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Secretory factors produced by adipose mesenchymal stem cells downregulate Th17 and increase Treg cells in peripheral blood mononuclear cells from rheumatoid arthritis patients

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Abstract

We aimed to assess the immunoregulatory effects of secretory factors produced by adipose tissue-derived MSC (AT-MSC) on Th17 and Treg subsets from patients with rheumatoid arthritis (RA). 17 patients with active disease matching the ACR/EULAR 2010 criteria for RA were included. Patients' peripheral blood mononuclear cells (PBMC) were cultured in AT-MSC-conditioned medium (AT-MSCcm) and in control medium. The cytokine production of AT-MSC and PBMC was quantified by ELISA. Th17 and Treg were determined by flow cytometry. AT-MSCcm contained: IL-6, IL-17, IL-21, CCL2, CCL5, IL-8, sVEGF-A and PGE_2. Cultivation of patients' PBMC with AT-MSCcm increased TGF-β1 (8318 pg/ml; IQR 6327–11,686) vs control medium [6227 pg/ml (IQR 1681–10,148, $p=0.013$)]. PBMC cultivated with AT-MSCcm downregulated TNF-α, IL-17A, and IL-21 compared to control PBMC: 5 pg/ml IQR (1.75–11.65) vs 1 pg/ml (IQR 0.7–1.9), $p=0.001$; 4.2 pg/ml (IQR 3.1–6.1) vs 2.3 pg/ml (IQR.75–5.42), $p=0.017$; 66.9 pg/ml (IQR 40.6–107.2) vs 53 pg/ml (IQR 22–73), $p=0.022$. Th17 decreased under the influence of AT-MSCcm: 10.13 ± 3.88% vs 8.98 ± 3.58%, $p=0.02$. $CD4^+FoxP3^+$, $CD4^+CD25^-FoxP3^+$, and $CD4^+CD25^+FoxP3^+$ was 11.35 ± 4.1%; 7.13 ± 3.12% and 4.22 ± 2% in control PBMC. Accordingly, $CD4^+FoxP3^+$, $CD4^+CD25^-FoxP3^+$, and $CD4^+CD25^+FoxP3^+$ significantly increased in PBMC cultured with AT-MSCcm: 15.6 ± 6.1%, $p=0.001$; 9.56 ± 5.4%, $p=0.004$ and 6.04 ± 3.6%, $p=0.001$. All these effects could define MSC-based approaches as adequate avenues for further treatment development in RA.

Keywords Mesenchymal stem cells · Immunosuppression · Rheumatoid arthritis · Th17 · Treg · Cytokines

Introduction

Maintenance of self-tolerance and immunological homeostasis is a finely tuned process that requires constant cross-talk between a multitude of immunocompetent cells from both innate and adaptive arms of immunity and a spectrum of cells of non-lymphoid origin such as mesenchymal stem cells (MSC) [1]. Comprehensive understanding of these precise mechanisms and their dysregulations are of great importance in terms of the development of more suitable and successful curative strategies for long-term management of a range of socially significant disabling immune-mediated diseases such as rheumatoid arthritis (RA).

Mesenchymal stem cells could be characterized as a heterogeneous population of undifferentiated, non-hematopoietic, multipotent fibroblast-like progenitor cells that could be isolated and easily expanded in vitro from array of adult and fetal tissues, among which adipose tissue (AT-MSC), bone marrow (BM-MSC), synovium, tendons, muscle, liver, placenta, etc. [2, 3]. Hallmark features of MSC are their clonogenicity and potential for proliferation and self-renewal [3, 4]. Moreover, MSC can undergo a process of multi-lineage differentiation into adipocytes, chondrocytes or osteocytes [3]. MSC normally reside in specialized settings termed as MSC niches. Two major types of stem cell niches have been delineated: the endosteal niche in bone marrow and the perivascular niche [2]. In bone marrow

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niche MSC differentiate in pericytes, myofibroblasts, and other stromal cells, thus creating a favorable microenvironment that supports hematopoiesis. In perivascular niches, stem cells outline the microcirculatory blood vessels from the outside. This abluminal disposal allows them to interact with a range of immunocompetent cells and also to gain access to almost all tissues. From this standpoint, complex interaction between immunocompetent cells and MSC arises in the context of the inflammatory conditions characteristic for RA. Their interactions become even more complicated due to the fact that synovial lining is also a stem cell niche [2] along with the proximity of synovial and subchondral inflammation with the bone marrow niche as well.

