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Lactate accumulation promotes immunosuppression and fibrotic transformation of bone marrow microenvironment in myelofibrosis

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Abstract

Background Clonal myeloproliferation and fibrotic transformation of the bone marrow (BM) are the pathogenetic events most commonly occurring in myelofibrosis (MF). There is great evidence indicating that tumor microenvironment is characterized by high lactate levels, acting not only as an energetic source, but also as a signaling molecule.

Methods To test the involvement of lactate in MF milieu transformation, we measured its levels in MF patients' sera, eventually finding a massive accumulation of this metabolite, which we showed to promote the expansion of immunosuppressive subsets. Therefore, to assess the significance of its trafficking, we inhibited monocarboxylate transporter 1 (MCT1) by its selective antagonist, AZD3965, eventually finding a mitigation of lactate-mediated immunosuppressive subsets expansion. To further dig into the impact of lactate in tumor microenvironment, we evaluated the effect of this metabolite on mesenchymal stromal cells (MSCs) reprogramming.

Results Our results show an activation of a cancer-associated phenotype (CAF) related to mineralized matrix formation and early fibrosis development. Strikingly, MF serum, enriched in lactate, causes a strong deposition of collagen in healthy stromal cells, which was restrained by AZD3965. To corroborate these outcomes, we therefore generated for the first time a TPO[high] zebrafish model for the establishment of experimental fibrosis. By adopting this model, we were able to unveil a remarkable increase in lactate concentration and monocarboxylate transporter 1 (MCT1) expression in the site of hematopoiesis, associated with a strong downregulation of lactate export channel

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MCT4. Notably, exploiting MCTs expression in biopsy specimens from patients with myeloproliferative neoplasms, we found a loss of MCT4 expression in PMF, corroborating changes in MCT expression during BM fibrosis establishment.

Conclusions In conclusion, our results unveil lactate as a key regulator of immune escape and BM fibrotic transformation in MF patients, suggesting MCT1 blocking as a novel antifibrotic strategy.

Keywords Lactate, Myelofibrosis, Tumor microenvironment, Monocarboxylate transporters

Introduction

Myelofibrosis (MF) is a severe subtype of myeloproliferative neoplasm (MPN) characterized by clonal proliferation of hematopoietic stem cells, bone marrow (BM) fibrosis, extramedullary hematopoiesis, splenomegaly, aberrant inflammation and an inherent risk of blastic transformation [1]. Currently, the treatment with JAK inhibitors (JAKi) has benefits in terms of spleen size control, and symptoms mitigation, eventually improving the quality of life, and prolonging patients' overall survival [2–4], especially in those classified as high risk according to standard prognostic scores [5]. However, the disease-modifying potential of JAKi therapy is limited, and the only potentially curative treatment is allogeneic hematopoietic stem cell transplantation [6], even if new helpful approaches are under evaluation above all in anemic [7] and JAKi refractory patients [8]. The disease arises *de novo* as primary M F (PMF), or it might occur post polycythemia vera (PV) or post essential thrombocythemia (ET). In this context, 90% of patients carry JAK2, CALR or MPL mutations, which are indeed defined as "driver" mutations [9]. Interestingly, MF pathophysiology is deeply influenced by alterations in the BM microenvironment, highlighted by BM fibrosis, neo-angiogenesis and osteosclerosis [10, 11]. The hallmark feature of MF is indeed the excessive extracellular matrix (ECM) deposition, causing a progressive loss of hematopoiesis and splenomegaly due to extramedullary hematopoiesis. In particular, BM fibrosis occurs as a cytokine-mediated secondary reaction toward the starting clonal malignant expansion, leading to overbalanced ECM deposition [12, 13].

In this context, mesenchymal stromal cells (MSCs) interact with megakaryocytes (MK), in turn releasing profibrotic cytokines as tumor growth factor beta (TGF-b). As a result, MKs prompt MSCs to differentiate into fibroblasts, acquiring a fibronectin-secreting phenotype [13, 14]. In particular, multipotent mesenchymal progenitor cells are the predominant type I collagen-producing cells in overt BM fibrosis [15]. Over time, all MSC subsets are functionally reprogrammed losing their hematopoietic supportive capacity and secreting non-collagenous ECM as a scaffold for collagen fibrosis [15]. Moreover, MSCs are pushed toward osteogenic differentiation supporting MF pathophysiology [12, 16].

Genetic and epigenetic changes are often coupled with metabolic reprogramming of cancer cells in order to fuel their increased energy demands [17, 18]. This leads to a nutrient competition between cancer cells and the surrounding stromal cells as well as the immune compartment, potentially promoting immune escape mechanisms and reprogramming of the whole BM niche in favor of tumor cell proliferation [19, 20]. Enhanced glycolysis, even under aerobic conditions, which is known as Warburg effect, is one of the hallmarks of cancer cells [18, 21]. This metabolic alteration is more closely related to the deregulations of molecular pathways controlling glucose uptake than mitochondria defects [22]. Lactate dehydrogenase A (LDHA), which catalyzes pyruvate-to-lactate conversion, has a central role in glycolysis, and, in several models, it has been related to tumor progression [23, 24]. Consistent with these observations, LDH levels are typically increased in MF patients' sera and predicts shorter overall and leukemia-free survival [25]. Moreover, JAK2-V617F mutant cells increase glucose uptake and glycolysis through STAT5 activation, required for JAK2-V617F mediated transformation [19, 26, 27]. Interestingly, selective inhibition of 6-phosphofructo-2-Kinase activity induced apoptosis in primary cells from patients and antagonized the disease phenotype of JAK2-mutant MPN mice [28]. The deregulation of intracellular and extracellular metabolites associated to cancer metabolic reprogramming also affects cellular transcriptome, secretome, and differentiation, eventually reshaping the whole tumor microenvironment (TME) [29]. In this context, the excess of lactate secreted by cancer cells as a consequence of the Warburg's effect itself, promotes angiogenesis, immune suppression and cancer-associated fibroblast (CAF) activation [20, 30–33]. Similarly, an association between plasmatic LDH and the severity of idiopathic pulmonary fibrosis has been described [34, 35]. Recently, it has been suggested that lactate from pancreatic cancer cells increases the number of CAF, which promote fibrotic tumor formation [36]. The role of lactate in TME reshaping also involves its role as epigenetic regulator. In hepatocellular carcinoma, lysine lactylation at specific sites, such as K28, plays a crucial role in promoting tumor proliferation and metastasis [37]. Additionally, lactylation influences critical cellular processes like glycolysis and macrophage polarization, further supporting tumor progression and immune modulation [38].

Although the characterization of tumor metabolism has been described, insights on how changed TME metabolic composition is in MF patients, is poorly understood. Here, we showed that the amount of circulating lactate increases in MF patients, eventually promoting the expansion of immunosuppressive subsets and fibrosis development. Accordingly, these effects are restrained by blocking lactate import channel monocarboxylate transporter 1 (MCT1). We also demonstrated that the expression of lactate export channel monocarboxylate transporter 4 (MCT4) in TME is deeply remodeled during fibrotic transformation, suggesting a link between lactate trafficking and pro-fibrotic establishment.

