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TRANSLATIONAL AND CLINICAL RESEARCH

Brief Report: Proteasomal Indoleamine 2,3-Dioxygenase Degradation Reduces the Immunosuppressive Potential of Clinical Grade-Mesenchymal Stromal Cells Undergoing Replicative Senescence

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ABSTRACT

Owing to their immunosuppressive properties, mesenchymal stromal cells (MSCs) obtained from bone marrow (BM-MSCs) or adipose tissue (ASCs) are considered a promising tool for cell therapy. However, important issues should be considered to ensure the reproducible production of efficient and safe clinical-grade MSCs. In particular, high expansion rate, associated with progressive senescence, was recently proposed as one of the parameters that could alter MSC functionality. In this study, we directly address the consequences of replicative senescence on BM-MSC and ASC immunomodulatory properties. We demonstrate that MSCs produced according to GMP procedures inhibit less efficiently T-cell, but not Natural Killer (NK)- and B-cell, proliferation after reaching senescence. Senescence-related loss-of-function is associated with a decreased indoleamine 2,3-dioxygenase (IDO) activity in response to inflammatory stimuli. In particular, although STAT-1-dependent IDO expression is transcriptionally induced at a similar level in senescent and nonsenescent MSCs, IDO protein is specifically degraded by the proteasome in senescent ASCs and BM-MSCs, a process that could be reversed by the MG132 proteasome inhibitor. These data encourage the use of appropriate quality controls focusing on immunosuppressive mechanisms before translating clinical-grade MSCs in the clinic. STEM CELLS *2017;35:1431–1436*

SIGNIFICANCE STATEMENT

Based on encouraging results from preclinical studies and early-phase clinical trials, mesenchymal stromal cells (MSCs) have gained strong interest for cell therapy purposes but their clinical use is challenged by conflicting results of some large clinical trials. Importantly, to reach sufficient cell numbers for therapeutic applications, large-scale expansion of MSCs is required, a process that emerges as a potential limiting factor for their clinical benefit. In this study, we have demonstrated that MSC replicative senescence reduces their immunosuppressive properties, a central feature supporting their pharmaceutical effect. This work suggests that senescence may affect MSC clinical efficacy and should be evaluated before administration of the therapeutic cell product.

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are considered a promising tool for cell-based therapeutic strategies in the context of immune disorders and tissue regeneration [1]. The possibility to expand ex vivo high numbers of clinical-grade MSCs from bone marrow (BM-MSCs) or adipose tissue (ASCs) has paved the way for their therapeutic use. In agreement, more than 500 clinical trials evaluating MSC therapy in multiple clinical settings are registered (http://www.clinicaltrials.gov) and encouraging results have been reported in phase I/II trials [1, 2]. Interestingly, the efficacy of transplanted MSCs mainly relies on their paracrine ability to produce trophic factors, reduce inflammation, and dampen innate and adaptive immune response [3, 4]. The increasing use of MSCs and their classification by the Regulatory Authorities as strictly-controlled cell-based medicinal products has led to the development of large-scale standardized production processes [5, 6]. Understanding the main determinants

that affect their immunomodulatory activity is crucial for the development of effective MSC strategies. For that purpose, the use of completely defined in vitro immunological assays is a prerequisite to limit uncontrolled technical variability [4, 7, 8].

Besides interindividual variability, the scale of culture expansion and the related gradual entry into replicative senescence emerge as critical culture-related parameters that could influence clinical-grade MSC safety and clinical efficacy [9, 10]. In particular, late-passage MSCs were shown to be less effective than early-passage MSCs to control acute graft-versus-host disease [11]. More generally, while academic groups essentially use a one-donor to one-recipient strategy with a minimal requirement for in vitro amplification, industry-driven trials are based on a one-donor to multiple-recipient strategy and involve MSCs with a high number of cumulative population doublings (PD) [9]. As senescence is observed in expanding human MSCs [10], it can be hypothesized that long-term culture affects MSC clinical efficacy due to replicative senescence.