Emerging data have demonstrated that MSC are well endowed with a vast repertoire of immunosuppressive tools [5, 6]. Their immunoregulatory machinery could modulate the immune response on several levels via the production of soluble factors (cytokines and chemokines) as by contact-dependent mechanisms [6]. For these reasons MSC have drawn considerable interest over the last 2 decades and novel cell-based approaches utilizing adult MSCs have emerged. Moreover, due to MSC immunomodulatory features and potential for self-renewal and tri-lineage differentiation, stem cell-based protocols have been spotlighted as promising multiple-hit treatment candidates for therapeutic intervention in a multitude of autoimmune diseases and debilitating conditions [7–10]. Large body of pre-clinical studies and clinical trials clearly demonstrated the curative properties of MSC of adult and fetal origin in RA [7], systemic lupus [8], multiple sclerosis [9] and life-threatening conditions such as graft vs host disease [10]. A brief reference to the official clinical trials database of U.S. National Library of Medicine [11] shows currently a number of 740 ongoing trials exploring the curative effects of MSC obtained from various sources in a variety of diseases, most of which are autoimmune in nature. Notably, 13 of them are only focused on the assessment of MSC-based agents' curative effects, efficacy and safety in RA.

Albeit these achievements, the precise immunosuppressive mechanism of MSC remains largely unclear. In line with the aforementioned and taking into consideration the current shift in Th1/Th2 paradigm suggesting a key role of Th17/Treg axis dysregulations in RA pathogenesis [12], we aimed to assess the immunoregulatory effects of secretory factors produced by adipose tissue-derived MSC (AT-MSC) on interleukin-17 (IL-17)-producing T helper (Th17) and regulatory T (Treg) cell subsets from peripheral blood mononuclear cells (PBMC) of RA patients with moderate to high disease activity. We did not only explore the changes in the pool of transcription factor forkhead box P3(FoxP3)$^+$ Tregs but also separated them based on CD25 surface expression and put emphasis on the kinetics of both classical CD25$^+$ and CD25$^-$ FoxP3$^+$ Treg subsets.

Methods

Study subjects, sample collection and clinical assessments

A total of 17 patients matching the ACR/EULAR 2010 Classification Criteria for RA were enrolled in the study. Selection and clinical evaluation of eligible subjects were performed in the Rheumatology Clinic of University Hospital "St. Iv. Rilski" in Sofia, Bulgaria over a period of 1 year. Disease Activity Score 28 calculated using CRP (DAS28-CRP) was applied for measurement of the disease activity of RA patients. The main inclusion criteria were age ≥ 18 years; symptom onset at least 6 months prior to the sample collection. For study purposes subjects with moderate to high disease activity defined as DAS28-CRP > 3.5 were selected. Main exclusion criteria were history of other inflammatory rheumatological or autoimmune disorders; history of malignancies; current treatment with biological disease modifying anti-rheumatic drugs (bDMARD) or targeted synthetic DMARDs; prior history of treatment with bDMARD less than 6 months before study; heart failure; active and latent tuberculosis; other active infections. With regard to drug therapy, 12 patients were being treated with conventional synthetic DMARDS (csDMARDs)—methotrexate (10–25 mg/week), and 10 patients were receiving low dose systemic glucocorticoids (10 mg/day). Parameters of studied population are summarized in Table 1.

The study was approved by the ethics committee of University Hospital "St. Ivan Rilski"-Sofia. Each subject signed a voluntarily informed consent in accordance with the ethical recommendations of the Helsinki Declaration. Peripheral venous blood-8 ml (Vacutainer CPT Cell Preparation Tube Sodium Citrate 0.1 ml and Ficoll, Beckton and Dickinson, USA) was collected from each patient. Thereafter, density gradient centrifugation technique was applied to isolate each patients' PBMC from peripheral venous blood.

