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14. ABSTRACT Despite more than three decades of therapies that selectively target the tumor cells, the 5-year survival rate for metastatic ovarian cancer patients remains at less than 30%. New strategies that can change this dismal scenario are urgently needed to improve or complement classical treatments for this malignancy. Targeting the tumor microenvironment (TME) represents one such new therapeutic strategy. Tumors depend on a supportive microenvironment to thrive and disseminate. Experimental clinical strategies that target the ovarian tumor microenvironment are beginning to show some promise. Mesenchymal stem cells (MSCs) are known to reside in TME or tumor stroma. Therefore, gene-modified MSCs that can act as "Trojan horses" and deliver anti-cancer therapeutics into the tumor stroma are being evaluated as a promising new specific cell-based therapy for cancer. We established that MSCs promoted ovarian tumor growth. We have also developed new methodology to induce the standard mixed pool of MSCs into two uniform but distinct phenotypes, <i>MSC1</i> and <i>MSC2</i> . In recent studies we found that when we delivered <i>MSC2</i> into mouse models of ovarian cancer, tumors grew and spread faster. Whereas surprisingly when we delivered <i>MSC1</i> into the mice there was an opposite anti-tumor effect. We do not yet know the mechanisms behind this <i>MSC1</i> mediated inhibition of tumor growth.				
15. SUBJECT TERMS BMSC, bone marrow-derived mesenchymal stem cell, MSC, mesenchymal stem cell, MSC1, pro-inflammatory phenotype, MSC2, anti-inflammatory phenotype				
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INTRODUCTION:

o **Background:** Currently, there are many promising clinical trials using mesenchymal stem cells (MSCs) in cell-based therapies of diseases ranging widely from graft-versus-host to joint and cartilage disorders. Increasingly, however, there is a concern over the clinical use of MSCs because they are also known to home to tumors and once resident in the tumor microenvironment (TME) to support tumor growth and spread. For instance, we established that MSCs in the ovarian tumor microenvironment promoted tumor growth and favored angiogenesis. We also developed new methodology to induce the standard mixed pool of MSCs into two uniform but distinct phenotypes, *MSC1* and *MSC2*. In recent studies we found that *MSC2* supported ovarian cancer growth and spread while surprisingly *MSC1* had an opposite anti-tumor effect. We do not yet know the mechanisms behind this *MSC1* mediated inhibition of tumor growth.

o **Objective/Hypothesis:** Our *long-term goal* is to determine the role that MSCs play in cancer growth and spread in order to design more effective tumor therapies. The *objective here* is to establish whether induction of MSCs into *MSC1* is a feasible new anti-tumor cell-based therapy approach, and to identify the molecular mechanisms behind the *MSC1* mediated anti-tumor effect. Our *central hypothesis* is that *MSC1* will home to the ovarian tumor microenvironment and shift the balance from a tumor promoting stroma to a tumor eradicating one that attenuates tumor growth and spread by influencing the secretion of defined soluble factors and extracellular matrix proteins as well as modifying the host immune response.

o **Specific Aims:**

Aim 1. Determine the effect of MSC-based therapies on ovarian tumor growth and spread.

Aim 2. Determine the anti-tumor mechanisms established by MSC1-treatment of MOSEC mice.

o **Study Design:**

Aim 1. Determine the effect of MSC-based therapies on ovarian tumor growth and spread. We will test the *hypothesis* that *MSC1*-based therapy can effectively attenuate ovarian tumor growth and spread in an established immune competent model (MOSEC) when compared to MSCs- or vehicle-treated animals. MSC engraftment, tumor growth and spread, as well as survival data will be collected.

Aim 2. Determine the anti-tumor mechanisms established by MSC1-treatment of MOSEC mice. The effect on TME and systemic immune and inflammatory responses will be evaluated. Changes in the secretion of inflammatory factors, ECM, and immune cells will be measured from the ovarian TME, ascites, draining lymph nodes and spleen of mice treated with standard MSCs, *MSC1*, or vehicle controls.

o **Impact:**

Our objectives in this TEAL Expansion award study were to show that *MSC1*-based therapy attenuates tumor growth and spread in a murine ovarian cancer model and to identify the specific *MSC1*-therapy driven anti-tumor mechanisms. **This approach has identified anti-tumor MSC1 therapy as a new cancer immunotherapy that safely and effectively switches the tumor-associated immunity from a pro-tumor one to an anti-tumor one able to attenuate cancer and spread.** The mechanisms identified include the enhanced secretion of tumor specific pro-apoptotic TRAIL, the increased secretion of pro-inflammatory factors, tumor associated mast cell degranulation, decreased collagen deposition and increased tumor associated leukocyte infiltration. We have developed a strategy for the next steps to cost-effectively manufacture allogeneic anti-tumor MSC1 products, we have developed diagnostic and quality control assays to measure the identity, purity and potency of our scaled up MSC1 product and have hired consultants to design the pre-clinical FDA-IND studies. We have also submitted complementary grant applications to achieve these goals.

We are well under way in developing a clinical grade anti-tumor MSC1 cancer immunotherapy that we hope to quickly translate into the clinic within 12-18 months post financing. We are very thankful to the DOD and the U.S. Army Medical Research and Materiel Command for funding this award and allowing us to advance our new cancer immunotherapy.

KEYWORDS:

ASC, adipose-derived mesenchymal stem cell
MSC, bone marrow-derived mesenchymal stem cell
CANCER IMMUNOTHERAPY
COX, cyclooxygenase
CTL, cytotoxic T lymphocyte
HLA, human leukocyte antigen
IDO, indoleamine 2,3-dioxygenase
IFN, interferon
IL, interleukin
iNOS, inducible nitric-oxide synthase
IBD, inflammatory bowel diseases
MHC, major histocompatibility complex
MSC, mesenchymal stem cell
MSC1, anti-tumor phenotype
MSC2, anti-inflammatory phenotype
NF, nuclear factor
PGE-2, prostaglandin E2
TGF, transforming growth factor
TLR, toll-like receptor
TNF, tumor necrosis factor
Th1 T helper cell 1
Treg, T regulatory lymphocyte

OVERALL PROJECT SUMMARY:

o **Background:** Currently, there are many promising clinical trials using mesenchymal stem cells (MSCs) in cell-based therapies of diseases ranging widely from graft-versus-host to joint and cartilage disorders. Increasingly, however, there is a concern over the clinical use of MSCs because they are also known to home to tumors and once resident in the tumor microenvironment (TME) to support tumor growth and spread. For instance, we established that MSCs in the ovarian tumor microenvironment promoted tumor growth and favored angiogenesis. We also developed new methodology to induce the standard mixed pool of MSCs into two uniform but distinct phenotypes, *MSC1* and *MSC2*. In recent studies we found that *MSC2* supported ovarian cancer growth and spread while surprisingly *MSC1* had an opposite anti-tumor effect. We were not sure of the mechanisms behind this *MSC1* mediated inhibition of tumor growth.

o **Objective/Hypothesis:** Our *long-term goal* is to determine the role that MSCs play in cancer growth and spread in order to design more effective tumor therapies. The *objective here* was to establish whether induction of MSCs into *MSC1* is a feasible new anti-tumor cell-based therapy approach, and to identify the molecular mechanisms behind the *MSC1* mediated anti-tumor effect. Our *central hypothesis* was that *MSC1* would home to the ovarian tumor microenvironment and shift the balance from a tumor promoting stroma to a tumor eradicating one that attenuates tumor growth and spread by influencing the secretion of defined soluble factors and extracellular matrix proteins as well as modifying the host immune response. This information was gained by these studies and is reported here.

o **Specific Aims:**

Aim 1. Determine the effect of MSC-based therapies on ovarian tumor growth and spread.

Aim 2. Determine the anti-tumor mechanisms established by MSC1-treatment of MOSEC mice.

o **Study Design:**

Aim 1. Determine the effect of MSC-based therapies on ovarian tumor growth and spread. We tested the *hypothesis* that *MSC1*-based therapy can effectively attenuate ovarian tumor growth and spread in an established immune competent model (MOSEC) when compared to MSCs- or vehicle-treated animals. MSC engraftment, tumor growth and spread, as well as survival data was collected.

Aim 2. Determine the anti-tumor mechanisms established by MSC1-treatment of MOSEC mice. The effect on TME and systemic immune and inflammatory responses will be evaluated. Changes in the secretion of inflammatory factors, ECM, and immune cells were measured from the ovarian TME, ascites, draining lymph nodes and spleen of mice treated with standard MSCs, *MSC1*, or vehicle controls.

o **Impact:**

Our objectives in this TEAL Expansion award study were to show that *MSC1*-based therapy attenuates tumor growth and spread in a murine ovarian cancer model and to identify the specific *MSC1*-therapy driven anti-tumor mechanisms. We identified new targets for preventive and therapeutic interventions of ovarian cancer, as well as determined the contributions of stromal components to ovarian cancer growth and spread. We also provide potential mechanisms behind the divergent tumor effects of conventional MSCs and *MSC1*, to shed some light on the growing controversy over whether MSCs are safe in cell-based therapies of human disease. Therefore, these studies laid the groundwork for safe cell-based therapies that inhibit tumor growth and spread that we are currently pursuing following the studies this enabling award allowed us to complete.

BODY:

We have included the study as it was described in our Statement of Work and following each section presented what we have accomplished based on the proposed work.

Statement of Work

Goals and Objectives: Our *long-term goal* is to determine the role that MSCs play in cancer growth and spread in order to design more effective tumor therapies. The *objective here* was to establish whether induction of MSCs into *MSC1* is a feasible new anti-tumor cell-based therapy approach, and to identify the molecular mechanisms behind the *MSC1* mediated anti-tumor effect. Our central hypothesis was that *MSC1* will home to the ovarian tumor microenvironment and shift the balance from a tumor promoting stroma to a tumor eradicating one that attenuates tumor growth and spread by influencing the secretion of defined soluble factors and extracellular matrix proteins as well as modifying the host immune response. *This work has allowed us to identify MSC1 cell therapy as a new cancer immunotherapy for ovarian cancer.*

Task 0. Obtain IACUC and ACURO approval for this project. (before start of project) **COMPLETED**

Task 1. Determine the effect of MSC-based therapies on ovarian tumor growth and spread:

Task 1.A COMPLETED

MSC1 Immunotherapy Attenuated Tumor Growth at All Disease Stages.

The effect of *MSC1*-therapy on tumor growth and spread was evaluated in the syngeneic immunocompetent, murine ovarian surface epithelium carcinoma (MOSEC) model. This model is considered “a transplantable, tumorigenic, murine ovarian carcinoma disease model that recapitulates the progression of the human disease, including slow progression, the development of late-stage ascites, and, importantly, immune cell recruitment and accumulation” [30]. This model was chosen as a starting point because cell-based therapies had a measureable impact on tumor growth and spread, as well as provided valuable information on host immune responses we also want to assess for *MSC1*-based therapies [31, 32]. Based on our preliminary work and the literature, we anticipated that *MSC1* would home to the tumor microenvironment, and shift the TME from tumor supportive to cancer eradicating.

Fig. 1. Optimized Anti-tumor *MSC1* Treatment Regimen

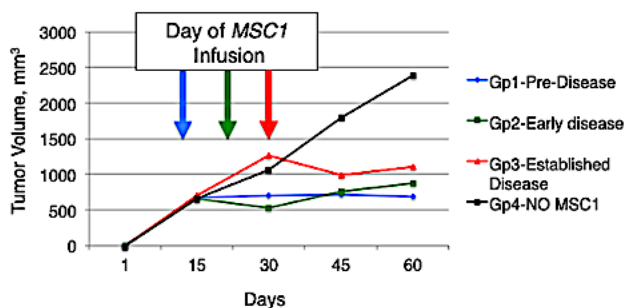


Fig.1. *MSC1* Immunotherapy Attenuated Tumor Growth at All Disease Stages. The murine ovarian surface epithelium carcinoma (MOSEC) inoculated mice or MOSEC model was treated intraperitoneally (IP) with a single injection of anti-tumor *MSC1* as indicated by the arrows ↓ with colors matching their corresponding group. MOSEC mice were infused with *MSC1* (1×10^6 cells), or vehicle (Gp4). The first group of MOSEC mice had *MSC1* treatment at day 15 (pre-clinical disease). Group 2 was treated with *MSC1* at day 20 (early disease). Group 3 was treated at day 30 (established disease). Mice were monitored every other day until termination at day 60. Tumor growth was measured at weekly intervals until day of mouse sacrifice (Day 60). Harvested tumors and metastasis were weighed, counted and processed. Accumulated ascites was collected, measured, and a sample was spun on cytopsin slides and stained by DiffQuick cytology stain by standard methods. Gp. 1 (2 mL), 2 (1.5 mL) and 3 (5mL) had accumulated some ascites. Gp4 the untreated control animals had more ascites(>10mL) and enlarged spleens, respectively. The average $\pm$ SEM is reported in fig. with at least 5 mice per treatment group. The average $\pm$ SEM is reported in fig. with at least 5 mice per treatment group.

The MOSEC mice were treated intraperitoneally (IP) with a single injection of anti-tumor *MSC1* as indicated by the arrows ↓ with colors matching their corresponding group. MOSEC mice were infused with *MSC1* (1×10^6 cells), or vehicle (Gp4) Fig. 1. The first group of MOSEC mice had *MSC1* treatment at day 15 (pre-clinical disease). Group 2 was treated with *MSC1* at day 20 (early disease). Group 3 was treated at day 30 (established disease). Mice were monitored every other day until termination at day 60. Tumor growth was measured at weekly intervals until day of mouse sacrifice (Day 60). Harvested tumors and metastasis were weighed, counted and processed. Accumulated ascites was collected, measured, and a sample was spun on cytopsin slides and stained by DiffQuick cytology stain by standard methods.

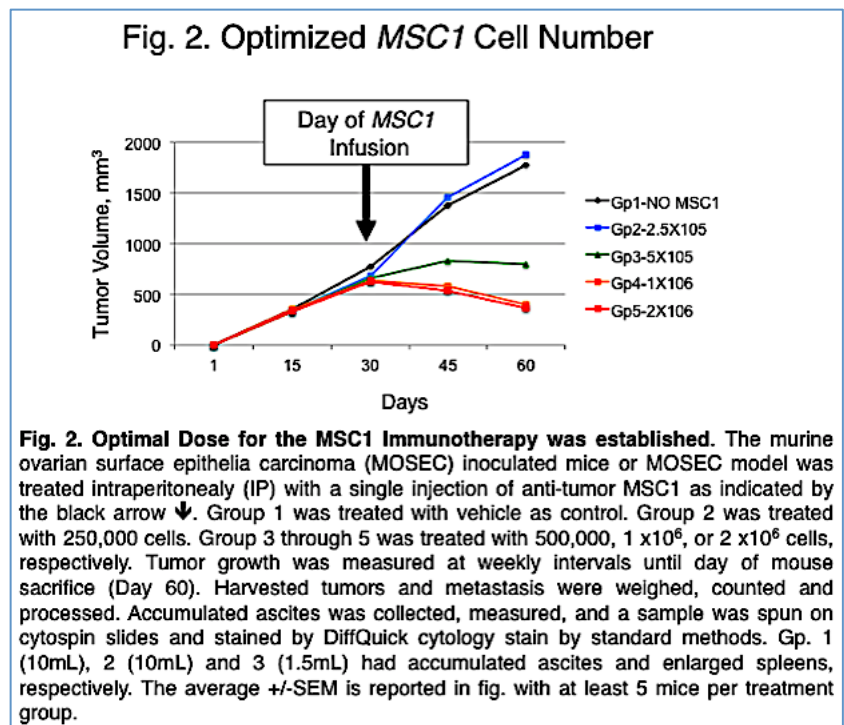
Gp. 1 (2 mL), 2 (1.5 mL) and 3 (5mL) had accumulated some ascites. Gp4 the untreated control animals had more ascites (>10mL) and enlarged spleens, respectively. The average +/-SEM is reported in the figure with at least 5 mice per treatment group.

From these experiments, we learned that although the earlier the treatment the better, our MSC1 immunotherapy attenuated cancer growth regardless of disease stage given Fig. 1. This is encouraging from a clinical standpoint because it means that the therapy will be effective regardless of staging.

Task 1.B COMPLETED

Optimal Dose for the MSC1 Immunotherapy was established.

Next we turned to establishing the optimal treatment dose for the MSC1 immunotherapy Fig. 2. Again the MOSEC mice were treated intraperitoneally (IP) with a single injection of anti-tumor MSC1 as indicated by the black arrow ↓. Group 1 was treated with vehicle as control. Group 2 was treated with 250,000 cells. Group 3 through 5 was treated with 500,000, 1×10^6 , or 2×10^6 cells, respectively. Tumor growth was measured at weekly intervals until day of mouse sacrifice (Day 60). Harvested tumors and metastasis were weighed, counted and processed as before. Also the accumulated ascites was collected, measured, and a sample was spun on cytopsin slides and stained by DiffQuick cytology stain by standard methods. Gp. 1 (10mL), 2 (10mL) and 3 (1.5mL) had accumulated ascites and enlarged spleens, respectively.



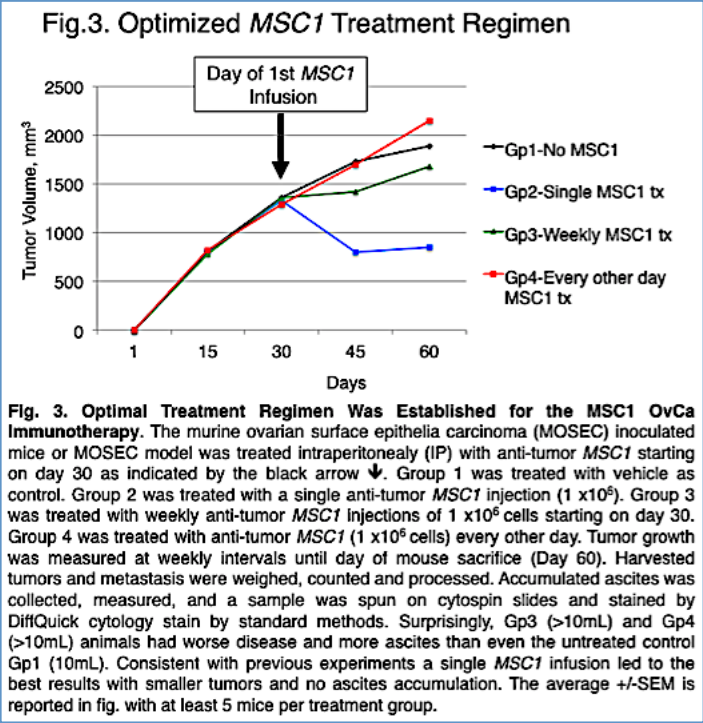
We found that a dose greater than 500,000 cells/animal was the most effective with about 1 million cells per animal being the most effective. Unexpectedly, the highest dose was

Optimal Treatment Regimen To Attenuate Tumor Growth Was Established for the MSC1 OvCa Immunotherapy

The dosing regimen was studied next to determine the optimal method for the MSC1 treatment Fig. 3. The MOSEC mice were treated intraperitoneally (IP) with anti-tumor *MSC1* starting on day 30 as indicated by the black arrow ↓. Group 1 was treated with vehicle as control. Group 2 was treated with a single anti-tumor *MSC1* injection (1×10^6). Group 3 was treated with weekly anti-tumor *MSC1* injections of 1×10^6 cells starting on day 30. Group 4 was treated with anti-tumor *MSC1* (1×10^6 cells) every other day. As before, tumor growth was measured at weekly intervals until day of mouse sacrifice (Day 60). Harvested tumors and metastasis were weighed, counted and processed. Finally, accumulated ascites was collected, measured, and a sample was spun on cytopsin slides and stained by DiffQuick cytology stain by standard methods.

From this set of experiments we learned that surprisingly, Gp3 (>10mL) and Gp4 (>10mL) animals had worse disease and more ascites than even the untreated control Gp1 (10mL). Consistent with previous experiments a

single *MSC1* infusion led to the best results with smaller tumors and no ascites accumulation.



Task 1.C COMPLETED

*Optimal Treatment Regimen Was Established for the *MSC1* OvCa Immunotherapy*

Lastly, we set out to test whether the optimized treatment regimen would be consistent regardless of MSC donor. We tested five different donors following priming by the optimized treatment regimen Fig. 4. Again the MOSEC inoculated mice were treated intraperitoneally (IP) with anti-tumor *MSC1* starting on day 30. Group 1 was treated with vehicle as control. Group 2 was treated with a single injection of unprimed naïve MSCs (1×10^6) as our optimized dose and regimen experiments indicated. Group 3 was treated with a single injection of anti-tumor *MSC1* (1×10^6 cells) starting on day 30. Tumor growth was measured as before, at weekly intervals until day of mouse sacrifice (Day 60). Tumor tissue and ascites were collected and processed for analysis as

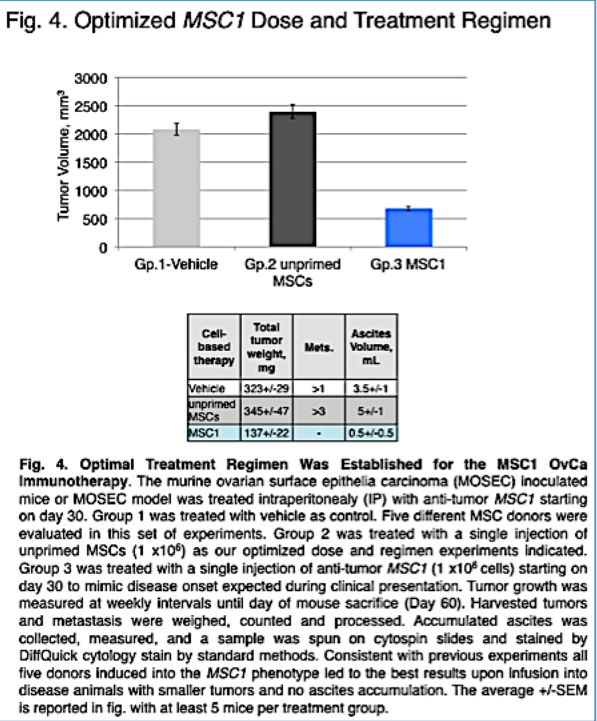
previously indicated.

