Regulation of Multiple Myeloma Cancer Cell Plasticity and Stemness by MIF-1

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By

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Abbreviations

4-IPP – 4-iodo-6-phenylpyrimidine – MIF-1 inhibitor

7AAD- 7-aminoactinomycin D

AML – acute myeloid leukemia

BCL-6 – B cell lymphoma protein 6

Blimp1 – B lymphocyte induced maturation protein-1

BM –bone marrow

BMSC – bone marrow stromal cells

CD138/ SDC1 – cluster of differentiation 138/ syndecan 1

CD38 – cluster of differentiation 38/ cyclic ADP ribose hydrolase

CFSE –carboxyfluorescein succinimidyl ester

CSC –cancer stem cell

CXCR4 – C-X-C chemokine receptor 4

DNA – deoxyribose nucleic acid

ECM- extracellular matrix

FACS – fluorescent activated cell sorting

HSC – hematopoietic stem cell

IRF4 –interferon regulatory factor 4

ISO-1 – MIF-1 inhibitor

LSC- leukemic stem cells

MFI –mean fluorescent intensity

MGUS – monoclonal gammopathy of undetermined significance

MIF-1 – macrophage migration inhibitory factor -1

MM – multiple myeloma

PC - plasma cell

PCL - plasma cell leukemia

Pre-PC – pre-plasma cell

PY –pyronin Y

RBCs – red blood cells

RNA – ribose nucleic acid

WBCs – white blood cells

Abstract

Multiple myeloma (MM) is characterized by the clonal proliferation of malignant plasma cells within the bone marrow. While advancements made in chemotherapeutic treatment regimens may prolong MM patient survival, relapse is almost always inevitable. This suggests that there exists a sub-population of MM cells that are capable of evading cytotoxic insult and that can recapitulate the tumor burden once this insult is removed. The human myeloma cell lines RPMI8226, U266-B1, and NCI-H929 are all comprised of a heterogenous make-up of $CD138^{+}CD38^{+}$ and $CD138^{-}CD38^{+}$ cells present in an ~ 90-98: 2-10 ratio within the 2D tissue culture environment. It has been suggested that the $CD138^{-}CD38^{+}$ MM cells have the characteristics of a cancer stem cell (CSC) in that they are slowly proliferating, are able to undergo long-term self-renewal, are chemoresistant, and are able to differentiate into the mature $CD138^{+}CD38^{+}$ MM progeny cells. Our data supports previous findings that $CD138^{+}CD38^{+}$ MM cells exhibit plasticity: the capability to de-differentiate, and give rise to their $CD138^{-}CD38^{+}$ precursor cell. This interconversion is auto-regulated by the MM cells themselves, and ensures proper proliferation and maintenance of the population. We found that pure populations of either $CD138^{+}CD38^{+}$ or $CD138^{-}CD38^{+}$ cells are able to regulate this interconversion through the secretion of MIF-1, an inflammatory cytokine. Additionally, we found that blocking MIF-1, using either the small molecule MIF-1 inhibitor 4-IPP, or MIF-1 neutralizing antibodies, favors de-differentiation of the $CD138^{+}CD38^{+}$ MM cells into their $CD138^{-}CD38^{+}$ progenitor population. Our results suggest that both sorted MM cell populations secrete MIF-1 in order to establish and maintain their heterogeneic ratio in vitro and that this may occur in vivo to maintain a CSC population. We additionally propose that the regulation of MIF-1 expression may affect not only the differentiation status of MM cells, but also their response to chemotherapeutic agent, Bortezomib. Our findings suggest that the general clinical approach used to treat MM should take into account the heterogenic make-up of the cancer and the presence of both $CD138^{+}$ and $CD138^{-}$ MM cells, which have intrinsically different responses to chemotherapy.

Introduction

MM characterization

Multiple myeloma (MM) is the second most common hematological cancer[1] and is more prevalent among African-Americans than other ethnic groups[2]. This cancer is twice as likely to affect men as women and predominantly occurs in those over the age of 50[3]. MM is characterized by the proliferation of clonal plasma cells within the bone marrow[4]. This aberrant proliferation causes lytic lesions of the bone and interferes with normal blood cell production, resulting in hypercalcemia, anemia, and also often causing renal insufficiency[1, 5]. Similar to normal plasma cells, MM tumor cells usually secrete immunoglobulin. The myeloma cell product, M protein, can be detected in the peripheral blood and urine of patients and is used in the diagnosis and detection of minimal residual disease[2, 6, 7]. The median survival of MM patients is between 5-10 years post diagnosis[1].

Patients can be treated with a combination of drugs including: dexamethasone (lymphocytotoxic), bortezomib (proteasome inhibitor), and lenalidomide (immunomodulator)[1, 8, 9]. Depending on MM stage, treatment can include a regimen systemic therapy followed by high dose chemotherapy and autologous stem cell transplant (ASCT), where the patient's own stem cells are first harvested and then re-administered. While many MM patients achieve remission, almost all eventually relapse[10]. This has led to the hypothesis that a population of cells exists, which is capable of evading cytotoxic insult and of re-initiating tumor growth. These cancer stem cells (CSC) remain dormant and chemoresistant in the bone marrow niche. The median overall survival of patients with relapsed, refractory MM, previously treated with bortezomib and lenalidomide, is between 5 to 9 months[10], suggesting that these cells become resistant to therapy, and are able to expand.

Cancer Stem Cell Hypothesis

The characteristics of the cancer stem cell (CSC) are predicated on the already established characteristics of the hematopoietic stem cell (HSC)[11]. The HSC is pluripotent and produces all cell types

detected in the blood. It has the ability to give rise to an HSC (self-renew), and further differentiates into the erythroid/megakaryocytic, myeloid, and lymphoid progenitors[12, 13]. The erythroid/megakaryocytic progenitor produces red blood cells and megakaryocytes that produce platelets[12]. The myeloid progenitors differentiate into the common granulocyte precursor that gives rise to two different lineages; granulocytes, which are neutrophils, basophils, and eosinophils; and monocytes and mast cells[13]. The lymphoid progenitor can differentiate into T cells (effector T cells, cytotoxic T cells, natural killer T cells) , NK cells and B cells (memory B cells and plasma cells)[12, 13] (Figure 3). Figure 1 illustrates the ability of the pluripotent stem cell to self-renew and differentiate into lineage-committed progenitors that also possess the potential to further differentiate. HSCs proliferate at a slower rate and/or are quiescent. The slow proliferative rate of these stem cells makes them more resistant to DNA damage caused by environmental factors or chemical agents[14]. The HSC resides in the bone marrow niche, which itself is influential in the maintenance of the HSC population[13, 14].

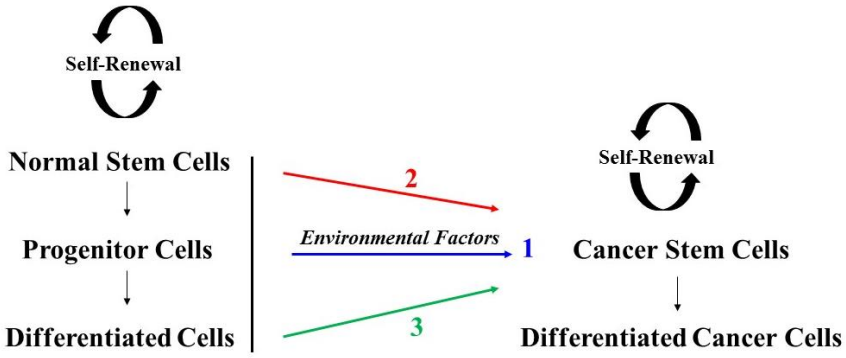

Figure 1. Possible origin of the cancer stem cell. It has been postulated that cancer stem cells can be derived from normal stem cells, or more differentiated cells that have acquired some genetic mutation to endow them with stemness characteristics. These malignant cells would regain the ability to self-renew, and differentiate which are hallmarks of cancer. This figure was adapted from Zheng, S., Xin, L., Liang, A., Fu, Y. Cytotechnology 65, 505-512 (2013).

Stimuli that cause DNA damage/instability can transform normal stem cells, progenitor cells, or differentiated cells to become a malignant tumor cell or to become a cancer stem cell[15]. Similar to the HSC, data suggest that the CSC is characterized by its ability to undergo long-term self-renewal, to give rise to differentiated progeny, to proliferate at a slower rate and/or to remain quiescent[15, 16]. The CSC also appears to be chemoresistant and may exist in a niche that provides a physical barrier to protect against cytotoxic insult[17]. These four characteristics define "stemness."[16, 18, 19] Chemotherapeutic agents that preferentially target rapidly dividing cells by inducing DNA damage and mitotic instability are ineffectual against CSCs, which do not proliferate as often as their rapidly dividing counterparts. When the therapeutic insult is removed, CSCs are free to produce

malignant progeny that can re-populate tumors *in vivo*. Data also suggest that CSCs can be resistant to agents, like bortezomib, which do not rely on the proliferative state and/or DNA damage to arrest cells, suggesting additional mechanisms are responsible[9, 20, 21]. The mechanism by which the CSC remains in its quiescent state is ill-defined, but may be due to extrinsic and intrinsic factors that are generated by autocrine or paracrine signaling from the CSC and/or its niche[17, 22].

Cancer	CSC Marker
AML	$CD34^{+}$ $CD38^{-}$
Breast	$CD44^{+}$ $CD24^{low/-}$ ESA^{+}
Colon	$CD133^{+}$
Lung	$CD133^{+}$
Prostate	$CD44^{-}$ $\alpha_2\beta_1^{hi}$ $CD133^{+}$
Liver	$CD133^{+}$
Brain	$CD133^{+}$ $CD15^{+}$

Table 1. Cancer Stem Cell Markers In Different Human Cancers. This table was adapted from Rahman, M., Deleyrolle, L., Vedam-Mai, V., Azari, H., Abd-El-Barr, M., Reynolds, B. Neurosurgery, 68(2), 531-545 (2011).

The concept of the CSC has been more fully defined in other blood cancers, such as acute myeloid leukemia (AML), which was the first cancer shown to have CSCs[23]. The CSCs in AML have been characterized by their specific cell-surface proteins and gene expression signatures, which frequently are shared between leukemic stem cells and normal HSCs, and have been used for targeted therapy[24, 25]. Evidence suggests that CSCs might also exist in several solid cancers as well, and can be identified by cell-surface markers, as seen in Table 1[26]. It has also been shown that targeting this population of cells through the use of monoclonal antibodies against specific cell surface markers or certain intracellular proteins has increased patient remission rates or overall survival in several cancers[23, 27]. In AML, the CSC population/leukemic stem cell can be quite heterogenous in its expression of several cell surface markers such as CD19, CD33, CD34, and CD38[23, 24]. The advent of gemutuzumab ozogamicin (GO), which is a monoclonal antibody against CD33, is an example of targeted therapy against the leukemic stem cell (LSC) in AML[28]. GO have proven to be effective in increasing the overall survival of patients with previously untreated *de novo* AML in combination with conventional high dose chemotherapeutic treatments[24, 28, 29]. The best way to identify a cancer stem cell would be to identify a cell that has the characteristics of stemness: the ability to undergo long-term self-renewal, to remain quiescent, to undergo differentiation, and to remain chemoresistant[26].

Characteristics of Stemness

Self-Renewal

The first characteristic, self-renewal, is the potential of the cell to give rise to clonal progeny that retain characteristics of stemness[30]. CSCs need to produce CSCs. Stem cells must maintain the ability to self-renew to provide for the survival and future replenishing of a specific cell type[13]. Self-renewal of the stem cell can be either symmetric or asymmetric. In symmetric cell division, both daughter cells generated after cell division are identical to the parent cell and this allows for the maintenance of the stem cell pool[31]. In asymmetric division, one daughter cell is identical to the parent stem cell, while the other has a differentiated cell fate[31] (Figure 2). The more differentiated progenitor often has less proliferative capacity than its precursor[32, 33].

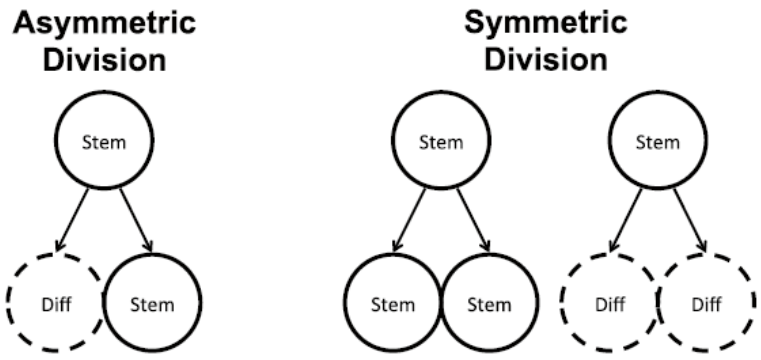

Figure 2. Asymmetric division vs Symmetric division. Types of stem cell division that illustrate its capacity to self-renew and differentiate. This figure was taken from Shahriyari, L., Komarova, N.L.PLoS ONE 8(10) e76195 (2013).

Differentiation

The purpose of the tissue specific stem cell is to maintain homeostasis and provide a source to replenish the tissue after cell death or injury[34]. This can only happen if the multipotent stem cell differentiates, produces a more specialized and mature progeny[12]. The progression from the HSC to the terminally differentiated plasma cell (Figure 3) is an example of differentiation and illustrates that with each progressive phase of differentiation, cell surface markers are gained or lost due to the transcriptional regulation of the proteins necessary for proper

development and function[12, 13]. These phenotypic changes can be analyzed by flow cytometry and can track the progression of differentiation.

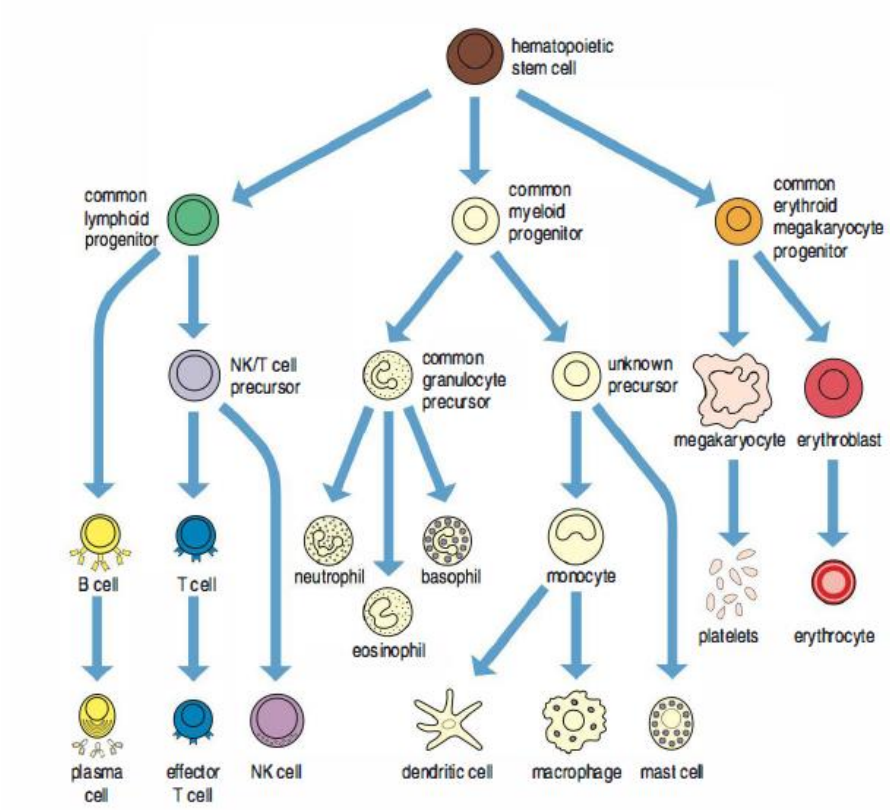

Figure 3. Lineage commitment of the pluripotent Hematopoietic Stem Cell. The HSC differentiates to give rise to all cell types of the blood and immune. This figure was taken from Parham, P. (2009) The Immune System. NY, NY: Garland Science.

Slowly Proliferating/ Quiescence

The cell cycle is separated into four phases[35]. The first phase, G1 phase, is a period of cell growth, where there is an increase in cellular organelles and the cell is transcriptionally active, preparing for cell division[36]. A cell in G1 phase has a 2N DNA content and a low, but steadily increasing RNA content. The second phase is S phase, also known as the synthesis phase, where DNA is replicated. A cell in S phase has DNA content between 2N-4N, indicative of the increasing DNA content of the cell. The third phase, G2 phase, is where the cell continues to be transcriptionally active and grow in size in preparation for cytokinesis. A cell in G2 phase has a 4N DNA content and a high level of RNA. The final phase, M phase, or mitosis, is where the cell divides. When a cell exits the cell cycle for extended periods of time, such as seen with dormant HSCs in the bone marrow, it exists in a quiescent phase also known as G0 phase. This phase is characterized by the lack of DNA replication and low transcription, and has a 2N DNA content and the lowest relative level of RNA[36, 37]. Using fluorescent dyes that bind or intercalate into DNA or RNA, these cell cycle phases can be resolved by flow cytometry[38]. Figure 4A is an example of cell cycle phase separation and shows

the resolution of proliferating cells based on their DNA content. Figure 4B shows the progression of cells from G0 phase (low DNA, low RNA) into the cell cycle and S phase (high DNA, high RNA).

Most tissue specific stem cells are maintained in a quiescent state until certain chemical or physical factors stimulate their proliferation and exit from G0 phase. Once in cycle, division will be asymmetric or symmetric, and differentiated progeny may be produced[31]. Cancer stem cells are typically detected in the G0 phase[14, 19].

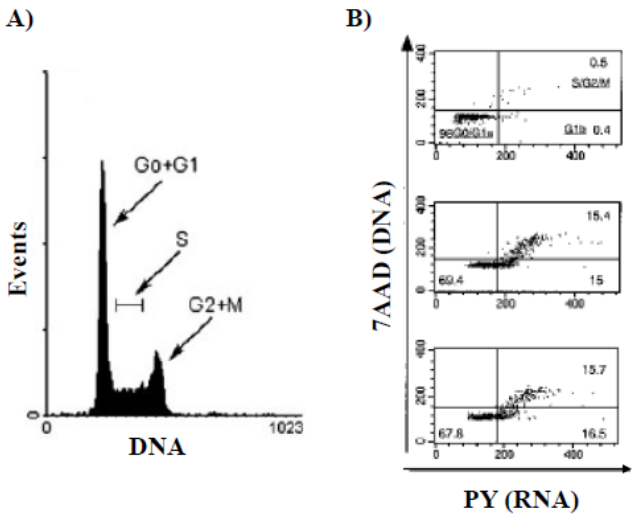

Figure 4. Cell cycle analysis using flow cytometry. A) Cell Cycle profile of mouse kidney proximal tubule cells stained with propidium iodide (PI). Panel A was taken from Price, P.M., Safirstein, R.L., Megyesi, J. Am J Physiol Renal Physiol 286: F378-F384 (2004). B) Cell cycle entry of naïve cord blood, naïve adult and memory T cells activated by IL-7. Panel B was taken from Verhoeyen,E et.al. Blood 101(6); 2167-2174 (2003).

Chemoresistance

In some cases, the fourth characteristic of stemness, chemoresistance is a result of quiescence, because most high dose chemotherapeutic drugs cause DNA instability[26]. For cells that are proliferating and progressing through the cell cycle, DNA damage triggers cell cycle arrest and/or apoptosis, measured as chemosensitivity[26]. Chemoresistance can be assessed by measuring the viability of cells after incubation with a specific chemotherapeutic drug. However, CSCs frequently appear resistant to non-DNA damaging agents as well. Data suggest that CSCs frequently overexpress multi-drug resistant transporters that actively pump out the drug, thereby preventing the cell from metabolizing the toxic agent and permitting them to remain viable in the presence of the chemotherapeutic agent[16]. CSCs may also evade toxicity due to their location, where the tumor microenvironment shelters these cells from exposure to chemotherapeutic drugs[17, 22]. It has been shown in a mouse model of AML that the endosteal region of the bone marrow acts as a protective niche for human AML stem cells resulting in a quiescent and chemoresistant population of cells[39].

These four characteristics define stemness, regardless of cell surface marker expression[26]. The concept of the CSC is controversial, and some do not believe that a distinct population of cells must exist[18]. It has been suggested that "stemness" might simply be a characteristic that is acquired during tumor progression[40, 41]. The proliferating tumorigenic cell in MM is the plasma cell and my work and that of others has demonstrated in both primary patient-derived tumor cells and in MM cell lines that a subset of plasma cells can be consistently detected that meets the characteristics of stemness[33, 42-44]. The examination of this population is the basis of my thesis work.

B Cell Development

B cells are generated in the bone marrow from the HSC via the lymphoid progenitor[45] (Figure 5). In the presence of chemokine gradients, the naïve B cell relocates to the secondary lymphoid tissues, such as the spleen and/or lymph nodes[45, 46]. Here, naïve B cells are activated through the binding of antigen to their BCR (or surface Ig), and co-stimulation by CD40 Ligand present on a helper T cell, which causes rapid cell division of the B cell to form a structure called the germinal center[45, 47]. Within the germinal center, B cells undergo class-switch recombination where rearrangement of the heavy chain locus constant regions will result in the switch of one immunoglobulin class to another, such as IgM to IgG[47]. The B cell also

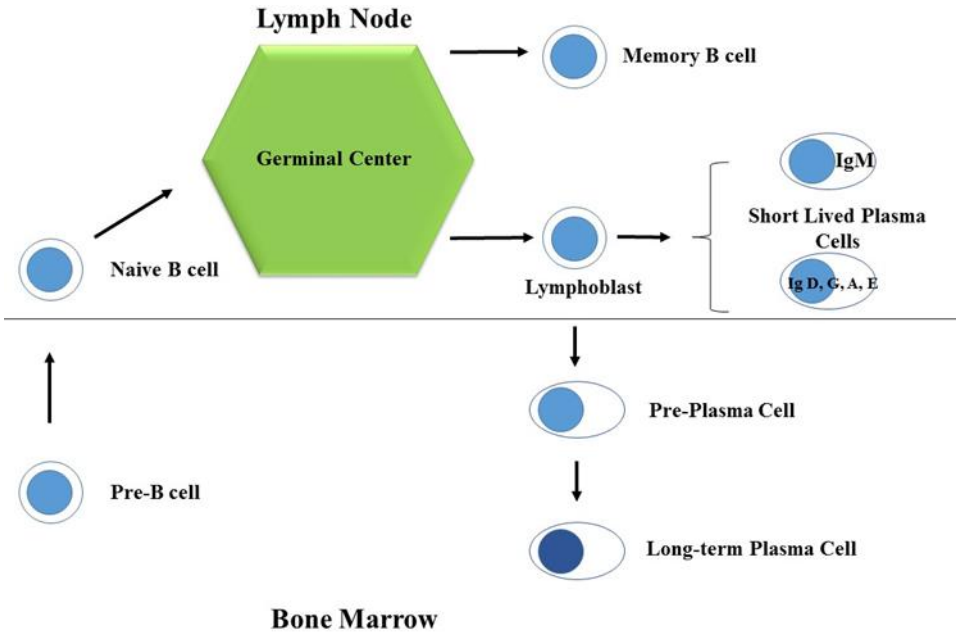

Figure 5. Illustration of the maturation and transport of the B cell into terminally differentiated plasma cells between first and second lymphoid tissues. This figure is adapted from Hallek, M., Bergsagel, P.L., Anderson, K.C. Blood, 91(1), 3-21 (1998).

undergoes the process of affinity maturation which consists of two interrelated processes; somatic hypermutation and clonal selection[45]. In somatic hypermutation, single point mutations, deletions, or insertions are introduced into the variable regions of both the heavy and light chains of the immunoglobulin gene[45]. In the process of clonal selection, follicular dendritic cells display antigen to B cells within germinal centers, and B cells that express immunoglobulin with the highest affinity to that antigen are able to bind and proliferate, (positive selection)[41, 45]. Ultimately, the full maturation of the B cell results in the cessation of cell cycle progression and the induction of the differentiation program that leads to synthesis and expression of the appropriate high affinity immunoglobulin, to produce an antibody with increased antigen specificity[41]. Antigen selected B cells exit the germinal center as either an affinity matured memory B cell or a pre-plasma cell/plasmablast[41] (Figure 6).

Memory B cells can be retained in the spleen or lymph nodes and serve as part of the secondary immune response, which provides the rapid proliferation and secretion of the antibody in the event the same antigen is later encountered[45, 47]. During B cell development, cell surface markers are transiently expressed at different stages and can be used to characterize each subpopulation. Some of the established and noted cell surface markers associated with post-germinal center B cells are CD19, CD21, CD27, and B220[47]. These B cell markers are gradually lost during maturation, concomitant with the upregulation of syndecan-1 (CD138), CD38, and CD44, which are hallmarks of mature, non-cycling, immunoglobulin-secreting plasma cells[41, 47]. Intermediate phases of the maturing plasma cell do exist; the prePC, the immature precursor is CD138-, CD38+ whereas the terminally differentiated antibody-secreting plasma cell is CD138+, CD38+[41]. Plasmablasts that exit the germinal center of secondary lymphoid tissues can either remain in those tissues or relocate to the bone marrow where stromal cell interaction along with chemokine availability promote differentiation into an antibody secreting plasma cell[41, 48]. As a part of the humoral branch of the immune system, the purpose of the plasma cell is to secrete functioning antibodies into circulation[49]. Normal plasma cells are maintained in a non-proliferative state and are found within the bone marrow and secondary lymphoid tissues dependent on their pre-determined life span[49]. Short lived plasma cells are found close to where antigen is encountered, only

secrete IgM and die between 7 -10 days[46, 49]. Long lived plasma cells secrete antibody that has high affinity toward antigen, are found within the bone marrow, and have a life span of more than 30 days[45, 49] (Figure 5).

The cell surface marker CD138 (syndecan-1) is an important indicator of the maturation of the pre-plasma cell into the terminally differentiated plasma cell, but it also has been shown to have a function in cell:cell and cell:matrix adhesion[50]. Normal plasma cells are maintained in the bone marrow through the interaction of CD138 and the stromal matrix via type I collagen[34, 49]. Cytokines can shift a cell's ability to adhere to extracellular matrices by causing the downregulation of cell surface markers important for cell:cell adhesion, as has been seen in B cell development where maturing pre-B cells downregulate the chemokine receptor, CXCR4, causing them to leave the bone marrow microenvironment to translocate to secondary lymphoid tissues[51].

