Minimal modulation of the host immune response to SIS matrix implants by mesenchymal stem cells from the amniotic fluid.

Flore Lesage¹, Savitree Pranpanus², Francesca Maria Bosisio^{3,4}, Maureen Jacobs¹, Simona Ospitalieri¹, Jaan Toelen⁵, Jan Deprest⁶

¹ KU Leuven – University of Leuven, Department of Reproduction and Regeneration, Leuven, Belgium

² Prince of Songkla University, Department of Obstetrics and Gynecology, Songkhla, Thailand

³ KU Leuven – University of Leuven, Department of Imaging and Pathology, Leuven, Belgium

⁴ Università Degli Studi di Milano-Bicocca, Milan, Italy

⁵ University Hospitals Leuven, Department of Pediatrics, Leuven, Belgium

⁶ University Hospitals Leuven, Department of Obstetrics and Gynecology, Leuven, Belgium

CORRESPONDING AUTHOR

Jan A. Deprest, MD, PhD, FRCOG

Department of Obstetrics and Gynecology, University Hospitals Leuven

Herestraat 49, 3000 Leuven, Belgium

Email: jan.deprest@uzleuven.be

Phone: +32 16 34 42 15, Fax: +32 16 34 42 05

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ABSTRACT

PURPOSE

Surgical restoration of soft tissue defects often requires implantable devices. The clinical outcome of the surgery is determined by the properties inherent to the used matrix. Mesenchymal stem cells (MSC) modulate the immune processes after *in vivo* transplantation and their addition to matrices is associated with constructive remodeling. Herein we evaluate the potential of MSC derived from the amniotic fluid, an interesting MSC source for cell therapeutic applications in the perinatal period, for immune modulation when added to a biomaterial.

METHODS

We implant cell free small intestinal submucosa (SIS) or SIS seeded with AF-MSC at a density of $1 \cdot 10^5/\text{cm}^2$ subcutaneously at the abdominal wall in immune competent rats. The host immune response is evaluated at 3, 7 and 14 days postoperatively.

RESULTS

The matrix-specific or cellular characteristics are not altered after 24 hours of *in vitro* co-culture of SIS with AF-MSC. The host immune response was not different between animals implanted with cell free or AF-MSC-seeded SIS in terms of cellular infiltration, vascularity, macrophage polarization or scaffold replacement. Profiling the mRNA expression level of inflammatory cytokines at the matrix-interface shows a significant reduction in the expression of the pro-inflammatory marker *Tnf- α* and a trend towards lower *iNos* expression upon AF-MSC-seeding of the SIS matrix. Anti-inflammatory marker expression does not alter upon cell seeding of matrix implants.

CONCLUSION

We conclude that SIS is a suitable substrate for *in vitro* culture of AF-MSC and fibroblasts. AF-MSC addition to SIS does not significantly modulate the host immune response after subcutaneous implantation in rats.

KEYWORDS:

Amniotic fluid-derived mesenchymal stem cells, small intestinal submucosa, immune modulation

1. INTRODUCTION

The remodeling response and ultimately the clinical outcome of surgical reconstruction of soft tissue defects is defined by the properties inherent to the implanted matrix material. This knowledge has fueled the search to alternative implant materials that do not only mimic the structure, but also the biological function of the native tissue in order to move towards constructive implant remodeling rather than fibrotic encapsulation.

The specific morphogenetic characteristics of the extracellular matrix (ECM) make it an attractive material for tissue engineering approaches. The ECM plays an important role, both during tissue homeostasis and in response to disease or injury, in maintaining the tissue's structure and function by providing environmental cues that influence cellular attachment, migration, proliferation, fate specification and three-dimensional organization. In addition, it is composed of a mixture of structural and functional proteins secreted by the various cells constituting a specific tissue of interest. ECM bioscaffolds, derived from various decellularized tissues, have been used successfully for tissue reconstruction in multiple clinical applications. For instance, Surgisis is routinely used in clinics for the reconstruction of the lower urinary tract [1], musculofascial structures [2], the cardiovascular system [3] and the skin [4]. Surgisis is a translucent layer of collagen and other ECM components obtained from porcine small intestinal submucosa (SIS) by mechanical decellularization. The success of this biomaterial is attributed to its three-dimensional organization, its inherent cytokines and growth factors which enable regeneration through the modulation of angiogenesis, fibrosis and inflammation, and its slow biodegradability allowing host tissue ingrowth [5].

