


Modulation of T-Cell Activation Markers Expression by the Adipose Tissue-Derived Mesenchymal Stem Cells of Patients with Rheumatic Diseases

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Abstract

Background: Activated T lymphocytes play an important role in the pathogenesis of rheumatic diseases (RD). Mesenchymal stem cells (MSCs) possess immunoregulatory activities but such functions of MSCs from bone marrow of systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and ankylosing spondylitis (AS) patients are impaired. Adipose tissue-derived MSCs (ASCs) are an optional pool of therapeutically useful MSCs, but biology of these cells in RD is poorly known. This study aimed at investigating the effect of ASCs from RD patients and healthy donors (HD) on the expression of the key T-cell activation markers. **Methods:** ASCs were isolated from subcutaneous abdominal fat from SLE ($n = 16$), SSc ($n = 18$), and AS ($n = 16$) patients, while five human ASCs lines from HD were used as a control. Untreated and cytokine (tumor necrosis factor α + interferon γ)-treated ASCs were co-cultured with allogenic, mitogen (phytohemagglutinin)-stimulated peripheral blood mononuclear cells (PBMCs) or purified anti-CD3/CD28-activated CD4⁺ T lymphocytes. Contacting and noncontacting ASCs-PBMCs co-cultures were performed. RD/ASCs were analyzed in co-cultures with both allogeneic and autologous PBMCs. Flow cytometry analysis was used to evaluate expression of CD25, HLA-DR, and CD69 molecules on CD4⁺ and CD8⁺ cells. **Results:** In co-cultures with allogeneic, activated CD4⁺ T cells and PBMCs, HD/ASCs and RD/ASCs down-regulated CD25 and HLA-DR, while upregulated CD69 molecules expression on both CD4⁺ and CD8⁺ cells with comparable potency. This modulatory effect was similar in contacting and noncontacting co-cultures. RD/ASCs exerted weaker inhibitory effect on CD25 expression on autologous than allogeneic CD4⁺ and CD8⁺ T cells. **Conclusion:** RD/ASCs retain normal capability to regulate expression of activation markers on allogeneic T cells. Both HD/ASCs and RD/ASCs exert this effect independently of their activation status, mostly through the indirect pathway and soluble factors. However, autologous CD4⁺ and CD8⁺ T cells are partially resistant to RD/ASCs inhibition of CD25 expression, suggesting weaker control of T-cell activation *in vivo*.

Keywords

systemic lupus erythematosus, systemic sclerosis, ankylosing spondylitis, adipose tissue-derived mesenchymal stem cells, T-cell activation

Introduction

Mesenchymal stromal/stem cells (MSCs), endowed with both regenerative potential and immunomodulatory activities, are currently being studied for therapeutic use in regenerative medicine and immune-mediated disorders, including rheumatic diseases (RD), characterized by an abnormal activation of B and/or T lymphocytes, chronic inflammation, progressive tissue, and/or organ damage^{1,2}. It should be stressed that despite the progress in therapeutic

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Table 1. Demographic and Clinical Characteristics of the Patients.

	SLE (n = 16)	SSc (n = 18)	AS (n = 16)
Demographics			
Age, years	43 (20–58)	49.5 (20–77)	42.5 (25–70)
Sex, female (F) male (M), n	15 F/1 M	12 F/6 M	7 F/10 M
BMI	26.6 (16.4–37)	25.8 (16.5–38.7)	26.6 (21.4–35.8)
Disease duration, years	6.5 (0–47)	4.5 (1–40) ^a /3 (1–23) ^b	5.5 (1.5–17)
Clinical data			
Disease activity*, score	8 (0–32) ^{***}	1 (0–8.5) ^{**}	6.15 (1.0–8.2) [*]
Laboratory values			
CRP, mg/l	4 (1–16) ^{&&}	6.5 (1–33)	7 (5–50) ^{&&}
ESR, mm/h	15.5 (3–73)	17.5 (4–59)	13.5 (1–59)
Proteinuria, mg/24 h	285 (0–7,550) ^{###}	0 (0–0.3) ^{###}	n/a
C3, mg/dl	78.3 (23.2–119) [#]	100.6 (65.8–141) [#]	n/a
C4, mg/dl	10.36 (4.94–23.7) [#]	16.65 (13–27.1) [#]	n/a
ANA, titer (1:x)	960 (160–10,240) ^{###}	5120 (320–20,480) ^{###}	n/a
Anti-dsDNA antibody, %	93.75	n/a	n/a
Anti-dsDNA antibody, IU/ml	85.15 (0–666.9)	n/a	n/a
Scl-70 antibody, %	n/a	55.5	n/a
Autoantibody specificities, no.	4 (2–7) [#]	3 (2–4) [#]	n/a
Medications, %			
NSAIDs	0	0	87.5
Immunosuppressive drugs	73.3	55.5	0
Nonbiologic DMARDs	33.3	50.0	25.0
Glucocorticosteroids	85.7	11.1	6.25

Except where indicated otherwise, values are the median (range).

^aDuration of Raynaud's syndrome.

^bDuration of skin/organ symptoms.

^{***}SLEDAI: SLE Disease Activity Index, ^{**}EUSTAR: The European Scleroderma Trials and Research group Revised Index, or ^{*}BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; ^{&&}*P* = 0.003 for SLE vs AS patients comparison; [#]*P* = 0.05–0.01, ^{###}*P* = 0.01–0.001, and ^{####}*P* = 0.0001 for SLE vs SSc comparisons.

ANA: antinuclear antibody; AS: ankylosing spondylitis; BMI: body mass index; C: complement components; CRP: C-reactive protein; DMARDs: disease-modifying antirheumatic drugs; ESR: erythrocyte sedimentation rate; NSAIDs: nonsteroid anti-inflammatory drugs; n/a: not applicable; Scl-70: anti-topoisomerase I antibody; SLE: systemic lupus erythematosus; SSc: systemic sclerosis.

opportunities, RD of autoimmune background, such as systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), are still incurable^{3,4}. Another RD, ankylosing spondylitis (AS), exhibiting certain autoinflammatory features, is better controlled, but disease accompanying pathological bone formation and joint damage are usually irreversible⁵. Accumulating evidence shows some defects of bone marrow-derived MSCs (BM-MSCs) obtained from RD patients^{6–8}. Therefore, it is proposed that in RD adipose tissue-derived MSCs (ASCs) may represent an optional pool of therapeutically useful autologous MSCs, but biology of these cells is poorly understood⁹. We have recently reported certain abnormalities of ASCs of SLE, SSc, and AS patients in the secretory activity and expression of surface molecules (CD90, ICAM-1, and VCAM-1)¹⁰. Thus, it is likely that in RD prolonged chronic inflammation, irrespective of the initial cause(s), may affect immunoregulatory functions of ASCs as well. To verify this supposition, we have presently evaluated the effect of ASCs of healthy donors (HD) and patients suffering from different RD (SLE, SSc, and AS) on the expression of the key T-cell activation markers, i.e., CD25, CD69, and HLA-DR. These surface molecules are

known to play an important role in mediating T-cell biological functions. CD69, a type II C-lectin receptor, a very early activation marker, which exerts a complex immunoregulatory role, is involved in the generation and function of different Treg types and determines T-cell migration–retention ratio^{11,12}. Another early activation marker, CD25, is the α chain of IL-2 receptor complex binding IL-2—a T-cell growth factor essential for cell proliferation, generation of effector and memory cells, and maintenance of Treg cells¹³. HLA-DR molecules are expressed during mid-to-late time periods after T-cell activation. By mediating homotypic and heterotypic T-cell interactions and triggering various intracellular signaling pathways, HLA-DR molecules contribute to T-cell homeostasis¹⁴.

