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In vitro immunomodulatory effects of microencapsulated umbilical cord Wharton jelly-derived mesenchymal stem cells in primary Sjögren's syndrome

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Abstract

Objective. Human umbilical cord Wharton jelly-derived mesenchymal stem cells (hUCMS) are easy to retrieve in bulk. They may interact with immune cells by either cell contact or soluble factors. Little evidence is currently available on potential therapeutic application of hUCMS to systemic autoimmune disorders such as primary SS (pSS). We have recently developed an endotoxin-free alginate gel that can be used to microencapsulate different cell types for graft into non-immunosuppressed hosts. We aimed to assess the *in vitro* effects of IFN-γ-pretreated microencapsulated (CpS)-hUCMS on T cells of pSS.

Methods. Ten pSS patients and 10 healthy donors were selected. Peripheral blood mononuclear cells (PBMCs) were obtained from venous blood to establish co-cultures with CpS-hUCMS. Lymphocyte proliferation and phenotypic analysis was performed by flow cytometry and real-time PCR on IFN-γ-pretreated hUCMS was performed before PBMCs co-culture.

Results. We found that CpS-hUCMS suppress pSS T cell proliferation and restore the Treg/Th17 ratio, thereby possibly positively impacting the pSS disease process.

Conclusion. We have developed a new biohybrid drug delivery system that now waits for clinical application in autoimmune diseases, including pSS.

Key words: human umbilical cord mesenchymal cells, Sjögren's syndrome, microencapsulation, Th17 cells, Treg cells.

Introduction

Primary SS (pSS) is a systemic autoimmune disorder characterized by chronic inflammation of exocrine glands leading to secretory function impairment and tissue damage [1] in which Th17 cells represent a main pathogenic effector subset involved in autoimmunity and glandular tissue injury [2]. An intriguing aspect of Th17 cell homeostasis is the relationship with Tregs, whose imbalance may lead to the development of autoimmune diseases [3, 4].

The course of the disease is rather mild in many patients, but a subgroup of subjects can experience severe extraglandular involvement and often currently available treatments in these cases are not efficacious [5]. Therefore the identification of therapeutic alternatives is desirable.

Human umbilical cord mesenchymal stem cells (hUCMS) are adult stem cells able to differentiate into several cell phenotypes [6]. They display homing properties, are immunoprivileged, owing to the lack of HLA-DR, and exhibit immunomodulatory properties. Their properties seem to depend on both humoral factors, including

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TGF-β1, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), IL-6, prostaglandin E2, hepatocyte growth factor and VEGF, and their interaction with target cells.

Despite the therapeutic potential of hUCMS, limitations associated with direct grafting procedures prevent effective hUCMS transplantation within the framework of safe and controlled therapeutic protocols [7]. We recently completed clinical application of human islets of Langerhans, microencapsulated in immunoprotective alginate-based microcapsules in patients with type 1 diabetes mellitus (T1D). This procedure allowed avoidance of the recipient's general immune suppression (Italian Ministry of Health no. 19382, PRE805; Clinical Trial Registration ISRCTN43557935) [8]. We employed the microencapsulation technique to arrange a co-culture system ensuring a separation between hUCMS and peripheral blood mononuclear cells (PBMCs) for further analysis upon IFN-γ incubation [9]. The aim of this study was to demonstrate whether microencapsulated (CpS)-hUCMS represent a possible and viable therapeutic alternative to conventional immunosuppressive agents in pSS.

