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Research article

FasL Promotes Proliferation and Delays Differentiation of ASCs

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Abstract

The use of stem cells as cellular therapy is most promising, however, many hurdles still exist. This process is usually lengthy, costly, bares the risk of cellular differentiation, highly variable and usually does not reach the desired outcome of sufficient quantity of cells that preserve homogeneity and “stemness”. Bone marrow transplantation is a good example where the lack of selection between the cells needed for transplantation and the cells resulting in the immune reaction results in life threatening conditions such as the Graft versus Host Disease (GvHD). To overcome the variability of cells in the cellular product, Cellect has recently introduced the Apoptosis-based functional cell selection technology. This technology is expected to deliver a broad range of next generation cell- and gene-therapies, which are reproducible, safer and more efficacious. The technology is based on our findings that cells are differentially sensitive to apoptotic signals mediated via the CD95 receptor. In the current study, we show that our technology is not limited to a specific stem cell type. Mesenchymal Stem Cells (MSC) treated with FasL for six passages show an increase in cell proliferation while maintaining their wide differentiation potential. In in-vivo studies for human pathologies, treated cells showed better outcomes. Hence, our technology overcomes hurdles in stem cell therapy by presenting a more rapid proliferation coupled with a more homogenous cell population retaining their stemness. These advantages have both health and economic implications for the fast-growing stem cell industry.

Keywords: Stem Cell; apoptosis-based cellular functional cell selection technology; stem cell property maintenance;FasL

Introduction

Stem cells are used in cellular therapy (CT) and regenerative medicine (RM) for replacing or "regenerating" human cells, tissues or organs to restore normal function [1]. Various types of stem cells are used for a wide variety of clinical indications. For example, hematopoietic stem cells (HSC) can be used for immune system reconstitution in diseases like cancer and autoimmune disorders and mesenchymal stem cells (MSC) are used in the treatment of inflammatory responses as well as skeletal defects and traumas [2].

In RM and CT, isolated stem cells can be introduced to the patient immediately after retrieval or after a period of time in culture. Cell culturing is used mainly for expanding the number of cells (while maintaining their differentiation potential) and in some cases for cellular manipulation such as gene manipulation for gene therapy [3]. Since these cells exist in very small numbers, using them for medical purposes requires a controlled process to obtain large quantities. Stem and progenitor cells tend to undergo differentiation once out of their normal environment. Therefore, inducing cellular proliferation while maintaining cellular properties is a double challenge [4].

According to the Alliance for Regenerative Medicine (https://alliancerm.org/), there are currently about 1,028 clinical trials worldwide for regenerative medicine. When human MSC (hMSCs) are used in RM clinical trials, it is essential to ensure that while cells propagate, they maintain their required properties. Cell quality poses a huge challenge for the industry. It requires the development of methods for cost effective scale-up of industrial cell growth while maintaining cellular properties such as functionality and safety. Thus, any technology that improves cell expansion while maintaining cellular properties (improving quantity and quality) will significantly impact the outcomes of studies in the field of regenerative medicine [4,5].

Cellect Biotechnology has developed a unique and potentially groundbreaking platform technology for functional selection of cells. Cellect uses a proprietary Apoptosis-based cell selection technology for the development of functional cell products that have the potential of delivering a broad range of next generation cell and gene therapies, which are reproducible, safer and more efficacious [6].

The technology is based on our findings that cells are differentially sensitive to apoptotic signals mediated via the CD95

receptor (Figure 1). While many mature cells die following exposure to CD95 ligands [6-8], the same signal can activate stem and progenitor cells. Hence, this potentially groundbreaking technology can be used for both negative selection of mature unwanted-differentiated cells and at the same time by promoting positive selection of cells, by preserving and proliferating stem and progenitor cells and improving their stemness [6].

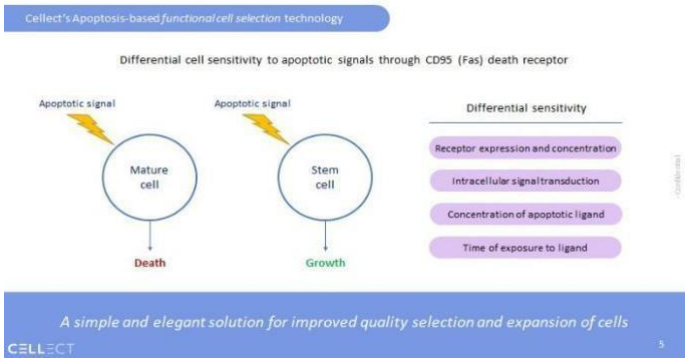

Figure 1. A Schematic representation of Cellect's Apoptosis-based functional selection technology. Due to different cellular sensitivity to apoptosis inducing signals, mature cells would be removed from culture, while stem cells can proliferate resulting in a more homogeneous stem cell population that can be used for human therapy.

Bone Marrow transplantation (BMT) is a very powerful CT treatment, widely used for immune related disorders. Currently BMT has a major safety barrier of Graft versus Host Disease (GvHD), which limits its use to only few life-threatening indications. In the context of BMT, Cellect's lead product (ApoGraft™) is utilized for the negative selection of Hematopoietic SCs (HSC). It is a non-invasive, short (~4 hours) ex-vivo process performed on mobilized peripheral blood cells (mPBCs). This process is designed to increase the safety of bone marrow transplantation (BMT) by depleting mature and activated toxicity-eliciting cells, while maintaining stem and progenitor cells composition and function in all cell lineages tested to date [9]. ApoGraft™ is now in phase I/II clinical trials for the prevention of GvHD (for information see NCT02828878; NCT04006652 on www.clinicaltrials.gov). Moreover, since ApoGraft™ treatment can potentially increase BMT safety while maintaining its efficacy, it can substantially widen BMT use in other indications, even where BMT is considered to be efficient but too risky, such as tolerance induction in Solid Organ Transplantation and treatment of Auto immune diseases.

