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ORIGINAL REPORT

Phase II Multicenter, Randomized, Double-Blind Controlled Study of Efficacy and Safety of Umbilical Cord–Derived Mesenchymal Stromal Cells in the Prophylaxis of Chronic Graft-Versus-Host Disease After HLA-Haploidentical Stem-Cell Transplantation

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See accompanying editorial on page 2812

ABSTRACT

Purpose

Although mesenchymal stromal cells (MSCs) possess immunomodulatory properties and exhibit promising efficacy against chronic graft-versus-host disease (cGVHD), little is known about the efficacy of MSCs in the prophylaxis of cGVHD after HLA-haploidentical hematopoietic stem-cell transplantation (HLA-haplo HSCT).

Patients and Methods

In this multicenter, double-blind, randomized controlled trial, we investigated the incidence and severity of cGVHD among patients, and the changes in T, B, and natural killer (NK) cells after the repeated infusion of MSCs.

Results

The 2-year cumulative incidence of cGVHD in the MSCs group was 27.4% (95% CI, 16.2% to 38.6%), compared with 49.0% (95% CI, 36.5% to 61.5%) in the non-MSCs control group ($P = .021$). Seven patients in the non-MSCs control group had severe lung cGVHD, but no patients in the MSCs group developed typical lung cGVHD ($P = .047$). After the MSC infusions, increasing memory B lymphocytes and regulatory T cells, as well as the ratio of type 1 T helper to type 2 T helper cells, were observed, whereas the number of NK cells decreased.

Conclusion

Our findings suggest that the repeated infusion of MSCs might inhibit cGVHD symptoms in patients after HLA-haplo HSCT, accompanied by changes in the numbers and subtypes of T, B, and NK cells, leading to the acquisition of immune tolerance.

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INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is currently the leading cause of long-term morbidity and mortality after allogeneic hematopoietic stem-cell transplantation (allo-HSCT),[1] occurring in 28% to 60% of patients who survive for more than 100 days after allo-HSCT.[2,3] It affects nearly all organs and tissues, and the clinical manifestations result from a highly complex immune pathology involving donor B cells and T cells, and other cells.[1,4] Although this complication is associated with a reduced risk of relapse in patients with leukemia, cGVHD is the leading cause of nonrelapse mortality in transplantation and has a significant impact on patient quality of life and the need for immunosuppressive medications.[5,6]

The incidence of cGVHD in HLA-haploidentical HSCT (HLA-haplo HSCT) is higher than that in matched HSCT. Fuchs et al[7] and Zhang et al[8] reported that the cumulative incidence of cGVHD was 56.7% to 63.3%, with an incidence of 26.9% to 27.2% for limited cGVHD and 29.5% to 36.7% for extensive cGVHD. Despite significant advances in the field of HSCT over the past 25 years, there has been little change in the incidence, morbidity, and

mortality of cGVHD.[9-11] Post-transplantation cyclophosphamide has proved to be effective as sole GVHD prophylaxis for myeloablative HLA-matched–related or –unrelated bone marrow transplantation.[12-14] In 2015, data from the Blood and Marrow Transplant Clinical Trials Network further revealed that administration of high-dose cyclophosphamide on days 3 and 4 after HLA-haplo transplantation could reduce the incidence of cGVHD.[15] Even so, there is still a need to explore new programs to further reduce the incidence of cGVHD.

Numerous studies have revealed that mesenchymal stromal cells (MSCs) have profound immunomodulatory functions both in vitro and in vivo.[16] MSCs are known to modulate the proliferation, activation, and maturation of T and B lymphocytes in vitro in a dose-dependent and time-limited manner.[17,18] Several clinical trials demonstrated that the cotransplantation of MSCs with hematopoietic stem cells (HSCs) is an effective and apparently safe therapy, without MSC-associated immediate or late infusion toxicities.[19-22] Zhou et al[23] and Weng et al[24] also suggested that the infusion of MSCs that were expanded in vitro is a safe and effective salvage therapy for patients with steroid-resistant cGVHD. MSCs have also been used for the prophylaxis of acute GVHD (aGVHD) and for the treatment of patients with steroid-refractory cGVHD.

On the basis of early results for MSCs in the treatment of steroid-refractory acute/chronic GVHD and an encouraging safety profile, we speculated that MSCs could help prevent cGVHD and thus designed a clinical study to determine whether infusion of umbilical cord–derived MSCs could prevent cGVHD in recipients after HLA-haplo HSCT. To our knowledge, this is the first study of the efficacy and safety of MSCs in the prophylaxis of cGVHD after HLA-haplo HSCT in China or elsewhere.

PATIENTS AND METHODS

Patients

This study was a phase II multicenter, double-blind, randomized controlled trial. The objective of the study was to evaluate the efficacy and safety of umbilical cord–derived MSCs in cGVHD prophylaxis in patients after HLA-haplo HSCT. Patients with hematologic malignancies who underwent HLA-haplo HSCT were enrolled in this study from five transplantation centers. Eligible patients met the following criteria: aGVHD did not occur or was controlled by more than 100 days after transplantation; lack of cGVHD at 100 days after transplantation; and the absence of uncontrolled infections and severe liver, renal, lung, and heart diseases.