Materials and methods

Sample collection, cell cultures and treatments

Peripheral blood (PB) samples were collected from MF patients and age-matched controls after written informed consent (Azienda Ospedaliero-Universitaria Policlinico "G.Rodolico-San Marco", n. 54/2022/PO). Clinical data of patients included in this study are shown in Table 1. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donor buffy coats after separation by Ficoll-Hypaque gradient.

Commercially available stromal cell lines HS-5 were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Osteoblastic differentiation was induced culturing HS-5 cells with a osteogenic induction medium (OIM): DMEM 10% FBS, 1%

Table 1 Clinical characteristic of PMF patients (*n* = 22) included in the study

Median years of age (range)	67 (40–76)
Sex	16 M (69%), 7 F (31%)
Blood count	
-Hb, g/dL (range)	10.2 (6.1–17.2)
-WBC, x10/mmc (range)	9.7 (1.2-149.1)
-Platelets, x10/mmc (range)	349 (70-1016)
Blast > 5%	2 (9.1%)
BM fibrosis	
-0	3 (13.04%)
-1	11 (47.82%)
-2	6 (26.09%)
-3	3 (13.04%)
Driver mutations	
-JAK2	15 (65.2%)
-CALR	3 (13.05%)
-MPL	0
-Triple negative	3 (13.05%)
-Not available	2 (8.7%)
IPSS/MYSEC-PM	
-Low	2 (8.7%)
-Intermediate-1	6 (26.08%)
-Intermediate-2	10 (43.48%)
-High	5 (21,74%)

Hb: hemoglobin; WBC = white blood cells; IPSS/MYSEC-PM = International Prognostic Scoring System/MYSEC Prognostic Model Risk Calculator

penicillin-streptomycin, 10 mM b-glycerophosphate (Calbiochem, San Diego, CA, USA), 0.2 mM ascorbic acid and 0.1 mM dexamethasone (Sigma-Adrich, Mylan, Italy). The medium was replaced every 3 days.

Sodium lactate (20 mM) and AZD3965 (10 μM) were obtained from Sigma-Adrich (Mylan, Italy) and Selleckchem (Cologne, Germany) respectively.

Lactate concentration measurement

Spectrophotometric determination of lactate was carried out as previously described [39]. Briefly, the reaction mixture contained 100 mM Tris–HCl, 1.5 mM N-ethyl-N-2-hydroxy-3-sulfopro- pyl-3-methylalanine, 1.7 mM 4-aminoantipyrine and 5 International Unit (IU) horseradish peroxidase. Fifty microliters of serum were added to the mixture. The reaction was started with the addition of 5 IU of lactate oxidase to the cuvette (finale volume = 1 ml) and it was considered ended when no change in absorbance was recorded for at least 3 min. To calculate lactate in serum samples, the difference in absorbance at 545 nm wavelength (Δabs) of each sample was interpolated with a calibration curve obtained by plotting Δabs measured in standard solutions of lactate with increasing known concentrations.

Flow cytometry

For regulatory T cells (Treg) analysis, immune cells were stained with CD4-PEVio770 (clone SFCI12T4D11), CD25-APC (clone B1.49.9) and FOXP3-PE (clone 259D), all from Beckman Coulter, and Treg were defined as $CD4^+CD25^{high}Foxp3^+$. For monocytic myeloid derived suppressor cells (M-MDSCs) analysis, immune cells were stained with CD14-FITC (clone 322 A.1, TLY4) and HLA-DR-APC (clone Immu-357) [40], all from Beckman Coulter, and M-MDSCs were defined as $CD14^+HLA-DR^-$.

qPCR

After RNA extraction, reverse transcription was performed by using the high-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Milan, Italy). Then the relative transcription of human genes MCT1 (Fw: TGTTGTTGCAAATGGAGTGT; Rw: AAGTCGA TAATT- GATGCCCATGCCAA), MCT4 (Fw: TATCCA GATCTACCTCACCAC; Rw: GGCCTGGCAAAGATG TCGATGA) 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: AGCCCTCCTAGAGC- TACCTG) using the $2^{-\Delta\Delta Ct}$ method.

Western blot

As previously described [41], extracted proteins were loaded onto a 12% polyacrylamide gel Mini- PROTEAN TGXTM (BIO-RAD, Milan, Italy) followed by electro-transfer to nitrocellulose membrane Trans-Blot TurboTM (BIO-RAD) using Trans-Blot SE Semi-Dry Transfer Cell (BIO-RAD) [42]. After blocking membranes were incubated with primary antibodies against human Col1A (sc-59772, Santa Cruz Biotechnology, Texas, USA), αSMA, PDGFRβ, FAP, β-actin (ab15734, ab32570, ab207178 and ab181602 respectively; Abcam, Mylan, Italy). Next day, infrared anti-mouse IRDye800CW and antirabbit IRDye700CW secondary antibodies (Licor Biotechnology, Cambridge, United Kingdom) were used. Proteins bands were visualized using Odyssey Infrared Imaging Scanner (Licor Biotechnology). The density of each band was quantified using ImageJ analysis software and normalized to protein levels of β-actin.

Cytokine and chemokine detection

Multiplex immunobead assay technology (procartaplex Cytokine/Chemokine Magnetic Bead Panel, of Thermo Scientific, MA, Usa and Magpix analytical test instrument of Luminex Corp., Austin, TX) was performed on culture medium to determine concentrations of selected cytokines (Bone morphogenetic protein 2 (BMP2), osteopontin, calcitonin, Receptor activator of nuclear factor kappa-B ligand (sRANKL), matrix metallopeptidase 2 (MMP2), matrix metallopeptidase 9 (MMP9) chitinase 3 like 1 (CHI3L1), Regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5).

Cell mineralization

HS-5 cells were incubated with 2% Alizarin Red S (Sigma-Aldrich) for 10 min. After washing, cell mineralization was evaluated by using a phase-contrast microscopy.

Mallory's trichrome staining

Cells were stained in 1% fuchsin acid (Sigma-Adrich) solution in distilled water; then, they were counterstained with a solution of 0.5% aniline blue (Sigma-Adrich), 2% orange G (Sigma-Adrich) and 2% oxalic acid (Sigma-Adrich) in distilled water. The standard methods were applied as previously described by Wołuń-Cholewa et al. [43].

Immunofluorescence

Cells were fixed using 4% paraformaldehyde (PFA) prior to permeabilization. Primary antibody against human Col1A1 (sc-59772) and MCT4 (Abcam; ab234728) were used. After 24 h, cells were washed and incubated with an anti-mouse secondary antibody Alexa Fluor 647 for 1 h at room temperature. After washing, nuclei were counterstained with DAPI 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).

Immunohistochemistry

The immunohistochemical analyses were performed as previously described [44]. After proper deparaffinization and pretreatments, the slides were incubated with primary antibodies against CD90 (Abcam; ab181469), MCT1 (Abcam; ab90582) and MCT4 (Abcam; ab234728). Sections were counterstained with hematoxylin and evaluated with a light microscope (Carl Zeiss, Oberkochen, Germany). Both MCT1 and MCT4 were assessed as positive if chromogen was found in the cellular cytoplasms with or without nuclear staining. CD90 was considered as positive if chromogen was found in the cellular membranes and/or cytoplasms.

In vivo zebrafish model

Ethics statement

The experimental procedures were approved by the Animal Studies Committee of Ministero della Salute Italy (Approval code: 30/2022-PR). All of the procedures were performed according to the relevant regulations.