Material and methods

Study population

Ten female patients with pSS classified according to the Euro-American criteria [10] and 10 sex- and age-matched healthy subjects [healthy donors (HDs)] were enrolled. None of the patients was receiving corticosteroids or immunosuppressants. Disease activity was assessed using the European League Against Rheumatism (EULAR) SS disease activity index [11]. The study was approved by the local ethics committee (CEAS Umbria) and written informed consent was obtained from participants in accordance with the Declaration of Helsinki.

hUCMS procurement, isolation and culture

Isolation of hUCMS was performed from post-partum human umbilical cords as described by Montanucci *et al.* [6].

hUCMS microencapsulation and stimulation with IFN

A 1.8% endotoxin-free sodium alginate solution, employed for cell encapsulation, was prepared according to our protocol (patent WO2009093184A1) [12]. Briefly, cell pellets were mixed in 1.8% alginate solution so as to make a final homogeneous suspension. The alginate:hUCMS ratio was adjusted so as to match 1 ml of alginate to 3×10^6 cells in order to avoid formation of empty capsules. CpS-hUCMS were triggered overnight with 300 U/ml IFN-γ (Sigma-Aldrich, St Louis, MO, USA) that was removed before setting up the co-culture with PBMCs.

Cell cultures

PBMCs were isolated by gradient separation, stained with carboxyfluorescein succinimidyl ester (CFSE), activated with anti-CD3/CD28-coated beads (Dynabeads, Invitrogen, Carlsbad, CA, USA) and plated with hUCMS at different ratios. Unstimulated and CD3-CD28-triggered PBMCs acted as negative and positive controls, respectively. CFSE dilution was analysed on day 5 using a FACSCalibur flow cytometer and CellQuestPro software (BD Biosciences, San Jose, CA, USA).

Phenotypic analysis

Th1, Th17 and Treg cells were identified by flow cytometry as described by Alunno *et al.* [4, 13].

Cytokine assessment

In the culture supernatant, concentrations of IL-17, IL-2, IL-10, TNF-α, IL-6 and TGF-β were assessed by the cytometric bead array technique (BD Bioscience) and analysed by flow cytometry.

Transcriptional expression analysis by RT-PCR and quantitative PCR

Total cellular RNA was extracted from the cultured cells using the TRI-reagent method (Bio-Rad Laboratories, Segrate, Italy). cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) and was used as a template in quantitative PCR (qPCR) (see supplementary Table S1, available at *Rheumatology* Online). qPCR amplifications were performed using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories) and MxPro 3000 system (Stratagene, La Jolla, CA, USA).

Western blotting

Total protein was extracted and assayed as described [6]. Protein samples (40 μg) were analysed on 10% or 12% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad Laboratories). Immunodetection was performed with the Immun-Star HRP Chemiluminescent kit (Bio-Rad Laboratories) (see supplementary Table S2, available at *Rheumatology* Online).