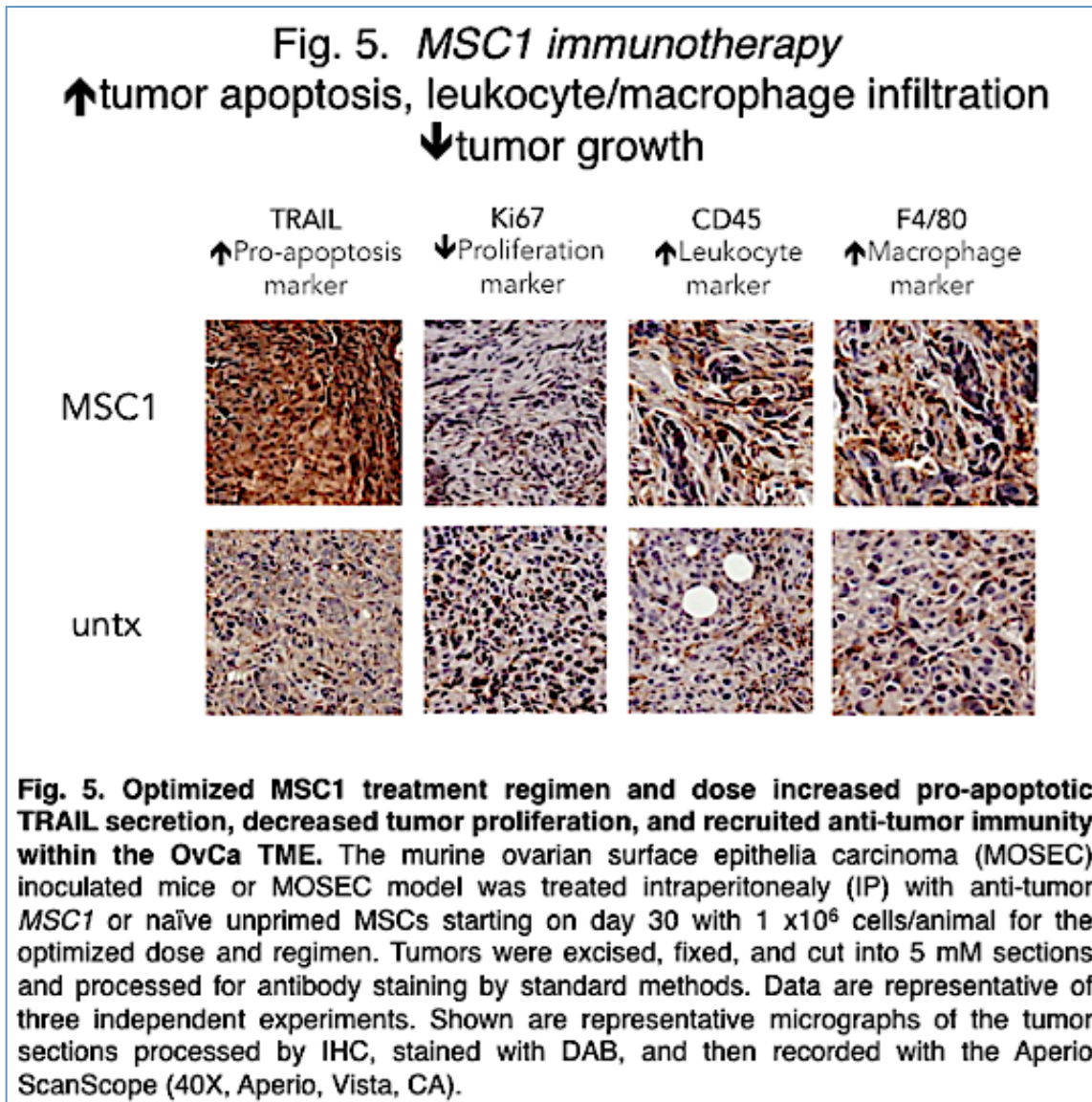
Consistent with previous experiments all five donors induced into the *MSC1* phenotype led to the best results upon infusion into disease animals with smaller tumors and no ascites accumulation. Notably, all donors tested resulted in the expected results that supports the notion that this *MSC1* immunotherapy will be able to be effective even when scaled to satisfy clinical use. Furthermore, as expected, the *MSC1* immunotherapy resulted in 30-50% tumor reduction and few if any metastasis when compared with the MSC-treated MOSEC mice.

Task 2. Determine the anti-tumor mechanisms established by *MSC1*-treatment of MOSEC mice.

Task 2. COMPLETED

In this aim we studied the changes at the molecular level imposed by the MSC-based therapies of MOSEC within the tumor microenvironment and on overall host immune responses. Based on our preliminary work and the literature, we expected the *MSC1* immunotherapy to promote an anti-tumor microenvironment [2]. Initially we identified changes in the secretion of inflammatory factors, ECM proteins, and immune cells measured from the ovarian TME, ascites, draining lymph nodes, and spleen of mice treated with pre-labeled standard MSCs, *MSC1*, or vehicle controls. With the potential aim for this approach to identify new targets for preventive and therapeutic interventions of ovarian cancer, as well as to determine the contributions of stromal components to ovarian cancer growth and spread.





*Optimized *MSC1* treatment regimen and dose increased pro-apoptotic TRAIL secretion, decreased tumor proliferation, and recruited anti-tumor immunity within the OvCa TME*

The MOSEC mice were treated with the optimal therapeutic regimen of the *MSC1* immunotherapy established in Task 1. Next the tumor tissues and ascites were collected and processed as described before to examine the inflammatory and ECM components driving the therapeutic benefit of *MSC1*. For this part, tumors were excised, fixed, and

cut into 5 mM sections and processed for antibody staining by standard methods. As we had noted before the increased expression of pro-apoptotic TNF-related apoptosis-inducing ligand, TRAIL, in *MSC1* treated MOSEC cells we repeated this here Fig. 5. We also examined the proliferation capacity of the treated tumors when compared with untreated tumor samples by measuring the nuclear Ki67 staining. Lastly, we measured the leukocyte (CD45) and macrophage (F4/80) infiltration of the tumor microenvironment by immunohistochemistry (IHC).

As the representative micrographs demonstrate the optimized *MSC1* therapeutic regimen led to increased pro-apoptotic TRAIL expression, decreased tumor cell proliferation (Ki67) and increased infiltration of CD45 positive and F4/80 positive macrophage inflammatory cells. These results suggest that the *MSC1* immunotherapy is indeed shifting the tumor microenvironment from a pro-tumor one to anti-tumor immunity.

*Bioactive Factor Secretion by the *MSC1* treated MOSEC Suggest Mechanism of the Immunotherapy*

We also measured the cytokines, chemokines, and other secreted bioactive factors (Table 1, [13,20]). In these experiments we have no means of distinguishing which cell; MSC or cancer, is contributing the bioactive factors, we can simply detect their net expression. *MSC1*-treated samples elaborated higher levels of pro-inflammatory factors including IL17, IL3, MIG, MIP1 β , and GM-CSF whereas *MSC*-treated samples had

marked increases in IL1RA, IL10, CXCL1, CCL5 and CXCL10 (Table 1). Interestingly, as before we saw TRAIL expression was dramatically induced in *MSC1*-treated samples when compared to untreated ones. By contrast, the expression of GM-CSF, LIF, and TRAIL was attenuated in these samples when compared to *MSC1*-treated ones.

Table 1. Bioactive Factor Secretion by the MSC1 treated MOSEC

Bioactive Factor	<i>MSC1</i>	<i>Naïve unprimed MSCs</i>
IL1RA	↓	↑↑
IL3	↑↑	↓
IL10	-	↑
IL12p40	↓	↑
IL17	↑↑	↑
CXCL1 (Groα)	↓	↑↑
CXCL10 (IP10)	↓	↑↑
CCL5 (RANTES)	-/↓	↑↑
MIG	↑	-
MIP1β	↑	↑↑
GM-CSF	↑↑	↓
HGF	↓	-
LIF	↑↑	↓
TRAIL	↑↑	↓

Table 1. Bioactive Factor Secretion by the MSC1 treated MOSEC. Treated tumor samples were analyzed by Bio-Plex Cytokine Assays following the manufacturer's instructions (Human Group I & II; Bio-Rad, Hercules, CA). Arrows represent relative normalized changes compared with unstained controls.

A New Role for Mast Cells and ECM in the TME Discovered by the MSC1 Immunotherapy of MOSEC

Mast cells (MCs) are known to affect the extracellular membrane (ECM) proteins, yet another component of the TME important to tumor growth and spread [23,24,30,38]. Thus, the changes in mast cells and collagen (ECM) levels among the MSC-treated tumor groups were measured Figure 6. We used a proteoglycan-specific stain (safranin O-fast green) to help visualize the mast cells (MCs) found within the MSC-treated tumor sections Figure 6. MCs are immune cells that are increasingly implicated in tumor growth, spread, and aggressiveness [22]. The metastatic potential of tumors is affected by the composition of the tumor associated extracellular matrix (ECM). MCs are known to promote ECM protein deposition and are associated with various human ECM disorders [23,24]. Lastly, MCs are also known to interact with MSCs [3,25]. Although we did not observe

obvious differences in the number of safranin O positive mast cells in each of the MSC-treated MOSEC samples, there appeared to be differences in the stained granules within the MCs among them. Specifically, while naïve unprimed-treated tumor sections appeared to contain mostly safranin O-positive granule laden MCs, *MSC1*-treated tumor sections contained mostly MCs that appeared degranulated (insets of Figure 6). We also noted that the MCs were distributed mostly throughout the stromal fibrovascular compartments of all tumors where they may also be acting to affect the ECM. This association was further implicated by comparison of the safranin O stained sections with those of the Verhoeff-Van Gieson (VVG) collagen stained sections, which revealed mast cells concentrated in areas with the darkest pink/red collagen stained regions Figure 7 and 8.

Fig. 6. Anti-tumor *MSC1* therapy affects mast cell degranulation in the TME

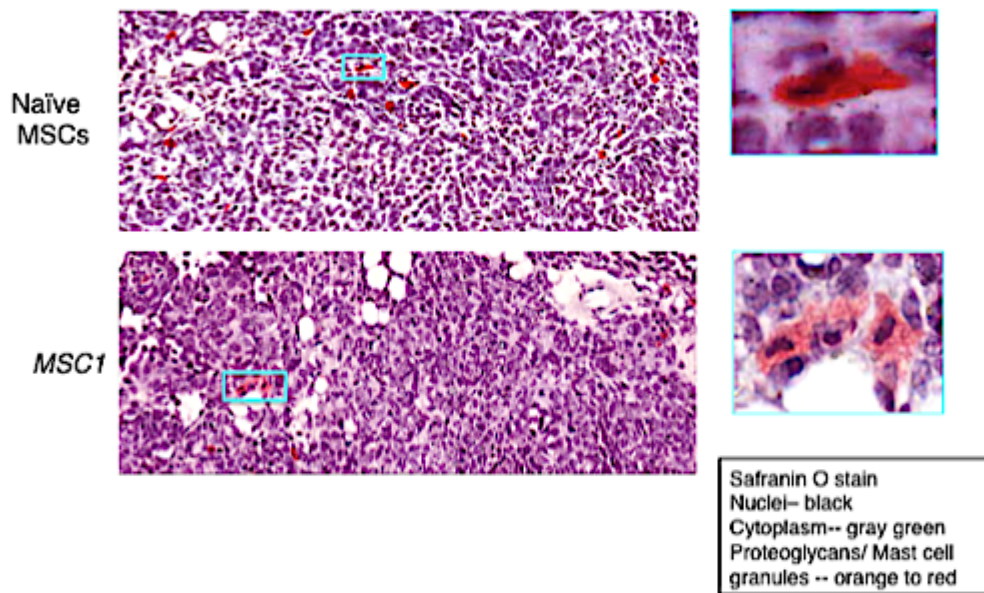


Fig. 6 Anti-tumor *MSC1* therapy affects mast cell degranulation in the TME. MOSEC tumors were established in C57BL/6 mice for 4 weeks. Naïve MSCs (control) or *MSC1* (1×10^6 in 0.5mL HBSS) were infused IP at day 30 and the mice were harvested after 65 days. Tumors were excised, fixed, and cut into 5 mM sections by standard methods [7]. Sections were processed for safranin O proteoglycan staining (www.ihcworld.com). Representative micrographs of MSC-treated tumor sections are included from images obtained from the Aperio ScanScope (200X, Aperio, Vista, CA). The expected color for each tissue element is described in the inset on the lower right hand side. 400X images are included in boxed insets. Data are representative of three independent experiments with at least 6 mice per treatment group.

Unexpectedly, we observed the opposite effect of *MSC1* on collagen levels (*in vivo*) than we previously reported for *MSC1* induction alone (*in vitro*) [13]. VVG stained tumor sections from *MSC1*-treated groups had less dark pink/red areas than the other samples, whereas *in vitro* *MSC1* had the greatest expression of collagen compared to the other samples. These differences may be explained by direct *in vivo* interactions between the MSCs and MCs that were recently discovered and that would be present in the TME but lacking in the *in vitro* setting [25].

From this set of experiments then we have demonstrated how *MSC1*-based therapy leads to attenuated tumor growth and spread. We found that *MSC1* shifts the ovarian TME from a pro-tumor to anti-tumor by increased secretion of IL6, IL8, IFN γ , reduced IL10 and TGF β , deposition of collagen ECM, increased mast cell degranulation, increased secretion of pro-apoptotic TRAIL, and lastly, inhibition of cancer growth (Ki67). We expect that the immunotherapy with *MSC1* will safely and effectively attenuate ovarian tumor growth and spread. The next steps are to manufacture a clinical grade *MSC1* product and complete all of the FDA IND enabling studies to initiate the first in man clinical trials of *MSC1* immunotherapy in ovarian cancer.

Fig. 7. *MSC1*-treated tumor samples have diminished levels of collagen

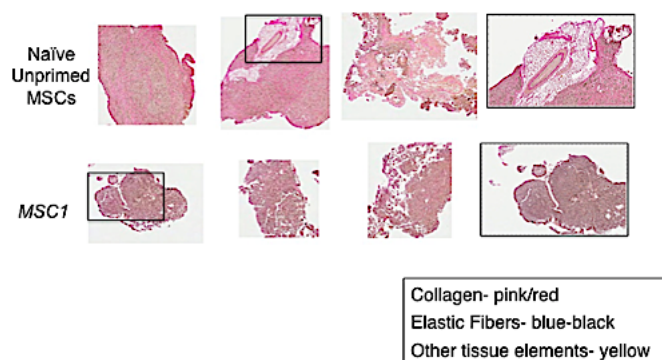


Fig. 7. *MSC1*-treated tumor samples have diminished levels of collagen within the TME compared with MSC-treated tumor groups. MOSEC tumors were established as before. Naïve unprimed MSCs or *MSC1* were infused IP by the optimized treatment regimen. Tumors were excised, fixed, and cut into 5 mM sections by standard methods [7]. Sections were processed for Verhoeff-Van Gieson (VVG) elastic fiber/collagen staining (www.ihcworld.com). Representative micrographs of several MSC-treated tumor sections are included from images obtained from the Aperio ScanScope (40X, Aperio, Vista, CA). The expected color for each tissue element is described in the inset on the lower right hand side. 80X images are included in boxed insets. Data are representative of three independent experiments with at least 6 mice per treatment group.

Fig. 8. Co-localization of tumor associated mast cells with collagen

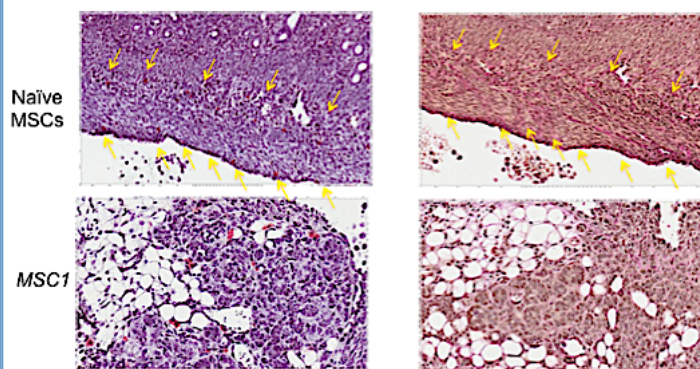


Fig. 8. Co-localization of tumor associated mast cells with collagen. MOSEC tumors were established as before. MSCs or *MSC1* treatments followed optimized regimen and dose as indicated before. Tumors were excised, fixed, and cut into 5 mM sections by standard methods [7]. Sections were processed for Verhoeff-Van Gieson (VVG) elastic fiber/collagen staining (left panels) or for safranin O proteoglycan staining (right panels, www.ihcworld.com). Representative micrographs of several MSC-treated tumor sections are included from images obtained from the Aperio ScanScope (40X, Aperio, Vista, CA). yellow arrows indicate comparable sections among the tumor tissue sections. Data are representative of three independent experiments with at least 6 mice per treatment group.

Development of a Diagnostic Multiplex qPCR Assay

In anticipation of the need to standardize the *MSC1* preparation during scale-up and clinical grade production we have developed multiplex qPCR assays with 5 genes and 4 miRNA that can predict with a high confidence interval (>99%) the anti-tumor potency of each *MSC1* preparation Figure 9. During each *MSC1* preparation we extract miRNA and RNA from a working cell stock and have designed specific primers to evaluate the levels of 5 unique genes and 3 miRNAs. This methodology will soon be submitted as a patent application and thus cannot disclose all of the genes and miRNAs. We will develop this assay as a new diagnostic kit for others interested in using our improved MSC technology. One of the genes used is revealed here since it is relevant to the goal of this work: trying to understand the anti-tumor mechanism of *MSC1* therapy. We are pursuing TRAIL as a key molecule in this process.

Fig. 9. Development of multiplex qPCR Assays

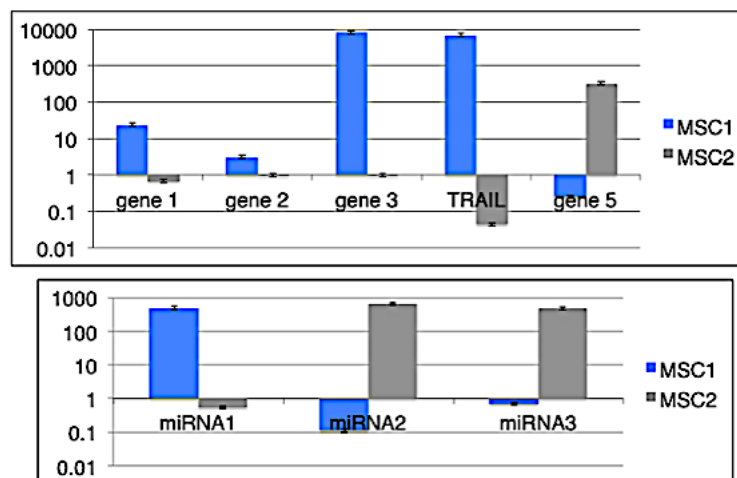


Fig. 9. Development of QC Multiplex qPCR Assay for Primed MSCs. Essential to the translation of our therapy to the clinic is having well characterized parameters to ensure lot-to-lot consistency of the primed *MSC1* cell products. We developed a multiplex qPCR assay based on our previous DOD funded studies that identified key differentially expressed miRNA and mRNA molecules among the primed and naïve MSCs. This included expression of *trail* mRNA as reported here.

Our objective here is to develop QC tests that ensure safe and consistent efficacy of our expanded and banked clinical grade anti-tumor *MSC1* immunotherapy for every manufacturing run. Additionally, this will help overcome one of the industry hurdles of inconsistent efficacy from mixed pools of MSC products.

Proposed FDA Target Product Profile for the MSC1 Immunotherapy of Ovarian Cancer

We have also begun to develop the parameters that we would propose to the FDA for designation of our MSC1 Immunotherapy of Ovarian Cancer as an Investigational New Drug (IND) Figure 10. Based on this work and other DOD funded work we have established sufficient parameters that ensure a safe and consistent MSC1 therapeutic for ovarian cancer. Our hypothesis is that we will be able to develop a battery of tests that ensures safety and efficacy of our MSC1 immunotherapy product. The proposed *MSC1* specification tests are based on our DOD funded pre-clinical experience that served to initially identify and describe the MSC1 phenotype and also modifications of those tests used by other industry leaders. We feel that the sum of this work will soon lead to a new ovarian cancer immunotherapy that is safe and effective unlike any other to this point.

Fig. 10. Target Product Profile Expected for MSC1 Immunotherapy Cell Products

Assay Description	MSC1 Specification	Comments
Cell Surface Marker Analysis	>90% positive: CD90, CD105, CD44, CD166, CD29 <10% positive: CD34, CD45, CD14, CD11b	Standard ISCT profile
Cytokine Profile	>5000pg/mL IL6 and IL8	By ELISA
qRT-PCR Assay (proprietary)	>2 fold change in 4 genes relative to unprimed MSCs >2 fold change in 5 miRNAs relative to unprimed MSCs	A proprietary multiplex qPCR assay and miRNA profile has been developed for characterization of MSC1
Potency Assay Mixed lymphocyte T cell activation Assay	<50% suppression of T cell activation	Standard In vitro T-cell activation assay
Viability	≥70%	
Sterility Test	Negative	USP <71>
Endotoxin	< 5.0 EU/mL	Use vendor method
Mycoplasma	Negative	Use vendor method

Fig. 10. Target Product Profile Expected for MSC1 Immunotherapy Cell Products. Expected parameters to define MSC1 immunotherapy cell products for FDA-IND enabling purposes.

KEY RESEARCH ACCOMPLISHMENTS:

We were the first to show that:

- Human bone marrow derived mesenchymal stem/progenitor cells (MSCs) can be induced into a more homogeneous and predictable anti-tumor *MSC1* phenotype.
- We have now consistently tested the anti-tumor *MSC1* cell therapy in a relevant immune COMPETENT mouse model of ovarian cancer.
- Studies in this project have now determined the optimal cell dose, time and regimen for the anti-tumor *MSC1* cell therapy.
- We have determined that the *MSC1* therapy can be advanced as a new type of safer tumor-specific cancer immunotherapy since the therapy switches the tumor immunity to an anti-tumor one without eliciting other non-tumor host immune responses.
- We have determined that the *MSC1* therapy drives tumor cell death through increased TRAIL secretion, pro-inflammatory factor secretion, tumor-associated mast cell degranulation, decreased collagen deposition and enhanced leukocyte recruitment to the tumor microenvironment.
- Complementary efforts to develop this therapy for the clinic are underway with my recent start-up WibiWorks Therapeutics, Inc., we have developed a diagnostic kit in anticipation of scaling up and manufacture of the *MSC1* cell therapy.
- We have submitted SBIR proposals that will help quickly translate the anti-tumor *MSC1* therapy from the lab to the clinic.

CONCLUSION:

In this TEAL Expansion Award project:

We have pre-clinically evaluated a new cell-based MSC therapy for ovarian cancer that has the potential to shift the ovarian tumor microenvironment from a pro-tumor one to a tumor eradicating one. There are over 20 ongoing or completed clinical trials that have established that mesenchymal stem cell (MSC)-based therapies are safe and effective in the treatment of many human diseases. MSCs derived from various *adult* tissues naturally track to inflamed sites and help to heal these sites by their anti-inflammatory properties. Both self (autologous) and non-self (allogeneic) MSC-based therapies are confirmed as safe and effective. As a result, many new and existing businesses are developing off-the-shelf allogeneic MSC-based products for the treatment of a wide-ranging set of human diseases including cancer. In this proposed work we will gain information and collect evidence for an improved method (phenotype induction) to prepare and deliver anti-tumor MSCs (*MSC1*) to the ovarian cancers.

We have identified the molecular details behind the contributions of tumor-resident MSCs to ovarian cancer growth and spread.

This approach has identified anti-tumor MSC1 therapy as a new cancer immunotherapy that safely and effectively switches the tumor-associated immunity from a pro-tumor one to an anti-tumor one able to attenuate cancer and spread. The mechanisms identified include the enhanced secretion of tumor specific pro-apoptotic TRAIL, the increased secretion of pro-inflammatory factors, tumor associated mast cell degranulation, decreased collagen deposition and increased tumor associated leukocyte infiltration. We have developed a strategy for the next steps to cost-effectively manufacture allogeneic anti-tumor MSC1 products, we have developed diagnostic and quality control assays to measure the identity, purity and potency of our scaled up MSC1 product and have hired consultants to design the pre-clinical FDA-IND studies. We have also submitted complementary grant applications to achieve these goals.

We are well under way in developing a clinical grade anti-tumor MSC1 cancer immunotherapy that we hope to quickly translate into the clinic within 12-18 months post financing to our start-up WibiWorks Therapeutics (figures below). We are very thankful to the DOD and the U.S. Army Medical Research and Materiel Command for funding this award and allowing us to advance our new cancer immunotherapy.