Zebrafish husbandry

Adult wildtype (5–8-months-old) AB zebrafish were used for this study. Fishes were housed at a density of five fishes per tank in mixed-sex groups in 2.5 L tanks on a recirculating system in 28 °C water in a room with a 14:10 h light: dark cycle. System water was carbon-filtered municipal tap water, filtered through a 20 μm pleated particulate filter, and exposed to 40 W UV light [45]. Standard feeding protocol was three meals daily of Tetra-Min (Tetra) in the CAPIR (University of Catania) facility.

TPOhigh zebrafish model, cell collection and flow cytometry

TPO[high] zebrafish cohort was exposed to 100 μg/kg Eltrombopag Olamine (Selleckchem, Cologne, Germany) every 3 day. Control and TPO[high] zebrafish were anaesthetized after 15 days with 0.02% tricaine before kidney marrow collection. After a ventral, midline incision was made, kidney marrow was dissected and placed into ice-cold PBS containing 5% FBS. Whole kidney marrow (WKM) cells were passed through a filter with a 40-μm pore size. Propidium iodide (Sigma) was added to exclude dead cells and debris. Flow cytometry analysis and sorting was based on propidium iodide exclusion, forward scatter and side scatter as previously described [39]. WKM cells were also transferred onto a slide by cytocentrifugation and stained with May-Grunwald-Giemsa.

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RT-qPCR

Total RNA was extracted from WKM by Trizol reagent. After reverse transcription, we evaluated expression of the following zebrafish mRNA: cd41 (FW: CTGAAGG CACTAACGTCAAC; RW: TCCTTCTTCTGACCAGA GTTA), mpl (FW: CGCCAACCAAAGCCAGAGTTA; RW: ACTTTTCAACAGGTGCATCCCA), tgfb1 (FW: TTCGTCTTCCAGCAAGCTCA; RW: TGGAGACAA AGCGAGTTCCC), col1a1 (FW: ACCCTTAAGTCCCT GAGCCA; RW: AATCCAGTACTCGCCGCTCT), mct1 (FW: TCATGTATGCTGGAGGACCAA; RW: AGTCG ACAAGAACACTGGGC), mct4 (FW: AGACCCTAGG AGAGTTGAGCC; RW: CTCCATCCGGTGCTTTGAC T). For each sample, the relative expression level of each studied mRNA was normalized using gapdh (FW: AGTG TCAGGACGAACAGAGGCT; RW: GCCAATGCGACC GAATCCGTTA) as invariant control.

Reticulum fiber staining method

Slides were cut to 4 micron and stained with a silver impregnation-based kit for reticulin staining (Bio-Optica Bo 04–040,801, Milan, Italy). The staining steps were followed according to the manufacturer's instructions [46].

Statistical analysis

The data are expressed as mean ± SD. Statistical analysis was carried out by Student's t-test, ANOVA test. A p-value < 0.05 was considered to indicate a statistically significant difference between experimental and control groups.

Results

Increased levels of circulating lactate sustain the expansion of immune suppressive subsets in MF patients

Lactate concentration was measured in PB sera from PMF ($n = 23$) compared to healthy matched controls (HC; $n = 16$). The amount of circulating lactate was significantly increased in sera from patients in comparison with HC (3.69 ± 2.2 vs. 1.08 ± 0.3 mmol/L, $p < 0.0001$; Fig. 1A).

Given that MPN patients exhibit immune alterations, including derangement of Treg and myeloid derived suppressor cells (MDSCs) [44, 47–49], we investigated the role of higher circulating lactate in promoting expansion of immunosuppressive subsets. After 48 h exposure of healthy PBMCs to medium conditioned with 20% sera from HC ($n = 10$) or PMF ($n = 16$), we found a significant increase of the percentage of both Treg and monocytic-MDSCs (M-MDSCs) in PBMCs cultured with PMF sera compared to the same cells grown with HC ones ($p < 0.001$; Fig. 1B, D). Of note, any significative difference was assessed by exposure of healthy PBMCs to media supplemented with FBS. To better define the role of lactate in the expansion of these immune suppressive subsets, we cultured PBMCs with PMF sera with or without

AZD3965, a selective inhibitor MCT1, to inhibit lactate import. The addition of AZD3965 decreased expansion of Treg and M-MDSCs of about 8.04 ± 0.2% and 6.98 ± 6.2% respectively ($p < 0.0001$ and $p = 0.0251$; Fig. 1C, E). Collectively these data demonstrate that increased circulating lactate contribute to immune deregulation observed in MF patients.

Lactate stimulates CAF phenotype and osteogenic differentiation in MSCs

A line of evidence suggests that lactate can activate a CAF phenotype in stromal cells sustaining formation of tumor niche [32, 36]. Therefore, we next evaluated the role of lactate in MSCs reprogramming by using an in vitro model of healthy MSCs (HS-5). The evaluation of CAF biomarkers expression after exposure to 20 mM lactate concentration unveiled a significant upregulation of alpha-smooth muscle actin (αSMA), platelet-derived growth factor receptor β (PDGFRβ) and fibroblast activation protein alpha (FAP) and after 48 h ($p < 0.001$ and $p < 0.0001$ respectively; Fig. 2A). Notably, chronic exposure to high lactate also raised the concentration of osteoblast differentiation markers. In particular, after 10 days, lactate exposure increased the amount of BMP2, osteopontin, calcitonin and sRANKL (respectively $p < 0.0001$, $p < 0.01$, $p < 0.0001$ and $p < 0.0001$ vs. untreated cells; Fig. 2B), similarly to cells cultured in osteogenic induction medium (OIM). Alizarin red staining confirmed the increased deposition of calcium phosphate after chronic exposure to lactate (Fig. 2C); interestingly, this effect was reverted by AZD3965 treatment. Together, these results suggest that lactate-induced HS-5 cells toward a CAF-activated phenotype and chronic exposure of these cells to the metabolite stimulates osteogenic differentiation.

Lactate stimulates stromal cells to produce collagen favoring fibrotic transformation of TME

