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Sarah Romanelli December 8, 2020

# Clinical Applications of Mesenchymal Stromal Cells

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Clinical Applications of Mesenchymal Stromal Cells

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Mesenchymal stromal cells (MSCs) are multipotent progenitor cells which have been shown to exhibit immunomodulatory properties as well as to preferentially migrate to areas of upregulated immune response, such as tumors and sites of inflammation. When modified with the cytokine interferon gamma (IFN-γ), the immunomodulatory properties of MSCs can be enhanced. Therefore, MSCs present as an attractive candidate for cell therapy. Osteosarcoma is the most common malignant primary bone tumor in children and adolescents. Because metastatic, chemotherapy resistant, and relapsed osteosarcoma often results in poor prognoses, development of a targeted treatment is most crucial. The tumor microenvironment (TME) has been shown to be composed of various cellular components including tumor associated macrophages (TAMs), which exhibit different tumor proliferation effects based on their phenotype along an M1 to M2 continuum. With knowledge of MSCs migration to the tumor site and immunomodulatory properties, this study aims to use genetically modified MSCs as a targeted treatment delivery system for IFN-γ in order to increase anti-tumor M1 polarization. Future research aims to determine if delivery of MSCs transduced with IFN-γ can allow for an increased ratio of M1 to M2 macrophages in order to improve patient prognosis. Additionally, MSCs can be used for the treatment and prophylaxis of acute Graft-versus-Host Disease (aGvHD). aGvHD is a complication of blood and marrow transplantation in which donor T cells attack the patients' healthy tissues. aGvHD is most effectively treated with glucocorticoids and various immunosuppressive drugs, however patients with steroid-resistant aGvHD often face poor prognosis. Therefore, this study aims to once again harness the migration of MSCs towards areas of high immunoactivity, such as activated T cells following transplantation. Consistent with our hypothesis, more MSCs were found in organs associated with the immune response, such as the spleen and mesenteric lymph nodes. It is hypothesized that increasing the immunosuppressive effects of MSCs via priming with IFN-γ will allow for the suppression of T cells and therefore reduced aGvHD. Through the lens of two different diseases, this study investigates the various clinical applications of MSCs.

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# INTRODUCTION

## Characteristics of Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are spindle shaped, plastic-adhered multipotent progenitor cells[1]. First identified by Friedenstein et al., 1987, MSCs can be derived from multiple tissues[2] including adipose tissue[3, 4], dental tissues[5], dermis[6], limb buds, menstrual blood, and perinatal tissues[7-10]. Since their discovery, MSCs have bene shown to act as hematopoietic supportive cells in the bone marrow microenvironment in order to support hematopoiesis. However, MSCs are non-hematopoietic cells: they are simply able to produce soluble proteins that critically support hematopoietic stem cells. Though originally thought to act as stem cells due to their ability to give rise to bone, cartilage, tendon, ligament, marrow stroma, adipocytes, dermis, muscle and connective tissue, MSCs are now classified as adult progenitor cells and are no longer thought of as a homogenous population of stem cells.

As of 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) defined the minimal criteria that MSCs should present, followed by several updates mainly focusing on the refinement of standards for therapeutic efficacy[11-14]. These criteria include: remaining plastic-adherent under standard culture conditions; expressing 95% of the surface markers CD105 (transforming growth factor β receptor III or endoglin), CD73 (ecto-5'-nucleotidase) and CD90 (Thy-1); lacking expression of CD45 (pan-leukocyte marker), CD34 (hematopoietic progenitor and endothelial cell marker), CD14 (macrophage marker), CD11b (monocyte marker), CD79a or CD19 (B-cell markers), and HLA-DR; and retain the ability to differentiate into osteoblasts, adipocytes, and chondrocytes in vitro. Other surface antigens generally expressed by MSCs include CD13, CD29, CD44, and CD10[15, 16].

Besides their differentiation potential, MSCs possess numerous other characteristics including *in vitro* and *in vivo* immunoregulatory properties[17, 18], trophic effects mediated by a wide range of growth factors and cytokines produced,[19] and the ability to migrate toward inflammatory sites[20-23]. The broad range of *in vivo* effects of MSCs have prompted relevant interest in several biomedicine fields such as immunology, regenerative medicine, and more recently, in gene therapy applications[24-27].

The immunomodulatory properties of MSCs have become a research topic of particular interest. Specifically, MSCs have previously been shown to suppress T cell and B cell proliferation[28] as well as to modulate both adaptive and innate immunity *in vitro* and *in vivo*[29, 30]. The mechanism by which MSCs mediate immunosuppression is yet to be deduced, however previous studies have suggested that the suppression of CD25 expression in T cells is involved. Previous research has also indicated that the cytokine interferon-gamma (IFN-γ) may upregulate the immunosuppression provided by MSCs[31].

Notably, MSCs have been shown to be hypoimmunogenic. In humans, MSCs express very low surface levels of Major Histocompatibility Complex (MHC) class I molecules.[32, 33] This minor expression of MHC class I molecules allows for MSCs to escape cell death by natural killer (NK) cells. Additionally, MSCs express neither MHC class II molecules nor costimulatory molecules, such as CD40, CD40L, CD80, and CD86. This lack of MHC class II expression allows MSCs to escape immune recognition by CD4 cells. Therefore, the ability of MSCs to escape from the immune response while also being able to modulate T cell proliferation makes MSCs an attractive candidate for cell therapy, even if the mechanism behind immunosuppression is still unknown.

Once migrated to the site of interest, MSCs can be manipulated to have a wide range of effects. Transduced MSCs could act as a targeted treatment delivery system – releasing a specific

therapeutic compound of interest into the cellular microenvironment. Alternatively, the various immunomodulatory properties of MSCs, as previously described, could be enhanced to suppress T cell activation. Therefore, it is clear that MSCs have many potential clinical applications.

The Horwitz lab specifically focuses on these many biological and clinical applications of MSCs. His research team was the first to infuse allogenic MSCs into patients and the first to infuse genetically modified MSCs into patients. Most recently, the Horwitz lab has shown that transduced, cytokine secreting MSCs can be injected into a solid tumor in a murine xenograft model and modify the tumor microenvironment which impairs tumor growth[34, 35]. In a second avenue of research, they are working to identify a novel mechanism of IFN-γ primed MSCs (γMSCs)-mediated immune modulation that may be especially important for the prevention of acute Graft-vs-Host Disease (aGvHD), a complication of blood and marrow transplantation in which donor T cells attack the patients' healthy tissues.

During this last year, my research activity in Horwitz's lab was focused on both fields of research. For the first 6 months, I investigated the migration of human MSCs to specific osteosarcoma patient cells lines and the significance of Tumor Associated Macrophages (TAMs) polarization on osteosarcoma viability *in vitro*. While, during the last months, I investigated the migration of intravenously injected γMSCs after syngeneic or allogeneic Bone Marrow Transplantation (BMT) for aGvHD prophylaxis and treatment. Therefore, this study aims to harness the migratory capabilities of MSCs for application toward two different clinical contexts: osteosarcoma and aGvHD.

**MSCs as a targeted anti-tumor vehicle for Osteosarcoma**

Osteosarcoma is the most common malignant primary bone tumor in children and adolescents and is prone to unfavorable prognosis. Due to its wide metastatic capabilities, an

entirely effective treatment has not yet been established. Osteosarcoma usually occurs in long bones, but in in 20-30% of cases it can metastasize to other bones or to the lungs. Of patients who enter metastasis or relapse, overall survival is around 10-30%[36]. By compassion, 70% of patients are able to achieve remission for localized osteosarcoma. Unfortunately, there have not been any significant advances in osteosarcoma treatment in the past 40 years despite considerable research effort[37]. Therefore, novel treatment development, particularly against osteosarcoma metastasis, is most crucial.

The tumor microenvironment (TME) is made up of stroma, vasculature, extracellular matrix (ECM), signaling molecules, and immune cells[38] and has been recognized as an important component of therapy resistance and tumor progression. Although each tumor builds its own unique TME, the critical components that comprise the TME and their roles in tumor progression are common between different cancer types. Recently, immunosuppressive cancer microenvironments have been recognized as major impediments and key factor to the efficacy of chemotherapy or checkpoint inhibitors of immunotherapy[39], due to the presence of tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs), which have an immune-mediated anti-tumor effect.

The immune context of the osteosarcoma microenvironment is mainly composed of TAMs, with a significant number of dendritic, lymphoid, and myeloid cells[40]. Macrophages are heterogenous cells that are of particular interest in the context of osteosarcoma due to their malleable phenotype in response to local environmental cues along an M1 to M2 phenotype continuum. Aghighi et al. found that bone sarcomas and lymphomas have significantly different MRI enhancement and TAM density, demonstrating that ferumoxytol-enhanced MRI can be used to differentiate tumors with different TAM content and monitor tumor response to TAM-

targeted immunotherapies *in vivo*[41]. Though it is widely accepted that different tumors exhibit different TAM densities, naturally occurring TAMs typically express an M2 macrophage phenotype. M2 macrophages physiologically promote tissue repair, but also tumor growth. They are associated with increasing tumor proliferation, angiogenesis, and metastasis. In contrast, M1-polarized macrophages foster inflammation and immune clearance of pathogens[42, 43], giving rise to an overall tumor suppressing affect. Specifically, M1 macrophages stimulate cytotoxic T cells to secrete IFN-γ, which then triggers tumor cell killing. Therefore, macrophage polarization is hypothesized to play a crucial role in osteosarcoma treatment. Because naturally occurring TAMs typically express an M2 phenotype[46], it is hypothesized that tilting macrophage polarization in favor of M1 macrophages could improve patient outcome.

In the early stages of tumors, TAMs have anti-tumor features. However, with tumor progression, the TME induces an M2 phenotype in TAMs. This increased infiltration of M2-like TAMs has been associated with more aggressive osteosarcoma and poor patient prognosis as more monocytes are recruited to the tumor site, thus leading to metastasis[44, 45] . Therefore, research aims to determine what specifically in the TME activates the M2 phenotype so this signal may be blocked. Potential antitumor therapies could include switching M2-like TAMs to M1 phenotype as well as generating M1 macrophages from monocytes.