Current Cancer Immunotherapies				
	Drug	Drug target	Clinical Indication	Side Effects
DC therapy	Provenge	DCs	Prostate Cancer	Chills, fatigue, fever, back pain, nausea, joint ache, and headache
Vaccine/T-cell therapy	IMA901	CTLs, Th1	Ovarian cancer	Fever, injection site tenderness
Antibody/ "checkpoint" Inhibitors	Ipilimumab , Tremelimumab , Nivolumab , MK-3475	CTLA-4-CD80/86 PD-1 PD-L1	Solid tumors and leukemias	On-target, off tumor - T cell toxicities
Tregs inhibitors	Dacizumab , Basiliximab	Tregs	Breast cancer	Auto-immunity potential
MSC1	proimunoceTM	TRAIL T regs DCs M1 macrophages Mast cells	Solid organ cancers and leukemias	Possible IV injection site tenderness, possible fever; NO T-cell toxicities or auto-immunity potential

Key features of anti-tumor MSC1 immunotherapy		
Features	Benefits	Bottom Line
Specific MSC1 migration to tumors	• TUMOR-TARGETED THERAPY	• Few side effects or off-target effects
Expression of pro-apoptotic tumor specific TRAIL	• SELECTIVE TUMOR KILL	• Few side effects or off-target effects
Potent and specific immune modulation ↑ Tu-Ag CTLs, ↓ Tregs, ↑ Mast cells and ↑ M1 MΦ	• SELECTIVE AND COORDINATED REAWAKENING OF ANTI-TUMOR IMMUNITY	• Absence of systemic related toxicities or unleashed auto-immunity
Unique TLR priming technology	• UNIFORM PRODUCT ENSURES EFFICACY	• SAFE Therapy • FIRST in CLASS opportunity

Ready to begin IND enabling studies



REPORTABLE OUTCOMES:

Publications:

1. Yang M, Stapor PC, Peirce SM, **Betancourt, A.M.** and Murfee, W.L. (2012) Rat Mesentery Exteriorization: A Model for Investigating the Cellular Dynamics Involved in Angiogenesis. J Vis Exp. 2012:e3954.
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Book Chapters:

1. **Aline M. Betancourt** and Ruth S. Waterman (2012). The Role of Mesenchymal Stem Cells in the Tumor Microenvironment, Tumor Microenvironment and Myelomonocytic Cells, Subhra K. Biswas (Ed.), ISBN: 978-953-51-0439-1, InTech, Available from: <http://www.intechopen.com/books/tumor-microenvironment-and-myelomonocytic-cells/the->

role-of-mesenchymal-stem-cells-in-the-tumor-microenvironment

2. **Aline M. Betancourt**. New Cell-Based Therapy Paradigms: Polarization of Bone Marrow-Derived Multipotent Stromal Cells into Pro-inflammatory (MSC1) and Anti-Inflammatory (MSC2) Phenotypes. *Advances in Biochemical Engineering/Biotechnology*, "Mesenchymal stem cells - origin and characteristics, functions and perspectives for clinical use" M. Dominici ed, **2012**, 1-35, DOI: 10.1007/10_2012_141 Springer Publishing Co.

Presentations:

- Building Towards a Standard for MSCs: a UK-US (NIH)-Canada-led Workshop Bethesda 2013
- 6th International Symposium on Mesenchymal Stem/Progenitor Cells Texas 2013
- MSC1: A new cancer immunotherapy, San Diego 2014

Submitted Research Support:

GRANT11542059 (Betancourt)	07/01/2014-03/31/2015	6.0 calendar
NIH PA-13-223	\$441,488.00	

The First Anti-Inflammatory Mesenchymal Stem Cell-Based Therapy for Pain

The *overall goal of this Phase I study* is to generate "proof of principle" data validating *MSC2* as a consistent anti-inflammatory therapy that safely improves disease outcomes in established murine models of pain. Further, by specifically acting on the afflicted systems many of the adverse effects seen with conventional systemic anti-inflammatory treatments will be avoided with this cell-based therapy. The *aims* we propose in pursuit of this goal are: 1. Determine the efficacy of *MSC2* over conventional treatments in murine models of pain, and 2. Determine the mechanisms behind decreased pain severity in *MSC2*-treated mice.

GRANT11538344 (Betancourt)	07/01/2014-03/31/2015	6.0 calendar
NIH PA-13-223	\$460,438.00	

Anti-Tumor Mesenchymal Stem Cell (MSC1)-Based Immunotherapy

At the end of the 9-month project we intend to have expanded and banked clinical grade *MSC1*, as well as to have tested the ability of the clinical grade *MSC1* to arrest tumor growth and spread in immune competent murine models of cancer. The *aims* we propose are: 1. Expand and bank clinical grade *MSC1*. Wibi+Works, LLC has hired an established and certified cGMP facility for MSC manufacturing and 2. Test the efficacy of clinical grade *MSC1* cell-based therapy in a murine ovarian cancer model. This study is the next logical step from our published work that demonstrated attenuated tumor growth and spread with laboratory grade *MSC1* in the same pre-clinical animal model.

RT3-07729 (Betancourt)	01/01/2015-12/31/2018	6.0 calendar
CIRM Tools and Technology RFA 13-05	\$900,000	

A Disruptive Technology & A New Tool: Scaled-up anti-inflammatory mesenchymal stem cells (MSC2) for the treatment of pain & identification of a robust potency assay for MSCs

There are significant hurdles to the immediate clinical translation of cell therapies. One major hurdle is large-scale manufacture of uniform well-characterized cells. Another is developing potency assays that can accurately predict the clinical effect that the products will have within the patient. Our goal is to tackle these hurdles with our two proposed aims. Aim 1: use our new *disruptive technology* that renders mesenchymal stem cell (MSC) preparations consistently uniform by inducing them into a discrete anti-inflammatory phenotype (*MSC2*). Aim 2: we propose to work along with Dr. Yaksh to identify a high throughput, robust murine model of pain that can accurately test the *in vivo* potency of any MSC.

Next Generation Safe and Effective Anti-Inflammatory Cell Therapy for Inflammatory Bowel Diseases

This proposal represents the next logical step in our goal of producing the first anti-inflammatory *MSC2*-based therapy for Crohn's disease with the translation from our research grade to clinical grade manufacture of *MSC2*. Our objective is to show that scaled up clinical grade *MSC2* therapy attenuates disease in established models of Crohn's disease like the research grade *MSC2* and to identify the mechanisms behind the therapeutic benefit that may also help find other therapeutic targets. Moreover, this cell therapy, by mainly acting at the affected sites, will avoid many of the adverse effects seen with other systemic anti-inflammatory treatments.

INVENTIONS, PATENTS AND LICENSES:

Nothing to report

REPORTABLE OUTCOMES:

This approach has identified anti-tumor MSC1 therapy as a new cancer immunotherapy that safely and effectively switches the tumor-associated immunity from a pro-tumor one to an anti-tumor one able to attenuate cancer and spread. The mechanisms identified include the enhanced secretion of tumor specific pro-apoptotic TRAIL, the increased secretion of pro-inflammatory factors, tumor associated mast cell degranulation, decreased collagen deposition and increased tumor associated leukocyte infiltration. We have developed a strategy for the next steps to cost-effectively manufacture allogeneic anti-tumor MSC1 products, we have developed diagnostic and quality control assays to measure the identity, purity and potency of our scaled up MSC1 product and have hired consultants to design the pre-clinical FDA-IND studies. We have also submitted complementary grant applications to achieve these goals.

We are well under way in developing a clinical grade anti-tumor MSC1 cancer immunotherapy that we hope to quickly translate into the clinic within 12-18 months post financing to our start-up WibiWorks Therapeutics, Inc. We are very thankful to the DOD and the U.S. Army Medical Research and Materiel Command for funding this award and allowing us to advance our new cancer immunotherapy.

OTHER ACHIEVEMENTS:

Nothing to report

REFERENCES:

(PMCID numbers follow references)

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Personnel that worked on the study include graduate student Derek Dashti and post-doctoral fellow Dr. Shuguang Wang, PhD

2 reprints follow- PLoS ONE paper and review with MSC1 26 pages

Mesenchymal Stem Cell 1 (*MSC1*)-Based Therapy Attenuates Tumor Growth Whereas *MSC2*-Treatment Promotes Tumor Growth and Metastasis

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Abstract

Background: Currently, there are many promising clinical trials using mesenchymal stem cells (MSCs) in cell-based therapies of numerous diseases. Increasingly, however, there is a concern over the use of MSCs because they home to tumors and can support tumor growth and metastasis. For instance, we established that MSCs in the ovarian tumor microenvironment promoted tumor growth and favored angiogenesis. In parallel studies, we also developed a new approach to induce the conventional mixed pool of MSCs into two uniform but distinct phenotypes we termed *MSC1* and *MSC2*.

Methodology/Principal Findings: Here we tested the *in vitro* and *in vivo* stability of *MSC1* and *MSC2* phenotypes as well as their effects on tumor growth and spread. *In vitro* co-culture of *MSC1* with various cancer cells diminished growth in colony forming units and tumor spheroid assays, while conventional MSCs or *MSC2* co-culture had the opposite effect in these assays. Co-culture of *MSC1* and cancer cells also distinctly affected their migration and invasion potential when compared to MSCs or *MSC2* treated samples. The expression of bioactive molecules also differed dramatically among these samples. *MSC1*-based treatment of established tumors in an immune competent model attenuated tumor growth and metastasis in contrast to MSCs- and *MSC2*-treated animals in which tumor growth and spread was increased. Also, in contrast to these groups, *MSC1*-therapy led to less ascites accumulation, increased CD45+leukocytes, decreased collagen deposition, and mast cell degranulation.

Conclusion/Significance: These observations indicate that the *MSC1* and *MSC2* phenotypes may be convenient tools for the discovery of critical components of the tumor stroma. The continued investigation of these cells may help ensure that cell based-therapy is used safely and effectively in human disease.

Citation: Waterman RS, Henkle SL, Betancourt AM (2012) Mesenchymal Stem Cell 1 (*MSC1*)-Based Therapy Attenuates Tumor Growth Whereas *MSC2*-Treatment Promotes Tumor Growth and Metastasis. PLoS ONE 7(9): e45590. doi:10.1371/journal.pone.0045590

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Competing Interests: Aline M. Betancourt is the inventor of the patent-pending "Mesenchymal Stem Cells and Related Therapies" (*MSC1* and *MSC2* methodology) US 61/391,749. Aline M. Betancourt is Founder and Chief Financial Officer for wibi+works, LLC without any salary, benefit or income. As inventor of the patent stated above there are no interests, products, benefits, compensations, or salary. Ruth S. Waterman is the Chief Executive Officer for wibi+works, LLC without any benefit, salary, or income. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Mesenchymal stem cells (MSCs, more accurately termed multipotent mesenchymal stromal cells) are increasingly being used in cell-based therapies of diseases ranging widely from graft-versus-host to joint and cartilage disorders [1,2]. There are many features that make these cells attractive and practical for use in human therapy. First, MSCs are easily obtained from various adult-derived tissues, quickly expanded, and stored *ex vivo* without significant impact to their capabilities. Second, once reintroduced, MSCs preferentially home to sites of injury or inflammation and support healing and repair mostly through the local secretion of bioactive factors and modulation of immune cells. Third, MSCs

from non-self (allogeneic) or self (autologous) donors can be used safely since they do not elicit harmful immune responses within the recipient host. Lastly, pre-clinical studies have demonstrated efficacy with MSCs genetically engineered to carry various therapeutics that reached their target with significant treatment benefit even in the xenogeneic setting (human cells to mouse host) (recently reviewed [3–5]).

Despite these promising features, there is a growing concern over the clinical use of MSCs since they are also known to home to tumors and once resident in the tumor microenvironment (TME) to support tumor growth and spread [4–8]. Conversely, other studies have reported that MSCs found in the TME diminish tumor growth, which has further generated some controversy in

this field (reviewed in [4,5]). Other noted concerns in the clinical use of MSCs, is the fact that we still do not have a general consensus of what defines them, and furthermore although one of their most profound clinical effects upon intravenous administration is the modulation of host immune responses, we do not yet truly understand all of their consequences upon introduction into the host [1,9,10]. Either way, as a result of the established clinical properties of MSC and their added propensity for the TME, modified MSCs that can act as “Trojan horses” and deliver anti-cancer therapeutics into the tumor stroma are being evaluated as a promising new targeted cell-based therapy for cancer [4,5].

MSCs targeted to cancers are expected to contribute many soluble factors such as mitogens, extracellular matrix (ECM) proteins, angiogenic, and inflammatory factors, as well as exosomes or microvesicles, once resident in the TME [3–5]. MSCs are also expected to affect tumor-associated leukocytes either directly by cell-cell contact or indirectly by the secretion of trophic factors [3–5]. MSCs are known to affect the proliferation and differentiation of dendritic cells, monocytes/macrophages, B and T cells, NK cells, and even mast cells [3–5]. Many reasons have been advanced to explain the contradictory MSC role in cancer including but not limited to the heterogeneity of MSC preparations, the age or health of the MSC donor, and the experimental model or condition [3–5].

Our group established that MSCs in the ovarian tumor microenvironment promoted tumor growth and favored angiogenesis [7,11,12]. We also developed new methodology to induce the conventional mixed pool of MSCs into two uniform but distinct phenotypes, *MSC1* and *MSC2* [13]. These phenotypes were recently and successfully tested in the therapy of a mouse model of painful diabetic peripheral neuropathy [14]. This study also demonstrates the stability of these newly defined phenotypes in cell-based treatment of an immune competent disease model. We initially based their classification on several parallel observations reported within the monocyte literature. Like MSCs, heterogeneous bone marrow-derived monocytes respond to stress or “danger” inflammatory signals and home to tissue injury. Monocyte polarization into the classically activated pro-inflammatory macrophages (M1) occurs early on in tissue repair, whereas monocyte polarization into alternatively activated macrophages (M2) follows later to help in tissue injury resolution [15,16]. Although, this is a very simplified view of what occurs in the complex process of wound healing and repair, it provides a convenient paradigm to begin to dissect critical components within this complex biological process [17–19].

In this study, we similarly took advantage of this convenient paradigm in MSCs as a way to potentially resolve some of the controversy surrounding the complex role of MSCs in cancer. Indeed, *MSC1* and *MSC2* were found to have divergent effects on cancer growth and metastasis by *in vitro* and *in vivo* methods. In our experiments, *MSC1* primarily had an anti-tumor effect, whereas *MSC2* promoted tumor growth and metastases. We suggest that further investigation of these cells may provide some guidance in designing safer and more efficacious MSC-based therapies.

Results

MSC1 do not Support *in vitro* Tumor Cell Growth Whereas *MSC2* Favor Tumor Cell Growth

To further extend our studies on the role of MSCs and ovarian tumors we initially investigated the effect of the recently described *MSC1* and *MSC2* phenotypes on various cancer cell lines [7,12,13,20]. The effect of MSCs, *MSC1*, or *MSC2* on the growth of various cancer cell lines was determined by traditional 2D-

colony forming units (CFU) and 3D- tumor spheroid formation assays (Figure 1). Please note that the ratio of cancer cells to MSCs used was 10 to 1 respectively. As expected co-culture with MSCs led to more breast (MDA-MB-231), pancreas (PANC-1) and ovarian (OVCAR, SKOV3, MOSEC) cancer cell colonies and larger tumor spheroids compared to untreated controls (Fig. 2A, B Figures S1 and S2, and data not shown). By contrast, *MSC1*-cancer co-culture consistently led to fewer colonies and much smaller tumor spheroids. Each cancer cell line exhibited their own unique morphology when grown in the CFU and tumor spheroids. It is expected that at a 10:1 cancer cell to MSC ratio the body of the colonies and spheroids are primarily composed of the cancer cells. This is supported by the observed unique morphologies recorded for each cancer cell line treated with the MSCs. *MSC2* co-culture resulted in the greatest number of CFUs and largest spheroids. We noted that typically the MSCs and *MSC2* co-cultures led to bigger and more diffuse colonies and spheroids whereas the *MSC1* resulted in smaller, tighter, and more compact CFUs and tumor spheroids. CellTracker green labeled MSCs and *MSC2* in the tumor spheroid assays mostly distributed throughout the spheroids (Figure S2). These *in vitro* assays' results suggest that MSCs and *MSC2* support tumor cell growth whereas *MSC1* seem to diminish tumor cell growth.

We also measured the cytokines, chemokines, and other bioactive factors secreted into the medium by the MSC-cancer cell co-cultures as before (Table 1, [13,20]). In these experiments we have no means of distinguishing which cell; MSC or cancer, is contributing the bioactive factors, we can simply detect the net effect of the co-culture conditions used here. SKOV3 ovarian cancer cells were plated on 24-well plates until they reached 50–70% confluence. *MSC1*, *MSC2*, (25,000 cells/insert) or medium control were then added into 0.4 μ M (no cancer cell-MSC contact) or 8 μ M transwell inserts and the co-cultures were allowed another 72 hr prior to collecting the conditioned medium and testing by BioPlex assay. *MSC1*-treated samples elaborated higher levels of pro-inflammatory factors including IL17, IL3, MIG, MIP1 β and GM-CSF whereas *MSC2*-treated samples had marked increases in IL1A, IL10, CXCL1, CCL5 and CXCL10 (Table 1). Interestingly, TNF-related apoptosis-inducing ligand (TRAIL) expression was dramatically induced in *MSC1*-treated co-cultures when compared to *MSC2*-treated ones. By contrast, the expression of GM-CSF, LIF, and TRAIL was attenuated in *MSC2*-treated samples when compared to *MSC1*-treated ones. We observed similar trends when we sampled the biofactor secretion from the 3D tumor spheroid co-cultures (data not shown).

Migration and Invasion of Cancer Cells Following MSC Phenotype Co-culture

We next examined the effect on the migration and invasion capabilities of these cancer cells following co-culture with the MSCs, *MSC1*, and *MSC2*. Similar to the previous report that conventionally derived MSCs promote MDA-MB-231 breast cancer cell migration and invasion [8], we also found that migration and invasion was promoted by MSCs and *MSC2* but not by *MSC1* (Figure 2). We observed about a two-fold increase in both migration and invasion assays by MSCs and *MSC2* co-culture (Figure 2A and B, respectively). In our experiments, all MSCs were added at a 10:1 ratio of cancer cells to MSC as before. We tested the effect of co-culture of the cells plated in traditional 2D dishes 72 hr prior to placing the dissociated cells within the transwell inserts. We also tested the effect of the MSCs on the 3D tumor spheroids grown cancer cells after subsequent dissociation and loading in transwell inserts for these assays (Figure 2A and B). We

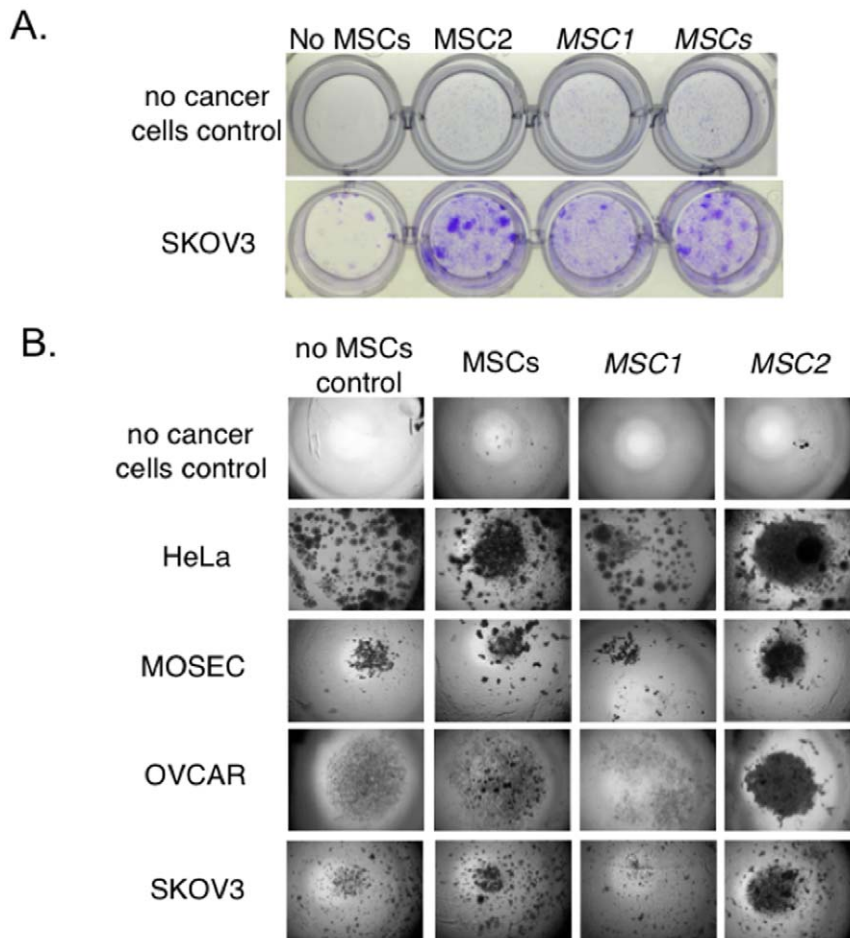


Figure 1. *MSC1* do not support tumor cell growth whereas *MSC2* favor tumor cell growth. **A.** Representative micrographs from colony forming units (CFU) assays performed by culturing human tumor cells (200 cells/well) mixed with MSCs, *MSC1*, or *MSC2* (2 cells/well) at a ratio of 10 cancer cells per 1 MSC and plated in 24-well plates in growth medium supplemented with 10% FBS as indicated in figure. Cultures were grown for 14 days at 37°C in a humidified atmosphere of 5% carbon dioxide balance air. Growth medium was changed every 3–4 days. Colonies were visualized by staining with a crystal violet solution (0.5% crystal violet/10% ethanol). The resulting colonies were enumerated by the colony counting macro in ImageJ software, SKOV3- ovarian adenocarcinoma cell lines. Colony counts are given below the micrographs. Data are representative of at least three independent experiments with at least four MSC donors. **B.** Representative micrograph of tumor spheroids formed by culturing tumor cells (200 cells/well) mixed without any other cells (–) or with MSCs, *MSC1*, or *MSC2* (20 cells/well) at a ratio of 10 cancer cells per 1 MSC and plated over 1.5% agarose in 96-well plates in growth medium supplemented with 10% FBS as indicated in figure. Cultures were grown for 14 days at 37°C in a humidified atmosphere of 5% carbon dioxide balance air. Growth medium was changed every 3–4 days. Micrographs shown represent 20X magnified field of the 96-well plate. Cancer cell lines used are: HeLa-human cervical adenocarcinoma, OVCAR-human ovarian adenocarcinoma, SKOV3-human ovarian adenocarcinoma, and MOSEC-murine ovarian surface epithelium carcinoma cells. Data are representative of at least three independent experiments with at least four MSC donors.
doi:10.1371/journal.pone.0045590.g001

recorded similar effects by the MSCs on the invasion and migration of the cancer cells regardless of culturing conditions.

Additionally, the effect of the MSCs, *MSC1*, and *MSC2* in these assays does not appear to correlate with their expression of matrix metalloproteinases (MMPs, Figure 2C and D). We consistently measured increased expression of several *mmps* following MSCs induction into the *MSC1* or *MSC2* phenotypes (Figure 2C). We also observed elevated secretion of activated MMP2 (MMP2*) into the condition medium of co-cultures of *MSC1* and *MSC2* with SKOV3 when compared with medium from cultures with MSCs or SKOV3 alone (Figure 2D). Although, these levels were slightly lower than those of the MSCs samples without cancer cell co-cultures (Figure 2D). These results indicate that the distinct MSC-mediated effects on cancer migration and invasion are more complex and perhaps not directly mediated by MMP2* in agreement with the studies of the report described earlier [8].

MSC1 Attenuate Tumor Growth Whereas *MSC2* Promote Tumor Growth and Metastasis

The anti-tumor *MSC1* and the pro-tumor *MSC2* *in vitro* effects were further supported in pilot studies with human ovarian cancer xenograft animal models treated with the MSC-based therapies as previously established ([7] and data not shown). We subsequently used the immune *competent* MOSEC model to verify these MSC-tumor effects (Figure 3, [21]). The tumors were established in the mice with 1×10^7 MOSEC (ID8) cells. After approximately 4 weeks a single dose of CellTracker fluorescently labeled human MSCs, *MSC1*, or *MSC2* (1×10^6 /per mouse) were injected IP. The small amount of remaining MSCs preparations within the syringes were again plated and observed for contamination and subsequent growth properties. No change was noted among these spent MSC preparations in growth properties even after 2-weeks of culture.

