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BLOCKING LACTATE TRAFFICKING IN MULTIPLE MYELOMA MICROENVIRONMENT MAY BE A POTENTIAL TARGET FOR ANTI-CANCER THERAPY

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1 INTRODUCTION

1.1 Pathophysiology of Multiple Myeloma

Multiple myeloma (MM) is a hematological malignancy of differentiated plasma cells caused by the uncontrolled proliferation of monoclonal plasma cells (PCs) in the bone marrow (BM), leading to the accumulation of non-functional immunoglobulins or immunoglobulin chains known as M proteins (1). MM represents the second most common hematological malignancy after non-Hodgkin lymphoma (2) accounting for about 10% of all blood cancers and males are affected more than females (3). In Western countries, the annual incidence is 5.6 cases per 100,000 people (4). Diagnosis occurs at median age of about 70 years; 37% of patients are younger than 65 years old, 26% are between the ages of 65 and 74 years old and 37% are 75 years of age or older (4)(5).

Insight into B cell development and plasma cell biology is essential for understanding multiple myeloma. Plasma cells develop from haematopoietic stem cells, which undergo several rounds of differentiation in the bone marrow and secondary lymphoid organs to B cells and eventually to plasma cells. In the bone marrow, immature B cells undergo V(D)J rearrangement, a process that generates their diverse primary immunoglobulin repertoire (6). B cells with a IgH–IgL complex (B cell receptor) on cell surface migrate to secondary lymphoid organs, such as lymphnodes or spleen. In these secondary lymphoid organs, B cells undergo several processes (affinity maturation, somatic hypermutation and class-switch recombination) that result in the production of antibodies, which have a high affinity for specific antigens and with different functional properties. Double-strand DNA breaks in the immunoglobulin loci are needed for recombination and somatic hypermutations. However, these DNA breaks can fuse with other breaks that occur in the genome, leading to aberrant fusions of DNA and chromosomal translocations. However, translocations involving specific oncogenes can give cells a growth advantage, which could lead to the development of pathological states, such as MGUS, SMM and eventually MM. Thus, chromosomal translocations are a possible initiating event for a subset of multiple myeloma cases.

Models of multiple myeloma development have contributed to our understanding of this disease (7)(8). Translocation t(11;14), which is found in 14% of all patients with multiple myeloma, results in increased expression of CCND1, whose product, cyclin D1, important for cell cycle progression. Other chromosomal defects observed in patients with multiple myeloma include loss of the short arm of chromosome 1 (del(1p)), gain of the long arm of chromosome 1 (gain(1q)), deletion of the long arm of chromosome 13 (del(13q)) and loss of the short arm of chromosome 17 (del(17p)) (9)(10). Epigenetic defects studied in MM include altered DNA methylation, chromatin structure and miRNA deregulation. Levels of hypermethylation are similar in MGUS and multiple myeloma, whereas levels of hypomethylation are increased in multiple myeloma, suggesting that this might play a part in disease development (11)(12).

The development of MM is not only due to uncontrolled proliferation of plasma cells (PCs), but also to changes in the bone marrow (BM) microenvironment (13), which supports and regulates tumor proliferation, survival, and migration of PCs. In the BM, PCs are hosted in special niches and receive multiple signals that maintain their long survival and exert a protective effect on drug-induced apoptosis, due to the secretion of soluble factors, such as IL-6 and extracellular vesicles (14-16).

In 2003, the International Working Group on Myeloma has established diagnostic criteria to classify the different stages in the development of multiple myeloma based on: proportion of plasma cells in bone marrow, M protein in serum and presence or absence of end-organ damage (17).

	MGUS	Smoldering myeloma	Symptomatic multiple myeloma
Proportion of plasma cells in bone marrow	<10%	≥ 10%	≥ 10%
M protein in serum	<30 g/L	≥ 30 g/L	Detectable in serum and/or urine
End-organ damage (CRAB)	No	No	Present

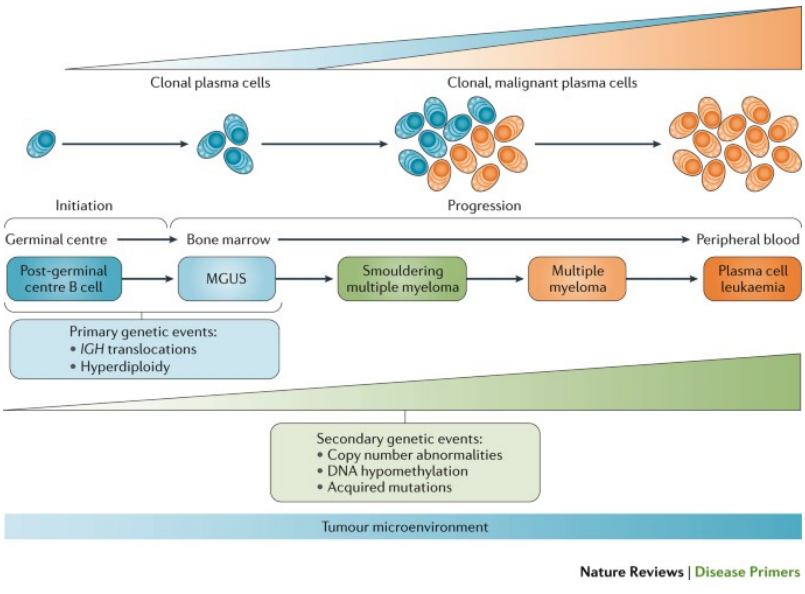
CRAB criteria: hypercalcemia, renal insufficiency, anemia, bone lesions
MGUS, monoclonal gammopathy of uncertain significance

***Table 1**: Diagnostic criteria of the International Myeloma Working Group (Table from: The Diagnosis and Treatment of Multiple Myeloma)*

The clinical manifestations of disease include signs of organ damage, which are collectively known as CRAB features (18):

- **Calcaemia**: it occurs in approximately 30% of patients; widespread bone destruction induced by tumor is the primary cause of the hypercalcemia (19).
- **Renal insufficiency**: this complication is present in 20 – 40% of patients; the main causes of renal failure are the accumulation of monoclonal light chains in distal and collecting renal tubules and hypercalcemia (20).
- **Anaemia**: it is diagnosed in 73% of patients and it is a consequence to the suppression of erythropoiesis by cytokine (21).
- **Bone disease**: it shows as lytic lesions or osteopenia and is usually combined with severe pain, pathological fracture, spinal cord compression, vertebral collapse; osteolytic bone disease is a consequence of bone homeostasis perturbation, which is physiologically regulated by osteoblasts and osteoclasts (22).

Myeloma evolves from an asymptomatic pre-malignant stage of plasma cells proliferation termed monoclonal gammopathy of undetermined clinical significance (usually known as MGUS) that can progress to an intermediate but more advanced pre-malignant stage defined smouldering myeloma (SMM) and, finally, to symptomatic myeloma (23).

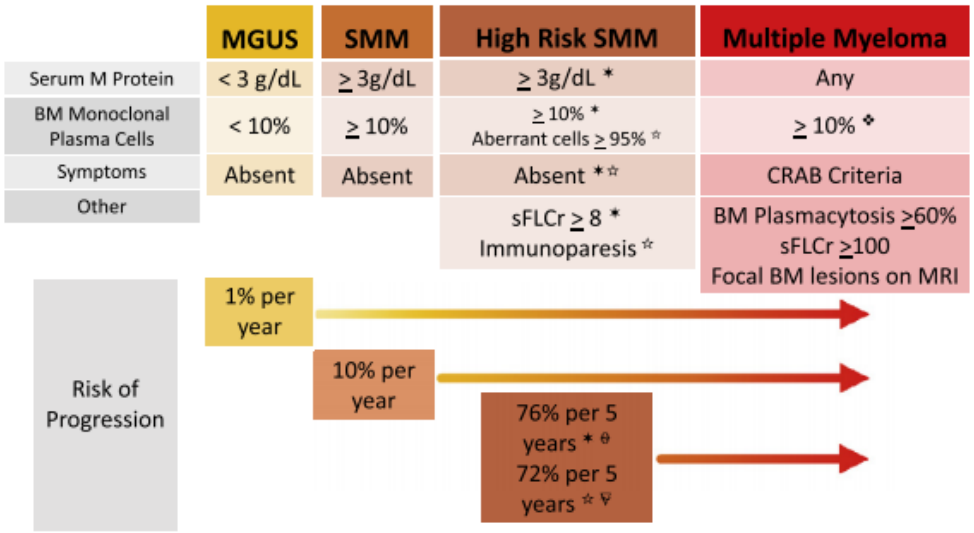


Picture 1: *The development of multiple myeloma is a multistep process, which starts with precursor disease states, such as monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM) and progresses to symptomatic myeloma (MM). (Image from: Kumar, S. et al. Multiple myeloma. Nat Rev Dis Primers 3, 17046 (2017)).*

MGUS patients are characterized by a serum M-protein concentration < 3 g/dL, <10% clonal plasma cells in the bone marrow and the absence of any organ damage (24). This disorder can evolve into symptomatic myeloma and overall rate of malignant progression to myeloma is about 1% of patients per year (25). The prevalence of MGUS increases with advancing age, with 3.2% of cases presenting in persons aged over 50 years, and 5.3% of cases in persons aged over 70 years (26).

The rate of progression to malignant disease for SMM patients is much higher, approximately 10% per year in the first 5 years after diagnosis (27). SMM is defined by laboratory criteria such as monoclonal protein level of > 3 g/dL or a clonal marrow plasmacytosis of 10%–60% and the absence of end-organ damage (2).

MM diagnosis requires the presence of one or more myeloma defining events (MDE) and 10% or more PCs on bone marrow examination or a biopsy proven plasmacytoma; MDE includes the CRAB criteria and three specific biomarkers: clonal bone marrow plasma cells ≥60%, serum free light chain (FLC) ratio ≥100 (provided involved FLC level is ≥100 mg/L), and more than one focal lesion on MRI studies (28).