Previous research has shown IFN-γ is highly effective in driving monocyte differentiation toward an M1 phenotype. However, systemic exposure to IFN-γ can cause negative side effects, necessitating a targeted treatment approach for delivering IFN-γ to the TME and associated macrophages[47]. Osteosarcoma has been shown to secrete certain signals into the TME that attract MSCs.[48] For this reason, MSCs present as a possible cell therapy to act as a targeted treatment delivery system.

The specific signal being released by osteosarcoma, which causes MSC chemotaxis, is yet to be identified. There are various molecules secreted by the TME and tumor itself that may cause MSCs to take on a pro-tumor phenotype, including: interleukin (IL)-6, transforming growth factor (TGF)-β, stromal-derived factor (SDF)-1, tumor necrosis factor (TNF)-α, macrophage inhibition factor (MIF), and IFN-γ[49]. However, the innate tendency of MSCs to migrate to malignant sites makes them an ideal carrier for treatment even if the specific signal released by osteosarcoma causing MSC chemotaxis is still unknown.

It should be noted, however, that the effect of MSCs on osteosarcoma is yet to be fully established. Zhang et al. and colleagues used an osteosarcoma murine model to inject MSCs intravenously and directly into the tumor at a surgical site[49]. It was determined that local administration directly into a tumor did not increase metastasis, but intravenous delivery of MSCs did. Furthermore, Zhou et al. injected Saos-2 nude mouse models with GFP labeled human MSCs into the caudal vein[45]. It was found that human MSCs targeted the tumor site, promoted tumor growth, and promoted pulmonary metastasis in vivo. Such research shows that wild type MSCs have an increased attraction to osteosarcoma. However, how they directly affect metastasis is not certain. Taking advantage of MSCs tropism toward the osteosarcoma stroma, research has shifted toward considering genetically modified MSCs to act as a treatment delivery system directly to osteosarcoma[50]. The Horwitz lab has already shown that transduced, cytokine secreting MSCs can be injected into a solid tumor in a murinexenograft model and modify the tumor microenvironment, thereby impairing tumor growth[34, 35].

With knowledge of MSCs chemoattractions to the tumor microenvironment, this study first aims to determine human MSCs migration to specific osteosarcoma patient cells lines and the pertinent chemokines involved in this chemotaxis. Furthermore, this study aims to investigate the

effect of TAMs polarization on osteosarcoma viability *in vitro*. It is hypothesized that if MSCs are indeed attracted to the TME and if IFN-γ does stimulate macrophage polarization toward an anti-tumor M1 phenotype, then MSCs could be the ideal cell type to use as vehicles for IFN-γ delivery directly to the tumor site, thereby preventing systemic IFN-γ exposure and osteosarcoma metastasis.

**MSCs for the treatment of acute Graft-versus-Host Disease**

Allogeneic Hematopoietic Cell Transplantation (HCT) provides the best chance for cure for many patients with malignant and nonmalignant hematologic disorders[51]. HCT can be used to treat a wide array of hematological diseases as well as to repopulate the patient with hematopoietic cells after chemotherapy and radiation. Conditioning regimen, immunosuppressive strategies, supportive care and prophylaxis for infectious disease are improving, reducing mortality related to transplant. However, aGvHD still remains as one of the most common complication of this potentially curative option for hematological disorders, leading to negative effects on disease prognosis and patient survival[52].

aGvHD is the body's response, a manifestation of the fight between the T cells of the donor and host's immune system. It presents as an inflammatory disorder triggered by recipient major or minor histocompatibility antigens and driven by donor T-cell cytotoxicity[52]. The "cytokine storm" that occurs after transplant brings about the development of aGVHD in three phases[53]: activation of Antigen Presenting Cells (APCs), donor T cells activation and the cellular inflammatory effector phase.

The initiation of aGvHD is triggered by host tissue injury and inflammation as a result of the cytotoxic effects of the preparatory conditioning regimens (radiation and/or chemotherapy), leading to increased exposure to damage or pathogen associated molecules that activate the host

APCs. The activation of host APCs primes the proliferation of alloantigen-specific donor T cells and their migration into target sites, leading to immune-mediated injury through a broad array of cytotoxic and cytokine dependent mechanisms, a process that is amplified by the recruitment of additional effector populations. This activation of donor T cells by host APCs subsequently results in alloreactive cytotoxicity of host tissues. aGvHD mostly affects the skin, gastrointestinal tract, and liver, but can have overall systemic effects[54].

Because aGvHD is an alloreactive immune reaction, it is most effectively treated using glucocorticoids and various immunosuppressive drugs[55, 56]. Specifically, corticosteroids[57] and various T cell immunosuppressive agents (cyclosporin, tacrolimus, and sirolimus)[55,58] are currently used for aGvHD prophylaxis and treatment. However, despite these prevention strategies, 40-80% of pediatric patients receiving HCT from an unrelated donor and 27% of pediatric patient receiving HCT from a human leukocyte antigen (HLA) identical sibling still develop aGvHD[59]. Therefore, the development of novel treatment is most crucial. In addition to HLA mismatch between donor and recipient, the use of an unrelated donor, age, and prior damage to the gastrointestinal (GI) tract are all significant risk factors for the development of aGvHD.

Because current treatment for aGvHD involves immunosuppression, and because MSCs have previously been shown to exhibit immunomodulatory properties, the use of MSCs for aGvHD prophylaxis and treatment has become an area of focus. The mechanism by which MSCs mediate immunosuppression is yet to be deduced, however it is most likely driven by cell to cell contact and paracrine signaling[32]. It is already known that after intravenous injection, MSCs become trapped in the lungs, and, subsequently, the cells are cleared from the lungs and distributed to other tissues such as secondary lymphoid organs (SLOs), and gut associated

lymphoid tissue (GALT)[34, 61]. However, the significance of migration and the *in vivo* chemotactic axis of intravenously-injected MSCs to immune and T cell populated organs, including SLOs and GALT, on immunosuppression and aGvHD prophylaxis is poorly understood.

SLOs, including spleen (SP), lymphoid nodes (LN), mesenteric lymphoid nodes (MLN), and Peyer's Patches (PP), are similarly organized with T cells, B cells, antigen presenting cells, stromal cells as well as a vascular supply. Therefore, SLOs are the niches to generate immune responses or tolerance.[58] The Horwitz lab has already shown that MSCs migrate preferentially toward activated T cells (*Burnham et al. in preparation*). Moreover, the Horwitz lab has demonstrated that, upon priming with IFN-γ, MSCs exhibit enhanced immunosuppressive functions and an increase in their secretion of anti-inflammatory and immunomodulatory factors.

With this knowledge of MSCs immunosuppressive properties and migration, this study first aims to evaluate the migration of intravenously injected γMSCs after syngeneic or allogeneic Bone Marrow Transplantation (BMT). To quantify γMSCs biodistribution, an Alu-based real-time PCR method was used for discriminating human cells from mouse cells. Once the migration of γMSCs to SLOs and GALT is determined, the second goal of this study is to elucidate the mechanism that guides the migration of γMSCs in order to enhance their aGvHD attenuating effect. It is hypothesized that increasing γMSCs migration to these tissues would increase MSCs-mediated immunosuppression and therefore improve aGvHD prophylaxis and treatment for patients with steroid-resistant aGvHD. Understanding the mechanism of intravenous MSCs migration during HCT may ultimatley aid in the development of more efficient and targeted cell therapies for the treatment and prevention of aGvHD.

# METHODOLOGY

## The effect of tumor associated macrophages and tumor microenvironment on osteosarcoma metastasis and treatment

### Human MSCs to Osteosarcoma Chemotaxis Migration Assay

*48-hour osteosarcoma conditioning*

Prior to beginning the conditioning treatment, an 80%+ confluency of osteosarcoma cells was ensured. Multiple different osteosarcoma cell lines with different complimentary media were used: human samples OS17 was cultured in RPMI 1640 media (Corning) with 1% glutamine, 1% antibiotic-antimycotic, and 10% fetal bovine serum (FBS) while 143B was cultured in EMEM media (Corning) with 1% glutamine, 1% antibiotic-antimycotic, and 10% FBS. Each cell line was at low passage and plated in a new T75 tissue culture treated dish (Corning) at 1 x $10^6$ cell density per 60mm plate. For each cell line, two tissue culture dishes were created: one plate for serum free media (experimental condition) and one for complete media (positive control). A third plate with only specific complimentary serum free media and no cells was also cultured as a negative control. All tissue culture plates were incubated for 48 hours to allow for the release of cytokines by osteosarcoma.

*Plating and Starving MSCs on Incucyte plate*

The same day as conditioning, human mesenchymal stem cells (MSCs) were passaged and plated at 500 MSCs per Incucyte well. For each cell line, three different variables were tested: complete media (positive control), conditioned serum free media, and nonconditioned serum free media (negative control). For each variable, triplicate samples were used to ensure standardized results. The MSCs were plated at the apical membrane of the Incucyte plate, along

with their complimentary complete media (DMEM, Corning) at the basal membrane. The Incucyte plate was incubated overnight to allow for MSC adherence. The following day, the complete media was removed from the apical and basal membranes and replaced with serum free media in order to starve the MSCs. The Incucyte plate was incubated overnight to allow ample time for MSC starving. This allows the MSCs to become more receptive to any cytokines in the conditioned media that will be added in the following step.

*Conditioning the Incucyte Plate*

The following day, serum free media was removed from the apical and basal membranes of the Incucyte plate and replaced with conditioned media at the basal membrane for each cell type. The Incucyte plate was then imaged for four days every hour. This real-time cell imaging technology allows MSC migration to be tracked from the apical membrane to the conditioned media at the basal membrane.