Combining stem cells, and more specifically mesenchymal stem cells (MSC), with extracellular matrix-based scaffolds has gained much interest in recent years as it is widely recognized that MSC mediate the host immune response. Therefore, MSC-seeded biomaterials are believed to result in improved implant integration and long-term performance. MSC have been shown to home to the site of injury and to modulate the inflammatory response of the host via paracrine signaling. They will create an anti-inflammatory environment and stimulate the resident cells to synthesize a new ECM, as well as to proliferate, differentiate and repopulate the injured tissue. MSC can be isolated from various adult tissues (bone marrow, adipose tissue) but also protocols for the isolation of MSC from tissues of fetal origin including the umbilical cord [6], placental tissue [7], amniotic membranes [8] and amniotic fluid [9, 10] have been described. Devices engineered with MSC derived from fetal sources are of particular interest for neonatal reconstruction of congenital defects such as diaphragmatic hernia or abdominal wall defects, and hold promise to overcome the current limitations in this field. Firstly, a biomaterial for pediatric surgery should accommodate the development of the child, meaning it has to provide enough strength during the patient's growth, without causing deformities, and it has to be able to

remodel itself within a continuously changing microenvironment. Secondly, the device should have very low immunogenicity as the immune system in children is not completely developed, which makes them more susceptible to immunological rejection of the implant [11]. In addition, fetal MSC could be employed as a generic MSC type for multiple applications within the field of regenerative medicine thanks to their low immunogenicity and the possibility to obtain them via non-invasive methods from medical waste at birth, as opposed to the more invasive harvesting methods necessary to obtain adult MSC.

The present study examined the potential of human amniotic fluid-derived mesenchymal stem cells (AF-MSC) cultured on a biodegradable matrix to modulate the host immune response upon implantation. Amniotic fluid is an easily accessible prenatal source of MSC, which enables the engineering of an implantable device containing patient-specific MSC by the time of birth or the early postnatal period. SIS was used as a well-studied biological scaffold for regenerative applications, however having currently variable success in its native form for pediatric soft tissue reconstruction [12, 13].

2. METHODS

2.1 Cell culture

The cell harvesting protocol was approved by the Ethics Committee of the University Hospital of Leuven (license ML4149), Belgium. The AF used herein was obtained from a diagnostic amniocentesis at 17 weeks of gestation from which MSC were isolated as previously reported [9]. Cells were grown in AF-MSC medium consisting of α -MEM supplemented with 15% fetal bovine serum (Gibco, ThermoFisher, Ghent, Belgium), 1% L-glutamine (Gibco), 1% penicillin/streptomycin (Gibco) and 18% Chang B and 2% Chang C (Irvine Scientific, Brussels, Belgium). Fibroblasts derived from human foreskin biopsies were kindly donated by Prof. Lambertus van den Heuvel. They were grown in fibroblast medium consisting of DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin.

2.2 Scaffold preparation

Triple layer small intestinal submucosa implants were kindly donated by Cook Biotech (West Lafayette, IN, USA). All were from the same production lot and delivered sterile and pre-cut measuring 1 x 1 cm or 2,5 x 2,5 cm. Prior to cell seeding, implants were prehydrated in growth medium while being fixed to the bottom of a twelve- or six-well plate with a metal ring with an internal diameter of 1 or 2 cm, for *in vitro* or *in vivo* studies, respectively (Figure 2A). Cells were seeded at a density of $1 \cdot 10^5$ cells/cm² and incubated at 37°C in 5% CO₂. For *in vitro* studies, samples were incubated in triplicate for 24h, 48h, 7d and 14 days after initial cell seeding. Cell free SIS scaffolds incubated in AF-MSC or fibroblast medium were included as control. Medium was replaced every 2-3 days and each experiment was repeated 3 times. For *in vivo* studies, samples were incubated for 24h at 37° in 5% CO₂ after cell seeding prior to implantation. Again, cell free SIS scaffolds incubated in both media were included as control.