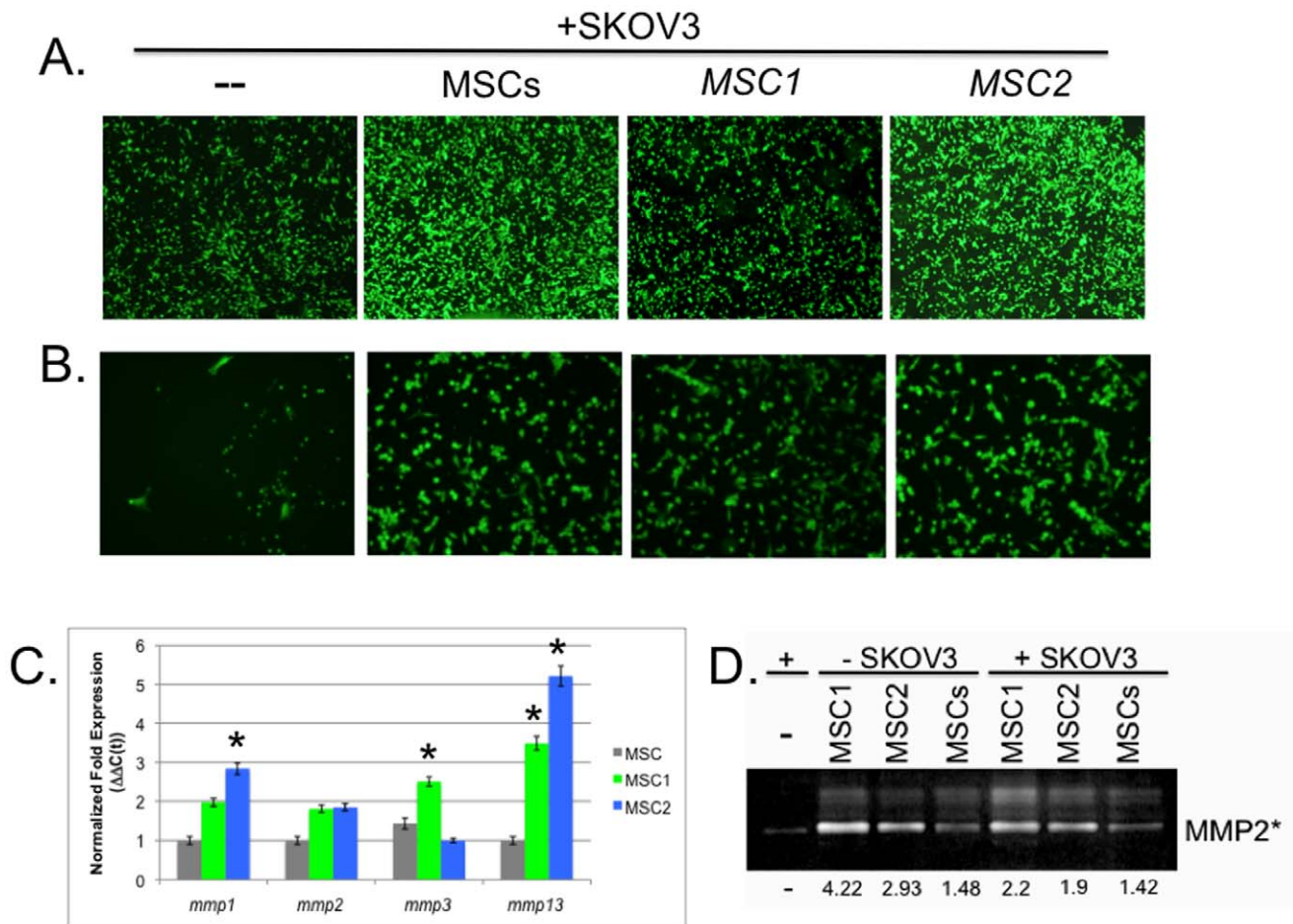


Figure 2. Migration and Invasion of Cancer Cells following MSC phenotype co-culture. Transwell migration and matrigel invasion assays were performed with 3 μ M Falcon fluoroblok transwell inserts as described previously [12,20,45]. MSCs were added at a 10:1 ratio of SKOV3 to MSC. These were co-cultured on traditional 2D dishes 72 hr prior to placing the dissociated cells within the transwell inserts. Representative micrographs of **A.** transwell migrating and **B.** matrigel invading cells were visualized and obtained on an inverted fluorescence microscope (A. 100X and B. 200X, Olympus, MetaMorph analysis software). Data are representative of duplicates in at least three independent experiments. **C.** Representative bar graph of quantitative real-time PCR (qPCR) assays carried out as previously described [39]. Gene expression of *mmps* among the MSC samples is expressed by the normalized cumulative threshold method ($\Delta\Delta C(t)$). * $P < 0.05$ versus the normalized values for MSC. Statistically significant differences were not measured among the other samples. Samples were run in triplicate for at least four different MSC donors. **D.** Representative micrograph following gelatin zymography of the condition medium from MSC-SKOV3 co-cultures (1:10) or SKOV3 and MSC samples cultured alone as indicated for 72 hr. Bands are of pro-MMP2 (72 kDa) and active MMP2* (62 kDa). The numbers below micrograph are the fold changes relative to SKOV3 alone sample obtained following densitometric analysis (ImageJ). Data are representative of at least three independent experiments. doi:10.1371/journal.pone.0045590.g002

Following 24 hr after the MSC-based treatments, one animal was sacrificed per treatment group to measure MSC engraftment to the primary tumor. All MSC-treated samples had similar detectable pre-labeled fluorescence MSCs within the tumor tissue trending towards more *MSC1* and *MSC2* measured than MSCs with approximately 15–25 cells versus 10–15 counted per 200X field after 24 hr of MSC-treatment (data not shown). Based on the literature and our previous experiments, MSC-based therapy of the tumor typically results in very little engraftment ($<0.5\%$) or local proliferation of MSCs at the tumor site [10,14]. Tumor growth was measured at weekly intervals until day of mouse sacrifice (Day 65). At harvest, the ascites accumulated in the tumor bearing mice was collected. The tumors and metastases were measured and processed for flow cytometry and IHC analyses [7].

The collected ascites samples were spun down on cytospin slides and stained with Diff Quick (Figure 3B). Notably, very little (<0.5 mL/mice) to no ascites accumulated in *MSC1*-treated

animals compared with MSCs- (1.25 mL/mice) and *MSC2*-treated (>5 mL/mice) animals (Figure 3C). Furthermore, *MSC2*-treated animals had the most tumor cell aggregates within the ascites followed by the MSC-treated samples, with few tumor aggregates found in *MSC1*-treated sample ascites (Figure 3B). In parallel, the tumor size and weights were biggest in *MSC2*-treated (~ 1500 mm³ and 375 mg) animals followed by MSCs-treated animals (~ 1000 mm³ and 283 mg) and *MSC1*-treated animals (~ 500 mm³ and 167 mg, Figure 3A and C). Metastasis was found only in MSCs- and *MSC2*-treated mice.

Tumor-associated Leukocytes Differ among the MSC-treated Groups

Flow cytometry and IHC analyses of harvested tumors demonstrated some interesting differences dependent upon the MSC-treatments (Figure 4). Based on both CD45+ flow cytometry and IHC analyses *MSC1*-treated groups appeared to have the

Table 1. Ovarian cancer cells co-cultured with *MSC1* differ from *MSC2* co-cultures in their secretion of bioactive factors.

Bioactive Factor	Contact dependent effect	<i>MSC1</i>	<i>MSC2</i>
IL1RA	–	↓	↑ ↑
IL3	–	↑ ↑	↓
IL10	+	–	↑
IL12p40	–	↓	↑
IL17	+	↑ ↑	↑
CXCL1 (Groα)	–	↓	↑ ↑
CXCL10 (IP10)	+	↓	↑ ↑
CCL5 (RANTES)	+	–/↓	↑ ↑
MIG	+	↑	–
MIP1β	+	↑	↑ ↑
GM-CSF	+	↑ ↑	↓
HGF	+	↓	–
LIF	–	↑ ↑	↓
TRAIL	+	↑ ↑	↓

SKOV3 ovarian cancer cells were plated on 24-well plates until they reached 50–70% confluence. *MSC1*, *MSC2*, (25,000 cells/insert) or medium control were then added into 0.4 μM (no cell-cell contact) or 8 μM transwell inserts and the co-cultures were allowed another 72 hr prior to collecting the conditioned medium and testing by Bio-Plex Cytokine Assays following the manufacturer's instructions (Human Group I & II; Bio-Rad, Hercules, CA). Arrows represent relative normalized changes compared with the SKOV3 alone control. Biofactor levels that were different between the MSCs grown in 0.4 μM (no cell-cell contact) versus 8 μM transwell inserts are represented by “+.” Those biofactor levels that were similar in both sample groups are represented by “–.” Data are representative of triplicate measurements with 4 MSC donors in at least 4 independent experiments.

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greatest recruitment of leukocytes to the TME compared to the other treatment groups (Figure 4A and B). *MSC2*-treated groups also had an increased number of tumor-associated CD45+leukocytes compared to *MSC*-treated groups. Representative micrographs of the ImageJ threshold analysis with CD45+cells colorized red demonstrate these differences (Figure 4A). Additionally, *MSC1*-treated groups had elevated levels of F4/80+ leukocytes (likely macrophages) compared to *MSC*- and *MSC2*-treated groups as determined by flow cytometry (Figure 4C). The *MSC*s-treated groups had the most tumor-associated neutrophils (~35%) whereas *MSC1*-treated groups had more monocytes (~40%) and *MSC2*-treated groups had close to equivalent numbers of neutrophils, monocytes and lymphocytes (~20%/each) based on differential flow cytometry analyses with specific antibodies to CD3, CD4, CD8, CD11b, CD45R, Ly-6G (Gr-1), and NKG2D (CD314) (http://phenome.jax.org/db/q?rt=projects/docstatic&doc=Jaxpheno6/Jaxpheno6_Protocol).

Next, we used a proteoglycan-specific stain (safranin O-fast green) to help visualize the mast cells (MCs) found within the *MSC*-treated tumor sections (Figure 5). MCs are immune cells that are increasingly implicated in tumor growth, spread, and aggressiveness [22]. The metastatic potential of tumors is affected by the composition of the tumor associated extracellular matrix (ECM). MCs are known to promote ECM protein deposition and are associated with various human ECM disorders [23,24]. Lastly, MCs are also known to interact with *MSC*s [3,25]. Although we did not observe obvious differences in the number of safranin O

positive mast cells in each of the *MSC*-treated groups, there appeared to be differences in the stained granules within the MCs among them. Specifically, while *MSC*- and *MSC2*-treated tumor sections appeared to contain mostly safranin O-positive granule laden MCs, *MSC1*-treated tumor sections contained mostly MCs that appeared degranulated (insets of Figure 5). We also noted that the MCs were distributed mostly throughout the stromal fibrovascular compartments of all tumors where they may also be acting to affect the ECM (Figures S3 and S4). These results indicate that the anti-tumor *MSC1*-effects and the pro-tumor *MSC2*-effects may be mediated by differences in their ability to distinctly affect various tumor-associated leukocytes as well as directly or indirectly affect the ECM content of the tumor microenvironment.

Discussion

The novel finding of this study is that mesenchymal stem cells (multipotent stromal cells, *MSC*s) induced into the *MSC1* phenotype attenuate cancer cell growth while *MSC*s induced into the *MSC2* phenotype mostly mimic conventional *MSC*s in promoting cancer cell growth and spread. Additionally, that once the *MSC1* and *MSC2* phenotypes are induced and reintroduced they appear to lead to distinct tumor effects. In another complementary study, we similarly tested for the stability of the induced phenotypes and their distinct therapeutic effects in a murine model of pain [14].

Recently, a shadow has been cast over the successful and increasing use of *MSC*-based therapies in many diseases, by the growing controversy of whether the *MSC*s used in the treatment might promote tumor growth as some preclinical studies, including ours, suggest [7]. By contrast, others have argued that *MSC*s attenuate tumor growth and spread. However, most agree that as a result of the propensity of *MSC*s to home to tumors, these cells used in cell therapies of cancer provide ideal cancer drug delivery vehicles [4–6,26]. In this study, we present evidence that might shed some light over these controversies and that may provide some guidance in the design of safer *MSC*-based therapies.

We extended our work on *MSC*s and ovarian cancer, as well as our study describing a new approach for the induction of *MSC*s into a pro-inflammatory *MSC1* and an immunosuppressive *MSC2* phenotype. Accordingly, we chose to focus our investigation on the distinct effect that *MSC1* and *MSC2* might have on tumor growth and spread compared to the established one with conventionally prepared *MSC*s [7,13]. Our initial *in vitro* experiments demonstrated that *MSC1* co-culture with various cancer cells diminished their capacity to form colonies in contrast to growth promoting *MSC*- or *MSC2*-co-cultures (Figure 1 and Figures S1 and S2). This effect remained constant even when tested by 3D tumor spheroid models. In this study we only tested cancer cells derived from solid organ tumors and not from leukemia or other blood-related malignancies. We also used *MSC* to cancer cell ratios of 1:10 throughout the study to more closely resemble the proportions that might be achieved in the clinic with *MSC*-based therapies and different to the 1:1 ratios used by other *MSC* and cancer studies (e.g. [8,27–29]).

*MSC*s targeted to cancers are expected to contribute many bioactive factors once resident in the TME, such as mitogens, extracellular matrix (ECM) proteins, angiogenic, and inflammatory factors, as well as exosomes or microvesicles. *MSC*s are also expected to affect tumor-associated leukocytes either directly by cell-cell contact or indirectly by these secreted factors [3]. Most of these parameters were measured in this study. We previously reported that there were differences among several of these

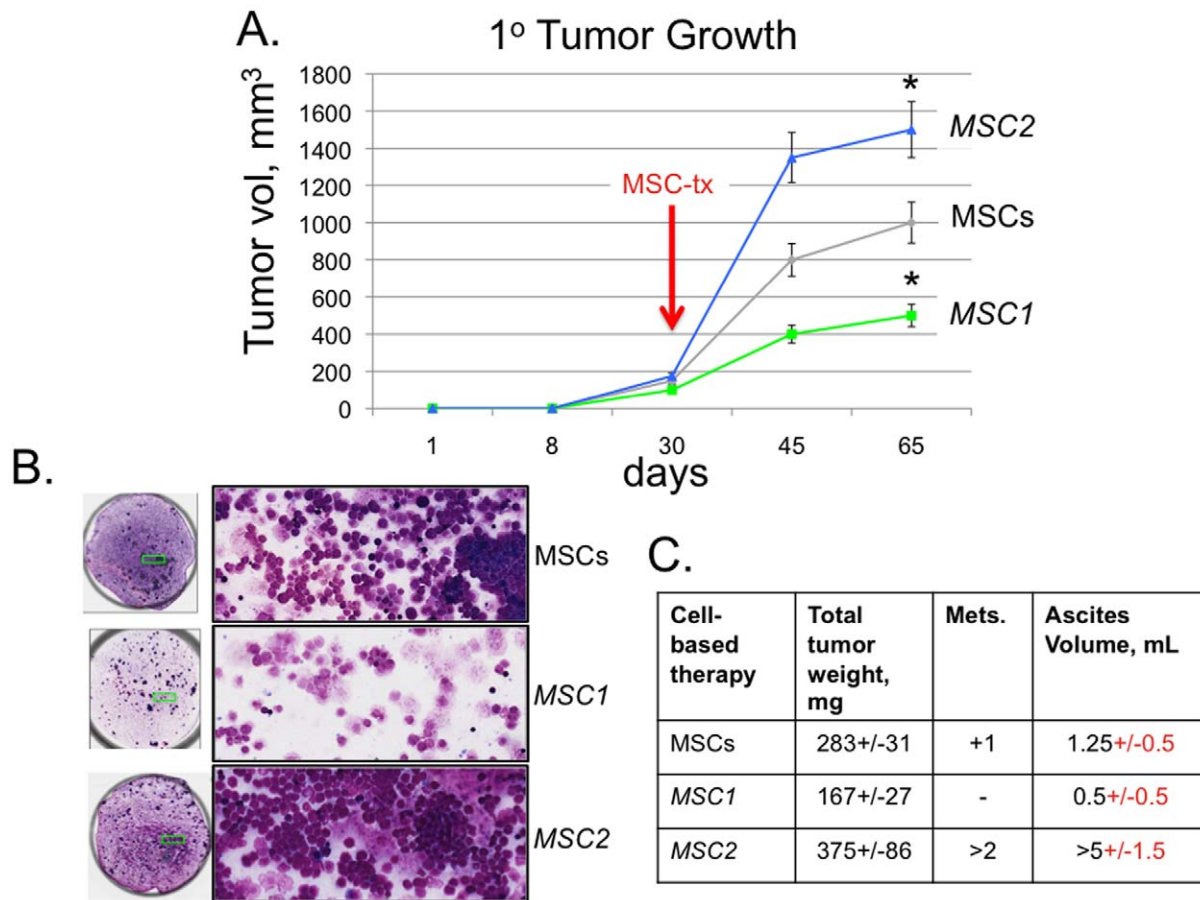


Figure 3. *MSC1* do not support tumor growth whereas *MSC2* favor tumor growth and metastasis. The established syngeneic mouse model for epithelial ovarian cancer used is based upon a spontaneously transformed mouse ovarian surface epithelial cell (MOSEC) line ID8 that has been previously described [21]. At approximately 4 weeks a single dose of human MSCs (MSCs), *MSC1*, or *MSC2* (1×10^6 /per mouse) were injected intraperitoneally (IP) as indicated by red arrow. **A.** Tumor growth was measured at weekly intervals until day of mouse sacrifice (Day 65). Harvested tumors and metastasis were weighed, counted and processed for flow cytometry and immunohistochemical analysis (IHC). * $P < 0.05$ versus the MSCs-treated tumors. **B.** Accumulated ascites was collected, measured, and a sample was spun on cytospin slides and stained by DiffQuick cytology stain by standard methods. Left circles are representative micrographs of cytospin slides (20X) with enlarged areas to the right marked by green box (100X). **C.** Table of average $\pm$ SEM results among the different MSC-treatment groups. Data are representative of three independent experiments with at least 6 mice per treatment group.
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secreted bioactive factors following the induction of MSCs into *MSC1* and *MSC2* [13]. Co-cultures of these MSC phenotypes with the cancer cells also reflected distinct effects on the secreted factors as summarized on Table 1. Both contact-dependent and independent effects were observed. Increases measured in the levels of CCL5 (RANTES) secreted by the pro-tumor *MSC2* groups are in agreement with previous reports [8,13]. By contrast, *MSC1* treatment groups had elevated levels of IL17, GM-CSF, and TRAIL that would suggest an overall inflammatory and pro-apoptotic effect by these cells. *MSC2* treatment groups also had elevated levels of secreted IL1RA, IL10 and most chemokines tested, which suggests a net tumor supportive immunosuppressive effect by this treatment group [27]. However, it is important to recognize that the expression of bioactive factors is by necessity a dynamic process, quickly changing at any given time and place and probably confined to communication across short intercellular distances. We are also not able to distinguish the source be it MSC or cancer cell of the factors elaborated in our established co-culture experimental conditions. Furthermore, what we are able to measure with the current technology is one snapshot of time and

thus it must be accordingly weighed and validated with other supportive experiments prior to drawing too many conclusions.

To this end, transwell migration and matrigel invasion capabilities were also studied (Figure 2). However, though we measured fewer migrating and invading cells for the *MSC1* sample groups compared to the other MSC sample groups, we could not attribute this difference to decreased expression of activated MMP2. Additionally, we have not been able to detect significant levels of either the zymogen or active forms of MMP9 in MSC phenotype *in vitro* cultures or co-cultures with cancer cells. These results are intriguing given the documented importance of MMP2 and 9 in tumor spread and invasion [30]. Further studies are needed to investigate this complex tumor process and how the MSCs might affect it.

Following these *in vitro* experiments, we next investigated the effects of the MSC-based therapies in an immune competent mouse model of ovarian cancer that has been useful in similar studies [21,31,32]. Since the most prevalent effect of MSC-based therapy reported in human clinical trials appears to be immune modulation, and the profile of bioactive factors primarily expressed by MSCs are immune modulatory, we thought it

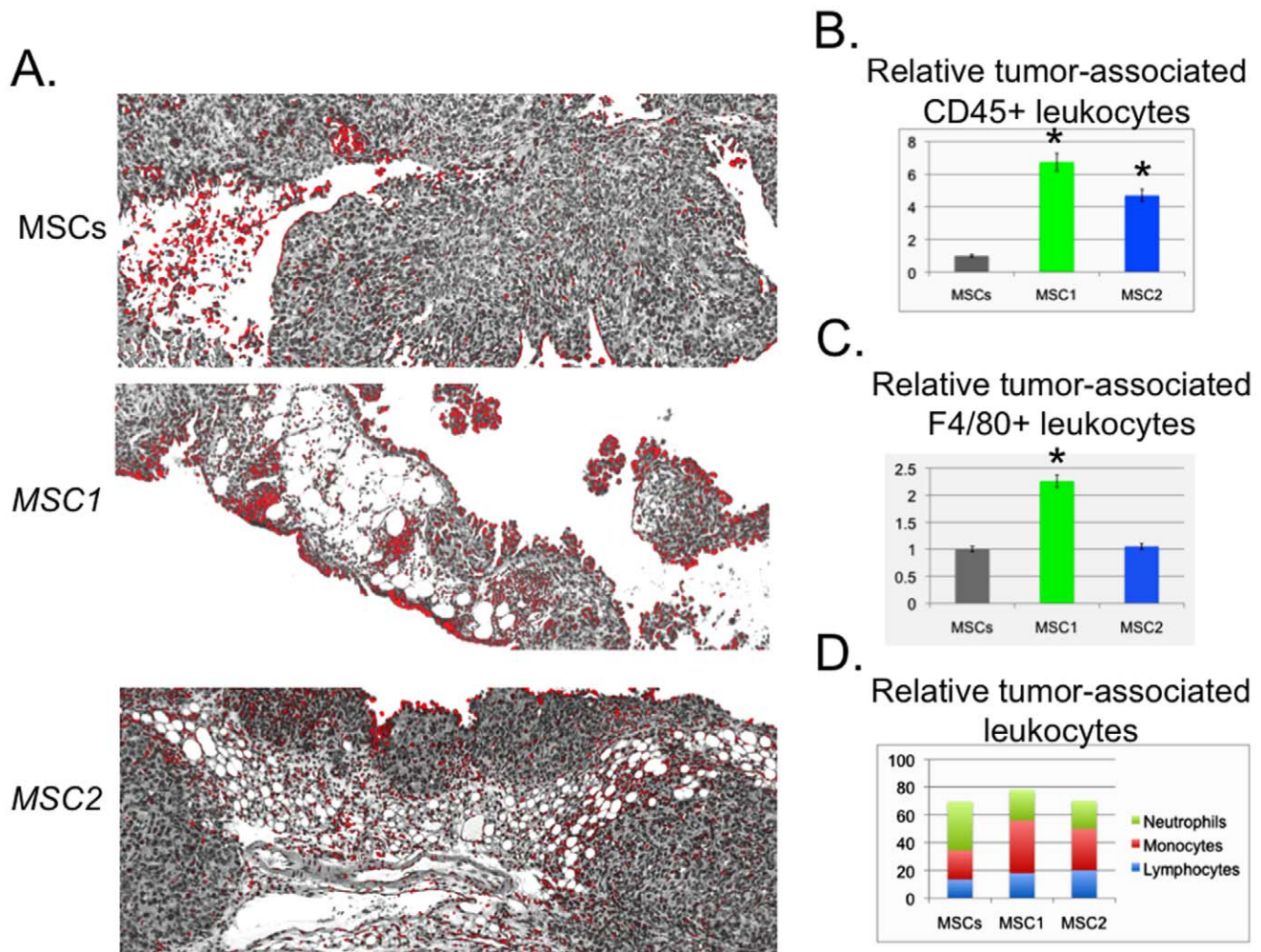


Figure 4. Tumor-associated leukocytes differ among the MSC-treated groups. MOSEC tumors were established in C57BL/6 mice for 4 weeks. MSCs, MSC1, or MSC2 (1×10^6 in 0.5 mL HBSS) were infused IP and the mice were harvested after 65 days. Tumors were excised, fixed, and cut into $5 \mu\text{m}$ sections and processed for antibody staining by standard methods or single cell suspensions were made from the tumors and processed for flow cytometry analysis [7]. Data are representative of three independent experiments with at least 6 mice per treatment group. **A.** Representative micrographs of the tumor sections processed by IHC, stained with DAB, and then recorded with the Aperio ScanScope (40X, Aperio, Vista, CA). Shown is the subsequent ImageJ threshold analysis with CD45+ cells colorized red. **B.** Bar graph depicting the results from the CD45+ flow cytometry analyses of the tumors relative to the MSC-treated tumors. * $P < 0.05$ versus the MSCs-treated tumors. Statistically significant differences were not measured between MSC1- and MSC2-treated tumor samples. **C.** Bar graph depicting the results from the F4/80+ flow cytometry analyses of the tumors relative to the MSC-treated tumors. * $P < 0.05$ versus the MSCs-treated tumors. Statistically significant differences were not measured between MSCs- and MSC2-treated tumor samples. **D.** Bar graph depicting the results from flow cytometry analyses to identify neutrophil, monocyte, and lymphocyte populations among the tumor samples as described in Materials and Methods. Flow cytometry data are representative of at least duplicate samples from at least three independent experiments. doi:10.1371/journal.pone.0045590.g004

important to use immune *competent* models [2,33]. Previous studies with human MSCs introduced into allogeneic or xenogeneic hosts have been similarly reported with success [1,9,34]. In this context, we consistently observed that the MSC1-treatment groups had smaller tumors without any detectable metastasis, and accumulated little to no ascites when compared to the MSCs- or MSC2-treated groups (Figure 3). Upon staining of the collected ascites, it was evident that there were large tumor aggregates or spheroids present in the MSCs- and MSC2-treatment groups but not in the MSC1 ones. MSC-based therapies of tumors or other diseased organs typically results in very low engraftment by the delivered MSCs. It is established that one hurdle in the translation of MSC-based therapies remains improving their survival in the recipient host [1,9,34].