*Mass Spectrometry and Bulk RNA-Sequencing*

Remaining conditioned media was frozen at -80C and sent for mass spectrometry in order to determine important chemokines released by each osteosarcoma cell line. Determining any overlapping chemokines secreted by both osteosarcoma patient samples could point to a future therapeutic target.

**M1 and M2 Macrophage Polarization**

*Stimulating Macrophage Polarization*

Frozen naïve murine monocytes (Jax) were removed from liquid nitrogen, thawed, and plated on an untreated petri dish at 2 x $10^6$ cell density per two 60mm non-cell cultured treated dishes using high glucose DMEM media (Corning) with 1% glutamine, 1% antibiotic-

antimycotic, and 10% FBS. After 48 hours of incubation with appropriate media changing, the macrophages were plated on six well plates (Corning) at 500,000 cells per well and allowed to become adherent overnight. Three groups were established: M1 polarization, M2 polarization, and an unstimulated group used for flow cytometry compensation and control. M1 polarization was induced using 20 ng/ml IFN-γ (Invitrogen) and 100ng/ml LPS (Invitrogen) while M2 polarization was induced using 20 ng/ml IL-4 (Biolegend). Macrophages were incubated for 24 hours following stimulation and then sorted via flow cytometry.

*Macrophage Flow Cytometry*

A fixable viability stain (FVS520, BD Horizon) was used as a viability marker in order to separate living and dead cells. Only viable cells were analyzed for the presence of specific macrophage antigens. An unstained macrophage population was used in order to determine the baseline level of autofluorescence in each sample so voltages and negative gates could be appropriately set. Macrophages tend to auto-fluoresce easily due to their large size, resulting in relatively low voltages being used.

CD11b (Invitrogen) and F4/80 (BioLegend)are antigens expressed by all macrophages and therefore serve as general macrophage identifiers. CD11b and F4/80 antibodies conjugated to a specific fluorophore (ex. PE, APC, etc.) were used in compensations in order to establish what a positive cell population should look like. Compensation of each fluorophore was performed in order to prevent any spillover fluorescence from the "wrong" channel and establish proper antigen density measurements.

Antibodies serves as a detector for an antigen of interest, which provides information into what type of cell population is being analyzed (M1 or M2), while its conjugated fluorophore

translates antibody detection into a quantifiable signal. Specific M1 and M2 antibody-fluorophore complexes (markers) were used based on specific antigens known to be expressed by each macrophage type (Table 1). M1 markers used include iNOS (Invitrogen) and CD38 (BD Pharmingen) while M2 markers used include Egr2 (Invitrogen) and CD206 (BioLegend). Each sample was analyzed using the Cytek Aurora flow cytometry system.

| **M1 Markers** | | **M2 Markers** | | **General Macrophage Markers** | |
|---|---|---|---|---|---|
| Antibody | Fluorophore | Antibody | Fluorophore | Antibody | Fluorophore |
| iNOS (intracellular) | PE | Egr2 (intracellular) | APC | F4/80 (surface) | APC-Cy7 |
| CD38 (surface) | PE-Cy7 | CD206 (surface) | PE-Dazzle 594 | CD11b (surface) | AF-700 |

**Table 1: Macrophage Markers for Flow Cytometry**
Cells exhibit unique intracellular and extracellular receptors based on their phenotype. A flow cytometry marker, or an antibody conjugated to a fluorophore, can bind to a specific cell receptor and allow for its expression to be quantified. This table indicates the general macrophage markers, M1 markers, and M2 markers used to identify macrophage phenotypes in each sample.

## The migration and immunosuppressive effects of interferon gamma primed MSCs to secondary lymphoid organs for GVHD prophylaxis and treatment

### Tracking the Migration of Human γMSCs to Murine Organs *in vivo*

*Priming MSCs to create* γMSCs

MSCs were plated in a T75 tissue culture treated flask (Corning) at a seed density of 1 x $10^6$ cells/$cm^2$ and cultured in DMEM media (Corning) with 1% glutamine, 1% antibiotic-antimycotic, and 10% FBS. MSCs were primed with IFN-γ (Invitrogen) by adding 25ng/1mL of IFN-γ directly to the DMEM media. Priming was allowed to occur over 48 hours.

*Bone Marrow Transplantation*

Beginning on Day -4, BALB/c mice (Jax)were treated with 2mg/mL of the antibiotic, Baytril (Bayer), to prepare for irradiation. On Day-1, BALB/c mice were irradiated (radiation:

4Gy x 2). On Day 0, C57BL/6 (Jax) bone marrow was prepared for T cell depletion following irradiation by using CD3 positive selection kit (Invitrogen). The bone marrow was prepared for both allogenic and syngeneic groups, with bone marrow being transferred from BALB/c to BALB/c for syngeneic transplantation and bone marrow being transferred from BALC/c to C57B/L6 for allogenic transplantation. Then, splenocytes were prepared for T cell isolation using a negative selection kit (StemCells) following the manufacturing instruction in order to confirm how many T cells to include in the bone marrow transplantation. The control group of mice did not receive any bone marrow transplantation. On Day +1, 1 x $10^6$ yMSCs in 200uL PBS (Corning) were injected intravenously through the tail vein. On Day +2, the mice were sacrificed, and the organs were harvested and stored at -80C. DNA extraction and Alu-based real-time PCR was subsequently performed.

*DNA Extraction of Harvested Murine Organs*

The DNA of the organs was extracted using the DNEasy Blood and Tissue kit (QIAGEN) by following the manufacturing instructions. The concentration of DNA was quantified using a nanodrop, and this concentration was used to inform the amount of DNA used for the PCR reaction. High quality DNA with minimal protein contamination was considered to have 260/280 ratios ranging from 1.8 to 1.9, 260/230 ratios ranging from 2.0 to 2.2, and a concentration (ng/uL) greater than 100.

*Alu-based real-time PCR*

Alu-based real-time Polymerase Chain Reaction (qPCR) was used to discriminate human MSCs from mice cells. qPCR was performed in a total volume of 20µl per reaction using 0.2µM forward and reverse primers (101F, IDT and 206R, IDT), 0.25µM hydrolysis probe (HuAlu144RH), 10µl of TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific),

and an appropriate amount of genomic DNA. A standard curve was created by preparing serial dilutions of 11.5µg/mL human genomic DNA mixed with 11.5µg/mL rodent genomic DNA. Each qPCR reaction was performed using a total of 100ng of genomic DNA. The reaction was performed on an Applied Biosystems 7500 real-time PCR instrument (ABI 7500; Thermo Fisher Scientific) and then conditions were as follows: 1 cycle of 95°C for 10min as the holding stage, followed by 50 cycles of 95 °C for 15s, 56 °C for 30s and 72 °C for 30s for the cycling stage.

The Ct value of each sample was determined, which is defined as the number of cycles of amplification required for the florescence of the human DNA to be detected as crossing a threshold. The Ct values were then used to determine how many human cells were present in each organ per 100,000 murine cells.

# RESULTS

## The effect of tumor associated macrophages and tumor microenvironment on osteosarcoma metastasis and treatment

### Human MSCs to Osteosarcoma Chemotaxis Migration Assay

Our first aim was to confirm that MSCs do in fact exhibit increased attraction to osteosarcoma. To elucidate the chemotaxis of human MSCs to osteosarcoma *in vitro*, the Incucyte live cell imagine migration assay was used. It is hypothesized that if osteosarcoma cells release specific cytokines involved in MSCs chemotaxis into the surrounding cellular microenvironment, then media conditioned with osteosarcoma should experience increased MSCs migration as compared to non-conditioned media.

An Incucyte live cell imaging migration assay was performed over the course of four days to determine any differences in MSC migration rate to serum free media versus osteosarcoma conditioned serum free media. It was found that MSCs had a 17x increase OS17 serum free conditioned media (figure 1A) and a 3x increase toward 143B serum free conditioned media (figure 1B). The underlying mechanism for this chemoattraction was not identified using mass spectrometry. Future work looks towards using a protein array profiler to determine specific cytokines released by osteosarcoma that could be responsible for MSC chemotaxis.

