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Stem Cells Translational Medicine, 2023, **XX**, 1–13
https://doi.org/10.1093/stcltm/szad010
Advance access publication 16 March 2023
Human Clinical Article

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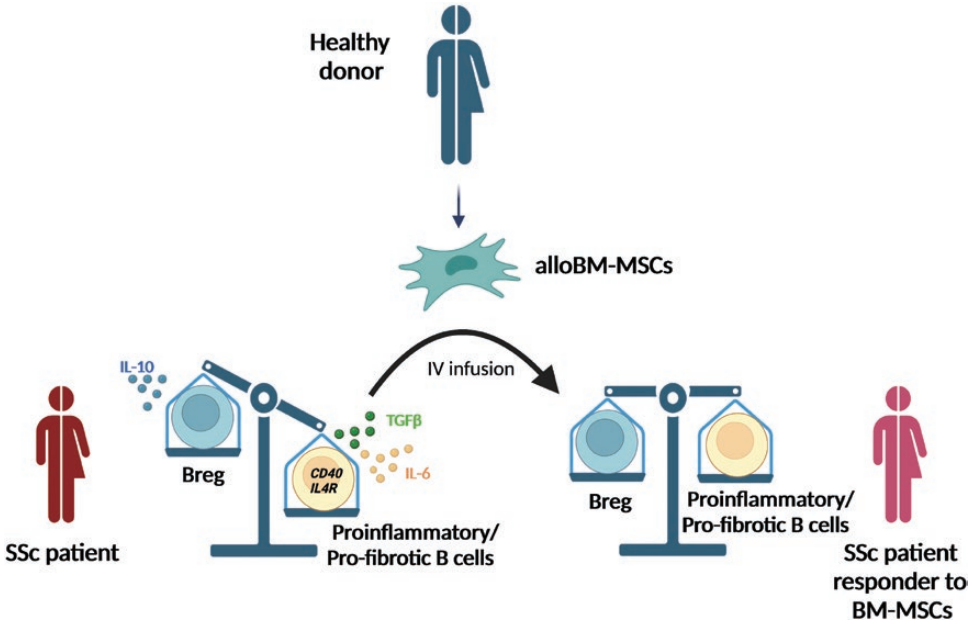
Abstract

Mesenchymal stromal cells (MSCs) have recently emerged as an interesting therapeutic approach for patients with progressive systemic sclerosis (SSc), a rare and life-threatening orphan autoimmune disease. Whereas MSC immunomodulatory potential is considered as a central mechanism for their clinical benefit, very few data are available on the impact of MSCs on immune cell subsets in vivo. In the current extended study of a phase I/II clinical trial exploring the injection of a single dose of allogeneic bone marrow-MSCs (alloBM-MSCs) in patients with severe SSc (NCT02213705), we performed a longitudinal in-depth characterization of circulating immune cells in 19 MSC-treated patients, including 14 responders and 5 non-responders. By a combination of flow cytometry and transcriptomic analyses, we highlighted an increase in circulating $CD24^{hi}CD27^{pos}CD38^{lo/neg}$ memory B cells, the main IL-10-producing regulatory B cell (Breg) subset, and an upregulation of *IL10* expression in ex-vivo purified B cells, specifically in responder patients, early after the alloBM-MSC infusion. In addition, a deeper alteration of the B-cell compartment before alloBM-MSC treatment, including a higher expression of profibrotic cytokines *IL6* and *TGF*β by sorted B cells was associated with a non-responder clinical status. Finally, BM-MSCs were able to directly upregulate IL-10 production in activated B cells in vitro. These data suggest that cytokine-producing B cells, in particular Breg, are pivotal effectors of BM-MSC therapeutic activity in SSc. Their quantification as activity biomarkers in MSC potency assays and patient selection criteria may be considered to reach optimal clinical benefit when designing MSC-based clinical trials.

Key words: Breg; mesenchymal stromal cells; immune monitoring; systemic sclerosis; clinical trial.

Received: 23 November 2022; Accepted: 9 February 2023.

Graphical Abstract



Significance Statement

The clinical use of mesenchymal stromal cells (MSC) is yet limited by our poor understanding of their mechanisms of action in vivo. This study evaluated the relationship between patient outcome and biological effects of MSC infusion in patients suffering from systemic sclerosis, a life-threatening orphan autoimmune disease, identifying the reprogramming of B cells as a key driver of MSC clinical activity. Such data illustrate how a detailed follow-up of patient immune status could help in designing MSC-based clinical trials, based on the identification of predictive biomarkers and MSC potency assays.

Introduction

Systemic sclerosis (SSc) is a rare and severe orphan autoimmune disease characterized by a diffuse vasculopathy, with innate immune cell activation, and dysfunctional T-cell and B-cell homeostasis including autoimmune reactivity, all resulting in progressive fibrosis of the skin and the internal organs.[1] Despite modest clinical improvement with the use of standard immunosuppressive drugs, targeted therapies against B cells or interleukin (IL)-6, or the antifibrotic nintedanib, all recently approved for treating SSc, diffuse SSc disease remains a major clinical challenge with a 5-year survival rate of 50%-70% depending on the extent of organ involvement. In severe progressive SSc patients, autologous hematopoietic stem cell transplantation (AHSCT) was proven to trigger a sustained survival benefit with effective regression of skin and lung fibrosis.[2,3] Clinical response to AHSCT is associated with a resetting of the immune system leading to a depletion of autoreactive clones and a renewal of the T-cell repertoire.[4,5] However, AHSCT remains contraindicated in patients with advanced visceral involvement and can be associated with significant toxicity. In this context, mesenchymal stromal cells (MSCs) were recently considered as an appealing immune reprogramming strategy in severe SSc patients.[6]

MSCs, obtained from bone marrow (BM-MSCs), adipose tissue (ASCs), or umbilical cord (UC-MSCs) produce proangiogenic and anti-fibrotic factors driving paracrine tissue regeneration,[7] and exert immunoregulatory functions on both the innate and adaptive immune responses, in particular after priming by inflammatory stimuli.[8,9] A wide set of mechanisms have been involved in MSC immunosuppressive functions, including the production of immunosuppressive enzymes, cytokines, and metabolites, the release of chemokines, or the expression of immune checkpoints.[10] Although a large number of preclinical studies were designed to characterize MSC immunomodulatory effects in vitro and in mouse models in vivo, MSC precise mechanisms of action in treated patients remain elusive. Indeed, the clinical response to MSC treatment may be affected by several parameters, including MSC tissue source and production process, recipient clinical features, and protocol design.[11,12] One strategy to improve our understanding of the effects of MSCs in vivo is to establish longitudinal bio-monitoring of MSC-treated patients, looking for biomarkers of the observed clinical response, to be translated into relevant potency assays. Clinical trials using MSC infusion in various clinical conditions had shown no major impact on circulating B- and T-cell numbers, even if an increase in the percentage of regulatory T cells (Treg) has been regularly reported.[13-17] Other MSC-induced immune alterations have been inconsistently described in clinical use, including the increase of NK or memory B-cell counts, the elevation of transitional B-cell percentage, or the decrease in circulating monocytes.[13,16,17] These data were obtained with very low numbers of highly heterogeneous patients and poorly correlated with the observed clinical outcome. Additional monitoring approaches, including functional studies, are needed to better capture the biological impact of MSCs in patients and how it could explain their clinical efficacy.