Given the significance of fibrotic tumor formation in MF patients, we next tested the role of high lactate concentration in promoting fibrosis. After 48 h lactate exposure, we found a significant upregulation of alpha-1 type I collagen (Col1A1; $p < 0.001$ vs. untreated cells; Fig. 3A). As showed in Fig. 3B, AZD3965 prevented lactate-induced Col1A1 expression ($p < 0.001$ vs. lactate treatment alone). Consistently, Mallory trichrome staining evidenced increased deposition of collagen in lactate treated stromal cells ($p < 0.01$ vs. untreated; Fig. 3C). Lactate-induced collagen deposition was partially reduced by MCT1 selective inhibitor AZD3965 ($p < 0.05$ vs. lactate treatment alone). These changes were sustained by a significant increase of key proteins involved in ECM degradation as MMP-2, MMP-9, RANTES and CHI3L1 (Fig. 3D). Expression of MMP-2 and RANTES raised already after 48 h lactate exposure (respectively $p < 0.0001$ and $p < 0.001$ vs.
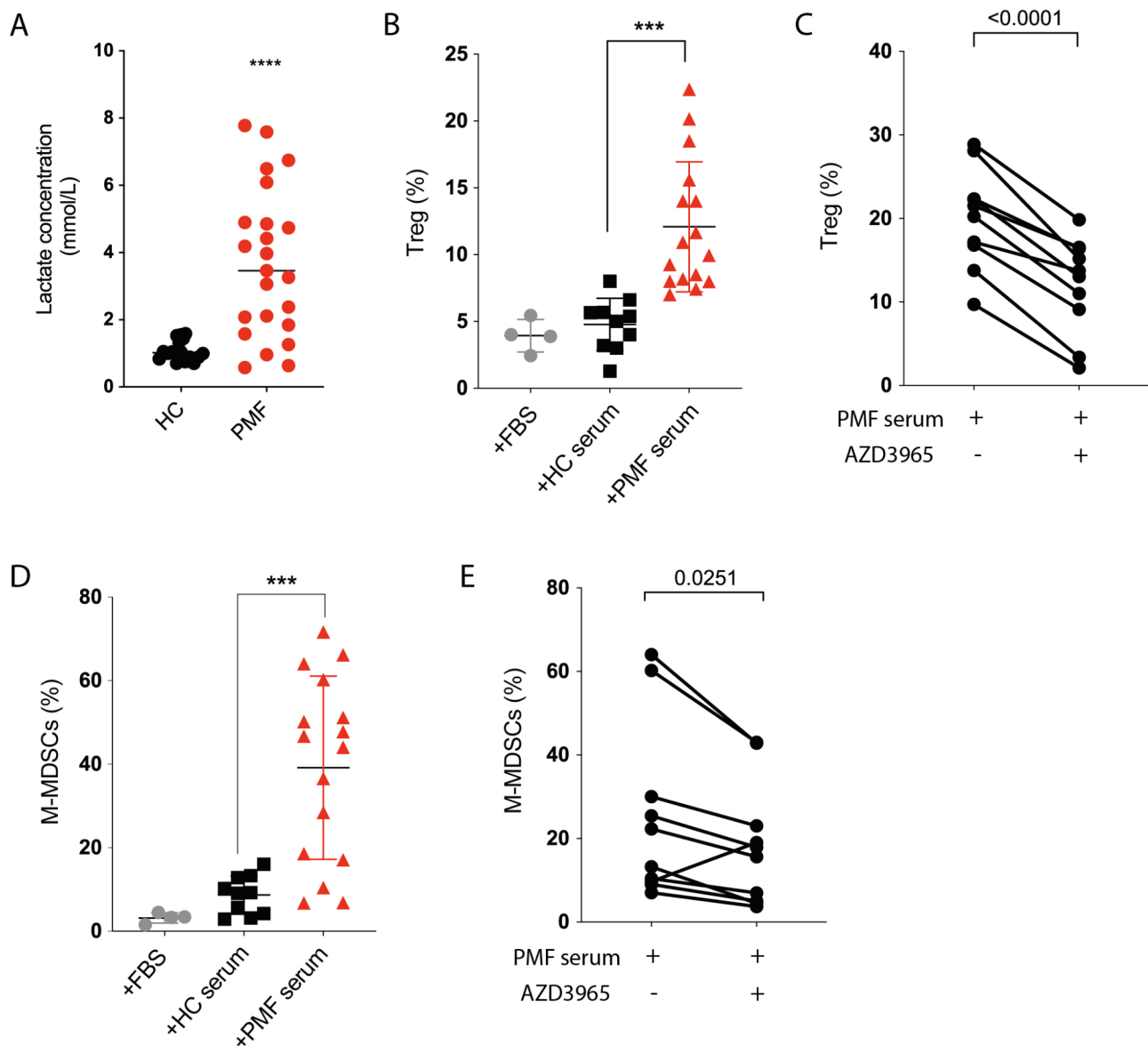
Fig. 1 High circulating lactate in MF sera favors immune deregulation. (**A**) Evaluation of Lactate concentration in PB from healthy and PMF patients. (**B**) Analysis of the percentage of Treg and (**D**) M-MDSCs after culturing healthy PBMCs with HC or PMF serum. (**C**) Evaluation of 10 μM AZD3965 effects on circulating lactate-induced Treg and (**E**) M-MDSC expansion. Data are presented as means ± SD. ***$p < 0.001$; ****$p < 0.0001$

untreated cells). To better elucidate the role of high circulating lactate in MF patients' sera in this context, we therefore compared the effect of healthy and PMF sera on collagen deposition. Following 24 h exposure to PMF sera, HS-5 cells showed a higher collagen accumulation compared to stromal cells treated with lactate (Fig. 3E). Of note, there was no significative increase when the culture medium was supplemented with healthy serum (Fig. 3E). Most importantly, the amount of collagen in cells treated with PMF sera or with lactate was similar after 48 h. Notably, PMF serum-induced collagen accumulation was inhibited by AZD3965 ($p < 0.0001$ vs. PMF serum condition; Fig. 3F). Collectively, these results

suggest that lactate increases collagen deposition in stromal cells contributing to ECM changes and fibrotic transformation of TME.

Alteration of lactate and its transporters in TPOhigh zebrafish model

To further validate the in vitro data, we generated for the first time a thrombopoietin (TPO) high zebrafish model based on repeated exposure of supra-pharmacological doses of Eltrombopag Olamine for 15 days (Fig. 4A). TPO is the principal physiologic regulator of platelet production. Studies suggest that TPO mimetics promote
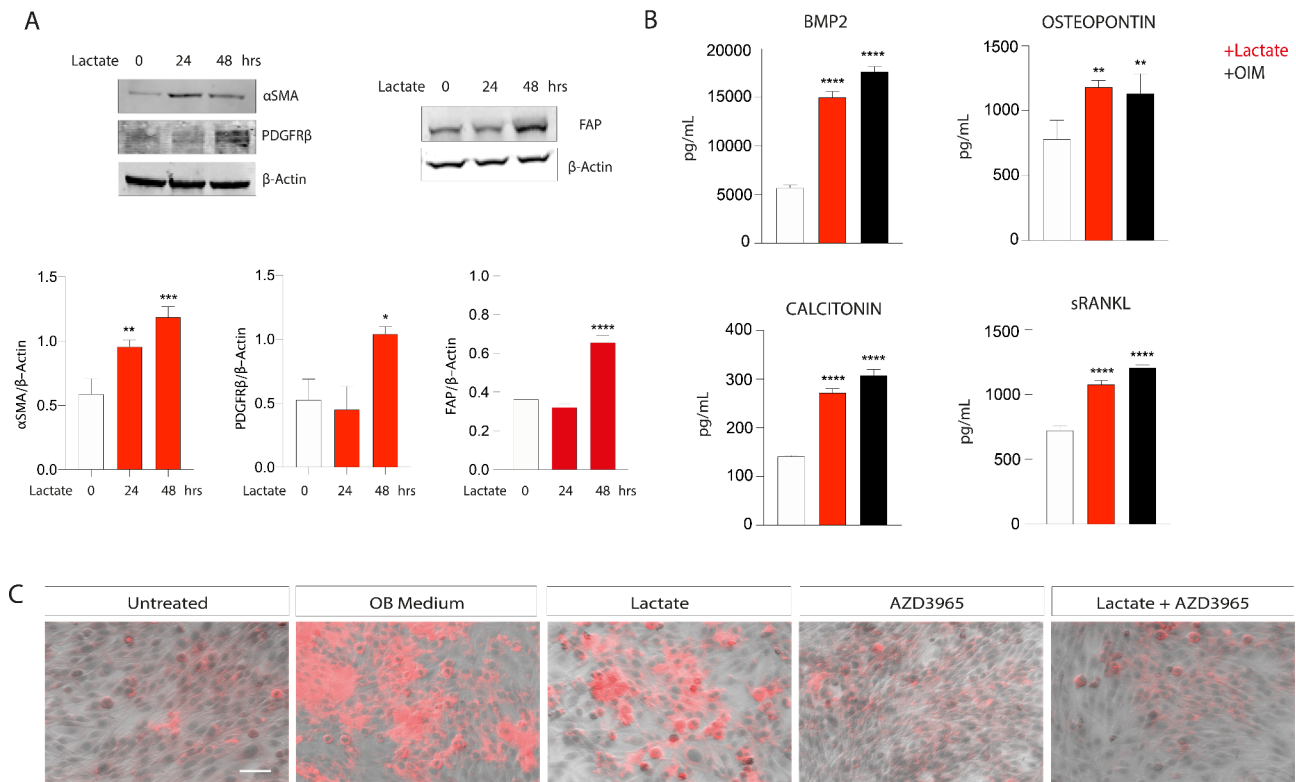
Fig. 2 Effect of lactate on stromal cell phenotype. (**A**) Cropped blots and densitometric analysis from western blot analysis on αSMA, PDGFRβ and FAP, normalized on β-actin. (**B**) Multiplex immunobead assay technology on culture medium from HS5 cells after 10 days of 20 mM lactate exposure to determine concentrations of indicated osteoblast differentiation markers. (**C**) Alizarin red S staining for mineralization. The calcified nodules appeared bright red in color. Scale bar: 20 μm. All the data are presented as means ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