2.3 Animals

Male Wistar rats of 3 months old, weighing between 370 and 500 g, were used in this study. Animals were housed in the animal facility of the Faculty of Medicine of KU Leuven with free access to water and food throughout the experiments. Animals were treated in accordance with current national guidelines on animal welfare. This part of the study was approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine of the KU Leuven (license 239/2014). There were five study groups as we aimed to control for multiple variables. Firstly, in order to be able to discriminate between effects evoked by the co-cultured AF-MSC and effects evoked by the addition of a cellular component to the matrix, we included a group of rats implanted with fibroblast-seeded matrices.

Secondly, to discriminate between the effects attributable to the different growth media necessary for AF-MSC and fibroblast culturing, Chang medium and complete DMEM respectively, we included two groups of rats to be implanted with unseeded matrices incubated *in vitro* either in Chang medium or in complete DMEM. We also included sham operated animals. Rats were randomly divided into different groups, with a total of 6 animals per implant group and time point[14]. There were in total five groups: (1) animals undergoing sham surgery (SHAM); (2) animals implanted with either AF-MSC-seeded SIS (AF-MSC), (3) with SIS soaked in AF-MSC medium (AF-MSC CTRL), (4) with fibroblast-seeded SIS (FB), or (5) with SIS soaked in fibroblast medium (FB CTRL). Harvesting was done at 3, 7 and 14 days to cover the early host response. Figure 2A displays the time line of the experiment.

2.4 Scaffold implantation

Prior to implantation, anaesthesia was induced by inhalation of 5% isoflurane (Iso-Vet, Eurovet, Heusden-Zolder, Belgium) with O₂ (1.5 L/min). Lidocaine 0.5% (Xylocaine, AstraZeneca, Brussels, Belgium) was injected subcutaneously at the site of incision and buprenorphine (Vetergesic® 10%; Ecuphar, Breda, the Netherlands) was given intraperitoneally. During surgery, anaesthesia was maintained using a nose-cone delivering 2% isoflurane with O₂ (1.5 L/min). A vertical skin incision of 3-4 cm was made ventrally, one cm next to the midline. A subcutaneous pocket was created permitting implantation of the scaffold with the cultured cells (if applicable) facing the abdominal wall. The scaffold was fixed by eight polypropylene 4/0 sutures (Prolene, Ethicon®, Zaventem, Belgium). Closure of subcutaneous layers was performed using polyglycolic acid 4/0 (Vicryl, Ethicon®) and the skin was closed with polyglecaprone 3/0 (Monocryl, Ethicon®). To avoid mutilation, rats received a plastic collar until 24h post operation.

2.5 Harvest and sample fixation

Anesthetized rats were euthanized by intracardiac injection of a solution consisting of embutramide 200 mg, mebezonium 50 mg and tetracaine hydrochloride 5 mg (T61®, Hoechst GmbH, Munich, Germany). First the abdominal skin over the initial implant area, which could be determined by the fixation sutures, was removed. The original implant area, and underlying abdominal wall structures, were harvested en bloc, and divided for either fixation in PFA 4% for 24 hours or OCT, or snap freezing.

2.6 Histomorphologic analysis

Paraffin embedded specimens were cut into 5 µm thick slices and mounted on Superfrost® plus glass slides. The specimens were deparaffinized with toluol followed by exposure to a graded series of ethanol dilutions (100-70%). Sections were stained either with hematoxylin and eosin or with Masson's

trichrome dyes and dehydrated to xylene prior to cover slipping. Histologic sections were evaluated by a pathologist using previously validated quantitative criteria [15, 16].

