-HCT are usually treated with immunosuppressants. Before using HuMoSC therapy for GvHD prevention or treating flares of autoimmune diseases, it is essential to check that these drugs do not affect the therapeutic potential of HuMoSC. HuMoSC co-cultured with PBMC were exposed to clinically relevant doses of anti-inflammatory and immunosuppressive agents, such as methylprednisolone, methotrexate, and cyclosporine, currently used for GvHD prevention and treatment in allo-HCT in leukemia patients. As these treatments themselves show certain levels of suppression against proliferating PBMC, the chosen dose was the highest dose of each drug which does not strongly inhibit T cell proliferation when stimulated with anti-CD3/CD28 microbeads. Of therapeutic relevance, our data demonstrate that the inhibitory function of HuMoSC against T cell proliferation was not affected by anti-inflammatory and immunosuppressive agents, such as methylprednisolone, methotrexate, and cyclosporine, which are currently used in clinical practice (Figure 2a). Moreover, the ability of HuMoSC to induce Treg differentiation, particularly CD8 Treg, which express CD39 and CD103 (data not shown), was preserved in the presence of immunosuppressants used to prevent GvHD (Figure 2 B-C). Furthermore, immunosuppressive treatments used for GvHD prevention did not impair the suppressive properties of HuMoSC *in vivo* (Figure 2d). The dose of 10 mg/Kg of cyclophosphamide (CTX) was chosen because this dose protected mice against GvHD with 80% of survival at day 60 whereas all the control mice were dead at the same time. However, CTX 10 mg/Kg did not induce a long-time protection of NSG (NOD/SCID/IL2-Rγ<sup>−/−</sup>) mice against xenogenic GvHD for a long time, because only approximately 20% of the mice were still alive at day 100. Interestingly, the addition of HuMoSC to CTX 10 mg/Kg significantly delayed GvHD occurrence and increased survival, with 65% of mice still alive at day 100 (Figure 3a). Therefore, we demonstrated that CTX did not inhibit the protective effect of HuMoSC and interestingly, the addition of HuMoSC with CTX showed a synergistic effect for controlling GvHD.

**HuMoSC control acute xeno-GvHD while preserving the GvL effect**

We have previously reported that HuMoSC reduce GvHD symptoms *in vivo*, resulting in prolonged animal survival. To further determine whether the HuMoSC-based treatment influences GvL activity of the adoptively transferred PBMC, HuMoSC were applied in a clinically relevant model of GvL. We therefore first established the model of leukemia using the human pDC leukemia cell line, CAL-1. CAL-1 cells acquire the pDC phenotype co-expressing BDCA4 and CD123 (data not shown) and are easily detectable in the spleen and bone marrow of NSG mice 15 days after intravenous inoculation at a dose of 5 × 10<sup>6</sup> cells (data not shown). In our tumor model, four groups of NSG mice were inoculated intravenously with different numbers of CAL-1 cells (from 1 to 10 × 10<sup>6</sup> per mouse) (Figure 3a).
Figure 1. HuMoSC properties (inhibition of PBMC proliferation and Treg induction) are not impaired by an inflammatory environment. Cell-Trace Violet stained PBMC stimulated with anti-CD3/CD28 microbeads were co-cultured with or without HuMoSC (ratio 4:1) in an inflammatory environment and assessed for their proliferation index. At day 5, PBMC were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD103, anti-CD39 and anti-FoxP3 antibodies. A. HuMoSC inhibitory effect on T cell proliferation in the presence or not of different doses of the following pro-inflammatory cytokines: IL-2 (20IU/ml), IFN-γ (20 ng/ml), TNF-α (20 ng/ml) and IL-1β (20 ng/ml) or a mix at the respective doses for 5 days. B. HuMoSC inhibitory effect on T cell proliferation in the presence or not of different doses of the following TLR ligands:
Tumor signs and survival were followed for 21 days. Mice injected with 1 and 2 x 10^6 leukemic cells showed mild disease symptoms while the groups injected with higher numbers of cells became paralyzed in a shorter period of time. Based on this observation, we chose to inject 5 x 10^6 leukemic cells per mouse, which, in the progressive tumor development, gives a large enough window for therapeutic intervention (Figure 3a-3b).