The protocol and informed consent form were approved by the ethics committee of all transplant centers. All of the patients or their legal guardians signed informed consent forms in accordance with the Declaration of Helsinki. This trial has been registered as ChiCTR-IOR-15006330 (www.chictr.org.cn).

Study Design, Treatment, and Study Objectives

The sample size estimation was obtained using a difference test for two proportions on the basis of the primary outcome of cGVHD incidence. In preliminary experiments, the incidences of cGVHD in the experimental and control group were 0.289 and 0.519, respectively. A sample size of a total of 112 patients was calculated with a one-side type I error of 0.05 and a statistical power of 80% (no patient in preliminary experiments was included in this study). Considering possible loss of patients to follow-up (10%), we decided to include 124 patients, with 62 patients in each arm. The sample size calculation was performed with PASS version 11 software (NCSS, Kaysville, UT). Forty-four patients from the lead institution and 20 patients from the other transplant centers were included.

Randomization was stratified by the center at enrollment. The included patients, in a 1:1 ratio, were randomly chosen to receive umbilical cord–derived MSCs (MSCs group; 3×10^7 cells/100 mL/ per month) or normal saline (non-MSCs group; 100 mL/mo) from more than 4 months after transplantation for cGVHD prophylaxis. The infusion was discontinued if cGVHD occurred, if the leukemia relapsed, or after four cycles of infusion. The random numbers were generated from a computer by a statistician who was not involved in this study.

Both preparations were produced by the same institute (Stem Cell Bank, Chinese Academy of Sciences, Beijing, China) to ensure identical volume, consistency, shape, and color. The observation period extended from enrollment to March 31, 2015, or the time of patient death. During follow-up, the incidence and severity of cGVHD, other symptoms, and adverse effects of the infusion were carefully recorded.

The primary objective was to determine the incidence and severity of cGVHD in the intent-to-treat (ITT) population. We used the 2005 National Institutes of Health (NIH) consensus criteria for organ scoring and the global assessment of cGVHD.[25,26] Secondary objectives included relapse, safety, overall survival (OS), and disease-free survival (DFS) in the ITT population.

Transplantation Procedure

All patients received a myeloablative conditioning regimen. Patients with acute lymphoblastic leukemia received total body irradiation plus arabinosylcytosine (Ara-C), cyclophosphamide (CY), and antithymocyte globulin (ATG). Patients with acute myeloid leukemia and those with myelodysplastic syndrome s received chlorethyl cyclohexyl nitrosourea, Ara-C, busulfan, CY, and ATG. The GVHD prophylaxis consisted of cyclosporine A (CsA), mycophenolate mofetil, and short-term methotrexate. Specific usage was according to reported criteria.[27-29]

Preparation of MSCs and Analysis of Lymphocyte Subset

Clinical-grade MSCs derived from umbilical cords with the informed consent of the mother were isolated by the Stem Cell Bank of the Chinese Academy of Sciences, as previously reported.[30-32] Lymphocyte subsets were routinely determined in the patients of the MSCs group and the non-MSCs control group[33] (Data Supplement).

Statistical Analysis

Mann-Whitney tests and χ^2 tests were used to compare the baseline characteristics between the MSCs group and the non-MSCs control group. χ^2 tests were also used for the difference tests of cGVHD incidence and the proportion of death course between the two groups. The 2-year cumulative probabilities of OS and DFS and the cumulative incidence of cGVHD and disease relapse were estimated using Kaplan-Meier analysis and are expressed as percentages with 95% CIs. Log-rank tests were used to compare the OS and DFS survival curves and the cumulative incidences of cGVHD and disease relapses between the two groups.

An analysis of variance was used to assess differences in the repeated measurement data from the lymphocyte subset analysis.

All reported *P* values are two-sided. Statistical analyses were performed using the STATA package (StataCorp, College Station, TX)[34] and the R 2.5.0 software package (R Project, http://www.r-project.org).[35]

RESULTS

Patient Population and Disposition

Between May 2009 and March 2013, 146 patients were screened in five transplant centers. Twelve failed to meet the inclusion criteria, 10 refused to participate in the study, and 124 were randomly assigned (MSCs, n = 62; control group, n = 62). Details of the study population and controls are reported in Table 1. Of these patients, 12 (9.7%) subsequently discontinued the study treatment and two were lost to follow-up. The most common reasons for treatment discontinuation were cGVHD and progressive disease (Fig 1).