Adipose tissue mesenchymal stem cell-conditioned medium

Tissue samples of adipose tissue were obtained from healthy donors after voluntarily signed informed consent. Subsequently, adipose tissue mesenchymal stem cells (AT-MSC) were isolated, cultured and phenotyped according to well-established and accepted protocols and adipose tissue mesenchymal stem cell-conditioned medium (AT-MSCcm) was derived. AT-MSCcm contained all the soluble factors, secreted by AT-MSC. Methods and protocols for the

Table 1 Demographic, clinical and immunological parameters of RA patients

	RA
n	17
Age (years)[a]	54 ± 10
Sex	
Male (%)	1 (6)
Female (%)	16 (94)
Disease duration (years)[b]	6 (1–11)
Duration of morning stiffness (min)[b]	30 (10–90)
Visual analog scale of pain (mm)[b]	70 (45–85)
DAS28-CRP[a]	5.3 ± 1.2
Patients with + CCP antibodies (%)	15 (88.2)
CCP antibodies (UI/ml)[a]	360 ± 260
Patients with +RF (%)	12 (70.6)
IgM RF (IU/ml)[a]	214 ± 54
IgG RF (IU/ml)[a]	150 ± 46
IgA RF (IU/ml)[a]	126 ± 45
CRP (mg/l)[a]	23 ± 15

CRP C reactive protein, *CCP antibodies* antibodies against cyclic citrullinated proteins, *DAS28-ESR* Disease Activity Score 28 calculated using CRP, *RA* rheumatoid arthritis, *RF* rheumatoid factor

[a]Normally distributed data are presented as mean ± standard deviation

[b]Non-normally distributed data are presented as median (min; max)

isolation, cultivation, and preparation of AT-MSCcm are described in detail in a previous publication of the authors [13].

Cell cultures

Thereafter, 1×10^6 PBMC from each RA patient were cultured at a concentration of 1×10^6 cells per well with AT-MSC-conditioned medium (AT-MSCcm). In parallel, 1×10^6 PBMC from each RA patient were also cultured at a concentration of 1×10^6 cells per well with control cell culturing medium-DMEM/F12 (Dulbecco's Modified Eagle's Medium, Merck, New Jersey, USA; containing 10% fetal bovine serum and antibiotic/antimycotic—penicillin, streptomycin and amphotericin B). Latter cell culture was designated as "Control". Cells were further cultivated for 72 h in standard conditions (37 °C, 5% CO2, and 95% humidity). Afterward, PBMC were resuspended and centrifugated at 280*g* for 10 min to separate cells from the culture medium. This experiment was performed with PBMC from 17 individual RA patients.

Flowcytometry

PBMC at a concentration of 1×10^6 cells were washed twice in BD cell wash (Beckton Dickinson, Pharmingen, USA) and tested for surface expression of CD3-FITC, CD4-PerCP, CD25-FITC, CD161-PE, CD196-Alexa Fluor 488 (Beckton Dickinson, Pharmingen, USA) and intracellular expression of FoxP3-PE with Cytofix/Cytoperm Fixation/Permeabilization kit (Beckton Dickinson Pharmingen, USA). Cells were further processed in line with the manufacturer's instructions, fixed with CellFix (BD, USA) and counted on FACSCalibur flowcytometer (BD, USA). To evaluate the percentage of cells in stages of early and late apoptosis, cells were tested for surface expression of phosphatidylserine and propidium iodide staining using Annexin V-FITC Apoptosis Detection Kit (Beckton Dickinson, Pharmingen, USA). For analysis of the data obtained, Software CellQuest and WinMDI 2 were used.

Enzyme-linked immunosorbent assay (ELISA)

AT-MSCcm and culture media from all 17 experimental settings were tested by immunoenzyme assay for the presence of the following cytokines, chemokines and soluble factors: interleukin (IL)-4, IL-6, IL-8, IL-10, IL-12p40, IL-17A, IL-18, IL-21, IL-23, IL-33, transforming growth factor (TGF)-β1, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, chemokine (C–C motif) ligand 2 (CCL2), soluble vascular endothelial growth factor (sVEGF-A), (Gen-Probe Diaclone SAS), prostaglandin E_2 (PGE_2) (Abcam UK), indoleamine-2,3-dioxygenase (R&D Systems, Minneapolis USA). The ELISA was performed according to the manufacturer's recommendations.