MSCs were reported to display anti-inflammatory and anti-fibrotic activities in several animal models of SSc.[18-20] However, very few clinical trials evaluated the systemic infusion of MSCs in patients with SSc. In the current extended study of a recently conducted phase I/II clinical trial exploring the injection of a single dose of allogeneic bone marrow-MSCs (alloBM-MSCs) in patients with severe SSc (NCT02213705),[21] we performed a longitudinal in-depth phenotypic and functional characterization of circulating immune cell subsets in alloBM-MSC treated patients, looking for both quantitative and qualitative modifications and correlation with clinical response. Altogether, our data shed new light on the mechanism of action of MSCs and would be useful for the design of new clinically relevant potency assays.

Patients, Materials, and Methods

Patients and Samples

This extended study was designed as the in-depth phenotypic and functional immunomonitoring analysis of the patients with severe diffuse SSc treated with a single intravenous injection of alloBM-MSCs as enrolled in an open-label, phase I/II, single-center study conducted at Saint-Louis Hospital in Paris (France) between March 24, 2014, and January 6, 2020 (NCT02213705).[21] Each patient received a unique batch of clinical-grade alloBM-MSCs obtained from a different BM donor and fully qualified at the phenotypic, transcriptomic, karyotypic, and functional levels. All alloBM-MSC products were used at the end of passage 1. As prespecified in the clinical study protocol, the clinical response was defined as a greater than 25% decrease in modified Rodnan skin score or a greater than 10% increase in forced vital capacity or pulmonary diffusion capacity for carbon monoxide, or both, without additional immunosuppressive treatment, except low-dose steroids. Considering the clinical response at 3 or 6 months after alloBM-MSC infusion, patients were classified as responders (R, n = 15) and as non-responders (NR, n = 5).[21]

Heparinized blood samples were collected before (D0), at 1 month (M1), and at 3 months (M3) after alloBM-MSC treatment for 19 out of 20 patients. Flow cytometry analyses were performed on fresh blood samples. When enough collected cells were available, peripheral blood mononuclear cells (PBMCs) obtained by density gradient centrifugation on lymphocyte separation medium (Eurobio) were frozen for further experiments (n = 10 patients with enough PBMCs at the 3 timepoints). The biological studies performed on each SSC patient included in the clinical trial and their respective clinical features have been detailed in Supplementary Tables S1 and S2. For this extended study, control blood samples from sex- and age-matched healthy donors (HD), who had provided written informed consent, were obtained from the *Etablissement Français du Sang*. For additional in vitro studies of interactions, BM aspirates were obtained from patients undergoing cardiac surgery following institutional review board approval and written informed content process and were used to generate research-grade BM-MSCs (rgBM-MSCs).

Blood Immune Cell Phenotyping

Circulating immune cell subpopulation frequencies and activation status were analyzed by flow cytometry using whole blood (for T-cell populations) or freshly prepared PBMCs (for B-cell subsets) using specific fluorochrome-conjugated antibodies (Supplementary Table S3). Following incubation with antibodies, red blood cells were lysed (Easylyse, DAKO) and cells were washed twice (PBS) before staining with fixable viability stain (BD Biosciences) and acquisition on a FACS LSR Fortessa X20 flow cytometer. T-, B-, and NK-cell absolute quantification was performed on whole blood using the Multitest reagent (CD3/CD16 + 56/CD45/CD19, BD Bioscience) and count beads (Biolegend).

After gating on viable $CD19^{pos}$ cells, we quantified $CD27^{pos}CD38^{neg/lo}$ memory B cells, including the $CD24^{hi}$ subset, $CD27^{hi}CD38^{hi}CD24^{neg}$ plasmablasts (PB)/plasma cells (PC), and $CD24^{hi}CD38^{hi}$ transitional B cells. Similarly, after gating on viable $CD3^{pos}$ T cells, we distinguished $CD4^{pos}$ from $CD8^{pos}$ T cells, and among them, we quantified $CD45RA^{pos}CCR7^{pos}$ naive T cells, $CD45RA^{neg}CCR7^{neg}$ effector memory (EM) T cells, $CD45RA^{neg}$ $CCR7^{pos}$ central memory (CM) T cells and $CD45RA^{pos}CCR7^{neg}$ terminally differentiated (EMRA) T cells. T-cell activation was also evaluated through quantification of HLA-DR expression and $CD3^{pos}CD4^{pos}CD25^{hi}Foxp3^{pos}$ Treg were followed as immunosuppressive cells of interest. Analyses were performed using Kaluza (Beckman Coulter) software.

Real Time Quantitative PCR (Q-PCR) Analysis

Viable B cells were sorted from frozen PBMCs of SSc patients (n = 9 selected only based on the frozen PBMC availability) and HD as $CD3^{neg}CD14^{neg}CD16^{neg}$ $CD19^{pos}DAPI^{neg}$ cells and from in vitro activated HD B cells maintained in the presence or not of rgBM-MSCs for 2 days as $DAPI^{neg}CD19^{pos}CD105^{neg}$ cells, both using FACSAria Fusion cell sorter (BD Biosciences) with a purity >99% (see Supplementary Table S3 for detailed antibody references and Supplementary Fig. S1 for gating strategies). Sorted B cells were then washed twice with PBS and resuspended in 200 µL of lysis buffer for storage at −20 °C before RNA extraction using RNA-XS Nucleospin kit (Machery-Nagel). Gene expression level was then assessed using the Fluidigm BioMark HD system. Briefly, cDNAs were obtained using the Fluidigm reverse transcription Master Mix and pre-amplified for 14 cycles in the presence of Pre-Amp Master Mix and pooled Taqman assay mix. Gene expression of 46 genes relevant to B-cell biology and interaction with MSCs (Supplementary Table S4) was measured with the TaqMan Gene Expression Master Mix (Thermo Fisher) on a 48.48 Dynamic Array IFC compatible with the BioMark instrument (Fluidigm). Gene expression was then quantified based on the ΔCT calculation method and PCR data were normalized to the geometric mean of *ABL1, B2M*, and *GAPDH* used as housekeeping genes. Results were next standardized by comparison to gene expression of a pool of tonsil B cells, thus generating standardized relative expressions. For genes undetectable at one point, the ΔΔCT was calculated with an arbitrary C_T value of 30.

Quantification of B-Cells Producing IL-10

B cells were purified from frozen PBMCs by negative selection using the B-cell isolation kit (Miltenyi Biotec, purity >96%) and activated in vitro by 200 ng/mL CD40 ligand (Immunex), 0.5 µg/mL CpG B (ODN 2006, Invivogen), and 50 IU/ml IL-2 (Proleukin) in RPMI supplemented with 10% fetal calf serum (FCS, Gibco). After 4 days of activation, B cells were re-stimulated for 5 h using 0.05 µg/mL

phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 µg/mL ionomycine (Sigma) in the presence or not of 1/1000 Golgi Plug (BD Biosciences), before fixation, permeabilization, and intracellular staining (Cytofix/cytoperm BD kit) with fluorochrome conjugated anti-CD19 and anti-IL-10 antibodies (BD Biosciences, Supplementary Table S3). B cells restimulated in the absence of Golgi Plus were used as a negative control.