Mesenchymal Stem Cells (MSC) are defined as cells that can differentiate into three distinct cell lineages (adipose, bone and cartilage) [10]. MSCs are also known to exert a paracrine immune suppression [11] and, therefore, induce anti-inflammatory response. Due to their differentiation potential, MSCs have been proposed as a potential resource of cells for a variety of needs, including inflammation control and tissue regeneration. To date, more than 36,000 patients have been treated with MSC in more than 1,000 registered clinical trials [5, 12]. However, these clinical trials frequently fail in advanced stages (phase III) due to inconsistent effects. Lack of good cell selection criteria and multiple expansions and loss of original cellular functionality seem to result in dramatic differences between patient outcomes.

Adipose Derived Stem Cells (ASC), a type of MSC, can be extracted by minimal invasive procedures, such as liposuction. However, they are less abundant than HSC and have fewer known characteristics that define them. The lack of good surface markers for MSC makes their isolation even more challenging and time consuming, resulting in an increased cost for therapy. In our previous work, we showed that our apoptosis-based functional selection technology is not limited to HSCs but also has a potential for CT treatment using ASCs [6, 13]. Specifically, we examined the effects of FasL, an apoptotic inducing ligand and member of the tumor necrosis factor (TNF) family, on the isolation of ASC. We showed that the exposure of ASC to FasL resulted in accelerated cellular proliferation while maintaining undifferentiated properties. In addition, upon the removal of FasL from the medium, cells showed better differentiation into bone or lipoid (depending on the differentiation medium) tissues and in forming fibroblast-like colonies [6]. Taken together, these findings demonstrate the potential of FasL treated ASC as a possible treatment for CT [6, 9]. These results support our hypothesis that the FasL functional selection technology has the potential for high quality and high quantity production of MSC and to help bridge current technological gaps in the production of such cells.

In a recent review, Olsen et al [5] showed that the demand for MSC is on the rise. New technologies using MSC (whole cells or parts of the cell) are not limited to the clinic and include their use in producing artificial meat, cosmetics and many more commercial products. They estimate that there is an annual need of $12.5x10^{18}$ cells in a multi-billion dollar growing industry. In contrast to the production of small molecules, the clinic and industry face difficulties in large quantity production of good quality living cells. Although many techniques for the expansion of cells have been developed (usually in 3D bioreactors), these processes are limited due to the risk of losing stem cells characteristics during cell growth and harvest. Therefore, new technologies are needed for both production of a large number of good quality cells able to differentiate, in a minimal time in culture, and removal of contaminating cells. Here-in, we set out to examine whether our technology of apoptosis-induced cell selection can provide a comprehensive solution for these requirements.

In the current study, we tested FasL's ability for quantity and quality expansion of ASC (over more than 1-2 passages), to determine whether this method could overcome hurdles essential for developing products for CT. For this purpose, we used our unique apoptosis-based functional selection technology in various quantitative and qualitative in-vitro assays as well as in two preliminary animal models. These results demonstrate that short exposure of freshly isolated ASC to FasL (Passage 1) or long exposure (Passage 6; about 52 days) resulted in enhanced cellular proliferation while strictly maintaining stemness properties. The results of preliminary in vivo experiments in mouse models reiterated the potential of ASC for CT. Using RNA-seq, we showed that genes involved in immunosuppressive pathways are overexpressed in these cells, suggesting a potential model for FasL induced apoptosis in differentiated cells, whilst causing the proliferation of the undifferentiated ASC.

Taken together, these findings indicate that apoptosis-based functional selection technology is not limited to one type of stem cells. Our technology has an advantage relative to other expansion technologies for clinical and industrial production of large quantities of high-quality cells. We show that treatment with apoptosis-inducing

cell selection results in increased proliferation rates while maintaining cells undifferentiated state, hence reducing the number of passages necessary to reach a sufficient quantity of cells. Future experiments will examine how to integrate this module into clinical batches, in clinical trials that use ASC for the treatment of human diseases.

Materials and Methods

All methods have been described previously [6] other than those specifically described below. All experiments were carried out in the presence of 5ng/ml FasL (AdipoGen, Cat. No AG-40B-0130) when compared to control (no FasL) unless otherwise specifically indicated.

2D and 3D differentiation

All 2D and 3D experiments were carried out as outsourced to biotech companies, which under agreement, cannot be disclosed.

Cell Proliferation and Alizarin Red staining of 2D cultures

24-well plates were coated with a 2D-matrix with or without FasL (10 ng/ml). Following overnight incubation at room temperature, the plates were washed once with PBS, aspirated and used for plating ASCs (p0) at the density of 5000 cells/cm2 Following 6 days in culture, at approximately 80% confluent, cells were harvested and counted.

Osteogenic differentiation was assessed by Alizarin Red. P0 ASC were cultured with or without FasL for 18 days. Cells were passaged, seeded in a 24-well plate coated or non-coated with a 2D-matrix, and induced to undergo bone differentiation using a StemPro osteogenesis Basal medium (Gibco, Cat. No A10069-01) with StemPro osteogenesis Supplement (Gibco, Cat. No A10066-01). After 21 days, the cells were fixed and stained with Alizarin Red (a commonly used dye to identify calcium containing Osteocytes). The dye was extracted and quantified using an ELISA plate reader. Unstained cells in control wells were counted.

3D differentiation of FasL treated cells

Qualitative viability assessment of ASCs immediately after plating and after four days in the injectable scaffold. ASCs with plasma or medium mixed with plasma within the scaffold, were incubated for four days at 37º C 10% CO_2 and loaded with PrestoBlue™ reagent. The color of the wells was assessed. Wells containing metabolically active cells appear pink whereas in the control wells there are no cells and the color remains dark blue.