Statistical processing of data

For the statistical and graphical processing of the data obtained, the Statistical Package for the Social Sciences (SPSS) version 21 and GraphPad Prism 8 were used. The normality of the distribution of the variables was investigated using the Shapiro–Wilk test. Due to the relatively small number of paired samples ($n = 17$), the hypotheses were tested using the non-parametric Wilcoxon signed-rank test and adjustment in compliance with Benjamini–Hochberg method was applied. Correlational analysis was performed using Pearson and Spearman's rho analysis. Level of significance was set up for $p < 0.05$.

Results

Cytokines and chemokines present in AT-MSCcm

AT-MSCcm obtained from ten healthy adult donors was repeatedly tested for the presence of 24 secretory molecules. Our results demonstrated that AT-MSCcm contained eight soluble factors with well-studied immunomodulatory effects: three cytokines (IL-6, IL-17A, IL-21), three chemokines

Table 2 Cytokines, chemokines, and soluble factors present in conditioned media from ATMSC obtained from 10 healthy donors

	pg/ml*
IL-6	250 (210–290)
IL-8	550 (100–1000)
IL-17A	2 (2.1–2.9)
IL-21	50 (40–60)
CCL2	950 (790–1110)
CCL5	1550 (1000–2100)
sVEGF-A	3069 (2499–3639)
PGE_2	249 (200–298)

* Data is presented as median (min – max)

(CCL2, CCL5, IL-8), sVEGF-A and PGE_2. CCL2, CCL5, IL-8, sVEGF-A, PGE_2 and IL-6 were detected in relatively high concentrations in AT-MSCcm. However, IL-17A was found in negligible concentrations in AT-MSCcm (Table 2).

PBMC from patients with RA upregulate TGF-β1 and diminish TNF-α, IL-17A, IL-21 cytokine secretion when cultured with AT-MSCcm

The results obtained showed that cultivation of RA patients' PBMCs with AT-MSCcm as opposed to control media led to significant upregulation of TGF-β1 secretion, 8318.2 pg/ml (IQR 6327–11,686 pg/ml) vs 6227 pg/ml; (IQR 1681–10,148 pg/ml), $p=0.019$. Furthermore, PBMC cultivated in AT-MSCcm had significantly diminished levels of TNF-α, IL-17A, and IL-21 compared to control PBMC, respectively: 5 pg/ml (IQR 1.75–11.65 pg/ml) vs 1 pg/ml (IQR 0.7–1.9 pg/ml), $p=0.002$; 4.2 pg/ml (IQR 3.1–6.1 pg/ml) vs 2.3 pg/ml (IQR 0.75–5.42 pg/ml), $p=0.021$; 66.9 (IQR 40.6–107.2 pg/ml) vs 53 pg/ml (IQR 22–73 pg/ml), $p=0.022$ (Fig. 1). Moreover, we demonstrated that the reduction in IL-17A levels positively correlated with the decrease of TNF-α (Spearman's rho = 0.921, $p=0.001$).

Secretory factors present in AT-MSCcm decrease percentage of Th17 in PBMC from patients with RA in vitro

Th17 cells were characterized phenotypically by the positive expression of CD3, CD4, CD161, CD196 (CCR6) surface markers. The mean (± SD) values of Th17 cells from all $CD4^+$ Th cells in PBMC cultured with AT-MSCcm, 8.98 ± 3.58% were diminished as opposed to Th17 in PBMC cultured with control media, 10.13% ± 3.88%, $p=0.022$ (Fig. 2a).

AT-MSCcm do not influence apoptosis rate of T helper cells in PBMC from patients with RA

We did not detect any significant changes in percentage values of T helper cells in stages of early and late apoptosis, based on their membrane expression of phosphatidylserine and propidium iodide staining under the influence of AT-MSC soluble factors (data not shown).