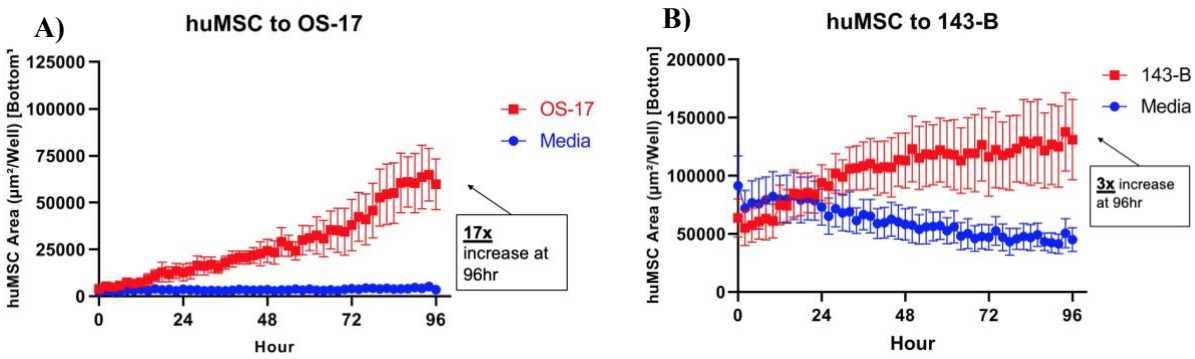
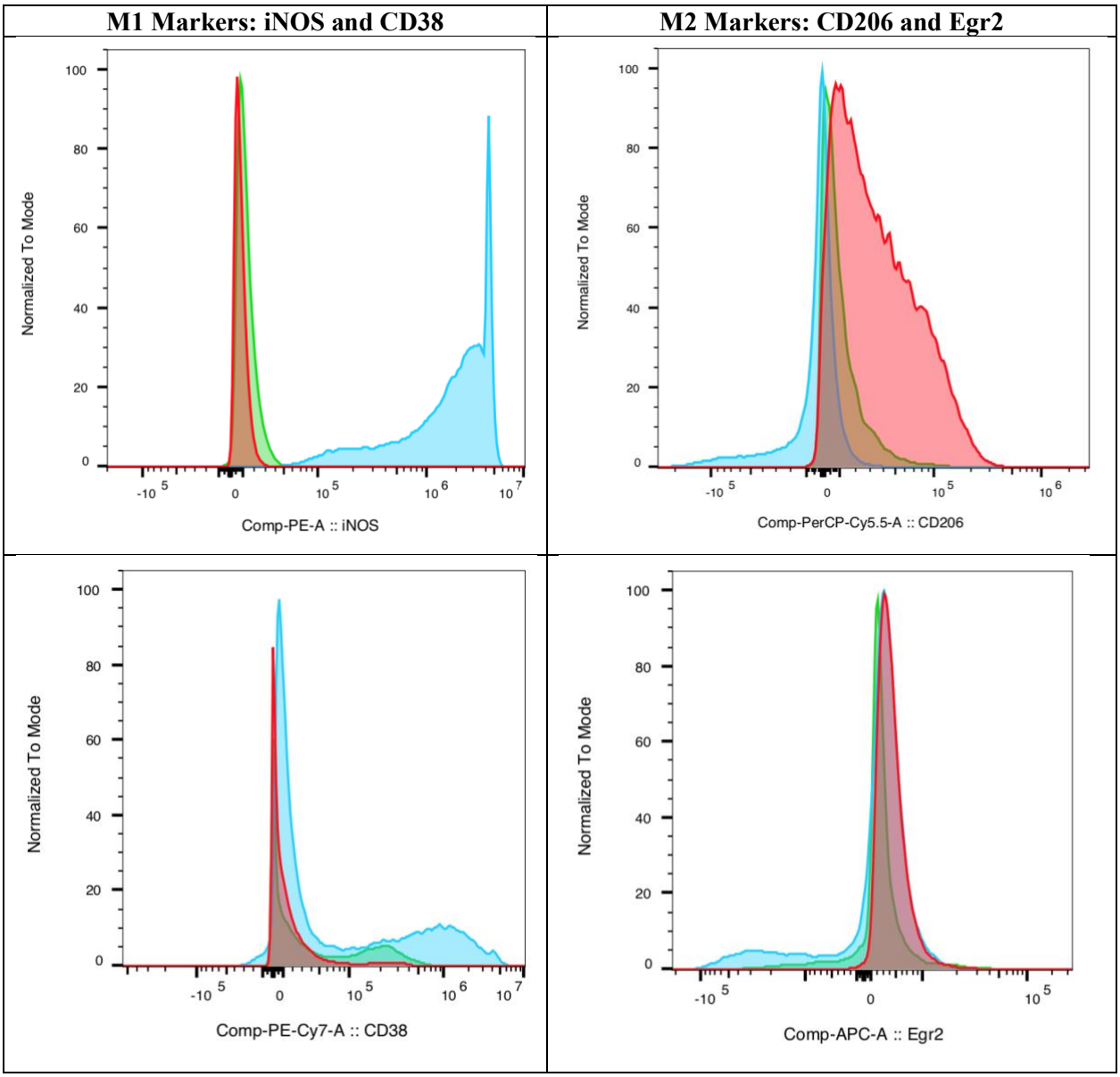



**Figure 1: MSCs show increased migration towards osteosarcoma**
Four-day hourly live cell imaging with Incucyte demonstrated that MSCs exhibit chemo-attractiveness to osteosarcoma. Specifically, a 17x migration increase was observed with OS-17 (**A**) osteosarcoma cells and a 3x migration increase was observed with 143-B (**B**).

## M1 and M2 Macrophage Polarization

The second aim of this experiment was to determine the effects of TAMs polarization on osteosarcoma viability *in vitro*. To do so, an effective protocol for the polarization and subsequent sorting of macrophages must first be established. Undifferentiated monocytes were polarized to an M1 or M2 phenotype and then separated from one another using flow cytometry. This experiment aims to determine two effective and specific M1 and M2 flow cytometry markers.

It was found that iNOS and CD38 proved to be effective M1 markers because they are selectively positive toward naïve monocytes polarized with IFN-γ + LPS, while also being mostly negative in other groups. However, more specific M2 markers must be established (figure 2, figure 3). Future research aims to culture M1 macrophage and M2 macrophage conditioned media with osteosarcoma in order to determine its effects on osteosarcoma viability.




| | iNOS | CD38 | CD206 | Egr2 |
|---|---|---|---|---|
| **M0** | -99.6%, +0.45% | -40.2%, +59.8% | -97.7%, +2.29% | -98.2%, +1.78% |
| **M1** | -15.2%, +84.8% | -66.0%, +34.0% | -99.3%, +0.68% | -99.9%, +0.05% |
| **M2** | -99/9%, +0.11% | -49.5%, +50.5% | -13.5%, +86.5% | -98.0%, +2.04% |

**Figure 2: The relative positivity of each marker for M0, M1, and M2 macrophages**
M0, M1, and M2 polarization was induced following treatment with no stimulation, IFN-γ and LPS, and IL-4, respectively. To ensure polarization was effective, specific M1 and M2 markers were chosen and flow cytometry was subsequently run on each sample. Macrophages intended to be M0 are shown in green, M1 shown in blue, and M2 shown in red. The percentage of cells that were negative for each marker and positive for each marker for each macrophage group (M0, M1, M2) are indicated in the table.

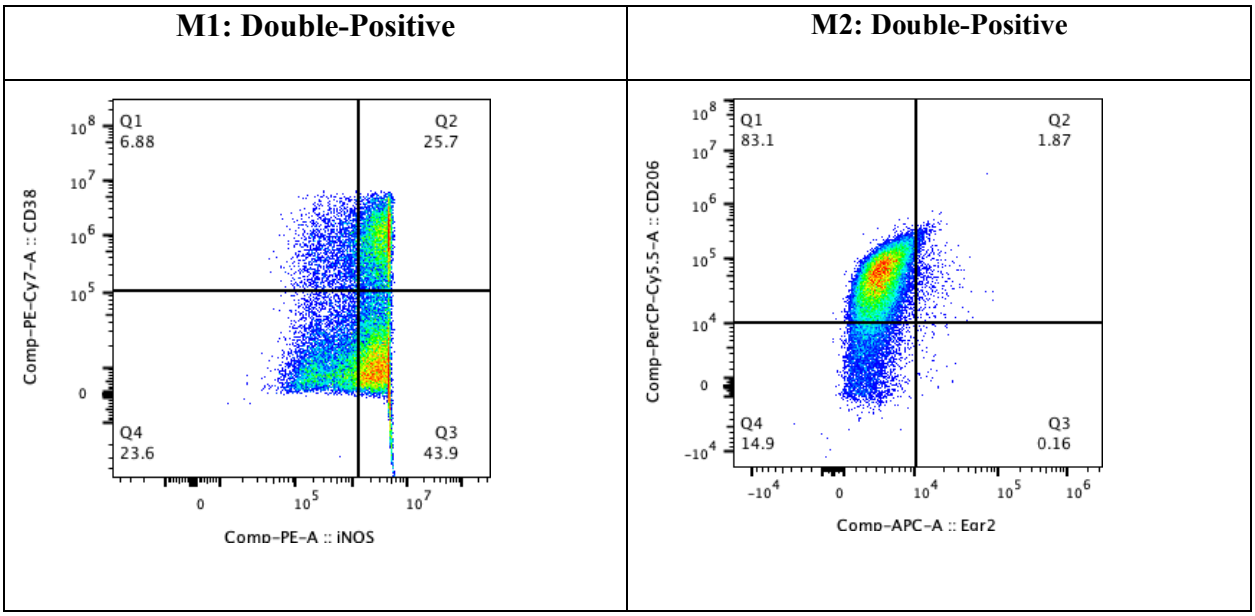



**Figure 3: Effective M1 markers are established**
Cells that are double-positive (top right quadrant) for both iNOS and CD38 are considered to be M1 macrophages while cells that are double-positive for both CD206 and Egr2 are considered to be M2 macrophages. It was found that iNOS and CD38 are effective M1 markers as the IFN-γ + LPS group is most double-positive for these markers while IL-4 and unstimulated groups are significantly less positive. Such specificity is not seen to the same extent with M2 markers Egr2 and CD206. Particularly, Egr2 seems to be the most unspecific. Subsequent experiments will aim to use a different M2 marker, such as Arg-1 or c-Myc.

**The migration and immunosuppressive effects of interferon gamma primed MSCs to secondary lymphoid organs for GVHD prophylaxis and treatment**

The migratory capabilities of γMSCs to SLOs and GALT, amongst other organs, was investigated using Alu-based real-time PCR. This experiment aims to determine the preferential migration of MSCs to specific organs and if immunosuppression of SLOs and GALT through the migration of γMSCs is necessary for aGvHD prophylaxis and treatment.

In contrast with the conventional PCR, where the amplified product is detected by an end-point analysis on a gel after the reaction has finished, the real-time PCR (qPCR) allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in "real time". In this study the real-time PCR was used for detecting the number of human γMSCs in various mice organs.

Alu-based real-time PCR was used as it allows for alu-elements to be detected, which are primate-specific short interspersed elements (SINEs). SINEs are about 300 nucleotides in length, and over 1 million copies of SINCEs are present in the human genome. Therefore, Alu elements are useful targets for quantifying the presence of human cells[62]. Through the use of Alu-based qPCR, it is possible to distinguish 1fg of human genome from 100ng mixed human and rodent genomes.

The sensitive and specific quantification of human DNA using qPCR has played important roles in various field, such as forensic science, cancer diagnostic and stem cell research. Here we examined the biodistribution of human BM-MSCs 16 hours after tail vein injection. Mice receive 1X106 human BM-MSCs after syngeneic or allogeneic BMT. Only the concentration of DNA was compared for the same organ in all three treatment groups.