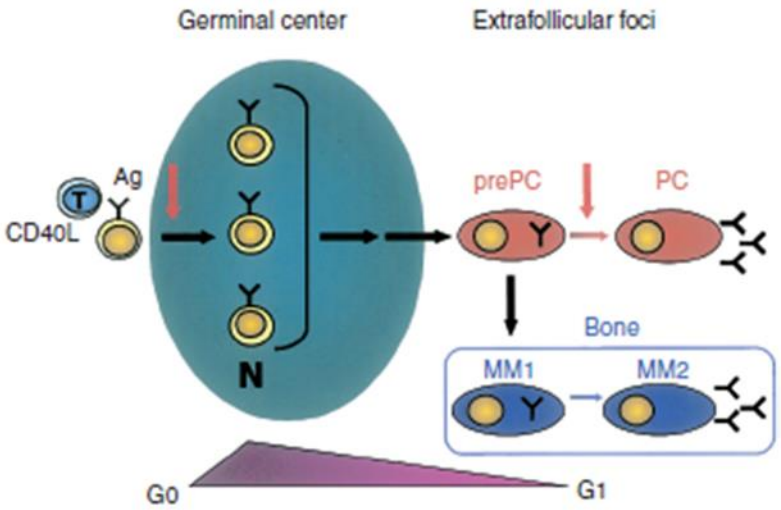

Figure 6. Proposed malignant Plasma Cell generation and MM cell heterogeneity model. After B cell interaction with CD40 ligand and antigen, B cells proliferate to form germinal centers. B cells that are selected to exit the germinal center are classified as a pre-plasma cell (prePC). These cells then arrest (big red arrow) and further differentiate into a functioning plasma cell (PC). Genetic instability or environmental factors (thick black arrow) can cause a normal prePC to differentiate into two malignant classes of PC: MM1 and MM2. This figure was taken from Chen-Kiang, S. Immunol Rev 194: 39-47 (2003).

This maturation process is also highly dependent on the presence or absence of two transcription factors, Bcl-6 (B cell lymphoma-6) and Blimp1 (B lymphocyte-induced maturation protein 1)[52, 53]. Pre-B cells and naïve B cells express high levels of Bcl6 and low levels of Blimp-1, whereas mature plasma cells have the inverse expression of both transcription factors[54]. It has been shown that these transcriptions factors repress each other during B cell development[55-57]. Bcl6 functions as a transcriptional repressor for multiple targets, including Blimp1, p27, and Bcl-2[58]. In the pre-B cell, Bcl-6 expression is at its highest and is down-regulated as the B cell develops into an antibody-secreting plasma cell where the expression of Blimp1 is at its highest level. Blimp-1 expression causes the differentiation of B cells into plasma cells and has been shown to

inhibit the expression of AID, Ku-70, Ku-86, DNA-PKcs and STAT6, which are required for class switching within the germinal center[41, 56]. In patient-derived MM tumor cells, both plasma cells and precursor cells express Blimp[6, 59]. IRF4 (IFN regulatory factor 4) is also an important transcription factor in B cell differentiation and has been shown to downregulate BCL-6 expression[60]. IRF4 is overexpressed in MM tumor cells and its expression is crucial to the homing of the MM cell to the BM as well as its survival[51].

Multiple Myeloma Development

In general, the majority of MM tumor cells isolated from patients are CD138+, terminally differentiated plasma cells. However, both CD138- and CD138+ MM cells can be detected in primary MM tumor samples and commercially available cell lines[33]. It has been suggested that the CD138+ MM cell arises from the prePC, CD138- MM cell through the acquisition of additional mutations, allowing further differentiation into two classes of aberrant plasma cells: MM1 and MM2[41]. According to the model posed by the Chen-Kiang lab, the MM1 cells are the non-cycling plasma cells (most likely the CSC) and the MM2 cells are the rapidly dividing plasma cells. These two classes of plasma cells home to the bone marrow where they cause the lesions attributed to this disease[41] (Figure 6).

Data suggest that the MM tumor cell, is a CD138- CD38+ prePC, or a cell that has phenotypically left the B cell classification and is transitioning into a mature, differentiated CD138+CD38+ plasma cell[42]. Both CD138+ and CD138- cells are found within the bone marrow. The Matsui lab found that CD138- cells were able to form more colonies than the differentiated CD138+ cells after serial re-plating in methylcellulose, suggesting that the CD138- cells possess long-term self-renewal capabilities[9]. When NOD/SCID mice were injected intravenously with either CD138+ or CD138- patient-derived cells, only CD138- cells were able to engraft into the bone marrow of these mice[9]. However, upon analysis of these bone marrow aspirates, they found that the cells had become CD138+ and expressed the same light chain restriction as the initial patient material[9]. This was the first suggestion that this prePC CD138- cell had the stem-like phenotype.

Other labs have since shown that both CD138- and CD138+ cells are able to engraft and colonize bone marrow if a different mouse model is used. Chaidos et al. (2013) was able to show that primary human CD138+ as well as CD138- cells were able to engraft NSG (NOD/SCID/ common cytokine receptor γ-chain deficient) mice, and human CD138- cells were detected in the bone marrow aspirates in both groups[42]. Kim, et al (2012) compared the engraftment capability of $CD138^+$ and $CD138^-$ primary MM cells injected into NOD/SCID, NSG, and $RAG2^-/\gamma c^-$ (recombinase-activating gene 2/ common cytokine receptor γ chain-deficient) mice injected either intravenously, intrafemorally with primary MM cells, or by subcutaneous placement of fetal human bone with primary MM cells[43]. This study showed that human fetal bone grafts injected with CD138+ MM primary cells resulted in the detection of human antibodies in the serum of 30% of immunocompromised mice. Only one of seventy one immunocompromised mice (NSG and $RAG2^-/\gamma c^-$) that had primary MM cells injected intravenously or intrafemorally was able to successfully engraft[43]. Additionally, more than 60% of these xenotransplantation mouse models showed expansion of the human MM primary cells resulting in both CD138+ and CD138- cells[43]. This suggests that the microenvironment may play a significant role in the cell fate of both the CD138- and CD138+ cell. However, only xenografts of CD138+ MM primary cells were serially transplantable in immunocompromised mice, suggesting that in this study CD138+ cells might be the source of the CSC population.

At first, these studies might seem conflicting. However, in Yaccoby et al. (2005), CD138+ CD38+ cells, isolated from primary bone marrow samples of patients with MM, were co-cultured with osteoclasts and the cell surface expressions of B cell and plasma cell markers were analyzed after 6 weeks[44]. These experiments showed that the CD138+ CD38+ CD45- mature plasma cell population gave rise to plasma cells that had reduced CD138, and CD38 expression and began to express CD45. CD138-CD45+ is indicative of a plasma precursor cell (pre-PC). This study suggests that CD138+ cells were able to de-differentiate into CD138- cells and the osteoclast microenvironment was responsible[44]. These newly dedifferentiated CD138- MM plasma cells were more chemoresistant displaying a decrease in Annexin V staining after treatment with chemotherapeutic agent, dexamethasone, consistent with the idea that CD138- cells were the CSC. Even

though there is a physical interaction between the MM cells and osteoclasts, the MM cells do not adhere to the osteoclasts suggesting that soluble factors present in the media are responsible for this de-differentiation. Osteoclasts secrete several cytokines, such as IL-6, which promote plasma cell survival. In the Yaccoby et al. (2005) study, neutralizing antibodies were used against both IL-6 and the IL-6 receptor to determine the effect on the differentiation status of MM plasma cells[44]. This method did not inhibit the de-differentiation of CD138+ MM plasma cells, suggesting that IL-6 was not the cytokine responsible for MM plasma cell de-differentiation.

The capacity of both the immature CD138- pre-PC and the mature CD138+ PC to initiate tumor growth was also suggested by Chaidos et al.[42] (2013). In this study, the CD138- pre-PCs were quiescent (detected in the G0/G1 phase) and were more morphologically similar to B cells than to fully differentiated CD138+ plasma cells. Upon engraftment of NSG mice, the CD138- pre-PC MM population gave rise to differentiated CD138+ MM cells. However, the mature CD138+ primary MM cells were also able to engraft into NSG mice and both CD138+ and CD138- human cells were found in the bone marrow, spleen and liver of these mice. Based on mathematical modeling, Chaidos et al. (2013) suggested that the CD138- cells detected in the CD138+ engrafted animals were likely to be derived from the CD138+ cells, suggesting a plasticity existed in the CD138+ MM cell population[42]. Here, the cell surface markers, in addition to CD138, which further characterized both the pre-PC and the PC, were CD319, CD19, and CD38. CD138-CD319+CD38++CD19- described the pre-PC and CD138+CD319+CD38++CD19- described the mature PC[42].

Role of Tumor Microenvironment and CSC maintenance

The tumor microenvironment may play a significant role in maintaining and enhancing tumor growth, especially in MM. The plethora of cell types that make up this specialized and nurturing environment could both shelter and influence the cancer stem cell. The bone marrow microenvironment associated with MM primarily consists of hematopoietic stem cells, osteoclasts, osteoblasts, fibroblasts, macrophages, adipocytes

and endothelial cells, which have been shown to interact with these malignant plasma cells[61, 62]. Osteoclasts associated with MM have been shown to secrete elevated levels of IL-6 and IL-8, which in turn activate MM tumor cells causing rapid proliferation[63]. Certain cytokines are readily released by MM tumor cells[48, 59, 64]. An important attribute for the preservation of the CSC is the ability to influence the surrounding microenvironment to support cell growth and proliferation. This is usually carried out through the secretion in an autocrine regulatory manner of certain cytokines that act as chemoattractants for the recruitment of specialized cells. Cytokines often associated with MM are IL-6, IL-8, TNF-α, IFN-γ, SDF1, and MIF-1[48, 65, 66] (Figure 7).

In 2008, the Kirshner lab created an in vitro three-dimensional model to investigate and track the clonal expansion of the chemoresistant MM cell[67]. They constructed a 3D pseudo-bone marrow microenvironment using a fibronectin/collagen I surface (called the reconstructed Endosteal layer), overlaid with bone marrow mononuclear cells isolated from MM patients in a fibronectin/matrigel mixture, and additionally covered in growth media containing patient serum[67]. They were able to stain the patient bone marrow mononuclear cells with CFSE (carboxyfluorescein succinimidyl ester) to track MM cell proliferation over time. After 3 weeks in culture, the cells were removed from the pseudo-bone marrow microenvironment and stained for their expression of CD138. They found that after extended culture, there were still MM PCs that were high for CFSE (non-dividing) as well as MM PCs that were low for CFSE (dividing). They observed that all of these CFSE^{hi} PCs localized to the endosteal niche of the 3D pseudo-BM, the CD20+ B cells localized to the middle layer, and the CD138+ CFSE^{low} PCs localized mainly to the top of

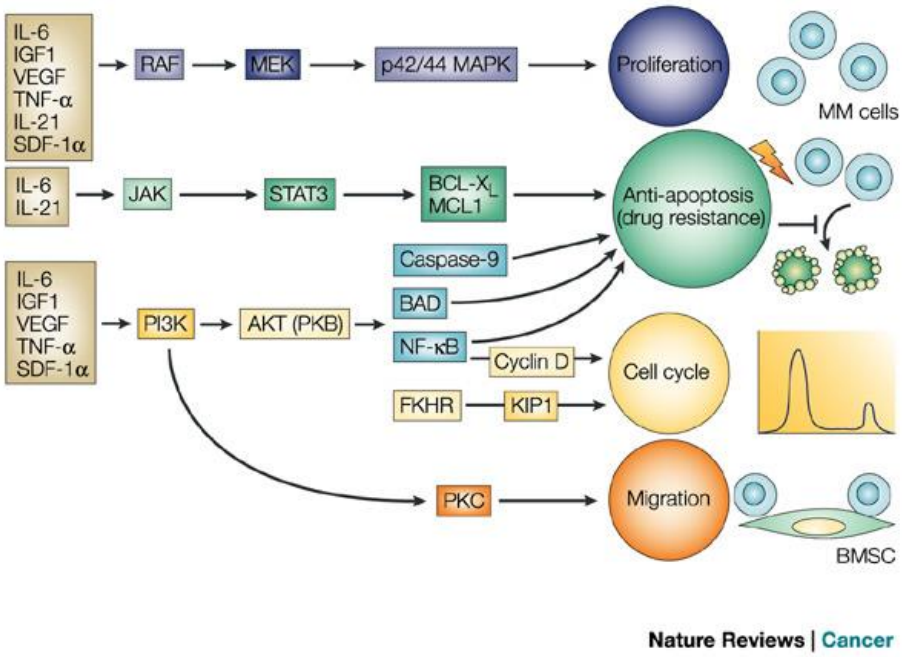

Figure 7. Cytokines associated with MM cell survival within the tumor microenvironment. This figure is from Hideshima, T., Anderson, K.C. Nat Rev 2, 927-937 (2002).

the 3D-pseudo-BM. When treated with melphalan, there was an increase in the percentage of $CFSE^{hi}$ PCs in comparison to the untreated 3D-pseudo BM, suggesting that these cells were chemoresistant and quiescent[67]. This data suggest that the MM CSC is a physiological population of cells that can maintain their survival by actively creating a niche when cultured in the presence of accessory cells. These MM CSCs were slowly proliferating, chemoresistant and had characteristics of stemness.

Taken together, MM PCs can be influenced by their microenvironment. Cytokines secreted by cells of the bone marrow stroma can cause the MM PC to re-program itself and become the MM CSC or the physical location of the MM PC within the bone marrow provides a protective barrier from cytotoxic insult.

MM Cell Lines In Multiple Myeloma Research

As advances in MM research continue, experimentation using higher order subjects, such as mice, rabbits, macaques, are especially valuable since they have the potential to mimic the cellular and systemic interactions that might happen in a patient suffering with MM. However, for the >30 years since they have been created, MM cell lines have been a powerful avenue to study the complexities of MM pathogenesis. Studying these transformed MM cells in vitro, in the absence of an extracellular matrix or niche, can still highlight novel characteristics within MM. Some of the most commonly used MM cell lines are RPMI8226, AMO-1, MOLP-8, KMS-12-BM, EJM, IM-9, U266-B1, NCI-H929, and OPM-29[33, 68]. The three MM cell lines used in my study are RPMI8226, U266-B1, and NCI-H929. There are several chromosomal translocations that can be found in patients, which are associated with disease progression and prognosis. There are five chromosomal translocation events between oncogenes and the IgH locus that occur in nearly 50% of all MM and MGUS (monoclonal gammopathy of undetermined significance) patient-derived clonal plasma cells[69]. These are: 1) 4p16 (MMSET and FGFR3), 2) 6p21 (Cyclin D3), 3) 11q13 (Cyclin D1), 4) 16q23 (c-MAF), and 5) 20q11 (MAFB)[6, 70]. These translocations are also found in the MM cell lines. The RPMI 8226 cell line was generated from cultures grown after leukapheresis was performed on a 61 year old Caucasian man with MM and this cell

line contains the MAF, IgH translocation[71]. MAF (avian musculoaponeurotic fibrosarcoma oncogene homolog) is a transcription factor that targets cyclin D2 and regulates lineage-specific hematopoiesis.

The RPMI 8226 cell line has been shown to be heterogeneous in that it consists of CD138+ CD38+ cells (~90-95%) and a subpopulation CD138-CD38+ (~5-10%)[9]. The U266-B1 cell line was generated from leukocytes isolated from peripheral blood of a 53 year old Caucasian man diagnosed with MM and this cell line contains the cyclinD1, IgH translocation[72]. Cyclin D is present in three isoforms; D1, D2, and D3. This protein is essential for progression through the G1 phase of the cell cycle by forming a complex with cyclin-dependent kinases (cdk4/6)[36]. The U266-B1 cell line is also heterogenous in that it consists of both mature plasma cells (CD138+, ~ 95-98%) and immature plasmablasts (CD138-, ~2-5%)[33]. However there is also differential expression of CD38 on U266 MM cells. The NCI-H929 cell line was generated from cells isolated from a pleural effusion of a 62 year old Caucasian woman, with relapsed MM[73]. This cell line contains the FGFR3/MMSET, IgH translocation. FGRFR3 is the gene encoding the fibroblast growth factor receptor 3 and has been shown to be upregulated in approximately 40% of MM patients. MMSET is the multiple myeloma SET domain protein, which is a histone methyltransferase and thereby has a role in regulating transcription. Both FGFR3 and MMSET are located on chromosome 4 and the t(4;14) translocation that occurs in MM results in overexpression of both FGFR3 and MMSET. The NCI-H929 cell line is also heterogeneous in that it contains both CD138+CD38++ (~95-98%) and CD138-CD38++ (~2-5%) MM cells[33]. Previous data has shown that of these three MM cell lines, only U266-B1 retains the capacity to secrete monoclonal Ig[71-74]. Thus, the

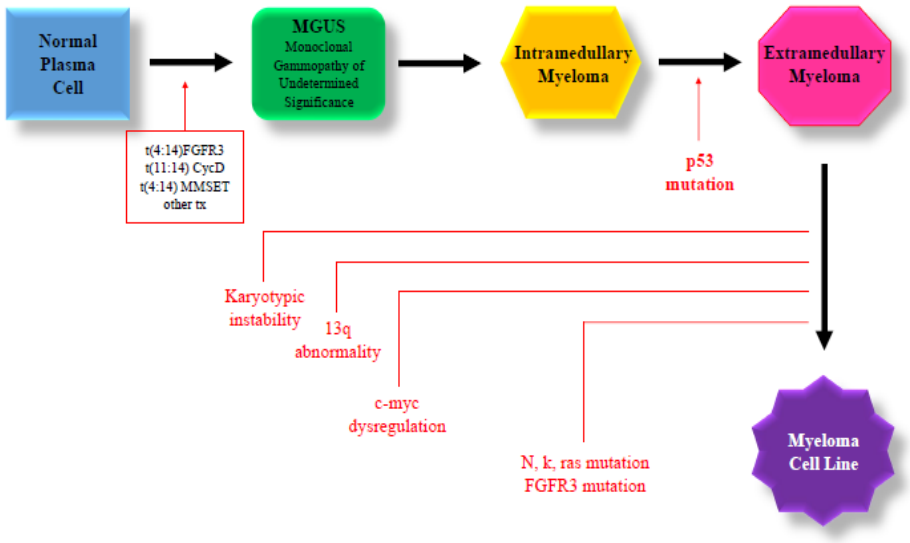

Figure 8. Karyotypic instability events that lead to the progression of malignant and immortalized plasma cells. This figure is adapted from Hallek, M., Bergsagel, P.L., Anderson, K.C. Blood 91(1), 3-21 (1998).

characterization of the karyotype of these MM cell lines is a good model system to use despite immortalization and the fact that they grow in the absence of accessory cells. These genetic differences underscore the observation that different translocations can result in malignant plasma cells and develop the tumorigenic heterogeneity and epigenetic plasticity indicative of MM that are the focus of my study.

Because of the controversy as to the nature of the MM CSC, I decided to investigate and identify the CSC based on the characteristics of stemness in addition to cell surface markers. Both primary patient material and MM tumor cell lines contain the subset of CD138- cells believed to be the MM CSC. We wanted to determine how this potential MM CSC could be an active participant in its own maintenance even in the absence of accessory cells. We hypothesize that we can identify the MM CSC population using assays to determine the capacity of a population to self-renew, to differentiate, to remain quiescent and to be chemoresistant. Using flow cytometry, both the immature CD138- MM cell population and the CD138+ MM cell population from the MM cell lines can be sorted to 99% purity, which allows us to characterize and analyze each population for cell surface marker expression, cell cycle phase, response to chemotherapy, and cytokine secretion. We further determined whether manipulating any of these secreted cytokines was able to alter the stem-like characteristics of the CD138- MM cell population or the plasticity of the mature CD138+ MM cell population. Thus, I was able to generate the goals of my specific aims as outlined below.

References

1. Mateos MV, Ocio EM, Paiva B, et al. Treatment for patients with newly diagnosed multiple myeloma in 2015. *Blood Rev*. 2015;29(6):387-403.
2. Zwick C, Held G, Auth M, et al. Over one-third of African-American MGUS and multiple myeloma patients are carriers of hyperphosphorylated paratarg-7, an autosomal dominantly inherited risk factor for MGUS/MM. *Int J Cancer*. 2014;135(4):934-8.
3. Greenberg AJ, Vachon CM, Rajkumar SV. Disparities in the prevalence, pathogenesis and progression of monoclonal gammopathy of undetermined significance and multiple myeloma between blacks and whites. *Leukemia*. 2012;26(4):609-14.
4. Mahindra A, Hideshima T, Anderson KC. Multiple myeloma: biology of the disease. *Blood Rev*. 2010;24 Suppl 1:S5-11.
5. Paiva B, van Dongen JJ, Orfao A. New criteria for response assessment: role of minimal residual disease in multiple myeloma. *Blood*. 2015;125(20):3059-68.
6. Bergsagel PL, Kuehl WM. Molecular pathogenesis and a consequent classification of multiple myeloma. *J Clin Oncol*. 2005;23(26):6333-8.
7. Bianchi G, Munshi NC. Pathogenesis beyond the cancer clone(s) in multiple myeloma. *Blood*. 2015;125(20):3049-58.
8. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res*. 2001;61(7):3071-6.
9. Matsui W, Wang Q, Barber JP, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. *Cancer Res*. 2008;68(1):190-7.
10. Rajkumar SV. Multiple myeloma: 2016 update on diagnosis, risk-stratification, and management. *Am J Hematol*. 2016;91(7):719-34.
11. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.
12. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med*. 2010;2(6):640-53.
13. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell*. 2008;132(4):631-44.
14. Nakamura-Ishizu A, Takizawa H, Suda T. The analysis, roles and regulation of quiescence in hematopoietic stem cells. *Development*. 2014;141(24):4656-66.
15. Kreso A, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell*. 2014;14(3):275-91.
16. Donnenberg VS, Donnenberg AD. Multiple drug resistance in cancer revisited: the cancer stem cell hypothesis. *J Clin Pharmacol*. 2005;45(8):872-7.
17. Castells M, Thibault B, Delord JP, Couderc B. Implication of tumor microenvironment in chemoresistance: tumor-associated stromal cells protect tumor cells from cell death. *Int J Mol Sci*. 2012;13(8):9545-71.
18. Zheng S, Xin L, Liang A, Fu Y. Cancer stem cell hypothesis: a brief summary and two proposals. *Cytotechnology*. 2013;65(4):505-12.
19. Lewis MT. Faith, heresy and the cancer stem cell hypothesis. *Future Oncol*. 2008;4(5):585-9.
20. Xu D, Hu J, Xu S, et al. Dll1/Notch activation accelerates multiple myeloma disease development by promoting CD138+ MM-cell proliferation. *Leukemia*. 2012;26(6):1402-5.
21. Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabrilovich DI. Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. *Blood*. 2004;103(9):3503-10.
22. Burness ML, Sipkins DA. The stem cell niche in health and malignancy. *Semin Cancer Biol*. 2010;20(2):107-15.

23. Pollyea DA, Gutman JA, Gore L, Smith CA, Jordan CT. Targeting acute myeloid leukemia stem cells: a review and principles for the development of clinical trials. *Haematologica*. 2014;99(8):1277-84.
24. Gao W, Estey E. Moving toward targeted therapies in acute myeloid leukemia. *Clin Adv Hematol Oncol*. 2015;13(11):748-54.
25. van Rhenen A, van Dongen GA, Kelder A, et al. The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood*. 2007;110(7):2659-66.
26. Rahman M, Deleyrolle L, Vedam-Mai V, et al. The cancer stem cell hypothesis: failures and pitfalls. *Neurosurgery*. 2011;68(2):531-45; discussion 45.
27. Saito Y, Kitamura H, Hijikata A, et al. Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. *Sci Transl Med*. 2010;2(17):17ra9.
28. Hills RK, Castaigne S, Appelbaum FR, et al. Addition of gemtuzumab ozogamicin to induction chemotherapy in adult patients with acute myeloid leukaemia: a meta-analysis of individual patient data from randomised controlled trials. *Lancet Oncol*. 2014;15(9):986-96.
29. Castaigne S, Pautas C, Terre C, et al. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. *Lancet*. 2012;379(9825):1508-16.
30. Tan BT, Park CY, Ailles LE, Weissman IL. The cancer stem cell hypothesis: a work in progress. *Lab Invest*. 2006;86(12):1203-7.
31. Shahriyari L, Komarova NL. Symmetric vs. asymmetric stem cell divisions: an adaptation against cancer? *PLoS One*. 2013;8(10):e76195.
32. Buick RN, Chang LJ, Messner HA, Curtis JE, McCulloch EA. Self-renewal capacity of leukemic blast progenitor cells. *Cancer Res*. 1981;41(11 Pt 2):4849-52.
33. Matsui W, Huff CA, Wang Q, et al. Characterization of clonogenic multiple myeloma cells. *Blood*. 2004;103(6):2332-6.
34. Kopp HG, Avecilla ST, Hooper AT, Rafii S. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology (Bethesda)*. 2005;20:349-56.
35. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer*. 2009;9(3):153-66.
36. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif*. 2003;36(3):131-49.
37. Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. *Cell Prolif*. 2003;36(3):165-75.
38. Darzynkiewicz Z, Halicka HD, Zhao H. Analysis of cellular DNA content by flow and laser scanning cytometry. *Adv Exp Med Biol*. 2010;676:137-47.
39. Saito Y, Uchida N, Tanaka S, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nat Biotechnol*. 2010;28(3):275-80.
40. Potten CS, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development*. 1990;110(4):1001-20.
41. Chen-Kiang S. Cell-cycle control of plasma cell differentiation and tumorigenesis. *Immunol Rev*. 2003;194:39-47.
42. Chaidos A, Barnes CP, Cowan G, et al. Clinical drug resistance linked to interconvertible phenotypic and functional states of tumor-propagating cells in multiple myeloma. *Blood*. 2013;121(2):318-28.
43. Kim D, Park CY, Medeiros BC, Weissman IL. CD19-CD45 low/- CD38 high/CD138+ plasma cells enrich for human tumorigenic myeloma cells. *Leukemia*. 2012;26(12):2530-7.
44. Yaccoby S. The phenotypic plasticity of myeloma plasma cells as expressed by dedifferentiation into an immature, resilient, and apoptosis-resistant phenotype. *Clin Cancer Res*. 2005;11(21):7599-606.
45. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. *J Allergy Clin Immunol*. 2013;131(4):959-71.
46. Hallek M, Bergsagel PL, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. *Blood*. 1998;91(1):3-21.