Materials and Methods

Patients

Three groups of patients, who fulfilled the criteria for SLE (*n* = 16), SSc (*n* = 18), or AS (*n* = 16), were included in the study^{15–17}. Patients' characteristics are given in Table 1.

Ethics Approval

This study meets all criteria contained in the Declaration of Helsinki and was approved by the Ethics Committee of the National Institute of Geriatrics, Rheumatology, and Rehabilitation, Warsaw, Poland (the approval protocol no: KBT-8/4/20016). All patients gave their written informed consent prior to enrolment.

ASCs Isolation and Culture

Specimens of subcutaneous abdominal fat were taken from the patients by 18 G needle biopsy. Fat samples from RD patients were taken as a normal part of diagnostic procedure toward amyloidosis. Tissue processing, ASCs isolation, and culture were performed as described previously¹⁸. Five human ASCs lines from HD (Lonza Group, Lonza Walkersville Inc., Walkersville, MD, USA; donor numbers: 0000440549, 0000410252, 0000535975, 0000605220, and 00005550179) were used as a control. All experiments were performed using ASCs at three to five passages. The complete medium used for ASCs culture was composed of Dulbecco's modified Eagle's medium (DMEM)/F12 (PAN Biotech UK Ltd., Wimborne, UK), 10% fetal calf serum (Biochrom, Berlin, Germany), 200 U/ml penicillin, 200 µg/ml streptomycin (Polfa Tarchomin S.A., Warsaw, Poland), and 5 µg/ml plasmocin (InvivoGen, San Diego, CA, USA). For some experiments, ASCs were stimulated for 24 h with recombinant human tumor necrosis factor (TNF α) and interferon γ (IFN γ) (TNF α + IFN γ treatment (TI treatment)). Both cytokines, each applied at 10 ng/ml, were from R&D Systems, Minneapolis, MN, USA.

Contacting and Noncontacting Co-cultures of ASCs with Peripheral Blood Mononuclear Cells

All co-cultures were performed in the complete DMEM/F12 medium. ASCs (5×10^4 /well/2 ml of medium) were seeded into 24-well plates and stimulated with IFN γ and TNF α (see above). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy male honorary blood donors (<60 years old) or RD patients, according to routinely applied procedure with the use of Ficoll-Paque (GE Healthcare, Uppsala, Sweden). After isolation, PBMCs (1.2×10^6 /well/2 ml of medium) were seeded either directly (contacting co-culture) or on a 0.4 µm pore size Transwell (TW) filters (MD24 with carrier for inserts 0.4 MY, Thermo Fisher Scientific, Waltham, MA, USA) (noncontacting co-culture) into 24-well plates with adherent ASCs (6×10^4 /well), and treated with 2.5 µg/ml of phytohemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO, USA). Cells for cytometric analysis were harvested after 5 days of co-culture. PBMCs cultured separately were used as control.

Co-Culture of ASCs with Purified Allogeneic CD4⁺ T Cells

The CD3⁺CD4⁺ cells were isolated from PBMCs using EasySep™ Human CD4⁺ T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). Then, the CD4⁺ T cells (1.2×10^6 /well/2 ml of medium) were seeded into 24-well plates with adherent ASCs (5×10^4 /well/2 ml of medium), and then were activated with Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher Scientific). After 5 days of co-culture, CD4⁺ T cells were harvested for cytometric analysis. The CD4⁺ T cells cultured separately were used as control.

Flow Cytometry Analysis

For the CD4⁺ cells or PBMCs analysis, harvested cells were resuspended in 50 µl of fluorescent-activated cell sorting buffer and stained for 30 min on ice for respective membrane antigens using fluorochrome conjugated monoclonal antibodies specific for human: CD4-FITC, CD25-PE, human leukocyte antigen (HLA-) DR-PerCP (all from BD Pharmingen, San Diego, CA, USA), CD8-PerCP, and CD69-Pe-Cy7 (both from eBioscience, San Diego, CA, USA). To assess viability, PBMCs were stained with 7-aminoactinomycin D (7AAD). After the washing step, cells were acquired and analyzed using a FACSCanto cell cytometer and Diva software. Appropriate isotype controls were used in all experiments. The gating strategy and applied isotype controls are shown in Supplemental Fig. S1.

Statistical Analysis

Data were analyzed using GraphPad PRISM 5 software. The Shapiro–Wilk test was used as a normality test. The results are shown as median \pm interquartile range or range. One-way analysis of variance with repeated measures and post hoc Tukey test were used to compare the effects of untreated and TI-treated ASCs, as well as the effects of ASCs on allogeneic and autologous PBMCs. The differences between ASCs lines from healthy donors (HD/ASCs), ASCs from SLE (SLE/ASCs), SSc (SSc/ASCs), and AS (AS/ASCs) patients were analyzed using the Kruskal–Wallis and Dunn's multiple comparison tests. *P* values less than 0.05 were considered significant.

Results

Characteristics of the Patients

The patients' cohort was heterogeneous with respect to demographic and clinical data (Table 1). There were no significant differences between patients' groups in body mass index, disease duration, and erythrocyte sedimentation rate values, but AS patients had slightly higher concentrations of C-reactive protein than SLE patients. All AS patients were HLA-B27 positive, and they were mostly treated with

nonsteroid anti-inflammatory drugs (NSAIDs). The majority of SLE and SSc patients had disease-specific autoantibody (anti-dsDNA or Scl70, respectively); some of them had also other types of autoantibodies, and the number of autoantibody specificities was higher in SLE than SSc patients. Both SLE and SSc patients received immunosuppressive drugs, mostly in combination with nonbiologic disease-modifying antirheumatic drugs (DMARDs) and glucocorticosteroids (SLE) or DMARDs only (SSc). Similar proportion of SSc patients had localized (44.5%) or diffused (55.5%) disease form. Previous treatment of rheumatic patients with glucocorticosteroids and antirheumatic DMARDs have not been found to affect the clonogenic and proliferation capabilities of bone marrow-derived MSCs¹⁹. Similarly, *in vitro* 3–9 days exposure of human MSCs to methotrexate (MTX) and cyclophosphamide does not affect the survival and functional characteristics of MSCs, while glucocorticosteroids may partly impair their proliferation potential²⁰. NSAIDs have been reported to promote proliferation and migration of MSCs at low (0.1–1 μ M), while inhibit it at high (10–200 μ M) concentrations^{21,22}, but the data evaluating the effects of these drugs on MSCs differentiation are inconsistent^{23,24}. Therefore, it is hard to conclude whether treatment of our patients have an impact on tested MSC activities. However, as it usually takes about 1 month to expand ASCs to the number high enough to run described experiments, it is likely that during this culture period drug-related effects disappear.

Viability of PBMCs in Co-culture with ASC

The viability of PBMCs in the ASC co-culture was assessed by incorporation of 7AAD by dead cells. Five-day co-culture of PBMCs with ASC did not have a significant effect on PBMC viability in most of the tested variants. The median of viable cells was about 70%. The only exception was the co-culture with ASC obtained from SSc patients, when the number of viable cells increased significantly to 82% (Fig. 1).