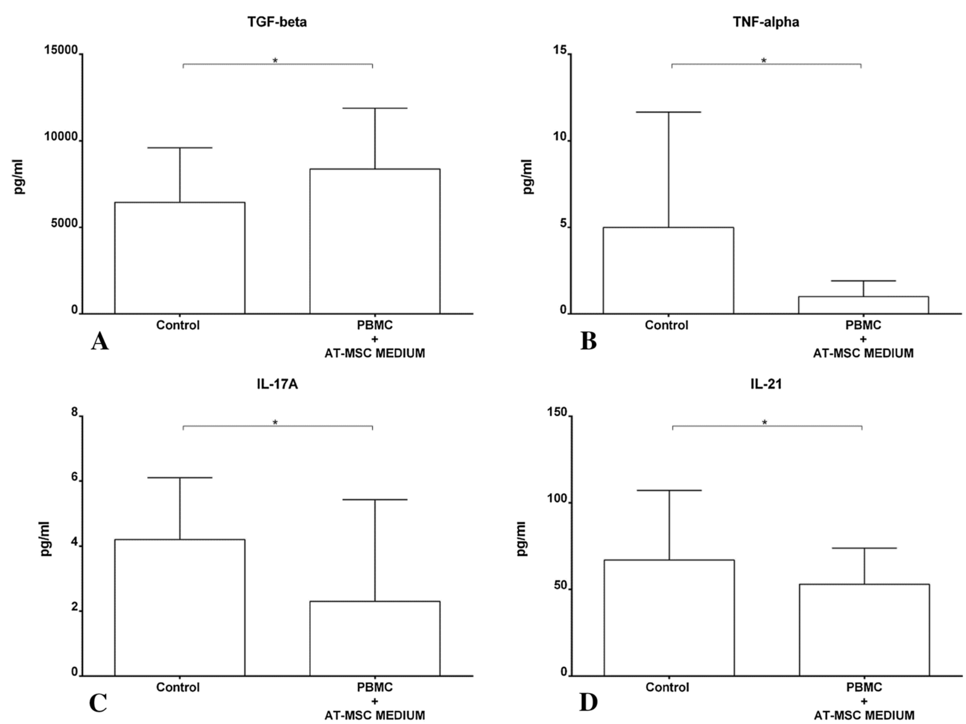

Fig. 1 Box plots displaying the changes in cytokine levels of TGF-β1 (**a**), TNF-α (**b**), IL-17A(**c**), and IL-21 (**d**) in paired samples of PBMCs obtained from RA patients cultured in DMEM medium (control) as opposed to PBMCs from the same patients cultured in AT-MSC conditioned medium (PBMC + AT-MSC medium). Data are expressed as median (IQR); *$p<0.05$ (Wilcoxon sign-rank test)