47. Hardy RR, Hayakawa K. B cell development pathways. *Annu Rev Immunol*. 2001;19:595-621.
48. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. *Nat Rev Cancer*. 2002;2(12):927-37.
49. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. *Nat Rev Immunol*. 2015;15(3):160-71.
50. Szatmari T, Otvos R, Hjerpe A, Dobra K. Syndecan-1 in Cancer: Implications for Cell Signaling, Differentiation, and Prognostication. *Dis Markers*. 2015;2015:796052.
51. Nie Y, Waite J, Brewer F, et al. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J Exp Med*. 2004;200(9):1145-56.
52. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol*. 2010;11(2):114-20.
53. Cattoretti G, Chang CC, Cechova K, et al. BCL-6 protein is expressed in germinal-center B cells. *Blood*. 1995;86(1):45-53.
54. Diehl SA, Schmidlin H, Nagasawa M, et al. STAT3-mediated up-regulation of BLIMP1 Is coordinated with BCL6 down-regulation to control human plasma cell differentiation. *J Immunol*. 2008;180(7):4805-15.
55. Dent AL, Vasanwala FH, Toney LM. Regulation of gene expression by the proto-oncogene BCL-6. *Crit Rev Oncol Hematol*. 2002;41(1):1-9.
56. Shaffer AL, Lin KI, Kuo TC, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity*. 2002;17(1):51-62.
57. Vasanwala FH, Kusam S, Toney LM, Dent AL. Repression of AP-1 function: a mechanism for the regulation of Blimp-1 expression and B lymphocyte differentiation by the B cell lymphoma-6 protooncogene. *J Immunol*. 2002;169(4):1922-9.
58. Scheeren FA, Naspetti M, Diehl S, et al. STAT5 regulates the self-renewal capacity and differentiation of human memory B cells and controls Bcl-6 expression. *Nat Immunol*. 2005;6(3):303-13.
59. Bataille R, Jego G, Robillard N, et al. The phenotype of normal, reactive and malignant plasma cells. Identification of "many and multiple myelomas" and of new targets for myeloma therapy. *Haematologica*. 2006;91(9):1234-40.
60. Willis SN, Good-Jacobson KL, Curtis J, et al. Transcription factor IRF4 regulates germinal center cell formation through a B cell-intrinsic mechanism. *J Immunol*. 2014;192(7):3200-6.
61. Zipori D. The hemopoietic stem cell niche versus the microenvironment of the multiple myeloma-tumor initiating cell. *Cancer Microenviron*. 2010;3(1):15-28.
62. Kumar S, Rajkumar SV, Greipp PR, Witzig TE. Cell proliferation of myeloma plasma cells: comparison of the blood and marrow compartments. *Am J Hematol*. 2004;77(1):7-11.
63. Abe M, Hiura K, Wilde J, et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood*. 2004;104(8):2484-91.
64. Merico F, Bergui L, Gregoretti MG, et al. Cytokines involved in the progression of multiple myeloma. *Clin Exp Immunol*. 1993;92(1):27-31.
65. Cheng Q, McKeown SJ, Santos L, et al. Macrophage migration inhibitory factor increases leukocyte-endothelial interactions in human endothelial cells via promotion of expression of adhesion molecules. *J Immunol*. 2010;185(2):1238-47.
66. Zheng Y, Wang Q, Li T, et al. Role of Myeloma-Derived MIF in Myeloma Cell Adhesion to Bone Marrow and Chemotherapy Response. *J Natl Cancer Inst*. 2016;108(11).
67. Kirshner J, Thulien KJ, Martin LD, et al. A unique three-dimensional model for evaluating the impact of therapy on multiple myeloma. *Blood*. 2008;112(7):2935-45.
68. Kawano Y, Fujiwara S, Wada N, et al. Multiple myeloma cells expressing low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. *Int J Oncol*. 2012;41(3):876-84.
69. Bergsagel PL, Kuehl WM. Chromosome translocations in multiple myeloma. *Oncogene*. 2001;20(40):5611-22.
70. Bergsagel PL, Kuehl WM, Zhan F, et al. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. *Blood*. 2005;106(1):296-303.

71. Matsuoka Y, Moore GE, Yagi Y, Pressman D. Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. *Proc Soc Exp Biol Med*. 1967;125(4):1246-50.
72. Nilsson K, Bennich H, Johansson SG, Ponten J. Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient. *Clin Exp Immunol*. 1970;7(4):477-89.
73. Gazdar AF, Oie HK, Kirsch IR, Hollis GF. Establishment and characterization of a human plasma cell myeloma culture having a rearranged cellular myc proto-oncogene. *Blood*. 1986;67(6):1542-9.
74. Huff CA, Matsui W. Multiple myeloma cancer stem cells. *J Clin Oncol*. 2008;26(17):2895-900.

Specific Aims

Specific Aim 1:

To characterize the MM CD138$^-$ precursor and the differentiated MM CD138$^+$ from the RPMI8226, U266-B1, and NCI-H929 cell lines for stemness characteristics: the abilities to self-renew, to produce differentiated progeny, to remain quiescent and chemoresistant.

This aim seeks to determine if a sub-population of MM cells exists within three MM cell lines (RPMI 8226, U266-B1, and NCI-H929) that is able to meet all the requirements of stemness. Both CD138$^-$ and CD138$^+$ MM cells from all three cell lines will be isolated as pure populations based on cell surface expression markers. We will assay the CD138$^-$ and CD138$^+$ populations to test whether either population meets our four defined characteristics of stemness: a) quiescence, b) differentiation, c) self-renewal, and d) chemosensitivity. Our data suggests that the CD138$^-$ cell is the precursor to the CD138$^+$ mature differentiated MM cell and meets the qualifications of a MM CSC, but there also exists an inherent plasticity in the CD138$^+$ population that allows for de-differentiation to occur, giving rise to the CD138$^-$ precursor.

Specific Aim 2:

To determine which cytokines are responsible for the plasticity that permits a tumor cell to gain or lose the stemness phenotype, and to target these pathways, demonstrating that blocking this interconversion will alter chemosensitivity.

This aim seeks to identify the impact of specific cytokines on MM cell fate. Both pure sorted CD138$^-$ and CD138$^+$ MM cells will be isolated and replated in standard tissue culture conditions to permit differentiation and de-differentiation respectively. We measured cytokine levels secreted into the media from these MM cell populations themselves using a cytokine array to track 36 different cytokines and their relative levels. We identified MIF-1 as being rapidly secreted from both the CD138$^+$ and CD138$^-$ MM cell populations. To validate the role of MIF-1 in influencing differentiation or dedifferentiation, neutralizing antibodies, and/or a known specific drug was used to block these processes. Ultimately the response of MM cells treated with neutralizing

antibodies or a specific drug to chemotherapy will be compared to determine if this impacts chemosensitivity of $CD138^-$ and $CD138^+$ MM cells.

Macrophage Inhibitory Factor-1 (MIF-1) Controls the Plasticity of Multiple Myeloma Tumor Cells

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Aim: To investigate the role of MIF-1 on the plasticity of MM plasma cells of 3 different commercially available MM cell lines and whether it alters chemosensitivity.

Authorship Contributions: DJ performed the experiments, SB performed experiments of primary MM samples, and SB and DJ wrote the manuscript.

Abstract

Multiple Myeloma (MM) is the second most common hematological malignancy with a median survival of 5-10 years. While current treatments initially cause remission, relapse almost always occurs, leading to the hypothesis that a chemotherapy-resistant cancer stem cell (CSC) remains dormant, undergoes self-renewal and differentiation to reestablish disease. Our finding is that the mature cancer cell (CD138+, rapidly proliferating and chemosensitive) has developmental plasticity; namely, the ability to dedifferentiate back into its own chemoresistant CSC progenitor, the CD138–, quiescent pre-plasma cell. In our 2D culture system, we observe multiple cycles of differentiation and dedifferentiation in the absence of niche or supportive accessory cells, suggesting that soluble cytokines secreted by the MM cells themselves are responsible for this bidirectional interconversion and that stemness and chemoresistance are dynamic characteristics that can be acquired or lost and thus are targetable. By examining cytokine secretion of CD138- and CD138+ RPMI-8226 cells, we identified that concomitant with interconversion, Macrophage Migration Inhibitory Factor (MIF-1) is secreted. The addition of a small molecule MIF-1 inhibitor (4-IPP) or MIF-1 neutralizing antibodies to CD138+ cells accelerated dedifferentiation back into the CD138- progenitor, while addition of recombinant MIF-1 drove cells towards CD138+ differentiation. As the CD138+ MM cell is chemosensitive, targeting MIF-1 and/or the pathways that it regulates could be a viable way to modulate stemness and chemosensitivity, which could in turn transform the treatment of MM.

Introduction

Multiple myeloma (MM) is the second most prevalent hematological malignancy in the United States, with a survival rate of 47% within the first five years post diagnosis [2, 3]. It is characterized by the proliferation of clonal plasma cells within the bone marrow, which can interfere with normal blood cell production and also secrete non-functioning monoclonal antibodies called M protein [4-6]. The tumor cells colonize the bone marrow, and symptomatically, this results in anemia, lytic bone lesions, hypercalcemia, in addition to renal failure [7, 8]. Together with autologous stem cell transplantation and the introduction of novel therapies such as proteasome inhibitors and immunomodulatory drugs, patients frequently show improvement, even to the point of remission. However, relapse is usually inevitable, suggesting that there exists a subset of MM tumor cells that are able to evade cytotoxic insult and eventually recapitulate tumor burden [9-11]. This has led to the hypothesis that a population of chemoresistant cancer stem cells (CSC) persists and remains dormant until a time post-treatment when they reinitiate growth and re-establish tumor burden [11-13].

In addition to cell surface markers, which have been used to identify CSC populations, "stemness" can be phenotypically defined. Similar to the hematopoietic stem cell (HSC), the CSC: 1) exists in a quiescent, slowly proliferating state, 2) possesses the ability to differentiate into more mature progeny, 3) is resistant to chemotherapy, and 4) has the ability to undergo long-term self-renewal in order to maintain the CSC population itself [14-16]. The CSC may be derived from a hematopoietic stem cell that has acquired oncogenic mutation(s) allowing it to become tumorigenic while maintaining stemness OR alternatively from a differentiated tumor plasma cell that dedifferentiates and reacquires stemness properties (i.e. self-renewal, differentiation, quiescence, chemoresistance). MM tumor cells are clonal plasma cells, which have isotype switched, suggesting that the progenitor arises from a post-germinal center B cell lineage, such as a plasma cell precursor or plasma cell itself [6, 17, 18]. A marker distinguishing an immature plasma cell (prePC) and a mature plasma cell [19] is the acquisition of CD138 and it is generally believed that the MM CSC population is CD138- as are pre-PCs. Matsui, et al. found that both primary tumor cells isolated from MM patients and MM cell lines are composed of a heterogeneous mixture of CD138- and CD138+ populations [5, 20, 21], but only the CD138- cells were able to engraft in the bone

marrow of NOD/SCID (non-obese diabetic/ severe compromised immunodeficient) mice [22]. Upon analysis of bone marrow aspirates from these mice, they found that the cells were all CD138+ and expressed the same light chain restriction as the initial patient material, suggesting that the CD138- cells were the CSC population that with time gave rise to the differentiated CD138+ cells. Other laboratories, however, have shown that both CD138- and CD138+ cells can engraft the bone marrow when a different mouse model, NOD/SCID γ-chain deficient (NSG), was used [23], and the authors suggested that the recipient bone marrow niche might play a role in altering stemness. Chaidos et al. showed that patient-derived purified CD138+ plasma cells could produce tumors in animals, but they detected both CD138- and CD138+ cells in the BM of mice, suggesting that the precursor CD138- cells were derived from or were the progeny of the CD138+ as a result of dedifferentiation [24]. These findings imply that a functional plasticity between differentiated progeny and dedifferentiated precursor cells might exist and reconcile the difficulties in clearly defining the CSC population. If interconversion provides a way to continually renew the chemoresistant CSC population, this would contribute to the eventual relapse seen in this disease.

To investigate this, we examined multiple MM cell lines to identify and isolate the CSC population. Our finding is that the ***mature*** myeloma cell (CD138+, rapidly proliferating and chemosensitive) has developmental plasticity; namely, the ability to dedifferentiate back into its own chemoresistant CSC progenitor, the $CD138^{-}$, quiescent plasma cell. As these cells are grown in the absence of accessory cells, this suggests that soluble cytokines secreted by the MM cells themselves are responsible for this bidirectional interconversion. We identified MIF-1 (macrophage migration inhibitory factor-1) as a secreted cytokine that controls this interconversion in MM cell lines, and show that by manipulating the activity or levels of MIF-1 present in the culture media, we can shift the set point towards more differentiation or more dedifferentiation.

Materials and Methods

Cells and Reagents. Human Multiple Myeloma Cell Lines RPMI 8226, U266-B1, and NCI-H929 obtained from ATCC (American Type Culture Collection) were grown in RPMI1640 media containing 10% FBS, 1% Penicillin Streptomycin, 0.5% Ampicilin B. RPMI 8226 cells were plated at a cell density of 250,000 cells/mL; U266-B1, 600,000 cells/mL; and NCI-H929, 450,000 cells/mL. Cells were split every 48 hours.

Flow Cytometry and Sorting. MM cell lines were stained with CD138 PE-Cy5 (Beckman Coulter) and CD38 PE (BD Pharmingen) and sorted using the BioRad S3 cell sorter in complete media (2.5 $x10^6$/mL). Analysis of additional cell surface proteins were analyzed: CD74 FITC (Abcam), Isotype Control IgG1 PE, CXCR4 PE, CD44 FITC (BD Pharmingen). Intracellular staining of Blimp1: Human Blimp1 Alexa-Fluor 488-conjugated antibody (R&D Systems). Cell cycle analysis: Vybrant DyeCycle Green Stain (Invitrogen) on sorted MM cells fixed with 70% ice cold ethanol. All flow cytometry analysis used the BD FACScan (BD Biosciences) and CellQuest software (BD Biosciences). Analyses were based on the acquisition of 10,000 events.

Differentiation and Clonogenicity Assays. Cells stained with CD138 and CD38 were sorted into $CD138^+CD38^+$ and $CD138^-CD38^+$ populations and re-plated in complete media at 37°C and 5% CO_2. Cells were harvested at days 5, 10 and 13, stained with CD138 and CD38, fixed in 4% paraformaldehyde, and stored at 4º C for later analysis. Freshly sorted $CD138^+CD38^+$ and $CD138^-CD38^+$ were resuspended in methylcellulose (Stem Cell Technologies) at 10^4 cells/ml. Colonies were counted manually and photographs were taken using a light microscope under 4X magnification. Colonies were measured using the annote tab to compare pixel number to the scale created by the Kodak film software.

Quantitation of mRNA expression by real-time PCR. Total mRNA was isolated from sorted MM cells using the RNeasy mini kit (Qiagen). cDNA for each sample was generated using the Verso cDNA kit (Thermo Scientific). Quanititative PCR was completed using gene-specific primers along with Absolute Blue SyBr Green ROX (Thermo Scientific) for Blimp1 (forward primer: 5'-GTGTCAGAACGGGATGAACA-3' and reverse primer: 5'-GCTCGGTTGCTTTAGACTGC-3'); IRF4 (forward primer: 5'-

ACCGAAGCTGGAGGGACTAC-3' and reverse primer: 5'-GTGGGGCACAAGCATAAAAG-3'); SDC1 (forward primer: 5'-AGGACGAAGGCAGCTACTCCT-3' and reverse primer: 5'-TTTGGTGGGCTTCTGGTAGG-3'); MIF1 (forward primer: 5'-CCGGACAGGTCTACATCAACTATTAC-3' and reverse primer: 5'-TAGGCGAAGGTGGAGTTGTTCC-3'); GAPDH (forward primer: 5'-GACAGTCAGCCGCATCTTCT-3' and reverse primer: 5'-GCGCCCAATACGACCAAATC-3'). The level of each gene was normalized to GAPDH expression.

Chemosensitivity Assay. Sorted populations (CD138+CD38+ and CD138-CD38+) were treated with 0.01μM Bortezomib for 24 h. Chemosensitivity was measured as the change in viability before and after treatment as measured by trypan blue exclusion. Viability was also measured using the MTT Assay (Promega) to measure the change in viability of sorted populations treated with either anti-MIF mAb (R&D Systems) or rMIF (R&D Systems) and consequently treated with 0.01μM Bortezomib for 24 hours.

Tracking Assay. Cells were treated with 5μM PD 0332991 (SelleckChem) for 72 h. These cells were washed with complete media and stained with 5μM CFSE (carboxyfluorescein succinimidyl ester) for 10 min. Cells were further stained with CD138 PE-Cy5 and CD38 PE and sorted into $CFSE^{hi}CD138^{+}CD38^{+}$ and $CFSE^{hi}CD138^{-}CD38^{+}$ and re-plated. Cells were harvested, and stained with CD138 PE-Cy5 and CD38 PE at days 2, 4 and 6, fixed with 4% paraformaldehyde and stored at 4°C for analysis on flow cytometer.

Cytokine Array and Analysis. RPMI8226 cells stained with CD138 and CD38 and sorted into $CD138^{+}CD38^{+}$ and $CD138^{-}CD38^{+}$ populations were re-plated in complete media. At days 1, 2, and 4, cells were harvested and stained for CD38 PE and CD138 PE-Cy5, fixed with 4% paraformaldehyde, and stored at 4ºC, while media for each time point was stored at -20ºC for later assessment using the Human Cytokine Array Panel A (R&D Systems). Each cytokine array was analyzed using IRDye 800CW Streptavidin (LICOR) and the LICOR Imaging System following vendor's instructions. Raw values for each cytokine detected in media alone were subtracted from values for each cytokine at each time point.

MIF-1 ELISA. Sorted CD138+ or CD138- populations were re-plated and media was harvested 1, 4, 12, and 24 h. post sort. Samples were stored at -80°C for future analysis. The presence of MIF was analyzed using the Human MIF Immunoassay (R&D Systems), all samples were diluted by a factor of 10. Quantitation of MIF in each population was completed by measuring absorbance using a 96-well spectrophotometer.

MIF inhibitor, 4-IPP. Sorted CD138+ or CD138- populations were re-plated in increasing concentrations of 4-IPP (4-iodo-6-phenylpyrimidine in DMSO) (Santa Cruz Biotech). CD138 and CD38 cell surface expression was measured at different time points for each concentration of 4-IPP. Assessment of U266-B1 and NCI-H929 and 4-IPP treatment was completed on unsorted cells. *Wash Out Assay*. $CD138^{+}CD38^{+}$ RPMI 8226 cells were treated with 4-IPP for 48 hours, the cells were washed and re-plated in complete media, and CD138 and CD38 cell surface expression was assessed at 48 and 96 h post wash out. PI was used to gate for viable cells during flow cytometry analysis.

MIF Neutralizing Antibody (Nab) and rMIF Assay. Sorted CD138+ or CD138- populations were treated with either increasing concentrations of MIF Neutralizing antibody (NAb) (R&D) (0, 100, 200, and 500 µg/mL) or recombinant MIF (rMIF) (R&D) (0, 50, 100, 200 ng/mL) and incubated for 24 h. CD138 expression and viability were dually assessed by flow cytometry by staining for cell surface antibodies and PI staining.

Primary MM patient samples. Bone marrow aspirates from MM patients at King's County Hospital, obtained by informed consent, was purified by passage through the MM enrichment kit (RosetteStep), Purified plasma cells were either stained with CD38 PE and CD138 PE-Cy5, fixed with 4% paraformaldehyde, and stored at 4ºC or plated +/- 4IPP for 24 and 72 h. Cells were grown using the patient's own serum, obtained from peripheral blood. Cells were then stained, fix and analyzed by flow cytometry as described.

Statistics. All data are shown as the mean ± SD. The statistical analyses were performed using the Exact Mann-Whitney 2-sided tests Figure 1D,E & 3B,C, a semi-parametric (SAS PROC MULTTEST with bootstrapping) approach was used to (1) apply attest of monotonic trend, allowing for possible non-normality; (2) correct p-values derived from pair-wise t-tests for possible non-normality and for multiple testing (Figure

4E). The Satterthwaite adjustment for heteroscedasticity was also applied. Selected pair-wise comparisons were made using t-tests (with Satterthwaite corrections to allow for possible unequal variances). The distribution of the dependent variable was checked for symmetry and for the presence of outliers (Figure 5D & 6C). All other statistics used student t-test (2 tail).

Results

CD138- cells have the characteristics of stemness

RPMI 8226 cells were stained with CD138 and CD38 antibodies, and sorted by flow cytometry. Unstained populations and single staining was used to identify doubly stained populations (Fig. 1A). Three distinct populations were consistently detected (Fig. 1B, dashed boxes, i-iii, Fig. S1A). Each was recovered by sorting with high efficiency and population "i" (hereafter referred to as CD138+CD38+) and "ii" (hereafter referred to as CD138-CD38+) were shown to be viable (Fig. 1B, inset). The "iii" population was non-viable in cell culture and was gated out of all future analysis (Fig. 1B, inset). The CD138+ and CD138- populations were consistently detected in a ~9:1 ratio, where ~90-95% of the cells were CD138+ and ~5-10% of the cells were CD138- (Fig. 1B). The viability of neither the CD138+ or CD138- cells changes with time, as measured by MTT at time points post plating of pure populations (Fig. S1B). Previous studies have suggested that these CD138- cells were the MM CSC population[25], and we analyzed these pure, sorted populations to further characterize "stemness" by assessing: proliferation rate (Fig. 1C), long-term self-renewal/clonogenic potential (Fig. 1D,E, G, H), chemoresistance (Fig. 1F) and expression of differentiation specific markers (Fig. 1I,J). Purified CD138- and CD138+ populations were stained with Vybrant Green to measure DNA content (Fig. 1C). The $CD138^{+}CD38^{+}$ cells were rapidly proliferating with only 56% in G0/G1 phase, and 20% and 24% of cells in S and G2/M phase, respectively (Fig. 1C, top). On the other hand, 88% of the $CD138^{-}CD38^{+}$ cells were present in G0/G1 phase, demonstrating that these cells were quiescent or only very slowly proliferating (Fig. 1C, bottom). The shift to the left for the G0/G1 peak seen in the CD138- cells in the presence of Vybrant Green suggests a more tightly compacted DNA, suggesting that these cells may be in G0 rather than G1 phase. To test the self-renewal capacity of each population, sorted $CD138^{+}$ or $CD138^{-}$ cells were plated in methylcellulose and the formation of colonies was assessed after 14 days. $CD138^{+}$ cells were able to form significantly more colonies than $CD138^{-}$ cells, suggesting that more colonies were able to initiate proliferation (Fig. 1D). However, these colonies were smaller than those derived from the $CD138^{-}$ precursors (Fig. 1E, G, H). This suggests that fewer CD138- cells entered cycle, but those that did were able to undergo more duplication events than the

CD138+ cells, resulting in larger colony width. To compare chemotherapeutic response, sorted CD138+ and CD138- populations were treated and mock treated for 24 h. with 0.01μM Bortezomib, a proteasome inhibitor used as frontline therapy in MM treatment. Viability was measured by trypan blue exclusion and chemosensitivity was defined as the difference in viability between mock and Bortezomib treated cells (Fig. 1F). CD138+ cells were more sensitive, with a greater loss of viability seen with Bortezomib treatment, than the CD138- cells, where Bortezomib had a reduced effect. Finally, we measured the expression of two transcription factors, Blimp1 and IRF4, which have been shown to be responsible for the differentiation of B cells into plasma cells [4, 26, 27]. CD138+ cells evaluated immediately after sorting have higher expression of Blimp1, as detected by intracellular staining (Fig.1I, blue) and RT-PCR analysis (Fig. 1J). IRF4 is also expressed preferentially in the CD138+ cells (Fig. 1J), suggesting that these are the differentiated progeny, and the CD138- cells are more immature. CD138 expression is regulated at least in part at the RNA level, as reduced expression of CD138 (SDC1) is detected in the CD138- cells (Fig. 1J). Together, these experiments suggest that the CD138- cells meet the qualifications of stemness: they are quiescent, chemoresistant, able to undergo long term self-renewal and do not express the differentiated cell specific markers, IRF4 and Blimp1.

Cells undergo interconversion, exhibiting plasticity.

An additional characteristic of stemness is the ability to undergo differentiation to produce mature progeny. To examine this, pure CD138+ and CD138- RPMI 8226 populations were re-plated and followed for up to 13 days (Fig. 2A, B). CD138+ cells proliferated faster than CD138- cells (Fig. S2A: with CD138+ cell doubling rate of 2.4 compared to the 1.5 doubling rate of CD138- cells during the first 5 days), and no significant loss of viability was seen in the populations over the 13 day time course, suggesting that the changes seen in CD138 expression were not reflective of cell death or dying. Cells were harvested at different time points, and CD138 expression was measured by flow cytometry (Fig. 2A, B, C). Within 5 days post plating, pure CD138- cells were able to differentiate and give rise to differentiated CD138+ cells: 50% of the total cells were CD138+ by

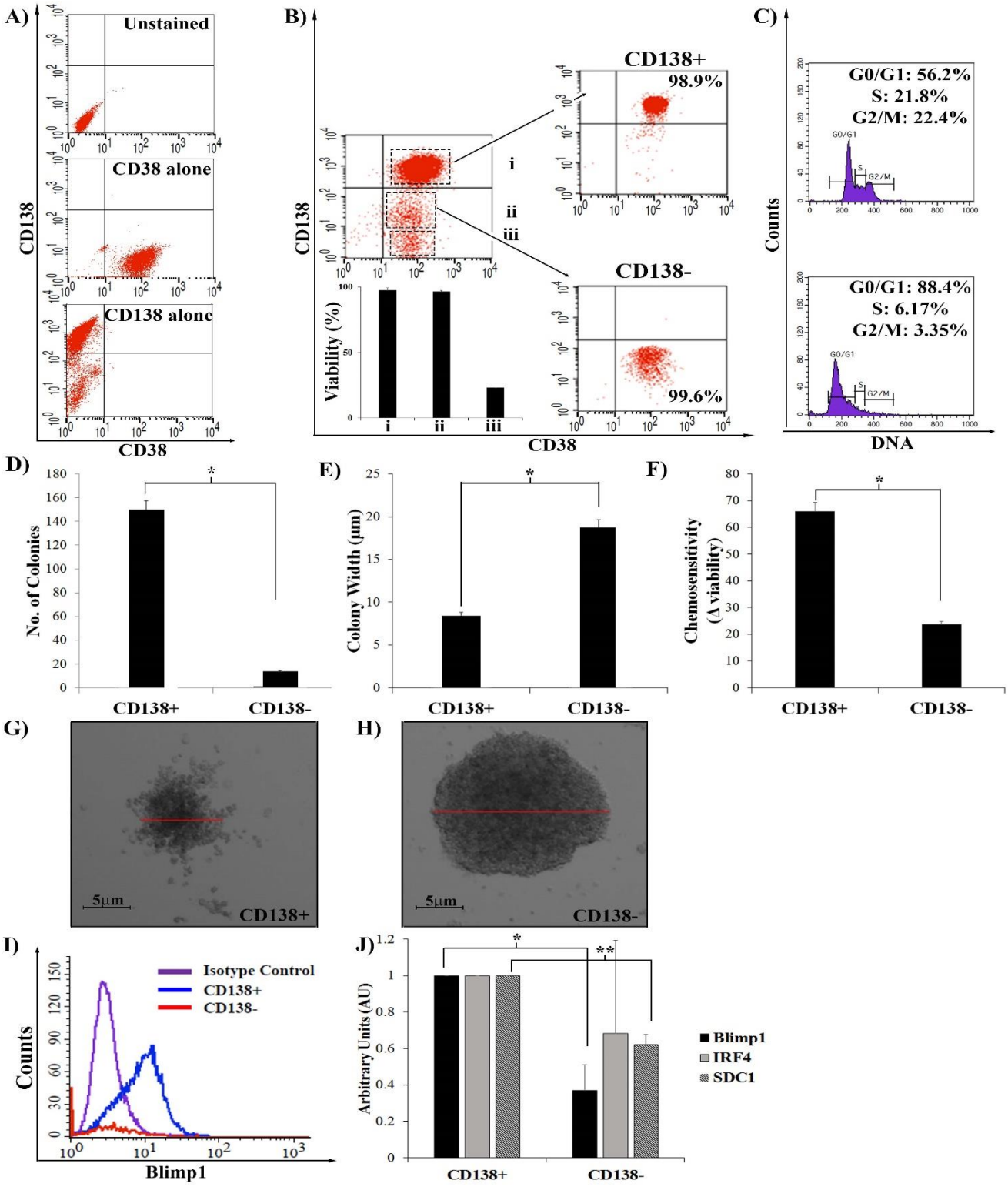


Figure 1. Stemnes of RPMI8226 cells. **A)** Cells analyzed by flow cytometry as unstained, CD38 or CD138 single stained populations to establish gates for double stained populations. **B)** Cells were co-stained with CD138 and CD38, and sorted into CD138+CD38+ (i) and CD138-CD38+ (ii), CD138—CD38+ (iii) populations with high efficiency. ***(Inset)*** Viability of each population. **C)** CD138+ and CD138- populations were independently fixed in ethanol and stained with Vybrant Green to measure cell cycle status. **D,E)** CD138+ and CD138- sorted cells were plated in methylcellulose for 14 days, colonies were counted and the width measured. (n=4) *p<0, Exact Mann-Whitney 2-sided t-test. **F)** Each population was treated with 0.01µM Bortezomib for 24 h and chemosensitivity measured as the difference in viability before and after treatment. (n=4) *p<0.001, student t-test **G,H)** Representative colonies formed

day 5 (Fig. 2A, red). The cells continued to differentiate and by day 13, the bulk of the culture was CD138+,

reestablishing the 9:1 ratio of CD138+:CD138- seen in unsorted cultures (Fig. 2A, pink). Raw values are presented in Fig 2C. When pure, sorted CD138+ cells were re-plated, they were able to give rise to CD138- cells, suggesting that these cells were able to de-differentiate (Fig. 2B). Only 40% of the cells remained CD138+ by day 5 (Fig. 2B, red). By day 10, the culture started to shift back and 87% of the cells now were CD138+, and by day 13, the 9:1 ratio of CD138+:CD138- cells seen in unsorted cultures had been reestablished (Fig. 2B, pink). Thus, the CD138- cells could differentiate (Fig. 2A), but the CD138+ cells could de-differentiate (Fig. 2B), suggesting that the cells could interconvert in culture.