Next, in the tumor-bearing mice, we optimized the irradiation dose (2 Gy) and the PBMC count (10 x 10^6 per mouse) to achieve successful engraftment (Figure 3c). Following the optimization steps, we carried out the complete experiment according to the timeline depicted in Figure 3d. Briefly, mice were inoculated with 5 x 10^5 of CAL-1 leukemic cells at day 0 and given low dose (2 Gy) irradiation at day 7 followed by the engraftment of 10 x 10^6 PBMC with or without HuMoSC co-administration at day 8. A group without PBMC engraftment was used as the control for leukemia recurrence post-irradiation. Tumor growth, GvHD symptoms and survival were followed in all groups for 40 days. All the mice which had received leukemic cells alone were found to be paralyzed and spleens and blood from five euthanized mice from this group contained high numbers of CD123+ leukemic cells; so, these mice were categorized as deaths due to leukemia. In the two other groups, no CD123 positive cells were found in the spleen and blood from five mice euthanized of both groups, leukemia cells + PBMC or leukemic cells + PBMC + HuMoSC. Moreover, most of the dead mice from these two groups presented clear signs of GVHD (>15% weight loss, hunched posture, ruffled fur, reduced mobility, tachypnea) and so these mice were considered as having died because of the GVHD (Table 1).

All mice receiving CAL-1 leukemic cells alone died from leukemia, whereas mice receiving PBMCs without HuMoSC also died quickly but from GVHD. Interestingly, PBMC injection efficiently provided a GvL effect, which significantly increased survival (p = 0.0305). At day 25, 90% of the mice receiving CAL-1 cells alone had died as had 60% of mice receiving CAL-1 cells + PBMC, whereas 80% of mice receiving CAL-1 cells + PBMC + HuMoSC showed long-term disease-free survival. Interestingly, none of the mice receiving PBMCs + HuMoSC died from leukemia, thus demonstrating that HuMoSC do not impair the GvL effect in this model. Altogether, we demonstrated that the co-administration of HuMoSC with PBMC efficiently delayed the development of GVHD in NSG mice without increasing the recurrence of leukemia, resulting in significantly improved overall survival (leukemic cells versus leukemic cells + PBMC + HuMoSC, p = 0.0002) (Figure 3e). These results also showed that HuMoSC maintain their GVHD-prevention effect in an in vivo model mimicking allo-HCT conditions.

**Clinical grade HuMoSC can be generated**

HuMoSC tolerate long-term cryopreservation with unaltered viability, phenotype, and function (Figure 4a-4b). The same qualities are preserved when the cell culture medium is replaced by clinically used albumin solution in cell transfusion procedures. Moreover, HuMoSC remain stable at room temperature or at 4°C for a long period of time, long enough for clinical transfusions (Figure 4c-4d). Of great clinical importance, HuMoSC can be generated in GMP conditions with a comparable CD33^+CD11b^ phenotype (Figure 4e) and strong suppressive function (>75% of inhibition of proliferation) against stimulated CD4 and CD8 T lymphocytes (figure 4f). Number of viable and isolated HuMoSC remained stable during time (Table 2).