Previous analyses of the impact of senescence on MSC immunological properties led to opposite results in relation with the use of unstandardized MSC production processes, senescence induction, and immunological assays [12, 13]. In addition, whereas MSC priming by inflammatory stimuli is a prerequisite to trigger their suppressive function [14], previous studies regarding MSC senescence only focused on resting MSCs. We investigate here the impact of replicative senescence, induced by long-term culture, on the ability of clinical grade BM-MSCs and ASCs to inhibit immune response. We highlight that senescent MSCs are less efficient than their nonsenescent counterpart to inhibit T-cell proliferation and identify the proteasomal degradation of indoleamine 2,3-dioxygenase (IDO) immunosuppressive enzyme as a supportive mechanism.

Materials and Methods

Complete Materials and Methods are available in the Supporting Information.

Results and Discussion

Senescent MSCs Display Reduced Capacity to Inhibit T-Cell Proliferation

To analyze the impact of replicative senescence on immunological properties of MSCs, we maintained clinical-grade MSC batches in culture until senescence and compared paired senescent (SEN+) and nonsenescent (SEN−) cells from the same donors.

Reduced cell proliferation occurred after 25-35 cumulative PD for BM-MSCs and 35-45 cumulative PD for ASCs, and was associated with classical senescence features including increased cell size, β-galactosidase (β-gal) staining, and expression of p16INK4 as previously described [10] (Supporting Information Fig. S1A-S1C). To ensure data homogeneity, SEN+ and SEN− MSC batches were defined by a combination of high cumulative PD/β-gal staining >25% versus low cumulative PD/β-gal staining <5% (Supporting Information Fig. S1D) rather than by the passage number, a highly variable parameter in culture. We then compared the effect of SEN+ and SEN− MSCs on activated purified immune effector cells instead of unfractionated peripheral blood mononuclear cells using robust and validated assays as proposed by the International Society for Cellular Therapy [8, 15]. Interestingly, SEN+ BM-MSCs and ASCs displayed a significantly decreased capacity to inhibit T-cell proliferation compared to their SEN− counterpart regardless of the MSC-to-immune cell ratio although they retained their NK-cell inhibitory function (Figure 1A, 1B and Supporting Information Fig. S2A). SEN− MSCs inhibited T-cell proliferation at a similar level upon transwell coculture (Supporting Information Fig. S2B), showing that the mechanism involved was contact-independent. *HGF* and *TGF-β* were significantly upregulated at the mRNA level in ASCs and BM-MSCs during senescence in both resting and inflammatory context, ruling out that the loss of T-cell inhibitory properties of SEN+ MSCs was related to a decreased production of HGF and TGF-β1 soluble immunosuppressive molecules (Supporting Information Fig. S2C). The capacity of human MSCs to inhibit T-cell, unlike NK-cell, proliferation in vitro was proposed to be dependent on their capacity to express functional IDO [4, 16]. We thus assessed IDO activity of SEN+ versus SEN− MSCs after licensing by weak (20 IU/ml interferon (IFN)-γ; Low IFN) versus strong (100 IU/ml IFN-γ +1.5 ng/ml tumor necrosis factor (TNF)-α; Hi IFN +TNF) inflammatory stimuli, as selected by the quantification of *IDO* mRNA (Fig. 1C). Of note, T cells produced similar to higher amounts of IFN-γ and TNF-α when cocultured with SEN+ MSCs compared to SEN− MSCs in agreement with the decreased T-cell suppressive activity of SEN+ MSCs, confirming that the lack of T-cell inhibition by SEN+ MSCs could not be attributed to a weaker inflammatory context (Supporting Information Fig. S2D). Interestingly, whereas SEN+ and SEN− MSCs responded to inflammatory cytokines by producing similar amount of *IDO* mRNA, SEN+ MSCs required a stronger priming than SEN− to trigger a full IDO activity (Fig. 1D). In agreement, priming of SEN+ MSCs by a strong inflammatory stimulus restored their capacity to inhibit T-cell proliferation (Fig. 1E), even upon cell separation by a transwell (Supporting Information Fig. S2B).