GSH levels

A correlation between the Stemness of the cells and the Glutathione (GSH) levels in the cells has been demonstrated [14] GSH is an anti-oxidant found in millimolar levels in the cells. Low levels of GSH indicate the cell is under oxidative stress, detected by Oxidative Stress Resistive Capacity (ORC) resulting in lower stemness potential of the cell. Cell2in (http://www.cell2in.com/) is a Korean Biotech company that developed the FreSHTracer (FT), a fluorescent dye that in living cells, can penetrate the membrane and bind GSH. Bound and free FT have different absorption and emission spectra.

Immunomodulatory and Immunosuppression activity of ASC
Immunosuppression potential

ASC cultured in the absence or presence of FasL for 14 days (2 passages) were seeded in 24 well-plates (100,000 cells/well). Next day, human T-cells were isolated from a buffy coat sample using RosetteSep-Human T-cell Enrichment Cocktail (StemCell Cat. No 15021) and labeled with CFSE (2μM, eBioscience 65-0850). The labeled T-Cells were then added to the ASC (100,000 T-cells/well). The cells were stimulated with the Human TActivator CD3/CD28 (111.31D, Dynabeads, Invitrogen, Carlsbad, CA, USA) at a 1:10 bead:T-cells ratio. Following 5 days of incubation at 37˚C, 5% CO_2 , the supernatant was collected and IFN-γ secretion was detected using ELISA assay (Quantikine, R&D systems, Minneapolis, MN, USA). In addition, T-cells were subjected to flow cytometry assay. Cell proliferation is presented as Proliferation Index: the average number of divisions all cells have undergone after being stained by a cell proliferation dye (CFSE).

Immunomodulatory of ASCs

ASC were isolated and maintained in the presence or absence of FasL, as described previously. On the day of the Passage, cells were plated in a 24-well plate, 40,000 cells/ml/well in Complete Medium (CM). Cells were incubated in a humidified chamber for 18-24 hours. Medium was replaced with CM containing 2% FBS with or without IFN-γ. The supernatant was collected after 18-26 hours, centrifuged at 1000xg for 10 min, aliquoted and stored at -80°C until use. (https://pubmed.ncbi.nlm.nih.gov/21934657/).

Gene expression using RNA-seq

Cells

SVF were extracted according to standard for 4 patients, each on a different day. Cells were seeded (8000 cell/cm^2) and grown during 13 days and then frozen. Cells were thawed simultaneously from all 4 patients, and grown 3 days to 100% confluence (P1). Cells were treated with 30 ng / ml MEGA FAS-L for 24 hours. Then FAS-L treated and untreated cells were lysed and RNA extracted according to manufacture protocol (High Pure RNA Isolation Kit, ROCHE)

RNA SEQ

Libraries were prepared at the Crown Genomics institute of the Nancy and Stephen Grand Israel National Center for Personalized Medicine, Weizmann Institute of Science. 500ng of total RNA for each sample was processed using the Inhouse poly A based RNA seq protocol. Libraries were evaluated by Qubit and TapeStation. Sequencing libraries were constructed with barcodes to allow multiplexing of 12 samples on one lane of Illumina HiSeq 2500 machine, using the Single-Read 60 protocol (v4), yielding a median of 20.6M reads per sample (Illumina; single read sequencing). Poly-A/T stretches, and Illumina adapters were trimmed from the reads using cutadapt [15]; resulting reads shorter than 30bp were discarded. Reads were mapped to the H. sapiens reference genome GRCh38 using STAR [16], supplied with gene annotations downloaded from Ensembl (release 92). The EndToEnd option was used and out Filter Mismatch Nover Lmax

was set to 0.04. Expression levels for each gene were quantified using htseq-count [17], using the gene annotations downloaded from Ensembl. Differentially expressed genes were identified using DESeq2 [18] with the betaPrior, cooksCutoff and independentFiltering parameters set to False. Raw P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. Pipeline was run using snakemake [19].

ICAM-1 Expiration: SVF were prepared as described in RNA-Seq section, after thawing, the cells were treated with 40 ng / ml FasL for 44 hours. Treated and control untreated cells were stained with anti-human CD54- PerCP/Cyanine5.5 antibody (BioLegend, San Diego, California), % CD54 (ICAM-1 receptor) was analyzed by flow cytometry.

In-vivo models

Collagen Induced Rheumatoid Arthritis Model

DBA-1 mice (40 males, 7-9 weeks old; Jackson Laboratory, Bar Harbor MN, USA) were housed in a pathogenfree facility and handled in accordance with the guidelines of the Animal Care and Use Committee of the Science in Action LTD (SIA), Ness Ziona, Israel. Collagen Type II (CII) from newborn calf articular joints (Missouri, USA, Cat No. CJ385) together with CFA Elastin emulsion was injected to 14 mice on Day 0 sub-cutaneous (SC) at the base of the tail (total volume of 0.05 ml/animal). On day 21 the animals received a booster consisting of the same amount of the emulsion SC at the base of the tail. Clinical score monitoring was carried out twice weekly as described by [20]. Paw swelling of the joints of the hind legs was assessed by measuring the mean thickness of paws with 0–10-mm calipers. Daily measurements begun at arthritis onset.

Treatment

ASCs were cultured in present of 5ng/ml FasL for 35 days (4 passages). On day 23 of mice treatment, 8 mice who did not developed clinical score, were administrated with 2.5x106 cells/mouse intraperitoneally (IP). The group of 3 untreated mice were used as Vehicle control. Mice were sacrificed in case of weight loss ≥20% of initial body weight, dehydration, motility, shaking or the presence of blood in feces.