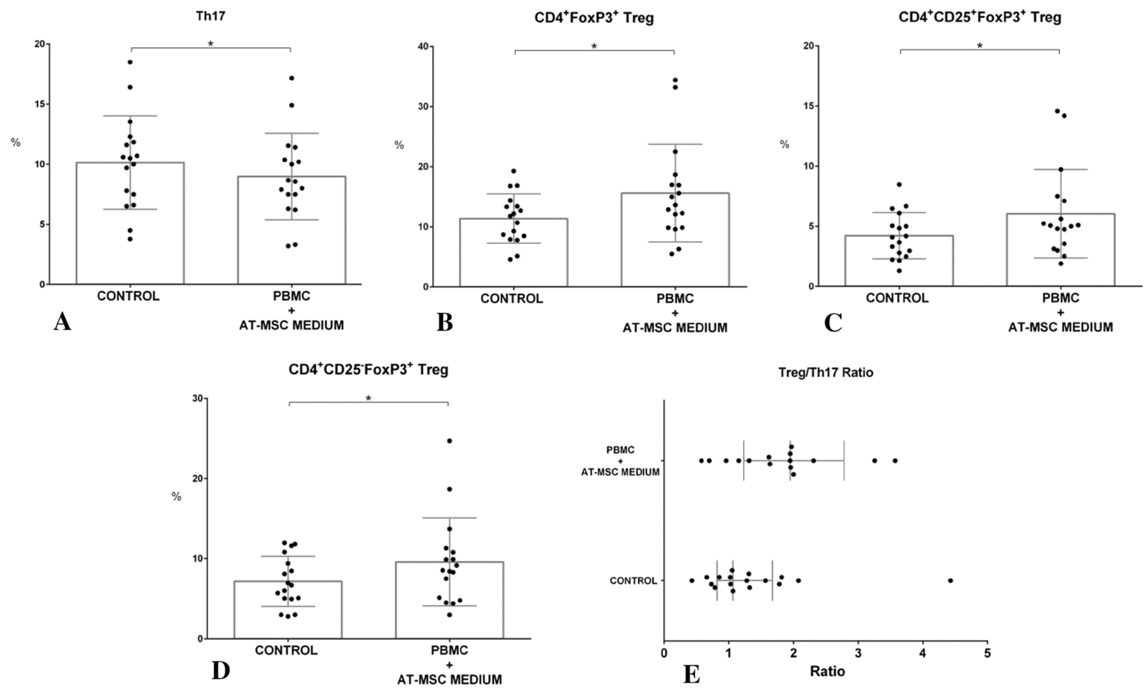

Fig. 2 Scatter plots displaying the changes in percentage values of Th17 cells (**a**), $CD4^+FoxP3^+$ Tregs (**b**), $CD4^+CD25^+FoxP3^+$ Tregs (**c**), $CD4^+CD25^-FoxP3^+$ Tregs (**d**) and overall Treg to Th17 ratio (**e**) in paired samples of PBMCs obtained from RA patients cultured in DMEM medium (control) as opposed to PBMCs from the same patients cultured in AT-MSC conditioned medium (PBMC + AT-MSC medium). Data are expressed as mean ± SD; $*p<0.05$ (Wilcoxon sign-rank test), **data are expressed as median (IQR)

Soluble factors secreted by AT-MSC increase the number of Treg and skews Treg/Th17 ratio in vitro in RA

The presence of Tregs and the effect of AT-MSCcm on them were investigated by flow cytometric analysis based on the expression of markers typical for this cell subset—CD4, CD25, and FoxP3. The mean (± SD) values of $CD4^+FoxP3^+$, $CD4^+CD25^-FoxP3^+$ and $CD4^+CD25^+FoxP3^+$ in control PBMC were 11.35 ± 4.1%, 7.13 ± 3.12%, and 4.22 ± 2%, respectively. On the other hand, the percentage of $CD4^+FoxP3^+$, $CD4^+CD25^-FoxP3^+$, and $CD4^+CD25^+FoxP3$ in PBMC cultured with AT-MSCcm were significantly increased (mean ± SD): 15.6 ± 6.1%, $p = 0.002$, 9.56 ± 5.4%, $p = 0.007$ and 6.04 ± 3.6%, $p = 0.002$, respectively. Moreover, significant positive correlation between $CD4^+CD25^-FoxP3^+$ and $CD4^+CD25^+FoxP3$ subsets was observed (Pearson's $r = 0.54$, $p = 0.024$) (Fig. 2b–d). We calculated the $CD4^+FoxP3^+$/Th17 ratio and our data pointed out a significant almost twofold elevation in Tregs to Th17 ratio under the influence of AT-MSCcm compared to control medium, 1.06 (IQR 0.81–1.67) vs 1.95 (IQR 1.23–2.78), $p = 0.002$ (Fig. 2e).

Discussion

In RA, current paradigm sets a central role of the Th17/Treg axis imbalances in the pathogenetic chain of events leading from initial loss of peripheral tolerance to full-blown disease establishment and further propagation [12]. One of the most considerable findings of our in vitro study were the decrease of Th17 cells along with the increase of $CD4^+FoxP3^+$ Tregs and reversal of overall Treg/Th17 ratio under the influence of AT-MSCcm. The results obtained demonstrated that the expansion of regulatory cells affected both $CD25^+$ and $CD25^-$ Treg subsets and also a strong positive correlation between them was to be observed. These phenotypic changes were accompanied by the increase of the immunosuppressive cytokine TGF-β1 and decrease of TNF-α and Th-17-related cytokines: IL-17 and IL-21 in parallel.

Elevated plasma levels of IL-17A, IL-21, and TNF-α are typical for RA [14]. However, the levels of IL-17A, IL-21, and TNF-α produced by RA patients' PBMC were diminished under the influence of MSC' soluble factors as opposed to the control medium. Furthermore, Th17 cells were significantly reduced when PBMC exposed to AT-MSCcm and this reduction was not a product of increased apoptosis rate amongst T helper cell compartment. This finding might be seen as a strong indicator that MSC secretory factors could act as potent blockers of major effector cytokine pathway: TNF-α, IL17, IL-21, and Th17 generation in particular in RA.