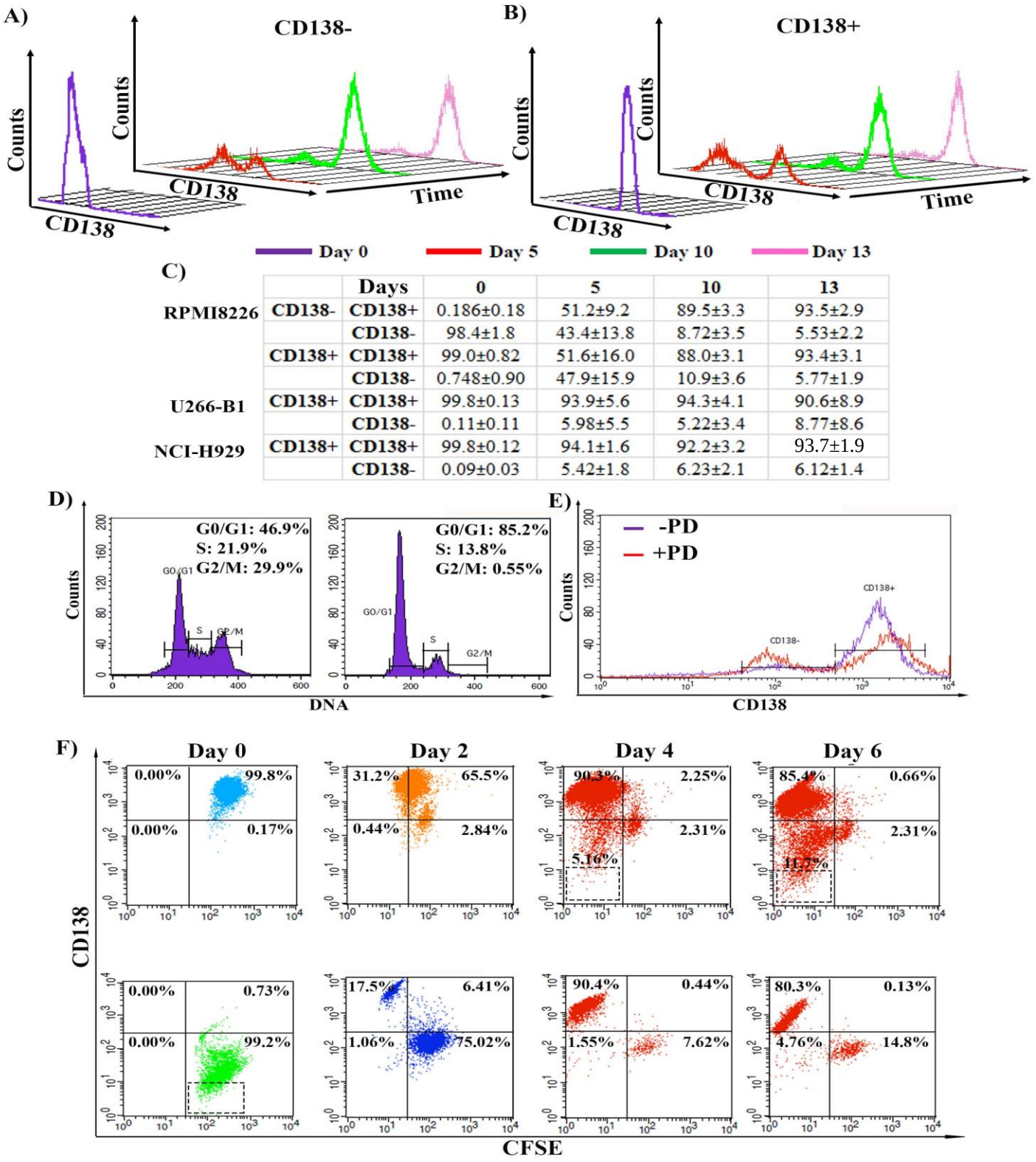
To analyze whether this plasticity was intrinsic to the RPMI8226 cell line, or was a quality of other human MM cell lines, NCI-H929 and U266-B1 cells were sorted into CD138+ populations. Unsorted NCI-H929 and U266-B1 cultures have slightly different set points, the NCI-H929 and U266-B1 cells contain approximately 95-98% of cells that are CD138+ and approximately 2-5% of the cells are CD138- (Figure S2B, S2C). When 99% pure CD138+ populations of NCI-H929 and U266-B1 cells were re-plated, with time, CD138- cells were detected (Fig. 2C, middle and bottom panel). By day 13, the cultures had reestablished the ~95-98:2-5 CD138+:CD138- ratio, suggesting that plasticity was a shared characteristic of MM cell lines.

To further investigate the interconversion of the CD138- and CD138+ populations, we synchronized cells to follow their differentiation or dedifferentiation. Unsorted RPMI8226 cells were treated with PD0332991 (Palbociclib), a specific inhibitor of cyclin-dependent kinase 4/6, to arrest cells in G1 phase. After 72 h. of PD0332991 treatment, cell cycle profiles were measured using the DNA stain Vybrant Green, in mock and PD0332991 treated cells, and PD0332991 treatment increased the G1 phase content, demonstrating arrest (Fig. 2D). These arrested cells were stained with CD138, and we found that PD0332991 treatment caused a change in the CD138-:CD138+ ratio, with an increase in the CD138- pool detected (Fig. 2E, red), suggesting that G1 phase arrest and CD138 status might be linked. PD0332991 arrested cells were then subsequently stained with

Figure 1 Continued: G,H) within methylcellulose by CD138+ and CD138- sorted cells (10^4cells/mL). **I)** Intracellular staining of Blimp1 expression in CD138+ (blue) and CD138- (red) cells as measured by flow cytometry, isotype control (purple). **J)** RT-PCR was used to measure the relative levels of Blimp1 (n=4) and IRF4 (n=3) and SDC1 (CD138) (n=3) between both CD138+ and CD138- populations levels in CD138+ set to 100, plotted as AU. *$p<0.05$. **$p<0.001$. Student t-test, error bars denote the mean ± SD.

the fluorescent cell permeable dye CFSE. This dye is retained in the cytoplasm, and decreases its fluorescent intensity by exactly half upon each cycle of cell division. This allows us to track cell proliferation as measured by loss of CFSE of both the CD138+ and CD138- populations in tandem with their differentiation status, as measured by CD138 expression. PD0332991 arrested, CFSE stained cells were sorted into two pure populations: $CD138^{+}CD38^{+}CFSE^{hi}$ and $CD138^{-}CD38^{+}CFSE^{hi}$ (Fig. 2F, Day 0). PD0332991 was removed from the culture through sequential washing with fresh media and the labeled cells were replated in 2D tissue culture. Cells were harvested every two days and analyzed for CD138 and CFSE. $CD138^{-}$ cells (bottom panel) were able to divide (reducing CFSE content) and differentiate (increasing CD138 content) and within two days 17.5% of the population had become $CD138^{+}CFSE^{lo}$ (Fig. 2F, bottom panel, Day 2). 75% of the population did not divide, and while there was a slight increase in CD138 expression when compared to Day 0, these cells remained within the CD138- gate. By days 4 and 6, the bulk of the culture had divided to become $CD138^{+}CFSE^{lo}$, while a small pool of cells remained $CFSE+CD138^{-}$ and did not divide (Fig. 2F, bottom panel, Days 4, 6). Thus, CD138- cells could give rise to CD138+ progeny, and this appeared linked to cell division.

When sorted, arrested, $CD138^{+}$ $CFSE^{hi}$ cells were replated and released from arrest (Fig. 2F, top panel), by day 2, the bulk of the cells are $CFSE^{mid}$ (between 10^{1}-10^{2}) but remain CD138+, suggesting that they are starting to divide but maintain the CD138 marker (top quadrants). However, a new population, $CD138^{mid}CFSE^{mid}$, is also detected, suggesting that some cells are losing expression of CD138 as they divided. This $CD138^{mid}CFSE^{mid}$ population continues to decrease in CD138 expression through the last two time points, becoming $CD138^{-}CFSE^{mid}$, without a further decrease in CFSE fluorescence, supporting that they are dividing slowly and dedifferentiating. By day 6, three populations are visible: 85.4% are $CD138^{+}CFSE^{lo}$ (CD138+ cells that divided into CD138+ cells); 2.3% are $CD138^{-}CFSE^{mid}$ (dedifferentiated CD138- cells that may have divided once but then stopped and has been maintained since day 2); 11.7 % are $CD138^{-}CFSE^{lo}$ (CD138+ cells that divided and then dedifferentiated into CD138- cells). All of these populations are viable and are outside of



C)

	Days	0	5	10	13
RPMI8226	CD138-	CD138+	0.186±0.18	51.2±9.2	89.5±3.3	93.5±2.9
		CD138-	98.4±1.8	43.4±13.8	8.72±3.5	5.53±2.2
	CD138+	CD138+	99.0±0.82	51.6±16.0	88.0±3.1	93.4±3.1
		CD138-	0.748±0.90	47.9±15.9	10.9±3.6	5.77±1.9
U266-B1	CD138+	CD138+	99.8±0.13	93.9±5.6	94.3±4.1	90.6±8.9
		CD138-	0.11±0.11	5.98±5.5	5.22±3.4	8.77±8.6
NCI-H929	CD138+	CD138+	99.8±0.12	94.1±1.6	92.2±3.2	93.7±1.9
		CD138-	0.09±0.03	5.42±1.8	6.23±2.1	6.12±1.4

Figure 2. Plasticity of RPMI8226 cells. A, B) Sorted CD138-CD38+ and CD138+CD38+ populations were plated, aliquots were analyzed for CD138 expression at days 5, 10, and 13. (Representative of n=5). **C)** Sorted CD138-CD38+ and CD138+CD38+ cells from RPMI8226 (n=5), U266-B1 (n=3) and NCI-H929 (n=3) MM lines were plated and aliquots were analyzed for percent CD138- at days 5, 10 and 13. (Mean ± SD) **D)** Cell Cycle status of RPMI8226 cells before (left) and after (right) treatment with PD0332991 (PD). **E)** CD138 expression of unsorted RPMI8226 cells before (purple) and after (red) treatment with PD for 3 days.

the non-viable population "iii" gate from Fig. 1. This suggests that not only are $CD138^+$ cells able to proliferate but are also able to de-differentiate, giving rise to the $CD138^-$ precursor.

Dedifferentiated CD138- cells exhibit characteristics of stemness.

To determine whether MM cells could undergo multiple cycles of differentiation and de-differentiation and remain phenotypically the same as CD138- or CD138+ cells isolated from the parent RPMI8226 cell line, pure $CD138^+$ cells were re-plated and maintained for 4 weeks (Fig. 3A). During this time, the three populations (i, ii, and iii) were again present (Fig. 3A). CD138+* and CD138-* populations were gated and sorted to >95% purity as illustrated before, with 93.8% being CD138+ and 6.3% being CD138- (Fig. S3A). These pure populations ($CD138^+$* and $CD138^-$*) were recovered from these cells and tested by the stemness assays described in Fig. 1. $CD138^+$* and $CD138^-$* cells were plated in methylcellulose and the formation of colonies was assessed. As seen with cells isolated from the parent cell line, the $CD138^+$* cells formed significantly more colonies than the CD138-* cells (Fig. 3B), but the $CD138^-$* population formed significantly larger colonies (Fig. 3C). Both populations were treated with Bortezomib or mock treated for 24 h. and chemosensitivity was measured as the difference in viability of the mock and treated populations by trypan blue exclusion. Similar to cells isolated from the parent line, $CD138^+$* cells showed increased sensitivity to chemotherapeutic treatment as indicated by the greater change in viability as compared to the more chemoresistant $CD138^-$* cells (Fig. 3D). The expression of Blimp1, IRF4 and SDC1 (CD138) in the doubly sorted CD138+* and CD138-* cells was measured by RT-PCR (Fig. 3E). As seen with cells isolated from the parent cell line, $CD138^-$* cells had lower levels of Blimp1 and SDC1 RNA (Fig. 3E), while IRF4 levels between the populations were more similar (Fig. 3E). The doubly sorted $CD138^-$* cells were replated and followed in the differentiation assay for up to 13 days (Fig. 3F). These cells were able to differentiate again, with kinetics similar to those seen in cells isolated from the parent line, re-establishing the ~9:1 ratio of $CD138^+$:$CD138^-$ cells by day 13 (Fig. 3G). Doubly sorted

Figure 2 Continued: F) Cells were treated with 5μM PD for 3 days, stained with CFSE, washed, sorted, then followed in culture for 6 days. Aliquots were analyzed for CFSE and CD138 over this time course. Dashed boxes are of non-viable cells (population "iii") were not included in the overall percentages of each quadrant.

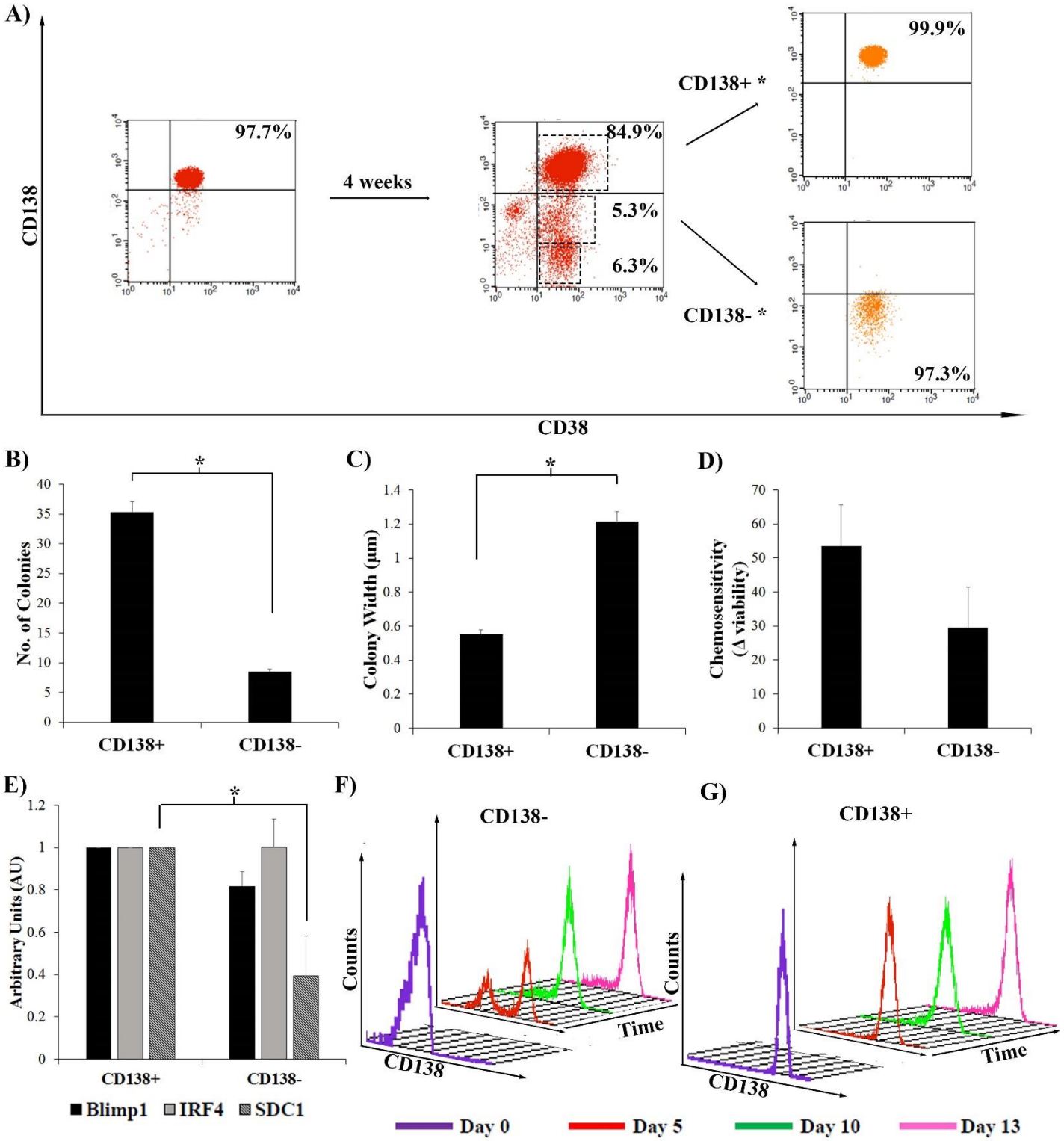


Figure 3. **Authenticity of newly dedifferentiated CD138- cells. A)** CD138+CD38+ sorted cells were plated for four weeks, and then re-sorted based on CD138 status, to yield CD138+* and CD138-* populations. Representative of n=3. Dashed boxes are of non-viable cells (population "iii") that are not included in the overall percentages of each quadrant. **B,C)** CD138+* and CD138-* cells were plated in methylcellulose for 14 days, colonies were counted and measured (n=3) *p<0.05. **D)** CD138+* and CD138-* cells were treated with 0.01µM Bortezomib for 24 h. Chemosensitivity is measured as the difference in viability before and after treatment (n=3). **E)** RT-PCR on CD138+* and CD138-* populations to assess the levels of Blimp1, IRF4, and SDC1 (CD138) (n=3) levels in CD138+ set to 100, plotted as AU *p<0.05. Error bars denote the mean ± SD. **F,G)** CD138+* and CD138-* populations were plated, and aliquots were analyzed for the expression of CD138 by flow cytometry at days 5, 10, and 13.

$CD138^{+}$* cells were able to de-differentiate by day 5 and re-establish the ~9:1 ratio of $CD138^{+}$:$CD138^{-}$ sorted cells by day 13 (Fig. 3F). The kinetics of dedifferentiation of the $CD138^{+}$* cells was different in that there was no large $CD138^{-}$ population by day 5 as seen in the parent $CD138^{+}$ cells (Fig. 2B). However, in general, both of the doubly sorted populations maintained plasticity and could differentiate and/or dedifferentiate respectively.

Modulation of MIF-1 activity alters interconversion.

While interconversion of MM cells has been reported before [24, 28], this is the first time that plasticity has been reported to occur in 2D culture in the absence of accessory cells. This suggests that the MM cells themselves controlled differentiation or dedifferentation status and we hypothesized that the cells might secrete cytokines into the culture media that would influence interconversion. In support of this, two pilot experiments suggest that the kinetics of dedifferentiation could be altered by plating different concentrations of cells and/or removing the newly conditioned media after plating. When more CD138+ cells were plated in the same volume to potentially increase the concentration of secreted cytokines, dedifferentiation into CD138- cells increased (Fig. S4A, panel A). When a constant number of CD138+ cells were plated, but the media was replaced every 12 h, to reduce secreted cytokine concentration, dedifferentiation into CD138- cells was decreased (Fig. S4A,panel B). To examine cytokine secretion directly, CD138+ and CD138- cells were sorted and re-plated in complete media, conditioned media was harvested at days 1 and 2, and a cytokine array was used to detect the presence of 36 cytokines simultaneously (Fig. 4A). The cytokine values detected at time 0 (plating) and in the media alone were subtracted to generate the change in expression, plotted as AU (Fig. S4B). While several different cytokines were modestly increased in both CD138+ and CD138- cells, the expression of macrophage inhibitory factor 1 (MIF-1) increased rapidly and significantly in both populations. MIF-1 is an inflammatory cytokine, secreted by many cell types, and shown to increase survival and proliferation of several stem cell lineages [29-31]. We hypothesized that the burst of MIF-1 secreted into the media might promote interconversion of the individual populations, and blocking MIF-1 activity with 4-IPP (4-iodo-6-phenylpyrimidine), a small

molecule inhibitor of MIF-1 activity, might alter the kinetics of dedifferentiation or differentiation. Pure populations of CD138+ cells were treated with different concentrations of 4-IPP and viability was followed by PI staining (Fig. 4B) Because treatment with 4-IPP decreased cell viability with time and concentration, we only analyzed viable cells, as measured by PI staining, for CD138 status in future experiments (Fig. 4C, D, G). When pure CD138+ cells were plated and then mock treated, roughly 60% of CD138+ cells remained CD138+ at 24 h., suggesting that 40% had dedifferentiated during this time frame (Fig. 4C, compare T=0 to [0]). However, when treated with 30 or 60 μM 4-IPP, only 50 or 35% remained CD138+ respectively, suggesting that blocking MIF-1 activity increased dedifferentiation (50 to 65% became CD138- during the same time period). When $CD138^-$ populations were plated and treated for 24 h. with 4-IPP, 12% differentiated within the 48 h. time frame (88% remained CD138-) (Fig. 4D, T=0 to [0]). However, in the presence of 30 or 60 μM 4-IPP, differentiation was slowed, and only 8 and 4% of the PI- cells became CD138+ (92 and 96% of cells remained CD138-) (Fig. 4D). This suggested that blocking MIF-1 increased the pool of dedifferentiated cells.

The loss of viability seen in the CD138+ cells in the presence of 4-IPP (Fig. 4B) was due to apoptosis, as measured by increased Annexin V staining after 24 h with increasing concentrations of drug (Fig. 4E). Annexin V staining of utreated CD138+ cells did not change within the 24h. timepoint, demonstrating the stability of this population. Treatment of unsorted RPMI8226 cells with increasing concentrations of 4-IPP also caused a significant increase in the G0/G1 phase, as measured by Vybrant Green staining (Fig. 4F). While unsorted cells had a G0/G1 content of ~50%, 4-IPP treatment increased this to ~ 60%. As 4-IPP promotes increases in the CD138- pool, this increase in quiescence is consistent.