reticulin deposition through stimulation of cytokine production by megakaryocytes, including TGF-b [50].

Analyzing the WKM, which is equivalent to the hematopoietic BM of mammals [45], the major blood lineages were measured using combined scatter profiles [39]. Therefore, we first evaluated the effect of TPO mimetic treatment on the distribution of several blood cells. As shown in Fig. 4B-C, we found an increased percentage of progenitor cells in TPO^high zebrafish ($n = 9$) compared to controls ($n = 5$) ($p < 0.01$); on the other hand, the number of erythroid and myeloid cells decreased significantly ($p < 0.001$ and $p < 0.01$ respectively). The increased number of precursors in TPO^high zebrafish was also confirmed by morphologic analysis of WKM (Fig. 4D). Moreover, TPO^high zebrafish showed a prominent upregulation of the megakaryocytic marker cd41 and mpl ($p < 0.05$ compared to control; Fig. 4E) associated to a higher expression of col1a1 and tgfb1 (respectively $p < 0.001$ and $p < 0.05$ vs. untreated group; Fig. 4F). Consistently with these results, TPO^high zebrafish displayed increased deposition of Col1a1 and reticulum fibers in WKM suggesting establishment of fibrosis (Fig. 4G-H). Notably, levels of lactate in the WKM were higher in TPO^high zebrafish compared to untreated animals ($p < 0.05$; Fig. 5A). Moreover, the analysis of lactate transporters expression unveiled a significant upregulation of mct1 accompanied by a strong decrease of mct4 expression ($p < 0.001$ compared to control; Fig. 5B), also confirmed by immunofluorescence (Fig. 5C). Taken together, these results demonstrated that lactate and its transporters are skewed in TPO^high zebrafish.

MCTs expression in PMF patients

To validate MCT4 downregulation in myelofibrosis, we analyzed MCT expression in BM biopsies from PMF patients. Immunohistochemical analysis of three consecutive slides revealed that PMF-MSCs, identified as CD90^+ cells, expressed only MCT1 (Fig. 6A). Interestingly, evaluating expression of MCTs in PV, TE and PMF, we found that the loss of MCT4 is an exclusive feature of myelofibrosis TME (Fig. 6B).

Discussion

The evolution of MF is associated with the development of BM fibrosis, progressive splenomegaly and systemic symptoms. Concomitantly, metabolic alterations, occurring in cancer cells reshape TME through exchange of signaling molecules including metabolites which induce cancer-associated transformation and immune-escape [20, 51, 52]. Although metabolic alterations in MPN
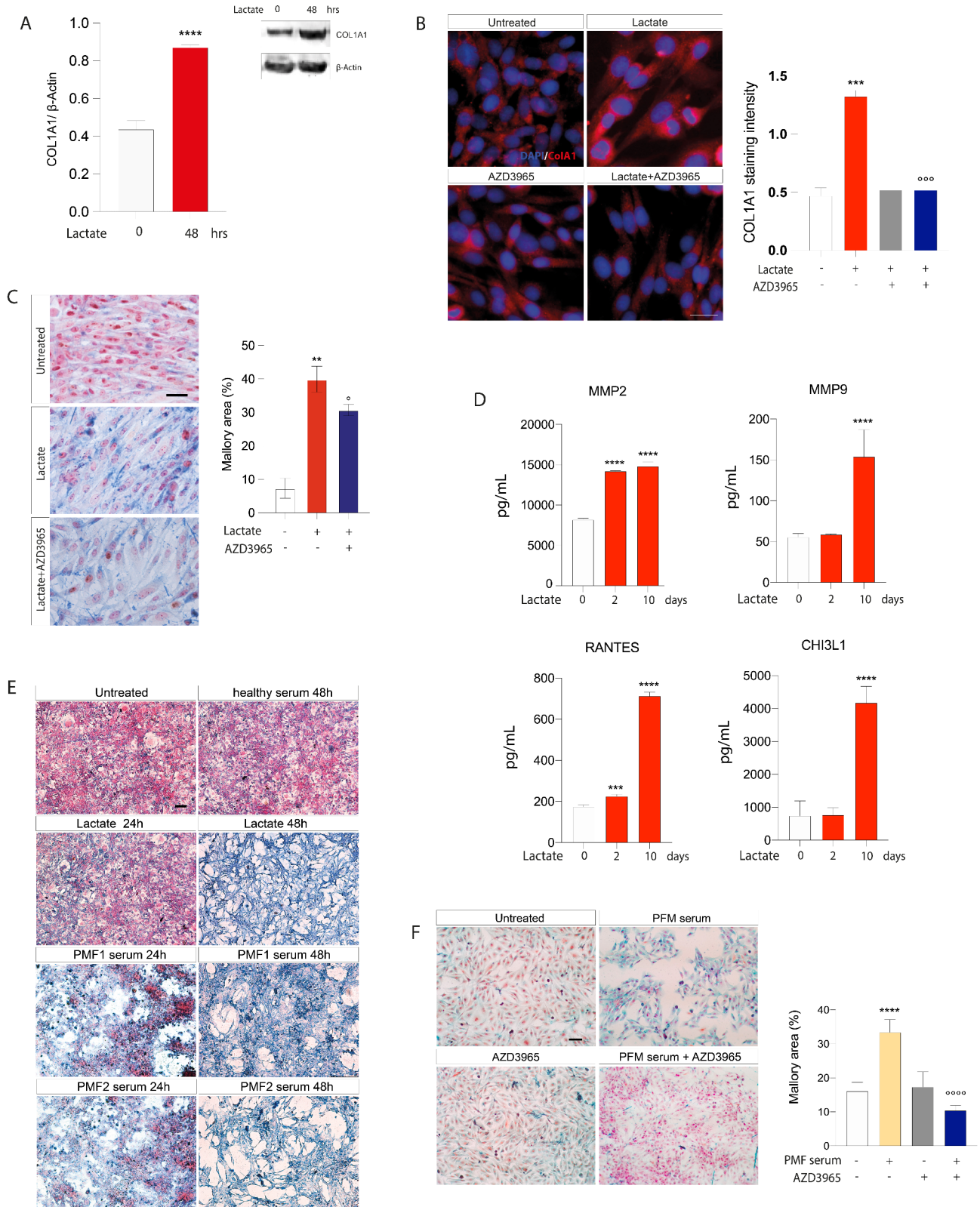
Fig. 3 (See legend on next page.)