To identify the key molecules responsible for the alterations observed, AT-MSCcm was repeatedly tested for a broad panel of cytokines and chemokines (Table 2). Our data showed relatively high levels of the pleiotropic cytokine IL-6 in AT-MSCcm at a comparative rate to other studies focused on stem cell-derived soluble factors [5, 6]. Park and colleagues have found that IL-6 mRNA has the highest expression profile in human MSC [15]. Two mechanisms of IL-6 have been delineated; the inflammatory effects of IL-6 have been more frequently linked to the classical signaling pathway and trans-signaling phenomenon has been associated merely with immunosuppression. This statement is somewhat partially true and is introduced to explain in particular the controversial effects of IL-6 signaling. Recent evidence has also shown that IL-6 is capable of 'generating' $CD8^+FoxP3^+$ regulatory cells, which have been identified as potent suppressors of Th17 cells [16]. According to the literature, IL-6 could downregulate pro-inflammatory cytokines such as TNF-α, IL-1, GM-CSF and IFN-γ [17] and on the other side upregulate production of glucocorticoids, IL-10, TNF-α soluble receptor, and IL-1 receptor antagonist. Indirect immunosuppressive mechanisms of IL-6 action impede monocytes differentiation into matured dendritic cells via production of STAT3 and upregulation of their IL-10 and PGE_2 production [18]. Up to this point, the double-edged nature of IL-6 in the mosaic of MSC's immunosuppression have become clearly evident as we demonstrated that the action of high levels of IL-6, along with the induced TGF-β1 on Th-17/Treg, could be contrary to their well-established features to induce Th17.

Based on our results, PGE_2 was found elevated in MSCcm and this is in consent with already published data [5, 6, 19]. PGE_2 have key suppressive features and immunomodulatory properties. PGE_2 could directly hamper T-helper proliferation via reduction of IL-2 and also decrease TNF-α and IFN-γ levels [19]. PGE_2 increases the numbers of Treg as shown by Kim et al. [20] and Tanaka et al. [21]. Major regulatory effects of PGE_2 are realized through induction of IL-10 and PGE_2 production. IL-10 could directly induce Treg cells or via modulation of antigen-presenting cells [22]. PGE_2 alongside with TGF-β1could induce Th17 polarization into Treg [23].

Further on, we also detected high concentrations of sVEGF-A in our AT-MSCcm. Several immunosuppressive features have been attributed to sVEGF-A. It was demonstrated that sVEGF-A has an inhibitory effect on nuclear factor kappa beta (NFkB) and nuclear factor of activated T cells (NFAT) signaling pathways [24] and ligation of sVEGF-A with VEGF-R2 could induce Treg proliferation [25]. Moreover, an interaction of sVEGF-A with neuropilin-1 [26], ubiquitously expressed by Tregs, was shown to induce surface expression of inhibitory checkpoint molecules, thus enhancing Tregs' immunosuppressive potential [26]. Additionally, we found three chemokines—CCL2, CCL5, and IL-8—in elevated concentrations in supernatants from AT-MSC. Although data in the literature, regarding their influence on Th17/Treg cells, are scarce, for CCL2 was shown to be engaged in inducing tolerogenic dendritic cells and Treg [27].

A number of alterations in the compartment of the regulatory $FoxP3^+$ T lymphocytes have been associated with RA [12, 28]. For example, intrinsic defects and acquired post-translational modifications affecting $FoxP3^+$ stability and expression are thought to contribute to the increased instability and conversion of Treg into Th17 cells [29]. Komatso et al. demonstrated that IL-17-expressing Treg cells, a product of $CD4^+Foxp3^+$ conversion into Th17, exert a more aggressive effect on synovial cartilage and bone destruction [30]. Despite their antagonism and diametrical opposite functions, Th17 and Treg bifurcate on their common developmental pathway and retain a certain degree of plasticity [28, 29]. Therapeutic interventions that exploit their plasticity and target restoration of impaired balance have been considered as promising avenues for further pharmaceutical development.