Picture 2: *Diagnosis and progression of multiple myeloma. (Image from State of science in smoldering myeloma: Should we be treating in the clinic?)*

1.2 Current treatment strategies for Multiple Myeloma

Survival of MM has improved strongly in the last two decades (29) thanks to many promising options for treatment, including proteasome inhibitors (PIs), immunomodulatory drugs (IMiDs) and monoclonal antibodies (30,31). Bortezomib (BTZ) is a dipeptidyl boronic acid that selectively inhibits the ubiquitin proteasome pathway, which plays a role in the degradation of many intracellular proteins. It is the first-in-class selective and reversible inhibitor of the 26S proteasome: a multisubunit protein that degrades proteins involved in multiple cellular processes, including cell-cycle regulation, transcription factor activation, and apoptosis (32). The sensitivity of multiple myeloma cells to the proteasome inhibitors is related to the balance between the load and the capacity of the proteasome. Indeed, plasma cells are antibody- producing cells, so they have a physiological induction of the unfolded protein response to accommodate for antibody production. Bortezomib treatment has been associated with induction of mitochondrial depolarization and apoptosis (33). BTZ realizes its antiproliferative and antitumor activity by inhibiting the proteasomal degradation of several ubiquitinated proteins. Bortezomib toxicity in myeloma PCs has also been associated with the inhibition of nuclear factor-κB activity, accumulation of misfolded proteins, the activation of c-Jun N-terminal kinase, stabilization of cell-cycle inhibitors (34) and induction of mitochondrial depolarization.

IMiDs are a group of compounds that includes thalidomide and its analogues Lenalidomide and Pomalidomide; they have pleiotropic antimyeloma properties including immune-modulating, anti-angiogenic, anti-inflammatory and antiproliferative effects (35).

Surface antigen CD38 is expressed on the malignant PCs of patients with MM (36) and the monoclonal antibody daratumumab was the first anti-CD38 approach to be approved by the FDA for relapsed/refractory MM (37). Daratumumab causes cell death through different mechanisms including complement-dependent and antibody-dependent cell-mediated cytotoxicity, antibody-dependent cellular phagocytosis, and apoptosis (38).

Elotuzumab, the second monoclonal antibody approved for treatment of MM, targets SLAMF7, activating antibody cell-mediated cytotoxicity, enhancing the cytotoxicity of NK cells, and inhibiting the interaction of myeloma cell with bone marrow stromal cells (39).

Autologous stem cell transplantation (ASCT) remains efficacious in eligible MM patients. this therapeutic approach occurs after treatment with varying combinations of proteasome inhibitors, alkylating agents, IMiDs, steroids and most recently, monoclonal antibodies until a maximal response is achieved (40).

Despite the therapeutic progress achieved with the introduction of the PIs, immunomodulators and monoclonal antibodies, MM remains a non-curable disease due to intrinsic or acquired primary and secondary drug resistance (41). Nearly all MM patients eventually relapse and approximately one third of them develops resistance to BTZ (42). Remission can be regained, but the depth and duration of response to subsequent lines of therapy diminishes with each relapse which also tend to be progressively more aggressive, ultimately culminating in refractory disease to all available treatments (43). Resistance has been attributed to several factors, including genetic mutations, clonal evolution of MM cells, and bone marrow microenvironment changes. Therefore, a better understanding of the disease biology including microenvironment interactions could facilitate deeper and longer remissions and even provide a potential for cure.

1.3 Microenvironment: role in tumor progression

MM tumor microenvironment is a heterogeneous tissue where PCs are physically constrained in a hypoxic niche containing mesenchymal stromal cells (MSCs), osteoblasts (OBs), osteoclasts (OCs), and immune cells (e.g. T effector, T regulatory cells, B lymphocytes, NK, myeloid derived suppressor cells (MDSC) and macrophages) (44). The bidirectional interaction between MM cells and surrounding cells transforms the host BM microenvironment into a tumor promoting and immune-suppressive milieu (45) playing a pivotal role for PCs growth, proliferation, survival, migration, and drug resistance.

The importance of tumor microenvironment is strongly confirmed by the low capacity of MM PCs to grow in a medium without a stromal support and by the fact that PCs from patients affected by refractory myeloma show drug sensitivity when cultured *in vitro* (46).

Haematopoietic cells such as B cells, T cells, natural killers, myeloid-derived suppressor cells (MDSCs), osteoclasts (which have a role in bone resorption) and non-hematopoietic cells, including bone marrow stromal cells and osteoblasts secrete several factors which contribute to proliferation of multiple myeloma cells, and can also contribute to bone damage. Both increased activity and number of osteoclasts and reduced activity and number of osteoblasts cause bone disease in patients with multiple myeloma. Indeed, the interaction of multiple myeloma cells with bone marrow stromal cells and osteoblasts causes increased production of RANKL and reduced levels of osteoprotegerin (47). RANKL binds to RANK (receptor activator of NF-Kb) which is expressed by preosteoclasts, resulting in increased differentiation to osteoclasts. Imbalance in the number and activity of osteoclasts and osteoblasts results in bone destruction and the development of bone disease (48). Also, factors produced in the microenvironment can be associated with angiogenesis. Indeed, vascular endothelial growth factor A (VEGFA), which is produced by bone marrow stromal cells, is a strong angiogenic factor, resulting in increased oxygen supply through increased and local abundance of blood vessels (49).

Upon stabilization of the MM niche, malignant plasma cells reprogram the local BM microenvironment, either by direct contact with stromal, endothelial or osteolineage cells, or involving the stimulation of supportive cytokines for MM cells, and by facilitating immune evasion through the expansion of immunosuppressive subsets such as myeloid derived suppressor cells (MDSCs) and Treg (50,51). These responses provide further expansion signals for MM cells, which become gradually independent from initial normal niche support, leading to the generation of a favourable TME.

1.4 Metabolic features of multiple myeloma

1.4.1 Role of Warburg effect

Reprogramming of tumor microenvironment by malignant PCs leads to cancer niche formation, tumor growth (52,53), promotes resistance to anticancer drugs (54,55) and maintains an immunosuppressive milieu (56,57). In this scenario, heterocellular communication and dysregulation of critical signalling axes, including metabolic pathways, are among the major contributors to progression and treatment failure.

MM is one of the numerous tumors characterized by increased glucose consumption (58), even in the presence of oxygen (Warburg effect), with a marked increase in lactate production in correlation with expression of the Lactate dehydrogenase (LDH) gene (59). Elevated LDH levels in MM patients correlate with advanced disease and inferior overall survival, even when they belong to a low or intermediate International Staging System (ISS) subgroup (60). LDH is comprised of two major subunits, LDH-A and LDH-B, which reversibly catalyses the conversion of pyruvate to lactate (LDH-A) or lactate to pyruvate (LDH-B). Elevated LDH-A is a negative prognostic biomarker not only because it is a key enzyme involved in cancer metabolism, but also because it allows neoplastic cells to suppress and evade the immune system by altering the tumor microenvironment.

Cancer cells might have a preference to produce energy reserves *via* mitochondrial oxidative phosphorylation (OXPHOS) rather than high glycolysis according to their surrounding condition (61). In tumor niche, a reverse Warburg effect has been recently described, consisting in metabolic changes occurring in stromal cells in the attempt to metabolically support adjacent cancer cells (62). The reverse Warburg effect explains the metabolic flexibility of cancer cells which educate MSCs to enhance aerobic glycolysis and produce lactate, which is converted to pyruvate and utilized for mitochondrial OXPHOS in cancer cells (63,64). As result, tumor cells and MSCs influence each other in energy metabolites for co-evolution in cancer progression. The increased glycolytic metabolism of MM-MSCs could contribute to high lactate secretion by which MSCs sustain PCs growth, drug resistance and favour immune escape.

1.4.2 Lactate-dependent regulation of immune response

Lactate is the ionic form of lactic acid, an organic acid physiologically produced in cells as a result of anaerobic fermentation of pyruvate following intense muscular exercise. The reason why this metabolite is produced is to regenerate NAD+ by reducing pyruvate with the aim to fuel glycolysis, a process which otherwise, in absence of oxygen, would stop, leading cells to energy depletion and death. Despite lactate was first recognized only as a waste product of anaerobic cell metabolism, it is now known that lactate can be incorporated into the tricarboxylic acid (TCA) cycle and be a source of energy. Moreover, lactate can act as an oncometabolite with signalling properties involved in sophisticated mechanisms that shutdown anti-tumor immune response (65). Physiological circulating lactate levels range from 0.5 to 2 mM and can increase up to 10 to 25 mM after intense exercise (66, 67) or during pathophysiologic conditions, where plasma lactate levels may increase to levels above 4 mM (68, 69). High glycolytic and lactate production rates have also been observed during pro-inflammatory responses (70). In human melanoma high lactate levels lead to rapid changes in immune cells metabolic pathways impairing the activity of effector T cells (T helper, T cytotoxic and T regulatory cells) (71). Naïve T cells are small and quiescent cells that require relatively small amounts of glucose, amino acids and fatty acids to maintain basic energetic and biosynthesis demands. Encounter with antigens triggers T cell activation and differentiation into effector cells (Teff), which rely on high intake of glucose and amino acids to support proliferation and effector functions such as cytotoxicity and cytokine production (72). While activated CD8+ T cells and CD4+ T cells require high levels of glucose metabolism, Treg cannot rely on high rates of glucose and can efficiently survive under high lactate conditions metabolizing it through the tricarboxylic acid cycle (TCA) to fuel their OXPHOS metabolism thus establishing an immunosuppressive environment (73-75). Increased lactate concentration also inhibits dendritic cells (DCs) function impairing their antigen presentation and induces a state of anergy in natural killer (NK) cells (76)(77). As reported in breast cancer, high lactate production also affects macrophages function inducing their polarization toward the M2 phenotype and arginase-1 expression (78).

Lactate can be taken up by cells via proton-couple transmembrane solute carriers called MCTs and might thereby directly affect cellular metabolism and function. Expression and modulation of MCTs have been shown to be important for immune cell function in T cells and macrophages (79,80) suggesting direct effects of lactate on cellular metabolism that might contribute to modulation of immune cell function.

Blocking lactate trafficking has been used to improve immune therapy in solid cancer (81), but it is still poorly investigated in the context of haematological malignancies.

1.4.3 Mitochondrial fitness and metabolism affect drug-efficacy in multiple myeloma

Growing evidence indicates that the metabolic reprogramming required in cancers to fuel the increased energy demand is coupled to the increased ability to evade apoptosis (82,83). Modulating ATP availability might be an essential strategy in inducing cell resistance and sustaining cancer progression and growth (83). Recent findings have demonstrated that cancer cells, including leukemias, lymphomas, pancreatic ductal adenocarcinoma, melanoma, and endometrial carcinoma, take advantage from high OXPHOS (84-95), while mitochondria can modulate their morphology regulating the intrinsic apoptotic pathway and participating in the resistance of cancer cells to apoptotic stimuli (96-106). Mitochondrial fitness may, at least in part, drive chemoresistance in cancer (107). Accordingly, induction of an energy metabolism shift toward a low oxidative phosphorylation system (OXPHOS), by targeting mitochondrial functions, markedly enhanced the antileukemic effects of chemotherapy in acute myeloid leukemia (108). Bioenergetic changes have also been implicated in the PI resistance of MM cells (109,110). These adaptations are characterized by an increase in mitochondrial biomass and an increased reliance on mitochondrial respiration (110). Increased OXPHOS has been found in MM cells from both relapsed and drug resistant patients (111). Recently, our group characterized the biochemical, metabolic and molecular features of a U266 clone resistant to BTZ (U266-R), both under resting conditions and after BTZ exposure (107,112).