GVHD Incidence and Severity

In the MSCs group, the average number of MSC infusions was 3.7 (range, 2 to 4). cGVHD developed in 17 patients (27.4%): 14 (22.6%) exhibited mild/moderate cGVHD when the CsA administration was gradually reduced at the scheduled time, whereas three (4.8%) showed severe cGVHD. In the non-MSCs control group, cGVHD occurred in 30 patients (48.4%): 22 (35.5%) exhibited mild/moderate cGVHD, and eight (12.9%) had severe cGVHD (Table 2; Fig 2). The cumulative incidence of cGVHD in the MSCs group was 27.4% (95% CI, 16.2% to 38.6%), compared with 49.0% (95% CI, 36.5% to 61.5%) in the non-MSCs control group ($P = .021$).

The clinical manifestations of mild/moderate cGVHD included the following: poikiloderma; depigmentation and erythema; nail splitting; mouth ulcers; dry, gritty, or painful eyes; genital ulcers; nausea, vomiting, and diarrhea; edema; and slight hepatic functional lesions. The manifestations of severe cGVHD included the following: onycholysis, bronchiolitis obliterans, restriction of the mouth, keratoconjunctivitis sicca, arthritis or arthralgia, polymyositis, dermatosclerosis, and severe hepatic functional lesions (Table 2). Seven patients in the non-MSCs control group developed severe lung cGVHD, all exhibiting symptoms of exertion, cough, and wheezing, as well as forced expiratory volume in 1 second/forced vital capacity ratio < 0.7, evidence of air trapping and small airway thickening on high-resolution chest computed tomography, and an absence of infection in the respiratory tract. None of the patients in the MSCs group developed typical lung cGVHD ($P = .047$).

Table 1. Patient, Donor, and Graft Characteristics

Characteristic	MSCs, No. (%)	Non-MSCs, No. (%)	Statistical Testing Result	*P*
Patients	62 (100.0)	62 (100.0)		
Age at transplantation, years			−0.042*	.967
< 18	15 (24.2)	13 (21.0)		
18-40	39 (62.9)	43 (69.4)		
> 40	8 (12.9)	6 (9.7)		
Sex,			0.032†	.857
Male	29 (46.8)	30 (48.4)		
Female	33 (53.2)	32 (51.6)		
Diagnosis of FAB			0.256†	.880
AML	43 (69.4)	42 (67.7)		
ALL	14 (22.6)	16 (25.8)		
MDS	5 (8.1)	4 (6.5)		
Disease status before HSCT			3.347†	.188
CR1	48 (77.4)	39 (62.9)		
CR2	8 (12.9)	15 (24.2)		
PR	6 (9.7)	8 (12.9)		
HLA compatibility			0.553†	.759
1-loci-mismatched	5 (8.1)	3 (4.8)		
2-loci-mismatched A, B	20 (32.3)	20 (32.3)		
3-loci-mismatched A, DRB1	37 (59.7)	39 (62.9)		
Donor-recipient sex match			0.199†	.978
Female-female	16 (25.8)	14 (22.6)		
Female-male	17 (27.4)	18 (29.0)		
Male-male	16 (25.8)	16 (25.8)		
Male-female	13 (21.0)	14 (22.6)		
Donor-recipient relationship			1.340†	.512
Mother-child	12 (19.4)	10 (16.1)		
Father-child	17 (27.4)	23 (37.1)		
Siblings	33 (53.2)	29 (46.8)		
Prognostic risk category			0.575†	.750
Favorable	10 (16.1)	13 (21.0)		
Intermediate	26 (41.9)	26 (41.9)		
Unfavorable	26 (41.9)	23 (37.1)		

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CR, complete remission; FAB, French-British-American; HSCT, hematopoietic stem-cell transplantation; MDS, myelodysplastic syndrome; MSC, mesenchymal stromal cell; PR, partial remission.
*Mann-Whitney test.
†χ^2 test.

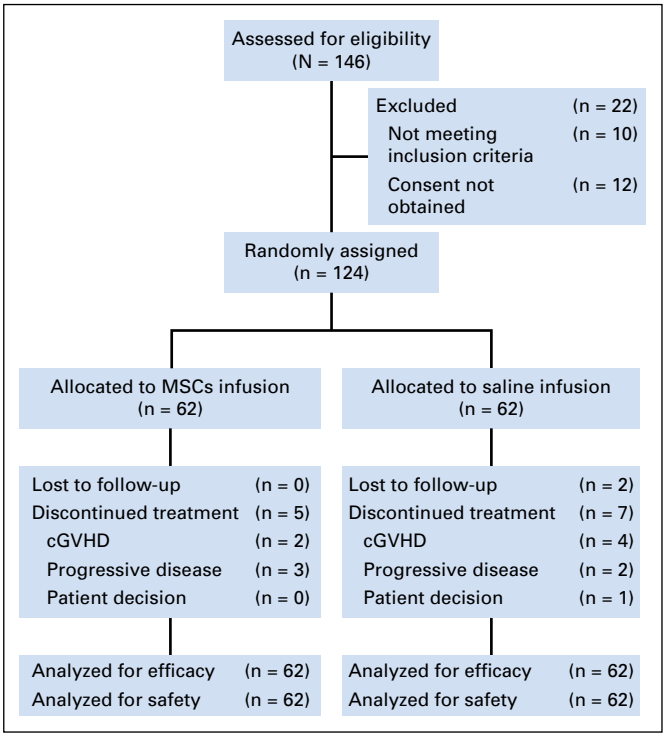

Fig 1. Flowchart of patients. cGVHD, chronic graft-versus-host disease; MSC, mesenchymal stromal cell.