We rejected increased apoptosis rate as a cause of reduction of Th17 cells. Therefore, these effects could be attributed to plasticity of Th17 and their conversion into other T helper subtypes, such as Treg cells. On the other hand, increase of Treg under the influence of AT-MSCcm may be explained by the conversion of other subpopulations including Th17 [30], Th1 [30] and/or differentiation of naïve T cells [30] or to proliferation of already available Tregs [31]. The observed changes regarding reversal in Th17/Treg balance could be linked to the increased TGF-β1levels. Since Treg has been identified as a major source of TGF-β1 in our experimental settings, a possible explanation for the increase in its levels is that AT-MSCcm enhances the immunosuppressive potential of Tregs already available in PBMC from patients with rheumatoid arthritis and this subsequently could be linked to their increased secretion of TGF-β1 [31]. The released TGF-β1 could further inhibit the activation, proliferation and cytokine secretion of other T helper lymphocyte subsets. Moreover, in combination with other soluble factors: PGE2, IL-6, sVEGF-A, CCL2 present in AT-MSCcm, TGF-β1 may direct them to differentiate into Treg.

Tregs are classically characterized by $CD4^+CD25^+FoxP3^+$ phenotype, but accumulating data show that $CD4^+CD25^-FoxP3^+$ termed as 'mysterious' could also exert potent immunosuppressive effects [29, 32]. In this study, we found positive correlation between the elevations in the percentage of $CD25^+$ and $CD25^-$ Tregs occurring under the influence of AT-MSC secretory factors. These findings may reflect the degree of functional interconnection between both subpopulations. Two theories have been introduced to explain the connection of $CD25^-$ Tregs with classical $CD25^+$ Tregs: according to some authors there is an intermediate cell subset, arising prior to $CD25^+$ Tregs in the process of induced Treg generation [33], or loss of CD25 could reflect a transitory fluctuation in Treg cell metabolism that is tidily connected to cellular division and expansion [34]. To our knowledge, this is the first report in the literature, describing such correlation in RA. It is also of interest because, in addition to their direct therapeutic benefits, the study of mechanisms of immunoregulation exerted by MSCs could provide a better understanding of the interrelation between different subsets in the pool of Tregs and their complex dynamics as well.

However, our study has certain limitations that need to be highlighted. First, due to the relatively small sample size, further research on larger patients' groups may be needed to increase statistical power for study observations. Second, our study lacks a drug naïve patients' group. Third, our findings, regarding Th17/Treg ratio and changes in cytokines should be interpreted and analyzed with caution since these findings were produced by an in vitro study. Despite the well-described immunoregulatory features of some secretory molecules found by us in AT-MSC supernatants, we should interpret them with caution constantly bearing in mind that these are pleiotropic cytokines capable of exerting opposite effects. Additionally, the soluble factors detected by us are only small fraction of complex mosaic of more than 30 different immunomodulatory molecules shaping MSC's "secretome" [34].

Conclusion

In conclusion, our study unambiguously demonstrates the immunosuppressive capacity of the secretory factors produced by AT-MSC to skew Th17/Treg balance in direction of Treg accumulation and also to downregulate major effector cytokine pathways in RA, thus identifying them as a potent multiple-target-hit tool. Since the current conventional and biological DMARDs have reached certain curative limitations, MSC-based treatment approaches should not be neglected in the light to develop novel pharmaceutical avenues in RA.

Author contributions Research concept and design: DK. Methodology: EI-T, MI. Data curation: GV. Investigation: GV, EK, KY, MI, RS. Data analysis and interpretation: DK, GV. Project administration: GV. Validation: DK. Writing the article: GV. Critical revision and editing of the article: DK, MI, EI-T, EK.

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Compliance with ethical standards

Conflict of interest Authors report no conflict of interest concerning this article.

Ethical approval All experiments performed in this study involving human participants were approved by the institutional and national research committee and complied with the Helsinki Declaration of 1964 and its later amendments (2008).

Informed consent Voluntarily signed informed consent was obtained from all participants in accordance with the ethical recommendations of the Helsinki Declaration before entering the study.

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