In comparison to the sensitive clone (U266-S), U266-R based their resistance to BTZ on changes of various genes and protein expressions, several of which are involved in mitochondrial dynamics and protein glycosylation. Metabolically, U266-R are characterized by high levels of triphosphate nucleosides (ATP, UTP, GTP, CTP), ATP/ADP ratio and compounds of the hexoseamine biosynthetic pathway (HBP) indicating, respectively, sustained energy metabolism, efficient mitochondrial phosphorylating capacity and a high rate of protein glycosylation (113).

Mitochondrial homeostasis is assured by two critical processes which are mitochondrial dynamics (repetitive cycles of fusion and fission) and mitophagy (114). Mitochondrial dynamics concerns to continual cycles of fusion and fission between mitochondria (115-117). These oppose processes are responsible for the architecture of the mitochondrial population of the cell and impact many aspects of mitochondrial functions, including respiration, calcium buffering, and apoptosis (118-122). Mitochondrial fusion results in a hyperfused network to counteract metabolic insults, preserve cellular integrity, and protect against autophagy (123). Unopposed fission induces mitochondrial fragmentation, which is generally related with metabolic dysfunction and disease. The interplay between mitochondrial dynamics (repetitive cycles of fusion and fission) and mitophagy assures cell homeostasis and is utilized by malignant PCs to promote drug resistance. Indeed, PI-resistant PCs activate mitochondrial fusion mechanisms and mitochondrial biogenesis, as demonstrated by mitofusin, optic atrophy 1 (OPA-1) and mitochondrial transcription factor A (TFAM) upregulation after exposure to BTZ. In particular, the upregulation of OPA1 impacts mitochondrial energy production favouring the remodelling of mitochondrial cristae and regulating ETC-linked OXPHOS (113). In addition, mitofusin prevents removal of impaired mitochondria allowing them to reengage in the fusion process despite their deprived energetic state so improving mitochondrial activity (124).

1.4.4 Monocarboxylate transporters (MCTs) and role of MCT1 and MCT4 in lactate shuttle in cancer cells

Lactate transport from glycolytic cells to oxidative cells is achieved in part by the cell-type specific expression MCTs (125).

Under physiological conditions, MCT1-4 cooperate to form a lactate shuttling system that maintains lactate homeostasis between glycolytic and oxidative cells (126). MCT1 is the most suitable for cell respiration because of its affinity with lactate.

MCTs are transmembrane proteins belonging to solute carrier (SLC) 16A family and are involved in the proton-coupled influx-efflux mechanisms of monocarboxylates like lactate and pyruvate (127). Due to their role in the transport of monocarboxylates, both across the plasma membrane and the mitochondrial membrane, members of the MCT family contribute to the functioning of vital metabolic pathways. The SLC16A family comprises 14 members, of which only MCT1 to MCT4 are characterized biochemically and play an important role in cancer (128).

Among MCT1 and MCT4, the latter has lower affinity for lactate, and it is responsible for efflux of glycolysis-derived lactate from hypoxic cells. In contrast, MCT1 has a higher affinity for lactate and is responsible for lactate uptake in normoxic tumour cells. To avoid lactate-mediated intracellular acidification induced cell death, the lactate is then effluxed by MCT4 from hypoxic cells and subsequently these exported lactate molecules are then taken up by the well-oxygenated cells through MCT1, utilizing this imported lactate as a substitute fuel for energy production (129,130). In tumor microenvironment, in which stromal cells (MSCs) produce high lactate levels and tumor cells uptake it (reverse Warburg effect), MSCs and tumor cells are characterized by high MCT4 and MCT1 expression respectively (131).

Overexpression of MCT1 and MCT4 have been reported in different types of cancers including breast cancer, colon cancer, pancreatic cancer, glioblastoma, prostate cancer and renal cell carcinoma, which then contribute to the development of multi-drug resistance (MDR) (132).

The transport of monocarboxylates take place through facilitative diffusion wherein protons are co-transported in order to maintain the intracellular neutral pH (132,133).

Hypoxic cancer cells with enhanced expression of GLUT1 overcomes ATP deficiency by accelerating glucose uptake and thereby increasing the overall ATP production. HIF-1 also facilitates the conversion of glucose to pyruvate, later converted to lactate by a well-expressed enzyme lactate dehydrogenase (LDH) (134). Aerobic tumor cells contain lower levels of HIF-1, leading to inefficient glycolysis. Hence, to meet the energy requirements, these cells use lactate produced from the hypoxic cells and oxidize it into pyruvate by LDH-1 with simultaneous reduction of NAD+ to NADH. The resulting pyruvate and NADH then enters the TCA cycle and ultimately undergoes OXPHOS for ATP generation. For oxygenated cells oxidative lactate metabolism could be more advantageous than the aerobic glycolysis as it results in up to 7.5 times more ATP produced (135). As glucose consumption of normoxic cells is reduced, more glucose becomes available to the hypoxic cells. This symbiosis developed between tumor cells enhance their survival and proliferation irrespective of oxygen availability.

The tumor survival is dependent on lactate shuttle between the cells and the transporters that mediate this transfer are MCT1 and MCT4. Therefore, these two MCTs are potential targets for anti-cancer therapy, which can be knocked down alone or simultaneously by chemotherapy, or in combination with radiotherapy (136). The rationale is that if the export of lactic acid in cancer cells is blocked or decreased, the resultant intracellular acidification will kill the cells. Several studies have provided proof-of-principle for this therapeutic approach, where selective inhibitors of MCT1/4 have been shown to have potency in cancer treatment in preclinical studies (137-139).

Specific inhibitors with greater inhibitory potency and selectivity towards MCT1 have been developed and these have been shown to influence lactate transport. Recent improvements on these compounds have resulted in the generation, by AstraZeneca, of AZD3965 (140). This compound selectively inhibits MCT1 with a binding affinity 1.6 nM and is currently in phase I clinical trials for treatment of solid tumors and B-cell lymphomas (140,141).

1.4.5 Lactate as an extracellular messenger: involvement of GPR81

The emergence of lactate as a signaling molecule has come full circle with the discovery of the lactate receptor GPR81 in the plasma membrane of adipocytes (142). GPR81 expression is also found in muscle cells, central nervous system, immune cells, endothelial cells, dendritic cells, macrophages and recently has been discovered also in tumor cells. GPR81 belongs to a subfamily of protein Gi/o-coupled receptors (GPCRs), termed the hydroxy carboxylic acid receptors (HCARs), consisting of three members: HCAR1 (GPR81), HCAR2 (GPR109A), and HCAR3 (GPR109B) (143). GPR81 is believed to be the most evolutionarily conserved of the three receptors, and it is found in all mammals and fish while GPR109A is found only in higher mammals. The HCAR family members are unique because they recognize metabolic intermediates as their endogenous agonists for which they show relatively low affinity (low to high millimolar range) in comparison to members of other GPCR families (143). The endogenous ligand for GPR81 is L-lactate (EC50 = ~ 5 mM), but it is also known that 3,5 dihydroxybenzoic acid (3,5 DHBA) activates the receptor although 3,5 DHBA is not physiologically present in circulation. For this reason, 3,5 DHBA is considered as a pharmacologic agonist of the receptor. As the cell-surface receptor for lactate, GPR81 might have an important role in tumor growth based on the fact that cancer cells generate lactic acid as the end product of glycolysis and export it out to the extracellular milieu. This role could be autocrine or paracrine where extracellular lactate acts on GPR81 expressed on tumor cells themselves or on surrounding cells promoting tumor growth, metastasis, angiogenesis, immune evasion and chemoresistance (144). GPR81 was shown to be upregulated in many cancers such as pancreatic adenocarcinoma (145), breast cancer (145-147), hepatocellular carcinoma, cervical squamous-cell carcinoma, and lung cancer (148,149) but very little is known about the effects of GPR81 activation in hematological malignancies, including MM. In theory, selective high-affinity antagonists of GPR81 are potential anticancer drugs. At present, only few blockers are available for testing the validity of this strategy.

Several studies provide overwhelming evidence that 3-hydroxybutiric acid (3-OBA) antagonizes the cellular effects of extracellularly applied lactate or those of 3,5-DHBA (150) in various model systems. In particular, it has been demonstrated that 3-OBA mimics the effect of GPR81 knockout (151). In support of using GPR81 inhibitors as anticancer molecules, it has been demonstrated that GPR81-null mice do not have any detrimental phenotype, suggesting that pharmacological blockade of the receptor as a strategy to treat cancer may limit any significant unwanted side effects. The blockade of GPR81, either with small-molecules or with antibodies, could also be used in combination with any of the currently available anticancer approaches.

1.4.6 Aim of the study

In this work we firstly aim to measure lactate concentration in MGUS, SMM and MM patients in order to evaluate whether patients at different stage of disease progression show any differences in circulating lactate levels. Our second objective is to evaluate MCT1 channel expression and lactate effects on HMCLs, particularly focusing on mitochondrial activity and resistance to PIs such as BTZ and CFZ in presence or not of MCT1 channel inhibitor AZD3965 and GPR81 inhibitor 3-OBA to understand the different involvement of MCT1 channel- and lactate/GPR81 pathway in drug resistance. Finally, since it has been reported that high lactate production in tumor microenvironment may affect antitumor immune response (152-157), we evaluated the effects of lactate on immune cells with a particular focus on the role of lactate in the establishment of an immunosuppressive milieu in MM.

2 MATHERIALS AND METHODS

2.1 Cell cultures and treatments

Human myeloma cell lines (HMCLs) were cultured in RPMI 1640 medium supplemented with 10% (OPM2, NCI–H929) or 20% (U266) FBS and 1% penicillin/streptomycin at 37 °C and 5% CO2. PBMCs were obtained by healthy donor buffy coat after separation by Ficoll-Hypaque gradient and cultured for 48h in RPMI-1640 medium supplemented with 10% FBS and 1% Penicillin/streptomycin in presence of 5 mg/ml Phytohemagglutinin (PHA) at 37 °C and 5% CO2 (13).