Lymphocyte Subset Analysis

Flow cytometry analysis was performed to measure the lymphocyte subsets in the two groups. There were no significant changes in the proportions of T or B cells in either the MSCs group or the non-MSCs control group ($P > .05$; Figs 3A and 3B). In contrast, NK cells decreased to a certain extent at different time points after MSC infusions (Fig 3C). An analysis of the T-lymphocyte subsets indicated that the number of $CD4^+CD25^+CD127^-$ regulatory T (Treg) cells in the MSCs group was higher than that in the non-MSCs control group ($P < .05$; Fig 3D), although the numbers of $CD3^+CD4^+$ cells were not significantly different between the two groups ($P > .05$; Fig 3E). Next, we analyzed $CD27^+$ memory B lymphocytes and $CD27^-$-naïve B lymphocytes. The results revealed that the $CD27^+$ memory B-lymphocyte numbers were significantly increased after MSC infusion compared with pretreatment values ($P < .05$; Fig 3F).

We also evaluated changes in the ratio of type 1 T helper (Th1) cells to Th2 cells before and after the infusion with MSCs. The results demonstrated that the Th1 (interferon [IFN]-γ^+):Th2 interleukin (IL)-4^+) cell ratio increased almost two-fold after four MSC infusions (from 1.37 to 2.68; $P < .05$; Figs 3G and 3H). This result

Table 2. Clinical Outcomes of the MSCs and Non-MSCs Groups After Haploidentical Hematopoietic Stem-Cell Transplantation

Outcome	MSCs	Non-MSCs	Statistical Test Result	*P*
cGVHD, No. (%)			−2.596*	.009
None	45 (72.6)	32 (51.6)		
Mild	8 (12.9)	9 (14.5)		
Moderate	6 (9.7)	13 (21.0)		
Severe	3 (4.8)	8 (12.9)		
Total	17 (27.4)	30 (48.4)	5.791†	.016
Cumulative incidence, % (95% CI)				
Total cGVHD	27.4 (16.2 to 38.6)	49.0 (36.5 to 61.5)	5.302‡	.021
Mild cGVHD	14.2 (5.0 to 23.4)	18.4 (7.4 to 29.4)	0.254‡	.614
Moderate cGVHD	10.5 (2.5 to 18.5)	25.5 (13.3 to 37.7)	3.486‡	.062
Severe cGVHD	4.9 (0 to 10.4)	15.2 (4.8 to 25.6)	2.573‡	.109
Signs and symptoms of cGVHD, No. (%)				
Skin	7 (11.3)	18 (29.0)		.307‡
Nails	5 (8.1)	7 (11.3)		.689‡
Scalp and body hair	6 (9.7)	7 (11.3)		.694‡
Mouth	9 (14.5)	12 (19.4)		.295‡
Eyes	4 (6.5)	5 (8.1)		.656‡
Genitalia	0	1 (1.6)		1.000‡
GI tract	1 (1.6)	2 (3.2)		1.000‡
Liver	3 (4.8)	4 (6.5)		.669‡
Lung	0	7 (11.3)		.047‡
Muscles	1 (1.6)	2 (3.2)		.538‡
Cause of death			0.641†	.726
Infection	3 (4.8)	5 (8.1)		
Relapse	18 (29.0)	19 (30.6)		
2-year OS, % (95% CI)	66.1 (54.3 to 77.9)	61.3 (49.1 to 73.5)	0.254‡	.614
2-year DFS, % (95% CI)	64.5 (52.5 to 76.5)	59.7 (47.5 to 71.9)	0.246‡	.620

Abbreviations: DFS, disease-free survival; cGVHD, chronic graft-versus-host disease; OS, overall survival.
*Mann-Whitney test.
†χ^2 test.
‡Kaplan-Meier analysis and log-rank test.

indicates that the Th0 cells developed a differentiation imbalance and tended to become Th2 cells before the treatment with MSCs. After four MSC infusions, the proportion of Th1 cells gradually increased and the proportion of Th2 cells gradually decreased. Specifically, these results indicate that infusion with MSCs plays a role in downregulating excessive Th0-to-Th2 differentiation and increasing the proportion of Th1 cells, thereby reversing the Th1:Th2 cell imbalance in patients after HSCT. However, the increasing trend in the Th1:Th2 cell ratio disappeared after day 28.