Xenogeneic GvHD Model

NOD-scid IL2Rgamma-null (NSG) mice (Jackson Laboratory, Bar Harbor MN, USA) were housed in a pathogenfree facility and handled in accordance with the guidelines of the Animal Care and Use Committee of the Rabin Medical Center, Petach Tikva, Israel. Ten female NSG mice (6-9 weeks) per group were irradiated with 2-2.75Gy (CLINACDBX linear accelerators, Varian Medical System, Palo Alto,CA, USA) 24h prior to intra venous (IV) infusion of 3x106 / mouse mobilized peripheral blood cells (MPBCs). Mice were randomized according to age and body weight.

ASC were cultured in the presence of 5ng/ml FasL for 32 days (4 passages), 1.2x106 FasL ASCs/mouse were injected IV 8 days after MPBCs administration.

Monitoring of GvHD clinical score was carried out twice weekly according to the murine clinical grading system described by [21].Weight loss, hunched posture, skin lesions, dull fur, and mobility were each assigned a score of 0 (absent), 1 (moderate) or 2 (severe). Scoring was blinded to the treated or control group. Mice were sacrificed in case of weight loss ≥20% of initial weight or upon reaching a clinical GvHD score of ≥7.

iPS treatment with FasL

All iPS experiments were carried out in conjunction with Accellta, an Israeli company (https://www.accellta.com/) according to company confidential protocols. MSCs derived from iPS were cultured at Accellta facility for seven days in medium without FasL or in the presence of 1ng/ml of FasL in static suspension.

The cells were harvested and seeded (passage from p1 to p2) at Accellta, 5000 cells/cm2 in Accellta Differentiation Medium in T25 flasks coated with 1mg/ml of Gelatin (Sigma) according to the company confidential protocol. The flasks were then transferred to Cellect and cultured in a humidified 37^{o} C 10% CO_2 incubator. Media was refreshed every 3-4 days. The cells were harvested at approximately 80% confluence- after 7 days from seeding (at p2) and counted using the Trypan blue exclusion test.

Results

The use of MSC for clinical trials requires a tissue culture step in which cells proliferate while maintaining their homing ability, multilineage differentiation potential and their immunomodulatory properties. These steps are usually lengthy and costly [5, 22]. Therefore, to reduce time in culture, while maintaining cellular properties, we examined the potential of FasL treatment as an enhancement platform for this process.

FasL treated isolated ASC proliferate while maintaining stemness properties

ASCs were isolated from liposuction procedures according to ethical approvals. Isolated cells were tested for changes in proliferation rates, surface marker expression and differentiation potential as a result of FasL treatment. We first reproduced our previous report [6], and showed that isolated ASCs at p0 proliferate in culture and incubation with 5ng/ml of FasL increased cell count by 1.3-fold over 14 days. These results were reproducible and demonstrated in cells isolated from over 30 patients (data not shown). Using FACS to assess ASC cell surface markers following FasL treatment, we examined the expression of CD105 and CD73. Low surface expression of CD105 and high surface expression of CD73 were previously correlated with increased bone differentiation capacity [23]. As expected from our previous work, we found a decrease in CD105 levels and increase in CD73 levels (not shown; [6]), indicating the improved osteogenic differentiation potential of ASC following incubation with FasL. We next verified the isolated cells' stemness potential to differentiate.

We observed an increase in Colony Forming Units Fibroblasts (CFU-F) like colonies (Figure 2a) in response to FasL treatment. In addition, treatment of p0-p1 isolated cells with FasL increased differentiation of ASCs into bone (Figure 2b). Cumulatively, these results indicate that isolated ASC treated with FasL proliferate and Maintain their stemness properties.

Prolonged treatment of ASCs with FasL as a source for cellular therapy

The use of ASCs for cell therapy treatment usually requires prolonged periods of ex vivo proliferation with the risk of differentiation [5,22]. Therefore, we examined whether prolonged FasL treatment of ASCs results in increased quantity and quality of the undifferentiated cell population. Isolated ASC were maintained in culture for at least 52 days (p6) with 5ng/ml FasL. As seen in Figure 2c, FasL treated ASCs proliferated more, as there were 4.6 more cells compared to control over 5 passages. This statistically significant increase in cell number was observed at all timepoints (Figure 2d). The cells maintained high viability (>90%) throughout the entire duration of the experiment. This increase in cell number was not related to karyotypic changes, as a normal karyotype was observed after 8 passages (data not shown). It should be emphasized that cellular quality measures following prolonged FasL exposure (described below) were not reduced and even potentially improved.

We next examined the quality of cells by assessing their differentiation potential following an extended period of FasL treatment. Differentiation was assessed by multiple methods:

Expression of cell surface markers

As described previously, high CD73 expression is associated with stemness properties. Therefore, we examined CD73 level of expression, as measured by FACS. We found that although both treated and untreated cells exhibited an increase in CD73 levels over passages, FasL treated ASC expressed even higher levels of this specific MSC surface marker (data not shown). This might indicate that under FasL treatment, a larger portion of the cells are stem cells.

Fibroblast-like colony forming units (CFU)

The capacity of FasL-treated and untreated ASCs ability to form CFU was examined. We found that while FasL treated cells maintained their ability to form fibroblast-like colonies, there was no statistically significant difference between the groups (CFU data for prolonged growth in culture is not shown).

Ability to differentiate in multi-dimensional matrixes Tissue engineering, such as for tendon repair, requires not only the differentiation of cells into target cells, but also their ability to grow on 2D and 3D matrix scaffolds. All of the experiments examining cell growth on matrixes were conducted in collaboration with two biotechnology company (not disclosed due to commercial restrictions).