ASCs Modify Activation Markers Expression on T Cells

Co-culture of ASCs with Purified Allogeneic CD4⁺ T Cells. To assess the effect of ASCs of patients suffering from rheumatic diseases (RD/ASCs) on the expression of activation markers on CD4⁺ T cells, we first co-cultured purified activated CD4⁺ T cells with untreated or TI-treated ASCs from SLE, SSc, and AS patients and compared their effects with HD/ASCs. Unstimulated, control CD4⁺ T cells did not express CD69, negligible to small proportion of these cells expressed HLA-DR [median (range) = 0.25 (0.2–0.3)] or CD25 [5.2 (2.7–7.7)] molecules (data not shown). Upon anti-CD3/CD28 stimulation, the majority of separately cultured, activated CD4⁺ T cells were CD25⁺ and CD69⁺, while less than 7% of cells expressed HLA-DR molecules (Fig. 2). In the presence of HD/ASCs, as well as SLE/ASCs and SSc/ASCs, the percentage of CD25⁺ declined

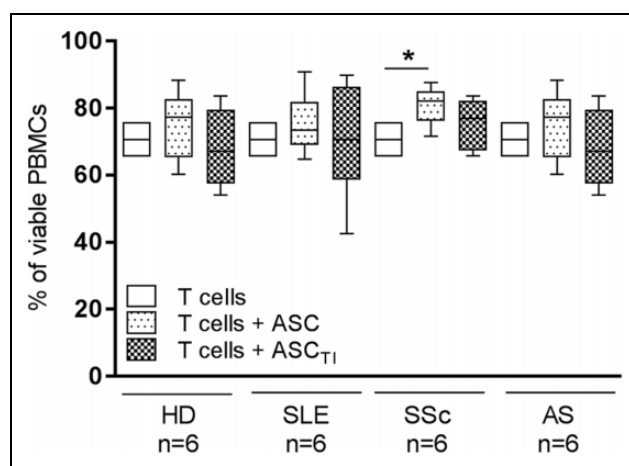


Figure 1. Modulation of viability of PBMCs in the co-cultures of activated PBMCs with ASCs. Cell co-cultures were performed using PBMCs obtained from two healthy blood volunteers and ASCs lines obtained from three HD, six SLE, six SSc, and six AS patients. PBMCs activated with PHA were cultured with untreated, or TNF α + IFN γ (TI)-treated ASCs. CD4⁺ T cells were analyzed by flow cytometry. * P = 0.05–0.01. AS: ankylosing spondylitis; ASCs: adipose tissue-derived MSCs; HD: healthy donors; IFN γ : interferon γ ; MSC: mesenchymal stem cell; PBMC: peripheral blood mononuclear cell; PHA: phytohemagglutinin; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; TNF α : tumor necrosis factor α .

significantly, while AS/ASCs failed to exert significant effect (Fig. 2A). The reduction of HLA-DR⁺ cells proportion was observed when CD4⁺ T cells were co-cultured with both HD/ASCs and RD/ASCs (Fig. 2B). Conversely, in these co-cultures the proportion of CD69⁺ T cells raised significantly (Fig. 2C). In general, untreated and TI-treated ASCs altered CD25, CD69, and HLA-DR expression in much the same way, and the effects exerted by HD/ASCs and RD/ASCs were similar. No significant changes of the expression levels of CD25 and HLA-DR, measured as the mean fluorescence intensity (MFI), were found, except reduction of HLA-DR MFI in the presence of HD/ASCs. There was also slight increasing trend of CD69 MFI on CD4⁺ T cells co-cultured with HD/ASCs and RD/ASCs (Fig. 2A–C, lower panel).

Co-culture of ASCs with Allogeneic PBMCs.

To verify the above effects of ASCs in conditions similar to *in vivo*, and to check whether other leukocyte populations contribute to observed changes, we performed the same analysis after co-culture of ASCs with PBMCs, followed by flow cytometry analysis of CD4⁺ (Fig. 3), and CD8⁺ T-cell subsets (Fig. 4). In unstimulated, separately cultured PBMCs the proportions of CD25⁺ cells among CD4⁺ T [median (range) = 7.8 (5.4–9.7)] and CD8⁺ T [0.7 (0.3–1.1)] lymphocytes, as well as the proportions of CD69⁺ [1.1 (0.4–3.3) and 2.9 (0.5–3.9), respectively] and HLA-DR⁺ [1.5 (0.7–2.0) and 2.1 (0.8–7.4), respectively] cells were low (data not shown). Upon PHA stimulation of PBMCs, the percentages of CD25⁺, CD69⁺, and HLA-DR⁺ cells in both CD4⁺