Overall these data provide evidences that senescent MSCs are specifically less suppressive toward T-cell immunity than nonsenescent MSCs, in association with a reduced IDO activity. Importantly, full senescence was obtained after only 4-9 passages for BM-MSCs versus 10-14 passages for ASCs (Supporting Information Fig. S1A), indicating that senescence evaluation could be a valuable quality control, in particular for clinical-grade BM-MSCs. In addition, these data strengthen the potential interest of the priming of MSCs with inflammatory stimuli before their clinical use [17].

IDO Activity Is Reduced in Senescent MSCs Through a Proteasome-Dependent Degradation Pathway

Given the discrepancy between IFN-γ-dependent IDO transcription versus activity in SEN+ MSCs, we further explored the molecular mechanisms underlying IDO regulation during senescence. We first underlined, through inhibition by validated siRNA (Supporting Information Fig. S3) that STAT1 but not STAT3 was required for the induction of *IDO* transcription by IFN-γ in MSCs (Fig. 2A), as described in other cell subsets [18, 19]. STAT1 signaling was retained during MSC senescence (Fig. 2B), in agreement with the induction of similar levels of *IDO* mRNA by IFN-γ in SEN+ versus SEN− cells (Fig. 1C). We next hypothesized that the decrease of IDO activity during MSC senescence could be due to a decrease of IDO protein stability. In fact, SEN+ MSCs contained less IDO protein than SEN− MSCs after