We found that ASCs could proliferate on the 2D-matrix (Figure 2E). Next, to asset cell differentiation potential, we embedded FasL into the 2D-matrix, seeded ASCs and maintained seeded cells in culture for 18 days. We found that while FasL could increase cell proliferation, both FasL treated and untreated cells exhibited an increase in differentiation potential when grown on a 2D-matrix compared to control, as assessed by Alizarin Red staining (data not shown). Next, ASC seeded on Collagen disc 3D-matrix for 6 days, maintained their viability and proliferation capacities (data not shown).

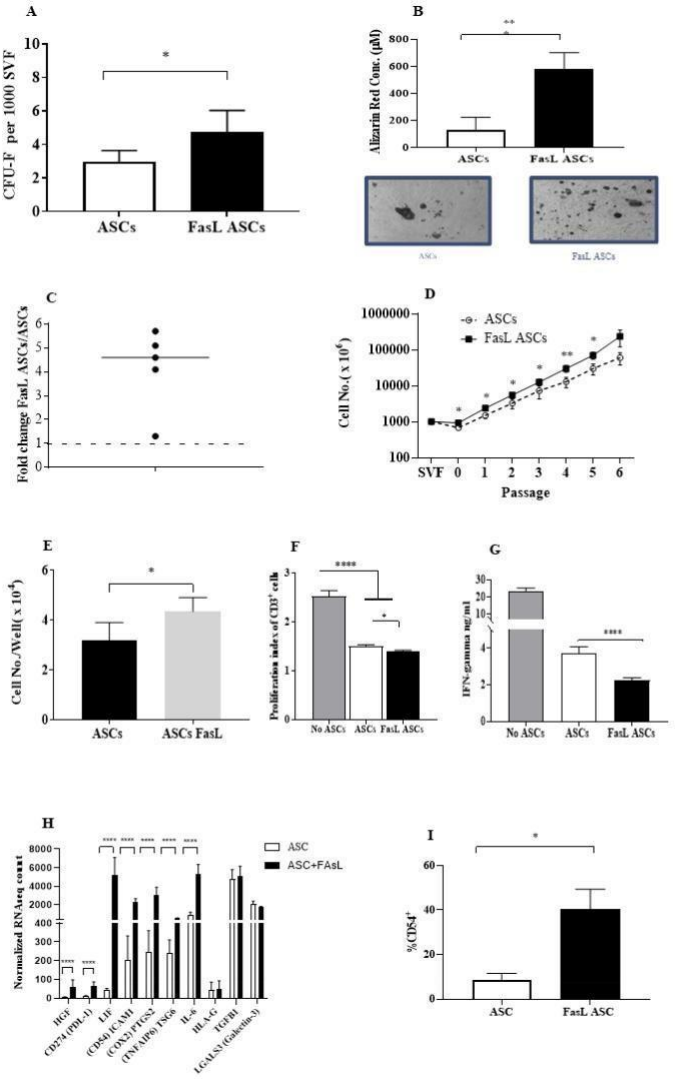

Figure 2. FasL treated SVFs for 6 passages show increased proliferation potential and maintain stem cell properties.

Isolated SVFs were cultured in presence or absence FasL for 11-14 days (P0) followed by an additional Passage (P1) and prolonged treatment for 52 days (P6). (A) An increase in Colony Forming Units (CFU) of Fibroblast like colonies was observed after P0. The results of 5 ASCs batches are presented as Mean ± SEM, *P<0.05 Ratio T-test. (B) After P1 ASCs treated with FasL depicted increased differentiation into bone as assessed by Alizarin Red concentration (upper panel; cells seen in lower panel). Results of 4 technical replicates of a representative experiment are presented as Mean ± SEM . ***P<0.001 T-test. (C) FasL treated ASC proliferated, as there were 4.6 more cells compared to control. The fold change was calculated as follows: Total FasL ASCs harvested/Total ASCs harvested. (D) This statistically significant increase in cell number was observed at all timepoints of cell growth for 6 passages. The results of 5 ASCs batches are presented as Mean ± SEM, Paired T-test *P ≤0.05, **P ≤0.001. (E) FasL increases ASCs growth on 2D- matrix. Cells treated with 10ng/ml for 6 days on matrix in triplicate. Results are presented as Mean ± SD, T test *P ≤0.001. (F) FasL ASCs exhibit improved Immunosuppressive Potential- FasL ASCs inhibited T cells proliferation significantly more than ASCs cultured without FasL. The number of $CD3^+$ cells was examined using FACS. (G) T cells co-cultured with FasL. ASCs

secreted 40% less IFN-ɤ compared to T cells cultured with ASCs only, as assessed by ELISA. Results are presented as Mean ± SD of 6 replicates of the representative experiment. *P< 0.05, **** P<0.0001, T-test. (H) ASCs treatment with FasL significantly increase expression of gens involved in an immunosuppressive process. 4 different ASCs samples after p1 were treated with 30 ng/ml FasL for 24 hours. Cells were lysed and RNA was extracted and processed to sequencing. Data presented as Mean ± SD; ****P<0.0001, Multiple testing. (I) ASCs treatment with FasL significantly increased ICAM-1 expression on cell surface. 4 different ASCs samples after p1 were treated with 40ng/ml FasL for 44 hours. Treated and control untreated cells were stained with anti-human CD54 antibody, % CD54 (ICAM-1 receptor) was analyzed by flow cytometry. Data are presented as Mean ± SD *P<0.05 T-test.

Glutathione levels

A correlation between stemness and the anti-oxidant Glutathione (GSH) levels has been reported [14]. Low levels of GSH are correlated with oxidative stress. In order to distinguish these low levels of GSH, Cell2in (http://www.cell2in.com/) developed specific dyes that can penetrate live cells and bind GSH. Bound and free GSH have different absorption and emission spectra. Cell2in have shown that in MSC, a higher bound/unbound ratio is correlated with an increase in cells stemness. We found higher GSH levels following FasL treatment (data not shown).