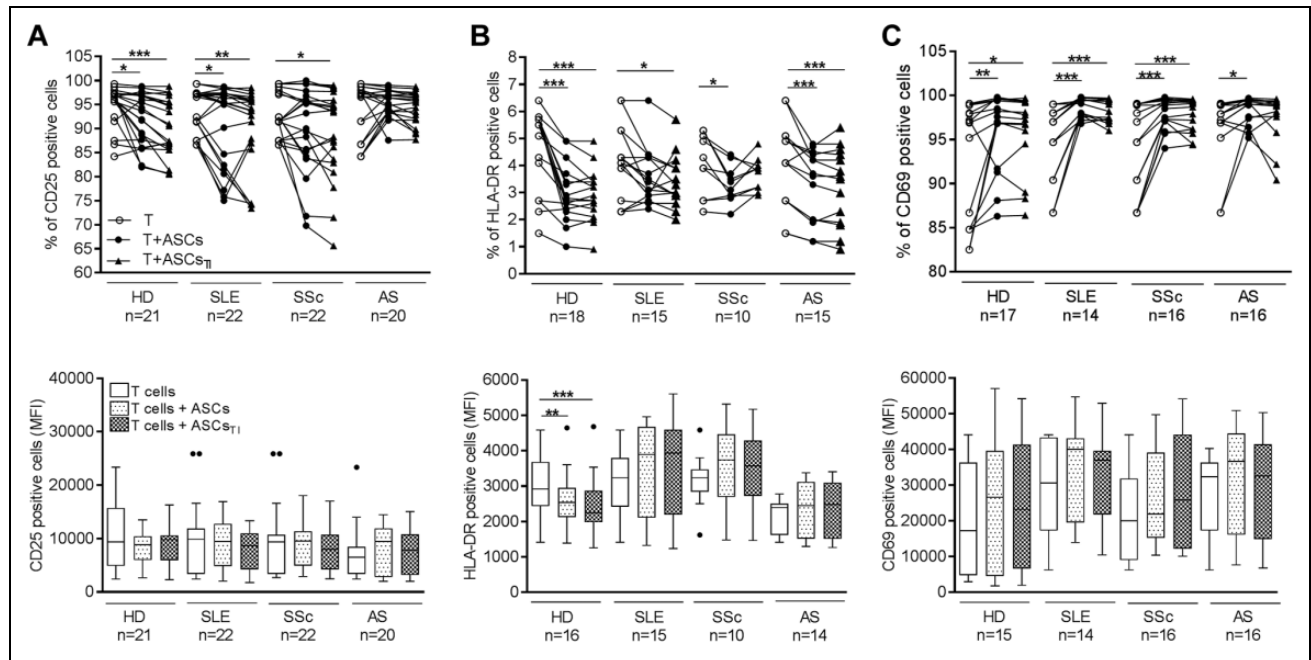


Figure 2. Modulation of activation markers expression on purified activated CD4⁺ T cells co-cultured with ASCs. Cell co-cultures were performed using CD4⁺ T cells purified from PBMCs of 18 healthy blood volunteers and ASCs lines obtained from 5 HD, 13 SLE, 16 SSc, and 10 AS patients. Lines between points in the upper panels identify cultures containing the same combination of CD4⁺ T cells and ASCs. Data in the lower panels are expressed as the median (horizontal line) with IQR (box), lower and upper whiskers (data within 3/2 × IQR) and outliers (points) (Tukey's box). CD4⁺ T cells activated with anti-CD3/CD28-specific antibodies were cultured with untreated, or TNF + IFN γ (TI)-treated ASCs. See Materials and Methods for details. The upper and lower panels show the percentage of cells positive for particular marker and MFI, respectively. * $P = 0.05-0.01$, ** $P = 0.01-0.001$, *** $P = 0.001-0.0001$ for comparison of T cells cultured separately, vs T cells co-cultured with untreated or TI-treated ASCs. AS: ankylosing spondylitis; ASCs: adipose tissue-derived MSCs; HD: healthy donors; IFN γ : interferon γ ; IQR: interquartile range; MFI: mean fluorescence intensity; MSC: mesenchymal stem cell; n: number of experiments performed; PBMC: peripheral blood mononuclear cell; PHA: phytohemagglutinin; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; TNF: tumor necrosis factor.

(Fig. 3) and CD8⁺ (Fig. 4) T-cell subsets raised significantly, but were much diverse than in activated purified CD4⁺ T-cell subset (Fig. 2), at least in the case of HLA-DR⁺ and CD69⁺ cell numbers. Consistently with the results obtained in co-cultures of ASCs with purified CD4⁺ T cells, cultivation of PHA-stimulated PBMCs (PBMCs_{PHA}) in the presence of HD/ASCs or RD/ASCs significantly reduced the percentage of CD25⁺ and HLA-DR⁺ cells, with concomitant increase of CD69⁺ cell number. These modulatory effects were noted in both CD4⁺ (Fig. 3, upper panel) and CD8⁺ (Fig. 4, upper panel) cell subsets, and there was no significant difference between HD/ASCs and RD/ASCs exerted impact. In addition, in the presence of HD/ASCs and RD/ASCs the expression levels (MFI) of CD25 on CD4⁺ and CD8⁺ cells, and HLA-DR on CD4⁺ cells were reduced, but CD69 MFI stayed unchanged, except some increase on CD4⁺ T cells in the presence of SLE/ASCs and SSc/ASCs (Figs. 3 and 4, lower panels).

ASCs-derived Effects are Mediated Mostly Through Soluble Factors

To discriminate between cell-to-cell contact and soluble factors contribution to observed modulatory effects of ASCs,

we used the same combinations of PBMCs and ASCs grown in co-cultures allowing direct cell-cell contact, or preventing it by physical separation of these cell types, using TW filters. In this part of experiments RD/ASCs isolated from fat tissue of five SLE, five SSc, and five AS patients were used. As shown in Fig. 5A-C, HD/ASCs and RD/ASCs exerted similar reducing effects on the proportion of CD25⁺ and HLA-DR⁺ cells in CD4⁺ T-cell subset, irrespective of the type of co-culture applied. However, exposure of PBMCs_{PHA} to TI-treated RD/ASCs in a contact co-cultures caused significantly higher increase of CD69⁺ cells proportion, compared to TW co-cultures, and similar tendency was noted in the presence of HD/ASCs. Comparable effects of ASCs on CD8⁺ T cells after contacting and noncontacting co-culture with PBMCs_{PHA} were observed (Fig. 5D-F).

Modulatory Effects of RD/ASCs on Autologous T Cells

To check whether modulatory effects of RD/ASCs are preserved toward autologous T cells, eight RD/ASCs lines, derived from two SLE, three SSc, and three AS patients, were co-cultured in a direct contact with allogeneic or autologous PBMCs_{PHA}, then CD4⁺ (Fig. 6) as well as CD8⁺ (Fig. 7) T cells were analyzed. In allogeneic and autologous