Cocultures of MM cell lines with PBMC were set using a 2:1 PCs/PBMCs ratio while cocultures of MM cell lines with HS-5 stromal cell lines were set using a 1:5 HS-5/PCs ratio. PBMCs for the measurement Treg and M-MDSC percentage were seeded in 6-well plates at a concentration of 5 x 10^5 cells/well.

Based on the previous literature data, 15 nM (U266) or 20 nM (NCI-H929) BTZ (Takeda, Rome, Italy) and 5nM CFZ (Amgen) were used. Used doses of Sodium lactate (Sigma-Aldrich), AZD3965 (Astra Zeneca), 3-OBA(Sigma-Aldrich) and 3,5 DHBA(Sigma-Aldrich) were respectively 20 mM, 10μM, 3mM and 150 μM.

2.2 Samples collection

After written informed consent, samples were collected from healthy donors (HD) at Division of Hematology, AOU Policlinico – OVE, University of Catania. Peripheral blood mononuclear cells (PBMC) were obtained after density gradient centrifugation on Ficoll and cultured in RPMI medium supplemented with 10% heat- inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% L-glutamine.

2.3 Lactate concentration measurement

After deproteinization, lactate concentration in sera was measured using a colorimetric assay (sigma-aldrich) and comparing results with a standard curve of known concentrations. Before starting the test, standards were prepared using scalar concentrations of lactate whilst samples were diluted 1:10 in distilled water. In each of the diluted sample 195 µL aqueous mix containing 17Mm Aminoantipyrine (AAP), 15mM TOOS, a hydrogen donor, 0.5 U/L peroxidase (POD) and 1M TRIS-HCl pH 8 were added. Background absorbance (Blank) of each well was read by measuring the absorption of light at 545 nm using a spectrophotometer. After the first reading, 2.5 mU/L Lactate oxidase (LOD), the limiting enzyme, were added to each sample and the solution was incubated at room temperature. The addition of LOD triggers the following reaction:

$$\text{L - Lactate} + O_2 \xrightarrow[\text{Oxidase}]{\text{Lactate}} \text{Pyruvate} + H_2O_2$$

$$2\,H_2O_2 + \text{TOOS} + 4\,\text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

After 30 minutes incubation, quinonimine (a violet chromogen) absorbance was read at 545 nm. Subtracting the blank absorbance of each well from the absorbance value after 30 minutes and comparing it with standard curve, it was possible to obtain lactate concentration in each sample considering the initial dilution factor.

2.4 Flow cytometry

Evaluation of apoptosis was performed by flow cytometry. Samples were washed and resuspended in 100 µL of phosphate-buffered saline (PBS). 1 µL of Annexin V-FITC solution and 5 µl of Propidium Iodide (Beckman Coulter, made in France) were added to cell suspension and mixed gently. Cells were incubated for 15 min in the dark. Finally, 400 µl of 1X binding buffer was added and cell preparation was analyzed by flow cytometry (MACSQuant Analyzer 10, Miltenyi Biotec).

In order to measure changes in the mitochondrial mass, cells were reacted with 200 nM MitoTracker Red CMXRos probe (Thermo Fisher Scientific) for 30 min at 37 °C and then washed three times with 1 ml of PBS buffer, according to the manufacturer's instructions.

Reactive oxygen species (ROS) were detected using 2′,7′-dichlorodihydrofluorescein acetate (H2-DCF; Sigma-Aldrich, St. Louis, MO, USA), and fluorescence intensity was measured according to the fluorescence detection conditions of FITC.

A membrane potential probe, the 3,3′-Diethyloxacarbocyanine Iodide (DiOC2(3)), was used to evaluate the mitochondrial membrane potential. Cells were incubated with 10 µM DiOC2(3) (Thermo Fisher Scientific, Milan, Italy) for 30 min at 37 °C, washed twice, resuspended in PBS and analyzed by flow cytometry through the detection of the green fluorescence intensity of FITC.

For Treg analysis, immune cells were stained with CD4-PEVio770, CD25-APC and FOXP3-PE, all from Beckman Coulter, and Treg were defined as $CD4^{+}CD25^{high}Foxp3^{+}$. For monocytic myeloid suppressor cells (M-MDSCs) analysis, immune cells were stained with CD14-FITC and HLA-DR-APC, all from Beckman Coulter, and M-MDSCs were defined as $CD14^{+}HLA\text{-}DR^{-}$.

Samples were analyzed by using the flowcytometer MACSQuant Analyzer 10, Miltenyi Biotec.

2.5 Immunofluorescence assay

Paraffin sections from biopsy specimens from relapse/refractory MM patients (n=3) and early-stage MM patients (n=4) were deparaffinized and rehydrated as previously described [25]. Sections were permeabilized using 0.3% Triton X and blocked to prevent nonspecific antibody binding using a 0,3% Triton X-10% NGS solution. The slides were then incubated overnight at 4 °C with the primary antibodies mouse anti-MCT1 (Abcam) and rabbit anti-CD138 (Abcam) at 1:100 dilution in 0,3% Triton-X.

Subsequently, cells were washed three times in PBS for 5 min and then incubated for 1 h at room temperature with the appropriate combination of fluorescence conjugated secondary antibodies donkey polyclonal anti-rabbit Alexa Fluor 647 (A32849, Thermo Fisher Scientific; 1:500) and subsequently with goat polyclonal anti-mouse Alexa Fluor 488 (A21247, Thermo Fisher Scientific 1:500). Samples were then washed in 0.3% Triton X in PBS and nuclei were counterstained with DAPI (1:1000) for 5 min, at room temperature. Slices were mounted with fluorescent mounting medium Permafluor (Thermo Fisher Scientific) and digital images were acquired using a Zeiss Axio Imager Z1 Microscope with Apotome 2 system (Zeiss, Milan, Italy).

2.6 Western blot analysis

Briefly, for western blot analysis 50 μg of proteins were loaded onto a 12% polyacrylamide gel Mini-PROTEAN® TGXTM (BIO-RAD, Milan, Italy) followed by electrotransfer to nitrocellulose membrane Trans- Blot® TurboTM (BIO-RAD) using Trans- Blot® SE Semi-Dry Transfer Cell (BIO-RAD). Subsequently, membrane was blocked in Odyssey Blocking Buffer (Licor, Milan, Italy) for 1 h at room temperature. After blocking, membrane was washed three times in PBS for 5 min and incubated with primary antibodies against human MCT1 (Abcam), and β-actin (Abcam), overnight at 4 °C.

Next day, membranes were washed three times in PBS for 5 min and incubated with Infrared anti-mouse IRDye800CW (1:3000) and anti-rabbit IRDye700CW secondary antibodies (1:3000) in PBS/0.5% Tween-20 for 1 h at room temperature. All antibodies were diluted in Odyssey Blocking Buffer. The blots were visualized using Odyssey Infrared Imaging Scanner (Licor, Milan, Italy).

2.7 qPCR

After RNA extraction, reverse transcription was performed by using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Then the relative transcription of human genes ATP syntase (ATPsynt; Fw: AGCTCAGCTCTTACTGCGG Rw: GGTGGTAGTCCCTCATCAAACT) Cytocrome B (CytB; Fw: TCCTCCCGTGAGGCCAAATATCAT Rw:AAAGAATCGTGTGAGGGTGGGACT), MCT1 (Fw: TGTTGTTGCAAATGGAGTGT Rw: AAGTCGATAATTGATGCCCATGCCAA), MCT4 (Fw: TATCCAGATCTACCTCACCAC Rw: GGCCTGGCAAAGATGTCGATGA) and GPR81 (Fw: TTCGTATTTGGTGGCAGGCA Rw:TTTCGAGGGGTCCAGGTACA) was determined by RTqPCR using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Milan, Italy) and 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). For each sample, the relative expression level of the mRNA of interest was determined by comparison with the control housekeeping gene B2M (Fw: AGCAGCATCATGGAGGTTTG; Rw: AGCCCTCCTAGAGCTACCTG) using the 2^−ΔΔCt method.

2.8 Seahorse analysis

Live cell analysis of oxygen consumption rate (OCR) was measured using Seahorse Extracellular Flux Analyzer XF24 (Seahorse Bioscience/ Agilent Milan Italy). Cells were cultured in the XF24-well plate overnight at 100.000 cells per well after wells polylysination and centrifuged at 200g for 3 minutes to let cells seed.

For the analysis, assay media was prepared similar to culture media (25 mM glucose, 1 mM sodium pyruvate, and 4 mM L-glutamine) and pH was adjusted to 7.4 ± 0.1.

Manufacturer's protocol was followed for the Cell Mito Stress Test kit using a XFp cartridge previously maintained overnight in an incubator at 37°C. XFp Cartridge ports were injected with Mitostress reagents with port B containing oligomycin (ATP-Synthase inhibitor) at 1.0 µM, port C with 1.5 µM FCCP (mitochondrial membrane depolarizer), and port D with a mixture of 0.5 µM of each rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) (final well concentration).

Port A on the XFp cartridge was designated for acute treatment of control (culture medium) or 20mM Lactate. Seahorse XF-24 Wave software was used to analyze the data and OCR detection was represented as pmoles/min.

2.9 Statistical analysis

All statistics were performed using GraphPad Prism (version 5.00 for Mac, GraphPad Software, San Diego, CA, USA). All data were tested for normality using Shapiro–Wilk test. Data that passed normality test were statistical analyzed using Student's t-test or ANOVA test where appropriate. A *p*-value < 0.05 was considered to indicate a statistically significant difference between experimental and control groups.

3 RESULTS

3.1 MM Patients show higher levels of circulating lactate

Lactate concentration was measured in PB sera of MGUS (n=12), SMM (n=6) and MM at diagnosis (n=14) compared to healthy matched controls (n=9). The amount of lactate was significantly higher in patients' sera (from 1.77 ± 0.43 of control to 6.34 ± 2.46 ($p<0.0001$), 5.4 ± 2.02 ($p<0.01$) and 5.11 ± 1.9 ($p<0.01$) of MGUS, SMM and MM patients respectively) although no difference was found comparing patients at the different stages of disease (**Fig. 1A**).

The increased levels of lactate found in patients' sera at different stage of disease were not correlated with LDH-A concentration ($p=0.98$) (**Fig. 1B).**

In order to better study the ability of MM cell lines to respond to lactate we subsequently evaluated the different expression of MCT1/MCT4 channels and GPR81 receptor in several MM cells lines (U266, NCI-H929, OPM2, MM1S). MCT1 channel showed higher levels of expression in all tested cell lines with an average Ct value of 17.9 ± 1.03, compared to the other genes which exhibited lower levels of expression with an average Ct value of 31.25 ± 0.93 for MCT4 and 31.41 ± 1,22 for GPR81 (**Fig.1C**). Concerning the expression of these genes in every single cell line, OPM2 showed the highest expression of MCT1 and MCT4 channels, whilst GPR81 showed no significant differences of expression among HMCLs (**Fig. 1D**).