Statistical analysis

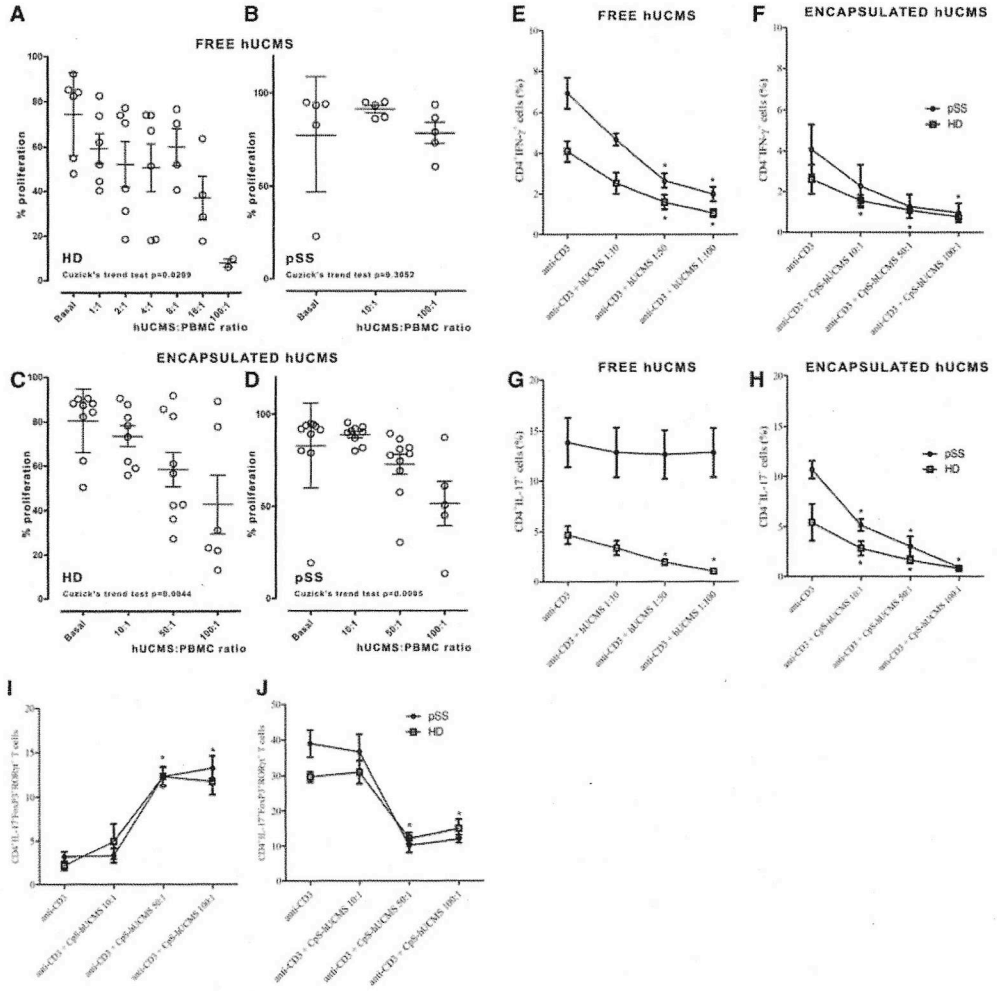
Cuzick's trend test, Kruskall–Wallis test with Dunn's test for multiple comparisons *post hoc* and Mann–Whitney *U* test were employed. The significance level was two-sided and set at $P < 0.05$. All data analysis was performed using SPSS version 20.0 (IBM, Armonk, NY, USA).

Results

In previously published studies, free hUCMS were employed at a 1:1 ratio or in excess with respect to PBMCs, revealing good suppressive activity. We confirmed that free hUCMS inhibit HD T cell proliferation at a 1:1 ratio, but most interestingly we demonstrated that such inhibition was even more evident by progressively reducing hUCMS in culture (Fig. 1A). Similarly, CpS-hUCMS to reduced HD T cell proliferation at a 1:1 ratio and such inhibition was progressively more pronounced by decreasing hUCMS number (Fig. 1B).

In pSS, free hUCMS did not display inhibitory effects (Fig. 1C), while hUCMS were able to reduce T cell proliferation (Fig. 1D). In particular, the inhibitory effect of

FIG. 1 CFSE dilution and phenotypic analysis in co-cultures of HD or pSS PBMCs and hUCMS



(**A**) Free hUCMS are able to inhibit HD but not pSS T cell proliferation. (**B**) Microencapsulated hUCMS are able to inhibit HD and to a greater extent pSS T cell proliferation. Percentages of proliferation refer to $CD3^+$ gated cells. Data were expressed as mean (S.D.) in at last three independent experiments. (**E** and **F**) HD and pSS Th1 cells are inhibited by both free and microencapsulated hUCMS. (**G**) HD but not pSS Th17 cells are inhibited by free hUCMS. (**H**) Microencapsulated hUCMS inhibit both HD and pSS Th17 cells. In each panel the mean (S.E.M.) of three different experiments are plotted. Asterisks indicate the comparison with stimulated cells in the absence of hUCMS in the same arm ($P < 0.05$). The expression of FoxP3 was assessed in (**I**) $CD4^+IL\text{-}17^-ROR\gamma t^+$ and (**J**) $CD4^+IL\text{-}17^+ROR\gamma t^+$ cells. In each panel the mean (S.E.M.) of three different experiments are plotted. Asterisks indicate the comparison with stimulated cells in the absence of hUCMS in the same arm ($P < 0.05$). CFSE: carboxyfluorescein succinimidyl ester; HD: healthy donor; pSS: primary SS; PBMCs: peripheral blood mononuclear cells; hUCMS: human umbilical cord Wharton jelly-derived mesenchymal stem cells.