FasL enhances immunomodulatory and immunesupp--ression activity of ASCs

MSC have been proposed as treatment due to their immunomodulation properties and ability to migrate to injury sites. In addition, it has been robustly demonstrated that MSC negatively regulate the activation and proliferation of T cells by the secretion of soluble factors [24-26]. Therefore, using FACS and comparing different subsets, we examined T-cell proliferation, and T-cell surface marker expression of FasL treated ASCs (p6) in a co-culture system of ASCs and T cells labeled with CFSE and activated with anti CD3/CD28 beads as described in the "Materials and Methods" section. We found a significant reduction in CD3 positive T- cell proliferation (Figure 2f). Next, we examined the immunosuppressive activity of FasL on p6 ASCs using ELISA. T cells co-cultured with FasL treated ASCs secreted 40% less IFN-γ compared to T cells cultured with ASCs without FasL (Figure 2g). Cumulatively, these results indicate ASCs immunosuppression enhanced by FasL and support the advantages of using FasL treated ASCs for therapy, as they do not evoke an immune response and can even suppress it.

Immunosuppressive gene expression following treatment with FasL using RNA-seq

In order to explore the underlying mechanism of FasL effects on ASCs, ASCs were treated for one passage with FasL for 24 hours and RNA was extracted and analyzed using RNA-seq. As depicted in Figure 2h, a statistically significant change in gene expression was observed for genes involved in immunosuppressive processes. These genes include CD54 (pro-inflammatory in Macrophages), PDL-1 (interaction of MSCs with T lymphocytes), TSG6 (Induction of M2 macrophage polarization), LIF (Inhibition of proliferation, cytokine secretion and cytotoxicity of T cells), and others (Figure 2h). Moreover, ICAM-1 (CD54) expression, which is known to promote MSC immunosuppression capacity, was upregulated following 48 hours treatment (Figure 2i).

FasL treated with human ASCs as a potential treatment of human pathologies

The use of MSCs as treatment requires the proliferation of cells in culture while maintaining their properties. As we have shown that FasL treatment in vitro of ASCs over many passages results in cell proliferation while maintaining stemness properties, we next examined activity of FasL treated cells in two murine models for potential human treatments: Rheumatoid Arthritis (RA) and GvHD. As depicted in Figure 3, a single administration of a relatively small number of treated human ASCs to both models improved the clinical score and survival of mice over time. Cells were able to decrease GvHD (Figure 3a and 3b) as well as reduce the thickness of hind paw in the RA model (Figure 3c and 3d) compared to control. Although both pathologies have an immune system component, our results show that the administration of FasL treated cells did not evoke an immune response. It should be emphasized that mice were administered with only a single dose of cells in both models. We anticipate that in future experiments, multiple administrations of cells would lead to even more pronounced effects. These results strengthen our claim for the potential of using FasL treated ASCs for human treatment.

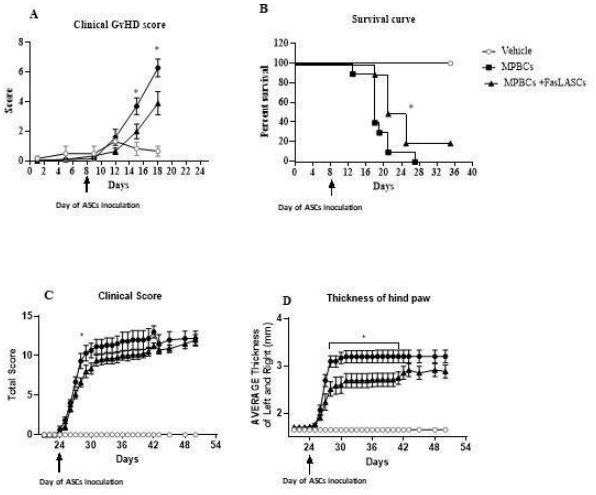

Figure 3. Activity of FasL treated cells in two murine models for potential human treatments

Xenogeneic GvHD Model (A and B) and Collagen Induced Rheumatoid Arthritis (RA) Model (C and D) were treated with human FasL treated cells. A single intravenously injection (IV) significantly improved clinical score (A) and prolonged mice survival (B) compared to control. N=10 for both control and treated groups, N=3 for the Vehicle group. A single intraperitoneal (IP) administration of FasL treated ASCs significantly reduced the clinical score (C) and thickness of hind paws (D) compared to control. N=10 for both control and treated groups. N=3 for the Vehicle group. Results are presented as Mean±SEM, T-test * P≤0.05. Long-rank test for survival curves *P≤0.05. For experiment details, see "Materials and Methods" section.

FasL treatment of BM and Umbilical cord MSC increases cellular stemness

As we described previously [14], GSH levels are correlated to stemness. GSH is an anti-oxidant found in millimolar levels in the cells. Low levels of GSH indicate the cell is under oxidative stress, detected by Oxidative Stress Resistive Capacity (ORC) resulting in lower stemness potential of the cell. Bone marrow (BM) and umbilical cord (UC) derived MSC were treated with FasL and the GSH levels were examined. As seen in Figure 4a, higher bound/unbound ratio of GSH levels was seen in FasL treated BM derived MSCs compared to untreated cells. A similar result was also observed with umbilical cord derived MSCs (Figure 4b) indicating that FasL enhances stemness represented by higher GSH levels.