2.7 Immunostaining

Following deparaffinization, the slides were placed in 3% H₂O₂ in methanol to block endogenous peroxidase activity. Slides were washed 3 times for 5' with Tris-Buffered Saline (TBS; 150 mM NaCl, 50 mM Tris, pH 7,6) and antigen retrieval was performed (for details see Table 1). The slides were then allowed to cool down and were washed prior to incubation with blocking solution (TBS with 1% BSA, 2% nfdm and 0,1% Tween 80) for 45' to inhibit non-specific binding of the primary antibody. Primary antibodies were dissolved in TBS and used as indicated in Table 1. Secondary antibodies (Dako, Leuven, Belgium) were either PO-labeled (vimentin, cluster of differentiation 80, CD80) or biotinylated and combined with a PO-labeled streptavidin (CD68, CD163). They were dissolved in TBS with 4% normal human serum (vimentin) or normal rat serum (CD68, CD80, CD163). After washing, the reaction was visualized with 3,3'-diaminobenzidine and Mayer's hematoxylin was used as counterstaining. Digital photographs from five non-overlapping fields per slide, randomly selected at the interface between scaffold and underlying tissue were taken at magnification 40x using an Axioskop microscope (AxioCam MRc5, Zeiss, Oberkochen, Germany). The average percentage of positive stained area relative to the total stained area was measured using ImageJ software.

2.8 PCR and qRT-PCR

RNA was isolated from snap frozen specimens using Tri-Pure (Sigma-Aldrich, Brussels, Belgium) in accordance with the manufacturer's instructions. Quality of the isolated RNA was checked using a spectrophotometer ND-1000 (Isogen Life Science, Utrecht, The Netherlands) and on a 1% agarose gel. cDNA was synthesized from 1 µg of RNA using Taq Man® Reverse Transcription Reagents (Applied Biosystem, Carlsbad, CA, USA). PCR was performed on a MasterCycler (Eppendorf, Rotselaar, Belgium) using GoTaq DNA Polymerase kit (Promega) to detect expression of *MKI67*, *CASP3* and *GAPDH*. qRT-PCR was performed on a StepOnePlus Real-Time PCR instrument (Thermo Fisher Scientific) using the Platinum® SYBR® Green qPCR Supermix-UDG with ROX (Thermo Fisher Scientific) to detect expression of inducible nitric oxide synthase (*iNos*), transforming growth factor-β (*Tgf-β*), tumor necrosis factor-α (*Tnf-α*) and cluster of *CD204*. The ribosomal protein L13A (*Rpl13a*), cyclophilin A (*Cypa*) and phosphoglycerate kinase 1 (*Pgk1*) were used as housekeeping genes to normalize mRNA levels. Relative quantitation was determined using the comparative Ct method. Primers were synthesized by Integrated DNA Technologies (Haasrode, Belgium). Primer sequences can be found in Table 2.

144 2.9 Statistical analysis

145 D'Agostino and Pearson omnibus normality test was used to verify normal distribution of the data.
146 One-way ANOVA followed by Bonferroni's multiple comparison test was used to determine significant
147 differences when normality was assumed. For data not normally distributed, Kruskal-Wallis non-
148 parametric testing was used, followed by the Dunn's multiple comparison test. A p-value less than 0.05
149 was considered as significant.

3. RESULTS

3.1 Short-term cell-matrix co-culture does not influence cellular or matrix-specific characteristics

Prior to *in vivo* implantation we assessed if cellular or matrix-specific characteristics were influenced by *in vitro* co-culture. Both AF-MSC and fibroblasts were able to adhere to the SIS matrix (Figure 1A). AF-MSC formed a dense monolayer of cells on top of the scaffold and retained their proliferative capacities without induction of apoptosis up to 14 days in co-culture (Suppl Figure 1A). Fibroblasts showed higher proliferative potential on SIS (Suppl Figure 1A) and started to form multilayers by 7 days of co-culture, which was also reflected in the amount of cells present on the scaffold (Figure 1A). At 7 and 14 days of co-culture fibroblast-seeded scaffolds contained significantly more cells than AF-MSC-seeded scaffolds.

Seeded cells retained their mesenchymal characteristics (Figure 1C), as well as their ability to produce ECM (Figure 1B). An extended AF-MSC/SIS co-culture for up to 14 days resulted in a significantly denser ECM on the collagen fibers of the matrix (vs 24h and vs 48h). A significantly denser ECM in extended fibroblast-SIS co-cultures was already observed at 7 days (vs 24h and vs 48h), as well as at 14 days (vs 24h and vs 48h).