CpS-hUCMS was more pronounced in pSS than in HD T cells. Supplementary Fig. S1 (available at *Rheumatology* Online) displays an evaluation of the morphology and viability of hUCMS co-cultured with PBMCs. In agreement with functional assays, a reduction in both Th1 and Th17 cell subsets was observed in the presence of CpS-hUCMS in HDs. Conversely, free hUCMS, which failed to inhibit pSS T cell proliferation, were able to reduce Th1 but not Th17 cells (Fig. 1E and G). Finally, CpS-hUCMS were able to decrease both Th1 and Th17 cells in pSS (Fig. 1F and H). As free hUCMS did not show suppressive activity on pSS Th17 cells, in subsequent experiments we only used CpS-hUCMS.

Since mesenchymal stem cells can up-regulate Treg transcription factor FoxP3 and Th17/Treg plasticity is well established [14], we speculated that Th17 cell

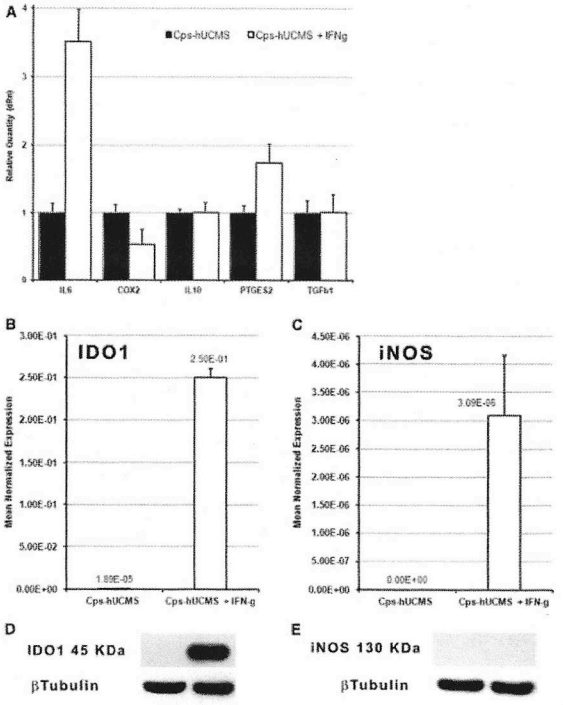
reduction induced by CpS-hUCMS may be due to their conversion into Tregs. We found that CpS-hUCMS increased the number of FoxP3$^+$ cells among CD4$^+$IL-17$^-$RORγt$^-$ cells in HDs and pSS patients (Fig. 1I). We also observed an increase in FoxP3$^+$ cells among CD4$^+$IL-17$^-$RORγt$^+$ cells but a decrease in FoxP3$^+$ cells among CD4$^+$IL-17$^+$RORγt$^+$ cells (Fig. 1J).

The evidence that several IFN-γ-inducible factors were unchanged in culture supernatants of pSS co-cultures (see supplementary Fig. S2 and supplementary material section S1, available at *Rheumatology* Online; evaluation of cytokines in culture supernatants) prompted us to investigate other IFN-γ-inducible mediators in CpS-hUCMS. TGF-β, IL-10 and cyclooxygenase 2 (COX-2) expression was not increased by IFN-γ, while prostaglandin E synthase 2 (PTGES2) expression was up-regulated (Fig. 2A). We also observed a significant IL-6 up-regulation in IFN-γ-stimulated hUCMS, which may explain the massive presence of this cytokine in the cell culture supernatants. HLA-G5 mRNA was undetectable in resting and IFN-γ-stimulated CpS-hUCMS. Intriguingly, IDO1 mRNA was massively up-regulated following IFN-γ stimulation (Fig. 2B and D). Finally, IFN-γ stimulation also induced an increase in inducible nitric oxide synthase (iNOS) mRNA, but not protein (Fig. 2C and E), as previously demonstrated [15].