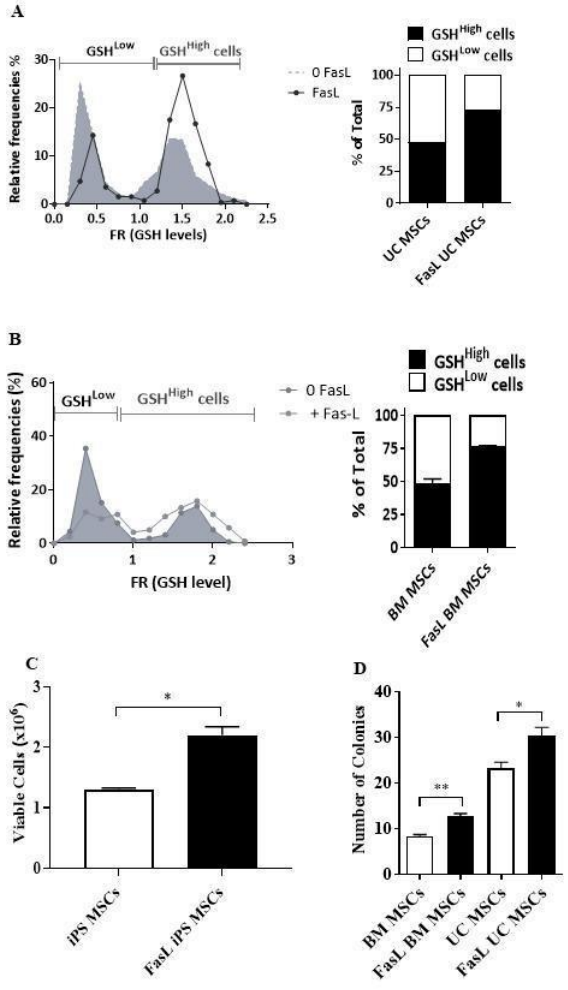

Figure 4. FasL effects on MSCs derived from Umbilical, BM and iPS cells. Effect of short exposure (4 days) of BM MSCs (A) and UC MSCs (A) to 0.5 ng/ml FasL on stemness potential detected by ORC levels. Results are presented as Mean ± SD of 3 replicates. For both (A) and (B), right panel is the quantification of the histogram presented on left. FasL Enhances MSCs Stemness exhibit higher levels of Glutathione (GSH). (C) FasL at the concentration of 1 ng/ml significantly enhanced iPS MSCs proliferation at passage 2. (D) Effect of short exposure (7 days) of P6 BM MSCs and UC MSCs to 0.5 ng/ml Fas L examined by the number of colonies formed (CFU). Results are presented as Mean ± SD of 3 replicates. * p <0.05. ** p <0.01

Apoptosis-based functional selection enhances Stemness of MSCs derived from Bone Marrow, Umbilical cord and iPS cells

Since many types of stem cells have been proposed as potential sources for cellular therapy [27], we examined the effects of FasL treatment on other types of stem cells. Inducible pluripotent cells (iPS) are differentiated cells that undergo an undifferentiation process, and therefore have been suggested as a source for stem cells for therapy. We treated iPS derived MSCs (iPS MSCs) with FasL, and found that treated cells had increased proliferation (Figure 4c) and maintained their differentiation potential (data not shown). As a measure for stemness potential, CFU were tested for bone marrow MSCs (BM MSCs) and Umbilical Cord MSCs (UC MSCs) grown with or without FasL. We found that MSCs grown in the presence of 0.5 ng/ml FasL for only 4 days produced a significant increase of colony compared to control MSCs grown without FasL (Figure 4d). Thus, apoptosis-inducing functional selection is not limited to HSC or ASC, but has the potential to serve as a platform for other types of cells as well.

Discussion

The International Society for Cell Therapy (ISCT) has outlined minimal criteria for defining Mesenchymal Stem Cells (MSCs). They are defined by their capacity for expansion in vitro, by specific surface markers expression profile [28-29] and their ability to adhere to plastic and form colonies (i.e., CFU-F cells). Cells can differentiate along adipogenic, chondrogenic and osteogenic lineages in vitro [30-32]. MSCs have been proposed for cellular therapy due to their immunosuppression properties, ability to migrate to injury sites and ability to differentiate into skeletal tissues. Over 1,000 registered clinical trials have been conducted using MSC for a variety of human pathologies [22]. Unfortunately, the majority of clinical trials have failed due to the non-reproducible efficiency resulting from the lack of distinct criteria for the necessary quality of cells used as a medical product.

To overcome this issue and produce a more functionally homogeneous cell population, cells are propagated in culture with very little means to assess their functionality and differentiation capacity. The main criterion for treatment is the number of cells and not the cells functional capacity. Eventually, most clinical trials fail, probably, due to inconsistency and functionally heterogenous cell populations used [33]. To overcome this problem, it is essential to develop methods and protocols that ensure the cells used in clinical trials are a functionally homogeneous cell population. These protocols could also reduce the length of time cells are maintained in culture, whilemaintaining function and when possible, enhancing proliferation. As a result, protocols should result in a more functionally homogeneous cell population and, consequently, better outcomes for clinical trials.