**Discussion**

Immunosuppressive cell-based therapy may make it possible to reduce doses of immunosuppressive drugs being administered to patients with auto-immune diseases and/or to patients who have undergone allogeneic transplantation. We have already developed a clinically relevant and feasible approach to generate *ex vivo* a subpopulation of HuMoSC that can be used as an efficient therapeutic tool to prevent inflammatory disorders. The therapeutic efficacy of HuMoSC was determined in the xenogeneic GvHD mouse model in which the disease was induced by injecting human PBMC. In the present report, we further investigated the practical potential of this HuMoSC therapy. It is evident that the overwhelming positive results of suppressive cell therapies reported from *in vitro* and preclinical animal studies have for the most part not yet translated into full clinical efficacy. Clearly, there is still much to be learned with regard to the *in vivo* interactions of cell therapies in human pathological states. The preservation of the suppressive capacity of cell therapies after injection into patients is crucial. Unlike other cell therapies, such as those with Treg, which converted into different types of ex-Treg cells under inflammatory conditions or autoimmune diseases, HuMoSC maintain their suppressive properties under inflammatory conditions. The suppressive properties of HuMoSC, such as the ability of HuMoSC to inhibit T cell proliferation, are not affected by LPS and other TLR ligands or inflammatory cytokines, whereas those of mesenchymal stromal cells (MSC) are. However, the effect of *in situ* inflammation on MSC activation status varies depending on the level and type of inflammation. Moreover, Treg induction by HuMoSC is not affected by TLR ligands.

Methotrexate, cyclosporine, and methylprednisolone are widely used immunosuppressive drugs to prevent/treat GVHD after alloHCT. The general immunosuppression following their administration may lead to the development of opportunistic infections and patient relapse. In this study, we demonstrated that HuMoSC can be used in the presence of these drugs and, more importantly, we showed synergistic effect that would allow to lower the doses of immunosuppressive drugs administered to patients. It has been shown that mouse MDSC cultured in the presence of cyclosporine are
Figure 2. HuMoSC properties (inhibition of T cell proliferation and Treg induction) are not impaired by currently used immunosuppressive treatments in GvHD. Cell-Trace Violet stained PBMC stimulated with anti-CD3/CD28 microbeads were co-cultured with or without HuMoSC (ratio 4:1) and methylprednisolone (MP) 25 ng/ml, methotrexate (MTX) 2.5 ng/ml, cyclosporine A (CsA) 5 ng/ml or a mix for 5 days and assessed for their proliferation index. At day 5, PBMC were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD103, anti-CD39 and anti-FoxP3 antibodies. A. HuMoSC inhibitory effect on T cell proliferation in the presence of immunosuppressive drugs B. HuMoSC-induced CD4 Treg in the presence of immunosuppressive drugs. C. HuMoSC-induced CD8 Treg in the presence of immunosuppressive drugs.
increased with no alteration of their phenotype and, moreover, these MDSC exhibited enhanced immunosuppression via the iNOS pathway. In our study, we showed in vitro that HuMoSC retained their suppressive effect against T cell proliferation and promoted Treg induction in the presence of cyclosporine. This result could be surprising because it has been demonstrated that cyclosporine inhibits induction of Ag-specific CD4+CD25+FoxP3+ Tregs in vivo, as the survival and suppressive activity of Treg depend on exogenous IL-2. This result suggests that as HuMoSC do not produce IL-2, they promote Treg expansion through another pathway. For instance, Ruppert et al showed that regulatory T cells were able to resist cyclosporine-induced cell death via CD44 cross-linking by hyaluronan, which promoted IL-2 independent FoxP3 expression. The impact of associating immunosuppressive cells and immunosuppressive drugs has also been evaluated in Treg therapy. In a GMP generation protocol of Treg, the presence of immunosuppressive drugs, i.e. tacrolimus, mycophenolic acid (active metabolite of mycophenolate mofetil) and methylprednisolone, affected the viability and proliferation of expanded Treg in vitro and in vivo. Here, we showed that HuMoSC do not lose their suppressive activity when exposed to these immunosuppressive drugs, which are routinely used in patients treated for GvHD or autoimmune diseases. We found similar data to that obtained with MSC, namely, that the co-administration of HuMoSC with classically used immunosuppressive drugs increased their protective effects and, thus, made it possible to reduce the dose of immunosuppressants without jeopardizing GvHD prevention. This result opens a new therapeutic era in which doses of immunosuppressant, and thus their iatrogenic damage, will be reduced thanks to HuMoSC therapy.