A standard curve was used to quantify the DNA found in the samples on a logarithmic scale, with the standard ranging from a high concentration of human DNA mixed with murine DNA to only murine DNA. The Ct value of each sample was determined, which is defined as the number of cycles of amplification required for the florescence of the human DNA to be detected as crossing a threshold. The Ct values were then used to determine how many human cells were present in each organ per 100,000 murine cells.

The organs of interest included in this analysis have been the lungs, because they act as an intravenous trap, SLOs including the spleen, skin draining lymph node (SDLN), GALTs including mesenteric lymph nodes (MLNs) and small bowel, and the kidney and liver, because they act as filtration organs. Due to the anatomical differences between organs, which affects how much blood and therefore intravenous additives the organ is exposed to, the concentration of human DNA between different tissues could not be compared. Rather, the concentration of DNA was compared for the same organ in all three treatment groups: syngraft, allograft, and normal (no bone marrow transplantation).

In the lungs, relatively abundant human genomic DNA was detected in all three groups followed by spleen, MLNs, kidney, liver, and small bowel (figure 4). Because MSCs traffic toward inflammation in order to suppress the immune response, increased MSCs to the spleen, an organ involved in the immune response, is consistent with our hypothesis. It should be noted that an increase in human DNA detected in the spleen and MLN was mostly observed in the graft treatments. This is likely because BMT mounts an immune response, which has been shown to attract MSCs. A larger immune response would be expected in the graft treatment groups, therefore finding more human DNA in these groups is consistent with our hypothesis. Additionally, the amount of MSCs found in filtration organs, such as the liver and kidneys, may

be due to anatomy as both the liver and kidney receive 25% of the cardiac output. By contrast, human DNA was not detected in SDLN for any of the groups.

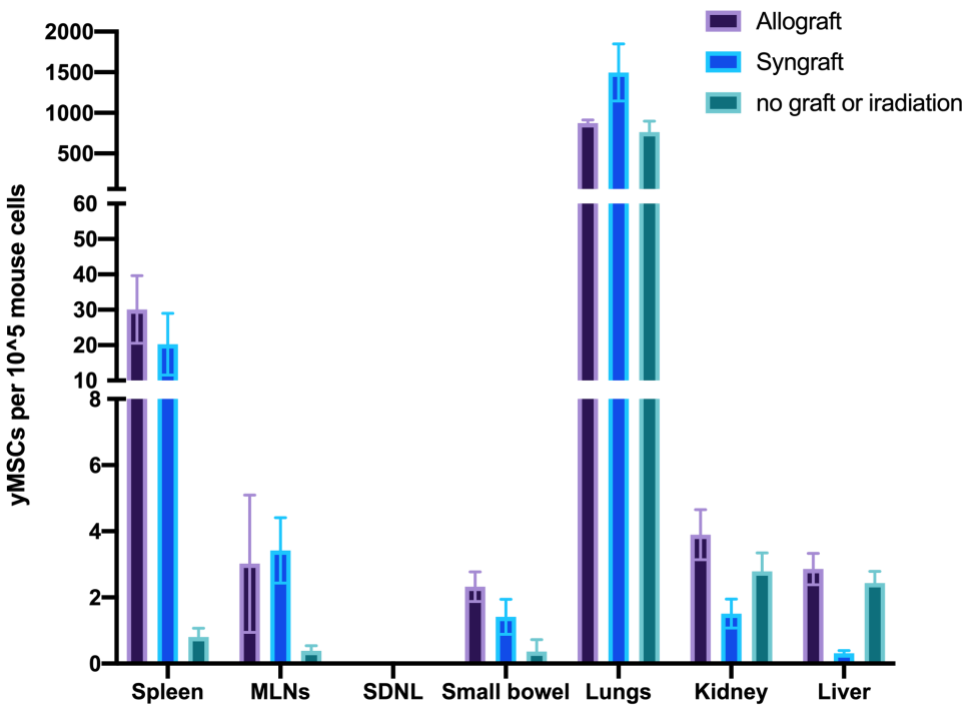



**Figure 4: γMSCs show increased migration to the spleen in allogenic and syngeneic mice** (*in collaboration with Burnham et al. in preparation*)
Following syngraft bone marrow transplant, the spleen, MLNs, SDNL, small bowel, lungs, kidney, and liver were harvested. DNA extraction was performed followed by Alu-based real-time PCR to track the migration of human γMSCs in syngraft BALB/c mice. In the lungs, relatively abundant human genomic DNA was detected in all three groups following by spleen, MLNs, kidney, liver, and small bowel. By contrast, human DNA was not detected in SDLN for any of the groups.

## DISCUSSION

This study aims to harness the migratory capabilities of MSCs in order to advance osteosarcoma and aGvHD treatment.

Osteosarcoma is the most frequent malignant bone tumor in adolescents and young adults. Of patients with localized, non-metastatic disease, up to 70% achieve persistent remission[36]. However, only 20-30% of patients with metastatic or relapsed osteosarcoma experience remission despite intensive chemotherapy. Thus, novel therapies, particularly against chemotherapy-resistant and metastatic disease, are needed.

Macrophages have been established as innate immune cells that can play a pro-tumor or antitumor role in osteosarcoma depending on their polarization. It has been established that naturally occurring TAMs tend to adapt a pro-tumor M2 phenotype instead of an anti-tumor M1 phenotype. Additionally, patients with a higher ratio of M1 to M2 macrophages at the time of diagnosis tend to have improved prognosis. Research efforts have therefore been focused toward shifting the polarization of TAMs to a more M1-like phenotype.

Pahl et al. was the first research group to describe that human macrophages can interfere with the growth of human osteosarcoma cells[63]. In this study, osteosarcoma was co-cultured with M1 conditioned media following monocyte activation with IFN-γ + LPS. After incubation for two days, viable tumor cell numbers were analyzed. It was found that the supernatants of activated M1-like macrophages inhibited osteosarcoma cell growth *in vitro*. Further analysis revealed a larger presence of TNF-α and IL-1β found in M1 conditioned media as compared to M2 conditioned media. However, blocking these cytokines did not inhibit the anti-tumor effect, suggesting that other soluble factors released by M1 macrophage activation are involved.

This study has helped establish the protocol for M1 macrophage and M2 macrophage polarization from IFN-γ + LPS and IL-4, respectively, and subsequent sorting via flow cytometry. Furthermore, this study shows that MSCs exhibit chemoattraction to osteosarcoma, which would allow them to migrate specifically to a patient's tumor. Therefore, transduced MSCs could potentially be used as a mechanism to specifically deliver IFN-γ to osteosarcoma TAMs in the TME without systemic effects. Such IFN-γ exposure would skew TAMs *in situ* toward an M1 phenotype, thereby maximizing the anti-tumor role of macrophages against osteosarcoma. Future research aims to test this *in vivo* by delivering MSCs transduced with IFN-γ directly to the primary bone tumor of mice.

This study also demonstrates how the migration of MSCs can be harnessed for the treatment and prophylaxis of aGvHD. The immunosuppressive activity of MSCs is well-documented, but their therapeutic benefit is rather unpredictable. However, the public registry of clinical trials at the U.S. National Institute of Health database (at ClinicalTrials.gov) shows a continuous increase in the number of new studies involving MSCs for the both treatment and prophylaxes of immune-mediated diseases.

Currently, there is no standardized treatment for patients with aGvHD who do not respond to steroids, and their prognosis is still very poor, with overall survival inferior to 20% at 2 years[64]. The interest in MSCs for the treatment of aGvHD has since raised the very encouraging results published in 2008 by the European Group for Blood and Marrow Transplantation Developmental Committee[65] where 55% of the evaluated patients with steroid resistant aGvHD showed complete response to MSCs. In addition, these responding patients had more than 50% overall survival at 2 years.

One of the unresolved challenges which limits our understanding of MSCs immunosuppression *in vivo* is that the vast majority of infused MSCs become undetectable a few hours after transiently residing in the lungs[66]. Nevertheless, MSCs appear to maintain their ability to deliver therapeutic activities.

Starting from these observations, this experiment tested γMSCs migration 16 hours after tail vein injection using qPCR to determine which organs MSCs are attracted to after their initial trap in the lungs. The findings of this study have suggested that the spleen is the major SLOs most populated by γMSCs after IV injection, followed by MLNs. These results highlight the specific immunoregulatory efficiency of human MSCs *in vivo*. Understanding the migration of MSCs, particularly their migration to organs that act as niches for immune initiation such as SLOs and GALT, may improve the clinical use of MSCs for aGvHD.

## CONCLUSION

While this study investigates two separate diseases, the distinct role of MSCs migration and use of IFN-γ allow for advancement in the treatment of both osteosarcoma and aGvHD.

Before expanding the osteosarcoma project closer to its ultimate goal, slight methodology alterations are needed. First, a more appropriate murine M2 intracellular marker must be established as Egr-2 was not effective. Possible candidates include Arg-1 and c-Myc, as identified in previous literature[45, 49]. Furthermore, in order to better control for nonspecific staining during flow cytometry, isotype controls will be used. Isotype controls are primary antibodies that lack specificity to the target but match the class and type of the primary antibody used. The most appropriate isotype controls match the host species, Ig class, and fluorophore of the primary antibody. By using an isotype control, we will be able to better determine the nonspecific binding of an antibody to Fc receptors found on the macrophage of interest.

After improving upon the previously established protocols for separating M1 from M2 macrophages using flow cytometry, future research aims to co-culture IFN-γ secreting MSCs with naïve monocytes in order to induce M1 polarization. A viability assay could also be performed to confirm previous research demonstrating that macrophages can affect osteosarcoma growth *in vitro*. It would be expected that osteosarcoma exposed to M1 macrophages rather than M2 macrophages will have decreased viability.