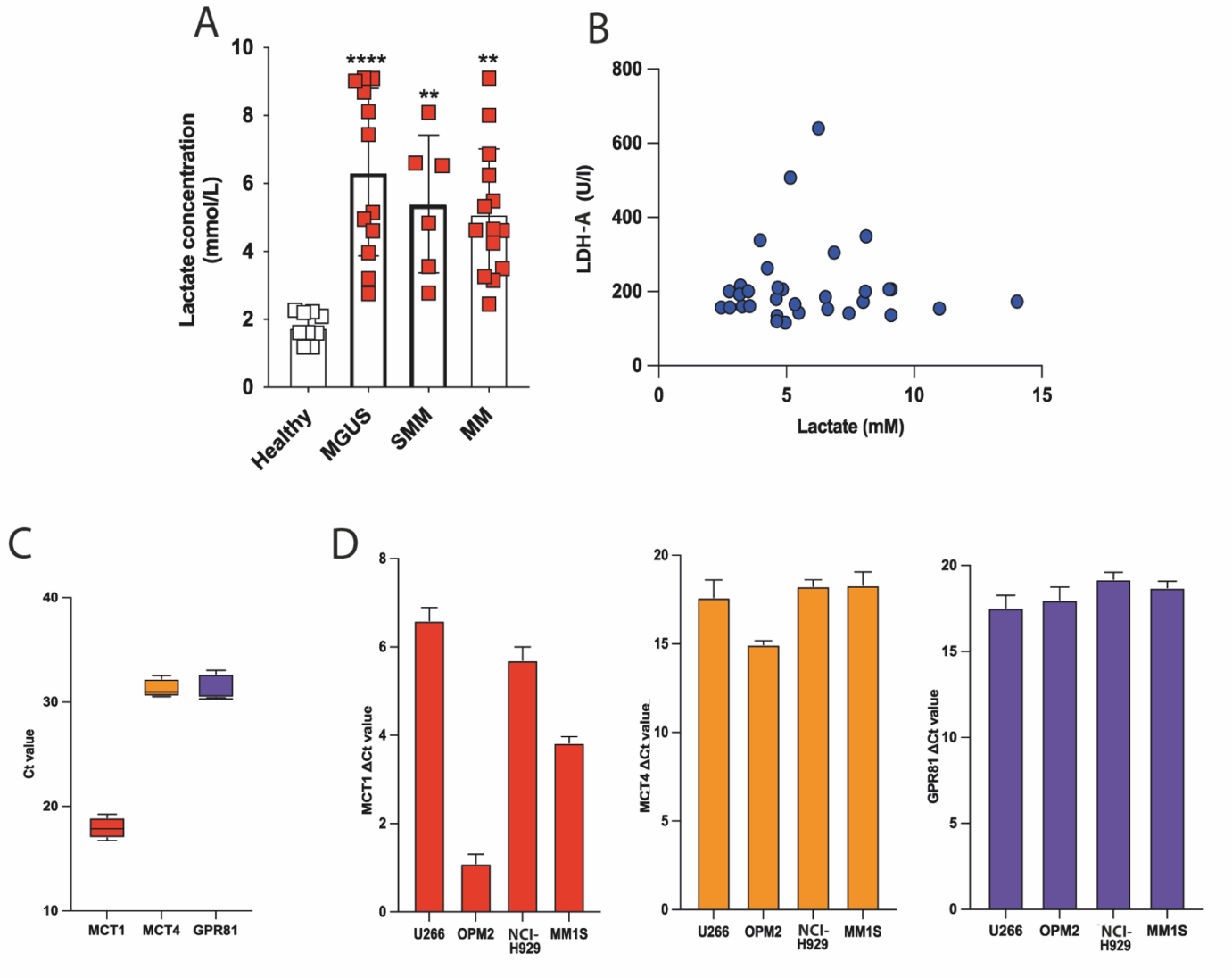


Figure 1. The amount of circulating lactate is increased in myeloma patients. ***(A)*** *Lactate concentration (mmol/L) in PB sera from patients at different stages of disease progression.* ***(B)*** *Correlation between LDH-A and Lactate concentrations in PB sera of MM patients.* ***(C)*** *Boxplot for the Ct values of MCT1, MCT4 and GPR81 of HMCLs (U266, NCI-H929, OPM2, MM1S).* ***(D)*** *ΔCt values of MCT1, MCT4 and GPR81 in HMCLs. Data are presented as means ± SD of three independent experiments**p<0.01; ****p<0.0001.*

3.2 Effects of lactate on MM PCs

3.2.1 Lactate treatment enhances mitochondrial metabolism in HMCLs

In order to evaluate the effects of the high levels of circulating lactate observed in MM patients, we cultured MM cell lines in presence of 20 mM lactate. Evaluating expression of MCT1 over time, we observed a significant increase of this transporter in U266 and OPM2 cell lines (**Fig. 2A**). Although the MCT1 expression is altered in some HMCLs, lactate exposure caused a significant rise of ROS levels both in U266, NCI-H929 and OPM2 cells (**Fig. 2B**) associated with increased expression of *GPR81* ($p<0.001$ compared to untreated; **Fig. 2C**). Moreover, we also found a significant upregulation of the OXPHOS-related genes *ATPsynt* (from 1.08 ± 0.21, 1.027 ± 0.3 and 1.00 ± 0.15 of control to 3.74 ± 0.11, 2.17 ± 0.17 and 1.82 ± 0.03 respectively for U266, NCI-H929 and OPM2; $p<0.01$, $p<0.05$ and $p<0.01$) and *CytB* (from 1.01 ± 0.21, 0.99 ± 0.07 and 1.00 ± 0.10 to 2.26 ± 0.17, 3.85 ± 0.72 and 1.88 ± 0.04; $p<0.05$, $p<0.01$ and $p<0.01$) (**Fig. 2D**). We then moved to confirm the ability of lactate to increase mitochondrial activity, using a Seahorse-assisted MitoStress Test. Our data showed that both U266 and NCI-H929 cell lines exhibited significantly increased OCR after acute injection of Lactate (**Fig. 2E, F**). Altogether, the effects reported here strongly suggest that lactate supplementation induces an increase in the expression of genes involved in the electron transport chain, which in turn increases OCR and ROS production.