To validate the specificity of 4-IPP to promote the dedifferentiation of $CD138^+$ cells by targeting MIF-1, we used a monoclonal neutralizing antibody (NAb) to block MIF-1 (Fig. 4G). Increasing concentrations of MIF NAb were added to pure sorted CD138+ cells for 24 h., and then viable cells, as measured by PI staining, were analyzed for CD138 status by flow cytometry (Fig. 4G). While approximately 50% of the mock treated CD138+ cells remained CD138+ (50% had dedifferentiated), only 30% of the NAb treated cells remained CD138+ (70% dedifferentatied into CD138- cells within the same time frame). Just like 4-IPP treatment, the

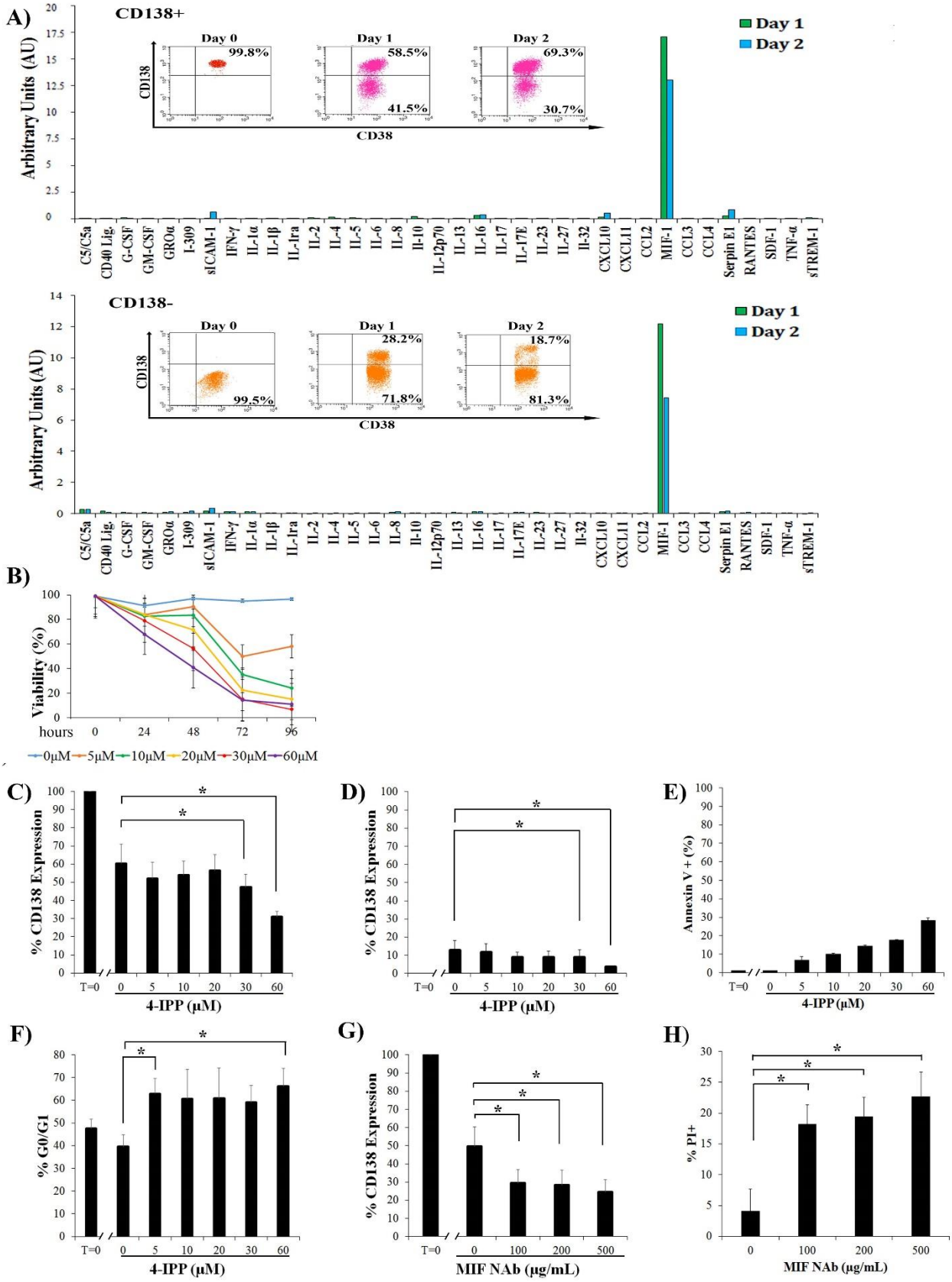


Figure 4. **MIF-1 regulates differentiation status. A)** Sorted CD138+ or CD138- cells were plated and media was harvested at T0, T24 and T48 and used in the cytokine array assay. AU plotted as the expression of 36 different cytokines. Inset: CD138 and CD38 status at each time point, assessed by flow cytometry. Representative of n=3 (CD138+) and n=2 of (CD138-).

viability of the NAb treated cells was reduced. Pure CD138+ cells were plated in the absence or increasing concentrations of MIF NAb for 24 h, stained with PI, and analyzed by flow cytometry (Fig 4H). A similar 20% decrease in viability was seen with NAb addition, suggesting that death might be a physiological response to forced, excessive dedifferentiation.

4-IPP or NAb treated cells behave like dedifferentiated CD138- cells.

Blocking MIF-1 activity appeared to drive or maintain dedifferentiation status in both the CD138+ and CD138- populations (Fig. 4C-G) and we wanted to determine whether CD138- and CD138+ cells generated by blocking MIF-1 activity behaved like untreated CD138- and CD138+ cells, in terms of chemotherapeutic response. Sorted parent $CD138^+$ cells were treated with 60 µM 4-IPP for 24 h to promote dedifferentiation, and 62.5% of these cells became CD138- (Fig. 5A). These cells were doubly stained with CD138 and PI to isolate viable $CD138^+$ and $CD138^-$ populations (Fig. 5A, bottom), which were then mock treated or treated with Bortezomib for 24 h. Chemosensitivity was measured as the difference in viability between the mock and Bortezomib treated populations (Fig. 5B). The 4-IPP derived CD138- cells were significantly less sensitive to chemotherapeutic treatment than the CD138+ cells, similar to what was seen with the parent derived CD138- populations (Fig.1F). To determine whether 4-IPP treated cells could recover, sorted CD138+ cells, treated with 4-IPP for 48 h. (to become CD138-) (Fig. 5C, red), were replated without drug and analyzed for CD138 expression at 48 h. (green) and 96 h (pink). Whereas 68% of 4-IPP treated cells were CD138- (red), before replating, but by 48 h. without drug, a large CD138+ population appeared, and by 96 h, 73% were CD138+.

Figure 4 Continued: B) Viability measured by PI staining of cells at different concentrations of 4-IPP and timepoints. (n=3). **C, D)** CD138 expression of viable (PI negative) ,CD138+ (C) and CD138- (D) cells treated with increasing concentrations of 4-IPP. (n=3). **E)** Flow cytometric analysis of Annexin V of CD138+ cells treated with increasing concentrations of 4-IPP (n=3) Test of trend p=0.008. **F)** Vybrant Green cell cycle analysis of unsorted cells treated with increasing concentrations of 4-IPP (n=3). **G**) CD138 expression of viable (PI negative), CD138+ cells treated with increasing concentrations of MIF NAb. (n=3). H) Viability measured by PI staining of cells at different concentrations of MIF NAb (n=3). *p<0.05. Error bars denote the mean ± SD.

Thus, the CD138- cells derived by 4-IPP treatment regained characteristics of stemness: they were chemoresistant and could differentiate.

MIF-1 NAb treatment also increased the pool of CD138- cells (Fig. 4G), and we wanted to determine whether this treatment also increased chemoresistance in these CD138- cells (Fig. 5D). Sorted $CD138^+$ were mock treated or treated with NAB for 24 h. to promote dedifferentiation (Fig. 5D, lane 4). After 24 h., the MIF-1 NAb treated cells were treated with Bortezomib for an additional 24 h to measure viability using the MTT assay. This was compared to the response seen with untreated CD138+ cells treated+/- Bortezomib (Fig. 5D, lanes 1, 2). Untreated CD138+ cells are sensitive to Bortezomib, and their viability decreases to 48% following treatment (lane 2). CD138+ cells treated with NAb, however, were less sensitive to Bortezomib and their viability was only reduced 17% with treatment (lane 4). Consistent with what was seen with 4-IPP treatment, CD138+ cells treated with MIF-1 NAb were more chemoresistant than untreated cells, consistent with the increase in CD138- populations.

rMIF promotes differentiation into $CD138^+$ cells.

Because blocking MIF-1 activity with either 4-IPP or MIF-1 NAb promoted dedifferentiation, we hypothesized that an excess of rMIF-1 might promote differentiation into CD138+ cells. Sorted $CD138^+$ cells were treated with rMIF-1 at increasing concentrations, and PI+, CD138 status was measured after 24 h (Fig. 6A). While approximately 60% of mock treated CD138+ cells remained CD138+ (40% had dedifferentiated to CD138- cells), in the presence of 50 ng/ml rMIF-1, almost 90% of cells remained CD138+, suggesting that rMIF-1 prevented dedifferentiation and allowed the maintenance of the $CD138^+$ population (Fig. 6A, compare T=0 to [0]). When sorted, pure $CD138^-$ cells were mock treated, 10% of cells differentiated and became CD138+ within 24 h. (90% remained CD138-), but when treated with different concentrations of rMIF-1, an increase in CD138+ cells was also seen (Fig. 6B). This suggests that the presence of rMIF-1 helped to maintain and/or generate a CD138+ population by promoting differentiation.

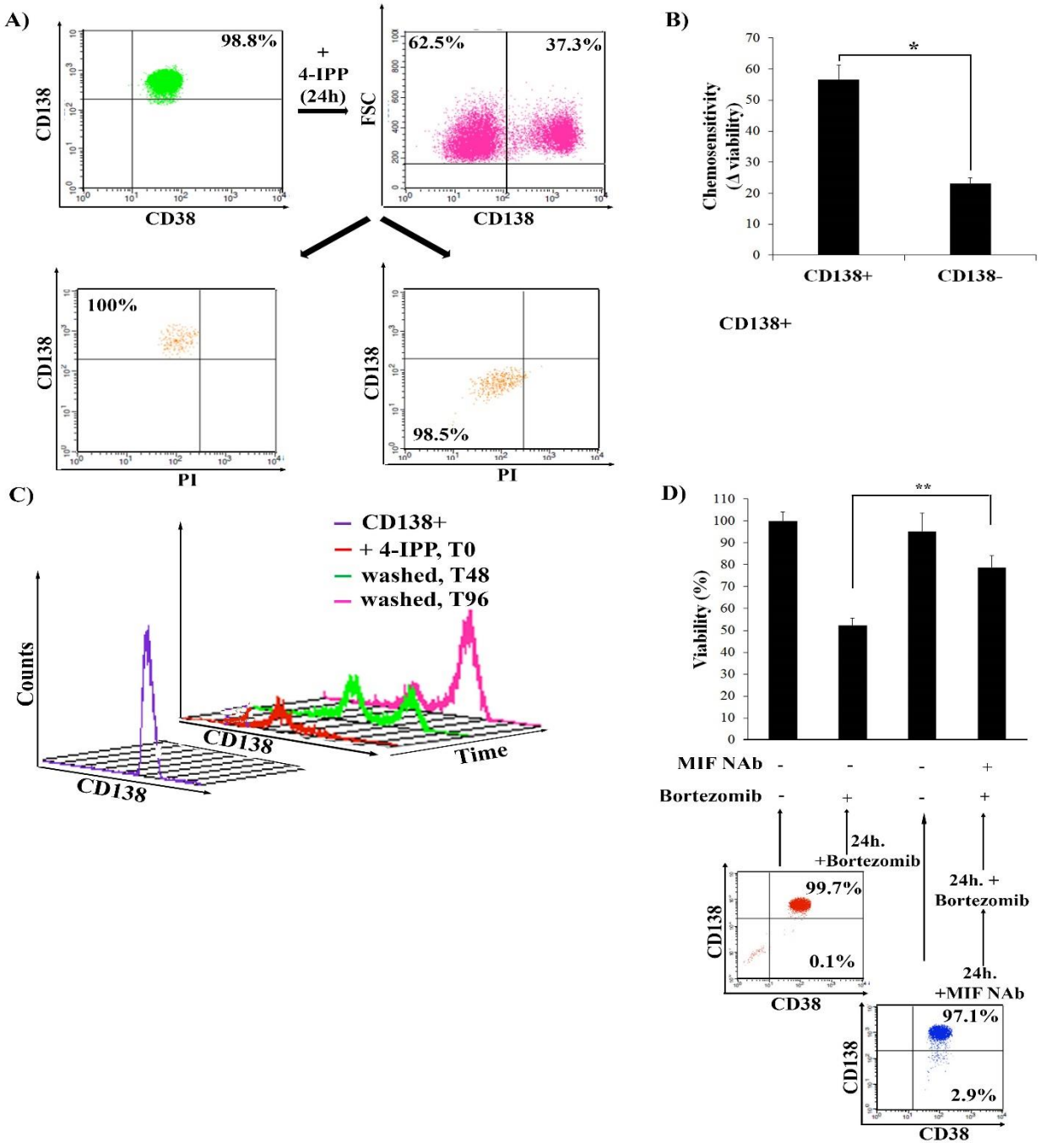


Figure 5. **4-IPP dedifferentiated CD138- cells are chemoresistant. A)** Sorted CD138+ cells were treated with 60µM 4-IPP for 24 h. Cells were then stained for CD138 and PI to recover viable CD138+ and CD138- cells. **B)** These cells were then treated with 0.01µM of Bortezomib for 24 h and chemosensitivity measured as the difference in viability before and after treatment by trypan blue exclusion. *p<0.01 **C)** Sorted CD138+ cells (purple) were treated with 60µM 4-IPP for 48 h (red). 4-IPP was then washed out and cells were re-plated and CD138 expression measured at 48 (green) and 96 h (pink) [1]. **D)** MTT assay used to assess viability after treatment with Bortezomib, mean absorbance values of lanes that were untreated at time point 24 hrs were set to 100 and plotted as AU (n=3). Sorted CD138+ cells were treated + MIF NAB for 24 h, followed by 0.01µM Bortezomib treatment for 24 h (lane 4) or untreated for 48 h. (lane 3). Freshly sorted CD138+ cells were treated with 0.01µM Bortezomib (lane 2) or untreated (lane 1) for 24 h. **p<0.001 t-test with Satterthwaite corrections to allow for possible unequal variances. Error bars denote the mean ± SD.

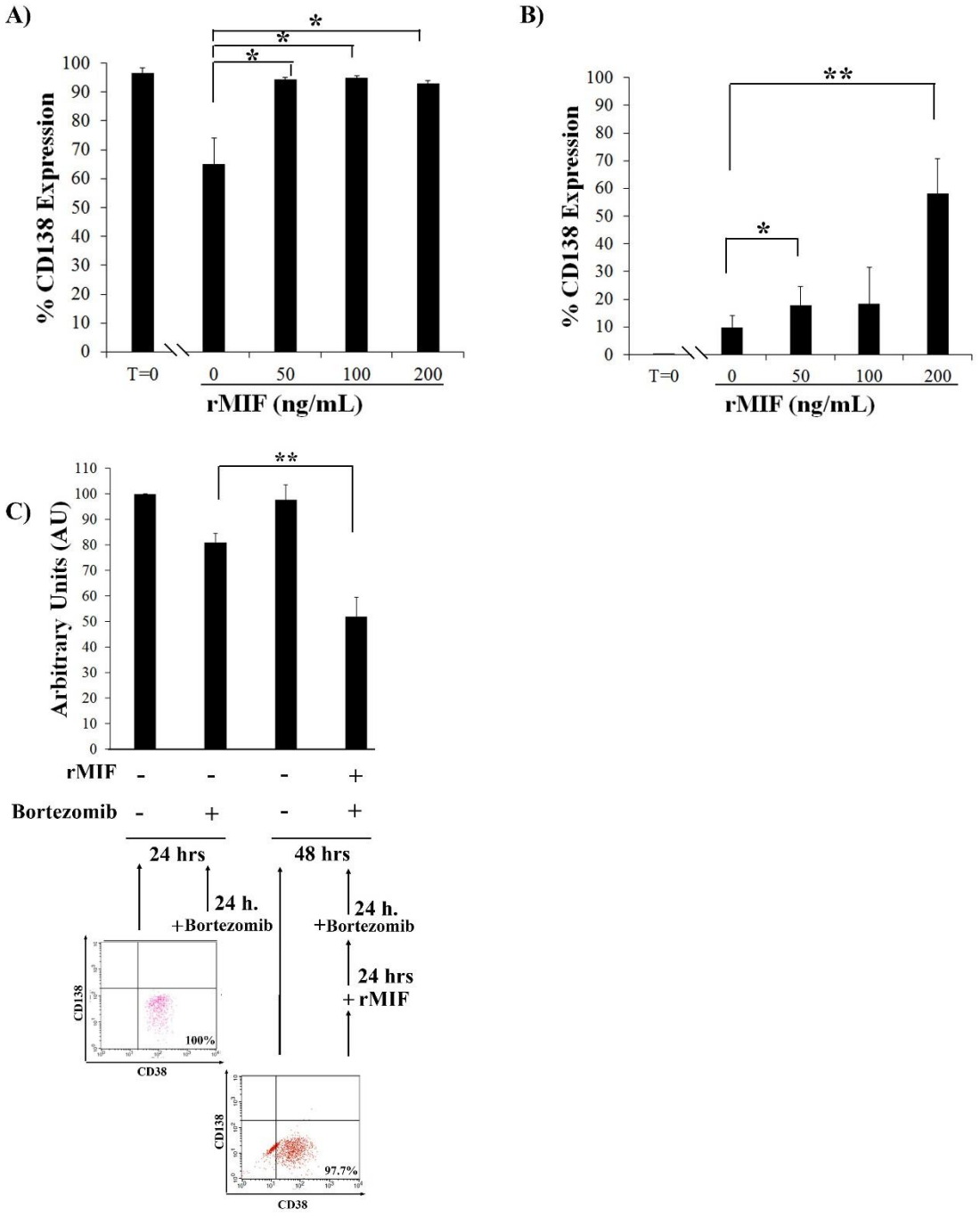


Figure 6. rMIF promotes the differentiation and maintenance of CD138+ cells. A,B) CD138 expression of viable (PI negative), CD138+ (A) and CD138- (B) cells treated with increasing concentrations of rMIF. (n=3). **C)** MTT assay used to assess viability after treatment with Bortezomib, mean absorbance values of lanes that were untreated at time point 24 hrs were set to 100 and plotted as AU of freshly sorted CD138- cells and CD138- cells previously treated with rMIF (n=3). CD138- cells were sorted and treated with rMIF for 24 hrs, followed by 0.01μM Bortezomib treatment for 24hrs (lane 4) or untreated for 48 hrs (lane 3). Freshly sorted CD138- cells treated with 0.01μM Bortezomib (lane 2) or untreated (lane 1) for 24 hrs. **p<0.001 t-test with Satterthwaite corrections to allow for possible unequal variances. Error bars denote the mean ± SD.

To determine whether the CD138+ cells generated by rMIF treatment were more sensitive to chemotherapeutic agents, sorted CD138$^-$ were mock treated (Fig. 6C, lane 3) or treated with rMIF for 24 h. to promote differentiation (lane 4), and then the rMIF treated population was additionally treated with Bortezomib for an additional 24 h to measure viability in the MTT assay (Fig. 6C, lane 4). This was compared to the chemotherapeutic response seen with pure CD138- cells derived from parent cultures in the presence (lane 2) or absence (lane 1) of Bortezomib (Fig. 6C). CD138- cells are relatively resistant to Bortezomib, and viability of treated cells was only reduced by 20% (lane 2). CD138- cells treated with rMIF, however, were more sensitive to Bortezomib and viability was now reduced 46% by treatment (lane 4).

CD138+ cells secrete more MIF-1 than CD138- cell

We had seen that NCI-H929 and U266-B1 cells also underwent differentiation and dedifferentiation when plated as pure populations (Fig. 2C). When CD138+ cells were treated with increasing concentrations of 4-IPP, U266-B1 cells also exhibited a marked decrease in the level of CD138+ cells (and an increase in dedifferentiated CD138- cells) (Fig. 7A). 4-IPP did not increase dedifferentiation of the NCI-H929 cells within this 48h. time frame (Fig. 7B). Thus, MIF-1 activity appeared to regulate interconversion in at least two MM cell lines. As we had previously seen interconversion after 5 days, 48 hrs may have been too short a timeframe to see an effect of 4-IPP on NCI-H929 cells.

The cytokine array assay had demonstrated that MIF-1 was secreted from both pure CD138- and CD138+ cells (Fig. 4A). We examined RNA levels in both CD138+ and CD138- RPMI8226 populations using RT-PCR and did not find any statistically significant differences (Fig. 7C). Using a quantitative MIF-1 ELISA kit, we were able to measure MIF-1 cytokine secretion at 1, 4, 12 and 24 h. post plating of pure populations (Fig. 7D, E,F). CD138+ cells secreted statistically significant more MIF-1 than CD138- cells in the RPMI8226 cell line (Fig. 7D). No significant differences in secretion were detected for U266-B1 cells, while more MIF-1 was secreted in the CD138+ cells than the CD138- cells in the NCI-H929 line (Fig. 7E, F).

MIF-1 has previously been shown to associate with multiple receptors, including CD74, CXCR2/4/7 and CD44[32, 33]. MIF can bind to CD74 alone, but requires association with a co-receptor, in order to initiate a signaling cascade. CD74 can form a complex with CD44 and/or CXCR2/4 or CXCR7, which contain intracytoplasmic signaling domains[34, 35]. To determine whether differences in receptor expression could be detected, unsorted RPMI8226, NCI-H929 and U266-B1 cells were stained for the presence of CD74, CXCR4 and CD44 in tandem with CD138 (Fig. 7G). Only 10% of RPMI 8226 cells expressed any of these markers (Fig. 7G, top panel). The majority of U266-B1 cells expressed CD44 and CXCR4, and ~50% expressed CD74 (middle panel). The majority of NCI-H929 cells expressed CD44, and ~50% and ~30% expressed CD74 or CXCR4 (bottom panel). This suggested that the expression of these markers varied from cell line to cell line.

MIF-1 interconversion in primary MM cells.

To examine whether MIF-1 regulates interconversion in vivo, bone marrow aspirates from MM patients were obtained with informed consent. MM tumor cells were purified using the Rosette Step MM purification kit, which removes other contaminating hematopoetic cells. This material was either stained immediately with CD138 and CD38 antibodies (Fig. 7G) or plated in tissue culture in the absence or presence of 4-IPP (Fig. 7I, J). Both CD138+ and CD138- populations could be detected from fresh bone marrow aspirates, although the percent of each pool varied between patients (Fig. 7G). When material was replated +/- 4-IPP, cells were harvested at 24 and 72 h., stained with CD138 and CD38 antibodies and analyzed by flow cytometry (Fig. 7I). After 24 and 72 h., the untreated cells were predominantly CD138+ (two patients, Fig. 7I, J). However, when plated in the presence of 4-IPP, the CD138- population increased at 24 and 72 h., suggesting that dedifferentiation in these cells had also occurred.

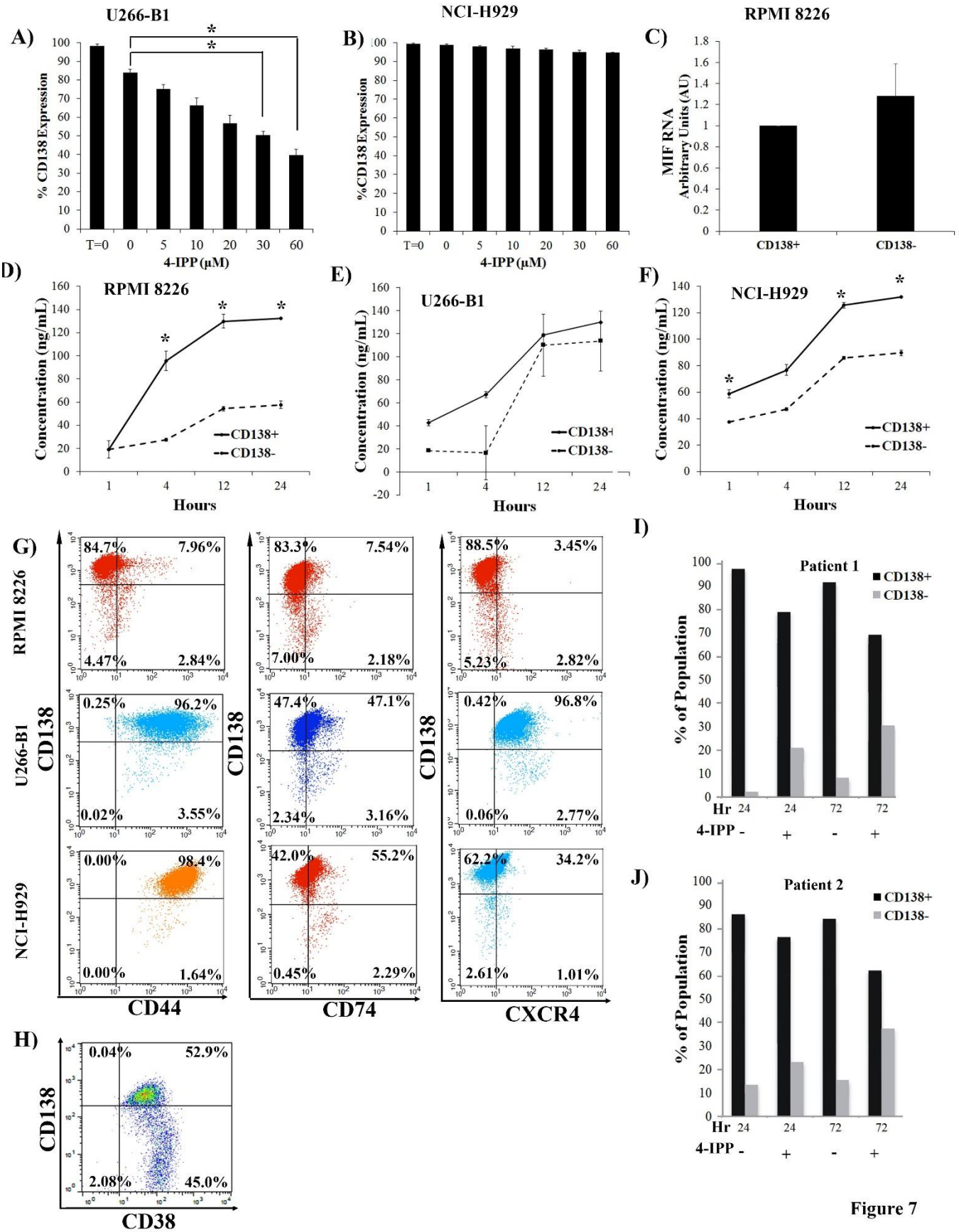


Figure 7

Figure 7. MIF-1 regulates differentiation in other cell lines and primary patient material. A,B) Sorted CD138+ U266 cells (A) and NCI-H929 CD138+ cells (B) treated with increasing concentrations of 4-IPP for 24 hours.

Discussion

While there has been some discrepancy in the field regarding the nature of the MM CSC, our data and that of others strongly suggest that the CD138- cell meets the definition of stemness, based on phenotypic characteristics: quiescence, chemotherapeutic resistance, the ability to self-renew and differentiate [24, 36]. This CD138- population is consistently detected in MM cell lines, suggesting that these lines are a mixture of the mature, rapidly dividing CD138+ cells, and immature, slowly proliferating CD138- cells [5, 20, 21, 24, 28]. The fact that these populations are maintained at a near constant ratio within the tissue culture system suggested that there had to be a mechanism to maintain this set point, because otherwise the quiescent CD138- cells should be lost from the culture during passaging. We show that the mature MM cancer cell detected in tissue culture has developmental plasticity, and can dedifferentiate back into its own chemoresistant, CD138- CSC progenitor. These newly born CSC regain all of the characteristics of the CSC demonstrating that stemness is a trait that is epigenetically controlled. Thus, instead of a CSC, we suggest that stemness is a dynamic, autoregulated phenotype that can be acquired or lost depending on the interaction with surrounding cells.

This interconversion occurs in the absence of support or accessory cells, suggesting that the MM cells themselves secrete cytokines or factors that mediate this plasticity, and in essence sense and make their own niche [24, 28]. We found that MIF-1 was secreted by pure CD138- and C138+ populations, although to different levels. The secretion of several other cytokines increased but to a much more modest extent, and by blocking or increasing MIF-1 activity, we were able to show that MIF-1 could alter interconversion, and more importantly chemosensitivity. Blocking MIF-1 drives cells to dedifferentiate into chemoresistant CD138- cells, and the addition of rMIF-1 drives cells to differentiate into chemosensitive CD138+ cells. These cells can be toggled back

Figure 7 Continued: CD138 expression of viable (PI negative), cells after 24 hrs as measured by CD138 flow cytometry. (n=3) *p<0.05. **C)** Steady state MIF-1 levels of sorted CD138+ and CD138- RPMI 8226 cells as measured by RT-PCR. **D, E, F)** Sorted CD138+ and CD138- cells from RPMI8226 (D), U266-B1 (E), and NCI-H929 (F) plated and media harvested at 1, 4, 12, and 24 hours for use in MIF ELISA assay. *p<0.001 **G)** Unsorted RPMI8226, U266-B1, and NCI-H929 dually stained with CD138/CD44 (left) or CD138/CD74 (middle), or CD138/CXCR4 (right). **H)** MM cells derived from human bone marrow aspirates and passaged through the Rosette MM purification kit, stained for CD138 and CD38. **I,J)** Unsorted BM derived MM cells were placed in culture +/- 4-IPP for 24 hrs and 72 hrs., then were gated for CD38 and analyzed for CD138 status percentage of population for two patients shown.

and forth depending on MIF-1 activity or levels, offering new insight into the epigenetic control of MM growth. Too little MIF-1 and cells dedifferentiate; too much MIF-1 and cells differentiate.