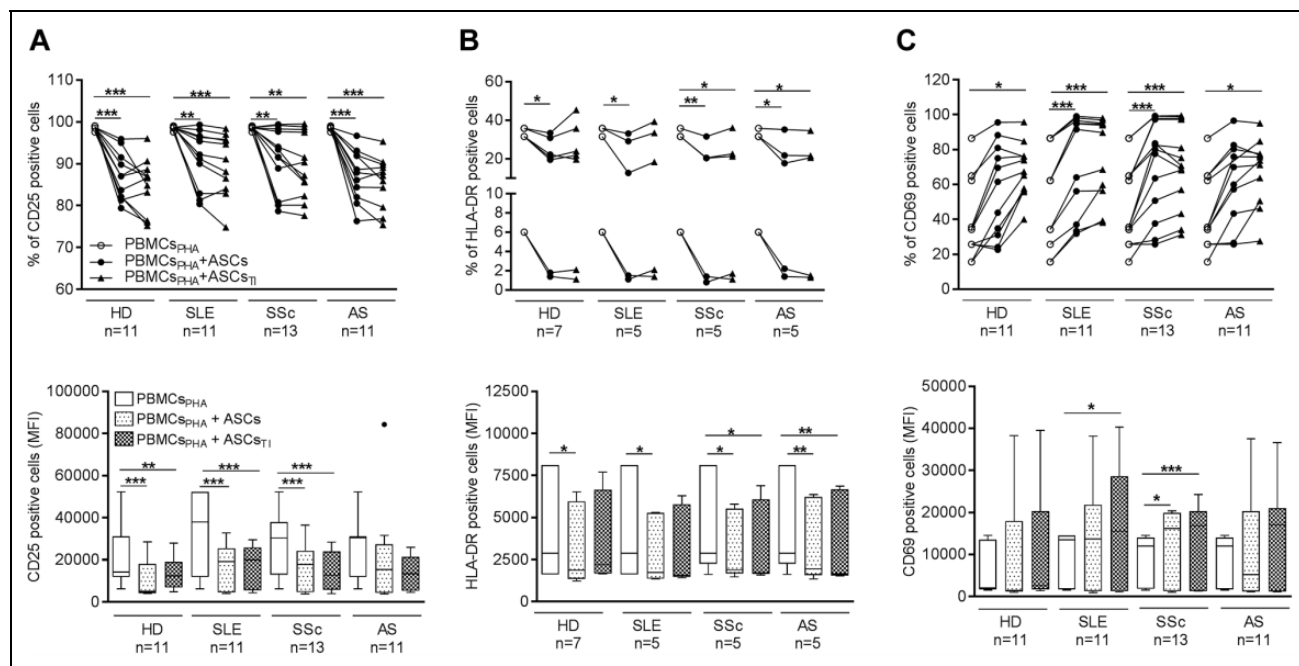


Figure 3. Modulation of activation markers on CD4⁺ T cells in the co-cultures of activated PBMCs with ASCs. Cell co-cultures were performed using PBMCs obtained from seven healthy blood volunteers and ASC lines obtained from five HD, five to eight SLE, five to nine SSc, and five to seven AS patients. Lines between points in the upper panels identify cultures containing the same combination of PBMCs and ASCs. Data in the lower panels are expressed as the Tukey's boxes. PBMCs activated with PHA, untreated, or TNF + IFN γ (TI)-treated ASCs were used. CD4⁺ T cells were analyzed by flow cytometry. See Materials and Methods for details. The upper and lower panels show the percentage of cells positive for particular marker and MFI, respectively. * $P = 0.05-0.01$, ** $P = 0.01-0.001$, *** $P = 0.001-0.0001$ for comparison of PBMCs_{PHA} cultured separately, vs PBMCs_{PHA} co-cultured with untreated or TI-treated ASCs. AS: ankylosing spondylitis; ASCs: adipose tissue-derived MSCs; HD: healthy donors; IFN γ : interferon γ ; MFI: mean fluorescence intensity; MSC: mesenchymal stem cell; n : number of experiments performed; PBMC: peripheral blood mononuclear cell; PHA: phytohemagglutinin; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; TNF: tumor necrosis factor.

systems RD/ASCs increased the percentage of CD69⁺ and decreased the percentage of HLA-DR⁺ cells to similar extent, and this modulatory effect was observed in both CD4⁺ and CD8⁺ T-cell subsets (Figs. 6B, C and 7B, C, upper panels). However, RD/ASCs exerted weaker inhibitory effect on the proportion of CD25⁺ cells in autologous than allogeneic CD4⁺ and CD8⁺ T-cell subsets (Figs. 6A and 7A, upper panels). Similar significant difference or trend was observed in CD25 MFI downregulation on CD8⁺ and CD4⁺ T-cell subset, respectively (Figs. 6A and 7A, lower panels), whereas neither CD69MFI nor HLA-DR MFI changed significantly (Figs. 6B, C and 7B, C, lower panels).

Discussion

Activated CD4⁺ and CD8⁺ T cells are essential participants of RD pathogenesis. In SLE and SSc, various CD4⁺ and CD8⁺ T-cell subsets play pathogenic role, allowing production of autoantibodies, promoting inflammation and/or fibrosis, while defective regulatory T cells (Treg) contribute to unchecked immune response²⁵⁻²⁸. Defects of Treg cells, along with T-cell subsets imbalance, increased numbers of circulating Th17 cells, and migration of activated cytotoxic

CD8⁺ T lymphocytes to affected joints, were also reported in AS²⁹⁻³².

Upon activation, T lymphocytes start to express the surface molecules (CD69, CD25, and HLA-DR) that are required to fulfil biological functions of these cells¹¹⁻¹⁴. MSCs of various origin, including human ASCs of HD, were reported to regulate these activation markers expression on T cells³³⁻³⁹. Present work focused on checking whether RD/ASCs, pre-exposed *in vivo* to proinflammatory microenvironment, possess similar ability. The immunosuppressive function of MSCs is thought to be triggered or enhanced by proinflammatory cytokines, mostly by TNF and IFN γ ^{40,41}. Moreover, accumulating evidence shows that MSCs exert immunomodulatory effects on T cells directly or via indirect, monocyte/macrophage-mediated pathway⁴²⁻⁴⁵. However, there is no agreement regarding contribution of direct and indirect pathways in the regulation of T-cell activation markers expression so far^{34,37}. For the above reasons, we co-cultured untreated and TNF α plus IFN γ (TI)-pre-treated ASCs with allogeneic target cells: (i) purified CD4⁺ T cells activated via CD3/CD28 signaling pathway (Fig. 2) and (ii) mitogen (PHA)-stimulated PBMCs (Figs. 3 and 4). Although preconditioning can alter various MSCs activities, its final outcome depends on the type of applied priming factor and

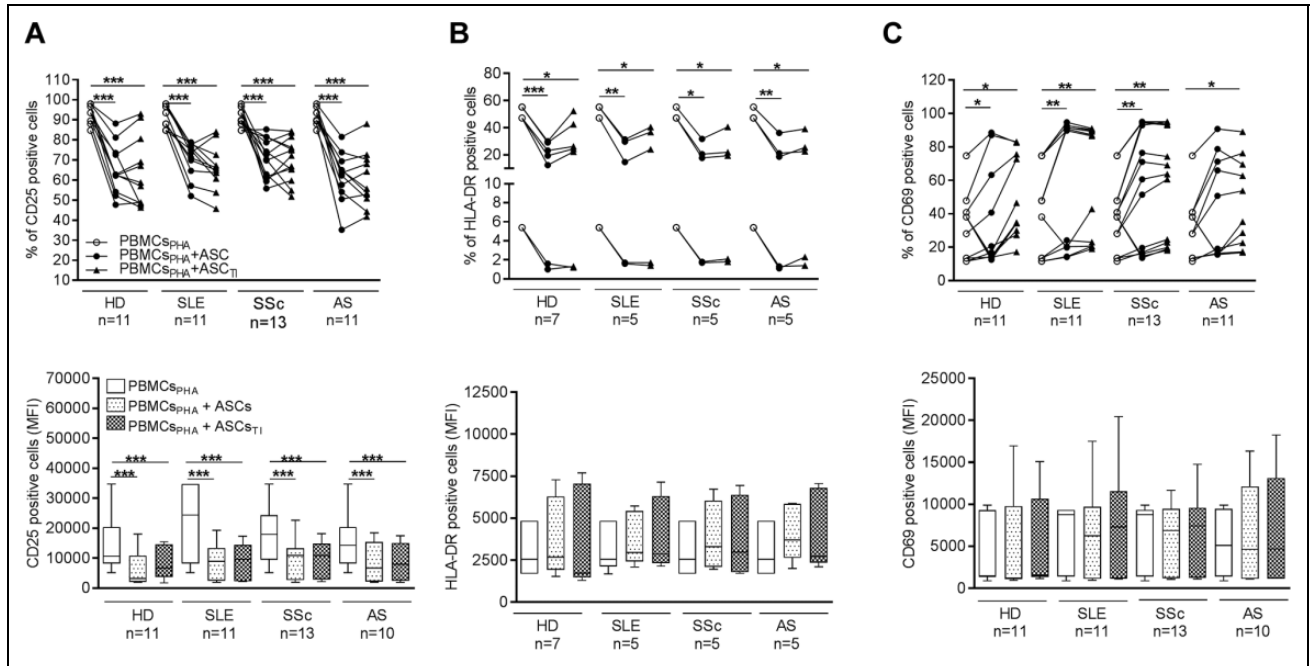


Figure 4. Modulation of activation markers on CD8⁺ T cells in the co-cultures of activated PBMCs with ASCs. Explanations as in Fig. 2. After co-culture CD8⁺ cells were analyzed by cytometry. ASCs: adipose tissue-derived MSCs; MSC: mesenchymal stem cell; PBMC: peripheral blood mononuclear cell.

combination of some of them, e.g., TNF α and IFN γ exerts usually additive effects⁴¹. We have previously reported that TI treatment upregulates ICAM-1, but not VCAM-1, expression on ASCs and increases secretion of some (kynurenines, IL-6, and LIF), while fails to modify release of other (IL-1Ra, sHLA-G, TSG-6, galectin-3, and TGF β) immunoregulatory factors, pointing out some selectivity of TI priming¹⁰. Importantly, untreated (naïve) MSCs are endowed with some immunoregulatory activities as well, including regulation of T-cell activation markers expression^{44,46,47,48}. Interestingly, to downregulate CD25 expression untreated MSCs^{47,48} seem to use other mechanisms than TNF α + IFN γ -pre-treated cells³⁵. Altogether these data show that both naïve and licensed MSCs may exert some similar immunoregulatory effects and that priming of MSCs with TI may alter selected activities of these cells, thus explaining the lack of TI-pre-treatment on ASCs immunomodulatory action observed in present study.