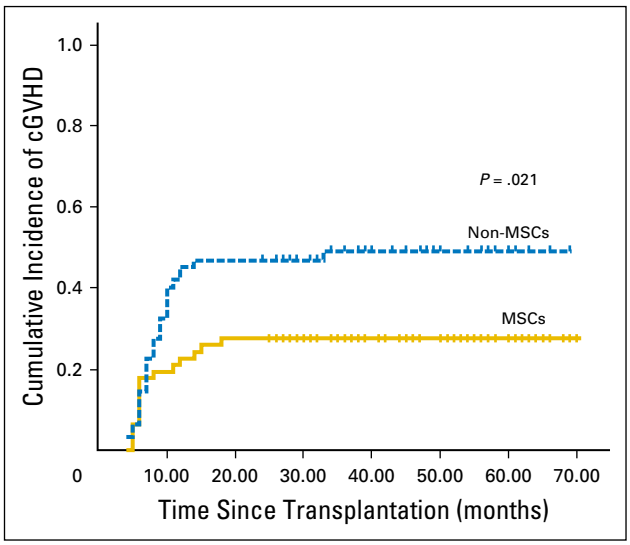

Fig 2. The cumulative incidence of chronic graft-versus-host disease (cGVHD). MSC, mesenchymal stromal cell.

Relapse Incidence

The estimated cumulative incidences of relapse were 30.6% (95% CI, 19% to 42.2%) and 32.3% (95% CI, 20.7% to 43.9%) in the MSCs group and the non-MSCs control group, respectively ($P > .05$; Fig 4A). The rescue therapy used to treat relapse included donor lymphocyte infusion (DLI) in nine patients, chemotherapy in seven patients, DLI and chemotherapy in 10 patients, and HLA-haplo HSCT from other haploidentical donors in four patients. Seven patients did not receive any treatment because of a rapid, aggressive relapse. Two patients achieved complete remission again after the second transplantation, whereas 35 patients died (25 died of leukemia, eight died of serious infections during the bone marrow suppression, and two died of transplantation-related mortality after a second transplantation).

Safety

A total of 230 infusions of MSCs were administered to 62 patients. All infusions were well tolerated, with no acute infusional toxicity and no adverse events associated with MSC infusions. Defined per Common Terminology Criteria for Adverse Events version 4, 85 patients experienced at least one treatment-emergent adverse event (TEAE): 45 (72.6%) in the MSCs group and 40 (64.5%) in the non-