Discussion

We demonstrated that IFN-γ-stimulated CpS-hUCMS exert positive immunological effects in pSS by reducing pathogenic T cell subsets and potentiating regulatory counterparts. Although the immunomodulatory effects of free hUCMS are well known [16], their administration for therapeutic purposes has shown several limits, mainly related to safety issues. In this study we used microbioreactors as a drug delivery system, which is already employed in patients with T1D [8]. Our technology provides a means for hUCMS transplantation without host immunosuppression, which would otherwise be necessary for free hUCMS grafting procedures. Additionally, the present data support the operative use of a very low CpS-hUCMS therapeutic mass. Finally, CpS-hUCMS represent a dynamic and immune-isolatory system that does not impair crosstalk with the surrounding environment. Unlike RA, free cell contact with pSS lymphocytes may weaken the suppressive activity of hUCMS, and this provides a rationale for microencapsulating hUCMS when faced with pSS-derived lymphocytes. Even though ligand-receptor interactions occurring between T cells and hUCMS may be the same in HDs and RA and pSS patients, differences may reside in the intracellular signalling apparatus, with negative feedback following hUCMS contact with pSS T cells. Alternatively, activated lymphocytes from patients express a set of peculiar surface molecules that may account for the different ligand-receptor interactions. Further experiments aimed at identifying mechanisms involved in this deranged crosstalk between free hUCMS and pSS T cells are required. In particular, we observed massive up-regulation of IDO1 at the mRNA and protein levels in IFN-γ-stimulated hUCMS. IDO1, a master regulator of tolerance [17], is normally expressed at low basal levels. One of the most powerful triggers of IDO1 expression is IFN-γ, but this by itself could not account for the stable long-term tolerance occurring, for instance, during pregnancy. In fact, an IFN-γ-dominated environment leads to rapid and intense tryptophan degradation, but it has been subsequently demonstrated that TGF-β is required to switch IDO activity into a self-maintaining form of intracellular signalling activity, eventually leading to a stable regulatory environment. The

FIG. 2 Main protein and mRNA expressed by microencapsulated hUCMS following IFN-γ treatment



(**A**) Quantitative PCR for the indicated genes in hUCMS upon IFN-γ stimulation. Expression of each marker, relative to its own untreated control, equals 1. HPRT1 was used for normalization. (**B** and **C**) Mean normalized expression of IDO1 and iNOS gene mRNA in comparison with HPRT1 gene mRNA. (**D** and **E**) Western blotting evaluation of IDO and iNOS proteins. The $2^{-\Delta\Delta Ct}$ method was used to quantify human IL-6, IL-10, COX-2, PTGES2, TGF-β, HLA-G5, and HPRT1 gene mRNA. QGene 4.3 was used to calculate the mean normalized expression of IDO1 and iNOS gene mRNA in comparison with HPRT1 gene mRNA. All results were expressed as the mean (S.D.) of three independent experiments. hUCMS: human umbilical cord Wharton jelly-derived mesenchymal stem cells; HPRT1: hypoxanthine phosphoribosyltransferase 1; IDO: indoleamine 2,3-dioxygenase 1; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase 2; PTGES2: prostaglandin E synthase 2.

IFN–IDO axis is crucial to minimizing immune-mediated tissue injury secondary to infections and chronic inflammation by depleting the essential amino acid tryptophan. Indeed, products of tryptophan metabolism such as kynurenines display immunoregulatory activity and, besides inhibiting T cell proliferation, mediate differentiation into Treg cells by interacting with aryl hydrocarbon receptor expressed on the naive T cell surface. As reported, it is conceivable that IFN-γ treatment leads to massive up-regulation of iNOS at the mRNA but not the protein level, as occurs in murine hUCMS and other cell types [15]. This provides a clue about different pathways used by hUCMS to exert their immunomodulatory properties as compared with their murine counterpart. Indeed, in the latter, NO appears to play a crucial role in mediating immunoregulation.