We used both flow cytometry and immunohistochemical analyses to determine the changes among the treatment groups in the tumor-associated leukocytes (Figures 4 and 5). Here, too, we found changes among the MSC-treated groups as was expected. The CD45+ population of cells present in the tumors were more numerous in MSC1- and MSC2- treatment groups than in MSCs-treated groups. Additionally we measured the greatest number of F4/80+ cells in the MSC1- treated group compared to the others. The significance of these findings remains to be elucidated. Tumor-associated macrophages (TAMs) are known to be educated from tumor eradicating cells to tumor promoting cells with F4/80 expression potentially changing from one population to the other [35–37]. It will be interesting to determine in future studies whether tumor-associated MSCs and TAMs directly affect each

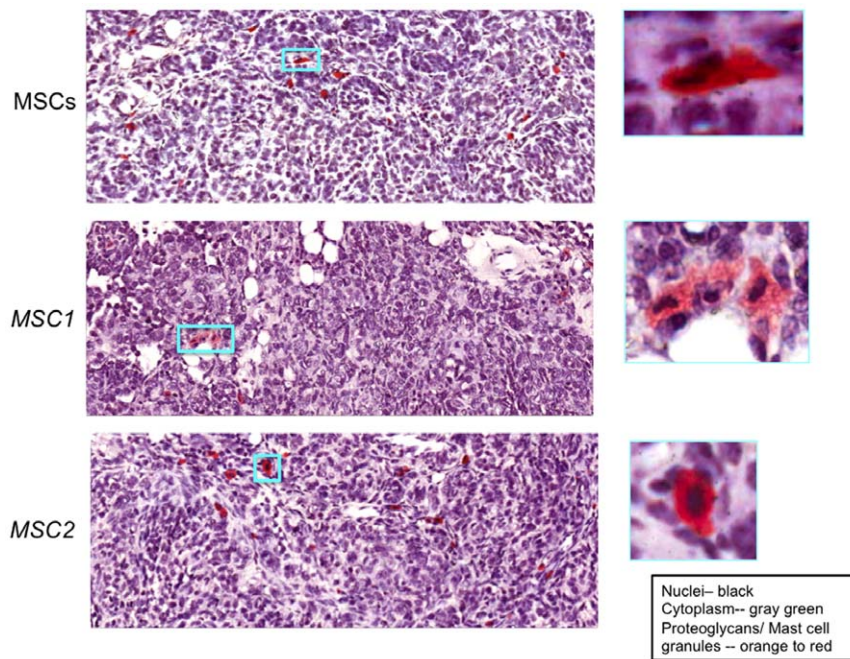


Figure 5. Proteoglycan-rich stained mast cells found in tumor sections from *MSC2*- and *MSC*-treated tumor groups but mostly degranulated ones found in *MSC1*-treated tumor groups. MOSEC tumors were established in C57BL/6 mice for 4 weeks. *MSCs*, *MSC1*, or *MSC2* (1×10^6 in 0.5 mL HBSS) were infused IP and the mice were harvested after 65 days. Tumors were excised, fixed, and cut into 5 μ M sections by standard methods [7]. Sections were processed for safranin O proteoglycan staining (www.ihcworld.com). Representative micrographs of several *MSC*-treated tumor sections are included from images obtained from the Aperio ScanScope (200X, Aperio, Vista, CA). The expected color for each tissue element is described in the inset on the lower right hand side. 400X images are included in boxed insets. Data are representative of three independent experiments with at least 6 mice per treatment group.
doi:10.1371/journal.pone.0045590.g005

other and can be “re-educated” from one form to the other following this interaction.

Macrophages, mast cells (MCs), and MSCs also affect ECM proteins, yet another component of the TME important to tumor growth and spread [23,24,30,38]. Thus, the changes in mast cells and collagen (ECM) levels among the *MSC*-treated tumor groups were measured (Figure 5 and Figure S3). Safranin O stains the proteoglycan-rich granules of mast cells and surprisingly revealed that the MCs of tumor sections of *MSCs*- and *MSC2*-treated groups were mostly loaded with these granules while the *MSC1*-treated groups were not. Furthermore, we observed localization of the MCs to the stromal compartments of the tumors, which may suggest an association of MCs and the ECM. This association was further implicated by comparison of the safranin O stained sections with those of the Verhoeff-Van Gieson (VVG) collagen stained sections, which revealed mast cells concentrated in areas with the darkest pink/red collagen stained regions (Figure S4). Unexpectedly, we observed the opposite effect of *MSC1* on collagen levels (*in vivo*) than we previously reported for *MSC1* induction alone (*in vitro*) [13]. VVG stained tumor sections from *MSC1*-treated groups had less dark pink/red areas than the other samples, whereas *in vitro* *MSC1* had the greatest expression of collagen compared to the other samples. These differences may be explained by direct *in vivo* interactions between the MSCs and MCs that were recently discovered and that would be present in the TME but lacking in the *in vitro* setting [25]. Further investigation of the interaction of MCs with MSCs within the TME will have to be added to those of MSCs and macrophages mentioned above. Adding to the complexity of the TME, MSCs, macrophages, and MCs seem to share many properties affecting

the secretion of bioactive factors and the tumor immunity [26,39–43].

More detailed analyses are required to complete our understanding of the effect that *MSC*-based therapies might have on all of the tumor-associated leukocytes including MCs and macrophages. In particular, it would be interesting to begin to dissect the contributions of each leukocyte population in the *MSC*-affected tumors by using specific mouse knockout models. We also expect that the study of other solid tumor and leukemia models as well as other strains of mice may identify subtle differences in the net effect of the *MSC*-based therapies that will be useful to our understanding of the TME and its contribution to tumor growth and spread. Important as well will be determining the most effective *MSC*-based cancer therapy. To this end, the optimal dose, frequency, and timing of the *MSC*-based therapy need to be determined for each cancer. We are encouraged that the *ex vivo* induced *MSC1* and *MSC2* phenotypes appear to remain stable when re-introduced into various animal disease models and were capable of mediating distinct results even 65 days after just a single *MSC* injection (Figure 3).

Cell-based therapies are undoubtedly gaining ground given their growing international use, regulatory agency approval (FDA and European Medicines Agency-EMA), billion dollar a year market, and proven efficacy in many human diseases [44]. Among these, *MSC*-based therapies are widely used because MSCs are thus far clinically safe, are easily obtained from adult tissues, can be expanded as well as stored, and are unique in their immune modulating capabilities. Additionally, their proclivity for the tumor microenvironment makes them ideally suited for the directed delivery of anti-cancer payloads. An ideal therapeutic approach for the complex pathology of cancer may be a complementary one

that employs conventional methods to target the cancer cells (seed) combined with MSC-based therapies that target the TME (soil). Finally, the new *MSC1*- and *MSC2*-therapy approach we have identified provides a convenient tool with which to begin to dissect the contribution of MSCs to tumors, and may help resolve some of the surrounding controversies to safely advance the use of MSC-based therapies in many human diseases including cancer.

Materials and Methods

Cells

Bone marrow-derived human MSC (MSCs) used for all studies were obtained from the Tulane Center for Stem Cell Research and Regenerative Medicine, New Orleans, LA or Lonza, Walkersville, MD and are IRB exempt as previously described [13]. MSCs from at least six different human donors were used in these experiments and cultured as previously described [12,45]. All experiments were conducted on MSCs at a passage ≤ 5 . HeLa human cervical adenocarcinoma, OVCAR-human ovarian adenocarcinoma, and SKOV3 (SKOV3AB) human ovarian adenocarcinoma were obtained from the American Type Tissue Collection (ATCC, Walkersville, MD). PANC-1 human pancreatic adenocarcinoma, and SKOV3FM were obtained as a gift from Dr. Frank C. Marini (Wake Forest Medical Center, NC and are also commercially available from ATCC, Walkersville, MD). Preparation of MSCs into a pro-inflammatory *MSC1* phenotype or an immunosuppressive *MSC2* phenotype was described previously [13], patent-pending US 61/391,749).

Animals

Animal care and use was pre-approved by the Tulane University Medical Center Advisory Committee for Animal Resources. 3–7-week-old female C57BL/6J *wt* mice were obtained from Jackson Laboratories (Bar Harbor, ME). The syngeneic mouse model for epithelial ovarian cancer based upon a spontaneously transformed mouse ovarian surface epithelial cell (MOSEC) line ID8 has been previously described [21,46]. ID8 cells were a generous gift from Dr. Katherine F. Roby (Kansas University Medical Center). At approximately 4 weeks post cancer cell introduction and tumor formation, 1×10^6 cells/per mouse of CellTracker fluorescently-labeled *wt* MSCs, *MSC1*, *MSC2* or mock control was infused IP (Molecular Probes, Life Technologies, Carlsbad, CA) [47]. The ability of the cells to reach their target was measured by flow cytometry analyses of collected tumors 24 hr after the MSC infusions [47]. The 24 hr window was chosen as optimal for MSC engraftment measurements based on previous studies [47]. Mice were monitored daily for changes in weight, morbidity, and mortality. Tumors were measured and at harvest, ascites, tumors and any metastases were weighed and documented as before [7]. Kaplan-Meier survival plots of mice were analyzed by the log rank test (Prism4, GraphPad Software Inc. CA). Greater than 6 mice per sample group was used in each of the experiments.

Flow Cytometry

Single cell suspensions of collected tumors were achieved by the method previously described [32]. Analysis of cell surface markers expressed from the obtained tumor samples was done by multi-color cell surface antibody staining as in that study, or as indicated for the specific cell subsets including anti-CD3, -CD4, -CD8, -CD11b, -CD11c, -CD19, -CD45R, -Ly-6G (Gr-1), and -NKG2D (CD314) [46,48]. Intracellular cytokine antibody staining was achieved after fixation and permeabilization of the cells. Isotype controls and untreated or unstained samples were routinely run in parallel as standard. End point flow cytometry analysis was

performed on a BD LSRII analyzer and analyzed with CellQuest software. Data are representative of at least duplicate samples from at least three independent experiments.

Colony Forming Units (CFU) and Tumor Spheroid Assays

CFU assay was performed by culturing human tumor cells (200 cells/well) mixed with conventionally prepared MSCs, *MSC1* or *MSC2* (2 cells/well) at a ratio of 10 cancer cells per MSC and plated in 24-well plates in growth medium supplemented with 10% FBS as indicated. Cultures were grown for 14 days at 37°C in a humidified incubator. Growth medium was changed every 3–4 days. Colonies were visualized by staining with a crystal violet solution (0.5% crystal violet/10% ethanol). The resulting colonies were enumerated by the colony counting macro in ImageJ software. Tumor spheroids were formed by culturing tumor cells (200 cells/well) mixed without any other cells (–) or with CellTracker labeled MSCs, *MSC1* or *MSC2* (20 cells/well) at a ratio of 10 cancer cells per 1 MSC and plated over 1.5% agarose in 96-well plates in growth medium supplemented with 10% FBS as indicated. Cultures were grown for 14 days at 37°C in a humidified incubator. Growth medium was changed every 3–4 days. Micrographs shown represent a 20-fold magnified field of the 96-well plate. CFU and tumor spheroid assays were performed in at least three independent experiments with duplicate wells.

Migration and Invasion Assays

Migration and invasion assays were performed with cells loaded on 3 μ M Falcon fluoroblok transwell inserts and allowed 16 hrs in a humidified CO₂ incubator as described previously [12,20,45]. Transwell migrating and matrigel invading cells were visualized on an inverted fluorescence microscope (Olympus). Image analyses were routinely performed with ImageJ. Data are representative of duplicates in at least three independent experiments.

qPCR

Quantitative Real-Time PCR (qPCR) was carried out as previously described using the following primers pairs [39]: matrix metalloproteinase 1 (*mmp1*)-forward (F) GGA GAT CAT CGG GAC AAC TC; *mmp1*-reverse (R)-ACC GGA CTT CAT ATG TCG; *mmp2*-F-CAA GTG GTC CGT GTG AAG TAT G; *mmp2*-R-CGT CAT CGT AGT TGG CTG TG; *mmp3*-F-GAC AAA GGA TAC AAC AGG GAC C; *mmp3*-R-TAT CAG AAA TGG CTG CAT CG; *mmp9*-F-CAA GGA TGG GAA GTA CTG GCG; *mmp9*-R- TCA ACT CAC TCC GGG AAC TC; *mmp13*-F-GAT ACG TTC TTA CAG AAG; *mmp13*-R GAC AAA TCA TCT TCA TCA CC; membrane-type matrix metalloproteinase-1 (*mt-mmp1*)-F-GTC TTC AAG GAG CGC TGG TTC TG *mt-mmp1*-R- TAG CCC GGT TCT ACC TTCA G; *18S rRNA* -F-GAG GGA GCC TGA GAA ACG G, *18S rRNA* -R-GTC GGG AGT GGG TAA TTT GC-3' (IDT, Coralville, IA). Samples from at least three independent experiments were run in triplicate.

Histology and Immunohistochemistry

The collected ascites samples were spun down on cytospin slides and processed for Diff Quick stain as described (http://www.ihcworld.com/_protocols/special_stains/diff_quick_ellis.htm). Tumors were fixed in 10% formalin solution and embedded in paraffin by standard methods. Sections were cut into 5 μ m sections and stained with hematoxylin and eosin (H&E), Verhoeff-Van Gieson (VVG)– elastic fiber/collagen staining and safranin O– proteoglycan staining were performed also as described (www.ihcworld.com). Immunostaining was performed using monoclonal anti-hCAP-18/LL-37, -CD45, -F4/80, and other relevant markers

as before [49]. All stained tissue sections were scanned with the Aperio ScanScope (Aperio, Vista, CA) at an initial magnification of 40X, and images were visualized and captured using the Aperio ImageScope program. Image analyses were routinely performed with ImageJ. For threshold analysis (percent DAB or safranin positive), the images were digitally adjusted to remove background and increase the contrast between the tissue and the background. The RGB images were stacked into separate R, G, B images and threshold determinations were used to digitally highlight all the stained tissue while dismissing the background. Finally, the percent of highlighted pixels (positive cells) was calculated relative to total area of the field. A similar ImageJ analysis method was used to determine collagen positive areas within the VVG stained tumor sections as detailed in http://cardprint.ucsd.edu/CV_Lab_Web_Page/HowToDocs/ImageJProtocol.pdf. Greater than 10 viewing fields were recorded and analyzed after three independent experiments for each sample group.

Statistical Analysis

Data are presented as average $\pm$ standard error of the mean (S.E.M.). Multiple group comparison was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni procedure for comparison of means. Comparison between any two groups was analyzed by the two-tailed Student's t-test or two-way ANOVA (Prism4, GraphPad Software Inc. CA). Values of $P < 0.05$ were considered statistically significant.

Supporting Information

Figure S1 MSC1 diminish tumor growth whereas MSC2 favor tumor growth. Tumor spheroids were formed by culturing tumor cells (200 cells/well) mixed without any other cells (–) or with CellTracker green labeled MSCs, *MSC1*, or *MSC2* (20 cells/well) at a ratio of 10 cancer cells per 1 MSC and plated over 1.5% agarose in 96-well plates in growth medium supplemented with 10% FBS as indicated in figure. Cultures were grown for 14 days at 37°C in a humidified atmosphere of 5% carbon dioxide balance air. Growth medium was changed every 3–4 days. Representative micrographs shown represent 20X magnified bright field of the 96-well plate. Cancer cell lines used are: HeLa- human cervical adenocarcinoma, PANC-1- human pancreatic adenocarcinoma, OVCAR-human ovarian adenocarcinoma, SKOV3-human ovarian adenocarcinoma, and MOSEC-murine ovarian surface epithelium carcinoma cells. (TIF)

Figure S2 MSC1 diminish tumor growth whereas MSC2 favor tumor growth. Fluorescence micrographs corresponding to those bright field micrographs presented in Figure S1. CellTracker green labeled MSCs, *MSC1*, or *MSC2* appear as the brighter spots in the images. It appears that the cells distribute

throughout the tumor spheroids—whose shadows are visible in these fluorescence micrographs. (TIF)

Figure S3 MSC1-treated tumor samples have diminished levels of collagen within the TME compared to MSC2- and MSC-treated tumor groups. MOSEC tumors were established in C57BL/6 mice for 4 weeks. MSCs, *MSC1*, or *MSC2* (1×10^6 in 0.5 mL HBSS) were infused IP and the mice were harvested after 65 days. Tumors were excised, fixed, and cut into 5 μ M sections by standard methods [7]. Sections were processed for Verhoeff-Van Gieson (VVG) elastic fiber/collagen staining (www.ihcworld.com). Representative micrographs of several MSC-treated tumor sections are included from images obtained from the Aperio ScanScope (40X, Aperio, Vista, CA). The expected color for each tissue element is described in the inset on the lower right hand side. 80X images are included in boxed insets. Data are representative of three independent experiments with at least 6 mice per treatment group. (TIF)

Figure S4 Co-localization of tumor associated mast cells with collagen. MOSEC tumors were established in C57BL/6 mice for 4 weeks. MSCs, *MSC1*, or *MSC2* (1×10^6 in 0.5 mL HBSS) were infused IP and the mice were harvested after 65 days. Tumors were excised, fixed, and cut into 5 μ M sections by standard methods [7]. Sections were processed for Verhoeff-Van Gieson (VVG) elastic fiber/collagen staining (left panels) or for safranin O proteoglycan staining (right panels, www.ihcworld.com). Representative micrographs of several MSC-treated tumor sections are included from images obtained from the Aperio ScanScope (40X, Aperio, Vista, CA). Yellow arrows indicate comparable sections among the tumor tissue sections. Data are representative of three independent experiments with at least 6 mice per treatment group. (TIF)

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Author Contributions

Conceived and designed the experiments: RSW AMB. Performed the experiments: RSW SLH AMB. Analyzed the data: RSW SLH AMB. Contributed reagents/materials/analysis tools: RSW SLH AMB. Wrote the paper: RSW AMB.

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The Role of Mesenchymal Stem Cells in the Tumor Microenvironment

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1. Introduction

Currently, there are many promising clinical trials using mesenchymal stem cells (MSCs) in cell-based therapies of diseases ranging widely from graft-versus-host to joint and cartilage disorders (Salem and Thiernemann 2010; Tolar, Le Blanc et al.). Increasingly, however, there is a concern over the clinical use of MSCs because they are also known to home to tumors and once resident in the tumor microenvironment (TME) to support tumor growth and spread (Karnoub, Dash et al. 2007; Kidd, Spaeth et al. 2008; Coffelt, Marini et al. 2009; Klopp, Gupta et al. 2010; Klopp, Gupta et al. 2011). Conversely, other studies have reported that MSCs found in the TME diminish tumor growth, which has further generated some controversy in this field (reviewed in (Klopp, Gupta et al. 2010; Klopp, Gupta et al. 2011). Either way as a result of the MSC propensity for the TME, genetically modified MSCs that can act as “Trojan horses” and deliver anti-cancer therapeutics into the tumor stroma are being evaluated as a promising new specific cell-based therapy for cancer.

Our group established that MSCs in the ovarian tumor microenvironment promoted tumor growth and favored angiogenesis (Zvezdaryk, Coffelt et al. 2007; Coffelt and Scandurro 2008; Coffelt, Marini et al. 2009). We also developed new methodology to induce the conventional mixed pool of MSCs into two uniform but distinct phenotypes, *MSC1* and *MSC2* (Waterman, Tomchuck et al. 2010). We based their classification on several parallel observations reported within the monocyte literature. Like MSCs, heterogeneous bone marrow-derived monocytes respond to stress or “danger” inflammatory signals and home to tissue injury. Monocyte polarization into pro-inflammatory macrophages (M1) occurs early on in tissue repair whereas, monocyte polarization into anti-inflammatory macrophages (M2) follows later to help in tissue injury resolution (Mantovani, Sozzani et al. 2002; Martinez, Gordon et al. 2006). Although, this is a much simplified view of what occurs in the complex process of wound healing and repair, it provides a convenient paradigm to begin to dissect critical components within this biological process (Mantovani, Sica et al. 2007; Mosser and Edwards 2008; Mosser and Zhang 2008). Likewise, we believe that pro-inflammatory *MSC1* and anti-inflammatory *MSC2* provide convenient tools with which to begin to interrogate the role of MSCs in the tumor microenvironment.

In recent studies we found that *MSC2* supported ovarian cancer growth and spread while surprisingly *MSC1* had an opposite anti-tumor effect (Waterman 2011). We suggest that by

more closely studying the distinct tumor effects observed for these MSC phenotypes we may figure out why in the studies mentioned above MSCs favor tumor growth while in others MSCs attenuate tumors. In other words, induction into each discrete but uniform phenotype may help resolve some of the controversies surrounding the use of MSCs in cell based-therapies.

It is known that MSCs resident in the TME contribute mitogens, extracellular matrix proteins, angiogenic, and inflammatory factors. These contributions are not trivial to tumor growth and spread and serve to recruit specific subsets of leukocytes and endothelia to the TME that profoundly influence tumors. *MSC1* in the TME are expected to attenuate tumor growth by secretion of anti-tumor factors and recruitment of anti-tumor immunity. *MSC2* found in TME should promote tumor growth and spread by secretion of mitogens and suppressing anti-tumor immune responses. We expect that by identifying the differences between these two phenotypes we will shed some light on the growing controversy on the role of MSCs in tumors, and provide a means to safely deliver MSCs in cell-based therapies. We have attempted to provide all relevant information that is available concerning these issues in the sections included in this chapter.

2. Current understanding of MSCs function in the TME

Mesenchymal stem cells (MSCs) are a group of heterogeneous multipotent cells that can be easily isolated from many tissues throughout the body. Though initially isolated from the bone marrow, they are now recognized to be mostly in perivascular regions throughout the body (Feng, Mantesso et al. ; Zvezdaryk, Coffelt et al. 2007; da Silva Meirelles, Caplan et al. 2008). The discovery of these cells dates back to the 1960s (Friedenstein, Piatetzky et al. 1966). In recent years, MSCs have been widely studied due to their ability to be expanded in culture and stored without losing their capacity to differentiate into many different cells of mesodermal origin such as osteoblasts, chondrocytes, and adipocytes (Bruder, Jaiswal et al. 1997; Jaiswal, Haynesworth et al. 1997; Digirolamo, Stokes et al. 1999; Phinney, Kopen et al. 1999; Pittenger, Mackay et al. 1999). MSCs can also transdifferentiate into cells of ectodermal (Kopen, Prockop et al. 1999) and endodermal (Sun, Chen et al. 2007; Ju, Teng et al. 2010) origins. As a result, many preclinical studies have focused on evaluating the capacity of MSCs to repair and replace injured or diseased tissues of all origins.