The Horwitz lab aims to expand these research efforts *in vivo* by injecting murine bone tumors with MSCs transduced with IFN-γ in order to determine their effect on macrophage polarization. Such an experiment could be carried out by harvesting the bone tumor following MSCs injection to obtain single cells, enriching the cells for macrophages, and then sorting via flow cytometry. It would be hypothesized that mice injected with IFN-γ transduced MSCs would

have a higher ratio of M1 to M2 macrophages and therefore prolonged survival. If this hypothesis is confirmed, these research efforts could eventually move on to a clinical trial with the hopes of injecting IFNγ primed MSCs directly into the primary tumor of patients in order to prevent osteosarcoma metastasis.

Future research regarding the use of γMSCs as an immunosuppressive agent to combat aGvHD aims to continue investigation *in vivo*. A mouse model with aGvHD could be created and then γMSCs could be delivered via tail vein injection to determine the effects on aGvHD. It would be expected that the mice given γMSCs would have reduced aGvHD and therefore should lose less weight, have less lymphocytes in their blood, and have less of a hunched back as compared to mice with aGvHD and no γMSCs treatment. Ultimately, research aims to use γMSCs intravenously in patients with the expectation of the immunosuppressive γMSCs trafficking to SLOs and GALT in order to prevent and treat aGvHD.

Future research also aims to elucidate the mechanism by which MSCs are able to cross the endothelium of SLOs and GALT. This study shows γMSCs migrate more to SLOs, such as the spleen, and GALT, such as the MLNs. Since these organs are involved in the immune system, we believe the migration of γMSCs involves the immune response, particularly inflammation following radiation and alloreactive cytotoxicity following transplant. However, it should be noted that γMSCs are not found in the SDLNs, despite the involvement of this organ in the immune response. Therefore, the Horwitz lab is currently investigating the mechanism behind γMSCs migration to specific immune organs. Mucosal Vascular Addressin Cell Adhesion Molecule 1 (MAdCAM-1) is a receptor expressed on the endothelium of GALT, MLNs, and spleen, but not expressed by SDLNs[67]. It is hypothesized that MAdCAM-1 expression is upregulated by an increased immune response following cytotoxicity caused by transplantation.

Additionally, MSCs express alpha4beta1, a ligand for MAdCAM-1[68]. Therefore, future research aims to investigate the trans-endothelial migration of MSCs from the blood vessels into the spleen and MLN versus the SDLNs via the proposed MAdCAM-1/alpha4beta1 interaction. Once migrated into the organ of interest, it is hypothesized that MSCs are attracted to T cells via a chemokine gradient. This chemotaxis allows MSCs to migrate toward T cell rich areas and exert immunosuppression upon activated T cells. Once again, immunosuppression is expected to be enhanced in γMSCs. The Horwitz lab aims to investigate this proposed mechanism of MSCs migration in order to determine if crossing the endothelium, rather than merely staying in the blood vessels, is necessary for γMSCs to have an immunosuppressive effect relevant for the treatment of aGvHD.

In conclusion, this study demonstrates the various applications of MSCs towards treating osteosarcoma and aGvHD. While both experiments aim to harness the migratory capabilities of MSCs to create a more targeted treatment, an important distinction to note is the differences in the way the MSCs are modified.

To act as a targeted treatment delivery system of IFN-γ to osteosarcoma in order to upregulate M1 polarization, MSCs are *transduced* with IFN-γ. Transduction is used in this context because it allows for the permanent modification of the genome of the MSCs, allowing the MSCs to possess the genes needed to produce and secrete IFN-γ into the TME.

By contrast, the immunomodulatory properties of MSCs and their preferential migration towards SLOs and GALT can be harnessed as potential treatment and prophylaxis for aGvHD using IFN-γ *primed* MSCs. IFN-γ has been already shown to suppress T cell activation, therefore priming the already immunosuppressive MSCs with IFN-γ increases their immunosuppressive affect against activated T cells which causes aGvHD. MSCs are primed with IFN-γ simply

though the addition of IFN-γ into the media during cell culture. However, unlike transduction, priming is a temporary affect. IFN-γ priming allows for MSCs to possess increased immunosuppressive affects, for approximately 72 hours, before the effect is lost. Therefore, γMSCs are able to mitigate aGvHD following HCT, allowing for improved engraftment, without creating permanent immunosuppression.

The Horwitz lab continues to study the various applications of MSCs migration and the use of IFN-γ on immune diseases. In January, I plan to begin working full-time with the Horwitz lab to carry out the future experiments related to the osteosarcoma project previously described.

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## SUPPLEMENTARY METHODS

### 1. MSCs TO OSTEOSARCOMA MIGRATION ASSAY

**DAY 1:**

*Passage / add osteosarcoma cells to small dishes and add specific conditioning media. Let sit for 48 hours to condition and become adherent*

**1) SPLIT OSTEOSARCOMA CELLS INTO DISHES→ 48 HOUR CONDITIONING**

1. Ensure 80% + confluence of osteosarcoma cells
2. Warm up reagents to 37C in water or bead bath: specific 10% FBS media, PBS, 0.05% trypsin
3. Aspirate out old media from the flask (hold at angle)
4. Wash adherent osteosarcoma cells in flask with 10mL PBS
    a. Swirl around PBS, aspirate out at an angle
5. Place 3mL trypsin into flask
    a. Swirl around trypsin, place flask in incubator for 3-5mins
    b. Visualize floating (detached) cells under microscope
6. Placed 4mL complete media into flask→ neutralize trypsin
    a. Swirl around media, collect all media + cells into serological pipette and decant into centrifugate tube
    b. Keep old flask to put leftover cells back into culture
7. Centrifuge at 400ref for 5 min (acceleration 9)
    a. Aspirate off supernatant without disturbing osteosarcoma cell pellet
8. Count cells:
    a. Resuspend pellet in 1mL complete media and *keep tube on bead bath (37C)*
    b. Add 30uL trypan blue dye to an Eppendorf tube, add to that 10uL of cell pellet resuspension (total = 40uL). Mix / resuspend thoroughly
    c. Of this 40uL, take out 10uL of cell-dye resuspension and inject int hemocytomer with clean coverslip on top
    d. Place on microscope and count cells for 2 quadrants→ calculate how much of cell resuspension to add to get 1 * $10^6$ cell density in each dish:
        - Average # cells in one quadrant * 4 * $10^4$ = # cells / 1mL
        - ***Ideal cell density for 60mm dish should be around 1 x $10^6$***
            - Ex. If you count an average of 160 cells per quadrant, 160 x 4 x $10^4$ = 6.4 x $10^6$ cells in 1mL of your cell resuspension→ 640 cells in 10uL
            - $\frac{6.4 * 10^6}{1000uL} = \frac{\mathbf{1 * 10^6}}{X\ uL}$ = 156uL of cell resuspension / 60mm dish
9. Aliquot out specific osteosarcoma SF and FBS medias to clean tubes to later add to dishes
10. Add specific media to corresponding labeled dish → need 3mL media per 60mm dish
    a. For each cell line have 3 plates:
        - Cells + FBS media
        - Cells + SF media
        - SF media (negative control)
11. Add XuL (ex. 156uL) cell resuspension to each dish

12. Place dishes in incubator for 48 hours
13. Put leftover osteosarcoma cell resuspension back into flask to culture / split if confluence is >80%
    a. Change passage number because cells were trypsinized

**DAY 2:**

*Add MSCs to Incucyte plate with complete media to become adherent O/N*

**2) LOAD MSCs TO INCUCYTE MIGRATION PLATE**

1. Ensure 80% + confluence of MSCs
2. Warm up reagents to 37C in water or bead bath: specific 10% FBS media, PBS, 0.05% trypsin
3. Aspirate out old media from the flask (hold at angle)
4. Wash adherent MSCs in flask with 10mL PBS
    a. Swirl around PBS, aspirate out at an angle
5. Place 3mL trypsin into flask
    a. Swirl around trypsin, place flask in incubator for 3-5mins
    b. Visualize floating (detached) cells under microscope
6. Place 4mL complete media into flask→ neutralize trypsin
    a. Swirl around media, collect all media + cells into serological pipette and decant into centrifugate tube
    b. Keep old flask to put leftover cells back into culture
7. Centrifuge at 400ref for 5 min (acceleration 9)
    a. Aspirate off supernatant without disturbing MSC pellet
8. Count cells:
    a. Resuspend MSC pellet in 1,000uL complete media and keep tube on bead bath
    b. Add 30uL trypan blue dye to an Eppendorf tube, add to that 10uL of cell pellet resuspension (total = 40uL). Mix / resuspend thoroughly
    c. Of this 40uL, take out 10uL of cell-dye resuspension and inject into hemocytomer with clean coverslip on top
    d. Place on microscope and count cells for 2 quadrants→ use the average to calculate number of cells in 1mL with equation:
        - Average # MSCs in one quadrant * 4 * $10^4$ = # MSCs in 1mL resuspension
            - Ex. 25 * 4 * $10^4$ = 1,000,000 MSCs / 1mL of resuspension
        - # Incucyte wells in use * 50uL media for top plate wells = media loading volume
            - ***Ideal density in each well is 500MSCs / 50uL well***
            - Ex. 2 rows * 12 wells pers row * 50uL per well = 1,200uL media needed→ used 2,000uL for extra
                - Add a little extra volume for reverse pipetting
        - Calculate # MSCs need to be added to the needed volume of loading media
            - $\frac{\mathbf{500}\boldsymbol{MSCs}}{\mathbf{50}\boldsymbol{uL\ media\ well}} = \frac{\text{X MSCs}}{\text{2,000uL loading media}}$ = 20,000 MSCs in 2000uL
        - Calculate volume of MSC resuspension to add to get correct MSC #

- $\frac{20{,}000\ \text{MSCs}}{\text{X Volume MSC resuspension}} = \frac{1{,}000{,}000\ \text{MSCs}}{1{,}000\ \text{uL}}$ = 20uL MSC suspension
    - SO, take 20uL MSC suspension and put it in 2mL MSC FBS media
        - Of this mixture, you will add 50uL per top well

| |
|---|
| **TOP: Large hole** 50uL MSC-media loading mixture |
| **BOTTOM: add directly** 200uL FBS media |