Consistently with the reports of others^{35–37,39}, we found significant decrease of the number of CD25⁺ and HLA-DR⁺ cells in the co-cultures of HD/ASCs and RD/ASCs with both activated CD4⁺ cells and PBMCs_{PHA}. The AS/ASCs-T cell co-cultures, where weak, if any, inhibitory effect on CD25⁺ cell number was observed, were the only exception (Fig. 2A). When HD/ASCs and RD/ASCs were co-cultivated with PBMCs_{PHA}, the decrease of CD25⁺ and HLA-DR⁺ cell numbers occurred in both CD4⁺ and CD8⁺ cell subsets, concomitantly with the lowering of CD25 (on both subsets) and HLA-DR (on CD4⁺ cells only) expression levels (MFI)

(Figs. 3 and 4). Therefore, the reduction of the number of HLA-DR⁺ and CD25⁺ T cells, and the diminution of these markers expression levels, seem to be mediated via direct and indirect pathway, respectively. As for AS/ASCs, the indirect effect appears to be more important in decreasing CD25⁺ T-cell proportion (compare Figs. 2A and 3A, upper panels). By contrast to CD25 and HLA-DR molecules, HD/ASCs and RD/ASCs increased the number of CD8⁺CD69⁺ and/or CD4⁺CD69⁺ cells, when co-cultured with allogeneic PBMCs_{PHA} or activated CD4⁺ T cells, respectively, without substantial change of CD69 expression levels (Figs. 2–4). There is no consensus in the literature about MSCs influence on CD69 expression by T cells. Although MSCs were mostly reported to upregulate CD69^{33,34,38,46}, which corresponds to our results, opposite effect was also shown^{36,39,42}. Regarding this discrepancy, MSCs origin and culture conditions seem to have critical impact on final results^{39,49}. It is worth mention that in SLE, SSc, and AS patients, quantitative and qualitative abnormalities of CD69⁺ effector and/or regulatory T cells were reported^{29,50,51} and upregulation of CD69 by MSCs restored impaired cell function⁵².

Based on the above results we conclude that in allogeneic system HD/ASCs and RD/ASCs act in a similar way: (i) reduce CD25⁺ and HLA-DR⁺, while increase CD69⁺ T-cell numbers, (ii) exert these effects independently of their own activation status (similar effects of untreated vs TI-treated ASCs), the type of T-cell stimulus (anti-CD3/CD28 vs mitogen), and, with the exception of AS/ASCs, independently of cellular context (Figs. 1–3). These effects

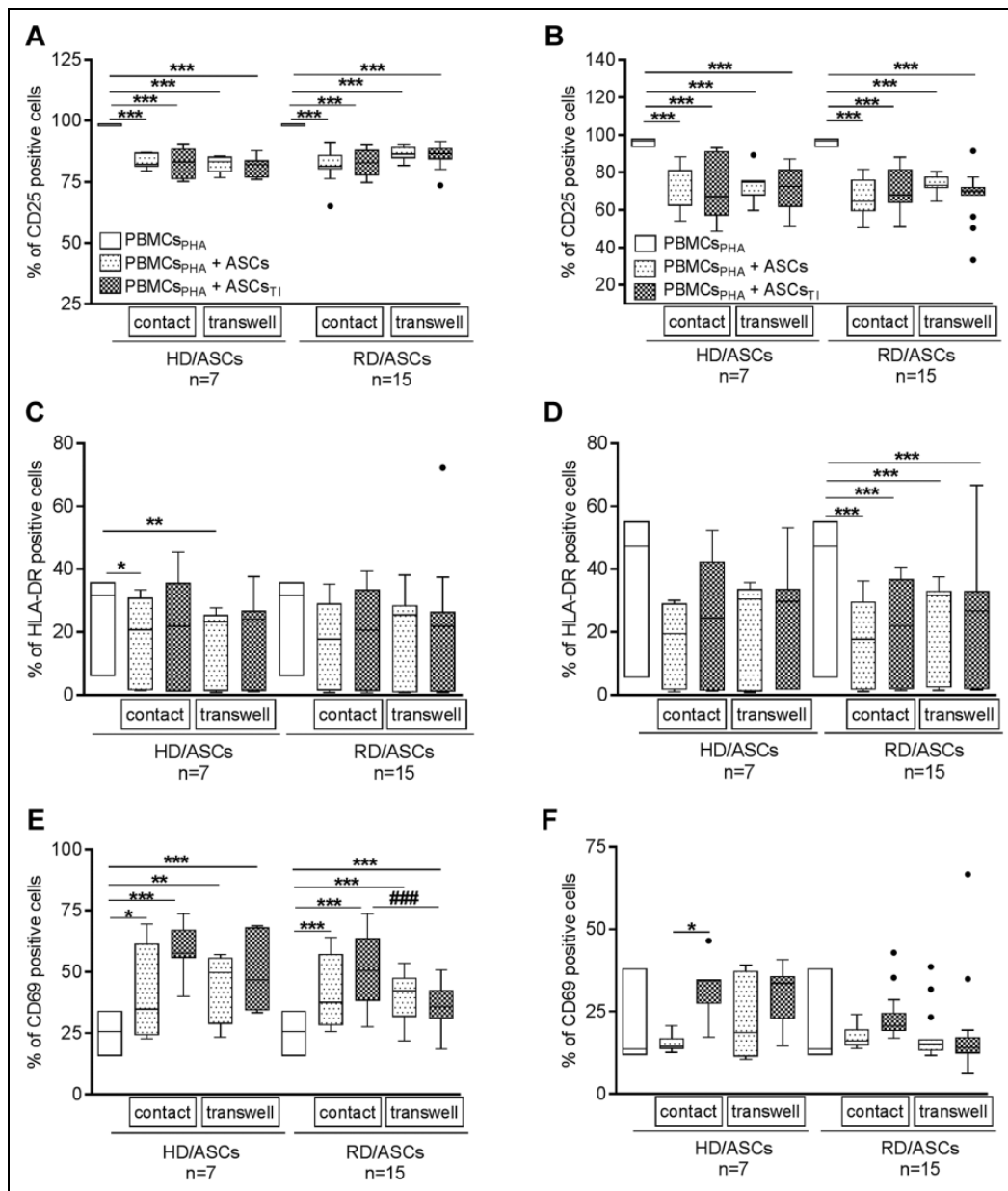


Figure 5. Modulatory effects of ASCs on the expression of activation markers on T cells are mediated through soluble factors. Untreated and TNF + IFN γ (TI)-treated ASCs from healthy donors (HD/ASCs, $n = 4$) and patients with rheumatic diseases (RD/ASCs, $n = 15$) were grown with PHA-activated PBMCs from healthy blood volunteers ($n = 3$), in the contacting and noncontacting (transwell) co-cultures, using the same combinations of cell types (n —number of experiments performed). CD4 $^{+}$ (A–C) and CD8 $^{+}$ (D–F) T cells were analyzed by flow cytometry. Data are expressed as the Tukey's boxes. * $P = 0.05$ – 0.01 , ** $P = 0.01$ – 0.001 , *** $P = 0.001$ – 0.0001 for comparison of PBMCs_{PHA} cultured separately, vs PBMCs_{PHA} co-cultured with untreated or TI-treated ASCs. #### $P = 0.001$ – 0.0001 for comparison of contact and transwell co-cultures. ASCs: adipose tissue-derived MSCs; IFN γ : interferon γ ; MSC: mesenchymal stem cell; PBMC: peripheral blood mononuclear cell; PHA: phytohemagglutinin; TNF: tumor necrosis factor.

apply to both CD4 $^{+}$ and CD8 $^{+}$ T-cell subsets, which is in line with other reports^{42,46}.