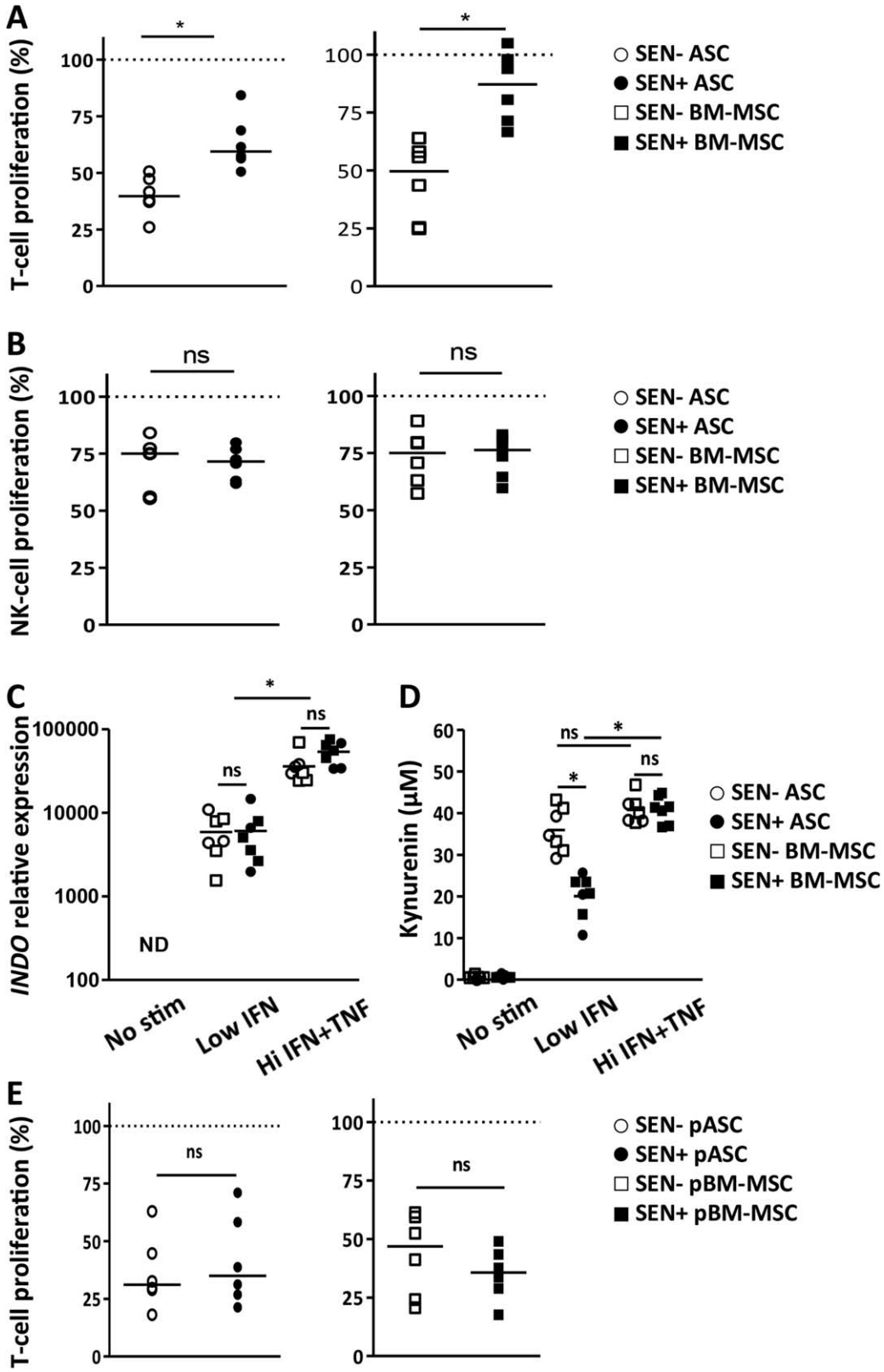

Figure 1. Senescent MSCs lose their ability to inhibit T-cell proliferation through indoleamine 2,3-dioxygenase (IDO) activity. **(A, B)**: ASCs (left panels) and BM-MSCs (right panels) were cocultured with either CFSE-labeled purified T cells stimulated with anti-CD3/anti-CD28 antibodies (MSC to T-cell ratio 1:10) (A) or CFSE-labeled purified NK-cells stimulated with IL-2 (MSC to NK-cell ratio 1:1) (B). Inhibition of immune cell proliferation by senescent (SEN+; black symbols) and nonsenescent MSCs (SEN−; opened symbols) was assessed by CFSE dilution to determine the proportion of cells that have undergone at least one cell division. Data are expressed as the percentage of proliferating cells after normalization to 100% for the proliferation obtained without MSCs (dotted lines). Six independent ASCs and BM-MSC batches were used. **(C, D)**: SEN+ (black symbols) and SEN− (opened symbols) ASCs (circles, $n = 3$) and BM-MSCs (squares, $n = 4$) were stimulated or not with 20 IU/ml IFN-γ (Low IFN) or with 100 IU/ml IFN-γ and 1.5 ng/ml TNF-α (Hi IFN + TNF). Expression of *INDO* (encoding for IDO protein) was measured by RQ-PCR after 24 hours of culture, normalized to three housekeeping genes, and represented in arbitrary units obtained by assigning the value of 1 to a pool of PBMC. *IDO* is not detectable in unstimulated MSCs (C); Culture supernatants were collected after 40 hours of culture for IDO activity evaluation through quantification of kynurenine level (D). **(E)**: T-cell proliferation inhibition assay was performed using pMSCs, previously stimulated for 48 hours with Hi IFN + TNF (strong inflammatory priming). Results are represented as in (A). *, $p < .05$; ns, not significant. Abbreviations: ASC, adipose tissue stromal cells; BM-MSCs, bone marrow-mesenchymal stromal cells; CFSE, carboxyfluorescein succinimidyl ester; IFN, interferon; pBM-MSCs, primed BM-MSCs; SEN+, paired senescent; SEN−, nonsenescent; TNF, tumor necrosis factor.