9. Carefully take off plastic Incucyte cover and place it on cell culture table flipped up
10. Carefully take off top portion of Incucyte plate (black) and set it down on the plastic
11. Add 200mL MSC FBS media to each bottom well using revere pipetting
12. Replace top portion of Incucyte plate (black)
    a. Put on at an angle
13. Add 50uL of MSC-media loading mixture to each top well using reverse pipetting
    a. Resuspend / mix mixture thoroughly before pipetting to ensure even MSC distribution
    b. Pipette into the **LARGE HOLE (top well)** at an angle to the side
        i. Do not stick pipette in too deeply to avoid damaging the membrane that separate the top and bottom well
14. Replace plastic plate cover and visualize MSCs under microscope
    a. Should see round cells because they were just plated
15. Place Incucyte in incubator overnight→ MSCs will become adherent to membrane
16. Put leftover MSC resuspension back into flask to culture
    a. Change passage number because MSCs were trypsinized

**<u>DAY 3:</u>**

*Take out FBS-M from bottom Incucyte well and replace with SF-M to starve MSCs*

**3) STARVE MSCs WITH SF-M**

1. Carefully remove Incucyte plate from incubator. Verify MSCs present under microscope (should be spread out and adherent). Place Incucyte plate in hood
2. Warm up reagents to 37C (specific osteosarcoma SF-M) in water bath or bead bath
3. Take off Incucyte plastic plate cover and put face up on the side
4. Take off black top well plate of Incucyte and hold in hand→ keep balanced
5. Aspirate out media from bottom wells (while holding black top well plate)
    a. Replace top well plate once all media is vacuumed out of bottom wells
6. Aspirate out media from **top wells**
    a. Place small pipet tip on end of aspirator and tilt migrate plate towards you to pool the media in the top wells
    b. Angle aspirator and slowly insert pipette tip into the **LARGE HOLE** to remove media→ do not touch membrane

| |
|---|
| **TOP: Large hole** 50uL SF media |
| **BOTTOM: add directly** 200uL SF media |

7. Portion out media to be used for both bottom and top well plates into a tube
8. Aliquot 50uL fresh SF-M specific to your osteosarcoma cell line into top wells (LARGE HOLE)
    a. Reverse pipette→ use unfiltered pipette tip and 200uL pipette
9. Aliquot 200uL fresh SF-M specific to your osteosarcoma cell line into bottom wells (SMALL HOLE)
    a. Reverse pipette→ use unfiltered pipette tip and 1000uL pipette
10. Visualize MSCs under microscope (should be adherent)

11. Place Incucyte plate in incubator for 24 hours

**DAY 4:**

*Add specific media conditions to bottom Incucyte well according to plate map (3 conditions / cell line: cells + FBS, cells + SF, SF)*

**4) LOAD CONDITIONED MEDIA IN INCUCYTE**

1. Warm up reagents to 37C in water or bead bath: specific osteosarcoma SF-M and FBS-M
2. Remove osteosarcoma conditioned dishes from incubator (SF-M and FBS-M) and verify >80% confluency of adherent osteosarcoma cells under microscope
3. Pipet up media from each plate (tilt at an angle to pool media) and place into separate labelled centrifuge tube. Throw away culture plates
4. Centrifuge at 4200 (max speed) for 5 min
    a. Prepare Eppendorf tubes during this time for loading and for storage (freeze down)

| **TOP: Large hole**<br>Do nothing |
|---|
| **BOTTOM: add directly**<br>200uL specific conditioning media |

- Ex. 143B: should have 4 conditions per osteosarcoma cell line
    - 143B conditioned EMEM-SF
    - 143B conditioned EMEM-FBS
    - EMEM-SF (- control)

5. Aliquot out needed volume of each media type based on # wells per condition into the loading Eppendorf tubes
6. Fill Eppendorf tubes used for storage with remaining media→ used for chemokine analysis
    a. Keep on ice bath until stored in -20C freezer
7. About 1 hour before desired time to load the Incucyte, take out Incucyte plate and ensure MSCs present
8. Aspirate off media in the bottom wells of the Incucyte plate while carefully balancing black top well plate in hand
9. Tape plate-map to the hood to use as guide and carefully load ***bottom wells*** with 200uL of their specific media condition ***(SMALL HOLE)***
    a. Leave black top well plate on, just take off plastic cover
    b. Reverse pipette: NO BUBBLES
        - Use unfiltered pipette tips and the 1000uL pipette
    c. Record the time that the bottom wells were loaded with specific media condition
10. Put plastic cover back on Incucyte plate and walk it to the Incucyte machine at HSRB on 3rd floor, second lab entrance
    a. Check on computer when next scan is / if there are other plates in the Incucyte before loading
        - Double check when next scan is once at the Incucyte machine
11. Press eject button on Incucyte to open tray and load the plate
    a. Keep one hand underneath to prevent falling, ensure that it "clicks in"
    b. Check that there is autoclaved water in the bottom of the incubator
12. Close the Incucyte tray and close the incubator door
13. Wait 10-15 minutes to allow condensation to form, then wipe the inside of the Incucyte plastic cover and the bottom of the Incucyte plate with a KimWipe

14. Quickly replace over and close the Incucyte tray and incubator door
    a. Record the time when the first scan occurs→ should be less than one hour from when the wells were loaded

## 2. MACROPHAGE FLOW CYTOMETRY

**DAY 1:** *Unfreezing and plating macrophages on Petri dish for growth*

1. Remove frozen undifferentiated macrophages from liquid Nitrogen (should be frozen at 2 x $10^6$)
2. Thaw quickly in 37C water path and plat onto 10cm untreated petri dish with 7mL BMDM DMEM (high glucose (4.5g/mL) + 20% FBS + 20% M-CSF + 1% glutamine + 1% antibiotics)
3. Incubate O/N to allow macrophages to become adherent
4. After 12 hours, remove old media, wash with 5mL PBS, add fresh 7mL BMDM DMEM
5. Incubate for 48 hours to allow for macrophage (macrophages double in 3-4 days)

**DAY 3:** *Plating macrophages on 6 well plate*

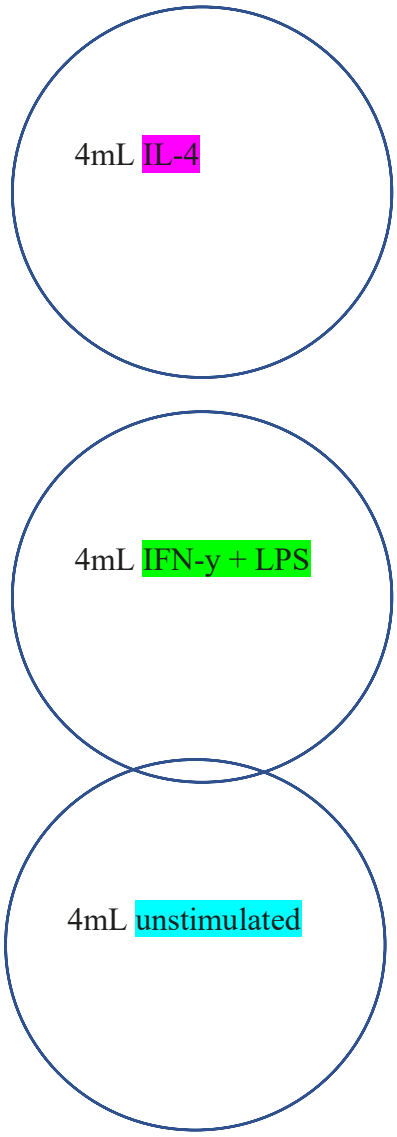
1. Remove media, wash with 5mL PBS, remove PBS
2. Add 5mL accutase (room temperature) to detach cells from untreated petri dish→ incubate at room temperature for 10-15 minutes
    - Check after 10-15 minutes if macrophages have been lifted (agitate against scope)→ if not yet lifted add another 3-5mL accutase and incubate another 10-15 minutes
3. Carefully collect cells in serological pipette and transfer to falcon tube
4. Centrifuge at 400rcf for 5 minutes (in cell culture centrifuge)
5. Aspirate off supernatant and resuspend macrophage pellet in 1mL BMDM DMEM
    - Keep macrophages on bead bath whenever possible
6. Count macrophages→ 1:10 dilution
    - Add 90uL tryphan blue + 10uL macrophage resuspension to an Eppendorf tube→ load to 10uL of this mixture to counting slide
        6 Average # macrophages counted on grid x 10 (dilution factor) x $10^4$ = # of macrophages in resuspension
7. Divide up macrophages so as to have 1 million cells (or 500,000 cells) per well
    - Use non tissue treated 6 cell plate, 500,000 macrophages per well→ used a total of 2 plates (12 wells)→ 2 IFNy + LPS (M1), 2 IL-4 (M2), 2 unstimulated, 6 for comps for experimental samples for 6 well plate and ½ million for comps.
8. Add 2mL BMDM media to each well
9. Incubate O/N to become adherent

**DAY 4:** *Stimulation*

1. Remove media, wash with 5mL PBS, remove PBS
2. Prepare conditioning:
    - Thaw LPS, IFNy, and IL-4 from -80C freezer at room temperature→ once liquid keep on ice
        - IL-4 and IFNy are in an orange labeled box in -80C (3rd row from bottom), and LPS is in the white box directly behind orange box

- Label each petri dish
    1. IFN-y + LPS
    2. IL-4
    3. Unstimulated

**Conditions with 3 petri dishes**




- Create three falcon tubes each with 8mL BMDM DMEM media in them (because each dish gets 8mL): One falcon tube for M1 (**LPS at concentration of 25ng/ml + IFNy at concentration of 25ng/ml**) and one for M2 (**IL-4 at concentration of 30ng/ml**), one falcon tube with just BMDM DMEM (unstimulated)
    - M1:
        - For IFN-y (stock concentration: 10 **ug**/ml→ 10ng/uL ; volume of 50 ul per aliquot, so each 50ul aliquot has 500**ng** of IFNy)