Numerous mechanism(s), including cell–cell contact and paracrine action of secreted factors, were proposed to mediate the immunoregulatory function of MSCs^{53,54}. Present results demonstrate that downregulation of the number of CD4 $^{+}$ and CD8 $^{+}$ T cells expressing CD25 and, to a lesser extent, HLA-DR molecules occurs when PBMCs_{PHA} are co-

cultivated with HD/ASCs and RD/ASCs even without cell–cell contact (TW cultures) (Fig. 5). As no significant differences between cell–cell contact and TW cultures were found, this implies that both types of ASCs exert this inhibitory effects through the release of bioactive soluble factors. In case of CD69 expression, significant increase of CD69 $^{+}$ CD4 $^{+}$ T-cell number was more pronounced in contact than TW co-cultures with TI-treated RD/ASCs,

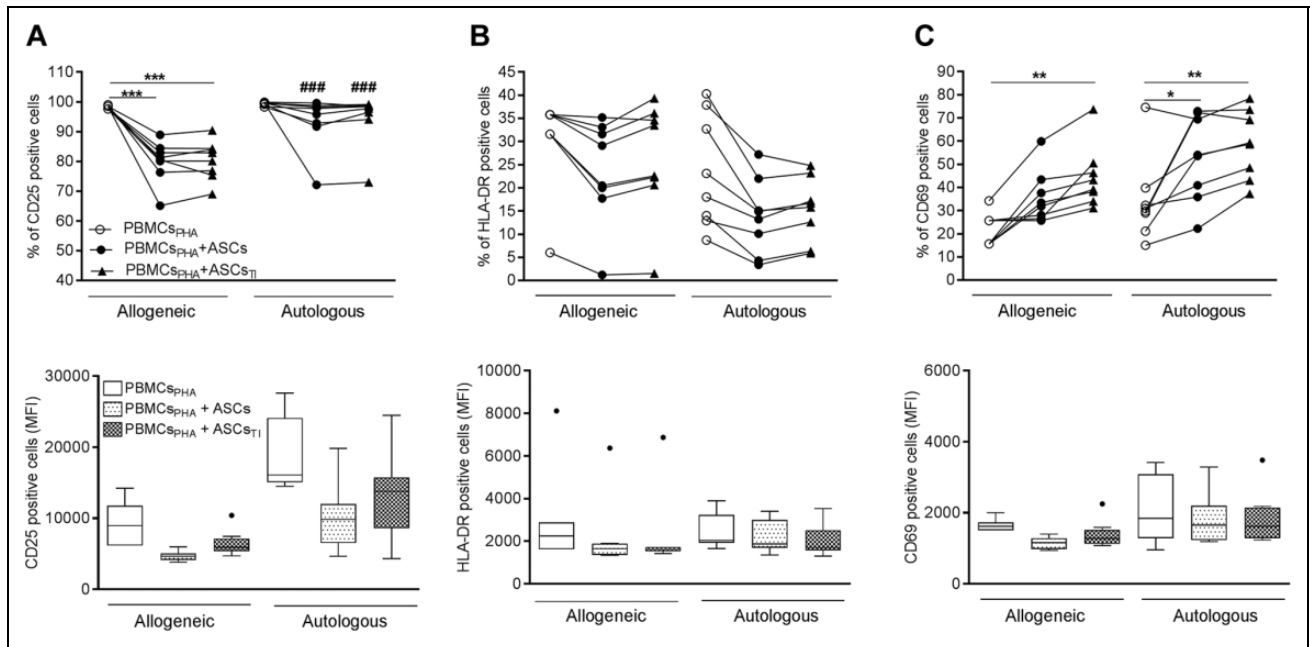


Figure 6. Modulatory effects of RD/ASCs on the expression of activation markers on allogeneic and autologous CD4⁺ T cells. Untreated and TNF + IFN γ (T1)-treated ASCs from eight patients with RD were co-cultured with PHA-activated either allogeneic PBMCs from healthy blood volunteers ($n = 3$) or autologous PBMCs. Expression of CD25, CD69, and HLA-DR molecules on CD4⁺ T cells was analyzed by flow cytometry. Lines between points in the upper panels point out cultures containing the same combination of PBMCs and ASCs. Data in the lower panels are expressed as the Tukey's boxes. * $P = 0.05-0.01$, ** $P = 0.01-0.001$, *** $P = 0.001-0.0001$ for comparison of PBMCs_{PHA} cultured separately, vs PBMCs_{PHA} co-cultured with untreated or T1-treated ASCs. #### $P = 0.001-0.0001$ for comparison of allogeneic and autologous co-cultures. ASCs: adipose tissue-derived MSCs; IFN γ : interferon γ ; MSC: mesenchymal stem cell; PBMC: peripheral blood mononuclear cell; PHA: phytohemagglutinin; RD: rheumatic diseases; TNF: tumor necrosis factor.