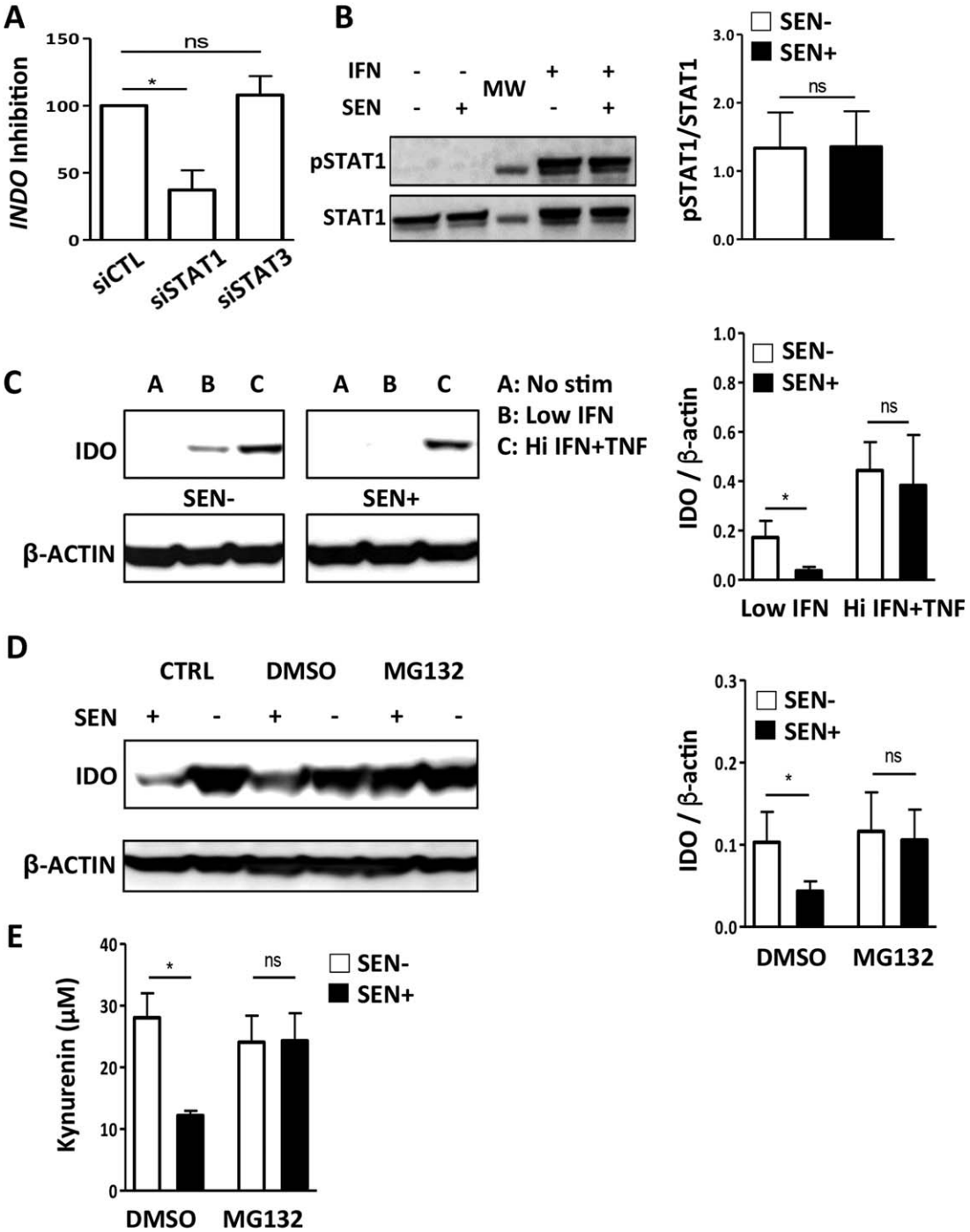

Figure 2. IDO protein is degraded by the proteasome in senescent MSCs. **(A)**: SEN− MSCs were transfected with control (siCTL), STAT3 (siSTAT3), or STAT1 (siSTAT1) siRNAs before stimulation by IFN-γ (20 IU/ml). *INDO* expression was measured by RQ-PCR after 24 hours of culture, normalized to three housekeeping genes, and represented in arbitrary units obtained by assigning the value of 100 to the siCTL condition. Bars: mean +/− SD from 6 independent experiments (3 with BM-MSCs and 3 with ASCs). *, $p < .05$; ns: not significant. **(B)**: SEN+ and SEN− MSCs were stimulated or not with 20 IU/ml IFN-γ for 45 minutes. Phospho-STAT1 (pSTAT1) expression was then determined by Western blot and normalized to STAT1. MW: molecular weight. Bars: mean +/− SD from 6 independent experiments (3 with BM-MSCs and 3 with ASCs). ns: not significant. **(C)**: SEN+ and SEN− MSCs were stimulated or not for 2 days with 20 IU/ml IFN-γ (Low IFN) or with 100 IU/ml IFN-γ and 1.5 ng/ml TNF-α (Hi IFN + TNF). IDO expression was determined by Western blot and normalized to β-actin. Bars: mean +/− SD from 6 independent experiments (3 BM-MSCs and 3 ASCs). *, $p < .05$; ns: not significant. **(D)**: SEN+ and SEN− MSCs were stimulated by IFN-γ (20 IU/ml) and exposed to MG-132 or its vehicle, DMSO, for the last 6 hours. IDO expression was determined by Western blot and normalized to β-actin. Bars: mean +/− SD from 6 independent experiments (3 BM-MSCs and 3 ASCs). *, $p < .05$; ns: not significant. **(E)**: SEN+ and SEN− MSCs were stimulated by IFN-γ (20 IU/ml) in the presence of MG-132 or DMSO, for 2 days. Culture supernatants were collected for IDO activity evaluation through kynurenin quantification. Bars: mean +/− SD from 5 independent experiments (3 BM-MSCs and 2 ASCs). *, $p < .05$; ns: not significant. Abbreviations: DMSO, dimethylsulfoxyde; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; pSTAT1, phospho-STAT1; RQ-PCR: real-time quantitative polymerase chain reaction; SEN+, paired senescent; SEN−, nonsenescent; TNF, tumor necrosis factor.