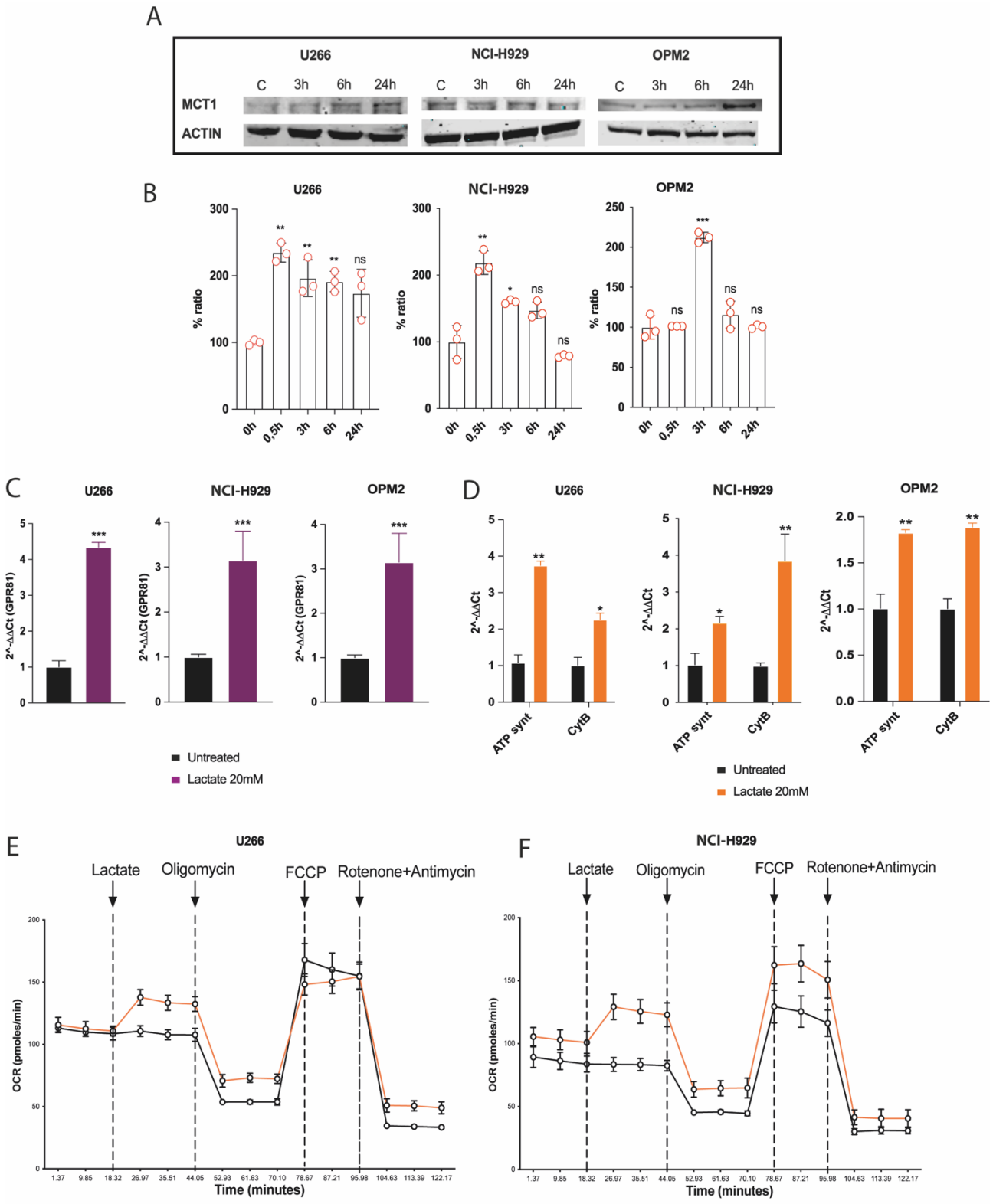


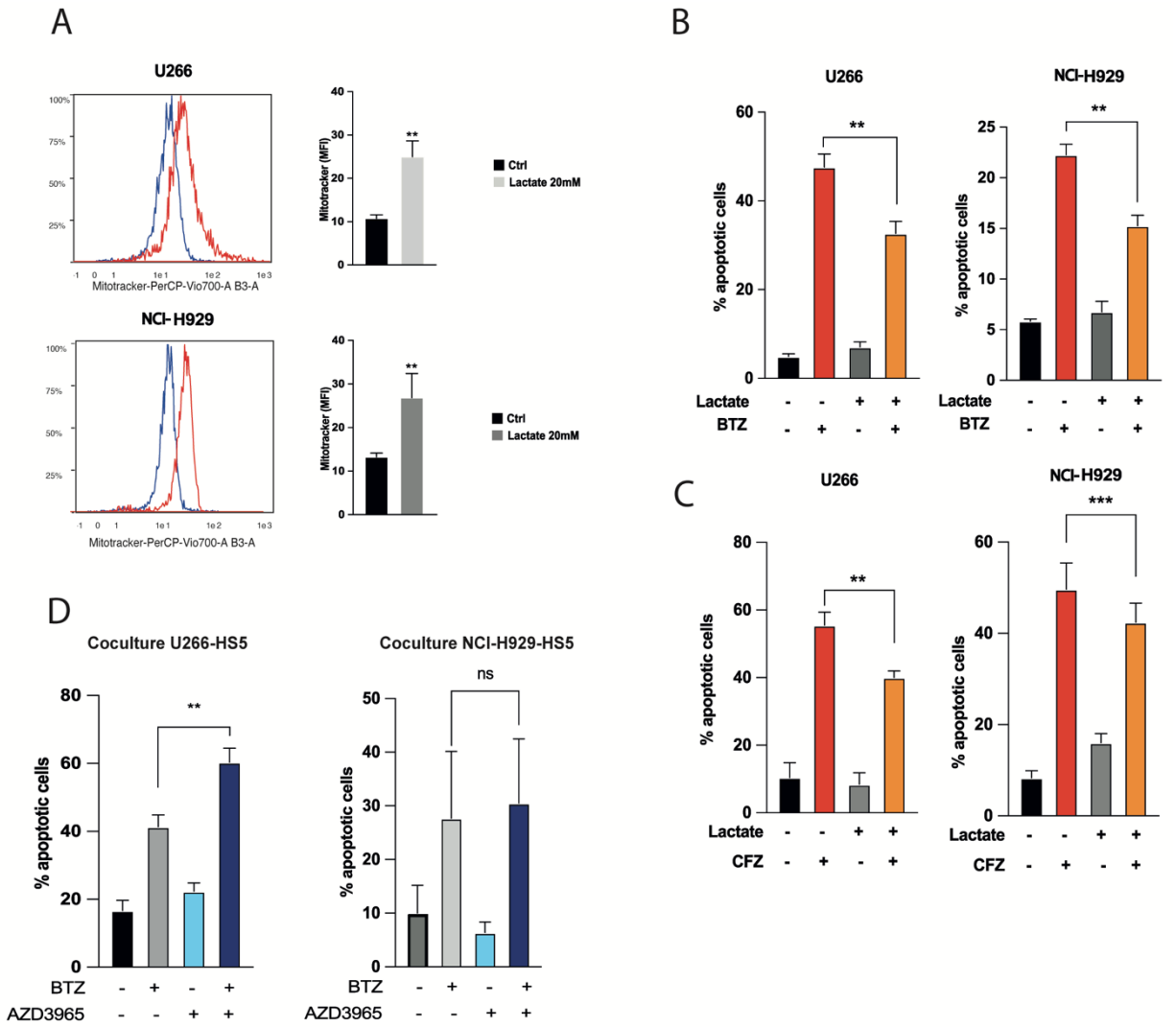
Figure 2. Lactate treatment enhances mitochondrial metabolism in HMCLs. *Western blot analysis of MCT1 protein after 3h, 6h, 24h lactate exposure* ***(A)****. Reactive oxygen species (ROS) production after lactate treatment measured by flow cytometry* ***(B).*** *RT-qPCR gene expression levels of GPR81* ***(C)****, ATPsynt and CytB genes* ***(D)*** *after 3h lactate treatment. Seahorse mitostress OCR measurement expressed in pmoles/min in U266* ***(E)*** *and NCI-H929* ***(F)*** *after acute injection of 20mM lactate. Data are presented as means ± SD of three independent experiments. *p<0.05; **p<0.01; ***p<0.001.*

3.2.2 Chronic exposure to high concentration of lactate reduces PCs sensitivity to PIs

To assess whether higher lactate concentration could affect PI efficacy, we cultured MM cells in presence of a high concentration of lactate for 72h. Chronic lactate exposure was able to significantly increase mitochondrial mass of about 2.5 and 2 times respectively in U266 and NCI-H929 (MFI: U266 Ctrl 10.75 ± 0.82 vs U266 Lac 24.97 ± 3.67 and H929 ctrl 13.26 ± 0.90 vs H929 Lac 26.85 ± 5.55) (**Fig. 3A**) and to reduce efficacy of BTZ of about 13.81 ± 1.88 % in U266 and 7.01 ± 1.5 % in NCI-H929 (**Fig. 3B**). The sensitivity to CFZ was reduced of about 21.16 ± 0.28% in U266 cells and of about 7.26 ± 1.78 in NCI-H929 after chronic exposure to lactate (**Fig. 3C**).

Since mesenchymal stromal cells protect myeloma PCs against BTZ-induced apoptosis, we co-cultured HMCLs with HS-5 stromal cell line for 48h alone or in combination with AZD3965, a selective inhibitor of MCT1, and subsequently cells were treated with BTZ for 24h. Interestingly, coculture with HS-5 in presence of AZD3965 increased the rate of apoptotic cells in U266 cell line compared to U266 cocultured without AZD3965. (**Fig. 3D**). No significant difference was observed in cocultured NCI-H929.

Next, we assessed whether MCT1 is differentially expressed in primary CD138+ cells from MM at diagnosis and resistant/refractory patients in BM biopsy specimens by using immunofluorescence. Interestingly, colocalization of MCT1 within CD138+ PCs was higher in refractory/relapse MM compared to patients at diagnosis (**Fig. 3E**).


A
U266
Mitotracker-PerCP-Vio700-A B3-A
Mitotracker (MFI)
Ctrl
Lactate 20mM
NCI-H929
B
U266
NCI-H929
% apoptotic cells
Lactate
BTZ
C
CFZ
D
Coculture U266-HS5
Coculture NCI-H929-HS5
ns
AZD3965

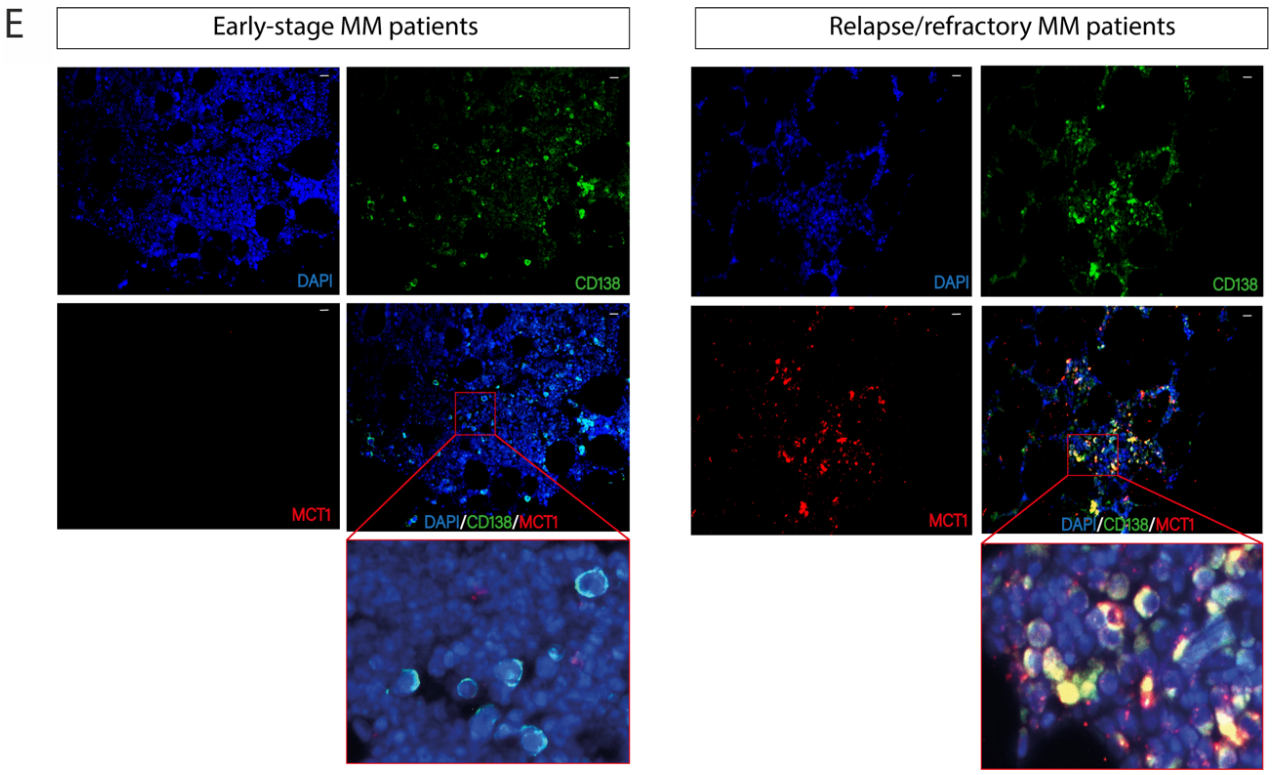

E
Early-stage MM patients
Relapse/refractory MM patients
DAPI
CD138
MCT1
DAPI/CD138/MCT1

***Figure 3*. *High concentration of lactate reduces the efficacy of PI treatment. (A)** Representative plots and quantification of mitochondrial mass analysis using MitoTracker and flow cytometry). **B-C** Quantification of apoptotic cells after BTZ and CFZ treatment in U266 and NCI-H929 cell lines after chronic exposure to high levels of lactate; **(D)** Analysis of BTZ-induced apoptosis in HMCLs cocultured with HS5 in presence or not of AZD3965. **(E)** Immunofluorescence analysis of MCT1 (red) in CD138+ plasma cells (green) of MM patients' biopsy specimens. Nuclei are stained with DAPI (blue). Scale bar: 50 μm.* Data are presented as means ± SD of three independent experiments. ***p<0.01.*