As biomaterials often induce calcification [17, 18], we assessed if co-culture with mesenchymal stem cells -which have by definition the potential to differentiate into osteogenic lineages- resulted in calcium deposits, yet none were detected up to 14 days of AF-MSC/SIS co-culture (Suppl Fig 1B). Co-culturing cells on matrices might affect the mechanical properties of the matrix. We measured cell-mediated change in scaffold surface in relation to the original scaffold size (Figure 1D, Suppl Table 1). SIS matrices seeded with AF-MSC underwent a slight yet significant surface reduction by 14 days of co-culture as compared to the early time points 24h and 48h. The surface reduction observed for matrices seeded with fibroblasts was not statistically significant ($p = 0,1037$). Minimal dimensional changes were observed for unseeded matrices incubated in culture medium (data not shown).

3.2 Local cellular infiltration, vascularity and replacement of the SIS matrix is not modulated by AF-MSC

No complications occurred during the surgical procedures or post-operative period (24h). Two animals were excluded from the study (1 at 3 days, 1 at 14 days) because of auto mutilation within 24 hours postoperatively. Abdominal implantation of both cellular and acellular SIS matrices resulted in a local large fluid accumulation at 7 days in 12 of the 24 animals. That fluid collection was less frequent (4/24) at the 14 days time point. Transient seroma formation has been observed with this product before [14, 19]. Sham operated animals did not develop a fluid accumulation. Also the cellular infiltrate in those wound areas was very mild. When characterizing the infiltrate around cell free SIS-matrix, polymorphonuclear (PMN) cells were more abundant at 3d, where after scores dropped (Figure 2B, D,

Suppl Fig 2). Monocytes were predominant at 14 days, however they were not scarce at the earlier time points (Suppl Fig 2). Around cell seeded matrices a similar cellular response was observed ($p > 0.05$ for AF-MSC/FB vs AF-MSC/FB ctrl groups). At individual time points the nature and degree of infiltration in sham operated controls was in the same range.

Vascularization was observed at 3 days post-surgery with small vessels that were mainly located around the matrix, whereas at 14 days the blood vessels had a greater caliber and were also observed within the outer collagen layers of the SIS device. Over time there were no significant differences observed between study groups and sham controls ($p > 0.05$; Figure 2B, D, Suppl Fig 2). Scaffold replacement was evaluated after 7 and 14 days. SIS either seeded with stem cells or immersed in Chang, were over this period progressively degraded, apparently from the interface into the matrix. However, fibroblast seeded matrices were completely degraded by 14 days, a process starting in a similar pattern a week before. This degradation process was not observed for SIS immersed in complete DMEM.

3.3 Macrophage phenotype and cytokine profile

Mononuclear macrophages were present in all study groups at each of the time points investigated (Figure 3A, Table 3). At 14 days following implantation the macrophage phenotype was determined by immunohistochemical staining (Figure 3A). Overall the infiltrate was dominated by M1 type macrophages (CD80), with fewer cells expressing M2 macrophage markers (CD163; CD206). Again no statistical differences were found between cell seeded and cell free matrices, and a similar pattern as in sham operated animals was observed.

At three days post implantation the mRNA expression level of selected pro-inflammatory and anti-inflammatory markers was comparable for all groups, with the exception of *Tnf- α* , which was consistently lower in stem cell seeded explants as compared to Chang immersed SIS. Of note is that for all other markers there was a quite heterogeneous response in the cell free explants, whereas cell seeded explants had a more uniform cytokine profile (Figure 3B).

4. DISCUSSION

The present study investigates the potential of clonal AF-MSC seeded onto a biological matrix to modulate the host immune response at early time points after implantation in rats. We confirmed *in vitro* the biocompatibility of SIS for culture of AF-MSC and fibroblasts. Following subcutaneous implantation in rats we did however not observe any measurable influence of the transplanted AF-MSC on inflammation or macrophage polarization, with the exception of a reduction in the pro-inflammatory marker *Tnf- α* early in time.