stimulation with weak inflammatory stimuli, a defect corrected by the use of a strong inflammatory priming (Fig. 2C). Proteasome has been proposed to regulate IDO protein stability in various cell models [20, 21]. In addition, the proteasome is involved in the regulation of senescence that, in turn, alters the expression of proteasome pathway components [22–24]. We thus tested the MG132 reversible proteasome inhibitor on the inflammation-dependent expression of IDO in MSCs. Interestingly, MG132 specifically increased the amount of IDO protein and activity in SEN+ MSCs (Fig. 2D, 2E), suggesting a crucial role for proteasome degradation pathway in the decrease of IDO activity, and subsequent reduction of immunosuppressive MSC properties during senescence. Identification of IDO-degrading factors and their inducers could provide new quality controls for clinical-grade MSC production as well as new potential targets to maintain IDO-dependent suppressive phenotype.

Conclusion

Our findings support the emerging idea that senescence modifies MSC immunosuppressive properties. Indeed, our results demonstrate a deleterious effect of senescence on MSC capacity to inhibit T-cell mediated immune response, in link with a proteasome-dependent degradation of IDO. Of note, although resting SEN+ MSCs maintain their ability to sustain B-cell proliferation as previously described for SEN− MSCs [4], priming by weak inflammatory stimuli was sufficient to convert them into a B-cell suppressive phenotype (Supporting Information Fig. S4), suggesting that IDO-independent mechanisms could trigger B-cell inhibition during senescence. These data encourage the definition of accurate qualification controls of MSCs, in particular focusing on immunosuppressive mechanisms, to optimize their use in the clinic.

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Author Contributions

S.L.: collection and assembly of data, data analysis and interpretation, manuscript writing; J.D. and C.M.: collection and assembly of data, manuscript editing; M.-L.R., N. Bertheuil, and E.F.: collection of human tissues and production of MSCs; N.M.: IDO activity quantification; I.B., M.L., and N. Bescher: collection and assembly of data, final approval of the manuscript; R.P.: final approval of the manuscript; L.S.: financial support, final approval of the manuscript; K.T.: conception and design of the study, financial support, data analysis and interpretation, manuscript writing and final approval.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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