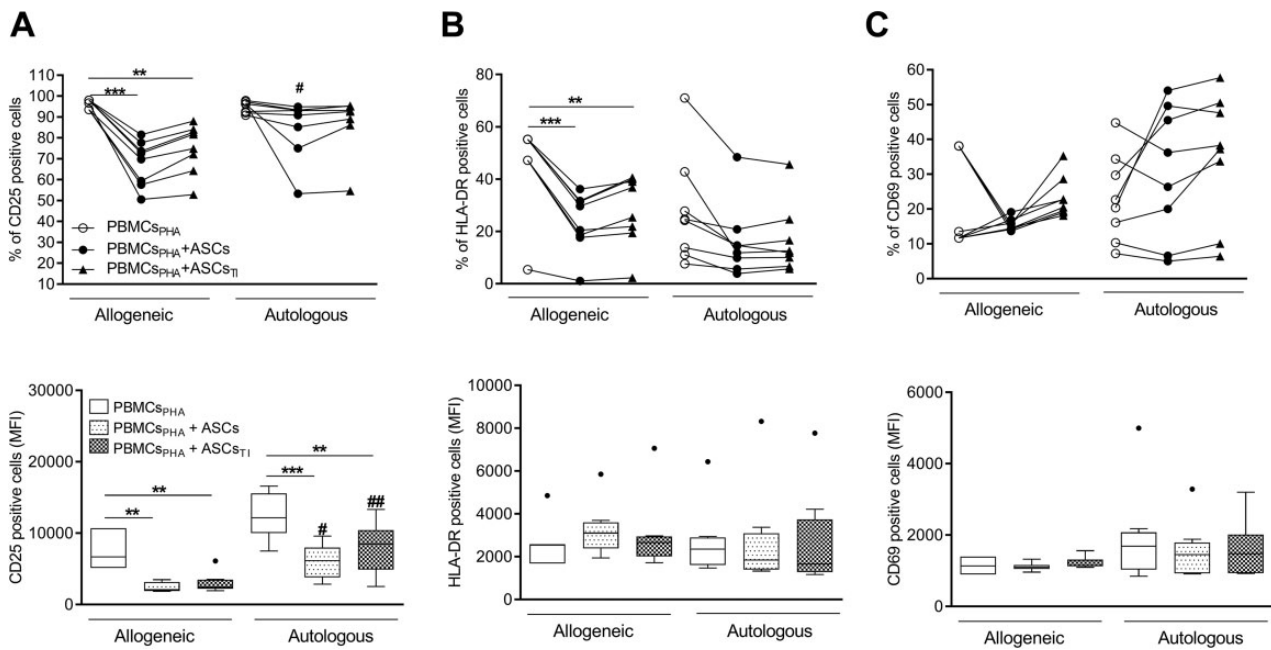


Figure 7. Modulatory effects of RD/ASCs on the expression of activation markers on allogeneic and autologous CD8⁺ T cells. Explanations as in Fig. 6. After co-culture CD8⁺ T cells were analyzed. ** $P = 0.01-0.001$, *** $P = 0.001-0.0001$ for comparison of PBMCs_{PHA} cultured separately, vs PBMCs_{PHA} co-cultured with untreated or T1-treated ASCs. ## $P = 0.05-0.01$, ### $P = 0.01-0.001$, #### $P = 0.001-0.0001$ for comparison of allogeneic and autologous co-cultures. ASCs: adipose tissue-derived MSCs; MSC: mesenchymal stem cell; PBMC: peripheral blood mononuclear cell; RD: rheumatic diseases.

suggesting involvement of both cell–cell interactions and soluble factors. However, no such difference was observed in CD8⁺ T-cell subset. Also HD/ASCs comparably upregulated CD69 in contact and TW co-cultures (Fig. 5), supporting the importance of soluble agents. Many MSCs released factors are known to exert suppressive effects on T cells⁵⁵. Some of them were reported to regulate T-cell activation markers expression. In human system, modulation of CD25 and CD69 on T cells was shown to be dependent on TGFβ released by BM-MSCs³⁶ or TGFβ and IL-10 secreted by MSCs-exposed macrophages⁴⁵. Moreover, MSCs-derived soluble forms of programmed death-1 ligands were demonstrated to downregulate CD25 by binding to respective receptors on T cells³⁵. In addition, human MSCs were found to downregulate CD25 through transcription inhibition and CD25 shedding⁴⁴, while murine MSCs reduced CD25 expression by enzymatic cleavage or by nitric oxide-triggered inhibition of CD25 translation^{47,48}. Thus, MSCs use various mediators to keep T-cell activation under control. Identification of factors implicated in presently observed ASCs effects exerted on T lymphocytes requires further studies, but our previous comparative characteristics of HD/ASCs and RD/ASCs secretory activity point out some candidates, including TGFβ¹⁰.

Apart from maintenance of normal ASCs functions, the sensitivity of target cells to ASCs suppression is another prerequisite for their application as a therapeutic tool. To check this, we evaluated the effects of RD/ASCs on T-cell activation markers expression also in autologous system. Importantly, we found that RD/ASCs exerted significantly weaker inhibitory effect on CD25 expression in co-cultures with autologous than allogeneic PBMC_{S_{PHA}} (Figs. 6 and 7). These differences were observed in both CD4⁺ and CD8⁺ T-cell subsets and consisted in smaller reduction of both the number of CD25⁺ cells and CD25 expression level per cell. By contrast, RD/ASCs similarly modified HLA-DR and CD69 expression on allogeneic and autologous CD4⁺ and CD8⁺ T cells. The reason for presented partial resistance of T cells from RD patients to ASCs-induced CD25 downregulation is unknown, and to our knowledge, such defect has not been reported so far. Because MSCs may use direct and indirect pathways to control CD25 expression, it is possible that observed abnormality results from the dysfunction of T cells and/or monocytes/macrophages^{42–45}. Regarding cells from healthy volunteers, there are studies showing that T-cell suppression, including downregulation of CD25, results from mutual interaction of MSCs and monocytes/macrophages. This cellular communication enhances MSCs suppressive activity and skews macrophage polarization into immunosuppressive M2-like type, leading to decrease in effector T-cell activity^{42,45,56}. Intriguingly, accumulating evidence shows that for autoimmune and autoinflammatory disorders the proinflammatory status of monocytes/macrophages and abnormal behavior of BM-MSCs is characteristic and might be induced by trained immunity^{57,58}.

Thus, contribution of “trained” cells to presently found defect is quite possible. Data demonstrate potential benefits of therapeutic application of autologous ASCs to reverse tissue damage in OA and SSc, but the knowledge about immunoregulatory functions of these cells is incomplete^{59,60}. In SLE, by analogy to bone marrow-derived MSCs, also ASCs are supposed to be functionally defective, but biology of these cells is poorly understood and data concerning immunoregulatory capabilities of autologous ASCs from AS patients are very limited. Present results suggest that immunoregulatory functions of RD/ASCs related to T-cell activation control are not compromised, but *in vivo* this regulatory mechanism may be weakened due to partial resistance of autologous T-cells to be a subject of such control. In patients with other autoimmune diseases, multiple sclerosis and myasthenia gravis, reduced sensitivity of T cells to immunoregulation by HD/ASCs was reported to be a consequence of IL-2 overproduction by lymphocytes, but not monocyte dysfunction⁴⁴. Whatever the reason of the defect in autologous RD system is, our *in vitro* data suggest that autologous ASCs may not be efficient enough to quiescent activated T cells in RD patients.

Conclusion

We report that in allogeneic system RD/ASCs retain normal capability to modify expression of activation markers (to downregulate CD25 and HLA-DR, while upregulate CD69) on both CD4⁺ and CD8⁺ T cells, acting independently of their own activation status, mostly through the indirect pathway and soluble factors. However, autologous CD4⁺ and CD8⁺ T cells are partially resistant to RD/ASCs inhibition of CD25 expression, suggesting weaker control of T-cell activation *in vivo*.

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Author Contributions

EK-W designed experiments, performed flow cytometry experiments, and data analysis. IJ was responsible for cell isolation and culture. KB, PG, PS, and MO helped with recruiting patients, acquired and analyzed clinical data. EK wrote the manuscript.

Data Availability

The data used to support the findings of this study are included in the article.

Ethical Approval

Ethical approval to report this case was obtained from Ethics Committee of the National Institute of Geriatrics, Rheumatology, and Rehabilitation, Warsaw, Poland (the approval protocol no: KBT-8/4/20016).

Statement of Human and Animal Rights

All procedures with human subjects in this study were conducted in accordance with the guidelines of Ethics Committee of the National Institute of Geriatrics, Rheumatology, and Rehabilitation, Warsaw, Poland. This article does not contain any studies with animals.

Statement of Informed Consent

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.


Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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