3.2.3 Blocking lactate import increases PI-induced cytotoxicity in myeloma PCs

To elucidate whether the protective effect of lactate was mediated by the activation of GPR81 signaling, NCI-H929 cells were cultured in medium supplemented with 20% sera from different MM patients (n=10) and treated with PI alone or in combination with AZD3965 or 3-OBA, an antagonist of GPR81. PCs treated with BTZ or CFZ in combination with AZD3965 showed higher apoptosis rate compared to PI-only treated cells (respectively of about 6.3 ± 1.45 % for BTZ/AZD3965 and 3.8 ± 2.93% for CFZ/AZD3965) (**Fig.4A and D**). In contrast to inhibition of MCT1, 3-OBA decreased the anti-myeloma effect of BTZ of 5.36 ± 2.75 ($p<0.01$; **Fig. 4B**) but not of CFZ (**Fig. 4E**). Since it is known that PIs exert their apoptotic effect by disrupting mitochondrial integrity and activity (112,113), we also measured mitochondrial polarization state. As expected, PCs treated both with BTZ and CFZ showed a percentage of mitochondrial depolarization of respectively about 63.05 ± 6.8 % ($p<0.0001$) and 19.58 ± 3.37% ($p<0.0001$) compared to untreated cells. Combination of 3-OBA with BTZ or CFZ increased the percentage of depolarization of about 10.08 ± 3.56 % ($p<0.01$) and 2.86 ± 2.04% ($p<0.01$) compared to BTZ or CFZ alone. On the contrary, mitochondrial depolarization increased significantly of 5.36 ± 2.75 in PCs treated with AZD3965/CFZ combination ($p<0.01$) compared to CFZ-treated cells. No difference was observed comparing AZD3965/BTZ treatment with BTZ alone (**Fig. 4C, F**).

These data suggest that circulating lactate in MM sera protects mitochondria against PI-induced cytotoxicity and this effect can be counteracted only blocking lactate import by MCT1 channel using AZD3965. Indeed, circulating lactate reduced PI-induced mitochondrial depolarization also after blocking lactate/GPR81-mediated signaling by 3-OBA.

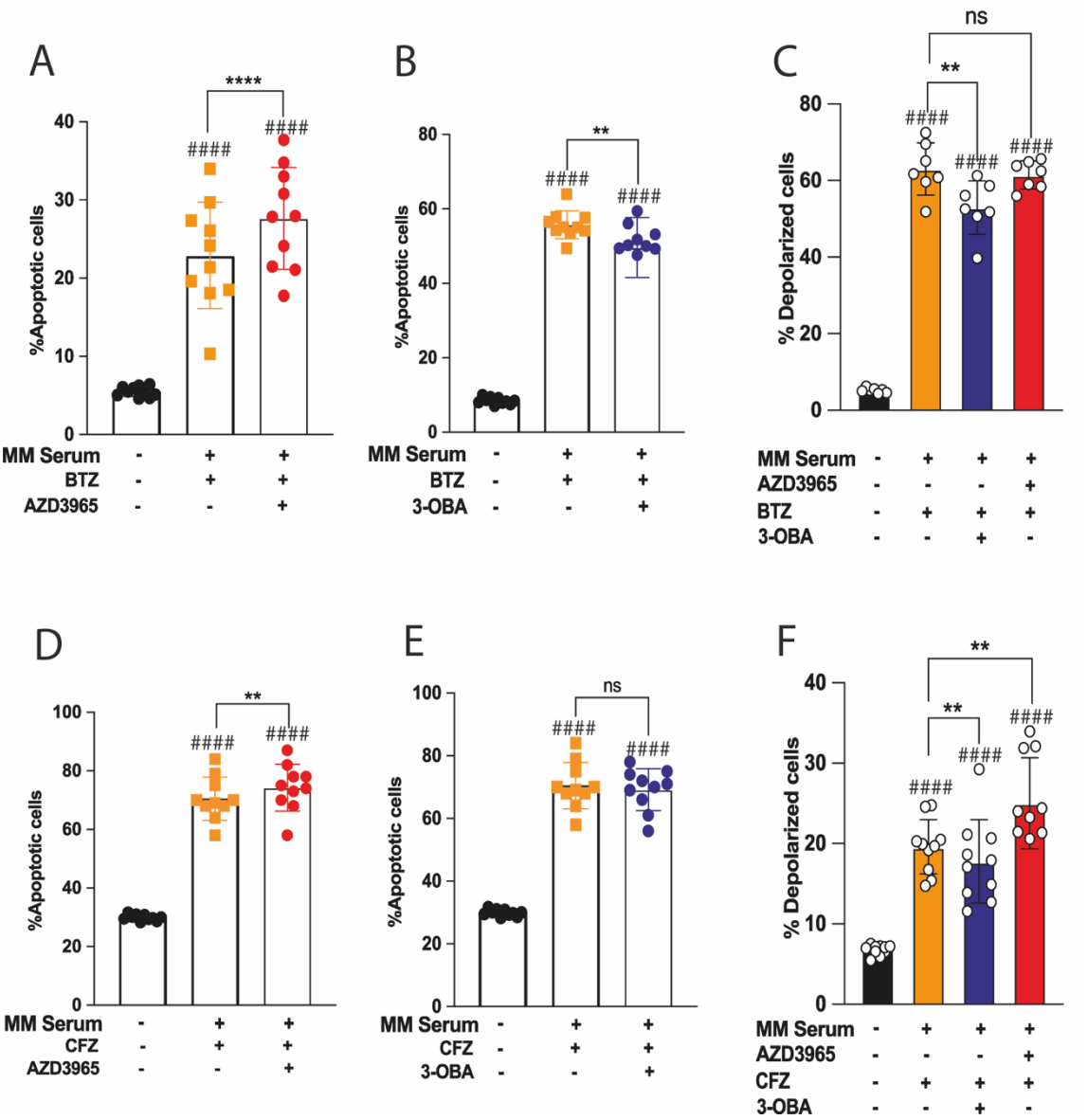


Figure 4. AZD3965 treatment increases PI-induced cytotoxicity in vitro. (***A-B-D-E)*** *Analysis of apoptosis* in *MM cells treated with BTZ* ***(A-B)*** *or CFZ* ***(D-E)*** *in presence of MM serum and AZD3965 or 3-OBA.* ***(C-F)*** *Mitochondrial polarization status measured by flow cytometry after treatment with PI alone or in combination with AZD3965 or 3-OBA in presence of 20% MM serum.* Data are presented as means ± SD of three independent experiments. $^{\#\#\#\#}p<0.0001$ *vs Ctrl;* $^{**}p<0.01$; $^{****}p<0.0001$.

3.3 Effects of lactate on TME

3.3.1 Lactate promotes immune-escape mechanisms which are reverted by MCT1 inhibitor AZD3965

Finally, we explored the effects of high concentration of lactate in promoting immune escape mechanisms in myeloma microenvironment. First, we exposed PBMCs to 20 mM lactate in presence or not of AZD3965. After 48h we observed a significant increase of the amount of M-MDSCs (CD14+/ HLA-DR$^-$) and Treg (CD4$^+$/CD25$^+$/FOXP3$^+$) percentages (respectively of about 8.49 ± 2.12% and 14.22 ± 0.31% compared to control; $p<0.01$; **Fig. 5A and E**). AZD3965 exposure significantly decreased respectively of 5.05 ± 1.76% and 7.68 ± 2.4% their amount in respect of lactate treatment alone ($p<0.05$ and $p<0.01$; **Fig. 5A and E**). In order to assay the involvement of lactate/GPR81 pathway in inducing the expansion of immune-suppressive populations, we exposed PBMCs to 3,5-DHBA, a selective agonist of GPR81(144). Data showed that 3,5 DHBA treatment did not induce any significant increase neither of M-MDSCs nor Treg (**Fig. 5B and F**), demonstrating that lactate did not promote immune-suppressive cell expansion in a hormone-like manner. To better define the immunosuppressive role of lactate in myeloma microenvironment, PBMCs were cultured in medium conditioned with 20% sera from healthy controls (healthy conditioned medium, h-CM; n=11) or MM patients at diagnosis (MM conditioned medium, MM-CM; n=15) for 48h. A significant increase of 27.98 ± 11.9% for M-MDSCs ($p<0.0001$) and 13.10 ± 5.5 % for Treg ($p<0.0001$) was found in PBMCs cultured with MM-CM compared to the same cells cultured in h-CM (**Fig. 5C and G**). Adding AZD3965 to cells cultured with MM-CM, we found a significant decrease of both M-MDSCs and Treg of about 10.5 ± 6% and 7.55 ± 6.16% compared to PBMCs cultured in presence of MM sera only ($p<0.01$**Fig. 5D and H**). As a last step, we investigated whether myeloma PCs could be directly involved in the expansion of immunosuppressive subsets through lactate secretion.

As expected, coculturing PBMCs with MM cell lines both M-MDSCs and Treg subpopulations resulted significantly increased after 48h but only Treg expansion was reduced in presence of AZD3965 (of about 9.01± 2.73% and 20.6 ± 2.92% for PBMC cocultured respectively with U266 and NCI-H929 ($p<0.01$ and $p<0.0001$; **Fig 5L**). No difference was observed after AZD3965 treatment on M-MDSCs expansion (**Fig. 5I**).