Despite these research efforts however, there is growing evidence that the clinical benefit of MSCs in cell-based therapies is not the replacement of the injured tissue, but rather their efficiency in modulating aberrant host immune responses (Pittenger, Mackay et al. 1999; Prockop 2003; Prockop 2009). Following the remarkable clinical observations by the Le Blanc group who used the successful delivery of MSCs as a last resort to stave off graft-versus-host disease in a young boy, the immune modulating capability of MSCs is now more widely recognized (Le Blanc, Rasmusson et al. 2004). Further evidence indicating that immunomodulation is the primary activity of MSCs can be gleaned from the observation in many studies that although infused MSCs home to sites of injury and provide treatment benefit in widely ranging diseases, they can rarely be detected within the repaired tissue. Subsequent research efforts are beginning to identify the myriad ways that MSCs affect host immune responses. These appear to be mediated both by direct cell-to-cell contact and indirectly by the secretion of inflammatory factors (further discussed below) (Aggarwal and Pittenger 2005; Abdi, Fiorina et al. 2008; Uccelli, Moretta et al. 2008; Nemeth, Mayer et al. 2009; Bunnell, Betancourt et al. 2010; Singer and Caplan 2011).

Thus far, the immune modulating effects of MSCs include inhibition of the proliferation of activated CD8+ and CD4+ T lymphocytes and natural killer (NK) cells, recruitment and support of regulatory T cells, suppression of Th17 lymphocytes and immunoglobulin production by plasma cells, inhibition of maturation of dendritic cells (DCs), as well as attenuation of mast cells (Aggarwal and Pittenger 2005; Abdi, Fiorina et al. 2008; Uccelli, Moretta et al. 2008; Nemeth, Mayer et al. 2009; Nemeth, Keane-Myers et al. 2010). MSCs secrete various inflammatory factors including TNF- α -induced protein 6 (TNAIP6 or TSG-6), prostaglandin E2 (PGE2), human leukocyte antigen G5 (HLA-G5), hepatocyte growth factor (HGF), inducible nitric oxide synthase (iNOS), indoleamine-2,3-dioxygenase (IDO), transforming growth factor β (TGF- β), leukemia-inhibitory factor (LIF), and interleukin (IL)-10 (Krampera, Pasini et al. 2006; Gur-Wahnon, Borovsky et al. 2009; Bunnell, Betancourt et al. 2010; Singer and Caplan 2011).

MSCs express low levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I, do not express co-stimulatory molecules (B7-1/CD80 and -2/CD86, CD40, or CD40L), and must be induced to express MHC class II and Fas ligand that likely allows the safe delivery of these cells in non-self (allogeneic) hosts (Aggarwal and Pittenger 2005; Bunnell, Betancourt et al. 2010). Indeed, MSCs stand alone among the other types of stem cells such as embryonic or induced pluripotent (iPS) cells being considered in regenerative medicine for their safe, non-immune provoking, allogeneic host delivery capability. This has prompted many new and established businesses to amass expanded stockpiles of MSCs ready for use in the treatment of many human diseases including cancer (Salem and Thiemermann 2010).

Given the ability to deliver expanded, stockpiled clinical grade MSCs, knowing that they specifically home to the TME, and that they secrete mitogens, extracellular matrix proteins, angiogenic and inflammatory factors, it is not hard to conceive that MSCs might on the one hand influence tumors, and on the other hand, be used as vehicles to deliver anti-cancer agents. At issue is that despite intense study over the past few years, the effect of MSCs on tumors or their function in the TME is far from clear. Some studies report that MSCs promote tumor growth and spread while others report that MSCs attenuate tumor growth (Table 1). The distinct effects by MSCs on tumors has recently been attributed to differences in the experimental cancer model, the heterogeneity of MSC preparations, the dose or timing of the delivered MSCs, the animal host, or some as yet unknown factor (Klopp, Gupta et al. 2010; Klopp, Gupta et al. 2011). Also at play may be that the primary immunomodulatory function of MSCs is not realized in the context of most of these studies, which rely on immune compromised animal models. It is clear however, that with all of their unique properties MSCs make attractive candidates in cell therapies of cancer. In fact, a few promising pre-clinical reports have shown the delivery by MSCs of several anti-cancer therapeutics such as interferon (IFN)- β , cytosine deaminase, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and oncolytic viruses to tumors (Pittenger, Mackay et al. 1999; Studeny, Marini et al. 2002; Prockop 2003; Studeny, Marini et al. 2004; Nakamizo, Marini et al. 2005; Ren, Li et al. 2007; Kim, Lim et al. 2008; Ren, Kumar et al. 2008; Ren, Kumar et al. 2008; Mader, Maeyama et al. 2009; Prockop 2009). Though it would seem from these reports that any pro-tumor MSC effect is outweighed by the anti-cancer strategy, it is important to fully understand all of the contributions that MSCs have in the TME of immune competent tumors to safely use them in cell-based therapies of human disease.

It is appreciated that MSCs contribute in a number of ways within the TME. As mentioned above, it has long been documented that MSCs elaborate a number of factors directly, after

stimulation, or after contact with adjacent cells. These include mitogens, extracellular matrix (ECM) proteins, angiogenic factors, and inflammatory factors, all of which could potentially influence tumor growth and spread. These are summarized below along with some of the pro-tumorigenic and anti-tumorigenic evidence for MSCs.

2.1 Pro-Tumorigenic evidence

There are a growing number of studies implicating a role for MSCs derived from various tissues in tumor growth and spread. Upon review of these studies and the anti-tumorigenic

Study	MSC Source	MSC:Tumor Ratio	Immune Status of animal model	Tumor Model	MSC Effect
(Muehlberg, Song et al. 2009)	Hu, Mu ASCs	10:1	-	Br	Larger tumor, increased SDF-1
(Karnoub, Dash et al. 2007)	Hu BMSCs	3:1	-	Br	Larger tumor, increased spread, CCL5-mediated
(Galie, Konstantinidou et al. 2008)	Mu ASCs	1:1	+	Br	Larger tumors, pro-angiogenesis
(Yu, Ren et al. 2008)	Hu ASCs	1:1, 1:2, 1:10	-	Lu, Glioma	Larger tumor, anti-apoptosis
(Djouad, Plence et al. 2003; Djouad, Fritz et al. 2005)	Mu BMSCs	1:1	+	Melanoma	Larger tumors, inflammation
(Kucerova, Matuskova et al.)	Hu ASCs	1:5-1:10	-	Melanoma Glioblastoma	Larger tumors, VEGF and SDF1-CXCR4
(Coffelt, Marini et al. 2009)	Hu BMSCs	1:10	-	Ova	Larger tumors, pro-angiogenesis
(Lin, Yang et al. 2010)	Hu ASCs	1:2	-	Pr	Larger tumors, pro-angiogenesis and CXCR4
(Prantl, Muehlberg et al.)	Hu ASCs	1:10	-	Pr	Larger tumors, pro-angiogenesis
(Zhu, Xu et al. 2006)	Hu BMSCs	10:1, 1:1	-	Co	Larger tumors, pro-angiogenesis
(Shinagawa, Kitadai et al.)	Hu BMSCs	1:2	-	Co	Larger tumors, anti-apoptosis

Abbreviations: Hu- human, Mu- murine, ASC- adipose-derived MSCs, BMSCs- bone marrow-derived MSCs, Immune Status of animal model- - immune compromised +- immune competent, Br- breast, Lu- lung, Ov- ovarian, Pr- prostate, and Co- colon cancer cell lines.

Table 1. Pro-tumorigenic evidence for MSCs in the TME

ones below it is tempting to speculate that cancers of endo- and ectodermal tissue origin are likely supported by MSCs whereas cancers of mesodermal tissue origin are likely inhibited by MSCs. However, as stated above, the fact that most of the studies are for technical reasons conducted in immune compromised animals greatly limits these conclusions and our understanding of the final outcome of MSCs in cancer. Evidence that MSCs promote tumor growth and their stated mechanism(s) is given by the studies summarized in Table 1. MSCs supported growth of breast, brain, lung, ovary, prostate, and colon, as well as lymphoma and melanoma (Kucerova, Matuskova et al. ; Shinagawa, Kitadai et al. ; Djouad, Plence et al. 2003; Djouad, Fritz et al. 2005; Zhu, Xu et al. 2006; Karnoub, Dash et al. 2007; Galie, Konstantinidou et al. 2008; Yu, Ren et al. 2008; Coffelt, Marini et al. 2009; Muehlberg, Song et al. 2009; Lin, Yang et al. 2010). The MSCs delivered at high ratios to the experimental tumor cell lines most commonly promoted tumor growth and metastasis. Most studies reported an increase in angiogenesis as a result of increased VEGF production by the MSCs in the TME. Some studies reported attenuation of tumor apoptosis. Chemokines such as Chemokine Ligand-5 (CCL5 or RANTES) and stromal-derived factor-1 (SDF-1)-C-X-C chemokine receptor-4 (CXCR4) axis effects by the MSCs were associated with elevated tumor migration and spread.

The secretion of pro-angiogenic molecules by the MSCs likely assist the tumors in capturing essential nutrients—perhaps also explaining the anti-apoptosis effects-- and in gaining the ability to spread to remote tissues—explaining the role of the chemokines. MSCs are known to secrete pro-angiogenic factors such as VEGF and possibly erythropoietin (Epo) thus this chief effect is not unexpected (Zvezdaryk, Coffelt et al. 2007; Singer and Caplan 2011). More studies are needed that focus on whether MSC conditioned medium is sufficient to elicit these responses and to test whether cell-to-cell contact by the MSCs, leukocytes, and/or cancer cells is required for the promotion of tumor growth and spread by MSCs.

2.2 Anti-Tumorigenic evidence

While the pro-tumorigenic activity of MSCs is largely characterized by the secretion of pro-angiogenic molecules, the anti-tumorigenic activity of these cells is exemplified by modulation of members of the Wnt-signaling family (Table 2). MSCs inhibited the growth of tumors in several different models (Maestroni, Hertens et al. 1999; Ohlsson, Varas et al. 2003; Khakoo, Pati et al. 2006; Lu, Yuan et al. 2008; Qiao, Xu et al. 2008; Qiao, Xu et al. 2008; Cousin, Ravet et al. 2009; Otsu, Das et al. 2009; Zhu, Sun et al. 2009; Dasari, Kaur et al. ; Dasari, Velpula et al. ; Secchiero, Zorzet et al.). For instance, in studies that used fetal tissue derived MSCs, their secretion of the Wnt-signalling inhibitor Dickkopf-related protein-1 (DKK-1) inhibited breast and liver cancer cell lines (Qiao, Xu et al. 2008; Qiao, Xu et al. 2008). When the researchers used a neutralizing antibody or small interfering RNA to block DKK-1 within MSCs, the inhibitory tumor effects were attenuated. In the DKK-1 associated inhibition of primary leukemia by adipose-derived MSCs (ASCs), the stem cell transcription factor NANOG was also implicated (Zhu, Sun et al. 2009).

Interestingly, in an immune competent model, MSCs typically believed to be immune suppressive, recruited leukocytes and appeared to favor pro-inflammatory monocyte/granulocyte infiltration, which promoted rat colon carcinoma growth (Ohlsson, Varas et al. 2003). In the other immune competent model studies, one reported lack of immune suppression or attenuation of T-cell activation by the admixed MSCs but did not report the changes in any other pro-inflammatory leukocytes, and the other study was

focused more on the effect on angiogenesis by the MSCs rather than on inflammatory cells (Lu, Yuan et al. 2008; Otsu, Das et al. 2009).

Study	MSC Source	MSC:Tumor Ratio	Immune Status of animal model	Tumor Model	MSC Effect
(Khakoo, Pati et al. 2006)	Hu BMSCs	1:1, 2:1	-	Kaposi's Sarcoma	Smaller tumors, E-cadherin dependent AKT-inhibition
(Secchiero, Zorzet et al.)	Hu BMSCs	1:2, 1:10	-	NH-Lymphoma	Smaller tumors, increased animal survival
(Lu, Yuan et al. 2008)	Mu BMSCs	2-4:1	+	Insulinoma Li	Decreased ascites, pro-apoptosis
(Zhu, Sun et al. 2009)	Hu ASCs	1:10	-	Leukemia	DKK-1 mediated anti-proliferation
(Cousin, Ravet et al. 2009)	Hu ASCs	10 ³ ASCs/mm ³ tumor	-	Pan	Smaller tumors
(Otsu, Das et al. 2009)	Mu BMSCs	10 ⁶ MSCs/700mm ³ tumor	+	Melanoma	Smaller tumors, anti-angiogenesis
(Maestroni, Hertens et al. 1999)	Hu BMSCs	1:1	-	Melanoma, Lu	Smaller tumors and mets with GM-CSF tx MSCs
(Dasari, Kaur et al. ; Dasari, Velpula et al.)	Hu UCSCs	1:4	-	Glioma	Smaller tumors, ↑PTEN, ↓PI3K,AKT
(Qiao, Xu et al. 2008)	Hu MSCs-TERT tx	1:100	-	Br	Smaller tumors, less mets, DKK-1 mediated Wnt1 inhibition
(Qiao, Xu et al. 2008)	Hu MSCs-TERT tx	1:1	-	Li	Smaller tumors, less mets, DKK-1 mediated Wnt1 inhibition
(Ohlsson, Varas et al. 2003)	Mu BpMSCs- <i>c-myc</i>	1:1-10	+	Co	Smaller tumors, ↑inflammation

Abbreviations: Hu- human, Mu- murine, ASC- adipose-derived MSCs, BMSCs- bone marrow-derived MSCs, UCSCs- umbilical cord-derived MSCs, MSCs-TERT tx -MSC cell line immortalized with telomerase vectors, BpMSCs-*c-myc*-bone marrow-derived MSC progenitor cells immortalized with *c-myc*, Immune Status of animal model- - immune compromised +/- immune competent, Br- breast, Co- colon, Li- liver, Lu- lung, NH- Non-Hodgkin's lymphoma, and Pan- pancreas cancer cell lines. DKK-1- dickkopf-related protein 1, GM-CSF-granulocyte/monocyte-colony stimulating factor, PTEN-phosphatase and tensin homolog 10, PI3K-phosphoinositol-3-kinase.

Table 2. Anti-tumorigenic evidence for MSCs in the TME

2.3 Controversies

Greater than a 100 clinical trials are underway or completed that investigate MSC-based therapy of human disease, and thus far the reports of adverse effects related to the therapy have been unremarkable (Salem and Thiemermann 2010; Tolar, Le Blanc et al. 2010; Singer and Caplan 2011). Therapy-related tumorigenicity has not been found, yet the preclinical studies presented above argue that we should carefully study this MSC potential. The question is why did MSCs promote cancer growth and spread in some studies, while in others MSCs diminished growth and spread? To begin to address this question there are a few important issues that have to be considered. First is the fact that surprisingly the chief effect of MSC-based therapies on disease is the modulation of the inflammatory host responses and not the replacement of injured tissue. Secondly, this observed therapeutic benefit is carried out by a few lingering MSCs that survive the relatively quick clearance of the cell bolus from the circulation—given that very small numbers of MSCs are ever detected at the sites of injury (Prockop 2009). Thirdly, it is known that both the adaptive and innate immune response arms profoundly influence tumor growth and spread by a complex interplay between inflammation and immunosurveillance (Frese and Tuveson 2007; Cheng, Ramesh et al. 2010). To resolve some of this controversy and to better understand the complex nature of the MSC-tumor interaction these issues need to be taken into account in future studies.

It is difficult to accurately model tumorigenesis with human tumor xenograft models in immunodeficient mice to finally resolve the effect that MSC-based therapy will have on cancer (Frese and Tuveson 2007; Cheng, Ramesh et al. 2010). Moreover, the number of MSCs interacting with the tumor must reflect more closely what is observed by the clinical experience. To more precisely model tumorigenicity attempts have been made at humanizing the murine immune system by eliminating the endogenous immune system followed by engraftment of human bone marrow or immune cells (Frese and Tuveson 2007). The problem with this approach has been that species-specific differences in both arms of the immune system confound interpretations. Immunocompetent autochthonous mouse models of human cancer provide a valuable tool that better addresses some of these issues. Though far from perfect, these models more closely parallel human carcinogenesis by allowing intrinsic tumor formation with immune surveillance and offer a better alternative system to study MSC-tumor interactions.

Apart from the limitations of current cancer models there are many other reasons that have been suggested to explain the divergent effects of MSCs in tumors (Klopp, Gupta et al. 2010; Klopp, Gupta et al. 2011). These include the heterogeneity of cells present in current MSC preparation protocols. Convention dictates that more homogeneous preparations of MSCs will also yield more consistent therapeutic outcomes with these cells. However, provided that we can overcome this hurdle and deliver more uniform cells, we may never get away from the variability that comes from the human donors. The age, gender, weight, and disease status of the donor may always affect efficacy outcomes and needs to be investigated more closely. Differences in the tissue source of the MSCs, whether bone marrow, adipose, umbilical cord, or other, also appear to affect a number of MSC functions (Sakaguchi, Sekiya et al. 2005; Hass, Kasper et al. 2011). Further complicating matters in all MSC-based therapy is the cell number and dosing frequency used to achieve a particular therapeutic efficacy. Cancer is a complex disease and to fully understand the contribution of MSCs, which are also intricate, more careful consideration of all these issues needs to be given. Despite these hurdles, MSCs remain an intriguing vehicle that can specifically target tumors.

3. Contributions by MSCs to tumors

In spite of all the limitations described, there is agreement about certain factors that MSCs elaborate that are important to tumorigenesis. It has long been known that MSCs synthesize a broad spectrum of growth factors, extracellular matrix proteins (ECM), cytokines, chemokines, and angiogenic molecules that have effects on cells in their vicinity. The effects of the bioactive molecules that MSCs secrete can be either direct, indirect, or even both: direct by causing intracellular signaling or indirect by causing another cell in the vicinity to secrete a bioactive factor. The indirect activity is typically termed "trophic", based on the original use of this word in neurobiology to distinguish neurotransmitters from other bioactive molecules released from nerve terminals (Caplan and Dennis 2006; Meirelles Lda, Fontes et al. 2009; Singer and Caplan 2011).

Typically, the bioactive molecules that are released from MSCs are reported to be relatively constant between different donors, regardless of age or health status of the donor. However, there can be some donor-specific differences in the levels of the secreted molecules-- that can be as high as a ten-fold difference. Moreover, the specific bioactive agents secreted by individual MSCs are also controlled by their functional status, level of differentiation, and the influence of their local microenvironments (Phinney, Kopen et al. 1999; Djouad, Fritz et al. 2005; Caplan and Dennis 2006; Krampera, Pasini et al. 2006; Tomchuck, Zvezdaryk et al. 2008; Nemeth, Mayer et al. 2009; Prasanna, Gopalakrishnan et al. ; Singer and Caplan 2011). It is expected that MSCs, as multipotent stem cells, will elaborate different levels and arrays of bioactive molecules as they differentiate into defined lineages. Additionally, the pattern and quantity of these secreted factors is well known to feed back on the MSC itself and change both its functional status and physiology.

These MSC paracrine and autocrine factors can have profound effects on local cellular dynamics. For instance, the marrow stroma derived from MSCs not only provides the matrix that supports cell anchorage, but also helps to maintain nearby endothelia and hematopoietic cells. In stroma poor niches within the marrow the hematopoietic stem cells (HSCs) will begin distinct programs of differentiation. The interdependence of MSCs and HSCs in the marrow is governed by the secretion of bioactive molecules such as the stromal-derived factor-1 (SDF1) to C-X-C chemokine receptor-4 (CXCR4) axis that helps support full hematopoietic lineage progression (Lopez Ponte, Marais et al. 2007).

3.1 Soluble, Extracellular Matrix (ECM), and angiogenic factors

The secretion of these broad range bioactive molecules is now believed to be the main mechanism by which MSCs achieve their therapeutic effect and that likely most affect the tumor microenvironment. These are typically divided by the processes they affect, such as mitogenic, angiogenic, apoptotic, or inflammatory/immune modulating (Table 3). We have added exosomes as a new category to these bioactive factors. Exosomes appear to be a previously unrecognized secretory vesicle that can affect neighboring cells. We include mitogens, Extracellular Matrix (ECM) proteins, and angiogens, exosomes and inflammatory/immune modulating bioactive factors as molecules potentially contributed by MSCs but caution that this is not an exhaustive list of all MSC products. Some of the molecules overlap in function, some of the molecules play greater roles in one species versus another (e.g.-mouse vs. human), and some of the molecules are released only following

specific stimulation or activation (Tomchuck, Zvezdaryk et al. 2008; Klopp, Gupta et al. 2010; Waterman, Tomchuck et al. 2010; Klopp, Gupta et al. 2011). These have been recently reviewed (da Silva Meirelles, Caplan et al. 2008; Klopp, Gupta et al. 2010; Klopp, Gupta et al. 2011; Singer and Caplan 2011).

Molecule Types	Molecules	Study
Mitogens	bFGF, G-CSF, GM-CSF, HGF, IGF-I, IL6, Leptin, LIF, SCF, SDF-1, stanniocalcin-1, TGF β , VEGF	(Zvezdaryk, Coffelt et al. 2007; Block, Ohkouchi et al. 2009; Meirelles Lda, Fontes et al. 2009; Tomchuck, 2008 #621; Klopp, Gupta et al. 2010; Waterman, Tomchuck et al. 2010; Klopp, Gupta et al. 2011)
Extracellular Matrix Proteins	Collagens, Fibronectin, Laminin	(Zuckerman and Wicha 1983; Hashimoto, Kariya et al. 2006; Zvezdaryk, Coffelt et al. 2007; Tomchuck, Zvezdaryk et al. 2008; Meirelles Lda, Fontes et al. 2009; Waterman, Tomchuck et al. 2010)
Angiogens	Angiopoetin-1, bFGF, IL6, IL8, Leptin, stanniocalcin-1, VEGF	(Zvezdaryk, Coffelt et al. 2007; Tomchuck, Zvezdaryk et al. 2008; Meirelles Lda, Fontes et al. 2009; Waterman, Tomchuck et al. 2010)
Exosomes	Pro-inflammatory molecules, miRNAs	(Anand 2010; Chen, Lai et al. 2010; Lai, Arslan et al. 2010)
Inflammatory/ Immune Modulating	galectin-3, galectin-1, HGF, HLA-G, IDO, IL1 β , IL1RA, IL6, IL8, IL12, iNOS, IP-10, LIF, MCP-1, MIP-1, PGE2, semaphorin-3A, RANTES, SDF-1, stanniocalcin-1, TGF β , TSG-6	(Zvezdaryk, Coffelt et al. 2007; Tomchuck, Zvezdaryk et al. 2008; Block, Ohkouchi et al. 2009; Meirelles Lda, Fontes et al. 2009; Bartosh, Ylostalo et al. 2010; Bunnell, Betancourt et al. 2010; Klopp, Gupta et al. 2010; Waterman, Tomchuck et al. 2010; Danchuk, Ylostalo et al. 2011; Klopp, Gupta et al. 2011)

Abbreviations: bFGF- basic fibroblast growth factor, CCL- C-C motif chemokine ligand, CXC- C-X-C- motif chemokine, CXCL-CXC-ligand, G-CSF-granulocyte-colony stimulating factor, GM-CSF-granulocyte-macrophage-colony stimulating factor, HGF-hepatocyte growth factor (scatter factor), HLA-G- human leukocyte antigen-G, IDO- indoleamine 2,3-dioxygenase, IGF-I-insulin-like growth factor-1, IL-interleukin, IL-1RA- interleukin-receptor 1 antagonist, iNOS-inducible nitric oxide synthase, IP-10-interferon-gamma-inducible protein 10 (CXCL10), LIF-leukemia inhibitory factor, MCP-1-monocyte chemoattractant protein-1 (CCL2), MIP1-macrophage inflammatory protein-1 (CCL3), PGE2-prostaglandin-E2, PlGF-placental-derived growth factor, RANTES- regulated upon activation normal T cell expressed and secreted (CCL5), SCF-stem cell factor, SDF-1-stromal-derived factor-1, TGF β -transforming growth factor- β , TSG-6- TNF-alpha stimulated gene/protein 6, VEGF-vascular-derived endothelial growth factor (vascular permeability factor, VPF).