We have already shown that HuMoSC can induce FoxP3+CD8+ and FoxP3+CD4+ Treg subpopulations in vitro and in vivo. Treg induction is already a well-known immunosuppressive pathway used by MDSC to alter the tumoral environment and may involve immunosuppressive cytokines, such as IL-10, which is actually secreted by HuMoSC. It has also been suggested that TGF-β is involved in this Treg induction, however, HuMoSC do not produce TGF-β and do not induce Treg proliferation, but rather induce Treg differentiation from naive T cells, which may exclude the role of TGF-β. HuMoSC not only induce Treg but also increase their suppressive ability. Although the Treg induction mechanism has not yet been deciphered, this peculiar property of HuMoSC makes them a promising therapy in inflammatory diseases. The GvL/GvHD balance is critical in allo-HCT. Immunosuppressive therapy should not inhibit the GvL effect, which is an expected goal of allo-HCT. We established a model mimicking allo-HCT in NSG mice, in which leukemia or GvHD do not kill the mice too quickly so as to leave time for a therapeutic intervention. Our experimental model tackles important limitations of mouse models of GvL/GvHD, since human cells were used to induce both xenogenic GvHD and allogeneic GvL. Even though this model enabled us to demonstrate that the injection of HuMoSC did not increase the percentage of leukemia recurrence or relapse in mice undergoing allo-HCT while delaying the onset of GvHD. These results allowed us to conclude that HuMoSC do not diminish the GvL effect. Every step of the clinical manufacturing process needs to respect local legislation, such as Advanced Therapy Medicinal Product (ATMP) legislation in all EU countries, and to comply with GMP requirements specific to the field. HuMoSC are expected to be generated from third-party healthy donors and administered in a completely HLA-mismatched allogeneic setting. HuMoSC have been shown to inhibit both autologous and allogeneic T lymphocytes. Moreover, the cryopreservation step in the manufacturing process allowing the use of cell therapy in clinical assays is necessary since cryopreservation enables rapid access to this cell therapy in acute conditions, such as the onset of GvHD. Large-scale manufacturing and banking of batches of HuMoSC are already validated. Unlike HuMoSC, MSC lose their suppressive properties after cryopreservation, which explains the unresponsiveness of some patients with severe acute GvHD to MSC infusion. Cryopreservation does not alter the HuMoSC phenotype or impair their functional suppressive properties. In this study, we demonstrated the stability of clinical grade HuMoSC. Our current manufacturing protocol contains only one freezing step and the frozen HuMoSC are thawed before CD33 sorting and infusion into the patient. HuMoSC remain viable and maintain their suppressive functions for at least 6 hours after thawing, which is sufficiently long to infuse the cells into patients; this has also been validated. Moreover, all of our in vivo experiments were carried out with freeze-thawed HuMoSC.