We have developed a novel method that addresses this crucial need for homogenous progenitor cell populations - Apoptosis-based functional selection technology. When applied to HSCs (the ApoGraft^{TM}) is based on a revolutionary approach, in which a short exposure of heterogenous Bone marrow cells to an

apoptosis-inducing Ex-Vivo environment causes selective cell death in specific sub-populations of differentiated cells, while not harming, stem and progenitor cells and the subsets of mature cells supporting engraftment and Graft Versus Tumor effects [9]. This apoptosis-based functional-selection technology harnesses the cell's differential sensitivity to extrinsic apoptotic signals, namely members of the TNFα family such as FasL. In a previous study, we showed that, while many mature cells die following exposure to CD95 ligands, stem and progenitor cells not only survive, but also receive an activating signal [6, 9]. This dual effect can be explained by the understanding that when mature cells encounter CD95 signaling they undergo apoptosis, while progenitor cells perceive an apoptotic rich microenvironment as a “call for duty”. The net result is an enriched population of undifferentiated stem and progenitor cells, with eventually drive reduction of the toxicity elicited by terminally differentiated cells. This differential mode of action can be explained by a number of mechanisms that may each operate alone or in synergy with each other: 1. mature cells and stem cells differ in their intracellular signaling pathways activated by the same membranal CD95 receptor. 2. Different concentrations and time of exposure to apoptosis-inducing agents (i.e., the strength of the signal) might affect cells differently due to differences in the number of cell surface receptors. The outcome of these effects can clearly be seen when inducing apoptosis in a heterogenous population of cells, as there is a clear correlation between the level of Fas receptor and apoptosis seen in mature subpopulations of hematopoietic cells. For example, in MPBCs who undergo functional selection by ApoGraft^{TM}, some activated T-cells and antigen-presenting cells are almost completely eliminated, while only minimal apoptosis was seen in T regulatory cells.

In the clinical settings, the differential apoptotic sensitivity may be used to eliminate (negatively select) immune hyperactive cells, such as cells responsible for the cytokine storm among CART manipulated cells. Concomitantly, the apoptosis inducing ligands can activate (positive effect) MSCs from various tissues (i.e., BM and adipose) leading to enhanced proliferation. Hence, cells treated by a single triggering molecule, FasL in this context, can result in the combination of these two effects with an outcome of increased numbers and improved purity and activity.

In this study we further support the positive selection effect of our functional selection platform. The need for expansion of MSCs in culture is already impacting their use in autologous transplantation let alone in the much more desired allogeneic transplantation.

The Pharma industry is searching for "off-the-shelf" cellular products. This will certainly increase the demand for efficient and robust expansion processes where the FasL technology may be applicable. We examined the outcomes of FasL treatment on freshly isolated liposuction cells (ASCs), and are now describing FasL’s trophic effects of proliferation, delayed differentiation and improved immunosuppression. We show that FasL treatment of ASCs resulted in improved cell proliferation. In addition, we found that FasL treatment resulting in increased growth rates is associated with improved stemness properties. This is important, as shorter time in culture to achieve desired number of cells which concomitantly preserve the undifferentiated status during the FasL incubation and improved differentiation when FasL is removed, is beneficial for both better reproducibility of the product as well as reduction of manufacturing costs. We show here that this enhanced proliferation did not cause chromosomal aberrations, as the cells maintained a stable karyotype, even in prolonged culture conditions. Treated cells also maintained their critical properties including migration, stemness (as measured by mitochondrial activity) and differentiation potential. Finally, we show that FasL treated ASCs maintains their effect when tested in vivo and can be used as a potential source for treatment of various pathologies, including RA and GvHD response, as assessed in murine in-vivo models.

When comparing mature cells to undifferentiated cells, differences can be found on the protein level (different cell surface markers), on the cellular level (different cellular activity), the apoptotic response (mature cells undergo apoptosis in response to FasL treatment) and on the "stemness" level (the ability to migrate, differentiate and exert immune suppressive effects). Therefore, as mature and progenitor cells differ from each other significantly, they would respond differently to a specific signal. Hence FasL, or any other apoptosis-based functional selection agent, may initiate a process causing cells in culture, depending on their maturity, to respond differently to the same cue.

To address the question whether the apoptosis-based functional selection is limited to MSC originating from bone marrow or from fat ([6] and current study), we examined additional stem cells that have been proposed as potential treatments. Both Inducible pluripotent cells (iPS) and umbilical stem cells have showed great potential as sources for cellular therapy [27]. We showed that both types of cells proliferated faster following treatment with FasL and could differentiate into fibroblast-like colonies. These results highlight the potential use of the ApoGraft platform as an agent to enhance growth of different types of stem cells in culture. The potential use of the platform seems to be of wide range and not limited to a unique cell origin.

Conclusion

Our results presented here, combined with our previous studies, demonstrate that FasL treatment of stem cells is a robust method for proliferating cells in culture while improving their stemness properties. Treatment with FasL results in a large quantity of ASCs of high quality - homogenous undifferentiated cell population with increased differentiation potential. Taken together, the combination of removing cells undergoing differentiation, while improving stemness and enhancing proliferation of the remaining cells, creates an amplification effect. Using this FasL treatment results in large numbers of highly activated homogenous populations. This method holds immense potential for cellular treatment not only with ASCs but has the potential to have a wide range of effects that will assist in functional selection of cells in many cellular models. This will lead to better selection of cells for cellular therapy which may result in more efficient clinical testing and therefore better outcomes of

clinical trial. Additional clinical trials in humans are needed to demonstrate the immense potential of this technology.

Abbreviations

SC: Stem Cell; MSC: Mesenchymal Stem Cells; HSC: Hematopoietic Stem Cells; CT: Cellular Therapy; RM: Regenerative Medicine; GvHD: Graft versus Host Disease; ASC: Adipose derived Stem Cells; TNF: Tumor Necrosis Factor; GSH: Glutathione; IDO: Indoleamine 2,3-Dioxygenase; SVF: Stromal Vascular Fraction; SC: Sub-cutaneous; IP: Intraperitoneal; IV: Intravenous; CFU-F: Colony Forming Unit - Fibroblast; BMT: bone marrow transplantation; MPBCs: mobilized peripheral blood cells; p: passage; iPSC: Induced Pluripotent Stem Cell; UC: umbilical cord; ORC: Oxidative Stress Resistive Capacity

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Conflicts of Interest

The authors declair there is no conflict of interest.

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