MIF-1 was secreted more rapidly by CD138+ cells, although lower secretion is still detected in the pure CD138- populations. MIF-1 is known to associate with multiple receptors, including CD74, CXCR2/4/7 and CD44 [37-39], but only 10% of RPMI8226 cells expressed any of these individual receptors. This might suggests that the receptor controlling differentiation status remains to be determined or alternatively that the 10% receptor + population is the one susceptible to changes in MIF-1 levels and starts the interconversion process. To examine this, careful analysis of receptor changes during the interconversion process will have to be examined. Differential gene expression in the CD138- and CD138+ MM cells has been described, further supporting the idea that they represent two different functional populations, and expression of genes associated with stemness, such as NANOG, Wnt, and Hedgehog, are preferentially detected in CD138- precursor cells [4, 26]. More work is required to connect the cell surface receptors and ligands that mediate interconversion with the final epigenetic outcomes. But, this study demonstrates that small changes in MIF-1 activity or levels can shift the set point in one direction or the other. The differentiation assays that we used are somewhat artificial and in vivo it is less likely that the CD138+ cells are present in the absence of the other counterpart and *vice versa*. Cytokines are likely also secreted from the BM microenvironment, and both chemical and physical cues, such as adhesion and oxygen tension, may influence interconversion [40-42]. MIF-1 has emerged as a candidate controlling plasticity and interconversion, and the logical extension of this work will be to validate this in more physiological in vivo settings [42-44].

How the set point of 9:1 CD138+:CD138- is reestablished is unclear, and suggests that additional feedback loops might exist. Decreased viability was seen in the presence of both 4-IPP and MIF-1 NABs, and while we initially thought this might be due to non-specific 4-IPP toxicity, the fact that apoptosis was also seen in both conditions suggests that this might be a physiological response to increased dedifferentiation or persistence of the CD138- state. Likewise, while cells are able to dedifferentiate at least twice, the kinetics are different in parent derived or once differentiated cells. However, the model suggests that perturbation or modulation of the MIF-1 levels could shift the balance in one direction or the other, and thus represents a viable

way to increase the percentage of CD138+, chemosenstive cells. In our limited analysis, the addition of MIF-1 inhibitors to bone marrow derived MM cells from patients altered the CD138-:CD138+, CSC:progeny ratio, suggesting that similar mechanisms are at play in vivo and might explain intrinsically different patient responses to chemotherapy. Additional, larger analysis of interconversion of primary material is required to address these issues.

Recently, MIF-1 has emerged as a factor involved in regulating the adhesion of MM tumor cells to BM. Higher levels of MIF-1 expression are seen in MM tumor cells than in normal plasma cells, and loss of MIF-1 reduced MM adhesion to BMSC in vitro. While MIF-1 expressing cells formed tumors in the bone when injected into SCID-mice, cells lacking MIF only formed tumors in the abdomen or extramedullary space, suggesting that MIF-1 enhances BM colonization[45]. While these authors did not directly examine interconversion in their assays, in the absence of MIF-1, there was an increase in the percentage of $CD138^-$ MM cells in the tumors taken from extramedullary space in the MIF-1 KD animals[45]. It is entirely possible that loss of MIF-1 and an increase in CD138- CSCs would affect BM homing and adhesion. Zheng, et al. also found that MIF-1 acted through CXCR4 and the adaptor COPS5 to increase the expression of adhesion molecules ALCAM, ITGAV, and ITGB5[45]. Interestingly, they also found that the inhibition of MIF-1 either through KD or 4-IPP treatment sensitized MM cells to the chemotherapeutic, Melphalan, while data suggest that 4-IPP treatment rendered cells more chemoresistant to Bortezomib. While the mechanism of action of Melphalan and Bortezomib are very different, our data is in agreement with theirs that altering MIF-1 levels affects chemotherapeutic response, highlighting MIF-1 as an important target.

Numerous new treatments have been introduced for the management of MM over the past several years, with four approved in 2015 alone [3, 46]. While these extend the remission period, “cure” as a goal has been consistently elusive even in the setting of hematopoietic stem cell transplants [11]. More and more, the regiments are keeping patients on continuous chemotherapy to prevent re-emergence of clinically significant disease [46-48]. This puts MM outcomes behind those for some acute leukemias where real cures have been realized in upwards of 60 % (APL) to 80% (pre-B ALL) of patients for the last 10 and 30 years, respectively. In those cases, the real

difference was the development of treatments aimed at the CSC. Our model suggests that when presented with the appropriate environment, any myeloma tumor cell might have the ability to acquire “stemness”, which increases the difficulty in treating a moving target. Current therapies are successful at killing differentiated tumor cells. However, as cells are removed from the tumor in vivo, the cytokine milieu would change due to loss of cytokine secreting cells, effectively changing the microenvironment and potentially permitting interconversion to occur. As it stands, our very use of chemotherapy and its change to the microenvironment may permit the reactivation of the chemoresistant clone. If interconversion provides a way to continually renew the chemoresistant CSC population, this would contribute to the eventual relapse seen in this disease.

References

1. O'Connell FP, Pinkus JL, Pinkus GS. CD138 (syndecan-1), a plasma cell marker immunohistochemical profile in hematopoietic and nonhematopoietic neoplasms. *Am J Clin Pathol*. 2004;121(2):254-63.
2. Bataille R, Jego, G., Robillard, N., Barille-Nion, S., Harousseau, J.L., Moreau, P., Amiot, M., Pellat-Deceunynck, C. The phenotype of normal, reactive and malignant plasma cells. Identification of "many and multiple myelomas" and of new targets for myeloma therapy. *Haematologica*. 2006;91(9):1234-40.
3. Mateos Mea. Treatment for patients with newly diagnosed multiple myeloma in 2015. *Blood Reviews*. 2015;29(6):387-403.
4. Bergsagel PL, Kuehl, M. Molecular Pathogenesis and a Consequent Classification of Multiple Myeloma. *Journal of Clinical Oncology*. 2005;23(26):6333-8.
5. Matsui Wea. Clonogenic Multiple Myeloma Progenitors, Stem Cell Properties, and Drug Resistance. *Cancer Research*. 2008;68:190-7.
6. Bianchi G, Munshi, N.C. Pathogenesis beyond the cancer clone(s) in multiple myeloma. *Blood* 2015;125(20):3049-58.
7. Mahindra A, Hideshima, T., Anderson, K.C. Multiple myeloma: biology of the disease. *Blood Review*. 2010;24:S5-S11.
8. Rajkumar SV. Multiple myeloma: 2014 Update on diagnosis, risk-stratification, and management. *Am J Hematol*. 2014;89(10):999-1009.
9. Hideshima T, Richardson, P., Chauhan, D., Palombella, V.J., Elliott, P.J., Adams, J., Anderson, K.C. The Proteasome Inhibitor PS-341 Inhibits Growth, Induces Apoptosis, and Overcomes Drug Resistance in Human Multiple Myeloma Cells. *Cancer Research*. 2001;61:3071-6.
10. Kawano Y, Fujiwara, S., Wada, N., Izaki, M., Yuki, H., Okuno, Y., Yama, K., Yamasaki, H., Sakai, A., Mitsuya, H., Hata, H. Multiple myeloma cells expressing low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. *Intl J Onc*. 2012;41:876-84.
11. Reghunathan R, Bi, C., Liu, S.C. et al. Clonogenic multiple myeloma cells have shared stemness signature associated with patient survival. *Oncotarget*. 2013;4(8):1230-40.
12. Boucher K, Parquet N, Widen R, et al. Stemness of B-cell progenitors in multiple myeloma bone marrow. *Clin, Cancer Res* 2012;8:6155-68.
13. Hajek R, Okubote SA, Svachova H. Myeloma stem cell concepts, heterogeneity and plasticity of multiple myeloma. *British J Haem* 2013;163:551–64.
14. Donnenberg VS, Donnenberg, A.D. Multiple Drug Resistance in Cancer Revisited: The Cancer Stem Cell Hypothesis. *J Clin Pharmacol*. 2005;45:872-7.
15. Saito T, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nature Biotechnology*. 2010;28(3):275-80.
16. Tan BT, Park, C.Y., Ailles, L.E., Weissman, I.L. The cancer stem cell hypothesis: a work in progress. *Lab Inv*. 2006;86:1203-7.
17. Matsui Wea. Characterization of clonogenic multiple myeloma cells. *Blood*. 2003;103(6):2332-6.
18. Yata K, Yaccoby S. The SCID-rab model: a novel in vivo system for primary human myeloma demonstrating growth of CD138-expressing malignant cells. *Leukemia*. 2004;18:1891-7.
19. Chaidos A, Barnes CP, Cowan G, et al. Clinical drug resistance linked to interconvertible phenotypic and functional states of tumor-propagating cells in multiple myeloma. *Blood*. 2013;121(2):318-28.
20. Matsui W, Huff, C.A. . Multiple Myeloma Cancer Stem Cells. *J Clin Oncol*. 2008;26(17):2895-900.
21. Matsui Wea. Characterization of clonogenic multiple myeloma cells. *Blood*. 2003;103(6):2332-6.
22. Matsui Wea. Clonogenic Multiple Myeloma Progeniotrs, Stem Cell Properties, and Drug Resistance. *Cancer Research*. 2008;68:190-7.
23. Chaidos A, Barnes C, Cowan G, et al. Clinical drug resistance linked to interconvertible phenotypic and functional states of tumor-propagating cells in multiple myeloma. *Blood*. 2013;12:18-28.
24. Chaidos A, Barnes, C.P. et al. Clinical drug resistance linked to interconvertible phenotypic and functional states of tumor-propagating cells in multiple myeloma. *Blood*. 2013;121(2):318-28.

25. Huff CA, Matsui W. Multiple myeloma cancer stem cells. *J Clin Oncol*. 2008;26(17):2895-900.
26. Diehl SA, Schmidlin, H. et al. STAT3-Mediated Up-Regulation of BLIMP1 is Coordinated with BCL6 Down-Regulation to Control Human Plasma Cell Differentiation. *J Immunol*. 2008;180:4805-15.
27. Shaffer ALea. Blimp-1 orchestrates plasma cell differentiation by extingushing the mature B cell gene expression program.

. *Immunity*. 2002;17:51-62.
28. Yaccoby S. The Phenotypic Plasticity of Myeloma Plasma Cells as Expressed by Dedifferentiation into an Immature, Resilient, and Apoptosis-Resistant Phenotype. *Clin Cancer Research*. 2005;21:7599-606.
29. Mun SH, Oh D, Lee SK. Macrophage migration inhibitory factor down-regulates the RANKL-RANK signaling pathway by activating Lyn tyrosine kinase in mouse models. *Arthritis Rheumatol*. 2014;66:2482-93.
30. Subbannayya T, Variar P, Advani J, et al. An integrated signal transduction network of macrophage migration inhibitory factor. *J Cell Communication and Signaling*. 2016;10:165-70.
31. Xia W, Zhang F, Xie C, Jiang M, Hou M. Macrophage migration inhibitory factor confers resistance to senescence through CD74-dependent AMPK-FOXO3a signaling in mesenchymal stem cells. *Stem Cell Research & Therapy*. 2015;6(82).
32. Buckner CM, Moir S, Kardava L, et al. CXCR4/IgG-expressing plasma cells are associated with human gastrointestinal tissue inflammation. *J Allergy Clin Immunol*. 2014;133(6):1676-85 e5.
33. Hosen N, Matsuoka Y, Kishida S, et al. CD138-negative clonogenic cells are plasma cells but not B cells in some multiple myeloma patients. *Leukemia*. 2012;26(9):2135-41.
34. Saito Y, Uchida N, Tanaka S, et al. Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nat Biotechnol*. 2010;28(3):275-80.
35. Shaffer AL, Lin KI, Kuo TC, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity*. 2002;17(1):51-62.
36. Hosen Nea. CD138-negative clonogenic cells are plasma cells but not B cells in some multiple myeloma patients. *Leukemia*. 2012;26(9):2135-41.
37. Buckner CMea. CXCR4/IgG-expressing plasma cells are associated with human gastrointestinal tissue inflammation.

. *Allergy Clin Immunol*. 2014;133(6):1676-85.
38. Burton JD, Ely, S., Reddy, P.K. CD74 Is Expressed by Multiple Myeloma and Is a Promising Target for Therapy. *Clin Cancer Research*. 2004;10:6606-11.
39. Sanchez-Nino MDea. MIF, CD74 and others parnters in kidney disease: tales of a promiscuous couple. *Cyto & Growth Factor Rev*. 2013;24:23-40.
40. Burness M, Sipkins, D.A. . The stem cell niche in health and malignancy. *Seminars in Cancer Biology*. 2010;20:107-15.
41. Fuhler G, Baanstra, M. et al. Bone marrow stromal cell interaction reduces Syndecan-1 expression and induces kinomic changes in myeloma cells. *Exp Cell Res*. 2010;316:1816-28.
42. Cheng Q, McKeown, S.J., Santos, L., Santiago, F.S., Khachigian, L.M., Morand, E.F., HIckey, M.J. Macrophage Migration Inhibitory Factor Increases Leukocyte-Endothelial Interactions in Human Endothelial Cells via Promotion of Expression of Adhesion Molecules. *J Immunol*. 2010;185:1238-47.
43. Tarnowski Mea. Macrophage Migration Inhibitory Factor Is Secreted by Rhabdomyosarcoma Cells, Modulates Tumor Metastasis by Binding to CXCR4 and CXCR7 Receptors and INhibits Recruitment of Cancer-Associated Fibroblasts. *Mol Cancer Res*. 2010;8(10):1328-43.
44. Winner Mea. A Novel Macrophage Migration Inhibitory Factor Suicide Substrate Inhibits Motility and Growth of Lung Cancer Cells. *Cancer Research*. 2008;68(18):7253-7.
45. Zheng Y, Wang Q, Li T, et al. Role of Myeloma-Derived MIF in Myeloma Cell Adhesion to Bone Marrow and Chemotherapy Response. *J Natl Cancer Inst*. 2016;108(11).
46. Nooka AK, Kastritis, E., Dimopoulos, M.A., Lonial, S. Treatment options for relapsed and refractory multiple myeloma. *Blood*. 2015;125(20):3085-99.

47. Paiva B, van Dongen, J. J.M., Orfao, A. New criteria for response assessment: role of minimal residual disease in multiple myeloma. *Blood*. 2015;125(20):3059-68.
48. Anderson KC, Hideshima, T. Molecular Mechanisms of Novel Therapeutic Approaches for Multiple Myeloma. *Nature Review*. 2002;2:927-37.

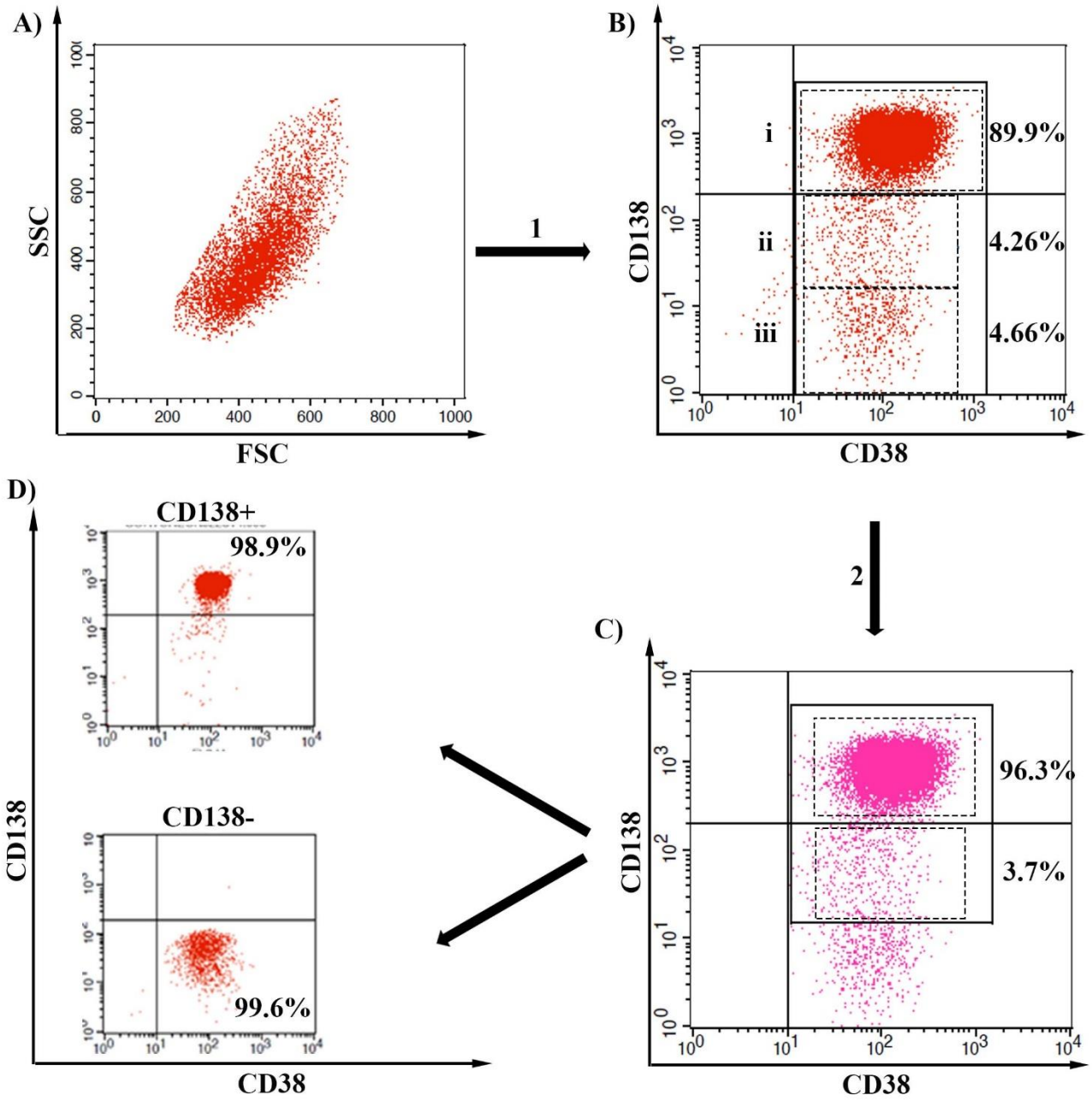


Figure S1A. Schematic Gating and Sorting Parameters of RPMI8226 CD138+ and CD138- sorted cells. A) RPMI8226 cells are gated based on forward scatter and side scatter. This gate is applied to **B)** RPMI8226 cells stained with CD138PE-Cy5 and CD38PE. Population "i" is a gate around the CD138+CD38+ RPMI cells, population "ii" is a gate around the CD138-CD38+ RPMI cells, population "iii" is a gate around the CD138- -CD38+, which are non-viable cells. Quadrants are created to distinguish CD138+ and CD138- cells along the y-axis and CD38+ and CD38- cells along the x-axis. Another gate is drawn around all CD38+ cells and is applied to **C)** RPMI8226 cells only positive for CD38. These cells are gated for CD138+ and CD138- cells to be sorted for future experiments. **D)** Dot plots of purely sorted CD138+CD38+ cells (top) and CD138-CD38+ cells (bottom). All experiments using sorted populations were assessed in this manner.

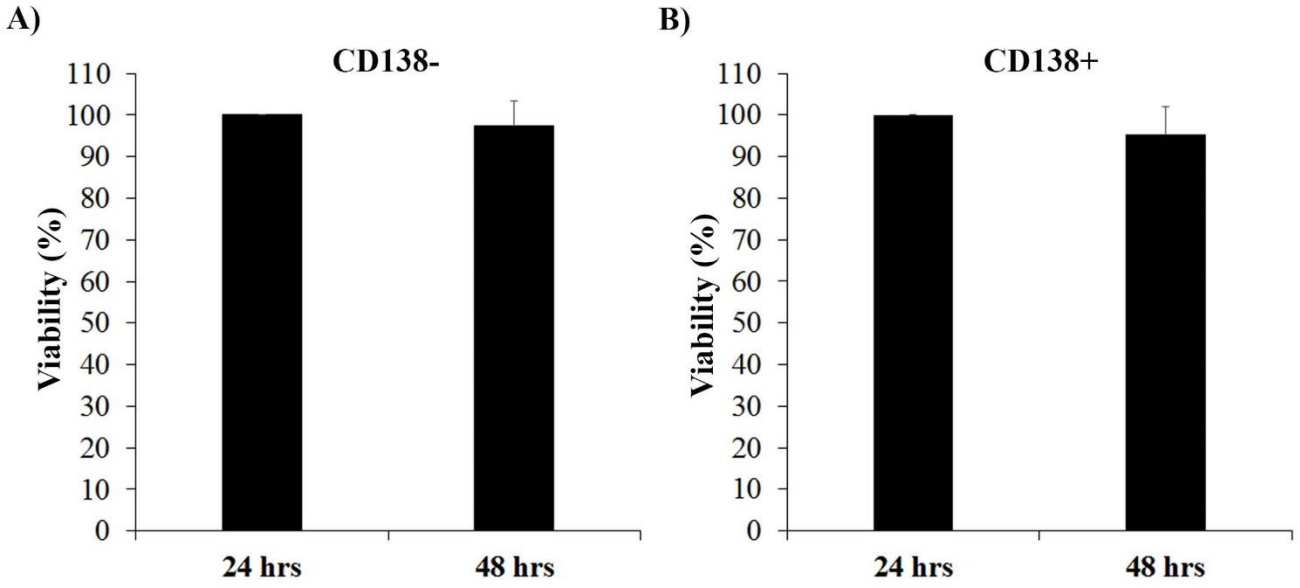


Figure S1B. Viability of Sorted CD138- and CD138+ RPMI 8226 cells. A) Viability as measured by MTT assay of sorted CD138- RPMI8226 cells 24 hours and 48 hours post sort. **B)** Viability as measured by MTT assay of sorted CD138+ RPMI8226 cells 24 hours and 48 hours post sort. No significant loss in viability after 24 hours in tissue culture was observed for either sorted population. N=3

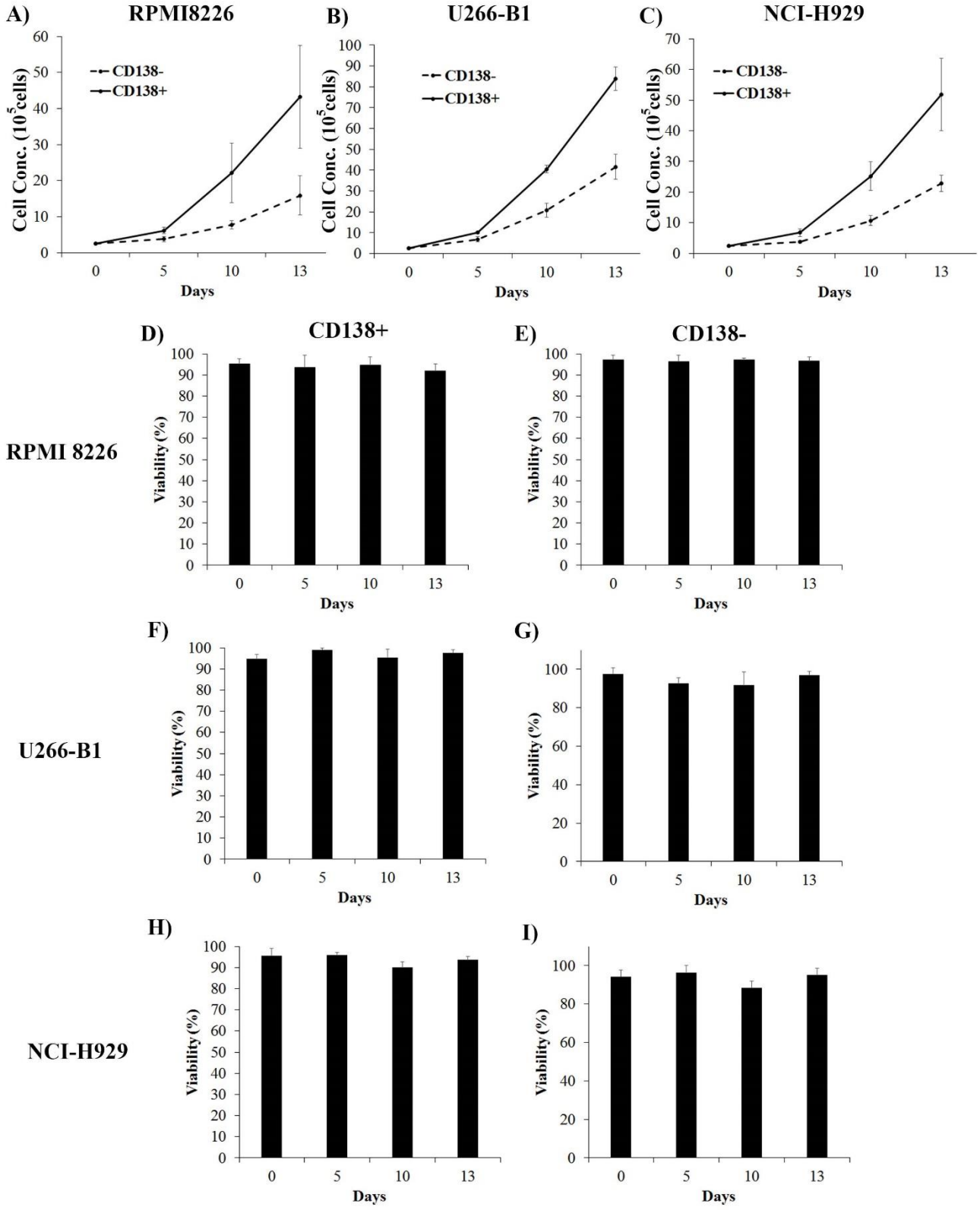


Figure S2A. Cell proliferation and viability of sorted populations of RPMI8226, U266-B1, and NCI-H929. A,B, C) Total cell numbers of CD138+ and CD138- isolated from RPMI8226 (A), U266-B1 (B), and NCI-H929 (C) at days 0, 5, 10 and 13 post sort. **D-I)** Percent viability of sorted CD138+ and CD138- from RPMI8226 (D-E), U266-B1 (F-G), and NCI-H929 (H-I) as measured by trypan exclusion at days 0, 5, 10, and 13 post sort. Each sorted population is proliferating from day 0 to day 13 and there is no significant change or loss in viability between CD138- and CD138+ populations for all three MM cell lines. Error bars denote mean ±SD. RPMI8226 (n=5), U266-B1 (n=3), NCI-H929 (n=3).