Table 3. Molecules Contributed by MSCs

3.2 Exosomes

A recently described form of intercellular communication that may also be important in MSC-tumor exchanges is exosomes. These are endosome-derived vesicles of about 40-100 nm that are formed by the involution of endosome membranes resulting in the formation of

multi-vesicular bodies (MVBs). Following certain physiological conditions, the MVBs fuse with the plasma membrane and release the exosomes into the circulation or tissue microenvironment. Exosomes have a "saucer-shaped" morphology as determined from electron microscopy analyses. Various methods have been developed to enrich for exosomes derived from a number of cell types including antigen-presenting cells (APCs), monocytes, T-lymphocytes, reticulocytes, mast cells, platelets, fibroblasts, tumor cells, and MSCs (Anand 2010; Lai, Arslan et al. 2010; Tan, De La Pena et al. 2010).

Investigators studying the cardioprotective effect of human embryonic stem cell-derived MSC-conditioned medium (CM) on myocardial ischemia/reperfusion injury reasoned based on proteomic analyses that exosomes were responsible for the beneficial effect (Sze, de Kleijn et al. 2007; Lai, Arslan et al. 2010). Their unbiased proteomic profiling of proteins secreted by MSCs revealed an abundance of membrane and cytosolic proteins. This suggested to them that the trophic effects of MSCs were not mediated by soluble growth factors and cytokines alone. Sze *et al.* proceeded to enrich for particles by size-exclusion fractionation on HPLC. Based on the size and the composition of the particles they figured exosomes were present in the condition medium of MSCs. Moreover they demonstrated that the enriched fraction of exosomes reduced infarct size in a mouse model of myocardial ischemia/reperfusion injury.

The particles could be visualized by electron microscopy and were shown to be phospholipid vesicles consisting of cholesterol, sphingomyelin, and phosphatidylcholine. Moreover, they were composed of known exosome-associated proteins-- CD81, CD9, and Alix. Exosomes are known to have a specific protein composition, including CD9, CD81, Alix, TSP-1, SOD-1, and pyruvate kinase. CD9 and CD81 are tetrapannin membrane proteins that are also localized in the membrane of exosomes. Consistent with the presence of exosomes in the CM of the MSCs this study further demonstrated that CD9 in the CM was a membrane-bound protein while SOD-1 was localized within a lipid vesicle. They eliminated the possibility of immune cells or platelets as sources of exosomes with an *ex vivo* mouse model of myocardial ischemia/reperfusion injury.

Similarly in human ESC-derived MSC conditioned medium other investigators found exosomes that contained small RNAs (less than 300 nt) encapsulated in cholesterol-rich phospholipid vesicles. The small RNAs were identified by a number of biochemical and genetic criteria to be microRNAs (miRNAs). Of interest the *Let-7* family of miRNAs figured prominently in these studies (Chen, Lai et al. 2010; Koh, Sheng et al. 2010). It is becoming increasingly clear that miRNAs are potent global gene regulators of many diverse cell functions including adaptation to mitogens, low oxygen (hypoxia), and inflammation. Perhaps this might explain why exosomes are potent immune modulators (Anand 2010). Apart from the molecules present inside the lumen of exosomes, it has been suggested that certain exosomal membrane molecules can interact with their surface receptors on the target cells thereby inducing an immunomodulatory response or activating the immune system. Consistent with this notion, exosome release is enhanced following pathologies where immune activation is required. It has been suggested that immunogenic molecules on the exosomal membrane can activate leukocytes. In support of this idea is the fact that exosomes are analogous to inverted endosomes and thus display inflammatory intracellular factors present normally within plasma membrane. Taking advantage of this inflammatory nature

of exosomes, clinicians are developing cancer vaccines based on loading dendritic cells (DCs) with tumor antigens, expanding the DCs *ex vivo*, and subsequently isolating their enriched exosomes (Tan, De La Pena et al. 2010). The tumor antigen loaded exosomes are then reintroduced into patients to elicit tumor specific anti-tumor immunity.

Lastly, highlighting the interactions of tumors and MSCs, exosomes derived from tumors appear to drive adipose-derived MSC differentiation toward tumor associated myofibroblasts that can then contribute to tumor growth and spread (Webber, Steadman et al. 2010; Cho, Park et al. 2011; Cho, Park et al. 2011). Interestingly and perhaps providing a mechanism for the Wnt-signaling mediated anti-tumor effect of MSCs mentioned above, β -catenin was found to be contained within exosomes (Chairoungdua, Smith et al. 2010). Furthermore, exosomal release of β -catenin antagonized Wnt-signaling in the recipient cell. These studies emphasize the need for more intense investigations that clarify the role of both tumor- and MSC-derived exosomes in tumorigenesis. Besides identifying new components of tumor biology such studies may identify new therapeutic interventional agents.

3.3 Immune modulation

Apart from the ability of MSCs to contribute mitogens, ECM proteins, pro-angiogenic molecules, inflammatory agents, and exosomes to the TME, their most significant contribution may be modulating specific subsets of immune cells (Table 4)(Fibbe, Nauta et al. 2007; Nauta and Fibbe 2007; Bunnell, Betancourt et al. 2010; Roddy, Oh et al. 2011; Singer and Caplan 2011; Weiss, Bertoncello et al. 2011). The specific mechanism for this MSC role is not completely understood and may involve direct immune cell-MSC cell contact or indirect effects such as by the contribution of the factors just described or both. However, knowing the importance of immune and inflammatory cells in cancer growth and metastasis, the manner that MSCs in the TME might influence this process deserves closer attention and study.

Though initially described as an *ex vivo* phenomena requiring the stimulation of the MSCs to lead to suppression of T-lymphocyte activation or proliferation, many clinical trials have asserted immune modulation to be a primary effect of MSC-based therapies (Di Nicola, Carlo-Stella et al. 2002; Krampera, Glennie et al. 2003; Le Blanc, Rasmusson et al. 2004; Aggarwal and Pittenger 2005). In addition, these early observations prompted a number of studies to explore the distinct immune modulatory effects of MSCs derived from a variety of sources and species. Of note, although MSCs influence many immune cells, part of what makes them attractive candidates in cell-based therapies is their muted host immune responses even when delivered into a non-self (allogeneic) host. This is partly due to the fact that MSCs express low levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I, do not express co-stimulatory molecules (B7-1/CD80 and B7-2/CD86, CD40, or CD40L), and express MHC class II and Fas ligand only after specific stimulation.

MSCs are now known to inhibit dendritic cell maturation, B and T cell proliferation and differentiation, attenuate natural killer cell and mast cell activity, as well as support the production of suppressive T regulatory cells (Tregs) while attenuating pro-inflammatory Th17 cells (Table 4) (Najar, Raicevic et al. ; Di Nicola, Carlo-Stella et al. 2002; Krampera, Glennie et al. 2003; Aggarwal and Pittenger 2005; Beyth, Borovsky et al. 2005; Ramasamy, Fazekasova et al. 2007; Ren, Zhang et al. 2008; Uccelli, Moretta et al. 2008; Gur-Wahnon, Borovsky et al. 2009; Meirelles Lda, Fontes et al. 2009; Nemeth, Mayer et al. 2009; Bunnell, Betancourt et al. 2010; Salem and Thiemermann 2010; Tolar, Le Blanc et al. 2010; Brown, Nemeth et al. 2011; Singer and Caplan 2011).

Immune Response Arm	Cells	MSC effects
Innate	Dendritic Cells (APC)	Inhibition of maturation (CD80/86 expression) by STAT3 and IL10 (Beyth, Borovsky et al. 2005; Gur-Wahnon, Borovsky et al. 2009; Mezey, Mayer et al. 2009; Nemeth, Leelahavanichkul et al. 2009)
	Monocyte/Macrophages (APC)	PGE2 mediated increased IL10 secretion and attenuation of maturation (Beyth, Borovsky et al. 2005; Gur-Wahnon, Borovsky et al. 2009; Mezey, Mayer et al. 2009; Nemeth, Leelahavanichkul et al. 2009)
	Natural Killer Cells	Inhibition of proliferation and cytolytic activity (Giuliani, Oudrhiri et al. 2011)
	Mast Cells	COX-2 mediated suppression (Brown, Nemeth et al. 2011)
Adaptive	Th1	Inhibition of proliferation/activation (class switching) by HLA-G5, HGF, iNOS, COX2, IDO, PGE2, TGFβ and indirectly through support of immature APCs reviewed in (Singer and Caplan 2011)
	Th2	Inhibition of proliferation/activation (class switching) by HLA-G5, HGF, iNOS, COX2, IDO, PGE2, TGFβ and indirectly through support of immature APCs reviewed in (Singer and Caplan 2011)
	Tregs	Recruited and support (class switching) IL10, TGFβ, LIF
	Th17	Inhibition of proliferation/activation (class switching) by COX-2 and PGE2 (Duffy, Pindjakova et al. 2011; Duffy, Ritter et al. 2011)
	B lymphocyte	Suppression of terminal differentiation to plasma cell (Asari, Itakura et al. 2009)

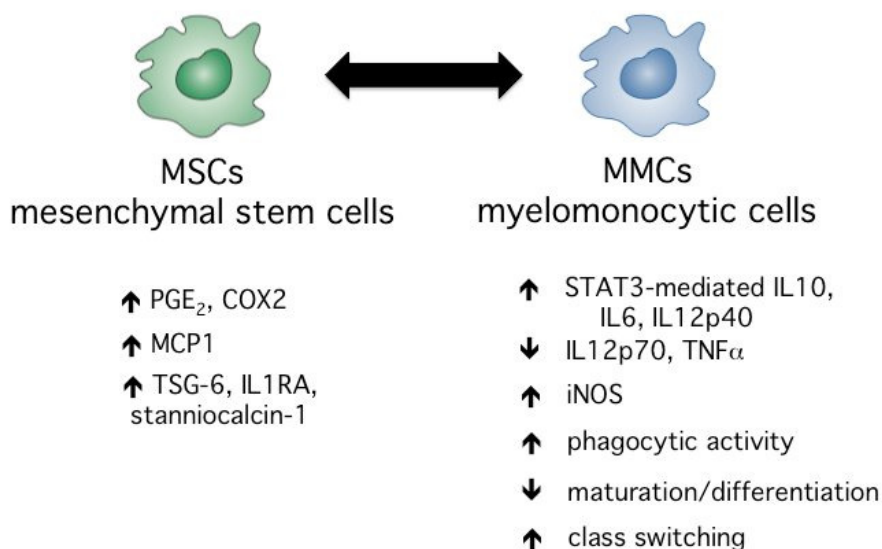
Abbreviations: COX-2- cyclooxygenase-2, HGF-hepatocyte growth factor (scatter factor), HLA-G- human leukocyte antigen-G, IDO- indoleamine 2,3-dioxygenase, iNOS-inducible nitric oxide synthase, IL10-interleukin-10, LIF-leukemia inhibitory factor, PGE2- prostaglandin-E2, STAT3- signal transducer and activator of transcription-3, TGFβ-transforming growth factor-β.

Table 4. Immune cells modulated by MSCs

3.3.1 MSCs and myelomonocytic cells

Although the details of the interactions of MSCs with T lymphocytes, B lymphocytes, natural killer cells, and dendritic cells have been investigated in some detail, the effects of MSCs on cells of myelomonocytic lineages (MMCs) observed early on by the Rachmilewitz group remained under investigated until recently (Figure 1. Beyth, Borovsky et al. 2005). The growing clinical evidence for MSCs as major regulators of immune and inflammatory processes and the central role played by MMCs (including monocytes and granulocytes) within them has sparked new interest in studies on the interplay between MSCs and MMCs. Kim and Hematti (2009) reported that human macrophages generated *in vitro* after co-culture with MSCs assume an immunophenotype defined as IL-10-high, IL-12-low, IL-6-high, and

Consequences of MSC-Myelomonocytic Cell Interaction



Abbreviations: COX-2- cyclooxygenase-2, IL-interleukin, IL-1RA- interleukin-receptor 1 antagonist, iNOS-inducible nitric oxide synthase, MCP-1-monocyte chemoattractant protein-1 (CCL2), PGE2-prostaglandin-E2, STAT3- signal transducer and activator of transcription-3, TSG-6- TNF-alpha stimulated gene/protein.

Fig. 1. The Consequences of the Interaction Between MSCs and Myelomonocytic Cells. Though still in their infancy the studies that have begun to identify the effect of the interactions between MSCs and MMCs whether cell-cell contact dependent or not have so far described those included in the figure. Please refer to the text for details.

TNF- α -low secreting cells (Kim and Hematti 2009). They proposed that these MSC-educated monocytes represent a unique and novel type of alternatively activated macrophage with a potentially significant role in tissue repair. Initially, Beyth *et al.* reported that human MSCs affect monocytes or dendritic antigen-presenting cell (APC) maturation in a contact-dependent manner (Beyth, Borovsky et al. 2005). Later, it was reported that the MSCs co-cultured with the APCs induced the expression of the anti-inflammatory IL10 and that activation of the signal transducer and activator of transcription 3 (STAT3) within APCs is linked to abnormal APC differentiation and function by a new contact-dependent mechanism, that plays a critical role in mediating the immunomodulatory effects of MSCs (Gur-Wahnon, Borovsky et al. 2007; Gur-Wahnon, Borovsky et al. 2009). In order to understand this process better, they further extended their studies to tumor cells since tumors secrete a variety of bioactive factors that activate STAT3 within infiltrating APCs. Their studies demonstrated that in at least certain cellular microenvironments, cell-to-cell dependent interactions represent a novel way to activate STAT3 signaling different from the activation of STAT3 seen with soluble bioactive factors. As such this observation suggests an uncoupling of APC activation events and that may consequently independently regulate

immunity and tolerance. In agreement with these studies, the Mezey group identified other pathways involved in MSC-murine macrophage interactions (Nemeth, Leelahavanichkul et al. 2009). They also showed that LPS-stimulated macrophages produced more IL-10 when cultured with MSCs, but this effect was eliminated if the MSCs lacked the genes encoding TLR4, myeloid differentiation primary response gene-88 (MyD88), TNF-receptor-1 α or cyclooxygenase-2 (COX-2). Their observations demonstrated that MSCs reprogram macrophages by releasing PGE2 that then acts on the macrophages through the prostaglandin EP2 and EP4 receptors. A unique population of MSCs isolated from human gingiva (GMSCs) with similar stem cell-like properties, immunosuppressive, and anti-inflammatory functions as bone marrow-derived MSCs were also studied in this context with similar effects (Zhang, Su et al. 2010).

When co-cultured with GMSCs, macrophages acquired an anti-inflammatory M2 phenotype similarly characterized by an increased expression of IL10 and IL6, mannose receptor (MR; CD206), a suppressed production of TNF α , and also decreased the ability to induce Th-17 cell expansion. Interesting to the discussion on tumors and their microenvironments, they demonstrated that systemically infused GMSCs could home to wounds-- specifically to sites where host macrophages were found-- promoted M2 polarization of the co-localized monocytes, significantly enhanced wound repair, and thus presumably could promote tumor growth by similar mechanisms. In addition, they noted that GMSC treatment suppressed local inflammation by reducing the infiltration of inflammatory cells and the production of IL6 and TNF α , and by increased expression of IL10. Another complementary study used murine macrophages stimulated with LPS and co-cultured with MSCs and found the suppression of TNF α , IL6, IL12p70 and interferon γ but increased levels of secreted IL10 and IL12p40. They noted that the murine MSC effect could be reproduced with MSC conditioned medium suggesting that bioactive factors constitutively released by the murine MSCs may be sufficient for the monocyte effect in this animal species (Maggini, Mirkin et al. 2010). They also found in cell-based therapy of mouse models that MSCs supported macrophages that showed a low expression of CD86 and MHC class II, and with a high ability to secrete IL10 and IL12p40, but not IL12 p70. They suggested in agreement with the other studies, that MSCs switch monocytes into a regulatory profile characterized by enhanced IL10 secretion, reduced inflammatory cytokine elaboration and enhanced phagocytic activity. Apart from elevated IL10 and related signaling mechanisms, other new players in the effects observed for MSCs on monocytes were recently advanced (Block, Ohkouchi et al. 2008; Block, Ohkouchi et al. 2009; Danchuk, Ylostalo et al. 2011; Prockop and Youn Oh 2011)]. Anti-inflammatory effects supported by MSC-monocyte interactions were suggested to also be partly mediated by elevated IL1 receptor antagonist (IL1RA) and by a negative feedback loop in which TNF α and other pro-inflammatory cytokines from resident macrophages activate MSCs to secrete the anti-inflammatory protein TNF α stimulated gene/protein 6 (TSG-6). These reports demonstrate that MSC derived TSG-6 acts to repress NF- κ B signaling in the resident macrophages causing attenuation of pro-inflammatory cytokine synthesis. The investigators of these studies also proposed that MSC secreted PGE2 promotes monocytes toward an IL10 secreting phenotype as well as, that anti-inflammatory effects may also be mediated by stanniocalcin-1.

Finally, in another recent report using pre-clinical murine models it was shown that MCP1 secreted by activated MSCs contributes to the bone marrow egress, trafficking, and

recruitment of monocytes towards remote sites (Shi, Jia et al. 2011). This elegant study demonstrated the intimate and complex cooperation that exists between MSCs and myelomonocytic cells that occurs not only in peripheral tissues or tumors but also in their originating bone marrow niche. It is widely recognized that tumor infiltrating cells can include macrophages, myeloid-derived suppressor cells (MDSCs), MSCs, and TIE2-expressing monocytes that are all mostly derived from the bone marrow. MDSCs represent a heterogeneous population of cells of myeloid origin that are expanded and activated in response to growth factors and cytokines released by tumors much like MSCs. The details of the effects of MDSCs on tumors are better understood. It is known that once MDSCs are activated, they accumulate in lymphoid organs and tumors where they exert specific T cell mediated immune suppression. However, not much is known about whether MDSCs and MSCs cooperate at tumor sites or the nature of that interaction. It is tempting to suggest that MSC-myelomonocytic cell interactions including MSC-MDSC ones represent an intriguing new target for cancer therapies that would break the anti-inflammatory tumor tolerance mechanisms established by these two cell types however, there is still much left to learn before this can come to fruition. Furthermore, while the vast majority of these reports demonstrate the ability of MSCs to suppress immune responses or act in an anti-inflammatory manner, there is emerging evidence that supports their contrasting ability to elicit pro-inflammatory responses-- which may also be mediated by their interaction with myelomonocytic cells. Both anti-inflammatory and pro-inflammatory effects will be important to know in dissecting their specific roles in tumors. This information will ultimately help in the design of more effective and targeted cancer therapeutics.

3.3.2 Immune suppressive or anti-inflammatory responses

The expression of IDO and iNOS by MSCs has been associated with its immune suppression of T-cell proliferation. Recently, secretion of IDO by MSCs therapeutically delivered in an experimental autoimmune myasthenia gravis model inhibited the proliferation of acetylcholine receptor-specific T cells and B cells and normalized the distribution of Th1, Th2, Th17 and Treg cells (Kong, Sun et al. 2009). IDO catalyzes the conversion of tryptophan, an essential amino acid for T-cell proliferation, into kynurenine. Immune suppression by IDO results from the local accumulation of tryptophan metabolites, rather than through tryptophan depletion (Ryan, Barry et al. 2007). Expression of IDO by MSCs was thought to be IFN- γ dependent (Krampera, Cosmi et al. 2006; Ryan, Barry et al. 2007; Bunnell, Betancourt et al. 2010). However, Opitz and colleagues recently demonstrated that IDO expression in MSCs can also be induced by activation of Toll-like receptor 3 (TLR3) and TLR4 via induction of an autocrine IFN- β signaling loop involving protein kinase R and independent of IFN- γ (Opitz, Litzenburger et al. 2009). Interestingly, when MSCs were treated with IFN- γ *in vitro*, they expressed extremely high levels of IDO and very low levels of iNOS, whereas mouse MSCs expressed abundant iNOS and very little IDO. These data suggest there is species variation in the mechanisms of MSC immunosuppression (Opitz, Litzenburger et al. 2009).

Prostaglandin E2 (PGE2) is emerging as a central mediator of many of the anti-inflammatory properties of MSCs (Nauta and Fibbe 2007; Uccelli, Moretta et al. 2008). PGE-2 is synthesized from arachidonic acid by cyclooxygenase (COX) enzymes COX-1 and COX-2.

COX-1 is constitutively expressed in MSCs and COX-2 expression can be induced by inflammatory cytokines such as IL-1 β , IL-6, IFN- γ , and TNF- α (Chen, Wang et al. 2010). Inhibitors of PGE2 synthesis attenuated MSC suppression of T cells and natural killer cells (Sotiropoulou, Perez et al. 2006; Chen, Wang et al. 2010). PGE2 is associated also with the MSC-mediated inhibition of dendritic cell maturation. Nemeth *et al.* reported that activated MSCs released PGE2 causing increased production of IL10 by macrophages, and decreased production of the pro-inflammatory cytokines TNF- α and IL-6 in a murine sepsis model (Sotiropoulou, Perez et al. 2006). Maggini *et al.* similarly reported macrophage alterations by PGE2 (Maggini, Mirkin et al. 2010).

Mezey's group demonstrated that COX-2 is also involved in MSCs ability to suppress mast cell activation (Brown, Nemeth et al. 2011). Mast cells (MCs) have a key role in the induction of allergic inflammation and contribute to the severity of certain autoimmune diseases. An increasing body of literature also implicates MCs in the TME to affect tumor inflammation, angiogenesis, and growth (Ribatti, Nico et al. 2011). To date, few studies have investigated the potential of mast cell-MSC interactions. Since MCs are critical effector cells in allergic inflammation and they represent an important cell type to therapeutically target using the immune modulatory properties of MSCs, Mezey's group set out to study murine MC-MSCs effects. They reported that MSCs effectively suppressed specific MC functions *in vitro* and in animal models. MCs co-cultured with MSCs in direct contact, had dampened MC degranulation, pro-inflammatory cytokine production, chemokinesis, and chemotaxis. They also found that MC degranulation within mouse skin or the peritoneal cavity was suppressed following delivery of MSCs. Lastly, they discovered that these inhibitory effects were dependent on COX2 in MSCs (Brown, Nemeth et al. 2011).

Transforming growth factor- β (TGF β) is an anti-inflammatory cytokine that is constitutively expressed by MSCs. The immune modulatory function of MSCs on T cells and natural killer cells can be impaired by treatment with neutralizing antibodies to TGF β (Di Nicola, Carlo-Stella et al. 2002; Sotiropoulou, Perez et al. 2006). In contrast, several studies have also established that TGF β had no effect on the immunosuppressive properties of MSCs (Tse, Pendleton et al. 2003; Xu, Zhang et al. 2007). These discrepancies are likely explained by differences in species or experimental conditions. The importance of TGF β in MSC therapy was recently established in a mouse model of ragweed-induced asthma. Mezey's group again demonstrated this assertion with neutralizing antibodies and the use of MSCs derived from TGF β knockout mice (Nemeth, Keane-Myers et al. 2010). Notably, the number of Tregs in this model was elevated by the MSC-therapy. However, the role of TGF β in this process was not directly studied, as was done by Patel *et al.* who showed that in co-cultures of peripheral blood mononuclear cells (PBMCs) with MSCs, TGF β produced by MSCs resulted in increased numbers of Tregs (Patel, Meyer et al. 2010).

Several other factors are associated with the potential anti-inflammatory properties of MSCs including HLA-G, hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), IL1 receptor antagonist (IL1RA), CCL2, galectin-3, galectin-1 and semaphorin-3A, most of which attenuate T lymphocyte activation and are highly expressed by MSCs (Di Nicola, Carlo-Stella et al. 2002; Ortiz, Dutreil et al. 2007; Di Ianni, Del Papa et al. 2008; Kang, Kang et al. 2008; Nasef, Ashammakhi et al. 2008; Rafei, Hsieh et al. 2008; Lepelletier, Lecourt et al. 2009; Selmani, Naji et al. 2009; Sioud, Mobergslien et al. 2010; Volarevic, Al-Qahtani et al. 2010). A recently advanced culprit is TNF- α -induced protein 6 TNAIP6 or TSG-6 (Lee, Pulin et al.