Numerous synthetic and biological scaffolds are currently used for surgical reconstruction of damaged or diseased tissue. However, limitations of the scaffolds include inflammation with foreign body reactions, failing reconstructive remodeling and mechanical material failure. Materials may be bioactivated by addition of e.g. growth factors, small molecules or several cell types to provide a microenvironment that improves tissue regeneration. MSC are considered as an ideal cell type as they modulate the immune response in many diseases and are already being used as anti-inflammatory agent for multiple applications [20]. Indeed, preclinical studies show that SIS matrices seeded with MSC result in improved regeneration of epithelial [21], abdominal wall [22], cardiovascular [23], urogynecological [24] or tracheal defects [25]. Reconstruction with unseeded scaffolds resulted in more fibrosis, whereas additional seeding with MSC decreased inflammation, increased vascularity and muscle formation, enhanced construct replacement and ultimately improved organ function. By labeling, the transplanted MSC were shown to participate in the formation of new tissue. Conversely, some other studies did not observe beneficial effects of MSC [26, 27]. All the above studies had remote and structural or functional endpoints, yet lacked in depth analysis of the host immune response.

Previously, MSC from different sources have been co-cultured with SIS with preservation of both cellular and matrix-specific characteristics [22, 23]. Herein we used MSC derived from the human AF as our research is focused on regeneration of congenital birth defects in the perinatal period. We previously established the *in vivo* anti-inflammatory and anti-fibrotic potential of clonally-derived human AF-MSC [28, 29]. In line with previous cell-seeding experiments with SIS, the matrix showed good attachment of both cell types without abrogating their ability to proliferate or inducing apoptosis. AF-MSC, as well as the fibroblasts used as control, maintained their mesenchymal characteristics and were able to deposit ECM onto SIS. Incubation with cells for 24h did not alter the layered structure of SIS and cell-mediated surface reduction was negligible. The latter is particularly important as the contraction of cell-seeded scaffolds is observed frequently, resulting in a changed microstructure of the matrix which will influence its mechanical properties and the ingrowth of host tissue upon implantation [30]. These results, combined with our primary interest in potential immunomodulation

by AF-MSC, prompted us to move forward to *in vivo* experiments with implants incubated for 24 hours.

Around the cell free implant we observed at early time points infiltration of PMN, decreasing later on. Indeed, shortly after implantation of a biomaterial, PMN cells, mostly neutrophils, are actively recruited as a first line of defense. Subsequently, cellular infiltration gradually decreased with a predominant mononuclear cell type, also in line with what has been described before [31]. In view of the potential relationship between the degree of inflammation and as such the ultimate remodeling response, and the macrophage type present in the infiltrate, we proceeded with their further characterization. Macrophages are plastic and capable of assuming several phenotypes along a spectrum depending on the temporal and environmental cues. The shift from an early pro-inflammatory M1 response towards an anti-inflammatory M2 response will determine whether the remodeling process is either inflammatory and fibrotic or rather constructive and functional [32]. Implantation of SIS, as any other acellular collagen matrix, for the restoration of an abdominal wall defect, is associated with a predominant M2 response [33]. In contrast with these findings, we observed mainly M1-macrophages at 14 days. However, in our study both cell free and cell seeded matrices were incubated in cell growth medium rather than immersed in saline (as the manufacturer instructs) prior to implantation. The protein content of these media might cause a delay in the M2 polarization of the infiltrating macrophages. The observed host response pattern; i.e. cellular infiltration and macrophage polarization, was *not* strikingly influenced when seeding SIS with AF-MSC, in contrast with the hypothesis of this study.

At the cytokine level, animals implanted with cell free scaffolds showed a comparable response as sham operated animals. We observed a significantly lower expression of pro-inflammatory cytokine *Tnf- α* and a trend towards lower *iNos* expression in AF-MSC seeded (but not in fibroblast seeded) scaffolds. This drop in pro-inflammatory cytokines was not associated with a difference in expression of anti-inflammatory cytokines. These molecular changes did however not result in a changed inflammatory response, as described above.

SIS typically gets replaced by 25-28 days post implantation [31]. Rats implanted with cell free SIS showed typical signs of beginning scaffold replacement at 14 days, however its extent was not influenced by the addition of AF-MSC to the scaffold. Conversely, when fibroblast were cultured on SIS, implanted rats showed features of intensive replacement after 14 days of implantation: degradation of the SIS fibers, massive infiltration of fibroblasts and production of fresh ECM. The fast replacement of SIS matrix with pre-seeded fibroblasts has been earlier observed. It was suggested this was due to modulation of the immune response, yet the nature and functionality of the replaced tissue was not investigated [34].