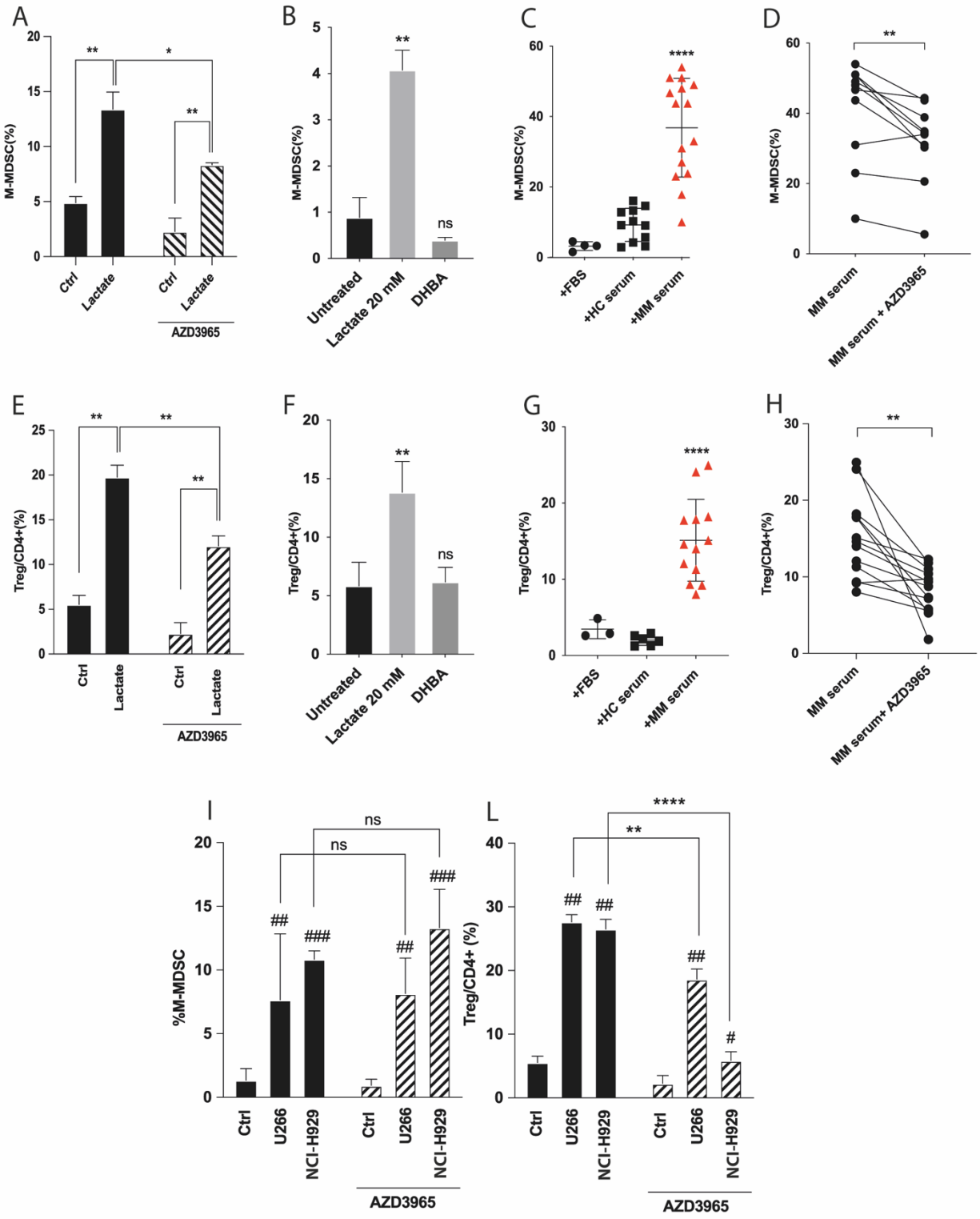


Figure 5. High lactate concentrations promote M-MDSCs and T-reg expansion in MM microenvironment. A-E *Evaluation of M-MDSC* ***(A)*** *and Treg* ***(E)*** *expansion in PBMCs after exposure to lactate alone or in combination to AZD3965.* ***(B, F)*** *3,5 DHBA does not affect expansion of M-MDSCs* ***(B)*** *and Treg* ***(F)****. (****C, G****) Analysis of M-MDSCs* ***(C)*** *and Treg* ***(G)*** *percentages after culturing PBMC in medium supplemented with 20% MM serum.* ***(D, H)*** *Evaluation of AZD3965 effects on circulating lactate-induced M-MDSCs* ***(D)*** *and Treg* ***(H)*** *expansion.* ***(I-L)*** *M-MDSCs and Treg analysis in PBMCs after coculture with HMCLs in presence or not of AZD3965.* Data are presented as means ± SD of three independent experiments *#p<0.05; ##p<0.01; ###p<0.001 vs ctrl; *p<0.05; **p<0.01; ****P<0.0001*

4 DISCUSSION

Metabolic reprogramming, a hallmark of human tumors, is important for MM. Metabolic features of tumor PCs are typical of the Warburg effect with an aerobic glycolysis and increased lactate production (158), but at the same time, MM cells can use the reverse Warburg effect taking up lactate from tumor microenvironment through MCT1 and utilizing it for mitochondrial metabolism (159, 160).

Both MCT1 and MCT4 show high substrate specificity for lactate; however, when MCT1 is expressed, it tends to be the first regulator of lactate flux in the cells (133). Walters and colleagues analyzed expression of lactate transporters on MM cells and found an overexpression of MCT1 in MM PCs compared to MGUS. Moreover, the authors have also demonstrated that MCT1 has a predominant role in lactate flux compared to MCT4 and plays an important role in regulating tumor growth (161). Here, we report that, compared to healthy subjects, MM patients have higher levels of circulating lactate which are, however, increased also in MGUS and SMM patients. It is therefore conceivable that myeloma PCs become more responsive to lactate and its flux, in accordance with the previously observation that MCT1 expression increases steadily with the progression state of plasma cell disorders (161, 162). Our data confirmed that MCT1 expression is higher than MCT4 in HMCLs. In general, MCT1 is preferentially expressed in normoxic cells, while hypoxia-inducible MCT4 has been primarily reported to be expressed in glycolytic/hypoxic cells (163). Exposing MM cell lines to high levels of lactate, we observed a different response in the regulation of MCT1 expression. Contrary to U266 cells, OPM2 cell line increased MCT1 expression only after 24h lactate exposure. NCI-H929 cells did not change levels of MCT1 protein. Nevertheless, all the three selected MM cell lines increased expression of the lactate receptor GPR81 which acts as a lactate sensor regulating genes involved in lactate uptake and metabolism (164).

Lactate can be taken up and used as a mitochondrial respiratory substrate due to mitochondrial lactate carriers and a mitochondrial LDH localized in the matrix/inner membrane component (165,166). Lactate into mitochondria is oxidized to pyruvate and then acetyl-CoA through mitochondrial lactate oxidation complex (mLOC) (167). Our data demonstrated that myeloma PCs exposed to high lactate concentration increased ROS production and upregulated OXPHOS-related genes. The lactate-induced oxidative metabolism was then confirmed by the higher basal respiration and spare respiratory capacity found in PCs exposed to lactate compared to untreated cells. OXPHOS stimulation takes part to the mechanisms used by PCs to adapt metabolically and maintain bioenergetic plasticity (168,169,170,112). Increased OXPHOS has been found in MM cells from relapsed and resistant patients (171) and impairment of mitochondrial fitness restores pharmacological response to PIs (113). Our results demonstrated that chronic exposure to lactate increased mitochondrial mass and reduced the apoptotic effect of BTZ and CFZ in MM cells. Interestingly, resistant/refractory patients expressed higher levels of MCT1 in CD138+ PCs in respect to patients at diagnosis.

Recently, MCT1 has been identified as a predictive marker for the efficacy of lenalidomide maintenance therapy in MM patients with a reduced PFS and OS in patients with higher expression of the lactate transporter (162). Furthermore, the overexpression of MCT1 protects myeloma PCs from the antimyeloma activity of lenalidomide (162). One approach to take advantage of the metabolic vulnerability of cancer cells has been through the development of AZD3965, an orally bioavailable inhibitor of MCT1, currently under phase I clinical trial (NCT01791595). Our data indicate that combination of PI with AZD3965 can be synergistic, increasing tumor PC death. Indeed, pharmacological inhibition of MCT1 prevented the import of lactate circulating in MM sera overcoming its metabolic protective effects. Inhibition of lactate/GPR81-mediated signaling by 3-OBA increased the efficacy of BTZ, but not of CFZ, in presence of high levels of circulating lactate. We have additionally demonstrated that combination of 3-OBA with both BTZ or CFZ decreased PI-induced mitochondrial depolarization probably augmenting lactate metabolic activity.

This hypothesis agrees with the fact that contrary to combination with the inhibitor of lactate/GPR81 pathway, AZD3965/CFZ treatment increased the percentage of mitochondrial depolarization compared to CFZ alone.

In addition to its uptake by tumor cells, lactate also induces immunosuppressive cell types in the tumor microenvironment (172, 173). For example, as a consequence of the high extracellular lactate levels, the expression of Foxp3, the key transcription factor for Treg function, increases. Foxp3 stimulates oxidation of lactate to fuel mitochondrial activity, providing Treg with a metabolic advantage in high-lactate conditions (174). It has been demonstrated that lactate downregulates FIP200 expression leading to naïve T cell apoptosis and autophagy impairment (175). Lactate is also able to impair the cytolytic functions of CD8+ T cells (176), to polarize macrophages towards an M2-like state (152) and to decrease cytotoxic activity of NK (154). Based on this, we evaluated the immunosuppressive effects of lactate in MM microenvironment, in particular on expansion of Treg and MDSCs which are significantly increased in MM microenvironment (56, 177, 178, 179). Exposing healthy PBMCs to high lactate concentration, we found not only an increase of the percentage of Treg but also of M-MDSCs. This lactate-mediated expansion was not dependent by lactate/GPR81 pathway as demonstrated by treatment of PBMCs with the selective agonist of GPR81, 3,5-DHBA. This datum was consistent with the observation that a significant reduction of both Treg and M-MDSC subtypes was found after blocking MCT1. To demonstrate the involvement of lactate in Treg and M-MDSCs expansion in MM, we then cultured PBMCs in presence of serum from HC or MM patients. In our system, both immunosuppressive subsets increased only in presence of MM sera and the high levels of circulating lactate are in part responsible of these induction as demonstrated by the reduction of the expanded percentage of Treg or M-MDSCs after adding AZD3965. The expansion of Treg and M-MDSCs can be directly regulated by tumor PCs (180, 181, 182). Therefore, we sought to examine whether tumor release of lactate may be involved. We found a significant decrease of the percentage of tumor-induced Treg, but not of M-MDSCs, after blocking MCT1.

Collectively, these results suggest that the highest levels of circulating lactate in MM patients favor expansion of Treg and MDSCs and this metabolite is a mediator of Treg activation induced by tumor PCs.

In conclusion, the current work demonstrates that circulating lactate levels are higher in MM patients and that this metabolite plays a key role both in tumor PCs and in the surrounding microenvironment. PCs take advantage from lactate by uploading it through MCT1 channel and using it as a fuel for OXPHOS, increasing their mitochondrial activity which in turn is involved in inducing resistance to both BTZ and CFZ. At the same time, lactate in tumor microenvironment favors the proliferation of those immune cells which can efficiently survive under high lactate conditions metabolizing it through the tricarboxylic acid cycle (TCA) to fuel their OXPHOS metabolism thus establishing an immunosuppressive environment such as Tregs and M-MDSCs. Taken together, the concerted effects of lactate on tumor niche components suggest that lactate acts as an immuno-modulatory molecule that can strongly repress anti-tumor immunity, favouring MM cell proliferation, immune-escape mechanisms and controlling resistance to anti-cancer therapy. Targeting lactate pathway may therefore represent a potential strategy for anti-cancer therapy in multiple myeloma.

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