tumor cells have been previously described, insights on how these changes impact TME are poorly understood. In this study, we found an increased amount of circulating lactate in sera from myelofibrosis patients. These data are in accordance with previous works demonstrating an increased glycolytic rate in JAK2-V617F mutant cells [26, 28] and higher lactate levels in platelets from MPN patients [53]. Lactate deeply modulates immune response through the expansion of immunosuppressive subsets [20], prompting naïve T cell apoptosis [54] and decreasing the cytotoxic activity of NK [55]. We found that the exposure of healthy PBMCs to PMF sera induces the expansion of Treg and M-MDSC immunosuppressive subtypes. This effect is in part lactate-mediated as demonstrated by the significant reduction of the percentage of Treg and M-MDSCs after MCT1 blocking.

The pathophysiology underlying BM fibrosis remains unclear despite intensive study, with lack of specific therapy [56]. It is the outcome of complex interactions between myelofibrosis tumor cells and stromal cells, which, after differentiation into myofibroblasts and fibroblasts, lay down specific classes of collagen and matrix protein, culminating in the fibrotic transformation of the BM niche [13]. Lactate metabolism alterations have been noted in Idiopathic Pulmonary Fibrosis [42] and liver fibrosis [57], but the relationship between lactate and BM fibrosis has not been elucidated. Here, we demonstrated that lactate confers CAF phenotypic features to healthy MSCs, as suggested by increased αSMA and FAP expression after lactate exposure. It is generally accepted that CAFs are the main participants in ECM remodeling and fibrosis [58]. Furthermore, lactate has been reported to promote TGF-b signaling, eventually triggering tumor fibrosis [59]. Interestingly, our data demonstrated that lactate-induced CAFs engage in the release of osteo-inductive growth factors including BMP2, osteopontin, calcitonin and sRANKL, which leads to mineralized matrix formation. Pharmacological inhibition of MCT1 by AZD3965 prevented osteoblast differentiation. Osteoblasts dysregulation might cause secretion of large amounts of type I collagen and ECM remodeling, culminating in a stiff, type I collagen-rich fibrotic matrix [60]. After lactate exposure, HS-5 cells showed increased expression of COL1A1 and collagen accumulation which decreased after MCT1 inhibition. This lactate-induced

early fibrosis development was corroborated by higher secretion of MMP2, MMP9, RANTES and CHI3L1. As a proof of context, when excessive remodeling of ECM occurs, both the quantity of collagen and levels of MMPs increase significantly [61]. In this context, MMP-9 is highly expressed during fibrosis process, and it has become a research focus to reduce ECM deposition and inhibit fibrosis [62]. Notably, PMF serum lactate causes a strong deposition of collagen which was not observed after incubation of stromal cells with serum from healthy controls. This circulating lactate-induced effect was significantly reverted after addition of AZD3965.