To our knowledge this is the first study investigating the *in vitro* biocompatibility of SIS with AF-MSC

and their *in vivo* immune modulatory effects. We provide a profound analysis of both the growth of AF-MSC on an acellular bioscaffold and the host immune response and macrophage polarization upon *in vivo* implantation of unseeded, or AF-MSC-seeded SIS. In this study, we explore the potential of *human* AF-MSC, as the *human* AF is the only clinically relevant source. Additionally, *human* AF-MSC are different from AF-MSC derived from other species because of the species-specific origin and composition of the AF [35]. As MSC have immune-modulatory capacities without being very immunogenic themselves, one could employ them across species without transplant rejection. However, a number of weaknesses of this study are to be recognized. Firstly, the experiments were performed in outbred Wistar rats, having a diverse genetic background by definition. This impedes conclusion drawing as we observed for various readouts a heterogeneous host response within the same study group. Using an inbred strain would lead to more consistent results, but on the other hand -and importantly in translational research- it would have the disadvantage of being less representative of its eventual clinical application. Secondly, we limited ourselves to using typical macrophage markers and cytokines rather than high-throughput array analysis. Additionally, that would have enabled discrimination between cytokines produced by the transplanted human cells or by the local rat tissue. Thirdly, local survival, retention and possible integration of the human cells seeded on the matrix was not monitored and possible mechanisms of action were not investigated. We conclude that (1) SIS is a suitable substrate for AF-MSC culture and (2) AF-MSC seeded onto an acellular matrix have no obvious short-term modulatory effects on local cytokine secretion, neither result in phenotypic changes of M1/M2 polarization or matrix replacement.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The SIS implants were kindly donated by Cook Biotech (West Lafayette, IN) within an agreement for independent research evaluation. This agreement was handled by the transfer office of the KU Leuven (Leuven Research and Development).

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TABLES

Table 1: Markers selected for immunostaining

Marker	Function	Time point	Heat retrieval	Primary antibody
Vimentin	Mesenchymal marker	24h, 48h, 7d, 14d	0.01M citrate, pH 6 1h 80°	Dako M0725 1:100 v/v, 2h, RT
CD68	Macrophage marker	3, 7, 14d	0.01M citrate, pH 6 1h 80°	Serotec MCA341R 1:200 v/v, ON, 4°
CD80	M1 marker	14d	0.01M TrisHCl, pH 8 30' 95°	Santa Cruz sc-9091 1:100 v/v, ON, 4°
CD163	M2 marker	14d	Pepsine 0.01% 10' 37°	Serotec MCA342R 1:300 v/v, 1h, RT

Table 2: Primer sequences of target genes

Gene	Forward primer	Reverse primer
<i>MKI67</i>	5'-CTTTGGGTGCGACTTGACG -3'	5'- GTCGACCCCGCTCCTTTT-3'
<i>CASP3</i>	5'-TGGTTCATCCAGTCGCTTTG -3'	5'- CATTCTGTTGCCACCTTTTCG -3'
<i>GAPDH</i>	5'-TGGTATCGTGGAAGGACTCATGAC -3'	5'- ATGCCAGTGAGCTTCCCGTTCAGC-3'
<i>iNos</i>	5'-CTCAGGCTTGGGTCTTGTTAG-3'	5'-TGTTGTTGGGCTGGGAATAG-3'
<i>Tgfβ</i>	5'-TGAACCAAGGAGACGGAATACAGG-3'	5'-GAGGAGCAGGAAGGGTCGGT-3'
<i>Tnfa</i>	5'-AAGGAGGAGAAGTTCCCAAATG-3'	5'-AGAGAACCTGGGAGTAGATAAGG-3'
<i>Cd204</i>	5'-GCAACAGGAGGACATCAGTAAG-3'	5'-GAGGCCCTTGAATTAAGGTGATA-3'
<i>Rpl13a</i>	5'-GGATCCCTCCACCCTATGACA-3'	5'-CTGGTACTTCCACCCGACCTC-3'
<i>Pgk1</i>	5'- ATGCAAAGACTGGCCAAGCTAC'-3	5'-AGCCACAGCCTCAGCATATTTTC-3'
<i>Cypa</i>	5'-TATCTGCACTGCCAAGACTGAGTG -3'	5'-CTTCTTGCTGGTCTTGCCATTCC-3'