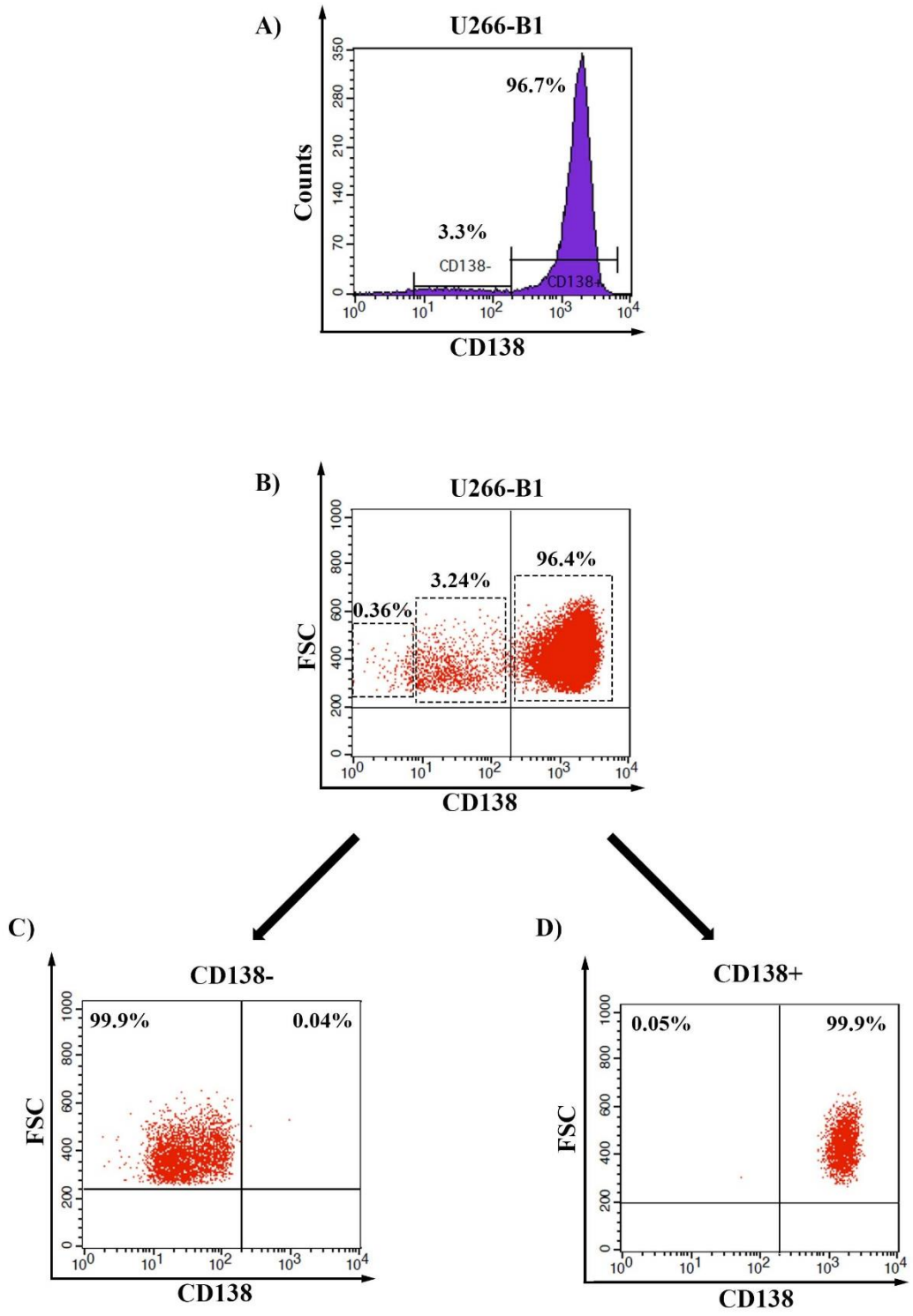


Figure S2B. U266-B1 MM cell line characterization using CD138. A) Histogram of unsorted U266-B1 MM stained with CD138. U266-B1 MM cells differentially express CD38, therefore U266-B1 cells were sorted on CD138 expression alone. **B)** Dot Plot of U266-B1 cells stained for CD138PE-Cy5 and gated on the CD138+ and CD138- cell populations. **C)** Purely sorted CD138- U266-B1 cells. **D)** Purely sorted CD138+ U266-B1 cells. All experiments using sorted populations were assessed in this manner.

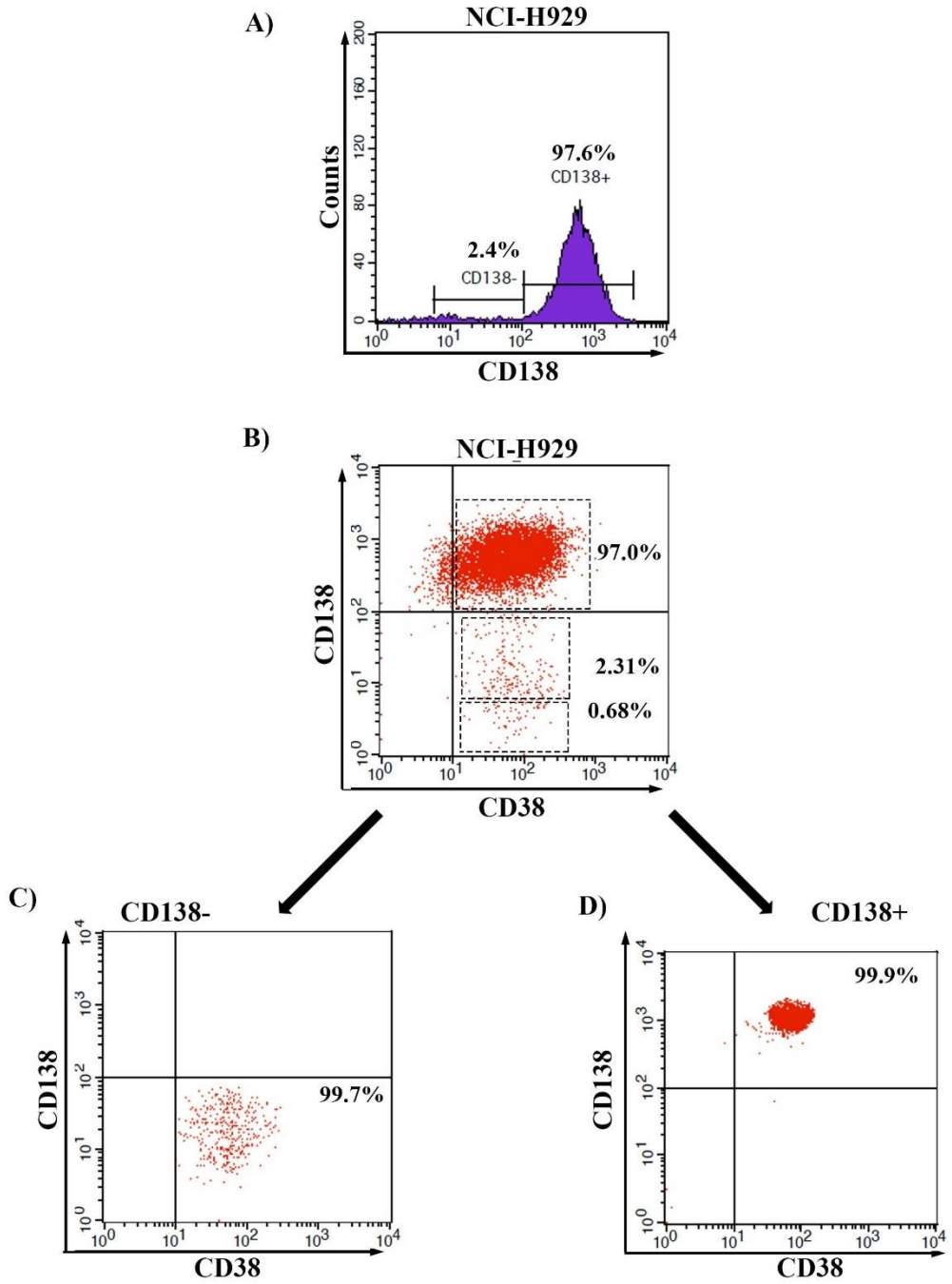


Figure S2C. Schematic of NCI-H929 MM cell line characterization using CD138 and CD38. A) Histogram of unsorted NCI-H929 cells stained with CD138. **B)** Gating parameters of NCI-H929 cells into CD138+CD38+ and CD138-CD38+ MM populations. C) Purely sorted CD138-CD38+ NCI-H929 cells. D) Purely sorted CD138+CD38+ NCI-H929 cells. All experiments using sorted cells were assessed in this manner.

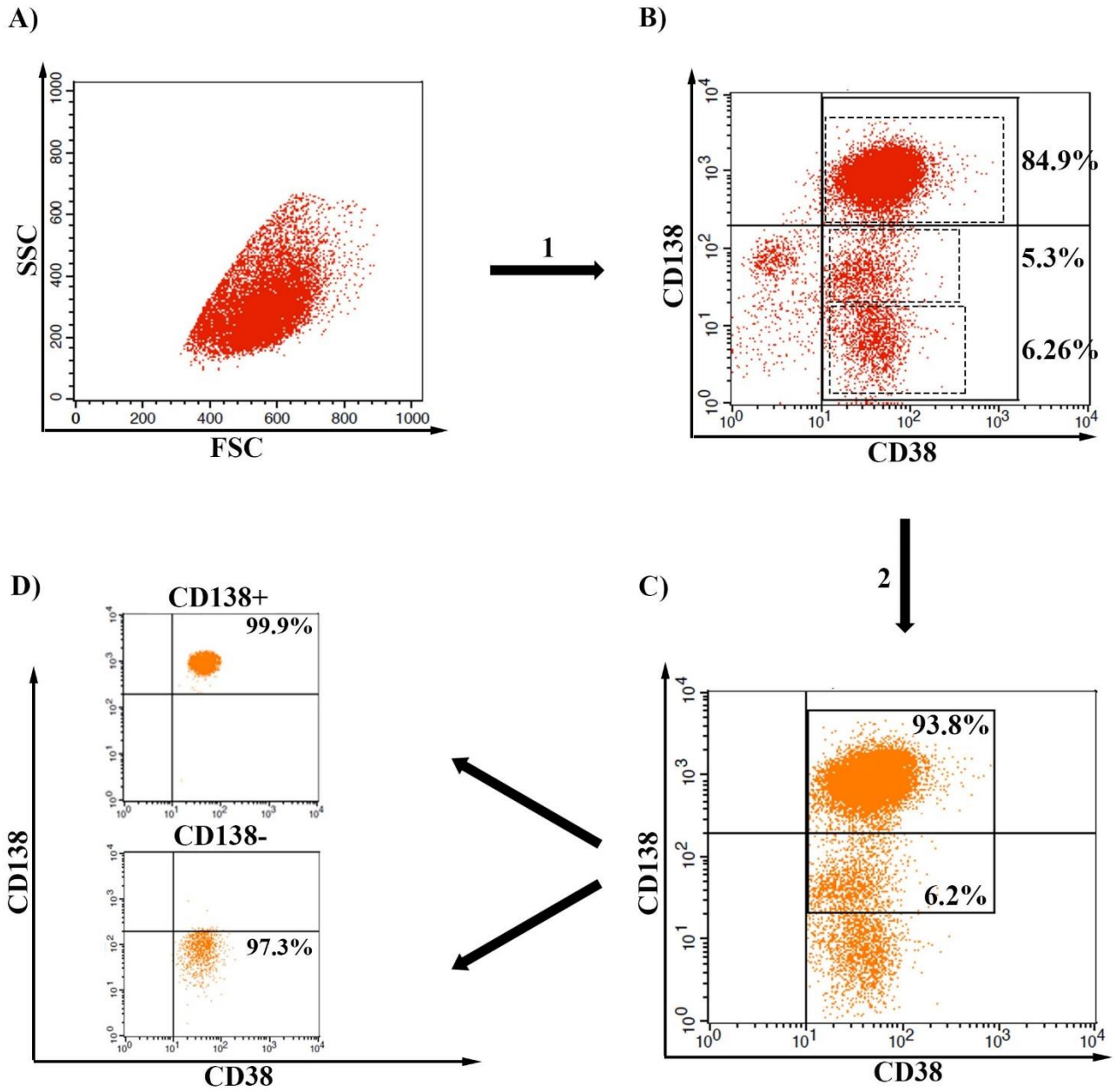


Figure S3A. Schematic of Gating and Sorting Parameters of ReSorted RPMI8226 cells. A) RPMI8226 cells were gated based on forward scatter (FSC) and side scatter (SSC). That gate was applied to **B)** RPMI8226 cells stained with CD38 and CD138. A gate is placed around all CD38+ cells. **C)** CD38+ RPMI8226 cells are assessed for the percentage of CD138+CD38+ cells and CD138-CD38+ cells. **D)** Purely sorted CD138+CD38+ population (top) and a purely sorted CD138-CD38+ population (bottom).

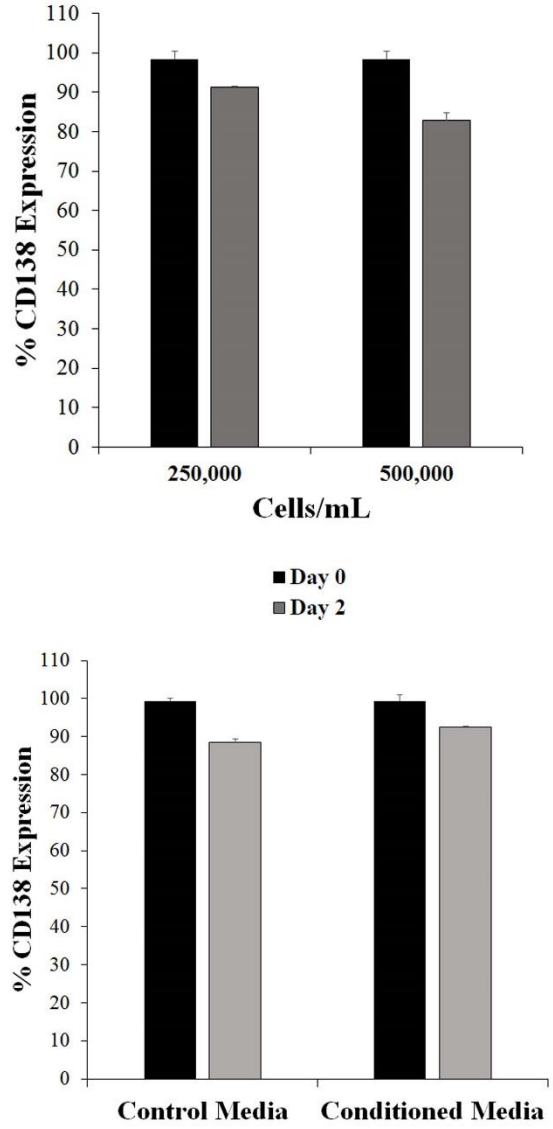


Figure S4A. Cell Density or Conditioned Media of RPMI8226 cells influences de-differentiation kinetics. A) Unsorted RPMI8226 cells were grown at two concentrations and CD138 expression was assessed by flow cytometry after 48 hours. **B)** CD138+ sorted RPMI8226 cells were plated in normal tissue culture conditions, cells were spun down and media was replaced with fresh media (Control) or kept in the same media every 12 hours for 48 hours. Cells were then analyzed for CD138 expression by flow cytometry. Statistical significance could not be achieved due to low experimental n. N=2.

A)

CD138- RPMI 8226	Media Alone	Day 1	Day 2
C5/C5a	34.405	-100.485	27.84
CD40 Lig.	165.98	-55.22	119.845
G-CSF	42.69	-82.265	30.775
GM-CSF	34.45	-88.675	16.565
GROα	54.025	-42.065	32.665
I-309	55.32	-16.915	33.81
sICAM-1	88.125	-19.565	28.205
IFN-γ	113.47	14.645	79.695
IL-1α	27.425	-103.35	27
IL-1β	14.32	-118.14	12.185
IL-1ra	43.705	-87.845	43.94
IL-2	28.865	-97.24	21.36
IL-4	22.215	-105.77	12.265
IL-5	21.225	-97.175	16.075
IL-6	33.35	-106.145	18.655
IL-8	35.23	-109.255	29.02
Il-10	7.65	-113.465	14.67
IL-12p70	20.035	-115.865	21.1
IL-13	97.295	-53.065	122.475
IL-16	45.405	-1.355	179.535
IL-17	18.625	-106.5	14.5
IL-17E	52.795	-87.62	48.24
IL-23	138.72	-43.85	137.625
IL-27	27.715	-112.745	24.46
Il-32	7.22	-54.6	11.82
CXCL10	15.99	-96.46	20.48
CXCL11	19.7	-90.305	19.39
CCL2	11.655	-96.915	16.91
MIF-1	41.47	8912.8	10668.07
CCL3	31.17	-83.28	25.285
CCL4	16.475	-95.415	15.985
SerpinE1	71.46	-6.34	91.555
RANTES	53.015	2.415	22.06
SDF-1	51.99	-55.8	17.515
TNF-α	87.21	-77.22	17.085
sTREM-1	89.13	-85.275	10.65

B)

CD138+ RPMI8226	Media Alone	Day 1	Day 2
C5/C5a	37.76	2.34	-19.065
CD40 Lig.	195.085	59.51	32.35
G-CSF	79.435	3.505	-9.22
GM-CSF	41.735	-8.115	-17.055
GROα	129.925	12.29	-6.57
I-309	119.655	11.765	-9.395
sICAM-1	26.52	136.135	548.36
IFN-γ	147.86	31.425	36.165
IL-1α	41.025	-4.545	-18.88
IL-1β	20.5	-13.695	-22.57
IL-1ra	91.595	5.805	-9.93
IL-2	59.81	-12.525	-18.205
IL-4	25.875	-18.8	-23.94
IL-5	12.965	-9.09	-24.755
IL-6	12.63	-19.58	-23.14
IL-8	29.89	16.465	34.46
Il-10	19.685	-9.42	-23.385
IL-12p70	31.685	-10.705	-18.535
IL-13	212.52	39.35	12.465
IL-16	276.08	126.285	109.95
IL-17	25.08	-15.81	-23.71
IL-17E	80.41	7.565	-4.77
IL-23	216.65	53.575	25.675
IL-27	25.23	-13.515	-19.795
Il-32	11.98	-13.795	-21.885
CXCL10	28.605	76.44	178.635
CXCL11	45.41	0.8	-24.445
CCL2	47.81	-6.6	-24.875
MIF-1	76.595	10402.15	8632.91
CCL3	29.19	-10.975	-18.15
CCL4	0.945	-24.665	-25.63
SerpinE1	119.695	674.125	958.845
RANTES	18.48	56.76	84.825
SDF-1	18.19	2.43	-9.975
TNF-α	37.9	107.495	16.735
sTREM-1	42.29	83.665	16.915

Figure S4B. Cytokine Array Data for sorted RPMI8226 populations. Raw values obtained by LICOR imaging system for cytokine arrays at each time point for both CD138- **(A)** and CD138+ **(B)** and media alone.

Discussion

Macrophage migration inhibitory factor-1 (MIF-1) is a ubiquitously expressed inflammatory cytokine[1]. It is constitutively secreted at low levels within the body and has been shown to be significantly elevated in conditions of inflammation, hypoxia and cancer[2]. MIF-1 promotes lymphocyte survival and activation, promotes angiogenesis, and has also been shown to promote the expression of adhesion molecules such as V-CAM and I-CAM necessary for cell: ECM mediated binding and cell migration[1, 3]. These pleiotropic effects of MIF-1 are mediated by its several binding partners, among them CXCR2/4/7 and CD74, which require co-receptor CD44 for cell-mediated signaling[1, 4, 5]. Signaling via these receptors can activate the ERK/MAPK, PI3K/Akt, and JNK pathways that result in cell proliferation and survival[1, 5]. Further, MIF-1 has also been shown to support the secretion of several pro-inflammatory cytokines such as IL-1, TNF-α, IFN-γ, IL-6, IL-8 and PGE_2[1].

MIF-1 has been implicated to have a role in the proliferation and survival of cancer cells[6, 7]. Utilizing the same chemokine receptors, MIF-1 mediated cell signaling results in the proliferation and survival of tumorigenic cells in certain types of cancer[1]. It has been suggested that this is due to the overexpression of, not only soluble MIF-1, but of one of its receptors, CD74[8]. CD74 is normally expressed only on lymphocytes, plasma cells, dendritic cells, thymus and some endothelial cells[1, 8]. Yet, research has shown that CD74 is expressed in non-small cell lung cancer[9], clear cell renal cell carcinoma[10], and gastric cancer[11]. More significantly, CD74 expression was shown to be greater on malignant cells as compared to their non-malignant counterparts in thymic epithelial neoplasms[12], B cell lymphomas and even MM[8]. This finding prompted the generation of a monoclonal antibody against CD74, milatuzumab, which would preferentially target tumorigenic cells. In 2013, the results of a clinical phase I trial using milatuzumab in patients with refractory or relapsed multiple myeloma showed that for 26% of patients with relapsed MM, milatuzumab was able to stabilize their disease for almost three months and over 17 months for one patient[13]. This was encouraging because it showed that targeting a receptor that binds MIF-1 could influence MM cell growth. Since that study, no further clinical trials using milatuzumab in multiple myeloma patients have been sponsored. However, this

result also highlights the challenge in selectively choosing one cell surface marker in a transformed cell population that has the capacity to evolve over time. A study of MM cells isolated from the bone marrow of 22 patients showed that 86% expressed CD74 to varying degrees as measured by flow cytometry[14]. This suggests that there is a heterogeneity within the MM cell population across patients and even within the same patient, which results in a difference in surface protein expression. Therefore, targeting one cell surface marker would give a differential response to treatment since it only targets cells that express CD74.

We found that the RPMI8226, U266-B1, and NCI-H929 MM cell lines differentially expressed CD74, its co-receptor CD44, and CXCR4. Less than 10% of the RPMI8226 cell line expressed CD74 as compared to over 50% of either the U266-B1 or NCI-H929 cell lines. The expression of CD44, the co-receptor of CD74, was also found to be on less than 10% of the RPMI8226 cell line as compared to the nearly 100% for both the U266-B1 and NCI-H929 MM cell lines. Lastly, the expression of CXCR4 in the RPMI8226 cell line was approximately 5%, in contrast with the U266-B1 cell line, which was approximately 100% positive and with the NCI-H929 cell line, which was almost 35% positive for CXCR4. Thus, the RPMI8226 cell line had the least cell surface expression of MIF-1 receptors relative to either the U266-B1 or NCI-H929 cell lines. However, there was an observable and significant change in viability and differentiation status of both sorted CD138+ and CD138- RPMI8226 cells after anti-MIF-1 treatment by either 4-IPP or neutralizing antibody. This suggests that these receptors might not be the primary receptors for MIF-1 mediated signaling for the RPMI8226 cell line. The U266-B1 cell line had the highest cell surface expression of all three receptors, however the change in the differentiation status of CD138+ cells was similar to that seen in the RPPMI8226 cell line. This might suggest that for the U266-B1 cell line, these receptors are sufficient to regulate MIF-1 mediated signaling. The NCI-H929 cell line did not exhibit a response to MIF-1 inhibition at the same concentrations or time frame sufficient to cause a change in viability or differentiation status in either RPMI8226 or U266-B1. Future experiments might include increasing the incubation time of NCI-H929 cells with 4-IPP or increasing the concentrations of 4-IPP, which would give us insight as to whether these receptors are capable of initiating the same response observed in the RPMI8226 and the U266-B1 cell lines. Further, all three cell lines could be treated with

monoclonal antibody against CD74, CD44, and CXCR4 in combination with 4-IPP to elucidate the necessity for these receptors in MIF-1 mediated signaling. It also would be advantageous to investigate the cell surface expression of the other two known MIF-1 receptors in all three MM cell lines. The cell surface levels of CD74 present on these three cell lines was particularly interesting, considering the encouraging evidence of previously published articles that suggested that targeting CD74 via milatuzumab in MM was a promising method of therapy[8, 14, 15]. However, given the differential expression seen in MM patients and the rather low percentage of milatuzumab-responsive patients in the phase 1 clinical trial, this data suggests that targeting the cytokine, MIF-1, rather than the receptor would result in a better clinical response.

Of the few MIF-1 antagonists that have been characterized, 4-IPP has emerged as being the least toxic and the most specific. ISO-1 and 6-PP were the initial analogs of 4-IPP and were used to inhibit MIF-1[16, 17]. 4-IPP covalently modifies the N-terminus of MIF-1, preventing MIF-1 from binding to its receptor and thereby preventing activation of the appropriate signaling cascade[16]. Recently, the Zheng lab has shown that inhibiting MIF-1 by 4-IPP can effectively alter the chemosensitivity of two MM cell lines[3]. Their data suggest that blocking MIF-1 causes MM cells to become more sensitive to the chemotherapeutic agent melphalan. The Zheng lab demonstrated that MIF-1 inhibition downregulates the expression of the adhesion molecules ITGAV, ITGB5, and ALCAM, allowing MM cell escape from the bone marrow niche and homing to other extramedullary sites[3]. Although the correlation between MIF-1 inhibition and different adhesion molecules has been shown, the expression of CD138[18], which is known to be involved in supporting cell:cell adhesion and cell:ECM interaction was not investigated in the Zheng study. They also show that the relative protein levels of phosphorylated NFκB and phosphorylated MEK1/2 are decreased after MIF-1 knockdown[3]. This further shows the participation of the known cell signaling proteins in response to MIF-1 inhibition.

Previously, published data showed that tumorigenic cells from different cancers can secrete cytokines that serve as chemoattractants, thereby making their microenvionment suitable for cell survival and proliferation[19-22]. Cytokines most associated with the microenvironment of the MM cell are IL-1β, IL-6, IL-8, IL-16, TNF-α, and GM-CSF, some of which can be secreted by both surrounding bone marrow stromal cells

and the malignant plasma cell[19, 23, 24]. Of the 36 different cytokines that we tested, we found that seven cytokines were secreted into the media of RPMI8226 MM cell line at a relatively higher level than the others, with the MIF-1 level being the highest. Piddock et al. suggested that MM cells secrete MIF-1 which causes the bone marrow stromal cells (BMSCs) to secrete IL-6 and IL-8 that in turn maintain the viability of the MM tumor cells and their resistance to chemotherapeutic agents[25]. Further, MIF-1 inhibition of MM cells co-cultured with BMSCs and dually treated with a proteasome inhibitor resulted in apoptosis[25]. This finding further validated the role of MIF-1 in MM cell tumor development and its impact on sensitivity to chemotherapeutic agents that was highlighted in this work. Collectively, this suggests that: 1) the presence of MIF-1promotes MM cell growth and proliferation and 2) the absence or removal of MIF-1 drives apoptosis of the MM cell[3, 25].