We also performed a detailed analysis of lactate amount and its transporter expression in WKM from adult zebrafish after establishing experimental fibrosis. Indeed, this study provides evidence that, after treatment with a TPO mimetic, zebrafish become prone to develop fibrosis in WKM, the site for hematopoiesis in adult zebrafish [63]. This is in keeping with the TPO[high] murine model known to recreate the phenotype of BM fibrosis shared by MF patients associated with increased megakaryocytes and splenomegaly [56, 64, 65]. After its binding to MPL receptor, TPO regulates megakaryopoiesis and induces proliferation of progenitor hematopoietic cells [50]. Moreover, thrombopoietin receptor agonists are reported to increase the risk for reticulin fiber deposition within bone marrow [66]. Consistently, TPO[high] zebrafish showed higher expression of megakaryocyte-related and fibrosis-related genes, which was corroborated by increased deposition of collagen and reticulum fibers in WKM. Performing a detailed analysis of the major blood lineages, we also found higher number of progenitor cells accompanied by a reduction of erythroid and myeloid cells. Besides, we unveil a remarkable increase in lactate concentration accompanied by a higher expression of MCT1 and a strong downregulation of MCT4 in WKM from TPO[high] zebrafish. To definitively unravel the expression of MCTs in myelofibrosis TME, we exploited their expression in biopsy specimens from MPN patients. Notably, our results demonstrate that, among the MPNs, myelofibrosis is the only characterized by the loss of MCT4 expression It is well known that MCTs are the most important elements in lactate metabolism, orchestrating metabolic remodeling of TME. As MCT4 mediates lactate export in highly glycolytic cells
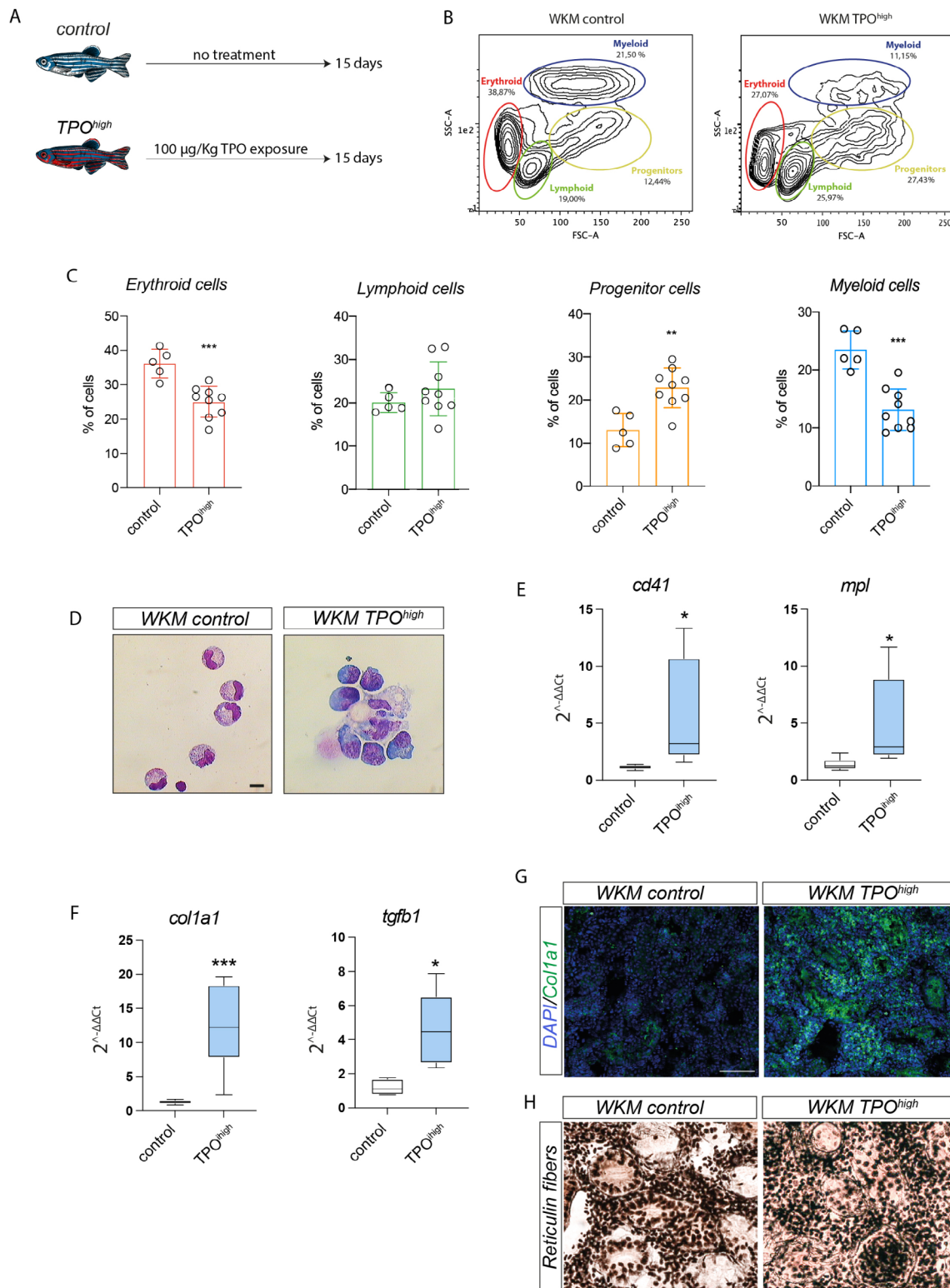
Fig. 4 TPO^high zebrafish as an in vivo model of megakaryocyte expansion and fibrosis development. (**A**) A schematic view of TPO^high zebrafish model. (**B**) Flow cytometry analysis of WKM in control and TPO^high zebrafish. (**C**) Evaluation of the percentage of each blood lineage after TPO treatment. (**D**) May-Grünwald Giemsa images of hematopoietic cells isolated from WKM of control and TPO^high zebrafish. Scale bar: 5 μm. (**E**) qPCR analysis of megakaryocytic markers and (**F**) col1a1 and tgfb1 expression in WKM. B2m was used as housekeeping gene. Calculated value of $2^{-\Delta\Delta Ct}$ in control was 1. (**G**) Representative immunofluorescence images of Col1A1 and (**H**) immunohistochemical images of reticulum fibers in control and TPO^high zebrafish. Scale bar: 40 μm; magnification: 40X. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$
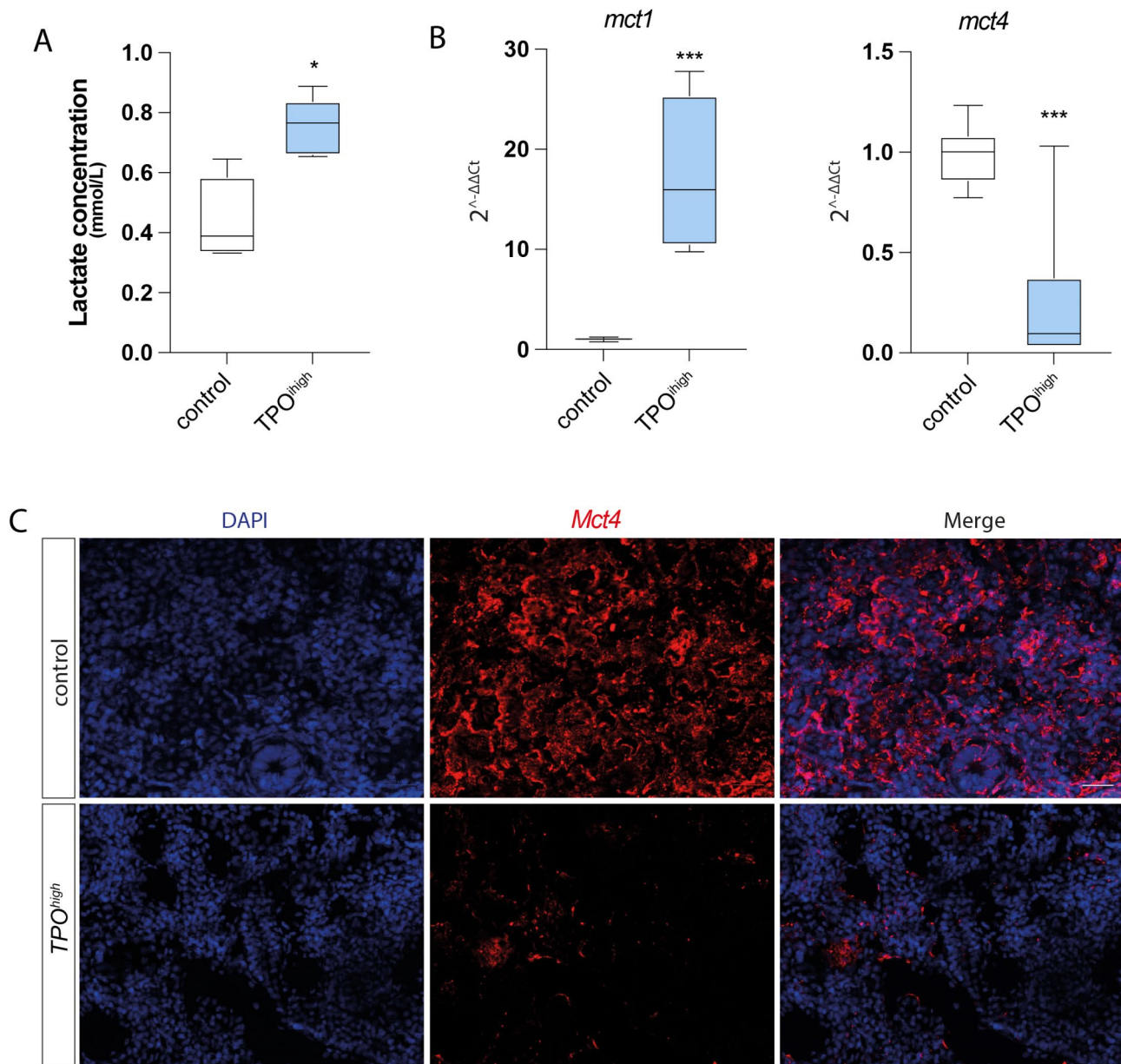
Fig. 5 Alteration of lactate and its transporters in TPO^high zebrafish model. (**A**) Evaluation of lactate concentration in WKM from control and TPO^high zebrafish. (**B**) qPCR analysis of mct1 and mct4 in WKM from control and TPO^high animals. (**C**) Representative immunofluorescence images for Mct4. Scale bar: 20 μm. All the data are presented as means ± SD of three independent experiments. *$p < 0.05$; ***$p < 0.001$

[67, 68], a deeply metabolic adaptation may occur in MF patients. Most importantly, the heterogeneity of MCT1, which is expressed in different cell populations, and the homogeneity loss of MCT4 expression in PMF biopsies might contribute to lactate accumulation in BM, supporting tumor fibrosis and progression. Further studies will be developed in order to investigate the potential role of MCT4 as a potential marker of clinical utility in the differential diagnosis between TE and early stage of myelofibrosis.