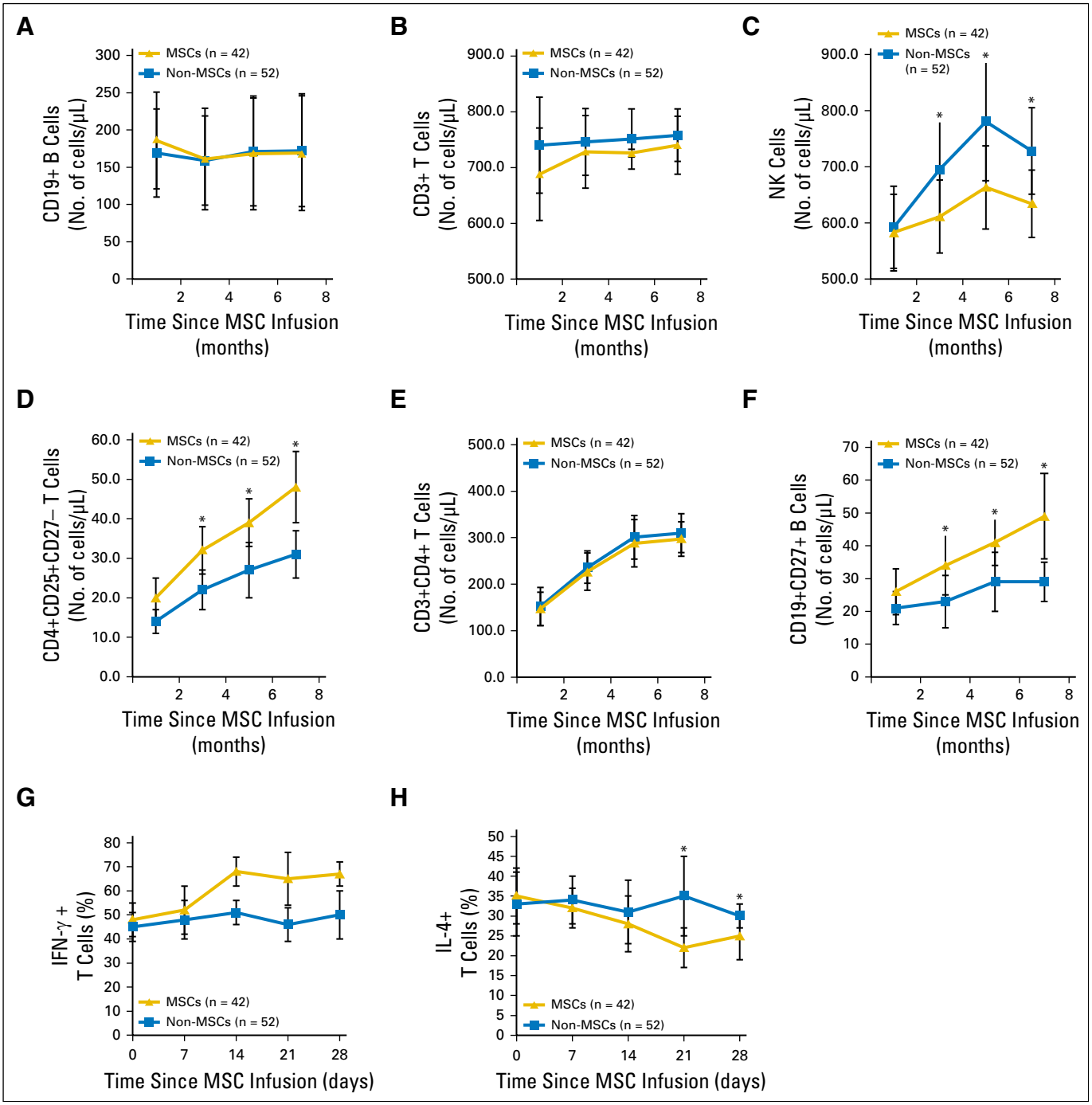

Fig 3. Changes in the mean absolute counts of lymphocyte subsets and the percentage of type 1 and type 2 T helper lymphocytes after mesenchymal stromal cell (MSC) infusion (n = 42 in the MSCs group and n = 52 in the non-MSCs control group). (A) $CD19^+$ B cells; (B) $CD3^+$ T cells; (C) NK cells; (D) $CD4^+CD25^+CD127^-$ T cells; (E) $CD3^+CD4^+$ T cells; (F) $CD19^+CD27^+$ B cells; (G)INF-γ^+ T cells; (H) IL-4^+ T cells. IFN, interferon; IL, interleukin; NK, natural killer.

MSCs control group. The most common TEAEs were related to infections and grade 1–2 liver dysfunction and renal impairment. There was no correlation between MSC dose and toxicity grade.

Survival

A total of 41 patients in the MSCs group and 38 patients in the non-MSCs control group were still alive at the median follow-up of 51 months (range, 24–70 months). The causes of transplant-related mortality included leukemia relapse in 37 cases and severe infection in eight cases. The 2-year probabilities of DFS and OS in the MSCs group compared with the non-MSCs control group were 64.5% (95% CI, 52.5% to 76.5%) versus 59.7% (95% CI, 47.5% to 71.9%) and 66.1% (95% CI, 54.3% to 77.9%) versus 61.3% (95% CI, 49.1% to 73.5%), respectively ($P > .05$; Table 2; Fig 4).