Table 3: Macrophage infiltration at 3, 7 and 14 days post implantation

	Sham	FB ctrl	FB	AF-MSC ctrl	AF-MSC
3 days	14.01±0.82%	7.91±1.28%	7.66±0.72%	8.53±1.44%	10.33±1.70%
7 days	8.31±0.85%	9.24±0.40%	11.06±2.13%	7.99±0.75%	8.23±1.36%
14 days	5.42±1.06%	8.51±1.64%	9.78±1.28%	18.34±2.78%	13.16±3.45%

The amount of CD68+ cells is represented as a fraction of the total cellular infiltration. Data: mean ± SEM.

FIGURE LEGENDS

Figure 1: *In vitro* analysis of cell growth on the SIS matrix

(A) Hematoxylin eosin staining and quantification of cell growth of AF-MSC and FB after 14 days of co-culture with the SIS matrix. (B) Movat staining to visualize the ECM secretion of AF-MSC and fibroblasts in co-culture with SIS. (C) Vimentin staining showing AF-MSC and fibroblasts retain their mesenchymal character in co-culture with SIS. (D) Scaffold area reduction represented as a fraction of the original scaffold area. Scale bars represent 50 μm . Data; mean $\pm$ SEM.

Figure 2: Cellular infiltration and matrix remodeling 14 days post implantation

(A) Graphical representation of the implantation protocol. (B) H&E staining of cellular infiltration at the matrix interface. (C) Masson staining showing matrix degradation and deposition of new collagen at the matrix interface. (D) Quantification of polymorphonuclear cell infiltration, vascularity and matrix replacement at 14 days post implantation according to previously described criteria. Scale bars represent 100 μm . Data; mean $\pm$ SEM. *** $P < 0.001$ vs FB ctrl.

Figure 3: Macrophage phenotype and molecular profile at the implant site

(A) Representative images of immunohistochemical staining for CD68, CD80, CD 163 and CD206 at the matrix interface 14 days after implantation with AF-MSC-seeded SIS. Quantification of the macrophage phenotype at the matrix interface. No statistical differences were observed between rats implanted with control SIS and cell-seeded SIS. (B) qRT-PCR results of M1/M2-specific markers 3 days post implantation. Scale bars represent 100 μm . Data; mean $\pm$ SEM. * $P < 0.05$ vs AF-MSC ctrl.

FIGURES

SUPPLEMENTARY MATERIAL

Supplementary Table 1: Cell-mediated scaffold contraction

	24h	48h	7d	14d
AF-MSC	1.96 ± 1.06	1.09 ± 1.70	7.60 ± 4.30	16.95 ± 4.49***
FB	1.22 ± 2.96	-0.10 ± 3.60	2.46 ± 2.15	7.20 ± 5.97

*Data: mean ± SEM; ***P < 0.001 vs 24h and vs 48h*

Supplementary Figure 1: AF-MSC co-cultured with SIS maintain their proliferative potential and do not induce mineralization *in vitro*

(A) Agarose gel electrophoresis (top) and densitometry analysis (bottom) of PCR for KI67, CASP3 and GAPDH of DNA isolated from AF-MSC and FB co-cultured with SIS for 24h, 48h, 7d and 14d. The 'adjusted relative density' is both relative to the housekeeping gene and the 24h time point (set at 100%).

(B) Alizarin Red staining of SIS co-cultured with AF-MSC for 14 days shows absence of mineralization (left), of AF-MSC grown in TC plates in Osteogenesis Differentiation Medium for 28 days shows calcium sites covered in red (right). Scale bars represent 50 μ m.

Supplementary Figure 2: Infiltration of inflammatory cells, angiogenesis and scaffold remodeling at the SIS implant site

Quantification of mononuclear (A) and polymorphonuclear (B) cell infiltration at the matrix interface after 3, 7 or 14 days. Histological quantification of neoangiogenesis at 3 or 7 days post implantation (C) and of scaffold remodeling at 7 days post implantation (D). Quantifications were performed following previously described criteria. Scale bar represents 50 μ m. Data; mean $\pm$ SEM. **P = 0.01 vs AF-MSC ctrl.