The effects of hUCMS on the Th17 arm of the immune system deserve some consideration. Increasing evidence supports plasticity of Treg/Th17 cells, and we showed that CpS-hUCMS reciprocally modulate FoxP3 and RORγt expression, eventually leading to the conversion of Th17 into Treg cells. Indeed, $CD4^+IL\text{-}17^+FoxP3^+ROR\gamma t^-$ T cells may represent an intermediate phenotype of Th17 cells turning into Treg cells.

A recent work reported that IDO blockade induced conversion of Tregs into Th17-like cells in rodent tumour-draining lymph nodes, while IL-6 seems to be crucial in such a scenario [14]. We know that IFN-γ-pretreated hUCMS up-regulate IL-6 expression, hence we cannot assess whether the cytokine we detect in culture supernatant comes from hUCMS or dendritic cells [18]. However, in our system, where IDO is strongly expressed by CpS-hUCMS, conversion of Th17 into Treg cells may occur, in spite of the presence of hUCMS-derived IL-6. The longstanding paradigm that IL-6 has only pro-inflammatory functions was recently challenged by the demonstration that it may also display different properties in organ-specific autoimmune diseases such as T1D [19]. Therefore we speculate that it is not a matter of the cytokine itself, but rather of the cellular source, in this case hUCMS. Also, IL-6 was not associated with the direct immunosuppressive effect of hUCMS on $CD4^+$ cells [18]. Although we observed reduced percentages of Th17 cells in both pSS patients and HDs, a parallel decrease in IL-17 concentration was observed only in HD co-cultures. These paradoxical findings could be explained by the fact that other IL-17-producing cells, insensitive to hUCMS, may be present within PBMCs. Double negative (DN) T cells are increased in autoimmune disorders such as SLE and pSS [20] and they infiltrate target organs, e.g. kidneys and minor salivary glands. These cells, isolated from pSS patients, are resistant to dexamethasone *in vitro*, while HD DN cells promptly reduced IL-17 expression in the presence of dexamethasone [13]. Here we have shown that DN Th17 cells are insensitive to the suppressive activity of CpS-hUCMS and maintain their resistance to dexamethasone. DN T cells are still being debated in immunology since conclusive data about their origin, function and fate are still missing. Therefore the overall beneficial effects of CpS-hUCMS against a lack of efficacy towards DN T cells in pSS requires further investigation *in vivo* in animal models of pSS.

In conclusion, we provided preliminary evidence that CpS-hUCMS represent a functional biohybrid artificial system where cellular molecular products are able to exert powerful immunomodulatory effects *in vitro* on T cells in pSS. Although our study focused on the evaluation of T cells as major players in pSS immunopathogenesis, additional *in vitro* studies assessing the effects of hUCMS on B cell subsets, and possibly on glandular epithelial cells, may be of interest. Although differences between *in vitro* settings and *in vivo* application are expected, we believe that *in vivo* therapeutic modalities using CpS-hUCMS may be built in preclinical animal models of autoimmune diseases. This may aid our understanding of whether the system devised in our laboratory represents a viable therapeutic alternative for pSS and other similar disorders.

Rheumatology key messages

- CpS-hUCMS but not free hUCMS have the capacity to reduce primary SS peripheral blood mononuclear cell proliferation.
- CpS-hUCMS represent a functional biohybrid artificial system able to exert powerful *in vitro* immunomodulatory effects in primary SS.
- CpS-hUCMS modulate FoxP3 and RORγt expression leading to the conversion of Th17 into Treg cells in primary SS.