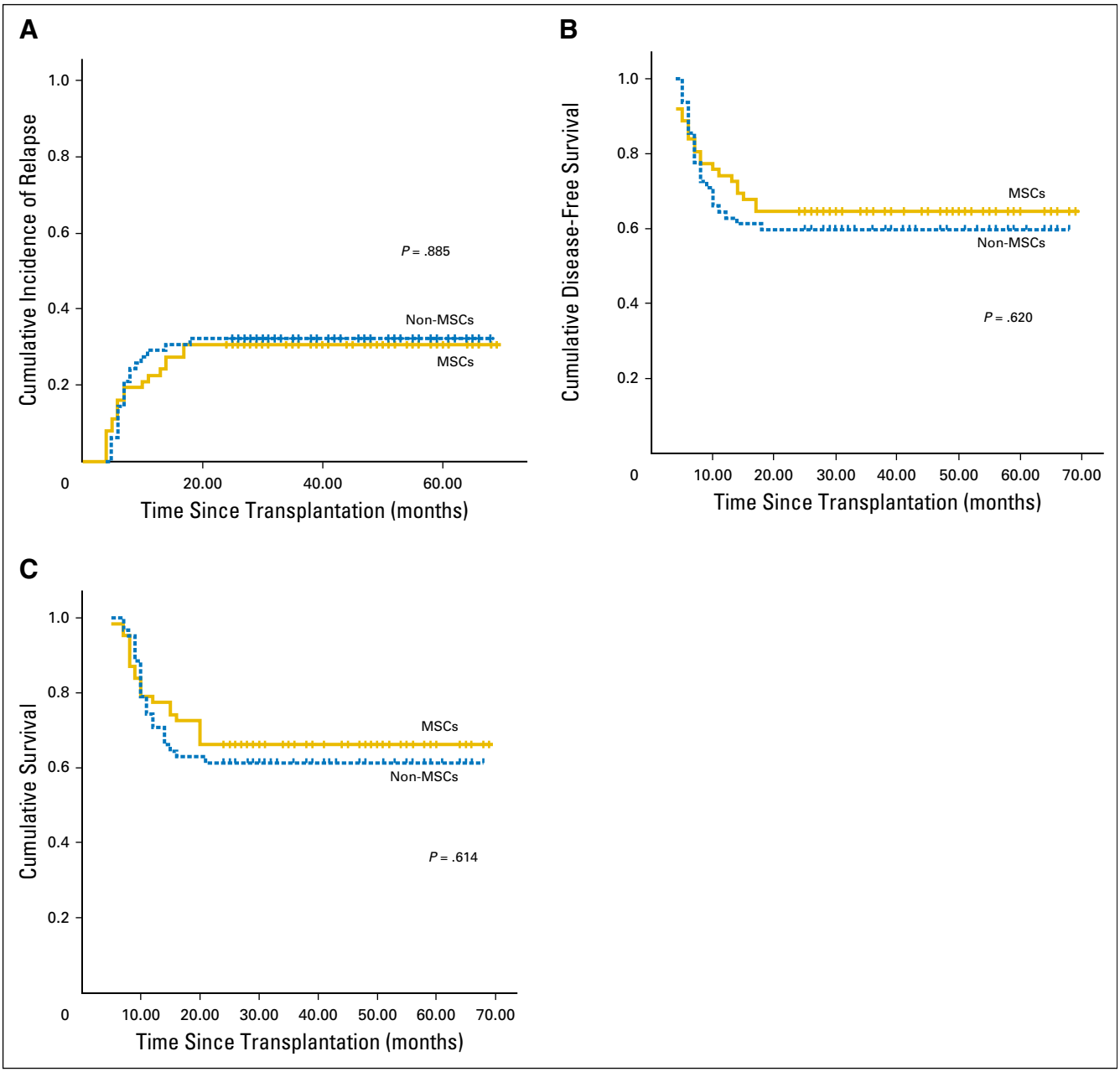

Fig 4. (A) Relapse rate, (B) disease-free survival, and (C) overall survival of patients in the two groups. MSC, mesenchymal stromal cell.

DISCUSSION

In this study, we investigated MSC infusions as a strategy for cGVHD prophylaxis. Our goal was to minimize the incidence of cGVHD, reduce the severity of cGVHD, and demonstrate the safety of MSC infusions. We performed repeated infusions of MSCs once a month for a total of four rounds for each patient. Over the median 47-month posttransplantation period, the incidence of cGVHD was lower in the MSCs group than in the non-MSCs control group.

Chronic GVHD is a common and often severe condition affecting long-term survivors of allo-HSCT. Although the condition has been the subject of extensive research, the pathogenesis behind the syndrome remains elusive.[36] Significant advances in our understanding of human cGVHD have been made in recent years. Data in humans support that both T and B cells are involved in a highly complex network leading to cGVHD. However, it is not clear how these T- and B-cell networks interact.[4,37]

In our study, although the number of T cells did not change after MSC infusion, we found that the number of Treg cells and the ratio of Th1:Th2 cells were increased. Currently, the relationship between the number of Tregs in the blood and the onset of cGVHD is controversial, with some reports demonstrating that reduced numbers of $Foxp3^+$ or $CD4^+CD25^+$ Tregs are associated with cGVHD,[38] whereas other studies indicate that higher absolute numbers of circulating $CD4^+CD25^+$ Tregs are found in patients with cGVHD after allo-HSCT.[39] Our study confirmed that the

infusion of MSCs increased the number of Treg cells and decreased the incidence of cGVHD. This result indicates that Treg cells may play an inhibitory role in cGVHD. The Th1:Th2 ratio is considered a major factor reflecting immune status.[40] Cell imbalances represent an important pathologic mechanism and has been receiving increasing attention in studies of immune system disorders. An increase in Th2 cells is an important factor in the development of fibrosis.[41,42] Transplantation of MSCs also has good therapeutic effects in certain Th2-increase-related immune disorders, such as acute-phase fibrosis.[43,44] cGVHD is an autoimmune-like systemic syndrome and is associated with fibroproliferative changes.[45] Some studies have suggested that the proportion of Th2 cells increases in patients with cGVHD.[46] In this study, we observed a decreased proportion of Th2 cells after prophylactic MSC infusion. This finding suggests that adjustment of the Th1:Th2 cell ratio is a potential mechanism by which MSC infusion inhibits cGVHD.

The role of B cells in the pathogenesis of cGVHD has attracted much more attention because the clinical manifestations of cGVHD are similar to those of autoimmune disorders.[47,48] Recent studies have proposed that B-cell reconstitution after allo-HSCT is involved in the development of cGVHD.[49,50] Patients who develop cGVHD exhibit a deficiency of memory $CD27^+$ B lymphocytes.[51] Recent studies have shown that an enhanced $CD27^+$ B-lymphocyte population is important for long-term allograft acceptance after renal transplantation.[52] We observed that MSC infusion significantly increased the frequency and the number of $CD27^+$ memory B lymphocytes, suggesting that MSC prophylaxis most likely affected the memory B-cell subpopulation. In addition, we found that the number of NK cells decreased after the infusion of MSCs. Zhao et al[53] demonstrated that a high concentration of NK cells in an allograft is associated with an increased risk of acute and chronic GVHD. The observed decrease in NK cells, thus, may be one of the reasons for the lower incidence of cGVHD in the MSCs group.