In vitro Co-Culture of B Cells With BM-MSCs

rgBM-MSCs were obtained from HD frozen BM mononuclear cells as previously described.[22] Briefly BM mononuclear cells were thawed and seeded at 10^5 cells/cm^2 in αMEM supplemented with 10% screened FCS (Hyclone) and 1 ng/mL FGF-2 (R&D Systems). The entire medium was renewed every 3 days until the cells reached near confluence (the end of passage 0, P0). Cells were then further expanded in the same culture medium at 500 cells/cm^2 and harvested when reaching 80% confluence (passage 1, P1) for co-culture with B cells. Phenotypic criteria for MSC definition were validated according to ISCT standards[23] including the expression of CD73, CD105, and CD90 (>90%), and the lack of CD45 (<1%) as determined by flow cytometry (Supplementary Table S3).

P1 rgBM-MSCs were seeded at 6 × 10^5 cells/cm² corresponding to a confluent monolayer before adding peripheral blood B cells (ratio of 1B/ 1MSC) purified from HD (B-cell isolation kit, Miltenyi Biotec). B cells were activated as previously described[22] by 125 ng/mL soluble CD40 Ligand, 1 µg/mL CpG B, 2.6 µg/mL F(ab′)2 anti-human IgM/IgA/IgG (Jackson Immunoresearch), and 50 IU/mL IL-2 in RPMI supplemented with 10% FCS. After 48 h, activated HD B cells cultured with or without rgBM-MSCs were harvested, washed, and stained with anti-CD19 and anti-CD105 fluorochrome-conjugated antibodies and a viability marker (Supplementary Table S3) for sorting and transcriptional analysis. On day 5, cell culture supernatants from HD B cells activated in the presence or not of rgBM-MSCs were harvested and centrifuged for 10 min at 1900*g* to remove debris before storage at −80 °C. The production of IL-10 in the supernatants of HD B cells activated in the presence or not of rgBM-MSCs was measured with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems).

Statistical Analysis

Statistical analyses were performed with R version 4.1.2, and GraphPad Prism version 9.1.2. For paired analyses, global differences were analyzed by Friedman tests and when a significant difference was detected, pair-wise comparisons of the differences were performed using paired Wilcoxon signed rank tests. Comparisons of unpaired samples were performed using Mann-Whitney test.

Concerning Q-PCR data, differentially expressed genes (DEG) between HD and SSc patients at D0 were identified using a Mann-Whitney test. An individual score was then generated based on the geometric means of the 13 DEG. This 13 DEG-score was calculated as proposed in the MetaIntegrator R package[24] as the difference of the geometric mean of the expression levels of the genes upregulated in SSc compared to HD minus the geometric mean of the expression levels of the genes downregulated in SSc compared to HD and was then normalized to a *z*-score to center the samples around zero. The heatmap was generated with pheatmap version 1.0.12 R package.

Results

Impact of alloBM-MSC Infusion on Peripheral T-Cell Subsets in SSc Patients

Based on the standard immunological analysis of our phases I-II study, aiming to evaluate the effect of a single infusion of alloBM-MSCs in severe SSc patients,[21] we initially reported no B-cell and T-cell count modification, whereas NK-cell counts were increased at M1 and M3 after infusion.[21] Here, we first analyzed T-cell subsets by flow cytometry with fresh whole blood samples to avoid the variability induced by freeze/thaw cycles. We detected a transient increase of CD8^{pos} T-cell frequency and count at M1 ($P < .05$, Fig. 1A; Supplementary Fig. S2A, $n = 16$) together with marks of T-cell activation including a decrease in naive CD8^{pos} T-cell frequency and count at M3 ($P < .01$ and $P < .05$ respectively, $n = 16$), and an early upregulation of HLA-DR on both CD45RA^{neg}CCR7^{neg} EM and CD45RA^{pos}CCR7^{neg} EMRA CD8^{pos} T cells ($P < .01$, $n = 16$). Concerning CD4^{pos} T cells, no variation in the frequency of cell subsets or HLA-DR expression was observed, except a transient decrease of EM CD4^{pos} T-cell frequency and count at M1 ($P < .05$ and $P < .001$ respectively, $n = 16$, Fig. 1B; Supplementary Fig. S2B). No correlation was found between the patient clinical response, classified as R or NR to alloBM-MSC infusion, and the T-cell subset count nor phenotype (Supplementary Fig. S3A, S3B). Altogether, these data confirm the activation of cytotoxic T cells, unlike CD4^{pos} T cells, in SSc patients receiving alloBM-MSCs, independently from the observed clinical response.

Impact of alloBM-MSC Infusion on Peripheral B-Cell Subsets in SSc Patients

B-cell characterization was performed on freshly prepared PBMCs with a specific interest for the 3 main subsets previously reported to display regulatory functions in mice and humans: CD38^{hi}CD27^{hi} PB/PC, CD24^{hi}CD38^{hi} transitional B cells, and CD24^{hi}CD27^{pos}CD38$^{neg/lo}$ memory B cells[25-27] (Fig. 2A). Whereas PB/PC frequency and count were not affected by BM-MSC infusion in the whole cohort nor when considering R versus NR patients (Fig. 2B; Supplementary Fig. S3C), the percentage and number of transitional B cells were increased at M1 ($P < .001$ and $P < .05$ respectively, $n = 19$, Fig. 2C) independently from the clinical response (Fig. 2D). CD27^{pos} memory B cells were not modified by alloBM-MSC infusion (Fig. 2E; Supplementary Fig. S3C). Conversely, whereas the CD24^{hi}CD27^{pos} Breg subset was not modified by alloBM-MSC infusion when considering all treated patients (Fig. 2F), its frequency within CD19^{pos} B cells was increased in R compared to NR patients ($P < .05$, 5 R versus 14 NR patients, Fig. 2G) and was increased at M1 in the R patient subgroup ($P < .05$, $n = 14$, Fig. 2H). Unlike their frequency, CD24^{hi}CD27^{pos} Breg count was not significantly increased at M1 in R patients (data not shown). Overall, 2 Breg subsets, considered as the human counterpart of murine IL-10 producing B cells (B10),[25] were significantly increased after injection of alloBM-MSCs in severe SSc patients, either in all recipients or in patients with clinical improvement.