Conclusions

In conclusion, our results unveil lactate as a key regulator of immune escape and BM fibrotic transformation in MF patients, reinforcing the idea that targeting metabolism can be a good strategy against cancer. Moreover, our data represent the first experimental evidence of a BM fibrotic phenotype in TPO^high zebrafish model, together with several hematological features of the disease observed in MF patients. Our results suggest that changes in MCT expression in TME is an effect occurring during fibrosis development, despite a causal link between the loss of MCT4 and pro-fibrotic phenotype needs to
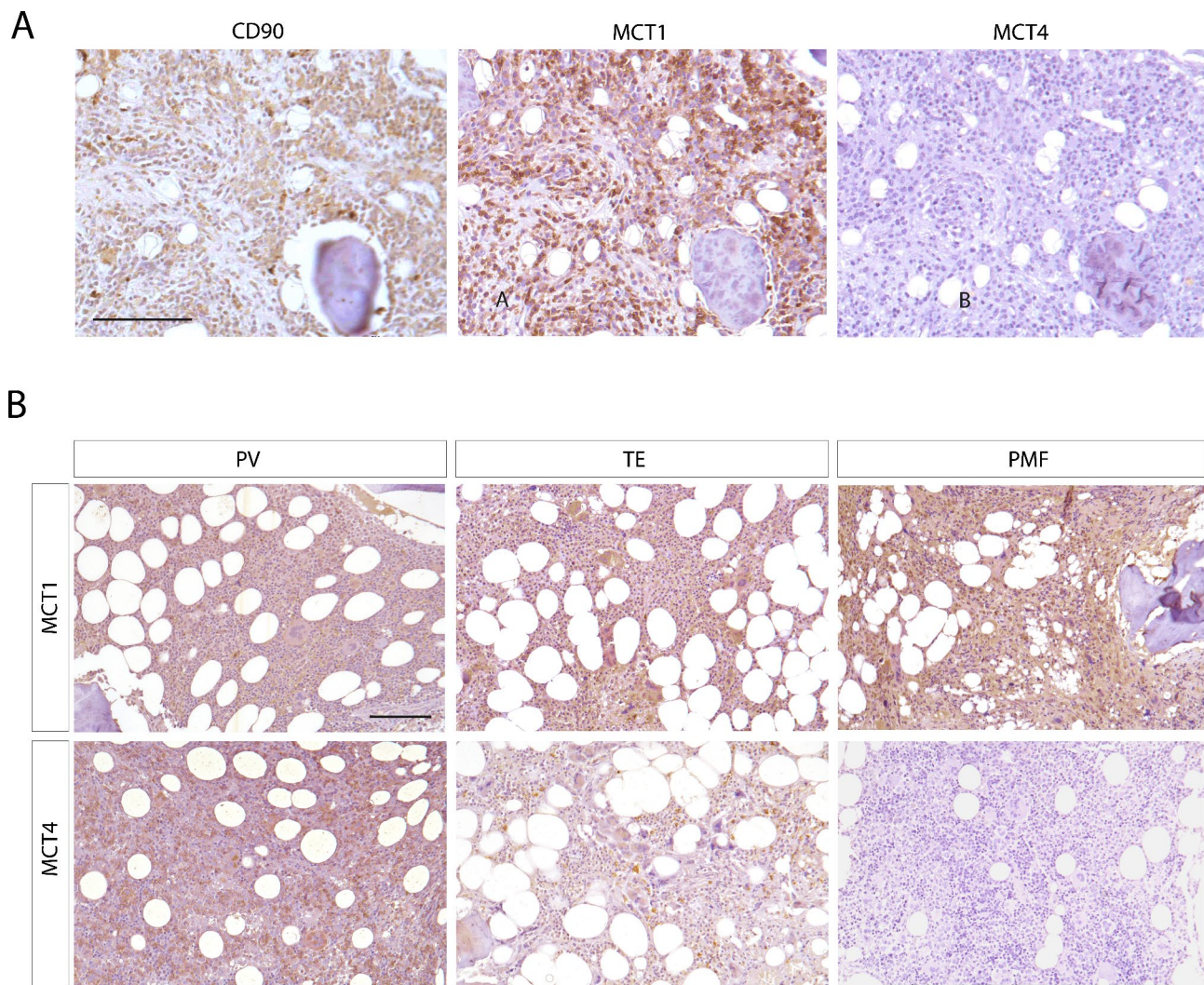
Fig. 6 MCTs expression in BM biopsies from MPN patients.(**A**) Immunohistochemical analysis of CD90, MCT1 and MT4 expression in three consecutive sections from a PMF BM biopsy. (**B**) Representative immunohistochemical images of MCT1 and MCT4 in BM slides from PV, TE and PMF patients. Scale bar: 500 px

be demonstrated. To date, identifying novel antifibrotic strategies is very important since the effectiveness of current therapies in reverting BM fibrosis is debated. Our results demonstrate that MCT1 might be a novel druggable target in MF paving the way to antifibrotic therapies based on the inhibition of lactate accumulation in tumor microenvironment.

Abbreviations

αSMA	Alpha-smooth muscle actin
BM	Bone marrow
BMP2	Bone morphogenetic protein 2
CAF	Cancer-associated phenotype
CHI3L1	Chitinase 3 like 1
Col1A1	Alpha-1 type I collagen
ECM	Extracellular matrix
ET	Essential thrombocythemia
FAP	Fibroblast activation protein alpha
JAKi	JAK inhibitors
LDHA	Lactate dehydrogenase A
MCTs	Monocarboxylate transporters
MDSCs	Myeloid derived suppressor cells
MF	Myelofibrosis
MMPs	Matrix metallopeptidases
MPN	Myeloproliferative neoplasm
MSCs	Mesenchymal stromal cells
OIM	Osteogenic induction medium
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PDGFRβ	Platelet-derived growth factor receptor β
PMF	Primary myelofibrosis
PV	Polycythemia vera
RANTES/CCL5	Regulated upon activation, normal T cell expressed and secreted
sRANKL	Receptor activator of nuclear factor kappa-B ligand
TGF-b	Tumor growth factotor beta
TME	Tumor microenvironment
TPO	Thrombopoietin
WKM	Whole kidney marrow

Supplementary Information

Supplementary Material 1

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Author contributions

Conceptualization: DT, CG, GAP and GLV; project administration: DT, CG, GAP, FDR, SG and GLV; methodology: MS, CG, ELS, AD, LL, RC, LS, ID, GB, VDF and AR; investigation: MS, CG, ELS, AD, LL, RC, LS, GB, AMDM, VDF and AR; formal analysis: CG, SG, DT, GAP, FDR and GLV; resources: MS, CG, SG, DT, GAP and GLV; supervision: CG, GAP, DT and GLV; writing-original draft: CG, SG, GAP, EPP, DT and GLV; writing-reviewing and editing: FDR, AR, RC, CG, SG, GAP, DT and GLV.

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Data availability

The datasets used and/or analyzed in this study are reported within the manuscript and/or additional files and are available from the corresponding authors.

Declarations

Ethics approval and consent to participate

Peripheral blood (PB) samples were collected from MF patients and age-matched controls after written informed consent (Azienda Ospedaliero-Universitaria Policlinico "G.Rodolico-San Marco", n. 54/2022/PO).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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