In this study, we found MSCs have potential applications in the prevention of lung cGVHD. Bronchiolitis obliterans (BO) is a typical manifestation of lung cGVHD, characterized by subepithelial inflammatory and fibrotic narrowing of the bronchioles.[54] To date, there has not been a specific study evaluating the prophylaxis of BO after HSCT. Evidence that MSCs attenuate BO in murine orthotopic tracheal transplantation may help to explain the potential mechanism. Guo et al[55] found that MSCs can decrease the innate inflammatory and prevent allograft rejection by downregulating the levels of IL-6 and tumor necrosis factor-α, and increasing IL-10 production, respectively. Zhao et al[56] also found that treatment with placenta-derived MSCs is protective against the development of BO in a heterotopic tracheal transplant model. We, therefore, conjecture that the mechanism by which MSC treatment impacts BO is associated with the regulation of donor T lymphocytes, inflammatory mediators, and cytokines.

It is a debatable clinical point whether the incidence of relapse increases after MSC infusion. Previous studies have indicated that MSC infusions can increase the risk of leukemia recurrence.[57] However, in this study, the estimated cumulative incidences of relapse were not significantly different between the MSCs group and the control group, from the initial MSC infusion to the end of the follow-up period. In addition, no MSC infusion-related adverse reactions or effects were observed, suggesting that MSC infusion is safe and reliable.

In conclusion, we demonstrate for the first time, to our knowledge, that reduplicative infusion of MSCs after HLA-haplo HSCT can reduce the incidence of cGVHD, accompanied by changes in the number of Treg cells, memory B lymphocytes, and NK cells, as well as the Th1:Th2 cell ratio. Future studies to adjust the MSC infusion program and understand the underlying mechanism will enable us to elucidate the in vivo effects of MSCs and help facilitate their broad application.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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Manuscript writing: All authors
Final approval of manuscript: All authors

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Phase II Multicenter, Randomized, Double-Blind Controlled Study of Efficacy and Safety of Umbilical Cord–Derived Mesenchymal Stromal Cells in the Prophylaxis of Chronic Graft-Versus-Host Disease After HLA-Haploidentical Stem-Cell Transplantation

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Appendix

Preparation of Mesenchymal Stromal Cells

Umbilical cord segments (5 to 10 cm) were sectioned longitudinally to expose Wharton's jelly. Incisions were made on the matrix with a sterile scalpel to expose a wider area of tissue to be in contact with the plastic surface. The cord sections were then transferred to a 10-cm^2 Petri dish and cultured for 5 days in Dulbecco's modified Eagle's medium (GE Healthcare, Logan, UT) with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) and 0.5% antibiotic–antimycotic solution (Sigma-Aldrich). Cultures were maintained in a humidified atmosphere with 5% carbon dioxide at 37°C. After 5 days, the cord segments were discarded, and the new medium was added. At 70% to 80% confluence, cells were detached by trypsin-EDTA and passaged at a ratio of one to three. Multiple individual donor preparations were performed, and second or third passage mesenchymal stromal cells (MSCs) were used for individual experiments. Before infusion, cells were examined for MSC characteristics. Isolated cells showed positivity for CD44, CD73, CD90, CD105, and CD166, and negativity for CD34, CD45, and CD11a. They inhibited the mixed-lymphocyte reaction and could be differentiated to fat cells, chondrocytes, and osteoblasts. Multicolor fluorescence in situ hybridization showed that the cells had no chromosomal abnormalities. No infectious agents, such as bacteria, mycoplasma, or viruses, were detected in the cell supernatants or the cells themselves. No endotoxins were detected in the supernatant.

Analysis of Lymphocyte Subset and Intracellular Cytokines

Peripheral blood mononuclear cells (PBMCs) were stained with the following antibodies: CD3, CD8, CD4, CD25, CD27, CD19, CD45, CD16, CD56, and CD127 (BD Biosciences Pharmingen, San Diego, CA). To stimulate interferon (IFN)-γ production, the PBMCs were stimulated for 6 hours with 5 ng/mL phorbol myristate acetate (PMA) and 500 ng/mL ionomycin in the presence of a protein-transport inhibitor containing monensin (GolgiStop; BD Biosciences, San Jose, CA). To stimulate interleukin (IL)-4 production, the PBMCs were stimulated for 2 days with 50 mg/mL of PHA and then cultured in medium containing 10 ng/mL of rhIL-2 and 20 ng/mL of rhIL-4 for 3 days (all from Sigma-Aldrich). Finally, the cells were harvested and restimulated for 4 hours with PMA and ionomycin in the presence of GolgiStop. Immunofluorescence staining of intracellular cytokines and flow cytometric analysis were also performed as routine method.