        - Add 20uL (200ng) of stock and place in 8mL BMDM DMEM in falcon tube, **have to discard the rest**
    - For LPS (stock concentration: **10ug/ml**→ 10ng/uL
        - Add 20uL (200ng) of stock and place into 8ml BMDM DMEM in falcon tube, **can put back in -80C afterwards**)
- M2:
    - For IL-4 (stock concentration: 10 ug/ml→ 10ng/uL; volume of 25 ul per aliquot, so each 25ul aliquot has 250ng of IL-4)
        - Add 24uL (240ng) IL-4 to 8mL BMDM DMEM in falcon tube, **have to discard the rest**

3. Add conditioning media to each petri dish
4. Incubate for 48 hours to allow for macrophage differentiation
2. Aspirate off old media from each well, add 2mL PBS to each well to wash, aspirate off
3. Add 2mL M1 conditioning media to each of its two respective wells
4. Add 2mL M2 conditioning media to each of its two respective wells
5. Add 2mL fresh BMDM DMEM media to each unstimulated well (8 total)
6. Incubate for 24 hours to allow for macrophage differentiation

## 3. REAGENTS USED

- Media
    - MSCs: DMEM
    - OS17: RPMI
    - 143B: EMEM
    - K7M2: DMEM 1g/L glucose
    - Macrophages: BMDM DMEM 4.5g/L glucose
        - Osteosarcoma and MSC media additives
            - 1% anti-anti→ -20C freezer
            - 1% Glutamax 100x Gibco→ Tissue culture fridge
            - 10% FBS→ -20C freezer
        - Macrophage additives
            - 1% anti-anti
            - 1% Glutamax 100x Gibco
            - 20% FBS
            - 20% M-CSF
- Lifting cells
    - MSCs and Osteosarcoma
        - 0.05% Trypsin (~3mL / 75cm$^2$) → tissue culture fridge, new in -20C freezer (bottom drawer)
    - Macrophages
        - Acutase (~5mL / 75cm$^2$)→ tissue culture fridge, new in -20C freezer
            - Do not warm actuase, do not incubate
- Plating flasks
    - MSCs and Osteosarcoma
        - Use tissue culture treated flasks

- Macrophages
    - Use non-tissue culture treated flasks (petri dish plates)

## SUPPLEMENTARY METHODS: GVHD

### 1. DNEASY BLOOD AND TISSUE KIT

1. Retrieve organs from -80C and put on ice
2. Retrieve DNEasy Blood and Tissue Kit (blue) and QIAschredder Kit (red) from over Andre's bench
3. Set up corresponding number of autoclaved Eppendorf tubes for each sample
4. Add 180uL **Buffer ATL** to each tube
5. Add 20uL **proteinaseK** to each tube
    a. Slightly resuspend, change tips between samples
6. Prepare scissors wash
    a. Add bleach to 50mL tube
        i. Bleach is under the sink in cell culture room
    b. Add 70% ethanol to another 50mL tube
        i. 70% ethanol is in red cabinet outside cell culture room
7. Get mouse tools from green bin above Linda's desk
8. Take a piece of the organ from each sample and put it in corresponding tube
    a. Keep remaining organs on ice
    b. Clean scissors in between samples!!
        i. First bleach, then ethanol
    c. When finished, rinse scissors in water, dry well, and put in a new bag to be autoclaved
9. Mash up organ pieces in each tube using **blue plastic crucible**
    a. Blue crucible in QIAshredder(250) red box
10. Vortex each sample and put in dry incubator at 56C
    a. Check back every 5 min to vortex again
    b. Total incubation time about 15 min for complete lysis
    c. Set up *purple QIAshredder tubes*
11. Vortex samples again right after completing incubation
12. Pipette all of each sample into the purple QIAshredder tube
    a. Centrifuge at max speed 1 min
    b. Discard filter, keep flow through (liquid)
13. Add 200uL **Buffer AL** to each tube
    a. Can use same tips but don't touch samples
    b. Cap tubes and vortex
14. Add 200uL of **100% ethanol**
    a. Can use same tips but don't touch samples
    b. Cap tubes and vortex
15. Transfer each sample into a *white DNeasy mini spin column* placed in a 2mL collection tube
    a. Centrifuge for 1 min at 6,000rcf
        i. Check if all the liquid flowed through → if there is still some liquid above the filter, spin for another 1 min

b. Discard the flow through (liquid) and collection tube, keep the filter

16. Put filter into a new 2mL collection tube
    a. Add 500uL **Buffer AW1** (make sure it has 100% ethanol added)
        i. Can use same tips but don't touch samples
    b. Centrifuge for 1 min at 6,000 rcf
    c. Discard the flow through (liquid) and collection tube, keep the filter
17. Put filter into a new 2mL collection tube
    a. Add 500uL **Buffer AW2** (make sure it has 100% ethanol added)
        i. Can use same tips but don't touch samples
    b. Centrifuge for 3 min at max speed
    c. Discard the flow through (liquid) and collection tube, keep the filter
18. Put filter into a new 2mL collection tube
    a. Centrifuge for 1 min at max speed
19. Discard collection tube and put filter into a labeled Eppendorf tube to elute DNA into
20. Add 100uL **Buffer AE** to each tube
    a. Cover the filter completely with buffer but do not touch filter with tip
    b. Incubate at room temp for 1 min
    c. Centrifuge tubers for 1 min at 6,000 rcf
        i. Be careful with caps: lay caps flat against the centrifuge and skip two spaces between each tube
21. Discard filter, keep liquid in Eppendorf tubes (DNA inside!)
    a. Store DNA at -20C
    b. Store RNA at -80C

2. **NANODROP**: Quantify DNA concentration
    1. Clean nanodrop with water from wash bottle and with kimtech wipe
    2. Add 1.5uL buffer AE to clean → put nanodrop down → click "OK"
    3. Clean off buffer AE with kimtech wipe
    4. Add 1.5uL buffer AE to blank → put down nanodrop → click "BLANK"
    5. Clean off buffer AE with kimtech wipe
    6. Run Samples
        a. Load 1.5uL of sample onto nanodrop
        b. Write in the name of the sample in sample ID
        c. Click "MEASURE"
            i. Ideal ratios:
                1. 260/280: 1.8 – 1.9
                2. 260/230: 2 – 2.2
                3. ng/uL > 100
        d. Wipe nanodrop with kimtech wipe in between samples
        e. Keep samples on ice!!
    7. Clean nanodrop by washing with water and kimtech wipe
    8. Store DNA in -20C fridge until time for PCR

**3. ALU-BASED REAL-TIME PCR**

1. Prepare MAstermix (based on reagents used in Funakoshi et al 2017)
    a. Reagents found in "Andre's box Alu PCR materials" in -20C

b. Taqman no UNG found in 4C

| | For 1 rnx (uL) | For 50 rns (uL) | For 76 rxns (uL) | For 1000 rxns (uL) |
|---|---|---|---|---|
| Taq | 10 | 500 | 760 | 1000 |
| Alu Forward primer | .4 | 20 | 30.4 | 40 |
| Alu Reverse primer | .4 | 20 | 30.4 | 40 |
| Probe | .5 | 25 | 38 | 50 |
| Total DNA | 8.7 | 8.7 | 8.7 | 8.7 |

- Master mix = Taq, AluF, AluR, Probe
    - Add 11.3uL Master Mix to each well
- DNA = DNA added from your sample
    - Add 8.7uL of DNA LAST to each well!!
    - You add 8.7uL DNA for stand curves well

2. Set up PCR calculation to determine how much DNA to add (C1V1 = C2V2)
    a. (nanodrop DNA)(V1) = (11.5)(35)

| Sample | **C1** Concentration of DNA from nanodrop (ng/uL) | **C2** (ng/uL) | **V2** (uL) | **V1** DNA to add (uL) | **Buffer AE** to add (final volume = 35uL) |
|---|---|---|---|---|---|
| 1 | | 11.5 | 35 | | |
| 2 | | 11.5 | 35 | | |
| 3 | | 11.5 | 35 | | |
| 4 | | 11.5 | 35 | | |

3. Create new Eppendorf tubes with C2 concentration of DNA and corresponding amount of buffer AE to get 35uL total
    a. Add buffer AE first, then add DNA
4. Label 6 Eppendorf tube 1-6 to make standard curve
    a. 1 = lowest [DNA]
    b. 6 = highest [DNA]
5. From the tube "MoDNA 11.5ug/mL," add 31.5uL to each standard tube (1 – 6)
    a. Vortex "MoDNA 11.5ug/mL" tube first!
6. From "Human DNA" tube (blue writing), add 3.5uL to standard tube 6
    a. SERIAL DILUTION:
        i. Mix tube 6 very well, then take 3.5uL from tube 6 and add it to standard tube 5
        ii. Mix tube 5 very well, then take 3.5uL form tube 5 and add it to standard tube 4
        iii. Continue for all tubes except DO NOT ADD ANY HUMAN DNA to tube 1
            1. Standard tube 1 has only mouse DNA!
7. Prepare plate setup
    a. Triplicate wells for each sample and for each standard

8. Create enough master mix based on number of reactions (number of wells being used)
    a. Master Mix = Taq, AluF, AluR, probe
    b. Always make a little extra master mix
9. Vortex all samples, standards, and master mix
10. Get applied biosystems "Microamp Fast Optical 96 well reaction plate with barcode" → found at Andre's bench
11. Prepare plate ON ICE → put plate over orange plastic so it doesn't get any ice in it
12. Add master mix to appropriate wells
13. Add DNA of samples to appropriate wells
14. Centrifuge plate to get rid of bubbles and cover plate with plastic covering
15. PCR parameters:
    a. Instrument used: 7500 Fast (96 wells)
    b. Experiment set-up: Quantitation – Comparative Cr
    c. Target sequence detected: SYBR Green Reagents
    d. Instrument speed: Standard (~ 2 hours to complete a run)