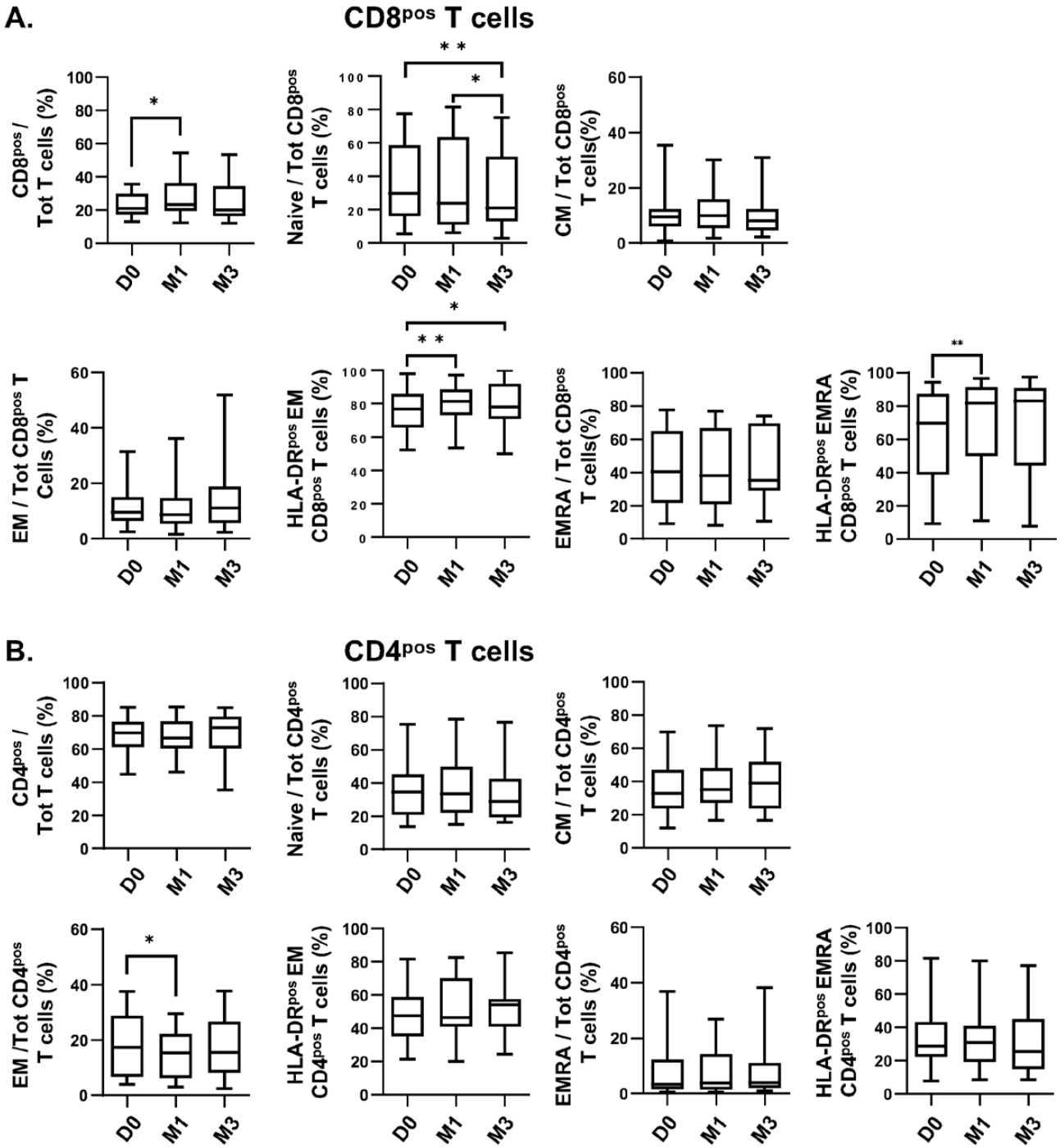


Figure 1. Impact of BM-MSC infusion on circulating T-cell subsets. (**A-B**) $CD8^{pos}$ (A) and $CD4^{pos}$ (B) T-cell subsets were analyzed by flow cytometry in the peripheral blood of SSc patients before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion ($n = 16$). The proportions of $CD45RA^{pos}CCR7^{pos}$ naive, $CD45RA^{neg}CCR7^{pos}$ central memory (CM), $CD45RA^{neg}CCR7^{neg}$ (EM), and $CD45RA^{pos}CCR7^{neg}$ (EMRA) subpopulations, as well as the expression of HLA-DR were studied. Boxplots represent the frequency (%) or numbers (per μL of blood) of the indicated subpopulations, with whiskers extended to the most extreme data points. $^{*}P < .05$; $^{**}P < .01$.

Upregulation of IL-10 Expression in B Cells From SSc Patients as a Marker of Clinical Response to Treatment by alloBM-MSCs

Given the potential involvement of B cells in both SSc disease activity and BM-MSC clinical impact, we performed a transcriptomic profiling by Q-PCR of B cells sorted from 9 SSc patients before treatment by alloBM-MSCs, using 7 age- and sex-matched HD B cells as a control. We selected 46 genes related to B-cell biology (Supplementary Table S4) and interaction with MSCs and identified a pattern of 13 DEG between B cells from severe diffuse SSc patients and HD ($P < .05$, Fig. 3A). In particular, 6 genes were found significantly upregulated in B cells from SSc patients, namely *IL6* and *TGFB*, previously involved in B-cell-dependent fibroblast activation in SSc,[28] as well as *CD40* and *IL4R*, supporting B-cell response to activation stimuli, and *MBP1/HIVEP1* and *IRF4*, simultaneously induced after anti-CD40/IL-4 stimulation.[29] Conversely, 7