2009; Prockop and Youn Oh 2011). TSG-6 secretion is known to suppress inflammation through the inhibition of the inflammatory network of proteases primarily by increasing the inhibitory activity of inter- α -inhibitor, sequestration of hyaluronan fragments, and decreasing neutrophil infiltration into sites of inflammation. In a model of acute inflammation induced by myocardial infarction, TSG-6 knockdown in MSCs significantly reduced their anti-inflammatory therapeutic effect. The administration of recombinant TSG-6 protein largely duplicated the therapeutic effects of the delivered MSCs on inflammatory responses and infarct size (Getting, Mahoney et al. 2002; Wisniewski and Vilcek 2004; Milner, Higman et al. 2006; Forteza, Casalino-Matsuda et al. 2007; Lee, Pulin et al. 2009). Together these results make TSG-6 an interesting new factor in the anti-inflammatory effects of MSCs.

3.3.3 Pro-Inflammatory MSC responses

Though we are beginning to better understand the many complex mechanisms associated with the secretion by MSCs of immune suppressive mediators like TSG-6, so far only a few reports have described a contrasting pro-inflammatory activity of MSCs that could be important in understanding the distinct role of MSCs in tumors. Indeed, the observation of this distinct MSCs immune effect came from studies primarily focused on the downstream consequences of TLR stimulation within these cells. TLRs are a conserved family of receptors that recognize pathogen-associated molecular patterns (PAMPs) and promote the activation of immune cells (Wright 1999-76; Triantafilou, Triantafilou et al. 2001; Sabroe, Read et al. 2003; Anders, Banas et al. 2004; Miggin and O'Neill 2006; West, Koblansky et al. 2006; Bunnell, Betancourt et al. 2010). Many TLRs (TLR1 to TLR13) have been identified and characterized in a variety of immune cell types and species. Agonists for TLRs include exogenous microbial components, such as LPS (TLR2 and 4), lipoproteins and peptidoglycans (TLR1, 2, 6), viral RNA (TLR3), bacterial and viral unmethylated CpG-DNA (TLR9), and endogenous molecules shed following cell injury, including heat shock proteins and extracellular matrix molecules (Wright 1999-77; Triantafilou, Triantafilou et al. 2001; Sabroe, Read et al. 2003; Anders, Banas et al. 2004; Miggin and O'Neill 2006; West, Koblansky et al. 2006; Bunnell, Betancourt et al. 2010). Specific agonist engagement of TLRs leads to the expression of inflammatory cytokines or co-stimulatory molecules by a MyD88 (a TLR adapter protein)-dependent or MyD88-independent signaling pathways and can promote chemotaxis of the stimulated cell. TLRs are differentially expressed on leukocyte subsets and non-immune cells and may regulate important aspects of innate and adaptive immune responses (Mempel, Voelcker et al. 2003; Hwa Cho, Bae et al. 2006; Nagai, Garrett et al. 2006; Pevsner-Fischer, Morad et al. 2006; West, Koblansky et al. 2006; Tomchuck, Zwezdaryk et al. 2008).

MSCs are among the cells that express an array of TLRs, including TLR2, 3, 4, 5, 6 and 9 (Hwa Cho, Bae et al. 2006; Pevsner-Fischer, Morad et al. 2006; Tomchuck, Zwezdaryk et al. 2008). Furthermore, studies by our group established that the stimulation of MSCs with TLR agonists led to the activation of downstream signaling pathways, including NF- κ B, AKT, and mitogen-activated protein kinase (MAPK). Consequently, activation of these pathways triggers the previously unreported induction and secretion of pro-inflammatory cytokines, chemokines, and related TLR gene products. Interestingly, the unique patterns of affected genes, cytokines, and chemokines measured identified the TLRs as potential players in the

established MSC immune modulatory properties, as well as their ability to migrate towards injured tissues. Surprisingly, we noted that TLR4 stimulation with LPS led to the secretion of primarily pro-inflammatory mediators, such as IL-1 β and IL6 (Tomchuck, Zwezdaryk et al. 2008). Though unexpected, previous observations reported by Beyth *et al.* recognized that LPS priming affected co-cultures of leukocytes with human MSCs and attenuated the expected human MSC- mediated inhibition of T-lymphocyte activation as well as affected their capacity to secrete interferon (Beyth, Borovsky et al. 2005). More recently, Romieu-Mourez *et al.* showed that TLR stimulation in murine MSCs similarly resulted in the production of inflammatory mediators, such as IL-1, IL-6, IL-8, and CCL5 (Romieu-Mourez, Francois et al. 2009). Furthermore, they demonstrated that TLR and IFN activated murine MSCs injected within Matrigel matrices into mice resulted in the formation of an inflammatory site attracting innate immune cells and resulting in a dramatic recruitment of neutrophils. Raicevic *et al.*, studying the effect of TLR activation within MSCs in an inflammatory milieu, observed that this environment shifted the cytokine profile to a pro-inflammatory one rather than the expected immunosuppressive one (Raicevic, Rouas et al. 2010). They similarly observed an increase in IL-1 β , IL-6, and IL-12 after TLR activation in this inflammatory context.

Though somewhat confounding, this recent body of work on the downstream consequences of TLRs provides emerging evidence for a new pro-inflammatory immune modulating role for MSCs. The identification of the molecular details for this new pro-inflammatory MSC role, and whether it is innate or just an *in vitro* artifact, awaits further investigation. However, this novel observation is important to consider given the accelerated use of MSCs in anti-inflammatory cell-based therapies. Additionally, as Raicevic *et al.* suggest targeting of TLRs in MSCs, may avoid deleterious consequences in their use as anti-inflammatory therapies (Raicevic, Rouas et al. 2010). By contrast, TLR-activated pro-inflammatory MSCs could prove useful in breaking tolerance in the therapy of immune evasive diseases, such as cancer.

4. New MSC paradigm: Pro-Inflammatory MSC1 and Anti-Inflammatory MSC2

Our recent studies are partly an attempt to resolve some of the controversy surrounding the potential of MSCs to be anti-inflammatory in some cases and pro-inflammatory in others or to be pro-tumor in some cancers and anti-tumor in others, as described above. These studies led us to propose a new paradigm for MSCs based on the premise that these heterogeneous cells can be induced to polarize into two distinct but homogeneously acting phenotypes--that we modeled after monocytes, the other heterogeneous bone marrow-derived cells (Figure 2. Verreck, de Boer et al. 2006).

It is established that stimulation of monocytes with known cytokines or agonists to their TLRs, including IFN- γ and endotoxin (LPS, TLR4-agonist), polarizes them into a classical M1 phenotype that participates in early pro-inflammatory responses. IL-4 treatment of monocytes yields the alternative M2 phenotype that is associated with anti-inflammatory resolution responses (Verreck, de Boer et al. 2006). We proposed that MSCs, like monocytes, are polarized by downstream TLR signaling into two homogeneously acting phenotypes, classified as MSC1 and MSC2, following the monocyte nomenclature. We reported that TLR4 agonists polarized MSCs toward a pro-inflammatory MSC1 phenotype while the downstream consequences of TLR3 stimulation of MSCs was a skewing toward an anti-inflammatory MSC2 phenotype. This novel MSC polarization paradigm is based on the

consistent but novel outcomes observed for *MSC1* when compared with *MSC2* for several parameters, including dissimilar patterns of secretion of cytokines and chemokines and differences in differentiation capabilities, extracellular matrix deposition, TGF- β signaling pathways, and Jagged, IDO and PGE-2 expression (Waterman, Tomchuck et al. 2010). The most compelling outcome was opposite effects of each cell type on T-lymphocyte activation (Waterman, Tomchuck et al. 2010).

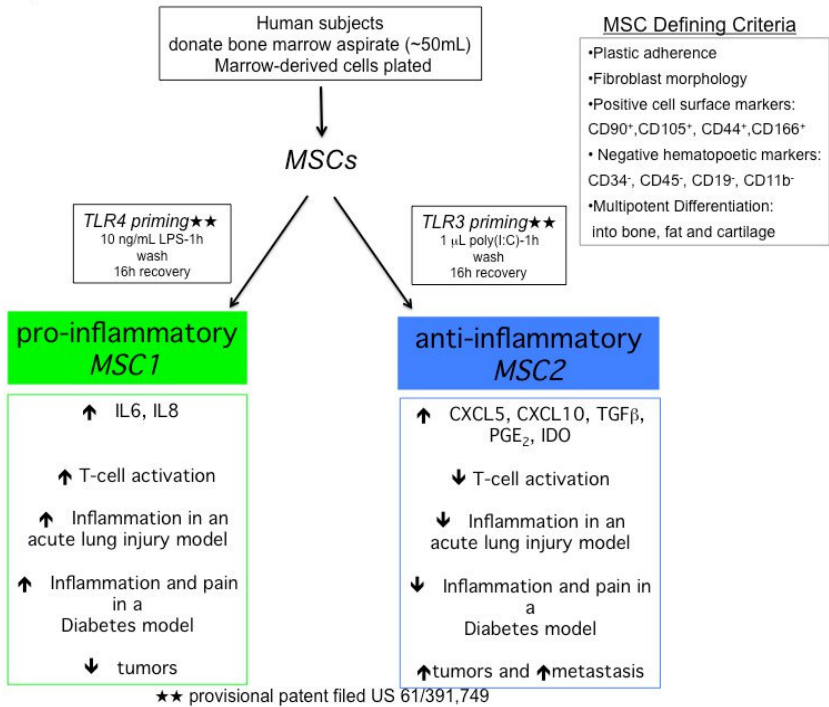


Fig. 2. Characteristics of the MSC1 and MSC2 Phenotypes. Short-term and low-level priming of TLR4 (left side) and TLR3 (right side) leads to the induction of heterogeneous hMSC preparations into a pro-inflammatory *MSC1* phenotype or an anti-inflammatory *MSC2* phenotype. (adapted from (Tomchuck, Zvezdaryk et al. 2008; Waterman, Tomchuck et al. 2010).

4.1 Evidence for *MSC1* and *MSC2*

Our previous work, as well as that of others, established that MSCs reside in TMEs or tumor stroma, provide structural support for the malignant cells, modulate the tumor microenvironment, and consequently promote tumor growth and spread. Therefore, gene-modified MSCs that can act as “Trojan horses” and deliver anti-cancer therapeutics into the tumor stroma are being evaluated as a promising new specific cell-based therapy for cancer. We also previously established that MSCs recruited to ovarian tumors by elevated secretion of LL-37 play a supportive role in ovarian tumor stroma. We found that specific induction of MSCs into *MSC1* causes the secretion of pro-inflammatory mediators rather than anti-

inflammatory ones, as well as promotes collagen rather than fibronectin deposition into the extracellular matrix (Figure 1)(Waterman, Tomchuck et al. 2010). Our preliminary studies support the notion that *MSC1* may be effective in new cell-based treatment of cancers. Indeed, ovarian cancer cell lines co-cultured with *MSC1* formed smaller tumor spheroids and had markedly reduced tumor colony forming potential; whereas, co-cultures with *MSC2* phenotype had the expected pro-tumor effect. Moreover, *MSC1*-treated ovarian cancer cells were less invasive than *MSC2*-treated ones in matrigel coated transwell migration assays. Pilot tests in murine ovarian cancer models were consistent with these findings. *MSC1* delivered in mice with established tumors had attenuated growth and spread. Mice treated with *MSC2* had larger and more metastatic tumors.

MSC1 and *MSC2* therapy has been successfully tested in several animal disease models and has resulted in predictable inflammatory responses and distinct effects on tumor growth and spread (Table 5).

Animal Disease Model	MSC-based Therapy	MSC Dose (cells)	Treatment Frequency (Time of treatment)	Disease Impact	Length of study	Adverse Effects
1. LPS-induced Acute Lung Injury (ALI) (BalbC and C57BL/6J, n=12)	MSCs	0.5X10 ⁶	1X (24hrs post-disease onset)	Mostly anti-inflammatory	1 week post-treatment	NONE
	<i>MSC1</i>	0.5X10 ⁶	1X (24hrs post-disease onset)	Pro-inflammatory	1 week post-treatment	NONE
	<i>MSC2</i>	0.5X10 ⁶	1X (24hrs post-disease onset)	Anti-inflammatory	1 week post-treatment	NONE
2. Streptozotocin-Induced Diabetes and neuropathic pain (C57BL/6J, n=30)	MSCs	1-3X10 ⁶	3X (given in 10-day intervals post-disease onset)	Mostly anti-inflammatory	70 days post-treatment	NONE
	<i>MSC1</i>	1-3X10 ⁶	3X (given in 10-day intervals post-disease onset)	Pro-inflammatory	70 days post-treatment	NONE
	<i>MSC2</i>	1-3X10 ⁶	3X (given in 10-day intervals post-disease onset)	Anti-inflammatory	70 days post-treatment	NONE
3. Immune-incompetent human tumor xenografts (Balb scid and nude n=60)	MSCs	0.5X10 ⁶	3X (given weekly post-disease onset)	Mostly anti-inflammatory	>120 days post-treatment	NONE
	<i>MSC1</i>	0.5X10 ⁶	3X (given weekly post-disease onset)	Pro-inflammatory	>120 days post-treatment	NONE
	<i>MSC2</i>	0.5X10 ⁶	3X (given weekly post-disease onset)	Anti-inflammatory	>120 days post-treatment	NONE
4. Immune-competent MOSEC (C57/BL6J n=20)	MSCs	0.5X10 ⁶	3X (given weekly post-disease onset)	Mostly anti-inflammatory	>70 days post-treatment	NONE
	<i>MSC1</i>	0.5X10 ⁶	3X (given weekly post-disease onset)	Pro-inflammatory	>70 days post-treatment	NONE

Table 5. Human MSC-based therapy of murine disease models.

Please NOTE that for all of the data presented MSCs represent conventionally prepared human MSCs, *MSC1* are defined as the hMSCs incubated for 1hr with 10 ng/mL LPS and washed prior to delivery. *MSC2* are defined as the hMSCs incubated for 1hr with 1 mg/mL poly(I:C) and washed prior to delivery (provisional patent filed US 61/391,749).

Cancer models: Pilot studies with the mouse ovarian cancer model (MOSEC) and with a xenograft model demonstrate our assertions. A single delivery of *MSC1*-based therapy resulted in slower growing tumors, whereas comparable therapy with *MSCs* or *MSC2* resulted in larger tumors and metastasis at the end of the study (day 65, Figure 3).

MSC1 do not support tumor growth whereas *MSC2* favor tumor growth and metastasis

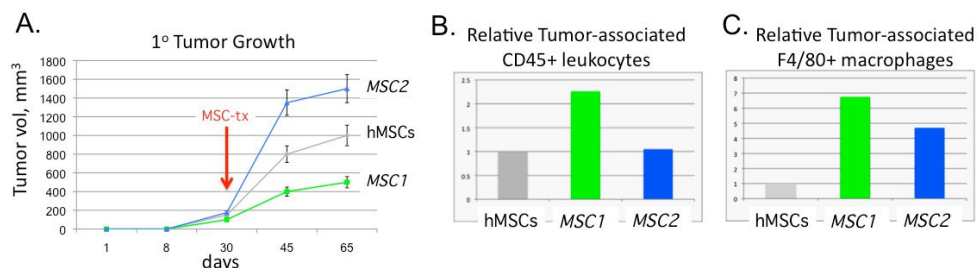


Fig. 3. *MSC1* do not support tumor growth whereas *MSC2* favor tumor growth and metastasis. The data show differences in tumor volume, CD45+leukocyte, and F4/80+ macrophage recruitment after the treatment of mice with established ovarian tumors, with human *MSC1*- and *MSC2*-based therapies. **Methods** The established syngeneic mouse model for epithelial ovarian cancer used is based upon a spontaneously transformed mouse ovarian surface epithelial cell (MOSEC) line ID8 that has been previously described (Roby, Taylor et al. 2000). 4-6 week-old female mice ($n > 10$ mice/MSC-treatment) were injected subcutaneously (s.c.) in the right hind leg with 1×10^7 MOSEC cells. At approximately 4 weeks a single dose of labeled human MSCs (hMSCs), *MSC1*, or *MSC2* (1×10^6 /per mouse) were injected intraperitoneally (IP) as indicated by red arrow $\downarrow$. (A.) Tumor growth was measured with callipers as standard at weekly intervals until day of mouse sacrifice (Day 65). Harvested tumors and metastasis were weighed, counted and processed for flow cytometry and immunohistochemical analysis (IHC, Coffelt et al., 2009). Metastasis was found only in *MSC2*-treated mice (data not shown). MSCs were detected by flow cytometry and IHC. All MSC-treated samples had similar detectable MSCs within the tumor tissue-trending towards more *MSC1* and *MSC2* measured than hMSCs: approximately 15-25 cells counted per 200X field after 24hr of MSC-treatment and 2-5 cells at time of tissue harvest (day 65, data not shown). Sectioned tumor sample slides were stained with murine CD45 (B.) or F4/80 (C.) antibodies and the number of positively stained immune cells per 200X field were scored as described previously (Coffelt et al., 2009). Data are expressed as average cells counted in 4 fields/slide relative to hMSC sample. Data indicate in vivo stability and predictably distinct effects by the *MSC1* and *MSC2*.

ALI model: In an established endotoxin-induced acute lung injury (ALI) mouse model, LPS, or endotoxin (0.1 mg/kg) was instilled intratracheally into adult Balb/C mice. After 24 hrs, mice were each treated with 0.5×10^6 MSCs, *MSC1*, *MSC2*, or HBSS vehicle. To characterize inflammation, the lungs of the animals were lavaged and bronchioalveolar lavage fluid (BALF) was analyzed after 24 hr for changes in neutrophil/monocyte recruitment (myeloperoxidase activity), total cell content by flow cytometry, and lung integrity by total protein leaked into the BALF ($n = 12$). *MSC1*-therapy aggravated the disease and resulted in

increased neutrophil recruitment and more compromised lungs than the conventional MSC or MSC2 therapy.

Diabetes Model: Streptozotocin (STZ)-induced diabetic mice were procured from Jackson Laboratory (Bar Harbor, Maine). Blood glucose levels and animal weights were measured by standard methods. A month post STZ-injection, mice received intraperitoneally (IP) 0.5×10^6 cells of MSCs, MSC1, MSC2, or HBSS vehicle for a total of 3 times in 10-day intervals. Established behavioral assays to evaluate hyperalgesia and allodynia were conducted one day prior to each MSC therapy, as well as prior to sacrifice. Inflammatory factors and immune cell changes were measured as before to characterize the treatment effects on inflammation ($n=30$). Again, all indicators were consistent with enhanced inflammation by MSC1-treatment and an improvement of disease by the MSC2- or MSC-treated animals. *Manuscript in preparation.*

Additionally *in vitro* studies show divergent effects of MSC1 and MSC2 on cancer cells. Co-culture of various human cancer cell lines with MSC1 and MSC2 in Colony Forming Units (CFU) assays and 3-D tumor spheroid assays agree with the *in vivo* tumor models with different MSC1 and MSC2 treatment effects (Figure 4).

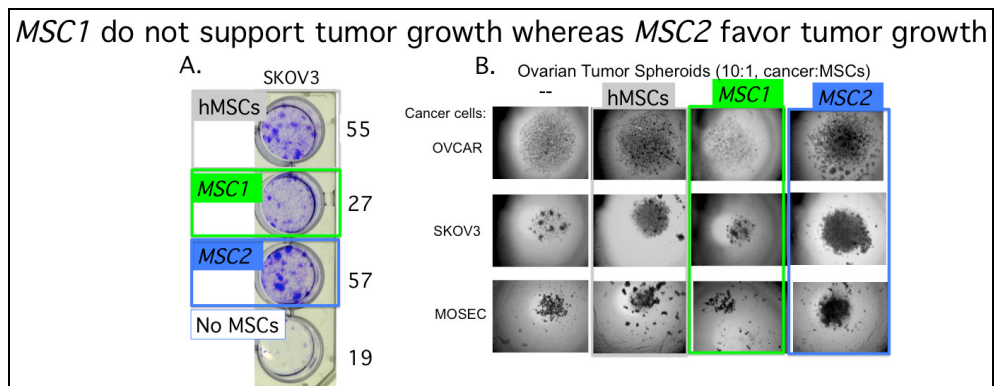


Fig. 4. MSC1 do not support tumor growth whereas MSC2 favor tumor growth: A. Data demonstrates that there are distinct effects on colony forming units (CFU) after coculture of different human cancer cell lines with untreated MSCs (hMSCs), MSC1, or MSC2. Methods: CFU assay was performed by culturing human tumor cells (200 cells/well) mixed with hMSCs, MSC1, or MSC2 (2 cells/well) at a ratio of 10 cancer cells per 1 MSC and plated in 24-well plates in growth medium supplemented with 10% FBS as indicated in figure. Cultures were grown for 14 days at 37°C in a humidified atmosphere of 5% carbon dioxide balance air. Growth medium was changed every 3-4 days. Colonies were visualized by staining with a crystal violet solution (0.5% crystal violet/10% ethanol). The resulting colonies were enumerated by the colony counting macro in ImageJ software, SKOV3-ovarian cancer cell lines. Micrographs of the stained plates are shown. Colony counts are at right. ($n=8$) B. Data demonstrates that there are distinct effects on tumor spheroids after coculture of different cancer cell lines with unprimed MSCs, MSC1, or MSC2. Methods: Tumor spheroids were formed by culturing tumor cells (2000 cells/well) mixed without any other cells (--) or with hMSCs, MSC1, or MSC2 (20 cells/well) at a ratio of 10 cancer cells per

1 MSC and plated over 1.5% agarose in 96-well plates in growth medium supplemented with 10% FBS as indicated in figure. Cultures were grown for 14 days at 37°C in a humidified atmosphere of 5% carbon dioxide balance air. Growth medium was changed every 3-4 days. Micrographs shown represent 20X magnified field of the 96-well plate. Cancer cell lines used are: OVCAR-human ovarian cancer, SKOV3-human ovarian cancer cell lines, and MOSEC-murine ovarian surface epithelium carcinoma cells. Data indicate distinct effects by MSC1 and MSC2 on cancer cell growth and spread.

5. Conclusion

The unique pathology of individual tumors presents a huge problem for conventional mono-specific therapies. New approaches aiming at developing effective treatments against cancer include the use of MSC-based therapies. There are many features that make this new strategy attractive and feasible. First, MSC-based therapies are already in clinical use and thus far have not been associated with adverse effects. Second, MSCs can be easily expanded and stored without any impact to their capabilities—a phenomenon that has triggered the creation of many new biotech start-ups. Third, once delivered, MSCs preferentially home to tumors and affect tumor growth and spread. Fourth, MSCs from non-self (allogeneic) or autologous (self) hosts can be safely delivered since they do not elicit immunity. Lastly, pre-clinical studies have demonstrated efficacy with genetically-engineered MSCs that carry anti-cancer therapeutics that reached the tumors and prevented their growth.

MSCs targeted to cancers are expected to contribute many soluble factors such as mitogens, extracellular matrix proteins, angiogenic and inflammatory factors, as well as exosomes with as yet poorly defined potentials, once resident in the TME. MSCs are also expected to affect tumor-associated leukocytes either directly by cell-cell contact or indirectly by the secretion of trophic factors. MSCs are known to affect the proliferation and differentiation of dendritic cells, monocytes/macrophages, B and T cells, NK cells, and even mast cells. There has been a great deal of debate in the field in trying to assert whether MSCs resident in the TME contribute to tumor growth and spread or prevent it, and if so, by what mechanisms. Many reasons have been advanced to explain the contradictory MSC role in cancer including the heterogeneity of MSC preparations, the age or health of the MSC donor, and the experimental model or condition, to name a few. Our group has suggested a new paradigm for MSCs that we believe will help resolve some of the conflicting issues. The induction of MSCs into uniform and consistently acting pro-inflammatory MSC1 or anti-inflammatory MSC2 phenotypes should provide convenient experimental tools that dissect the potential pro- and anti-tumor contributions of MSCs. MSC-based therapies stand to revolutionize medicine with the myriad ways that they can be manipulated and guided to reach pathologic tissue sites such as tumors. The continued investigation of these cells will ensure safe and effective therapy of human disease.

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