Acknowledgements

A.A. and P.M. designed and performed the experiments, wrote the manuscript, arranged the images and analysed the data. G.B. and O.B. designed the experiments and wrote the manuscript. S.C. performed the experiments and sampling. T.P. performed the hUCMS culture. I.P. performed the viability assay. V.B. the performed statistical analysis. E.B. recruited pSS patients and HDs. R.G. and R.C. critically reviewed the results and wrote the manuscript.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

References

1 Nocturne G, Mariette X. Advances in understanding the pathogenesis of primary Sjögren's syndrome. Nat Rev Rheumatol 2013;9:544–56.

2 Katsifis GE, Rekka S, Moutsopoulos NM *et al*. Systemic and local interleukin-17 and linked cytokines associated with Sjögren's syndrome immunopathogenesis. Am J Pathol 2009;175:1167–77.

3 Alunno A, Bartoloni E, Bistoni O *et al*. Balance between regulatory T and Th17 cells in systemic lupus erythematosus: the old and the new. Clin Dev Immunol 2012;2012: 823085.

4 Alunno A, Petrillo MG, Nocentini G *et al*. Characterization of a new regulatory CD4+ T cell subset in primary Sjogren's syndrome. Rheumatology 2013;52:1387–96.

5 Ramos-Casals M, Brito-Zerón P, Sisó-Almirall A, Bosch X, Tzioufas AG. Topical and systemic medications for the treatment of primary Sjögren's syndrome. Nat Rev Rheumatol 2012;8:399–411.

6 Montanucci P, Basta G, Pescara T *et al*. New simple and rapid method for purification of mesenchymal stem cells from the human umbilical cord Wharton jelly. Tissue Eng Part A 2011;17:2651–61.

7 Li L, Davidovich AE, Schloss JM *et al*. Neuronal lineage differentiation of embryonic stem cells within alginate microbeads. Biomaterials 2011;32:4489–97.

8 Calafiore R, Basta G, Luca G *et al*. Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: first two cases. Diabetes Care 2006;29:137–8.

9 Ben-Ami E, Berrih-Aknin S, Miller A. Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases. Autoimmun Rev 2011;10:410–5.

10 Vitali C, Bombardieri S, Jonsson R *et al*. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554–8.

11 Seror R, Ravaud P, Bowman SJ *et al*. EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. Ann Rheum Dis 2010;69:1103–9.

12 Montanucci P, Pennoni I, Pescara T *et al*. The functional performance of microencapsulated human pancreatic islet-derived precursor cells. Biomaterials 2011;32: 9254–62.

13 Alunno A, Bistoni O, Bartoloni E *et al*. IL-17-producing $CD4^{-}CD8^{-}$ T cells are expanded in the peripheral blood, infiltrate salivary glands and are resistant to corticosteroids in patients with primary Sjogren's syndrome. Ann Rheum Dis 2013;72:286–92.

14 Sharma MD, Hou DY, Liu Y *et al*. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. Blood 2009;113:6102–11.

15 Luss H, Li RK, Shapiro RA *et al*. Dedifferentiated human ventricular cardiac myocytes express inducible nitric oxide synthase mRNA but not protein in response to IL-1, TNF, IFNgamma, and LPS. J Mol Cell Cardiol 1997; 29:1153–65.

16 Liu Y, Mu R, Wang S *et al*. Therapeutic potential of human umbilical cord mesenchymal stem cells in the treatment of rheumatoid arthritis. Arthritis Res Ther 2010; 12:R210.

17 Fallarino F, Grohmann U, Puccetti P. Indoleamine 2,3-dioxygenase: from catalyst to signaling function. Eur J Immunol 2012;42:1932–7.

18 Wang D, Ji YR, Chen K *et al*. IL-6 production stimulated by CD14(+) monocytes-paracrined IL-1β does not contribute to the immunosuppressive activity of human umbilical cord mesenchymal stem cells. Cell Physiol Biochem 2012;29:551–60.

19 Kristiansen OP. Mandrup-Poulsen T Interleukin-6 and diabetes: the good, the bad, or the indifferent? Diabetes 2005;54(Suppl 2):S114–24.

20 Crispín JC, Oukka M, Bayliss G *et al*. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. J. Immunol 2008;181:8761–6.