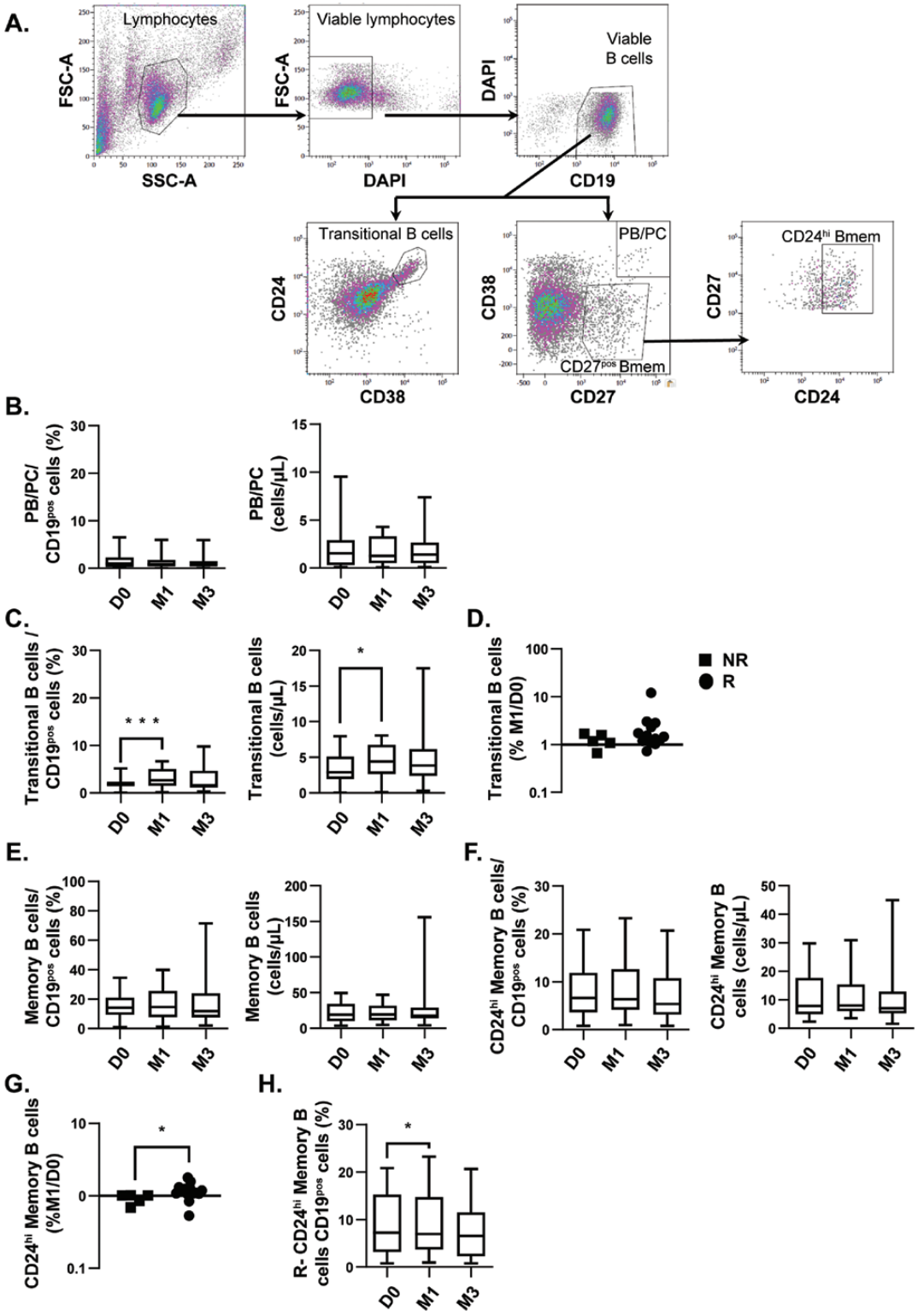


Figure 2. Impact of BM-MSC infusion on circulating B cell subsets. (**A**) Gating strategy for the analysis by flow cytometry of B cell subsets with regulatory functions. (**B**) The percentage (left) and number (right) of $CD27^{hi}CD38^{hi}$ plasmablasts/plasma cells (PB/PC) were evaluated before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion ($n = 19$). (**C**) The percentage (left) and number (right) of $CD24^{hi}CD38^{hi}$ transitional B cells were evaluated before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion ($n = 19$). (**D**) Distribution of the M1/D0 ratio of the frequency of $CD24^{hi}CD38^{hi}$ transitional B cells in non-responder (NR, $n = 5$) versus responder (R, $n = 14$) patients. (**E**) The percentage (left) and number (right) of total $CD27^{pos}$ memory B cells were evaluated before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion ($n = 19$). (**F**) The percentage (left) and number (right) of $CD24^{hi}CD27^{pos}$ memory B cells were evaluated before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion ($n = 19$). (**G**) Distribution of the M1/D0 ratio of the frequency of $CD24^{hi}CD27^{pos}$ memory B cells in non-responder (NR, $n = 5$) versus responder (R, $n = 14$) patients. (**H**) The percentage of $CD24^{hi}CD27^{pos}$ memory B cells were evaluated before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion in responder patients (R, $n = 14$). Boxplots represent the frequency (%) or numbers (per µL of blood) of the indicated subpopulations, with whiskers extended to the most extreme data points. $^{*}P < .05$; $^{**}P < .01$.

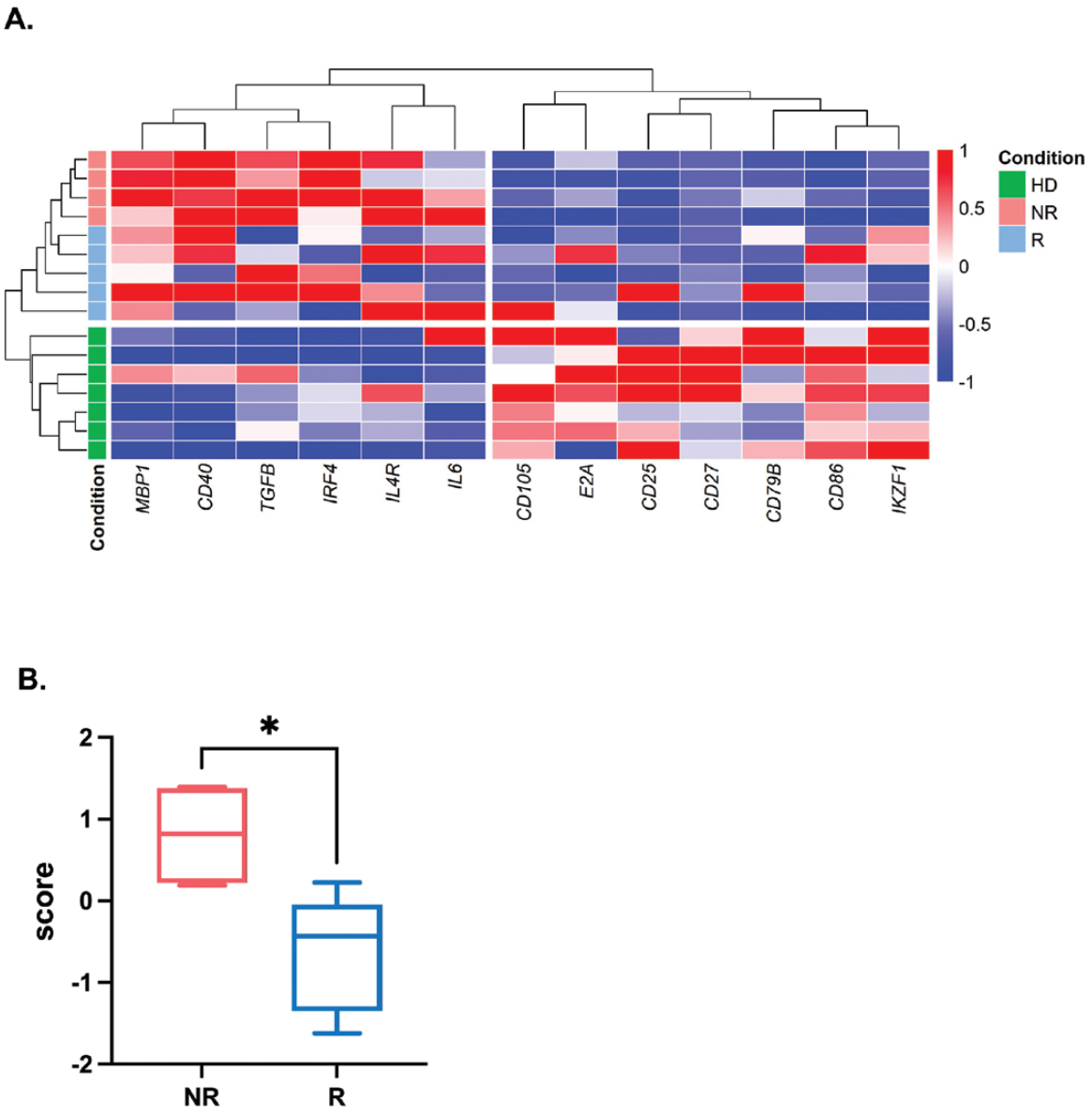


Figure 3. Alteration of the B-cell transcriptional profile in SSc patients before BM-MSC infusion. (**A**) B cells were sorted from frozen PBMCs obtained from healthy donors (HD, $n = 7$) or SSc patients ($n = 9$) before alloBM-MSC infusion, including 5 responders (R) and 4 non-responders (NR). The 13 differentially expressed genes (among the 46 tested by Q-PCR) between HD and SSc B cells (Mann-Whitney test, $P < .05$) are represented through a heatmap. Six genes *CD40, IL4R, IL6, IRF4, MBP1/HIVEP1,* and *TGFB* are upregulated in SSc patients compared to HD while 7 genes *CD105, CD25, CD27, CD79B, CD86, E2A,* and *IKZF1* are downregulated in SSc patients versus HD. (**B**) Boxplot of the gene score established for B cells from responder (R, $n = 5$) and non-responder (NR, $n = 4$) D0 SSc patients calculated based on the 13 differentially expressed genes identified between SSc patients at D0 and HD, with whiskers extended to the most extreme data points. *$P < .05$.