In characterizing the three MM cell lines, we have supported previous findings that these cell lines consist of a heterogeneous mixture of both mature CD138+CD38+ MM cells and the precursor CD138-CD38+ MM cell. These populations are always present in the cell culture in at approximately 9:1 ratio of CD138+:CD138- cells respectively in all three cell lines. After isolating to >95% purity, each population was found to exhibit its own unique characteristics. These characteristics have also been previously published by Matsui et al and have been attributed to the cancer stem cell like phenotype: the ability to undergo long-term self-renewal, to differentiate, to remain quiescent, and to be chemoresistant. In testing the "stemness" characterstics of the CD138- cell we were able to show that the CD138- cell can undergo long-term self-renewal as indicated by the methylcellulose colony forming assay. This semi-solid medium provides each cell an environment to generate colonies based solely on its own intrinsic ability. Methylcellulose inhibits cross-talk between cells by hampering the diffusion of cytokines or preventing the physical interactions that might elicit a cellular response as would freely occur in tissue culture plates with fluid media. In this assay, CD138- cells formed large colonies while the mature CD138+ MM cell formed many small colonies. This suggests that the colonies generated by the CD138+ MM cells had exhausted their proliferative potential, while the CD138- MM cells were able to proliferate to produce large sized colonies. Analysis of the DNA content of the CD138- precursor MM cell showed that ~90% of its population was held in the G0/G1 phase, as opposed to the mature

CD138+ MM cell that had less than 60% of its population in the G0/G1 phase. Additionally, treatment of either population with Bortezomib showed that the CD138- MM precursor cell was less chemosensitive as compared to the CD138+ MM population. Chemosensitivity is measured as the change in viability before and after treatment with bortezomib. It was not surprising that the CD138- MM cells were able to differentiate into the CD138+ MM plasma cell and the same 9:1 ratio of CD138+:CD138- was again re-established 13 days post sort. However, more surprising was the result that mature CD138+ MM cells were able to give rise to their precursor, CD138- MM cells. This suggests that in certain conditions the mature CD138+ MM plasma cell exhibits an epigenetic plasticity that allows it to change its differentiation status. Both CD138+ and CD138- MM cells consistently re-established the 9:1 ratio 13 days post sort, demonstrating that the populations have methods to re-equilibrate themselves. Taken together, this work supports previously published scientific data that defined the CD138- precursor MM cell as the MM CSC. One of the main contributions of my work was due to the fact that all of the data was derived from highly enriched populations, sorted by flow cytometry, so for the first time, we can trace these population origins precisely and their resulting cell fates.

The consistent re-establishment of the 9:1 ratio after isolation of nearly pure populations suggested that there might be soluble factor(s) secreted by the MM plasma cells that regulated this process. When placed in media in isolation, a certain concentration of that secreted protein must be reached in order for that plasma cell to re-establish itself in the *in vitro* microenvironment. Given that these MM cell lines are grown in the absence of accessory cells or a pseudo-extracellular matrix, the plasticity of the two populations seems to be intrinsically regulated. We have shown in this MM plasma cell system that the RPMI8226 cells secrete six identifiable cytokines in addition to MIF-1: CD54 (sICAM), IL-8, IL-16, CXCL10, SerpinE1, and RANTES. This finding was encouraging because each of these cytokines has been shown to be involved in MM disease progression. CD54 is a soluble adhesion molecule that is elevated in the sera of patients with MM as compared to sera from healthy individuals[26]. Moreover, this serum concentration can be correlated to the stage of MM disease. IL-8 has angiogenic activity and is associated with active MM disease[19]. IL-16 acts as a chemoattractant of CD4+ lymphocytes and has been detected at a concentration three times higher in the sera of patients with MM as

compared to healthy individuals[27]. These elevated levels correlate with poor survival rates in patients with MM. Serpin E1 has been shown to be expressed at significantly higher levels in MM patient sera, as well as to have anti-apoptotic effects on MM tumor cells, and to cause increased angiogenesis in the MM BM[28]. RANTES has been implicated in contributing to osteoclast resorption of bone in MM, and serum levels of this chemokine are highest in patients with stage 3 MM[29]. Interestingly, CXCL10 promotes cell proliferation and growth as well as the induction of apoptosis[30]. It has been suggested that this is regulated by the particular isoform of CXCR3 receptor present on the cell that mediates its downstream signaling[30]. The secretion of MIF-1, however, was by far the highest in our cultures and we decided to focus on this cytokine. We showed that MIF-1 was able to regulate this plasticity. However, it is unlikely that MIF-1 is the only factor involved, in part because inhibition of MIF-1 did not completely alter the set point distribution. In the presence of 4-IPP, for example 69.2% of CD138+ MM cells became CD138-, but 30.8% did not. That suggests that either we did not completely block MIF-1 because it was continually being secreted or other factors are involved, such as the difference in receptor expression and secretion.

As discussed earlier, an increased sensitivity to chemotherapeutic agents after MIF-1 inhibition in MM plasma cells has been suggested by both Zheng et al, and Piddock et al, and at first glance might seem contradictory to our results in that we see a reduced chemosensitivity in the presence of MIF-1 inhibition. However, we believe that the difference in experimental design is responsible. Both the Zheng, et al 2016 and Piddock, et al 2015 studies illustrated the effect of MIF-1 inhibition on

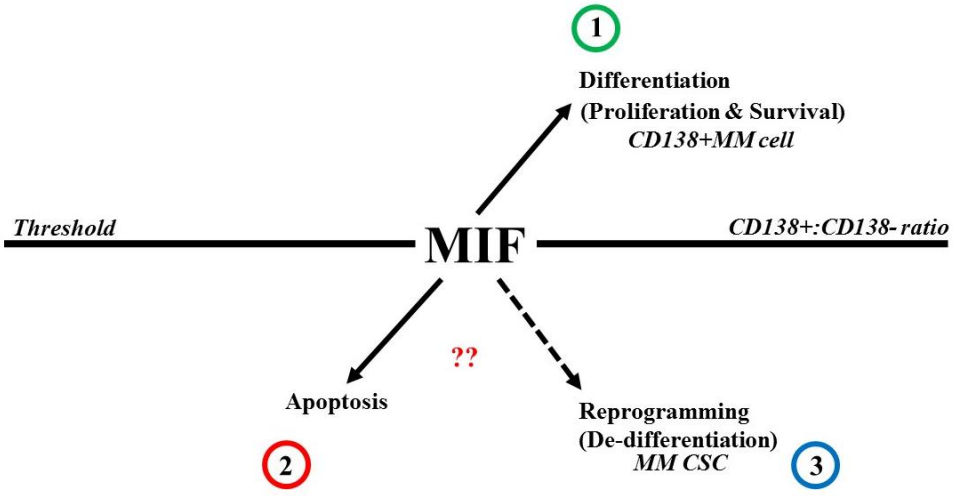


Figure D1. A diagram of MM cell in response to MIF-1 concentration. MM plasma cell plasticity mediated by the presence of MIF-1 in the tissue culture. At threshold levels, 9:1 of CD138+:CD138- is maintained. If MIF concentration is above the threshold, MM plasma cell favors differentiation. If MIF concentration is below the threshold, MM plasma cell can either undergo apoptosis or de-differentiate favoring the CD138- status.

chemosensitivity on "mixed" populations of MM plasma cells, and not on pure, sorted CD138+ or CD138- MM plasma cells. The dual effect of MIF-1 inhibition and chemotherapeutic treatment on the heterogeneous population of cells in the Zheng, et al 2016[3] and Piddock, et al 2015[25] experiments allow cross-talk between the CD138+ MM cells and CD138- MM cells, at least at the initiation of their experiment, whereas our do not. Additionally, the heterogeneous MM cell population was cultured in the presence of BMSCs, which also secrete cytokines that can impact the overall result. Though their experiments might better simulate what might happen *in vivo*, they do not fully test the capacity of an individual MM cell to be tumorigenic. This is an important concern, because it is the potential of a single cell, the MM CSC, which can home to a niche, influence its microenvironment, and re-initiate tumor growth. While our system is artificial, these experiments focus on the intrinsic tumorigenic capacity, epigenetic plasticity and regulatory feedback loops intrinsic to the CD138- and CD138+ MM cell populations.

While inhibition of MIF-1 or the addition of rMIF-1 affects plasticity, we were not able to completely "force" 100% of cells into one state over the other. This might suggest that one or a combination of the six other highlighted cytokines from the cytokine array might be involved in the cytokine "cocktail" that mediates the complete de-differentiation of the mature MM plasma cell population or the complete differentiation of the precursor MM cell population. Preliminary data showed that, of the seven cytokines that were present at higher levels relative to the other cytokines within the array between CD138+ and CD138- RPMI 8226 cells, only RANTES was undetectable after 4 days of 4-IPP treatment (Appendix Fig1). Several other cytokines are also undetectable after 4-IPP treatment that are present in the media of untreated RPMI8226 cells: G-CSF, GM-CSF, IL-2, IL-5, CXCL11, CCL2, TNF-α, and sTREM-1. This data suggests that MIF-1 inhibition can affect the secretion of other cytokines. It is unclear whether this is a direct result of MIF-1 inhibition or a secondary consequence of de-differentiation. However, this is still an encouraging finding and suggests that this experiment should be performed on both sorted CD138+ and CD138- MM cells.

As previously stated, the MM cell lines were grown in the absence of accessory cells, which suggests that we can effectively alter intrinsic chemosensitivity independent of a physical tumor microenvironment. However, as also stated, this in vitro system does not reflect the normal physiological environment. Since we have shown that we can induce differentiation of the MM cells in the absence of accessory cells, it might be informative to see if this same phenotype occurs when MM cells are treated with rMIF in the presence of an extracellular matrix and microvasculature as would occur in a mouse model. The NOD/SCID γ-irradiated mouse model could be used to subcutaneously inject 1) CD138+ sorted MM cells, 2) CD138- MM sorted MM cells, and 3) unsorted MM cells. Each of these groups would have several conditions: untreated, rMIF-1 alone (at different concentrations), Carfilzomib alone, and rMIF-1 and Carfilzomib combined. Carfilzomib is a proteasome inhibitor that irreversibly binds to the active site of the proteasome. The rationale of this experiment is that if MIF-inhibition in purely sorted MM cell populations encourages the chemoresistant MM CSC population, then adding MIF-1 to the system should effectively encourage the generation of the chemosensitive MM CD138+ cell population. The expected result should be that for all MM cells dually treated with rMIF and Carfilzomib, tumors should not be present or if they are, they should be significantly smaller as compared to those that were not dually treated. It would also be informative to excise the tumor of a mouse that received CD138- sorted MM cells before and after treatment with rMIF-1 alone, and perform immunohistochemical staining with antibodies against CD138. The expected result might resemble a heterogeneous mixture of primarily CD138+ MM cells. This would validate the concept that rMIF-1 causes differentiation resulting in a CD138+ chemosensitive tumor.

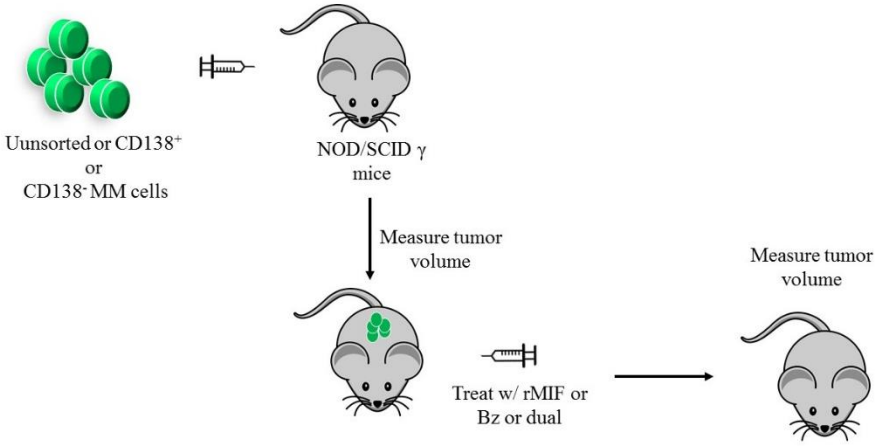


Figure D2. Experimental Schematic of Subcutaneous injection of sorted MM cells in NOD/SCID γ mice.

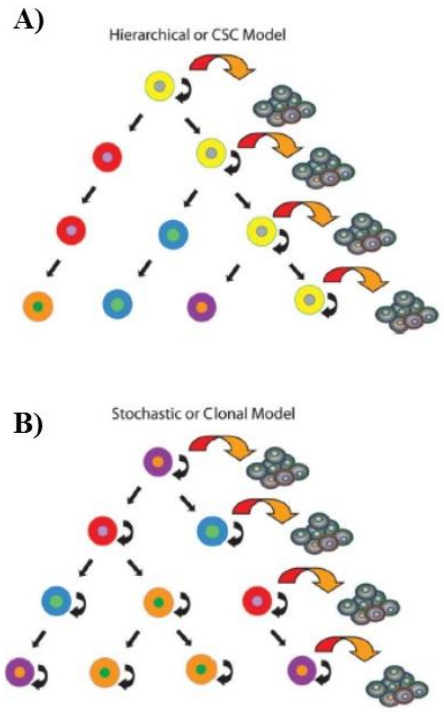


Figure D3. Hierarchical vs stochastic model of tumor heterogeneity. A) The hierarchical model dictates that a CSC (yellow cells) can self-renew (black curved arrows) and give rise to malignant cells of different stages of maturation (different color cells) that populate the tumor. **B)** The stochastic model dictates that every tumor cell has the same capacity to self-renew (black curved arrows) and give rise to malignant cells of different stages that populate the tumor. This figure was taken from Rahman, M., Deleyrolle, L., Vedam-Mai, V. et al. Neurosurgery 68(2):531-45 (2011).

This proposed future experiment seeks to eliminate the "MM CSC" by encouraging a desired cellular phenotype, susceptibility to chemotherapy, rather than trying to identify a specific cell surface marker and then target it. The evasiveness of the cancer stem cell is that it often times can become interchangeable with a tumor-initiating cell. The cancer stem cell model, suggests that the CSC has specialized characteristics that allow it to give rise to clonal progeny that have the potential to differentiate, undergo long-term self-renewal, are quiescent and chemoresistant (Figure D3, Panel A). All of these characteristics are intrinsic to and maintain the cancer stem cell pool, much like the HSC pool, after which the CSC is modeled. Yet a tumor-initiating cell is a cell that, in the right microenvironment, can proliferate extensively and is still susceptible to cytotoxic injury by chemotherapeutic agents, and thus can be eradicated. This agrees with the stochastic model of tumor heterogeneity (Figure D3, Panel B).

Our work is consistent with the stochastic model and extends beyond that, where the interconvertible plasticity of both the immature CD138- MM cell and the CD138+ MM cell can create at any time a cell with "stemness". Our work further suggests that either population of MM cell is susceptible to the presence or absence of MIF-1, resulting in altered chemosensitivity. Though the CD138- MM cell meets the characteristics of "stemness", the de-differentiation of the CD138+ MM cell generates a fully functioning MM cell that can also meet the characteristics of "stemness," in that it has the ability to self-renew, and is chemoresistant. Thus, the distinctive feature of our model is that our data postulates that any CD138- or CD138+ MM cell has the potential to act as a tumor-initiating cell.

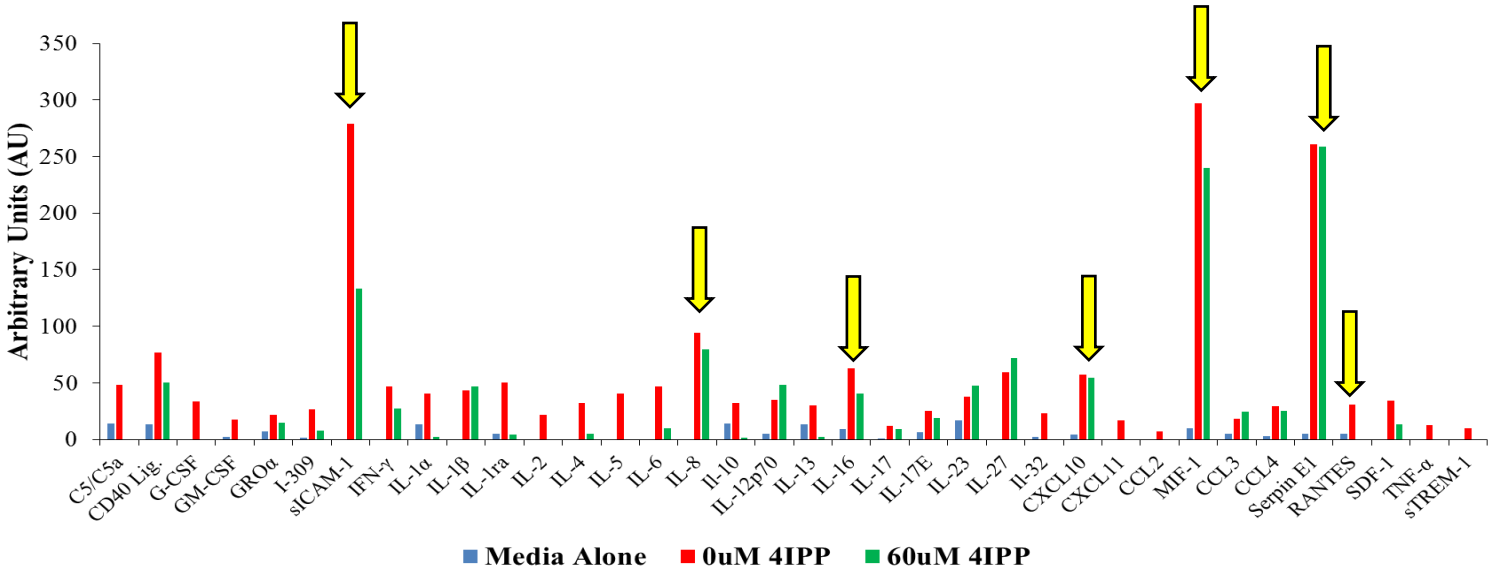
Preliminary data suggest that primary MM patient plasma cells contain both the immature CD138- MM cell and the mature CD138+ MM cell to varying degrees. We also found that the plasma cells isolated from an MM patient were able to be cultured *in vitro* for two weeks, supplanting fetal bovine serum with the patient's own serum. Additionally, we observed that primary-patient derived MM cells were sensitive to MIF-inhibition by 4-IPP, causing de-differentiation. This is encouraging because it validates our *in vitro* findings that used commercially available MM cell lines. We would further like to isolate the CD138- and CD138+ MM cells from patient bone marrow samples and dually treat with 4-IPP and bortezomib. Given the de-differentiation by 4-IPP observed in the primary MM cells, we would also expect to see an increase in chemoresistance after dual treatment with bortezomib in these primary tumors.

In conclusion, we have shown that MIF-1 can regulate the plasticity of MM tumor cells, which ultimately alters chemosensitivity. Through the extensive use of MM cell lines and preliminary data using primary patient material, it can be suggested that MIF-1 promotes de-differentiation. Both CD138+ and CD138- MM cells can secrete MIF-1 and thus are independently capable of influencing and creating their own optimal microenvironment. This work has suggested that the conventional methods for treating MM by isolating and targeting a CSC population based on cell surface markers may be futile. However, manipulating the levels of the cytokine, MIF-1, and pathways responsive to it which promotes sensitivity to chemotherapeutic agents might be the more favorable method of treatment and could circumvent the CSC hypothesis since each cell can acquire stemness.

References

1. Sanchez-Nino MD, Sanz AB, Ruiz-Andres O, et al. MIF, CD74 and other partners in kidney disease: tales of a promiscuous couple. *Cytokine Growth Factor Rev*. 2013;24(1):23-40.
2. Bernhagen J, Calandra T, Bucala R. Regulation of the immune response by macrophage migration inhibitory factor: biological and structural features. *J Mol Med (Berl)*. 1998;76(3-4):151-61.
3. Zheng Y, Wang Q, Li T, et al. Role of Myeloma-Derived MIF in Myeloma Cell Adhesion to Bone Marrow and Chemotherapy Response. *J Natl Cancer Inst*. 2016;108(11).
4. Schwartz V, Lue H, Kraemer S, et al. A functional heteromeric MIF receptor formed by CD74 and CXCR4. *FEBS Lett*. 2009;583(17):2749-57.
5. Shi X, Leng L, Wang T, et al. CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity*. 2006;25(4):595-606.
6. Fingerle-Rowson G, Petrenko O. MIF coordinates the cell cycle with DNA damage checkpoints. Lessons from knockout mouse models. *Cell Div*. 2007;2:22.
7. Lue H, Thiele M, Franz J, et al. Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. *Oncogene*. 2007;26(35):5046-59.
8. Burton JD, Ely S, Reddy PK, et al. CD74 is expressed by multiple myeloma and is a promising target for therapy. *Clin Cancer Res*. 2004;10(19):6606-11.
9. McClelland M, Zhao L, Carskadon S, Arenberg D. Expression of CD74, the receptor for macrophage migration inhibitory factor, in non-small cell lung cancer. *Am J Pathol*. 2009;174(2):638-46.
10. Young AN, Amin MB, Moreno CS, et al. Expression profiling of renal epithelial neoplasms: a method for tumor classification and discovery of diagnostic molecular markers. *Am J Pathol*. 2001;158(5):1639-51.
11. Tamori Y, Tan X, Nakagawa K, et al. Clinical significance of MHC class II-associated invariant chain expression in human gastric carcinoma. *Oncol Rep*. 2005;14(4):873-7.
12. Datta MW, Shahsafaei A, Nadler LM, Freeman GJ, Dorfman DM. Expression of MHC class II-associated invariant chain (Ii;CD74) in thymic epithelial neoplasms. *Appl Immunohistochem Mol Morphol*. 2000;8(3):210-5.
13. Kaufman JL, Niesvizky R, Stadtmauer EA, et al. Phase I, multicentre, dose-escalation trial of monotherapy with milatuzumab (humanized anti-CD74 monoclonal antibody) in relapsed or refractory multiple myeloma. *Br J Haematol*. 2013;163(4):478-86.
14. Stein R, Mattes MJ, Cardillo TM, et al. CD74: a new candidate target for the immunotherapy of B-cell neoplasms. *Clin Cancer Res*. 2007;13(18 Pt 2):5556s-63s.
15. Stein R, Smith MR, Chen S, Zalath M, Goldenberg DM. Combining milatuzumab with bortezomib, doxorubicin, or dexamethasone improves responses in multiple myeloma cell lines. *Clin Cancer Res*. 2009;15(8):2808-17.
16. Winner M, Meier J, Zierow S, et al. A novel, macrophage migration inhibitory factor suicide substrate inhibits motility and growth of lung cancer cells. *Cancer Res*. 2008;68(18):7253-7.
17. Leng L, Chen L, Fan J, et al. A small-molecule macrophage migration inhibitory factor antagonist protects against glomerulonephritis in lupus-prone NZB/NZW F1 and MRL/lpr mice. *J Immunol*. 2011;186(1):527-38.
18. O'Connell FP, Pinkus JL, Pinkus GS. CD138 (syndecan-1), a plasma cell marker immunohistochemical profile in hematopoietic and nonhematopoietic neoplasms. *Am J Clin Pathol*. 2004;121(2):254-63.
19. Kline M, Donovan K, Wellik L, et al. Cytokine and chemokine profiles in multiple myeloma; significance of stromal interaction and correlation of IL-8 production with disease progression. *Leuk Res*. 2007;31(5):591-8.
20. Zipin-Roitman A, Meshel T, Sagi-Assif O, et al. CXCL10 promotes invasion-related properties in human colorectal carcinoma cells. *Cancer Res*. 2007;67(7):3396-405.

21. Fusek M, Vetvickova J, Vetvicka V. Secretion of cytokines in breast cancer cells: the molecular mechanism of procathepsin D proliferative effects. *J Interferon Cytokine Res*. 2007;27(3):191-9.
22. Yamamoto T, Kimura T, Ueta E, Tatemoto Y, Osaki T. Characteristic cytokine generation patterns in cancer cells and infiltrating lymphocytes in oral squamous cell carcinomas and the influence of chemoradiation combined with immunotherapy on these patterns. *Oncology*. 2003;64(4):407-15.
23. Merico F, Bergui L, Gregoretti MG, et al. Cytokines involved in the progression of multiple myeloma. *Clin Exp Immunol*. 1993;92(1):27-31.
24. Atanackovic D, Hildebrandt Y, Templin J, et al. Role of interleukin 16 in multiple myeloma. *J Natl Cancer Inst*. 2012;104(13):1005-20.
25. Piddock REA-A, A.M,; Auger, M.J; Bowles, K.M.; Rushworth, S.A. Macrophage Migration Inhibitory Factor Drives Multiple Myeloma IL-6/8 Pro-survival signals in the Tumor Microenvironment. 2015.
26. Alexandrakis MG, Passam FH, Sfiridaki K, et al. Clinical significance of circulating endothelial adhesion molecules (sE-selectin and sICAM) in untreated multiple myeloma patients. *Clin Chim Acta*. 2004;349(1-2):39-43.
27. Alexandrakis MG, Passam FH, Kyriakou DS, et al. Serum level of interleukin-16 in multiple myeloma patients and its relationship to disease activity. *Am J Hematol*. 2004;75(2):101-6.
28. Purdue MP, Lan Q, Menashe I, et al. Variation in innate immunity genes and risk of multiple myeloma. *Hematol Oncol*. 2011;29(1):42-6.
29. Hayashi K, Nomura, S., Hashimoto, S., Hideshima, T. CCL5 (RANTES) Is Suspected to be the Key Chemokine to Understand the Bone Pathology of Multiple Myeloma. *Blood*. 2008.
30. Liu M, Guo S, Stiles JK. The emerging role of CXCL10 in cancer (Review). *Oncol Lett*. 2011;2(4):583-9.



Appendix Figure 1. Raw Values of CD138+ RPMI8226 cells after for 4 days with and without treatment of 60μM 4-IPP. Sorted CD138+ cells were plated in complete media with 4-IPP (green) or without 4-IPP (red) for four days. Blue bars are cytokines in media alone without cells. Of the prior 7 cytokines (yellow arrows) that were detected at high relative levels compared to the other measured cytokines in the media of CD138+ and CD138- RPMI8226 cells, only RANTES is completely undetectable in media of CD138+ RPMI8226 cells after MIF-1 inhibition (N=1).