Our study has some limitations because of a lack of mechanistics and controls. We clearly demonstrated the suppressive properties of the HuMoSC, however, we did not decipher the exact immunosuppressive mechanisms. Because of the complexity of the experimental model, we were not able to use different in vivo knock-out mice, to decipher the key molecules expressed or produced by the HuMoSC that inhibit GvHD but keep the GvL effect, and to control the leukemia progression by bioluminescence imaging. The leukemia progression was measured by the onset of clinical signs such as paralysis, survival, and spleen and blood analyses of euthanized mice. However, we demonstrated that these cells are strongly immunosuppressive in vitro and in vivo and also in inflammatory environments by keeping their immunosuppressive functions contrary to other immunosuppressive myeloid cells that lost their immunosuppressive function in inflammatory environment due to inflamasome activation.
Figure 3. HuMoSC increase the survival of tumor-bearing mice by protecting them from GvHD and leukemia. A. Different models of leukemia using Cal-1 were tested in order to determine the best model. Model using $5 \times 10^5$ Cal-1 by I.V. on D0 cells was finally chosen. B. Survival of NSG mice with different doses of Cal-1 (1, 2, 5 and $10 \times 10^5$ Cal-1). C. Optimization of irradiation doses (2 and 4 Gy) and PBMC count (10 or $20 \times 10^6$ per mouse). D. Chosen timeline for experiment and evaluated parameters. E. Survival was assessed for tumor group (n = 15), tumor + PBMC group (n = 15) and tumor+PBMC+HuMoSC group (n = 15) Mice received an intravenous injection of $5 \times 10^5$ Cal-1 cells followed by low-dose irradiation (2 Gy) at day 7 and an intraperitoneal injection of either $10 \times 10^6$ PBMC alone or PBMC+ $2.5 \times 10^6$ HuMoSC at day 8. Data were censored after 40 days of follow-up. P values are the result of log-rank test: ns = non-significant, *p ≤0.05, **p ≤0.01, ***p ≤0.001.
Figure 4. HuMoSC phenotype and function are not impaired by long-term cryopreservation or room temperature. HuMoSC phenotype and immunosuppressive function estimated by the inhibition of proliferation of Cell-Trace Violet stained PBMC stimulated with anti-CD3/CD28 microbeads. A. Phenotype after cryopreservation at −80°C for 0, 7, 30, 60, or 100 days. B. Function after cryopreservation at −80°C for 0, 7, 30, 60, or 100 days. C. Phenotype after preservation at room temperature (RT) or at 4°C for 6 hours. D. Function after preservation at RT or at 4°C for 6 hours. Data are shown as means ± SEM of three representative experiments. P values are the result of Student t test: ns = non-significant. E. Phenotype of two different batches after 2 months of cryopreservation. F. Function of two different batches after 2 months of cryopreservation. Results are shown by dot plot and histogram of representative experiments.

Even though more robust clinical data showing the efficacy of HuMoSC in controlling GvHD in leukemia patients undergoing allo-HCT are needed, HuMoSC-based cell therapy represents a promising targeted approach in the prevention of
human acute GvHD and in the treatment of auto-immune diseases.

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Authors’ contributions

NJ, CG, MT, AB, CB, CC, and MC conducted the experiments; NJ, CG, MT, AB, and CB analyzed data; NJ, CG, and AB performed statistical analysis; NJ, CG, MT, and AB assisted with in vivo experiments. NJ, CG, and BB wrote the manuscript, and HG, SO, TG, MS, SA, and PS commented on the manuscript. NJ and BB supervised the research. All authors provided input and edited and approved the final version of the manuscript.

Disclosure statement

The authors declare that they have no conflict of interest.

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Table 1. Causes of death, pre-mortem clinical signs from all mice in each group (n = 15), and blood and spleen analyses from five euthanized mice in each group.

| Pre-mortem clinical signs and blood and spleen analyses from euthanized mice | Group 1: leukemic cells | Group 2: leukemic cells + radiation + Human PBMC | Group 3: leukemic cells + radiation + Human PBMC + HuMoSC |
|---|---|---|---|
| Paralysis | 15/15 | 0/15 | 0/15 |
| Clinical signs of GvHD | 0/15 | 14/15 | 10/15 |
| CD123+ cells in the spleen | 5/5 | 0/5 | 0/5 |
| CD123+ cells in the blood | 5/5 | 0/5 | 0/5 |

Table 2. Percentage of HuMoSC viability after cryopreservation.

| Sample 1 viability | Sample 2 viability |
|---|---|
| Day 0 | Day 7 | Day 30 | Day 60 | Day 100 |
| >85% | >85% | >85% | >85% | >85% |
| >85% | >85% | >85% | >85% | >85% |

Table 2. Percentage of HuMoSC viability after cryopreservation.

| Day 0 | Day 7 | Day 30 | Day 60 | Day 100 |
|---|---|---|---|---|
| >85% | >85% | >85% | >85% | >85% |
| >85% | >85% | >85% | >85% | >85% |

Table 1. Causes of death, pre-mortem clinical signs from all mice in each group (n = 15), and blood and spleen analyses from five euthanized mice in each group.

Table 2. Percentage of HuMoSC viability after cryopreservation.
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