genes were significantly downregulated in B cells from SSc patients including the *CD27* marker, reflecting the reduced frequency of circulating memory B cells and PB/PC in SSc patients,[30] and others involved in B-cell differentiation, like *E2A* and *IKZF1*, or activation, like *CD25* and *CD86*. Of note, among the 9 SSc patients analyzed at D0, clinical NR patients segregated together based on the level of expression of the 13 DEG (Fig. 3A). We therefore calculated a gene score based on these 13 DEG, as the geometric mean of the expression levels of the 6 genes significantly upregulated in SSc patients at D0 compared to HD minus the geometric mean of the expression levels of the 7 genes significantly downregulated in SSc patients at D0 compared to HD. This gene score was significantly higher in NR ($n = 4$) than in R ($n = 5$) patients, essentially related to the overexpression of profibrotic and activation genes, ($P < .05$, Fig. 3B; Supplementary Fig. S4A, S4B). Altogether, these data highlight an overexpression of profibrotic factors in severe SSc patients, and suggest that a deeper alteration of B-cell homeostasis is associated with a lack of response to alloBM-MSC infusion.

To better capture the effect of BM-MSCs on B-cell behavior, we next studied how their gene expression profile was modified at M1 and M3 after alloBM-MSC infusion in the 9 SSc treated patients. Among the 46 genes tested, only 4 were differentially expressed at M1 ($P < .01$ for all of them, Fig. 4A). *CD40* and *IL4R*, overexpressed in B cells from SSc patients compared to HD (Fig. 3A), were

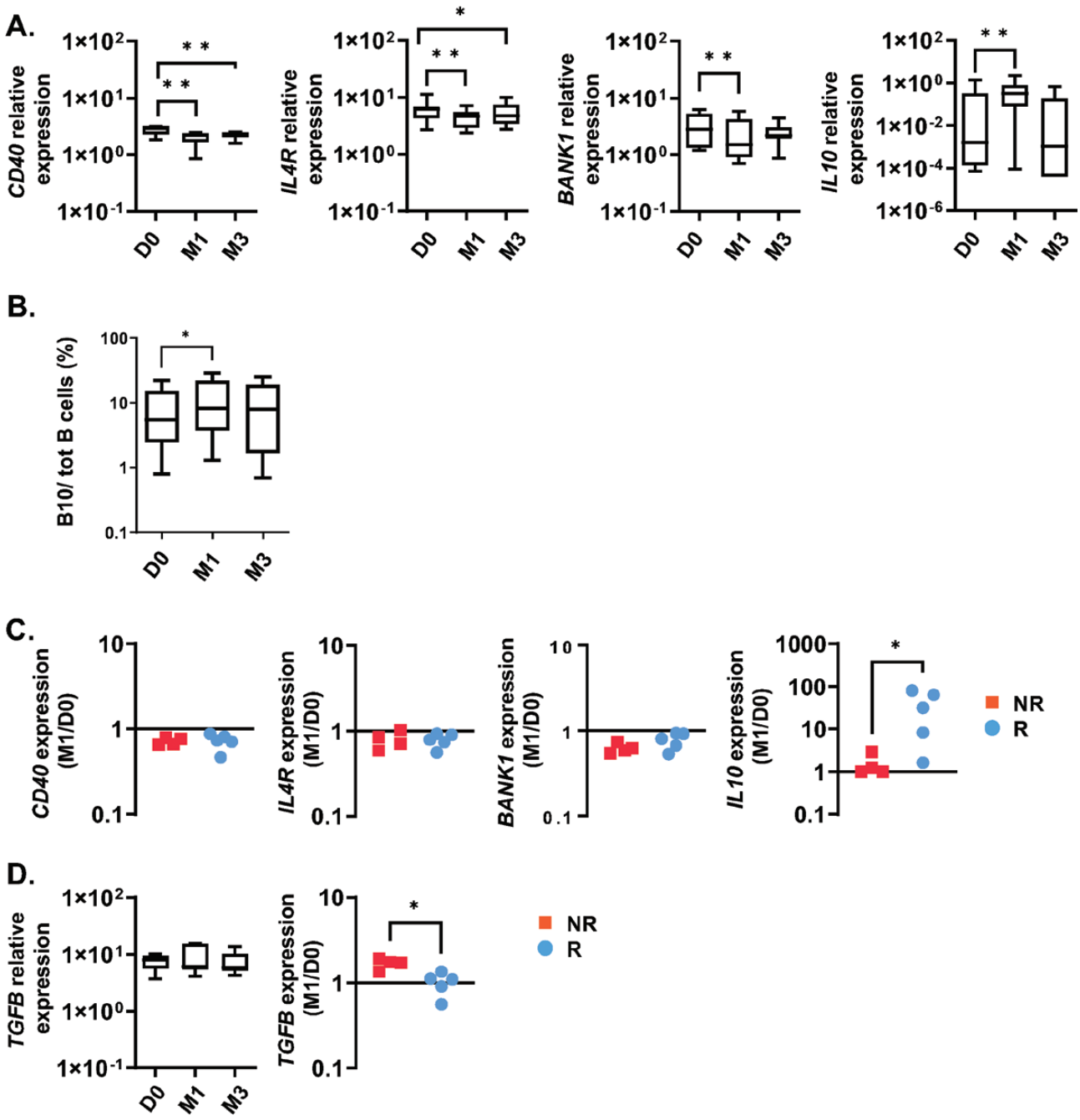


Figure 4. BM-MSCs reprogram circulating B cells in vivo. (**A**) B cells were sorted from frozen PBMCs obtained from SSc patients (n = 9, including 5 responders (R) and 4 non-responders (NR)) before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion and studied by Q-PCR for a panel of 46 genes. The 4 genes significantly affected at M1 after alloBM-MSC infusion are presented as boxplots, with whiskers extended to the most extreme data points. (**B**) B cells were purified from SSc patients and were restimulated in vitro for detection of IL-10 production by flow cytometry before (D0), 1 month (M1), and 3 months (M3) after alloBM-MSC infusion (n = 6). Boxplot represents IL10-producing B-cell frequency, with whiskers extended to the most extreme data points. (**C**) Distribution of the M1/D0 expression levels of the 4 genes affected by alloBM-MSC infusion in B cells sorted from non-responder (NR, n = 4) versus responder (R, n = 5) SSc patients. (**D**) *TGFB* relative expression at D0, M1, and M3 (left), and repartition between NR (n = 4) and R (n = 5) of the M1/D0 expression levels (right) have been determined in sorted B cells. *P < .05; **P < .01.

decreased at M1, and also at M3 after alloBM-MSC infusion. The *BANK1* gene, involved in BCR-, CD40-, and TLR-signaling and poorly expressed in Breg specifically,[31] was transiently downregulated, whereas *IL10*, the Breg hallmark, emerged as the unique upregulated gene at M1 post alloBM-MSC treatment. In 6 out of the 9 SSc treated patients with enough available samples of frozen PBMCs, we performed a B10 functional assay based on the quantification of the percentage of IL-10-producing B cells within in vitro restimulated purified B cells. This experiment revealed a transient increase of B10 at M1 after alloBM-MSC infusion (P < .05) (Fig. 4B), thus confirming the data obtained on *IL10* expression by ex vivo sorted B cells. Importantly, *CD40*, *IL4R*, and *BANK1* downregulation were not related to the observed clinical response, while early *IL10* upregulation in B cells was significantly higher

in R (n = 5) compared with NR (n = 4) patients (P < .05, Fig. 4C), identifying B10 induction as one of the potential mechanisms of alloBM-MSC effect in SSc. Of note, *TGFB* was significantly increased in NR compared to R patients at M1 (P < .05, Fig. 4D) suggesting that the lack of clinical response to BM-MSCs in SSc patients may be associated with a lack of control of *TGFB* expression by B cells.

BM-MSCs Directly Upregulate IL-10 Production by B Cells In Vitro

Having demonstrated that BM-MSCs could amplify in vivo a pool of B cells with Breg phenotype and function in SSc patients, we planned to evaluate whether such activity could be mediated by the direct contact between B cells and BM-MSCs. For that purpose, we performed in vitro co-cultures of activated HD B cells with rgBM-MSCs and analyzed sorted viable B cells after 2 days and culture supernatants after 5 days. Both *IL10* and *CD38*, 2 markers associated with Breg phenotype and function, were found upregulated in B cells activated in the presence of rgBM-MSCs (n = 6, P < .01, Fig. 5A). We confirmed the increase of IL-10 production at the protein level by ELISA assay (P < .05, Fig. 5B). None of the other tested genes, including *TGFB* (Fig. 5C), was affected by the coculture of activated B cells with BM-MSCs. These data suggest that the induction of functional B10 in SSc patients could rely, at least partly, on a direct effect of BM-MSCs on the B-cell compartment.

Discussion

Identification of biomarkers of clinical activity in MSC-treated patients, allowing the development of relevant potency assays to qualify MSC productions and the definition of criteria for recipient selection, remains a major unmet need. This gap still limits the capacity to design large clinical trials to conclude on MSC clinical efficacy in selected applications and to optimize MSC-based therapies. However, whereas the immunomodulatory activity of MSCs has been

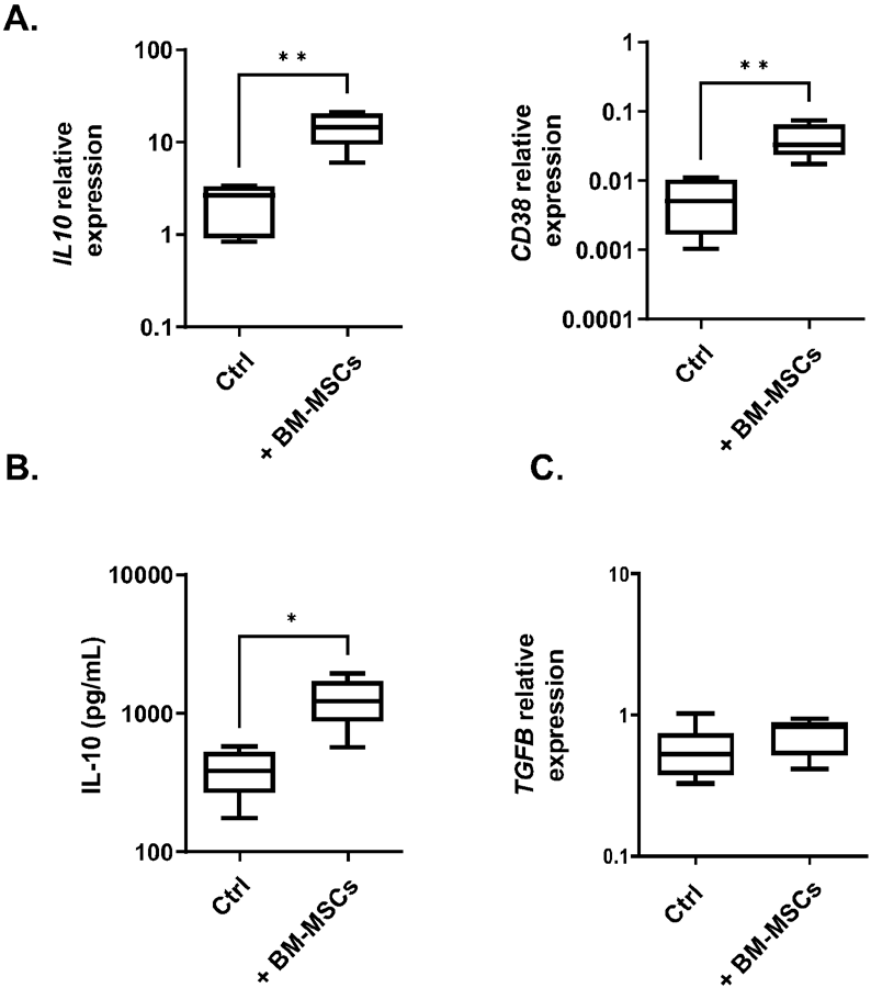


Figure 5. BM-MSCs induce IL-10-producing cells in vitro. (**A**) Peripheral blood B cells purified from healthy donors were stimulated for 2 days in the presence or not (Ctrl) of rgBM-MSCs (ratio 1/1) before sorting of DAPI^{neg}CD19^{pos}CD105^{neg} B cells and analysis by QPCR for a panel of 46 genes (n = 6). The 2 differentially expressed genes (*IL10* and *CD38*) are shown as boxplots with whiskers extended to the most extreme data points. (**B)** Peripheral blood B cells purified from healthy donors were stimulated for 2 days in the presence or not (Ctrl) of rgBM-MSCs (ratio 1/1) before collection of culture supernatants at day 5 and quantification of IL-10 by ELISA (n = 6). Data are presented as boxplots with whiskers extended to the most extreme data points. (**C)** Peripheral blood B cells purified from healthy donors were stimulated for 2 days in the presence or not (Ctrl) of rgBM-MSCs (ratio 1/1) before sorting of DAPI^{neg}CD19^{pos}CD105^{neg} B cells and analysis by QPCR for a panel of 46 genes (n = 6). Expression of *TGFB* is shown as boxplots with whiskers extended to the most extreme data points. * P < .05; ** P < .01.

repeatedly proposed as their primary mechanism of action, very few early clinical trials have proposed a thorough longitudinal characterization of immune cell subsets, looking for the impact of MSCs on their frequency, activation, and function.

T cells were first considered as the primary target of MSC suppressive activity, with the initial in vitro demonstration that both BM-MSCs and ASCs can inhibit T-cell proliferation and trigger Treg expansion. Due to a limited volume of available blood samples, Treg follow-up was performed for 9 patients only and no impact of alloBM-MSCs infusion on Treg percentage or count was found (data not shown). More recent evidence has shown that MSCs are also able to affect B-cell behavior by regulating B-cell proliferation and differentiation and by inducing Breg expansion.[32] Modulation of B-cell functions by MSCs is associated with benefits in numerous murine models of autoimmune diseases,[33-35] and in graft versus host disease (GvHD).[36,37] Moreover, clinical improvement after BM-MSC infusion in refractory chronic GvHD patients was proposed to rely on the induction of IL-10-producing $CD5^{pos}$ B cells.[36] Inhibition of B-cell proliferation has been linked to the production of indoleamine-2,3 dioxygenase (IDO) by MSCs primed by inflammatory stimuli,[38-40] whereas the chemokine CCL2 produced by resting MSCs suppresses terminal B-cell differentiation and PC immunoglobulin secretion.[41] In agreement, BM-MSCs from lupus-like mice and from patients with systemic lupus erythematosus produce reduced amount of CCL2, leading to a decreased capacity to inhibit B-cell activation.[42] The quality control of alloBM-MSC batches used for treating the SSc patients enrolled in the clinical trial described here revealed that a low inducible IDO activity and low constitutive CCL2 production were associated with a lack of clinical response in corresponding recipients.[21] Although the production of IDO and CCL2 predicts T-cell suppression by MSCs in vitro,[22,43] the capacity of alloBM-MSC batches to inhibit T-cell proliferation, assessed by mixed lymphocyte reaction, was not found predictive of the observed clinical response in these alloBM-MSC-treated SSc patients.[21] Altogether, data from the present expanded study strongly argue for B cells being pivotal effectors of BM-MSC therapeutic effect in SSc. Whether new potency assays, capturing the capacity of MSCs to reprogram B cells towards a suppressive functional phenotype, will be predictive of their clinical efficacy remains to be established in larger series of patients.

Several recent studies highlighted the key role of B cells in the pathogenesis of SSc. While the direct contribution of SSc-specific autoantibodies to tissue damage is not clearly demonstrated,[44] autoreactive B cells play an antibody-independent role in SSc. In particular, the increase in the affinity of topoisomerase I-reactive B cells triggers a switch from suppressive (IL-10, IL-35) to inflammatory (IL-6, IL-23) cytokine production, associated with a differential impact on Th differentiation and the development of fibrosis in a SSc murine model and SSc patients.[45] More generally, cytokine-producing B cells are quantitatively and functionally impaired in SSc patients. A major increase in IL-6 and TGFβ production by B cells from SSc patients have been previously described, whereas IL-10-producing Breg is decreased.[28,46,47] In vitro, the blockade of TGFβ and IL-6 activity abrogates the fibroblast proliferative effect of SSc B-cell supernatants.[28] In bleomycin-induced SSc murine model, B-cell-specific IL-6-deficient mice showed attenuated skin and lung fibrosis whereas B-cell-specific IL-10-deficient mice exhibit exacerbated fibrosis.[48] In this model, B cells trigger macrophage profibrotic polarization besides their direct activity on fibroblasts.[49] Finally, the ratio of IL-6/IL-10-producing B cells is associated with the severity of SSc disease, including the extent of skin sclerosis score and interstitial lung disease.[50] In the present study, we identified SSc patients with $IL\text{-}6^{hi}TGF\beta^{hi}$ B cells as NR to alloBM-MSC therapy, suggesting that a deep B-cell defect could not be reversed by a single BM-MSC infusion. We previously reported a high amount of circulating TGFβ as a marker of poor clinical response after alloBM-MSC infusion in these SSc patients.[21] Further studies will be required to validate whether the quantification of profibrotic cytokine production by B cells could be used as a selection marker to include patients in MSC clinical trials.

Regulatory B-cell function is supported by several B-cell subsets with variable phenotypes. Among them, $CD24^{hi}CD27^{pos}$ memory B cells have been proposed as the major IL-10-producing B cells, able to inhibit T-cell proliferation and IFN-γ/IL-17 production.[25] Interestingly, percentages of B10 inversely correlated with those of IL-17- and IFN-γ-producing T cells in SSc patients.[51] Herein, we identified for the first time the increase of $CD24^{hi}CD27^{pos}$ memory B-cell frequency and the increase of *IL10* expression by B cells as associated with the observed clinical response to alloBM-MSCs, highlighting Breg as key targets of MSCs in SSc. Changes in the composition of the B-cell compartment, including increased production of IL-10, were also shown to be associated with clinical improvement in SSc patients treated by classical immunosuppressive drugs[52] and AHSCT,[53] suggesting a general mechanism for immune-reprogramming strategies in this disease. Therapeutic approaches targeting B cells or B-cell-derived factors have provided some interesting preliminary clinical results in SSc, even if large randomized trials are still needed to definitively conclude on this aspect.[44]

The main limitations of this study are the limited number of patients included in phase I/II clinical trial and the limited volume of peripheral blood that could be ethically collected in these severely-ill patients, making it impossible to perform all the analyses on all patients at the 3 time points. Based on our phenotypic data, we decided to focus our prospective transcriptomic and functional study on B cells, but other cell subsets would also be relevant to characterize. In addition, a deeper analysis of B-cell subsets, including through currently available single-cell approaches, would provide additional insights into the impact of MSC on B-cell biology and deserve attention for further translational studies. In our in vitro co-culture assay, we could not use any remaining clinical-grade alloBM-MSC batches, but we produced rgBM-MSC with a very similar clinical-grade-like process including a use at the end of P1. In vivo, MSC/B cell crosstalk probably also relies on the effect of MSC on T-cell or myeloid cell polarization that could indirectly affect B-cell behavior as recently proposed in a mouse model of acute colitis.[54] Additional in vitro experiments would be required to explore this possibility.

Conclusion

We report here the impact of a single injection of BM-MSCs on B-cell phenotypic, transcriptomic, and functional profiles in SSc patients, with a shift from profibrotic to regulatory B

cells in clinical responders. These data support a new concept for MSC-based clinical trials, where the quantification of cytokine-producing B cells in MSC potency assays and patient selection criteria may be helpful to reach optimal clinical benefit in SSc and potentially in other autoimmune diseases.

Acknowledgments

We thank Céline Delaloy and Juliette Ferrant for their insightful scientific comments on the manuscript. Cell sorting was performed at the Biosit Flow Cytometry and Cell Sorting Facility CytomeTRI (UMS 6480 Biosit).

Funding

This study was funded by the French Ministry of Health (National Hospital Clinical Research Program AOM 11–250), the Fonds de Dotation de l'AFER pour la Recherche Medicale, the Groupe Francophone de Recherche sur la Sclerodermie (GFRS), and the Infrastructure Program Ecell France (ANR-11-INSB-005).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

S.L.: conception and design of the biological study, collection and/or assembly of data, data analysis and interpretation, contribution to manuscript writing, final approval of manuscript. P.L.: conception and design, final approval of manuscript. D.R: data analysis and interpretation, final approval of manuscript. J.D, C.Me., C.Mo., N.B., I.B., M.L.: collection and/or assembly of data, final approval of manuscript. A.C: managed the production of cell therapy product for the clinical trial, final approval of manuscript. D.F: conception and design of the clinical trial, provision of study material and patients, revised the manuscript, financial support, final approval of manuscript. K.T: conception and design of the biological study, collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support final approval of manuscript.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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